STUDIES ON *THIOBACILLUS FERROOXIDANS* ATCC 33020 *ATP* GENES AND GENE PRODUCTS, USING *ESCHERICHIA COLI.*

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CERTIFICATION OF SUPERVISOR.

In terms of papragraph GP 8 in "General Rules for the Degree of Doctor of Philosophy (PhD)", I as supervisor of the candidate, Lynn Dryden Brown, certify that I approve of the incorporation in this dissertation, of material that has already been published, or submitted for publication.

Signed by candidate

Signature Removed

Professor Douglas E. Rawlings
Department of Microbiology
University of Cape Town
Cape Town

THE PURSUIT OF SCIENCE.

1934.

"You will hardly find one among the profounder sort of scientific minds without a peculiar religious feeling of his own. But it is different from the religion of the naive man. For the latter, God is a being from whose care one hopes to benefit and whose punishment one fears; a sublimation of feeling similar to that of a child for its father, a being to whom one stands to some extent in a personal relation, however deeply it may be tinged with awe.

But the scientist is possessed by the sense of universal causation. The future to him, is every whit as necessary and determined as the past. There is nothing divine about morality; it is a purely human affair. His religious feeling takes the form of a rapturous amazement at the harmony of natural law, which reveals an intelligence of such superiority that, compared with it, all the systematic thinking and acting of human beings is an utterly insignificant reflection. This feeling is the guiding principle of his life and work, insofar as he succeeds in keeping himself from the shackles of selfish desire. It is beyond question closely akin to that which has possessed the religious geniuses of all ages."

Albert Einstein, (1934).

1987.

"The confusion besetting society, when dealing with some of the fruits of present-day scientific research, is not surprising. It was the wont to rank science, until far into our century, among the highest and purest pursuits of mankind. Science was the never-ending search for truth about nature, a quest that would help us understand the workings of our world. That era has ended, I believe, with the splitting of the atomic nucleus, with the manipulation of the cellular nucleus, with the ability to modify the heriditary apparatus. A new era has begun: science is now the craft of the manipulation, modification, substitution and deflection of the forces of nature."

Erwin Chargaff, (1987).

1993.

"The (Human Genome Project) itself doesn't have any ethical problems. It is only the implications and applications of that knowledge within society. Society has to decide what it is prepared to have happen."

Linda Evans, Scientific Administrator of the Human Genome Organisation, Europe.
The Guardian Weekly, April 16-22, 1993.

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ABSTRACT.

An *atp* gene cluster from the extreme acidophile *Thiobacillus ferrooxidans* ATCC 33020 was cloned by complementation of *Escherichia coli unc* mutants. Eight different *E. coli unc* mutants were screened with *T. ferrooxidans* ATCC 33020 p*Eco*R251 plasmid and pHC79 cosmid gene banks. The ability of the transformants/transductants to grow on succinate as the sole non-fermentable carbon source was used to select mutants with a functional F_1F_0 ATPsynthase. Many F_1-complementing plasmids and cosmids were isolated from the four *E. coli* F_1 *unc* point mutants screened. No plasmids or cosmids which complemented an *E. coli* Δ*unc* strain or any of the three *E. coli* F_0 mutants screened, were isolated.

Sequencing of a 5.946 kb *Sau*3A-*Bgl*II *T. ferrooxidans* ATCC 33020 chromosomal fragment present on one F_1-complementing plasmid isolate (p*Tfatp*2) showed the presence of seven complete open reading frames, and one incomplete unidentified reading frame cloned in reverse orientation with respect to the p*Eco*R251 vector λ promoter. The predicted translation products of the seven complete open reading frames showed marked homology to F_1F_0 ATPsynthase subunits c, b, δ, α, γ, β, and ε from other organisms. The incomplete unidentified reading frame was homologous to an unidentified reading frame of unknown function which occurs downstream of the *unc/atp* operons of *E. coli* and *Vibrio alginolyticus* respectively.

The *atp* genes from *T. ferrooxidans* ATCC 33020 were arranged in the order *EFGHADC* as a contiguous cluster. The incomplete unidentified reading frame was downstream of the *atpC* gene. The arrangement of the *atp* genes was most like that of the γ-proteobacteria *E. coli* and *V. alginolyticus*, where the F_0 cluster occurs immediately upstream of the F_1 group. A detailed computer analysis of the nucleotide sequence of the seven *T. ferrooxidans atp* genes showed that codon usage patterns were typical of that recorded previously for *T. ferrooxidans* ATCC 33020 cistrons. However, certain genes showed unusual trends; in the case of *atpE* there was a notably high occurrence of U in the third 3' (wobble) position of the codons, possibly related to a high rate of *atpE* expression. In genes associated with catalytic mechanism viz. *atpD, A* and *G*, the use of common codons was more frequent than in the remaining four cistrons. A comparative analysis of the intergenic regions of *T. ferrooxidans atp* and *E. coli unc* operons showed similarities and differences between the two organisms. The *T. ferrooxidans atp* operon was terminated by a region showing a marked probability to form two highly stable tandem transcript loops. Whereas the function of the initial 5'-loop was unknown, the second loop was typical of a *rho*-independent terminator.

A comparison between the nucleotide sequence-derived polypeptides of the seven *T. ferrooxidans* F_1F_0 ATPsynthase polypeptides and those from other organisms was carried

out. This analysis showed that subunits b, δ, α, γ, β and ε of the *T. ferrooxidans* enzyme were most like that of the two γ-proteobacteria included in the comparisons, viz. *E. coli* and *V. alginolyticus*. The c subunit of *T. ferrooxidans* was similar in predicted size, charge (and possibly tertiary structure) to the proteolipid from other organisms. However at the level of primary structure, the c subunit was not sufficiently similar to other bacterial species compared to be grouped within any defined phylogenetic clusters. The predicted primary sequence of all seven *T. ferrooxidans* F_1F_0 ATPsynthase subunits indicated that generally, regions known to be associated with catalytic mechanism were conserved. However, unusual domains and/or isolated residues were noted for *T. ferrooxidans* c, δ, γ, ε and even β. The c subunit had many features unique to *T. ferrooxidans*, collectively thought to indicate an unusual proton pathway within the F_0 oligomer. Unusual N- and/or C-termini were noted for δ, γ, ε and β. These domains could represent mechanisms of proton gating associated with energy coupling in an extreme acidophile.

An *E. coli*-derived *in vitro* transcription/translation system was used to identify polypeptides produced from cloned *T. ferrooxidans atp* genes. This showed that all seven *T. ferrooxidans* F_1F_0 ATPsynthase polypeptides could be produced by an *E. coli* system. The five F_1 gene-products were synthesised from p*Tfatp*2. As the genes present on p*Tfatp*2 were cloned in reverse orientation with respect to the vector promoter, it was likely that an internal promoter located upstream of the *atpH* gene was recognised by *E. coli*. However, primer extension experiments failed to identify the location of this promoter.

A series of studies was performed to determine the extent of cross-complementation between the F_1F_0 ATPsynthase of an acidophile and neutrophile. This was done by subcloning various combinations of *T. ferrooxidans atp* genes behind plasmid vector promoters recognised by *E. coli*. The *atpE* and *F* genes, although expressed *in vitro*, did not complement *E. coli* F_0 mutants. However, several observations suggested that the degree of complementation by the *T. ferrooxidans* F_1 subunits was sufficient for some of the F_1F_0 ATPsynthase components to be interchangeable. A plasmid construct which expressed only the *T. ferrooxidans* β and ε subunits, complemented the corresponding *E. coli* mutants. When expressed on its own, *T. ferrooxidans* ε did not complement an *E. coli uncC* mutant. All five *T. ferrooxidans* F_1 subunits together formed a functional association with the F_0 portion of *E. coli* in an *E. coli unc* deletion strain.

Studies on an *E. coli unc* deletion strain which carried a hybrid *T. ferrooxidans* F_1/*E. coli* F_0 enzyme showed that the growth rate and yield of the transformant was less than that of wild-type *E. coli*. ATPase assays of the hybrid enzyme demonstrated that the specific activity of the enzyme was markedly lower than that of wild-type *E. coli* F_1F_0 ATPsynthase. The hybrid showed notable resistance to known F_1F_0 ATPsynthase inhibitors viz. *N,N'*-dicyclohexylcarbodiimide and sodium azide, which suggested that the

enzyme was impaired with regard to both coupling and multisite catalysis. The pH profile of the ATPase activity of the hybrid was similar to that of wild-type *E. coli* F_1F_0 ATPsynthase. Membrane preparations from *T. ferrooxidans* ATCC 33020 showed no ATPase activity. The enzyme was apparently destroyed during preparative procedures.

Despite many attempts, the *T. ferrooxidans* ATCC 33020 operon promoter (*P*), *atpI* and *atpB* were not cloned. No F_1-complementing plasmids contained *T. ferrooxidans* genomic inserts which extended upstream of the *Sau*3AI-*Bgl*II chromosomal fragment present on p*Tfatp*2. Over 24 *uncA*-complementing plasmids terminated at the same upstream *Sau*3AI site. Fifty cosmids were isolated which complemented an *E. coli uncA* mutant. Several of these cosmids which were selected for further study did not appear to extend upstream of the *atpE* gene. It was possible that the *T. ferrooxidans atpPIB* and/or the entire *atpBEF* regions were harmful and/or unstable when expressed in *E. coli*. A *T. ferrooxidans* ATCC 33020 2.2 kb *Eco*RV-*Xho*I genomic fragment thought to contain the *atpB* gene was identified. All attempts to isolate this fragment either by cloning in reverse orientation with respect to an inducible vector promoter (to minimise *atpB* expression), or by the polymerase chain reaction were unsuccessful.

RNA was extracted from *E. coli unc* mutants harbouring *T. ferrooxidans atp* plasmid constructs and from *T. ferrooxidans* ATCC 33020. Although the RNA from the organisms appeared to be of satisfactory quality, no discrete *atp* transcripts were identified by Northern blotting.

ABBREVIATIONS.

2-N_3-ANP	2-azido-ANP
2-N_3-ATP	2-azido-ATP
8-N_3-ANP	8-azido-ANP
8-N_3-ATP	8-azido-ATP
A	adenosine
ACMA	9-amino-6-chloro-2-methoxyacridine
ADP	adenosine diphosphate
AK	adenylate kinase
AMP-PNP	adenylyl-β,-τ-imidodiphosphate
ANP	ATP or ADP
Ap	ampicillin
ATCC	American Type Culture Collection
ATP	adenosine 5'-triphosphate
bp	base pair/s
B_zATP	3'-*O*-(4-benzoyl)benzoyl-ATP
C	cytosine
C-	carboxy terminal (end of protein)
Cm	chloramphenicol
Da	Daltons
DCCD	*N,N'*-dicyclohexylcarbodiimide
DEPC	diethyl pyrocarbonate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease I
DNP	2,4 dinitrophenol
dNTP	deoxyribonucleoside triphosphate
DTT	dithiothreitol
EDTA	disodium ethylene-diaminetetra-acetate
EF-Tu	elongation factor Tu of *Escherichia coli*
FSBA	5'-*p*-fluorosulfonylbenzoyladenosine
FSBI	5'-*p*-flurosulfonylbenzoylinosine
G	guanine
GTP	guanosine 5'-triphosphate
h	hour/s
IPTG	isopropyl-β-D-thiogalactopyranoside
ITP	inosine 5'-triphosphate
K_a	acid dissociation constant
kDa	kilodalton
Km	kanamycin
LB	Luria Bertani broth
LBA	Luria Bertani agar
MES	2-(*N*-morpholino)ethanesulphonic acid
min	minute/s
MLB	modified Luria Bertani broth
MMS	minimal succinate medium
MOPS	3-(*N*-morpholino)propanesulphonic acid
M_r	relative molecular mass

mRNA	messenger RNA
N-	amino terminal (end of protein)
Nbf-Cl	7-chloro-4-nitrobenzofurazan
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect
nt	nucleotide/s
OD_x	optical density at x nm
ORF	open reading frame
OSCP	oligomycin sensitivity conferring protein
P	promoter
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEG	polyethylene glycol
P_i	Inorganic phosphate
pK_a	log K_a
PLP-ADP	pyridoxal 5'-triphosphoadenosine
PLP-AMP	pyridoxal 5'-diphosphoadenosine
psi	pounds per square inch
Ras	*ras*p21 protein
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal RNA
s	second/s
S	Svedburg sedimentation co-efficient
SD	standard deviation
SDS	sodium dodecyl sulphate
T	thymine
TID	3-trifluoromethyl-*m*-(iodophenyl)-diazirine
Tris	Tris(hydroxymethyl)aminomethane
tRNA	transfer RNA
U	units of enzyme activity
U	uracil
URF	unidentified reading frame
V-ATPase	vacuolar proton-translocating ATPase
w/v	weight volume
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
α	alpha
β	beta
γ	gamma
δ	delta
ε	epsilon
Δ	delta
$\Delta\mu_{H+}$	proton motive force
ΔpH	transmembrane proton gradient
$\Delta\Psi$	transmembrane electrochemical gradient
ΔG	change in Gibbs function
E	electrode reduction potential
E_0'	standard electrode reduction potential
ΔE	$E_{cathode} - E_{anode}$

CHAPTER 1.

GENERAL INTRODUCTION.

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"The organism's (T. ferrooxidans) importance to man cannot be overemphasised; on the negative side, the organism is associated with one of the major water pollution problems of the world. From a positive viewpoint, the organism is economically important in mineral conservation and in hydrometallurgical extraction for low-grade ores." Lundgren *et al.* (1974).

1.1. The discovery of *Thiobacillus ferrooxidans.*

In 1943, Taylor and Whelan presented a paper summarizing the results of an exhaustive study of the Rio Tinto copper mines in Spain. The authors' primary objective was to establish the basis of the exponential formation of soluble copper sulphate in acid waters, which drained from residual ore waste dumps after the annual rains. The copper content of the effluent waters was so high that it had become economically worthwhile to recover the copper by precipitation using scrap and pig iron. Although the authors did not realise it at the time, their paper was amongst the first published references to *T. ferrooxidans.* Instead, Taylor and Whelan attributed the exponential dissolution of copper in the waste dumps to a chemical phenomenon, in which "some constituent of the dust (of) the leaching liquors (was) particularly effective in promoting" the leach reactions. Five years later in 1947, Colmer and Hinckle published a paper describing a study on the origins of severe pollution problems associated with acid mine drainage waters in the United States of America (U.S.A). These authors suggested that microorganisms in the waters were responsible. Subsequently in 1951, Temple and Colmer identified *T. ferrooxidans,* isolated from the acidic waters. Later papers established beyond doubt that *T. ferrooxidans* was one of the organisms responsible for both the solubilisation of copper noted at Rio Tinto and the generation of the acid mine drainage waters associated with various mining areas in the U.S.A (Lundgren and Silver, 1980). These phenomena were a direct result of the unique physiology of *T. ferrooxidans.*

1.2. The physiology of *T. ferrooxidans.*

T. ferrooxidans is an obligate chemolithotrophic acidophilic mesophile. The optimum external pH (pH_o) range for growth is 2-2.5, and the preferred environmental temperature is between 30-35°C. The organism is a small gram-negative rod, measuring 0.5-1.0 μm. It is motile by means of a single polar flagellum. The mol% GC of the type species, *T. ferrooxidans* ATCC 23270 is 58-59 (Kelly and Harrison, 1989). For a long time the nutritional status of the organism was confused, and early reports in the literature frequently noted that the organism was mixotrophic/facultatively heterotrophic (eg. see

Tabita and Lundgren, 1971). However, studies on the DNA base composition of supposedly pure heterotrophic strains of *T. ferrooxidans* showed conclusively that most of these claims regarding heterotrophic growth were erroneous, as the cultures were contaminated by heterotrophic acidophiles, such as *Acidiphilium cryptum* and *Thiobacillus acidophilus* (Guay and Silver, 1975; Guay *et al.*, 1976; Harrison *et al.*, 1980).

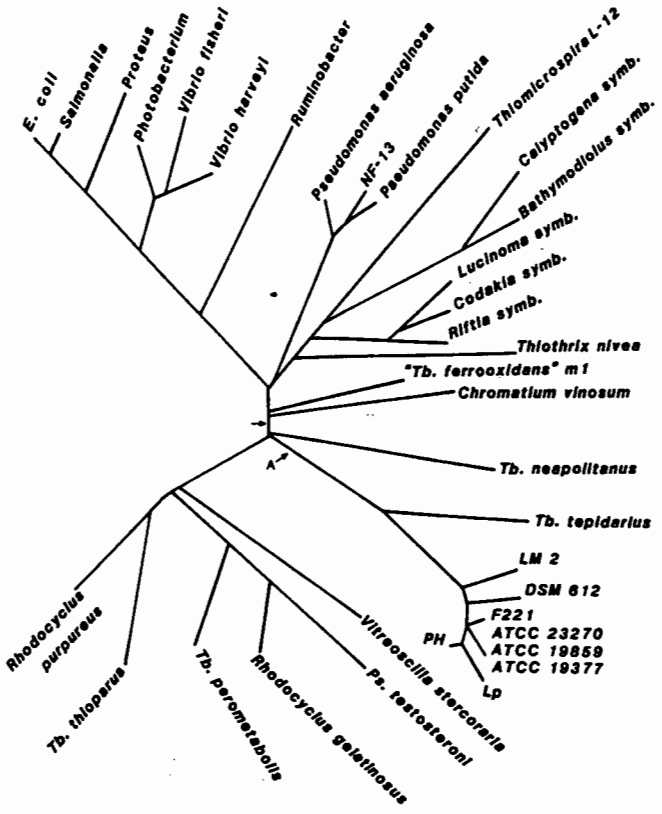


Fig. 1.1. The 16S rRNA relationships between the beta and gamma subdivisions of the proteobacteria. The branch lengths are proportional to evolutionary distances. *T. ferrooxidans* strains listed are ATCC 23270, ATCC 19859, F221, Lp, PH, and LM2. Note the close relationship of the *T. ferrooxidans* strains to *T. thiooxidans* DSM 612 and ATCC 19377, and the position of *T. ferrooxidans* m1, no longer thought to be a member of the species (after Lane *et al.*, 1992).

Recent studies incorporating 5S and 16S rRNA base sequence have been useful in predicting possible phylogenetic relationships among the sulphur and iron-oxidising eubacteria, including *T. ferrooxidans*. Most of the *T. ferrooxidans* and *Thiobacillus thiooxidans* (mol% GC, 52) strains form a tight cluster in the β subdivision, toward the root of the β/γ subdivision of the proteobacteria (Lane *et al.*, 1992) (Fig. 1.1). The proteobacteria consist of five subdivisions of gram-negative bacteria, and were formerly classified as the Purple bacteria (Woese, 1987) (Fig. 1.2). The γ subdivision includes most of the commonly encountered gram-negative bacteria, such as the enteric bacteria, vibrios, fluorescent pseudomonads, photosynthetic purple sulphur bacteria (eg. *Chromatium*), *Legionella*, and others (Woese, 1987). The topology of the β subdivision is not yet well-characterised, as its

members are still poorly represented in both 5S and 16S rRNA sequence collections (Lane *et al.*, 1992). The heterotrophic "contaminants" of the *T. ferrooxidans* cultures, viz. *Acidiphilium* species and *T. acidophilus*, are phylogenetically far removed from the iron- and sulphur-oxidising autotrophic *Thiobacillus* species, and form a distinct cluster within the α subdivision (Lane *et al.*, 1992).

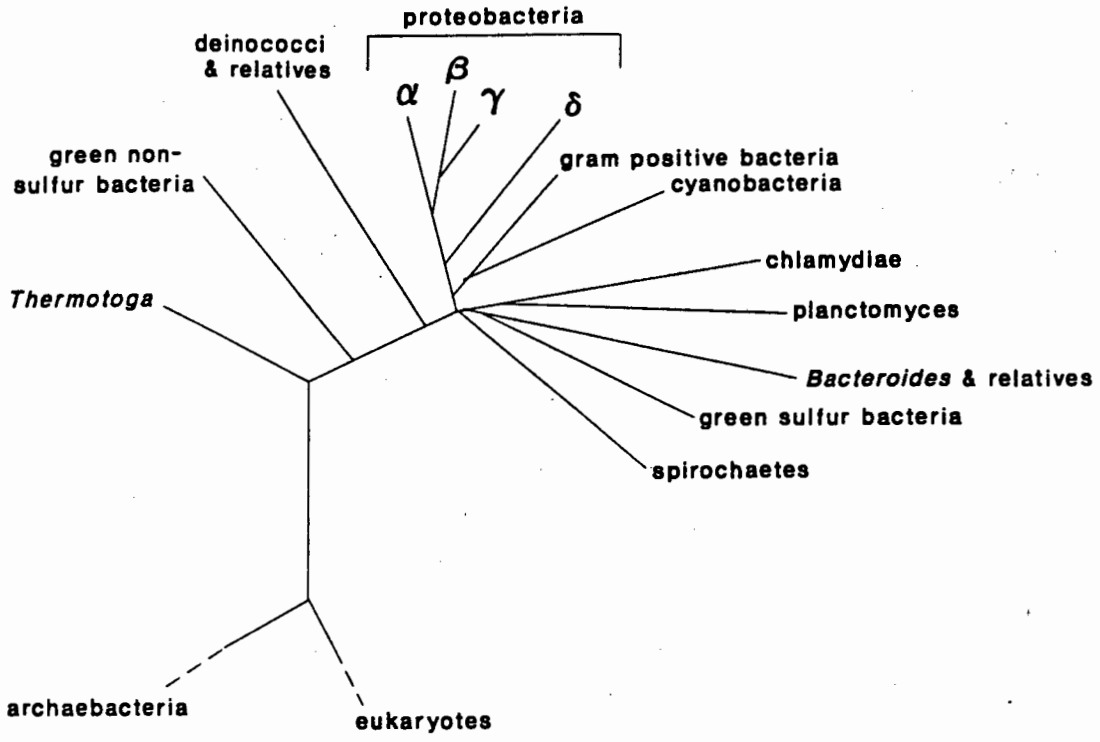


Fig. 1.2. Phylogenetic relationships among the eubacteria, based on 16S rRNA sequences. (after Woese, 1987).

T. ferrooxidans occupies a restricted and potentially hostile environmental niche. Yet within that niche, this remarkable prokaryote has evolved extraordinary physiological strategies which enable the organism to exploit all aspects of the environment to its own advantage. This is particularly apparent with regard to the dissimilatory and assimilatory metabolism. As these latter strategies concern the generation and utilisation of ATP, they will be reviewed. Also of significance is the tolerance of *T. ferrooxidans* to a wide variety of heavy metal cations, and the ability of the organism to adapt to changing environmental conditions. Both of these properties may require ATP, and/or affect rates of ATP synthesis.

The dissimilatory (ATP-generating) metabolism of *T. ferrooxidans* involves the oxidation of inorganic substrates in an acidic *milieu*, external to the cytoplasmic membrane (Bodo and Lundgren, 1974). The pH of the cytosol remains at 6.5 (Ingledew, 1982). The preferred substrates are iron, and reduced sulphur compounds. The bio-oxidation of these

substrates may be acidogenic, and result in the production of sulphuric acid. Energy is generated from iron and sulphur oxidation and is conserved by the production of ATP during oxidative phosphorylation (Ingledew, 1982). The mechanism of ATP generation in *T. ferrooxidans* will be discussed in Section 1.5.2.4.

The oxidation of ferrous iron requires oxygen as the final electron acceptor. The overall reaction for ferrous oxidation by *T. ferrooxidans* may be summarised by the following equations:-

$$FeS_2 + 3.5O_2 + H_20 \longrightarrow FeSO_4 + H_2SO_4 \quad (1) \text{ and,}$$

$$2FeSO_4 + 0.5O_2 + H_2SO_4 \longrightarrow Fe_2(SO_4)_3 + H_20 \quad (2)$$

(Lundgren and Silver, 1980)

The mechanism of ferrous iron oxidation by *T. ferrooxidans* has been extensively studied, and will be discussed in Section 1.5.2.4.

The mechanism/s of the bio-oxidation of elemental sulphur, and reduced inorganic sulphur compounds by *T. ferrooxidans* are not yet fully understood. However, existing experimental data suggest that the dissimilatory metabolism of sulphur by *T. ferrooxidans* is both complex and remarkable. It was reported to occur aerobically and anaerobically (Silver and Lundgren, 1968; Brock and Gustafson, 1976). The oxidation of various sulphur forms by the organism may be summarised as :-

$$S + 1.5O_2 + H_20 \longrightarrow SO_4^{2-} + 2H^+ \quad (3)$$

$$S^{2-} + 2O_2 \longrightarrow SO_4^{2-} \quad (4)$$

$$S_2O_3^{2-} + H_20 + 2O_2 \longrightarrow 2SO_4^{2-} + 2H^+ \quad (5)$$

$$S^0 + 6Fe^{3+} + 4H_2O \longrightarrow HSO_4^{2-} + 6Fe^{2+} + 7H^+ \quad (6)$$

Silver and Lundgren (1968) reported the existence of a sulphur:oxygen oxidoreductase which utilised oxygen as a final electron acceptor during the oxidation of sulphur. This reaction was inhibited by the presence of 1 mM Fe^{2+} or 1 mM Fe^{3+} (Sugio *et al.*, 1985). The laboratories of Sugio demonstrated the presence of both a hydrogen sulphide:ferric iron oxidoreductase (SFORase) and a sulphite:ferric iron oxidoreductase in a number of strains of *T. ferrooxidans*, including ATCC 33020 (Sugio *et al.*, 1985, 1988a, 1989 and 1992b). SFORase and the sulphite:ferric iron oxidoreductase catalyse the oxidation of elemental sulphur and sulphite respectively, using ferric ions as the terminal electron acceptor. An unusual feature of the ferric ion reducing system in *T. ferrooxidans*, is that the enzymes involved functioned under both the aerobic and anaerobic conditions. Aerobic sulphur oxidation in *T. ferrooxidans* occurs by the following three steps.

(i). SFORase catalyses the oxidation of elemental sulphur, with ferric ions as the electron acceptor to produce sulphite and ferrous ions.

(ii). The suphite is further oxidised by sulphite:ferric iron oxidoreductase using ferric ions as the electron acceptor.

(iii). Oxygen is required for the oxidation of ferrous to ferric ions by an iron oxidase (Sugio *et al.*, 1985; 1988c and 1992b).

SFORase is also able to reduce Mo^{6+} and Cu^{2+} with elemental sulphur (Sugio *et al.*, 1988b and 1990b).

The dissimilation of other inorganic substrates by *T. ferrooxidans* was variously reported. The oxidation of cuprous copper and stannous tin by washed cell suspensions of the organism was demonstrated, but the authors were unable to show whether these oxidations were coupled to phosphorylation (Nielson and Beck, 1972; Lewis and Miller, 1977). Sugio *et al.* (1990a) demonstrated that SFORase was directly involved in the solubilisation of copper from copper concentrate by *T. ferrooxidans*. Di Spirito and Tuovinen (1982) reported that in the presence of oxygen, certain strains of *T. ferrooxidans* were able to oxidise reduced uranium, and used the energy derived from this reaction to drive carbon dioxide fixation . Drobner *et al.* (1990) demonstrated that *T. ferrooxidans* ATCC 23270 and two other strains were able to utilise hydrogen as a sole energy source, via a cytoplasmic inducible hydrogenase. During the oxidation of hydrogen, the organism became less acid tolerant and would not grow below pH_o 3. The oxidation was inhibited by the presence of sulphur, ferrous iron, and sulphidic ores. Sugio *et al.* (1992a) reported the oxidation of molybdenum blue (Mo^{5+}) by *T. ferrooxidans* AP19-3. The oxidation was catalysed by both a molybdenum oxidase, and cytochrome oxidase.

Assimilatory (ATP-dependent) metabolism by *T. ferrooxidans* involves the incorporation of carbon, nitrogen, sulphur and phosphorous into organic compounds of cellular constituents.

All of the carbon needs of *T. ferrooxidans* are met by the fixation of atmospheric carbon dioxide via the Calvin Benson cycle (Ingledew, 1982). The principal enzyme involved is D-ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCase) which in *T. ferrooxidans*, is most like that of the RuBPCase of the photosynthetic bacterium, *Chromatium vinosum* (Kusano *et al.*, 1991a). One strain, *T. ferrooxidans* ATCC 21834, was shown to utilise low concentrations of formate as a carbon-source (Pronk *et al.*, 1991). The carbon dioxide released from formate oxidation was utilised during carbon dioxide fixation. The formate dehydrogenase of the organism was $NADP^+$-independent, and the oxidation occurred simultaneously with high rates of ferrous iron oxidation. It remains to be determined whether formate utilisation is common within the species.

The assimilation of nitrogen by *T. ferrooxidans* occurs by diverse means. The organism is able to utilise ammonium salts and nitrate as nitrogen sources (Tuovinen *et al.*, 1979).

T. ferrooxidans is also able to fix atmospheric dinitrogen (Mackintosh, 1978). The circumstances which induce dinitrogen-fixation remain to be clarified, as it is known that the high concentrations of oxygen necessary for ferrous iron oxidation are inhibitory to the process (Mackintosh, 1978). It was suggested that when oxygen is limiting, and SFORase-induced oxidation of sulphur compounds results, where Fe^{3+}, Mo^{6+} or Cu^{2+} are the terminal electron acceptors, simultaneous dinitrogen-fixation might occur (Berger, 1990; Rawlings and Kusano, 1994). Sources of organic nitrogen are inhibitory to *T. ferrooxidans*, and cannot be assimilated (Tuovinen *et al.*, 1979).

Mechanisms for the assimilation of sulphur into methionine and cysteine by *T. ferrooxidans* are currently being investigated. *T. ferrooxidans* has the ability to assimilate sulphate into the cell, by the production of the sulphate-assimilatory enzymes, ATP sulfurylase (ATP:sulphate adenylyl transferase) and APS kinase (ATP:adenosine 5'-phosphosulphate 3'-phosphotransferase) (Fry and Garcia, 1989). The manner in which the organism shifts metabolism between the diametrically opposed pathways of sulphate reduction during assimilation and the production of sulphate by dissimilatory processes is unknown.

Little is known about the mechanism of phosphorous assimilation by *T. ferrooxidans.* The organism is capable of phosphate uptake by a 40 kDa outer membrane protein, the production of which is stimulated under conditions of phosphate starvation. It was proposed that this protein, which has the ability to form channels across a lipid membrane, is part of a phosphate-scavenging system in *T. ferrooxidans* (Jerez *et al.*, 1992).

T. ferrooxidans is able to tolerate high concentrations of heavy metal cations. In addition to those referred to previously, these include zinc, arsenic, cadmium, lead, cobalt, nickel, antimony, gallium and bismuth (Lundgren and Silver, 1980; Ingledew, 1982; Rawlings *et al.*, 1991). The mechanism of tolerance is not fully understood; whether this requires expending energy on the part of the organism, is unknown. It was suggested that the active generation of an electrochemical gradient across the cytoplasmic membrane of *T. ferrooxidans* (poised inside positive to outside) could inhibit cation uptake by the organism (Alexander *et al.*, 1987). The genome is involved in mercury resistance. Many strains of *T. ferrooxidans* are suseptible to mercury, but recently, mercury-resistant strains were isolated. These strains carry chromosomally-encoded mercury-resistance genes (Shiratori *et al.*, 1989; Inoue *et al.*, 1989 and 1990).

T. ferrooxidans has a remarkable ability to adapt to adverse environmental changes, and this was suggested to occur by natural selection of phenotypes within a population, incorporating both advantageous mutations (Rawlings *et al.*, 1991) and phenotypic switching (Holmes and Ul Haq, 1989). In response to changes in environmental pH, *T. ferrooxidans* synthesises differing sets of membrane-associated proteins (Amaro *et al.*,

1991). The organism is also able to fluctuate the cytoplasmic membrane proton gradient and electrochemical charge, in response to external pH perturbations (Cox *et al.*, 1979) (Section 1.5.3).

Within its environment, *T. ferrooxidans* is inhibited by certain substances. Silver is highly toxic to the organism; the reason for this is unknown (Ingledew, 1982). *T. ferrooxidans* is also sensitive to a wide range of monovalent anions including Cl^-, Br^-, I^-, and NO_3^-. Depending on pH_o, varying concentrations of these anions become toxic to *T. ferrooxidans* (Ingledew, 1982; Alexander *et al.*, 1987). This exerts a direct effect on oxidative phophorylation in *T. ferrooxidans*, and is discussed further in Section 1.5.3. Generally, organic substances are considered to be inhibitory to *T. ferrooxidans* (Matin, 1978). Particularly interesting is the fact that although the organism is an acidophile, "weak" organic acids are inhibitory at low concentrations. Organic acids accumulate in the cell matrix of the organism in response to the large transmembrane pH difference, cause lethal acidification of the cytosol, disrupt the proton motive force and cause ATP synthesis to cease (Ingledew, 1982; Alexander *et al.*, 1987).

1.3. The economic significance of *T. ferrooxidans*.

The discovery of *T. ferrooxidans* has had both beneficial and negative implications for the mining industry. The positive aspect has been that through exploitation of the organism's metabolism, lucrative new processes in extractive metallurgy, known as biomining/bioleaching, emerged. On the negative side, is the realisation that through the disturbance of the Earth's crust by the mining industry, the associated stimulation of the growth of *T. ferrooxidans* has resulted in severe environmental pollution problems.

As a result of *T. ferrooxidans* being able to oxidise iron and reduced sulphur compounds, and to tolerate high metal ion concentrations and low acidity, the organism is ideally suited for use by the mining industry. *T. ferrooxidans* is one of a variety of microorganisms used to assist in the extraction of precious metals such as copper, nickel, zinc, molybdenum, uranium and gold, in processes collectively known as bioleaching. Although the iron- and sulphur-oxidising bacteria, such as *T. ferrooxidans*, *T. thiooxidans* and *Leptospirillum ferrooxidans* constitute the major microbiological flora of mesophilic bioleaching processes, heterotrophic organisms are also involved. Heterotrophs isolated include *Acidiphilium* species and *T. acidophilus* (Harrison, 1984). For effective bioleaching to occur, it is essential that a mixed culture or "consortium" of bacteria be present. The reasons for this are unknown, but it is considered likely that mutualistic interrelationships operate (reviewed in Brierley, 1982; Harrison, 1984; Lundgren and Silver, 1980; Norris, 1990).

Bioleaching is a combination of "direct" and "indirect" processes of microbiological origin. In direct processes, bacteria oxidise the components of sulphidic ores, and these may be summarised as follows:-

$$MS + 2O_2 \text{ ----> } MSO_4 \quad (7),$$

where M represents a bivalent metal (Lundgren and Silver, 1980). During the direct attack, the more oxidised product is often more soluble, and is recovered during subsequent extraction processes.

Indirect leaching occurs where bacterial activity on ferrous ores results in the generation of ferric ions and sulphuric acid, as summarised in equations (1) and (2) above. Ferric iron is a powerful oxidising agent that reacts with other metals, transforming them into the soluble oxidised form in an sulphuric acid solution. Both direct and indirect processes may operate simultaneously in a given bioleaching process.

A disadvantage of bioleaching is that it is slow when compared to alternative physico-chemical extractive procedures. However, with the continued depletion of high-grade mineral resources, bioleaching offers an attractive and viable method for solublising metals from a variety of low-grade ores, which would otherwise be economically unworkable. Furthermore, industrially controlled bioleaching is less polluting, and more energy-efficient than physico-chemical techniques. Bio-oxidation procedures are being used principally for the efficient extraction of copper, uranium and gold from sulphidic ores (Rawlings *et al.*, 1991).

The environmental pollution arising from mining and resultant uncontrolled *T. ferrooxidans* bio-leaching is considerable. In undisturbed areas, the presence of *T. ferrooxidans* goes unnoticed, and the organism has played an important part in the formation of the Earth's crust. Particularly significant has been its role in the formation of pure iron ore deposits resulting from a natural microbial leaching process occurring over millions of years (Schlegel, 1988). However, when disturbed by mining activities, the metabolism of *T. ferrooxidans* is artificially stimulated, and is responsible for pollution of the Earth's surface on a massive scale. In 1946, in Western Pennsylvania alone, workings and abandoned mines contributed to a million tons of sulphuric acid being deposited in the drainage area of the Ohio River. In 1972, the U.S.A. Department of the Interior estimated that 10 000 square miles of streams, and 29 000 surface acres of impoundments and reservoirs were seriously affected by the microbiological activity associated with surface mining operations (Lundgren *et al.*, 1972). In England, water flowing from CuS waste dumps contained at least 50 ppm copper in solution in water with a pH of 2.4 (Le Roux, 1969). The acid content of these waters is not the sole reason for concern. Another is the formation of extensive ferric iron precipitates in the water, as a result of the activity

of *T. ferrooxidans*. One of the most common precipitates is "yellow-boy", or jarosite, which is formed microbiologically as :-

$$11CuFeS_2 + 45.5O_2 + 11H_20 \rightarrow 11CuSO_4 + 5FeSO_4 + 2(H_3O)Fe_3(SO_4)_2(OH)_6 + 2H_2SO_4 \quad (8)$$

(Lundgren *et al.*, 1972).

1.4. Molecular microbiology and *T. ferrooxidans*.

The economic significance of *T. ferrooxidans* to the mining industry warrants a thorough understanding of all aspects of the physiology of the organism. In this respect, the molecular biological research of *T. ferrooxidans* is of crucial significance. Insight into the genes responsible for ATP-generation, and those regulating and governing the metabolism of the organism would contribute to the understanding of assimilatory and dissimilatory pathways in the organism. The mechanisms of tolerance to a wide range of heavy metal cations could also be revealed through a knowledge of any genes involved. A thorough knowledge of gene structure and regulation will assist towards attaining the long-term objective of recombinant DNA studies on *T. ferrooxidans* viz. the genetic manipulation of the organism to improve strain performance during bioleaching processes. Implicit in this is the development of genetic systems for *T. ferrooxidans*.

A survey of relevant journals will reveal that molecular biology has dominated microbiological research over the past 15 years. As a result, molecular mechanisms of many micro-organisms are well understood, and their subsequent genetic manipulation was successfully achieved. However, although recombinant DNA technology has progressed over the past ten years in *T. ferrooxidans*, the process has been slow when compared to a genetically well-characterised organism such as *Escherichia coli*. The principal reasons for this are to be found with *T. ferrooxidans* itself, which as an experimental system has few redeeming features. As a result, few laboratories are willing to undertake genetic research, using *T. ferrooxidans* as the test organism.

Growth rates associated with *T. ferrooxidans* are notoriously slow, with the organism having an average generation time of 6-7 h in ferrous iron-containing batch cultures. Furthermore, *T. ferrooxidans* oxidises up to 30 times its own weight in ferrous iron/h, and growth yields are low (Kelly, 1978, cited by Rawlings *et al.*, 1991). Due to the inhibitory effects of organic substances, *T. ferrooxidans* is difficult to culture on growth media solidified with agar, and minute colonies take up to seven days to develop. As a result, most *T. ferrooxidans* cultures are grown in ferrous liquid 9K medium (Silverman and Lundgren, 1959). As growth yields are only between 11-33 mg/l, this necessitates the preparation of large volumes of costly and corrosive medium (Ingledew, 1982). A further problem encountered during the growth of the organism, is the precipitation of ferric hydroxides in both liquid and solid media. As noted by Apel *et al.* (1980) these

precipitates impede the centrifugal recovery of cells, as the ferric salts co-sediment with the cells. Extensive washing of harvested cells with water acidified with sulphuric acid is required to ensure all traces of both precipitated and non-precipitated iron are removed, prior to further experimental procedures, that would otherwise be hampered by the presence of excessive amounts of iron.

Efficient procedures are available for the recovery of DNA from *T. ferrooxidans*, although yields of the nucleic acid are low. However, research groups have constructed *T. ferrooxidans* genomic libraries which are commonly used for conventional molecular techniques, and have contributed substantially to knowledge on gene structure of the organism. For example, these libraries made possible the isolation of *T. ferrooxidans* genes by using DNA probes which are homologous to highly conserved regions in the gene of interest. Probes used are constructed from the genomic or plasmid DNA of heterologous organisms which possess the gene. Screening of *T. ferrooxidans* gene banks using such DNA probes has resulted in the isolation of the *T. ferrooxidans* genes associated with nitrogen fixation (Rawlings *et al.*, 1991), carbon dioxide fixation (Kusano *et al.*, 1991a; 1991b and 1993a; Shively *et al.*, 1989), mercury resistance (Shiratori *et al.*, 1989), rRNA (Salazar *et al.*, 1989) and tRNA (Venegas and Sanchez, 1988).

Of significance to this dissertation, are firstly, that no *T. ferrooxidans* mutants which would allow the identification of cloned *T. ferrooxidans* genes by expression have been characterised. Secondly, there is no readily usable genetic system for *T. ferrooxidans*. This necessitates the use of a heterologous host, typically *E. coli*, as an alternative genetic system. The impact that this has had on molecular biological studies of *T. ferrooxidans*, and the implications for this study, are discussed in Chapter 2 (Sections 2.1 and 2.4) and in Chapter 5 (Section 5.4).

There has been progress in the field of protein biochemistry in *T. ferrooxidans*, particularly in the isolation of periplasmic and cytoplasmic-located proteins. Proteins involved in both iron oxidation and electron transport were well characterised (Ingledew, 1982 and 1986; Ronk *et al.*, 1991; Yamanaka *et al.*, 1991). The extraction and purification of proteins involved in iron and sulphur metabolism has made possible the use of the technique of "reverse genetics" in *T. ferrooxidans* (Rawlings *et al.*, 1991). This approach is used when neither appropriate *E. coli* mutants, nor homologous DNA from a heterologous organism for probe construction, is available. Reverse genetics initially involves *in vivo* synthesis of a protein of interest. The amino acid sequence of the polypeptide is partially determined. Using this sequence, and incorporating codon preference data for the organism concerned, degenerate oligonucleotide probes are then constructed. In the case of *T. ferrooxidans*, these probes are used to screen a gene bank of the organism, and the gene of interest is identified. In this manner, the *T. ferrooxidans iro* gene, encoding the Fe(II) oxidase protein

was cloned (Kusano *et al.*, 1992b). Fortuitously, the *purA* gene was cloned simultaneously in the same study (Kusano *et al.*, 1993b).

Much progress has been made in the field of molecular biology, using RNA-based techniques. The availability of mRNA from organisms under study has yielded significant advances. Difficulties associated with the physiology of *T. ferrooxidans* have resulted in few successes being reported for the isolation of mRNA from the species (eg. see Drolet and Lau, 1992). However, Inoue *et al.* (1991) and Kusano *et al.* (1991b, 1992b and 1993b) reported extraction of mRNA from *T. ferrooxidans* strains E-15 and Fe-1, which was used for Northern hybridisation and primer extension.

The development of polymerase-chain-reaction (PCR)-based technologies for the furthering the progress in the field of the molecular biology of *T. ferrooxidans* would be of great benefit. As yet, little has been published on the use of PCR with *T. ferrooxidans*. This technique offers the possibility of cloning *T. ferrooxidans* genes, such as those associated with cytoplasmic membrane proteins, without the use of an intermediary host. Holden and Brown (1991) reported cloning the RuBisCO LSU gene fragments from *T. ferrooxidans*, using PCR. Kusano *et al.* (1993a) utilised PCR-amplified fragments of *T. ferrooxidans* Fe1 chromosomal DNA, which were then used for gel mobility shift and DNA-footprinting assays, incorporating regulation studies of the *rbc* operon.

1.5. Oxidative phosphorylation in *T. ferrooxidans*.

Metabolic pathways implicated in both ATP generation and utilisation in *T. ferrooxidans* were reviewed above. In common with any other living organism, *T. ferrooxidans* must be able to generate ATP and reducing power for its anabolic processes. As an obligate chemoautotroph, respiratory metabolism in *T. ferrooxidans* is restricted to the oxidation of inorganic compounds, where a major function of substrate oxidation is to provide ATP by oxidative phosphorylation, and to provide reducing power.

During oxidative phosphorylation, ATP is generated by cytoplasmic membrane-associated electron-transport processes. The mechanism by which ATP is generated as a result of the passage of electrons through a transport chain is unknown, but at least four hypotheses were proposed, of which the two most relevant will be described. These are the chemical coupling process, and the chemiosmosis hypothesis. The former hypothesis proposed that when a pair of electrons is passed from one carrier to the next, a third component generates a high-energy link. This high energy link forms a precursor for the high energy bond in ATP. Although adequate in some respects, the chemical coupling hypothesis has two fundamental weaknesses. To date, no third component has been identified, and the theory does not explain why phosphorylation occurs only if the cytoplasmic membrane is

fairly intact (reviewed in Mahler and Cordes, 1966). The chemiosmotic hypothesis of Mitchell (1961) is now the far more widely accepted model. It accounts for the presence of an intact membrane, and does not require the presence of a third high-energy component.

1.5.1. The chemiosmotic theory (Mitchell, 1961 and 1966).

According to the theory, oxidative phosphorylation and photophosphorylation in bacteria, mitochondria and chloroplasts are associated with an electron transport chain orientated in the cytoplasmic membrane, such that the oxidation of the electron carriers is accompanied by the stoichiometric translocation of protons across the membrane. The cytoplasmic membrane is largely impermeable to protons and hydroxyl ions, and has low electrical conductivity. As a result of the membrane properties, the translocation of protons across the membrane and the movement of electrons down the electron transport chain, two gradients are established viz. a transmembrane proton gradient (ΔpH) and a transmembrane electrical charge gradient ($\Delta\Psi$). Internally therefore, all bacteria are alkaline and most (acidophiles excluded) are electrically negative with respect to the suspending medium. Together, both ΔpH and $\Delta\Psi$ represent an energy source, known as the proton motive force ($\Delta\mu_{H+}$). The energy associated with $\Delta\mu_{H+}$ drives a number of membrane-associated processes. These include solute transport systems, bacterial motility, transhydrogenase activity, reverse electron transport, and of particular relevance to this study, ATP synthesis by a proton-translocating ATPsynthase (Cobley and Cox, 1983; Krulwich and Guffanti, 1983). The chemiosmotic theory is illustrated in the figure below.

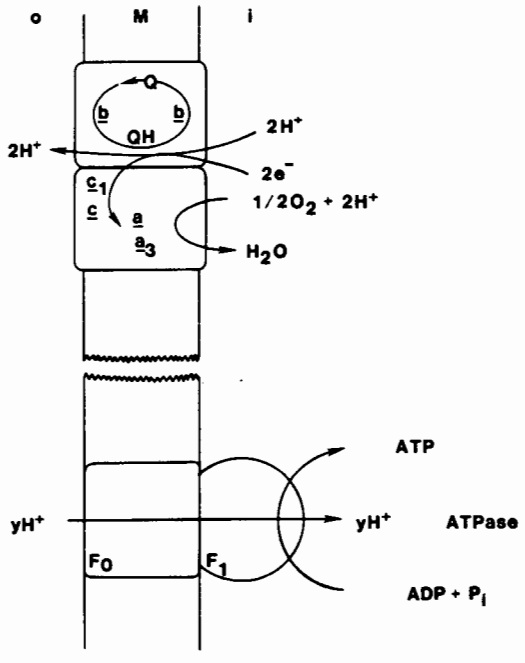


Fig. 1.3. Scheme for chemiosmotic coupling of a proton electrochemical gradient generated by a proton motive cytochrome system to the phosphorylation of ADP in heterotrophic prokaryotes. M refers to the cytoplasmic membrane, and o and i refer to the inner and outer bulk phases respectively (after Cobley and Cox, 1983).

The $\Delta\mu_{H+}$ is calculated from the following formula:-

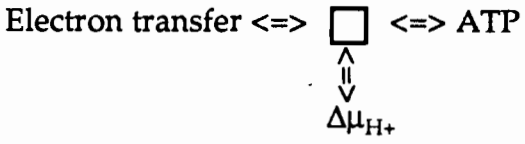
$\Delta\mu_{H+}/F = \Delta\Psi - Z\Delta pH$ (in mV).

F = Faraday constant (96,903 $J \cdot V^{-1} \cdot equivalent^{-1}$)

Z = 2,303RT/F R = gas constant (8.35 $J \cdot mol^{-1} \cdot K^{-1}$)

T = absolute temperature (K) (after Bakker, 1990).

It should be stressed that the manner in which $\Delta\mu_{H+}$ is coupled to ATP synthesis is not understood, and there are those involved in bioenergetic research who are of the opinion that $\Delta\mu_{H+}$ is not an obligatory intermediate in electron-transfer-linked phosphorylation, and have abandoned the chemiosmotic hypothesis. Alternative proposals can be summarised by the scheme:-



The nature of the closed box is controversial and at present, is described by three hypotheses. These are localised proton circuits within the membrane, localised $\Delta\mu_{H+}$, and energised protein conformations of the "collision"/fluid membrane hypothesis (reviewed in Slater, 1987).

It is also notable that Mitchell's concept of energy transduction by proticity has evolved to recognise the integral role of other ion transport processes, such as those for potassium and sodium, in the formation and utilisation of ion gradients for energetic processes. Some of these, as they relate to acidophilic bacteria, are briefly discussed in Section 1.5.3.

This thesis will present the view, that whilst the generation of $\Delta\mu_{H+}$ by chemiosmotic mechanism is significant, so too are changes in conformation of proteins, which indirectly couple $\Delta\mu_{H+}$ to oxidative phosphorylation (Boyer, 1993) (Section 1.6).

1.5.2. Chemiosmotic coupling in *T. ferrooxidans.*

Prior to discussing chemiosmotic mechanisms proposed for *T. ferrooxidans*, the energy production and demands associated with its mode of life will be briefly reviewed. As discussed above (Section 1.2) the preferred source of energy is the aerobic oxidation of either ferrous iron or reduced sulphur compounds at pH_o 2.8 (Cobley and Cox, 1983). The oxidation of sulphur compounds is energetically more favourable (Ingledew, 1982) but is less well studied than the oxidation of ferrous iron by the organism. Indeed, it is the fact that the biological oxidation of ferrous iron is so energetically unfavourable, that has probably resulted in bioenergetic research interest being centered almost solely on the Fe^{2+}/Fe^{3+} couple in *T. ferrooxidans*. This latter couple represents one of the narrowest thermodynamic limits at which life is known to occur. The two half reactions are:-

$$2Fe^{2+} \longrightarrow 2Fe^{3+} + 2e^- \quad (9)$$

$$2e^- + 0.5O_2 + 2H^+ \longrightarrow H_2O \quad (10)$$

$$\text{Sum: } 2Fe^{2+} + 0.5O_2 + 2H^+ \longrightarrow 2Fe^{3+} + H_2O. \quad (11)$$

The amount of free energy produced from the two half reactions depends on their location relative to the *T. ferrooxidans* cell. It is now widely accepted that the Fe^{2+}/Fe^{3+} couple occurs external to the cytoplasmic membrane, in the periplasm (Bodo and Lundgren, 1974). However, the site of the O_2/water couple is still debatable. Ingledew (1982 and 1986) contended that the latter couple occurs at the inner surface of the cytoplasmic membrane (pH 6.5) and as such the ΔG for Fe^{2+} oxidation is -2.3 kcal/2 moles Fe^{2+}. Others suggested that the O_2/water couple occurs in the periplasm of the cell at pH 2. This would yield a far higher amount of free energy, with ΔG equal to -16 kcal/two moles Fe^{2+} (Yamanaka *et al.*, 1991). The site of oxygen reduction will be discussed further in Section 1.5.2.4.

The anabolic demands of the organism are extreme. Energy demands for carbon dioxide fixation are high. Assuming that two moles Fe^{2+} are required to generate sufficient redox power per mole ATP, and knowing that the fixation of a mole of CO_2 via the Calvin cycle requires three moles ATP and two moles NADPH, implies the oxidation of 22.4 moles Fe^{2+} by the organism (Ingledew, 1982). Oxygen demands are therefore extreme (Equation 11). The provision of NADPH in *T. ferrooxidans* cultured in ferrous-based media is problematic. Thermodynamically it is impossible for the Fe^{2+}/Fe^{3+} couple to directly reduce $NADP^+$. Hence the organism has to conserve energy from ferrous oxidation, either as ATP, or as $\Delta\mu_{H+}$ to permit $NADP^+$ reduction by reverse electron transport (Ingledew, 1982). Under conditions where *T. ferrooxidans* fixes nitrogen, 28 moles ATP per mole N_2 are required (Postgate, 1982).

It is also possible that maintenance of ΔpH and/or $\Delta\Psi$ in *T. ferrooxidans* both require energy, particularly if protons are extruded from the cell, against the transmembrane proton gradient, as is the case in many acidophilic bacteria (Booth, 1985; Bakker, 1990).

Considering both the energy demands of the organism, and the acidic environment in which it lives, it is likely that *T. ferrooxidans* has evolved unique strategies to conserve available energy. In this context therefore, it is possible that strategies for $\Delta\mu_{H+}$ generation and associated oxidative phosphorylation in an obligate acidophilic chemoautotroph are unusual, and may possibly even differ from those utilised by heterotrophic obligate acidophiles (Cobley and Cox, 1983).

In order for oxidative phosphorylation to occur by chemiosmosis in *T. ferrooxidans*, the organism must possess the following:-

i). a respiratory electron transport chain

ii). proton-translocating ATP synthase

iii). an intact, largely proton-impermeable cytoplasmic membrane.

1.5.2.1. The respiratory electron transport chain. The respiratory chain in *T. ferrooxidans* consists of two parts, a minor and a major section. The minor section, through which 10% of the electrons pass, is not thought to be involved in the generation of $\Delta\mu_{H+}$. Instead, it utilises energy available from either $\Delta\mu_{H+}$ and/or ATP to drive reverse electron transport to reduce the pyridine nucleotide pool (Ingledew, 1982). The major section, through which 90% of electrons pass, is that which is responsible for the passage of electrons from ferrous iron to oxygen, and is the section of relevance to this review. It couples respiratory electron transport to ATP synthesis by oxidative phosphorylation (Ingledew, 1982).

The qualitative make-up of the *T. ferrooxidans* respiratory chain is not unusual. It consists of cytochromes, quinones and iron-sulphur and copper centres. However, in keeping with the high oxygen demand of the organism, the quantitative amounts are most unusual, and represent a manner in which a lithotrophic organism has adapted to meet anabolic demands. The amounts of cytochrome and rusticyanin in *T. ferrooxidans* account for 5-10% of the total cell protein (Ingledew, 1982). Two possible topographical models for the constituents of the major portion of the chain were proposed, and will be discussed below in Section 1.5.2.4. (Ingledew, 1982 and 1986; Yamanaka *et al.*, 1991).

1.5.2.2. Proton-translocating ATPsynthase. Experimental data accumulated by Apel *et al* (1980) suggested the presence of a proton-translocating ATPsynthase in *T. ferrooxidans*. In *T. ferrooxidans* vesicles, ATP generation occurred when vesicles were loaded with ADP+P_i and subjected to artificially imposed proton gradients similar to those of the cell's normal environment. The optimum internal pH (pH_i) was 7.0-7.8, and that for pH_o was 2.8. Oxidative phosphorylation was indicated by the fact that the uncouplers 2,4 dinitrophenol (DNP) and pentachlorophenol inhibited ATP synthesis in the vesicles by dissipating the proton gradient. Nigericin, which dissipated the proton gradient by introducing the electrically neutral exchange of potassium ions and protons, similarly inhibited ATP synthesis. As is typical for oxidative phosphorylation, valinomycin addition to the vesicles enhanced the process by potassium ion efflux balancing proton influx. The enzyme activity was consistent with that of a proton-translocating ATPsynthase in that it was strongly magnesium-dependent, and was inhibited by *N,N'*-dicyclohexylcarbodiimide (DCCD).

1.5.2.3. Proton movement across the cytoplasmic membrane. Apart from establishing the fact that protons enter the *T. ferrooxidans* cytoplasmic membrane via a proton-translocating ATPsynthase, little is known about the movement of protons across the cytoplasmic membrane in the organism. Both Ingledew (1982) and Cobley and Cox (1983) referred to the fact that it is not possible to use a pH electrode to measure the extrusion of protons across the cytoplasmic membrane in acidophilic organisms where the pH_o is 2-3, as the number of protons translocated outwards would be insufficient to increase the already high external concentration of protons significantly.

Work by Apel and Dugan (1978) indicated that protons move into and out of the *T. ferrooxidans* cell. When ferrous iron was added to a culture of *T. ferrooxidans* at a pH_o 2.4, the external pH increased. The authors ascribed this to an initial influx of protons that correlated directly with the concentration of ferrous ions in the medium. After 20 min, the pH_o decreased, which implied proton efflux. The authors proposed that during the oxidation of Fe^{2+} to Fe^{3+} by *T. ferrooxidans*, an electron is transported into the cell, and that to maintain a balance of electrical charge, a proton from the environment moves into the cell, where it is utilised for oxygen reduction. It was suggested that protons are an essential nutrient for the cell (Apel and Dugan, 1978). However, it is equally possible that the movement of protons reflected a $\Delta\Psi$ initially poised inside negative, and the influx of protons occurred until the $\Delta\Psi$ was inverted by the organism and poised correctly i.e. inside positive to outside (Section 1.5.3).

In keeping with the requirements of chemiosomosis, it is evident that proton movement across the cytoplasmic membrane in *T. ferrooxidans* is carefully controlled. This is evident from the fact that pH_i of the organism is maintained at 6.5 when pH_o values vary between 1 and 8 (Cox *et al.*, 1979) (Table 1.1).

1.5.2.4. Chemiosmotic models described. Models for the generation of $\Delta\mu_{H+}$ in *T. ferrooxidans* grown on ferrous iron, sulphur and sulphite were described by Ingledew (1982) and Cobley and Cox (1983). Although thermodynamically sound, the authors pointed out that there are no experimental data to support the models, other than some which substantiate the topography of the components of the respiratory chain.

In the model illustrated (Fig. 1.4) the first step of ferrous oxidation is catalysed by Fe(II) oxidase coupled to ferrocytochrome c_{552} reduction at pH_o 2. This enzyme is iron oxidase (Iro) and is located in the periplasm. In the second step, ferrocytochrome c_{552} transfers electrons to a cytochrome oxidase complex, situated deep in the cytoplasmic membrane. It is thought that rusticyanin, a blue copper protein, also participates in the ferrous oxidation pathway, as it is reduced during ferrous oxidation. At the inner surface of the membrane, the oxidase complex catalyses the reduction of oxygen to water. The nature of the oxidase

complex is debatable. Ingledew (1982 and 1986) reported that the complex was typical of cytochrome a_1. Kai *et al.* (1989) reported that the oxidase of *T. ferrooxidans* had properties more like that of cytochrome aa_3 than the a_1-type. Further work is in progress to characterise the precise nature of the complex. The protons required for the reduction of molecular oxygen enter the cell via a proton-translocating ATPsynthase and catalyse the production of ATP.

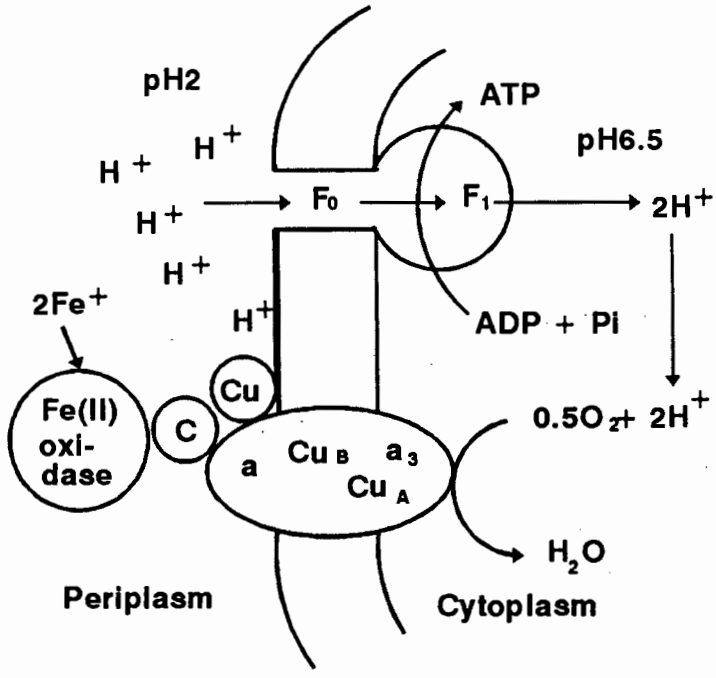


Fig. 1.4. The chemiosmotic model for *T. ferrooxidans*, proposed by Ingledew (1982) and modified by Yamanaka *et al.* (1991). Fe^{2+} oxidase denotes Fe(II)-cytochrome *c*-552 (Iro); *c*, soluble cytochrome *c*-552; Cu, rusticyanin. *a*, a_3, Cu_A and Cu_B are components of cytochrome oxidase. F_1 and F_0 represent F_1F_0 ATPsynthase.

This model does not provide for the extrusion of protons from the cell. Although protons are not translocated *per se* by the cell, $\Delta\mu_{H+}$ is generated since protons are consumed in the cytoplasm and the charge is separated, as the two half reactions occur on either side of the cytoplasmic membrane. The model accounts for protons which may accidentally leak into the cytoplasm, where they are scavenged by the cytochrome oxidase (Ingledew, 1982).

The extreme feature of the above model is the fact that the inorganic oxidisable substrate (ferrous iron) is oxidised outside the cytoplasmic membrane, in the periplasm. Overall, $\Delta\mu_{H+}$ in this model is postulated to be actively maintained by internal proton consumption, as opposed to proton extrusion (Ingledew, 1982). This is different from the heterotrophic acidophiles, where an organic substrate is oxidised internally. Therefore unlike the lithotrophic *T. ferrooxidans*, heterotrophic acidophiles have to generate a transmembrane electrochemical gradient. This is done by the active extrusion of protons

from the cell. It is a crucial difference, and will influence chemiosmotic dynamics in both types of organisms (Cobley and Cox, 1982) (Section 1.5.3).

The work on *T. ferrooxidans* by Apel and Dugan (1978) cited in Section 1.5.2.3 suggested that protons may be extruded across the cytoplasmic membrane. Ingledew (1982) acknowledged that there is a possibility in *T. ferrooxidans*, that both half reactions of the Fe^{2+}/Fe^{3+} and O_2/water couples may occur on the outside of the cell membrane. If this were to occur, the organism would have to generate a transmembrane electrical gradient by active proton extrusion against the proton gradient. The nature of the proton pump is speculative. Cytochromes are unable to transport protons; they transfer electrons only (Schlegel, 1988). Ingledew (1982) suggested that the cytochrome oxidase complex could translocate the two protons across the cytoplasmic membrane during electron transfer.

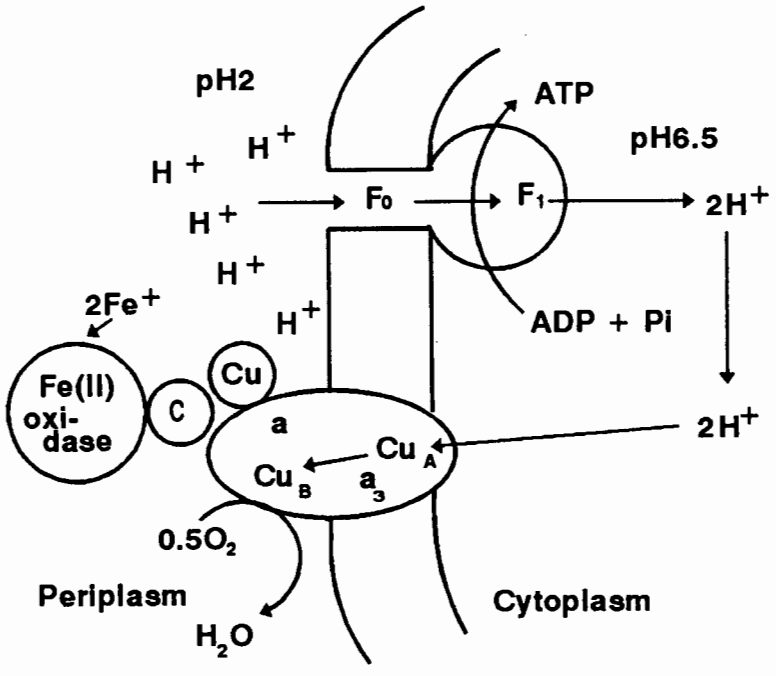


Fig. 1.5. A chemiosmotic model for *T. ferrooxidans*, with both half-reactions occuring outside the cytoplasmic membrane (after Yamanaka *et al.*, 1991). Fe^{2+} oxidase denotes Fe(II)-cytochrome *c*-552 (Iro); *c*, soluble cytochrome *c*-552; Cu, rusticyanin. *a*, a_3, Cu_A and Cu_B are components of cytochrome oxidase. F_1 and F_0 represent F_1F_0 ATPsynthase.

There is experimental evidence supportive of both half-reactions occurring external to the cytoplasmic membrane. Recent work on the constituents of the respiratory transport chain of *T. ferrooxidans* in the laboratories of Yamanaka and Sato demonstrated that the optimum pH for the oxidation of ferrocytochrome *c*-552 by the oxidase complex was 3.5 (Kai *et al.*, 1989). This lead to the proposal that the oxidase complex was located in the periplasm, and not deep within the cytoplasmic membrane. The authors suggested that

oxygen might be reduced in the periplasm and proposed the model depicted in Fig. 1.5 (Yamanaka *et al.*, 1991). It must be stressed however, that the most rapid reduction of oxygen by the oxidase complex measured by these authors was at pH 6, and the significance of this remains to be clarified (Kai *et al.*, 1989). Such pH optima could indicate that whilst the oxidation of the ferrocytochrome *c* by the oxidase complex could occur at the outer face of the cytoplasmic membrane, the reduction of oxygen may be catalysed by the oxidase at the inner surface, adjacent to the cytosol. As such, this would be supportive of the model shown in Fig. 1.4.

A criticism of the model as shown in Fig. 1.4 proposed by Ingledew (1982) was that thermodynamically, it is unsound for the generation of a favourable $\Delta\mu_{H+}$ (Cox and Brand, 1984, cited by Ingledew, 1986). The free energy yield for Fe^{2+} oxidation is far higher when both half reactions occur outside the cell, at pH 2 (Section 1.5.2). Hence, the model depicted in Fig. 1.5 would appear to be thermodynamically more favourable for the cell. To accept the latter model would require a revision of the mechanism of ATP formation in *T. ferrooxidans*. However, Ingledew (1982 and 1986) proposed that the small driving force available from the two separated half-cells of the ferrous oxidation depicted in Fig. 1.4 is not the sole force for the generation of $\Delta\mu_{H+}$ in the organism. Instead, the driving force for $\Delta\mu_{H+}$ is the ΔE between the two couples in the bulk phase. The difference between the ΔE in the bulk phase and the ΔE for the electron transfer reaction would be stored directly as transmembrane chemical potential of protons, or ΔpH. This means that in the electron transport scheme depicted in Fig. 1.4, work is already being done against the pH difference (Ingledew, 1986). Hence thermodynamically, the separation of the oxidation of Fe^{2+} by the coupling cytoplasmic membrane is favourable, and ATP synthesis can occur.

Of importance to the chemiosmotic models proposed, is the number of protons entering the cell. Experimentally determined values obtained for the $\Delta\mu_{H+}$ in *T. ferrooxidans* suggested that there is a possibility that more than one proton is translocated per Fe^{2+} oxidised. A tenuous figure of two protons per Fe^{2+} oxidised was proposed (Ingledew, 1982). If this is the case, the above models could be accommodated to incorporate proton extrusion. In the case of Fig. 1.4, the translocation of additional protons can be superimposed from the inside to the outside, whilst in Fig. 1.5, it is achieved by increasing the number of protons translocated across the cytoplasmic membrane (Ingledew, 1982).

The role of the proton-translocating ATPsynthase in the above models is of significance as it is implicated in the flow of protons into the cell. In most organisms, a classic ratio of two protons per mole ATP synthesised during coupled respiratory chain transport is hypothesised. This is a direct result of the initial oxidation of NADPH+H (Schlegel, 1989). Ingledew (1982) postulated that in *T. ferrooxidans*, two protons are similarly required to generate one mole of ATP, and that a stoichiometric balance is obtained by neutralising

the charge of two electrons from $2Fe^{2+}$ oxidation. It has been suggested that the production of a mole of ATP by proton-translocating ATPsynthase in *E. coli*, requires three protons (Maloney, 1987, cited by Fillingame, 1990) (Section 1.6.3). Should the ATPsynthase of *T. ferrooxidans* be similar to that of *E. coli*, and should the enzyme be capable of functioning in the latter organism, then it is not inconceivable that a similar stoichiometry of three protons per mole ATP could apply in *T. ferrooxidans*. This would therefore indicate that to balance the number of protons entering the *T. ferrooxidans* cell per mole ATP synthesised, three moles Fe^{2+} are oxidised. This increase could be stoichiometrically accommodated by the above models.

Experimental data are needed to assist in determining whether *T. ferrooxidans* is able to extrude protons from the cell or not. It is of particular relevance to record that recent reviewers are of the opinion that the ΔpH in aerobic acidophilic bacteria is almost wholly dependent on the extrusion of protons across a proton-impermeable membrane (Booth, 1985; Bakker, 1990). Most interesting, and of relevance to *T. ferrooxidans*, is the recent finding that in the iron-oxidising archaebacterium *Sulfolobus acidocaldarius*, protons were extruded during electron transport against the proton gradient, and that efficiency of the extrusion was dependent on potassium ion concentration (Moll and Schafer, 1988). The nature of the proton pump is still unknown, but a cytochrome type aa_3 was identified (Anemuller and Schafer, 1989). Furthermore, *S. acidocaldarius* is known to possess a proton-translocating ATPsynthase, which has similarities to both V-type and eubacterial F-type ATPsynthases (Schafer and Meyering-Vos, 1992). However, in the studies cited, *S. acidocaldarius* was grown on heterotrophic medium.

It is also of interest to note that during active metabolism by *T. acidophilus*, protons are extruded from both cells and sphaeroplasts (Matin and Matin, 1982). *T. acidophilus* was isolated from a culture of *T. ferrooxidans*, and is capable of both chemoautotrophic and heterotrophic growth (Guay and Silver, 1975).

All the acidophilic bacterial species reviewed by Booth (1985) and Bakker (1990) were heterotrophically grown, where proton extrusion for $\Delta\mu_{H+}$ generation is obligatory. The chemiosmotic models proposed for ferrous iron-grown *T. ferrooxidans* were not discussed by either reviewer. It would be of interest to obtain data on generation and maintenance of $\Delta\mu_{H+}$ in chemoautotrophically grown *T. acidophilus* and *S. acidocaldarius*. As Cobley and Cox (1983) pointed out, it may be that the mechanisms of chemiosmosis differ between heterotrophic and chemoautotrophic acidophiles, with the latter not extruding protons, which may represent an energy-saving for these organisms. As will be discussed below, the $\Delta\mu_{H+}$ in ferrous iron-grown *T. ferrooxidans* is substantially larger than that reported for any heterotrophic acidophile, which could indicate lack of active proton extrusion during electron transport in *T. ferrooxidans* (Table 1.1). In fact, the inability to extrude protons

may be linked to obligate chemoautotrophy in acidophilic lithotrophs (Cobley and Cox, 1983).

No serious alternatives for the chemiosmotic models proposed by Ingledew (1982) for ferrous iron-grown *T. ferrooxidans* have been suggested. Until proved otherwise, it may be assumed that in *T. ferrooxidans*, chemiosmotic principles operate by respiration-driven proton consumption, and not by proton extrusion across the cytoplasmic membrane.

Very little work has been done on the bioenergetics of sulphur-grown *T. ferrooxidans*. Cobley and Cox (1983) suggested that $\Delta\mu_{H+}$ in acidic thiobacilli utilising inorganic sulphur compounds could be generated as described in Fig. 1.6. As in the ferrous iron-grown cells, the two half reactions are separated by the cytoplasmic membrane. Particularly relevant is the fact that all protons generated by the oxidation of the sulphur compounds are released outside the cell. Hence, as is the case in ferrous oxidation, the electrochemical gradient is maintained without having to resort to proton extrusion. However, this represents a loss of potential reducing power by the cell, which could be used to reduce the pyridine nucleotides. Hence these cells would then still have to utilise reverse electron transport to generate a supply of NADPH. In this early model, no provision was made for the inclusion of the SFORase $Fe^{3+}/Mo^{6+}/Cu^{2+}$-reducing system proposed for the aerobic oxidation of sulphur by *T. ferrooxidans* (Sugio *et al.*, 1985, 1988b and 1990b) (Section 1.2).

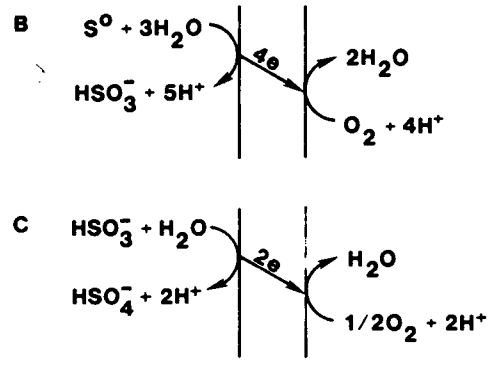


Fig. 1.6. Mechanism for the generation of proton electrochemical gradients by sulphur and sulphite oxidation in *T. ferrooxidans*, as proposed by Cobley and Cox (1983) for *T. thiooxidans*.

1.5.3. The magnitude and maintenance of the proton-motive force in *T. ferrooxidans*.

The ability of *T. ferrooxidans* to generate and maintain $\Delta\mu_{H+}$ is relevant for a number of reasons. Firstly, the implications for the established chemiosmotic models proposed by Ingledew (1982) where $\Delta\mu_{H+}$ is proposed to be actively maintained by internal proton consumption (Section 1.5.2.4). Secondly, the generation of ATP by oxidative phophorylation at neutral pH_i is dependent on the existence of the $\Delta\mu_{H+}$ composed of ΔpH

and $\Delta\Psi$ of the correct magnitude (Ingledew, 1982). Thirdly, it is possible that a proton-translocating ATPsynthase may be implicated in determining the magnitude of the $\Delta\mu_{H+}$. Experimental data relating to the generation and maintenance of ΔpH and $\Delta\Psi$ in *T. ferrooxidans* will therefore be discussed. Where relevant, similar data from a range of other acidophilic bacteria will also be included.

Methods used to estimate $\Delta\mu_{H+}$ in acidophilic bacteria are open to criticism. Most reviewers are of the opinion that available methods yield at best crude results (Cobley and Cox, 1983; Booth, 1985; Bakker, 1990). Artefacts are notorious, and as a result, reviewers suggested that in acidophilic bacteria, published results pertaining to $\Delta\mu_{H+}$ and its components should not be unequivocally accepted.

Ideally, measurement of ΔpH and $\Delta\Psi$ in bacteria requires the use of micro-electrodes, but the small size of prokaryotic cells precludes this. This has resulted in the use of the "second best method" (Bakker, 1990) which involves measuring ΔpH and $\Delta\Psi$ with radioactively-labelled probes. The distribution of the latter across the cytoplasmic membrane is dependent on ΔpH and $\Delta\Psi$.

The probes used are not without considerable problems themselves. In certain studies with acidophiles, probes are used below their pK_a values. When this happens, it becomes impossible to measure ΔpH accurately, particularly when ΔpH is small (Booth, 1985). Certain probes routinely used for determinations of $\Delta\mu_{H+}$ in acidophiles, such as acetylsalicylic acid, bind to the cell constituents and hence subsequent corrections for probe binding must be made. This can introduce errors. Furthermore, it is known that *E. coli* actually has a transport system for this probe, and it remains to be seen whether the same system operates in acidophiles (Booth, 1985).

Measurements of $\Delta\mu_{H+}$ using flow dialysis to measure $\Delta\mu_{H+}$ are also suseptible to error. If cell bulk in the flow chamber is too large, anaerobiosis can result, which will give incorrect results. It is critical that in measuring $\Delta\mu_{H+}$ in aerobic acidophiles, that aerobic conditions are maintained throughout incubation periods (Cobley and Cox, 1983).

Errors in estimating cell volume, cell mass and quench correction in liquid scintillation counting have all been cited as being responsible for introducing inaccuracies in the assessment of $\Delta\mu_{H+}$ in acidophiles (Booth, 1985). It has also been noted that the choice of suspending buffer in determining $\Delta\mu_{H+}$ is critical. The presence of permeant ions in a buffer exerts great influence on the magnitude of $\Delta\mu_{H+}$ (eg. see Goulbourne *et al.*, 1986 and Michels and Bakker, 1985).

Table 1.1. Experimental data obtained for the value of $\Delta\mu H^+$ in acidophilic bacteria.

Species	pH_0	pH_i	$-\Delta pH$	$\Delta\psi$(mV)	$\Delta\mu H^+$(mV)
T. ferro-	1	6-7	>5	-70	270
oxidans [a,b]	2	6-7	4.5	0/-10	256
	3	6-7	3.3	50	240
	8	6-7	>1.0	170	0
T. acido-	2	5.6	3.5	-105	110
philus [c]	3	5.6	2.6	-73	83*
	4	5.8	1.8	-46	68
PW2 [d]	3	5.9	2.7	-90	75
	4	5.9	1.9	0	114*
B.acido-	3.5	5.9	2.4	-36	117*
caldarius [e]					
T. acido-	2.0	6.3	4.5	-120	176
philum [f]					

[a] - Ingledew, (1982) [b] - Cobley and Cox, (1983)

[c] - Matin *et al.* (1982) [d] - Goulbourne *et al.* (1986)

[e] - Krulwich *et al.* (1978) [f] - Hsung and Haug (1975 & 1977)

* - Bakker, (1990): Alternate values: *T. acidophilus* $\Delta\mu H^+$, 119mV

PW2 $\Delta\mu H^+$, 154mV

Michels and Bakker, (1985): *B. acidocaldarius* $\Delta\mu H^+$, 200mV

It is not within the ambit of this review to give a detailed analysis of results obtained by various groups working on establishing the kinetics of $\Delta\mu_{H+}$ in acidophiles. There is considerable controversy in the literature as to accuracy of reported values. Booth (1985) and Bakker (1990) stated that the discrepancies observed in establishing ΔpH and $\Delta\Psi$ in

acidophiles are due to experimental error, rather than unusual physiologies of the organisms involved. Bakker (1990) detailed improved methods available for determining $\Delta\mu_{H+}$ in acidophiles. Problems relating to the measurement of $\Delta\mu_{H+}$ in bacteria are not restricted to acidophiles alone. The $\Delta\Psi$ of neutrophilic bacteria cannot yet be determined with great accuracy (Bakker, 1990).

Experimental data provided evidence for the existence of a large $\Delta\mu_{H+}$ in *T. ferrooxidans*. Some of the values reported are given in Table 1.1. Estimated values for other acidophiles are also included.

From Table 1.1 it is evident that at low pH_o, *T. ferrooxidans* generates a large $\Delta\mu_{H+}$, and as is typical for all acidophiles for which data are available, the bulk of the $\Delta\mu_{H+}$ is composed of a large ΔpH. The $\Delta\Psi$ is small, and below pH_o 3, is poised at inside positive to outside. The magnitude of ΔpH in *T. ferrooxidans* enables the organism to utilise the favourable chemical potential of protons thus generated (Ingledew, 1982). The principal function of the $\Delta\Psi$ poised inside positive is to enable acidophilic bacteria to generate and maintain the large ΔpH (Garland, 1977, cited by Booth, 1985). Two other trends are evident in the table. Firstly that the values of ΔpH and $\Delta\Psi$ are dependent on pH_o, and secondly that pH_i remains constant over a wide range of pH_o in *T. ferrooxidans*. Therefore the ability to interconvert ΔpH and $\Delta\Psi$ is important not only for influencing magnitude of $\Delta\mu_{H+}$ but also for pH homeostasis (Bakker, 1990). It was suggested that the ability of bacteria to interconvert $\Delta\Psi$ and ΔpH maybe of advantage in adaptation of bacteria to different environments (Futai and Kanazawa, 1983). As mechanisms of ΔpH and $\Delta\Psi$ interconversion could influence energy coupling in *T. ferrooxidans*, they will be briefly reviewed below.

It should be initially pointed out that the mechanisms of generation and maintenance of the components of $\Delta\mu_{H+}$ are poorly understood in acidophiles.

There is considerable controversy in the literature as to whether ΔpH is actively or passively maintained and the issue remains unresolved. The observation that energetically compromised/non-respiring cells of *T. ferrooxidans* (Beck, 1960, cited by Krulwich and Guffanti, 1983) *T. acidophilus* (Zychlinsky and Matin, 1983a) and the heterotrophic acidophile PW2 (Goulbourne *et al.*, 1986) all maintain a residual ΔpH in the presence of a collapsed $\Delta\mu_{H+}$ supported the suggestion that ΔpH is largely passively maintained in acidophiles (Cobley and Cox, 1983). In addition, it was observed that when treated with ionophores, ΔpH in many acidophiles, including *T. ferrooxidans* (Cobley and Cox, 1983) did not collapse, and pH_i did not decrease, although $\Delta\Psi$ became large and positive. This was interpreted to mean that acidophiles possess a membrane of unusual ionic impermeablity, and a cytosol of unusually high buffering capacity (Cobley and Cox,

1983; Ingledew, 1982; Matin *et al.*, 1982; Matin and Matin. 1982; Goulbourne *et al.*, 1986). Booth (1985) disagreed and claimed that the residual ΔpH remaining after treatment with ionophores is artefactual. Cobley and Cox (1983) and Bakker (1990) provided suitable alternative explanations for this phenomenon. Collapse of ΔpH was reported for *B. acidocaldarius* and *Thermoplasma acidophilum* after ionophore treatment (Krulwich *et al.*, 1978; Michels and Bakker, 1987). The cytoplasmic membrane of *B. acidocaldarius* is not unusually impermeable to protons and does not have an unusually high buffering capacity associated with the cytosol (Guffanti and Hou, 1987). *B. acidocaldarius* vesicles were shown to have proton-permeability and protonophore/ionophore sensitivity similar to non-acidophiles (Krulwich and Guffanti, 1983). If these properties apply to all acidophilic bacterial cytosol and cytoplasmic membranes, the observation that pH_i does not decrease after ionophore treatment would only be possible if proton influx was accompanied by either cation efflux or anion influx, and implies the existence of active antiporters/symporters that maintain ΔpH (Bakker, 1990; Booth, 1985; Krulwich and Guffanti, 1983).

Reaction of ΔΨ in acidophiles to ionophores is immediate. It becomes large and positive, and $\Delta\mu_{H+}$ is collapsed (Bakker, 1990; Cobley and Cox, 1983; Krulwich and Guffanti, 1983). Therefore ΔΨ is actively maintained and generated. The ΔΨ observed for acidophiles is of reversed polarity to that recorded for neutrophiles (Cobley and Cox, 1983). The mechanism for the generation of an inverted ΔΨ remains unknown. Three theories were proposed. Firstly, the Donnan potential theory (Hsung and Haug, 1977; Goulbourne *et al.*, 1986; McLaggan *et al.*, 1989; Zychlinsky and Matin, 1983b) which was questioned by Cobley and Cox (1983) and Krulwich and Guffanti (1983). Secondly, it was proposed that inversion of ΔΨ might be a result of influx of protons down their concentration gradient (Matin *et al.*, 1982; Goulbourne *et al.*, 1986). However, Booth (1985) suggested that continued uptake of protons would be self-defeating, in that it would abolish the established trans-membrane proton gradient. Furthermore, uptake of protons could not continue indefinitely with a ΔΨ, inside positive, unless it were charge-compensated by the efflux of cations from the cell, or the influx of anions (Booth, 1985). Bakker (1990) pointed out that thermodynamically the generation of a trans-membrane electrical field that is larger than that formed by proton pumps, by backward diffusion of protons, is impossible. The third theory was suggested by Booth (1985) who proposed that as in *E. coli*, ion transport mechanisms in acidophiles may be a means of depolarising the cytoplasmic membrane.

The interconversion of ΔpH and ΔΨ in response to pH_o is not unique to acidophiles. Neutrophilic bacteria also maintain cytoplasmic pH homeostasis by control of ΔpH and ΔΨ (Bakker, 1990; Booth, 1985). It could therefore be argued that in acidophiles and

neutrophiles, these interconversions are carried out in a similar manner, but, dependent on the organism, on a different scale of magnitude (Booth, 1985).

One of the currently held views is that in acidophilic and neutrophilic bacteria, the pH-dependent interconversion of ΔpH and $\Delta\Psi$ cannot be satisfactorily explained in terms of proton translocation only. Rather, this is attained by coupling a primary transmembrane proton flux to the secondary cycling of alkali cations, such as K^+ and Na^+, across the cytoplasmic membrane (Booth, 1985; Bakker, 1990). The transport of ions other than protons is thought to occur by the activity of the primary electrogenic pumps linked to the respiratory chain. Without such regulation of ΔpH and $\Delta\Psi$ in response to pH_o, the chemiosmotic mechanism would not be possible (Booth, 1985; Michels and Bakker, 1987; Bakker, 1990). Obviously crucial to the acceptance of such a hypothesis for acidophiles is proof of the existence of electrogenic and electroneutral alkali-cation transport systems in these bacteria. If many such systems operate simultaneously, this will not be easy to do (Bakker, 1990). An investigation of the ion transport systems in acidophiles would assist in establishing whether such mechanisms do operate, and also in understanding energy coupling in these organisms.

Potassium ion uptake systems were demonstrated for *B. acidocaldarius* (Bakker, 1990; Bakker *et al.*, 1987; Hafer *et al.*, 1989). It was suggested that during proton extrusion by *S. acidocaldarius*, there is electrogenic uptake of potassiums ions (Moll and Schafer, 1988). However, it is considered unlikely that in those acidophiles for which data are available, potassium ion uptake is the principal method of cytoplasmic membrane depolarisation (Bakker, 1990).

A role for sodium ions was suggested. Michels and Bakker (1987) found that during potassium ion uptake in *B. acidocaldarius*, sodium ions were extruded. This has lead to the suggestion of a dual Na^+-H^+ and K^+-H^+ antiporter operating. Further experimentation is in progress (Bakker, 1990). It is known that the presence of sodium ions in suspending buffers can cause the collapse of ΔpH in acidophiles in the presence of ionophores (Michels and Bakker, 1985).

Booth (1985) and Bakker (1990) proposed that anions might well be involved in development and maintenance of $\Delta\mu_{H^+}$ in acidophiles. With the $\Delta\Psi$ poised inside positive, electrogenic movement of anions into the cell is a possibility. However, to prevent collapse of $\Delta\Psi$, anion uptake would have to be rigidly controlled. Experimental data on the movement of anions across the cytoplasmic membrane of acidophiles is scarce. Goulbourne *et al.* (1986) demonstrated that the cytoplasmic membrane of *T. acidophilus* was permeable to chloride ions, and suggested that the cytoplasmic membrane of *B. acidocaldarius* may also be. McLaggan *et al.* (1990) showed both electrogenic chloride

transport and an energy-dependent mechanism for chloride exclusion in the obligate acidophile *Bacillus coagulans*. These results lead the authors to tentatively suggest the existence of either a H^+-Cl^- cotransport driven by ATP, or the active closure of chloride ion channels by the bacterium, or both.

Mechanisms of transmembrane ion movements in *T. ferrooxidans* are not well understood. Although it is known that *T. ferrooxidans* is tolerant of a wide range of cations (Section 1.2) there is no published information on the existence of secondary alkali-cation transport systems in the organism. At this stage, the possible role of either potassium or sodium ions in influencing values of ΔpH and $\Delta\Psi$ in response to change in pH_o is unknown. However, it is well-known that many monovalent anions exert an inhibitory effect on iron oxidation in *T. ferrooxidans* (Ingledew, 1982).

An interesting study on the relationship between chemiosmotic parameters and sensitivity to inorganic anions in *T. ferrooxidans* demonstrated that at certain concentrations the monovalent anions Cl^-, Br^-, I^-, and NO_3^- became inhibitory to Fe^{2+} oxidation by the organism (Alexander *et al.*, 1987). The concentration at which the anion became inhibitory was dependent on pH_o. As pH_o decreased, the toxic effect of the anions was more marked. For example, at pH_o 3 and $\Delta\Psi$ -40 mV inside, chloride ion concentration of 150 mM inhibited ferrous oxidation by 50%. However, at pH_o 0.94 and $\Delta\Psi$ +70 mV inside, chloride ion concentration of 10 mM inhibited ferrous oxidation by 50%. This was a result of the permeability of the cytoplasmic membrane to the anions and the magnitude and charge of $\Delta\Psi$, which became more inside positive as pH_o decreased. A similar trend was noted when $\Delta\Psi$, poised positive, was increased in de-energised *T. ferrooxidans* cells, or by the use of DNP. As $\Delta\Psi$ became increasingly positive, electrophoretic exclusion of anions decreased, anions accumulated inside the cell and acidification of the cytoplasm occurred. The authors demonstrated that the accumulation of anions in the cytosol was unaffected by ΔpH. To explain acidification of the cytoplasm as a result of anion entry (at inhibitory concentations of the anion), the authors proposed the following. In the steady state, $\Delta\Psi$ and ΔpH are balanced. A decrease in $\Delta\Psi$ (inside positive) caused by the influx of anions means that ΔpH becomes greater than the opposing $\Delta\Psi$, and protons slowly enter the cell. Once the two forces are again balanced, proton uptake ceases. Entry of protons may be slow and when coupled respiration is occurring, respiring cells are able to remove the protons. Hence collapsing of $\Delta\Psi$ by anions either in the presence of high concentrations of the anions, or when cells are de-energised, results in the cytosol becoming acidified. It is interesting to note that the poor growth obtained with *T. ferrooxidans* in various leaching operations in Australia has been attributed to the high chloride ion content of the brackish water in certain areas (D. E. Rawlings, personal communication).

Relevant to the development and maintenance of $\Delta\mu_{H+}$ in *T. ferrooxidans* is the anion SO_4^{2-}. It is known that the presence of sulphate is essential for cell growth and ferrous oxidation in this organism (Ingledew, 1982 and 1986). A direct role for the involvement of sulphate in the mechanism of ferrous oxidation was proposed (Dugan and Lundgren, 1965). Cell-free ferrous iron oxidising systems are not as dependent on sulphate, neither are they as sensitive to the presence of other anions (Ingledew, 1982). It could be postulated that it is the presence of an intact membrane, which is required. Bakker (1990) suggested that sulphate might be implicated in the maintenance and generation of $\Delta\mu_{H+}$ in *T. ferrooxidans*. He proposed that a transmembrane transport cycle could exist, which is composed of sulphuric acid uptake, and sulphate extrusion, resulting in a situation similar to the transport cycles of sodium and potassium ions across the cytoplasmic membrane. However, until experimental evidence to support this hypothesis becomes available, it can only be viewed with caution.

Ingledew (1982) indicated that at pH_o 2, the concentration of protonated sulphate will be high. When fully protonated, the structure will retain a large dipole, and as such, may not be readily permeant. In the study by Alexander *et al.* (1987) cited above, none of the toxic effects associated with the monovalent anions tested was noted for sulphate ions; in fact, it was the only anion tested which did not cause acidification of the cytosol in *T. ferrooxidans*, even at high concentration and when $\Delta\Psi$ was strongly inside positive. This suggested that the cytoplasmic membrane of the organism was impermeable to the divalent anion. Dugan and Lundgren (1965) demonstrated that radio-actively labelled sulphate was bound to the cell. Uptake of the label in the cell was not reported. They proposed that ferrous iron is auto-oxidised in an oxygen, ferric ion and sulphate-chelate coat surrounding the cell.

1.5.4. Role of F_1F_0 ATPsynthase in $\Delta\mu_{H+}$ control in acidophiles.

Little consideration has been given to the possible role of proton-translocating ATPsynthases in acidophiles as a means of regulating cytoplasmic pH homeostasis. Data collected from energetically compromised acidophilic bacteria showed that the enzyme is not solely responsible for the maintenance of ΔpH (Beck, 1960, cited by Krulwich and Guffanti, 1983; Goulbourne *et al.*, 1986; Zychlinsky and Matin, 1983a). However, there is little experimental evidence to suggest that proton-translocating ATPsynthase is not involved in the generation of ΔpH in the steady state. The enzyme appears to be involved in the generation and maintenance of $\Delta\Psi$ in PW2. When starving cells of PW2 were deprived of magnesium, a rapid increase in $\Delta\Psi$ was immediately noted. However, in cells starved in an identical medium, but with magnesium present at 0.2 g/l, $\Delta\Psi$ increased only after 48 h, and did not attain as high a value as in the magnesium-deprived culture (Goulbourne *et al.*, 1986). The presence of magnesium is required for a functional F_1F_0 ATPsynthase (Boyer, 1993). Hence a lack of magnesium would prevent both ATP

synthesis and coupled proton transport by F_1F_0 ATPsynthase. This result indicated that it is possible that in the steady state, the combined activities of the enzyme are implicated in the maintenance and generation of $\Delta\Psi$. It would be of interest to determine the effect of DCCD-inhibition of F_1F_0 ATPsynthase on the components of $\Delta\mu_{H+}$ in acidophiles.

Work with energised *B. acidocaldarius* vesicles demonstrated that the vesicles were unable to invert $\Delta\Psi$. It is notable that although ΔpH in the vesicles was present at pH_o 3, it was absent at pH_o 6 (Guffanti *et al.*, 1984). Hence, in *B. acidocaldarius*, F_1F_0 ATPsynthase is probably not the major contributor to $\Delta\Psi$ inversion.

1.5.4.1. Effect of F_1F_0 ATPsynthase turnover and expression. When F_1F_0 ATPsynthase was induced such that the enzyme was overexpressed fifteen-fold in *E. coli*, $\Delta\mu_{H+}$ collapsed; however, a five-fold increase in F_1F_0 ATPsynthase production had negligible effects on cell growth and physiology (Senior, 1990). Recent research on F_1F_0 ATPsynthase in *E. coli* grown on succinate demonstrated that enzyme concentration could influence the size of $\Delta\mu_{H+}$ in that organism. In *E. coli* mutants where the concentration of the enzyme was reduced to 40% of wild-type levels during respiration, an increase in enzyme turnover was noted due to an increased driving force caused by a reduction in total flux through the enzymes. Growth rate remained unaffected; this was related to turnover of the enzyme, which increased by 60%. Only when F_1F_0 ATPsynthase concentration was reduced to 15% of the wild-type, did it become limiting to growth rate (Jensen *et al.*, 1993). One of the reasons offered to explain the increase in enzyme turnover was that a decrease in concentration of F_1F_0 ATPsynthase lead to an increase in $\Delta\mu_{H+}$, because of a decreased influx of protons. Respiration rate in *E. coli* mutants with enhanced turnover of F_1F_0 ATPsynthase was increased and it was suggested that this was a result of a reduction in backflow of protons through the cytoplasmic membrane which would increase membrane potential (Jensen *et al.*, 1993; Jensen and Michelsen, 1992). These studies indicated that *E. coli* may change the magnitude of $\Delta\mu_{H+}$ (without affecting growth rates) by altering the number of F_1F_0 ATPsynthase operating at any one time. If the number of protons required per catalytic cycle per enzyme is constant, it is possible that in acidophiles, by increasing or decreasing the number of proton-translocating ATPsynthases within acceptable limits in response to pH_o, fluctuations in the components of $\Delta\mu_{H+}$ could result. The ability to alter turnover rates of proton-translocating ATPsynthase might also alter the magnitude of proton fluxes across the cytoplasmic membrane.

Gibson *et al.* (1983) isolated an *E. coli unc* mutant where the F_1F_0 ATPsynthase was unable to synthesise ATP coupled with respiration dependent on NADH, but could synthesise ATP with $\Delta\mu_{H+}$ in an artificial system. It was suggested by Futai and Kanazawa (1983) that the mutant was defective in energy coupling, possibly related to interconversion of $\Delta\Psi$ and ΔpH. Detailed studies of bacterial F_1F_0 ATPsynthase were recommended as a means

of understanding interconversion of the two components of $\Delta\mu_{H+}$ (Futai and Kanazawa, 1983).

Biochemical and genetic studies showed that in the anaerobic *Enterococcus hirae* (formerly *Streptococcus faecalis*) which lacks a respiratory chain, F_1F_0 ATPsynthase functioned exclusively to regulate internal cytoplasmic pH, by dissipating membrane potential. Significantly, it was demonstrated that *atp* gene expression in that organism was regulated by pH_i. When the latter became acidic, *atp* gene expression was induced and F_1F_0 ATPsynthase levels increased. Accompanying this increase, was an increase in proton extrusion through F_1F_0 ATPsynthase, and pH_i increased. As pH_i became alkaline, F_1F_0 ATPsynthase synthesis decreased (Kobayashi, 1985; Kobayashi *et al.*, 1984; 1986; Shibata *et al.*, 1992). It would be of interest to determine whether in acidophiles, F_1F_0 ATPsynthase synthesis is similarly induced.

It was demonstrated that facultatively anaerobic dental plaque bacteria which tolerate acidic environments and which lack a respiratory electron transport chain, viz. *Streptococcus mutans, Streptococcus sanguis* and *Lactobacillus casei,* maintained ΔpH across the cytoplasmic membrane and a neutral pH_i, by proton extrusion during ATP hydrolysis by F_1F_0 ATPsynthase. Association of F_1 with F_0 enhanced pH tolerance to acid and alkaline conditions (Sturr and Marquis, 1992).

To conclude this section on chemiosmotic mechanism in *T. ferrooxidans,* it is apparent that little is known about the way in which the organism regulates the magnitude of $\Delta\mu_{H+}$. It is not yet certain whether ΔpH is largely actively or passively maintained in the steady state. The organism maintains an inverted $\Delta\Psi$ at below pH_o 3 values, and is able to alter $\Delta\Psi$ in response to changes in pH_o. It is not known how this is achieved; it may be the result of respiration-driven proton consumption or extrusion (Cobley and Cox, 1983). Alternatively, compensatory ion movements may also be implicated. A proton-translocating ATPsynthase in *T. ferrooxidans* may be important in influencing generation, size, and maintenance of the components of $\Delta\mu_{H+}$. Indeed, it is difficult to preclude a role for the enzyme in this regard as it is closely implicated in proton movement across the cytoplasmic membrane. As such, it could also be indirectly involved in determining the extent of compensatory movement of ions across the cytoplasmic membrane. The energy generated by proton-translocating ATPsynthase in *T. ferrooxidans* may be used to drive primary ion-transport pumps required for the generation and maintenance of $\Delta\mu_{H+}$ in the bacterium. It is possible that by regulating levels of enzyme turnover and expression, in response to variation in pH_o, F_1F_0 ATPsynthase may regulate both ΔpH and $\Delta\Psi$. Experiments where F_1F_0 ATPsynthase is specifically inhibited by DCCD or mutation are required to determine whether the enzyme is important in regulating size of $\Delta\mu_{H+}$ in response to changes in pH_o in *T. ferrooxidans.*

1.6. Proton-translocating ATPsynthases.

1.6.1. The occurrence, structure and function of proton-translocating ATPsynthases.

As discussed previously, according to the chemiosmotic theory, ATP synthesis by eubacteria, chloroplasts and mitochondria is accomplished using the energy provided by $\Delta\mu_{H+}$. The enzyme of relevance is the multimeric complex known as F_1F_0 ATPsynthase (E.C. 3.6.1.34). One known exception is the F_1F_0 ATPsynthase from *Propionigenium modestum*, where ATP synthesis is coupled to the movement of sodium ions and/or protons (Dimroth, 1992; Fillingame, 1990; Kluge and Dimroth, 1992; Walker *et al.*, 1990). It remains controversial as to whether the F_1F_0 ATPsynthase of *Vibrio alginolyticus* conducts both sodium ions and protons, or protons only (Dmitriev *et al.*, 1991; Krumholz *et al.*, 1990). *Vibrio parahaemolyticus* utilises sodium ions rather than protons as the coupling cation for oxidative phosphorylation under alkaline conditions, but it is thought that this is the function of a specific sodium-translocating ATPase, and not the proton-translocating ATPase (Sakai-Tomita *et al.*, 1992). Other families/classes of proton-translocating ATPsynthases are known. These include the vacuolar proton-ATPsynthase (V-ATPase), the proton-translocating ATPsynthase of the archaebacteria, which is considered a chimeric form of F- and V- type ATPsynthases (Nuomi *et al.*, 1991; Schafer and Meyering-Vos, 1992), and the P-, or E_1-E_2, ATPases, which include the cation-specific pumps, such as the Na^+/K^+-, H^+/K^+- and Ca^+-ATPases (Nelson and Taiz, 1989). The P-type ATPases will not be discussed further; they are distinctly different from the V-, F-, and VF-types. The V- and F-types show extensive primary sequence and tertiary structural homology, and are considered to have a common ancestral form (Nelson and Taiz, 1989). However, in the interests of brevity, the V-type will not be discussed in detail. Instead, in subsequent chapters, where there is an indication of a relevant common structural/functional aspect, the V-type archaebacterial proton-translocating enzymes will be referred to when necessary.

All F_1F_0 ATPsynthases show remarkable homologies in both structure and function, which indicates a phylogenetic relatedness, and a common general mechanism of energy transfer from the electrochemical gradient of protons and/or sodium ions to the energy-rich phosphoric anhydride bond of ATP. There is a great variation in the amount of energy supply; certain bacterial species have the problem of synthesising ATP under less-than favourable energy conditions. Nevertheless, these organisms have evolved remarkable mechanisms to ensure that $\Delta\mu_{H+}$ is such that ATP synthesis occurs. In aerobic acidophiles, this has required the evolution of mechanisms to invert $\Delta\Psi$. In aerobic alkaliphilic bacteria, a reversed ΔpH occurs, the magnitude of which depends on pH_o; this may reduce $\Delta\mu_{H+}$ to very low levels (Dimroth, 1992). Nevertheless *Bacillus alcalophilus* utilises protons by conventional chemiosmotic mechanisms to synthesise ATP (Dimroth, 1992). The

mechanism of oxidative phosphorylation in the alkaliphile *Bacillus firmus* OF4 is controversial. It was suggested for the latter organism that conventional chemiosmosis occurs at pH_o 8-9, but that above pH_o values of 9, non-chemiosmotic energisation of oxidative phosphorylation occurs (Guffanti and Krulwich, 1992). However, Dimroth (1992) favoured a unifying common mechanism of chemiosmosis in *B. firmus* OF4, regardless of pH_o.

The function/s of F_1F_0 ATPsynthases include the net synthesis or hydrolysis of ATP. ATP synthesis encompasses the utilisation of the energy derived from substrate oxidation, or, in phototrophs, from exposure to light, and involves coupling the movement of protons/sodium ions across a cytoplasmic membrane to catalytic processes. Under anaerobic conditions in bacteria, F_1F_0 ATPsynthase is able to hydrolyse ATP generated by glycolysis to generate $\Delta\mu_{H+}$. Certain bacteria, notably those which lack a respiratory electron transport chain, regulate pH_i via F_1F_0 ATPsynthase (Section 1.5.4.1).

The subunit stoichiometry of F_1F_0 ATPsynthase is remarkable. The enzyme is unique in having multiple copies of subunits known to be involved in catalysis and may contain up to 12 copies of individual subunits (Boyer, 1993; Fillingame, 1990). It was suggested that the stoichiometry is linked to high catalytic turnover rates associated with F_1F_0 ATPsynthase (Boyer, 1993).

All known F_1F_0 ATPsynthases consist of two distinct portions. viz. F_1 and F_0. The F_0 portion is a composite of a number of different polypeptides, which together combine to form a membrane-intrinsic proton-translocating channel. Attached to the F_0 channel is the membrane-extrinsic cytoplasmic-soluble F_1 portion (Fig. 1.3). The F_1 portion forms the distinct hexagonal "head" associated with F_1F_0 ATPsynthase, and houses the catalytic domains (Fillingame, 1990; Futai *et al.*, 1989; Senior, 1990; Walker *et al.*, 1990). The two portions are readily dissociated from one another. Isolated F_1 is capable of ATP hydrolysis, and when F_1 is removed from F_0, the latter forms a passive proton-conduction channel through the membrane (Fillingame, 1990; Futai *et al.*, 1989). Unfortunately, the detailed knowledge of enzyme structure required for the full understanding of catalytic mechanism is not yet available. However, recent detailed analyses of the F_1F_0 ATPsynthase in bacteria and mitochondria, using electron microscopic and X-ray diffraction techniques provided useful information about the topographical arrangement of the F_1 moiety of F_1F_0 ATPsynthases associated with bacteria and mitochondria (Fig. 1.8) (Amzel *et al.*, 1992; Capaldi *et al.*, 1992; Kagawa *et al.*, 1992; Thomas *et al.*, 1992a).

The F_0 portion of all known F_1F_0 ATPsynthases is integral to a cytoplasmic membrane system, and it forms a proton channel which is functionally coupled to the catalytic F_1 domain. The complexity of the topology of F_0 varies according to the organism in which it

occurs. The most complex form of F_0 occurs in bovine mitochondria, where it is comprised of at least five different subunits (Walker *et al.*, 1990). The simplest form of F_0 characterised to date, is that associated with *E. coli*, where the proton channel consists of three distinct types of subunits viz. a, b, and c (Fillingame, 1990). Despite the difference in constitution of the F_0 moiety from different F_1F_0 ATPsynthases, the three subunits in *E. coli* F_0 have homologues in higher organisms. In the interests of brevity, this discussion will concentrate largely on the F_0 of *E. coli*. The three subunits of *E. coli* F_0, a, b and c, occur in the stoichiometry of 1:2:9-12 (Fillingame, 1990; Senior, 1990).

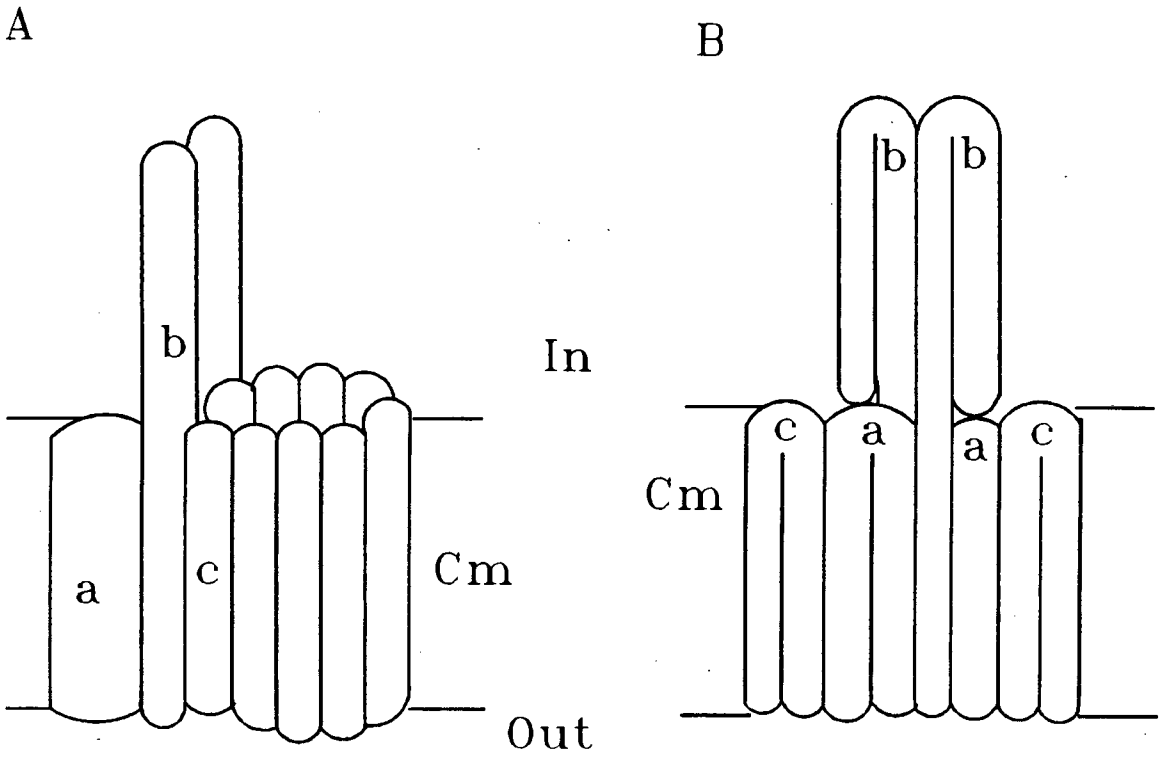


Fig. 1.7. Two hypothetical models for the arrangement of the *E. coli* F_1F_0 ATPsynthase F_0 subunits a, b and c. Cm = cytoplasmic membrane, In = interior of cell, Out = exterior to cell (after Cox *et al.*, 1986; Hoppe and Sebald, 1984). (Not drawn to scale).

Whilst it is known that all three subunits are required for a functional F_0 *in vivo*, the manner in which the *E. coli* F_0 subunits are arranged to form the F_0 channel is not understood (Fillingame, 1992b; Vik and Dao, 1992). Two different models were proposed. These are depicted in Fig. 1.7. A and B. Hoppe and Sebald (1984) proposed an oligomer of c subunits adjacent to an a-b_2 complex (Fig. 1.7. A). Cox *et al.* (1986) proposed an a-b_2 complex surrounded by a ring of c subunits, shielding them from the lipids (Fig. 1.7 B). TID (3-[trifluoromethyl]-3-*m*[I]iodophenyldiazirine)-labelling experiments (Hoppe and

Sebald, 1984) and more recent analyses, using variational and hydrophobic moment methods (Vik and Dao, 1992) supported the former model. The structure and function of individual F_0 subunits are reviewed further in Chapter 3.

The F_1 moiety of ATPsynthases in bacteria and chloroplasts consists of five different subunits α, β, γ, δ and ε (Walker *et al.* 1990). Similarly, in bovine mitochondria, subunits α, β, γ, δ and ε make up F_1. However, whilst subunits α, β and γ are homologous to bacterial and chloroplast homonyms, bovine δ and ε are not. Instead, bovine δ is equivalent to bacterial/chloroplast ε; bovine ε has no bacterial or chloroplast equivalent. The mitochondrial equivalent of bacterial/chloroplast δ is OSCP (oligomycin sensitivity conferring protein) which is not released from the membrane complex as a component of F_1; however, functionally, it is similar to bacterial δ. A small basic protein which binds to the F_1 sector of bovine mitochondrial F_1, has also been identified, and is known as the inhibitor protein (Walker *et al.*, 1990). Mitochondrial inhibitor protein has limited homology with bacterial ε (Futai *et al.* 1989). The detailed structure and function/s of the individual bacterial F_1 subunits and their homologues in chloroplasts and mitochondria, including a review of relevant literature, may be found in Chapter 3.

The topology of the F_1 subunits from *E. coli* (Capaldi *et al.*, 1992) and rat liver mitochondria (Amzel *et al.*, 1992; Bianchet *et al.*, 1992) was studied by means of electron microscopy, and/or X-ray diffraction techniques. A common general arrangement was reported. Together, the F_1 subunits form a distinct hexagonal head, situated in the cytoplasm, linked to the F_0 channel. A recently-proposed model for the topography of the *E. coli* F_1 moiety is illustrated in Fig. 1.8.

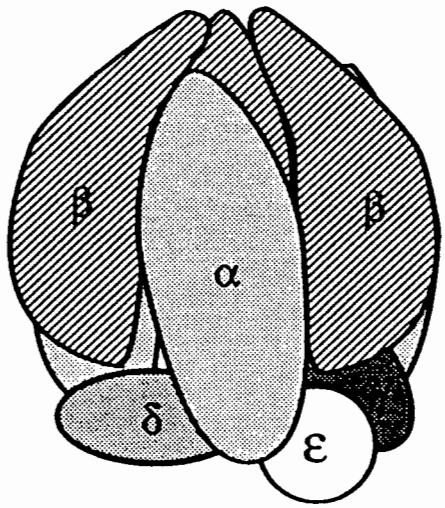


Fig. 1.8. A schematic representation of the *E. coli* F_1 subunits (after Capaldi *et al.*, 1992).

The M_r of the *E. coli* F_1 complex is 385 000 Da. The large "major" subunits, α and β, occur in three copies each and are ellipsoidal in shape. They alternate with one another, and interdigitate along their 6 nm length within the F_1 oligomer to form a hexagonal head, surrounding a central cavity, with a diameter of approximately 1.5 nm. The β subunits lie to the outside of the hexagon (Capaldi *et al.*, 1992). A similar arrangement for α and β was also reported for mitochondrial F_1 (Bianchet *et al.*, 1991). It is known that α-β and possibly α-α make contact within F_1; β-β interaction is no longer thought to occur (Bianchet *et al.*, 1991; Gromet-Elhanan, 1992; Thomas *et al.*, 1992a). This model of F_1 replaces the version suggested by Tiedge *et al.* (1985) which proposed an F_1 where there were α-α and β-β linkages. Located towards the base of the hexagonal head in *E. coli* F_1 are the three single-copy "minor" subunits, γ, δ and ε. The γ subunit is the more centrally located minor subunit. The γ, δ and ε subunits all interact with both an α and β subunit, and the γ and ε subunits interact with each other (Aggeler *et al.*, 1992; Capaldi *et al.*, 1992; Mendel-Hartvig and Capaldi, 1991a). The distribution of the single-copy F_1 subunits within mitochondrial F_1 is unknown, but is likely to be similar to that of *E. coli* (Bianchet *et al.*, 1991; Thomas *et al.*, 1992a).

The asymmetric arrangement of F_1 is a feature of the enzyme, and has significant implications for catalytic mechanism. The asymmetry is conferred by the core of minor subunits, which are considered to be off-centre within the enzyme, and by the large α and β subunits themselves, which are non-identically arranged within F_1 (Boyer, 1993).

Electron-microscope studies on *E. coli* F_1F_0 ATPsynthase demonstrated that the F_1 and F_0 subunits are connected by a stalk of approximately 4 nm in length. This stalk was suggested to be comprised of b and δ subunits, and is thought to be important in the mechanism of catalysis (Capaldi *et al.*, 1992).

The overall topology of the F_1F_0 ATPsynthase in *E. coli* has lead to the suggestion that there are three distinct but interlinked functional domains, viz. the proton-conducting machinery of $a_1c_{9\text{-}12}$ interacts with the connector/transmittor device of $b_2\delta$, which interacts with the catalytic unit associated with F_1 (Senior, 1990).

1.6.2. Nucleotide binding sites in F_1F_0 ATPsynthase.

The two large subunits α and β, each contain a single nucleotide-binding domain; hence there are six such domains in F_1. The location and nature of these nucleotide-binding domains is uncertain. The current hypothesis favoured by some is that these domains are located at the interface of interacting αβ pairs (Chapter 3, Fig. 3.20). There are two categories of nucleotide-binding sites, viz. catalytic and "non-catalytic". The former sites, which total three in number, are thought to be located largely on the β subunit, 1-2 nm from F_0, and to be the site of catalysis. Based on analogy with other known nucleotide-

binding proteins such as adenylate kinase (AK), Ras, and *E. coli* elongation factor EF-Tu, a model was proposed for the three-dimensional folding of the catalytic-binding sites (Duncan and Cross, 1992); this is presented in Chapter 3, Section 3.4.3.2.d, Fig. 3.21. The three "non-catalytic" domains are thought to be situated largely on α subunits, and recently, a role was demonstrated for them in both chloroplast and mitochondrial F_1F_0 ATPsynthase multi-site catalysis (reviewed by Allison *et al.*, 1992; Amzel *et al.*, 1992; Boyer, 1993; Capaldi *et al.*, 1992; Fillingame, 1990; Futai *et al.*, 1989; Senior, 1990 and 1992; Slater, 1987; Thomas *et al.*, 1992a). (Refer also Chapter 3, Section 3.4.3.2.d).

1.6.3. Catalysis in F_1F_0 ATPsynthase.

The preferred physiological substrates of F_1F_0 ATPsynthase are ATP, and ADP, but GTP, GDP, ITP and IDP are used. Magnesium ions are required by the enzyme for catalysis, and cobalt and manganese act as co-factors (Senior, 1990). Amongst certain eubacteria, notably *V. parahaemolyticus*, and strain PS3, the presence of sulphate significantly increases catalytic turnover (Sakai *et al.*, 1990; Sakai-Tomita *et al.*, 1992; Takeda *et al.*, 1982, cited by Sakai *et al.*, 1990).

A number of inhibitors of catalytic mechanism are known. These include the antibiotics, aurovertin, citreoviridin, venturicidin, efrapeptin, and tentoxin, which act on F_1, and oligomycin, which reacts with F_0. DCCD and 7-chloro-4-nitrobenzofurazan (Nbf-Cl) are inactivators; DCCD acts both on F_0 and F_1, and Nbf-Cl on F_1. Steady state multi-site catalysis is prevented by azide; uni-site mode is not affected (reviewed by Senior, 1990; Gromet-Elhanan, 1992). A large number of amphipathic cations inhibit ATPase activity; these are discussed further in Chapter 3 (reviewed by Allison *et al.*, 1992). Studies on mechanisms of inhibition have contributed substantially to understanding the mechanisms of catalysis.

Detailed arguments for and against the many hypotheses which have been presented to explain catalytic mechanisms of F_1F_0 ATPsynthase will not be reviewed. These were extensively discussed elsewhere in the literature. Some more recent reviews include, Boyer (1993); Fillingame (1990 and 1992a and b); Futai *et al.* (1989); Mitchell (1985); Penefsky and Cross (1991); Senior (1990 and 1992); Slater (1987); Thomas *et al.* (1992a) and Walker *et al.* (1990). Mechanism of catalysis forms the subject of a number of heated scientific debates, particularly between Mitchell and Boyer, and Mitchell and Slater. All catalytic models suggested to date which include participation of $\Delta\mu_{H+}$, are largely hypothetical. It has still not been settled whether net ATP synthesis can be driven by oxidations without proton translocation across the cytoplasmic membrane. Slater (1987) is of the opinion that this may indeed occur, and that bulk $\Delta\mu_{H+}$ is not an obligatory intermediate of oxidative phosphorylation. The passage of protons through the enzyme complex has not yet been elucidated (Sections 1.7 and 1.8). It may be that all that is

required to transduce energy in membranes, is a charge separation in a membrane-spanning protein which can itself lead to a membrane potential, and to the creation of fixed negative charges on the surface of the membrane. These mechanisms which might result in the formation of ATP by F_1F_0 ATPsynthase, were reviewed by Slater (1987).

The following discussion will present criteria which should be incorporated into any catalytic model, and having reviewed experimental data pertinent to the topic, a currently favoured hypothesis, the binding change mechanism, will be discussed (Boyer, 1993). Although full understanding of catalytic mechanism will depend on the elucidation of three-dimensional structure of the enzyme, it is not necessary to know the three-dimensional structure of the synthase at high resolution to make meaningful assessments of the validity of the binding change mechanism (Boyer, 1993). Alternate experimental data provided substantial data from which to suggest how ATP synthesis may be coupled to proton translocation by F_1F_0 ATPsynthase. Experimental data encompasses that from isotope-labelling studies (particularly ^{18}O) enzyme kinetics, chemical affinity, and photoaffinity labelling/derivitisation, and mutational analyses.

Any model of catalysis for F_1F_0 ATPsynthase should take into account the following features of the enzyme:- (after Boyer, 1993; Capaldi *et al.*, 1992; Fillingame, 1990 and 1992; Futai *et al.*, 1989; Kagawa *et al.*, 1992; Penefsky and Cross, 1991; Senior, 1990 and 1992; Slater, 1987).

i). A catalytic site with high affinity for ATP is formed only in catalytically competent αβ complexes; the K_A is 10^{12} M^{-1}, and 10^{10} M^{-1}, in mitochondrial and *E. coli* F_1 respectively. At any one time, only one such site is active within a given F_1F_0 ATPsynthase complex. In *E. coli* F_1F_0 ATPsynthase, this site shows two distinct conformations; one which is hydrophobic and highly sequestered, and favours ATP binding, and the other which is more hydrophilic in nature and favours ADP binding.

ii). ATP hydrolysis or synthesis is not accompanied by the formation of any co-valent intermediates. At the catalytic site, in a four-stage reaction, the following occurs:-

$$ADP + P_i <=> ATP.$$

The equilibrium constant is close to unity, i.e. the reaction is freely reversible. Magnesium ions are essential. The rate-limiting step is product-release. ATP formation is spontaneous, and the presence of water at the catalytic site is important in the transduction of energy required for ATP hydrolysis or synthesis. A small change in the activity of water at the catalytic site would be required to drive ATP synthesis in F_1F_0 ATPsynthase. During net ATP synthesis, an exchange of phosphoryl oxygens with water oxygens was conclusively demonstrated by two

independent laboratories. During the catalytic step, hydrolysis takes place, but the products remain bound in the catalytic site. ATP hydrolysis involves inversion of the O atoms about the γ-P atom to yield ADP+P_i. In ATP synthesis, ADP-O is the phosphate acceptor. In this respect, F_1F_0 ATPsynthase is different from E_1E_2 enzymes, where a co-valent phosphorylated intermediate is formed during hydrolysis. Experimental data, accumulated from intact membrane-bound F_1F_0 ATPsynthase, and from isolated F_1-ATPases revealed that bulk proton motive force (energy) is not required at the catalytic site for the formation of tightly bound ATP from ADP+P_i. This indicated that there is not a direct participation of protons in the esterification reaction. Energy for the reaction is required for ATP release, and the binding of ADP+P_i, in a manner competent to form ATP.

iii). The distance of the catalytic site from the proton-conducting channel of the cytoplasmic membrane can be as much as 1-2 nm and the F_0/F_1 connecting stalk is not thought to provide an insulated proton circuit. Yet, the movement of protons through F_0 is immediately coupled to the concomitant binding of ADP+P_i at a distant catalytic site. For the rapid catalytic rates associated with intact F_1F_0 ATPsynthase, this argues against long-range proton conduction to the catalytic site.

iv). When ATP is bound in F_1, such that only one catalytic site is occupied, hydrolysis is slow (uni-site mode) at a rate of $10^{-3}s^{-1}$ and $10^{-2}s^{-1}$ for mitochondrial and *E. coli* F_1 respectively. **As soon as a second catalytic site is occupied by nucleotides, ATP hydrolysis is markedly increased (multi-site mode)** to 600 s^{-1} and 50 s^{-1} for mitochondrial and *E. coli* F_1 respectively. This shows positive catalytic co-operativity and negative nucleotide-binding co-operativity between nucleotide-binding sites.

v). There is extensive evidence of F_0-to-F_1-to-F_0 subunit co-operativity during catalysis, without which catalysis is markedly reduced. This is reviewed in Chapter 3.

vi). Asymmetry is a feature of the enzyme. At any one time individual α and β subunits show conformational asymmetry. In addition, electron microscope studies showed that the single copy F_1 subunits δ, γ and ε undergo conformational change within F_1 during catalysis which contribute to asymmetry. In the case of γ and ε, these changes are dependent on nucleotides at the binding sites (Chapter 3, Section 3.4.3.2).

1.6.3.1. The current model of catalysis for F_1F_0 ATPsynthase. The catalytic model which best accommodates the features of the enzyme referred to above is the binding change mechanism (Boyer, 1993). This proposes that conformational changes induced in F_0 by the translocation of protons are transmitted to the catalytic centres. These induced conformational changes at the catalytic site cause the release of tightly bound ATP from its site of synthesis, and simultaneously promote tight binding of ADP+P_i at a second site. Similarly, conformational changes of the F_1 subunits induced by the binding of nucleotides

at appropriate binding sites are transmitted back to F_0 such that proton translocation is carefully modulated. According to this hypothesis, protons which enter F_0 need never traverse the F_1 part of the enzyme. Electrical potential at the catalytic site is not envisaged to play any role in altering reaction affinities. In support of this hypothesis, it is well known that ligand binding in proteins can cause three-dimensional changes in structure, and that these changes are transmitted through proteins where they can cause change in dissociation constants/binding affinities of several orders of magnitude; however, this does not *per se* prove the binding change mechanism. Rather, central to this hypothesis are two requirements:-

-the principal function of the energy input is to promote the competent binding of ADP+P_i and the release of tightly bound ATP;

-there must be strong co-operativity between catalytic sites that alternate between binding and release of substrates (Boyer, 1993).

The indirect mode of coupling of proton translocation to ATP synthesis/hydrolysis by long range transformational changes transmitted from F_0 to F_1, as proposed by the binding change mechanism, is in contrast to the direct mechanism proposed by Mitchell (1985). The latter proposed that translocated protons in F_1F_0 ATPsynthase reach the catalytic site region where they combine with phosphoryl oxygen atoms to form water. Mitchell suggested that an electrical potential gradient across the active centre could in some manner push ADP+P_i into, and pull ATP out of the catalytic domain. However, as referred to above, there is conclusive experimental evidence to suggest that there is no direct participation of translocated protons in the formation of ATP (Boyer, 1993; Penefsky and Cross, 1991). Furthermore, in the marine anaerobic bacterium, *P. modestum*, F_1F_0 ATPsynthase couples the transport of sodium ions, not protons, to ATP synthesis. Significantly, the F_0 channel of *P. modestum* is able to functionally complement *E. coli* F_0 mutants. This suggested that there is a unifying mechanism for the coupling of either sodium ions and/or proton transport to ATP synthesis by F_1F_0 ATPsynthase (Dimroth, 1992; Kaim *et al.*, 1992; Kluge and Dimroth, 1992).

Whilst definitive experimental proof of the binding change mechanism is still lacking, the observations pertinent to catalysis in F_1F_0 ATPsynthases listed above do favour the hypothesis. Incorporating many of these facts, and including a recent observation that in chloroplast F_1, ATP formation at the high affinity tight binding catalytic site increases the affinity for ADP at another catalytic site, the following binding change mechanism model was proposed by Zhou and Boyer (1993).

As depicted in Fig. 1.9, three binding changes per ATP molecule synthesised or hydrolysed are proposed. Each step is coupled to associated proton translocation at a distant site. In Step 1, loosely bound ADP+P_i are converted to tightly bound ATP at a catalytic site. In Step 2, a conformational change occurs such that ATP becomes loosely

bound. In Step 3, a third conformational change occurs in which ADP+P_i binding is favoured, and there may also be a promotion of residual ATP release. Unlike earlier models of the binding change mechanism, ADP+P_i do not appear as a required intermediate stage. The model accommodates the situation wherein either substrate or $\Delta\mu_{H+}$ becomes limiting, and reversible conversion of tightly bound ATP+H_20 to tightly bound ADP+P_i can occur. This reaction results in exchange of phosphate oxygen. A reversal of Step 1 also results in oxygen exchange (Boyer, 1993; Zhou and Boyer, 1993).

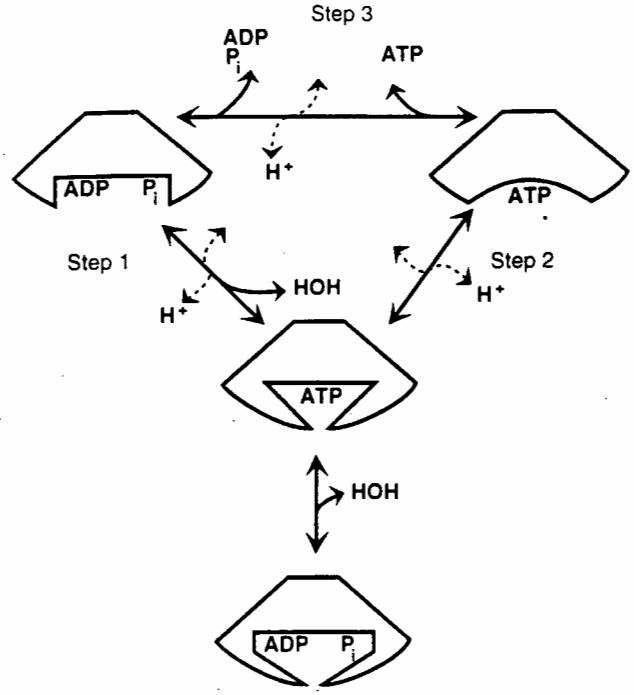


Fig. 1.9. Depiction of proposed conformational changes, associated proton translocation and catalytic steps during the binding change mechanism for ATP synthase. A single catalytic site, located on β, is shown going through conformations as catalysis of ATP proceeds (after Zhou and Boyer, 1993).

The number of protons per mole ATP synthesised is unknown (Section 1.5.2.4). Both Fillingame (1990) and Boyer (1993) suggested a minimum of three protons/mole ATP, but figures as high as nine protons/mole ATP were suggested (reviewed in Boyer, 1993). To accommodate such conflicting values, Boyer (1993) has suggested that the ratio of protons translocated/mole ATP synthesised or cleaved may depend on opposing phosphate potential or $\Delta\mu_{H+}$.

It is still a matter of some debate as to the number of catalytic sites which co-operate at any one moment. Proposals were put forward which suggest that there may be two, three or four co-operating catalytic sites; a single site model has also been proposed (reviewed in Boyer, 1993). Mutational analyses in *E. coli* support the notion that optimal catalytic rates are only attained when there are three fully functional αβ pairs (reviewed in Fillingame,

1990; Senior, 1990). Kinetic data for ATP synthesis or hydrolysis from F_1F_0 ATPsynthases from various sources support a three-site model (reviewed by Boyer, 1993). It is known from many studies, that at any given time, all three proposed catalytic sites are assymetric and non-equivalent (Boyer, 1993; Futai *et al.*, 1989; Senior, 1992). Certain authors (eg. Senior, 1992; Boyer, 1993) were of the opinion that at any one moment, catalysis occurs at one site only, with the progression of this catalysis-competent site around the three β subunits in a cyclical fashion, i.e. all three sites are sequentially involved in co-operative catalysis at any one time. For all three sites to return to their original conformation would therefore require the synthesis of three moles ATP, and translocation of nine protons. A model of the the three participating sites, on three β subunits in the three different conformations at a given time, is depicted in Fig. 1.10.

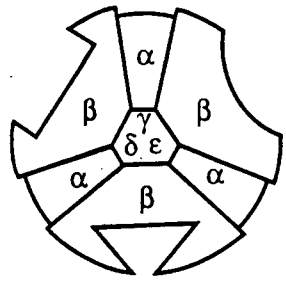


Fig. 1.10. A depiction of the F_1-ATPase, emphasising the circular interconversions of conformations of β subunits, and the importance of interactions with the α and the single-copy subunits (after Boyer, 1993).

This model allows for the interaction of the β subunits with other F_1 subunits, but not with each other; hence the binding change mechanism accommodates co-operativity between catalytic sites and other F_1 subunits, which results in the formation of the high affinity catalytic site. The co-operativity implied also accommodates a recently reported function of the non-catalytic nucleotide-binding sites, i.e. that the presence of bound nucleotides at the non-catalytic sites of an interacting αβ pairs is required for transmitting conformational changes necessary for multisite catalysis on non-communicating β subunits (reviewed by Allison *et al.*, 1992; Gromet-Elhanan, 1992; Jault and Allison, 1993). The model could also accommodate the view that the catalytic site is itself situated at the interface of an interacting αβ pair.

The binding change mechanism has important implications for the chemiosmotic coupling models proposed for *T. ferrooxidans* (Section 1.5.2.4). Most of these relate to the translocation of protons in the organism. Firstly, the binding change mechanism, other than to exclude the direct involvement of protons at the catalytic site, via reduction to water, does not explain what happens to translocated protons. The fate of protons beyond

the F_0 channel is unknown. The only requirement of the binding change mechanism is that proton translocation at some point within F_0, must induce a conformational change. The models proposed for oxidative phosphorylation in *T. ferrooxidans*, which are based on Mitchell's "direct mechanism" suggest that protons entering F_1 are reduced to water at the catalytic site (Figs 1.4 and 1.5). However, it is highly unlikely that this occurs, and the neutralisation of protons, if they enter the cell through F_0, must occur elsewhere. Whether this occurs by a reduction to water, or by translocation out of the cell across the membrane remains to be determined. The second point is that it is likely that at least three protons/mole ATP are necessary; the implications of this for *T. ferrooxidans* were discussed (Section 1.5.2.4) . Of relevance is Boyer's suggestion that the ratio of protons translocated:ATP formed or cleaved is variable, depending on opposing phosphate potential or, particularly in the case of acidophiles, on the value of $\Delta\mu_{H+}$. Until there is further understanding of the movement of protons into and out of the *T. ferrooxidans* cell, the models proposed for chemiosmotic coupling remain largely speculative.

1.6.4. The mode of proton conductance through F_1F_0 ATPsynthase.

The manner in which protons traverse F_1F_0 ATPsynthase is unknown, but there are two hypotheses which attempt to explain the mechanism of active proton translocation through F_0 in *E. coli*. It was proposed that this occurs by mechanisms which involve either a proton relay/wire, or a water-lined ion conducting channel, both of which require an interacting a-c oligomer.

The proton relay theory incorporates the movement/relay of protons across the membrane by the sequential interaction of a series of charged buried side-chains within subunits a and c. However, such a mechanism could not explain the mode of sodium ion conductance by the F_1F_0 ATPsynthase of *P. modestum* (Fillingame, 1990). Furthermore, a continuous proton wire, using only the single face of an amphipathic helix, such as occurs in subunits a and c, would not be viable without the continual backwards and forwards movement of charged groups to transport protons (Fillingame, 1990).

More plausible is the formation of a narrow water-filled pore across the cytoplasmic membrane, which would provide a continuum for the conduction of both ions and protons between charged groups located on a and c. The constitution of subunit c provides support for this latter mechanism; the subunit has a number of small water sequestering residues interspersed amongst hydrophobic amino acids which traverse the membrane as an N-terminal α-helix (See Chapter 3, Figs. 3.7, 3.8, 3.9). Such an arrangement is thought to provide a thin water-lined channel. The existence of an appropriately charged "filter" would allow the translocation of protons through the channel; in all F_0 pores studied to date, this residue is highly conserved as either Glu or Asp on the C-terminal α-helix of subunit c (Fillingame, 1990; Senior, 1990) (Chapter 3, Figs.

3.7, 3.8 and 3.9). By altering the size of the water-lined ion channel, by varying the the number of polar groups, and therefore the amount of water in the channel, ion-specificity of the channel could be altered to accommodate the conductance of sodium ions (Fillingame, 1990; Lear *et al.*, 1988, cited by Fillingame, 1990).

1.6.5. Coupling proton-translocation to ATP synthesis.

Subunit stoichiometry in *E. coli* F_0 is thought to be closely related to mechanism. Both a and c are directly implicated in proton transport, whereas b is apparently not (Fillingame, 1990; Senior, 1990). A number of models were proposed to explain how active proton translocation is related to both *E. coli* F_0 topology and stoichiometry and F_0/F_1 interaction. All involve a mechanism of rotational catalysis and conformational change. After extensive experimentation, Cox *et al.* (1986) suggested a mechanism involving a rotating a-b_2 core inside a circular $c_{9\text{-}10}$ oligomer. Hoppe and Sebald (1984) suggested that proton translocation may occur at the surface of an inner core formed by oligomers of subunit c, possibly at the interface with residues of the a and b subunits, and that the oligomeric core may then rotate against the subunit a or b during proton translocation (Fig. 1.7). More recently, Fillingame (1992b) suggested that in *E. coli*, the c oligomer consists of three units of subunit c trimers, and each trimer occurs beneath one of three interacting $\alpha\beta$ catalytic pairs. At least three (perhaps four) protons must be translocated per mole ATP synthesised. This translocation is coupled to conformational change/s in F_0, which is/are transmitted to F_1, thereby promoting product release. The coupling mechanism involves the three subunits of a given c trimer being sequentially protonated by subunit a, at the a/c interface. The three protons are released simultaneously by an event which is coupled to conformational change.

Although it has been clearly established that F_0 provides a channel for protons into the cytoplasmic membrane, it is not clear how protons reach the aqueous phase. The path of protons beyond F_0 remains to be established. The mechanism of transmitting conformational change from F_0 to F_1 and vice versa is not understood. Many highly speculative models were suggested, all of which incorporate a rotation of various F_0 and/or F_1 subunits in relation to each other (reviewed in Boyer, 1993; Futai *et al.*, 1989; Penefsky and Cross, 1991). It is not relevant to include a review of these models in this discussion. It is pertinent to include Boyer's comment that, "If some type of rotational catalysis occurs, likely it will be found that nature is cleverer than amateur biological machinists" (Boyer, 1993).

1.6.6. Gene clusters for F_1F_0 ATPsynthase, and their diversity.

The operon coding for *E. coli* F_1F_0 ATPsynthase was cloned by the use of mutant cells, where ATP synthesis was uncoupled from oxidative phosphorylation, and mutants could not grow on non-fermentable carbon sources, such as succinate (reviewed in Brusilow,

1993). For this reason, the term *unc* was proposed to describe the cluster of genes which transcribes F_1F_0 ATPsynthase in *E. coli*. As there are many bacterial mutants of F_1F_0 ATPsynthase, in which uncoupling does not occur, alternative descriptive terms for the F_1F_0 were described. These are *pap* (proton-translocating ATPase subunit protein), *bcf* (bacterial coupling factor) and *atp* (ATPase-translocating proteins) (reviewed in Gibson, 1983; Futai and Kanazawa, 1983). For the purposes of this thesis, the term *unc* will be used to describe the *E. coli* F_1F_0 ATPsynthase operon, whilst *atp* will be used to describe the genes in other prokaryotes.

The *unc* operon maps at 83 min on the *E. coli* chromosome, and is situated 3-10 kb to the left of the *oriC*, and to the right of the *glmS* gene. (Bachmann, 1990; Walker *et al.*, 1984b). The operon consists of nine genes which are preceded by a single promoter, 73 bp upstream of the first 5' gene, *uncI*. The nine genes occur as a single cluster, and throughout this thesis, the terminology following in this paragraph will be used to describe the *unc* genes. At the extreme 5'-end, is *uncI* which, *in vitro*, transcribes a small 13-14 kDa basic hydrophobic protein, i (Walker *et al.*, 1984a; Scneppe *et al.*, 1990). *In vivo*, the concentration of i is substoichiometric relative to that of any other *unc* gene products (Mc Carthy *et al.*, 1991) and its function is unknown. Cells defective in *uncI* show slower rates of catalysis than the wild-type (von Meyenburg *et al.*, 1982). It was suggested that i may be required for efficient enzyme assembly (Walker *et al.*, 1984a). The existence of two internal promoter-like sequences within *uncI* was reported; hence a translational/transcriptional regulatory role might be possible (Schneppe *et al.*, 1990). Apart from *E. hirae* (Shibata *et al.*, 1992) all eubacterial *atp* operons have *atpI* at the 5'-end; there therefore appears to have been evolutionary pressure to conserve this gene (Walker *et al.*, 1984a). The *uncI* gene is succeeded at the 3'-end by the F_0 gene cluster of *uncB, E,* and *F*, which code for subunits a, c and b, respectively. The five genes which code for F_1 occur to the 3'-end of *uncF* in the order *uncH, A, G, D* and *C*. These genes code for subunits δ, α, γ, β and ε respectively. The *unc* operon is terminated by a *rho*-independent terminator, six nucleotides downstream of *uncC* (Walker *et al.*, 1984a).

The arrangement of the *atp* operon follows two trends amongst bacteria. Either, all the genes form a single cluster, as in *E. coli*, with the F_0 genes preceding the F_1 subcluster, or the genes are separated on the chromosome. These trends are fully discussed in Chapter 3, where the arrangement of the *T. ferrooxidans atp* operon is aligned against, and compared with, that from a number of different bacterial species (Chapter 3, Fig. 3.5 and related discussion).

Amongst the eukaryotes, the arrangement of genes coding for F_1F_0 ATPsynthases within either chloroplasts and/or mitochondria is fairly complex. This topic will not be discussed

in detail here, other than to mention that the cistrons occur on both organellar and nuclear genomes (reviewed in Futai *et al.*, 1989; Walker *et al.*, 1990).

1.6.7. Assembly of F_1F_0 ATPsynthase *in vivo*.

There are two aspects to be considered regarding assembly of F_1F_0 ATPsynthase within bacteria. The first concerns the actual assembly of the subunits into the final form. The second concerns mechanisms of control used to ensure that subunit stoichiometry is such that the enzyme is assembled in the correct multimeric form.

Little is known regarding enzyme assembly. It is important that within the cell/organelle, F_1F_0 ATPsynthase is assembled and inserted into the cytoplasmic membrane without the formation of harmful intermediates which might disrupt the transmembrane proton gradient, or deplete ATP levels, or both. Early models describing F_1F_0 ATPsynthase assembly in *E. coli* indicated that F_0 subunits could not be assembled into the cytoplasmic membrane without certain F_1 subunits, suggesting that neither F_0 nor F_1 could be assembled in the absence of the other (Cox *et al.*, 1981, cited by Walker *et al.*, 1984a). However, it is now known that both F_0 and F_1 can be assembled in a reconstitutable form, independently of each other. In the absence of F_1, F_0 subunits form a proton channel of low proton-conductivity in the cytoplasmic membrane, i.e. in an "immature" form. Only in the presence of other F_1 subunits, notably δ, α and γ, does F_0 become fully functional i.e. "mature". The formation of the "mature" form of F_0 which is associated with normal levels of proton translocation, is irreversible (Brusilow, 1993; Futai *et al.*, 1989; Monticello *et al.*, 1992; Pati and Brusilow, 1991)

Under normal conditions, haploid *E. coli* cells express and synthesisze F_1F_0 ATPsynthase constitutively in the amount of 1-2% total cell protein (Senior, 1990). In *E. hirae* the promoter is induced at low pH_i values (reviewed in section 1.5.3.7). The *atp* promoter in *V. parahaemolyticus*, and *V. alginolyticus* is subject to catabolite repression, as the region has a consensus sequence for a cAMP binding site (Sakai-Tomita *et al.*, 1992). Whilst levels of F_1F_0 ATPsynthase in the cell may be under control of the single promoter, the stoichiometries of the subunits within the holoenzyme are not. The *unc* operon is transcribed as a single 7 kb mRNA message from the single promoter (Senior, 1990). In *E. coli* control of subunit stoichiometry is achieved via complex mechanisms. The regulation is achieved primarily at the level of post-transcription (McCarthy, 1990). This topic is reviewed in Chapter 3.

1.7. Aims and objectives of this study.

The aims of the study were:-

i). to isolate the *T. ferrooxidans* ATCC 33020 *atp* genes (Chapters 2 and 5);

ii). a comparison of the deduced primary amino acid sequence of the *T. ferrooxidans* F_1F_0 ATPsynthase subunits to identify features that may represent adaptations to proton translocation and/or energy coupling in an extremely acidophilic environment and contribute to the magnitude of the ΔpH (Chapter 3);

iii). a comparison of *atp* operon structures (Chapter 3);

iv). a comparison of intergenic regions of the *T. ferrooxidans atp* operon with the well-characterised *E. coli* regions to assess possible mechanisms of control of *atp* gene expression (Chapter 3);

v). to assess whether the primary sequence of the *T. ferrooxidans* F_1F_0 ATPsynthase polypeptides, particularly the β catalytic subunit, could be used to verify the current taxonomic status of *T. ferrooxidans* (Chapter 3);

vi). a series of cross-complementation studies and specific hydrolytic activity assays of a hybrid *T. ferrooxidans*/*E. coli* F_1F_0 ATPsynthase to determine the extent to which hybrid enzymes may be functionally reconstituted in the neutrophilic host *E. coli* (Chapter 4);

vii). measurement of specific ATPase activities of *T. ferrooxidans* F_1F_0 ATPsynthase to determine whether the enzyme shows unusual pH activity profiles and a dependence on sulphate (Chapter 4);

viii). isolation of mRNA from *T. ferrooxidans* ATCC 33020 to identify the size of the *atp* operon transcript expressed *in vivo*, and isolation of mRNA from *E. coli* to locate transcriptional start site/s and sizes of *T. ferrooxidans atp* transcripts expressed in *E. coli unc*⁻ mutants (Chapter 5).

This study reports on the characterisation of seven of the *atp* genes of *T. ferrooxidans* ATCC 33020 and on various aspects related to the functioning of these genes and gene-products in the heterologous host, *E. coli*.

CHAPTER 2.

THE ISOLATION OF *T. FERROOXIDANS ATP GENES.*

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THE ISOLATION OF *T. FERROOXIDANS ATP* GENES.

2.0 Summary.

Eight *E. coli unc* mutant strains were screened with *T. ferrooxidans* ATCC 33020 gene banks. Mutants used were AN727 (a^-), AN943 (c^-), AN1440 (b^-), AN730 (α^-), AN1273 (γ^-), AN818 (β^-), AN802 (ε^-) and DK8 (Δunc). Using the ability to grow on minimal succinate agar plates as the selection method, many *T. ferrooxidans* F_1-complementing plasmids and cosmids were isolated from the *E. coli* AN mutants. Two plasmids, p*Tfatp*1 and p*Tfatp*2, which contained overlapping fragments of *T. ferrooxidans* chromosomal DNA cloned in opposite orientations in the vector p*Eco*R251, were selected for further study. No plasmids or cosmids which complemented *E. coli unc* AN F_0, or DK8 mutants were isolated.

"(*E. coli*) *is responsible for few infections but probably for more scientific papers than any other living organism.*" Chargaff, (1978).

2.1. Introduction.

The lack of an efficient genetic system for *T. ferrooxidans* and the fact that there are no mutants characterised for the species, had a marked impact on the isolation and subsequent study of the *T. ferrooxidans* ATCC 33020 *atp* genes. Therefore, these two aspects will be briefly reviewed and the implications for the current study discussed.

The absence of a well-developed genetic system for *T. ferrooxidans* precludes the use of routine molecular biological techniques such as transformation, transduction and conjugation with the organism. Three requirements are necessary for the development of a genetic system in *T. ferrooxidans*. These are the availability of a suitable plasmid vector, selectable markers and vector transfer (Rawlings *et al.*, 1991).

A number of naturally-occurring plasmids have been isolated from populations of *T. ferrooxidans*. In the development of a genetic system for *T. ferrooxidans*, such plasmids could be used to construct shuttle vectors to replicate in *E. coli* and *T. ferrooxidans*. Two plasmids isolated from *T. ferrooxidans* viz. pTF1 and pTF-FC2, were extensively studied in the laboratories of Lau and Rawlings respectively. In common with all *T. ferrooxidans* plasmids isolated to date, the plasmids appear to be cryptic and have no distinct phenotype (reviewed in Rawlings and Kusano, 1994).

The development of a selective marker for a genetic system in *T. ferrooxidans* has proved to be difficult. Commonly used markers in heterotrophic bacteria are antibiotic resistance genes but to date, all antibiotics screened have been susceptible to the combined effects of low pH and high inorganic ions associated with the growth media of *T. ferrooxidans* (Rawlings *et al.*, 1991). An alternate possibility is the use of plasmid-borne inorganic-ion resistance genes such as mercury, arsenic and silver. Spontaneous mutation in *T. ferrooxidans* creates a problem in the choice of a reliable genetic marker in *T. ferrooxidans*. Cations which were once thought to be toxic to the organism, are now no longer considered to be so, as resistant strains of *T. ferrooxidans* were isolated. Resistance to arsenic, mercury, uranium and molybdenum has been frequently reported (Di Spirito and Tuovinen, 1982; Rawlings *et al.*, 1991; Shiratori *et al.*, 1989; Sugio *et al.*, 1988b and 1992a). The use of mercury-resistance genes as markers in mercury-sensitive *T. ferrooxidans* strains was reported. However, the use of mercury resistance is not stringent enough, as the difference in threshold values reported for $HgCl_2$ tolerance between the original host and the transformant was small (Kusano *et al.*, 1992a).

Vector transfer in *T. ferrooxidans* has proved to be a major problem. Common procedures for transferring DNA into bacterial cells include transduction, transformation and conjugation. No bacteriophages which infect *T. ferrooxidans* have been reported, hence transduction of the species has not been possible (Rawlings *et al.*, 1991).

Attempts to transform either *T. ferrooxidans* whole cells and/or sphaeroplasts by conventional means were unsuccessful (Rawlings *et al.*, 1991). Transformation of most *T. ferrooxidans* strains by electroporation proved fruitless. Recently, 30 independent strains of mercury-sensitive *T. ferrooxidans* were electroporated with a mercury-resistant shuttle vector. A low frequency of transformation of one strain, Y4-3 was noted. The transformed plasmid was stably inherited and replicated in *T. ferrooxidans* Y4-3 (Kusano *et al.*, 1992a). To date, this is the only strain for which transformation has been successful.

No *T. ferrooxidans* mutants have been characterised, which would allow the identification of cloned *T. ferrooxidans* genes by expression in mutated phenotypes. As an alternative, the genetically well-characterised *E.coli* is often used as a heterologous host system for cloned *T. ferrooxidans* genes. Both *T. ferrooxidans* and *E. coli* are gram-negative eubacteria, and are phylogenetically related. Both genera occur within the proteobacteria; *E. coli* within the γ and *T. ferrooxidans* within the β subdivisions (Chapter 1, Fig. 1.1). A commonly used approach is to screen *T. ferrooxidans* gene banks by complementation in defined *E.coli* mutants. *T. ferrooxidans* genes isolated in this manner include some of those associated with nitrogen metabolism (reviewed in Rawlings *et al.*, 1991; Rawlings and Kusano, 1994), the DNA repair gene, *recA* (Rawlings *et al.*, 1991) and genes associated with sulphur assimilation (Fry and Garcia, 1989). The use of *E.coli* as a heterologous host for the study of *T. ferrooxidans* genes is limited. Control of gene expression cannot be meaningfully pursued, as the manner of gene expression in *T. ferrooxidans* may be entirely different from the way in which these genes are expressed in *E. coli*. Furthermore, the heterotrophic *E. coli* cannot be used to isolate the *T. ferrooxidans* genes associated with nitrogen and carbon dioxide fixation, and the important iron and sulphur dissimilatory pathways, as appropriate *E. coli* mutants do not exist. Of particular relevance to this study is the fact that *T. ferrooxidans* genes associated with the production of cytoplasmic membrane proteins common to both *T. ferrooxidans* and *E. coli* may not be functional in *E. coli*, due to inherent differences between acidophilic and neutrophilic cytoplasmic membrane systems.

For the purposes of the current study, in the absence of both a genetic system for *T. ferrooxidans* and suitable *T. ferrooxidans atp* mutants, the best alternatives are either screening a *T. ferrooxidans* gene bank using a probe from a previously cloned *atp/unc* gene (some of which are highly conserved), or attempting to complement *E. coli unc* mutants. Unlike *T. ferrooxidans*, many *E. coli* mutants deficient in all the subunits of F_1F_0

ATPsynthase are available for use in screening by complementation. This is the approach that was used. There have been many reports on the successful use of *E. coli unc* mutants as heterologous hosts for the isolation *atp* genes from other prokaryotic species. These reports are reviewed in Section 2.4.

A selection method used for a heterotrophic bacterium possessing a functional F_1F_0 ATPsynthase involves the ability of the cell to metabolise succinate as a sole carbon source. Certain *E. coli* mutants deficient in F_1F_0 ATPsynthase are unable to utilise the tricarboxylic acid cycle (TCA) to generate ATP via oxidative phosphorylation, although the respiration rate of these cells is normal. These organisms cannot grow on minimal medium with succinate as the sole non-fermentable carbon source. If the organism concerned is a facultative anaerobe (such as *E. coli*), glucose may be used as the sole fermentable carbon source (reviewed in Futai and Kagawa, 1983). During the current study, the restoration of the ability of defined *E. coli unc* mutants transformed with *T. ferrooxidans* ATCC 33020 gene banks, to utilise succinate as the sole non-fermentable carbon source, was used as the selection method for *T. ferrooxidans atp* genes.

The disadvantage of *E. coli* as the selected system for this study is the fact that the bacterium is a neutrophile. This could present problems for the isolation of those subunits of F_1F_0 ATPsynthase which are associated with the passage and regulation of protons through the cytoplasmic membrane. Also of significance is the fact that *E. coli* is heterotrophic, and as such has to generate $\Delta\mu_{H+}$ by the active extrusion of protons and/or potassium ions across the cytoplasmic membrane (Booth, 1985). Hence mechanisms of generation of $\Delta\mu_{H+}$ and associated pH_i homeostasis between *T. ferrooxidans* and *E. coli* are possibly different. This could influence functional complementation of *E. coli unc* mutants by *T. ferrooxidans atp* genes, particularly if F_1F_0 ATPsynthase in *T. ferrooxidans* is involved in the generation and maintenance of $\Delta\mu_{H+}$, and the associated mechanisms of pH_i homeostasis (i.e. interconversion of ΔpH and $\Delta\Psi$) between the two organisms are incompatible.

This chapter describes the isolation of *T. ferrooxidans atp* genes by functional complementation of *E. coli* F_1 mutants. Significantly, functional complementation of *E. coli* F_0 mutants used in this study by *T. ferrooxidans atp* genes, was not observed.

2.2. Materials and Methods.

2.2.1. Bacterial strains, plasmids, and media.

Bacterial strains, plasmids and their relevant genotypes are listed in Table 1, Appendix A. In brief, *E. coli unc* mutant strains used were; *E. coli unc* point mutants, AN727 (a^-), AN943 (c^-), AN1440 (b^-), AN730 (α^-), AN1273 (γ^-), AN818 (β^-) and AN802 (ε^-); and strain DK8 from which the entire *unc* operon had been deleted (Δunc). *T. ferrooxidans* ATCC 33020 was grown in 9K inorganic medium (Silverman and Lundgren, 1959) and was used as the source of chromosomal DNA. *E. coli* strains were routinely maintained on Luria-Bertani medium (LB) solidified with 1.5% agar (LBA) (Appendix B). When necessary, LBA was supplemented with ampicillin (Ap) at a concentration of 100 µg/ml. Minimal succinate medium (MMS) supplemented with Ap at 50 µg/ml, made as described by Gibson *et al.* (1977) was used for the selection of *E. coli* AN *unc* mutants transformed or transduced with *T. ferrooxidans atp* plasmids or cosmids; MMS medium for *E. coli* DK8 was that described by Klionsky *et al.* (1984) (Appendix B). Where *E. coli unc* mutants were transformed with pAN45 (*uncBEFHAGD*), chloramphenicol (Cm) was used in the growth media at 25 µg/ml.

2.2.2. General techniques.

E. coli AN cells were made competent by the standard rubidium chloride method described by Maniatis *et al.* (1982) and *E. coli* DK8 by the dimethyl sulfoxide (DMSO) method of Chung and Miller (1988). For the latter method, bacterial cells were grown in 25 ml LB (Appendix B) at 37°C, to OD_{600} 0.3-0.6. Cells were harvested by centrifugation at 5 000 rpm in a Beckman JA-21 rotor for 10 min at 4°C, and were resuspended in 0.1 volume of TSB, at 4°C for 10 min. TSB was constituted as follows:- LB (pH 6.1) with 10% PEG (polyethylene glycol) (M_r = 3 350), 5% DMSO (dimethyl sulfoxide), 10 mM $MgCl_2$ and 10 mM $MgSO_4$. To transform, 100 µl aliquots of chilled cells/TSB were pipetted into chilled microfuge tubes and mixed with 100 pg plasmid DNA. The mix was returned to ice for 30 min and was expressed for an hour at 37°C in 0.9 ml TSB before being plated out onto appropriate agar selection plates.

The preparation, restriction endonuclease mapping and manipulation of plasmids and cosmids were carried out using standard procedures (Ausubel *et al.*, 1993; Sambrook *et al.*, 1989). All enzymes and buffers used were supplied by Boehringer Mannheim, and were used according to manufacturer's instructions.

2.2.3. Construction of the *T. ferrooxidans* genomic library.

Chromosomal DNA was prepared from *T. ferrooxidans* ATCC 33020 as follows. *T. ferrooxidans* ATCC 33020 was inoculated into 10 l of sterile 9K medium (Silverman and Lundgren, 1959). The culture was vigorously aerated at 30°C, for approximately ten days until the ferrous iron was oxidised. The cells were harvested by centrifugation at

10 000 rpm in a Beckman JA-14 rotor. The pellets of cells were resuspended and washed twice in water/H_2SO_4, pH 1.8. The pellets were resuspended in 4 ml of a 25% sucrose, 2 mM EDTA, 50 mM Tris-Cl buffer (pH 8.0) and frozen at -20°C for 1 h. Proteinase K (20 mg/ml final concentration) was added to the frozen cells and the samples were shaken at ambient temperature until they had thawed. Sodium dodecyl sulphate (SDS) was added to a final concentration of 1%, and the samples were held on ice for 15 min, followed by RNase (50 µg/ml) digestion for 20 min at 37°C. The viscous opaque, brown-coloured samples were dialysed at room temperature for 24-60 h against numerous changes of TE buffer (pH 8.0) until they became translucent. Protein was removed from the samples by three phenol-chloroform-*iso*-amyl alcohol (25:24:1) extractions, followed by two diethyl-ether extractions. The DNA purification procedure was completed by an overnight dialysis against TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0) at room temperature.

A plasmid gene bank of *T. ferrooxidans* ATCC 33020 chromosomal DNA was prepared by cloning sized (4-10 kb) partial *Sau*3AI fragments into the *Bgl*II site of the p*Eco*R251 vector (Ramesar *et al.*, 1988). A cosmid bank of larger (36-45 kb) chromosomal *Sau*3AI fragments was prepared by cloning the fragments into the *Bam*HI site of pHC79 (Ramesar, 1988).

2.2.4. Isolation of *T. ferrooxidans atp* genes.

Competent *E. coli* AN and DK8 cells were transformed with the plasmid gene bank. Vegetatively growing *E. coli* AN and DK8 strains were transduced with the cosmid bank, using standard procedures (Sambrook *et al.*, 1989). Bacterial transformants/transductants containing *T. ferrooxidans atp* genes were selected by the ability to grow at 37°C on MMS plates, supplemented with Ap. Prior to plating onto the minimal medium, the transformed/transduced cells were washed twice in sterile 0.1% NaCl. To confirm that the ability to grow on succinate was plasmid/cosmid-associated, plasmids and cosmids were re-isolated by the small scale alkaline lysis method (Sambrook *et al.*, 1989) and used to re-transform the *E. coli* AN mutants.

2.2.5. Southern hybridisation.

To confirm the origin of the cloned *T. ferrooxidans atp* genes, Southern hybridisation of two of the plasmids, p*Tfatp*1 and p*Tfatp*2 was carried out against *Bgl*II and *Pst*I digests of *T. ferrooxidans* chromosomal DNA. Standard procedures were followed for the digestion of chromosomal DNA, and preparation of the plasmid ^{32}P probes (Ausubel *et al.*, 1993; Sambrook *et al.*, 1989). Hybridisation and blotting was done according to the methods recommended by Amersham for their Hybond N^+ membranes.

2.3. Results.

2.3.1. The complementation of *E. coli unc* mutants by *T. ferrooxidans atp genes.*

After an incubation period lasting approximately 7-10 days at 37°C, a number of colonies from *E. coli unc*⁻ mutants transformed or transduced with the *T. ferrooxidans* gene banks, developed on MMS medium supplemented with Ap. Such colonies were selected for further study. Approximately 40 different plasmids and 50 cosmids that complemented at least one of the *E. coli* F_1 mutants AN730 (α^-), AN1273 (γ^-), AN818 (β^-), and AN802 (ε^-), were isolated. No plasmids or cosmids capable of complementing any of the three *E. coli* F_0 mutants, AN727 (a^-), AN943 (c^-) and AN1440 (b^-), or the *E. coli unc* deletion strain DK8 (Δ*unc*) were found (Table 2.1).

Table 2.1. Genetic complementation of *E. coli unc*⁻ mutants with plasmid-borne *T. ferrooxidans atp* DNA.

Growth (+), or no growth (-) on minimal succinate medium was scored after 4-10 days incubation at 37°C. Positive control was pAN45. Negative controls were pACYC184 and p*Eco*R251.

	Mutants							
Plasmids	**AN727 (a^-)**	**AN943 (c^-)**	**AN1440 (b^-)**	**AN730 (α^-)**	**AN1273 (γ^-)**	**AN818 (β^-)**	**AN802 (ε^-)**	**DK8 (Δ*unc*)**
p*Tfatp*1	—	—	—	—	—	+	+	—
p*Tfatp*2	—	—	—	+	+	+	+	—
pAN45	+	+	+	+	+	+	+	+

Restriction endonuclease digestion of the complementing plasmids revealed the presence of a common 3.4 kb *Bgl*II fragment. Several of the plasmids isolated from one or other of the F_1 mutant strains were tested to determine whether they would complement the rest of the F_1 mutant strains. Two plasmids were chosen for further study. These were p*Tfatp*1, which complemented the β^- and ε^- strains, and p*Tfatp*2, which complemented the α^-, γ^-, β^-, and ε^- strains (Table 2.1). These two plasmids were mapped using various restriction endonucleases (Fig. 2.1).

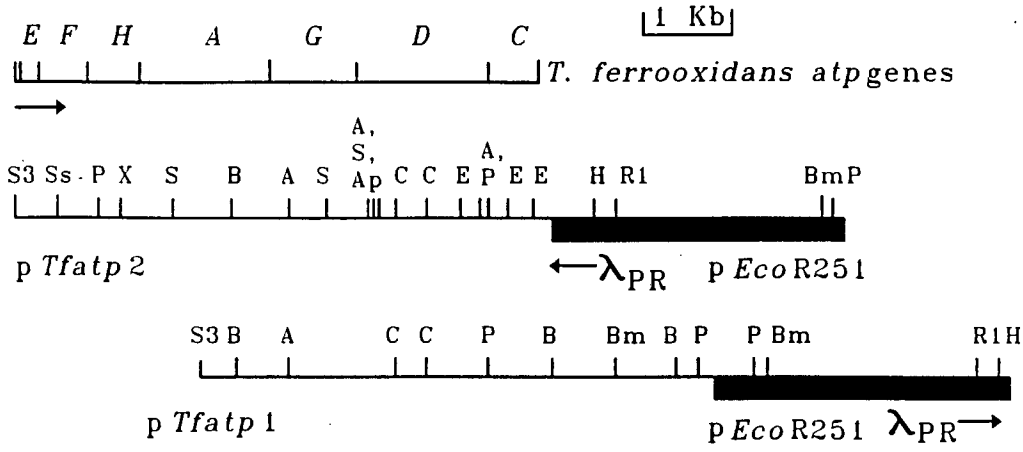

Fig. 2.1. Partial restriction endonuclease maps of p*Tfatp*1 and p*Tfatp*2 aligned against the *T. ferrooxidans* ATCC 33020 *atp* genes. Key to restriction endonucleases: A=*Ava*I; Ap=*Apa*I; B=*Bgl*II; Bm=*Bam*HI; C=*Cla*I; E=*Eco*RV; H=*Hin*dIII; P=*Pst*I; R1=*Eco*R1; S=*Sal*I; S3=*Sau*3AI; Ss=*Sst*I; X=*Xho*I. Arrows indicate direction of transcription; λ_{PR}, phage lambda rightward promoter.

From the restriction map, it was evident that the two plasmids contained overlapping fragments of *T. ferrooxidans* chromosomal DNA, cloned in opposite orientations in the p*Eco*R251 vector.

Using results from the restriction enzyme mapping and Southern hybridisation experiments of p*Tfatp*1 and p*Tfatp*2, the 50 F_1-complementing cosmids were studied to determine whether they had a fragment of *T. ferrooxidans* chromosomal DNA which extended upstream of the *Sau*3AI site mapped at the 5'-end of p*Tfatp*2. Details of this study, where it was shown that selected cosmids did not extend beyond this point, are described in Chapter 5.

2.3.2. Southern hybridisation of p*Tfatp*1 and p*Tfatp*2 against *T. ferrooxidans* chromosomal DNA.

The source of the cloned DNA was confirmed by hybridisation of labelled p*Tfatp*1 and p*Tfatp*2 to *T. ferrooxidans* ATCC 33020 chromosomal DNA. The 3.4 kb and 1.35 kb *Bgl*II fragments internal to the cloned *T. ferrooxidans* fragment of p*Tfatp*1 corresponded exactly to the cloned *T. ferrooxidans* fragment on the *T. ferrooxidans* chromosome. Likewise, the 4.3 kb *Pst*I fragment internal to the cloned fragment of p*Tfatp*2 corresponded to a fragment of equal size in a *Pst*I digest of the *T. ferrooxidans* chromosome (Fig. 2.2).

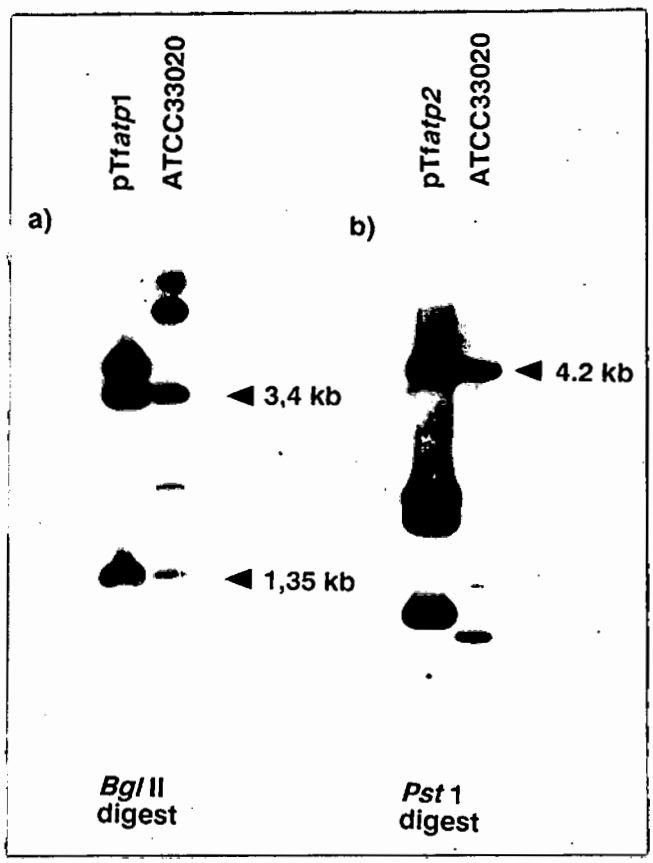

Fig. 2.2. Hybridisation of p*Tfatp*1 and p*Tfatp*2 to total DNA from *T. ferrooxidans* ATCC 33020. (a) ^{32}P-labelled p*Tfatp*1 hybridised to *Bgl*II fragments of total DNA from *T. ferrooxidans*, and a *Bgl*II digest of p*Tfatp*1. (b) ^{32}P-labelled p*Tfatp*2 hybridised to *Pst*I fragments of total DNA from *T. ferrooxidans* and a *Pst*I digest of p*Tfatp*2.

2.4. Discussion.

The use of *E. coli unc* mutants as the heterologous host for the cloning of various *atp* genes from other bacteria has been frequently reported. F_1F_0 ATPsynthase genes for *Salmonella typhimurium* (Hsu *et al.*, 1984, cited by Kauffer *et al.*, 1987), *Klebsiella pneumoniae* (Kauffer *et al.*, 1987), *E. hirae* (Shibata *et al.*, 1992), and strain PS3 (reviewed in Futai *et al.*, 1989) were cloned in *E. coli*. Complementation of *E. coli unc* mutants by *atp* genes from other organisms has also been reported. These include, in addition to species mentioned above, *Streptococcus mutans* (Quivey *et al.*, 1991), *Enterobacter aerogenes* and *Flavobacterium ferrugineum* (Amann *et al.*, 1988a), *P. modestum* (Kaim *et al.*, 1992), *V. alginolyticus* (Krumholz *et al.*, 1990), *Bacillus megaterium* (Hawthorne and Brusilow, 1986; Scarpetta *et al.*, 1991), and chloroplasts (reviewed by Engelbrecht and Junge, 1992). Mukhopadhyay *et al.* (1992) obtained the heterologous expression of *Saccharomyces cerevisiae* F_1F_0 ATPsynthase OSCP in *E. coli*. The expressed protein was indentical to yeast mature OSCP, and was fully re-constitutable in OSCP-depleted membranes.

During the present study it was found that in spite of the acidophilic nature of *T. ferrooxidans*, genes encoding the F_1 subunits of F_1F_0 ATPsynthase were readily isolated by selection for the ability of *E. coli* AN F_1 point mutants to grow on MMS. It was noted however, that the time required for the growth of the transformants was far longer (up to 10 days) than was the case for *E. coli unc* mutants transformed with pAN45 (*unc*+), (3-5 days). This indicated that the complementation of *E. coli unc* mutants was such that the F_1F_0 ATPsynthase functioned inefficiently. Possible reasons for this are discussed in Chapter 4. As all of the AN mutants used were *recA*-, growth of the transformed strains on MMS was a result of complementation by *T. ferrooxidans* F_1 gene products, rather than by homologous recombination. It was however possible that α-complementation between the mutant *E. coli* F_1F_0 ATPsynthase subunits and the homologous *T. ferrooxidans* polypeptides may have occurred. Further complementation studies, where it was demonstrated that the *T. ferrooxidans* F_1 gene products were functional in *E. coli*, are described in Chapter 4.

Restriction endonuclease mapping of p*Tfatp*1 and p*Tfatp*2 showed that the *T. ferrooxidans atp* genes were cloned in opposite directions in *pEco*R251 (Fig. 2.1). Alignment of the 5.946 kb *Sau*3AI-*Bgl*II *T. ferrooxidans* chromosomal fragment of p*Tfatp*2 against the *E. coli unc* operon indicated that the *T. ferrooxidans atp* operon promoter had probably not been cloned. It was therefore likely that in *E. coli* F_1 mutants complemented by p*Tfatp*2, which was cloned against the p*Eco*R251 λ promoter, the *T. ferrooxidans* F_1 genes were expressed off a promoter located within the p*Tfatp*2 fragment, upstream of *atpH*. Attempts to map this promoter sequence on the *T. ferrooxidans atp* chromosomal fragment are described in Chapter 5.

Despite many attempts, no *T. ferrooxidans* ATCC 33020 plasmids were isolated which were able to complement *E. coli* F_0 mutants during this study. Preliminary studies with F_0 AN mutants reported a similar result (Dennehy, 1989; D.E. Rawlings, personal communication). It was considered possible that the results reported from the latter studies could have been due to the low transformation frequencies (1×10^2 transformants/μg DNA) routinely reported for *E. coli* AN mutants using either the DMSO (Chung and Miller, 1988) or routine calcium chloride (Sambrook *et al.*, 1989) methods for preparing competent cells. However, despite improving the transformation frequency of the mutants to 4×10^6 colonies/μg supercoiled plasmid DNA by using the rubidium chloride method (Maniatus *et al.*, 1982), no F_0-complementing plasmids were isolated during the current study. Transformation frequencies obtained for *E. coli* DK8 (Δ*unc*) prepared by the DMSO method (Chung and Miller, 1988) were consistently high at 2×10^9 transformants/μg supercoiled plasmid DNA. No complementation of this strain on MMS by *T. ferrooxidans* ATCC 33020 plasmids was observed.

Using results obtained from either *Bam*HI or *Bgl*II restriction endonuclease mapping, common band patterns were used to divide the AN F_1-complementing plasmids into six different groups. In the current and preliminary studies (Dennehy, 1989; D. E. Rawlings, personal communication) extensive restriction enzyme mapping and Southern hybridisation studies of representative plasmids from each group showed that none of the plasmids extended to the 5'-end of the *Sau*3AI site present on p*Tfatp*2 (Fig. 2.1) (results not shown). Extensive screening of *E. coli* AN and DK8 mutants with a *T. ferrooxidans* ATCC 33020 cosmid gene bank resulted in the isolation of 50 AN F_1-complementing cosmids; no cosmids were isolated which complemented either *E. coli* AN F_0 or DK8 mutants. Preliminary studies with *E. coli* AN mutants reported similar results (D. E. Rawlings, personal communication). Experiments with cosmids isolated during the current study, where it was shown that those chosen for further investigation did not appear to extend beyond the 5'-end of the *Sau*3AI site on p*Tfatp*2 (Fig. 2.1) are described in Chapter 5.

It is possible that the "missing" *atp* gene/s were not represented in the *T. ferrooxidans* ATCC 33020 gene banks used. However, both the plasmid and cosmid libraries used for screening the *E. coli* AN and DK8 mutants were reported to have a confidence level several-fold higher than that required for a library of 99% confidence prepared from the *T. ferrooxidans* genome (Ramesar, 1988). Therefore, the libraries used may be considered representative of the *T. ferrooxidans* chromosome, and it is unlikely (although not definite) that the "missing" genes were not present in the banks.

It is likely that because of the difference in the transmembrane pH gradient between the two bacterial species, the *T. ferrooxidans* F_0 subunits were not functional in a neutrophilic organism such as *E. coli*. This is discussed further in Chapters 4 and 5. In a study on the isolation of *E. hirae atp* genes by complementation of *E. coli unc* mutants, Shibata *et al.* (1992) noted a similar phenomonen. They reported that regions of the *E. hirae atp* operon, notably upstream of *atpB* and in an *atpG-D-C* region, were unstable in *E. coli* and could not be cloned by complementation on MMS. Even after isolating these regions by PCR-based methods and cloning them into a suitable vector, the DNA was unstable in transformed *E. coli* cells. Furthermore, the nucleotide sequence of the upper region of *atpE* gene was the same in all clones isolated independently, which suggested a site-specific deletion. The interpretation offered was that these genes may be harmful to *E. coli*, and that the organism possesses some system/s to remove such genes at specific sites; however, a common sequence was not found in the two regions reported in the study. Similarly, Quivey *et al.* (1991) demonstrated that *Streptococcus mutans atpD* plasmids which complemented *E. coli uncD* mutants on MMS were unstable on selective medium in the host background used. These authors had to use PCR as an alternative screening method for cloning the ATPase genes from the streptococci. In the present and preliminary studies (D.E. Rawlings, personal communication) it was of interest to note that when 24

T. ferrooxidans uncA mutant-complementing plasmids were mapped, several plasmids terminated at the same *Sau*3AI position recorded for p*Tfatp*2 (results not shown). It is therefore possible that a mechanism similar to that noted by Shibata *et al.* (1992) was operative.

In order to expedite the possible isolation of the entire *T. ferrooxidans atp* operon, it was decided to continue studies with p*Tfatp*2, which contained a larger upstream *T. ferrooxidans atp* chromosomal fragment than did p*Tfatp*1 (Fig. 2.1). Sequencing of the *T. ferrooxidans* ATCC 33020 chromosomal insert present on p*Tfatp*2 was used to identify which of the *T. ferrooxidans atp* genes had been cloned. Based on results obtained from this, further strategies were devised to attempt to isolate the entire *T. ferrooxidans atp* operon. (Chapters 3 and 5).

The complementation of *E. coli unc* mutants by *T. ferrooxidans atp* genes reported on in this chapter suggested that control of proton translocation in *T. ferrooxidans* F_1F_0 ATPsynthase was largely a function of the F_0 subunits, which did not complement the neutrophilic *E. coli.* Since observed growth rates on MMS in the F_1-complementing *T. ferrooxidans* plasmids in AN point mutants were noticeably slower than *E. coli* DK8 (pAN45), a role for the *T. ferrooxidans* F_1 subunits cannot be entirely excluded (Chapter 4). Results presented in this section demonstrated the disadvantages of the lack of a suitable genetic system for *T. ferrooxidans* ATCC 33020. Whilst the use of *E. coli* as a heterologous host for isolating genes coding for common cytoplasmically-located proteins is possible, it would appear that functional complementation of the organism cannot be successfully used to clone genes which transcribe membrane-associated proteins associated with proton translocation, even if the proteins are functionally homologous.

CHAPTER 3.

DNA SEQUENCE OF THE *T. FERROOXIDANS ATP* GENES, AND AMINO ACID COMPARISONS OF THE GENE PRODUCTS WITH *ATP* PROTEINS FROM OTHER ORGANISMS.

CHAPTER 3.

DNA SEQUENCE OF THE *T. FERROOXIDANS ATP* GENES, AND AMINO ACID COMPARISONS OF THE GENE PRODUCTS WITH *ATP* PROTEINS FROM OTHER ORGANISMS.

3.0. Summary.

The nucleotide sequence of the entire 5.946 kb *T. ferrooxidans Sau*3AI-*Bgl*II chromosomal fragment which complemented *E. coli* AN F_1 *unc* mutants was determined. Seven complete open reading frames were identified. These open reading frames had high homology to known F_1F_0 ATPsynthase subunits, and were identified as c (*atpE*), b (*atpF*), δ (*atpH*), α (*atpA*), γ (*atpG*), β (*atpD*) and ε (*atpC*). The seven genes occurred as a contiguous group, and the F_0 genes were located to the 5'-end of the F_1 cluster. At the 3'-end of the F_1 genes, the operon was terminated by a typical *rho*-independent terminator. Hence the operon arrangement was similar to that recorded for *E. coli unc*. Downstream of the *atp* operon terminator was an incomplete unidentified reading frame (URF) of 42 amino acids. Analysis of the nucleotide sequence showed that in general, codon usage by the *atp* genes was typical of that recorded for *T. ferrooxidans*. There were some unusual trends noted, particularly for *atpE*. Examination of the nucleotide sequence of the translation intiation regions of the *T. ferrooxidans atp* operon indicated that at the post-transcriptional level, control of gene expression could be similar to that exercised by *E. coli*. An internal putative σ^{70} promoter, which could have been recognised by *E. coli* for transcription of the *T. ferrooxidans* F_1 genes, was tentatively identified towards the 3'-end of *atpF*.

Analysis of the predicted primary sequence for the seven open reading frames showed that despite the fact that *T. ferrooxidans* is an obligately acidophilic chemoautotroph, six of the F_1F_0 ATPsynthase subunits (b, δ, α, γ, β and ε) were most like those of the γ-proteobacteria *E. coli* and *V. alginolyticus*. The exception was the c subunit which, when compared with the primary sequence of the proteolipid from 14 other organisms, formed a distinct out-group. Further analyses of the deduced primary sequence of the seven *T. ferrooxidans* F_1F_0 ATPsynthase subunits demonstrated that overall, regions of the enzyme known to be concerned with catalytic mechanisms, were conserved. However, certain differences in the qualitative constitution of the subunits suggested an adaptation to an acidophilic mode of life. These differences were observed in both the F_0 and F_1 subunits. The c (F_0) subunit had many features unique to *T. ferrooxidans*, collectively thought to indicate an unusual mode of proton translocation. Amongst the F_1 subunits, δ, γ, β and ε had N- and/or C- termini which were unique, and could indicate an unusual mode of interaction between the F_1 and/or F_0 subunits, related to the gating of protons in an extreme acidophile.

"The evidence presented supports the belief that a nucleic acid of the desoxyribose type is the fundamental unit of the transforming principle of Pneumococcus Type II." Avery *et al.* (1944). (The elucidation of the chemical nature of heredity).

3.1. Introduction.

The ability to determine the nucleotide sequence of the DNA molecule has provided a means of ascertaining the structure and frequently, the function of genes. Moreover, the sequence of a given gene may be used to deduce the amino acid/primary sequence of the protein transcribed by that gene. Hence DNA sequencing is of great significance in the linking of genotype to phenotype.

The nucleotide sequences for the *atp* genes encoding proton-translocating ATPsynthase has been determined from a wide variety of eukaryotic and prokaryotic organisms. Eukaryotic genes include those encoding mitochondrial and chloroplast F_1F_0 ATPsynthase (eg. Walker *et al.*, 1985; Bird *et al.*, 1985). Particularly relevant to this study has been the sequencing of *atp* genes which encode part or all of the operon for F_1F_0 ATPsynthase in prokaryotes. Bacteria studied encompass a wide range of interesting phenotypes. Some eubacterial species include:-

i). Gram-negative, heterotrophic, neutrophilic, facultative anaerobes, such as *E. coli* (Walker *et al.*, 1984a), *Enterobacter aerogenes* (Amann *et al.*, 1988a) and the halophilic *V. alginolyticus* (Krumholz *et al.*, 1989).

ii). The gram-negative heterotrophic anaerobe, *Flavobacterium ferrugineum* (Amann *et al.*,1988a).

iii). The heterotrophic aerobic acidophile, *Acido caldarius*, origin unknown (Hoppe and Sebald, 1984).

iv). The gram-positive, heterotrophic, aerobic neutrophile, *Bacillus megaterium* (Brusilow *et al.*, 1989).

v). Gram-positive, heterotrophic, aerobic alkaliphiles, *Bacillus firmus* RAB (Mack Ivey and Krulwich, 1990) and *Bacillus firmus* OF4 (Mack Ivey and Krulwich, 1991).

vi). The gram-positive, heterotrophic, aerobic, thermophilic *Bacillus*, strain PS3 (Ohta *et al.*, 1988).

vii). The gram-positive, heterotrophic anaerobic neutrophile, *E. hirae* (Hoppe and Sebald, 1984; Shibata *et al.*, 1992).

viii). The gram-negative photosynthetic autotrophs, *Rhodospirillum rubrum* (Falk *et al.*, 1985; Falk and Walker, 1988), *Rhodopseudomonas blastica* (Tybulewicz *et al.*, 1984), *Anabaena* sp. Strain PCC 7120 (Curtis *et al.*, 1987; McCarn *et al.*, 1988), *Synechococcus* 6301 (Cozens and Walker, 1987) and *Synechocystis* 6803 (Werner *et al.*, 1989).

ix). Certain species which have an unusual F_1F_0 ATPsynthase. These include that from *V. parahaemolyticus* which is stimulated by the presence of high concentrations of anions such as sulphate (Sakai *et al.*, 1990; Sakai-Tomita *et al.*, 1992), and the F_1F_0 ATPsynthase of the obligately anaerobic marine *P. modestum* (Esser *et al.*, 1990; Krumholz *et al.*, 1992, Kaim *et al.*, 1992) which translocates sodium ions (Dimroth, 1992).

From amongst the archaebacterial species, the *atp* operon encoding the proton-translocating ATPsynthase from *S. acidocaldarius* was sequenced in its entirety (Denda *et al.*, 1989 and 1990).

The collective nucleotide sequence data from the various *atp* genes sequenced has provided detailed information regarding gene arrangement and qualitative make-up of the individual genes. Moreover, the primary structure of the protein products of the *atp* genes of many organisms has been predicted. Much has been learned regarding transcriptional and translational control of the *unc* operon, protein domains associated with catalysis, proton conductance, and enzyme assembly and structure in *E. coli*, by utilising the data available from the nucleotide sequence.

The nucleotide sequence for the *unc/atp* operons from many of the organisms listed above is now available in the GENEMBL/Genbank/DDBJ data bases. Comparative analyses of this data have been invaluable in providing information regarding genetic regions which have a regulatory function, and F_1F_0 ATPsynthase domains/residues which are involved in catalysis, enzyme structure/assembly, and proton conductance in organisms additional to *E. coli*. Comparative analyses provided guides as to evolutionary relationships between organisms, and supported the endosymbiotic theory of mitochondrial and chloroplast origin.

Prior to this study, the nucleotide sequence for *atp* genes encoding the proton-translocating ATPsynthase of an obligately acidophilic aerobic chemoautotroph was not available. This chapter reports on the sequencing of the *T. ferrooxidans* ATCC 33020 *atp* genes cloned in *E. coli unc* mutants, as described in Chapter 2. The objectives of this section of the study were:-

i). to confirm whether the cloned genes were those responsible for encoding the proton-translocating ATPsynthase in *T. ferrooxidans* ATCC 33020

ii). to establish the genetic constitution of the *atp* operon in *T. ferrooxidans* ATCC 33020,

iii). to examine codon frequency and usage data of the cloned genes, to determine whether it was similar to, or different from that recorded for other *T. ferrooxidans* ATCC 33020 chromosomal genes sequenced to date,

iv). by comparing the nucleotide sequence data available in the databases to,

-ascertain whether the operon arrangement and predicted enzyme primary structure were typical of the eubacterial and/or archaebacterial types;

-identify regions in the operon which may have regulatory functions;

-establish whether primary protein sequences of individual proteins subunits could be used to categorise *T. ferrooxidans* phylogenetically/taxonomically within the bacteria;

-locate domains in the protein products which may have a catalytic function;

-identify domains/residues in the protein subunits which may represent unusual modes of translocation and gating of protons and/or energy coupling, related to the acidophilic environment.

3.2. Materials and Methods.

3.2.1. Bacterial strains.

E. coli strains LK111 (Appendix A) or JM109 (Appendix A) were used to propagate and subclone all the *T. ferrooxidans* DNA fragments of p*Tfatp*2 required for nucleotide sequencing.

3.2.2. Media, buffers and enzymes.

Restriction endonucleases, T4 ligase, S1 nuclease and exonuclease III were purchased from Boehringer Mannheim. All buffers used were those supplied or recommended by the manufacturers.

3.2.3. Nucleotide sequencing.

In order to sequence the entire p*Tfatp*2 fragment, *T. ferrooxidans* ATCC 33020 chromosomal DNA was digested into four separate but contiguous fragments. The plasmid vectors used for ligation of the DNA segments were, pUC18 (Norrander *et al.*, 1983), pUCBM21 (Boehringer Mannheim) and pBluescriptSK+ (Stratagene, California) (Appendix D). The constructs were labelled as p*Tfatp*100, p*Tfatp*200, p*Tfatp*300, and p*Tfatp*500 (Fig. 3.1). Details of plasmid construction are given in Appendix A. All plasmids were caesium chloride purified, and prepared from the host cell by the standard large scale alkaline lysis method (Sambrook *et al.*, 1989; Ausubel *et al.*, 1993).

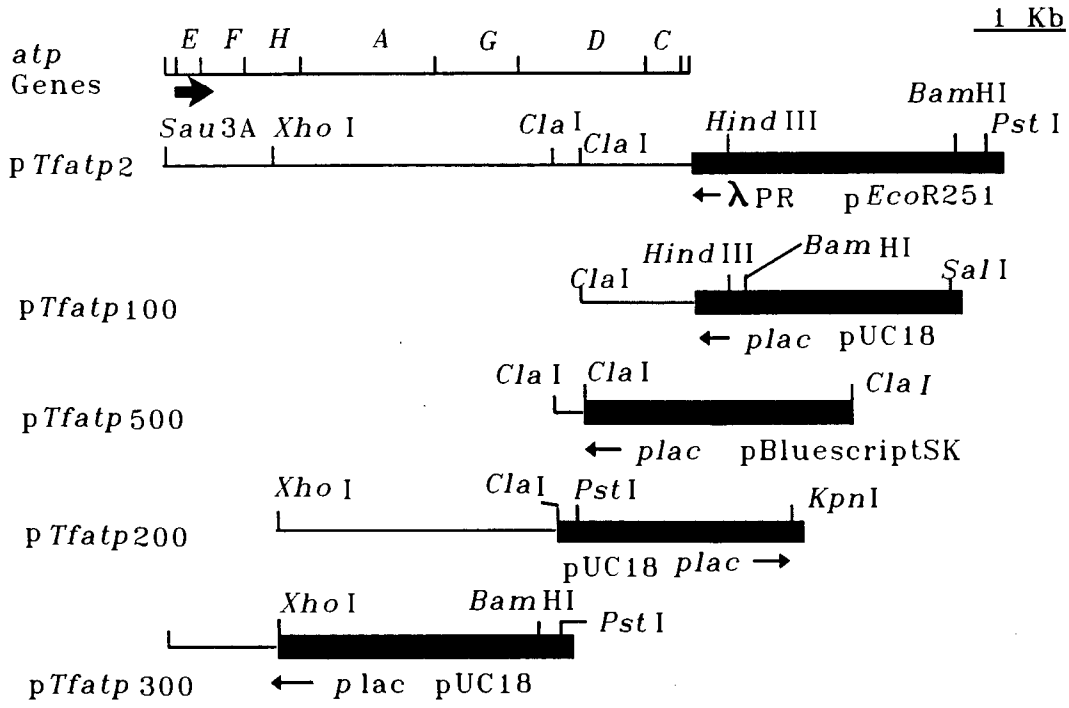

Fig. 3.1. The construction of fragments of p*Tfatp*2, aligned against the *T. ferrooxidans atp* genes, for ExoIII shortening and subsequent sequencing. Direction of transcription of the wild-type *T. ferrooxidans atp* genes is indicated by the broad black arrow, and that of the cloning vectors by narrow black arrows. λ_{PR} = phage lambda promoter; *plac* = β-galactosidase promoter. Details of construction of plasmids are given in Appendix A.

The technique of Henikoff (1984) was used to generate sets of unidirectional shortenings of p*Tfatp*100, p*Tfatp*200, and p*Tfatp*300. The small 0.324 kb *Cla*I-*Cla*I fragment of p*Tfatp*500 was not shortened. The nucleotide sequence of both strands was determined by the standard di-deoxynucleotide termination method of Sanger *et al.* (1977) using a Sequenase kit (United States Biochemical Corp., Cleveland, Ohio). Double stranded plasmid template was prepared, and then sequenced according to the instructions provided with the Sequenase kit. Standard gel electrophoretic and autoradiographic methods were used (Sambrook *et al.*, 1989).

3.2.4. Sequence analysis.

The DNA and amino acid sequences of p*Tfatp*2 were analysed using the IBM XT computer Genepro (Version 4.1) programme and a VAX computer, using the UWGCG (Version 7) sequence analysis software package (Devereaux *et al.*, 1984) with associated databases. A Table of one- and three- letter codes for amino acids is given in Appendix C.

3.3. Results.

3.3.1. Nucleotide and amino acid sequence of the cloned *T. ferrooxidans atp* genes.

The nucleotide sequence of the entire 5.946 kb *T. ferrooxidans* chromosomal fragment of p*Tfatp*2 is given in Fig. 3.2.

Analysis of the sequence data revealed seven complete continuous open reading frames. Data base searches, using the UWGCG FASTA subroutine, indicated significant nucleotide sequence homology to known *atp* genes from other organisms (data not shown). These were *atpE, atpF, atpH, atpA, atpG, atpD,*and *atpC* respectively (Fig. 3.2). Although the four plasmid constructs prepared for sequencing (Fig. 3.1) did not have marked regions of overlapping sequence, there was no break in homology of the *T. ferrooxidans atp* genes with those from other species. Data base searches, using the UWGCG TFASTA subroutine similarly indicated significant amino acid homology of the protein products of the cloned *T. ferrooxidans atp* genes, to seven F_1F_0 ATPsynthase subunits. These were, subunits c, b, δ, α, γ, β and ε respectively (data not shown).

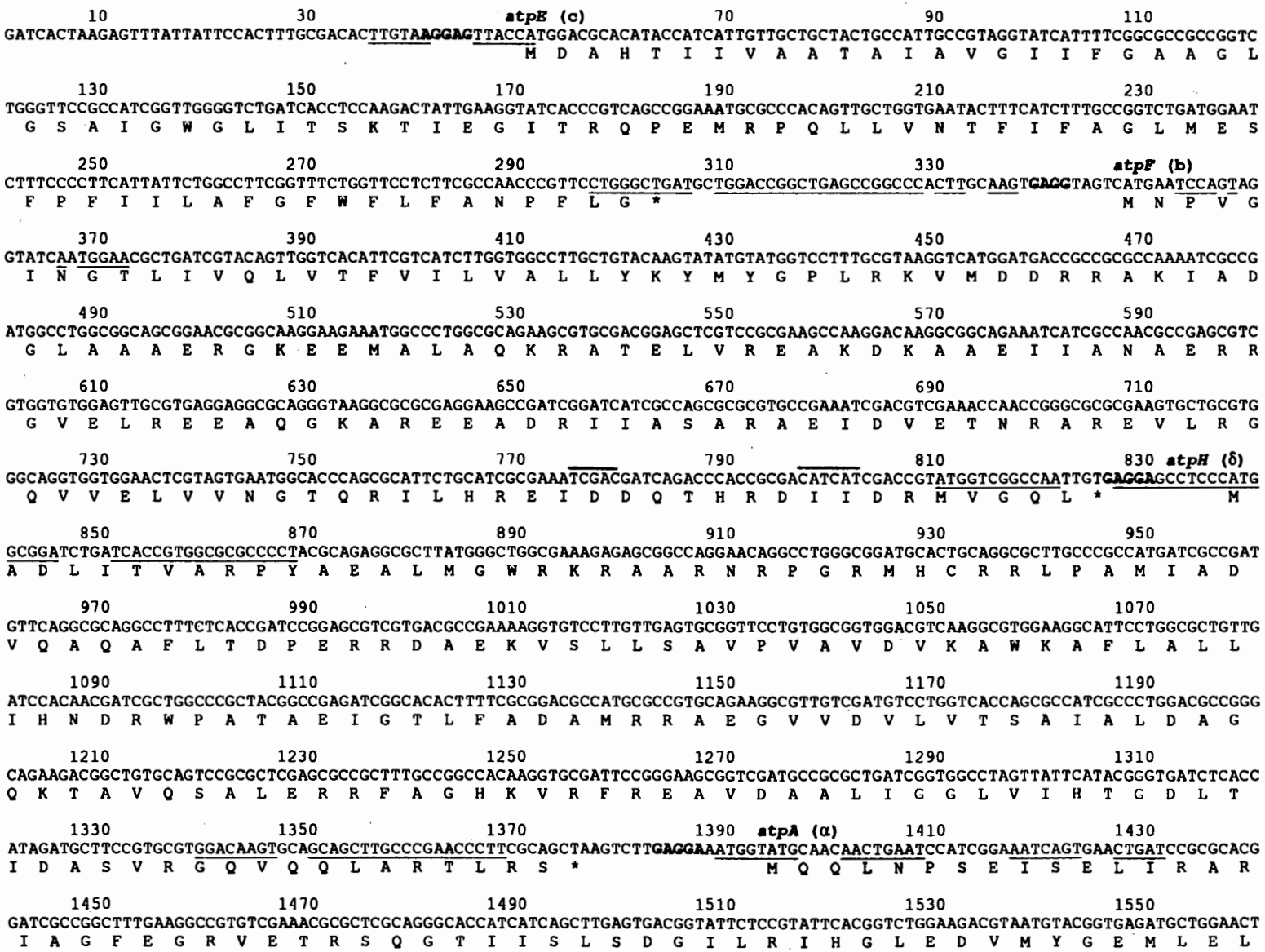

Fig 3.2. page 1

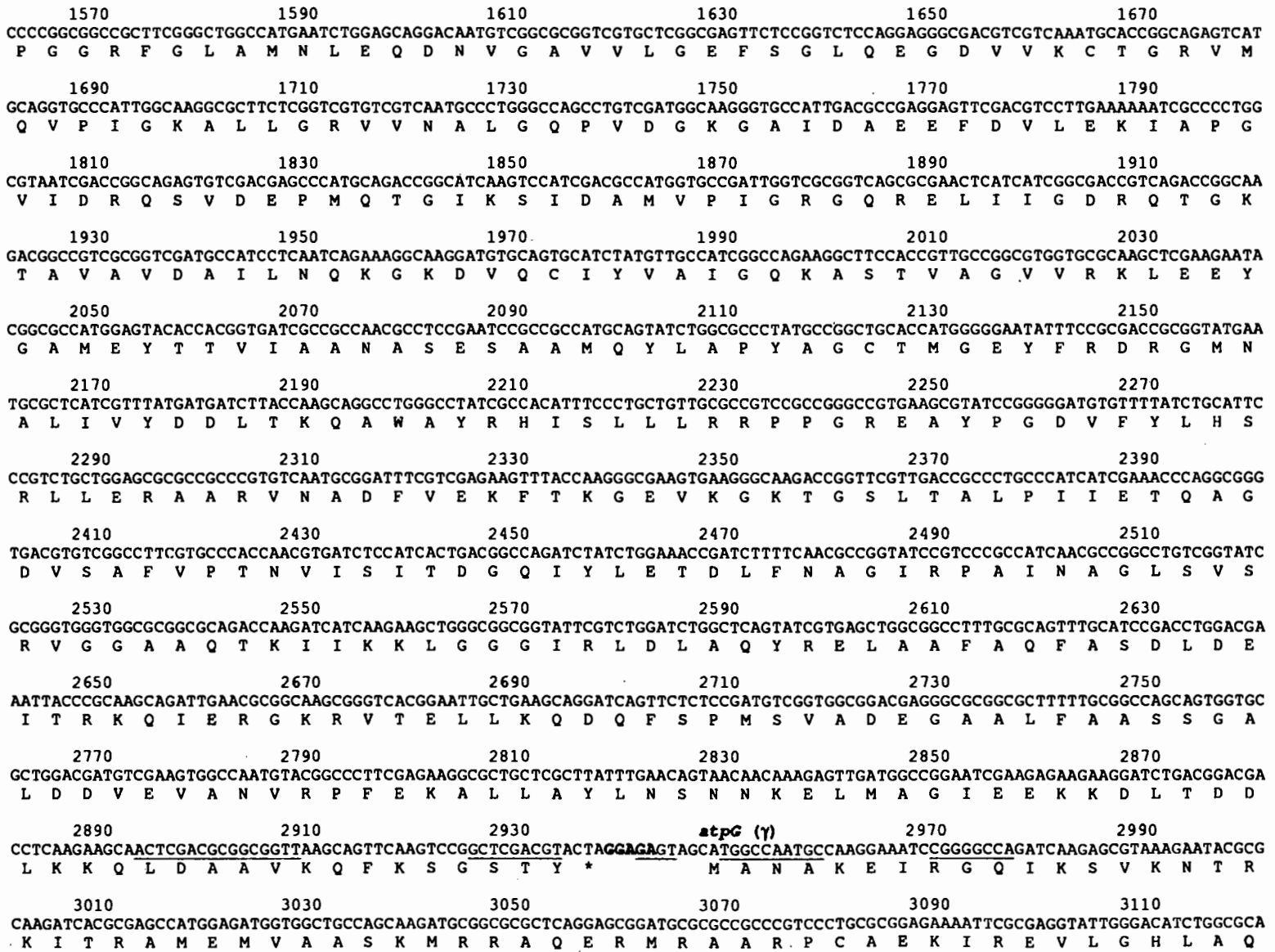
1570 1590 1610 1630 1650 1670
CCCCGGCGGCCGCTTCGGGCTGGCCATGAATCTGGAGCAGGACAATGTCGGCGCGGTCGTGCTCGGCGAGTTCTCCGGTCTCCAGGAGGGCGACGTCGTCAAATGCACCGGCAGAGTCAT
P G G R F G L A M N L E Q D N V G A V V L G E F S G L Q E G D V V K C T G R V M
1690 1710 1730 1750 1770 1790
GCAGGTGCCCATTGGCAAGGCGCTTCTCGGTCGTGTCGTCAATGCCCTGGGCCAGCCTGTCGATGGCAAGGGTGCCATTGACGCCGAGGAGTTCGACGTCCTTGAAAAAATCGCCCCTGG
Q V P I G K A L L G R V V N A L G Q P V D G K G A I D A E E F D V L E K I A P G
1810 1830 1850 1870 1890 1910
CGTAATCGACCGGCAGAGTGTCGACGAGCCCATGCAGACCGGCATCAAGTCCATCGACGCCATGGTGCCGATTGGTCGCGGTCAGCGCGAACTCATCATCGGCGACCGTCAGACCGGCAA
V I D R Q S V D E P M Q T G I K S I D A M V P I G R G Q R E L I I G D R Q T G K
1930 1950 1970 1990 2010 2030
GACGGCCGTCGCGGTCGATGCCATCCTCAATCAGAAAGGCAAGGATGTGCAGTGCATCTATGTTGCCATCGGCCAGAAGGCTTCCACCGTTGCCGGCGTGGTGCGCAAGCTCGAAGAATA
T A V A V D A I L N Q K G K D V Q C I Y V A I G Q K A S T V A G V V R K L E E Y
2050 2070 2090 2110 2130 2150
CGGCGCCATGGAGTACACCACGGTGATCGCCGCCAACGCCTCCGAATCCGCCGCCATGCAGTATCTGGCGCCCTATGCCGGCTGCACCATGGGGGAATATTTCCGCGACCGCGGTATGAA
G A M E Y T T V I A A N A S E S A A M Q Y L A P Y A G C T M G E Y F R D R G M N
2170 2190 2210 2230 2250 2270
TGCGCTCATCGTTTATGATGATCTTACCAAGCAGGCCTGGGCCTATCGCCACATTTCCCTGCTGTTGCGCCGTCCGCCGGGCCGTGAAGCGTATCCGGGGGATGTGTTTTATCTGCATTC
A L I V Y D D L T K Q A W A Y R H I S L L L R R P P G R E A Y P G D V F Y L H S
2290 2310 2330 2350 2370 2390
CCGTCTGCTGGAGCGCGCCGCCCGTGTCAATGCGGATTTCGTCGAGAAGTTTACCAAGGGCGAAGTGAAGGGCAAGACCGGTTCGTTGACCGCCCTGCCCATCATCGAAACCCAGGCGGG
R L L E R A A R V N A D F V E K F T K G E V K G K T G S L T A L P I I E T Q A G
2410 2430 2450 2470 2490 2510
TGACGTGTCGGCCTTCGTGCCCACCAACGTGATCTCCATCACTGACGGCCAGATCTATCTGGAAACCGATCTTTTCAACGCCGGTATCCGTCCCGCCATCAACGCCGGCCTGTCGGTATC
D V S A F V P T N V I S I T D G Q I Y L E T D L F N A G I R P A I N A G L S V S
2530 2550 2570 2590 2610 2630
GCGGGTGGGTGGCGCGGCGCAGACCAAGATCATCAAGAAGCTGGGCGGCGGTATTCGTCTGGATCTGGCTCAGTATCGTGAGCTGGCGGCCTTTGCGCAGTTTGCATCCGACCTGGACGA
R V G G A A Q T K I I K K L G G G I R L D L A Q Y R E L A A F A Q F A S D L D E
2650 2670 2690 2710 2730 2750
AATTACCCGCAAGCAGATTGAACGCGGCAAGCGGGTCACGGAATTGCTGAAGCAGGATCAGTTCTCTCCGATGTCGGTGGCGGACGAGGGCGCGGCGCTTTTTGCGGCCAGCAGTGGTGC
I T R K Q I E R G K R V T E L L K Q D Q F S P M S V A D E G A A L F A A S S G A
2770 2790 2810 2830 2850 2870
GCTGGACGATGTCGAAGTGGCCAATGTACGGCCCTTCGAGAAGGCGCTGCTCGCTTATTTGAACAGTAACAACAAAGAGTTGATGGCCGGAATCGAAGAGAAGAAGGATCTGACGGACGA
L D D V E V A N V R P F E K A L L A Y L N S N N K E L M A G I E E K K D L T D D
2890 2910 2930 atpG (γ) 2970 2990
CCTCAAGAAGCAACTCGACGCGGCGGTTAAGCAGTTCAAGTCCGGCTCGACGTACTAGGAGAGTAGCATGGCCAATGCCAAGGAAATCCGGGGCCAGATCAAGAGCGTAAAGAATACGCG
L K K Q L D A A V K Q F K S G S T Y * M A N A K E I R G Q I K S V K N T R
3010 3030 3050 3070 3090 3110
CAAGATCACGCGAGCCATGGAGATGGTGGCTGCCAGCAAGATGCGGCGCGCTCAGGAGCGGATGCGCGCCGCCCGTCCCTGCGCGGAGAAAATTCGCGAGGTATTGGGACATCTGGCGCA
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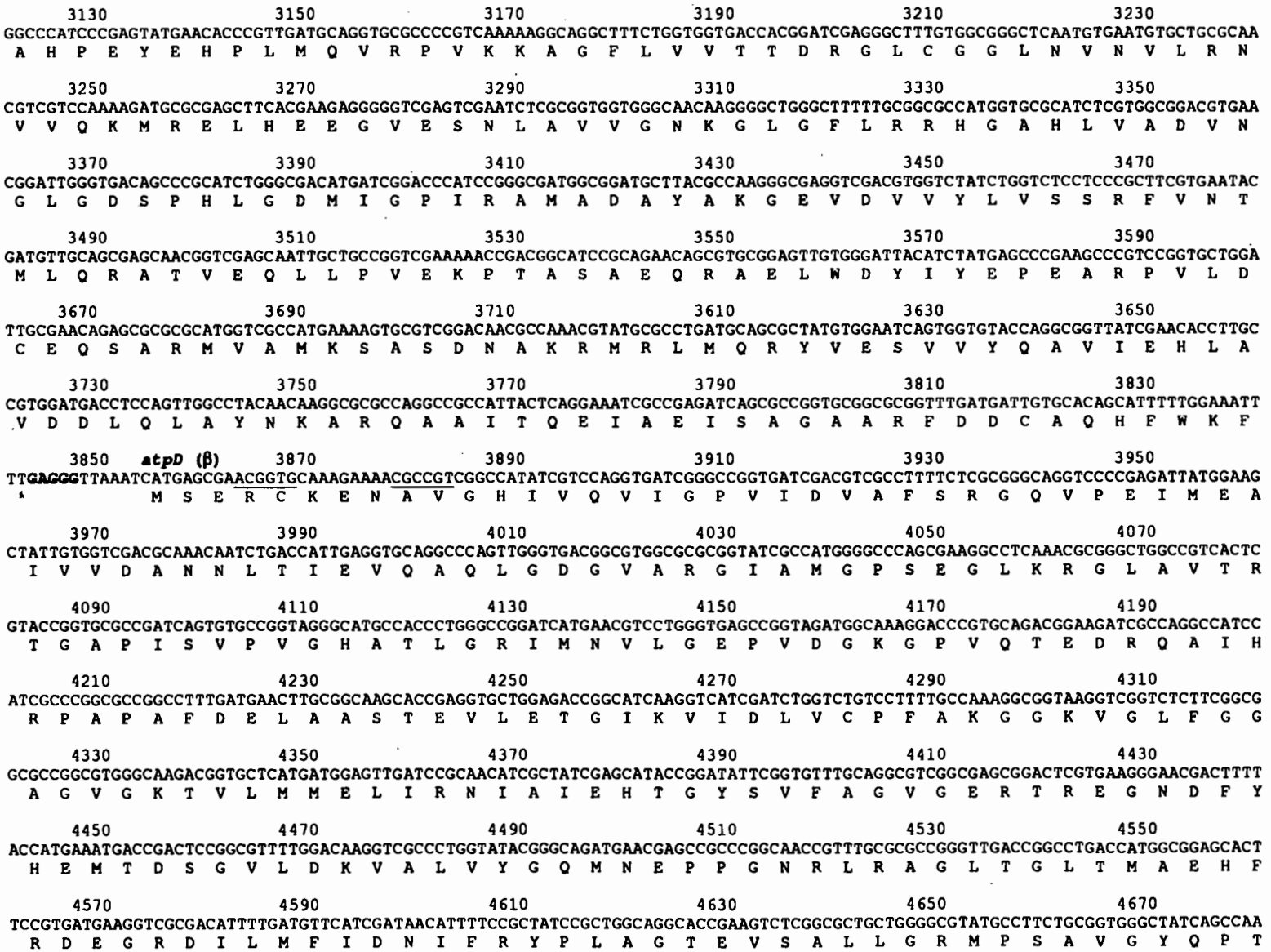
Fig. 3.2. page 2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A H P E Y E H P L M Q V R P V K K A G F L V V T T D R G L C G G L N V N V L R N
3250 3270 3290 3310 3330 3350
CGTCGTCCAAAAGATGCGCGAGCTTCACGAAGAGGGGGTCGAGTCGAATCTCGCGGTGGTGGGCAACAAGGGGCTGGGCTTTTTGCGGCGCCATGGTGCGCATCTCGTGGCGGACGTGAA
V V Q K M R E L H E E G V E S N L A V V G N K G L G F L R R H G A H L V A D V N
3370 3390 3410 3430 3450 3470
CGGATTGGGTGACAGCCCGCATCTGGGCGACATGATCGGACCCATCCGGGCGATGGCGGATGCTTACGCCAAGGGCGAGGTCGACGTGGTCTATCTGGTCTCCTCCCGCTTCGTGAATAC
G L G D S P H L G D M I G P I R A M A D A Y A K G E V D V V Y L V S S R F V N T
3490 3510 3530 3550 3570 3590
GATGTTGCAGCGAGCAACGGTCGAGCAATTGCTGCCGGTCGAAAAACCGACGGCATCCGCAGAACAGCGTGCGGAGTTGTGGGATTACATCTATGAGCCCGAAGCCCGTCCGGTGCTGGA
M L Q R A T V E Q L L P V E K P T A S A E Q R A E L W D Y I Y E P E A R P V L D
3670 3690 3710 3610 3630 3650
TTGCGAACAGAGCGCGCGCATGGTCGCCATGAAAAGTGCGTCGGACAACGCCAAACGTATGCGCCTGATGCAGCGCTATGTGGAATCAGTGGTGTACCAGGCGGTTATCGAACACCTTGC
C E Q S A R M V A M K S A S D N A K R M R L M Q R Y V E S V V Y Q A V I E H L A
3730 3750 3770 3790 3810 3830
CGTGGATGACCTCCAGTTGGCCTACAACAAGGCGCGCCAGGCCGCCATTACTCAGGAAATCGCCGAGATCAGCGCCGGTGCGGCGCGGTTTGATGATTGTGCACAGCATTTTTGGAAATT
V D D L Q L A Y N K A R Q A A I T Q E I A E I S A G A A R F D D C A Q H F W K F
3850 atpD (β) 3870 3890 3910 3930 3950
TTGAGGGTTAAATCATGAGCGAACGGTGCAAAGAAAACGCCGTCGGCCATATCGTCCAGGTGATCGGGCCGGTGATCGACGTCGCCTTTTCTCGCGGGCAGGTCCCCGAGATTATGGAAG
M S E R C K E N A V G H I V Q V I G P V I D V A F S R G Q V P E I M E A
3970 3990 4010 4030 4050 4070
CTATTGTGGTCGACGCAAACAATCTGACCATTGAGGTGCAGGCCCAGTTGGGTGACGGCGTGGCGCGCGGTATCGCCATGGGGCCCAGCGAAGGCCTCAAACGCGGGCTGGCCGTCACTC
I V V D A N N L T I E V Q A Q L G D G V A R G I A M G P S E G L K R G L A V T R
4090 4110 4130 4150 4170 4190
GTACCGGTGCGCCGATCAGTGTGCCGGTAGGGCATGCCACCCTGGGCCGGATCATGAACGTCCTGGGTGAGCCGGTAGATGGCAAAGGACCCGTGCAGACGGAAGATCGCCAGGCCATCC
T G A P I S V P V G H A T L G R I M N V L G E P V D G K G P V Q T E D R Q A I H
4210 4230 4250 4270 4290 4310
ATCGCCCGGCGCCGGCCTTTGATGAACTTGCGGCAAGCACCGAGGTGCTGGAGACCGGCATCAAGGTCATCGATCTGGTCTGTCCTTTTGCCAAAGGCGGTAAGGTCGGTCTCTTCGGCG
R P A P A F D E L A A S T E V L E T G I K V I D L V C P F A K G G K V G L F G G
4330 4350 4370 4390 4410 4430
GCGCCGGCGTGGGCAAGACGGTGCTCATGATGGAGTTGATCCGCAACATCGCTATCGAGCATACCGGATATTCGGTGTTTGCAGGCGTCGGCGAGCGGACTCGTGAAGGGAACGACTTTT
A G V G K T V L M M E L I R N I A I E H T G Y S V F A G V G E R T R E G N D F Y
4450 4470 4490 4510 4530 4550
ACCATGAAATGACCGACTCCGGCGTTTTGGACAAGGTCGCCCTGGTATACGGGCAGATGAACGAGCCGCCCCGGCAACCGTTTGCGCGCCGGGTTGACCGGCCTGACCATGGCGGAGCACT
H E M T D S G V L D K V A L V Y G Q M N E P P G N R L R A G L T G L T M A E H F
4570 4590 4610 4630 4650 4670
TCCGTGATGAAGGTCGCGACATTTTGATGTTCATCGATAACATTTTCCGCTATCCGCTGGCAGGCACCGAAGTCTCGGCGCTGCTGGGGCGTATGCCTTCTGCGGTGGGCTATCAGCCAA
R D E G R D I L M F I D N I F R Y P L A G T E V S A L L G R M P S A V G Y Q P T

Fig. 3.2. page 3

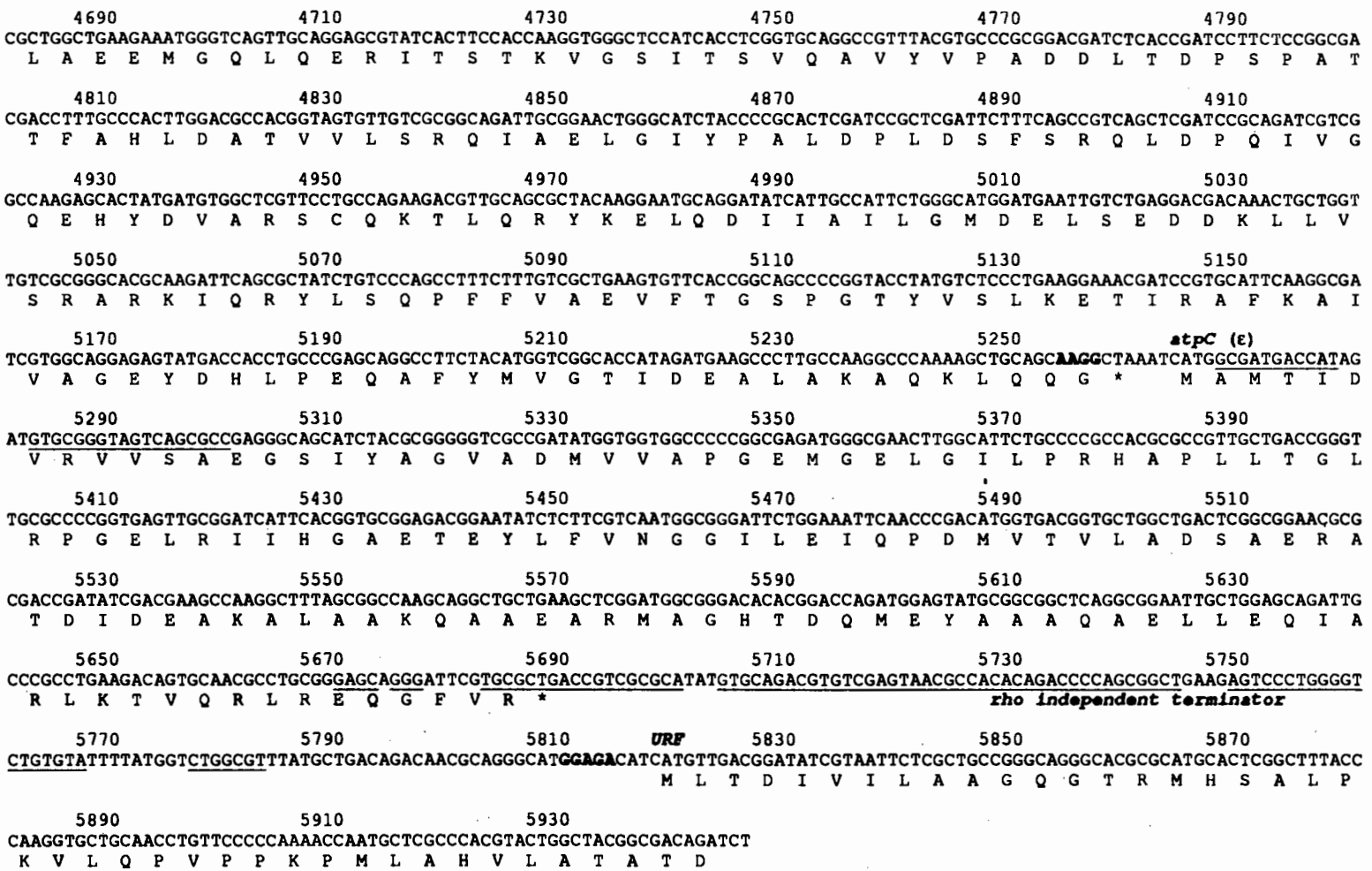

Fig. 3.2. page 4

Fig. 3.2. Nucleotide sequence for the 5946 nucleotide *Sau*3AI-*Bgl*II fragment of p*Tfatp*2, containing the *atpEFHAGDC* genes and an incomplete *URF*. Amino acid sequences, deduced from the nucleotide sequence, are shown below the DNA sequence by one-letter symbols. Ribosome binding sites are shown in **boldface**, a putative σ^{70}-like promoter by an overline and translation initiation regions (TIRs) which show a probability of forming stemloop structures are underlined.

The use of the UWGCG CODONPREFERENCE subroutine confirmed that the open reading frames on the 5.946 kb fragment of *T. ferrooxidans* DNA cloned for this study had a codon utilisation preference profile typical of that recorded for other *T. ferrooxidans* ATCC 33020 reading frames sequenced to date. CODONPREFERENCE utilises a codon usage data file to analyse all six reading frames of a DNA sequence, to identify open reading frames (ORFs) that have a similar codon usage to that provided in the data file. Fig. 3.3 shows the CODONPREFERENCE analysis using a *T. ferrooxidans* ATCC 33020 codon usage file compiled by Rawlings *et al.* (1991). The latter file is a composite of 2820 codons from seven previously sequenced *T. ferrooxidans* ATCC 33020 chromosomal genes. These genes are, *recA, glnA, nifHDK, ntrA* and *ORF1*. For convenience, this data file will be referred to as *Tf*DNA7 hereafter.

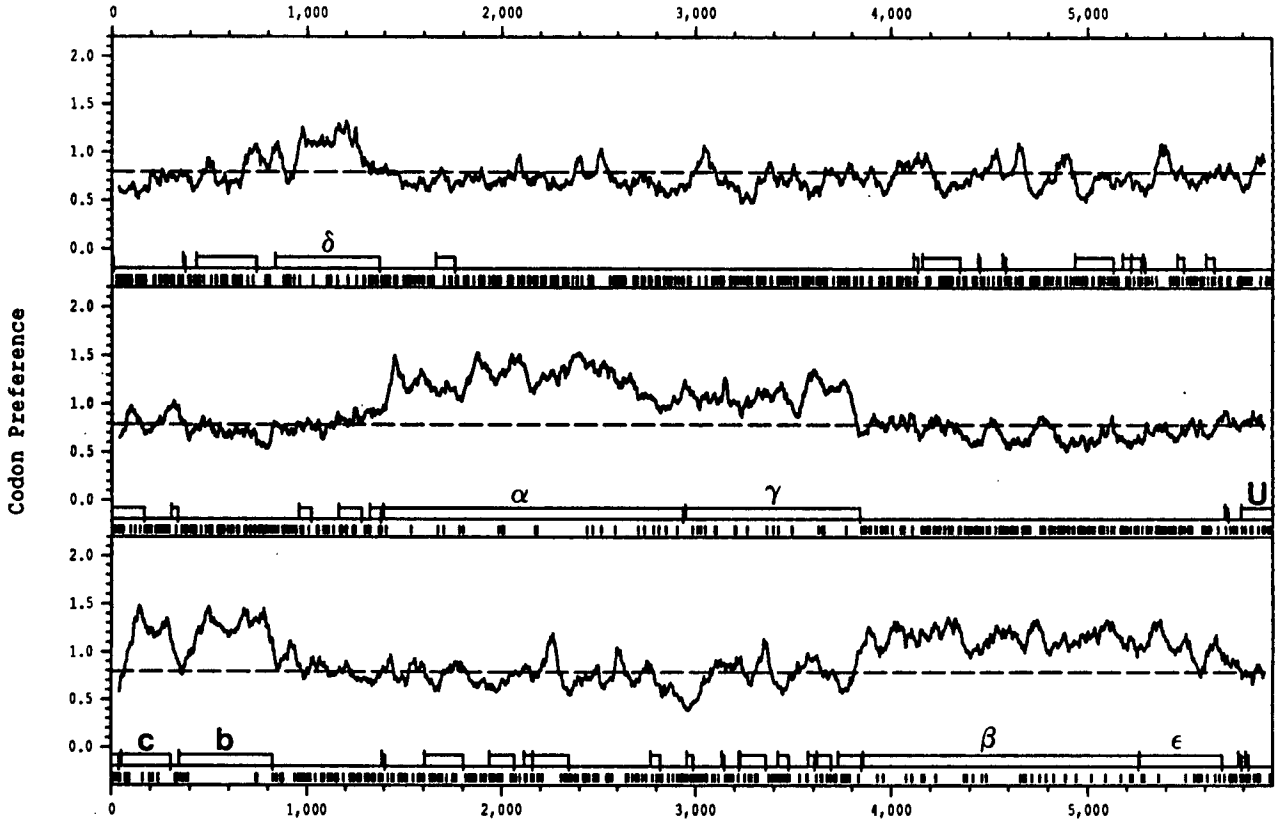

Fig. 3.3. CODONPREFERENCE analysis of the 5946 nucleotide fragment of p*Tfatp*2, with the predicted protein-coding open reading frames, c, b, δ, α, γ, β, and ε, shown as open boxes. U indicates the incomplete URF. The vertical axis shows the CODONPREFERENCE plots of all of the three forward frames. The positions of the rare codons (i.e. those codons which appear in the *Tf*DNA7 codon usage file at a frequency of less than 10%) are drawn as vertical bars within the open boxes. Any plot above the dotted line in any of the three reading frames identifies a protein-coding sequence with a similar codon usage as the *Tf*DNA7 datafile.

Fig. 3.3 clearly indicates that the seven *T. ferrooxidans atp* genes correlated well with the data provided by the *Tf*DNA7 data file. At the extreme 3'-end of the 5.946 kb insert was an

incomplete unidentified reading frame (URF) which had a codon usage profile similar to that of the *Tf*DNA7 data file. The GC content of the entire 5.946 kb *Sau*3AI-*Bgl*II chromosomal fragment was 59%, which is typical of the 58-59% value recorded for the type species ATCC 23270 by Kelly and Harrison (1989).

It was therefore apparent that seven *T. ferrooxidans atp* genes which code for the proton-translocating ATPsynthase, had been cloned by using a heterologous *E. coli unc* mutant host system. Gene sequence clearly indicated that the enzyme was of the F_1F_0 ATPsynthase-type. Despite the lack of complementation of *E. coli* F_0 mutants by p*Tfatp*2, two of the cloned genes, *atpE* and *atpF* were categorised as F_0. The remaining five genes, viz. *atpH, atpA, atpG, atpD* and *atpC*, were classified as the F_1 gene-set of *T. ferrooxidans* F_1F_0 ATPsynthase. The sequence has been lodged with the Genbank Nucleotide Sequence Database under the accession number M81087.

3.3.2. Codon usage in the *T. ferrooxidans atp* operon.

Table 3.1 details the codon usage pattern of the *T. ferrooxidans atp* genes. The two extreme right hand columns provide a means of comparing the codon usage pattern of the *Tf*DNA7 file with a data file *Tfatp*, which is a composite file of the 1851 *T. ferrooxidans atp* codons recorded during this study. Generally, the codon usage and bias of *Tfatp* was similar to that recorded for *Tf*DNA7. However, there were a few differences noted for both bias and frequency of usage by *Tfatp* genes.

Altered codon bias in the *Tfatp* data set was noted for the GAC and GAU, coding for Asp. On average, both codons were used with almost equal frequency, whereas in *Tf*DNA7, GAC and GAU occurred at frequencies of 61% and 39% respectively.

The distribution of the Leu codons in the *Tfatp* file was interesting. There was a marked increase in the usage of the UUG codon (25%) as opposed to the 12% noted for *Tf*DNA7. This trend in the *T. ferrooxidans atp* genes resulted in a simultaneous 10% decrease in the frequency of use of the major Leu codon, CUG.

It was also noted that the AAA (Lys) codon in *Tfatp* was used 10% less frequently than the *Tf*DNA7 genes. Hence there was increased usage of the alternate Lys codon, AAG.

Although the composite data for the *Tfatp* codon usage file indicated few differences from the *Tf*DNA7 data set, unusual trends recorded observed in individual *atp* genes were obscured by the average values of *Tfatp*. Individually, certain *atp* genes revealed unique codon usage patterns which are highlighted in bold face in Table 3.1.

Table 3.1. Codon usage of *T. ferrooxidans* genes, calculated using the CODONFREQUENCY subroutine of the UWGCG Version 7 package.

Codon	*atpE*	*atpF*	*atpH*	*atpA*	*atpG*	*atpD*	*atpC*	*Tfatp*[b]		*Tf DNA7*[a]	
	No[c]	No	No	No	No	No	No	No	%[d]	No	%
Ala GCA	1	2	3	2	5	**8**	0	21	9	29	12
GCC	**8**[e]	10	**13**	31	17	20	**6**	105	46	128	51
GCG	**0**	9	13	20	14	11	**11**	78	35	78	31
GCU	*2	0	*3	*3	*3	*5	***6**	***22**	**10**	15	6
Arg AGA	0	0	*1	*1	0	0	0	*2	2	2	1
AGG	0	0	*3	0	0	0	0	*3	2	4	3
CGA	0	0	*3	0	*3	0	0	*6	5	4	3
CGC	1	9	**7**	13	13	12	**6**	61	46	77	49
CGG	0	**2**	**2**	5	6	5	4	24	18	34	22
CGU	1	**8**	4	**11**	4	9	**0**	37	28	35	22
Asn AAC	1	**2**	2	7	**5**	**8**	0	25	56	55	62
AAU	1	**3**	0	8	**6**	1	**1**	20	44	33	38
Asp GAC	1	6	**4**	20	**6**	**12**	4	**53**	**52**	110	61
GAU	0	4	**8**	12	**7**	**15**	3	**49**	**48**	70	39
Cys UGC	0	0	1	3	2	2	0	8	73	22	76
UGU	0	0	0	0	2	1	0	3	27	7	24
Gln CAA	0	1	1	3	2	3	2	12	14	**17**	16
CAG	2	6	6	23	13	21	5	76	86	89	84
Glu GAA	**3**	11	**3**	21	**10**	18	7	73	55	108	56
GAG	0	6	**4**	15	**13**	15	7	60	45	85	44
Gly GGA	0	*1	*1	*1	*3	*3	*2	*11	7	15	6
GGC	**2**	**4**	5	32	7	25	5	80	**51**	146	60
GGG	0	*1	*2	*3	*4	*9	*3	*22	**14**	25	10
GGU	**8**	**4**	2	15	3	9	2	43	28	56	23
His CAC	0	1	3	2	3	4	3	16	52	45	56
CAU	**1**	1	1	1	**6**	5	**0**	15	48	35	44
Ile AUA	0	0	1	0	0	*1	*1	*3	3	7	4
AUC	**6**	**12**	6	26	9	20	**3**	82	69	125	75
AUU	**6**	**1**	1	9	2	9	**5**	33	28	34	20

Table 3.1. (continued).

Codon	*atpE*	*atpF*	*atpH*	*atpA*	*atpG*	*atpD*	*atpC*	*Tfatp*[b]		*Tf* DNA7[a]	
	No [c]	No	No	No	No	No	No	No	%[d]	No	%
Leu CUA	0	0	*1	0	0	0	0	*1	1	3	1
CUC	1	2	3	12	4	7	1	30	18	50	21
CUG	**6**	**6**	**6**	25	9	20	7	**79**	**47**	138	57
CUU	0	0	*4	*5	*3	*2	*1	*15	9	17	7
UUA	0	0	0	0	0	0	*1	*1	1	6	2
UUG	1	**6**	**4**	6	**8**	**12**	**4**	**41**	**25**	28	12
Lys AAA	0	1	0	4	6	5	0	**16**	**20**	45	30
AAG	1	**7**	**6**	**25**	10	12	**3**	**64**	**80**	106	70
Met AUG	3	5	5	13	14	14	7	61	100	92	100
Phe UUC	**9**	0	**2**	10	1	**9**	2	35	63	74	70
UUU	**1**	1	**3**	6	**5**	**7**	0	21	38	31	30
Pro CCA	*1	*1	0	*1	0	*1	0	*4	6	10	8
CCC	2	0	3	8	5	8	**4**	29	43	64	49
CCG	1	0	1	5	**5**	11	1	25	37	42	32
CCU	0	**1**	**2**	2	**0**	4	0	9	13	14	11
Ser AGC	0	1	2	2	**5**	5	2	17	23	44	26
AGU	0	0	1	5	1	1	0	8	11	22	13
UCA	0	0	1	0	*1	0	0	*1	1	6	4
UCC	2	0	**3**	10	3	6	0	24	33	53	31
UCG	0	0	0	8	2	5	1	16	22	32	19
UCU	*1	0	0	*1	0	*5	0	*7	10	14	8
Thr ACA	0	*1	*1	0	0	0	*1	*3	3	6	5
AAC	3	3	5	16	**1**	17	3	48	56	79	61
ACG	**0**	2	3	6	**6**	7	3	27	**31**	33	26
ACU	**3**	0	0	*1	*1	*3	0	*8	9	11	9
Trp UGG	2	0	3	1	2	0	0	8	100	26	100
Tyr UAC	0	**1**	1	4	4	**6**	**1**	**17**	**40**	55	60
UAU	0	**2**	0	11	4	**7**	**2**	**26**	**60**	37	40
Val GUA	*1	*3	0	*4	*2	*4	*1	*15	9	12	6
GUC	0	**6**	5	**17**	10	17	3	58	36	55	30
GUG	1	**6**	8	**15**	17	20	**7**	74	47	102	55
GUU	*1	0	*4	*4	*1	*2	0	*12	8	16	9

Table 3.1. (continued).

Codon	*atpE*	*atpF*	*atpH*	*atpA*	*atpG*	*atpD*	*atpC*	*Tfatp*[b]		*Tf* DNA7[a]	
	No [c]	No	No	No	No	No	No	No	%[d]	No	%
End TGA	1	1	0	0	1	0	1	4	-	4	-
TAG	0	0	0	1	0	0	0	1	-	0	-
TAA	0	0	1	0	0	1	0	2	-	3	-

[a] - Composite codon usage of 7 previously sequenced *T. ferrooxidans* genes viz. *recA, glnA, nifHDK, ntrA, ORF1* (Rawlings *et al.* 1991)

[b] - Codon usage of all 7 *T. ferrooxidans atp* genes sequenced in this study.

[c] - No = number of times a codon appears in each data set.

[d] - % = frequency of each codon, expressed as a %

[e] - Numbers typed in boldface indicate unusual trends, discussed in the text.

* - rare codons

The frequency of use of rare codons varied within the seven *atp* genes, and was recorded at 10.7%, 4.4%, 13.4%, 4.7%, 7.0%, 7.5%, and 11.3%, for *atpE, F, H, A, G, D,* and *C,* respectively. Three genes, *atpE, atpH* and *atpC* used rare codons at frequencies in excess of the average 7.3% recorded for the entire *T. ferrooxidans atp* gene set. Low frequencies for rare codon usage were recorded for *atpF* and *atpA.* Rare codons were either randomly distributed within a codon, or in the case of *atpE, F, H* and *C,* were grouped together as short clusters or "strings" (Fig. 3.3).

The UWGCG CORRESPOND subroutine was used to compare codon frequency tables, and provided information as to how similar or different codon usage tables for each gene were to each other. The lower the statistic (D) given, the more similar the patterns of codon usage. The codon frequency tables of individual *T. ferrooxidans atp* genes were compared with the composite *Tfatp* and *Tf*DNA7 data files. Results are shown in Table 3.2.

In *T. ferrooxidans,* codon usage by the smallest gene, *atpE* was the most different from the composite files compared. However, this may simply be a reflection of an inadequate codon usage sample from a small gene (84 codons). Codon usage in the three largest *atp* reading frames viz. *atpA, D* and *G* was most similar to the composite data files. Codon usage of the *atpH* gene was more similar to that of the *Tf*DNA7 file than to the *Tfatp* file. Of the remaining *T. ferrooxidans atp* genes, the codon frequency of the *atpD* was most like that of the *Tf*DNA7 data set.

Table 3.2. Comparison of the codon usage of individual *T. ferrooxidans atp* genes, with that of the *Tfatp* and *Tf*DNA7 data files, using the UWGCG CORREPOND subroutine.

Gene	*Tfatp*[a]	*Tf* DNA7[b]
atpE	3.5[c]	3.6
atpF	2.1	2.3
atpH	2.0	1.6
atpA	0.4	0.7
atpG	1.3	1.8
atpD	0.4	0.5
atpC	2.6	2.9

[a] - *T. ferrooxidans atp* operon, composite usage file. [b] - *T. ferrooxidans* composite usage file for *T. ferrooxidans recA, glnA, nifHDK, ntrA* and *ORF1* genes (Rawlings *et al.*, 1991). [c]-Numbers represent the statistic D^2 as described in the text.

3.3.3. Bias in the third 3' position (wobble) position in the *T. ferrooxidans atp* genes.

As unusual codon usage trends were observed in some of the *T. ferrooxidans atp* genes, an analysis was done to determine the identity of the purine/pyrimidine occurring at the third 3' (wobble) position of the codons of both individual *atp* genes, and the composite *Tfatp* file. Results are presented in Table 3.3.

Rawlings *et al.* (1991) recorded from data collected from the *Tf*DNA7 data file, that the probability of a G or a C occurring in the wobble position for *T. ferrooxidans* ATCC 33020 was 73.3%, and that this was in the range expected for an organism with a GC mole% value of 59. The frequency of A or U occurring in the third position in *Tf*DNA7 was 9.8% or 15.9% respectively. The combined values for the wobble position of *Tfatp* codons was average. G+C was measured at 74.3%, whilst that of A and U was 9.2% and 17.8% respectively. However, a large variation from these values was evident in *atpE*. The number of U recorded in the wobble position was high, giving a value of 29.8%., whilst the G content was considerably lower at 21.4%. The combined pyrimidine (U+C) content of the *atpE* codon wobble position was 71.5%, compared to the average of 57.8% for *Tf*DNA7, and 55.1% for *Tfatp*. As a comparison, the wobble position of *E. coli uncE* was determined. The % G, A, U, and C for *uncE* were 35.4%, 8.8%, 32.9%, and 22.7% respectively (calculated from data in Walker *et al.*, 1984a). Hence, the U content of the wobble in the *uncE* codon was similar to *T. ferrooxidans atpE*. However, the combined pyrimidine content of 55.6% was lower, as may be expected for an organism such as *E. coli* with a G+C mole% composition of 48-52 (Krieg, 1984). Also of note in Table 3.3 was the reversal in bias of G and C in the wobble position in the *atpG* and *atpC* genes, compared to the averages recorded for the *Tfatp* and *Tf*DNA7 files.

Table 3. 3. Bias of the third (3') position, ("wobble") of *T. ferrooxidans atp* genes, and the composite *Tf* DNA7 datafile.

	Third 3' Purine/Pyrimidine in codon								
	G		A		U		C		
Gene	**No**	**%**	**No**	**%**	**No**	**%**	**No**	**%**	**% G+C**
atpE	**18**	**21.4**	6	7.1	**25**	**29.8**	35	41.7	**63.1**
atpF	56	35.2	21	13.2	24	15.1	58	36.4	71.6
atpH	66	36.9	15	8.4	32	17.8	66	41.9	78.8
atpA	170	32.4	37	9.8	94	15.9	213	41.9	74.3
atpG	**123**	**41.2**	32	10.7	48	16.1	95	**31.8**	73.0
atpD	162	34.6	43	9.2	85	18.2	178	38.0	72.6
atpC	**63**	**44.7**	15	10.6	20	14.2	43	**30.5**	75.2
Tfatp[a]	658	35.7	169	9.2	328	17.8	688	37.3	74.3
Tf DNA7[b]	—	32.4	—	9.8	—	15.9	—	41.9	73.3

[a] - Total of all *T. ferrooxidans* ATCC 33020 *atp* genes sequenced.

[b] - Total of 7 *T. ferrooxidans* ATCC 33020 genes sequenced; *recA*, *glnA*, *nifHDK*, *ntrA*, and *ORF1* (Rawlings *et al.* 1991).

No/% - Number or % of times a purine or pyrimidine occurs within a gene.

Numbers typed in boldface indicate unusual trends, referred to in the text.

3.3.4. Probable ribosomal binding sites, start and stop codons, and intergenic regions of the *T. ferrooxidans atp* genes.

Probable starts of all seven of the *T. ferrooxidans atp* reading frames were identified using the codon-useage patterns of the organism, and by aligning the amino acid sequence of homologous polypeptides from different organisms, including the heterologous host, *E. coli*. As these regions have been particularly well characterised in the *E. coli unc* operon, a comparison of several features in the *E. coli unc* and *T. ferrooxidans atp* operons is given in Table 3.4.

Consensus ribosome binding sites for the seven *T. ferrooxidans atp* genes were located at appropriate distances upstream of the probable starts of the open reading frames (see also Fig. 3.2). Unlike *E. coli*, all the *T. ferrooxidans atp* genes started with an AUG codon. All three stop codons were used to terminate the *T. ferrooxidans atp* genes, whereas in *E. coli*, the predominant stop codon was UAA. The tandem double stop of UAG.UAA, which terminated the *E. coli uncE* gene, was not observed to terminate *atpE* in *T. ferrooxidans*.

The comparison of the intergenic regions of the *T. ferrooxidans atp* and *E. coli unc* genes was interesting. The intergenic distances of the *F-H* and *H-A* regions was similar in both organisms, but the *E-F*, *A-G*, *G-D*, and *D-C* regions in *T. ferrooxidans* were noticeably shorter than those recorded for *E. coli*.

Table 3.4. Comparison of the data of the intergenic regions, start and stop codons and ribosome binding sites of the *atp/unc* operons of *T. ferrooxidans* and *E. coli*[a].

	Ribosomal binding site		Start codon		Stop codon		Intergenic region	Size of intergenic region (nt)	
Gene	*Tf*	*Ec*	*Tf*	*Ec*	*Tf*	*Ec*		*Tf*	*Ec*
atpE	GGAG(-5)	GGAG(-7)	AUG	AUG	UGA	UGA:UAA	B-E	—	46
atpF	GAGG(-5)	GAGG(-5)	AUG	GUG	UGA	UAA	E-F	42	58
atpH	GAGGA(-7)	AGGAGG(-10)	AUG	AUG	UAA	UAA	F-H	12	14
atpA	GAGGA(-7)	GGAG(-2)	AUG	AUG	UAG	UAA	H-A	16	12
atpG	GGAG(-6)	GAGGA(-8)	AUG	AUG	UGA	UAA	A-G	9	50
atpD	GAGGG(-7)	GAGGA(-7)	AUG	AUG	UAA	UAA	G-D	10	26
atpC	AAGG(-7)	GGAGG(-6)	AUG	AUG	UGA	UAA	D-C	3	20

[a]-*E. coli* data from Walker *et al.* (1984a); nt-nucleotides; *Ec-E. coli*; *Tf-T. ferrooxidans*.

The translation initiation regions (TIRs) of the *T. ferrooxidans atp* operon were analysed using various subroutines of the UWGCG package. A TIR in the *unc* operon is a region of nucleotide sequence which occurs on either side of a given start codon. It encompasses both the non-coding intercistronic region upstream of the start, and a defined length of coding cistronic sequence immediately downstream of the start codon (Mc Carthy, 1990). In the *E. coli unc* operon, the TIRs have been shown to be implicated in regulation of gene expression (Hellmuth *et al.*, 1991; McCarthy, 1990; Pati *et al.*, 1992). Computer subroutines were STEMLOOP and FOLD. STEMLOOP indicates areas of invert repeat nucleotide sequence which have the ability to form stemloop structures, both on the DNA and in the mRNA transcript. FOLD finds a secondary structure of minimum free energy for RNA molecules, based on published values of stacking and loop destabilising energies (Zuker and Stiegler, 1981). The programme does have limitations associated with energy minimising algorithims in predicting real secondary structures. Obviously values for FOLD will vary with the length of DNA specified for a given TIR region. As a result, both STEMLOOP and FOLD were therefore used merely as guides to indicate possible mRNA secondary structure and associated stabilities in the *T. ferrooxidans atp* operon TIRs.

TIRs analysed were:-

The *B-E* region (nucleotides 27-97)
The *E-F* region (nucleotides 280-400)
The *F-H* region (nucleotides 790-870)
The *H-A* region (nucleotides 1330-1440)
The *A-G* region (nucleotides 2890-2990)
The *G-D* region (nucleotides 3810-3890) and
The *D-C* region (nucleotides 5230-5300).

Regions which revealed a high probability for stemloop structures were, *E-F*, *F-H* and *H-A* (Fig. 3.2). In all these three regions, the FOLD data indicated that the mRNA transcribed from these regions could have a relatively stable secondary structure; *E-F* (ΔG = -28.3 kcal/mol); *F-H* (ΔG = -20.1 kcal/mol); *H-A* (ΔG = -29.6 kcal/mol). Regions associated with less stable mRNA secondary structures were *B-E* (ΔG = -12.9 kcal/mol); *G-D* (ΔG = -12.8kcal/mol) and *D-C* (ΔG = -13.6 kcal/mol). The *A-G* TIR region had regions of invert repeat sequence, and the predicted stability of the mRNA secondary structure was ΔG = -18.5 kcal/mol.

3.3.5. The transcriptional terminator of the *T. ferrooxidans atp* operon.

The *T. ferrooxidans atp* operon was terminated by a *rho*-independent terminator with an 18-nucleotide stem-loop region (ΔG = -24.7kcal/mol; Salser, 1977) followed by an eight-nucleotide poly U-rich tract, 26 nucleotides downstream of the *atpC* gene. In *E. coli*, the

unc operon is similarly terminated by a *rho*-independent terminator, six nucleotides downstream of the *unc*C gene (Walker *et al.*, 1984a).

STEMLOOP and FOLD analyses of the termination region (nucleotides 5670-5790) of the *T. ferrooxidans atp* operon indicated that the region was rich in invert repeat sequences with the propensity to form stemloop structures. FOLD predicted a highly stable secondary structure of the mRNA, consisting of two distinct hairpin loops (ΔG = -46.8 kcal/mol). These are indicated in Fig. 3.4, which was generated using the SQUIGGLES subroutine of the UWGCG programme using data from the FOLD analysis of the region.

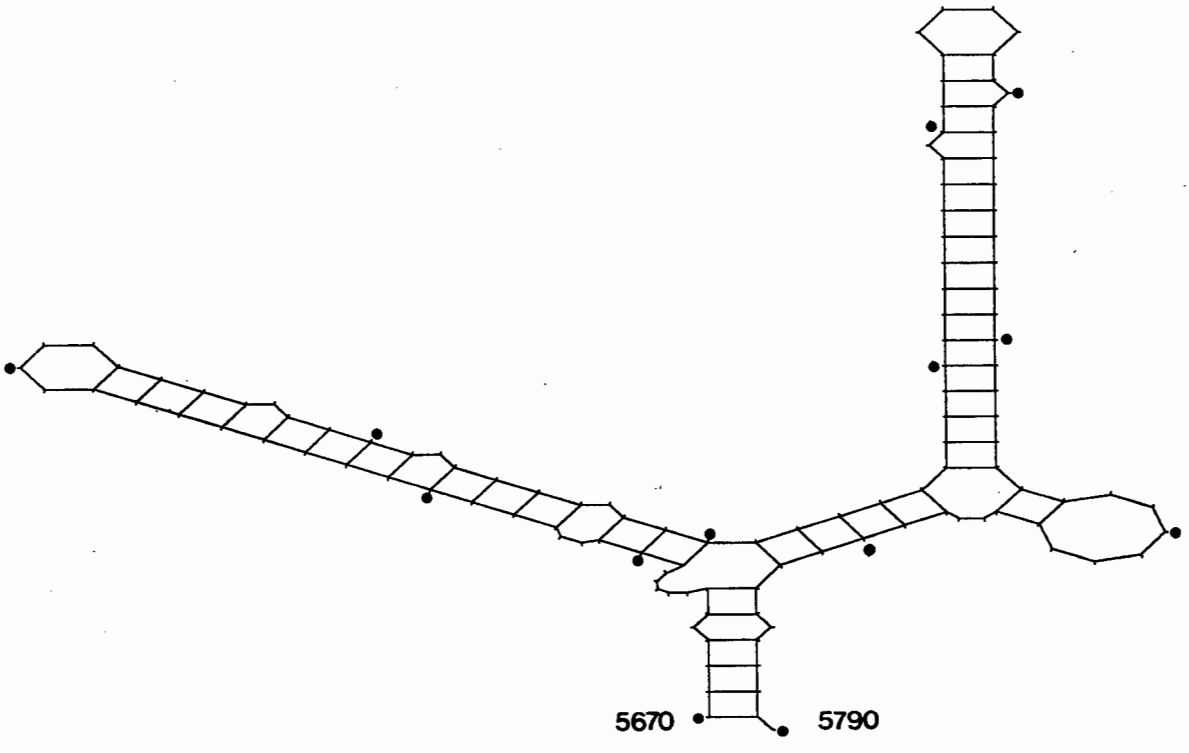

Fig. 3.4. The predicted folding of the *T. ferrooxidans atp* operon terminator region, nucleotides 5670-5790. ΔG= -46.8 kcal/mol (data from UWGCG FOLD analyses). Solid black dots mark every ten nucleotides. The second stemloop is typical of a *rho*-independent terminator.

3.3.6. The location of an internal promoter-like sequence in the *T. ferrooxidans* atp operon.

Despite being cloned against the λ promoter of p*Eco*R251, the *T. ferrooxidans* F_1 genes were expressed in *E. coli unc* mutants (Chapter 2). This implied that the F_1 *atp* genes were expressed from an internal promoter recognised by *E. coli*. The most likely location of the promoter was to the 5'-end of the *atpH* gene start codon. Examination of the sequence (Fig. 3.2) showed at least one putative σ^{70}-type promoter (Harley and Reynolds, 1987). To determine whether this putative promoter is functional in either *E. coli* or *T. ferrooxidans*

would require the isolation of mRNA from both organisms for primer extension experiments. Attempts to do so are discussed in Chapter 5.

3.3.7. The primary amino acid sequence of the *T. ferrooxidans atp* operon.

Using the GAP facility of UWGCG, the *T. ferrooxidans* F_1F_0 ATPsynthase subunits were aligned, pairwise, against those of a number of other prokaryotic organisms. GAP uses the algorithm method of Needleman and Wunsch (Devereaux *et al.*, 1984) to find the alignment of two complete sequences that maximises the number of matches, and minimises the number of gaps. This subroutine indicates the % similar and identical amino acids in the aligned pair of sequences. Results are shown in Table 3.5.

In general, the F_1F_0 ATPsynthase polypeptides of *T. ferrooxidans*, were most like those of the two gram-negative heterotrophic species, *E. coli* and *V. alginolyticus*. The exception to this was the c subunit of the F_0 particle, where the proteins from *V. alginolyticus* and strain PS3 were approximately equally homologous to *T. ferrooxidans*. Homologies of the subunits associated with catalysis, viz. α, β, and γ, were higher than those recorded for the remaining non-catalytic subunits. When compared with the photosynthetic autotrophic species in the table, it was of interest to note that the b' (rather than the b subunit) was more like the *T. ferrooxidans* b subunit.

Table 3.5. Amino acid similarities between *T. ferrooxidans* F_1F_0ATPsynthase subunits, and those from other eubacterial species.
Numbers represent % identical amino acids, and % similar amino acids (in brackets)[1].

Bacterial species	F_1F_0 ATP synthase subunit							
	c	b'	b	δ	α	γ	β	ε
E. coli[a]	32	-	39	29	67	53	79	44
	(65)	-	(64)	(58)	(83)	(77)	(88)	(69)
V. alginolyticus[b]	50	-	43	32	68	57	72	40
	(72)	-	(66)	(56)	(82)	(75)	(85)	(62)
R. blastica[c]	-	-	-	24	58	40	68	35
				(48)	(76)	(60)	(80)	(52)
R. rubrum[d]	27	24	21	27	58	38	70	39
	(54)	(51)	(44)	(54)	(76)	(59)	(81)	(59)
Thermus PS3[e]	50	-	29	21	58	42	67	37
	(69)		(51)	(45)	(75)	(61)	(81)	(59)
B. megaterium[f]	43	-	30	23	58	40	65	39
	(60)		(58)	(47)	(75)	(64)	(80)	(58)
B. firmus OF4[g]	39	-	28	24	57	37	65	37
	(65)		(57)	(47)	(74)	(60)	(80)	(57)
P. modestum[h]	31	-	30	23	63	35	68	25
	(65)		(60)	(46)	(80)	(59)	(80)	(50)
E. hirae[i]	36	-	29	21	56	33	66	
	(63)		(56)	(48)	(74)	(56)	(79)	(59)
Synechococcus 6301[j]	31	33	25	26	59	42	67	38
	(54)	(56)	(48)	(45)	(75)	(62)	(79)	(58)

Table 3.5. (continued).

Bacterial species	F_1F_0 ATP synthase subunit							
	c	b'	b	δ	α	γ	β	ε
Anabaena PCC7120[k]	31 (55)	26 (48)	23 (47)	27 (42)	59 (75)	41 (62)	68 (81)	31 (58)

[a] - Walker, *et al.* (1984a)
[b] - Krumholz *et al.* (1989)
[c] - Tybulewicz *et al.* (1984)
[d] - Falk *et al.* (1985); Falk and Walker (1988)
[e] - Ohta *et al.*, (1988)
[f] - Brusilow *et al.* (1989)
[g] - Mack Ivey and Krulwich (1991)
[h] - Esser *et al.* (1990)
[i] - Hoppe and Sebald, (1984) Shibata *et al.* (1992)
[j] - Cozens and Walker, (1987)
[k] - Curtis, (1987); Mc Carn *et al.* (1988)

[l] - Determined with the algorithm of Needleman & Wunsch (Devereaux *et al.* 1984).

A comparison was made between the nucleotide sequence-derived polypeptides of the *T. ferrooxidans* and *E. coli* F_1F_0 ATPsynthase. Results generated using the PEPTIDESORT facility of UWGCG are shown in Table 3.6.

Table 3.6. Comparison of amino acid number, molecular weight (M_r), and isoelectric points (pI) of the F_1F_0 ATPsynthase subunits and *E. coli*. (Calculated using the UWGCG PEPTIDESORT subroutine).

Subunits	*T. ferrooxidans*			*E. coli*[a]		
	No of residues	M_r (Da)	pI	No of residues	M_r (Da)	pI
a(*atpB*)	—	—	—	271	30285	ND
c(*atpE*)	84	8989	5.68	79	8264	4.29
b(*atpF*)	159	17884	7.69	156	17230	6.11
δ (*atpH*)	179	19573	11.03	177	19328	4.78
α (*atpA*)	514	55600	5.12	513	55282	6.23
γ (*atpG*)	298	33318	9.58	286	32559	9.42
β (*atpD*)	468	50609	4.92	459	50286	4.74
ε (*atpC*)	141	15169	4.92	138	15051	5.60

ND - no determination; [a] - Data from Walker *et al.* (1984a).

All of the *T. ferrooxidans* subunits showed a remarkable similarity in size and pI to those of *E. coli*. An exception was recorded for the pI of the δ protein of *T. ferrooxidans*, which was considerably more electropositive than the *E. coli* homologue. Further results generated by the PEPTIDESORT analyses will be discussed in Section 3.4.3.

Additional UWGCG subroutines were utilised to analyse primary sequence data. These included PEPPLOT, PEPTIDESTRUCTURE and PILEUP. PEPPLOT yields information, derived from primary amino acid sequence relating to the hydrophobicity and possible structural conformations of a given protein. PEPTIDESTRUCTURE predicts likely folding patterns of proteins, incorporating domains of α-helical, β-sheet and likely turns. It also indicates regions of hydrophobicity and hydrophilicity. PILEUP is an extension of the GAP programme, and creates a multiple sequence alignment from a group of related sequences using progressive pairwise alignments. The programme also plots a tree/dendrogram, showing the clustering relationships used to create the final multiple sequence alignments. The clustering strategy incorporates the use of UPGMA (unweighted pair-group method using arithmetic averages) developed by Sneath and Sokal (1973) cited by Devereaux *et al.* (1984). The dendrogram generated is **not** a phylogenetic reconstition; it represents only the clustering order used to create the final alignment. Data obtained from these subroutines is discussed in Section 3.4.3.

3.4. Discussion.

The absence of a workable genetic system for *T. ferrooxidans* constitutes a substantial problem for a scientifically sound interpretation of the qualitative nature of nucleotide and deduced primary sequence data. The best alternative means available is a comparative analysis of the *T. ferrooxidans atp* sequence data with that from other organisms, particularly *E. coli*. The results presented in this study showed that the *T. ferrooxidans atp* gene cluster and primary sequence of individual F_1F_0 ATPsynthase subunits were generally most like that of *E. coli*. In addition, *E. coli* represents the most thoroughly studied of the bacterial F_1F_0 ATPsynthases at both genetic and biochemical levels. The discussion will be presented in the following manner. It will commence with a comparative analysis of various *atp* operon structures, including that from *T. ferrooxidans*. This will be followed by an interpretation of nucleotide sequence data of the *T. ferrooxidans atp* operon, by analogy with the well-characterised *E. coli unc* operon. Finally, the deduced primary sequence of the seven *T. ferrooxidans* F_1F_0 ATPsynthase subunits will be discussed. This latter section will concentrate on the structure/function aspects of the polypeptides. In the interests of brevity, it was decided to present a detailed analysis of two of the *T. ferrooxidans* subunits, viz. c and β. Subunit c, because of its role in proton translocation, could be expected to be markedly different from that of other non-acidophilic bacteria. Subunit β, because of its catalytic function, could be predicted to similar to homologues in

both prokaryotic and eukaryotic organisms. The remaining five *T. ferrooxidans* F_1F_0 ATPsynthase subunits will only be briefly dealt with and unusual features which could be related to gating of protons will be emphasised. The discussion should also be considered as a literature review of the topics mentioned, covering aspects which were not sufficiently dealt with in Chapter 1, as their inclusion in Chapter 3 was considered to be of particular relevance with regard to interpreting *T. ferrooxidans* data.

3.4.1. Structure and composition of the *T. ferrooxidans atp* operon.

Fig. 3.5 illustrates the arrangement of the *atp/unc* operon from a number of bacterial species, including that of *T. ferrooxidans*.

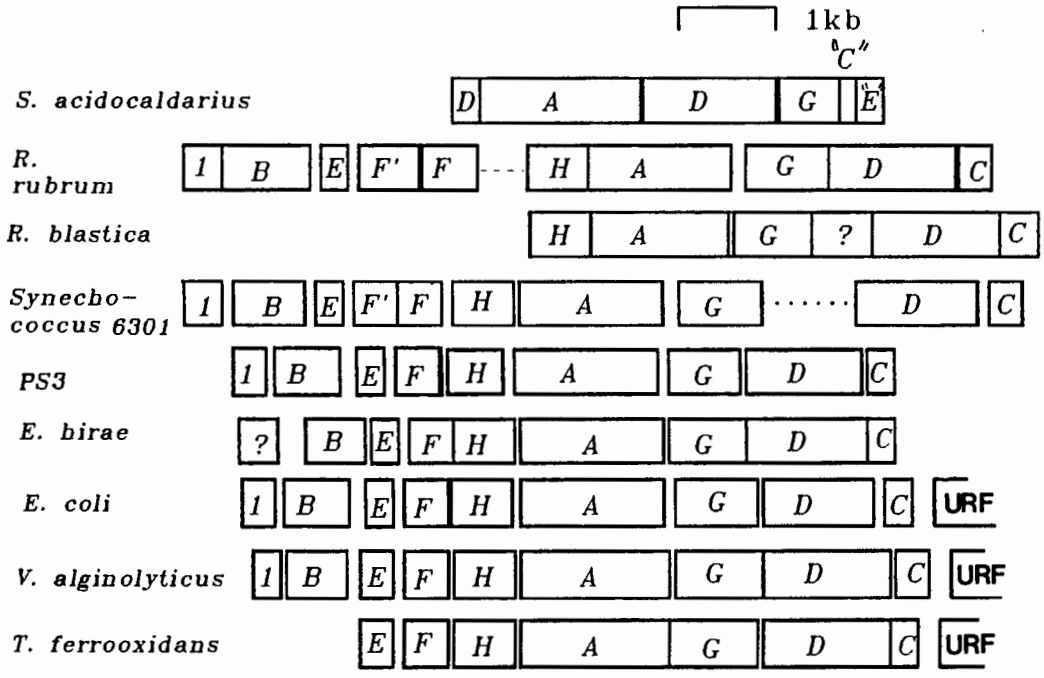

Fig. 3.5. The arrangement of the *atp/unc* operons from a number of prokaryotic organisms. ? refers to a region of DNA sequence of unknown function in *E. hirae*. The broken line indicates the separation on the chromosome, by at least 15 kb, of the *atp* genes in *Synechococcus* 6301. URF = unidentified reading frame. Note: *S. acidocaldarius* "C" equivalent is known as *atpE*, and "E", as *atpP*. (Refs:- *S. acidocaldarius*, Denda *et al.*, 1989 and 1990; *R. rubrum*, Falk *et al.*, 1985, and Falk and Walker, 1988; *Synechococcus* 6301, Cozens and Walker, 1987; *PS3*, Ohta *et al.*, 1988; *E. hirae*, Shibata *et al.*, 1992; *E. coli*, Walker *et al.*, 1984a and b; *V. alginolyticus*, Krumholz *et al.*, 1989; *T. ferrooxidans*, this study).

The *T. ferrooxidans atp* operon was constituted in a manner most like that of the heterotrophic bacterial species, such as *E. coli*, *V. alginolyticus*, *Thermus* PS3, and *E. hirae*, and the sodium ion-translocating ATPsynthase of *P. modestum*. In all of the latter organisms, the *atp* genes occur as a single cluster in a highly conserved manner, where, apart from *E. hirae* (Fig. 3.5) the F_0 genes are preceded by *atpI* and the promoter sequence, and occur to the 5' end of the F_1 cluster. The *T. ferrooxidans atp* genes were not arranged as in the other autotrophic eubacteria viz. the photosynthetic *Synechococcus* 6301, *Anabaena*

sp. Strain PCC 7120, *R. rubrum*, and *R. blastica* included in Fig. 3.5. In these latter organisms, the *atp* genes are not grouped as a single cluster, but are separated on the chromosome. Furthermore, the photosynthetic prokaryotes exhibit gene duplication in the *atpF* gene, which results in the production of subunits b' and b in the F_1F_0 ATPsynthase of these organisms. The order of the seven *T. ferrooxidans atp* genes was very different from that recorded for the archaebacterial iron-oxidising acidophile, *S. acidocaldarius*.

The *atpI* and *atpB* genes for *T. ferrooxidans* ATCC 33020 are not represented in Fig. 3.5. The reason for this, discussed fully in Chapters 2 and 5, is that despite many attempts, neither these genes, nor the *T. ferrooxidans atp* operon promoter was cloned. Using Southern hybridisation, it was established that the (probable) *atpB* gene was located immediately upstream of the *T. ferrooxidans atpE* gene (Chapter 5). Analysis of the first 42 nucleotides sequenced upstream of *atpE* in *T. ferrooxidans* suggested that the extreme 3'-end of the *atpB* gene might be present.

An URF located to the 3'-end of the *atp* operon was common in *T. ferrooxidans*, *E. coli* and *V. alginolyticus*. The incomplete *T. ferrooxidans* URF consisted of 42 residues, which had a 50% and 63% sequence identity to an URF of unknown function immediately downstream of the *E. coli unc* (Walker *et al.*, 1984b) and *V. alginolyticus atp* (Krumholz *et al.*, 1989) operons. In *T. ferrooxidans*, the *glmS* gene is located to the 3'-end of the URF (D.E. Rawlings, personal communication). Hence it is likely that the location of the *T. ferrooxidans atp* operon on the chromosome is similar to that of *E. coli* i.e. between the *oriC* at the 5'-end and *glmS* at the 3'-end (Bachmann, 1990; Walker *et al.*, 1984b).

The conserved arrangement of genes coding for F_1F_0 ATPsynthase from a variety of different organisms was commented on by Walker *et al.* (1984a). It was suggested that such striking order has important functional connotations. Although the function of the *atp/uncI* gene is unknown (Chapter 1, Section 1.6.6) it is apparent that the remaining *atp/unc* genes are clustered according to function; the channel-forming proton conducting F_0 gene products are clustered together, as are the F_1 genes associated with the production of subunits involved in catalysis.

It was suggested that the clustering of genes may reflect evolutionary origins of modern assembly of genes by tandem duplication and divergence of ancestral genes.(Casjens and Hendrix, 1974, cited by Walker *et al.*, 1984a). In this regard, evolutionary relationships between *unc/atp* genes are apparent. For example, Walker *et al.* (1984a) commented on related sequences observed between the protein products of *uncA* and *uncD*, the *uncH* and *uncC*, and *uncC* and *uncF* in *E. coli*. The relationship between the F- and V- type ATPases was referred to in Chapter 1, Section 1.6.1. A conserved operon arrangement would

facilitate cross-species transfer during evolution of all the genes for the subunits of the ATPase. Hence operon arrangement may have resulted from an evolutionary advantage (Nelson, 1992, cited by Brusilow, 1993).

The order of the genes in a given operon may be related to order of assembly of their final enzyme product (Katsura, 1980, cited by Walker *et al.*, 1984a). Brusilow (1993) suggested that the arrangement of the *unc* operon would facilitate *cis* assembly of F_1F_0 ATPsynthase *in vivo* from a single transcript.

3.4.2. The nucleotide sequence of the *T. ferrooxidans atp* gene cluster.

This section of the discussion, by analogy from data obtained from other organisms, particularly *E. coli*, will concentrate on the possible influence that the nucleotide sequence may have on regulating both transcription and translation of the *T. ferrooxidans atp* operon.

In *E. coli*, the *unc* operon transcript is transcribed as a single 7 kb polycistronic message from the promoter located 73 bp upstream of *uncI* (Senior, 1990; Walker *et al.*, 1984a). Each *unc* gene is present in only one copy on the transcript, yet the final stoichiometry of the F_1F_0 ATPsynthase subunits is $ab_2c_{10\text{-}12}\alpha_3\beta_3\gamma\delta\varepsilon$ (Fillingame 1990). *In vitro* and minicell experiments showed that the *unc* polypeptides were not synthesised in equimolar amounts; rather they were produced in relative amounts approximating their final stoichiometry. There was no proteolysis of excessive amounts of subunits (reviewed in Senior, 1990; McCarthy *et al.*, 1985). Fig. 3.6 illustrates how the arrangement of the individual genes implies that expression of individual genes must rise and fall from one end of the operon to the other. Many studies demonstrated that in the *unc* operon, genes are differentially expressed (reviewed in McCarthy, 1990).

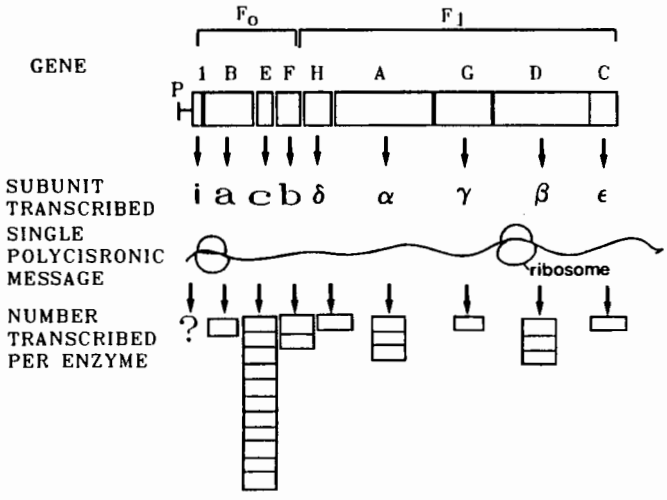

Fig. 3.6. The relationship between the genes, gene-products, and stoichiometries of the *unc* operon in *E. coli*. P = *unc* promoter. Each empty box represents one subunit in the final F_1F_0 ATPsynthase complex. ? represents the 13-14 kDa *uncI* gene product (i) of unknown stoichiometry *in vivo*.

Early statistical studies showed that codon bias among the *unc* genes varied. The more highly expressed genes showed a bias towards common codon usage, whilst in those less frequently translated, the frequency of rare codon usage increased. It was suggested that these trends could contribute toward control of gene expression (Futai and Kanazawa, 1983; Kanazawa *et al.*, 1982; Walker *et al.*, 1984a). It was proposed that the degeneracy of the genetic code may be exploited to modulate performance of the translation system in unicellular organisms; however, whilst it is known that there is a significant correlation between the abundance of tRNA species, common codons and highly expressed genes, the same correlation does not exist between infrequently expressed genes and their cognate tRNA species (reviewed in Andersson and Kurland, 1990). In most instances, rare codons do not influence speed of translation. Only when a "string" of rare codons occurs towards the extreme 5'-end of a gene, is it likely that translation rate might be slowed (Andersson and Kurland, 1990). As far as the *unc* operon is concerned, it is now known that codon bias plays little part in control of the gene expression pathway. Instead, post-transcriptional control mechanisms at two levels are involved. The molecular basis of these mechanisms is poorly understood (McCarthy, 1990).

At one level, segmental differences in stability of the polycistronic *unc* mRNA are important. It was demonstrated that the *uncIB* and *uncH* regions of the transcript have regions of instability which could influence rates of expression of these genes; other regions of the transcript are more stable (McCarthy, 1990; McCarthy *et al.*, 1991; Schaeffer *et al.*, 1989). The second, and possibly major level of control of *unc* gene expression, is related to the efficiency of initiation of translation of each gene. It is known that the intercistronic length and secondary structure of mRNA to the 5'- and 3'-end of the initiation codon (collectively known as the TIR) influence rate of *unc* gene translation. Much experimental data has accumulated which shows that when a TIR is manipulated, translation rate of the gene located to the 3'-end is altered (Dunn and Dallman, 1990; Gerstel and McCarthy, 1989; Hellmuth *et al.*, 1991; McCarthy, 1990; Pati *et al.*, 1992). Particularly interesting is the fact that there is no predictable correlation between length of the intercistronic region, TIR secondary structure and rate of gene expression. For example, the lengthy *uncB-E* TIR region associated with an mRNA of predicted unstable intramolecular structure, is a region where gene expression rate is markedly increased (Fig. 3.6). In contrast, the 58 bp tract between *uncE* and *uncF*, associated with a TIR of intramolecular stability is a region where gene expression is markedly decreased. Short intergenic distances noted for *uncF-H*, and *uncH-A* (Table 3.4) associated with mRNA of predicted stable secondary structure, are regions where gene expression is decreased and increased respectively (McCarthy, 1990). Hence this points to complex control mechanisms which are as yet poorly understood.

Whilst the proximal (*uncBE*) and distal (*uncGDC*) cistrons are relatively independently expressed, control of expression rates of cistrons in the central region of the operon is the result of *in cis* translational coupling of the genes. In all cases, translation of the gene at the 3'-end is dependent on the presence of a translationally active cistron at the 5'-end (McCarthy, 1990; Hellmuth *et al.*, 1991). The translationally coupled genes, listed in order of tightness (with intercistronic bp distances given in parentheses) are:- *uncH-A* (12 bp) > *uncF-H* (14 bp) > *uncE-F* (58 bp) > *uncA-G* (50 bp) (Hellmuth *et al.*, 1991). The mRNA from the TIR of these coupled genes is predicted to form a relatively stable secondary structure, thought to inhibit ribosome binding (McCarthy, 1990). The mechanism is not fully understood, but it is suggested that as the distance between two genes gets smaller, the likelihood that the influence of secondary structure on TIR performance will be modulated by ribosomes translating the upstream gene becomes greater i.e. the secondary structure could influence re-initiation of the ribosomes (McCarthy, 1990). Where re-intiation is prevented, gene expression rate is down-graded. However, this does not explain the tight translation couple noted for the short *uncH-A* region, where gene expression rate increases in spite of a TIR with stable secondary structure. In the latter case, it was suggested that "facilitated binding" occurs. Here it is predicted that initiation at the coupled downstream start codon is dependent on binding of free 30S subunits to an intrinsically sound TIR sequence which has been opened up by ribosomes translating the upstream gene (Hellmuth *et al.*, 1991). It is possible that both re-initiation and facilitated binding mechanisms occur simultaneously in the centrally coupled *unc* cistrons (Hellmuth *et al.*, 1991).

Codon bias of the entire *T. ferrooxidans* ATCC 33020 gene cluster was generally typical of that recorded for the *Tf*DNA7 data file. However, within individual genes, differences in codon bias were noted (Tables 3.1, and 3.2). The three *atp* genes associated with catalysis, *atpA, D* and *G*, had a codon bias that was associated with the use of more common codons (Fig. 3.3 and Tables 3.1 and 3.2). As this was unlikely to be related to the speed with which these cistrons were translated (see above) the reason for this bias must lie elsewhere. It was suggested that the use of more abundant/stable codons by genes coding for catalytic subunits is a strategy devised by an organism to reduce the likelihood of mutations in these open reading frames (Andersson and Kurland, 1990).

All of the *T. ferrooxidans atp* genes had rare codons; in four of the genes, rare codon usage was below the average of 7.3% calculated for the gene cluster; in others, *atpE, H* and *C*, the rare codon frequency was above average at 10.7%, 13.4% and 11.3% respectively. In *atpE, F, H* and *C*, some of the rare codons were grouped in "strings" (Fig. 3.3). In one instance (*atpF*) the string was at the extreme 5'-end of the gene, and could therefore possibly represent a means of downgrading the translation rate of *atpF*. In the other genes, the strings were well within the open reading frame; in such instances a function of the

"string" could be to facilitate the folding of nascent proteins by slowing the progress of ribosome transit (Andersson and Kurland, 1990).

The comparison of the length of *T. ferrooxidans atp* and *E. coli unc* intercistronic differences showed similarities and differences between the two organisms (Table 3.4). Three similar regions were *H-A, F-H* and *E-F*. However, in the case of the *A-G, G-D* and *D-C* regions, *T. ferrooxidans* intercistronic regions were noticeably shorter. As there appears to be little predictable correlation between intercistronic distance and speed of gene translation or translational coupling of genes, no reasonable interpretation of this data can be offered.

The analysis of the suggested *T. ferrooxidans atp* TIR regions, using the STEMLOOP and FOLD facilities of UWGCG predicted that the order of the intramolecular stability of the regions was *atpH-A* (ΔG = -29.6 kcal/mol) > *atpE-F* (ΔG = -28.3 kcal/mol) > *atpF-H* (ΔG = -20.1 kcal/mol) > *atpA-G* (ΔG = -18.5 kcal/mol) > *atpD-C* (ΔG = -13.6 kcal/mol) > *atpB-E* (ΔG = -12.9 kcal/mol) > *atpG-G* (ΔG = -12.8 kcal/mol). It was notable that the predicted intramolecular stability and presence of invert repeat sequences of the TIRs (Fig. 3.2) associated with the central regions of the *atp* operon was higher than the cistrons found at the proximal and distal ends of the operon. This was similar to the trends noted for the *E. coli unc* transcript (see above). Hence it is possible that the centrally located regions of the *T. ferrooxidans atp* operons are translationally coupled, whilst the proximal and distal genes may not be.

The TIRs of the *atpE* and *atpD* genes were interesting. Although the *atpB-E* region cannot be defined as *atpB* was not isolated, it was noted that nucleotide sequence located to the 5'-end of the start codon was U-rich (Fig. 3.2). Also notable was the cisronic U-content immediately downstream of the initiation codon (Fig. 3.2 and Table 3.3). A similar U-rich region was noted to the 5'-end of the highly efficient TIR of the *uncE* cistron, and this was predicted to be positively correlated with the high expression rate of the *atpE* gene (McCarthy *et al.*, 1985). Furthermore, the proposed ribosome binding site for *T. ferrooxidans atpE* was UAAGGAGUUA (Fig. 3.2) which was similar to that predicted to represent an ideal translational initiation site (Scherer *et al.*, 1980, cited by McCarthy *et al.*, 1985). The predicted ideal UAAGGAGGUAUA is homologous to the 3'-end of 16S rRNA of *E. coli*. It would be of interest to determine whether the *T. ferrooxidans atpE* domain referred to was homologous to the 3'-end of *T. ferrooxidans* ATCC 33020 16S rRNA. Nevertheless, in the absence of this data, the homology to the 3'-end of *E. coli* 16S rRNA suggested that as in *E. coli*, the *T. ferrooxidans atpE* gene represents a highly expressed cistron. This would have to be experimentally verified (Chapter 6). Hellmuth *et al.* (1991) reported that the rate of expression in the *uncD* gene was capable of independently directing efficient translation; it was not translationally coupled to *uncG*. The *T. ferrooxidans atpG-D* distance was approximately half of the *E. coli uncG-D* region (Table

3.4). Located to the extreme 3'-end of *atpG*, and immediately adjacent to the predicted *atpD* ribosome binding site, was the sequence UUUUUGGAAAUUUU. This region represented a pyrimidine-rich tract, interrupted by a purine-rich GAAA, which was suggested to be implicated in the efficient translation of frequently expressed genes, including *uncE* (McCarthy *et al.*, 1985). If the motif noted for *T. ferrooxidans atpD* represented a means of increasing rate of translation, then there would be *in cis* coupling of *atpG* and *atpD*.

The termination region of the *T. ferrooxidans atp* operon was characterised by regions with the ability to form two highly stable stemloop structures in the transcript, with a combined ΔG of -46.8 kcal/mol (Fig. 3.4). In the *E. coli unc* transcript, the single *rho*-independent terminator occurs only six nucleotides downstream of *uncC*. The operon termination region is therefore different between the two species. The loop structure 26 nucleotides downstream of *T. ferrooxidans atpC*, at the 3'-end of the region, was typical of a *rho*-independent terminator (ΔG = -24.7 kcal/mol) similar to that reported for the *unc* operon (Walker *et al.*, 1984a). The reason for the possible presence of the 3' weaker hairpin loop in the 5682-5721 nucleotide region (Figs. 3.2 and 3.4) is unknown. However, it was demonstrated that the *uncC* transcript was stabilised by the structure of the operon transcript to the 3'-end of the cistron (McCarthy, 1990; Patel *et al.*, 1990). Therefore in view of its proximity to the 3'-end of the *atpC* gene, it may be that this initial loop influences stability of the *atpC* transcript, which in the *E. coli unc* operon, has the longest half-life of >13min (McCarthy *et al.*, 1991).

3.4.3. The primary sequence of the *T. ferrooxidans atp* operon.

3.4.3.1. The F_0 subunits. In most eubacterial F_1F_0 ATPsynthases, the F_0 cytoplasmic membrane channel consists of three polypeptides, viz. a, b and c (Chapter 1, Section 1.6.1. and Fig. 1.7).

Despite many attempts (Chapters 2 and 5) it was not possible to clone *T. ferrooxidans atpB*. Subunit a (coded for by *uncB*) is the largest of the three *E. coli* F_0 subunits, and mutational studies showed that the hydrophobic polypeptide is necessary for F_0 assembly and F_0/F_1 binding (Cain and Simoni, 1989; Vik *et al.*, 1988 and 1991). Particularly significant is the involvement of highly conserved C-terminal residues in proton conduction. These latter residues are thought to interact with subunit c during proton translocation through F_0 (Fillingame, 1992a and b; Lewis and Simoni, 1992; Vik and Dao, 1992). In view of its role in proton translocation, it was unfortunate that *T. ferrooxidans atpB* was not cloned. Indeed, it is possible that the protonophoric property of *T. ferrooxidans* F_1F_0 ATPsynthase subunit a was the reason it was not isolated in the neutrophilic heterologous *E. coli* host systems used in this study. However, two of the F_0 subunits, viz. c and b of *T. ferrooxidans* F_1F_0 ATPsynthase were isolated.

3.4.3.1.a. Subunit c. The nucleotide sequence of F_1F_0 ATPsynthase subunit c has been determined for approximately 40 broadly distributed organisms and genomes. The homologues of c in the higher organisms are known as III and 9 for chloroplasts and mitochondrial F_1F_0 ATPsynthases respectively (Walker *et al.*, 1990).

In all eukaroytic and eubacterial organisms studied to date, the subunit consists of only 70-85 amino acid residues (Hoppe and Sebald, 1984) (Fig. 3.9); amongst the archaebacteria, the equivalent of c is larger, with approximately 100 residues (Denda *et al.*, 1989). In the interests of brevity, the structure of the archaebacterial "c" will not be discussed in detail. Amongst the eukaryotes and eubacteria, subunit c/III/9 is soluble in organic solvents, and it is known as the proteolipid. In all these diverse organisms, despite an extensive variation in primary structure, a number of common features are observed. All c/III/9 subunits consist of two hydrophobic stretches, separated by a central polar loop. Within the C-terminal hydrophilic domain is a highly conserved acidic residue, Glu or Asp, which reacts with DCCD, and the result is a cessation of proton translocation via F_0. Catalysis in F_1 also ceases. In *E. coli*, this residue is Asp-61. (Fillingame, 1990; Futai *et al.*, 1989; Senior, 1990). In many bacteria a highly conserved Gly-rich region occurs within the N-terminal hydrophilic terminus; an exception is *B. firmus* OF4 where certain Gly residues are conservatively replaced by Ala (Mack Ivey and Krulwich, 1991). In *E. coli*, the domain referred to is Gly-23, Gly-27 and Gly-29. In addition, there is always a region of uniformly small amino acids, which typically consist of Gly and Ala. These residues overlap with the Gly-rich region (Figs. 3.7 and 3.9).

There have been extensive studies to determine the topology of *E. coli* subunit c within the cytoplasmic membrane. All of these indicate that the subunit has a hairpin-like structure within the membrane. In support of this hypothesis are the following:-

i). subunit c consists of two hydrophobic segments, separated by a polar loop region (Hoppe and Sebald, 1984; Fillingame 1992a and b; Senior, 1990).
ii). TID-labelling (reviewed in Schneider and Altendorff, 1987) indicated that in *E. coli*, hydrophobic regions Leu-4 to Leu-19, and Phe-53 to Phe-76 are membrane integrated.
iii). Recent nuclear magnetic resonance (NMR) studies on *E. coli* c confirmed earlier data which suggested that the N- and C- hydrophobic regions are extensively α-helical, and that the C-terminal hydrophobic domain is comprised of two α-helices, disrupted by Pro-64 (Norwood *et al.*, 1992).
iv). Nuclear Overhauser effects (NOEs) studies in *E. coli* c showed that the protein must be folding such that the two α-helices are within 0.5 nm of each other (Fillingame, 1992a and b).

v). Studies on the *E. coli* DCCD-resistant mutants, Ile-28->Thr/Val, and an Asp-61->Gly, Ala-24->Asp double mutant, in which the lethal Asp-61->Gly mutation was suppressed (Table 3.8) demonstrated that Ile-28 and Ala-24 must be close to Asp-61.

vi). The C-terminus is located at the periplasmic side of the membrane, as indicated by chemical modification studies (Schneider and Altendorff, 1987).

vii). Subunit c has antigenic determinants on both sides of the cytoplasmic membrane, and antibodies to the polar loop region showed the latter domain is exposed to the cytoplasm (Schneider and Altendorff, 1987; Girvin *et al.*, 1989).

A topographical model of *E. coli* c, is shown in Fig. 3.7. Some of the residues referred to in the text are shown on the diagram. The N- and C-terminal α-helices will be referred to as Helix 1 and Helix 2 respectively.

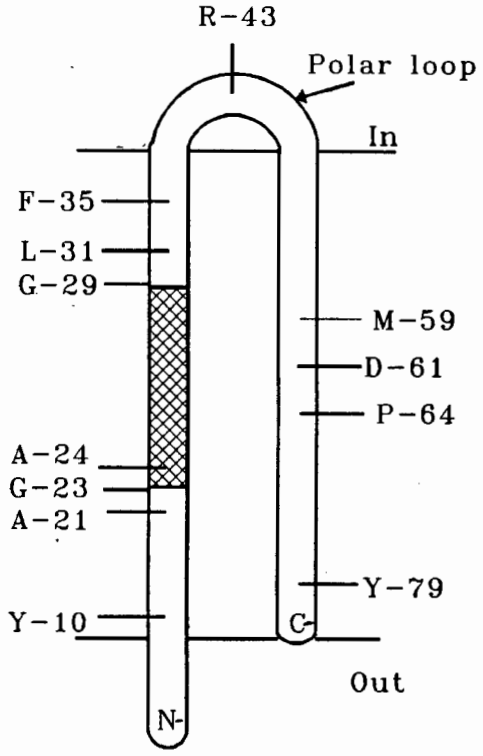

Fig. 3.7. A proposed transmembrane topological model of the c subunit of *E. coli* F_1F_0 ATPsynthase. The positions of important residues in the N-terminal α-helix (N-), (Helix 1), hydrophilic loop, and C-terminal α-helix (C-), (Helix 2), are indicated. Amino acids are indicated by a single-letter code (Appendix C); In = cell interior; Out = cell exterior (after Senior, 1990). The hatched area in Helix 1 represents the Gly-rich flexible domain of the subunit (Fillingame, 1992a and b).

The arrangement of the subunits in the c oligomer of F_0 is not understood (Fig. 1.7). However, Vik and Dao (1992) proposed that in the c-subunit oligomer, the amino terminal α-helix contacts two carboxy-terminal helices on opposite faces, while the carboxy-terminal helix contacts two amino-terminal helices on a single face. The authors claimed

that such a model is consistent with TID-labelling experiments, which predicted that in *E. coli* c, residues Tyr-10, Ala-24 and Asp-61 all face the outside of the oligomer. However, Schneider and Altendorff (1987) indicated that in right-side-out vesicles, where Tyr-10 and Tyr-73 were modified by tetranitromethane, only nitrotyrosine-73 was converted to the amino form by $Na_2S_2O_4$; nitrotyrosine-10 was not. This demonstrated that the C-terminal region faces the periplasm, and that the N-terminal end is buried within the membrane. As TID labelled both Tyr-10 and Tyr-73, both residues must be in contact with the cytoplasmic membrane; the actual orientation of the helices remains to be clarified. If the oligomer is dynamic, this may be difficult to establish *in situ*.

A number of studies indicated the functional significance of subunit c in F_1F_0 ATPsynthase. In summary, the functions of c are to:-

i). catalyse proton translocation, and to harness the energy from the translocation by conformational changes that are transmitted to F_1, thereby promoting catalysis (Fillingame, 1992 a and b). Hence for a functional c subunit, there must be regions implicated in proton translocation, and conformational change.

ii). assist in competent functional F_0 assembly (reviewed in Futai *et al.*, 1989).

iii). promote F_0/F_1 binding; certain antibodies directed against c prevent F_0/F_1 binding (Deckers-Hebestreit and Altendorff, 1992a)

Extensive genetic and physical analyses pinpointed residues and/or domains in *E. coli* c, which are responsible for F_0 assembly, proton translocation and conformational change. Generally, mutations in the hydrophobic α-helices result in faulty enzyme assembly and/or inhibition of proton translocation and associated conformational changes. Mutations in the hydrophilic loop domain indicated that one residue, Arg-41 is critical for the transmission of conformational change to F_1 (Table 3.8).

Mutations which disrupt proton translocation in *E. coli* c suggested an intriguing mechanism. Co-operativity among individual c subunits within the oligomer is known to occur. This was concluded from the fact that the inactivation of only one c subunit with DCCD results in the inactivation of the entire enzyme complex (Fillingame, 1992 a and b).

The DCCD-binding Asp-61 is critical for proton translocation, and directing conformational change. Asp-61 is thought to lie at the centre of a protonation-deprotonation "pocket" within subunit c. This pocket spans the mid-region of both Helix 1 and Helix 2, and the ensemble of the residues in this pocket is such that it optimises the functioning of the DCCD-binding residue (Fillingame, 1992a and b). For example, the conservative replacement of Glu for Asp-61 in *E. coli*, drastically decreases ATPase activity in that organism (Miller *et al.*, 1990). In *E. coli*, proton-resonance studies incorporating the nitroxide analogue of DCCD demonstrated that the centre of the pocket lies between

Met-57 and Val-60 on Helix 2. Another face of the binding pocket is provided by the side chain of Leu-31, extending from the opposite transmembrane helix. Other residues in Helix 1, shown to be of significance in proton-translocation are Gly-23, Ala-24, Ala-25, Lys-34 and Phe-35 (Figs. 3.7 and 3.9) (Fillingame, 1992a and b; Fillingame *et al.*, 1991)). The NMR study of Norwood *et al.* (1992) reported that the pK_a value of Asp-61 within the pocket was elevated by 1 unit. Such an observation could be of relevance to mechanisms of proton translocation.

Domains in *E. coli* subunit c which are thought to be crucial for the transmission of conformational change from F_0 to F_1, and possibly from F_1 to F_0, are flexible regions which occur between Gly-23 to Gly-29, the hydrophilic loop, and the region between Asp-61 and the C-terminus (Fillingame, 1992a and b; Norwood *et al.*, 1992).

Incorporating most of the data from mutational, NMR and NOEs studies, Fillingame (1992a and b) proposed that the two helices of the subunit come together as a unit during the process of proton translocation. Either helix could serve as a scaffold to anchor the essential carboxyl group at the same position within the pocket in the middle of the membrane. Hence the pocket could provide both proton-deprotonation, and the coupled pK_a-altering conformational change, within the two interacting helices. Furthermore, Fillingame (1992a and b) cited experimental evidence to suggest that during proton translocation, a helical-helical interaction occurs between the helical unit of subunit c that anchors the essential carboxyl group of Asp-61, and a transmembrane helix of subunit a which includes residues 217-224 of the latter subunit. Important in this interaction is the conserved stretch of Gly residues occuring between Gly-23 and Gly-29 of Helix 1. This hypothesis forms the basis of the model for proton-translocation proposed by Fillingame (1992a and b). The role of the essential Arg-41 in the hydrophilic loop in transmitting conformational changes is unknown.

The primary sequence data for the *T. ferrooxidans* F_1F_0 ATPsynthase c subunit indicated that the polypeptide was typical of an F_0 proteolipid. The subunit was a small hydrophobic protein, comprised of 84 residues with a combined M_r of 8 989. (Tables 3.6 and 3.7). PEPPLOT data indicated that the subunit consisted of two hydrophobic domains of high α-helical potential. These domains were situated between residues 1-38 and 53-84 (data not shown). Between these two domains was a hydrophilic stretch of sequence between residues 38-52. Hence it is highly likely that the overall tertiary structure in the cytoplasmic membrane was similar to that recorded for the *E. coli* c subunit. By analogy with *E. coli* c, a model for the proposed folding of *T. ferrooxidans* c is depicted in Fig. 3.8.

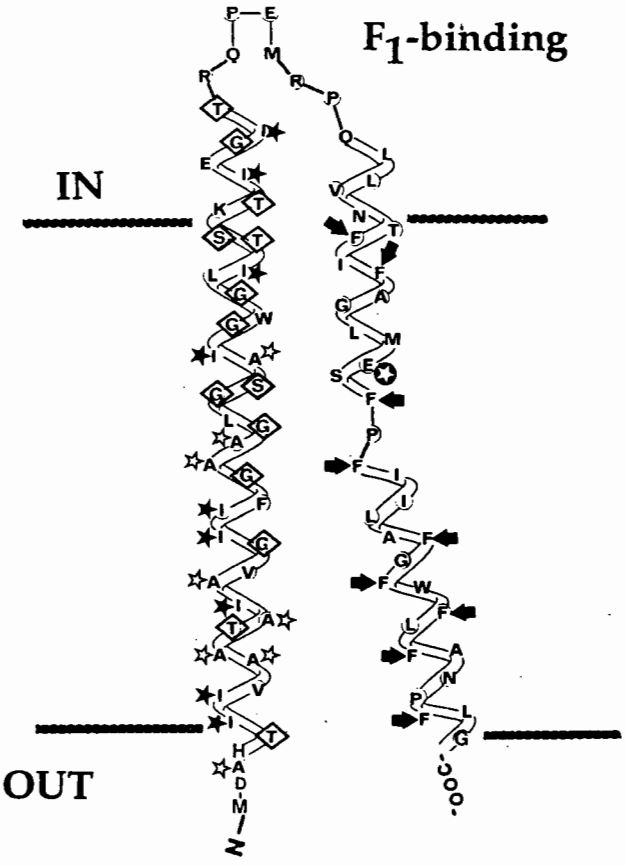

Fig. 3.8. The hypothesised orientation of *T. ferrooxidans* c in the cytoplasmic membrane modelled on that proposed for *E. coli* by Miller *et al.* (1990). To emphasise their distribution, certain residues discussed in the text are marked. These include Ala, Gly, Ile, Thr and Ser in the N-terminal α-helix (Helix 1) and Phe and DCCD-binding Glu-63 in the C-terminal α-helix (Helix 2). In = cell interior; Out = cell exterior.

Despite the probable similarity in overall tertiary structure, it was not possible to complement *E. coli uncE* mutants with the *T. ferrooxidans atpE* gene. *T. ferrooxidans atpE* was expressed *in vitro* by an *E. coli*-derived translation system using an *E. coli in vitro* translation kit (Chapter 4). Therefore it seemed likely that qualitative differences in primary structure between the *E. coli* and *T. ferrooxidans* c subunits were such that the latter subunit could not functionally complement the *uncE* mutant used in this study.

GAP analyses had indicated that *T. ferrooxidans* subunit c was very different from that of *E. coli*, and from any other c subunit compared (Table 3.5). Table 3.7 summarises the primary sequence data from a number of bacterial c subunits, obtained using the PEPTIDESORT facility of UWGCG.

Table 3.7. Amino acid composition, (mole %), of *T. ferrooxidans* F_1F_0 ATPsynthase subunit c, compared with subunit c from other bacterial species. (Calculated using UWGCG Version 7 PEPTIDESORT).

		Amino Acid[c] (Mole %)							
		Aliphatic	Hydroxyl	Acidic	Acid + acid amide	Basic	Charged	Hydro-phobic	Aromatic
Bacterial Species[a]	No[b]	A,G	S,T	D,E	D,E,N,Q	H,K,R	D,E,H,K,R	I.L.M.V	F,W,Y
T. ferrooxidans	84	25.0	10.0	4.7	9.5	4.8	9.5	31.0	14.3
S. acidocaldarius	101	31.7	6.9	5.0	7.9	5.0	9.9	34.7	11.0
A. caldarius	82	29.3	6.1	6.1	9.8	6.1	12.2	37.8	8.5
P. modestum	89	33.7	9.0	5.6	10.1	4.5	10.1	34.8	4.5
B. megaterium	70	31.4	10.0	4.3	8.6	5.7	10.0	38.6	2.9
Thermus PS3	72	27.8	8.3	4.2	8.3	5.6	9.7	40.3	5.6
B. firmus OF4	69	30.4	8.7	4.3	7.2	4.3	8.7	40.5	4.3
V. alginolyticus	84	27.4	7.1	4.8	9.5	3.6	8.3	39.2	8.3
E. coli	79	29.1	1.3	6.3	11.4	3.8	10.1	43.0	7.6
Ave.protein[d]	-	16.9	13.1	11.5	19.8	13.5	25.1	20.2	8.3

[a] - For references, see Fig.3.9.

[b] - No. of amino acid residues/subunit.

[c] - Amino acids referred to single letter code

[d] - Values after Dayhoff et al. (1978).

Generally, data indicated that the mole% content of categories of amino acids in *T. ferrooxidans* c was typical of other c subunits, with one notable exception. This was the high content of the aromatic amino acids (14.3%). It was of interest to note that the other extreme acidophile included, the archaebacterium *S. acidocaldarius*, also had a high content of aromatic amino acids (11%). Comparison of the amino acid content of *T. ferrooxidans* and *E. coli* showed that the content of hydroxy-amino acids was markedly higher in *T. ferrooxidans*. The *E. coli* c subunit was more hydrophobic than that of *T. ferrooxidans*. It is possible that these differences collectively prevented functional complementation of *E. coli* F_0 mutants. However, the sodium ion-translocating F_0 channel of *P. modestum* functionally complements *E. coli* F_0 mutants; the hydroxyamino acid content of *P. modestum* c subunit (Kaim *et al.*, 1992) is similar to that of *T. ferrooxidans* (Table 3.7).

Multiple sequence alignment data clearly indicated the variability in position amongst subunit c/III/9 residues. Amongst the species compared, only two positions were invariant (Fig. 3.9). These were, in *T. ferrooxidans* c, Arg-43 and Pro-45. The common occurrence of small Ala and Gly residues interspersed amongst hydrophobic residues of Helix 1 in eubacterial c subunits, is clearly evident. The hydrophilic loop domain is relatively well conserved amongst bacteria. Apart from the DCCD-binding residue, which occurred as either Glu or Asp, there were few observable common trends in primary sequence structure in Helix 2. Notable was the presence of a conserved Pro amongst the bacteria (Fig. 3.9).

Primary sequence alignment data of c/III/9 subunits from a number of organisms indicated that in certain regions the *T. ferrooxidans* c subunit was unique; however, in other areas, known to be implicated in proton translocation and conformational changes, *T. ferrooxidans* was remarkably similar to other c subunits (Fig. 3.9).

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          1                 # #     # # ## #   # #          ### # *#*46
  Bov  .......DID TAAKFIGAGA A.TVGVAGSG AGIGTVPGS. LIIGYARNPS
  Sac  ......MQLV LAALYIGAGI S.TIGLLGAG IGIAIVFAA. LINGVSRNPS
  Rru  .......MDA EAAKMIGAGL A.AIGMIGSG IGVGNIWAAN LISTVGRNPA
  Spi  .....MNPLI AAASVIAAGL AVGLASIGPG VGGGTAAGQA V.EGIARQPE
  Wht  .....MNPLI AAASVIAAGL AVGLASIGPG VGQGTAAGQA V.EGIARQPE
  Syn  .....MDSLT SAASVLAAAL AVGLAAIGPG IGQGSAAGQA V.EGIARQPE
  Pmo  MDMVLAKTVV LAASAVGAGA AM.IAGIGPG VGQGYAAGKA V.ESVARQPE
  Eco  .....MENLN MDLLYMAAAV MMGLAAIGAA IGIGILGGKF L.EGAARQPD
  Val  .....METL. LSFSAIAVGI IVGLASLGTA IGFALLGGKF L.EGAARQPE
  Bme  .......... ..MGLIASAI AIGLAALGAG IGNGLIVSKT I.EGTARQPE
  Ps3  .......... MSLGVLAAAI AVGLGALGAG IGNGLIVSRT I.EGIARQPE
 Bfir  .......... ..MAFLGAAI AAGLAAVAGA IAVAIIVKAT I.EGTTRQPE
  Ehi  .......... ..MNYIAAAI AIMGAAIGAG YGNGQVISLT IEEGMARGPE
 Acido ....MQLDMV KAIYNIAVAL LLGLAAVGSG VGDGMVMSKY V.EGVARQPE
  Tfe  ...MDAHTII VAATAIAVGI IFGAAGLGSA IGWGLITSKT I.EGITRQPE

       47  #       ##  # ## #    #                84
  Bov  LKQQLFSYAI LGFALSEAMG LFCLMVAFLI LFAM..... .
  Sac  IKDTVFPMAI LGFALSEATG LFCLMVSFLL LFGV..... .
  Rru  AKSTVGLYGW IGFAVTEAIA LFALVVALIL LFAA..... .
  Spi  AEGKIRGTLL LSLAFMEALT IYGLVVALAL LFANPFV... .
  Wht  AEGKIRGTLL LSLAFMEALT IYGLVVALAL LFANPFV... .
  Syn  AEGKIRGTLL LSLAFMEALT IYGLVVALVL LFANPFA... .
  Pmo  AKGDIISTMV LGQAIAESTG IYSLVIALIL LYANPFVGLL G
  Eco  LIPLLRTQFF IVMGLVDAIP MIAVGLGLYV MFAVA..... .
  Val  MAPMLQVKMF IIAGLLDAVP MIGIVIALLF TFANPFVGQL G
  Bme  ARGTLTSMMF VGVALVEALP IIAVVIAFMV QGK..... .
  Ps3  LRPVLQTTMF IGVALVEALP IIGVVFSFIY LGR..... .
 Bfir  LRGTLQTLMF IGVPLAEAVP IIAIVISLLI LF..... .
  Ehi  MSGQLRTTMF IGVALVEAVP ILGVVIALIL VFAV..... .
 Acido ARGSIFGSAL LGVALVEAFP VIALAFGIII LFTKGAF... .
  Tfe  MRPQLLVNTF IFAGLMESFP FIILAFGFWF LFANPFLG.. .
```

Fig. 3.9. Alignment of the deduced amino acid sequence of the *T. ferrooxidans* subunit c with other c/III/9 subunits, obtained by using the PILEUP facility of UWGCG vax programme. Numbers refer to *T. ferrooxidans* c residues. * denotes invariant residues; # denotes semi-conserved residues. *E. coli* residues in **boldface** indicate sites of missense mutations (Table 3.8). *Bov* = bovine mitochondrial subunit 9 (Hoppe and Sebald, 1984); *Sac* = *Saccharomyces cerevisiae* mitochondrial subunit 9 (Hoppe and Sebald, 1984); *Rru* = *R. rubrum* (Falk *et al.*, 1985); *Spi* = spinach chloroplast subunit III (Hudson *et al.*, 1987) *Wht* = wheat chloroplast subunit III (Howe *et al.*, 1982); *Syn* = *Synechococcus* 6301 (Cozens and Walker, 1987); *Pmo* = *P. modestum* (Esser *et al.*, 1990); *Eco* = *E. coli* (Walker *et al.*, 1984a); *Val* = *V. alginolyticus* (Krumholz *et al.*, 1989) *Bme* = *B. megaterium* (Brusilow *et al.*, 1989); *Ps3* = strain PS3 (Ohta *et al.*, 1988); *Bfi* = *B. firmus* OF4 (Mack Ivey and Krulwich, 1991) *Ehi* = *E. hirae* (Hoppe and Sebald, 1984); *Acido* = *Acido caldarius* (Hoppe and Sebald, 1984); *Tfe* = *T. ferrooxidans* (this study).

Mutational analyses of *E. coli* c residues have been useful for describing functions for certain residues within the subunit. Table 3.8 lists most of the results obtained from various studies involving the mutation of *E. coli* c residues. Equivalent *T. ferrooxidans* c residues are also given.

Table 3.8. A summary of the mutations[a], and their resultant effects in *E. coli* F_1F_0 ATP synthase subunit c. Equivalent *T. ferrooxidans* residues are given

Location in proteolipid	Mutation	Result	Equivalent *T. ferrooxidans* residue
N- terminal	Ala20→Pro	Suppresses Pro64→Leu/Ala mutation	Ala-22
α- helix	Ala21→Val	No catalysis	Gly-23
(Helix 1)	Gly23→Asp	Assembly fault	Gly-25
	Ala24→Ser	Confers DCCD resistance	Ser-26
	Ala24→Asp	Suppresses Asp61→Gly mutation	
	Ala25→Thr	Inhibits H^+ translocation	Ala-27
	Ile28→Thr/Val	Confers DCCD resistance	Trp-30
	Leu31→Phe	DCCD resistance; no H^+ translocation	Ile-33
Hydrophilic	Ala39→Gly/Pro	No catalysis	Ile-41
loop	Ala39→Gly/Ser/Thr	Slows catalysis	
	Ala40→Glu	No catalysis	Thr-42
	Ala40→Val/Ser/Pro	Slow catalysis	
	Arg41→Cys/Ser/ Leu/Trp/His/Lys	Proton leaky: uncoupled ATPase	Arg-43
	Gln42→Glu/Arg	Proton leaky: uncoupled ATPase	Gln-44
	Gln42→Leu/His/ Gly/Ala/Val	Defective coupling	
	Pro43→Ser/Ala/His/Thr	Minor defects in coupling	Pro-45
	Pro43→Leu	No catalysis	
	Asp44→Asn/His	Slow catalysis	Glu-46
	Asp44→Tyr	No catalysis	
	Leu45→Pro/Arg	Slow catalysis	Met-47

Location in proteolipid	Mutation	Result	Equivalent *T. ferrooxidans* residue
C-terminal	Gly58→Asp	Defective assembly	Gly-60
α-helix	Asp61→Asn/Gly	DCCD-resistant	Glu-63
(Helix 2)	Asp61→Glu	Slow catalysis	
	Asp62→Ser	Inhibition of c/b binding	Ser-64
	Ala62→Pro	Suppresses subunit b Gly9→Asp mutation	Ser-64
		mutation	
	Pro64→Leu/Ala	Inhibition of H^+translocation; DCCD resistant	Pro-66

[a] - Mutations reviewed in:
Fillingame, (1990, 1992a; 1992b); Futai *et al* (1989); Fraga and Fillingame, (1991); Miller *et al.* (1990); Senior, (1990).

At most of the sites where mutation affected function in *E. coli*, the equivalent *T. ferrooxidans* c residue was usually identically or semi-conserved. Hence in these areas, structure/function relationships in *T. ferrooxidans* c are possibly similar. However, there were some unusual substitutions in *T. ferrooxidans* c, which will be referred to in the following.

Collectively, the amino acid composition of the transmembrane helices of *T. ferrooxidans* c subunit were of particular interest.

The extreme N-terminus of the subunit was predicted to lie within the acidic periplasm of the organism (Fig. 3.8). Despite this, this region of the c subunit was not noticeably different from other organisms compared (Fig. 3.9). Within the acidic milieu of the periplasm it is likely that most of the amino acids would occur as cations, where only the α-amino group is ionised, and may therefore act to repel protons. An exception would be Asp-2, which would occur as a zwitterion. The presence of His-4 at the extreme N-terminus was interesting. Apart from *Acido caldarius*, in which Lys occurs at position 7, *T. ferrooxidans* c was the only subunit to have a basically charged residue in this region. It is possible therefore that the inclusion of a basic residue, which would exert a neutralising effect, represented an adaptation to acidophily.

Within the region predicted to be in the cytoplasmic membrane, the composition of the *T. ferrooxidans* subunit c Helix 1 was typical, in that small amino acid residues were interspersed among hydrophobic Ile residues (Fig. 3.8). Whilst the content of Gly and Ala was representative of the subunit as a whole, the presence of six Thr, and two Ser was unusual (Figs. 3.8 and 3.9). Small relatively hydrophilic Gly residues may attract water molecules into the interfaces of the hydrophobic transmembrane helices, thereby participating in proton conductance (Nuomi *et al.*, 1991). The presence of a narrow water-lined ion channel, rich in Gly and Ala may be of significance in proton conductance in *E. coli* (Fillingame, 1990) (Chapter 1). Nuomi *et al.* (1991) in a study on the proteolipid from yeast vacuolar proton-translocating ATPase, suggested that the protonated group associated with Thr and Ser residues was required at certain positions for control of proton flow. The protonated groups associated with these residues may represent an unusual channeling of protons through Helix 1 in *T. ferrooxidans* c. Research on model amphipathic peptides, which were constructed to mimic natural ion channels, demonstrated that the number of Ser residues included in a Leu/Ser peptide chain influenced both the size and associated ion-conducting capacity of the water-lined channel (Lear *et al.*, 1988, cited by Fillingame, 1990). Mack Ivey and Krulwich (1991) in interpreting their data for the alkaliphilic *B. firmus* OF4 commented on unusual substitutions in Helix 1 in that organism, and suggested that these could represent an unusual proton passage, related to the environment in which *B. firmus* OF4 occurs. It is possible that the hydroxy amino acids associated with Helix 1 of *T. ferrooxidans* c, represented an unusual water-lined proton-conducting channel related to the acidic environment; the presence of both Ser and Thr in Helix 1 of *T. ferrooxidans* c subunit would decrease the hydrophobicity of the Helix (Table 3.7).

Fillingame (1992a and b) indicated that in eubacterial c subunits (apart from *B. firmus* OF4; Mack Ivey and Krulwich, 1991) the region equivalent to Gly-23 to Gly-29 in *E. coli*, has a consensus sequence of Gly-X-Gly/Ala-X-Gly-X-Gly/Ala. Mutations in this region confer DCCD resistance (See Table 3.8). Fillingame (1992b) proposed that the Gly residues in this region introduce a compact but flexible structure, separated by bulky hydrophilic residues, and form a screw-like groove around the helix. This groove is thought to be the site of sequential protonation of trimers of c subunits by interaction with subunit a, and conformational change within F_0 (Fillingame 1992a and b; Fillingame *et al.*, 1991). The *T. ferrooxidans* c subunit in this region was conserved as Gly_{25}-Ser-Ala-Ile-Gly-Trp-Gly_{31} (Figs. 3.8 and 3.9). Of interest in this domain was the presence of the bulky Trp-30 residue. This was unique to *T. ferrooxidans* c. If *T. ferrooxidans* c was folded in a similar manner to *E. coli* c, this would place the Trp-30 almost opposite to the DCCD-binding Glu-63 charged residue (Fig. 3.8). As such, Trp-30 would be located within the proposed protonation-deprotonation pocket. A mutation of the residue equivalent to *T. ferrooxidans* c Trp-30 in *E. coli*, viz. Ile-28, to either Thr or Val conferred DCCD resistance on the mutant (Table 3.8)

(Senior, 1990). A mutation which introduced an aromatic residue at Leu-31 in *E. coli* c rendered the membrane proton-impermeable (Table 3.8). Hence Trp-30 could be implicated in proton translocation in *T. ferrooxidans*. Alternatively, this residue could serve to introduce rigidity and therefore stabilise the inner surface of the F_0 channel.

Another three residues possibly implicated in proton movement in the binding pocket of *E. coli* c Helix 1 are, Leu-31, Lys-34 and Phe-35 (Fillingame, 1992b). In *T. ferrooxidans*, these were represented by Ile-33, Lys-36 and Thr-37, respectively. Overall, the amino acid residues in *T. ferrooxidans* subunit c Helix 1 indicated that the organism has evolved its own unique "ensemble" of amino acids to optimise proton conduction in that organism.

The C-terminal Helix 2 of *T. ferrooxidans* c subunit was notable for both common and unique features. The proposed DCCD-binding residue was conserved as Glu-63. Also conserved was *T. ferrooxidans* c Pro-66. In *E. coli*, the equivalent Pro-64 is thought to introduce a disruption of the α-helix of Helix 2 in this region (Fillingame. 1992a and b; Norwood *et al.*, 1992; Senior, 1990). Mutation of Pro-64->Leu/Ala resulted in inhibition of proton translocation and DCCD resistance in *E. coli*. This mutation was suppressed by mutating the residue opposite to Pro-64, Ala-20, to Pro (Table 3.8). This was interpreted to indicate that the Pro residue, by disrupting the α-helix, confers a kink in this region between Helix 1 and Helix 2 which is necessary for the translocation of protons (Senior, 1990).

Of interest, and unique to *T. ferrooxidans* c, was the presence of a substantial number of aromatic residues in Helix 2 (Table 3.7). In the extreme C-terminal 29 residues, Phe occurred nine times, and Trp, once (Fig. 3.8). The reason for this constitution is unknown, but it is possible that the aromatic amino acids are implicated in both stabilisation of the F_0 channel, and in proton conductance.

Of particular interest to this study was the mutation of *E. coli* c Leu-31->Phe (Table 3.8). The mutant cells were DCCD resistant, due to the membrane being rendered proton-impermeable by the single Phe-31 residue. Yet, the mutants were capable of normal ATPase and oxidative phosphorylation activity. It was demonstrated that the cytoplasmic membrane of the mutant was fully energised, even when stripped of ATPase. In wild-type *E. coli*, stripping the membrane of ATPase results in an unenergised membrane, due to proton-permeability of the cytoplasmic membrane. It was concluded that in the mutants, membranes were energised by protons from electron transport, and that oxidative phosphorylation was occurring in the absence of $\Delta\mu_{H+}$ (Cox *et al.*, 1983). Certain authors suggested that this experiment provided strong support for the theory that $\Delta\mu_{H+}$ is not an obligatory intermediate of oxidative phosphorylation (Slater, 1987). In *T. ferrooxidans* c, the residue Ile-33 was equivalent to *E. coli* c Leu-31. Located opposite

Ile-33 was a unique Phe-55 (Figs. 3.8 and 3.9). Hence this Phe residue in *T. ferrooxidans* c occurred in the vicinity of the *E. coli* c Leu-31->Phe mutant. It is possible that *T. ferrooxidans* c Phe-55, adjacent to the DCCD-binding Glu-60, and close to Trp-30, is implicated in control of proton flow in F_0. It would be of interest to mutate the *E. coli* residue equivalent to Phe-55 viz. Val-56, to Phe, and to assess the implications for oxidative phosphorylation in that organism.

Regarding a possible structural role for *T. ferrooxidans* c aromatic residues, Nuomi *et al.* (1991) in their study of yeast vacuolar proton-ATPase suggested that Phe and Tyr residues at certain positions in that enzyme imposed structural constraints on the proteolipid and when altered, resulted in inactive proteolipid. The location of the aromatic residues in the C-terminus of *T. ferrooxidans* c was interesting. Two Phe residues were on either side of the conserved Pro-66 and six of the remaining aromatic residues were located to the 3'-end of Pro-66 (Fig. 3.8) The presence of the Leu_{77}-Phe-Ala-Asn-Pro-Phe_{83} motif in *T. ferrooxidans* c is recorded in other c/III subunits (Fig. 3.9). PEPPLOT data indicated that the $Asn_{80}Pro_{81}$ residues could be interpreted to introduce a break in an α-helical domain, which stretched from Phe-67 to Leu-83. Also of significance was the high probability of the $Asn_{80}Pro_{81}$ tandem to form a turn in the subunit at the extreme 3'-end (data not shown). It is possible that the Leu_{77}-Phe-Ala-Asn-Pro-Phe_{82} motif is important in conferring structural constraints in this region of the subunit. *E. coli* subunit c does not have this motif. Hence the tertiary structure of the c subunit from the two organisms is likely to be different at the extreme C-terminus; this difference is not shown in Fig. 3.8.

The striking differences in the primary sequence data recorded for the *T. ferrooxidans* c subunit transmembrane helices could indicate distinctive functions. The N-terminal Helix 1 could be interpreted to lie at the inner face of the channel, and be implicated in the formation of a water-lined channel for proton conduction, possibly stabilised by the presence of Trp-30. The C-terminal Helix 2, possibly located at the outer face of the channel (i.e. periplasmic-facing) through its possession of a high number of aromatic residues, could provide a stable "backbone" for the presentation of the DCCD-binding residue to protons, such that protons move through F_0 through a narrow water-filled channel, to the "active site" of the Glu-63 proton pump. Such a mechanism for proton conduction via a water-lined channel was suggested for *E. coli* c by Fillingame (1990).

The hydrophilic loop region (residues 37-51) of *T. ferrooxidans* c was generally homologous to that of other bacterial species and could have structure/function relationships similar to those of other c/III/9 subunits (Figs. 3.8 and 3.9). Mutational studies of the polar loop region of *E. coli* subunit c showed that this region is important in binding F_0 to F_1, together with subunit b (Miller *et al.*, 1989; Fraga and Fillingame, 1989). The polar loop of subunit c plays a special role in coupling proton translocation to ATP synthesis. The

$Arg_{43}Gln_{44}Pro_{45}$ sequence in *T. ferrooxidans* c is highly conserved amongst species, with no variation recorded for either Arg or Pro (Fig. 3.9). An extensive mutational analysis of the *E. coli* c Glu-37 to Leu-45 region was done (Fraga and Fillingame, 1991). It should be noted that in this study, the effect of mutations was gauged solely on the ability of an Δ*uncE* mutant transformed with plasmids carrying the mutant gene, to grow on MMS; no attempt was made to assess the precise effect of any the 30 mutant *uncE* genes generated. Although growth on MMS might have occurred, enzyme activity might have been impaired; the latter possibility was not investigated. Fraga and Fillingame (1991) showed that only Arg-41 (Arg-43 in *T. ferrooxidans* c) was absolutely essential for growth on MMS. Any substitution of this residue resulted in the uncoupling of ATP hydrolysis from proton translocation. All other residues were tolerant to some extent, of limited changes, although sufficiently drastic changes in any one of the residues resulted in loss of function (Table 3.8). Fillingame (1992b) suggested that the function of the conserved Arg is associated with proton translocation and transmission of conformational change to F_1. He further proposed that the collective "ensemble" of amino acids in the hydrophilic loop of subunit c is important in maintaining the essential features of the domain. Futai *et al.* (1989) referred to a study in which it was shown that in yeast, the presence of a positively charged Arg-39 residue, equivalent to *T. ferrooxidans* c Arg-43, was essential for a functional F_1F_0 ATPsynthase and was probably required for interaction with other subunits.

An interesting residue in the *T. ferrooxidans* hydrophilic loop region was the hydroxyamino acid, Thr-42. The only other organism to possess Thr in the identical position is *B. firmus* OF4 (Mack Ivey and Krulwich, 1991) (Fig. 3.9). In *E. coli*, substitution of the equivalent Ala-40 with Glu is lethal, whereas mutation to Val, Ser or Pro retards growth rate on succinate minimal medium (Table 3.8) (Fraga and Fillingame, 1991). The result with the hydroxyamino acid Ser is interesting, as it indicates that the Thr residue in *T. ferrooxidans* and *B. firmus* could be of significance in establishing an optimum ensemble of residues in the hydrophilic loop domain of these two organisms.

The tolerance of mutational change in the hydrophilic loop region of *E. coli* c, and the fact that the critical Arg residue was conserved in *T. ferrooxidans*, makes it likely that this entire domain of *T. ferrooxidans* c subunit could functionally complement *E. coli*.

Analysis of the primary sequence data from the *T. ferrooxidans* c subunit showed that the polypeptide has evolved some unique features, which may be representative of an adaptation to an acidophilic mode of existence. However, in regions known to be implicated in proton translocation and/or conformational change, whether within the subunit itself, or between subunits c and a, *T. ferrooxidans* c was remarkably conserved; hence, the proton-translocating mechanism of *T. ferrooxidans* c is probably similar to that

proposed for *E. coli* F_0. The detailed analysis of the *T. ferrooxidans* c subunit primary structure did not show unequivocally why *T. ferrooxidans* c subunit was unable to function in various *E. coli unc* mutants. It is likely that collectively, the primary structure of the *T. ferrooxidans* subunit was sufficiently different from that of *E. coli* c to prevent F_0 complementation of a neutrophilic organism.

A dendrogram generated by the PILEUP multiple sequence alignment facility of the UWGCG programme (See Fig. 3.10). The distance along the vertical axes is proportional to the differences between the sequences; the horizontal axes have no significance at all.

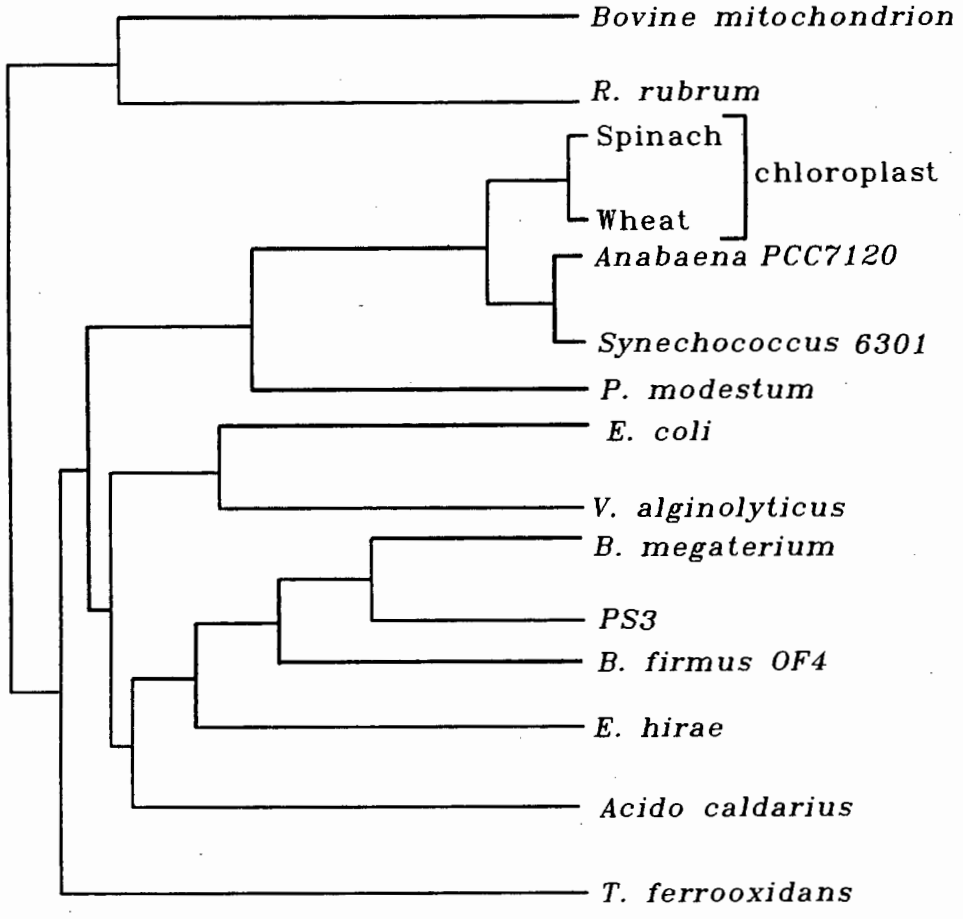

Fig. 3.10. A dendrogram showing the clustering of various c/III/9 subunits from a variety of organisms, generated from the multiple sequence alignment data (PILEUP) of F_1F_0 ATPsynthase subunit c/III/9 illustrated in Fig. 3.9. For references, see Fig. 3.9.

The dendrogram indicated that the *T. ferrooxidans* c subunit was considerably different from others included in the alignment; the subunit formed a distinct outgroup, and was not grouped with the c subunit from any other of the other species compared.

Although the dendrogram is not a phylogenetic reconstitution, it was interesting to note that the F_1F_0 ATPsynthase c subunit from *R. rubrum* was clustered with subunit 9 from bovine mitochondria. *R. rubrum* and mitochondria are considered to have a common ancestral form. Similarly, the predicted phylogenetic relatedness of the cyanobacteria and the chloroplast of higher plants was evident by the clustering of the respective c/III subunits (reviewed in Recipon *et al.*, 1992). Amongst the non-photosynthetic eubacterial species, the two γ-proteobacteria, *E. coli* and *V. alginolyticus* were grouped together, as were gram-positive *B. megaterium*, strain PS3, *B. firmus*, and *E. hirae*. The acidophilic species, *Acido caldarius*, of unknown origin, was loosely clustered with the gram-positive group. *P. modestum* of unknown phylogenetic affiliation (Esser *et al.*, 1990) was distantly clustered with the cyanobacterial/chloroplast group. A recent study of 38 different subunit c/III/9 was undertaken with the aim of using the primary sequences as a tool for interkingdom and metaphytes molecular phylogenies, as a backup to rRNA data (Recipon *et al.*, 1992). In the bacterial group, the primary sequences of eight bacterial species c subunits were compared. These included *Anabaena* sp. Strain PCC 7120, *B. megaterium*, *E. coli*, strain PS3, *R. rubrum*, *Synechococcus* 6301, *V. alginolyticus* and *S. acidocaldarius*. Data generated by these authors for the bacteria was considered to be in agreement with the rRNA data of Woese (1987). It would be of interest to include the *T. ferrooxidans* c subunit data in their programme, as results from this study indicate that primary sequence from *T. ferrooxidans* c may not be suitable for phylogenetic analysis of this organism, as the polypeptide has shown adaptation to acidophilic environmental constraints.

3.4.3.1.b. Subunit b. Although the primary sequence of the b subunit and its homologues I and b, in chloroplast and mitochondrial F_1F_0 ATPsynthases respectively, is not well conserved, the subunit does have strikingly common features amongst the different organisms (Senior, 1990; Walker *et al.*, 1987a). Towards the N-terminal, the first 25-30 residues are amphipathic, and form a distinctly hydrophobic domain. This is followed by a highly charged polar α-helical domain, consisting of the remaining residues, which number in the region of 120. The homologue of b in the photosynthetic bacteria, b' (subunit II in chloroplast F_1F_0 ATPsynthase) is probably similarly constituted (Falk and Walker, 1988; McCarn *et al.*, 1988; Walker *et al.*, 1990).

The b subunit in *E. coli* F_0 has been well characterised, and the transmembrane topology is established. The predicted folding of the subunit is shown in Fig. 3.11.

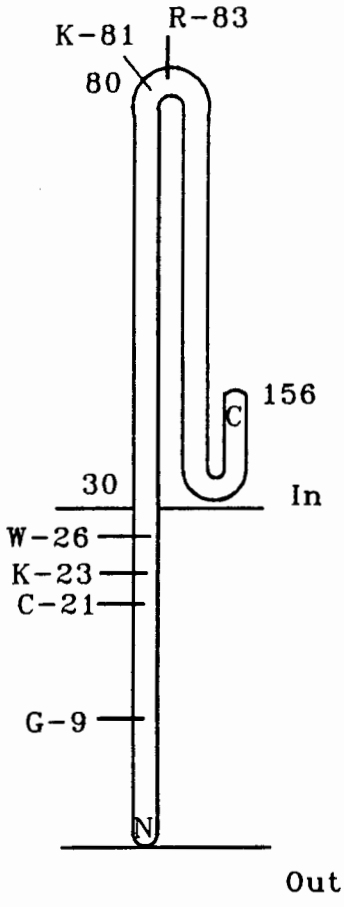

Fig. 3.11. A structural model of the b subunit of *E. coli* F_1F_0 ATPsynthase within the cytoplasmic membrane. The N- and C-termini are indicated, as are the positions of certain residues discussed in the text. In = cell interior; Out = cell exterior (after Senior, 1990).

Typically, the first 30 residues of the 156-residue polypeptide are predicted to span the cytoplasmic membrane as a single transmembrane α-helix. The polar domain of the protein extends into the cytoplasm to form two extended α-helices, which are formed by a bend in the subunit in the region of residues Glu-77 to Gln-85 (reviewed in Senior, 1990). Studies indicated that the two b subunits in *E. coli* F_0 exist as a dimer, and that this is mediated by interaction of the polar portions of the subunit, regardless of any interaction in the membrane-spanning N-terminal domain (Dunn, 1992, Senior, 1990). As a dimer, the polar regions of the subunit are thought to form an extended four-helix bundle (Dunn, 1992). It is possible that this structure forms at least part of the interconnecting stalk structure which is seen in electron-micrographs of the F_1/F_0 complex (Fillingame, 1992b).

The function of the b subunit remains to be finally established. In *E. coli*, it is required for the formation of a functional F_0 *in vivo*, and subunits a and c will not assemble in the absence of b. It was proposed that the hydrophobic residues which form the single membrane-spanning α-helix, anchor the polar region, and interact with other F_0 subunits in the membrane bi-layer. This region is known to interact with subunits a and c (reviewed in Fillingame, 1990; Senior, 1990). The polar domain is thought to be required

for high-affinity binding of F_0 to F_1. It was proposed that the group of four α-helices enters the hollow cavity of the F_1 complex, and interact with β subunits; b-β cross-links were demonstrated (Dunn, 1992). After the assembly of F_0, the membrane-protruding polar domains can be removed by proteolysis, the F_0 channel remains intact, and still supports proton translocation. However, binding to F_1 is abolished, and the digested fragments of the b subunits are unable to reconstitute into the F_0 channel (Senior, 1990). It is unlikely that b/I/9 subunits are involved in the translocation of protons (Fillingame, 1990; McCormick and Cain, 1991; Senior, 1990; Vik and Dao, 1992; Walker *et al.*, 1987a).

GAP analyses of the b subunit of *T. ferrooxidans* indicated that it was most like that of the b subunits from *E. coli* and *V. alginolyticus*. However, homologies amongst b subunits in general were not high (Table 3.5). PEPTIDESORT analyses of the primary structure from the b subunits of a number of bacterial species showed that the *T. ferrooxidans* b subunit was typical (Tables 3.6 and 3.9). It consisted of 159 residues with a combined M_r of 17 884 Da. The pI was 7.69, which was within the range recorded. The protein was highly polar, and exhibited hydrophobic tendencies. No unusual trends were noted in *T. ferrooxidans* b.

PEPPLOT data demonstrated that the first 26 N-terminal residues of *T. ferrooxidans* subunit b were hydrophobic, and demonstrated both α-helical and β-sheet potential. Another short hydrophobic domain was noted between residues Arg_{124}-Val_{132}, towards the C-terminus (Fig. 3.12). Otherwise, the remainder of the protein was extremely polar, and exhibited strong α-helical tendencies (data not shown). It is likely that the tertiary structure of *T. ferrooxidans* b is similar to that noted for other b subunits. Like the *T. ferrooxidans* c subunit, the b subunit of *T. ferrooxidans*, despite a predicted common tertiary structure, was unable to complement *E. coli uncF* mutants, although *T. ferrooxidans atpF* was expressed by an *E. coli in vitro* translation kit (Chapters 2 and 4). This suggested that the qualitative make-up of the *T. ferrooxidans* b subunit was such that complementation of *E. coli uncF*$^-$ was not possible.

Results generated from the multiple primary sequence data alignment indicated that only two residues were conserved amongst bacterial b subunits. In *T. ferrooxidans* these were Arg-39 and Ala-82. Many residues were semiconserved and in these domains, bacteria appeared to cluster according to extant taxonomic groupings. (Figs. 3.12 and 3.13).

Table 3.9. Amino acid composition, (mole %) of the *T. ferrooxidans* F_1F_0 ATP synthase b subunit, compared with subunit b from other eubacterial species[1].

		Amino Acid (mole %)							
		Aliphatic	Hydroxyl	Acidic	Acid + Acid amide	Basic	Charged	Hydro-phobic	Aromatic
Bacterial species[a]	No[b]	A,G	S,T	D,E	D,E,N,Q	H,K,R,	D,E,H,K,R	I,L,M,V,	F,W,Y
T. ferrooxidans	159	19.5	4.4	17.0	24.5	18.2	35.2	29.6	2.5
E. coli	156	21.8	5.1	16.7	25.6	16.7	33.3	25.6	3.2
V. alginolyticus	156	20.5	5.8	17.3	28.8	16.7	34.0	22.4	3.8
B. firmus OF4	153	14.4	5.9	16.3	30.1	15.0	31.4	28.8	5.2
B. megaterium	172	16.7	4.6	19.2	30.2	15.1	34.3	29.1	3.5
Thermus PS3	163	17.7	8.0	16.0	27.0	16.6	32.5	26.4	3.7
E. hirae	174	13.2	12.1	16.1	27.6	13.8	29.9	28.7	2.9
P. modestum	168	15.5	4.2	17.9	28.6	17.9	35.7	26.8	5.4
Synechococcus 6301	171	22.2	10.5	14.0	25.1	11.7	25.7	26.9	3.5
*Anabaena*PCC7120	187	26.7	7.5	13.4	27.3	10.7	24.1	25.7	2.1
R. rubrum	182	29.1	4.4	14.8	21.4	17.0	31.9	23.1	3.8
Ave.protein[d]	—	16.9	13.1	11.5	19.8	13.5	25.1	20.2	8.3

a - For references, see Fig. 3.12.

b - No. of amino acid residues/subunit

c - Amino acids referred to by single letter code

d - Values after Dayhoff *et al.* (1978).

l - Data generated using UWGCG Version 7 PEPTIDESORT.

```
                                    1              #    # #     26
 Ana  MGTFLLLMAE ASAVGGELAE GGAEGGFGLN TNILDTNLIN LAIIITVLFV
 Syn  MSSWILL... .........A HAETSGFGLN LDLFETNLIN LAIIIGLLVY
 Bme  .......... ...MAVSNMF VLGAA..GIN GGDILFQLVM FLILLALLQK
 Ps3  .......... ..VLWKANVW VLGEAAHGIS GGTIIYQLLM FIILLALLRK
Bfir  .......... .......... ....MGFDIN WGSALYQLLA FSVLLFFLSK
 Efa  .......... .....MLNQL AIAEVGNPM. LGNIIVVSGS FLILMFLLKH
 Eco  .......... .......... .......VNL NATILGQAIA FVLFVLFCMK
 Val  .......... .......... .......VNI NATLLGQAIS FALFVWFCMK
 Tfe  .......... .......... ....MNPVGI NGTLIVQLVT FVILVALLYK
 Pmo  .......... .........L APQNMPAVSI DINMFWQIIN FLILMFFFKK
 Rru  ......MISL ALAAETAEHG GEAASHGGLF ADPAFWVSIA FLMVVGFVYI

      27                                                    76
        #       *   #                                       #
 Ana  FGRKVLGNTL KTRRENIETA IKNAEQRAAD AAKQLKEAQQ KLEQAQAEAE
 Syn  AGRGFLGNLL SNRRAAIEAE IREVEEKLAS SAQALSQAQT QLKEAEAEAA
 Bme  FAFGPVMGIM KKREEHIAGE IDEAEKQNEE AKKLVEEQRE ILKQSRQEVQ
 Ps3  FAWQPLMNIM KQREEHIATK STRRKNDRQE AEKLLEEQRE LMKQSRQEAQ
Bfir  FALKPLLGIM EKREQMINEQ ISSADKNRKD L.KLLLRQRQ ALEQARMEAN
 Efa  FAWGPISDIL KKREDKIAND LDSAEKSRIN SAKMEQEREQ QLLASRSDAA
 Eco  YVWPPLMAAI EKRQKEIADG LASAERAHKD LDLAKASATD QLKKAKAEAQ
 Val  YVWPPLMKAI EERQKKIADG LQAAERAAKD LDLAQANASD QLKEAKRTAT
 Tfe  YMYGPLRKVM DDRRAKIADG LAAAERGKEE MALAQKRATE LVREAKDKAA
 Pmo  YFQKPIAKVL DARKEKIAND LKQAEIDKEM AAKANGEAQG IVKSAKTEAN
 Rru  KAKNKILGAL DGRGAAVKAK LDEARKLRDD AQALLAEYQR RQRDAMKEAD

      77                                                   126
       #   *           #          #     #  ##          #
 Ana  RIKKSAQDNA QTAGQAIIAQ AAVDIERLQE AGAADLNAEL DRAIAQLRQR
 Syn  RLLVEAKARA AAVRQEILDK AAADVERLKA TAAQDVSTEQ QRVLDELRRY
 Bme  VMMENARKSA EDKKEEIVAA AREESERLKA AAKQEIEQQK DQAVAALREQ
 Ps3  ALIENAASLA EEQKEQIVAS ARAEAERVKE AAKKEIEREK EQAMAALREQ
Bfir  EIIQNAKKLS EQQGQDIVKA ARNDA..... ....EIHREK EQAVSALREQ
 Efa  DIIKNAKESG ELSRQNILKE TQEEVARLKS KAQTDIMLER DTALNSVKDD
 Eco  VIIEQANKRR SQILDEAKAE AEQERTKIVA QAQAEIEAER KRAREELRKQ
 Val  EIIEQANKRK SQILDEAREE AQAERQKILA QAEAELEAER NRARDDLRKQ
 Tfe  EIIANAERRG VELREEAQGK AREEADRIIA SARAEIDVET NRAREVLRGQ
 Pmo  EMLLRAEKKA DERKETILKE ANTQREKMLK SAEVEIEKMK EQARKELQLE
 Rru  EIIRHAKDEA ARLRAKAEAD LEASIRRREQ QAVDRIAQAE AQALAQVRNE

      127                                 159
      #             #           #
 Ana  VVALALQKVE SELQGGISED AQKTLIDRSI AQLGGGV .. .
 Syn  AVAQALSRVE TQLSQQLDEA AQQRLIDRSL ATL..... .
 Bme  VASLSVLIAS KVIEKELSEQ DQEKLIHEYI QEVGDVR... .
 Ps3  VASLSVLIAS KVIEKELTEQ DQAAS..... ..... .
Bfir  VAGLSVLIAT KVIEKELNEA DQEKLVQEYL KEVGEEL... .
 Efa  VADLSLQIAA KILNKELSPE MHESLINQYI EGLGSSNETR
 Eco  VAILAVAGAE KIIERSVDEA ANSDIVDKLV AEL..... .
 Val  VATLAVAGAE KILERSIDKD AQKDILDNIT AKL..... .
 Tfe  VVELVVNGTQ RILHREIDDQ THRDIIDRMV GQL..... .
 Pmo  VTDLAVKLAE KMINEKVDAK IGANLLDQFI GEVGEEK... .
 Rru  AVDVAVSAAR SLMAGSLAKA DQNRLIDAAI ADLPGKLH.. .
```

Fig. 3.12. Alignment of deduced amino acid sequence of the *T. ferrooxidans* F_1F_0 ATPsynthase b subunit with the b protein from other bacterial species, obtained by using the PILEUP facility of UWGCG vax programme. Numbers refer to *T. ferrooxidans* b residues. * denotes invariant residues; # denotes semi-conserved residues. *E. coli* residues in **boldface** indicate sites of missense mutations. *Ana* = *Anabaena* sp. Strain PCC 7120 (McCarn *et al.*, 1988); *Syn* = *Synechococcus* 6301 (Cozens and Walker, 1987); *Bme* = *B. megaterium* (Brusilow *et al.*, 1989); *Ps3* = strain PS3 (Ohta *et al.*, 1988); *Bfi* = *B. firmus* OF4 (Mack Ivey and Krulwich, 1991); *Ehi* = *E. hirae* (Hoppe and Sebald, 1984); *Eco* = *E. coli* (Walker *et al.*, 1984a); *Val* = *V. alginolyticus* (Krumholz *et al.*, 1989); *Tfe* = *T. ferrooxidans* (this study); *Pmo* = *P. modestum* (Esser *et al.*, 1990); *Rru* = *R. rubrum* (Falk *et al.*, 1985).

The primary sequence of the b subunit of *T. ferrooxidans* F_1F_0 ATPsynthase appeared to be fairly typical of the non-photosynthetic gram-negative group (Fig. 3.12). One interesting feature was the Asn_2Pro_3 motif at the extreme 5'-end of the N-terminus. This motif was also noted in *E. hirae* b (Fig. 3.12). The AsnPro tandem was present at the 3'-end of the *T. ferrooxidans* c subunit (Fig. 3.9) and was postulated to be the site of a turn in the α-helix of c at that point. PEPPLOT data for *T. ferrooxidans* b indicated the same feature was likely at the N-terminus of the subunit (data not shown).

Regions and/or isolated residues for which functions were suggested in *E. coli* b were clearly homologous in *T. ferrooxidans* b. Hoppe and Sebald (1984) and Deckers-Hebestreit *et al.* (1992a) suggested that the following domains in *E. coli*, with the equivalent *T. ferrooxidans* domains given in parentheses, are important for F_0/F_1 binding: 53-82 (57-85), 85-105 (89-108), 84-98 (87-101) and 101-122 (104-125) (Fig. 3.12). Mutational analyses have identified functions for some of the *E. coli* b residues. These *E. coli* b residues, with the *T. ferrooxidans* b equivalent in brackets are: Gly-9 (Val-12), implicated in F_0 assembly (Futai *et al.*, 1989); Trp-26 (Tyr-29), stabilises the proton pathway (Futai *et al.*, 1989); Gly-131 (Gly-134), Glu-155 (Gln-156) and Leu-156 (Leu-159), important for F_0/F_1 binding (Futai *et al.*, 1989; Senior, 1990). Certain mutational studies were unable to describe a function for *E. coli* b residues. These include, with the *T. ferrooxidans* residue/domain in brackets: Lys-23 (Lys-29), Cys-21 (Leu-24) and the Glu_{77}-Gln_{85} (Ala_{80}-Glu_{88}) domain (Senior, 1990; Kauffer *et al.*, 1991; McCormick and Cain, 1991).

From the analysis of the primary sequence data for *T. ferrooxidans* b, there were no conclusive data to suggest that any modifications of the subunit were a result of the acidophilic environment which the organism inhabits. There was no conclusive information to indicate why the *T. ferrooxidans* b subunit was unable to complement either the *E. coli unc* point or deletion mutants (Chapters 2, 4 and 5). In many areas known to be implicated in F_0/F_1 binding, *T. ferrooxidans* b was homologous to *E. coli* b. At this stage, it can only be suggested that as for subunit c, the collective primary structure of *T. ferrooxidans* b was such that it was unable to function in *E. coli*. Whether this was due to inability to form a functional F_0 pore, and/or to bind to F_1 *in vivo*, is undetermined.

The dendrogram generated from the multiple primary sequence data for various b subunits is presented in Fig. 3.13.

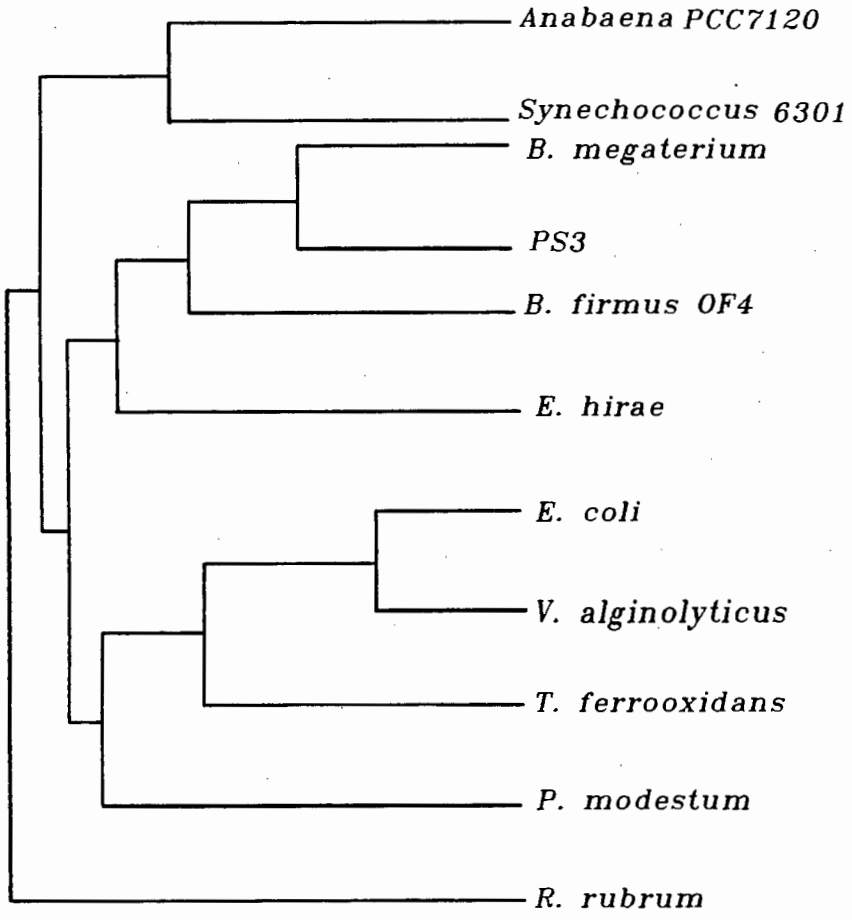

Fig. 3.13. A dendrogram showing the clustering of subunit b from a number of bacterial species generated from the multiple sequence alignment data (PILEUP) of F_1F_0 ATPsynthase subunit b illustrated in Fig. 3.12. For references, see Fig. 3.12.

The cyanobacteria, gram positive, gram negative, and photosynthetic heterotrophic *R. rubrum* formed distinct clusters, consistent with current phylogenetic groupings. Unlike subunit c, the *T. ferrooxidans* b subunit was clustered, albeit distantly, with that of *E. coli* and *V. alginolyticus*. It was of interest to note that the b subunits of the two latter γ-proteobacteria were grouped together, whereas the position of the *T. ferrooxidans* b subunit was approximately that expected of a β-group proteobacterium.

3.4.3.2. The F_1 subunits. All five *T. ferrooxidans* F_1 subunits complemented *E. coli unc* mutants (Chapters 2 and 4). Table 3.6 demonstrates that the subunits were remarkably similar in size to those from *E. coli*. Apart from δ, the pI values for the subunits were also similar between the two organisms. GAP analyses of all five subunits indicated that the *T. ferrooxidans* F_1 subunits were most like those of *E. coli* and *V. alginolyticus* (Table 3.5). Sequence homology was most conserved amongst the major F_1 subunits known to be

directly implicated in catalysis, viz. α and β. Homology amongst the "minor" subunits was lower, with that of the *T. ferrooxidans* δ subunit being as low as for that recorded for the F_0 subunits. Sequence was moderately conserved amongst the ε and γ subunits of the gram-negative group. PEPTIDESORT data indicated that all of the *T. ferrooxidans* F_1 subunits were typical globular proteins, and an extensive comparison of the mole% amino acid composition of the *T. ferrooxidans* F_1 subunits with those from other bacteria, did not reveal any significant differences (data not shown).

Of the seven *T. ferrooxidans* F_1F_0 ATPsynthase genes sequenced for this study, only four had Cys residues. All of these subunits were located in F_1; they were, δ (1), α (3), γ (4) and β (3) (Table 3.1). Thiol groups in F_1F_0 ATPsynthase may play an important role in the linking of F_0 to F_1, thereby influencing proton conduction and may also be involved in intersubunit crosslinking within F_1. Oxidation of certain dithiol residues in mitochondrial F_1F_0 ATPsynthase results in enhanced proton flow through F_0 (Zanotti *et al.*, 1992). It is possible that within *T. ferrooxidans* F_1F_0 ATPsynthase, δ, α, γ, and β, may be involved in maintenance of the higher order structure of the enzyme, control of proton translocation from F_0, and the coupling of proton-induced conformational change to catalysis.

For the purposes of this discussion, the primary sequence data of the *T. ferrooxidans* F_1 single copy "minor" core subunits will be discussed first, followed by the two "major" catalytic subunits.

3.4.3.2.a. Subunit δ. Subunit δ of bacterial and chloroplast F_1F_0 ATPsynthases, and its mitochondrial homologue, OSCP, is a globular protein consisting of between 180-190 amino acids (Walker *et al.*, 1990). The topology of δ/OSCP within the F_1F_0 complex is uncertain. Electron microscope studies on the structure of the *E. coli* F_0F_1 structure led to speculation that δ/OSCP forms part of a "stalk" linking F_1 to F_0 (Lucken *et al.*, 1990; Capaldi *et al.*, 1992). However, contrary to this view, and based on experimental evidence, is the proposal that δ/OSCP is actually embedded within the F_0F_1 complex (Boyer, 1993; Engelbrecht and Junge, 1990; Fillingame,1990; Senior, 1990). In bovine mitochondrial F_1, OSCP does not appear to be a component of the F_1-ATPase (Walker *et al.*, 1990). The currently favoured conformation of δ within *E. coli* is shown in Fig. 1.8 (after Capaldi *et al.*, 1992). Whatever the final topology, it was suggested that δ/OSCP is at the "interface of proton flow and ATP synthesis" within the enzyme complex (Engelbrecht and Junge, 1990).

The functions of δ within F_1F_0 ATPsynthase have not been conclusively established. There is a notable lack of mutational data on δ subunits, and until such data become available, the precise functions of residues/domains within δ will remain largely speculative. It was

suggested that δ/OSCP could be involved in both protonic flow and/or conformational change by interacting with proton-conducting subunits (Engelbrecht and Junge, 1990; Senior, 1990). There are experimental data to to support this hypothesis.

There is evidence that in *E. coli*, δ interacts with other subunits within the enzyme. It was suggested that δ mediates F_1/F_0 binding, although no direct link between δ and F_0 was shown (Fillingame, 1990; Senior, 1990). However, either purified δ or to a certain extent, ε is capable of promoting the attachment of an $\alpha_3\beta_3$ complex to F_0 in *E. coli* (Tuttas-Dorschung and Hanstein, 1989). Within *E. coli* F_1, δ binds to the N-terminal of the α subunit (Engelbrecht and Junge, 1990; Senior, 1990). In wild-type *E. coli*, δ also binds to β, probably at an αβ interface. This linking is independent of nucleotide conditions used (Aggeler *et al.*, 1992; Capaldi *et al.*, 1992; Mendel-Hartvig and Capaldi, 1991a). Electron microscope studies of *E. coli* F_1F_0 ATPsynthase provided evidence that δ is involved in conformational coupling within the enzyme, as the position of the subunit alters during catalysis (Gogol *et al.*, 1990; Capaldi *et al.*, 1992). In chloroplasts, δ is not involved in F_0/F_1 binding (Engelbrecht and Junge, 1990; Fillingame, 1990). In bovine mitochondria, OSCP together with a second protein F_6, is required for correct binding of F_1 to the membrane sector (reviewed in Walker *et al.*, 1990).

A possible role for δ in control of proton flow was suggested. In chloroplasts, when δ is added to F_1-depleted thylakoid membranes, proton conduction is blocked through F_0 possibly by δ acting as a plug (Engelbrecht and Junge, 1990; Lill *et al.*, 1988, cited by Fillingame, 1990). Although *E. coli* δ partially fulfils the function of blocking protons within chloroplast F_0F_1 complexes, this property is not observed in *E. coli* F_0 complexes (Engelbrecht and Junge, 1990). In contrast, *E. coli* δ is required for the opening of the F_0 channel; in the presence of δ, F_0 channels assembled in the cytoplasmic membrane become increasingly proton-permeable. This effect is enhanced by the presence of α, and blocked by the presence of γ (Angov *et al.*, 1991; Brusilow, 1993; Monticello *et al.*, 1992; Pati and Brusilow, 1991). In the thermophilic strain PS3, passive proton conduction through F_0 is blocked by the combined action of δ, γ and ε (Yoshida *et al.*, 1977, cited by Futai *et al.*, 1989). In mitochondrial F_1F_0 ATPsynthase, δ modifies NADPH-dependent proton flux in the presence of F_1 (Penin *et al.*, 1986, cited by Engelbrecht and Junge, 1990). Together with γ, OSCP acts to gate protons (Zanotti *et al.*, 1992).

In addition to its proposed function as a connector/transmitter device, *E. coli* δ may direct the assembly of the F_1F_0 ATPsynthase complex (Angov *et al.*, 1991; Brusilow, 1987; Solomon and Brusilow, 1989).

```
                1                                                  42
                                  #**  *#       #
    Eco  ....... SE FITVARPYAK AAFDF....A VEHQSVERWQ .DMLAFAAEV
    Val  .......MSD LTTIARPYAK AAFDF....A LEKDQLDQWG .QMLSFAAEV
    Tfe  .......MAD LITVARPYAE ALMGWRKRAA RNRPGRMHCR .RLPAMIADV
    Rbl  ...MAEAASI SQGIAERYAT ALFEL....S KETGALKTLE TDIDALKDVL
    Rru  ...MSSHKAG VTGVAERYAT ALYEL....A EDRGALDQVS ADLRSLKAML
Bovoscp  FAKLVRPPVQ IYGIQGRYAT ALYSA....A SKQNKLEQVE KELLRVGQIL
    Bme  ........MS QPAVAKRYAL ALFQL....A TEKQMIDEMQ DQLQIVEEVF
    Ps3  ........MN QEVIAKRYAS ALFQI....A LEQGQLDRIE EDVRAVRQAL
    Ana  ....MTSKVA NTEVAQPYAQ ALLSI....A KSKSLTEEFG TDARTLLNLL
    Syn  ....MTS... TSQLFDPYAE ALMAI....A REQGLEDRFG EDAALFRSTL
    Spi  .......... VDSTASRYAS ALADV....A DVTGTLEATN SDVEKLIRIF

            43                                                 87
                                                      # ## #  *
    Eco  TKNEQMAELL SGA.LAPETL AESFIAVCGE QLDENG.QNL IRVMAENGRL
    Val  AKNEQMNELL TGS.VSADKM AEIFVAVCGE QVDTHG.QNL LKVMAENGRL
    Tfe  ....QAQAFL TDP.ERRDAE KVSLLSAVPV AVDVKAWKAF LALLIHNDRW
    Rbl  AGSPDLGAMI ASPVISRGDQ AKAVAAIAGK MGLSPLMTNT LALMSEKRRL
    Rru  DESGDLRRVI ASPVIGRDDQ RKALTALAEK AGFHEIVRNF LGVVAAKHRS
Bovoscp  KE.PKMAASL LNPYVKRSVK VKSLSDMTAK EKFSPLTSNL INLLAENGRL
    Bme  AKTPELMDVL THPKIT.IER KKQFVSEAF. AELSPTVQHT VLLLLERHRI
    Ps3  AENGEFLSLL SYPKLS.LDQ KKALIREAF. AGVSTPVQNT LLLLLERHRF
    Ana  TENQQLRNFI DNPFIA.AEN KKALIKQIL. SEASPYLRNF LLLLVDKRRI
    Syn  AASADLRHLL ENPTLF.SSQ KKAVLNQVFG SSVHPLVLNF LNLLVDRNRI
    Spi  SEEP.VYYFF ANPVISIDNK RSVLDEIITT SGLQPHTANF INILIDSERI

            88                                                135
                #
    Eco  NALPDVLEQF IHLRAVS.EA TAEVDVISAA ALSEQQLAKI SAAMEKRL..
    Val  AALPDVCTEF YTLKKEH.EK EIDVEVISAT ELSDEQLANI GSKLEKRL..
    Tfe  PATAEIGTLF ADAMRRA.EG VVDVLVTSAI ALDAGQKTAV QSALERRF.A
    Rbl  FALPQVLSAL AGLIAEE.KG EVTAEVTAAT KLSAAQAKKL AETLKA..KV
    Rru  FAVPGMIGAF LERLAAR.RG EVTARIVSAT ALTSAQKSAL TTALNK..AT
Bovoscp  TNTPAVISAF STMMSVH.RG EVPCTVTTAS ALNEATLTEL KTVLKSFLKK
    Bme  QIVSEMVKEY .RFLANEVRG VADATVYSVK PLSADEKRAI SQSFASKVGK
    Ps3  GLVPELAGTV SRPRSTTARG IAKAVAYSGA ASTDEELRAL SDVFAQKVGK
    Ana  FFLPEILQQY LALL.RQLNQ TVLAEVTSAV ALTEDQQQAV TEKVLALTKA
    Syn  AFLDGIADRY QALL.RKLRN VVRADVSSAV PLTEAQVQVI TEKVKQLTGA
    Spi  NLVKEILNEF EDVF.NKITG TEVAVVTSVV KLENDHLAQI AKGVQKITGA

            136                                               179
              #     # *  ###*# # #    *  #  * * # ##   #   #
    Eco  SRKVKLNCKI DKSVMAGVII RA...GDMVI DGSVRGRLER LADVLQS...
    Val  ERKVKLNCSV DETLLGGVII RA...GDLVI DDSARGRLNR LSDALQS...
    Tfe  GHKVRFREAV DAALIGGLVI HT...GDLTI DASVRGQVQQ LARTLRS...
    Rbl  GKTVKLNTTV DESLIGGLIV KL...GSTMI DTSVKSKLAS LQNAMKEVG.
    Rru  GNTVTIDASV DPALLGGMVV RV...GSRMV DSSLSTKLKR LQLAMKGVG.
Bovoscp  GQVLKLEVKI DPSIMGGMIV RI...GEKYV DMSAKTKIQK LSRAMRQIL.
    Bme  .HTLNISNIV DKTVIGGVKL RI...GNRIY DGSISSKLET IHRGLLAHRS
    Ps3  .QTLEIENII DPELIGGVNV RI...GNRIY DGSVSGQLER IRRQLIG...
    Ana  .RQVELATKV DSDLIGGVII KV...GSQVI DSSIRGQLRR LSLRLSNS..
    Syn  .AGVEIESQV DADLLGGVII KV...GSQVL DASLRGQLKR ISISLAA...
    Spi  .KNVRIKTVI DPSLVAGFTI RYGNEGSKLV DMSVKKQLEE IAAQLEMDDV
```

Fig. 3.14. Multiple sequence alignment of the deduced primary amino acid sequence of *T. ferrooxidans* δ subunit, complied using the PILEUP facility of UWGCG. Numbers refer to *T. ferrooxidans* δ residues. * denotes invariant residues, # denotes semi-conserved residues. *Eco* = *E. coli* (Walker *et al.*, 1984a); *Val* = *V. alginolyticus* (Krumholz *et al.*, 1989); *Tfe* = *T. ferrooxidans* (this study); *Rbl* = *R. blastica* (Tybulewicz *et al.*, 1984); *Rru* = *R. rubrum* (Falk *et al.*, 1985); Bovoscp = bovine mitochondrial OSCP (Walker *et al.*, 1985); *Bme* = *B. megaterium* (Brusilow *et al.*, 1989); *Ps3* = strain PS3 (Ohta *et al.*, 1988) *Ana* = *Anabaena* sp. Strain PCC 7120, (McCarn *et al.*, 1988); *Syn* = *Synechococcus* 6301 (Cozens and Walker, 1987); Spi = spinach chloroplast (Hermans *et al.*, 1988).

In *T. ferrooxidans*, δ consisted of 179 residues with a combined *Mr* of 19 573 Da. Hence the protein was similar in size to that from *E. coli*. The subunit was highly charged, and had an isoelectric point of 11.03 (Table 3.6). Although this value was different from the electronegative values of δ in *E. coli* and *V. alginolyticus*, PEPTIDESORT analyses indicated that the subunit was electropositive in gram-positive bacteria, the non-sulphur photosynthetic bacteria, *Anabaena* and mitochondria (data not shown). The differences recorded for the isoelectric points of various δ/OSCP subunits are curious, but this could be due to differences in surface charges of the protein which are related to neither attachment sites, nor to function (Engelbrecht and Junge, 1990).

The δ subunit from *T. ferrooxidans* and *E. coli* shared only 29% identical and 57% similar residues. Despite this, it was demonstrated during the current study that *T. ferrooxidans* δ functionally complemented an *E. coli* Δ*unc* mutant, where the F_0 channel was comprised of *E. coli* subunits, and the F_1 moiety of *T. ferrooxidans* subunits (Chapter 4). It is possible that unlike the F_0 subunits, secondary and tertiary structure of the single-copy F_1 subunits are more important to enzyme function than is primary structure (Engelbrecht and Junge, 1992).

Aligning the primary sequence data from 11 different organisms (Fig. 3.14) showed that the δ subunit was poorly conserved. Only nine residues were absolutely conserved throughout. Five of these occurred toward the C-terminal, and two at the N-terminus. Only one, *T. ferrooxidans* δ Arg-86, occurred away from either of the two termini. *T. ferrooxidans* δ had a short domain that was unique amongst the δ subunits sequenced to date. The residues occurred adjacent to the universally conserved Ala-23, and were comprised of Arg19-Lys-Arg-Ala22. The basically charged nature of this N-terminal hydrophilic domain extended to Arg-26.

The dendrogram compiled from the multiple primary sequence data of various δ subunits in some cases reflected phylogenetic trends of these organisms (Fig. 3.15). *T. ferrooxidans* δ was distantly clustered with the δ from the two gram negative heterotrophs, *E. coli* and *V. alginolyticus*. The *R. blastica*/*R. rubrum*/mitochondrial group formed a cluster, as did the gram positive and cyanobacterial groups. The phylogenetic relationship of the chloroplast/cyanobacterial group was not reflected in this dendrogram. Cyanobacterial δ was more similar to δ from the gram-positive bacteria, than it was to chloroplast δ. The latter subunit formed an isolated outgroup.

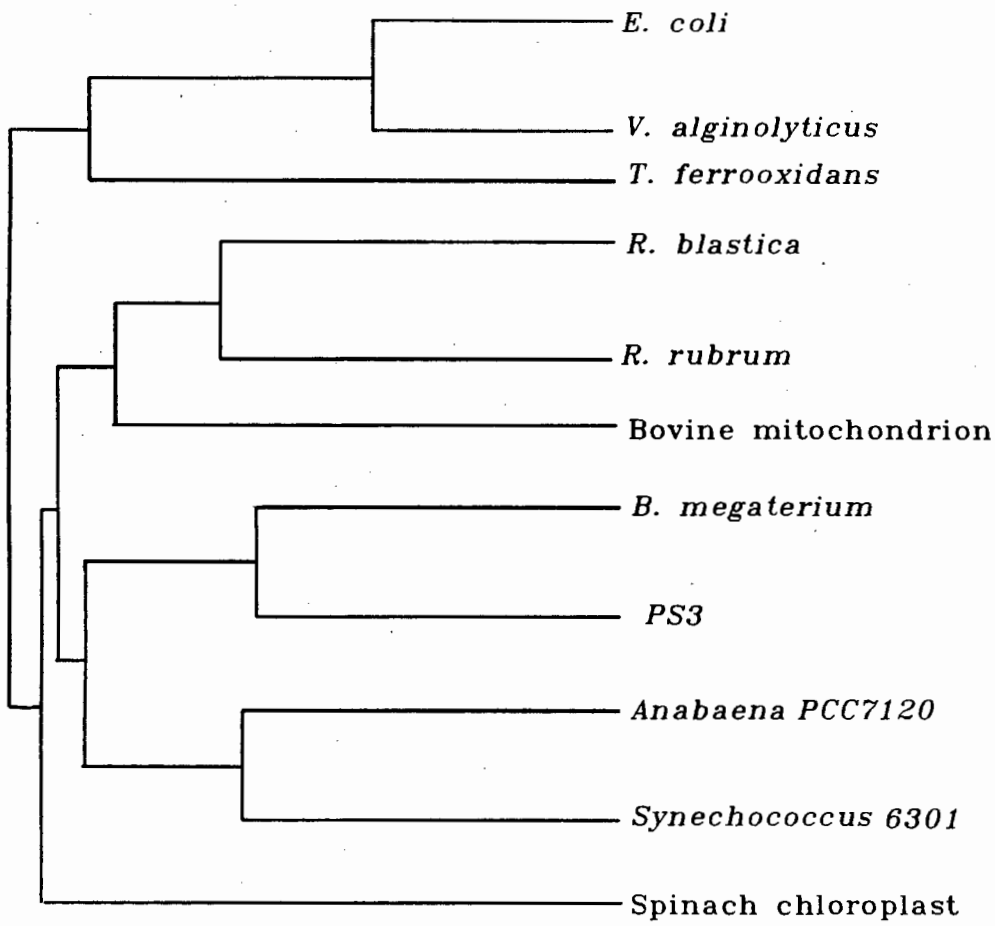

Fig. 3.15. A dendrogram showing the clustering of subunit δ/OSCP from a number of organisms, generated from the multiple sequence alignment data (PILEUP) of F_1F_0 ATPsynthase subunit δ/OSCP illustrated in Fig. 3.14. For references, see Fig. 3.14.

3.4.3.2.b. Subunit γ. The topology of the γ subunit within the F_1 moiety of F_1F_0 ATPsynthases remains controversial but there is substantial evidence to suggest that in *E. coli*, γ is assymetrically located within, and extends from, the cavity of F_1 (Fig. 1.8) (Lucken *et al.*, 1990; Gogol *et al.*, 1989a and b; 1990; Capaldi *et al.*, 1992). In *E. coli*, γ is thought to fold such that the midregion, C- and N-termini are all in close contact (Nakamoto *et al.*, 1992).

Functionally, the role of γ is also controversial. Most authors agree that γ is essential for a functional F_1 complex (reviewed in Fillingame, 1990; Futai *et al.*, 1989; Senior, 1990). ATPase activity in isolated $\alpha_3\beta_3$ complexes is significantly increased when, in the presence of magnensium ions, γ binds to the hexamer (reviewed in Gromet-Elhanan, 1992). The

structural asymmetry in F_1, resulting from the inclusion of a single γ monomeric unit, could be implicated in catalysis by introducing functional asymmetry (Senior, 1990). Boyer (1993) was of the opinion that experimental evidence is supportive of prominent conformational interaction between γ and catalytic events. He suggested that one of the functions of γ could be to transmit nucleotide-binding induced conformational changes in F_1 back to F_0. Electron microscopic, trypsin cleavage and crosslinking studies provided convincing evidence that during catalysis the conformation of γ in *E. coli* alters. Dependent on nucleotides, magnesium ions, inorganic phosphate and EDTA presence, the subunit cross-links to β or ε or αβε complexes (Aggeler and Capaldi, 1992; Capaldi *et al.*, 1992; Gogol *et al.*, 1990; Senior, 1990). In *E. coli*, γ regulates ATPase activity (Iwamoto *et al.*, 1990; Shin *et al.*, 1992) (Table 3.10). In strain PS3, catalytic rate of a $\alpha_3\beta_3\gamma$ complex is greater than that of isolated $\alpha_3\beta_3$ complexes (Kagawa *et al.*, 1989: Kagawa *et al.*, 1992).

Of interest to this study is the speculation in the literature as to whether γ regulates proton flow. If αβ pairs alternate in catalysis, then it is likely that the juxtaposition of the γ subunit would change as sites alternate and thereby act in a gating capacity (Fillingame, 1990). *E. coli uncG* mutants are resistant to aminoglycoside antibiotics, due to the proton-permeability of the cytoplasmic membrane which prevents antibiotic uptake. This ATPase-related permeability is dependent on a mutation in *uncG*, together with the presence of other wild-type F_1 genes (Humbert and Altendorf, 1989). Mutational studies demonstrated the participation of *E. coli* γ in energy coupling in F_1F_0 ATPsynthase by influencing proton-flow (Table 3.10) (Iwamoto *et al.*, 1990; Nakamoto *et al.*, 1993; Shin *et al.*, 1992). The γ subunit could serve a proton-gating function in strain PS3 and chloroplast ATPase (Engelbrecht and Junge, 1992). Futai *et al.* (1989) proposed that a complex of γδε subunits acts to gate protons in strain PS3. In mitochondria, γ cross-links to F_0 and this complex acts in a proton-gating capacity (Zanotti *et al.*, 1992).

The γ subunit may be implicated in F_1F_0 ATPsynthase assembly. In *E. coli*, during enzyme assembly, γ, δ and α interact with F_0 subunits to regulate the proton pathway (Brusilow, 1987, 1993; Pati and Brusilow, 1989). However, other experimental data precluded a direct role for γ in the assembly of a functional F_0 in the cytoplasmic membrane of *E. coli* (Miki *et al.*, 1988, cited by Futai *et al.*, 1989).

Table 3.10. A summary of mutational analyses of the *E. coli* F_1F_0 ATPsynthase γ subunit. The equivalent *T. ferrooxidans* γ residues are given.

Domain	Mutation	Result	Ref.	*T.ferrooxidans* equivalent
N-terminus	ΔK_{21}-A-E-M-V-A-A_{27}	Unstable F_1; No F_1 assembly	a	R_{22}-A-M-E-M-V-A-A_{28}
	Ile19→Glu	Catalysis, but $\Delta\mu H^+$ reduced	b	Ile-20
	Thr20→Val	No effect	b	Thr-21
	Lys21→Leu	No effect	b	Arg-22
	Met23→Lys/Arg	Catalysis, but low levels of H^+ pumping	a	Met-24
		Suppressed Gln269→Arg and Thr272 →Ser mutations	b	
	Glu24 →Leu	No effect on catalysis	b	Glu-25
	Met 25→Lys	"	b	Met-26
	Val26→Glu	"	b	Val-27
	Lys30→Glu/Leu	"	b	Lys-31
	Lys33→Glu/Leu	"	b	Arg-34
Midregion	Asp83→Lys/Val	No effect on catalysis	b	Asp-84
	Arg84→ Glu/Leu	"	b	Arg-85
	Leu86→Glu	"	b	Leu-87
	Cys87→Ala	"	b	Cys-88
	Asp165→Lys/Asn/ Ala/Glu	"	b	Asp-166
Carboxy-terminus	Arg242→ Cys	Suppressed Met23→Lys mutation	d	Arg-243
	Gln269→Leu/Glu	Catalysis reduced; weaker H^+ gradient	c	Gln-270
	Gln269→Arg	Reduced oxidative phosphorylation; suppressed Met23→Lys mutation	d	
	ΔGln269→end	Unstable F_1	a	Gln270→ end
	Ala270→Val	Suppressed Met23→Lys mutation	d	Ala-271
	Ile272→Thr	"	d	Ile-273
	Thr273→Ser/Val	Reduced catalysis; suppressed Met23→ Lys mutation	c,d	Thr-274
	Glu275→Lys	Catalysis reduced; weaker H^+ gradient	c	Glu-276
	ΔThr277→end	"	c	Thr278→end
	Glu278→Gly	Suppressed Met23→Lys mutation	d	Glu-279
	Ile279→Thr	"	d	Ile-280
	Val280→Ala	"	d	Ser-281
	ΔA_{284}-A-A-V_{287}	No effect on assembly or catalysis	a	A_{285}-A-R-F_{288}

a - Futai *et al.* (1989)
b - Shin *et al.* (1992)
c - Iwamoto *et al.* (1990)
d - Nakamoto *et al.* (1992)

Fig. 3.16. Page 1.

```
          1                                                      47
           # ## #   ## ####* ##*###* #*  ### ****       *** *
Rbl   ...MPSLKDL KNRIGSVKNT RKITKAMQMV AAAKLRRAQD SAEAARPYAE
Rru   ...MASLKDL RSRITSVKST QKITSAMKMV AASRLRRAQD TAEAARPYTQ
Bov   ....ATLKDI TRRLKSIKNI QKITKSMKMV AAAKYARAER ELKPARVYG.
Ana   ...MPNLKSI RDRIQSVKNT KKITEAMRLV AAARVRRAQE QVIATRPFAD
Syn   ...MANLKAI RDRIKSVRNT RKITEAMRLV AAAKVRRAQE QVLSTRPFAD
Spi   ....ANLREL RDRIGSVKNT QKITEAMKLV AAAKVRRAQE AVVNGRPFSE
Bme   ...LASLRDI QTRITSTKKT SQITKAMEMV SAAKLNRAEQ NAKSFVPYME
Ps3   MKPLASLRDI KTRINATKKT SQITKAMEMV LTSKLNRAEK R.EIVRPYME
Eco   ... AGAKEI RSKIASVQNT QKITKAMEMV AASKMRKSQD RMAASRPYAE
Val   ...MAGAKEI RNKIGSVKST QKITKAMEMV AASKMRRSQD AMEASRPYAE
Tfe   ...MANAKEI RGQIKSVKNT RKITRAMEMV AASKMRRAQE RMRAARPCAE

      48                                                      94
       #  ##                          #     #      ***#*  *
Rbl   RMGAVIASLA SGQG..AGAP RLLAGNGRDQ IHLLVVMTSE RGLCGGFNST
Rru   RMERMLGNLA ASTAGMAGAS PLLGGTGKDN VHLIVALTAN RGLCGGFNGS
Bov   .....VGSLA .....LYEKA DIKTPEDKKK .HLIIGVSSD RGLCGAIHSS
Ana   RLAQVLYGLQ TRLRFEDVDL PLLKKREVKS VGLLV.ISGD RGLCGGYNTN
Syn   RLAQVLAGLQ QRLQFENVDL PLLQRREVKT VALLV.VSGD RGLCGGYNSN
Spi   TLVEVLYNMN EQLQTEDVDV PLTKIRTVKK VALMV.VTGD RGLCGGFNNM
Bme   KIQEVVSSVA ..LGSRGASH PMLTARSVKK TGYIV.ITSD RGLAGAYNSN
Ps3   KIQEVVANVA ..LAAR.ASH PMLVSRPVKK TGYLV.ITSD RGLAGAYNSN
Eco   TMRKVIGHLA ..HGNLEYKH PYLEDRDVKR VGYLV.VSTD RGLCGGLNIN
Val   TMRKVIGHVA ..NANLEYRH PYLEEREAKR VGYII.VSTD RGLCGGLNIN
Tfe   KIREVLGHLA ..QAHPEYEH PLMQVRPVKK AGFLV.VTTD RGLCGGLNVN

      95                                                     143
      # #             #        #* ##    #
Rbl   IVRLARQRAN ELVAQGKTVK ILTVGKKGRE QLKRDWASAF VGHVDLSDVR
Rru   IIRATRTLVR ELEAQGKTVK LLCIGKKGRD GLKREFPKQI IGGIADQSSK
Bov   VAKQMKSEAA NLAAAGZEVK IIGVGDKIRS ILHRTHSDQF LVTFKEVGRR
Ana   VIRRAENRAK ELKAEGLDYT FVIVGRKAEQ YFRR.REQPI DASYTGLEQI
Syn   VIRRAEQRAR ELSAQGLDYK FVIVGRKAGQ YFQR.REQPI EATYSGLEQI
Spi   LLKKAESRIA ELKKLGVDYT IISIGKKGNT YFIR.RPEIP VDRYFDGTNL
Bme   ILRKVSQAIE ERHQSPDEYG VIAIGRVGRD FFVK.RGIPV LLEITGLADQ
Ps3   VLRLVYQTIQ KRHASPDEYA IIVIGRVGLS FFRK.RNMPV ILDITRLPDQ
Eco   LFKKLLAEMK TWTDKGVQCD LAMIGSKGVS FFNS.VGGNV VAQVTGMGDN
Val   VFKKAVTDMQ TWREKGAEIE LAVVGSKATA FFKH.GGAKV AAQVSGLGDN
Tfe   VLRNVVQKMR ELHEEGVESN LAVVGNKGLG FLRR.HGAHL VADVNGLGDS
      144                                                    191
                              #   *                           #
Rbl   RLGYSNAQGI AREVLAAFEA G.EADVVTIF YNRFQSVISQ VPTAQQVIPA
Rru   AIGFSDADRF SRLILDMFQA G.EFDVCTLV YNRFQSAISQ VVTRQQIIPF
Bov   PPTFGDASVI A...LELLNS GYEFDEGSII FNRFRSVISY KTEEKPIFSL
Ana   P.TADEANKI ADELLSLFLS E.KVDRIELV YTRFVSLVSS RPVIQTLLPL
Syn   P.TAQEANDI ADELLSLFLS G.TVDRVELV YTKFLSLVAS NPVVQTLLPL
Spi   P.TAKEAQAI ADDVFSLFVS E.EVDKVEML YTKFVSLVKS DPVIHTLLPL
Bme   P.AFADIQGI ASQTVQMFAD G.TFDELYLY YNHFINTISQ EVTEKKLLPL
Ps3   P.SFADIKEI ARKTVGLFAD G.TFDELYMY YNHYVSAIQQ EVTERKLLPL
Eco   P.SLSELIGP VKVMLQAYDE G.RLDKLYIV SNKFINTMSQ VPTISQLLPL
Val   P.SLEDLIGS VGVMLKKYDE G.ELDRLYVV FNKFVNTMVQ QPTIDQLLPL
Tfe   P.HLGDMIGP IRAMADAYAK G.EVDVVYLV SSRFVNTMLQ RATVEQLLPV
```

Fig. 3.16. Page 2.

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      192                                                        208

Rbl   KFE..... ....AAETNA L..... ..... .....YD
Rru   AVPTTVAAGN DNDRTAGPKA I..... ..... .....YE
Bov   ..... ..DTISSAES M..... ..... .....SI
Ana   DTQG..... ...LEAADDE IFRLTTRGGQ FQVERQTVTS QARPLPRDSI
Syn   DPQG..... ...LASSDDE IFRLTTRGGS FTVEREKLTS EVAPLPRDMI
Spi   SPKGEICDIN GKCVDAAEDE LFRLTTKEGK LTVERDMIKT ETPAFSPILE
Bme   TDLQP..... ..... ..... .....SG KLV....GYE
Ps3   TDLAE..... ..... ..... .....NK QRT....VYE
Eco   PASDD..... ..... ..... .....DD L.KHKSWDYL
Val   PKSDS..... ..... ..... .....EE MQREHSWDYI
Tfe   EXPTA..... ..... ..... .....SA EQRAELWDYI

      209                                                        258
      ###    #*   #          #     #  # #*###** **   ** *#
Rbl   YEPSEEAILA DLLPRGVATQ IFTALLENAA SEQGARMSAM DNATRNAGDM
Rru   YEPSEEEILA DLLPKNVAIQ VFRGMLESFA SEQGARMTAM DNATRNAGDM
Bov   YDDIDADVLR NYQEYSLANI IYYSLKESTT SEQSARMTAM DNASKNASEM
Ana   FEQDPVQILD SLLPLYLSNQ LLRALQESAA SELAARMTAM SNASENAGEL
Syn   FEQDPAQILS ALLPLYLSNQ LLRALQEAAA SELAARMTAM NSASDNANAL
Spi   FEQDPAQILD ALLPLYLNSQ ILRALQESLA SELAARMTAM SNATDNANEL
Bme   FEPSQEEILE VLLPQYAESL IYGGLLDGKA SEHAARMTAM KSATDNAKDL
Ps3   FEPSQEEILD VLLPQYAESL IYGALLDAKA SEHAARMTAM KNATDNANEL
Eco   YEPDPKALLD TLLRRYVESQ VYQGVVENLA SEQAARMVAM KAATDNGGSL
Val   YEPEPKPLLD TLLVRYVESQ VYQGVVENLA CEQAARMIAM KAATDNATNL
Tfe   YEPEARPVLD RLMQRYVESV VYQAVIEHLA CEQSARMVAM KSASDNAKRM

      259                                                  298
      #  * # #*#  *** **#*#  *#  ** #
Rbl   INKLTIQYNR SRQAAITKEL IEIISGAEAL .....
Rru   IKKLSLTYNR TRQAQITKEL IEIISGAEAI .....
Bov   IDKLTLTFNR TRQAVITKEL IEIISGAAAL .....
Ana   IKSLSLSYNK ARQAAITQEL LEVVGGAEAL T.....
Syn   VGQLTLVYNK ARQAAITQEL LEVVAGAEAL NG.....
Spi   KKTLSINYNR ARQAKITGEI LEIVAGANAC V.....
Bme   INNLTLSYNR ARQAAITQEI TEIVGGAAAL E.....
Ps3   IRTLTLSYNR ARQAAITQEI TEIVAGANAL Q.....
Eco   IKELQLVYNK ARQASITQEL TEIVSGAAAV .....
Val   IDDLELVYNK ARQAAITQEL SEIVGGAAAV .....
Tfe   VDDLQLAYNK ARQAAITQEI AEISAGAARF DDCAQHFWKF
```

Fig. 3.16. Multiple alignment of deduced primary sequence for the γ subunits from a number of organisms, generated by the PILEUP facility of UWGCG. Numbers refer to *T. ferrooxidans* γ residues. * denotes invariant residues, # denotes semiconserved regions. Residues in boldface indicates the positions of mutated residues in *E. coli* γ. *Rbl* = *R. blastica* (Tybulewicsz *et al.*, 1984); *Rru* = *R. rubrum* (Falk *et al.*, 1985); Bov = bovine mitochondrion (Dyer *et al.*, 1989); *Ana* = *Anabaena* sp. Strain PCC 7120 (McCarn *et al.*, 1988); *Syn* = *Synechococcus* 6301 (Cozens and Walker, 1987); Spi = spinach chloroplast (Miki, 1988); *Bme* = *B. megaterium* (Brusilow *et al.*, 1989); *Ps3* = strain PS3 (Ohta *et al.*, 1988); *Eco* = *E. coli* (Walker *et al.*, 1984a); *Val* = *V. alginolyticus* (Krumholz *et al.*, 1989); *Tfe* = *T. ferrooxidans* (this study).

Primary sequence data obtained for the γ subunit from *T. ferrooxidans* indicated that the subunit was a globular protein comprised of 298 residues, with a combined *Mr* of 32 482 Da. The estimated pI value was 9.58. It was therefore similar in size, and charge, to the γ subunit from *E. coli* (Table 3.6). GAP analyses indicated that primary sequence of γ in *T. ferrooxidans* was most like that of *E. coli* and *V. alginolyticus* (Table 3.5). Alignment of the deduced primary sequence data from a number of γ subunits (Fig. 3.16) showed that there were considerable domains of conserved residues amongst the *T. ferrooxidans/E. coli/V. alginolyticus* group. Hence it is not surprising that *T. ferrooxidans* γ was able to functionally complement *E. coli uncG* mutants. The multiple sequence alignment from 11 different organisms showed three areas where γ subunits were highly conserved. In *T. ferrooxidans* γ, these domains corresponded to residues 3-43 towards the N-terminal, 83-89 centrally located, and 238-284 towards the carboxy terminus (Fig. 3.16). Residues in the conserved N- and C- domains are postulated to form α-helices in *E. coli* γ (Fillingame, 1990; Senior, 1990). In all three domains, certain residues were universally conserved (Fig. 3.16). The possible functions of these domains in *T. ferrooxidans* γ, as deduced from mutational analyses in *E. coli*, are presented in Table 3.10.

Amongst the γ subunits aligned (Fig. 3.16), the carboxy-terminal region (residues 286 -> end) in *T. ferrooxidans* γ comprised a unique domain which was situated immediately adjacent to the conserved and catalytically significant C-terminal domain. It had a unique Cys, and both charged and bulky aromatic residues. In this regard, the studies of Nakamoto *et al.* (1992) on *E. coli* γ, and Zanotti *et al.* (1992) on mitochondrial γ are of interest. Nakamoto showed the closeness of the extreme C-terminus of *E. coli* γ to the N-terminus. By substituting the conserved Met-23 with a positively charged Lys or Arg residue, the flow of protons was perturbed (Table 3.10). If *T. ferrooxidans* γ folded in a manner similar to that of *E. coli* γ, it would mean that the polar residues of the C-terminus would be close to Met-23, and may be implicated in the control of proton flow. Zanotti *et al.* (1992) suggested that the conserved C-terminus of mitochondrial γ binds to F_0, and that this complex is implicated in the gating of proton movement in both directions through the proton channel. Acidic and basic residues at the C-terminus were considered to be important in the linking of γ to protolytic residues in mitochondrial F_0, by the formation of salt-bridges. Dithiol groups in γ and F_0 were also involved via the formation of disulphide bonds, as was the OSCP subunit. It is therefore possible that the unique C-terminus of *T. ferrooxidans* γ is implicated in an unusual means of gating protons. It would be of value to determine whether *E. coli* γ or *T. ferrooxidans* γ, from which the extreme terminus had been deleted, was able to complement a *T. ferrooxidans atpG* mutant. Unfortunately, the lack of a workable genetic system for *T. ferrooxidans* precludes this.

The dendrogram compiled from the primary sequence alignment data of the compared γ subunits was in agreement with predicted phylogenetic trends. The γ of *T. ferrooxidans*

in *E. coli* (Sternweiss, 1978, cited by Senior, 1990). Residues implicated occur in the amino-terminal of ε (Jounouchi *et al.*, 1992; Kuki *et al.*, 1988; La Roe and Vik, 1992). It is well-established that in chloroplast, *E. coli* and mitochondrial F_1F_0 ATPsynthases, ε/δ inhibits multi-site ATPase activity (reviewed in Boyer, 1993; Fillingame, 1990; Futai *et al.*, 1989; Senior, 1990). In *E. coli* F_1F_0 ATPsynthase, tightly bound ε inhibits product release from catalytic sites in the enzyme complex by decreasing the off-rate of inorganic phosphate (Dunn *et al.*, 1987). Initially, this inhibition was recorded only in isolated $\alpha_3\beta_3\gamma$ and $\alpha_3\beta_3\gamma\delta$ complexes, and was not observed in intact, membrane-bound F_1F_0 complexes (Sternweiss and Smith, 1980, cited by Senior, 1990). However, recent work has clearly indicated that ε does affect the rate of ATP hydrolysis in *E. coli* F_1F_0 ATPsynthase, but not as dramatically as in dissociated *E. coli* F_1 (Mendel-Hartvig and Capaldi, 1991a and 1991b). These authors showed that the terminal 40 residues of ε contained the site important for inhibition. Electron-microscope and cross-linking studies demonstrated that *E. coli* ε undergoes nucleotide/Pi-induced crosslinks to other F_1 subunits. In the presence of ADP, magnesium ions and inorganic phosphate, ε attached to an αβ pair which linked to γ. When ATP and EDTA were present, the ε/αβ crosslink did not occur; however, an εγ link was observed. In the presence of magnesium ions, ε altered β conformation. When *E. coli* subunit c was inhibited by DCCD, no nucleotide/Pi conformational changes were observed with ε (Aggeler *et al.*, 1992; Capaldi *et al.*, 1992; Dallman *et al.*, 1992; Dunn *et al.*, 1987; Gogol *et al.*, 1990; Mendel-Hartvig and Capaldi, 1991a and b). By switching between two conformations or two binding sites (i.e. γ or α/β binding), ε could influence the rate-limiting step of ATP hydrolysis (Mendel-Hartvig and Capaldi, 1991b). Skakoon and Dunn (1993a and b) suggested that by moving about a flexible hinge region between residues 80-85, *E. coli* ε is able to undergo conformational change, enabling it to interact with an αβ interface at an exposed surface of the enzyme. Similar catalytically-induced structural changes were observed for chloroplast ε (Richter and McCarty, 1987; Soteropoulos *et al.*, 1992).

Specific proton-gating functions were suggested for certain ε subunits. In strain PS3, ε together with δγ, appears to gate protons (Yoshida *et al.*, 1977, cited by Futai *et al.*, 1989). In *E. coli* ε, the N-terminus may be important in regulating proton-translocation, possibly in combination with γ (Jounouchi *et al.*, 1992). Capaldi *et al.* (1992) were of the opinion that conformational changes of ε and γ are together critical for coupling catalytic site events with proton pumping in the enzyme complex in *E. coli*.

```
                  1       #   #    #     #          * *  38
   Tob  .......... ...MTLNLSV LTPNRIVWDS .EVEEIVLST NSGQIGILPN
   Ana  .......... ...MTLTVRV ISPDKTVWDA .EVDEVILPS TTGQLGILSG
   Syn  .......... ...MSLTVRV IAPDRTVWDA .PAQEVILPS TTGQLGILPG
   Bme  .......... ..MKTIHVSV VTPDGPVYES .EVEMVSTRA QSGELGILHG
   Ps3  .......... ..MKTIHVSV VTPDGPVYED .DVEMVSVKA KSGELGILPG
   Bfi  .......... ..MSTIRVNV VTPDGKVYDG .DVDLVVVRT VEGELGIFRK
   Eco  .......... . AMTYHLDV VSAEQQMFSG .LVEKIQVTG SEGELGIYPG
   Val  .......... MAAITFHLDV VSAEKKIFSG .RVETFQVTG SEGELGIFHG
   Tfe  .......... .MAMTIDVRV VSAEGSIYAG .VADMVVAPG EMGELGILPR
Bovdel  AEAAAAQAPA AGPGQMSFTF ASPTQVFFNS ANVRQVDVPT QTGAFGILAA
   Rbl  .......... .MAATLQFDL VSPERRLA.S VQATEVQIPG AAGDMTAMQG
   Rru  .......... .MAETTEFEL VSPERLLF.S EPVEMVVVPG TDGDFGAMPR

        39                                          85
        * *                          # * *          ###  #
   Tob  HAPIATAVDI GILRIRLN.. DQWLTMALMG GFARIGNN.E ITVLVNDAEK
   Ana  HAPLSTALDT GVLRVRTSKS QNWQAIALLG GFAEVEED.E VTILVNGGER
   Syn  HAPLLSALDT GVLRVRADK. .EWLAIAVLG GFAEVENN.E VTVLVNAAER
   Bme  HIPMVAPLQI GAVR..LKKA SSTELVAVSG GFLEVRPD.K VTILAQAAET
   Ps3  HIPLVAPLEI SAAR..LKKG GKTQYIAVSG GFLEVRPD.N VTILAQAAER
   Bfi  HIPLVAPVTV GRVR..LKKG NSEEQVAVSG GFVEVRAD.Q VTILAEAAEL
   Eco  HAPLLTAIKP GMIRI.VKQH GHEEFIYLSG GILEVQPG.N VTVLADTAIR
   Val  HTPLLTAIKP GMVRI.VKQH GHEEIIYVSG GMVEIQPG.T ATVLADTAIR
   Tfe  HAPLLTGLRP GELRI.I.HG AETEYLFVNG GILEIQPD.M VTVLADSAER
Bovdel  HVPTLQVLRP GLVVVHAEDG TTSKYF.VSS GSVTVNADSS VQLLAEEAVT
   Rbl  HAPTITTLRP GILRAV.SAE GTKAYV.VTG GFAEISAT.G VSVLAERAVP
   Rru  HAPLLSTVRP GVISTYNGGK VQRRIF.VAG GFAEVTED.R CTVLADEAFD

        86      #       #   #                           133
   Tob  GSDIDPQEAQ QTLELAEANV K..KAEGRRQ KIEANLALRR ARTRVEAINP
   Ana  GDTINLEEAR TAYSQAQTKL NQVPAGDRQA QIQANQAFKR ARARFQATGG
   Syn  GDKIDLEEAR AAFSQADERL KGVKEDDRQG KFQATQAYRR ARARLQAAGG
   Bme  AEEIDVARAE EAKKRAEMRL DS.KQDDVDV K.RAEIALKR AVNRLDISQR
   Ps3  AEDIDVLRA. KARKSGRTPL QS.QQDDIDF K.RAELALKR AMNRLSVAEM
   Bfi  PSAIDVDRAR AAKERAESRL NSTKQDAVDF K.RAELALKR AINRLDVTGK
   Eco  GQDLDEARAM EAKRKAEEHI SS.SHGDVDY A.QASAELAK AIAQLRVIEL
   Val  GEELDAAKAE EAKRRAEEQI QN.QHGDMDF A.QAASELAK AIAQLRVIEL
   Tfe  ATDIDEAKAL AAKQAAEARM AG.HTDQMEY A.AAQAELLE QIARLKTVQR
Bovdel  LDMLDLGAAK ANLEKAQSEL LGAADEATRA EIQIRIEANE A..ALVKALE
   Rbl  LDEMDAKLMD QLVADASA.. .ASSVGVDKD TAEKAMSDLQ AMKAAAGF..
   Rru  LASLSEEAVR ARLQAADDRL KEATSEAEKA EAAQAKAIAE ALLAARKG..

        134
   Tob  IS......
   Ana  LA......
   Syn  LVSV....
   Bme  KF......
   Ps3  K.......
   Bfi  ........
   Eco  TKKAM...
   Val  TKKRR...
   Tfe  LREQGFVR
Bovdel  ........
   Rbl  ........
   Rru  ........
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Fig. 3.18. Multiple sequence alignment of deduced primary sequence of the F_1F_0 ATPsynthase ε/δ subunit from a number of organisms, generated using the PILEUP facility of UWGCG. Numbers refer to *T. ferrooxidans* ε residues. * denotes invariant residues, and # denotes semi-conserved positions. *Tob* = tobacco chloroplast (Shinozaki *et al.*, 1983); *Ana* = *Anabaena* sp. Strain PCC 7120 (Curtis, 1987); *Syn* = *Synechococcus* 6301 (Cozens and Walker, 1987); *Bme* = *B. megaterium* (Brusilow *et al.*, 1989); *Ps3* = strain PS3 (Ohta *et al.*, 1988); *Eco* = *E. coli* (Walker *et al.*, 1984a); *Val* = *V. alginolyticus* (Krumholz *et al.*, 1989); *Tfe* = *T. ferrooxidans* (this study); Bovdel = bovine mitochondrial δ (Walker *et al.*, 1985); *Rbl* = *R. blastica* (Tybulewicz *et al.*, 1984); *Rru* = *R. rubrum* (Falk *et al.*, 1985).

In *T. ferrooxidans*, ε was a typical globular protein, as indicated by PEPPLOT analyses (data not shown). It consisted of 141 residues, with a combined M_r of 15 369 Da, and an isoelectric point of 4.66. It was therefore similar to the ε subunit of *E. coli* (Table 3.6). *T. ferrooxidans* ε was able to functionally complement various *E. coli unc* mutants (Chapters 2 and 4). GAP analyses showed that *T. ferrooxidans* ε was most like that of *E. coli* and *V. alginolyticus*; however the two organisms shared only 29% identical and 58% similar residues (Table 3.5). Alignment of multiple primary sequence data for various ε subunits indicated that overall, primary sequence was not well-conserved (Fig. 3.18). However, sequence homology amongst the non-photosynthetic gram-negative group was fairly consistent, particularly towards the N-termini.

Mutational and cross-linking studies on *E. coli* ε are summarised in Table 3.11. *T. ferrooxidans* ε was homologous to *E. coli* ε at most domains and residues listed.

Table 3.11. A summary of mutational and cross-linking studies on the *E. coli* F_1F_0ATPsynthase ε subunit. Equivalent *T. ferrooxidans* ε domains or residues are given

Mutation	**Result**	**Ref.**	***T.ferrooxidans equivalent***
ΔM_1-V_{78}	No F_1F_0 binding; ε still inhibitory	a	M_1 - V_{77}
ΔM_1-A_{93}	No ATPase inhibition by ε	a	M_1 -A_{92}
ΔA_{93}-M_{138}	ε still inhibitory in ATPase	a	A_{92} - R_{141}
Ser10→Cys	No effect on ATPase activity; γ-binding residue	b	Ser-11
Glu32→Arg/Pro	Diminished H^+- translocation; intact, coupled F_1F_0	c	Glu-31
Glu32→Ile	No effect	c	
His39→Pro/Arg	Growth on succinate retarded; distuption of F_1F_0 binding	c	His-39
Gly48→Asp	Enzyme inactivated; suppressed Gly48→Asp mutation	d	Gly-49
Ser108→Cys	No inactivation of enzyme; binds to α and β	b; e	Gly-107

a - Kuki *et al.* (1988)
b - Aggeler *et al.* (1992)
c - La Roe and Vik , (1992)
d - Futai *et al.* (1989)
e - Dallmann *et al.* (1992)

As was observed for the *T. ferrooxidans* γ subunit, the extreme C-terminus of *T. ferrooxidans* ε was unusual. This region of the subunit was elongated, when compared to other ε/δ subunits aligned, and was characterised by the presence of charged residues and an aromatic Phe-139 (Fig. 3.18). In *E. coli* ε, the 16 extreme N-terminal residues, which include the γ-binding residue, interact with the four extreme C-terminal residues and are important for efficient oxidative phosphorylation (Jounouchi *et al.*, 1992). Therefore, if these terminal residues in *T. ferrooxidans* ε were involved with a conformational interaction with the γ-binding N-terminal residue/s, it could indicate an unusual mode of interaction/gating in *T. ferrooxidans*.

The dendrogram complied from the multiple sequence alignment data is presented below (Fig. 3.19). Clusterings presented were a reflection of phylogenetic trends within the organisms represented, with *T. ferrooxidans* ε being loosely clustered with the γ-proteobacterial representatives, *E. coli* and *V. alginolyticus*.

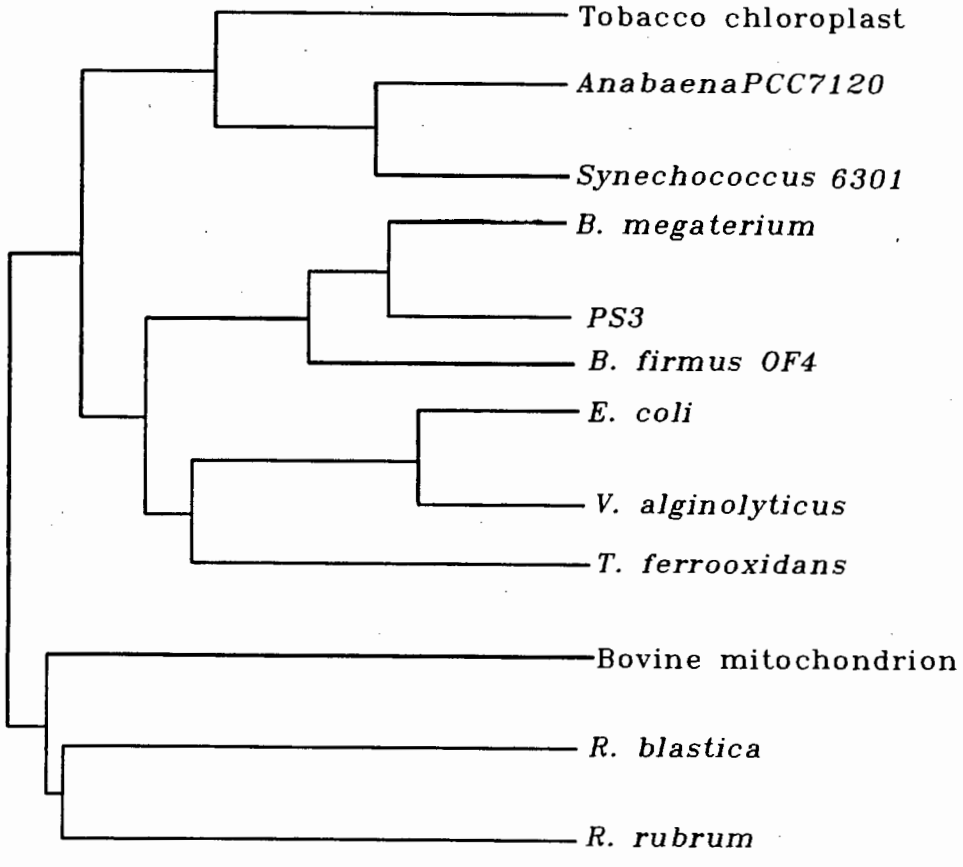


Fig. 3.19. A dendrogram showing the clustering of subunit ε/δ from a number of organisms, generated from the multiple sequence alignment data (PILEUP) of F_1F_0 ATPsynthase subunit ε/δ illustrated in Fig. 3.18. For references, see Fig. 3.18.

3.4.3.2.d. Subunits α and β. The two largest subunits of F_1F_0 ATPsynthase are α and β. The currently accepted arrangement of α and β within F_1 is that proposed by Capaldi *et al.* (1992) and Amzel *et al.* (1992) and was described in Chapter 1, Section 1.6.3, Fig. 1.8. The trimeric arrangement and asymmetry of α and β in F_1F_0 ATPsynthase is associated with the rapid turnover rate of the enzyme (Boyer, 1993).

The α and β subunits are intimately associated with catalysis (Chapter 1, Section 1.6.3.1) and this is reflected by the highly conserved nature of the primary sequence of the polypeptides among diverse species. In addition, the α and β subunits generally share a 25% identity in primary sequence, which suggests that they arose as a duplication of β, followed by a loss of catalytic function of one of the genes (reviewed in Penefsky and Cross, 1991; Walker *et al.*, 1982).

The evolution of two similar but non-identical subunits within the enzyme complex is an unusual feature of F_1F_0 ATPsynthase and raises interesting questions regarding catalysis. Undoubtedly, the heterogeneity of the major subunits is more advantageous than a homogeneous array. This is evident from the fact that neither isolated α nor β from any F_1F_0 ATPsynthase studied to date has ATP synthetic or hydrolytic activity. However, as soon as the two subunits associate to form αβ complexes, catalysis occurs. Furthermore, in intact F_1 αβ complexes, a high affinity catalytic site develops which does not occur on isolated α or β (Boyer, 1993; Penefsky and Cross, 1991; Senior, 1992) (Chapter 1, Section 1.6.3).

Active αβ complexes were isolated from chloroplasts, strain PS3 and *E. coli* (reviewed by Gromet-Elhanan, 1992; Senior, 1992). Isolated α and β subunits from strain PS3, in the absence of either nucleotides or magnesium ions, reconstitute into a catalytically competent $\alpha_3\beta_3$ hexamer, with a hydrolytic activity at 15% of an isolated $\alpha_3\beta_3\gamma$ complex (Kagawa *et al.*, 1992). It is not known how this reconstitution occurs, but chaperonin-like activity was demonstrated in isolated α subunits from mitochondria and chloroplasts (Avni *et al.*, 1991; Luis *et al.*, 1990). The strain PS3 hexamer constitutes the "catalytic core" of the holoenzyme (Miwa and Yoshida, 1989). In the presence of MgADP, the $\alpha_3\beta_3$ complex dissociates into an $\alpha_1\beta_1$ dimer. In the presence of MgATP, $\alpha_1\beta_1$ rapidly associates into $\alpha_3\beta_3$. This reaction is freely reversible. The hexamer has two distinct sequential covalent nucleotide-binding patterns. These remarkable studies demonstrated firstly, that the hexamer has all the properties required for the assembly of isolated α and β subunits into assymmetrical catalytically active complexes, in the absence of other F_1 subunits, and secondly, that α and β cooperate and interact as functional pairs (Kagawa *et al.*, 1992). The minimum subunit composition which supports ATPase activity in *E. coli* is an (αβ)-oligomer which has approximately 10% ATPase activity of isolated F_1 or $\alpha_3\beta_3\gamma$ complexes (reviewed in Senior, 1992). Boyer (1993) stated that the mere binding of

nucleotide to either α or β is enough to confer the assymmetry required for, and associated with catalysis. However, it is obvious that the catalytic rates associated with isolated complexes are orders of magnitude lower than native F_1 complexes, and these αβ complexes do not show the functional heterogeneity observed in F_1F_0 ATPsynthases. Interactions of α and β with the single-copy F_1 subunits, and possibly F_0 subunits (described in the foregoing) are necessary for optimal catalytic rates (Gromet-Elhanan, 1992).

Three types of functional sites were identified in F_1F_0 ATPsynthase, all of which are associated with α and β. These interacting sites are, three catalytic and three non-catalytic nucleotide-binding sites, and sites which bind inhibitory amphipathic cations (Allison *et al.*, 1992).

Both α and β subunits have primary sequence domains known as the Walker Homology A and Homology B regions. These domains have a consensus sequence diagnostic of other nucleotide-binding proteins, such as AK, EF-Tu, Ras and the *recA* gene product (Duncan and Cross, 1992; Senior, 1992; Walker *et al.*, 1982). Although both α and β bind to nucleotides, the exact location of the nucleotide-binding sites within an active αβ F_1 complex is the subject of some controversy. It was thought that the catalytic sites were exclusively located on β, and the non-catalytic on α. However, recent opinion was that neither subunit has a complete catalytically competent nucleotide binding site. Instead, these sites are considered to lie close together at the interface of associated αβ pairs (Allison *et al.*, 1992; Boyer, 1993; Gromet-Elhanan, 1992; Ida *et al.*, 1991; Jault and Allison, 1993; Penefsky and Cross, 1991; Vogel and Cross, 1991; Zhou *et al.*, 1992). To accommodate this view, Gromet-Elhanan (1992) presented a model for the arrangement of the catalytic and non-catalytic sites at the interface of associated αβ pairs. This is depicted in Fig. 3.20.

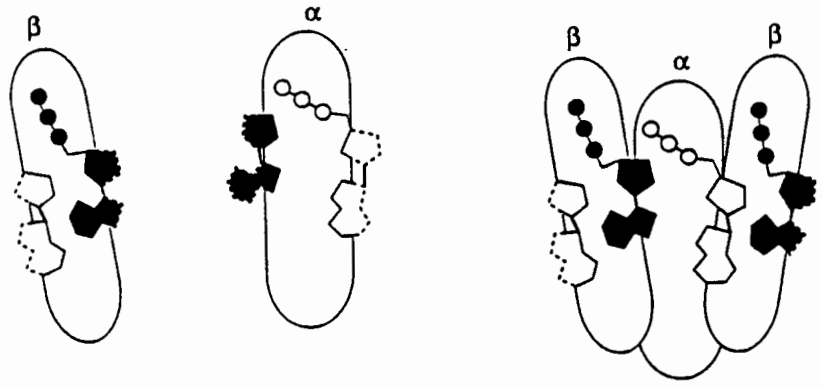


Fig. 3.20. A speculative model for the location of the catalytic (dark-shaded) and non-catalytic (un-shaded) nucleotide-binding sites on each isolated F_1 α and β subunit, and on a partially assembled F_1 complex (after Gromet-Elhanan, 1992).

Most of the catalytic binding-site is represented on β, with only a small part of the adenine moiety on an isolated α. In the non-catalytic site, only a small part of the adenine moiety occurs on α, with most of the adenine moiety on β. Neither subunit has a complete nucleotide-binding site. If correct, this model could explain a number of poorly understood observations, such as why all isolated β subunits cannot hydrolyse ATP by themselves, but $\alpha_3\beta_3$ and $\alpha_1\beta_1$ can, and reported indiscrepancies as to the nature of β nucleotide-binding sites (reviewed by Gromet-Elhanan, 1992). The orientation of the bound nucleotides at either site is controversial (eg. see Vogel and Cross, 1991).

The catalytic and non-catalytic nucleotide-binding sites show common and unique properties. Both exhibit co-operativity and heterogeneity. Both sites show negative co-operativity of nucleotide-binding, and at any one time are non-equivalent with respect to nucleotide-binding affinites. Both sites exhibit tight binding of nucleotides, although tight binding is mainly confined to non-catalytic sites (Allison *et al.*, 1992; Boyer, 1993; Penefsky and Cross, 1991).

The non-catalytic sites have a high specifity for adenine nucleotides, although chloroplast F_1 non-catalytic sites were shown to bind to GTP (Milgrom *et al.*, 1991). Nucleotides bound at non-catalytic sites are tightly bound and exchange extremely slowly with medium nucleotides (Boyer, 1993; Senior, 1992). As a result, it was suggested that these binding sites are uninvolved in catalysis (Boyer, 1993; Wise and Senior, 1985). The functional role of these sites remains poorly understood, but recent work suggested that the non-catalytic sites are important for multi-site catalysis by controlling negative co-operativity in nucleotide-binding (Allison *et al.*, 1992; Jault and Allison, 1993).

The catalytic sites are unspecific regarding the nature of the nucleotide base and bind to ATP, GTP, and ITP. Nucleotides at the catalytic sites are bound in conformations known as tight, open and loose (Walker *et al.*, 1990). As opposed to the non-catalytic sites, bound nucleotides are exchanged rapidly with medium nucleotides. Catalytic sites show positive co-operativity during catalysis necessary for the formation of a single high affinity catalytic site (Duncan and Cross, 1991). For a review of properties of catalytic sites as related to mechanism of catalysis, refer Chapter 1, Section 1.6.3.

To conclude this section on the nucleotide-binding domains, it should be pointed out that although primary sequence data of isolated α and β subunits indicate six potential nucleotide-binding sites, few workers have been able to directly demonstrate this binding. Only when photoaffinity or hydrophobic nucleotide analogues are used, is there evidence of nucleotide binding at separate sites *in situ*. When more natural substrates or analogues are used, only very tight binding sites are detected, and the stoichiometry falls below six moles nucleotide per mole F_1 (Futai *et al.*, 1989; Thomas *et al.*, 1992a). This raises the

disturbing possibility of artefactual results *in vitro* and *in vivo*, due to the use of unnatural enzyme substrates, and every effort should be made to determine the validity of the existence of three catalytic and three "non-catalytic" sites *in vivo*.

A large number of amphipathic cations act as non-competitive, mixed or uncompetitive inhibitors, and prevent ATPase activity in both isolated and membrane-bound F_1. These cations include dequalinium, the bee venom peptide, and substituted xanthenes, acridines, phenothiazines, and alkyl guanidines (Allison *et al.*, 1992). These binding sites, which are in conformational equilibrium with the catalytic sites, are located in highly conserved C-terminal subdomains of both α and β. As the amount of inhibition caused by these amphipathic cations is greater in membrane-bound F_1F_0 ATPsynthase, it was suggested that these sub-domains are the site of F_1/stalk binding, and are sites of transmitting conformational changes between F_0 and F_1, and vice versa (Allison, *et al.*, 1992). It was proposed that the amphipathic cation binding region in β contains the site of magnesium ion-induced β-ε interaction (Dallman *et al.*, 1992).

In *T. ferrooxidans* F_1, the α and β subunits were comprised of 514 and 468 residues respectively. The calculated M_r for α was 55 512 Da, and that of β, 51 012 Da. These values, together with the estimated pI, were similar to those reported for *E. coli* α and β (Table 3.6). GAP analyses indicated that *T. ferrooxidans* α was most like that of *E. coli*, sharing 67% identical and 83% similar residues. A similar trend was noted for the *T. ferrooxidans* β subunit, which shared 79% identical and 88% similar residues with *E. coli* F_1 β (Table 3.5). The high homology noted amongst these subunits from various organisms is a reflection of the conserved nature of protein domains with a catalytic function. PEPPLOT and PLOTSTRUCTURE analyses indicated that both the α and β subunits from *T. ferrooxidans* could be folded in a manner similar to that of the *E. coli* subunits (data not shown). It is therefore not surprising that the *T. ferrooxidans* α and β subunits were able to complement *E. coli unc* mutants.

There has been extensive research by many laboratories into the structure and function of F_1 α and β from bacteria, chloroplasts and mitochondria. Studies involved in the combined approaches of introducing mutations, and chemical modifications (or affinity labelling) yielded much information, in the absence of knowledge regarding higher order structure of the enzyme, on the significance of isolated amino acid residues within F_1 Genetic studies adopted three approaches viz. random mutagenesis, directed mutagenesis and the isolation of pseudorevertants of mutant enzymes. Attempts have been made to correlate results from these studies with a proposed three-dimensional structure of the catalytic domain of F_1 β, by analogy with other nucleotide-binding proteins for which higher order structure is known. Together, all the results regarding the structure and/or function of residues and/or domains in F_1 α and β are of formidable volume, and often

conflicting. Hence, for the purposes of this discussion, it was decided to present much of the information in the form of a summary in diagrammatic maps. As the *T. ferrooxidans* α and β subunits are homologous in both structure and function to each other, and to other F_1 α and β subunits from which the information was derived, such a map makes possible a valuable comparison of the *T. ferrooxidans* α and β subunits. These maps are presented in Figs. 3.23 and 3.25. In both, the primary sequence of the *T. ferrooxidans* α or β subunits is aligned against the *E. coli* homologue. In these maps, conserved residues/domains amongst the subunits from various sources are clearly indicated, as are mutated residues, and those which are chemically modified by nucleotide analogues. The legend to the maps explains mutations and these are referred to as follows: C137Y/S indicates that a Cys residue, which normally occurs at position 137 within a subunit was mutated to either Tyr or Ser. In the interests of brevity, the detailed multiple sequence alignments from which some of the data for the maps was obtained, are not shown. Instead, dendrograms arising out of multiple primary sequence alignments are given, as Figs. 3.24 and 3.26 for β and α respectively.

Fig. 3.23 shows the extent of the homology of the β subunit of *T. ferrooxidans* and *E. coli* F_1. It also indicates the highly conserved nature of certain domains of the subunit amongst the organisms compared in the sequence alignment. These included β subunits from mammalian and plant mitochondria, plant choloroplasts, and heterotrophic and autotrophic bacteria (Fig. 3.24). The *T. ferrooxidans* β subunit was typical. Some unique substitutions were noted, and these are referred to in the text to follow. However, in residues for which mutational and chemical modification data are available, *T. ferrooxidans* β was identically conserved; hence identical functions can be assumed at these positions. As far as tertiary structure is concerned, Chou and Fasman, and Garnier predictions were made for various domains within β (after Duncan and Cross, 1992). In all these regions, the primary sequence of *T. ferrooxidans* β was either highly or semi-conserved, and is therefore likely to be similar to that predicted for *E. coli*.

Senior (1990 and 1992) predicted on the basis of combined mutational and chemical modification data, that in *E. coli* β, the region between residues 137-335 is important for the formation of the high affinity catalytic site in F_1, and is involved in nucleotide-binding, and uni- and multi-site catalysis. In *T. ferrooxidans* β, this region corresponded to residues 143-341. Senior (1992) proposed that given the primary sequence homology occurring between this domain and that from other nucleotide-binding proteins, it is likely that the region forms a typical nucleotide-binding fold with probably six parallel β-strands. Duncan and Cross (1992), by analogy to known tertiary structure for AK, Ras and EF-Tu, proposed a folding model for the *E. coli* domain which occurs between residues β141-331. As the corresponding domain in *T. ferrooxidans* β was almost identically conserved between residues β147-340, tertiary structure would be likely to be similar. This schematic

model is presented in Fig. 3.21. The location of all the proposed (numbered) β-strands and α-helices are indicated on the map (Fig. 3.23). The nucleotide-binding sites in F_1 are thought to be at the interface of an interacting αβ pair; this is not indicated satisfactorally in the Duncan and Cross model. Therefore, the proposed juxtapostion of the two subunits in *E. coli*, as far as is known, is depicted in Fig. 3.22 (Futai *et al.*, 1992).

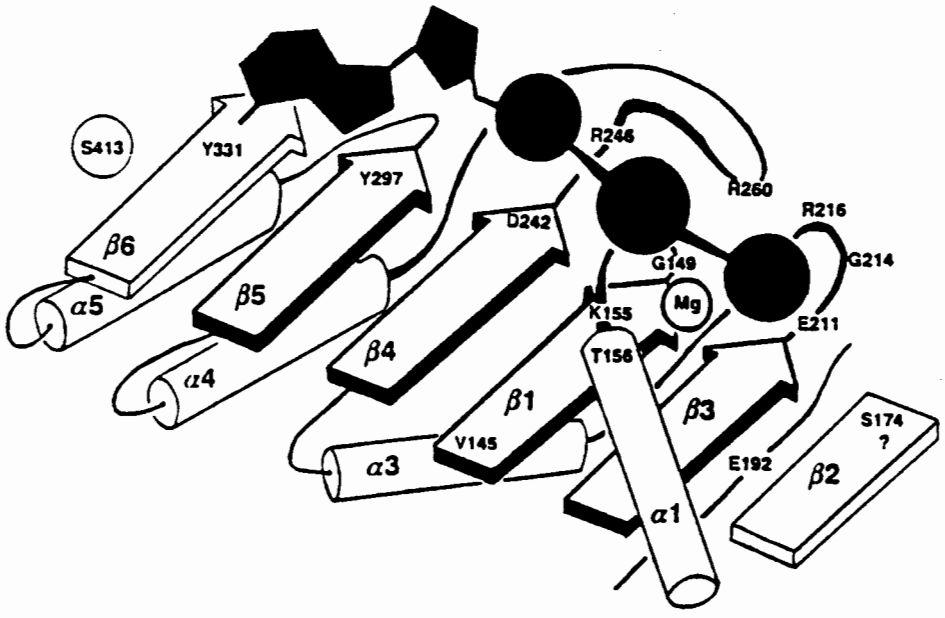


Fig. 3.21. A schematic representation of the proposed folding of the catalytic nucleotide site of F_1 β subunits. Topology of secondary structural elements and the positions of bound ATP (solid black), and magnesium ions (circled) are analogous to AK, EF-Tu and Ras. The "core" β-strands (5-4-1-3) are outlined in bold. Some segments of protein sequence are not shown. Domains are discussed in the text and are also indicated in Fig. 3.23 (after Duncan and Cross, 1992).

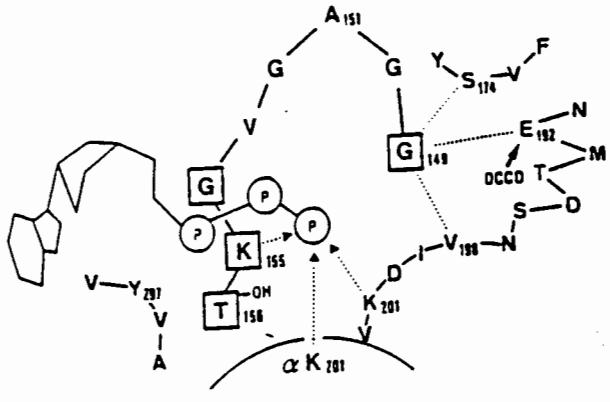


Fig. 3.22. A model of the proposed domain of *E. coli* F_1 αβ interaction, showing details of the orientation of the F_1 β glycine-rich flexible loop (Walker A) domain and bound ATP. Note positions of F_1 αLys-201 and F_1 β DCCD-binding Glu-192 (after Futai *et al.*, 1992; Iwamoto *et al.*, 1993).

Fig. 3.23. Page 1.

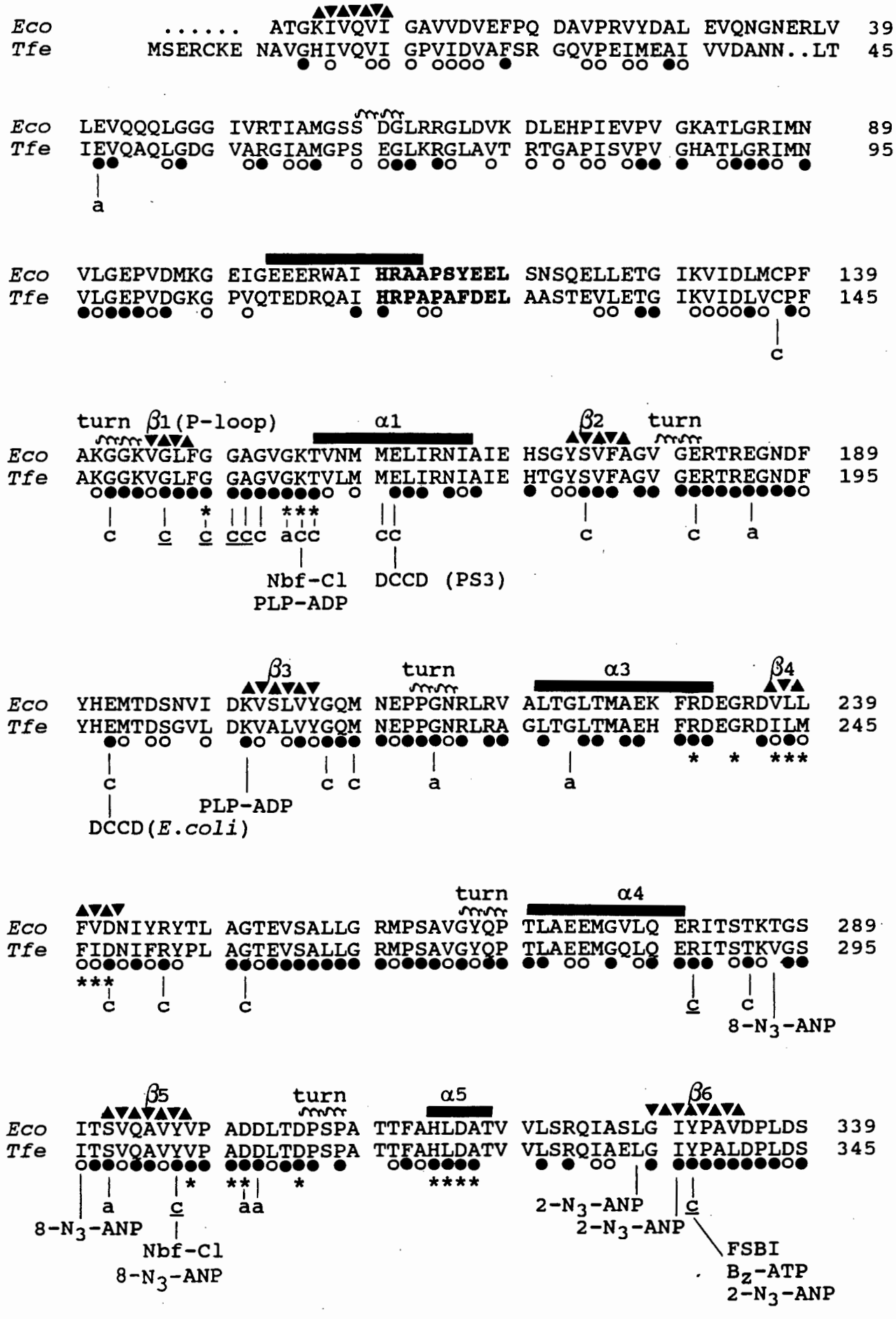


Fig. 3.23. Page 2.

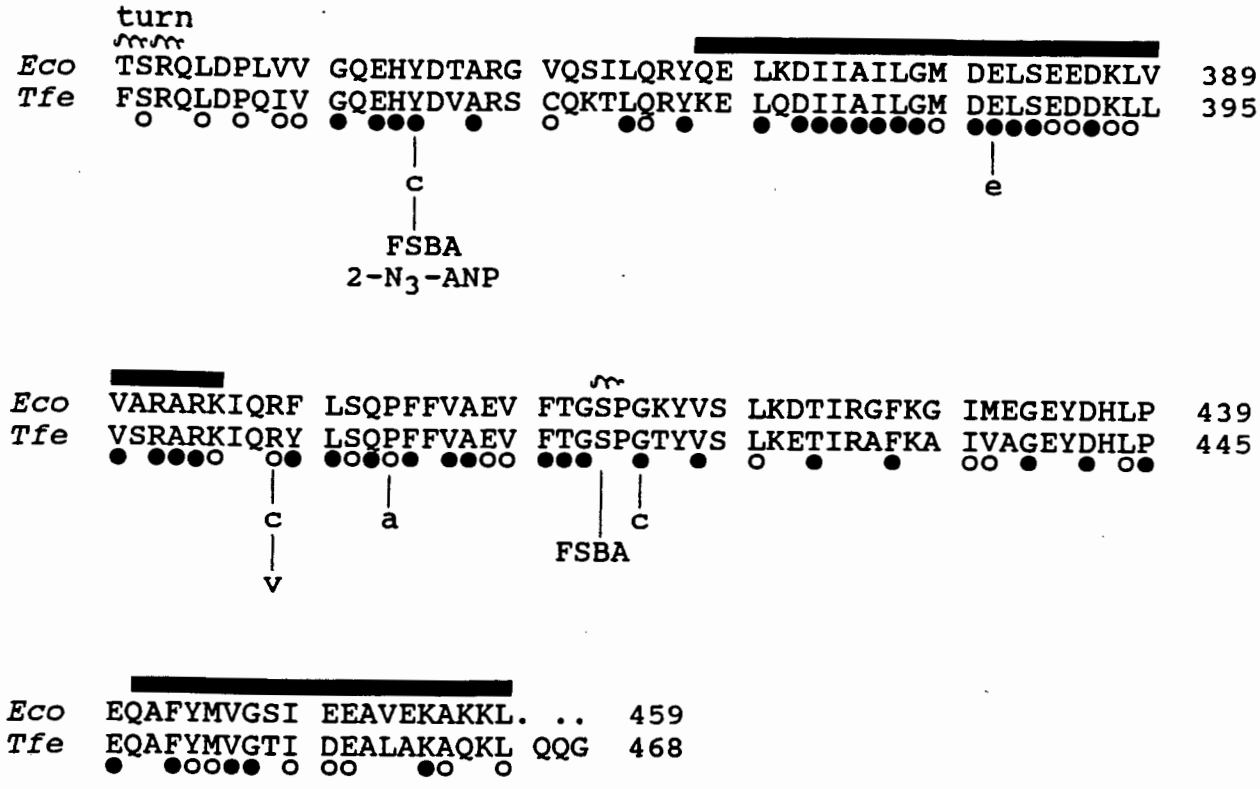


Fig. 3.23. The β subunit of *T. ferrooxidans* F_1F_0 ATPsynthase (*Tfe*) aligned against that from *E. coli* F_1F_0 ATPsynthase (*Eco*). The *E. coli* sequence is taken from Walker *et al.* (1984a). Minimal elements of predicted secondary structure are shown above the sequence, and were derived by comparing predictions made by the method of Mc Lachlan (see Walker *et al.*, 1984a) and Garnier *et al.* (1978) cited by Penefsky and Cross (1991). Only those α-helices (▬) and β-strands (▼▲▼) that are strongly predicted by both algorithms are shown here. All β-turns shown (ᔓ) are predicted by the second method, all but two, by the first, and all show highly conserved residues in β of other species. The β-strands thought to form the β-sheet of the nucleotide-binding domain are labelled β1 through β6; each α-helix thought to be part of that domain is numbered after the preceding β-strand in the sequence. Residues typed in **boldface** represent a domain with homology to molecular chaperonins. *Notations below the sequence:* •, invariant residues, o conservative residue replacements of the species compared (see Fig. 3.24 -archaebacteria excluded); (note, in all other species, *T. ferrooxidans* Ile-37 is Leu, Ala-58 is Val or Ile, Ala-223 is Val, Cys-366 is Val, Tyr-405 is Phe); * denotes consensus of Walker A, Walker B and "C" regions; a, "assembly" defects caused by a missense mutation at that residue, c, "catalytic" defects were caused by missense mutations at most of these sites, c, indicates that at least one mutation at that site yielded enzyme with >25% of normal ATPase activity, (non-essential for catalysis); residues which are of covalently modified (in one or more species) by nucleotide analogues are indicated (Duncan and Cross, 1992); v, aurovertin-binding residue in *E. coli*, (Weber *et al.*, 1992b); e, epsilon-binding residue in *E. coli* (Dallman *et al.*, 1992); DCCD, DCCD-binding residues in PS3 and *E. coli* (reviewed in Duncan and Cross, 1992; Futai *et al.*, 1992). Details of mutagenesis for *E. coli* β: E41K; C137Y/S; G142D/S; G146S; G149I/S/A/C/T; G150S, A151V/P, G152D/R, G154I, K155E/Q/A/T/S, K155T + T156K double mutant, T156A/C/D/S; T156A + V157T double mutant; E161Q/R; S174F; E181Q; E185K; E192Q; G207D; M209I; G214R; G223D; D242N/V; R246C/H; G251D; T285D; S292F; Y297F; D301V; D302V; Y331A/C/F/G/S/E/L; Y354F; R398C/W; P403S; P403S + G415D double mutant. Mutagenesis for PS3 β, (PS3 numbers in parentheses), K155I (164); D242N (252); E181Q (190); E192Q (201); Y297C/F (307); Y331C/F (341); Y354C/F (364). Mutagenesis for yeast mitochondrial β, (yeast numbers in parentheses), T156S (197); M160Y (Q201); R281A/K (328). (Mutations cited from Duncan and Cross, 1992; Fillingame, 1990; Futai *et al.*, 1989 and 1992; Iwamoto *et al.*, 1991; Lee *et al.*, 1991; Omote *et al.*, 1992; Penefsky and Cross, 1991; Senior and Al-Shawi, 1992; Senior, 1992; Weber *et al.*, 1992a). (*E. coli* map after Duncan and Cross, 1992; Penefsky and Cross, 1991).

The significance of the domains depicted in the models in Figs. 3.21 and 3.22 will be discussed, as they relate to *T. ferrooxidans* β. In all cases, when an individual residue is discussed, the *T. ferrooxidans* residue will be cited first, with the *E. coli* equivalent residue in brackets afterwards. This is in order that the references involving mutated and/or chemically modified numbered residues for *E. coli* β cited in Fig. 3.23, can be easily compared.

i) Turn-β-Strand1 through α-Helix 1. (*T. ferrooxidans* β147-173). (Figs. 3.21, 3.22, and 3.23). This region is highly conserved amongst β subunits, and contains the Walker A consensus region, characteristic of nucleotide-binding proteins, which occurs as Gx_4GKT/S, where x may be any residue; it also contains the P-loop motif, which is conserved among purine-binding proteins (Duncan and Cross, 1992; Senior, 1992; Thomas *et al.*, 1992b; Walker *et al.*, 1982). Extensive mutational analyses demonstrated the significance of this domain in catalysis. The Walker A consensus, *T. ferrooxidans* β156-163, is thought to form a glycine flexible loop, and is known to interact with the pyrophosphate moiety of either ATP or ADP (Futai *et al.*, 1992; Omote *et al.*, 1992; Senior, 1992). Any mutation in this domain which alters the orientation of essential residues in the Gly-rich loop, drastically affects uni- and multi-site catalytic rates (Futai *et al.*, 1992; Senior, 1992). Two residues were identified as critical. These are Lys-162 (155) and Thr 163 (156). Senior (1992) demonstrated that ionisable residues in this domain played an essential part in switching the binding affinity of the domain from ATP to ADP and vice versa. It was suggested that the charged group responsible was the ε-amino group of Lys-162 (155) (Senior, 1992). In a remarkable series of studies, wherein the Walker A domain in *E. coli* was replaced by the equivalent domain from either AK or Ras, it was demonstrated that the orientation of the Thr-163 (156) side chain was critical for ATPase function in *E. coli*; it was suggested that Thr-163 (156) was the site of magnesium ion binding in F_1 (Futai *et al.*, 1992; Omote *et al.*, 1992; Takayama *et al.*, 1990). Suppressor mutations showed that Lys-162 (155) is close to βTyr-178 (172) and significantly, to αLys-202 (201) (Futai *et al.*, 1992). Further suppressor mutation studies indicated that Gly-156 (149) is situated close to β Gly-178 (172), Ser-180 (174), the DCCD-binding residue Glu-198 (192) and Val-204 (198) (Futai *et al.*, 1992; Iwamoto *et al.*, 1993). These interactions are not satisfactorily accommodated by the Duncan and Cross model, but are shown in the Futai model (Figs. 3.21 and 3.22).

ii) β-Strand 2 and the next connecting segment. (*T. ferrooxidans* β180-205). (Figs. 3.21, 3.22 and 3.23). The orientation of this part of the β catalytic site in the Duncan and Cross model is not clear. Incorporating the extensive mutational data from the laboratories of Futai, this β-strand should be close to the Walker A Gly-flexible loop. It is also known that Ser-180 (174) interacts with magnesium ions, and should therefore be near the magnesium ion-binding site; Arg-188 (182) is predicted to be at an αβ interface, possibly close to

αLys-202 (201) and αCys-194 (193) (reviewed by Duncan and Cross, 1992). Within this domain is the DCCD-binding residue, Glu-198 (192) which influences multi-site (co-operative) catalysis (Fillingame, 1990). A recent study by Tozawa *et al.* (1992) on the β subunit of strain PS3 indicated that this region formed a water-exposed domain in the tertiary structure of the protein. As will be referred to below, this particular study indicated that domains of β suspected of being at an αβ interface nearly all formed water-exposed loops. It is therefore possible that this region of β forms an αβ interface, which interacts with the Gly-flexible loop.

iii) β-Strand 3 and a conserved turn or loop, through to α-Helix 3. (*T. ferrooxidans* β207-238). (Figs. 3.21, 3.22, and 3.23). This region is highly conserved, and contains many residues associated with uni- and multi-site catalysis and enzyme assembly. The residue Lys-207 (201) is close to αLys-202 (201) and was shown to be labelled by pyridoxal 5'-triphosphoadenosine (PLP-ADP). The extent of this labelling was magnesium ion-dependent (Ida *et al.*, 1991). Hence it must be close to the Gly-flexible loop, and to an αβ interface. This domain also includes the beginning of the Walker B consensus sequence viz. Arg-237 (231). There was an interesting substitute in *T. ferrooxidans* β; this was Ala-225, which in all other β subunits is conserved as Val, and is flanked by residues known to be important for enzyme assembly.

iv) β-Strand 4 through α-Helix 4. (*T. ferrooxidans* β243-287). (Figs. 3.21 and 3.23). The domain is highly conserved and mutated residues indicate that the region is critical for uni- and multi-site activity. It contains most of the Walker B consensus sequence, $Rx_{6-8}h_4D$, characteristic of nucleotide-binding proteins, where x corresponds to any residue, and h to hydrophobic residues (Walker *et al.*, 1982). The Walker B domain in *T. ferrooxidans* was between Arg-237 and Glu-248. The role of this region in catalysis is not known and a recent study by Thomas *et al.* (1992b) on purified mitochondrial β subunits showed that it had no role in nucleotide-binding. In *E. coli*, residue Glu-248 (242) is implicated in magnesium ion-binding (Senior and Al-Shawi, 1992). Tazawa *et al.* (1992) demonstrated that in strain PS3 F_1 β, this domain formed a water-accessible loop in the tertiary structure of the protein. Overall, the domain must be situated close to the magnesium ion-binding site and could form part of and αβ interface, important in communicating changes required for efficient catalysis.

v) β-Strand 5 through α-Helix 5. (*T. ferrooxidans* β296-324). (Figs. 3.21 and 3.23). This domain is highly conserved, and is associated with catalysis and enzyme assembly. It is situated close to the Gly-rich loop, and two residues in the domain viz. Val-291 (Thr-288) and Ile-296 (290) are associated with binding to nucleotide analogues. The region contains the "C" domain, consensus $VxADx_3Dx_8HLDA$, homologous to AK, where x is any residue (Thomas *et al.*, 1992b). In *T. ferrooxidans* β, this corresponded to Val-304 to Ala-323.

Thomas *et al.* (1992b) suggested that the "C" region plays an important but unidentified role in nucleotide binding in mitochondrial β. The study by Tozawa *et al.* (1992) on strain PS3 β showed that the region incorporating Leu-263 to Lys-292 formed an exposed loop on the subunit. This domain is homologous to α-342 (341) - α-381 (380) and it was suggested to be a region of α-β interaction (Table 3.12) (Allison *et al.*, 1992).

vi) β-Strand 6 through to a turn. (*T. ferrooxidans* β335-348). (Figs. 3.21 and 3.23). This domain is highly conserved, and is critical for nucleotide binding. Many studies showed that in intact F_1, Tyr-337 (331) binds to the adenine moiety of the nucleotide (eg. Weber *et al.*, 1992a). However, Thomas *et al.* (1992b) working with purified over-expressed mitochondrial β subunits, as opposed to intact F_1, could not ascribe a role in nucleotide binding for Tyr-337 (331). Tozawa *et al.* (1992) showed that this region formed a water-exposed loop in strain PS3 F_1. It is possible that adenine binding at Tyr-337 (331) is dependent on interaction of α and β, and occurs at an αβ interface (Fig. 3.20)

vii) Regions not covered by the models depicted in Figs. 3.21. and 3.22. Neither the N- nor C- terminal regions is shown in either of the models illustrated above. The function of the N-terminus of F_1 β is unknown, despite the fact that certain residues are highly conserved. Only one residue, Glu-47 (41) has been mutated in *E. coli* and shown to be implicated in enzyme assembly (Fig. 3.23). In mitochondrial β, the N-terminal was postulated to be close to the nucleotide-binding site, and could therefore be important in influencing binding affinity changes necessary for multi-site catalysis (Thomas *et al.*, 1992b). Homology between residues 8-107 (1-100) of *T. ferrooxidans* and *E. coli* was generally high, which implies structural/functional similarity. However, the extreme N-terminus in *T. ferrooxidans* β had seven additional residues over that of *E. coli* (Fig. 3.23). Although both the cyanobacteria compared in the multiple alignment analysis, viz. *Anabaena* sp. Strain PCC 7120 and *Synechococcus* 6301 (Cozens and Walker, 1987; Curtis, 1987), had a similar domain, it was unlike that of *T. ferrooxidans*. The highly charged nature of the domain and the Cys-5 residue were unique to *T. ferrooxidans* β.

The C-terminal domain of F_1F_0 ATPsynthase β subunits is particularly interesting. In *T. ferrooxidans*, this domain which commenced at Leu-350 and terminated at Gly-468, was characterised by many absolutely conserved residues (Fig. 3.23). Mutational, inhibitory amphipathic cation-, nucleotide analogue- and aurovertin-binding studies showed the region to be important in catalytic mechanism and enzyme assembly (Aggeler *et al.*, 1992b; Allison *et al.*, 1992; Duncan and Cross, 1992; Weber *et al.*, 1992a and 1992b; Zhou *et al.*, 1992). The D_{381}ELSEDD$_{372}$ motif is highly conserved and is thought to be the site of F_1/F_0 stalk interaction, and β-ε binding (Allison *et al.*, 1992; Dallman *et al.*, 1992b). Generally, the C-terminus in *T. ferrooxidans* β was typical. However, the substitution of Cys-366 for the conserved Val, and the constitution of the extreme C-terminus were unique to the subunit.

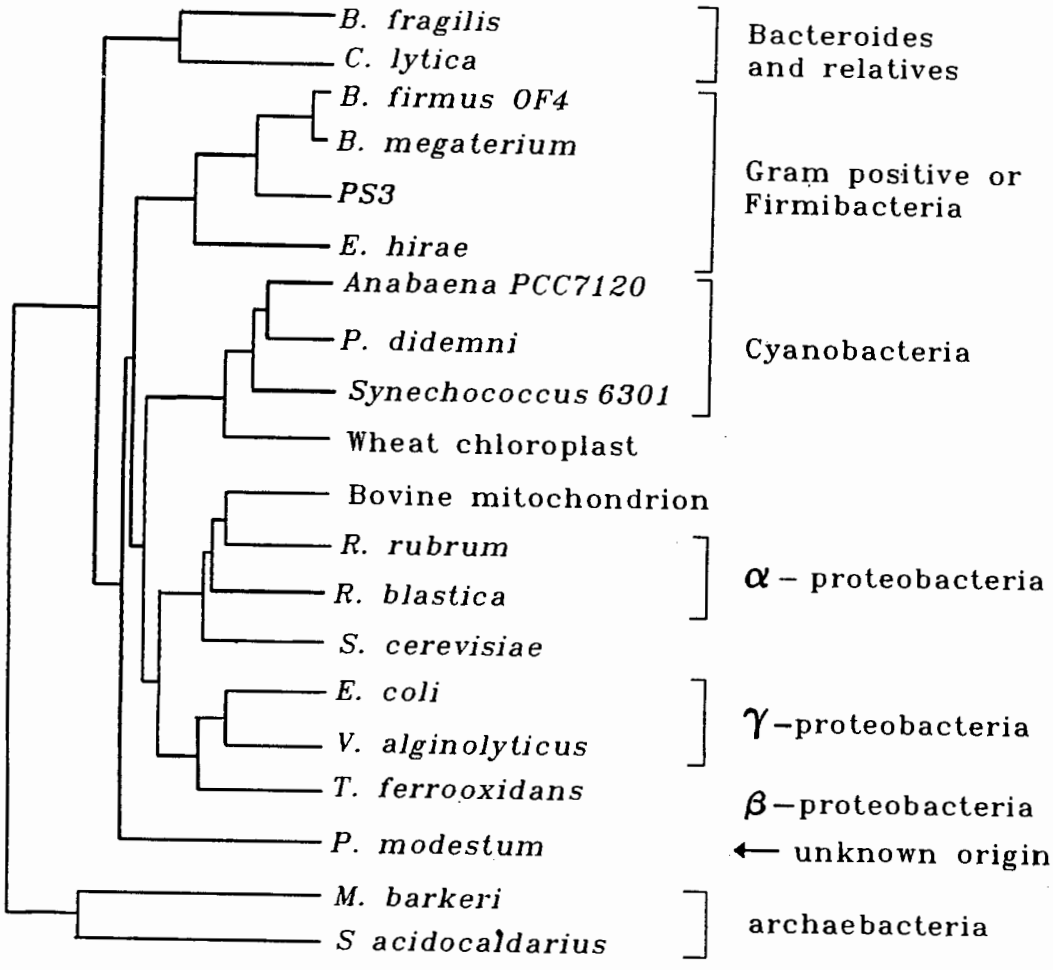


Fig. 3.24. Dendrogram compiled from the multiple sequence alignment of F_1F_0 ATPsynthase β subunits from various organisms, using the PILEUP facility of UWGCG. Refs:- *Bacteroides fragilis* (Amann *et al.*, 1988b); *Cytophaga lytica* (Amann *et al.*, 1988b); *B.firmus* OF4 (Mack Ivey and Krulwich, 1991); *B. megaterium* (Brusilow *et al.*, 1989); strain PS3 (Ohta *et al.*, 1988); *E. hirae*, (Shibata *et al.*, 1992); *Anabaena* sp. Strain PCC 7120 (Curtis, 1987); *Synechococcus* 6301 (Cozens and Walker, 1987); *Prochloron didemni* (Lockhart *et al.*, 1992); Wheat chloroplast (Howe *et al.*, 1985); bovine mitochondrion (Walker *et al.*, 1982); *R. rubrum* (Falk *et al.*, 1985); *R. blastica* (Tybulewicz *et al.*, 1984); *Saccharomyces cerevisiae* (Takeda *et al.*, 1985); *E. coli* (Walker *et al.*, (1984a); *V. alginolyticus* (Krumholz *et al.*, 1989); *T. ferrooxidans* (this study); *P. modestum* (Krumholz *et al.*, 1992); *Methanococcus barkeri* (Inatomi *et al.*, 1989); *S. acidocaldarius* (Denda *et al.*, 1990).

It was suggested that the primary sequence of the F_1F_0 ATPsynthase β subunit may be a useful means of establishing phylogenetic and taxonomic relationships amongst various organisms (Amann *et al.*, 1988a and b; Nelson and Taiz, 1989). The dendrogram presented in Fig. 3.24, compiled from the multiple primary sequence alignment of 20 β subunits, indicates clearly that prokaryotic β subunits were grouped in a manner which accurately reflected proposed phylogenetic trends as revealed by 5S and 16S rRNA analyses (Chapter 1, Fig. 1.2) (Woese, 1987). In addition, predicted phylogenetic trends amongst the α-proteobacteria and mitochondria, and cyanobacteria and chloroplasts was evident.

Typically, *T. ferrooxidans* was clustered within the proteobacteria, forming a lineage distinct from that of the γ group. This was in agreement with rRNA data of Lane *et al.* (1992) which indicated that *T. ferrooxidans* is part of the β-proteobacteria (See Chapter 1, Fig. 1.1). In the case of *T. ferrooxidans*, analysis of the β subunit was of more value than compiling a phylogenetic relationship based on the primary sequence of the c subunit, as proposed by Recipon *et al.* (1992) for diverse organisms, and discussed in the foregoing. This is probably because the β subunit, during the course of its evolution, is unlikely to have been extensively influenced by the environmental constraints associated with acidophily, as the membrane-associated F_0 c subunit has been.

The extensive primary sequence homology which occurs between *T. ferrooxidans* and *E. coli* α subunits, and among α subunits in general is clearly demonstrated in Fig. 3.25. Therefore, α from a wide variety of organisms is probably structurally and functionally similar. Tertiary structures were predicted for some *E. coli* α domains, and these are illustrated in Figure 3.25 (after Pagan and Senior, 1990).

Mutations at residues of *E. coli* α result in either faulty enzyme assembly, or alterations in multi-site catalytic rates (Fig. 3.25). There is no correlation between mutation site and effect. As yet, no mutation in α has affected the high affinity catalytic site associated with uni-site catalysis (Pagan and Senior, 1990; reviewed in Fillingame, 1990; Futai *et al.*, 1989; Senior, 1990 and 1992).

Based on results from various mutational and nucleotide analogue binding studies, Senior (1990 and 1992) proposed three functional regions for *E. coli* α. These are:-

(i). a proposed F_1F_0 binding domain associated with the first 30 N-terminal residues of the subunit,

(ii). a typical nucleotide-binding domain between residues 160-340, homologous to the domain in β, and

(iii). an α/β signal transmission region between residues 345-375 important for catalytic co-operativity.

Both domains (ii) and (iii) are thought to be at an αβ interface in *E. coli* and mitochondrial F_1 (Bianchet *et al.*, 1991; Duncan and Cross, 1992; Ida *et al.*, 1991; Futai *et al.*, 1992; Zhou *et al.*, 1992). In all these domains, *T. ferrooxidans* α was highly homologous to *E. coli* α (Fig. 3.25).

The C-terminal domain of α subunits is conserved. Although no distinct function has been demonstrated for this region, it was suggested on the basis of both inhibitory amphipathic cation binding and sequence homology to the equivalent domain in β, that this region in α is the site of α/β/stalk interaction (Allison *et al.*, 1992) (Table 3.12). It may also be the site of α/β/ε interaction (Aggeler *et al.*, 1992; Dallman *et al.*, 1992).

Fig. 3.25. Page 1.

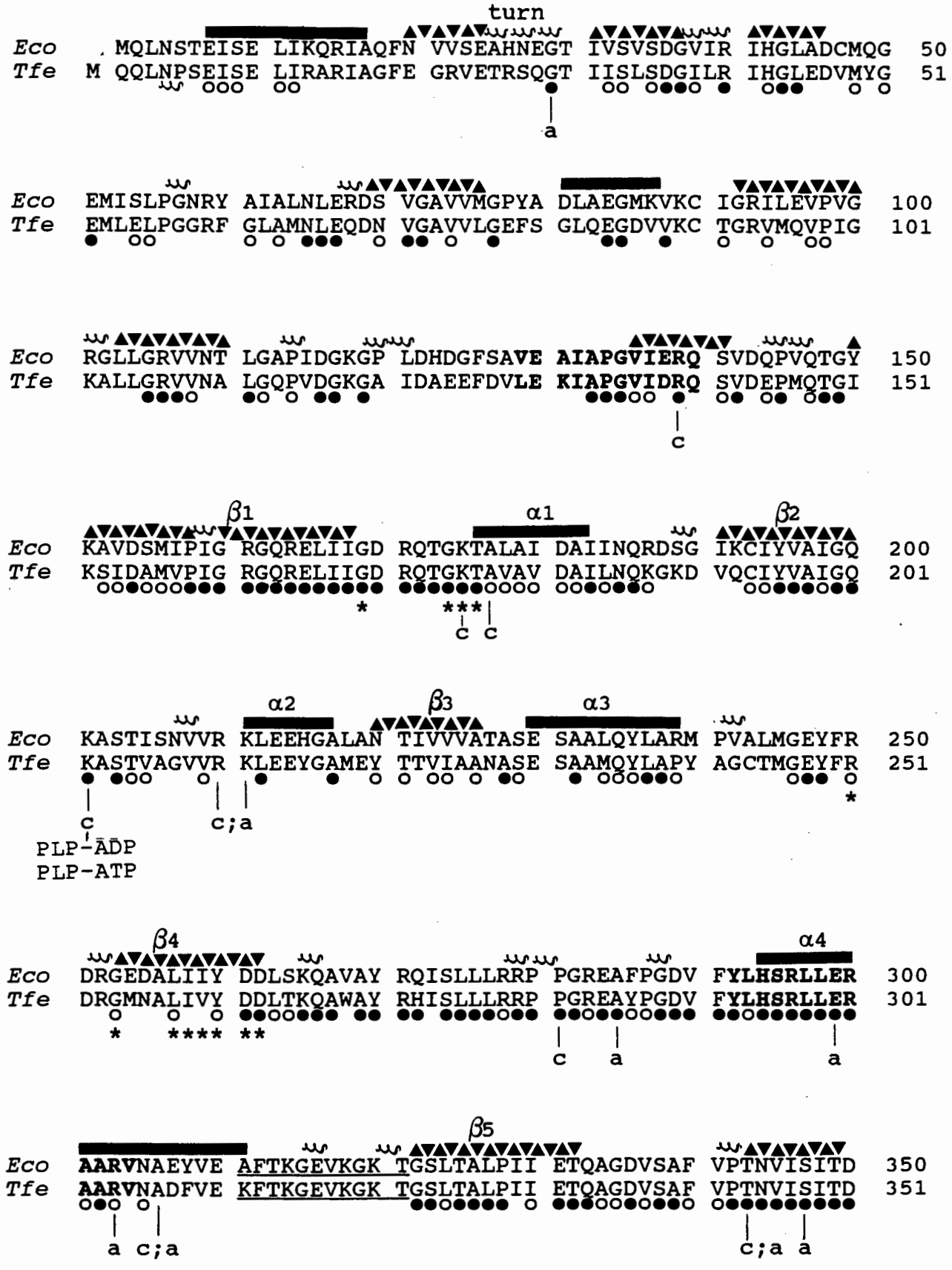


Fig. 3.25. Page 2.

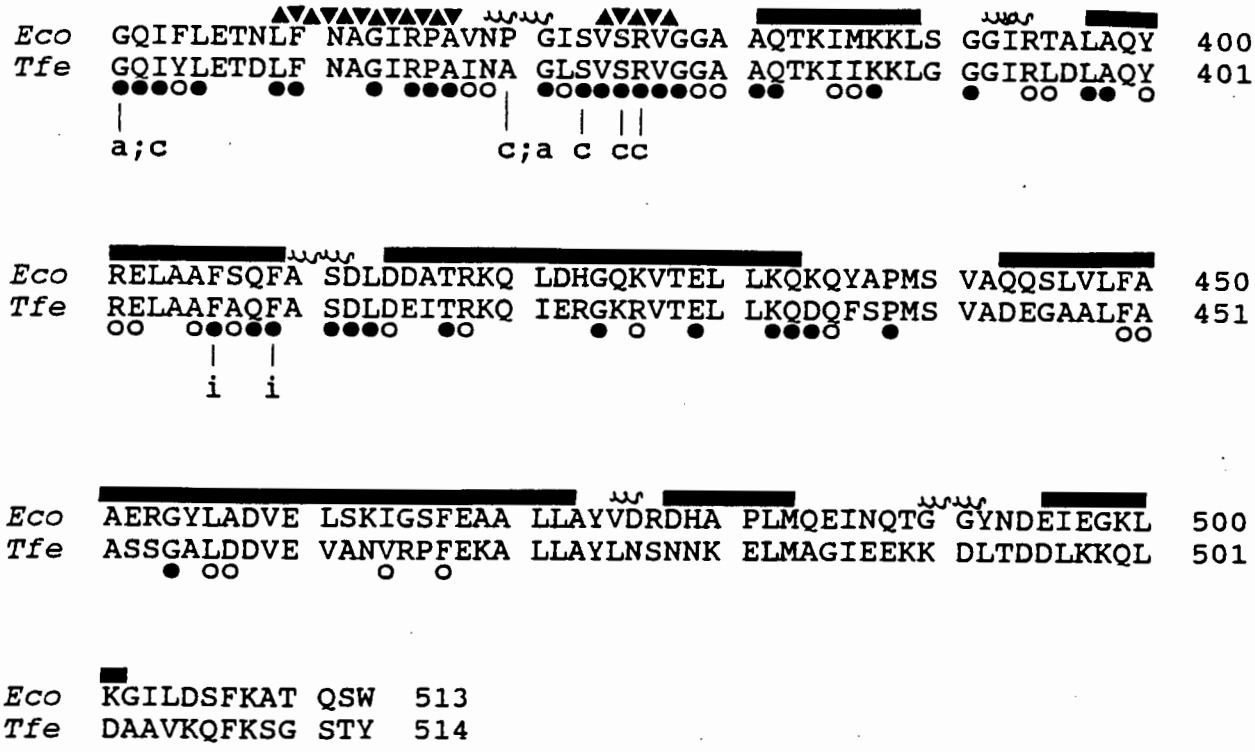


Fig. 3.25. The α subunit of *T. ferrooxidans* F_1F_0 ATPsynthase (*Tfe*) aligned against that from *E. coli* F_1F_0 ATPsynthase (*Eco*). The *E. coli* sequence is taken from Walker *et al.* (1984a). Minimal elements of predicted secondary structure are shown above the sequence and were derived using the method of Chou and Fasman, (1978) (cited by Pagan and Senior, 1990 and Devereaux *et al.*, 1984). α-helices (▬); β-strands (▲▼▲); β-turns (⌇⌇⌇). The nucleotide-binding domain was predicted to comprise residues 160-340, approximately, containing β-strands 1-5, and α-helices 1-4 in crossover connections (after Pagan and Senior, 1990). Amino acid domains in **boldface** are those which show homology to molecular chaperonins, and those underlined represent a domain unique to *T. ferrooxidans*, *E. coli* and *V. alginolyticus* α. *Notations below the sequence:* • , invariant residues, o conservative residue replacements of the species compared (see Fig. 3.27); (note, in all other species, *T. ferrooxidans* Tyr-269 is Val, Ser, Ala or Gln, His-272 is Gln or Glu, Ala-456 is Phe, Tyr or Leu); * denotes consensus of Walker A and Walker B sequence; a, "assembly" defects caused by a missense mutation at that residue, c, multisite "catalytic" defects were caused by missense mutations at these sites; a residue in *E. coli* α which is covalently modified by nucleotide analogues is indicated (reviewed in Futai *et al.*, 1992); i, residues implicated in binding to inhibitory amphipathic cations (Allison *et al.*, 1992). Details of mutagenesis for *E. coli* α: G29D; R139H; K175E/I; A177V; K201Q; R210C; P281L; A285V; E299K; R303C; A306V; T343I; S347F; G351D/S; P370L; S373F; S375F; R376C. (Mutations cited from Fillingame, 1990; Futai *et al.*, 1989; Pagan and Senior, 1990; Senior, 1990; Senior, 1992).

Chaperonin-like activities were reported for mitochondrial and chloroplast F_1 α subunits, together with sequence homology to heat shock proteins and molecular chaperonins (Arni *et al.*, 1991; Luis *et al.*, 1990). *T. ferrooxidans* α had two domains of primary sequence homology to the *E. coli* groEL sequence located on either side of the proposed nucleotide binding domain. The first domain in *T. ferrooxidans* α, also homologous to a domain in *T. ferrooxidans* β was:-

T. ferrooxidans α130-141	L-E-K-I-**A-P-G**-V-I-**D-R**-Q-
E. coli groEL 274-285	A-A-V-K-**A-P-G-F**-G-**D-R**-R-
T. ferrooxidans β115-126	I-H-R-P-**A-P-A-F**-D-**E**-L-A-

The second *T. ferrooxidans* α domain, which did not have a homologous domain in β was:-

T. ferrooxidans α293-305	**Y**-L-H-S-R-**L**-L-**E-R-A-A-K-M**-
E. coli gro-EL 350-362	**Y**-D-R-E-K-**L**-Q-**E-R-V-A-K-L**-

(*E. coli* sequence from Hemmingson *et al.*, 1988).

It is possible that these domains are implicated in folding of the subunit/s into catalytically competent conformations.

A domain which was unique to *T. ferrooxidans, E. coli* and *V. alginolyticus* α subunits was noted toward the C-terminal end of the nucleotide-binding domain (Fig. 3.25). No mutations have been reported for this domain in *E. coli;* hence its functional significance remains unknown. The domain had close homology to the Walker A Gx_4GKT consensus.

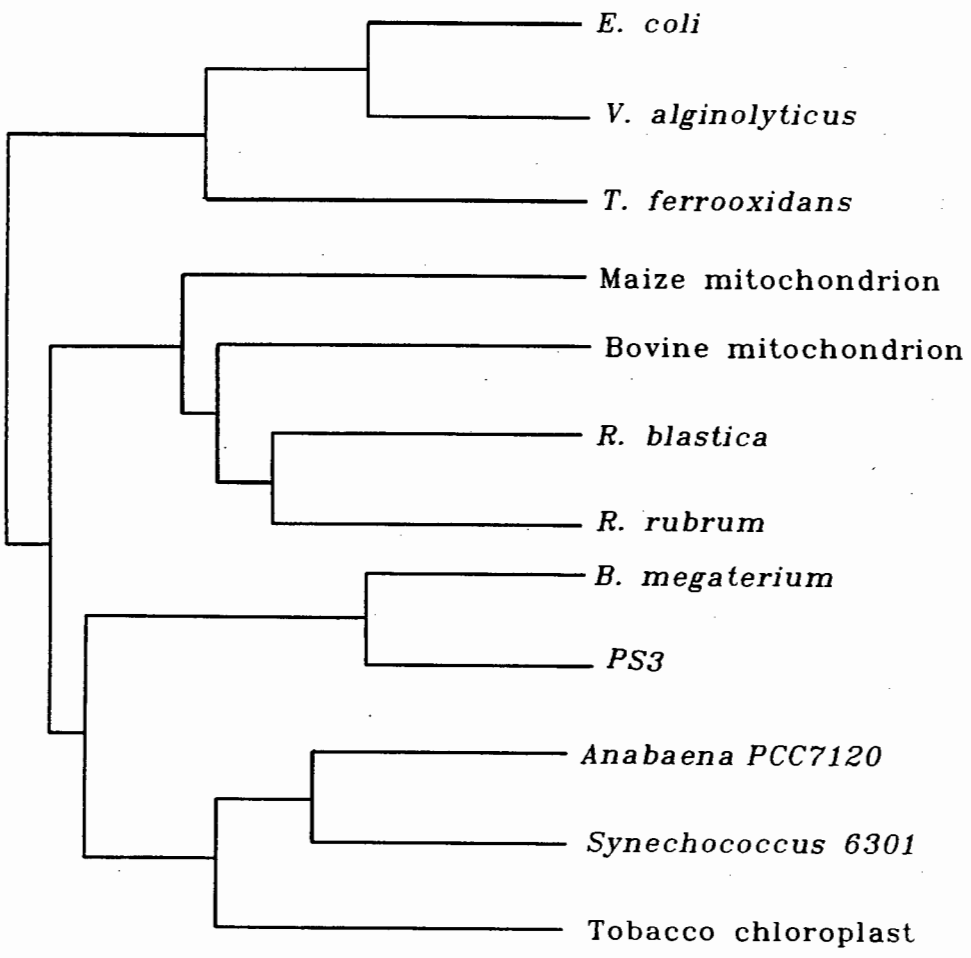


Fig. 3.26. A dendrogram compiled from the multiple sequence alignment of various F_1 α subunits, using the PILEUP facility of the UWGCG programme. Refs :- *E. coli* (Walker *et al.*, 1984a); *V. alginolyticus* (Krumholz *et al.*, 1989); *T. ferrooxidans* (this study); maize mitochondrion (Braun and Levings, 1985); bovine mitochondrion (Walker *et al.*, 1985); *R. blastica* (Tybulewicsz *et al.*, 1984); *R. rubrum* (Falk *et al.*, 1985); *B. megaterium* (Brusilow *et al.*, 1989); strain PS3 (Ohta *et al.*, 1988); *Anabaena* sp. Strain PCC 7120 (McCarn *et al.*, 1988); *Synechococcus* 6301 (Cozens and Walker, 1987); tobacco chloroplast F_1 (Deno *et al.*, 1987).

The dendrogram resulting from the multiple sequence alignment of 14 differing F_1 α subunits is depicted in Fig. 3.26. Although not as extensive as the alignment done for the F_1 β subunit, the clustering of the various α subunits was according to predicted phylogenetic groupings.

Table 3.12. Major domains of primary sequence homology between *T. ferroxidans* F_1F_0ATPsynthase α and β subunits, as indicated by sequence alignment[1].

Domain of subunit	Residues	Function
N-terminal region	αLeu68 - Glu126 βLeu53 - Glu126	Unknown
Midregion	αIle161 - Ser 341 βPhe145 - Thr310	Nucleotide binding. Possible control of protein folding
C terminal regions	αVal342 - Ala381 βSer313 - Pro352	α/β signal transmission (Multisite catalysis)
	αArg402 - Gln436 βTyr373 - Ser407	Amphipathic cation binding; possible F_1/stalk and β-ε-α interaction

[1] - Sequence aligned using UWGCG Version 7 GAP subroutine

The F_1 α and β subunits of *T. ferrooxidans* were aligned using the GAP facility of UWGCG. As expected, this indicated that the two subunits had 52% similar, and 24% identical amino acid residues, reflecting their common origin/gene duplication (Walker *et al.*, 1982). Certain domains within the two subunits exhibited more homology than others, and these are presented, together with proposed functional significance when known (discussed in the foregoing) in Table 3.12. Extensive homology was noted for the N-termini of both *T. ferrooxidans* F_1 α and β. However, as there are little mutational data recorded for these regions, the functional significance is unknown.

CHAPTER 4.

IN VITRO TRANSLATION OF *T. FERROOXIDANS ATP* GENES, COMPLEMENTATION STUDIES OF *E. COLI UNC* MUTANTS, *IN VIVO* GROWTH RATES OF TRANSFORMANTS AND ENZYME ACTIVITY ASSAYS ASSOCIATED WITH A HYBRID F_1F_0 ATP SYNTHASE.

CHAPTER 4.

IN VITRO TRANSLATION OF *T. FERROOXIDANS ATP* GENES, COMPLEMENTATION STUDIES ON *E. COLI UNC* MUTANTS, *IN VIVO* GROWTH RATES OF TRANSFORMANTS AND ENZYME ACTIVITY ASSAYS ASSOCIATED WITH A HYBRID F_1F_0 ATP SYNTHASE.

4.0. Summary.

In vitro SDS-PAGE analysis of the polypeptide products of the *T. ferrooxidans atp* genes demonstrated that all seven, including the two F_0 proteins could be synthesised in an *E. coli*-derived transcription/translation system. Data from these analyses demonstrated that the F_1 genes of p*Tfatp*2 cloned against the λ promoter of the vector, p*Eco*R251, were expressed *in vitro* by an *E. coli*-derived system. This indicated that *E. coli* recognised an internal promoter possibly located upstream of the *T. ferrooxidans atpH* gene. The *atpE* and *atpF* genes on p*Tfatp*2 were not expressed *in vitro*. However, by cloning these genes behind a *lacZ* promoter, on p*Tfatp*3001, the respective polypeptide products (c and b) were expressed *in vitro*. *T. ferrooxidans* F_1 products, β and ε were strongly expressed *in vitro* from the p*Eco*R251 promoter by p*Tfatp*1; although present on p*Tfatp*1, a subunit corresponding in size to γ was not detected on SDS-PAGE gels. When cloned behind the *lacZ* promoter on pUC18 (p*Tfatp*2001), *T. ferrooxidans atpA* and *atpG* were not synthesised *in vitro*.

A series of complementation studies was done with various combinations of the seven *T. ferrooxidans atp* genes cloned into plasmid expression vectors. The *T. ferrooxidans* F_0 genes for subunits c and b, although expressed *in vitro*, when transformed into *E. coli* AN943 (c⁻) or AN1440 (b⁻) did not functionally complement either F_0 mutant on minimal succinate medium. Complementation data from AN F_1 mutants indicated that functional hybrid *T. ferrooxidans* F_1/*E. coli* F_1/F_0 ATPsynthases were formed *in vivo*. Whilst a *T. ferrooxidans* βε combination present on p*Tfatp*1 functioned with *E. coli* F_1F_0 ATPsynthase subunits, ε when produced on its own, did not. Functional F_1F_0 ATPsynthases were noted for *E. coli* AN730 (p*Tfatp*2), *E. coli* AN1273 (p*Tfatp*2), *E. coli* AN818 (p*Tfatp*2) and *E. coli* AN802 (p*Tfatp*2). A plasmid, pAN45F_0, containing the *uncBEF* and a portion of the *uncH* genes from *E. coli*, was constructed from pAN45. This plasmid, together with p*Tfatp*2, when transformed into *E. coli* DK8 (Δ*unc*) formed a partially functional hybrid F_1F_0 ATPsynthase, which complemented *E. coli* DK8 on MMS.

Growth curves of *E. coli* DK8 (p*Tfatp*2, pAN45F_0) indicated that when compared to *E. coli* *unc*⁺ strains, growth rates and yields were reduced and were similar to those of the *E. coli* Δ*unc* strain. ATPase activity assays, determined by measuring the amount of inorganic phosphate released by various *E. coli* membrane preparations, demonstrated that the hybrid F_1F_0 ATPsynthase had a low specific activity. The recombinant enzyme showed resistance to ATPase inhibitors DCCD and NaN_3, indicating that the enzyme was severely impaired with regard to both coupling and multisite catalysis. The ATPase activity of the hybrid *T. ferrooxidans* F_1/*E. coli* F_0 enzyme had a pH profile similar to that of wild-type F_1F_0 ATPsynthase.

"Irrespective of the many future studies that are required to gain an adequate understanding of the ATP synthase, I feel sure that it will continue to deserve the designation as a remarkable enzyme". Boyer, (1993).

4.1. Introduction.

It was suggested that reconstitution of hybrid forms of enzymes with subunits from different species, might be a tool to evaluate phylogenetic relationships, as well as to obtain information about essential features of enzyme function (Steffens *et al.*, 1987). Hybrid F_1F_0 ATPsynthases from a variety of prokaryotes have been reported, and this is reviewed in Section 4.4. The data obtained from the nucleotide sequence of the *T. ferrooxidans atpEFHAGDC* genes were used to devise a series of complementation studies, which were designed to produce hybrid *T. ferrooxidans/E. coli* F_1F_0 ATPsynthases. Results from such studies, which combined results from the ability of transformants to grow on MMS with those from the SDS-PAGE analyses, extended complementation data presented in Chapters 2 and 5 and provided information as to the nature and the extent to which cross-complementation could occur between the F_1F_0 ATPsynthases from an obligately acidophilic and a neutrophilic organism. By incorporating additional data from growth curves and ATPase activities of *E. coli* strains harbouring recombinant plasmids, the efficacy and biochemical properties of *T. ferrooxidans/E. coli* hybrid F_1F_0 ATPsynthases were studied.

Proteins synthesised in heterologous systems can be detected by assaying for a particular activity, or by employing assays that are independent of such activity. For the purposes of this study, functional complementation of *E. coli unc* mutants by *T. ferrooxidans atp* genes, by selecting for growth on solid MMS agar plates, was used to indicate that the transformed organism had a biologically active F_1F_0 ATPsynthase. To determine specific activities of such ATP synthases, it was decided to assay the activity of the certain enzyme preparations by measuring the ATP hydrolysis of isolated bacterial cytoplasmic membrane systems. This analysis provided a useful guide as to the functionality of the proteins synthesised by cloned genes. However, such an assay does have certain limitations. Firstly, where the protein is synthesised in minute amounts, the assay might not be sensitive enough. Secondly, host cells which harbour a protein which is similar to the foreign protein, or a protein with biological activity similar to the one being assayed, might interfere with the accuracy of such assays. Thirdly, the assay does not explain the reasons for observed biological activity levels, particularly if the protein concerned is in a heterologous host. For the purposes of this study, and in common with many other workers in the field, ATP hydrolysis was measured by assaying the nanomolar amount of inorganic phosphate released during a defined time by membrane preparations, in the presence of ATP and magnesium ions. As will become apparent, these ATPase specific

assays served as an indication of the extent to which F_1F_0 ATPsynthase subunits from an acidophile can be functionally reconstituted in a neutrophilic organism.

4.2. Materials and Methods.

4.2.1. Bacterial strains and media used.

Bacterial strains and their relevant genotypes are listed in Table 1, Appendix A. *E. coli* AN strains and *E. coli* DK8 were maintained routinely as described in Chapter 2, Section 2.2.1. When necessary, after transformation of *E. coli unc* mutants, LBA was supplemented with Ap and/or Cm. MMS was prepared for the selection of *E. coli unc* mutants. Details of media preparation are described in Chapter 2, Section 2.2.1 and in Appendix B. *E. coli* JM109 (Table 1, Appendix A) was routinely used to maintain and prepare plasmids.

4.2.2. Plasmids used.

Plasmids used, their construction and genotype are described in Table 1, Appendix A. For convenience, those of relevance are aligned and diagrammatically illustrated in Fig. 4.1. The source and isolation of the *T. ferrooxidans atp* genes on p*Tfatp*1 and p*Tfatp*2 was described in Chapter 2, Sections 2.2.3. and 2.2.4.

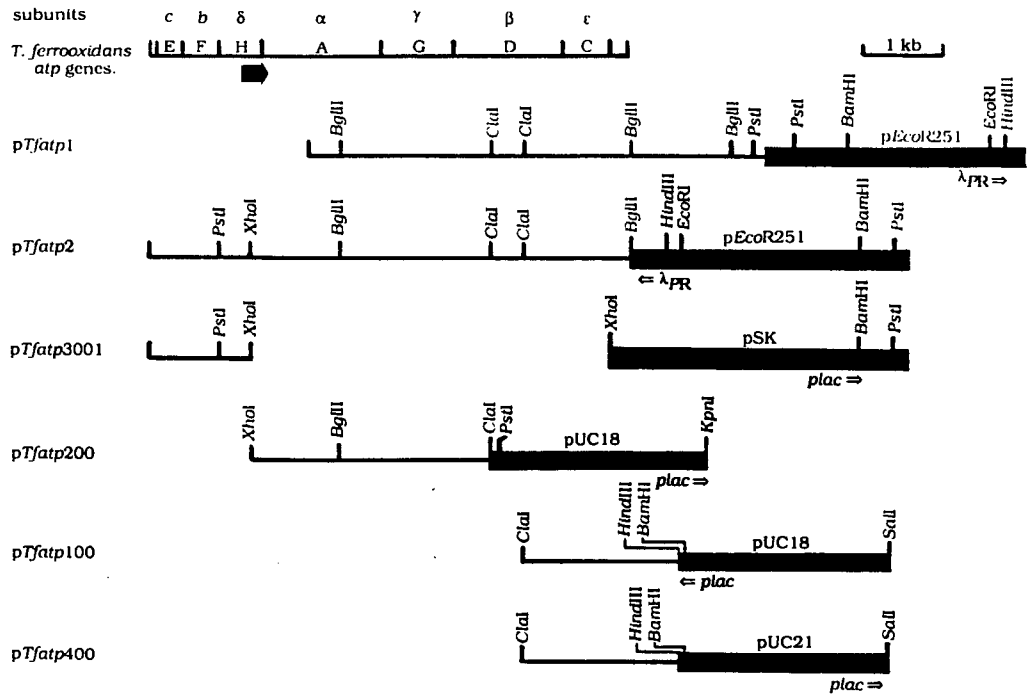


Fig. 4.1. Genetic and physical map of cloned *T. ferrooxidans* ATCC 33020 plasmid constructs used for *in vitro* protein analyses and *in vivo* complementation studies. Direction of transcription of wild-type *T. ferrooxidans atp* genes is indicated by the solid black arrow, and that of the cloning vectors, by the unshaded arrow. λ_{PR} = phage lambda promoter; *plac* = β-galactosidase promoter.

4.2.2.1. The construction of pAN45F_0. Plasmid pAN45F_0 (Table 1, Appendix A) which contained the *E. coli* F_0 genes, was constructed from a *Cla*I deletion of the 19.3 kb pAN45 plasmid (Fig. 4.2). A *Cla*I digest of pAN45 yielded 0.6 kb, 6.2 kb and 12.5 kb fragments, which were separated on a 1% agarose gel in Tris-acetate buffer (Sambrook *et al.*, 1989) (Fig. 4.2). The 6.2 kb *Cla*I fragment, which contained the *E. coli uncB, uncE, uncF,* and a portion of the *uncH* gene linked to the Cm resistance marker and the replicon of pACYC184, was excised from the gel and eluted from the agarose. The linear DNA thus obtained was ligated and transformed into *E. coli.*

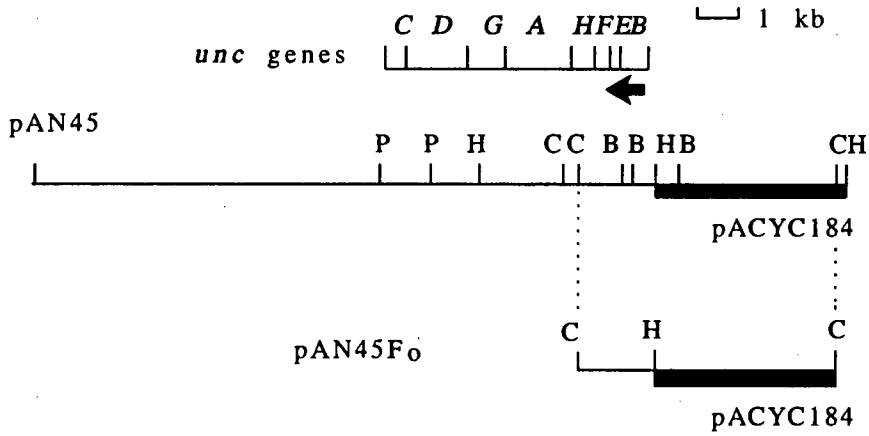


Fig. 4.2. The construction of pAN45F_0 from pAN45. The plasmids are aligned against the *E. coli unc* operon to show the position of *unc* genes on the plasmids. Key to restriction endonucleases; B = *Bam*HI; H = *Hin*dIII; C = *ClaI;* P= *Pst*I. The arrow indicates direction of transcription of wild-type *E. coli unc* genes.

4.2.3. General techniques.

Refer Chapter 2, Section 2.2.2.

4.2.4. *In vitro* synthesis of F_1F_0 ATPsynthase proteins.

The *in-vitro* synthesis of proteins from the F_1F_0 ATPsynthase-containing plasmid clones was done using the prokaryotic DNA-directed transcription kit, code L4500 *E. coli* S30 system of Promega Corporation, Madison, U.S.A. All reactions were performed according to manufacturer's specifications. Proteins were labelled using ^{35}S-methionine, and were separated by SDS-PAGE as described by Laemmli (1970).

4.2.5. The determination of the rate of growth of *E. coli* K12 (*unc*$^+$) and *E. coli* DK8 (Δ*unc*) mutants.

Growth curves were determined for the following cultures:-

E. coli DK8 (Δ*unc*); *E. coli* K12 (*unc*$^+$);

E. coli DK8 (p*Tfatp*2, pAN45F_0); *E. coli* DK8 (pAN45).

Five ml of each of the above cultures, initially grown to a uniform OD_{600} of 0.75 in LB medium, were suspended to OD_{600} 0.3 in sterile LB. 100 µl was removed from each of the

suspensions to determine the number of viable cells/ml. (Approximately 1250 viable cells/100µl were present in the OD_{600} 0.3 suspension). Three ml of the remaining suspension (OD_{600} 0.3) was immediately inoculated into 250 ml of "modified" Luria Bertani broth (MLB) (Appendix B) which had been pre-warmed to 37°C. Where necessary, Ap and/or Cm were added at 60 µg/ml and 25 µg/ml respectively. Ap was used in cultures containing p*Tfatp*2, and Cm in cultures transformed with pAN45 or pAN45F_0.

After inoculation, the cultures were vigorously shaken at 37°C. To determine growth curves, 500 µl was aseptically removed from each culture every 30 min and the OD_{600} of the aliquot was determined. Samples were blanked against uninoculated MLB. Readings were continued until the cultures were into stationary phase.

4.2.6. The preparation of *E. coli* membranes for ATPase assay (after Fillingame, 1986; Pati and Brusilow, 1989; Scarpetta *et al.*, 1991).

Each *E. coli* culture was vigorously shaken at 37°C in 2 X 500 ml of MLB. Strains used were:-

E. coli DK8 (Δ*unc*); *E. coli* DK8 (pAN45); *E. coli* DK8 (p*Tfatp*2, pAN45F_0);
E. coli K12 (*unc*+).

Once the OD_{600} reached 0.9, the cells were harvested by centrifugation at 10 000 Xg for 20 min in a Beckmann J2-21M/E centrifuge. The pellet of cells from one of the 500 ml cultures was resuspended in 20 ml chilled 50 mM MOPS, 10 mM $MgCl_2$, pH 7.0 (Harvest buffer) at 4°C. The pellet of cells from the other 500 ml volume was given a "boost" by resuspending the cells in 200 ml of liquid minimal succinate medium described by Klionsky *et al.* (1984) (Appendix B) to which yeast extract and tryptone had been added at a concentration of 1 g/l (w/v). The cells were shaken at 37°C for 3 h, and were then re-harvested by centrifugation. The resulting pellet was resuspended in 20 ml chilled Harvest buffer. Both sets of cells were lysed separately by two passages through a French Press at 18 000 psi. Intact cells and debris were removed by centrifugation at 12 000 Xg at 4°C for 10 min. The supernatent was collected and spun at 100 000 Xg for 90 min in a Beckman 50.2 VTi rotor, in a Beckman L7-65 ultracentrifuge operating at 4°C. After rinsing twice with chilled Harvest buffer, the membrane pellet was disrupted and resuspended in chilled Harvest buffer by vortexing and passing the suspension through a 21 gauge needle ten times. The pellet was diluted in Harvest buffer at a concentration of 5 mg membrane protein/ml, and was stored at -70°C until required for assay.

4.2.7. The preparation of *T. ferrooxidans* membranes for ATPase assay.

T. ferrooxidans ATCC 33020 was grown in 10 l of 9K medium (Silverman and Lundgren, 1959) at 30°C with vigorous aeration. After five days when the medium had started to oxidise, cells were harvested by centrifugation at 10 000 Xg for 10 min. The pellet of cells was resuspended in 10 ml of chilled water, acidified with H_2SO_4, pH 2. The cells were left overnight to allow precipitated ferric salts to settle. The supernatent was carefully

decanted, and the cells were re-sedimented and suspended in 10 ml of the chilled acidified water. One ml volumes of cells were aliquoted into ten Eppendorf tubes and pelleted by spinning for 60 s in a bench-top microfuge. One ml of fresh acidified water was added to the pellet, and the pink cells were carefully resuspended with a Gilson micro-pipette, without dislodging the precipitated yellow residual ferric salts. The suspension of cells was transferred to a fresh Eppendorf, and the washing procedure was repeated twice more. Once the pellet of cells was seen to be free of precipitates, it was suspended in 1 ml 50 mM Tris-SO_4, 10 mM $MgSO_4$ buffer (pH 7) to which a sterile sucrose solution had been added at a final concentration of 10% w/v. The cells were sedimented and washed twice more in the Tris-SO_4/sucrose buffer. Finally, the contents of the ten microfuges were pooled, and chilled to 4°C. Cells were lysed and membranes were harvested as described above for *E. coli*, except that the buffer used throughout was chilled 50 mM Tris-SO_4, 10 mM $MgSO_4$ (pH 7) with 5% sucrose (w/v). To prevent possible loss of viability, *T. ferrooxidans* membrane preparations were not stored at -70°C, but were assayed immediately. The supernatent remaining after the membrane fraction had been pelleted was kept for ATPase assay.

4.2.8. Quantification of proteins in the prepared membranes.

Protein content of the membranes was determined colorimetrically at 37°C, using the Pierce BCA protein assay kit 23225X (Pierce, Rockford, IL, USA). All assays were done in triplicate. Standard protein curves at OD_{562} were obtained using the albumen stocks supplied with the kit.

4.2.9. The assay for ATPase activity.

T. ferrooxidans and *E. coli* membrane preparations, which had been stored at -70°C, were thawed on ice. They were diluted in respective harvest buffers, such that protein concentration was between 350-700 ng/µl. The higher range of dilution was used for membranes with high ATPase activity (eg. *unc*$^+$) and lower dilution ranges were used for membranes with little activity (eg. *unc*$^-$). A 25 µl aliquot of the membranes was added to a reaction-mix, prewarmed to 37°C. The reaction was run for precisely 3 min at 37°C and was terminated by the addition of 75 µl 0.2 N HCl. All reactions were done in triplicate. The reaction mix consisted of 50 mM buffer, 5 mM ATP and 5 mM Mg^{2+}, made to 275 µl.
Buffers used for *E. coli* membrane preparations were:-
MES, pH 4 and pH 5; Tris-Cl, pH 6, pH 7 and pH 8.
Buffers used for *T. ferrooxidans* membranes were:-
Tris-SO_4, pH 5, 6 and 7; MOPS, pH 7; Tris-Cl, pH 5, 6, and 7.

Negative controls were 300 µl reaction mix without added cells, and 25 µl cells suspended in 275 µl of the appropriate buffer from which ATP and magnesium had been omitted.

The effect of two inhibitors of ATPase on membranes prepared from cells grown in MLB, was determined. A 25 µl membrane aliquot was added to 255 µl of Tris-Cl (pH 7), which contained either 100 µM DCCD or 1 mM NaN_3, pre-warmed to 37°C. The mix was equilibrated for 2 min at 37°C, and the reaction was started by adding ATP and $MgCl_2$ to a final concentration of 5 mM each. The final volume of the mix was 300 µl. The reaction was run for 3 min at 37°C, and terminated as described above. All reactions were done in triplicate, and controls consisted of 300 µl of reaction mix without membranes, and 300 µl reaction mix with membranes from which ATP and $MgCl_2$ were omitted.

F_1F_0 ATPase activity was determined by measuring the amount of inorganic phosphate (P_i) released. One unit of ATPase activity was defined as that amount which released 1 µmol P_i/min/mg membrane protein (Krumholz *et al.*, 1990; Scarpetta *et al.*, 1991).

The method of Lanzetta *et al.* (1979) was used to measure nanomolar amounts of inorganic phosphate released. The following stock solutions were made:-

A. 0.045% malachite green hydrochloride,
B. 4.2% ammonium molybdate in 4 N HCl,
C. 34% sodium citrate.$2H_20$ (w/v),
D. 100 µl Triton N in 20 ml de-ionised water.
E. 10 mM K_2HPO_4 (initially dried overnight at 100°C), from which appropriate dilutions were made to give inorganic phosphate concentrations of 1-10 nM for standard curve determinations.

The colorimetric reagent was made with a 3:1 mix of A:B. This solution was stirred for 20 min, and was then filtered through a Millipore 0.45 µm membrane filter. Solution D was added at a concentration of 300 µl/5ml filtrate, and the solution was stirred for 30 min at room temperature.

The amount of inorganic phosphate released by the membrane reaction mixes was measured by adding 50 µl of the terminated mix to 800 µl of the colorimetric reagent in 1 ml disposable spectrophotometric tubes. After 1 min, the reaction was quenched by adding 100 µl of Solution C. The contents of the tubes were briefly vortexed and left at ambient temperature for 30 min. The OD_{660} was read. Blanks used were the negative controls referred to above. The amount of inorganic phosphate released was determined by reference to a standard curve, obtained using P_i prepared from Solution D, at 1, 2, 5, 8, 9 and 10 nM.

4.3. Results.

4.3.1. Expression of the cloned *T. ferrooxidans atp* genes *in vitro*.

An *E. coli*-derived *in vitro* transcription-translation system was used to identify polypeptides produced from the cloned *T. ferrooxidans atp* genes. Results are presented in Figs. 4.3, 4.4 and 4.5.

Protein bands corresponding to all five F_1 subunits (δ, α, γ, β and ε) were produced from p*Tfatp*2. Sizes of the polypeptides were in agreement with those predicted from nucleotide sequence (Table 3.6; Figs. 4.3 and 4.4, Lanes 2 and 3). As the *T. ferrooxidans atp* genes were cloned in reverse orientation with respect to the p*Eco*R251 λ promoter, it was likely that the *T. ferrooxidans* F_1 *atp* genes were expressed from an internal promoter recognised by *E. coli* and probably located upstream of *atpH* (Fig. 3.2). Although both *atpE* and *atpF* were present on p*Tfatp*2 (Fig. 4.2), they were not expressed *in vitro*.

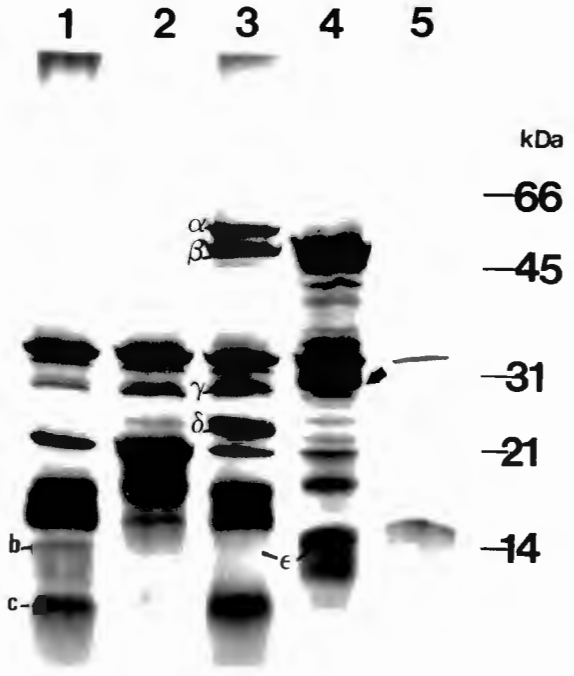


Fig. 4.3. *In vitro* analysis of *T. ferrooxidans* ATCC 33020 F_1F_0 ATPsynthase products, run on a 12% SDS-PAGE gel. The positions of individual subunits are labelled. Lane 1, p*Tfatp*3001; Lane 2, pBluescriptSK+, Lane 3, p*Tfatp*2; Lane 4. p*Tfatp*1; Lane 5, p*Eco*R251.

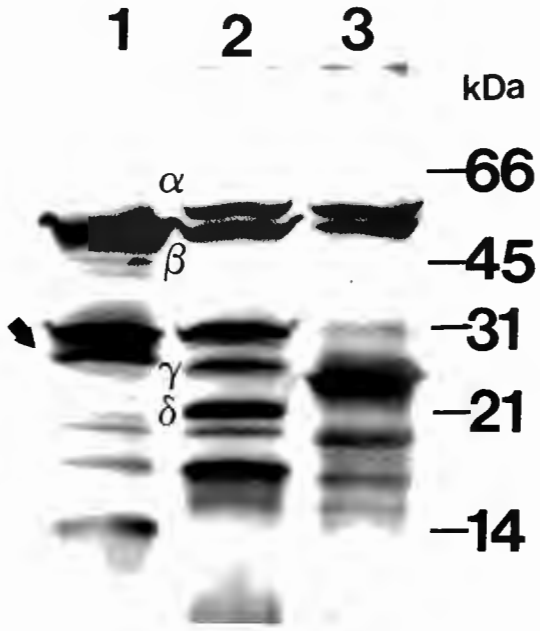


Fig. 4.4. *In vitro* analysis of the *T. ferrooxidans* ATCC 33020 F_1F_0 ATPsynthase proteins, run on a 12% SDS-PAGE gel, compared with the large α and β subunits of *E. coli* F_1F_0 ATPsynthase. Lane 1, p*Tfatp*1; Lane 2, p*Tfatp*2; Lane 3, pAN45. Where present, the positions of individual F_1F_0 ATPsynthase subunits are labelled. The solid black arrow indicates a polypeptide of uncertain origin in p*Tfatp*1.

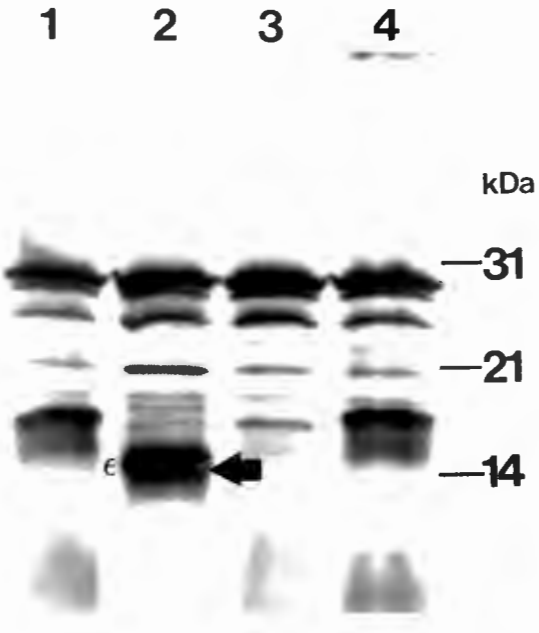


Fig. 4.5. *In vitro* analysis of the *T. ferrooxidans* ATCC 33020 F_1F_0 ATPsynthase proteins, run on a 15% SDS-PAGE gel. Where present, the positions of subunits are indicated in the figure. Lane 1, p*Tfatp*100; Lane 2, p*Tfatp*400; Lane 3, p*Tfatp*200; Lane 4, pUC18.

Protein bands corresponding to F_1 subunits β and ε were produced by p*Tfatp*1, strongly expressed from the p*Eco*R251 λ promoter (Lanes 4 and 1, Figs. 4.3 and 4.4). Although *atpG* was present on p*Tfatp*1 (Fig. 4.1) there was no band equivalent in size to the γ-band detected for p*Tfatp*2. An unidentified polypeptide product, slightly larger than the γ-band detected for p*Tfatp*2, was transcribed from p*Tfatp*1; the origin of this band, indicated by the solid black arrow in Figs. 4.3 and 4.4, was unresolved. Sequencing of the *atp* genes present on p*Tfatp*2 identified an incomplete URF downstream of *atpC* (Chapter 3). If the protein product of the entire URF (present on p*Tfatp*1) is approximately the same size as that of the *E. coli* homologue, then the unidentified band was too small for the complete polypeptide product. In *E. coli*, the M_r of the URF downstream of *uncC* is 49 163 Da (Walker *et al.*, 1984b).

As predicted from nucleotide-derived primary sequence (Table 3.6), the size of the *T. ferrooxidans* F_1F_0 ATPsynthase α and β subunits was similar to those of the *E. coli* homologues (Lane 3, Fig. 4.4), where the polypeptide products of pAN45 were expressed *in vitro*. As pAN45 has a 15 kb insert of *E. coli* chromosomal DNA, it was not possible to identify, with certainty, any other *E. coli* F_1F_0 ATPsynthase subunits *in vitro*.

The protein band corresponding to ε in p*Tfatp*2 was very feint (Figs. 4.3 and 4.4, Lanes 3 and 2). However, when a fragment containing the *atpC* gene was cloned behind a *lacZ* promoter in p*Tfatp*400 (Fig. 4.2), a strong protein band of about 14 kDa, corresponding to ε, was synthesised (Fig. 4.5, Lane 2). This band was absent when the same fragment was cloned in the opposite direction with respect to *atpC*, p*Tfatp*100 (Fig. 4.5., Lane 1). In p*Tfatp*200, the *atpA* and *atpG* genes for the α and γ subunits were cloned behind a *lacZ* promoter (Fig. 4.2). No polypeptides corresponding to the α or γ subunits were detected (Fig. 4.5, Lane 3). As referred to above, the *atpE* and *atpF* genes present on p*Tfatp*2 were not expressed *in vitro*. However, when cloned behind a *lacZ* promoter in p*Tfatp*3001 (Fig. 4.2), protein bands corresponding to subunits c and b were detected (Fig. 4.3, Lane 1).

4.3.2. *In vivo* complementation of *E. coli unc* mutants by *T. ferrooxidans atp* genes.

Using both p*Tfatp*1 and p*Tfatp*2, and constructs derived from p*Tfatp*2, a series of complementation studies was done on various *E. coli unc* mutants. Complementation was tested for by the ability of transformants to grow on solid MMS supplemented with appropriate antibiotic, as described in Chapter 2. Results are reported in Table 4.1.

Table 4.1. Genetic complementation of *E. coli unc* mutants with plasmid-borne *T. ferrooxidans atp* DNA. Growth (+) or no growth (—) on minimal succinate medium was scored after 4-10 days incubation at 37°C. Positive control was pAN45. Negative controls were pACYC184, and *pEco*R251.

The *T. ferrooxidans atp* or *E. coli unc* gene products carried by each plasmid are shown in parentheses.

	Mutants							
Plasmids[a]	**AN727(a^-)**	**AN943(c^-)**	**AN1440(b^-)**	**AN730(α^-)**	**AN1273(γ^-)**	**AN818(β^-)**	**AN802(ε^-)**	**DK8 (Δ*unc*)**
pTfatp 1(γβε)	—	—	—	—	—	+	+	—
*pTfatp*2(cbδαγβε)	—	—	—	+	+	+	+	—
*pTfatp*2 + pAN45F_0	ND	ND	ND	ND	ND	ND	ND	+
*pTfatp*400(ε)	ND	ND	ND	ND	ND	ND	—	ND
pTfatp 200(αγ)	ND	ND	ND	—	—	ND	ND	ND
*pTfatp*3001(cb)	ND	—	—	ND	ND	ND	ND	ND
pAN45F_0(acb)	ND	ND	ND	ND	ND	ND	ND	—
pAN45(acbδαγβε)	+	+	+	+	+	+	+	+

[a] - For details of plasmid construction, description, description and genotype, see Appendix A, Table 1.

ND - no determination

p*Tfatp*1 complemented both *E. coli uncD*- (AN818) and *uncC*- (AN802). This indicated that a hybrid F_1, consisting of *T. ferrooxidans* β and ε, and *E. coli* δ, α and γ was functional, together with the *E. coli* F_0 subunits. Results with p*Tfatp*400 suggested that when produced on its own, *T. ferrooxidans* ε was unable to functionally complement *E. coli* F_1F_0 ATPsynthase mutants. Although *atpG* was present on p*Tfatp*1, the *E. coli uncG*- mutant (AN1273) was not complemented.

Results from p*Tfatp*2 transformed into *E. coli recA*- AN mutants demonstrated that *T. ferrooxidans* F_1 α, γ, β and ε subunits were able to functionally complement *E. coli* F_1 *unc* mutants. However, it was possible that α-complementation was occurring. Therefore, p*Tfatp*2 was transformed together with the F_0 genes for *E. coli* present on pAN45F_0, into *E. coli* DK8 (Δ*unc*). After seven days growth at 37°C, colonies developed on MMS, supplemented with Ap and Cm. Plasmid DNA from these colonies was miniprepped, and run on a 0.9% agarose gel. Two plasmids, corresponding in size to p*Tfatp*2 and pANF$_0$, were visible (Fig. 4.6).

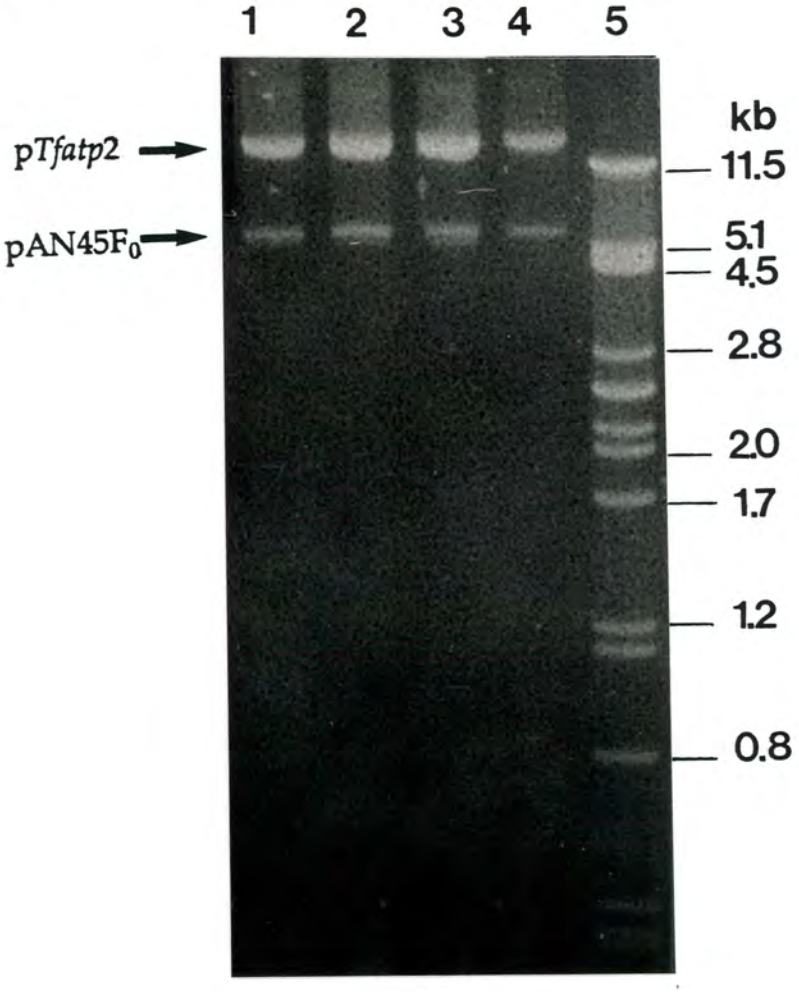


Fig. 4.6. Plasmid DNA extracted from *E. coli* DK8 (*pTfatp*2, pAN45F_0) colonies which developed on MMS, supplemented with Ap and Cm. Lanes 1-4 show p*Tfatp*2 and pAN45F_0; Lane 5, 3 μg λ DNA (*Pst*I), used as a molecular weight marker.

These results indicated that *T. ferrooxidans* F_1 subunits functionally complemented *E. coli unc*⁻ mutants and that all five *T. ferrooxidans* F_1 subunits formed a functional hybrid F_1F_0 ATPsynthase, where the *E. coli* F_0 subunits complexed with *T. ferrooxidans* F_1.

Plasmid p*Tfatp*3001, which carried the *T. ferrooxidans* F_0 *atpE* and *atpF* genes, both of which were expressed off pBluescriptSK⁺ *lacZ in vitro* (Fig. 4.3, Lane 1) was transformed into *E. coli* AN943 (c⁻) and AN1440 (b⁻). No complementation was observed (Table 4.1).

It was reported that in *E. coli*, there is strong translational coupling of the *uncH* and *uncA* genes (McCarthy, 1990; Hellmuth *et al.*, 1991). To test whether this coupling might also apply to *T. ferrooxidans atpH* and *atpA* genes expressed in *E. coli unc* mutants *in vivo*, p*Tfatp*200, which carried part of *atpH* and all of *atpA* and *atpG*, was transformed into *E. coli* AN 1273 (γ) and AN730 (α⁻). No complementation was observed.

4.3.3. Growth curves observed for *E. coli* DK8 (p*Tfatp*2, pAN45F_0) and *E. coli* DK8 (pAN45).

Growth curves observed for *E. coli* DK8 (p*Tfatp*2, pAN45F_0) and *E. coli* DK8 (pAN45) grown in MLB, are shown in Fig. 4.7. As controls, growth curves for both *E. coli* DK8 (Δ*unc*) and *E. coli* K12 (*unc*⁺) are included.

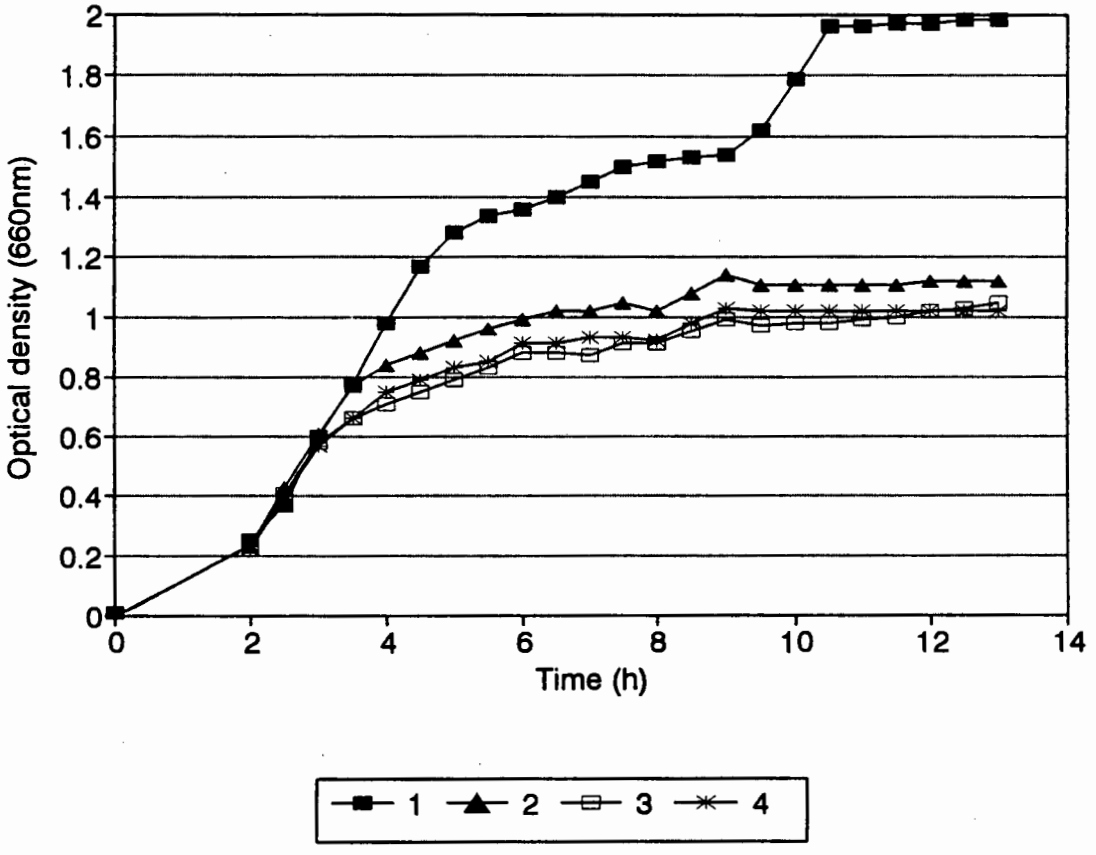


Fig. 4.7. Growth curves for various *E. coli* cultures. The vertical axis represents the OD_{600}, and the horizontal axis, time in hours. (1) *E. coli* K12 (*unc*⁺); (2) *E. coli* DK8 (pAN45); (3) *E. coli* DK8 (Δ*unc*); (4) *E. coli* DK8 (p*Tfatp*2, pANF$_0$).

Results indicated that growth rates and yields were most efficient for *E. coli* K12. Exponential growth recorded for this strain was rapid, with the OD_{600} doubling every 60 min. Late log phase commenced after five hours, once the OD_{600} reached 1.2. Lowest rates and yields were noted for *E. coli* DK8 and *E. coli* DK8 (p*Tfatp*2, pAN45F_0). The growth curve of the latter culture was almost identical to that of the Δ*unc* strain, DK8. In both cultures, although growth curves were similar to that of *E. coli* K12 for the first three hours, rapid exponential growth ceased after four hours, and cultures entered late log phase with an OD_{600} of 0.66; hence cell yield toward the end of log phase was half of that of *E. coli* K12. Maximum culture density (OD_{600} 1.0) was attained only after nine hours. Slightly better growth yields were noted for *E. coli* DK8 (pAN45). This culture entered late log phase after approximately four hours, with an OD_{600} 0.85. Maximum culture density (OD_{600} 1.1) was reached after eight hours.

4.3.4. Membrane-bound ATPase activity, measured for *E.coli* K12 (*unc*$^+$); *E. coli* DK8 (Δ*unc*); *E. coli* DK8 (p*Tfatp*2, pAN45F_0); *E. coli* DK8 (pAN45); and *T. ferrooxidans* ATCC 33020.

Results recorded for the determination of the ATPase activities of various *E. coli* membrane preparations, in the absence of inhibitory substances are given in Table 4.2.

Observations noted for both the slow growth rates of *E. coli unc* mutants transformed with *T. ferrooxidans atp* genes on MMS, and the growth curves of these transformants in MLB, indicated that growth yields obtained from *E. coli* DK8 with *E. coli/T. ferrooxidans* hybrid F_1F_0 ATPsynthases were low. Hence, it was decided to isolate cell membranes from all cultures (excluding untransformed *E. coli* DK8) which had been grown in two different media types, as described in the Methods, Section 4.2.6. This was done to determine whether the synthesis and production of F_1F_0 ATPsynthase could be stimulated by a "boost" in modified MMS. Enzyme assays were done on both sets of membranes, and results compared. Untransformed *E. coli* DK8 membranes (Δ*unc*) were prepared from cells grown only in MLB.

Highest ATPase activity was recorded from *E. coli* DK8 (pAN45) which had been "boosted" in MMS. ATPase activity for the hybrid F_1F_0 ATPsynthase in *E. coli* DK8 was low. The "boost" in MMS had no stimulatory effect on ATPase activity of these membranes. Cell membranes prepared from *E. coli* K12 showed high ATPase activity and was not stimulated by the MMS boost. In all the membranes with F_1F_0 ATPsynthase, enzyme activity levels were maximal at pH 7 and 8. Below pH 6, ATPase activity was reduced to at least half of the value measured at pH 7 and/or 8. There was no evidence from the data presented here to suggest that the F_1 from *T. ferrooxidans* enabled the ATPase activity of the enzyme to show an improved tolerance to a lower pH.

Table 4.2. Membrane-bound ATPase activities determined from membranes prepared from various *E. coli* strains, at different pH levels. Activities in two different culture media are reported, viz. cells grown in MLB[a], and cells grown in MLB and "boosted" on MMS[b].

	***E.coli* membrane type and growth medium**						
Buffer	**DK8 (Δ*unc*)**	**DK8 (p*Tfatp*2, pAN45F$_0$)**		**DK8 (pAN45)**		**K12 (*unc*$^+$)**	
(50mM)	MLB	MLB	MMS	MLB	MMS	MLB	MMS
MES pH4	—	—	0.03 ± 0.006	—	0.19 ± 0.02	0.12 ± 0.04	0.16 ± 0.01
MES pH5	0.019 ± 0.001[c]	0.03 ± 0.006	0.03 ± 0.006	0.10 ± 0.01	0.25 ± 0.01	0.22 ± 0.06	0.34 ± 0.04
Tris-Cl pH6	0.05 ± 0.005	0.07 ± 0.006	0.04 ± 0.006	0.25 ± 0.02	0.80 ± 0.01	0.45 ± 0	0.47 ± 0.02
Tris-Cl pH7	0.06 ± 0	0.13 ± 0.01	0.07 ± 0.006	0.35 ± 0.01	0.98 ± 0.04	0.53 ± 0.06	0.50 ± 0.03
Tris- Cl pH8	0.08 ± 0.006	0.14 ± 0.06	0.12 ± 0.006	0.69 ± 0.04	1.3 ± 0.06	0.79 ± 0.01	0.74 ± 0.04

[a] - MLB - "modified" Luria broth

[b] - MMS - minimal succinate medium "boost"

[c] - Numbers represent units of F_1F_0 ATPase specific activity (± SD);
(1U ATPase activity releases 1μmol P*i*/min/mg membrane protein)

Results from the study on the effects of two inhibitors of ATPase activity viz. DCCD and NaN_3, on various *E. coli* membrane preparations are presented in Table 4.3.

Table 4.3. The effects of inhibitors on membrane-bound ATPase activity assays in 50 mMTris-Cl + 5mM ATP + 5mM $MgCl_2$, pH 7.0.

E. coli **membrane type**	**% ATPase activity**		
	Additions to buffer		
	Nil	100μM DCCD	1mM NaN_3
DK8 (p*Tfatp*2, pAN45F_0)	100	62	73
DK8 (pAN45)	100	12	14
K12	100	16	20

The membranes assayed for ATPase activity in the presence of known inhibitors were prepared from cells grown in MLB medium. Maximum activity (100%) was recorded from the ATPase activity in Tris-Cl (pH 7) without either DCCD or NaN_3. The membranes prepared from *E. coli* K12 and *E. coli* DK8 (pAN45) showed typical inhibition of ATP hydrolysis in the presence of both inhibitory substances (Table 4.3). In contrast, the recombinant F_1F_0 ATPsynthase on the membranes prepared from *E. coli* DK8 (p*Tfatp*2, pAN45F_0) showed resistance to both DCCD, and to NaN_3. In the presence of 100 μM DCCD, or 1 mM NaN_3, the hybrid ATPase retained 62% and 73% hydrolytic activity respectively (Table 4.3). It should be noted that all values reported in Table 4.3 were corrected for "background" activity recorded from *E. coli* DK8 membranes.

Despite repeated attempts, membrane pellets isolated from *T. ferrooxidans* ATCC 33020, as described above, had no ATPase activity in any of the buffer systems described. It was considered possible that inhibitory substances in the *T. ferrooxidans* preparation may have caused the lack of ATPase activity noted. Hence, a 25 μl aliquot of the *T. ferrooxidans* membrane preparation, with a protein concentration of 1 mg/ml, was added to the *E. coli* DK8 (pAN45) membranes, and the enzyme activity was assayed in Tris-Cl (pH 7). No inhibitory effect on the *E. coli* ATPase activity was noted. Supernatant which had been collected after ultracentrifugation to isolate the *T. ferrooxidans* membrane pellet demonstrated no ATPase activity.

4.4. Discussion.

In vitro expression studies showed that in an *E. coli*-derived transcription/translation system, polypeptides were produced from all seven of the cloned *T. ferrooxidans atp* genes including the F_0 *atpE* and *F*. The study demonstrated that in the absence of the predicted *T. ferrooxidans atp* promoter, *T. ferrooxidans atp* genes could be expressed in the *in vitro* system, and that in the case of p*Tfatp*2, the F_1 genes were probably transcribed in *E. coli* from an internal promoter located upstream of *atpH*. *In vitro* expression helped to identify which polypeptides could be produced by *E. coli* from p*Tfatp*1 and p*Tfatp*2, as well as several other subclones. These results were useful in interpreting the *in vivo* complementation data.

The reconstitution of hybrid enzymes from different species could be useful in evaluating proposed phylogenetic relationships, as well as providing a means of obtaining information regarding essential features of enzyme function (Steffens *et al.*, 1987). In this context, it is relevant to briefly review experimental data relevant to genetically reconstituted hybrid F_1F_0 ATPsynthases, and to relate this data to results obtained in the current study, which describes cross-complementation between an acidophile and a neutrophile.

Pioneering studies on the synthesis of reconstituted F_1F_0 ATPsynthases were done by Yoshida *et al.* (1977) and Futai *et al.* (1980) cited by Futai *et al.* (1989) where functionally reconstituted hybrid F_1F_0 ATPsynthases were demonstrated in gram-negative *E. coli*, and gram-positive strain PS3. It was demonstrated that either $\gamma\alpha\beta$ or isolated γ in combination with $\alpha\beta$ complexes were functionally interchangeable between the two organisms. This was despite the fact that the γ subunit from the two species shared only 38% identical residues. Steffens *et al.* (1987) demonstrated that the F_1 and F_0 oligomers of F_1F_0 ATPsynthase of *E. coli* and strain PS3 were fully interchangeable, and could be reconstituted to form an enzyme complex which was fully functional. Antibodies to *E. coli* β and c subunits cross-reacted with strain PS3 homologues (Steffens *et al.*, 1987). Hence F_1F_0 ATPsynthase subunits from a thermophilic organism, which might be expected to show structural adaptation to high temperatures, are able to complement the mesophilic *E. coli*.

Certain *atp* genes from another gram-positive bacterium, *B. megaterium*, were shown to complement *E. coli uncD* and *uncA* mutants. In the *E. coli uncD* mutant, the *atpD* subunit from *B. megaterium* was sufficient to form a functional hybrid enzyme, whereas in order to complement an *uncA* mutant, the α,γ, β, and possibly the ε subunits from *B. megaterium* were required (Hawthorne and Brusilow, 1986). In a more recent study, Scarpetta *et al.* (1991) demonstrated that the genotype of *E. coli unc* mutants influenced the degree of

complementation by *B. megaterium* F_1F_0 ATPsynthase subunits. In both *uncA* and *uncD* mutants, complementation was dependent on the cloned genes, the vector and the bacterial strain used. With the assembly defective mutant AN818 (used in the current study), complementation was observed only when *B. megaterium atpD* and *atpC* genes are present. The *uncD* mutation of AN818 was not complemented when *B. megaterium* β was produced in the presence of *B. megaterium* γ. The hybrid enzyme produced in AN818 was functionally impaired (see below).

Amongst gram-negative bacteria, experimental data were presented for the reconstitution of hybrid F_1F_0 ATPsynthases. Hsu *et al.* (1984) cited by Futai *et al.* (1989) demonstrated that in the closely related *E. coli* and *Salmonella typhimurium*, the F_1F_0 ATPsynthase α, γ and β subunits were mutually interchangeable, and formed active hybrid ATPases. Bragg and Hou (1975) cited by Kauffer *et al.* (1987) demonstrated that *S. typhimurium* F_1 could rebind to F_1-depleted membranes in *E. coli*, and reconstituted ATP-dependent proton-translocation. The F_0 subunits from *K. pneumoniae* formed a functional F_1F_0 ATPsynthase with *E. coli* F_1 subunits on everted *K. pneumoniae* vesicles stripped of wild-type F_1 (Kauffer *et al.*, 1987). Krumholz *et al.* (1990) were able to complement an *E. coli* Δ*unc* strain with the *atp* operon from *V. alginolyticus*. Shibata *et al.* (1992) reported complementation of *E. coli unc* mutants with *E. hirae atpE, F, H* and *A* genes. No complementation was reported for the *E. hirae atpB, G, D* and *C* genes. The β subunit from *E. coli* F_1F_0 ATPsynthase reconstituted a functional ATPase from β-less chromatophores of *R. rubrum* (Gromet-Elhanan *et al.*, 1985 cited by Futai *et al.*, 1989). The F_0 genes from *P. modestum* were shown to functionally complement an *E. coli* F_0 deletion *atpI-atpA* mutant. This *in vivo* complementation was based on integration of the corresponding *P. modestum* genes into the genome of the *E. coli* mutant. The reconstituted enzyme was functional, and was able to couple the flow of sodium ions and/or protons to ATP synthesis (Kaim *et al.*, 1992; G.W. Kaim, personal communication). The F_1 δ, γ, β and ε subunits from *P. modestum* reconstituted into a functional *E. coli/P. modestum* hybrid ATPase. The α subunit in that enzyme appeared to be comprised of both *E. coli* and *P. modestum* residues, as neither the *E. coli unc* mutant nor the *P. modestum atp* plasmid used had a complete *unc/atpA* gene (Kluge and Dimroth, 1992).

Hence, it appears that amongst the prokaryotes, hybrid ATPases may be formed from a diverse array of organisms, and that many of these hybrid enzymes are functional. This would tend to support the hypothesis that identical subunits with different amino acid sequences, which may have conserved amino acids at specific positions important for enzyme function, may be able to form complexes with structures similar enough to allow specific interaction between heterologous oligomers (Steffens *et al.*, 1987).

It is interesting that the formation of functional F_1F_0 ATPsynthase complexes between a number of prokaryotes and chloroplasts of eukaryotes has also been reported (reviewed in Engelbrecht and Junge, 1992; Bar Zvi *et al.*, 1985, cited by Steffens *et al.*, 1987; Richter *et al.*, 1986, cited by Futai *et al.*, 1989). Little is known regarding the interchangeablity of mitochondrial F_1F_0 ATPsynthase subunits with those from other species. It is known that the OSCP (δ) subunit from mitochondria cannot substitute for chloroplast δ (Engelbrecht and Junge, 1990).

Results obtained during the current study from various *E. coli unc* mutants transformed with p*Tfatp*1 and p*Tfatp*2 indicated that the degree of complementation on MMS was sufficient to suggest that several F_1F_0 ATPsynthase components were interchangeable between an acidophile and a neutrophile (Table 4.1). The growth of *E. coli* DK8 (p*Tfatp*2, pAN45F_0) on solid MMS medium demonstrated that a hybrid *T. ferrooxidans* F_1/*E. coli* F_0 ATPsynthase was functionally reconstituted independent of possible α-complementation. Similarly, results with *E. coli* AN F_1 (p*Tfatp*2) transformants indicated the presence of a hybrid enzyme, although the degree of heterogeneity within the holoenzyme was not clear. Growth of *E. coli* AN818 (p*Tfatp*1) and *E. coli* AN802 (p*Tfatp*1) on MMS showed the presence of an F_1 oligomer in which the *T. ferrooxidans* β and ε subunits formed a functional F_1F_0 ATPsynthase with subunits from *E. coli*. This latter result was similar to that recorded by Scarpetta *et al.* (1991) where a *B. megaterium atpDC* combination complemented *E. coli* AN818.

Complementation studies with various plasmid derivatives of p*Tfatp*2 were interesting. *E. coli* AN730 (α$^-$) and AN1273 (γ), transformed with p*Tfatp*200, did not grow on MMS (Table 4.1). In p*Tfatp*200, a portion of the *atpH* and the entire *atpA* and *atpG* genes were cloned behind a *lacZ* promoter. No polypeptides corresponding in size to *T. ferrooxidans* α and γ were detected *in vitro* in this construct (Fig. 4.5, Lane 3). In *E. coli*, there is translational coupling between both the *uncH/uncA* and the *uncA/uncG* gene pairs (Hellmuth *et al.*, 1991) (Chapter 3). Since the start of *atpH* was missing from p*Tfatp*200, this result may indicate that the cloned *T. ferrooxidans* genes were also coupled when expressed in an *E. coli*-derived system.

Although the *T. ferrooxidans* βε combination on p*Tfatp*1 was able to complement *E. coli* AN802, the ε subunit on its own, was not. This was inferred from the fact that although p*Tfatp*400 (*atpC*) expressed the 15 kDa *T. ferrooxidans* ε subunit *in vitro* (Fig. 4.5, Lane 2) the plasmid construct did not functionally complement *E. coli* AN802 on MMS (Table 4.1). However, it is possible that the lack of complementation was due to over-expression of subunit ε by the high copy number plasmid vector, and not to the inability to form a hybrid F_1F_0 ATPsynthase.

Although instances (cited above) were reported for the functional complementation of *E. coli unc* mutants by the F_0 oligomer from other bacterial species, no *T. ferrooxidans uncBEF*-complementing plasmids or cosmids were isolated during the current study (Chapters 2 and 5). The *T. ferrooxidans atpEF* genes were fortuitously cloned in reverse orientation on F_1-complementing p*Tfatp*2. *In vitro* expression assays showed that whilst *T. ferrooxidans atpE* and *F* were not expressed by p*Tfatp*2, when cloned behind the *lacZ* promoter of pBluescriptSK+, *T. ferrooxidans* b and c subunits were synthesised by p*Tfatp*3001 (Fig. 4.3, Lane 1). However, neither *E. coli* AN943 (p*Tfatp*3001) nor *E. coli* AN1443 (p*Tfatp*3001) grew on MMS (Table 4.1). These results could indicate that when overexpressed by a high copy number plasmid, *T. ferrooxidans* c and b subunits cause general growth inhibtion in *E. coli*. This was reported for other over-expressed *E. coli* hydrophobic proteins including F_1F_0 ATPsynthase subunit a (reviewed in Brusilow, 1993). However, taken together with the consistent failure to complement any *E. coli* F_0 mutant when screened with *T. ferrooxidans* genomic libraries prepared in low-copy number vectors (Chapters 2 and 5), the failure of p*Tfatp*3001 to complement *E. coli* AN943 and AN1440 could also infer that even if expressed in *E. coli*, *T. ferrooxidans* c and b subunits would not form a functional hybrid F_0 pore. The reasons for this are unknown, but could relate to the inability of *T. ferrooxidans* F_0 subunits to form a functional F_0/F_1 link, and/or to the differences between F_0 proton translocation in an acidophilic and neutrophilic organism. This latter possibility is further discussed in Chapter 5.

The complementation of *E. coli* F_1 mutants by *T. ferrooxidans* F_1 subunits could indicate that control of proton translocation/gating in *T. ferrooxidans* F_1F_0 ATPsynthase was not due to any inherent features of the *T. ferrooxidans* F_1 subunits. However, the ability to grow on MMS does not accurately reflect the degree of functionality of hybrid F_1F_0 ATPsynthases. It was noted that *E. coli unc* strains with hybrid enzymes grew more slowly on MMS than did *E. coli* K12 (*unc*+) or *E. coli* DK8 (pAN45). Therefore further experiments were done in an attempt to determine whether the hybrid F_1F_0 ATPsynthases were functionally impaired.

Scarpetta *et al.* (1991) showed that *E. coli* AN818 transformed with *B. megaterium* β or βε subunits became an obligate aerobe, possibly due to ATPase activity of the hybrid being insufficient to maintain a viable $\Delta\mu_{H+}$ under anaerobic conditions, related to energy coupling by the hybrid complex. To establish whether a *T. ferrooxidans*/*E. coli* F_1F_0 ATPsynthase was similarly impaired, *E. coli* AN818 (p*Tfatp*1), *E. coli* AN818 (p*Tfatp*2), *E. coli* AN730 (p*Tfatp*2) and *E. coli* DK8 (p*Tfatp*2, pAN45F_0) were tested for anaerobic growth on the rich glucose medium described by Scarpetta *et al.* (1991). In all instances, transformants grew anaerobically. Therefore, these hybrid F_1F_0 ATPsynthases supported coupled ATP hydrolysis under anoxic conditions.

Growth curves (Fig. 4.7) comparing the growth rates of *E. coli* K12, *E. coli* DK8, *E. coli* DK8 (pAN45) and *E. coli* DK8 (p*Tfatp2*, pAN45F_0) indicated that in the medium used, the culture containing the *T. ferrooxidans/E. coli* recombinant F_1F_0 ATPsynthase developed at the same rate as untransformed *E. coli* DK8 (Δ*unc*). Growth rates of the wild-type and of *E. coli* DK8 (pAN45) were more efficient. These observations suggested that impaired oxidative phosphorylation rates of hybrid *T. ferrooxidans/E. coli* F_1F_0 ATPsynthases were possibly responsible for the lower growth rates and yields noted.

Enzyme assays of ATPase activity confirmed that the activity of the recombinant enzyme was low, reaching a maximum of 0.14 U at a pH of 8, and was not stimulated by a three hour "boost" on MMS (Table 4.2). The activity of the recombinant enzyme was therefore only 10% and 17.5% of that of the maximum rate reached by *E. coli* DK8 (pAN45) and *E. coli* K12 membranes respectively. Results pertaining to the influence of pH on ATPase activity demonstrated that the *T. ferrooxidans* F_1 oligomer of the recombinant enzyme was not more capable of ATP hydrolysis at low pH values. Even though the cytoplasmic pH of *T. ferrooxidans* is neutral, there was a possibility that the ATPase of *T. ferrooxidans* was more acid-tolerant. However, in their study on comparative acid tolerances of oral lactic acid bacteria, Sturr and Marquis (1992) reported that association of F_1 with F_0 and possibly other membrane components, enhanced the tolerance of the F_1F_0 ATPsynthases to both acid and alkaline media. In the absence of F_0, pH activity profiles for F_1 enzymes were distinctly narrower.

ATPase activities recorded in this study for *E. coli* K12 were in agreement with those recorded for *E. coli* wild-type membranes reported elsewhere by Scarpetta *et al.* (1991) and Krumholz *et al.* (1990). Values presented for the Δ*unc* strain used by Krumholz *et al.* (1990) were similar to those recorded in this study for *E. coli* DK8. It was of interest to note that the ATPase activity of the *T. ferrooxidans/E. coli* recombinant enzyme was in the range of that reported for the *E. coli* AN818/*B. megaterium* F_1F_0 ATPsynthase hybrid (Scarpetta *et al.*, 1991). These authors recorded values between 0.04 and 0.11 U for the hybrid enzyme (5-14% of the wild type). Krumholz *et al.* (1990) reported that when expressed in an *E. coli* Δ*unc* strain, the F_1F_0 ATPsynthase of *V. alginolyticus* was approximately 14% of that of the wild-type *E. coli* membranes.

The reason/s for the low ATPase activity of the *T. ferrooxidans/E. coli* hybrid enzyme could be due to difficulties associated with the production of an unstable complex, and/or to difficulties associated with actual catalytic mechanism itself.

Studies on the effects of known F_1F_0 ATPsynthase inhibitors on the *T. ferrooxidans/E. coli* F_1F_0 ATPsynthase hybrid suggested that catalytic mechanism was impaired. The hybrid enzyme showed a marked resistance to both NaN_3 and to DCCD (Table 4.3). Azide is a

general potent inhibitor of membrane-bound and soluble F_1F_0 ATPsynthase. It was reported to inhibit multi-site but not uni-site ATP hydrolysis by F_1, and was therefore thought to block catalytic co-operativity (Futai *et al.*, 1989). Active isolated *E. coli* αβ core complexes showed resistance to azide; hence they lacked the functional heterogeneity observed in F_1-ATPases. Therefore, as co-operativity is probably a function of the single-copy F_1 subunits (δ, ε and γ), azide possibly exerts its effect at this level (reviewed in Gromet-Elhanan, 1992). The result obtained with NaN_3 for the *T. ferrooxidans/E. coli* hybrid F_1F_0 ATPsynthase indicated that multi-site activity of the recombinant enzyme was notably reduced. Co-operativity, possibly from F_0-to-F_1, within F_1 itself, and from F_1-to-F_0 was impaired.

DCCD combines with both the c and β subunits of *E. coli* F_1F_0 ATPsynthase (refer Figs. 3.7, and 3.23) where it blocks proton-conduction, and thus inhibits energy coupling (reviewed in Fillingame, 1990). In the hybrid enzyme in *E. coli* DK8, the two DCCD-binding residues occurred as *E. coli* F_0 cAsp-61, and *T. ferrooxidans* F_1 βGlu-198 respectively. The fact that the *T. ferrooxidans/E. coli* recombinant F_1F_0 ATPsynthase retained 62% ATPase activity in the presence of 100 μM DCCD, indicated that possibly the enzyme was poorly coupled, when compared to wild-type levels (Table 4.3).

The combined results with F_1F_0 ATPsynthase inhibitors could explain the lower ATPase activities recorded for the hybrid, when compared to wild-type levels (Table 4.2). The inefficiency of the enzyme would also explain the trends observed in the growth curves (Fig. 4.7). This study has shown that whilst the F_1 oligomer from *T. ferrooxidans* F_1F_0 ATPsynthase formed a functional enzyme complex with the F_0 moiety from *E. coli* F_1F_0 ATPsynthase, the enzyme function *in vivo* was severely impaired. This observation was not unique to a hybrid F_1F_0 ATPsynthase formed from an acidophile and neutrophile. Similar observations were recorded for the *E. coli* AN818/*B. megaterium* F_1F_0 ATPsynthase, where partial and complete resistance to DCCD and azide respectively were reported (Scarpetta *et al.*, 1991). Factors directly responsible for the functional impairment of the *T. ferrooxidans/E. coli* F_1F_0 ATPsynthase are unknown. They could relate to phylogenetic and/or metabolic differences between *T. ferrooxidans* and *E. coli*. These differences could influence, for example, the mode of F_1/F_0 linking, and/or difficulties associated with co-operativity in the hybrid, due to the unusual primary structure noted for the termini of the *T. ferrooxidans* F_1 γ, ε, δ and β subunits (Chapter 3). As the latter features could possibly be related to the gating of protons, a role for certain *T. ferrooxidans* F_1 subunits in controlling proton flow cannot be entirely discounted.

The *T. ferrooxidans* membrane preparations, even with a protein concentration at 1 mg/ml, showed no ATPase activity. OD_{660} readings of the assayed membranes were no different from the control blanks. No definite reason can be given to explain this result. It is

possible that the mechanism of membrane preparation destroyed the F_1F_0 ATPsynthase during extractive procedures, such as the use of a French press. Other authors have reported successful preparation of membrane-associated proteins associated with electron-transport in *T. ferrooxidans* by rupturing cells by passing them through a French press, and using a combination of both a French press and sonication (Ingledew, 1982; Kai *et al.*, 1989). If *T. ferrooxidans* F_1F_0 ATPsynthase was destroyed during cell lysis, then the protein content of the *T. ferrooxidans* membranes measured for this study could be largely due to the presence of components of the electron transport chain which occur in unusual abundance in *T. ferrooxidans* membranes (Ingledew, 1982). The buffer selected for the preparation of *T. ferrooxidans* membranes was that prescribed by Apel *et al.* (1980) for the preparation of *T. ferrooxidans* membrane vesicles. These authors recommended the use of Tris-sulphate, probably because of the toxic effect of chloride ions exerted on *T. ferrooxidans* by disruption of $\Delta\mu_{H+}$, reviewed in Chapter 1. Therefore, inactivation of *T. ferrooxidans* cells/membranes which could have occurred with the use of Tris-chloride as the buffering medium, was avoided during this study. The lack of any enzyme activity on the *T. ferrooxidans* membranes was unfortunate, as it was hoped to determine whether enzyme ATPase activity was stimulated by the presence of sulphate, as was reported for *V. parahaemolyticus*, and strain PS3 (Sakai *et al.*, 1990; Sakai-Tomita *et al.*, 1992; Takeda *et al.*, 1982, cited by Sakai *et al.*, 1990). Alternative methods will have to be pursued to induce *T. ferrooxidans* ATCC 33020 cell lysis and subsequent membrane preparation for ATPase assay by less harsh means. Preliminary attempts to lyse *T. ferrooxidans* ATCC 33020 cells incorporating the harvest, wash and sonication methods successfully used by Shiratori *et al.* (1989) to lyse various *T. ferrooxidans* strains isolated from Japanese domestic mining sites, were unsuccessful. The production of *T. ferrooxidans* ATCC 33020 sphaeroplasts accompanied by cell lysis induced by short bursts of sonication or osmotic shock may be a suitable alternative.

CHAPTER 5.

ATTEMPTS TO ISOLATE THE EXTREME 5'-END OF THE *T. FERROOXIDANS ATP* OPERON, TO DETERMINE THE SIZE OF THE *ATP* OPERON TRANSCRIPT AND TO LOCATE AN INTERNAL PROMOTER UPSTREAM OF THE *atpH* GENE, RECOGNISED BY *E. COLI* FOR THE TRANSCRIPTION OF *T. FERROOXIDANS* F_1 GENES *IN VIVO*.

CHAPTER 5.

ATTEMPTS TO ISOLATE THE EXTREME 5'-END OF THE *T. FERROOXIDANS ATP* OPERON, TO DETERMINE THE SIZE OF THE *ATP* OPERON TRANSCRIPT, AND TO LOCATE AN INTERNAL PROMOTER UPSTREAM OF THE *ATPH* GENE RECOGNISED BY *E. COLI* FOR THE TRANSCRIPTION OF *T. FERROOXIDANS* F_1 GENES *IN VIVO*.

5.0. Summary.

Southern hybridisation of *T. ferrooxidans* ATCC 33020 chromosomal digests against p*Tfatp*2 identified 8 kb *Cla*I, 6 kb *Eco*RV and 9 kb *Bgl*II genomic fragments thought to lie upstream of and adjacent to the 5'-end of the *atpE* gene. In an attempt to isolate the *T. ferrooxidans atpPIB* fragment, *E. coli* AN 730 (α^-) was screened with a representative *T. ferrooxidans* ATCC 33020 chromosomal cosmid library, and 50 cosmids were isolated which complemented the mutant on MMS+Ap. Restriction enzyme digests and Southern hybridisation analyses demonstrated that none of the cosmids selected for further study extended upstream of the *T. ferrooxidans atpE* gene.

Southern hybridisation of *T. ferrooxidans Eco*RV, *Xho*I and *Eco*RV/*Xho*I chromosomal digests, using a 500 bp fragment from the the extreme 5'-end of the *T. ferrooxidans* genomic insert on p*Tfatp*2 as a probe, identified 5.2 kb *Xho*I, 6 kb *Eco*RV and 2 kb *Eco*RV/*Xho*I fragments which extended upstream beyond the 5'-end of the *atpE* gene. Attempts to isolate the 2 kb *Eco*RV/*Xho*I fragment thought to contain the *atpBEF* genes, either by cloning the fragment in a reverse orientation with respect to the pBluescriptKS$^+$ *lacZ* promoter, or by PCR, were unsuccessful. Further extensive ligation-mediated PCR experiments which were used as attempts to "walk" along the *T. ferrooxidans* chromosome into the region to the 5'-end of the *atp* genes present on p*Tfatp*2, did not amplify *T. ferrooxidans atpPIB*.

RNA was extracted from *E. coli* K12 (*unc*$^+$), *E. coli* DK8 (Δ*unc*), *E. coli* DK8 (pAN45), *E. coli* DK8 (p*Tfatp*2) and *E. coli* DK8 (p*Tfatp*2, pAN45F_0). The RNA was prepared using a hot-phenol extraction method and was used in an attempt to gauge *T. ferrooxidans* F_1 transcript sizes in *E. coli* DK8 by Northern hybridisation. By using primer extension, it was hoped to locate an internal promoter in the *T. ferrooxidans atp* insert on p*Tfatp*2, recognised by *E. coli* for the transcription of the *T. ferrooxidans* F_1 genes. Although RNA was extracted, no conclusive results were obtained either from Northern hybridisation or primer extension. RNA was extracted in large quantities from actively growing cultures of *T. ferrooxidans* ATCC 33020. This was used to attempt to ascertain the *atp* operon transcript size, by using a ^{32}P-labelled 4.35 kb *Pst*I fragment of p*Tfatp*2 as a probe. Although the RNA extracted from *T. ferrooxidans* appeared to be in a satisfactory condition as regards rRNA appearance, no discrete *atp* transcript was identified.

"I do not know what I may appear to the world, but to myself, I seem to have been only a boy playing on the sea-shore, and diverting myself in now and then finding a smoother pebble or a prettier shell than ordinary, whilst the great ocean of truth lay undiscovered before me." Isaac Newton. From: The Concise Oxford Dictionary of Quotations; World Books, London (1964).

5.1. Introduction.

During the course of the current study, a number of attempts were made to isolate the entire *T. ferrooxidans atp* operon. The objective of these experiments was to identify and isolate a common *T. ferrooxidans* ATCC 33020 genomic fragment located immediately upstream of the 5'-end of the *atpE* gene.

Extensive screening of *E. coli* AN727 (a$^-$), AN943 (c$^-$), AN1440 (b$^-$), or *E. coli* DK8 (Δ*unc*) strains with *T. ferrooxidans* ATCC 33020 gene banks had not resulted in the isolation of *T. ferrooxidans* F_0 *atpBEF* genes. However, screening *E. coli* F_1 mutants resulted in the fortuitous cloning of *T. ferrooxidans atpEF* on p*Tfatp*2, where the *atpEFHAGDC* genes were cloned in reverse orientation with respect to the p*Eco*R251 λ promoter (Chapter 2). The plasmid gene bank used consisted of *T. ferrooxidans* chromosomal inserts of between 4-10 kb cloned into p*Eco*R251 (Ramesar, 1988). It was hoped that by screening the *E. coli* F_1 mutant AN730 (α$^-$) with larger 36-45 kb *T. ferrooxidans* genomic fragments present in a cosmid gene bank (Ramesar, 1988), the likelihood of isolating the entire *atp* operon would be increased. Prior to screening *E. coli* AN730 with the cosmid bank, *T. ferrooxidans* genomic restriction fragments thought to include the *atp* operon promoter (*P*) and the *atpIBEFHA* genes, were identified. Any cosmid which complemented *E. coli* AN730 on MMS was to be screened for these fragments which might indicate the presence of the *atpPIBEFHA* fragment. These studies were conducted before sequencing the *T. ferrooxidans atp* genomic insert on p*Tfatp*2.

After sequencing *T. ferrooxidans atpEFHAGDC*, further experimentation was devised in an attempt to isolate the 5'-end of the *atp* operon. Initially, using plasmids generated from the ExoIII shortening of the *T. ferrooxidans atp* gene cluster and by exploiting the availability of the *Xho*I site present in the *T. ferrooxidans atpH* cistron (Fig. 2.1), a discrete *T. ferrooxidans* 2.0 kb chromosomal fragment, thought to contain the *T. ferrooxidans atpBEFH* gene cluster, was identified. To isolate this fragment, two approaches were used. The first was to clone the *T. ferrooxidans* genomic fragment in an opposite orientation to the IPTG (isopropylthio-β-D-galactoside)-inducible *lacZ* promoter of a plasmid cloning vector, such that transcription of the *T. ferrooxidans* genes would be less likely in the *E. coli* host system. The second approach was to avoid the use of *E. coli* as a heterologous host system by using PCR. Over the past few years, PCR has become a choice method for amplifying a DNA

fragment. The requirement for PCR is that the nucleotide sequence of the DNA at the extreme 5' and 3' termini of the fragment to be amplified is known, in order to design oligonucleotide primers which bracket the DNA fragment. Where the nucleotide sequence at either one of the two extremities of the DNA fragment is unknown, amplification of such a fragment is problematic. However, there are techniques available to circumvent this and for the purposes of the current study, strategies were used which were designed to amplify uncloned double-stranded DNA, where only the sequence of one end of the fragment to be amplified was known. Two methods were attempted, based on ligation-mediated PCR, which, if successful, would enable "walking" along the *T. ferrooxidans* chromosome to the 5'-end of *atpEFHAGDC* fragment cloned on p*Tfatp*2.

The expression of the *T. ferrooxidans* F_1 *atp* genes on p*Tfatp*2 by *E. coli unc*$^-$ mutants indicated that as the five genes were cloned in opposite orientation to the p*Eco*R251 λ promoter, there was an internal promoter on the p*Tfatp*2 *atp* genes located upstream of *atpH*, which was recognised by *E. coli* (Chapters 2 and 4). The site of a putative σ^{70} promoter was identified in the 3'-end of *atpF* on p*Tfatp*2, by a comparison of known *E. coli* σ^{70} consensus sequences (Harley and Reynolds, 1987) with the *T. ferrooxidans atp* nucleotide sequence (Fig. 3.2). It was hoped to identify the precise location of the internal promoter site located upstream of *T. ferrooxidans atpH* using primer extension, by isolating mRNA from *E. coli* DK8 (p*Tfatp*2, pAN45F_0) or *E. coli* DK8 (p*Tfatp*2) cells. To do this, it was necessary to establish the size of the *T. ferrooxidans atp* F_1 transcript when synthesised in *E. coli* DK8. Transcript size would assist in deciding on the location and design of an oligonucleotide primer suitable for primer extension.

Whilst it is relatively easy to harvest mRNA from the experimentally amenable *E. coli*, the harvesting of representative mRNA species from actively growing cultures of *T. ferrooxidans* is fraught with problems. This was noted, for example, by Drolet and Lau (1992). To obtain high quality yields of mRNA from prokaryotes requires the rapid killing of exponentially grown cells to prevent degradation of mRNA *in vivo*. To harvest *T. ferrooxidans* during exponential phase of growth and to prepare the cells for lysis such that mRNA is not degraded during subsequent extractive procedures, is a lengthy process (Chapter 1 and Chapter 4, Section 4.2.7). Hence obtaining high quality mRNA species from *T. ferrooxidans* cultures is not easy. Inoue *et al.* (1991) and Kusano *et al.* (1991b, 1992b, and 1993b) reported the successful isolation of mRNA from *T. ferrooxidans* strains E-15 and Fe1. The mRNA was successfully used for both Northern Blotting and primer extension experiments. As it had not been possible to clone the entire *T. ferrooxidans atp* operon during the current study, it was hoped to establish the size of the *T. ferrooxidans atp* operon transcript. This would give a general idea of the size of the DNA fragment needed to carry the extreme 5'-end of the operon.

5.2. Materials and Methods.

5.2.1. Bacterial strains and media.

Bacterial strains, and their relevant genotypes are listed in Table 1, Appendix A. *E. coli* AN730 (*uncA*-) was used for screening a *T. ferrooxidans* cosmid genomic library. *E. coli* JM109 was the host system used for the isolation of a *T. ferrooxidans atpBEFH* genomic fragment, cloned in reverse orientation with respect to the pBluescriptKS+ *lacZ* promoter. *E. coli* GM41 (*dam*-) was used to prepare cosmid DNA for *Cla*I restriction analyses. *T. ferrooxidans* ATCC 33020 was used as the source of chromosomal DNA, prepared as described in Chapter 2, Section 2.2.3. *E. coli* strains were routinely maintained on LBA (Appendix B) and when necessary, LBA was supplemented with Ap at 100 µg/ml. Minimal succinate medium, supplemented with Ap at 50 µg/ml, was made as described by Gibson *et al.* (1977) (Appendix B).

5.2.2. General techniques.

See Chapter 2, Section 2.2.2.

5.2.3. Construction of the *T. ferrooxidans* genomic library.

The cosmid bank of *T. ferrooxidans* chromosomal fragments used was that described in Chapter 2, Section 2.2.3.

5.2.4. Identification of *T. ferrooxidans atpIBEFHA* genomic fragments.

To locate a *T. ferrooxidans* ATCC 33020 chromosomal *atpIBEFHA* fragment, 5 X 10 µg genomic DNA were cut with single digests of *Pst*I, *Bgl*II, *Eco*RI, *Cla*I, and *Eco*RV. Fifty ng of p*Tfatp*2 was digested with *Pst*I. These digests were electrophoresed together with λDNA (*Pst*I) as a molecular weight marker on an 0.8% agarose gel. This was transferred to an Amersham Hybond N+ membrane following manufacturer's recommendations. p*Tfatp*2 and λ DNA (Boehringer Mannheim) were ^{32}P-labelled by nick-translation (Sambrook *et al.*, 1989). The section of the filter containing the λDNA (*Pst*I) was sliced off and hybridisation was carried out separately from the remainder of the filter. Hybridisation was done according to the recommendations of Amersham for their Hybond N+ membranes.

5.2.5. Isolation of *T. ferrooxidans atp* genes in *E. coli* AN730.

Vegetatively growing *E. coli* AN730 cells were transduced with the cosmid bank (Sambrook *et al.*, 1989). Methods used were as described in Chapter 2, Section 2.2.4.

5.2.6. An analysis of *E. coli* AN730-complementing cosmids.

Thirty cosmid isolates which complemented *E. coli* AN730 on MMS+Ap were digested with *Eco*RV to establish whether a fragment of approximately 6-7 kb was present. Any cosmids which had such a fragment were further digested with both *Xho*I and *Eco*RV, to determine whether the large *Eco*RV fragment had an internal *Xho*I site, which could

represent the *Xho*I site mapped in the *T. ferrooxidans atpH* gene (Figs. 2.1 and 5.2). A cosmid isolate 689.25 which had such a fragment, was then digested with a number of enzymes (see Results section below) in an attempt to ascertain whether an internal 6 kb *Eco*RV fragment located on the cosmid extended beyond the 5'-end of the *T. ferrooxidans atpE* gene. In addition, 4 X 10 µg samples of cosmid 689.25 DNA were digested with *Eco*RV, *Cla*I, *Eco*RV/*Xho*I, and *Cla*I/*Xho*I. Two 10 µg aliquots of cosmid 689.25 which had been prepared in *E. coli* GM41 were cut with *Cla*I and *Xho*I/*Cla*I. Southern hybridisation of these six digests was carried out using the *Xho*I-*Bam*HI fragment of p*Tfatp*3001 as a probe (Fig. 4.1 and Table 1, Appendix A). This fragment was gel-purified three times to remove all traces of the vector Ap resistance gene, which was common to the cosmid isolate. λDNA (*Pst*I) was used as a molecular weight marker. Labelling of the probes, hybridisation and blotting was done as outlined in 5.2.4.

5.2.7. Assignment of a discrete *T. ferrooxidans* genomic fragment adjacent to the 5'-end of the *Xho*I site in the *atpH* gene.

T. ferrooxidans chromosomal DNA (3 X 20 µg) was digested with *Xho*I, *Eco*RV and an *Eco*RV/*Xho*I combination. Southern hybridisation of these digests was was carried out using p*Tfatp*3150 as a probe, which consisted of 500 bp from the extreme 5'-end of the *T. ferrooxidans* chromosomal insert present on p*Tfatp*2, upstream of the *Xho*I site, cloned into pUC18. p*Tfatp*3150 contained all of *atpE* and a small fragment from the 5'-end of *atpF*, and was generated from p*Tfatp*300 (Fig. 3.1) during the ExoIII shortening procedure required for sequencing of p*Tfatp*2. Preparation for and completion of Southern hybridisation using λDNA (*Pst*I) as a molecular weight marker was as described in 5.2.4.

5.2.8. Attempts to isolate and amplify a 2.0 kb *T. ferrooxidans Xho*I/*Eco*RV fragment, located adjacent to the 5'-end of the *Xho*I site in *atpH*.

5.2.8.1. Cloning of a 2.0 kb *Xho*I/*Eco*RV fragment into an expression vector in reverse orientation. *T. ferrooxidans* ATCC 33020 chromosomal DNA (20 µg) was digested with *Xho*I/*Eco*RV. The resultant digest was electrophoresed on a 0.9% agarose gel (Sambrook *et al.*, 1989). A library of fragments between 1.7-2.5 kb was prepared by excising these fragments from the gel, and eluting the DNA from the gel-slices. The fragments were ligated with T4 ligase into pBluescriptKS+, cut with *Xho*I and *Eco*RV. After ligation, representative aliquots of the ligation mix were either used to transform competent *E. coli* JM109 cells or were removed for PCR procedures (described below). Transformants were plated out onto X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) + 40 µM IPTG + Ap (100 µg/ml) agar plates (Sambrook *et al.*, 1989). The small-scale alkaline lysis method (Ausubel *et al.*, 1993) was used to prepare plasmid DNA from all white transformed colonies and a representative 4 µl volume from each preparation was digested with *Xho*I/*Sst*I. This digest was electrophoresed on 0.9% agarose gels (Sambrook *et al.*, 1989). In addition, an aliquot of 0.5 µl was removed from each plasmid preparation, which had been phenol-cleaned and made to 30 µl volume with sterile de-ionised water. These

aliquots were pooled in lots of 20, and each 10 µl volume was used for PCR analysis, described below.

5.2.8.2. PCR attempts to amplify *T. ferrooxidans* ATCC 33020 genomic DNA adjacent to the 5'-end of the *Xho*I site in *atpH*. Two ligation-mediated PCR methods were utilised to locate the 5'-end of the *T. ferrooxidans atp* operon.

The first method was the single-specific-primer polymerase chain reaction (SSP-PCR) devised by Shyamala and Ames (1989). A library of 1.7-2.5 kb *T. ferrooxidans Xho*I/*Eco*RV chromosomal fragments, separated on an agarose gel, was made in compatibly digested pBluescriptKS$^+$ as described above. Further complete double digests of 5 µg of *T. ferrooxidans* chromosomal DNA were done, using *Xho*I and a randomly selected second restriction enzyme. These were *Xho*I/*Cla*I, *Xho*I/*Hin*dIII, *Xho*I/*Eco*RI, and *Xho*I/*Bam*HI. The digests were then ligated into pBluescriptKS$^+$, cut with compatible enzymes. Ligations were performed in a total reaction volume of 20 µl, using T4 ligase (Sambrook *et al.*, 1987).

All oligonucleotide primers required for PCR studies were synthesised in the Dept. of Biochemistry, University of Cape Town, using an Autogen 6500 DNA synthesiser and were purified by a reverse phase phenyl cartridge purification process.

To "walk" along the *T. ferrooxidans* chromosome located to the 5'-end of *atpE*, the entire ligated mix was amplified by a PCR reaction as follows. Two gene-specific primers, P1 and P2, were synthesised such that they were complementary to nucleotides 168-187 and 459-475 of p*Tfatp*2 (Fig. 3.2) respectively. The nucleotide sequence of P1 was,

5'-CGCCGGATCCGGCTGACGGGTGATACC-3', and P2,

5'-CGCCGGATCCATTTTGGCGCGGCGGTC-3'.

The underlined sequence at the 5'-end of the primers represented a *Bam*HI site included to use as a means of cloning any amplified *T. ferrooxidans* chromosomal fragment which might result after SSP-PCR. Using the 20 complementary nucleotides of P1, the estimated temperature of annealing (Tm) to p*Tfatp*2, was calculated to be 60^{0}C. The formula used to calculate Tm:-

$$81.5 + 16.6(\log 0.053) + 0.41(\%GC) - (500/n),$$

where n = number of complementary nucleotides of the primer, and 0.053 is the approximate molarity of salt (excluding Mg^{2+}) in the amplification buffer used (Ausubel *et al.*, 1993). Similarly, the Tm of P2, considering the 17 complementary nucleotides, was calculated to be 56.8^{0}C. As others (eg. see Sambrook *et al.*, 1989) recommended different methods to estimate Tm, the value was used as a guide for PCR annealing temperatures.

Two further primers ("non-specific") were synthesised which were homologous to the 5'->3' T7 and T3 promoter regions of pBluescript vectors. These were T7,

5'-TACGACTCACTATAGGGCGAATTGGG-3' and T3,

5'-GCTCGGAATTAACCCTCACTAAAGGG-3'.

The Tm of these primers was calculated to be approximately 61°C.

The chilled reaction mix for SSP-PCR was constituted as follows:-
20 µl ligation mix, 1 µl 10 pmol/µl non-specific primer, 1 µl 10 pmol/µl gene-specific primer, 0.8 µl 25 mM dNTP, 20 µl 5X amplification buffer, 54.2 µl water. The mix was kept on ice, and immediately prior to starting PCR cycling, 3 µl of chilled Vent mix was added.

The 5X amplification buffer was that recommended for use with $Vent_R^{Tm}$ polmerase (Kit 254S, New England Biolabs, Beverly, MA 01915/USA) by Ausubel *et al.* (1993) and was constituted as follows:-
200 mM NaCl, 100 mM Tris-Cl (pH 8.9) 25 mM $MgSO_4$, 0.05% gelatin and 0.5% Triton X-100, and was stored at -20°C.

Vent mix (Ausubel *et al.*, 1993) was made up as:-
0.6 µl 5X Amplification buffer, 1.9 µl water (well-chilled) and 0.5 µl (1.0 U) $Vent_R^{Tm}$ polymerase.

Only one gene-specific primer (P1 or P2) was added to any reaction mix at any one time and the non-specific primer depended on the pBluescript vector used. T7 was used with KS^+ and T3 with SK^+.

The PCR regime was:-
Denaturation, 95°C, 1 min; annealing at temperatures between 45-60°C, 1.5 min; and extension, 76°C, 3 min. The final extension was 5 min. The total number of cycles was 25.

After completion of the reaction, a 10 µl sample was removed from the chilled mix, electrophoresed and visualised on an agarose gel, following standard procedures.

Positive controls were:-
200 ng of p*Tfatp*3001, which had the entire 1.3 kb *Xho*I-*Bam*HI fragment of p*Tfatp*2 cloned into pBluescriptSK^+ (Fig. 4.1), primed with either P1 or P2, together with T3 and,
200 ng of p*Tfatp*2001 which had the entire 3.05 kb *Xho*I-*Cla*I fragment of p*Tfatp*2 cloned into pBluescriptKS^+ (Table 1, Appendix A), primed with T3 and T7.

Negative controls were:-
p*Tfatp*3001 and p*Tfatp*2001 without any added primers; p*Tfatp*3001 annealed to either P1 or P2 and T7, and reaction mixes from which all plasmids were omitted.

The second ligation-mediated PCR method used was an abbreviated variation of the "ligation-mediated PCR for genomic sequencing and footprinting" (LMPCR). Techniques, reagents and solutions used were those recommended for LMPCR by standard procedures for $Vent_R^{Tm}$ polymerase (Mueller *et al.*, 1992, in Ausubel *et al.*, 1993, Part 15.5.26-Suppl.20). Primers LMPCR1 (Tm = 64.5°C) and LMPCR2 (Tm = 30°C) which were annealed to synthesise the staggered linker required for the reaction, were constituted as recommended by Ausubel *et al.* (1993). The sequence of LMPCR1 was,

5'-GCGGTGACCCGGGAGATCTGAATTC-3', and that of LMPCR2,

5'-GAATTCAGATC-3'.

The gene-specific primers used were P1 and P2.

Suitably digested *T. ferrooxidans* ATCC 33020 chromosomal DNA was prepared as follows. Ten µg of genomic DNA was cut to completion with one of the following restriction enzymes:- *Sst*I, *Kpn*I, *Stu*I, *Mlu*I, *Apa*I, *Eco*RI, and a combination of *Xho*I and *Eco*RV. A partial *Sau*3AI digest of 15 µg of *T. ferrooxidans* chromosomal DNA was done after calibration to give a digest in which the bulk of the fragments was 5-1.2 kb. Included as a positive control was 500 ng of p*Tfatp*3001 (Fig. 4.1) which had been linearised with *Bam*HI. After digestion, all preparations were heat-inactivated, and the DNA was re-precipitated and dissolved in first strand synthesis mix, as described for LMPCR (Ausubel *et al.* 1993). First strand synthesis was carried out using gene-specific primer P2, as recommended. Thereafter, the staggered unidirectional linker consisting of annealed LMPCR1 and LMPCR2 was ligated to the first strand synthesis mix overnight. The ligated DNA was precipitated and cleaned, and the pellet was dissolved in 100 µl amplification mix, as recommended by the protocol. Using gene-specific primer P1, and linker-primer LMPCR1, the ligated mix underwent 20 amplification cycles as:- Denaturation, 95°C for 1 min; annealing, at either 50°C or 55°C for 2 min; extension, 76°C for 3 min. The final extension proceeded for 10 min, as prescribed. The 100 µl reaction mix was constituted as recommended by Ausubel *et al.* (1993). A 10 µl aliquot from each amplified mix was electrophoresed on 0.9% agarose, and visualised with ethidium bromide. Fifty µl of the remaining mix was removed, the DNA reprecipitated and phenol-cleaned (Ausubel *et al.*, 1993). DNA from the *Eco*RV/*Xho*I, *Sau*3AI and positive control sample was redissolved in appropriate buffer and sequenced, using a Sequenase kit (Version 2) from US Biochemical Corp, Cleveland, Ohio. The primer used for DNA synthesis for sequencing was P1.

Prior to any "experimental" amplification, all specific and non-specific oligonucleotide primers were checked for efficacy by carrying out amplification reactions at varying

annealing temperatures with p*Tfatp*3001 or p*Tfatp*2001 as described above. In addition, all primers were used, together with p*Tfatp*3001 or p*Tfatp*2001 in standard di-deoxy sequencing reactions using a Sequenase kit (Version 2). This was to ascertain whether primers were annealing at the correct sites on the *atp* genes or pBluescript vectors.

5.2.9. The preparation of perspex, glassware and chemicals for RNA extractive procedures.

All glassware and chemicals prepared for RNA extraction procedures were soaked/prepared with 0.01% DEPC (diethyl pyrocarbonate) for 24 h at ambient temperature, and autoclaved prior to use. Tris-based buffers were not treated with DEPC; rather they were made up with de-ionised water which had been pre-treated with 0.01% DEPC, and subsequently autoclaved, prior to adding Tris. Perspex electrophoretic buffer tanks, and glass gel supports were soaked with 10% sodium hypochlorite for 24 h prior to use. They were then thoroughly rinsed with de-ionised water. These precautions were taken to eliminate any possibility of contaminating RNases.

5.2.10. The extraction of mRNA from *E. coli* K12, and *E. coli* DK8 (p*Tfatp*2, pAN45F_0) (after Aiba *et al.*, 1981).

E. coli cells were grown in vigorously shaken 25 ml MLB (Appendix B) at 37°C until OD_{650} 0.5 was reached. The entire culture volume was poured over 40 ml loosely packed ice in a 50 ml centrifuge tube, and was pelleted at 4 000 rpm for 8 min in a Beckman JA20 rotor. The bacterial pellet was resuspended in 125 µl ice-cold 300 mM sucrose, 10 mM sodium acetate (pH 4.5) and transferred to a 1.5 ml microfuge tube. To this were added 125 µl 10 mM sodium acetate (pH 4.5) and 2% SDS (sodium dodecyl sulphate). The resulting suspension was heated to 65°C in a water bath for 1.5 min. Hot phenol (65°C, 250 µl) which had been equilibrated with unbuffered water, was added. The mix was vortexed and heated at 65°C for 3 min in a water bath. The suspension was then chilled in a -70°C bath for 15 s and microfuged for 5 min. The aqueous layer was decanted and re-extracted with hot unbuffered phenol twice more. The RNA was precipitated by the addition of 30 µl 3 M sodium acetate and 900 µl ethanol. The RNA was pelleted by centrifugation in a microfuge for 5 min, washed with 70% ethanol, dried and dissolved in 180 µl RNA storage buffer (20 mM sodium phosphate, pH 6.5, 1 mM EDTA), plus 20 µl 10x DNase buffer (20 mM sodium acetate, pH 4.5, 10 mM $MgCl_2$, 10 mM NaCl). RNase-free DNase (30 U) (Boehringer Mannheim) was added and contaminating DNA in the preparation was digested for 30 min at ambient temperature. The DNase was inactivated by the addition of 20 µl of 250 mM EDTA and extracted with phenol-chloroform (1:1, equilibrated with TE buffer). The RNA was ethanol precipitated, re-dissolved in RNA storage buffer and stored at -70°C. Prior to storage, a 5 µl aliquot of the RNA preparation was electrophoresed at 100V on a 1.2% agarose/formaldehyde gel (Sambrook *et al.*, 1989). To estimate the molecular weights of the RNA bands on the gel, the GIBCO BRL 0.24-9.5 kb RNA ladder

(Catalogue No. 5620SA) was used. The gel was stained and visualised with ethidium bromide (Ausubel *et al.*, 1993).

5.2.11. The extraction of RNA from *T. ferrooxidans*.

Twenty ml of a *T. ferrooxidans* ATCC 33020 culture, grown to exponential phase in sterile 9K medium (Silverman and Lundgren, 1959) was added to 15 l of sterile 9K. The culture was vigorously aerated at 30°C for 5 days, until ferrous oxidation indicated that the cells were in exponential phase of growth. The entire 15 l culture was batch-centifuged at 12 000 Xg in a Beckman J2-21M/E centrifuge for 10 min. The resulting pellets were pooled, resuspended in 800 ml 9K medium and shaken for 1 h, until the medium started to oxidise. The culture was divided into four volumes of 200 ml each and the cells were harvested as described above. Two of the pellets were resuspended in 10 ml water acidified with H_2SO_4 (pH 1.5) placed into microfuges in 1 ml volumes and immediately frozen at -70°C. The remaining cells were suspended in a total volume of 10 ml water, acidified with H_2SO_4 (pH 1.8). The suspension was dispensed into microfuge tubes in 1 ml volumes. The cells were given three rapid washes with acidified water to remove precipitated iron salts (Chapter 4, Section 4.2.8). Finally, the cells were washed once with sterile de-ionised water without acid. This procedure from the time of harvest, took approximately 30 min. The RNA was extracted from the pelleted cells as described for *E. coli* preparations, except that volumes of all solutions used were doubled. In addition, the hot phenol used was buffered to pH 8.0 (Sambrook *et al.*, 1989). This was to minimise the effects of residual acids in subsequent RNA extractive procedures. The resultant RNA in the pellet was visualised as described above for *E. coli* RNA extracts.

After 14 days storage at -70°C, the remaining pellets of *T. ferrooxidans* were thawed and inoculated into 200 ml sterile 9K medium at 30°C. After four days of gentle shaking, the culture entered exponential phase, was harvested and the cells washed as described above. This washing procedure took approximately 15 min. RNA was extracted and visualised as described.

5.2.12. Northern hybridisation.

RNA (50-100 µg) prepared from *E. coli* K12, *E. coli* DK8, *E. coli* DK8 (p*Tfatp*2, pAN45F_0), *E. coli* DK8 (pAN45) and *T. ferrooxidans* was run on denaturing formaldehyde/formamide agarose gels (Sambrook *et al.*, 1989). Also run on the gel were 2 µg undigested *T. ferrooxidans* chromosomal DNA, 30 ng of the probe DNA, and as a molecular weight marker, a sample of the GIBCO BRL 0.24-9.5 RNA ladder (Catalogue No. 5620SA) was used. RNA transfer and subsequent hybridisation was done according to methods recommended by Amersham for their Hybond N^+ membranes. Hybridisation was for 20 h at either 64°C or 68°C in a total volume of 100 ml. The probe used was the 4.35 kb

internal *Pst*I fragment of p*Tfatp*2 (Figs. 2.1. and 2.2) which had been gel-purified three times to remove all vector DNA and labelled with ^{32}P by nick-translation (Sambrook *et al.*, 1989).

5.2.13. The analysis of *E. coli* DK8 RNA preparations by primer extension.

RNA was extracted from *E. coli* DK8 (p*Tfatp*2, pAN45 F_0) and *E. coli* DK8 (p*Tfatp*2) (Section 5.2.10). Prior to proceeding with primer extension, 4 µl aliquots of the RNA from these preparations were run on an agarose-formaldehyde gel (Ausubel *et al.*, 1993) to gauge the quality of the RNA.

The 18-mer oligonucleotide primer used was synthesised by the Dept. of Biochemistry, University of Cape Town. The sequence of the primer (PE1) complementary to nucleotides 858-875 of the non-coding DNA strand depicted for p*Tfatp*2 in Fig. 3.2 was, 5'-TCTGCGTAGGGGCGCGCC-3'. The calculated Tm of the primer, according to the formula 4(G+C) + 2(A+T) (Sambrook *et al.*, 1989) was 64°C. To label the primer at the 5'-terminus, 1 µg of primer was added to 2 µl of 1 M Tris-Cl (pH 8.0); 1 µl 100 mM dithiothreitol (DTT); 2 µl 100 mM $MgCl_2$; 8 µl $\gamma^{32}P$-ATP; 1 µl (10 U) of polynucleotide kinase (Boehringer Mannheim). The volume of the mix was made to 20 µl with de-ionised sterile water. The reaction was incubated at 30°C for 45 min. The labelled primer was stored at -20°C for up to two weeks. The primer extension reaction was carried out using 150 µg of RNA and following the protocol of Sambrook *et al.* (1989) recommended for murine reverse transcriptase (Boehringer Mannheim). A deviation from the standard protocol was the use of the Ambion RNA hybridisation buffer supplied by Ambion, Woodward St., Austin, Texas, with their Ribonuclease Protection assay kit. Hybridisation temperatures used were: 72°C, 64°C, 57°C, 50°C and 45°C, and hybridisation time was at least 16 h. The length of the resulting end-labelled cDNA was to be measured by electrophoresis through a standard 6% polyacrylamide gel under denaturing conditions against a sequencing ladder generated from p*Tfatp*2 primed with PE1, using a Sequenase kit, Version 2 from U.S. Biochemical Corp., Cleveland, Ohio.

5.3. Results.

5.3.1. Attempts to identify a *T. ferrooxidans* ATCC 33020 *atpIBEFHA* chromosomal fragment.

Southern hybridisation of p*Tfatp*2 to *T. ferrooxidans* chromosomal single restriction enzyme digests of *Pst*I, *Bgl*II, *Eco*RI, *Cla*I and *Eco*RV is shown in Fig. 5.1.

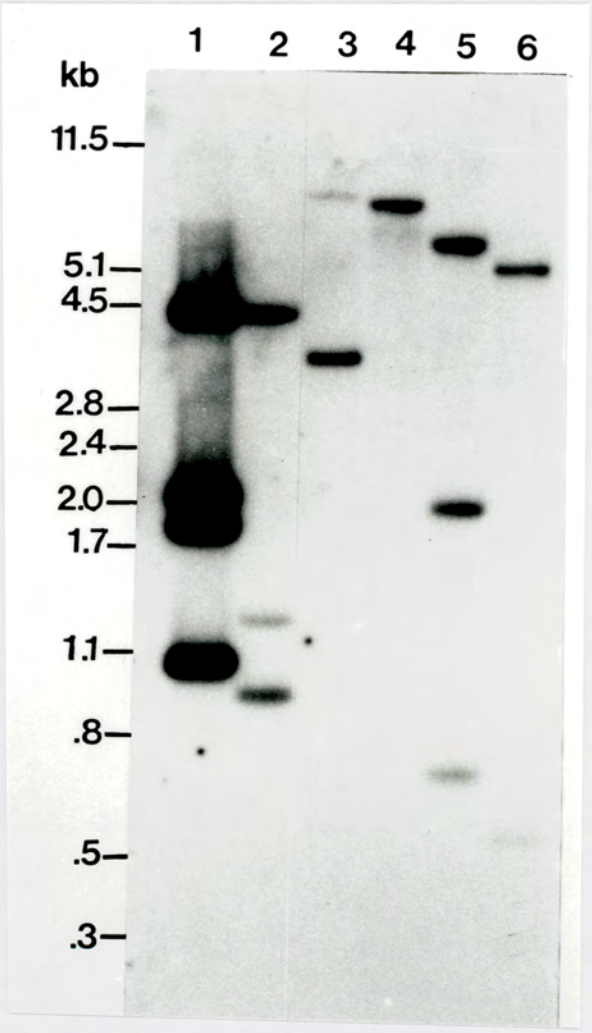


Fig. 5.1. Southern hybridisation of p*Tfatp*2 to the *T. ferrooxidans* ATCC 33020 chromosome. Lane 1, 40 ng p*Tfatp*2 *Pst*I digest; Lanes 2-6, *T. ferrooxidans* ATCC 33020 chromosomal digests; Lane 2, *Pst* I; Lane 3, *Bgl*II; Lane 4, *Eco*RI; Lane 5, *Cla*I; Lane 6, *Eco*RV. Fragment sizes (kb) were estimated from a λDNA (*Pst*I) digest.

The *Pst*I digest of the *T. ferrooxidans* chromosome (Lane 2) confirmed results of the earlier Southern hybridisation (Fig. 2.2) and indicated an internal 4.3 kb *Pst*I fragment present on p*Tfatp*2. Two smaller fragments were also visible. As there was no suitable *Pst*I site downstream of the 5.25 kb site on p*Tfatp*2 (Figs. 2.1 and 5.2), these fragments were interpreted to lie upstream of the 0.93 kb *Pst*I site on p*Tfatp*2 (Fig. 2.1), with the larger fragment probably representing a partial digest. Therefore it was likely that a *Pst*I site occurred at the extreme 3'-end of the *T. ferrooxidans atpB* gene, and a *T. ferrooxidans* ATCC

33020 *Pst*I genomic DNA fragment would not be suitable for cloning the entire *atpPIB* region.

The *Eco*RI digest (Lane 4) showed a fragment of approximately 8.5 kb in size. As there were no internal *Eco*RI sites on the *atp* genes of p*Tfatp*2 sites (Fig. 2.1) this result indicated that an *Eco*RI site probably occurred in the *atpB* gene; hence *Eco*RI was not suitable for cloning the *atpPIB* region. The *Bgl*II digest (Lane 3) showed the internal 3.4 kb fragment of the *T. ferrooxidans* ATCC 33020 chromosome noted previously (Fig. 2.2). A feint signal was noted in the 9 kb region. Southern hybridisation of p*Tfatp*1 against a *Bgl*II digest of the *T. ferrooxidans* chromosome had confirmed a 1.35 kb *Bgl*II fragment adjacent to the 3'-end of the 3.4 kb fragment (Fig. 2.2). Therefore, if the feint band noted (Fig. 5.1, Lane 3) represented a fragment from a complete *T. ferrooxidans* genomic *Bgl*II digest, a large 9 kb fragment lay upstream of the internal *Bgl*II site mapped at 2.45 kb on p*Tfatp*2 (Figs. 2.1. and 5.2). The *T. ferrooxidans* chromosomal *Cla*I digest (Lane 5) gave three fragments; approximately 600 bp, 2 kb and 8 kb. Restriction enzyme digests showed two *Cla*I sites present on p*Tfatp*2 (Fig. 2.1). The origin of the smaller fragments on the blot was difficult to assign. As the small 324 bp fragment internal to p*Tfatp*2 (Fig. 2.1) was not visible on the blot, it was possible that the approximately 600 bp fragment was a partial digest. The 2 kb fragment should lie to the 3'-end of the internal *Cla*I sites on p*Tfatp*2. However, a restriction enzyme digest of p*Tfatp*1 did not place a *Cla*I site in the expected region (Figs. 2.1 and 5.2). One possible explanation is that as p*Tfatp*1 was not prepared in *E. coli dam*$^-$ and *dcm*$^-$ strains (Sambrook *et al.*, 1989), a *Cla*I site on the plasmid was methylated. The presence of this site would have to be confirmed either by preparation of p*Tfatp*1 in *E. coli dam*$^-$ and *dcm*$^-$ strains or by sequencing the *T. ferrooxidans* chromosomal insert on the plasmid. The large 8 kb *Cla*I fragment noted in Lane 5 could lie to the 5'-end of the 4.27 kb *Cla*I site mapped on p*Tfatp*2 (Fig. 2.1) and should have at least one internal *Xho*I site. The *Eco*RV digest (Lane 6) indicated a strong positive signal in the 6 kb size range. There were three *Eco*RV sites internal to the *T. ferrooxidans atp* chromosomal fragment on p*Tfatp*2, which gave fragments of 305 bp and 537 bp in size (Figs. 2.1, and 5.2). These were barely visible on the Southern blot. It was considered likely that the approximately 6 kb *Eco*RV fragment on the *T. ferrooxidans* chromosome lay upstream of the 4.99 kb *Eco*RV site mapped on p*Tfatp*2. If so, this fragment would have at least one internal *Xho*I site.

5.3.2. An analysis of the *E. coli* AN730-complementing cosmids.

A screening of *E. coli* AN730 strain with the *T. ferrooxidans* ATCC 33020 cosmid gene bank resulted in the isolation of 30 cosmids which complemented the mutant on MMS+Ap plates. Results obtained from screening the cosmids for an internal *Eco*RV fragment of approximately 6 kb demonstrated that on the basis of common *Eco*RV fragments, the cosmid isolates fell into six different groups. *Eco*RV and *Eco*RV/*Xho*I digests of five of the groups showed that none of these *uncA*-complementing cosmids had a fragment which

could have corresponded to the 6 kb *Eco*RV band mapped on the *T. ferrooxidans* chromosome (results not shown). Cosmids from groups 1-5 were therefore not examined further.

One cosmid, isolate 689.25, representative of group 6 appeared to have at least one *Eco*RV band of approximately 6 kb and was selected for further study. Details of restriction enzyme digests of cosmid 689.25 are presented in Fig. 5.2.

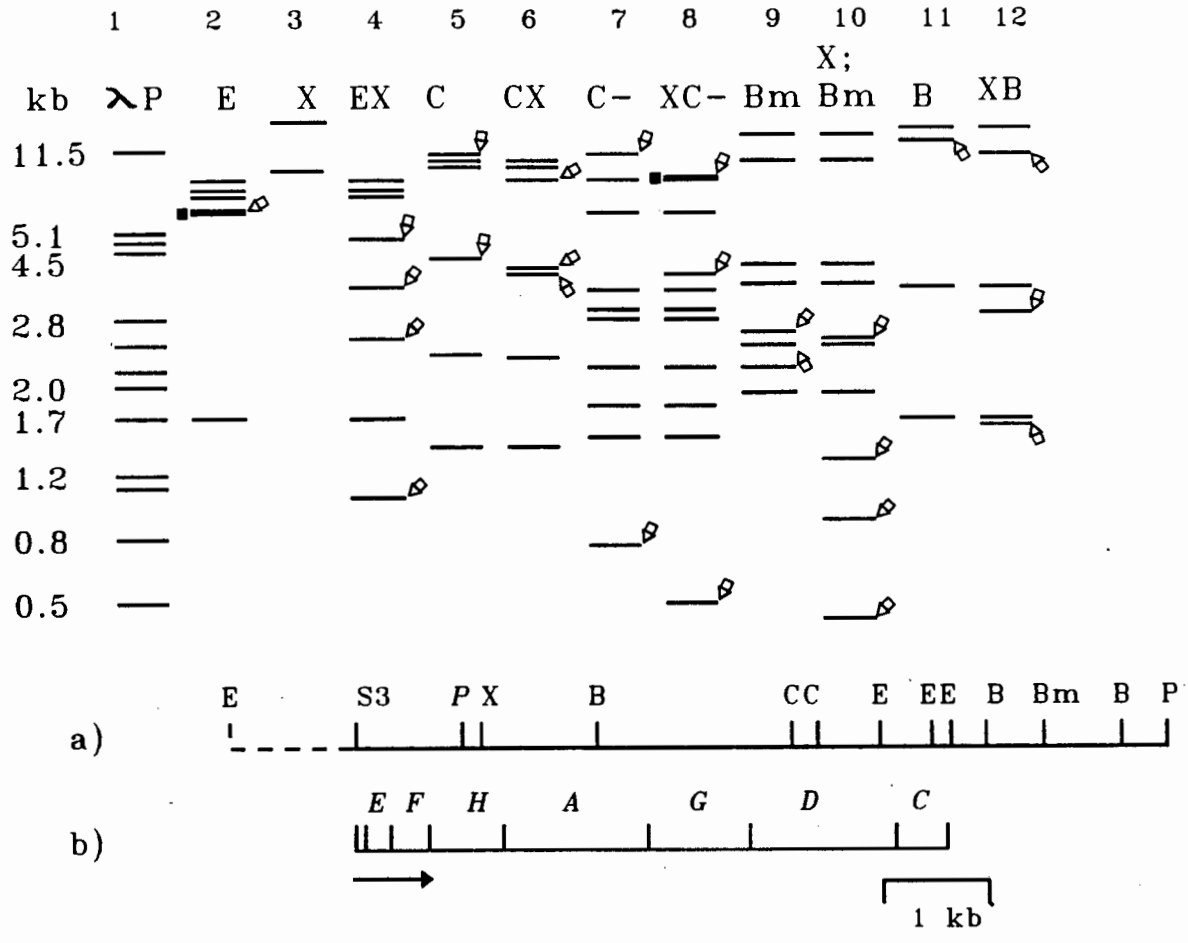


Fig. 5.2. Diagrammatic representation of fragments yielded from various restriction endonuclease digests of cosmid 689.25. Only bands of interest are shown. Key to enzymes; B=*Bgl*II, Bm=*Bam*HI, C=*Cla*I, C⁻=*Cla*I sites in cosmid 689.25 prepared in *E. coli* GM41 (*dam*⁻), E=*Eco*RV, P=*Pst*I, S3=*Sau*3AI, X=*Xho*I. Bands marked with ⊏▷ are referred to in the text. Bands marked with ■ represented doublets. Lane 1, *Pst*I digest of phage λ DNA, with band sizes indicated in kb. (a) A partial restriction enzyme map of the *T. ferrooxidans* ATCC 33020 chromosomal inserts cloned on p*Tfatp*2 and p*Tfatp*1. The broken line indicates the possible orientation of a DNA fragment adjacent to the 5'-end of the *Sau*3AI site upstream of *atpE*. (b) The *T. ferrooxidans atp* genes aligned against the restriction enzyme map; arrow indicates direction of transcription of wild-type *T. ferrooxidans* ATCC 33020 *atp* genes.

An *Eco*RV digest of cosmid 689.25 (Lane 2, Fig. 5.2) indicated the presence of two bands in the 6-7 kb region. An *Xho*I digest showed the presence of two *Xho*I sites in 689.25 (Lane 3, Fig. 5.2). A double *Eco*RV/*Xho*I digest (Lane 4, Fig. 5.2) produced four bands between 0.9 and 4.7 kb. Hence both of the 6 kb *Eco*RV fragments had an internal *Xho*I site. The band in the 3.8 kb range could have corresponded to the internal *Eco*RV/*Xho*I fragment present on the *T. ferrooxidans atp* genomic fragment (Fig. 5.3a). A *Cla*I digest of the cosmid (Lane 5, Fig. 5.2) demonstrated the presence of a large *Cla*I band, which could have corresponded to the large *Cla*I fragment mapped on the *T. ferrooxidans* ATCC 33020 chromosome thought to contain the entire upstream region of the *atp* operon (Fig. 5.1). The cosmid also had a *Cla*I fragment of approximately 2.4 kb, which was larger than the 2 kb *Cla*I site mapped on the *T. ferrooxidans* chromosome (Fig. 5.1). The large *Cla*I fragment had an *Xho*I site (Lane 6, Fig. 5.2). To test the *Cla*I sites further, cosmid 689.25 was prepared in an *E. coli dam*⁻ strain (GM41) (Table 1, Appendix A) and results of these digests are shown in Lanes 7 and 8 (Fig. 5.2). A large *Cla*I fragment had an internal *Xho*I site. Notably, a smaller *Cla*I band of approximately 2 kb was present in the digest. These results suggested that 689.25 could have the upstream region of the *T. ferrooxidans atp* operon. Digests with *Bam*HI were inconclusive. Lanes 9 and 10 (Fig. 5.2) showed that both of the 689.25 *Xho*I sites were flanked by a *Bam*HI site. No internal *Bam*HI sites were mapped in the vicinity of the *Xho*I site on the *T. ferrooxidans atp* DNA chromosomal insert (Fig. 5.3). This raised three possibilities. Firstly, that both *Bam*HI sites were upstream of the *Sau*3AI site on p*Tfatp*2 (Fig. 5.2a). Secondly, that methylation of *Bam*HI sites (Sambrook *et al.*, 1989) by the *E. coli* host system used to prepare the cosmid DNA had resulted in incomplete restriction of all prospective *Bam*HI sites present on p*Tfatp*2. Thirdly, that if all *Bam*HI sites were represented on p*Tfatp*2, the 689.25 *Eco*RV/*Xho*I fragment was not the one hoped for. The *Bgl*II digest of 689.25 (Lane 11, Fig. 5.2) showed a 3.5 kb fragment which could have corresponded to the internal *Bgl*II fragment noted for the *T. ferrooxidans* chromosomal fragments cloned on p*Tfatp*2 and p*Tfatp*1 (Figs. 2.1 and 5.2). The internal downstream 1.35 kb *Bgl*II fragment noted for p*Tfatp*1 (Figs. 2.1, 2.2 and 5.2) was not present. An *Xho*I/*Bgl*II digest (Lane 12) indicated that a large *Bgl*II fragment had two *Xho*I sites. Had this carried *atpIBEFHA*, the *Xho*I/*Bgl*II digest would have yielded a 1.25 kb fragment, internal to the *T. ferrooxidans atp* genes (Fig. 5.2a). This was not observed. It therefore seemed that the 689.25 *Eco*RV fragment was not the internal 6-7 kb *T. ferrooxidans* genomic fragment which extended upstream of the *atpE* gene. In an attempt to resolve apparent discrepancies, various restriction digests of 689.25 (Section 5.2.6) were hybridised to the ^{32}P-labelled *Xho*I/*Bam*HI fragment of p*Tfatp*3001. Although the probe hybridised strongly to a *Pst*I digest of p*Tfatp*3001, there was no hybridisation of this upstream region of the *T. ferrooxidans atp* genes to any of the 689.25 digests (results not shown). This proved that the cosmid isolate did not extend beyond the *Xho*I site mapped on the *T. ferrooxidans atp* chromosomal insert, and was either composed of more than one non-contiguous fragment

of *T. ferrooxidans* chromosomal DNA, or had been subjected to re-arrangement in *E. coli*. The cosmid was therefore not investigated further.

A second screening of *E. coli* AN730 was done using the cosmid bank as described above. In this case, 20 *uncA*-complementing cosmids were digested with *Bgl*II. The rationale behind this experiment was to isolate cosmids which had the 3.4 kb, but not the 1.35 kb *Bgl*II fragment internal to the *T. ferrooxidans* chromosomal fragment present on p*Tfatp*1 and p*Tfatp*2 i.e. the cosmid should extend upstream of the 2.48 kb *Bgl*II site on p*Tfatp*2 (Figs. 2.2 and 5.2a). On this basis, four cosmids were chosen for further study. However, restriction enzyme digests and a Southern hybridisation showed that these cosmids were all probably spurious re-arrangements of *T. ferrooxidans* chromosomal DNA in the *E. coli* host system used (results not shown).

Finally, after sequencing p*Tfatp*2, when it became possible to construct primers homologous/complementary to the *T. ferrooxidans atp* genes, attempted annealing of P1 (Section 5.2.8.2) to both 689.25 and one of the cosmids from a *Bgl*II digest (isolate 818.1) in order to generate a sequencing ladder of DNA synthesised towards the 5'-end of *atpE*, gave no result. This showed that neither of these cosmids had DNA complementary to P1 and confirmed the earlier results that the upstream fragment of the *T. ferrooxidans* operon was not present. In this experiment, the positive control used was p*Tfatp*2 annealed to P1, where a sequencing ladder was generated in the correct upstream direction (results not shown).

5.3.3. Assignment of a discrete 2 kb *T. ferrooxidans* chromosomal fragment adjacent to the 5'-end of the *Xho*I site in *atpH*.

The results of the Southern hybridisation of p*Tfatp*3150 to *Xho*I, *Eco*RV and *Xho*I/*Eco*RV digests of the *T. ferrooxidans* chromosome are shown in Fig. 5.3.

All three *T. ferrooxidans* ATCC 33020 chromosomal digests hybridised to p*Tfatp*3150 (Fig. 5.3). Positive signals were from 5.2 kb *Xho*I (Lane 3), 6-7 kb *Eco*RV (Lane 4), and 2 kb *Eco*RV/*Xho*I, indicated by the arrow (Lane 5) fragments. As the probe was designed to hybridise to complementary DNA upstream of the *atpH Xho*I site, all three fragments had common DNA adjacent to the 5'-end of *atpE*.

For the current study, it was decided to continue with the 2 kb *Xho*I/*Eco*RV fragment for two reasons. Firstly, it was too small to have the *T. ferrooxidans atp* operon promoter, which suited the rationale of the reverse orientation cloning experiments. Secondly, as the small fragment would have approximately 900 bp of DNA of unknown sequence, it was an ideal size for PCR amplification. It was of interest to note that cosmid 689.25 did not carry the 2 kb *Eco*RV/*Xho*I fragment (Fig. 5.2, Lane 4).

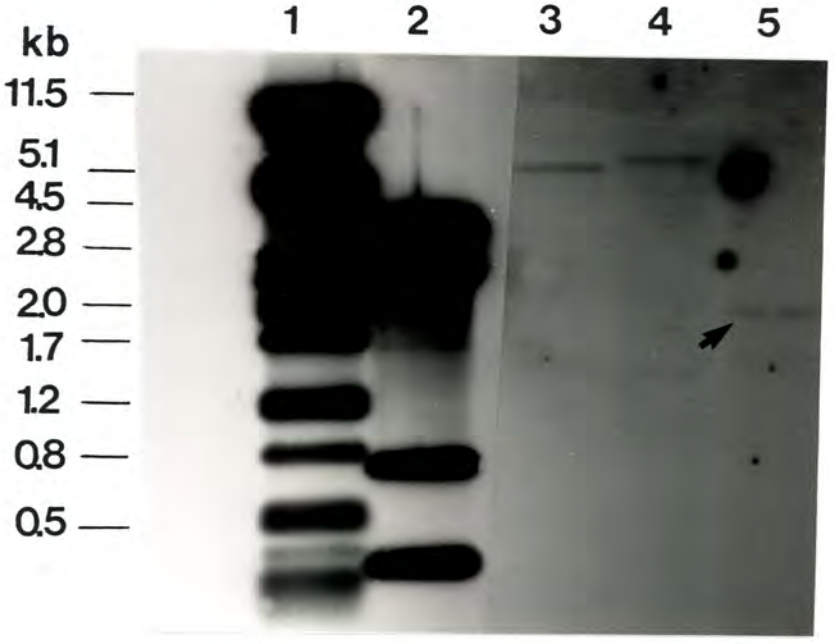


Fig. 5.3. Southern hybridisation of p*Tfatp*3150 to total DNA from *T. ferrooxidans* ATCC 33020. Lane 1, λDNA (*Pst*I) (kb fragment sizes indicated alongside); Lane 2, 40 ng p*Tfatp*3150, partial *Eco*RI/*Pst*I digest. Lanes 3-5, *T. ferrooxidans* ATCC 33020 chromosomal digests; Lane 3, *Xho*I; Lane 4, *Eco*RV; Lane 5, *Xho*I/*Eco*RV.

5.3.4. Attempts to clone the 2 kb *Eco*RV/*Xho*I fragment against the *lacZ* promoter ("antisense") of pBluescriptKS+.

Attempts to clone the 2.0 kb *Eco*RV/*Xho*I fragment into pBluescriptKS+ digested with compatible enzymes yielded 229 "white" colonies on X-Gal/IPTG/Ap plates. Extensive restriction enzyme analyses of plasmids isolated from the colonies, using an *Xho*I/*Sst*I double digest showed that not one plasmid carried a 667 bp *Xho*I/*Sst*I fragment internal to p*Tfatp*2 (Fig. 2.2). In addition, PCR-attempted amplification of aliquots from either the ligation mix or pooled micro-aliquots of phenol-cleaned miniprepped plasmid DNA, using either gene-specific primers P1 or P2 together with with T7, gave no result. However, in all PCR positive controls at amplification temperatures between 45-60°C, fragments of the correct sizes were amplified. P1 together with T7, annealed to p*Tfatp*3001 and amplified the expected 680 bp fragment of which 180 bp was complementary to the *atp* gene insert at the extreme 5'-end of p*Tfatp*2. The remaining 500 nucleotides were complementary to the *Bgl*II/*Bam*HI p*Eco*R251 fragment included in p*Tfatp*3001 (Table 1, Appendix A; Fig. 4.1). Primer P2 together with T7, annealed to p*Tfatp*3001 amplified a fragment of approximately 970 bp of which 470 bp were complementary to the 5'-end of the *T. ferrooxidans atp* gene insert on p*Tfatp*2. The origin of the remaining approximately 500 bp was as described for P1. When primers T3 and T7 were annealed to p*Tfatp*2001, the entire 3.05 kb *Xho*I-*Cla*I fragment internal to p*Tfatp*2 was amplified. Negative controls, including either P1 or P2 and T7 annealed to p*Tfatp*3001, showed no specific identifiable

amplified DNA fragments (results not shown). Conventional sequencing gels demonstrated that the oligonucleotide primers were annealing to the correct sites, and in the case of the p*Tfatp*3001 control, DNA synthesis was occuring off the correct (non-coding) DNA strand with both gene-specific primers.

5.3.5. PCR-mediated attempts to amplify the *T. ferrooxidans* chromosomal fragment adjacent to the 5'-end of the *atpE* gene.

Extensive SSP-PCR experiments using representative aliquots from the ligations described viz. *T. ferrooxidans* ATCC 33020 total genomic digests of *Xho*I/*Eco*RI, *Xho*I/*Cla*I, *Xho*I/*Hin*dIII, *Xho*I/*Bam*HI and an *Xho*I/*Eco*RV 1.7-2.2 kb library of fragments ligated into pBluescriptKS$^+$ gave no result with either P1 or P2 in combination with T7. Varying annealing temperatures, magnesium ion, primer and dNTP concentrations, or the addition of bovine serum albumin or DMSO (dimethyl sulfoxide) to the amplification mix, produced no positive results. In most cases, varying these parameters had no influence on positive controls, which consistently amplified the expected fragments described above. Assuming that perhaps minute quantities of the correct inserts had been amplified, a second round of PCR amplification was done. To do this, 20 µl aliquots from the mix resulting from the first 25 amplification cycles underwent a further 25 cycles of PCR. No positive results were obtained. The mix resulting from the amplification of the 1.7-2.2 kb *Eco*RV/*Xho*I library of fragments ligated into pBluescriptKS$^+$ was run on a low melting point agarose gel, and discrete plugs of agar were excised from the 1.7-2.4 kb region of the electrophoresed sample using Gilson micropipette tips from which the ends had been removed. The plugs of agarose underwent a further 25 cycles of PCR, as described (Section 5.2.8.2) using P1 and T7 as primers. No positive results were obtained.

The LMPCR method, described above gave no positive result. None of the randomly digested *T. ferrooxidans* chromosomal preparations viz. *Sst*I, *Kpn*I, *Stu*I, *Mlu*I, *Apa*I, *Eco*RI, *Sau*3AI or *Xho*I/*Eco*RV resulted in the amplification of any fragment of DNA which might have been cloned or sequenced. Neither did the positive control included, viz. p*Tfatp*3001 linearised with *Bam*HI, give the expected 680 bp fragment. A second round of PCR, using 20 µl samples from the mixes generated after the first 20 amplification cycles, gave no positive results.

5.3.6. RNA yields obtained from *E. coli* and *T. ferrooxidans*.

The RNA extracted from *E. coli* cells, using the method described in Section 5.2.10 above gave high yields from all cultures. The RNA was dissolved in RNA storage buffer such that the concentration was between 1-2 mg/ml. High yields of RNA were also obtained from cultures of *T. ferrooxidans*. *T. ferrooxidans* RNA was dissolved in RNA storage buffer at a concentration of between 0.8-1.5 mg/ml. The RNA from both *E. coli* and *T. ferrooxidans*, when visualised on a formaldehyde-agarose gel demonstrated that the RNA was not totally degraded as the 23S, 16S and 5S rRNA were clearly visible (Fig. 5.4).

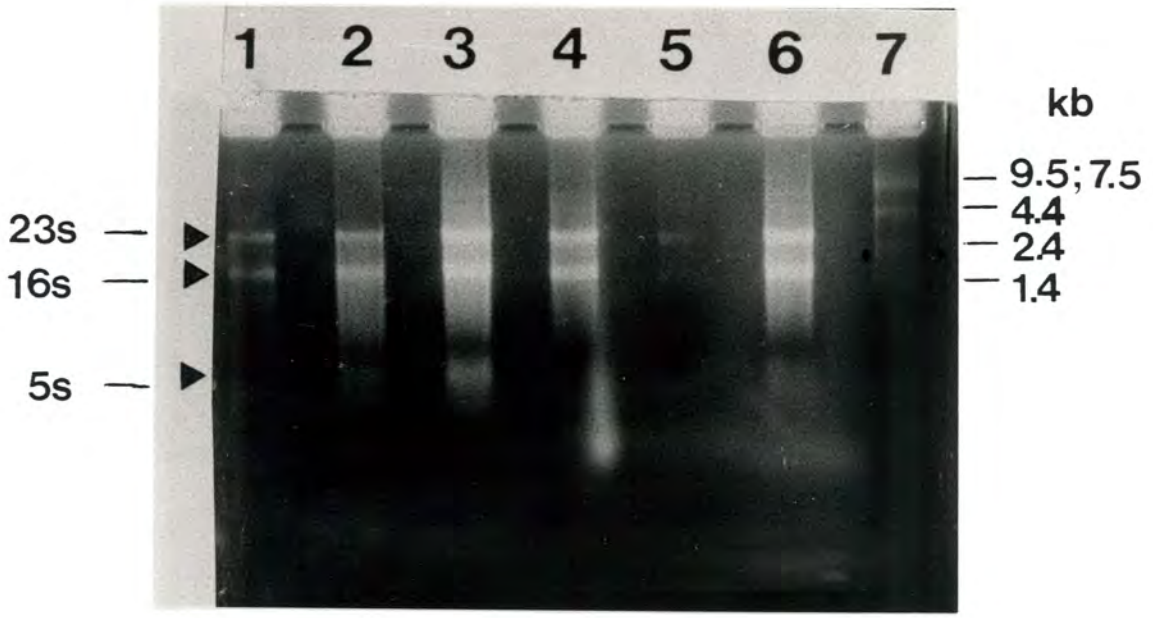


Fig. 5.4. RNA formaldehyde-agarose gels of RNA extracted from *T. ferrooxidans* and *E. coli* cultures. Four µl volumes of each RNA preparation (i.e. non-equivalent amounts of RNA) were loaded into each well. Lanes 1 and 2, *T. ferrooxidans* ATCC 33020 RNA; Lane 3, *E. coli* DK8 (p*Tfatp*2); Lane 4, *E. coli* DK8 (pAN45); Lane 5, *E. coli* K12; Lane 6, *E. coli* DK8; Lane 7, RNA molecular weight markers (GIBCO BRL 0.24-9.5 kb RNA ladder). The arrow-heads indicate the positions of the 23S, 16S and 5S rRNA.

5.3.7. Northern hybridisation of *T. ferrooxidans* and *E. coli* RNA.

Typical results obtained from two Northern hybridisation studies are shown in Fig. 5.5.a and b.

Results indicated that with the conditions used, the DNA probe prepared from p*Tfatp*2 hybridised to itself (Lane 2, Fig. 5.5b) and to the *T. ferrooxidans* chromosome (Lane 1, Fig.5.5b). Negative controls, i.e. RNA from *E. coli* DK8 only, *E. coli* DK8 (pAN45) and *E. coli* K12, were free of any signal which suggested that the probe was highly specific. However, results with the RNA preparations wherein a specific signal was expected, showed general non-specific hybridisation (Fig. 5.5a, Lanes 3, 4, 5, and 6; Fig. 5.5b Lanes 5, 6, and 7). At best, there was a broad band between approximately 7-9 kb for *T. ferrooxidans* RNA in Lanes 5 and 6, Fig. 5.5a (indicated by the arrow). However, this was not clear enough to be conclusive. Furthermore, in Fig. 5.5b, Lane 6 where hybridisation was at 68^{0}C, no such "band" was visible. None of the *E. coli* DK8 (p*Tfatp*2) cultures gave a discrete signal in the expected 4.4 kb zone; instead, there was overall general hybridisation.

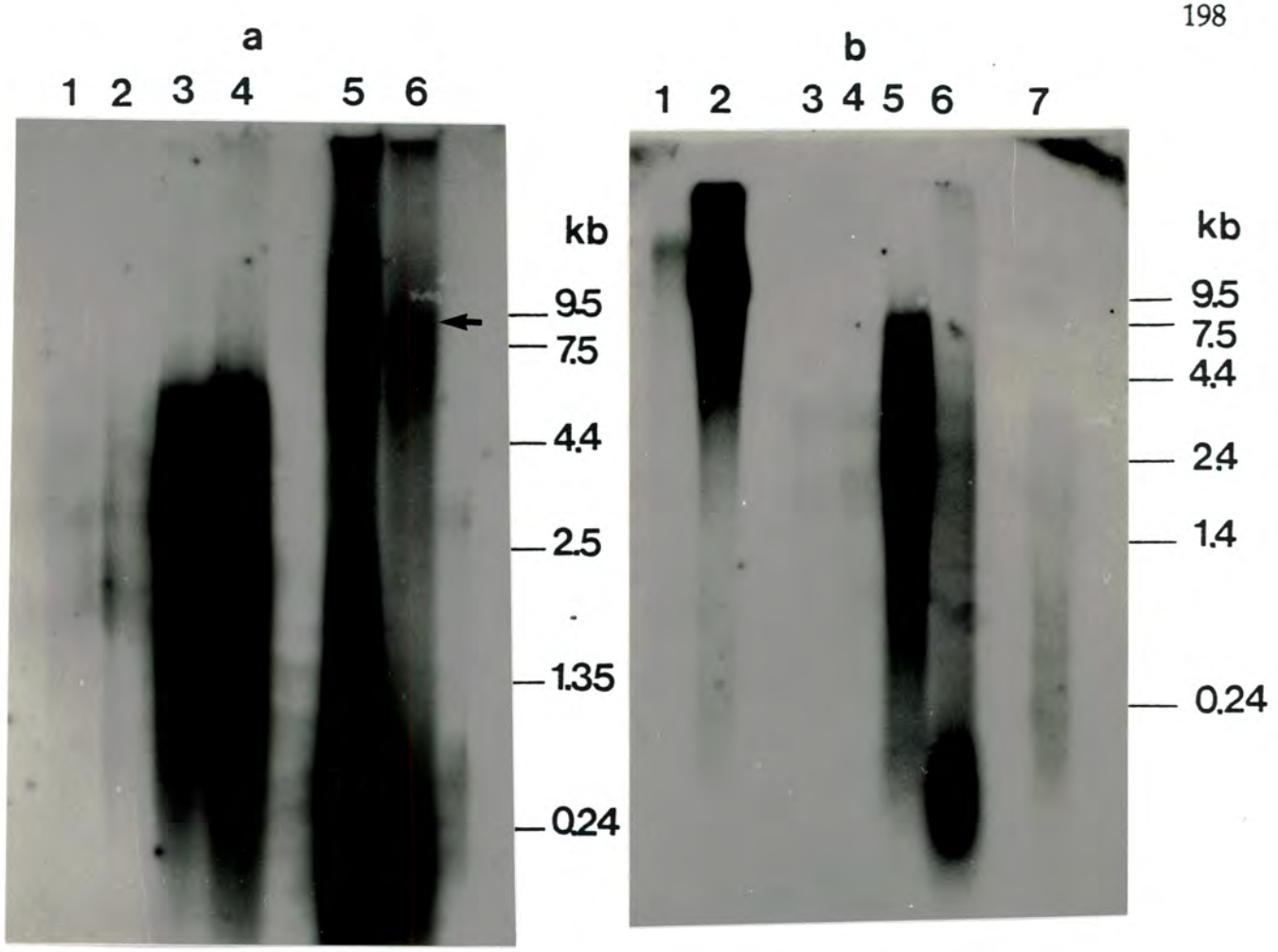


Fig. 5.5. Northern hybridisation of *E. coli* and *T. ferrooxidans* ATCC 33020 mRNA, probed with the 4.35 kb *Pst*I-*Pst*I fragment of p*Tfatp*2.;(a) Hybridisation at 64°C. Lane 1 (75 µg) *E. coli* K12 (*unc*+); Lane 2 (100 µg) *E. coli* DK8 (pAN45); Lanes 3 and 4 (100 µg) *E. coli* DK8 (p*Tfatp*2); Lanes 5 (100 µg) and 6 (50 µg) *T. ferrooxidans* ATCC 33020 RNA. (b) Hybridisation at 68°C. Lane 1, 2 µg *T. ferrooxidans* ATCC 33020 chromosomal DNA; Lane 2, 40 ng of the p*Tfatp*2 probe fragment; Lane 3 (75 µg) *E. coli* DK8 only; Lane 4 (100 µg) *E. coli* DK8 (pAN45); Lanes 5 (100 µg) and 6 (50 µg) *E. coli* DK8 (p*Tfatp*2); Lane 7 (75 µg) *T. ferrooxidans* ATCC 33020 RNA.

5.3.8. Primer extension.

Results from a primer extension study are shown in Fig. 5.6.

Despite using hybridisation temperatures of 72°C, 64°C, 57°C, 50°C and 45°C, and repeating the experiment a number of times with freshly prepared RNA from the *E. coli* cultures, no positive signal was generated in the area in which a transcriptional start recognised by *E. coli* upstream of the *T. ferrooxidans atpH* gene was hypothesised. Even at lower hybridisation temperatures, there was no signal due to non-specific hybridisation. Results with the sequencing ladder generated from p*Tfatp*2 primed with PE1 indicated that the primer was annealing to the DNA at the correct site, and that DNA synthesis was occuring off the correct DNA (non-coding) strand.

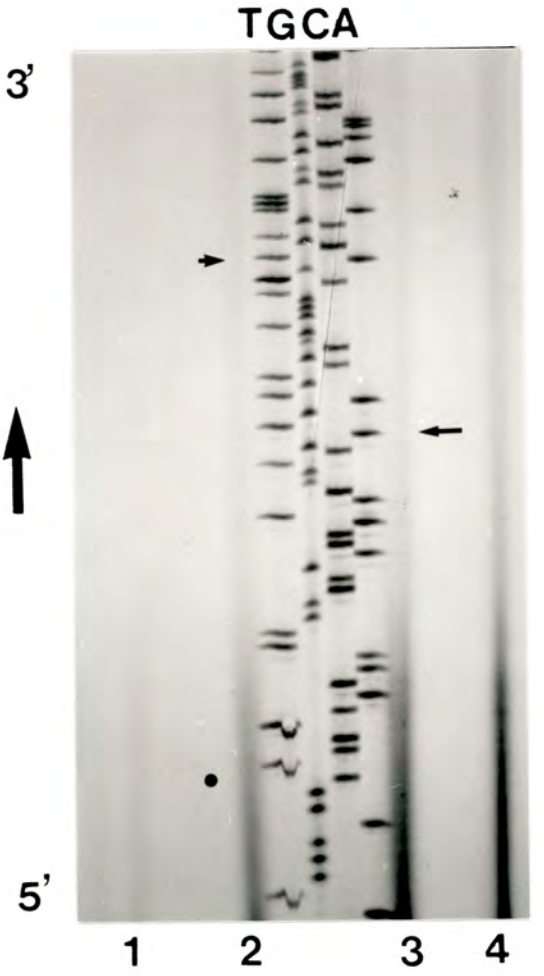


Fig. 5.6. Primer extension, using primer PE1, with RNA from *E. coli* DK8 (p*Tfatp*2) and *E. coli* DK8 (p*Tfatp*2, pAN45F_0), hybridised at various temperatures. Lanes 1 and 2, *E. coli* DK8 (p*Tfatp*2) RNA annealed at 45°C and 57°C respectively; Lanes 3 and 4, *E. coli* DK8 (p*Tfatp*2, pAN45F_0) annealed at 45°C and 57°C respectively. The sequencing ladder was generated from p*Tfatp*2, primed with PE1. The solid dot indicates the putative *atpH* ribosome binding site on the complementary strand; small arrows mark regions of *T. ferrooxidans* ATCC 33020 DNA with homology to *E. coli* σ^{70} promoter regions (Fig. 3.2). The large arrow indicates the 5'->3' direction of DNA synthesis, occurring from p*Tfatp*2 primed with PE1.

5.4. Discussion.

T. ferrooxidans ATCC 33020 chromosomal digests (Fig. 5.1) indicated that the extreme 5'-end of the *T. ferrooxidans atp* operon was probably located on large *Cla*I, *Bgl*II and *Eco*RV fragments. Screening *E. coli* AN730 with a *T. ferrooxidans* ATCC 33020 cosmid bank resulted in the isolation of fifty *uncA*-complementing cosmids. Results from the studies with those cosmids selected for further investigation showed it was unlikely that they extended to the 5'-end of the *Xho*I site internal to *atpH*. Possible reasons for this are that firstly, the *T. ferrooxidans atpPIB* genomic fragment was not present in the cosmid bank screened; secondly that regions within the discrete *T. ferrooxidans atpPIB* cluster were spontaneously deleted, intrinsically unstable or lethal in *E. coli;* thirdly, that the entire *T. ferrooxidans* F_0 oligomer was non-functional in *E. coli unc* mutants tested.

With reference to the absence of the *T. ferrooxidans atpPIB* fragment in the cosmid library screened, it was reported that both the cosmid and plasmid banks used during this study represented a library of *T. ferrooxidans* chromosomal fragments of greater than 99% confidence (Ramesar, 1988). Therefore it is likely (though not certain) that the *T. ferrooxidans atpPIB* fragment was represented and reasons for the inability to clone the fragment by functional complementation of *E. coli unc* mutants probably lay elsewhere.

The consistent inability to clone *T. ferrooxidans atpPIB* by physical linkage to the *atpEFHAGDC* fragment during the current study, suggested that regions within *atpPIB* were either deleted, unstable or lethal in the *E. coli* host systems used. Of particular relevance in this regard was the fact that during the current and preliminary studies with *T. ferrooxidans atp/uncA*-complementing plasmids, 24 were isolated which terminated in the same region as the *Sau*3AI site mapped upstream of *atpE* on p*Tfatp*2 (Chapter 2; Dennehy, 1988; D.E. Rawlings, unpublished data). Shibata *et al.* (1992) reported that *E. coli* appeared to delete out regions of the *E. hirae atp* operon, particularly in the region of *atpB*. It was postulated that the protonophoric properties of the *atpB* gene product (subunit a) might be harmful to *E. coli* (reviewed in Chapter 2, Section 2.4). It was demonstrated by others that when overexpressed in *E. coli,* subunit a causes inhibition of cell growth (discussed below). It is not yet clear as to whether this inhibition is a result of increasing membrane proton-permeability or not. Kanazawa *et al.* (1984) demonstrated subunit a-induced lethality, but did not address cytoplasmic membrane proton permeablity. von Meyenburg *et al.* (1985) reported that subunit a-dependent growth inhibition also produced proton-leaky membranes in strains which were deleted for F_0 genes, but which still carried the genes for the γ, β and ε subunits. Eya *et al.* (1989) also demonstrated growth inhibition in *E. coli,* due to over-production of subunit a. In the latter study, the inhibiton could not be correlated with the ability of subunit a to form a proton pathway. Inhibiton was rather due to disorganised enzyme assembly, or to the synthesis of other

membrane components. Monticello *et al.* (1992) confirmed that inducing transcription of plasmids containing *uncB* resulted in significant inhibiton of growth, but this was not due to increased proton permeability of the cytoplasmic membrane. Therefore the question as to whether the a subunit alone can cause proton-permeability is not decided. It may be that in the studies cited above, the over-expression of a hydrophobic protein in *E. coli* caused non-specific growth inhibition (Brusilow, 1993). Therefore at this stage it cannot be concluded that protonophoric activity of *T. ferrooxidans* subunit a was solely responsible for the failure to isolate *T. ferrooxidans atpB*.

The inability to clone any *T. ferrooxidans* F_0 gene by complementation of the *E. coli* F_0 point and Δ*unc* mutants used during the current study suggested that the entire *T. ferrooxidans* F_0 oligomer was non-functional in *E. coli*. This observation was supported by the fact that the *T. ferrooxidans atpEF* genes even though expressed *in vitro* by the *lacZ* promoter present on p*Tfatp*3001, after transformation into *E. coli* AN943 (c-) or AN1440 (b-), did not functionally complement the strains on MMS *in vivo* (Chapter 4). It was suggested that this was due to differences associated with the cytoplasmic membrane of acidophilic and neutrophilic bacteria, and the associated role of proton translocation of the F_0 channel (Chapter 4, Section 4.4). *E. coli* F_0 is probably assembled in the cytoplasmic membrane in a non-proton permeable form, independently of any F_1 subunits (reviewed in Brusilow, 1993). When this immature form interacts with the δ and α subunits, F_0 assumes an irreversible proton-permeable form. Only in the presence of δ and α, does lethal proton-permeability result in *E. coli unc*- mutants which contain F_0 plasmids. This lethality is countered by the presence of γ (Pati and Brusilow, 1991; Monticello *et al.* 1992). If the mature form of *T. ferrooxidans* F_0 was lethal in *E. coli*, it may be that *T. ferrooxidans* F_0, in the presence of certain F_1 subunits will never complement *E. coli* F_0 mutants. As such, it would only be possible to clone all three *T. ferrooxidans* F_0 subunits in *E. coli* when they are assembled in the cytoplasmic membrane in the immature "non-leaky" form. Therefore to isolate *T. ferrooxidans* F_0 genes in *E. coli*, would only be possible by transforming *E. coli* DK8 (Δ*unc*) with a *T. ferrooxidans* gene bank prepared in a low-copy number vector and growing all transformants on LBA supplemented with appropriate antibiotic. All colonies would have to be screened with a DNA probe prepared from the 5'-end of the *T. ferrooxidans atp* genes present on p*Tfatp*2. Any colonies which gave a positive hybridisation signal may harbour the *T. ferrooxidans* F_0 genes. However, there is no guarantee that such an approach would work and other methods to isolate the entire *T. ferrooxidans atpPIB* fragment may provide more suitable alternatives.

Two methods designed to be independent of complementation of *E. coli unc* mutants were devised to isolate the *T. ferrooxidans* genomic fragment upstream of *atpE*. One was to clone a 2 kb *Eco*RV/*Xho*I *atpBEF* fragment (probably lacking the *atp* promoter), identified by

Southern hybridisation, against the *lacZ* promoter present on pBluescriptKS+ to reduce *atp* gene expression; the other was to amplify the fragment by PCR.

Numerous attempts to clone the *T. ferrooxidans* 2 kb *Xho*I/*Eco*RV *atpBEF* fragment in reverse orientation in pBluescriptKS+ in the heterologous host *E. coli* JM109, were unsuccessful. Although the ligation yielded only 229 insert-bearing transformants, this number was considered to be fairly representative of the fragment library generated from the genome, as *Xho*I, unlike *Eco*RV, does not cut the *T. ferrooxidans* ATCC 33020 chromosome extremely frequently (D.E. Rawlings and C. Kilkenny, personal communication). However, *Sst*I/*Xho*I digests of the inserts suggested that the correct fragment had not been cloned. Extensive SSP-PCR attempts to amplify the desired clone either from the ligation mixes prepared, or from pools of the recombinant plasmids generated, gave no result. As positive controls had indicated that PCR conditions were conducive to amplification of fragments both larger and smaller than the 2.1 kb *Xho*I/*Eco*RV fragment, these results suggested that the *Xho*I/*Eco*RV fragment had not been isolated by cloning in reverse orientation in pBluescriptKS+.

Other SSP-PCR attempts using various restriction endonuclease digests of the *T. ferrooxidans* chromosome gave no specific results. Extensive attempts to amplify fragments of unknown sizes in *T. ferrooxidans* chromosomal digests, including possibly large 4-5 kb *Xho*I/*Cla*I, and small *Xho*I/*Eco*RI inserts (Sections 5.2.8.2 and 5.3.1) consistently failed to produce any positive result from experimental ligation mixes.

The reasons for the failure at any level to clone any *T. ferrooxidans* genomic fragment to the extreme 5'-end of the *atp* genes present on p*Tfatp*2 whether by an "antisense" or SSP-PCR method, remain unknown. Varying experimental conditions such as restriction enzyme concentrations, vector:insert ratios in ligation mixes, or PCR parameters, did not prove successful.

The variation of the LMPCR methodology (Section 5.2.1) was completely unsuccessful in the current study. Even the positive control (linearised p*Tfatp*3001) failed to produce a fragment of the expected size. This clearly indicated that either PCR parameters were too stringent or that the method had failed elsewhere. Correct annealing temperatures are critical to the method (Ausubel *et al.*, 1993). As all oligonucleotide primers used successfully amplified fragments from both p*Tfatp*3001 and p*Tfatp*2001 at temperatures as high as 60°C, it is likely that the reasons for the failure lay elsewhere. The method relies on the production of blunt-ended DNA fragments by $Vent_R$ polymerase after the first strand synthesis step, which are then ligated to an annealed staggered primer at the 5'-end of the fragments generated. If failure occurs at this stage, then the method will not succeed. It is imperative that LMPCR1 and LMPCR2 are purified and annealed prior to

the ligation step. As far as could be ascertained by the use of gels, cartridge-purified LMPCR1 and LMPCR2 had annealed satisfactorally. The method as prescribed by Ausubel *et al.* (1993) was developed for the analysis of mammalian DNA based on Maxam-Gilbert chemistry, but in principle, any site-specific restriction endonuclease which yields cleaved DNA with a 5'-phosphate can be used. Therefore in theory, the ligation step if carried out under the correct conditions, should not be a problem. However, it is possible that ligation did not occur and refinement of optimal conditions required for this step of the protocol, at least for the experiment under review, may be required.

Chromosome "walking" by PCR was successfully used elsewhere to amplify *E. hirae atp* genes which were presumably harmful to *E. coli* (Shibata *et al.*, 1992). Although the authors did not specify their precise method, it is possible that they used the single-sided PCR method known as "inverse PCR" as described by Triglia *et al.* (1988). As other ligation-mediated attempts to amplify any *T. ferrooxidans atpPIB* fragment were unsuccessful, inverse PCR might provide a suitable alternative. Obviously suitable restriction enzyme sites must be available. In this regard, it could be of value to utilise the 5.3 kb *Xho*I *T. ferrooxidans* genomic fragment mapped during this study and known to lie upstream of the internal *atpH Xho*I site (Fig. 5.4). The disadvantage would be that the fragment size to be amplified (approximately 4 kb) lies at the outer limits suitable for PCR (Triglia *et al.*, 1988). Alternatively, more suitable smaller *T. ferrooxidans* genomic fragments will have to be identified.

A further instance reporting the cloning of the unknown 5'-end of an *atp* operon by the use of PCR was reported. In this case, the organism was *V. parahaemolyticus*. Primers were designed which were homologous to a promoter region or complementary to an *atpB* region of the *V. alginolyticus atp* operon (Sakai-Tomita *et al.*, 1992). The authors were fortunate in that homology of the promoter and *atpB* regions of the *atp* operons of the two species was similar enough to result in successful amplification of the desired fragment. No sequence has been reported for the *atp* operon from any other *Thiobacillus* species; therefore it is not possible to design a specific oligonucleotide primer for this region of the *T. ferrooxidans* chromosome.

Shibata *et al.* (1992) reported that oligonucleotide probes designed to be homologous or complementary to highly conserved regions of *E. coli uncD* were successfully annealed to the *atpD* on the *V. parahaemolyticus* chromosome. Therefore if a highly conserved sequence occurred in the *atpIB* regions amongst differing bacterial genera, a primer could be designed to amplify the region in the *T. ferrooxidans atp* operon. However, the fact that *atpIB* regions are characterised by poorly conserved primary sequence (reviewed in Senior, 1990) makes this approach unsuitable.

The large *Bgl*II and *Cla*I fragments mapped in the current study (Fig. 5.1) which were possibly located to the 5'-end of the *T. ferrooxidans atp* genes on p*Tfatp*2, provide a base from which to continue attempts to isolate the *T. ferrooxidans atpPIB* region. The precise location of these fragments still requires confirmation. This could be done by probing appropriate *Cla*I and *Bgl*II chromosomal digests with p*Tfatp*3150. Further restriction enzymes might provide more suitable alternative *T. ferrooxidans* genomic fragments which lie upstream of *atpE*. As a *T. ferrooxidans* chromosomal *Xho*I site was identified approximately 5 kb upstream of the internal *atpH Xho*I site (Fig. 5.3) the use of this enzyme as an "anchor" to identify various upstream *Xho*I/? restriction endonuclease double digests could provide sized fragments ideal for further isolation attempts by PCR or "antisense" cloning.

It is recommended that any recombinant plasmids which might carry the 5'-end of the *T. ferrooxidans atp* operon cloned in reverse orientation in a plasmid vector, should be transformed into *E. coli* DK8 (Δ*unc*). For reasons explained above, in the event of translation of these genes, use of this strain could overcome possible harmful effects of *T. ferrooxidans* F_0 polypeptides.

RNA extraction procedures for both *E. coli* and *T. ferrooxidans* ATCC 33020 gave high yields of the product. In the case of *T. ferrooxidans*, best yields were those from cultures from which RNA was extracted immediately, as opposed to those which had been frozen at -70°C and then revived (Section 5.2.4). Nevertheless, the storage of viable *T. ferrooxidans* cells at -70°C does offer the means to harvest RNA from the organism without having to initially run a bulk culture.

The RNA extracted from both *E. coli* and *T. ferrooxidans* by the hot-phenol methods described above, appeared to be of satisfactory quality (Fig. 5.4). In *T. ferrooxidans*, the method described was similar to that of Inoue *et al.* (1991) and Kusano *et al.* (1991b and 1992b) who also utilised a hot-phenol extraction procedure for ferrous iron-grown cells. Therefore, the results obtained in the Northern hybridisation experiments were disappointing, as it appeared that mRNA degradation had occurred, resulting in the non-specific radioactivity noted in Fig. 5.5. Improved results might have been obtained had the RNA been glyoxylated and electrophoresed through gels containing glyoxal/DMSO prior to nitrocellulose transfer (Sambrook *et al.*, 1989). However, other groups which isolated mRNA from *T. ferrooxidans* worked routinely with formaldehyde gels (Inoue *et al.*, 1991; Kusano *et al.*, 1991b and 1992b).

In a study on the relative stability of the *E. coli unc* operon transcript, McCarthy *et al.* (1991) deduced approximate chemical half lives of specific regions as being 4 min for *atpI*,

>8 min for *atpE* and 13 min for *atpC*. The authors reported consistent difficulty in being able to detect the full-sized 7 kb transcript due to the rapid degradation of the *uncI-B* transcript. It was found that by expressing *unc* genes in an *E. coli ams* (altered mRNA stability) strain HAK117, where the chemical half-life of specific messages was extended, the full-size 7 kb transcript was visible in Northern hybridisation studies. It was suggested that the *ams* mutation prevented RNase E cleavage of the *uncIB* transcript. The study by McCarthy *et al.* (1991) is of relevance to the extraction of the *T. ferrooxidans atp* transcript because if stability of the *T. ferrooxidans* transcript was similar to that of *E. coli*, the time between cell harvest and lysis in the current study was too long. Alternative methods will have to be devised such that there is rapid killing of *T. ferrooxidans* cells or forced cessation of the cell activity immediately after harvest and prior to washing. It would therefore better to work with maximum subculture volumes of 25 ml after the initial bulk harvest. The addition of rifampicin to the *T. ferrooxidans* growth medium prior to harvest might retard mRNA decay, as reported for *E. coli* transcripts (McCarthy *et al.*, 1991), but Rawlings *et al.* (1991) reported that rifampicin was unstable at the low pH and high metal ion concentrations of the *T. ferrooxidans* growth medium. Shibata *et al.* (1992) reported that for *E. hirae*, quality mRNA was obtained by pouring actively growing cultures over an equal volume of frozen "killing buffer" (20 mM Tris-HCl, 5 mM $MgCl_2$, 20 mM NaN_3, pH 7.3). Rapid chilling of exponentially growing *T. ferrooxidans* cells may assist in alleviating mRNA degadation. However, the major problem with *T. ferrooxidans* prior to killing, is to free the cells of iron salts, and then to suspend the cells in a solution at a pH of approximately 4, which is equivalent to the pH of the lysis buffer used in the current study. To overcome the problem of iron precipitates, axenic cultures of *T. ferrooxidans* could be grown in iron-free media, where inorganic sulphur serves as the energy source. For the future extraction of mRNA from *T. ferrooxidans* ATCC 33020, a killing buffer with a maximum pH of 2 should be developed. Such a buffer should be frozen at -70°C and a 25-50 ml subculture of actively growing cells poured over. The killed, unlysed cells should be pelleted by centrifugation at 4°C and only then washed, prior to lysis. Neither Inoue *et al.* (1991) nor Kusano *et al.* (1991b, 1992b and 1993b) explained how they prepared iron-grown *T. ferrooxidans* Fe1 or E-15 cultures, prior to cell lysis and hot phenol extraction. It was of interest to note that in the current study, a hot-phenol extraction of RNA from *T. ferrooxidans* cells harvested and immediately stored at -70°C for two weeks, yielded RNA which was totally degraded (results not shown). Therefore, although the cells remained viable at -70°C, RNA degradation still occurred. As there is no *ams* mutant for *T. ferrooxidans*, the use of such a strain is not possible to improve chemical stability of the RNA transcripts.

From an assessment of transcript size it was hoped to design a primer and by primer extension, identify a promoter on the *T. ferrooxidans* F_1 genes possibly located at the extreme 3'-end of *atpF* (Fig. 3.2) and recognised by *E. coli* DK8 for the transcription of

T. ferrooxidans F_1 genes. However, Northern blotting did not identify a discrete transcript. Instead, there was general hybridisation of the probe to the RNA prepared from *E. coli* DK8 (p*Tfatp*2) (Fig. 5.5). This indicated that either the transcript was not present, or if present, was at levels too low to be detected, or was degraded/unstable. Another possibility was that different multiple-sized transcripts were produced, many of which hybridised to the probe. In the absence of a discrete identifiable transcript, an oligonucleotide primer was designed 54 bp downstream of the putative σ^{70} -10 region tentatively identified in *atpF* (Fig. 3.2). However, despite using a range of experimental annealing temperatures, no transcriptional start site was mapped for *E. coli* DK8 (p*Tfatp*2) or *E. coli* DK8 (p*Tfatp*2, pAN45F_0) (Fig. 5.6). This implied that the hypothetical transcript may not have been produced by the cells in the growth medium used or if produced, was expressed at levels too low to detect and/or was highly unstable. A suitable alternative might be to culture *E. coli* DK8 (p*Tfatp*2, pAN45F_0) cells on solid MMS agar plates, to force production of F_1F_0 ATPsynthase, prior to RNA extraction. When attempts were made to do this in liquid MMS medium, harvesting and growth of cells was consistently hampered by the formation of precipitates in the shaken medium. The formation of magnesium-phosphate precipitates in glucose minimal medium was noted by Fillingame and Foster (1986) for growth of *E. coli* cells for F_1F_0 ATPsynthase production.

CHAPTER 6.

GENERAL DISCUSSION.

"In everything I did I was impressed by the marvel of the cell, in which I saw nothing but order and beauty." Chargaff, (1978).

A major function of this study was to isolate the genes responsible for the production of F_1F_0 ATPsynthase of *T. ferrooxidans* ATCC 33020, and having done so, to attempt to establish any features/adaptations of genes or gene products which might indicate unusual mechanisms of proton translocation and/or gating associated with the F_1F_0 ATPsynthase of an obligate extreme acidophile.

In the absence of both suitable *T. ferrooxidans* mutants and a genetic system for the organism, the use of *E. coli*, for which there are available a wide variety of *unc* mutants and a classic genetic system, provided a suitable heterologous host system for the current study. It should be noted that even if the means became available to produce *T. ferrooxidans atp* mutants, the screening of such mutants using the minimal succinate method would not be suitable. This is a direct result of the obligately chemoautotrophic nature of the organism and the fact that organic substances inhibit *T. ferrooxidans* growth. Therefore, an alternative method would have to be devised. During the current study, screening several *E. coli unc* mutants with *T. ferrooxidans* ATCC 33020 gene-banks resulted in the isolation of the *T. ferrooxidans atpEFHAGDC* genes.

The interpretation of the predicted primary sequence of the seven *T. ferrooxidans atp* open reading frames was valuable in that it provided structural and/or functional information of specific residues and domains in *T. ferrooxidans* F_1F_0 ATPsynthase. In the absence of any suitable test system for *T. ferrooxidans*, only speculative interpretations can be given. Fortunately, the extensive studies at the genetic level in the host system used viz. *E. coli*, offered an invaluable source from which to interpret the *T. ferrooxidans* ATCC 33020 *atp* sequence data.

Computer analyses predicted that generally, *T. ferrooxidans* F_1F_0 ATPsynthase subunits were probably folded in a manner similar to that predicted for the *E. coli* homologues. Residues/domains in *T. ferrooxidans* F_0 and F_1 subunits known to be critical in *E. coli* homologues for either inducing conformational change and/or catalysis, were identically conserved in the two organisms. Hence at these sites, the transmission of conformational change necessary for enzyme catalysis, and actual catalytic mechanism are probably similar in both organisms. However, various domains and/or isolated residues in certain *T. ferrooxidans* F_1F_0 ATPsynthase subunits, notably c, δ, γ, ε and even β were markedly

different from the homologous subunits in *E. coli* and other organisms. In some instances, these differences might be related to a unique mode of proton translocation and gating in an extreme acidophile.

By comparing primary sequence data of *T. ferrooxidans* subunit c with those from a number of other organisms, including the extensively studied proteolipid from *E. coli*, several unusual features were noted for *T. ferrooxidans* c. The *T. ferrooxidans* c subunit was comprised of an ensemble of amino acids which may indicate an unusual proton pathway within the F_0 channel. Certain domains/residues within *T. ferrooxidans* F_1 subunits were unique to the organism. As most of these unusual features were associated with regions which in *E. coli* F_1 are known to be important for proton gating and/or subunit interaction, it is possible that these mechanisms are unusual in *T. ferrooxidans*. Domains referred to are the N-terminus of δ, the extreme C-termini of γ and ε, and the extreme N- and C- termini of β. Typically, these domains were comprised of either charged and/or aromatic residues. The constitution of the *T. ferrooxidans* γ residue was particularly interesting and may imply a unique form of proton gating. To prove any function for an isolated residue or domain of a *T. ferrooxidans* F_1F_0 ATPsynthase subunit, would require detailed mutagenesis of the residues and screening of defined *T. ferrooxidans atp* mutants to assess the function of such mutations in a homogeneous system. At this stage, such an analysis is not possible. It would be of little value to screen defined *E. coli unc* mutants with mutated *T. ferrooxidans atp* genes, as the system is heterologous.

Complementation studies of *E. coli unc* mutants by *T. ferrooxidans* suggested that the control of proton translocation in *T. ferrooxidans* F_1F_0 ATPsynthase is excercised primarily at the level of F_0. Despite the fact that the *T. ferrooxidans atpE* and *F* genes were isolated and expressed *in vitro* by an *E. coli*-derived transcription/translation system, these genes did not functionally complement *E. coli uncE* and *F* mutants *in vivo*. Furthermore, *T. ferrooxidans atpB* was not isolated. It was not possible during the course of this study, to obtain either heterogeneous *T. ferrooxidans*/*E. coli* F_0 or homogeneous *T. ferrooxidans* F_0 oligomers which were functional in *E. coli unc* mutants. Although F_1F_0 ATPsynthase is common to both *T. ferrooxidans* and *E. coli*, and eight different *E. coli unc* mutants were available for screening as suitable genetic markers, it was striking that the entire *T. ferrooxidans* F_0 gene cluster was not cloned by physical linkage to the F_1 genes through functional complementation of any of these mutants (Chapters 2 and 5). It is significant that F_1F_0 ATPsynthase is intimately associated with transmembrane proton movement. It is possible that the overall mechanism of proton translocation within the F_0 channel and the subsequent coupling of this mechanism to oxidative phosphorylation in F_1 is sufficiently different between an obligate acidophile and a neutrophile to make the F_0 oligomers functionally incompatible. However, it is notable that while there are many reports on functional heterogeneous F_1 particles in *E. coli unc* mutants (reviewed in

Chapters 2 and 4), none reports a functional heterogeneous F_0 particle in the organism. Whether this is due to a lack of experimentation in this area, or to the fact that heterogeneous F_0 particles are generally non-functional in *E. coli*, is unknown. Complementation of various *E. coli* F_0 mutants by other prokaryotic genes has only been reported where the F_0 particle was entirely composed of subunits from the heterologous organism. Unlike *T. ferrooxidans*, the F_0 particle from *V. alginolyticus*, in combination with the *V. alginolyticus* F_1 oligomer, functionally complemented an *E. coli* Δ*unc* strain (Krumholz *et al.*, 1990). It has not been determined whether the *V. alginolyticus* F_0 particle can form a functional F_1F_0 ATPsynthase with an *E. coli* F_1 oligomer in a suitable *E. coli unc* mutant. Kauffer *et al.* (1987) reported a functional hybrid *K. pneumoniae* F_0/*E. coli* F_1 ATPsynthase in *K. pneumoniae* F_1-stripped vesicles. Although the sodium-ion translocating F_0 oligomer from *P. modestum* can function within an *E. coli* F_0 mutant (Kaim *et al.*, 1992) (see below), results from the complementation of defective single F_0 subunits in *E. coli* by corresponding *P. modestum* subunits were not unequivocal. It was suggested that the a subunit from *P. modestum* could interfere with the b and c subunits of *E. coli* (G.W. Kaim, personal communication). Therefore as far as the current study is concerned, it is notable that the entire *T. ferrooxidans* F_0 oligomer (as opposed to single *T. ferrooxidans* F_0 subunits) appeared to be non-functional in the *E. coli unc* mutants screened.

Prior to this study, there had been no reports of cross-complementation studies between acidophilic and neutrophilic F_1F_0 ATPsynthases. Complementation of *E. coli* F_1 mutants by *T. ferrooxidans* F_1 genes indicated that certain hybrid F_1F_0 ATPsynthases, which consisted of either a homogeneous *T. ferrooxidans* F_1, or a heterogeneous *T. ferrooxidans*/*E. coli* F_1, together with an *E. coli* F_0 oligomer, were functionally active. It was demonstrated in two instances in *E. coli* β^- and ε^- mutants, that together, *T. ferrooxidans* F_1 β and ε, in combination with the a, c, b, δ, and α *E. coli* subunits, formed active F_1F_0 ATPsynthases. However, the *T. ferrooxidans* ε subunit when expressed on its own, was unable to complement an *E. coli uncC* mutant. No conclusions could be made to determine whether the γ subunit in these two latter hybrids was of *T. ferrooxidans* or *E. coli* origin. The extent to which hybrid acidophilic/neutrophilic F_1 oligomers can be functionally reconstituted has not yet been fully exploited and could form the basis of future studies. Using plasmids generated during the ExoIII shortening of p*Tfatp*2, it should be possible to clone specific *T. ferrooxidans* F_1 *atp* genes behind a promoter (recognised by *E. coli*) in a low-copy number vector such that they are expressed in *E. coli*. These constructs would extend the cross-complementation data produced during the course of the current study. It would be particularly interesting in view of the unique features noted for the *T. ferrooxidans* γ subunit, to determine whether this subunit when expressed on its own, can form a functionally recontitutable F_1F_0 ATPsynthase in an *E. coli* γ^- mutant. If not, the minimal functional *T. ferrooxidans* γ/? subunit combination required to provide a functional hybrid F_1F_0 ATPsynthase in *E. coli* could be determined. Similar studies could

be conducted for the *T. ferrooxidans* α and δ subunits. The studies could be extended to gauging efficacy of ATPsynthase activity by the preparation of membrane vesicles and associated ACMA (9-amino-6-chloro-2-methoxyacridine) fluorescence quenching data, to determine proton-pumping capacity of the recombinant enzymes in *E. coli*. In the absence of a workable *in situ* situation for *T. ferrooxidans*, such studies could provide insight into the mechanism of the F_1F_0 ATPsynthase of an acidophilic organism, and possibly further explain functional impairments noted for the hybrid F_1F_0 ATPsynthases generated during this study.

Growth rates of *E. coli unc* mutants harbouring hybrid F_1F_0 ATPsynthases were notably slower than *unc* mutants transformed with the *E. coli unc* operon. ATPase specific activity assays demonstrated low hydrolytic activity of a *T. ferrooxidans* F_1/*E. coli* F_0 hybrid, and that this was probably a result of impaired coupling and multi-site (co-operative) catalysis. Therefore, although control of proton translocation is probably primarily a function of *T. ferrooxidans* F_0 subunits, it cannot be discounted that the F_1 subunits are not involved at a secondary level. This was predicted from the unusual features of primary sequence noted for various *T. ferrooxidans* F_1 subunits which indicated that these subunits would interact optimally with *T. ferrooxidans* F_0. It would be of value to extend the experiments attempted during the current study, by isolating active *T. ferrooxidans* ATCC 33020 membranes and everted membrane vesicles, in order to measure both ATP -ase and synthase catalytic activity of the wild-type enzyme. It would also be of interest to strip *T. ferrooxidans* vesicles of F_1, and determine whether F_1F_0 ATPsynthase activity could be reconstituted in these vesicles by using purified *E. coli* F_1. With active membrane preparations, the effect, if any, of sulphate on *T. ferrooxidans* F_1F_0 ATPsynthase could be determined. As reviewed in Chapter 1, the hydrolytic activity of F_1F_0 ATPsynthase of certain bacteria is enhanced by the presence of sulphate. Although it is known that the latter divalent anion is necessary for *T. ferrooxidans* growth, the reason for this requirement has not yet been finally established. Bakker (1990) suggested that sulphate might be important in mechanisms of cytoplasmic homeostasis; others suggested that the function is of a physico-chemical nature (Dugan and Lundgren, 1965; Ingledew, 1982) (Chapter 1). Fluorescense quenching of *T. ferrooxidans* ATCC 33020 vesicle preparations would be particularly valuable to determine the proton-pumping capacity of the F_1F_0 ATPsynthase of the species, about which little is known.

From the data generated during the current study pertinent to *T. ferrooxidans* F_1F_0 ATPsynthase structure and function, it was not possible to positively identify any features in seven of the enzyme subunits which may be involved in the active regulation of the components of $\Delta\mu_{H+}$. It does seem likely that there is an unusual mechanism of proton translocation and/or gating, and it is possible that this could influence the magnitude of ΔpH. During active metabolism, the entry of protons into the *T. ferrooxidans* cell through

the F_1F_0 ATPsynthase must be tightly regulated to prevent collapse of ΔpH. However, from data generated elsewhere (Beck, 1960, cited by Krulwich and Guffanti, 1983), it appears that as starving *T. ferrooxidans* cells maintain a residual ΔpH even when $\Delta\mu_{H+}$ has collapsed, F_1F_0 ATPsynthase activity is not solely responsible for magnitude of the ΔpH component of $\Delta\mu_{H+}$ (reviewed in Chapter 1). The precise effect of *T. ferrooxidans* F_1F_0 ATPsynthase on $\Delta\mu_{H+}$ during steady state growth would require the determination of the magnitude of ΔpH and $\Delta\Psi$ in response to changes in pH_o in *T. ferrooxidans* membrane vesicles and/or entire cells in which the F_1F_0 ATPsynthase has been specifically inactivated either by the use of DCCD or mutation.

A further objective of the current study was to determine whether an analysis of the nucleotide sequence of the seven *T. ferrooxidans atp* open reading frames could provide information regarding the regulation of translation of the operon. Codon usage patterns of most open reading frames was generally typical of that noted previously for strain ATCC 33020. An unusual codon usage pattern was noted for *atpE*, although the small size of the gene made these data statistically unreliable. The analysis of the nucleotide sequence of the intergenic regions and TIRs of the *T. ferrooxidans atp* operon, in which equivalent regions of the heterologous host system *E. coli* were compared, indicated regions of similarity and difference between the operons of the two species. Where similarities were observed, it is likely that similar mechanisms of control of gene expression at the level of post-transcription might operate. Sequence in the *atpE* TIR region suggested an upgrading in the rates of gene expression between adjacent cistrons on the operon. The TIR was unusually high in U, and in *E. coli* a similar phenomonen was demonstrated to be highly efficient in the upgrading of *uncE* translation rate (McCarthy *et al.*, 1985). This could be tested for the *T. ferrooxidans atpE* TIR by fusing this region upstream of an AUG start codon of a gene normally expressed at low frequency in *E. coli*, and assaying for an increase in levels of the gene product. The terminator region of the *T. ferrooxidans atp* operon was characterised by the presence of nucleotide sequence which computer analysis predicted could form two highly stable tandem stemloop structures. The loop to the 3'-end of the pair was typical of a *rho*-independent terminator. The function of the 5'-end loop is unknown, but it could be implicated in control of *atpC* gene expression. Unfortunately, the lack of any workable genetic system for *T. ferrooxidans* ATCC 33020 precludes experimental testing of any of these observations *in situ*, as has been extensively done for *E. coli* in the laboratories of McCarthy (Hellmuth *et al.*, 1991; reviewed in McCarthy, 1990).

F_1F_0 ATPsynthase is considered to be an evolutionarily ancient enzyme (Nelson and Taiz, 1989). As a result, various authors were of the opinion that derived primary amino acid sequence of certain F_1F_0 ATPsynthase subunits, notably β and c, could be used to predict phylogenies of organisms (Amann *et al.*, 1988a and b; Nelson and Taiz, 1989; Recipon *et al.*,

1992). Therefore, an objective of the current study was to determine whether the sequence of the seven *T. ferrooxidans atp* genes could be interpreted likewise. Data obtained from 5S and 16S rRNA for *T. ferrooxidans* showed the organism to be a member of the poorly characterised β-proteobacteria (Lane *et al.*, 1992; Woese, 1987). Predicted polypeptide sequence for the *T. ferrooxidans* ATCC 33020 *glnA, ntrA, ntrB, ntrC* and *recA* gene products clustered *T. ferrooxidans* within the β-proteobacteria. However, the *nifH* polypeptide showed a close relationship to the homologous protein from the α-proteobacterium, *Bradyrhizobium* (D.E. Rawlings, personal communication). The arrangement of the *T. ferrooxidans atp* gene cluster was most like that of the γ-proteobacteria, *V. alginolyticus* and *E. coli*, both in constitution and chromosomal location (Fig. 3.5). Analysis of the primary sequence of *T. ferrooxidans* F_1F_0 ATPsynthase b, δ, α, γ, β, and ε, clustered the organism as a separate branch (β) of the proteobacteria. Predicted phylogenies of 20 different organisms including *T. ferrooxidans*, based on a comparative analysis of the β subunit, was a reflection of that predicted by rRNA data. However, the *T. ferrooxidans* c subunit in a comparison with 14 other organisms formed a distinct outgroup. There was no indication of the phylogenetic relatedness to any of the other organisms included. This is most likely a result of the adaptation the subunit has undergone to the constraints of extreme acidophily, and the function of the subunit in regulating transmembrane proton movement. It would be of interest to include the data for the *T. ferrooxidans* c subunit in the computer analysis developed by Recipon *et al.* (1992) wherein it was predicted that the primary sequence from the proteolipid from a wide variety of organisms could be used to establish phylogenetic trends.

It remains a challenge to develop an alternative means to isolate the entire *T. ferrooxidans* F_0 gene fragment. As far as the author is aware, this study represented the first attempt to clone a gene for a *T. ferrooxidans* membrane-associated protein by complementation in a heterologous host, and demonstrated the restrictions of this approach. The F_1 subunits of the *T. ferrooxidans* enzyme located in the neutral cytosol and associated with catalytic mechanism, were isolated by complementation of *E. coli unc* mutants. However, as discussed above, and elsewhere (Chapters 2 and 5), the F_0 subunits appeared unable to complement the *unc* mutants screened. It is possible that this is due to structural and/or functional adaptations that acidophilic bacterial membrane-associated proteins have undergone such that they have become acid-compatible (Bakker, 1990) and are no longer neutrophile-compatible. Therefore, it may not be possible to use complementation of *E. coli unc* mutants to isolate the entire *T. ferrooxidans* F_0 gene cluster, even though the F_0 oligomers from the two organisms are (in theory) generally functionally equivalent.

To attempt to isolate the extreme 5'-end of the *T. ferrooxidans atp* operon promoter, the following methods could be used:-

i). Isolate the upstream fragment on the *T. ferrooxidans* chromosome immediately upstream of the 5'-end of the *atpE* gene, and clone it in reverse orientation with respect to an IPTG-inducible vector promoter in *E. coli*.

ii). Amplify a suitably sized fragment identified as probably carrying the *atpPIB* region using PCR.

iii). Use an *E. coli* Δ*unc* mutant to clone a non-functional *T. ferrooxidans* F_0 particle.

iv). Use an *E. coli* F_0 mutant not yet screened with *T. ferrooxidans* gene banks during the current study.

v). Screen a suitable heterologous host system other than *E. coli*, with *T. ferrooxidans* gene banks.

Methods (i)-(iii) were described in detail in Chapter 5. With reference to method (iv), it is notable that in a study on the complementation of *E. coli unc* mutants transformed with a plasmid carrying the *P. modestum* F_0 genes, the efficacy of complementation depended on the *E. coli* strain used. Better results were obtained with *E. coli* CM1470 (Δ*atpIBEFA*) than with *E. coli* CM2080 (Δ*atpIB*) (Kaim *et al.*, 1992). It has since been shown that functional complementation of the former strain was dependent on the integration of the *P. modestum atpIBEFA* genes into the genome of *E. coli* CM1470 (G.W. Kaim, personal communication). It may be worthwhile attempting to establish whether *E. coli* CM1470 can be similarly complemented by *T. ferrooxidans atp* genes. Studies cited elsewhere in the text (Chapter 4, Section 4.4) demonstrated that the extent of functional complementation of *E. coli unc* mutants by *B. megaterium atp* genes was partially dependent on the genotype of the mutant used (Scarpetta *et al.*, 1991).

With reference to method (v), it is possible that in another heterologous host system, *T. ferrooxidans* F_0 genes which may be harmful when expressed in *E. coli*, may not be in the alternative host. In this regard, it may be worthwhile to screen the gram-positive *B. megaterium*, for which an *atp* mutant has been described (Decker and Lang, 1977, cited by Hawthorne and Brusilow, 1986) with a *T. ferrooxidans* ATCC 33020 gene bank, and to test for functional complementation on MMS.

It was hoped during the course of the current study to develop a routine procedure for mRNA extraction from *T. ferrooxidans* ATCC 33020. Although RNA was extracted, it appeared that the mRNA fraction of the *atp* operon transcript was degraded during the long washing interval between harvesting and lysing *T. ferrooxidans* cells. Although the principal aim of this section of the study was to determine transcript size, the availability of high quality mRNA from *T. ferrooxidans* ATCC 33020 could be used to expand the current study in other areas. Assuming that the complete *T. ferrooxidans* ATCC 33020 *atp* operon will in time be isolated and sequenced, the entire mRNA transcript would enable primer extension studies, as was reported for *T. ferrooxidans* strains Fe1 and E-15 (Inoue *et*

al., 1990; Kusano *et al.*, 1991b; 1992b; 1993a). With the technology becoming available to study prokaryotic gene regulation *in vivo* by the synthesis of cDNA libraries (Chuang *et al.*, 1993), the influence of various environmental factors on the expression of the *T. ferrooxidans* ATCC 33020 operon could be studied. For example, qualititative and quantitative levels of *atp* gene transcripts as influenced by pH_o could be determined; it could then be established whether a decrease in pH_o stimulates levels of *atp* gene expression in *T. ferrooxidans*, as was reported for the acido-tolerant lactic acid bacteria, which utilise F_1F_0 ATPsynthase to regulate pH_i (Kobayashi, 1985; Kobayashi *et al.*, 1984 and 1986; Sturr and Marquis, 1992). Methods which involve rapid killing of exponentially growing cultures of *T. ferrooxidans* ATCC 33020 cells, before proceeding to cell washing and lysis for RNA extraction were described in Chapter 5, Section 5.4. The routine availability of mRNA from *T. ferrooxidans* ATCC 33020 could be of value for many future molecular biological studies with this strain.

Table 1. Bacterial strains and plasmids used.

Strains	Genotype/characteristics	Source
Thiobacillus ferrooxidans ATCC33020	Wild type	Rockville, Md.
Escherichia coli		
K12	Wild type	Gibson *et al.* (1977)
LK111	*lac*^{q} *lac*^{z} ΔM15 *lacy*$^+$ *thi-1*	Zabeau and Stanley, (1982)
JM105	*supE endA sbcB*15 *hsdR*4 *rpsL thi*Δ (*lac - proAB*)	Yanisch-Perron *et al.*(1985)
JM109	*recA*1 *supE*44 *endA1 hsdR17 gyrA96 relA*1 *thi*Δ (*lac-proAB*)	Promega Corp., USA
GM41	*dam thi*-1 r*el*-1 HfrH	Marinus, (1973)
DK8	1100Δ (*uncB - uncC*)*ilv*::*TN10 bglR thi-1 HfrP01*	Klionsky *et al.* (1984)
AN727 (a^-)	*uncB402 argH pyrE entA recA nalA*	Gibson *et al.* (1977)
AN943 (c^-)	*uncE429 argG pyrE entA recA nalA*	Downie *et al.* (1980)
AN1440 (b^-)	*uncF469 argH pyrE entA recA nalA*	L. Hatch, John Curtin Med. School, Canberra
AN730 (α^-)	*uncA401 argH pyrE entA recA nalA*	Cox *et al.* (1978)
AN1273 (γ^-)	*uncG428 argH pyrE entA recA nalA*	Downie *et al.* (1980)
AN818 (β^-)	*uncD409 argG pyrE entA recA nalA*	Cox *et al.* (1978)
AN802 (ε^-)	*uncC424 argH pyrE entA recA nalA*	Gibson *et. al.* (1977)
Plasmids		
T. ferrooxidans	Ap^R	
p*Tfatp*1	Orientation forward. *atpGDC* on p*Eco*R251	This study
p*Tfatp*2	Orientation reverse *atpEFHAGDC* on p*Eco*R251	This study
p*Tfatp*12	0.842 kb *Eco*RV deletion of p*Tfatp*1 (*atp* $G^+D^-C^-$)`	This study

Plasmids	Genotype/characteristics	Source
p*Tfatp*1001	1.5kb *Cla*I - *HindIII* fragment from p*Tfatp*2, cloned into ρBluescriptSK (*atpC*$^+$), against *lacZ.*	This study
p*Tfatp*100	1.5kb *ClaI-Hind*III fragment from p*Tfatp*1001, cloned into puc18, using *Bam*HI and *Sal*I sites from p*Tfatp*1001, against *lacZ.* (*atpC*$^+$)	This study
p*Tfatp*2001	3.05kb *Xho*I-*ClaI* fragment from p*Tfatp*2, cloned into pBluescriptSK, against *lacZ* (*atpA*$^+$*G*$^+$*D*$^-$*C*$^-$)	This study
p*Tfatp*200	3.05kb *Xho*I-*Cla*I fragment from p*Tfatp*2001, cloned into pUC18, using the *Kpn*I and *Pst*I sites from p*Tfatp*2001, expressed off *lacZ.* (*atpA*$^+$*G*$^+$*D*$^-$*C*$^-$)	This study
p*Tfatp*3001	1.3kb *Bam*HI-*Xho*I fragment from p*Tfatp*2, cloned into pBluescriptSK, expressed off *lacZ* (*atpE*$^+$*F*$^+$*H*$^-$)	This study
p*Tfatp*300	1.3 kb *Bam*HI-*Xho*I fragment from p*Tfatp*3001,cloned into pUC18, against *lacZ,* using the *Kpn*I and *Bam*HI sites from p*Tfatp*3001 (*atpE*$^+$*F*$^+$*H*$^-$)	This study
p*Tfatp*400	1.5 kb *Cla*I-*Hind*III fragment from p*Tfatp*100, cloned into pUCBm21, using the *Sal*I and *Bam*HI sites from pUC18. expressed off *lacZ* (*atpC*$^+$)	This study
p*Tfatp*500	0.324kb *Cla*I-*Cla*I fragment from p*Tfatp*2, cloned into the *Cla*I site of pBluescriptSK. (Fragment of *atpD*)	This study

Plasmids	Genotype/characteristics	Source
E. coli		
pAN45	*uncBEFHAGDC*, cloned into pACYC184 (19.3kb Cm^RTc^S)	L. Hatch
pAN45Fo	6.6kb *Cla*I deletion of pAN45 (*unc* $B^+E^+F^+H^-$, Cm^RTc^S)	This study
General		
pUC18 and 19	Ap^R, cloning vectors	Norrander *et al.* (1983).
pUCBm21	Ap^R, cloning vector	Boehringer Mannheim.
pHC79	Ap^R, cloning vector	Boehringer Mannheim
p*Eco*R251	Cm^R, Ap^R, cloning vector	M. Zabeau, Plant Genetic Systems.
pBluescriptSK and KS	Ap^R, cloning vectors	Stratagene, California
pACYC184	Cm^RTc^R, cloning vector	Chang and Cohen, (1978).

APPENDIX B.

CULTURE MEDIA USED.

B.1.1. Minimal succinate medium (MMS) for *E.coli* AN mutants (after Gibson et al., 1977)

The minimal medium used contained 60 mM K_2HPO_4, 40 mM $NaH_2PO_4.2H_2O$, and 15 mM $(NH_4)_2SO_4$. The pH was adjusted to 7. This was sterilised by autoclaving and then 1 ml of concentrated trace-element solution (14 mM $ZnSO_4$, 1 mM $MnSO_4$, 4.7 mM H_3BO_3, 0.7 mM $CaSO_4$, 2.5 mM $CaCl_2$ and 1.8 mM $FeCl_3$)/l, was added. The following sterile solutions were added/l:-

30 mM sodium succinate; 1 mM $MgSO_4$; 0.2 µM thiamine hydrochloride; 40 µM 2,3,-dihydroxybenzoate; 0.8 mM L-arginine hydrochloride; 0.2 mM uracil; 0.15 mM adenine hydrochloride; 0.3 mM L-isoleucine; 0.3 mM L-valine.

Medium was solidified by the addition of 2% (w/v) Oxoid agar No 1. At times, sodium succinate was replaced by 30 mM glucose.

B.1.2. Minimal succinate medium (MMS) for *E.coli* DK8 (after Klionsky *et al.*, 1984).

The minimal medium used contained 50 mM K_2HPO_4, 50 mM K_2HPO_4, and 15 mM $(NH_4)_2SO_4$, pH 7.1. After sterilisation, 1 ml/l of the trace element mix described for the AN MMS medium was added. This was followed by the addition of sterile/l:-

12 mM potassium succinate; 1 mM $MgSO_4$; 0.2 µM thiamine hydrochloride; 0.3 mM L-isoleucine; 0.3 mM L-valine.

Medium was solidified by the addition of 1% (w/v) Oxoid agar No 1. At times, potassium succinate was replaced by 12 mM glucose.

B.1.3. Luria Bertani medium (LB) (Sambrook *et al.*, 1989).

To make 1000 ml of liquid medium, the following were mixed;

10 g Bacto tryptone; 5 g yeast extract; 5 g NaCl; 1000 ml distilled water.

Solid medium **(LBA)** contained 1.5% agar.

B.1.4. Modified LB medium (MLB)

To make 1000 ml medium, the following were mixed;

10 g Bacto tryptone; 5 g yeast extract; 5 g NaCl; 200 mM KCl; 2 g glucose; 900 ml water (pH 7.5). This solution was sterilised and 100 ml sterile 100 mM $MgSO_4$ was added.

B.2. Media preparation.

Heat-labile substances were filter-sterilised by passage through 0.22 µm membranes; otherwise all media solutions were sterilised by autoclaving at 121°C for 20 min.

APPENDIX C.

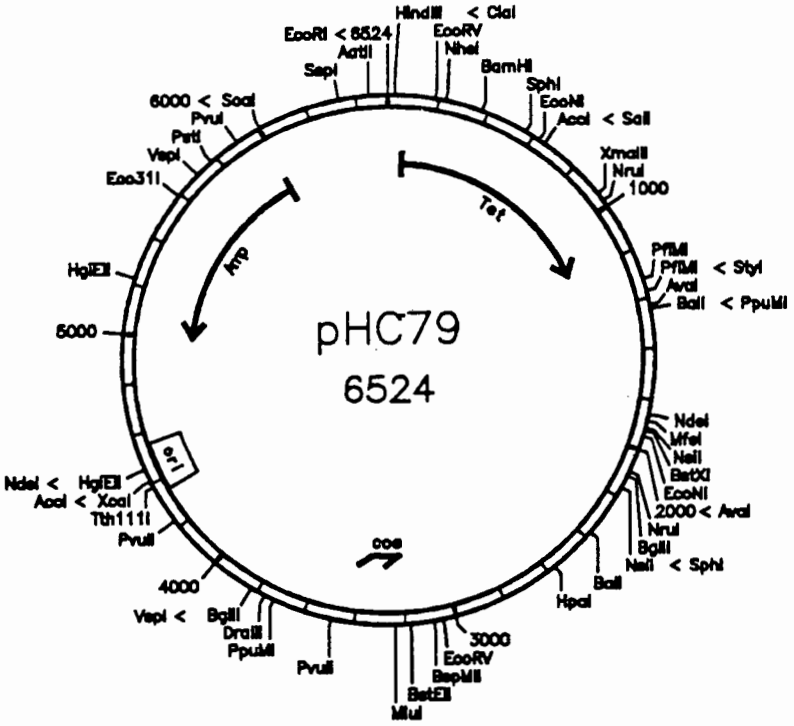
ONE- AND THREE-LETTER CODES FOR AMINO ACIDS.

Amino acid	Codes		Code	Amino acid
Alanine	Ala	A	A	Alanine
Arginine	Arg	R	C	Cysteine
Asparagine	Asn	N	D	Aspartic acid
Aspartic acid	Asp	D	E	Glutamic acid
Cysteine	Cys	C	F	Phenylalanine
Glutamine	Gln	Q	G	Glycine
Glutamic acid	Glu	E	H	Histidine
Glycine	Gly	G	I	Isoleucine
Histidine	His	H	K	Lysine
Isoleucine	Ile	I	L	Leucine
Leucine	Leu	L	M	Methionine
Lysine	Lys	K	N	Asparagine
Methionine	Met	M	P	Proline
Phenylalanine	Phe	F	Q	Glutamine
Proline	Pro	P	R	Arginine
Serine	Ser	S	S	Serine
Threonine	Thr	T	T	Threonine
Tryptophan	Trp	W	V	Valine
Tyrosine	Tyr	Y	W	Tryptophan
Valine	Val	V	Y	Tyrosine

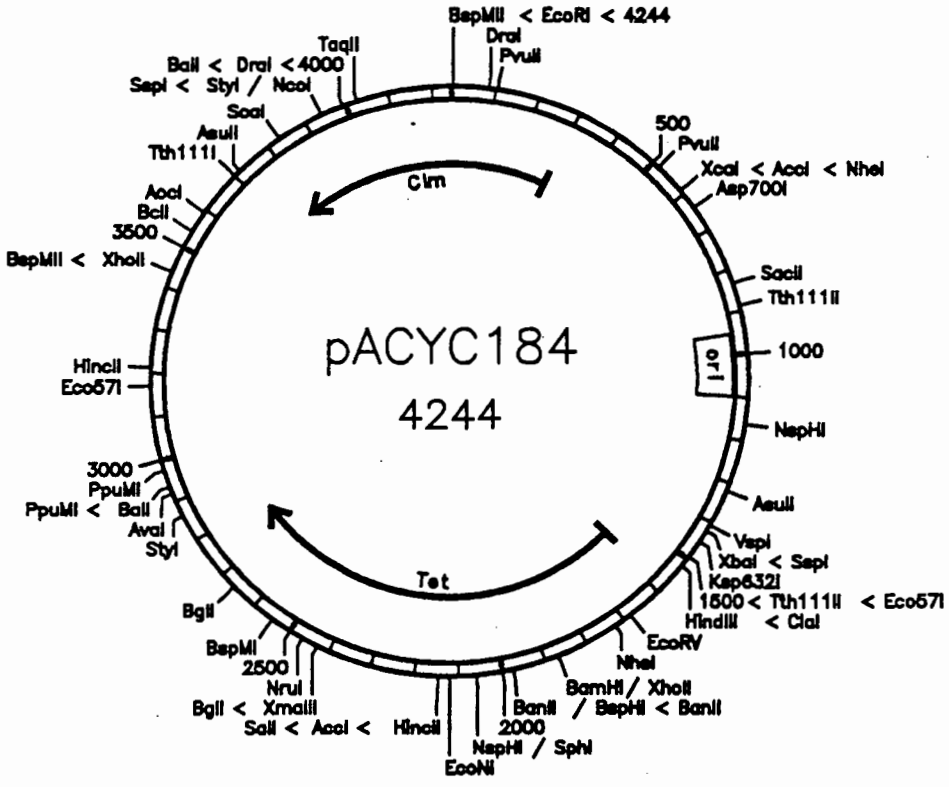
APPENDIX D.

PLASMID VECTOR MAPS

Maps show restriction enzymes (6 bp recognition) with a maximum of two sites.

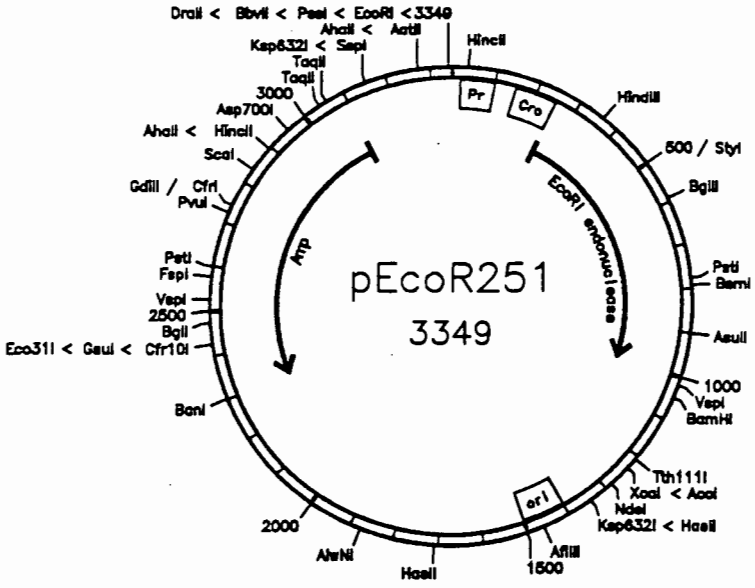


Hohn and Collins (1980)

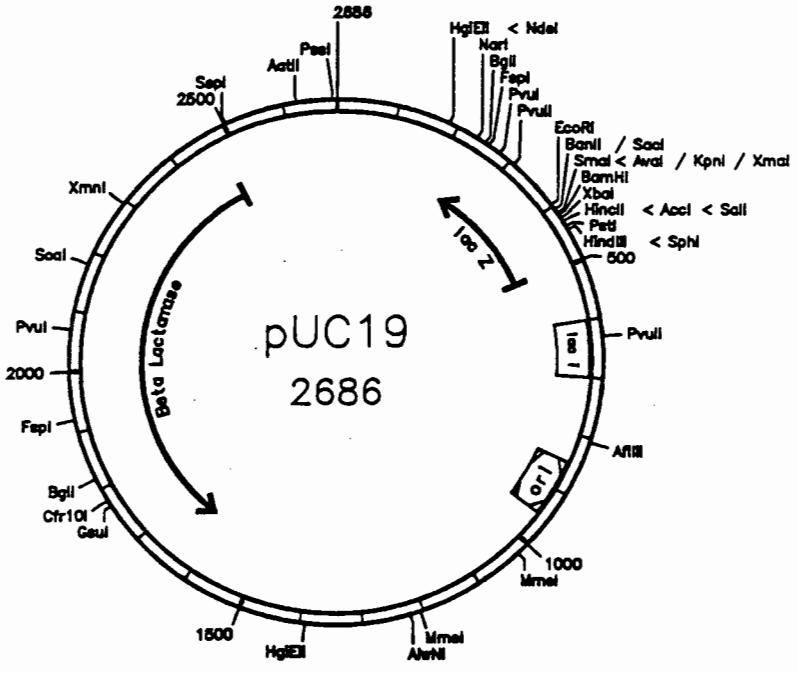


Chang and Cohen (1978)

APPENDIX D.

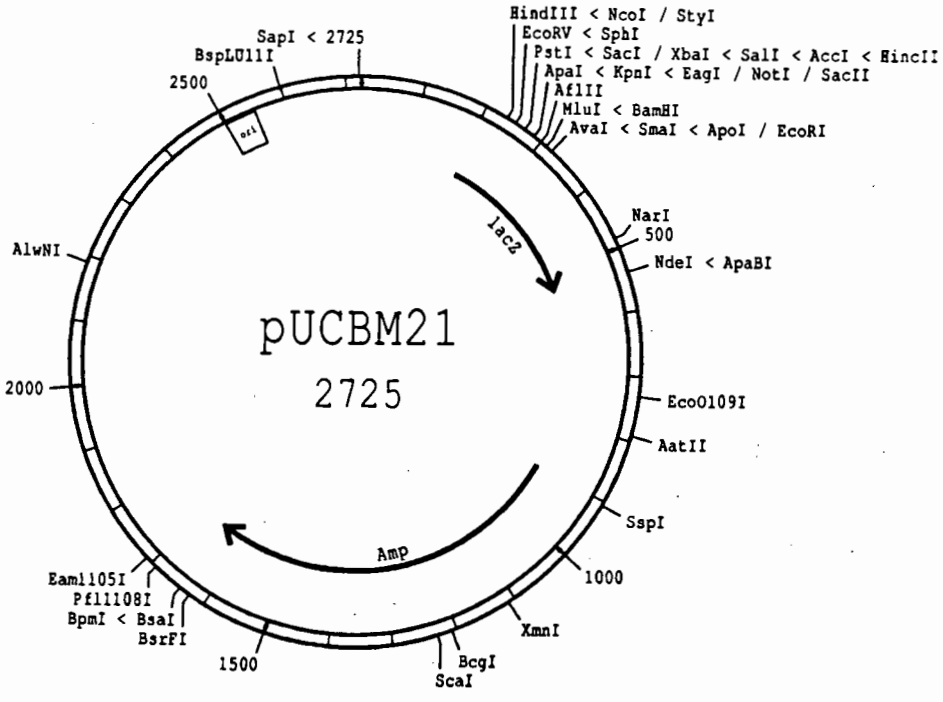


pEcoR251 is a positive selection vector used routinely by insertional activation of the *Eco*RI endonuclease gene at the *Bgl*II or *Hind*III site.
Zabeau and Stanley (1982).

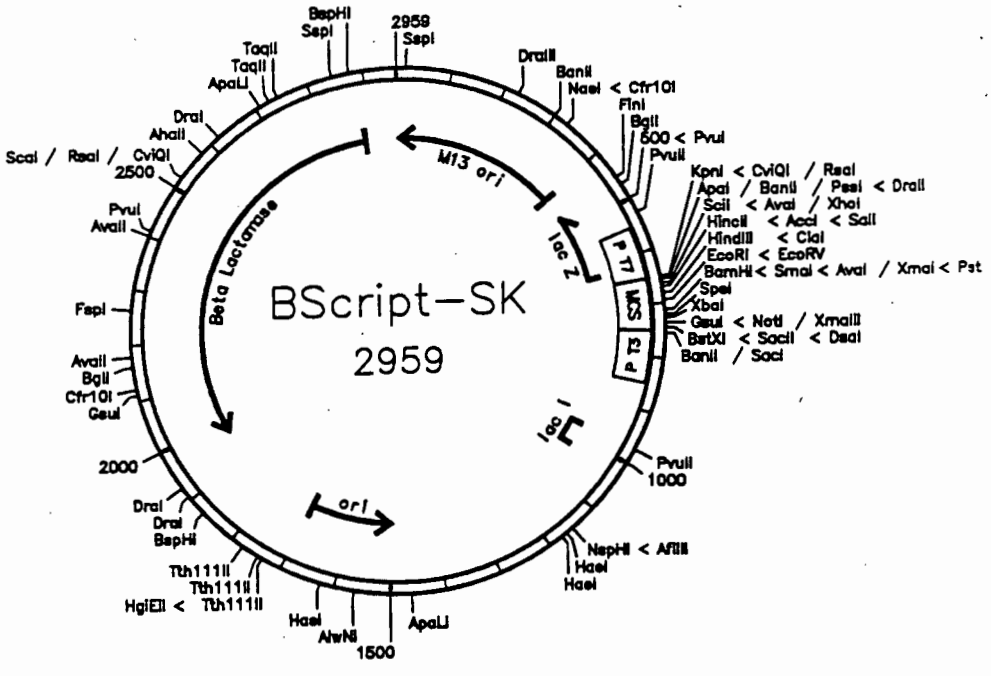


pUC18 differs from **pUC19** in the orientation of the multiple cloning site (MCS) polylinker between the *Eco*RI and *Hind*III sites.
The forward primer (-40) (5'-GTT TTC CCA GTC ACG AC-3') is situated on the *Eco*RI side of the MCS in **pUC19**. Norrander *et al.* (1983).

APPENDIX D.



Map of **pUCBM21.** A derivative of pUC19 which contains a different MCS.



Map of **Bluescript SK+**. **Bluescript KS+** differs in the orientation of the MCS polylinker between the *KpnI* and *SacI* sites. The forward primer (-40) is situated on the *KpnI* side of the MCS of **SK+**. Stratagene, San Diego.

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