THE STUDY OF TWO GENES INVOLVED IN THE MAINTENANCE OF INTRACELLULAR REDOX POTENTIALS IN *THIOBACILLUS FERROOXIDANS.*

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CERTIFICATION OF SUPERVISOR

In terms of paragraph 9 of "General regulations for the degree of Ph.D." I as the supervisor of the candidate Rosamund Powles, certify that I approve of the incorporation in this thesis of material that has already been published or submitted for publication.

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ABSTRACT

The thioredoxin and γ-glutamylcysteine synthetase genes of *Thiobacillus ferrooxidans* were isolated by complementation of an *Escherichia coli trxA gshA* double mutant for growth on minimal medium lacking glutathione. Transduction of a *T. ferrooxidans* genome cosmid library into the *E. coli* mutant resulted in the identification of two groups of complementing cosmids. One of these groups was able to complement the $TrxA^+$ requirement of an *E. coli trxA met* mutant for conversion of methionine sulfoxide to methionine.

The nucleotide sequence of a 1.1 kbp *Hin*dIII-*Pst*I *T. ferrooxidans* chromosomal DNA fragment containing the cloned *trxA* gene, was determined. An open reading frame (ORF) corresponding to the thioredoxin gene and part of an ORF corresponding to the N-terminal region of the rho gene were identified. A 14 kDa protein corresponding to the *T. ferrooxidans* thioredoxin was synthesised in an *E. coli*-derived *in vitro* transcription/translation system. The predicted amino acid sequence of the *T. ferrooxidans trxA* gene showed 70.6% identity to that of *E. coli*.

The cloned *T. ferrooxidans trxA* gene was able to support T7 phage replication in an *E. coli trxA* mutant to almost the same level as a $TrxA^+$ wild type strain. However, it was not able to satisfy the thioredoxin requirement of M13 phage.

Crude extracts of the *E. coli trxA gshA* mutant containing the cloned *T. ferrooxidans trxA* gene were able to reduce insulin far more rapidly those that did not contain the cloned gene, indicating that the *T. ferrooxidans* thioredoxin has disulfide reductase activity.

DNA:RNA hybridisation analysis was carried out on transcripts prepared from the *E. coli trxA gshA* double mutant, the mutant containing the cloned *trxA* gene and *T. ferroxidans* cells. A single transcript of about 0.5 kbp was obtained for RNA from *T. ferrooxidans* cells. Several transcripts were produced from the cloned *T. ferrooxidans trxA* gene in *E. coli*. One of these transcripts corresponded in size to the 0.5 kbp transcript produced by *T. ferrooxidans* cells, whereas the other transcripts were larger (1.35, 1.40, 1.60 and 1.80 kbp) and presumably represented transcription products originating from the *lac* promoter of the vector.

Primer extension analysis of the thioredoxin gene with RNA prepared from *T. ferrooxidans* gave three transcription start sites. RNA derived from the cloned *trxA* gene in *E. coli* only gave two transcription start sites, which were identical to two of the three found with *T. ferrooxidans* RNA.

The second group of cosmids which was able to complement the *gshA trxA* double mutant for growth on minimal media but which did not allow growth of the *E. coli trxA met* mutant on minimal media plus methionine sulfoxide was also examined. The nucleotide sequence of a 2.3

kbp *Cla*I-*Bam*HI *T. ferrooxidans* chromosomal DNA fragment containing the complementing DNA, was determined. Two ORFs, separated by 9 bp, corresponding to a citrate synthase gene and a γ-glutamylcysteine synthetase gene were identified. The predicted amino acid sequence of the *T. ferrooxidans gltA* gene showed 37% identity to that of *E. coli*, whereas the predicted amino acid sequence of the *T. ferrooxidans gshA* gene only showed 18% identity to that of *E. coli*. The low homology of the *gshA* gene products is not abnormal, as the γ-glutamylcysteine synthetases studied to date differ widely in amino acid sequence and structure. Partial sequencing of the cloned *T. ferrooxidans* chromosomal DNA upstream of the *gltA* gene indicated regions of homology to the genes of the pyruvate dehydrogenase complex.

Synthesis of proteins corresponding to the *T. ferrooxidans* citrate synthase (43 kDa) and γ-glutamylcysteine synthetase (49 kDa) enzymes were confirmed using an *E. coli*-derived *in vitro* transcription/translation system. The identity of the proposed citrate synthase gene was confirmed by complementation of an *E. coli gltA* mutant. Similarly, the DNA upstream of the *gltA* gene, was found to complement an *E. coli* mutant for the pyruvate dehydrogenase complex, confirming that this region contained the *T. ferrooxidans* pyruvate dehydrogenase complex, as predicted from the partial sequence analysis.

The identity of the *T. ferrooxidans* γ-glutamylcysteine synthetase gene was confirmed by determining the levels of glutathione in crude extracts of the *E. coli gshA trxA* double mutant, the mutant containing the cloned *T. ferrooxidans gshA* gene, and an *E. coli* parental strain. Crude extracts prepared from *E. coli* double mutant contained much lower levels of glutathione than crude extracts prepared from the *E.coli* mutant containing the cloned *gshA* gene, indicating that the gene from *T. ferrooxidans* encoded a protein with γ-glutamylcysteine synthetase activity.

RNA:DNA hybridisation analysis was carried out using probes specific to the *gshA* gene, the *gltA* gene and the *aceF* gene (pyruvate dehydrogenase transacetylase gene). All three probes gave transcripts of 9 kbp and a number of smaller transcripts with RNA derived from *T. ferrooxidans* cells. The 9 kbp transcripts indicated transcriptional linkage of the γ-glutamylcysteine synthetase gene, the citrate synthase gene and the genes of the pyruvate dehydrogenase complex in *T. ferrooxidans*.

ABBREVIATIONS

A	-adenosine
A	-alanine
Ala	-alanine (A)
Ap	-ampicillin
Arg	-arginine (R)
Asn	-asparagine (N)
Asp	-aspartic acid (D)
ATCC	-American Type Culture Collection
ATP	-adenosine 5'-triphosphate
bp	-base pair(s)
BSA	-bovine serum albumin
C	-cytosine
C	-cysteine
C-	-carboxy terminal (end of protein)
cAMP	-cyclic adenosine 3',5'-monophosphate
Ci	-Curie
Cm	-chloramphenicol
cpm	-counts per minute
CsCl	-cesium chloride
Cys	-cysteine (C)
^{o}C	-degrees Celcius
d	-deoxyribo
D	-aspartic acid
DMSO	-dimethylsulphoxide
DNA	-deoxyribonucleic acid
DNase	-deoxyribonuclease
dNTP	-deoxyribonucleotide triphosphate
ddNTP	-dideoxyribonucleotide triphosphate
ds	-double strand(ed)
DTT	-dithiothreitol
E	-glutamic acid
EDTA	-ethylene-diaminetetra-acetic acid
F	-phenylalanine
FAD	-flavin adenine dinucleotide
FBP-ase	-fructose 1,6-bisphosphatase
g	-gram
G	-guanine
G	-glycine
Gln	-glutamine (Q)
Glu	-glutamic acid (E)
Gly	-glycine (G)
GSH	-glutathione
GSSG	-oxidised glutathione
h	-hour(s)
H	-histidine
His	-histidine (H)
HPLC	-high pressure liquid chromatography
I	-isoleucine
Ile	-isoleucine (I)

IPTG	-isopropyl-β-D-thiogalactopyranoside
K	-lysine
kbp	-kilobase pair(s) or 1000 bp
kDa	-kilodalton
K_m	-Michaelis constant
L	-leucine
LA	-Luria-Bertani (agar)
LB	-Luria-Bertani (medium)
Leu	-leucine (L)
Lys	-lysine (K)
M	-methionine
Met	-methionine (M)
min	-minute(s)
mM	-milli-mole
MM	-minimal medium
M_r	-relative molecular mass (dimensionless)
mRNA	-messenger RNA
N	-asparagine
N-	-amino terminal (end of protein)
NAD	-nicotinamide-adenine dinucleotide
NADH	-reduced form of NAD
NADP	-nicotinamide-adenine dinucleotide phosphate
NADPH	-reduced form of NADP
nm	-nanometers
NMR	-nuclear magnetic resonance
OD_{600}	-optical density at 600 nm
oligo	-oligodeoxyribonucleotide
O/N	-overnight
ORF	-open reading frame
ori	-origin of DNA replication
P	-proline
PAGE	-polyacrylamide-gel electrophoresis
Phe	-phenylalanine
P_i	-inorganic phosphate
Pro	-proline (P)
Q	-glutamine
R	-arginine
R	-(superscript) resistance/resistant
RBS	-ribosome binding site
RNA	-ribonucleic acid
RNase	-ribonuclease
rpm	-revolutions per minute
RT	-room temperature
s	-second(s)
S	-(superscript) sensitivity/sensitive
SD	-Shine-Delgarno (sequence)
SDS	-sodium dodecyl sulphate
Ser	-serine (S)
ss	-single stranded

T	-thymine
T	-threonine
TAE	-tris-acetate EDTA buffer
TBE	-tris-borate EDTA buffer
Thr	-threonine (T)
Tris	-Tris (hydroxymethyl) aminomethane
Trp	-tryptophan
TRX	-thioredoxin
TRX-$(SH)_2$	-reduced thioredoxin
Tyr	-tyrosine
UV	-ultraviolet
U	-unit of enzyme activity
V	-valine
Val	-valine (V)
W	-tryptophan
wt	-wild type
w/v	-weight/volume
α	-alpha
β	-beta
Δ	-delta (indicates a change in measured quantity)
γ	-gamma
λ	-lamda
μ	-micro (10^{-6})
σ	-sigma

CHAPTER 1

GENERAL INTRODUCTION

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1.1 Bioleaching bacteria

The increased use of microorganisms in industrial mineral leaching operations and their potential use in waste treatment and coal desulphurization, have generated considerable interest in the unusual physiological capabilities of these organisms. Industrial bioleaching operations are usually carried out using an assortment of bacteria, as conditions are not sterile and mixed cultures of bacteria are often more efficient than any one species alone (Norris, 1990). At temperatures between 20°C and 40°C, the bioleaching bacteria may include *Thiobacillus ferrooxidans*, *Thiobacillus thiooxidans*, *Leptospirillum ferrooxidans* and acidophilic, heterotrophic bacteria of the genus *Acidiphilum* (Harrison, 1984; Kelly, 1988). At temperatures greater than 45°C other bacteria such as the *Thiobacillus* TH strains and members of the genus *Sulfobacillus* are predominant, and at even higher temperatures extremely thermophilic Archaea such as *Sulfolobus* and *Acidianus* species are found (Norris, 1990). The potential for the improvement of bacterial leaching systems by genetic manipulation, has contributed greatly to the interest in the molecular biology of these bacteria.

The bacterium considered to play a principal role in most bioleaching operations was *T. ferrooxidans*, and it has therefore been studied more extensively than other organisms associated with metal extraction. However, recent evidence has indicated that *T. ferrooxidans* may not be the predominant organism in the bioleaching of gold from arsenopyrite ores in a continuous flow system. *T. ferrooxidans* appears to have been inadvertently selected for under the batch culture conditions employed to isolate bacteria from the arsenopyrite ore leachate in the laboratory, and was thus thought to be the predominant organism (Rawlings, 1995; Pizarro *et al.*, 1996).

T. ferrooxidans attacks sulfide-containing minerals and converts the insoluble sulfides of metals such as copper, lead, zinc or nickel to their soluble metal sulfates, which can then be recovered from the leachate. In addition *T. ferrooxidans* can obtain energy from the oxidation of ferrous iron or reduced sulfur compounds. In contrast, *T. thiooxidans* can use only reduced sulfur compounds, and *L. ferrooxidans* can only use ferrous iron (Kelly & Harrison, 1989). The three metals recovered in the largest quantities through direct or indirect bacterial action are copper (Brierley, 1978), uranium (McCready *et al.*, 1990) and gold (Livesey-Goldblatt *et al.*, 1983).

1.1.1 Bioleaching of copper ores

The use of bioleaching to recover copper is growing as remaining ore bodies are depleted and conventional extraction technology becomes uneconomic (Rawlings & Silver, 1995). In 1990, it was estimated that at least 15% of the world production of copper was recovered using

bioleaching. Chemolithotrophic bacteria attack and oxidize a variety of copper sulfide ores converting them to their soluble metal sulfates.

$$4CuFeS_2 + 17O_2 + 2H_2SO_4 \rightarrow 4CuSO_4 + 2Fe_2(SO_4)_3 + 2H_2O$$

chalcopyrite

$$CuS + 2O_2 \rightarrow CuSO_4$$

covellite

Copper is usually leached from waste dumps, by irrigation of the dump. In some cases the leachate is collected and recycled, thus speeding up the process. The solubilized copper is then recovered from the leachate.

1.1.2 Bioleaching of uranium ores

Uranium can be solubilized from ores by direct microbial oxidation of the insoluble tetravalent uranium to the soluble hexavalent uranyl ion.

$$2UO_2 + 2H_2SO_4 \rightarrow 2UO_2SO_4 + 2H_2O$$

DiSpirito & Touvinen (1982), showed that *T. ferrooxidans* was capable of direct uranium oxidation and demonstrated a linkage between CO_2 fixation and uranium oxidation. However most uranium deposits are found underground in oxygen-depleted environments. In these situations uranium is solubilized indirectly. Pyrite is often found associated with these uranium ores, and thus *T. ferrooxidans* and other chemolithotrophic bacteria are able to convert the pyrite to ferric iron and sulfuric acid. The ferric iron and sulfuric acid can then react with the insoluble uranium oxides to produce soluble uranyl sulfates (Kelly *et al.*, 1979).

$$UO_2 + Fe_2(SO_4)_3 \rightarrow UO_2SO_4 + 2FeSO_4$$

$$UO_3 + H_2SO_4 \rightarrow UO_2SO_4 + H_2O$$

The spent ferrous iron containing leach liquors may be regenerated by aeration in the presence of *T. ferrooxidans* and the ferric iron recycled to the leaching process.

$$4FeSO_4 + 2H_2SO_4 + O_2 \rightarrow 2Fe_2(SO_4)_3 + 2H_2O$$

1.1.3 Leaching of gold-bearing arsenopyrite ores

A considerable amount of gold is found in recalcitrant ores. These are ores in which the gold is encased in a matrix of pyrite and arsenopyrite and as a result cannot be solubilized by the usual process of cyanidation. Traditionally, pressure leaching or roasting of the recalcitrant ores to

decompose the arsenopyrite was required to render the gold accessible to cyanide extraction. However, these methods are very energy intensive. Oxidation of the arsenopyrite using *T. ferrooxidans* is a low energy alternative, and is used commercially to expose the gold for cyanide extraction in South Africa, Brazil, Western Australia and Ghana. The leaching is carried out in a tank or vat. Greater than 95% of the gold can be recovered in this way.

$2FeAsS + 7O_2 + 2H_2O \rightarrow 2FeAsO_4 + 2H_2SO_4$
arsenopyrite

1.1.4 Physiology of *T. ferrooxidans*

T. ferrooxidans is a Gram-negative, rod-shaped, mesophilic bacterium, that has a physiology which is well suited for growth in an acidic, inorganic, metal-rich, mining environment. It obtains its energy by the oxidation of either ferrous ions to ferric ions or reduced-sulfur compounds to sulfuric acid. The bacterium is acidophilic with an optimum pH within the range pH 1.5 to 2.5. It grows best in an aerobic environment with oxygen as an electron acceptor (Rawlings & Kusano, 1994). However, if oxygen is lacking, reduced-sulfur compounds or formate are used as the electron donor and ferric ions can serve as the electron acceptor (Pronk *et al.*, 1991). Other compounds besides iron and sulfur may be oxidised. Three *T. ferrooxidans* strains, including the type strain ATCC 23270, grow on basal-salts medium when incubated in the presence of CO_2 and O_2, with H_2 as the sole energy source (Drobner *et al.*, 1990).

T. ferrooxidans obtains its carbon by fixing atmospheric carbon dioxide and is obligately autotrophic (Kelly & Harrison, 1989). The reduction of pyridine nucleotides (NAD, NADP), provides *T. ferrooxidans* with reducing power, required for CO_2 fixation, and these reactions are energised by the oxidation of ferrous iron or reduced sulfur-compounds (Lundgren & Silver, 1980). One strain has been reported to grow mixotrophically in the presence of iron and glucose (Barros *et al.*, 1984) but in general, organic compounds inhibit growth (Alexander *et al.*, 1987; Touvinen, 1971). Formic acid can replace carbon dioxide as a carbon source in most, but not all, *T. ferrooxidans* strains examined (Pronk *et al.*, 1991).

T. ferrooxidans is also capable of fixing atmospheric nitrogen (Mackintosh, 1978). Of 15 *T. ferrooxidans* strains examined, every one had the genes for nitrogen fixation (Pretorius *et al.*, 1986; Rawlings, 1988).

The direct physical attachment of *T. ferrooxidans* to pyrite (FeS_2) crystals has been observed (Lundgren & Silver, 1980), and results in localised oxidation of pyritic sulfide to sulfate (1), and ferrous iron to ferric (2):

$$2FeS_2 + 7O_2 + 2H_2O \rightarrow 2FeSO_2 + 2H_2SO_4 \qquad (1)$$

$$4FeSO_4 + O_2 + 2H_2SO_4 \rightarrow 2Fe_2(SO_4)_3 + 2H_2O \quad (2)$$

These reactions generate ferric iron, a strong oxidising agent, which participates in the indirect oxidation of pyrite, generating sulfate and elemental sulfur (3):

$$FeS_2 + Fe_2(SO_4)_3 \rightarrow 3FeSO_4 + 2S \quad (3)$$

On aeration this ferrous iron is reoxidised to ferric iron. The elemental sulfur may be reoxidised to generate additional sulfuric acid (4) (Monticello & Finnerty, 1985):

$$2S + 3O_2 + 2H_2O \rightarrow 2H_2SO_4 \quad (4)$$

The production of sulfuric acid during microbial leaching of ores continually lowers the pH of the leach solution. The cytoplasmic pH of *T. ferrooxidans* is neutral, thus maintaining a transmembrane gradient of 4.5 pH units (Lundgren & Silver, 1980). The movement of protons across the membrane, against the transmembrane gradient, is chemiosmotically coupled to ATP production, with oxygen usually serving as the terminal electron acceptor.

T. ferrooxidans also has an inherent resistance to high concentrations of metallic and other ions (Tuovinen *et al.*, 1971), and has been grown in media containing, for example Zn (120g/l), Ni (72g/l), Co (30g/l), Cu (55g/l), U_3O_8 (12g/l) and Fe^{2+} (113g/l) (Torma, 1977). Another feature is the remarkable adaptability of the organism when faced with adverse growth conditions. Previously sensitive strains have become adapted to high concentrations of arsenic (Rawlings & Woods, 1995) and copper (Holmes & Ul Haq, 1989), lower-than-optimum pH (Vian *et al.*, 1986) and a number of other factors.

Recent studies incorporating 5S and 16S rRNA base sequences have been useful in predicting phylogenetic relationships among the sulfur and iron-oxidising eubacteria, including *T. ferrooxidans*. Most of the *T. ferrooxidans* and *T. thiooxidans* strains form a tight cluster in the β subdivision, towards the root of the β/γ subdivision of the proteobacteria (Lane *et al.*, 1992). An exception was the *T. ferrooxidans* strain m-1, which falls within the proteobacteria γ subdivision.

1.1.5 Molecular genetics of *T. ferrooxidans*

The economic significance of bacteria to the mining industry warrants a thorough understanding of all aspects of the physiology of these organisms. In this respect, the molecular biological research of these organisms is of great importance. To improve the economic viability of biomining, the rates of cell growth and ore oxidation by microbes need to be increased. The growth rates of *T. ferrooxidans*, for example, are slow, 6-7 hour average generation time in ferrous iron-containing batch cultures. Furthermore *T. ferrooxidans* oxidises up to 30 times its

own weight in ferrous iron/h and its cell yields are low (Kelly *et al.*, 1979). Since this organism does not settle, there is no ready means of recycling and the minimum retention time in a leaching vat is restricted by the low growth rate of the bacteria. It may be possible to solve these problems by increasing our knowledge of the nutrition and energy generation mechanisms of leaching bacteria, and developing a genetic system to carry out physiological studies and manipulate commercial strains of the bacteria. Studies of the molecular genetics of *T. ferrooxidans* were usually carried out with these objectives in mind.

Several native plasmids have been isolated from *T. ferrooxidans*. Martin *et al.* (1981) reported that 11 out of 15 strains studied contained 1 to 5 plasmids per strain, ranging in size from 7.4 to 75 kbp. In a survey of more that 100 strains from six Japanese mining sites, 73% were found to carry one or more plasmids ranging in size from 2.0 to 30 kbp (Shiratori, 1991). Plasmids have also been reported in *T. ferrooxidans* strains in other countries (Lau *et al.*, 1993). Two *T. ferrooxidans* plasmids pTF1 (Holmes *et al.*, 1984) and pTF-FC2 (Rawlings *et al.*, 1984; Rawlings *et al.*, 1993) have been studied in some detail.

Two families of repetitive DNA sequences in the genome of *T. ferrooxidans* have been identified (Yates & Holmes, 1987). It has been suggested that transposition of family 1 insertion sequences (IS*T1*) might be involved in phenotypic switching of *T. ferrooxidans*, including the reversible loss of its iron-oxidising capacity (Schrader & Holmes, 1988).

A number of genes have been cloned from *T. ferrooxidans* and expressed in *E. coli*. These include genes involved in carbon dioxide fixation, nitrogen fixation and regulation, iron oxidation, mercury resistance and energy production (Rawlings & Kusano, 1994).

1.1.5.1 Gene transfer studies

In the absence of confirmed reports of bacteriophages and numerous unsuccessful attempts at producing a state of transformation competence, conjugation and electroporation are the most likely means of transferring DNA into *T. ferrooxidans* (Rawlings & Kusano, 1994).

(i) Conjugation systems

The growth requirements of *T. ferrooxidans* and *E. coli* are so different that the lack of a mating medium in which both bacteria could meet their energy needs, may have been the cause of previous failures to demonstrate transfer between the two bacteria (Peng *et al.*, 1994). However, recently the conjugal transfer of plasmids from *E. coli* to *T. ferrooxidans* has been reported (Peng *et al.*, 1994). The broad-host-range IncP plasmids RP4, R68.45, RP1::Tn*501*, and pUB307 were transferred to *T. ferrooxidans* from *E. coli* by conjugation (Peng *et al.*, 1994). The broad-host-range IncQ vector pJRD215 (Km^r, Sm^r, IncQ, Mob^+) was also mobilized into *T. ferrooxidans* with the aid of the plasmid RP4 integrated in the chromosome of *E. coli* SM10. The transfer frequency ranged from 1.1×10^{-7} to 2.8×10^{-5}, for the different plasmids. By use

of the suicide vector pSUP1011, transposon Tn5 was introduced into *T. ferrooxidans*. More recently two arsenic-resistant plasmids were introduced into *T. ferrooxidans* by conjugation (Peng *et al.*, 1994). The plasmids were stable and the arsenic resistance genes were expressed in *T. ferrooxidans*.

(ii) Electroporation

Kusano *et al.* (1992) electroporated mercury-sensitive *T. ferrooxidans* cells with shuttle vectors consisting of the *T. ferrooxidans mer* resistance determinant cloned into four natural *T. ferrooxidans* plasmids, as well as plasmid pKT240, a plasmid containing an IncQ-type replicon. Of 30 *T. ferrooxidans* strains electroporated, only 1 gave transformants at a low efficiency of 120 to 200 colonies per mg of plasmid DNA. In addition, selection for mercury resistance was not a very stringent selection procedure and only 13 of the 22 colonies which grew on mercury contained the recombinant plasmid.

1.2 Thioredoxin

Thioredoxins are small (Mr 11 700), ubiquitous, heat-stable proteins with conserved redox-active sites **-Trp-Cys-Gly-Pro-Cys-** (Holmgren, 1968). Thioredoxin participates in redox reactions through the reversible oxidation of its active center dithiol, to a disulfide. Two cellular reductants, reduced ferredoxin and NADPH, supply the equivalents for reduction via different enzymes. In the first system, thioredoxin is reduced by ferredoxin via the iron-sulfur enzyme, ferredoxin-thioredoxin reductase (ferredoxin is reduced by the electron transport chain of illuminated chloroplast thylakoid membranes). Whereas in the NADP/thioredoxin system the reduction of thioredoxin is linked to NADP via a flavin enzyme NADP-thioredoxin reductase.

Thioredoxin was first purified in 1964 from *Escherichia coli* as an *in vitro* hydrogen donor for ribonucleotide reductase (Laurent et al., 1964). Since then research has revealed numerous functions of thioredoxin in many important biological phenomena. The reduced form of the protein is a strong disulfide oxidoreductase that is involved in the reduction of enzymes that reduce sulfate and methionine sulfoxide (Gonzalez Porqué *et al.*, 1970). Thioredoxin is also a highly efficient disulfide reductase of wide specificity, catalyzing many dithiol-disulfide redox reactions. It has been identified as an essential subunit of the T7 DNA polymerase (Mark & Richardson, 1976), and is also required for filamentous phage assembly (Russel & Model, 1986). In plant cells, thioredoxin is involved in the regulation of enzymes of carbon dioxide fixation (Buchanan, 1980).

1.2.1 General properties of thioredoxin

Thioredoxin negative mutants of *E. coli* are viable, making the precise cellular, physiological functions of thioredoxin unknown. *Anacystis nidulans*, a photosynthetic bacterium, is the only

organism in which a single thioredoxin gene has been shown to be essential for cell viability (Muller & Buchanan, 1989).

The reduced form of thioredoxin acts as a hydrogen donor for the reductive enzymes ribonucleotide reductase (Laurent *et al.*, 1964), methionine sulfoxide reductase (Ejiri *et al.*, 1980) and PAPS reductase (Tsang, 1981). However, glutaredoxin can also act as a hydrogen donor for all the above enzymes, except methionine sulfoxide reductase, which has a specific requirement for thioredoxin.

Thioredoxin can effectively catalyse the reduction of exposed disulfides in proteins and is thus a protein disulfide reductase. Reduction of insulin has been used as an assay for thioredoxin. The reduction of two interchain disulfide bonds of insulin leads to the precipitation of the B-chain which can easily be monitored as an increase in turbidity (Holmgren, 1979). Thioredoxin is thought to operate via a ping-pong mechanism, governed by the reduction of TRX-S_2 and the oxidation of TRX-$(SH)_2$ (Gleason & Holmgren, 1988). Thioredoxin can also act as a protein disulfide isomerase (Gleason & Holmgren, 1988).

Thioredoxin is involved in the redox control of various enzymes. For example, the reduction and oxidation of critical disulfide bonds of certain proteins is known to regulate the activity of various photosynthetic enzymes, such as malate dehydrogenase (Scheibe *et al.*, 1986), and fructose-1,6-bisphosphatase (Buchanan, 1980).

There is a growing body of evidence that thioredoxin occupies a central position in controlling the division and proliferation of animal cells (Buchanan *et al.*, 1994). Principal sites of thioredoxin action lie at the levels of transcription (Mathews *et al.*, 1992), cellular proliferation (Biguet *et al.*, 1994), hormone action (Grippo *et al.*, 1985) and removal of oxidative stress (Fernando *et al.*, 1992). In addition, thioredoxin was demonstrated to be required for oogenesis in *Drosophila* (Salz *et al.*, 1994), and early embryonic division in both *Drosophila* (Salz *et al.*, 1994) and *Xenopus* (Hartman *et al.*, 1993), and is implicated in the developmental cycle of *Dictyostelium discoideum* (Wetterauer *et al.*, 1992).

1.2.2 *Escherichia coli* thioredoxin

1.2.2.1 Location

Thioredoxin is present at a relatively high concentration of 10 000 to 20 000 molecules per cell in *E. coli* (Holmgren *et al.*, 1978). Separation of the membrane and soluble fractions of *E. coli* by gentle lysis and centrifugation showed that thioredoxin was located in the soluble fraction. However, cytochemical studies have indicated that thioredoxin is associated with the bacterial cell membrane (Nygren *et al.*, 1981). A peripheral localization at the inner membrane was also indicated from the quantitative release of thioredoxin by osmotic shock and its partial association with a membrane fraction prepared after gentle lysis (Lunn & Pigiet, 1982).

Immunoelectron microscopy showed thioredoxin localized close to the membrane, however, in some cells a localization in the nucleoid region was also observed. Similar studies using cross-linking agents suggested a more precise localization on the inner membrane and adhesion sites where inner and outer membranes are contiguous (Bayer *et al.*, 1987).

Localization of thioredoxin at the cell membrane could be related to its function in filamentous phage assembly and possibly disulfide bond formation in proteins exported into the periplasmic space. Soluble or nucleoid-associated thioredoxin may be involved in deoxyribonucleotide metabolism, although the localization of ribonucleotide reductase has not been determined (Gleason & Holmgren, 1988).

1.2.2.2 Structure of thioredoxin

Thioredoxins, from Archaea to humans, have 27-69% sequence identity to *E. coli* thioredoxin. However, despite large differences in amino acid sequence all the proteins have similar three-dimensional structures, and as expected, residues in the active site region are highly conserved (Eklund *et al.*, 1991).

E. coli thioredoxin consists of a single polypeptide chain of 108 amino acid residues (Holmgren, 1968). The active site is formed by a 14 membered disulfide ring. Amino acids Cys-32 and Cys-35 form the disulfide bridge. Crystallization of TRX-S_2 was obtained close to the isoelectric point at pH 4.5, with alcohol, in the presence of copper ions (Holmgren & Söderberg, 1970). The TRX-S_2 crystals were formed from dimers linked into layers of molecules by the copper ions (Söderberg *et al.*, 1974). The 3-D structure of the oxidised thioredoxin has been solved to 2.8Å by X-ray crystallography (Söderberg *et al.*, 1974; Holmgren *et al.*, 1975) and more recently refined to 1.68Å (Katti *et al.*, 1990). The molecule consisted of two conformational domains, $\beta\alpha\beta\alpha\beta$ and $\beta\beta\alpha$, connected by a single-turn α-helix and a 3_{10} helix. There were also four reverse turns in the molecule. Most of the residues (90%) were in α-helices, β-sheets or reverse turns, which explains the very high stability of the protein. The two Cys residues of the active site formed a protruding loop between the C-terminal end of the β_2 sheet and the α_3 helix, which was only accessible from one side of the structure (Fig. 1.1). A flat hydrophobic surface was formed on this side of the molecule by residues Gly-33, Pro-34, Ile-75, Pro-76, Val-91, Gly-92 and Ala-93. This area is thought to be involved in binding of thioredoxin to other protein molecules (Eklund *et al.*, 1984).

No crystals of TRX-$(SH)_2$ have been obtained. However the 3-D solution structure has been determined by NMR spectroscopy. The molecule also consisted of central a five-strand β-sheet and 3 α-helices. The β strands were packed in the sheet in the order β_1, β_3, β_2, β_4, β_5, with β_1, β_3 and β_2 parallel and β_4 and β_5 arranged in an antiparallel fashion. The α_1 helix connected β_1 and β_2 strands and the α_2 helix connected the β_2 and β_3 strands. Strands β_4 and β_5 were connected by a short loop that contained a β bulge. Strands β_3 and β_4 were connected by a long

loop that contained a series of turn-like or 3_{10} helical structures. As in the oxidized form the active site formed a protruding loop between strands β_2 and helix α_2. The structure was very similar to the oxidized form, but differed slightly around the active site loop. The distance between the sulfurs of Cys-32 and Cys-35 increased from 2.05Å in the disulfide bridge to approximately 6.8Å in the dithiol of the reduced thioredoxin as a result of the rotation of the side chain of Csy-35 and a significant change in the position of Pro-34 (Dyson *et al.*, 1990).

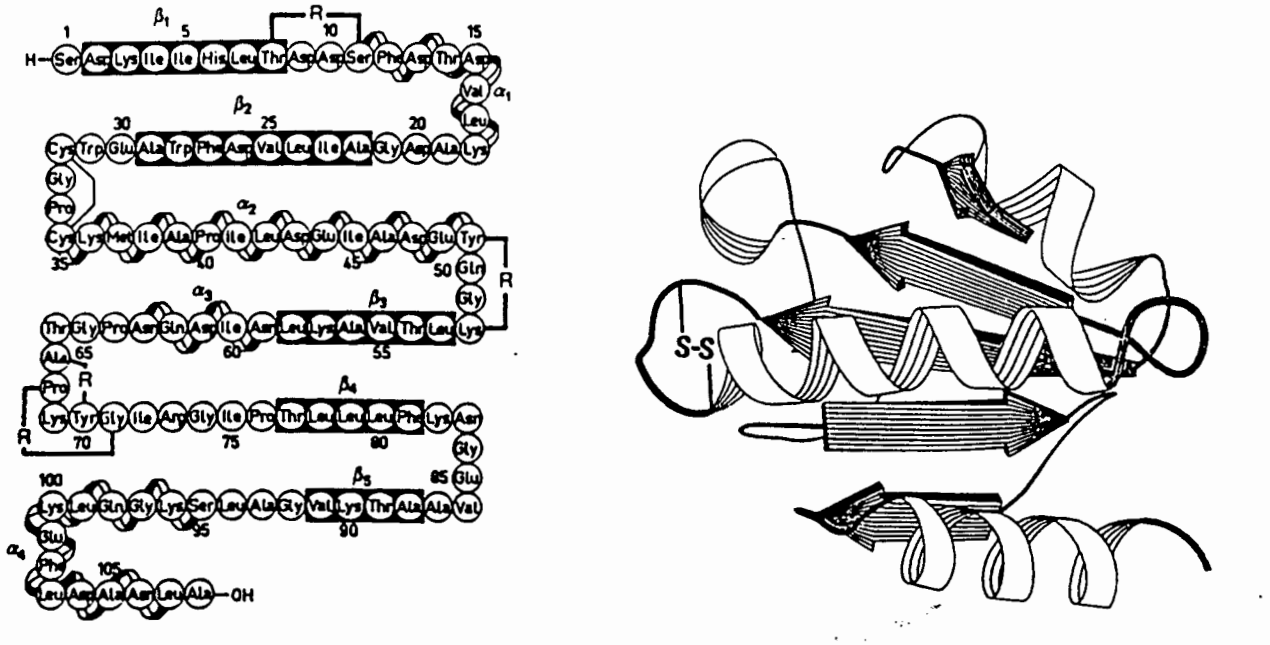


Fig. 1.1 (a) Primary structure of *E. coli* thioredoxin. R = reverse turn. (b) Schematic drawing of three-dimensional structure of oxidized *E. coli* thioredoxin.

Langsetmo *et al.*, (1989) examined urea-induced denaturation of *E. coli* TRX and TRX variants, by electrophoresis on urea gradient slab gels. TRX-S_2 and TRX-$(SH)_2$ each had two folded isomers with different stability to urea denaturation. The data suggested that these were cis and trans isomers of the Pro-76 peptide bond. Urea gel experiments with the mutant P76A, corroborated this interpretation. Since cis TRX-S_2 was much more stable than trans, folded TRX-S_2 was essentially all cis, and in the absence of urea, only a very low percentage of the folded TRX-$(SH)_2$ existed as the trans isomer.

1.2.2.3 Structure/function relationship

Subtle increases in the polypeptide backbone dynamics were observed for TRX-$(SH)_2$ compared to TRX-S_2 (Stone *et al.*, 1993). This was interpreted as an increased flexibility for the active site sequence and its contacting loops, consistent with the reported decreased thermal stability of TRX-$(SH)_2$ compared to that of TRX-S_2 (Hiraoki *et al.*, 1988). The flexibility was suggested as a possible explanation for the differential reactivity of the oxidized and reduced

forms of the TRX, particularly in complex formation with the gene 5 protein of the T7 DNA polymerase (Hiraoki *et al.*, 1988).

Jeng *et al.* (1995), studied the hydrogen exchange behaviour of reduced and oxidized thioredoxin. Significant changes were observed in the active site sequence and in the regions of the protein that were close to this sequence, including portions of the β-strand and α-helical sequences immediately adjacent to the active site. In particular the exchange rate of the Cys-35 amide proton was significantly slowed for oxidized thioredoxin compared with the reduced form. In general, the amide proton exchange rates were consistently lower for the oxidized form of thioredoxin. The rather generalized loosening of the protein structure upon reduction indicated by the hydrogen exchange measurements, provides further evidence that the functional differences observed between the two forms of thioredoxin can most likely be ascribed to the greater flexibility of the reduced form of the protein.

In contrast, circular dichromism (Reutimann *et al.*, 1981. Brown *et al.*, 1987) and optical density dispersion spectra (Stryer *et al.*, 1967) showed no change between the oxidized and reduced states. Nor were any detectable changes found between the two forms for backbone amide vibrations by Raman spectroscopy (Li *et al.*, 1993).

Characterization by ^{1}H NMR of a C32S, C35S double mutant of *E. coli* thioredoxin confirmed its resemblance to the reduced form of the wild type protein (Dyson *et al.*, 1994). The mutant protein was able to substitute for the reduced thioredoxin in T7 phage and filamentous phage assembly systems. This work also confirmed that the major titrating group near the active site was most likely to be the Asp-26 carboxyl group. The ionization and hydrogen bonding states of the Cys-32 and Cys-35 residues in reduced TRX were determined by Raman spectroscopy (Li *et al.*, 1993). The results indicated; (i) one sulfhydryl was a stronger H-bond donor than the other (ii) the weaker H-bond donor (Cys-32) was preferentially titrated to the thiolate ion as the solution pH was increased from pH 4 to 7. Water or hydroxyl oxygens were the possible acceptors for the Cys-32 S-H donor (iii) Cys-35 resisted ionization until approximately 50% of the more acidic sulfhydryl had been titrated. A carbonyl oxygen was proposed as the likely acceptor of the Cys-35 S-H donor (iv) Raman titration data indicated $pK_1 = 7.1$ and $pK_2 = 7.9$ for the two thiol-thiolate equilibria. The lower pK_a was thought to be assigned to Cys-32. Conformation-sensitive Raman bands of Trp, Asp and Glu carboxylate groups and aliphatic side chains indicated significant changes in the environment and interactions of these side chains with thiol titrations, although the secondary structure was largely conserved (Li *et al.*, 1993).

Elofsson *et al.* (1991), determined the picosecond fluorescence of the four aromatic amino acid residues (Trp-28, Trp-31, Tyr-49 and Tyr-70) in wild type thioredoxin and a mutant thioredoxin with Trp-31 replaced by Phe. The major features of the lifetime distribution of tryptophan fluorescence were unchanged in the mutant. However the mutation changed the mobility of

residue Trp-28, but not Tyr-49 or Tyr-70. The mobility of the two tyrosines increased on reduction of the active site disulfides indicating a looser structure with reduction. This increased motion could also be seen from molecular dynamics simulation (Elofsson *et al.*, 1991).

A comparison of backbone and side chain dynamics of TRX-$(SH)_2$ using ^{15}N-NMR relaxation measurements revealed that Trp-31 was more mobile that Trp-28 and lay on the surface of the molecule, while Trp-28 was buried in the hydrophobic core (Stone *et al.*, 1993). Backbone regions that were more mobile than average included; the N-terminus, the C-terminus, residues 20-22 (forms a linker between α_1 helix and β_2 strand), and residues 73-75 and 93-94 which are located adjacent to the active site. Oxidation of both Trp residues by N-bromosuccinimide inactivated TRX, whereas modifications of only Trp-31 lead to partial activity as a substrate for thioredoxin reductase. Thus Trp-28 seems to be an essential part of the surface required for interactions with other proteins.

Tryptophan emission of TRX-S_2 was strongly quenched over the pH range 2-10 (Holmgren, 1972). In contrast the fluorescence of TRX-$(SH)_2$ was strongly pH dependent with a maximum at pH 5 (Holmgren, 1972) and an apparent titration curve for a quenching group with a pK_a of 6.35 (Reutimann *et al.*, 1981). This quenching group was proposed to be the deprotonated form of the sulfhydryl group of Cys-32, which has an estimated pH value of 6.7 judged from the chemical modifications by iodoacetate (Kallis & Holmgren, 1980). The alkylation of cysteine residues using iodoacetamide, below pH 8, lead to labelling of only Cys-32, and the reaction was strongly pH dependent. Both cysteine residues were alkylated when the protein was denatured with guanidine hydrochloride, showing that the folded structure was responsible for the reactivity of TRX-$(SH)_2$. It was proposed that the thiolate ion of Cys-32 may be stabilized and its activity enhanced by interaction with the ε-amino group of Lys-36. However, Nikkola *et al.* (1993) showed that the conserved positive charge at position 36 was not essential for *E. coli* TRX activity, as a TRX mutant that had Lys-36 substituted with a negatively charged glutamic acid residue could still function with approximately 75% of the catalytic efficiency of the wild type protein. The authors concluded that the pK_a of Cys-32 was lowered by its interaction with the α_2 helix dipole (α-helix structures create a positive charge at their N-termini), and that the positively charged residue at position 36 was conserved to optimize protein interactions and did not increase the reactivity of the active site thiol group.

In addition, site-directed mutagenesis of Lys-36 in human thioredoxin to either glutamic acid or leucine, affected the reduction rates and growth stimulation but was not essential for the redox protein's biochemical or biological properties (Oblong *et al.*, 1995).

Gly-92 has been found to be essential for the function of TRX-$(SH)_2$ as a subunit of T7 phage DNA polymerase (Nordström *et al.*, 1981, Holmgren *et al.*, 1981). The altered thioredoxin

isolated from *E. coli* 7007 had Gly-92 changed to an Asp residue. It had about 20% activity in its reduced form with ribonucleotide reductase and altered activity with thioredoxin reductase.

A Pro to His mutation in the active site increased disulfide isomerase activity of thioredoxin 10-fold (Lundström *et al.*, 1992). This mutation mimicked the proposed protein disulfide isomerase active site -W-C-G-H-C- (Lundström *et al.*, 1992). However, a Pro-34 to Ser-34 mutation had little effect on the redox activity of thioredoxin (Gleason *et al.*, 1990). The authors proposed that Pro-34 maintains the stability of the protein rather than being involved in the redox function. This theory was supported by Russel & Model (1986), whose results showed that the P34S thioredoxin mutant supported f1 phage replication at 37°C, but not at 42°C.

Asp-26 in *E. coli* thioredoxin is located at the bottom of a hydrophobic cavity, near the redox active disulfide of the active site. Examination of the sequence from a number of species revealed that Asp-26 is a highly conserved residue (Eklund *et al.*, 1991). The titration behaviour of both oxidized and reduced TRX appeared to implicate Asp-26 in the electron-proton transfer in thioredoxin, consistent with its close proximity to the active site (Dyson *et al.*, 1991). Langsetmo *et al.* (1991) have determined the pK_a of Asp-26 by a novel electrophorectic method based on the relative electrophoretic mobilities of wild type thioredoxin and of the mutant D26A (Asp replaced with Ala). The pK_a was found to be 7.5, more than 3 units above the pK_a of a solvated carboxyl side chain. The titration of Asp-26 was found to be thermodynamically linked to the stability of TRX. The ΔG of denaturation of the wild type TRX varied with pH in the vicinity of pH 7.5, but the ΔG of denaturation of the mutant D26A was independent of pH, indicating that the stabilization of D26A TRX compared to the wild type was primarily due to the removal of the Asp-26 residue. More recently Wilson *et al.* (1995), found the pK_a of TRX-$(SH)_2$ to be greater than 9. The authors proposed that the increase in pK_a of Asp-26 in TRX-$(SH)_2$ accounts for the lower stability of TRX-$(SH)_2$ compared to TRX-S_2. The higher pK_a of Asp-26 in TRX-$(SH)_2$ also offers an explanation for the requirement of thioredoxin in the reduced conformation by phages. The binding of a phage factor may be inhibited by a partial negative charge of Asp-26 at physiological pH, which is neutralised in the reduced form.

Cleavage of TRX with cyanogen bromide resulted in two structureless fragments: TRX-C(1-37) and TRX-C(38-108) (Holmgren & Reichard, 1967). The TRX-C(1-37) containing the active site was inactive, but on mixing with TRX-C(38-108) it was reconstituted, non-covalently to an active form. The reconstituted form showed 50% activity with thioredoxin reductase on comparison to the native form (Slaby & Holmgren, 1975). However, it was not active as a H-donor for ribonucleotide reductase or as an insulin disulfide reductant. In immunoprecipitation it showed full activity as an antigen. These results suggested that Met-37 and its peptide bond

are essential for the conformational changes taking place in the oxidation of TRX-$(SH)_2$ (Slaby & Holmgren, 1975).

Cleavage of TRX by trypsin, after reversible blocking of the lysine residues by citraconic anhydride, generated two structureless and inactive fragments: TRX-T(1-73) and TRX-T(74-108) (Slaby & Holmgren, 1975; Holmgren & Slaby, 1979). Mixing the two fragments generated a complex with full immunoprecipitation activity with antibodies against TRX (Slaby & Holmgren, 1979), but low activity with thioredoxin reductase, and ribonucleotide reductase (Slaby & Holmgren, 1979). Spectroscopic studies showed that it was induced to fold to a structure similar to TRX-S_2 at low pH (Reutimann *et al,* 1981). Therefore it appeared that the area around Arg-73 was of critical importance for the interaction with both thioredoxin reductase and ribonuleotide reductase (Holmgren & Slaby, 1979).

1.2.3 Genetics

The thioredoxin gene from *E. coli* has been cloned, sequenced and studied in several laboratories (Wallace & Kushner, 1984; Höög *et al.*, 1984; Lim *et al.*, 1985). The *trxA* gene was located at 84.7 min, directly upstream of *rho*, the gene encoding the transcription termination factor Rho (Matsumoto *et al.*, 1986). The gene order in this region of the *E. coli* chromosome is: *ilvC...rep...trxA-rho...cya...metE* (Aldea *et al.*, 1988).

The position of the *E. coli trxA* gene promoter has been reported by three different groups. Wallace & Kushner (1984) suggested that the start site of the *trxA* promoter was located 63 nucleotides upstream from the *trxA* start codon, based on the size of RNA transcribed from *trxA in vitro*. On the other hand Lim *et al.* (1985) identified two transcription initiation sites located approximately 114 and 74 nucleotides upstream from the *trxA* start codon, by S1 mapping analysis of RNA isolated from a strain containing *trxA* cloned on a multicopy plasmid. Matsumoto *et al.* (1986) located the *trxA* promoters at 111 and 114 nucleotides upstream of the start codon using S1 mapping and only at 111 nucleotides upstream of the start codon using *in vitro* transcription analysis. They suggested that initiation from the upstream site (nucleotide 114) may be hindered under *in vitro* conditions due to an unfavourable helical configuration of the DNA as linearised DNA fragments were used. Thus both Lim *et al.* (1985) and Matsumoto *et al.* (1986) detected transcription start sites at 114 nucleotides upstream of the start codon. However Matsumoto *et al.*'s results suggest that transcription from the second promoter reported by Lim *et al.* is undetectably weak if it functions at all. The helical configuration of the plasmid DNA used by Lim *et al.* may also have affected transcription from the second promoter, leading to differing results. Matsumoto *et al.* proposed that the discrepancies between their results and those of Wallace & Kushner may be due to differences in conditions used for *in vitro* transcription and subsequent analytical procedures.

A near consensus cyclic-AMP binding protein (CRP) recognition site (AATGTGTTTTGCTCATAGT) was located between the -35 and -10 sequences. Binding of a cyclic AMP-CRP complex at this site would interfere with the entry of RNA polymerase to the *trxA* promoters (Wallace & Kushner, 1984; Matsumoto *et al.*, 1986). Such a situation may explain the observed enhancement of *trxA* transcription in a *cya*$^-$ strain, in which the assumed interference should be reduced by the absence of the cofactor, cyclic AMP (Matsumoto *et al.*, 1986). In addition, the apparent level of thioredoxin has been found to decrease about 4-fold in rich medium as opposed to minimal medium (Holmgren, 1983).

The terminator for *trxA* was located so close to the *rho* promoter that it was situated between its -35 and -10 regions (Matsumoto *et al.*, 1986). The possibility that transcription from *trxA* might influence initiation from the *rho* promoter was excluded by the observation that *rho* transcription was almost unchanged when the *trxA* promoter was deleted. A small amount of *trxA* terminator read-through gave rise to a 2.1 kb mRNA encoding both the *trxA* and *rho* genes. About 10% of *rho* mRNA seemed to be made this way. This read-through tended to increase in a temperature-sensitive *rho* mutant, suggesting that the *trxA* terminator is at least partially Rho dependent (Matsumoto *et al.*, 1986).

Lim *et al.* (1985) reported a Rho-independent terminator-like sequence at the 3' end of the *trxA* gene. However, Matsumoto *et al.* (1986) found no increase in transcription of *rho* when they deleted this putative terminator, nor did they detect any RNA terminating at this site in S1 mapping experiments. In addition to the terminator located within the *rho* promoter, 5 other termination sites downstream of the *rho* promoter were also located. The sequence proceeding these terminator sites invariably contained potential stem-loop structures. The first two sites lay within the leader region of *rho* and appeared to be Rho dependent. The remaining 3 sites were localized within the structural gene of *rho* and were therefore thought to be intracistronic terminators.

The genes encoding thioredoxin (TRX) and thioredoxin reductase (TR) have been characterised for a number of bacteria, and from these studies it has become clear that the genes are organised on the genome in a different fashion in different bacterial species. In *E. coli*, for example, the genes are widely separated on the genome, whereas in *Streptomyces clavuligerus* (Cohen *et al.*, 1993) and *Eubacterium acidaminophilum* (Lübbers & Andreesen, 1993), both genes are closely linked. In mycobacteria at least three modes of organisation of thioredoxin and thioredoxin reductase genes can exist within a single bacterial genus. (i) In the majority of mycobacterial strains the genes encoding TRX and TR are located at separate sites on the genome. (ii) However, in all pathogenic mycobacteria from the *Mycobacterium tuberculosis* complex group both genes are found on the same locus. The start codon of TRX overlaps with the stop codon of TR by one nucleotide, requiring the ribosomes to shift frames. (iii) In the pathogen *Mycobacterium leprae*, TR and TRX are encoded by a single gene. The N-terminal part of the

protein corresponds to TR and the C-terminal part to TRX (Wieles *et al.*, 1995). The authors postulate that the various types of gene organisation may be associated with different modes of expression of the TRX and TR proteins.

1.2.4 Thioredoxin in bacteriophage life cycles

1.2.4.1 Filamentous phage

Filamentous phage assembly *in vivo* shows an absolute requirement for thioredoxin and a partial requirement for thioredoxin reductase (Russel & Model, 1985). Mutants in which one or both of the thioredoxin active site cysteine residues were changed to alanine or serine were still able to support filamentous phage assembly (Russel & Model, 1986). The mutants were inactive in an assay which couples oxidation of NADPH to reduction of 5,5'-dithiobis-(2-nitrobenzoic) acid via thioredoxin reductase and thioredoxin. These results suggest filamentous phage assembly requires the participation of thioredoxin in the reduced conformation but not as a reductant. The authors isolated a variety of mutants that were unable to support filamentous phage assembly. These mutants had alterations at amino acid positions Pro-34(trxA2), Gly-74(trxA11), Pro-76(trxA76) and Gly-92(trxA12, trxA13). These mutations fall within the "molecular surface area" that Eklund *et al.* (1984) identified as the binding surface for redox-interactions between thioredoxin and other proteins. This area is comprised of three regions, the first amino acids 33-34, the second 75-76 and the third 91-93. Revertants of mutants in these regions that were able to support filamentous phage assembly were identified. Changes in the amino acids that connect two of the conserved domains (residues 64-70) restored competence in phage assembly to mutant trxA12. One revertant of trxA12, and all four of trxA11 and trxA13 had regained the wild type Gly codons. As in both mutants trxA11 and trxA13 Gly residues had been altered to Asp residues, the authors proposed that the positively charged Asp residue perturbed the "molecular surface area" (Russel & Model, 1986). Mutants in Gly-33 or Pro-34 were tested for the ability to support growth of T7 and f1 phages, and it was found that amino acids with smaller side-chain groups were indispensable for the growth of phages (Minarik *et al.*, 1993).

Thioredoxin does not appear to be required for DNA synthesis, as DNA synthesis of f1 and T7 phages was normal in *trxA* mutants. The conversion of the single-stranded DNA-gene *V* complex to phage required two events; the removal of the gene *V* DNA binding protein and the binding of the major coat protein to the DNA. This process required the action of the gene *I* and *IV* products (neither of which are phage structural components), since mutations in either of these genes blocked filamentous phage assembly but did not affect synthesis of either the DNA or protein components of the phage (Webster & Lopez, 1984). Despite strong evidence for an interaction between thioredoxin and the product of gene *I*, efforts to demonstrate complex formation by immune precipitation of extracts of gently lysed, phage f1-infected cells with anti-thioredoxin serum were unsuccessful (Russel & Model, 1983).

1.2.4.2 T7 phage

Two classes of *E. coli* mutants designated *tsn*B and *tsn*C, were isolated (Chamberlain, 1974). These mutants grew normally but were unable to support growth of T7 phage. *E. coli tsn*C mutants were found to be defective in cell-free replication of duplex T7 DNA and T7 DNA polymerase activity. Modrich & Richardson (1975) identified a 12 kDa protein designated TsnC protein, that was necessary for viral DNA synthesis. The TsnC protein was found to be identical to thioredoxin. The T7 DNA polymerase complex was found to be composed of a 1:1 complex of an 84 kDa protein specified by the gene *5* protein of the phage and a 12 kDa "TsnC" protein from *E. coli* (Mark & Richardson, 1976). The polymerase activity of the complex did not depend on reducing agents, suggesting that the active site merely plays a conformational role in a protein:protein interaction (Alder & Modrich, 1983). A mutant thioredoxin Ser-32, Ser-35 was able to support T7 phage DNA replication to the same level as the wild type thioredoxin if present at about 100-fold higher concentration (Huber *et al.*, 1986). Thus neither a reduction step nor an intermolecular disulfide bridge were necessary for a functional polymerase complex. In the absence of thioredoxin the T7 DNA polymerase dissociated from the primer after the incorporation of 1 to 50 nucleotides, depending on the salt concentration. In contrast, in the presence of thioredoxin, synthesis was highly processive. The gene *5* protein-thioredoxin complex could incorporate 1000s of nucleotides from a given primer without dissociation (Tabor *et al.*, 1987). Dissociation was accelerated by excess single stranded DNA in an apparent second order reaction, indicating transfer of the polymerase between DNA fragments. High salt concentrations also increased dissociation, indicating that thioredoxin stabilized the primer-template complex mainly through additional charge-charge interactions (Huber *et al.*, 1987). A highly specific interaction between complementary surfaces of thioredoxin and the gene *5* protein appeared to be required, as mutants of thioredoxin affecting the conserved, flat, hydrophobic surface were inactive as T7 DNA polymerase subunits (Huber *et al.*, 1986). Furthermore, only reduced thioredoxin from *E. coli*, *T. ferrooxidans*, *Rhodobacter sphaeroides* and a few other bacteria, has been found to activate the T7 gene *5* protein. Reduced thioredoxins from spinach chloroplasts, mammals, T4 phage, *Anabaena*, *C. nephridii* and many other bacteria cannot activate the gene *5* protein.

There is extensive amino acid homology between the Klenow fragment of *E. coli* DNA polymerase I, the T7 gene *5* protein and other DNA-dependent DNA polymerases. The strongest homology between the Klenow fragment and the T7 gene *5* protein lies in the residues that form the putative DNA-binding groove of the Klenow fragment (Ollis *et al.*, 1985). In the crystals of the Klenow fragment a "thumb-like" protrusion over the DNA-binding crevice was postulated to close the cleft, reducing the DNA dissociation rate of the subunits (Ollis *et al.*, 1992). Himawan *et al.* (1992) suggested that the thioredoxin binds the gene *5* protein at the edge of this crevice in the T7 polymerase such that the two proteins together clamp the primer-template into position. A thioredoxin mutant in which Gly-74 was replaced by Asp could not

support T7 phage replication. The authors analysed the interaction between the gene *5* protein and thioredoxin by selecting revertant T7 phage that were able to suppress this thioredoxin mutation. The authors proposed that the suppressor mutations compensated for the thioredoxin defect either by creating a conformational change in the gene *5* protein or by relieving ionic repulsion between Asp-74 and amino acids in the gene *5* protein.

1.2.4.3 T4 phage

T4 phage was found to encode its own ribonucleotide reductase and thioredoxin but the thioredoxin was reduced by NADPH and the host thioredoxin reductase (Berglund, 1969). Reduced T4 thioredoxin was a hydrogen donor for the T4 ribonucleotide reductase; this enzyme showed no activity with *E. coli* thioredoxin, and T4 thioredoxin was not active with *E. coli* ribonucleotide reductase.

T4 thioredoxin contained 87 amino acids and its primary structure showed no homology with *E. coli* thioredoxin (Sjöberg & Holmgren, 1972). The three-dimensional structure of T4 thioredoxin has been solved by X-ray crystallography to 2.8Å resolution (Eklund *et al.*, 1984). The protein had a similar folding pattern to *E. coli* TRX. It had two simple folding units: a βαβ unit from the N-terminal end (residues 1-36) and a ββα unit from the C-terminal end (residues 67-87). The redox active disulfide bridge formed part of a protrusion from the molecule as in *E. coli*. The T4 thioredoxin lacked the first 22 residues and therefore the α_1 and β_1 of the *E. coli* thioredoxin. Both molecules have a similar flat hydrophobic surface that is thought to be involved in binding to thioredoxin reductase and other protein molecules (Eklund *et al.*, 1984).

T4 thioredoxin was able to catalyze glutathione-dependent ribonucleotide reduction and thus also has glutaredoxin activity (Holmgren, 1978). In addition, glutaredoxin from *E. coli* was a hydrogen donor for T4 ribonucleotide reductase. T4 thioredoxin also had a GSH-disulfide transhydrogenase activity similar to glutaredoxin and 32% identity to *E. coli* glutaredoxin (Höög *et al.*, 1983). T4 thioredoxin has the active site sequence **-Cys-Val-Tyr-Cys-** which is closer to the glutaredoxin active site sequence **-Cys-Pro-Tyr-Cys-** than that of *E. coli* thioredoxin **-Cys-Gly-Pro-Cys-**. Immunologically, T4 thioredoxin and *E. coli* thioredoxin showed no cross-reactivity (Holmgren & Sjöberg, 1972). Holmgren (1985) proposed that T4 thioredoxin had resulted from a modification of a gene for "glutaredoxin" to include reactivity with thioredoxin reductase, as T4 ribonucleotide reductase could not function with *E. coli* thioredoxin and the low amounts of glutaredoxin in *E. coli* were insufficient for reduction of the viral ribonucleotide reductase. This adapted system therefore enabled T4 phage to utilize all available reducing power for T4 replication.

1.2.5 Thioredoxins of Anaerobic bacteria

The obligately anaerobic, fermentative bacteria have been reported to lack glutathione (Fahey *et al.*, 1978). In the absence of glutathione, thioredoxin might be a particularly important cellular reductant. A thioredoxin system was found in the classical saccharolytic fermenter *Clostridium pasteurianum* (Hammel & Buchanan, 1981). It was found that reduced ferredoxin rather than NADPH served as the physiological reductant. In this respect, this anaerobic bacterium resembled the oxygenic photosynthetic cells rather than aerobic heterotrophic cells. However, the clostridial thioredoxin reductase required hydrogen and hydrogenase to provide reduced ferredoxin for the reaction (Hammel *et al.*, 1983). Also, unlike the plant reductase, the bacterial protein had a typical flavin spectrum and presumably contains no iron. It was also highly specific for *C. pasteurianum* thioredoxin and would not reduce *E. coli*, *Chromatium* or *Rhizobium* thioredoxins. There is no known function for the clostridial thioredoxin system, as activation of higher plant enzymes was the only reaction studied by these workers. However, since the organism lacks glutathione, it is possible that thioredoxin is essential for ribonucleotide reduction (Gleason & Holmgren, 1988).

E. acidaminophilum and *Clostridium litoralis* are physiologically closely related anaerobic organisms, specialized with respect to fermentation of glycine and serine if no additional hydrogen donor or acceptor is present. In *E. acidaminophilum* dihydrolipoamide dehydrogenase activity was found associated with a protein of exceptionally small molecular size (68kDa) that was attached to the cytoplasmic membrane and formed a complex with the selenoprotein P_A of glycine reductase (Freudenberg *et al.*, 1989). This protein and the corresponding electron-transferring flavoprotein in *Clostridium sporogenes* exhibited strong homology to *E. coli* thioredoxin reductase but not to dihydrolipoamide dehydrogenases of other anaerobic bacteria (Dietrichs *et al.*, 1990). *C. litoralis* contained two enzymes for oxidizing dihydrolipoamide. The first was found to be a genuine dihydrolipoamide dehydrogenase, but the second consisted of two proteins, a thioredoxin reductase and a thioredoxin (Dietrichs *et al.*, 1990; Meyer *et al.*, 1991). The thioredoxin system was found to be responsible for most of the dihydrolipoamide dehydrogenase activity in cell extracts of *C. litoralis*. The thioredoxin did not bind to DEAE, was heat stable, and had a molecular mass of about 15kDa. N-terminal amino acid sequence data of the *E. acidaminophilum* and *C. litoralis* thioredoxins provided convincing evidence that both proteins were thioredoxins. However, two differences were noted at the strongly conserved redox-active site, a Gly residue replaced Trp-31 of *E. coli* and Val replaced Gly-33 (Meyer *et al.*, 1991).

Lübbers & Andreesen (1993), cloned and sequenced the genes for thioredoxin reductase, thioredoxin, protein P_A of glycine reductase and the first 23 amino acids of the large subunit of protein P_C of glycine reductase from *E. acidaminophilum*. The organisation of the genes corresponded exactly to the predicted order of their physiological involvement. The thioredoxin

reductase gene was separated from the thioredoxin gene by 85 base pairs, and the thioredoxin gene was followed by the protein P_A after a 91 base pair intergenic region. The thioredoxin system was found to reduce the oxidized selenoprotein P_A, enabling it to participate in a new cycle of reductive deamination. The *trxA* gene contained 110 codons corresponding to a protein of 11.7 kDa and exhibited 36% amino acid identity to the *E. coli* thioredoxin (Lübbers & Andreesen, 1993).

The thioredoxin and thioredoxin reductase proteins were also found in high amounts in sarcosine and betaine grown cells of *E. acidaminophilum*. It therefore seems quite possible that the thioredoxin system is involved in the three corresponding reductase systems specific for glycine, sarcosine and betaine (Lübbers & Andreesen, 1993).

1.2.6 Thioredoxins of Gram positive bacteria

Corynebacterium nephridii is a facultative organism, which can grow anaerobically using nitrate as its terminal electron acceptor. A thioredoxin was purified from *C. nephridii* (C-1) that had 50% amino acid identity to *E. coli* thioredoxin but had no His or Tyr residues (Meng & Hogenkamp, 1981). *C. nephridii* thioredoxin (C-1) could be reduced by *E. coli* thioredoxin reductase, and was able to act as a reducing agent for yeast methionine sulfoxide reductase, *Lactobacillus leichmannii* ribonucleotide reductase, spinach chloroplast fructose-1,6-bisphosphatase and NADP-malate dehydrogenase. However *C. nephridii* thioredoxin (C-1) was unable to serve as a reducing agent for its own ribonucleotide reductase. The ribonucleotide reductase of *C. nephridii* did not fit into either the category of the iron-containing protein found in *E. coli* or the adenosylcobalamin-dependent ribonucleotide reductase characteristic of *L. leichmannii*, cyanobacteria and other "primitive" bacteria. The *C. nephridii* ribonucleotide reductase also required adenosylcobalamin but like the *E. coli* protein it was composed of two subunits and reduced nucleotides at the diphosphate level. The authors suggested that *C. nephridii* thioredoxin C-1 is a general protein disulfide reductase and not an obligatory reductant for deoxyribonucleotide synthesis.

A second thioredoxin was cloned and characterised from *C. nephridii* by complementation of an *E. coli trxA* mutant (Lim *et al.*, 1987). The second thioredoxin, designated C-2, showed approximately 50% identity to both *E. coli* and the first *C. nephridii* thioredoxin isolated. Thioredoxin C-2 was able to support filamentous phage assembly but it was unable to support T7 phage replication. The active site of thioredoxin C-2 differed from all known thioredoxins as Gly-33 was replaced by an Ala residue. However, this probably did not affect the overall conformation as both are small, neutral amino acids. C-2 thioredoxin was found to be a hydrogen donor for yeast and *E. coli* methionine sulfoxide reductase, but not for its own ribonucleotide reductase. This is the first example of a second thioredoxin found in a prokaryotic, non-photosynthetic organism. Thioredoxin C-2 may not have been detected

initially as thioredoxin C-1 was isolated anaerobically whereas C-2 was isolated aerobically. So far no function has been established for thioredoxin in the denitrifiers.

Streptomyces clavuligerus produces penicillin and cephalosporin antibiotics. The activity of isopenicillin-N-synthetase (IPNS), a key enzyme in the biosynthesis of these β-lactam compounds, and its tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) are dependent on the redox state of their cysteine amino acid residues (Aharonowitz *et al.*, 1992). A disulfide reductase able to reduce bis-ACV to its thiol form and modulate the activity of IPNS was identified. The disulfide reductase was composed of a flavoprotein, containing two 35 kDa subunits and a 12 kDa, heat stable protein. The 35 kDa protein was identified as thioredoxin reductase and the 12 kDa protein as thioredoxin (Cohen *et al.*, 1993). On cloning and sequencing, the *S. clavuligerus* thioredoxin and thioredoxin reductase genes were found to be separated by 33 nucleotides and orientated in the same direction. The thioredoxin was unable to complement the *E. coli trxA* mutation for f1 phage growth or conversion of methionine sulfoxide to methionine. However, it was closely related to other thioredoxins with which it shared 48-56% amino acid identity.

1.2.7 Thioredoxins of Photosynthetic bacteria

Rhodobacter sphaeroides, is a purple non-sulfur bacterium which belongs to the group *Rhodospirillaceae*. It can grow anaerobically in the light and aerobically both in the light and the dark. Light intensity and molecular oxygen are the most prominent environmental factors that effect synthesis of bacterio-chlorophyll. The thioredoxin system in *R. sphaeroides* is involved in oxygen regulation of bacteriochlorophyll synthesis by the redox control of the activity of 5'-aminolevulinic acid synthetase, the first enzyme that occurs in the bacteriochlorophyll synthesis pathway. Thioredoxin has been shown to activate purified preparations of this enzyme (Clément-Métral, 1979).

R. sphaeroides thioredoxin was able to act as a hydrogen donor for *E. coli* methionine sulfoxide reductase and to support the growth of phages f1 and T7. It had 49% amino acid identity to *E. coli* thioredoxin and the active sites were identical except *R. sphaeroides* contained an Arg residue at position 33 instead of a Gly residue. The protein had a MW of 10.8 kDa and contained 102 amino acid residues (Pille *et al.*, 1990)

Thioredoxin has also been purified and characterized from another member of the group, *Rhodospirillum rubrum* (Johnson *et al.*, 1988). The amino acid sequence was determined by high performance tandem spectrometry. *R. rubrum* thioredoxin exhibited 53% amino acid identity to that of *R. sphaeroides* which was not much greater than its homology to the thioredoxins from other photosynthetic bacteria or *E. coli*. The data suggested the presence of a NADPH-dependent thioredoxin reductase in *R. rubrum* rather than the ferredoxin-thioredoxin reductase system found in plant chloroplasts.

Chromatium vinosum a purple, non-sulfur bacterium is an obligate anaerobe, which uses reduced sulfur compounds such as hydrogen sulfide as a source of electrons for photosynthetic electron transport. Thioredoxin was detected in extracts of this organism by its ability to activate chloroplast NADP-malate dehydrogenase using dithiothreitol (DTT) as a reductant (Johnson *et al.*, 1984). The protein was found to have a MW of 13 kDa. Unlike other thioredoxins it was found to be heat sensitive.

Antibodies prepared against *E. coli* thioredoxin cross-reacted with *C. vinosum* thioredoxin as well as those of other bacteria. By contrast antibodies prepared against spinach chloroplast thioredoxin m cross-reacted with *E. coli* and *N. muscorum* thioredoxins but only showed marginal reactivity with *C. vinosum* thioredoxin (Johnson *et al.*, 1984). A NADPH-dependent thioredoxin reductase was also isolated from *C. vinosum*. These results support the conclusion that *C. vinosum* thioredoxin is of the bacterial type. This seems appropriate since the organism utilizes ATP-driven reverse electron transport rather than direct photoreduction to reduce electronegative receptors such as NAD^+ and ferredoxin; and thioredoxin reduction via NADP would be energetically less costly than via ferredoxin. The role of thioredoxin in anoxygenic, photosynthetic organisms seems to lie outside the regulation of photosynthesis and carbon dioxide assimilation and in an area as yet unknown (Johnson *et al.*, 1984).

1.2.8 Thioredoxins of Archaea

A small redox-active protein was purified from *Methanobacterium thermoautotrophicum* (McFarlan *et al.*, 1992). The protein was initially thought to be a glutaredoxin as it had the redox-active site **-Cys-Pro-Tyr-Cys-** typical of glutaredoxins. However, the protein did not function as a glutathione-disulfide oxidoreductase in the presence of glutathione and glutathione reductase, nor did it interact with the thioredoxin reductases from *E. coli* or *C. nephridii* or the ribonucleotide reductase from *C. nephridii*. It was able to catalyse the reduction of insulin and serve as a hydrogen donor for *E. coli* ribonucleotide reductase. The authors proposed that this glutaredoxin-like protein may be a component of the ribonucleotide reductase system distinct from the described systems utilizing thioredoxin or glutaredoxin. It was similar to T4 phage "glutaredoxin" in that it had properties of both thioredoxins and glutaredoxins but did not conform completely to either group of proteins (McFarlan *et al.*, 1992).

Recently a thioredoxin was isolated from the extreme thermophilic Archaea *Sulfolobus solfataricus* (Guagliardi *et al.*, 1994). The protein was found to be highly heat resistant (no loss of activity after 3 hours at 90°C), it had a typically acid pI value of 4.5, but it differed from classical thioredoxins in molecular size. A molecular weight of 24.8 kDa was estimated under strongly denaturing conditions, furthermore the molecular weight determined by amino acid composition analyses agreed with that determined by SDS-PAGE. Other high MW forms of thioredoxin have been identified in plant species (Jacquot *et al.*, 1978), unicellular green algae

Scenedesmus obliquus (Langlotz *et al.*, 1986) and cyanobacterium *Anabaena* PCC7119 (Whittaker & Gleason, 1984).

1.2.9 Thioredoxins of Fungi

Penicillium chrysogenum is a fungus that produces penicillins which are sulfur-containing β-lactam compounds. As in *S. clavuligerus*, thioredoxin and thioredoxin reductase are responsible for the redox state of isopenicillin-N-synthetase (a key enzyme in the biosynthetic pathway of β-lactam compounds) and its tripeptide ACV. The *P. chrysogenum* thioredoxin was found to be a heat-stable 12 kDa protein that showed strongest homology to the thioredoxins of *Aspergillus niger* (59.6% identity; Le Marechal *et al.*, 1992) and *Saccharomyces cerevisiae* (53% identity; Gan, 1991). It contained the active site sequence **-W-C-G-P-C-** as well as several other highly conserved residues common to most thioredoxins. The *trxA* gene consisted of 2 exons and 1 intron (Cohen *et al.*, 1994). At present little is known about the regulatory mechanisms operating on the thioredoxin-thioredoxin reductase system and less about their involvement in the synthesis of antibiotics. Analysis of the roles of the glutathione and thioredoxin systems in penicillin production in *P. chrysogenum* and their regulation is required.

A thioredoxin that showed maximum identity (45%) to that of yeast and minimum identity to the spinach thioredoxin m, was isolated from a filamentous fungus, *Aspergillus nidulans* (Le Marechal *et al.*, 1992). The authors' results indicated that the *A. nidulans* thioredoxin belonged to the "h" type of thioredoxins, which are more eukaryotic-like. It exhibited an unusual fluorescence emission spectrum, characterized by a high number of Tyr residues. However, no fluorescence peak at 340nm, corresponding to a Trp residue was observed, as *A. nidulans* thioredoxin only contained one Trp residue and this was quenched by the disulfide bridge. The amino acid corresponding to Trp-28 of *E. coli* thioredoxin was a Phe residue in *A. nidulans*. The amino acid sequence contained the characteristic disulfide active site and some well-conserved amino acids; Pro-40, Pro-76, Lys-82 and Gly-92 (*E. coli* numbering). A third cysteinyl residue was present close to the active site. Such a thiol could form disulfide intermolecular bonds, as reported for *Dictyostelium discoideum* thioredoxin (Wetterauer *et al.*, 1992). The thioredoxin from *A. nidulans* could not serve as a substrate for *E. coli* thioredoxin reductase, nor could it activate fructose-1,6-bisphosphatase, but it could activate corn NADP-malate dehydrogenase.

cDNA clones for three different thioredoxins have been isolated from *D. discoideum* and multiple genes were identified on cross-hybridisation of cDNA with oligonucleotide probes (Wetterauer *et al.*, 1992). The three different *D. discoideum* thioredoxins showed 80% mutual identity. *D. discoideum* is a cellular slime mould and its life cycle can be divided into two phases, a vegetative growth phase and a developmental phase. The expression of the mRNA coding for the three thioredoxins was coregulated during development and maximal mRNA levels were seen in the phase of the life cycle when growth was arrested. This is of interest as

many of the known functions of thioredoxin, for example ribonucleotide reduction or sulfur assimilation are linked to growth. Since the expression of *D. discoideum* thioredoxin does not fit this pattern the authors decided to compare the enzymatic capacity of the *D. discoideum* thioredoxin to other thioredoxins that have been studied (Wetterauer *et al.*, 1992).

The *D. discoideum* thioredoxin TRX1 was found to be most similar to the cytoplasmic vertebrate thioredoxin (43% identity), whilst it was equally distant (30% identity) from *E. coli*, yeast and chloroplast f and m thioredoxins. The protein was found to be heat stable, like other thioredoxins but on a reverse-phase HPLC column, irrespective of its redox state, TRX1 separated into two peaks. This was unlike *E. coli* TRX which migrated as a homogeneous sample. The authors proposed that this was due to an additional cysteine residue at the N-terminus. The *D. discoideum* TRX1 was unable to substitute for *E. coli* TRX during filamentous phage assembly and was found to be a 100-fold less reactive with the *E. coli* thioredoxin reductase than the *E. coli* TRX. However, it was highly reactive with insulin, chloroplast NADPH-malate dehydrogenase and fructose-1,6-bisphosphatase (FBP-ase). This is unusual as apart from chloroplast thioredoxin f, most thioredoxins interact poorly with FBP-ase. A comparison of amino acid sequences of *D. discoideum* thioredoxin, thioredoxin f and human thioredoxin, revealed that most changes between *D. discoideum* thioredoxin and human thioredoxin occurred in the C-terminal region, rendering the human thioredoxin more basic. The discovery that conversion of Asp-61 to Asn increased the reactivity of *E. coli* thioredoxin with FBP-ase supported the theory that more acidic residues are important for interaction with FBP-ase (de Lamotte-Guéry *et al.*, 1991). Thus *D. discoideum* thioredoxin appeared to be a promiscuous protein, reacting with a variety of substrates and reductants, and it was not possible to group it with human cytoplasmic thioredoxin or *E. coli* thioredoxin (Wetterauer *et al.*, 1992).

1.2.10 Thioredoxins of Yeast

Characterization of thioredoxin deletion mutants of *Saccharomyces cerevisiae* (Muller, 1991) revealed that the two thioredoxin genes, *TRX1* and *TRX2*, were interchangeable and loss of either one alone had no effect on cell growth. However, deletion of both thioredoxin genes prolonged S phase, the period of DNA synthesis, by 3-fold and lead to a 33% increase in cell generation time. Since thioredoxin is a hydrogen donor for ribonucleotide synthesis (Laurent *et al.*, 1964), the inhibition of DNA synthesis was predicted to be caused by a reduction in the deoxyribonucleotide pools. However, the levels of dNTPs were either unchanged or slightly greater in the thioredoxin mutant (Muller, 1994). Thioredoxin mutants C30S and C33S were also unable to sustain normal rates of DNA synthesis, indicating that the redox capacity of thioredoxin was required for DNA synthesis (Muller, 1995). Muller proposed two alternative models incorporating these results. In one model it was suggested that thioredoxin reduces a multiprotein complex channeling nucleotides to the replication apparatus. In the second model it was proposed that thioredoxin regulates the tempo of DNA replication directly by activation of a component of the replication machinery.

A 25 kDa antioxidant enzyme that provided protection against oxidation systems capable of generating reactive oxygen and sulfur species was identified in yeast (Kim *et al.*, 1989) and rat brain (Kim *et al.*, 1988). The antioxidant enzyme was later shown to be a peroxidase that reduced hydrogen peroxide and alkyl hydroperoxides with the use of hydrogens provided by thioredoxin, thioredoxin reductase and NADPH (Chae *et al.*, 1994). The antioxidant enzyme was therefore named thioredoxin peroxidase. Thioredoxin peroxidase existed as a dimer of identical 25 kDa subunits that contained two cysteine residues, Cys-47 and Cys-170. Reduced Cys-47 appeared to be the site of oxidation by peroxides, and the oxidized Cys-47 probably reacted with reduced Cys-170 of the other subunit to form an intermolecular disulfide. Mutants lacking either Cys residue did not exhibit thioredoxin-coupled peroxidase activity.

1.2.11 Thioredoxin in Plants and Cyanobacteria

In plants light acts as a switch, changing metabolism from oxidative in the dark to photosynthetic in the light by modulating the catalytic activity of selective enzymes of degradative and biosynthetic pathways. Thioredoxin mediates the activation of some of these enzymes (Wolosuik & Buchanan, 1977). In the light, electrons are transferred from chlorophyll to ferredoxin (F_d), a strongly reducing iron-sulfur protein, and then via the enzyme ferredoxin-thioredoxin reductase to thioredoxin. Reduced thioredoxin can activate many enzymes. These include FBP-ase (Schürmann *et al.*, 1981), sedoheptulose-1,7-bisphosphatase (Nishizawa & Buchanan, 1981), NADP-malate dehydrogenase, phenylalanine ammonia lyase, ribulose bisphosphate carboxylase, and NADPH-glyceraldehyde-3-phosphate dehydrogenase and phosphoribulokinase of the pentose phosphate pathway (Jacquot *et al.*, 1978). The cyanobacteria are the simplest organisms with an oxygen-evolving photosynthetic system like that of plants. As in eukaryotic algae and higher plants, these prokaryotes contain chlorophyll a and use water as a source of electrons for photosynthetic electron transport, and are able to fix carbon dioxide via the Calvin cycle with ribulose-1,5-bisphosphate carboxylase serving as the key carboxylating enzyme.

In plant chloroplasts and cyanobacteria there appear to be two types of thioredoxins. Those capable of activating fructose-1,6-bisphosphatase and certain other key enzymes of carbon dioxide assimilation are called f-type thioredoxins, while those preferentially activating NADP-dependent malate dehydrogenase are termed m-type thioredoxins. Unlike cyanobacteria, thioredoxins f and m of plants appeared to have signal peptides that allowed import into chloroplasts (Kamo *et al.*, 1989; Wedel *et al.*, 1992). A third type of thioredoxin which was found in the cytoplasm of plants but not cyanobacteria and which was specifically reduced by a NADP/thioredoxin system was called the h-type thioredoxin (Florencio *et al.*, 1988).

The type-f thioredoxins appear to be highly divergent in activity and amino acid sequence, unlike most thioredoxins which are highly conserved. Their only similarity appears to be the ability to activate fructose-1,6-bisphosphatase. Comparison of numerous thioredoxin amino

acid sequences showed that apart from the conserved active site thioredoxin f of plants was not very similar to other thioredoxins (Hodges *et al.*, 1994). This was shown directly by the fact that pea thioredoxin f antibodies did not recognise any of the other thioredoxins tested (Hodges *et al.*, 1994). Similarly antibodies to T-2 thioredoxin of *Anabaena* PCC 7120 (f-type thioredoxin) did not cross react with *Anabaena* PCC 7120 T-1, *E. coli* or spinach m thioredoxins, but did react with T4 phage, *Synechocystis* sp. strain 6803 and *Synechococcus* sp. strain R2 thioredoxins (Gleason, 1992). Thioredoxin T_f from *Anabaena* PCC 7119 was found to have a molecular weight of 25 kDa, which is much higher than most other thioredoxins. In addition it was not a general protein disulfide reductase and could not act as a reductant for ribonuleotide reductase (Whittaker & Gleason, 1984). However, thioredoxin T-2 of *Anabaena* PCC 7120 was able to reduce both *E. coli* and *Anabaena* ribonucleotide reductases and insulin (Alam *et al.*, 1989). T-2 could also be reduced by glutathione, like the glutaredoxins (Gleason, 1992). The T-2 thioredoxin gene did not complement the TrxA mutation in *E. coli* (Alam *et al.*, 1989). Transformed *E. coli* cells were not able to use methionine sulfoxide as a methionine source or support replication of T7 phage or the filamentous phages.

Thioredoxin m of plants and cyanobacteria and thioredoxin Ch-2 of *Chlamydomonas reinhardtii* had considerable sequence homology and shared a similar spectrum of activities with the *E. coli* thioredoxin (Tsugita *et al.*, 1983; Yee *et al.*, 1981; Gleason & Holmgren, 1981; Alam *et al.*, 1989; Gleason, 1992; Decottignies *et al.*, 1990). Thioredoxin T-1 of *Anabaena* PCC 7120 (m-type thioredoxin) was found to be essential for photosynthetic growth (Muller & Buchanan, 1989). Thioredoxin T_m of *Anabaena* PCC 7119 complemented the *E. coli* $TrxA^-$ phenotype in every respect except T7 phage replication (Lim *et al.*, 1986). In plants NADP-malate dehydrogenase undergoes reductive activation in the light. This process was mediated by thioredoxin and involved the reduction of a N-terminal and a C-terminal disulfide (Issakidis *et al.*, 1992). Spinach thioredoxin m could exist as one of two very similar isomers m_b and m_c; the only difference was an additional N-terminal lysine residue in m_b (Schürmann *et al.*, 1981).

Both *Synechococcus* and *Anabaena cylindrica* have also been found to contain two thioredoxin species. Immunocytochemical techniques indicated the presence of one thioredoxin species predominantly in the nucleoplasm and a second species in the vicinity of the thylakoid membranes (chromatoplasm) of vegetative cells of *A. cylindrica* (Cossar *et al.*, 1985). A thioredoxin of MW 11.68 kDa and high sequence similarity to the m-type thioredoxins had previously been isolated from *A. cylindrica* (Ip *et al.*, 1984). The cyanobacteria, therefore, seem to resemble chloroplasts in containing more than one species of thioredoxin. Thus, it appears that most photosynthetic organisms contain two dissimilar thioredoxins, suggesting that the unusual thioredoxin (f-type) plays a specific role in photoautotrophic metabolism (Gleason, 1992).

The other thioredoxin system (thioredoxin h) found in plants, the NADP/thioredoxin reductase system, was found in the mitochondria, endoplasmic reticulum and cytosol (Kobrehel *et al.*, 1992). The seed was the only tissue for which thioredoxin h has been ascribed physiological activity. Thioredoxin h reduced members of several different soluble seed proteins; thionins, α-amylase and trypsin inhibitors (Johnson *et al.*, 1987; Kobrehel *et al.*, 1991). It also reductively activated PP*i* fructose-6-P,1-phosphotransferase (PFP) an enzyme of the carbohydrate metabolism (Kiss *et al.*, 1991). Thioredoxin h is thought to function as a signal in germination to enhance metabolic processes, such as the mobilization of storage proteins and activation of enzymes (Kobrehel *et al.*, 1992). Recently thioredoxin h was found to be one of the major proteins in rice phloem sap (Ishiwatari *et al.*, 1995). The authors proposed that the thioredoxin identified in this study may participate in maintenance of the sieve-tube proteins, such as ATPases, carriers, and transporters. Immunoblotting and Southern hybridization analysis suggested that rice plants contain another form of thioredoxin h as well as the cloned thioredoxin. A cytosolic h-type thioredoxin (Ch1) was also isolated from *C. reinhardtii* (Stein *et al.*, 1995) and two forms of thioredoxin h have been identified in spinach and tobacco (Florencio *et al.*, 1988; Brugidou *et al.*, 1993).

1.2.12 Thioredoxin in Mammalian cells

Thioredoxin has been purified from rats (Luthman & Holmgren, 1982), calves (Engström *et al.*, 1974), rabbits (Hopper & Iurlano, 1983) and humans (Wollman *et al.*, 1988). All these mammalian cells appeared to contain a classical thioredoxin system with one thioredoxin and a NADPH-thioredoxin reductase. The thioredoxins had a molecular weight of approximately 12 kDa and the same highly conserved thioredoxin active site as *E. coli*. They were also able to reduce *E. coli* ribonucleotide reductase and, apart from rabbit bone marrow thioredoxin, their own ribonucleotide reductases. Mammalian thioredoxins had, in addition to the active site disulfide, two additional structural half-cysteine residues in the C-terminal half of the molecule (Ren *et al.*, 1993). Mutation of the half-cysteinyl residues to serine indicated that Cys-72 was critical for the potential regulation of activity of thioredoxin via the formation of intra- or intermolecular disulfides, which caused aggregation or dimerization and inactivation of thioredoxin (Wollman *et al.*, 1988). High levels of thioredoxin mRNA were detected in actively dividing cell lines, but very low levels were found in resting cells. The coding region of human thioredoxin spanned over 13 kb and was organised into five exons separated by four introns. The transcription start point, located 22 base pairs downstream of a TATA box, defined a 5' untranslated region of 74 base pairs (Kaghad *et al.*, 1994). The promoter contained many possible regulatory elements compatible with both basal, constitutive expression and regulated, inducible expression, especially by cytokines such as interleukin-6 and interferons. Using Southern hybridization only one active gene encoding human thioredoxin was detected (Kaghad *et al.*, 1994).

Antisera prepared against pure rat liver thioredoxin and thioredoxin reductase, were used in immunohistochemical studies of adult rats to show that these two proteins to be widely distributed in tissues and organs (Rozell *et al.*, 1985). However, variations were seen between cell types; epithelial cells, neuronal cells and secretory cells (exocrine and endocrine) showed high immunoreactivity whereas mesenchymal cells showed low activity. Surface epithelial cells and keratinizing cells showed high activity. Thioredoxin was localised in the cytoplasm of cells with higher levels in the plasma membrane or subplasma membrane zone (Rozell *et al.*, 1985). High levels of thioredoxin and thioredoxin reductase were found in the endoplasmic reticulum which was consistent with its function in protein processing, secretion and the formation of nascent protein disulfides (Rozell *et al.*, 1988).

Thioredoxin was found to be secreted by normal and transformed cells despite its lack of a signal sequence (Rubartelli *et al.*, 1992). It was not secreted by the normal endoplasmic reticulum-Golgi route, but shared several features with the alternative pathway described for interleukin-1b, such as the slow kinetics of secretion, the enhancing effect on secretion of several unrelated drugs and the sensitivity to methylamine (Rubartelli *et al.*, 1992).

However, not all cells that synthesized thioredoxin secreted it, even within the same lineage. Whether this was due to some form of regulation or the absence of some components of machinery for secretion was not known (Rubartelli *et al.*, 1992).

Thioredoxin has been found to regenerate proteins inactivated by oxidative stress in endothelial cells (Fernando *et al.*, 1992). Björnstedt *et al.* (1994), found that NADPH, thioredoxin reductase and thioredoxin were efficient electron donors for human plasma peroxidase. Human plasma glutathione peroxidase is a member of the selenium-dependent peroxidases, which are usually very specific for glutathione as an electron donor. However, the plasma level of glutathione is very low, suggesting that the peroxidase is dependent on other electron donors. Glutaredoxin was also able to act as an electron donor for the peroxidase.

A soluble growth factor isolated from the supernatants of certain leukemic T cells, named acute T cell leukemia-derived growth factor (Tagaya *et al.*, 1989), and a factor which seemed to promote the growth of Epstein-Barr virus-immortalized lymphocytes (Wakasugi *et al.*, 1990), was shown to be identical to human thioredoxin (Wollman *et al.*, 1988). This factor has been shown to up-regulate the expression of the IL-2 receptor and might be involved in autocrine control of certain transformed T and B cells (Rubartelli *et al.*, 1992). Later Biguet *et al.* (1994), discovered that thioredoxin increased the proliferation of a B-cell line via the activation of protein kinase C through its translocation to the membrane. The authors suggested that enhanced phosphoinositide-specific phospholipase C activity induced by the dithiol form of thioredoxin was associated with protein kinase activation, and thus played a role in the permanent growth of Epstein-Barr virus-infected B cells.

Human thioredoxin was also found to be capable of stimulating cellular proliferation and DNA synthesis of murine fibroblasts (Oblong *et al.*, 1994). The mitogenic effect of thioredoxin was dependent on the active site or structural features of thioredoxin imparted by the active site cysteines since mutants C32S, C35S and C32S/C35S were not able to stimulate cell proliferation. The ability of thioredoxin to stimulate cellular proliferation could not be duplicated by either dithiothreitol or glutathione (Oblong *et al.*, 1994). Added I^{125}-thioredoxin was taken up by cells in culture and therefore could have an intracellular action (Powis *et al.*, 1994). In addition, competitive inhibitors of thioredoxin reductase were shown to inhibit thioredoxin-dependent cell proliferation, supporting the theory that thioredoxin can stimulate proliferation of a variety of normal and tumor cell lines (Powis *et al.*, 1994).

Another function of thioredoxin and thioredoxin reductase is the redox regulation of gene expression through the interaction with transcription factors. For example thioredoxin was proposed to stimulate DNA-binding of transcription factor NF-κB, by reduction of a disulfide bond involving the cysteine 62 residue of the p50 subunit of NF-κB (Matthews *et al.*, 1992). Similar observations of redox control by thioredoxin have been made with the transcription factors TFIIIC (Cromlish & Roeder, 1989), BZLF (Bannister *et al.*, 1991) and AP-1 (Abate *et al.*, 1990).

Similarly thioredoxin is involved in the activation of glucocorticoid receptor to a steroid binding state (Grippo *et al.*, 1985), and in the activation of rat brain cytosolic 3,5,3'-triiodo-L-thyronine-binding protein (CCTBP) (Lennon, 1992). Thyroid hormones regulate a number of biological responses through the binding of CCTBP to a nuclear protein acceptor and subsequent transcriptional regulation of target genes (Oppenheimer & Samuels, 1986).

Thioredoxin derived from activated macrophages was found to be a potent neurotrophic factor for central cholinergic neurons (Endoh *et al.*, 1993).

Loss of sensitivity to growth inhibitory polypeptides is thought to be one of the events that leads to tumor formation and might be caused by inactivation or loss of genetic elements that transduce the extracellular signals (Deiss & Kimchi, 1991). One of these genetic elements was isolated by random inactivation of genes with an anti-sense complementary DNA expression library followed by direct selection for growth in the presence of an inhibitory peptide. Thus a gene whose inactivation conveyed growth resistance to interferon-γ was isolated. Sequence analysis showed this factor, implicated in the interferon-γ-mediated growth arrest of HeLa cells, to be thioredoxin (Deiss & Kimchi, 1991). Both normal liver cells and hepatocarcinoma cell line HepG2 synthesize thioredoxin. Only the former, however, secrete abundant thioredoxin extracellularly. Under mild reducing conditions, HepG2 cells but not normal hepatocytes increased the rate of thioredoxin secretion and underwent growth inhibition accompanied by morphological changes (Rubartelli *et al.*, 1995). Recombinant thioredoxin was also found to

inhibit proliferation of HepG2 cells. This was in contrast to the previous examples of thioredoxin stimulating proliferation of leukemic T-cells, Epstein Barr virus lymphocyctes and murine fibroblasts, indicating that different cell types respond differently to variations in the extracellular redox potential (Rubartelli *et al.*, 1995).

Thioredoxin has been identified as the key component of the early pregnancy factor system. A system of components present in pregnancy sera which express a lymphocyte modifying activity in an assay known as the rosette inhibition assay (Tonissen *et al.*, 1993). Conversion of the two active site cysteine residues (Cys-32 and Cys-35) to serine, results in a protein devoid of classical redox activity; however this protein was still able to express lymphocyte modifying activity in the rosette inhibition assay. Further study revealed that lymphocyte modifying activity was solely dependent on Cys-74 of thioredoxin.

1.3 Glutathione

This ubiquitous tripeptide (γ-Glutamyl-L-cysteinylglycine), usually the most prevalent intracellular thiol, plays a pivotal role in the control of redox status, the storage of reduced sulfur and cysteine, the reduction of protein thiol groups, the synthesis of DNA, and the removal of reactive oxygen species and free radicals (Meister, 1983; Arrick *et al.*, 1982; Williamson *et al.*, 1982). In *E. coli* 25% of the sulfur is found in glutathione (Kosower, E. M. & Kosower, N. S., 1969).

Biosynthesis of glutathione takes place in two steps. In the first step the enzyme γ-glutamylcysteine synthetase, catalyzes the formation of γ-glutamylcysteine from glutamate and L-cysteine. In the second step, glycine is added to yield glutathione; this reaction is catalyzed by glutathione synthetase. Both reactions require ATP. The first reaction is usually the rate-limiting step in glutathione synthesis, and is feed-back inhibited by glutathione.

L-Glu + L-Cys + ATP → L-γ-Glu-L-Cys + ADP + P_i

L-γ-Glu-L-Cys + Gly + ATP → L-γ-Glu-L-Cys-Gly + ADP + P_i

Much less is known about glutathione metabolism in bacteria and plants than in mammalian systems. Observations on human diseases associated with glutathione deficiency indicated that glutathione was essential for normal health (Meister & Anderson, 1983). However, a number of bacteria, including certain anaerobes, do not contain glutathione (Fahey *et al.*, 1978), and at least one organism (*E. coli*) does not require glutathione for growth. The metabolism of glutathione in mammalian cells has been studied extensively. I shall therefore give an overview of the metabolism of glutathione in mammalian cells.

1.3.1 Metabolism of glutathione in mammalian cells

Meister & Anderson (1983) summarized glutathione metabolism in mammals in the cycle shown in Fig. 1.2. Glutathione is synthesized intracellularly by the consecutive actions of γ-glutamylcysteine synthetase (Reaction 1) and glutathione synthetase (Reaction 2). The breakdown of glutathione (and also of oxidized glutathione and S-substituted glutathione) is catalyzed by γ-glutamyl transpeptidase, which catalyzes transfer of the γ-glutamyl moiety to acceptors - amino acids (cysteine, glutamine and methionine), certain dipeptides, water and glutathione itself - (Reaction 3). Glutathione occurs mainly intracellularly and a major fraction of the transpeptidase is on the external surface of the cell membrane. Glutathione transported across cell membranes interacts with γ-glutamyl transpeptidase. γ-Glutamyl amino acids formed by γ-glutamyl transpeptidase are transported into cells. Intracellular γ-glutamyl amino acids are substrates of γ-glutamyl cyclotransferase (Reaction 4), which converts these compounds into the corresponding amino acids and 5-oxo-L-proline. The ATP-dependent conversion of 5-oxo-L-proline to L-glutamate is catalyzed by the intracellular enzyme 5-oxo-prolinase (Reaction 5). The cysteinylglycine formed in the transpeptidase reaction is split by dipeptidase (Reaction 6). These six reactions constitute the γ-glutamyl cycle, which thus accounts for the synthesis and degradation of glutathione. Two enzymes of the cycle also function in the metabolism of S-substituted glutathione derivatives, which may be formed non-enzymatically by reaction of glutathione with certain electrophilic compounds or by glutathione S-transferases (Reaction 7). The γ-glutamyl moiety of such conjugates is removed by the action of γ-glutamyl transpeptidase (Reaction 3), a reaction facilitated by γ-glutamyl amino acid formation. The resulting S-substituted cysteinylglycines are cleaved by dipeptidase (Reaction 6a) to yield the corresponding S-substituted cysteines, which may undergo N-acylation (Reaction 8) or an additional transpeptidation reaction to form the corresponding γ-glutamyl derivative (Reaction 3a).

Intracellular glutathione is converted to oxidized glutathione by selenium-containing glutathione peroxidase, which catalyzes the reduction of H_2O_2 and other peroxides (Reaction 9); there is evidence that certain glutathione S-transferases can also catalyze such reactions. Glutathione is also converted to oxidized glutathione by transhydrogenation (Reaction 10). Reduction of glutathione to oxidized glutathione is mediated by the widely distributed enzyme oxidized glutathione reductase which uses NADPH (Reaction 11). Extracellular conversion of GSH to GSSG has also been reported; the overall reaction requires O_2 and leads to the formation of H_2O_2 (Reaction 12). GSSG is also formed by the reaction of GSH with free radicals.

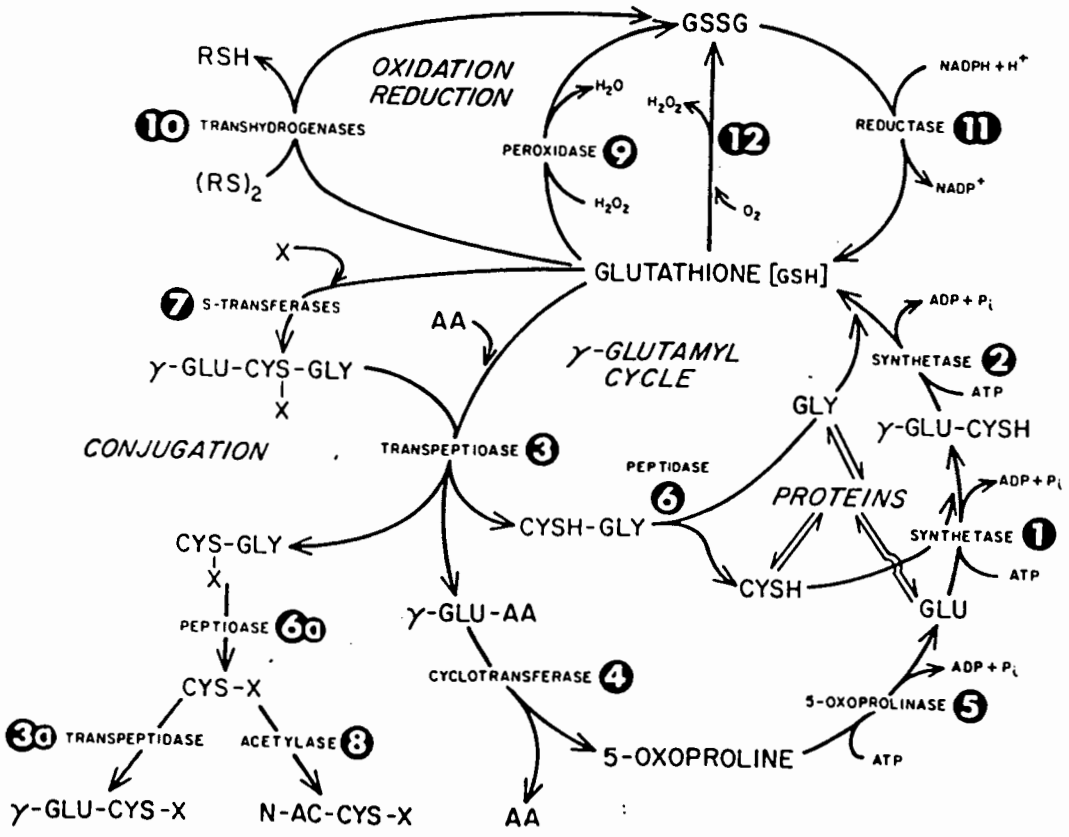


Fig. 1.2 Summary of glutathione metabolism in mammals.

1.3.2 Some functions of glutathione

As indicated in the above section glutathione is involved in the transport of amino acids, especially cysteine (via γ-glutamyl transpeptidase). The activities of many enzymes are influenced by glutathione and by other thiols (e.g. thioredoxin), and glutathione may therefore be involved in the regulation of these enzymes. There are also several reactions in which glutathione participates as a coenzyme.

Characterization of viable mutants of *E. coli* lacking thioredoxin, resulted in the discovery of glutaredoxin and glutathione as a hydrogen donor for NADPH-dependent ribonucleotide reduction (Holmgren, 1979). In this mutant glutaredoxin, glutathione and glutathione reductase functioned in the reduction of ribonucleotide reductase (Holmgren, 1976). In addition to ribonucleotide reduction, glutaredoxin also catalyzed the glutathione-dependent reduction of small disulfide containing compounds like hydroxyethyl disulfide (HED); i.e. it exhibits glutathione disulfide transhydrogenase activity (Holmgren, 1979). As discussed above thioredoxin and/or glutaredoxin have been assumed to be required as hydrogen donors for ribonucleotide reductase which is essential for DNA synthesis, yet an *E. coli* double mutant lacking thioredoxin and glutaredoxin (Grx1) could be constructed (Russel *et al.*, 1990). This implied the presence of a third hydrogen donor. Åslund *et al.* (1994), reported the purification of two proteins (Grx2 and Grx3), with glutaredoxin activity from an *E. coli* mutant lacking Trx

and Grx1. Their evidence indicated that Grx3 is likely to substitute for Trx and Grx1 in the double mutant.

Glutathione has been proposed to protect cells from oxidative damage and ionizing radiation (Meister & Anderson, 1983). Studies have shown that cells can maintain and even increase cellular glutathione content in response to sublethal oxidative stress. Shi *et al.* (1994) were able to show that quinone-induced oxidative stress increased the rate of transcription of the gene for γ-glutamylcysteine synthetase in rat lung cells. Induction of the γ-glutamylcysteine synthetase heavy subunit gene transcription in rat cells did not appear to require an electrophile-glutathione conjugate, as 2,3-dimethoxy-1,4-naphthoquinone (DMNQ) was able to induce γ-glutamylcysteine synthetase transcription, but unlike simpler quinones, could not form a glutathione conjugate (Shi *et al.*, 1994). More recently Mulcahy & Gipp (1995), identified a possible antioxidant response element (ARE) in the 5'-flanking region of the human γ-glutamylcysteine synthetase heavy subunit gene, suggesting that γ-glutamylcysteine synthetase expression in human cells may be regulated in response to oxidative challenge via an ARE. Depletion of cellular glutathione or inhibition of γ-glutamylcysteine synthetase enhanced the sensitivity of *S. cerevisiae* to hydrogen peroxide, suggesting that glutathione plays an important role in the adaptive response of *S. cerevisiae* to oxidative damage (Izawa *et al.*, 1995). Glutathione synthesis and the transport of glutathione metabolites also seemed to be responsive to heat shock (Kondo *et al.*, 1993), indicating that glutathione may play a role in the defense system of cells against several different stresses.

In humans, glutathione peroxidases catalyze the glutathione-dependent reduction of H_2O_2, and are important enzymes in the detoxification of hydrogen peroxide, lipid peroxides and organic hydroperoxides (Björnstedt *et al.*, 1994). However, human plasma glutathione peroxidase uses extracellular thioredoxin reductase, thioredoxin or glutaredoxin as reductants rather than glutathione, as plasma levels of glutathione are very low (Björnstedt *et al.*, 1994).

Conflicting results have been obtained as to whether glutathione protects *E. coli* cells from oxidative damage or not. Greenberg *et al.* (1986) showed that *E. coli* deficient in glutathione had normal resistance to H_2O_2, cumene hydroperoxide, heat and ionizing radiation. However, this *E. coli gshA* mutant was hypersensitive to thiol-specific reagents and highly resistant to toxic agents thought to require thiols for their activation. This data indicated that glutathione may not protect *E. coli* from oxidative damage. In contrast, an *E. coli gshA* mutant was found to be sensitive to 8-hydroxyquinoline, unless glutathione was provided in the media or the mutant was transformed with a γ-glutamylcysteine synthetase gene able to complement the *gshA* mutation (May & Leaver, 1994). *E. coli* apparently lacks the enzyme glutathione peroxidase, which catalyzes the reduction of hydrogen peroxide and other peroxides (Smith & Schriff, 1979).

The role of glutathione in the prevention of oxygen toxicity is closely related to phenomena associated with highly reactive oxygen species formed as a result of radiation. There are indications that diminished levels of glutathione render mammalian cells more sensitive to radiation (Bump *et al.*, 1982; Edgren *et al.*, 1981). However, in other experiments glutathione depletion had no effect on mammalian cells (Clark *et al.*, 1984). A strain of *E. coli* enriched in its content of γ-glutamylcysteine synthetase and glutathione synthetase activities by DNA recombination techniques was found to be more resistant to the lethal effects of gamma-radiation than the corresponding wild type strain (Moore *et al.*, 1989). The observed radioresistance appeared to be associated with the increased capacity of the gene-enriched *E. coli* strain to synthesize glutathione, rather than the actual cellular levels of glutathione, as buthionine sulfoximine (an inhibitor of γ-glutamylcysteine synthetase) abolished resistance, although glutathione levels remained constant.

Reduction of glutathione concentrations can sensitize drug-resistant tumour cells *in vivo* as well as *in vitro*, suggesting that elevated levels of glutathione may play a prominent role in drug resistant phenotypes (Suzukake *et al.*, 1982; Ono *et al.*, 1986; Ozols *et al.*, 1987). One of the most common biochemical changes detected in tumor cells resistant to the cytotoxic effects of alkylating or platinating agents or anthracyclines, such as doxorubicin, was a significant increase in the intracellular concentration of glutathione (Wolf *et al.*, 1987). In yeast, the yAP-1 transcriptional regulatory protein, has been found to cause profound alterations in cellular drug and metal resistance. The γ-glutamylcysteine synthetase gene of yeast appears to be one of several genes that are transcriptionally regulated by yAP-1 and influence drug resistance (Wu & Moye-Rowley, 1994). Tumor cells depleted of glutathione by treatment with buthionine sulfoximine also exhibited increased susceptibility to cytolysis by reactive oxygen intermediates (Arrick *et al.*, 1982). These findings indicated, as suggested by Griffith & Meister (1979), that buthionine sulfoximine, because it inhibits γ-glutamylcysteine synthetase, may be an important adjuvant in a wide range of chemo- and radiation therapies (Meister & Anderson, 1983).

Administration of certain carcinogens increases the levels of glutathione and of γ-glutamyl transpeptidase. Most chemical carcinogens are electrophiles and thus good candidates for detoxification by reactions catalyzed by glutathione-S-transferases. A substantial regression of aflatoxin-induced liver tumors in rats treated with large oral doses of glutathione, was noted by Novi (1981). Trypanosomes which lack catalase and have high intracellular H_2O_2 levels, are more susceptible to the effects of glutathione depletion than are most cells. Treatment of mice infected with trypanosomes with buthionine sulfoximine, which inhibits glutathione synthesis, led to prolonged survival of the mice, indicating death of the trypanosomes (Arrick *et al.*, 1981).

These studies have provided significant evidence for the proposed functions of glutathione in destroying free radicals, and in the reduction of reactive oxygen intermediates.

Glutathione-S-transferases catalyze the nucleophilic conjugation of glutathione with a very large number and variety of foreign compounds to form glutathione conjugates (Habig, *et al.*, 1974). Initial degradation of the glutathione conjugate proceeds via γ-glutamyltranspeptidase to the cysteinylglycine conjugate and then is hydrolysed by a carboxypeptidase to the cysteine conjugate (Zablotowicz *et al.*, 1995). Glutathione conjugation is a major mechanism of detoxification in mammals (Habig *et al.*, 1974) and is involved in detoxification of at least six major herbicides in plants (Lamoureux & Rusness, 1989). It is postulated that glutathione transferase also plays a role in the detoxification of xenobiotics in rhizosphere bacteria (Zablotowicz *et al.*, 1995). A crucial role for glutathione has thus been proposed in the detoxification of air pollutants such as sulfur dioxide and ozone (Sen-Gupta *et al.*, 1991), in the removal of xenobiotics such as herbicides (Zablotowicz *et al.*, 1995) and heavy metals (Jackson *et al.*, 1987), and in the adaptation of plants to drought (Dhindsa & Matowe, 1981) and extremes of temperature (Nieto-Sotelo & Ho, 1986).

The metabolism of glutathione in plant and bacteria has not been studied as extensively as in mammalian systems. However, various functions of glutathione in plants and bacteria have been identified. Interestingly both microorganisms and plants (Meister, 1980; Rennenberg, 1982) seem to export glutathione, at least in a few instances thus far studied. As stated above, glutathione-S-transferase activity was identified in 36 species of rhizosphere bacteria in detoxification of xenobiotics (Zablotowicz *et al.*, 1995). *E. coli* accumulates the novel compound glutathionyl-spermidine (Tabor & Tabor, 1972), and homoglutathione (γ-glutamyl-cysteinyl-β-alanine) has been found in mung beans (Buchanan, 1983). Thioredoxin, and possibly glutathione, function in photosynthesis (Buchanan, 1983). Plants also contain glutathione S-transferases (Lamoureux & Rusness, 1989). Thus, further investigation of the metabolism of glutathione in plants and bacteria may reveal further similarities to that of mammals.

γ-Glutamylcysteine and bis-γ-glutamylcysteine reductase in halobacteria were found to function in an analagous fashion to glutathione and glutathione reductase in other cells (Sundquist & Fahey, 1989). All species of halobacteria examined produced millimolar levels of γ-glutamylcysteine, whilst glutathione was not detected. Under halophilic conditions, γ-glutamylcysteine is as least as stable as glutathione, which may indicate why halobacteria are able to utilize γ-glutamylcysteine rather than glutathione (Sundquist & Fahey, 1989).

1.4 γ-Glutamylcysteine synthetases from different organisms

γ-Glutamylcysteine synthetase has been purified from rat kidney (Orlowski & Meister, 1971), bovine lens (Wendel *et al.*, 1972), human erythrocytes (Majerus *et al.*, 1971), tobacco (Hell & Bergmann, 1990), *Arabidopsis thaliana* (May & Leaver, 1994), *Candida biodinii* (Dennda & Kula, 1986), *Saccharomyces cerevisiae* (Ohtake & Yabuuchi, 1991), *Escherichia coli*

(Apontoweil & Berends, 1975) and *Proteus mirabilis* (Kumagai *et al.*, 1982). The different γ-glutamylcysteine synthetase enzymes vary widely in primary structure but all appear to follow the same reaction mechanism.

1.4.1 Mammalian γ-glutamylcysteine synthetases

The enzyme purified from rat kidney has been studied the most and has 89% amino acid identity to that of humans. Rat kidney γ-glutamylcysteine synthetase will be discussed as an example of mammalian γ-glutamylcysteine synthetase. Rat kidney γ-glutamylcysteine synthetase is composed of two subunits (M_r 73 kDa and 27.7 kDa), which can be dissociated by native electrophoresis after treatment with DTT (Seelig *et al.*, 1984). Partial reassociation occurs after DTT removal, suggesting that this phenomenon is associated with reformation of disulfide linkages between subunits. The heavy subunit exhibited all the structural requirements for enzyme activity and feedback inhibition by glutathione (Richman & Meister, 1975). The light subunit was recently shown to have a physiologically important regulatory function (Huang *et al.*, 1993). The light chain also lowered the K_m for glutathione and increased feedback inhibition by glutathione of the heavy catalytic subunit. Feedback inhibition by glutathione appeared to involve reduction of the enzyme (Huang *et al.*, 1993).

The acceptor amino acid specificity of the enzyme is rather broad, and it can interact with several glutamate analogs, including β-aminoglutarate and α-aminomethylglutarate (Sekura & Meister, 1977). Evidence has also been obtained that γ-methylene-D-glutamate, which inactivates the enzyme, binds in the glutamate-binding site of the active center and forms a covalent linkage with the active site thiol through a Michael-type addition reaction (Simondsen & Meister, 1986).

Feedback inhibition by glutathione was thought to be "non-allosteric" as inhibition by glutamate was competitive (Richman & Meister, 1975). However, only glutathione, and not γ-glutamyl-α-aminobutyrylglycine, inhibited γ-glutamylcysteine synthetase. This suggested that glutathione binds not only at the glutamate binding site of the active center but also at another site that interacts with the thiol moiety of glutathione (Huang *et al.*, 1988), as the thiol group of glutathione is replaced by a methyl group in γ-glutamyl-α-aminobutrylglycine.

γ-Glutamylcysteine synthetase was strongly inactivated by cystamine. However, the inactivation could be reversed by treatment with DTT, suggesting that cystamine forms a mixed disulfide with an enzyme thiol (Seelig & Meister, 1984). Treatment of the enzyme with cystamine prevented interaction with the sulfoximines. Titration of the enzyme with 5,5'dithiobis(2-nitrobenzoate) revealed that the enzyme had a single exposed thiol that reacted with this reagent without affecting activity (Seelig & Meister, 1982). Thus cystamine must interact with another enzyme thiol group.

γ-Glutamylcysteine synthetase bound covalently to cystamine-Sepharose, an interaction that was facilitated by ATP and inhibited by Mg^{2+} plus glutamate (Seelig & Meister, 1982). A large fraction of the enzyme applied to such columns apparently bound by forming a disulfide bond between cystamine-Sepharose and a sulfhydryl group at or near the active site. The enzyme could be released by treatment with dithiothreitol. The enzyme did not bind to columns of S-(S-methyl)cysteamine-Sepharose, but S-(S-methyl)cysteamine was a potent inhibitor of γ-glutamylcysteine synthetase. A cysteamine-S-disulfide moiety derived from the external cysteamine residue of cystamine-Sepharose, seemed to be the critical group recognized by the enzyme. Studies with a number of cystamine analogs supported this conclusion and led to the further conclusion that a disulfide (or diselenide) moiety and a single free amino group are required for inhibition (Seelig & Meister, 1984).

The enzyme was also inactivated by S-sulfocysteine and S-sulfohomocysteine; these inactivators were tightly but noncovalently bound and their interaction with the enzyme was postulated to involve effects of the active site thiol (Moore *et al.*, 1987).

The heavy subunit was inactivated by incubation with L-2-amino-4-oxo-chloropentanoate. Studies with the ^{14}C-labelled chloroketone showed that it bound stoichiometrically to the enzyme, and it bound exclusively to the heavy subunit (Sekura & Meister, 1977). Inhibition by the chloroketone was competitive with L-glutamate. Like cystamine the chloroketones are thought to interact with a thiol group at the active site. A low concentration of divalent metal ions was required for inhibition by the chloroketones. The authors suggested that a divalent ion such as Mg^{2+} may be involved in the binding or reaction, or both, of the chloroketone, and possibly also of the natural substrate, glutamate. It must be noted that while L-glutamate protected the enzyme against inactivation by the chloroketone, D-glutamate did not. Although the enzyme was inactivated by incubation with the less specific alkylating agent, iodoacetamide, higher concentrations of the latter reagent were required for inhibition and L-glutamate did not protect the enzyme against such inhibition as effectively as it did against the chloroketone (Sekura & Meister, 1977).

Meister (1974) proposed that the reaction catalysed by γ-glutamylcysteine synthetase proceeds via an enzyme-bound γ-glutamylphosphate intermediate, which interacts with the amino group of cysteine. Kinetic investigations were consistent with this possibility, but did not demonstrate γ-glutamyl phosphate as a discreet covalent complex (Yip & Rudolph, 1976).

Methionine sulfoximine was an effective, irreversible inhibitor of γ-glutamylcysteine synthetase and, in the presence of Mg^{2+} and ATP, was converted to methionine sulfoximine phosphate, which bound tightly to the enzyme (Richman *et al.*, 1973). Under certain conditions the methionine sulfoximine could be released with the restoration of catalytic activity. These findings are consistent with the formation of an enzyme-bound glutamyl phosphate

intermediate, since phosphorylation of methionine sulfoximine seems to reflect phosphorylation of glutamate in the normal reaction (Richman *et al.*, 1973). Buthionine sulfoximine was a more potent inhibitor of γ-glutamylcysteine synthetase (Griffith & Meister, 1979), and was also phosphorylated at the active site of γ-glutamylcysteine synthetase (Griffith, 1982). It is thought that the S-alkyl moiety of the sulfoximines binds at the enzyme site that normally binds the acceptor amino acid (Griffith & Meister, 1979).

1.4.2 Yeast

Dennda & Kula (1986), purified the γ-glutamylcysteine synthetase enzyme from *Candida boidinii*. Their studies indicated that the yeast enzyme was very similar to the mammalian γ-glutamylcysteine synthetase. Both seem to be regulated by feedback inhibition. They exhibited similar temperature and pH optima and seemed to possess SH groups essential for maintenance of enzymatic activity, as reagents reacting with sulfhydryl groups such as *N*-ethylmaleimide (NEM), *p*-chloromercuribenzoate (PCMB) and iodoacetamide (IAA) had a strong inhibitory effect on the yeast enzyme. However, the mammalian enzyme was inactivated by treatment with DTE, while DTE was used in the extraction of the yeast enzyme and did not appear to affect the activity of the enzyme. The yeast enzyme also differed from the mammalian enzyme in the size of its subunits. The native yeast enzyme had a molecular weight of approximately 124 kDa, and appeared to be composed of two 60 kDa subunits, whereas the mammalian enzyme was formed from 73 kDa and 27 kDa subunits. Therefore, the main differences between the two enzymes appeared to be the sizes of the subunits and the effect of DTE on enzyme activity.

The γ-glutamylcysteine synthetase gene of *Saccharomyces cerevisiae* was cloned and sequenced. The deduced amino acid sequence was reported to have 45% identity to that of rat kidney and only 26% identity to that of *E. coli* (Ohtake & Yabuuchi, 1991).

1.4.3 Plants

1.4.3.1 Higher Plants

γ-Glutamylcysteine synthetase activity was analyzed from partially purified cell extracts of *Nicotiana tabacum* (Hell & Bergmann, 1990). The enzyme had a relative molecular mass of 60 kDa and exhibited maximal activity at pH 8 and an absolute requirement for Mg^{2+}. Treatment with DTE led to a heavy loss of activity and to dissociation into subunits (M_r 34 kDa). The enzyme was completely inhibited by glutathione and the inhibition was competitive with respect to L-glutamate.

Buthionine sulfoximine and L-methionine sulfoximine were effective inhibitors of the plant enzyme, indicating that the reaction may proceed via a γ-glutamylphosphate intermediate (Hell & Bergmann, 1990).

γ-Glutamylcysteine synthetase activity was found both in the cytoplasm and the chloroplasts. Hell & Bergmann (1990) proposed that the rate of glutathione synthesis could be linked to light, from the following observations. When chloroplasts are illuminated the stroma pH is shifted from pH 7.0 to pH 8.0 and at the same time the stromal Mg^{2+} concentration increases to about 2mM. The alkalinization of the stroma and the increase in Mg^{2+} concentration are in a range where they can effectively influence the rate of γ-glutamylcysteine synthesis and the rate of glutathione synthesis in the chloroplasts, as glutathione synthetase also shows 50% of its maximal activity at pH 7.0 and a broad pH optimum between pH 8.0 and pH 9.0 (Hell & Bergmann, 1988). Such an increase in the rate of glutathione synthesis during illumination could be involved in the light-dependent diurnal fluctuations of glutathione concentrations observed in leaves (Koike & Patterson, 1988; Schupp & Rennenberg, 1988).

1.4.3.2 *Arabidopsis thaliana*

The *A. thaliana* γ-glutamylcysteine synthetase gene was cloned by complementation of an *E. coli gshA* mutant for growth on 8-hydroxyquinoline (May & Leaver, 1994). The *E. coli gshA* mutant was unable to synthesize glutathione and in the absence of glutathione was sensitive to 8-hydroxyquinoline. The derived amino acid sequence of the *A. thaliana* γ-glutamylcysteine synthetase gene constituted a polypeptide of 59.9 kDa. The predicted amino acid sequence had only 15-19% identity to other γ-glutamylcysteine synthetase genes. To determine the extent of sequence conservation among higher plants, the authors carried out Southern hybridization, using cDNA derived from the *A. thaliana* γ-glutamylcysteine synthetase gene as a probe. Surprisingly, this probe did not hybridize to tobacco DNA and only a weak signal was detected against maize DNA. This suggested that plant γ-glutamylcysteine synthetase molecules are structurally highly divergent. Differences in kinetic properties of the recombinant *A. thaliana* γ-glutamylcysteine synthetase (May & Leaver, 1994), maize γ-glutamylcysteine synthetase (Ruegsegger & Brunold, 1993) and that of tobacco (Hell & Bergmann, 1990), were also found.

In direct contrast to tobacco and mammals the *A. thaliana* γ-glutamylcysteine synthetase activity was stable upon storage and was not inactivated by reductants (May & Leaver, 1994). The tobacco enzyme was found to be dimeric, while *A. thaliana* enzyme activity suggested it was monomeric since it was not susceptible to dissociation and subsequent inactivation by reductants. However, the maize enzyme was active in the presence of DTT and, therefore, may be more similar to the *A. thaliana* enzyme, as indicated by the weak hybridization of *A. thaliana* γ-glutamylcysteine synthetase cDNA to maize genomic DNA (May & Leaver, 1994).

As with all the other γ-glutamylcysteine synthetase enzymes studies so far, the *A. thaliana* γ-glutamylcysteine synthetase enzyme was feedback inhibited by glutathione in a nonallosteric manner, and inhibited by sulfoximine analogs of γ-glutamylphosphate.

1.4.4 Prokaryotes

1.4.4.1 *Escherichia coli*

The *E. coli* γ-glutamylcysteine synthetase enzyme had a molecular weight of 56 kDa and was found to be homogenous on polyacrylamide gel electrophoresis in the presence or absence of SDS (Huang *et al.*, 1988). The enzyme had a pH optima of 8.5 and was stable after storage at -20°C for two months (Apontoweil & Berends, 1975).

The catalytic properties of the γ-glutamylcysteine synthetase from *E. coli* and rat kidney were very similar. This was evident from the similarities in turnover number, substrate specificity and apparent K_m values. Furthermore both enzymes were inactivated to the same extent by buthionine sulfoximine in the presence of ATP. In contrast to the mammalian enzyme, the *E. coli* enzyme was insensitive to cystamine, γ-methylene glutamate and S-sulfo amino acids, indicating that it did not have an active site thiol (Huang *et al.*, 1988). It seems from these results that although the rat kidney and *E. coli* enzymes catalyze the same overall reaction, their catalytic functions may be performed by use of different active site structures. The antigenic determinants of the rat kidney and *E. coli* enzymes were also different; polyclonal antibodies obtained from rabbits immunized against the one or other of the purified enzymes did not cross-react on Ouchterlony plates (Huang *et al.*, 1988). Thus the quaternary structures of the two enzymes must differ. The *E. coli* enzyme appeared to consist of a single 56 kDa subunit, whilst the mammalian enzyme has been found to be composed of two subunits differing in size and activity.

1.4.4.2 *Proteus mirabilis*

A γ-glutamylcysteine synthetase enzyme isolated from *Proteus mirabilis* (Kumagai *et al.*, 1982) had similar properties to the rat kidney and *E. coli* enzymes in optimum pH and K_m values for substrates, but it showed a sigmoidal dependence of the reaction rate on L-cysteine which has not been observed in other γ-glutamylcysteine synthetases. The molecular weight of the enzyme was determined by ultracentrifugation and gel filtration to be between 62 and 64 kDa, and a single band at 62 kDa was obtained on SDS polyacrylamide gel electrophoresis, after cross-linking with dimethylsuberimidate. However, several bands were obtained on SDS (without cross-linking) and disc polyacrylamide gel electrophoresis.

1.5 Motivation for the isolation and characterisation of the thioredoxin and γ-glutamylcysteine synthetase genes.

The redox potentials in the environment of an iron-oxidising bacterium such as *T. ferrooxidans* is a function of the Fe^{3+}/Fe^{2+} ratio. When ferrous iron is oxidised to ferric iron the redox potential of the surrounding medium increases correspondingly and bacteria which grow in such an environment require the ability to maintain their cytoplasmic redox potential within certain limits. Iron-oxidising bacteria like *T. ferrooxidans* might, therefore, be expected to have a

greater capacity for controlling their internal redox potential than most bacteria. This study was undertaken to investigate intracellular systems that are likely to play a role in the ability of *T. ferrooxidans* to cope with large changes in its redox potential. The thioredoxin and γ-glutamylcysteine synthetase genes were chosen for investigation, as thioredoxin and glutathione are two molecules that play a role in the control of redox potential within a cell.

The isolation and analysis of these genes was also of interest in terms of increasing the knowledge of the genetics and physiology of *T. ferrooxidans*.

CHAPTER 2

THE ISOLATION AND CHARACTERIZATION OF THE *T. FERROOXIDANS* THIOREDOXIN GENE

CHAPTER 2

THE ISOLATION AND CHARACTERIZATION OF THE *T. FERROOXIDANS* THIOREDOXIN GENE

2.0 Summary

The *T. ferrooxidans* thioredoxin gene was isolated by complementation of the *E. coli trxA* mutant strains BH5262 and BH2012. The source of the thioredoxin complementing DNA was confirmed by Southern hybridisation as originating from the *T. ferrooxidans* ATCC 33020 chromosome. The nucleotide sequence of a 1.1kbp *Hind*III-*Pst*I *T. ferrooxidans* chromosomal DNA fragment was determined from both strands. An open reading frame (ORF) corresponding to the thioredoxin gene and part of an ORF corresponding to the N-terminal region of the *rho* gene were identified. Synthesis of a 14 kDa protein corresponding to the predicted size of the *T. ferrooxidans* thioredoxin was confirmed using an *E. coli*-derived *in vitro* transcription-translation system.

DNA:RNA hybridisation was carried out on RNA transcripts prepared from *E. coli* BH5262 *trxA* (deletion mutant), *E. coli* BH5262 containing the cloned *trxA* gene and *T. ferrooxidans* cells. The *T. ferrooxidans trxA* gene was found to be transcribed independently of the downstream *rho* gene. Primer extension analysis was used to determine the transcriptional start sites of the *trxA* gene in *T. ferrooxidans* and that of the cloned gene in *E. coli*. Three transcription start sites were identified with RNA prepared from *T. ferrooxidans*. Using RNA derived from the cloned gene in *E. coli*, two transcription start sites were identified that were identical to those of *T. ferrooxidans*-derived RNA.

The cloned *T. ferrooxidans* thioredoxin gene was able to support T7 phage growth in the *E. coli trxA* mutant, BH2012. A growth curve of phage T7 was determined in *E. coli* host strains MC1061, BH2012 and BH2012(pTRX6). The *T. ferrooxidans* thioredoxin was also tested for the ability to support filamentous phage assembly, however, it was not able to satisfy the thioredoxin requirement of M13 phage.

Crude extracts of *E. coli trxA* mutants with and without the *T. ferrooxidans trxA* gene were compared for the ability to reduce insulin. Crude extracts of the *E. coli trxA* mutant, containing the cloned *T. ferrooxidans trxA* gene were able to reduce insulin far more rapidly than those that did not contain the cloned gene, indicating that the *T. ferrooxidans* thioredoxin has disulfide reductase activity.

2.1 Introduction

As discussed in Chapter 1 thioredoxin has many different functions, and the role it plays in *T. ferrooxidans* is unclear. There are numerous possible functions thioredoxin may have in relation to the unusual habitat occupied by *T. ferrooxidans*. These may include the maintenance of redox potential in the cell, enzyme regulation or acting as a cofactor to specific enzymes. When growing in arsenopyrite ores *T. ferrooxidans* is able to tolerate high concentrations of arsenic. In other arsenic resistant bacteria thioredoxin is required for the maintenance of the cysteine residues of arsenate reductase (ArsC) in the reduced state (Ji & Silver, 1992). Neither glutaredoxin nor glutathione were able to replace thioredoxin in this reaction. A similar mechanism of arsenic resistance may operate in *T. ferrooxidans* to that of ArsC but this has not been elucidated yet. In addition reduced thioredoxin may react directly and irreversibly with arsonous acid to form very stable dithioesters thus removing the highly toxic arsonous acid from the cell (Knowles & Benson, 1983).

$$TRX\text{-}SH_2 + HAs(OH)_2 \rightarrow TRX\text{-}S_2AsH + 2H_2O$$

Despite *T. ferrooxidans* being an acidophilic, obligate chemoautolithotroph, and *E. coli* being a heterotrophic bacterium, *E. coli* and *T. ferrooxidans* share a remarkable amount of similartiy at the genetic level. A number of *T. ferrooxidans* genes have been isolated by complementation of *E. coli* mutants (Rawlings & Kusano, 1994). Thioredoxin genes from other bacteria have also been isolated by complementation of *E. coli trxA* mutants, as stated in Chapter 1. The ability of *T. ferrooxidans* genetic material to be expressed, and the proteins to function in *E. coli* enabled the isolation of the thioredoxin gene from *T. ferrooxidans*.

2.2 Materials and Methods

2.2.1 Bacterial strains and media

The strains and plasmids used in this study are described in Appendix A. Complex medium used for growth of *E. coli* was Luria-Bertani medium (LB), and M9 minimal medium was used for growth of *E. coli* on selective medium (Sambrook *et al.*, 1989). When required, antibiotics were added at the following concentrations: ampicillin 100 µg/ml and chloramphenicol 30 µg/ml. *T. ferrooxidans* was grown on tetrathionate medium for DNA and RNA extractions. Tetrathionate medium was made from mineral salts solution (g/l); $(NH_4)_2SO_4$, 3.0; KCl, 0.1; K_2HPO_4, 0.5; and $Ca(NO_3)2$, 0.01, adjusted to pH 2.5 with H_2SO_4 and autoclaved. Trace elements solution (mg/l); $FeCl_3.6H_2O$, 11.0; $CuSO_4.5H_2O$, 0.5; HBO_3, 2.0; $Na_2MoO_4.2H_2O$, 0.8; $CoCl_2.6H_2O$, 0.6 and $ZnSO_4.7H_2O$, 0.6, was filter sterilized. One ml of trace elements solution was added to 100 ml mineral salts solution and to this was added 50 mM sterile $K_2S_4O_6$ and the pH adjusted such that the final pH was 2.5.

2.2.2 Preparation of DNA

The alkaline lysis method (Ish-Horowicz & Burke, 1981; Birnboim & Doly, 1979) was used for both small and large scale preparations of *E. coli* DNA. Chromosomal DNA was prepared from *T. ferrooxidans* ATCC 33020 grown on tetrathionate medium by the DNA extraction method as outlined below. Cells from a 1 litre culture were harvested, concentrated down to 1.5 ml and pelleted by centrifugation in an Eppendorf microfuge tube for 1 min. The pellet was resuspended in 0.5 ml of a 25% sucrose, 2 mM EDTA, 50 mM Tris-Cl buffer (pH 8.0) and frozen at -20°C for 1h. Proteinase K was added to the frozen cells (0.1 mg/ml final concentration) and the samples shaken at ambient temperature until they thawed. Sodium dodecyl sulphate (SDS) was added to a final concentration of 0.5 %, and the samples held on ice for 15 min, followed by RNase (50 µg/ml) digestion for 20 min at 37°C. The viscous, opaque 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-isoamyl alcohol (25:24:1) extractions, followed by two diethyl-ether extractions. The DNA purification procedure was completed by an overnight dialysis against TE buffer at room temperature.

Construction of the gene bank of *T. ferrooxidans* ATCC 33020 DNA in the cosmid vector pHC79 was carried out by Raj Ramesar (Ramesar, R. PhD Thesis, University of Cape Town, South Africa, 1988).

2.2.3 DNA manipulations

Standard methods (Sambrook *et al.*, 1989; Ausubel *et al.*, 1993) were used for restriction digests, gel electrophoresis, purification of DNA fragments from agarose gels and ligations. All enzymes and buffers were used according to the manufacturer's specifications. *E. coli* cells

were made competent by the standard rubidium chloride method described by Sambrook *et al.* (1989).

2.2.4 Transduction of *E. coli* cells

The *T. ferrooxidans* pHC79 cosmid gene bank was stored as a phage lysate above chloroform at 4°C. Recipient *E. coli* cells were grown overnight in 25 ml LB medium containing 0.25% (w/v) maltose. A sample of 1 ml was sedimented by centrifugation in an Eppendorf microfuge, resuspended in 10 mM $MgSO_4$, and placed at room temperature for 1 h. A 10 µl sample of an appropriate dilution (routinely 10^{-7} or 10^{-6} in SM buffer; Sambrook *et al.*, 1989) of the cosmid gene bank phage lysate was mixed with 200 µl of cells. The sample was incubated at 37°C for 30 min, followed by addition of 1 ml of LB and a further 60 min incubation at 37°C. Samples of 100 µl were plated onto the appropriate agar plates.

2.2.5 Screening for the *T. ferrooxidans trxA* gene by complementation of *E. coli trxA* mutants

The *T. ferrooxidans* ATCC 33020 cosmid library was transduced into the *E. coli trxA gshA* mutant BH5262. The expression mixes were washed with 0.8% sodium chloride before plating onto M9 minimal medium containing 20 µg/ml arginine and 100 µg/ml ampicillin. Possible thioredoxin positive clones were identified by their abilitiy to grow on M9 minimal medium lacking in glutathione. Positive clones were then miniprepped and retransformed into *E. coli* BH2012 competent cells and plated on minimal medium containing methionine sulfoxide, isoleucine, valine and leucine (20 µg/ml of each) plus ampicillin (100 µg/ml). Positive colonies were identified by their ability to convert methionine sulfoxide to methionine. Methionine sulfoxide reductase specifically requires thioredoxin as a reductant for this reaction (Gonzalez Porqué *et al.*, 1970).

2.2.6 Subcloning of cosmid 32

Cosmid 32 DNA was digested with *Pst*I and ligated into the vector pBluescriptSK (*Pst*I). The ligation mixes were transformed into *E. coli* BH5262 and expressed in M9 minimal medium. The transformants were selected for complementation of the *trxA* phenotype. Subclone pTRX32A was isolated. A restriction map of pTRX32A was constructed (Fig. 2.1). All subclones are shown in Fig. 2.1. Plasmid pTRX32 was constructed from pTRX32A by cloning a 1.6 kb *Sac*II-*Pst*I fragment into pBluescriptSK. A 850 bp *Sfu*I-*Pst*I fragment from pTRX32 was cloned into pBluescriptSK to produce pTRX6. Plasmid pTRX13A is the result of an insertion of a 960 bp *Sac*II-*Sac*I fragment from pTRX32 into pBluescriptSK. Plasmid pTRX14A was a *Sac*I deletion of pTRX32. Plasmid pTRX9 was constructed by cloning a 682 bp *Hin*dIII fragment into pBluescriptSK, whilst pTRX24 is the result of inserting a 192 bp *Hin*dIII-*Sfu*I fragment from pTRX32 into pBluescriptSK.

All subclones of plasmid pTRX32A shown in Fig. 2.1 were tested for the ability to complement *E. coli* strains BH2012 and BH5262 as described above.

2.2.7 DNA hybridisation

In order to confirm the origin of the cloned thioredoxin-complementing DNA, DNA hybridisation was performed. *T. ferrooxidans* chromosomal DNA (15-20µg), cosmid 32 (800ng), plasmid pTRX32 (20ng) and plasmid pTRX6 (20ng) were digested with restriction enzymes *Kpn*I-*Sac*I and *Hin*dIII and electrophoresed together with lambda DNA digested with *Pst*I, as a marker, on a 0.8% agarose gel. Blotting was carried out according to the method recommended by Amersham for Hybond N^+ membranes. A 517 bp *Kpn*I-*Sac*I fragment from pTRX32 was used as a probe. Labelling of the probe, hybridisation and detection were carried out according to the dioxygenin-dUTP non-radioactive DNA labelling and detection kit (Boehringer-Mannheim).

2.2.8 Nucleotide sequencing

Recombinant plasmids generated by subcloning were sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977), using the Sequenase kit (version 2.0) from the United States Biochemical Corp. The sequence of the 1.05 kbp *Hin*dIII-*Pst*I fragment from pTRX32 (Fig. 2.3) was determined from both strands. The Genetic Computer Group Inc. (GCG) sequence analysis software package (version 7.1) was used for sequence analysis (Devereux *et al.*, 1984).

2.2.9 *In vitro* synthesis of thioredoxin

The *in vitro* synthesis of polypeptides was determined using the prokaryotic DNA-directed transcription kit, *E. coli* S30 system of the Promega Corp., Madison USA. Reactions were performed according to the manufacturer's specifications. The proteins were separated by SDS polyacrylamide gel electrophoresis using a 10 to 20% gradient gel. The gel was poured using a standard gradient pourer, and SDS polyacrylamide gel electrophoresis carried out according to the method outlined by Promega.

2.2.10 Phage growth

A growth curve of phage T7 was determined in *E. coli* host strains MC1061, BH2012 and BH2012(pTRX6). Overnight cultures of the host strains grown in Luria-Bertani medium were diluted to an OD_{600} = 0.6 in a 20 ml volume. This was done to ensure that each culture had approximately the same number of cells per ml of culture. Each 20ml culture was inoculated with 100µl T7 phage (10^{10} pfu/ml). At different time intervals 10µl samples were taken and the appropriate dilutions made. Ten microlitres of the diluted sample was added to 4 ml of sloppy agar (40°C) plus 100µl plating cells and poured onto Luria agar plates. The plates were incubated at 37°C for 8 hours and the plaques counted. Plating cells consisted of an overnight culture of *E. coli* MC1061 diluted to an OD_{600} of 0.6 in Luria-Bertani medium.

The growth of T7 phage was also monitored using different *E. coli* host strains; MC1061, BH2012 and BH2012(pTRX6), as plating bacterium.

It was not possible to determine a growth curve of M13 phage in *E. coli* host strains JF510 and JF510(pTRX6), as M13 phage showed no increase in titre over the 4 hour interval studied. Cultures of *E. coli* strains 71/18, JF510 and JF510(pTRX6) were, therefore, innoculated with M13 phage to 5 x 10^8 pfu/ml and grown overnight with vigorous aeration at 37°C. Titres were taken at the time of inoculation and 20 hours later using *E. coli* 71/18 as a plating bacterium.

2.2.11 Insulin Assay

The insulin assay of Holmgren (1979), was used to measure the rate of disulfide reduction, by recording the rate of precipitation at 650nm. Crude extracts were prepared as follows. Midlog bacterial cells were harvested from 100ml M9 minimal medium and resuspended in 1ml assay buffer (100mM potassium phosphate pH 7.5, 1mM EDTA, 1mM DTT and 1mM Pefabloc). Pefabloc [4-(2-aminoethyl)benzoylsulfonyl-fluoride, hydrochloride] was obtained from Boehringer Mannheim. After sonication, lysed cells were precipitated and the supernatant heated to 85°C for 5 minutes. Most thioredoxins are heat stable and are, therefore, not denatured. Denatured proteins were removed by centrifugation and the supernatant used in the assay. Insulin was added to 900µl crude extract (final concentration 1mg/ml), and the reaction initiated by adding 3µl 100mM DTT. The rate of precipitation of the insulin B chain was monitored by measuring the increase in turbidity at 650nm. Crude extract prepared from *E. coli trxA* mutant BH2012 was compared to that prepared from *E. coli* BH2012(pTRX6), which contained the cloned *T. ferrooxidans* thioredoxin gene,.

2.2.12 The preparation of glassware, perspex and chemicals for RNA extraction and analysis.

All chemicals used for RNA extraction procedures were treated with 0.01% DEPC (diethyl pyrocarbonate) for 2 hours at 37°C, and autoclaved prior to use. Tris-based buffers were not treated with DEPC; rather they were made up with deionized 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 in 10% sodium hypochlorite for 24 hours prior to use, and thoroughly rinsed with DEPC-treated de-ionized water. Glassware and metal utensils were baked at 200°C for 8 hours prior to use. These precautions were taken to eliminate any possibility of contaminating RNases.

2.2.13 Preparation of RNA

T. ferrooxidans total RNA was prepared from cultures grown on tetrathionate medium (see 2.2.1), and *E. coli* total RNA from cultures grown on M9 minimal medium, by the method of Aiba *et al.* (1981).

2.2.14 DNA:RNA hybridisation

The *Hin*dIII fragment of pTRX9 labelled with (α-^{32}P)CTP (3000 Ci/mmol) using the Random Primed DNA labelling kit from Boehringer Mannheim, was used as a probe for RNA hybridisation. Total RNA from *E. coli* BH5262, *E. coli* BH5262(pTRX6) and *T. ferrooxidans* was separated on a 1.5% agarose gel containing 6% formaldehyde. The RNA was transferred to an Amersham Hybond N^+ membrane and hybridisation carried out. RNA separation, blotting and hybridisation were carried out according to the Amersham Hybond N^+ protocol.

2.2.15 Primer extension analysis

Transcript start sites of the thioredoxin mRNA from *T. ferrooxidans* and the cloned gene in *E. coli* BH5262 were determined by primer extension analysis. A synthetic 18 mer oligonucleotide primer GACACATAAAGAATGGCG was designed to anneal 8 bp from the start of the *trxA* gene. The primer was end-labelled with γ-^{32}P using polynucleotide kinase according to the method of Ausubel *et al.* (1993). Primer extension was carried out using total RNA derived from *E. coli* BH5262(pTRX6) and total RNA from *T. ferrooxidans* according to the method of Ausubel *et al.* (1993). The resulting cDNA and a sequencing ladder generated from pTRX6 primed with the above 18 mer primer were analysed on a 6% polyacrylamide/urea sequencing gel. The length of the cDNA and, therefore, the transcription start site was estimated by comparison to the pTRX6 sequencing ladder.

2.3 Results

2.3.1 Screening for the *T. ferrooxidans trxA* gene by complementation of *E. coli trxA* mutants.

Transduction of the *T. ferrooxidans* genome cosmid library into *E. coli* BH5262 resulted in approximately 100 colonies that were able to grow on minimal media lacking glutathione. DNA was prepared from 16 of these colonies. These cosmids could be divided into two groups that had several restriction fragments in common and appeared to contain overlapping pieces of two regions of the *T. ferrooxidans* chromosome. On transforming into *E. coli* BH2012 only one of the groups of cosmids enabled cells to grow on minimal media plus methionine sulfoxide. One cosmid from this group (cosmid 32) was chosen for further study.

All subclones of cosmid 32 were tested for complementation of *E. coli* mutant BH2012 for growth on methionine sulfoxide and *E. coli* mutant BH5262 for growth on minimal medium lacking glutathione. Plasmids pTRX32A, pTRX32 and pTRX6 complemented the *E. coli trxA* mutants, while plasmids pTRX13A; pTRX14A, pTRX9 and pTRX24 did not (Fig. 2.1). This indicated that the thioredoxin-complementing DNA was located between the *Sfu*I and *Pst*I sites of pTRX32.

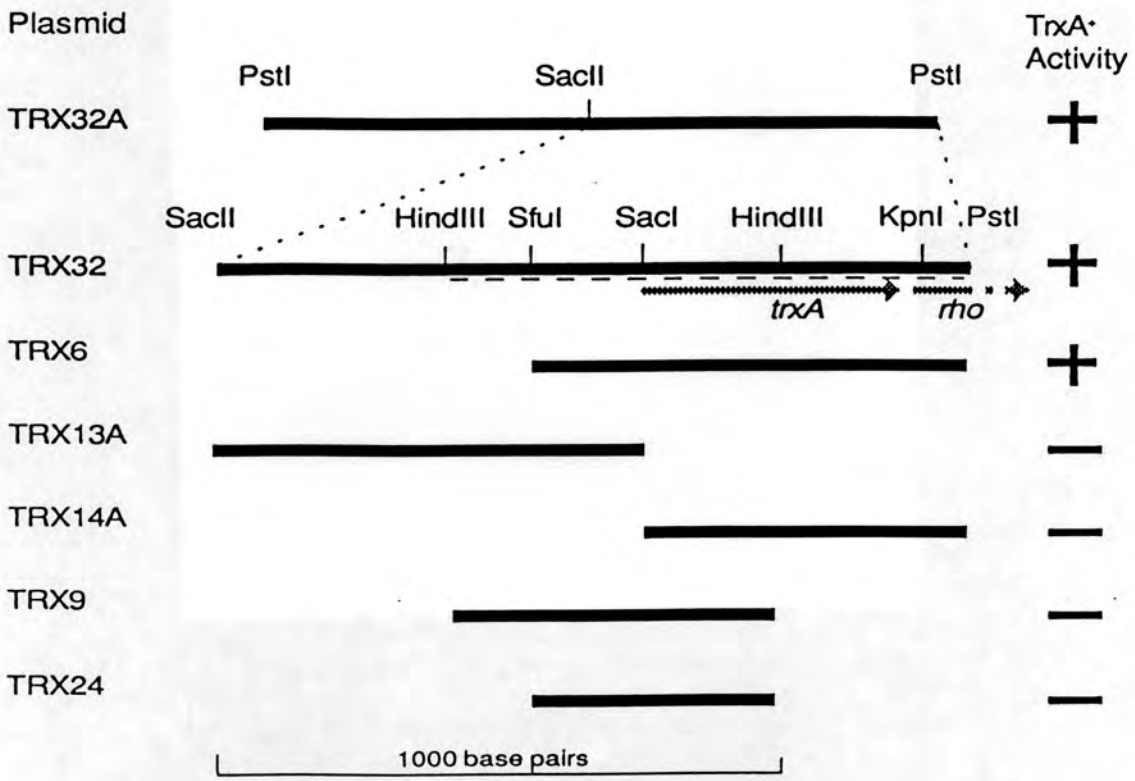


Fig. 2.1 Restriction endonuclease map of plasmid pTRX32A and derivatives. The *trxA* gene and the start of the *rho* gene are indicated by arrows. The dotted line indicates the region of DNA sequenced from both strands. $TrxA^+$ activity indicates that the transformed plasmid was able to complement the thioredoxin mutant *E. coli* BH2012 for growth on methionine sulfoxide.

2.3.2 DNA hybridisation

The source of the cloned thioredoxin-complementing DNA was confirmed by hybridisation of the labelled *Sac*I-*Kpn*I fragment from pTRX32 to *T. ferrooxidans* ATCC 33020 chromosomal DNA, cosmid 32, pTRX32 and pTRX6 digested with *Hin*dIII (Fig. 2.2 lanes 1-4) and *Sac*I-*Kpn*I (Fig. 2.2 lanes 6-9). The 685bp *Hin*dIII fragment that is internal to the cloned *T. ferrooxidans* chromosomal DNA present on pTRX32 corresponded exactly to the *Hin*dIII fragment present on the *T. ferrooxidans* chromosome, cosmid 32 and pTRX32. The hybridisation signals at 1.8kb (Fig. 2.2, lanes 1 and 2) represented the adjacent *Hin*dIII fragment, which contained part of the *trxA* and *rho* genes and was present in the chromosome and cosmid 32 only. The hybridisation signal at approximately 3.0 kb in the *Hin*dIII digest of pTRX32 (Fig. 2.2, lane 3) corresponded to the region flanking the 685bp *Hin*dIII fragment and included the vector. Plasmid pTRX6 has a single *Hin*dIII site and, therefore, gave a single hybridisation signal at 3.9kb (Fig. 2.2, lane 4) when probed with the *Sac*I-*Kpn*I fragment. Similarly, when the *T. ferrooxidans* chromosomal DNA was digested with both *Sac*I and *Kpn*I, a single hybridisation signal at 530bp, also present on cosmid 32, pTRX32 and pTRX6 was detected.

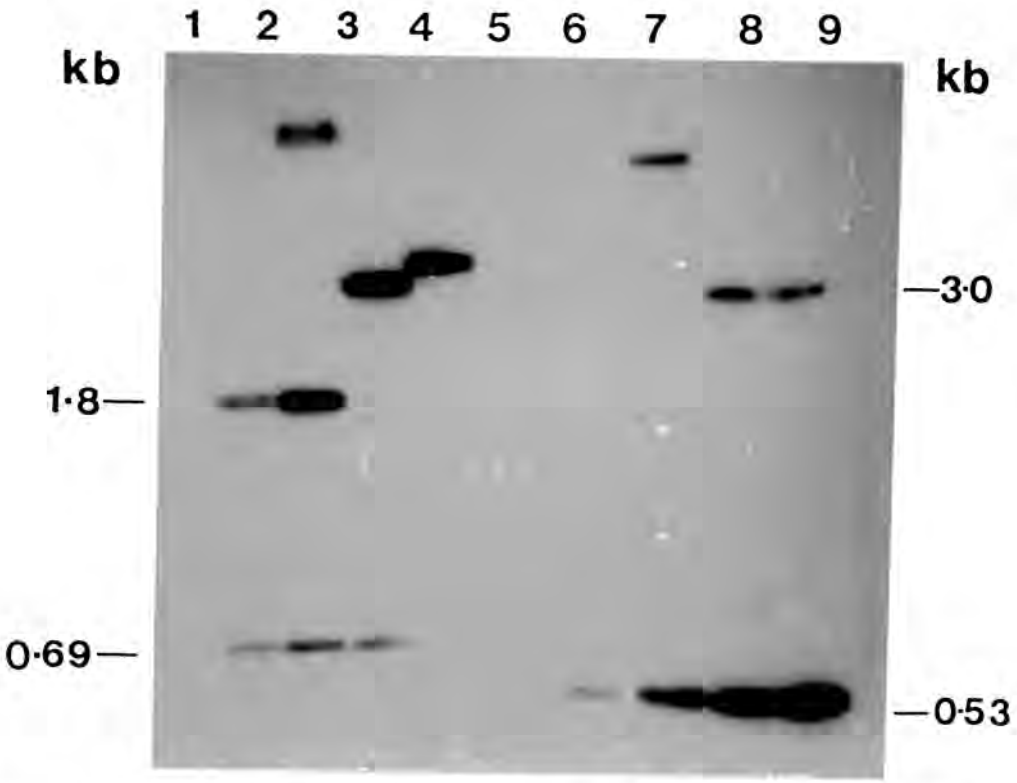


Fig. 2.2 Hybridisation of the labelled 517 bp *Kpn*I-*Sac*I fragment of pTRX32 to *Hin*dIII digests (lanes 1 to 4) and *Kpn*I-*Sac*I digests (lanes 6 to 9) of: lane 1, *T. ferrooxidans* chromosomal DNA; lane 2, cosmid 32; lane 3, plasmid pTRX32; lane 4, plasmid pTRX6; lane 5, blank; lane 6, *T. ferrooxidans* chromosomal DNA; lane 7, cosmid 32; lane 8, plasmid pTRX32; lane 9, plasmid pTRX6.

The faint upper bands in lanes 2, 7, 8 and 9 (Fig. 2.2), were due to a low concentration of vector DNA which remained in the incompletely purified *Sac*I-*Kpn*I probe. Overexposure of the autoradiogram (not shown) failed to indicate any additional chromosomal bands. This indicated

that there was one copy of the thioredoxin gene per genome and that the cloned thioredoxin-complementing DNA originated from *T. ferrooxidans* ATTCC 33020.

2.3.3 Nucleotide and amino acid sequence of the *T. ferrooxidans trxA* gene and flanking regions

The nucleotide sequence of the 1.05 kbp *Hin*dIII-*Pst*I fragment of pTRX32 is shown in Fig. 2.3. Analysis of the sequence data revealed one complete ORF. Data base searches, using the BLASTNCBI package revealed significant amino acid homology of the ORF to known thioredoxin genes of other organisms (data not shown).

```
1    AAGCTTTGCCAAACGCAGATGTTTACCATACGCCAAGCCCCGAACGGCGACTATA

56   CCGATCTCCAGGACAAGGGCGGCAGATCGGGTTTTGACCTGAGCCCGTGCGACAG

111  GTTAGAATCCGCTACCGGAGAGATGGCCGAGCGCTGAAGGCGCTCCCCTGCTAAG

166  GGAGTATGGGGCTAAACACTCCATCGAGGGTTCGAATCCCTCTCTCTCCGCCACC
                                                    -35      «
221  CTTACTGGCTTAAAATCTTAACGAAAGTGACCATCCATCGCTCTTGCATCCCTGT
                  -10       «
276  TGTATCGTGCTATCCTTCAATCATCTTATGGACTGAAAGAAGTACTTATCTGCGG
                 «
331  GCGCCATCCGGCGCATGTGGTCTCGGGTTAAATCCCTCGAACGATAAAAATCTCA

386  TGAATATCCACCGAAGCGTTGTACCCAACTGACTGAGGAGCTCTCTCCAATGAGT
                                start of trxA gene →  M  S

441  GACGCCATTCTTTATGTGTCTGATGACAGTTTTGAAACGGATGTATTGAAGTCCT
      D  A  I  L  Y  V  S  D  D  S  F  E  T  D  V  L  K  S

496  CCAAACCGGTATTGGTCGATTTCTGGGCCGAATGGTGCGGACCTTGCAAGATGAT
     S  K  P  V  L  V  D  F  W  A  E  W  C  G  P  C  K  M  I

551  CGCCCCCATTCTGGAAGAGATCGCTGATGAATATGCCGATCGCCTGCGGGTTGCC
       A  P  I  L  E  E  I  A  D  E  Y  A  D  R  L  R  V  A

606  AAATTCAACATCGACGAAAATCCCAATACTCCCCCTCAGTACGCGATTCGCGGTA
      K  F  N  I  D  E  N  P  N  T  P  P  Q  Y  A  I  R  G

661  TTCCCACACTGCTCCTTTTCAAGGCTGGCAAGCTTGAAGCGACCAAAGTGGGTGC
     I  P  T  L  L  L  F  K  A  G  K  L  E  A  T  K  V  G  A

716  CCTGAGCAAAGCCCAGCTGACCGCATTCCTGGATAGCCAACTTTGAATACGTCCC
       L  S  K  A  Q  L  T  A  F  L  D  S  Q  L  *

771  GCCACCGCCGTAAACCCCCCCGGACAGCCCAAGTCCTTTCCCAACGAAGATATTC

826  TGGTAGACGAAGACGAGAACGCGCTTTGTCCGTCACCTGGCGCTGCGGCGATGAA
                                start of rho gene →  M  N

881  TCTGACCGACCTCAAACGCAAAACGGCCGCTGATCTGGCGGTGATCTGCCAGGAC
       L  T  D  L  K  R  K  T  A  A  D  L  A  V  I  C  Q  D
```

```
936  ATGGGGTTGGAGGGTACCGCCCGTCAGAAGAAACAGGAAATCATTTTCAATATCC
      M  G  L  E  G  T  A  R  Q  K  K  Q  E  I  I  F  N  I

991  TCAACGCACGCGCGCAATGCGACGCCATTTACGGTGAGGGGGTGCTGGAAATTCT
     L  N  A  R  A  Q  C  D  A  I  Y  G  E  G  V  L  E  I  L

1046 GCAG
       Q
```

Fig. 2.3 Nucleotide and deduced amino acid sequence of the *Hin*dIII-*Pst*I fragment of pTRX32A, containing the *trxA* gene and the N-terminal region of the *rho* gene. A putative ribosome binding site is indicated in bold and -10 and -35 regions of a σ^{70}-like promoter are underlined. 5' transcription start sites are indicated by arrows.

2.3.3.1 Codon usage in the *T. ferrooxidans trxA* gene.

The table in Appendix C details the codon usage pattern of the *trxA* gene. As can be seen from the table the codon usage of the *trxA* gene is very similar to that in Tfchrom.cod, with a bias towards the G or C in the "wobble" position.

The UWGCG CODONPREFERENCE application ultilizes a codon usage data file to analyze all 6 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. Figure 2.4 shows the CODONPREFERENCE analysis of the 1.05kbp *Hin*dIII-*Pst*I fragment of pTRX32 using the *T. ferrooxidans* codon usage file (Tfchrom.cod). The latter file was compiled from 14 previously sequenced *T. ferrooxidans* genes, namely; *glnA* (Rawlings *et al.*, 1987), *nifHDK* (Pretorius *et al.*, 1987; Rawlings, 1988), *recA* (Ramesar *et al.*, 1989), *ntrA* (Berger, 1990), ORF1 (Berger, 1990) and the 7 ATPase genes (Brown *et al.*, 1994).

The central ORF seen in Fig. 2.4, frame 1, was found to code for the *trxA* gene, and the incomplete ORF at the 3' end of the 1.05kb fragment, for the N-terminal region of the *rho* gene. Both genes show a codonpreference and absence of rare codons which correlates with the Tfchrom.cod codon usage data file. The GC composition of the *trxA* gene was 50.92%.

The codon usage data was analysed using the UWGCG CORRESPOND subroutine. The program CORRESPOND is designed to identify similar patterns of codon usage by comparing codon frequency, which is calculated by dividing the number of incidents of the codon in question by the total number of codons specifying the amino acid or terminator in each table. The lower the statistic (D), the more similar the patterns of codon usage. The codon frequency table of the *T. ferrooxidans trxA* gene was compared to the composite codon frequency table of *T. ferrooxidans* (Tfchrom.cod) as well as the codon frequency tables for highly expressed (UWGCG datafile "Ecohigh.cod") and the weakly expressed (UWGCG datafile "Ecolow.cod") *E. coli* genes. The results are shown in Table 2.1.

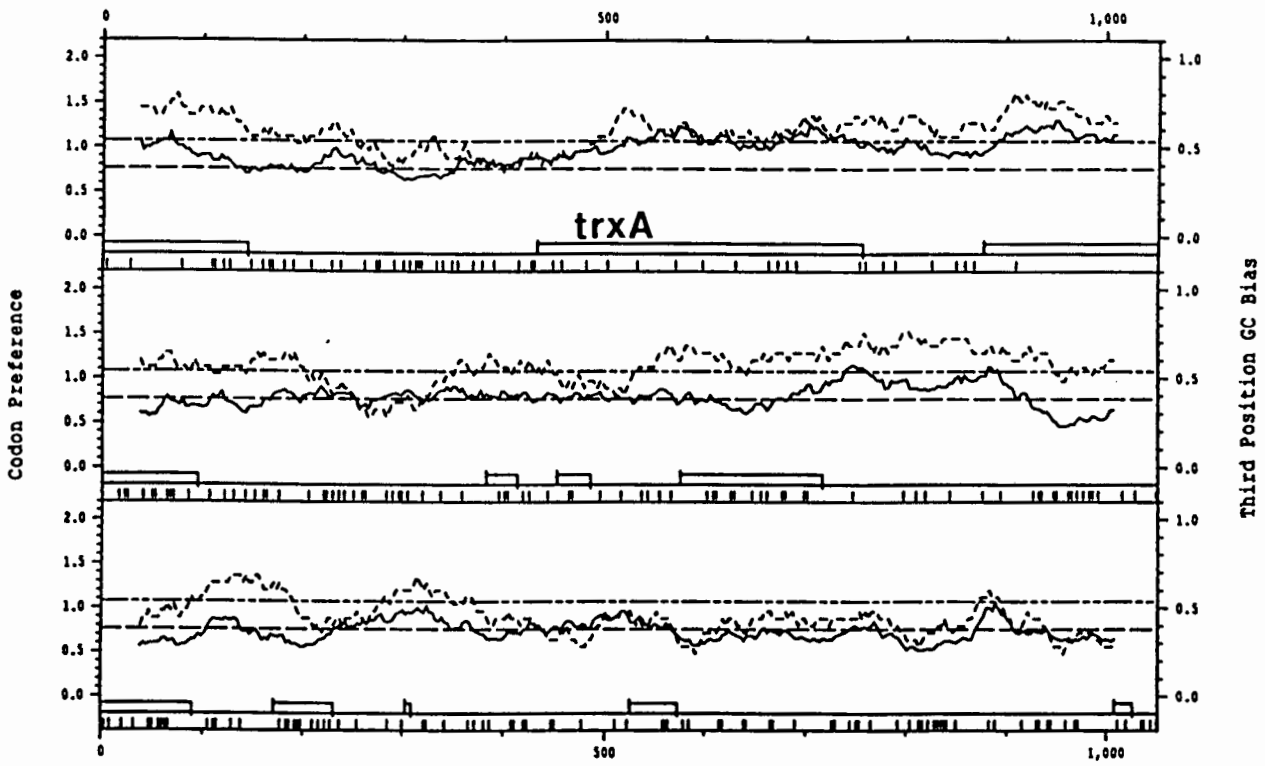


Fig. 2.4 CODONPREFERENCE analysis of the *Hin*dIII-*Pst*I fragment of pTRX32A. The predicted protein-coding open reading frames, *trxA* and *rho*, are shown as open boxes. The vertical axis shows the CODONPREFERENCE plots of all of the three forward frames, with ORFs and the positions of rare codons (defined as those codons which appear in the Tfchrom.cod codon usage datafile at a frequency less than 10%) drawn below as open boxes and vertical bars, respectively. Any plot above the dotted line in any of the three reading frames identifies a protein-coding sequence with a similar codon usage to the Tfchrom.cod datafile.

Table 2.1. Comparison of codon usage of the *T. ferrooxidans trxA* gene with that of *T. ferrooxidans* and *E. coli* genes using the UWGCG program CORRESPOND[a]. Numbers represent the statistic D^2 as described in the text.

	Tfchrom.cod	Ecohigh.cod	Eclow.cod
trxA.cod	1.34	3.33	1.11

a Tfchrom.cod is a composite codon usage frequency table of 14 *T. ferrooxidans* genes as stated earlier. Ecohigh.cod and Ecolow.cod refer to the codon usage frequency tables of *E. coli* weakly and *E. coli* highly expressed genes respectively. *trxA*.cod refers to the codon usage frequency table of the *T. ferrooxidans trxA* gene.

The codon usage of the *T. ferrooxidans trxA* gene is most similar to that of the weakly expressed *E. coli* genes and the *T. ferrooxidans* genes.

2.3.3.2 Amino acid similarity between *T. ferrooxidans* TrxA and other TrxA proteins

The amino acid sequence of the *trxA* ORF was aligned with other TrxA proteins using the multiple sequence alignment UWGCG application PILEUP (Fig. 2.5).

```
Ectrx  ...MSDKIIHLTDDSFDTDVLKADG...AILVDFWAEWCGPCKMIAP
Tftrx  ...MSDAILYVSDDSFETDVLKSSK...PVLVDFWAEWCGPCKMIAP
Rstrx  ......STVPVTDATFDTEVRKSDV...PVVVDFWAEWCGPCRQIGP
Bstrx  .....MAIVKATDQSFSAE..TSEG...VVLADFWAPWCGPCKMIAP
Hstrx  .....MVKQIESKTAFQEALDAAGD..KLVVVDFSATWCGPCKMIKP
Sctrx  .....MVTQFKTASEFDSAI..AQD..KLVVVDFYATWCGPCKMIAP
Nttrx  MAEEGQVIGVHTVDAWNEHLQKGIDDKKLIVVDFTASWCGPCKFIAS

Ectrx  ILDEIADEYQGKLTVAKLNIDQNPGTAPKYGIRGIPTLLLFKNGEVA
Tftrx  ILEEIADEYADRLRVAKFNIDENPNTPPQYAIRGIPTLLLFKAGKLE
Rstrx  ALEELSKEYAGKVKIVKVNVDENPESPAMLGVRGIPALFLFKNGQVV
Bstrx  VLEELDQEMGDKLKIVKIDVDENQETAGKYGVMSIPTLLVLKDGEVV
Hstrx  FFHSLSEKYSN.VIFLEVDVDDCQDVASECEVKCMPTFQFFKKGQKV
Sctrx  MIEKFSEQYPQ.ADFYKLDVDELGDVAQKNEVSAMPTLLLFKNGKEV
Nttrx  FYAELAKKMPT.VTFLKVDVDELKSVATDWAVEAMPTFMFLKEGKIV

Ectrx  ATKVGALSKGQLKEFLDANLA.....
Tftrx  ATKVGALSKAQLTAFLDSQL*.....
Rstrx  SNKVGAAPKAALATWIASAL......
Bstrx  ETSVGFKPKEALQELVNKHL......
Hstrx  GEFSGA.NKEKLEATINELV......
Sctrx  AKVVGA.NPAAIKQAIAANA......
Nttrx  DKVVGA.KKDELQQTIAKHISSTSTA
```

Fig. 2.5. Alignment of deduced amino acid sequence of the *T. ferrooxidans* TrxA with other TrxA proteins. Amino acids conserved in all seven TrxA proteins are shaded in black. Ectrx = *E. coli* TrxA (GenBank M87049), Tftrx = *T. ferrooxidans* TrxA, Rstrx = *R. sphaeroides* TrxA (GenBank M33806), Bstrx = *B. subtilis* (GenBank J03294), Hstrx = *H. sapiens* (GenBank X54540), Sctrx = *S. cerevisiae* (Trx1)(GenBank M62647), Nttrx = *Nicotiana tabacum* (GenBank Z11803).

To give a comparison of thioredoxins across a broad range of different organisms representatives were chosen from plants (*Nicotiana tabacum*), mammals (*Homo sapiens*), fungi (*Saccharomyces cerevisiae*), Gram-positive bacteria (*Bacillus subtilis*), Gram-negative bacteria (*E. coli*) and *Rhodobacter sphaeroides*. Thioredoxins are usually highly conserved proteins, and their comparison is thus thought to be of evolutionary significance. The high degree of conservation among thioredoxins is clearly illustrated in Fig. 2.5. All the proteins studied here have the highly conserved active site sequence -W-C-G-P-C-. There are also a number of other highly conserved residues which are indicated by shading.

The *T. ferrooxidans* TrxA shows highest identity to those of *E. coli* (70.6%) and *Salmonella typhimurium* (GenBank M10424) (70.6%), followed by *Chromatium vinosum* (GenBank P09857) (69.2%). It has lowest identity to the *Eubacterium acidaminophilum* TrxA (GenBank L04500) (29.4 %), followed by that of *N. tabacum* (29.9 %).

2.3.3.3 Identification of the downstream *rho* ORF

An incomplete ORF, 126 bp downstream of the thioredoxin gene was identified (Fig. 2.6). The predicted amino acid sequence showed homology to the *rho* transcriptional termination protein of other prokaryotes. A multiple sequence alignment of the first 58 amino acids of the *T. ferrooxidans rho* gene with those of *E. coli* (GenBank J01673), *S. typhimurium* (GenBank D10015), *Neisseria gonorrhoeae* (GenBank Z21790), *Pseudomonas fluorescens* (GenBank L27278) and *C. vinosum* (GenBank L27275) was carried out using the UWGCG PILEUP application (Fig. 2.6). The first 58 amino acids of the *T. ferrooxidans* rho protein were not as homologous as the rho proteins of *E. coli*, *S. typhimurium* and *N. gonorrhoeae* were to each other (*T. ferrooxidans* 53.6 % identity to *E. coli*). Opperman & Richardson (1994) carried out a phylogenetic analysis of possible *rho* genes in a range of bacterium. Their results indicated that the *rho* gene can be split into three regions; the first region, stretching from amino acids 1 to 116 was denoted the RNA-binding domain. This region was found to be the most divergent. However, their analysis revealed a region of higher similarity within the RNA-binding domain from amino acids 51 to 141. Although the region is very short, *T. ferrooxidans* shows increased homology to the other rho proteins from amino acid 51 onwards (Fig. 2.6), consistant with this analysis. From these sequence data and its position downstream of the *trxA* gene (similar to *E. coli*) this incomplete ORF appears to code for the rho transcriptional terminator protein.

```
T.f. MNLTDLKRKTAADLAVICQDMGLEGTARQKKQEIIFNILNARAQ.CDAIYGEGVLEILQ
E.c. MNLTELKNTPVSELITLGENMGLENLARMRKQDIIFAILKQHA.SGEDIFGDGVLEILQ
S.t. MNLTELKNTPVSELITLGESMGLENLARMRKQDIIFAILKQHAKSGEDIFGDGVLEILQ
N.g. MNLTELKNTPVSELITLGESMGLENLARMRKQDIIFAILKQHAKSGEDIFGDGVVEILQ
P.f. MNLTELKQKPITDLLQLAEEMGIENMARSRKQDVIFSLLKKHA.SGEEISGDGVLEILQ
C.v. MNLTELKKMPVPNLVALAQSMDIEGVGRSRKQDLIFAILKAQAK.GEDIYGDGVLEKIL
```

Fig. 2.6. Comparison of the N-terminal amino acid sequence of *T. ferrooxidans* rho protein (T.f) to that of *E. coli* (E.c) GenBank J01673, *S. typhimurium* (S.t) GenBank D10015, *N. gonorrheae* (N.g) GenBank Z21790, *Pseudomonas flourescens* (P.f) GenBank L27278 and *C. vinosum* (C.v) GenBank L27275.

2.3.4 *In vitro* protein synthesis

To establish a correlation between the predicted ORF, derived from sequence analysis, and the polypeptide produced, protein products from cosmid 32 and various subclones were examined. A protein of approximately 14 kDa was produced from cosmid 32 and plasmids pTRX32 and pTRX6, but not by plasmid pTRX14A or the vector pBluescriptSK (Fig. 2.7). A slightly larger protein was synthesized from plasmid pTRX9 (Fig. 2.7, lane 5). However, this construct lacked the stop codon of the thioredoxin gene and the larger protein was probably the result of transcriptional and translational read-through. These results strongly support the contention that the thioredoxin gene is expressed in an *E. coli*-derived *in vitro* system.

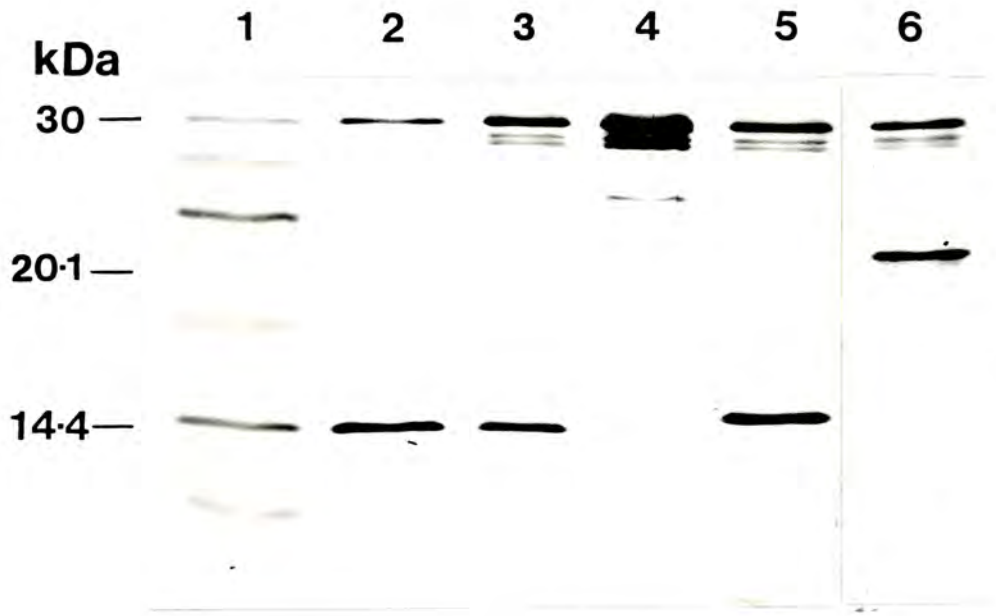


Fig. 2.7. SDS PAGE analysis of proteins expressed from cosmid 32 and subclones using an *E. coli*-derived *in vitro* transcription-translation system. Lanes: 1, cosmid 32; 2, pTRX32; 3, pTRX6; 4, pTRX14A; 5, pTRX9; 6, pBluescriptSK.

2.3.5 Phage Growth

The ability of thioredoxin expressed from pTRX6 in *E. coli* BH2012 to support T7 phage replication was compared to the thioredoxin expressed from the chromosome of *E. coli* parental strain MC1061 (Fig. 2.8). As can be seen from Fig. 2.8 titres of phage T7 reached almost the same level when the *T. ferrooxidans* thioredoxin was provided, as the titres obtained with the *E. coli* chromosomal thioredoxin gene. When *E. coli* BH2012(pTRX6) was used as a plating bacterium the T7 plaques were more variable in size and slightly smaller that when strain MC1061 was used (Fig. 2.9). No plaques were formed when *E. coli* BH2012 was used as plating bacterium. The cloned *T. ferrooxidans trxA* gene was clearly able to complement the *E. coli trxA* mutant to support growth of T7 phage, although with slightly reduced efficiency. The *T. ferrooxidans* thioredoxin, therefore, appears to be able to form a functional association with the gene 5 protein of the phage T7 DNA polymerase complex.

The ability of the *T. ferrooxidans* thioredoxin to support the growth of M13 phage is shown in Table 2.2. Although there was a slight increase in titre of phage M13 in *E. coli* JF510(pTRX6) relative to the *E. coli* JF510 control when grown in liquid media no plaques were detected on solid media, when *E. coli* JF510(pTRX6) was used as a plating bacterium. The *T. ferrooxidans* thioredoxin, therefore, does not appear to be able to satisfy the thioredoxin requirement of the filamentous phage.

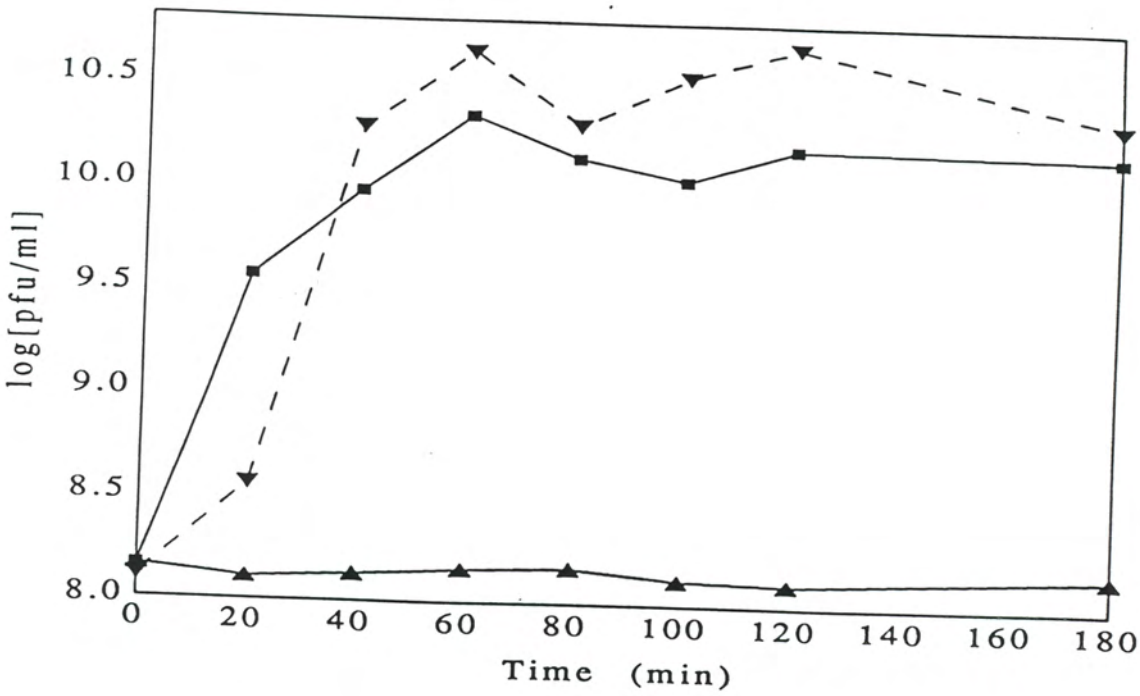


Fig. 2.8. Growth of T7 Phage on *E. coli* cells. *E. coli* hosts: MC1061 — — ▼ — — ; BH2012(pTRX6) ——■—— ;BH2012 ——▲——. The data represent the means of three experiments.

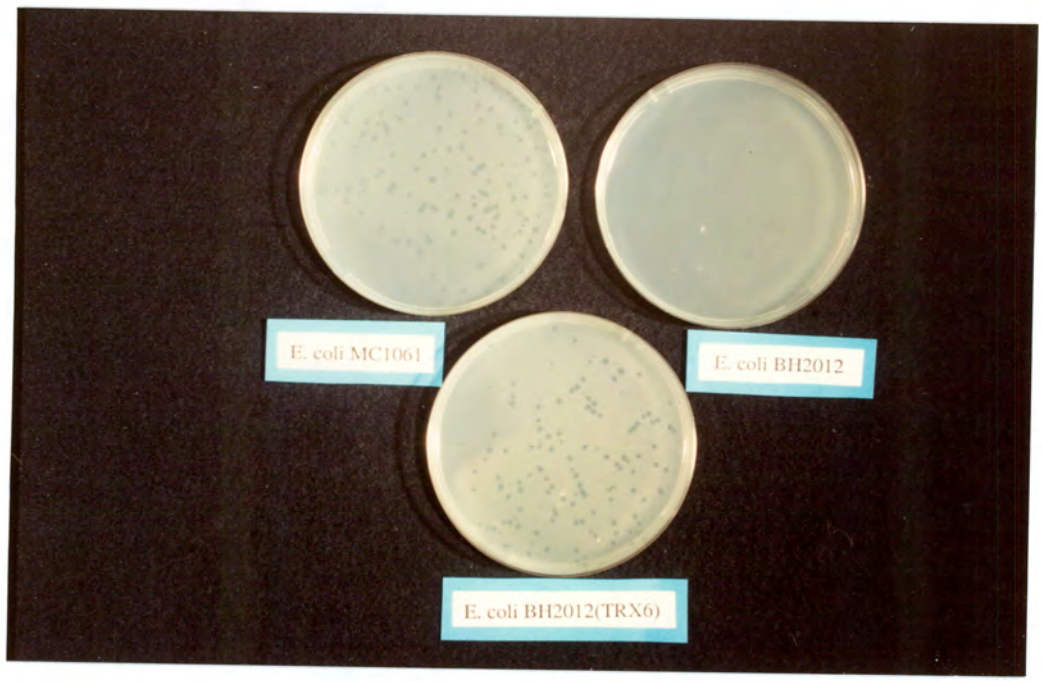


Fig. 2.9. A comparison of the size and number of plaques formed when T7 phage is grown on *E. coli* cells. *E. coli* hosts: **A** MC1061; **B** BH2012(pTRX6); C BH2012.

Table 2.2. Growth of M13 phage in different *E. coli* hosts. Values are expressed in plaque forming units per ml. The data represent the mean of three experiments. Standard deviations are shown in brackets.

	Time (h.)	
E. coli Host	0	20
71/18	$1.69(0.13) \times 10^8$	$1.71(0.51) \times 10^{11}$
JF510(pTRX6)	$1.55(0.39) \times 10^8$	$2.63(1.38) \times 10^8$
JF510	$1.52(0.45) \times 10^8$	$1.45(1.03) \times 10^8$

2.3.6 Insulin reduction

Thioredoxin has been shown to catalyze the reduction of the insulin disulfide bridge by dithiothreitol (Holmgren, 1979). This reduction resulted in the precipitation of the insulin B chain which could be readily measured as an increase in turbidity.

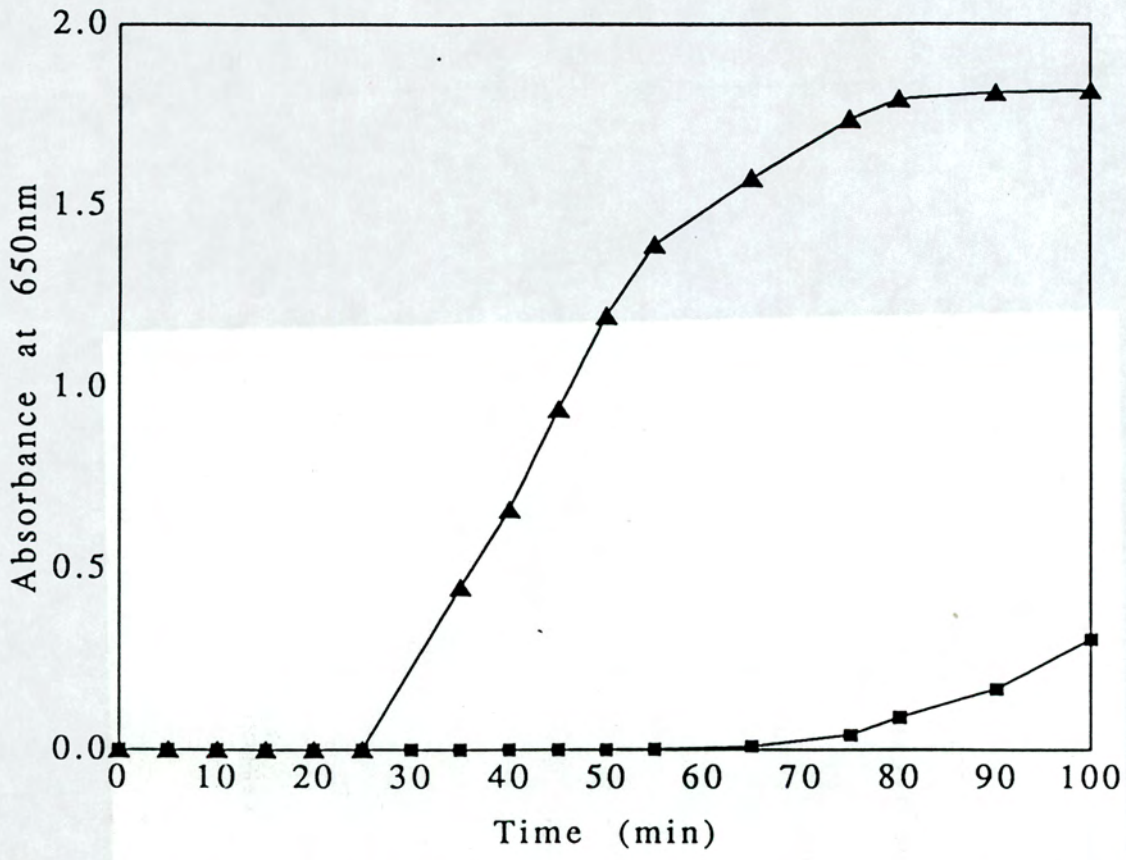


Fig. 2.10. Reduction of disulfide bonds of insulin by DTT and crude extracts of *E. coli* cells containing the cloned *T. ferrooxidans* thioredoxin gene. *E. coli* BH2012(pTRX6), —▲— *E. coli* BH2012 —■— . The data represent the means of three assays.

We compared crude extracts of *E. coli trxA* mutants with and without the cloned *T. ferrooxidans trxA* gene for the ability to reduce insulin (Fig. 2.10). Extracts prepared from *E. coli*

BH2012(pTRX6) cells were able to reduce insulin at a greatly enhanced rate compared to extracts from *E. coli* BH2012 cells. This clearly indicated that the thioredoxin from the cloned *T. ferrooxidans trxA* gene was active in *E. coli*.

2.3.7 Transcript analysis

To determine whether the *T. ferrooxidans trxA* gene was independently transcribed, or cotranscribed with an unidentified upstream gene or with the downstream *rho* gene, RNA:DNA hybridisation analysis was carried out on RNA transcripts prepared from *E. coli* BH5262 *trxA* mutants, *E. coli* BH5262(pTRX6) and *T. ferrooxidans* cells, probed with the *Hin*dIII-*Hin*dIII fragment from pTRX9 (Fig. 2.11). A single transcript of about 0.5kb was obtained for RNA from *T. ferrooxidans* cells (Fig. 2.11, lanes 3 and 4), which corresponded to the predicted size of the thioredoxin gene and indicated that the *T. ferrooxidans trxA* gene was transcribed on its own. Several transcripts were produced from *E. coli* BH5262(pTRX6) cells (Fig. 2.11, lane 2) and one of these corresponded exactly in size to the 0.5 kb signal from *T. ferrooxidans* while the other transcripts were much larger (approximate sizes 1.35, 1.40, 1.60 and 1,80 kb). These presumably represented transcription products that originated from the *lacZ* promoter of the vector. A very weak signal at about 0.45 kb was produced from RNA isolated from *E. coli* BH5262 cells which was smaller than the *T. ferrooxidans trxA* transcript and may represent a low amount of *trxA* transcription from the *E. coli* BH5262 *trxA* mutant (Fig. 2.11, lane 1).

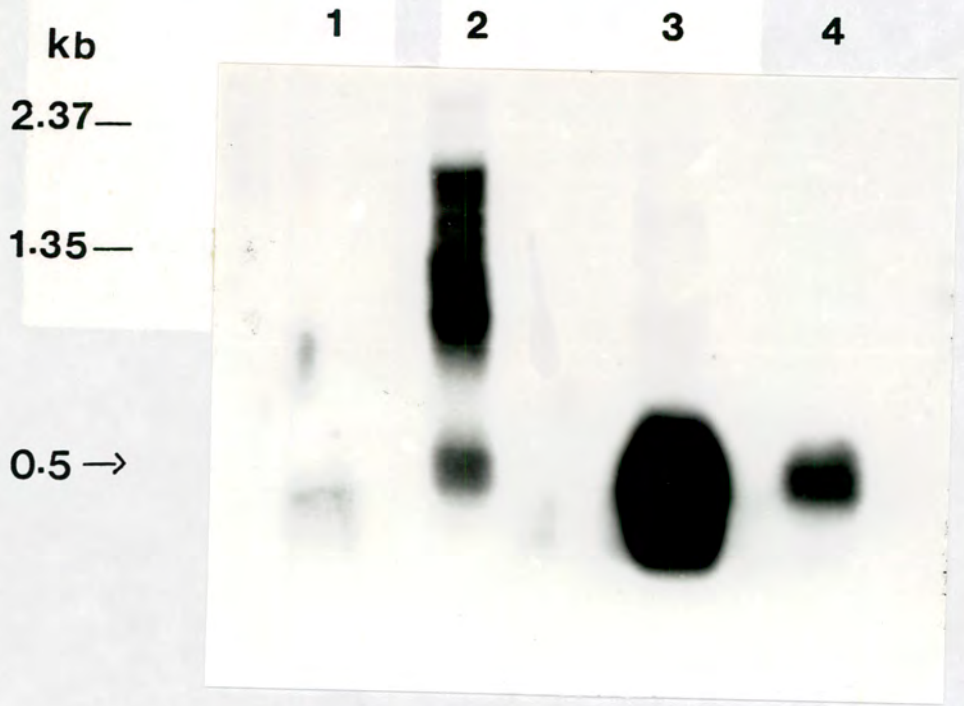


Fig. 2.11. Hybridisation of labelled *Hin*dIII fragment of pTRX9 to total RNA prepared from: lane 1, *E. coli* BH5262 (~20 µg); lane 2, *E. coli* BH5262(pTRX6) (~40 µg); lane 3, *T. ferrooxidans* (~25 µg); lane 4, *T. ferrooxidans* (~5 µg).

Primer extension analysis was used to compare the transcriptional start sites of the *T. ferrooxidans trxA* gene in its natural host with those of the gene cloned in *E. coli*. RNA prepared from *T. ferrooxidans* gave three possible transcriptional start sites, at positions 274, 298 and 345 (Fig. 2.12, lane 2). In the case of RNA derived from *E. coli* BH5262(pTRX6), only two transcription start sites were detected which were identical to those of *T. ferrooxidans*, at positions 298 and 345 (Fig.2.12, lane 1).

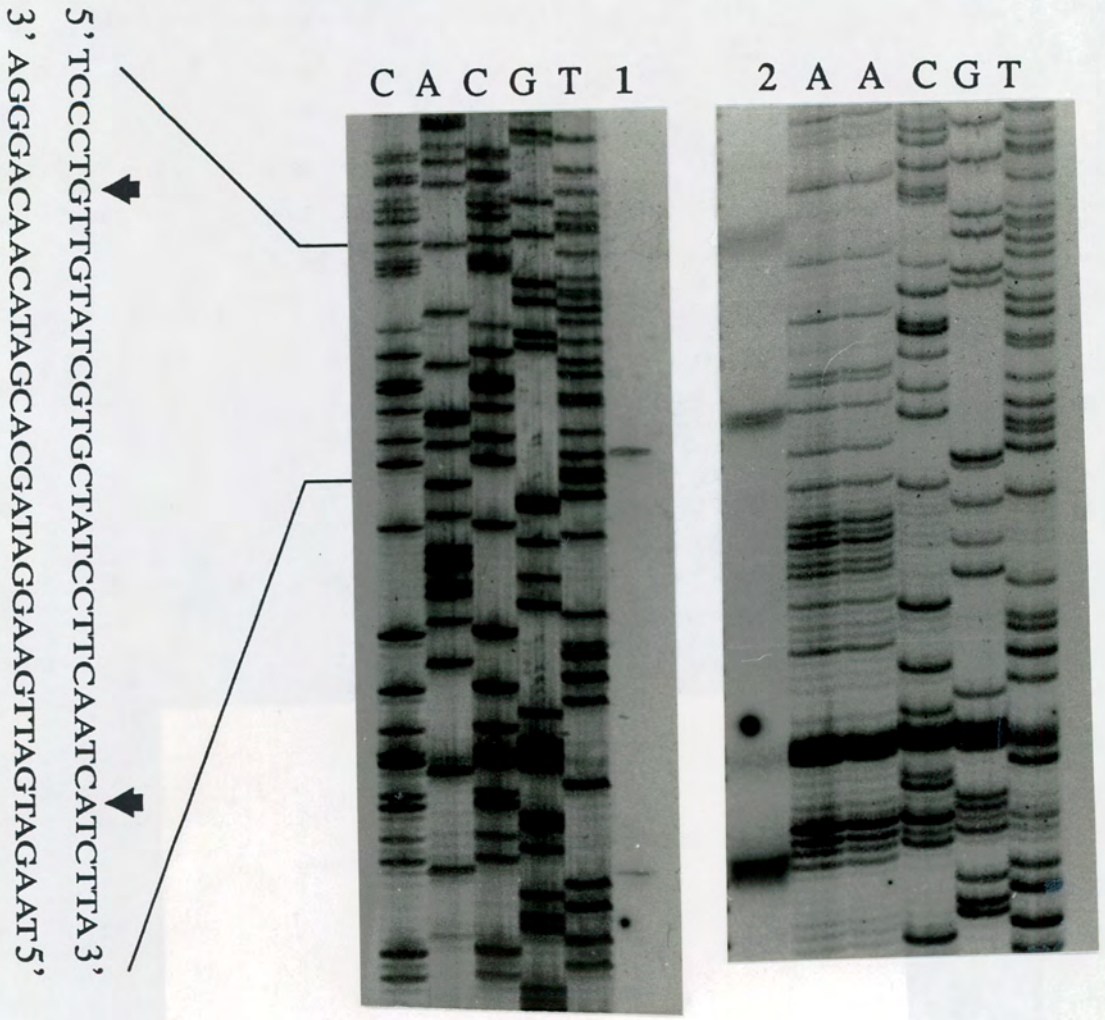


Fig. 2.12. Primer extension analysis of the 5' transcription start sites of the *trxA* gene cloned in *E. coli* and in *T. ferrooxidans*. The letters above each lane indicate the dideoxynucleotide used to terminate the sequencing reaction. Lane 1, extension products from *E. coli* BH5262(pTRX6) RNA and lane 2, extension products from *T. ferrooxidans* RNA. The first two transcription start sites of the gene in *T. ferrooxidans* are indicated by black arrows next to the text.

2.4 Discussion

In spite of the differences in physiology between *T. ferrooxidans* and *E. coli*, the two bacteria share a remarkable amount of similarity at the genetic level (Rawlings & Kusano, 1994). Analysis of the *trxA* gene and its flanking regions is an illustration of this. The *trxA* genes of both bacteria are independently transcribed (Wallace & Kushner, 1984), present in a single copy and have a *rho* gene located immediately downstream. Furthermore, two of the 3 transcriptional start sites detected in *T. ferrooxidans* were also functional in *E. coli*. A minor difference is that in *E. coli* approximately 10% of the *rho* gene mRNA occurs as a 2.1 kbp transcript (Matsumoto *et al.*, 1986), which is a result of transcriptional read-through from the *trxA* gene. This does not appear to be the case in *T. ferrooxidans*. The *T. ferrooxidans* thioredoxin was clearly functional in *E. coli* as it enabled growth of the *E. coli* BH5262 *gshA trxA* mutant on minimal medium lacking glutathione and the *E. coli* BH2012 *trxA met* mutant on minimal medium containing methionine sulfoxide. Thus the *T. ferrooxidans* thioredoxin was able to serve as a cofactor for methionine sulfoxide reductase in *E. coli*.

A more illustrative representation of the relatedness of the TrxA proteins is shown in the phylogram (Fig. 2.13). As thioredoxins are usually approximately 100 amino acids in length, they are too small to predict phylogenetic relatedness between sequences, however it is of interest to compare the relatedness of thioredoxins to each other. This analysis shows the *T. ferrooxidans* thioredoxin to be most closely related to the thioredoxins of *E. coli* and *S. typhimurium*. The TrxA proteins analysed in Figure 2.13 form four clusters. The prokaryotes and the eubacteria form one cluster. The two Gram-positive bacteria, *B. subtilis* and *S. clavuligerus*, are loosely clustered with the prokaryotes and eubacteria. *A. nidulans*, *Anabaena* PCC 7119, *C. reinhardtii* (Ch2), *P. yezoensis* and *C. caldarium* form a second cluster. A third cluster is formed by *E. coli*, *S. typhimurium*, *T. ferrooxidans*, *C. vinosum* and *R. rubrum*. Lastly, *C. bacterium*, *C. nephridii* and *S. aureofaciens* form a fourth cluster, to which the thioredoxin of *R. capsulatus* is also very loosely clustered.

As stated earlier, *E. coli* and *T. ferrooxidans* show a remarkable amount of similarity at the genetic level, and thus it is not surprising that the two thioredoxins show a high degree of relatedness. The thioredoxins of *C. vinosum* and *R. rubrum* are probably clustered together as both are photosynthetic, purple, non-sulfur bacteria. Unlike the thioredoxins of other photosynthetic organisms, such as cyanobacteria and plants, their thioredoxins are reduced by NADPH and thioredoxin reductase rather than ferredoxin-thioredoxin reductase. *T. ferrooxidans* is also an autotrophic bacterium and, like the TrxAs of *C. vinosum* and *R. rubrum*, the *T. ferrooxidans* TrxA appears to be reduced by NADP-thioredoxin reductase, as it is functional in *E. coli*. In addition, *T. ferrooxidans* and *C. vinosum* both utilize ATP-driven reverse electron transport to reduce electronegative acceptors such as NAD^+ (E_0 = -320mV) and

possibly ferredoxin. Reduction via NADP is therefore energetically less costly to these bacteria than via ferredoxin.

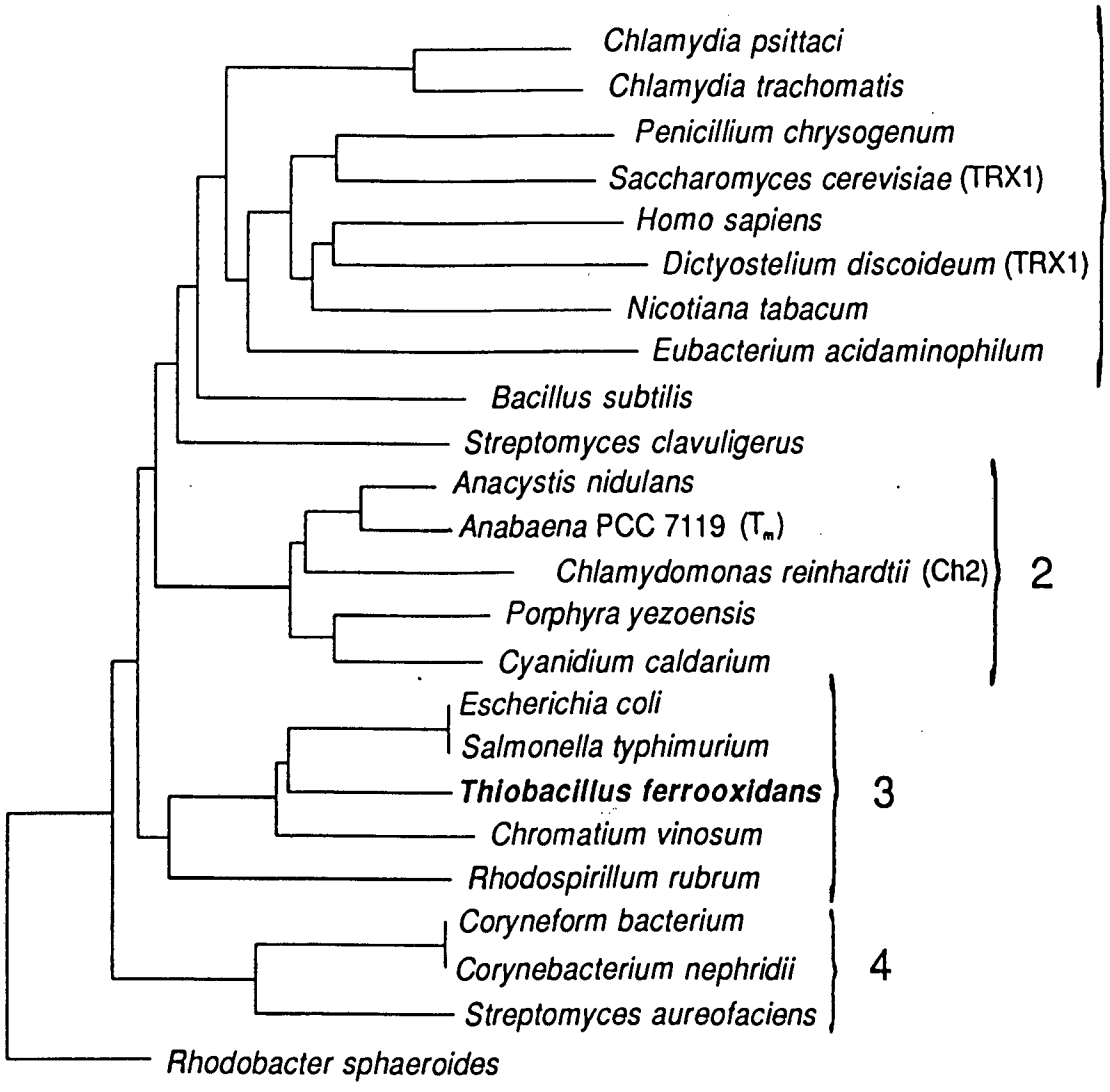


Fig. 2.13. A phylogram based on thioredoxin amino acid sequences. The GCG software packages Pileup and GrowTree were used to calculate and draw the phylogram, using the neighbour-joining method (Swofford & Olsen, 1990). The GenBank accession numbers of the sequences used were as follows: *C. psittaci* L3982; *C. trachomatis* L40369; *P. chrysogenum* X76120; *S. cerevisiae* M62647; *H. sapiens* X54540; *D. discoideum* M91384; *N. tabacum* Z11803; *E. acidaminophilum* L04500; *B. subtilis* J03294; *S. clavuligerus* Z21946; *A. nidulans* M14736; *C. reinhardtii* X62335; *P. yezoensis* X76612; *C. caldarium* X21723; *E. coli* M87049; *S. typhimurium* M10424; *C. vinosum* P09857; *R. rubrum* P10473; *C. bacterium* P00275; *C. nephridii* J02801; *S. aureofaciens* P33791 and *R. sphaeroides* M33806.

The amino acids forming the flat, hydrophobic surface around the active site of thioredoxin that is thought to interact with other proteins are highly conserved. These amino acids include Asp-26, Ala-29, Trp-31, Cys-32, Gly-33, Pro-34, Cys-35, Asp-61, Pro-76 and Gly-92 (Eklund *et al.*, 1991). Pro-40 provides a kink in the alpha-2 helix, ensuring the proper position of the active site at the amino end of this helix, whilst Pro-76 appears to be important in maintaining the native structure of the molecule. In addition, residues Phe-12, Val-25 and Phe-27 which form

the internal contact surfaces between the secondary structural elements are generally unchanged (Eklund *et al.*, 1991). These amino acids are all conserved in the *T. ferrooxidans* thioredoxin.

The ability of the *T. ferrooxidans* thioredoxin in *E. coli* to support growth of phage T7 but not the filamentous phage M13 is different to that which was found for the thioredoxin of *Anabaena* PCC 7119. No growth of wild type phage T7 occurred in the presence of the *Anabaena* thioredoxin and the protein was unable to form an active protein with the DNA polymerase gene 5 protein *in vitro* (Lim *et al.*, 1986). However, the *Anabaena* thioredoxin was able to support filamentous phage assembly. It has been suggested that the regions around amino acids 74 to 77 and 91 to 93 of the *E. coli* protein are critical for the interaction of thioredoxin with the gene 5 protein (Huber *et al.*, 1986). Amino acid Gly-74 of the *E. coli* thioredoxin has been found to be essential for the formation of an active T7 DNA polymerase complex. *T. ferrooxidans*, *E. coli* (Mark & Richardson, 1976) and *R. sphaeroides* (Pille *et al.*, 1990) all possess a glycine residue at position 74 and are all able to support T7 phage replication. However, in the *C. nephridii* (Lim *et al.*, 1987) and *Anabaena* (Lim *et al.*, 1986) thioredoxins, which are both unable to support T7 replication, Gly-74 is replaced by a serine residue.

CHAPTER THREE

THE ISOLATION AND CHARACTERIZATION OF THE γ-GLUTAMYLCYSTEINE SYNTHETASE GENE OF *T. FERROOXIDANS* AND THE UPSTREAM CHROMOSOMAL DNA.

CHAPTER THREE

THE ISOLATION AND CHARACTERIZATION OF THE γ-GLUTAMYLCYSTEINE SYNTHETASE GENE OF *T. FERROOXIDANS* AND THE UPSTREAM CHROMOSOMAL DNA.

3.1. Summary

The γ-glutamylcysteine synthetase gene of *T. ferrooxidans* was isolated by complementation of the *E. coli gshA trxA* double mutant, strain BH5262. An 8 kbp region of chromosomal DNA lying upstream of the γ-glutamylcysteine synthetase-complementing DNA was also cloned. It was confirmed by Southern hybridisation that the source of the cloned DNA was *T. ferrooxidans* ATCC 33020 chromosomal DNA and that the cloned DNA was contiguous on the chromosome. The nucleotide sequence of a 2.3 kbp *Cla*I-*Bam*HI *T. ferrooxidans* chromosomal DNA fragment was determined from both strands. Two ORFs, separated by 9 bp, corresponding to a citrate synthase gene and a γ-glutamylcysteine synthetase gene were identified on the 2.3 kbp *Cla*I-*Bam*HI fragment. Partial sequencing and analysis of the cloned chromosomal DNA upstream of the *Cla*I site indicated regions of homology to the genes of the pyruvate dehydrogenase complex.

Synthesis of proteins corresponding to the *T. ferrooxidans* citrate synthase (43 kDa) and γ-glutamylcysteine synthetase (49 kDa) enzymes were confirmed using an *E. coli*-derived *in vitro* transcription-translation system. The identity of the proposed citrate synthase gene was also demonstrated by complementation of an *E. coli gltA*⁻ mutant. Similarly, the DNA upstream of the citrate synthase gene, was found to complement an *E. coli* mutant for the pyruvate dehydrogenase complex, confirming that this region contained the *T. ferrooxidans* pyruvate dehydrogenase complex, as predicted from the sequence analysis.

RNA:DNA hybridisation was carried out on RNA transcripts prepared from *E. coli* JM109, *E. coli* JM109(cosmid 5.1) and *T. ferrooxidans*. The transcripts were probed with probes specific to the *gshA* gene, the *gltA* gene and the *aceF* gene. All three probes gave transcripts of 9 kbp and a number of smaller transcripts. The 9 kbp transcripts indicate transcriptional linkage of the γ-glutamylcysteine synthetase gene, the citrate synthase gene and the genes of the pyruvate dehydrogenase complex.

The *T. ferrooxidans* γ-glutamylcysteine synthetase gene had very low homology to previously cloned genes. The predicted amino acid sequence of the *T. ferrooxidans gshA* gene had only 18

% identity to those of *E. coli*, *A. thaliana*, and *S. cerevisiae*; and 15 % identity to that of human and 16 % identity to that of rat. The identity of the gene product was, therefore, confirmed by determining the levels of glutathione in crude extracts of the *gshA* mutant (*E. coli* BH5262), the *gshA* mutant containing the cloned *T. ferrooxidans* γ-glutamylcysteine synthetase gene (*E. coli* BH5262(pTHIO7)) and a parental strain (*E. coli* MC1061). Crude extracts prepared from *E. coli* BH5262(pTHIO7) contained far higher levels of glutathione than crude extracts prepared from *E. coli* BH5262, indicating that the gene from *T. ferrooxidans* encoded a protein with γ-glutamylcysteine synthetase activity.

3.2. Introduction

As discussed in Chapter 2 a group of cosmids was isolated that was able to complement the *E. coli trxA gshA* double mutant, strain BH5262, but was unable to complement the *E. coli trxA* mutant, strain BH2012. It was, therefore, thought possible that these cosmids might encode a *gshA* gene or some other gene from *T. ferrooxidans*, which enabled growth of the *E. coli trxA gshA* mutant on minimal medium lacking glutathione.

The *gshA* gene encodes the enzyme γ-glutamylcysteine synthetase which catalyses the first step in the synthesis of the tripeptide thiol glutathione. Glutathione plays a pivotal role in the metabolism of a cell. It has previously been proposed that γ-glutamylcysteine synthetase has evolved independently in different organisms, and yet functionally similar active centers have been achieved (May & Leaver, 1994). Functionally, γ-glutamylcysteine synthetase enzymes from all organisms catalyse the same reaction and exhibit the same substrate specificity and apparent K_m values; all enzymes are feedback inhibited in a nonallosteric manner by GSH (May & Leaver, 1994; Hell & Bergmann, 1990; Meister, 1983; Huang *et al.*, 1988) and all are inhibited by sulfoximine analogs of γ-glutamyl phosphate (May & Leaver, 1994; Hell & Bergmann, 1990; Griffith & Meister, 1979). However, sequence analysis suggests that the five *gshA* genes sequenced to date are structurally dissimilar. It was, therefore, of interest to compare the *gshA* gene of *T. ferrooxidans* to the other *gshA* genes studied.

This is only the second *gshA* gene to be sequenced from a bacterium, and the first *gshA* gene found to be transcriptionally linked to the citrate synthase gene.

3.3. Materials & Methods

3.3.1. Bacterial strains, plasmids and media

The bacterial strains and plasmids used in this study are described in Appendix A. Complex media used for growth of *E. coli* was Luria-Bertani medium (LB), and M9 minimal medium was used for growth of *E. coli* on selective medium (Sambrook *et al.*, 1989). *T. ferrooxidans* was grown on tetrathionate medium (see Chapter 2). When required antibiotics were added at the following concentrations; ampicillin 100μg/ml and chloramphenicol 30μg/ml.

3.3.2. Preparation of DNA

The alkaline lysis method (Birnboim & Doly, 1979; Ish-Horowicz & Burke, 1981) was used for both small and large scale plasmid preparations. Chromosomal DNA was prepared from *T. ferrooxidans* ATCC 33020 by the DNA extraction method outlined in Chapter 2. Construction of the gene bank of *T. ferrooxidans* ATCC 33020 DNA in the cosmid vector pHC79 was carried out by Ramesar (1988).

3.3.3. DNA manipulations

Standard methods were used for restriction digests, gel electrophoresis, purification of DNA fragments from agarose gels and ligations (Sambrook *et al.*, 1989; Ausubel *et al.*, 1993). All enzymes and buffers were used according to the manufacturer's specifications. *E. coli* cells were made competent by the standard rubidium chloride method described by Sambrook *et al.* (1989). Transduction of *E. coli* cells with the *T. ferrooxidans* cosmid gene bank was carried out according to the method outlined in Chapter 2.

3.3.4. Isolation of the g-glutamylcysteine synthetase gene by complementation of an *E. coli gshA trxA* mutant.

The *T. ferrooxidans* ATCC 33020 cosmid library was transduced into *E. coli* BH5262, a *gshA trxA* double mutant. The expression mixes were washed with 0.8% NaCl before plating onto M9 minimal medium containing 20 μg/ml arginine and 100 μg/ml ampicillin. Possible positive γ-glutamylcysteine synthetase clones were identified by their ability to grow on M9 minimal medium lacking glutathione. *E. coli* strain BH5262 is unable to grow on minimal medium lacking glutathione whereas $TrxA^+$ or $GshA^+$ colonies can (Lim *et al.*, 1986). The positive clones were miniprepped and retransformed into *E. coli* BH5262 to confirm the $GshA^+$ or $TrxA^+$ complementation.

3.3.5. Subcloning of cosmid 5.1

Cosmid 5.1 DNA was restriction digested with *Bam*HI and ligated with the vector pBluescriptSK (digested with *Bam*HI). The ligation mixes were transformed into *E. coli*

BH5262 competent cells and expressed in M9 minimal media plus 20 μg/ml arginine. The expression mixes were then plated on M9 minimal medium and transformants selected for complementation of the $GshA^+$ or $TrxA^+$ phenotype. The subclone cosmid 4.1, a *Bam*HI deletion of cosmid 5.1, was isolated. A 5 kbp *Hin*dIII-*Hin*dIII fragment from cosmid 4.1 was further subcloned into the vector pUCBM21 (digested with *Hin*dIII) to give the subclone pTHIOD.

3.3.6. Subcloning of pTHIOD

All subclones are shown in Fig. 3.1. A 4.6 kbp *Hin*dIII-*Sac*I fragment from pTHIOD was cloned into pUCBM21 to form plasmid pTHIO6. Plasmid pTHIO5 was constructed by cloning of a 2.6 kbp *Hin*dIII-*Cla*I fragment from pTHIOD into pBluescriptSK (*Hin*dIII-*Cla*I). Plasmid pTHIO7 was constructed by cloning of a 1.9 kbp *Hin*dIII-*Bgl*II fragment from pTHIOD into pBluescriptSK (*Hin*dIII-*Bam*HI). A 1.8 kbp *Sma*I-*Eco*RV fragment from pTHIOD was cloned into the *Sma*I and *Eco*RV sites of pBluescript to produce pTHIO28. As both *Sma*I and *Eco*RV are blunt-ended restriction sites the 1.8 kbp fragment could ligate to the vector in either orientation. It was found that the *Sma*I site of the insert had ligated with the *Eco*RV site of the vector and vice versa. Plasmid pTHIO82 was constructed by cloning the same *Sma*I-*Eco*RV fragment in the opposite orientation with respect to the *lac* promoter. A 1.3 kbp *Eco*RV fragment from pTHIOD was cloned into the *Eco*RV site of pBluescriptSK to produce pTHIO15. Plasmid pTHIO9 was constructed by insertion of a 1.1 kbp *Hin*dIII-*Sal*I fragment from pTHIOD into pBluescriptSK (*Hin*dIII-*Sal*I). A 0.4 kbp *Eco*RI-*Cla*I fragment from pTHIO7 was cloned into pBluescriptSK (*Eco*RI-*Cla*I) to produce pTHIO8. Plasmid pTHIO13 was constructed from a 1.3 kbp *Xba*I-*Eco*RI fragment of pTHIO7 cloned into pBluescriptSK (*Xba*I-*Eco*RI). Plasmid pTHIO4 was constructed from a 0.8 kbp *Sal*I-*Xba*I fragment of pTHIO7 cloned into pBluescriptSK (*Sal*I-*Xba*I). A 0.6 kbp *Bam*HI-*Eco*RV fragment from pTHIOD was cloned into pBluescriptSK (*Bam*HI-*Eco*RV) to produce pTHIO21. Plasmid pTHIO12 was constructed from a 1.9 kbp *Kpn*I-*Sac*I fragment of pTHIO7 cloned into pUCBM21 (*Kpn*I-*Sac*I). A 0.5 kbp *Bgl*II fragment from pTHIOD was cloned into pUCBM21 to form pTHIO36. Plasmid pTHIO14 was constructed from a 0.9 kbp *Cla*I fragment of pTHIOD cloned into pBluescriptSK. A 3.0 kbp *Hin*dIII-*Bam*HI fragment from pTHIOD was cloned into pBluescriptSK to produce pTHIO17. All subclones were tested for the ability to complement the *E. coli trxA gshA* double mutant, BH5262.

3.3.7. Exonuclease shortening and cloning of shortened fragments

Plasmid pTHIO7 was digested with *Hin*dIII-*Apa*I to generate a template for unidirectional Exonuclease III digestion by the method of Henikoff (1987). This enabled shortening from the *Bgl*II end of pTHIO7. Plasmid pTHIO12 was digested with *Kpn*I-*Sac*I to enable shortening with Exonuclease III from the *Hin*dIII end of pTHIO7. All subclones generated by Exonuclease shortening are shown in Fig. 3.2.

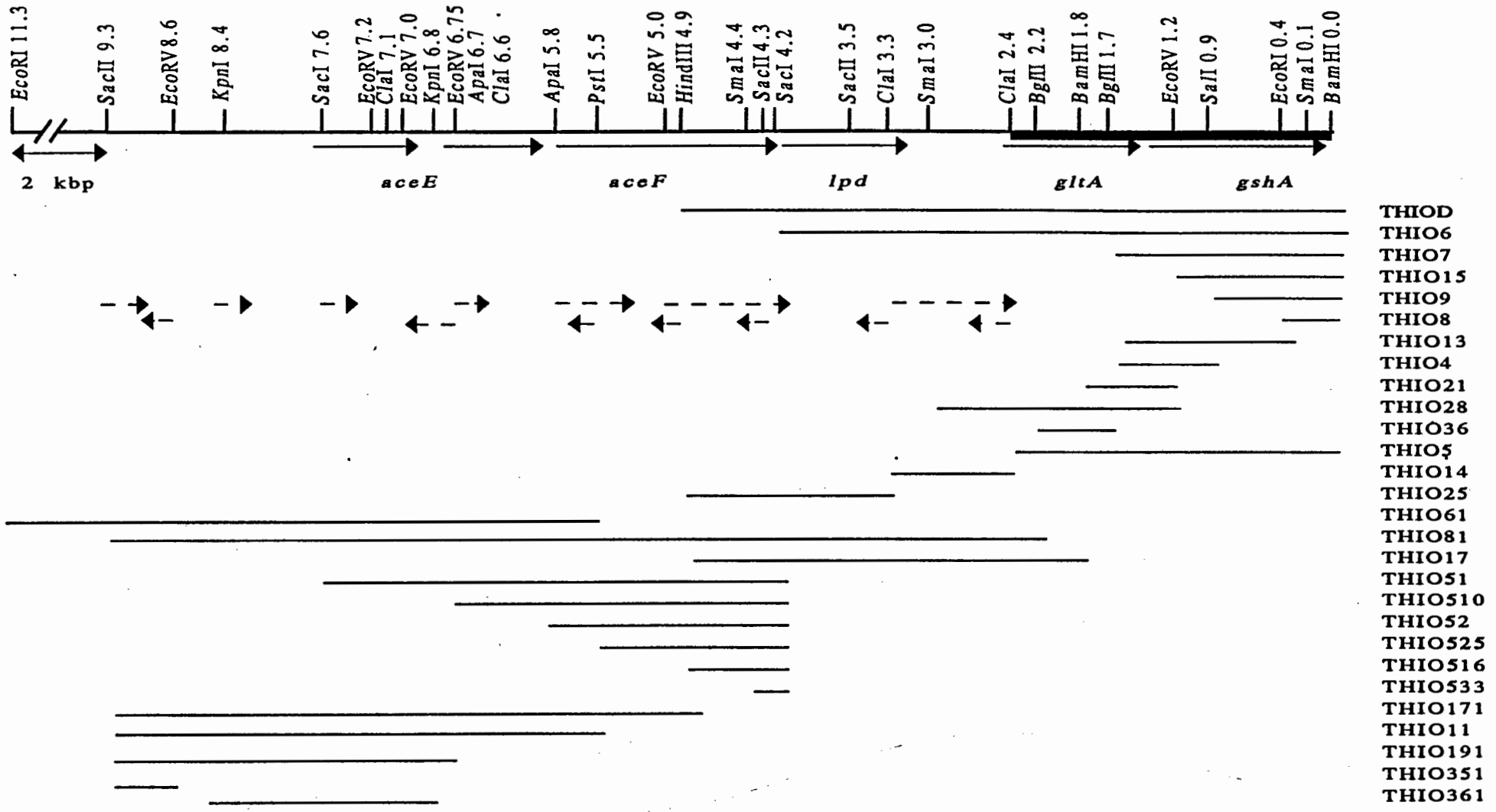


Fig. 3.1 Restriction endonuclease map of plasmid pTHIOD and derivatives. The *gshA*, *gltA* and possible *aceE*, *aceF* and *lpd* genes are indicated by arrows. The solid line represents the region of DNA sequenced from both strands. The arrows with dotted lines indicate regions of single stranded sequence. Numbers proceeding restriction enzyme sites indicate the distance from the *Bam*HI (0.0) site in kbp.

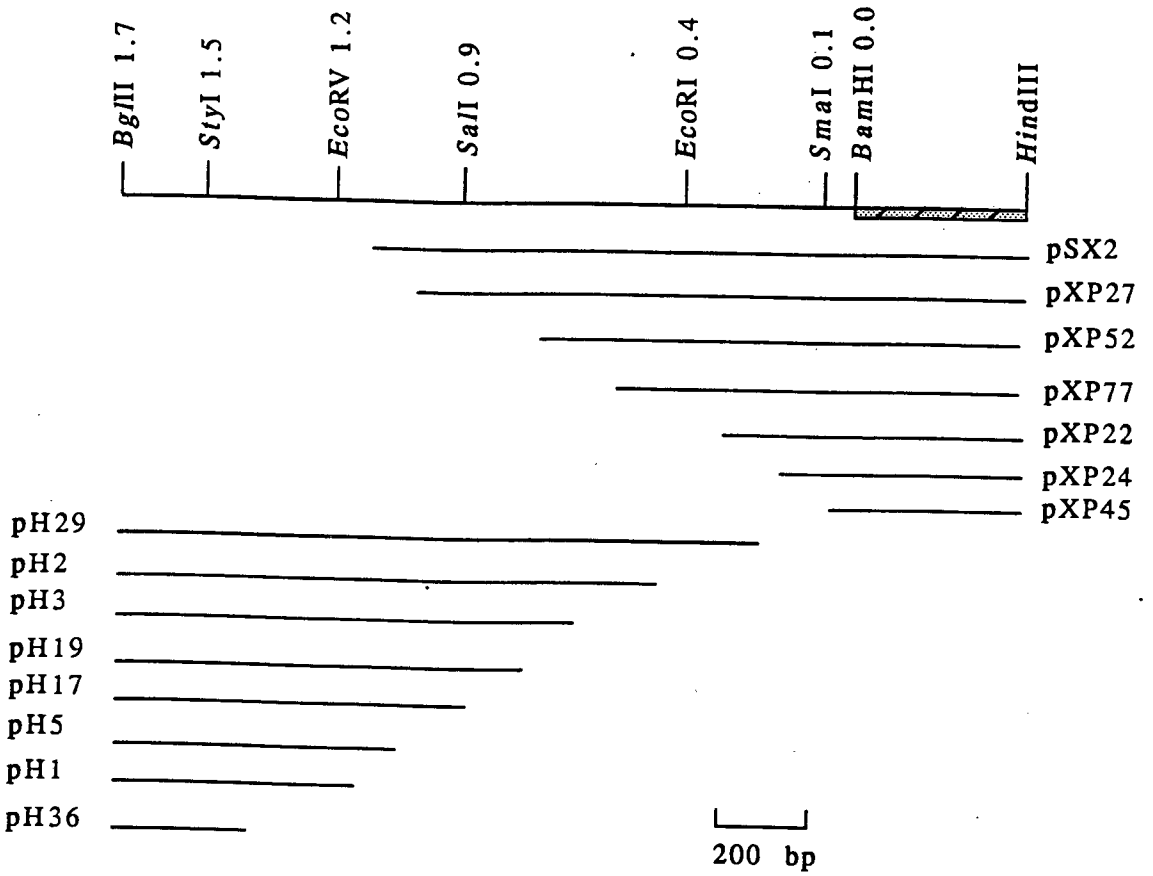


Fig. 3.2 Restriction endonuclease map of subclones generated by exonuclease III shortening of pTHIO7. Cross-hatched line indicates cosmid vector pHC79, numbers indicate distance from *Bam*HI (0.0) site in kbp.

3.3.8. Subcloning the upstream region

All subclones are shown in Figure 3.1. A 3.4 kbp *Sac*I fragment from cosmid 5.1 was subcloned into pBluescriptSK (*Sac*I) to produce pTHIO51. Plasmids pTHIO510, pTHIO52, pTHIO525, pTHIO516 and pTHIO533 were *Cla*I, *Apa*I, *Pst*I, *Eco*RV and *Sma*I deletions of pTHIO51, respectively. Plasmid pTHIO61 was constructed from a 6.0 kbp *Eco*RI-*Pst*I fragment of cosmid 5.1 cloned into pUCBM21 (*Eco*RI-*Pst*I). A 3.8 kbp *Sac*II-*Pst*I fragment from pTHIO61 and a 3.3 kbp *Pst*I-*Bgl*II fragment from cosmid 4.1 were ligated and cloned into pBluescriptSK (*Sac*II-*Bam*HI) to form pTHIO81. Plasmids pTHIO171, pTHIO111, pTHIO191 and pTHIO351 were *Hin*dIII, *Pst*I, *Apa*I and *Eco*RV deletions of pTHIO81, respectively. Plasmid pTHIO361 was constructed from a 1.3 kbp *Kpn*I fragment of cosmid 5.1 cloned into pUCBM21 (*Kpn*I). A 1.6 kbp *Hin*dIII/*Cla*I fragment from pTHIOD was cloned into pBluescriptSK to produce pTHIO25.

3.3.9. Southern hybridisation of plasmid pTHIO7 to *T. ferrooxidans* chromosomal DNA

In order to confirm the origin of the cloned γ-glutamylcysteine synthetase-complementing DNA and the region cloned upstream, DNA hybridisation was performed. *T. ferrooxidans* chromosomal DNA, cosmid DNA and plasmid DNA were digested with restriction enzymes and electrophoresed together with lambda DNA (*Pst*I) as a molecular weight marker, on a 0.8%

agarose gel. Blotting was carried out according to the method recommended by Amersham for Hybond N^+ membranes. Hybridisation and detection were carried out according to the dioxygenin-dUTP non-radioactive DNA labelling and detection kit (Boehringer Mannheim).

3.3.10. Nucleotide sequencing

Recombinant plasmids generated from Exonuclease III shortening (Fig. 3.2), or from subcloning (Fig. 3.1), were sequenced by the dideoxy chain terminating method (Sanger *et al.*, 1977), using the Sequenase kit (version 2.0) from United States Biochemical Corp. The sequence of a 2.4 kbp *Cla*I-*Bam*HI fragment was determined from both strands. Regions of single and double-stranded sequence were determined upstream of the *Cla*I restriction site (Fig. 3.1). The Genetic Computer Group Inc. (GCG) sequence analysis software package (version 7.1) was used for sequence analysis (Devereux *et al.*, 1984). Multiple sequence alignments and phylogenetic trees were calculated using the software package Clustal W (Thompson *et al.*, 1994). The software package TreeView was utilised to draw the phylogenetic tree (Page, 1995).

3.3.11. *In vitro* protein synthesis from the entire cloned region

The *in vitro* syntheis of polypeptides from cosmid 5.1, plasmids, pTHIO81, pTHIO191, pTHIO51, pTHIOD, pTHIO28, pTHIO7 and pBluescriptSK was determined using the prokaryotic DNA-directed transcription kit, *E. coli* S30 system of Promega Corp., Madison USA. Reactions were performed according to the manufacturer's specifications. The proteins were separated by SDS-PAGE using a 5-20% gradient gel.

3.3.12. Complementation of the *gltA* phenotype

The plasmids pMW264, pBluescriptSK, pTHIO6, pTHIO28 and pTHIO82 were transformed into the *E. coli gltA* mutant, strain MOB150. The transformants were plated onto Luria-Bertani agar containing 100 µg/ml ampicillin and placed at 37°C until colonies were visible (approximately 36 hours). Individual colonies were then streaked onto both M9 minimal media plates containing no amino acids and M9 minimal media plates containing 20 µg/ml glutamate. Possible citrate synthase positive clones were identified by their ability to grow on M9 minimal medium lacking glutamate. *E. coli* MOB150 is unable to grow on minimal medium lacking glutamate, whereas $GltA^+$ colonies can (Wood *et al.*, 1987). Positive clones were selected, and the DNA isolated, retransformed and tested for the ability to complement *E. coli* MOB150, as above, to confirm the $GltA^+$ phenotype.

3.3.13. Complementation of the pyruvate dehydrogenase complex

Constructs to be tested were transformed into *E. coli* JRG1342, the *aroP-aceEF-lpd* deletion mutant. The expression mixes were plated on Luria-Bertani agar plates and the appropriate antibiotics and placed at 37°C for approximately 36 hours or until colonies were visible. Individual colonies from each plate were then streaked out onto M9 minimal media agar plates, with glucose (10 mM), potassium succinate (50 mM) or potassium acetate (50 mM) as carbon

sources and supplements of acetate (2 mM and 4 mM for glucose and succinate carbon sources respectively), succinate (2 mM), and L-methionine (20 µg/ml) (see Table 3.5).

3.3.14. Preparation of RNA

All glassware, apparati and chemicals used for RNA extraction and analysis were treated as outlined in Chapter 2. *T. ferrooxidans* total RNA was prepared from cultures grown on tetrathionate medium, and *E. coli* total RNA from cultures grown on M9 minimal medium by the method of Aiba *et al.* (1981).

3.3.15. DNA:RNA hybridisation

All DNA probes were labelled with [^{32}P]CTP (3000Ci/mmol) using the Random Primed DNA labelling kit from Boehringer Mannheim. Total RNA from *T. ferrooxidans*, *E. coli* JM105(cosmid 5.1) and *E. coli* JM105 was separated on a 1.5% agarose gel containing 6% formaldehyde (Amersham Hybond N$^+$ protocol). The RNA was transferred to an Amersham Hybond N$^+$ membrane and hybridisation and washes were carried out according to the manufacturer's specifications.

Three different DNA:RNA hybridisations were carried out. In the first, the RNA was probed with a 900 bp *Sal*I-*Bam*HI ^{32}P-labelled fragment from pTHIO7. The second probe was derived from a 1.1 kbp *Bam*HI-*Sma*I fragment of pTHIO28 and the third probe from a 900 bp *Hin*dIII-*Apa*I fragment from pTHIO52.

3.3.16. Assay for glutathione

A modification of the fluorometric assay described by Hissin & Hilf (1976) was used to measure the endogenous GSH content of the bacteria. Overnight cultures of the strains were grown in M9 minimal medium plus the appropriate amino acids and antibiotics. The cultures were pelleted (5000rpm for 10 minutes), and resuspended to an approximate concentration of 1g wet cells per 5 ml of minimal medium. The bacterial suspensions were sonicated for 8 X 30s at 0^{o}C, centrifuged for 10 minutes at 12 000 rpm and the resulting supernatant fractions used immediately or stored at -70^{o}C for up to a month.

Fifteen µl of 25% trichloroacetic acid were added to 100 µl of bacterial extract, and the extract centrifuged to remove precipitated protein. Forty µl of the supernatant were added to 960 µl of fresh phosphate-EDTA buffer (0.1M potassium phosphate pH 8, 0.005M EDTA). The final reaction mixture contained 0.1 ml of the diluted extract, 1.8 ml of phosphate-EDTA buffer and 0.1 ml of o-ophthalaldehyde (OPT) solution (1mg/ml o-opthalaldehyde in absolute methanol, made up fresh). After thorough mixing and incubation at room temperature for 15 minutes, the solution was transferred to a quartz cuvette, and the fluorescence at 420nm was determined with activation at 350nm.

A standard curve was plotted using known concentrations of glutathione (Appendix B). To account for background interference due to the reaction of o-opthalaldehyde with compounds other than GSH in the bacterial extract, 4 µl of undiluted bacterial extract (treated with trichloroacetic acid), derived from the negative control *E. coli* BH5262, was added to the standards.

3.3.17. Cloning, expression and purification of maltose-binding protein fusion

The maltose-binding protein (MBP) vectors allow the expression and purification of a protein encoded by a cloned gene, by fusing it to the MBP, encoded by the *malE* gene of *E. coli*. This method uses the strong, inducible *tac* promoter and the *malE* translation initiation signals to give high levels of expression of the cloned gene, followed by affinity purification for the MBP to facilitate isolation of the fusion protein (Kellerman & Ferenci, 1982).

As there were no restriction sites available for cloning the target gene in frame with the *malE* gene of the vector pMal-c2, the target gene was cloned by PCR. A synthetic oligonucleotide primer was designed containing a *Pvu*II restriction site for in frame cloning into the *Stu*I site of the pMal vector, followed by the nucleotide sequence corresponding to the 5' end of the target gene (nucleotides 1346 -1363, Fig. 3.6). The primer had the following sequence:

*Pvu*II

5'-CGATCGCAGCTGATGACGGAAGTGATACGG-3'
M T E V I R

The oligonucleotide primer was synthesized by the Dept. of Biochemistry, University of Cape Town, using an Autogen 6500 DNA synthesizer and it was purified by a reverse phenyl cartridge purification processs.

The second primer used for PCR, was the T7 sequencing primer, which annealed to the pBluescriptSK vector of plasmid pTHIO7, at the 3' end of the target gene (*Hin*dIII end). The reaction mix for PCR was constituted as follows:

0.5 µg pTHIO7 DNA or less	2 µl
10 X PCR/*Taq* buffer	10 µl
10 X $MgCl_2$ (25mM)	10 µl
0.25 µM of each primer	5 µl
2.0 mM each mixed dNTPs (final conc. 200 µM each)	10 µl
2 U *Taq* (diluted 5 X)	1 µl
Distilled water	57 µl
Total volume	100µl

The reaction mix was overlayed with 100 µl of mineral oil and placed in the PCR machine. The PCR regime was as follows;

1. 60s at 94°C
2. 30s at 94°C
3. 30s at 50°C
4. 90s at 72°C
5. 120s at 72°C
6. 60s at 20°C

Steps 2 to 4 were repeated for 30 cycles.

A 10 µl aliquot was removed and electrophoresed on a 0.8 % agarose gel to check that a PCR product of the predicted size had been produced. The aqueous phase of the PCR reaction mix was then removed, precipitated and resuspended in 50 µl Tris/EDTA buffer (Sambrook *et al.*, 1989). The PCR product was digested with the restriction enzymes *Pvu*II/*Bam*HI and the pMal vector with *Stu*I/*Bam*HI and the two fragments ligated. The ligation mix was transformed into *E. coli* TBI competent cells and the DNA isolated from the transformants mapped with restriction enzymes.

The MBP-fusion protein was then purified according to the method of Ausubel *et al.* (1993). A pilot experiment indicated that the fusion protein was located in the crude extract and not in the osmotic shock fluid, so the standard purification procedure was followed. Maltose was removed from the extract after Factor X_a cleavage by dialysis in 2 litres of column buffer for 24 hours (with replacement of the buffer every 5 hours). The dialysed cleavage products were passed through the amylose resin column to remove the MBP. The purified protein was concentrated to 1.0 mg/ml using a Diaflo PM10 membrane (Amicon) and an Amicon filtration apparatus.

3.3.18. Assay for γ-glutamylcysteine synthetase activity

The standard assay for determination of *gshA* activity contained; 100mM Tris-HCl at pH 8.2, 10mM L-glutamate, 10mM L-α-aminobutyrate, 40mM $MgCl_2$, 5mM ATP and the enzyme extract to a final volume of 50 µl. The reaction mix was incubated at 30°C for 15 minutes. Ten µl 25% trichloroacetic acid was added and the precipitated proteins removed by centrifugation. As a negative control enzyme storage buffer was used in place of the enzyme extract. The amount of inorganic phosphate in the supernatant was measured according to the method of Lanzetta *et al.* (1979). The following stock solutions were made:

A. 0.045% malachite green hydrochloride
B. 4.2% ammonium molybdate in 4N HCl
C. 34% sodium citrate.$2H_2O$ (w/v)
D. 100µl Triton N in 20ml deionized water

E. 10mM 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 minutes, and was then filtered through a 0.45 µm Millipore membrane filter. Solution D was added at a concentration of 300µl/5ml filtrate, and the solution stirred for 30 minutes at room temperature.

The amount of inorganic phosphate released by the enzyme reaction mix was measured by adding 50µl of the terminated mix to 800µl of the colorimetric reagent in 1ml disposable spectrophotometric cuvettes. After 1 min, the reaction was quenched by adding 100µl of solution C. The contents of the tubes were briefly vortexed and left at ambient temperature for 30 minutes. The OD_{660} was read. To account for background interference, the absorbance reading obtained for the negative control was substracted from that obtained for each sample. The amount of inorganic phosphate released was determined by reference to a standard curve, obtained using P_i prepared from solution D, containing 1, 2, 5, 8, and 10nm P_i and 5% trichloroacetic acid. Five percent trichloroacetic acid was added as it was found to affect the absorbance reading of the samples and therefore had to be added to the standards also.

3.3.19 Dabsylation and HPLC analysis of DABS-amino acids

The following samples were prepared for HPLC analysis: (i) the standard assay mixture for determination of *gshA* activity, as in section 3.3.19. (ii) the standard assay mixture with enzyme storage buffer instead of enzyme extract, (iii) the standard assay mixture without the substrates. The samples were placed at 30°C for 30 minutes. Proteins and contaminants were removed from the samples prior to HPLC analysis using Sep-pak C18 columns, according to the manufacturer's specifications (Millipore). The above samples and the standards L-glutamate, α-aminobutyrate, GSH and water were dabsylated according to the following procedure: the samples were hydrolysed by freeze drying and dissolved in 25 µl of 50mM sodium bicarbonate (pH 9.0). Next, 50 µl DABS-Cl solution (4 mM in acetonitrile; freshly prepared) was added to each sample. The samples were sealed and heated at 70°C for 12 minutes (the mixture becomes completely soluble after heating). After dabsylation, the samples were diluted with 125 µl of diluting solution (50 mM sodium phosphate pH 7.0/ethanol, 1:1 v/v) and approximately 50 µl injected directly for HPLC analysis.

The dabsylated samples and standards were separated on a reverse phase C-18 column (4.6mm x 15 cm, TSK-gel ODS-80Tm, manufactured by TosoHaas), using as eluting solvents 38 mM potassium phosphate, pH 6.5 (solvent A) and 80 % acetonitrile (solvent B) at a flow rate of 1 ml/minute. Both solvents were degased prior to use. The detector wavelength used was 436nm. Following injection the column was eluted as follows:

Time (mins)	% Solvent A	% Solvent B
0	100	0
15	50	50
30	20	80
32	20	80
38	100	0

3.4. Results

3.4.1. Isolation of the *gshA* gene by complementation of the *E. coli gshA trxA* mutant.

As described in Chapter 2, transduction of the *T. ferrooxidans* genome cosmid library into *E. coli* BH5262 resulted in approximately 100 colonies that were able to grow on minimal medium lacking glutathione. DNA was prepared from 16 of these colonies and restriction endonuclease mapping carried out. These cosmids could be divided into two groups that had several fragments in common and appeared to contain overlapping pieces of two regions of the *T. ferrooxidans* chromosome. Only one of the groups of cosmids, on transforming into *E. coli* BH2012, enabled cells to grow on minimal medium plus methionine sulfoxide. This group of cosmids was found to contain the thioredoxin gene. A representative of the second group of cosmids, that were unable to complement *E. coli* BH2012, cosmid 5.1, was chosen for further study.

3.4.2. DNA hybridisation of *gshA*.

The source of γ-glutamylcysteine synthetase-complementing DNA was confirmed by the hybridisation of labelled pTHIO7 (linearised with *Hin*dIII), to *T. ferrooxidans* ATCC 33020 chromosomal DNA, cosmid 5.1, cosmid 4.1 and plasmids pTHIOD and pTHIO7 digested with *Sma*I, and to cosmid 5.1 digested with *Bam*HI (Fig. 3.3).

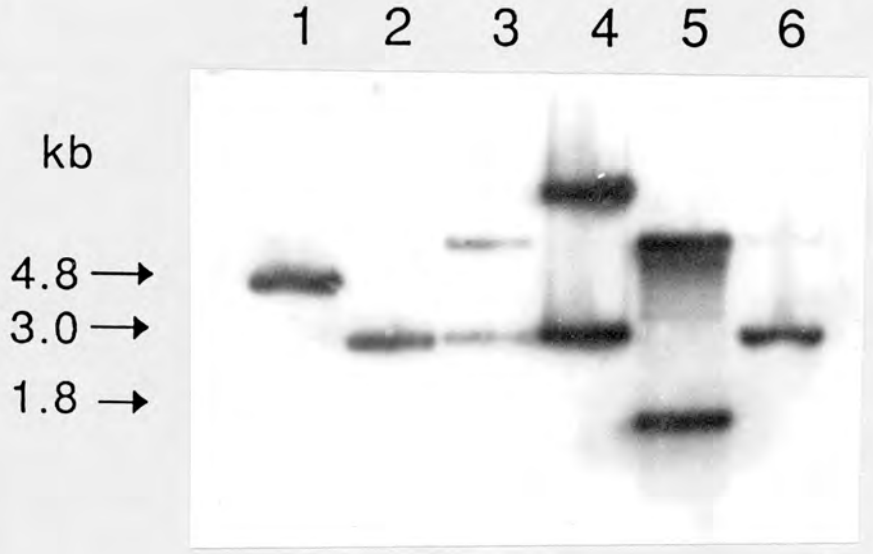


Fig. 3.3 Hybridisation of labelled plasmid pTHIO7 to: *Sma*I digests of lane 1, plasmid pTHIO7; lane 2, plasmid pTHIOD; lane 3, cosmid 4.1; lane 4, cosmid 5.1 and lane 6, *T. ferrooxidans* ATCC 33020 chromosomal DNA, and a *Bam*HI digest of cosmid 5.1, lane 5.

The 3.0 kbp *Sma*I fragment that is internal to the cloned *T. ferrooxidans* chromosomal DNA present on pTHIOD corresponded exactly to the *Sma*I fragment present on the *T. ferrooxidans* chromosome, cosmid 5.1, cosmid 4.1 and pTHIOD. Similarly the 1.8 kbp *Bam*HI fragment present on cosmid 5.1, corresponded to the size predicted from restriction endonuclease analysis. This indicated that the cloned genes originated from *T. ferrooxidans* ATCC 33020, and that no rearrangements had occurred in the region between the *Sma*I (3.0) and *Bam*HI (1.0) sites during cloning. The hybridisation signal at 4.8 kbp (Fig. 3.3, lane 1) of pTHIO7 corrresponded to the linearised plasmid, as pTHIO7 only contains a single *Sma*I site. The hybridisation signals above the 3.0 kbp *Sma*I signal, for cosmids 4.1 (lane 3) and 5.1 (lane 4) corresponded to hybridisation of the *Sma*I-*Bam*HI flanking region and vector DNA. Digestion of pTHIOD with *Sma*I gives a doublet at approximately 3.0 kbp and single fragments at 1.8 kbp and 0.4 kbp. The probe hybridised to both the internal 3.0 kbp *Sma*I fragment and the 3.0 kbp vector-*Sma*I-*Bam*HI fragment.

3.4.3. DNA hybridisation of the upstream region

It was confirmed that the subclones pTHIO61, pTHIO81 and pTHIO51 were composed of contiguous pieces of DNA, by comparison of hybridisation signals of the plasmids to those of the *T. ferrooxidans* ATCC 33020 chromosome and cosmid 5.1, probed with linearised plasmid pTHIO51 (Fig. 3.4). The 3.4 kbp *Sac*I fragment that was cloned into pTHIO51 corresponded exactly to the fragment present on the *T. ferrooxidans* chromosome (lane 1), cosmid 5.1 (lane 2) and plasmids pTHIO81 (lane 4) and pTHIO51 (lane 10). The digest of plasmid pTHIO51 was included to check that the probe was labelled correctly. Plasmid pTHIO61 contains a single *Sac*I restriction site and therefore gave a hybridisation signal at 9.0 kbp corresponding to the linearised plasmid (Fig. 3.4 lane 3). Additional hybridisation signals at approximately 14.0 kbp (cosmid 5.1, lane 2), 5.4 kbp (pTHIO81, lane 4) and 2.9 kbp (pTHIO51, lane 10), corresponded to the hybridisation of vector DNA. When the *T. ferrooxidans* chromosomal DNA was digested with *Sac*II a strong hybridisation signal at 5.1 kbp and a weaker one at 0.8 kbp, which were also present on cosmid 5.1 and plasmid pTHIO81, were detected (Fig. 3.4, lanes 6, 7 and 9). Plasmid pTHIO61 contains a single *Sac*II restriction site and therefore gave a single hybridisation signal at 9.0 kbp (Fig. 3.4 lane 8). The hybridisation signal at 14.0 kbp in the *Sac*II digest of cosmid 5.1 (Fig. 3.4 lane 7) and at 5.1 kbp in the *Sac*II digest of pTHIO81 (Fig. 3.4 lane 9) corresponded to the hybridisation of vector DNA. Digestion of pTHIO81 with *Sac*II gave a doublet at 5.1 kbp and a single fragment at 0.8 kbp. Thus the probe (pTHIO51) hybridised to both fragments of the pTHIO81 (*Sac*II) doublet. These results indicated that the cloned DNA originated from *T. ferrooxidans* ATCC 33020, and that no rearrangements had occurred between the *Sac*II (9.3) and *Sac*II (3.5) sites.

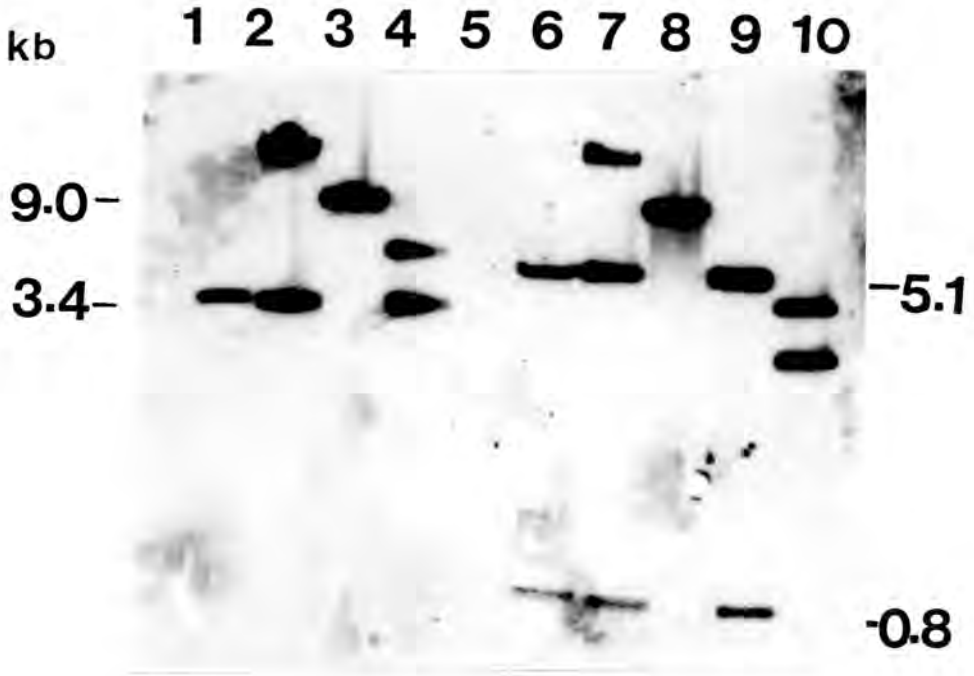


Fig. 3.4 Hybridisation of labelled plasmid pTHIO51 to: *Sac*I digests of lane 1, *T. ferrooxidans* ATCC 33020 chromosomal DNA; lane 2, cosmid 5.1; lane 3, plasmid pTHIO61; lane 4, plasmid pTHIO81; lane 10, plasmid pTHIO51; and *Sac*II digests of lane 6, *T. ferrooxidans* ATCC 33020 chromosomal DNA; lane 7, cosmid 5.1; lane 8, plasmid pTHIO61 and lane 9 plasmid pTHIO81.

3.4.4. Nucleotide Sequence Analysis

The nucleotide sequence from 154 bp upstream of the *Cla*I (2.4) site to the *Bam*HI (0.0) site is shown in Fig. 3.5.

```
1     AGTGCCCCAAGGGGAGGCTGACGGCCTCCCTTTTATTTTACGGGCAGGTTATGATGAT

59    CCGGCGGCTTGTCGTCCGGACGCCTTCGTGCAGGACGCTGCCCGTGAGCAGTGCCCCG

117   ACTGGCCGACCGCCGAAGGTCCGGGCAGTCAAACACCAGAATGATTAAAGGAGTTCTG
                                          start of gltA gene ⟶

175   TATGGCGGAACCGAACTTTGCGCCAGGTCTGGAGGGTGTGGCTGCAACCCAGTCCAGC
        M  A  E  P  N  F  A  P  G  L  E  G  V  A  A  T  Q  S  S

233   ATTTCCAACATCGATGGCGCTGCCGGCCTGCTGAGTTACCGTGGTTTTGCCATTGCGG
        I  S  N  I  D  G  A  A  G  L  L  S  Y  R  G  F  A  I  A

291   ATCTTGCGGCGCACAGCAGTTTCGAGGAGGTGGCGCTCTTGCTGCTGGATGGTGTCCT
      D  L  A  A  H  S  S  F  E  E  V  A  L  L  L  L  D  G  V  L

349   GCCCGGCGCCGCAGATCTGGAACGGTTCGACCACGGTCTGCGTGCGCACCGCCAAGTC
        P  G  A  A  D  L  E  R  F  D  H  G  L  R  A  H  R  Q  V
```

```
407   AAATATAATGTCCGGGAAATCATGAAGTTCATGCCCGTGACCGGACACCCCATGGATA
        K  Y  N  V  R  E  I  M  K  F  M  P  V  T  G  H  P  M  D

465   TGCTGCACTGTGCCGTGGCCAGTCTGGGCATGTTCTACCCGCAGCAGGAGCTTTCCGA
      M  L  H  C  A  V  A  S  L  G  M  F  Y  P  Q  Q  E  L  S  D

523   TGCCGAACGCGGAAATACGCTCCATTTGGACGCCATGGCGATGCGGATTATCGCGCGC
        A  E  R  G  N  T  L  H  L  D  A  M  A  M  R  I  I  A  R

581   ATGCCCACCATTGTCGCGATGTGGGAGCAGATGCGTTTCGGCAATGATCCTATTTCAC
        M  P  T  I  V  A  M  W  E  Q  M  R  F  G  N  D  P  I  S

639   CTCGCCCGGATCTCAGCCATGCGGCCAACTTTCTCTATATGCTGTCGGGTCGCGAACC
      P  R  P  D  L  S  H  A  A  N  F  L  Y  M  L  S  G  R  E  P

697   TGATCCGGCCCATACCAAAATCCTCGACTCCTGCCTGATTCTGCATGCCGAGCACACC
        D  P  A  H  T  K  I  L  D  S  C  L  I  L  H  A  E  H  T

755   ATCAATGCCAGTACCTTCTCGGTACTGGTGACCGGATCCACCCTGACCAATCCTTACC
        I  N  A  S  T  F  S  V  L  V  T  G  S  T  L  T  N  P  Y

813   ATGTCATCGGGGGGGCGATCGGAACCCTGGCCGGCCCGTTGCATGGTGGTGCCAATCA
      H  V  I  G  G  A  I  G  T  L  A  G  P  L  H  G  G  A  N  Q

871   GAAGGTGGTGGAAATGCTGGAAGAAATCAGCTCCGTCCAGCAGGTGGGTGCCTATCTC
        K  V  V  E  M  L  E  E  I  S  S  V  Q  Q  V  G  A  Y  L

929   GACAGGAAGATGGCCAACAAGGAGAAGATCTGGGGTTTCGGGCATCGCATCTACAAAA
        D  R  K  M  A  N  K  E  K  I  W  G  F  G  H  R  I  Y  K

987   CCCGCGATCCGCGTGCAGTGATTCTCAAGGGGATGATGGAGGATATGGCCAGTCATGG
      T  R  D  P  R  A  V  I  L  K  G  M  M  E  D  M  A  S  H  G

1045  AAATCTGCGGCATAGCAGCCTCTTTGAAATTGCCATCGAAGTGGAACGCCAGGCTACG
        N  L  R  H  S  S  L  F  E  I  A  I  E  V  E  R  Q  A  T

1103  GAGCGGCTCGGTGCCCAAGGGATTCACGCCAATGTGGATTTCTATTCGGGCGTGCTGT
        E  R  L  G  A  Q  G  I  H  A  N  V  D  F  Y  S  G  V  L

1161  ATCACGAGATGGGCATCAAAGCGGACCTTTTTACGCCTATTTTTGCTATGGCTCGTTC
      Y  H  E  M  G  I  K  A  D  L  F  T  P  I  F  A  M  A  R  S

1219  TGCGGGCTGGCTGGCTCACTGGCGGGAGCAACTGGCGGATAACCGGATCTTCCGGCCT
        A  G  W  L  A  H  W  R  E  Q  L  A  D  N  R  I  F  R  P

1277  ACGCAGGTGTATACAGGGGAACAGGATCGACGCTATGTGCCTGTGGCCCAACGTACTT
        T  Q  V  Y  T  G  E  Q  D  R  R  Y  V  P  V  A  Q  R  T
```

start of *gshA* gene

```
1335  AGGAAAGACGCATGACGGAAGTGATACGGGATATTCCTTTCCTGGCCACGGATCGGGT
      *                 M  T  E  V  I  R  D  I  P  F  L  A  T  D  R  V

1393  GGAGGCTCTGCTGGATATCGAGCGCCACCTGATCGTCGAGCAATCCACGATTGAGCGC
        E  A  L  L  D  I  E  R  H  L  I  V  E  Q  S  T  I  E  R

1451  TGGTTTCGGGGACAATGGCAACGGACGCCACCGCCTTTCTATGCCTCTGTGGACTTGC
        W  F  R  G  Q  W  Q  R  T  P  P  P  F  Y  A  S  V  D  L

1509  GCAATGCCGGTTTCAAGTTGGCGCCCGTGGACACCAATCTGTTTTCGGCGGGCTTTAA
      R  N  A  G  F  K  L  A  P  V  D  T  N  L  F  S  A  G  F  N
```

```
1567  TAACCTCAATCCGGAGTTTTCGGCGCTCTATGTTTCGGCTATTCAGCATTATCTGAAC
        N  L  N  P  E  F  S  A  L  Y  V  S  A  I  Q  H  Y  L  N

1625  CAGTACCATCCAGGTCTGGAGCGGGTACTGCTGATTCCTGAAAACCACACGCGTAACC
        Q  Y  H  P  G  L  E  R  V  L  L  I  P  E  N  H  T  R  N

1683  TTTTTTATCTGGAGAGCGTGGCACGATTGCGTGAGTTGTTGGAACTCGCCGGATTGGA
        L  F  Y  L  E  S  V  A  R  L  R  E  L  L  E  L  A  G  L  D

1741  CGTGCGAGTGGGCTCCCTGATTCTGGAAGACCGTACGGTCTATGATCTGCCTTCAGGT
        V  R  V  G  S  L  I  L  E  D  R  T  V  Y  D  L  P  S  G

1799  GGCCAGTTGCTGCTGGAACCACTGTGTCGCGACGACGGGCGGCTGTCGACGAGGGGTT
        G  Q  L  L  L  E  P  L  C  R  D  D  G  R  L  S  T  R  G

1857  TTGATGCACAACTGATCTTACTGAACAATGATTTATCTGGTGGTGTGCCGGAAATATT
        F  D  A  Q  L  I  L  L  N  N  D  L  D  G  G  V  P  E  I  L

1915  GGCAGGCCTGGAGCAACCTATTCTGCCCCCCCTCGCGGCAGGTTGGCGTAACCGGCGT
        A  G  L  E  Q  P  I  L  P  P  L  A  A  G  W  R  N  R  R

1973  AAATCCCAGCACTTCACCCATTACCGTGCGGTGGCACAGGAGCTGGCGGAGGTGATTG
        K  S  Q  H  F  T  H  Y  R  A  V  A  Q  E  L  A  E  V  I

2031  GCATCGATCCCTGGCTGATTGACCCCGTTTTTCGGCGTTGTCAGGGCATTGACTTCAT
        G  I  D  P  W  L  I  D  P  V  F  R  R  C  Q  G  I  D  F  M

2089  GCGCTCCGAAGGCCGCGAGTGCCTGGTTGCGAACGTGGATGCCGTACTGGAGATCACC
        R  S  E  G  R  E  C  L  V  A  N  V  D  A  V  L  E  I  T

2147  CGCGAGCGTTATGCCCACTATGGTATTCATCAGCGTCCTTTTGTGATCGTCAAGGCGG
        R  E  R  Y  A  H  Y  G  I  H  Q  R  P  F  V  I  V  K  A

2205  ATGCGGGTACTTACGGTATGGGTATTATGACCGCCTATTCGGGTGAGGAATTCCTTGA
        D  A  G  T  Y  G  M  G  I  M  T  A  Y  S  G  E  E  F  L  D

2263  CCTCAACCGTAAGGAACGCACCCGGATGGCGAAGAGTAAAGAGGGGTTGCCGGTCAGT
        L  N  R  K  E  R  T  R  M  A  K  S  K  E  G  L  P  V  S

2321  GACGTCTTTATTCAAGAGGGTGTATATACCTTCGAACAGACCGCGCAGGAGGCAGTCG
        D  V  F  I  Q  E  G  V  Y  T  F  E  Q  T  A  Q  E  A  V

2379  CAGAGCCGGTGGTCTATATGATCGGTCAGCAGGTGCTGGGAGGGTTTTACCGGGTGCA
      A  E  P  V  V  Y  M  I  G  Q  Q  V  L  G  G  F  Y  R  V  H

2437  TACGGAGCGTGGACGGGATGAGAATCTCAACGCTCCGGGTGCGCATTTTGAGCCAATG
        T  E  R  G  R  D  E  N  L  N  A  P  G  A  H  F  E  P  M

2495  GCCTTTGGCCAGACCTGCGTGCTCCCTTGCCGGAAGTCGCCACCGGATGCGCCGGCCA
        A  F  G  Q  T  C  V  L  P  C  R  K  S  P  P  D  A  P  A

2553  ATCGTTACTATGCCTATGGCGTCATCGCGCGCCTCACTCTGGTGGCGGCGGCCCGGGA
      N  R  Y  Y  A  Y  G  V  I  A  R  L  T  L  V  A  A  A  R  E

2611  AATGGCAGACTGGCGGCGGCAAGGTACACTGGAGACAGGCCAATGAGTGCGCTCAAGG
        M  A  D  W  R  R  Q  G  T  L  E  T  G  Q  *
```

Fig. 3.5 Nucleotide sequence from 154 bp upstream of *Cla*I site (2.4) to *Bam*HI site (0.0) and the deduced citrate synthase and γ-glutamylcysteine synthetase amino acid sequences. Putative ribosome binding sites are indicated in bold.

Fig. 3.6 shows the CODONPREFERENCE analysis of the sequence using a Tfchrom.cod codon usage data file generated from previously sequenced *T. ferrooxidans* genes. Two ORFs can be clearly seen in the second reading frame. Both ORFs show a codon preference and absence of rare codons which correlates to the Tfchrom.cod codon usage data file.

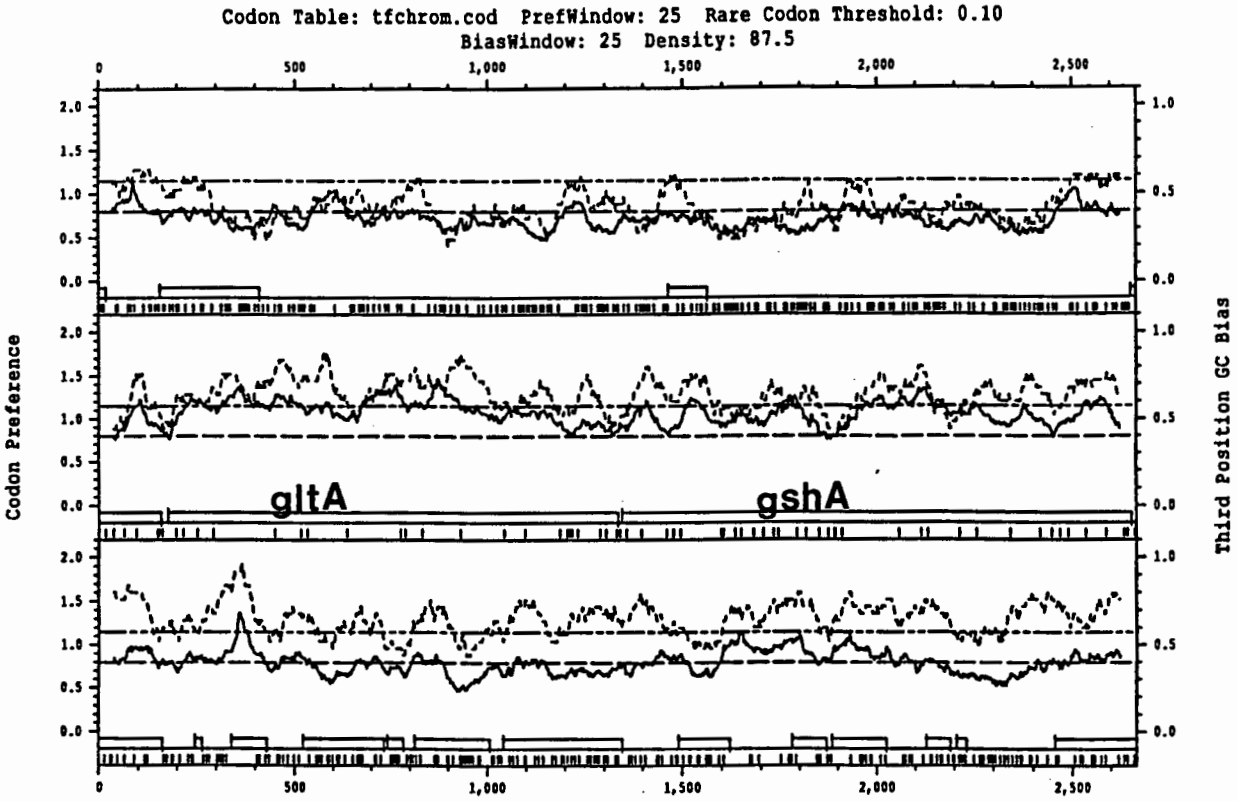


Fig. 3.6 Codonpreference analysis of the amino acid sequence from 154 bp upstream of the *Cla*I site (2.4) to the *Bam*HI site (0.0). The vertical axis shows the CODONPREFERENCE plots of all of the three forward frames, with ORFs and the positions of rare codons (defined as those codons which appear in the Tfchrom.cod codon usage data file at a frequency of less than 10%) drawn below as open boxes and vertical bars, respectively. Any plot above the dotted line in any of the three reading frames identifies a protein-coding sequence with a similar codon usage to the Tfchrom.cod data file.

Further evidence for the probability of the two ORFs coding for proteins was provided by analysis of the sequence data using the UWGCG application TESTCODE. TESTCODE produces a graph which predicts coding regions to a 95% level of confidence, represented by any plot above a level of 9.5 TESTCODE units. Fig. 3.7 shows the TESTCODE analysis in which both ORFs predicted from the CODONPREFERENCE application correspond to coding regions using the TESTCODE analysis.

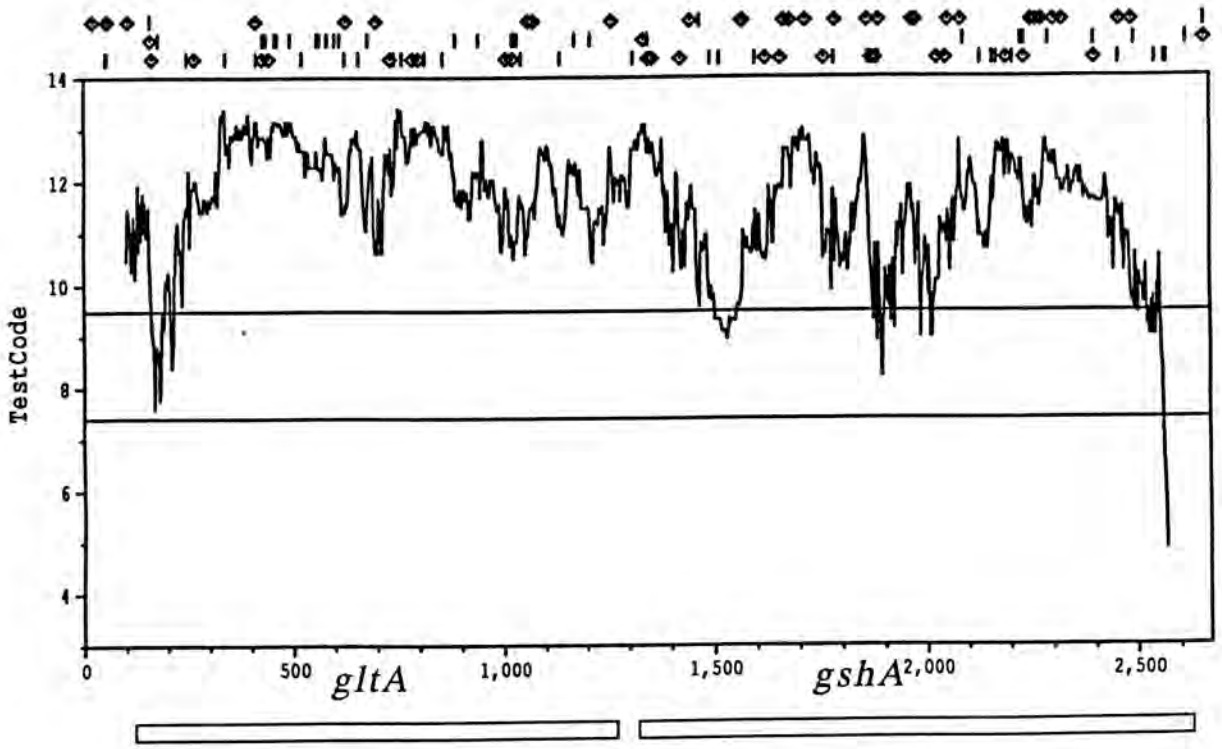
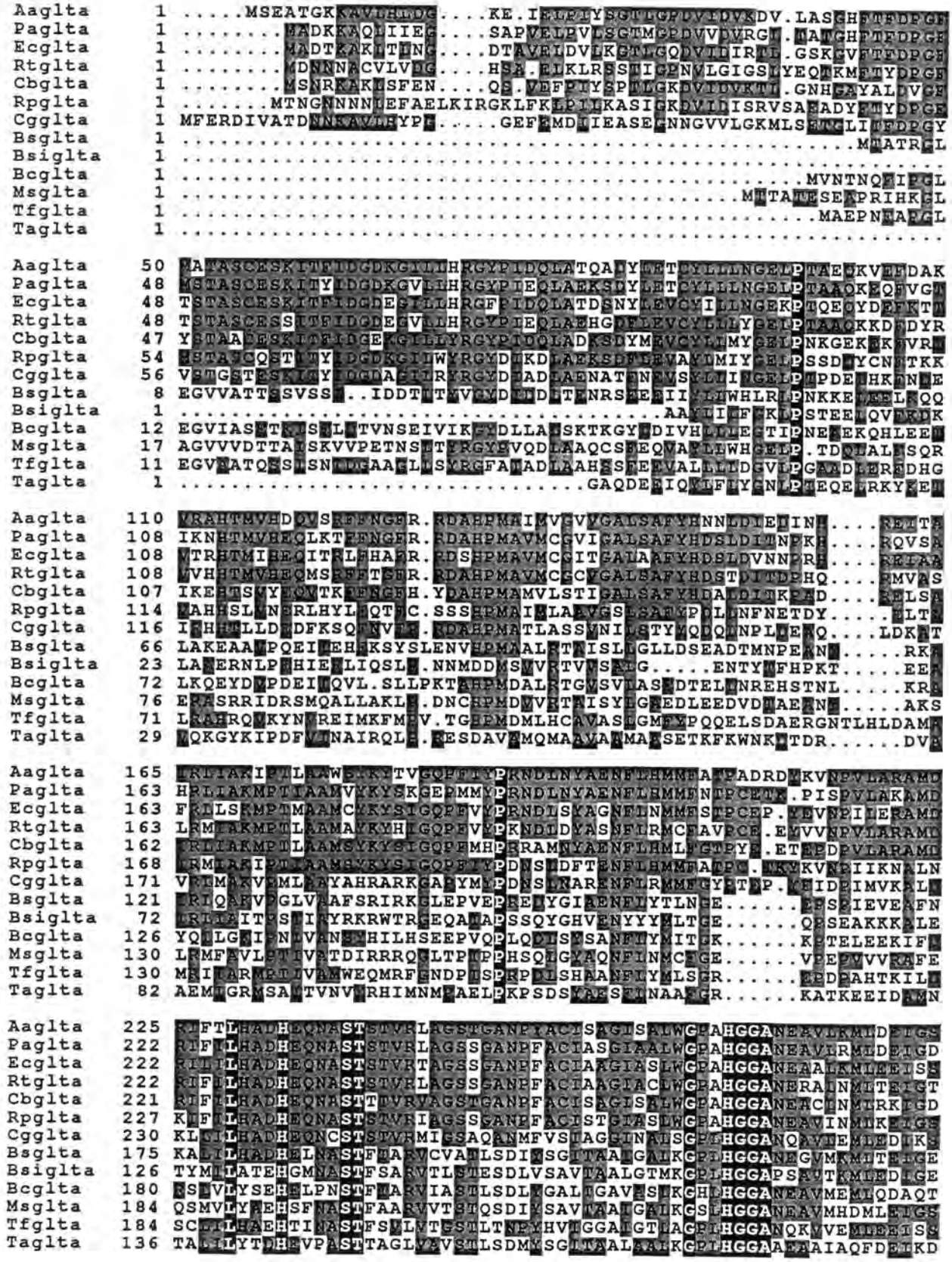


Fig. 3.7 TESTCODE analysis of the amino acid sequence from 154 bp upstream of the *Cla*I site (2.4) to the *Bam*HI site (0.0). The ORFs predicted from the nucleotide sequence are shown as open boxes. The vertical axis is in TESTCODE units, with two horizontal lines demarcating the three "windows" of probability for protein-coding sequences: < 7,5 units = low probability; 7.5-9.5 units = intermediate probability; >9.5 units = 95% probability.

The first ORF is preceeded by a strong ribosomal binding site and encodes a polypeptide of 386 amino acids, corresponding to a protein of approximately 43 kDa. The second ORF encodes a polypeptide of 436 amino acids, corresponding to a protein of 49 kDa. The predicted amino acid sequences were analysed for homology to sequences in the GenBank data base using the program NCBI BLAST and the first ORF was found to be closely related to a number of citrate synthases (37% identity to that of *E. coli* GenBank accession no. J01619). The predicted amino acid sequence of the *T. ferrooxidans* citrate synthase gene (*gltA*) was aligned with previously sequenced *gltA* gene products (Fig. 3.8), using the multiple sequence alignment UWGCG application PILEUP.



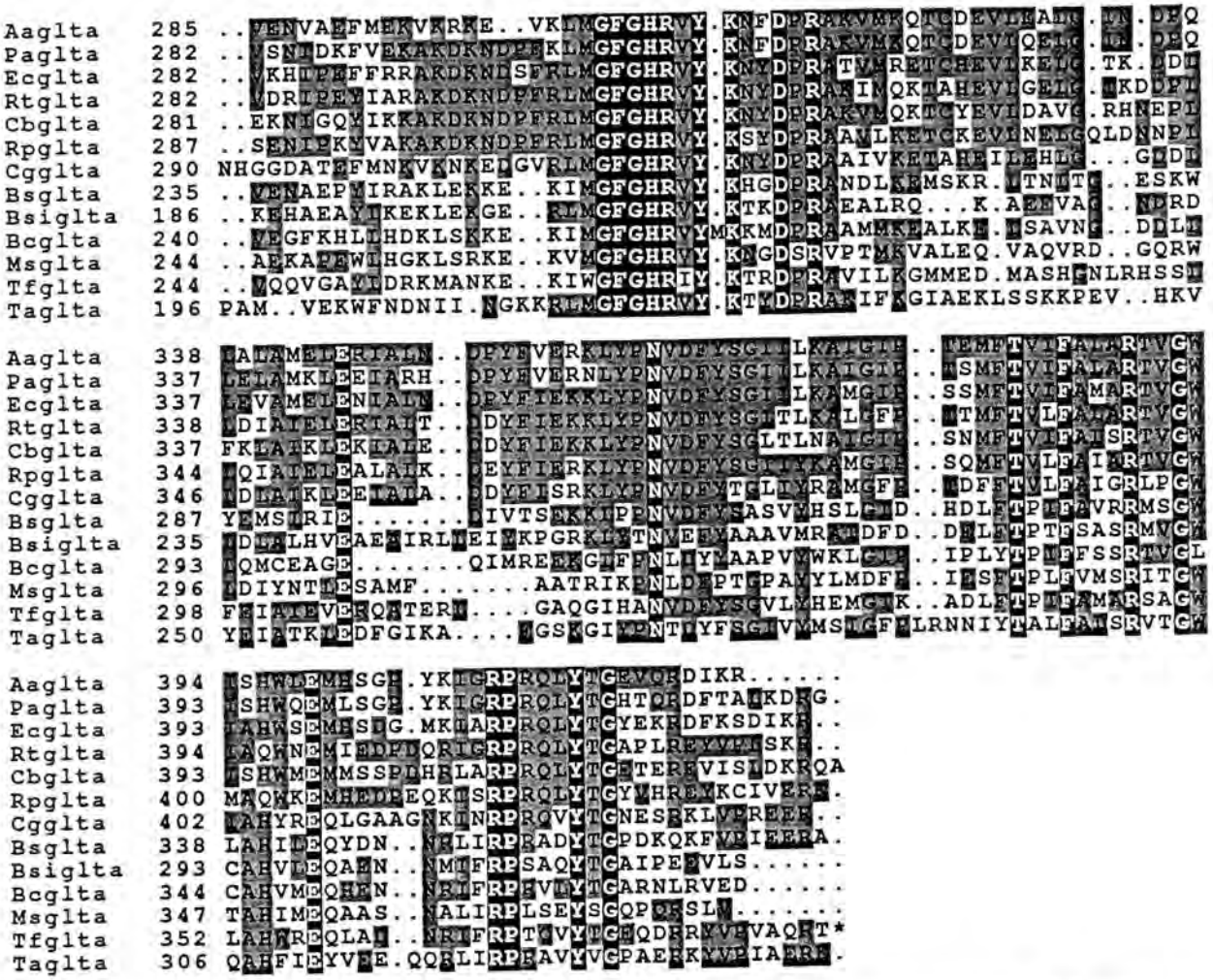


Fig. 3.8 Alignment of the deduced amino acid sequence of the *T. ferrooxidans gltA* gene with other *gltA* gene products. Aa = *Acetobacter anitratum* (GenBank no. M33037), Pa = *Pseudomonas aeruginosa* (GenBank no. M29728), Ec = *E. coli* (GenBank no. V01501), Rt = *Rhizobium tropici* (GenBank no. Z34516), Cb = *Coxiella burnetti* (GenBank no. M36338), Rp = *Rickettsia prowazekii* (GenBank no. M17149), Cg = *Corynebacterium glutamicum* (GenBank no. X66112), Bs = *Bacillus subtilis citZ* gene (GenBank no. U05257), Bsi = *B. subtilis citA* gene (GenBank no. U05256), Bc = *Bacillus coagulans* (GenBank no. M74818), Ms = *Mycobacterium smegmatis* (GenBank no. X60513) and Ta = *Thermoplasma acidophilum* (GenBank no. X55282).

Regions of high sequence homology can be seen, identifying the ORF as a *T. ferrooxidans* citrate synthase gene. The key position of citrate synthase as the first enzyme of the Kreb's cycle, has lead to it being studied in a number of different organisms. These studies have identified two main types of citrate synthase: Gram-positive and eukaryotic organisms possess "small" dimeric citrate synthases (M_r 100 kDa), which are inhibited by ATP and isosterically regulated, whereas Gram-negative bacteria in general have "large", hexameric forms (M_r 250 kDa) which are allosterically inhibited by NADH, and in facultative anaerobes, by a-ketoglutarate. However, the subunits of both citrate synthase types are approximately the same size (M_r 40-50 kDa) and display fairly high amino acid identity in some regions. Thus, although the *T. ferrooxidans* citrate synthase is clustered with Gram-positive bacteria such as the Bacilli, this may be due to similarity at the amino acid level only (Fig 3.9). Further analyses

would be required to determine whether the enzyme belongs to the "small", hexameric type found in Gram-negative bacteria or to the "large" dimeric type found in Gram-positive bacteria.

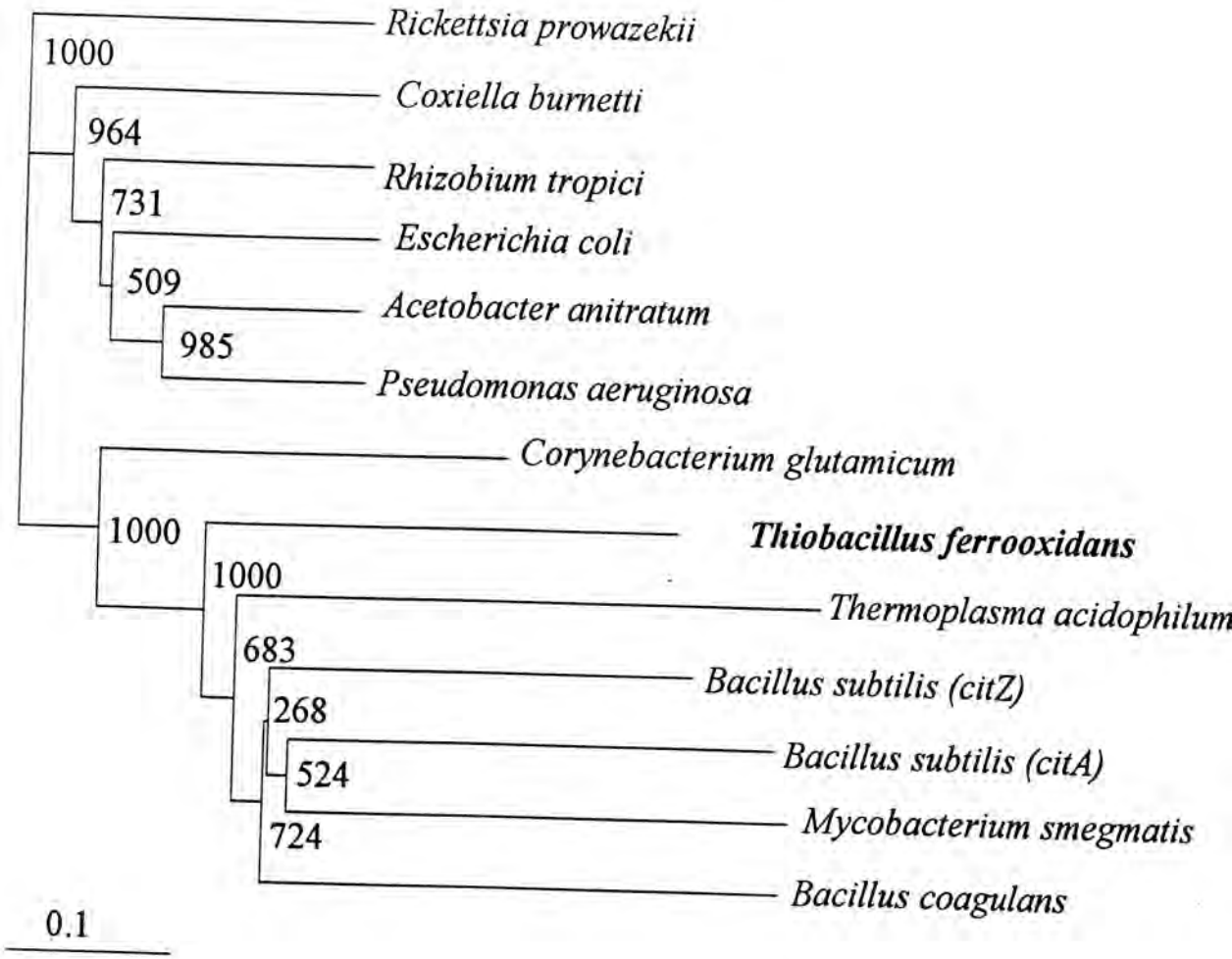


Fig. 3.9 A phylogram based on citrate synthase amino acid sequences. The software packages Clustal W and TreeView were used to calculate and draw the phylogram. Bootstraping values are indicated at the branch points (values are out of 1000). The scale of 0.1 indicates 10% amino acid divergence. The GenBank accession numbers are the same as those indicated in Fig. 3.8.

The percentage similarity and identity of the *T. ferrooxidans gltA* gene product to other *gltA* gene products is shown in Table 3.1.

Table 3.1. Percentage identity and similarity of *T. ferrooxidans gltA* predicted amino acid sequence to other *gltA* gene products. Values were determined using the UWGCG program GAP.

Bacterium	% Identity	% Similarity
B. subtilis citZ	42.59	60.11
C. glutamicum	41.69	62.80
R. prowazekii	39.37	59.58
A. anitratum	38.03	59.57
R. tropici	37.53	59.32
M. smegmatis	37.50	57.34
E. coli	37.23	59.04
B. subtilis citA	36.91	58.36
P. aeruginosa	36.74	58.00
C. burnetti	36.55	58.23
B. coagulans	35.95	60.27
T. acidophilum	32.74	56.25
S. cerevisiae[a]	25.73	52.26
Porcine[b]	24.60	49.20

[a] GenBank accession no. Z23259
[b] GenBank accession no. M21197

The second ORF showed no significant homology to any proteins in the GenBank data base using the program NCBI BLAST. Since the gene was known to complement an *E. coli gshA trxA* mutant, but not an *E. coli trxA* mutant, it was probable that the ORF was complementing the *gshA* mutation. The amino acid sequence was, therefore, compared to the amino acid sequence of the *E. coli gshA* gene, using the UWGCG application GAP (Fig. 3.10). The ORF was found to have 47% amino acid similarity and 18% amino acid identity to the *E. coli gshA* gene. This low level of homology was considered significant as the *gshA* genes are not highly homologous. A comparison of the % amino acid identities and similarities of the gene product of the second ORF to those of several other organisms was carried out using the UWGCG application GAP (Table 3.2). The rat and human GshA proteins are highly homologous (89 % identity), and the yeast protein is fairly homologous to the human and rat proteins (46 and 45% respectively). However, the *A. thaliana*, *E. coli* and *T. ferrooxidans* proteins differ widely, with identities ranging between 15 and 18% to the other γ-glutamylcysteine synthetase proteins.

```
T.f   MTEVIRDIPFLATDRVEALLDIERHLIVEQSTIERWFRGQWQRTPPPFYASVDL  54
E.c  LIPDVSQALAWLE.KHPQALKGIQRGL..ERETLRVNADGTLATTGHP.....EA  47

T.f RNAGFKLAPVDTNLFSAGFNNLNP.....EFSALYVSAIQHYLNQYHPGLERVL.  103
E.c LGSALTHKWITTDFAEALLEFITPVDGDIEHMLTFMRDLHRYTAR.NMGDERMLP  101

T.f .....LIPENHTRNL..FYLESVARLRELLELAGLDVRVGSLILEDRTVYDLPSG  151
E.c LSMPCYIAEGQDIELAQYGTSNTGRFKTLYR.EGLKNRYGALMQTISGVHYNFSL  155

T.f GQLLLEPLCRD......DGRLSTRGFDA.....QLILLNNDLSGGVPEILAGLEQ  195
E.c PMAFWQAKCGDISGADAKEKISAGYFRVIRNYYRFGWVIPYLFGASPAICSSFLQ  210

T.f PILPPLA.....................AGWRNRRKSQHFTHYRAVAQELAEV..  227
E.c GKPTSLPFEKTECGMYYLPYATSLRLSDLGYTNKSQSNLGITFNDLYEYVAGLKQ  265

T.f ...........IGIDP...........WLIDPVFRRCQGIDFMRSEGREC...L  256
E.c AIKTPSEEYAKIGIEKDGKRLQINSNVLQIENELYAPIRPKRVTRSGESPSDALL  320

T.f VANVD..AVLEITRERYAHYGIHQRPFVIVKADAGTYGMGIMTAYSGEEF..LDL  307
E.c RGGIEYIEVRSLDINPFSPIGVDEQQVRFLDLFMVWCALADAPEMSSSELACTRV  374

T.f NRKERTRMAKSKEGLPVSDVFIQEGVYTFEQTAQEAVAEPVVYMIGQQVLGGFYR  362
E.c NW.NRVILEGRKPGLTL.GIGCETAQFPLPQVGKD......LFRDLKRVAQTLDS  422

T.f VHTERGRDENLNAPGAHFEPMAFGQTC.VLPCRKSPPDA..PANRYYAYGVIARL  414
E.c INGGEAYQKVCDELVACFDNPDLTFSARILRSMIDTGIGGTGKAFAEAYRNLLRE  476

T.f TLVAAARE...MADW....RRQGTLETGQ.............  436
E.c EPLEILREEDFVAEREGFERRQQEMEAADTEPFAVWLEKHA  518
```

Fig. 3.10 Comparision of the amino acid sequences of the *T. ferrooxidans* (T.f.) and *E. coli* (E.c.) (GenBank X03954) γ-glutamylcysteine synthetase proteins.

Table 3.2 Amino acid identities and similarities between γ-glutamylcysteine synthetases from different species.

		% Similarity					
		Rat	Human	Yeast	*A. t.*	*E. c.*	*T. f.*
	Rat	-	97	63	44	42	46
	Human	94	-	65	44	43	43
% Identity	Yeast	45	46	-	44	46	45
	A. t	15	17	17	-	45	45
	E. c	15	15	16	16	-	47
	T. f	16	15	18	18	18	-

A. t = A. thaliana, E. c = E. coli, T. f = T. ferrooxidans. The GenBank numbers of the sequences used were as follows: Rat P19468, human M90656, *S. cerevisiae* D90220, *A. thaliana* Z29490 and *E. coli* X03954.

The Table in Appendix C shows the codon usage pattern of the *gshA* gene, the *gltA* gene and that of Tfchrom.cod, a codon usage file composed from 14 previously sequenced *T. ferrooxidans* genes. The codon usage of the *gshA* and *gltA* genes are similar to that of the other *T. ferrooxidans* genes and show a bias towards a G or a C in the "wobble" position.

The UWGCG program CORRESPOND is designed to identify similar patterns of codon usage by comparing codon frequency tables. The frequency which is compared is calculated by dividing the number of incidents of the codon in question by the total number of codons specifying the amino acid or terminator in each table. The lower the statistic (D), the more similar the patterns of codon usage.

Table 3.3. Comparison of the codon usage of the *T. ferrooxidans gshA* gene with that of *T. ferrooxidans* and *E. coli* genes, using the UWGCG program CORRESPOND[a]. Numbers represent the statistic D^2 as described in the text.

	Tfchrom.cod	Eco.low	Eco.high
TfgshA	0.12	1.24	1.77
TfgltA	2.12	1.18	4.11

a *TfgshA*, *TfgltA*, Ecol.low and Eco.high refer to the codon frequency tables of *T. ferrooxidans gshA*, *T. ferrooxidans gltA*, *E. coli* weakly expressed and *E. coli* highly expressed genes respectively. Tfchrom.cod is the composite codon usage frequency table of *T. ferrooxidans*.

The codon frequencies of the *T. ferrooxidans gshA* and *gltA* genes were compared to that of the composite codon frequency table for *T. ferrooxidans* (Tfchrom.cod) and to the codon frequency

tables for the highly expressed (UWGCG data file "eco.high") and weakly expressed (UWGCG data file "eco.low") *E. coli* genes. The codon usage of the *T. ferrooxidans gshA* gene was most similar to the *T. ferrooxidans* composite codon usage file (Tfchrom.cod) as would be expected (Table 3.3). The codon usage of the *gltA* gene, however, was most similar to that of the weakly expressed *E. coli* genes.

Analysis of portions of single-stranded sequence derived from subclones upstream of the *Cla*I-*Bam*HI fragment (see Fig. 3.1) using the NCBI BLAST application revealed homology to a number of pyruvate dehydrogenase complexes.

3.4.4.1. Analysis of partial, single-stranded sequence obtained upstream of the *gltA* gene.
Portions of sequence obtained upstream of the *Cla*I (2.4) site (Fig. 3.1) were compared directly to the amino acid sequences of the pyruvate dehydrogenase complex genes of *B. subtilis* and *E. coli*. Pyruvate dehydrogenase complexes consist of three components: the pyruvate decarboxylase component (E1), the dihydrolipoyl transacetlyase component (E2) and the dihyrolipoamide dehydrogenase component (E3). The E2 component forms the structural core of the complex, to which the peripheral subunits E1 and E3 bind (Oliver & Reed, 1982). Pyruvate dehydrogenases from Gram-negative bacteria have a core of 24 E2 subunits arranged with octahedral symmetry, whereas those from Gram-positive bacteria and eukaryotes have a larger E2 core of 60 subunits arranged with icosahedral symmetry (Mattevi *et al.*, 1992). The E1 component can be one of two forms. In the octahedral complex (Gram-negative bacteria), E1 exists as a homodimer, α_2, with a subunit molecular weight of approximately 100 kDa, whereas in the icosahedral complex it is found as a heterotetramer, $\alpha_2\beta_2$, with subunit molecular weights of approximately 41 and 36 kDa respectively. Partial *T. ferrooxidans* amino acid sequence obtained upstream of the *Cla*I (2.4) site (Fig. 3.1) was compared to that of pyruvate dehydrogenase complexes from a Gram-positive bacterium, *B. subtilis* (Fig. 3.11) and a Gram-negative bacterium, *E. coli* (Fig. 3.12). Interestingly, preliminary sequence of the *T. ferrooxidans* E1 component showed homology to the heterotetramer E1 component found in Gram-positive bacteria (Fig. 3.11a) and eukaryotes and no homology to the E1 component of *E. coli* (Fig. 3.12a). The E2 component is well conserved amongst all pyruvate dehydrogenase complexes, and the E2 component of *T. ferrooxidans* shows homology to both that of *B. subtilus* (Fig. 3.11b) and of *E. coli* (Fig. 3.12b). The E3 component is also homologous to both *B. subtilus* (Fig. 3.11c) and *E. coli* (Fig. 3.12c).

```
Bs  MAAKTKKAIVDSKKQFDAIKKQFETFQILNEKGEVVNEAAMPDLTDDQLKELMRRMVFTRVL
Tf

Bs  DQRSISLNRQGRLGFYAPTAGQEASQIATHFALEKEDFVLPGYRDVPQLIWHGLPLYQAFLF
Tf              GFLHLYPGEEAVHRCAGEGSTGSDYVVTGYRDHIH           ELF

Bs  SRGHFRGNQMPDDVNALSPQIIIGAQYIQTAGVALGLKKRGKKAVAITYTGDGGRSQGDFYE
Tf  GKETGCSKGRGGSMHLFDPDVHFMGGYHWSAAPARWTAGFAKAYQLRGSKEISYRFLGDGRQ

Bs  GINFAGAYKAPAIFVVQNNRYAISTPVEKQSAAETIAQKAVAAGIVGVQVDGMDPLAVYAAT
Tf  TFHE

Bs  AEARERAINGEGPTLIETLTFRYGPHTMAGDDPTKYRTKEIENEWEQKDPLVRFRAFLENKG
Tf              PYFLELMTYRLRGHSMS--DSGAYRTKEEVEEWAQSRSHRHLQAP

Bs  LWSEEEEAKVIEDAKEEIKQAIKKADAEPKQKVTDLMKIMYEKMPHNLEEQFEIYTQKESK
Tf
```

Fig. 3.11a(i) Amino acid sequence of the pyruvate decarboxylase gene (*pdhA*), E1α subunit, of the *Bacillus subtilis* pyruvate dehydrogenase complex.

```
Bs  MAQMTMIQAITDALRTELKNDENVLVFGEDVGVNGGVFRATEGLQKEFGEDRVFDTPLAESG
Tf        RESCGSAHDEEMAAGPPGFCHGGYRVAGGTY-KATSGLFAKYGEQRVIDTPISENS

Bs  IGGLALGLGLNGFRPVMEIQFFGFVYEVMDSVSGQMARMRYRSGGRWTSPVTIRSPFGGGVH
Tf  YTGIGVGAAMIGARPIVEIMSVNFAWLAMDQLMNNAAKIHYMCGGRMRCAFVMF

Bs  TPELHADSLEGLVAQQPGIKVVIPSTPYDAKGLLISAIRDNDPVVFLEHMKLYRSFRQEVPE
Tf

Bs  EEYTIELGKADVKREGTDLSIITYGAMVHESLKAADELEKDGISAEVVDLRTVSPLDIDTII
Tf

Bs  ASVEKTGRAIVVQEAQKQAGIAANVVAEINDRAILSLEAPVLRAAPDTVFPFSQAESVWLL
Tf

Bs  PNHKDVETARKVLEF
Tf
```

Fig. 3.11a(ii) Amino acid sequence of the pyruvate decarboxylase gene (*pdhB*), E1β subunit, of the *B. subtilis* pyruvate dehydrogenase complex.

```
Bs  MAFEFKLPDIGEGIHEGEIVKWFVKPNDEVDEDDVLAEVQNDKAVVEIPSPVKGKVLELKVE
Tf  EGYAVKMPQLSDTMTEGVLVSWEKAPGDRIQRGDVVATVETDKAIMDVEVFREDVEVFREDT

Bs  EGTVATVGQTIITFDAPGYEDLQFKGSDESDDAKTEAQVQSTAEAGQDVAKEEQAQEPAKAT
Tf  CRSFGGRGCGGARRDGHRWLVESPEQVSHE

Bs  GAGQQDQAEVDPNKRVIAMPSVRKYAREKGVDIRKVTGSGNNGRVVKEDIDSFVNGGAQEAA
Tf                                  DINGLRGTGPAGVIVAADVLGAAGGRAAPVA

Bs  PQETAAPQETAAKPAAAPAPEGEFPETREKMSGIRKAIAKAMVNSKHTAP-HVTLMDEVDVT
Tf  SSGTAEPAVPG---NAAPRPSKERSA--RAMTAIERAISQAMAASLSIPVFHVTVQVRPDA
```

```
Bs  NLVAHRKQFKQVAADQGIKLTYLPVYVVKALTSALKKFPVLNTSIDDKTDEVIQKHYFNIGI
Tf

Bs  AAADTEKGLLVPVVKNADRKSVFEISDEINGLATKAREGKLAPAEMKGASCTITNIGSAGGQ
Tf              VLRGVEGKTPEQLQTEWTSLLEKARKRRLSPPEYTNPTFTISNMGMYALP

Bs  WFTPVINHPEVAILGIGRIAEKAIVRDGEIVAAPVLALSLSFDHRMIDGATAQNALNHIKRL
Tf  SSMP                                    MPITITADHRVVNGAEAALFLNDLKQA

Bs  LNDPQLILMEA
Tf  IEKPENWLGSG
```

Fig. 3.11b Amino acid sequence of the dihydrolipoyl transacetylase gene (*pdhC*), subunit E2, of the *B. subtilis* pyruvate dehydrogenase complex.

```
Bs  MVVGDFPIETDTLVIGAGPGGYVAAIRAAQLGQKVTVVEKATLGGVCLNVGCIPSKALINAG
Tf  PVIPEGDYDVQVLVIGAGPVVEDCAR                        CLWRGCIPSKAWRAAA

Bs  HRYENAKHSDDMGITAENVTVDFTKVQEWKASVVNKLTGGVALLKGNKVDVVKGEAYFVDSN
Tf  DRIRPGARR-AMGITLGTPRLDWAQLEQHRRG

Bs  SVRVMDENSAQTYTFKNAIIATGSRPIEPNFKYSERVLNSTGALALKEIPKKLVVIGGYIGT
Tf                    VVIATGAPAFVPPIPIPCIQDALKSGAAVTSDTVWNLK

Bs  ELGTAYANFGTELVILEGGDEILPGFEKQMSSLVTRRLKKKGNVEIHTNAMKGVEERPDGVT
Tf

Bs  VTFEVKGEEKTVDADYVLITVGRRPNTDELGLEQVGIEMTDRGIVKTDKQCRTNVPNIYAIG
Tf                          GKRPDTSGLNLLAAGVALGDRAVIASMCSGRTNVPHIYAVG

Bs  DIIEGPPLAHKASYEGKIAAEAIAGEPAEIDYLGIPAVVFSEPELASVGYTEAQAKEEGLDI
Tf  DVIGGYMLAHTAGQQGRVAAASLLGHSARYEAAKDCGVTFTRPQCAFVGLSLEQARAEGIDA

Bs  VAAKFPFAANGRALSLNETDGFMKLITRKEDGLVIGAQIAGASASDMISELSLAIEGGMTAE
Tf  VEVKVPLSIDAKAMMTGETDGLIKIVADKISHRIVGVHFLADHADTLVGEA

Bs  DIAMTIHAHPTLGEITMEAAEVAIGSPIHIVK
Tf
```

Fig. 3.11c Amino acid sequence of the dihydrolipoamide dehydrogenase gene (*pdhD*), subunit E3, of the *B. subtilis* pyruvate dehydrogenase complex.

```
Ec  MSERFPNDVDPIETRDWLQAIESVIREEGVERAQYLIDQLLAEARKGGVNVAAGTGISNYIN

Ec  ISNYINTIPVEEQPEYPGNLELERRIRSAIRWNAIMTVLRASKKDLELGGHMASFQSSATIY

Ec  DVCFNHFFRARNEQDGGDLVYFQGHISRGVYARAFLEGRLTQEQLDNFRQEVHGNGLSSYPH

Ec  PKLMPEFWQFPTVSMGLGPIGAIYQAKFLKYLEHRGLKDTSKQTVYAFLGDGEMDEPESKGA

Ec  ITIATREKLDNLVFVINCNLQRLDGPVTGNGKINELEGIFEGAGWNVIKVMWGSRWDELLRK

Ec  DTSGKLIQLMNETVDGDYQTFKSKDGAYVREHFFGKYPETAALVADWTDEQIWALNRGGHDP

Ec  KKIYAAFKKAQETKGKATVILAHTIKGYGMGDAAEGKNIAHQVKKMNMDGVRHIRDRFNVPV

Ec  SDADIEKLPYITFPEGSEEHTYLHAQRQKLHGYLPSRQPNFTEKLELPSLQDFGALLEEQSK

Ec  EISTTIAFVRALNVMLKNKSIKDRLVPIIADEARTFGMEGLFRQIGIYSPNGQQYTPQDREY

Ec  KEDEQVAYKGQILQEGINELGAGCSWLAAATSYSTNNLPMIPFYIYYSMFGFQRIGDLCWAA

Ec  GDQQARGFLIGGTSGRTTLNGEGLQHEDGHSHIQSLTIPNCISYDPAYAYEVAVIMHDGLER

Ec  MYGEKQENVYYYITTLNENYHMPAMPEGAEEGIRKGIYKLETIEGSKGKVQLLGSGSILRHV

Ec  REAAEILAKDYGVGSDVYSVTSFTELARDGQDCERWNMLHPLETPRVPYIAQVMNDAPAVAS

Ec  TDYMKLFAEQVRTYVPADDYRVLGTDGFGRSDSRENLRHHFEVDASYVVVAALGELAKRGEI

Ec  DKKVVADAIAKFNIDADKVNPRLA
```

Fig. 3.12a Amino acid sequence of the pyruvate decarboxylase gene (*aceE*), E1 subunit, of the E. *coli* pyruvate dehydrogenase complex.

```
Ec  MAIEIKVPDIGADEVEITEILVKV--GDKVEAEQSLITVEGDKASMEVPSPQAGIVKEIKVS
Tf  EGYAVKMPQLSDTMTEGVLVSWEKAPGDRIQRGDVVATVETDKAIMDVEVFREDTCRSFGGR

Ec  VGDKTQTGALIMIFDSADGAADAAPAQAEEKKEAAPAAAPAAAAAKDVNVPDIGSDEVEVTE
Tf  GCGGARRD

Ec  ILVKVGDKVEAEQSLITVEGDKASMEVPAPFAGTVKEIKVNVGDKVSTGSVFEVAGEAGAAA
Tf  WEKAPGDRIQRGDVVATVETDKAIMDVECSVRIPVG                FRHGGDRKAAS

Ec  PAAKQEAAPAAAPAPAAGVKEVNVPDIGGDEVEVTEVMVKVGDKVAAEQSLITVEGDKASME
Tf  QALHRHPLVNAAYQPVDKIVERSQHDIGIG

Ec  AKVPAPFAGVVKELKVNVGDKVKTGSLIMIFEVEGAAPAAAPAKQEAAAPAPAAKAEAPAAA
Tf

Ec  PAAEGKSEFAENDAYVHATPLIRRLAREFGVNLAKVKGTGRKGRILREDVQAYVKEAIKRAE
Tf

Ec  AAPAATGGGIPGMLPWPKVDFSKFGEIEEVELGRIQKISGANLSRNWVMIPHVTHFDKTDIT
Tf

Ec  ELEAFRKQQNEEAAKRKLDVKITPVVFIMKAVAAALEQMPRFNSSLSEDGQRLTLKKYINIG
Tf

Ec  VAVDTPNGLVVPVFKDVNKKGIIELSRELMTISKKARDGKLTAGEMQGGCFTISSIGGLGTT
Tf                VLRGVEGKTPEQLQTEWTSLLEKARKRRLSPPEYTNPTFTISNMGMYALP
```

```
Ec  HFAPIVNAPEVAILGVSKSAMEPVWNGKEFVPRLMLPISLSFDHRVIDGADGARFITIINNT
Tf  SSMP                                        MPITITADHRVVNGAEAALFNDLKQAI

Ec..LSDIRRLVM
Tf  EHPENWLGS
```

Fig. 3.12b Amino acid sequence of the dihydrolipoyl transacetylase gene(*aceF*), subunit E2, of the *E. coli* pyruvate dehydrogenase complex

```
Ec  MMSTEIKTQVVVLGAGPAGYSAAFRCADLGLETVIVERYNTLGGVCLNVGCIPSKALLHVA
Tf  IPEGDYDVQVLVIGAGPGGEDCARE                          CLWRGCIPSKAWRAAA

Ec  KVIEEAKALAEHGIVFGEPKTDIDKIRTWKEKVINQLTGGLAGMAKGRKVKVVNGLGKFTG
Tf  DRIRPCARRA-MGITLGTPRLDWAQLEQHRRG

Ec  ANTLEVEGENGKTVINFDNAIIAAGSRPIQLPFIPHEDPRIWDSTDALELKEVPERLLVMGG
Tf                        VVIATGAPAFVPPIPGIQDALKSGAAVTSDTVWNLK

Ec  GIIGLEMGTVYHALVVEMFDQVIPAADKDIVKVFTKRISKKFNLMLETKVTAVEAKEDGIYV
Tf

Ec  TMEGKKAPAEPQRYDAVLVAIGRVPNGKNLDAGKAGVEVDDRGFIRVDKQLRTNVPHIFAIG
Tf                        ATGKRPDTSGLNLLAAGVALGDRAVIASMCSGRTNVPHIYAVG

Ec  DIVGQPMLAHKGVHEGHVAAEVIAGKKHYFDPKVIPSIAYTEPEVAWVGLTEKEAKEKGISY
Tf  DVIGGYMLAHTAGQQGRVAAASLLGHSARYEAAKDCGVTFTRPQCAFVGLSLEQARAEGIDA

Ec  ETATFPWAASGRAIASDCADGMTKLIFDKESHRVIGGAIVGTNGGELLGEIGLAIEMGCDAE
Tf  VEVKVPLSIDAKAMMTGETDGLIKIVADKISHRIVGVHFLADHADTLVGEA

Ec  DIALTIHAHPTLHESVGLAAEVFEGSITDLPNPKAKKK
Tf
```

Fig. 3.12c Amino acid sequence of the lipoamide dehydrogenase gene (*lpd*), subunit E3, of the *E. coli* pyruvate dehydrogenase complex.

3.4.5. *In vitro* synthesis of *gltA*, *gshA* and possible pyruvate dehydrogenase genes.

To establish the correlation between a predicted ORF, derived from sequence analysis, and the polypeptide produced, protein products from various subclones of cosmid 5.1 were examined using an *E. coli*-derived *in vitro* transcription-translation system (Fig. 3.13). A protein of approximately 49 kDa, corresponding to the GshA protein, was produced from cosmid 5.1 and plasmids pTHIOD and pTHIO7 (Fig. 3.13 lanes 1, 5 and 7) and a protein of approximately 43 kDa, corresponding to the GltA protein, was produced from cosmid 5.1 and plasmids pTHIOD and pTHIO28 (Fig. 3.13 lanes 1, 5 and 6).

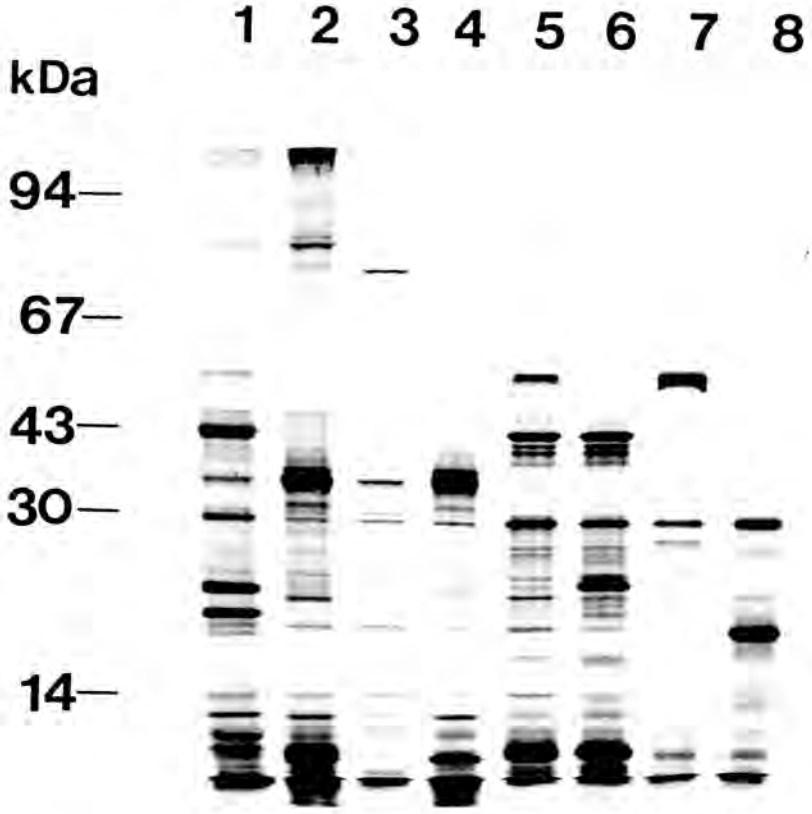


Fig. 3.13 SDS-PAGE analysis of proteins expressed from cosmid 5.1, plasmid pTHIO81, pTHIO51, pTHIO191, pTHIOD, pTHIO28, pTHIO7 and pBluescriptSK (lanes 1 to 8 respectively) using an *E. coli*-derived *in vitro* transcription/translation system.

Proteins of approximately 110 kDa, 80 kDa, 49 kDa, 43 kDa, 36 kDa, 34 kDa, 25 kDa, 22 kDa, 19 kDa and about 6 proteins smaller than 14 kDa were produced by cosmid 5.1 (Fig. 3.13 lane 1) and by the various plasmid subclones analysed, but not by cosmid vector pHC79 (unpublished results). There is insufficient data to clearly identify the location of the genes encoding all these proteins. However, it is possible to identify the approximate location of some of the genes. The 110 kDa and 80 kDa proteins were produced by cosmid 5.1 and plasmid pTHIO81, but not by plasmids pTHIO51, pTHIO191, pTHIOD, pTHIO28, pTHIO7, pBluescriptSK or pTHIO61 (unpublished results). These results indicate that the starts of the genes encoding the 110 kDa and 80 kDa proteins probably lie between the *Apa*I (6.7) and the

the *Hin*dIII (4.9) sites (Fig. 3.1). The 80 kDa protein could be a degradation product of the 110 kDa protein. A protein of approximately 36 kDa was produced by cosmid 5.1 and plasmids pTHIO81 and pTHIO191 only. The gene encoding this protein must, therefore, lie between the *Sac*II (9.3) and *Apa*I (6.7) sites (Fig. 3.1), as this is the overlapping region of DNA between plasmids pTHIO81 and pTHIO191. The 34 kDa protein was only produced by cosmid 5.1, plasmids pTHIO81, pTHIO51 and pTHIO191, thus the gene encoding this protein must lie between the *Sac*I (7.6) and *Apa*I (6.7) sites. Plasmid pTHIO51 also produced a protein of about 69 kDa which may be a truncated form of either the 110 kDa or 80 kDa proteins or is encoded by a gene lying between the *Sac*I (7.6) and *Sac*I (4.2) sites (Fig. 3.13 lane 3). The 30 kDa protein produced by cosmid 5.1 and all the plasmids corresponds to the β-lactamase protein. It is not possible to clearly identify the positions of the genes encoding the proteins smaller than 30 kDa. Some of the proteins produced may be artefacts of the *in vitro* transcription/translation system.

3.4.6. Complementation of the *gshA* phenotype.

E. coli strain BH5262 is unable to grow on minimal medium lacking glutathione, whereas GshA$^+$ or TrxA$^+$ colonies can (Lim *et al.*, 1986). Plasmids pTHIOD and pTHIO7 complemented the *E. coli gshA trxA* double mutant. In comparison plasmids pTHIO1 and pTHIO15 weakly complemented *E. coli* BH5262, whilst plasmids pTHIO5, pTHIO9, pH29 and the vectors pHC79 and pBluescriptSK were unable to complement the mutant. Plasmid pTHIO1 (not shown in Fig. 3.1) is the same subclone as pTHIO7, but the insert lies in the opposite orientation with respect to the *lac* promoter of the vector. This result together with the observation that pTHIO5, which contains the entire *gshA* gene but lacks the start of the *gltA*, produced a GshA$^+$ phenotype is evidence that the *gshA* gene was operating off the *lac* promoter of the vector in the pTHIO7 construct.

3.4.7. Complementation of the *gltA* phenotype

Various plasmids containing the *T. ferrooxidans* citrate synthase gene were tested for the ability to complement the *gltA*$^-$ phenotype of *E. coli* strain MOB150. *E. coli* strain MOB150 is unable to grow on minimal medium lacking glutamate whereas GltA$^+$ colonies can (Wood *et al.*, 1987). Plasmid pMW264, which contains the *Rickettsia prowazekii* citrate synthase gene, was used as a positive control. Plasmids pMW264, pTHIO6, pTHIO28 and pTHIO82 were able to complement the *E. coli gltA* mutant, while the vector, pBluescriptSK, did not. Cosmid 5.1 complemented the *E. coli gltA* mutant very weakly. As pTHIO82 and pTHIO28 lie in opposite orientations with respect to the vector *lac* promoter, the *gltA* gene of *T. ferrooxidans* appears to be able to operate off its own promoter. Plasmids pMW264, pTHIO6, pTHIO28 and pTHIO82 were reisolated from *E. coli* MOB150, and retransformed into fresh *E. coli* MOB150 competent cells, to ensure that the GltA$^+$ was not due to a mutation in the host strain. All four plasmids were able to complement *E. coli gltA* mutant on retransformation, whilst the negative control pBluescriptSK was not.

3.4.8. Complementation of the pyruvate dehydrogenase complex

The pyruvate dehydrogenase complex catalyses the following reaction:

pyruvate + CoA + NAD $\rightarrow$ acetyl-CoA + NADH + CO_2

Firstly, pyruvate decarboxylase converts pyruvate to hydroxyethylthiamine pyrophosphate. Then the transacetylase shifts the two-carbon fragment from thiamine pyrophosphate to lipoic acid and thence to coenzyme A, forming acetyl-coenzyme A. Lastly, the flavoprotein dihydrolipoamide dehydrogenase reoxidises the reduced form of lipoic acid. Citrate synthase then catalyses the Claisen condensation of oxalacetate and acetyl-CoA to form citrate (Rawn, 1983).

E. coli strain JRG1342 is an *aceEF-lpd* deletion mutant, and therefore lacks the pyruvate decarboxylase, dihydrolipoyl transacetylase and dihydrolipoamide dehydrogenase activities. It is thus unable to grow on minimal medium lacking acetate (Guest *et al.*, 1983; Guest & Lewis, 1985). However, *E. coli* JRG1342 transformants containing the pyruvate dehydrogenase complex can grow on minimal medium lacking acetate. In *E. coli* the product of the *lpd* gene, lipoamide dehydrogenase, also functions as the E3 component of the analogous α-ketoglutarate dehydrogenase multienzyme complex. This multienzyme complex catalyses the conversion of α-ketoglutarate to succinyl-coenzyme A. The Lpd^+ phenotype confers succinate-independence (but not acetate-independence) on glucose minimal medium as *E. coli* JRG1342 Lpd^+ colonies are able to form a functional α-ketoglutarate dehydrogenase multienzyme complex. The results of the complementation of the *E. coli pdhR-lpd* mutant JRG1342 are shown in Table 3.4.

Different carbon sources were used in the M9 minimal medium, as indicated. Plasmid pGS87 contained the cloned *E. coli pdhR-lpd* region and was used as a positive control. The data in Table 3.4, indicated that cosmid 5.1 and plasmid pTHIO81 were able to complement the *E. coli pdhR-lpd* mutant (shown by the ability to utilise glucose as the sole carbon source), whereas plasmids pBluescriptSK and pTHIO251 were not. The *T. ferrooxidans lpd* gene product, lipoamide dehydrogenase, also appeared to be functional, as the *E. coli pdhR-lpd* mutant was able to grow in the absence of succinate. This indicated that the cloned *T. ferrooxidans* pyruvate dehydrogenase complex lies between the *Sac*II and *Bgl*II sites of cosmid 5.1, and is functional in *E. coli*. The orange pigmented colonies obtained on growing *E. coli* JRG1342 containing pGS87, cosmid 5.1 and pTHIO81 on minimal media with succinate as the sole carbon source were not contaminants (tested for lysis by T4 phage). When the experiment was repeated twice, colonies with the same orange appearance grew on each occasion.

Table 3.4. Genetic complementation of *E. coli pdhR-lpd* mutant, JRG1342.

	Carbon Source				
Plasmid	**G+A+S**	**G+A**	**G**	**S+A**	**S**
None	++++	++++	-	+++	-
pSK	++++	++++	-	+	-
pGS87	++++	++++	++++	+++	+++*
cosmid5.1	++++	++++	+++	+++	+*
pTHIO81	++++	++++	+++	+	+*
pTHIO251	+++	+++	-	+	-

G+A+S = 10mM glucose, 2mM acetate, 2mM succinate; G+A = 10mM glucose, 2mM acetate; G = 10mM glucose; A+S = 4 mM acetate, 50 mM succinate; S = 50 mM succinate; ++++ = normal growth; + = very poor growth; - = no growth; +* = growth of orange pigmented colonies after 6-7 days; +++* = growth of orange pigmented colonies after 2 -3 days.

3.4.9. Transcript analysis

To determine whether the γ-glutamylcysteine synthetase gene, the citrate synthase gene and the pyruvate dehydrogenase genes were part of an operon or were independently transcribed total RNA isolated from *E. coli* JM109, *E. coli* JM109(cosmid 5.1) and *T. ferrooxidans* cells was examined by RNA:DNA hybridisation. The transcripts were probed with probes specific to the *gshA* gene, the *gltA* and the *aceF* gene (transacetylase gene of the pyruvate dehydrogenase complex). With all three probes a small quantity of transcript of about 9 kbp was obtained for RNA from *T. ferrooxidans* cells (lane 1 Figs 3.14, 3.15 and 3.16). RNA obtained from *T. ferrooxidans* cells probed with the *gshA* and *gltA*-specific probes also gave a transcript of about 2.4 kbp. RNA derived from *E. coli* JM109(cosmid 5.1) gave a small quantity of transcript of about 4 kbp and a strong transcript of about 2.4 kbp for both probes (Fig. 3.14, lanes 2 and 4; Fig. 3.15 lanes 2 and 4). RNA derived from *E. coli* JM109(cosmid 5.1) probed with the *aceF*-specific probe gave a transcript of approximately 4.0 kbp (Fig. 3.16 lane 4). Smaller RNA transcripts which probably resulted from degradation were detected with all three probes and with both sources of RNA. No transcripts were detected from RNA obtained from *E. coli* JM109, the negative control. The major transcript in both *T. ferrooxidans* and *E. coli* is about 2.4 kbp. This transcipt size is sufficient to encompass both the *gshA* and *gltA* genes suggesting that they may be transcriptionally linked in both *E. coli* and *T. ferrooxidans*. The 9 kbp

transcript visible in the RNA preparation from *T. ferrooxidans* (lane 1, Figs 3.14, 3.15 and 3.16) suggests transcriptional linkage of the *aceE*, *aceF*, *lpd*, *gltA* and *gshA* genes.

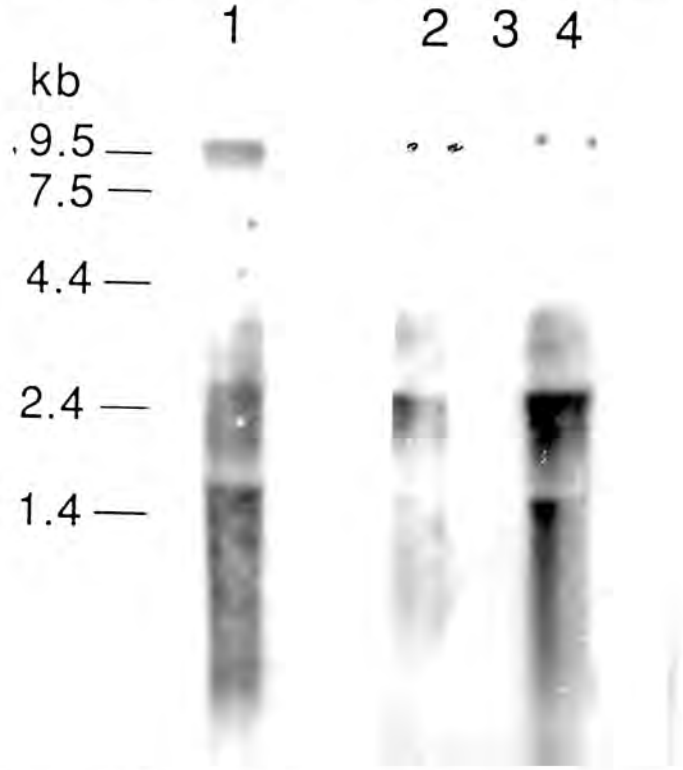


Fig. 3.14 Hybridisation of labelled *Bam*HI-*Sal*I fragment of pTHIO7 to total RNA prepared from: lane 1, *T. ferrooxidans*; lane 2, *E. coli* JM109(cosmid 5.1); lane 3, *E. coli* JM109; lane 4, *E. coli* JM109(cosmid 5.1).

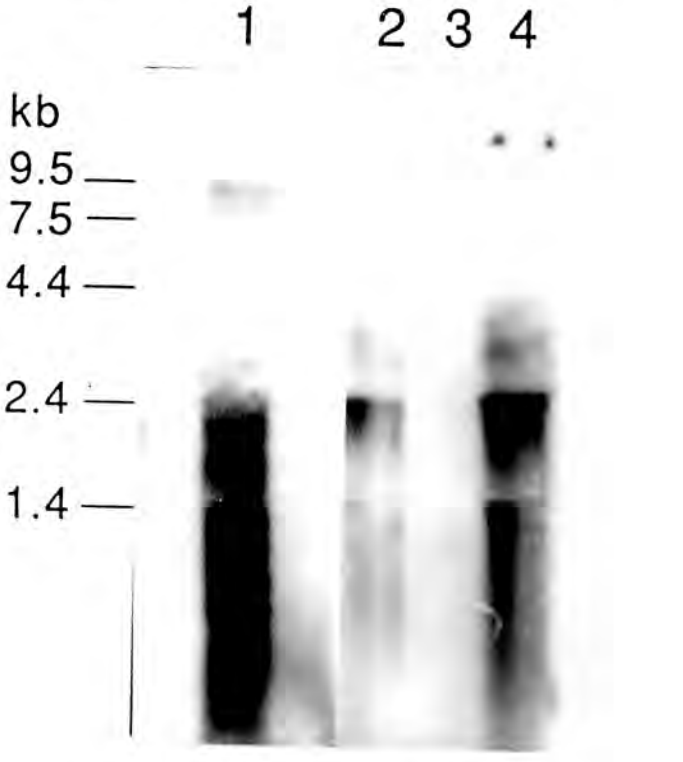


Fig. 3.15 Hybridisation of labelled *Bam*HI-*Sma*I fragment of pTHIO28 to total RNA prepared from: lane 1, *T. ferrooxidans*; lane 2, *E. coli* JM109(cosmid 5.1); lane 3, *E. coli* JM109; lane 4, *E. coli* JM109(cosmid 5.1).

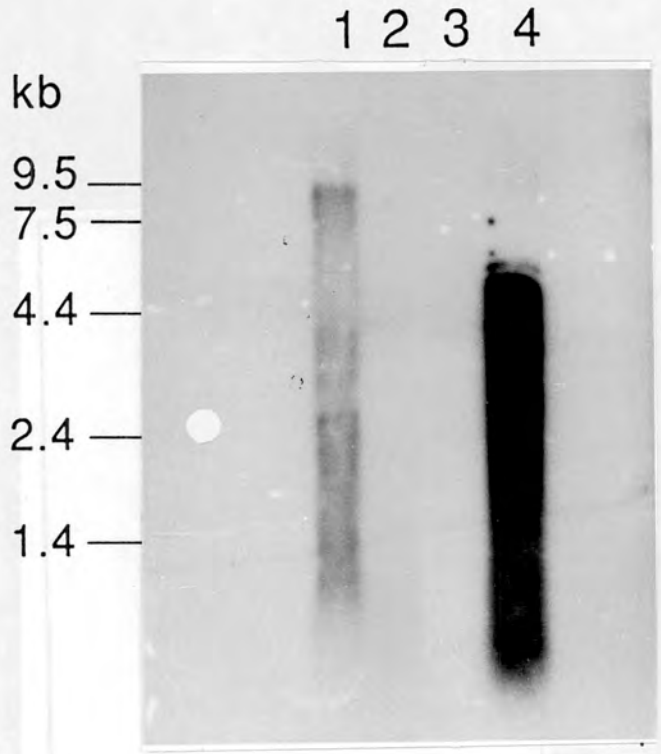


Fig. 3.16 Hybridisation of labelled *Hin*dIII-*Apa*I fragment from pTHIO52 to total RNA prepared from: lane 1, *T. ferrooxidans*; lane 2, *E. coli* JM109; lane 3, *E. coli* JM109; lane 4, *E. coli* JM109(cosmid 5.1).

3.4.10. Glutathione assay

GSH was shown to react specifically with o-opthalaldehyde at pH 8.0, yielding a highly fluorescent product that could be activated at 350nm with an emission peak at 420nm (Hissin & Hilf, 1976). Extracts from *E. coli* BH5262(pTHIO7) were shown to contain GSH concentrations comparable to the wild type strain *E. coli* MC1061 and much higher concentrations than *E. coli* BH5262 (Table 3.5). These results indicate that the cloned *T. ferrooxidans* γ-glutamylcysteine synthetase gene was able to complement the *E. coli gshA* mutant to produce glutathione at almost the same level as the *E. coli* chromosomal *gshA* gene (*E. coli* MC1061).

Table 3.5. Cellular glutathione content of *E. coli* MC1061, *E. coli* BH5262 and *E. coli* BH5262(pTHIO7). The results represent the means of three assays. Standard deviations are indicated in brackets.

Bacterial cell extract	**nmoles GSH/mg protein**
E. coli MC1061	31.67(4.04)
E. coli BH5262	9.00(1.73)
E. coli BH5262(pTHIO7)	29.67(3.06)

3.4.11. Cloning, expression and purification of the MBP-fusion

The pilot experiment indicated that the fusion protein (90 kDa) was largely in the cell extract (Fig. 3.17, lane 2), however some of the fusion protein was also found in the insoluble matter (Fig. 3.17, lane 3). This was probably due to binding of the fusion protein to the membrane fraction. As expected the fusion protein was found in the shocked cells (Fig. 3.17, lane 4), but not in the osmotic shock fluid (Fig. 3.17, lane 5). This indicates that the protein is not located in the periplasm. The partially purified fusion protein (Fig. 3.18, lane 1), and the products after cleavage with Factor X_a for 2, 4, 8 and 24 hours (Fig. 3.18, lanes 2, 3, 4 and 5, respectively) indicated that the fusion protein was cleaved as the 90 kDa protein band disappears. Protein bands corresponding in size to the target (GshA) protein (49 kDa) and the maltose-binding protein (40 kDa) could be seen, as well as low concentrations of other proteins.

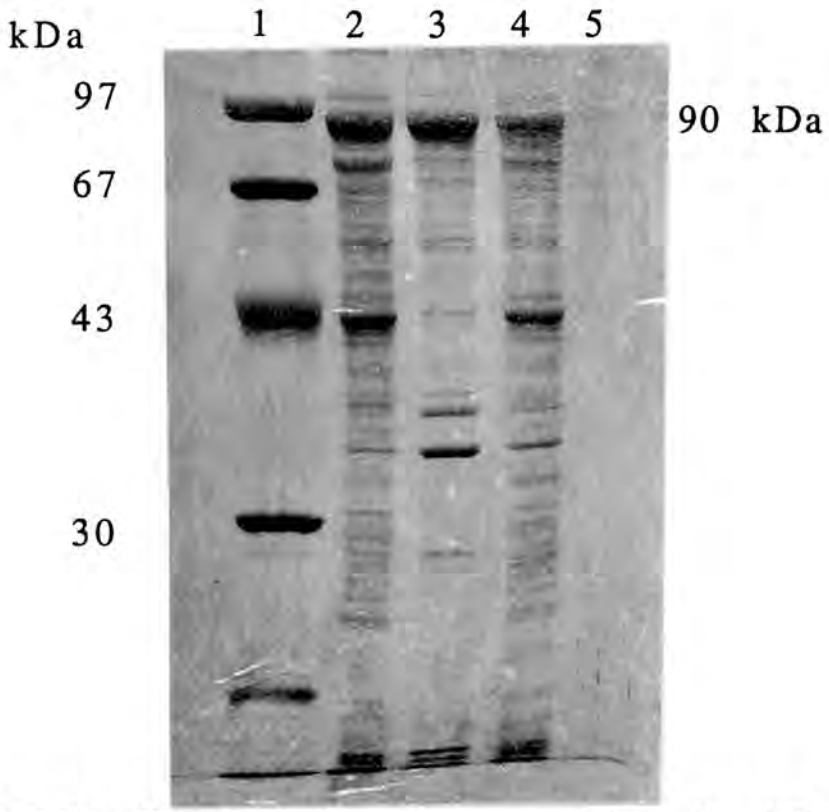


Fig. 3.17 SDS-PAGE analysis of proteins expressed from *E. coli* TBI cells containing the pMal-fusion protein. Lane 1, molecular weight markers; lane 2, cell free extract; lane 3, insoluble cell matter; lane 4, shocked cells and lane 5, osmotic shock fluid.

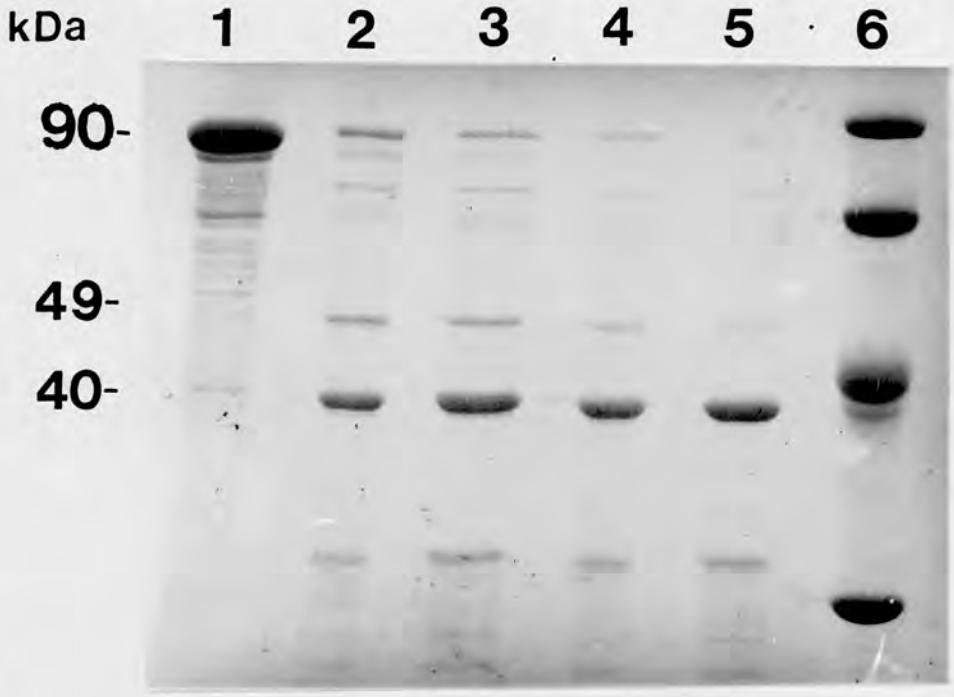


Fig. 3.18 SDS-PAGE analysis of the partially purified fusion protein (lane 1) and the partially purified fusion protein after cleavage with Factor X_a for 2, 4, 8 and 24 hours (lanes 2, 3, 4 and 5 respectively). Molecular weight markers (lane 6).

3.4.12. Assay for γ-glutamylcysteine synthetase activity.

γ-Glutamylcysteine synthetase catalyses the ATP-dependent formation of the dipeptide γ-glutamylcysteine from L-glutamate and L-cysteine, as indicated by the reaction below;

$$\text{L-Glu} + \text{L-Cys} + \text{ATP} \rightarrow \text{L-}\gamma\text{-Glu-L-Cys} + \text{ADP} + P_i$$

γ-Glutamylcysteine synthetase activity was determined by measuring the release of P_i from ATP in the presence of the substrates L-glutamate and L-α-aminobutyrate. While L-cysteine is the natural substrate for γ-glutamylcysteine synthetase, it is convenient to replace it with L-α-aminobutyrate, since the latter amino acid does not undergo spontaneous oxidation and is an effective substrate for the enzyme (Hell & Bergmann, 1990). P_i release was found to be approximately the same whether the substrates, glutamate or α-aminobutyrate, were omitted or added to the assay, indicating that the ligation of L-glutamate and L-α-aminobutyrate by the enzyme to form γ-glutamylaminobutyrate, was not necessary for ATP degradation to occur. Fresh enzyme extracts were prepared to confirm these results and the same results were obtained. P_i release was not detectable in assays in which enzyme extract or ATP were omitted. To try and obtain activity above the spurious background P_i release, the assays were also carried out at a range of pHs between 7.0 and 9.0. However, no increased level of P_i release was obtained. In addition L-cysteine was used in the reaction instead of L-α-aminobutyrate to ensure that L-α-aminobutyrate was an effective substrate for the enzyme. The cleavage factor X_a was substituted for the enzyme extract in the standard assay to confirm it had no ATPase activity. HPLC was used to analyse the reaction products, as measuring the

release of P_i in the assay above did not indicate whether small amounts of γ-glutamylcysteine/g-glutamylaminobutyrate were being formed.

3.4.13. HPLC Analysis of Dabsylated products

The samples and standards were dabsylated, as the undabsylated samples all had very low retention times on the reverse phase column and were thus difficult to separate. Dabsyl chloride is a large, hydrophobic group which reacts with the amino group of amino acids making them more hydrophobic. More hydrophobic amino acids are eluted less rapidly on a reverse phase column and are thus easier to separate. Dabsylated standards of L-glutamate, L-α-aminobutyrate and glutathione were run to determine retention times on the reverse phase column, and to estimate the retention time of γ-glutamylcysteine. A dabyslated standard of γ-glutamylcysteine was not run as it was not commercially available. No new peak at the predicted retention time for γ-glutamylcysteine was observed, and there was no detectable decrease in the concentration of either the L-glutamate or the L-α-aminobutyrate in the complete reaction mix analysed. These results indicate that no L-glutamate or L-α-aminobutyrate was being converted to γ-glutamylcysteine by the enzyme in this reaction, and thus no γ-glutamylcysteine was being formed.

3.5. Discussion

The organisation of the *T. ferrooxidans* pyruvate dehydrogenase, citrate synthase and γ-glutamylcysteine synthetase genes as a single transcript on a contiguous piece of chromosomal DNA has not been found before. The pyruvate dehydrogenase genes and citrate synthase genes of both *E. coli* and *B. subtilis* are widely separated on the chromosome (Fig 3.19). The *E. coli* γ-glutamylcysteine synthetase gene is also located on a different portion of the *E. coli* chromosome. However, as discussed in section 3.4.8., pyruvate dehydrogenase provides the link between glycolysis and the Kreb's cycle, as it catalyses the oxidative decarboxylation of pyruvate to acetyl-SCoA. Citrate synthase catalyses the condensation of acetyl-SCoA with oxaloacetate to form citrate. Experiments have shown that citrate synthase binds to the dihydrolipoyl transacetylase core of the pyruvate dehydrogenase complex and to other components of the pyruvate dehydrogenase complex (Sümegi & Alkonyi, 1983). As physiologically the two enzymes are linked it is likely that the genes would be contiguous.

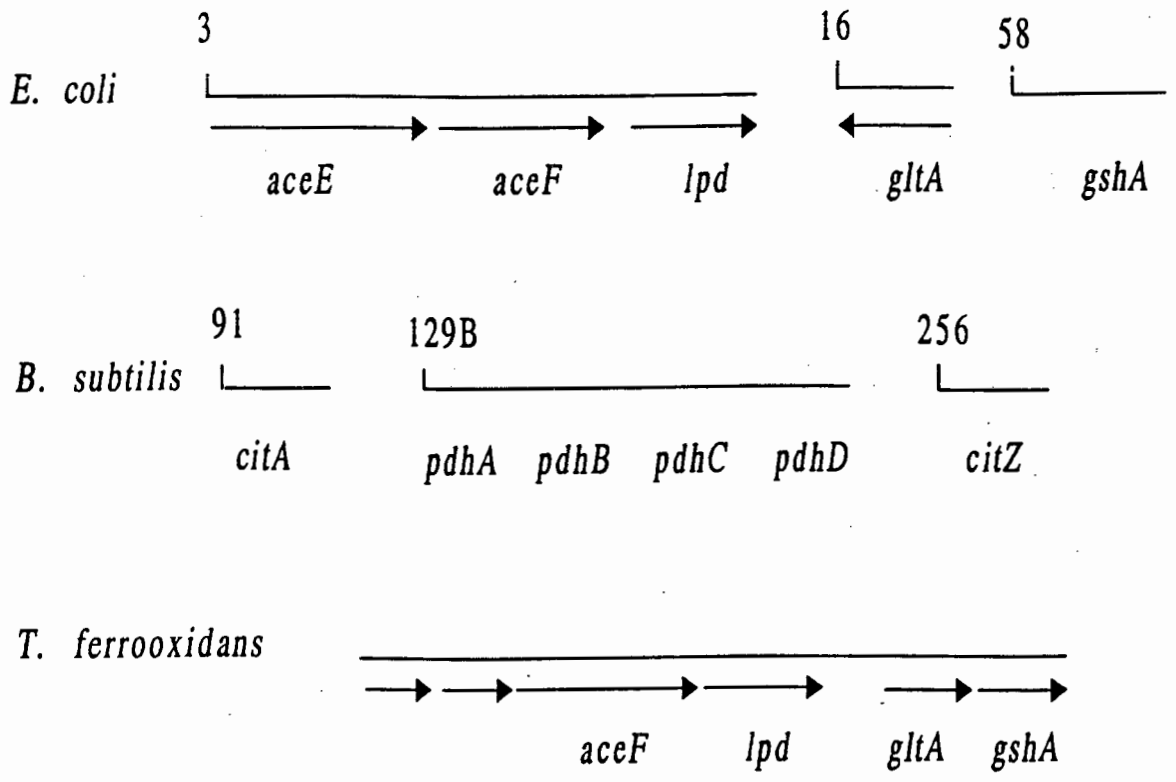


Fig. 3.19 The location of the γ-glutamylcysteine synthetase, citrate synthase and pyruvate dehydrogenase genes on the *E. coli*, *B. subtilis* and *T. ferrooxidans* chromosomes. The genes have the following nomenclature: pyruvate carboxylase, *aceE* in *E. coli* and *T. ferrooxidans* and *pdhA* (α subunit) and *pdhB* (β subunit) in *B. subtilis*; dihydrolipoyl transacetylase, *aceF* in *E. coli* and *T. ferrooxidans* and *pdhC* in *B. subtilis*; lipoamide dehydrogenase, *lpd* in *E. coli* and *T. ferrooxidans* and *pdhD* in *B. subtilis*; citrate synthase, *gltA* in *E. coli* and *T. ferrooxidans* and *citA* and *citZ* in *B. subtilis*; γ-glutamylcysteine synthetase, *gshA* in *E. coli* and *T. ferrooxidans*. The arrows indicate the direction of transcription of the genes (where it is known) and the numbers indicate the location of the genes on the chromosome.

The Kreb's cycle has a two-fold metabolic function: (i) to oxidise metabolites and generate NADH, which on conversion to NAD^+ may be coupled to ATP formation (ii) to generate metabolites required for biosynthesis, such as the amino acids glutamine, proline and arginine. *T. ferrooxidans* is a chemolithoautotroph, and therefore, would not require the Kreb's cycle for energy generation, but it would require portions of the cycle to generate certain metabolites required for biosynthesis. This would explain the presence of the pyruvate dehydrogenase and citrate synthase genes in *T. ferrooxidans.*

There are three pieces of evidence indicating that the *T. ferrooxidans gltA* and *gshA* genes are transcriptionally linked, and that the cloned *gltA* and *gshA* genes are linked in *E. coli.* Firstly, the DNA:RNA hybridisation experiments (section 3.4.9) gave similar transcript signals when a probe from each of the genes was used. A similar result was achieved with an *aceF* probe, suggesting that the *aceF*, *gltA* and *gshA* are all linked in *T. ferrooxidans.* Secondly, the *gshA* gene does not appear to function off its own promoter in *E. coli*, as subclones in which the *gshA* gene is transcribed in the opposite orientation to the *lac* promoter were unable to complement the *gshA* mutation of *E. coli* BH5262. The citrate synthase gene, however, was able to complement *gltA* mutant *E. coli* MOB150 irrespective of its orientation to the *lac* promoter. These results suggest that the *gshA* gene is transcribed from a promoter upstream of the citrate synthase gene. Thirdly, the two genes are separated by only 9 bp, the close proximity of genes is frequently a feature of genes that are transcriptionally coupled. Transcriptional linkage of the *aceE, aceF, lpd* and *gltA* genes is logical since, as discussed above, the enzymes they encode are all involved in the transfer of an acetate group to oxaloacetate during the synthesis of citrate. However, the reason for the linkage between the *gltA* and *gshA* genes is unclear.

It is possible that glutathione may be utilised in the pyruvate dehydrogenase complex of *T. ferrooxidans* instead of lipoic acid, which would explan the transcriptional linkage of the whole *aceEF-lpd-gltA-gshA* cluster. In *E. coli* lipoic acid is found in two multienzyme complexes (pyruvate dehydrogenase and α-ketoglutarate dehydrogenase) where it serves as an acyl carrier and as a two electron carrier. It is covalently bound to a lysyl residue of dihydrolipoyl transcacetylase. The disulfide bridge of lipoic acid is 1.5 nm from the peptide backbone, and this miniature "arm" serves to swing an acyl group from one part of the enzyme complex to another (Rawn, 1983). This function is based on the presence of two sulfur atoms that exist as either two sulfhydryl groups (-SH) or as a single disulfide linkage (-S-S-). The tripeptide glutathione can exist as a dimer (G-S-S-G) or as two monomers (GSH) and thus may be able to substitute for lipoic acid in the pyruvate dehydrogenase complex.

The γ-glutamylcysteine synthetases studied to date differ widely in amino acid sequence and structure. The mammalian proteins are composed of two subunits whereas the bacterial proteins

(*E. coli* and *P. mirabilis*) are composed of a single subunit. The *T. ferrooxidans* protein also consists of a single 47 kDa subunit which is considerably smaller than the 60 kDa and 64 kDa proteins of *E. coli* and *P. mirabilis* respectively. As can be seen from the phylogram (Fig. 3.20) the two mammalian proteins are closely related (89% identity), and that of *S. cerevisiae* lies within the same cluster. However, the γ-glutamylcysteine synthetases of *A. thaliana*, and the two bacterial proteins form deeply branched separate clusters, indicating that these proteins are not closely related.

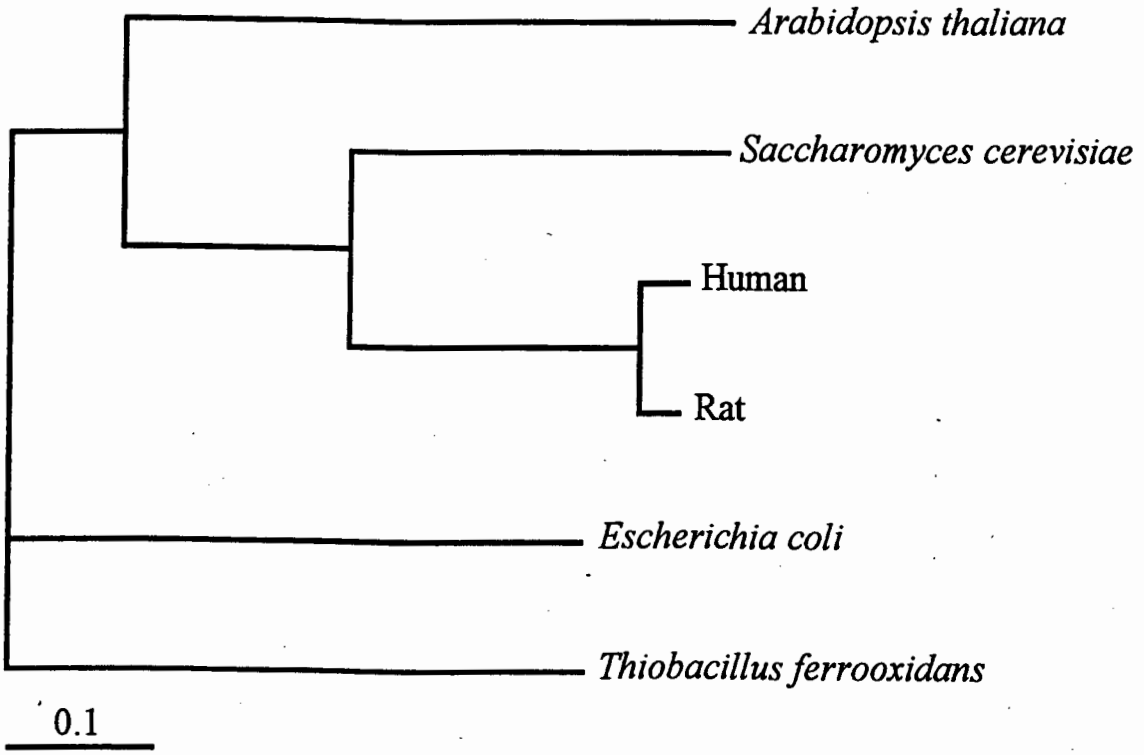


Fig. 3.20 A phylogram based on γ-glutamylcysteine synthetase amino acid sequences. The software packages Clustal W and TreeView were used to calculate and draw the phylogram. Bootstrapping values were all 100% and the scale of 0.1 indicates 10% amino acid divergence. The GenBank accession numbers of the sequences used were as follows: *E. coli* X03954; *A. thaliana* Z29490; *S. cerevisiae* D90220; Rat P19468 and Human M90656.

In spite of the low sequence homology to other *gshA* genes, there is evidence to indicate that the *gshA* gene of *T. ferrooxidans* has been isolated. Firstly, the cloned gene from *T. ferrooxidans* was able to complement the *E. coli gshA* mutant for growth on minimal medium. Secondly, concentrations of glutathione produced by the cloned gene in the *E. coli gshA* mutant were comparable to those of the wild type *E. coli* strain, indicating the presence of a functional γ-glutamylcysteine synthetase enzyme. Furthermore, lack of homology between γ-glutamylcysteine synthetases is common as, with the exception of human and rat, γ-glutamylcysteine synthetase genes are generally poorly conserved.

The reason for the lack of enzyme activity obtained from the partially purified γ-glutamylcysteine synthetase enzyme remains to be addressed. There are numerous possible

explanantions for the production of an inactive enzyme using the maltose binding protein expression system. The problem might be that although the enzyme appears to function *in vivo*, once linked to the maltose binding protein, or purified, *in vitro* activity is lost. The enzyme may have been unable to bind the substrates due to incorrect folding of the active site of the enzyme when it is translated as a fusion protein.

It was not possible to assay crude extracts of the cloned gene in *E. coli* BH5262 using the assay described in section 3.4.11, as the breakdown of ATP to ADP and P_i, by components of the crude extracts (other than the γ-glutamylcysteine synthetase enzyme) was too high. It was also not possible to assay for γ-glutamylcysteine production in crude extracts using HPLC due to the impurity of the samples which would have made peak interpretation too difficult.

The inactivity of the γ-glutamylcysteine synthetase enzyme *in vitro* could be due to a variety of factors. However, there is good evidence that the *T. ferrooxidans* γ-glutamylcysteine synthetase gene has been cloned in this study, as its product is clearly active *in vivo*.

CHAPTER FOUR

GENERAL DISCUSSION

During the biooxidation of a mineral such as arsenopyrite, large quantities of air are pumped into a reactor vessel and the redox potential of the solution may rise from less than +300mV before oxidation to greater than +700mV when oxidation is complete (Lindström *et al.*, 1992). The redox potential in the environment of an iron-oxidising bacterium such as *T. ferrooxidans* is a function of the Fe^{3+}/Fe^{2+} ratio. When ferrous iron is oxidised to ferric iron, the redox potential of the surrounding medium increases correspondingly, and bacteria which grow in such an environment require the ability to maintain their cytoplasmic redox potential within certain limits. This implies that an organism such as *T. ferrooxidans* must have considerable redox-controlling capacity and the ability to withstand the toxic affects of oxygen. As discussed in Chapter 1, glutathione and thioredoxin are two molecules that play a role in the control of redox potential within a cell and in the removal of free radicals generated by oxygen.

In this study the thioredoxin and γ-glutamylcysteine synthetase genes were cloned and characterised as part of an investigation of intracellular systems that are likely to play a role in the ability of *T. ferrooxidans* to cope with large changes in its external redox potential.

It is interesting to note that transposon Tn*5467*, which is located on *T. ferrooxidans* plasmid pTF-FC2, has recently been shown to encode a protein with highest amino acid sequence homology to a glutaredoxin isolated from *Methanobacterium thermoautotrophicum* (Fig. 4.1; Clennel *et al.*, 1995). This glutaredoxin-like protein might be expected to participate in internal redox control. The gene for the glutaredoxin-like protein was expressed in *E. coli* and enabled growth of the *E. coli trxA gshA* double mutant strain BH5262 on minimal medium lacking glutathione. In addition, the glutaredoxin-like gene was able to complement the *E. coli trxA met* mutant for growth on minimal medium plus methionine sulfoxide. Methionine sulfoxide reductase usually specifically requires thioredoxin as a hydrogen donor for the conversion of methionine sulfoxide to methionine (Gonzalez Porqué, 1970), however, in this case the glutaredoxin-like protein was able to substitute for thioredoxin.

```
Grx          VVKIEVFTSPT CPYC PMAIEVVDEAKKEFGDKIDVEKIDIMV   42
               :.::: :  :: :  :::.:.       .   ..  :: :
Tn5467    MTTKRKVEVF.SAG CPSC QTAIELVNRLACGSCEVSILDMNDINV   44

Grx       DREKAIDYGLMAVPAIAIDG.VVRFVGAPGREELFEAISDEI       83
           . .:: : :   :::.::.: .    .. : ::
Tn5467    AK.RARDLGVRSVPAVAINGQLASCCSGSGIEEQALRWPRQA       85
```

Fig. 4.1 A comparison of the amino acid sequences of the *M. thermoautotrophicum* glutaredoxin gene (Grx) and of the glutaredoxin-like protein from Tn *5467*. The active sites are indicated in bold.

Glutaredoxins, like thioredoxins, are ubiquitous, heat-stable proteins which catalyse thiol/disulfide exchange reactions (both proteins can act as H-donors for ribonucleotide reductase) but glutaredoxins receive their reducing equivalents from glutathione not thioredoxin reductase (Holmgren, 1979). The active site of the glutaredoxin contains two redox-active cysteine residues Cys-Pro-Tyr(Phe)-Cys and is well-conserved from *E. coli* to mammals. The glutaredoxin-like protein isolated from the *T. ferrooxidans* transposon contains this highly conserved active site (Fig. 4.1). There is little similarity in primary structure between glutaredoxins and thioredoxins apart from the 14-membered disulfide ring, but they are thought to have a similar tertiary structure (Eklund *et al.*, 1984).

The discovery of a glutaredoxin-like gene encoding a redox-active protein of this type on a transposon is unusual (Fig. 4.2, ORF8). Proteins of this type have important biosynthetic functions, and the genes encoding these proteins are not normally found on plasmids. Plasmid pTF-FC2 is a broad-host-range, mobilizable plasmid that has a copy number of 12 in *E. coli*. *T. ferrooxidans* could, therefore, have 12 copies of the glutaredoxin gene, depending on the copy number of plasmid pTF-FC2 in *T. ferrooxidans*. It may be advantageous for *T. ferrooxidans* to have the glutaredoxin gene on a multicopy plasmid to generate large quantities of glutaredoxin for the maintenance of redox potential within the cell. *T. ferrooxidans* also may have more than one glutaredoxin gene as *E. coli* has been found to have at least three different copies (Åslund *et al.*, 1994).

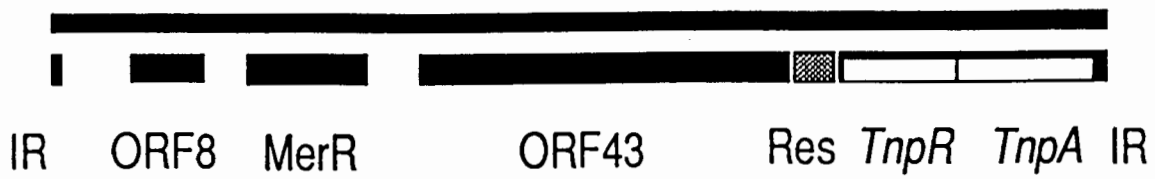


Fig. 4.2 A map of transposon Tn*5467*. IR, 38 bp inverted repeats (proximal and distal) with 37 out of 38 and 38 out of 38 matches to the IR sequences of Tn*21*. ORF8 encodes the glutaredoxin-like gene. MerR, ORF with similarity to regulators of the mercury reductase operons. Res, region with similarity to resolution sites of Tn*21*. TnpR and TnpA show homology to the resolvase and transposase proteins, respectively, of Tn*21*.

An additional function of thioredoxin may be to act as a cofactor in the arsenic resistance mechanism of *T. ferrooxidans*. As discussed in Chapter 1 and 2, *T. ferrooxidans* is able to tolerate high concentrations of arsenic, when growing on arsenopyrite ores, and thus must have some form of resistance to arsenic. Arsenic resistance in *S. aureus* (Novick & Roth, 1968), *E. coli* (Hedges & Baumberg, 1973) and *P. aeruginosa* (Cervantes & Chavez, 1992) is due to genes located on plasmids of these bacteria. Arsenic resistance genes have also been located on the chromosome of *E. coli* (Diorio *et al.*, 1995). DNA:DNA hybridisation experiments using the *arsBC* genes of *E. coli* plasmid R773 as a probe have indicated that both *Thiobacillus caldus* (Hallberg, 1995) and *T. ferrooxidans* (Hallberg personal communication) contain chromosomal

DNA that is homologous to the *arsBC* genes of *E. coli*. The plasmid-determined bacterial resistance system for arsenic and antimony consists of five genes in *E. coli* (Chen *et al.*, 1986; San Francisco *et al.*, 1990; Wu & Rosen, 1993) (*arsR*, *arsD*, *arsA*, *arsB* and *arsC*) but only three genes (*arsR*, *arsB* and *arsC*) in *S. aureus* (Ji & Silver, 1992) (Fig. 4.3).

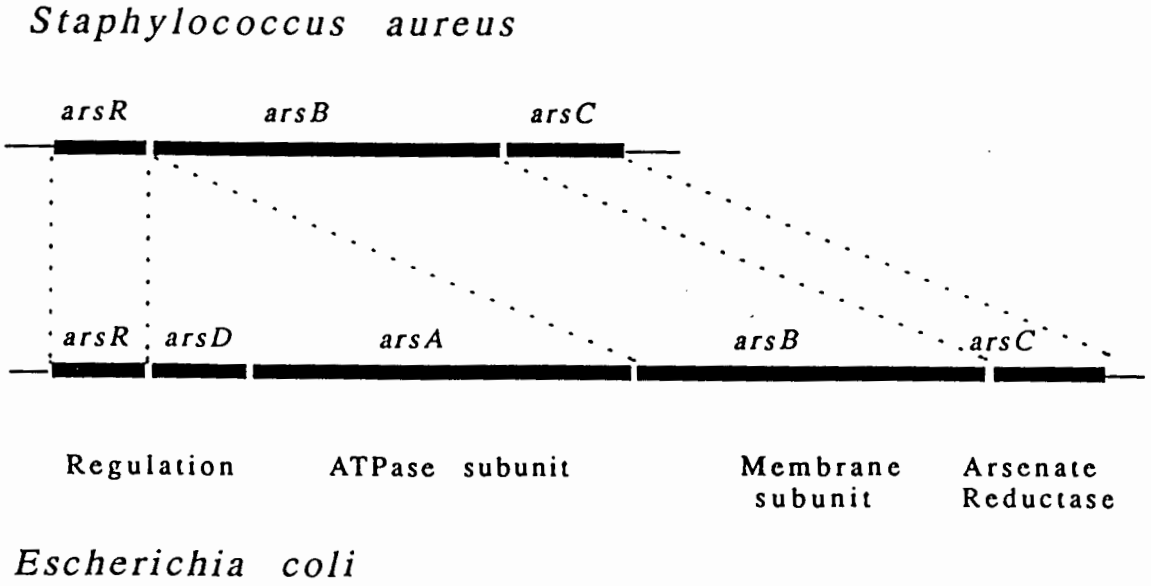


Fig. 4.3 Structure of the arsenic/antimony resistance determinants of *S. aureus* plasmid pI258 and *E. coli* plasmid R773 (updated from Silver *et al.*, 1993).

In both bacteria, the overall process is very similar. The systems are regulated by the repressor protein ArsR. A membrane complex consisting of ArsB and ArsA in *E. coli* (but only ArsB in *S. aureus*) carries out the energy-dependent efflux of arsenite. The ArsC protein reduces arsenate to arsenite, which is then exported from the cell by the ArsB membrane protein (Fig. 4.4). In both *S. aureus* and *E. coli* thioredoxin is specifically required as a cofactor by arsenate reductase (ArsC) for the maintenance of the cysteine residues of ArsC in the reduced state (Ji & Silver, 1992; Silver *et al.*, 1993). A similar mechanism of arsenic resistance that requires thioredoxin to reduce ArsC may operate in *T. ferrooxidans* but this has not been elucidated yet. In addition reduced thioredoxin may react directly and irreversibly with arsonous acid to form very stable dithioesters thus removing the highly toxic arsonous acid from the cell (Knowles & Benson, 1983).

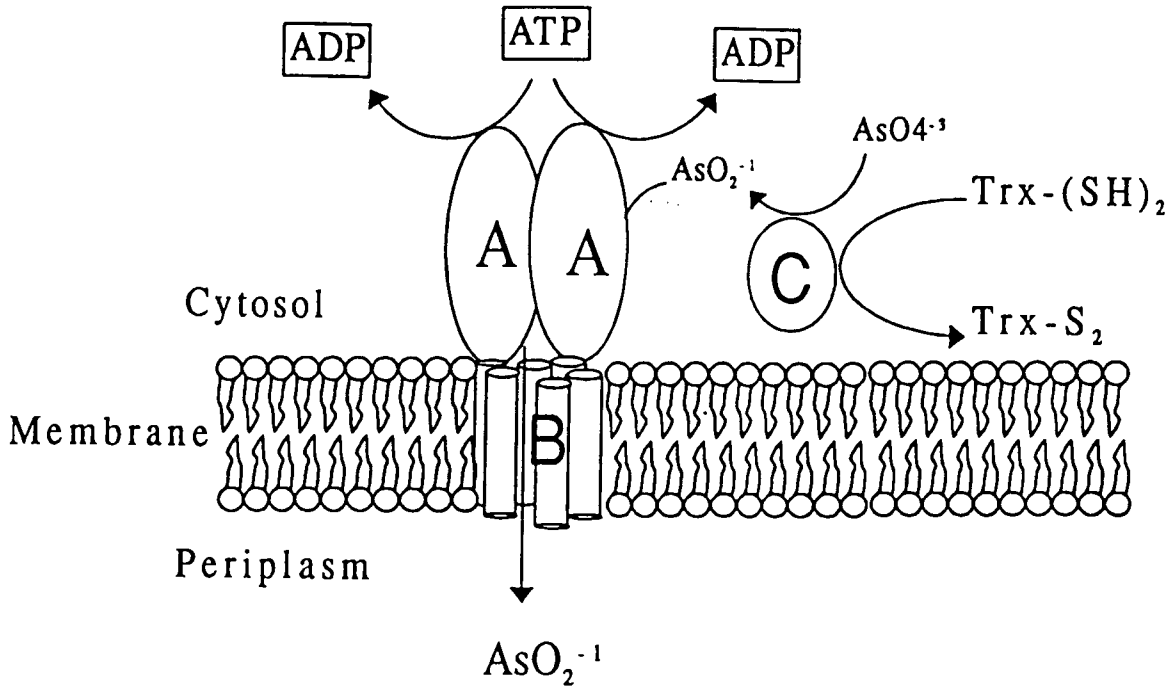


Fig. 4.4 Diagram indicating the Ars efflux system of *E. coli* (updated from Silver *et al.*, 1993). A = ArsA protein, B = ArsB protein, C = ArsC protein, Trx-$(SH)_2$ = reduced thioredoxin, Trx-S_2 = oxidised thioredoxin.

In order to determine the functions of thioredoxin and glutathione in *T. ferrooxidans*, such as maintenance of intracellular redox potentials or the inactivation of toxic compounds that result from high concentrations of oxygen, the genes encoding these molecules would have to be inactivated in *T. ferrooxidans*. The affect of the loss of thioredoxin or glutathione on *T. ferrooxidans* would then have to be assessed. This could be done by insertional inactivation of these genes. A procedure for introducing DNA into *T. ferrooxidans* by direct conjugation with *E. coli* has recently been reported, as discussed in the Introduction (Peng *et al.*, 1994). Unfortunately at present attempts to introduce DNA into *T. ferrooxidans* ATCC 33020 have been unsuccessful.

A comparison of *T. ferrooxidans* with well-studied heterotrophic bacteria at the genetic level is interesting from an evolutionary point of view. The ecological niche inhabited by the chemolithoautotrophic *T. ferrooxidans* is populated by relatively few other bacterial species and there would seem to be limited opportunity for genetic exchange with other organisms. Thus it is of interest to determine to what extent operon structures differ from more extensively studied bacteria such as *E. coli*. The *trxA* gene of *T. ferrooxidans* is located upstream of the *rho* gene, as in *E. coli*. However, the position of the γ-glutamylcysteine synthetase gene in *T. ferrooxidans* differs from that of *E. coli* and all other bacteria studied to date. In *E. coli*, the γ-glutamylcysteine synthetase gene, the citrate synthase gene and the pyruvate dehydrogenase genes are all located at different positions on the chromosome. In *T. ferrooxidans* these genes

are located on a contiguous piece of DNA and their transcripts appear to be linked in a physiologically logical manner.

It is surprising that no other redox-active genes able to complement the *trxA* or *gshA* mutations of the *E. coli* strains used in this study were isolated. However, 100 cosmids that were able to complement the *E. coli trxA gshA* double mutant were initially isolated in this study, of which only 16 were chosen for further study. Further characterisation of the remaining 84 cosmids may reveal additional redox-active proteins, such as other glutaredoxins and thioredoxins.

APPENDIX A continued

Plasmid	Description	Reference/origin
pG587	derivative of pBR322 containing *aceEF-lpd* region of *E. coli*	John Guest, Universtiy of Sheffield, England
pMW264	derivative of pBR322 containing *gltA* of *R. prowazekii*	Wood *et al.* (1987)
pBluescriptSK	Ap^R, cloning vector	Stratagene, California
pBluescriptKS	Ap^R, cloning vector	Stratagene, California
pUCBM21	Ap^R, cloining vector	Boehringer Mannheim
pUCBM20	Ap^R, cloning vector	Boehringer Mannheim
pHC79	Ap^R, cosmid pBR322 derivative	Boehringer Mannheim

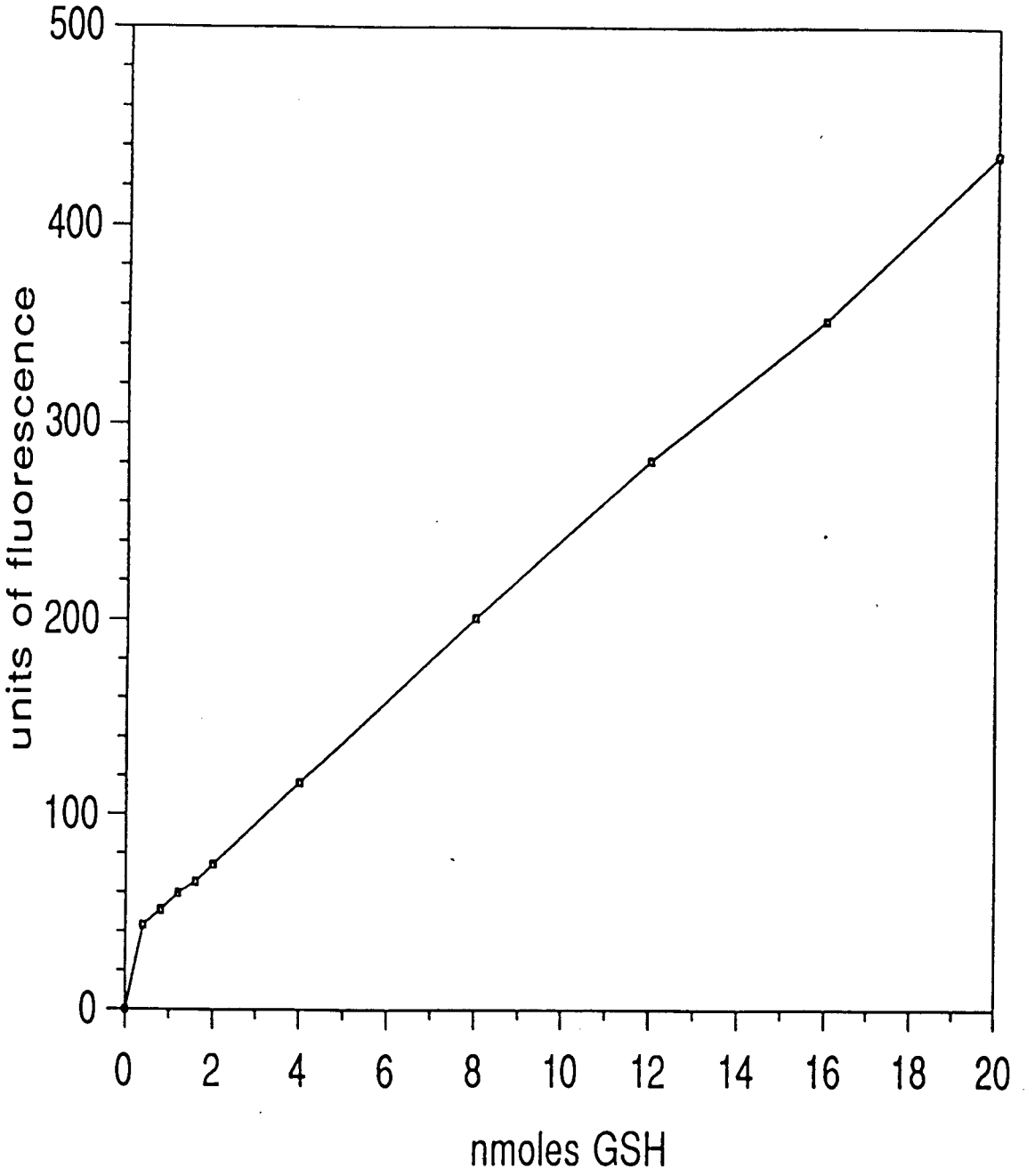
APPENDIX A

Strains	Genotype	Reference/origin
T. ferrooxidans ATCC33020	Wild type	Rockville, Md.
E. coli JM109	*recA*1 *supE*44 *endA hsdR*17 *gyrA*96 *relA*1 *thi* Δ(*lac-proAB*)[F' *trad*36 *proAB lacI*^{q}ZδM15]	Promega Corp. USA
BH5262	K-12 *araD*139? *galU galK hsdR rpsL argH*1 *trxA*7004 *ilvC*::Tn10 *gshA*	Lim *et al.*(1985)
BH2012	K-12 *araD*139? *galU galK hsdR rpsL metA*46 *argH*1 *trxA*7004 *ilvC*::Tn5	Lim *et al.* (1985)
71/18	Δ(*lac-proAB*) *thi supE* [F'*proAB lacI*^{q}*lacZ*δM15]	Heidecker *et al.* (1980)
JF510	Δ(*lac-proAB*) *thi supE trxA*7004 *ilvC*::Tn5 [F'*proAB lacI*^{q}*lacZ*δM15]	Lim *et al.* (1986)
MC1061	K-12 F^{-} *araD*139? *galU galK hsdR rpsL* Δ(*araABC-leu*)7679 D*lacX*74	Britt Persson, University of Umea, Sweden
MOB150	F^{-} *gltA*5 *lct thi lacY galK hsdR xyl mtl tsx tfr rpsL supE*	Wood *et al.*, (1987)
JRG1342	*recA rpsL* [Δ18(*aroP-lpd*)] *metB thyA azi pps ton*	John Guest, University of Sheffield, England

APPENDIX B

Graph indicating the change in fluorescence with changes in glutathione (GSH) concentration. Glutathione is complexed with the flourescent compound o-opthalaldehyde. The data represents the means of three experiments.

Standard Curve for GSH



APPENDIX C

CODON USAGE OF *T. FERROOXIDANS* GENES.

Codon		Tfchrom	*gshA*	*gltA*	*trxA*
Gly	GGG	0.99	0.69	1.55	0.98
	GGA	0.56	0.92	1.29	0.98
	GGT	2.13	3.66	2.84	1.30
	GGC	4.85	2.29	2.33	0.98
Glu	GAG	3.12	5.26	2.84	2.29
	GAA	3.84	2.52	3.10	4.58
Asp	GAT	2.54	2.52	3.62	2.61
	GAC	3.45	2.52	1.29	3.27
Val	GTG	3.73	3.89	3.89	1.63
	GTA	0.56	0.69	0.26	1.96
	GTT	0.60	0.69	0.00	0.98
	GTC	2.41	1.83	1.55	0.65
Ala	GCG	3.36	3.66	3.89	2.61
	GCA	1.08	1.83	0.78	0.65
	GCT	0.80	0.69	1.55	1.63
	GCC	5.02	2.52	5.17	3.92
Arg	AGG	0.17	0.23	0.26	0.65
	AGA	0.09	0.00	0.00	0.65
Ser	AGT	0.65	0.46	1.29	0.65
	AGC	1.31	0.23	1.55	0.65
Lys	AAG	3.69	1.14	1.55	2.61
	AAA	1.31	0.46	1.03	3.27
Asn	AAT	1.12	1.60	2.07	1.31
	AAC	1.75	2.06	1.29	1.63
Met	ATG	3.28	1.83	4.91	1.63
Ile	ATA	0.22	0.46	0.00	0.33
	ATT	1.42	2.75	2.58	2.61
	ATC	4.40	1.83	3.36	3.60
Thr	ACG	1.29	1.83	1.03	0.98
	ACA	0.19	0.46	0.26	0.65
	ACT	0.41	0.46	0.26	0.98
	ACC	2.69	1.83	2.84	2.29
Trp	TGG	0.78	1.14	1.03	1.96

continued..

Codon		Tfchrom	*gltA*	*gshA*	*trxA*
End	TGA	0.15	0.23	0.00	0.65
Cys	TGT	0.22	0.46	0.26	0.65
	TGC	0.69	0.69	0.26	1.96
End	TAG	0.04	0.00	0.26	0.00
	TAA	0.11	0.00	0.00	0.65
Tyr	TAT	1.36	2.75	1.81	1.63
	TAC	1.55	1.14	1.03	0.98
Leu	TTG	1.47	2.16	0.78	0.98
	TTA	0.15	0.46	0.00	0.65
Phe	TTT	1.12	2.75	1.55	0.65
	TTC	2.35	1.60	2.33	1.96
Ser	TCG	1.06	1.37	0.78	0.33
	TCA	0.15	0.23	0.26	0.65
	TCT	0.45	0.46	0.26	2.29
	TCC	1.66	0.91	1.55	1.96
Arg	CGG	1.25	3.43	2.07	0.98
	CGA	0.21	0.46	0.26	0.65
	CGT	1.51	2.75	1.55	0.65
	CGC	2.91	2.06	2.33	2.94
Gln	CAG	3.49	2.75	2.58	2.29
	CAA	0.60	1.83	1.03	0.98
His	CAT	1.06	1.37	2.33	0.98
	CAC	1.29	0.92	2.33	0.65
Leu	CTG	4.63	6.41	5.43	4.58
	CTA	0.09	0.00	0.00	0.00
	CTT	0.69	0.46	0.78	1.96
	CTC	1.70	1.83	2.33	2.61
Pro	CCG	1.44	1.83	1.55	0.98
	CCA	0.30	1.14	0.26	0.98
	CCT	0.50	1.60	1.81	2.29
	CCC	2.00	1.14	1.03	2.94

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