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STUDIES OF THE CLONING AND EXPRESSION OF

THIOBACILLUS FERROOXIDANS

PLASMID AND NITROGENASE GENES

INGE-MARTINE PRETORIUS

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These things, these things were here and but the beholder
Wanting; which two when they once meet,
The heart réars wings bold and bolder
And hurls for him, O half curls earth for him off
under his feet.

Gerard Manley Hopkins

Pour les roses qui ont apparu mystérieusement pour marquer le début de cette étude, et pour toutes les autres de la vie que j'ai reçues, je dédie cette thèse à mes parents, à K et à P.

Je serai toujours redevable à mon amie et mentor LA, autant qu'à SM et GMK qui ont constamment tenu compagnie à Inge-Martine.

ERRATA

Throughout this thesis "autotrophic DNA" and "autotrophic genes" should be read as "DNA isolated from an autotroph" , and "genes from an autotroph". Similarly for "heterotrophic DNA or genes".

CONTENTS

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ABBREVIATIONS

A	adenine
Ala	L-alanine (A)
Ap	ampicillin
Arg	L-arginine (R)
Asn	L-asparagine (N)
Asp	L-aspartic acid (D)
ATCC	American Type Culture Collection
bp	base pair(s)
C	cytosine
Cb	carbenicillin
Ci	Curie
Cln	clindamycin
Cm	chloramphenicol
CPM	counts per minute
CsCl	caesium chloride
Cys	L-cysteine (C)
D	Dalton(s)
d	day(s)
DNase	deoxyribonuclease
ds	double stranded
G	guanine
Gln	L-glutamine (E)
Glu	L-glutamic acid (Q)
Gly	L-glycine (G)
h	hour(s)
His	L-histidine (H)
Ile	L-isoleucine (I)
Kan	kanamycin
kb	kilobase(s) or kilobase pairs
LA	Luria agar
LB	Luria broth
Leu	L-leucine (L)
Lys	L-lysine (K)
MCS	multiple cloning site
Met	L-methionine (M)
MIC	minimum inhibitory concentration
min	minute(s)
mRNA	messenger RNA
mw	molecular weight ≡ relative molecular mass (M_r)
N	any nucleotide
NA	nutrient agar
Nal	nalidixic acid
NB	nutrient broth
Neo	neomycin
NFDM	nitrogen-free-Davis-Mingioli liquid medium
ORF	open reading frame

ori	origin of replication
p	plasmid
PAGE	polyacrylamide gel electrophoresis
Pen	penicillin
Phe	L-phenylalanine (F)
Pro	L-proline (P)
r	(superscript) resistance
RBS	ribosome binding site
RF	replicative form
RNase	ribonuclease
rpm	revolutions per minute
rRNA	ribosomal RNA
S	Svedberg sedimentation constant
s	(superscript) sensitivity
SD	standard deviation
SDS	sodium dodecyl sulphate
sec	second(s)
Ser	L-serine (S)
ss	single stranded
Str	streptomycin
T	thymine
Tc	tetracycline
Thr	L-threonine (T)
Trp	L-tryptophan (W)
Tn	transposon
Tyr	L-tyrosine (Y)
u	units of enzyme activity
UV	ultraviolet light
V	volts
Val	L-valine (V)
wt	wild type
::	novel joint

ABSTRACT

This dissertation forms part of a fundamental investigation into the molecular biology of the industrially important bacterium, Thiobacillus ferrooxidans. The expression of T. ferrooxidans plasmid encoded functions, as well as the identification, cloning, sequencing and expression in a variety of heterotrophic bacteria and in vitro systems of the T. ferrooxidans nitrogenase structural genes were studied.

Recombinant DNA vehicles were constructed between cryptic plasmids of T. ferrooxidans and Escherichia coli. The recombinant plasmids were characterised. The origin of replication of pBR325 was deleted and one recombinant plasmid was shown to replicate in E. coli using an origin of replication located on the T. ferrooxidans DNA. This plasmid had a relatively broad host range which increased its suitability as a potential cloning vector for T. ferrooxidans. The expression of autotrophic T. ferrooxidans genes in a heterotrophic E. coli cell-free system was demonstrated.

The presence of DNA sequences which were homologous to the Klebsiella pneumoniae nifHDK, nifX, nifUSV and nifJ genes, was demonstrated in total cellular DNA preparations from five different iron oxidising T. ferrooxidans strains. A non-iron oxidising Thiobacillus novellus strain, and a

heterotrophic Acidiphilium strain which occurs in close association with T. ferrooxidans did not contain DNA homologous to the nif genes. The entire T. ferrooxidans ATCC33020 nifHDK operon was cloned on a 6.7 kb insert on pIMP16. The arrangement of the T. ferrooxidans nifHDK genes was similar to that of K. pneumoniae.

The T. ferrooxidans recombinant plasmids pIMP16 (T. ferrooxidans nifHDK), pIMP11 (T. ferrooxidans nifH), and pIMP5 (T. ferrooxidans nifDK) reduced diazotrophic growth and nitrogenase activity in a K. pneumoniae Nif^+ strain. Plasmids pIMP16 and pIMP11 did not allow diazotrophic growth, or restore nitrogenase activity to the transformed K. pneumoniae $nifH^-$, $nifD^-$ or $nifK^-$ mutant strains. Plasmid pIMP5 did not allow diazotrophic growth, but did restore low levels of nitrogenase activity to transformed K. pneumoniae $nifK^-$ mutants. No nitrogenase activity was detectable in K. pneumoniae $nifH^-$ or $nifD^-$ strains containing pIMP5. In K. pneumoniae in vivo transcription assays, only pIMP11 (nifH) produced an mRNA transcript which was detected by pIMP16 [^{32}P]DNA. In a heterotrophic in vitro translation-transcription system, the T. ferrooxidans insert on pIMP16 (nifHDK) produced polypeptides with apparent molecular masses of approximately 33, 56, 58 and 60 kD, pIMP11 (nifH) produced a 33 kD protein, and pIMP5 (nifDK) produced two proteins of 56 and 60 kD.

The DNA sequence was determined for the cloned T. ferrooxidans nifH and part of the nifD gene. The T.

ferrooxidans *nifH* promoter was identified and showed perfect consensus with *K. pneumoniae* *nif* promoter sequences. The amino acid sequence was deduced from the DNA sequence. The *T. ferrooxidans* Fe-protein, encoded by *nifH*, contains five cysteine residues and 296 or 298 amino acids (depending on which of two ATG codons initiates transcription). In a comparison of *nifH* DNA sequences from *T. ferrooxidans* and eight other diazotrophs, *Parasponia rhizobium* showed the greatest homology (74%) and *Clostridium pasteurianum* (*nifH1*) the least homology (54%). In a comparison of the amino acid sequences of the Fe-proteins, *P. rhizobium* and *Rhizobium japonicum* showed the greatest homology (both 86%), and *C. pasteurianum* (*nifH1* gene product) the least homology (56%) to *T. ferrooxidans*. The codon usage in *nifH* of *T. ferrooxidans* was very similar to the other Gram-negative diazotrophs under comparison.

CHAPTER ONE

GENERAL INTRODUCTION

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1.1 MICROBIAL MINING

Hydrometallurgical processes involving bacterial leaching are playing an increasingly important role in the extraction of metals from low grade mineral ore. Numerous characteristics of the biomining process account for the increased interest, including: the relative absence of land and water pollution; the need to mine increasingly lower grade ores which cannot be economically processed by conventional operations; and the ease of implementation and lower capital costs of hydrometallurgical operations as compared to conventional processing.

Bacterial hydrometallurgy, which consists of the dissolution of metals from mineral ores and the recovery of the desired elements, has a long history. As early as 1 000 BCE, the recovery of copper from the drainage water of mines was a widespread practice in the Mediterranean basin. The large scale leaching of copper was well established by the 18th century at the Rio Tinto mines in Spain (Brierley, 1982). The presence of iron and copper in the effluents of copper mines was considered to be solely the result of chemical reactions until 1922, when reports of bacterial leaching of

zinc and iron sulfides were published (Torma and Banhegyi, 1984). Unidentified autotrophic bacteria were involved in these studies and suggested a possible role for bacteria in the extraction of metals from low grade ores.

1.1a Methods of leaching. The principal physical methods of leaching are dump, heap, vat and in situ leaching. In commercial dump-leaching operations the uncrushed, low grade oxide and sulfide ores, which contain less than 0.4% copper (Brierley, 1978), are transported to the dump site. Valleys with naturally impermeable bases provide ideal dump sites by maintaining the stability of the pile, and facilitating the recovery of the leach solutions. Because leaching bacteria are ubiquitous, the dumps need not be inoculated, but merely sprinkled with acidified water to promote bacterial growth. The solutions percolate through the dump and are collected in catchment basins where the copper is extracted from the leach liquor by a variety of methods. The barren solution is reapplied to the heap and such a leach cycle is measured in years.

Heap leaching is used to extract copper, uranium and gold from ores of a higher grade than those subject to dump leaching. The ore is crushed, placed on prepared surfaces and aerated to reduce the leach cycle to a few months. Placing the crushed ores in confined tanks, known as vat leaching, accelerates the leaching process which may be completed in a few days.

In situ leaching has the potential for the recovery of metals from low grade ores in inaccessible sites with a minimal impact on the environment. This process is applicable to oxide and sulfide minerals of copper and uranium. The hydrology of such deposits must be well characterised to enable accurate predictions and control of the path followed by the leach liquor. Compounding the difficulties of handling large volumes is the potential danger of contamination of ground waters by the acid leach liquor.

1.1b The chemistry of leaching. Bacterial leaching is divided, by convention, into direct and indirect leaching. Direct leaching involves an enzymatic attack by the bacteria on components of the mineral sulphide which are susceptible to oxidation. The inorganic ions never enter the bacterial cell but their electrons, released by the oxidation reaction, enter the cell and generate energy-rich adenosine triphosphate (ATP). In aerobic leaching organisms, oxygen is the final electron acceptor. Iron- and sulphur-rich substrates constitute the principle components of mineral sulphides which can be oxidised. The oxidation reactions of these substrates are shown by the following equations (Kelly *et al.*, 1979):

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

$$S_8 + 12O_2 + 8H_2O \longrightarrow 8H_2SO_4$$

$$H_2S + 2O_2 \longrightarrow H_2SO_4$$

Indirect or bacterial-assisted leaching does not proceed through bacterial attack on the atomic structure of the mineral. Instead, the powerful oxidising agents, ferric iron (Fe^{3+}) and sulphuric acid, produced during direct leaching react with other metals, transforming them into the oxidised, soluble form. In this reaction ferrous iron (Fe^{2+}) is again produced and is rapidly reoxidised by the bacteria. In an acidic solution without bacteria, ferrous iron is stable, and leaching mediated by ferric iron would be slow. The presence of leaching bacteria can accelerate such an oxidation reaction by a factor of more than a million (Brierley, 1982). The direct leaching products, ferric sulphate and sulphuric acid, form soluble uranyl sulphate in the oxidation of uranium-containing ores (Tuovinen and Kelly, 1974):

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

$$UO_3 + H_2SO_4 \longrightarrow UO_2SO_4 + H_2O$$

1.1c Bacteria involved in leaching. In practice, the leaching process is exceedingly complex, involving numerous other reactions in additon to direct enzymatic oxidation and bacterial generation of oxidising agents. Furthermore, direct and indirect leaching are difficult to differentiate quantitatively because most minerals include some iron. The microorganisms involved in the leaching process are

extremely diverse with respect to their nutritional requirements and ability to withstand the acidic conditions of their environment. The chemolithotrophs can be classified as moderately or extremely thermophilic according to the temperature at which they thrive. The most robust of the leaching microorganisms are the extremely thermophilic and acidophilic species which flourish at hot springs and volcanic fissures where temperatures can exceed 60°C. These microorganisms belong to the Archaebacteria, and this includes the genus Sulfolobus which oxidises iron and sulphur. The electrons generated during the oxidation process are accepted by oxygen under aerobic conditions, while molybdenum and ferric iron serve as ultimate electron acceptors in the absence of air.

The largest number of leaching microorganisms are moderately thermophilic and acidophilic, thriving at temperatures up to 50°C. Although the obligate autotroph Thiobacillus ferrooxidans is the dominant bacterium of this group, bacteria requiring organic supplements are also found. Thiobacillus TH1 requires yeast extract, glutathione or cysteine for growth on inorganic substrates (Brierley and Le Roux, 1977), while Thiobacillus thermosulfidooxidans is facultatively heterotrophic on sugars (Golovacheva and Karavaiko, 1977). Heterotrophic bacteria and fungi also occur in leaching environments, but their contribution to leaching is not known (Kelly et al., 1979). Mixed populations of bacteria have been well documented as more efficient at rendering minerals soluble than pure cultures.

A mixed culture of the facultatively chemolithotrophic Thiobacillus organoparus and Leptospirillum ferrooxidans can grow on, and degrade, both pyrite and chalcopyrite, which neither organism alone can metabolise. Associations between T. ferrooxidans and Thiobacillus thiooxidans resulting in enhanced leaching have been well documented (Kelly et al., 1979). No anaerobic iron-oxidising bacteria have been found, although Sulfolobus (anaerobically), T. ferrooxidans (microaerophilically) and T. thiooxidans (aerobically) can oxidise sulphur with the coupled reduction of ferric to ferrous iron, as an alternative to the reduction of oxygen (Brock and Gustafson, 1976).

1.1d T. ferrooxidans. This is the most important bacterium in the leaching process. It is a motile, Gram-negative, rod-shaped organism having approximate cell dimensions of 0.5 x 1.5 - 2 μm. T. ferrooxidans is acidiphilic (pH 1.3 - 2.5) and mesophilic (20 - 35°C). This autotrophic chemolithotroph uses CO_2 as the sole source of carbon and obtains energy from the oxidation of iron or sulphur. Ferrous iron (Fe^{2+}) is converted to ferric iron (Fe^{3+}), while several reduced forms of sulphur, including soluble and insoluble sulphides and elemental sulphur, can be attacked by the bacteria. The organism is strongly aerobic, with oxygen acting as the final electron acceptor during the oxidation process. Recent studies of the sulphur oxidation of T. ferrooxidans have suggested a new oxidation route involving an electron acceptor other than molecular oxygen (Sugio et al., 1985). This work showed that the reduction

of ferric ion by elemental sulphur was catalysed by a heat-labile ferric ion-reducing system. This occurred under conditions in which the concentration of oxygen available to the cells was lower than that of optimal growth conditions, or when compounds inhibiting the iron oxidase of the cells were present in their environment.

T. ferrooxidans obtains nitrogen in the form of ammonia or nitrate. Mackintosh (1978) using $^{15}N_2$ label, showed that at least one strain of T. ferrooxidans was able to fix atmospheric nitrogen by incorporating it into cellular material. However, in situ studies have not demonstrated nitrogen fixing activities in heap leaching (Khalid and Ralph, 1977).

1.2 NITROGEN FIXATION

The ability to fix atmospheric dinitrogen (N_2), termed diazotrophy, is an important faculty for any organism inhabiting environments which lack fixed nitrogen. This ability is widely distributed amongst divergent prokaryotic taxonomic groups (Burns and Hardy, 1975). These include Azotobacteriaceae, Enterobacteriaceae, Rhodospirillaceae, Bacillaceae, Rhizobiaceae, Actinomycetaceae and Cyanobacteria. Biological nitrogen fixation is the process whereby these free-living or symbiotic microorganisms reduce N_2 to ammonia (NH_3), which is subsequently incorporated in other cell constituents. The enzyme involved is

nitrogenase.

1.2a Genes involved in nitrogen fixation. The facultative aerobe Klebsiella pneumoniae is the best studied N_2 fixing organism. Genes specifically required for the fixation of N_2 (referred to as nif genes) are clustered on the genetic map near the operator of the histidine biosynthesis operon (MacNeil et al., 1978). Physical mapping techniques (Riedel et al., 1979), which have been confirmed by genetic mapping involving transposon mutagenesis (Merrick et al., 1980), have resulted in a fine-structure map of the nif gene cluster. This consists of 17 contiguous genes arranged in 7 or 8 operons within a 24 kb length of DNA (Fig. 1.1).

In addition to the protein components of nitrogenase itself, which are specified by 3 genes (nifH, nifD and nifK, termed the structural genes), many of the other gene products appear to be catalysts concerned with electron transport, metal processing, and maturation of the nitrogenase precursor proteins into the functioning nitrogenase enzyme (Orme-Johnson, 1985). Table 1.1 lists the nif gene products and their properties.

1.2b The nitrogenase enzyme. Although 17 or 18 gene products are required for the production, regulation and maintenance of nitrogenase activity in cells, only 3 protein components are required for the in vitro demonstration of N_2 reduction to NH_3. These are the Fe-protein and the Mo-Fe-proteins (Eady et al., 1972), encoded by the nifHDK

Fig. 1.1 Transcription map of the *nif* gene cluster of *K. pneumoniae* (taken from Beynon *et al.*, 1983).

(a) Restriction map of the cluster;

(b) the physical map of the *nif* genes;

(c) the previously published *nif* transcription map;

(d) the revised map of *nif* transcripts.

Arrows indicate the direction of transcription.

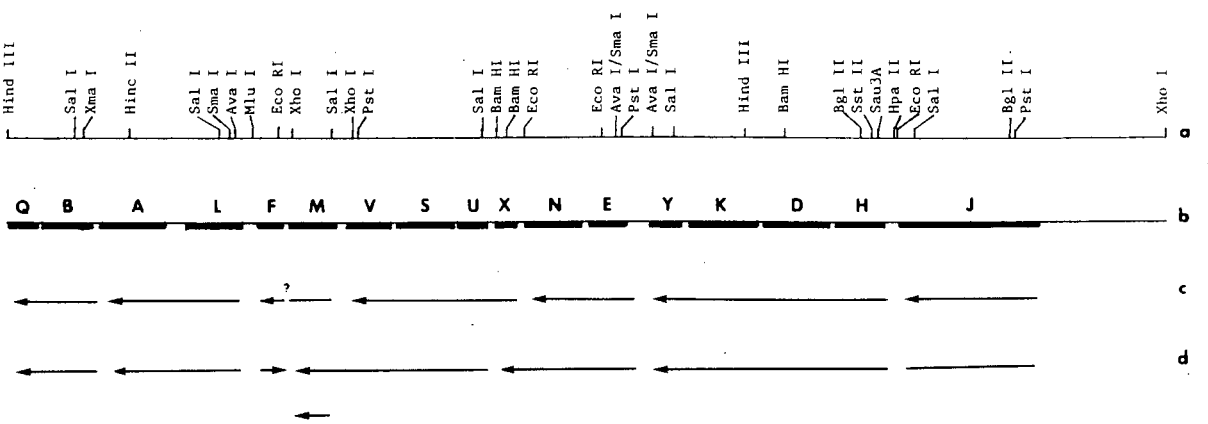


Figure 1. Transcription Map of the *nif* Gene Cluster of K. pneumoniae

(a) Restriction map of the cluster showing only those sites relevant to this work; (b) the physical map of the *nif* genes; (c) the previously published *nif* transcription map; (d) the revised map of *nif* transcripts as determined in this study. Arrows indicate the direction of transcription.

Table 1.1 The *nif* gene products and their properties (taken from Orme-Johnson, 1985).

Gene	Peptide mw (kD)	Proposed function/comment
Q	-	Apparently involved in Mo processing
B	60	Synthesis of FeMoco
A	57-66	Thermosensitive *nif*-specific activator
L	50	Mediates O_2 and amino acid repression of *nif* expression
R,W	-	These "genes" are likely to be regions of DNA that do not encode proteins
F	10-26	Flavoprotein shuttles electrons between *nifJ* and the Fe protein
M	27-28	Processing of Fe protein
V	38-24	Processing of FeMoco
S	42-45	Possible role in the Fe protein stability or MoFe maturation
U	22-28	Apparently dependent on *nifS* for stability. Possible role in MoFe protein maturation
X	18	Posible role in FeMoco synthesis due to location in *nifNE* operon
N	50	Synthesis of FeMoco. Mutually dependent on *nifE* product for stability
E	40-46	Synthesis of FeMoco
Y	19-24	Possible role in MoFe protein maturation
K	60	α-subunit of nitrogenase MoFe protein
D	56	β-subunit of nitrogenase MoFe protein
H	31-35	Fe protein subunit
C	13.3	Possible role in FeMoco synthesis or insertion. Existence of gene disputed
J	120	Pyruvate:flavodoxin oxido reductase

operon. This operon is transcribed from a single promoter located upstream of the N-terminal end of the nifH gene. The larger Mo-Fe-protein (Dinitrogenase, Component I or Kpl) is a tetramer ($\alpha_2 \beta_2$) of molecular mass 220 000, containing molybdenum (Mo), iron and acid labile sulphur (Eady et al., 1972). This protein consists of two non-identical subunits: the α subunit of 60 kD encoded by nifK, and the β subunit of 56 kD encoded by nifD (Dixon et al., 1977; Roberts et al., 1978; Cannon et al., 1979; Roberts and Brill, 1980). The smaller component, termed the Fe-protein (Dinitrogenase reductase, Component II or Kp2), is a dimeric protein of molecular mass 67 000, which contains iron and sulphur atoms (Eady et al., 1972). The dimer consists of identical polypeptides of 31 - 35 kD encoded by the nifH gene (Dixon et al., 1977; Roberts et al., 1978; Cannon et al., 1979; Roberts and Brill, 1980).

Neither protein alone catalyses any of the known nitrogenase reactions: reduction of N_2, reduction of alternative substrates, reduction of protons, or ATP hydrolysis. To be enzymatically active, the subunits must be combined in the presence of Mg-ATP and a low-potential electron donor (Emerich and Burris, 1978). The mode of action of the nitrogenase proteins can be summarised in the following steps (Postgate, 1982):

a) flavodoxin donates electron to reduce Fe-protein;
b) activation of the reduced Fe-protein by Mg-ATP;
c) electron transfer from Fe-protein to the Mo-Fe-protein

accompanied by loss of adenosine diphosphate (ADP), inorganic phosphate (Pi), and Mg^{2+} as well as regeneration of oxidised Fe-protein;

d) Mo-Fe-protein binds the N_2 substrate (or other reducible substrates, including acetylene) at a Mo atom;

e) sequential electron transfer to the Mo-Fe-protein-N_2 complex via hydrazido-2^- and nitrido intermediates;

f) release of NH_3 and regeneration of the active form of the Mo-Fe-protein.

1.2c Physiological regulation of nitrogen fixation. The physiology of diazotrophy can be separated into the external environmental pre-requisites, and the internal genetic regulation of the system within the appropriate environment. Several characteristics of nitrogenase determine the physiology of the diazotroph. The most important physiological constraint imposed by nitrogenase arises from its oxygen sensitivity (Kelly, 1969). The oxygen lability is attributed to the irreversible oxidation of the essential iron-sulphur (Fe-S) groups present in both proteins (Petering *et al.*, 1971). Diazotrophs have evolved various methods for protecting the enzyme system from O_2 denaturation. Enteric bacteria such as *K. pneumoniae* which are facultative aerobes, will only fix N_2 under obligate anaerobic conditions (St. John *et al.*, 1974). Crude nitrogenase extracts of the obligate aerobe *Azotobacter chroococcum* are O_2 tolerant due to a mechanism of conformational protection of the enzyme, probably dependent

on the relative orientation of the proteins within the complex (Robson, 1979). Some obligate aerobes, including T. ferrooxidans (Kelley et al., 1979), behave like microaerophiles (organisms able to grow only at low dissolved O_2 tensions) when obliged to grow diazotrophically. Other oxygen protection mechanisms include gum formation, bacterial clustering and compartmentation. The most sophisticated compartments for restricting access of oxygen to nitrogenase are the nodules of leguminous plants. The characteristic pink colour of these nodules is due to a haemoprotein, leghaemoglogin which is an oxygen binding protein. The function of leghaemoglobin is to act as a reservoir of oxygen and a protective agent simultaneously: to supply oxygen to the bacteroids (rhizobia which have colonised the root nodules), but at a concentration low enough not to damage the nitrogenase (Postgate, 1982).

Additional characteristics which regulate diazotrophic growth include the need for ATP and molybdenum. Iron and sulphur are also required, although supplies are usually adequate in nature (Postgate, 1982). Numerous other external factors regulate diazotrophic growth. In K. pneumoniae, nitrogenase synthesis is strongly repressed: (a) in the presence of fixed nitrogen compounds such as NH_4^+ (Roberts et al., 1978); (b) under conditions ot aerobic growth (St. John et al., 1974); (c) by certain amino acids (Shanmugam and Morandi, 1976); and (d) under high growth temperatures above 37°C (Hennecke and Shanmugam,

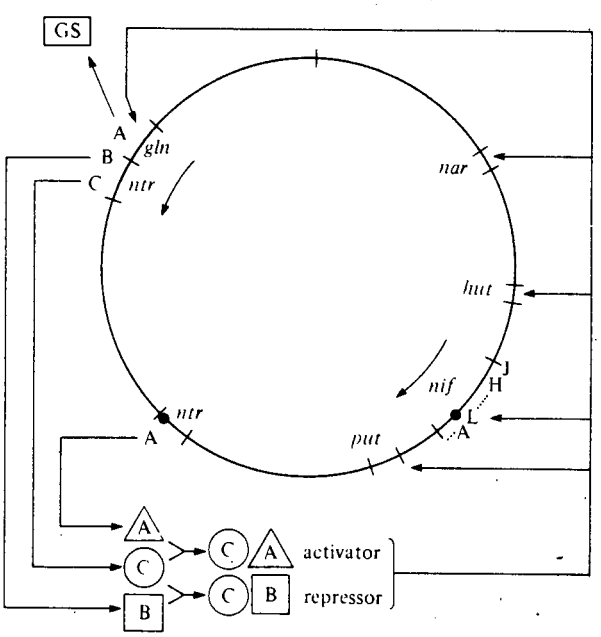
1979). Recent studies of the rhizosphere diazotroph of rice plants, Enterobacter cloacae have revealed an exception to the regulation of diazotrophic growth by high temperatures. The nifA-like gene product of E. cloacae was less temperature sensitive than the K. pneumoniae nifA gene product, enabling E. cloacae to fix N_2 at 39°C, while K. pneumoniae could not do so (Zhu et al., 1986).

1.2d Regulation of nif genes by externally specified gene products. K. pneumoniae nif genes are subject to two levels of positive genetic regulation and at least one level of negative regulation, in response to oxygen and ammonia (Fig. 1.2). The first level is mediated by the products of the nitrogen regulation (ntr) genes: ntrA, ntrB and ntrC (also known as glnF, glnL and glnG, respectively). The ntrBC genes are part of a complex operon glnA-ntrBC (gln denotes genes involved in the synthesis of glutamine synthetase), while the ntrA gene is not linked to ntrBC. The ntr system regulates transcription of a number of genes in K. pneumoniae including glnA, ntrBC and the nif genes (for a review see Magasanik, 1982). Under conditions of NH_4^+ starvation, the ntrC + ntrA products activate the nifLA operon (Ow and Ausubel, 1983). Other gene systems involved in nitrogen assimilation under control of the ntrC + ntrA products include glnA, hut (histidine utilisation), aut (arginine utilisation), and put (proline utilisation) (Magasanik, 1982) (Fig. 1.2).

1.2e Internal regulation of nif genes. The second level of

Fig. 1.2 The regulation of <u>nif</u> and other genes by externally specified gene products (taken from Postgate, 1982).

Regulation of *nif* and other genes by externally specified gene products. The circle is an hypothetical enterobacterial chromosome with an origin of replication at the top. Genes involved in glutamine synthetase (GS) synthesis (*gln*A), and regulation (*ntr*), proline oxidation (*put*) and nitrate reduction (*nar*) are placed at plausible relative positions judged from maps of *K. pneumoniae*, *S. typhimurium* and *E. coli*. The individual gene products are depicted in variously shaped boxes. The *ntr*C and *ntr*A products combine to form an activator, and the *ntr*C and *ntr*B products combine to form a repressor, of expression of *put*, *nif*, *hut*, *nar* and *gln*A. In *nif* they are shown as acting at the *nif*LA operon. Arrows within the circle indicate directions of transcription.



genetic control is *nif*-specific and is mediated by the *nifLA* operon (Fig. 1.1). Regulation of all the *nif* operons, except that of *nifLA* is mediated by the products of the *nifLA* operon (Buchanan-Wollaston *et al.*, 1981a). The *nifA* product is a transcriptional activator of the other *nif* transcriptional units (Roberts *et al.*, 1978; Dixon *et al.*, 1980; Buchanan-Wollaston *et al.*, 1981a). More recently, however, it has been shown that the product of *nifA*, as well as that of *ntrC*, can mediate activation of the *nifLA* promoter. Furthermore, this activation involving the *nifA* product, at this and other promoters requires the *ntrA* gene product (Ow and Ausubel, 1983). Repression by oxygen and fixed nitrogen involves the *nifL* product (Roberts *et al.*, 1978; Roberts and Brill, 1980; Hill *et al.*, 1981). The *nifL* product appears to antagonise the action of the *nifA* product in the presence of oxygen, and this action takes place at the transcriptional level (Buchanan-Wollaston *et al.*, 1981b). Studies have suggested that the *nifL* protein represses transcription by inactivating the *nifA* gene product, and that this repression does not involve DNA binding (Beynon *et al.*, 1983).

1.2f Nitrogen fixation in other bacteria. *K. pneumoniae* is one of the best studied N_2 fixing organisms. Most of what is known about other diazotrophs has been learned by comparison and extrapolation from the relatively detailed understanding of the *nif* genes in *K. pneumoniae*. The availability of numerous well characterised *K. pneumoniae* Nif$^-$ mutants, unable to fix N_2, has greatly facilitated

studies of the *nif* genes of other organisms. Such Nif$^-$ mutants have been widely used in complementation studies to investigate the genetic control and expression of *nif* genes. The advent of the acetylene (C_2H_2) test for N_2 fixation (in the mid-1960s) facilitated ecological, physiological and genetic studies of N_2 fixation. It is based on the observations of Dilworth (1966) and Schöllhorn and Burris (1967) that preparations of nitrogenase reduce C_2H_2 specifically to ethylene (C_2H_4), and has been widely used because of its ease and sensitivity.

Another approach to isolating and characterising genes consists of looking for DNA-DNA hybridisation with heterologous probes. This approach requires conservation of DNA sequences. The *nif* genes, particularly the structural *nifHDK* genes have been shown to be highly conserved in a large variety of phylogenetically unrelated diazotrophs (Ruvkun and Ausubel, 1980). The *nifHDK* genes of *K. pneumoniae* have been used to provide information about the organisation and to initiate cloning of comparable genes from a number of organisms. The conserved nature of the *nifHDK* DNA sequence is reflected in the translation products of these genes. Detroy *et al.* (1968) were the first to demonstrate that the Mo-Fe-protein from one organism, when added to the Fe-protein from a different organism, could generate an active N_2 fixing complex. These studies have been extended to different organisms, and alternative substrates by other researchers (for a review see Postgate, 1982).

1.2g DNA homology of the nitrogenase genes. Various *nif* genes cloned from *K. pneumoniae* have been widely used in hybridisation studies to identify and characterise the *nif* genes of other organisms. This is particularly the case for plasmid pSA30 which contains the entire *K. pneumoniae* operon for the three structural genes for nitrogenase, *nifHDK* (Cannon *et al.*, 1979). Comparative *nif* gene studies have revealed differences amongst organisms, including the location, arrangement and degree of DNA homology of the genes.

The *nif* genes of diazotrophs, symbiotically associated with the roots of higher plants, have been widely studied. In fast growing *Rhizobium* species *nif* genes are located on large plasmids (Rosenberg *et al.*, 1981) but slow growing *Rhizobium japonicum* species probably have *nif* genes on the chromosomes (Masterson *et al.*, 1982). Barbour *et al.* (1985) have presented evidence for plasmid- and chromosome-borne multiple copies of *nif* genes in *Rhizobium fredii*. In one strain, *R. fredii* USDA206, *nif* genes appeared to be present on the chromosome, the 197 mD plasmid and one or both of the smaller plasmids.

The *nifHDK* genes of *Rhizobium leguminosarum* have been cloned and found to be contiguous (Schetgens *et al.*, 1984). Similarly, other fast growing rhizobia, *Rhizobium meliloti* (Corbin *et al.*, 1982) and *R. japonicum* (Prakash and Atherly, 1984) also have a contiguous *nifHDK* gene cluster. By contrast, in the slow growing *Parasponia rhizobium* the *nifDK*

and nifH genes are separated (Weinman et al., 1984). In the slow growing R. japonicum, the nitrogenase structural genes are also organised on two separated transcriptional units: nifDK and nifH. Located near to these operons, new DNA regions (named nifB-, fixBC- and fixA-like genes) likely to be involved in N_2 fixation, have been identified and mapped (Fuhrmann et al., 1985).

Further studies of diazotrophs isolated from the roots of plants have characterised five N_2 fixing Enterobacter agglomerans strains which all have the nif structural genes located on plasmids (Singh et al., 1983). More recently, Väisänen et al. (1985) have identified two different biotypes among N_2 fixing E. agglomerans strains based on the nutritional and oxygen requirements of the strains. Whereas all the strains of biotype 1 carried nifHDK genes on a large plasmid, no plasmid was detected in any strains belonging to biotype 2.

DNA regions homologous to K. pneumoniae nifHDK probes were different in A. chroococcum and Azotobacter vinelandii strains, and inconsistent with the arrangement of the homologous sequences as a contiguous cluster of unique genes (Jones et al., 1984). In A. chroococcum two nifH-like sequences were present in the genome; one copy was closely linked to nifD and nifK and the order of genes was as for K. pneumoniae. There was no evidence suggesting reiteration of the other nitrogenase genes, and the biological significance of the reiteration is unknown. In Rhodospeudomonas

capsulata multiple copies of some nitrogenase genes, which are functionally interchangeable, have been reported (Scolnik and Haselkorn, 1984).

The non-heterocystous and heterocystous cyanobacteria have characteristic and fundamentally different nif gene arrangement which might serve different functions. Five non-heterocystous strains (unicellular and filamentous) had a contiguous nifHDK gene cluster, whereas all heterocystous strains examined showed separation of nifK from contiguous nifDH genes (Kallas et al., 1985). Recently, Golden et al. (1985) have reported an orderly gene rearrangement which is environmentally triggered in Anabaena strain 7120. An 11 kb DNA fragment separating nifK from nifHD becomes excised and circularised within differentiating heterocysts of Anabaena filaments starved of combined nitrogen. The rearranged, contiguous genes are then transcribed as a 4.7 kb polycistronic message, which appears in both aerobic and anaerobic N_2 fixing filaments. A gene required for the regulation of the 11 kb DNA excision event in Anabaena has been identified and sequenced (Lammers et al., 1986). The function of the non-continuous nif gene pattern remains obscure, but may be a regulatory phenomenon.

A gene arrangement, different from the heterocystous cyanobacteria (nifHD separated from nifK) but similar to slow growing rhizobia (nifH separated from nifDK) has been reported for methanogenic bacteria. Methanogens were previously not known to be N_2 fixing organisms. In four

methanogenic strains examined, evidence suggested that nifH and nifDK were not adjacent (Sibold et al., 1985).

1.2h DNA homology of nif genes other than nifHDK. Whereas the nifHDK genes were highly homologous, Ruvkun and Ausubel (1980) predicted that the other genes of the K. pneumoniae nif cluster would exhibit little, if any, homology to corresponding genes from other diazotrophs. This assertion was supported by studies on two methylotrophs where the interspecies nif homology was limited to DNA fragments encoding the nitrogenase structural proteins, nifHDK (Toukdarian and Lidstrom, 1984). In other diazotrophs however, DNA homology between various other nif genes has been reported. Norel et al. (1984) reported hybridisation of K. pneumoniae nifNE and nifJ to DNA from a Rhizobium species ORS571. The nifF and nifA genes of K. pneumoniae were found to hybridise to DNA from R. leguminosarum (Hontelez et al., 1984). Homology with K. pneumoniae nifA was also found in R. meliloti (Szeto et al., 1984a), Rhizobium trifolii (Scott et al., 1984) and a slow growing R. japonicum (Adams et al., 1984). Recently nifB was detected in a slow growing R. japonicum by interspecies hybridisation, and its position located between the nifDK and nifH genes (Fuhrmann et al., 1985).

No homology was detected between A. chroococcum genomic digests, and K. pneumoniae nifB, nifA, or nifL DNA but weak homology was detected for K. pneumoniae nifM, nifV and nifS (Jones et al., 1984). Although azotobacters contain a gene

analogous to nifA (Kennedy and Robson, 1983), no homology could be detected between K. pneumoniae nifA and A. chroococcum DNA (Jones et al., 1984).

In Anabaena 7120, homology with K. pneumoniae nifV DNA was detected (Rice et al., 1982), although no hybridisation was observed with nifA, nifNE or nifJ in the same strain (Sibold et al., 1985).

1.2i Recent developments. As indicated above, the numerous recent studies have revealed much about the structural organisation and regulation of nif genes from numerous diazotrophs. This information has elucidated aspects of nitrogen fixation and revealed unique characteristics of the system. These will be discussed in turn as follows:

(i) nifA and ntrC genes are evolutionarily related;

(ii) DNA sequence data available;

(iii) K. pneumoniae nif promoters have a characteristic structure;

(iv) interspecies conservation of nif promoter sequence;

(v) upstream activator sequences are present in the promoters of nitrogen fixation genes.

1.2i (i) The nifA and ntrC genes are evolutionarily related. Similarities between nifA and ntrC have been observed (Merrick, 1983; Ow and Ausubel, 1983). (a) The nifA product can activate ntrC-regulated promoters. (b) The nifA product, like the ntrC product, requires the ntrA product as co-activator. (c) The nifA and ntrC proteins

are similar in size and charge. (d) Both genes are the downstream gene of a two-gene regulatory operon. Ow and Ausubel (1983) have proposed that the chromosomal duplication event, which is believed to have occurred during the evolution of enteric bacteria, had at one point, provided an additional copy of *ntrC*, from which evolved a *nif*-specific regulator. The rationale for a *nif*-specific regulator is the advantage of an additional level of stringent control over this ATP-intensive pathway, for obtaining fixed nitrogen. In addition, because the *nifA* product has the capacity to activate other nitrogen metabolism genes, it may act as a general regulator of these genes during active nitrogen fixation.

The ancestral relationship between the *nifA* product of *K. pneumoniae* and the *ntrC* product of enteric bacteria is further reflected by *nifA*-like or *ntrC*-like *nif* regulatory systems in other N_2 fixing bacteria. In *A. vinelandii*, the *K. pneumoniae nifA* product can complement *nif* regulatory mutants, suggesting a *nifA*-like product in the wild type host (Kennedy and Robson, 1983). However, whereas the *K. pneumoniae nifLA* and *nifF* promoters are expressed when introduced into this host, the *K. pneumoniae nifH* promoter is not. In this respect, the *Azotobacter nif* gene activation bears a resemblance to the *nifC* product of enteric bacteria. An analogous situation is found in *R. meliloti* (Szeto *et al.*, 1984b). This work suggested that, whereas a strict requirement for the *K. pneumoniae nifA* product was the case for the *K. pneumoniae nifH* promoter,

the R. meliloti nifH promoter could respond to a number of nifA- and ntrC-like products. In E. coli the R. meliloti nifH promoter behaved as a typical ntrC-activated promoter.

The ability of the nifA product to substitute for the ntrC product in the activation of the nifL, glnA, aut and put genes led to the discovery of two consensus sequences located in the promoter regions of nifA/ntrC activated genes. In the case of the K. pneumoniae nifL and R. meliloti nifH genes, which are activated by either nifA or ntrC, the consensus sequence is **TTTTGCA** (5' - 3'), and is located in the -10 to -15 bp region. In contrast, the K. pneumoniae nif promoters which can only be activated by nifA contain a subset of heptameric sequence; **TTGCA** (5' - 3') at the same position (Beynon et al., 1983; Sundaresan et al., 1983).

1.2i (ii) DNA sequence data available. The recent DNA sequencing of several nitrogenase genes has revealed information about the promoters, transcriptional terminators and ribosome binding sites (RBS) of the putative mRNA products. From the DNA sequence, the amino acid sequences of the polypeptides have been predicted enabling the organisms to be characterised with respect to their preferred triplet codon usage. It must be cautioned that, because of the conserved nature of the nitrogenase genes, the codon usage for these genes does not necessarily reflect that for other genes in the organism. Interspecies comparisons of the amino acid sequences are of invaluable

help in detecting functionally important domains within the primary structure of the nitrogenase polypeptides.

As iron-sulphur proteins, the cysteine residues of nitrogenase are of particular interest. In K. pneumoniae, the Fe-protein has five conserved cysteines located in highly homologous regions, with cysteines 97 and 132 as probable ligands for the binding of the single [4Fe:4S] cluster, which may be bound symmetrically between the subunits (Hausinger and Howard, 1984). The exact number of functionally important cysteine residues in the Mo-Fe-protein is not yet known. The α subunit has five conserved cysteines but the number for the β subunit is uncertain due to insufficient sequence information (Lammers and Haselkorn, 1983). Structural and functional homology between the α and β subunits of the nitrogenase Mo-Fe-protein of R. japonicum has recently been reported (Thöny et al., 1985). The β subunit has three strongly conserved cysteine residues (at positions 69, 94 and 152) which are located in an area sharing amino acid sequence homology with the α subunit, which in turn has analogous cysteines at positions 67, 93 and 159. This could reflect the structural requirements of the α and β polypeptides for the binding of [4Fe:4S] clusters, and may indicate that nifD and nifK have evolved from a common ancestral gene.

The complete nucleotide sequence for the entire nifHDK structural gene cluster is available for:

A. vinelandii (Brigle et al., 1985).

Complete nucleotide sequences have been published for nifH from the following organisms:

R. meliloti (Török and Kondorosi, 1981),

R. japonicum (Fuhrmann and Hennecke, 1984),

P. rhizobium (Scott et al., 1983a),

Cyanobacterium (Mevarech et al., 1980),

K. pneumoniae (Hausinger and Howard, 1980; Scott et al., 1981; Sundaresan and Ausubel, 1981),

R. trifolii (Scott et al., 1983b),

Clostridium pasteurianum (Chen et al., 1986).

Nucleotide sequences for nifD are available for:

Anabaena (Lammers and Haselkorn, 1983),

R. japonicum (Kaluza and Hennecke, 1984),

a cowpea Rhizobium strain (Yun and Szalay, 1984),

P. rhizobium (Weinman et al., 1984).

The DNA sequence of nifK has been determined for:

Anabaena (Mazur and Chui, 1982),

R. japonicum (Thöny et al., 1985),

P. rhizobium (Weinman et al., 1984).

Related genes which have been sequenced include:

nifE from A. vinelandii (Dean and Brigle, 1985),

nifA and ntrC from K. pneumoniae (Drummond et al., 1986).

Complete amino acid sequences have been established for purified Fe-proteins from:

C. pasteurianum (Tanaka et al., 1977),

A. vinelandii (Hausinger and Howard, 1982),

Rhizobium phaseoli (Quinto et al., 1985),

and for the purified Mo-Fe-protein α subunit from:

C. pasteurianum (Hase et al., 1984).

1.2i (iii) K. pneumoniae nif promoters have a characteristic structure. The DNA sequences and transcription initiation sites of the different K. pneumoniae nif promoters have been determined (Fig. 1.3). These promoters have a similar structure, characterised by the consensus sequence (Beynon et al., 1983):

5' - **TTGCA** - 3' at -15 to -1 bp and

5' - **CTGG** - 3' at -26 to -23 bp.

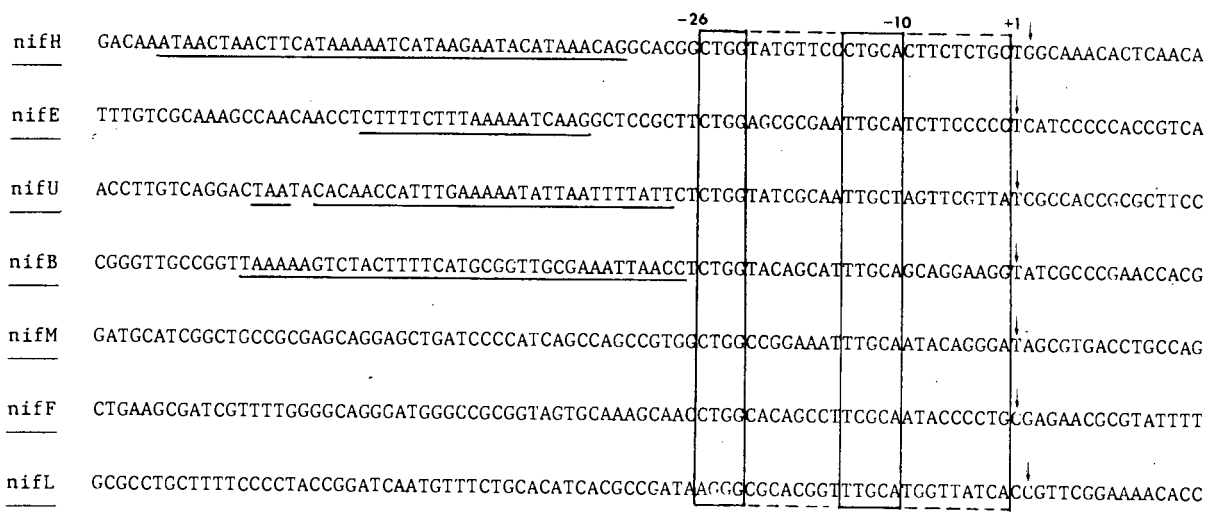
The K. pneumoniae nif promoters exhibit poor homology to the consensus promoters of Escherichia coli and other enteric bacteria:

	-35 ▽		-10 ▽		
E.coli:	TTGACA	N_{16-19}	TATAAT	N_{6-9}	+1
		-23 ▽	-10 ▽		
nif:		CTGG N_8	TTGCA	N_9	+1

(By convention, DNA sequences are given in the 5' to 3' direction, N represents an unspecified bp, and the start of transcription is denoted position 1). Beynon et al. (1983) have suggested that for nif promoters the -10 homology is

Fig. 1.3 Comparison of *nif* promoter sequences (taken from Beynon *et al.*, 1983).

By convention the DNA sequences are given in the 5' to 3' direction. Bases at which transcription is initiated are marked by arrows. The characteristic -26 and -10 bp structures of the *nifA* activated promoters are indicated in the boxes. Areas protected from DNase digestion are underlined.



equivalent to a Pribnow box for promoters expressed under nitrogen-starved conditions, and that the -23 homology confers activator specificity on the *nif* promoters.

1.2i (iv) Interspecies conservation of *nif* promoter structure. Sundaresan *et al.* (1983) compared the *R. meliloti* and *K. pneumoniae* *nifH* promoter sequences, and observed extensive regions of homology. These homologous regions coincided with those already observed amongst the various *nif* promoters of *K. pneumoniae*. Similar conserved sequences were suggested for *A. vinelandii* (Kennedy and Robson, 1983). Despite the DNA sequence similarities observed in these genera, *nif* promoter structure, and by extension *ntr* promoter structure, does not appear to be universally conserved. In the heterocystous cyanobacterium *Anabaena* 7120, the *nifH* promoter has the sequence **TCTAC** at the -14 to -10 bp region instead of **TTGCA** (Tumer *et al.*, 1983).

1.2i (v) Upstream activator sequences are present in the promoters of nitrogen fixation genes. As described previously, the *nif* promoters lack the canonical -35 and -10 promoter elements, but require for their activation nucleotide sequences located around the -26 and -10 bp regions. Buck *et al.* (1986) have identified and characterised another essential promoter sequence in the *K. pneumoniae* *nifH*, *nifU*, *nifB* and open reading frame (ORF) promoters, located more than 100 bp from the transcription initiation site. The promoter element is required for

nifA- (Brown and Ausubel, 1984) but not for *ntrC*-mediated activation, and for the inhibition of chromosomal *nif* expression observed when cells harbour multiple copies of certain promoters (Buchanan-Wollaston *et al.*, 1981b). The upstream sequence was shown to be conserved among the ten *Rhizobium* and two *Azotobacter* *nif* promoters (Buck *et al.*, 1986). This study showed further that the positioning and orientation of the upstream sequence was not critical for promoter activity up to a distance of 2 kb, and that the upstream sequence itself was transcriptionally inactive, probably acting in *cis* with the downstream sequences to produce a fully active promoter. The conserved sequence conforms to a consensus sequence for protein binding sites on DNA (Gicquel-Sanzey and Cossart, 1982) and could be a site at which the *nifA* product interacts with *nif* promoters. The upstream activation sequences are characterised by invariant **TGT** and **ACA** regions as indicated (Buck *et al.*, 1986):

nif consensus: $\begin{matrix}G\\A\end{matrix}$ N_7 TGT N_4 T N_5 ACA

protein binding site: TGTGT N_{6-10} ACACA

1.3 GENETICS OF *T. FERROOXIDANS*

1.3a Establishing a genetic system. Although its unusual metabolism has been extensively studied (Brierley, 1978),

little is known about the genetics of T. ferrooxidans. The development of a genetic system is of fundamental importance to the understanding of the molecular biology of any microorganism. Various requirements must be fulfilled in order to establish a genetic system. Firstly, there must be at least one, genetically inherited, readily detectable, phenotypic difference between strains. Secondly, a system must exist to transfer genes between these strains. Vector DNA molecules which can replicate in T. ferrooxidans, are able to accommodate and thus transfer inserted DNA, must be identified and characterised. Thirdly, a method must exist enabling the recognition of the phenotypic change, resulting from the transferred genetic material. No method exists at present for introducing genetic material into the organism. Recent conjugation studies, suggesting the use of Thiobacillus novellus as an intermediary host, represents the most plausible method to date, of transferring DNA from E. coli to T. ferrooxidans (Rawlings et al., 1985).

1.3b Aim of this study. Molecular genetic studies rely on a basic understanding of gene expression. Since little is known about gene expression in T. ferrooxidans, the aim of this study was to investigate the expression and regulation of genes of T. ferrooxidans. The characterised gene pool available for genetically manipulating T. ferrooxidans is derived almost entirely from heterotrophic bacteria making it imperative to ascertain the extent of similarity between the bacteria. The gene expression of plasmid and chromosomal genes was investigated. Natural plasmids of T.

ferrooxidans were isolated, cloned into suitable vectors and characterised for plasmid-encoded gene functions.

Although T. ferrooxidans is extremely efficient in scavenging nitrogen in the form of ammonia, the scarcity of nitrogen in leach liquors may limit the efficiency of bacterial leaching operations. The presence and organisation of genes involved in nitrogen fixation was investigated in a number of strains. The structural genes for the nitrogenase enzyme were cloned from T. ferrooxidans ATCC33020, and the expression and regulation of these genes were investigated. Finally, functionally important domains were identified within the DNA primary structure, by determining the DNA sequence of the cloned T. ferrooxidans nitrogenase genes.

CHAPTER TWO

GENE EXPRESSION OF *T. FERROOXIDANS* RECOMBINANT PLASMIDS

CHAPTER TWO

GENE EXPRESSION OF T. FERROOXIDANS RECOMBINANT PLASMIDS

Summary. Recombinant DNA vehicles were constructed between cryptic plasmids of T. ferrooxidans and E. coli. The recombinant plasmids were characterised. The ori of pBR325 was deleted and one recombinant plasmid was shown to replicate in E. coli using an ori located on the T. ferrooxidans DNA. This plasmid had a relatively broad host range which increased its suitability as a potential cloning vector for T. ferrooxidans. The expression of autotrophic T. ferrooxidans genes in a heterorophic E. coli cell-free system was demonstrated.

2.1 INTRODUCTION

The genetic manipulation of bacteria depends to a large extent on plasmids. They serve as vehicles for the introduction and propagation of new genetic material in the development of genetically altered bacterial strains.

Numerous isolates of T. ferrooxidans have been shown to contain plasmid DNA (Mao et al., 1980; Rawlings et al., 1983; Martin et al., 1983). To date, no identifiable

phenotype has been conclusively linked to the presence of these plasmids. In order to investigate whether any T. ferrooxidans plasmid functions are expressed in heterotrophic Gram-negative bacteria, several T. ferrooxidans plasmids were cloned into suitable E. coli plasmid vectors pBR322 and pBR325 (Rawlings et al., 1983; 1984).

A unique feature of the natural habitat of T. ferrooxidans is the high concentration of toxic inorganic ions. Genes encoding resistance to various heavy metal ions and antibiotics were shown to be plasmid encoded in Staphylococcus aureus (Novick and Roth, 1968). Resistance to arsenate, arsenite and antimony are genetically linked on the plasmids of Gram-positive and Gram-negative bacteria, and the mechanism of resistance to these toxins has been greatly elucidated in studies of E. coli and S. aureus (Foster, 1983). Plasmid determined resistance to cadmium, mercury and organomercury has been studied extensively in S. aureus and in Gram-negative bacteria (Foster, 1983). T. ferrooxidans strains which are inherently tolerant to various metals have been isolated (Holmes et al., 1983), and the genes conferring this resistance could be plasmid borne. Establishing a correlation between the presence of a particular size plasmid and uranium resistance was attempted in T. ferrooxidans strains isolated from a uranium mine in Canada. Concurrent with the loss of uranium resistance, the plasmid could no longer be detected (Martin et al., 1983). Such circumstantial evidence is not sufficient, however, to

establish a causal relationship, and at present all T. ferrooxidans plasmids are cryptic. Metal ion tolerance is a particularly attractive marker for genetic studies in T. ferrooxidans since it is a useful laboratory marker for which plasmid mediated resistance is known to occur, and also has the potential for conferring an industrially significant characteristic on the organism (Brierley, 1982). Screening the recombinant T. ferrooxidans plasmids for plasmid encoded metal ion resistance in E. coli and Pseudomonas aeruginosa formed the first investigation into T. ferrooxidans gene expression. Antibiotic resistance, which can be plasmid mediated, has been widely used as selectable markers in heterotrophic bacteria, and although the acidic natural environment would be expected to inhibit most antibiotic activity, the T. ferrooxidans recombinant plasmids were screened for possible plasmid mediated antibiotic resistance.

For plasmids to serve as vehicles for the propagation of new genetic material they must be transferable to the new strain, and stably maintained within the cells. Although a few broad host range plasmids exist, the majority of plasmids are able to replicate only within a single species or closely related bacteria. Davidson and Summers (1983) and Kulpa et al. (1983) have demonstrated the transfer of the broad host range plasmid RP4 from E. coli to T. novellus and Thiobacillus neopolitanus respectively, and from these thiobacilli back to E. coli. These strains are however, genetically unrelated to T. ferrooxidans (Lane et al., 1985)

and no foreign plasmids have so far been shown to replicate in T. ferrooxidans. The nature of the origins of replication of the hybrid plasmids was investigated. Cloning experiments were conducted to localise sites on the T. ferrooxidans DNA necessary for replication of the recombinant plasmids. Their ability to be stably maintained in a variety of host strains was determined.

All known extrachromosomal elements exist in well defined intracellular copy numbers. For many such elements, genetic evidence exists supporting the postulation that plasmid-specified information is involved in copy number control (Nordström et al., 1972). For some plasmids this number may be very low (1 - 5 copies per cell), yet they are stably inherited and plasmid-free cells are rarely formed. The suggested plasmid determined factors which control plasmid copy number per cell include:

a) incompatibility of plasmids which can result in some instability;
b) size; larger plasmids tend to be less stable than smaller plasmids;
c) selection for a gene on a plasmid may increase the copy number and stability of the plasmid (Futcher and Cox, 1984);
d) efficiency of plasmid replicon (the replicon is defined as the smallest part of the plasmid which carries all information needed for replication) (Nordström et al., 1980).

In addition to being stably propagated in different bacterial strains, desirable characteristics of cloning vehicles include being present in multiple copies in the host cell. The copy numbers of four derivatives of one of the T. ferrooxidans recombinant plasmids were determined in E. coli to investigate further gene expression and their potential use as cloning vectors.

When DNA segments are cloned into plasmid vectors, it is often desirable to examine the protein products encoded by the inserted fragment. Such studies can yield information about the regulation of transcription and translation of the cloned DNA, and are of value where no phenotypic expression can be detected, as was the case for the T. ferrooxidans recombinant plasmids. It is thus important to determine whether any genes from T. ferrooxidans are expressed in E. coli. For this reason, the plasmid encoded polypeptides of the T. ferrooxidans recombinant plasmids were investigated in a heterotrophic bacterial system.

Several methods have been used to study plasmid products including minicells, maxicells and cell-free DNA-directed transcription-translation systems. All three procedures involve the selective labelling of proteins using radioactive amino acids, followed by gel electrophoresis and autoradiography.

In minicell producing strains, abortive cell division results in the generation of relatively stable, anucleate

minicells. Since plasmids present in the cell during growth are segregated uniformly to the minicells, the exclusive production of polypeptides encoded by these plasmids can be monitored (Meagher *et al.*, 1977). Maxicells are *uvrA*, *recA* mutants of *E. coli* which degrade host chromosomal, but not plasmid DNA following low doses of UV light irradiation. Plasmid coded protein synthesis continues 18 - 24 h after irradiation facilitating their study (Sancar *et al.*, 1979). Problems frequently encountered with these techniques include: difficulty in transforming the strains and high background labelling of host encoded polypeptides. These *in vivo* associated problems can be circumvented by using an *in vitro* DNA-directed transcription-translation system, the components of which can be purified or obtained in kit form. The *T. ferrooxidans* plasmid gene products were studied in an *E. coli in vitro* system.

2.2 MATERIALS AND METHODS

2.2a Bacterial strains and plasmids. The bacterial strains and plasmids are listed in Table 2.1. The *T. ferrooxidans* recombinant plasmids are described in Table 2.2.

2.2b Construction and restriction mapping of *T. ferrooxidans* plasmids. Plasmid DNA was isolated from the *T. ferrooxidans* strains TF35, FC and ATCC33020 and cloned into the *E. coli* vectors pBR325 or pBR322 by Dr D E Rawlings as reported by Rawlings *et al.* (1983; 1984). Unless otherwise stated, the

Table 2.1 Bacterial strains and plasmids.

Strain or plasmid	Genotype	Reference or source
T. ferrooxidans FC	wt	Fairview Mine, South Africa
T. ferrooxidans TF35	wt	C Nicolau[a]
T. ferrooxidans	wt	ATCC33020
E. coli HB101	*pro*$^-$ *leu*$^-$ Ap^s	Maniatis *et al.* (1982)
E. coli ED8654	*hsdR hsdM*	K. Ellis[b]
K. pneumoniae KP5022	*hisD2 hsdR1* *nif*$^+$	R. Robson[c]
P. aeruginosa PA01162	*leu*-38 *rmo*-11	Bagdasarian and Timmis (1982)
pBR322	Ap^r Tc^r	Bolivar *et al.* (1977) (Appendix D)
pBR325	Ap^r Tc^r Cm^r	Bolivar (1978) (Appendix D)
R46	Ap^r Tc^r Kan^r	Brown and Willetts (1981)

a C. Nicolau: Centre de Biophysique Moleculaire, C.N.R.S., Orlèans, France.

b K. Ellis: University of London, UK.

c R. Robson: University of Sussex, Brighton, UK.

Table 2.2 *T. ferrooxidans* recombinant plasmids.

T. ferro-oxidans plasmid	Size kb	Cloning site	Cloning vector	Selectable marker(s)	Recombinant plasmid designation[a]
pTF35	19.6	HindIII	pBR322	Ap^r	pDR301 pDR302
pTF35	19.6	HindIII	pBR325	Ap^r Cm^r	pDR330 pDR331
pTF-FC2	12.4	PstI	pBR325	Tc^r Cm^r	pDR401 pDR403
pTF33020-1	6.7	HindIII	pBR325	Ap^r Cm^r	pDER501
				Tc^r Ap^r Cm^r	pDER502
pTF33020-2	11.5	HindIII	pBR325	Ap^r Cm^r	pDER502
				Tc^r Ap^r Cm^r	pDER602

[a] The different designations are the two orientations of the *T. ferrooxidans* plasmid in the cloning vector.

standard molecular genetics techniques compiled by Maniatis et al. (1982) were followed. Transformants were selected on Luria agar (LA) (Appendix C) using antibiotics at the following concentrations (μg/ml) Ap 100, Cm 25, Tc 25. The T. ferrooxidans plasmids (Table 2.2) were denoted interchangeably pDR or pDER, but the number code for a particular plasmid is consistent. Transformants were screened until both orientations were found of the T. ferrooxidans DNA in the cloning vector. Restriction maps were determined for the plasmids by single and double digestion analysis as described in Appendix A (Figs. 2.1 and 2.2). The cloning of plasmids pDR404 and pDR420 (Fig. 2.3) was described by Rawlings et al. (1984) and involved the 7.5 kb arsenic resistant (As^r) and tetracycline resistant (Tc^r) fragment of plasmid R46.

2.2c Screening for metal and antibiotic resistance. The T. ferrooxidans recombinant plasmids (Table 2.2) were transformed into the E. coli strains HB101 or ED8654. The minimum inhibitory concentration (MIC) values for the E. coli recipients, for each metal and antibiotic, were established by incorporating metal salt or antibiotic solutions into LA and inoculating with a standardised E. coli suspension. LA plates were poured containing the metal salt or antibiotic at concentrations ranging around the determined MIC. The resistance of the E. coli cells containing each of the T. ferrooxidans recombinant plasmids was compared with E. coli containing only pBR322 or pBR325. Resistance to the following metal salts was tested in this

Fig. 2.1 Restriction maps of pDER330, pDER331, pDER401 and pDER403, indicating their cloning orientation in the vector pBR325. The bold lines represent pBR325 vector DNA. The extent of the XhoI-SalI deletion of pDR401, which generated plasmid pDR412, is indicated as well as the site (a) which is essential for replication of pDR412, 0: ori of pBR325.

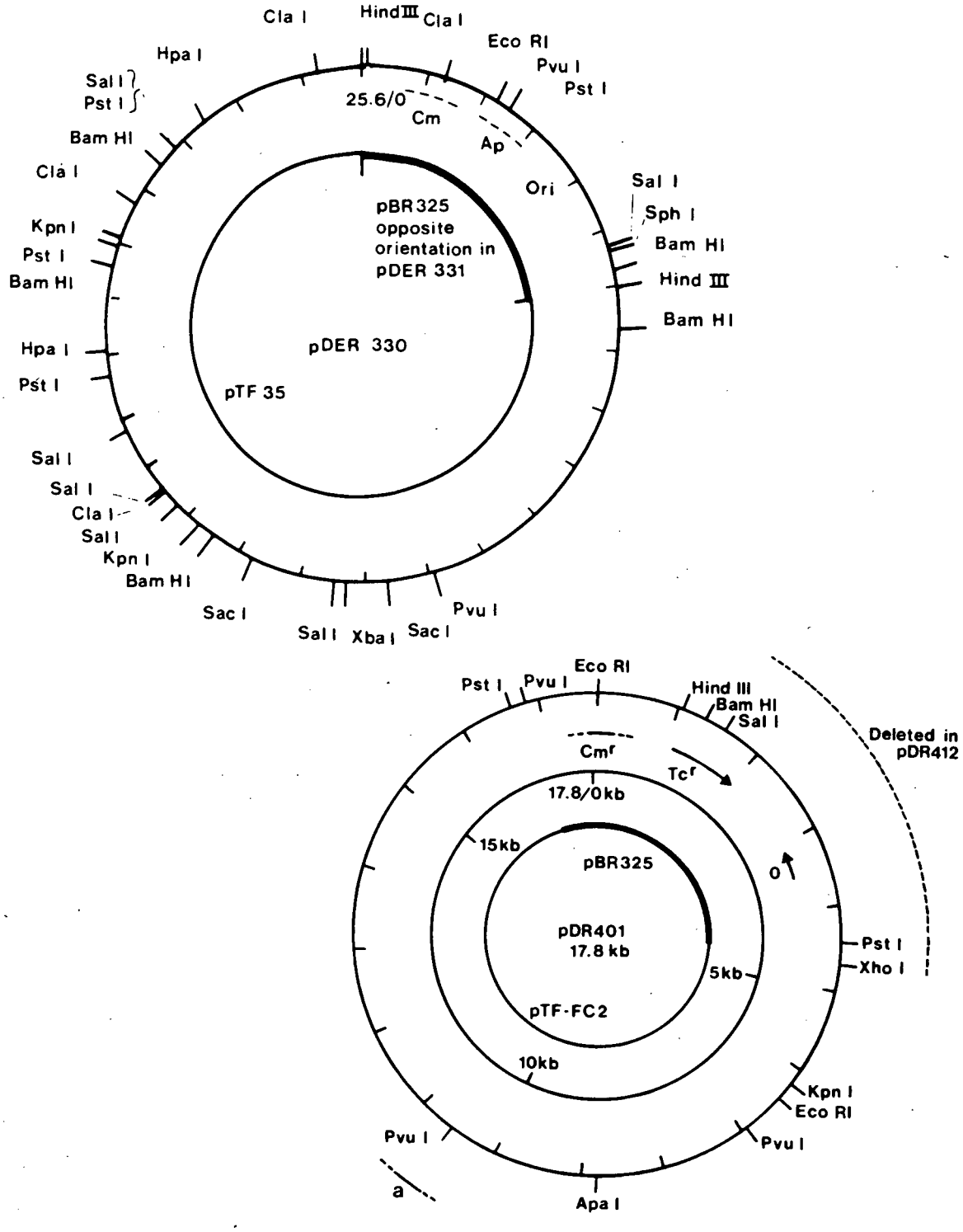


Fig. 2.2 Restriction maps of plasmids pDER501, pDER502, pDER601 and pDER602 indicating their cloning orientation in the vector pBR325. The bold lines represent pBR325 vector DNA.

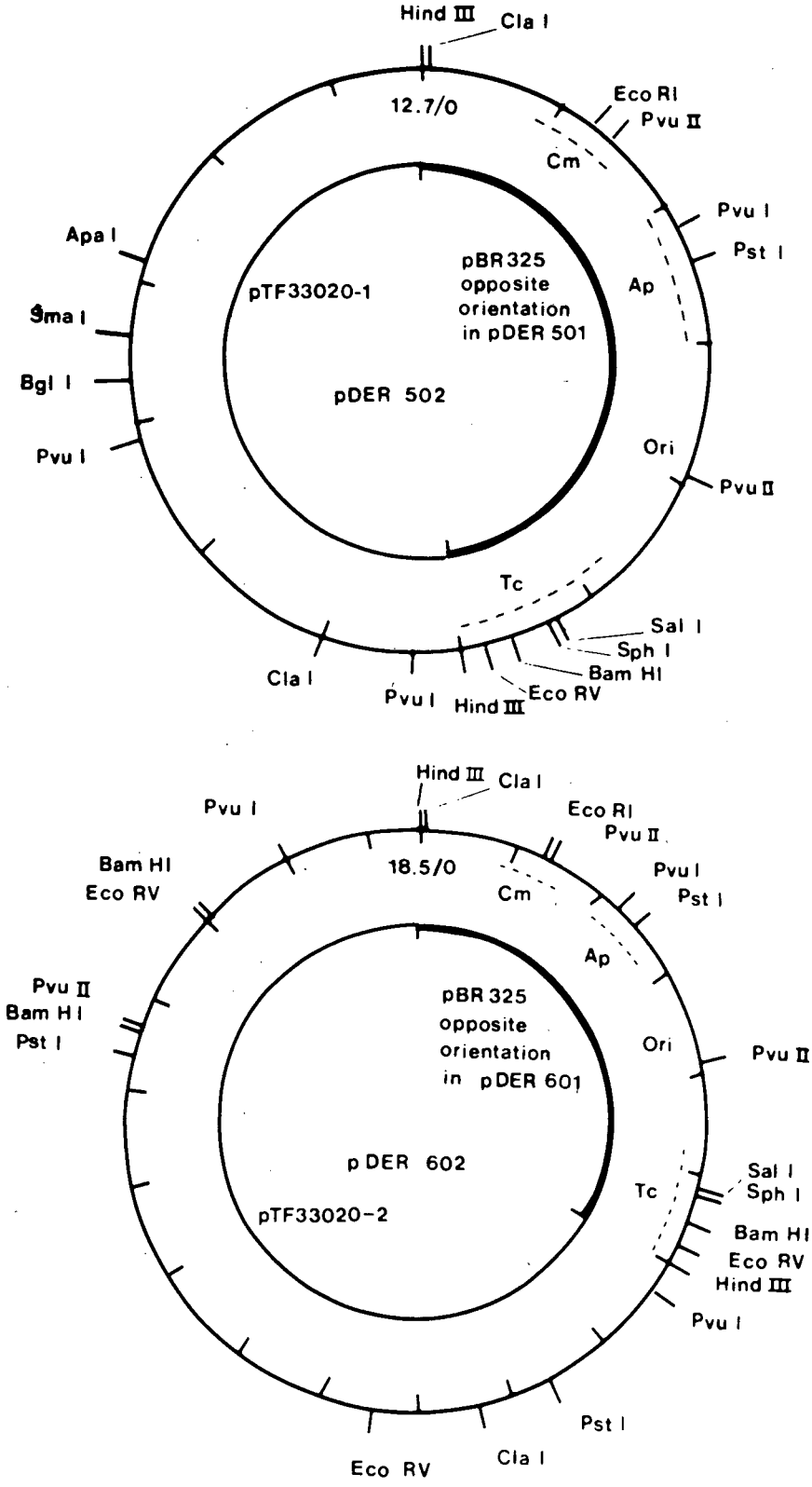
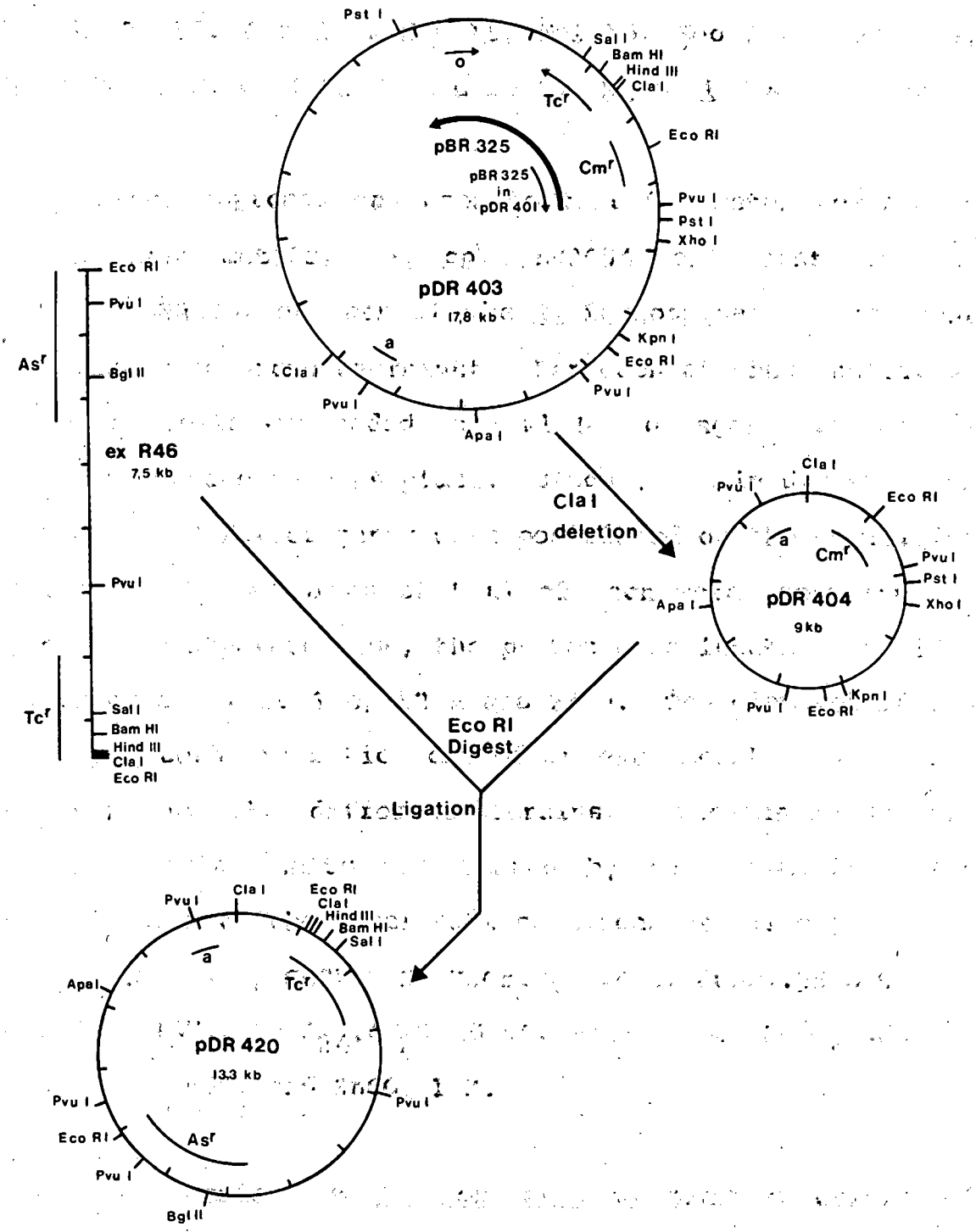


Fig. 2.3 Construction of recombinant plasmids from *T. ferrooxidans* and *E. coli* plasmids. The deletion plasmid pDR404 was ligated to the EcoRI fragment of the IncN plasmid R46 which conferred arsenic resistance (As^r) to the recombinant plasmid pDR420. a: *T. ferrooxidans* site essential for replication in *E. coli*, 0: ori of pBR325.



2.1 and 2.2. Experiments were carried out to localise sites on the T. ferrooxidans DNA which were essential for replication of plasmids pDR401, pDR330, pDR331, pDER501, pDER502, pDER601 and pDER602. Since the position of the origin of replication (ori) of pBR325 is known, it was possible to delete this region and test whether the recombinant T. ferrooxidans plasmid was able to replicate in the absence of the pBR325 ori.

2.2e Host range of T. ferrooxidans recombinant plasmids. The pDER plasmids were transformed into competent P. aeruginosa cells according to the E. coli transformation method (Appendix A) and into competent K. pneumoniae cells by the method described in Chapter 4.2b. Transformants were selected by the appropriate antibiotic markers and plasmid isolation, followed by restriction endonuclease analysis, verified their presence in the host cells.

2.2f Determination of plasmid copy number. The plasmids pBR325, pDR401, pDR404, pDR412 and pDR420 were transformed into E. coli ED8654 and grown in Luria broth (LB) (Appendix C) without antibiotic selection, to a standardised cell density. From each strain, total cellular DNA and plasmid DNA were isolated as described in Appendix A, but the plasmid DNA isolation involved two successive CsCl gradients to obtain high purity plasmid DNA. The DNA concentration was determined using a Beckman DU-8 spectrophotometer. DNA dilution series were made in a standardised minimum volume for total cellular DNA (15 - 0.15 µg) and plasmid DNA (0.5 -

0.015 µg), and these were dotted onto pre-wetted GeneScreen membrane. The membrane was dried, baked and prehybridised as described in Appendix B. Since the plasmids investigated were derivatives of pDR401, this plasmid could be used as the homologous probe throughout the experiment. Approximately 1 x 10^6 CPM of ^{32}P-labelled probe was added per membrane, and the hybridisaton, washing and autoradiography conditions were as described in Appendix B.

2.2g ***In vitro*** **transcription and translation studies of recombinant plasmids.** The synthesis of proteins by plasmids pDR401, pDR403, pDR404, pDR412 and pBR325 was investigated in an *E. coli* DNA-directed cell-free system (Enzo Bio Probe, New York, NY, USA) by the method of Yang *et al.* (1980). The procedure followed was as specified by the manufacturers, except that half quantities were used throughout. pBR325 DNA (2.5 µg) was used, while 2.5 - 8.5 µg DNA was used for the other plasmids depending on plasmid size and estimated promoter efficiency relative to pBR325. For each plasmid, the translated polypeptides were labelled with 15 µCi L-[^{35}S]methionine (specific activity 1040 Ci/m mol). The samples were precipitated and prepared for resolution by SDS-PAGE as specified by the manufacturers. Approximately 2 x 10^5 CPM of radio-labelled sample were loaded per well of a 15% SDS-polyacrylamide gel (Laemmli, 1970; O'Farrell, 1975) which was prepared and processed as described in Appendix B. Polypeptide molecular mass markers covering a range of 14.4 - 94 kD were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden) and used as specified by the manufacturers.

The gels were dried and autoradiography was continued for 24 - 60 h (as described in Appendix B), to visualise the plasmid encoded polypeptides.

2.3 RESULTS AND DISCUSSION

2.3a Construction and restriction mapping of *T. ferrooxidans* recombinant plasmids. The *T. ferrooxidans* plasmid sizes, the *E. coli* plasmid vectors and cloning sites used in the construction of the recombinant plasmids are given in Table 2.2. *E. coli* ED8654 transformants displaying the expected antibiotic resistance were screened for insertional inactivation of the antibiotic gene containing the cloning site. This was followed by restriction endonuclease analysis and the resulting plasmid maps are given in Figs. 2.1 and 2.2.

In the case of plasmids pTF33020-1 and pTF33020-2, the Tc^r gene of pBR325 was inactivated when the plasmid was cloned in one orientation, but functional (MIC > 20 µg/ml) when cloned in the opposite orientation. The *Hin*dIII site is situated within the promoter region of the Tc^r gene of pBR325 (Sutcliffe, 1979), and cloning into the *Hin*dIII site does not always inactivate the Tc gene expression (Bolivar *et al.*, 1977). The most probable explanation for the functioning of the Tc^r gene in the case of pDER502 and

Fig. 2.4 (on following page) Plasmids investigated to locate the T. ferrooxidans site(s) necessary for plasmid replication. Only restriction sites relevant to this study are indicated. Fragment sizes are given in kb, the bold lines indicate pBR325 vector DNA and the thin lines represent T. ferrooxidans plasmid DNA. Full explanations are given in the Results and Discussion (2.3c) of:

(a) pDR401 was digested with SalI and XhoI to form the deletion plasmid pDR412;

(b) pDER601 and pDER602 were analysed by PstI digestions;

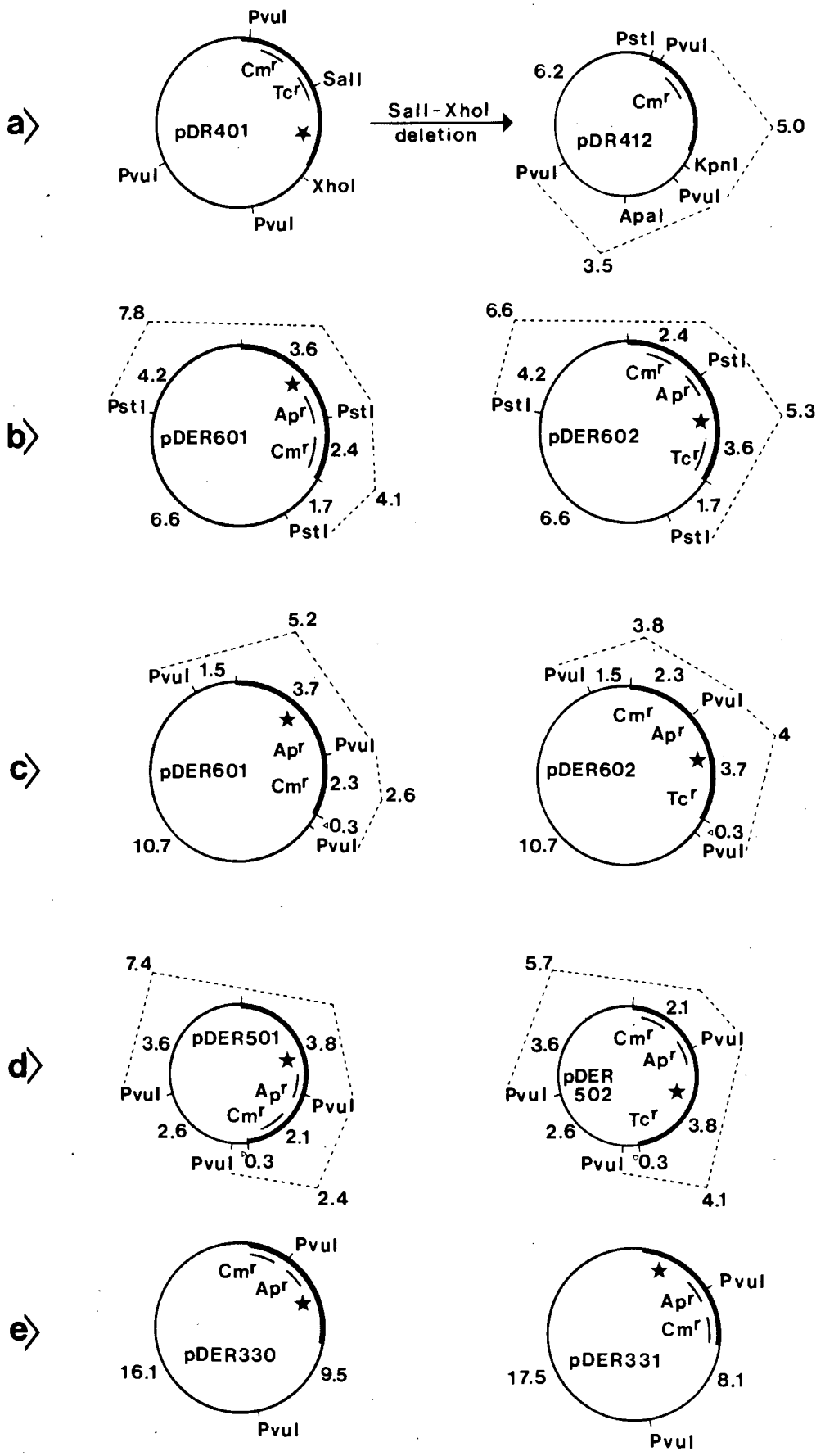
(c) pDER601 and pDER602 were analysed by PvuI digestions;

(d) pDER501 and pDER502 were analysed by PvuI digestions;

(e) pDER330 and pDER331 were analysed by PvuI digestions.

*: ColE1 ori of pBR325.

Fig. 2.4 (Legend on previous page)



pDER602, is that the gene is able to be transcribed from regulatory DNA sequences situated within the T. ferrooxidans plasmid insert. This implies that E. coli is able to recognise a T. ferrooxidans promoter or regulatory sequence.

2.3b Screening for metal and antibiotic resistance. The recombinant T. ferrooxidans plasmids were transformed into E. coli and the resistance of transformants to Ag, As^{III}, As^{V}, Cd, Co, Cr, Cs, Cu, Hg, Li, Mo, Ni, Sb, Te, Pb, U and Zn was tested. The recombinant plasmids did not affect the metal ion tolerance of the transformants. Furthermore, the plasmids did not confer resistance to the ten commonly used antibiotics which were tested: Ap, Cb, Cm, Cln, Kan, Nal, Neo, Pen-G, Str and Tc.

2.3c Location of sites on T. ferrooxidans DNA necessary for plasmid replication in E. coli. Two experimental approaches were used to test whether the T. ferrooxidans recombinant plasmids, not containing the pBR325 ori could replicate in E. coli. The first experimental approach involved pDER401 where it was possible to construct a deletion plasmid from which the pBR325 ori had been removed. As digestion of DNA with the restriction enzymes SalI and XhoI produces identical overlapping sticky ends, it was possible to construct a SalI-XhoI deletion plasmid from pDR401 (Figs. 2.1 and 2.4a). Removal of the SalI-XhoI 3.1 kb fragment would result in a new plasmid which had lost the pBR325 ori, approximately half of the Tc gene and a 0.3 kb fragment of T. ferrooxidans pTF-FC2 DNA. Plasmid pDR401 was digested

with SalI and XhoI, ligated and Cm^r, Tc^s and Ap^s E. coli transformants were shown to contain the 14.7 kb deletion plasmid pDR412 (Fig. 2.4a).

Experiments were carried out to localise site(s) essential for replication in pDR412. Complete digestion of pDR412 with PvuI produced two fragments of 3.5 and 6.2 kb which were composed entirely of T. ferrooxidans DNA (pTF-FC2) and a 5.0 kb fragment which contained the pBR325 Cm^r gene and approximately 2 kb of T. ferrooxidans plasmid DNA. These three fragments were religated and transformed into E. coli. Cm^r transformants were obtained at a very low frequency (20 transformants/μg DNA). Unligated PvuI control digests yielded no transformants. The isolation and restriction analysis of the plasmids from 32 E. coli transformants indicated that all three PvuI retriction fragments were always present (Fig. 2.4a). Smaller plasmids without a full complement of PvuI sites were never obtained. The 5.0 kb fragment contains the Cm^r marker and will always be present since it is selected. The requirement for both the 3.5 and 6.2 kb fragments suggests that the T. ferrooxidans ori is located at the PvuI site which joins these two fragments. If this suggestion is correct then the 3.5 and 6.2 kb fragments, joined by the PvuI site, must always be present in the same orientation. However the 5.0 kb fragment containing the Cm^r gene may be present in either orientation.

In order to confirm this suggestion the pDR412 plasmids

obtained by religation of the three PvuI fragments were restricted with a PstI-ApaI double digest. In all cases the religated plasmids contained a 7.7 kb fragment which indicated that the 6.2 kb PvuI fragment had been religated in the same orientation as in the uncut parent pDR412 plasmid. KpnI-ApaI double digests of the religated pDR412 plasmids generated either 2.5 and 12.2 kb fragments or 6.2 and 8.5 kb fragments which demonstrated that the 5 kb PvuI fragment could be present in either orientation. These two orientations occurred in approximately equal proportions amongst the transformants. Since the ApaI site is approximately in the middle of the 3.5 kb PvuI fragment, a PstI-ApaI double digest would produce a 7.7 kb fragment for either orientation of this fragment. However as the orientation of the 6.2 kb fragment was always fixed with respect to the 3.5 kb fragment it is concluded that the site of an essential gene for replication incorporates the PvuI site which joins them, and it is likely that the 3.5 kb PvuI fragment is also present in the reconstituted plasmids in the same orientation as the parent plasmid.

No similar, convenient strategy existed to construct deletion plasmids which lacked the pBR325 ori in the case of pDR330, pDR331, pDER501, pDER502, pDER601 or pDER602. A different experimental approach was therefore adopted to localise sites on the T. ferrooxidans DNA necessary for plasmid replication. For each plasmid pair a restriction enzyme was chosen which generated three fragments (two fragments in the case of pDR330 and pDR331) such that the

gene for the selected resistance was not situated on the same fragment as the pBR325 ori. After complete digestion, a ligation would randomly link combinations of the fragments. Selection for the particular antibiotic marker would ensure that all transformants contained a minimum of that fragment bearing the antibiotic gene. Any unselected fragment always found present in transformants, could be interpreted as carrying a site(s) required for the replication of the plasmid.

A PstI digestion of pDER601 generated three fragments: a 4.1 kb fragment carrying a selectable Cm^r marker linked to a 1.7 kb T. ferrooxidans DNA fragment; a 7.8 kb fragment bearing the pBR325 ori linked to a 4.2 kb T. ferrooxidans DNA fragment; and a 6.6 kb fragment consisting entirely of T. ferrooxidans DNA (Fig. 2.4b). After complete PstI digestion, the fragments were ligated in the Cm^r E. coli ED8654 transformants selected and analysed. If the unselected 7.8 kb fragment (bearing the pBR325 ori linked to a 4.2 kb T. ferrooxidans DNA fragment) was always present, two interpretations of this result would be possible, namely: (a) that the pBR325 ori was necessary for plasmid replication; or (b) that the T. ferrooxidans 4.2 kb DNA fragment carried the necessary origin of replication site. To elucidate this possible ambiguous result, pDER602 was analysed by the same procedure. Since pDER602 differs from pDER601 with respect to the cloning orientation of pBR325, the PstI fragment (5.3 kb) bearing the pBR325 ori was linked to the 1.7 kb T. ferrooxidans DNA fragment (which was linked

to the Cm^r gene in pDER601) (Fig. 2.4b).

The PstI digestions of pDER601 and pDER602 were ligated and resulted in a high frequency of Cm^r E. coli transformants. Unligated PstI digested controls yielded no transformants. In addition to the DNA fragment bearing the selected Cm^r marker, all pDER601 and pDER602 transformants contained the DNA fragment bearing the pBR325 ori, suggesting its presence necessary for plasmid replication. Approximately 50% of the 30 transformants investigated displayed Ap^r indicating that the ligation reaction had regenerated the PstI inactivated Ap^r gene. Further restriction endonuclease analysis confirmed that, although both fragments were always present, their orientation to each other had no apparent significance. In both the pDER601 and pDER602 experiments, transformants did not contain the third PstI fragment which contained T. ferrooxidans DNA. This suggested that this fragment did not contain DNA necessary for plasmid replication.

The possibility existed that the PstI cleavage of pDER601 and pDER602 could inactivate the T. ferrooxidans ori. To circumvent this possibility, pDER601 was digested with PvuI, the fragments ligated and Cm^r E. coli transformants were selected and analysed (Fig. 2.4c). A PvuI digestion of pDER601 resulted in three fragments: a 2.6 kb fragment bearing the selected Cm^r gene linked to a 0.3 kb T. ferrooxidans DNA fragment; a 5.2 kb fragment bearing the pBR325 ori linked to a 1.5 kb T. ferrooxidans DNA fragment;

and a 10.7 kb fragment consisting entirely of T. ferrooxidans DNA. Similarly, a PvuI digestion analysis was applied to pDER602 (Fig. 2.4c). An advantage of the PvuI digestion was that the T. ferrooxidans DNA fragment linked to the Cm^r gene in pDER601, and linked to the pBR325 ori in pDER602, was only 0.3 kb in size as opposed to the corresponding 1.7 kb fragment in the PstI digestions.

The PvuI digestions of pDER601 and pDER602 yielded results comparable with the PstI digestion experiments. The DNA fragment carrying the pBR325 ori was always present, but could be ligated in either orientation to the selected Cm^r fragment. The third PvuI digestion fragment containing only T. ferrooxidans DNA was not present in transformants.

Following the same analysis approach, in separate experiments pDER501 and pDER502 were PvuI digested, the fragments ligated and Cm^r transformants selected (Fig. 2.4d). The PvuI digestion of pDER501 generated three fragments: a 7.4 kb fragment carrying the pBR325 ori linked to a 3.6 kb T. ferrooxidans DNA fragment; a 2.4 kb fragment carrying the selected Cm^r marker linked to a 0.3 kb T. ferrooxidans DNA fragment; and a 2.6 kb T. ferrooxidans DNA fragment (Fig. 2.4d). The same observations were made in the PvuI digestion analyses of pDER501 and pDER502 as had been made in the analyses of pDER601 and pDER602, namely, that in addition to the presence of the selected Cm^r DNA fragment, the DNA fragment carrying the pBR325 ori was always present in transformants.

In the case of pDR330 and pDR331, PvuI digestion resulted in two fragments, one bearing the selected Cm^r marker linked to T. ferrooxidans DNA, the other carrying the pBR325 ori linked to a fragment of T. ferrooxidans DNA (Fig. 2.4e). After complete PvuI digestion, the fragments were ligated and Cm^r E. coli transformants were selected and analysed. Results, similar to those previously obtained, indicated that the fragment carrying the pBR325 ori was always present in transformants.

Thus, with the exception of the deletion plasmid pDR412, all the T. ferrooxidans recombinant plasmids analysed required the presence of the pBR325 ori, in order to replicate in E. coli. The demonstration that pDR412 could replicate in E. coli using a T. ferrooxidans ori indicates that some signals for gene expression are similar in autotrophs and heterotrophs.

2.3d Host range of T. ferrooxidans recombinant plasmids. Since pBR325 is unable to replicate in P. aeruginosa it was possible to determine whether any of the T. ferrooxidans plasmids were able to replicate in P. aeruginosa by transforming competent Pseudomonas cells with each of the recombinant plasmids. After selection for either Tc, Ap or Cm resistance, only pDER401 and pDER403 were able to transform and be re-isolated from P. aeruginosa. Plasmids pDER330, pDER331, pDER501, pDER502, pDER601 and pDER602 were unable to transform and replicate in P. aeruginosa.

In contrast to P. aeruginosa, the ColE1-type ori of pBR322 and pBR325 is able to function in K. pneumoniae. All recombinant plasmids carrying this ori were able to transform and be re-isolated from the K. pneumoniae cells. The T. ferrooxidans recombinant plasmids pDR404, pDR412, pDR420 from which the pBR325 ori had been deleted, were also able to transform and replicate in K. pneumoniae.

Thus, the ability of autotrophic DNA to function in heterotrophic cells was demonstrated by the T. ferrooxidans recombinant plasmid pDR412. This plasmid was able to replicate in E. coli, P. aeruginosa and K. pneumoniae from an ori located on the T. ferrooxidans DNA, whereas the other recombinant plasmids were unable to do so. This is in agreement with similar observations made with other naturally occurring plasmids where the replication of most is restricted to the species or close relatives from which they were isolated, while a few are able to replicate in a variety of other bacteria.

2.3e Determination of plasmid copy number. The autoradiograms revealed dots of decreasing intensity according to the DNA concentration loaded onto the GeneScreen membrane. The relative intensity of the positive hybridisation signal for each dot was estimated visually and determined spectrophotometrically by scanning the autoradiograms in a Beckman DU-8 spectrophotometer. By comparing the plasmid DNA dot intensity to the total DNA dot (containing E. coli cellular DNA plus plasmid DNA), it was

possible to estimate the proportion of plasmid DNA present in the total cellular DNA dot. Since the plasmid size and E. coli chromosome size are known as well as the concentration of DNA of each dot, it was possible to estimate the number of plasmids present per chromosome. The procedure was followed for each plasmid dilution series. The approximate plasmid copy number per cell of each T. ferrooxidans recombinant plasmid, as well as the standard pBR325 is given in Table 2.3. Plasmid pBR325, the cloning vector of the T. ferrooxidans plasmids had a copy number of 50 - 100 per cell. The smallest recombinant plasmid pDR404 (9 kb) had the highest copy number of 75 - 150, which was expected since these two factors are inversely linked. The presence of the ColE1 ori did not seem to affect copy number since pDR401 containing both the ColE1 and T. ferrooxidans origins of replication, and pDR420 only containing the T. ferrooxidans ori had approximately the same plasmid copy number (30 - 60 and 25 - 50, respectively). The recombinant plasmid pDR412 had the lowest copy number of 10 - 20 per cell (Table 2.3). It is interesting to note that pDR412 had a lower copy number per cell than either of its deletion plasmids, pDR404 or pDR420. This could be the result of some negative regulatory sequence which was removed from pDR412 when the deletion plasmids were constructed.

2.3f T. ferrooxidans plasmid DNA-directed protein synthesis. The polypeptides synthesised from pBR325, pDR401, pDR403 and the deletion plasmids pDR404 (Fig. 2.3) and pDR412 (Fig. 2.1) were separated on SDS-PAGE. The

Table 2.3 Plasmid copy number per cell in *E. coli*.

Plasmid	Size kb	Ori(s) present	Approximate copy number per cell
pBR325	6	ColE1	50 - 100
pDR401	17.8	ColE1 and *T. ferrooxidans*	30 - 60
pDR404	9	*T. ferrooxidans*	75 - 150
pDR412	14.7	*T. ferrooxidans*	10 - 20
pDR420	13.3	*T. ferrooxidans*	25 - 50

autoradiograms indicated that two major polypeptides with apparent molecular masses of approximately 49 and 48 kD (proteins e and f) were produced by pBR325 but not by any of the hybrid plasmids (Fig. 2.5). (For a full discussion of the polypeptide products associated with the Ap, Tc and Cm resistance genes of pBR325, refer to Chapter 4.3d). All the recombinant plasmids produced two major polypeptides with apparent molecular masses of approximately 126 and 46 kD (proteins a and g). pDR401 and pDR403 differ only with respect to the orientation of the pBR325 fragment and are the largest recombinant plasmids without any deletions in the pTF-FC2 region. In addition to polypeptides a and g these two recombinant plasmids also produced major polypeptides with apparent molecular masses of 94 and 55 kD (proteins b and c). These two polypeptides were produced by the deletion plasmid pDR412 but were not produced by the smallest plasmid pDR404. This suggests that polypeptides b and c were produced by genes located on the 5.3 kb PstI to ClaI fragment of pDR401 and pDR403 which is present in pDR412 but is absent in pDR404. A polypeptide with an apparent approximate molecular mass of 54 kD (protein d) was produced by pDR401 and its deletion plasmid pDR412 but not by pDR403 or its deletion plasmid pDR404. This suggests that the orientation of the inserts with respect to the pBR325 fragment may play a role in gene expression of the recombinant plasmids. This suggestion is supported by a previous observation where the Tc^r gene expression of plasmids pDER502 and pDER602 was determined by the cloning orientation of the *T. ferrooxidans* DNA in pBR325. The *E. coli in vitro* translation system

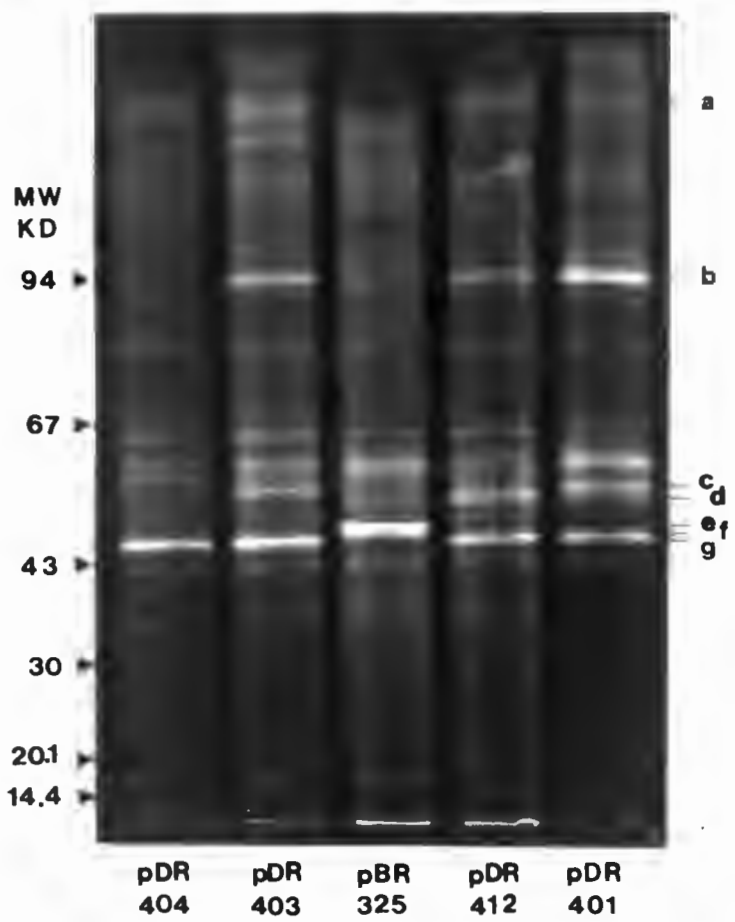
revealed other minor polypeptide bands which were produced by some plasmids but not by others. Although no phenotype or function could be ascribed to the T. ferrooxidans plasmid encoded polypeptides, their presence confirmed the ability of E. coli to recognise T. ferrooxidans control signals. These observations confirm the previous indications that some regulatory signals on an autotrophic plasmid DNA molecule are recognised by a heterotrophic system.

2.4 CONCLUSION

Although no selectable genetic markers on any of the recombinant T. ferrooxidans plasmids were detected in either E. coli or P. aeruginosa, there is substantial evidence that several gene functions are expressed in these bacteria. The ability of heterotrophic DNA to recognise autotrophic DNA regulatory signals was demonstrated. Transformation and replication of the recombinant plasmids in T. ferrooxidans has not yet been shown, but numerous results from this study suggest the potential for these plasmids to be used as cloning vehicles. These results include the following:

a) the recombinant plasmids contain unique restriction sites which can be used as cloning sites;
b) a T. ferrooxidans ori which can be expressed in E. coli has been located (on pDR412);
c) the recombinant plasmids are stably maintained in E. coli;

Fig. 2.5 Autoradiogram of a 15% SDS-PAGE of proteins produced by plasmids pDR404, pDR403, pBR325, pDR412 and pDR401 in an *E. coli* cell-free system. Molecular masses (kD) a, b, c, d, e, f and g indicate the positions of major proteins synthesised by the different plasmids, which are explained fully in the Results and Discussion (2.3f).



d) one *T. ferrooxidans* plasmid (pDR412) has a relatively broad host range;

e) some signals for gene expression are similar in autotrophs and heterotrophs;

f) the recombinant plasmids contain Cm and/or Tc resistance genes. These are suitable selectable genetic markers for *T. ferrooxidans* (Rawlings *et al.*, 1983).

Rawlings and Woods (1985) have subsequently reported that additional *T. ferrooxidans* plasmid functions are expressed in *E. coli*, including the oriT, nic and mob functions. These results enhance the suitability of the recombinant plasmids as potential vectors in future genetic manipulations of biomining bacteria.

CHAPTER THREE

IDENTIFICATION AND CLONING OF *T. FERROOXIDANS* STRUCTURAL *NIF* GENES IN *E. COLI*

CHAPTER THREE

IDENTIFICATION AND CLONING OF T. FERROOXIDANS STRUCTURAL NIF GENES IN E. COLI

Summary. The presence of DNA sequences which were homologous to the K. pneumoniae nifHDK, nifX, nifUSV and nifJ genes, was demonstrated in total cellular DNA preparations from five different iron oxidising T. ferrooxidans strains. A non-iron oxidising T. novellus strain, and a heterotrophic Acidiphilium strain which occurs in close association with T. ferrooxidans did not contain DNA homologous to the nif genes. The entire T. ferrooxidans ATCC33020 nifHDK operon was cloned on a 6.7 kb insert on pIMP16. The arrangement of the T. ferrooxidans nifHDK genes was similar to that of K. pneumoniae.

3.1 INTRODUCTION

Although T. ferrooxidans is extremely efficient at scavenging nitrogen in the form of ammonia, the scarcity of nitrogen or oxygen in leach liquors may limit the efficiency of bacterial leaching operations. Sugio et al. (1985) have proposed a new sulphur-oxidising route not requiring oxygen as the terminal electron acceptor, and which occurs under microaerobic conditions, or in the presence of compounds

inhibiting the iron oxydase enzyme in the T. ferrooxidans cells. Mackintosh (1971) suggested that under microaerophilic conditions the cells fixed atmospheric nitrogen, and showed that certain T. ferrooxidans strains reduced acetylene when grown in a nitrogen-free medium. The acetylene reduction test gives only presumptive evidence for the presence of the nitrogenase enzyme and the ability of the microorganism to fix atmospheric nitrogen (Hardy et al., 1968). Mackintosh (1978), using $^{15}N_2$-label, subsequently showed that at least one strain of T. ferrooxidans was able to fix atmospheric nitrogen by incorporating it into cellular material. It has not been established whether the ability to fix nitrogen is widely distributed among T. ferrooxidans isolates. In situ studies have not demonstrated nitrogen fixing activity in heap leaching operations (Khalid and Ralph, 1977).

The strong interspecies homology which exists for the nitrogenase genes (Ruvkun and Ausubel, 1980), has facilitated their identification and cloning from numerous free-living and symbiotic diazotrophs. The presence of genes homologous to the various K. pneumoniae nif genes was investigated in five different T. ferrooxidans isolates, a closely associated Acidiphilium strain, and the facultatively autotrophic bacterium T. novellus. DNA homologous to the K. pneumoniae nifHDK operon was identified in T. ferrooxidans ATCC33020 and cloned in a two-step cloning procedure. The structural organisation and linkage of the T. ferrooxidans nifHDK genes were determined.

3.2 MATERIALS AND METHODS

3.2a Bacterial strains, plasmids and media. The bacterial strains and plasmids are listed in Table 3.1. The *T. ferrooxidans* strains were grown on the inorganic medium (TK medium) of Tuovinen and Kelly (1973) (Appendix C). The heterotrophic *Acidiphilium* strain was grown on GYE medium (Shafia and Wilkinson, 1969), while *T. novellus* and the *E. coli* strains were grown in LB (Appendix C). Transformants were selected on LA using antibiotics at the following concentrations (μg/ml) Ap 100, Cm 25, Tc 25. Plasmid pEcoR251, which was a gift from M M Zabeau (Plant Genetic Systems, Gent, Belgium), is a positive selection vector containing the *E. coli* *Eco*RI gene under the control of the lambda rightward promoter, as well as an Ap resistance gene and the pBR322 ori. It was derived from the pCL plasmids described by Zabeau and Stanley (1982). The *Eco*RI gene product expressed at high levels by the lambda promoter on pEcoR251 is lethal unless insertionally inactivated or regulated by plasmid pC1857 which contains a temperature sensitive lambda repressor gene (Remaut *et al.*, 1983). The *Eco*RI gene has a single *Bgl*II cloning site, as shown on the restriction map of pEcoR251 in Appendix D.

3.2b Preparation of DNA. Plasmids used in cloning experiments or as probes were isolated by the CsCl buoyant density gradient method of Ish-Horowicz and Burke (1981) as described in Appendix A. Total cellular DNA was prepared from *Thiobacillus* cultures (2 litre) and the *Acidiphilium*

Table 3.1 Bacterial strains and plasmids.

Strain or plasmid	Genotype	Reference or source
T. ferrooxidans FD1	wt	Barros *et al.* (1984)
T. ferrooxidans TF29	wt	C Nicolau[a]
T. ferrooxidans TF35	wt	C Nicolau[a]
T. ferrooxidans	wt	ATCC33020
T. ferrooxidans	wt	ATCC19859
Acidiphilium sp.	wt	Barros *et al.* (1984)
T. novellus	wt	ATCC8093
E. coli HB101	*pro*$^-$ *leu*$^-$ Ap^s	Maniatis *et al.* (1982)
pEcoR251	Ap^r *Eco*RI	M M Zabeau[b] (Appendix D)
pSA30	Tc^r *nifHDKY*	Cannon *et al.* (1979)
pBR325	Cm^r Ap^r Tc^r	Bolivar (1978) (Appendix D)
pCRA37	Tc^r *nifXUSVMFLABQ*	Cannon *et al.* (1977)
pMC16	*nifJ*	R Jones[c]
pACYC184	Tc^r Cm^r	Chang and Cohen (1978) (Appendix D)

[a] C Nicolau: Centre de Biophysique Moléculaire, C.N.R.S., Orléans, France.

[b] M M Zabeau: Plant Genetic Systems, Gent, Belgium.

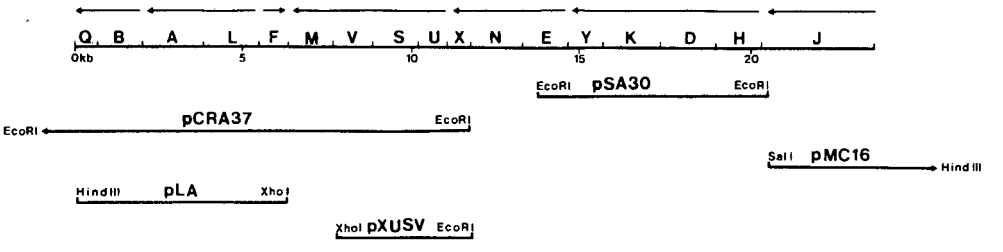
[c] R Jones: University of Chicago, Chicago, IL, USA.

species (1 litre) by the DNA isolation method described in Appendix A. The method of Davidson and Summers (1983) (Appendix A) was used to isolate total cellular DNA from T. novellus (200 ml), and the procedure for E. coli (500 ml) is described in Appendix A.

3.2c Restriction endonuclease reactions and electrophoresis of DNA. Restriction enzymes were obtained commercially and used in accordance with the specifications of the manufacturers. The standard molecular genetics techniques compiled by Maniatis et al. (1982) were followed as described in Appendix A.

3.2d Construction of plasmids pLA and pXUSV. The 23 kb plasmid pCRA37 (Cannon et al., 1977) was restricted with HindIII and XhoI, resulting in four DNA fragments. One fragment of approximately 6.2 kb contained the nifLA and nifBQ operons of K. pneumoniae, while another of approximately 4.7 kb contained the nifX, nifU, nifS and nifV genes (Fig. 3.1). As digestions of DNA with the restriction enzymes SalI and XhoI produce identical overlapping sticky ends, the vector pACYC184 (Chang and Cohen, 1978) was restricted with SalI and HindIII. The ligation reaction contained vector DNA to insert DNA in a ratio of 1 : 1. The DNA fragments were ligated and Cm^r HB101 transformants were selected on LA. Since the restriction sites SalI and HindIII lie within the Tc gene of vector pACYC184, the Cm^r transformants were checked for insertional inactivation of the Tc genes, and verified by restriction endonuclease

Fig. 3.1 Genetic map of the *K. pneumoniae nif* gene cluster. The vertical lines on the map demarcate the individual genes, and the arrows indicate the direction of transcription for the specified operons (Beynon *et al.*, 1983). DNA probes were prepared from plasmids pSA30, pCRA37, pMC16, pLA and pXUSV. Each plasmid is positioned relative to the *K. pneumoniae nif* gene map to indicate the area of the *K. pneumoniae* DNA, which the plasmid contained, defined by the specified restriction endonucleases.



analysis. The resulting plasmids were denoted pLA (*nifLABQ*), 9.7 kb in size, and pXUSV (*nifXUSV*) which was 8.2 kb in size (Fig. 3.1).

3.2e Preparation of DNA probes. The following plasmids were used as DNA probes: pSA30, pCRA37, pMC16, pLA, pXUSV (Fig. 3.1) and pIMP5 (constructed and described in this study). In addition to these plasmids, four DNA fragments of the *K. pneumoniae nifHDK* genes were used as probes (Fig. 3.9). Fragment purification involved the restriction of pSA30 DNA with the appropriate enzymes, separation of the fragments by agarose gel electrophoresis, and their recovery by the method of Dretzen *et al.* (1981) as described in Appendix A. The DNA was ^{32}P-labelled by nick-translation as described in Appendix B.

3.2f Hybridisation. After electrophoresis, DNA was transferred to GeneScreen membrane (Smith and Summers, 1980) and the membranes were hybridised and washed as described in Appendix B. Approximately 1 x 10^6 CPM of denatured probe were used per membrane. The autoradiography was continued for 12 h - 2 weeks.

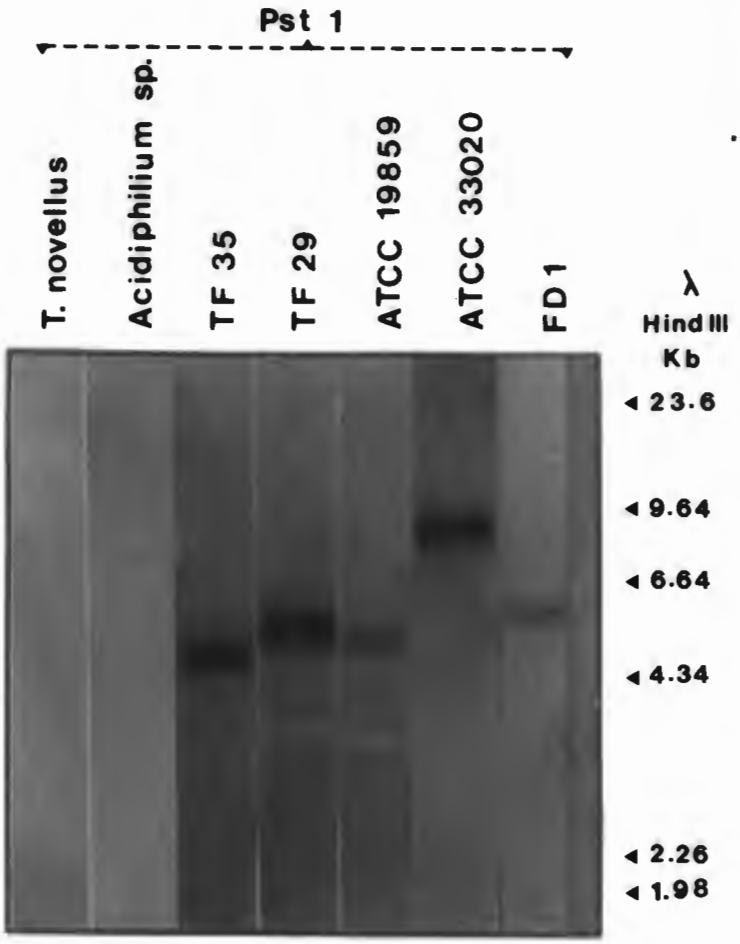
3.3 RESULTS

3.3a Identification of nitrogen fixation genes in *T. ferrooxidans*. Plasmid pSA30 contains the *nifHDKY* and part of the *nifE* gene of *K. pneumoniae* (Cannon *et al.*, 1979).

Since the structural genes (*nifHDK*) have been shown to be conserved between different genera of nitrogen fixing organisms (Ruvkun and Ausubel, 1980), pSA30 [^{32}P]DNA was used as a hybridisation probe for homologous sequences in the total cellular DNA of five *T. ferrooxidans* strains as well as *T. novellus* ATCC8093 and an *Acidiphilium* isolate (Fig. 3.2). After autoradiography, DNA fragments which showed homology with pSA30 were identified in all five *T. ferrooxidans* strains but no homologous sequences were detected in the DNA of *T. novellus* or the *Acidiphilium* strain. The DNA fragments which gave positive hybridisation signals were located on different *Pst*I fragments of the total DNA digests from the five different *T. ferrooxidans* strains.

In their original experiment, Ruvkun and Ausubel (1980) pointed out that the *K. pneumoniae nif* genes, other than *nifHDK* genes would exhibit little, if any, homology to corresponding genes from other diazotrophs. To investigate these predictions, the *Thiobacillus* strains were tested for sequences homologous to the genes of the *K. pneumoniae nif* gene cluster, other than the structural genes for nitrogenase. These genes (except for part of *nifE*) are contained on the plasmids pCRA37, pMC16, pLA and pXUSV as indicated in Fig. 3.1. As in the procedure for probing with pSA30, these plasmids were radiolabelled and used to probe total cellular DNA from the five *T. ferrooxidans* strains, *T. novellus* and the *Acidiphilium* strain. After autoradiography, DNA fragments which showed homology with

Fig. 3.2 Autoradiogram of pSA30 [^{32}P]DNA containing the *K. pneumoniae nifHDK* genes hybridised to *Pst*I digested total cellular DNA from five *T. ferrooxidans* strains (TF35, TF29, ATCC19859, ATCC33020 and FD1), a *T. novellus* strain and an *Acidiphilium* strain.

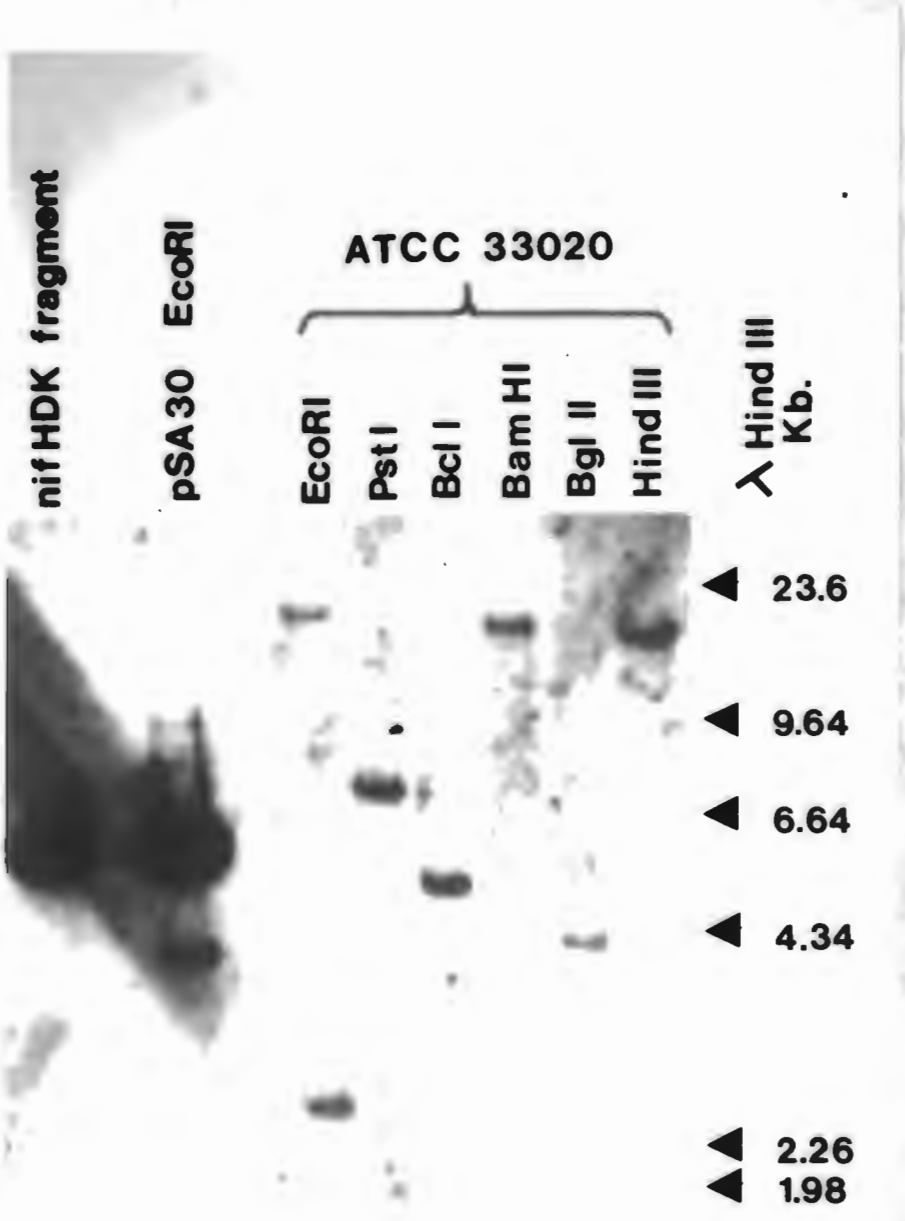


pCRA37 were identified in all five T. ferrooxidans strains, but not in T. novellus or the Acidiphilium strain. (No satisfactory photographic reproduction could be made of the autoradiograms to illustrate these results). The positive hybridisation signals were much fainter, and the autoradiography was continued for much longer periods than where pSA30 was used as a probe. When probing with pLA and pXUSV, which contained subfragments of the K. pneumoniae nif insert of pCRA37, only pXUSV displayed extremely faint positive hybridisation signals. As expected, homology was observed with the five T. ferrooxidans strains, but not with the other two strains. This suggests that the positive hybridisation signals obtained for pCRA37 were due to DNA homology existing between the T. ferrooxidans DNA and the nifX, nifU, nifS and nifV DNA region of K. pneumoniae.

When pMC16 containing the nifJ gene of K. pneumoniae was used as a probe, homologous DNA sequences were identified in all five T. ferrooxidans strains, but not in the other two strains. The intensity of the positive hybridisaton signals obtained for pMC16 was comparable to those obtained for pCRA37.

3.3b Cloning of T. ferrooxidans nifHDK genes. Total cellular DNA of T. ferrooxidans ATCC33020, restricted with a variety of enzymes, was probed with the EcoRI nifHDK fragment of pSA30 (Fig. 3.3). All the digestions displayed one DNA fragment homologous to the probe except for the EcoRI cellular DNA digestion, suggesting that the T.

Fig. 3.3 Autoradiogram of ^{32}P-labelled *nifHDK* fragment of pSA30 DNA hybridised to *T. ferrooxidans* ATCC33020 total cellular DNA which was digested with the specified restriction endonucleases. Plasmid pSA30 DNA digested with *Eco*RI as well as the *Eco*RI-*Eco*RI *nifHDK* DNA fragment (purified for use as a probe) were used as controls.

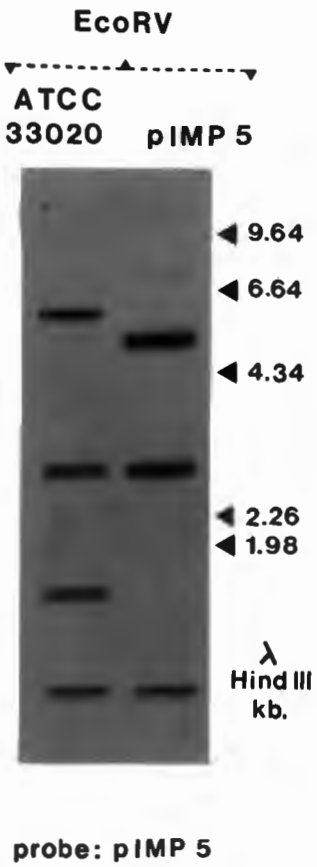


ferrooxidans DNA sequences homologous to the *nifHDK* were in a single copy per cell. Furthermore, none of the positive hybridisation signals occurred at the positions expected for the two natural plasmids of *T. ferrooxidans* ATCC33020 (discussed in Chapter Two), suggesting that the *T. ferrooxidans* DNA sequences displaying homology were chromosomal. Hybridisation studies with the *Eco*RI *nifHDK* fragment of pSA30 indicated that sequences homologous to the *K. pneumoniae nifHDK* genes were located on a 5.1 kb *Bcl*I fragment of *T. ferrooxidans* ATCC33020 (Fig. 3.3). *Bcl*I fragments of *T. ferrooxidans* ATCC33020 total cellular DNA in the 4.5 - 6.0 kb size range were excised from an agarose gel and cloned into the *Bgl*II site of pEcoR251. The 25 µl ligation reaction contained 0.25 pM vector DNA and 0.25 pM *T. ferrooxidans* insert DNA (1 pM = [0.662 x kb DNA] µg DNA). The other components of the ligation reaction, as well as the various controls used, are described in Appendix A. After transformation of *E. coli* HB101, approximately 1 200 Ap^r colonies with insertionally inactivated *Eco*RI genes of pEcoR251 were isolated. The partial gene bank was divided into 44 pools and each pool was probed with the *nifHDK* fragment of pSA30. The 28 individual colonies from the one pool which showed positive hybridisation were re-probed with the *nifHDK* fragment and two colonies showed positive hybridisation. Two pEcoR251 recombinant plasmids, pIMP5 and pIMP6, were isolated from these colonies. Restriction endonuclease analysis showed pIMP5 and pIMP6 to be identical clones, and pIMP5 was chosen for further study.

3.3c Characterisation of the recombinant plasmid containing *T. ferrooxidans nif* DNA. A restriction map of pIMP5 was obtained by single and double digestions with restriction endonucleases (Fig. 3.9). Since the *Bcl*I sites of the insert were destroyed by cloning into the *Bgl*II site of the vector, the three *Eco*RV sites of the insert were used to generate DNA fragments to confirm that the cloned DNA was of *T. ferrooxidans* origin. Digestion of pIMP5 DNA with *Eco*RV produced three DNA fragments, two of which (0.85 and 2.9 kb) were internal to the insert. *T. ferrooxidans* chromosmal DNA and pIMP5 DNA were digested with *Eco*RV, the fragments were separated on an agarose gel, blotted onto GeneScreen membrane and hybridised with pIMP5 [^{32}P]DNA (Fig. 3.4). The 0.85 and 2.9 kb internal fragments of pIMP5 correspond exactly to two of the four fragments of the *T. ferrooxidans* chromosomal digest which gave a positive hybridisation signal. The other two chromosomal fragments which gave a positive hybridisation signal correspond to the fragments from the *Eco*RV sites extending beyond the *Bcl*I site used in the cloning of pIMP5.

3.3d Location of the *nifH*, *nifD* and *nifK* genes on pIMP5. To characterise further the *T. ferrooxidans* chromosomal insert, the position of the *nifH* *nifD* and *nifK* genes were located by the hybridisation of restriction fragments of pIMP5 with [^{32}P]DNA probes derived from the *K. pneumoniae* *nifHDK* genes of pSA30. DNA probe A was the *Bam*HI-*Eco*RI fragment of pSA30 which contained the *K. pneumoniae* *nifK* *nifY* and part of the *nifD* genes. Probe B was the *Eco*RI-*Sal*I

Fig. 3.4 Hybridisation of pIMP5 [^{32}P]DNA to total cellular T. ferrooxidans ATCC33020 DNA and pIMP5 DNA digested with EcoRV. Digestion of pIMP5 with EcoRV generated two DNA fragments of 2.9 and 0.85 kb which were within the T. ferrooxidans DNA in pIMP5 and were detected by the probing experiment.



fragment of pSA30 which included the nifH and a portion of the nifD gene (Fig. 3.9). Plasmid pIMP5 DNA was restricted with the specified restriction enzymes and probed separately with DNA probe A and DNA probe B (Fig. 3.5). The autoradiograms indicated which of the fragments of pIMP5 were homologous to the particular probe used. By comparing the intensity of the hybridisation signals and relating the homologous fragments back to the restriction map of pIMP5, the nifH, nifD and nifK genes could be assigned to particular DNA regions on pIMP5 (and subsequently pIMP16, the construction and characterisation of which, will follow). Homology between probe A and the T. ferrooxidans nif insert of pIMP5 was detected in all fragments extending from the EcoRV site (2.6 kb on pIMP16) up to the end of the T. ferrooxidans insert at the BclI site (6.7 kb on pIMP16) (Fig. 3.9). The degree of homology was greatest in the DNA fragment extending from the EcoRV site (2.6 kb on pIMP16) to the SalI site (3.6 kb on pIMP16). This region of greatest homology corresponded to the nifD gene of the K. pneumoniae probe. The cloned T. ferrooxidans chromosmal DNA which hybridised with the DNA probe B extended from the end of the insert at the BclI site (1.5 kb on pIMP16) to the SalI site (3.6 kb on pIMP16). Again, the greatest degree of homology corresponded to the nifD gene of the probe.

3.3e Cloning of T. ferrooxidans DNA adjacent to the nifH gene. With the exception of certain slow growing Rhizobia and the cyanobacterium Anabaena (discussed in the General Introduction), the arrangement of the nifHDK operon has been

Fig. 3.5 (on following page) Hybridisation of fragments A (*nifK* gene and part of *nifD* gene) and B (*nifH* gene and part of *nifD* gene) from pSA30 to digested pIMP5 DNA. Fragment A [^{32}P]DNA was hybridised with pIMP5 digested with (a) *Sph*I-*Eco*RI; (b) *Eco*RV-*Sal*I; (c) *Eco*RV; and (d) *Pst*I. Fragment B [^{32}P]DNA was hybridised with (a) pSA30 digested with *Eco*RI-*Sal*I; (b) p*Eco*R251 digested with *Pst*I; and pIMP5 digested with (c) *Eco*RV-*Sal*I; (d) *Eco*RV; and (e) *Pst*I. The agarose gels are represented diagramatically alongside their corresponding autoradiograms.

Fig. 3.5 (legend on previous page)

found to be conserved between different genera of nitrogen fixing organisms (Ruvkun and Ausubel, 1980). It was assumed that a similar situation is likely to exist in the T. ferrooxidans with the promoter of the nifHDK operon located at the start of the nifH gene. Since it was not certain whether there was sufficient coding sequence between the nifD gene and the end of the T. ferrooxidans insert to code for the entire nifH gene, including the likely promoter of the operon, the T. ferrooxidans chromosomal DNA immediately adjacent to the nifH insert in pIMP5 was cloned.

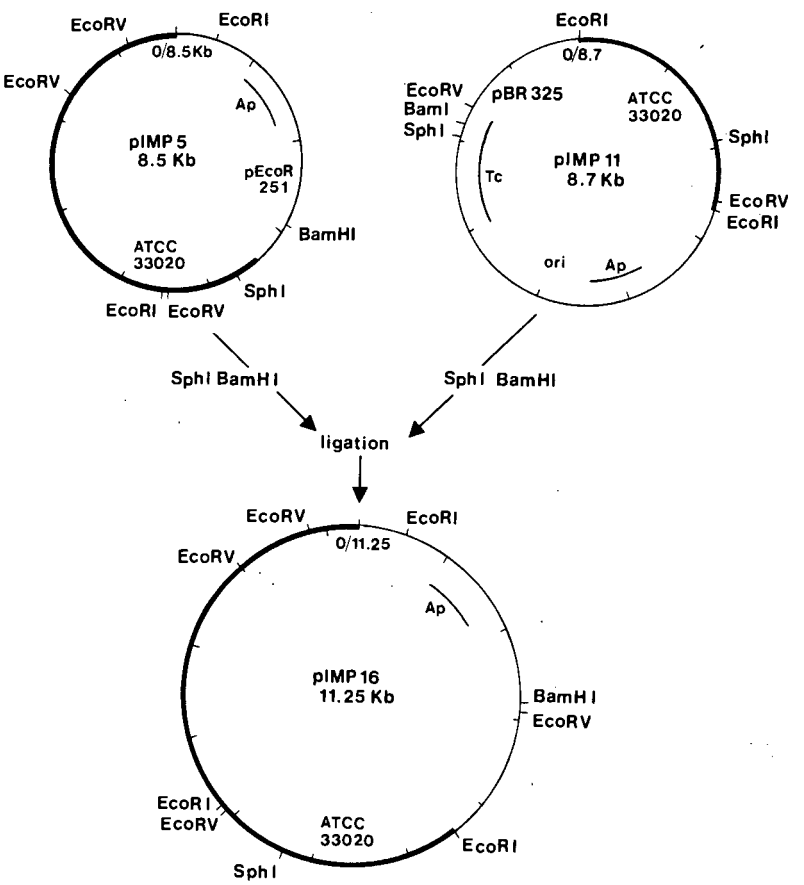
Previously it was shown that two EcoRI fragments of approximately 20 and 2.5 kb of a T. ferrooxidans chromosomal digest gave a positive signal with the pSA30 [^{32}P]DNA probe (Fig. 3.3). There was a single EcoRI site within the already cloned nif fragment situated 4.0 kb from one end of the fragment and 1.1 kb from the end encoding the nifH gene. The 2.5 kb DNA fragment of the EcoRI T. ferrooxidans chromosomal digest which showed homology with the pSA30 probe must therefore extend 1.4 kb further along the T. ferrooxidans chromosome. If it is assumed that the T. ferrooxidans nifH gene is similar in size to the K. pneumoniae nifH gene (approximtely 1 kb, Orme-Johnson, 1985), this additional fragment should encompass the whole of the T. ferrooxidans nifH gene. This 2.5 kb EcoRI fragment was excised from an agarose gel and cloned into the EcoRI site of pBR325. The 25 µl ligation reaction contained 2 pM vector DNA and 8 pM T. ferrooxidans insert DNA, as well as the other components described in Appendix A. Since the

EcoRI site of pBR325 is within the Cm^r gene, the plasmids from 22 Cm^s colonies were isolated and hybridised with pSA30 subfragment B [^{32}P]DNA. A single colony showing homology to the probe was identified and denoted pIMP11. Restriction endonuclease analysis of the plasmid confirmed the presence of single BclI, ClaI, EcoRV, StuI, and SphI sites as expected from the restriction map of pIMP5 (Fig. 3.9).

3.3f Construction of the extended cloned *T. ferrooxidans* *nifHDK* gene region. A recombinant plasmid pIMP16 was constructed from pIMP5 and pIMP11 (Fig. 3.6). Plasmids pIMP5 and pIMP11 were digested with BamHI and SphI and the DNA fragments separated on an agarose gel. The 7.75 kb fragment of pIMP5 and the 3.5 kb fragment of pIMP11 were excised from the gel, ligated and transformed into *E. coli* HB101. The ligation reaction contained vector DNA to insert DNA in a ratio of 1 : 1. From the transformants, a plasmid, pIMP16, was isolated which had the restriction pattern expected from the extended *nifHDK* region. The restriction map for the entire cloned *T. ferrooxidans* *nifHDK* region is shown in Fig. 3.9. No restriction sites were detected for ClaI, PvuII, SmaI, XbaI, HindIII or PaeR71.

3.3g Confirmation of the presence and relative positions of *nifH* and *nifK* genes on pIMP16. Confirmation of the presence of the *nifH* gene, and location of *nifH* and *nifK* genes on pIMP16 was obtained by probing with isolated *K. pneumoniae* *nifH* and *nifK* genes. Probe H was the portion of the *nifH* gene included on the EcoRI-BglII fragment, and probe K, the

Fig. 3.6 Cloning strategy for the construction of pIMP16 containing the extended *T. ferrooxidans nifHDK* region. The 7.75 kb SphI-BamHI fragment of pIMP5 was ligated to the 3.5 kb SphI-BamHI fragment of pIMP11 to yield pIMP16. The bold lines indicate the *T. ferrooxidans* plasmid DNA, while the thin lines indicate the vector DNA of the recombinant plasmids.



portion of the *nifK* gene included on the *Hin*dIII-*Sal*I fragment of pSA30 (fig. 3.9). Plasmid pIMP16 was digested with a variety of restriction enzymes and probed separately with probe K (Fig. 3.7) and probe H (Fig. 3.8). Regions homologous to these fragments of the *nifH* and *nifK* genes could be assigned to pIMP16. Homology between probe K and the *T. ferrooxidans nif* insert of pIMP16 was detected in all fragments extending from the *Stu*I site to the *Eco*RV site, as indicated in Fig. 3.9. The cloned *T. ferrooxidans* DNA, which hybridised with the DNA probe H, extended from the *Stu*I site to slightly beyond the unique *Xho*I site on pIMP16, as indicated on Fig. 3.9. These results confirmed the location of the *nifH*, *nifD* an *nifK* genes as found previously when pIMP5 was probed with fragments A and B, and demonstrated the linkage which exists between the *nifH*, *nifD* and *nifK* genes in *T. ferrooxidans*.

Similarly, in a separate experiment, pIMP16 DNA was restricted with a variety of enzymes, and the separated and blotted DNA fragments were probed with pMC16 [^{32}P]DNA. Plasmid pMC16 contains the *nifJ* gene of *K. pneumoniae*, which is located in front of *nifH* at the extremity of the *nif* gene cluster of *K. pneumoniae* (MacNeil *et al.*, 1978) (Fig. 3.1). No homology was detected between the *T. ferrooxidans nif* insert of pIMP16 and the *nifJ* gene on plasmid pMC16.

3.7 Hybridisation of fragment K (nifK fragment of pSA30) to digested DNA of pIMP16. Fragment K [^{32}P]DNA was hybridised to: (a) pSA30 digested with HindIII and SalI, and to pIMP16 digested with enzyme combinations as specified in (b), (c) and (d). The agarose gel is represented diagramatically alongside the autoradiogram.

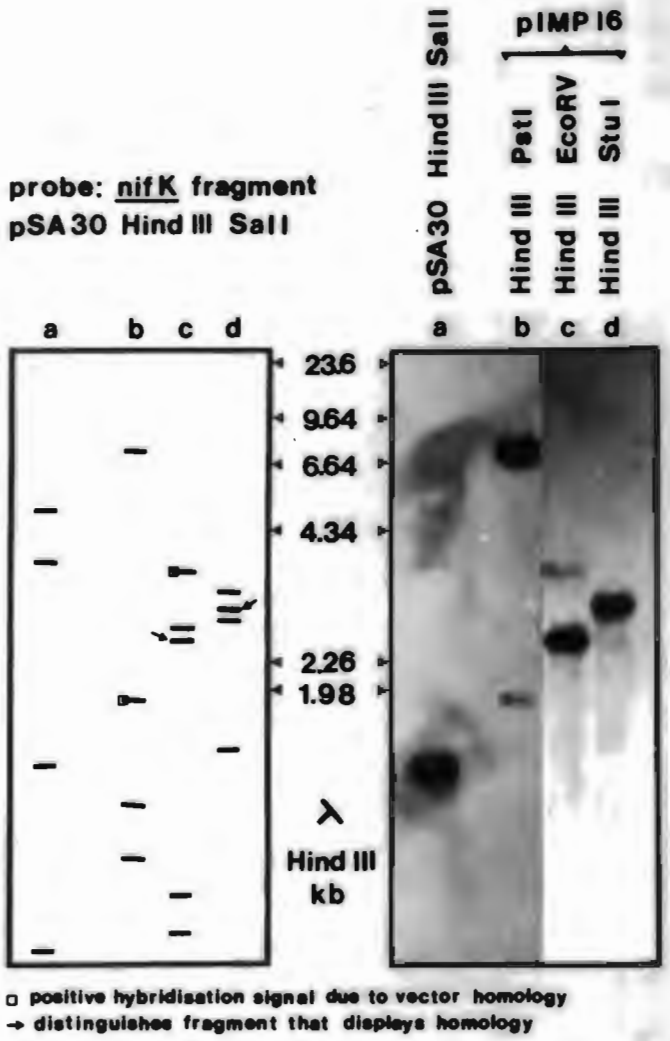
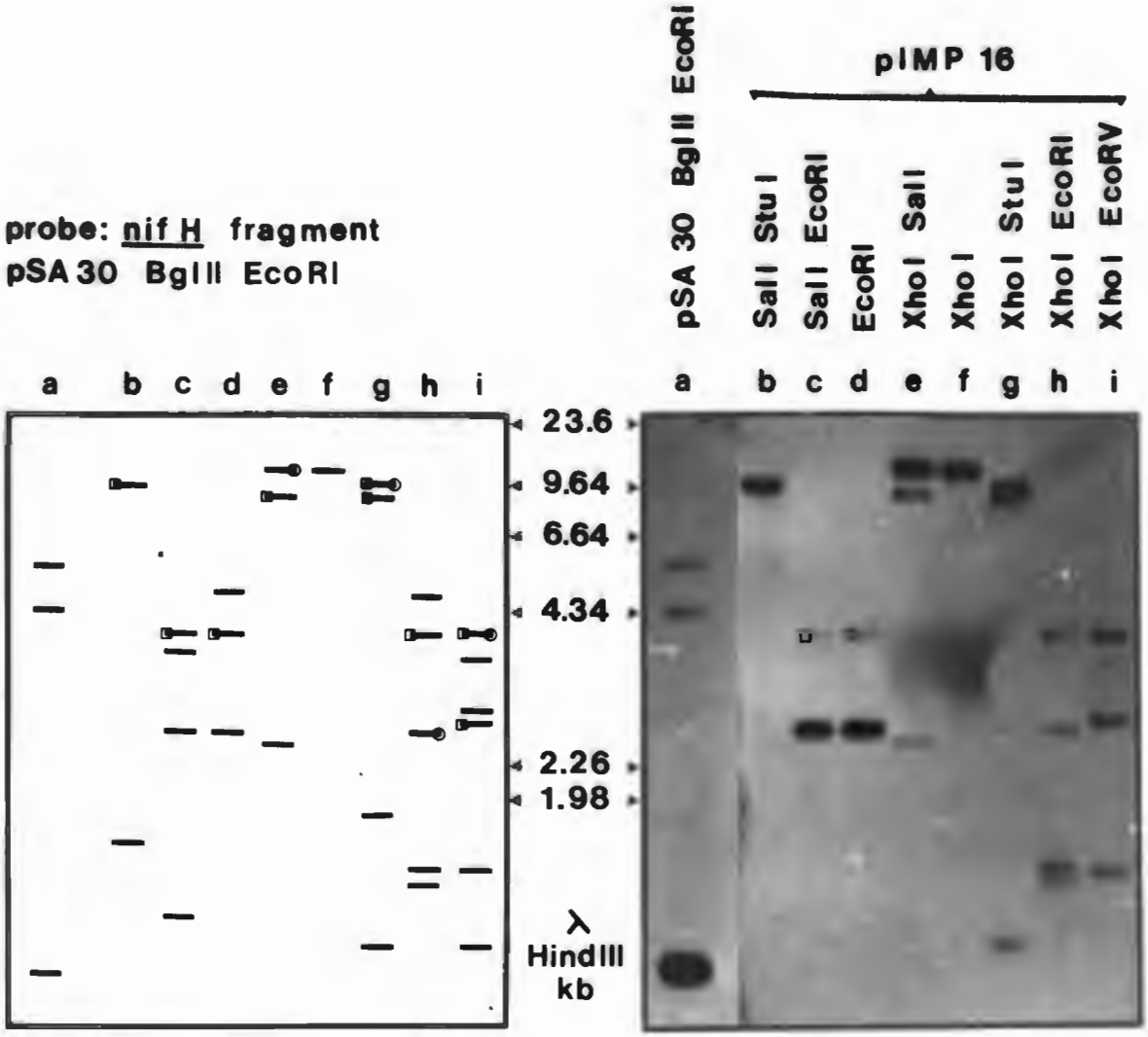


Fig. 3.8 Hybridisation of fragment H (*nifH* fragment of pSA30) to digested pIMP16 DNA. Fragment H [^{32}P]DNA was hybridised to: (a) pSA30 digested with BglII and EcoRI and to pIMP16 digested with specified enzymes as indicated in (b) to (i). The agarose gel is represented diagramatically alongside the autoradiogram.



□ positive hybridisation signal due partially or entirely to vector homology
○ partial digestion product

Fig. 3.9 (on following page) Restriction maps and relative positions of the T. ferrooxidans insert of DNA of pIMP5, pIMP11 and pIMP16. Regions of homology between the T. ferrooxidans insert and DNA fragments A, B, H and K which are subfragments of the nifHDK insert of the pSA30 DNA are indicated. These regions of homology were deduced from hybridisation experiments with the individual DNA fragments A, B, H and K, shown in Figs. 3.5, 3.8 and 3.7, respectively.

1) The restriction map of the T. ferrooxidans insert of pIMP5. T. ferrooxidans plasmid DNA was inserted into the vector pEcoR251 at the BclI site. The vertical lines indicate the restriction sites on the DNA and these correspond to the sites labelled on pIMP16 (see line 3).

2) The restriction map of the T. ferrooxidans insert of pIMP11. T. ferrooxidans plasmid DNA was inserted into the vector pBR325 at the EcoRI site. The vertical lines indicate the restriction sites on the DNA and these correspond to the sites labelled on pIMP16 (see line 3).

3) The restriction map of the T. ferrooxidans plasmid DNA of pIMP16 indicating the restriction sites.

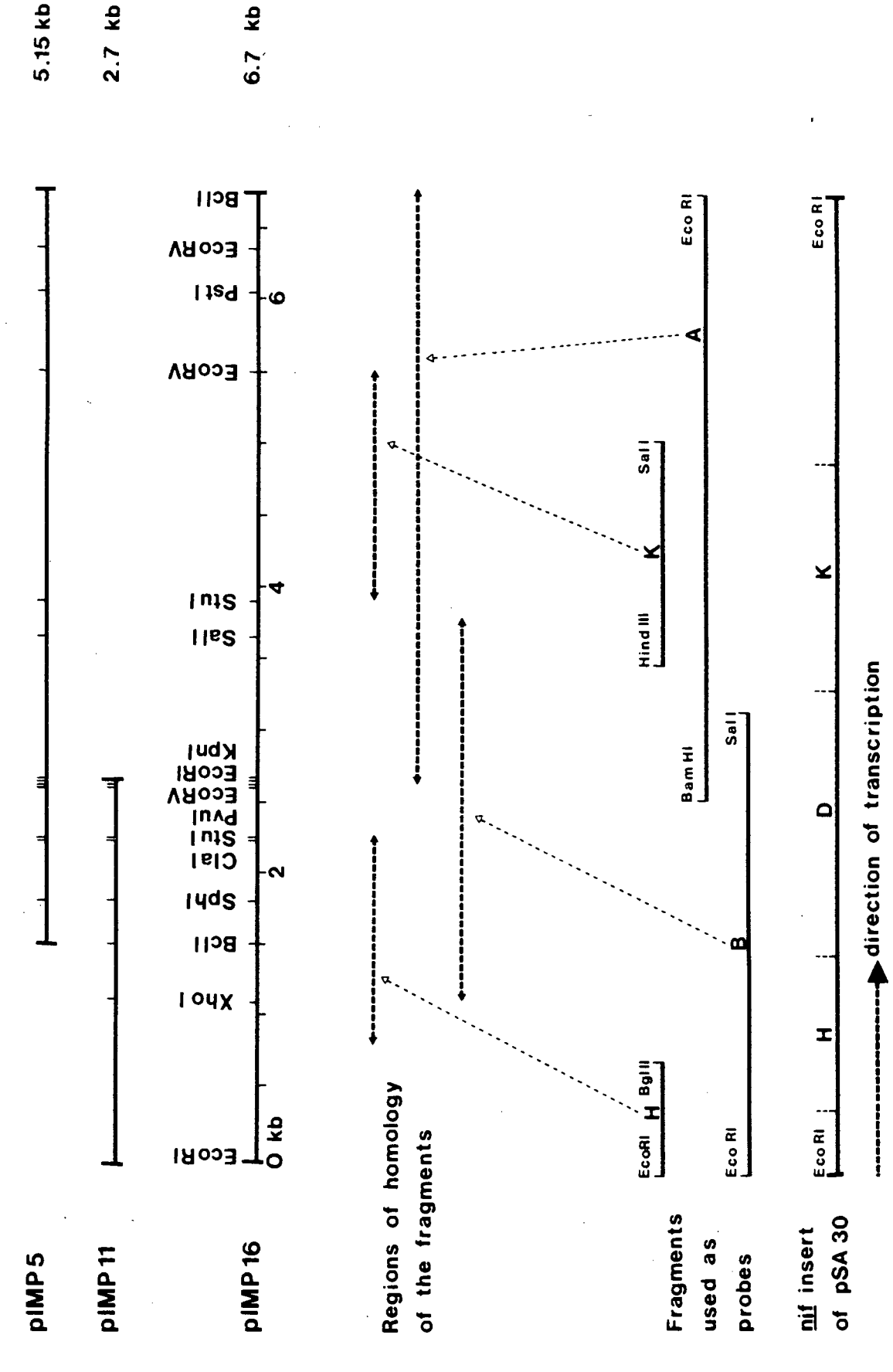
Fig. 3.9 (continued)

4) Regions of homology between fragments of pSA30 (H, K, A and B) and pIMP16 DNA. Each dotted line indicates the extent of the DNA area on pIMP16 which showed positive hybridisation to the H, K, A and B DNA fragments (indicated in 5).

5) The DNA fragments (H, K, A and B) of pSA30 which were used as probes against pIMP16 DNA in separate hybridisation experiments shown in Figs. 3.8, 3.7 and 3.5, respectively. The individual fragments are positioned according to their location on the pSA30 restriction map.

6) The map of the *K. pneumoniae* *nifHDK* insert of pSA30 indicating the direction of transcription of the operon from the promoter, which is situated upstream of the N-terminal end of the *nifH* gene. The dashed lines demarcate the *nifH*, *nifD* and *nifK* genes on the pSA30 insert.

Fig. 3.9 (Legend on previous page.)



3.4 DISCUSSION

The presence of DNA sequences which were homologous to the *nifHDK* genes of *K. pneumoniae* in total DNA preparations from five different strains of *T. ferrooxidans* was shown. The location of DNA homologous to the *K. pneumoniae nifHDK* genes on *Pst*I restriction fragments of different sizes indicated that the five *T. ferrooxidans* strains were all different. This suggests that the ability to fix nitrogen is probably widespread in *T. ferrooxidans* strains. The non-iron oxidising *T. novellus* strain did not contain DNA homologous to *K. pneumoniae nifHDK* DNA, and it remains to be established whether the absence of *nif* genes is a general property of non-iron oxidising thiobacilli. The heterotrophic acidiophile, *Acidiphilium* sp., also did not contain *nifHDK* gene sequences.

Following these results, it was expected that any detectable homology to the other *K. pneumoniae nif* genes would be found in the five *T. ferrooxidans* strains only. This proved to be the case, and positive hybridisation was detected for *T. ferrooxidans* DNA corresponding to the *nifXUSV* and *nifJ* DNA regions of *K. pneumoniae*. No DNA homology was detectable for *nifBQ*, *nifLA*, or *nifF*. This is an interesting observation since the *nifLA* genes regulate the *nifHDK* operon in all diazotrophs studied, and so *nifLA*-like genes must be present in the *T. ferrooxidans* genome. A comparable situation has been reported for Azotobacter. Kennedy and Robson (1983) demonstrated the presence of a gene analogous

to *nifA*, but no homology could be detected between *K. pneumoniae nifA* and *A. chroococcum* DNA (Jones *et al.*, 1984).

The *nifHDK* genes of *T. ferrooxidans* are arranged as one contiguous gene cluster, as is the case of the *K. pneumoniae nifHDK* operon. The probing of pIMP16 with pMC16 (containing *K. pneumoniae nifJ*) revealed however, that DNA homologous to the *K. pneumoniae nifJ* was not located immediately alongside *nifH* in *T. ferrooxidans*. Yet, probing with pMC16 [^{32}P]DNA had indicated that homologous sequences did exist in total cellular *T. ferrooxidans* DNA. This suggests that either the *T. ferrooxidans nifJ* gene is not located in the approximately 1 kb DNA fragment situated immediately alongside the *nifK* gene in pIMP16, or that the C-terminal end of the *nifJ* gene is present in pIMP16, but is less homologous to the *K. pneumoniae nifJ* gene than the N-terminal end, and a positive hybridisation signal was thus not detected in pIMP16. The degree of homology between the *nifHDK* genes of *K. pneumoniae* and the same genes from *T. ferrooxidans* varied between different regions of the operon. A region corresponding to the *nifD* gene of *T. ferrooxidans* appeared to have a greater degree of homology with *K. pneumoniae* DNA than any other region. This is similar to the finding of Bishop *et al.* (1985), who reported that the *nifD* gene of *A. vinelandii* had a greater degree of homology with the corresponding *nifD* gene of *K. pneumoniae* than either the *nifK* or *nifH* genes.

An interesting feature of *T. ferrooxidans* physiology is that

when the organism obtains its energy by the oxidation of ferrous iron, oxygen is the final electron acceptor and the organism is an obligate aerobe. The nitrogenase enzyme is, however, oxygen labile and the system responsible for nitrogen fixation has been found to function only when protected from oxygen (Roberts and Brill, 1981). Mackintosh (1978) demonstrated that T. ferrooxidans was able to fix atmospheric nitrogen using ferrous iron as an energy source, but oxygen had to be supplied in limited concentrations such that it was sufficient to allow iron oxidation to support measurable $^{15}N_2$-fixation, but insufficient to inhibit the expression of the nitrogenase system. Positive $^{15}N_2$-fixation occurred only in an environment where microaerophilic conditions prevailed. How T. ferrooxidans is able to resolve the apparently conflicting requirement of oxygen for energy generation and its absence for nitrogen fixation, has still to be determined.

CHAPTER FOUR

EXPRESSION OF CLONED *T. FERROOXIDANS* NITROGENASE GENES IN *K. PNEUMONIAE* AND IN A HETEROTROPHIC CELL-FREE SYSTEM

CHAPTER FOUR

EXPRESSION OF CLONED *T. FERROOXIDANS* NITROGENASE GENES IN *K. PNEUMONIAE* AND IN A HETEROTROPHIC CELL-FREE SYSTEM

Summary. The *T. ferrooxidans* recombinant plasmids pIMP16 (*T. ferrooxidans nifHDK*), pIMP11 (*T. ferrooxidans nifH*), and pIMP5 (*T. ferrooxidans nifDK*) reduced diazotrophic growth and nitrogenase activity in a *K. pneumoniae* Nif^+ strain. Plasmids pIMP16 and pIMP11 did not allow diazotrophic growth, or restore nitrogenase activity to the transformed *K. pneumoniae* $nifH^-$, $nifD^-$ or $nifK^-$ strains. Plasmid pIMP5 did not allow diazotrophic growth, but did restore low levels of nitrogenase activity to transformed *K. pneumoniae* $nifK^-$ mutants. No nitrogenase activity was detectable in *K. pneumoniae* $nifH^-$ or $nifD^-$ strains containing pIMP5. In *K. pneumoniae in vivo* transcription assays, only pIMP11 (*nifH*) produced an mRNA transcript which was detected by hybridisation to pIMP16 [^{32}P]DNA. In a heterotrophic *in vitro* translation-transcription system, the *T. ferrooxidans* insert on pIMP16 (*nifHDK*) produced polypeptides with apparent molecular masses of approximately 33, 56, 58 and 60 kD, pIMP11 (*nifH*) produced a 33 kD protein, and pIMP5 (*nifDK*) produced two proteins of 56 and 60 kD.

4.1 INTRODUCTION

Methods for the measurement of N_2 fixation include growth and morphological determinations, isotopic N-analysis and reduction of alternate nitrogenase substrates (Hardy *et al.*, 1973). The simplest method is to measure growth as optical density increments in N-free medium. Mutants of *K. pneumoniae*, unable to reduce N_2, have been well characterised by genetic and biochemical techniques (MacNeil *et al.*, 1978; Merrick *et al.*, 1980) and extensively used in complementation studies of *nif* genes from other organisms. Cloned DNA, introduced into Nif^- mutants can be screened for their ability to restore the wild type (wt) phenotype to the host strain by assessing bacterial growth in N-free medium.

The presence of the enzyme nitrogenase can be investigated by the sensitive acetylene reduction test. Dilworth (1966) and Schöllhorn and Burris (1967) observed that preparations of nitrogenase reduced C_2H_2 specifically to C_2H_4. No other known biological system conducts this reaction. Alternative substrates for nitrogenase exist, including cyanides and isocyanides (for a review see Postgate, 1982), but because of its relative lack of toxicity and ease of preparation, acetylene is the substrate of choice. Ethylene may be detected with great sensitivity by gas chromatography using a flame ionisation detector. Experimental aspects of the acetylene test have been well documented, including its limitations and the interference by oxygen in the test (Drozd and Postgate, 1970). The cloned *T. ferrooxidans*

nifHDK genes were transformed into *K. pneumoniae* Nif$^-$ mutants, and their ablity to partially or fully restore bacterial growth or acetylene reduction to these mutants, was investigated.

Since phenotypic expression could not be detected in all instances, for the various *T. ferrooxidans* recombinant plasmids, the transcription products of the cloned genes were investigated in *K. pneumoniae* cells. Nitrogenase production is controlled on two levels, by the *ntr* system, and by the products of the *nifLA* operon (see General Introduction). In *K. pneumoniae* expression of *nifLA* is not affected by oxygen or high temperatures (Dixon *et al.*, 1980), but is repressed by ammonia through the action of *ntr*-encoded factors (Magasanik, 1982; Ow and Ausubel, 1983). Kaluza and Hennecke (1981) showed that *nifHDK* mRNA synthesis was inhibited by oxygen, ammonia and at 39^{o}C in *K. pneumoniae*. Transcription of certain *nif* operons was inhibited in cells harbouring multicopy plasmids containing the *nifL* gene (Buchanan-Wollaston *et al.*, 1981b). This suggested that *nif* gene expression is subject to control at the transcription level and that the *nifL* product plays a role in this control. However, *nif* gene expression was also inhibited by many multicopy hybrid *nif* plasmids, some of which did not contain *nifL* (Riedel *et al.*, 1983). In recent studies on the control of *K. pneumoniae nif* mRNA synthesis, Collins and Brill (1985) have confirmed the respective roles of the *nifLA* gene products. The *nifA* product activates while the *nifL* product represses transcription of *nif*

operons (see General Introduction).

In addition to investigating transcriptional products as described above, gene expression may be studied at the translation level. The polypeptides specified by the *K. pneumoniae nif* genes have been well characterised with respect to size, isoelectric point and proposed function (see General Introduction). The component proteins of nitrogenase have been isolated from several bacteria, and their ability to complement each other functionally has been demonstrated (Emerich and Burris, 1978). An *in vitro* transcription and translation system offers an efficient method for identifying protein products. Such a cell-free system had previously demonstrated the ability of *T. ferrooxidans* plasmid DNA to function in a heterotrophic system, and was employed to investigate the cloned *T. ferrooxidans nifHDK* gene products.

4.2 MATERIALS AND METHODS

4.2a Bacterial strains, plasmids and media. The bacterial strains and plasmids are listed in Table 4.1. The strains were maintained aerobically at 37°C in LB or nutrient broth (NB) (Appendix C), or anaerobically at 30°C in the nitrogen-free-Davis-Mingioli medium (NFDM) described by Cannon *et al.* (1974) (Appendix C).

4.2b Transformation and selection of strains. *E. coli*

Table 4.1 Bacterial strains and plasmids

Genotype or phenotype		Reference or source
K. pneumoniae		
KP5022	*hisD2 hsdR1* Nif^+	Robert Robson
CK260	*hisD2 hsdR1 rspL4 nifH*	University of Sussex,
UNF842	*hisD2 nifD2560*::Tn5	Brighton, UK
UNF1149	*hisD2 hsdR1 nif2189*::Tn7	
UNF1150	*hisD2 hsdR1 nifH2191*::Tn7	
UNF1155	*hisD2 hsdR1 nifK2185*::Tn7	
UC164	$nifH^-$	Robert Haselkorn
UC165	$nifH^-$	University of Chicago,
		Chicago, IL, USA

Plasmids		Reference or source
pSA30	Tc^r *K. pneumoniae nifHDKY*	Cannon *et al.* (1979)
pLA	Cm^r *K. pneumoniae nifLABQ*	Constructed
pIMP5	Ap^r *T. ferrooxidans nifDK*	and described
pIMP11	Ap^r Tc^r *T. ferrooxidans nifH*	in
pIMP16	Ap^r *T. ferrooxidans nifHDK*	Chapter Three

strains were transformed with plasmid DNA according to the $CaCl_2$ method of Dagert and Ehrlich (1979), described in Appendix A. The same procedure was followed for the transformaton of K. pneumoniae strains except for the temperature shock treatment which was modified to -70°C for 10 min, 42°C for 10 min, followed by 10 min on ice. Transformants were selected on LA (Appendix C) containing the appropriate antibiotics at the following concentrations (µg/ml): Cm 20, Tc 25, Ap 100. Where K. pneumoniae strains showed inherent resistance to Ap, plasmids carrying the β-lactamase gene were selected on nutrient agar (NA) (Appendix C) containing Ap and Cb both at 400 µg/ml. Rapid plasmid isolation followed by restriction endonuclease digestion and agarose gel electrophoresis, confirmed the transformations (Appendix A).

4.2c Growth in N-free medium. Liquid NFDM under N_2 in Hungate tubes, was inoculated with the K. pneumoniae transformants and incubated under stringent anaerobic conditions at 30°C, with gentle shaking. Bacterial growth was determined visually and turbidometrically 3 - 7 d after inoculation.

4.2d Nitrogenase assays. The gases were obtained as follows: N_2 and He from Air Products (Johannesburg, SA) and C_2H_2 from Afrox (Johannesburg, SA). To induce nitrogenase activity, exponential phase cells (200 µl) in LB containing appropriate antibiotics were inoculated into 4 ml NFDM under N_2 in a glass bijou bottle, and incubated anaerobically for

17 - 20 h at 30°C with gentle shaking. The gas phase was replaced with He and 500 µl C_2H_2 was injected into the bottle. After incubation for 3 h at 30°C with gentle shaking, 50 µl samples of gas were extracted using a 100 ul Pressure-Lok Hamilton gas syringe (Supelco Inc. Bellefonte PA, USA). C_2H_4 formation was measured by gas chromatography, using a flame ionisation detector. A Hewlett Packard gas chromatograph (Hp 5880A series) equipped with a Carbosieve B column (60/80 mesh size, Supelco Inc.) was used. Before each series of samples was analysed, the gas chromatograph was calibrated with Scotty II Analysed Gases (Alltech Ass. Inc., Applied Science Laboratories, Deerfield, IL, USA). In every series of C_2H_2 reduction assays, the wt *K. pneumoniae* Nif^+ strain KP5022, as well as KP5022 containing plasmid pSA30 were included as positive controls. Over a total of eight experiments the C_2H_2 reduction results showed a standard deviation (SD) of 5% for the *K. pneumoniae* wt strain and 3% for the wt strain containing plasmid pSA30.

4.2e Isolation of total cellular RNA. RNA was isolated from *K. pneumoniae* UNF1150 *nifH*::Tn7 cells which contained the plasmids pIMP5 (*T. ferrooxidans nifDK*), pIMP11 (*T. ferrooxidans nifH*) or pIMP16 (*T. ferrooxidans nifHDK*). For comparison RNA was also isolated from *K. pneumoniae* UNF1150 containing no plasmid DNA, or containing pSA30 (*K. pneumoniae nifHDK*). The precautions taken to ensure that glassware, plasticware and all solutions were RNase-free, are described in Appendix C. RNA was isolated according to

the method of Krol et al. (1982). Exponential phase cells (6 ml), grown in LB containing appropriate antibiotics were harvested and washed twice in solution B of NFDM (Appendix C). The pellets were resuspended in 2 ml NFDM under N_2 in a Hungate tube, and incubated anaerobically at 30°C with gentle shaking. To induce nitrogenase activity incubation was continued for 1, 2, 2.5, 5, 8 and 24 h before the addition of 200 µl of lysis buffer (0.5 M Tris-HCl, 0.2 M EDTA, 10% w/v SDS, pH 8.0). The tubes were swirled gently several times, and the lysate was immediately extracted with 1 volume phenol-cresol (phenol:m-cresol:8-hydroxyquinoline, 1 000:140:0.4 w/w/w). After centrifugation (3 000 rpm for 10 min), the aqueous phase was transferred to a sterile, disposable Falcon Tube (2098 polypropylene conical tubes, Becton Dickinson and Co., Oxnard, CA, USA), and 2 volumes of 95% ethanol were added. The tubes were inverted several times and stored at -20°C overnight or until required. Extracts were transferred to Eppendorf tubes and the nucleic acid pelleted by centrifugation for 10 min in a microfuge. The ethanol was decanted, the pellet dried in a vacuum centrifuge, and resuspended in 20 µl distilled water.

4.2f Agarose gel electrophoresis of RNA. A denaturing gel system was prepared as described by Maniatis et al. (1982). Gloves were worn at all times, and a fumehood was used. The RNA was denatured by incubation at 65°C for 5 min in the following solution: 9 µl RNA, 20 µl deionised formamide (Appendix C), 7 µl of a 37% solution of formaldehyde and 4 µl of 10 x MOPS buffer (Appendix C). The samples were

chilled on ice and 2 µl sterile loading buffer (50% glycerol, 1 mM EDTA, 0.4% bromophenol blue, 0.4% xylene cyanol) was added. Samples were loaded on a 1.2% agarose gel prepared as follows: 1.2 g agarose was autoclaved in 10 ml of 10 x MOPS buffer and 75 ml distilled water. When this had cooled to 50°C, the volume was checked, 17 ml formaldehyde added and the gel was cast immediately. Electrophoresis was continued in 1 x MOPS buffer. As molecular mass markers, ribosomal RNA from K. pneumoniae was electrophoresed in the same gel. The lanes containing the markers were cut from the gel and the 16 S (molecular mass 0.55×10^6D) and 23 S (molecular mass 1.07×10^6D) rRNA bands visualised by EtBr staining.

4.2g Transfer of RNA to membranes. Electrophoresed RNA fragments were transferred to Hybond-N membrane as described by the manufacturers (Amersham Int., UK). The untreated gel was placed on a flat surface and a piece of membrane, cut to the gel dimensions presoaked in distilled water then 2 x SSPE buffer (Appendix C), was carefully laid on the gel. Dry paper towels and a weight were placed on top of this as described for DNA blotting (Appendix B), and transfer continued for 18 - 24 h. The membrane was air dried, placed in a plastic bag, and shortwave UV (254 nm) irradiated for 4 min, to crosslink the nucleic acids.

4.2h Preparation of probes. Plasmid pIMP16 DNA was ^{32}P labelled by nick translation as described in Appendix B. Reaction conditions were varied until high DNA specific

activity was obtained of approximately 2 x 10^8 CPM/µg DNA.

4.2i Hybridisation of RNA. The Hybond-N membrane manufacturer's procedure was followed (Amersham Int., UK). The membrane was presoaked in 5 x SSPE buffer (Appendix C) then placed in a plastic container or heat sealable plastic bag. An excess volume of prehybridisation fluid was added (5 x SSPE buffer, 50% v/v deionised formamide, 5 x Denhardt's solution, 100 µg/ml denatured herring sperm DNA) (Appendix C). Prehybridisation was continued overnight at 42°C, with constant agitation. The excess volume was removed, the denatured probe added, and hybridisation continued overnight under the same conditions. Solutions of increasing stringencies were used to wash the filters. The first two washes were carried out at 42°C for 15 min each, using an excess of 5 x SSPE buffer. One subsequent wash using 1 x SSPE buffer and 1% SDS was carried out at 42°C for 30 min before the wet membranes were sealed in plastic bags. The autoradiography procedure followed has been described in Appendix B.

4.2j <u>In vitro</u> transcription and translation of recombinant plasmids. The polypeptide products of the cloned <u>T. ferrooxidans</u> fragments of pIMP5, pIMP11 and pIMP16 were investigated using an <u>in vitro</u> <u>E. coli</u> DNA-directed translation system (Amersham Int., UK). The procedure followed was as specified by the manufacturers, but quarter quantities were used throughout. The polypeptides were labelled with L-[^{35}S]methionine (specific activity 1040 Ci/m

mol), and the radioactivity incorporation was determined by liquid scintillation counting (Appendix B). Sample volumes, containing 1 - 2 x 10^5 CPM of incorporated radioactivity were resolved by 15% SDS-PAGE (Laemmli, 1970; O'Farrell, 1975) as described in Appendix B. The *in vitro* translated polypeptides were visualised by autoradiography.

4.3 RESULTS

4.3a Growth on N-free medium. Well characterised Nif$^-$ mutants of *K. pneumoniae* were used to investigate the ability of the cloned *T. ferrooxidans nifHDK* genes to complement the corresponding gene mutations in *K. pneumoniae*. The plasmids pIMP5 (*T. ferrooxidans nifDK*), pIMP16 (*T. ferrooxidans nifHDK*) or pSA30 (*K. pneumoniae nifHDK*) were transformed into a *K. pneumoniae* Nif$^+$ strain, *nifH*$^-$ or *nifK*$^-$ mutant strains. The transformants were inoculated into NFDM and their growth monitored (Table 4.2). All three plasmids markedly reduced the growth of the *K. pneumoniae* Nif$^+$ strain. Plasmid pSA30 complemented two of the three *nifH*$^-$ mutants, and both *nifK*$^-$ mutants, and enabled these strains to grow in media with N_2 gas as the sole source of nitrogen. Plasmids pIMP5 and pIMP16 failed to complement any of the *nifH*$^-$ in *nifK*$^-$ mutants and did not allow the growth of these strains in the absence of fixed nitrogen.

4.3b Nitrogenase acetylene reduction assay. Nitrogenase

Table 4.2 Growth of *K. pneumoniae* Nif$^+$, *nifH*$^-$ and *nifK*$^-$ mutants in NFDM after transformation with pSA30 (*K. pneumoniae nifHDK*), pIMP5 (*T. ferrooxidans nifDK*) or pIMP16 (*T. ferrooxidans nifDK*). Cells were inoculated into NFDM with N_2 gas as the sole source of nitrogen. Samples were incubated anaerobically at 30°C with gentle shaking.

+ indicates growth assessed 3 to 7 d after inoculation

- indicates no detectable growth

		PLASMID TRANSFORMED			
Bacterial strain		None	pSA30	pIMP5	pIMP16
KP5022	Nif$^+$	++	+	+	+
UC164	*nifH*$^-$	-	+	-	-
UC165	*nifH*$^-$	-	-	-	-
UNF1150	*nifH*::Tn7	-	+	-	-
UNF1149	*nifK*::Tn7	-	+	-	-
UNF1155	*nifK*::Tn7	-	+	-	-

activity was assayed in *K. pneumoniae* *nif*$^+$, *nifH*$^-$, *nifD*$^-$ and *nifK*$^-$ strains containing pSA30 (*K. pneumoniae nifHDK*), pLA (*K. pneumoniae nifLABQ*), pIMP5 (*T. ferrooxidans nifDK*), pIMP11 (*T. ferrooxidans nifH*) or pIMP16 (*T. ferrooxidans nifHDK*). The effect on nitrogenase activity by co-transforming strains containing pSA30, pIMP5, pIMP11 or pIMP16 with pLA was also determined (Table 4.3). Plasmid pLA (constructed and described in Chapter Three), contains the *K. pneumoniae nifLABQ* genes, and has a p15A-type ori which is compatible with the ColE1-type ori on the pIMP plasmids. Since pACYC184 was the cloning vector for pSA30 (Tc^r) and pLA (Cm^r), both contained the p15A-type ori, but they could be comaintained in strains on LA, by antibiotic selection for both plasmids (Tc 25 μg/ml, Cm 25 μg/ml). Rapid plasmid isolations, followed by restriction enzyme and agarose gel analysis were regularly performed to verify that the Cm^r, Tc^r transformed strains contained both pLA and pSA30.

The *K. pneumoniae* wt strain KP5022 as well as the wt strain containing pSA30 were included as positive controls in each series of C_2H_2 reduction assays. Over a total of eight experiments the C_2H_2 reduction results showed a SD of 5% for the *K. pneumoniae* wt strain, and 3% for the wt strain containing pSA30. The introduction of pSA30 (*K. pneumoniae nifHDK*) or pIMP5 (*T. ferrooxidans nifDK*) resulted in a 20% reduction in nitrogenase activity in KP5022 *nif*$^+$ (Table 4.3). The presence of the entire *T. ferrooxidans* nitrogenase operon on pIMP16 caused a 70% decrease in

Table 4.3 Effect of pSA30 (*K. pneumoniae nifHDK*), pLA (*K. pneumoniae nifLABQ*), pIMP5 (*T. ferrooxidans nifDK*), pIMP11 (*T. ferrooxidans nifH*) and pIMP16 (*T. ferrooxidans nifHDK*) on nitrogenase activity of *K. pneumoniae* Nif^+ and Nif^- strains. Nitrogenase activity was assayed by the reduction of acetylene and is expressed as a % of the *K. pneumoniae* KP5022 wt strain. The SD of acetylene reduction results for *K. pneumoniae* KP5022 was 5%, and for KP5022 containing pSA30 was 3% over eight determinations. ND indicates not done.

	PLASMID TRANSFORMED									
Strain and phenotype	None	pSA30	pLA	pIMP5	pIMP11	pIMP16	pLA + pIMP5	pLA + pIMP11	pLA + pIMP16	pLA + pSA30
KP5022 Nif^+	100	80.0	ND	79.7	5.8	26.6	ND	ND	ND	ND
UC164 *nifH*$^-$	0	0.8	ND	0	0	0	ND	ND	ND	ND
UC165 *nifH*$^-$	0	0	ND	0	0	0	0	0	0	4.2
UNF1150 *nifH*::Tn7	0	13.0	ND	0	0	0	ND	ND	ND	ND
UNF842 *nifD*::Tn5	0	0.5	0	0	0	0	0	0	0	0.01
UNF1149 *nifK*::Tn7	0	8.8	ND	5.1	0	0	ND	ND	ND	ND
UNF1155 *nifK*::Tn7	0	12.8	0	8.5	0	0	0	0	0	0

nitrogenase activity, whereas pIMP11 containing only the *nifH* gene repressed nitrogenase activity in *K. pneumoniae* KP5022 *nif*$^+$ to the greatest extent (approximately 94% reduction).

Low levels of nitrogenase activities were obtained in *K. pneumoniae* UC164 *nifH*$^-$, UNF1150 *nifH*$^-$, UNF842 *nifD*$^-$, UNF1149 *nifK*$^-$ and UNF1155 *nifK*$^-$ strains containing pSA30 (Table 4.3). Complementation of nitrogenase activity in *K. pneumoniae* UC165 *nifH*$^-$ by pSA30 required the presence of pLA. In contrast, the levels of nitrogenase activity measured in strains UNF842 *nifD*$^-$ and UNF1155 *nifK*$^-$ containing pSA30 were reduced when pSA30 plus pLA were present in these strains.

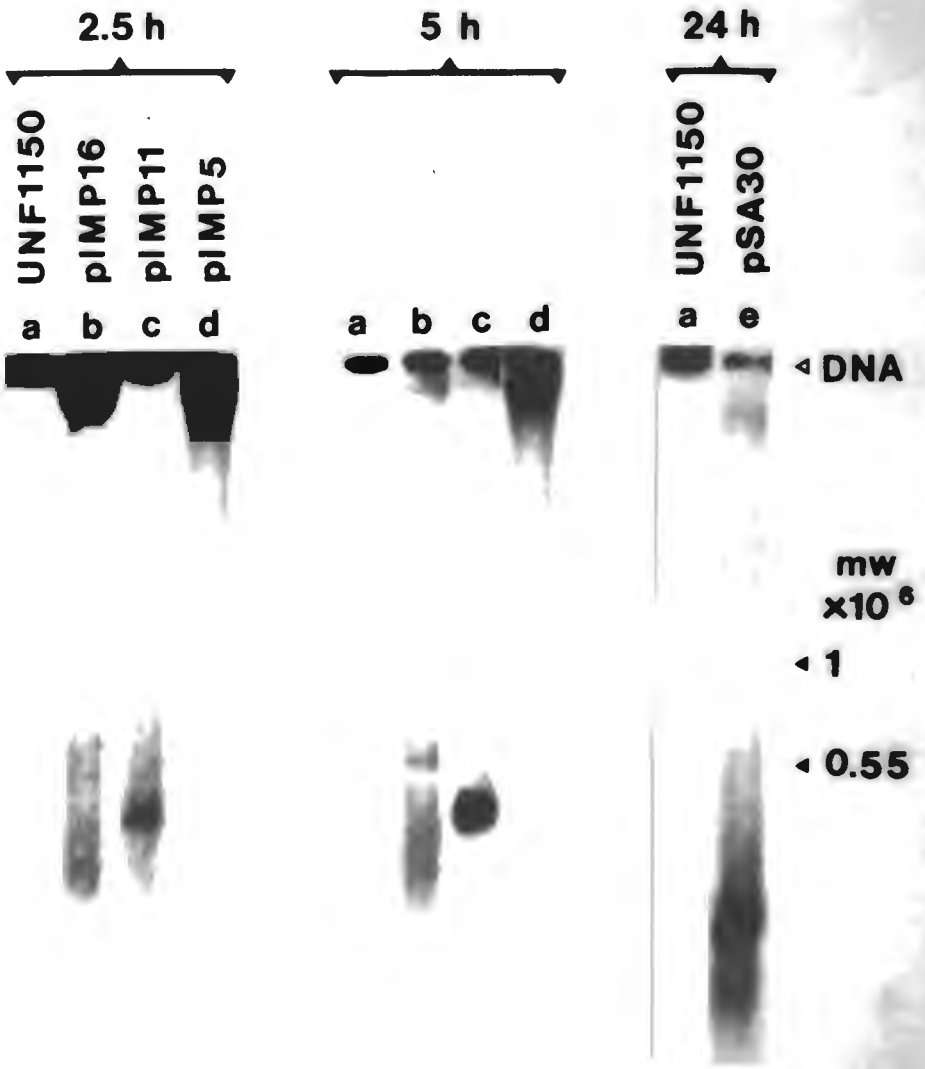
Plasmids pIMP16 and pIMP11 failed to complement any of the *K. pneumoniae* Nif$^-$ mutants, and zero nitrogenase activity was detected in any of the mutants (Table 4.3). When pIMP5 (*T. ferrooxidans nifDK*) was introduced into the two *K. pneumoniae nifK*$^-$ mutants, low, but highly reproducible levels of nitrogenase activity were detected. Nitrogenase activity was not detected in the *K. pneumoniae nifH*$^-$ or *nifD*$^-$ strains containing pIMP5.

The presence of pLA (*K. pneumoniae nifLABQ*) in conjunction with the pIMP plasmids did not result in nitrogenase activity in the *K. pneumoniae* Nif$^-$ mutants (Table 4.3). In contrast, pLA inhibited nitrogenase activity in *K. pneumoniae* UNF1155 *nifK*$^-$ containing pIMP5 (*T. ferrooxidans*

nifDK).

4.3c Total cellular RNA isolation. The K. pneumoniae UNF1150 cells containing the plasmids pSA30, pIMP5, pIMP11 or pIMP16 were cultured under conditions which derepress nitrogenase activity. The RNA was isolated from these cells at the specified time intervals, electrophoresed and immobilised on Hybond-N membrane. Plasmid pIMP16 [^{32}P]DNA was used as a probe to detect complementary mRNA transcripts. No positive hybridisation signals were detected in the total cellular RNA isolated from the host strain UNF1150 nifH::Tn7 into which no plasmid had been transformed (Fig. 4.1). The lane containing RNA isolated at 24 h, from UNF1150 cells containing pSA30 showed a high background of hybridisation in the low molecular mass region. Within this area two broad bands which hybridised to pIMP16 [^{32}P]DNA could be distinguished. No bands were detectable when the nitrogenase induction period of these cells was shorter. No positive hybridisation signals were detected in cells containing pIMP5 at any of the time intervals at which RNA was isolated. No distinct bands were visible for pIMP16 either, although a high background hybridisation, particularly in the low molecular mass region was detectable. In contrast, after 2.5 h of nitrogenase induction, UNF1150 cells containing pIMP11 produced a faint RNA band which hybridised to pIMP16 [^{32}P]DNA (Fig. 4.1). After a 5 h induction period this band gave a markedly stronger positive hybridisation signal, and was visible in all subsequent RNA isolations from this strain.

Fig. 4.1 Detection of nitrogenase mRNA isolated from (a) *K. pneumoniae* UNF1150 *nifH*::Tn7, and UNF1150 cells containing (b) pIMP16 (*T. ferrooxidans nifHDK*), (c) pIMP11 (*T. ferrooxidans nifH*), (d) pIMP5 (*T. ferrooxidans nifDK*), and (e) pSA30 (*K. pneumoniae nifHDK*). Total cellular RNA was isolated from the cells after nitrogenase activity had been induced for 2.5, 5 and 24 h. Plasmid pIMP16 [^{32}P]DNA was used as a probe for complementary mRNA transcripts. *K. pneumoniae* rRNA, electrophoresed on the same agarose gel provided the molecular mass markers. The high molecular mass bands showing positive hybridisation to the probe are cellular DNA.

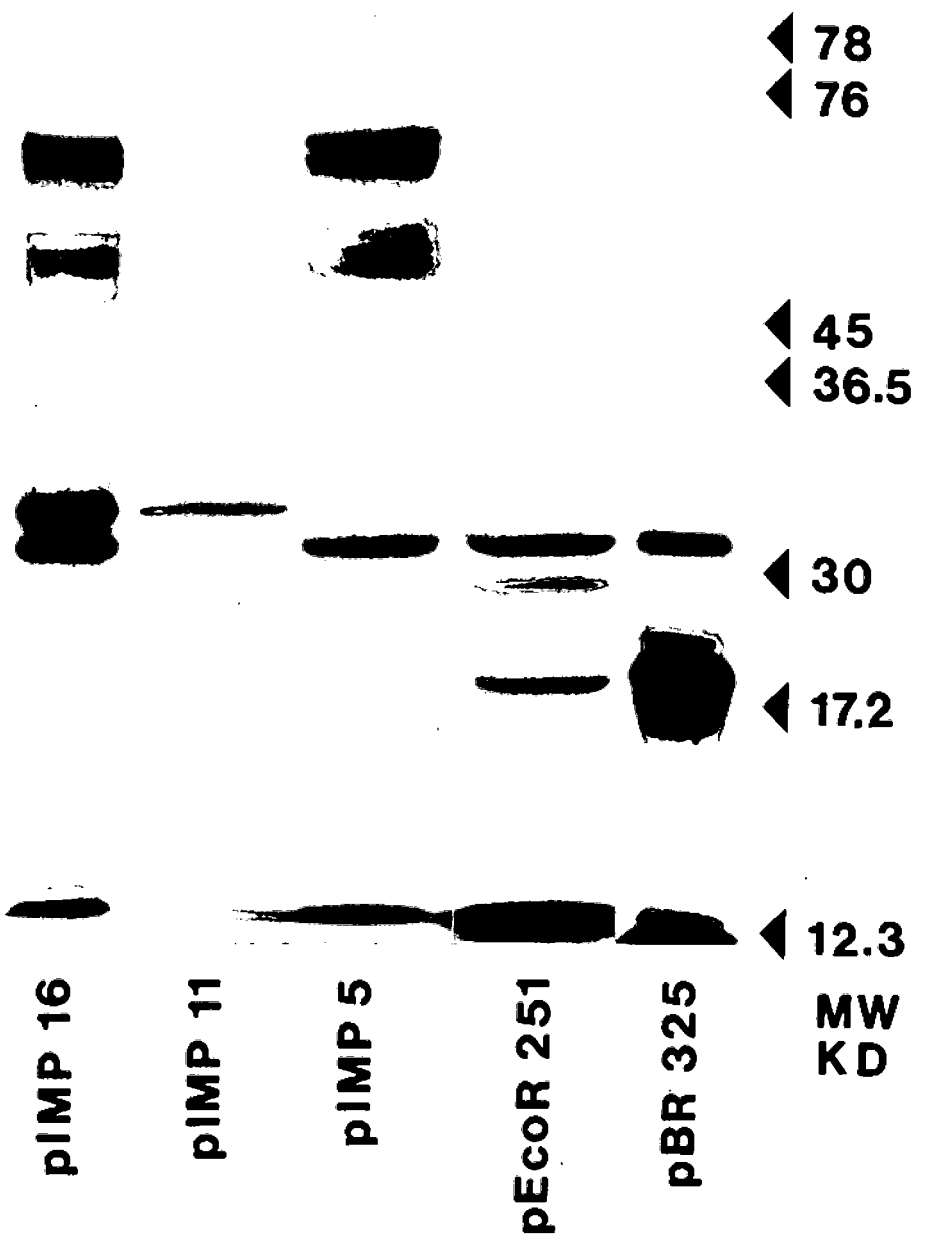


4.3d *In vitro* **transcription-translation products.** The plasmids pBR325 and pEcoR251 were the cloning vectors used in the construction of the pIMP plasmids (Bolivar, 1978; Zabeau and Stanley, 1982, respectively) and were included as controls to determine vector associated polypeptides (Fig. 4.2). The faint band of approximately 50 - 60 kD detectable in the pEcoR251 sample has been explained by the manufacturers of the DNA-directed translation kit (Amersham Int., UK). This band, sometimes detectable in samples, including control reactions to which no DNA was added, is a property of experiments involving L-[^{35}S]methionine, and may be due to the presence of a protein which binds methionine or methionyl-tRNA.

Numerous β-lactamase associated polypeptides have been ascribed to the Ap resistance gene of plasmids pBR325 and pEcoR251. These include polypeptides of apparent molecular masses of 28 an 31 kD (Sancar *et al.*, 1979), and 30, 28 and 25 kD (Covarrubias *et al.*, 1981). The polypeptide of apparent molecular mass of approximately 31 kD, produced by pBR325, pEcoR251, pIMP16, and pIMP5 was the β-lactamase gene product. The additional polypeptide products detectable in the pEcoR251 control sample are associated with the *Eco*RI gene, and are not detectable in pIMP16 or pIMP5 since the cloning procedure of these plasmids inactivated the *Eco*RI gene.

In addition to the β-lactamase gene product found in the pBR325 control sample, a polypeptide of approximately 22 kD

Fig. 4.2 In vitro transcription-translation products of pIMP16 (T. ferrooxidans nifHDK), pIMP11 (T. ferrooxidans nifH), pIMP5 (T. ferrooxidans nifDK), and the cloning vectors pEcoR251 and pBR325. A full discussion of the polypeptides associated with the Ap, Tc, Cm and EcoRI genes is given in Results 4.3d. The insert on pIMP16 produced four major polypeptides with apparent molecular masses of approximately 33, 56, 58 and 60 kD proteins. The insert on pIMP11 produced a single polypeptide with apparent molecular mass of 33 kD.



is visible. This polypeptide corresponds to the Cm-acetyltransferase encoded by the Cm resistance gene (Meagher *et al.*, 1977; Bolivar, 1978; Covarrubias *et al.*, 1981). This polypeptide was not detected in pIMP11 or pIMP16 since the cloning procedure of these plasmids inactivated the gene.

Numerous polypeptide products have been ascribed to the Tc resistance gene of pBR325, including polypeptides of 34 kD (Covarrubias *et al.*, 1981), 19, 17, 14 and 12 kD (Meagher *et al.*, 1977). The polypeptides of approximately 17 - 19 kD visible in pBR325 are most probably due to the Tc resistance gene, since a faint band of this molecular mass was detectable in the autoradiograms of pIMP11 (Tc^r). (This band was not photographically reproducible, but is visible in the original autoradiograms.)

The insert on pIMP16 (*T. ferrooxidans nifHDK*) produced four major additional polypeptides in the *in vitro* system (Fig. 4.2). One polypeptide had an apparent molecular mass of approximately 33 kD. The high molecular mass polypeptides appear as a doublet, but on closer examination, three polypeptides are distinguishable in this region. These have apparent molecular masses of approximately 56, 58 and 60 kD. The insert on pIMP5 (*T. ferrooxidans nifDK*) produced two major polypeptides with approximate molecular masses of 56 and 60 kD, but did not produce the 33 kD or 58 kD proteins. The insert on pIMP11 (*T. ferrooxidans nifH*) produced a single polypeptide with apparent molecular mass of 33 kD.

Both pIMP5 and pIMP16 showed minor polypeptides with apparent molecular masses of 46 - 50 kD.

It is concluded that the expression of the *nif* genes on the pIMP recombinant plasmids is from promoter regions situated on the *T. ferrooxidans* DNA insert and not due to read through from a vector promoter, for the following reasons:

a) the relative fidelity of transcription-translation with respect to the molecular masses of the nitrogenase polypeptides in the three different recombinant plasmids which contain different vector DNA (pEcoR251 and pBR325);
b) the direction of transcription of the *nifH* gene in pIMP11 was in the opposite direction to that of the Cm gene of pBR325 into which the *T. ferrooxidans* DNA fragment was cloned;
c) similarly in pIMP5, the direction of transcription of the cloned DNA is opposite to that of the *Eco*RI gene into which it was cloned.

4.4 DISCUSSION

Jones *et al*. (1984) reported that multiple copies of the *A. chroococcum nifH* promoter, cloned on a high copy number plasmid, inhibit the expression of the single *nifHDK* operon carried by the *K. pneumoniae* chromosome. Similar results were obtained in this study, and it is suggested that this

inhibition of nitrogenase activity was probably due to competition for limited amounts of the *nifA* gene product between the various *nif* promoters of the cluster (Buchanan-Wollaston *et al.*, 1981b). Plasmid pSA30, containing the *K. pneumoniae nifHDK* operon cloned into pACYC184 (copy number approximately 20 copies per cell) (Chang and Cohen, 1978; Cannon *et al.*, 1979), inhibited the growth, and decreased the nitrogenase activity by 20% (SD = 3%, for 8 determinations), of the *K. pneumoniae* wt strain in NFDM. Previous studies have reported a greater decrease of nitrogenase activity when pSA30 was present in the wt strain; Cannon *et al.* (1979) observed a 79% decrease, Riedel *et al.* (1983) a decrease greater than 90%, and Buchanan-Wollaston *et al.* (1981b) a decrease of 99%.

Plasmids pIMP11 and pIMP16, which contained the *T. ferrooxidans nifH* gene inhibited the growth of the *K. pneumoniae* nif^+ strain and caused a marked reduction in nitrogenase activity (approximately 94% and 73%, respectively) in comparison to pSA30. Since pIMP11 and pIMP16 have relaxed copy number control of greater than 40, the variation in nitrogenase repression by these two plasmids in comparison with pSA30, may be due to an effect of copy number. Similarly, the variation in nitrogenase repression among the three pIMP plasmids could be attributed to a copy number effect since the construction of the plasmid involved different vectors (pEcoR251 was used for pIMP5, pBR325 for pIMP11 while pIMP16 is a hybrid plasmid of pIMP5 and pIMP11). Nevertheless, these results suggest that

the T. ferrooxidans nifH gene is able to titrate out a positive effector of nitrogen fixation, which could be the K. pneumoniae nifA gene product.

In contrast to pIMP11 and pIMP16, pIMP5 (T. ferrooxidans nifDK) which does not contain the T. ferrooxidans nifH gene promoter region, repressed nitrogenase activity by approximately 20%. The reason for this repression in nitrogenase activity is not known but a hypothesis implicating a second promoter on the nifDK fragment of pIMP5 with lower affinity for the nifA gene product could explain the repression of nitrogenase activity.

The presence of a promoter on the T. ferrooxidans nifDK fragment of pIMP5 is suggested by the experiments on the complementation of K. pneumoniae nif$^-$ mutants and the expression of nitrogenase activity. Plasmid pIMP5 was the only T. ferrooxidans recombinant plasmid which partially complemented any of the K. pneumoniae nif$^-$ mutants and produced nitrogenase in two K. pneumoniae nifK$^-$ mutants. The level of nitrogenase activity to K. pneumoniae nifK$^-$ strains containing pIMP5 was similar to that obtained in the nifK$^-$ mutants containing pSA30. Although pIMP5 contained the nifD gene it did not complement or restore nitrogenase activity in the K. pneumoniae nifD$^-$ mutant. Since pSA30 (K. pneumoniae nifHDK) restored nitrogenase activity at extremely low levels (0.5%) to the K. pneumoniae nifD::Tn5 mutant, complementation by the heterologous T. ferrooxidans DNA is likely to be undetectable.

Plasmid pSA30 partially complemented the *K. pneumoniae* *nifK*$^-$ and *nifD*$^-$ strains, and all but one *nifH*$^-$ strain, and nitrogenase activities of 0.5 - 13% of the wt were detected. These values are greater than those of Cannon *et al.* (1979) who found that pSA30 partially complemented most *nifH*$^-$, *nifD*$^-$ and *nifK*$^-$ strains tested at nitrogenase levels of 0.6 - 3.5% of a wt *RecA*$^+$ *K. pneumoniae* strain. These results were proved to be inconclusive by Riedel *et al.* (1983), because the strains used by Cannon *et al.* (1979) were *RecA*$^+$ strains (containing deletions in the *nifHDK* operon), and it was therefore not possible to distinguish between a low level of complementation and a high rate of recombination. Riedel *et al.* (1983) demonstrated further that pSA30 contained DNA which inhibited the Nif$^+$ phenotype, and claimed that any complementation of which pSA30 might be capable, would be masked by this Nif inhibition phenomenon. Their results supported the hypothesis that if pSA30 restored the Nif$^+$ phenotype to *nif* mutant strains (observed by Cannon *et al.*, 1979; and in this study), it was by recombination, followed by curing of the plasmid, and not by complementation. The deletion mutant *K. pneumoniae* strains used by Cannon *et al.* (1979) might allow recombination to occur at a higher frequency than the *K. pneumoniae* strains used by Riedel *et al.* (1983) and in this study, where inactivation of the specified *nif* gene was caused by transposon insertion. Nonetheless, the possibility of recombination between the *nifK* of *T. ferrooxidans* (on pIMP5) with the *nifK* of *K. pneumoniae* UNF1155 *nifK*::Tn7, which resulted in the low level of nitrogenase activity observed,

cannot be ruled out.

As mentioned earlier, nitrogenase activity was inhibited in a *K. pneumoniae* Nif^+ strain when multiple copies of the *A. chroococcum* *nifH* promoters were present (Jones *et al.*, 1984). This inhibition could be alleviated, however, by the provision of multiple copies of the *K. pneumoniae* *nifA* gene product, and the plasmid pCK1, which carries the *nifA* gene expressed from a constitutive promoter (constructed by Kennedy and Robson, 1983) was used for this purpose by Jones *et al.* (1984). The ability of the *K. pneumoniae* *nifA* gene product to activate *nif* gene promoters of other organisms has previously been reported for both *A. vinelandii* and *A. chroococcum* (Kennedy and Robson, 1983). Similarly, the *nifH* promoter of *R. meliloti* was activated by the *K. pneumoniae* *nifA* gene product (Sundaresan *et al.*, 1983), and again, the wide host range multicopy plasmid pCK1 (Kennedy and Robson, 1983) carrying the *nifA* gene expressed from a constitutive promoter was used.

To investigate whether a comparable situation existed for *T. ferrooxidans*, plasmid pLA (*K. pneumoniae* *nifLABQ*) was introduced into *K. pneumoniae* nif^- strains containing pSA30 or the pIMP plasmids. In *K. pneumoniae* UC165 $nifH^-$ the presence of pSA30 plus pLA allowed partial complementation, where pSA30 alone had not. By contrast, in the $nifD^-$ strain, the presence of pLA plus pSA30 reduced the nitrogenase activity detected when only pSA30 was present in that strain. The presence of pLA with the pIMP plasmids did

not enhance nitrogenase activity, but rather inhibited the partial complementation by pIMP5 in the *K. pneumoniae nifK*$^-$ mutant.

A notable difference exists between this attempt to allow the *K. pneumoniae nifA* gene product to activate the *T. ferrooxidans nif* promoters, and the reported analogous studies. Where the *A. chroococcum nifH* promoter inhibitory effect was alleviated by the presence of the *K. pneumoniae nifA* gene product, plasmid pCK1 provided the *nifA* gene (Jones *et al.*, 1984). Plasmid pCK1 is a wide host range, multicopy plasmid carrying the *K. pneumoniae nifA*, portions of the *nifL* and *nifB* genes on a *Sal*I-*Sal*I fragment, and expressing the *nifA* gene from a constitutive promoter (Kennedy and Robson, 1983). For *T. ferrooxidans*, plasmid pLA was used which contained the entire *K. pneumoniae nifLABQ* genes and part of the *nifF* gene on a *Xho*I-*Hin*dIII fragment (constructed in Chapter Three). None of the *nif* genes of pLA were expressed from a constitutive promoter. The presence of the entire *nifL* gene, including its promoter, on pLA might account for the inability of the *K. pneumoniae nifA* product to activate *T. ferrooxidans nif* promoters. Over-production of the *K. pneumoniae nifL* gene product inhibited *nif* transcription, particularly of *nifHDK*, under physiological conditions in which *nif* was normally expressed (Buchanan-Wollaston *et al.*, 1981b). Furthermore, Riedel *et al.* (1983) identified the *K. pneumoniae nifFLABQ* genes as containing DNA which inhibited the Nif$^+$ phenotype. Their plasmid pGR112 (*K. pneumoniae* 8 kb DNA fragment

containing nifFLABQ genes inserted into the EcoRI site of pACYC184) is very similar to the plasmid used in this study, pLA (K. pneumoniae 6.2 kb DNA fragment containing nifLABQ genes inserted into the HindIII-SalI sites of pACYC184). Riedel et al. (1983) claimed that any complementation or which plasmids containing this DNA were capable, would be masked by the inhibitory phenomenon associated with the DNA. Acetylene reduction studies are envisaged, using the pIMP plasmids in conjunction with a plasmid containing the K. pneumoniae nifA gene product expressed from a constitutive promoter.

Since only pSA30 and pIMP5 restored nitrogenase activity to certain K. pneumoniae nif mutants, and at extremely low levels, diazotrophic growth of these strains would not be expected to be detectable. Jones et al. (1984) obtained similar results when certain A. chroococcum nif genes were present in K. pneumoniae nif$^-$ strains, and suggested that the K. pneumoniae nif products responsible for the maturation of the Mo-Fe-protein were not fully functional in a heterologous system.

In an attempt to determine at which level the T. ferrooxidans nif gene expression was inhibited, the transcription products were studied. Plasmid pIMP11 (T. ferrooxidans nifH) which had not demonstrated complementation in any K. pneumoniae nif mutants, was the only plasmid to give positive results in the RNA study. The appearance of the pIMP11 mRNA transcript which hybridised

positively to pIMP16 [^{32}P]DNA did not appear before a 2.5 h *nif* induction period had elapsed. Plasmid pIMP16 resulted in areas of high hybridisation background, but no broad band was distinguishable, as was the case for pIMP11.

Evidence for a possible second promoter on the *T. ferrooxidans nifHDK* operon was obtained from the *in vitro* transcription-translation studies. The homology studies between the *nifHDK* genes from *K. pneumoniae* and *T. ferrooxidans* had indicated the presence of all three nitrogenase genes on pIMP16. This plasmid produced four proteins, three of which had similar molecular masses to the proteins comprising the *K. pneumoniae* nitrogenase (33, 56 and 60 kD proteins produced by *nifH*, *nifD* and *nifK*, respectively). The significance and possible function of the fourth polypeptide (58 kD) is unknown. The complete nucleotide sequence of the *A. vinelandii nifHDK* cluster revealed the presence of an unknown additional polypeptide (Brigle *et al.*, 1985). It was suggested that this might be the *A. vinelandii nifY* gene product. It is unlikely that this is the case for pIMP16 since neither pIMP5 nor pIMP11, which contain subfragments of the DNA in pIMP16, produced the fourth polypeptide.

A surprising result was the production of the 56 and 60 kD proteins by pIMP5, which had been shown by hybridisation studies to lack the *nifH* promoter region. The presence of a second promoter, which can promote transcription of *nifD* and *nifK* in the absence of *nifH*, is suggested. This contrasts

with the organisation of the nifHDK operon in K. pneumoniae, which contains only a single promoter situated before the nifH gene, although the arrangement of the three genes is the same for both organisms. The nitrogenase genes of certain slow growing Rhizobium strains contain two promoters, one controlling nifH, the other nifDK (see General Introduction) which is comparable to the proposed situation in T. ferrooxidans. In such Rhizobium strains, however, the nif genes are separated into two operons necessitating two promoters, whereas hybridisation studies have shown the T. ferrooxidans nitrogenase genes to be contiguous.

Although both the complementation and the in vitro expression studies indicate that the T. ferrooxidans nifDK may be regulated by its own promoter, there are other possible explanations:

a) The genes could be regulated by a remote promoter in the plasmid which is present on pIMP5, but absent from pIMP16. This, however, is unlikely, since the plasmids are well characterised. Although this could explain the complementation results, it would not explain the in vitro expression results.

b) Homologous recombination between the T. ferrooxidans nifK and K. pneumoniae genes could explain the complementation results, but not the in vitro expression results.

Obtaining the DNA sequences could elucidate the contradictory results obtained in the expression studies of the cloned *T. ferrooxidans nif* genes.

CHAPTER FIVE

DNA SEQUENCE OF *T. FERROOXIDANS* NITROGENASE GENES AND DEDUCED AMINO ACID COMPOSITION OF THE FE-PROTEIN

CHAPTER FIVE

DNA SEQUENCE OF *T. FERROOXIDANS* NITROGENASE GENES AND DEDUCED AMINO ACID COMPOSITION OF THE FE-PROTEIN

Summary. The DNA sequence was determined for the cloned *T. ferrooxidans nifH* and part of the *nifD* genes. A presumptive *T. ferrooxidans nifH* promoter was identified, which showed perfect consensus with *K. pneumoniae nif* promoter sequences. The amino acid sequence was deduced from the DNA sequence. The *T. ferrooxidans* Fe-protein encoded by *nifH* contains 5 cysteine residues and 296 or 298 amino acids (depending on which of two ATG codons initiates transcription). In a comparison of *nifH* DNA sequences from *T. ferrooxidans* and eight other diazotrophs, *P. rhizobium* showed the greatest homology (74%) and *C. pasteurianum* (*nifH1*) the least homology (54%). In a comparison of the amino acid sequences of the Fe-proteins, *P. rhizobium* and *R. japonicum* showed the greatest homology (both 86%), and *C. pasteurianum* (*nifH1* gene product) the least homology (56%) to *T. ferrooxidans*. The codon usage in *nifH* of *T. ferrooxidans* was very similar to the other Gram-negative diazotrophs under comparison.

5.1 INTRODUCTION

Knowledge of the primary structure of a gene can reveal much about the molecular genetics of an organism, including genetic control signals, and the preferred codon usage within the cell. Apart from evolutionary considerations, the comparison of DNA sequences are of invaluable help in detecting functionally important domains within the DNA primary structure.

As discussed fully in the General Introduction, similarity on numerous levels exists between the nitrogenase structural genes of different organisms. Recent DNA sequencing data have revealed unique structures specific to *nif* genes. The *nif* promoters have a characteristic structure of 26 bp containing two regions of conserved sequences at -10 and -23 bp (Beynon *et al.*, 1983). In addition to this, *nifA*-, but not *ntrC*-activated promoters, have an essential promoter element situated more than 100 bp upstream from the transcription initiation site (Buck *et al.*, 1986).

The nitrogenase proteins from different species have been shown to be closely related by the evolutionary conservation of both the DNA and amino acid sequences. Furthermore, the nitrogenase components from several different bacteria are able to complement each other to form enzymatically active hybrid complexes. The purified Fe-proteins from different species have been found to differ with respect to cold lability, sensitivity to oxygen and number of Cys residues

(Eady and Smith, 1979). Comparisons of Fe-proteins have revealed highly conserved regions of amino acid sequences, which coincide with functionally significant domains (Sundaresan and Ausubel, 1981; Chen *et al.*, 1986). Such comparisons rely on the availability of DNA or amino acid sequences of several nitrogenase genes or proteins. Since *T. ferrooxidans* inhabits an ecological niche very different from other diazotrophs, a comparison of nitrogenase proteins could reveal characteristics unique to *T. ferrooxidans*.

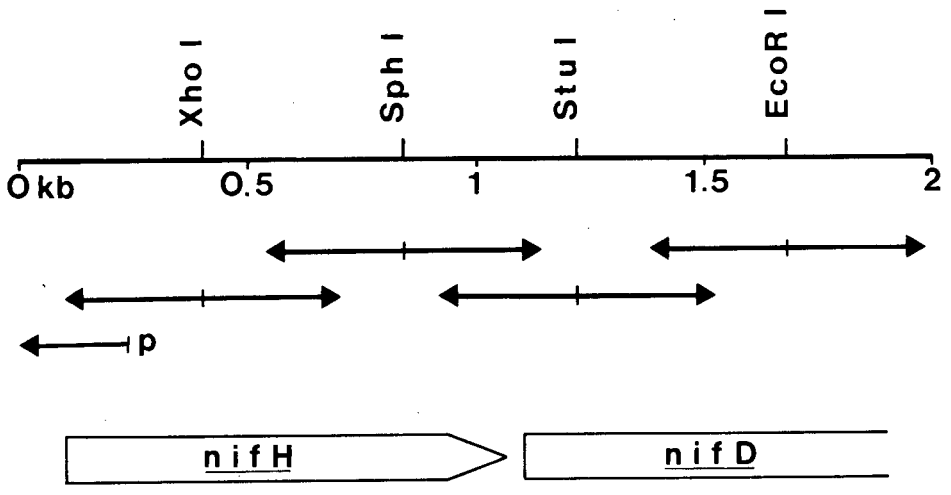
5.2 MATERIALS AND METHODS

5.2a Bacterial strains, vectors and plasmids. Plasmid pIMP16, which contained the *T. ferrooxidans nifHDK* genes, was used as the primary source of DNA. The filamentous phage vectors described by Messing (1983) were used. These vectors, M13mp18 and M13mp19 (Appendix D), were obtained from the Bethesda Research Laboratories (BRL Inc., Rockville, MA, USA). The double stranded replicative form (RF) of the phage vectors, as well as plasmid pIMP16 DNA were prepared according to the CsCl density gradient method (Appendix A). The *E. coli* strain JM103 Δ *lacpro*, *thi*, *strA*, *supE*, *endA*, *sbcB15*, *hsdR4*, F'*traD36*, *proAB*, *lacI*^{q} Z Δ M15 (Messing *et al.*, 1981) was obtained from BRL. This strain was maintained on glucose/minimal medium agar (Appendix C) to select for the F pilus, and was grown at 37^{o}C in TY broth (Appendix C).

5.2b Cloning of T. ferrooxidans DNA into M13mp vectors. The sequencing strategy is outlined in Fig. 5.1. The forced orientation cloning approach was used to prevent recircularisation of the vector and to reduce the background (blue plaques). Approximately 5 µg of pIMP16 DNA was digested with two different restriction endonucleases which produced termini compatible with M13mp sites. The M13mp vector pair (5 µg DNA) was digested with the same, or compatible restriction endonucleases. Agarose gel electrophoresis analysis verified that the digestions were complete before the T. ferrooxidans DNA fragments were ligated into the vector (Appendix A). Optimum results were obtained from dilute ligations which contained approximately 1 µg DNA per 100 µl ligation reaction, and in a vector DNA : insert DNA ratio of 1 : 1.

5.2c Preparation and transformation of competent host cells. E. coli JM103 cells, grown in TY broth were made competent as described in Appendix A. Simultaneously, JM103 cells from a overnight culture were diluted into TY broth such that, at the time of transformation, these cells would be in exponential phase and could serve as the lawn host cells. The appropriate controls were included in the experiment as described for the transformation of E. coli cells in Appendix A. A ligation volume containing 100 - 500 ng of DNA was added to 200 µl of JM103 competent cells. The mixture was placed on ice for 40 min, temperature shocked at 42^{o}C for 3 min and placed on ice for 3 min. To a tube containing 3 ml of melted H top agar (Appendix C) the

Fig. 5.1 Restriction map (5' - 3') of the nifH and nifD region of pIMP16 showing the sequencing strategy. The arrows indicate the direction and extent of sequencing from each restriction site. The arrow marked with p indicates where primer-assisted sequencing was done using synthetic T. ferrooxidans-specific oligonucleotides. Approximately 300 bp were determined from each sequencing reaction, and in all cases, by more than one sequencing reaction. The boxes indicate the relative positioning of the T. ferrooxidans nifH and nifD genes based on the sequencing data.



following components were added: 40 µl IPTG (isopropyl-β-D-thiogalactopyranoside, 100 mM stock solution made in distilled water), 40 µl X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside, 2% stock solution made in dimethylformamide) and approximately 200 µl of exponential phase JM103 cells. A volume (20 - 100 µl) of transformed JM103 cells was added to each tube, the tube was mixed gently and the contents spread evenly on an H agar plate (Appendix C). After the top agar had solidified, the plates were incubated at 37°C overnight to enable the white and blue plaques to form.

5.2d Identification of recombinants and preparation of template DNA. Exponential phase E. coli JM103 cells were infected with a white plaque and incubated for 5 h at 37°C. Centrifugation separated the cell pellet which contained double stranded (ds) M13mp RF recombinant DNA, and the supernatant which contained single stranded (ss) M13mp recombinant virus. The RF DNA of the recombinant phage was obtained from the pelleted cells by the rapid plasmid isolation method (Appendix A). Restriction endonuclease analysis, followed by agarose gel electrophoresis verified the size of the insert DNA. From the culture supernatant, ss M13mp DNA containing the insert was isolated and prepared to serve as the ss DNA template for the sequencing reaction (as described in the BRL manual).

5.2e Sequencing reactions. All DNA sequences were determined using the dideoxy chain termination procedure

(Sanger et al., 1977). For the DNA sequencing reactions, the reagents were obtained in a kit form (BRL kit M13 C/SS I) and used according to the manufacturer's specifications. Two M13-specific sequencing primers were used : a 14-base primer : 5'-TCCAGTCACGACGT-3' (Boehringer Mannheim GmbH-Biochemica, West Germany), and a 17-base primer : 5'-GTAAAACGACGGCCAGT-3' (BRL, MD, USA). In addition to these, a custom-made 15-base primer was synthesised and used to extend the T. ferrooxidans nifH DNA sequence into the promoter region. The synthetic primer had the sequence : 5'-TCCCATTTCCGCCAG-3' and was a gift from Prof D Botes (Dept of Biochemistry, University of Cape Town, SA). The T. ferrooxidans-specific primer was synthesised in an Applied Biosystems 381A DNA synthesiser (Forster City, CA, USA), using their proprietary reagents and solvents. The oligonucleotides were purified by PAGE and UV shadowing. These primers were annealed to the ss M13mp DNA template and synthesis was continued according to the BRL manual. The DNA chains were radiolabelled with [α-^{32}P]dCTP (3000 Ci/ m mol) or [α-^{35}S]dCTP (400 Ci/m mol) obtained from Amersham Int., UK.

5.2f Polyacrylamide gel electrophoresis. The dideoxy sequencing reactions were analysed on denaturing polyacrylamide gels. A 6% polyacrylamide wedge gel (0.4 mm spacers at top, 0.8 mm spacers at bottom) was cast in the BRL model SO apparatus (34 x 40 cm). A sharktooth comb was used (BRL 1045 SC, 24 teeth, 0.4 mm thick, 6 mm point-to-point). Although this had the capacity for 24

samples (6 templates), only 4 templates were routinely loaded in the central wells of the gel, to improve the resolution of fragments.

5.2g Autoradiography. After electrophoresis, gels containing [^{32}P]DNA were placed under Cronex 4 film (Röntgen autoradiographic film) in an X-ray cassette and exposed for 5 - 24 h. Gels containing [^{35}S]DNA were autoradiographed under Kodak XAR-5 X-ray film for 20 h to 4 days.

5.2h Analysis of sequences. The DNA sequences were analysed using the IBM XT computer DNA Tools programme. The deduced amino acids were analysed and compared, using the IBM AT Microgenie Version 2, Protein Alignment Subroutine.

5.3 RESULTS AND DISCUSSION

The DNA sequence of the 1.76 kb DNA region of pIMP16 is given in Fig. 5.2. From the DNA homology studies, the *nifH* and part of the *nifD* genes had been assigned to this area of *T. ferrooxidans* DNA. A comparison of the sequences of *T. ferrooxidans* DNA with the *nifH* and *nifD* sequences of *K. pneumoniae* (Sundaresan and Ausubel, 1981) and *A. vinelandii* (Brigle *et al.*, 1985) revealed extensive regions of homology. These known DNA sequences assisted in allocating the different *T. ferrooxidans* sequences to regions of the *nifH* gene, and ensuring that there were no sequence deletions when sequencing away from a cloning site in both

Fig. 5.2 The complete nucleotide sequence of the *T. ferrooxidans* *nifH* and part of the *nifD* genes. Only the coding strand (5' - 3') is shown, with the direction of transcription from left to right. Restriction endonucleases having 6 bp recognition sites are indicated. The deduced amino acid sequences for the Fe-protein (first encoded polypeptide) and part of the Mo-Fe-protein β subunit (second encoded polypeptide) are shown below the coding sequences. The regions upstream from the Fe-protein (encoded by *nifH*) which show perfect consensus with *K. pneumoniae* *nif* promoters are underlined. The two sequences preceding the *nifD* gene (which encodes the β subunit of the Mo-Fe-protein) which show limited homology to *K. pneumoniae* *nif* promoter consensus sequences, are also underlined. The two potential RBS are indicated by bold type.

```
1    TATAGTGTTAAATAGGCCATGATGAACTTGGCACGGCCCTTGCAACAGCGAGGACGGAAC

61   GCGACTCGTCCCTTTTGGGGGGCTTCCATCTGGCAAGCTAGTCATTTTTAAATAGGAGAC

121  ATGGCAATGAGTGACAAACTAAGACAAATCGCCTTTTATGGTAAAGGGGGCATTGGCAAG
     MetAlaMetSerAspLysLeuArgGlnIleAlaPheTyrGlyLysGlyGlyIleGlyLys

181  TCCACGACCTCGCAGAAACACCTGGCGGCACTGGCGGAAATGGGACAGAAAATTCTCATC
     SerThrThrSerGlnLysHisLeuAlaAlaLeuAlaGluMetGlyGlnLysIleLeuIle

241  GTCGGCTGCGATCCCAAGGCCGACTCCACCCGACTGATCCTGCATTCCAAGGCGCAAGAC
     ValGlyCysAspProLysAlaAspSerThrArgLeuIleLeuHisSerLysAlaGlnAsp

                                                        XhoI
301  ACCGTGCTTAGTCTGGCGGCCGAAGCCGGCAGTGTGGAGGATCTCGAGCTTGAAGATGTC
     ThrValLeuSerLeuAlaAlaGluAlaGlySerValGluAspLeuGluLeuGluAspVal

361  ATGAAGGTGGGGTATCGCGACATCCGCTGCGTCGAGTCCGGTGGCCCTGAGCCGGGCGTG
     MetLysValGlyTyrArgAspIleArgCysValGluSerGlyGlyProGluProGlyVal

                          BclI
421  GGTTGCGCAGGTCGTGGTGTGATCACCTCCATCAACTTCCTGGAAGAAAACGGGGCCTAT
     GlyCysAlaGlyArgGlyValIleThrSerIleAsnPheLeuGluGluAsnGlyAlaTyr

        NarI
481  GATGGCGCCAACTATGTCTCCTACGACGTGTTGGGAGACGTGGTCTGCGGCGGCTTTGCC
     AspGlyAlaAsnTyrValSerTyrAspValLeuGlyAspValValCysGlyGlyPheAla

                                  BglII
541  ATGCCCATCCGGAAACAGGCGCAGGAGATCTACATCGTCATGTCCGGCGAAATGATGGCC
     MetProIleArgLysGlnAlaGlnGluIleTyrIleValMetSerGlyGluMetMetAla
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Fig. 5.2 (continued)

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601  ATGTACGCGGCCAACAACATCTCCAAGGGCGTGCTCAAGTATGCCAACTCCGGCGGCGTA
     MetTyrAlaAlaAsnAsnIleSerLysGlyValLeuLysTyrAlaAsnSerGlyGlyVal

661  CGTCTGGGCGGCCTCATCTGTAACGAGCGTCAGACCGACAAGGAACTGGAACTGGCCGAG
     ArgLeuGlyGlyLeuIleCysAsnGluArgGlnThrAspLysGluLeuGluLeuAlaGlu

721  GCATTGGCCGGCAAACTGGGCACCAAGCTCATTCATTTCGTACCCCGCGACTTCATCGTG
     AlaLeuAlaGlyLysLeuGlyThrLysLeuIleHisPheValProArgAspPheIleVal

          SphI
781  CAGCATGCCGAATTGCGGCGCATGACGGTGCTGGAATACGCACCGGAATCCAAGCAGGCG
     GlnHisAlaGluLeuArgArgMetThrValLeuGluTyrAlaProGluSerLysGlnAla

841  CAAGAATACCGGACTCTGGCGGAAAAAATTCATGCCAATGCCGGCAACCCGGCTATCCCC
     GlnGluTyrArgThrLeuAlaGluLysIleHisAlaAsnAlaGlyAsnProAlaIlePro

                              BglII
901  ACCCCGATCACCATGGACGAGTTGGAAGATCTGCTTATGGACTTCGGCATCATGCAGAAG
     ThrProIleThrMetAspGluLeuGluAspLeuLeuMetAspPheGlyIleMetGlnLys

961  GAAGACACCAGCATCATCGGCAAGACTGCTGCCGAATTGGCGGCTGCGGGAATGTAATGA
     GluAspThrSerIleIleGlyLysThrAlaAlaGluLeuAlaAlaAlaGlyMetEndEnd

1021 ACGGTGGCGCGGGTTGTTCACCGTCCCCACCAGGATGTGGCACCTAATTGAAGCAAGGAG

                              BglII
1081 TACACCAAAATGAGTATATCAGCGGAAGATCTCAGCACACAGCCACAGCGGAGAAAACTG
          MetSerIleSerAlaGluAspLeuSerThrGlnProGlnArgArgLysLeu

                    ClaI              StuI
1141 CCAGAAATCGCGGAACTGATCGATGAGACGCTCAAGGCCTATCCAGAGAAGTTCGCCAAG
     ProGluIleAlaGluLeuIleAspGluThrLeuLysAlaTyrProGluLysPheAlaLys

1201 CGGCGCGCCAAGCACCTCAATGTCTATGAAGAGGGCAAGAGCGAGTGCGACTGCAAGTCC
     ArgArgAlaLysHisLeuAsnValTyrGluGluGlyLysSerGluCysAspCysLysSer

                                   SacII
1261 AACATCAAATCCGTTCCCGGCGTGATGACCATCCGCGGCTGCGCCTACGCCGGTTCTTAC
     AsnIleLysSerValProGlyValMetThrIleArgGlyCysAlaTyrAlaGlySerTyr

1321 GGCGTGGTCTGGAGCCCAGTCAAGGACATGATCCATATCAGCCATGGTCCGGTCGGCTGC
     GlyValValTrpSerProValLysAspMetIleHisIleSerHisGlyProValGlyCys

1381 GGCCACTACGCGCGCGCTGGACGGCGCGCCTACTACATCGGCACCACCGGGGTGGACACC
     GlyHisTyrAlaArgAlaGlyArgArgAlaTyrTyrIleGlyThrThrGlyValAspThr

1441 TACACGACCATGCACTTCACCTCGGACTTCCAGGTCAAGGACATCGTTTTCGGCGGCGAC
     TyrThrThrMetHisPheThrSerAspPheGlnValLysAspIleValPheGlyGlyAsp

1501 AAGAAACTCGCCAAGCTGATGGACGAGTTGGAAGAACTGTTTCCAATGTCCAAGGGCATC
     LysLysLeuAlaLysLeuMetAspGluLeuGluGluLeuPheProMetSerLysGlyIle

                         PvuI                    EcoRV
1561 ACGGTGCAATCAGAATGTCCGATCGGGCTGATCGGCGACGATATCGAGGCGGTTTTCAAG
     ThrValGlnSerGluCysProIleGlyLeuIleGlyAspAspIleGluAlaValPheLys

           SacIIEcoRI            KpnI
1621 AAAAAGGCCGCGGAATTCGGCAAGCCGGTGGTACCCAATCGCTGTGAGGGTTTCCGTGGT
     LysLysAlaAlaGluPheGlyLysProValValProAsnArgCysGluGlyPheArgGly

1681 GTGTCGCAGTCGCTTGGCCACCACATCGCCAACGATAGTATCCGCGACTGGGTGCTGGAC
     ValSerGlnSerLeuGlyHisHisIleAlaAsnAspSerIleArgAspTrpValLeuAsp

1741 CCCGCCGCAGACAAGC
     ProAlaAlaAspLys
```

Fig. 5.3 Comparison of *nif* promoter sequences. The -10 and -23 bp regions of conserved sequence of the *K. pneumoniae* (Kp) *nifH* promoter are given. The underlined nucleotides indicate areas of absolute consensus between the *K. pneumoniae* *nifH* promoter, and the DNA sequences which precede the *T. ferrooxidans* (Tf) *nifH* gene.

	-23 ▽		-10 ▽
Kp *nifH*:	C T G G T A T	N_5	C T G C A
Tf *nifH*:	T T G G C A C	N_5	T T G C A

Fig. 5.4 Comparison of *K. pneumoniae* *nif* promoter consensus sequences with the two *T. ferrooxidans* sequences (X and Y) which precede the *T. ferrooxidans* *nifD* gene. The invariant nucleotides of *nif* consensus promoters are underlined.

	-23 ▽		-10 ▽
nif consensus promoter:	C T G G	N_8	T T G C A
Tf sequence X:	T C G G	N_8	C T G C C
Tf sequence Y:	C T G G	N_{10}	A A G C A

directions. The restriction endonuclease sites revealed by the DNA sequence (Fig. 5.2) were in agreement with the restriction maps obtained previously by agarose gel electrophoresis techniques.

5.3a Nucleotide sequence of *T. ferrooxidans nifH* and the flanking regions. The complete nucleotide sequence of the *T. ferrooxidans nifH* gene is given in Fig. 5.2. The DNA region preceding the *nifH* gene contains sequences which show perfect consensus with the *nifH* promoter sequences of *K. pneumoniae* (Beynon *et al.*, 1983) (Figs. 5.2 and 5.3). The sequence 5'-AGGAGA-3' is apparent just preceding the proposed *nifH* ATG start codon. This sequence shows perfect homology to the Shine-Dalgarno sequence (AGGAG) (Shine and Dalgarno, 1975) or the ribosome binding site (RBS). Two ATG codons, encoding Met separated by a GCA codon encoding Ala, appear directly after the RBS. The Fe-protein encoded by *nifH*, consists thus of 296 or 298 amino acids, depending on which of the two ATG codons initiates translation of the gene. Translation of the *nifH* gene is terminated by tandem stop codons.

5.3b Nucleotide sequence of *T. ferrooxidans nifD* and the flanking regions. The nucleotide sequence for part of the *T. ferrooxidans nifD* gene is given in Fig. 5.2. A potential RBS of sequence 5'-AGGAG-3' precedes the proposed ATG translation initiation codon for the *T. ferrooxidans nifD* gene. The presence of RBS preceding the *T. ferrooxidans nifH* and *nifD* genes is in agreement with Brigle *et al.*

(1985), who reported the presence of RBS preceding each of the the nifH, nifD and nifK genes of A. vinelandii. Chen et al. (1986) have identified putative RBS preceding the nifH and the nifD genes of C. pasteurianum.

The results previously obtained in the complementation and in vitro expression studies suggested the presence of a promoter regulating the T. ferrooxidans nifDK genes. If this suggestion is correct, the nucleotide sequence upstream of the nifD gene should contain promoter sequences. The DNA sequences preceding the nifD gene revealed two possible areas which showed limited homology to the -23 and -10 bp nif promoter consensus sequences (these areas are referred to as sequence X and Y) (Figs. 5.2 and 5.4).

Sequence X showed consensus with the K. pneumoniae nif promoters with respect to the 10 bp distance separating the canonical GG at -23 bp and the GC at -12 bp. The nucleotide sequence homology of the T. ferrooxidans DNA was limited to only two nucleotides in each of the -23 and -10 bp nif promoter consensus regions (Fig. 5.4). Sequence X is situated within the coding region of the T. ferrooxidans nifH gene which enables a sufficiently large leader region to exist between the trancription initiation site and the putative RBS. It must be noted however, that statistically, the likelihood of generating a GG-N_{10}-GC sequence in an organism with a 60% GC content (Harrison, 1984), is 1 in 123 bp. It is thus highly likely that such a sequence could be found in the DNA region preceding the T. ferrooxidans nifD

gene.

Sequence Y is situated in the intercistronic region separating the *T. ferrooxidans nifH* and *nifD* genes (Fig. 5.2). This sequence showed homology to three nucleotides in each of the -23 and -10 bp *nif* promoter consensus sequences (Fig. 5.4). In sequence Y however, the conserved GG at -23 bp and the GC at -12 bp were separated by 12 bp as opposed to 10 bp in *K. pneumoniae nif* promoters. Since 10 bp forms one helical turn in the DNA molecule, the distance of 10 bp separating the two consensus sequences of a *K. pneumoniae nif* promoter is highly significant. The *nif* promoter consensus -12 and -23 bp sequences would be located directly alongside each other in the secondary DNA structure, whereas in the *T. ferrooxidans* sequence Y these regions would not correspond. The location of sequence Y does not allow sufficient leader region between the transcription initiation site, and the putative RBS of the *nifD* gene.

Despite the conservation of *nif* promoter structure in at least three N_2 fixing genera, *nif* promoter structure does not appear to be universally conserved. In the heterocystous cyanobacterium *Anabaena* 7120, the *nifH* promoter has the sequence TCTAC at the -14 to -10 bp region, instead of the canonical TTGCA (Tumer *et al.*, 1983). More recently, Chen *et al.* (1986) have reported that the multiple structural *nifH* genes (*nifH1* and *nifH2*), encoding the Fe-protein in *C. pasteurianum* do not contain *nif* consensus promoter sequences. Instead, a sequence resembling the *E.*

coli consensus promoter preceded nifH2, and a TATAAT sequence preceded the nifH1 gene. No E. coli consensus promoter sequences were detectable in the DNA preceding the T. ferrooxidans nifD gene, and the two sequences, X and Y showed the most homology to nif promoter consensus sequences. The presence of a promoter preceding the T. ferrooxidans nifD gene was suggested, however, by the results of the gene expression studies (Chapter Four), where the T. ferrooxidans nifD and nifK genes were translated in the absence of the nifH promoter.

5.3c T. ferrooxidans nifH and nifD intercistronic region. The amino acid sequences which were deduced from the DNA sequence revealed a 68 bp area corresponding to the intercistronic region between the nifH and nifD genes (Fig. 5.2). This region corresponded to that of the A. vinelandii nifH and nifD genes (Brigle et al., 1985), and confirmed the contiguous nature of the T. ferrooxidans nifH and nifD genes in the operon. No inverted-repeat DNA sequences were detectable upstream of the nifD gene, suggesting that transcription of the nifH gene is not terminated between the nifH and nifD genes.

5.3d Comparison of DNA sequences. In Fig. 5.5 a comparison was made of the DNA sequences of the amino acid encoding region of the nifH genes of T. ferrooxidans, A. vinelandii (Brigle et al., 1985), C. pasteurianum (the nifH1 structural gene, Chen et al., 1986), K. pneumoniae (Sundaresan and Ausubel, 1981), R. meliloti (Török and Kondorosi, 1981), P.

Fig. 5.5 Comparison of DNA sequence alignment of *nifH* from *T. ferrooxidans* (Tf), an *Anabaena* sp. (As), *K. pneumoniae* (Kp), *R. japonicum* (Rj), *R. trifolii* (Rt), *C. pasteuriaum* (*nifH1*, Cp) *P. rhizobium* (Pr), *A. vinelandii* (Av), and *R. meliloti* (Rm) (references are given in Results and Discussion 5.3d). Only the coding strand (5' - 3') is shown with the direction of transcription from left to right, beginning at the translation initiation codon (ATG) and ending at the termination codon (UAA, UAG or UGA) of the *nifH* gene of each organism. The bold type nucleotides are homologous to nucleotides in the *T. ferrooxidans* *nifH* DNA sequence.

```
123Tf       ATGGCAATGAGTGACAAACTAAGACAAATCGCCTTTTATGGTAAAGGGGGCATTGGC

Av          ATGGCTATGCGT              CAATGCGCCATCTACGGCAAAGGTGGTATCGGT
As                ATG       GCTATGCGTCAATGCGCCATCTACGGCAAAGGTGGTATCGGT
Cp                ATG             AGACAGGTAGCTATTTATGGAAAAGGTGGAATAGGA
Kp                ATG             CGTCAATGCGCTATTTACGGTAAAGGCGGTATCGG
Rm          ATGGCA          GCTCTGCGTCAGATCGCGTTCTACGGTAAGGGGGGTATCGGC
Pr                ATG  T CTTCACTGAGACAAATCGCGTTCTACGGAAAGGGCGGCATCGGC
Rt                ATG   GCTGCTCTGCGTCAGATCGCGTTTTACGGAAAGGGAGGCATTGGC
Rj                ATG   GCTTCACTAAGACAAATCGCCTTCTACGGGAAGGGCGGAATCGGC

180Tf      AAGTCCACGACCTCGCAGAAACACCTGGCGGCACTGGCGGAAATGGGACAGAAAATTCTC

Av         AACTCCACCACTACTCAGAACCTGGTGGCAGCCCTGGCTGAGATGGGCAAGAAGGTCATG
As         AAGTCCACCACTACTCAGAACCTGGTGGCAGCCCTGGCTGAGATGGGCAAGAAGGTCATG
Cp         AAATCAACTACAACACAAAACTTAACATCAGGTCTTCATGCAATGGGTAAGACTATAATG
Kp         AAATCCACCACCACGCAGAACCTCGTCGCCGCGCTGGCGGAGATGGGTAAGAAAGTGATG
Rm         AAGTCCACGACCTCCCAAAATACACTCGCCGCGCTTGTCGACCTGGGGCAAAAGATCCTT
Pr         AAGTCGACCACGTCCCAGAATACGTTGGCGGCACTGGCCGAGATGGGCCAGAAAATCCTG
Rt         AAATCCACTACATCCCAGAATACGCTCGCTGCCCTGGTCGAACTTGGGCAGAAAATCCTC
Rj         AAGTCCACCACTTCGCAGAACACGCTAGCGGCGCTGGCAGAGATGGGTCAGAAGATCCTG

240Tf      ATCGTCGGCTGCGATCCCAAGGCCGACTCCACCCGACTGATCCTGCATTCCAAGGCGCAA

Av         ATCGTTGGTTGTGACCCGAAAGCTGACTCCACCCGCCTGATCCTGCACTCCAAGGCCCAG
As         ATCGTTGGTTGTGACCCGAAAGCTGACTCCACCCGCCTGATCCTGCACTCCAAGGCCCAG
Cp         GTAGTAGGTTGTGATCCTAAGGCAGATTCAACAAGATTATTACTTGGAGGTCTTGCACAG
Kp         ATCGTCGGCTGCGATCCGAAGGCGGACTCCACCCGTCTGATTCTGCACGCCAAAGCACAG
Rm         ATTGTCGGCTGCGATCCGAAAGCGGACTCCACGCGCCTCATCCTGAACGCAAAGGCACAG
Pr         ATCGTCGGATGCGATCCTAAGGCGGACTCGACGCGCCTCATCCTGCACGCGAAGGCGCAG
Rt         ATCGTCGGCTGCGACCCAAAAGCTGATTCGACGCGATTGATCCTGAACTCCAAAGCGCAG
Rj         ATTGTAGGGTGCGATCCGAAAGCGGACTCGACTCGCCTTATTCTGCACGCCAAGGCTCAA

300Tf      GACACCGTGCTTAGTCTGGCGGCCGAAGCCGGCAGTGTGGAGGATCTCGAGCTTGAAGAT

Av         AACACCATCATGGAAATGGCTGCCGAAGCCGGTACCGTGGAAGATCTGGAGCTGGAAGAC
As         AACACCATCATGGAAATGGCTGCCGAAGCCGGTACCGTGGAAGATCTGGAGCTGGAAGAC
Cp         AAATCAGTTCTTGATACATTAAGAGAAGAAGG A     GAAGACGTTGAATTAGATTCC
Kp         AACACCATTATGGAGATGGCCGCGGAAGTCGGCTCGGTCGAGGACCTCGAACTCGAAGAC
Rm         GACACCGTACTGCATCTTGCGGCAACCGAAGGTTCGGTCGAAGATCTCGAGCTCGAGGAC
Pr         GACACGATTTTGAGCCTTGCAGCGAGCGCTGGCAGCGTGGAAGACCTCGAACTCGAGGAC
Rt         GGCACGGTTCTGGATCTAGCCGCAACGAAGGGTTCAGTTGAAGATCTGGAACTCGGCGAT
Rj         GACACGATTTTGAGTCTTGCCGCGAGCGCCGGCAGCGTGGAGGATCTGGAGCTCGAGGAC
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Fig. 5.5 (continued)

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360Tf    GTCATGAAGGTGGGGTATCGCGACATCCGCTGCGTCGAGTCCGGTGGCCCTGAGCCGGGC

Av       GTGCTGAAGGCTGGCTACGGCGGCGTCAAGTGCGTTGAGTCCGGTGGTCCGGAGCCGGGC
As       GTGCTGAAGGCTGGCTACGGCGGCGTCAAGTGCGTTGAGTCCGGTGGTCCGGAGCCGGGC
Cp       ATATTAAAAGAAGGATATGGCGGAATTAGATGTGTTGAATCCGGTGGTCCAGAACCAGGA
Kp       GTGCTGCAAATTGGCTACGGCGATGTGCGCTGCGCGGAATCCGGCGGCCCGGAGCCAGGC
Rm       GTGCTCAAAGTGGGTTACAGAGGCATCAAGTGCGTGGAGTCCGGTGGCCCAGAGCCGGGC
Pr       GTGATGAAGGTCGGCTACAAGGACATCCGATGCGTGGAGTCCGGTGGTCCCGAGCCGGGT
Rt       GTGCTCAAAACTGGCTACGGCGGCATCAAATGTGTGGAGTCCGGCGGCCCTGAACCCGGC
Rj       GTAATGAAGGTTGGCTACCAGGACATTCGCTGCGTTGAGTCCGGTGGCCCTGAGCCAGGT

420Tf    GTGGGTTGCGCAGGTCGTGGTGTGATCACCTCCATCAACTTCCTGGAAGAAAACGGGGCC

Av       GTTGGCTGCGCCGGCCGTGGTGTTATCACAGCAATCAACTTCCTGGAAGAGGAAGGCGCC
As       GTTGGCTGCGCCGGCCGTGGTGTTATCACAGCAATCAACTTCCTGGAAGAGGAAGGCGCC
Cp       GTAGGATGTGCAGGAAGAGGAATAATCACTTCAATAAACATGCTTGAACAATTAGGAGCT
Kp       GTCGGCTGCGCCGGACGCGGCGTGATCACGGCGATCAACTTTCTTGAAGAAGAAGGCGCC
Rm       GTCGGCTGCGCCGGACGCGGCGTTATCACCTCGATCAACTTCCTGGAAGAGAACGGCGCT
Pr       GTCGGCTGCGCGGGCCGCGGCGTCATCACCTCGATCAATTTCCTGGAGGAGAACGGCGCC
Rt       GTCGGCTGCGCCGGACGGGGGGTCATAACGTCGATCAACTTCTTGGAAGAAAACGGCGCC
Rj       GTCGGCTGCGCCGGCCGCGGTGTCATCACCTCGATCAATTTTCTTGAAGAGAACGGAGCC

480Tf    TATGATGGCG CC  AACTATGTCTCCTACGACGTGTTGGGAGACGTGGTCTGCGGCGGC

Av       TACGAAGACGATCTGGACTTCGTATTCTACGACGTCCTGGGCGACGTGGTGTGTGGCGGC
As       TACGAAGACGATCTGGACTTCGTATTCTACGACGTCCTGGGCGACGTGGTGTGTGGCGGC
Cp       TATACAGACGATTTAGACTATGTATTCTACGATGTACTTGGAGACGTTGTTTGTGGTGGA
Kp       TACGAGGACGATCTCGATTTCGTGTTCTATGACGTGCTCGGCGACGTGGTCTGCGGCGGC
Rm       TACAACGATG TC  GATTACGTCTCATACGACGTGCTAGGGGACGTAGTATGCGGCGGC
Pr       TATGAGAAC ATT  GACTATGTCTCATATGACGTGCTCGGCGACGTCGTTTGCGGTGGC
Rt       TACGACGATG TC  GACTACGTCTCCTATGACGTGCTCGGAGACGTGGTGTGCGGGGGC
Rj       TACGAGAACA TT  GACTATGTTTCTTACGATGTGCTTGGCGACGTTGTTTGCGGTGGC

537Tf    TTTGCCATGCCCATCCG GA AAC AGGCGCAGGAGATCTACATCGTCATGTCCGGCGAA

Av       TTCGCCATGCCGATCCGCGAGAACAAGGCCCAAGAAATCTACATCGTCTGCTCCGGTGAG
As       TTCGCCATGCCGATCCGCGAGAACAAGGCCCAAGAAATCTACATCGTCTGCTCCGGTGAG
Cp       TTCGCAATGCCAATCAGAGAAGGAAAAGCTCAGGAAATATATATAGTAGCAAGTGGAGAA
Kp       TTCGCCATGCCGATCCGCGAAAACAAAGCCCAGGAGATCTACATCGTCTGCTCCGGCGAA
Rm       TTTGCGATGCCTATTCGCGAAAACAAGGCTCAGGAAATCTACATCGTCATGTCCGGTGAG
Pr       TTTGCGATGCCGATCCGGGGAAAACAAGGCGCAGGAGATCTATATCGTGATGTCTGGAGAA
Rt       TTTGCTATGCCCATCCGCGAGAACAAGGCTCAGGAAATCTACATCGTCATGTCCGGTGAG
Rj       TTTGCGATGCCAATCCGCGAAAACAAGGCGCAGGAGATCTACATCGTGATGTCTGGTGAA

594Tf    ATGATGGCCATGTACGCGGCCAACAACATCTCCAAGGGCGTGCTCAAGTATGCCAACTCC

Av       ATGATGGCCATGTACGCCGCCAACAACATCTCCAAGGGCATCGTGAAGTATGCCAACTCC
As       ATGATGGCCATGTACGCCGCCAACAACATCTCCAAGGGCATCGTGAAGTATGCCAACTCC
Cp       ATGATGGCACTATATGCTGCTAATAACATATCAAAAGGTATCCAAAAATATGCTAAGAGC
Kp       ATGATGGCGATGTACGCGGCCAACAATATCTCCAAAGGGATCGTTAAATACGCCAAATCC
Rm       ATGATGGCGCTCTATGCCGCCAACAACATCGCGAAGGGTATCCTGAAGTACGCCCATGCG
Pr       ATGATGGCAATGTATGCCGCAAACAATATCTCCAAAGGTATCCTGAAATACGCCAACTCT
Rt       ATGATGGCTCTGTATGCTGCAAACAACATCGCGAGGGCATCCTCAAATACGCCAGCGCC
Rj       ATGATGGCAATGTATGCCGCAAACAATATTTCCAAGGGGATCCTGAAATACGCGAACTCA

654Tf    GGCGGCGTACGTCTGGGCGGCCTCATCTGTAACGAGCGTCAGACCGACAAGGAACTGGAA

Av       GGCAGCGTGCGTCTGGGCGGCCTGATCTGCAACAGCCGTAACACCGACCGCGAAGACGAG
As       GGCAGCGTGCGTCTGGGCGGCCTGATCTGCAACAGCCGTAACACCGACCGCGAAGACGAG
Cp       GGTGGAGTTAGACTTGGTGGTATCATCTGTAACAGTAGAAAAGTTGCAAATGAATATGAA
Kp       GGCAAGGTGCGCCTCGGCGGCCTGATCTGTAACTCACGTCAGACCGACCGTGAAGACGAA
Rm       GGCGGCGTGCGGCTGGGGGGGTTGATTTGCAACGAGCGCCACACCGATCGGGAGCTCGAC
Pr       GGCGGCGTGCGGCTGGGCGGCCTGATCTGCAACGAGCGGCAGACCGATAAGGAGCTGGAG
Rt       GGAAGCGTGCGGCTGGGCGGCCTGATTTGTAATGAGCGGCAGACCGACCGAGAATTAGAC
Rj       GGTGGGGTGCGGTTGGGCGGCCTGATCTGCAACGAGCGGCAGACCGACAAGGAATTGGAA
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Fig. 5.5 (continued)

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714Tf     CTGGCCGAGGCATTGGCCGGCAAACTGGGCACCAAGCTCATTCATTTCGTACCCCGCGAC

Av        CTGATCATCGCTCTGGCCAACAAGCTGGGCACCCAGATGATCCACTTCGTGCCGCGTGAC
As        CTGATCATCGCTCTGGCCAACAAGCTGGGCACCCAGATGATCCACTTCGTGCCGCGTGAC
Cp        TTACTTGATGCTTTGCTAAAGAACTAGGAAGTCAATTAATACATTTCGTACCAAGAAGC
Kp        CTGATTATTGCCCTGGCGGAAAAGCTCGGTACCCAGATGATCCACTTTGTGCCCCGCGAC
Rm        CTCGCCGAGGCACTTGCCGCCCGCCTCAATTCCAAGCTCATCCACTTCGTGCCGCGCGAC
Pr        CTGGCGGAGGCGCTGGCCAAGAAGTTAGGTACTCAGCTGATCTACTTCGTGCCGCGCGAC
Rt        CTCGCTGAAGCGCTGGCGCAAAGCTCAATTCAAAGCTCATTCACTTCGTGCCGCGAGAC
Rj        CTGGCGGAAGCGTTGGCCAAGAAGCTTGGCACTCAACTGATCTACTTCGTGCCGCGTGAC


774Tf     TTCATCGTGCAGCATGCCGAATTGCGGCGCATGACGGTGCTGGAATACGCACCGGAATCC

Av        AACGTCGTGCAGCGCGCCGAAATCCGCCGCATGACCGTGATCGAATACGATCCGAAAGCC
As        AACGTCGTGCAGCGCGCCGAAATCCGCCGCATGACCGTGATCGAATACGATCCGAAAGCC
Cp        CCAATGGTTACAAAAGCAGAAATCAATAAGCAAACTGTTATTGAATATGATCCTACTTGT
Kp        AACATCGTGCAGCGCGCGGAGATCCGCCGCATGACGGTTATCGAGTACGACCCCGCCTGT
Rm        AATATCGTTCAGCACGCAGAGCTCAGAAAGATGACAGTGATCCAATATGCGCCGAACTCT
Pr        AATGTGGTGCAGCATGCCGAGCTACGGCGCATGACGGTGCTGGAGTATGCCCCTGAGTCG
Rt        AACATCGTCCAGCACGCGCGAGCTTAGAAAGATGACCGTGATCCAATACGCGCCACGCTCC
Rj        AATGTGGTGCAGCATGCAGAGCTGCGTCGCATGACGGTGCTTGAATATGCACCCGATTCC


834Tf     AAGCAGGCGCAAGAATACCGGACTCTGGCGGAAAAAATTCATGCCAATGCCGGCAACCCG

Av        AAGCAAGCCGACGAATACCGCGCTCTGGCCCGCAAGGTCGTCGACAA  CAAAC TGCTG
As        AAGCAAGCCGACGAATACCGCGCTCTGGCCCGCAAGGTCGTCGACAA  CAAAC TGCTG
Cp        GAACAGGCTGAAGAATACAGAGAATTAGCTAGAAAAGTAGATGCAAATG AATTATTC
Kp        AAACAGGCCAACGAATACCGCACCCTGGCGCAGAAGATCGTCAACAACACCATGAAAGTG
Rm        AAGCAAGCCGGGGAATATCGCGCCCTGGCTGAAAAGATCCATGCAAATTCCGGCCGAGGC
Pr        CAGCAGGCCGATCACTATCGCAATCTTGCGACCAAGGTTCACAACAATGGCGGCAAAGGC
Rt        AAACAGGCGGCGGAATATCGATGGCTGGCCGAGAAGATCCACTCCAATTCGGGGAAGGGC
Rj        AAGCAGGCTGATCACTATCGGAAACTAGCGGCCAAGGTTCACAATAATGGCGGCAAGGGC


894Tf     GCTATCCCCACCCCGATCACCATGGACGAGTTGGAAGATCTGCTTATGGACTTCGGCATC

Av        GTCATCCCGAACCCGATCACCATGGACGAGCTCGAAGAGCTGCTGATGGAATTCGGTATC
As        GTCATCCCGAACCCGATCACCATGGACGAGCTCGAAGAGCTGCTGATGGAATTCGGTATC
Cp        GTTATACCAAAGCCAATGACTCAAGAAAGACTTGAAGAAATATTAATGCAATATGGTTTA
Kp        G  TGCCGACGCCCTGCACCATGGATGAGCTGGAATCGCTGCTGATGGAGTTCGGCATC
Rm        ACCGTCCCTACACCGATCACTATGGAGGAACTGGAGGACATGCTGCTCGACTTTGGAATC
Pr        ATCATTCCGACTCCGATCTCCATGGATGAGCTCGAGGACATGCTGATGGAGCATGGCATT
Rt        ACTATCCCTACTCCTATCACTATGGAGGAGCTGGAGGACATGCTACTCGACTTCGGAATC
Rj        ATCATTCCGACCCCGATCTCAATGGATGAGCTCGAGGACATGCTGATGGAGCATGGCATT


953Tf     ATGCAGAAGGAAGACACCAGCATCATCGGCAAGACTGCTGCCGAATTGGCGGCTGCGGGA

Av        ATGGAAGTCGAAGA CGAATCCATCGTCGGCAAAACCGCCGAAGAAGTCTGA
As        ATGGAAGTCGAAGA CGAATCCATCGTCGGCAAAACCGCCGAAGAA GTCTGATAG
Cp        ATGGATCTATAA
Kp        ATGGAAGAGGAAGA CACCAGCATCATTGGCAAAACCGCCGCCGAA          GA
Rm        ATGAAGAGCGACGAGCAGATGCTTGCCGAACTCCAC GCCAAGGAAGCCAAGGTAA
Pr        ATGAAGCCCGTCGACGAATCCATCGTCGGCAAGACCGCCGCCGAACTCGCGGCCTCGTAA
Rt        ATGAAGTCGGACGAGCAGATGCTTGAAGAACTTCTCGCCAAAGAGGTGCAGGCGGCCGTG


1012Tf    ATGTAA

Av
As
Cp
Kp        AAACGCGGCCTGA
Rm
Pr        GCGATAA
Rt
```

Table 5.1 Comparison of DNA and amino acid sequences of the Fe-proteins from the specified diazotrophs. The references for the DNA and amino acid sequences are given in Results and Discussion 5.3d and 5.3e, respectively. The % homologies are given relative to the *T. ferrooxidans* sequences. aa: amino acid, NA: data not available.

	Number of aa	Number of Cys	% aa homology	% DNA homology
T. ferrooxidans	298	5	100	100
A. vinelandii	289	7	73	73
Anabaena sp.	301	6	70	73
C. pasteurianum	273	6	56	54
K. pneumoniae	289	9	74	71
R. meliloti	292	5	79	71
P. rhizobium	301	5	86	74
R. trifolii	297	5	77	64
R. japonicum	294	5	86	73
R. phaseoli	297	5	83	NA

rhizobium (Scott et al., 1983a), R. japonicum (Fuhrman and Hennecke, 1984), R. trifolii (Scott et al., 1983b), and an Anabaena sp. (Mevarech et al., 1980).

The extent of homology between the nifH DNA sequences is given in Table 5.1. The nifH DNA sequence of P. rhizobium showed greatest homology to the corresponding T. ferrooxidans sequence (74% homology). The lowest DNA homology of 54% existed between the nifH genes of C. pasteurianum (nifH1) and T. ferrooxidans. With the exception of C. pasteurianum (54%) and R. trifolii (64%), the nifH DNA sequences of the other organisms were closely related to T. ferrooxidans, ranging between 71% and 74% homology.

5.3e Comparison of the Fe-proteins. A comparison was made between the Fe-proteins from T. ferrooxidans and nine other diazotrophs (Fig. 5.6). The nomenclature used is according to Eady et al. (1972), which denotes the Fe-protein as protein 2, and the Mo-Fe-protein as protein 1. Thus, Tf2 is the T. ferrooxidans Fe-protein, and similarly for the deduced or determined amino acid sequences for the other organisms in the comparison: Av2 for A. vinelandii (Brigle et al., 1985), As2 for the Anabaena sp. (Mevarech et al., 1980), Cp2 for C. pasteurianum (Tanaka et al., 1977), Kp2 for K. pneumoniae (Scott et al., 1981; Sundaresan and Ausubel, 1981), Rm2 for R. meliloti (Török and Kondorosi, 1981), Pr2 for P. rhizobium (Scott et al., 1983a), Rt2 for R. trifolii (Scott et al., 1983b), Rj2 for R. japonicum

Fig. 5.6 Comparison of amino acid sequence alignment of Fe-proteins from T. ferrooxidans (Tf), A. vinelandii (Av), Anabaena sp. (As), C. pasteurianum (nifHl, Cp), K. pneumoniae (Kp), R. meliloti (Rm), P. rhizobium (Pr), R. trifolii (Rt), R. japonicum (Rj) and R. phaseoli (Rp) (references are given in Results and Discussion, 5.3e). The amino acids are identified by the single-letter code, and the sequences are read from the amino to the carboxyl terminal. The bold type residues are homologous to amino acids in the T. ferrooxidans Fe-protein. The Cys residues are underlined.

```
1Tf     MAMSDKLRQIAFYGKGGIGKSTTSQKHLAALAEMGQKILI

1Av      AM    RQCAIYGKGGIGKSTTTQNLVAALAEMGKKVMI
1As     MT DENIRQCIAFYGKGGIGKSTTSQNTLAAMAEMGQRIMI
1Cp     M      RQVAIYGKGGIGKSTTTQNLTSGLHAMGKTIMV
1Kp      TM    RQCAIYGKGGIGKSTTTQNLVAALAEMGKKVMI
1Rm     MA A  LRQCIAFYGKGGIGKSTTSQNTLAALVDLGQKILI
1Pr     MS S  LRQIAFYGKGGIGKSTTSQNTLAALAEMGQKILI
1Rt     MA A  LRQIAFYGKGGIGKSTTSQNTLAALVELGQKILI
1Rj     MA S  LRQIAFYGKGGIGKSTTSQNTLAALAEMGQKILI
1Rp     M  SD LRQIAFYGKGGIGKSTTSQNTLAALVDLGQKILI

41Tf    VGCDPKADSTRLILHSKAQDTVLSLAAEAGSVEDLELEDV

36Av    VGCDPKADSTRLILHSKAQNTIMEMAAEAGTVEDLELEDV
40As    VGCDPKADSTRLMLHSKAQTTVLHLAAERGAVEDLELHEV
35Cp    VGCDPKADSTRLLLGGLAQKSVLDTLREEG EDVELDSI
36Kp    VGCDPKADSTRLILHAKAQNTIMEMAAEVGSVEDLELEDV
38Rm    VGCDPKADSTRLILHAKAQDTVLHLAATEGSVEDLELEDV
38Pr    VGCDPKADSTRLILHAKAQDTILSLAASAGSVEDLELEDV
38Rt    VGCDPKADSTRLILNSKAQGTVLDLAATKGSVEDLELGDV
38Rj    VGCDPKADSTRLILHAKAQDTILSLAASAGSVEDLELEDV
38Rp    VGCDPKADSTRLILNAKAQDTVLHLAAQEGSVEDLELEDV

81Tf    MKVGYRDIRCVESGGPEPGVGCAGRGVITSINFLEENGAY

76Av    LKAGYGGVKCVESGGPEPGVGCAGRGVITAINFLEEEGAY
80As    MLTGFRGVKCVESGGPEPGVGCAGRGIITAINFLEENGAY
73Cp    LKEGYGGIRCVESGGPEPGVGCAGRGIITSINMLEQLGAY
76Kp    LQIGYGDVRCAESGGPEPGVGCAGRGVITAINFLEEEGAY
78Rm    LKVGYRGIKCVESGGPEPGVGCAGRGVITSINFLEENGAY
78Pr    MKVGYKDIRCVESGGPEPGVGCAGRGVITSINFLEENGAY
78Rt    LKTGYGGIKCVESGGPEPGVGCAGRGVITSINFLEENGAY
78Rj    MKVGYQDIRCVESGGPEPGVGCAGRGVITSINFLEENGAY
78Rp    LKAGYKGIKCVESGGPEPGVGCAGRGVITSINFLEENGAY
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Fig. 5.6 (continued)

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121Tf      DGANYVSYDVLGDVVCGGFAMPIRKQ AQEIYIVMSGEM

116Av      EDDLDFVFYDVLGDVVCGGFAMPIRENKAQEIYIVCSGEM
120As      QD LDFVSYDVLGDVVCGGFAMPIREGKAQEIYIVTSGEM
113Cp      TDDLDYVFYDVLGDVVCGGFAMPIREGKAQEIYIVASGEM
116Kp      EDDLDFVFYDVLGDVVCGGFAMPIRENKAQEIYIVCSGEM
118Rm      NDVD YVSYDVLGDVVCGGFAMPIRENKAQEIYIVMSGEM
118Pr      ENID YVSYDVLGDVVCGGFAMPIRENKAQEIYIVMSGEM
118Rt      DDVD YVSYDVLGDVVCGGFAMPIRENKAQEIYIVMSGEM
118Rj      ENID YVSYDVLGDVVCGGFAMPIRENKAQEIYIVMSGEM
118Rp      DDVD YVSYDVLGDVVCGGFAMPIRENKAQEIYIVMSGEM


159Tf      MAMYAANNISKGVLKYANSGGVRLGGLICNERQTDKELEL

156Av      MAMYAANNISKGIVKYANSGSVRLGGLICNSRNTDREDEL
159As      MAMYAANNIARGILKYAHSGGVRLGGLICNSRKVDREDEL
153Cp      MALYAANNISKGIQKYAKSGGVRLGGIICNSRKVANEYEL
156Kp      MAMYAANNISKGIVKYAKSGKVRLGGLICNSRQTDREDEL
157Rm      MALYAANNIARGILKYAHAGGVRLGGLICNERHTDRELDL
157Pr      MAMYAANNISKGILKYANSGGVRLGGLICNERQTDKELEL
157Rt      MALYAANNIARGILKYASAGSVRLGGLICNERQTDRELDL
157Rj      MAMYAANNISKGILKYANSGGVRLGGLICNERQTDKELEL
157Rp      MALYAANNIAKGILKYAHSGGVRLGGLICNERQTDRELDL


199Tf      AEALAGKLGTKLIHFVPRDFIVQHAELRRMTVLEYAPESK

196Av      IIALANKLGTQMIHFVPRDNVVQRAEIRRMTVIEYDPKAK
199As      IMNLAERLNTQMIHFVPRDNIVQHAELRRMTVNEYAPDSN
193Cp      LDAFAKELGSQLIHFVPRSPMVTKAEINKQTVIEYDPTCE
196Kp      IIALAEKLGTQMIHFVPRDNIVQRAEIRRMTVIEYDPACK
197Rm      AEALAARLNSKLIHFVPRDNIVQHAELRKMTVIQYAPNSK
197Pr      AEALAKKLGTQLIYFVPRDNVVQHAELRRMTVLEYAPESQ
197Rt      AEALAAKLNSKLIHFVPRDNIVQHAELRKMTVIQYAPRSK
197Rj      AEALAKKLGTQLIYFVPRDNVVQHAELRRMTVLEYAPDSK
197Rp      SEALAARLNSKLIHFVPRDNIVQHAELRKMTVIQYAPDSK


239Tf      QAQEYRTLAEKIHANAGNPAIPTPITMDELEDLLMDFGIM

236Av      QADEYRALARKVVDNKLLV IPNPITMDELEELLMEFGIM
239As      QGQEYRALAKKI NNDKLT IPTPMEMDELEALKIEYGLL
233Cp      QAEEYRELARK V              DANE L   FVIP
236Kp      QANEYRTLAQKIVNNTMKV VPTPCTMDELESLLMEFGIM
237Rm      QAGEYRALAEKIHANSGRGTVPTPITMEELEDMLLDFGIM
237Pr      QADHYRNLATKVHNNGGKGIIPTPISMDELEDMLMEHGIM
237Rt      QAAEYRWLAEKIHSNSGKGTIPTPITMEELEDMLLDFGIM
237Rj      QADHYRKLAAKVHNNGGKGIIPTPISMDELEDMLMEHGII
237Rp      QAGEYRALAEKIHANSGQGTIPTPITMEELEDMLLDFGIM


279Tf      QKEDTSIIGKTAAELAAAGM

275Av      EVEDESIVGKTA EEV
277As      DDDDT    KHS EIIGKPA AEATNRSCRN
254Cp      KPMTQERLEEILMQYGLMDL
275Kp      EEEDTSIIGKTAAE    E
277Rm       KSDEQMLA    ELHAKE  AA
277Pr      KPVDESIVGKTAAELAAS   AKVIAPH
277Rt       KSDEQMLE    ELLAKEVQAAV AP
277Rj      KAVDESIIGKTAAELAAS
277Rp      K SDEQMLA    ELQAKES  AVVAAQ
```

(Fuhrmann and Hennecke, 1984), and Rp2 for R. phaseoli (Quinto et al., 1985).

From the amino acid alignment in Fig. 5.6, the following observations can be made (summarised in Table 5.1):

a) Length. Tf2 consists of either 296 or 298 amino acids depending on which ATG codon initiates translation. This polypeptide length falls within the range of 273 (for Cp2) to 301 amino acids (for As2 and Pr2) for the other organisms.

b) Cysteine residues (underlined in Fig. 5.6). Tf2 has only five Cys residues located at positions 43, 90, 102, 136 and 187. In Tf2 there are no Cys residues located at positions unique to Tf2, but all five residues are found at the same positions in all the other Fe-proteins under comparison. Futhermore, these Cys residues are within highly conserved amino acid regions, and are candidates for possible ligands of the [4Fe:4S] cluster. The five Rhizobium species each contain only five Cys residues in their Fe-proteins as opposed to the other species which all contain more than five Cys residues: Av2 7, As2 6, Cp2 6 and Kp2 9. This is significant since the Fe-proteins of the Rhizobium species as a group, show a greater percentage homology to Tf2 than the other diazotrophs under comparison.

c) Conserved regions. In addition to the highly conserved regions containing the five Cys residues, there are numerous

other regions of extensive amino acid homology (indicated by bold face typing in Fig. 5.6). Apart from the first few amino acids, the amino termini of the Fe-proteins show highly conserved regions, whereas the carboxyl termini contain few conserved regions. Tf2 has a deletion of two residues. The deletion at residue 147 is unique to Tf2, and all the other Fe-proteins contain a Lys residue at this position. The other amino acid deletion in Tf2 (position 121) is shared by all the Rhizobium species Fe-proteins (position 121), but not by any of the other Fe-proteins under comparison. This is in accordance with the previous observation that Tf2 was more closely related to the Fe-proteins of the Rhizobium species.

d) Degree of homology. In Fig. 5.6, the Fe-protein sequences are aligned for maximum homology. The percentage homology of the Fe-proteins of the various diazotrophs relative to Tf2 are given in Table 5.1. The Fe-proteins of the Rhizobium species as a group have the highest homology with Tf2. In particular R. japonicum and P. rhizobium, both showing 86% homology with Tf2, are the most closely related to T. ferrooxidans. It is interesting to note that both R. japonicum and P. rhizobium are slow growing species, and P. rhizobium colonises the non-legume Parasponia (for a review see Postgate, 1982). Tf2 has the lowest amino acid homology with Cp2 of 56%.

5.3f Codon usage. Table 5.2 gives a comparison of the codon usage in the nifH gene of the specified nine

Table 5.2 Comparison of codon usage in *nifH* of *T. ferrooxidans* (Tf), *K. pneumoniae* (Kp), *A. vinelandii* (Av), an *Anabaena* sp. (As), *C. pasteurianum* (*nifH1*, Cp), *R. japonicum* (Rj), *R. trifolii* (Rt), *P. rhizobium* (Pr), and *R. meliloti* (Rm) (references are given in Results and Discussion 5.3d).

Amino acid	Codon	Number of times codon appears in *nifH* gene								
		Tf	Kp	Av	As	Cp	Rj	Rt	Pr	Rm
Arg	CGA	1	0	0	0	0	0	5	1	2
	CGC	4	9	8	5	0	5	2	5	7
	CGG	3	0	0	0	0	3	4	4	2
	CGU	3	4	5	11	0	2	1	0	1
	AGA	1	0	0	1	12	1	1	1	2
	AGG	0	0	0	1	0	0	1	0	0
Leu	CUA	1	0	0	4	3	3	2	1	1
	CUC	5	7	1	2	0	3	12	6	13
	CUG	13	11	20	2	0	10	11	14	10
	CUU	3	1	0	2	9	6	4	2	5
	UUA	0	0	0	7	14	0	1	1	1
	UUG	5	0	0	7	0	4	2	2	1
Ser	UCA	0	1	0	1	6	4	2	2	1
	UCC	10	6	8	5	2	4	7	5	7
	UCG	1	1	0	0	0	4	6	5	2
	UCU	0	0	0	4	0	2	0	3	1
	AGC	1	1	2	2	2	4	2	3	3
	AGU	3	0	0	0	3	1	0	0	0
Thr	ACA	0	0	2	1	7	0	1	0	3
	ACC	9	12	9	14	0	5	2	5	7
	ACG	2	4	0	0	0	3	5	5	2
	ACU	2	0	2	1	6	3	5	2	1
Pro	CCA	0	1	0	3	7	2	3	0	1
	CCC	4	3	0	2	0	1	3	2	1
	CCG	4	4	8	0	0	4	1	5	6
	CCU	1	0	0	4	2	1	3	2	2

Table 5.2 (continued)

Amino acid	Codon	Number of times codon appears in *nifH* gene								
		Tf	Kp	Av	As	Cp	Rj	Rt	Pr	Rm
Ala	GCA	5	1	2	8	10	6	3	4	7
	GCC	16	14	19	2	0	13	10	11	14
	GCG	10	13	0	1	0	10	10	13	9
	GCU	3	1	7	16	10	3	9	1	4
Gly	GGA	3	1	0	0	18	2	6	3	3
	GGC	20	20	18	3	1	17	18	21	14
	GGG	3	1	0	0	0	4	4	0	6
	GGU	5	5	10	26	13	7	3	6	7
Val	GUA	2	0	1	11	9	2	0	0	4
	GUC	6	8	11	0	0	2	8	7	8
	GUG	10	12	10	0	0	9	10	11	7
	GUU	0	2	4	6	10	6	2	2	2
Lys	AAA	7	11	5	6	11	4	9	4	3
	AAG	11	5	11	8	5	14	10	13	16
Asn	AAC	8	11	14	14	4	7	7	9	12
	AAU	1	1	0	2	4	5	4	6	5
Gln	CAA	3	2	3	9	7	3	1	1	4
	CAG	8	8	4	1	4	7	10	9	5
His	CAC	1	2	2	7	0	3	4	3	5
	CAU	4	0	0	1	2	2	1	2	3
Glu	GAA	16	18	19	22	24	9	16	6	10
	GAG	8	11	10	3	0	15	11	19	13
Asp	GAC	11	11	14	16	4	9	11	11	11
	GAU	5	5	3	1	10	7	5	4	5
Tyr	UAC	5	8	8	7	2	7	6	4	7
	UAU	5	1	1	2	10	4	4	7	3

Table 5.2 (continued)

Amino acid	Codon	Number of times codon appears in *nifH* gene								
		Tf	Kp	Av	As	Cp	Rj	Rt	Pr	Rm
Cys	UGC	4	7	5	4	0	5	3	5	5
	UGU	1	2	2	2	6	0	2	0	0
Phe	UUC	4	4	6	6	4	2	4	3	3
	UUU	2	2	0	0	1	2	2	1	2
Ile	AUA	0	0	0	1	11	1	2	0	1
	AUC	17	17	22	15	6	15	17	18	17
	AUU	4	7	0	6	3	8	3	4	3
Met	AUG	14	16	15	15	11	13	11	14	11
Trp	UGG	0	1	0	0	0	0	1	0	0

diazotrophs. As expected from the DNA sequence comparisons which had revealed extensive DNA homology between the various diazotrophs, the codon usage pattern in their nifH genes is very similar. For most amino acids there is one codon which is preferentially used by the majority of the organisms.

The codon usage in nifH of T. ferrooxidans was very similar to the Gram-negative bacteria, but rather different from the Gram-positive C. pasteurianum and the Anabaena sp. While the nifH of the Anabaena sp. showed a unique codon usage, it was the organism which showed the second greatest DNA homology to the nifH of T. ferrooxidans.

A comparison of the codon usage in nifH of T. ferrooxidans and K. pneumoniae could indicate whether the cloned T. ferrooxidans nifH gene would be functional in K. pneumoniae transformants. The expression of a gene is controlled at the level of transcription as well as at the level of translation of which codon usage in the gene, is a determining factor. Having a very different codon usage from the host cell could prevent the expression of the gene when transformed into that host cell. Since the codon usage in nifH is very similar in T. ferrooxidans and K. pneumoniae, codon usage is unlikely to prevent the expression of the cloned T. ferrooxidans nifH gene in K. pneumoniae transformants. For gene expression predictions to be meaningful a greater statistical sample is necessary. As discussed in the General Introduction, since nif genes

have been shown to be evolutionarily conserved in divergent prokaryotic groups, the codon usage in the nif genes of an organism will not necessarily reflect the codon usage in the other genes of that organism.

C. pasteurianum nifH1 shows a very biased codon usage, as observed by Chen et al., (1986). Five amino acids are coded by a single codon: Arg, Cys, Glu, His and Met. In the comparisons of the DNA and amino acid sequences between the various diazotrophs, C. pasteurianum showed the least homology to T. ferrooxidans. A. vinelandii nifH also shows fairly biased codon usage with Pro, Asn, His, Phe and Ile being coded by single codons. In the Anabaena sp. nifH gene, only Phe is coded by a single codon. The amino acid Trp is present in the nifH gene product of K. pneumoniae and R. trifolii, but is absent from the other organisms under comparison. In all the nifH genes, except for C. pasteurianum, UGC was the codon used preferentially for Cys.

5.4 EVOLUTIONARY CONSIDERATIONS

Several interesting observations can be made from the comparisons of the Fe-protein amino acid sequences, and the nifH DNA sequences. In most cases the percentage homology was lower at the DNA level than at the amino acid level, for each organism compared to T. ferrooxidans. The percentage homology on the DNA level ranged from 54 - 74%, while at the

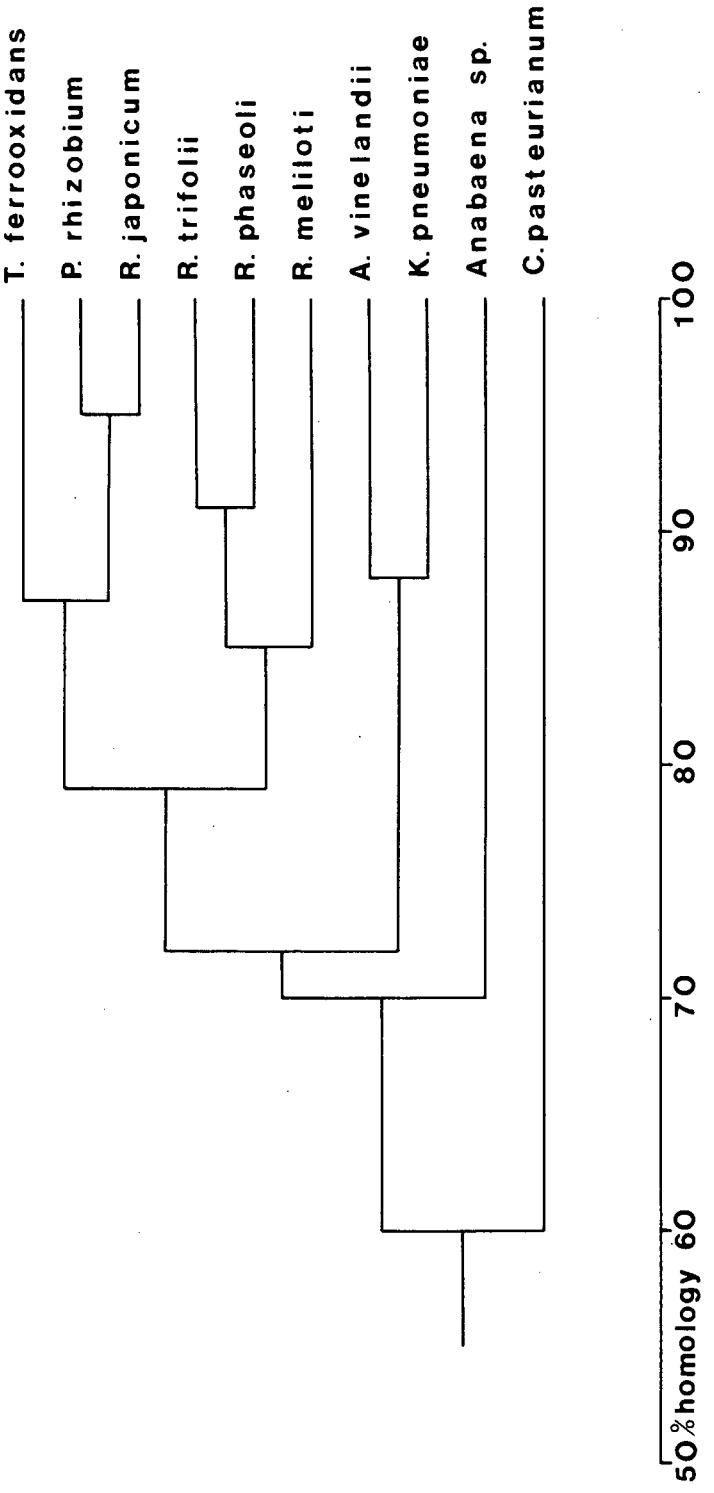
amino acid level it showed a far greater range of 56 - 86% for the various diazotrophs relative to *T. ferrooxidans*. This difference is partially accountable by the marginally different codon usage (except for *C. pasteurianum* which showed very biased codon usage) in the *nifH* genes of the various diazotrophs. The homology differences also suggest that a stronger evolutionary selection exists on the amino acid sequence level than on the DNA sequence level of *nif* genes. This would be expected, since the activity of an enzyme is determined directly by its amino acid sequence, and, due to the degenerate nature of the DNA code, is determined less directly by the DNA sequence of its coding gene.

The homology comparisons throughout this chapter have been based on calculations relative to *T. ferrooxidans*. A homology matrix was constructed to enable direct comparisons between the Fe-protein amino acid sequences of the diazotrophs (Fig. 5.7). From this, a dendogram, illustrating the phylogenetic relationships between the organisms was deduced by the unweighted-pair group method of cluster analysis with arithmetic means (UPGMA) (Sneath and Sokal, 1973) (Fig. 5.7).

Fig. 5.7 Homology matrix and deduced phylogenetic relationships between the specified diazotrophs. The Fe-protein amino acid sequence comparisons are expressed as % homology in the matrix. The references for the amino acid sequences are given in Results and Discussion 5.3e for the *Anabaena* sp. (As), *A. vinelandii* (Av), *C. pasteurianum* (*nifHl*, Cp), *K. pneumoniae* (Kp), *R. meliloti* (Rm), *P. rhizobium* (Pr), *R. trifolii* (Rt), *T. ferrooxidans* (Tf), *R. phaseoli* (Rp), and *R. japonicum* (Rj).

	As	Av	Cp	Kp	Rm	Pr	Rt	Tf	Rp	Rj
As	-									
Av	69	-								
Cp	58	66	-							
Kp	69	88	64	-						
Rm	69	67	60	67	-					
Pr	69	75	60	75	77	-				
Rt	69	69	60	67	90	77	-			
Tf	71	73	52	75	79	88	78	-		
Rp	72	70	61	69	79	79	91	83	-	
Rj	72	74	58	75	77	95	77	86	84	-

Fig. 5.7 (continued)



CHAPTER SIX

GENERAL CONCLUSIONS

CHAPTER SIX

GENERAL CONCLUSIONS

This study was aimed at obtaining an understanding of the gene expression of *T. ferrooxidans*. Both plasmid and chromosomal genes were studied, and the ability of these autotrophic genes to function in heterotrophic systems was investigated.

Although no phenotype could be ascribed to the *T. ferrooxidans* plasmids which were cloned, the results indicated that some regulatory signals are similar in autotrophic and heterotrophic systems. In an *E. coli in vitro* transcription-translation system, the four *T. ferrooxidans* plasmids tested produced novel polypeptides. Several characteristics of the *T. ferrooxidans* recombinant plasmid pDR412 revealed that derivatives of this plasmid could be used as shuttle vectors between *T. ferrooxidans* and heterotrophic bacteria. These characteristics include the plasmid's ability to replicate and be stably maintained in a relatively broad host range, using a *T. ferrooxidans* ori. Subsequent work has shown that additional *T. ferrooxidans* plasmid functions are expressed in *E. coli*, including the oriT, nic and mob functions.

The *T. ferrooxidans* genome was shown to contain a single

copy of chromosomal genes for nitrogenase (*nifHDK*) arranged in a contiguous gene cluster. In a two-step cloning experiment involving pIMP5 (*nifDK*) and pIMP11 (*nifH*), the entire *T. ferrooxidans nifHDK* gene cluster was cloned on a 6.7 kb fragment in pIMP16.

Using the acetylene reduction test, the cloned *T. ferrooxidans nif* genes were tested for their ability to affect the nitrogenase activity in *K. pneumoniae* Nif^+ and Nif^- strains. In a *K. pneumoniae* wt strain KP5022 nif^+, pIMP5 reduced nitrogenase activity by approximately 20%, pIMP16 by approximately 75% and pIMP11 had the most drastic affect, reducing nitrogenase activity by approximately 94%. These results demonstrated the ability of regulatory signals on the *T. ferrooxidans* DNA to be recognised in *K. pneumoniae*. Furthermore, the results suggested that the *T. ferrooxidans nifH* gene is able to titrate out a positive effector of nitrogen fixation, which could be the *K. pneumoniae nifA* gene product.

When the cloned *T. ferrooxidans nif* gene expression was investigated in *K. pneumoniae* $nifH^-$, $nifD^-$ and $nifK^-$ mutants, only pIMP5 (*nifDK*) was able to restore low levels of nitrogenase activity to *K. pneumoniae* $nifK^-$ strains. Future acetylene reduction assays of *K. pneumoniae* Nif^+ and Nif^- strains containing the pIMP plasmids in conjunction with a plasmid containing a constitutively controlled *nifA* gene from *K. pneumoniae* or from another diazotroph, could reveal whether this gene product was able to activate the *T.*

ferrooxidans *nifH* promoter. Isolating and cloning the *T. ferrooxidans* *nifA* gene and using this in conjunction with the pIMP plasmids could elucidate its effect on nitrogenase production in *K. pneumoniae* *nif*$^-$ mutants.

In an *E. coli* *in vitro* transcription-translation system, the cloned *T. ferrooxidans* *nifHDK* genes produced polypeptides which corresponded in molecular mass to the subunits of the *K. pneumoniae* Fe- and Mo-Fe-proteins. This reaffirmed the ability of this autotrophic DNA to function in a heterotrophic system. The *in vitro* studies suggested the presence of two promoters in the *T. ferrooxidans* *nifHDK* genes; one preceding *nifH*, and another preceding *nifD*. The DNA sequence of the cloned / *T. ferrooxidans* *nif* genes revealed the presence of a *T. ferrooxidans* *nifH* promoter which showed perfect consensus to the *K. pneumoniae* *nifH* promoter. No sequences homologous to the *nif* consensus promoter sequences were apparent in the DNA region preceding the *T. ferrooxidans* *nifD* gene. Experiments involving transposon mutagenesis and fusion proteins could further elucidate the structure and regulation of the *T. ferrooxidans* *nifHDK* genes.

The amino acid sequence of the Fe-protein was deduced from the *T. ferrooxidans* *nifH* DNA sequence. This protein contained five Cys residues, located at the same positions within the Fe-proteins of nine other diazotrophs. In a comparison of *nifH* DNA, and Fe-protein amino acid sequences between ten diazotrophs, *P. rhizobium* was shown to be most

closely related to *T. ferrooxidans* (74% homology on DNA level, and 86% on amino acid level).

The codon usage in *nifH* of *T. ferrooxidans* was shown to be very similar to *K. pneumoniae* as well as to the other Gram-negative diazotrophs under comparison. Since the *nif* genes have been evolutionarily conserved in numerous divergent prokaryotic groups, the codon usage in *nifH* does not necessarily reflect codon usage in the other genes of a particular organism. Nonetheless, the codon usage comparisons suggest that *T. ferrooxidans* genes would be functional in *K. pneumoniae* and other heterotrophic cells.

APPENDIX A

GENERAL DNA TECHNIQUES

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GENERAL DNA TECHNIQUES

A.1 EXTRACTION OF DNA

a) Small scale plasmid DNA isolation. Plasmid DNA was isolated from a 1 ml saturated culture of LB (Appendix C) by the method of Ish-Horowicz and Burke (1981). The cells were harvested for 20 sec in an Eppendorf microfuge, resuspended

in 100 µl of Solution I (50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA, pH 8.0) and held at room temperature for 5 min. Solution II was added (200 µl of 0.2 N NaOH, 1% SDS, fresh preparation made each week), the microfuge tube contents mixed gently and the tube was placed on ice for exactly 5 min. Precooled Solution III was added (150 µl of 5 M potassium acetate, pH 4.8), mixed gently, and allowed to stand on ice for a further 10 min. Precipitated protein, SDS and chromosomal DNA was removed by centrifugation at 12 000 g for 5 min. Two volumes of 95% ethanol were added to the supernatant fluid, the mixture kept at room temperature for 2 min and the nucleic acid pelleted by centrifugation for 3 min in an Eppendorf microfuge. The pellet was resuspended in 100 µl TE buffer (Appendix C) and the nucleic acid reprecipitated by the addition of sodium acetate to a final concentration of 300 mM, and 2 volumes of 95% ethanol. After centrifugation, the ethanol was decanted, the pellet dried in a vacuum centrifuge and resuspended in 20 µl TE buffer. Plasmid DNA prepared in this way was sufficiently pure for restriction endonuclease digestion and 5 µl of DNA was routinely used for each digestion.

b) Large scale *E. coli* plasmid DNA isolation. Plasmid DNA was prepared from 400 ml of an overnight culture of *E. coli* in LB, grown at 37°C in the presence of the appropriate antibiotic. The procedure used for plasmid isolation was that of Ish-Horowicz and Burke (1981) scaled up for larger volumes. The procedure remained the same while the volumes of the solutions were increased 40-fold. The nucleic acid

pellet, after ethanol precipitation, was redissolved in 4.2 ml of TE buffer to which 4.2 g of caesium chloride (CsCl) was added. Ethidium bromide (EtBr) was added to a final concentration of 250 µg/ml. The refractive index was adjusted to 1.398, the sample sealed in a Beckman Quickseal ultracentrifuge tube and centrifuged overnight at 55 000 rpm at 15°C in a Beckman VTi rotor. The plasmid band was visualised with long wave ultraviolet (UV) light (350 nm) and the fluorescent band removed in as small a volume as possible. The EtBr was removed by repeated extraction with one volume of NaCl-saturated isopropanol until the pink colour had disappeared. The sample was purified of CsCl by an overnight dialysis against TE buffer at room temperature. The DNA concentration was assessed spectrophotometrically by monitoring its absorbance at the wavelength of 260 nm and by calculating the concentration using the relationship:

A_{260} = 1 for 50 µg/ml DNA (Maniatis et al., 1982).

c) **Extraction of total cellular DNA from E. coli.** The method of Kirby and Wotton (1979) was followed. Cells were grown in LB overnight, shaking at 37°C, then harvested by centrifugation at 5 000 rpm for 10 min. The pellet was resuspended in 10 ml of a 25% sucrose, 10 mM Tris-HCl, 10 mM EDTA buffer (pH 8.0). Lysozyme (Sigma Grade III, from chicken egg white, dissolved in distilled water) was added to a final concentration of 1 mg/ml and the sample was incubated for 1 h at 37°C. After chilling on ice, 5 ml of 0.25 M EDTA (pH 8.0) was added, and the incubation on ice continued a further 5 min. The cells were lysed by the

solution. Lysozyme (10 mg/ml) was added, and the samples were incubated for 1 h at 37°C, shaking gently. The cells were kept on ice while the following solutions were added at the time intervals indicated: 0.5 ml of 0.25 M EDTA, 5 min incubation; 1 ml of 2% SDS, 10 mM Tris, 0.2 M EDTA solution (pH 8.0), 5 min incubation; RNase (20 µg/ml) 15 min incubation. Volumes of 0.5 ml of 25% SDS were added at 3 min intervals until cell lysis was visible. Approximately 1.5 ml of 25% SDS was routinely required for complete cell lysis to occur. Thereafter, 1 ml of a 1.5 M NaCl solution was added and the incubation continued on ice for a further 5 min. Cell debris and SDS were removed by centrifugation at 18 000 rpm for 10 min. Two phenol-chloroform-isoamyl alcohol (25:24:1) extractions were performed, followed by two diethyl-ether extractions before the DNA was ethanol precipitated and resuspended in TE buffer.

e) Extraction of total cellular DNA from acidiphilic *T. ferrooxidans*. Cells were inoculated into 2 litres of TK medium (Appendix C) and incubated at 30°C with constant aeration until the iron was completely oxidised (approximately 10 d). The cells were harvested by centrifugation at 10 000 rpm for 10 min and then washed twice in acidified TK salts (Appendix C). The pellets were resuspended in 4 ml of a 25% sucrose, 2 mM EDTA, 50 mM Tris-HCl solution (pH 8.0) and incubated at -20°C for 1 h. To the frozen suspension, 1 mg/ml of Proteinase K was added and the samples shaken gently at room temperature until they thawed. SDS was added to a final concentration of 1% and

the samples kept on ice for 15 min, followed by RNase (50 µg/ml) digestion for 20 min at 37°C. The viscous, opaque and often brown-coloured samples were dialysed at room temperature against numerous changes of TE buffer until they became translucent (24 - 60 h). The samples were purified of proteins by three phenol-chloroform-isoamyl alcohol (25:24:1) extractions followed by 2 diethyl-ether extractions. The DNA purification procedure was completed by an overnight dialysis against TE buffer, at room temperature.

A.2 RESTRICTION ENDONUCLEASE DIGESTION

The established techniques compiled by Maniatis *et al.* (1982) were followed for general recombinant DNA work unless otherwise specified. Restriction endonucleases were obtained from Boehringer Mannheim GmbH-Biochemica, West Germany; Amersham Int., UK; Anglian Biotechnology Limited, UK; and New England Biolabs, Inc., MA, USA. Restriction endonuclease digestions of plasmid and total cellular DNA were carried out using either low, medium or high salt restriction buffers according to the salt preference of the particular enzyme as specified by the suppliers. *Sma*I, however, was an exception requiring the *Sma*I-specific buffer recommended by the suppliers. Double and triple digestions could often be carried out simultaneously if the salt and temperature requirements of the enzymes were compatible. If the conditions were dissimilar, digestion was carried out sequentially using the enzyme with the lowest salt optimum and the highest temperature tolerance first. The salt

concentrations and temperature of incubation were then adjusted for the second (and third) enzyme. All digestions were incubated at 37°C except for PstI (30°C) and BclI (65°C). Plasmid DNA digestion volumes were standardly 20 µl using 4 u of restriction enzyme per 1 µg of DNA. All volumes were scaled up for total cellular DNA digestions to use 10 µg of DNA. Digestion times were 1 h for plasmid DNA restrictions, and approximately 4 h for total cellular DNA digestions. For electrophoretic analysis of digestion products, the digestion reaction was terminated by addition of approximately 10 µl DNA sample loading buffer (Appendix C). The sample could be stored indefinitely. If the sample was to be used for subsequent enzyme reactions (eg. ligation), the sample was extracted three times with TE-equilibrated phenol (Appendix C), and twice with diethyl-ether. The DNA was ethanol precipitated and resuspended in TE buffer, and could then be dephosphorylated or ligated efficiently.

A.3 DNA GEL ELECTROPHORESIS

Electrophoresis of DNA was carried out using a horizontal gel system with TBE buffer, or TAE buffer (Appendix C) if DNA was to be immobilised onto a membrane. Sigma Type I (low EEO) agarose made up in TBE or TAE buffer containing 0.5 µg/ml EtBr, was used. The concentration of agarose varied from 0.3% for the analysis of large DNA fragments to 1.2% for the detection and sizing of smaller DNA fragments (Maniatis et al., 1982). An agarose concentration of 0.8% was used for routine applications. DNA was electrophoresed

through these gels at 2.7 V/cm overnight. The DNA was visualised using a 254 nm wavelength Transilluminator (Chromato-Vue Model TS-15; UV Products Inc., San Gabriel, CA, USA). The gels were photographed using a Polaroid CU-5 Land camera and Polaroid Land Pack 667 film. Exposures were generally of 1 - 2 sec duration at an aperture of f-4.5. The sizes of the DNA fragments were calculated by extrapolation from a standard curve. In this, the mobility of a standard series of fragments were plotted against the log of their molecular masses. DNA standards used were lambda DNA digested with HindIII or EcoRI-HindIII double digested lambda DNA. The amount of DNA loaded per lane was about 200 ng for every fragment expected from digests of phage lambda or plasmid DNA, and about 10 µg of total cellular DNA was loaded per lane for detection of homologous sequences by hybridisation.

A.4 RECOVERY OF DNA AFTER AGAROSE GEL ELECTROPHORESIS

The method of Dretzen et al. (1981) was modified to use DEAE membrane (NA45, 0.45 µm membranes, Schleicher and Schuell, Dassel, Germany). After electrophoretic separation, the DNA fragments were visualised by EtBr fluorescence using long wave UV light (350 nm). Horizontal slits were made directly below the bands to be recovered, and pieces of membrane cut to size, were inserted into each slit. The insertion of a piece of membrane, or removal of the gel above the fragment to be eluted, protected it against contamination by fragments of higher molecular mass. Electrophoresis was resumed until the DNA had entered the membrane; this could

be verified by observation under UV illumination. Using forceps, the membrane was transferred to a sterile microfuge tube to which was added 400 µl elution buffer (20 mM Tris-HCl, 1 mM EDTA, 1.5 M NaCl, pH 7.5). After a 2 - 15 h incubation at 37°C, the membrane was carefully removed, the nucleic acid ethanol precipitated from the solution, and resuspended in TE buffer. An appropriate volume of the sample was always verified electrophoretically.

A.5 LIGATION REACTIONS

The methods compiled by Maniatis et al. (1982) were followed. A variety of concentrations and ratios of vector DNA to insert DNA were used depending on the sizes of the fragments to be cloned, and the restriction endonucleases which had generated them. Ligation reactions continued overnight at 14°C in a sterile microfuge tube and consisted of DNA, 1 mM ATP, 10 mM DTT, 1 x ligation buffer and 0.25 - 5 u of T4 DNA ligase.

A.6 PREPARATION OF *E. COLI* COMPETENT CELLS

Competent cells of the various strains were prepared by the method of Cohen et al. (1972) with modifications as described by Dagert and Ehrlich (1979). A 1/100 dilution of an overnight culture of cells in LB, was made into fresh LB. This was incubated at 37°C, with shaking, until early exponential phase was reached (OD_{600} = 0.25). The cells were harvested by centrifugation at 5 000 g for 10 min. Cell pellets were resuspended in 1/2 the growth volume in cold 100 mM $CaCl_2$ and the cells kept on ice for at least 20

min. After this time had elapsed, the cells were harvested once more and resuspended in 1/100 the growth volume of a cold 100 mM $CaCl_2$, 15% glycerol solution. The cells were stored on ice for 15 h to improve their competency (Dagert and Ehrlich, 1979). These cells were then used the same day or quick frozen in liquid nitrogen and stored at -70°C for later use. Competent cells prepared in this manner and stored at -70°C were found to be suitable for use for more than 12 months.

A.7 TRANSFORMATION OF *E. COLI* COMPETENT CELLS

Plasmid DNA (100 - 500 ng) or a volume of ligation reaction (40 - 100 ng DNA) was added to 100 µl of competent cells. After 10 min on ice, the cells were induced to take up the DNA by heat-shocking the transformation mix for 2 min at 42°C, before returning the cells to ice. The transformation mix was diluted into 1 ml of LB and incubated at 37°C with shaking for 1 h, to allow expression of the transformed DNA. Various volumes of the ligation mix were spread (10 - 200 µl per plate) on LA (Appendix C) plates containing the appropriate antibiotics to select for the transformed DNA. The plates were incubated overnight at 37°C. The controls used included: competent cells with no DNA added; uncut pBR325 for plasmid DNA transformation experiments, or uncut vector for ligation experiments to monitor transformation; cut and religated vector to assess ligation efficiencies. Randomly chosen transformants were tested for the presence of plasmids (or hybrid plasmids) by the small scale plasmid isolation method.

APPENDIX B

DNA HYBRIDISATION AND POLYACRYLAMIDE GEL ELECTROPHORESIS TECHNIQUES

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DNA HYBRIDISATION AND POLYACRYLAMIDE GEL ELECTROPHORESIS TECHNIQUES

B.1 RADIOACTIVE LABELLING OF DNA PROBES

All DNA probes used in this study were labelled to high specific activities by nick translation (Rigby et al., 1977). The reagents were supplied in a kit form by Amersham (Amersham Int., UK, PB.5025) and the reaction was carried out according to the supplier's instructions. The progress of the reaction could be monitored by trichloro-acetic acid (TCA) precipitation (addition of TCA to a final concentration of 10%) and recovery of insoluble material by filtration on fibre glass filters (Whatman GFC). Radioactivity on these filters was measured by Cherenkov

counting on a Packard scintillation counter. The reaction was stopped when more than 50% of the nucleotides had been incorporated into TCA precipitable material (about 2 h). The reaction was stopped by the addition of an excess of 0.2 M Na_2EDTA and the labelled DNA was eluted in the void volume of a Sephadex G-50 spin column (Maniatis et al., 1982). Specific activities of approximately 1 x 10^7 CPM/µg DNA were routinely obtained. Radioactive probes were stored in lead canisters at 4°C until used. Just prior to use the probe was denatured by boiling for 10 min in a fume hood.

B.2 TRANSFER AND HYBRIDISATION OF DNA

a) Transfer of DNA from gels to GeneScreen membrane. DNA fragments resolved by agarose gel electrophoresis were transferred to GeneScreen hybridisation tranfer membrane (New England Nuclear Corp., Boston, MA, USA) by the method of Smith and Summers (1980). The gel was placed in 2 volumes of 0.25 M HCl for 15 min, before being rinsed in distilled water. The DNA fragments were denatured by soaking the gel twice for 15 min in 2 volumes of 0.5 M NaOH, 1.5 M NaCl, then neutralised by soaking in 2 volumes of 1 M ammonium acetate, 0.02 M NaOH, twice for 30 min. The gel was placed on a clean flat surface. A piece of membrane, cut to the gel dimensions was soaked in 1 M ammonium acetate, 0.02 M NaOH and laid carefully on the gel surface taking care not to trap any bubbles between the membrane and the gel. Three pieces of Whatman 3MM filter paper, also cut to fit the gel and neutralised in the same buffer, were laid on top of the membrane. A 4 cm layer of dry paper towel was

placed on the filter paper. A light weight placed on top of this, ensured even contact. Transfer continued for 2 - 12 h before the membrane was air dried and baked at 80°C for 2 h *in vacuo*.

b) Hybridisation of DNA. Numerous hybridisation conditions were experimented with, which involved varying the stringencies of the hybridisation and washing procedures. Parameters that were manipulated included the temperature of hybridisation, the sodium ion concentration, the duration and temperature of the subsequent membrane washes. In the procedure routinely followed the baked membrane was soaked in 6 x SSC buffer (Appendix C) and placed in a plastic container or heat sealable plastic bag containing an excess volume of prehybridisation fluid (6 x SSC 0.5% SDS, 5 x Denhardt's solution, 0.01 M EDTA, 100 µg/ml denatured Herring sperm DNA) (Appendix C). Prehybridisation was continued for 4 h at 60°C, with constant agitation. The excess fluid volume was removed, the denatured probe added, and hybridisation was continued overnight at 60°C, with constant agitation. Solutions of increasing stringencies were used to wash the filters. The first two washes were carried out at room temperature for 5 min each, using an excess volume of 0.3 M NaCl, 0.06 M Tris-HCl (pH 8.0), 0.002 M EDTA. Subsequent washes were carried out at 60°C in 0.2 x SSC, 0.1% SDS (2 x 30 min washes), 0.01 x SSC, 0.1% SDS (1 h) and 0.05 x SSC, 0.1% SDS (1 h). Each filter was sealed into a plastic bag while still wet, and could be washed further after autoradiography, if desired. The bag was

taped flat under Kodak XAR-5 autoradiographic film in an X-ray cassette equipped with a Fuji X-ray intensifying screen . Exposure was generally continued for 1 - 10 d at -70°C. The film was processed using Kodak GBX X-ray developer and fixer according to the manufacturer's instructions.

B.3 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

a) Gel preparation. SDS-polyacrylamide gels (10 and 15%) were prepared according to the method of Laemmli (1970) and O'Farrell (1975) using a Hoeffer gel apparatus SE600, and 1.5 mm spaces. The glass plates were sealed and the apparatus assembled. The resolving gel was prepared according to the table below, degassed and poured taking care not to trap air bubbles. Water was layered on the gel to promote clear interface formation. After the resolving gel had set (30 min at 22°C) the water was removed and the stacking gel, prepared according to the table below, was cast. The comb, which was presoaked in ammonium persulphate was inserted until the gel had set. Prior to loading the samples, any excess acrylamide was removed from the wells or the sides of the gel.

Samples were prepared in SDS sample buffer (4 µl 0.125 M HCl, 2.5 µl 10% SDS, 2 µl Glycerol, 1.5 µl distilled water) boiled for 2 min and 10 µl of 0.1% Bromophenol Blue solution was added, before being loaded onto the gel. Samples were run at 100 V through the stacking gel and at 10 mA, constant V through the resolving gel. After electrophoresis was

complete, the gels were stained for 1 h in 0.05% Coomassie Brilliant Blue in destain (90 ml acetic acid, 810 ml distilled water, 300 ml propan-2-ol). The gels were destained and dried. The protein molecular mass markers were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden (molecular mass range 14 400 - 94 000 D) and from British Drug Houses, Poole, England (molecular mass range 12 300 - 145 900 D).

b) SDS-PAGE buffers.

Acrylamide-bis-acrylamide stock solution

Acrylamide	29.2 g
Bis-acrylamide	0.8 g

These components were dissolved in distilled water and made up to 100 ml. Active charcoal (5 g) was added to the solution and the solution stirred for 8 h before filtered through Whatman paper (No. 1).

Running gel buffer (pH 8.8)

Tris	1.5 M
SDS	0.4% (w/v)

Reservoir buffer

Tris	0.067 M
Glycine	0.238 M

APENDIX C

MEDIA, BUFFERS AND SOLUTIONS

APPENDIX C

MEDIA, BUFFERS AND SOLUTIONS

Acidified TK salts (Tuovinen and Kelly, 1974)

$(NH_4)_2SO_4$	3 g
KCl	0.1 g
K_2HPO_4	0.5 g
$MgSO_4.7H_2O$	0.5 g
$Ca(NO_3)_2$	0.01 g
Distilled water	500 ml

Adjust to pH 1.6 and autoclave solution.

Deionised formamide (Maniatis et al., 1982)

Approximately 100 ml of formamide was mixed with 20 g of Amberlite MB-1 mixed bed resin (14 - 52 mesh size, Unilab). After being stirred overnight at room temperature, the formamide was filtered twice through Whatman No. 1 filter paper and used immediately.

DNA sample loading buffer

Bromophenol blue	0.25 % (w/v)
Glycerol	50% (v/v)
EDTA	100 mM

Denatured Herring sperm DNA (Maniatis et al., 1982)

Herring sperm DNA (lyophilised sodium salt, Boehringer

Mannheim, GmbH-Biochemica, West Germany) was dissolved in distilled water at a concentration of 10 mg/ml. The DNA was sheared by passing it several times through an 18-gauge hypodermic needle. The DNA was boiled for 10 min, immediately placed on ice, then stored at -20°C in 1 ml aliquots. Just prior to use, the DNA was boiled for 5 min and placed on ice.

Denhardt's solution (10x) (Maniatis et al., 1982)

Ficoll	1% (w/v)
Polyvinylpyrrolidone-40	1% (w/v)
BSA (Bovine serum albumen) (Pentax fraction V)	1% (w/v)

Filter sterilise through a 0.22 µm millipore filter, and store at -20°C

Glucose/Minimal medium agar

Autoclave the reagents separately and allow to cool before mixing aseptically.

M9 salts	1 000 ml
Bacto agar	15 g
1 M $MgSO_4$	1 ml
0.1 M $CaCl_2$	1 ml
1 M Thiamine HCl	1 ml
20% Glucose	10 ml

GYE Liquid Medium (Shafia and Wilkinson, 1969)

Solution A:	$(NH_4)_2SO_4$	2 g
	$MgSO_4.7H_2O$	4 g

	KH_2PO_4	1 g
	Distilled water	900 ml
Solution B:	Glucose	5 g
	Yeast extract	1 g
	Distilled water	100 ml

Autoclave solutions A and B separately and allow to cool before mixing aseptically. After mixing adjust to pH 4.0 with H_2SO_4.

H agar

Bacto tryptone	10 g
NaCl	8 g
Agar	12 g
Distilled water	1 000 ml

H top agar

Bacto tryptone	10 g
NaCl	8 g
Agar	8 g
Distilled water	1 000 ml

Luria agar (LA)

Tryptone	10 g
Yeast extract	5 g
NaCl	5 g
Agar	15 g
Distilled water	1 000 ml

Luria agar (LA) top agar

Tryptone	10 g
Yeast extract	5 g
NaCl	5 g
Agar	7.5 g
Distilled water	1 000 ml

Luria broth (LB)

Tryptone	10 g
Yeast extract	5 g
NaCl	5 g
Distilled water	1 000 ml

M9 salts

Na_2HPO_4	6 g
KH_2PO_4	3 g
NH_4Cl	1 g
NaCl	0.5 g
Distilled water	1 000 ml

MOPS buffer (pH 8.0)

MOPS morpholinopropanesulphonic acid (pH 7.0)	20 mM
CH_3COONa	5 mM
EDTA (pH 8.0)	1 mM

Nitrogen-free-Davis-Mingioli liquid medium (NFDM) (Cannon et al., 1974)

Solution A:	$MgSO_4$	0.1 g

	$Na_2MoO_4.2H_2O$	25 mg
	$FeSO_4.7H_2O$	25 mg
	Glucose	20 g
	Distilled water	500 ml
Solution B:	K_2HPO_4	12.06 g
	KH_2PO_4	3.4 g
	Distilled water	500 ml

Autoclave solutions A and B separately. Just prior to use, place both solutions in a boiling water bath for 10 min, and while still hot, combine them aseptically under N_2 gas.

Nutrient agar (NA)

Bacto nutrient agar	23 g
Distilled water	1 000 ml

Nutrient broth (NB)

Bacto nutrient broth	8 g
Distilled water	1 000 ml

RNase-free glassware, plasticware and solutions (Maniatis et al., 1982)

Where possible, sterile, disposable plasticware was used without pretreatment. General laboratory plasticware was soaked overnight at 37°C in a solution of 0.1% diethylpyrocarbonate before being autoclaved. Whenever possible new glassware was used, and all glassware was pretreated by baking at 260°C overnight. Gloves were worn at all times. All solutions were prepared using glass distilled, autoclaved water. Chemicals were reserved for

work with RNA and were handled with baked spatulas. Wherever possible, the solutions were treated with 0.1% diethylpyrocarbonate overnight before being autoclaved. Diethylpyrocarbonate was not used in solutions containing Tris, since it is highly unstable in the presence of Tris.

SSC buffer (pH 7.0)

NaCl	0.15 M
Sodium citrate	0.015 M

SSPE buffer (20 x) (pH 7.7)

NaCl	3.6 M
NaH_2PO_4	0.2 M
EDTA	0.02 M

TAE buffer (pH 8.0)

Tris	40 mM
Glacial acetic acid	20 mM
EDTA	2 mM

TBE buffer (pH 8.0)

Tris	89 mM
Boric acid	89 mM
EDTA	2.5 mM

TE buffer (pH 8.0)

Tris	10 mM
EDTA	1 mM

APPENDIX D

CLONING VECTOR RESTRICTION MAPS

APPENDIX D

CLONING VECTOR RESTRICTION MAPS

pBR322 Bolivar *et al.* (1977)

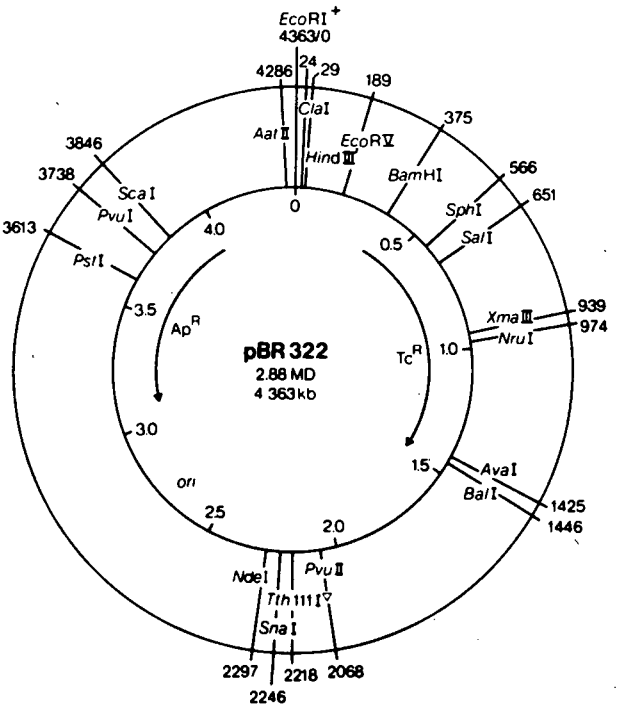
pBR325 Bolivar (1978)

pACYC184 Chang and Cohen (1978)

pEcoR251 Zabeau and Stanley (1982), Remaut *et al.* (1983)

M13mp18 Messing (1983)

M13mp19 Messing (1983)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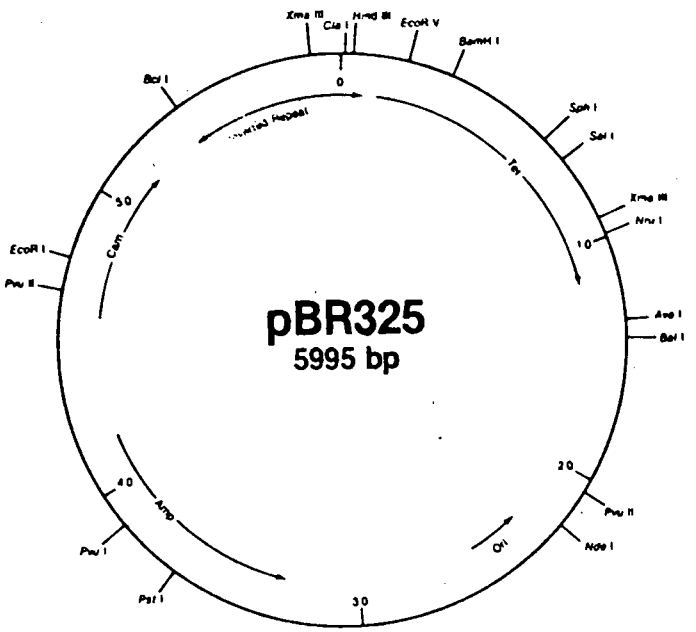
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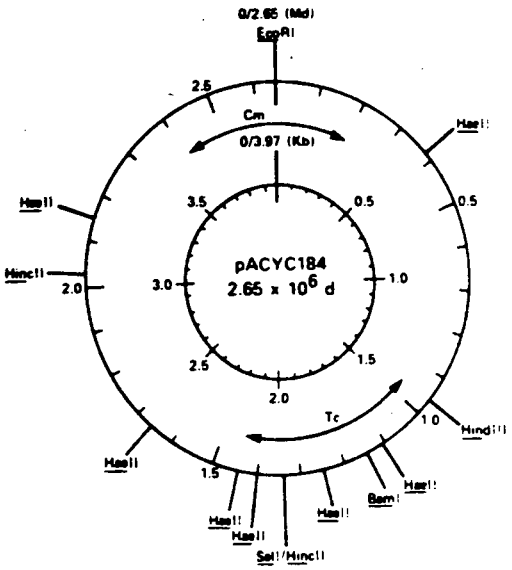
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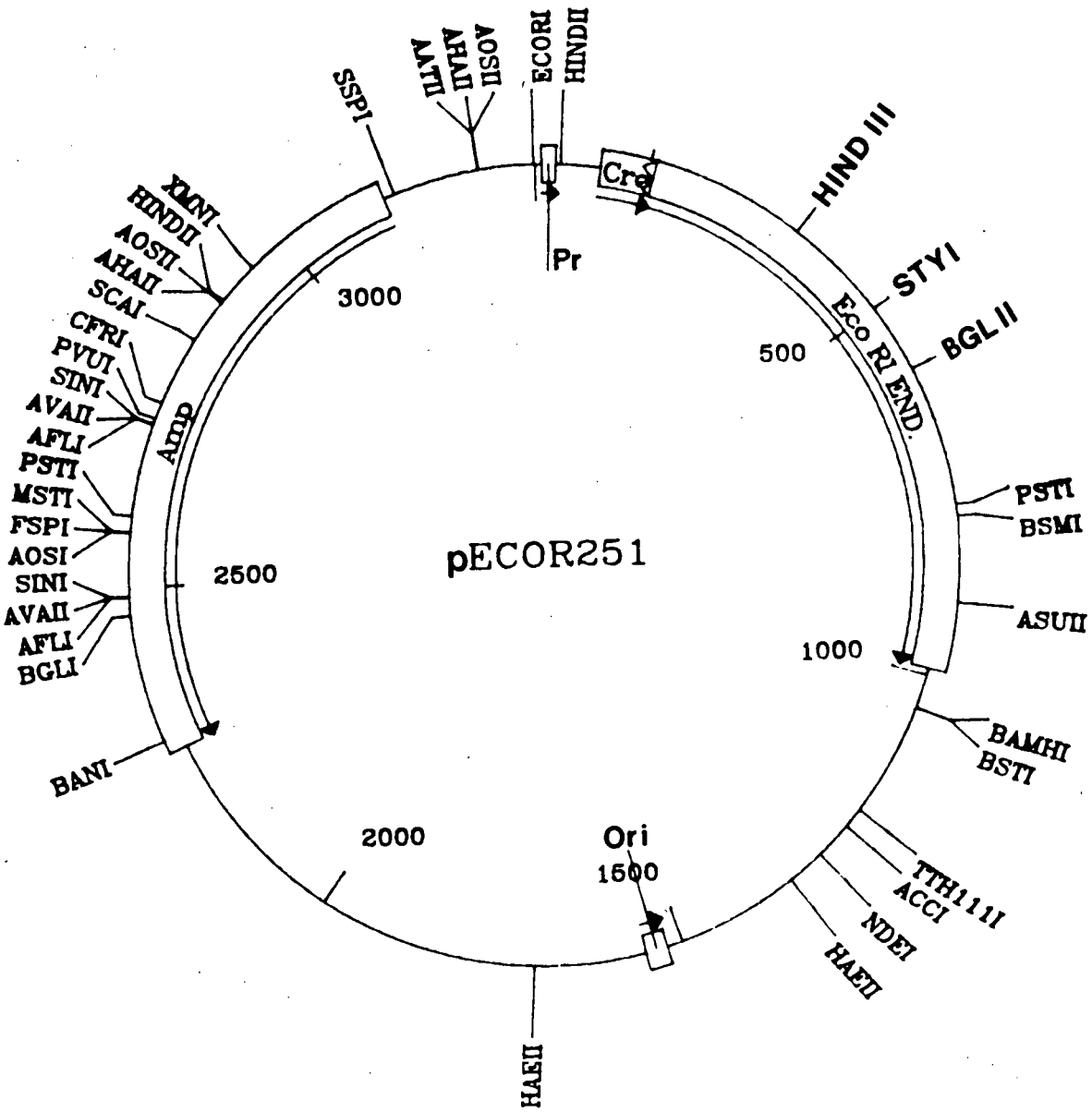
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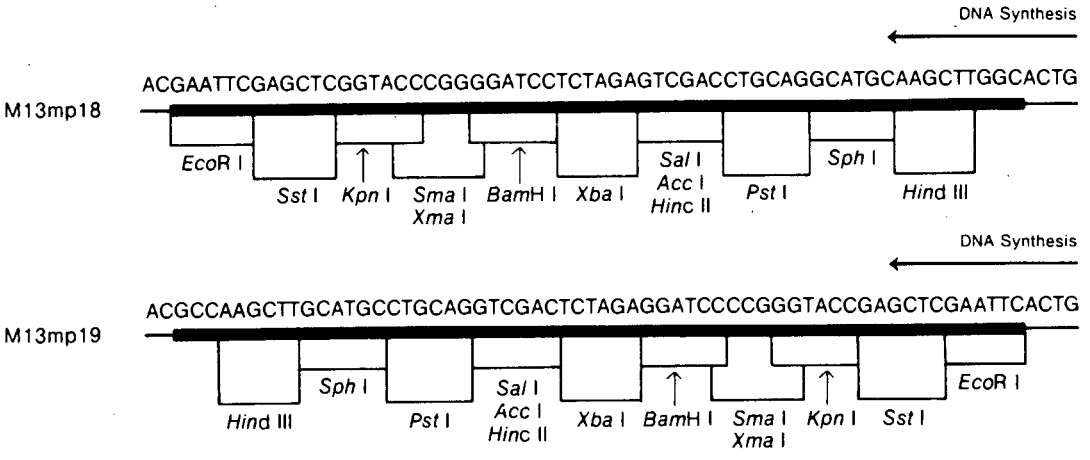
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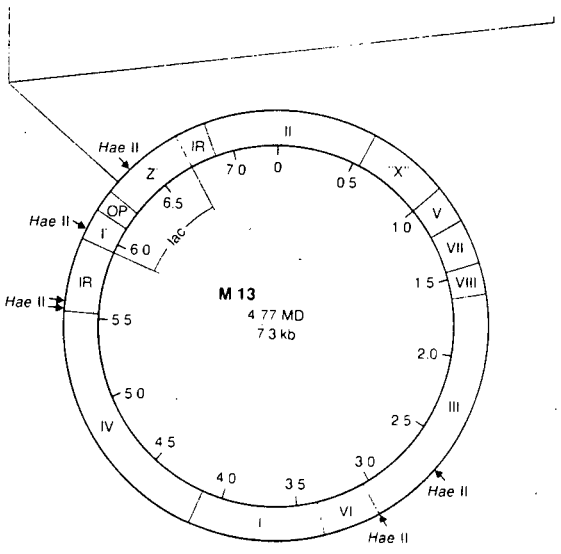
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APPENDIX E

LITERATURE CITED

APPENDIX E

LITERATURE CITED

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