A geographically widespread plasmid from *Thiobacillus ferrooxidans* has genes for ferredoxin-, FNR-, prismane- and NADH-oxidoreductase-like proteins which are also located on the chromosome

Clifford N. Dominy, Shelly M. Deane and Douglas E. Rawlings

**INTRODUCTION**

*Thiobacillus ferrooxidans*, *Thiobacillus thiooxidans* and *Leptospirillum ferrooxidans* are Gram-negative, acidophilic, mesophilic, chemoautolithotrophic bacteria which are frequently isolated from bioleaching processes used for the commercial extraction of metals such as copper, uranium and gold from sulphide-containing ores (Lundgren & Silver, 1980; Brierley, 1982; Rawlings & Silver, 1995). These bacteria grow as a consortium (often together with acidophilic heterotrophs, such as those of the genus *Acidiphilium*) in an inorganic, metal-rich, low-pH (typically 1.5–2.0) environment. *T. ferrooxidans* is the most extensively studied of the bio-
leaching bacteria from both a physiological and genetic point of view. A number of plasmids have been isolated from strains of *T. ferrooxidans*, several of which have been studied in detail (Rawlings & Kusano, 1994). Plasmids frequently contain non-essential genes which may increase the ability of their hosts to grow in a particular environment (Eberhard, 1989). Since *T. ferrooxidans* grows in an unusual environment, we have been particularly interested in investigating *T. ferrooxidans* plasmids in an attempt to identify the accessory genes they might possess.

Plasmid pTF-FC2 is the only *T. ferrooxidans* plasmid for which a functional accessory gene has been found (Clennel et al., 1995). Plasmid pTF-FC2 is a 12.2 kbp broad-host-range, mobilizable plasmid with a replicon which is similar to the IncQ plasmid RSF1010 and a mobilization region with clear homology to the TraI region of the IncP plasmids RK2 and R751 (Dorrington & Rawlings, 1990; Rohrer & Rawlings, 1992). A transposon with 38 bp inverted repeat sequences which are identical to those of Tn21 is located on pTF-FC2. This transposon, Tn5467, contains three accessory genes which encode a glutaredoxin, a MerR-like regulator protein and a 43 kDa protein with similarity to multidrug resistance transport proteins. Although it has not been proven what properties the plasmid confers on *T. ferrooxidans*, when cloned in *Escherichia coli* the gene for the glutaredoxin has been shown to complement *E. coli* thioredoxin mutants for several thioredoxin-dependent functions (Clennel et al., 1995).

Several authors have noted that some plasmids found in *T. ferrooxidans* have a very wide geographic distribution (Sanchez et al., 1986; Valenti et al., 1989, 1990; Chakravarty et al., 1995). Members of one widely distributed *T. ferrooxidans* plasmid family share a highly conserved replicon, part of which is present on a 2.2 kbp *Sac*I fragment (Chakravarty et al., 1995). One particular *T. ferrooxidans* plasmid has been isolated by workers in three different laboratories and although the estimates of its size vary slightly (19.6-20.0 kbp), the plasmids appear to have an identical restriction enzyme map to the previously reported and widely distributed plasmids, pTF35 and pTF0. We here present an analysis of a 60 kbp region of plasmid pTF5 which contained genes encoding products with homology to redox-active proteins and show that this region is also present on the chromosome. An additional unexpected finding was that this region was not associated with an increase in the sensitivity of *E. coli* F19 to metronidazole.

**METHODS**

**Bacterial strains, plasmids and growth conditions.** Bacterial strains and plasmids are shown in Table 1. *T. ferrooxidans* and *L. ferrooxidans* strains were grown in 9K liquid medium at 30 °C (Silverman & Lundgren, 1959). *T. thioxidans* was grown in medium containing 30 mM K₂S₂O₃, pH 2.5 (Powles et al., 1995). *E. coli* strains were grown in Luria broth at 30 °C and were appropriate ampicillin (100 μg ml⁻¹) was added. The *T. ferrooxidans* ATCC 33020 cosmid library was prepared using cosmid vector pHC79 and contained inserts of between 30 and 44 kbp (Ramases, 1988).

**Anaerobic assays.** Clones to be tested for MICs of metronidazole were transformed into *E. coli* F19 (Santangelo et al., 1991) and grown on Luria agar supplemented with 0.5% (w/v) NaN₃ and 0.5% (w/v) glucose. A 10 mg ml⁻¹ stock of metronidazole (Sigma) was diluted to test for growth of transformants at 0, 5, 10, 15, 20 and 35 μg ml⁻¹ final concentration. Clones positive for the ability to complement an *E. coli fmr* mutant phenotype were identified and plasmid DNA extracted. This DNA was transformed into *E. coli* strain RML02 and incubated anaerobically (CO₂/NaHCO₃, 85:15, by vol.) for 4 d at 37 °C on minimal media containing glycerol (0.5%, v/v), NaN₃ (0.5%) and ampicillin (100 μg ml⁻¹) (Birkmann et al., 1987), and then examined for growth.

**PFGE.** This was conducted using a Beckman Geneline Transverse Alternating Field system. Total genomic DNA samples were prepared by resuspending cell cultures in SET buffer (50 g sucrose l⁻¹, 0.5 g EDTA l⁻¹ and 10 g Tris l⁻¹) to an OD₅₀₀ of 1. Cells were lysed with 50 μg Proteinase K ml⁻¹ (Merck) for 30 min at 37 °C. Samples were embedded in a low-temperature-gelling agarose (Hispanagar) at 1:1 % (w/v) final concentration. Samples were sliced into 20 × 6 x 2 mm plugs and Tris/EDTA containing 1% (w/v) SDS and 50 μg Proteinase K ml⁻¹ added. Samples were incubated for 48 h at 45 °C and washed (3 x 15 min) in ES solution (10 g sodium lauryl sarcosine l⁻¹, 168 g EDTA l⁻¹, pH 8) and transferred to Tris/EDTA. Plugs were incubated in 2 mM Pefabloc proteinase inhibitor (Boehringer Mannheim) for 16 h at 4 °C and then washed in Tris/EDTA. Samples for digestion with *Xba*I were preincubated in 1 × restriction buffer H (Boehringer Mannheim) containing 1 μg BSA ml⁻¹ for 1 h, followed by digestion in 1 × H buffer containing 30 units *Xba*I for 4 h. Digested samples were washed in Tris/EDTA and the plugs stored at 4 °C until used. Slices were electrophoresed in 1 × PFGE running buffer (250 μl glacial acetic acid l⁻¹, 0.16 g EDTA l⁻¹ and 1.2 g Tris l⁻¹) prior to use. Agarose gels, 1% (w/v), were made with 1 × PFGE running buffer. Gels were electrophoresed...
Table 1. Bacterial strains and plasmids

<table>
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<th>Strain or plasmid</th>
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Phoresed in 1 × PFGE running buffer at 150 mA for 14 h with an 8 s pulse time.

**Determination of plasmid copy number.** Dot blot analysis of T. ferrooxidans ATCC 33020 total genomic DNA and cloned pTF5 DNA (pCD200) was used to determine the number of plasmids per genome in T. ferrooxidans. The amounts of genomic and plasmid DNA to be hybridized were standardized according to the relative sizes of the T. ferrooxidans chromosome and plasmid pTF5 assuming a copy number of...
one. Twofold dilutions of each sample were bound to Hybond-N+ membrane (Amersham) and hybridized with pCD200 digoxigenin-dUTP-labelled DNA. Copy number was determined by comparing the hybridization signal intensities of the genomic and plasmid dilution series.

**General methods.** DNA extraction and manipulation was carried out according to Sambrook et al. (1989). In Southern hybridization experiments, dUTP-digoxigenin-labelled DNA probes were labelled with the randomly primed DNA labelling kit (Boehringer Mannheim) and non-radioactive detection was carried out with the DIG Nucleic Acid Detection kit (Boehringer Mannheim). DNA sequencing was done by the dideoxy chain-termination method (Sanger et al., 1977) and templates were labelled using the Sequitherm kit (US Biochemical) with Cy5 fluorescent label. Automated sequencing was carried out on a Pharmacia Alf Express according to the manufacturer’s instructions. In vitro proteins were generated using an E. coli transcription-translation kit (Promega) using [35S]methionine label. SDS-PAGE was done according to Laemmli (1970). Polyacrylamide gels were run at 120 mA constant current for 12 h. Sequence was analysed using the UWGCG (Devereux et al., 1984) set of programs. Homology searches were performed using the BLAST algorithm (Altschul et al., 1990) and **BEAUTY** post-processing (Worley et al., 1995).

**RESULTS**

**Plasmid isolation using a metronidazole reduction assay**

Plasmid pTF5 was isolated from a *T. ferrooxidans* total DNA cosmid library during a screening procedure designed to select genes which express redox-active proteins. The *E. coli* mutant F19 is partially resistant to metronidazole and lacks the ability to reduce the drug (Santangelo et al., 1991). Several cosmids were isolated
Geographically widespread *T. ferrooxidans* plasmid

![Diagram](image)

**Fig. 2.** Restriction enzyme cleavage map of pTF5 showing the location and genetic map of the region which is present on both plasmid and chromosome. The subclones used for location of the region which resulted in metronidazole sensitivity of the *E. coli* F19 mutant are indicated in the centre of the map.

which restored wild-type levels of metronidazole sensitivity to *E. coli* F19. These cosmids were digested with a number of restriction enzymes and the sizes of several of the fragments were found to be similar (data not shown). Two of the cosmids, pMET7 and pMET17 were selected for further study. To confirm that pMET7 and pMET17 contained overlapping clones of the same region of *T. ferrooxidans* DNA, a 1.0 kbp fragment (probe C, Fig. 1) common to both cosmids was labelled and probed against a blot of digested and undigested total DNA isolated from *T. ferrooxidans*. When undigested total DNA is separated using PFGE, chromosomal DNA remains in the well but plasmid DNA migrates into the gel in a pulse-independent manner (Hightower et al., 1987). Unexpectedly, a positive hybridization signal was obtained for undigested plasmid DNA (Fig. 1, lane 3) and two signals at 20 kbp and approximately 60 kbp were obtained for total DNA digested with XbaI (Fig. 1, lane 4). This indicated that DNA common to pMET7 and pMET17 was present on a plasmid and that DNA with homology to the probe was also present on the chromosome. This finding was confirmed using probes C–G (Fig. 1), which indicates that only this region is also present on the chromosome.

Using probes C–G, the relative intensities of the two bands remained constant irrespective of probe or washing temperature, indicating that the plasmid and chromosomal copies were identical or nearly identical.

**Cloning and restriction enzyme map of pTF5**

The Southern hybridization experiment indicated that part of the insert DNA of pMET7 and pMET17 originated from a plasmid. Surprisingly, the restriction enzyme maps of portions of the two cosmids were identical to the map of pTF35, a plasmid from a different *T. ferrooxidans* strain (TF35) that had been cloned previously (Rawlings et al., 1983; Rawlings & Woods, 1985). As plasmid pTF5 contained unique HindIII and XbaI restriction sites, intact pTF5 was reassembled and cloned by ligation of a 6.9 kbp XbaI–HindIII fragment from pMET17 to a 12.9 kbp XbaI–HindIII fragment from pMET7 by inserting the 19.8 kbp HindIII–XbaI–HindIII fragment into the HindIII site of the cloning vector, pBluescript KS (pBS-KS). The resultant construct, pCD1, was digested with a number of restriction enzymes and the sizes of the fragments compared with pTF35, which had previously been cloned into pBR322 (pDER301) (Rawlings & Woods, 1985). Plasmids pCD1 and pDER301 were digested with HindIII to free pTF5 and pTF35 from their respective cloning vectors and then digested with a second restriction endonuclease (either BamHI, Clal, EcoRI, HpaI or SalI). Plasmids pTF5 and pTF35 gave fragments of identical size (not shown) and the sizes of the fragments corresponded to the restriction map reported for pTFO (Valenti et al., 1989).

**Identification of the region conferring metronidazole sensitivity**

The MIC of metronidazole for *E. coli* F19 containing cosmids, cloned pTF5 (pCD1) and relevant subclones was determined. *E. coli* containing pBS-KS or pUC-BM21 plasmid or pHC79 cosmid vectors was resistant to metronidazole (MIC 20 mg l⁻¹) while cosmids pMET7 and pMET17 conferred metronidazole sensitivity (MIC 10 mg l⁻¹). Cloned pTF5 (pCD1) as well as subclones pCD100 and pCD300 (Fig. 2) conferred metronidazole sensitivity, while subclones pCD200, pCD400 and pCD500 were metronidazole-resistant. A 319 bp XbaI–SalI fragment subclone of pCD300 was on its own responsible for full metronidazole sensitivity and this insert fragment was sequenced. It was unexpectedly found to have extensive nucleotide homology (98%) to part of a recently described origin of vegetative replication (oriV) from the *T. ferrooxidans* plasmid pTF191 (Chakravarty et al., 1995).

**DNA sequence analysis**

The region of pTF5 from the HindIII site (position 1 bp) to the *Sphl* site (5650 bp) was sequenced on both strands and is shown in Fig. 3. Four complete ORFs and two
Fig. 3. Relevant portions of the HindIII-SphI fragment of pTF5. Dotted lines indicate gaps in the nucleotide sequence. The amino acid sequence of the predicted proteins and products of partial ORFs are shown below the nucleotide sequence. Potential ribosome-binding sites are underlined, the FNR consensus binding site is in bold type, inverted repeat sequences between *psmA* and *redA* are represented as an inverted pair of arrows, the transposition scar is italicized, the directions of transcription are indicated by short arrows and the putative ferredoxin promoter is shown as bold and underlined.
truncated ORFs were identified within the 5-65 kbp HindIII–Sphl region (Fig. 2). Predicted amino acid sequences of the ORFs were compared with sequences in the non-redundant GenEMBL database using the BLAST program (Altschul et al., 1990). The ORF closest to the HindIII site consisted of 122 amino acids (14 kDa) with highest sequence homology to a [3Fe-4S,4Fe-4S] ferredoxin of 106 amino acids from Pseudomonas putida (Fig. 4a) (Hase et al., 1978). A putative α-like promoter sequence was identified 93 bp from the ATG start of the ferredoxin gene, fdxA (Fig. 3). This promoter appeared to be functional in E. coli as a lacZ reporter gene fusion to the ferredoxin N-terminus was expressed in E. coli (data not shown) and a protein product of the size predicted for the ferredoxin was detected in an E. coli-derived in vitro transcription-translation system (Fig. 6).

Adjacent to the ferredoxin gene was a region with an incomplete ORF of 82 amino acids which had 48% amino acid sequence identity to part of the transketolase (tktA gene) from E. coli (Sprenger, 1993) and which appeared to have been fused to part of an IST2-like insertion sequence (Figs 2, 3). The IST2-like insertion sequence element had 81% nucleotide sequence homology to the IST2 insertion sequence from T. ferrooxidans ATCC 19859 (Yates & Holmes, 1987; Yates et al., 1988). It contained an ORF with homology to part of the presumptive IST2 transposase except that the first 48 bp were deleted and the ORF terminated after 462 bp. The truncated transketolase-like ORF and IST2-like element are possibly the result of insertion and deletion events associated with transposon activity such that only a transpositional ‘scar’ remains.

Next to the IST2-like element was another complete ORF of 26-5 kDa which had highest amino acid sequence homology to the FNR-like protein from Pseudomonas aeruginosa (28.9% identity) (Araki et al., 1995) and Paracoccus denitrificans (27.5% identity) (Fig. 4b) (Van Spanning et al., 1995). FNR proteins are regulators of gene transcription and bind to a specific nucleotide consensus sequence in the promoter regions of genes they regulate (Spiro, 1994). The FNR regulator subfamily possess a C-terminal DNA recognition helix-turn-helix domain containing a conserved E-X$_2$-SR motif which was also present in the FNR-like protein encoded by pTF5 (Fig. 4b). However, cysteines equiva-

lent to the three E. coli FNR N-terminal Cys residues and the Cys$_{104}$ residue, which are thought to function in redox sensing and FNR intramolecular coupling (Spiro & Guest, 1988; Green et al., 1993), were not present. A perfectly conserved FNR-binding consensus sequence (TTGAT-N$_x$-ATCAA) was found 61 bp upstream of an ORF of 556 amino acids, designated psmA (predicted size 60-2 kDa) which was positioned upstream of the FNR-like ORF but translated in the opposite direction (Figs 2, 3).

The 60-2 kDa ORF had clear amino acid sequence similarity to the predicted amino acid sequences of the three prismane genes in the GenEMBL database (Fig. 4c). Prismane proteins have an unusual [6Fe–6S] pris-
DNA in the lanes digested with XbaI (Fig. 1, lanes 2, 4 and 6).

The experiment shown in Fig. 1 also enabled the approximate position of the junction between plasmid-specific DNA and the DNA present on both pTF5 and the chromosome to be determined. Since DNA probe B was the closest probe to the ferredoxin-like ORF which gave a hybridization signal to plasmid DNA only and as probe C gave a signal to both plasmid and chromosomal DNA, the junction was localized to the region within probe C, most likely in the 215 bp DNA EcoRI–HindIII.
Polypeptides produced from pTF5 using the
identification method were subjected to PFGE and a Southern hybridization experiment. The proteins at approximately 28 kDa were spurious proteins produced from the lacZ vector promoter which (in this particular construct) was facing in the opposite direction to the fdxA gene. A 60 kDa protein was expressed from the clone of the prismane-like ORF, pCD119 (lane 6) and a 37 and 39 kDa protein doublet corresponding to the NADH-oxidoreductase-like ORF, pCD163 (lane 7). The 60 kDa prismane-like protein and the 37 and 39 kDa doublet corresponding to the NADH-oxidoreductase-like proteins were synthesized when expressed behind a vector tac promoter, pCD164 (lane 8). All proteins corresponded closely to the sizes predicted from the DNA sequence analysis. No additional polypeptides were produced from the insert in clone pCD120 and no 22 kDa protein corresponding in size to the FNR-like protein was detected (not shown). In addition, attempts to complement an E. coli RM102 fnr mutant for nitrate reductase induction (Birkmann et al., 1987) with the pTF5 FNR-like gene (pCD120) were negative. The prismane- and NADH-oxidoreductase-like proteins were only expressed when in the same orientation as a vector promoter. A 37 and 39 kDa protein doublet was produced when the insert of pCD150 was cloned in the same orientation as the lacZ vector promoter (lane 5), but not when cloned in the reverse direction (lane 4). Likewise the prismane- and NADH-oxidoreductase-like proteins appeared not to be expressed from pTF5 promoters in E. coli (pCD1, lane 2); however the 14 kDa polypeptide corresponding in size to the ferredoxin-like protein was expressed from pTF5 (lane 2) or when cloned in either orientation relative to a vector promoter (not shown). With the exception of the 14 kDa protein, the proteins expressed from pCD1 appeared to have originated from DNA found on the part of pTF5 that was not present on the chromosome.

### Presence of plasmids with homology to pTF5 in T. thiooxidans, L. ferrooxidans and other strains of T. ferrooxidans

The presence of pTF5-like plasmids among five T. ferrooxidans strains not previously tested, including four strains which were isolated in South Africa, was examined. Undigested and XbaI-digested total DNA was subjected to PFGE and a Southern hybridization experiment carried out using probe G (Fig. 1). Positive hybridization signals to plasmids present in T. ferrooxidans strains ATCC 23270 (USA), ATCC 33020...
Fig. 6. SDS-PAGE analysis of polypeptides translated in vitro from pCD1 (pTF5 cloned into pBS-KS) and pTF5 subclones. The location of the constructs tested is shown below the PAGE gel and the direction of vector promoters indicated by one (lacZ promoter) and two (tac promoter) arrowheads. Lanes: 1, pBS-KS vector control; 2, pCD1; 3, pCD421; 4 and 5, pCD150 in both vector orientations; 6, pCD119; 7, pCD163; 8, pCD164.

Fig. 7. Southern hybridization of total DNA separated by PFGE from a number of bacterial strains. (a) Undigested total DNA hybridized to probe G; (b) total DNA digested with XbaI which hybridized to probe G; (c) undigested total DNA probed with pCD300. Lanes: 1, L. ferrooxidans DSM 2705; 2, T. thioprasovans ATCC 19377; 3, T. ferrooxidans ATCC 23270; 4, T. ferrooxidans ATCC 33020; 5, molecular mass markers; 6, T. ferrooxidans FC1; 7, T. ferrooxidans FC6; 8, T. ferrooxidans Black mountain Pb; lane 9, T. ferrooxidans Bateman Reno.
The hybridization signal to DNA remaining in the wells of each of these strains was due to non-mobile undigested chromosomal DNA and possibly some entrapped plasmid DNA. In the case of \textit{T. ferrooxidans} strains Black Mountain Pb and Bateman Reno (Fig. 7a, lanes 8 and 9), a hybridization signal for DNA remaining in the wells was obtained but there was no evidence of plasmid DNA. The \textit{XbaI}-digested DNA samples from all strains confirmed these results. Two bands were obtained for \textit{T. ferrooxidans} strains ATCC 23270, ATCC 33020 and FC1 (Fig. 7b, lanes 3, 4 and 6), one signal due to the copy of the \textit{redA} gene on the plasmid and the other due to the chromosomal copy. In the case of strain ATCC 23270 (lane 3), the chromosomal DNA migrated only a short distance from the well as it was on an \textit{XbaI} fragment of greater than 500 kb and fragments of this size do not penetrate the gel under the running conditions used to separate smaller fragments. From the \textit{XbaI}-digested samples it may be seen that the plasmid in strain ATCC 23270 was clearly of larger size than the plasmids in strains ATCC 33020 or FC1 (Fig. 7b, lanes 3, 4 and 6). \textit{T. ferrooxidans} strains Black Mountain Pb and Bateman Reno gave only a single hybridization signal at about 30 kb (Fig. 7b, lanes 8 and 9), slightly larger than the digested plasmids in strains ATCC 33020 and FC1 and smaller than the signal from the chromosomal copy in those strains (Fig. 7b, lanes 4 and 6). No signal was obtained for either \textit{L. ferrooxidans} type strain DSM 2705, \textit{T. thioparus} type strain ATCC 19377 or \textit{T. ferrooxidans} FC6 (Fig. 7a, b, lanes 1, 2 and 7).

The same membrane filters shown in Fig. 7(a, b) were stripped and probed with labelled DNA from the plasmid origin of replication (pCD300; Fig. 2). Only the result of reprobing the filter shown in Fig. 7(a) is presented in Fig. 7(c). As predicted, a hybridization signal to a single plasmid band in strains ATCC 23270, ATCC 33020 and FC1 was obtained (Fig. 7c, lanes 3, 4 and 6). Unexpectedly, hybridization signals to strains Black Mountain Pb and Bateman Reno were obtained to DNA in the wells (Fig. 7c, lanes 8 and 9). This implies that a plasmid with homology to pTF5 had become integrated into the chromosome of these strains.

**DISCUSSION**

The initial aim of this study was to identify \textit{T. ferrooxidans} electron transport genes which reductively activate metronidazole. For example, reduced ferredoxin has been reported to transfer electrons directly to metronidazole and produce the reduced toxic derivative (Edwards & Mathison, 1970). Furthermore, an identical screening procedure had been used to screen a gene bank from \textit{Clostridium acetobutylicum} and a gene for a flavodoxin which rendered the \textit{E. coli} F19 mutant sensitive to metronidazole was isolated (Santangelo et al., 1991). The pTF5 genes whose products have homology to redox-active proteins are exactly the type of genes that we would have expected to have isolated using the metronidazole activation procedure. However, the region of pTF5 which encoded these proteins was not associated with increased metronidazole sensitivity (Table 1). Instead, a 319 bp region from the origin of replication which was almost identical to the previously sequenced replicon of plasmid pTFI91 (Chakravarty et al., 1995) and which did not contain an ORF was the region of pTF5 associated with increased metronidazole sensitivity. In at least two other studies similar unexpected results have been obtained. In these studies two fragments of DNA from \textit{C. acetobutylicum} which resulted in increased sensitivity of \textit{E. coli} F19 mutants to metronidazole and which did not encode metronidazole-reducing proteins were isolated. One of the fragments had sequence homology to tRNA genes (Sealy, 1993) whilst the other encoded the \textit{regA} gene, which produced a 35 kDa protein with homology to the \textit{Lacl} family of repressor proteins (Davison et al., 1995). Some members of the \textit{Lacl} family are repressors of tRNA genes (Leclerc et al., 1990) and it is possible that the cloned tRNA genes had sites which bound and titrated members of this repressor family. We speculate that DNA fragments and proteins which do not themselves have metronidazole-reducing activity may confer increased metronidazole sensitivity via an indirect mechanism. For example, these DNA fragments could possess a binding site for a repressor of one of the \textit{E. coli} genes, the product of which is able to reduce metronidazole. This would have the effect of derepressing this gene and result in an increased metronidazole sensitivity.

Two features of plasmid pTF5 that are especially noteworthy are its wide geographical distribution among \textit{T. ferrooxidans} strains and the discovery that it has a segment of DNA which is also present on the chromosome. In this study, the geographical distribution of the plasmid was extended from \textit{T. ferrooxidans} strains found in the Northern hemisphere to strains found in South Africa. Even though it is widely distributed, pTF5 is not completely ubiquitous as \textit{T. ferrooxidans} strain FC6 (Fig. 7a, b, c, lane 7) and other \textit{T. ferrooxidans} strains (Valenti et al., 1989) do not possess the plasmid.

In \textit{T. ferrooxidans} strain ATCC 23270 a plasmid with homology to the NADH oxidoreductase region of pTF5 (probe G) was of a larger size than pTF5. No hybridization signal to plasmid DNA in \textit{T. ferrooxidans} strains Black Mountain Pb and Bateman Reno was detected when either a pTF5 replicon probe or a NADH-oxidoreductase probe (probe G) was used. However, positive hybridization to chromosomal DNA from both these strains with both probes was obtained. The most likely explanation for this is that DNA with homology to the pTF5 replicon (probe pCD300) and the NADH oxidoreductase (probe G) had become integrated into the chromosome. Particularly interesting was the observation that \textit{T. ferrooxidans} strain FC6 did not have DNA with homology to probe G on either a plasmid or its chromosome (a similar result was obtained for two other South African isolates of \textit{T. ferrooxidans}; data not
shown). This implies that the genes present on pTF5 are not essential for the growth of *T. ferrooxidans*.

Studies on whether genes present on both the chromosome and plasmid are expressed in *T. ferrooxidans* are complicated by the difficulty in determining whether RNA transcripts or translation products originate from the plasmid or chromosomal copies of the gene. With the exception of the ferredoxin, no polypeptides corresponding in size to the FNR-like, prismane-like or NADH-oxidoreductase-like ORFs were detected in the *E. coli*-derived *in-vitro* transcription-translation system in the absence of an *E. coli* vector promoter. However, when cloned behind an *E. coli* vector promoter, products corresponding to the latter two ORFs were detected. It is likely that the ORFs of the ferredoxin-, prismane- and NADH-oxidoreductase-like proteins represent functional genes but that some of the factors required for their expression in *E. coli* were absent in the *in-vitro* system. Although a product corresponding to the FNR-like protein regulator was not detected in the *E. coli in-vitro* system nor in the *in-vivo* complementation assay, it is probable that an FNR-like protein is involved in the expression of the prismane-like protein as a consensus FNR-binding site was found upstream of the prismane protein gene. The presence of the E-X₅-SR motif in the predicted DNA-binding C-terminal domain of the FNR-like protein suggests that this protein may be the regulator of the prismane gene.

The ferredoxin-, prismane- and NADH-oxidoreductase-like proteins are likely to be part of an electron transfer system. The combination of proteins has similarity to the electron transport chain involved in the degradation of aromatic and other compounds, in which electrons are transferred from NADH via a flavin (and/or ferredoxin) to a terminal oxygenase (Mason & Cammack, 1992). The NADH-oxidoreductase-like subunit on pTF5 was most similar to that of subunit c of the methane monoxygenase from *Methylcococcus capsulatus*. The methane monoxygenase of this bacterium consists of three subunits, with subunits a and b together forming the terminal oxygenase and subunit c the NADH reductase (Stainton et al., 1990). No oxygenase is apparent on pTF5 (unpublished) and the key to finding the role of the electron transport system on the plasmid might be found in discovering the function of the prismane-like polypeptide.

Prismane proteins were first discovered in sulphate-reducing bacteria where Southern hybridization experiments using the *Desulfovibrio vulgaris* prismane protein gene as a probe showed that prismane genes were common among sulphate-reducing bacteria (Stokkermans et al., 1992a). These proteins are known to be redox-active and possess a putative [6Fe-6S] prismatic cluster which is able to exist in four different redox states ranging from [6Fe-6S]³⁺ to [6Fe-6S]²⁺ (Marrit et al., 1995). Prismane proteins were reported to have no significant similarity to other proteins except within their N-terminal regions where there is some similarity to CO dehydrogenases of *Methanothrix soehngenii* and *Clostridium thermoaceticum* (Stokkermans et al., 1992a, b). A number of activity measurements have been carried out to investigate the physiological function of the prismane protein. Hydrogen-producing hydrogenase activity, fumarate-, sulphite-, nitrite-, thiosulphate- and adenosine-5'-phosphosulphate reductase activities, lactate- and formate dehydrogenase activities and NADH or NADPH reactivities were all negligible (Pierik et al., 1992). The discovery of a prismane-like homologue on pTF5 has extended the biological distribution of this unusual protein, but its function and role in *T. ferrooxidans* is still unknown.

Since the genes discussed in this paper are present on both chromosome and plasmid, one might expect recombinative integration of the plasmid into the chromosome. As discussed earlier, integration of a pTF5 homologous plasmid into the chromosome does appear to have occurred in the case of *T. ferrooxidans* strains Black Mountain Pb and Bateman Reno. This raises the question of what advantage there is to the host for plasmid pTF5 to be maintained independently of the chromosome in such a large number of *T. ferrooxidans* strains from widespread geographical locations. The answer to this is not clear. The whole of pTF5 has recently been sequenced (unpublished) but all ORFs on the exclusively plasmid-associated part have homology to proteins associated with replication, inverrase or recombine functions. No ORFs have been identified which are likely to give a selective advantage to *T. ferrooxidans* strains which contain pTF5 as an independent plasmid rather than when integrated into the chromosome.

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