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

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During a search for genes encoding electron transport proteins from a *Thiobacillus ferrooxidans* ATCC 33020 gene bank, a 19.8 kb plasmid, pTF5, which conferred increased sensitivity to the antimicrobial agent metronidazole upon an *Escherichia coli* mutant, was isolated and cloned in *E. coli*. The plasmid had an identical restriction enzyme map to a plasmid which has been found in *T. ferrooxidans* strains isolated from many different parts of the world. The plasmid was present at between two and four copies per genome and contained a region of approximately 5.6 kb which was also found on the chromosome. This region was sequenced and found to have four complete ORFs, which when translated had high percentage amino acid similarity to [3Fe–4S, 4Fe–4S] ferredoxins, proteins of the FNR regulator family, prismane-like proteins and the NADH oxidoreductase subunit of a methane monooxygenase. *In vitro* protein analysis using an *E. coli*-derived transcription-translation system indicated that three of the four products (FdxA, PsmA and RedA) were expressed in the heterologous system. Ferredoxins, prismane-like proteins and NADH oxidoreductases are redox-active proteins and it is likely that the proteins on pTF5 represent an electron transport system of as yet unknown function. Surprisingly, although genes for redox-active proteins have been isolated from other bacteria by screening gene banks for increased sensitivity to metronidazole, the region of pTF5 containing the genes for these proteins was not responsible for the increase in metronidazole sensitivity conferred by the plasmid. The region of pTF5 which did confer increased metronidazole sensitivity to an *E. coli* metronidazole-resistant mutant was a 319 bp region of DNA close to the origin of plasmid replication. This region contained no ORFs and was identical to that previously reported for the replicon of a 9.8 kb *T. ferrooxidans* plasmid, pTFI91.

Keywords: *Thiobacillus ferrooxidans*, prismane, FNR protein, ferredoxin, NADH oxidoreductase

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-

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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 multi-drug 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 *SacI* 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 pTF35 (Rawlings *et al.*, 1983). This plasmid has been found in *T. ferrooxidans* strains which have originated from a bituminous coal mining effluent in Pennsylvania (Leathen *et al.*, 1956; Chakravarty *et al.*, 1995), a chalcopyrite mine in New Mexico (Valenti *et al.*, 1990), a variety of sulphur spring and river water samples in the vicinity of Rome (Valenti *et al.*, 1990; Polidoro *et al.*, 1993) and a uranium deposit in Japan (this paper). Because of its wide geographical distribution it is possible that this 20 kbp plasmid contains genes which are beneficial to the survival of its host.

In parallel to ongoing plasmid studies we attempted to isolate genes encoding electron transport proteins from *T. ferrooxidans* ATCC 33020 using a procedure which involved screening for genes which are able to activate the antimicrobial drug metronidazole. Metronidazole is active only once it has been reduced and has previously been used successfully for this purpose (Wall *et al.*,

1984; Santangelo *et al.*, 1991). Our studies on plasmid biology and the search for genes for redox-active proteins converged when, using the metronidazole sensitivity screening procedure, we unexpectedly isolated a 19.8 kbp plasmid which conferred metronidazole sensitivity to an *E. coli* mutant. Furthermore, the plasmid had an identical restriction enzyme map to the previously reported and widely distributed plasmids, pTF35 and pTF0. We here present an analysis of a 6.0 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. thiooxidans* was grown in medium containing 50 mM $K_2S_4O_6$, pH 2.5 (Powles *et al.*, 1995). *E. coli* strains were grown in Luria broth at 30 °C and where 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 (Ramesar, 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) NaNO₃ 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* *fur* mutant phenotype were identified and plasmid DNA extracted. This DNA was transformed into *E. coli* RM102 and incubated anaerobically (CO₂/N₂/H₂, 85:10:5, by vol.) for 4 d at 37 °C on minimal media containing glycerol (0.5%, v/v), NaNO₃ (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 OD₆₀₀ 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 × 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 × 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 *XbaI* 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 *XbaI* for 4 h. Digested samples were washed in Tris/EDTA and the plugs stored at 4 °C until used. Slices were equilibrated 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 electro-

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
<i>L. ferrooxidans</i> DSM 2705	Type strain, Armenia	DSM, Darmstadt, Germany
<i>T. thiooxidans</i> ATCC 19377	Type strain, Libya	ATCC, Rockville, MD, USA
<i>T. ferrooxidans</i> ATCC 33020	U ^r isolate, Japan	ATCC, Rockville, MD, USA
ATCC 23270	Type strain, USA	ATCC, Rockville, MD, USA
FC1	As ^s strain, South Africa	Gencor
FC6	As ^r strain, South Africa	Gencor
Bateman Reno	South Africa	Gencor
Black Mountain Pb	South Africa	Gencor
<i>E. coli</i> F19	CC118 (TnPhoA::yehB TnPhoA::pgk)	Santangelo <i>et al.</i> (1991)
RM102	MC4100 Δ (srl-recA)306::Tn10	Birkmann <i>et al.</i> (1987)
Plasmids		
pTF5	<i>T. ferrooxidans</i> ATCC 33020	This study
pTF35	<i>T. ferrooxidans</i> TF35	Rawlings <i>et al.</i> (1983)
pCD1	pTF5 cloned into pBS-KS	This study
pDER301	pTF35 cloned into pBR322	Rawlings & Woods (1985)
pCD100	12.9 kb <i>Xba</i> I– <i>Hind</i> III pTF5 subclone in pBS-KS	This study
pCD119	2.3 kb <i>Eco</i> RV subclone in pBS-KS	This study
pCD120	1.3 kb amplified DNA cloned into pKK223-3	This study
pCD150	5.2 kb <i>Cla</i> I subclone in pBS-KS	This study
pCD163	1.0 kb amplified DNA cloned into pKK223-3	This study
pCD164	2.8 kb amplified DNA cloned into pKK223-3	This study
pCD200	6.9 kb <i>Xba</i> I– <i>Hind</i> III pTF5 subclone in pBS-KS	This study
pCD300	2.2 kb <i>Sac</i> I pTF5 subclone in pBS-KS	This study
pCD313	0.319 kb <i>Xba</i> I– <i>Sal</i> I pTF5 subclone in pBS-KS	This study
pCD400	2.5 kb <i>Sph</i> I pTF5 subclone in pUC-BM21	This study
pCD421	1.05 kb <i>Hind</i> III– <i>Sph</i> I subclone in pUC-BM21	This study
pCD500	11.8 kb <i>Sac</i> I Δ of pCD100 in pBS-KS	This study
pMET7	<i>T. ferrooxidans</i> cosmid library clone in pHc79	This study
pMET17	<i>T. ferrooxidans</i> cosmid library clone in pHc79	This study
pBS-KS	Cloning vector	Stratagene
pUC-BM21	Cloning vector	Boehringer Mannheim
pHc79	Cosmid cloning vector	Boehringer Mannheim
pKK223-3	Expression vector containing <i>tac</i> promoter	Pharmacia

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

Fig. 1. Southern hybridization of *T. ferrooxidans* ATCC 33020 total genomic DNA separated by PFGE. Lanes: 1, 3 and 5, undigested genomic DNA; 2, 4 and 6, genomic DNA digested with *Xba*I. The location of probes B–H are indicated above the restriction map. The centre panel represents hybridization of probes C–G since the result for each probe was identical.

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 [35 S]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) with 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

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 *Xba*I (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 *Hind*III and *Xba*I restriction sites, intact pTF5 was reassembled and cloned by ligation of a 6.9 kbp *Xba*I–*Hind*III fragment from pMET17 to a 12.9 kbp *Xba*I–*Hind*III fragment from pMET7 by inserting the 19.8 kbp *Hind*III–*Xba*I–*Hind*III fragment into the *Hind*III 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 *Hind*III to free pTF5 and pTF35 from their respective cloning vectors and then digested with a second restriction endonuclease (either *Bam*HI, *Cla*I, *Eco*RI, *Hpa*I or *Sal*I). 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 *Xba*I–*Sal*I 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 (*ori*V) from the *T. ferrooxidans* plasmid pTFI91 (Chakravarty *et al.*, 1995).

DNA sequence analysis

The region of pTF5 from the *Hind*III site (position 1 bp) to the *Sph*I 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 *Hind*III–*Sph*I 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 *Hind*III 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 σ^{70} -type 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) (Arai *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-X₂-SR motif which was also present in the FNR-like protein encoded by pTF5 (Fig. 4b). However, cysteines equivalent to the three *E. coli* FNR N-terminal Cys residues and the Cys₁₂₂ 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-N₄-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-

moidal cluster and have no known function. The pTF5 prismane-protein-like ORF had highest amino acid identity to the prismane proteins from the genus *Desulfovibrio* (43–46%) (Stokkermans, 1992a, b) and slightly less homology to the protein from the archaeon *Methanobacterium jannaschii* (41%) (<http://www3.ncbi.nlm.nih.gov>). The three non-pTF5 prismane proteins have nine cysteine residues, four of which occur in a Cys-Xaa₂-Cys-Xaa₇-Cys-Xaa₅-Cys motif located near the N-terminus (Fig. 4c). The pTF5 prismane-protein-like ORF had all four of the conserved N-terminal Cys residues; however, the spacing (Cys-Xaa₂-Cys-Xaa₁₁-Cys-Xaa₆-Cys) was different.

Downstream of the prismane-protein-like ORF was an ORF of 343 amino acids (38.1 kDa) (Fig. 3). This ORF had highest amino acid sequence similarity to subunit c of the methane monooxygenase of *Methylococcus capsulatus* (40.5% amino acid identity) which functions as an NADH oxidoreductase (Fig. 4d) (Stainthorpe *et al.*, 1990). A short intergenic region separated the prismane-protein-like and NADH-oxidoreductase-like ORFs, the most notable feature of which was a 10 bp inverted repeat sequence which could form a stem-loop structure of $\Delta G -14.6 \text{ kcal mol}^{-1}$ ($-61.3 \text{ kJ mol}^{-1}$) and was situated immediately upstream of the ribosome-binding site of the NADH-oxidoreductase-like ORF (Fig. 3).

Identification of the region of pTF5 which is also located on the chromosome of *T. ferrooxidans* ATCC 33020

As both chromosomal and plasmid DNA gave a positive hybridization signal to a probe from the region containing the ferredoxin-like ORF (probe C, Fig. 1), we wished to determine whether only a portion of pTF5 was present on the chromosome or whether the entire plasmid was integrated into the chromosome. Undigested total DNA from *T. ferrooxidans* ATCC 33020 cells and fragments of total DNA digested with *Xba*I were separated using PFGE. The gels were blotted and probed with digoxigenin-labelled fragments of pTF5 (Fig. 1). Probes C, D, E, F and G gave identical hybridization signals to undigested plasmid (Fig. 1, lane 3), and also to the 20 kbp linearized plasmid and a 60 kbp chromosomal fragment (lane 4). Probes B and H hybridized to a band corresponding to covalently closed circular plasmid DNA in the undigested samples and to the 20 kbp linearized plasmid band in the *Xba*I-digested DNA. No hybridization to the chromosomal band at 60 kbp was observed with probes B and H. This indicated that the DNA extending from probes C to G, which included the ferredoxin-, the FNR-, the prismane- and NADH-oxidoreductase-like ORFs, was present on both plasmid pTF5 and the chromosome of *T. ferrooxidans* ATCC 33020. The signal obtained from the DNA in the well of the undigested samples (Fig. 1, lanes 1, 3 and 5) was due to plasmid DNA becoming entangled in the chromosomal DNA and was absent from the

Fig. 5. Dot blot hybridization to determine pTF5 copy number in *T. ferrooxidans*. Upper row, *T. ferrooxidans* ATCC 33020 total genomic DNA; lower row, pCD200 DNA. Samples of DNA were standardized according to the relative sizes of the *T. ferrooxidans* chromosome and plasmid pTF5 assuming a pCD200 copy number of one in each sample.

fragment immediately downstream of the ferredoxin-like ORF. Similarly, analysis of the signals obtained from probes G and H enabled localization of the right-hand junction between plasmid and chromosome to a region within probe G, most likely the 531 bp *SphI*–*Bam*HI fragment downstream of the NADH-oxidoreductase-like ORF (Fig. 1). No transposon termini, inverted repeats, direct repeats or features frequently identified with transposon activity were obvious within either junction region.

Determination of plasmid copy number in *T. ferrooxidans*

The copy number of pTF5 was estimated by hybridization of a plasmid probe, pCD200 (Fig. 2), to known amounts of purified plasmid and total DNA prepared from *T. ferrooxidans* in a dot blot experiment. Based on the relative intensities of the hybridization signals, pTF5 was estimated to be present at between two and four copies per chromosome (Fig. 5). This estimate was supported by the observation that the intensity of the signal to hybridization probes C–G was consistently three- to fourfold greater than to the chromosomal bands (Fig. 1).

Expression of proteins from pTF5

We wished to determine whether any of the ORFs from the region of pTF5 that was present on both chromosome and plasmid were expressed. An *E. coli*-derived *in vitro* transcription-translation system was used to detect expression of plasmid-encoded proteins even though it had the disadvantage that not all factors required for expression might be present in the heterologous system. It was used because even if protein-specific antibodies had been available, they would not have assisted in identifying whether antibody-specific proteins produced by *T. ferrooxidans* originated from the plasmid or the chromosome since the same genes were present on both. Polypeptides produced from pTF5 using the *E. coli*

system are shown in Fig. 6. Comparison of polypeptides produced from pCD1 (lane 2) with those produced by the vector pBS-KS (lane 1) indicated that additional proteins of approximately 90, 40, 26, 17, and at least two between 14 and 9 kDa were produced from pTF5. To identify whether any of these polypeptides were expressed from the region of pTF5 which was also present on the chromosome, small fragments containing each of the ORFs were cloned into pBS-KS so that they were in the same orientation as the *lacZ* vector promoter. A 14 kDa polypeptide was detected from the clone pCD421 containing the ferredoxin-like ORF (lane 3). 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 from the clone of 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 *Xba*I-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 *Xba*I which hybridized to probe G; (c) undigested total DNA probed with pCD300. Lanes: 1, *L. ferrooxidans* DSM 2705; 2, *T. thiooxidans* 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.

(Japan) and FC1 (South Africa) were obtained (Fig. 7a, lanes 3, 4 and 6). The hybridization signal to DNA remaining in the well of each of these strains was due to non-mobile undigested chromosomal DNA and possibly some entrapped plasmid DNA. In the case of *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 *Xba*I-digested DNA samples from all strains confirmed these results. Two bands were obtained for *T. ferrooxidans* strains ATCC 23270, ATCC 33020 and FC1 (Fig. 7b, lanes 3, 4 and 6), one signal due to the copy of the *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 *Xba*I fragment of greater than 500 kbp and fragments of this size do not penetrate the gel under the running conditions used to separate smaller fragments. From the *Xba*I-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). *T. ferrooxidans* strains Black Mountain Pb and Bateman Reno gave only a single hybridization signal at about 30 kbp (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 *L. ferrooxidans* type strain DSM 2705, *T. thiooxidans* type strain ATCC 19377 or *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 *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 *Clostridium acetobutylicum* and a gene for a flavodoxin which rendered the *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 *C. acetobutylicum* which resulted in increased sensitivity of *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 *regA* gene, which produced a 35 kDa protein with homology to the LacI family of repressor proteins (Davison *et al.*, 1995). Some members of the LacI 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 *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 *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 *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 *T. ferrooxidans* strain FC6 (Fig. 7a, b, c, lane 7) and other *T. ferrooxidans* strains (Valenti *et al.*, 1989) do not possess the plasmid.

In *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 *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 *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 *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-X₂-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 monooxygenase from *Methylococcus capsulatus*. The methane monooxygenase of this bacterium consists of three subunits, with subunits a and b together forming the terminal oxygenase and subunit c the NADH reductase (Stainthorpe *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.

Prismene 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] prismane cluster which is able to exist in four different redox states ranging from [6Fe-6S]⁶⁺ to [6Fe-6S]³⁺ (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 *Methanotrix*

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, invertase or recombinase 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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