The gene for γ -glutamylcysteine synthetase from *Thiobacillus ferrooxidans* has low homology to its *Escherichia coli* equivalent and is linked to the gene for citrate synthase

Rosamund Powles, Shelly Deane and Douglas Rawlings

Author for correspondence: Douglas Rawlings. Tel: +27 21 650 3261. Fax: +27 21 650 4023.
e-mail: doug@molbiol.uct.ac.za

Department of
Microbiology,
University of
Cape Town,
Rondebosch 7700,
South Africa

The gene for γ -glutamylcysteine synthetase (*gshA*) from *Thiobacillus ferrooxidans* was isolated from a family of cosmids by its ability to complement an *Escherichia coli* *gshA trxA* double mutant which was unable to grow on minimal medium lacking glutathione. The predicted sequence of the γ -glutamylcysteine synthetase was found to have only 18% amino acid sequence identity to the equivalent enzyme from *E. coli*. In spite of this low sequence homology, concentrations of GSH in a cell extract prepared from the *E. coli* *gshA trxA* mutant containing the cloned gene were almost as high as in a cell extract prepared from a wild-type *E. coli* strain. The *gshA* gene was found to be physically and transcriptionally linked to the *T. ferrooxidans* gene for citrate synthase (*gltA*). The *T. ferrooxidans* and *E. coli* citrate synthases shared 37% amino acid sequence identity and the cloned *T. ferrooxidans* citrate synthase gene was able to complement an *E. coli* *gltA* mutant.

Keywords: *Thiobacillus ferrooxidans*, γ -glutamylcysteine synthetase, citrate synthase, molecular cloning

INTRODUCTION

Thiobacillus ferrooxidans is an autotrophic, N_2 -fixing, acidophilic (pH 1.5–3.5), chemolithotrophic bacterium which obtains its energy from the oxidation of either Fe^{2+} to Fe^{3+} or reduced forms of sulphur to sulphate. There has been much interest in *T. ferrooxidans* because of its use in industrial mineral processing and because of its unusual physiology.

During the commercial biooxidation of a mineral such as arsenopyrite, large quantities of air are pumped into the reactor vessels and the redox potential of the solution may rise from less than +300 mV before oxidation to greater than +700 mV when oxidation is complete (Lindström *et al.*, 1992). This implies that the organism must have considerable redox-controlling capacity and the ability to withstand the toxic effects of oxygen. Glutathione is one of the major low-molecular-mass thiol components of nearly all cell types and is involved in the control of redox status and is a scavenger of free radicals. Other gluta-

thione-associated intracellular functions include its role in transpeptidation reactions, in the reduction of protein thiol groups and as an enzyme cofactor in DNA synthesis (Meister & Anderson, 1983).

Glutathione synthesis takes place in two ATP-dependent steps. In the first, γ -glutamylcysteine synthetase catalyses the formation of the dipeptide, γ -glutamylcysteine, from L-glutamate and L-cysteine:

Glycine is then added to the C-terminal site of the dipeptide to yield glutathione; this reaction is catalysed by glutathione synthetase:

The γ -glutamylcysteine synthetase enzyme has been isolated from, and studied in, a number of organisms. The mammalian enzymes (rat and human) are composed of two subunits (73 kDa and 27 kDa) that can be reversibly dissociated using DTT (Seelig *et al.*, 1984). The heavy subunit exhibits catalytic activity and feedback inhibition

The GenBank accession number for the sequence reported in this paper is L75931.

by GSH (Seelig *et al.*, 1984; Richman & Meister, 1975), whereas the small subunit has been shown to have a regulatory function (Huang *et al.*, 1993). The γ -glutamylcysteine synthetase of *Nicotiana tabacum* is composed of two identical 34 kDa subunits (Hell & Bergmann, 1990) whereas all other γ -glutamylcysteine synthetases studied, those in yeast (Dennda & Kula, 1986), *Arabidopsis thaliana* (May & Leaver, 1994) and the bacteria *Proteus mirabilis* (Kumagai *et al.*, 1982) and *Escherichia coli* (Huang *et al.*, 1988), contain a single polypeptide of approximately 60 kDa. Five of the γ -glutamylcysteine synthetase (*gshA*) genes have been sequenced.

We have been investigating intracellular systems that might play a role in the ability of *T. ferrooxidans* to cope with large changes in its external redox potential. Here we report the isolation of the *T. ferrooxidans* gene for γ -glutamylcysteine synthetase and its transcriptional linkage to the gene for citrate synthase. This is only the second *gshA* gene to be sequenced from a bacterium.

METHODS

Bacterial strains and plasmids. Genotypes of the strains used are as follows: *E. coli* JM109, *recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 λ Δ (lac-proAB)* (*F'* *traD36 proAB lacI^q ZAM15*); *E. coli* BH5262 K-12, *F⁻ araD139⁺ galU galK hsr rpsL argH1 trxA7004 gshA srl::Tn10*; *E. coli* MC1061 K-12, *F⁻ araD139⁺ galU galK hsdR rpsL*; *E. coli* MOB150, *F⁻ gltA5 let-1 thi-1 lacY1 galK2 hsdR4 xyl-5 mtl-1 tsx-57 tfr-5 rpsL20 supE44*. *E. coli* strain BH5262 was kindly donated by J. A. Fuchs (University of Minnesota, USA). D. O. Wood (University of South Alabama College of Medicine, USA) provided *E. coli* MOB150 and plasmid pMW264. Plasmids pBluescript SK, pUCMB21 and pUCMB20 were used for subcloning and sequencing.

Media. Tetrathionate medium was made from mineral salts solution (g l^{-1}): $(\text{NH}_4)_2\text{SO}_4$, 3.0; KCl, 0.1; K_2HPO_4 , 0.5; $\text{Ca}(\text{NO}_3)_2$, 0.01; the pH was adjusted to 2.5 with H_2SO_4 and it was then autoclaved. Trace elements solution consisted of (mg l^{-1}): $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 11.0; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.5; HBO_3 , 2.0; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.8; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.6; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.9; filter sterilized. One millilitre of trace elements solution was added to 100 ml mineral salts solution and to this was added either 50 mM $\text{K}_2\text{S}_2\text{O}_8$ or 100 mM FeSO_4 ; the final pH was adjusted to 2.5. Luria-Bertani medium was used as a complete medium and M9 minimal medium as a selective medium (Sambrook *et al.*, 1989).

Cloning and genetic manipulations. A cosmid bank of the *T. ferrooxidans* genome (Ramesar, 1988) was transduced into the *E. coli* mutant BH5262 according to the method of Sambrook *et al.* (1989). Possible *gshA*- or *trxA*-positive transfectants were identified by their ability to grow on minimal medium. Strain BH5262 is unable to grow on minimal medium lacking glutathione, whereas *TrxA*⁺ or *GshA*⁺ colonies can (Lim *et al.*, 1986). Cosmids were isolated from positive colonies. Subclones of one of the *GshA*⁺ cosmids were made and tested for the ability to complement *E. coli* BH5262.

DNA techniques, sequencing and analysis. Standard methods were used for plasmid preparation, restriction enzyme digests, gel electrophoresis, ligations and DNA-DNA hybridizations (Sambrook *et al.*, 1989). Labelling of probes, hybridization and detection were carried out according to the digoxigenin-dUTP non-radioactive DNA labelling and detection kit (Boehringer

Mannheim). DNA sequencing was carried out by the dideoxy chain-termination method (Sanger *et al.*, 1977) using the Sequenase kit (version 2.0) from United States Biochemical. The sequence of the 2.4 kbp *Clal*-*Bam*HI fragment shown in Fig. 2 was determined for both strands. The Genetics Computer Group (GCG) software package was used for sequence analysis (Devereux *et al.*, 1984).

Complementation of the *E. coli* mutants for citrate synthase.

Various plasmid constructs were tested for the ability to complement *E. coli gltA* mutant MOB150 by streaking transformants onto M9 minimal medium (10 mM glucose). Strain MOB150 is unable to grow on minimal medium lacking glutamate, whereas *GltA*⁺ colonies can.

In vitro synthesis of *gshA* and *gltA*. The synthesis of polypeptides from cosmid 5.1, plasmids pTHIOD, pTHIO28 and pTHIO7 and the vector pBluescript SK was determined using the prokaryotic-DNA-directed transcription/translation kit, *E. coli* S30 system from Promega. Reactions were performed according to the manufacturer's specifications and the proteins were separated by SDS-PAGE using 5–20% (w/v) gradient gels.

Analysis of transcripts. *T. ferrooxidans* total RNA was prepared from cultures grown on tetrathionate medium and *E. coli* total RNA was prepared from cultures grown on M9 minimal medium, by the method of Aiba *et al.* (1981). For DNA-RNA hybridizations, total RNA was separated on a 1.5% agarose gel containing 6% (v/v) formaldehyde. The RNA was transferred to an Amersham Hybond N⁺ membrane and hybridization and washes were carried out according to the protocol of the manufacturers. Labelling of probes was carried out with ³²P using a Random Primed DNA labelling kit (Boehringer Mannheim).

GSH assay. The fluorometric method described by Hissin & Hilf (1976) was used to measure the endogenous GSH content of *E. coli* BH5262, *E. coli* BH5262(pTHIO7) and *E. coli* MC1061 cells, grown on M9 minimal medium with the necessary media supplements.

RESULTS

Isolation and localization of the *gshA* gene

E. coli BH5262 is a *trxA gshA* double mutant which is unable to grow on minimal medium lacking glutathione whereas either *TrxA*⁺ or *GshA*⁺ colonies can (Lim *et al.*, 1986). Complementation of the *gshA* gene would enable the *E. coli* mutant to synthesize glutathione and permit growth on minimal medium. Transduction of the *T. ferrooxidans* genome cosmid library into *E. coli* BH5262 resulted in approximately 100 colonies that were able to grow on minimal media lacking glutathione. DNA was prepared from 16 of these colonies. These cosmids had several fragments in common and could be divided into two groups that appeared to contain overlapping pieces of two regions of the *T. ferrooxidans* chromosome. One of the groups of cosmids was found to permit growth of an *E. coli metA46 trxA* mutant on minimal medium containing methionine sulfoxide and contained the thio-redoxin gene (Powles *et al.*, 1995). A representative from the other group of cosmids, cosmid 5.1, was chosen for further study.

A 5 kbp *Hind*III fragment from cosmid 5.1 which complemented *E. coli* BH5262 was subcloned into the

Fig. 1. Restriction endonuclease map of plasmid pTHIOD and derivatives. The *gltA* and *gshA* genes are indicated by arrows. The dotted line represents the region of DNA sequenced from both strands. GshA⁺ or GltA⁺ activity indicates that the transformed plasmid was able to complement the γ -glutamylcysteine synthetase mutation of *E. coli* BH5262 or the citrate synthase mutation of *E. coli* MOB150, respectively. The arrows at the end of each plasmid indicate the direction of the *lac* promoter of the vector.

Fig. 2. Hybridization of labelled plasmid pTHIO7 to pTHIO7 (lane 1), plasmid pTHIOD (lane 2), cosmid 5.1 (lane 3) and *T. ferrooxidans* ATCC 33020 chromosomal DNA (lane 5) and a *Bam*HI digest of cosmid 5.1 (lane 4).

vector pUCMB21 to give plasmid pTHIOD (Fig. 1). A restriction map of pTHIOD was constructed, and smaller fragments were subcloned into vectors pUCBM20, pUCBM21 or pBluescript SK and tested for their ability to complement *E. coli* BH5262 for growth on minimal medium lacking glutathione (Fig. 1).

Confirmation of the source of the *gshA*-complementing DNA was shown by hybridization of the labelled plasmid pTHIO7 to pTHIO7, pTHIOD, cosmid 5.1 and *T. ferrooxidans* ATCC 33020 chromosomal DNA digested with *Sma*I (Fig. 2, lanes 1, 2, 3 and 5, respectively) and to cosmid 5.1 digested with *Bam*HI (Fig. 2, lane 4). The 3.0 kb *Sma*I fragment that is internal to the cloned *T. ferrooxidans* DNA present on pTHIOD (lane 2) corresponded exactly to an *Sma*I fragment present on the *T. ferrooxidans* chromosome (lane 5) and cosmid 5.1 (lane 3). Similarly, the 1.8 kb *Bam*HI fragment present on cosmid

Fig. 3. Nucleotide sequence of the 2657 bp *Cla*I-*Bam*HI fragment and the deduced citrate synthase and γ -glutamylcysteine synthetase amino acid sequences. Putative ribosome-binding sites are indicated in bold.

5.1 (lane 4) corresponded to the size predicted from the restriction endonuclease map. The hybridization signal of pTHIO7 at 4.8 kb (lane 1) corresponded to the linearized plasmid, as pTHIO7 only contains a single *Sma*I site. The

T. f.	MYEVIRDIPLATDVEALLDIHFLIVEOSTIERMFGQWRTTPTPTASVDLRNAGFKIAPVDNLFSGAGNNMP.....EFS	82
E. c.	LIPVSGALNLE..KHFQALRGORGL..IRETLVWACGYLATGHP.....TALGSALTERTMTTDFARALLFTITPTVGDIEHML	80
T. f.	LYVSAIGYINQYHGLERVL.....LIPENSTINL..FYLESVARLRELELAGLDVVRVSSLTURDQTVYDLPSSGGQLLEPLCR	161
E. c.	TYHRLRYTAR..NGDEHMLPLSMCTIAGGGDILAGYGTSTNGFETYSR..EGLKRYGALMOTIKOVHMPSTLPMQFNGKCG	165
T. f.	D.....DNLSTSTQDPA.....QLILLNDISGVPRILAGLEQILPFLA.....AGWNNKNSQNTB	216
E. c.	DISGADKERTSAGYPRVIRNYRFGWVPLVFGSPATCESPLQKPTSLPFRKTECGHYLLPYATSLKLSDGGITNKSQSLGIT	252
T. f.	YRVAQELAEV.....IGIDF.....MLIDPFVRQDQIDEMSEGRDC...LVANVD..AVLEITREYAR	273
E. c.	FNOLYEVAGLQAKITPSESTAKIGISKGGKQINSNVLQIENELFAEIRKRVTRSGSDALLGGSEYIEVRSIDINSESP	329
T. f.	YSHIGRFVIVKADASTYNGINTAYSGEEF..LDLNKERTSNKSGREGVSDVFIOGVYTFEQTAQSAVAPVVMIGQVVG	358
E. c.	IGVDEQVRLDLFMWALACADENGSSSLACTHVM..KRVTLGGHFGCL..GICETAQFPLQVQK.....LFRDLKRYAD	418
T. f.	GFYRHTERGRDSELMANPGRFPRMAG..QTCVLPCKKSPPRAPANNY..AYGVIAKLTVNAARE...NADN.....RGGTLET	435
E. c.	TLDSINGEAYQVQVDELVACFONELIFBARIDNSMTDICTGCKGFAEAYNNLLKREPLELREDEFAVREGEFERQDGEAR	506
T. f.	Q	436
E. c.	DTKPPAVNLKHA	519

Fig. 4. Comparison of the amino acid sequences of the *T. ferrooxidans* (T. f) and *E. coli* (E. c) (GenBank/EMBL accession no. X03954) γ -glutamylcysteine synthetase proteins.

hybridization signals to the upper bands of cosmid 5.1 (lanes 3 and 4) corresponded to hybridization of the *Sma*I–*Bam*HI flanking region joined to vector DNA. This result indicated that the source of the 3.0 kb *Sma*I insert was from the *T. ferrooxidans* ATCC 33020 chromosome and that no rearrangements had occurred during cloning.

Identification of GltA and GshA

Analysis of the sequence from the *Cla*I site to the *Bam*HI site revealed two complete ORFs separated by 9 bp (Fig. 3). The first ORF is preceded by a strong ribosome-binding site and encodes a polypeptide of 386 amino acids, corresponding to a protein of 42.7 kDa. The predicted amino acid sequence was closely related to that of a number of citrate synthases (37% identity and 59% similarity to *E. coli*; GenBank/EMBL accession no. J01619). The second ORF encodes a polypeptide of 436 amino acids, corresponding to a protein of 49.3 kDa. The predicted amino acid sequence was matched against all sequences present in the GenBank and EMBL databases using the NCBI BLAST program. Weak but clear homology (18% amino acid identity; Fig. 4) to the γ -glutamylcysteine synthetase gene of *E. coli* was detected. No clear homology to the other four γ -glutamylcysteine synthetases present in the databases was apparent.

Synthesis of proteins corresponding to the *T. ferrooxidans* γ -glutamylcysteine synthetase (49.3 kDa) and citrate synthase (42.7 kDa) enzymes was confirmed using an *E. coli*-derived *in vitro* transcription/translation system. A protein of approximately 49 kDa was produced by cosmid 5.1 and plasmids pTHIOD and pTHIO7, but not by the vector pBluescript SK, plasmid pTHIO28 or the cosmid vector pHCT9 (results not shown) (Fig. 5). Cosmid 5.1 and plasmids pTHIOD and pTHIO28 produced a protein of approximately 43 kDa, whilst the vectors pBluescript SK and cosmid pHCT9 (results not shown) and plasmid pTHIO7 did not (Fig. 5).

GSH assay

Because of the low sequence homology and the difference in size between the 49 kDa polypeptide and the *E. coli* γ -glutamylcysteine synthetase (60 kDa), an assay for γ -

Fig. 5. SDS-PAGE analysis of proteins expressed from cosmid 5.1, plasmids pTHIOD, pTHIO28 and pTHIO7 and pBluescript SK (lanes 1–5, respectively) using an *E. coli*-derived *in vitro* transcription/translation system.

glutamylcysteine synthetase activity was carried out. GSH was shown to react specifically with *o*-phthalaldehyde at pH 8.0, yielding a highly fluorescent product that could be activated at 350 nm with an emission peak at 420 nm (Hissin & Hilf, 1976). Concentrations of GSH in crude extracts of *E. coli* BH5262, BH5262(pTHIO7) and the parental strain *E. coli* MC1061 were compared. Extracts prepared from *E. coli* BH5262(pTHIO7) contained far higher levels of GSH [29.67 ± 3.06 nmol GSH (mg protein) $^{-1}$] than extracts from *E. coli* BH5262 cells [9.00 ± 1.73 nmol GSH (mg protein) $^{-1}$], and almost the same levels of GSH as extracts prepared from a wild-type *E. coli* strain MC1061 [31.67 ± 4.04 nmol GSH (mg protein) $^{-1}$]. This clearly indicates that γ -glutamylcysteine is produced by the cloned *T. ferrooxidans* *gshA* gene, which is then converted to GSH by the host glutathione synthetase (*gshB*) gene.

Complementation of *E. coli* *gltA* mutants

Various plasmids thought to contain the *T. ferrooxidans* citrate synthase gene were tested for the ability to complement the *gltA* phenotype of *E. coli* strain MOB150. Plasmid pMW264, which contains the *Rickettsia prowazekii* citrate synthase gene (Wood *et al.*, 1987), was used as a positive control. Plasmids pMW264, pTHIO6, pTHIO28 and pTHIO82 enabled the *E. coli* *gltA* mutant

Fig. 6. Hybridization of a labelled *Bam*HI-*Sma*I fragment of pTHIO28 (lane 1) and a labelled *Bam*HI-*Sal*I fragment of pTHIO7 (lane 2), to total RNA prepared from *T. ferrooxidans*.

to grow on minimal medium, while the vector pBluescript did not. Cosmid 5.1 complemented *E. coli* MOB150 very weakly. As the *gltA* gene in pTHIO28 and pTHIO82 lies in an opposite orientation with respect to the *lac* promoter, and both plasmids are able to complement *E. coli* MOB150, the cloned *T. ferrooxidans* *gltA* gene appears to be expressed in *E. coli* from its own promoter.

Transcript analysis

To determine whether the citrate synthase and the γ -glutamylcysteine synthetase genes were part of an operon, total RNA isolated from *T. ferrooxidans* cells was examined by RNA-DNA hybridization. The transcripts were probed with the *Bam*HI-*Sma*I fragment from pTHIO28 (specific to the *gltA* gene) and the *Bam*HI-*Sal*I fragment from pTHIO7 (specific to the *gshA* gene). With both probes, a small quantity of transcript of about 9 kb and a transcript of 2.5 kb was obtained (Fig. 6), irrespective of which probe was used. No signal was obtained when either probe was hybridized to *E. coli* JM109 control mRNA (result not shown).

DISCUSSION

The redox potential in the environment of an iron-oxidizing bacterium such as *T. ferrooxidans* is a function of the $\text{Fe}^{3+}/\text{Fe}^{2+}$ ratio. When ferrous iron is oxidized to ferric iron, the redox potential of the surrounding medium

increases correspondingly and bacteria which grow in such an environment require the ability to maintain their cytoplasmic redox potential within certain limits. Iron-oxidizing bacteria like *T. ferrooxidans* might therefore be expected to have a greater capacity for controlling their internal redox potential than most bacteria. Glutathione and thioredoxin are two molecules that play a role in the control of redox potential within a cell. It is interesting that transposon Tn5467, which is located on *T. ferrooxidans* plasmid pTF-FC2, has recently been shown to encode a functional glutaredoxin-like protein (Clennel *et al.*, 1995) which might be expected to participate in internal redox control.

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

Several pieces of evidence support the view that the *T. ferrooxidans* citrate synthase and γ -glutamylcysteine synthetase genes are transcriptionally linked. DNA-RNA hybridization experiments indicated similar-sized transcription products irrespective of whether the *gltA* or the *gshA* gene was used as a probe. Furthermore, the *gshA* gene does not appear to be transcribed from its own promoter in *E. coli*, as subclones in which the *gshA* gene is transcribed in the opposite orientation to the *lac* promoter were unable to complement the *gshA* mutation of *E. coli* BH5262. The citrate synthase gene, however, was able to complement *gltA* mutant *E. coli* MOB150, irrespective of its orientation to the *lac* promoter. These results suggest that the *gshA* gene is transcribed from a promoter upstream of the citrate synthase gene. In addition, the two genes are separated by only 9 bp, which is frequently a feature of genes that are transcriptionally coupled. It has recently been discovered that in *T. ferrooxidans* ATCC 33020 the genes for lipoamide dehydrogenase (*lpd*) and pyruvate dehydrogenase (*aceE*, *aceF*) lie immediately upstream of the *gltA* gene for citrate synthase (R. Powles, unpublished data). The 9 kb transcript visible in the RNA preparation from *T. ferrooxidans* (Fig. 6, lanes 1 and 2) also hybridizes to a probe prepared from the region upstream of the *gltA* gene (unpublished). Transcriptional linkage of the *aceE*, *aceF*, *lpd* and *gltA* genes is not unexpected since the enzymes they encode are all involved in the transfer of an acetate group to oxaloacetate during the biosynthesis of citrate. However, the reason for the linkage between the *gltA* and *gshA* genes is unclear.

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

whereas the bacterial proteins (*E. coli* and *P. mirabilis*) are composed of a single subunit. The *T. ferrooxidans* protein also consists of a single subunit, of 49 kDa, which is considerably smaller than the 60 kDa and 64 kDa proteins of *E. coli* and *P. mirabilis*, respectively. The two mammalian γ -glutamylcysteine synthetases (rat and human) are very homologous (94% identity), and clearly related to that of *Saccharomyces cerevisiae* (45–46% identity). However, the amino acid sequences of the γ -glutamylcysteine synthetases of *A. thaliana*, *T. ferrooxidans* and *E. coli* are poorly related to each other and also to the rat, human and yeast enzymes (15–18% identity). The location of the gene for glutathione synthetase, the second step in glutathione synthesis, is still unknown.

ACKNOWLEDGEMENTS

We thank James Fuchs for *E. coli* strain BH5262, David O. Wood for *E. coli* strain MOB150 and plasmid pMW264 and Di James for assistance with DNA sequencing. This work was supported by grants from the Foundation for Research Development and Gencor (South Africa).

REFERENCES

- Aiba, H., Adhaya, S. & de Crombrugge, B. (1981). Evidence for two functional *gal* promoters in intact *E. coli* cells. *J Biol Chem* **256**, 11905–11910.
- Clennel, A.-M., Johnston, B. & Rawlings, D. E. (1995). Structure and function of Tn5467, a Tn21-like transposon located on the *Thiobacillus ferrooxidans* broad-host-range plasmid pTF-FC2. *Appl Environ Microbiol* **61**, 4223–4229.
- Dennda, G. & Kula, M.-R. (1986). Purification and evaluation of the glutathione-synthesizing enzymes of *Candida boidinii* for cell-free synthesis of glutathione. *J Biotechnol* **4**, 143–158.
- Devereux, J., Haeberli, P. & Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res* **12**, 387–395.
- Hell, R. & Bergmann, L. (1990). γ -Glutamylcysteine synthetase in higher plants: catalytic properties and subcellular localization. *Planta* **180**, 603–612.
- Hissin, P. J. & Hilf, R. (1976). A fluorometric method for determination of oxidized and reduced glutathione in tissues. *Anal Biochem* **74**, 214.
- Huang, C.-S., Moore, W. R. & Meister, A. (1988). On the active site thiol of γ -glutamylcysteine synthetase: relationships to catalysis, inhibition, and regulation. *Proc Natl Acad Sci USA* **85**, 2464–2468.
- Huang, C. S., Anderson, M. E. & Meister, A. (1993). Amino acid sequence and function of the light subunit of rat kidney gamma-glutamylcysteine synthetase. *J Biol Chem* **268**, 20578–20583.
- Kumagai, H., Nakayama, R. & Tochikura, T. (1982). γ -Glutamylcysteine synthetase from *Proteus mirabilis*. *Agric Biol Chem* **46**, 1301–1309.
- Lim, C.-J., Gleason, F. K. & Fuchs, J. A. (1986). Cloning, expression and characterization of the *Anabaena* thioredoxin gene in *Escherichia coli*. *J Bacteriol* **168**, 1258–1264.
- Lindström, E. B., Gunneriusson, E. & Tuovinen, O. H. (1992). Bacterial oxidation of refractory sulfide ores for gold recovery. *Crit Rev Biotechnol* **12**, 133–155.
- May, M. J. & Leaver, C. J. (1994). *Arabidopsis thaliana* γ -glutamylcysteine synthetase is structurally unrelated to mammalian, yeast, and *Escherichia coli* homologs. *Proc Natl Acad Sci USA* **91**, 10059–10063.
- Meister, A. & Anderson, M. E. (1983). Glutathione. *Annu Rev Biochem* **52**, 711–760.
- Powles, R. E., Deane, S. M. & Rawlings, D. E. (1995). Molecular and genetic analysis of a thioredoxin gene from *Thiobacillus ferrooxidans*. *Microbiology* **141**, 2175–2181.
- Ramesar, R. S. (1988). *Developmental genetic studies on Thiobacillus ferrooxidans*. PhD thesis, University of Cape Town, South Africa.
- Richman, P. G. & Meister, A. (1975). Regulation of γ -glutamylcysteine synthetase by nonallosteric feedback inhibition by glutathione. *J Biol Chem* **250**, 1422–1426.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74**, 3650–3654.
- Seelig, G. F., Simonsen, R. P. & Meister, A. (1984). Reversible dissociation of γ -glutamylcysteine synthetase into two subunits. *J Biol Chem* **259**, 9345–9347.
- Wood, D. O., Williamson, L. R., Winkler, H. H. & Krause, D. C. (1987). Nucleotide sequence of the *Rickettsia prowazekii* citrate synthase gene. *J Bacteriol* **169**, 3564–3572.

Received 29 January 1996; revised 18 April 1996; accepted 7 May 1996.