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

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The gene for \(\gamma\)-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 \(\gamma\)-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, \(\gamma\)-glutamylcysteine synthetase, citrate synthase, molecular cloning*

**INTRODUCTION**

*Thiobacillus ferrooxidans* is an autotrophic, \(\text{N}_2\)-fixing, acidophilic (pH 1.5–3.5), chemolithotrophic bacterium which obtains its energy from the oxidation of either \(\text{Fe}^{2+}\) to \(\text{Fe}^{3+}\) 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 glutathione-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, \(\gamma\)-glutamylcysteine synthetase catalyses the formation of the dipeptide, \(\gamma\)-glutamylcysteine, from \(\text{l}-\text{glutamate and l-cysteine:}

\[
\text{l-glutamate + l-cysteine + ATP} \rightarrow \gamma\text{-glutamylcysteine + ADP + P}_1
\]

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

\[
\gamma\text{-glutamylcysteine + glycine + ATP} \rightarrow \text{glutathione + ADP + P}_1
\]

The \(\gamma\)-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...
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 \textit{N. tabacum} is composed of two identical 34 kDa subunits (Holl & Bergmann, 1990) whereas all other γ-glutamylcysteine synthetases studied, those in yeast (Dennda & Kula, 1986), \textit{Arabidopsis thaliana} (May & Leaver, 1994) and the bacteria \textit{Proteus mirabilis} (Kumagai et al., 1982) and \textit{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 \textit{T. ferrooxidans} to cope with large changes in its external redox potential. Here we report the isolation of the \textit{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: \textit{E. coli} JM109, recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 [M(lac-proAB) (F' traD36 pro-AB lacF'ZAM15)]: \textit{E. coli} BH5262 K-12, F' araD139 galU galK his rplL argH1 trxA7004 gshA lrl-1: Tn10; \textit{E. coli} MC1061 K-12, F' araD139 galU galK his rplL; \textit{E. coli} MOB150, F' gltA5 lrl-1 thi-1 lacY1 galK2 hisD4 xyl-5 met-l trxA; \textit{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 \textit{E. coli} MOB150 and plasmid pMW264. Plasmids pBlueScript SK, pUCM21 and pUCMB20 were used for subcloning and sequencing.

**Media.** Tetrathionate medium was made from mineral salts solution (g l-1): (NH₄)₂SO₄, 30; KCl, 0.1; K,HPO₄, 0.5; Ca(NO₃)₂, 0.01; the pH was adjusted to 2.5 with H₂SO₄ and it was then autoclaved. Trace elements solution consisted of (mg l-1): FeCl₃, 0.01; CuSO₄, 0.01; CoCl₂, 0.01; ZnSO₄, 0.01; filter sterilized. One milliliter of trace elements solution was added to 100 ml mineral salts solution and to this was added 50 mM K₂SO₄ or 100 mM FeSO₄; 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 \textit{T. ferrooxidans} genome (Ramesar, 1988) was transduced into the \textit{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 \textit{TrxA}⁺ or \textit{GshA}⁺ colonies can (Lim et al., 1986). Cosmids were isolated from positive colonies. Subclones of one of the \textit{GshA}⁺ cosmids were made and tested for the ability to complement \textit{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 localization of the gene to be sequenced from a bacterium.

**Analysis of transcripts.** \textit{T. ferrooxidans} total RNA was prepared from cultures grown on tetrathionate medium and \textit{E. coli} total RNA was prepared from cultures grown on M9 minimal medium, by the method of Alba 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]dCTP 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 \textit{E. coli} BH5262, \textit{E. coli} BH5262(pTHIO7) and \textit{E. coli} MC1061 cells, grown on M9 minimal medium with the necessary media supplements.

**RESULTS**

**Isolation and localization of the gshA gene** \textit{E. coli} BH5262 is a \textit{trxA} gshA double mutant which is unable to grow on minimal medium lacking glutathione whereas either \textit{TrxA}⁺ or \textit{GshA}⁺ colonies can (Lim et al., 1986). Complementation of the gshA gene would enable the \textit{E. coli} mutant to synthesize glutathione and permit growth on minimal medium. Transduction of the \textit{T. ferrooxidans} genome cosmid library into \textit{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 \textit{T. ferrooxidans} chromosome. One of the groups of cosmids was found to permit growth of an \textit{E. coli metA46 trxA} mutant on minimal medium containing methionine sulfoxide and contained the thioredoxin gene (Powles et al., 1995). A representative from the other group of cosmids, cosmid 5.1, was chosen for further study.

A 5 kbp HindIII fragment from cosmid 5.1 which complemented \textit{E. coli} BH5262 was subcloned into the...
vector pUCM21 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 pTHIOD to pTHIOD, pTHIOD, cosmid 5.1 (lane 2) and *T. ferrooxidans* ATCC 33020 chromosomal DNA digested with *SmaI* (Fig. 2, lanes 1, 2, 3 and 5, respectively) and to cosmid 5.1 digested with *BamHI* (Fig. 2, lane 4). The 30 kb *SmaI* fragment that is internal to the cloned *T. ferrooxidans* DNA present on pTHIOD (lane 2) corresponded exactly to an *SmaI* fragment present on the *T. ferrooxidans* chromosome (lane 5) and cosmid 5.1 (lane 3). Similarly, the 1.8 kb *BamHI* fragment present on cosmid 5.1 (lane 4) corresponded to the size predicted from the restriction endonuclease map. The hybridization signal of pTHIOD at 4.8 kb (lane 1) corresponded to the linearized plasmid, as pTHIOD only contains a single *SmaI* site. The
Comparison of the amino acid sequences of the T. ferrooxidans (T. f) and E. coli (E. c) (GenBank/EMBL accession no. X03954) \(\gamma\)-glutamylcysteine synthetase proteins.

Identification of CltA and GshA

Analysis of the sequence from the ClaI site to the BamHI 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 \(\gamma\)-glutamylcysteine synthetase gene of E. coli was detected. No clear homology to the other four \(\gamma\)-glutamylcysteine synthetase genes was apparent.

Synthesis of proteins corresponding to the T. ferrooxidans \(\gamma\)-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 pTHI028, but not by the vector pBluescript SK or plasmid pTHI07 (results not shown) (Fig. 5). Cosmid 5.1 and plasmids pTHIOD and pTHI028 produced a protein of approximately 43 kDa, whilst the vectors pBluescript SK and cosmid pHC79 (results not shown) and plasmid pTHI07 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 \(\gamma\)-glutamylcysteine synthetase (60 kDa), an assay for \(\gamma\)-glutamylcysteine synthetase activity was carried out. GSH was shown to react specifically with \(\alpha\)-opthalaldehyde 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 (pTHI07) and the parental strain E. coli MC1061 were compared. Extracts prepared from E. coli BH5262 (pTHI07) 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 \(\gamma\)-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, pTHI06, pTHI028 and pTHI082 enabled the E. coli gltA mutant

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

**Fig. 5.** SDS-PAGE analysis of proteins expressed from cosmid 5.1, plasmids pTHIOD, pTHI028 and pTHI07 and pBluescript SK (lanes 1–5, respectively) using an E. coli-derived in vitro transcription/translation system.
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 y-glutamylcysteine synthetase genes are part of an operon, total RNA isolated from T. ferrooxidans cells was examined by RNA-DNA hybridization. The transcripts were probed with the BamHI-SmaI fragment from pTHIO28 (specific to the gltA gene) and the BamHI-SalI 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 Fe³⁺/Fe²⁺ 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 (Clennell 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 y-glutamylcysteine synthetase. Furthermore, lack of homology between y-glutamylcysteine synthetases is common, as with the exception of human and rat, y-glutamylcysteine synthetases are generally poorly conserved.

Several pieces of evidence support the view that the T. ferrooxidans citrate synthase and y-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 y-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. thalasia, 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.

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