Thiobacillus ferrooxidans Tyrosyl-tRNA Synthetase Functions In Vivo in *Escherichia coli*

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The tyrosyl-tRNA synthetase gene (*tyrZ*) from *Thiobacillus ferrooxidans*, an acidophilic, autotrophic, gram-negative bacterium that participates in bioleaching of minerals, was cloned and sequenced. The encoded polypeptide (TyrRZ) is 407 amino acids in length (molecular mass; 38 kDa). The predicted protein sequence has an extensive overall identity (44%) to the sequence of the protein encoded by the *Bacillus subtilis tyrZ* gene, one of the two genes encoding tyrosyl-tRNA synthetases in this microorganism. Alignment with *Escherichia coli* TyrRS revealed limited overall identity (24%), except in the regions of the signature sequence for class I aminoacyl-tRNA synthetases. Complementation of an *E. coli* strain with a thermosensitive mutation in TyrRS showed that the protein encoded by the *T. ferrooxidans tyrZ* gene is functional and recognizes the *E. coli* tRNA^{Tyr} as a substrate. TyrZ is a single-copy gene as revealed by Southern blot analysis. The gene was localized upstream from the putative promoters of the *rnmT2* ribosomal RNA operon. Although no rho-independent transcription terminator was found between the two genes, a 1.3-kb RNA hybridized to a DNA probe derived from the *tyrZ* gene. The functional relationship between these two transcription units is discussed.

Aminoacyl-tRNA synthetases are key enzymes ensuring the fidelity of protein biosynthesis. They catalyze the esterification of amino acids to the 3'-end of their cognate tRNA species. Despite performing the same overall function, the synthetases have a variety of sizes and subunit compositions (7) and can be divided into two classes on the basis of their structural domains which bind ATP (11).

Bacterial tyrosyl-tRNA synthetases (TyrRS) have been well characterized (for a review, see reference 3). All of them belong to class I aminoacyl-tRNA synthetases (11). The crystal structure of *Bacillus stearothermophilus* TyrRS has been determined at 0.23-nm resolution (5). The results of extensive mutational analysis together with the knowledge of the crystal structure of *B. stearothermophilus* TyrRS led to proposals for the interaction of TyrRS and the substrates tyrosine, ATP, and the intermediate tyrosine adenylate (see reference 3). The interaction between the enzyme and the tRNA^{Tyr} is less well understood. From mutational analysis, a model for the interaction of one molecule of tRNA with two subunits (α_2 dimer) of the enzyme has been proposed (4). Additional information may come from phylogenetic data through knowledge of the TyrRS protein sequences of the enzymes from other organisms. Multiple alignment of the sequences might provide insights into the more-conserved amino acids, which are, therefore, probably the most important for function, since it has been used with other class I synthetases (10, 11, 23).

The sequences of several other TyrRS have been reported, including those from *Escherichia coli* (1), *Bacillus caldotenax* (16), and *Bacillus subtilis* (12, 14). Unexpectedly, in *B. subtilis*, two genes encoding for TyrRS have been found. The encoded proteins were designated TyrRS (14) and TyrRZ (12), analogous to the threonyl-tRNA synthetase system from *B. subtilis* (19) in which the same pattern of two genes was found. TyrRS from *B. subtilis* is equivalent to the TyrRS from *E. coli* and *B.*

stearothermophilus enzymes (55 and 70% identities, respectively, at the amino acid level). The *tyrZ* gene encodes a protein (TyrZ) that is only 27% identical to its partner in *B. subtilis* and 29% identical to the protein from *B. stearothermophilus*. No function has been assigned to the *tyrZ* gene since no expression was detected at the RNA level in growing cells. Also, no altered phenotype was visible when the gene was deleted from the genome (12).

Thiobacillus ferrooxidans is an acidophilic, autotrophic, and chemolithotrophic bacterium that participates in the bioleaching of metals from minerals (9). Because of its industrial importance, interest in the basic aspects of the genetics, biochemistry, and physiology of this organism is increasing. In previous work from our laboratory, we have described the presence of two rRNA operons from this organism (20). A recombinant plasmid carrying the 5'-end of operon *rnmT2* led us to identify a region encoding the transcription promoter and the flanking DNA encoding for an unknown open reading frame (27). In this work, we report that the gene adjacent to the *rnmT2* operon encodes for a novel tyrosyl tRNA synthetase. The predicted amino acid sequence of the gene product is 44% homologous to the predicted product from the *tyrZ* gene of *B. subtilis* (12). A functional analysis of the gene product (TyrRZ) is described. From the analysis of the predicted amino acid sequence, a structure-function relationship of TyrRZ is discussed.

MATERIALS AND METHODS

Bacterial strains and phages. *T. ferrooxidans* Torma was grown in Mackintosh medium (17). *E. coli* C600 or DH5 α was used as a recipient of recombinant plasmids. *E. coli* HB2109 carrying a temperature-sensitive mutation in the *tyrS* gene was used in the complementation assays (4). *E. coli* BL21(DE3), carrying the phage T7 RNA polymerase gene under the control of the *lac* promoter, was used as the host in the

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FIG. 1. Schematic representation of the cloning strategy of the *tyrZ* gene in the expression vector pET22b. The rectangle represents the *tyrZ* coding region cloned in pTR-1. PCR primers are indicated by hyphens below the pTR-1 scheme. Dark lines represent the sequences encoding 16S rRNA. Restriction enzymes recognition sites are represented by the conventional names. Initials (E and B) represent the cohesive sites created after the digestion by the corresponding restriction enzyme.

overexpression of *tyrZ* (25). M13mp18 and -19 were used in DNA sequencing.

Plasmids and DNA manipulations. Plasmid pTR-1 carrying the *tyrZ* gene, isolated from a gene library from *T. ferrooxidans*, was described previously (20). pET22b, containing the phage T7 promoter under the control of the *lac* operator, was used as a vector for the overexpression of the *tyrZ* product fused to the *pelB* leader (25). A schematic representation of the cloning procedure of *tyrZ* in the pET22b is shown in Fig. 1. A 430-bp DNA fragment spanning the amino terminus of the TyrRZ product was amplified by the PCR in a Perkin-Elmer thermocycler with the following temperature program: (i) two cycles of 5 min at 95°C for denaturation, 30 s at 40°C for annealing, and 30 s at 72°C for elongation; and (ii) 28 cycles of 30 s at 95°C for denaturation, 30 s at 60°C for annealing, and 30 s at 72°C for elongation. The sequences of the oligonucleotides used as primers were: (i) 5' GAGAATTCTATGAAGCATCAGG 3' and (ii) 5' GCGCAATCTGGATCAGCTCC 3'. Primer (i) created an *EcoRI* site in the 5' flank of the *tyrZ* gene that allowed in-frame cloning in the pET vector. The amplified 140-bp fragment was digested with *BamHI* and *EcoRI* and purified by agarose gel electrophoresis. This DNA was ligated to the construct made of the 2-kb *BamHI-EcoRI* fragment from pTR-1 ligated to the pET22b vector digested with *EcoRI*. pET81 and pET82 carrying the *tyrZ* gene from *T. ferrooxidans* in the correct and opposite orientations, respectively, relative to the T7 promoter were obtained. The identity of the amplified DNA segment was confirmed by determination of the nucleotide sequence.

Plasmid pGP1-2 is a derivative of pACYC177 carrying the T7 RNA polymerase under the control of the inducible λ *p_L* promoter and the gene for the heat-sensitive *cI857* λ repressor (26). This plasmid was used as a source of T7 polymerase when cotransformed with pET81 or -82 in some complementation experiments (see below).

Chromosomal DNA from *T. ferrooxidans* was prepared by

the method of Marmur (18). All recombinant DNA constructions were carried out by conventional procedures (21). All enzymes were obtained from either New England Biolabs, Boehringer Mannheim, or Bethesda Research Laboratories. DNA sequencing was performed by the dideoxy chain termination method using M13 vectors to generate the single-stranded DNA (19). The Sequenase sequencing kit was supplied by United States Biochemicals Co., and [³⁵S]dATP was from New England Nuclear.

Hybridization analysis. The 0.68-kb *BamHI-PstI* fragment from plasmid pTR-1 was radioactively labeled by random priming and used as a probe in the DNA hybridization procedures. Chromosomal DNA was digested with the appropriate restriction enzyme and resolved by electrophoresis on 1% agarose gels, transferred to nylon membranes (Amersham), and hybridized to the probes by the Southern procedure (24) in a mixture of 50% formamide, 4× SSPE (1× SSPE is 0.15 M NaCl, 0.01 M NaH₂PO₄, and 0.001 M EDTA), 5× Denhardt solution, 0.5% sodium dodecyl sulfate (SDS), and 100 µg of salmon sperm DNA at 42°C or of 4× SSPE, 5× Denhardt solution, 0.5% SDS, and 100 µg of salmon sperm DNA at 30°C. Washes were carried out at 42°C in 2× SSPE-0.1% SDS or at 55°C in 0.1× SSPE-0.1% SDS.

Total RNA from *T. ferrooxidans* was prepared by the procedure described by Hagen and Young (13), resolved by electrophoresis in formaldehyde-agarose gels, transferred to nylon membranes, and hybridized essentially under the same conditions as those in the Southern experiments to the radioactively labeled 0.68-kb *BamHI-PstI* or the 1.2-kb *BamHI-BamHI* fragment from plasmid pTR-1 probes.

Complementation analysis. *E. coli* HB2109 carrying the temperature-sensitive mutation in *tyrS* (4) was transformed by electroporation with recombinant plasmids carrying the *T. ferrooxidans tyrZ* DNA or vectors as a control. Transformants were grown at 30°C on Luria broth (LB) plates in the presence of 50 µg of ampicillin per ml. Complementation assays were carried out by replating the colonies, incubating them at 42°C, and testing them for growth after 16, 24, 36, and 48 h. Plasmid pGP1-2 was cotransformed by electroporation with the pET derivatives, and the selection was carried out at 30°C on LB plates in the presence of 50 µg each of ampicillin and kanamycin per ml. Transformants were tested for complementation at 42 and 37°C in the presence of antibiotics at 16, 24, 36, and 48 h.

In vitro charging activity. (i) **Cell extract preparation.** *E. coli* cell extracts were prepared as follows. Five milliliters of LB-ampicillin medium was inoculated with 1 colony of the cells and grown for 14 h at 30 or 34°C. One milliliter of cells was resuspended in fresh medium and inoculated into 50 ml of medium, and the cells were grown to an optical density at 600 nm of 0.4. Cells were collected by centrifugation for 5 min at 3,000 × *g* in an International refrigerated centrifuge and resuspended in 1 ml of 50 mM Tris-HCl (pH 8.0)-20 mM 2-mercaptoethanol. Disruption of cells was carried out by sonication in an Ultrasonic sonicator with four 10-s pulses at 200 W. *T. ferrooxidans* cell extract was prepared essentially by the same procedure except that cells were grown in Mackintosh medium. Sonication was four 20-s pulses. Cell extracts were spun at 12,000 × *g* for 10 min in an International refrigerated centrifuge.

(ii) **TyrRS activity.** A 0.1- to 5.0-µg amount of protein from the cell extract was incubated for 5 min at 37°C in a buffer containing 50 mM Tris-HCl (pH 8.0), 5 mM MgCl₂, 2.6 mM ATP, 2 mM reduced glutathione, 4.0 mg of total *E. coli* tRNA (Sigma Co.) per ml, and 8 µM [¹⁴C]tyrosine (New England Nuclear; specific activity, 100 mCi/mmol). Reactions were

1 CACGTCGCTCCCTCGCCGAAACCGGATCGCTGGGCACATACACCTGGTATTTTCTCGCTGGTCTGCTCCTCGCCTGGCTGATTATATCTTCGCCGTGATGGTGTGGGCCGGTGC
121 CTGGGGCTGCCGCTGGCATCCGTGCAGACCTGGATTTCCTGGACTGGTATTTTCGGCCTGGCCAAATGTTTTTGGTGGCGGAGCGTGGCCATCTCTGGCGCTCAGTCCCGGTGCG
241 TTCCTGCTCTGGGCGAGCCTCGCGGATATTCTGGTGGTGGGCGGTCTGGCTGCGATGGGTGGTGGTATGCGGCCCTTACCGATGCCGATTATCTGCGGCCCTGCTCTGGCGACGATGGTC
361 TACACCTGATCTCTGACCAGATCAAGGTGCCTTTGTTCGGGCGACTAACCACTGCTGACCGTGACCATGGCGCCAATCCGGTGTGCGGCAAGAGCATTACGGCGAGAGCCCGCCGA
M T M K H Q D A F E Q I A F G T V D M L P E G E M L A
481 TGCTAATATGCAGCCTCATCACAAGACACCGGCCAATTTATGACTATGAAGCATCAGGATGCATTGCAACAAATCGCGTTGGCACCGTCGACATGCTGCCGAAGGCCGAAATGCTGGC
R L A A A Q R D N R P L R I K L G M D P T A P D L H L G A Y V L L H K A R Q F Q
601 CGGCTCGCAGCGGCGAGCGCATACCGCCCTACGGATCAAGCTGGCATGGATCCACCGCCCGGATCTGCACCTTGGGCAATACGTGCTACTCCATAAGGCCCGTCAATTCAG
D L G H R L L F V I G D F T A M I G D P T G K S V T R K A L S R E E V V A N A A
721 GATCTCGGCCATCGCTGCTGTTCTGTTATCGGCGATTTCACGGCATGATCGGCGACCCACGGGCAAGAGCGTTACCCGAAGAGCGTTGAGTTCGCGAAGAGGTGGTGGCCAAATGCCGCG
T Y R P Q V F K I L D P E R T E V M F N S E W L G A L R P E E L I Q I A A C Y T
841 ACGTATCGGCCGAGGTATTCAAGATACTGGACCCGAGCGTACCGAAGTGATGTTCAACTCGGAGTGGTGGCGCCCTGCGTCTGAGGAGCTGATCCAGATTGCCGCGTGTATACC
V A R M L E R D D F N K R Y S A N Q P I A I H E F L Y P L L Q G Y D S V A I K A
961 GTGGCGGCATGCTGGAGCGCGATGACTTCAACAAGCGTTACAGTGCGAATCAGGCCATCGCATTACAGGATTTCTCTATCCTTTGCTACAAGGTTATGACTCGGTGCGTATCAAAGCG
D V E L G G T D Q R F N L L V G R E L Q R E Y G Q K P Q L V L T M P I L E G L D
1081 GACGTGAGCTGGGCGGCACGGACCGCTTCAACCTGCTGGTGGGTGGGAATTACAACGAGAGTACGGCCAGAAACCGCAGTTGGTGCTCACCATGCCATTCTCGAGGGTCTGGAC
G V Q K M S K S L G N F I A V E D P P A E M F G K I M S I S D F L M W R Y Y A L
1201 GGTGTGCAAAAGATGTCCAATCCCTGGGCAATTCATCGCAGTAGAAGACCCGCGCAGAGATGTTCCGCAAGATCATGTCTATCTCGGACTTCTCGATGTGGCGCTTACTACGGCTA
L S R V P A V E Q T R L Q K E A A S G A R N G D P R D I K L D L A G E L V R F H G
1321 CTTTCCGTGTACCTGCGGTGAGGACGACGCGCTGCAGAAAGAGCGGGCAGTGGGCGCGCAATCCGCTGATATCAAGCTGGATCTGGCTGGTGAGTTGGTGGCTCGTTTACGGCG
T A A A Q E A H I A F L A R F Q R H E T P E D L P L Q A I K L S E A P R L S Q L
1441 ACCGCGCGGCTCAGGAAGCGCATATCGCCTTCCTGCACGGTTTCAGCGCCACGAACTCCTGAAGATCTGCCGTGCAAGCCATAAAGCTGTCCGAGGCCCTCGACTCAGTCAGCTA
L V Q V H L A A S T S E A M R K M K E G A V R V D W R R V V D P A T I L A L D A
1561 TTGGTACAGGTACACCTTGTGCCAGTACCAGCGAGGCGATGCGTAAGATGAAGAGGGCGCTGTGCGGGTGCATGGCGAAGGTTGTGCACCTGCGACAATCTGGCTCTGGATGCG
V Y L L Q F F G K R H F A R V A L Q K G E *
1681 GTATATCTGCTGCAATTTGGTAAACGGCACTTCGCCCGCTCGCTCGCAGAAAGCGGAATGACGCTCTAATTGCTGGTTCCATATGGGATGGTGCCACATAACCATGGTGCAGGGGCG
-35 -10
1801 GGAAGTCGAAATTTGGTGTGACACCTTCGGGACCTCCGTATAATCGCGCCTCACGACGCGCGCTGAGCCGAGGACTCCGGTCCGAATTTGTTCTTTCCAGCCGAGTGGAGATGTGT
1921 GGGGTTTAGGCCGAGGGGATATGAAGCGCTGAGCGCTGGATGATCGCCCTGAGCAATATAGTTGAATGGATTGAACCTTAAGAGTTGATCCT.....
16S ----->

FIG. 2. Sequences of the *tyrZ* gene and the predicted protein (TyrRZ) from *T. ferrooxidans*. The 16S rRNA encoding sequence is indicated in bold print. The promoter and possible Shine-Dalgarno elements are underlined and marked as -10 and -35 or SD, respectively. Signatures for tyrosyl tRNA synthetase are underlined in the protein sequence.

stopped by the addition of 5 ml of cold 5% trichloroacetic acid. Samples were collected by filtration on glass fiber filters (Whatman), washed with cold trichloroacetic acid, and dried, and the radioactivity was counted in a Delta 300 (Searle Analytical Inc.) liquid scintillator counter.

Sequence analysis. Sequence analyses were performed with the programs of the PCGENE package. The EMBL data bank accession number of the *tyrZ* gene is X79010.

RESULTS

Sequence analysis of the *tyrZ* gene from *T. ferrooxidans*. Sequencing of the entire segment upstream from the *rrnT₂* promoters from *T. ferrooxidans*, present in plasmid pAEB (27), led us to identify an open reading frame limited by the *Bam*HI site at the end of the cloned fragment. Searching for homologies in the PIR database showed that the predicted protein had homology to TyrRS of bacterial origin. The potential amino terminus of the protein was found subsequently in the adjacent *Bam*HI-*Bam*HI fragment from plasmid pTR-1, previously isolated from a gene library of *T. ferrooxidans* (20). Certain conserved regions of class I aminoacyl-tRNA synthetases (11) are also present in the *T. ferrooxidans* predicted protein. These include the HIGH and KMSK segments that are involved in the nucleotide binding site.

The DNA sequence of a 2,015-bp fragment and the predicted protein sequence are shown in Fig. 2. The DNA sequence overlaps (and corrects) the sequence of the 5' flanking end of the rRNA operon published previously (26).

Putative -35 and -10 promoter elements were found starting at positions 304 and 327, respectively. A possible ribosomal binding site is underlined. A DNA segment about 110 bp in length separates the stop codon and the possible initiation nucleotide(s) of the rRNA transcript. No rho-independent transcription terminator was found in this DNA segment.

The predicted protein is 407 amino acids in length and shows approximately 24% individual identity to TyrRS from *E. coli*, *B. stearotheophilus*, and *B. subtilis*. A multiple alignment of all five TyrRS of bacterial origin shows an overall identity of only 16%, which increases to 20% when functional conservation of amino acid residues (similarity) is considered (Fig. 3). On the other hand, a 44% identity and 77% functional similarity was found by alignment of the predicted protein and the one encoded by the *tyrZ* gene from *B. subtilis* (Fig. 3). For this reason, we named the protein encoded by the *tyrZ* gene from *T. ferrooxidans* TyrRZ. Some of the amino acids that have been shown to be critical for the *B. stearotheophilus* TyrRS activity by mutational and structural analysis were also found either conserved or functionally replaced in the predicted protein (Fig. 3).

Gene organization and expression. Southern blot analysis of the genome from *T. ferrooxidans*, with the 0.68-kb *Bam*HI-*Pst*II fragment of plasmid pTR-1 as the probe, revealed only one hybridization signal (Fig. 4A). No additional signals were detected under less-stringent hybridization conditions. These data strongly suggest the presence of only one *tyrZ* gene in the genome of *T. ferrooxidans*.

Tferr	M-----TMKHQDAFE-QIAF---GTVDMLPEGEMLARLAAAQRDNRLR	40
BsuZ	MRTFEQLTASQQKEVERQLQLYMTGAHEVIPPEELKAKLVKSISTGTPLK	50
BsuS	MT-----NLLEDLSF-RGLIQQMTDEEGLNKQLNEE-----K	31
Bstear	M-----DLAELQW-RGLVNQTTDEEDGLRKLLNEE-----R	30
Ecoli	ASS-----NLIKQLQE-RGLVAQVTDEEALAEERLAQG-----P	32
Tferr	IKL--GMDPTAPDLHLGAYVLLHKARQFQDLGHRLLFVIGDFTAMIGDPT	88
BsuZ	IKL--GLDPSAPDVHLGHTVVLNKLRFQENGHIVQLLIGDFTGKIGDPT	98
BsuS	IRLYSGFDPTADSLHIGHLLPILTLRRFQLAGHHPIALVGGATGLIGDPS	81
Bstear	VTLYCGFDPTADSLHIGHLATILTMRRFQQAGHRPIALVGGATGLIGDPS	80
Ecoli	IALLYCGFDPTADSLHLGHLVPLLCLKRFQQAGHKPVALVGGATGLIGDPS	82
Tferr	GKSVTRKALSREEVVANAATYRPQVFKILD-----PERTEVMFNSEWLG	132
BsuZ	GKSAARKQLTDEEVQHNAKTYFEQFGKVL-----PEKVELHYNKWLK	142
BsuS	GKKAERTLNTADIVSEWSQKIKNQLSRFLDFEAAENPAVIANNFD--WIG	129
Bstear	GKKSERTLNAKETVEAWSARIKEQLGRFLDFEADGNPAKIKNNYD--WIG	128
Ecoli	FKAAERKLNTETVQEWVDKIRKQVAPFLDFDCGENSAIAANNYD--WFG	130
Tferr	ALRPEELI-QIAACYTVARMLERDDFNKRYSANQP-IAIHEFLYPLLQGY	180
BsuZ	TLNLEDVI-ELAGKITVARLMERDDFEERIAMQKP-ISLHEFFYPLMQGY	190
BsuS	KMNVIDFLRDVGKNFGINYLAKDVTSSRI---ESGISYTEFSYMLQSY	176
Bstear	PLDVITFLRDVGKHFVSNYMMAKESVQSRI---ETGISYTEFSYMLQAY	175
Ecoli	NMNVLTFLRDIGKHFVSNQNMKEAVKQRLNREDQGISYTEFSYNLQGY	180
Tferr	DSVAIKAD----VELGGTDQRFNLLVGRELQREYQGK--POLVLTMPILE	224
BsuZ	DSVVLESD----IELGGTDQHFNVLMGRHFQERYNKE--KQVVILMPLE	234
BsuS	DFLNLYRDKNCKLQIGGSDQWGNITAGLELIRKSEEEGAKAFGLTIPLVT	226
Bstear	DFLRLYETEGCRLQIGGSDQWGNITAGLELIRKTKGE-ARAFGLTIPLVT	224
Ecoli	DFACLNKQYGVVLQIGGSDQWGNITSGIDLTRRLHQN--QVFGLTVPLIT	228
Tferr	GLDGVQKMSKSLGNFIAVEDP---PAEMFGKIMSISDFLMWRYALLSRV	271
BsuZ	GLDGVKMSKSKHNYIGINEH---PNDMYGKTMSLPDSLMKKYIHLATDL	281
BsuS	KADGT-KFGKTEGGAIWLDKEKTSPEYFYQFWINTDDRDVVKYLKYFTFL	275
Bstear	KADGT-KFGKTESGTIWLDEKTSPEYFYQFWINTDDRDVIRYLKYFTFL	273
Ecoli	KADGT-KFGKTEGGAVWLDPKKTSPEYFYQFWINTADADVIRFLKFFTFM	277
Tferr	PAVEQTRLQKEAASGARNPRDIKLDLAGELVRRFHGTARAQEAHIAFLAR	321
BsuZ	ELEKKQLVKDLETGAVHPRDAKMLLARTIVRMHGEKAAEAAEHSFKTV	331
BsuS	SKEEIEAYA EK-TEAPEK-REAQKRLAEVTSLVHGREALAQAINISQAL	323
Bstear	SKEEIEALEQELREAPEK-RAAQKTAAEVTKLVHGEEALRQAIRISEAL	322
Ecoli	SIEEINALEEEDKNSGKA-PRAQYVLAEQVTRLVHGEEGLQAAKRITECL	326
Tferr	FQ-----RHETPEDLPQAIKLSEAPRLSOLLVQVHLAASTSEA	360
BsuZ	FQ-----ENSLPEDIPAVNWKGEKTIAMIDLLVKKLLSSKSEA	370
BsuS	FSGNIKELSAQDVKVGFKD-VPSMEVDSTQELSLVDVLVQSKLSPSKRQA	372
Bstear	FSGDIANLTAAEIEQGFKD-VPSF-VHEGGDVPLVELLVSAGISPSKRQA	370
Ecoli	FSGSLSALSADFEQLAQDGVPMVEMEKAD--LMQALVDSQLQPSRGQA	374
Tferr	MRMKKEGAVRVWDVRRVDPATILALDAVY-----LLQFGKRHFARVALQK	405
BsuZ	RRMIQNGGVRIDGEKVTDVHAKAEIRENM-----IIQVGKRKFLKL---	411
BsuS	REDIQNGAVYINGERQTEINYTLSGEDRIENQFTVLRRGKKKYF---LVT	419
Bstear	REDIQNGAIYVNGERLQDVGAILTAEHRLEGRFTVIRRGKKKYF---LIR	417
Ecoli	RKTIASNAITINGEKQSDPEYFFKEEDRLFGRTLLRRGKKNYC---LIC	421

FIG. 3. Multiple alignment of the amino acid sequences from bacterial tyrosyl tRNA synthetases. The alignment was carried out by using the Clustal program from a PCGene package and improved by hand. Asterisks represent identical amino acids in all five sequences, and dots represent conserved amino acids. Abbreviations: Tferr, *T. ferrooxidans*, BsuZ and BsuS, TyrRZ and TyrRS, respectively, from *B. subtilis*; Bstear, *B. stearothermophilus*.

The potential expression of the *tyrZ* gene was investigated by Northern (RNA) blot analysis using the 0.68-kb fragment from pTR-1. A 1.3- to 1.4-kb RNA hybridized to the probe (Fig. 4B). The same results were obtained when the 1.2-kb *Bam*HI-*Bam*HI fragment from pTR-1 was used as the probe (data not shown). The transcription initiation site was investigated by primer extension analysis. Preliminary data, from analysis using the oligonucleotide 5' CAGGCACTGGTTAGTCGC CGC 3' as primer, revealed that a possible transcription starting point is localized at nucleotide 156 (Fig. 1) of the

reported sequence (data not shown). It is consistent with the predicted size of the transcript, assuming that transcription termination takes place within the 110 nucleotides separating *tyrZ* and the rRNA operon. No consensus sequences for bacterial promoters were found in this region. There was no evidence for the functionality of the possible promoter found in positions 304 and 327.

Complementation of a *tyrS* mutation in *E. coli*. To test whether the cloned *T. ferrooxidans* fragment encoded a TyrRZ protein that could function in *E. coli*, complementation of the

FIG. 4. Autoradiogram of the Southern and Northern hybridization experiments. (A) Plasmid pTR-1 (lane 1) and chromosomal DNA from *T. ferrooxidans* (lanes 2 and 3) were digested with *Bam*HI-*Pst*II (lanes 1 and 2) or *Eco*RI (lane 3) and transferred to nylon membranes. (B) Total RNA from *T. ferrooxidans* (lane 1) or *E. coli* (lane 2) was electrophoresed under denaturing conditions on agarose gels and transferred to nylon membranes. Filters from A and B were hybridized to the radioactive labeled 0.68-kb *Bam*HI-*Pst*II fragment from plasmid pTR-1. Numbers on the right side of each blot represent λ *Hind* DNA size markers (A) or rRNA markers (B).

thermosensitive mutation in *tyrS* in *E. coli* HB2109 (4) was carried out. The mutant strain was transformed with plasmid pTR-1, and visible colonies were observed after 24 h of incubation at 42°C (cells transformed with the *tyrS* wild-type gene grew at 14 h of incubation). Plasmid pBR322, used as a control, did not permit the cells to grow at the nonpermissive temperature (Table 1). This result indicates that the fragment cloned in pTR-1 encodes a TyrRZ protein that functions in *E. coli*.

To confirm that the *tyrZ* gene product is a TyrRZ and to develop a system for further biochemical analysis of the enzyme, we fused the coding region of *tyrZ* to the pET22b vector. This vector carries the T7 polymerase promoter under the control of the *lac* repressor, making the expression of the foreign gene inducible by galactoside analogs such as IPTG

(isopropyl- β -D-thiogalactopyranoside), and the *pelB* leader region that allows the expressed product to be exported to the periplasmic compartment. An *Eco*RI site was created adjacent to the predicted amino terminus of TyrRZ by PCR to allow the in-frame fusion of *tyrZ* to the *pelB* leader coding region (see Materials and Methods). The new recombinant plasmid was termed pET81. A recombinant plasmid carrying the *tyrZ* gene in the opposite orientation relative to the T7 promoter was named pET82. *E. coli* HB2109 was transformed with the recombinant plasmids, and the transformants were tested for the ability to grow at the nonpermissive temperature (42°C). Growth was observed in both cases (Table 1). No growth was observed at the nonpermissive temperature when cells were transformed with the pET22b vector. Therefore, the construct containing the *tyrZ* gene encodes a functional TyrRZ protein that is weakly expressed from either the T7 promoter or σ^{70} promoters present in the vector. To test the possibility that the complementation with the constructs is the result of the expression of *tyrZ* from the T7 promoter, the following procedure was carried out. Plasmid pET81 or pET82 was cotransformed with pGP1-2, a compatible plasmid that carries the T7 RNA polymerase gene under the control of the *cI*(Ts) repressor that is inactive at 42°C, into the *tyrS*(Ts) *E. coli* mutant strain HB2109. Transformed cells were selected for their ability to grow at 30°C in LB in the presence of ampicillin and kanamycin. Transformed cells were transferred and tested for their ability to grow at 42 and 37°C. Growth was observed after 24 (pET81) and 36 (pET82) h of incubation at 37°C but not at 42°C (Table 1). No growth was observed at either 37 or 42°C when the pET81 or pET82 was replaced by the pET22b vector.

Overexpression of TyrRZ from *T. ferrooxidans* in *E. coli*. Plasmid pET81 was transformed into *E. coli* BL21(DE3) carrying the T7 RNA polymerase gene in a lysogen phage under the control of the *lac* repressor. Cells were grown at 37°C and induced by 1 mM IPTG. After 3 h of induction, cells were collected and proteins were analyzed by SDS-polyacrylamide gel electrophoresis. Under these conditions, more than 60% of the total protein present in the cell was derived from the *tyrZ* expressed gene. A gene product of about 35 kDa, close to the 38 kDa predicted from the protein sequence, was observed. Under these induction conditions, almost all proteins, including the endogenous cellular proteins, were present in the insoluble fraction. When the cells were grown and induced at 29°C, and a time course of induction was followed, a small amount of the expressed protein was observed in the soluble fraction after 30 to 60 min of induction (Fig. 5).

In vitro charging activity. To test the in vitro amino acylation of *E. coli* tRNA^{Tyr} by the *T. ferrooxidans* TyrRZ, three

TABLE 1. Complementation of *E. coli* HB2109 with *T. ferrooxidans* *tyrZ*

Plasmid	Antibiotic ^a	Characteristic	Growth at temp of	
			37°C	42°C
pBR322	AMP		++	—
pTR-1	AMP	<i>T. ferrooxidans</i> <i>tyrZ</i> in pBR322	ND	—
ptyr	AMP	<i>E. coli</i> <i>tyrS</i>	ND	+++
pET22b	AMP		ND	—
pET81	AMP	<i>T. ferrooxidans</i> <i>tyrZ</i>	ND	++
pET82	AMP	<i>T. ferrooxidans</i> <i>tyrZ</i> opposite to T7 promoter	ND	+
pET22b/pGP1-2	KAN + AMP		—	—
pET81/pGP1-2	KAN + AMP	<i>T. ferrooxidans</i> <i>tyrZ</i> /T7 polymerase	+++	—
pET82/pGP1-2	KAN + AMP	<i>T. ferrooxidans</i> <i>tyrZ</i> opposite to T7 promoter/T7 polymerase	++	—

^a AMP, ampicillin; KAN, kanamycin.

^b Symbols: +++, growth in 14 to 16 h; ++, growth in 24 h; +, growth in 36 h; —, no growth. ND, not determined.

FIG. 5. Time course of expression of TyrRZ upon IPTG induction. SDS-polyacrylamide gel electrophoresis of total (A), supernatant (B), and pellet (C) proteins from *E. coli* BL21 transformed with pET81 induced by 1 mM IPTG. Lanes (time after induction): 1, 0 min; 2, 30 min; 3, 60 min; 4, 90 min; 5, 120 min; 6, 180 min.

sources of enzyme were used: (i) a whole extract from *T. ferrooxidans*, (ii) an extract from *E. coli* HB2109 transformed with pTR-1, and (iii) an extract from *E. coli* BL21 transformed with pET81. Total tRNA from *E. coli*, used as the substrate, was amino acylated by the *T. ferrooxidans* and the *E. coli* HB2109 extracts (Table 2), indicating that the TyrRZ present in these enzyme preparations recognizes the *E. coli* tRNA as a substrate. No activity above the endogenous activity was observed in *E. coli* BL21 transformed with pET81 (data not shown). This result might indicate that most of the protein expressed from pET81 is in the insoluble form (Fig. 5) and that the fraction capable of complementing the mutation in *E. coli* HB2109 (Table 1) is not detectable in the in vitro assay.

DISCUSSION

This study describes the isolation and characterization of the gene encoding a TyrRZ from the acidophilic bacterium *T. ferrooxidans*. The predicted sequence of the encoded protein showed considerable degrees of identity and similarity (44 and 77%, respectively) to the gene product of the *tyrZ* gene from *B. subtilis*, one of the two genes encoding for TyrRS in this microorganism (12). On the other hand, alignments to the TyrRZ protein sequences to the other described bacterial TyrRS showed limited individual identity (not more than 25% of amino acids are conserved). The overall identity, measured by a multiple alignment among the five sequences of TyrRS (or

TyrRZ) of bacterial origin, showed that about 16% of the amino acids are conserved.

Although the primary structure of the TyrRS and TyrRZ proteins differ, all them recognize *E. coli* tRNA as a substrate, as evidenced by either in vivo complementation of *E. coli* mutations in the *tyrS* gene or in vitro charging assays (see also reference 3). The multiple-alignment analysis confirmed previous observations that the majority of conserved residues are localized in the amino terminus of the proteins, the segment involved in the ATP and amino acid binding. Very little homology was observed at the carboxy-terminal portion of the proteins, the tRNA-binding segment. It is remarkable that several of the amino acids that have been shown to be involved in the substrate recognition and/or binding, evidenced by either structural or mutational analysis of the *B. stearrowthermophilus* or *E. coli* enzymes (see reference 3), are conserved among the TyrRS and TyrRZ proteins (Fig. 3). On the other hand, some other amino acids also shown to be important in the ATP binding are changed or missing in the TyrRZ family. This is the case with the tyrosine 34 and cysteine 35 from *B. stearrowthermophilus* TyrRS which are absent in the TyrRZ enzymes. Additionally, histidine 48 is replaced by an alanine in the *T. ferrooxidans* enzyme. Therefore, because of the polymorphism among the different synthetases, the multiple alignment provides an adequate tool in the search of the residues that are conserved. This methodology has been used in the analysis of other class I aminoacyl-tRNA synthetases (23). It provides an especially important tool for the analysis of the amino acids involved in the tRNA recognition by the TyrRS since the carboxy terminus of TyrRS from *B. stearrowthermophilus* shows a disordered crystal structure and, therefore, is not informative about its interaction with tRNA (5; see also reference 3).

Aminoacyl-tRNA synthetases are encoded by single-copy genes in the genome of *E. coli*, with the exception of *lysS* and *lysU*, which encode for two different lysyl-tRNA synthetases (8, 15). Two genes have been described for the threonyl (*thrS* and *thrZ*) (19) and tyrosyl (*tyrS* and *tyrZ*) (12, 14) tRNA synthetases from *B. subtilis*. Although both the S and Z forms of each gene potentially encode for a functional synthetase, only the S form is expressed under laboratory conditions. Furthermore, a deletion of the *tyrZ* gene shows no detectable phenotype. The *tyrZ* gene from *T. ferrooxidans* also encodes a functional gene product as is evidenced by the complementation assays. Our data show that the *T. ferrooxidans* genome contains a single copy of the *tyrZ* gene and that it is probably transcribed as a single unit. Further work will be conducted to determine the possible existence of an additional TyrRS gene as well as whether *tyrZ* is expressed in a protein product in this organism.

Our previous work revealed the presence of two rRNA operons in the genome of *T. ferrooxidans* (20). Several rRNA operons of bacterial origin have been described in detail. Open reading frames adjacent to the promoter sequences of other rRNA operons have been found (2, 6). To our knowledge, the *tyrZ* gene is the first case in which the function of such an open reading frame is described. In addition to this feature, it is interesting to note that there is no apparent *E. coli* type rho-independent transcription termination signal in the 110-nucleotide DNA sequence connecting the two genes. Additional information is required to identify the presumed termination signal in this region of the *T. ferrooxidans* genome. It also raises the possibility that under certain physiological conditions, transcription might not terminate in this region and the synthetase and rRNA genes might behave as a single transcription unit.

TABLE 2. In vitro activity of *T. ferrooxidans* TyrRZ

TyrRZ source	Plasmid (culture condition) ^a	Sp act (U/mg of protein) ^b
<i>E. coli</i> HB2109	pBR322 (34°C)	111
	pBR322 (42°C)	15
	pTR-1 (34°C)	1,423
	pTR-1 (42°C)	501
<i>T. ferrooxidans</i>		496

^a *E. coli* HB2109 harboring the indicated plasmid was cultured at 34°C to an optical density of 0.4 and then transferred to the indicated temperature for 2.5 h. After the cells were chilled, the extract was prepared as described in Materials and Methods.

^b One unit is defined as the amount of enzyme that amino acylates 1 pmol of tRNA in 5 min at 37°C.

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