Evidence for Two Sets of Structural Genes Coding for Ribulose Biphosphate Carboxylase in *Thiobacillus ferrooxidans*

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Previously, we reported the cloning of the ribulose-1,5-bisphosphate carboxylase genes (*rbcL1-rbcS1*) of *Thiobacillus ferrooxidans* Fe1 (T. Kusano, K. Sugawara, C. Inoue, and N. Suzuki, Curr. Microbiol. 22:35–41, 1991). With these genes as probes, a second set of ribulose-1,5-bisphosphate carboxylase genes (*rbcL2-rbcS2*) was identified in the same strain and cloned. *rbcL1* and *rbcL2* encode the large subunits, and *rbcS1* and *rbcS2* encode the small subunits. Similar restriction patterns between these gene sets suggested a high level of sequence homology. In fact, sequence analysis showed that a 2.2-kb region, including the entire large and small subunit structural genes, was totally conserved in *rbcL1-rbcS1* and *rbcL2-rbcS2*. The *rbcL1* (*rbcL2*) and *rbcS1* (*rbcS2*) genes were 1,422 and 333 bp in length and encoded 473- and 110-amino-acid proteins, respectively. The genes were separated by a 90-bp spacer sequence and were preceded by possible ribosome-binding sites. The N-terminal amino acid sequences of the subunit proteins, synthesized in *Escherichia coli*, were determined by Edman degradation and found to agree with the deduced amino acid sequences, except for the N-terminal methionine residue. The transcriptional start site of the *rbc* genes was determined by primer extension, and the size of the *rbc* transcript was estimated to be about 2.1 kb, suggestive of the cotranscription of *rbcL1-rbcS1* and/or *rbcL2-rbcS2* mRNAs. Comparisons of amino acid sequences of both subunits with those of other organisms revealed that the ribulose-1,5-bisphosphate carboxylase of *T. ferrooxidans*, a chemoautotrophic bacterium, is phylogenetically closer to the photosynthetic bacterium *Chromatium vinosum* than to another chemoautotrophic bacterium, *Alcaligenes eutrophus*.

Thiobacillus ferrooxidans, an iron-oxidizing bacterium, is an obligate chemolithotroph. The organism fixes atmospheric carbon dioxide to build up its cellular carbon skeleton by using energy derived from oxidation of ferrous ions to ferric ions or inorganic sulfur to sulfuric acid. The initial step of this carbon assimilation process is catalyzed by the enzyme D-ribulose-1,5-bisphosphate carboxylase (RuBPCase) (23). RuBPCase has been isolated from a wide range of taxa including higher plants, algae, and photo- and chemoautotrophic bacteria. In general, the enzyme takes a hexadecameric form, with eight large subunits of M_r 50×10^3 to 55×10^3 and eight small subunits of M_r 12×10^3 to 18×10^3 (36, 53), as also is found in the case of *T. ferrooxidans* (20, 28). The apparent K_m values (80 μ M for ribulose biphosphate and 28 μ M for CO_2) of the enzyme prepared from *T. ferrooxidans* Torma are similar to those of plants and green algae but four- to fivefold lower than those of most other bacteria (20).

Genes coding for both the large and the small subunits of RuBPCase have also been cloned and sequenced from diverse species, including the cyanobacteria *Anacystis nidulans* (48, 49) and *Anabaena* sp. strain 7120 (8, 38), the eukaryotic alga *Chlamydomonas reinhardtii* (9, 12), and tobacco (34, 47). The genes encoding the form II enzyme, present in *Rhodospirillum rubrum* and *Rhodobacter sphaeroides*, have also been sequenced (37, 58). Comparative studies of amino acid sequences have revealed that, although the large subunit is highly conserved, the small subunit has undergone significant divergence. In addition to such comparative analyses, site-directed mutagenesis and chemical modification methods have identified the functional

essentiality of certain amino acid residues of the RuBPCase gene products (3, 17, 18, 42).

In prokaryotes, it is known that the genes encoding the large and small subunits of RuBPCase are very closely linked and that both genes are cotranscribed (38, 48), differing from the case in higher plants.

The presence of two sets of RuBPCase genes has been reported in certain organisms; for example, in *R. sphaeroides* (11, 53, 58), *Alcaligenes eutrophus* (2, 21, 26), *Chromatium vinosum* (27, 56, 57), and *Nitrobacter hamburgensis* (15). In *A. eutrophus*, one set is located chromosomally and the other is on the megaplasmid (2, 26).

Previously, we reported the cloning of one set of the entire large and small subunit genes (*rbcL1-rbcS1*) of *T. ferrooxidans* Fe1 and on the expression of the genes when under the control of a *tac* promoter in *Escherichia coli* (28). We now report the cloning of a second set (*rbcL2-rbcS2*) of RuBPCase genes from the same strain of *T. ferrooxidans*, and we present the nucleotide sequence of the two sets and their deduced amino acid sequences. *T. ferrooxidans* RuBPCase genes are more closely related to those from a γ -purple photosynthetic bacterium, *C. vinosum*, than to a chemolithotrophic bacterium, *A. eutrophus*. Evidence is also presented that the sequences over a 2.2-kb region covering the *rbcL1-rbcS1* and *rbcL2-rbcS2* genes are completely identical and that the large and small subunit genes are cotranscribed as in other prokaryotes.

MATERIALS AND METHODS

Bacterial strains and plasmids. Strains DH5 α (24) and JM101 (61) of *E. coli* were grown in Luria broth or Luria agar (35) and used as transformation hosts. Competent *E. coli* cells were prepared by the method of Hanahan (14). *T.*

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ferrooxidans Fe1 cells, kindly provided by H. Shiota (Iwate University, Japan), were grown in inorganic 9K medium (51) and used as a source of chromosomal DNA and total RNA (59). The multicopy plasmids pUC18 and pUC19 were used as cloning vectors (40). The region from pTR11 (28), covering the *T. ferrooxidans rbcL1-rbcS1* genes, was placed under the control of the *tac* promoter in pKK223-3 (Pharmacia), resulting in pKSS14 (28).

DNA manipulations. Plasmids were isolated from *E. coli* as described by Birnboim and Doly (5). Chromosomal DNA of *T. ferrooxidans* Fe1 was prepared as described previously (50). Restriction endonuclease digests were performed as recommended by the suppliers. Restriction mapping and subcloning were carried out with standard methods (43).

Colony or Southern blot hybridization. Preparation, hybridization, and washing of blots were performed as described previously (43). Nylon membranes, Biodyne A (Pall Co. Ltd.) for colony blots and Hybond N (Amersham) for Southern blots, were used in all experiments.

DNA sequencing. DNA sequencing was conducted on both strands with denatured plasmid templates (19) by the dideoxy-chain termination method of Sanger et al. (44). To cover a 3- to 4-kb fragment, internal fragments of pTR11 and pTR21 were subcloned into pUC18, and a series of overlapping deletion plasmids were created by using exonuclease III (61). Computer-assisted sequence analysis was carried out with a SDC-GENETYX genetic information processing program (Software Development Co., Ltd., Tokyo, Japan).

Northern hybridization. *T. ferrooxidans* total RNA prepared by the procedure of Chatteraj et al. (7) was separated in a 1.5% agarose-formaldehyde gel and transferred to a nylon membrane (Hybond N). Blots were hybridized at 42°C in 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaPO₄, and 1 mM EDTA [pH 7.7])–5× Denhardt solution–100 µg of salmon sperm DNA per ml–50% formamide–0.5% sodium dodecyl sulfate (SDS) and washed first in 2× SSPE–0.1% SDS at 42°C for 15 min and then in 1× SSPE–0.1% SDS at 42°C for 30 min.

Oligonucleotide synthesis. A 30-mer primer (5'-TACACC GGCCTCATACGTTTTTACGGCCAT-3'), complementary to the sequence from nucleotides +30 to +1 in Fig. 3, was synthesized by using a Cyclone DNA synthesizer (Milligen/Bioscience) and purified on a 15% polyacrylamide gel containing 7 M urea.

N-terminal amino acid sequence determination. N-terminal amino acid sequences were determined by using automated Edman degradation on an Applied Biosystems 477A sequencer equipped with an on-line 120A phenylthiohydantoin analyzer. Samples to be sequenced were run on SDS–15% polyacrylamide gels with the buffer system of Laemmli (30), electroblotted onto a polyvinylidene difluoride membrane (Immobilon; Millipore Corp.), and stained with Coomassie dye R-250. The bands corresponding to the small or large subunits were excised for direct analysis.

Chemicals and enzymes. Restriction enzymes, DNA-modifying enzymes, 7-deaza sequence kits, and kilo-sequence deletion kits were purchased from Takara Shuzo Co., Ltd., (Kyoto, Japan). RNase A from Sigma and RNase-free DNase I from Boehringer Mannheim were used. The reagents required for oligonucleotide synthesis and amino acid sequencing were purchased from Milligen/Bioscience (San Rafael, Calif.) and Applied Biosystems Inc. (Foster City, Calif.), respectively. [α -³²P]dCTP (3,000 or 400 Ci/mmol) and [γ -³²P]ATP (3,000 Ci/mmol) were purchased from Amersham Corp. (Arlington Heights, Ill.) and New England Nuclear Corp. (Boston, Mass.), respectively. All other

FIG. 1. Southern blot analysis of *T. ferrooxidans* chromosomal DNA with its own RuBPCase genes as probes. Total DNA from the organism was digested with *Pst*I, *Sal*I, *Eco*RV, or *Bam*HI, as indicated above the lanes. After agarose gel electrophoresis, blots were made by the procedure of Reed and Mann (41). The left blot (A) was probed with the 2.6-kb *Pst*I-*Eco*RI fragment (Fig. 2) of the 4.0-kb insert DNA of pTR11 (28), whereas the right blot (B) was hybridized with the residual 1.4-kb *Eco*RI-*Pst*I fragment (Fig. 2). The former fragment contained the 5'-flanking region and most of the *rbcL* regions, and the latter contained a 41-bp 3' region of *rbcL*, the intergenic sequence, and the entire *rbcS* and 3'-flanking regions. The lowest band indicated by an arrowhead (in the *Pst*I lane in panel A) is an artifact caused by an overflow from an adjacent positive control lane. The positions of DNA size markers (lambda phage DNA digested with *Hind*III, in kilobases) are indicated.

chemicals, of analytical grade, were obtained from Wako Chemicals (Osaka, Japan).

Nucleotide sequence accession number. The nucleotide sequence shown in Fig. 3 has been submitted to GenBank (accession number D90113).

RESULTS

Presence of two sets of *rbc* genes in *T. ferrooxidans*. We previously reported the cloning of the *rbc* genes from *T. ferrooxidans* Fe1 in *E. coli* and demonstrated their expression when under the control of a *tac* promoter (28). The recombinant plasmid thus obtained, pTR11, contained a 4.0-kb *Pst*I fragment covering the whole region of the *rbc* genes. Since the presence of two sets of *rbc* genes has been reported in several other organisms, we examined this possibility in *T. ferrooxidans*. After repeated single colony isolation onto 9K (51)-containing colloidal silica plates to avoid cross-contamination with other organisms, chromosomal DNA from a single *T. ferrooxidans* clone was prepared and Southern blot analysis was performed. A 9.3-kb *Pst*I fragment, in addition to the 4.0-kb fragment mentioned above, hybridized with a 2.8-kb *Pst*I-*Eco*RI (*rbcL1*-specific) fragment and also with a 1.2-kb *Eco*RI-*Pst*I (*rbcS1*-specific) fragment (Fig. 1, lanes 1; Fig. 2). In other digests, the number of positive signals could not be explained by a single set of *rbc* genes (Fig. 1, lanes 2 through 4). Further restriction enzyme analyses revealed that the 9.3-kb *Pst*I fragment had a single *Bam*HI site and that the smaller 3.8-kb *Bam*HI-*Pst*I fragment hybridized with both *rbcL1*- and

FIG. 2. Detailed physical maps of the two sets of RuBPCase genes from *T. ferrooxidans* Fe1 and their sequencing strategy. The top solid line represents the 4.0-kb *Pst*I fragment, redrawn from reference 28. The fragments used as hybridization probes in Fig. 1 are indicated. The lower solid line represents the 9.4-kb *Pst*I fragment; the 5.6-kb *Pst*I-*Bam*HI region (dotted line) has been omitted. The lower 9.4-kb fragment was aligned in reference to the similarity in restriction pattern of the top 4.0-kb fragment. The direction and extent of sequencing determinations are indicated by arrows. The positions of *rbc* open reading frames for *rbcL1* (*rbcL2*) and *rbcS1* (*rbcS2*) are indicated with thick bars.

rbcS1-specific probes (data not shown). Subsequently, an attempt was made to clone this 3.8-kb *Bam*HI-*Pst*I fragment. The *Pst*I-digested *T. ferrooxidans* Fe1 chromosomal DNA was fractionated on a preparative 0.7% agarose gel; fragments ranging from 7 to 10 kb were recovered by electroelution, further digested with *Bam*HI, and then ligated with *Bam*HI-*Pst*I-cleaved pUC18. After transformation and colony hybridization of over 2,000 transformants with the *rbcL1*-specific probe, three positive clones were obtained. Because the plasmids residing in these colonies were of identical size and each contained the 3.8-kb *Bam*HI-*Pst*I fragment, they were subsequently referred to as pTR21. Interestingly, *E. coli* carrying pTR21 was unable to grow on LB (35) plates in the absence of glucose (0.1%), and this may partly explain why we were unable to obtain the 9.3-kb *Pst*I clone in our initial experiment. The 3.8-kb *Bam*HI-*Pst*I insert of pTR21 was recloned into *Bam*HI-*Pst*I-cleaved pUC19, yielding pTR22. The *lacZ* promoter was in the forward orientation, toward the *rbc* genes, in pTR21 but in the reverse orientation in pTR22. Since only pTR21 conferred ribulose-1,5-bisphosphate-dependent carbon dioxide-fixing activity to *E. coli* cells (data not shown), it appeared that the *T. ferrooxidans rbc* promoter did not function in *E. coli*. The RuBPCase produced in *E. coli* JM101(pTR21) may also have had a multimeric (possibly hexadecameric) form, as in *E. coli* JM101(pKSS14) (see Materials and Methods), since both their activities were detected in the same fractions of sucrose density gradient centrifugation.

The *rbc* genes in pTR21, referred to as *rbcL2* and *rbcS2*, encode the large and small subunits of RuBPCase, respectively. The insert DNA of pTR21 was mapped with several restriction enzymes and aligned with pTR11 (Fig. 2). The restriction patterns of both inserts were surprisingly similar except for the extreme 5' region, suggesting a high degree of nucleotide sequence homology.

Localization of *rbc* genes in *T. ferrooxidans*. To determine the location of the *rbc* genes in this bacterium, the plasmid fraction was prepared by either the alkaline SDS method (5) or by the method of Kado and Liu (25) for possibly large plasmids. These preparations, containing at least one visible plasmid (ca. 30 kb in size), were run on a 0.5% agarose gel, depurinated, and then transferred to a nylon filter. After hybridization with the 2.8-kb *rbcL1*-specific probe (Fig. 2), only a signal corresponding to the residual chromosomal band was observed (data not shown). Therefore, both sets of *rbc* genes were considered to be localized on the chromosomal DNA.

Nucleotide sequences of *rbcL1-rbcS1* and *rbcL2-rbcS2*. The 2,223-bp nucleotide sequence of pTR11, covering *rbcL1-rbcS1*, is presented in Fig. 3A. The *rbcL1* and the *rbcS1* genes were 1,422 and 333 bp in length, respectively, were separated by a 90-bp intergenic sequence, and were preceded by possible ribosome-binding sites (Fig. 3A). The *rbcL1* gene encodes a deduced protein of 473 amino acid residues with an estimated molecular mass of 52,500, which

A -180
T T T A G T T G T G C A A T G T A G G T G G G G A T A G A C A T G T C A A T A G T A A C C A T G T G A A T C A T A A T A G T A A A T A T A T T T A C T T T T A T A A A

-90
T T A G A G A T C A T C C A C A A T C C A A T C A A G C A T T G A A C C A A T C A G T G C T T C A A T G T G T T G T C T A C C A T G A C A C A A G G A G C C A A A C

1
A T G C C G T A A A A C G T A T G A G G C C G G T G T A A G G A C T A C C C C A G A C T A T T G C C C C C C G A G T A C G T C C T T T G A T T C C G A T A T C T A
N A V K T Y E A G V K D Y R Q T Y W A P E Y V P L D S D I L

90
G C T G T T C A A G A T T A C C C C A A C C A G G G T G G A T G T G A G A A C C C C C C C G T A C C C C C G A G T C T T C A C C C C A C A T G A C
A C F K I T P Q P G V D R E E A A A A V A A E S S T G T W T

180
A C G T G T G A C C G A T T G T T G A C C A C A T G A T T A C T A C A A G C C C C C C C T A T C C A T T G A A G A T G T C C C C C C C G A G A C C T G C T T T
T V W T D L L T D N D Y Y K G R A Y R I E D V P G D D T C F

270
T A C C C G T G G T G C C T A T C C C A T A G A T T G T T T G A G A G G G T C C G T G T T A C G T C T T C A C C T C C T G T G C G T A M C G T G T C C G C T T C
Y A F V A Y P I D L F E E G S V V N V F T S L V G N V F G F

360
A A C C C C T G C G T G C C T G C C C T G G A G A T G T G C G A T T C C C C T C C C C T A T G T C A A G A C T T C C A A T G A C C C C C C C A C G A T T C C A G T C
K A V R A L R L E D V R F P L A Y V K T C H G P P H G I Q V

450
G A C C C C A A A A T G A C A A T A T G C C C C C C A T C C C C T G C C A C C A C C A A C T C G T C T A T C C C C A A G A A C T A T G T G T
E R D K N N K Y G R P N L G C T I K P K L G L S A K N Y G R

540
G C A G T C A C A G T G T C T C C C C G T G C C T G A T T C A C A A G A C A T G A G A M C T C A C T G C A C C C T C A T C C C G T G C C C A T C C G
A V Y E C L R G G L D F T K D D E N V N S Q P F I A V R D R

630
T T T C T G T G T G C C G A T G C G A T C A C C C C G A G C T G A A C C C C G A G C C A A G G A C A C T A T T T A A C G T T A C A C C C C A T C C C C
F L F V A D A I N T A E A E T G E R K G N Y L N V T A P S P

720
G A G A A T G T A T G A C C C C A G A G T T C C C A A G A A C T C A A T A T C C C A T C A T C A C A G A C T T C T C A C C C C C C T C T G T G C C A T
E E N Y E R A E F A K E L N M P I I M N D F L T G G F C A N

810
A C C G T C T G C C G T T G T G C C G A A A A C C C G T C C T G T T C A C A T T C A T G T C C A T C A C C C G T G T G A C C C A A T C C C C A T C A C
T G L A R V C R K N G V L L N I N R A M H A V V D R N P N N

900
G C A T T C A C T T C C C C G T C T G C C A A G C C C T C C G T C G T C A G C C G T G A T C A C T C A C C C C A C C G T G T T G C C A C T G G A G T
G I N F R V L A K A L R L S G G D N L H T G T V V G K L E G

990
G A C C G T C C C C A C T C A G C C T G G T G A C C T C T C C C C A G T C A T T G T C C C G A G A C C C C G T C C C C A T C T T C T G A C C A G A C
D R A A T Q G V V D L L R E S F V P E D A G R G I P F D Q D

1080
T C C C C T C A T G C C C C G T C T T C C C G T C C T T C C C C C A T C A G T C T G C C A T C C C C C C T T A G C A T C T G C G A C A T
W G S N P G V F A V A S G G I N V W N M P A L L A I F G D D

1170
G C A A T A T C A G T T G G T G C C G T A C C T G C C C A T C C G T G C C A A C C T C C C C G A C C C T C C A C C C C G T G C A C T G A A C C T G T
A I P Q F G G G T L G N P V G N A A G A A A N R V A L E A C

1260
G T G A A C C C C A A T G A G C C C G A T C T G A G C C C G A G C A A G A C A T C T C A C A C C C C G A A G A C A G T C C C G A C T G A A A T T
V E A R N E G R D L E R E G K D I L T N A A K D S P E L K I

1350
G C C C T G A A C C T G A A A G A G A T C A A G T T G A A T T C A T A C C G T G A C A A T T G A C G T G T G A A T C C T G A T T G C T A C C G T G A T C C
A L E T V K E I K F E F D T V D K L D V V N R *

1440
A A G C C C A A C A A A A C C C C C C G T G A C T T G A C C C T C C T C C C C C C A C A A A G A A T A G A A

1512
1513
A T G C T G A C A T T C A G A C T A C A C T C A C A C C A G T A C A A A C T T C T C T A T T T C C C C A A T G G A C C G A A A A A T C C C C G T C A G
N A D I Q D Y N S T P K Y E T F S Y L P A N G P E K N R R Q

1603
A T C C C T A T C T A T C A A T C A G G C T G A A C C C C C A T G A C A T G T G A A C C T G A A C C C A T C A C A T A C T A C T G T A C A T G T A A G
I A Y L I N Q G V N P G I E N V E P E R A S T Y Y W Y N Y K

1693
T T A C C A T G T T C C C A C A G T C C G T G A C A C C G T A T C A T G A G T T G A A C A T C A T C C C T C A C C C C C A T C A C G T C C C T G
L P H F G E Q S V D T V I N E L E A C N R A N P G N H V R L

1783
G T C C G T A T A C A A T T A C T C C A G A C C A G C C A C C C T T T G T G T G T T T C C C C C C T A A G C A A G C C C T C C G A T T A T C C C C
V G Y D N Y S Q S Q G S A F V V F R G R *

1873
G A T C C C C C C A C C C G T T G A T G A T A C G A A G A T C A A C C A T G A C C A C T C T A T C C A A C A A T A C C C G T C C C C C C T G A C A C
1963
T C C C C C C C C T C C C A C C C C C C C G T T T G C A C A C C G T C C C C C C C C T G C C C C C A A G T C C C C C A C C A C A A

2043

FIG. 3

B

	<u>SspI</u>	<u>EcoRV</u>	<u>Eco47III</u>	
pTR11	TGAAATATTCAGTCGATCCAGGAT	ATCGTGGCAAGCCGACGTCAGCGCTTCCCC		-340
	* * * * *	*****	* * * * *	
pTR21	CGCCTTGTTCATTCGGGAGGATGCCACATTGGCGCAAACGAGTATTGACCTCTTGC			-347
	CGCCGCTGTCAGATAGATTTTTTAACTAT	TTGATCAAAAAGTCGGCCTGGCCAAAC		-282
	* * * * *	* * * * *	* * * * *	
	AAGAGATCGGGATGAGTCGATCTGAGGTGTGTTATTCGACACGAGCTTTCAGGTTACC			-287
	GTCGGCAAGTTGTCGGACCTGGATGGACAGGGCGGGAGGTGTCAAATGCAGTTCTTCAGC			-222
	* * * * *	*****	* * * * *	
	GCGTACCTGCAATCAGCGGATATGGTCTCATCTTCACCGCTCATCCAGTACTTCTTG			-227
		<u>ScaI</u>		
	AGCGGGCAAAGC-TCATGTGTCGAGCTACGGC-AGCGAAGATTTTG-AGTTGGTGA-A			-166
	** * * * *	* * * * *	* * * * *	
	AGGAACACCGCGCTTCATCATTGCCGAACCCCTCGCTCATATAGCGAACGCGACGCACA			-167
		<u>NcoI</u>		
	T-GTAGCGTGGCGGATAGACATGTCAATAGTAAACCATGGTAAATCATAATAGTAAATAT			-107
	* * * * *	*****	* * * * *	
	TCGTAGCGTGGCGGATAGACATGTCAATAGTAAACCATGGTAAATCATAATAGTAAATAT			-107
		<u>NcoI</u>		
	ATTTTACTTTTATAAATTAGAAGATCATCCACAATGCCAATCAAGCATTGGAACCCCAATC			-47
	*****	*****	*****	
	ATTTTACTTTTATAAATTAGAAGATCATCCACAATGCCAATCAAGCATTGGAACCCCAATC			-47
	AGTGGCTTCAATCGTCGTTGTCTACCATGACACAAGGAGAGCAAACATGCGCGTAAAAAC			14
	*****	*****	*****	
	AGTGGCTTCAATCGTCGTTGTCTACCATGACACAAGGAGAGCAAACATGCGCGTAAAAAC			14

FIG. 3. Nucleotide sequence of the *T. ferrooxidans* *rbc* genes. (A) *rbcL* and *rbcS* in pTR11. The nucleotide sequence of the noncoding strand of the *T. ferrooxidans* *rbc* region (pTR11) and the deduced amino acid sequences of the RbcL1 and RbcS1 polypeptides are shown. Numbering of the sequence begins from the *rbcL1* start codon. The positions of the putative ribosome-binding sites are double underlined. Stop codons are indicated by asterisks. The transcription start site is indicated by an arrowhead. Direct repeat or inverted repeat sequences in the 5' region of *rbcL1* are shown by arrows. (B) Upstream sequences of *rbcL1* (*rbcL2*) genes in pTR11 and pTR21. Identical bases are indicated by asterisks. The ATG initiation codons of the *rbcL1* and *rbcL2* genes are boxed. Restriction enzyme recognition sites (Fig. 2) are over- or underlined along the sequence.

is in good agreement with that estimated from SDS-polyacrylamide gel electrophoresis (28), whereas the *rbcS1* gene encodes a protein of 110 amino acids with an estimated molecular mass of 12,879. We then determined the nucleotide sequence of *rbcL2-rbcS2* in pTR21 and found that the *rbcL2*-intergenic-*rbcS2* sequence was completely identical with the *rbcL1*-intergenic-*rbcS1* sequence. The upstream regions of *rbcL1* and *rbcL2*, 164 bp from their start codons, were totally conserved, whereas regions further upstream were quite divergent (Fig. 3B). The downstream regions of *rbcS1* and *rbcS2*, 90 bp from their termination codons, were identical, whereas regions 200 bp further downstream were conserved more than 90% (data not shown). An imperfect palindromic sequence like those found in the intergenic regions of *C. vinosum* (56) and *A. nidulans* (48, 49) was not observed in *T. ferrooxidans*.

N-terminal amino acid sequence of the Rbc proteins synthesized in *E. coli*. The RuBPCase synthesized in *E. coli* containing pKSS14 or pTR21 was purified by sucrose density gradient centrifugation and fast protein liquid chromatography (Superose 6 chromatograph; Pharmacia) as described previously (28). The enzymatic active fraction from fast protein liquid chromatography was concentrated in Centricon tubes (Amicon) and loaded onto an SDS-15% polyacrylamide gel, and the separated proteins were blotted onto a polyvinylidene difluoride membrane. After staining with Coomassie blue, bands corresponding to the RbcL1 (RbcL2) and RbcS1 (RbcS2) proteins were excised and used for direct amino acid sequencing. For RbcL1 and RbcL2, the

predicted amino acid sequences were M-A-V-K-T-Y-E-A-G-V-K-D-Y- and the determined sequences were A-V-K-X-Y-E-A-G-V-. For RbcS1 and RbcS2, the predicted amino acid sequences were M-A-D-I-Q-D-Y-N-S-T- and the determined sequences were A-D-I-Q-X-Y-N-. The fourth residue of RbcL1 and RbcL2 and the fifth residue of RbcS1 and RbcS2 were not identified. These results verified the start codons of both *rbc* genes.

Transcription of *rbc* genes in *T. ferrooxidans*. It is known that the *rbc* genes in prokaryotes are tandemly located and cotranscribed (36, 53). To determine whether this is also the case in *T. ferrooxidans*, Northern hybridization was performed with a 1.3-kb *EcoRV-EcoRI* (*rbcL1*-specific) fragment and a 0.34-kb *EcoRI-SphI* (containing 41 bp of *rbcL1* and most of *rbcS1*) fragment of pTR11 as probes. Two bands of 2.1 and 1.5 kb were hybridized with the *rbcL* probe (Fig. 4, lane 1). In addition to the 2.1-kb band, the 3.0-kb band was hybridized with the *rbcS* probe (Fig. 4, lane 2). Three possible stem-and-loop structures were observed in the 3' region of the small subunit gene (Fig. 3A), and these might serve as transcriptional termination signals.

Mapping of the 5' end of the *rbc* transcript. As mentioned above, the 164-bp regions upstream of the *rbcL1* and *rbcL2* genes were identical (Fig. 3B). The *rbc* mRNA transcription start site, if located within this region, can thus be clearly specified. Under this assumption, determination of the 5' end of the *rbc* transcripts was performed with primer extension analysis. A single autoradiography signal revealed that *rbc* mRNA transcription started from an A residue, 64 bases

FIG. 4. Northern blot analysis of the *rbc* transcript. A 10- μ g sample of total RNA from *T. ferrooxidans* Fe1 was separated in a 1.5% agarose-formaldehyde gel. After transfer of the RNA, membranes were probed with either a 32 P-labeled 1.3-kb *EcoRV-EcoRI* fragment (lane 1) or a 0.34-kb *EcoRI-SphI* fragment (lane 2) of pTR11. The positions of 23S (2.9-kb) and 16S (1.5-kb) rRNA s are indicated. The positions of the *rbc* transcripts, which are discussed in the text, are indicated by arrowheads.

upstream of the *rbcL1* and/or *rbcL2* initiation codon (Fig. 5). Therefore, it was concluded that the *rbcL1-rbcS1* and/or *rbcL2-rbcS2* genes were cotranscribed in *T. ferrooxidans*. A search for potential *E. coli* promoter motifs showed a potential -10 hexamer sequence (positions -77 to -72; CACAAT); however, there were no sequences corresponding to a -35 consensus motif. The -140 to -87 region (Fig. 3A) was relatively A+T rich. Within this region, there were two pairs of imperfect direct repeats, TAAAT^{AT}CAT and TAAAT^{AT}TA and two pairs of inverted repeats (Fig. 3A).

Comparison of the *T. ferrooxidans* *rbc* genes and their products with those of various other organisms. When the *T. ferrooxidans* *rbcL1* (*rbcL2*) gene was compared at the nucleotide sequence level with *rbcL* genes from different organisms, the highest homology (78.9%) was scored with that of a photosynthetic bacterium, *C. vinosum* (Table 1). High levels of homology (over 60%) were also observed with cyanobacterial, eukaryotic algal, and plant sequences, although a lower (49.5%) level of homology was found with the *R. rubrum* sequence. On the other hand, the small subunit gene (*rbcS1* or *rbcS2*) exhibited 49 to 68% nucleotide sequence homology with those from different sources (Table 1); the highest identity (68.0%) was with that of *C. vinosum*. Although the hydrogen bacterium *A. eutrophus* is an chemoautotroph, as is *T. ferrooxidans*, their *rbc* genes shared little nucleotide sequence homology (Table 1). The G+C contents of the *rbcL1* (*rbcL2*)- and *rbcS1* (*rbcS2*)-coding regions were 59.6 and 54.9%, respectively, which are consistent with the overall G+C content of total *T. ferrooxidans* DNA (16). There was no strong bias in codon usage, and both genes generally favored C or G in the third codon position. Only four codons, CUU (for Leu), AGC (for Ser), and AGA and AGG (for Arg), were not used in the *T. ferrooxidans* *rbc* genes (data not shown).

The *T. ferrooxidans* large subunit protein exhibited 73.9 to 85.5% homology with those from tobacco (*Nicotiana tabacum*), *C. reinhardtii*, *Anabaena* sp., *A. nidulans*, and *C. vinosum*, 56.8% with that of *A. eutrophus*, and 30.1% with that of *R. rubrum* (Table 1). Several amino acid residues, including Glu-53 (17), Lys-168 (18), Asp-191 (13), Lys-194 (3,

FIG. 5. Mapping the 5' end of the *rbc* transcript. A 5'-end-labeled 30-mer (see Materials and Methods) that was complementary to the *rbcL1* or *rbcL2* mRNA was hybridized to 20 μ g of total RNA from *T. ferrooxidans* Fe1. The primers were extended by Rous-associated virus 2 reverse transcriptase in the presence of four unlabeled deoxynucleotide triphosphates at 37°C for 60 min. The products were analyzed on a 6% sequencing gel with the sequencing ladders of the DNA from plasmid pTR11 starting with the same 30-mer.

10, 32), His-291 (22, 39), and Lys-327 (18, 45, 52) (numerals are in reference to the *T. ferrooxidans* amino acid positions), which have been identified as essential in the active and catalytic sites of the enzyme, were conserved in *T. ferrooxidans* (Fig. 6). The *T. ferrooxidans* and *C. vinosum* large subunit proteins consisted of 473 and 472 amino acids, respectively, and showed 85.5% homology. The Ser-104 residue, which participates in holoenzyme assembly (4), was also conserved except in *R. rubrum* (37) and *R. sphaeroides* (58).

With regard to the small subunit protein, there was a large degree of sequence variation (Table 1). Only 16 residues were conserved in all of the comparisons that were made. Again, the *T. ferrooxidans* small subunit protein showed a higher (59.8%) level of homology with that of *C. vinosum* than with those of all other organisms (31.2 to 37.1%) (Table 1).

DISCUSSION

In this report, we show that *T. ferrooxidans* has two complete sets of genes for RuBPCase, and we present their primary structures. The existence of a second set of *rbc*

TABLE 1. Sequence homology between the *T. ferrooxidans* *rbcL1* and *rbcS1* genes and genes for the large and small subunits of other organisms

Species ^a	% of sequence homology ^b		
	Nucleotides	Amino acids	
		Identical	Related ^c
Large subunit			
<i>C. vinosum</i>	78.9	85.5	96.4
<i>A. nidulans</i>	72.2	77.1	93.7
<i>Anabaena</i> sp.	67.9	76.2	92.2
<i>C. reinhardtii</i>	65.5	76.2	91.6
<i>N. tabacum</i>	65.0	73.9	91.6
<i>A. eutrophus</i>	62.9	56.8	85.5
<i>R. rubrum</i>	49.5	30.1	66.0
Small subunit			
<i>C. vinosum</i>	68.0	59.8	97.1
<i>C. reinhardtii</i>	51.4	37.1	65.5
<i>A. nidulans</i>	52.7	36.2	81.0
<i>Anabaena</i> sp.	49.0	36.2	81.9
<i>N. tabacum</i>	51.3	35.8	72.5
<i>A. eutrophus</i>	53.0	31.2	82.8

^a Species are listed consecutively from that with the highest amino acid homology.

^b The percentage of sequence homology was calculated by comparing nucleotides or amino acids at identical positions in the sequences (ignoring insertions and deletions). The sequence alignments shown in Fig. 6 were used for the calculations.

^c Related residues are those with scores higher than 0.12 in the mutation matrix MDM₇₈ of Schwartz and Dayhoff (46).

genes was proved by (i) Southern analysis with the pTR11 insert DNA as a probe (Fig. 1) and (ii) direct cloning of pTR21. In the same strain of *T. ferrooxidans*, the *purA* gene encoding adenylosuccinate synthetase exists as one unit (29). This result may eliminate a possible artifact due to contamination with another organism. So far, similar pieces of evidence for two sets of *rbc* genes have been obtained in several other organisms, i.e., *R. sphaeroides*, *A. eutrophus*, *C. vinosum*, and *N. hambergensis* (2, 15, 21, 26, 53, 56). In contrast to *A. eutrophus*, in which one set of the genes is located on the chromosome and the other is on a plasmid, both sets of the *rbc* genes in *T. ferrooxidans* are located chromosomally. It is interesting to note that the nucleotide sequences of both sets of *rbc* genes in *T. ferrooxidans*, including the large and small subunit structural genes and spacer regions, were identical (Fig. 3). Therefore, it is uncertain whether both sets of *rbc* genes are expressed in this strain. The total identity over the whole *rbc* region suggests that duplication of these genes occurred quite recently in this strain. Although an insertion sequence, IST2, has been identified in *T. ferrooxidans* (62, 63), the element is thought not to participate in this duplication event because no homology is observed in the flanking sequences of the *T. ferrooxidans* *rbc* genes and the terminal inverted repeat sequence or the target sequence of IST2.

The *T. ferrooxidans* RbcL and RbcS proteins exhibited 73.9 and 35.8% homology with the respective proteins of *N. tabacum*. These data explain the previous immunological observations (28) that the *T. ferrooxidans* RbcL protein cross-reacted with a heterologous anti-tobacco RuBPCase serum, whereas the RbcS protein did not show any cross-reactivity with the serum. Both the RbcL and RbcS proteins of *T. ferrooxidans* exhibited a high level of homology with those of a photosynthetic bacterium, *C. vinosum* (Table 1).

Lane et al. (31) illustrated the phylogenetic relationship between the thiobacilli and the related purple photosynthetic bacteria by using the oligonucleotide matrix method; they reported that *T. ferrooxidans* m1 belongs to group III, in which *Chromatium* spp. are also classified. Higher homology in the amino acid sequences of RuBPCase and also in 5S rRNA sequences suggested a close phylogenetic relationship between *T. ferrooxidans* and *C. vinosum*, although the former is photoinsensitive and the latter is photosensitive. A recent report showed that the RbcS protein of the marine chromophytic alga *Olisthodiscus luteus* had 56% identity with that of *A. eutrophus* (6). Since the RbcS proteins show a high level of sequence divergence, any homology between the sequences may indicate a close evolutionary relationship.

The small subunit proteins from *C. reinhardtii* and *N. tabacum* contain 18 and 12 additional amino acid residues in the middle of the sequences, respectively (Fig. 6). A 16-amino-acid sequence, GFVYREHNKSPRYDYG, in the pea RbcS protein, which showed some overlap with the above additional sequences, has been reported as a holoenzyme assembly domain (60). However, in all RbcS sequences reported to date, including ours, the RuBPCases, which take a hetero-oligomeric form, do not have most of the assembly domain sequences (60). Therefore, the role of these additional sequences described above needs careful reevaluation. A point mutation causing the change of Ser-111 to Phe-111 in the large subunit of *N. tabacum* RuBPCase resulted in holoenzyme assembly failure (4). In keeping with this, amino acid sequence comparisons (Fig. 6) show that the Ser residue at position 104 in *T. ferrooxidans* is conserved in all the organisms that have a hexadecameric form of RuBPCase, whereas in *R. rubrum* and *R. sphaeroides*, in which the RuBPCase is composed only of large subunits (54), the residue is replaced with an Ile residue (37, 58). The amino acid comparison data also support the hypothesis that Ser-104 participates in holoenzyme assembly. Several other amino acid residues have been identified as essential for activity by chemical modification, affinity labeling, and site-specific mutagenesis studies. Such residues, Glu-53 (17), Lys-168 (18), Asp-191 (13), Lys-194 (3, 10, 32), His-291 (22, 39), and Lys-327 (18, 45, 52), are conserved in all RuBPCase large subunit proteins that have been sequenced, including the ones from *T. ferrooxidans*. On the other hand, the role of two cysteinyl residues, Cys-165 and Cys-459, that have been modified by affinity reagents in the spinach enzyme (45) is unclear, because one of these, Cys-165, is also conserved in *T. ferrooxidans* but is replaced with Ala and Thr residues in *A. eutrophus* (2) and *R. rubrum* (37), respectively, and the Cys-459 residue is also replaced by different residues in most organisms (Fig. 4). In the small subunit proteins, conserved sequences are clustered into three regions: amino acid positions 12 through 20, 55 through 65, and 88 through 95 (in reference to *T. ferrooxidans* positions). In addition to these three regions, Tyr-33, Gly-38, Glu-44, and Glu-75 residues are also conserved in all species.

In Northern analysis, two bands of 2.1 and 1.5 kb were hybridized with the *rbcL* probe, but only the former band was detected with the *rbcS* probe (Fig. 4). The 2.1-kb RNA is thought to be a cotranscript, because only this RNA species hybridized with both *rbcL* and *rbcS* probes. The smaller 1.5-kb band is supposed to be derived from *rbcL*, because it was detected with *rbcL* but not with *rbcS*. A similar result was observed by Valle et al. (55), who detected three transcripts of 2.0, 1.5, and 1.3 kb in the *C. vinosum* *rbc* genes. They found typical transcriptional termination signals in the DNA sequence of *Chromatium rbcL-rbcS* (56), and

Large subunit

	1	50	*
Tf	MAVKTYEAGVKDYRQ-TYWAPEYVPLDSILACFKITPQPGVDREAAAAVAASSTGTWTV		
Cv	..-...S....E..E-...M.N.T.K.T.....A..P.....		
An	MPKTQSAAG.K.....KL--YT.D.T.K.T.L..A.RFS.....PAD..G..I.....		
Cr	MVPQTETK.GAGFK.....L--YT.D..VR.T....A.RM...L..PP..CG.....		
Nt	MSPQTETK.SVGFK....E.KL--YT...QTK.T...A.RV.....PP..G.....		
Ae	MNAPETIQAKPR.R.D...MK.KEMG..DGD...K.T.V..L.R...D...PV.....G...A...V.		
Rr	MDQSSRYVNLAL.EE MK.KA.YGYVAT..HF.....NVE.		
	100		
Tf	WTDLLTMDYYKGRAYRIEDVPGDDTCFYAFVAYPIDLFEEGSVVNVFTSLVGNVFGFKAVRALRLLEDVRFP		
Cv	L.....A.....I.....		
An	R...K.C.H..P.Q.EENSIFY..I...L.....T.IL..I.....I.S....I...		
Cr	...G..SL.R...C.D..P...E.NQYI.Y.....T.M...I.....L.....L.I.		
Nt	...G..SL.R...C...R.V.EKDQYI.Y...L.....T.M...I.....L.....L.I.		
Ae	...R..AS.M.RAK...VDP..NNPEQ.FCY...DLS.....IA.LTA.II...S..PIK.A...M...		
Rr	TKI...VA..DRNI-TDGK-AMIASFLTLTMGNQGMG..EYA		
	150	*	* * 200
Tf	LAYVKTGNGPPHGIQVERDKMKNYGRPMLGCTIKPKLGLSAKNYGRAVYECLRGGLDFTKDDENVNSQPFIA		
Cv	I...M.....A.....I.....MR		
An	V.L...FQ.....LL.....I.....QR		
Cr	P.....FV.....L.....GL.....MR		
Nt	P.....FQ.....L.....L.....MR		
Ae	V.....FA..ST..I...ERLD.F..L..A.T.....GR...V...G.K....M....I....MH		
Rr	L...E TI.....RP.PFAE.CHAFWL...-I.N..PQGN...AP		
	250		
Tf	WRDRFLFVADAIHTAEAEETGERKGHYLNVTAPEEMYERAEFAKELNMPIMHDFLTGGFCANTGLARWCR		
Cv	..Q..D..M...DK..R...T...K...Y...IGA.....YI.....Q...		
An	KSQ.....I.....TC..MK.....G.....A.T...T...K...		
Cr	E..YK.Q....V.....A..GTC...MK..VC...GV.....Y...T...S..IY..		
Nt	C.E.LYK.Q....I.....A..GTC...IK..V..R..GV..V...Y...T...S..HY..		
Ae	M..VNK.S.A...V..S.....GTM....R.....S.GSV...V.LIV.WT.IQS-MSN...		
Rr	L..TIAL....MRR.QD....A.LFSA.I..DD.F.IIA.G.YVL.T GYVA.A		
	* 300	*	350
Tf	KNGVLLHIHRAMHAVVDRNPHHGIHFRVLAKALRLSGDHLHTGTVVGKLEGDRAATQGWVDLLRESFVPED		
Cv	D.....L.....T.I.....S.L..I.....YIK..		
An	D.....I..QRN.....C.....S.....K.S.L.F...M..DHIEA.		
Cr	D..L.....I..QRN.....M.....S.....E.EV.L.F...M.DDY.EK.		
Nt	D..L.....I..QKN.....M.....I.S.....E.DI.L.F....DD..EQ.		
Ae	Q.DMI..L...G.GTYT.QKN..VS...I..W...A.V..M...A.....PLTV..YYNVC.DAYTQT.		
Rr	D.FL---Y...G.GA.TS C.MA..Q.ASGI...MG..M..ESSDRAIAYM		

FIG. 6. Alignment of amino acid sequences of the large and small (mature) subunit proteins of RuBPCase from *T. ferrooxidans* (Tf) with those of other organisms. Large and small subunits from the photosynthetic bacterium *C. vinosum* (Cv) (56), the cyanobacterium *A. nidulans* (An) (48, 49), the eukaryotic alga *C. reinhardtii* (Cr) (9, 12), the higher plant *N. tabacum* (Nt) (34, 47), the chemolithotrophic bacterium *A. eutrophus* (Ae) (1), and the photosynthetic bacterium *R. rubrum* (Rr) (37) were compared. Residue numbers are in reference to the *T. ferrooxidans* sequence. Dots indicate the presence of the same residue at that position. For the *R. rubrum* large subunit, only regions homologous to the *T. ferrooxidans* large subunit are presented. Insertions are indicated by dashes. The conserved residues, including three active Lys residues, are shown by asterisks. The sequences that are believed to be parts of the catalytic site in the tobacco enzyme are underlined.

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