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Isolation and nucleotide sequence of the *Thiobacillus ferrooxidans* genes for the small and large subunits of ribulose 1,5-bisphosphate carboxylase/oxygenase

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The genes encoding for the large (*rbcL*) and small (*rbcS*) subunits of ribulose-1,5-bisphosphate carboxylase (RuBisCO) were cloned from the obligate autotroph *Thiobacillus ferrooxidans*, a bacterium involved in the bioleaching of minerals. Nucleotide sequence analysis of the cloned DNA showed that the two coding regions are separated by a 30-bp intergenic region, the smallest described for the RuBisCO genes. The *rbcL* and *rbcS* genes encode polypeptides of 473 and 118 amino acids, respectively. Comparison of the nucleotide and amino acid sequences with those of the genes for *rbcL* and *rbcS* found in other species demonstrated that the *T. ferrooxidans* genes have the closest degree of identity with those of *Chromatium vinosum* and of *Alvinoconcha hessleri* endosymbiont. Both *T. ferrooxidans* enzyme subunits contain all the conserved amino acids that are known to participate in the catalytic process or in holoenzyme assembly.

CO_2 fixation; Nucleotide sequence; Amino acid sequence; *Thiobacillus ferrooxidans*

1. INTRODUCTION

Thiobacillus ferrooxidans is an acidophilic, chemolithotrophic, Gram-negative bacterium that participates in bacterial leaching of minerals [1]. It is an obligate autotroph fixing atmospheric CO_2 via the Calvin cycle [2]. The key enzyme of this pathway, ribulose bisphosphate carboxylase-oxygenase (RuBisCO) from *T. ferrooxidans* has been isolated and characterized in our laboratory [3]. The enzyme has a native M_r of 570 000 and is composed of M_r 54 000 and M_r 15 500 subunits, corresponding to a type I enzyme with its characteristic hexadecameric structure, L_8S_8. The enzyme has apparent K_m values for CO_2 and for ribulose bisphosphate of 28 μM and 80 μM, respectively, and it can be inhibited by 6-phosphogluconate. It was estimated that RuBisCO accounts for 2–5% of the total protein present in a whole extract, and its specific activity increases when the percentage of the CO_2 is raised in the air bubbling through the culture [3].

RubisCO is present in a wide range of autotrophic organisms, from prokaryotes to higher plants. The L_8S_8 structure for the holoenzyme is found in the majority of the bacteria, algae and higher plants studied. A less common form of RuBisCO which contains only large subunits, corresponding to the type II enzyme, is found in some purple non-sulfur photosynthetic bacteria such as *Rhodospirillum rubrum* [4]. *Rhodobacter sphaeroides* and *Rhodobacter capsulatus* synthesize both type I and type II RuBisCO [5,6].

Some of the genes encoding for the large subunit of this enzyme in different organisms have been sequenced, showing a high degree of sequence similarity [7–13]. However, a great heterogeneity in nucleotide sequences has been found in genes encoding for the small subunit [8,12–15].

The growth of *T. ferrooxidans*, an economically important microorganism, appears to be regulated by its capacity to fix CO_2. For this reason, the study of the RuBisCO genes and the regulation of their expression has special biotechnological interest. Recently, Kusano et al. reported the cloning and expression of *T. ferrooxidans* RuBisCO in *E. coli*, confirming the hexadecameric form of the enzyme [16]. This group, however, did not publish the sequence of the genes which they cloned. In this communication we present the cloning and sequencing of *T. ferrooxidans rbcL* and *rbcS* genes.

2. MATERIALS AND METHODS

2.1. *Bacterial strains and phages*

E. coli JM105, maintained in minimal medium supplemented with glucose was used as a recipient for recombinant M13 DNA. *E. coli* C600 transformed with recombinant plasmids was grown in Luria broth supplemented with 100 μg of ampicillin per ml of culture.

T. ferrooxidans strain ATCC 19859 was grown in Mackintosh medium [17]. All recombinant DNAs were constructed according to standard methods [18].

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2.2. *Chromosomal DNA library*

A *T. ferroxidans* strain ATCC 19859 gene library was constructed by total digestion of chromosomal DNA with *Bam*HI and ligation of the resulting fragments to pBR322 previously linearized by digestion with *Bam*HI and dephosphorylated by treatment with calf intestine alkaline phosphatase [18].

2.3. *Screening of the DNA library*

The nearly complete *rbcL* gene from *A. nidulans*, cloned in pCS751 plasmid (kindly provided by Dr F.R. Tabita [19]), was labelled with [α-^{32}P]dATP by nick translation as described [18] and was used as a probe to screen the *T. ferrooxidans* gene library by the method described by Hanahan et al. [20].

10×10^3 recombinant colonies were screened by the colony hybridization method [20], and several colonies from the first screening were purified through secondary and tertiary screening.

Hybridizations were performed overnight at 42°C in a solution containing 5×SSPE (50 mM sodium phosphate, 5 mM EDTA, 0.9 M NaCl), 1×BFP (0.02% BSA, 0.02% Ficoll, 0.02% PVP), 20% formamide, 0.5% SDS and 25 μg per ml of heat-denatured salmon sperm DNA.

Filters were washed four times at 42°C in 4×SSPE and then twice at 52°C for 15 min, before exposing to autoradiographic films at −70°C for 2–3 days.

2.4. *DNA sequencing*

Appropiate restriction fragments from recombinant plasmids were subcloned into M13mp18 or M13mp19 vectors, according to the instructions from the manufacturer (Bethesda Research Laboratories). Nucleotide sequences were determined using the dideoxy chain termination method [21]. Sequencing reactions were carried out using the Sequenase Kit supplied by USB Corp. [α-^{35}S]dATP (Amersham) was used as the radiolabelled nucleotide. Synthetic oligonucleotides were used as primers.

3. RESULTS AND DISCUSSION

3.1. *Screening of* T. ferrooxidans *chromosomal DNA library*

The isolation and identification of the genes coding for RuBisCO was carried out using a *T. ferrooxidans* gene library and the *rbcL* gene from *A. nidulans*. Approximately 10^4 colonies were screened and 20 positive clones were obtained. Eight of these clones were purified by secondary and tertiary screening. DNA was prepared from three purified clones, digested with different restriction enzymes, blotted into nitrocellulose membranes and hybridized to the labeled *rbcL* gene from *A. nidulans*.

The restriction map of one recombinant plasmid, pRB-1, is presented in Fig. 1. Similar analysis of the other recombinants showed that the three purified clones were identical. The 3.3 kbp *Eco*RI-*Bam*HI fragment was subcloned in pBR322 (pRB-2) and some restriction sites were identified. DNA sequencing was performed on fragments derived from both recombinant plasmids, the sequencing strategy being summarized in the lower part of Fig. 1.

3.2. *Nucleotide sequences of* rbcL *and* rbcS *genes*

The complete *rbcL* and *rbcS* genes from *T. ferrooxidans* were present in the DNA fragment cloned in pRB-1 and its entire nucleotide sequence as well as the deduced amino acid sequences are shown in Fig. 2. The 2209 bp sequence predicts two consecutive open reading frames. The large and small subunit sequences are 1422 and 357 nucleotides long, respectively, leaving an intergenic region of 30 bp, the smallest described for prokaryotic RuBisCO genes. In prokaryotes the two subunit genes are co-transcribed, while in higher plants the large subunit is encoded by chloroplast DNA and the small subunit is nuclear encoded [22].

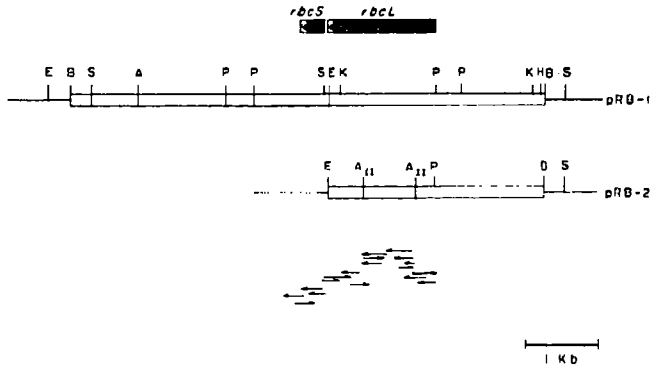

Fig. 1. Physical maps and the strategy for sequence analysis of the *T. ferrooxidans rbcL* and *rbcS* genes. A physical map of the 6.6-kbp *Bam*HI fragment (pRB-1) and of the 3.3-kbp *Eco*RI-*Bam*HI fragment (pRB-2) are shown. The arrows indicate the extent and direction of sequences determined and the solid bars show the regions encoding *rbcL* and *rbcS*. The direction of transcription, and some restriction sites are presented. A, *Aval*; AII, *Ava*II; B, *Bam*HI; E, *Eco*RI; *H*, *Hin*dIII; K, *Kpn*I, P, *Pst*I and S, *Sal*I.

The *rbcL* gene encodes for a protein of 473 amino acid residues while *rbcS* encodes for a polypeptide of 118 amino acid residues, with calculated molecular weights of 52.7 and 13.6 kDa, respectively.

Possible *rbcL* and *rbcS* ribosome-binding sequences at positions −12 to −7 and 1442 to 1446, respectively, were detected (underlined in Fig. 2), but no consensus sequences for transcription promoters were found in the cloned fragment.

Two potential stem-loop sequences were found adjacent to the 3' end of *rbcS* (arrows in Fig. 2), the last one being followed by the sequence TTTT (dotted line in Fig. 2), which could represent a rho-independent transcriptional termination signal.

3.3. *Comparison of nucleotide and amino acid sequences*

Table I shows the results of nucleotide and amino acid sequence comparisons of the RuBisCO genes from *T. ferrooxidans* with those of other bacteria such as *C. vinosum* [8], *A. hessleri* endosymbiont [12], *A. nidulans* [7,14], *A. eutrophus* [13] and *R. rubrum* [10]. *rbcL* and *rbcS* nucleotide sequences from *T. ferrooxidans* exhibit the highest similarity with *C. vinosum* and *A. hessleri* endosymbiont sequences.

Predicted *rbcL* and *rbcS* gene products from *T. ferrooxidans* also showed the highest percentage of similarity at amino acid level with *C. vinosum* and *A. hessleri*

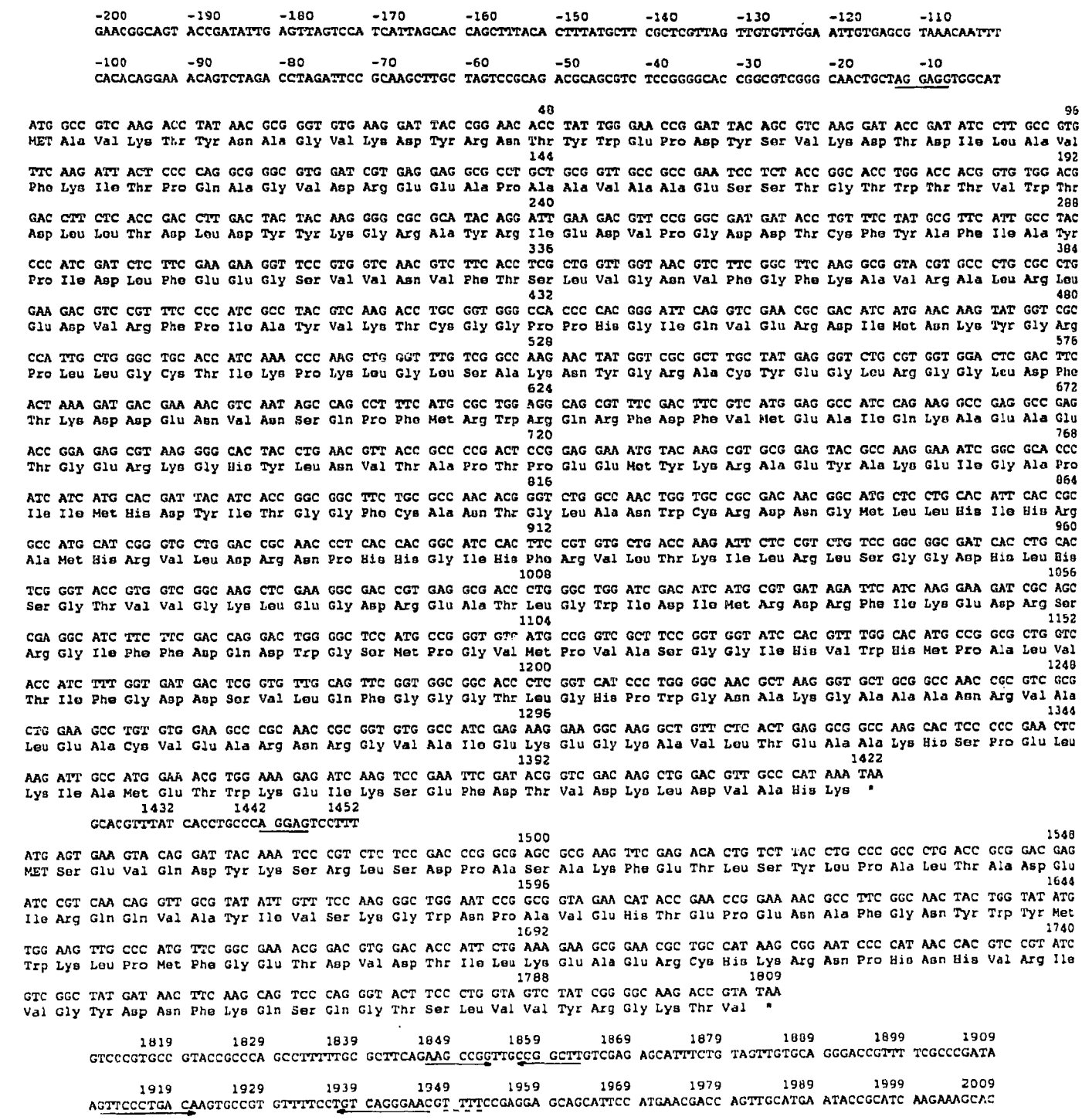

Fig. 2. DNA sequences of *T. ferrooxidans rbcL* and *rbcS* and deduced amino acid sequences of the large and small subunits of RuBisCO. The first reading frame (1–1422) corresponds to *rbcL*, and the following one (1453–1809) to *rbcS* (see text for details).

endosymbiont gene products. An even higher degree of similarity may be obtained when comparisons are made on the basis of functional groups of the amino acids (see Table I). It is interesting to note that the highest percentage of similarity both at nucleotide and deduced amino acid levels of *rbcL* and *rbcS* genes from *T. ferrooxidans* is observed with metabolically related bacteria, since *C. vinosum* and *A. hessleri* endosymbionts are also sulfur oxidizing autotrophic bacteria. In contrast, a lower percentage of similarity both at nucleotide and amino acid levels was found for genes and gene products from two purple non-sulfur bacteria, *A. eutrophus* and *R. rubrum* (see Table I). It may be recalled that the *R. rubrum* enzyme has a type II structure [4].

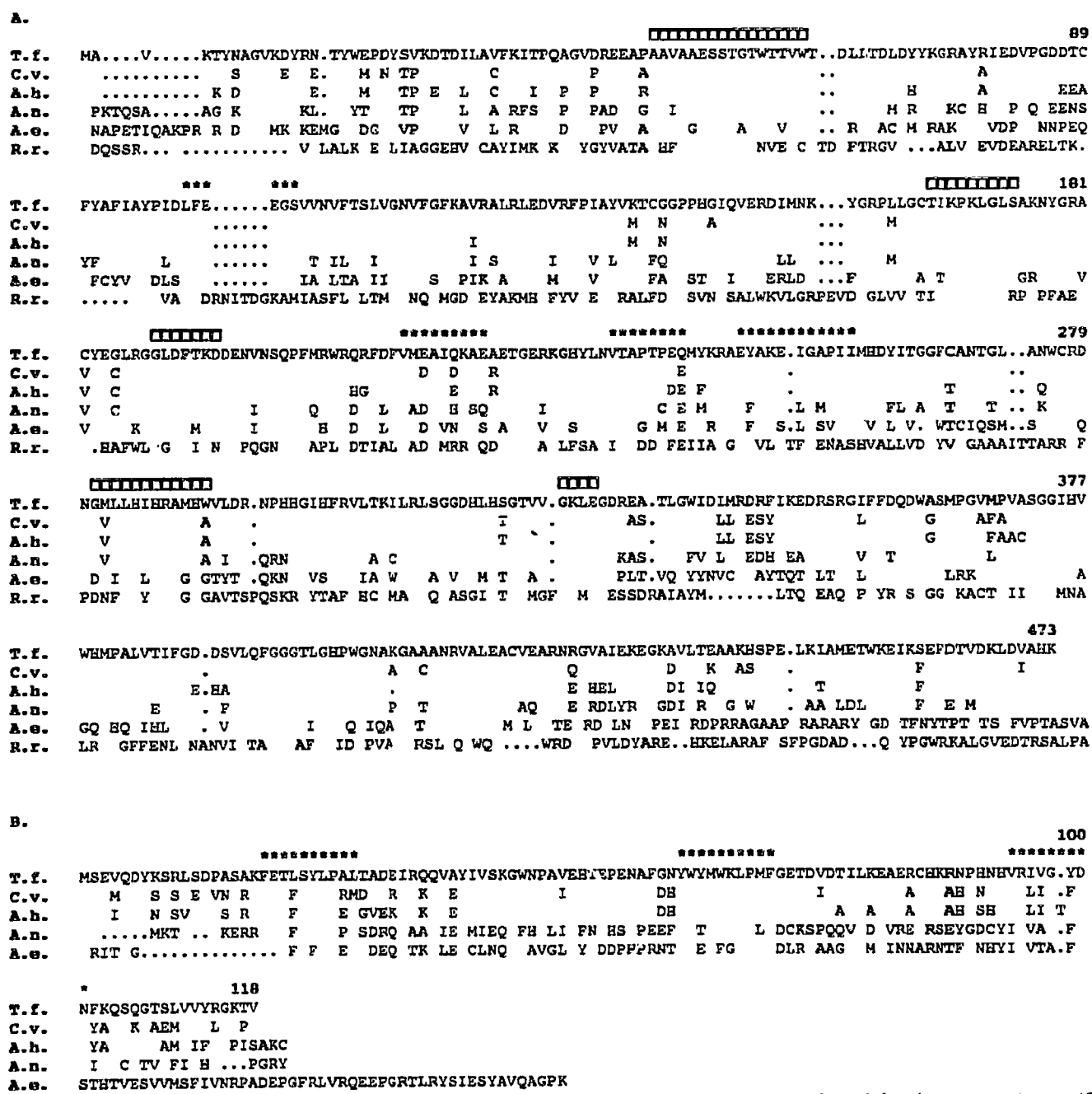

Fig. 3. Comparison of amino acid sequences of the *RbcL* (A) and *RbcS* (B) proteins from *T. ferrooxidans* (T.f), *Chromatium vinosum* (C.v), *Alvinoconcha hessleri* endoxymbiont (A.h), *Anacystis nidulans* (A.n), *Alcaligenes eutrophus* (A.e) and *Rhodospirillum rubrum* (R.r). Only residues that differ from *T. ferrooxidans* sequence are presented in the one letter code for the amino acids. Residue numbers refer to the *T. ferrooxidans* sequence. Deletions are indicated by dots. Conserved regions containing amino acid residues participating in catalysis (□) and in assembly of the holoenzyme (***) are indicated.

3.4. *Comparison of the deduced RbcL amino acid sequence of* T. ferrooxidans *with the sequences found in other organisms*

Several highly conserved regions became evident by the alignment of amino acid sequences of different bacterial RuBisCO large subunits (Fig. 3). They include amino acid residues which participate both in the active site and in the assembly of the holoenzyme.

The amino acids participating in the active site of the large subunit of RuBisCO from several bacteria, as reported by Tabita et al. [23], were compared with those from *T. ferroxidans* (see Fig. 3A). Glu[53], a highly conserved residue involved in catalysis [22] defines a conserved region from Ala[48] to Thr[64]. Lys[171] and Lys[173] belong to a second conserved region starting at Thr[169]. The Lys residues have been implicated at the active site of RuBisCO by affinity labeling [22]. The conserved Asp[191] associated to a CO_2 binding site and Lys[194] identified as

Table I

Sequence comparisons of the *rbcL* and *rbcS* genes of *T. ferrooxidans* and other prokaryotes. The percent similarity is calculated for amino acids and nucleotides at identical positions in the sequences being compared (aligned as shown in Fig. 3).

Species	*rbcL*			*rbcS*		
	Nucleotides	Amino acids		Nucleotides	Amino acids	
		Identical	Related*		Identical	Related
C. vinosum	82.0	90.0	94.7	72.0	72.0	81.3
A. hessleri (endosymbiont)	81.3	87.5	93.0	72.3	71.2	82.2
A. nidulans	70.8	73.1	86.2	58.6	36.9	55.8
A. eutrophus	64.6	55.0	72.1	54.0	22.0	31.3
R. rubrum	54.4	19.7	29.4			

*Residues belonging to the same functional group are considered related. These groups are: neutral, slightly hydrophobic: P, A, G, S, T; hydrophilic, acid amine: E, D, Q, N; hydrophilic basic: H, R, K; hydrophobic: L, I, V, M; hydrophobic aromatic: F, Y, W; cross-link forming: C.

the site of carbamylation during activation of the enzyme [24], correspond to another conserved region. Another constant region starting at Gly^{281} includes His^{291}, a highly conserved residue also involved in CO_2 binding [22]. The region that includes Lys^{327} was also highly conserved. Affinity-labeling experiments have shown that this residue participates in the active site [25]. Conserved regions starting at positions 100, 214, 235 and 248 corresponded with assembly domains of the large subunit of RuBisCO from several other bacteria [22]. (Fig. 3B).

3.5. *Comparison of the deduced RbcS amino acid sequence of* T. ferrooxidans *and other organisms*

Fig. 3B shows the alignment of amino acid sequences of several bacterial small subunits of RuBisCO. With the *rbcS* there is higher divergence than that observed among the large subunits, especially at the amino and carboxy termini. Three highly conserved regions containing amino acid residues related with the oligomer assembly: Glu^{20}, Ser^{23}, Leu^{28}, Tyr^{61}, Trp^{62}, Pro^{68} and Tyr^{99} [26,27], are shown in Fig. 3B. As expected, the conserved sequence of 16 amino acids that have been proposed to constitute a domain required to facilitate assembly of the holoenzyme from higher plants [15], is absent in *T. ferrooxidans*.

Experiments to study the regulation of the expression of *rbcL* and *rbcS* genes in *T. ferrooxidans* are now in progress.

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