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Characterization of the two rRNA gene operons present in *Thiobacillus ferrooxidans*

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The organization of rRNA genes from the autotrophic, acidophilic bacterium *Thiobacillus ferrooxidans* has been examined. Two rRNA operons were found in this microorganism by means of genomic hybridization studies. Recombinant plasmids, pTR-3 and pTR-1 that carry a portion of 16/23 S rDNA from one operon and the 5'-flanking region of the second operon, respectively, were identified and characterized.

Acidophilic bacteria; rRNA gene; Genome; Bioleaching

1. INTRODUCTION

Thiobacillus ferrooxidans is an autotrophic, chemolithotrophic and acidophilic bacterium that participates in bioleaching of metals from minerals. It obtains its energy from the oxidation of ferrous ions as well as from reduced sulfur compounds. The potential improvement of the leaching process depends, in part, on the understanding of gene expression of *T. ferrooxidans*. Recently, the expression in *E. coli* [1] and the sequence of some of the genes involved in nitrogen fixation [2] as well as the glutamine synthetase gene [3] from this microorganism have been reported. It is also known that the size of *T. ferrooxidans* genome is 2.8×10^6 bp, about two thirds the size of the *E. coli* genome [4] and contains about 6% repetitive sequences. Some plasmids from *T. ferrooxidans* strains have been isolated and cloned in *E. coli* [5]. One of them allows the replication in *E. coli* of a pBR325 derivative lacking its own origin of replication [6]. Analysis and manipulation of operon promoters isolated from *T. ferrooxidans* may be of value in modifying some of the properties of the bioleaching process at the level of the bacteria and also contribute to the general understanding of regulation of gene expression in autotrophic microorganisms.

We are interested in the genomic organization and molecular mechanisms responsible for gene expression in *T. ferrooxidans*, and have initiated a study of rRNA genes from this microorganism. Ribosomal RNA genes have been widely characterized in prokaryotic microorganisms. Seven operons, where 16, 23 and 5 S rRNA genes are closely linked, have been found and mapped in *E. coli* [7,8], but such a reiteration and organization of rRNA gene operons is not always present in all microorganisms as seen in *Thermus thermophilus* [9], *Vibrio fisherie* [10] or in mycoplasmas [11]. Here we report a structural characterization of cloned DNA segments as well as the organization of chromosomal DNA that encodes for rRNA sequences from *T. ferrooxidans*. Our data show the presence of two rRNA operons where 16 and 23 S genes are closely linked. Both operons are similar in their coding sequences but largely divergent in their flanking regions. A recombinant plasmid carrying a *T. ferrooxidans* DNA segment that probably encodes an rRNA gene promoter was identified and characterized.

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2. MATERIALS AND METHODS

2.1. *Bacterial strains and phages*

E. coli JM 105, maintained in minimal media supplemented with glucose was used as a recipient for recombinant M13 DNA and strains HB 101 or C 600 were grown in Luria broth supplemented with 100 μg per ml of ampicillin if recombinant plasmids were present. *T. ferrooxidans*, strain Torma, was grown in Mackintosh media [12]. All recombinant DNAs were constructed according to standard methods [13,14]. Chromosomal DNA was prepared according to the method of Marmur [15] with some minor modifications.

2.2. *Nucleic acid probes*

T. ferrooxidans and *E. coli* rRNA were labeled at their 5'-end with [^{32}P]ATP and polynucleotide kinase (Boehringer Mannheim) as described in [13,14]. Potential 23 S degradation products contaminating 16 S rRNA probe were considered for the interpretation of results.

2.3. *rDNA probes*

The 2.5 kb (probe 1), 0.7 kb (probe 2) and 1.2 kb (probe 3) *Eco*RI-*Sal*I fragments spanning a portion of 23 and 16 S, 16 S or 23 S rDNA, respectively, present in plasmid pTR-3 (see section 3, fig.2A) were subcloned in M13 mp18 and single-stranded DNA was prepared. Each recombinant phage DNAwas labeled by elongation of the universal primer used for DNA sequencing [16] in the presence of all three 10 μM dATP, dGTP and dCTP and 2.5 μM Biotin 11-d UTP (BRL) and DNA polymerase I. The reaction was initiated by the addition of 1 unit of enzyme and incubations were extended for 1.5 h. One more unit of enzyme was added after 60 min of incubation.

2.4. *Hybridization*

Plasmid or chromosomal DNA were digested with different restriction enzymes (Boehringer Mannheim). Resulting fragments were resolved by agarose gel electrophoresis and transferred to nitrocellulose (plasmid DNA) or nylon (chromosomal DNA) membranes by Southern blotting [17]. Hybridizations were carried out for 16 h at 42°C in 50% formamide and 4 × SSC. DNA fragment lengths were determined by comparison of their relative migrations in agarose gel electrophoresis to lambda DNA markers.

2.5. *Sequencing*

Nucleotide sequences were determined using the dideoxy chain termination method [18]. Sequencing reactions were carried out using the Sequenase reagents kit supplied by USB Corp. [^{35}S]dATP (Amersham) was used as radiolabeled nucleotide.

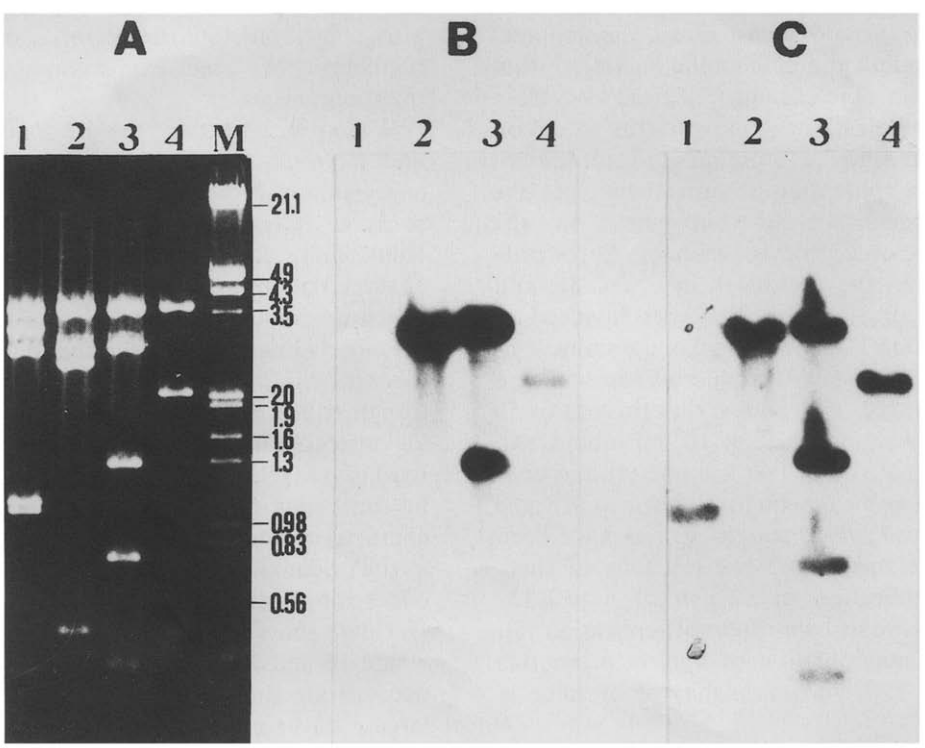

Fig.1. Analysis of recombinant plasmid DNA. Plasmids pTR-1 (lane 1), pTR-2 (lane 2), pTR-3 (lane 3) and pTR-4 (lane 4) were digested with a mixture of *Eco*RI and *Sal*I and subjected to agarose gel electrophoresis (A). DNA was transferred to nitrocellulose filters and hybridized to 5'-end labeled 23 S (B) and 16 S (C) rRNA from *E. coli*. Lane M, lambda DNA digested with *Eco*RI/*Hin*dIII.

3. RESULTS AND DISCUSSION

3.1. *Identification of cloned* T. ferrooxidans *rDNA*

A *T. ferrooxidans* gene library, constructed by partial digestion of chromosomal DNA with *Sau*3A1 and ligation of the resulting fragments to pBR322, linearized by digestion with *Bam*HI was used in the identification of rDNA segments. rRNA was isolated from purified *T. ferrooxidans* ribosomes, labeled at the 5′-end with ^{32}P (see section 2) and used as probe for the screening of the gene library under stringent conditions, in order to avoid potential background hybridization of *E. coli* rRNA genes. Several positive signals were detected and some of such clones were further purified. Recombinant plasmid DNA was isolated from four such clones, termed pTR 1, 2, 3 and 4, carrying inserts of 7.0, 4.5, 4.2 and 1.0 kb, respectively. The presence of rRNA gene sequences was confirmed by Southern hybridization of digested plasmids to the *T. ferrooxidans* rRNA probe.

In order to analyze the organization of rRNA genes in *T. ferrooxidans*, all four plasmids carrying rDNA from this microorganism were subjected to digestion with restriction enzymes and hybridization of the resulting fragments to 5′-end ^{32}P-labeled *E. coli* 16 S or 23 S rRNA. All of them hybridized to the 16 S rRNA probe but only pTR-2, pTR-3 and pTR-4 hybridized to 23 S rRNA (fig.1). In addition to the hybridization experiments, plasmid pTR-3 was subjected to partial DNA sequencing (not shown). More than 80% homology was found between *T. ferrooxidans* rDNA sequences and the operon rrnB, one of the seven rRNA operons of *E. coli* [19]. These results showed that sequences encoding 16 and 23 S rRNA are present in the 4.2 kb insert of *T. ferrooxidans* DNA in plasmid pTR-3 (fig.2A). By comparison of the relative positions of rDNA sequences found in pTR-3 and the *E. coli* rrnB operon, we concluded that 16 and 23 S rRNA genes from *T. ferrooxidans* are organized as in the *E. coli* operon (fig.2A). Experiments relevant to 5 S rRNA gene

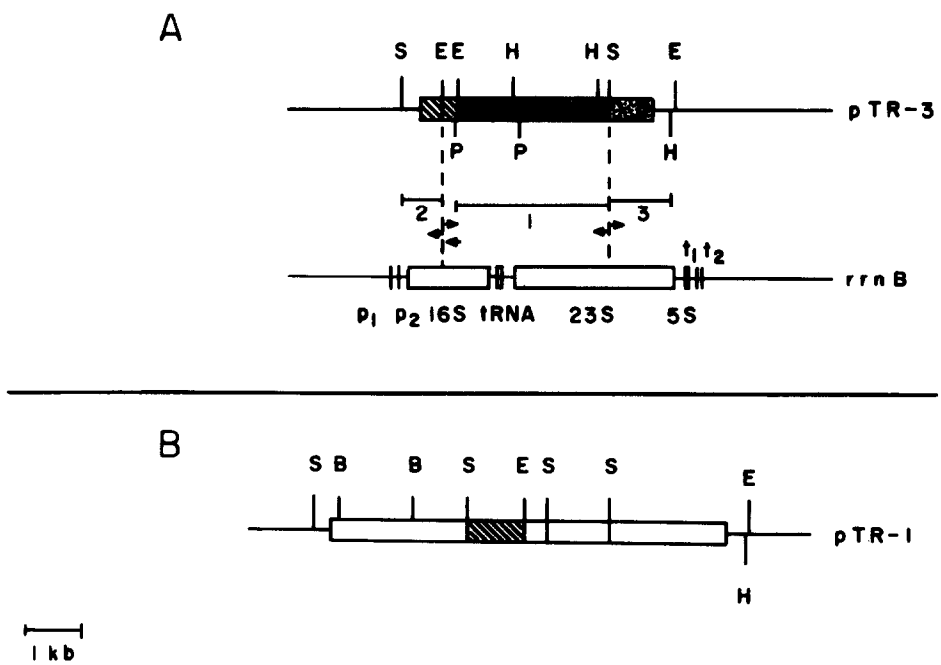

Fig.2. Schematic representation of recombinant plasmids. *T. ferrooxidans* insert DNA is represented by bars. Solid lines represent pBR322 sequences. Hybridization to 16 S (hatched areas) and 23 S (dotted areas) rRNAs are shown. Letters are restriction enzyme recognition sites: E, *Eco*RI; S, *Sal*I; P, *Pst*I; H, *Hin*dIII. (A) pTR-3: plasmid pTR-3. Arrows show approximately the sequenced regions of insert DNA (see section 2). Numbered segments represent DNA from pTR-3 used as probes for chromosomal DNA analysis (see text). rrnB represents the corresponding operon from *E. coli*. P_1, P_2 and t_1,t_2 are rrnB promoters and terminators, respectively. Broken vertical lines indicate the position of equivalent regions between *T. ferrooxidans* and *E. coli* rDNA. (B) pTR-1: plasmid pTR-1.

localization are in progress. Sequencing of the 400 nucleotides spacer region between 16 and 23 S rDNA of *T. ferrooxidans* strain A-4 (a native chilean strain) showed the presence of two tRNA gene sequences ($tRNA^{ile}$, $tRNA^{ala}$, Venegas, A., personal communication). In *E. coli*, all of these genes are transcribed as a single transcriptional unit and processed to yield mature RNAs [20]. Understanding whether *T. ferrooxidans* rRNA genes are expressed in a similar way requires the identification of transcriptional signals as well as the analysis of primary transcripts synthesized in vivo.

A second interesting cloned plasmid was pTR-1 (fig.2B). This plasmid hybridized to 16 S rRNA of *E. coli* but not to 23 S rRNA. Restriction pattern of the 2 kb *Eco*RI-*Bam*HI hybridizing DNA segment was identical to the chromosomal region spanning the 5′-flanking region of one of the rRNA operons found in this microorganism (as seen below in fig.4). If we assume that the organization of rRNA operons is similar to the rrnB operon from *E. coli*, as is indicated from the analysis of plasmid pTR-3, all these data strongly suggest that the 2 kb *Eco*RI-*Bam*HI insert DNA, present in pTR-1, carries the promoter(s) for such an operon in *T. ferrooxidans*. Sequencing and functional experiments on the cloned fragment carrying the potential promoter(s) are currently in progress. Although we expected the presence of rDNA sequences in the 4 kb insert DNA adjacent to the *Eco*RI site of the vector DNA in pTR-1 (fig.2B) we did not observe any hybridization of such a DNA to the rRNA probes. We suspect that a DNA rearrangement occurred during the gene library construction.

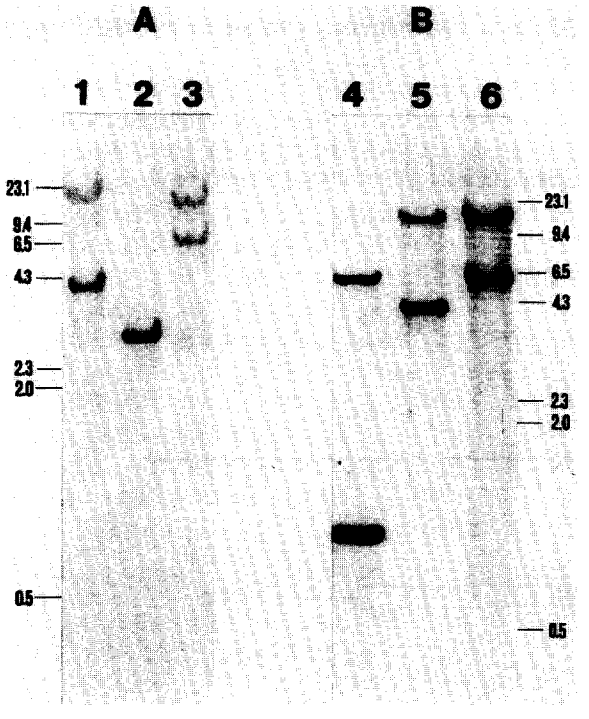

Fig.3. Analysis of chromosomal DNA from *T. ferrooxidans*. Chromosomal DNA was completely digested with *Sal*I (lanes 1,5), *Eco*RI (lanes 3,6) or *Eco*RI and *Sal*I (lanes 2,4). Fragmented DNA was resolved by agarose gel electrophoresis and transferred to Nylon membranes. Hybridization of transferred DNA to probe 1 (panel A) or probe 2 (panel B) was accomplished. Numbers represent lambda DNA markers.

3.2. *Chromosomal DNA analysis*

In order to quantitate the extent of potential reiteration of rRNA genes, the number of copies in *T. ferrooxidans* genome was studied by genomic hybridization to rDNA probes. Three probes, derived from pTR-3 insert DNA (fig.2A), were constructed. Chromosomal DNA was digested with different restriction enzymes and subjected to Southern blotting and hybridization to the three different rDNA probes (fig.3). A compilation of all the data obtained is shown in table 1. Our results led us to conclude that there are two rRNA operons in *T. ferrooxidans* chromosomal DNA (fig.4). A restriction map of both operons, termed

Table 1

Sizes of fragments carrying rDNA generated by digestion of *T. ferrooxidans* chromosomal DNA with restriction enzymes[a]

Probe	E[b]	S	H	P	E/S	S/H	B/E
1	12.0	16.4	20.0	2.8	2.5	3.0	11.2
	6.3	4.0	7.3	1.0		2.8	7.0
			3.1			1.5	
			1.5				
2	16.0	16.0	7.3		5.6	3.0	2.2
	5.6	4.0	3.1		0.9	2.8	2.0
3	12.0	3.5			3.5		
	6.3	2.0			2.0		

[a] Sizes in kilobases. Electrophoresis and calculation of fragment lengths were carried out as is described in section 2
[b] Letters represent restriction enzymes as in fig.2

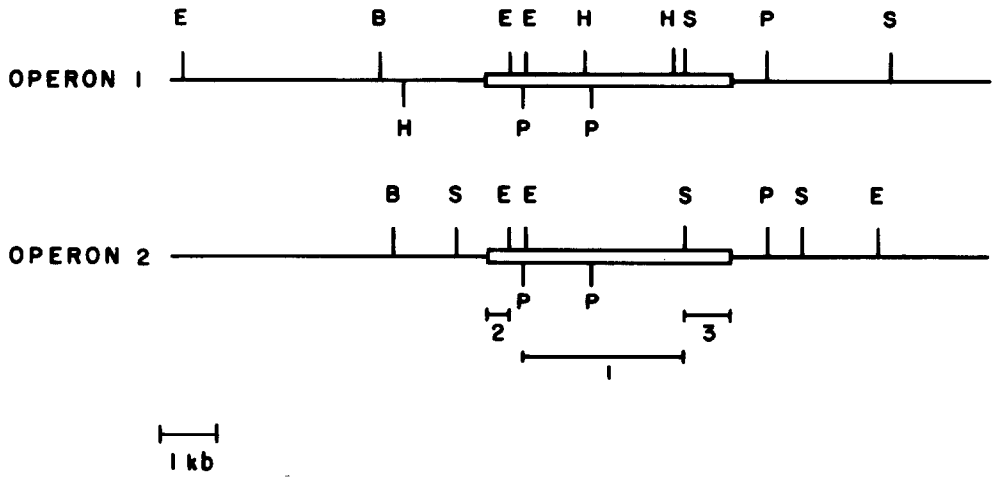

Fig.4. Schematic representation of *T. ferrooxidans* rRNA operons. Bars represent the coding regions detected by the rDNA probes (numbered segments) in both operons. 16 S rDNA is oriented to the left and 23 S to the right side of the bar. Symbols as in fig.2. B, *Bam*HI.

1 and 2, showed that they are similar in their coding sequences with the exception of *Hin*dIII sites (fig.4). Some *Hin*dIII sites were also found in operon 2, but they have not been localized yet. As was expected, both operons showed marked divergences at their flanking regions. As was mentioned above, an interesting feature is that the 2 kb *Eco*RI/*Bam*HI fragment at the 5′-flanking region of operon 2 is identical in size and restriction mapping to the 16 S rRNA hybridizing segment of plasmid pTR-1 (fig.4, see also fig.2B) and probably encodes the operon promoter(s). *T. ferrooxidans* rRNA gene promoters, supposedly found in this work would allow us to analyze the structural and functional properties of expression signals in autotrophic microorganisms and evaluate their utilization in the expression of genes from such a microorganism being of value in the potential modification of the bioleaching process by genetic engineering of *T. ferrooxidans*.

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