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Identification and structural analysis of a ribosomal RNA gene promoter from *Thiobacillus ferrooxidans*

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The 5'-terminus of a rRNA operon ($rrnT_2$) from *Thiobacillus ferrooxidans* was characterized. The rRNA promoters from this microorganism were identified by means of a functional assay in *Escherichia coli*. DNA sequencing of the promoter region, upstream the 16 S rRNA gene, showed the presence of a consensus sequence for bacterial ribosomal promoters. Other features such as a 'discriminator' sequence, antiterminator elements and an upstream hexanucleotide common to several rRNA operons were also found. Two other putative transcription promoters were also identified.

Acidophilic bacterium; Ribosomal RNA; Promoter

1. INTRODUCTION

Thiobacillus ferrooxidans is a Gram negative, acidophilic and autotrophic bacterium that can derive its energy from the oxidation of ferrous iron and several reduced sulfur compounds. These properties make this microorganism one of the most important in the bioleaching of mineral ores. Genetic manipulation of *T. ferrooxidans* may improve the bioleaching of minerals by the production of a bacterium with desirable properties for the industrial process. Achievement of this goal requires the understanding of genetic and molecular events involved in growth and adaptation of the microorganism to the natural environment. With this perspective, we have been interested in the structure of ribosomal RNA genes from *T. ferrooxidans* and the regulatory signals for their expression because they are directly related to cell growth.

In *E. coli*, rRNA gene expression plays a crucial role in the regulation of the synthesis of the translational machinery [1,2]. Therefore, rRNA synthesis is controlled by the nutritional environment since the transcription of these genes is influenced by the cell growth rate and the stringent response of the bacterium to amino acid starvation. *E. coli* rDNA is organized in seven operons in which 16, 23 and 5S rRNA genes are closely linked [1]. Several other microorganisms show a similar rRNA gene organization, an exception being *T. thermophilus* where the 16 S rRNA gene is transcribed separately from the 23 and 5 S genes [3,4]. Previously, we isolated two recombinant plasmids carrying portions of the rRNA genes from *T. ferrooxidans* [5]; pTR3 carried a portion of the 16 S gene, the spacer region and a portion of the 23 S rRNA genes and pTR1 a portion of the 16 S rRNA gene. By using these plasmids as probes for hybridization, we found two rRNA gene operons in the genome of *T. ferrooxidans*. (We renamed these operons, previously numbered as 1 and 2, as $rrnT_1$ and $rrnT_2$, respectively). The organization of these genes was found to be similar to that observed in *E. coli*. In this communication we report the identification by a functional assay and the sequence of the promoter(s) of one of the rRNA operons from *T. ferrooxidans*. Our data show that a consensus sequence for bacterial promoters is present at the 5'-flanking region of 16 S rRNA gene from $rrnT_2$ operon. This putative regulatory signal shows a striking similarity to *E. coli* rRNA gene promoters. Additional possible promoters were identified from the sequenced DNA.

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2. MATERIALS AND METHODS

2.1. *Bacterial strains and phages*

E. coli JM 105, maintained in minimal medium 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 [6]. All recombinant DNAs were constructed according to standard methods [7,8].

2.2. *Sequencing*

Nucleotide sequences were determined using the dideoxy chain termination method [9]. Sequencing reactions were carried out using the Sequenase reagents kit supplied by USB Corp. [^{35}S]dATP (Amer-

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sham) was used as radiolabelled nucleotide. Sequences were analyzed using the PCS computer program, a DNA sequence analysis package developed by L.M. Lagrimini and S.T. Bretano (University of Iowa, USA).

2.3. *Functional promoter assay*

Plasmid pKK232-8 (Pharmacia), a derivative of pBR322, that carries the chloramphenicol acetyltransferase gene lacking a promoter sequence was used as a reporter DNA [10]. *T. ferrooxidans* DNA segments from plasmid pAEB1 (see text) were filled at the ends by the incorporation of deoxynucleotides using the Klenow fragment of *E. coli* DNA polymerase I. Next, the fragments were cloned into the *Sma*I site of the multicloning site in the vector. *E. coli* C 600 was transformed with the DNA constructions and plated on LB solid media in the presence of ampicillin 100 μg/ml and the appropriate concentration of chloramphenicol (cam) ranging from 0 to 500 μg/ml.

3. RESULTS AND DISCUSSION

3.1. *Identification of rRNA gene promoters*

As described previously [5]. *T. ferrooxidans* insert DNA, cloned in plasmid pTR-1, contained a *Bam*HI/*Eco*RI segment that hybridized to 16 S rRNA from *T. ferrooxidans* and *E. coli* and its restriction pattern was identical to the 5'-terminus of $rrnT_2$ operon from *T. ferrooxidans*. To test for the presence of transcription promoters in the insert DNA, the 2.0 kb *Eco*RI/*Bam*HI DNA segment from pTR1 that bears about 1 kb of the 5'-flanking region of $rrnT_2$, was subcloned in pBR322, creating the plasmid pAEB1. A restriction map of this new plasmid was constructed (Fig. 1). A functional approach was used to test the possible promoter activity of different segments derived from the insert DNA in pAEB1. Making use of the plasmid pKK232-8, that carries the chloramphenicol acetyl-transferase gene lacking its own promoter as a reporter gene, the different DNA segments from pAEB1 were assayed for promoter activity as was described in section 2. *E. coli* cells were transformed with the genetic constructions and plated on LB in the presence of 20 μg/ml chloramphenicol (cam). Cell growth was observed only when a 0.4 kb fragment, limited by *Pst*I/*Pst*I restriction sites was inserted in the vector DNA. Four colonies were obtained under these conditions. They were grown in liquid media in the presence of increasing cam concentrations in order to test the minimum amount required to inhibit cell growth. Two of them were able to grow in media containing up to 500 μg/ml of cam (Table I). This is fifty-fold the concentration that inhibits growth in cells transformed with the vector DNA only. The presence of recombinant DNA containing the proper insert from *T. ferrooxidans* was analyzed. Plasmid DNA from *E. coli* cells transformed with the constructions containing the *Pst*I/*Pst*I fragment, grown in the presence of cam, was isolated, digested with restriction enzymes and analyzed by electrophoresis on agarose gels (data not shown).

3.2. *Sequencing of the 5'-flanking region of $rrnT_2$*

Different segments derived from the 2.0 kb insert DNA from *T. ferrooxidans*, cloned in pAEB1, were subcloned in vectors derived from bacteriophage M13 and subjected to sequencing by the dideoxy chain termination method. The sequenced DNA (Fig. 2) confirmed the presence of the 5'-flanking region from $rrnT_2$. The first 76 nucleotides of the 16 S rRNA gene were found and compared to the equivalent region of rrnB operon from *E. coli* [11] showing 79% of similarity between these two sequences.

A structural analysis of the region flanking the 16 S rRNA gene showed the presence of sequences that

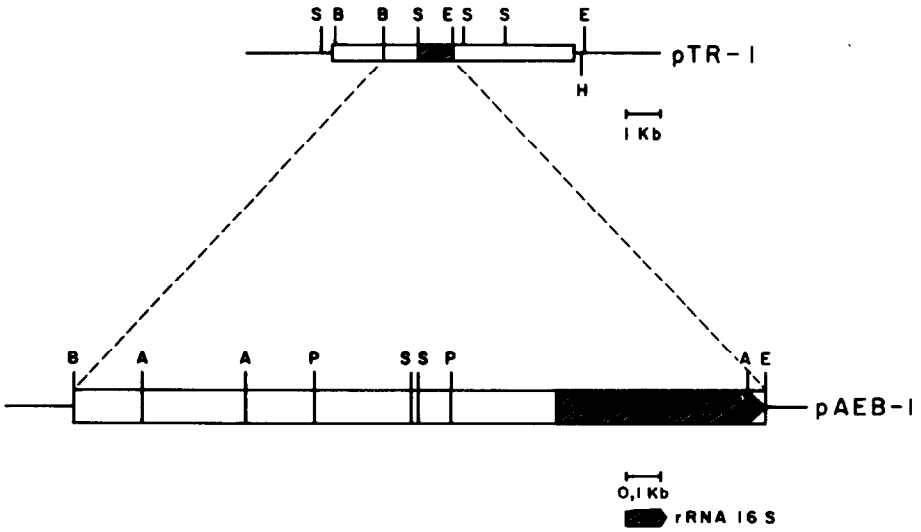

Fig. 1. Diagrammatic representation of recombinant plasmids carrying rRNA sequences from *T. ferrooxidans*. pTR-1 was isolated from a *T. ferrooxidans* gene library [5]. The hatched area represents the segment that hybridized to 16 S rRNA. pAEB-1 was obtained after the subcloning of a 2 kb *Bam*HI/*Eco*RI fragment from pTR-1 in pBR322. The hatched area represents the position of the 5'-terminus of 16 S rRNA gene (see text for details).

resembled the consensus transcription promoter from bacteria. A −35 box (TTGACT) starting at position 470 and a −10 (TATAAT, Pribnow box) at position 491 were found. The consensus sequence for ribosomal gene promoters from *E. coli* [12] was aligned with *T. ferrooxidans* promoter sequence (from nucleotides 456 to 513) showing 31 out of 58 matching nucleotides. The −35 and −10 elements described are homologous to the equivalent elements of P2 and P1 promoters respectively from *E. coli* rRNA operons [2]. The separation between the two promoter elements was found to be 16 nucleotides. In *E. coli*, ribosomal promoters show a 16 base pairs separation between these two elements [14]. This separation, despite its effect in lowering promoter strength [13], is crucial for growth rate-dependent regulation of rRNA gene transcription [14] in this microorganism. According to O'Neill's rules for promoter structure and function [12], the transcription starting point should be the A nucleotide located 9 bp downstream the Pribnow box. We are in progress in the determination of the in vivo transcription initiation point in *T. ferrooxidans*.

Adjacent to the 3'-end of the Pribnow box, a sequence identical to the 'discriminator' of stable RNA genes was found. In *E. coli*, this sequence is apparently involved in the stringent response of cells to amino acid starvation [1,2]. The presence of such a regulatory signal in this microorganism is interesting since *T. ferrooxidans* is an autotrophic bacterium and certain organic compounds such as glucose, lactose and yeast extract among others, that could be used as carbon sources, inhibit cell growth [15]. Upstream the −35 element, a hexanucleotide (AAATTT) identical to those present in *E. coli* operons rrnA, B and C was found in

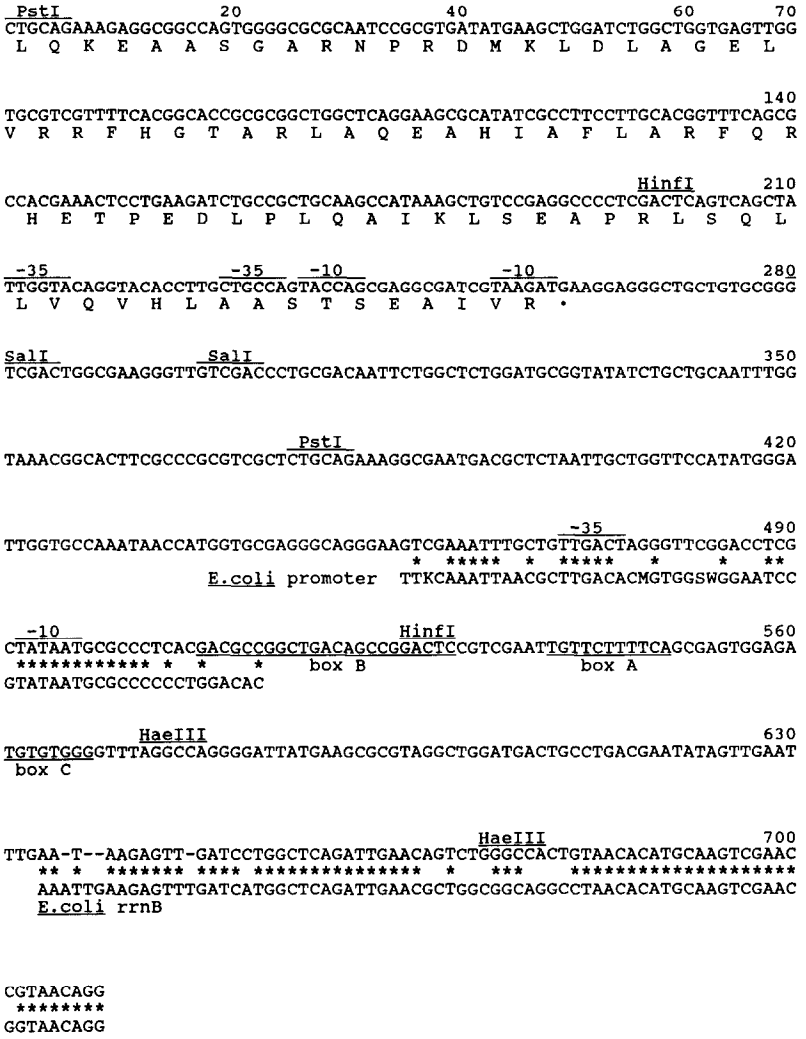

Fig. 2. Sequence of the 5'-terminus of rrnA. The sequence of the DNA segment from the *Pst*I site located to the left of the *Sal*I site in pAEB-1 (see Fig. 1) is shown. −35 and −10 promoter elements and antiterminator sequences are indicated by a solid line above and below the sequences, respectively. The consensus sequence for ribosomal promoters [12] and the 16 S rRNA gene sequence of rrnB [10] from *E. coli* were aligned to *T. ferrooxidans* DNA sequence. Asterisks indicate matching nucleotides. Capital letters and a dot indicate the amino acids (one letter code) and stop codon, respectively, encoded in this DNA segment. K, M, S and W in the *E. coli* promoter consensus sequence represent G or T, A or C, G or C and A or T, respectively.

the same relative position. This is the only similarity in this region of the operons.

It has been proposed that the region upstream the P1 promoter from *E. coli* rRNA operons is a second binding site for RNA polymerase [16,17] and that this binding might be involved in the enhancement of the promoter activity [16,17]. Although the promoter is very similar to *E. coli* promoters, the differences observed in the region upstream from the promoter make it very interesting to know whether there is any regulatory mechanism for rRNA gene expression in *T. ferrooxidans*. Also it would be important to identify any protein that might bind to this region as well as its effect on the activity of the promoter. Rawlings et al. [20] reported the sequence of the glutamine synthetase gene from *T. ferrooxidans*. A nucleotide sequence resembling the glnAp1 promoter from *E. coli* was identified. To our knowledge, this is the only promoter sequence reported for this bacterium to date. This sequence contained 8 out of the 12 nucleotides of the consensus −35 and −10 elements from *E. coli*.

The putative RNA leader sequence contained 126 nucleotides (around 50 nucleotides shorter than *E. coli* sequences). No extended similarity was observed between these two sequences. Some remarkable features were the presence of segments similar to the antiterminator box A and box C elements from *E. coli* operons [1]. No homologous sequence to box B element was found, but a hypothetical secondary structure might be formed in the primary transcript by pairing two consecutive segments that are partially complementary and located in a relative position equivalent to other rRNA operons. This structure could reflect the result of the conservation in rRNA genes of a secondary structure rather than a sequence for an unknown function. Also, a seven nucleotide segment identical to the putative RNase III cleavage site from *E. coli* [18] was identified in this region. No other similarities relevant to the stem formed between the leader and the 3′-flanking segments of the 16 S rRNA precursor were found.

Two other putative transcription promoters were found in the sequenced DNA segment. Such promoters were located from nucleotides 211 to 242 and 229 and 259, respectively. Both promoters showed seven out of twelve nucleotides matching the consensus promoter elements from *E. coli*. The separation between these sequences was 20 and 18 nucleotides, respectively. Although these promoter sequences might be in that position at random, it is interesting to note that they are located within the active fragment (a 0.4 kb fragment limited by *Pst*I restriction sites) identified by the functional assay in *E. coli* (see Table I). Preliminary data, obtained by S1 nuclease mapping of the transcription starting point showed that the first of these two putative promoters might be active in vivo in *T. ferrooxidans* (not shown).

Table I

Functional analysis in *E. coli* of rRNA gene promoters from *T. ferrooxidans*

Fragment[a]	Size (kb)	Colony growth[b] (+ chloramphenicol (μg/ml)		
		20	200	500
*Bam*HI/*Eco*RI	2	nd	nd	nd
*Sal*I/*Eco*RI	1	nd	nd	nd
*Pst*I/*Pst*I	0.4	+[b]	+(+)	(+)

[a] DNA segments from pAEB-1 subcloned into the *Sma*I site of plasmid pKK232-8 and subjected to functional analysis

[b] Colony growth after 16 h: +, colony growth after 48 h: (+); nd: not detected

An open reading frame for 84 amino acids was identified in the initial part of the sequenced fragment. The terminal portion of protein coding segment overlapped the two promoter-like sequences found between nucleotides 211 and 259. A protein encoded by this sequence would be highly hydrophobic but no homology was found to any sequence filed in NBRF data bank. No transcription terminator was found at the end of this reading frame. Open reading frames upstream rRNA promoters have been found in *E. coli* [11] and other microorganisms [4,19]. All encode a highly hydrophobic protein, but no function has been identified for the gene products.

In conclusion, our results showed that several features observed in bacterial ribosomal RNA gene promoters were present in the transcription signal of $rrnT_2$ from the acidophilic bacterium *T. ferrooxidans*. Among them we found the −10 and −35 promoter elements, the discriminator and antitermination sequences, an RNase III cleavage site and an upstream open reading frame encoding a highly hydrophobic polypeptide. Particularly interesting was the presence of two putative overlapping promoters located in the region encoding the terminal portion of the open reading frame. They were active in vivo in *E. coli*. It might be of interest to study the functionality of these promoters and to correlate it with possible relationships between the nutritional environment, where the cells grow, and the expression of rRNA genes.

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