Small cytoplasmic RNA of *Bacillus brevis*: transcriptional and phylogenetic analysis

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Using a DNA fragment of *Bacillus subtilis* scRNA as a probe, a *Bacillus brevis* gene encoding the small cytoplasmic RNA was cloned and characterized. *B. brevis* scRNA consists of 273 nucleotides; the sequence has comparatively low homology (approximately 70%) with other *Bacillus* sequences. Phylogenetic analysis indicated that *B. brevis* forms a line of descent distinct from other *Bacillus* species. However, despite the low overall homology, both functional nucleotide sequence and secondary structural features defined among signal recognition particle (SRP) RNA family members were well conserved.

**Keywords:** small cytoplasmic RNA, signal recognition particle, *Bacillus brevis*, phylogenetic analysis

INTRODUCTION

Signal recognition particle (SRP) is a cytoplasmic ribonucleoprotein which targets the ribosomes translating nascent secretory proteins to the endoplasmic reticulum membrane (Walter & Lingappa, 1986). The extensively studied canine particle is composed of one RNA molecule (here referred to as SRP RNA) and six polypeptides (Walter & Blobel, 1980, 1982). SRP RNA-like RNAs have been identified in a wide range of eukaryotes, including humans, plants and yeast, and in a wide variety of archaeobacteria (Larsen & Zwieb, 1991).

The small cytoplasmic RNA (scRNA; 271 nucleotides) of *Bacillus subtilis* is considered to be a member of the SRP RNA family. Although dissimilar in size and overall primary structure to mammalian SRP RNA, it contains domains of which the secondary structures correspond to domains I, II and IV of the SRP RNA-like RNAs of archaeobacteria and eukaryotes. The nucleotide sequence in domain IV is highly conserved (Poritz et al., 1988; Kaine, 1990). Functional analyses have shown that this RNA is essential for the cell viability of *B. subtilis* (Nakamura et al., 1992a). To identify the functional domains of scRNA, knowledge of its structure is required, and phylogenetic comparisons between homologous RNAs have proved useful in elucidating a higher-order RNA structure (Romero & Blackburn, 1991; Haas et al., 1991). It is therefore useful to compare scRNAs from more closely related species. The scRNA cataloguing studies have so far been restricted to two *Bacillus* species, *B. subtilis* and *B. steaothermophilus* (Struck & Erdmann, 1990). Consequently, it has not been possible to draw reliable conclusions about the phylogenetic structure of the genus. To identify the conserved nucleotides and structural elements of scRNA, we determined the nucleotide sequence of scRNA from various bacilli (Nakamura et al., 1992b). DNA fragments corresponding to the mature scRNA of ten *Bacillus* species were obtained by poly-nucleotide chain reaction (PCR), using primers based upon comparisons of the nucleotide sequence of scRNAs between *B. subtilis* and *B. steaothermophilus*. A positively amplified DNA fragment was not obtained in *B. brevis*.

In this report, we describe the cloning and nucleotide sequencing of the entire structural portion of the *B. brevis* scRNA gene. We also determined the 5' and 3' ends of mature scRNA. A pairwise comparison of scRNA sequences from 13 *Bacillus* species indicates that *B. brevis* forms a distinct line of descent in the genus *Bacillus*.

METHODS

**Bacterial strains.** *Escherichia coli* JM109 (Yanisch-Perron et al., 1985) and NM514 [hidR514 (r6mC) argH galE galX strA tycB7 (Hi-)'] were used as cloning hosts. *B. brevis* ATCC 5246 was obtained from the Bacillus Genetic Stock Center (Ohio State University). The cells were cultured in L broth at 37 °C.

**DNA and RNA preparation.** Chromosomal DNA of *B. brevis* ATCC 5246 was prepared by the method of Saito & Miura.
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from diethyl pyrocarbonate (DEPC).

DNA Southern hybridization. DNA preparations digested with restriction enzymes were separated by agarose gel electrophoresis and transferred onto Gene Screen Plus nylon membranes (NEN Research Products). Hybridization under low stringency proceeded as described previously (Nakamura et al., 1990). The 271 bp DNA fragment, corresponding to mature scRNA, was amplified from the chromosomal DNA of B. subtilis 168 using PCR with the synthetic oligonucleotide primers 5’ TTTGCCGTGCTAAGCGGG 3’ and 5’ CGGCACATGAGAGGTAATTCCCAT- GGAATTCACACCCTGGCTATT 3’) used in primer extension was synthesized automatically (Applied Biosystems). Total DNA was sequenced by dideoxy chain termination (Sanger et al., 1977). Both strands were entirely sequenced, and all ends of the fragments overlapped.

Primer extension. The oligonucleotide (NK1; 5’ AGGATAGGAATTTCAACCCCTGGCTATT 3’) used in primer extension was synthesized automatically (Applied Biosystems). Total B. brevis RNA (40 μg) and 5 x 10⁶ c.p.m. of the ³²P-labelled oligonucleotide primer were hybridized at 40 °C overnight. RAV-2 (Rous associated virus 2) reverse transcriptase was added and the mixture was incubated at 42 °C for 1 h. The reaction products were analysed on DNA sequencing gels. The length of extended DNA fragment was estimated by comparison with sequencing ladders generated from M13mp10.

RNAse protection assay. A BamHI–EcoRI fragment, including a 350 bp 3’ portion of the B. brevis scRNA gene, was inserted between the BamHI and EcoRI sites of Bluescript II SK(−) under the control of the bacteriophage T3 promoter. T3 RNA polymerase and [α-³²P]CTP were used to synthesize the labelled probe, which encompasses the 3’ portion of B. brevis scRNA, and to which it is complementary. The RNase protection assay was performed as described previously (Nakamura et al., 1992a), using total RNA (10 μg) and 3-9 x 10⁶ c.p.m. of the ³²P-labelled probe.

RESULTS AND DISCUSSION

Cloning and sequencing of the scRNA gene region

To verify the existence of the scr gene in chromosomal DNA of B. brevis ATCC 5246, Southern hybridization was performed using the B. subtilis scRNA gene without domain IV as a probe. A 3.8 kb PvuII–HincII fragment specifically hybridized with the probe. PvuII–HincII-digested chromosomal DNA from B. brevis ATCC 5246 was resolved by agarose gel electrophoresis. Fragments of 3–5 kb were extracted from the gel and ligated with the EcoRI–NotI adaptor (5’ dAATTCGCGGCCGCT 3’, 5’ dAGCGGCCGCG 3’) to create EcoRI sites at both ends. The resultant DNA fragments were inserted into λgt10, packed in vitro, then transfected into E. coli NM514. Among 5000 plaques, 25 carried the 3.8 kb insert. Plasmid pTUE825 was constructed by subcloning the 3.8 kb NotI fragment from the phage DNA into the NotI site of Bluescript II KS(−). Physical mapping followed by Southern analysis of pTUE825 revealed that a NotI– DraI fragment of about 1-kb contains the scRNA coding region (Fig. 1) and the entire region of this fragment was sequenced (Fig. 2a). The sequences were aligned with the mature form of B. subtilis scRNA (Fig. 2b). The scRNA coding region of B. brevis deduced from this alignment, underlined in Fig. 2a, is about 70% identical to that of B. subtilis scRNA. About 40 bp upstream from the putative scRNA coding region, a possible promoter region was identified (−35 box, TTGCTA; −10 box, TATTCT), which was highly homologous with the consensus sequence recognized by the B. subtilis σ⁵ RNA
Characterization of *B. brevis* scRNA

Figure 2. (a) Nucleotide sequence of the chromosomal segment containing the *B. brevis* scRNA gene. The nucleotide sequence of the Dral–Not1 fragment in pTUE825 was determined and numbered arbitrarily. The sequence of mature scRNA of *B. subtilis* deduced by alignment with that of *B. subtilis* is underlined. The presumed promoter elements (−10 and −35) are boxed. The positions of the 5' and 3' ends of the mature scRNA determined in Figure 3 are indicated by single and double asterisks, respectively. Palindromic sequences which may serve as transcriptional terminator are indicated by converging arrows. A nucleotide sequence complementary to the synthetic oligonucleotide primer used in primer extension is overlined.

(b) A comparison of the primary structure of *B. subtilis* scRNA (SUB) and the nucleotide sequence determined here for *B. brevis* scRNA (BRE). Asterisks indicate identical nucleotides between the two sequences.

Mapping the 5' and 3' ends of scRNA

To examine the *in vivo* functionality of these transcriptional regulatory elements, both the 5' and 3' ends of scRNA were mapped. In addition, we investigated whether processing events occurred in *B. brevis* scRNA, since RNA processing is involved in the maturation of the *B. subtilis* scRNA. The 5' end was identified by primer extension. Using the NK1 primer, only a 75 nucleotide band appeared (Fig. 3a), indicating that transcription starts from the T residue at nucleotide position 473 (Fig. 2a). This position matched well with the 5' end predicted by polymerase (−35 box, TTGACA; −10 box, TATAAT). About 30 bp downstream from the scRNA coding region there is an almost perfect palindromic sequence comprising 26 bp (ΔG = −25.7 kcal mol⁻¹; 107.5 kJ mol⁻¹) as calculated by the method of Tinoco *et al.* (1973), followed by a run of thymidine residues (Fig. 3c). This region resembles a characteristic *E. coli* p-independent transcription terminator (Adhya & Gottesman, 1978).
Table 1. Similarity and nucleotide distance data among scRNA gene sequences of Bacillus spp.

The upper right half of the table gives similarity values, $H$, for all pairwise comparisons of the scRNA sequences from 13 Bacillus species. $H$ is defined as in Sogin et al. (1986), where $H = m/(m + u + g/2)$; $m$ is the number of sequence positions with matching nucleotides in the two sequences, $u$ is the number of sequence with nonmatching nucleotides, and $g$ is the number of sequence positions that have a gap in one sequence opposite a nucleotide in the other. The absolute number of base changes and gapped positions is shown in the lower half of the table. The species names are abbreviated as follows: B. subtilis 168 (B. sub), B. amyloleiquefaciens H (B. amy), B. polymyxa ATCC 842 (B. pol), B. sphaericus 1593 (B. sp), B. pumilus PI (B. pum), B. macerans BKM B-51 (B. mac), B. megaterium 899 (B. meg), B. thuringiensis B4039 (B. thu), B. cereus T (B. cer), B. circulans ATCC 4513 (B. cir), B. stearothermophilus 799 (B. ste), alkalophilic Bacillus C-125 (B. C125) and B. brevis ATCC 5246 (B. bre).

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alignment with the B. subtilis mature scRNA sequence (Fig. 2b). No other minor pre-scRNA was detected by primer extension.

For 3' end mapping, a 0.6-kb BamHI-EcoRI fragment encompassing the 3' portion and the 3' flanking region of the B. brevis scRNA gene (Fig. 1) was cloned into the riboprobe plasmid pBluescript II SK(−) and used to generate a 32P-labelled antisense RNA probe. RNA was prepared 3, 6, 9 and 12 h after inoculation. Fig. 3(b) shows three major and several minor protected RNA species. The three major species comprised 210, 206 and 204 nucleotides, among which the longest (210 nucleotides) may represent the 3' end of mature scRNA. The 3' end heterogeneity is probably an artifact caused during RNA preparation or the RNase protection assay, since the smaller protected RNA fragments varied in length with the experiment and RNA preparation. This suggests that the transcription of the scRNA gene terminates at the A residue at nucleotide position 745. The 5' and 3' mapping study shows that the mature form of B. brevis scRNA consists of 273 nucleotides.

Phylogeny of Bacillus scRNA

Including the nucleotide sequence of B. brevis scRNA reported here, sequence data of scRNAs from 13 Bacillus species are now available. Gram-positive, rod-shaped, aerobic or facultatively anaerobic spore-forming bacteria have traditionally been assigned to the genus Bacillus. This genus as constituted in Bergey's Manual of Systematic Bacteriology (Claus & Berkeley, 1986) is phenotypically heterogeneous, demonstrating an extremely wide range of nutritional requirements, growth conditions, metabolic diversity and DNA composition. A phylogenetic matrix study of the scRNA sequence also shows that the genus Bacillus is phylogenetically very heterogeneous (Table 1). B. brevis scRNA exhibited remarkably low sequence homologies with the other Bacillus groups. Based upon a comparison of the sequences of small-subunit-ribosomal RNA, Ash et al. (1991) have reported that the genus Bacillus can be separated into phylogenetically distinct groups. Among these, a group consisting of B. brevis and B. laterosporus forms a distinct line of descent exhibiting low levels of relatedness to all other Bacillus groups. This phylogenetic relationship agreed well with that deduced from scRNA. In contrast, a pairwise comparison of small-subunit-ribosomal RNA reveals the very wide percentage sequence homology variation (range approximately 88–99%). Therefore, the overall rate of divergence for scRNA is greater than that for 16S rRNA.

Despite the relatively low sequence homology of the scRNA sequence between B. brevis and other Bacillus species, almost all nucleotide substitutions in scRNA are covariant and do not affect the secondary structure. That is, mutations that individually affect base pairing are often compensated by base changes on the other strand. Consequently, a strikingly similar secondary structure can be drawn for B. brevis scRNA based on that of B. subtilis (Struck & Erdmann, 1990). The presence of a terminal loop, corresponding to domains I and II of human SRP RNA, is restricted to spore-forming Bacillus species among eubacteria. In domain I of B. subtilis scRNA,
nucleotides 24–28 (5′ AGCGG 3′) can pair with nucleotides 46–50 (5′ CCGGU 3′). A potential tertiary interaction has been detected within this region (Fig. 4a). This interaction can be maintained in B. brevis scRNA by complementary base changes at nucleotide positions 24 and 50 (Fig. 4a). Therefore, this interaction is considered to also be important for the function of scRNA. It is notable that the same pairing can form in human SRP RNA and archaeobacterial 7S RNA (Fig. 4b, c), but not in yeast homologues.

On the other hand, the 22-nucleotide sequence in domain IV is completely identical among all Bacillus scRNAs. In eukaryotic SRP RNA and E. coli 4-5s RNA, this conserved structure serves as the binding site for SRP54/P48 proteins and has a pivotal role in RNA function in vitro (Selinger et al., 1993; Wood et al., 1993). Recently, we have cloned the B. subtilis gene encoding a homologue of SRP54/P48 (Honda et al., 1993). These results suggest that the corresponding region of scRNA of B. subtilis species functions in the same manner as eukaryotic and E. coli RNAs. Biochemical study and determination of the effects caused by mutations in domain IV may help in understanding of the function of this region.

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