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. stearothermophilus* (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 polynucleotide chain reaction (PCR), using primers based upon comparisons of the nucleotide sequence of scRNAs between *B. subtilis* and *B. stearothermophilus*. 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 [*hsdR514* ($r_K^- m_K^-$) *argH galE galX strA lyaB7* (*Hfl⁺*)] 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

Abbreviations: scRNA, small cytoplasmic RNA; SRP, signal recognition particle.

The GenBank accession number for the nucleotide sequence reported in this paper is D14472.

Fig. 1. Physical map and location of the *scr*RNA coding region (*scr*) in the subcloned 3.8 kb *NotI* fragment of pTUE825. The arrow indicates the direction of transcription of the *scr*. B, *Bam*HI site; E, *Eco*RI site; N, *NotI* site. The location of the *scr*RNA coding region was determined by Southern hybridization, using the *B. subtilis* *scr*RNA gene as the probe. +, +/- and - indicate the strength of hybridization signals.

(1963) and stored at 4 °C in TE buffer. Total RNA was isolated from *B. brevis* ATCC 5246 grown to the mid-exponential phase (OD_{600} , 0.5–0.6) as described previously (Nakamura *et al.*, 1992a) and stored at –80 °C in distilled water treated with 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 *scr*RNA, was amplified from the chromosomal DNA of *B. subtilis* 168 using PCR with the synthetic oligonucleotide primers 5' TTTGCCGTGCTAAGCGGG 3' and 5' TACCGTGCACCTTCTGTC 3'. The amplified DNA fragment was inserted at the *Sma*I site of M13mp10. To prepare the specific probe which cannot hybridize to the 4.5S RNA gene of *E. coli* but can hybridize to *Bacillus* *scr*RNA, 42 nucleotides in domain IV, corresponding to +144 to +185, were deleted by oligonucleotide-directed mutagenesis, using the mutagenic oligonucleotide 5' CGGCACATGAGAGGTAATTTCCCAT-TGCGCA 3'. After *Kpn*I–*Bam*HI digestion of the double-stranded M13 phage DNA, the 234-nucleotide fragment of *Bacillus subtilis* *scr*RNA gene without domain IV was isolated by polyacrylamide gel electrophoresis. This fragment was labelled with [α - 32 P]dCTP using a random primer DNA labelling kit (Takara Shuzo Co.) and used as the probe.

DNA sequence analysis. 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' AGGATAGGAATTCACACCCTGGCTATT 3') used in primer extension was synthesized automatically (Applied Biosystems). Total *B. brevis* RNA (40 µg) and 5×10^4 c.p.m. of the 32 P-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 *Bam*HI–*Eco*RI fragment, including a 350 bp 3' portion of the *B. brevis* *scr*RNA gene, was inserted

between the *Bam*HI and *Eco*RI sites of Bluescript II SK(–) under the control of the bacteriophage T3 promoter. T3 RNA polymerase and [α - 32 P]CTP were used to synthesize the labelled probe, which encompasses the 3' portion of *B. brevis* *scr*RNA, 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 3.9×10^6 c.p.m. of the 32 P-labelled probe.

RESULTS AND DISCUSSION

Cloning and sequencing of the *scr*RNA 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* *scr*RNA gene without domain IV as a probe. A 3.8 kb *Pvu*II–*Hinc*II fragment specifically hybridized with the probe. *Pvu*II–*Hinc*II-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 *Eco*RI–*Not*I adaptor (5' dAATTTCGCGCCGCT 3', 5' dAGCGGCCGCG 3') to create *Eco*RI sites at both ends. The resultant DNA fragments were inserted into λ gt10, 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 *Not*I fragment from the phage DNA into the *Not*I site of pBluescript II KS(+). Physical mapping followed by Southern analysis of pTUE825 revealed that a *Not*I–*Dra*I fragment of about 1.1 kb contains the *scr*RNA 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* *scr*RNA (Fig. 2b). The *scr*RNA coding region of *B. brevis* deduced from this alignment, underlined in Fig. 2(a), is about 70% identical to that of *B. subtilis* *scr*RNA. About 40 bp upstream from the putative *scr*RNA 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* σ^A RNA

Fig. 2. (a) Nucleotide sequence of the chromosomal segment containing the *B. brevis* scRNA gene. The nucleotide sequence of the *DraI*-*NotI* fragment in pTUE825 was determined and numbered arbitrarily. The sequence of mature scRNA of *B. brevis* 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 Fig. 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.

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 ($\Delta G = -25.7$ 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

Fig. 3. Determination of the 5′ and 3′ ends of *B. brevis* scRNA. (a) Mapping the 5′ terminus by primer extension using primer NK1 (Fig. 2a). RNA samples were prepared from *B. brevis* cultured for 6 (lane 1), 9 (lane 2) and 12 h (lane 3) after inoculation. Sequencing reactions (G, A, T and C) served as size markers. (b) Mapping the 3′ terminus by means of an RNase protection assay of the scRNA gene transcript. RNA samples were prepared from *B. brevis* cultured for 3 (lane 1), 6 (lane 2), 9 (lane 3) and 12 h (lane 4). Sequencing reactions (G, A, T and C) served as size markers. The lengths of some fragments are indicated. Protected major and minor fragments are indicated by arrows. (c) Suggested secondary structure of the 3′ portion of the *scr* gene, highlighting the secondary structure that may serve as the *p*-independent terminator. Arrows are according to (b).

region resembles a characteristic *E. coli* *p*-independent transcription terminator (Adhya & Gottesman, 1978).

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

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. amyloliquefaciens* H (*B. amy.*), *B. polymyxa* ATCC 842 (*B. pol.*), *B. sphaericus* 1593 (*B. sph.*), *B. pumilus* BP1 (*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.*).

	<i>B. sub</i>	<i>B. amy</i>	<i>B. pol</i>	<i>B. sph</i>	<i>B. pum</i>	<i>B. mac</i>	<i>B. meg</i>	<i>B. thu</i>	<i>B. cer</i>	<i>B. cir</i>	<i>B. ste</i>	<i>B. C125</i>	<i>B. bre</i>
<i>B. sub</i>	—	1.0	0.998	0.985	0.969	0.958	0.895	0.875	0.879	0.877	0.868	0.850	0.732
<i>B. amy</i>	0	—	0.998	0.985	0.969	0.958	0.895	0.875	0.879	0.877	0.868	0.850	0.732
<i>B. pol</i>	1	1	—	0.983	0.967	0.960	0.889	0.873	0.881	0.879	0.862	0.848	0.729
<i>B. sph</i>	1	4	5	—	0.954	0.947	0.880	0.863	0.875	0.866	0.852	0.842	0.721
<i>B. pum</i>	9	9	10	13	—	0.952	0.885	0.869	0.877	0.882	0.869	0.836	0.705
<i>B. mac</i>	12	12	11	15	14	—	0.856	0.866	0.870	0.871	0.851	0.837	0.716
<i>B. meg</i>	29	29	31	33	31	40	—	0.850	0.866	0.847	0.865	0.862	0.695
<i>B. thu</i>	35	35	36	38	36	38	41	—	0.978	0.839	0.852	0.842	0.711
<i>B. cer</i>	36	36	35	37	36	38	39	9	—	0.844	0.853	0.848	0.727
<i>B. cir</i>	34	34	33	37	33	35	43	44	45	—	0.847	0.822	0.728
<i>B. ste</i>	37	37	38	40	36	41	37	41	43	42	—	0.801	0.708
<i>B. C125</i>	43	43	44	45	45	47	39	44	42	51	55	—	0.705
<i>B. bre</i>	74	74	74	77	81	78	84	80	75	76	78	80	—

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 kbp *Bam*HI-*Eco*RI 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,

Fig. 4. Schematic drawing of two stem-loops in the domain I region of *B. subtilis* scrRNA (a), *Sulfolobus solfataricus* 7S RNA (b), and human SRP RNA (c). Dashed lines show the possible tertiary interaction in each RNA. Circles denote the nucleotide changes in the *B. brevis* scrRNA sequence, compared with that of *B. subtilis*. U·G and A·G base pairs are indicated by dots.

nucleotides 24–28 (5' AGCGG 3') can pair with nucleotides 46–50 (5' CCGCU 3'). A potential tertiary interaction has been detected within this region (Fig. 4a). This interaction can be maintained in *B. brevis* scrRNA 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 scrRNA. 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* scrRNAs. 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 vivo* (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 scrRNA 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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