Small cytoplasmic RNA (scRNA) gene from *Clostridium perfringens* can replace the gene for the *Bacillus subtilis* scRNA in both growth and sporulation

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Small cytoplasmic RNA (scRNA) is a member of an evolutionarily conserved signal-recognition-particle-like RNA family. Using a DNA fragment of *Bacillus subtilis* scRNA gene as a probe, we cloned and characterized a *Clostridium perfringens* gene encoding the scRNA. Mapping the 5' and 3' ends of scRNA revealed that *C. perfringens* scRNA consists of 269 nucleotides: the sequence has about 70% primary sequence homology with *B. subtilis* scRNA. The predicted secondary structure appeared to be similar to that of *B. subtilis* scRNA, indicating that there are domains I and II in *C. perfringens* scRNA, in addition to domain IV. Functional analysis showed that *C. perfringens* scRNA could compensate for vegetative growth and allow the formation of heat-resistant spores in an scRNA-depleted *B. subtilis* strain, whereas *Escherichia coli* 4-5S RNA could not maintain sporulation. Since both *E. coli* 4-5S RNA and *C. perfringens* scRNA have the same binding specificity to *B. subtilis* Ffh protein, the difference in complementation activity reflects the function of domains I and II.

**Keywords:** *Clostridium perfringens*, small cytoplasmic RNA, SRP-like RNA, sporulation

**INTRODUCTION**

Several species of RNA, such as mRNA, rRNA and tRNA, and many small RNA forms (~300 nucleotides) are present in cells (Fournier & Maxwell, 1993; Guthrie, 1991; Inouye & Delilas, 1988). Although there is a growing list of small RNAs, their functions are little understood. Among them, signal-recognition-particle (SRP)-like RNAs have been identified in a wide range of eukaryotes, including *Homo sapiens* (Ullu & Weiner, 1984), plants (Campos et al., 1988), *Schizosaccharomyces pombe* (Brennwald et al., 1988), and several species of Archaea (Kaine, 1990). The most studied SRP-like RNA is the mammalian 7SL RNA that is a component of the SRP (Walter & Blobel, 1982). SRP is essential for targeting a subset of nascent presecretory proteins emerging from the ribosome to the endoplasmic reticulum membrane (Gilmore, 1993; Nunnari & Walter, 1992; Rapoport, 1991, 1992; Sanders & Schekman, 1992; Walter & Lingappa, 1986). It has been proposed that mammalian SRP-like RNA consists of four domains (domains I–IV). In **vivo**, six proteins (9, 14, 19, 54, 68 and 72 kDa) bind to each domain (Walter & Lingappa, 1986). Among them, the 54 kDa GTPase protein (SRP54) binds to both the signal sequence and domain IV (High & Dobberstein, 1991; Krieg et al., 1986; Kurzchalia et al., 1986; Romish et al., 1990; Samuelsson & Olsson, 1993; Zopf et al., 1990). An evolutionary comparison has revealed that almost all eukaryotic and archaeal SRP-like RNAs are similar in size and can be folded into four functional domains. In contrast, bacterial SRP-like RNAs vary in size and secondary structure (Larsen & Zwieb, 1991). *Escherichia coli* 4-5S RNA (114 nucleotides), which was first identified as a translational modulator (Bourgaize & Fournier, 1987; Brown, 1987, 1989; Brown & Fournier, 1984), folds into a single hairpin. The primary and secondary
structures of E. coli 4-5S RNA have significant homology with that of domain IV in Homo sapiens SRP-like RNA (Poritz et al., 1988). However, this RNA lacks structures resembling domains I and III, and more than half of domain II is missing. Five other bacterial RNAs, including a 105 nucleotide RNA from the phylogenetically deepest branching Thermus thermophilus (Struck et al., 1988a), also fold into a structure similar to that of E. coli RNA. The Mycoplasma mycoides RNA (77 nucleotides) is most extensively diverged (Samuelsson & Guindy, 1990). On the other hand, small cytoplasmic RNA (scRNA; 271 nucleotides) from Bacillus subtilis contains structures corresponding to domains I and II of the mammalian RNA (Struck & Erdmann, 1990; Struck et al., 1988b), in addition to domain IV. We found that this structure is conserved among thirteen Bacillus species (Nakamura et al., 1992b). Functional analyses have shown that depletion of scRNA in B. subtilis led to defects in the production of extracellular enzymes and in β-lactamase translocation as well as to dramatic morphological changes (Nakamura et al., 1992a). These deficient characteristics could be compensated by introducing SRP-like RNAs from humans or E. coli, indicating that the essential function of SRP-like RNAs is evolutionarily conserved and that an essential function locates in domain IV. There is a discrepancy between the structural features of SRP-like RNAs and eubacterial phylogeny based upon 16S rRNA sequence comparisons, since T. thermophilus has been placed in the earliest branches of the eubacterial evolutionary tree, whereas bacilli are thought to have arisen considerably later in eubacterial evolution (Althoff et al., 1994). This lack of congruence between an established rRNA phylogeny and the size of eubacterial SRP-like RNAs begged to identify the structural determinants of B. subtilis scRNA, we constructed mutants in which individual domains or conserved nucleotides were deleted, and their importance was assayed in vivo (Nishiguchi et al., 1994). The results showed that domain IV is necessary to maintain cell viability, whereas domains I and II are not essential for vegetative growth but are needed for the formation of heat-resistant spores. Therefore, SRP-like RNA of other spore-forming eubacteria may have a similar structure to that of B. subtilis scRNA.

Here, we report that the secondary structure of scRNA from Clostridium perfringens appears to be similar to that of B. subtilis scRNA and that sporulation in scRNA-depleted B. subtilis can be compensated by introducing a Clostridium homologue. Therefore, it is likely that SRP-like RNA has undergone a dramatic reduction several times during bacterial evolution. However, the domains essential for sporulation remained because they conferred advantages.

**METHODS**

**Bacterial strains and vector plasmids.** Clostridium perfringens NCTC8237 was derived by H. Hayashi, Institute of Basic Medical Sciences, University of Tsukuba, Japan. B. subtilis SC200NA (purB6 trpB3 metB5 scr::pTUE807) was constructed as described by Nakamura et al. (1992a). In this strain, intact scRNA gene expression is regulated by the IPTG-inducible promoter P\textsubscript{Pspac}. This strain has been maintained in the presence of 3 mM IPTG. The expression plasmid pTUBE809 is an E. coli–B. subtilis shuttle vector. Plasmid pTUBE809 contains a synthetic 98 bp protein A promoter of Staphylococcus aureus. This promoter is constitutive and expressed even in the absence of IPTG. Plasmid PCR1000, into which primary products of the PCR can be cloned, was purchased from Invitrogen. The vectors pTUBE822 and pTUBE811, which can constitutively produce scRNA of B. subtilis and 4-5S RNA of E. coli, respectively, were constructed as described by Nakamura et al. (1992a).

**Determination of the efficiency of sporulation.** The efficiency of sporulation was determined as described by Nishiguchi et al. (1994). The bacterial cells were vigorously shaken in modified Schaeffer medium and harvested after 24 h. The spores were heated at 80 °C for 10 min and plated. After 12 h, the number of cells on the plate was counted.

**Construction of the plasmid vector pTUBE850.** A 269 bp DNA fragment, encoding mature C. perfringens scRNA, was amplified from the chromosomal DNA by means of PCR with the synthetic oligonucleotides 5' TGGCGTGTAGATGGGGA 3' and 5' AACCGTGACCTCACTTCTGTC 3'. Purified PCR product was inserted into the HphI site of the PCR1000 vector to create plasmid pTUBE840. A 315 bp Ncol–HindIII fragment was isolated from pTUBE840 and blunt-ended. The purified fragment was inserted between the protein A promoter and the trp transcriptional terminator. The resultant plasmid, pTUBE850, was confirmed by physical mapping and DNA sequencing.

**DNA Southern hybridization.** Chromosomal DNA of C. perfringens was prepared by the method of Saito & Miura (1963). DNA preparations digested with restriction enzymes were separated by agarose gel electrophoresis and transferred onto a Gene Screen Plus nylon membrane (NEN Research Products). Hybridization under low stringency proceeded as described by 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 oligonucleotides primers 5' TTTGGCCGTGTAGATGGGGA 3' and 5' TACCGTGACCTCACTTCTGTC 3'. The amplified DNA fragment was isolated at the HphI site of PCR1000 fragment was labelled with [α-32P]dCTP using a random primer labelling kit (Takara Shuzo) and used as a probe.

**DNA sequencing analysis.** The nucleotide sequence of the entire 2-7 kb HindIII fragment containing the scRNA gene was determined. The fragment was first degraded by sonic oscillation, then 0.5–1 kb fragments were isolated. After repairing both ends with Klenow large fragment and T4 DNA polymerase, the fragments were inserted into the Smal site of pUC119. DNA fragments in the recombinant plasmids were amplified and sequenced by dideoxy-chain termination using an automatic DNA sequencer type 373A (Perkin-Elmer Japan). Both strands were sequenced at least three times, and all ends of the fragments overlapped. The primary sequences were edited and analysed by GENETYX-MAC (Software Development Company, Tokyo, Japan). The DNA sequences were compared by sending them to the blast-mail server at the Human Genome Center at Tokyo University.

**Mapping of both ends of scRNA transcripts.** Total RNA of C. perfringens was extracted as described by Nakamura et al. (1992a).
Poly(d1-dC). After a 40 min incubation at room temperature, acrylamide gel containing 45 mM Tris/HCl (pH 8.3) and were incubated with various amounts of purified protein (0, 0.2, 0.4, 0.6, and 0.8 pg, as indicated in the legend to corresponding to domain IV of each bacterial SRP-like RNA, and used as a transcriptional template. The transcription single band after PAGE.

RNA mobility shift assay. The specific activity of the reaction contained 40 mM Tris/HCl (pH 8), 6 mM MgCl2, 5 mM DTT, 1 mM spermidine, 0.01% Triton X-100, 50 µg BSA ml-1, 2 mM each of ATP, GTP, and UTP, 0.2 mM CTP, 20 mM KC1, 5 mM MgCl2, 0.1 mM each dNTPs, and 1 mM DTT). Fifty units of RAV-2 (Rous associated virus 2) reverse transcriptase was added and the mixture was incubated at 42 °C for 1 h. The length of extended DNA fragment was determined by a sequencing ladder generated from M13mp10 containing the C. perfringens scRNA gene using the same oligonucleotides. The 3′ end of scRNA was estimated by the RNase protection assay. A 332 bp Apal–EcoRI fragment, including the 3′ portion of the C. perfringens scRNA (Fig. 1), was inserted between the Apal–EcoRI sites of Bluescript SK(−). T3 RNA polymerase and [α-32P]CTP were used to synthesize a radioactive probe which encompassed the 3′ portion of the scRNA gene and to which it is complementary. The RNase protection assay was performed as described by Nakamura et al. (1992a) using total RNA (10 µg) and 3–9 × 106 c.p.m. of the 32P-labelled probe.

Synthesis of 32P-labelled SRP-like RNAs from B. subtilis, E. coli and C. perfringens. The DNA fragments including domain IV of SRP-like RNAs from three bacteria were placed under the control of the SP6 promoter. A 114 bp Aal–HindII fragment, corresponding to positions +109 to +222 of B. subtilis scRNA, was isolated and inserted into the Smal site of pSP64 in the correct direction. A 138 bp DNA fragment which encodes mature E. coli 4SR RNA was amplified with the synthetic oligonucleotides 5′-AAGCTTAAAGCTTATGTAA-CCCTGCCAGCT-3′. These four primers were designed to create HindIII and BamHI sites at the 5′ and 3′ ends of the resulting PCR products, respectively. Purified products were first digested with both HindIII and BamHI, then inserted between the HindIII and BamHI sites in pSP64. To prepare a radioactive probe used for the RNA mobility shift assay, the resulting plasmids were linearized by digestion with BamHI, and synthesized as a transcriptional template. The transcription reaction contained 40 mM Tris/HCl (pH 8), 6 mM MgCl2, 5 mM DTT, 1 mM spermidine, 0.01% Triton X-100, 50 µg BSA ml-1, 2 mM each of ATP, GTP, and UTP, 0.2 mM CTP, 20 units RNasin, 50 µCi (1.85 MBq) [α-32P]CTP (3000 Ci mmol−1; 11 TBq mmol−1), 25 pmol DNA fragment, and 200 units SP6 RNA polymerase (Takara Shuzo). The specific activity of the radioactive probe which encompassed the 3′ portion of the scRNA gene and to which it is complementary. The RNase protection assay was performed as described by Nakamura et al. (1992a) using total RNA (10 µg) and 3–9 × 106 c.p.m. of the 32P-labelled probe.

RESULTS

Cloning of the scRNA gene of the C. perfringens chromosome

We identified the scRNA gene from C. perfringens by means of Southern hybridization using a 312 nucleotide DNA fragment encoding the mature B. subtilis scRNA

Fig. 1. Physical map and gene organization in the cloned 2.7 kb HindIII fragment from C. perfringens chromosomal DNA. The locations of the four genes (tRNA-Ser, scr, orf1, and braB) are indicated by the rectangles above the physical map. The shadowed rectangle represents the scr gene. Arrows indicate the direction of transcription in each gene.

Fig. 2. A 1 kb nucleotide sequence of the 2.7 kb HindIII fragment cloned. The nucleotides are numbered from the 5′ end, the first being taken as a HindIII restriction site. The presumed promoter elements (−10 and −35) are boxed. The potential ribosome binding sites (RBS) are underlined. Palindromic sequences that may serve as transcriptional terminators are indicated by converging arrows. The 5′ and 3′ ends of scRNA, shown in Fig. 4, are marked by a dot above the corresponding nucleotides (+284 and +552). The sequences exhibiting homology with tRNA-Ser and scRNA of B. subtilis are thickly underlined.

scRNA of Clostridium perfringens

Primer P-1 (5′-CAACAGTTAGACCGCTCTATG-3′), positions 352–371 (Fig. 2), was used to precisely map the 5′ end of mature scRNA. Total C. perfringens RNA (40 µg) and 5 × 106 c.p.m. of the 32P-labelled oligonucleotide primer were hybridized at 40 °C overnight. After hybridization, the sample was precipitated by adding two vols ethanol and being stored at 20 °C for 1 h. The pellet was collected by centrifugation and dissolved in 20 µl reverse transcriptase buffer (50 mM Tris/HCl, pH 7–8, 60 mM KCl, 10 mM MgCl2, 1 mM each dNTPs, 1 mM DTT). Fifty units of RAV-2 (Rous associated virus 2) reverse transcriptase was added and the mixture was incubated at 42 °C for 1 h. The length of extended DNA fragment was determined by a sequencing ladder generated from M13mp10 containing the C. perfringens scRNA gene using the same oligonucleotides. The 3′ end of scRNA was estimated by the RNase protection assay. A 332 bp Apal–EcoRI fragment, including the 3′ portion of the C. perfringens scRNA (Fig. 1), was inserted between the Apal–EcoRI sites of Bluescript SK(−). T3 RNA polymerase and [α-32P]CTP were used to synthesize a radioactive probe which encompassed the 3′ portion of the scRNA gene and to which it is complementary. The RNase protection assay was performed as described by Nakamura et al. (1992a) using total RNA (10 µg) and 3–9 × 106 c.p.m. of the 32P-labelled probe.
gene as a probe. A 2.7 kb HindIII fragment specifically hybridized with the probe (data not shown). DNA fragments of 2–4 kb were extracted from the gel and inserted into the HindIII site of pUC119. Among 400 plasmids that we purified, we found two clones showing positive bands. Restriction mapping the plasmids in the two positive clones revealed that both contained a fragment of about 2.8 kb, oriented in the same direction as the vector. One of them was used for determining the sequence.

Sequencing and characterization of the chromosome region around the scRNA gene of C. perfringens

We sequenced the entire 2.7 kb fragment to which the B. subtilis scRNA gene was hybridized. The physical map of the 2.7 kb fragment is shown in Fig. 1. The nucleotide sequence and deduced amino acid sequence of a 1000 bp fragment around the scRNA gene is shown in Fig. 2. Two ORFs were found in this fragment. They were aligned in the same direction and no sizeable ORF was found in the opposite direction. The first ORF, starting with an ATG codon at positions 716–718, consisted of 167 codons and would encode a protein of 18.4 kDa. This protein exhibited no significant homology to known proteins in the protein-sequence library at the Human Genome Center at Tokyo University. The second ORF begins with an ATG codon at positions 1659–1661. However, no termination codon was found in the downstream region. Therefore, we could not isolate the entire ORF. The amino acid sequence of this truncated protein showed 34.8% amino acid identity with the N-terminal portion of the product of the sodium-coupled, branched-chain amino acid carrier gene (braB) of Pseudomonas aeruginosa (Hoshino et al., 1990). Therefore, this gene was designated as braB in C. perfringens. There are typical sequences for the ribosome-binding sites in front of these ORFs.

From a database search of the DNA sequences, the nucleotide sequence from positions 284 to 552 showed 73.2% identity to that of the B. subtilis scRNA gene. We also found that a secondary structure of a gene juxtaposed to the scRNA gene had a typical clover-leaf structure conserved in the tRNA structure and that all determinant nucleotides conserved in tRNA-Ser (Normany et al., 1986), including the discriminator base, are well preserved in this tRNA gene. The sequence of the anti-codon of this
### Alignment of C. perfringens scRNA with other eubacterial cognates

The sequence of C. perfringens scRNA was aligned with SRP-like RNAs from B. subtilis (accession no. X06802), T. thermophilus (X12643), E. coli (X01074) and P. aeruginosa (M31829). The identical residues among all sequences are indicated by reversed-out letters. Dashes indicate gaps for maximal homology. The nucleotide sequence was aligned with other sequences for maximal homology, using the multiple alignment feature of the GENETYX-MAC software.

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Alignment</th>
</tr>
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<tbody>
<tr>
<td><strong>C. perfringens</strong></td>
<td>GAGT-TGAGGCCCATTTTTTTATATAG-----GTG-GTGAGCTAATGCTGATA 173</td>
</tr>
<tr>
<td><strong>B. subtilis</strong></td>
<td>AGGTTTTGCTGGGATGAAAA-TTCTGAGATAATATAG 175</td>
</tr>
<tr>
<td><strong>T. thermophilus</strong></td>
<td>GCCGCCCT-G-CATCGAGGCCGCGAGGGAACCTATATAG 60</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td>TCTTTGCTGCTCCCTAACATACATCTGATATAG 65</td>
</tr>
<tr>
<td><strong>P. aeruginosa</strong></td>
<td>CGGTGGTGCTCGCCAAAATATACATCGAGGTAGTATA 64</td>
</tr>
</tbody>
</table>

### Mapping of 5' and 3' ends for scRNA of C. perfringens

To verify the structure of scRNA from C. perfringens, both the 5' and 3' ends were determined. In addition, we investigated whether or not processing events occurred in C. perfringens scRNA, since the processing at the 5' and 3' ends is involved in the maturation of the B. subtilis scRNA. To determine the 5' end of scRNA, we isolated RNA from C. perfringens cells cultured in GAM broth (Nissui) under anaerobic conditions 1, 2 and 3 h after inoculation. The P-1 primer detected a major band with

- **tRNA is GGA**, demonstrating that this gene encodes tRNA-Ser. About 30 bp upstream from the tRNA-Ser gene a potential promoter was identified (−35 box, TTGAAT; −10 box, TAGAAT), which was highly homologous with the consensus sequence recognized by the E. coli and B. subtilis σ70 RNA polymerase (−35 box, TTGACA; −10 box, TATAAT) (Gitt et al., 1985).
- About 12 bp downstream from the scRNA coding region there is an almost perfect palindrome sequence comprising 29 bp \[ \Delta G = -13.4 \text{ kcal mol}^{-1} (-56.28 \text{ kJ mol}^{-1}) \] as calculated by the method of Tinoco et al. (1973) followed by a run of thymidine residues (Fig. 3c). This structure resembled that of a characteristic E. coli ρ-independent transcriptional terminator. Furthermore, between the tRNA-Ser and scRNA genes there was no gap, indicating that these two genes are organized into an operon and that the two genes are transcribed as one primary transcript.

### Mapping of 5' and 3' ends for scRNA of C. perfringens (continued)

To verify the structure of scRNA from C. perfringens, both the 5' and 3' ends were determined. In addition, we investigated whether or not processing events occurred in C. perfringens scRNA, since the processing at the 5' and 3' ends is involved in the maturation of the B. subtilis scRNA. To determine the 5' end of scRNA, we isolated RNA from C. perfringens cells cultured in GAM broth (Nissui) under anaerobic conditions 1, 2 and 3 h after inoculation. The P-1 primer detected a major band with
the 5′ end located at the G residue at position 285 (Fig. 3a). Moreover, a minor band (about 10% of the intensity of the major signal) that has a 5′ end located 1 nucleotide upstream of the major band was also detected. These data indicated that the 5′ end of scRNA starts from the T residue at nucleotide position 284. This lack of 1 nucleotide at the 5′ end is probably an artifact caused during RNA preparation or during the primer extension reaction. For the 3′-end mapping, a 332 bp ApaI-EcoRI fragment (Fig. 1) was inserted into Bluescript SK(−). The radioactive antisense RNA was prepared as described in Methods. Fig. 3(b) shows that only one major protected band, comprising 146 bp, appeared in the RNA sample prepared at 3 h after inoculation (Fig. 3b, lane 3). This result indicates that the 3′ end of scRNA is a T residue at nucleotide position 552 in Fig. 2. A 5′ and 3′ mapping study showed that the mature scRNA of *C. perfringens* scRNA consists of 269 nucleotides. In contrast to the SRP-like RNAs from *E. coli*, *T. thermophilus* and *P. aeruginosa*, the scRNA of *C. perfringens* can be significantly aligned with the entire region of *B. subtilis* scRNA (Fig. 4), with which it has about 70% homology. Moreover, based upon the secondary structure predicted in *B. subtilis* scRNA, most of the nucleotide substitutions observed in *C. perfringens* scRNA are covariant. Therefore, a similar secondary structure can be shown for *C. perfringens* scRNA (Fig. 5). In addition to the evolutionarily conserved domain IV, scRNA of *C. perfringens* has both domains I and II. This is a second example of a bacterial SRP-like RNA that contains these domains.

**C. perfringens** scRNA can compensate for the vegetative growth and sporulation of scRNA-depleted *B. subtilis* cells

Because of the structural resemblance, we examined whether or not *C. perfringens* scRNA functionally relates to *B. subtilis* scRNA. The test strain, a *B. subtilis* SC200NA, contains two scRNA gene (*scr*) loci. One allele is the inducible Pspac-7: :*scr* gene introduced by fusing the chromosomal *scr* locus with the spac-1 promoter by homologous recombination. The other is an inactive variant of the normal *scr* gene that lacks the 3′ one-third of the gene. Repression of the Pspac-1: :*scr* gene introduced by fusing the chromosomal *scr* locus with the spac-1 promoter by homologous recombination. The other is an inactive variant of the normal *scr* gene that lacks the 3′ one-third of the gene. Repression of the Pspac-1 allele is strengthened in the absence of IPTG. Without IPTG, this cell is not viable and has aberrant morphological features during the early period of incubation. We introduced *B. subtilis*–*E. coli* shuttle plasmids, containing the gene for RNA homologues from *B. subtilis* (pTUBE822), *E. coli* (pTUBE811) and *C. perfringens* (pTUBE850), into *B. subtilis*.
scRNA of Clostridium perfringens

Fig. 6. Complementation of cell growth of B. subtilis strains in the depletion of intrinsic scRNA by introduction of eubacterial SRP-like RNA homologues. B. subtilis SC200NA strains harbouring vector pTUBE809 (■), pTUBE822 (●), pTUBE811 (□) or pTUBE850 (△) were cultured in L-broth at 37°C in the absence of IPTG. The data shown are the means of two experiments performed on different days.

subtilis SC200NA. As shown in Fig. 6, the growth rates at the exponential and early stationary phases are indistinguishable among the three transformants, indicating that these RNAs are functionally interchangeable. This result is consistent with those of previous studies demonstrating compatibility within the SRP-like RNA family (Brown et al., 1989; Brown, 1991; Nakamura et al., 1992a; Simoneau & Hu, 1992). However, we also showed that a B. subtilis SC200NA transformant containing mutant scRNA, in which domain I and/or II is depleted, can no longer sporulate with the same frequency as the wild-type (Nishiguchi et al., 1994). Therefore, it is of interest to determine whether C. perfringens and E. coli RNAs can compensate for formation of heat-resistant spores of SC200NA in the absence of IPTG. To determine the sporulation efficiency, each transformant was cultivated in Schaeffer sporulation medium for 24 h without IPTG. As indicated in Table 1, sporulation of the pTUBE822 transformant was heavy, while that of the pTUBE809 transformant was suppressed (Table 1). Under these conditions, the pTUBE850 transformant sporulated and had nearly the same frequency of heat-resistant spores as the pTUBE822 transformant. On the other hand, the frequency of that of the pTUBE811 transformant, which expresses E. coli 4·5S RNA, was reduced to about 40% of that of the pTUBE822 transformant. This frequency is similar to that of the pTUBE823 transformant, which expresses an scRNA variant lacking domain I (Nishiguchi et al., 1994). Quantification of the RNAs expressed in the B. subtilis transformants confirmed that E. coli 4·5S RNA accumulated as well as B. subtilis and C. perfringens scRNAs (data not shown), indicating that this reduced frequency in the pTUBE811 transformant is not due to instability of the RNA.

Table 1. Efficiency of heat-resistant spore formation in SC200NA containing each scRNA gene

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Origin of scRNA</th>
<th>Viable cells (10⁴ x c.f.u. ml⁻¹)</th>
<th>Heat-resistant spores (10⁴ x c.f.u. ml⁻¹)</th>
<th>Frequency (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>pTUBE809</td>
<td>None</td>
<td>2·5</td>
<td>0·1</td>
<td>4</td>
</tr>
<tr>
<td>pTUBE822</td>
<td>B. subtilis</td>
<td>4·2</td>
<td>2·5</td>
<td>60</td>
</tr>
<tr>
<td>pTUBE850</td>
<td>C. perfringens</td>
<td>3·9</td>
<td>2·3</td>
<td>59</td>
</tr>
<tr>
<td>pTUBE811</td>
<td>E. coli</td>
<td>3·2</td>
<td>0·8</td>
<td>25</td>
</tr>
</tbody>
</table>

* Value for heat-resistant spores/value for viable cells x 100.

Ability of SRP-like RNAs from three bacteria to bind to B. subtilis Ffh protein

We elucidated that scRNA binds to the GTPase protein Ffh in vivo to form a stable ribonucleoprotein complex (Nakamura et al., 1994). Ffh protein is a homologue of mammalian SRP54 (Bernstein et al., 1989; Hann et al., 1989; Honda et al., 1993; Samuelsson, 1992). In contrast to mammalian SRP54, bacterial homologues have an intrinsic GTPase activity (Samuelsson & Olsson, 1993). Since complex formation between Ffh and scRNA is considered to be important for scRNA to function properly, it is possible that the depleted ability of E. coli 4·5S RNA to bind to Ffh led to a reduction in the efficiency of sporulation. To test this hypothesis, the ability of each RNA to bind to Ffh protein was determined by the RNA mobility shift assay. Since full size B. subtilis and C. perfringens scRNAs are considered too long for use...
as a probe for this assay and truncated \textit{B. subtilis} scRNA corresponding to domain IV can efficiently bind to Ffh protein (data not shown), we used the radioactive truncated form of each RNA, namely \textit{B. subtilis} scRNA-114 and \textit{C. perfringens} scRNA-117. These RNAs correspond to positions +109 to +222 of \textit{B. subtilis} scRNA (\textit{B. subtilis} scRNA-114) and positions +100 to +216 of \textit{C. perfringens} scRNA (\textit{C. perfringens} scRNA-117), respectively. The RNA secondary structure predicted using the Zucker minimal free energy program (Zucker, 1989) revealed that these RNAs can form a single hairpin structure just like \textit{E. coli} 4-5S RNA. Furthermore, the expression of \textit{B. subtilis} scRNA-114 and \textit{C. perfringens} scRNA-117 complemented the growth of \textit{B. subtilis} SC200NA in the absence of IPTG (data not shown). Fig. 7 demonstrates that \textit{B. subtilis} scRNA-114 32P-labelled RNA that mimics domain IV bound to Ffh protein in the mobility shift assay (lanes 1–5). Unlabelled \textit{B. subtilis} scRNA-114, but not tRNA, diminished this complex formation (data not shown), exhibiting binding specificity in this assay. Both \textit{E. coli} 4-5S RNA and \textit{C. perfringens} scRNA-117 also associate with Ffh protein and brought about the appearance of a complex form (lanes 6–10 and lanes 11–15). The amount of complex formed was dependent on the amount of protein added. The binding specificity of this complex was demonstrated with various unlabelled RNAs (data not shown). Quantitative densitometry of autoradiograms showed that the intensity of the complex that appeared in \textit{E. coli} 4-5S RNA and \textit{C. perfringens} scRNA-117 was about 95\% of that in \textit{B. subtilis} scRNA-114. These results demonstrated that \textit{E. coli} 4-5S RNA and \textit{C. perfringens} scRNA-117 interacted with \textit{B. subtilis} Ffh protein in vitro and exhibited the same apparent specificity for \textit{B. subtilis} scRNA-114.

\textbf{DISCUSSION}

The genus \textit{Clostridium} consists of a diverse collection of obligatory anaerobic Gram-positive bacteria that all sporulate (Smith & Hobbs, 1974). Based upon a phylogenetic study derived from a comparison of the 16S and 23S rRNA sequences, \textit{Clostridium} is related to the enlarged \textit{Bacillus} cluster (van der Meer et al., 1993; Woese, 1987). \textit{Clostridium} spp. have a dG + dC content varying from 24 to 55\%. \textit{C. perfringens} has been studied intensively because of its importance in human pathogenesis (Finegold, 1977) and it has a high dA + dT content (~ 75\%). Here, we reported that \textit{C. perfringens} has a DNA sequence that is significantly homologous with the entire region of \textit{B. subtilis} scRNA. A functional analysis showed that this encodes an scRNA in \textit{C. perfringens}. The scRNAs of \textit{C. perfringens} and \textit{B. subtilis} were almost the same size (269 and 271 nucleotides) and shared a comparatively high degree of identical nucleotides (73-2\%). This relationship led us to construct a common secondary structural model for the two scRNAs. This model indicated that \textit{C. perfringens} scRNA has both domains I and II, in addition to domain IV. The secondary structure is supported by several compensatory base-changes identified by a phylogenetic comparison. Moreover, as shown in Fig. 5, there is a potential tertiary interaction between two loops located within domain I of \textit{B. subtilis}. This interaction can be maintained in \textit{C. perfringens} scRNA and 6 nucleotides are involved in this interaction.

As shown in Results, the tRNA-Ser and scRNA genes are organized into an operon. Haas et al. (1990) have reported that the 7S RNA gene locates upstream of the tRNA-Ser gene and they detected transcripts of the 7S RNA and tRNA-Ser genes in the extremely thermophilic archaeo-
bacterium *Methanothermus fervidus*. On the other hand, in trypanosomes, upstream tRNA genes are essential for 7S RNA gene expression (Nakaar et al., 1994). However, since the scRNA gene of *B. subtilis* is a monofunctional transcriptional unit (Struck et al., 1989), we cannot underscore the significance of the association of tRNA and SRP-like RNA genes. The endoribonuclease P is responsible for generating the 5' termini of tRNAs (Altman, 1989). On the other hand, knowledge of the 3' processing of the tRNA precursor is severely limited (Deutscher, 1990). Early in vitro studies of the processing of a few *E. coli* tRNA precursors led to suggestions that the generation of a mature 3' terminus requires several multiple exoribonuclease activities. Several studies with mutant tRNA precursors or with extracts deficient in exoribonucleases supported the notion that the generation of the mature 3' terminus of tRNA involves an initial endonucleolytic cleavage downstream of the encoded -CCA sequence, followed by exoribonucleolytic trimming to expose these residues (Bikoff & Gefter, 1975; Sekiya et al., 1979). In contrast, there is no spacer region between the tRNA-Ser and scRNA genes of *C. perfringens*. Our data indicated that only a one-step endonucleolytic cleavage after the A residue at +283 (Fig. 3) is required to create the mature 3' end of tRNA. Therefore, although the ribonucleases involved in the processing are unknown, this structural feature represents the unique manner of the 3'-end processing of the tRNA transcript.

In contrast to the unification in the size and secondary structure of SRP RNA in eukaryotes, except for the yeast homologue, that of bacterial SRP-like RNAs has diverged (Larsen & Zwieb, 1991), i.e. *E. coli* 45S RNA is 114 nucleotides, *P. aeruginosa* is 113, *T. thermophilus* is 105, *B. subtilis* is 271, *C. perfringens* is 269 and *M. mycoides* is 77. Among them, both *B. subtilis* and *C. perfringens* contain structures corresponding to domains I, II and IV of mammalian 7SL RNA. Since both organisms are Gram-positive and can sporulate, the simplest explanation for these data is that the persistence of these structures reflects the close relationship between *B. subtilis* and *C. perfringens*. However, our data show that *S. aureus*, being more closely related to *Bacillus* species based on both 16S and 23S rRNA phylogeny, has SRP-like RNAs, the size of which are intermediate between *B. subtilis* and *E. coli* (E. Hashizume and others, unpublished). Moreover, T. Barry and others (personal communication) have determined the sequence of *Listeria monocytogenes* SRP-like RNA. *L. monocytogenes* is a Gram-positive bacterium that does not sporulate. The size and secondary structure of *Listeria* spp. RNA resembles that of *B. subtilis* scRNA, but there are several base substitutions that disrupt the secondary structure of domains I and II. Furthermore, the potential interaction between two loops within domain I has vanished. As a result, the SRP-like RNA of *L. monocytogenes* contains three domains but the secondary structure of domain I is ambiguous. To examine the functions of domains I and II, we constructed mutant scRNAs of *B. subtilis* in which individual domains or conserved nucleotides were deleted, and examined their effects in vivo. The results demonstrated that domains I and II have distinct functions in the formation of heat-resistant spores (Nishiguchi et al., 1994). Here, we showed that introducing *C. perfringens* scRNA in the conditional scRNA-deficient mutant *B. subtilis* SC200NA can compensate for not only the vegetative growth but also for heat-resistant spore formation, whereas *E. coli* 45S RNA was significantly less effective in supporting sporulation. This difference in activity is not due to the different ability of each RNA to bind to *B. subtilis* Ffh protein. The conservation of domains I and II throughout eubacterial evolution is exclusively related to the requirement for their functions in both organisms. We thus suggest a plausible scenario for the evolution of SRP-like RNA in cells of bacterial lineage. If eukaryotic and archaeobacterial SRP-like RNAs represent features of their prototype (Althoff et al., 1994), then SRP-like RNA has undergone several dramatic size reductions during bacterial evolution. Moreover, this phenomenon could occur at each step of evolution, even after the species *B. subtilis* *E. coli* and *Clostridium* appeared. However, the domains needed for efficient sporulation conferred selective advantages in *B. subtilis* and *C. perfringens*, whereas a reduction of such domains was tolerated in non-spore-forming bacteria.

In mammalian SRP, the 9 (SRP9) and 14 kDa (SRP14) subunits of SRP are required to confer elongation arrest activity to the particle. SRP9 and SRP14 form a heterodimer which specifically binds to Alu-like sequences, including domains I and II in 7SL RNA (Bovia et al., 1994; Strub & Walter, 1990; Strub et al., 1991). In eubacteria, proteins other than Ffh that bind to scRNA have not been identified. According to the procedure for the purification of mammalian SRP (Walter & Blobel, 1980), we are now purifying the RNP complex consisting of scRNA and the Ffh protein from *B. subtilis*. To characterize this complex should help to elucidate the functions of domains I and II.

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