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Structural Requirements of *Bacillus subtilis* Small Cytoplasmic RNA for Cell Growth, Sporulation, and Extracellular Enzyme Production

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Bacillus subtilis **small cytoplasmic RNA (scRNA; 271 nucleotides) is a member of the signal recognition particle (SRP) RNA family, which has evolutionarily conserved primary and secondary structures. The scRNA consists of three domains corresponding to domains I, II, and IV of human SRP 7S RNA. To identify the structural determinants required for its function, we constructed mutant scRNAs in which individual domains or conserved nucleotides were deleted, and their importance was assayed in vivo. The results demonstrated that domain IV of scRNA is necessary to maintain cell viability. On the other hand, domains I and II were not essential for vegetative growth but were preferentially required for the RNA to achieve its active structure, and assembled ribonucleoprotein between Ffh and scRNA is required for sporulation to proceed. This view is highly consistent with the fact that the presence of domains I and II is restricted to sporeforming *B. subtilis* scRNA among eubacterial SRP RNA-like RNAs.**

Signal recognition particle (SRP) RNA-like RNAs have been identified in a wide range of eukaryotes, including humans (31), plants (7), *Yarrowia lipolytica* (19), *Schizosaccharomyces pombe* (3), and a wide variety of archaebacterial species (14). SRP RNA of higher eukaryotes is an RNA component of the SRP, which is thought to be essential for targeting nascent secretory proteins to the endoplasmic reticulum membrane (31). It has been proposed that SRP RNA consists of four domains (domain I to IV), based on the predicted secondary structure of human SRP RNA. Phylogenetic studies have revealed that the overall secondary structures of the various RNAs are almost the same and that the primary sequences and secondary structures of domain IV are highly conserved, especially among those organisms (20). Mammalian SRP54 can bind in vitro to *Escherichia coli* 4.5S RNA (21), which is homologous to domain IV of human SRP 7S RNA. Thus, it is likely that this domain IV is also the binding site for SRP54 within the mammalian SRP. Genes encoding a SRP54 homolog have been isolated from *E. coli* (6, 22), *Bacillus subtilis* (11), yeasts (9, 10), and *Mycoplasma mycoides* (25). Therefore, it is also plausible that SRP RNA-like RNA forms ribonucleoprotein complexes associated with the SRP54 homolog within these organisms (1, 10, 18, 21).

Small cytoplasmic RNA (scRNA; 271 nucleotides) is an abundant and stable RNA of the gram-positive bacterium *B. subtilis* (30). It is transcribed as a 354-nucleotide primary transcript and then processed to 271-nucleotide RNAs at both the 5′ and 3′ ends (28). It can be folded into a secondary structure strikingly similar to that of eukaryotic SRP RNA, although it lacks domain III. *E. coli* and many eubacteria contain small stable RNA that can form a hairpin-like structure corresponding to domain IV (12, 26). With respect to size and secondary structure, *B. subtilis* scRNA is considered to be intermediate between the prokaryotic and eukaryotic SRP RNA families. To elucidate the role of SRP RNA and to confirm the presence of SRP in *B. subtilis*, we developed *B. subtilis* SC200NA, in which expression of the scRNA gene depends on isopropyl-β-D-thiogalactopyranoside (IPTG). Depletion of scRNA in this strain led to defects in production of extracellular enzymes and in β-lactamase translocation as well as to drastic morphological changes as shown previously (17). We also found that these characteristic features caused by the depletion of scRNA could be functionally compensated for by the expression of human SRP RNA or *E. coli* 4.5S RNA. The ability of the smaller 4.5S RNA of *E. coli* to compensate for the loss of scRNA in *B. subtilis* raises the issue of whether the extra terminal region of scRNA is required for its activity. It would be of interest to learn what function, if any, the extra domains of the scRNA have. The most striking features of the consensus structure of human SRP RNA, *E. coli* 4.5S RNA, and *B. subtilis* scRNA are the hairpin motif and the highly conserved bases in the loop region of domain IV. The presence of the strictly conserved 22-base sequence in the loop of domain IV in each RNA provides powerful evidence that this element has a key role in scRNA function.

To analyze the structural requirements of scRNA for its function, mutants carrying deletions in domain I only, domains I and II, and consensus residues in the loop of domain IV were constructed, and the importance of each domain for cell growth, spore formation, and the production of extracellular proteases was investigated in vivo.

MATERIALS AND METHODS

Bacterial strains and vector plasmids. *B. subtilis* SC200NA (*purB6 trpB3 metB5 scr*::pTUE807) was constructed from *B. subtilis* NA20 as previously described (17). In this strain, the intact scRNA gene (*scr*) is regulated by the IPTG-inducible promoter P*spac-1*. This strain has been maintained and cultured in medium containing 5 μg of chloramphenicol per ml and 3 mM IPTG. The expression plasmid pTUBE809 is an *E. coli-B. subtilis* shuttle vector which contains a 98-bp synthetic protein A promoter and is a derivative of pHY300PLK (Takara Co., Ltd., Kyoto, Japan). The protein A promoter was

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expressed constitutively in the absence of IPTG. Plasmid pCR1000, into which primary PCR products can be cloned, was purchased from Invitrogen Co. Ltd. (San Diego, Calif.).

Growth conditions and determination of the efficiency of sporulation. To maintain each plasmid in cells, bacteria were cultivated in medium containing 10 μg of tetracycline per ml. For sporulation, the bacterial cells were cultivated in Schaeffer medium (27) with vigorous shaking. The efficiency of sporulation was determined by harvesting spores from 1 ml of a bacterial suspension cultured for 24 h in the medium and washing them three times with cold water. The spore pellet was resuspended in an equal volume of 50 mM Tris-HCl buffer (pH 7.5), and the number of viable cells was determined. The spores were then heated at 80°C for 10 min and plated to determine the number of survivors.

Construction of plasmids. Plasmids containing deletions in the scRNA gene were constructed by using DNA fragments synthesized by PCR (24) and oligonucleotide-directed mutagenesis (15). The constructed plasmids were confirmed by physical mapping and DNA sequencing.

The 271-bp DNA fragment, corresponding to mature scRNA, was amplified from the chromosomal DNA of *B. subtilis*, using PCR with oligonucleotides a (18-mer; 5′-TTT GCCGTGCTAAGCGGG-3′) and b (18-mer; 5′-TACCGTG CACCTTCTGTC-3′). The 60-bp DNA fragment, corresponding to positions +135 to +194 of mature scRNA, was amplified with oligonucleotides c (18-mer; 5′-ATGGGAATTCATGA ACCA-3′) and d (18-mer; 5′-ATGAGAGGTTTCACTTAA -3′). Purified PCR products were inserted into the *Hph*I site of the pCR1000 vector to create plasmids pTUE812 and pTUE813. *Not*I-*Hin*dIII fragments of 317 and 106 bp were isolated from pTUE812 and pTUE813, and the purified DNA fragments (sc271 and sc60) were blunt ended. For DNA fragment sc104, corresponding to +116 to +219, a 104-bp-*Alu*I-*Hin*cII fragment was isolated from sc271. DNA fragments scD22 and scD42 were constructed by deleting 22 and 42 bases, respectively, from domain IV of sc271 by oligonucleotide-directed mutagenesis, using the mutagenenic oligonucleotides g (30-mer; 5′-GAGGTTTCACTTAATATGGTTCATGAAT TC-3′) and h (30-mer; 5′-CGGCACATGAGAGGTAATTCC CATTGCGCA-3′). By inserting blunt-ended sc271, sc104, sc60, scD22, and scD42 fragments between the protein A promoter and *trp* transcription terminator pTUBE809, pTUBE822, pTUBE823, pTUBE824, pTUBE832, and pTUBE 833, respectively, were obtained. To construct another series of mutant scRNA in which various lengths of nucleotides were deleted from domain IV, pTUBE822 was digested with *Acc*III followed by *Bal* 31 nuclease S (Takara Shuzo Co. Ltd., Kyoto, Japan), blunt ended, religated, and then introduced into *B. subtilis* cells. The deletions were confirmed by DNA sequencing.

Assay of protease activities. At various times, cultures were centrifuged at 4,000 rpm for 10 min. The supernatants were saved to measure extracellular enzyme production. Units of proteolytic activity in preparations were determined by the method of Chavira et al. (8), using Azocoll (Sigma Chemical Co., St. Louis, Mo.) as the substrate.

Purification of spores and determination of their heat resistance. Dormant spores of *B. subtilis* transformants cultured for 24 h at 37°C in Schaeffer medium were purified by washing the spore pellet three times with deionized water at 4°C, suspending it in 0.5 times the original culture volume of 50 mM Tris-HCl (pH 7.5) containing 50 mg of lysozyme per ml, incubating it for 60 min at 37°C, and then subjecting it to one wash with 0.1 M NaCl and three washes with deionized water. The final spore pellets were suspended in 0.5 times the original culture volume of deionized water. The heat resistance of spores was determined by incubating the purified preparation at various temperatures for 15 min and calculating the ratio of survivors from viable cell counts. The assays were performed in triplicate, and two independently purified spore preparations were tested for each transformant.

Electron microscopy. After centrifugation of cultures at 37°C for 24 h, the pellets were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 5 min, cut into small pieces, and fixed again in the same solution for 1 h at room temperature. The samples were then washed in the buffer and postfixed in 1% osmium tetroxide in cacodylate buffer for 1 h. Alcohol-dehydrated specimens were embedded in Spurr's resin. Thin sections were prepared on an LKB ultramicrotome and stained with uranyl acetate and lead citrate. A JEOL 100CXII electron microscope was used for observations.

RNA extraction and dot blot hybridization analysis of scRNA. *B. subtilis* SC200NA containing each plasmid was grown in the absence of IPTG. Total RNAs were extracted as described previously (17). To isolate RNAs bound to Ffh protein in *B. subtilis*, lysates were prepared by following steps. Cells were collected, washed and resuspended in 150 mM NaCl–5 mM $MgCl_2$–20 mM Tris-HCl (pH 8)–0.05% Nonidet P-40–4 mM vanadyl-ribonucleoside complex–0.1 mM phenylmethylsulfonyl fluoride, and treated with lysozyme (1 mg/ml) for 30 min at 30°C. Half volumes of glass beads (Sigma G-4649) were added, and the suspension was vortexed for 5 min at 4°C. Lysates were separated from glass beads by centrifugation. The prepared lysates were incubated overnight with antiserum against the *B. subtilis* SRP54 homolog (Ffh). Protein A–Sepharose CL-4B was added, and the mixture was incubated for 1 h at 4°C. After centrifugation, the pellet was washed three times in the same buffer. RNA was recovered by heating to 65°C in 1:1 mixtures of 4 M guanidine thiocyanate and phenol. Then, 0.05, 0.2, 0.8, and 3.2 μg of denatured RNAs were dot blotted onto a GeneScreen Plus filter with a manifold apparatus. 5′-^{32}P-labeled double-stranded DNA fragment sc271, which corresponds to mature scRNA, was prepared and used as the probe for DNA-RNA hybridization.

RESULTS

An in vivo complementation assay system dependent on a plasmid-borne scRNA. We developed a strain, SC200NA, of *B. subtilis* in which the expression of scRNA is dependent on IPTG by the presence of a P*spac-1* promoter cassette containing *scr* in the chromosome (17). In the absence of IPTG, the growth of SC200NA is greatly inhibited. By using derivatives of plasmid pTUBE809, a second scRNA gene, mutated or wild type, can be introduced into the strain. Consequently, in the absence of IPTG, the growth of a strain that harbors a recombinant plasmid is dependent on the additional scRNA expressed by that plasmid (Fig. 1). To test this system, a plasmid vector containing either no insert (pTUBE809) or a wild-type scRNA gene (pTUBE822) was introduced into *B. subtilis* SC200NA. Plasmid pTUBE809 without an insert resulted in a lack of transformant growth in the absence of IPTG, whereas the strain with pTUBE822 survived (data not shown). These results indicate that the phenotype of a plasmid-borne mutant scRNA can be assessed by this in vivo system.

Deletion of the conserved nucleotides in domain IV caused loss of function. A phylogenetic study previously demonstrated that the nucleotide sequences in the loop of domain IV are highly conserved (20), perhaps reflecting their interaction with specific proteins for its function. Especially among eubacteria, there is a block containing complete primary sequence identity

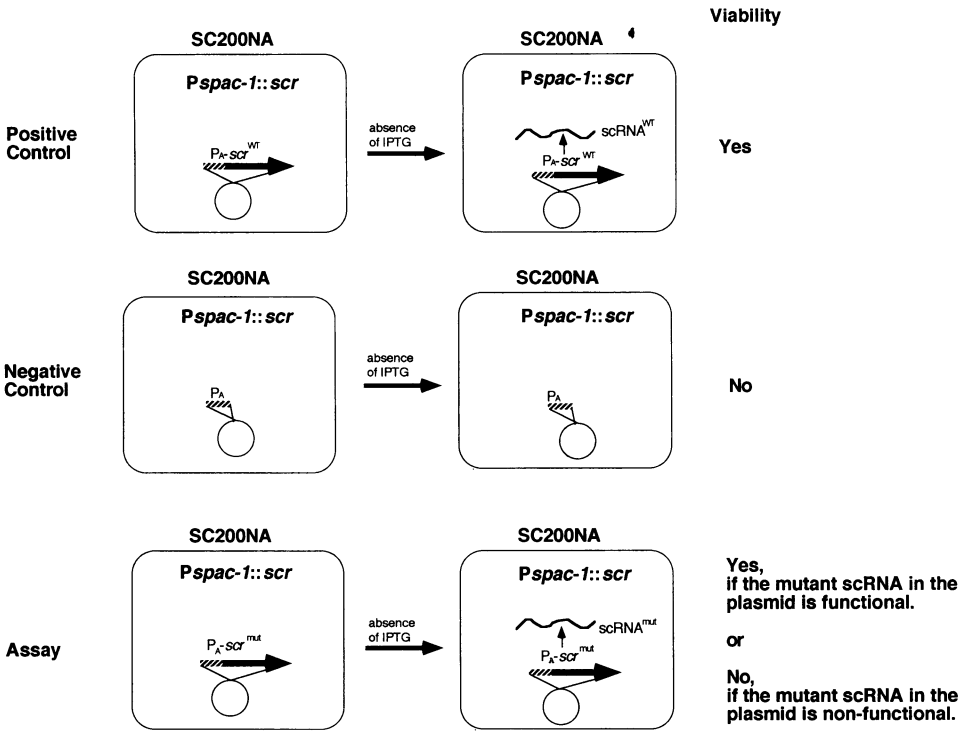

FIG. 1. In vivo assay system for mutant scRNA genes in *B. subtilis*. *B. subtilis* SC200NA has two scRNA genes on the chromosome. One gene codes for an intact scRNA and is regulated by the IPTG-inducible promoter P*spac-1*. The other gene is truncated and codes for about a 200-bp 5′ portion of scRNA. The truncated scRNA gene is regulated by the original promoter. The viability of the strains can be maintained by adding IPTG. Using the expression plasmid pTUBE809 and its derivatives, plasmid-encoded scRNA, mutated or wild type, can be introduced into SC200NA. Thus, in the absence of IPTG, growth of the transformants is dependent on plasmid-borne scRNA. ⟶, wild-type or mutant *scr* gene region; ▨, protein A promoter region (P_A).

in domain IV. To assess the importance of the stem-loop of domain IV, scRNA mutants with different lengths of nucleotide deletion encompassing an *Acc*III site, which is located at position 160 of wild-type scRNA, were constructed. Among six deletion mutants obtained, three in which 53 to 75 nucleotide were deleted should have most of domain IV missing and have no effect on the secondary structure of the remainder as a result of the deletions. None of the mutants restored the growth of SC200NA in the absence of IPTG (Table 1). To localize the nucleotide sequence responsible for the function of scRNA, the 22 (positions 154 to 175) and 42 (positions 144 to 185) nucleotides containing the evolutionarily conserved nucleotide sequences were deleted from the wild-type scRNA gene by site-directed mutagenesis (Fig. 2A). As shown in Fig. 2B, the mutant cells have a slower growth rate and reach half of the final cell density of wild-type cells.

TABLE 1. Effects of nucleotide deletions in domain IV of scRNA on the ability to restore the growth of SC200NA in the absence of IPTG

Plasmid	Nucleotide positions at start and end sites of deletion (start/end)	Growth[a] (A_{660})		Fold[b]
		−IPTG	+IPTG	
pTUBE826	145/197	0.26	0.86	0.30
pTUBE827	134/196	0.31	0.91	0.34
pTUBE828	140/214	0.24	0.90	0.27
pTUBE822	Wild type	0.86	0.86	1.0
pTUBE809	1/271	0.21	0.86	0.24

[a] Transformants harboring each plasmid were grown in liquid culture with or without IPTG, and A_{660} was measured at 12 h after inoculation.

[b] Ratio of A_{660} of cell culture grown without IPTG to A_{660} of cell culture grown with IPTG.

Deletion of domain I or both domains I and II affected sporulation but not vegetative growth. Since both domains I and II are restricted to the scRNA of *B. subtilis* among SRP RNA homologs of eubacteria, it would be of interest to determine their functions. We therefore examined the effects of depleting domain I or both domains I and II on vegetative cell growth and spore formation. When cultivated in L broth, pTUBE823 and pTUBE824 transformants (deletion regions in scRNA are shown in Fig. 3A) grew distinguishably from the pTUBE822 transformant (data not shown). To monitor the growth and sporulation of both transformants, cells were grown in Schaeffer medium supplemented with 10 μg of tetracycline per ml, and the results were compared with those for the pTUBE822 transformant. The growth rates of both pTUBE823 and pTUBE824 transformants growing exponentially at 37°C were identical to that of the pTUBE822 transformant (Fig. 3B), and there were no apparent differences in morphology (data not shown). In contrast, from the end of the exponential phase, the growth rates of both mutant strains appeared to be significantly inhibited.

After cultivation for 24 h in Schaeffer medium, gross sporulation of the pTUBE822 transformant occurred, while that of the pTUBE809 transformant was suppressed (Table 2). Under these conditions, pTUBE823 and pTUBE824 transformants sporulated but had the frequency of heat-resistant spores was reduced to 44 and 22%, respectively, of the level in the pTUBE822 transformant. To examine whether sporulation is

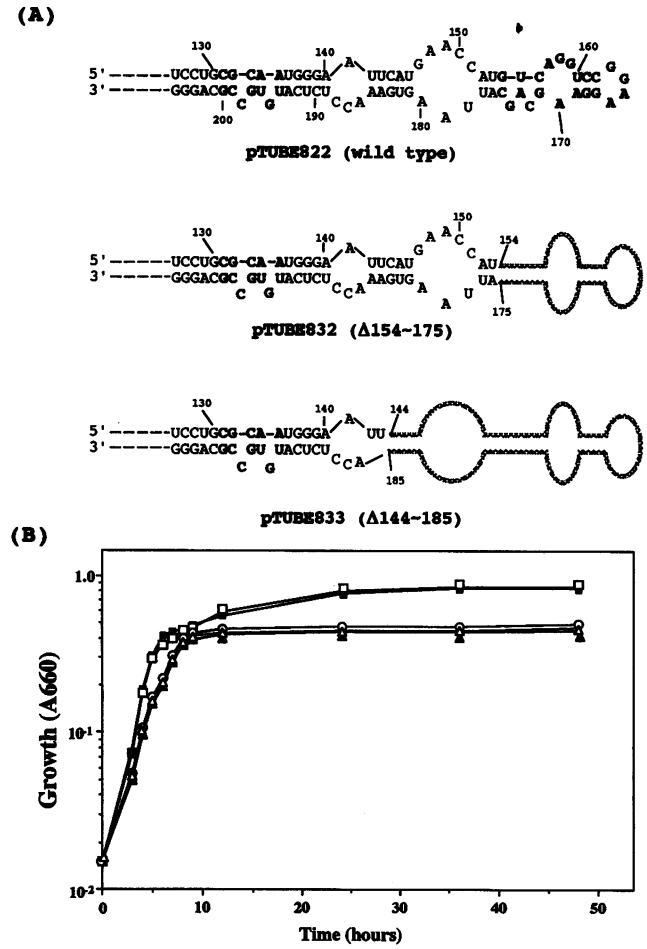

FIG. 2. Effects of 22- and 42-nucleotide deletions in domain IV on the ability of scRNA to restore the growth of strain SC200NA in the absence of IPTG. (A) Schematic drawing of the secondary structures of domain IV of wild-type and mutant scRNAs. The structures were numbered according to the wild-type scRNA structure. The deleted nucleotides are shown by gray lines. Boldface represents the nucleotides conserved among eubacterial SRP RNAs. (B) Growth curves of *B. subtilis* SC200-NA transformants harboring pTUBE822 (wild-type scRNA) (□), pTUBE832 (○), pTUBE833 (△), and pTUBE809 (▲) that were cultivated in Schaeffer medium at 37°C in the absence of IPTG. As a positive control, the pTUBE809 transformant was cultured in the presence of 3 mM IPTG (■).

delayed or blocked in pTUBE823 and pTUBE824 transformants, the frequency of heat-resistant spores was determined after cultivation for 48 h in Schaeffer medium (data not shown). No significant increase in the number of heat-resistant spores per ml was observed in either transformant compared with the level at 24 h. Moreover, the number of viable cells was also reduced. Therefore, it is plausible that the effect on sporulation is not delay but block.

On the other hand, at 24 h, the proportions of cells that contained forespores, which were observed by phase-contrast microscopy, were almost the same in pTUBE822, pTUBE823, and pTUBE824 transformants (70 to 80%). These results indicate that the poor sporulation observed in pTUBE823 and pTUBE824 transformants is attributed to the reduction of heat resistance of released dormant spores.

Alterations in heat resistance and ultrastructure of pTUBE823 and pTUBE824 transformant spores. The vegetative growth of pTUBE823 and pTUBE824 transformants was not remarkably affected. In addition, the cells sporulated and produced refractive spores when examined by phase-contrast microscopy. However, the spores of both cells were quite sensitive to heat. Almost all of the purified spores of pTUBE823 and pTUBE824 transformants were killed during a 15-min incubation at 95°C, while 60% of those of the pTUBE822 transformant survived (Fig. 4). The heat sensitivity of pTUBE823 and pTUBE824 transformant spores was apparent at 80°C. About 10 and 0.1% survival was obtained in pTUBE823 and pTUBE824 transformants, respectively, after 15 min at 80°C, in contrast to 70% for the pTUBE822 transformant. In addition, spores of the pTUBE824 transformant suffered a 30% drop in viability even at 50°C, whereas there was 100% survival of pTUBE822 and pTUBE823 transformants.

These data prompted us to examine the ultrastructure of pTUBE823 and pTUBE824 transformant spores. Typical examples for comparison among the spores of these transformants are shown in electron micrographs in Fig. 5. The cortex of the pTUBE822 transformant is well matured, and the core is highly contracted. The coat of this spore consists of an electron-dense outer layer and a lamella-like inner layer. In contrast, spores of the pTUBE823 and pTUBE824 transformants are premature, and the spore cortex is significantly reduced.

A deletion of domain I or both domains I and II affected the synthesis of extracellular protease. In mammalian cells, SRP RNA is an essential component of the SRP for protein translocation across the membrane of endoplasmic reticulum (31). In particular, functional roles of domains I and II of mammalian SRP RNA in elongation arrest have been proposed, in addition to functions as scaffolds for binding SRP proteins. Moreover, we previously showed that depletion of scRNA considerably reduced the production of extracellular α-amylase and β-lactamase in *B. subtilis* cells. To investigate whether the deletion of domain I or both domains I and II had any effects on extracellular protein production in vivo, the production of extracellular enzymes in the *B. subtilis* transformants harboring pTUBE823 or pTUBE824 was compared with that of pTUBE809 or pTUBE822 transformants. As shown in Table 3, in the absence of IPTG, the amount of protease activity in the culture medium of the pTUBE809 transformant was decreased to about 20% of that of the pTUBE822 transformant. The total activity of extracellular protease was reduced in parallel with the increased number of deleted nucleotides. The pTUBE823 and pTUBE824 transformants produced 60 and 28% of the activity of the pTUBE822 transformant after 48 h of cultivation. The alkaline protease inhibitor phenylmethylsulfonyl fluoride (1 mM) inhibited the extracellular activities of pTUBE823 and pTUBE824 transformants by about 60% as well as that of the pTUBE822 transformant. These results suggest that deletion of domain I or both domains I and II affected not only alkaline protease but also neutral protease. After the initiation of sporulation, *B. subtilis* simultaneously synthesizes intracellular protease. To date, at least two major intracellular proteases have been characterized in sporulating cells of *B. subtilis*: a major serine protease of the subtilisin type (protease I) and a less prevalent trypsin-like activity (protease II) (23). The production of total intracellular protease activity in pTUBE823 and pTUBE824 transformants in the absence of IPTG was similar to that in the presence of IPTG (data not shown). Furthermore, the activities were similar to that of the pTUBE822 transformant. These results suggest that the net protein synthesis in each transfor-

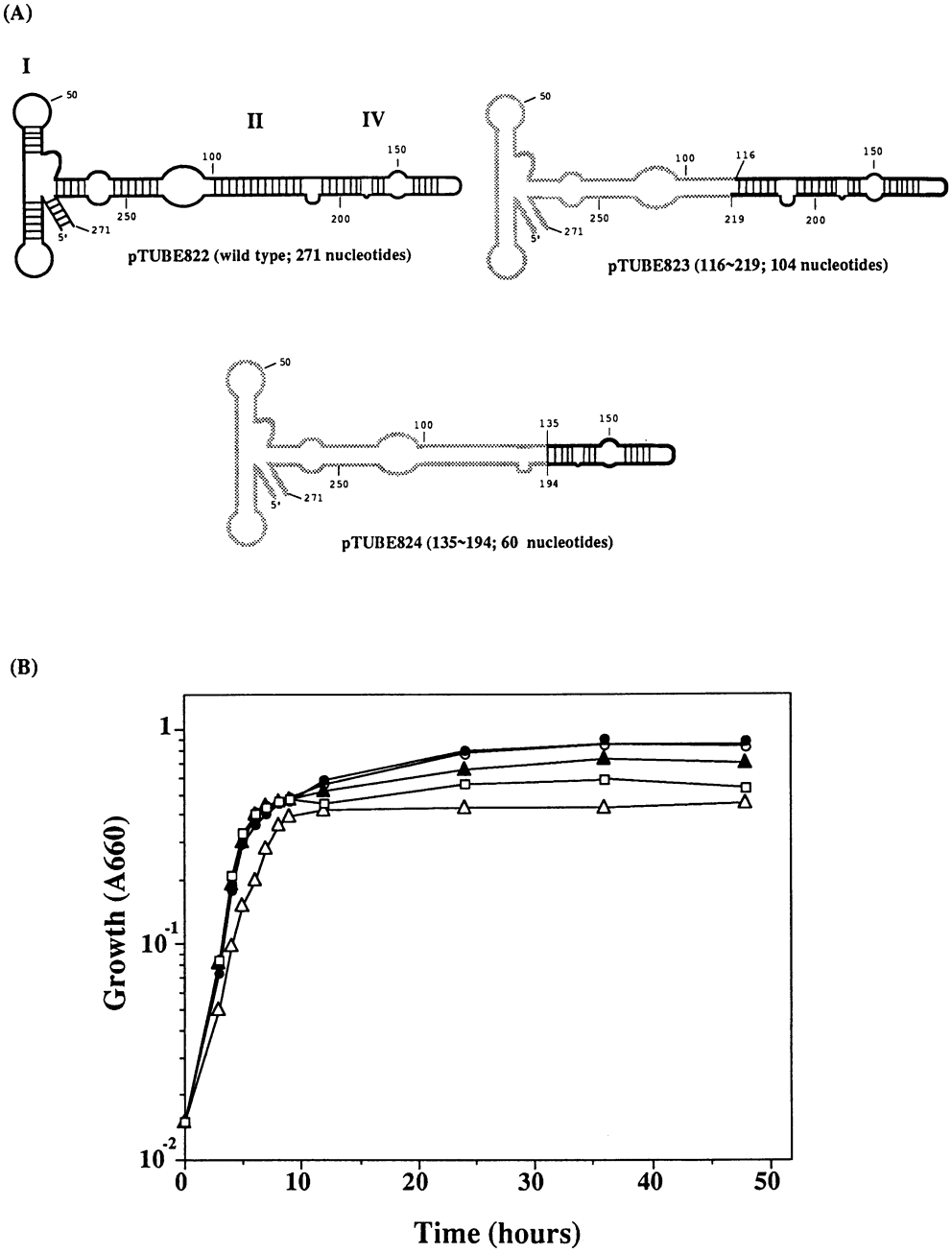

FIG. 3. Effects of deleting domain I or both domains I and II on the ability of scRNA to restore the growth of strain SC200NA in the absence of IPTG. (A) Predicted secondary structures of wild-type and mutant scRNAs were generated with the RNA structure-editing computer program DNASIS. Domains I, II, and IV indicated were deduced from a structural comparison with human SRP RNA. Black and gray lines represent the coding and deleted regions, respectively. (B) Growth curves of *B. subtilis* SC200NA transformants harboring pTUBE822 (○), pTUBE823 (▲), pTUBE824 (□), and pTUBE809 (△) that were cultivated in Schaeffer medium in the absence of IPTG. As a positive control, the pTUBE809 transformant was cultured in the presence of IPTG (●).

mant harboring each mutant scRNA was not affected by deleting domain I or both domains I and II.

To rule out the possibility that the lower levels of extracellular protease were initiated at the transcriptional level, alkaline protease mRNA levels were compared among pTUBE822, pTUBE823, and pTUBE824 transformants after 18 h of cultivation. Total RNA from each cell was extracted, and Northern (RNA) blots were probed with a labeled alkaline protease gene (*aprE*) fragment. Densitometry with autoradiograms showed that the levels of *aprE* mRNA present in pTUBE823 and pTUBE824 transformants were similar to that of the pTUBE822 transformant (data not shown). Therefore, the decreased level of production of extracellular alkaline protease is not accounted for by a deficient transcriptional ability.

Effects of deletions of each domain on stability and ability to associate with Ffh protein. In pTUBE832 and pTUBE833 transformants, deletions of 22 and 42 nucleotides in domain IV caused drastic effects and loss of function of scRNA. The

TABLE 2. Effects of deletion of domain I or both domains I and II of scRNA on sporulation[a]

Plasmid	Addition of IPTG	10^8 CFU/ml		Frequency (%)[b]
		Viable cells (10^8)	Heat-resistant spores (10^8)	
pTUBE809	+	3.9	2.7	69
	−	2.5	0.1	4
pTUBE822	−	4.2	2.1	50
pTUBE823	−	3.2	0.7	22
pTUBE824	−	3.8	0.4	11

[a] The transformants were cultured in the presence and absence of IPTG at 37°C for 24 h, and then the efficiency of heat-resistant spore formation was determined.

[b] Determined as (value for viable cells/value for heat-resistant cells) × 100.

secondary or higher structures of RNA molecules contribute to stability and activities to achieve its active structure. To examine whether the observed effects of pTUBE832 and pTUBE833 transformants are due to domain-specific deletion or result from altered levels of the RNA, we measured the amounts of scRNA. As shown in Fig. 6A, the amount of mutant scRNA in each transformant was almost identical to that of the pTUBE822 transformant.

On the other hand, our preliminary data suggest that scRNA binds to Ffh of *B. subtilis* in the domain IV region (16a). Therefore, mutant scRNAs which lack domains I or both domains I and II are considered to have binding sites for Ffh. To examine whether deletions in domains I and II affect interaction with Ffh, we measured the amounts of mutant scRNA which bind to Ffh. Figure 6B shows that the amounts of scRNA in Ffh-bound form were decreased to about 30 and 5%, respectively, of the amount found for the pTUBE822 transformant. The amounts of Ffh in pTUBE822, pTUBE823, and pTUBE824 transformants at 8 and 18 h after inoculation were determined by immunoblotting with an anti-*B. subtilis* Ffh antiserum. The amounts of Ffh in the pTUBE823 and pTUBE824 transformants were the same as that in the pTUBE822 transformants (data not shown).

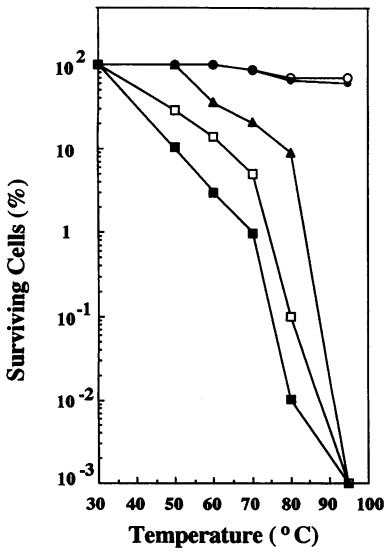

FIG. 4. Effects of deleting domain I or both domains I and II on the heat resistance of purified spores. *B. subtilis* SC200NA transformants harboring pTUBE822 (○), pTUBE823 (▲), pTUBE824 (□), and pTUBE809 (■) were grown in Schaeffer medium in the absence of IPTG at 37°C for 24 h. The spores were purified from each, and their heat resistance was assayed. As a positive control, spores from the pTUBE809 transformant grown in the presence of IPTG were also assayed (●).

DISCUSSION

scRNA of *B. subtilis* is a small RNA species, consisting of domains I, II, and IV of SRP RNA. Several mutant *B. subtilis* scRNAs have been constructed to identify the structural elements required for its function.

A mutant lacking 22 nucleotides in domain IV could not restore the growth of SC200NA in the absence of IPTG, suggesting that an essential region of scRNA lies between nucleotide positions 154 and 175. This region corresponds exactly to a domain extremely well conserved in SRP RNA during evolution. Figure 6A shows that deletion of domain IV does not affect the stability of RNA. These results show that certain conserved nucleotides of domain IV are important determinants for appropriate function of scRNA. Cell death may be, at least in part, a consequence of disrupting the activity of scRNA in protein synthesis, since our previous study showed that a depletion of scRNA primarily caused a decreased ability to synthesize protein and led to eventual cell death. In contrast to a mutant 4.5S RNA in *E. coli* which resulted in a dominant lethal phenotype (18), all mutant scRNAs presented in Table 1 have a recessive phenotype in *B. subtilis*. In the case of a dominant lethal 4.5S RNA mutant, phylogenetically conserved domain IV has been duplicated (18). In contrast, the mutant scRNAs in Table 1 contain nucleotide deletions in domain IV. Analysis of RNA indicates that mutant scRNAs can no longer associate with Ffh in *B. subtilis* (data not shown). Therefore, if both wild-type and mutant scRNAs are produced at the same time, wild-type scRNA can associate with Ffh to form a functional complex. This may be a reason why the mutant scRNAs presented in Table 1 have recessive phenotypes in *B. subtilis*.

On the other hand, deletion of domain I or both domains I and II did not affect exponentially growing SC200NA cells. In this state, the ability of protein synthesis, as estimated by incorporation of radioactive methionine, was not affected in pTUBE823 and pTUBE824 transformants (data not shown). In contrast, from the end of exponential growth, the growth of pTUBE823 and pTUBE824 transformants was significantly affected. Concomitantly, at 24 h after inoculation, the efficiency of heat-resistant spore formation was greatly reduced in these strains. In addition, the production of extracellular proteases was reduced. Therefore, scRNA is essential for cells growing in the stationary state as well as for heat-resistant spore formation, and domains I and II have pivotal roles in its function.

With respect to the function of scRNA in vivo, we propose that one of the functions of *B. subtilis* scRNA is to act as a translational modulator. Domain IV could be sufficient to maintain this activity, and neither domain I nor domain II may be required, since the pTUBE824 transformant can grow normally during exponential state. This view is highly consistent with the finding that the smaller *E. coli* 4.5S RNA, which corresponds to only domain IV, could maintain vegetative growth of *B. subtilis* SC200NA in the absence of IPTG (17). Phylogenetic studies revealed that the primary sequences and secondary structures of the domain IV region of SRP RNA-like RNAs are highly conserved among several organisms. The functional relationship between the mammalian and bacterial RNAs is demonstrated by the ability of heterologous bacterial RNAs to complement the loss of 4.5S RNA in *E. coli* cells (29)

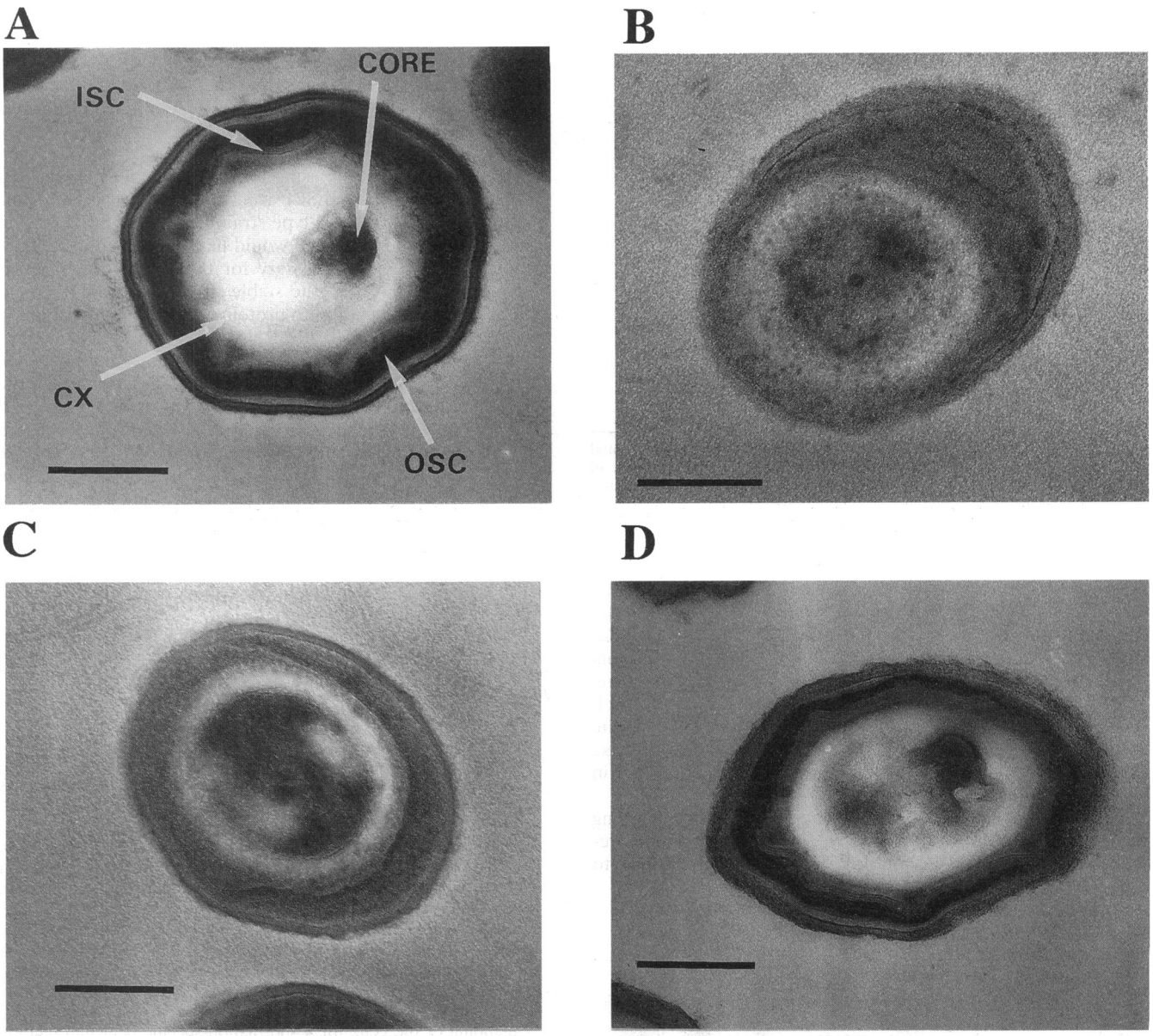

FIG. 5. Ultrastructure analysis of spores. *B. subtilis* SC200NA transformants harboring pTUBE822 (A), pTUBE823 (B), and pTUBE824 (C) were grown in Schaeffer medium in the absence of IPTG for 24 h. As a positive control, the pTUBE809 transformant was grown in Schaeffer medium in the presence of IPTG (D). Spores were prepared and sectioned as described in Materials and Methods. Spores of the pTUBE822 transformant show the dense outer spore coat (OSC), the multilayered inner spore coat (ISC), and the closely covering cortex (CX). The cytoplasmic regions (CORE) are highly contracted. Bars, 0.2 μm.

and by the ability of the human SRP 7S RNA to complement the loss of scRNA in *B. subtilis* (17). Therefore, the function of domain IV has remained conserved. Since 7 of 22 residues in domain IV of the mammalian and bacterial RNAs differed, phylogenetic comparisons are useful for identifying the specific residues required for each function.

Spores from pTUBE823 and pTUBE824 transformants lacked a large amount of cortex (Fig. 5). Imae and Strominger have shown that the amount of spore cortex is closely related to the heat resistance of *B. sphaericus* spores (13). This deficiency could be due to the reduced spore cortex. Recently, Buchanan and Ling (5) characterized a novel penicillin-binding protein, PBP5*, in *B. subtilis*. This protein, which has D,D-carboxypeptidase activity, is located in the outer forespore membrane and is specifically required for synthesis of the spore form of the peptidoglycan known as cortex. The mutant spores, in which the PBP5* gene (*dacB*) was disrupted, were quite sensitive to heat, but vegetative growth was not affected (4). These phenotypes closely resemble those of the pTUBE823 and pTUBE824 transformants. PBP5* could be considered to belong to a set of proteins whose biosynthesis depends on scRNA function. Thus, the amount of PBP5* protein in pTUBE823 and pTUBE824 transformants should be determined. Along with a decreased level of sporulation, the production of extracellular proteases declined in pTUBE 823 and pTUBE824 transformants compared with level in the

TABLE 3. Effects of deleting domain I or both I and II on the production of extracellular protease

Plasmid	Addition of IPTG	Time (h)	Extracellular protease sp act[a] (U/ml at A_{660})
pTUBE809	+	12	7.1
	+	24	20.6
	+	48	38.2
	−	12	6.1
	−	24	7.0
	−	48	7.0
pTUBE822	−	12	12.1
	−	24	20.6
	−	48	39.4
pTUBE823	−	12	10.3
	−	24	17.0
	−	48	24.2
pTUBE824	−	12	10.0
	−	24	10.3
	−	48	11.2

[a] The SC200NA strains harboring plasmids pTUBE822, pTUBE823, and pTUBE824 were grown in Schaeffer medium in the presence and absence of IPTG. Protease activity in the supernatants was assayed at the indicated times.

pTUBE822 transformant. A simple explanation for the reduced level of extracellular proteases is that the deletion caused a deficiency in net protein synthesis. However, under the same growth conditions, the production of intracellular proteases was not affected. Furthermore, the transcriptional level of extracellular protease is not affected. Therefore, scRNA functions in the biosynthesis of some sets of extracellular proteins, which are more efficiently expressed during the stationary and sporulating growth states. However, from these data, we cannot speculate further on the function of scRNA in protein translocation.

The 22-nucleotide sequence in domain IV is identical among eubacterial RNA species. Moreover, the corresponding regions of *E. coli* 4.5S RNA and mycoplasma RNA are known to serve as binding sites for each SRP54 homolog (25, 32). SRP54 and *E. coli* Ffh can recognize the signal sequences of nascent polypeptides (2, 16). Recently, we have cloned and characterized the *B. subtilis* gene encoding the SRP54 homolog (Ffh) (11). Preliminary data suggest that scRNA binds to Ffh in vivo (16a). Figure 6B shows that deletions in domains I and II of scRNA lead to gross misfolding of the RNAs. Moreover, immunoblotting analysis using anti-*B. subtilis* Ffh shows that pTUBE823 and pTUBE824 transformants have the same amount of Ffh as the wild-type strain does at 8 and 18 h after a inoculation. Therefore, we would like to conclude that both domains I and II are necessary for the RNA to achieve its active structure and that the stable ribonucleoprotein formation between scRNA and Ffh protein is needed for sporulation to proceed. In domain I of scRNA, two stem-loop structures exist, and possible tertiary interaction can be formed between them. This characteristic structural feature is restricted to the scRNA and human SRP 7S RNA. In the mammalian SRP complex, the 9,000- and 14,000-molecular-weight proteins bind to the corresponding region of mammalian SRP RNA and are essential for elongation arrest in the protein translocation pathway. Identification of the putative homologs of such proteins in *B. subtilis* may help to elucidate the function of scRNA.

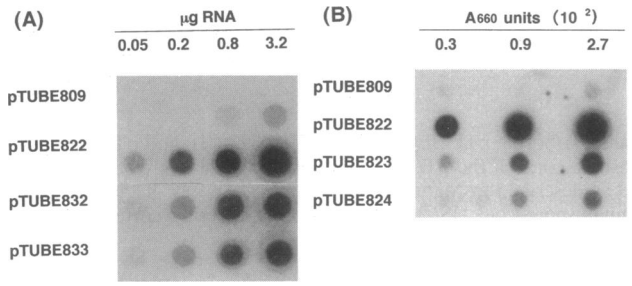

FIG. 6. Dot blot hybridization analysis of scRNAs in *B. subtilis* cells. (A) Effects of deletions in domain IV on the stability of mutant scRNA in vivo. Total RNA was extracted from *B. subtilis* SC200NA harboring each plasmid after 18 h of cultivation in Schaeffer medium in the absence of IPTG. Then, 0.05, 0.2, 0.8, and 3.2 μg of the total RNA from each transformant were blotted onto a GeneScreen Plus filter. Thereafter, DNA-RNA hybridization was performed. (B) Association of wild-type and mutant scRNAs with Ffh in vivo. Lysates were prepared from corresponding amounts of cultures of each transformant after 18 h of cultivation in Schaeffer medium in the absence of IPTG. Ffh was immunoprecipitated with antiserum, and the level of coprecipitated scRNAs was analyzed by dot blot hybridization. The three different RNA samples were prepared from three different amounts of cell lysates indicated by the A_{660} unit values at the top. The amounts of scRNA coprecipitated were quantitated densitometric analysis of the autoradiograms.

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REFERENCES

1. **Amaya, Y., and A. Nakano.** 1991. SRH1 protein, the yeast homologue of the 54 kDa subunit of signal recognition particle, is involved in ER translocation of secretory proteins. FEBS Lett. **283:**325–328.
2. **Bernstein, H. D., M. A. Poritz, K. Strub, P. J. Hoben, S. Bernstein, and P. Walter.** 1989. Model for signal sequence recognition from amino acid sequence of 54K subunit of signal recognition particle. Nature (London) **340:**482–486.
3. **Brennwald, P., X. Liao, K. Holm, G. Porter, and J. A. Wise.** 1988. Identification of an essential *Schizosaccharomyces pombe* RNA homologous to the 7SL component of signal recognition particle. Mol. Cell. Biol. **8:**1580–1590.
4. **Buchanan, C. E., and A. Gustafson.** 1992. Mutagenesis and mapping of the gene for a sporulation-specific penicillin-binding protein in *Bacillus subtilis*. J. Bacteriol. **174:**5430–5435.
5. **Buchanan, C. E., and M. L. Ling.** 1992. Isolation and sequence analysis of *dacB*, which encodes a sporulation-specific penicillin-binding protein in *Bacillus subtilis*. J. Bacteriol. **174:**1717–1725.
6. **Bystrom, A. S., K. J. Hjalmarsson, P. M. Wikstrom, and G. R. Bjork.** 1983. The nucleotide sequence of an *Escherichia coli* operon containing genes for the tRNA (m^1G) methyltransferase, the ribosomal proteins S16, S19 and a 21K polypeptide. EMBO J. **2:**899–905.
7. **Campos, N., J. Palau, M. Torrent, and D. Ludevid.** 1988. Signal recognition particles are present in maize. J. Biol. Chem. **263:**9646–9650.
8. **Chavira, R., T. J. Burnett, and J. H. Hageman.** 1984. Assaying proteinases with azocoll. Anal. Biochem. **136:**446–450.
9. **Hann, B. C., M. A. Poritz, and P. Walter.** 1989. *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* contain a homologue to the 54kD subunit of the signal recognition particle that in *S. cerevisiae* is essential for growth. J. Cell Biol. **109:**3223–3230.
10. **Hann, B. C., and P. Walter.** 1991. The signal recognition particle in *S. cerevisiae*. Cell **67:**131–144.
11. **Honda, K., K. Nakamura, M. Nishiguchi, and K. Yamane.** 1993. Cloning and characterization of a *Bacillus subtilis* gene encoding a homolog of the 54 kilodalton subunit of mammalian signal recog-

nition particle and *Escherichia coli* Ffh. J. Bacteriol. **175:**4885–4894.
12. **Hsu, L. M., J. Zagorski, and M. J. Fournier.** 1984. Cloning and sequence analysis of the *Escherichia coli* 4.5S RNA gene. J. Mol. Biol. **178:**509–531.
13. **Imae, Y., and J. L. Strominger.** 1976. Relationship between cortex content and properties of *Bacillus sphaericus* spores. J. Bacteriol. **126:**907–913.
14. **Kaine, B. P.** 1990. Structure of the archaebacterial 7S RNA molecule. Mol. Gen. Genet. **221:**315–321.
15. **Kunkel, T. A., J. D. Roberts, and R. A. Zakour.** 1987. Rapid and efficient site specific mutagenesis without phenotypic selection. Methods Enzymol. **154:**367–382.
16. **Luirink, J., S. High, H. Wood, A. Giner, D. Tollervey, and B. Dobberstein.** 1992. Signal sequence recognition by an *Escherichia coli* ribonucleoprotein complex. Nature (London) **359:**741–743.
16a. **Nakamura, K., et al.** Unpublished data.
17. **Nakamura, K., Y. Imai, A. Nakamura, and K. Yamane.** 1992. Small cytoplasmic RNA of *Bacillus subtilis*: functional relationship with human signal recognition particle 7S RNA and *Escherichia coli* 4.5S RNA. J. Bacteriol. **174:**2185–2192.
18. **Poritz, M. A., H. D. Bernstein, K. Strub, D. Zopf, H. Wilhelm, and P. Walter.** 1990. An *E. coli* ribonucleoprotein containing 4.5S RNA resembles mammalian signal recognition particle. Science **250:**1111–1117.
19. **Poritz, M. A., V. Siegel, W. Hansen, and P. Walter.** 1988. Small ribonucleoprotein in *Schizosaccharomyces pombe* and *Yarrowia lipolytica* homologous to signal recognition particle. Proc. Natl. Acad. Sci. USA **85:**4315–4319.
20. **Poritz, M. A., K. Strub, and P. Walter.** 1988. Human SRP RNA and *E. coli* 4.5S RNA contain a highly homologous structural domain. Cell **55:**4–6.
21. **Ribes, V., K. Romisch, A. Giner, B. Dobberstein, and D. Tollervey.** 1990. *E. coli* 4.5S RNA is part of a ribonucleoprotein particle that has properties related to signal recognition particle. Cell **63:**591–600.
22. **Romisch, K., J. Webb, J. Herz, S. Prehn, R. Frank, M. Vingron, and B. Dobberstein.** 1989. Homology of 54K protein of signal recognition particle, docking protein and two *E. coli* proteins with putative GTP-binding domains. Nature (London) **340:**478–482.
23. **Ruppen, M. E., G. L. Alstine, and L. Band.** 1988. Control of intracellular serine protease expression in *Bacillus subtilis*. J. Bacteriol. **170:**136–140.
24. **Saiki, R. K., S. Scharf, F. Faloona, K. Mullis, G. Horn, H. A. Erlich, and N. Arnheim.** 1985. Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science **230:**1350–1354.
25. **Samuelsson, T.** 1992. A *Mycoplasma* protein homologous to mammalian SRP54 recognizes a highly conserved domain of SRP RNA. Nucleic Acids Res. **20:**5763–5770.
26. **Samuelsson, T., and Y. Guindy.** 1990. Nucleotide sequence of a Mycoplasma mycoides RNA which is homologous to *Escherichia coli* 4.5S RNA. Nucleic Acids Res. **18:**4938.
27. **Schaeffer, P., J. Millet, and J. P. Aubert.** 1965. Catabolite repression of bacterial sporulation. Proc. Natl. Acad. Sci. USA **54:**704–711.
28. **Struck, J. C. R., R. K. Hartmann, H. Y. Toschka, and V. A. Erdmann.** 1989. Transcription and processing of *Bacillus subtilis* small cytoplasmic RNA. Mol. Gen. Genet. **215:**478–482.
29. **Struck, J. C. R., R. A. Lempicki, H. Y. Toschka, V. A. Erdmann, and M. J. Fournier.** 1990. *Escherichia coli* 4.5S RNA gene function can be complemented by heterologous bacterial RNA genes. J. Bacteriol. **172:**1284–1288.
30. **Struck, J. C. R., D. W. Vogel, N. Ulbrich, and V. A. Erdmann.** 1988. The *Bacillus subtilis* scRNA is related to the 4.5S RNA from *Escherichia coli*. Nucleic Acids Res. **16:**2719.
31. **Ullu, E., and A. M. Weiner.** 1984. Human genes and pseudogenes for the 7SL RNA component of signal recognition particle. EMBO J. **3:**3303–3310.
32. **Wood, H., J. Luirink, and D. Tollervey.** 1992. Evolutionary conserved nucleotides within the *E. coli* 4.5S RNA are required for association with P48 *in vitro* and for optimal function *in vivo*. Nucleic Acids Res. **20:**5919–5925.