Identification and characterization of novel small RNAs in the *aspS*–*yrvM* intergenic region of the *Bacillus subtilis* genome

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A novel RNA species was isolated from *Bacillus subtilis*, and its sequence was determined and mapped to its genetic position. This RNA was termed BS190 RNA from the length of its mature form (190 nt), and the gene encoding it is located within the *aspS*–*yrvM* intergenic region of the *B. subtilis* genome. Northern blotting revealed that the novel RNA species is transcribed in vegetative cells as a larger precursor (BS201 RNA, 201 nt). After transcription, the 5' end of the precursor is processed to generate the mature form, BS190 RNA. A computer-aided prediction of the secondary structure of BS190 RNA showed that it can be folded into a single hairpin structure with some bulge structures. The authors found that the growth rate of a Δ BS190 mutant strain of *B. subtilis* was reduced when compared to the wild-type. A phylogenetic comparison of the sequence of the BS190 RNA gene with sequences from the databases suggests that RNA related to BS190 RNA appears to be encoded in the genomes of *Bacillus halodurans* and *Listeria monocytogenes*.

Keywords: non-coding RNA, RNA processing, RNA secondary structure

INTRODUCTION

Bacterial and eukaryotic cells contain small RNAs (sRNAs) that are not translated (Wassarman *et al.*, 1999; Wightman *et al.*, 1993; Lankenau *et al.*, 1994). These sRNAs function as RNA molecules instead of encoding proteins. Non-coding sRNA genes are quite numerous and their functions vary (Eddy, 1999; Erdman *et al.*, 2001; Storz, 1999). Most of these non-coding sRNAs can be defined as regulatory molecules that seem to fine-tune the cellular responses to stress by integrating environmental signals into global regulation (Altuvia *et al.*, 1997; Zhang *et al.*, 1998). sRNAs act via several mechanisms, such as RNA–RNA base-pairing, RNA–protein interactions and intrinsic RNA activity (Altuvia & Wagner, 2000; Wassarman & Storz, 2000; Kirsebom, 2001); they also regulate diverse cellular functions, including RNA processing, mRNA stabilization, translation, protein stabilization and protein secretion. At least 10 sRNAs are encoded within the *Escherichia coli*

genome (Wassarman *et al.*, 1999). Recently, Wassarman *et al.* (2001) used sequence conservation coupled with micro-array expression analysis to identify 17 new non-coding RNAs in *E. coli*. Argaman *et al.* (2001) used sequence conservation coupled with promoter and *p*-independent terminator prediction and identified 14 new non-coding RNAs in the same species. Moreover, Rivas *et al.* (2001) predicted 275 candidates for structural RNA loci in *E. coli* using computational analysis and empirically found that at least 11 loci express small, apparently non-coding RNAs. Extensive experimental characterization of the new loci is now under way.

Signal recognition particle (SRP) RNA is involved in the translocation of proteins across the endoplasmic reticulum (Stroud & Walter, 1999). SRP RNAs are ubiquitous in nature and contain evolutionarily conserved structural features (Poritz *et al.*, 1988; Gorodkin *et al.*, 2001). The 4-5S RNA of *E. coli* and the small cytoplasmic RNA (scRNA) of *Bacillus subtilis* belong to a family of SRP RNAs that function in protein secretion as an integrated component of the SRP-like particle along with an Ffh protein that is a homologue of one of the mammalian SRP components, SRP54 (Bernstein *et al.*,

Abbreviations: scRNA, small cytoplasmic RNA; sRNA, small RNA; tmRNA, transfer-message RNA.

Fig. 1. Mapping and sequencing of the *aspS*–*yrvM* intergenic region. (A) Schematic representation of the organization of the genomic region around the BS190 RNA gene. Solid box, the gene encoding BS190 RNA (*bsrA*); cross-hatched boxes, upstream (UP) and downstream (DOWN) DNA fragments used to construct the BS190 RNA null-mutant strain (Δ BS190 RNA). Numbering is according to that of the *B. subtilis* whole-genome database and shows the distance from the origin of replication. (B) Nucleotide sequence of a 0.9 kb DNA fragment that includes the BS190 RNA gene (*bsrA*). The RNA-coding region corresponds to nucleotide positions 18815–18626 in the *B. subtilis* whole-genome database (GenBank accession no. Z99118). The region encoding BS201 RNA is underlined. The nucleotide at the 5' end of BS190 RNA, as determined by primer extension, is boxed (a). Two ORFs that are equivalent to portions of the *aspS* and *yrvM* genes are enclosed by dotted lines. Possible promoter sequences (–35 and –10) at the front of the BS190 RNA gene are enclosed by boxes.

1989). However, the earliest defect seen in cells conditionally depleted of 4·5S RNA or scRNA is the inhibition of protein synthesis – both RNA molecules are normally associated with ribosomes (Brown, 1987; Bourgaize & Fournier, 1987; Nakamura *et al.*, 1999). These results suggest a role for scRNA and 4·5S RNA in translation. Elongation factor G (EF-G) is a 4·5S RNA-binding

protein, and a depletion of 4·5S RNA causes an increase in the amount of EF-G associated with ribosomes, suggesting that 4·5S RNA is involved in EF-G recycling (Brown & Fournier, 1984; Jovine *et al.*, 2000; Shibata *et al.*, 1996). Therefore, 4·5S RNA affects both protein synthesis and secretion, but the exact mechanism of its action in each pathway is, as yet, unclear.

In contrast to *E. coli*, only three sRNAs besides 5S rRNA and tRNAs have been identified in *B. subtilis*. Genomic sequence data and new algorithms have allowed the development of systematic screens for non-coding sRNA genes (Le *et al.*, 1989; Chen *et al.*, 1990; Dandekar & Hentze, 1995; Lowe & Eddy, 1997). The present study identifies and characterizes two novel sRNAs in *B. subtilis* – named BS190 RNA and BS201 RNA – and shows that the smaller, mature BS190 RNA is generated by processing its precursor, BS201 RNA, at the 5' end. This study also shows that a reduction in the level of BS190 RNA expression leads to defective vegetative growth.

METHODS

Bacterial strains. *B. subtilis* 168 (*trpC2*) was used to construct a Δ BS190 RNA strain (a mutant with a BS190 RNA gene deletion).

Preparation and purification of RNAs from *B. subtilis*. Total RNA was isolated from frozen cells essentially as described by Sambrook *et al.* (1989) and Nuyts *et al.* (2001). Frozen cells (0.1 g, wet weight) were vigorously shaken in 2 ml phenol/chloroform (1:1, v/v) containing 2.4 g glass beads. Total RNA was loaded into the wells of 6% denaturing polyacrylamide gels (48 cm height) in 6 M urea and then resolved by electrophoresis for 14 h at 500 V. After this time, the gels were stained with ethidium bromide and visualized under a UV light. Two bands of about 200 nt in size (estimated by comparison with molecular markers) were excised from the gels and dialysed against 0.8 ml of 0.5 × TBE buffer [45 mM Tris/HCl (pH 8.0), 45 mM boric acid, 1 mM EDTA]. RNAs were electro-eluted from the gel and purified as described above.

Cloning and sequencing of the BS190 RNA and BS201 RNA genes. Both the BS190 RNA and the BS201 RNA, which had been purified from denaturing polyacrylamide gels and polyadenylated at their 3' termini using poly(A) polymerase (Takara-Shuzo), were used as templates for the reverse transcription of single-stranded DNA copies using the oligomer (dT)₂₅ as a primer. The first strand of DNA was synthesized using RNA-dependent DNA polymerase (reverse transcriptase; Takara-Shuzo). The product of the first-strand synthesis was treated with RNase H (Takara-Shuzo), and the second strand of DNA was constructed using *E. coli* DNA polymerase I (Takara-Shuzo). The DNA fragment generated was purified and then inserted into the *Hinc*II site of pUC118. The hybrid plasmid was used to transform *E. coli* JM109 as described above. Clones containing the BS190 RNA gene or the BS201 RNA gene were designated pTUE190 and pTUE201, respectively. Both strands of the DNA fragment were shotgun sequenced by chain termination using the Dye Terminator Cycle Sequencing Kit (Applied Biosystems). The two gene fragments that were cloned into pTUE190 and pTUE201 covered the 5' ends of BS190 RNA and BS201 RNA, respectively, according to a comparison with the 5' end of each RNA determined by primer extension.

Mapping the 5' ends of the BS190 RNA and BS201 RNA genes. This was done by primer extension. Primer P1 (5'-CGCCATTTAAAAATGCGGGC-3'; positions 542–523, Fig. 1B) was labelled by 5'-end phosphorylation using [γ -³²P]ATP in the presence of polynucleotide kinase (TOYOBO); the subsequent extension was done essentially as described previously (Nakamura *et al.*, 1994) in a reaction mixture containing 1 pmol of labelled P1, 10–90 μ g of total RNA or

Fig. 2. Effects of BS190 RNA depletion on cell growth. (A) Northern blots of BS201 RNA (precursor) and BS190 RNA (mature). Total RNAs were prepared from both the wild-type (lane 1) and the Δ BS190 RNA (lane 2) strains at 4 h after inoculation [indicated by an arrow in (B)]. The transcripts (precursor and mature) were identified based on their sizes. (B) Growth curves of the wild-type strain (◆) and the Δ BS190 RNA strain (●) in LB broth at 37 °C.

0.2 μ g of gel-purified RNA, 1 mM of each dNTP and 25 U of AMV reverse transcriptase (Takara-Shuzo) in 20 μ l of the buffer supplied by the manufacturer. The oligonucleotide and the RNA were mixed and then ethanol precipitated. The precipitate was resuspended in 10 μ l TE buffer [Tris/HCl (pH 7.5), 1 mM EDTA] and incubated at 70 °C for 5 min and slowly cooled to 42 °C. The other components of the reaction mixture were added to the suspension, and the mixture was incubated at 42 °C for 60 min. The reaction was stopped by a 5 min incubation at 95 °C. Formamide loading buffer was added to the samples; the reaction products were then separated through polyacrylamide sequencing gels containing 6 M urea. The gels were dried and the bands were visualized by autoradiography. Primer-extension products were identified on the gels by comparing the bands generated with those of sequencing reactions generated with the same labelled oligonucleotide and a Sequencing Kit (Takara-Shuzo). The templates for sequencing reactions were M13 phage clones containing the wild-type *B. subtilis* BS190 RNA gene.

Sucrose-gradient centrifugation. Vegetative cells (2 g wet weight) were collected by centrifugation and homogenized with sand quartz in 20 ml of buffer A [50 mM TEA (pH 8.0), 5 mM Mg(CH₃COO)₂, 0.5 mM PMSF, 1 mM DTT]. The homogenate was centrifuged in COREX tubes (30 ml) at 12000 g for 10 min. The supernatant was then loaded into a 5–20% sucrose density gradient prepared in buffer A and separated by centrifugation at 60000 g (8 h at 4 °C) using a Beckman SW41Ti rotor. Samples were collected from the top of the gradient as 21 fractions of 500 μ l each; the polysomal

Fig. 3. Detection of sRNA species from *B. subtilis* 168 (*trpC2*). Total RNA was isolated as described in Methods, then separated through a 6% polyacrylamide gel under denaturing conditions (lane 2). The two unidentified bands (X-RNA and Y-RNA) were purified from the gel and resolved by electrophoresis (lanes 3 and 4). The identities of the transcripts (RNaseP M1 RNA, tmRNA, scRNA and 5S rRNA) were based on their size and responses to DNA probes encoding each RNA gene in Northern blots (data not shown). Lane 1, molecular mass marker.

fraction was recovered from the bottom of the tube. Aliquots (400 μ l) of each fraction were extracted with phenol/chloroform/isoamyl alcohol (24:24:1, v/v/v) and precipitated with 2 vols ethanol. The RNAs were collected and dried for Northern hybridization.

Northern blotting. RNA was isolated from cell lysates prepared from *B. subtilis* cells at the vegetative phase of growth. After electrophoresis on a sequencing gel, the RNA was electrotransferred onto Gene Screen nylon membranes (NEN Research Products) and hybridized with the 5'-end 32 P-labelled 300 bp DNA fragment containing the BS190 RNA coding sequence that was amplified by using the synthetic primers P3 (5'-ATTTGCAGTTCGATTC-3') and P4 (5'-ATAAAATGGCTGATCC-3').

Construction of the deletion mutant. The Δ BS190 RNA mutant strain was constructed as follows. A 378 bp fragment (UP; from +48 to +411, Fig. 1B) of the BS190 RNA locus, including the 3' region of *aspS*, and a 576 bp fragment (DOWN; from +663 to +1218, Fig. 1B) in front of the 5'

Fig. 4. Determination of the 5' ends of *B. subtilis* BS201 RNA and BS190 RNA. Lanes T, G, C and A show DNA sequence ladders primed with the oligonucleotide used for the primer extension of RNA from *B. subtilis*. Total RNA samples were prepared from *B. subtilis* 168 (*trpC2*) that had been cultured for 4 h after inoculation (lane 1). Gel-purified BS190 RNA and BS201 RNA were also analysed (lanes 2 and 3) using the same primer. Primer-extension products for the BS190 RNA and BS201 RNA are indicated by arrows. The positions in the DNA sequence ladder of the non-coding strand corresponding to the 5' terminus of BS201 RNA (**) and BS190 RNA (*) are indicated.

region of *yrvM* were amplified from *B. subtilis* chromosomal DNA using P_{up}-1 (5'-GATCAAGCTTATGACCTCGTCTT-AAAC-3') and P_{up}-2 (5'-GATCGAATTCCTGCAAATTCAGTTCTTAAC-3'), and P_{down}-1 (5'-GATCGAATTCGTGCG-CAGAAAAACGGCTG-3') and P_{down}-2 (5'-GATCGGATCCTATCAGCGATTGGGAAGC-3'), respectively (Fig. 1). After amplification, the UP fragment was digested with *Hind*III and *Eco*RI and the DOWN fragment was digested with *Eco*RI and *Bam*HI – restriction sites for these enzymes were located within each primer used for PCR (shown in bold). The two fragments were ligated and inserted into the *Hind*III and *Bam*HI sites of pBluescript II SK⁺. The resulting recombinant plasmid DNA was digested with *Eco*RI and then ligated with a kanamycin-resistance cassette that was prepared from pDG783 (Guerout-Fleury *et al.*, 1995) by digestion with *Eco*RI. Plasmid DNA from positive clones selected in *E. coli* for kanamycin-resistance were isolated. The DNA fragment containing the BS190 RNA gene locus in which the BS190 RNA gene was replaced with the kanamycin-resistance gene was isolated by PCR using primers P_{up}-1 and P_{down}-2 and used to transform competent *B. subtilis* wild-type cells. Isolates exhibiting kanamycin-resistance resulted from double-cross-over events and were confirmed by Southern hybridization (data not shown). Northern blots demonstrated the absence of BS190 RNA in these isolates (Fig. 2A), which were referred to as the Δ BS190 RNA mutant.

In vitro synthesis of 32 P-labelled BS190 RNA. The 215 bp DNA fragment encoding BS190 RNA was amplified using two oligonucleotides, 5'-GTACAAGCTTTAAAGTCCTGATG-TGTTAGT-3' and 5'-GATCGGATCCAAAGTCCCAATA-

GTGCCGTT-3'. These two oligonucleotides were designed to create *Hind*III and *Bam*HI sites at the 5' and 3' ends of the PCR product (shown in bold). This fragment was digested with *Hind*III and *Bam*HI, and inserted into the *Hind*III-*Bam*HI sites of the pGEM-3Zf(+) vector (Promega). The resulting plasmid was linearized by digestion with *Bam*HI and used as the template for transcription. BS190 RNA was synthesized using 50 U of SP6 RNA polymerase (Takara-Shuzo) and Riboprobe System Buffers (Promega) in the presence of 10 μ Ci (370 kBq) of [α - 32 P]CTP (Amersham Pharmacia Biotech). The product was precipitated with ethanol, dissolved in sterile de-ionized water and renatured by a 10 min incubation at 70 °C followed by slowly cooling to room temperature.

RESULTS AND DISCUSSION

Identifying sRNAs

We attempted to isolate and characterize novel sRNAs with unknown functions in *B. subtilis*. Several distinct bands of <500 nt were detectable in total RNA. We assigned several known species of sRNAs, such as scRNA, M1 RNA (component of RNase P) and tmRNA, to individual bands by Northern blotting (data not shown). Two RNAs that migrated close together were

clearly separated at around the 200 nt mark (Fig. 3, lane 2). Based on the migration of the size markers used, we estimated that the lengths of these two RNAs were 190 and 201 nt. That these molecules were indeed RNAs was confirmed by incubating the separation gel with RNase in the presence of 25 mM EDTA: this caused the disappearance of the BS190 and BS201 RNA bands and of all the other bands (data not shown). The two new RNA species were named BS190 RNA (190 nt) and BS201 RNA (201 nt). To map the gene encoding them on the genome, the two RNAs were isolated separately from denaturing 6% polyacrylamide gels (Fig. 3, lanes 3 and 4). A polyadenylation tail was added to the RNAs using poly(A) polymerase, then two DNA fragments complementary to BS190 RNA and BS201 RNA were constructed, cloned into pUC118 and sequenced. The coding region of BS190 RNA is completely included in that of the BS201 RNA, and it was shown to be located within the *aspS*-*yrvM* intergenic region on the *B. subtilis* genome (Fig. 1A). The 3' ends of the two RNAs were identical, as judged from the cDNA sequence located immediately before the poly(A) tail. Conversely, the 5' end of BS201 RNA was located 11 nt upstream of that of BS190 RNA (Fig. 4). These mapping data confirmed that

Fig. 5. Processing of BS201 RNA to generate BS190 RNA. (A) The potential secondary structure of the predicted sequence of BS201 RNA. G-U base-pairs are indicated by $\circ$. (B) Northern blots showing the steady-state levels of BS201 RNA (precursor) and BS190 RNA (mature) in exponentially growing wild-type *B. subtilis*. Rifampicin (300 μ g ml $^{-1}$) was added to culture medium, then total RNAs were prepared after various time intervals (0, 10, 30 and 60 min). The Northern blots included 32 P-labelled 300 bp DNA fragments that contained the BS190 RNA gene.

Fig. 6. Subcellular localization of BS190 RNA. Cell lysates of *B. subtilis* 168 (*trpC2*) prepared from samples taken in the vegetative phase of growth were applied to a 5–20% sucrose gradient. Samples were collected from the Top of the gradient as 21 fractions of 500 μ l each (lanes 1–21, bottom panel), and the polysomal fraction was recovered from the Bottom of the tube (inset). The BS190 RNA (mature) and BS201 RNA (precursor) in each fraction were detected after electrophoresis through denaturing polyacrylamide gels containing 6 M urea followed by Northern-blotting using 5'-end 32 P-labelled DNA fragments that encoded BS201 RNA. The polysomal fraction was recovered from the Bottom of the tube and RNAs were collected for Northern blotting (lane 2, inset), as described in Methods. BS190 RNA (mature) and its precursor, BS201 RNA, were identified according to the bands seen in the sample of total RNA before sucrose-gradient centrifugation (lane 1, inset) and size markers.

BS190 RNA and BS201 RNA are 190 and 201 nt in length, respectively. The difference in size between the two RNAs is due to the heterogeneity at the 5' end. A promoter structure was identified (Fig. 1B) upstream of the 5' end of the BS201 RNA gene, which showed sequence homology with the consensus for other promoters in *B. subtilis* that are regulated by RNA polymerase containing σ^A . Moreover, Northern blots of total RNA that was separated on denaturing agarose gels and probed with a BS201-RNA-gene-specific DNA fragment did not produce any RNA molecules larger than the two sRNAs described above (data not shown). This finding suggests that BS201 RNA and BS190 RNA are independently expressed rather than them being the stable degradation products of a larger polycistronic RNA encoded by the *aspS* cluster, or processed leaders of downstream genes. Fig. 1 shows that the RNA-encoding region is located in the front-half of the *aspS*–*yrvM* intergenic region. However, when the DNA fragment encompassing nucleotides 720–1020 (Fig. 1B) was used as a probe no band was obvious (data not shown). Therefore, it is unlikely that BS190 RNA arises from a larger RNA, but is instead derived from BS201 RNA. Larger RNAs lack a protein-coding capacity and a Shine–Dalgarno sequence and it seems that they exert their action mainly or exclusively at the RNA level. Here, the gene encoding both BS190 RNA and BS201 RNA is designated as *bsrA* (*B. subtilis* small RNA-A).

Processing of BS201 RNA at its 5' end to form BS190 RNA

Computer-aided analysis predicted that BS190 RNA and BS201 RNA can be folded into the tight double helix structure shown in Fig. 5A. Two stem-loop structures are found in the middle of the hairpin structure. The 5' end of BS201 RNA contains an 11 nt sequence that does

not form base-pairs and which is absent in BS190 RNA. The different lengths of the two RNAs at the 5' end raises the notion that RNA processing from the primary transcript (BS201 RNA) produces BS190 RNA. To monitor the processing of BS201 RNA, exponentially growing *B. subtilis* cells were exposed to rifampicin. Total RNA was then extracted from these cultures and Northern blotted. BS190 RNA and BS201 RNA were actively expressed under exponential growth conditions (Fig. 5B, lane 1), as deduced from the promoter sequence. After the addition of rifampicin, the BS201 RNA band disappeared while the intensity of the BS190 RNA band concomitantly increased. Densitometry demonstrated that 30 min after the addition of rifampicin, 90% of BS201 RNA had been converted into BS190 RNA (Fig. 5B, lane 3). This processing of BS201 RNA was not inhibited in the *B. subtilis* RNaseIII conditional mutant (BG218) in which the RNaseIII gene functions only partially (data not shown). Moreover, the 11 nt at the 5' end of BS201 RNA had no significant homology with the consensus sequence for recognition by the RNaseP enzyme. Therefore, the enzyme participating in BS201 RNA processing remains unknown.

Effects of depletion of BS190 RNA on cell growth

To study the cellular functions of BS190 RNA, we constructed a null-mutant in which the gene encoding BS190 RNA was replaced with the kanamycin-resistance gene, as described in Methods. The colony size of the Δ BS190 RNA mutant on LB agar is smaller than that of the wild-type (data not shown). Neither BS190 RNA nor BS201 RNA was expressed under exponential growth conditions (Fig. 2A, lanes 1 and 2). The absence of intracellular BS190 RNA (BS201 RNA) resulted in a decreased growth rate (Fig. 2B) and a lower maximum cell yield in LB broth (Fig. 2B).

Subcellular localization of BS190 RNA

Among the sRNAs that have been characterized previously, several have been implicated in translation (Altuvia *et al.*, 1998; Majdalani *et al.*, 1998; Muto *et al.*, 1998; Tetart & Bouche, 1992). To examine the possibility that either BS190 RNA or BS201 RNA is associated with ribosomes, cell lysates prepared from exponentially growing cells were separated by sucrose density-gradient centrifugation. RNA was prepared from each fraction and Northern blotted to detect BS190 RNA and BS201 RNA. Quantitative densitometric data indicated that approximately 60% of the total BS190 RNA sedimented with polysomes (Fig. 6B, inset) and about 30% was detected in the 70S monomeric ribosomes (Fig. 6). No band corresponded to BS201 RNA in neither the 70S monosomal (Fig. 6) nor the polysomal fraction (Fig. 6, lane 2, inset). These results suggest that BS190 RNA specifically associates or interacts with ribosomes during translation. Consequently, BS190 RNA, rather than BS201 RNA, is active or functional.

In this study, we have identified and characterized a novel sRNA from *B. subtilis* (BS190 RNA) that is generated by 5'-end processing of a larger precursor (BS201 RNA) that accumulates during vegetative growth. A search of the non-redundant nucleic-acid-sequence database using BLAST revealed that RNAs related to BS201 and BS190 appear to be encoded in the genomes of *Bacillus halodurans* (nucleotide nos 1350677–1350868) and *Listeria monocytogenes/innocua* (nucleotide nos 1550993–1550786/1546540–1546331, respectively). For example, the nucleotide sequence of the corresponding region in *B. halodurans* shows 65% identity to that of BS190 RNA. Moreover, this region is located in front of the BH1255 gene that is a homologue of the *B. subtilis* *yrvM* gene.

To date, at least 10 sRNAs have been identified in *E. coli*. Recently, 24 candidate intergenic regions in *E. coli* have been proposed as possibly encoding novel sRNAs (Wassarman *et al.*, 2001). Of these 24 intergenic regions, Wassarman *et al.* (2001) confirmed that 14 encode sRNAs, by using micro-array technology and Northern hybridization. Based on the DNA sequence data for the whole genome of *B. subtilis*, we selected 123 regions of >500 nt in length as candidates for non-coding RNA-encoding regions in *B. subtilis*. These regions were located between two ORFs, but they lacked a protein-coding capacity and a Shine–Dalgarno sequence. Among these regions, we have found (by using Northern blotting) that the *yocI–yocJ* intergenic region can produce an sRNA of 203 nt (Ando *et al.*, 2002). However, we have not found a sequence corresponding to this RNA adjacent to the *E. coli* homologues of *yocI* and *yocJ*.

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