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Bacillus subtilis RNase III Gene: Cloning, Function of the Gene in *Escherichia coli*, and Construction of *Bacillus subtilis* Strains with Altered *rnc* Loci

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The *rnc* gene of *Bacillus subtilis*, which has 36% amino acid identity with the gene that encodes *Escherichia coli* RNase III endonuclease, was cloned in *E. coli* and shown by functional assays to encode *B. subtilis* RNase III (Bs-RNase III). The cloned *B. subtilis rnc* gene could complement an *E. coli rnc* strain that is deficient in rRNA processing, suggesting that Bs-RNase III is involved in rRNA processing in *B. subtilis*. Attempts to construct a *B. subtilis rnc* null mutant were unsuccessful, but a strain was constructed in which only a carboxy-terminal truncated version of Bs-RNase III was expressed. The truncated Bs-RNase III showed virtually no activity in vitro but was active in vivo. Analysis of expression of a copy of the *rnc* gene integrated at the *amy* locus and transcribed from a p_{spac} promoter suggested that expression of the *B. subtilis rnc* is under regulatory control.

mRNA decay in prokaryotes is thought to initiate with one or more endonucleolytic cleavages. The resulting RNA fragments are degraded by enzymes with 3′-to-5′ exonucleolytic activity. In *Escherichia coli*, two endonucleases are known to cleave mRNAs. One of these is RNase III, an endonuclease that cleaves specific double-stranded RNA structures. In several cases, it has been demonstrated that cleavage by RNase III can have significant positive or negative effects on the stability of a mRNA (3, 21–23). Nevertheless, *E. coli* strains with mutations in the *rnc* gene (encoding RNase III) do not show a general defect in mRNA decay (1). This is unlike the case of strains with a mutation in RNase E, the other *E. coli* endonuclease, which show a significant increase in bulk mRNA half-life (13). These observations suggest that while *E. coli* RNase III is involved in the control of a small number of mRNAs, it is not a major component of the mRNA decay machinery.

RNase III activity is also important for rRNA processing. The 30S rRNA precursor molecule is cleaved by RNase III to give intermediates in the pathway to 23S and 16S rRNA synthesis (4, 8, 17, 25). Nevertheless, *E. coli* strains lacking RNase III activity grow well and contain functional ribosomes, suggesting the existence of an alternative pathway for rRNA processing.

We have been studying the RNase III-like activity of *Bacillus subtilis*, designated Bs-RNase III. This enzyme was first characterized by Panganiban and Whiteley (19, 20), who found that early RNAs encoded by *B. subtilis* phage SP82 were cleaved by Bs-RNase III. We showed that endonucleolytic cleavage of an mRNA by Bs-RNase III, at a site that was encoded by a cloned SP82 early RNA sequence, can result in a decrease in its mRNA half-life (6). Thus, this endonuclease could play a role in mRNA processing in *B. subtilis*, an area about which little is known. Initial studies on the substrate specificity of purified Bs-RNase III showed similarities to the *E. coli* enzyme but also some significant differences (15).

Recently, Oguro et al. (18) discovered a *B. subtilis* gene whose sequence similarity to the *E. coli* RNase III gene suggested that it encoded Bs-RNase III. This gene, designated *rnc*, was reported to be the first gene in an operon that contains three coding sequences (Fig. 1), i.e., the putative Bs-RNase III coding sequence, an SMC protein coding sequence (specifying a protein that is thought to be involved in chromosome compaction), and the Srb coding sequence (specifying a homolog of the mammalian SRP receptor α subunit). With the putative Bs-RNase III gene sequence available, we were able to clone the gene, test it for function, and construct *B. subtilis* strains with different levels of Bs-RNase III activity.

MATERIALS AND METHODS

Bacterial strains and plasmids. A list of strains used in this study is shown in Table 1; a list of plasmids used is shown in Table 2. To clone the *rnc* coding sequence, chromosomal DNA from BG1 was amplified by use of PCR primers that were complementary to nucleotides (nt) 503 to 528 and 788 to 811 of the sequence described by Oguro et al. (18). The amplified fragment included the *rnc* ribosome binding site (RBS) and the complete coding sequence. The primers contained additional nucleotides that encoded *Eco*RI cleavage sites. The amplified fragment was cloned into the *Eco*RI site of pGEM-7Zf(+) (Promega) in two orientations. The plasmid with the *rnc* sequence inserted such that it could be transcribed from the *lacZ* promoter gave Bs-RNase III activity in *E. coli*, and this was designated pBSR2.

To construct the strain that contained the *rnc* gene integrated at the *amyE* locus and under p_{spac} promoter control, plasmid pDR67 was used (11). Plasmid pDR67 contains *amyE* front and back fragments flanking a chloramphenicol resistance (Cm^r) gene as well as the p_{spac} promoter located upstream of a multicloning site. PCR primers were used to amplify the *rnc* RBS and coding sequence, with additional nucleotides containing *Xba*I and *Bgl*II sites. The amplified fragment was cloned into *Xba*I-*Bgl*II-digested pDR67, giving plasmid pBSR12. Plasmid pBSR12 DNA was linearized with *Nru*I and used to transform BG1 to a Cm^r Amy^- phenotype (giving strain BG188).

For cloning of the *rnc* operon under control of the p_{spac} promoter, plasmid pDH88 was used (10). Cloning of a *B. subtilis* chromosomal DNA fragment in this plasmid allows integration by a Campbell-type recombination event and puts the DNA downstream of the site of integration under control of the p_{spac} promoter. First, the full-length *rnc* coding sequence, on the same *Xba*I-*Bgl*II fragment as that described above for pBSR12, was cloned into the multicloning site of pDH88, which is downstream of the p_{spac} promoter. This gave plasmid pBSR17, which was used to transform BG1 to a Cm^r phenotype, giving strain BG217 (see Fig. 6). In BG217, there are two copies of the *rnc* gene, one under control of a native, upstream promoter, and one under control of the p_{spac} promoter. This latter promoter also drives expression of the SMC and *srb* genes. To construct a disruption of the *rnc* gene, a 600-bp *Hin*dIII fragment located within the *rnc* coding sequence was cloned into the *Hin*dIII site of pDH88 such

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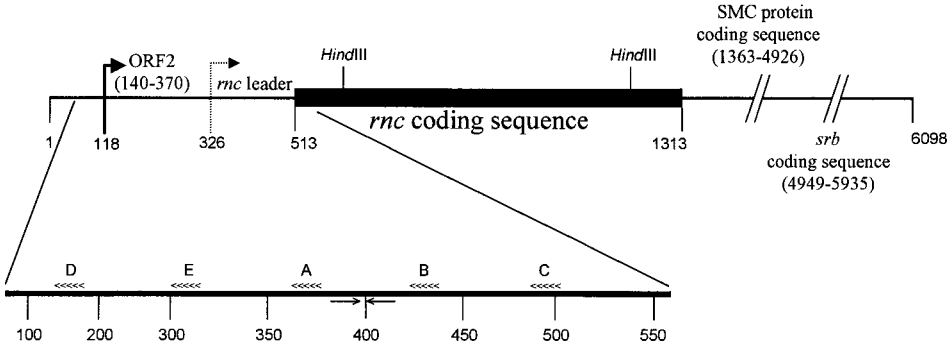

FIG. 1. Schematic diagram of *rnc* region of the *B. subtilis* chromosome. The upper line represents a diagram of the 6.1-kb chromosomal fragment cloned by Oguro et al. (18), which contains the *rnc* coding sequence. The locations of the upstream ORF2 and downstream SMC and *srb* coding sequences are indicated. Numbering is that of Oguro et al. (18). The reported *rnc* transcription start site, as mapped by Oguro et al., is indicated by a dashed arrow at nt 326. The start site mapped in this study is indicated by an arrow at nt 118. In the lower line of the diagram, the leader region is expanded to show the location of oligonucleotide primers (arrowheads A to E) that were used for reverse transcriptase analyses. The location of a predicted stem-loop structure around nt 400 is indicated by facing arrows. The internal *Hin*dIII fragment that was used to construct BG218 is shown.

that the sense strand was transcribed by the p_{spac} promoter (plasmid pBSR18). This was used to transform BG1 to give strain BG218 (see Fig. 6), in which there is no complete copy of the *rnc* gene. Southern blot analysis was used to confirm these constructions (data not shown).

E. coli DH5α (9) was the host for plasmid constructions.

His-tagged Bs-RNase III. The plasmid encoding carboxy-terminal, His-tagged Bs-RNase III (pBSR7) was constructed by insertion of the PCR-amplified *rnc* coding sequence between the *Nco*I and *Bgl*II sites of pQE60 (Qiagen). The strain carrying pBSR7 was induced for 60 min with 2 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and then purified by protocol 5, as detailed in the Qiagen kit. Late-eluting fractions from a 0 to 0.5 M imidazole gradient elution contained large amounts of a 29-kDa protein but also some higher-molecular-weight species. The Bs-RNase III was further purified by DEAE-Sephacel anion-exchange chromatography. Fractions were analyzed on a silver-stained gel, and a fraction in which only the 29-kDa band was detectable was dialyzed against 20 mM Tris (pH 7.0)–10% glycerol and stored in the presence of 200 μg of bovine serum albumin per ml. The Bs-RNase III concentration in this fraction was 3 μg/ml. This fraction was used for subsequent assays of purified Bs-RNase III.

Standard procedures. The preparation of *B. subtilis* growth media and competent *B. subtilis* cultures was as described previously (7). Reverse transcriptase and Northern blot analyses were as described previously (6), except that for the blot shown in Fig. 7, the RNA was separated in a 5% denaturing polyacrylamide gel and electroblotted first at 20 V for 1 h and then at 40 V for 2 h in Tris-acetate-EDTA buffer (pH 7.8).

Test for Bs-RNase III expression in *E. coli*. Strain WJW158 (constructed by H. Wilson in the laboratory of D. L. Court, Laboratory of Chromosome Biology, National Cancer Institute—Frederick Cancer Research and Development Center) contains a chromosomal *N* leader-*lacZ* fusion under control of the λ *c*I857 temperature-sensitive repressor. Expression of the *lacZ* gene is dependent on cleavage of *N* leader RNA by RNase III. Strain WJW158 has a transposon insertion in the endogenous *rnc* gene, so that β-galactosidase activity is dependent on RNase III activity from a plasmid-borne *rnc* gene. Into this strain was introduced plasmid pZH124, which is a spectinomycin-resistant (Sp^r) plasmid that contains a λ *N* gene under control of a *lac* promoter (24), to give strain EG287. The plasmid-encoded λ N protein binds *N* leader region RNA and is required to negatively regulate translation even in the absence of RNase III activity. EG287 was transformed either with pBSR2, the plasmid carrying the *B. subtilis rnc* gene, or pBSR7, the plasmid carrying the His-tagged *rnc* gene. Strains were grown overnight in LB medium with selection for the resident plasmids and then plated on MacConkey indicator agar, again with the appropriate selection. Appearance of red colonies after overnight incubation at 42°C indicated RNase III activity.

RESULTS

Cloning of the Bs-RNase III coding sequence. An 820-bp fragment of the *B. subtilis* chromosome, containing the putative Bs-RNase III coding sequence and its RBS, was amplified by PCR and cloned into the pUC19-derived vector pGEM-7Zf(+) in two orientations. In one of these, designated pBSR2, the direction of the Bs-RNase III coding sequence was such that it could be transcribed from the *lacZ* promoter.

To test whether the cloned *B. subtilis* DNA fragment could encode Bs-RNase III activity, the λ *N* gene system was used. Cleavage of the λ *N* gene leader region by *E. coli* RNase III results in activation of *N* gene expression at the posttranscriptional level (12). We first determined whether Bs-RNase III protein could cleave *N* leader region RNA in vitro. Plasmid pLKG002, which contained the *N* leader region cloned downstream of a T7 RNA polymerase promoter (12), was linearized

TABLE 1. Bacterial strains used in this study

Bacterial strain	Relevant characteristic(s)	Comment (reference)
B. subtilis		
BG1	*thr-5 trpC2*	Wild type
BG188	BG1 *amyE*::p_{spac}-*rnc*	Integration of *rnc* coding sequence under p_{spac} control at *amyE* locus
BG217	BG1 *rnc*::p_{spac}-*rnc* operon	Integration of p_{spac}-*rnc* plasmid at *rnc* locus, with *rnc* operon under p_{spac} control (wild-type *rnc* coding sequence)
BG218	BG1 *rnc*::p_{spac}-*rnc* operon	Integration of p_{spac}-*rnc* plasmid at *rnc* locus, with *rnc* operon under p_{spac} control (truncated *rnc* coding sequence)
BE480	BG217(pYH184)	BG217 strain with *ermC*::SP82 plasmid
BE481	BG218(pYH184)	BG218 strain with *ermC*::SP82 plasmid
E. coli		
EG287	W3110 *lac*Δ*U169* λ[*imm-21* p_L-*N*::*lacZ* *c*I857 Δ(*cro-bio*)] *rnc*::Tn*10* (pZH124)	β-Galactosidase expression from *N*::*lacZ* fusion dependent on plasmid-borne RNase III activity
SK7622	*thyA715* Δ*rnc-38* Km^r	Replacement of *rnc* coding sequence with Km^r gene (2)

TABLE 2. Plasmids used in this study

Plasmid	Description (reference)
pBSR2	*rnc* RBS and coding sequence cloned into pGEM-7Zf(+) vector
pBSR7	*rnc* coding sequence cloned in pQE60 (Qiagen) for expression with carboxyl hexahistidine tag
pBSR12	*rnc* RBS and coding sequence cloned downstream of the p_{spac} promoter in pDR67
pBSR17	*rnc* RBS and coding sequence cloned downstream of the p_{spac} promoter in pDH88
pBSR18	Internal *Hin*dIII fragment of *rnc* coding sequence cloned downstream of the p_{spac} promoter in pDH88
pDH88	Cm^r vector for cloning *B. subtilis* chromosomal DNA and integration into the corresponding locus (10)
pDR67	*amyE* integration vector with Cm^r gene and p_{spac} promoter upstream of multicloning site (11)
pJFD4	SP82 A site for Bs-RNase III cleavage cloned into pGEM-3Zf(+) (15)
pLKG002	Contains λ DNA fragment with *N* leader region cloned downstream of T7 RNA polymerase promoter (12)
pLK120	*E. coli rnc* gene cloned into pACYC184 (12)
pYH184	*B. subtilis-E. coli* shuttle plasmid containing the *ermC* gene with an inserted SP82 sequence encoding a Bs-RNase III cleavage site.

and transcribed to give labeled *N* leader RNA. This RNA was incubated with either purified *E. coli* RNase III (from the laboratory of A. W. Nicholson) or partially purified *B. subtilis* Bs-RNase III enzyme (15). The results (Fig. 2) show that Bs-RNase III cleaved the *N* leader RNA at the same positions as the major cleavage sites for *E. coli* RNase III. (The additional bands observed with *E. coli* RNase III cleavage were also observed previously [12].) Thus, it was predicted that gene expression that was dependent on cleavage of the *N* leader RNA could be used to test for Bs-RNase III activity in vivo.

The test system for in vivo RNase III function is described in Materials and Methods. In this system, expression of β-galactosidase from a chromosomally integrated *N*::*lacZ* fusion gene is dependent on cleavage of the *N* leader region by RNase III. A strain, designated EG287, has an *N*::*lacZ* fusion that is transcribed from the λ p_L promoter under control of the temperature-sensitive *c*I857 repressor. Strain EG287 was transformed with plasmid pBSR2, which contained the cloned putative Bs-RNase III coding sequence. When plated on MacConkey indicator medium, this strain gave yellow colonies when grown at 30°C but red colonies when grown at 42°C. When transformed with the vector DNA (pGEM-7Zf[+]), strain EG287 gave yellow colonies at both 30 and 42°C. Thus, it was demonstrated that the cloned *B. subtilis* chromosomal DNA fragment on plasmid pBSR2 contained the gene encoding Bs-RNase III.

Complementation of rRNA processing in an *E. coli rnc* mutant. The product specified by the cloned *B. subtilis rnc* sequence was tested for its ability to process rRNA. *E. coli* SK7622 contains a disrupted *rnc* gene (2). Unprocessed 30S rRNA, which is normally not observed because it is cleaved rapidly by RNase III, is readily detectable in strain SK7622. This strain was transformed with plasmid pBSR2, which encodes Bs-RNase III activity. As a control, this strain was also transformed with plasmid pLK120, which encodes *E. coli* RNase III activity. The results shown in Fig. 3 clearly demonstrate that the cloned *B. subtilis rnc* gene could complement loss of endogenous RNase III function. 30S rRNA was no longer observed in either the pLK120 or the pBSR2 transformant.

Isolation of His-tagged Bs-RNase III. As described in Materials and Methods, the *rnc* gene was cloned such that it was expressed with a hexahistidine sequence at its carboxyl terminus. The functionality of the Bs-RNase III protein with a histidine tag was tested in vivo by transforming EG287 with the plasmid containing the *rnc*-His tag sequence, as described above for testing of pBSR2. The results showed that the His-tagged Bs-RNase III was functional in vivo. The His-tagged

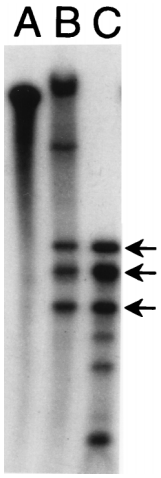

FIG. 2. Cleavage of *N* leader RNA. The transcription product of linearized plasmid pLKG002, encoding the *N* gene leader RNA, is shown (lane A). This RNA was incubated in the presence of a partially purified Bs-RNase III preparation (lane B) or purified *E. coli* RNase III (lane C). Arrows on the right indicate specific RNase III cleavage products (12).

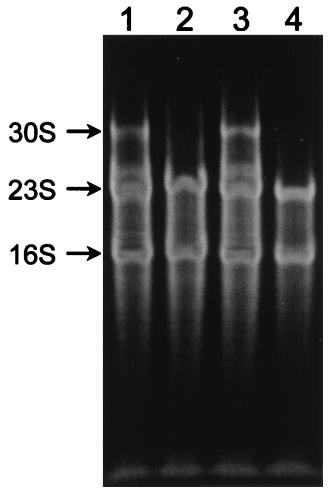

FIG. 3. Analysis of rRNA. rRNA processing in *E. coli* SK7622 (*rnc* deletion mutant) containing the following plasmids: pACYC184 vector (lane 1), pLK120 (*E. coli* rnc^+) (lane 2), pGEM-7Zf(+) vector (lane 3), pBSR2 (*B. subtilis* rnc^+) (lane 4). Migration of rRNA species is indicated on the left.

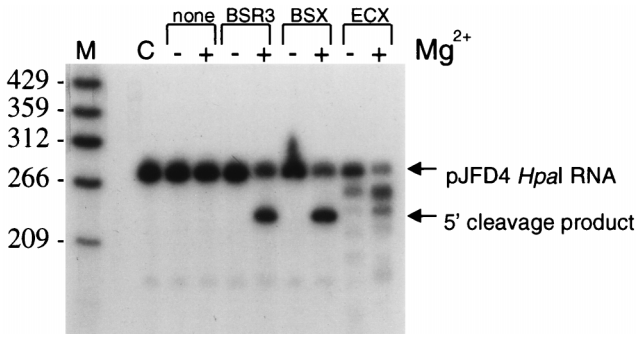

FIG. 4. Activity of His-tagged Bs-RNase III. Labeled pJFD4 *Hpa*I RNA was incubated in the presence or absence of Mg^{2+} with either no added protein (none), 3 ng of His-tagged Bs-RNase III (BSR3), 5 μg of *B. subtilis* protein extract (BSX), or 5 μg of *E. coli* protein extract (ECX). Migration of the full-length substrate and the Bs-RNase III 5′ cleavage product is indicated on the right. The control lane (C) contained the pJFD4 *Hpa*I RNA substrate with no buffer. The marker lane (M) contained 5′-end-labeled DNA fragments of a plasmid pSE420 (5) *Taq*I digestion. Fragment sizes (in nucleotides) are indicated on the left.

Bs-RNase III protein was purified to apparent homogeneity (see Materials and Methods) and used for cleavage of a Bs-RNase III substrate in vitro. Plasmid pJFD4 contains the A cleavage site of SP82 phage early RNA (19). When linearized with *Hpa*I, pJFD4 DNA can be transcribed by T7 RNA polymerase to give a 280-nt Bs-RNase III substrate that is termed pJFD4 *Hpa*I RNA (15). Labeled pJFD4 *Hpa*I RNA was prepared and incubated in the presence or absence of Mg^{2+} with the addition of protein (Fig. 4). The results showed that purified, His-tagged Bs-RNase III cleaved this substrate at the same site where it is cleaved when incubated with a *B. subtilis* extract. Interestingly, incubation with an *E. coli* extract (from a strain that contained a wild-type *rnc* gene) did not result in specific cleavage. We have reported previously that purified *E. coli* RNase III does not cleave the pJFD4 *Hpa*I RNA (15).

Regulation of *rnc* expression in *B. subtilis*. It is known that expression of the *E. coli rnc* gene is autoregulated by RNase III itself (3). Cleavage of *rnc* leader RNA by RNase III appears to result in mRNA instability that lowers the level of *rnc* expression. Cloning of the *B. subtilis rnc* gene provided an opportunity to begin to explore possible regulation of its expression.

The *B. subtilis rnc* coding sequence was placed under control of the IPTG-inducible p_{spac} promoter and integrated into the chromosome at the *amyE* locus to give strain BG188 (see Materials and Methods). Induction of *rnc* transcription in this strain was analyzed by Northern blotting (Fig. 5A). An identical pattern of *rnc*-specific bands was detected in the wild-type strain (BG1) grown with or without IPTG and in BG188 grown without IPTG. The appearance of multiple bands may be due to processing of the *rnc* operon transcript or to the existence of multiple promoters in this operon (18). In the BG188 strain that had been induced with IPTG, a new band which was undetectable in the absence of induction was clearly observed. The level of induction of transcription was relatively high, although it could not be quantitated since this band was not detectable in the absence of induction. The pJFD4 *Hpa*I RNA was used to assay for activity in extracts prepared from BG1 and BG188 (Fig. 5B). Surprisingly, only a 2.5- to 3-fold induction of activity was observed in the extract from BG188 that had been treated with IPTG. These data suggested that the induction of *rnc* transcription did not result in a comparable level of induction of protein synthesis.

We wished to test whether the *rnc* leader region RNA was a

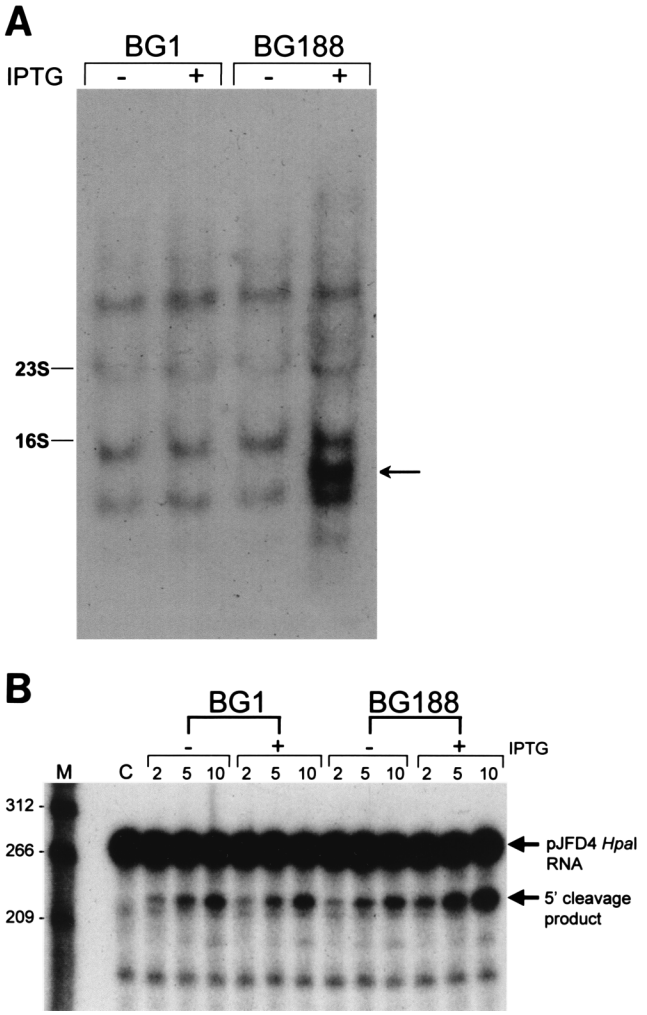

FIG. 5. Induction of *rnc* expression. (A) Northern blot analysis of *rnc* mRNA isolated from BG1 (wild type) and BG188, which contained the *rnc* gene integrated at the *amyE* locus and transcribed from the p_{spac} promoter, grown in the presence (+) or absence (−) of 1 mM IPTG. The probe was a 600-bp *Hin*dIII fragment from the *rnc* coding sequence (Fig. 1). The band representing the induced *rnc* transcript is indicated by an arrow on the right. Positions of migration of rRNAs are indicated on the left. (B) Induction of Bs-RNase III activity in BG188 by IPTG. Protein extracts were prepared from BG1 and BG188 grown in the presence (+) or absence (−) of 1 mM IPTG. Uniformly labeled pJFD4 *Hpa*I RNA was incubated at 37°C in the presence of 2.5 ng of extract for 2, 5, and 10 min, and the appearance of a 5′ cleavage product was analyzed by denaturing polyacrylamide gel electrophoresis. Radioactivity in the bands was quantified with a PhorphorImager (Molecular Dynamics). The control lane (C) had no extract added. See legend to Fig. 4 for marker lane (M).

substrate for Bs-RNase III cleavage in vitro, since the *E. coli rnc* leader is a substrate for *E. coli* RNase III (3). A predicted strong stem-loop structure is located in the *B. subtilis rnc* leader region, about 100 nt upstream of the translational initiation codon (18) (Fig. 1). First, reverse transcriptase analysis was performed on in vivo-isolated total RNA to map the transcriptional start site of the *rnc* operon. Three oligonucleotide primers were used, as shown in Fig. 1 (primers A to C). We found that primer A gave a reverse transcription product whose 5′ end mapped to nt 338 (near the start site proposed by Oguro et al. [18], which was at nt 326.) However, reverse transcriptase analysis with primers B and C gave no detectable product. Oguro et al. reported similar findings. To test for nonspecific reverse transcription products, reverse transcrip-

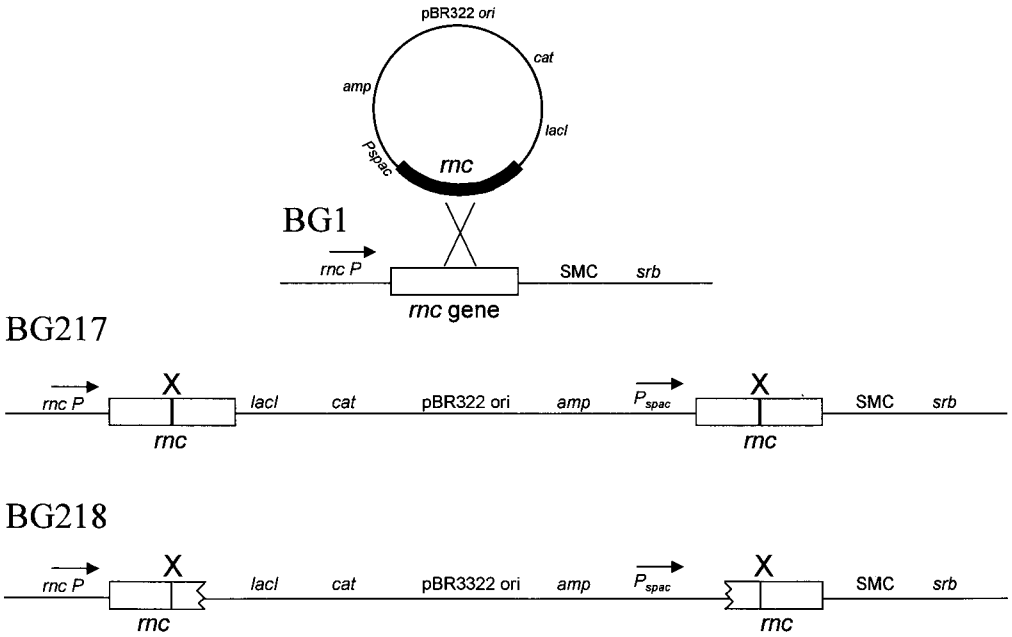

FIG. 6. Disruption of the *rnc* operon. A schematic diagram of the *rnc* operon in BG1 is shown. The integrative plasmid carrying all or part of the *rnc* gene is depicted at the top of the figure. This pBR322-based plasmid contains the p_{spac} promoter for transcription of the inserted sequence, the gram-positive *cat* gene for selection of Cm^r in *B. subtilis*, the ampicillin resistance gene for selection in *E. coli*, and the *lacI* gene for Lac repressor synthesis. The crossover point for Campbell-type integration is marked with an X. Below the integrative plasmid diagram are the results of integration into the *rnc* locus of either a plasmid containing the complete *rnc* coding sequence (BG217) or a plasmid containing an internal *Hin*dIII fragment of the *rnc* coding sequence (BG218). X's mark hypothetical crossover points between the plasmid and the chromosome. The jagged line in BG218 indicates partial *rnc* coding sequence. In BG217 and BG218, transcription of the *rnc* operon is under p_{spac} control.

tase analyses were performed on RNA that had been transcribed in vitro from a DNA template that encoded the first 800 nt of the cloned *rnc* operon fragment. The same results were obtained with the in vitro-transcribed RNA as with the in vivo-isolated RNA, i.e., a product at nt 338 when primer A was used but no products (except for full-length RNA) when primers B and C were used. We believe, therefore, that the previously mapped start site is actually a pause site for reverse transcriptase and that the true 5′ start site lies well upstream of the *rnc* gene.

Additional primers (primers D and E in Fig. 1), complementary to sequences upstream of nt 338, were used in reverse transcriptase analyses. Using these primers, we mapped an in vivo 5′ end to nt 118 (data not shown). This product was not observed when in vitro-transcribed RNA was used as the template for reverse transcriptase, so it is not a reverse transcriptase pause site. This start site is 7 to 8 nt upstream of the RBS sequence that precedes open reading frame 2 (ORF2). The predicted −35 and −10 promoter sequences (TTTCTC and ATCCAC, respectively) would give a poor match with consensus promoter sequences, and it is possible that this mapped 5′ end is a result of processing rather than transcription initiation.

To test for processing of *rnc* RNA by Bs-RNase III, the RNA molecule representing the first 800 nt of the cloned *rnc* fragment was transcribed in the presence of labeled UTP and tested for Bs-RNase III cleavage. Both the purified, His-tagged Bs-RNase III and extracts from wild-type *B. subtilis* strains were used. No cleavage of this RNA was observed (data not shown). We conclude that, at least under our in vitro conditions, Bs-RNase III is not capable of cleaving RNA located within 500 nt upstream of the *rnc* coding sequence.

Attempted disruption of the *B. subtilis rnc* gene. We wished to construct an *rnc* null strain to test the effect on growth and rRNA processing. We constructed plasmids that contained a replacement of part of the *rnc* coding region with an antibiotic resistance gene, maintaining flanking *rnc* sequences that would be targets for integration by double crossover. Several attempts to disrupt the chromosomal *rnc* gene by recombining the plasmid sequence into the chromosome were unsuccessful. We could demonstrate, however, that these plasmids were capable of directing integration of the resistance marker into an *rnc* gene. The BG188 strain, which contains an additional copy of the *rnc* gene at the *amy* locus (see above), was used as the recipient for a plasmid that, by recombination, would replace most of the *rnc* coding region with a kanamycin resistance (Km^r) gene. Many Km^r recombinants were isolated, and in all cases the insertion was in the *rnc* gene at the *amy* locus. Thus, this plasmid was capable of generating an *rnc* disruption but could not do so at the native locus. We hypothesized that our failure to obtain an *rnc* disruptant was due to effects on the expression of the downstream genes in the *rnc* operon. Therefore, we attempted to disrupt the *rnc* gene by Campbell integration of a plasmid carrying a p_{spac} promoter that could drive expression of the downstream genes (see Materials and Methods) (Fig. 6). First, we demonstrated that a strain in which expression of the *rnc* operon was under control of the p_{spac} promoter was viable. A strain, designated BG217, was constructed by integration of a plasmid containing the *rnc* RBS and complete coding sequence. BG217 contains an intact *rnc* gene with its native promoter, located upstream of the selected marker (Cm^r), and a complete *rnc* operon transcribed from the p_{spac} promoter, located downstream of the selected marker (Fig. 6). We then attempted to construct a similar strain with a plasmid that contained only an internal, 5′-proximal fragment of the *rnc* gene. Integration of such a plasmid at the *rnc* locus

would result in a strain with no intact *rnc* coding sequence. These attempts were unsuccessful.

Construction of a Bs-RNase III-deficient strain. One of the plasmids that was designed to yield, upon integration, an *rnc* null mutant did give Cm^r transformants that showed the expected pattern upon analysis by Southern blotting. The plasmid in this case contained an internal *Hin*dIII fragment of the *rnc* gene (Fig. 1), and the resulting transformant was designated BG218 (Fig. 6). In this case, the downstream *rnc* operon was expressed from the p_{spac} promoter but contained a nonfunctional *rnc* coding sequence that lacked an RBS and a translational initiation codon. Upstream of the integrated plasmid DNA was a copy of the *rnc* gene that was wild type until the distal *Hin*dIII site, which is located 100 bp 5′ from the end of the *rnc* coding sequence. Based on the published *rnc* sequence, expression of this truncated *rnc* gene would be expected to give a protein that was missing the C-terminal 28 amino acids. In BG218, these final 28 codons are replaced by 9 codons derived from the vector sequence.

Extracts prepared from strains BG1 (wild type), BG217, and BG218 were tested for Bs-RNase III activity in vitro on the SP82 RNA substrate (Fig. 7A). The results showed that the cleavage activity present in BG1 and BG217 appeared to be completely absent in BG218. To test for in vivo Bs-RNase III function, BG217 and BG218 were transformed with a plasmid (pYH184) that specifies an *ermC* RNA with an internal SP82 Bs-RNase III cleavage site (Fig. 7B). Specific cleavage in vivo by Bs-RNase III at this site has been demonstrated previously (6). The BG217 and BG218 strains with the plasmid encoding the *ermC*::SP82 RNA were designated BE480 and BE481, respectively. The data in Fig. 7B show that the *ermC*::SP82 RNA was cleaved in both BG217 and BG218 backgrounds. Reverse transcriptase mapping confirmed that the sites of cleavage were identical in both strains. Thus, despite the apparent absence of Bs-RNase III activity in a BG218 extract, the activity was clearly present in vivo. In fact, when autoradiograms of the type shown in Fig. 7A are greatly overexposed, a faint band representing the Bs-RNase III cleavage product can be observed in the BG218 lane.

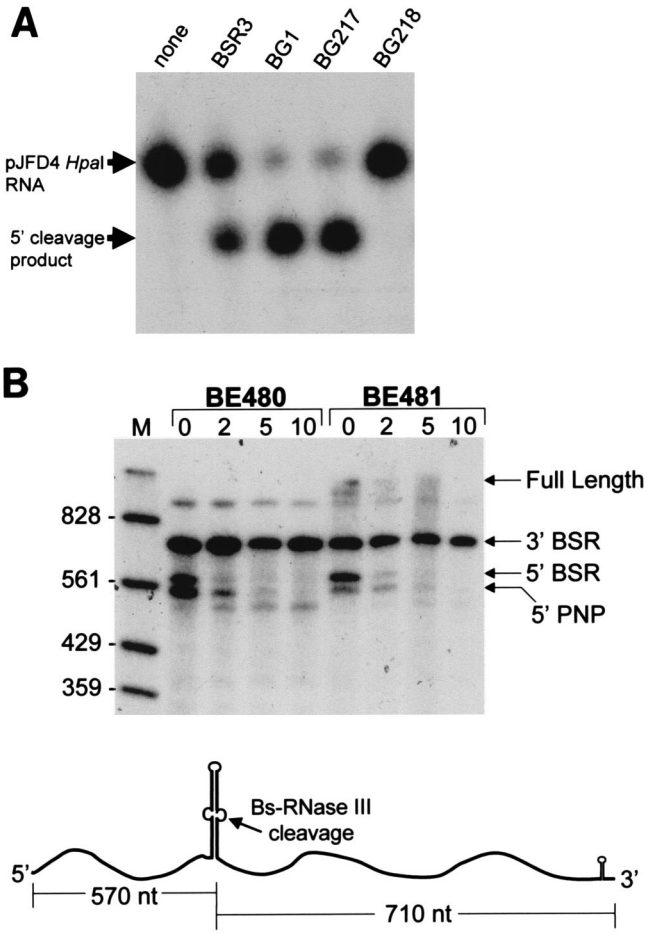

FIG. 7. (A) Bs-RNase III activity from *B. subtilis* strains. Labeled pJFD4 *Hpa*I RNA was incubated at 37°C for 20 min in the presence of 3 mM Mg^{2+} and either no protein (none), 3 ng of purified Bs-RNase III (BSR3), or 5 μg of protein extract from the indicated strains (BG1, BG217, and BG218). (B) Northern blot analysis to detect in vivo Bs-RNase III activity. The probe was labeled SP82 antisense RNA, transcribed from linearized pJFD4 with SP6 RNA polymerase. The time (in minutes) after rifampin addition is indicated above each lane. Migration of full-length *ermC*::SP82 RNA, 5′ and 3′ Bs-RNase III (5′ and 3′ BSR) cleavage products, and a 5′ Bs-RNase III fragment after polynucleotide phosphorylase processing (5′ PNP) (16) are indicated on the right. It has been shown previously that the 3′ Bs-RNase III cleavage product is stable (6). See legend to Fig. 4 for marker lane (M). A schematic diagram of the *ermC*::SP82 RNA and the predicted sizes of Bs-RNase III cleavage products are indicated at the bottom of the figure.

DISCUSSION

We cloned the putative *rnc* coding sequence, which was recently identified by Oguro et al. (18). Functional analysis of the cloned sequence in *E. coli*, both in vivo and in extracts, indicated that it encoded Bs-RNase III activity. Furthermore, the purified, His-tagged protein product of the cloned *rnc* sequence gave Bs-RNase III cleavage activity that was identical to the partially purified activity from *B. subtilis*. Most interestingly, the cloned *B. subtilis rnc* gene could complement the rRNA processing defect in an *E. coli rnc* mutant. In the course of characterizing Bs-RNase III activity, Panganiban and Whiteley (20) tested for cleavage activity on in vitro-transcribed rRNA from cloned rRNA operons of both *B. subtilis* and *E. coli*. They found that the purified Bs-RNase III could cleave *B. subtilis* rRNA but not *E. coli* rRNA. We show here that Bs-RNase III is capable of processing *E. coli* rRNA (Fig. 3). It is likely that certain in vivo conditions are required to enable cleavage of rRNA by Bs-RNase III, and these are not reproduced in vitro. The fact that Bs-RNase III can cleave *E. coli* rRNA and the homology of predicted *B. subtilis* rRNA precursor to that of *E. coli* (14) together suggest that Bs-RNase III is involved in rRNA processing in *B. subtilis.*

The cloned Bs-RNase III coding sequence was used in attempts to disrupt the *B. subtilis rnc* gene. The only transformant that could be isolated (BG218) contained a truncated *rnc* gene that specified Bs-RNase III activity in vivo but not in vitro. It appears that our in vitro assay conditions are missing an element(s) that supports efficient cleavage by the truncated Bs-RNase III. In any event, the extremely low level of Bs-RNase III activity in the BG218 extract suggests that the cloned *rnc* sequence is the only gene encoding this activity.

We assume that the low level of Bs-RNase III activity in BG218 is due to the truncation of the *rnc* gene at the 3′ end in this construct. The same procedures that were used to construct BG218 have been used with a smaller internal fragment of the *rnc* gene that ends about half way through the coding sequence (data not shown). No transformants could be recovered. This indicates that it is the presence of an almost full-length *rnc* coding sequence in BG218 that allowed its recovery.

While the inability to obtain an *rnc* null mutant constitutes negative evidence, the results suggest that Bs-RNase III activity is required for viability. If Bs-RNase III is involved in rRNA

processing, as our complementation of the *E. coli rnc* mutant suggests, then it could be this activity that makes the *rnc* gene essential. The alternative 30S rRNA processing pathway that allows *E. coli rnc* mutants to produce functional rRNA may not exist in *B. subtilis*. We are currently attempting to construct a *B. subtilis* strain that expresses *rnc* conditionally.

In growth experiments (data not shown), we found that strains BG217 (wild-type *rnc*) and BG218 (truncated *rnc*) grew as well as BG1 (wild type) did, whether in the presence or absence of IPTG. This was unexpected, since the *rnc* operon is under control of the p_{spac} promoter in both BG217 and BG218 and since, as shown by Oguro et al. (18), disruption of the *srb* gene results in a growth defect. We expected that these strains would grow poorly unless p_{spac}-driven transcription of the *rnc* operon was induced. Perhaps a low level of uninduced transcription of the *srb* gene is enough to allow normal growth. Alternatively, other promoters may exist for transcription of the downstream SMC protein and *srb* genes.

Analysis of expression of an *rnc* coding sequence integrated at the *amyE* locus and under p_{spac} control (in strain BG188) suggested that expression of Bs-RNase III is regulated (Fig. 5). We could not detect cleavage of the putative *rnc* leader RNA with purified Bs-RNase III. This could be due either to in vitro conditions that do not support cleavage or to an absence of the real target in our experiments. Our results with reverse transcriptase analysis of *rnc* operon transcription suggest that the true promoter region for *rnc* transcription is upstream of ORF2. Further work on the signals required for *rnc* gene expression could lead to the elucidation of a regulatory mechanism that the current work suggests may exist. If Bs-RNase III is required for rRNA processing, one might predict that regulation of *rnc* gene expression is coordinated with ribosome synthesis.

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