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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 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' exonuclease 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 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 EcoRI cleavage sites. The amplified fragment was cloned into the EcoRI 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 a Spac promoter control, plasmid pDR67 was used (11). Plasmid pDR67 contains amyE front and back fragments flanking a chloramphenicol resistance (Cm') gene as well as the Spac promoter located upstream of a multidonating site. PCR primers were used to amplify the rnc RBS and coding sequence, with additional nucleotides containing XbaI and BglII sites. The amplified fragment was cloned into XbaI-BglII-digested pDR67, giving plasmid pBSR12. Plasmid pBSR12 DNA was linearized with HinfI and used to transform BGI to a Cm’ Amy’ phenotype (giving strain BG188).

For cloning of the rnc operon under control of the 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 Spac promoter. First, the full-length rnc coding sequence, on the same XbaI-BglII fragment as that described above for pBSR12, was cloned into the multidonating site of pDH88, which is downstream of the Spac promoter. This gave plasmid pBSR17, which was used to transform BGI to a Cm’ 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 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 HindIII fragment located within the rnc coding sequence was cloned into the HindIII site of pDH88 such
that the sense strand was transcribed by the pBSR7 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 mc 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 (pBSR7) was constructed by insertion of the PCR-amplified rnc7380 fragment that was used to construct BG218 is shown.

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 N leader RNA by RNA III. Strain WW158 has a transposon insertion in the endogenous N leader RNA in vitro. Plasmid pLKG002, which contained the N leader region cloned downstream of a T7 RNA polymerase promoter (12), was linearized into 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 Bs subtilis rnc gene, or pBSR7, the plasmid carrying the His-tagged mc 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.

TABLE 1. Bacterial strains used in this study

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Relevant characteristic(s)</th>
<th>Comment (reference)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. subtilis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BG1</td>
<td>thr-5 trpC2</td>
<td>Wild type</td>
</tr>
<tr>
<td>BG188</td>
<td>BG1 amyE::pBSR7-rnc</td>
<td>Integration of rnc coding sequence under pBSR7 control at amyE locus</td>
</tr>
<tr>
<td>BG217</td>
<td>BG1 mc::pBSR7-rnc operon</td>
<td>Integration of pBSR7-rnc plasmid at mc locus, with mc operon under pBSR7 control (wild-type rnc coding sequence)</td>
</tr>
<tr>
<td>BG218</td>
<td>BG1 mc::pBSR7-rnc operon</td>
<td>Integration of pBSR7-rnc plasmid at mc locus, with mc operon under pBSR7 control (truncated mc coding sequence)</td>
</tr>
<tr>
<td>BE480</td>
<td>BG217(pYHI84)</td>
<td>BG217 strain with emc::SP82 plasmid</td>
</tr>
<tr>
<td>BE481</td>
<td>BG218(pYHI84)</td>
<td>BG218 strain with emc::SP82 plasmid</td>
</tr>
<tr>
<td>E. coli</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EG287</td>
<td>W3110 lacΔΔU169 λimm-21 p3-N::lacZ c1857 Δ(cro-bio)</td>
<td>β-Galactosidase expression from N::lacZ fusion dependent on plasmid-borne RNase III activity</td>
</tr>
<tr>
<td>SK7622</td>
<td>thyA713 Δmc-38 Km'</td>
<td>Replacement of mc coding sequence with Km' gene (2)</td>
</tr>
</tbody>
</table>
A strain, designated EG287, has an N transcribed from the lNtosidase from a chromosomally integrated bMaterials and Methods. In this system, expression of RNA could be used to test for Bs-RNase III activity in vivo.

is dependent on cleavage of the E. coliational bands observed with Bs-RNase III cleaved the E. coliRNase III enzyme (15). The results (Fig. 2) show that B. subtilisshuttle plasmid containing the B. subtilis-E. coli rnc

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)
pHY184 ................................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:λacZ fusion gene is dependent on cleavage of the N leader region by RNase III. A strain, designated EG287, has an N:λacZ fusion that is transcribed from the λ p1 promoter under control of the temperature-sensitive cI857 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...
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 HpaI, pJFD4 DNA can be transcribed by T7 RNA polymerase to give a 280-nt Bs-RNase III substrate that is termed pJFD4 HpaI RNA (15). Labeled pJFD4 HpaI RNA was prepared and incubated in the presence or absence of Mg²⁺ with either no added protein (none), 3 ng of His-tagged Bs-RNase III 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 HpaI RNA substrate with no buffer. The marker lane (M) contained 5'-end-labeled DNA fragments of a plasmid pSE420 (5) TaqI digestion. Fragment sizes (in nucleotides) are indicated on the left.

**FIG. 4.** Activity of His-tagged Bs-RNase III. Labeled pJFD4 HpaI RNA was incubated in the presence or absence of Mg²⁺ 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 HpaI RNA substrate with no buffer. The marker lane (M) contained 5'-end-labeled DNA fragments of a plasmid pSE420 (5) TaqI digestion. Fragment sizes (in nucleotides) are indicated on the left.

**FIG. 5.** Induction of mrc expression. (A) Northern blot analysis of mrc mRNA isolated from BG1 (wild type) and BG188, which contained the mrc gene integrated at the amyE locus and transcribed from the pgo f promoter, grown in the presence (+) or absence (−) of 1 mM IPTG. The probe was a 600-bp HindIII fragment from the mrc coding sequence (Fig. 1). The band representing the induced mrc 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 HpaI 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 PhosphorImager (Molecular Dynamics). The control lane (C) had no extract added. See legend to Fig. 4 for marker lane (M).
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′) gene. Many Km′ 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 Pspac 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 Pspac 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′), and a complete rnc operon transcribed from the Pspac 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<sup>t</sup> transformants that showed the expected pattern upon analysis by Southern blotting. The plasmid in this case contained an internal HindIII 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<sub>spac</sub> promoter but contained a non-functional 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 HindIII 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.

**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 activity in vitro 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 3OS 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 BGI (wild type) did, whether in the presence or absence of IPTG. This was unexpected, since the rnc operon is under control of the p\textsubscript{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\textsubscript{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\textsubscript{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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**REFERENCES**


