

The *yvaJ* Gene of *Bacillus subtilis* Encodes a 3'-to-5' Exoribonuclease and Is Not Essential in a Strain Lacking Polynucleotide Phosphorylase

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Studies of *Bacillus subtilis* RNases that are involved in mRNA degradation reveal a different pattern from that of *Escherichia coli*. A strain lacking polynucleotide phosphorylase, the major 3'-to-5' exoribonuclease activity in cell extracts, is viable. Here, we show that the *B. subtilis yvaJ* gene encodes a second 3'-to-5' exoribonuclease. A strain lacking both of these RNases grows slowly but is viable. The existence of another, as yet unknown, 3'-to-5' exoribonuclease in *B. subtilis* is suggested.

The short half-life of bacterial mRNAs is thought to be required for rapid changes in gene expression programs. In *Escherichia coli*, mRNA exonucleolytic decay is accomplished by two 3'-to-5' exoribonucleases, RNase II and polynucleotide phosphorylase (PNPase). The essential nature of mRNA turnover is inferred from the inviability of an RNase II^{ts} PNPase⁻ double mutant at the nonpermissive temperature (5).

While hydrolytic decay by RNase II constitutes the major 3'-to-5' exonuclease activity in *E. coli*, Deutscher and Reuven (4) reported that there was no RNase II-like activity in *Bacillus subtilis* extracts. Instead, phosphorolytic degradation catalyzed by a putative PNPase of *B. subtilis* was primarily responsible for poly(A) decay. A minor, Mn²⁺-stimulated hydrolytic activity was also detectable in the *B. subtilis* extract.

We cloned the *B. subtilis* PNPase gene (*pnpA*) and constructed a *pnpA* deletion strain (10). This strain displayed several phenotypes: slightly slower growth at 37°C, cold sensitivity, competence deficiency, tetracycline sensitivity, and altered mRNA processing (1, 8, 10). If 3'-to-5' exonucleolytic decay of mRNA is essential for *B. subtilis*, the isolation of a PNPase⁻ *B. subtilis* strain suggested that another 3'-to-5' exonuclease must fulfill this function. Analysis of decay of labeled substrates in the *pnpA* deletion strain extract indicated an Mn²⁺-stimulated hydrolytic activity (10).

We initiated a biochemical study to purify the “other” 3'-to-5' exoribonuclease from *B. subtilis*, using decay of ³H-labeled total RNA as the assay (10). The *pnpA* deletion strain,

BG119, was used to prepare extracts for purification of the Mn²⁺-stimulated RNase activity. After growth in YT medium (0.8% yeast extract, 0.8% tryptone, 0.25% NaCl) to 250 to 300 Klett units, cells were collected, washed with buffer A (20 mM Tris-HCl [pH 7.75], 100 mM KCl, 10% glycerol, 0.2 mM EDTA), and frozen at -80°C. Cell pellets were washed in 200 ml of buffer A (without glycerol) containing 1 mM phenylmethylsulfonyl fluoride and 1 mM dithiothreitol. Lysozyme was added to 0.2 mg/ml. After incubation at 37°C for 20 min, cells were passed through a French press and cell debris was pelleted at 12,000 × g. The supernatant from this low-speed spin was used as the “extract.”

For further purification, the low-speed-spin supernatant was pelleted at 200,000 × g, and nucleic acid-binding proteins were precipitated by addition of streptomycin sulfate (130 mg/ml), followed by centrifugation at 27,000 × g. More than 90% of the Mn²⁺-stimulated activity coprecipitated with the bulk nucleic acid. The pellet was dissolved in buffer A, dialyzed overnight against the same buffer, and diluted to a concentration of 0.2 mg of nucleic acid/ml. Nucleic acids were digested by addition of micrococcal nuclease (Worthington Biochemical) to 1.5 U per μg of nucleic acid and incubation for 20 min at 30°C in the presence of 1 mM CaCl₂. After dialysis in a 20-fold volume of buffer A containing 1 mM CaCl₂, protein was loaded onto a DEAE-Sepharose CL-6B (Sigma) column, which was equilibrated with buffer A. The column was washed with 10 bed volumes of buffer A, and protein was eluted with a linear

TABLE 1. Growth rate of RNase mutant strains

Strain	Genotype	Doubling time (min) ^a
BG1	Wild type	19.5 ± 1.0
BG119	<i>pnpA</i>	27.0 ± 0.0
BG295	<i>yvaJ</i>	20.0 ± 0.7
BG296	<i>pnpA yvaJ</i>	34.0 ± 1.7

^a Data are averages of measurements from at least four independent growth experiments.

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TABLE 2. RNA degradative activity in protein extracts^a

Divalent cation	Activity for strain:		
	BG1 (wild type)	BG119 (<i>pnpA</i>)	BG295 (<i>yvaJ</i>)
None	0.4	0.6	0.9
Mg ²⁺	14.8	18.0	1.7
Mn ²⁺	25.6	35.5	15.7

^a Decay of ³H-labeled total RNA was measured in the presence of 1 mM MgCl₂ or 1 mM MnCl₂. Values are nt released in nanomoles per minute per milligram of protein. Values are from a single experiment (average of duplicate samples), using 22 nmol of labeled RNA per assay, but similar results were obtained from numerous experiments using different concentrations of labeled RNA.

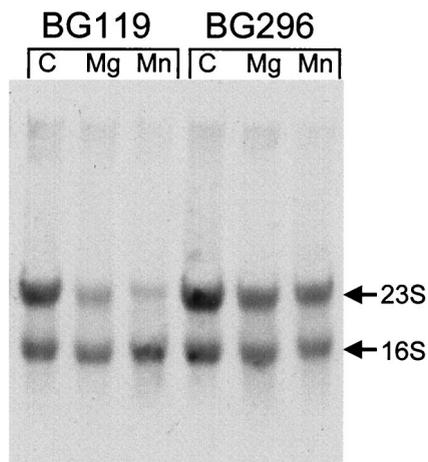


FIG. 1. Decay of ^3H -labeled total RNA. Migration of 23S and 16S rRNAs is indicated. Substrate RNA was incubated in the presence of the most active BG119 fraction from the DEAE-Sepharose column and the corresponding BG296 fraction. Incubation at 37°C for 80 min was in the absence of divalent cation (C) and in the presence of 1 mM Mg^{2+} or 1 mM Mn^{2+} .

gradient of KCl (100 to 500 mM). This protocol yielded a >50 -fold purification of the activity, assayed using ^3H -labeled total RNA.

In the course of these studies, Cheng et al. reported that the *vacB* gene of *E. coli* encodes the 3'-to-5' exonuclease RNase R (2) and proposed that the *vacB* gene be named *mr*. Since the *yvaJ* gene of *B. subtilis* showed significant homology (37% identity) with the *E. coli mr* gene, we investigated possible RNase activity encoded by *yvaJ*. A strain was constructed (BG295) in which about half of the *yvaJ* coding sequence was replaced with a spectinomycin resistance gene. In addition, a

pnpA yvaJ double mutant strain (BG296) was constructed. The growth rates of these strains in Luria-Bertani medium are shown in Table 1. Disruption of the *yvaJ* gene had no effect on the growth rate. However, the *pnpA yvaJ* strain grew even slower than the *pnpA* strain. Thus, the effect on growth rate conferred by the absence of PNPase was exacerbated by the absence of the *yvaJ* gene product. Northern blot analysis of mRNA encoded by the erythromycin resistance gene *ermC* showed no difference in the decay pattern between the *pnpA* and *pnpA yvaJ* strains (data not shown). The rate of bulk mRNA decay may be affected in the *pnpA yvaJ* strain, but this has not been measured.

Extracts of BG1 (wild type), BG119, and BG295 strains were prepared to assess RNase activity. For the RNA decay assay, 100- μl reaction mixtures contained 0.2 to 12 μg of protein, 10 to 40 nmol of RNA (1,000 to 4,000 $\mu\text{Ci}/\text{mmol}$), 50 mM Na-Tricine (pH 8.0), and 100 mM KCl, with or without addition of 1 mM MgCl_2 or MnCl_2 . After 20 min at 37°C , 300 μl of 0.5-mg/ml *E. coli* tRNA and 400 μl of 20% trichloroacetic acid were added. Samples were incubated on ice for 10 min and then centrifuged in the cold for 10 min. Four hundred microliters of the supernatant was removed and counted in 5 ml of Ecoscint A (National Diagnostics).

Addition of Mn^{2+} to BG1 or BG119 extracts resulted in a twofold increase in activity, relative to the activity in the presence of Mg^{2+} (Table 2). Activity in the BG295 extract in the presence of Mg^{2+} was only 11% of the wild type (Table 2). Thus, disruption of the *yvaJ* gene caused a substantial loss of RNase activity. Interestingly, activity in the BG295 extract in the presence of Mn^{2+} was 60% that of the wild type. This suggested that an Mn^{2+} -stimulated RNase activity was still present in the *yvaJ* mutant strain.

Earlier (10), we had assumed that the bulk of the ^3H -labeled RNA used to assay RNase activity was mRNA, since the RNA was isolated from a late-logarithmic-phase culture following a 10-min pulse of [^3H]uridine. However, when the labeled RNA

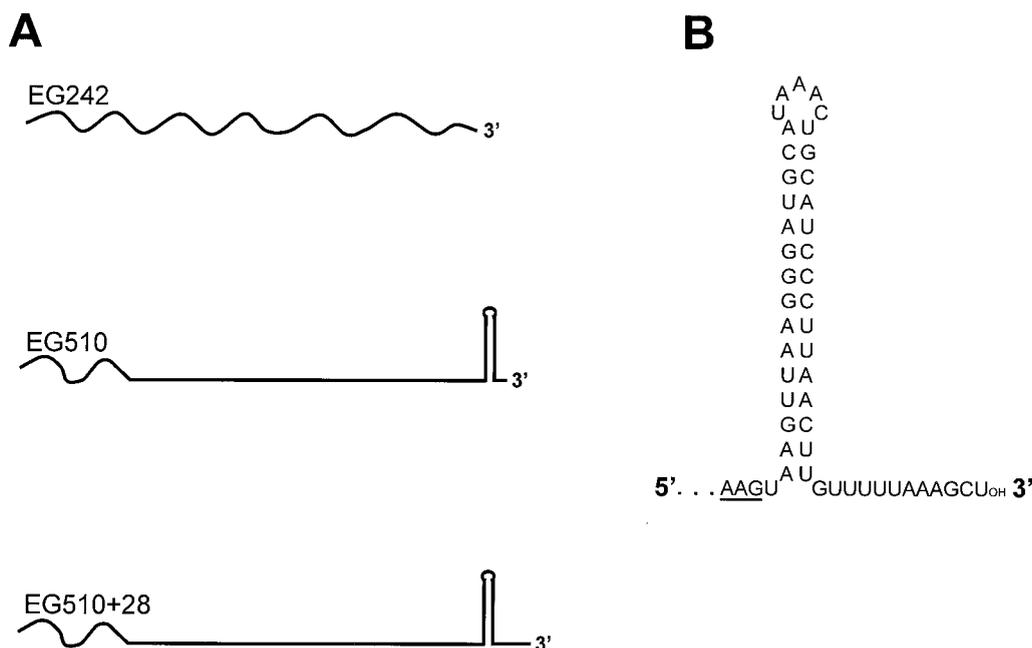


FIG. 2. (A) Schematic diagram of ^{32}P -labeled RNAs used to test for 3'-sensitive exoribonucleolytic decay. Wavy lines are SP82 sequences; straight lines are *ermC* sequences. (B) Predicted secondary structure of the *ermC* terminator sequence, which is present at the end of EG510 RNA. The *ermC* coding sequence ends with the AAG codon (underlined), followed by a UAA stop codon.

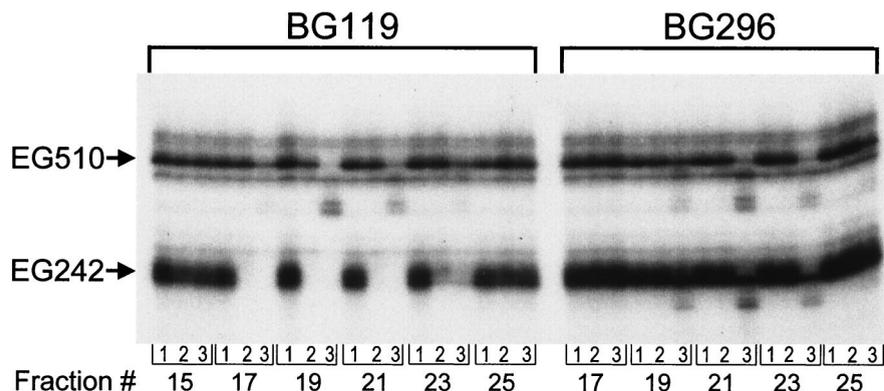


FIG. 3. Decay of ^{32}P -labeled RNAs. EG242 RNA and EG510 RNA were incubated with DEAE-Sepharose fractions from BG119 (*pnpA*) and BG296 (*pnpA yvaJ*). The samples contained either no divalent cation (lane 1), 1 mM Mg^{2+} (lane 2), or 1 mM Mn^{2+} (lane 3). The products were electrophoresed on a high-resolution 6% denaturing polyacrylamide gel. Bands other than the major ones indicated by "EG242" and "EG510" are minor transcription products.

was run on a morpholinopropanesulfonic acid-formaldehyde gel to directly observe RNase activity, fluorography (Autofluor; National Diagnostics) revealed that the bulk of this labeled RNA was rRNA (Fig. 1). 23S rRNA was degraded upon incubation with the most active DEAE fraction from BG119, but to a much lesser degree with the corresponding fraction extract from BG296 (*pnpA yvaJ*) (Fig. 1). This suggested that the major activity detected in our assays was an RNase that could degrade rRNA. Such an activity was reminiscent of *E. coli* RNase R, so named due to its nonspecific degradation of rRNA (3, 7).

Three ^{32}P -labeled RNA substrates (shown schematically in Fig. 2A) were prepared, as described previously (6), to test for 3'-to-5' exoribonuclease activity. The 187-nucleotide (nt) EG242 RNA contained sequences of SP82 bacteriophage, as described previously (9). In EG510 RNA (222 nt), the 5' terminal 42 nt were the same as in EG242 RNA, followed by the sequence of the 3' end of *ermC* mRNA, which contains a strong terminator structure (predicted free energy of -20.9 kcal/mol) located 12 nt from the 3' end (Fig. 2B). EG510+28 RNA was prepared from the same template as EG510 RNA, but such that the transcribed product contained an additional 28 nt at the 3' end.

Peak fractions from the DEAE-Sepharose column were assayed for degradation of ^{32}P -labeled EG242 and EG510 RNAs in the presence of 1 mM Mg^{2+} or Mn^{2+} (Fig. 3). For the BG119 fractions in the presence of Mg^{2+} or Mn^{2+} , complete degradation of EG242 RNA was observed in fractions 17, 19, and 21, with partial degradation in fraction 23. EG510 RNA was not degraded in the presence of Mg^{2+} but was cleaved in the presence of Mn^{2+} . The latter activity is likely due to non-specific *B. subtilis* RNase III endonuclease activity, which copurifies in these fractions (D. H. Bechhofer, unpublished results). The differential activity on substrates with (EG510) and without (EG242) a 3'-proximal stem-loop structure suggested that the RNase activity was degrading from the 3' end.

Corresponding fractions from the BG296 extract were tested for activity, and no degradation was detected for either substrate in the presence of Mg^{2+} (Fig. 3). Thus, deletion of the *yvaJ* gene resulted in a strain that was missing the 3'-sensitive RNase activity.

To further test the effect of 3' structure on the RNase activity present in BG119, the EG510+28 RNA substrate was used (Fig. 2A). While the activity present in BG119 could not degrade EG510 RNA, it was able to degrade EG510+28 RNA

(Fig. 4). The same fraction from the BG296 strain was unable to degrade either EG510 or EG510+28 RNA (data not shown). The sensitivity of EG510+28 RNA (but not EG510 RNA) to decay suggested strongly that the activity observed in the BG119 fraction, which was absent from BG296, was initiating at the 3' end.

To test whether the activity was exonucleolytic, degradation products from EG242 RNA were run on a 20% polyacrylamide gel. The only product observed was a mononucleotide (Fig. 5A), and this accumulated inversely with the decay of the full-length RNA (Fig. 5B). Taken together, these results demonstrate that the *yvaJ* gene codes for a 3'-to-5' exoribonuclease that is able to degrade rRNA. We propose that the *B. subtilis yvaJ* gene be renamed *mnr*.

We assume that 3'-to-5' exoribonucleolytic activity that can degrade mRNA is an essential function in *B. subtilis*. We found that a *B. subtilis* strain lacking both RNase R and PNPase was viable, suggesting that at least one other 3'-to-5' exoribonuclease is present that is involved in mRNA decay. The remaining ribonuclease activity in the BG296 extract is now a focus of our interest.

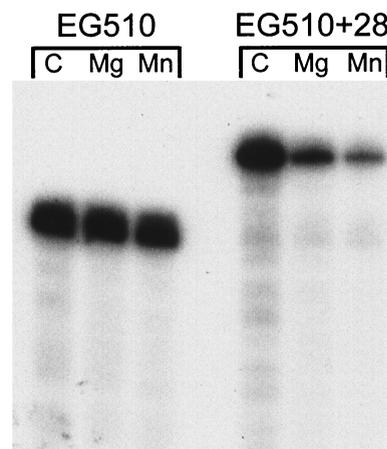


FIG. 4. Decay of ^{32}P -labeled RNAs in the presence of a purified fraction from BG119. EG510 RNA contains 12 unpaired nt at the 3' end, and EG510+28 RNA contains 40 unpaired nt at the 3' end. Incubation at 37°C for 20 min was in the absence of divalent cation (C) and in the presence of 1 mM Mg^{2+} or 1 mM Mn^{2+} .

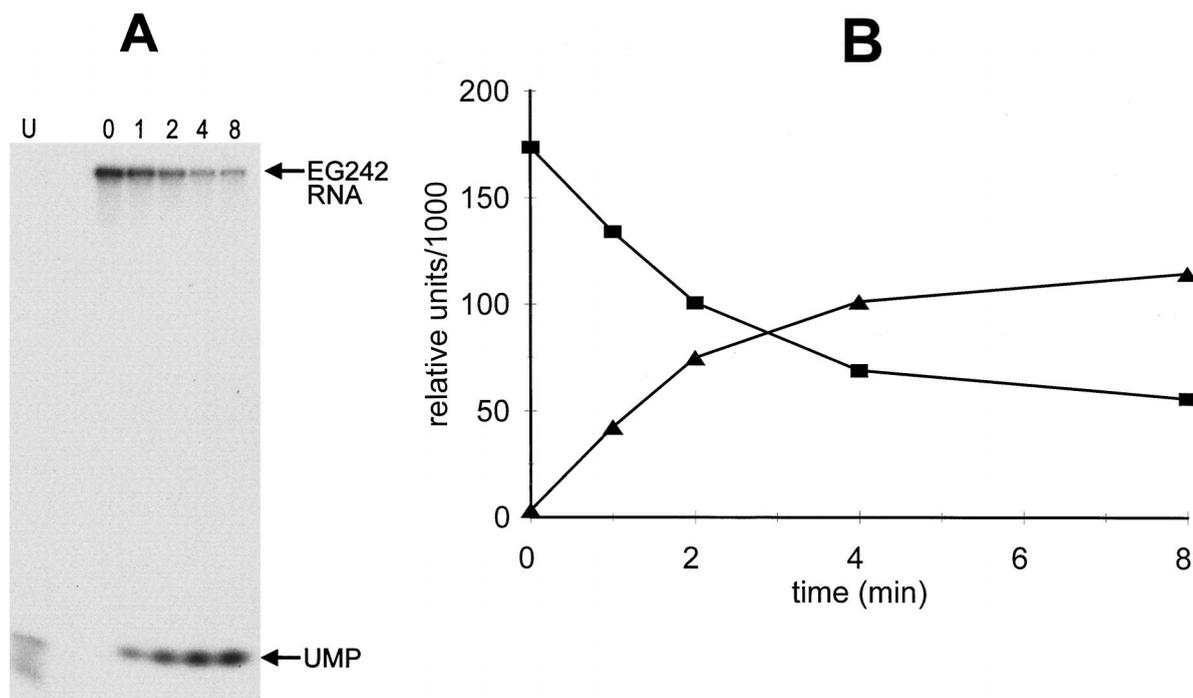


FIG. 5. Generation of monophosphate nucleosides from EG242 RNA. (A) Degradation products were separated on a 20% denaturing polyacrylamide gel. Time (in minutes) of incubation at 37°C are indicated at the top. The control lane (U) contained [^{32}P]UTP. (B) Plot of EG242 decay (squares) and generation of mononucleotides (triangles) versus time. Relative units represent volumes as measured by a PhosphorImager scan (Molecular Dynamics).

This work was supported by Public Health Service grant GM-48804 from the National Institutes of Health.

We thank David Hicks for advice on protein purification and George Mackie for suggesting the use of micrococcal nuclease.

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