Expression of multiple *Bacillus subtilis* genes is controlled by decay of *slrA* mRNA from Rho-dependent 3′ ends

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**ABSTRACT**

Timely turnover of RNA is an important element in the control of bacterial gene expression, but relatively few specific targets of RNA turnover regulation are known. Deletion of the *Bacillus subtilis pnpA* gene, encoding the major 3′ exonuclease turnover enzyme, polynucleotide phosphorylase (PNPase), was shown previously to cause a motility defect correlated with a reduced level of the 32-gene fla/che flagellar biosynthesis operon transcript. *fla/che* operon transcript abundance has been shown to be inhibited by an excess of the small regulatory protein, SlrA, and here we find that *slrA* mRNA accumulated in the *pnpA* deletion mutant. Mutation of *slrA* was epistatic to mutation of *pnpA* for the motility-related phenotype. Further, Rho-dependent termination was required for PNPase turnover of *slrA* mRNA. When the *slrA* gene was provided with a Rho-independent transcription terminator, gene regulation was no longer PNPase-dependent. Thus we show that the *slrA* transcript is a direct target of PNPase and that regulation of RNA turnover is a major determinant of motility gene expression. The interplay of specific transcription termination and mRNA decay mechanisms suggests selection for fine-tuning of gene expression.

**INTRODUCTION**

Levels of bacterial gene expression depend on the rate of transcription initiation, translation initiation, as well as the rate of messenger RNA decay. In *Bacillus subtilis* — the best-studied Gram-positive species in terms of mRNA turnover — initiation of mRNA decay is thought to begin most often with endonucleolytic cleavage catalyzed by RNase Y (1–3). Intra-transcript cleavage generates an upstream fragment that is degraded by polynucleotide phosphorylase (PNPase) or another 3′ exonuclease (4), and a downstream fragment that is subject to additional RNase Y-mediated cleavages or processive decay by RNase J1, a 5′ exonuclease (5,6). Decay from a transcript’s 5′ end can also occur by the action of RNase J1, provided the 5′-triphosphorylated end has been converted to a monophosphorylated form by an RNA pyrophosphohydrolase (7). Exonucleolytic decay from a transcript’s 3′ end is normally hindered by the strong secondary structure that is part of the Rho-independent transcription termination mechanism. Rho-dependent termination, which could generate 3′ ends without this strong structure, is not thought to play a significant role in *B. subtilis* transcription termination (8). Unlike in *Escherichia coli*, where about half of the transcription terminators are Rho-dependent and the rho gene is essential (9), the *B. subtilis rho* gene is not essential (10).

Biochemical evidence suggests that PNPase is the major mRNA turnover enzyme in *B. subtilis* (11,12). A strain that is deleted for the gene encoding PNPase, the Δ*pnpA* strain, shows several interesting phenotypes, including growth as non-motile chains of cells in liquid culture, competence deficiency and tetracycline sensitivity (12,13). However, at least in laboratory conditions, the Δ*pnpA* strain grows only slightly slower than the wild-type, perhaps suggesting that other exonuclease activities compensate in the mRNA turnover process when PNPase is absent. A recent RNA-Seq study analyzed the pattern of decay intermediates in *B. subtilis* strains that were either wild-type or deleted for the PNPase gene, and found altered levels for many mRNAs in the Δ*pnpA* strain (14). While many of the changes in gene expression in the Δ*pnpA* strain are likely due to indirect effects, a direct effect of the lack of PNPase was observed for about 10% of expressed genes, for which there was a significant increase in the level of 5′-proximal reads relative to the level of 3′-proximal reads.

Based on data from the RNA-Seq study, we were able to explain the chain growth phenotype of the Δ*pnpA* strain by an effect on the *fla/che* operon, a 27-kb operon containing 32 genes, of which the Sigma D transcription factor gene is the penultimate gene. RNA-Seq analysis revealed a 2-4-fold decrease in *fla/che* operon read levels overall, with a 3-fold decrease in *sigD* expression. This, in turn, caused depression

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of the sigD regulon, including the autolysis genes that are required for separation of daughter cells upon cell division (15,16).

We have now examined the ultimate cause of decreased fla/che operon expression in the ΔpnpA strain. An earlier report demonstrated that fla/che operon RNA levels are controlled by SlrA, a small, 52-amino-acid protein. Insertion in the chromosome of a single extra copy of the slrA gene caused a severe decrease in expression of the fla/che operon (17). The effect was shown to be at a post-transcriptional level, although the mechanism remains undetermined. Here, we show that the level of slrA mRNA increases in the absence of PNPase, resulting in slrA overexpression, as evidenced by chain growth.

MATERIALS AND METHODS

Bacterial strains

The wild-type strain, BG1, is a trpC2 thr-5 derivative of B. subtilis strain 168. BG546, the ΔpnpA strain, was described previously (14). For cell chaining assays, derivatives of the NCIB3610 strain (3610') were constructed by SPP1 phage transduction. The ΔslrA and (slrA⁺) strains were described previously (17). For construction of the Pspac-slrA allele, the slrA transcription unit, from 167 bp upstream of the CDS to 383 bp downstream of the coding sequence (CDS), was amplified with primers containing HindIII and SalI restriction endonuclease sites and cloned downstream of the Pspac promoter in plasmid pDR66 (18), giving pBL24. (The sequences of oligonucleotides used in this study are provided in Supplementary Table S1.) pBL24 DNA was linearized with restriction endonuclease NruI and used to transform the ΔslrA strain. The Pspac-slrA-lacZ β-galactosidase reporter gene was constructed as follows: a polymerase chain reaction (PCR) product containing the Pspac promoter was amplified from B. subtilis 3610 chromosomal DNA using the primers containing EcoRI and BamHI sites, and cloned into plasmid pDG268 (19), giving plasmid pDP422. pDP422 DNA was linearized with restriction endonuclease NruI for integration into the amyE locus.

The Δrho strain, a kind gift of J. Helmann, was described previously (20), as was the Δrrn strain (21). For construction of slrAATT, complementary 41-mer oligonucleotides representing the ermC transcription terminator sequence were inserted 285 bp downstream of the slrA CDS in pBL24, according to a previously-described mutagenesis procedure (22).

Northern blotting

RNA was isolated from B. subtilis strains grown to mid-logarithmic phase in Luria-Bertani (LB) broth (10 g tryptone, 5 g yeast extract, 5 g NaCl per L), as described (23). RNA was fractionated either on a 1% denaturing agarose MOPS gel and blotting by wicking, or on a 6% denaturing polyacrylamide gel and electrophorated. 5’-end-labeled oligonucleotides were used as probes. The slrA probe was complementary to nts 88-119 of the slrA CDS. For Pspac-slrA transcripts, the probe was complementary to nts 1-27 of the lacO sequence. To control for RNA loading in Northern blot analyses, membranes were stripped and probed for 5S RNA as described (24) or 16S rRNA using an oligonucleotide complementary to nts 1405-1424 (25). To determine the half-life of full-length RNA, exponential regression analysis (R² > 0.9) was performed on percentage of RNA remaining versus time. Since decay intermediates are being degraded and simultaneously generated from decay of full-length RNA, the half-life of decay intermediates was corrected based on an approach described previously (26).

3’-RACE

The 3’-RACE protocol was a slight modification of a published method (27). Total RNA from pnpA⁺ and ΔpnpA strains was isolated as described above. 3’ ends were ligated to pre-adenylated linker (5’-rAppCTGTTAGGCAACCATCAAT-ddC-3’) by incubation for 2 h at 25°C with truncated T4 RNA ligase 2 (New England BioLabs). The ligated RNA was purified with RNeasy MinElute Cleanup Kit (Qiagen), and the 3’-proximal slrA sequence was amplified by using QIAGEN OneStep RT-PCR Kit with a primer complementary to the 3’ linker and a primer consisting of slrA sequence. PCR products were separated on 1.5% agarose gel and appropriately-sized bands were excised and cloned into pGEM-T (Promega). For the band from the pnpA⁺ strain, six clones were sequenced; for the band from the ΔpnpA strain, 12 clones were sequenced.

Phenotype assays

Overnight growth in tetracycline was performed as described (12). Competence was measured by a standard transformation protocol (28), using 1 µg of plasmid pYH250 DNA (24) or 1 µg of chromosomal DNA from a strain that had a chloramphenicol-resistance marker in the amyE locus, and selection for colonies resistant to 4 µg/ml chloramphenicol. Assay of β-galactosidase activity was as described previously (29).

Microscopy

Cells were grown in LB broth to 1 OD₆₀₀. Isopropyl β-D-thiogalactopyranoside (IPTG, Sigma) was added to the medium at the indicated concentration when appropriate. Phase-contrast microscopy was performed with a Nikon 80i microscope, using a phase-contrast objective Nikon Plan Apo 100×. Images were captured with a Photometrics Coolsnap HQ2 camera and Metamorph image software.

RESULTS

Absence of PNPase affects slrA mRNA levels, causing cell chaining

An RNA-Seq analysis of mRNA levels in wild-type and ΔpnpA strains showed decreased levels of fla/che operon mRNA in the strain lacking PNPase (14). As it had been shown earlier that an increased SlrA level correlates with reduced fla/che operon transcript abundance (17), we examined the RNA-Seq data for the slrA transcription unit (Figure 1A). Although the slrA CDS is only 156 nts, the RNA-Seq read data suggested that the transcription unit is about
600 nts, with ~170-nt 5' untranslated region (UTR) and ~330-nt 3' UTR. The results in Figure 1A showed that slrA read levels were significantly elevated in the ΔpnpA strain, with a 3.4-fold read increase in the part of the transcription unit that includes the CDS, and a smaller increase in the reading unit that includes the CDS, and a smaller increase in the steadystate amount of full-length slrA mRNA in the ΔpnpA strain (average of two independent experiments), as well as an accumulation of decay intermediates, which were long enough to contain the complete slrA CDS (Figure 1B, lanes 1 and 2). Similar results were obtained in the B. subtilis 3610 strain (Figure 1B, lanes 3 and 4), which was used to assay chaining during cell growth (see below). We hypothesized that turnover of slrA mRNA by PNPase affects flac/che operon expression and sigD regulon expression.

As reported previously, depression of flac/che operon expression in the ΔpnpA mutant, and specifically the 3-fold decrease in sigD expression, was able to explain the chaining phenotype of the ΔpnpA mutant (14). If the effect of the loss of PNPase was due to elevated slrA mRNA, then deletion of the slrA gene should alleviate this effect. We used a previously-constructed ΔslrA strain (17) as well as a newly-constructed double deletion ΔpnpA ΔslrA strain to assay the chaining phenotype. Phase contrast microscopy of logarithmic phase cultures (Figure 2A) showed mostly dividing cells and a few short chains for the pnpA* strain, while there was extensive chaining in the (slrA') strain, which contains an additional chromosomal copy of the slrA gene (17). Similar extensive chaining was observed in the ΔpnpA strain, but deletion of the pnpA gene did not lead to chaining when the slrA gene was also deleted (Figure 2A; ΔpnpA ΔslrA strain). Thus, mutation of slrA was epistatic to mutation of pnpA for the cell-chaining phenotype.

Two other phenotypes reported for the ΔpnpA strain are competence deficiency (13) and tetracycline sensitivity (12). We tested whether slrA was epistatic to pnpA for these phenotypes as well. The data for overnight growth in the presence of tetracycline, shown in Figure 2B, indicated no significant difference in tetracycline sensitivity between the ΔpnpA and ΔpnpA ΔslrA strains. Thus, slrA was not epistatic to pnpA for tetracycline sensitivity. On the other hand, the data for competence indicated a partial suppression of the competence deficiency by deletion of slrA (Table 1). For chromosomal and plasmid DNA transformation, the ΔpnpA ΔslrA strain showed 9.8- and 2.7-fold, respectively, higher competence than the ΔpnpA strain (see ‘Discussion’ section).

The effect of PNPase on slrA mRNA is post-transcriptional

We determined whether the effect of PNPase on slrA mRNA was at the transcriptional or post-transcriptional level. First, Northern blot analysis of lacZ mRNA transcribed from the slrA promoter showed no difference in lacZ mRNA levels in the presence or absence of PNPase (data not shown). Assay of β-galactosidase expression from the PslrA-lacZ construct also showed no significant difference in the presence or absence of PNPase (Table 2). To facilitate analysis of slrA mRNA, which is present at low levels when expressed from the wild-type allele, we constructed a ΔslrA strain that contained a wild-type copy of slrA under control of the IPTG-inducible Pspac promoter integrated at the amyE locus. Phenotypes associated with deletion of pnpA were the same for the Pspac-slrA strain as for strains with native slrA (data not shown). The effect of the pnpA deletion on accumulation of slrA mRNA was recapitulated with the Pspac-slrA construct (Figure 3A), where the amount of full-length slrA mRNA was 2.4-fold higher in the ΔpnpA strain than in the pnpA* strain, and a similar pattern of decay intermediates was observed. slrA mRNA half-life was measured in the presence of rifampin, and the results showed ~2-fold increase in the half-life of full-length slrA mRNA in the absence of PNPase (Figure 3B, band 1), as well as in-
Figure 2. slrA mutation is epistatic to pnpA mutation for cell chaining. (A) Phase-contrast microscopy of cell chaining for the indicated strains. pnpA+, wild-type 3610 strain; slrA+, additional copy of slrA gene; ΔpnpA, deletion of gene encoding PNPase; ΔslrA, deletion of gene encoding SlrA; ΔpnpA ΔslrA, deletion of genes encoding PNPase and SlrA. (B) Overnight growth of strains in the presence of increasing concentrations of tetracycline.

Table 1. Percent transformants in ΔpnpA strains relative to pnpA+ straina

| Strains                      | Chromosomal DNA | Plasmid DNA |
|-----------------------------|-----------------|-------------|
| ΔpnpA/pnpA+                 | 1.1 (1.1, 1.1)  | 9.0 (8.0, 10.0) |
| ΔpnpA ΔslrA/pnpA+ ΔslrA    | 10.8 (10.4, 11.1) | 24.4 (27.8, 21.0) |

aValues are average of two independent experiments. Results from the two independent experiments are shown in parentheses.

Table 2. lacZ expression driven by slrA promoter

| Strain          | β-galactosidase activity per mg proteina |
|-----------------|----------------------------------------|
| pnpA+           | 241 ± 16                                |
| ΔpnpA           | 232 ± 10                                |

aMean ± standard deviation of three experiments.

increased half-life for two prominent decay intermediates that contain the slrA CDS (Figure 3B, bands 3 and 4).

slrA expression from the Pspac promoter afforded the opportunity to demonstrate conclusively that the effect of PNPase was not at the level of transcription from the slrA promoter. The chaining phenotype of pnpA+ and ΔpnpA strains was assayed in the presence of increasing concentrations of IPTG. As can be seen in Figure 3C, no chaining was evident in either strain in the absence of IPTG. For the pnpA+ strain, short chains and dividing cells were observed in the presence of 0.01 and 0.03 mM IPTG, while full chaining was observed when 0.10 mM IPTG was present. In the ΔpnpA strain, on the other hand, full chaining was observed even in the presence of 0.01 mM IPTG. These results confirmed that the chain growth that is observed in the absence of PNPase was the result of changes in slrA mRNA levels, independent of transcription from the slrA promoter.

Rho-dependent slrA mRNA 3′ ends

A previous analysis of rpsO mRNA decay in B. subtilis suggested that decay is unlikely to initiate from a 3′ end that is formed by Rho-independent transcription termination (30). Furthermore, in vitro experiments with purified PNPase showed that this enzyme is unable to degrade through the strong secondary structure of a Rho-independent transcription terminator (31). To address how PNPase could be controlling the full-length slrA mRNA half-life, we first used 3′ RACE to map the 3′ ends of slrA mRNA in the pnpA+ and ΔpnpA strains. In the pnpA+ strain, a single 3′ end was mapped at 301 nt downstream of the slrA stop codon (Figure 4A, open arrow). In the ΔpnpA strain, on the other hand, multiple 3′ ends were mapped, ranging from 301 to 333 nts downstream of the slrA stop codon (Figure 4A, short arrows). Immediately upstream of the 301-nt 3′ end is a predicted stem-loop structure that has a ΔG0 value
of −18 kcal/mol. The predicted structure is not typical of *B. subtilis* Rho-independent transcription terminators, almost all of which have completely base-paired, shorter stems (5-12 bp) followed by a 15-nt sequence containing 7–10 U residues (32). We therefore hypothesized that *slrA* transcription termination might be Rho-dependent. Northern blot analysis of *Pspac*-*slrA* RNAs in *rho*<sup>+</sup> and Δ*rho* strains is shown in Figure 4B. The absence of Rho resulted in a decreased intensity of the full-length band in the *pnpA*<sup>+</sup> and Δ*pnpA* backgrounds, as well as the appearance of higher molecular-weight RNA that is suggestive of transcriptional read-through (Figure 4B, lanes 3 and 4). Together, these results indicated that *slrA* transcription termination is Rho-dependent and terminates at multiple sites downstream of the stem-loop structure shown in Figure 4A. In the *pnpA*<sup>+</sup> strain, PNPase degrades *slrA* mRNA from these 3′ ends, although there is a partial block to PNPase processivity 5 ntS from the edge of the stem-loop structure (nt 301). In the Δ*pnpA* strain, *slrA* mRNAs with various 3′ ends are not efficiently degraded and they accumulate, resulting in an increase in ‘full-length’ *slrA* mRNA, which is actually a collection of mRNAs of different sizes due to slightly different termination sites. Indeed, higher resolution Northern blot analysis showed several, closely-spaced ‘full-length’ *slrA* mRNA bands in the Δ*pnpA* strain that were larger than in the *pnpA*<sup>+</sup> strain (Figure 4D, lanes 1 and 2).

We asked whether the unusually long 5′ and 3′ UTRs of *slrA* mRNA were relevant to PNPase-mediated decay. Deletion constructs were made in the *slrA* 5′ UTR (Δ1 and Δ2) and 3′ UTR (Δ3), as shown in Figure 4C. Northern blot analysis of a higher resolution gel (Figure 4D) showed for all constructs the presence of a single full-length band in the *pnpA*<sup>+</sup> strain, which was the predicted length based on the size of the deletion, and additional bands of slightly larger

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**Figure 3.** IPTG-induced *slrA* expression. (A) Northern blot analysis of *slrA* mRNA transcribed from the *Pspac* promoter. Ten microgram of total RNA was fractionated on a 6% denaturing polyacrylamide gel. Migration of 5′-end-labeled fragments of TaqI-digested pSE420 indicated at left. (B) Northern blot analysis of *slrA* mRNA half-life. Above each lane is time (min) after rifampin addition. Measured half-lives (average of two experiments) of specific bands are indicated on the right. Bands 1–4 are large enough to include the full *slrA* CDS. (C) Phase-contrast microscopy of cell chaining with increasing IPTG concentrations.
size in the ΔpnpA strain. Furthermore, a higher level of slrA mRNA in the ΔpnpA background was observed for all deletion constructs. The data suggested that the 5′- and 3′-UTR sequences do not play a role in slrA mRNA decay.

We next tested whether the other known processive 3′ exonucllease of B. subtilis, RNase R, was involved in slrA mRNA turnover. Northern blot analysis of Pspac-slrA mRNA was performed in ΔermC and ΔermC ΔpnpA strains. As shown in Figure 4E, there was little difference in the steady-state patterns of slrA mRNA, with or without RNase R; full-length slrA mRNA accumulated in the absence of PNase, whether or not RNase R was present. We conclude that primarily PNase is responsible for efficient turnover from the 3′ ends of slrA mRNA generated by Rho-dependent transcription termination.

Rho-independent termination of slrA transcription phenocopies the ΔpnpA strain

To test the hypothesis that Rho-dependent termination of slrA transcription allows access to PNase-mediated turnover from the 3′ end, a derivative of the Pspac-slrA construct was created that had a Rho-independent transcription terminator sequence inserted near the end of the 3′ UTR. The terminator was that of the ermC gene, which is predicted to form a strong secondary structure (ΔGθ = −24.1 kcal/mol) followed by five U residues (Figure 5A). The slrA gene with the ermC transcription terminator sequence is referred to as slrATT. The expectation was that the presence of 3′-terminal structure would block PNase decay, and even in the pnpAΔ strain there would be an accumulation of slrA mRNA and inhibition of fla/che operon expression. Northern blot analysis showed that slrATT mRNA was detected as a single band in both pnpAΔ and ΔpnpA strains (Figure 5B). Importantly, slrATT mRNA was present at a higher level than slrA mRNA in the pnpAΔ strain (∼8-fold), and, unlike slrA mRNA that was terminated in a Rho-dependent manner, there was no difference in steady-state mRNA level between the pnpAΔ and ΔpnpA strains. These results suggested that slrATT mRNA was not a substrate for PNase-mediated decay, which likely resulted in a higher level of SlrA protein. To show the effect of protecting slrA mRNA from decay, the chain growth phenotype of the Pspac-slrATT strain was tested. We showed above that when the Pspac-slrA strain was grown in the presence of 0.01 mM IPTG, there was limited chaining when PNase was present and a high degree of chaining when PNase was absent (Figure 3C). In contrast, the Pspac-slrATT strain showed massive chaining even when only 0.01 mM IPTG was added and PNase was present (Figure 5C). Thus, Rho-mediated termination of slrA transcription, in concert with PNase-mediated mRNA decay, is required to set a suitable level of slrA expression that allows motile B. subtilis growth.

DISCUSSION

The results presented here indicate that timely turnover of slrA mRNA is required for precise regulation of the fla/che operon (32 genes) and, indirectly, the rest of the sigD regulon (50 genes). Thus, the expression level of over 80 genes is determined by the ability of PNase to degrade slrA mRNA and confer on it a relatively short half-life. PNase-mediated decay of bacterial regulatory RNAs, such as small RNAs (sRNAs) and leader RNAs, has been documented previously: the decay (or stability) of a number of E. coli

**Figure 4.** Rho-dependent termination of slrA transcription. (A) Sequence and predicted secondary structure (52) of 3′-proximal region of slrA 3′ UTR. Open arrow indicates location of major 3′ end at nt 301, mapped by 3′ RACE in the pnpAΔ strain. Location of additional 3′ ends mapped in the ΔpnpA strain indicated by short arrows. Numbering is from downstream of the slrA CDS. (B) Northern blot analysis of slrA mRNA in rho+ and Δrho strains. Ten microgram of total RNA was fractionated on a 1.0% MOPS-formaldehyde agarose gel. RT, read-through. Migration of unlabeled RNA size markers indicated at left. (C) slrA gene schematic and location of slrA deletion constructs. Scale below is in base-pairs. (D) Northern blot analysis of Pspac-driven slrA mRNA from wild-type slrA gene and from deletion constructs. Ten microgram of total RNA was fractionated on a 6% denaturing polyacrylamide gel. (E) Northern blot analysis of slrA mRNA in the presence and absence of RNase R. Ten microgram of total RNA was fractionated on a 6% denaturing polyacrylamide gel.
and *Salmonella typhimurium* sRNAs are regulated by PN-Pase (see (33) and references therein); control of *E. coli* C biofilm formation by PN-Pase is hypothesized to occur via degradation of small regulatory RNAs (34); autoregulation of the *E. coli* *pnp* gene relies on efficient degradation of the *pnp* leader region RNA by PN-Pase (35); and we have shown that regulation of *B. subtilis* *trp* operon gene expression requires efficient degradation of *trp* leader RNA by PN-Pase (36). However, we are not aware of another report in which PN-Pase-mediated decay of a full-length mRNA is crucial for the control of many genes.

We show here that *slrA* mRNA decay by PN-Pase depends on Rho-dependent transcription termination (Figures 4 and 5). A recent transcriptome analysis suggested that deletion of the *rho* gene causes extended transcription of many mRNAs (37), and, indeed, the *slrA* gene is one of these. However, only a few transcripts acted on by Rho have been examined in any detail: the *rho* gene itself (8), the *trp* operon (38), and, very recently, the *rplJL* operon (39). In these cases, Rho acts either in a leader region or in the first gene of an operon to terminate transcription before synthesis of protein coding sequences. For *slrA*, Rho apparently binds downstream of a coding region to cause transcription termination that leaves 3′ ends susceptible to PN-Pase decay. PN-Pase is unable to degrade strong stem-loop structures *in vitro* (31), and it is not known whether *in vivo* association with the RNA helicase CshA (40) allows it to degrade Rho-independent transcription terminator sequences. It is also not known whether an iterative polyadenylation process, which in *E. coli* is catalyzed by poly(A) polymerase and confers susceptibility of 3′-terminal RNA fragments to decay (41), exists in *B. subtilis*. Experiments to test this have not yet been possible, since the gene encoding a *B. subtilis* poly(A) polymerase remains elusive (42). Recent evidence in our laboratory suggests that 3′-terminal mRNA fragments are degraded for the most part by RNase J1 (our unpublished data). It is therefore assumed that initiation of decay for mRNAs with a Rho-independent terminator occurs either by endonucleolytic cleavage catalyzed by RNase Y, or by 5′-to-3′ exonuclease activity of RNase J1 acting on a 5′-monophosphate end (6). It is expected that only for mRNAs whose transcription termination is Rho-dependent could efficient decay occur by a 3′ exonuclease activity starting from a native 3′ end. Although *B. subtilis* contains at least

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**Figure 5.** *slrA* with Rho-independent terminator. (A) Predicted secondary structure of the *ermC* transcription terminator. (B) Northern blot analysis of *slrA* mRNA carrying the *ermC* transcription terminator (*slrATT*). Ten microgram of total RNA was fractionated on a 6% denaturing polyacrylamide gel. (C) Phase-contrast microscopy of *slrATT* strains with increasing IPTG concentrations.
four 3′ exoribonucleases (4), in the case of slrA mRNA it appears that primarily PNPase engages in this degradative function (Figure 4E).

An interesting aspect of slrA mRNA is its unusually long 3′ UTR. A survey of the location of Rho-independent transcription terminators, relative to an upstream stop codon, determined that 93% occur within 100 bp of the stop codon, and many of the remaining terminators may be functioning in transcription termination of convergently transcribed genes (32). For slrA, termination occurs ∼330 nts downstream of the stop codon, and the next predicted gene is in the same orientation and starts 100 bp away. A 208-nt deletion starting 18 nts downstream of the slrA CDS (Δ3, Figure 4C) gave the same pattern of multiple ‘full-length’ slrA mRNA bands as the wild-type gene and as two deletions in the 5′ UTR (Figure 4D). The implication is that a Rho binding site exists in the remaining 75 nts of the Δ3 construct. For E. coli, the primary characteristics of Rho binding sites are ribosome-free, unstructured and C-rich (43). B. subtilis Rho has similar biochemical characteristics to E. coli Rho (8), suggesting that it will have similar requirements for binding. However, we were unable to recognize specific attributes of the 3′-proximal 75-nt sequence that would explain Rho binding. This sequence has an equal distribution of the four deoxyribonucleotides, and 56 nts of the sequence can be predicted to form a stable secondary structure (Figure 4A). The presence of a ‘C > G bubble,’ defined as a relatively C-rich and G-poor region over a length of 78 nts that precedes a Rho-dependent transcription termination site in many cases (44), was not observed in the corresponding region of the slrA gene (see Supplementary Figure S1). Clearly, there is much to be learned about the mechanism of B. subtilis Rho activity. Our discovery here of Rho-dependent transcription termination for a full-length mRNA, as well as the recent finding that the rho gene affects expression of genes involved in antibiotic resistance (20), should prompt additional study of the requirements for Rho activity in B. subtilis.

We showed that slrA was epistatic to pnpA for the chaining phenotype (Figure 2A). This, as well as chaining in the presence of increasing IPTG concentration for P_pnpA-promoted slrA (Figure 3C), are strong evidence for the hypothesis that the cause of the chaining in the absence of PNPase is due to a post-transcriptional effect on slrA expression. Two other phenotypes caused by the loss of PNPase did not appear to involve slrA mRNA. Tetracycline-sensitivity of the ΔpnpA strain, the basis of which is unknown, was not affected by the deletion of slrA (Figure 2B). Interestingly, competence deficiency of the ΔpnpA strain was partially suppressed by the slrA knockout (Table 1). Characterization of com gene expression in the ΔpnpA strain by Dubnau et al. suggested that loss of PNPase affected regulated expression of several competence genes, including comG, comK and srFAA (comS) (13). A simple explanation for the observed partial suppression in the ΔslrA strain is that the protein complex required for DNA uptake is located at the cell poles (45,46). Cells growing in long chains would have fewer poles available for presentation of transforming DNA to the DNA-binding apparatus. Alleviation of the chain growth by deletion of slrA may thus explain partial restoration of the competence defect caused by loss of PNPase.

The finding that efficient decay of an mRNA figures prominently in the regulation of a large number of genes supports the concept that models of gene expression networks must take into account not only transcriptional and translational control, but also control at the level of mRNA decay (47). In the case of slrA, the involvement of PNPase as a controlling factor relies on the generation of PNPase-susceptible 3′ ends by the action of Rho. slrA is not only required for fla/che operon regulation, but is part of a gene network that controls biofilm formation (48–50). Fine-tuning of slrA expression is likely necessary for its functions, and there has been selection for this control to involve an unusual form of mRNA decay. We expect that more examples of the interplay of Rho-dependent transcription termination and mRNA turnover will be discovered.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.

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