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Cell-cycle regulated degradation of tmRNA is controlled by RNase R and SmpB

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Abstract

The production and removal of regulatory RNAs must be controlled to ensure proper physiological responses. SsrA RNA (tmRNA), a regulatory RNA conserved in all bacteria, is cell-cycle regulated and is important for control of cell cycle progression in *Caulobacter crescentus*. We report that RNase R, a highly conserved 3'–5' exoribonuclease, is required for the selective degradation of SsrA RNA in stalked cells. Purified RNase R degrades SsrA RNA *in vitro*, and is kinetically competent to account for all SsrA RNA turnover. SmpB, a tmRNA-binding protein, protects SsrA RNA from RNase R degradation *in vitro,* and the levels of SmpB protein during the cell cycle correlate with SsrA RNA stability. These results suggest that SmpB binding controls the timing of SsrA RNA degradation by RNase R. We propose a model for the regulated degradation of SsrA RNA in which RNase R degrades SsrA RNA from a non-tRNA-like 3' end, and SmpB specifically protects SsrA RNA from RNase R. This model explains the regulation of SsrA RNA in other bacteria, and suggests that a highly conserved regulatory mechanism controls SsrA activity.

Keywords

Caulobacter; cell cycle; ribonuclease; RNA-binding protein; tmRNA

INTRODUCTION

Regulatory RNAs play a prominent role in a number of physiological processes in both prokaryotes and eukaryotes, including regulation of gene expression (Szymanski and Barciszewski, 2003), remodeling and modification of chromatin structure (Akhtar, 2003), modulating the activity of proteins (Wassarman and Storz, 2000), and controlling mRNA stability, processing, and translation (Bartel, 2004; Gottesman, 2004; Storz et al., 2004). In addition, most regulatory RNAs are expressed in highly specific patterns in relation to development and certain environmental conditions, suggesting that they play important roles in the cell (Bartel, 2004). The expression of regulatory RNAs under specific physiological conditions implies that these RNAs are produced and removed in a coordinated fashion to ensure proper signaling and physiological responses. While there are many examples of specific production of a regulatory RNA, there are far fewer cases of specific removal of a regulatory RNA. One example of regulatory RNA degradation is sRNAs in *Escherichia coli*. sRNAs base-pair with target mRNAs and attract RNase E for the coupled degradation of the sRNA and its target. The RNA chaperone Hfq is required to regulate this process

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(Gottesman, 2004). Here we report a mechanism for specifically removing a highly conserved regulatory RNA, SsrA, at a single point in the cell cycle. Analogous to proteolysis of regulatory proteins, SsrA RNA is specifically degraded by a conserved ribonuclease, RNase R, but stabilized by an RNA-binding protein, SmpB.

SsrA (also known as tmRNA and 10Sa RNA) is a small, highly structured RNA that is found in all bacteria and in chloroplasts and mitochondria of some eukaryotes (Felden et al., 1999; Gueneau de Novoa and Williams, 2004; Jacob et al., 2004; Keiler et al., 2000). SsrA interacts with selected ribosomes to add a peptide tag to nascent polypeptides that are subsequently recognized and degraded by intracellular proteases (Gottesman et al., 1998; Karzai et al., 2000; Keiler et al., 2000). SsrA activity is proposed to regulate gene expression and provide a quality-control mechanism for translation (Abo *et al.*, 2000; Hayes *et al.*, 2002a, 2002b; Karzai *et al.*, 2000; Keiler *et al.*, 1996; Ranquet *et al.*, 2001; Roche and Sauer, 1999, 2001; Sunohara *et al.*, 2002). The phenotypes of *ssrA* deletion stains in several bacterial species suggest that SsrA functions are important in bacterial physiology (Ebeling *et al.*, 1991; Julio *et al.*, 2000; Keiler and Shapiro, 2003a; Withey and Friedman, 1999).

In *Caulobacter crescentus*, SsrA is required for coordinated cell cycle progression (Keiler and Shapiro, 2003a), and both the synthesis and degradation of SsrA are tightly controlled during the cell cycle (Keiler and Shapiro, 2003b). The cell cycle of *C. crescentus* is linked to developmental progression such that initiation of DNA replication is coincident with differentiation from the swarmer cell stage (G_1 phase) to the stalked cell stage (S phase) (Hung et al., 1999). SsrA RNA is transcribed just before this G_1-S transition, specifically degraded during early S phase, and re-synthesized late in S phase (Keiler and Shapiro, 2003b). Deletion of the *ssrA* gene results in a disruption of the cell cycle at the G_1-S transition, consistent with an important role for the timing of SsrA RNA synthesis and degradation (Keiler and Shapiro, 2003a). The striking temporal expression pattern of SsrA RNA during the cell cycle raises the question of what regulatory factors control SsrA RNA expression and activity.

In *C. crescentus*, as in all other α-proteobacteria, SsrA RNA is composed of two RNA molecules due to a circular permutation in the *ssrA* gene (Keiler et al., 2000). The *ssrA* gene is transcribed as a single RNA, pre-SsrA, which is predicted to form a similar secondary structure to the mature SsrA except that the tRNA-like 5' and 3' ends are connected by a closed loop. This loop is then excised to produce mature SsrA, composed of the coding RNA and the acceptor RNA (Fig. 1A). In other bacteria such as *E. coli*, SsrA is made as a single transcript and the ends are processed in the same manner as for tRNAs. In all cases, processing of pre-SsrA to the active mature form is mechanistically and temporally distinct from the regulatory removal of SsrA, just as post-translational proteolytic processing is distinct from regulatory protein degradation.

Several proteins are required for SsrA activity, including the general translation factors EF-Tu and alanine-tRNA synthetase, and the SsrA-binding protein SmpB. SmpB binds with high affinity to the tRNA-like domain of SsrA RNA (Barends et al., 2001; Karzai et al., 1999), and is required for interaction of SsrA RNA with the ribosome (Karzai et al., 1999). In *C. crescentus*, SmpB is required for normal steady-state levels of SsrA RNA, and a deletion of *smpB* has the same phenotype as a deletion of *ssrA* (Keiler and Shapiro, 2003a). SmpB is widely distributed in bacteria, and SmpB homologues have been identified in almost all species in which SsrA has been found.

RNase R co-purifies at sub-stoichiometric levels with the SsrA-SmpB complex from *E. coli* but is not required for SsrA activity, and the significance of this interaction is unknown (Karzai and Sauer, 2001). RNase R, encoded by the *rnr* or *vacB* gene, was originally

identified as a residual 3' to 5' exoribonuclease activity in an *E. coli* strain devoid of RNase II (Kasai et al., 1977), and is a member of the RNR superfamily of exoribonucleases (Zuo and Deutscher, 2001). RNase R is highly processive and can degrade RNAs with significant secondary structure, such as rRNA and mRNAs containing stable stem-loops (Cheng and Deutscher, 2005), although tRNAs are poorly degraded. Despite biochemical association with the *E. coli* SsrA-SmpB complex, RNase R has not been implicated in the degradation of SsrA RNA or damaged mRNAs, and its role in the SsrA pathway has been unclear. The data presented here demonstrate that SsrA RNA in *C. crescentus* is specifically degraded by RNase R at a specific point in the cell cycle, and that this timing may be regulated by SmpB.

RESULTS

C. crescentus RNase R specifically degrades SsrA RNA *in vitro*

RNase R was identified as a candidate for the nuclease responsible for complete degradation of SsrA RNA in stalked cells because RNase R co-purifies with SsrA RNA in *E. coli* (Karzai and Sauer, 2001), and it is a highly processive 3' to 5' exonuclease that is capable of degrading RNAs with significant secondary structure (Cheng and Deutscher, 2005; Cheng and Deutscher, 2002, 2003). The *C. crescentus* homologue of *rnr* was identified by sequence similarity to the *E. coli* gene. The predicted amino acid sequences of the *C. crescentus* and *E. coli* RNase R are over 32% identical and 48% similar, and both contain a conserved RNase II family signature in the central region and a C-terminal S1 RNA-binding domain.

To determine if RNase R can directly degrade SsrA RNA, a histidine-tagged variant of *C. crescentus* RNase R was purified and assayed *in vitro*. When incubated with mature SsrA RNA, RNase R rapidly degraded both the coding and acceptor RNAs. More than 70% of the mature SsrA RNA was completely degraded within 5 min, corresponding to a minimum initial rate of 14 min^{-1} (Fig. 1B & C). The remaining RNA is significantly more stable, suggesting that there may be two RNA populations in the mature SsrA RNA preparation. In contrast to the mature SsrA RNA, when either the SsrA acceptor RNA alone or the SsrA coding RNA alone was incubated with RNase R, neither was degraded (Fig. 1B). These data indicate that mature SsrA RNA, but not its individual components, is recognized by RNase R as a substrate. The residual RNA observed after 5 min incubation of mature SsrA RNA with RNase R may represent a sub-population of SsrA acceptor RNA and SsrA coding RNA that is not correctly folded, and therefore is not recognized by RNase R. Like mature SsrA RNA, pre-SsrA RNA was a substrate for RNase R, but was degraded at a slightly slower rate. For comparison, the degradation of a 17-mer oligo(A) RNA, a good substrate for *E. coli* RNase R (Cheng and Deutscher, 2002), and tRNA, a poor substrate for *E. coli* RNase R (Cheng and Deutscher, 2002), were assayed under the same conditions as SsrA RNA. Oligo(A) RNA was degraded 3-fold slower than mature SsrA RNA, whereas very little activity against tRNA was observed. To obtain more detailed kinetic parameters, the degradation of pre-SsrA RNA and poly(A) RNA was measured using a TCA precipitation assay and the apparent kinetic constants were determined (Fig. 1C). Both substrates were efficiently degraded by RNase R, with very similar k_{cat} and K_M values. These data indicate that pre-SsrA RNA is as good a substrate for RNase R as poly(A) RNA, and mature SsrA RNA is the best substrate yet identified.

The degradation of SsrA RNA appears to be processive, because no degradation intermediates were observed for any substrate in the electrophoresis-based assay (Fig. 1B). Of particular interest, no processing of pre-SsrA to the mature form could be detected. To ensure that the observed degradation of pre-SsrA is in fact complete degradation and not processing, aliquots of radiolabeled pre-SsrA were removed during incubations with RNase R and resolved on a polyacrylamide gel. Again, no degradation intermediates were observed at any point in the time course (Fig. 1B), indicating that pre- SsrA was degraded into

fragments <32 nucleotides in length. These data are consistent with degradation of SsrA RNA and other substrates to individual nucleotides, as would be expected for a highly processive exonuclease.

RNase R is required for cell-cycle dependent degradation of SsrA RNA

To determine if RNase R degrades SsrA RNA *in vivo* as well as *in vitro*, a deletion of *rnr* was constructed in *C. crescentus* and the stability of SsrA RNA was measured in the Δ*rnr* strain. In log-phase cultures of the Δ*rnr* strain, both coding and acceptor SsrA RNAs have half-lives longer than 30 min (Fig. 2A). This rate represents a significant stabilization over the 4–5 min half-life observed for the mature SsrA RNAs in wild-type cells (Keiler and Shapiro, 2003b). The decay of pre-SsrA RNA is also stabilized in the Δ*rnr* strain, with a half-life of 4.7 ± 0.1 min compared to 2.5 ± 0.2 min for wild type. The half-lives of two mRNAs, *pilA* and a variant of λ repressor, were 1–2 min in the Δ*rnr* strain (not shown), the same as in wild type. These data indicate that deletion of *rnr* does not result in a general stabilization of all RNAs, but SsrA RNA is specifically stabilized.

In principle, the extended half-life of SsrA RNA in log-phase populations of the Δ*rnr* strain could be due to loss of specific degradation of SsrA RNA in stalked cells, or to partial stabilization of SsrA RNA throughout the cell cycle. To distinguish between these possibilities, the RNA degradation assays were repeated in pure populations of swarmer cells and stalked cells. In swarmer cells isolated from the Δ*rnr* strain, the mature SsrA RNAs have half-lives >30 min, just as in the wild-type strain (Fig. 2B). However, in stalked cells the half-lives are also >30 min, compared to 4–5 min for wild type (Fig. 2C). Thus, in the Δ*rnr* strain the cell-type specific degradation of SsrA is eliminated and SsrA RNA is stable in all cell types.

If RNase R is the sole nuclease responsible for the turnover of mature SsrA RNA *in vivo*, then it must degrade SsrA RNA with a rate sufficient to account for the observed turnover. During the *C. crescentus* stalked cell stage, SsrA RNA is almost completely degraded within 30 min, corresponding a 4–5 min half-life. Estimates for the concentration of SsrA RNA in *C. crescentus* indicate that there are approximately 2000 molecules per cell (KCK, unpublished observations). In this case, the degradation of mature SsrA RNA occurs with a rate of approximately 67 min^{-1}. Since the observed rate of degradation of SsrA RNA by RNase R *in vitro* is 14 mol SsrA RNA/ min / mol RNase R, there must be at least 5 molecules of RNase R per cell to account for the observed degradation of SsrA RNA. Western blots calibrated with purified RNase R protein indicate that there are at least 1000 RNase R molecules per cell (not shown). Therefore, assuming the conditions *in vitro* approximate the conditions *in vivo*, RNase R is kinetically competent to be the sole ribonuclease degrading SsrA RNA. Taken together, these *in vivo* and *in vitro* results suggest that RNase R is directly responsible for degrading SsrA RNA in a cell-type specific manner.

Lack of RNase R alters cell-cycle expression of *ssrA*

To determine if loss of cell-type specific degradation leads to over-accumulation of mature SsrA RNA, total RNA was isolated from log-phase cultures of wild type and the Δ*rnr* strain, and the amount of SsrA RNA was measured by Northern blotting. The amount of mature SsrA RNA was the same in the Δ*rnr* strain as wild type (Fig. 3A). Therefore, loss of SsrA RNA degradation does not alter the steady-state level of SsrA RNA. Even if the total amount of SsrA RNA is the same in a mixed culture, it is possible that the cell-cycle regulation of expression is altered. To investigate whether RNase R-mediated degradation is necessary for control of the pattern of *ssrA* expression through the cell cycle, the amount of SsrA RNA in synchronous cultures of the Δ*rnr* strain was assayed by Northern blotting. In the Δ*rnr* strain the cell-cycle expression pattern of SsrA RNA was disrupted (Fig. 3B & C).

There was <2-fold change in steady-state level over the course of the cell cycle, compared to a 5-fold change for wild type (Fig. 3C; Keiler and Shapiro, 2003b), and there was no decline during the stalked cell stage (30 min to 60 min after synchronization). Therefore, RNase R is necessary for degradation of SsrA RNA and this degradation is required for cell-cycle regulation of SsrA RNA levels.

Phenotype of the *rnr* deletion strain

To investigate whether lack of RNase R and the resulting constitutive expression of SsrA are detrimental to the cell, the morphology, growth, and cell cycle progression of the Δ*rnr* strain were assayed. The Δ*rnr* strain showed no changes in cellular morphology when examined by light microscopy. In addition, the growth rate of the Δ*rnr* strain during log-phase in complex and defined media and the timing of cell cycle events such as initiation of DNA replication, expression of the cell-cycle regulated proteins CtrA and McpA, loss of motility, and cell division, were not significantly different from those of wild type *C. crescentus* (Table I). Therefore, there is no significant defect in the cell cycle in the absence of RNase R. These data indicate that RNase R activity is not essential under culture conditions, and constitutive expression of SsrA RNA does not disrupt the cell cycle under the growth conditions assayed. Because a deletion of *ssrA* causes a significant defect in the cell cycle (Keiler and Shapiro, 2003a) and SsrA RNA is normally cell-cycle regulated (Keiler and Shapiro, 2003b), the lack of a phenotype when SsrA RNA is constitutively expressed suggests that there may be redundant mechanisms to control SsrA activity.

SmpB protects SsrA RNA from RNase R degradation

SsrA RNA is specifically degraded in the stalked cell. One possible mechanism for stalked cell-specific degradation would be to limit expression of RNase R to this cell type. However, Western blots showed that the level of RNase R does not fluctuate through the cell cycle in a pattern that would explain cell-type specific degradation of SsrA RNA (Fig. 4). Instead, RNase R protein accumulates through the cell cycle. These results exclude a mechanism of temporal separation of SsrA RNA and RNase R, and indicate that RNase R degradation of SsrA RNA is regulated by additional factors *in vivo*.

One candidate for a regulator of SsrA RNA degradation is the SsrA-binding protein SmpB. The steady-state level of SsrA RNA is decreased by 90% in a Δ*smpB* strain, consistent with an increase in SsrA RNA degradation in the absence of SmpB (Fig. 3A; Keiler and Shapiro, 2003a). To determine if SmpB can directly protect SsrA RNA from degradation by RNase R, the interactions among these molecules were assayed *in vitro*. First, direct association of SmpB with SsrA RNA was examined by equilibrium filter-binding assays. Purified SmpB bound to SsrA RNA with a $K_d = 1.8 \pm 0.1$ nM (not shown), an affinity similar to that reported for *E. coli* SmpB and SsrA RNA (Jacob et al. 2005; Sundermeier, et al., 2005). Second, the effect of SmpB on degradation of pre-SsrA RNA by RNase R was assayed *in vitro*. As shown in Table II, incubation of SsrA RNA with SmpB prior to the addition of RNase R protected SsrA RNA from degradation. When SsrA RNA was pre-incubated with 20 μM SmpB, no SsrA RNA degradation could be detected after 2 h, indicating at least a 14-fold decrease in the degradation rate. The degradation of oligo(A) RNA was not affected by pre-incubation with SmpB (Table II), indicating that SmpB is not a general inhibitor of RNase R, but specifically inhibits the degradation of SsrA RNA.

Western blotting of lysates from synchronous cultures showed that the amount of SmpB protein fluctuates during the cell cycle (Fig. 4), with a similar pattern to SsrA RNA (Fig. 3C). SmpB levels are low in early swarmer cells, but increase dramatically during the G_1-S transition, between 15 and 30 min after synchronization. During this interval, SsrA RNA accumulates in the cell. SmpB is rapidly removed from the cell after the initiation of DNA

replication, between 30 and 45 min after synchronization, when SsrA RNA is being degraded. SmpB protein levels increase again after 60 min, when SsrA RNA levels also increase. Thus the pattern of SmpB protein level throughout the cell cycle corresponds precisely to the stability of SsrA RNA. The pattern of SmpB protein level is unchanged in the Δ*rnr* strain, indicating that regulation of SmpB does not require RNase R activity (not shown). Moreover, because SsrA RNA levels are constitutive in the Δ*rnr* strain, regulation of SmpB abundance does depend on the expression pattern of SsrA RNA. Because SmpB binds with high affinity to SsrA RNA and protects SsrA RNA from degradation by RNase R *in vitro*, and SmpB has the same cell-cycle regulated expression pattern as SsrA RNA *in vivo*, it is likely that SmpB is required for SsrA RNA stability as well as activity.

DISCUSSION

The data presented here demonstrate that RNase R is the nuclease responsible for cell-type specific degradation of SsrA RNA. In the absence of RNase R there is no degradation of SsrA RNA, and RNase R is kinetically competent to account for the observed degradation of SsrA RNA. The cell-cycle regulation of this degradation *in vivo* is likely to be controlled by SmpB binding to SsrA RNA. The model presented in Figure 5 is sufficient to explain the data reported here, as well as known phenotypes of mutations in genes encoding SsrA, SmpB, and RNase R from other bacteria. In this model, SmpB protects SsrA RNA from degradation by RNase R at a non-tRNA-like 3'-end.

The substrate specificity of RNase R indicates that degradation of SsrA RNA requires an element of the folded structure, and is likely to initiate at the 3' end of the SsrA coding RNA *in vivo*. RNase R rapidly degrades both mature SsrA RNA and pre-SsrA RNA, but will not degrade the SsrA coding RNA or the SsrA acceptor RNA alone. This selectivity indicates that RNase R requires some feature of the folded SsrA RNA structure that is not present in the isolated coding and acceptor RNAs. One possibility is that RNase R specifically binds to part of the SsrA RNA folded structure. Alternatively, an important sequence determinant, such as the 3' end, may be obscured when the acceptor RNA and the coding RNA are not properly folded. In either case, degradation of folded SsrA RNA is likely to initiate at the 3' end of the SsrA coding RNA, because RNase R degrades both mature SsrA RNA and pre-SsrA RNA substantially faster than tRNA. The 3' end of tRNA and the 3' end of the acceptor RNA in mature SsrA have similar sequence and structure, and are largely constrained by base-pairing interactions. In contrast, pre-SsrA RNA and the coding RNA in mature SsrA have a non-tRNA-like 3' end that may be accessible to RNase R. Therefore, the stability of mature SsrA RNA *in vivo* could be regulated by protecting or exposing the 3' end of the SsrA coding RNA.

SmpB could limit access of RNase R to the 3' end of the SsrA coding RNA either by directly binding this region or by promoting a three-dimensional RNA structure that buries this 3' end. Biochemical and structural studies on SsrA-SmpB complexes from other species demonstrate that SmpB binds to the tRNA-like domain of SsrA RNA, but do not rule out a second contact with another portion of SsrA RNA (Barends et al., 2001; Gutmann et al., 2003; Valle et al., 2003). The cell-cycle regulation of SmpB protein levels could then control the susceptibility of SsrA RNA to RNase R degradation. When SmpB is present in swarmer cells and pre-divisional cells, SsrA RNA is protected from RNase R and is stable, but after the G_1-S transition SmpB levels decrease and SsrA RNA is degraded by RNase R. If this model is correct, the key event to degrading SsrA RNA in stalked cells is proteolysis of SmpB, and persistence of SmpB protein through the cell cycle should produce constitutive expression of SsrA RNA. Future studies using mutations that alter SmpB proteolysis will be required to confirm the role of SmpB in SsrA RNA stability.

The cell-cycle regulation of SmpB abundance also explains why a deletion of *rnr* that results in constitutive *ssrA* expression has no phenotype. SmpB is required for association of SsrA RNA with the ribosome (Karzai et al., 1999), so SsrA activity will be properly controlled, even if SsrA RNA is ectopically produced, as long as SmpB levels are cell-cycle regulated. The dual regulation of SsrA RNA activity by SmpB binding and degradation of SsrA RNA by RNase R provides a redundant regulatory network to control SsrA activity through the cell cycle, analogous to the control of key protein cell-cycle regulators such as CtrA (Ryan and Shapiro, 2003).

Why is SsrA RNA stable in *E. coli* even though RNase R co-purifies with SsrA (Karzai and Sauer, 2001)? In the context of the model presented in Figure 5, the lack of degradation of *E. coli* SsrA RNA by RNase R can be explained by the absence of a non-tRNA-like 3' end. If RNase R can not recognize the tRNA-like 3' end of SsrA, then *E. coli* SsrA RNA would have to unfold or be cleaved by another nuclease before RNase R could degrade it. Significantly, this model is consistent with the observed specificity of RNase R for its other known substrates *in vivo*, rRNAs and mRNAs with significant secondary structure. RNase R degrades intact rRNAs with a rate 5-fold lower than poly(A) RNA (Cheng and Deutscher, 2002). Likewise, mRNAs containing stable stem-loop structures are degraded rapidly by RNase R after polyadenylation by poly(A) polymerase (Cheng and Deutscher, 2005). Thus, *E. coli* SsrA RNA would only be a good substrate for RNase R if it is damaged, but the two-piece construction of *C. crescentus* SsrA RNA would provide access to a 3' end without the involvement of another nuclease. Interestingly, *E. coli* SsrA RNA is cleaved internally in a reaction that is stimulated by toxins such as RelE (Christensen and Gerdes, 2003) and MazF (Christensen et al., 2003). The proposed model predicts that cleaved SsrA RNA generated under stress conditions would be rapidly degraded by RNase R. Furthermore, the opportunity to regulate SsrA RNA by RNase R-mediated degradation may have contributed to the selective advantage that has produced at least three lineages of circularly permuted *ssrA* genes (Sharkady and Williams, 2004).

It has been suggested that RNase R is required for the processing of pre-SsrA in *E. coli* under cold shock conditions (Cairrao et al., 2003). RNase R is not induced under cold shock conditions in *C. crescentus*, and is not required for processing pre-SsrA RNA under cold shock or heat shock conditions, during log-phase growth, or during stationary phase (Fig. 3A and not shown). RNase R degrades pre-SsrA RNA *in vitro*, but no intermediate products that would be consistent with processing to the mature form were observed *in vitro* or *in vivo*. Instead, pre-SsrA RNA is completely degraded by RNase R in a processive manner.

SsrA and RNase R are each required for virulence in pathogenic bacteria, and the results presented here raise the possibility that these two molecules function together during pathogenesis. SsrA is required for virulence in *Salmonella typhimurium* (Julio et al., 2000), whereas RNase R is essential for virulence in *Shigella flexneri* and enteroinvasive *E. coli* (Cheng et al., 1998). If RNase R is required because it must degrade SsrA RNA in these species during pathogenesis, then both the presence and the removal of SsrA would be critical for pathogenic processes. This dependence would put SsrA at the center of an uncharacterized pathway for regulation of processes important for pathogenesis, and suggests that the SsrA pathway might be a useful target for antibacterial drugs. It will be crucial to examine the role and regulation of SsrA, SmpB, and RNase R during pathogenesis to test these possibilities.

EXPERIMENTAL PROCEDURES

Bacterial strains and plasmids

The wild-type *C. crescentus* strain used in this study is CB15N (Evinger and Agabian, 1977). *C. crescentus* strains were grown at 30°C in M2G, M2X, or PYE medium (Ely, 1991) supplemented with 1–2 μg/ml chloramphenicol, 5–20 μg/ml kanamycin, or 25–50 μg/ml spectinomycin as necessary, and monitored by optical density at 660 nm. The Δ*rnr* strain was constructed by engineering an in-frame deletion of all but 6 codons of the *rnr* open reading frame using the two-step recombination method as previously described (Gay et al., 1985), and verified by PCR analysis and Southern blotting. *E. coli* strains were grown at 37°C in Luria-Bertani broth (Sambrook et al., 1989) supplemented with 50–100 μg/ml ampicillin, 20–30 μg/ml chloramphenicol, or 30–50 μg/ml kanamycin as necessary, and monitored by optical density at 600 nm. pSmpBa was constructed by amplifying the coding sequence of the *smpB* gene from *C. crescentus* genomic DNA by PCR and cloning the product into the plasmid pET28a (Novagen) to produce a gene encoding N-terminal His_6-tagged SmpB. To generate pSmpBb, the *smpB* gene was amplified by PCR using primers to add a His_6 tag at the 5' end of the s*mpB* coding sequence, and the product was cloned into plasmid pML81 under control of the xylose-inducible promoter (Meisenzahl et al., 1997). To produce RNase R with a His_6 tag at the C terminus, plasmid pRNR was constructed by amplifying the coding region of *rnr* from *C. crescentus* genomic DNA by PCR and cloning the product into pET-21a (Novagen).

Cell-cycle experiments

Synchronized cultures of *C. crescentus* were obtained by isolation of swarmer cells from Ludox density gradients (Evinger and Agabian, 1977). Aliquots of synchronized cultures were removed every 15 min for analysis by flow cytometry, Western blotting, or Northern blotting. The timing of loss of motility and cell division in these cultures was estimated by visual inspection using light microscopy. The levels of SmpB, RNase R, CtrA, and McpA were analyzed by Western blotting followed by quantification using ImageQuant software (Molecular Dynamics). Flow cytometry assays for DNA content and initiation of replication were performed as previously described (Winzeler and Shapiro, 1995).

Northern blotting and RNA turnover

Total RNA was isolated using the hot phenol method (Sambrook et al., 1989). Northern blotting was performed after separating equal quantities of total RNA on polyacrylamide-urea gels. Specific RNAs were visualized by hybridization with [^{32}P]-labeled DNA probes generated from PCR products using the QuickPrime protocol (Amersham Biosciences), and quantified using a PhosphorImager with ImageQuant software. As a control for loading and transfer of RNA, the blots were re-probed with 5S rRNA, which does not fluctuate through the cell cycle (Keiler and Shapiro, 2003b). RNA decay experiments were performed by inhibiting transcription with rifampicin (15 μg/ml final concentration) and assaying the fate of existing RNAs by Northern blotting as previously described (Keiler and Shapiro, 2003b).

Protein purification and antisera production

His_6-SmpB for binding assays was produced from log-phase cultures of *E. coli* strain BL21(DE3)/pLys (Novagen) bearing plasmid pSmpBa by growth in the presence of 1 mM isopropyl-β-D-thiogalactopyranoside for 3 h. The culture was quickly cooled on ice, cells were harvested by centrifugation, and the cell pellet was suspended in 5 ml buffer A (50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl, 5 mM β-mercaptoethanol, 10 mM imidazole) per gram wet weight and lysed by sonication. The lysate was cleared by centrifugation at 10,000 × *g* for 30 min and the supernatant was added to 0.5 ml Ni-NTA resin (Qiagen) equilibrated in

buffer A. After mixing for 1 hr at 4°C, the resin was packed into a column, washed with 100 ml buffer A, 100 ml buffer A20 (A buffer with 20 mM imidazole), and eluted with 10 ml buffer B (50 mM MES (pH 6.5), 300 mM NaCl, 5 mM β-mercaptoethanol, 250 mM imidazole). The salt concentration of eluted protein was diluted by the addition of 5 volumes of 50 mM MES (pH 6.5) and applied to a MonoS HR 5/5 column (Amersham Biosciences) equilibrated in buffer S100 (50 mM MES (pH 6.5), 0.1 M KCl), and developed with a 50 ml gradient from 0.1–1 M KCl. Fractions containing the purified protein were identified by SDS-PAGE, quickly frozen in liquid nitrogen, and stored at −80°C.

His_6-SmpB for antibody production was produced as described above with the following exceptions. The cell pellet was suspended in buffer C (10 mM Tris-HCl (pH 8.0), 100 mM NaH_2PO_4, 8 M urea, 5 mM imidazole). The cleared supernatant was added to 1 ml Ni-NTA resin equilibrated in buffer C. After mixing for 1 h at room temperature, the resin was packed into a column, washed with 100 ml buffer C, 100 ml buffer C20 (C buffer with 20 mM imidazole), and eluted with 10 ml buffer C containing 250 mM imidazole. Fractions were separated on a preparative SDS-polyacrylamide gel and the band corresponding to the His_6-SmpB protein was excised and used to immunize rabbits. Immunization and sampling of the sera were performed by Josman LLC. (Napa, CA). The antisera were affinity-purified using the original antigen coupled to AminoLink Plus Coupling Gel (Pierce) and used for Western blotting.

RNase R-His_6 for enzyme assays was produced and purified by Ni-NTA chromatography as described for His_6-SmpB from *E. coli* strain BL21(DE3)/pLys bearing plasmid pRNR with the following exceptions. Buffer A for RNase R-His_6 purification was prepared without β-mercaptoethanol. The salt concentration of eluted protein from Ni-NTA was diluted by the addition of 5 volumes of 10 mM Tris-HCl (pH 7.6) and applied to a MonoQ HR 5/5 column equilibrated in buffer Q100 (10 mM Tris-HCl (pH 7.6), 0.1 M KCl, 1 mM DTT, 0.5 mM EDTA), and developed with a 50 ml gradient from 0.1–1 M KCl. Fractions containing the purified protein were identified by SDS-PAGE, pooled, and dialyzed against buffer Q500 (10 mM Tris-HCl (pH 7.6), 0.5 M KCl, 1 mM DTT, 0.5 mM EDTA, 10% glycerol). The dialyzed sample was quickly frozen in liquid nitrogen and stored at −80°C. Antisera against RNase R-His_6 were generated as described above.

RNA purification and labeling

Mature SsrA RNA was purified by affinity to SmpB. Log-phase cultures of *C. crescentus* bearing pSmpBb were induced by the addition of xylose to 0.03%, and grown for 3 h at 30°C in PYE broth. Cells were harvested by centrifugation, resuspended in 5 ml buffer D (50 mM NaH_2PO_4 (pH 8.0), 150 mM NaCl, 5 mM imidazole) per gram wet weight and lysed in a French press. The lysate was cleared by centrifugation at 10,000 × *g* for 30 min and added to 2 ml Ni-NTA resin equilibrated in buffer D. After mixing for 1 h at 4°C, the resin was packed into a column, washed with 200 ml buffer D, and bound protein was eluted with 10 ml buffer D containing 250 mM imidazole. The mature SsrA RNA was extracted from fractions containing SmpB with acid phenol (pH 4.5)-chloroform, precipitated with ethanol (Sambrook et al., 1989), and desalted with mini Quick Spin RNA columns (Roche Applied Science). To remove contaminating RNA, the mature SsrA RNA was separated by polyacrylamide-urea gel electrophoresis and the bands corresponding to mature SsrA RNA were excised and soaked in Tris-EDTA buffer (pH 7.0) containing 250 mM NaCl. The supernatant was extracted with acid phenol (pH 4.5)-chloroform, and the RNA was precipitated with ethanol and resuspended in water. Mature SsrA RNA was dephosphorylated with alkaline phosphatase, labeled at the 5'-ends with [γ-^{32}P]ATP and T4 polynucleotide kinase, and purified using mini Quick Spin RNA columns.

C. crescentus pre-SsrA was transcribed from a PCR-generated DNA fragment using the T7 RiboMax Large Scale RNA Production System (Promega) as described in the manufacturer's instructions, and the transcribed RNA was separated from unincorporated nucleotides and DNA fragments using the RNeasy MinElute Cleanup Kit (Qiagen). The presence of predicted secondary structures was confirmed by chemical probing, and the presence of unprocessed, pre-SsrA RNA was confirmed by Northern blotting. Pre-SsrA, poly(A) RNA (Sigma), and *E. coli* tRNA (Sigma) were labeled at the 5' end as described for mature SsrA RNA. 3'-end labeled pre-SsrA was prepared by ligation of [α-^{32}P]pCp using T4 RNA ligase, and purified using RNeasy MinElute Cleanup Kit. 17-mer oligo(A) RNA was synthesized (IDT) and labeled at the 5' end with [γ-^{32}P]ATP and T4 polynucleotide kinase, and purified using mini Quick Spin Oligo columns (Roche Applied Science).

RNA binding assays

100 pM 5'-end labeled pre-SsrA was incubated with varying amounts of purified His_6-SmpB protein in 20 μl reactions containing buffer E (5 50 mM MES (pH 6.5), 200 mM KCl, 5% glycerol, 5 mM β-mercaptoethanol, 0.01% NP-40, and 0.1 mg/ml BSA, 10 U of RNasin (Promega)) for 30 min at room temperature. The reaction products were passed under vacuum through a 0.45 μm nitrocellulose membrane filter (Millipore) that had been presoaked with buffer E. The filter was washed with 500 μl of buffer E under vacuum and dried, and radioactivity was determined by scintillation counting.

Ribonuclease assays

RNase R degradation assays were performed at 37°C in 10 μl total volume containing labeled substrate, buffer R (20 mM Tris-HCl (pH 8.2), 100 mM KCl, 0.5 mM $MgCl_2$), and 11–223 nM purified RNase R. At each time point a 1 μl aliquot of the reaction mixture was added to 250 μl ice-cold 5% TCA with 25 μg salmon testes DNA, incubated on ice for 15 min, and passed under vacuum through a glass microfiber filter (VWR) that had been presoaked with ice-cold 5% TCA. The filter was washed twice with 10 ml of ice-cold 5% TCA under vacuum and dried, and acid-insoluble radioactivity was determined by scintillation counting. The fraction RNA remaining was plotted *versus* time, and the initial rate of degradation was obtained from a linear fit to the data. Apparent steady-state kinetic parameters were obtained from non-linear curve fitting of plots of initial rate *versus* RNA concentration using Prism software (GraphPad).

RNase R degradation assays using polyacrylamide-urea gel analysis were performed at 37°C in 80 μl total volume containing 5–10 μM 5' -end labeled substrates, buffer R, and 50–100 nM purified RNase R. At each time point a 9 μl aliquot of the reaction mixture was added to 2 volumes RNA loading buffer (Sambrook et al., 1989) to stop the reaction, analyzed on polyacrylamide-urea gels and quantified using a PhosphorImager with ImageQuant software.

For assays in the presence of SmpB, 5 μM pre-SsrA labeled at the 5' end or 10 μM oligo(A) RNA labeled at the 5' end was incubated in the presence of 0–20 μM SmpB for 30 min at room temperature in buffer R, and RNase R was added to 50–100 nM final concentration. The reaction was incubated at room temperature for 2 hours, and degradation was monitored as above, except that acid-soluble radioactivity was determined by scintillation counting. Relative activity was calculated by subtracting background counts observed in the absence of RNase R, and normalizing the rate to that observed in the presence of RNase R with no SmpB.

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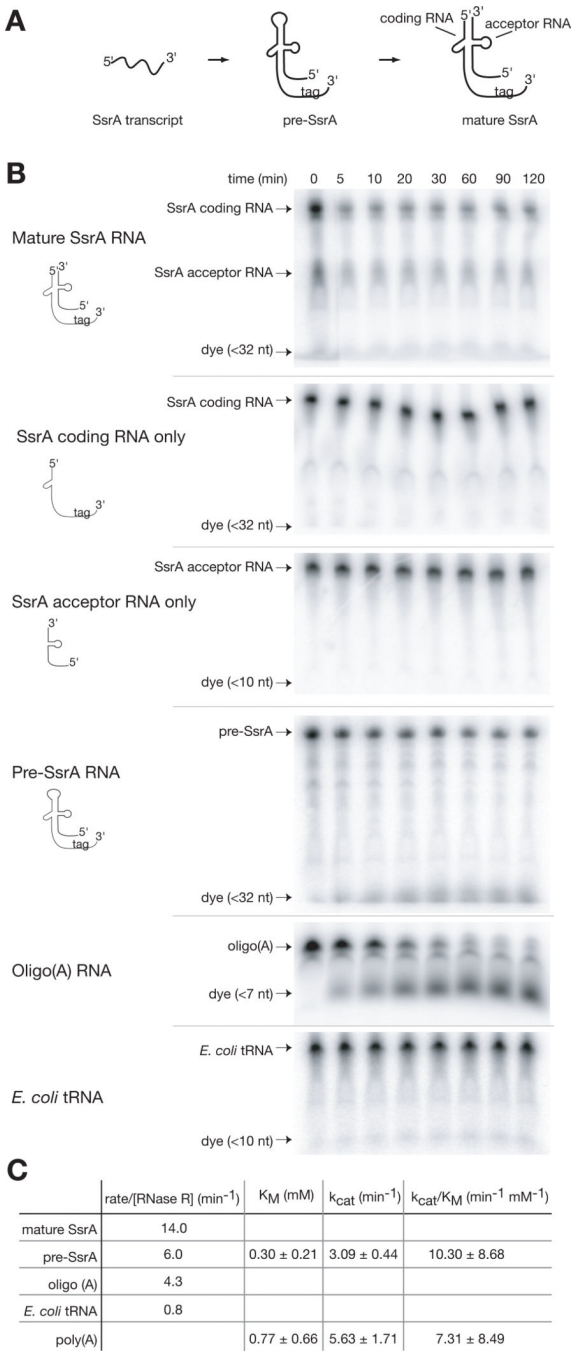

	rate/[RNase R] (min^{-1})	K_M (mM)	k_{cat} (min^{-1})	k_{cat}/K_M (min^{-1} mM^{-1})
mature SsrA	14.0			
pre-SsrA	6.0	0.30 ± 0.21	3.09 ± 0.44	10.30 ± 8.68
oligo (A)	4.3			
E. coli tRNA	0.8			
poly(A)		0.77 ± 0.66	5.63 ± 1.71	7.31 ± 8.49

Figure 1.

RNase R activity *in vitro*. (A) Cartoon depicting forms of SsrA RNA in *C. crescentus*. The *ssrA* gene is transcribed as a single RNA, pre-SsrA, in which the tRNAlike 5' and 3' ends are connected by an internal loop. Excision of the internal loop produces mature SsrA composed of a coding RNA and an acceptor RNA. (B) RNase R degradation of 5' [^{32}P]-labeled RNA substrates was analyzed on polyacrylamide-urea gels, and representative gels are shown. The mobilities of intact RNA molecules and the dye front are indicated, and the approximate RNA size expected to comigrate with the dye is noted in parentheses. (C) Rates and kinetic parameters for RNase R degradation of RNAs *in vitro*. Rate/[RNase R] was calculated based on the fraction of RNA that remained after 5 min incubation with RNase R

in assays as shown in panel B. K_M and k_{cat} were determined from TCA precipitation assay data by non-linear curve fitting of plots of initial rate *versus* substrate concentration.

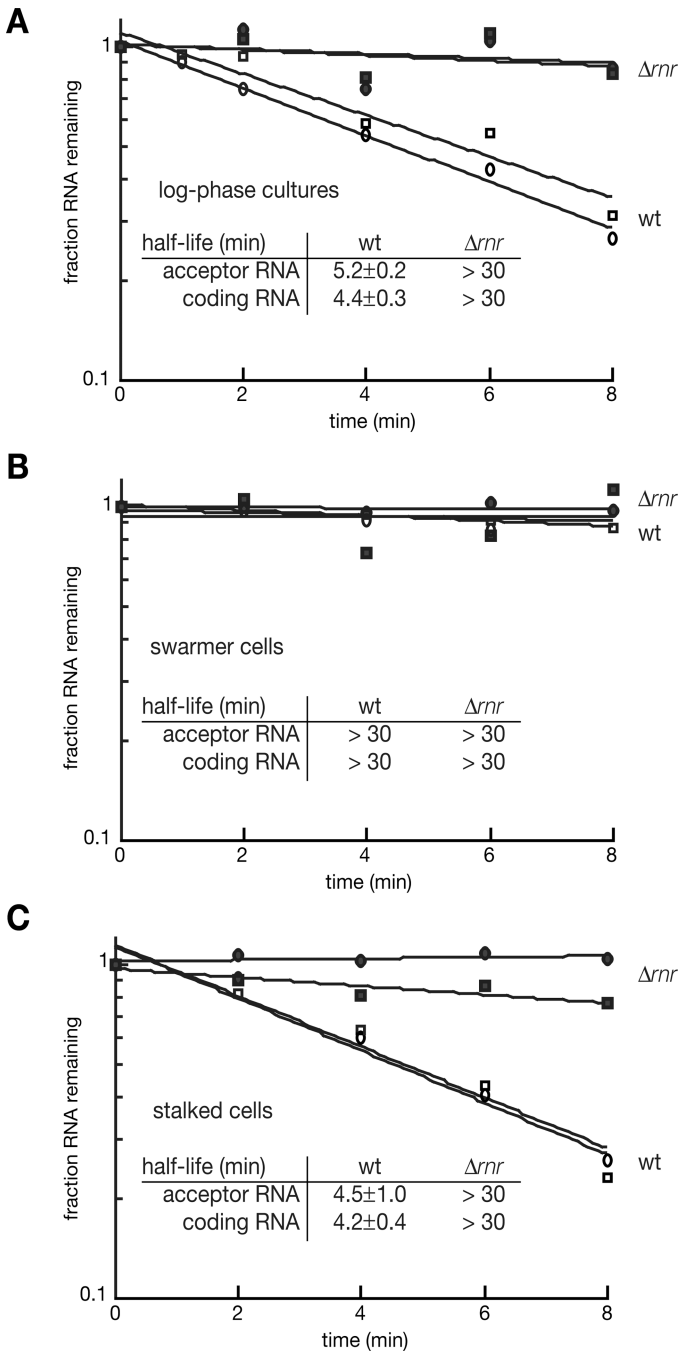

Figure 2.

Degradation of SsrA RNA in wild-type and Δ*rnr* strains. (A) The decay of SsrA coding RNA (squares) and SsrA acceptor RNA (circles) in log-phase cultures of Δ*rnr* (filled symbols) was assayed by inhibiting transcription and measuring the loss of SsrA RNAs by Northern blotting. Half-lives of SsrA RNAs were obtained by fitting the data to single exponential functions. Assays were repeated in pure populations of swarmer cells (B) and stalked cells (C). Published data (Keiler and Shapiro, 2003b) from wild type (open symbols) are shown for comparison.

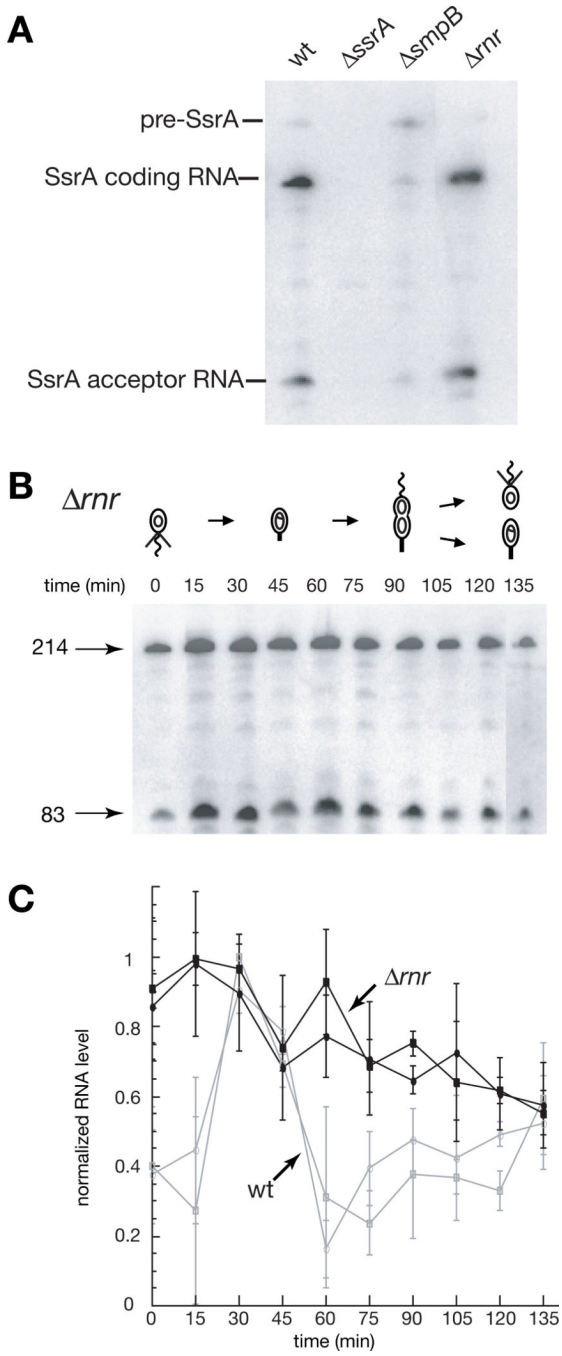

Figure 3.

Expression of SsrA RNA in the Δ*rnr* strain. (A) Equal amounts of total RNA 25 isolated from the wild-type (wt), Δ*ssrA*, Δ*smpB*, and Δ*rnr* strains were analyzed by Northern blots probed for SsrA RNA. A representative Northern blot with bands corresponding to pre-SsrA RNA, SsrA coding RNA, and SsrA acceptor RNA is shown. Results for wt, Δ*ssrA*, and Δ*smpB* are from published data (Keiler and Shapiro, 2003a). (B) A representative Northern blot of total RNA isolated from synchronized cultures of the Δ*rnr* strain probed for SsrA RNA. The stages of the cell cycle are indicated schematically (top), and arrows indicate the bands corresponding to the SsrA coding RNA and the SsrA acceptor RNA. (C) The amounts of SsrA coding RNA (squares) and SsrA acceptor RNA (circles) from 3 independent

experiments as in panel B were quantified and normalized to the peak level of SsrA RNA. Published data (Keiler and Shapiro, 2003b) from wild type (open symbols) are shown for comparison.

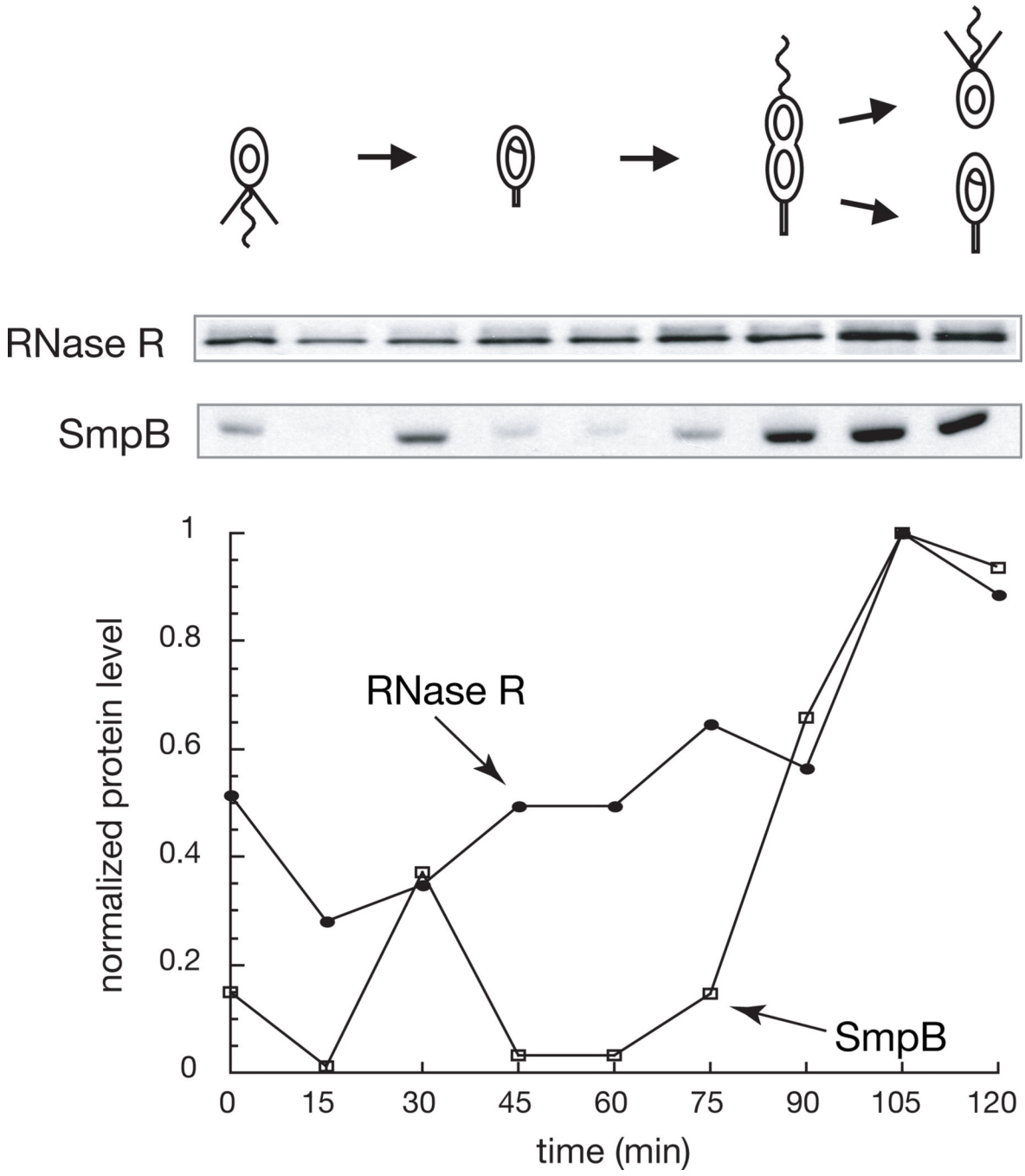

Figure 4.
Cell-cycle regulation of RNase R and SmpB protein levels. Steady-state protein levels were determined in synchronized cultures by Western blots probed with antibodies specific to RNase R or SmpB. A representative Western blot for each protein is shown. The stages of the cell cycle are indicated schematically (top). Western blots were quantified, normalized to the 105 min time point, and the average of at least 3 experiments was plotted versus time after synchronization.

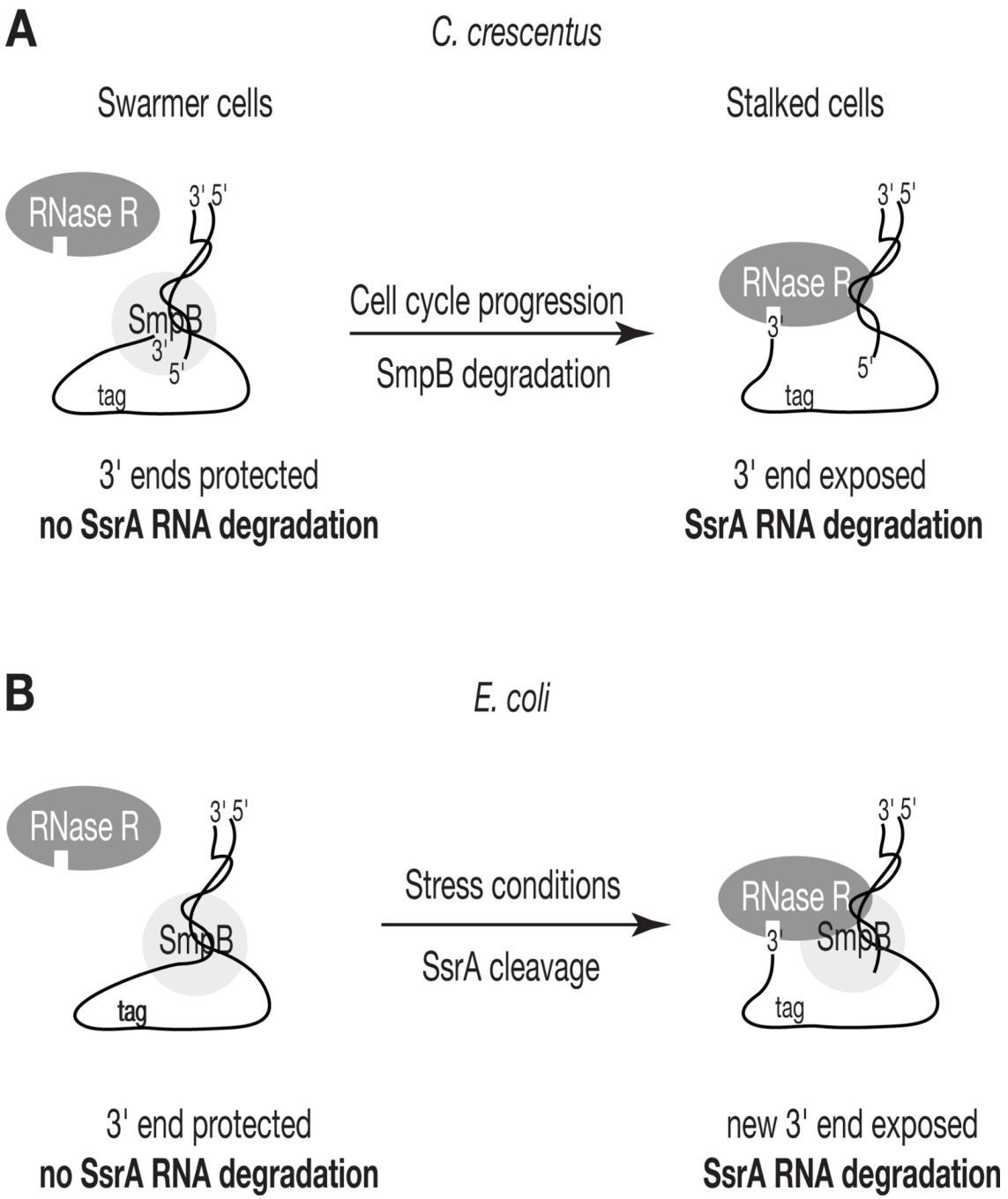

Figure 5.

Model for regulation of SsrA RNA by RNase R. (A) *C. crescentus* SsrA RNA is abundant in swarmer cells because RNase R cannot recognize either the tRNA-like 3' - end of the SsrA acceptor RNA or the 3'-end of the SsrA coding RNA. The 3' -end of the coding RNA may be protected by SmpB. In stalked cells, SmpB is absent and the 3'-end of SsrA coding RNA is exposed, resulting in recognition and degradation by RNase R. (B) *E. coli* SsrA RNA contains only a tRNA-like 3'-end that is not accessible to RNase R. If SsrA RNA is damaged or cleaved under stress conditions, the new 3'-end may be recognized by RNase R. Unfolding of the tRNA-like acceptor arm may lead to degradation by RNase R in either species.

Table I

Growth parameters of wild type and Δrnr strains.

	wild type	Δ*rnr*
doubling time in PYE medium[1]	102 ± 4	110 ± 5
doubling time in M2G medium[1]	153 ± 11	173 ± 11
initiation of DNA replication[2]	22 ± 3	28 ± 2
length of S-phase[3]	90	90
loss of motility[4]	15–30	15–30
cell division[4]	135–150	135–150
CtrA degradation/synthesis[5]	16 / 76	22 / 83
McpA degradation/synthesis[5]	40 / 91	42 / 98

[1]The doubling time (min) during exponential growth with the standard deviation.

[2]Time (min) at which 50% of cells have initiated DNA replication as assayed by flow cytometry after treatment of cultures with rifampicin.

[3]Time (min) required after initiation for the average DNA content to reach the level corresponding to two chromosomes.

[4]Time (min) of cell division and loss of motility are estimated from light microscopy study of synchronized cultures as interval during which more than 80% of the cells lost motility or divided.

[5]Time (min) of protein degradation/resynthesis monitored by Western blotting.

Table II

SmpB specifically inhibits RNase R degradation of SsrA RNA.

substrate	pre-SsrA RNA						oligo(A) RNA		
RNase R	−	+	−	+	+	+	−	+	+
SmpB (μM)	0	0	2.5	2.5	10.0	20.0	0	0	10
% activity[1]	0	100	0	31	10	0	0	100	100

[6]The counts released from 5' [^{32}P]-labeled substrates after 2 h incubation with RNase R expressed as a percentage of the value in the absence of SmpB for each substrate.