6S RNA Function Enhances Long-Term Cell Survival

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6S RNA was identified in *Escherichia coli* >30 years ago, but the physiological role of this RNA has remained elusive. Here, we demonstrate that 6S RNA-deficient cells are at a disadvantage for survival in stationary phase, a time when 6S RNA regulates transcription. Growth defects were most apparent as a decrease in the competitive fitness of cells lacking 6S RNA. To decipher the molecular mechanisms underlying the growth defects, we have expanded studies of 6S RNA effects on transcription. 6S RNA inhibition of σ^{70}-dependent transcription was not ubiquitous, in spite of the fact that the vast majority of σ^{70}-RNA polymerase is bound by 6S RNA during stationary phase. The σ^{70}-dependent promoters inhibited by 6S RNA contain an extended −10 promoter element, suggesting that this feature may define a class of 6S RNA-regulated genes. We also discovered a secondary effect of 6S RNA in the activation of σ^{S}-dependent transcription at several promoters. We conclude that 6S RNA regulation of both σ^{70} and σ^{S} activities contributes to increased cell persistence during nutrient deprivation.

For many years, it was thought that the wealth in number and function of untranslated small RNAs (sRNAs) was unique to eukaryotes. However, searches for sRNA genes in *Escherichia coli*, together with growing insight into their functions, have revealed that sRNAs are numerous, integral components of bacterial metabolism (17, 20, 33, 44, 45). The sophistication of bacterial sRNAs is exemplified by those that regulate gene expression, often providing critical changes in response to diverse environmental growth conditions (33, 45). For example, Spot42 RNA and CsrB RNA have roles during growth on various carbon sources (37, 42), and RyhB RNA responds to changes in iron availability (32). Other sRNAs respond to more immediate stresses, including hydrogen peroxide (OxyS RNA) (3), cold (DsrA RNA) (28, 41), and osmotic shock (RprA RNA and DsrA RNA) (30, 31).

6S RNA was the first sRNA to be sequenced >30 years ago (8). 6S RNA is processed out of a dicistronic transcript (22), although the mechanism for processing is unknown. The function of *ygfA*, an open reading frame within the 3′ end of the transcript, is also unknown. It was not until the discovery that 6S RNA interacts with RNA polymerase (RNAP) that the first hints of the 6S RNA function were revealed (47). 6S RNA interacts specifically with the σ^{70}-containing form of RNAP ($E\sigma^{70}$), where the RNA makes direct contact with the σ^{70} subunit. The 6S RNA accumulates to high levels during stationary phase, at which time >75% of $E\sigma^{70}$ is complexed with 6S RNA (47). Therefore, the 6S RNA-RNAP complex is the predominant form of $E\sigma^{70}$ in cells following their transition into stationary phase.

Bacterial RNAP is a multisubunit enzyme composed of β, β′, ω, and two α subunits, in addition to a σ subunit required for transcription initiation. Many RNAP interactions with promoter DNA are well defined based on >3 decades of biochemical, genetic, and biophysical analyses, including several high-resolution structures of RNAP (reviewed in references 6, 38, and 49). σ subunits are key players in the recognition and binding of RNAP to promoters during transcription initiation and directly mediate several of the interactions between RNAP and DNA (18). At specific promoters, the contribution of each individual RNAP-DNA interaction to overall transcription efficiency, especially in combination with *trans*-acting factors, is variable and allows intricate control of the expression of individual promoters with or without *trans*-acting factors. For instance, promoters with "extended −10" sequence elements contain an additional conserved sequence (TGN) immediately upstream of the −10 element and exhibit decreased dependence on the −35 promoter element (7, 9, 36).

In addition to the constitutively abundant σ^{70}, *E. coli* contains six sigma factors, many of which function in response to environmental cues (18). σ^{S} is an important regulator for establishing and surviving stationary phase (19, 23). σ^{S} remains at least 3-fold less abundant than σ^{70}, and σ^{70}-dependent transcription is down-regulated at least 10-fold in stationary phase (24). $E\sigma^{70}$ and $E\sigma^{S}$ activities respond differently to chemical environments, DNA supercoiling, modification of RNAP, and *trans*-acting proteins (19, 23), suggesting that these variables contribute to relative $E\sigma^{S}$ and $E\sigma^{70}$ activities in stationary phase. Other studies support direct competition in which σ^{S} outcompetes σ^{70} for core binding (11, 27); however, questions regarding relative abundances and core affinities suggest that the full complexity of σ^{70} versus σ^{S} regulation remains unknown.

During the transition from exponential to stationary phase, cells undergo a variety of morphological and physiological changes that assist them in survival as preferred nutrient pools are depleted (14, 15, 19, 39). Upon continued incubation, *E. coli* is able to survive for exceptionally long periods (years) without input of exogenous nutrients (reviewed in references 14 and 15). It has been shown that long-term survival is due in part to population heterogeneity arising from genetic mutations that confer increased fitness on dominant subpopulations of cells until other, more competitive mutants arise. This process of "waves of takeover" of cells with increased fitness has

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TABLE 1. Bacterial strains

Strain name	Genotype	Reference
ZK1142	W3110 ΔlacU169 tna-2 [Nal^r]	51
KW370	ZK1142 ssrS1 [Nal^r Amp^r]	This work
ZK1143	W3110 ΔlacU169 tna-2 [Str^r]	51
KW371	ZK1143 ssrS1 [Str^r Amp^r]	This work
KW72	Laboratory wild-type strain background	47
GS075	KW72 ssrS1 [Amp^r]	47
RLG3499	MG1655 pyrE^+ lacI lacZ [VH1000]	16
KW372	RLG3499 λrsdP2(−149+91)-lacZ	This work
KW373	KW372 ssrS1	This work
RLG4998	RLG3499 λlacUV5(−59+36)-lacZ	5
KW234	RLG4998 ssrS1	This work
RLG6358	RLG3499 λrrnBP1(−41+1)-lacZ	21
KW238	RLG6358 ssrS1	This work
RLG6641	RLG3499 λ_i^21 lambdaP_R(−40+20)-lacZ	4
KW325	RLG6641 ssrS1	This work
RLG5079	RLG3499 λRNA1(−60+1)-lacZ	M. M. Barker and R. L. Gourse, unpublished data
KW321	RLG5079 ssrS1	This work
KW374	RLG3499 λgalP1(−94+45)(−19G-T)-lacZ	This work
KW375	KW374 ssrS1	This work
KW376	RLG3499 λgalP2(−89+50)(−37C-T)-lacZ	This work
KW377	KW376 ssrS1	This work
RLG3760	RLG3499 λbolA1(−54+16)-lacZ	16
KW378	RLG3760 ssrS1	This work
RLG5852	RLG3760 rpoS::Tn10	16
KW380	KW378 rpoS::Tn10	This work
RLG5852	RLG3499 λcc-35con(−40+2)-lacZ	16
KW381	RLG5852 ssrS1	This work
RLG5857	RLG5852 rpoS::Tn10	16
KW383	KW381 rpoS::Tn10	This work
KW384	RLG3499 λhya (−279+27)-lacZ	This work
KW385	KW384 ssrS1	This work
KW386	KW384 rpoS::Tn10	This work
KW387	KW385 rpoS::Tn10	This work
KW388	RLG3499 λappY(−685+12)-lacZ	This work
KW389	KW388 ssrS1	This work
KW390	KW388 rpoS::Tn10	This work
KW391	KW389 rpoS::Tn10	This work
KW392	RLG3499 λrsdP1(−95+33)-lacZ	This work
KW393	KW392 ssrS1	This work
KW394	KW392 rpoS::Tn10	This work
KW395	KW393 rpoS::Tn10	This work
SG30013	MG1655 Δlac λrpoS-lacZ	46
KW396	SG30013 ssrS1	This work

been called the growth advantage in stationary phase (GASP) phenotype (50).

We show here that 6S RNA function leads to increased cell survival during nutrient deprivation. In stationary phase, 6S RNA interaction with RNAP leads to inhibition of σ^{70}-dependent transcription with a high degree of promoter specificity. Additionally, cells containing 6S RNA have increased σ^S-dependent transcription, although this increase is likewise promoter specific. We have established 6S RNA as a gene-specific trans-acting regulator of transcription, as well as an additional player involved in control of the relative utilization of σ^{70} and σ^S during stationary phase. We suggest that a combination of inappropriate σ^{70} and σ^S activities in 6S RNA-deficient cells is responsible for their decreased ability to survive extended nutrient deprivation.

MATERIALS AND METHODS

Strains and plasmids. Strains were grown in Lennox Luria broth (LB) or M9 minimal medium supplemented with 0.4% glucose and 0.002% vitamin B$_1$ (43).

LB was unsupplemented unless otherwise indicated. For buffered LB, a final concentration of 100 mM MOPS [3-(4-morpholino)propanesulfonic acid], pH 7.0, was used. Ampicillin (50 μg/ml), chloramphenicol (25 μg/ml), tetracycline (10 μg/ml), streptomycin (10 μg/ml), or nalidixic acid (15 μg/ml) was added when needed, as indicated. All cultures were grown on rotating platform shakers at ∼250 rpm. Cell culture volumes of 5 to 10 ml were grown in 16-mm-long tubes shaken at a 45° angle to the platform shaker; cell culture volumes of 50 ml were grown in 250-ml flasks.

P1 transductions were carried out as described previously (43) to move ssrS1 (29) and rpoS::Tn10 (2) into various strain backgrounds. Although the 6S RNA null mutation used here was originally called ssr1 (29), for clarity we will refer to it as ssrS1 (since the 6S RNA-encoding gene is ssrS). See Table 1 for the names and genotypes of the strains used. pKK* and pKK-6S* are an empty vector and a 6S RNA-expressing vector, respectively (47). For single-copy chromosomal reporter constructs, promoter regions were amplified by PCR from genomic DNA and cloned into pMSB1, followed by generation of λ phage lysogens using system II as described previously (40). Two previously constructed promoter-lacZ fusions (RLG6641 and RLG4418) were generated using λ system I (40). The extents of the promoter regions examined are indicated as numbers of nucleotides upstream and downstream of the +1 transcription start site (Table 1). For appY-lacZ and hya-lacZ, the distances are from the predicted start sites (1, 34). The galP1 and galP2 constructs contain point mutations to allow analysis of each promoter individually (Table 1) (10).

Competition assay. For standard competition experiments (see Fig. 1), approximately equal numbers of cells from fresh overnight cultures of each cell type were used to inoculate a 5-ml culture, similar to methods used to assess the relative cell fitness of various mutants (13, 48). Overnight cultures were diluted to an optical density at 600 nm (OD_{600}) of 0.05 ($\sim 5 \times 10^7$ cells), which was generally an $\sim 1:100$ dilution. The actual number of input cells was determined by plating of dilutions from the inoculated culture (zero time) to verify OD_{600} measurements, as it was imperative to know the precise starting number of each cell type. For ≤ 24-h cultures, we found good agreement between the cell numbers determined by OD_{600} and plating assays. Cocultures were grown at 37°C, and the number of each cell type was determined by plating, usually once per day for 5 days.

In competition experiments, two strain backgrounds (ZK1142 and ZK1143) differing only in distinguishable drug markers, Nal^r and Str^r, respectively, were typically used so that cell populations could be detected independently by plating them on nalidixic acid- or streptomycin-containing plates. For example, in competitions between KW371 and ZK1142, colonies formed on streptomycin-containing plates indicated the number of KW371 cells in the culture, while colonies formed on nalidixic acid plates indicated the number of ZK1142 cells. We did not detect any difference in growth or survival of the marked strain backgrounds (ZK1142 and ZK1143) when they were cultured alone or in direct competition (data not shown) as previously reported (51). In addition, all competition experiments were done in both combinations of strain backgrounds (i.e., ZK1143 versus KW370 and ZK1142 versus KW371) with identical results. To test any strain background variations, and in particular to examine growth phenotypes in the cells previously used for 6S RNA molecular and biochemical experiments (47), competition experiments also were done using GS075 (ssrS1) and KW72 (wild type). KW72 does not carry a drug resistance gene; therefore, viable-cell counts were monitored by plating cells on LB-ampicillin plates (ssrS1 cells) and LB plates (wild-type plus ssrS1 cells), and wild-type cell numbers were inferred as the difference.

Changes to this general protocol included inoculation with unequal numbers of competing cells (10:1 or 100:1). Once again, the number of cells used for inoculation was based on OD_{600} readings but also verified by plating to determine the actual input cell numbers. Cocultures were initiated by mixing equal numbers of cells from stationary-phase cultures (24 to 48 h) without dilution in fresh medium. Conditioned medium from 24-h cultures was removed from the cells by centrifugation (3,000 $\times$ g; 10 min at 4°C) or by killing cells in medium by heating them at 65°C for 2 h (12), with similar results. Conditioned media were free of living cells as determined by plating.

For competition experiments using different-age cultures (see Fig. 3), the same number of each cell type was used to inoculate cocultures ($\sim 5 \times 10^7$ cells). The cell density of the 10-day cultures was estimated to be the same as the cell density of the cultures determined at 9 days by plating. The cell density of 1-day cultures was estimated from the OD_{600} reading. Actual input cell numbers were determined by plating of the inoculated cultures at time zero. Note that these competition experiments were initiated with equal numbers of cells to more closely resemble our standard competition experiments, which is in contrast to the original experiments describing GASP mutants, in which aged cells were a minority population (51).

Plating assay. For each time point, 10 to 40 μl was removed from the cocultures, serially diluted in M9 salts, and spread on LB plates containing the appropriate antibiotics (see above), and the number of viable cells was determined as the number of colonies formed. Plates from dilutions that gave ~ 100 to 250 colonies per plate were used to minimize statistical variation due to small sample sizes. These numbers allow accuracy down to at least twofold changes. Typically, three to five replicates of each coculture were done per experiment, and experiments were repeated a minimum of three times. The experiments exhibited a high degree of reproducibility, so that the standard deviation was too small to be easily visible when graphed on a log scale (see Fig. 1 to 3).

Long-term growth. To monitor cell persistence, colonies from fresh plates were used to inoculate 50- or 10-ml cultures in LB. The cells were grown at 37°C on a continuously shaking platform. Viable-cell counts were determined periodically by plating cells onto LB plates (without antibiotics). Sterile water was added to cultures as needed to combat evaporation with no apparent immediate effect on cell survival. For example, in one experiment (see Fig. 2), water was added in the following manner to maintain a constant volume of 50 ml: 5 ml at 99 days, 8 to 12 ml at 135 days, and 7 to 10 ml at 170 days.

The overall cell survival, especially the timing and pattern of changes in cell counts, was very reproducible from experiment to experiment with no detectable dependence on the initial culture volumes (10 or 50 ml) or the different strain backgrounds reported here (ZK1142, ZK1143, or KW72) (data not shown). Our long-term patterns of cell survival are similar to published growth patterns of the

ZK1142, ZK1143, or parental ZK126 strain (13, 15, 48, 51), although minor variations in the exact timing and extent of cell density decreases were observed, probably due to slight differences in aeration of cultures, medium preparation, or general laboratory environments beyond our control over the time course of this type of experiment.

β-Galactosidase assays. β-Galactosidase activity assays were done at 30°C as previously described, and activity is expressed in Miller units (ΔOD_{420} per min per OD_{600} unit) (35). Cells were grown from a single colony in LB medium at 30°C for 24 h, diluted 1:100 in fresh LB medium, and assayed after continued growth for 18 h at 30°C. At least three independent cultures per strain were used for each experiment, and experiments were repeated at least three times. When cells contained a plasmid (Fig. 4A), LB supplemented with chloramphenicol was used.

Primer extension assays. RNA was isolated from cells grown for 18 h at 30°C (the same as for β-galactosidase activity) using Trizol reagent (Invitrogen) as previously described (47). Primer extension was done using SuperscriptII (Invitrogen) according to the manufacturer's protocol for first-strand synthesis, except that 10 μg of total RNA and 2 ng of oligonucleotide (AGGGTTTTCC CAGTCACGACGTTG), after 5'-end labeling with polynucleotide kinase (New England Biochemicals) and [γ-^{32}P]ATP (46), were used per reaction. Extension products were separated on 8% polyacrylamide denaturing gels and visualized and quantitated using a Storm 860 PhosphorImager (Molecular Dynamics).

RESULTS

Cells without 6S RNA are at a competitive disadvantage in stationary phase. 6S RNA binds to the majority of Eσ^{70} during stationary phase (47), suggesting a physiological role for 6S RNA at this time. However, growth phenotypes of 6S RNA null cells (ssrS1) have not been detected in entry, exit, or maintenance of stationary phase for up to 10 days (29, 47). In addition, ssrS1 cell morphology is indistinguishable from the wild type in exponential-phase to 24-h cultures as visualized by microscopy (data not shown). Here, we expand on these studies by examining competitive growth and longer periods of nutrient deprivation.

Cocultures were inoculated with equal numbers of ssrS1 and wild-type cells containing neutrally marked chromosomes (KW370 and ZK1143; KW371 and ZK1142) (Fig. 1A) in LB medium at 37°C to address the competitive fitness of ssrS1 cells. The number of each cell type remaining over time was monitored by differential plating (see Materials and Methods). ssrS1 and wild-type cells maintained equal numbers through the first day of competitive growth, but ssrS1 cells decreased 20-fold compared to wild-type cells by 2 to 3 days in coculture and ~ 70-fold after 3 days, indicating that ssrS1 cells are at a disadvantage. ssrS1 cells and wild-type cells cultured independently exhibit indistinguishable growth curves (Fig. 1B). Similar profiles of competitive and noncompetitive growth were seen when cocultures or independent cultures were inoculated with the isogenic ssrS1 and wild-type cells (GS075 and KW72) (data not shown).

To examine whether competitive growth through exponential phase was required for the observed growth defect, cocultures were initiated by mixing equal numbers of ssrS1 and wild-type cells (KW371 and ZK1142) from independent 48-h cultures without addition of fresh medium. ssrS1 cells decreased compared to wild-type cells (data not shown), indicating that the initiation of competition in exponential phase was not necessary. Likewise, specific growth medium was not required, as ssrS1 cells decreased relative to wild-type cells when cocultured in buffered LB medium (pH 7.0) or minimal media (M9-glucose and M9-glycerol) (KW371 and ZK1142; KW370 and ZK1143) (data not shown). To test the possibility that
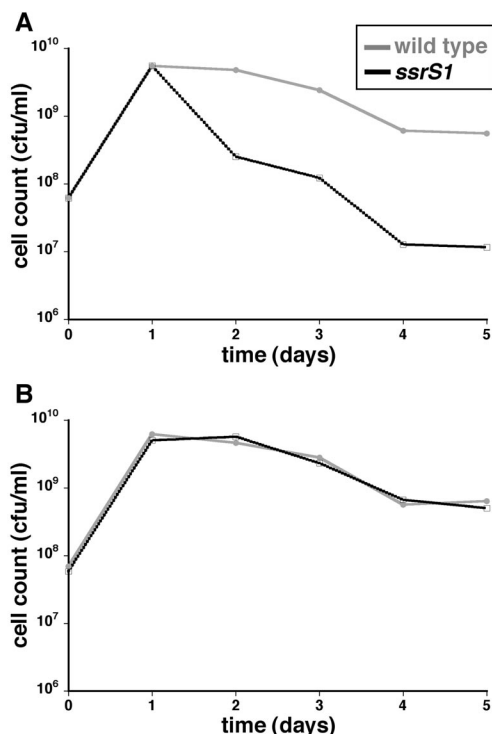
FIG. 1. 6S RNA-deficient cells (*ssrS1*) are at a competitive disadvantage after 2 days of growth in stationary phase. (A) Equal numbers of 6S RNA null cells and wild-type cells were inoculated into LB medium and incubated at 37°C, and viable-cell counts were measured over time by plating. The data presented are averages for five duplicate cultures of KW370 (*ssrS1* Nal^r) cocultured with ZK1143 (wild type, Str^r) and five duplicate cultures of KW371 (*ssrS1* Str^r) and ZK1142 (wild type, Nal^r). Standard deviations were small enough not to be visible on the log scale. (B) 6S RNA-deficient cells and wild-type cells were inoculated into individual cultures (not in competition) and incubated at 37°C, and cell densities were measured over time by plating. The data shown are from a representative experiment containing two duplicates of each cell type: ZK1142, ZK1143, KW370, and KW371.
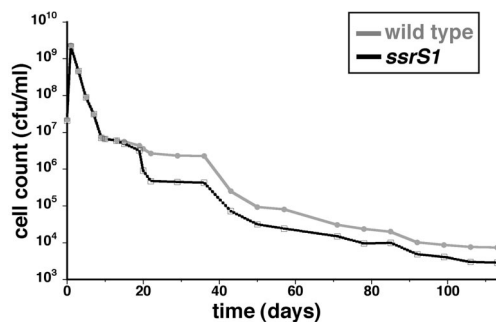
FIG. 2. 6S RNA-deficient cells are defective for persistence after 3 weeks in batch culture. KW72 (wild-type) or GS075 (*ssrS1*) cells were inoculated into LB medium and grown at 37°C without addition of exogenous nutrients. At the times indicated, viable-cell counts were measured by plating. The data shown are from one representative experiment with three duplicate cultures of each strain from 1 to 57 days, at which time one of the *ssrS1* cultures was lost. Therefore, for >57 days, there were two duplicate cultures of *ssrS1* and three of the wild type. Similar patterns of cell persistence were seen with ZK1142, ZK1143, KW30, and KW371 examined from 1 to 60 days in three independent experiments.

6S RNA is required for maximal persistence in stationary phase for >20 days. We next examined *ssrS1* and wild-type cell survival when cells were cultured independently in LB medium at 37°C for months to test if a growth defect in 6S RNA-deficient cells would be revealed at later times (Fig. 2). *ssrS1* (KW370, KW371, or GS075) and wild-type (ZK1142, ZK1143, or KS72) cultures were indistinguishable for at least 2 weeks after the initial inoculation. Viable-cell counts were determined by plating assays and revealed that *ssrS1* cells decreased ~5-fold relative to wild-type cells from 3 to 5 weeks. After 5 weeks, *ssrS1* cells maintained ~3-fold lower cell counts than wild-type cells for many months (we have followed cultures for up to 8 months). Although there were minor variations in absolute cell counts from experiment to experiment, the overall patterns of changes for *ssrS1* and wild-type cells were consistent, especially in the timing and magnitude of changes.

6S RNA-deficient cells maintain the ability to exhibit a GASP phenotype. The long time frame for appearance of *ssrS1* growth defects and their disadvantage in competitive growth led us to consider whether *ssrS1* cells were deficient in acquiring GASP phenotypes (reviewed in references 14 and 15). GASP phenotypes were originally described as the ability of 10-day cultures of wild-type cells to outcompete a 1-day culture of wild-type cells (51). We found that 10-day *ssrS1* cells cocultured with 1-day *ssrS1* cells (KW370$_{10\ day}$-KW371$_{1\ day}$ or KW371$_{10\ day}$-KW370$_{1\ day}$) resulted in the 1-day *ssrS1* cells decreasing in relative abundance, indicating that *ssrS1* cells are capable of GASP behavior (Fig. 3A). For comparison, we examined wild-type cells and found that 10-day wild-type cells efficiently outcompeted 1-day wild-type cells (ZK1142$_{10\ day}$-ZK1143$_{1\ day}$ or ZK1143$_{10\ day}$-ZK1142$_{1\ day}$), similarly to *ssrS1* (Fig. 3B) and as previously reported (51). We next cocultured 10-day *ssrS1* cells with 1-day wild-type cells (KW370$_{10\ day}$-ZK1143$_{10\ day}$ or KW371$_{10\ day}$-ZK1142$_{10\ day}$) and found that the 10-day *ssrS1* cells were not able to overtake the 1-day wild-type cells (data not shown). However, after 5 days of coculture, the *ssrS1*$_{10\ day}$ cells were only 3-fold underrepresented, compared

wild-type cells were making and secreting a factor responsible for decreased fitness of *ssrS1* cells, we compared levels of growth in media conditioned from 24-h cultures. Specifically, cells (GS075 or KW72) collected by centrifugation from a 24-h culture were resuspended in medium removed from a 24-h culture (KW72 or GS075), and the number of cells remaining was monitored over four additional days. *ssrS1* and wild-type cell numbers were indistinguishable from one another whether the cells were grown in *ssrS1*-conditioned medium or wild-type-conditioned medium (data not shown), suggesting that the *ssrS1* disadvantage in competition was not due to a stable factor excreted by wild-type cells.

The *ssrS1* cells were at a disadvantage even when cocultures were inoculated with a 10:1 or 100:1 ratio of *ssrS1* cells to wild-type cells (KW370-ZK1143 and KW371-ZK1142). In each case, wild-type cells gained in relative abundance and reached equal representation with *ssrS1* cells by 2 to 3 days (data not shown). In contrast, wild-type cells inoculated at 100:1 ratios into cocultures (ZK1142-ZK1143 and ZK1143-ZK1142) resulted in the minority cells remaining at least 100-fold underrepresented in the population (data not shown).
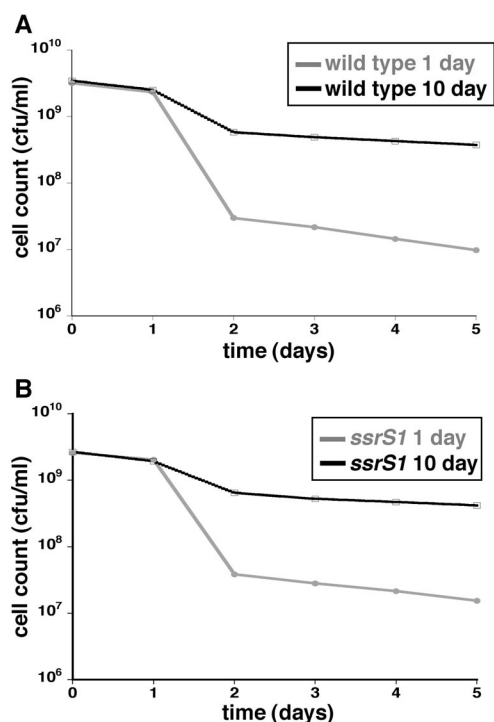
FIG. 3. Both wild-type and *ssrS1* cells exhibit GASP phenotypes; 10-day cultures are able to outcompete 1-day cultures from matched strain backgrounds. Wild-type (A) or *ssrS1* (B) cultures were inoculated into LB medium and grown at 37°C for 10 days. On the ninth day, fresh wild-type or *ssrS1* cultures were inoculated into fresh LB medium so that a 1-day culture and a 10-day culture would be ready at the same time. Equal numbers of 10-day and 1-day cells were mixed into a single culture and grown at 37°C, and viable-cell counts were monitored over time by plating. The data shown are averages from one experiment each with ZK1143 (10 day) versus ZK1142 (1 day) and ZK1142 (10 day) versus ZK1143 (1 day) (A), or three experiments with KW370 (10 day) versus KW371 (1 day) and one with KW371 (10 day) versus KW370 (1 day) (B).
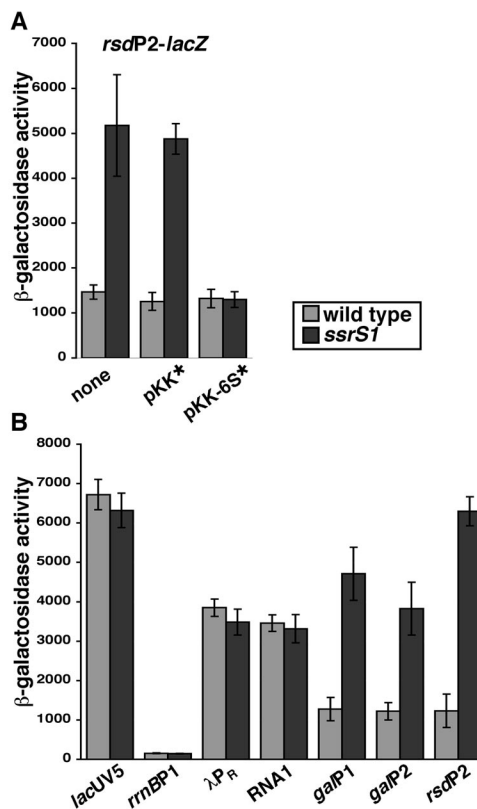

FIG. 4. 6S RNA inhibits transcription from several, but not all, σ^{70}-dependent promoters. The β-galactosidase activities of various promoters were measured in wild-type or *ssrS1* strain backgrounds. (A) Expression from *rsd*P2 at 18 h of growth in LB medium at 30°C in wild-type or *ssrS1* strain backgrounds alone, in the presence of an empty vector (pKK*), or in the presence of a 6S RNA-expressing vector (pKK-6S*). (B) The β-galactosidase activities of several σ^{70}-dependent promoters (*lac*UV5-*lacZ*, *rrn*BP1-*lacZ*, λP$_R$-*lacZ*, RNA1-*lacZ*, *gal*P1-*lacZ*, *gal*P2-*lacZ*, and *rsd*P2-*lacZ*) in wild-type or *ssrS1* strain backgrounds grown for 18 h in LB medium at 30°C were examined. Data are averages (with error bars corresponding to ± standard deviations from the averages) of at least three experiments, with three duplicates per experiment. β-Galactosidase activity is expressed in Miller units.

to ~70-fold for the *ssrS1*$_{1 \text{ day}}$ cells cocultured with 1-day wild-type cells (Fig. 1A).

6S RNA alters transcription from some, but not all, σ^{70}-dependent promoters. It was previously discovered that 6S RNA inhibits σ^{70}-dependent transcription, at least as monitored by expression from the *rsd*P2 promoter of the endogenous *rsd* gene (47) and by expression from *rsd*P2 in vitro (K. M. Wassarman, unpublished data). To probe the extent of 6S RNA inhibition of transcription that might account for the described growth defects, we examined 6S RNA effects on transcription at several different promoters (see below). All promoters examined were single-copy promoter-*lacZ* fusions to allow monitoring of expression over time via β-galactosidase activity. Expression from *rsd*P2-*lacZ* in wild-type cells (KW372) remained constant throughout 24 h of growth. However, in *ssrS1* cells (KW373), expression from *rsd*P2-*lacZ* was similar to that from the wild type in exponential phase but increased upon transition into stationary phase and remained elevated thereafter (data not shown). At 18 h, *rsd*P2-*lacZ* expression was increased by three- to fourfold in *ssrS1* compared to the wild-type strain background (Fig. 4A), which was the

largest difference observed. The three- to fourfold change was comparable to the 6S RNA-dependent change in expression observed from the chromosomal *rsd*P2 promoter using an RNase protection assay (47), and a three- to fivefold change in mRNA generated from the *rsd*P2-*lacZ* reporter in *ssrS1* compared to wild-type strain backgrounds as examined by primer extension (data not shown). Therefore, subsequent experiments examining other promoter activities were done at 18 h. Wild-type expression of *rsd*P2-*lacZ* was restored in *ssrS1* cells by introduction of a 6S RNA-expressing plasmid (pKK-6S*), but not a plasmid control (pKK*), confirming that changes in *rsd*P2-*lacZ* expression were due to changes in 6S RNA levels (Fig. 4A). Western analysis revealed that there was no change in core RNAP subunit or σ^{70} protein levels in *ssrS1* (GS075) compared to the wild type (KW72) over several days of growth (data not shown).

As 6S RNA interacts with the vast majority of Eσ^{70} in
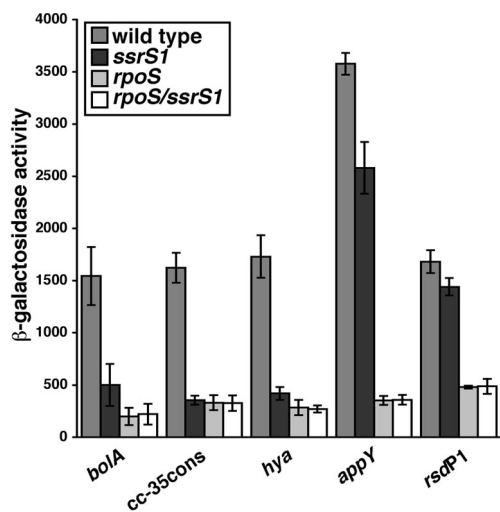
FIG. 5. Presence of 6S RNA leads to activation of some σ^S-dependent promoters. Shown are the β-galactosidase activities of σ^S-dependent promoters (bolA-lacZ, cc-35con-lacZ, hya-lacZ, appY-lacZ, and rsdP1-lacZ) in the wild-type, ssrS1, rpoS, and ssrS1/rpoS strains grown for 18 h at 30°C in LB medium. All data are averages (with error bars corresponding to ± standard deviations from the averages) of at least three experiments, with three duplicates per experiment. β-Galactosidase activity is expressed in Miller units.

stationary phase, the possibility existed that general σ^{70}-dependent transcription would be decreased by 6S RNA at this time. However, expression of several well-studied σ^{70}-dependent promoters (lacUV5-lacZ, rrnBP1-lacZ, λP$_R$-lacZ, and RNA1-lacZ) revealed equivalent levels of expression in ssrS1 and wild-type strain backgrounds at 18 h (Fig. 4B). To ensure that β-galactosidase activity was representative of mRNA levels and thus transcription activity at the times tested, primer extension analysis was done to examine mRNA from lacUV5-lacZ and RNA1-lacZ in wild-type and ssrS1 strain backgrounds at 18 h of growth. In both cases, the levels of extension product for each reporter gene were equal in ssrS1 and wild-type cells, indicating no change in mRNA levels and correlating with the lack of change in β-galactosidase activity. Therefore, 6S RNA does not inhibit transcription of all σ^{70}-dependent promoters in stationary phase.

rsdP2 contains an extended −10 promoter element (26), so we tested additional extended −10-containing promoters (galP1 and galP2) to examine whether this promoter element might define a class of promoters inhibited by 6S RNA. Indeed, expression from galP1-lacZ and galP2-lacZ, which each contain extended −10 elements (7), increased by >3-fold in ssrS1 cells compared to wild-type cells (Fig. 4B).

6S RNA also affects σ^S-dependent transcription. To expand our understanding of potential 6S RNA-dependent alterations in transcription, we next examined whether 6S RNA affects σ^S-dependent transcription, as σ^S is a key regulator during stationary phase when 6S RNA function appears to be most important. We observed that expression from the σ^S-dependent promoter, bolA-lacZ, was decreased in ssrS1 cells by three- to fourfold compared to wild-type cells, suggesting that the presence of 6S RNA activated its transcription (Fig. 5). Expression of 6S RNA from a plasmid (pKK-6S*) but not a

plasmid control (pKK*) restored wild-type expression (data not shown). Similarly, expression from the σ^S-dependent promoters cc-35con-lacZ (an artificial promoter [16]) and hya-lacZ was decreased in the ssrS1 strain background. However, another σ^S-dependent promoter, appY-lacZ, exhibited a smaller, if any, decrease in the ssrS1 strain background. Previously, the expression from the endogenous rsdP1 (a σ^S-dependent promoter) had been found to be insensitive to the presence of 6S RNA (47), although the experiments reported may not have been sensitive to small changes, as they were designed to evaluate the relative utilization of the σ^{70}-dependent promoter (rsdP2) compared to the σ^S-dependent promoter (rsdP1). Reexamination of this promoter via expression from rsdP1-lacZ showed that levels of expression are comparable in ssrS1 and wild-type strains (Fig. 5), consistent with earlier findings. Expression from bolA-lacZ, cc-35con-lacZ, hya-lacZ, appY-lacZ, and rsdP1-lacZ was dramatically reduced in rpoS strain backgrounds, confirming their σ^S dependence (Fig. 5).

To determine whether changes in σ^S-dependent transcription might reflect changes in σ^S protein levels in ssrS1 strain backgrounds, expression of rpoS was examined in ssrS1 and wild-type backgrounds from an rpoS-lacZ operon fusion reporter. No difference in activity was detected, indicating that transcription of rpoS was not altered by 6S RNA (data not shown). In addition, Western analysis of σ^S protein in ssrS1 (GS075) and wild-type strain backgrounds (KW72) revealed that σ^S protein levels were comparable (data not shown) and indicated that observed changes in σ^S-dependent transcription were not a result of changes in σ^S protein levels.

DISCUSSION

We have identified a growth phenotype for 6S RNA-deficient cells: they are at a competitive disadvantage compared to wild-type cells after 2 days of growth, and they exhibit decreased persistence in stationary phase after ~3 weeks of growth when not in competition. Given that 6S RNA interacts with RNAP and alters transcription, we speculate that these growth defects result from 6S RNA-dependent changes in gene expression. We have determined that σ^{70}-dependent promoters are not equally inhibited by 6S RNA in spite of the fact that the majority of Eσ^{70} is bound by 6S RNA in stationary phase. A common feature of σ^{70}-dependent promoters inhibited by 6S RNA identified here is an extended −10 promoter element. In addition to the direct effect of 6S RNA on Eσ^{70}, we have discovered that Eσ^S activity is activated when cells contain 6S RNA, although 6S RNA does not interact directly with Eσ^S (47; Wassarman, unpublished). We conclude that growth defects of 6S RNA-deficient cells most likely result from the inappropriate control of both σ^{70}- and σ^S-dependent gene expression.

Loss of 6S RNA leads to decreased survival compared to wild-type cells. When grown independently, 6S RNA null and wild-type cells appear to enter stationary phase and maintain stationary phase for many days indistinguishably from one another; however, when they are grown in direct competition, the 6S RNA null cells are at a distinct disadvantage within 2 days. The mechanism responsible for the decreased fitness of 6S RNA-deficient cells is expected to occur during stationary

phase, as competitive growth in exponential phase is not required for the observed defects. It is also possible that the timing of the growth phenotypes represents a time frame of cell sensitivity to defects established earlier. The loss of competitive fitness is not due to a stable factor secreted from wild-type cells, as *ssrS1* cells were unaffected by medium conditioned by wild-type cells at a time when mixture with the full wild-type culture is deleterious. One hypothesis for the direct consequences of competitive growth is that *ssrS1* cells are unable to efficiently compete for the remaining resources.

6S RNA null cells also are defective in long-term persistence (>20 days) when grown independently. We observed substantial 6S RNA-dependent changes in transcription by 18 h, raising important questions as to why growth defects are delayed. One possibility is that the timing of the growth defects represents sensitivity to environmental conditions at that time but that this sensitivity was established much earlier as a consequence of altered gene products. For instance, if a primary nutrient source at 3 weeks of growth cannot be properly utilized by *ssrS1* cells, this could account for the decrease in cell numbers. However, *ssrS1* cells are able to persist similarly to wild-type cells in conditioned medium prepared from 20-day wild-type cells (A. E. Trotochaud and K. M. Wassarman, unpublished data), suggesting that this is probably not the case.

A second possibility is that the decrease in *ssrS1* cell numbers at 20 days might be due to the presence of a deleterious by-product of metabolism that would normally not be generated or that cannot be properly disposed of in the *ssrS1* cells. Alternatively, the *ssrS1* cells may be less efficient at utilization of available nutrients and energy throughout growth, which might result in depletion of these sources earlier, leading to decreased cell densities. In either case, one would predict that 20-day *ssrS1*-conditioned medium would not support cell persistence as well as 20-day wild-type-conditioned medium, and preliminary data support this possibility (Trotochaud and Wassarman, unpublished). Further work is needed to distinguish between the potential presence of deleterious compounds and the absence of comparable nutrients as the cause for decreased cell numbers. It is also intriguing to speculate whether the decrease in numbers of *ssrS1* cells at ~3 weeks might be for reasons similar to those that normally cause wild-type cells to decrease at ~5 weeks.

A third possibility is that 6S RNA-deficient cells are defective in processes that allow cells to gain fitness by genetic modification. For instance, changes in mutation rates could directly alter the time required for the generation of advantageous mutations. Although we have shown that *ssrS1* cells are capable of generating GASP-phenotypic cells (Fig. 3), this may not reflect the efficiency with which these mutant cells arise and multiply. Further study of the timing and identity of GASP alleles generated between wild-type and *ssrS1* cells may elucidate such differences.

6S RNA alters the transcription of some, but not all, genes in stationary phase. We have discovered that specific σ^{70}-dependent promoters respond distinctively to 6S RNA; some promoters are inhibited, while others remain unchanged. Therefore, 6S RNA is a transcriptional regulator during stationary phase. Our data suggest that one class of promoters inhibited by 6S RNA consists of promoters with extended −10 elements. One proposed model for 6S RNA action is that its

binding to RNAP blocks normal RNAP binding to promoter DNA (45). The predicted 6S RNA structure is largely double stranded with a single-stranded bulged loop in the center, reminiscent of the DNA structure within the open complex. It is intriguing that one characteristic of extended −10 promoters is that they often have poor −35 sequence elements compared to the consensus and have a decreased dependence on the −35 element for recognition and binding of RNAP (7). Perhaps 6S RNA binding to RNAP alters the −10 region recognition or affinity, which cannot be overcome without strong −35 element contacts. Alternatively, 6S RNA-sensitive promoters may use a common *trans*-acting factor that is affected by 6S RNA or may contain additional defined sequences outside the core promoter elements. Identification of specific components responsible for 6S RNA inhibition, as well as further understanding of how 6S RNA alters RNAP physically, await future dissection of individual promoters and 6S RNA modification of RNAP.

6S RNA also can lead to activation of σ^{S}-dependent transcription from several, but not all, tested promoters. 6S RNA does not interact directly with $E\sigma^{S}$ or σ^{S}, as shown by the inability to copurify σ^{S} with 6S RNA from stationary-phase cells when endogenous 6S RNA-$E\sigma^{70}$ complexes are efficiently purified (47). Additionally, interactions between 6S RNA and $E\sigma^{S}$ or σ^{S} are not detectable by cross-linking experiments (47) or by in vitro reconstitution experiments that readily observe 6S RNA-$E\sigma^{70}$ complexes (Wassarman, unpublished). Therefore 6S RNA effects on σ^{S}-dependent transcription must be indirect. One model is that the effectiveness of σ^{S} competition for core RNAP is normally increased during late stationary phase by the presence of 6S RNA binding to the majority of $E\sigma^{70}$. Another possibility is that there is a *trans*-acting factor that is required for efficient σ^{S}-dependent transcription of sensitive promoters, which is decreased in 6S RNA-deficient cells, presumably through direct changes in σ^{70}-dependent transcription.

Uncovering further characteristics of promoters responsive to 6S RNA inhibition or activation will lead to global identification of sensitive promoters. The next question will be how alterations in gene products controlled by these promoters contribute to decreased persistence of 6S RNA-deficient cells. Already, a potential link exists between 6S RNA function and 6S RNA regulation of *rsd* expression. The Rsd protein is an anti-sigma factor which specifically inactivates free σ^{70} (25), although Rsd levels are not high enough to account for full σ^{70} inhibition during stationary phase. It is possible that 6S RNA and Rsd activities coordinate to reach appropriate levels of σ^{70} activity, in part through 6S RNA regulation of *rsd* transcription. Interestingly, in addition to the upregulation of the σ^{70}-dependent *rsd*P2 promoter, the *rsd*P1 promoter is the only σ^{S}-dependent promoter tested to date that is insensitive to 6S RNA, thus allowing maximal *rsd* expression from both promoters in the absence of 6S RNA. Recently, 106 promoters that contain extended −10 elements have been identified (36). If this promoter feature truly defines a class of 6S RNA-inhibited promoters, future work toward evaluating the overall contribution of 6S RNA alterations in expression of these and other genes may ultimately determine their roles in long-term cell survival.

ACKNOWLEDGMENTS

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