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Role and Regulation of σ^S in General Resistance Conferred by Low-Shear Simulated Microgravity in *Escherichia coli*

S. V. Lynch,[1] E. L. Brodie,[2] and A. Matin[1]*

Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford,[1] and Department of Environmental Science, Policy, and Management, University of California, Berkeley,[2] California

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Life on Earth evolved in the presence of gravity, and thus it is of interest from the perspective of space exploration to determine if diminished gravity affects biological processes. Cultivation of *Escherichia coli* under low-shear simulated microgravity (SMG) conditions resulted in enhanced stress resistance in both exponential- and stationary-phase cells, making the latter superresistant. Given that microgravity of space and SMG also compromise human immune response, this phenomenon constitutes a potential threat to astronauts. As low-shear environments are encountered by pathogens on Earth as well, SMG-conferred resistance is also relevant to controlling infectious disease on this planet. The SMG effect resembles the general stress response on Earth, which makes bacteria resistant to multiple stresses; this response is σ^s dependent, irrespective of the growth phase. However, SMG-induced increased resistance was dependent on σ^s only in stationary phase, being independent of this sigma factor in exponential phase. σ^s concentration was some 30% lower in exponential-phase SMG cells than in normal gravity cells but was twofold higher in stationary-phase SMG cells. While SMG affected σ^s synthesis at all levels of control, the main reasons for the differential effect of this gravity condition on σ^s levels were that it rendered the sigma protein less stable in exponential phase and increased *rpoS* mRNA translational efficiency. Since σ^s regulatory processes are influenced by mRNA and protein-folding patterns, the data suggest that SMG may affect these configurations.

Space flights, space stations, and eventual colonization of space—the core missions of National Aeronautics and Space Administration—entail exposure of humans and microbes to diminished-gravity environments. Since gravity is a permanent feature on Earth and life on this planet evolved in its presence, diminished gravity might affect biological processes. In fact, detrimental effects of space flight on the immune system of astronauts are well documented (5, 20, 33). Studies involving Earth-based systems to simulate microgravity have shown that many factors may contribute to a diminished immune response. Examples include altered proportion of circulating lymphocytes, reduction in peripheral blood mononuclear and erythroid cells, and defective or depressed dendritic T-cell activity (2, 3, 13, 27, 37).

A commonly used Earth-based system to generate a low-shear simulated microgravity (SMG) environment is the high-aspect-ratio vessel (HARV) bioreactor (Synthecon, Inc., Houston, Tex.)(Fig. 1). In the vessel rotated about a horizontal axis, the cells reach a steady-state terminal velocity at which the gravitational force is mitigated by equal and opposite hydrodynamic forces, which include shear, centrifugal, and Coriolis forces. This generates an overall time-averaged gravity of $10^{-2} \times g$ on the cells in culture and is referred to as SMG (7, 9, 29, 34). A HARV vessel rotated about a vertical axis provides a normal gravity (NG) control. Aeration is achieved through a semipermeable membrane at the back of the vessel.

While diminished gravity compromises the human immune system, studies conducted using HARV bioreactors show that it bolsters bacterial virulence. Studies of mice orally infected with SMG-cultured *Salmonella enterica* serovar Typhimurium show that the organism colonizes liver and spleen more effectively and kills mice more rapidly; such cells also exhibit in-

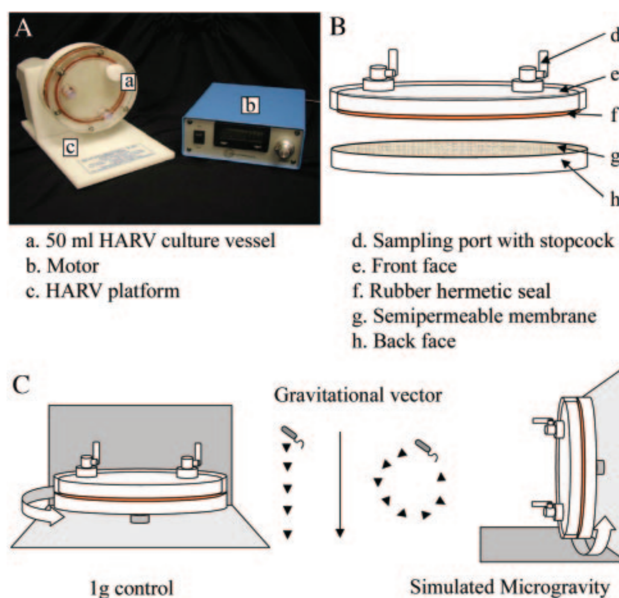

FIG. 1. (A)HARV system used to generate an SMG environment in ground-based investigations (reproduced with permission from Synthecon, Inc., Houston, Tex.). (B) Components of the HARV vessel. (C) Differential rotation of HARV vessels perpendicular to or parallel with the gravitational vector generates a 1-g (control) or SMG environment, respectively.

* Corresponding author. Mailing address: Department of Microbiology and Immunology, D317 Sherman Fairchild Science Building, Stanford University School of Medicine, 299 Campus Dr., Stanford, CA 94305. Phone: (650) 725-4745. Fax: (650) 725-6757. E-mail: a.matin@stanford.edu.

creased resistance to thermal, osmotic, and acid stresses (23, 35). This comprehensive increase of cellular robustness and virulence resembles the general stress response under NG conditions, whereby exposure to one stress, such as starvation, heat shock, or osmotic or oxidative stress, confers resistance against stresses in general (11, 16). This suggests that SMG may activate the general stress response.

The central regulating element of general stress response under NG in proteobacteria is the alternate sigma factor, σ^s (product of the *rpoS* gene). Bacterial cells exposed to individual stresses show increased levels of this sigma factor; the consequent increase in $E\sigma^s$ (RNA polymerase holoenzyme combined to σ^s) results in transcription of genes involved in increased cellular resistance (16).

This investigation was undertaken to determine if SMG confers a generalized stress resistance on *Escherichia coli* as well and the role of σ^s in this phenomenon. We included an examination of stationary growth phase in these studies, as this state is generally more representative of bacterial existence in nature (15, 16, 17); this phase was not examined in previous studies. The results show that SMG does indeed increase *E. coli* resistance in both exponential and stationary phases, making the latter superresistant. Further, while this increase parallels elevated σ^s levels in stationary phase, the opposite is true for exponential phase. We report, moreover, that SMG affects σ^s synthesis at all levels of control.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and β-galactosidase measurement. The bacterial strains used were as follows: *E. coli* AMS6 (K-12 λ^- F^- Δ*lac*; 28) and isogenic mutant strains AMS150 (like AMS6 but *rpoS*::Tn*10*; 19) and AMS171 (λOLS2 Kan^r). The last-mentioned strain contains a *lacZ* transcriptional fusion to the +82 nucleotide of the *pexB* coding region (14).

Bacteria were cultured in pairs of HARV reactors rotated about either a horizontal (SMG conditions) or vertical (NG conditions) axis to determine the effect of SMG. Fifty milliliters of 0.3% glucose–M9 minimal medium was used, which was supplemented with kanamycin (50 μg/ml) when required. Overnight conventional flask cultures grown in glucose-M9 medium served as inocula. The starting A_{660} in the HARV vessels was 0.1, and the incubation was at 37°C and 25 rpm. Under these conditions, as confirmed by counts of viable cells, the HARV cultures reached mid-exponential (A_{660}, 0.4) and stationary (A_{660}, 1.2) phases at 4 and 24 h of incubation, respectively.

β-Galactosidase activity was measured according to the method of Lomovskaya et al. (14) using CRPG as substrate; activity was calculated using the Miller equation (22).

Stress tests. Ten milliliters of exponential- or stationary-phase cultures grown under SMG or NG conditions was mixed with 10 ml of either 200 mM citrate buffer (pH 3.5) or 5 M NaCl (final NaCl concentration, 2.5 M) and incubated at 37°C. Viability was determined by serial dilution on Luria-Bertani plates, as described previously (24).

σ^s concentration and its half-life determination. σ^s was quantified by Western analysis as previously described (30, 38). Cells were mixed with sodium dodecyl sulfate (SDS)-lysis buffer (2% SDS in 0.5 M Tris-HC [pH 6.8]), and protein was quantified by the Bio-Rad Dc assay. Protein loadings were standardized at 75 μg (exponential phase) or 20 μg (stationary phase) of the total cellular protein from NG and SMG cultures, boiled for 3 min, and loaded on an SDS–12.5% polyacrylamide gel (Criterion; Bio-Rad, Hercules, Calif.). These were electroblotted onto Hybond polyvinylidene difluoride membranes, which were blocked overnight at 4°C with 5% nonfat dried milk, and washed (phosphate-buffered saline plus 1% Tween 20). A polyclonal anti-σ^s antibody (30) was used to probe the membranes. Blots were developed with the ECL-Plus system (Amersham, Piscataway, N.J.), and σ^s was quantified by densitometry (Image Quant; Amersham), with a standard curve relating purified σ^s concentration to signal intensity.

To determine σ^s half-life ($\tau_{1/2}{}^{\sigma s}$), chloramphenicol (500 μg/ml) was added to the 50-ml HARV cultures. Samples were removed at indicated intervals, and their σ^s concentration was determined by Western analysis as described above. This method was used in preference to immunoprecipitation because it permitted determination of the protein half-life under HARV conditions; as it halts translation immediately, it permits an accurate half-life measurement (1).

Quantification of *rpoS* mRNA and its half-life determination. Total RNA was isolated from cells grown under the specified conditions with the Masterpure kit (Epicentre, Madison, Wis.), and quantified by A_{260} measurements. Reverse transcription was performed on 1 μg of total RNA from each sample with the Omniscript RT kit (QIAGEN, Valencia, Calif.) and a reverse *rpoS* primer (5′ TTACTCGCGGAACAGCGCTT 3′). Primers for quantitative real-time PCR (qPCR) were designed (Beacon Designer software package; Premier Biosoft, Int.) to amplify a 131-bp internal *rpoS* fragment (*rpoS*629F, 5′ GCCGTATGCT TCGTCTTAAC 3′; *rpoS*759R, 5′ GTCATCTTGCGTGGTATCTTC 3′), and qPCR was performed with an iCycler iQ real-time detection system (Bio-Rad) with the QIAGEN QuantiTect Sybr Green PCR kit. Reaction mixtures (final volume for each, 20 μl) contained 1× QuantiTect MasterMix, 6 pmol of each primer, 1 μl of RT reaction mixture, and DNase-RNase-free water. Following hot start (95°C, 15 min), 40 cycles of 95°C for 15 s, 50°C for 30 s, and 72°C for 20 s were performed. A data acquisition step (81.5°C for 10 s) was used and set above the T_m of potential primer dimers to minimize any Sybr Green absorbance due to the dimers. Melting-curve analyses displayed a single peak at 84.5°C, indicating specific *rpoS* amplification. *rpoS* mRNA was quantified by comparing cycle thresholds to a standard curve (in the range of 10^3 to 10^8 copies), run in parallel, of the PCR-generated full-length *rpoS* gene; regression coefficients for the standard curves were consistently >0.99.

To determine *rpoS* mRNA half-life, 500 μl of rifampin (50 mg/ml dissolved in dimethyl sulfoxide) was added to the 50-ml HARV cultures, and 0.5 ml of samples was removed at the indicated intervals. Samples were processed, and *rpoS* mRNA was quantified by qPCR, following reverse transcription as described above. The copy number was determined and plotted against time on a semilogarithmic plot, and half-life ($\tau_{1/2}{}^{rpoS}$) was determined from the slope of a least-squares linear fit to the plot.

Calculation of σ^s and *rpoS* mRNA synthesis rates and mRNA translational efficiency. These were calculated as described previously (38), using the following equations. To calculate the *rpoS* messenger synthesis rate, where $\tau_{1/2}{}^{rpoS}$ is the *rpoS* mRNA half-life, the following equation was used.

$$K_S{}^{rpoS} = \ln 2(rpoS)/\tau_{1/2}{}^{rpoS} \qquad (1)$$

To calculate the σ^s synthesis rate, where $\tau_{1/2}{}^{\sigma s}$ is the σ^s protein half-life, the following equation was used.

$$K_S{}^{\sigma s} = \ln 2(\sigma^s)/\tau_{1/2}{}^{\sigma s} \qquad (2)$$

To calculate *rpoS* translational efficiency, where $K_E{}^{\sigma s}$ is the number of molecules of σ^s synthesized per copy of *rpoS* mRNA, the following equation was used.

$$K_E{}^{\sigma s} = K_S{}^{\sigma s}/rpoS \qquad (3)$$

RESULTS

There was no significant difference in the growth rate of *E. coli* under NG and SMG conditions in the HARVs with the generation time of ca. 3 h. In both cases, cessation of growth was due to the exhaustion of glucose from the medium. In contrast, the generation time in conventional flask cultures in this medium was 1 h (38), indicating that HARV conditions differed from those in the flask. Nevertheless, between the two differently rotated HARVs, SMG was the primary variable.

The effect of SMG on *E. coli* resistance to two individual stresses—hyperosmosis or low pH—was examined in exponential- and stationary-growth phases, using the wild-type strain AMS6 and the isogenic σ^s-deficient strain AMS150 (19). In exponential phase, while NG-grown cells of both strains showed an almost complete loss of viability upon exposure to 2.5 M NaCl, ca. 50% of the SMG-grown cells survived (Fig. 2A). Upon transition to stationary phase, NG wild-type cells exhibited markedly greater increase in resistance than AMS150 (Fig. 2B). However, while the wild type in this phase showed a further increase in resistance upon cultivation under

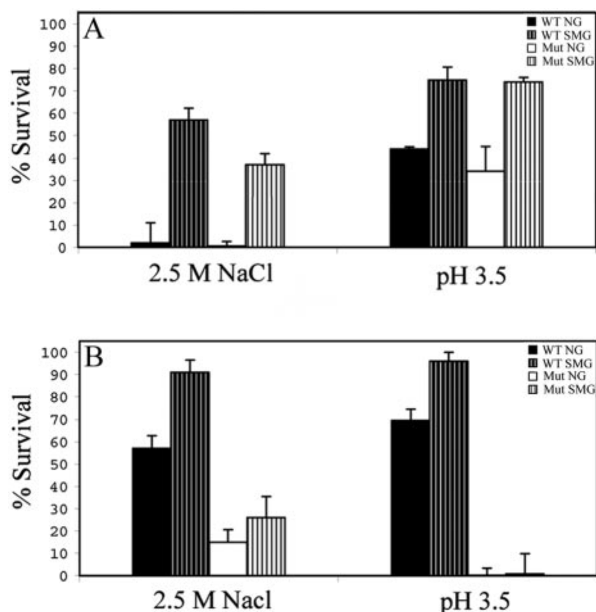

FIG. 2. Percent survival following exposure for 1 h to hyperosmotic (2.5 M NaCl) or acid (pH 3.5) stress in the exponential (A) or stationary (B) phase of growth. WT, wild type (AMS6); Mut, *rpoS* mutant (AMS150). Results represent an average of two independent measurements, each analyzed in triplicate; error bars represent standard errors of the mean. A viability of 100% corresponds to ca. 5×10^7 cells/ml.

SMG, AMS150 did not (Fig. 2B). Note that the small difference exhibited by AMS150 grown under the two gravity conditions is not statistically significant ($P > 0.05$). Exposure to pH 3.5 gave similar results: exponential-phase SMG-grown cells of both strains were ca. twofold more resistant than their NG-grown counterparts (Fig. 2A); but in stationary phase, SMG conferred further resistance only on the wild type, with nearly 100% survival (Fig. 2B). Consequently, the further reinforcement of robustness made SMG-grown stationary-phase wild-type cells almost completely resistant to the two stresses.

Thus, while SMG confers resistance to the two stresses in both growth phases, this resistance is independent of σ^s in exponential phase but dependent on it in the stationary phase. The former situation is the first instance of general stress resistance development in *E. coli* without σ^s involvement.

The changing roles of σ^s in the two growth phases under SMG raised the possibility that, in the wild type, SMG might affect σ^s levels differently in exponential and stationary phases. We therefore quantified the sigma levels in the two phases by Western analysis. In exponential-phase SMG cells, σ^s levels were consistently 30% lower than NG cells (Fig. 3A), but were 100% higher than in NG cells in stationary phase (Fig. 3B). The expression level of a σ^s-dependent gene (*pexB*) served as a further test of σ^s concentration: in both growth phases, the pattern of β-galactosidase synthesis of a *pexB-lacZ* transcriptional fusion strain (AMS171; isogenic with AMS6) (14) paralleled the measured σ^s levels (Fig. 3). Thus, under SMG, the paradigm that increased cellular general resistance parallels increased σ^s levels applied only to the stationary phase and was reversed in exponential phase, since in the latter phase increased cellular resistance coincided with lowered σ^s levels.

The regulation of σ^s under NG conditions is complex and can occur at transcriptional, posttranscriptional, translational, or posttranslational levels, depending on the stress involved (11, 16). To determine what level of control caused SMG to decrease σ^s concentration in one phase of growth while increasing it in the other, we conducted a detailed analysis of the effect of SMG on molecular regulation of σ^s synthesis. The steady-state *rpoS* mRNA copy number was quantified by qPCR. In NG- and SMG-grown cells, this number was nearly identical regardless of the growth phase. Under both gravity conditions, the copy number showed a 10-fold decrease upon transition from exponential to stationary phase (Table 1).

Since steady-state levels of an mRNA could reflect its synthesis rate, stability, or both, the *rpoS* messenger half-life ($\tau_{1/2}^{rpoS}$) was measured (Materials and Methods). As the half-life of the message was the same in exponential phase under the two gravity conditions (Fig. 4A), it follows that the transcriptional rate (equation 1) of the *rpoS* gene (K_s^{rpoS}) in this phase was not affected by SMG (Table 1). This is consistent with the microarray analysis of Wilson et al. (36), who found that SMG growth does not affect *rpoS* transcription in exponential phase. The messenger stability increased in stationary phase under both gravity conditions, but was twofold higher in SMG- than in NG-grown cells (Fig. 4B), indicating that the

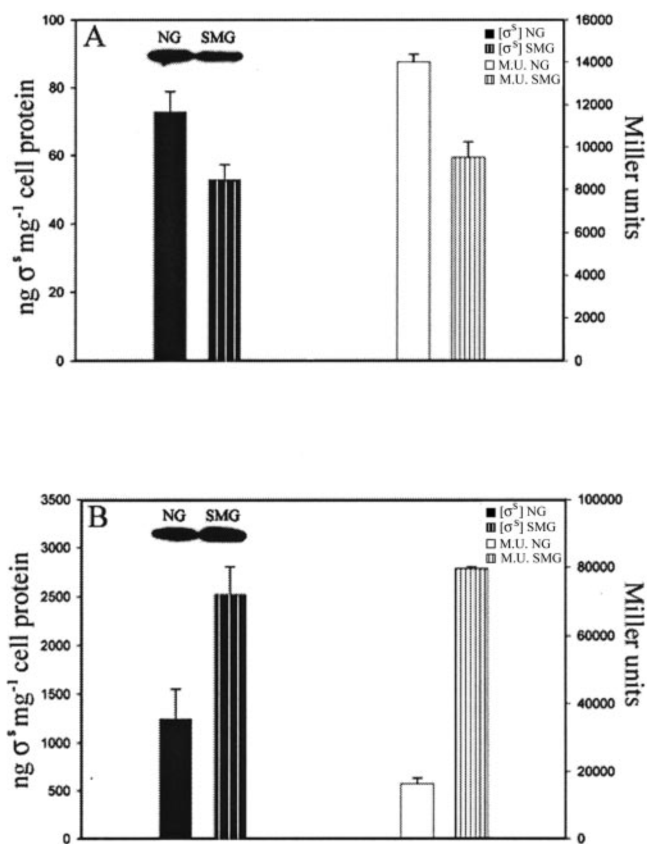

FIG. 3. σ^s concentrations determined by quantitative Western analysis or inferred from the levels of β-galactosidase synthesized by a *pexB-lacZ* transcriptional fusion strain (*E. coli* AMS171) in NG or SMG cultures during exponential (A) and stationary (B) phases of growth. M. U., Miller units. Data represent an average of three independent measurements; error bars represent standard errors of the mean.

TABLE 1. Transcriptional parameters of *rpoS* synthesis of exponential- and stationary-phase NG and SMG cultures.[a]

Growth phase	*rpoS* mRNA[b] [*rpoS*]		mRNA half-life[c] ($\tau_{1/2}^{rpoS}$)		Transcriptional rate[d] (K_s^{RNA})	
	NG	SMG	NG	SMG	NG	SMG
Exponential	1.3×10^8	1.3×10^8	5.4	5.4	1.6×10^7	1.6×10^7
Stationary	1.4×10^7	1.3×10^7	34.5	72.1	2.8×10^5	1.2×10^5

[a] Each value is an average of at least two independent determinations with analytical triplicates; standard error of the mean was <8%.
[b] Copies of *rpoS* mRNA per microgram of total RNA.
[c] Message half-life (minutes).
[d] Copies of *rpoS* synthesized per microgram of total RNA per minute.

rate of *rpoS* transcription decreased by twofold in stationary-phase SMG compared to NG cells (Table 1).

Regulation at the transcriptional level can therefore not account for the differential effect of SMG on σ^s levels in the two growth phases. We thus determined SMG effect on other levels of control of σ^s synthesis. σ^s stability was determined by measuring its half-life, as described in Materials and Methods. SMG markedly shortened the half-life of the sigma protein in exponential-phase cells (Fig. 5A and Table 2). In stationary phase, the sigma protein became more stable under both conditions; however, σ^s remained less stable in SMG than in NG by a small, but reproducible, value (Fig. 5B and Table 2).

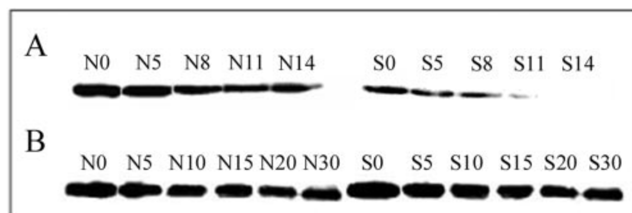

FIG. 5. σ^s protein half-life determined by quantitative Western analysis during the exponential (A) or stationary (B) phase of growth; representative Western blots are shown. N values (N0, N5, etc.) and S values (S0, S5, etc.) represent sampling time points in minutes under NG and SMG conditions, respectively.

From the steady state σ^s concentrations, its half-life, and the *rpoS* mRNA copy number, the *rpoS* mRNA translational rate (equation 2) and efficiency (equation 3) were calculated. Both the translational rate and efficiency increased markedly in stationary phase compared to exponential phase; in both phases, these parameters were higher in SMG than in NG-grown cells (Table 2). Thus, the main reasons for the differential effect of SMG on σ^s levels in the two phases are that it renders the sigma protein much more labile in the exponential than in the stationary phase and increases rpoS mRNA translational efficiency.

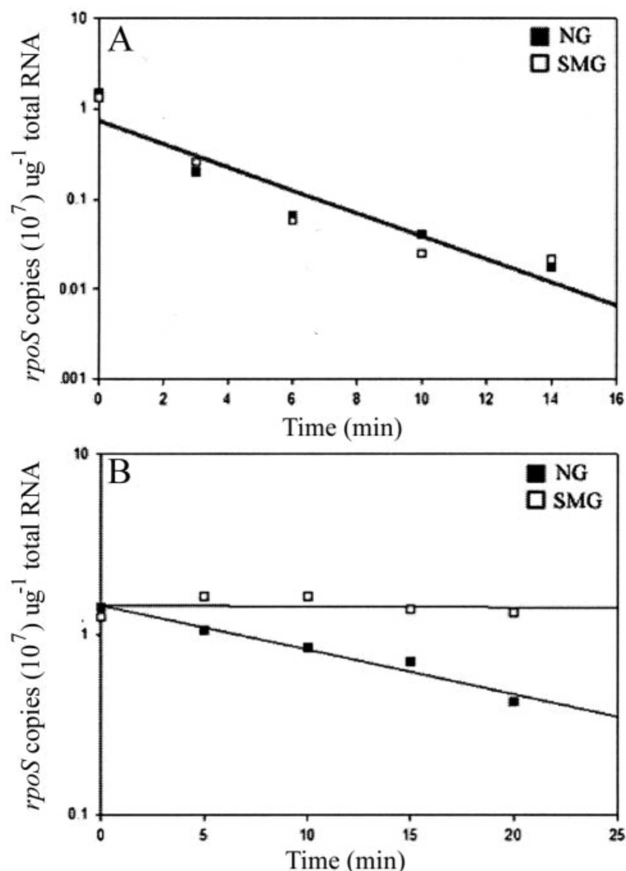

FIG. 4. *rpoS* message half-life of NG and SMG cells in exponential (A) and stationary (B) growth phases. Data represent an average of at least two independent qPCR measurements, with each time point analyzed in triplicate.

DISCUSSION

The HARV-grown NG cells of *E. coli* showed σ^s-dependent increased resistance in stationary phase to the stresses tested and elevated σ^s concentration in this phase, due largely to the stabilization of this protein. In both respect, the HARV cultures resembled conventional flask cultures in responding to stress (11, 24, 30), indicating that the use of the HARV reactors per se did not influence the stress response and that therefore these reactors are suitable for investigating SMG effects.

SMG enhanced *E. coli* resistance to both osmotic and acid stresses. That resistance developed simultaneously to two very different stresses strongly suggests that SMG conferred comprehensive cellular resistance. This conclusion is bolstered by the fact that Gao et al. (6) have shown that SMG also makes *E. coli* more resistant to ethanol stress.

The SMG effect in *S. enterica* serovar Typhimurium has been previously examined, but only in the exponential phase. The bacterium developed general resistance under this gravity con-

TABLE 2. Translational parameters of σ^s synthesis of exponential- and stationary-phase NG and SMG cultures[a]

Growth phase	σ^s protein[b] [σ^s]		σ^s half-life[c] ($\tau_{1/2}^{\sigma s}$)		Translational rate[d] ($K_s^{\sigma s}$)		Translational efficiency[e] ($K_E^{\sigma s}$)	
	NG	SMG	NG	SMG	NG	SMG	NG	SMG
Exponential	73	53	16.4	5.2	3.1	7.0	3.6×10^{11}	8.4×10^{11}
Stationary	1,263	2,530	28.8	24.6	30.4	71.2	3.3×10^{13}	8.5×10^{13}

[a] Each value is an average of at least two independent determinations with analytical triplicates; standard error of the mean was <8%.
[b] Nanograms σ^s per milligram of protein.
[c] Protein half-life (minutes).
[d] Nanograms of σ^s synthesized per milligram of cell protein per minute.
[e] Molecules of σ^s synthesized per copy of *rpoS* mRNA per minute.

dition in this phase independently of σ^s, since an *rpoS* mutant behaved like the wild type (35). Further, resistance developed without the induction of σ^s-regulated genes, such as *dnaK*, *groEL*, and *pexB* (*dps*) (36) that, by preventing cell macromolecular damage and promoting repair, mechanistically contribute to comprehensive cellular resistance in conventional flask cultures (10, 16). The findings with *E. coli* reported here not only reinforce the conclusion of Wilson et al. (36) that exponential-phase SMG-conferred general resistance is independent of σ^s, but they show further that in the wild-type *E. coli*, it is accompanied by diminished levels of this sigma factor. Thus, there appears to be an as-yet-undiscovered mechanism of comprehensive cellular resistance, which is not accompanied by increased σ^s levels and may not involve increased expression of known stress resistance genes.

SMG-grown *E. coli* cells were also more resistant than their NG-grown counterparts in stationary phase; since the latter are already quite robust, this results in superresistant cells. In contrast to exponential phase, the more normal situation of a direct relationship between σ^s levels and increased resistance prevails in the stationary phase (10, 16), as the highly resistant SMG stationary-phase cells possess higher σ^s concentrations than NG cells.

Conditions resembling the stationary phase are the norm in nature (15, 16, 17), and since SMG renders such cells superresistant, it follows that the SMG effect on bacterial resistance is even a greater cause for concern for space exploration than previously indicated by studies with exponential-phase cells (23, 35, 36). SMG conditions resemble low-shear environments on Earth, such as the brush border microvilli of the respiratory, gastrointestinal, and urogenital tracts (4, 8, 31). These are common routes of microbial infection, and therefore an understanding of the mechanism of SMG-conferred resistance will also contribute to a better control of infectious disease on this planet.

Apart from altering the dependence of stress resistance on σ^s in the two growth phases, SMG also profoundly affects σ^s regulation with respect to its stability and the translational efficiency of its mRNA. Studies conducted with conventional flask cultures have shown that σ^s stability is governed by its cleavage by ClpXP protease in which the phosphorylated form of a "tethering" response regulator protein is believed to play a role (12, 25, 30). As the extent of RssB phosphorylation may be a factor in determining σ^s stability (10), it is conceivable that SMG promotes RssB phosphorylation, thereby increasing σ^s instability. An additional possibility regarding the effect of SMG on the stability of this sigma factor relates to the fact that ClpXP protease activity is greatly affected by the folding pattern of its substrate (12). Previous studies show that space microgravity influences protein crystal formation (21), which suggests that diminished gravity and shear may influence protein-folding patterns. If so, σ^s folding under SMG might be altered, making it a more suitable target for ClpXP cleavage.

What might underlie the increased translational efficiency of the *rpoS* mRNA under SMG conditions in the two growth phases? *rpoS* translational efficiency under NG conditions, as obtained with conventional flask cultures, is thought to be regulated by two types of secondary structures, one formed within the untranslated region of the messenger (26) and the other between the anti-sense element in the coding region and the translational apparatus (18). Proteins such as Hfq and several small RNAs play a role in promoting secondary structures of the *rpoS* mRNA which possess different translational competence. SMG can conceivably affect this phenomenon. The lack of gravity may minimize tendency of the mRNA towards secondary structure formation and/or promote interaction between Hfq, *rpoS* mRNA, and the small RNA, such as *DsrA*, which acts as a positive regulator of translation of this messenger (32). These hypotheses are under investigation.

While the mechanistic basis of SMG effect on cell resistance at this stage must remain speculative, it is clear that this gravity condition introduces a new comprehensive cellular resistance paradigm and promises to shed light on basic mechanisms that regulate translational control and proteolysis. Insights into these phenomena are important not only for the safety of space travel, but also in enhancing our understanding of fundamental biological processes on Earth.

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