

RelA Inhibits *Bacillus subtilis* Motility and Chaining

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The nucleotide second messengers pppGpp and ppGpp [(p)ppGpp] are responsible for the global downregulation of transcription, translation, DNA replication, and growth rate that occurs during the stringent response. More recent studies suggest that (p)ppGpp is also an important effector in many nonstringent processes, including virulence, persister cell formation, and biofilm production. In *Bacillus subtilis*, (p)ppGpp production is primarily determined by the net activity of RelA, a bifunctional (p)ppGpp synthetase/hydrolase, and two monofunctional (p)ppGpp synthetases, YwaC and YjbM. We observe that in *B. subtilis*, a *relA* mutant grows exclusively as unchained, motile cells, phenotypes regulated by the alternative sigma factor SigD. Our data indicate that the *relA* mutant is trapped in a SigD “on” state during exponential growth, implicating RelA and (p)ppGpp levels in the regulation of cell chaining and motility in *B. subtilis*. Our results also suggest that minor variations in basal (p)ppGpp levels can significantly skew developmental decision-making outcomes.

Genetic regulation makes it possible for cells possessing identical genomes to differentiate into subpopulations of phenotypically and physiologically distinct cell types. The Gram-positive bacterium *Bacillus subtilis* is an excellent model system in which to study the molecular mechanisms underlying differentiation because it produces several well-characterized cell types, including spores, competent cells, biofilm formers, nonmotile chained cells, and motile unchained cells. These different cell types are produced through both stochastic and responsive phenotypic-switching mechanisms (1–3). For example, the proportion of chained, nonmotile cells versus unchained, motile cells in a culture of exponentially growing wild-type *B. subtilis* bacteria is driven by stochastic switching between the “on” and “off” states of SigD (4, 5), an alternative sigma factor that drives expression of the genes responsible for daughter cell separation and motility. However, stochastic determinants are only one part of SigD regulation, as the proportion of cells in the SigD on state also increases as cells transition out of exponential growth (6–9). In this study, we focus on the role of a conserved, intracellular signaling molecule in triggering the switch to a SigD on state.

The bacterial “alarmone” (also called “magic spot”), consisting of pppGpp and ppGpp [here referred to as (p)ppGpp], acts as an important second-messenger molecule linking both intra- and extracellular environmental cues with global changes in transcription, translation, and DNA replication (10–12). In *Escherichia coli* and *B. subtilis*, (p)ppGpp levels are estimated to be less than 20 pmol per optical-density unit during exponential growth (13–16) but rapidly rise to millimolar levels in response to adverse growth conditions, such as amino acid starvation (15). High levels of (p)ppGpp lead to the arrest of DNA replication and cell division, reduced lipid synthesis, a global reduction in transcription and translation (10–12, 17), and increased tolerance for cell stress (11, 18, 19). Levels of (p)ppGpp are strongly correlated with the growth rate (13) and ribosomal pool size (20), and one study found that various substringent concentrations of (p)ppGpp regulate different sets of genes, suggesting that the (p)ppGpp concentration may be associated with graded responses in gene expression (21).

In *B. subtilis*, (p)ppGpp levels are predominately determined by the net activity of RelA, a dual-function (p)ppGpp synthetase and hydrolase, and two additional (p)ppGpp synthetase enzymes,

YwaC and YjbM (22). (p)ppGpp is synthesized at low but detectable levels during exponential growth, primarily through the activity of RelA (22). RelA is also responsible for the high levels of (p)ppGpp that accumulate in *B. subtilis* during stringent response (22, 23). Less is known about the contribution of YwaC and YjbM activity to (p)ppGpp accumulation. *ywaC* is under the control of at least three stress response sigma factors, SigW, SigV, and SigM, and encodes the protein responsible for the increase in (p)ppGpp observed during alkaline shock (22, 24–26). Based on microarray data, *yjbM* is the second gene in an operon of otherwise experimentally uncharacterized genes in *B. subtilis* (27). Expression profiles suggest that the level of *yjbM* transcription peaks during rapid growth and transition states in rich media (22, 27). In addition, YjbM contributes, albeit less substantially than RelA, to the basal levels of (p)ppGpp present during exponential growth (22).

When *B. subtilis* has *relA* deleted or is depleted of a RelA variant that possesses hydrolase but not synthetase activity (RelA_{D264G}), it grows slowly (22) and is prone to growth rate suppressors (22, 28, 29). RelA mutants have also been shown to exhibit pleiotropic phenotypes, including reduced competence (30) and delayed sporulation (30). Absolute levels of (p)ppGpp are likely elevated in the *relA* deletion and hydrolase mutants, since these mutants lack the major activity responsible for breaking down (p)ppGpp synthesized by the remaining synthetases, YwaC and YjbM (22, 28). Consistent with the idea that (p)ppGpp levels are elevated, the slow-growth phenotype of the *relA* mutant can be complemented by introduction of an allele of *relA* that encodes only the (p)ppGpp hydrolase and through loss-of-function mutations in

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ywaC and/or *yjbM* (22). It is likely that small differences in (p)ppGpp levels during exponential growth account for the observed phenotypes; however, any variances in (p)ppGpp levels present in the *relA* mutant and wild type during exponential growth (when stringent response is not induced) are not quantifiable using current methodologies.

In this study, we show that the *relA* mutant grows exclusively as swimming, unchained cells during the exponential growth phase; this unchained phenotype contrasts sharply with the wild-type domesticated strain PY79, which exhibits a primarily chained phenotype during exponential growth in rich laboratory media (31). We also find that the *relA* mutant exhibits an accelerated swim front in a swim plate assay, consistent with the increased population of swimming cells we observe microscopically. Analysis of SigD synthesis and activity indicate that the *relA* mutant is trapped in a SigD on state during exponential growth. Thus, our results show that cell chaining and motility in *B. subtilis* are regulated by RelA through its activity as a (p)ppGpp hydrolase.

MATERIALS AND METHODS

General methods. A description of strains, plasmids, and oligonucleotides used in this study and a detailed description of plasmid constructions can be found in Tables S1, S2, and S3 in the supplemental material. The *B. subtilis* strains used in this study were grown at 37°C in CH medium (32) or Luria-Bertani (LB) broth (10 g/liter tryptone, 5 g/liter yeast extract, 5 g/liter NaCl). All samples were grown in volumes of either 25 ml or 50 ml in 250-ml baffled flasks in a shaking water bath set at 280 rpm. LB plates were supplemented with 1.5% Bacto agar. *E. coli* strains DH5 α and TG-1 were used for isolation of plasmids and were grown in LB medium. When needed, antibiotics were included at the following concentrations: 100 μ g ml⁻¹ spectinomycin, 7.5 μ g ml⁻¹ chloramphenicol, 10 μ g ml⁻¹ tetracycline, and 1 μ g ml⁻¹ erythromycin (Erm) plus 25 μ g ml⁻¹ lincomycin (MLS) for *B. subtilis* strains and 100 μ g ml⁻¹ ampicillin for *E. coli* strains.

Swim plate assay. *B. subtilis* strains were grown at 37°C in baffled flasks containing CH medium to mid-log phase. The cells were pelleted and resuspended at an optical density at 600 nm (OD₆₀₀) of 10 in phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄) containing 0.5% India ink (Higgins). Ten microliters of the cell suspension was spotted on top of 150- by 15-mm LB plates fortified with 0.25% Bacto agar (100 ml/plate), dried for 30 min at room temperature on the benchtop to allow time for absorption, and incubated at 37°C in a loosely covered glass dish containing paper towels soaked with water to generate humid conditions. The swim radius was measured relative to the edge of the origin marked by the India ink.

Microscopy. All fluorescence microscopic analyses were performed with a Nikon Ti-E microscope equipped with a CFI Plan Apo lambda DM 100 \times objective and a Prior Scientific Lumen 200 Illumination system; C-FL UV-2E/C DAPI (4',6-diamidino-2-phenylindole), C-FL GFP (green fluorescent protein) HC HISN Zero Shift, and C-FL Texas Red HC HISN Zero Shift filter cubes; and a CoolSnap HQ2 monochrome camera. Membranes were stained with either TMA-DPH [1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene *p*-toluenesulfonate; 0.02 mM] or FM4-64 (3 μ g ml⁻¹) (Life Technologies) and imaged with exposure times of 200 to 800 ms. All images were captured, analyzed, and processed using NIS Elements Advanced Research (version 4.10) and Adobe Photoshop (version 12.0).

Flagella were visualized by using the cysteine-reactive dye Alexa Fluor 488 C₅ maleimide (Life Technologies) using a previously described method (33). Briefly, 1-ml samples of mid-logarithmic-phase cultures were collected at an optical density at 600 nm (OD₆₀₀) of 0.5, and the cells were pelleted by centrifugation. The pellet was then washed with 1 ml of PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 2 mM KH₂PO₄), resuspended in 50 μ l of PBS containing 5 μ g ml⁻¹ Alexa Fluor 488 C₅ maleimide, and incubated at room temperature for 5 min. The cells were

then washed twice with 0.5 ml PBS. The pellet was resuspended in 50 μ l of PBS containing 5 μ g ml⁻¹ FM4-64 and on a glass slide with a poly-L-lysine (Sigma)-treated coverslip.

Western blot analysis. *B. subtilis* strains were grown to logarithmic growth phase (OD₆₀₀, 0.4 to 0.7), and 1- or 2-ml samples were collected. The cells were pelleted by centrifugation, resuspended in 50 μ l lysis buffer (20 mM Tris, pH 7.0, 10 mM EDTA, 1 mg ml⁻¹ lysozyme, 10 μ g ml⁻¹ DNase I, 100 μ g ml⁻¹ RNase A, with 1 mM phenylmethylsulfonyl fluoride [PMSF]), and incubated for 15 min at 37°C. Fifty microliters of sample buffer (0.25 M Tris, pH 6.8, 4% SDS, 20% glycerol, 10 mM EDTA) containing 10% 2-mercaptoethanol was added to the lysates, and the samples were boiled for 5 min prior to loading. Proteins were separated by SDS-PAGE on precast 4 to 20% Tris-HCl gels (Bio-Rad), transferred onto a nitrocellulose membrane (Pall) at 100 V for 1 h in transfer buffer (20 mM Tris, 15 mM glycine, and 20% methanol [vol/vol]), and then blocked in 5% nonfat milk in PBS containing 0.5% Tween 20. The membranes were incubated overnight at 4°C with a 1:5,000 dilution of anti-SigD peptide antibody (CIRDDKNVPPEEKIM; Genscript), a 1:20,000 dilution of anti-SigA (provided by Masaya Fujita, University of Houston, Houston, TX), or a 1:40,000 dilution of anti-Hag antibody (provided by Daniel B. Kearns, Indiana University) and washed and incubated with a 1:10,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Bio-Rad). After washing, the blots were incubated with SuperSignal West Femto Chemiluminescent substrate (Thermo) according to the manufacturer's instructions.

Flagellin labeling. *B. subtilis* strains were grown to logarithmic growth phase (OD₆₀₀, 0.5), and 1-ml samples were collected. The samples were labeled by Alexa Fluor 488 C₅ maleimide labeling as described in "Microscopy" above, and cell lysates were prepared for SDS-PAGE analysis as described in "Western blot analysis" above. Proteins were separated by SDS-PAGE on precast 4 to 20% Tris-HCl gels (Bio-Rad) and visualized by scanning the gels with a Typhoon Trio (GE Healthcare) at 100- μ m resolution and an excitation wavelength of 488 nm.

β -Galactosidase assays. To assay expression from *lacZ* transcriptional fusions, *B. subtilis* strains were grown at 37°C in baffled flasks containing CH medium. One- or 2-ml samples were harvested at an OD₆₀₀ of 0.5 by centrifugation in a room temperature tabletop centrifuge, and the cell pellets were frozen at -20°C or -80°C. To perform the assays, the pellets were thawed on ice, resuspended in 500 μ l of Z buffer (40 mM NaH₂PO₄, 60 mM Na₂HPO₄, 1 mM MgSO₄, 10 mM KCl, 38 mM β -mercaptoethanol, and 0.2 mg ml⁻¹ lysozyme), and incubated for 15 min at 30°C. The reaction was started by adding 100 μ l of 4-mg ml⁻¹ *O*-nitrophenyl β -D-galactopyranoside (in Z buffer) and stopped with 250 μ l of 1 M Na₂CO₃ after a yellow color developed. The optical densities of the reaction mixtures were measured at 420 and 550 nm and used to calculate the β -galactosidase specific activity according to the following equation: $1,000 \times \{[OD_{420} - (1.75 \times OD_{550})]/(\text{time} \times OD_{600})\}$.

RESULTS

The *relA* mutant grows slowly but exponentially in liquid culture. Previous studies showed that replacement of *B. subtilis relA* with an antibiotic resistance cassette leads to a slow-growth phenotype. *relA* is in an operon with *ddd* (27), encoding D-amino acid tRNA deacylase (34, 35), an enzyme that removes D-amino acids from mischarged tRNAs. In order to test if the slow-growth phenotype was attributable to loss of *relA* alone and not due to polar effects on *ddd*, we introduced $\Delta relA$ into a strain harboring an intact copy of *relA* and its putative promoter region at an ectopic locus (*amyE::P_{relA}-relA*) and examined the growth rate of the resultant strain. The $\Delta relA$ mutant grew more slowly than the wild type on LB plates, consistent with previous reports (22) (Fig. 1A). We determined that the $\Delta relA$ mutant also grew more slowly than the wild type in liquid culture (Fig. 1B), with a generation time in liquid CH medium at 37°C of 50 min for the $\Delta relA$ mutant com-

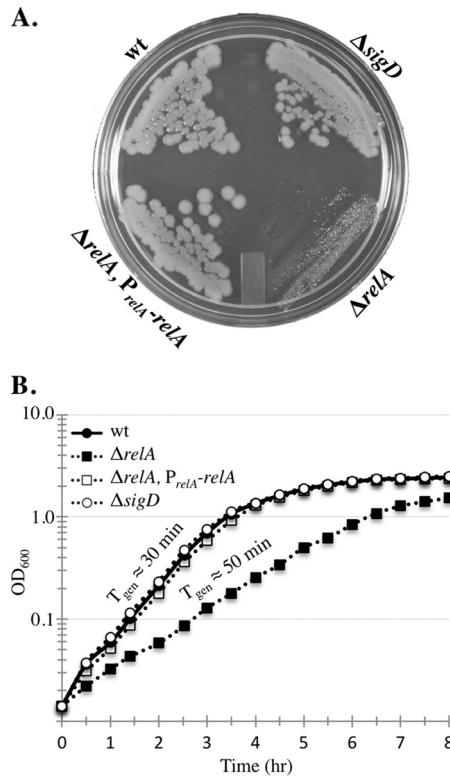


FIG 1 The *relA* mutant grows slowly and exponentially. (A) The indicated strains were streaked on an LB plate and incubated for 24 h at 37°C. (B) Growth curves of the same strains shown panel A grown in liquid CH medium. The average of three independent replicates is plotted for each strain. wt, wild type (BJH001); $\Delta relA$, BQA009; $\Delta relA P_{relA}-relA$, BQA068; $\Delta sigD$, BQA022.

pared to 30 min for the wild type (Fig. 1A). Introduction of *amyE::P_{relA}-relA* into the $\Delta relA$ strain complemented the slow-growth phenotype both on plates and in liquid medium (Fig. 1A and B), indicating that the slow-growth phenotype was solely attributable to the loss of *relA* and not related to polar effects on *dtb*. Despite the longer generation time of the $\Delta relA$ mutant, the linear phase of exponential growth mirrored that of the wild type, showing a decrease in the rate around an OD₆₀₀ of 0.8 to 1.0. This result suggests that the cues triggering the transition out of exponential growth are intact in the $\Delta relA$ mutant background.

The slow-growing $\Delta relA$ mutant was previously shown to be prone to the accumulation of growth rate suppressors in *ywaC* and *yjbM*, two other (p)ppGpp synthetase genes (22, 28, 29). When constructing and growing the $\Delta relA$ strain, we also observed that spontaneous growth rate suppressors arose frequently on plates; sequencing of these suppressors revealed that the faster-growing strains always possessed mutations in *ywaC* and/or *yjbM* (data not shown), consistent with prior reports (22, 28, 29). Since the $\Delta relA$ mutant frequently accumulates growth rate suppressors in *ywaC* and *yjbM*, the restoration of wild-type growth we observed in the presence of *amyE::P_{relA}-relA* could be attributable to the accumulation of one or more suppressor mutations in these genes as opposed to true complementation. To rule out this possibility, we sequenced the *ywaC* and *yjbM* regions of the complemented $\Delta relA$ mutant and found that the regions contained the wild-type alleles (data not shown).

A $\Delta relA$ mutant grows exclusively as unchained cells. While

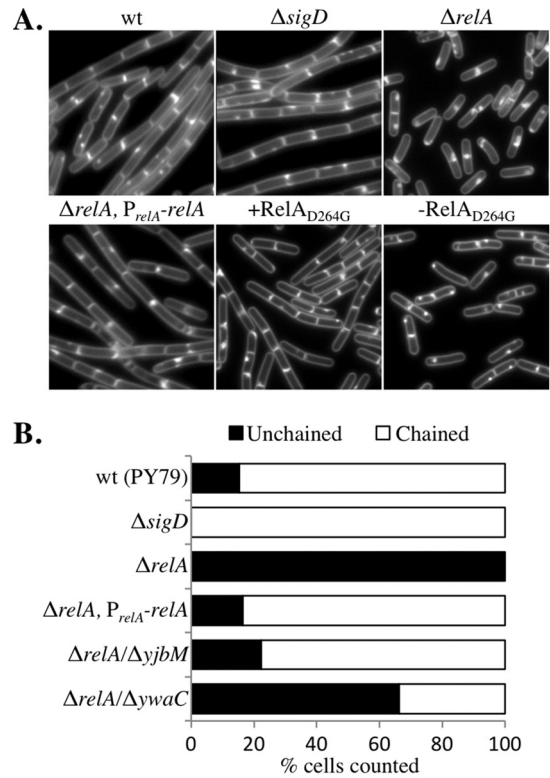


FIG 2 The $\Delta relA$ mutant grows exclusively as single cells. (A) Representative images of wt (BJH001), $\Delta relA$ (BQA009), $\Delta relA P_{relA}-relA$ (BQA068), $\Delta sigD$ (BQA022), and $P_{spac}-relA_{D264G}$ (EUB004) cells grown to mid-log phase in CH medium. For EUB004, cells grown in the presence (+RelA_{D264G}) and absence (-RelA_{D264G}) of 1 mM IPTG for 120 min are shown. The membranes were stained with TMA. (B) Frequencies of chained (three or more cells linked) and unchained (single and doublet) cells across a population. The cells in images from at least three independent cultures were counted, and no fewer than 1,500 cells were quantitated for each strain represented in the graph. (C) Representative images of $\Delta relA \Delta sigD$ (BQA059) and $\Delta relA P_{hy}-sigD \Delta sigD$ (BQA083) cells grown to exponential phase in CH medium. The membranes were stained with TMA.

examining the role of RelA in sporulation, we fortuitously observed that the $\Delta relA$ mutant grew as a homogeneous population of unchained cells during exponential growth in rich medium (Fig. 2A and B). This unchained phenotype is in sharp contrast to PY79, the wild-type domesticated strain used in this study, which grows as a mixed population of 85% chained and 15% unchained cells under the same growth conditions (Fig. 2A and B). The chaining phenotype was restored in the *relA* complementation strain, and sequencing of genomic DNA from the same culture pictured in Fig. 2A confirmed that *ywaC* and *yjbM* had not accumulated suppressor mutations. These results indicate that the unchained phenotype is due to the absence of the *relA* gene product

and not due to polar effects on *dtb*. Moreover, an inducible allele of *relA* that encodes the (p)ppGpp hydrolase activity but lacks synthetase activity (a D264G substitution in RelA) (22), restored not only the wild-type growth rate, as previously observed (22), but also the cell chaining in a $\Delta relA$ background (Fig. 2A). In contrast, depletion of RelA_{D264G} in the $\Delta relA$ background resulted in growth as single cells (Fig. 2A). This result is consistent with the unchained phenotype being attributable to the loss of RelA's (p)ppGpp hydrolase function. The presence of chains in all the *relA* mutant cultures examined throughout this study correlated with growth rate suppressor mutations in the other (p)ppGpp synthetases (data not shown). Therefore, we found that the distinctive, nonchaining phenotype of the *relA* mutant served as a convenient marker to monitor the integrity of *relA* mutant strains used in our experiments. To determine if the unchained phenotype could be attributed to the slow growth of the $\Delta relA$ mutant as opposed to a RelA-dependent activity, we examined wild-type cells grown at room temperature in identical media. Wild-type cells grown at room temperature had a generation time of ~50 min, comparable to that of the $\Delta relA$ mutant, but grew primarily as chained cells (data not shown), suggesting that a lower growth rate alone does not account for the unchained phenotype.

Deletion of *ywaC* or *yjbM* in a $\Delta relA$ mutant restores cell chaining during exponential growth. The $\Delta relA$ mutant lacks the major (p)ppGpp hydrolase activity, so synthesis of (p)ppGpp from YwaC and/or YjbM should lead to a net accumulation of (p)ppGpp in a $\Delta relA$ mutant background (22). We hypothesized that the unchained phenotype we observed in the $\Delta relA$ mutant might be due to increased (p)ppGpp levels. Since the basal levels of (p)ppGpp present in the wild-type and $\Delta relA$ mutant backgrounds fall outside the linear range of detection, we tested this idea genetically using (p)ppGpp synthetase mutants. We hypothesized that if higher levels of (p)ppGpp resulted in loss of chaining, then the $\Delta relA \Delta yjbM$ and $\Delta relA \Delta ywaC$ double mutants should be more chained than the $\Delta relA$ single mutant. We observed that in comparison to the wild type (85% chained) and the *relA* mutant strain (0% chained), the $\Delta relA \Delta yjbM$ and $\Delta relA \Delta ywaC$ mutants were 78% and 34% chained, respectively (Fig. 2B). These results are consistent with the idea that accumulation of (p)ppGpp promotes the switch from chained to unchained cells and also suggest that YjbM is a more significant contributor to (p)ppGpp pools during exponential growth than YwaC.

The proportion of chained versus unchained cells in a population varies with strain and culture conditions and was previously shown to be regulated by the level of an activated form of the alternative sigma factor SigD (4). Active SigD stimulates transcription of several genes, including the cell wall amidase LytF, which promotes postseptational hydrolysis of the peptidoglycan connecting daughter cells (36). As previously shown, a *sigD* mutant grows exclusively as chains (Fig. 2A and B). In order to determine if the unchained phenotype observed in the *relA* mutant was dependent on SigD activity, we generated a $\Delta sigD \Delta relA$ double mutant by introducing $\Delta relA$ into the $\Delta sigD$ background using transformation. We observed, using control DNA, that the transformation efficiency of the $\Delta sigD$ mutant was reduced more than 10-fold compared to the wild type. For unknown reasons, the efficiency of introducing the $\Delta relA$ mutation into the $\Delta sigD$ mutant was even lower, so that we were able to obtain only one transformant after 15 attempts. In contrast, $\Delta relA$ could be readily introduced into the $\Delta sigD$ background harboring a copy of P_{relA}

at an ectopic locus. These results hinted that the combination of $\Delta sigD$ and $\Delta relA$ could be synthetically lethal. To further test this idea, we introduced $\Delta relA$ into a SigD depletion strain in which the only copy of *sigD* is under the control of an IPTG (isopropyl- β -D-thiogalactopyranoside)-regulated promoter. To test for synthetic lethality, the resulting strain was inoculated on media with and without inducer. Surprisingly, the strain grew similarly to the $\Delta relA$ parent on plates whether *sigD* expression was induced with IPTG or not (data not shown). This result suggests that the reduced transformation efficiency we observed is due not to synthetic lethality but rather to a reduced ability to obtain transformants. Consistent with this, whole-genome sequencing of the viable $\Delta sigD \Delta relA$ mutant revealed no second-site suppressor mutations. Microscopic analysis of the $\Delta sigD \Delta relA$ mutant revealed an intermediate chaining phenotype different from that of either the $\Delta sigD$ or the $\Delta relA$ single mutant (Fig. 2C). Moreover, the SigD depletion strain harboring the $\Delta relA$ mutation appeared similar to the $\Delta relA$ strain when SigD was expressed (Fig. 2C) and similar to the $\Delta sigD \Delta relA$ mutant when SigD was depleted (Fig. 2C). These results indicate that SigD is required for the homogeneous single-cell phenotype of the $\Delta relA$ mutant.

The $\Delta relA$ mutant displays flagella on the cell surface. We observed that the unchained *relA* mutants observed by microscopy (Fig. 2A) appeared highly motile in wet mounts (data not shown), indicating that the unchained cells also possessed active flagella. As mentioned previously, accumulation of active SigD leads to the upregulation of genes responsible for the separation of chained cells into single cells (5, 36–38). Active SigD also coordinately upregulates the synthesis of late flagellar genes (39), including *hag*, the gene encoding flagellin (40). We hypothesized that since the *relA* mutants appeared exclusively unchained and motile by microscopy, the *relA* mutant would also be associated with more flagella than the wild type. In order to directly visualize flagella on intact cells, we engineered strains harboring a Hag_{T209C} change in the flagellin subunit, incubated intact cells with a cysteine-reactive dye that stains surface-exposed cysteine residues, and imaged the flagella by epifluorescence microscopy using methodology described previously (39). The *relA* mutant was unchained (as shown in Fig. 2A and B) and presented numerous peritrichous flagella (Fig. 3A). In contrast, our wild-type strain grew as both unchained cells that presented flagella and chained cells that generally lacked flagella (Fig. 3A). We were unable to determine the numbers of flagella present on the cell surfaces due to difficulty resolving individual filaments among the dense mass on the cell surface. In addition, we observed the presence of significant numbers of shed flagella in the samples that made it difficult to resolve whether the flagella were cell associated. We believe that the shed flagella likely break off due to the shear forces required to wash and centrifuge the samples during cysteine labeling (see below). We conclude that the presence of flagella on the surfaces of the unchained *relA* mutant cells suggests that SigD is more active in this strain background than in the wild type grown under identical conditions.

Although the use of cysteine-reactive dye to observe *B. subtilis* flagella was previously published (39) and we found that no fluorescent signal was associated with wild-type cells lacking Hag_{T209C} (data not shown), we performed an additional control to visualize the complement of protein species labeled by the cysteine-reactive dye. Intact cells were treated with dye as they would be prior to microscopy and then subjected to SDS-PAGE and laser scanning.

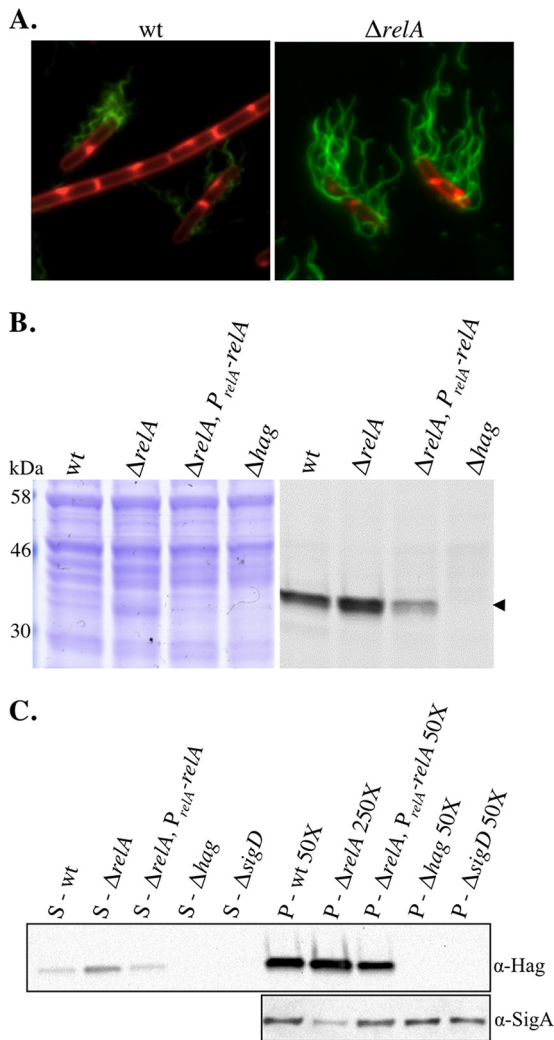


FIG 3 The *relA* mutant is comprised of mostly flagellated cells (A) Representative images of mid-log-phase cultures of wt (BQA057) and $\Delta relA$ (BQA062) strains. The membranes were stained with FM4-64 (red), and flagellin (Hag) was stained with Alexa Fluor 488 C₅ maleimide (green). Both strains harbor a point mutation in the chromosomal copy of *hag* that creates Hag_{T209C}. (B) Comparison of flagellin (Hag) protein levels in wt (BQA057), $\Delta relA$ (BQA062), $\Delta relA P_{relA-}relA$ (BQA080), and Δhag (BQA076) strains. Samples were collected from mid-log-phase cultures grown in CH medium and stained with Alexa Fluor 488 C₅ maleimide. Proteins from cell lysates were separated by SDS-PAGE, stained with Coomassie blue (left), and scanned with a laser scanner to visualize fluorescently labeled protein (right). The arrowhead indicates flagellin. (C) (Top) Western blot analysis showing flagellin levels associated with the culture supernatants (left; S) and cell pellets (right; P) of the indicated strains grown in CH medium to mid-log phase: wt (BJH001), $\Delta relA$ (BQA009), $\Delta relA P_{relA-}relA$ (BQA068), $\Delta sigD$ (BQA022), and Δhag (BQA076). It was necessary to dilute the cell pellet lysates to the indicated dilutions in order to assess the levels of protein associated with each sample without overexposure. (Bottom) SigA protein served as a loading control for the cell pellet samples.

A predominant band emitting in the green channel and running at the approximate molecular mass of the flagellin monomer (32 kDa) was present in strains harboring Hag_{T209C} for the wild type, the *relA* mutant, and the *relA*-complemented strain, but the band was absent from the flagellin mutant (Δhag) and the wild-type

PY79 strain, which lacks the Hag_{T209C} variant (Fig. 3B and data not shown). The amounts of flagellin varied greatly even between identical strains in independent trials, despite the fact that samples were normalized by optical density; total-protein staining confirmed that approximately equal amounts of protein were loaded for each sample (Fig. 3B). We believe the variability seen in flagellin levels is due to shearing of the flagella during the sample handling required for the labeling reaction. We conclude that the fluorescence observed by microscopy is due primarily to labeled flagella and that, while analysis of labeled proteins using SDS-PAGE followed by laser scanning is useful for determining the apparent molecular weight of stained proteins, it is not useful as a quantitative measure of total flagellin levels in *B. subtilis*.

The $\Delta relA$ mutant produces more flagellin than the wild type. In order to quantitate the amount of flagellin associated with the *relA* mutant relative to the wild type, we collected cells and supernatants from exponentially growing cultures and examined flagellin levels using Western blot analysis (Fig. 3C). The supernatants were collected so that we could also assess flagellin that might accumulate in the supernatants due to shedding, shearing, or cell lysis. A predominant band running at the approximate molecular mass of the flagellin monomer (32 kDa) was present in both supernatants and cell lysates of the wild type, the *relA* mutant, and the *relA* complemented strain but was absent from the flagellin mutant (Δhag) and the *sigD* mutant ($\Delta sigD$). The amount of flagellin associated with the supernatants was negligible compared to the amount associated with the cell bodies, so it was necessary to dilute the cell body lysates 50-fold to visualize all of the samples on the same Western blot without overexposure (Fig. 3C). Since the amount of flagellin present in the supernatant was minor compared to that in the cell bodies, we did not analyze these data further. The amounts of flagellin present in the wild-type control and the complemented *relA* strain were similar in each of four independent trials and showed no statistically significant difference in flagellin levels. The *relA* mutant, in contrast, showed a 5-fold increase in flagellin levels compared to the wild type, and it was necessary to dilute the sample 5-fold to avoid overexposure (Fig. 3C). The SigA antibody served as a loading control, confirming that the diluted sample was loaded in the *relA* mutant lane. These results are consistent with what we observed microscopically (Fig. 3A) and suggest that the unchained *relA* mutant population consists primarily of flagellated cells in a SigD on state.

The $\Delta relA$ mutant strain exhibits increased motility on swim plates. Since the *relA* mutant population is composed exclusively of unchained, flagellated cells, we expected that it would also produce a more motile population than the wild type. The laboratory strain used in this study (PY79) does not swarm (33), a type of surfaced-based group motility usually facilitated by a surfactant produced by bacteria (33, 41). Therefore, in order to obtain a quantitative assessment of the motility behavior of the population, we performed a swim plate assay that utilizes a lower concentration of agar and does not require surfactant production (42). All cultures were grown to mid-log phase and spotted in equal volumes and densities on swim plates. The visible edge of the culture, which we refer to as the “swim front,” was measured relative to the edge of the original spot over a time course (Fig. 4A). An example of a swim plate at the 3.5-h time point is shown in Fig. 4B. The absolute distance between the edge of the original spot and the swim front (the “swim radius”) varied slightly from assay to assay (standard deviations are shown in 4A); however, the max-

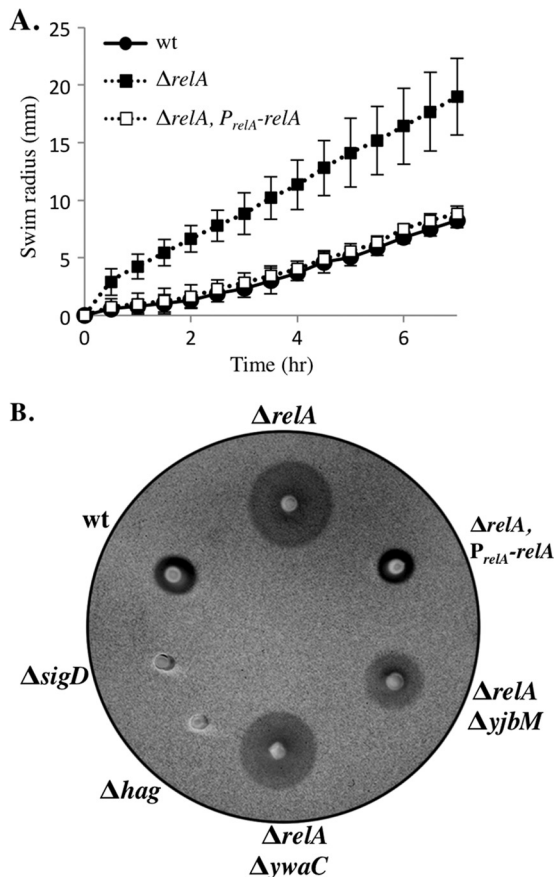


FIG 4 Loss of *relA* leads to increased mobility on swim plates. (A) Quantitation of swim expansion from plate assays performed with wt (BJH001), $\Delta relA$ (BQA009), and $\Delta relA P_{relA}-relA$ (BQA068) strains on LB fortified with 0.25% agar. Each symbol indicates the average of measurements from five independent experiments with standard deviations. Measurements were recorded every 30 min for 8 h. (B) wt (BJH001), $\Delta relA$ (BQA009), $\Delta relA P_{relA}-relA$ (BQA068), $\Delta sigD$ (BQA022), Δhag (BQA076), $\Delta relA \Delta yjbM$ (BQA081), and $\Delta relA \Delta ywaC$ (BQA082) strains were inoculated on LB fortified with 0.25% agar. After 3.5 h of incubation at 37°C, the plates were scanned against a black background. The images were inverted to better show contrast.

imal rate of migration, calculated from linear regions of the data in Fig. 4A, remained essentially constant from experiment to experiment.

The *relA* mutant exhibited two notable phenotypes in the swim plate assay. First, unlike the wild type, which exhibited an ~2-h lag before reaching its maximal swim front migration rate of 2.8 mm/h, the *relA* mutant had a constant migration rate throughout the course of the experiment (30 min to 8 h) of 4.9 mm/h. Wild-type cells from exponential-phase cultures, early transition phase (OD_{600} , 1.2), and mid-transition phase (OD_{600} , 1.9) all exhibited similar lags (data not shown). Lag periods for swarming had also been observed previously and may represent a physiological adjustment to growth on a more solid surface (41). The second observable phenotype was a maximal migration rate of the *relA* mutant of 4.9 mm/h, significantly higher than that of the wild type at 2.8 mm/h. The complemented *relA* strain behaved similarly to the wild type (Fig. 4A). These results suggest that both the absence of the lag period and the overall increase in the maximal swim front migration rate can be attributed to *relA* function. As expected, the

swim fronts of strains deficient in either SigD ($\Delta sigD$) or flagellin (Δhag) production did not migrate a measurable distance from the culture origin (Fig. 4B). The SigD and flagellin nonswimming controls exhibited wild-type growth rates in liquid culture (Fig. 1A and data not shown), suggesting that the growth rate has little effect on the distance traveled by the swim front in the 8-h time course. We conclude that, consistent with the unchained and flagellated phenotype we observed microscopically, the *relA* mutant population is poised to swim during the exponential growth phase.

The increased chaining we observed in the $\Delta relA \Delta yjbM$ and $\Delta relA \Delta ywaC$ double mutants (Fig. 2C) is consistent with a shift toward a SigD off state relative to the *relA* single mutant. If there is reduced SigD activity in the double mutants, then we would also expect a decrease in their ability to swim compared to the *relA* single mutant. To test this hypothesis, we assayed the swim front migration of the $\Delta relA \Delta yjbM$ and $\Delta relA \Delta ywaC$ strains. We observed that both the $\Delta relA \Delta ywaC$ and $\Delta relA \Delta yjbM$ strains possessed smaller swim radii than the *relA* parent alone but larger radii than the wild type (Fig. 4B; see Fig. S1A in the supplemental material). The $\Delta relA \Delta ywaC$ strain, which possesses an intact copy of exponentially expressed *yjbM*, consistently exhibited a larger swim radius than the $\Delta relA \Delta yjbM$ strain (Fig. 4B; see Fig. S1A in the supplemental material), indicating that it more closely resembles the *relA* strain than the $\Delta relA \Delta yjbM$ strain. These results are consistent with the idea that the switch from the nonmotile (SigD off) to the motile (SigD on) state is promoted by increasing (p)ppGpp levels; at the same time, we do not rule out the possibility that the switch is stimulated by another mechanism unrelated to (p)ppGpp accumulation.

Loss of *relA* leads to an increase in SigD levels and activity. The *relA* mutant grows exclusively as unchained, motile cells during exponential growth, consistent with the hypothesis that the *relA* mutant possesses higher levels of active SigD than the wild type during exponential growth. In order to test this hypothesis, we first examined the levels of SigD protein by performing Western blot analysis on strains grown to mid-log phase. Relative to the wild type, the *relA* mutant consistently showed a nearly 10-fold increase in SigD levels (Fig. 5A). Approximately wild-type SigD levels were restored in the *relA* complementation strain (Fig. 5A), indicating that the increase in SigD levels in the *relA* mutant are due to the absence of a functional RelA protein. We conclude that a RelA-dependent function is required to prevent the accumulation of SigD protein during exponential growth.

Although SigD protein levels are correlated with SigD activity, the relationship is not linear, and several levels of posttranslational regulation ultimately determine actual SigD activity (41, 43). In order to determine if the increase in SigD levels we observed also corresponded to an increase in SigD activity, we analyzed expression from two SigD-dependent promoters using transcriptional fusions to a *lacZ* reporter gene. Based on the unchained, flagellated phenotype, the increase in mobility observed on swimming plates, and the increase in SigD abundance, we expected that the *relA* mutant would exhibit significantly higher levels of SigD activity than the wild type. The results of β -galactosidase assays performed on samples collected from mid-log-phase growth are shown in Fig. 5B. As expected, both P_{hag} , the flagellin promoter and P_{lytA} , which drives expression of proteins important for flagellar activation through peptidoglycan hydrolysis (37), showed significantly higher levels of transcription in the *relA* mutant than in the wild-

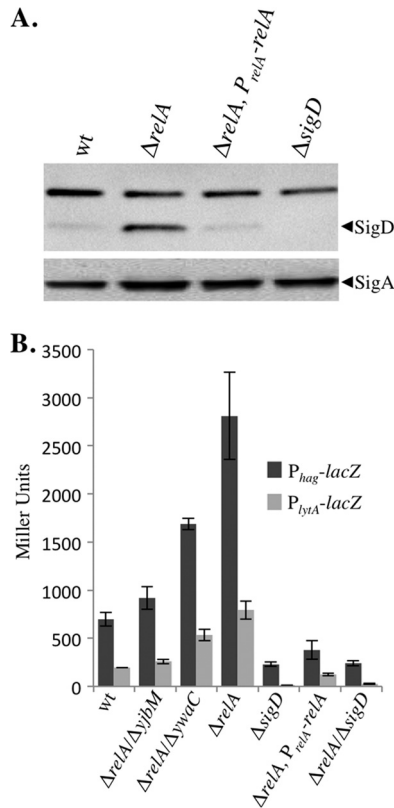


FIG 5 Loss of *relA* leads to an increase in SigD levels and activity. (A) Comparison of SigD protein levels in wt (BJH001), $\Delta relA$ (BQA009), $\Delta relA P_{relA}\text{-}relA$ (BQA068), and $\Delta sigD$ (BQA022) strains. Samples were collected from mid-log-phase cultures grown in CH medium. Proteins from cell lysates were separated by SDS-PAGE and analyzed by Western blot analysis by probing with either anti-SigD or anti-SigA antibody, as indicated. (B) β -Galactosidase assays of $P_{hag}\text{-}lacZ$ and $P_{lytA}\text{-}lacZ$ transcriptional activities conducted on wt (BJH046 and BJH047), $\Delta relA$ (BQA050 and BQA051), $\Delta relA \Delta yibM$ (BQA086 and BQA087), $\Delta relA \Delta ywaC$ (BQA088 and BQA089), $\Delta sigD$ (BQA071 and BQA072), $\Delta relA P_{relA}\text{-}relA$ (BQA073 and BQA074), and $\Delta relA \Delta sigD$ (BQA084 and BQA085) strains. Samples were collected from mid-log-phase cultures grown in CH medium. The data shown are the means of three independent replicates with standard deviations.

type control. The increased expression in the *relA* mutant was dependent on the presence of *sigD* (Fig. 5B, $\Delta relA \Delta sigD$). In contrast, expression was reduced to approximately wild-type levels in the *relA*-complemented strain. The $\Delta relA \Delta yjbM$ and $\Delta relA \Delta ywaC$ mutants exhibit intermediate levels of SigD activity (Fig. 5B), consistent with the intermediate chaining (Fig. 2B) and swimming motility (Fig. 4B) phenotypes we observed. We conclude that a RelA-dependent activity regulates the amount of active SigD that accumulates during the exponential growth phase and that the $\Delta relA$ mutant is shifted to a SigD on state during exponential growth.

DISCUSSION

Nucleotide second messengers, like cyclic di-GMP, cyclic AMP (cAMP), and the bacterial alarmone (p)ppGpp, are well-characterized examples of intracellular signaling molecules that play important roles in modulating gene expression in response to environmental and physiological fluxes in bacteria (11, 44, 45). (p)ppGpp is responsible for the global downregulation of transcription, translation,

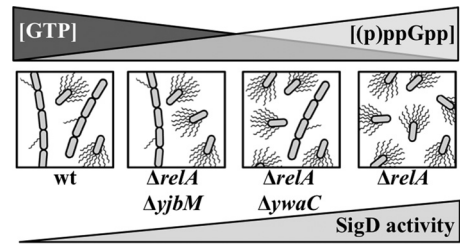


FIG 6 Model depicting the observed relationships between cell motility and chaining, SigD activity, GTP concentration (22), and putative (p)ppGpp levels in various (p)ppGpp synthetase/hydrolase backgrounds.

DNA replication, and growth rate that occurs during the stringent response to amino acid limitation (10–12). In more recent years, (p)ppGpp has been implicated in a number of nonstringent processes, including virulence (46–48), persister cell formation (49–51), biofilm production (52), maintaining a balanced growth rate (53), and regulating GTP pools in response to nutrient downshift (54).

A role for (p)ppGpp in modulating cell motility has also been previously reported for several Gram-negative bacteria, including *Vibrio cholerae* (55), *Legionella pneumophila* (56), and *E. coli* (57–59). For example, (p)ppGpp and the regulatory protein DksA have been shown to act directly on flagellar biosynthesis promoters to negatively regulate flagellum production during *E. coli* stationary-phase growth (59). In this study, we observed that a *B. subtilis relA* mutant deficient for (p)ppGpp hydrolysis (22) grows as a homogeneous population of unchained, motile cells during exponential growth in rich medium, a time when our wild-type laboratory strain is predominantly chained and nonmotile. These results suggest that increased (p)ppGpp levels or decreased GTP levels promote differentiation of *B. subtilis* (Fig. 6).

Since the levels of (p)ppGpp present in the strain backgrounds and under the growth conditions presented in this study fall outside the current quantitative-detection range, we tested our model genetically using (p)ppGpp synthetase mutants. We found that an increase in cell motility (Fig. 4B) and loss of cell chaining (Fig. 2B) directly correlate with the (p)ppGpp levels predicted to be produced in $\Delta relA$ (highest), $\Delta relA \Delta ywaC$ (intermediate), and $\Delta relA \Delta yjbM$ (lowest) mutants during exponential growth (Fig. 2 and 6). In contrast, the increase in cell motility and loss of chaining inversely correlate with the GTP levels measured in the $\Delta relA$ (lowest), $\Delta relA \Delta ywaC$ (intermediate), and $\Delta relA \Delta yjbM$ (highest) backgrounds (22). Our data also demonstrate that a $\Delta relA$ mutant has elevated levels of SigD activity compared to the wild type (Fig. 5B), consistent with the homogeneous population of unchained and flagellated cells we observed microscopically. These results support a model in which (p)ppGpp levels act as a modulator of SigD activity and, subsequently, cellular differentiation (Fig. 6). More specifically, our data suggest that even slight increases in (p)ppGpp may promote a SigD on activation state during exponential growth phase (Fig. 6). The absence of RelA activity clearly has a profound effect on bacterial decision making, dramatically shifting the bias of exponentially growing *B. subtilis* cells from a nonmotile, chained wild-type population (85%) to a homogeneous population of unchained, motile cells in the $\Delta relA$ mutant (Fig. 2A and B).

How might changes in (p)ppGpp levels and/or a decrease in GTP levels promote the activation of SigD and thus promote the

increased frequency of unchained, swimming cells we observed in *relA* mutant backgrounds? SigD activity is regulated at the level of protein synthesis (43) and by being held in an inactive state through the action of the anti-sigma factor FlgM (60, 61). We showed that, compared to the wild type, SigD is both more abundant and more active in the *relA* mutant (Fig. 5A and B). Since the global regulator DegU has been shown to repress SigD production (9) and promote FlgM synthesis (62), determining the role of DegU in the single-cell, swimming phenotype we observed is a potential avenue for future investigations. Interestingly, we observed that a $\Delta relA \Delta sigD$ mutant does not grow as long chains like the $\Delta sigD$ parent (Fig. 2C). This result suggests that the absence of *relA* promotes activation of some SigD-independent autolysins (63). One attractive idea that could explain both the $\Delta relA$ and the $\Delta relA \Delta sigD$ phenotypes is that elevated (p)ppGpp levels in *B. subtilis* may promote sigma factor switching such as has been reported in *E. coli* (64–66). In *E. coli*, (p)ppGpp has been shown to bind to RNA polymerase and, in concert with DksA, to directly regulate gene transcription (15). *B. subtilis* lacks a known DksA homolog, and direct regulation of RNA polymerase by (p)ppGpp has not been observed (66). Moreover, *B. subtilis* lacks conservation in the RNA polymerase (RNAP) residues that make contact with ppGpp in *E. coli* (67), suggesting that if (p)ppGpp directly regulates RNAP in *B. subtilis*, then the mechanisms will diverge significantly.

The *B. subtilis* $\Delta relA$ mutant harbors reduced levels of GTP (22, 28), which may also contribute to the motile, unchained phenotype we observed. (p)ppGpp synthesis affects GTP levels through at least two independent mechanisms. First, GDP, GTP, and ATP are the substrates for (p)ppGpp-synthetic enzymes, and (p)ppGpp synthesis is correlated with a subsequent drop in GTP levels (8, 15, 30, 68). Second, a recent study in *B. subtilis* showed that (p)ppGpp can also negatively regulate cellular GTP levels by binding directly to two key enzymes in the GTP synthesis pathway, HptR and Gmk, thereby negatively regulating their ability to synthesize GMP and GDP precursors, respectively (54).

It is possible that lower GTP levels present in the *relA* mutant relieve transcriptional repression of some promoters in the *SigD* regulon through CodY derepression. CodY is a GTP-sensing protein that binds DNA and positively or negatively regulates the expression of over 100 *B. subtilis* gene products (69). Although CodY has been shown to repress expression from *hag* (7), a recent global analysis found no evidence for binding of CodY to the *hag* promoter *in vivo* (69). Previous studies also showed that transcription can be affected by shifts in the GTP and ATP pools of cells. In *B. subtilis* undergoing stringent response through amino acid starvation, (p)ppGpp and ATP levels rise while GTP levels fall (54, 70). Promoters beginning with a +1 G are apparently particularly sensitive to GTP pools, as GTP is rate limiting for initiation (70, 71). Therefore, it is possible that the lower GTP levels of the *relA* mutant lead to decreased expression from +1 G promoters. Both of the *fla-che* promoters P_{D-3} and P_A , which regulate the bulk of *sigD* expression, begin with a +1 A (72), while the *flgM* promoter begins with a +1 G (70).

In summary, our results show that during exponential growth, RelA activity leads to the modulation of gene expression in *B. subtilis* and support the idea that even minor fluxes in (p)ppGpp levels may greatly influence cellular decision making. More specifically, we show that the *B. subtilis* $\Delta relA$ mutant is trapped in a SigD on state, biasing cells toward a motile, unchained phenotype

during exponential growth. Future experiments will be aimed at elucidating the molecular mechanisms underlying this developmental outcome.

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