Robust Stoichiometry of FliW-CsrA Governs Flagellin Homeostasis and Cytoplasmic Organization in *Bacillus subtilis*

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ABSTRACT Flagellin (Hag) is one of the most abundant proteins in *Bacillus subtilis*. Here we show that each flagellar filament is assembled from ~12,000 Hag monomers and that there is a cytoplasmic pool of Hag that is restricted to 5% of the total. Hag is thought to be restricted at the level of translation by a partner-switching mechanism involving FliW and the homodimeric RNA-binding protein CsrA (CsrA\textsubscript{dimer}). We further show that the mechanism of translation inhibition is hypersensitive due to a 1:1 ratio of Hag to FliW, a 1:1 inhibitory ratio of FliW to CsrA\textsubscript{dimer}, and a nearly 1:1 ratio of CsrA\textsubscript{dimer} to *hag* transcripts. Equimolarity of all components couples single-molecule detection of Hag export to compensatory translation and causes cytoplasmic Hag concentrations to oscillate around the level of FliW. We found that stoichiometry is ensured by genetic architecture, translational coupling, and the ability of CsrA\textsubscript{dimer} to restrict *hag* transcript accumulation. We further show that homeostasis prevents Hag hyperaccumulation that would otherwise cause severe defects in intracellular architecture, perhaps due to increased molecular crowding. We note that FliW-CsrA-mediated structural homeostasis has similarities to that seen with some toxin-antitoxin systems.

IMPORTANCE The intracellular concentration of flagellar filament protein Hag is restricted by the Hag-FliW-CsrA system in *B. subtilis*. Here we show that the Hag-FliW-CsrA\textsubscript{dimer} system functions at nearly 1:1:1 stoichiometry and that the system is both robust with respect to perturbation and hypersensitive to the Hag intracellular concentration. Moreover, restriction of cytoplasmic Hag levels is important for maintaining proper intracellular architecture, as artificial Hag hyperaccumulation led to generalized spatial defects and a high frequency of minicell production. The Hag-FliW-CsrA\textsubscript{dimer} system is conserved in the deeper branches of bacterial phylogeny, and we note that the Hag-FliW-CsrA “homeostasis module” resembles a toxin-antitoxin system where, by analogy, CsrA is the “toxin,” FliW is the “antitoxin,” and Hag is the target.

KEYWORDS FtsZ, flagella, fliC, homeostasis, minicell, motility, toxin-antitoxin

Homeostasis is the process in which a dynamic system is held in robust equilibrium to avoid extreme states that could be potentially wasteful, inefficient, or toxic. Negative feedback is the mechanism that generally governs homeostatic control. A classic example is the metabolic feedback inhibition in which the activity of the committed-step enzyme in a biosynthetic pathway is inhibited by the final product when that product accumulates above a certain threshold (1–3). Accordingly, the concentration of metabolite oscillates over a narrow range such that the cell immediately compensates for levels below the threshold by increasing synthesis and synthesis is inhibited when the concentration exceeds the threshold. Structural and highly abundant proteins, such as ribosome components and pilin, are also homeostatically controlled.
regulated by feedback inhibition (4–6). Here we explore the feedback inhibition controlling a structural subunit of the bacterial flagellum (7).

Bacterial flagella are primarily composed of long, helical extracellular polymeric filaments which, when rotated, act like propellers to push cells through the environment (8–10). Each filament is assembled from monomers of the protein flagellin (also known as Hag or FliC) that are synthesized in the cytoplasm and secreted by a dedicated type III secretion system within the flagellar basal body (11–14). Each flagellin monomer is secreted through the lumen of the nascent flagellum and polymerized at the tip of the extending structure by an extracellular chaperone, FlID (15–18). It has been estimated that approximately 20,000 flagellin monomers are needed to synthesize a single flagellar filament of Salmonella enterica, and the metabolic cost of filament synthesis is thought to be substantial (19). Consistent with a high synthetic cost, the flagellin primary sequence has evolved to disfavor energetically expensive amino acids, and complex regulatory pathways restrict flagellin expression (20, 21).

One way in which flagellin expression is restricted is by autoinhibition at the level of translation. In Bacillus subtilis, the Hag flagellin protein autoinhibits its translation through a partner-switching mechanism centered on the proteins FliW and CsrA (7) (Fig. 1A). Cytoplasmic Hag binds and inhibits FliW, and inhibition of FliW is relieved when Hag is exported for polymerization into the filament (22–24). Free FliW then binds and noncompetitively inhibits homodimers of the RNA-binding protein CsrA (CsrA\textsuperscript{dimer}), preventing CsrA\textsuperscript{dimer} from binding and occluding the Shine-Dalgarno (SD) sequence of the hag transcript to promote Hag translation (25–27). Thus, Hag secretion activates Hag translation for the duration of filament assembly (Fig. 1A and B). Moreover, when filament assembly is complete, secretion is reduced and Hag levels are thought to rise in the cytoplasm, causing Hag to bind to FliW and release CsrA\textsuperscript{dimer}, thereby restoring the ability of CsrA to repress hag translation (7). In toto, Hag-FliW-CsrA constitutes a three-node negative-feedback loop predicted to homeostatically restrict Hag levels within a narrow range of concentrations in the cytoplasm (7, 28). The homeostatic level of Hag relative to that of FliW-CsrA\textsuperscript{dimer} and the biological function for Hag homeostasis are poorly understood.

Here we explore the model of Hag homeostasis by measuring the levels of each protein involved in the negative-feedback loop. We show that low levels of Hag are maintained by equimolar amounts of FliW, CsrA\textsuperscript{dimer}, and hag transcript such that the system rests at a 1:1:1:1 ratio. The stoichiometric equilibrium was robust with respect to perturbations in flagellar secretion and was ensured by genetic architecture and translational coupling. Thus, Hag feedback is governed by strict stoichiometry poised to increase Hag translation when cytoplasmic Hag levels decrease to below a homeostatic threshold set by the titration of FliW. We further found that the feedback loop is hypersensitive such that even undetectable increases of CsrA levels above equilibrium inhibit motility due to a decrease in hag transcript abundance. The Hag-FliW-CsrA system is conserved in the deeper branches of bacterial phylogeny, but the generalized form of structural homeostasis, in which a protein autoinhibits its own synthesis through direct interaction with a simple regulatory module, may be widespread (29–31). We note that the molecular architecture and regulatory logic of FliW-CsrA resemble those of toxin-antitoxin systems (32). Thus, toxin-antitoxin systems could be “homeostasis modules” for essential proteins and limit potentially toxic accumulation of polymerases, ribosomes, or FtsZ.

RESULTS

Five percent of the flagellin pool is cytoplasmic. The flagellar filament is assembled from subunits of the Hag flagellin protein. Two approaches were taken to estimate the number of flagellin molecules per filament. First, cells encoding a cysteine-modified form of the Hag protein (Hag\textsuperscript{T209C}) were fluorescently labeled with a maleimide dye, and the average length of 40 filaments was measured by microscopy to be 7 ± 1 μm (33). Multiplying the average length (7 μm) by the value of 2,174 Hag monomers/μm determined by structural analysis of the B. subtilis filament (34) resulted in a value of

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In parallel, quantitative Western blot analysis was conducted on lysates of exponentially grown *B. subtilis* using an antibody raised against the Hag protein and an infrared-based Licor detection system. A standard curve was generated with purified Hag protein that was diluted with a cell lysate of a hag mutant to maximize transfer similarity to the whole-cell lysates with which it would be compared (see Fig. S1A in the supplemental material). An average of 130,000 Hag molecules was calculated per cell (Fig. 2A; see also Table S1 in the supplemental material). Dividing the quantitative Western blotting-based count (130,000 Hag/cell) by an estimated 15 filaments/cell results in a value of approximately 9,000 Hag proteins/filament. Averaging the two approaches, we estimated that each filament has ~12,000 Hag subunits. We conclude that Hag is highly abundant.
The Hag protein is found in two types of cellular locations: extracytoplasmic locations (as a polymerized flagellar filament) and cytoplasmic locations (as a soluble pool). To determine the number of Hag molecules in the cytoplasm, extracytoplasmic polymerization was abolished by two different approaches. In one approach, secretion of Hag was abolished by disrupting the \textit{flgE} gene encoding the flagellar hook protein FlgE (37, 38). In another approach, secretion of Hag was made constitutive but polymerization was abolished by disrupting the \textit{fliD} gene encoding the filament cap chaperone FliD (23, 39). Quantitative Western analysis indicated that there were \(\sim 2,000\) Hag monomers per \textit{flgE} mutant cell and \(\sim 6,000\) Hag monomers per \textit{fliD} mutant cell (Fig. 2A; see also Table S1). We suspect that the cytoplasmic concentration of Hag in the wild-type (WT) strain is probably closer to that observed in the \textit{fliD} mutant as motile cells would need to continuously synthesize new flagella during growth. Thus, by dividing the value of 6,000 monomers in the \textit{fliD} cytoplasm by the value of 130,000 monomers in whole-cell lysates of the wild type, we estimated that the cytoplasmic pool of Hag represents \(\sim 5\%\) of the total.

We speculate that the amount of Hag in the cytoplasm of the \textit{flgE} mutant is lower than that in the theoretical pool in the wild type because Hag transcription and translation are coupled to Hag secretion. When cells cannot complete the flagellar hook (FlgE), the anti-sigma factor FlgM inhibits \textit{hag} dependent transcription of the \textit{hag} gene, and \textit{hag} expression is further inhibited at the translational level by the RNA-binding protein CsrA (Fig. 1A) (28, 40–43). To determine the relative contributions of the regulators to the intracellular levels of Hag, the genes \textit{flgM} and \textit{csrA}, encoding FlgM and CsrA, respectively, were mutated in a background that was also mutated for \textit{flgE}. The \textit{flgE flgM} double mutant had \(\sim 8,000\) Hag monomers per cytoplasm, likely restricted by CsrA. The \textit{flgE csrA} double mutant had \(\sim 24,000\) Hag monomers per cytoplasm, despite the fact that transcription was impaired by FlgM (Fig. 2A; see also Table S1). A \textit{flgE flgM csrA} triple mutant had \(\sim 250,000\) Hag monomers, a value greater than that seen with either mutant alone and on par with the total number of subunits observed in the wild type, with the exception that they were entirely trapped within the cytoplasm. We conclude that FlgM and CsrA synergize to prevent cytoplasmic accumulation of Hag and that CsrA is the dominant inhibitor.

\textbf{CsrA\textsubscript{dimer} and FliW are present at a 1:1 ratio in the cytoplasm.} CsrA is part of a three-node negative-feedback loop that restricts Hag translation in a manner dependent on the level of cytoplasmic Hag (7, 28) (Fig. 1A). CsrA binds RNA as a dimer and CsrA\textsubscript{dimer} proteins are noncompetitively inhibited when bound by FliW (25, 26, 44, 45). FliW switches binding partners between CsrA and Hag to sense Hag levels and to
Fig. 3A

regulate Hag translation accordingly. As the model runs on protein-protein interactions, the relative numbers of those proteins should be important. To determine the amount of FliW and CsrA in the cytoplasm, quantitative Western blot analysis was conducted. Wild-type cells had ~8,000 CsrA dimer and ~9,000 FliW monomers in the cytoplasm, representing a nearly equimolar ratio (Fig. 2B and C; see also Table S1). Furthermore, the levels of CsrA dimer and FliW were robust with respect to perturbation as their cytoplasmic concentrations remained relatively unchanged even when flagellar assembly was disrupted by mutation of flgE or flgE and flgM or flgD. We infer that Hag levels in the cytoplasm are restricted by the equilibrium concentrations of CsrA dimer and FliW monomers such that the entire system is homeostatically maintained near a 1:1:1 ratio.

Structural analysis indicated that each CsrA dimer was bound by two separate molecules of FliW (FliW2:CsrA dimer), but the quantitation described above suggested that FliW levels in vivo were subsaturating (FliW1:CsrA dimer) (27). To determine whether one molecule of FliW was sufficient to antagonize a dimer of CsrA, we purified CsrA as a heterodimer (CsrA hetero) in which one monomer was proficient (CsrA WT-His6) and one monomer was deficient (CsrA N55D-strep [Strep-tag]) in binding FliW (26). To generate CsrA hetero, both the CsrA WT-His6 and CsrA N55D-strep fusions were expressed from the same vector and purified using sequential affinity purifications. The heteromeric complex was confirmed by the presence of two bands of similar intensities by the use of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. S2). To determine the RNA-binding affinity of CsrA hetero to the 5′ untranslated region (5′ UTR) of the hag transcript (+1 to +100), an RNA electrophoretic mobility shift assay (EMSA) was performed. CsrA hetero was found to bind the hag 5′ UTR with an apparent dissociation constant (Kd, app) of 460 nM, a value that was intermediate between the values determined for the CsrA WT-His6 homodimer (Kd, app = 290 nM) and the CsrA N55D-strep homodimer (Kd, app = 960 nM) (Fig. S3A, B, and C). We conclude that CsrA hetero was functional for RNA binding.

We next sought to determine whether the addition of the FliW protein would antagonize the RNA-binding activity of CsrA hetero, which has only one FliW binding site. As shown previously, addition of FliW alone resulted in an electrophoretic mobility shift of the hag 5′ UTR, but the FliW-mRNA affinity was 500-fold weaker than the affinity of FliW for CsrA (7) (Fig. 3A; see also Fig. S3D). Addition of FliW reduced the amount of CsrA WT bound to RNA under conditions of roughly equimolar concentrations, and excess FliW was bound as FliW levels further increased (Fig. 3B; see also Fig. S4A). In contrast, addition of FliW did not appear to decrease the abundance of CsrA N55D-bound RNA under conditions of roughly equimolar concentrations but rather appeared to compete for RNA binding at higher concentrations (Fig. 3A and C; see also Fig. S4B). Finally, the shift pattern seen after addition of FliW to CsrA hetero resembled the shift pattern observed with the wild type and was indicative of inhibition (Fig. 3D; see also Fig. S4C). We conclude that while each dimer of CsrA has the capacity to bind two molecules of FliW, the presence of only one molecule of FliW is necessary and sufficient to inhibit the interaction between CsrA dimer and RNA.

The amounts of FliW and CsrA dimer appear to be kept at a precise stoichiometric ratio. One way in which the stoichiometric ratio might be maintained is by translational coupling as the reading frame of fiw is upstream of and overlaps the reading frame of csrA (Fig. 4A). To determine whether translation of CsrA was coupled to that of FliW, a nonsense mutation was introduced into the middle of the fiw reading frame to create a premature stop codon (FiwE71*). Consistent with translational coupling, the premature stop codon in fiw abolished accumulation of both FliW and CsrA, resulting in a resemblance to a fiw csrA double deletion mutant (Fig. 4B). That the CsrA levels in the FiwE71* allele were undetectable was not a consequence of proteolysis in the absence of FliW, as CsrA was abundant in a strain containing an in-frame markerless deletion of fiw that preserved coupling (Fig. 4B). Moreover, overexpression of CsrA alone from an ectopic locus in the FiwE71* mutant resulted in motility inhibition, indicating that CsrA expression does not specifically require the presence of FliW (Fig. 4C). We conclude that expression of CsrA is translationally coupled to that of FliW.

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Translational coupling often involves internal Shine-Dalgarno sequences, and *csrA* is preceded by what appears to be a separate putative SD sequence (Fig. 4A). To determine the contribution of the *csrA* SD sequence, the sequence was mutated away from the consensus (GGAGG/GGTGG), simultaneously introducing a silent mutation in *fliW* (*fliW*139G; Fig. 4A). The *csrA* SD mutant (*fliW*139G) appeared to result in a slight qualitative reduction in the amount of CsrA protein in Western blot analysis, while *FliW* protein levels appeared to be unaffected (Fig. 4B). While the effect of the CsrA SD sequence mutation appeared minor, slight changes in the level of CsrA might have phenotypic consequences. To determine the biological consequence of a minor reduction in CsrA levels, CsrA was artificially expressed from an ectopic site in various mutant backgrounds. As previously reported, artificial ectopic expression of CsrA inhibited swarming motility when the native copy of *csrA* was also present in merodiploid (26) (Fig. 4D; see also Fig. S5). Artificial ectopic expression of *csrA* in the *csrA* SD sequence mutant (*fliW*139G) background resulted in only partial inhibition of swarming motility, suggesting that wild-type levels of CsrA from the native site were necessary for full inhibition (Fig. 4D). We conclude that the SD sequence in front of *csrA* plays a seemingly minor but nonetheless important role in maintaining CsrA levels, and we infer that the ratio of FliW to CsrA dimer must be precisely maintained.

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To further explore the importance of FliW:CsrA dimer stoichiometry, we titrated CsrA by gradual induction of an ectopically integrated, IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible *csrA* construct in merodiploid with the native copy. Swarming motility was partially inhibited when the construct was induced with 0.1 mM IPTG and was fully inhibited at 1 mM IPTG as previously reported (26) (Fig. 5A, left; see also Fig. S5, left). Quantitative Western blot analysis showed, however, that the levels of CsrA dimer and FliW appeared constant at all levels of IPTG induction, whether or not motility was inhibited (Fig. 5A, right; see also Table S2). We confirmed that the titers of
the IPTG-inducible promoter were nonetheless determinable, as CsrA levels increased with increasing amounts of IPTG in a background in which the native copy of $csrA$ was deleted (Fig. 5B, right; see also Table S2). In the native $csrA$ deletion, maximal IPTG induction elevated CsrA to wild-type levels, but, as previously reported, motility was not inhibited perhaps because native FliW is sufficient (26) (Fig. 5B, left; see also Fig. S5 right). We conclude that maintenance of both the levels and the ratio of FliW to CsrA dimer is robust with respect to artificial perturbation and appears to be restricted to a maximum. We further conclude that motility inhibition from ectopic CsrA expression was not correlated with either an increase in the total complex or an excess of CsrA dimer.

One way to explain the need for the native copy of $csrA$ to inhibit motility when $csrA$ was artificially expressed from an ectopic locus is that the location of the native copy was critical and somehow inhibited $hag$ translation in cis. A cis effect is plausible, as the genes encoding CsrA and Hag are adjacently positioned at the native locus. To test the relevance of gene location, the $hag$ gene was deleted at the native site and complemented at the ectopic $thrC$ site in the chromosome ($thrC::P_{hag-hag}$). Artificial ectopic expression of $csrA$ from the $amyE$ site in the chromosome resulted in inhibition of the strain containing the $csrA$ gene at the native locus even when the $hag$ gene was separated by relocation to $thrC$ (Fig. S6). Thus, we conclude that genetic adjacency of $csrA$ and $hag$ was irrelevant for inhibition and that translation inhibition by CsrA was
not due to a cis effect. We further conclude that ectopic expression of CsrA inhibits motility in trans, likely by titrating levels of FliW expressed from the native locus. Moreover, inhibition is not due to detectable changes in CsrA protein levels and we infer that inhibition is accomplished by another, as-yet-untested variable.

\[\text{hag} \text{ transcripts/cell} \]

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One variable that could account for the motility inhibition seen under conditions of ectopic expression of CsrA in merodiploid is the stoichiometric ratio between the hag transcript and CsrA\text{dimer} (7, 25) (Fig. 1A). To measure the number of hag transcripts per cell, quantitative reverse transcriptase PCR (qRT-PCR) was conducted. To generate a standard curve that would account for extraction efficiency, defined amounts of hag transcript were added to a lysate of a hag mutant, reextracted, serially diluted, and used as a template for qRT-PCR (Fig. 5D). Artificial expression of CsrA in merodiploid reduced the levels of hag transcripts 3-fold relative to the wild-type levels, and the hag

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One variable that could account for the motility inhibition seen under conditions of ectopic expression of CsrA in merodiploid is the stoichiometric ratio between the hag transcript and CsrA\text{dimer} (7, 25) (Fig. 1A). To measure the number of hag transcripts per cell, quantitative reverse transcriptase PCR (qRT-PCR) was conducted. To generate a standard curve that would account for extraction efficiency, defined amounts of hag transcript were added to a lysate of a hag mutant, reextracted, serially diluted, and used as a template for qRT-PCR (Fig. 5D). Artificial expression of CsrA in merodiploid reduced the levels of hag transcripts 3-fold relative to the wild-type levels, and the hag
transcript levels were restored when csrA was artificially expressed in a strain deleted for the native copy of csrA (Fig. 5C; see also Table S3). We conclude that artificial expression of CsrA in merodiploid inhibited motility, not because of an increase in total CsrA dimers but rather because of a decrease in the abundance of the hag transcript target (Fig. 5A).

To explore whether a single copy of CsrA encoded at the native site could restrict hag transcript levels, qRT-PCR was used to count hag transcripts in a variety of mutant backgrounds. Wild-type cells had ~22,000 copies of the hag transcript, representing a slight, 2-to-3-fold excess of the number of CsrA dimers and FliW monomers, which was perhaps consistent with active flagellar filament assembly (Fig. 2D; see also Table S1). When flagellar filament assembly was disrupted by mutation of FliD or FlgE, the levels of hag transcripts increased to ~140,000 copies or decreased to ~500 copies, respectively (Fig. 2D; see also Table S1). The dramatic changes in copy number were likely due to transcriptional regulation, as cells mutated for flID constitutively secrete both flagellin and FlgM whereas cells mutated for FlgE constitutively accumulate FlgM in the cytoplasm (43). Consistent with transcriptional inhibition by FlgM, mutation of flgM in the flgE mutant background increased hag transcript numbers 48-fold, but hag transcript levels did not rise to those observed in the flID mutant (Fig. 2D; see also Table S1). CsrA also appeared to inhibit hag transcript levels as transcript numbers increased 28-fold in the flgE csrA double mutant and 174-fold in the flgE flgM csrA triple mutant to phenocopy the constitutively secreting flID mutant state (Fig. 2D; see also Table S1). Thus, FlgM and CsrA synergize to repress hag transcript accumulation and we conclude that CsrA inhibits hag transcript accumulation by a mechanism independent of, and in parallel to, that of FlgM.

CsrA is unlikely to inhibit transcription initiation of hag, as mutation of csrA was shown to have no effect on a P_hag transcriptional reporter construct lacking the CsrA binding site (7). CsrA could inhibit hag transcript levels, however, by a mechanism dependent on its RNA binding activity. To determine if hag transcript reduction was dependent on direct interaction with CsrA, a sow3 allele which contains a mutation in the high-affinity binding site for CsrA and reduces the CsrA binding affinity 25-fold was introduced (7). The sow3 background had amounts of hag transcript equivalent to those seen with the wild type and prevented the reduction in hag transcript abundance when CsrA was artificially expressed in merodiploid (Fig. 5C; see also Table S3). We conclude that binding of CsrA to the hag 5’ untranslated leader sequence both inhibits hag translation and restricts hag transcript accumulation to maintain nearly equivalent stoichiometries of hag transcript, CsrA dimer, FliW, and, ultimately, Hag in the cytoplasm.

Hag homeostasis preserves cellular spatial organization. FlgM and CsrA synergize to restrict the cytoplasmic level of Hag protein, but the physiological reason that Hag levels are tightly restricted is unclear. Compared to the wild type, the flgE flgM csrA triple mutant suffered minor growth defects, including a modest increase in lag period, a decrease in growth rate, and a decrease in growth yield (Fig. 6A). The growth-related defects were primarily due to expression of the Hag protein, as a wild-type growth profile was restored by mutating the hag gene in the flgE flgM csrA triple mutant background (Fig. 6A). Phase-contrast microscopy indicated, however, that the flgE flgM csrA triple mutant produced what appeared to be minicells at high frequency and that the minicell production was Hag dependent (Fig. 6B).

Minicells are anucleoid products of aberrant FtsZ ring placement (46–48). To test whether FtsZ rings were mislocalized, a functional mNeonGreen-FtsZ fusion was integrated into various genetic backgrounds (65). Consistent with minicell formation, FtsZ placement in the flgE flgM csrA mutant was often polar and the small division products were found to lack nucleoids under conditions of staining with the fluorescent dye DAPI (4’,6-diamidino-2-phenylindole) (Fig. 6C). Moreover, FtsZ fluorescence intensity was reduced in the flgE flgM csrA triple mutant, perhaps due to frequent loss of the FtsZ pool to the inert minicell population. Finally, nucleoids of the flgE flgM csrA triple mutant appeared to be highly compacted and often centered in the cytoplasm.
The cytological defects observed in the \textit{flgE flgM csrA} triple mutant were corrected when \textit{hag} was also deleted (Fig. 6C). We conclude that hyperaccumulation of Hag in the cytoplasm caused generalized defects in intracellular organization and that Hag homeostatic control is necessary to ensure proper cell growth and division in motile cells.

**DISCUSSION**

The bacterial flagellum is an elaborate multisubunit complex assembled from dozens of different structural proteins in numbers that range from 6 copies of a
particular rod subunit to the \(\sim 12,000\) copies of flagellin measured here (34, 49). How the relative stoichiometries of flagellar structural subunits are rigorously governed over 4 orders of magnitude is poorly understood. Here we show that Hag levels in the cytoplasm are restricted to approximately 5% of the total pool by a roughly 1:1:1:1 ratio of Hag/FliW/CsrAdimer/hag transcript at equilibrium, relieved by Hag export during filament assembly (Fig. 1A). Strict equimolar ratios permit hypersensitive detection of Hag by FliW titration and an equally sensitive response in Hag translation as CsrAdimer titrates hag transcripts. We predict that the homeostatic level of Hag oscillates in the cytoplasm over a narrow concentration range that is ultimately set by the amount of FliW (Fig. 1B).

FliW binds either one molecule of Hag or one dimer of CsrA in a partner-switching mechanism. If the affinity of the FliW-CsrA interaction dominated over or was even equal to that of Hag \(\textit{in vivo}\), the levels of free hag transcript should be abundant (Fig. 1C, top and middle). An abundance of free hag transcript would cause Hag levels in the cytoplasm to increase and significantly exceed those of FliW before equilibrium would be restored, but that was not observed. Instead, we found that Hag and FliW levels were nearly equivalent \(\textit{in vivo}\), and thus we infer that the Hag-FliW interaction likely dominates (Fig. 1C, bottom). If FliW is primarily sequestered by Hag, then CsrAdimer proteins primarily bind to and inhibit translation of hag transcripts (Fig. 1C bottom). Domination of the FliW-Hag interaction would be advantageous to permit detection of nearly single-molecule variations in the Hag protein pool; for every molecule of Hag by which the amount of FliW exceeds that of Hag, one molecule of hag transcript is released to support compensatory translation (Fig. 1B). Seemingly inconsistent with a dominating FliW-Hag interaction is that FliW appears to have a higher affinity for CsrAdimer than Hag \(\textit{in vitro}\) (7), but the relative pairwise affinity does not take into account the other members of the \(\textit{in vivo}\) system, including the hag transcript. Thus, based on the \(\textit{in vivo}\) quantitation performed here, we conclude that FliW provides hypersensitive detection of Hag and that Hag oscillates around the FliW concentration at steady state.

Just as one molecule of Hag inhibits one molecule of FliW, here we show that only one molecule of FliW is needed to antagonize a dimer of CsrA. Three-dimensional (3D) structure analysis of the complex clearly indicates, however, that a second molecule of FliW can bind to a CsrAdimer, and secondary binding may be a mechanism to dampen the derepression of Hag translation (27). During steady-state Hag oscillation, relatively few FliW molecules are released, and likely each binds to free CsrA dimers. When Hag concentrations drop rapidly, however, perhaps when two or more flagellar basal bodies simultaneously draw from the pool to assemble filaments, an excess of FliW might be released that could bind to unbound CsrA dimers or to CsrA dimers already bound to one FliW. As a consequence, all of the FliW\(_2\):CsrAdimer pairings would create equal numbers of CsrAdimer bound transcripts and dampening would occur (Fig. 1C, bottom). The critical 1:2 stoichiometric ratio of FliW to CsrA is governed, at least in part, by genetic architecture and translational coupling such that even small reductions in the level of CsrA, such as those caused by mutation of an internal ribosome binding site (RBS), have biological consequences on motility. We predict that other systems must also be present to restrict FliW-CsrA abundances, as their levels were constant and CsrA could not be artificially forced to hyperaccumulate.

Ectopic transcription of CsrA from a strong artificial promoter inhibited motility only when it was expressed in merodiploid (24); the motility inhibition was not due to CsrA hyperaccumulation and was instead due to a reduction in the level of hag transcript. CsrA was found to be inhibitory with respect to hag transcript levels in general, as a csrA mutation elevated hag transcript levels in a flgE mutant nearly as much as mutation of the transcription-level anti-sigma factor FlgM. The mechanism by which CsrA restricts hag transcript levels is unknown but requires the CsrA binding site within the RNA target, and CsrA may control transcript stability such as it does for some transcripts in other organisms (50–54). The amount of hag transcript corresponding to the 5’ UTR is important and is regulated at multiple levels, and we note that FliW also binds the hag
leader sequence for as-yet-unknown reasons. Ultimately, hag transcript can be made to hyperaccumulate either in a strain in which FlgM and CsrA are antagonized by constitutive secretion of flagellin (fliD) or in a strain that cannot secrete flagellin and that is simultaneously mutated for both FlgM and CsrA (flgE flgM csrA). In the case of the flgE flgM csrA triple mutant, derepression of both transcription and translation caused hyperaccumulation of the Hag protein in the cytoplasm such that its levels exceeded the amount normally polymerized on the outer side of the cell.

The biological reason for Hag homeostasis is unknown, but one reason could be a need to limit the energetic expenditure on flagellar assembly. We tend to think that energy conservation is of minor advantage, at least in nutrient-rich environments, as the flgE flgM csrA triple mutant produced more flagellin than the wild type but suffered only minor defects in growth. Instead, flagellin hyperaccumulation resulted in cells with severe defects in spatial organization, including, but perhaps not limited to, high-frequency FtsZ-ring misplacement, compacted asymmetrically positioned nucleoids, and aberrant cell morphology. While Hag could bind to and interfere with a specific cell division target, the generality of the defect suggests a nonspecific mechanism. One mechanism by which Hag hyperaccumulation could cause generalized space defects is by an increase in molecular crowding. The volume of a 4-μm-by-1-μm B. subtilis cell is roughly $4 \times 10^9$ nm$^3$, and a structure-based approximation of the volume of a single Hag subunit is $8.0 \times 10^4$ nm$^3$ (34). The homeostatic level of 6,000 subunits of Hag occupies approximately 0.06% of the wild-type cytoplasm, but that level increases to 2.5% of the total volume in the hyperaccumulating flgE flgM csrA triple mutant. The 62-fold volumetric increase in the level of the Hag pool may be significant in an already crowded cytoplasm, and Hag homeostasis may thus be critical to minimize molecular crowding. While nutrients may or may not be limiting, depending on the environment, we suggest that cytoplasmic volume is always at a premium.

Structural homeostasis may be a common mechanism to restrict hyperaccumulation of enzymes and structural proteins in the cell. While FliW-CsrA is highly specific for flagellin, we note that the genetic and molecular architecture of the module is remarkably similar to that of some type II toxin-antitoxin systems (32) (Fig. 1D). As with the genes encoding FliW-CsrA, toxin-antitoxin genes are often translationally coupled, the stoichiometric ratio of toxin to antitoxin is thought to be critical for function, and antagonism of the protein products is mediated by direct interaction (55–57). Moreover, both systems have mechanisms to restrict their own synthesis, and some toxins have very specific targets (58–60). All that remains to complete the analogy with FliW-CsrA is to have the target of the toxin bind and inhibit antitoxin activity (Fig. 1D). While other binding partners for antitoxins have not been reported, the possibility that other targets might exist may not have been considered (Fig. 1D). Thus, FliW could be considered an “antitoxin,” and CsrA could be considered a “toxin” that happens to act on a nonessential target, in this case, Hag. Conversely, some toxin-antitoxin systems could be homeostasis modules that restrict accumulation of essential proteins that could be harmful when synthesized in excess.

MATERIALS AND METHODS

Strains and growth conditions. B. subtilis strains were grown in lysogeny broth (LB) broth (10 g tryptone, 5 g yeast extract, 5 g NaCl per liter) or on LB plates fortified with 1.5% Bacto agar at 37°C. When appropriate, antibiotics were included at the following concentrations: 10 μg/ml tetracycline (tet), 100 μg/ml spectinomycin (Spec), 5 μg/ml chloramphenicol (Cm), 5 μg/ml kanamycin (Kan), and 1 μg/ml erythromycin plus 25 μg/ml lincomycin (mls). Isopropyl β-D-thiogalactopyranoside (IPTG; Sigma) was added to the medium at the indicated concentration when appropriate.

For quantitative swarm expansion assay, strains were grown to mid-log phase (optical density at 600 nm [OD$_{600}$] of 0.3 to 1.0) and concentrated to an OD$_{600}$ of 10 in pH 7.4 phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, 2 mM KH$_2$PO$_4$) containing 0.5% India ink (Higgins). LB plates containing 0.7% Bacto agar with or without various concentrations of IPTG were dried for 10 min in a laminar flow hood, centrally inoculated with 10 μl of the cell suspension, dried for another 10 min, and incubated at 37°C in a humid chamber. The swarm radius was measured along the same axis every 30 min.

Microscopy. Fluorescence microscopy was performed with a Nikon 80i microscope with a phase-contrast objective (Nikon Plan Apo; 100×) and an Excite 120 metal halide lamp. FM4-64 dye [N-(3-
TABLE 1 Strains

| Strain     | Genotype                                      |
|------------|-----------------------------------------------|
| 3610       | Wild type                                     |
| DK1042     | comF<sup>12</sup> (62)                        |
| DK1522     | Δcra amyE::P<sub>hag</sub>-<sub>cra</sub> spec (26) |
| DK2665     | ΔfliWcrA comF<sup>12</sup>                   |
| DK4205     | com<sup>F12</sup> cra<sup>REDO</sup>         |
| DK4476     | com<sup>F12</sup> ΔcraΔhag flgM::tet ΔflgE  |
| DK4659     | ΔflgE<sup>F12</sup> com<sup>F12</sup>       |
| DK4660     | ΔflgE<sup>F12</sup>                          |
| DK5092     | mNeonGreen-fltsZ comP<sup>F12</sup>          |
| DK5224     | Δhag amyE::P<sub>hag</sub>-hag<sup>290C</sup> spec swrA::tet |
| DK6073     | hag<sup>awt</sup> comP<sup>F12</sup>         |
| DK6082     | hag<sup>awt</sup> amyE::P<sub>hag</sub>-<sub>cra</sub> spec comP<sup>F12</sup> |
| DK6083     | flgM::tet ΔcraΔhag ΔflgE mNeonGreen-fltsZ comP<sup>F12</sup> |
| DK6084     | ΔflgE flgM::tet craA::kan mNeonGreen-fltsZ comP<sup>F12</sup> |
| DK7072     | Δhag thrC::P<sub>hag</sub>-hag<sup>290C</sup> mls amyE::P<sub>hag</sub>-<sub>cra</sub> spec |
| DS1677     | Δhag (33)                                     |
| DS2569     | ΔflgE<sub>S32</sub> (62)                     |
| DS4681     | ΔflgE (38)                                    |
| DS4940     | amyE::P<sub>hag</sub>-<sub>cra</sub> (7)      |
| DS6188     | Δcra (7)                                      |
| DS7646     | ΔflgE Δcra                                    |
| DS7791     | ΔfliD (23)                                    |
| DS5178     | ΔfliW                                         |
| DS5622     | ΔflgE flgM::tet                               |
| DS5975     | ΔflgE flgM::tet craA::kan                    |

The FliW-CsrA system was utilized with a C-FL HYQ Texas Red filter cube (excitation filter wavelength of 532 to 587 nm, barrier filter wavelength of >590 nm). DAPI was visualized using a UV-2E/C DAPI filter cube (excitation filter wavelength of 340 to 380 nm, barrier filter wavelength of 345 to 485 nm). mNeonGreen was visualized using a C-FL fluorescein isothiocyanate (FITC) filter cube (FITC; excitation filter wavelength of 460 to 500 nm, barrier filter wavelengths of 515–550 nm). Images were captured with a Photometrics Coolsnap HQ2 camera in black and white, subjected to false coloring, and superimposed using Meta-morph image software.

To stain membranes and nucleoids, cells were grown at 37°C to an OD<sub>600</sub> of 0.6 to 1.0, and 1 ml of culture was centrifuged, resuspended in 50 μl PBS containing 5 μg/ml FM4-64 (Molecular Probes) and 1 μg/ml DAPI (Molecular Probes), and incubated at room temperature for 10 min. Samples were observed by spotting 4 μl of suspension on either a microscope slide (followed by immobilization with a poly-L-lysine-treated coverslip) or an agarose pad (which was then covered with a glass coverslip). Agarose pads were creating by making a 1% solution of electrophoresis-grade agarose (Fisher Scientific) mixed in PBS and applying the molten solution to a slide, covering the molten solution with a glass microscope slide, and allowing the molten solution to cool for 5 min prior to use.

For fluorescence microscopy and measurement of flagellar filaments, a strain (DK5687) with a reduced number of flagella and encoding the modified Hag protein Hag<sup>290C</sup> was collected as described above but was resuspended in 50 μl PBS buffer containing 5 μg/ml Alexa Fluor 488 C<sub>5</sub> maleimide (Molecular Probes), and incubated for 5 min at room temperature (33°C). Cells were then washed twice with 500 μl PBS buffer and were stained for membranes as described above. Four microliters of suspension was placed on a glass microscope slide and immobilized with a poly-L-lysine-treated coverslip. Approximate filament lengths were measured using the measurement tool of ImageJ (61).

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**Strain construction.** All constructs were either introduced into a 3610-derived natural competent strain DK1042 (62) or introduced first by natural competence into a domesticated strain (PY79) or a site-directed mutations at the native site were performed by allelic replacement. Site-directed mutations at the native site were performed by allelic replacement.
assembly (64), subjected to restriction digestion using BamHI and KpnI, and introduced into the respective sites of pMiniMAD, which carries a temperature-sensitive origin of replication and an erythromycin resistance cassette, to generate pRO25, pRO30, pRO33, and pRO68. The plasmids were subsequently subjected to passage through recA-positive (recA+) *Escherichia coli* strain TG1 and transformed into DK1042. Plasmid pMiniM (and its derivatives pRO25, pRO30, pRO33, and pRO68) encodes an mls resistance cassette and a temperature-sensitive origin that is active at room temperature but not at 37°C. Plasmid integration was selected for by mls resistance at 37°C. To evict the plasmid, the strain was subsequently incubated at a room temperature overnight without antibiotic selection. Cells were then serially diluted and plated on LB agar at 37°C. Individual colonies were patched onto LB plates containing or not containing mls to identify mls-sensitive colonies that had evicted the plasmid. Chromosomal DNA from colonies that had excised the plasmid was purified, and an amplicon for sequencing was PCR amplified using primer pair 1341/4641 and sequenced for the appropriate mutation using primers 1541/1544 for pRO25, pRO30, and pRO33 and primers 1873/4641 for pRO68.

**In-frame deletions.** To generate the Δ*flgW* and Δ*flICsrA* in-frame markerless deletion constructs in the DK1042 background, plasmid pIP87 and pSG36, respectively, were introduced and excised following the protocol described above. Deletions of *flgW* and *flICsrA* were verified by PCR using primers 1871/1907.

To generate strains with a Δ*flgE* in-frame markerless deletion, plasmid pDP306 was introduced and excised following the protocol described above. Deletion of *flgE* was verified by PCR using primers 1483/1486. (i) Insertion-deletion alleles. The Δ*csrA:kan* (7) and Δ*flgM:tet* (43) insertion-deletion alleles was generated by long-flanking homology PCR (7, 43) and introduced into strain PY79 by natural competence and further introduced into appropriate strain backgrounds by SPP1-mediated transduction. (ii) Δ*ftsZ::mNeonGreen-ftsZ*. The Δ*ftsZ::mNeonGreen-ftsZ* construct was a gift from Ethan Garner and was introduced following a protocol that was described previously (65). Briefly, *erm-flsA-mNeonGreen-ftsZ-cat* was introduced into the appropriate strain backgrounds by SPP1-mediated transduction. After integration, plasmid pDR24 (containing Cre recombinase and a temperature-sensitive origin of replication) (a gift from David Rudner) was introduced by SPP1-mediated phage transduction, grown at 30°C, and selected for on Spec. Transductants were selected on LB, LB-Cm, LB-MLS, and LB-Spec and grown at 37°C overnight to enable us to look for colonies that grew only on LB. (iii) Δ*m integration. The Δ*m integration complementation construct at the ectopic site *lacA* (the T209C mutation is functional and allows labeling of the flagellar filament with cysteine-reactive maleimide dyes) was generated by restriction digestion of plasmid pNE4 with BamHI and SalI to excise *FlaI* and subjected to Coomassie staining to verify purification of CsrAN55D-strep. Final purification, storage, and subjected to Coomassie staining to verify purification of CsrAN55D-strep. Final purification, storage,
and concentration determination of CsrAN55D-strep protein were conducted following the protocol described above.

CsrAN55D-strep/CsrA-His6 heterodimer purification. To purify a CsrA heterodimer (CsrAN55D-strep; CsrA-His6), CsrAN55D-strep and CsrA-His6 were expressed simultaneously from dual-expression vector pRO76 by transformation into Rosetta-gami E. coli. The CsrA heterodimer was affininity purified following the His6 tag protocol described above. Eluted proteins were separated by SDS/PAGE and stained with Coomassie brilliant blue to verify purification of both CsrAN55D-strep and CsrA-His6. Fractions containing both constructs were pooled and concentrated to ~5 ml, and an equal volume of CsrA wash buffer was added to dilute out the imidazole. The pooled fractions were subsequently affininity purified following the strep-tag protocol described above. Eluted proteins were separated by SDS/PAGE and subjected to Coomassie staining to verify the presence of both CsrAN55D-strep and CsrA-His6. Final purification, storage, and concentration determination of CsrA Heterodimer were conducted by following the purification protocols described above.

His6-SUMO-FliW and -Hag protein purification. SUMO-FliW protein expression vector pSM12 and SUMO-Hag protein expression vector pSM56 were transformed into Rosetta-gami E. coli. Purification of the SUMO-FliW protein was performed by following the purification protocol described above for CsrA-His6. Purification of SUMO-Hag protein followed a protocol similar to that described above for CsrA-His6, but differed with respect to the buffer conditions by use of the following buffers: lysis buffer (50 mM Na2HPO4, 300 mM NaCl, 10 mM imidazole), wash buffer (50 mM Na2HPO4, 300 mM NaCl, 30 mM imidazole), and elution buffer (50 mM Na2HPO4, 300 mM NaCl, 500 mM imidazole). Eluted proteins were separated by SDS/PAGE and subjected to Coomassie staining to verify purification of the SUMO-FliW/Hag fusion. Purified SUMO-FliW/Hag was combined with ubiquitin ligase (protease) and cleavage buffer and incubated overnight at 4°C to cleave the SUMO tag (66). The cleavage reaction mixture was then centrifuged, incubated for 2 h at 4°C, and centrifuged to pellet the resin. Supernatant for purified Hag protein was dialyzed in 1× PBS (pH 8.0) supplemented with 50% glycerol. Purified FliW protein was further cleaned following the same final purification step as that described for CsrA-His6. FliW and Hag were stored at −20°C. Removal of the SUMO tag was verified by SDS/PAGE and Coomassie staining.

Quantitative Western blotting. A standard curve was generated for each protein using purified Hag, FliW, or CsrA protein. A 2-μl volume of purified protein was mixed with 10 μl of appropriate deletion strain lysate (collected using qualitative Western blotting methods as described above) and mixed with 6× loading dye (1× final concentration). Samples were collected and prepared following the protocol described above but were also serially diluted and plated for CFU counts; testing of each strain was done in triplicate. Cell lysates were diluted 1:10 for the samples probed by anti-Hag, anti-SigA, and anti-EFP antibodies (67) (1:40,000). The standard curves were determined and the samples were separated by 15% SDS-PAGE, each gel had a standard curve. The proteins were electroblotted onto nitrocellulose and developed with anti-CsrA (1:5,000) (24), anti-FliW (1:10,000) (24), anti-Hag (1:80,000) (7), or anti-SigA (generous gift of Masaya Fujita, University of Houston) (1:8,000) and a 1:10,000 dilution secondary antibody (horsearshied peroxidase [HRP]-conjugated goat anti-rabbit immunoglobulin G). The immunoblot was developed using an Immun-Star HRP developer kit (Bio-Rad).

Calculating levels of protein per cell. The signal from each sample was compared to the corresponding standard curve to determine the amount of protein of interest in that sample. The amount of protein was converted to picomole values using Promega Biomath Calculator molar conversions (http://www.promega.com/a/apps/biomath/).

The number of cells loaded per lane was calculated by the following equations: (CFU/milliliter) *(sample volume) / (CFU/ml) = CFU. To calculate the amount of protein of interest in that sample. The amount of protein was converted to picomole values using Promega Biomath Calculator molar conversions (http://www.promega.com/a/apps/biomath/).

RNA electrophoretic mobility shift assay. The +1 to +100 region relative to the hag transcription start site was in vitro-transcribed using a PCR-derived template with a T7 promoter and internally labeled with [α-32P]ATP. The template was generated using 3610 genomic DNA and primer pair 6690/6691, and the template was subjected to phenol-chloroform extraction. The template was incubated with T7 RNA polymerase, nucleoside triphosphates (NTPs) (100 mM [each] CTP, GTP, and UTP; 5 mM ATP; and radioactive [α-32P]ATP) (68). Unincorporated nucleotides were removed by the use of a Chroma-spin column (Clontech), and the RNA was further cleaned by gel purification. RNA concentrations were calculated by the use of a scintillation counter. Various concentrations of CsrA proteins were incubated...
with 20 pM of radiolabeled RNA for 30 min at 37°C in CsrA binding reaction buffer (40 mM Tris-HCl [pH 7.5], 4 mM MgCl$_2$, 100 mM KCl, 20 ng/μg yeast RNA [Invitrogen], 20 mM dithiothreitol [DTT], 10% glycerol, 4 U of RNase inhibitor [Promega]) (69). Reactions that contained FIWI, CsrA, and the radiolabeled probe were incubated as described above; the FIWI protein was then added at various concentrations, and the reaction mixture was incubated for an additional 5 min at 37°C. After incubation, the reaction mixtures were fractionated on native 15% polyacrylamide gels (Bio-Rad) (19:1 acrylamide/bisacrylamide) at room temperature at 24 mA (constant) for 2 to 3 h with Tris-glycine-EDTA running buffer (30 mM Tris base, 190 mM glycine, and 1 mM EDTA, disodium salt). Gels were dried and exposed to phosphorimager screen overnight and were subjected to autoradiography (Typhoon FLA 9500). ImageQuant TL (ID; v8.1) was used to quantify the bound and free RNA. RNA-binding isotherms were obtained using GraphPad Prism 6 with a curve fit of specific binding with Hill slope.

**RNA isolation.** *B. subtilis* strains were grown in LB with or without 1 mM IPTG, when appropriate, to an OD$_{600}$ of ~1.0; 20 ml of culture was added to an equal volume of cold methanol (~80°C) and harvested by centrifugation at 5,000 g for 10 min at 4°C, and the supernatant was removed and stored at ~80°C (70). For quantitative RT-PCR, cultures were serially diluted onto LB plates to determine CFU levels per milliliter. Pellets were resuspended in 3.2 ml hot LETS buffer (10 mM Tris-HCl [pH 7.4], 50 mM LiCl, 10 mM EDTA [pH 8.0], 1% SDS, 75°C), subsequently added to a mixture of ~2.6-g acid-washed glass beads (Sigma) and 2.4 ml hot acid-saturated phenol (Fisher Bioreagents) (pH 4.6, 75°C), and subjected to vortex mixing for 3 min. Chloroform (Macron Fine Chemicals) (2.4 ml) was added, and the reaction mixture was subjected to vortex mixing for 30 s followed by centrifugation at 3,200 × g for 10 min at 4°C to separate the different phases. A 2.4-ml volume of the aqueous layer (top layer) was added to 3.2 ml of hot phenol-chloroform (Sigma) (pH 8.0; 1% SDS, 75°C; subsequently added to a mixture of ~2.6-g acid-washed glass beads (Sigma) and 2.4 ml hot acid-saturated phenol (Fisher Bioreagents) (pH 4.6, 75°C), and subjected to vortex mixing for 3 min, and centrifuged at 3,200 × g for 10 min at 4°C. An equal volume of the aqueous phase was added to 0.7 ml isopropanol in 4 separate microcentrifuge tubes, inverted, and left at room temperature for 10 min. The tube was processed by microcentrifugation for 25 min at maximum speed at 4°C, the supernatant was removed, and the pellet was washed in 1 ml cold 75% ethanol at maximum speed at 4°C. The supernatant was removed, the pellet was air dried for 10 min, and the pellet was rehydrated in 20 μl of nuclease-free water by heating at 55°C for 5 min. The four parallel samples were combined to reach a final volume of 80 μl TRIzol (Ambion) (1.2 ml) was added, and the reaction mixture was subjected to vortex mixing for 15 s and was then allowed to stand at room temperature for 5 min. Chloroform (0.24 ml) was added to the tube, and the tube was mixed by inversion for 15 s, allowed to stand for 2 min, and pelleted by microcentrifugation at maximum speed for 15 min at 4°C. The aqueous layer (0.7 ml) was mixed with an equal of isopropanol, inverted for mixing, and allowed to stand at room temperature for 10 min. The tubes were processed by microcentrifugation for 25 min at maximum speed at 4°C, the supernatant was removed, and the pellet was washed in 1 ml 75% ethanol at 4°C. The pellet was recollected by centrifugation, the supernatant was discarded, and the pellet was air dried for 10 min at room temperature. Finally, the pellet was resuspended in 80 μl of nuclease-free water by heating at 55°C for 5 min. Isolated RNA was treated with DNase (Turbo DNA-free kit; Ambion). RNA was reextracted from the DNase reaction mixture following the protocol described above starting at the TRizol step.

**Quantitative reverse transcriptase PCR.** To generate a standard curve, the *hag* transcript was transcribed in vitro using a PCR-derived template with a T7 promoter and primer pair 5373/5374. The template was used with a MEGAscript T7 kit (Invitrogen) to generate *hag* transcripts following the protocol described by the manufacturer. The *in vitro*-transcribed *hag* transcript was spiked into the Δ*hag* lysate (DS1677) at a concentration of 500 ng/μl in a total volume of 80 μl prior to the addition of chloroform at the beginning of the RNA purification protocol and was reextracted as described above. The amount recovered was presumed to be the same as the input, less a proportional loss due to recovery from lysates, similarly to the loss expected for native samples.

For both standard curve and native samples, cDNA was generated following the manufacturer protocol for a High Capacity cDNA reverse transcriptase kit (Invitrogen). Quantitative PCR was performed with primer pair 1564/1565 (71) and either cDNA or a negative control (the samples that were processed in parallel but were not treated with reverse transcriptase) using SYBR Select Master Mix (Applied Biosystems) with an Applied Bioscience StepOnePlus qRT-PCR machine (Life Technologies). The *hag* standard curve was serially diluted 9 times prior to being added to the quantitative PCR. The number of *hag* transcripts was determined using a standard curve (as described above) and the following calculations.

The number of *hag* transcript molecules per comparative threshold cycle (C$_T$) value for the standard was determined using a calculator for quantifying the number of copies of a template [http://cels.uri.edu/gsc/cndna.html](http://cels.uri.edu/gsc/cndna.html).

**Calculating *hag* transcripts per cell.** The comparative C$_T$ value determined for each sample was compared to the corresponding standard curve to determine the number of transcripts in that sample per nanogram of RNA. The number of transcripts per cell was calculated by the following equations: (transcript amount/nanogram) × (total nanograms of RNA isolated) = total transcripts; total transcripts/CFU = number of transcripts/cell.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at https://doi.org/10.1128/mBio.00533-19.

**FIG S1**, PDF file, 0.1 MB.

**FIG S2**, PDF file, 0.1 MB.
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