A quorum-sensing molecule acts as a morphogen controlling gas vesicle organelle biogenesis and adaptive flotation in an enterobacterium

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Gas vesicles are hollow intracellular proteinaceous organelles produced by aquatic Eubacteria and Archaea, including cyanobacteria and halobacteria. Gas vesicles increase buoyancy and allow taxis toward air–liquid interfaces, enabling subsequent niche colonization. Here we report a unique example of gas vesicle-mediated flotation in an enterobacterium; *Serratia* sp. strain ATCC39006. This strain is a member of the *Enterobacteriaceae* previously studied for its production of prodigiosin and carbapenem antibiotics. Genes required for gas vesicle synthesis mapped to a 16.6-kb gene cluster encoding three distinct homologs of the main structural protein, GvpA. Heterologous expression of this locus in *Escherichia coli* induced copious vesicle production and efficient cell buoyancy. Gas vesicle morphogenesis in *Serratia* enabled formation of a pellicle-like layer of highly vacuolated cells, which was dependent on oxygen limitation and the expression of *ntrB/C* and *cheY*-like regulatory genes within the gas-vesicle gene cluster. Gas vesicle biogenesis was strictly controlled by intercellular chemical signaling, through an *N*-acyl homoserine lactone, indicating that in this system the quorum-sensing molecule acts as a morphogen initiating organelle development. Flagella-based motility and gas vesicle morphogenesis were also oppositely regulated by the small RNA-binding protein, RsmA, suggesting environmental adaptation through physiological control of the choice between motility and flotation as alternative taxis modes. We propose that gas vesicle biogenesis in this strain represents a distinct mechanism of mobility, regulated by oxygen availability, nutritional status, the RsmA global regulatory system, and the quorum-sensing morphogen.

vacuole | ecological adaptation | microcompartment | intercellular signaling | macromolecular assembly

Bacteria have evolved several adaptive mechanisms enabling taxis into niches for pathogenesis, propagation, and survival. Flagella and pili allow bacteria to swim in aqueous environments or swarm, twitch, and glide across solid surfaces (1). In a process resembling primitive multicellularity, bacteria can implement cooperative strategies to swarm across surfaces as a concerted population event. A less well-understood mechanism of bacterial movement is that of regulated flotation through the production of buoyant, intracellular gas vesicles (2). Gas vesicle production in photosynthetic cyanobacteria, for example, can facilitate colonization of the air–liquid interface in stagnant waters, leading to the accumulation of toxic cyanobacterial blooms (3).

Gas vesicles are hollow, intracellular proteinaceous structures that self-associate into large "gas vacuole" conglomerate organelles, visible by light microscopy. In all species examined, gas vesicles are assembled from homologous proteins, namely the primary gas-vesicle subunit, GvpA, and the secondary strengthening protein, GvpC (2). GvpA is a small (~7–8 kDa), highly hydrophobic protein that assembles into a macromolecular crystalline array, forming a water-impermeable cylindrical shell that fills with gas by diffusion. The GvpC protein attaches to the hydrophilic exterior, increasing structural rigidity and resistance to collapse of the gas vesicle under pressure (2). Although there is a paucity of mechanistic information regarding the regulation of gas vesicle production, in some species it has been shown to be influenced by light intensity (cyanobacteria) or oxygen concentration (halobacteria), suggesting that gas vesicle regulation has evolved to enable migration to specific positions within the water column (2).

Serratia species are Gram-negative and members of the *Enterobacteriaceae* and are often causal agents of nosocomial infections (4). *Serratia* sp. strain ATCC 39006 (*Serratia* 39006) produces the carbapenem antibiotic, 1-carbapen-2-em-3-carboxylic acid and the bioactive red pigment, prodigiosin (2-methyl-3-pentyl-6-methoxyprodigiosin), a molecule with immunosuppressive and anticancer properties (5, 6). *Serratia* 39006 is also virulent in plant and animal models (7) and exhibits both swimming and swarming motility (8). Motility, secondary metabolism, and virulence in this strain are under control of quorum sensing (QS)—via production of the signaling molecule *N*-butanoyl-L-homoserine lactone (BHL)—and posttranscriptional regulation by the small RNA-binding protein RsmA.

In this article we describe the identification of QS-regulated gas vesicle morphogenesis in *Serratia* 39006, a unique example, thus far, of this phenomenon in a member of the *Enterobacteriaceae*. Gas vesicle production required a 16.6-kb cluster of genes encoding numerous putative structural and regulatory genes, and transfer of this cluster to *Escherichia coli* conferred gas vesicle synthesis capability. Gas vesicle morphogenesis in *Serratia* was upregulated in stationary phase and in static culture and required a region encoding NtrB/C- and CheY-like regulatory proteins for activation. Genetic screens revealed QS and RsmA positively regulated gas vesicle production. We propose that in *Serratia* 39006, gas vesicle synthesis, by providing buoyancy, allows it to migrate to and persist at the air–liquid interface in conditions where flagella-based migration, a more energy-dependent process, may be less efficient.

Results

Identification of Gas Vesicles in *Serratia* 39006. The production of gas vesicles causes opaque colony morphotypes in some bacteria (2), similar to those we had noted previously for *Serratia* 39006 (9, 10). Transposon mutagenesis screens were performed to identify mutants affected in the opaque colonial phenotype. Fifteen transposon insertions that resulted in loss of the opaque phenotype were found within a 16.6-kb region encompassing 19 co-orientated ORFs that included 11 genes predicted to encode

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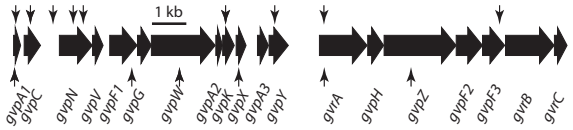

Fig. 1. Genetic organization of the *Serratia* 39006 gas-vesicle gene cluster. The 16.7-kb DNA region encoding gas-vesicle proteins. Transposon insertions causing colony translucency are indicated by arrows above (TnKRCPN1 insertions) or below the genes (TnDS1028 insertions). A summary of the predicted functions of each gene is listed in Table S1.

proteins involved in gas vesicle synthesis (Fig. 1). The locus contained three genes (*gvpA1*, *gvpA2*, *gvpA3*) encoding homologs of the primary gas vesicle structural protein GvpA. In addition, the gas-vesicle gene cluster encoded homologs of gas-vesicle proteins GvpC, GvpL/GvpF, GvpG, GvpH, GvpK, and GvpN and, by association, five other candidate gas-vesicle proteins of unknown function: GvpV, GvpW, GvpX, GvpY, and GvpZ. Additionally, putative regulatory proteins encoded by this cluster showed similarity to the σ^{54}-dependent transcriptional regulator NtrB (GvrA), the sensor histidine kinase NtrC (GvrB) (11), and the single-domain response-regulator protein CheY (GvrC) (12). Eleven of the 16 predicted proteins from this gas-vesicle locus produced top BLASTP hits with proteins encoded by the gas-vacuolated Alaskan sea-ice isolate, *Psychromonas ingrahamii*. A summary of the predicted functions of proteins encoded by the cluster are summarized in Table S2.

Gas vacuoles appear bright under phase-contrast microscopy (PCM) and have a propensity to collapse and disappear when subjected to abrupt pressure increase (2). PCM revealed phase-bright structures within most *Serratia* 39006 cells (WT) harvested from plate or liquid cultures. These structures disappeared upon pressurization, consistent with the nature of gas vesicles. Transmission electron microscopy (TEM) revealed abundant conical-ended cylindrical gas vesicles in *Serratia* 39006 WT that were absent in cells of the JRGVP strain (deleted for the gas-vesicle cluster). In pressurized samples, only smaller, diamond-shaped structures remained, indicating either that these residual vesicles were more pressure-resistant or that they were rapidly formed de novo, after pressurization (Fig. 2). Interestingly, gas-vesicle diameters varied considerably, even within single cells, showing that *Serratia* 39006 can assemble several distinct gas-vesicle structures (Fig. 2). Variations in GvpA sequence have been shown to effect changes in vesicle diameter (13), and so this gas vesicle morphological heterogeneity in *Serratia* 39006 might be controlled by differential expression of the three distinct GvpA homologs encoded by the cluster.

Dependence of *gvpA1* Expression on the *gvrA-gvrC* Operon and Oxygen Limitation. The temporal control of gas-vesicle gene expression was investigated in aerated flask cultures using a strain carrying a *gvpA1*::*uidA* reporter gene fusion, isolated in the transposon mutagenesis screen. Following inoculation, high β-D-glucuronidase activity was observed and this declined during late exponential growth, before increasing in stationary phase. The initially high *gvpA* expression in early exponential growth led us to analyze expression in the seeding culture. Expression in the seeding cultures (grown in 5-mL LB in sealed 25-mL vessels) was three times the maximum level of expression [148 ± 13 relative fluorescent units (RFU)/min normalized to OD_{600}] observed in flask cultures. Suspecting this affect was caused by reduced aeration, aliquots of the flask-grown cultures (at 12 h) were transferred to sealed vessels, covered with mineral oil, and incubated alongside the parent cultures. Expression from the *gvpA1*::*uidA* reporter in these conditions quickly increased and finally doubled that of the parent culture at 30 h (Fig. 2). Additionally, even higher expression from the *gvpA1*::*uidA* reporter was observed in static cultures (233 ± 5 RFU/min normalized to

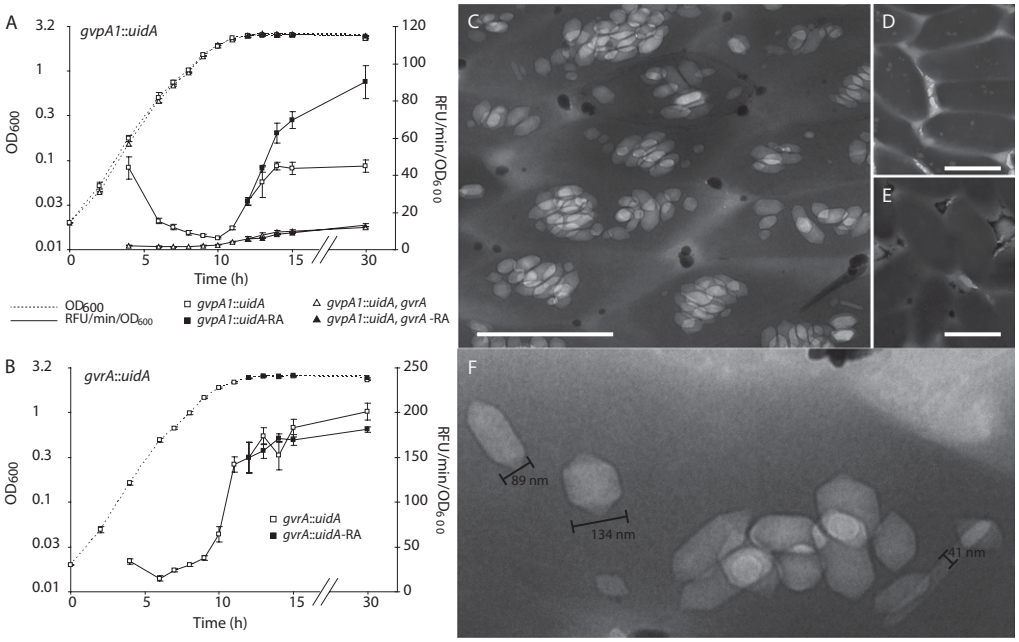

Fig. 2. Gas vesicle production in *Serratia* 39006. (*A*) Expression from the *gvpA1*::*uidA* transcriptional fusion throughout growth in *Serratia* 39006 (WT) and *gvrA* backgrounds. β-Glucuronidase expression was measured by the RFUs per minute, produced from cleavage of 4′-Methylumbelliferyl-β-D-glucuronide, normalized to the optical density of the culture (RFU/min normalized to OD_{600}) (values are average of three biological replicates ± SD). Filled symbols indicate subcultures incubated with reduced aeration. (*B*) Expression from the *gvrA*::*uidA* transcriptional fusion throughout growth. (*C*) TEM of WT cells, (*D*) pressurized WT cells, and (*E*) JRGVP cells (harvested from solid media). (*F*) TEM of individual WT cells showing vesicles of difference widths. (Scale bars, 1 μm unless indicated otherwise.)

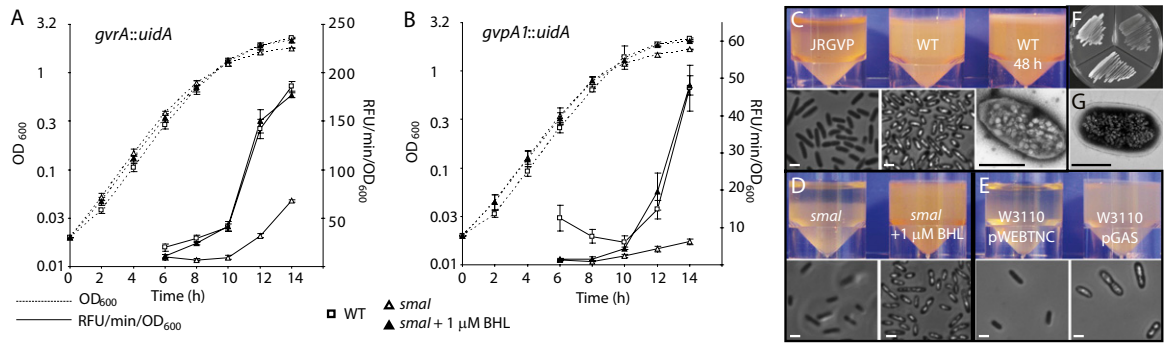

Fig. 3. Quorum-sensing regulated flotation and gas vesicle production. (*A*) Expression of the *gvrA*::*uidA* and (*B*) *gvpA1*::*uidA* reporter fusions in *Serratia* 39006 (WT), *smaI* background, and *smaI* supplemented with BHL (values are average of three biological replicates ± SD). (*C*) (*Upper*) Flotation of JRGVP and WT cultures after 24 and 48 h. (*Lower*) PCM of JRGVP and WT taken from the top layer of cells after 24-h static culture are shown below. TEM of cells harvested from the top white layer of cells formed in the 48-h WT culture is shown below the 48-h culture. (*D*) Flotation of the *smaI* strain with and without supplemented BHL, after static culture for 24 h; PCM of cells taken from the top layer of cells are shown below each culture. (*E*) Flotation of *E. coli* W3110 transformed with pWEBTNC or pGAS after static culture for 24 h; PCM of cells taken from the top layer of cells are shown below each culture. (*F*) Opaque morphology of *Serratia* strains [prodigiosin (–) background]; (*Upper Left* portion) WT; (*Upper Right* portion) *smaI*; (*Lower*) *smaI* + 1μM BHL. (*G*) TEM of *E. coli* W3110(pGAS). (Scale bars, 1 μm.)

OD_{600}), suggesting that gas vesicle synthesis was stimulated in conditions with reduced [O_2].

To test if *Serratia* 39006 gas vesicles increased cell buoyancy, overnight cultures of WT and JRGVP were allowed to stand in static culture for 24 h or more. Although the JRGVP strain began to sediment, producing a visibly clear layer near the air–water interface, the WT remained buoyant for at least 10 d. After 48 h, WT cultures formed a white layer of highly vacuolated cells at the air–water interface, reminiscent of a thin pellicle or biofilm formation (14). PCM of cells from this layer revealed nearly all cells were highly vacuolated and TEM confirmed cells were densely packed with vesicles (Fig. 3).

The similarity of the predicted products encoded by the *gvrA-gvrC* genes to proteins involved in nitrogen assimilation and chemotaxis in *E. coli* (11, 12) led us to investigate the role of this region in the expression of *gvpA1*. Therefore, a *gvrA*::*uidA* reporter-gene fusion strain was used to assess expression from the *gvrA* promoter. Low expression was observed in early-to-mid exponential phase but expression increased rapidly during the transition into stationary-phase (9–12 h), before the stationary-phase increase in *gvpA1* expression. When *gvpA1* expression was analyzed in a *gvrA* mutant background, only a basal level of *gvpA1* expression was observed throughout growth, indicating a dependence of *gvpA1* expression on one or more genes within the *gvrA-gvrC* locus. Interestingly, although reduced culture aeration had a dramatic affect on *gvpA1* expression, it had no effect on *gvrA* expression. Furthermore, no induction of *gvpA1* was observed under reduced aeration conditions in the *gvrA* mutant background (Fig. 2). Taken together, these results indicate that *gvrA-gvrC* genes are required for expression of *gvpA1* and gas vesicle morphogenesis and that proteins encoded by *gvrA-gvrC* likely transduce the response to reduced aeration.

Functional Expression of *Serratia* 39006 Gas Vesicles in *E. coli*. To determine if the *Serratia* 39006 gas-vesicle genes were functional in a heterologous background, a cosmid library of *Serratia* 39006 DNA was screened for genes in the gas-vesicle gene cluster, leading to isolation of pGAS. Transfer of pGAS to *E. coli* strain W3110 produced distinctly opaque colonies and microscopy revealed that gas vesicles were abundant throughout the cell (Fig. 3). Furthermore, *E. coli* W3110 (pGAS) cells remained buoyant in static culture when allowed to stand for 24 h, although the control strain sedimented (Fig. 3). The abundance of gas vesicles produced in *E. coli* suggested deregulation of the *Serratia* 39006 gas-vesicle gene cluster, presumably because of the absence of regulatory and physiological inputs that act during natural, controlled assembly in the cognate host.

Gas Vesicle Production Is Regulated by BHL, a Quorum-Sensing Morphogen Signal. In *Serratia* 39006, production of the prodigiosin pigment, the carbapenem antibiotic, virulence factor production, and swarming motility are tightly regulated by QS (8, 15, 16), which restricts full expression of these phenotypes to high cell density. Cells produce and respond to the autoinducer, BHL, by derepressing the LuxR-type transcriptional repressor, SmaR. The stationary phase increase in *gvpA1* and *gvrA* expression, along with the late-stage development of the opaque colony phenotype, suggested a possible QS input in gas vacuole development. Analysis of a *smaI* (BHL^-) mutant strain revealed that opaque colony morphology, *gvrA* and *gvpA1* expression, gas vesicle production, and buoyancy were all heavily reduced or absent. All these phenotypes were completely restored by addition of 1 μM BHL (Fig. 3). To confirm that the BHL dependence of gas vesicle morphogenesis was because of repression by SmaR, *smaR* and *smaR*, *smaI* double-mutants were analyzed. In these strains the profile of *gvrA* and *gvpA1* expression reflected that of WT and there was a loss of dependence on BHL, consistent with a lack of repression by SmaR (Figs. S1 and S2). Interestingly, expression of *gvpA1* and *gvrA* genes was neither increased nor precociously induced in the *smaR* background, nor in strains with added BHL, indicating that regulation by QS is conditional upon additional regulatory factors required for temporal activation, as we observed for other QS-regulated phenotypes in this strain of *Serratia* (15, 16). In summary, these results show a dependence on QS and BHL for initiation of gas vesicle biogenesis, indicating that this signaling molecule functions as a morphogen.

Inverse Regulation of Gas Vesicle Production and Flagella-Dependent Motility by the RsmA System. The previous results demonstrated that gas vesicle production by *Serratia* 39006 enhanced buoyancy and migration of cells to the air–liquid interface, consistent with the hypothesis that it constitutes an alternative form of mobility. We wondered if gas vesicle production might also come under the control of the RNA binding protein and global regulator, RsmA, which negatively regulates flagella-based swarming motility in *Serratia* 39006 through repression of surfactant and flagella gene expression (*rhlA* and *flhC*, respectively) (8). To test the impact of RsmA, expression of *gvpA1* and *gvrA* was analyzed in strain backgrounds carrying transposon insertions in *rsmA* and

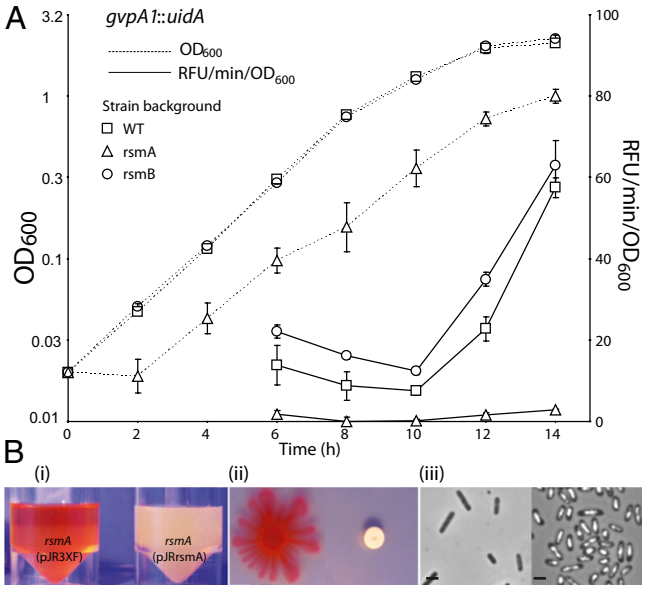

Fig. 4. Regulation of gas vesicle production and swarming motility by RsmA. (*A*) Expression of the *gvpA1*::*uidA* reporter fusion in *Serratia* 39006 (WT), *rsmA*, and *rsmB* backgrounds (values are average of three biological replicates ± SD). (*B*) Flotation (*i*), swarming motility (*ii*), and PCM of gas vesicles in the *rsmA* mutant backgrounds carrying vector-only plasmid pJR3XF (*Left*) or pJRrsmA (*Right*). (Scale bars, 1 μm.)

in other regulators of the RsmA pathway, including the small RNA RsmA-antagonist *rsmB* (8), the *E. coli csrD* homolog, *pigX* (17, 18), and the *rsmB* transcriptional activator homologue, *pigQ* (10) (Fig. 4, and Figs. S3 and S4). In the *rsmA* background, expression of both *gvrA* and *gvpA1* was almost eliminated, indicating that RsmA plays a strong positive role in gas vesicle expression (Fig. 4). Expression of *gvrA* and *gvpA1* was modestly reduced in the *pigX* mutant strains, consistent with the role of PigX, a negative regulation of RsmA activity (8) (Figs. S3 and S4). A slight but significant increase in *gvrA* and *gvpA1* expression was observed in the *rsmB* (Fig. 4 and Fig S4) and *pigQ* (Figs. S3 and S4) backgrounds, consistent with their role in antagonizing RsmA activity (8). Next, we analyzed the effect of ectopic expression of *rsmA* from plasmid pJRrsmA (where RsmA was expressed as a C-terminal 3XFLAG-tagged protein), on flotation, gas vesicle production, and swarming motility. As previously demonstrated (8), mutation of *rsmA* stimulated swarming motility, but expression of *rsmA in trans* resulted in its repression (Fig. 4). In contrast, flotation was repressed in the *rsmA* background, but restored in the strain carrying pJRrsmA. PCM of these same cells revealed that the majority of *rsmA* cells lacked visible gas vesicles, but the complemented strain was highly vacuolated. Taken together, these experiments demonstrated that RsmA simultaneously induced gas vesicle production and flotation, yet repressed swarming motility.

Discussion

This study is unique in demonstrating native production of gas vesicles in a member of the *Enterobacteriaceae*, *Serratia* 39006. Although gas vesicles are abundant in nature and have been found in both eubacteria and archea, their observation and analysis has been largely restricted to aquatic and halophilic organisms (2). It has been suggested that gas vesicles facilitate migration to the more oxygenated air–water interface, an advantageous migration for a facultative anaerobe (2). The discovery of gas vesicles in *Serratia* 39006 might therefore imply adaptation of this organism to an aquatic lifestyle. The discovery that the *Serratia* 39006 gas vesicles are activated in minimally aerated and static cultures gives further support to the notion that gas vesicle production is a form of mobility that evolved to facilitate colonization of air–liquid interfaces. Furthermore, flotation of *Serratia* 39006 to the air–liquid interface could promote killing—or reduced fitness—of competing aerobes that cannot rise in a water column, through competitive oxygen consumption.

Serratia 39006 gas vesicle production required a large gene cluster containing multiple homologs of previously identified gas vesicle proteins, and several unique proteins predicted to have a role in regulation. The 3′ portion of the cluster contained the *gvrA-gvrC* operon, which was required for activation of *gvpA1* expression. Mutation of *gvrA* resulted in loss of *gvpA1* activation in less-aerated or static cultures, suggesting proteins encoded by this operon might respond to gas concentrations to control gas vesicle expression. Consistent with this hypothesis, the GvrA and GvrB proteins show sequence similarity to NtrB and NtrC proteins, which transduce the response to environmental nitrogen limitation in *E. coli* (11). The GvrC protein contains only a single phosphoryl receiver domain, suggesting it might be phosphorylated, possibly as part of a signal transduction system. One possibility is that this protein may act in an analogous manner to the homologous protein CheY, which integrates multiple signals from chemoreceptors to control flagella-mediated chemotaxis (12).

The diameter of the gas-vesicle structure can be affected by variations in the sequence of both GvpA and GvpC. Wider diameter gas vesicles are more cost-effective, holding a larger volume of gas for fewer protein subunits; however, they are also weaker than narrow gas vesicles, which can withstand higher changes in pressure (13, 19). In lake populations of *Planktothrix rubescens*, recurring selective sweeps alternately select for narrow strong gas vesicles following periods of deep-water mixing and wider, less-costly gas vesicles in more stable conditions (20). The *Serratia* 39006 gas-vesicle gene cluster encodes three distinct homologs of the main subunit, GvpA. Although several species have been shown to encode multiple GvpA homologs, these are generally near-identical in sequence (2), suggesting they might contribute to the overall expression level of GvpA rather than producing significantly distinct gas-vesicle morphotypes. In contrast, the GvpA homologs in *Serratia* 39006 show only 31% to 51% amino acid identity, indicating they are divergent paralogues. It is tempting to speculate that the wide distribution of gas-vesicle diameters observed in individual *Serratia* 39006 cells is because of the assembly of different gas vesicles composed of distinct GvpA isoforms. To our knowledge, this observation of such a wide variety of gas-vesicle diameters found to occur naturally within a single strain is unique, and so the physiological and ecological significance of this heterogeneity warrants further investigation.

We have clearly demonstrated that natural gas-vesicle assembly is activated by QS and, indeed, that BHL is a morphogen in this bacterial strain. Why should gas vesicle morphogenesis be QS-dependent? Social theory predicts that QS may regulate phenotypes that are more beneficial when expressed cooperatively, for example, particularly in the production of "public goods" (21). However, gas vesicles are intracellular, so it is not immediately obvious how their production would benefit the population as a whole, unless cell–cell association is able to modulate buoyancy. As predicted by Stokes law, large colonies of gas vacuolated cells can achieve much higher flotation velocities than single cells (2); therefore, QS may activate gas vesicle production only when the colony size is sufficient to promote an efficient floating velocity. Alternatively, QS may be useful as an indicator of cell density and confinement, and so act as an early warning of impending starvation and oxygen deprivation, thereby encouraging cells to float away from a zone of heavy competition for adaptive survival. If large numbers of cells float upwards, they may be able to access new nutrients in another niche. Further-

more, the QS-dependent elaboration of bioactive secondary metabolites, coincident with flotation to a new niche, may be an adaptive response that allows defense of the new niche against bacterial competitors (via the carbapenem antibiotic) while possibly protecting *Serratia* 39006 against protozoan grazing (via the prodigiosin) (6, 22).

Strikingly, gas vesicle production and swarming motility were oppositely regulated by the small RNA binding protein RsmA, implying that the two methods of taxis may represent a physiological or developmental fork, leading to mutually exclusive production of either flagella or gas vesicles. It has been argued previously (2) that the sustained energy costs of powering flagella, coupled with the advantages of flexibility of direction of motion, make flagella more suitable to fast-growing heterotrophs, but for slower-growing autotrophs (needing only to maintain position or climb in the water column), gas vesicle production is a much more efficient (and possibly faster) method of taxis. Therefore, it would make sense that bacteria possessing the machinery for both mechanisms of mobility should carefully integrate signals from various environmental and physiological cues before making the decision on which taxis mechanism was more appropriate. Given the metabolic costs of gas vesicle morphogenesis or the considerable bioenergetic costs of assembly and functioning of flagella, the simultaneous biogenesis of both taxis systems could be counterproductive. In *E. coli*, the RsmA (CsrA) regulon encompasses over 700 mRNAs, including those involved in carbon metabolism and the stringent response (23). Therefore, RsmAB involvement in regulating this key behavioral switch between energy-draining flagella-dependent taxis, and the more passive, less energy-demanding taxis provided by gas vesicle-driven flotation, seems physiologically appropriate.

In summary, we propose that gas vesicle production in *Serratia* 39006 is a highly evolved alternative mechanism of mobility that responds to oxygen tension, the morphogen-like QS signal BHL, and metabolic status, to facilitate migration to—and colonization of—the air–liquid interface. The observations made in this study now provide an opportunity to characterize gas vesicle production and regulation comprehensively in this highly genetically tractable bacterium. Finally, these discoveries (including the efficient engineering and reconstruction of gas vesicle production in *E. coli*) could enable facile and obvious routes to exploitation of gas vesicles for diverse biotechnological processes, including uses in gas transfer in mammalian cell culture, engineering of new antigen presentation nanotechnology systems, and for ecological control of toxic blooms (3, 24, 25).

Materials and Methods

Bacterial Strains, Plasmids, Phage, and Culture Conditions. Bacterial strains and plasmids used in this study are listed in Table S1. *Serratia* 39006 and *E. coli* strains were grown in LB broth or agar and supplemented with antibiotics, as previously described (17). *E. coli* β2163 was supplemented with 300 μM 2,6-Diaminopimelic acid (DAPA). Transfer of plasmids into *Serratia* 39006 was by conjugation from *E. coli* β2163 (26). For signaling molecule "complementation," 1 μM of BHL was added upon inoculation. Transduction of chromosomal markers between *Serratia* 39006 was done with phage ϕOT8, as previously described (27). Growth studies in aerated conditions were in 250-mL Erlenmeyer flasks in 25 mL LB with an initial optical density of 0.02 (OD_{600}) and incubated at 30 °C with shaking at 300 rpm ("reduced shaking" refers to 150 rpm). For flotation experiments with *Serratia* 39006 and *E. coli*, 5-mL cultures grown upright in 25-mL sealed universals with 150 rpm shaking at 30 °C, were used to inoculate a second 5-mL broth that was then cultured for 24 h under the same conditions. Cultures were then mixed by vortexing and allowed to stand at 30 °C. Swarming assays were carried out as previously described (8), using Bacto agar.

Transposon Mutagenesis. Transposon mutagenesis of strain NWA19 (a prodigiosin pigment negative derivative of WT used for screening) was performed as described previously (8). Transfer of pKRCPN1 or pDS1028uidA into NWA19 by conjugation from *E. coli* β2163 was performed by mixing 10 μL of each strain followed by overnight incubation on LB agar containing DAPA. The mixture was suspended in LB, serially diluted, and plated onto LB containing appropriate antibiotics. Translucent NWA19 transconjugants were selected for further study. Transposon insertion sites were determined using random-primed PCR, as previously described (28).

β-Glucuronidase Assay. Samples of culture (150 μL) were taken at each time point and frozen at –80 °C until required. β-Glucuronidase activity was determined using 4′-Methylumbelliferyl-β-D-glucuronide. Ten-microliter aliquots of each sample culture were frozen at –80 °C for 10 min and then thawed at room temperature. Next, 100-μL reaction buffer (PBS, 400 μg/mL lysozyme, 250 μg/ ml 4′-Methylumbelliferyl-β-D-glucuronide) was added and samples were immediately monitored in a Gemini XPS plate reader using the following parameters: excitation 360 nm, emission 450 nm, cut-off 435 nm, eight reads per well, measured every 30 s for 30 min. RFUs produced min^{-1} were calculated from a period of linear increase in fluorescence normalized to the OD_{600} of the sample.

DNA Manipulation and Plasmid, Cosmid, and Mutant Construction. Plasmid construction, PCR, and cloning techniques were performed as described previously (16). For construction of pJRGVP, a region 5′ of the gas-vesicle cluster was amplified by PCR using primers GVP_5F_bamHI and GVP_5R_mfeI and a region 3′ of the cluster was amplified using GVP_3F_mfeI and GVP_3R_xbaI. The PCR products were joined using overlap extension PCR and cloned into pKNG101. Construction of strains JRGVP and NWA19 was carried out by marker-exchange mutagenesis using plasmids pJRGVP and pNRW54 (respectively), as previously described (29), except that donor *E. coli* β2163 was used for conjugation. The *Serratia* 39006 cosmid library was a gift from Tamsin Gristwood (University of Cambridge, Cambridge, United Kingdom) and was constructed using the pWEB-TNC Cosmid cloning kit as per the manufacturer's instructions (Epicentre Technologies). pGAS was identified by PCR using primers specific for the *gvpA1* promoter region (GvpA1proFedClaI and GvpA1prorev), *gvrA* (NtrC5BamHI and NtrC3′hindII), and *gvrB-gvrC* (NtrB5′bamHI and NtrREC3′InVrnaHindII). The gas-vesicle gene cluster sequence was assembled from preliminary genome shotgun sequencing. Plasmid pJRrsmA was constructed by amplifying *rsmA* using primers 5′RsmA_FwdEcoBam and 5′RsmA_RevClaI, which was then cloned into pJR3XF as an EcoRI-ClaI fragment.

Microscopy. Cells from plates were scraped off and suspended in PBS. Samples from liquid culture were analyzed directly without any further processing (for light microscopy) or collected by gravity-flow filtration in a cellulose acetate filter (0.2-μM pore size), washed with PBS, and eluted into 50 to 100 μL PBS. Gas vesicles were collapsed by compression within a sealed syringe (air was compressed to one-fifth of the original syringe volume). PCM was done using an Olympus BX-51 microscope with a 100× oil-immersion lens. Images were acquired using a QICAM monochrome camera and QCapture Pro-6 software. For TEM, cell suspensions were absorbed for 1 min onto carbon-coated, Formvar film grids that had been glow discharged (Quorum Emitech K100×) and treated with 0.01% polylysine. Grids were rinsed with distilled water and stained for 30 s with 2% phosphatungstic acid neutralized with KOH. Grids were rinsed with distilled water, dried, and viewed in an FEI Tecnai G2-operated at 120 Kv. Images were captured with an AMT XR60B digital camera running Deben software. Gas vesicle measurements were made using the ImageJ software distributed within the Fiji package (http://pacific.mpi-cbg.de/).

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