Overproduction of individual gas vesicle proteins perturbs flotation, antibiotic production and cell division in the enterobacterium Serratia sp. ATCC 39006

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Gas vesicles are intracellular proteinaceous organelles that facilitate bacterial colonization of static water columns. In the enterobacterium Serratia sp. ATCC 39006, gas vesicle formation requires the proteins GvpA1, GvpF1, GvpG, GvpA2, GvpK, GvpA3, GvpF2 and GvpF3 and the three gas vesicle regulatory proteins GvrA, GvrB and GvrC. Deletion of gvpC alters gas vesicle robustness and deletion of gvpN or gvpV results in small bicone vesicles. In this work, we assessed the impacts on gas vesicle formation when each of these 14 essential proteins was overexpressed. Overproduction of GvpF1, GvpF2, GvrA, GvrB or GvrC all resulted in significantly reduced gas vesicle synthesis. Perturbations in gas vesicle formation were also observed when GvpV and GvpA3 were in excess. In addition to impacts on gas vesicle formation, overproduction of GvrA or GvrB led to elevated biosynthesis of the tripyrrole pigment, prodigiosin, a secondary metabolite of increasing medical interest due to its antimalarial and anticancer properties. Finally, when GvpG was overexpressed, gas vesicles were still produced, but the cells exhibited a growth defect. Further analysis showed that induction of GvpG arrested cell growth and caused a drop in viable count, suggesting a possible physiological role for this protein linking gas vesicle biogenesis and binary fission. These combined results demonstrate that the stoichiometry of individual gas vesicle proteins is crucially important for controlled organelle morphogenesis and flotation and provides evidence for the first link between gas vesicle assembly and cell division, to our knowledge.

INTRODUCTION

Gas vesicles are intracellular proteinaceous structures that facilitate flotation throughout aquatic niches in many different micro-organisms (Pfeifer, 2012). These hollow cylindrical structures are permeable only to dissolved gas in the medium, and thus, gas vesicles reduce the density of bacterial cells, allowing them to fully colonize a static water column through flotation (Walsby, 1994). Gas vesicles are sensitive to pressure and production is often regulated in response to environmental and nutritional inputs from the organism’s surroundings (Hechler & Pfeifer, 2009; Pfeifer et al., 2002; Ramsay et al., 2011; Tashiro et al., 2016). To date, all gas vesicles identified are composed of the small hydrophobic protein GvpA, which self-assembles into a ribbed array structure forming the gas vesicle walls (Buchholz et al., 1993; Hayes et al., 1988; and reviewed extensively in Walsby, 1994). The protein GvpC forms a mesh structure covering the surface of the vesicle, thereby providing additional strength (Buchholz et al., 1993; Dunton et al., 2006; Offner et al., 1996). The specific functions of the remaining proteins are largely unclear, though some have been shown to associate with gas vesicles or form minor components of the structure (Tavlaridou et al., 2013, 2014; Xu et al., 2014).

The genetic clusters responsible for gas vesicle production have been widely reported in Archaea, cyanobacteria and recently in the enterobacterium Serratia sp. ATCC 39006 (S39006) (Dunton & Walsby, 2005; Halladay et al., 1993; van Keulen et al., 2005; Mlouka et al., 2004; Ramsay et al., 2011). S39006 is a Gram-negative bacillus that produces the intracellular red tripyrrole prodiginine antibiotic, 2-methyl-
3-pentyl-6-methoxyprogdinine (prodigiosin; Williamson et al., 2006) and the β-lactam antibiotic, 1-carbapenem-2-em-3-carboxylic acid (a carbapenem; Coulthurst et al., 2005). Prodigines have antitumor, immunosuppressant and antimarial properties and are of interest due to their pro-apoptotic activities (Hsieh et al., 2012; Marchal et al., 2014; Espona-Fiedler et al., 2012; Lu et al., 2012; Liu et al., 2013). S39006 is also a plant pathogen that produces plant cell wall degrading enzymes such as pectinases and cellulases (Fineran et al., 2005, 2007) but is also capable of killing the nematode worm Caenorhabditis elegans (Coulthurst et al., 2004; Wilf et al., 2011). Many of these phenotypes, including production of gas vesicles, are regulated in a manner dependent on cell concentration through the SmalR quorum sensing system (Fineran et al., 2005; Ramsay et al., 2011). Finally, in addition to movement due to the presence of gas vesicles, S39006 can swim using flagella and can swarm across the surface of media facilitated by the production of a biosurfactant (Williamson et al., 2008).

In S39006, the gas vesicle genetic locus contains 19 genes arranged in two operons (Fig. 1), the first starting with gvpA1 (an orthologue of gvpA) and the second with gvpC (a regulator of gas vesicle synthesis in S39006). The genetic cluster contains three closely related isoforms of GvpA, designated GvpA1, GvpA2 and GvpA3, and three isoforms of GvpF, designated F1–F3. Recently, we determined that 11 of the 19 genes in the genetic locus were required for robust gas vesicle synthesis (Tashiro et al., 2016). That is, when any one of these genes was removed, either production of gas vesicles was abolished or, in the case of gvpV or gvpN, only small bicone gas vesicles were produced and these never developed into larger, cylindrical, mature forms. Additionally, in a gvpC mutant, while gas vesicles were produced, they were significantly less robust under pressure, suggesting that GvpC-S39006 acts to strengthen gas vesicles, as it does in other systems (Tashiro et al., 2016). To demonstrate that, in each case, it was loss of the specific protein that prevented gas vesicle production, we expressed the WT genes from a plasmid in the cognate mutants to show restoration of gas vesicle production. This complementation confirmed genetically that the plasmid-based copy of the protein was expressed and functional in S39006.

While analysis of individual gene deletion mutants defined whether the corresponding proteins were essential for gas vesicle formation, it has been reported in other systems that stoichiometry of individual proteins is also important for gas vesicle production (Chu et al., 2011; Shukla & DasSarma, 2004; Tavlaridou et al., 2013), though this has not been examined in S39006. For example, a recent study from Halobacterium salinarum demonstrated that overexpression of GvpG and GvpH resulted in a significant decrease in cells containing gas vesicles (Tavlaridou et al., 2013). Further, overexpression of GvpM reduced the number of cells containing gas vesicles, with producing cells showing reduced vesicle numbers but larger organelles (Tavlaridou et al., 2013).

As we had previously demonstrated that plasmid-based expression of each essential gas vesicle protein was capable of complementation (Tashiro et al., 2016), we decided to examine further the impacts of overexpression of each protein essential for gas vesicle production in WT S39006.

**METHODS**

**Bacterial strains, plasmids and growth conditions.** All bacterial strains, plasmids and bacteriophages used are listed in Table 1. Serratia sp. ATCC 39006 LacA was used as a WT background in all experiments. Overnight cultures of S39006 and Escherichia coli strains were grown in lysogeny broth (LB) – Lennox (10 g tryptone l ̶ 1; 5 g NaCl l ̶ 1; 5 g yeast extract l ̶ 1) in sealed plastic universals or lysogeny broth agar (LBA) plates (1.5 %) at 30 °C and 37 °C, respectively. When necessary, strains were supplemented with ampicillin (100 µg ml ̶ 1) or chloramphenicol (25 µg ml ̶ 1). Although indicated, the inducer IPTG was added, in different concentrations, to plates and liquid culture. Generalized transduction with bacteriophage φOT8 was used to move plasmids between S39006 strains (Evans et al., 2010).

Growth studies were performed in 250 ml flasks containing 25 ml LB, inoculated to an initial OD 600 of 0.05 and grown under aerobic conditions with shaking at 215 r.p.m. Under microaerophilic conditions, the same culture conditions were used but 25 ml of sterile mineral oil was placed on top of the LB, and flasks were then shaken at 80 r.p.m. to restrict O2 availability. During growth studies, the OD600 was ascertained by measurement on a Helios Zeta spectrophotometer and viable colony counts were determined by serial dilution and plating onto LBA.

**Plasmid construction.** The plasmid pQE80-GvpGdomain was constructed by amplifying the sequence encoding the GvpG conserved domain region using oligonucleotides oREM602 (5¢-CCCCGAATTCGGCCTGG TACTCTCTGTACGAG-3¢) and oREM603 (5¢-CCCGGATCCTCA TTCATCCAGTGCTAATCTAC-3¢) by PCR. This fragment was digested with BamHI/EcoRI and ligated with compatibly digested pQE80-oriT to create pQE80-GvpGdomain. The recombinant plasmid sequence was confirmed by sequencing (GATC Biotech).

**Gas vesicle formation assays.** Assessment of gas vesicles on plates and flotation assays in liquid culture were performed largely as described.
Table 1. Bacterial strains, plasmids and bacteriophages used in this study

<table>
<thead>
<tr>
<th>Strain, plasmid or bacteriophage</th>
<th>Genotype or description</th>
<th>Reference</th>
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<tbody>
<tr>
<td><strong>Bacteria</strong></td>
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<tr>
<td>Serratia sp. ATCC 39006 LacA</td>
<td>Lac− derivative of ATCC 39006</td>
<td>Parker et al. (1982)</td>
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<tr>
<td>JRGVP (referred to as GV−)</td>
<td>Marker exchange mutant of LacA deleted for the gas vesicle genetic cluster</td>
<td>Ramsay et al. (2011)</td>
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<td>GPA1</td>
<td>gvpA1:: Tn-DS1028-uidA mutant in a LacA background</td>
<td>Ramsay et al. (2011)</td>
</tr>
<tr>
<td>ΔgvpG</td>
<td>In-frame mutant of gvpG in a LacA background</td>
<td>Tashiro et al. (2016)</td>
</tr>
<tr>
<td>E. coli strain W3110</td>
<td>F−, Λ−, IN(rmd−rmE)1, rph−1</td>
<td>Bachmann (1972)</td>
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<td><strong>Plasmids</strong></td>
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<td>pQE80-oriT</td>
<td>pQE80L carrying the RK2 origin of transfer</td>
<td>Ramsay et al. (2011)</td>
</tr>
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<td>pQE80-gvpA1</td>
<td>pQE80-oriT carrying the SD sequence and ORF of gvpA1</td>
<td>Tashiro et al. (2016)</td>
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<tr>
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<td>pQE80-oriT carrying the SD sequence and ORF of gvpA2</td>
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<td>pQE80-oriT carrying the SD sequence and ORF of gvpA3</td>
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<td>pQE80-oriT carrying the SD sequence and ORF of gvpC</td>
<td>Tashiro et al. (2016)</td>
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<td>pQE80-oriT carrying the SD sequence and ORF of gvpK</td>
<td>Tashiro et al. (2016)</td>
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<td>Tashiro et al. (2016)</td>
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<td>pQE80-gvrC</td>
<td>pQE80-oriT carrying the SD sequence and ORF of gvrC</td>
<td>Tashiro et al. (2016)</td>
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<td>This study</td>
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<td><strong>Bacteriophage</strong></td>
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<tr>
<td>φOT8</td>
<td>S39006 generalized transducing bacteriophage</td>
<td>Evans et al. (2010)</td>
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Pressure nephelometry. Pressure nephelometry on S39006 was performed as described in Tashiro et al. (2016), and cultures were grown in LB carrying the indicated concentration of IPTG.

Bioinformatics. Domain architecture of GvrA, GvrB, GvrC and GvpG was determined by searching the pfam database (Finn et al., 2015). Alignments of amino acid sequences were performed using Clustal Omega (Sievers et al., 2011). Phylogenetic trees were generated using the following settings in NCBI: Fast Minimum Evolution, Max Sequence Difference of 0.85 and Grishin protein distribution. Trees were plotted using NJPlot (Perrière & Gouy, 1996).

RESULTS

Overexpression of some gas-vesicle-associated proteins perturbs gas vesicle formation

Previously, we showed that deletion of any one of gvpA1, gvpA2, gvpA3, gvpF1, gvpF2, gvpF3, gvpC, gvpN, gvpV, gvpG, gvrA, gvrB or gvrC resulted in impaired biosynthesis of gas vesicles – in terms of size, strength or production. We were able to complement the deletion mutants by expression of the corresponding protein from a plasmid and thus were confident that in trans plasmid-based expression of each of these proteins produced a functional protein. Nonetheless, a recent study demonstrated that perturbing the relative amounts of individual proteins can impact on gas vesicle formation.
production (Tavlaridou et al., 2013). We expressed each essential gas vesicle protein from a plasmid within WT S39006. This plasmid system (pQE80-oriT* based) induced expression on addition of IPTG. For each overexpression strain, we assessed gas vesicles on plates with no induction, mid-level induction (0.1 mM IPTG) and full induction (1 mM IPTG). Gas vesicles refract light, and thus, colonial opacity was used as a facile monitor of gas vesicle production in individual colonies (Ramsay et al., 2011; Walsby, 1994). Furthermore, under PCM, intracellular groups of gas vesicles produce phase bright ‘gas vacuoles’ that are easy to identify (Ramsay et al., 2011). Using these two techniques, we observed that overexpression of GvpF1, GvpA3, GvrA, GvpF2, GvrB and GvrC resulted in little or no gas vesicle production (Fig. 2). An excess of GvpA1, GvpC, GvpN, GvpV, GvpA2, GvpK and GvpF3 did not visibly alter gas vesicle formation.

During these experiments, we noted that patches of strains overexpressing GvpG were slow growing, although gas vesicles were produced in these strains (see Fig. 2 and following sections). PCM analysis also showed that overexpression of GvpV resulted in a significant number of cells with no gas vesicles (Fig. 2). After extended incubation (5 days instead of 2) of the colonies (Fig. S1, available in the online Supplementary Material) while we still observed that gas vesicle production was perturbed as above, the patch morphology of cells overexpressing GvpV was mottled and showed a bull’s-eye pattern where gas vesicles (more opaque parts) were visible on the edges. Furthermore, translucent cells were visible toward the middle of the patch overexpressing GvpV. When cells from these older patches were observed by PCM, a significant population did not appear to produce gas vesicles (Fig. S1). A similar pattern of morphology was observed when GvrA was overexpressed at the highest levels, and the outer ring of the patch appeared white, suggesting that cells in this area were expressing gas vesicles but without prodigiosin.

Colony or patch opacity and PCM analysis are indicators of gas vesicle biosynthesis. However, microbes are known to grow differently on agar plates than in liquid medium (Jansson et al., 2015; Mikkelsen et al., 2007). As gas vesicles facilitate flotation in aquatic environments, we also wanted to examine gas vesicle production in liquid culture. To assess whether perturbation of individual gas vesicle genes affected flotation, we overexpressed each gene required for gas vesicle synthesis in liquid culture and observed flotation of cells in liquid culture (Fig. 3). Our results were similar to those seen on plates (Figs 2 and 3). Flotation was not observed when GvpF1, GvpF2, GvrA, GvrB and GvrC were overexpressed. However, gas vesicles were visible in a small population of cells overproducing GvpF2, suggesting that the lack of flotation was likely due to the majority of cells lacking any visible phase bright structures. Additionally, we observed a much more pronounced phenotype for cells overexpressing GvpA3 than we saw on plates. Cells were unable to float and almost no cells containing gas vesicles were visible by PCM (Fig. 3). Furthermore, in liquid culture, while cells expressing GvpG appeared to float, the cells appeared more red (Figs 2 and 3), suggesting that an excess of GvpG impacts on the production of prodigiosin.

**Overexpression of GvrA, GvrB or GvrC impacts gvpA1 transcription**

The gas vesicle gene cluster in S39006 is divided into two operons (Fig. 1). During our earlier experiments (Tashiro et al., 2016), we found that overproduction of any one of the three regulatory proteins GvrA, GvrB or GvrC, as well as all regulatory proteins in the second operon, resulted in almost no visible gas vesicles either on plates or in liquid media (Figs 2 and 3, see 1 mM IPTG conditions). GvrA, GvrB and GvrC all contain conserved motifs suggesting that they form parts of a two-component system. GvrA contains a response regulator receiver domain (pfam00072), an AAA+ ATPase domain (pfam00158), characteristic of a σ54 interaction domain and helix–turn–helix motif (pfam02954) characteristic of bacterial regulatory proteins such as Fis. GvrB contains a PAS domain (pfam13426), a histidine sensor kinase domain (pfam00512), again characteristic of two-component systems, and a histidine kinase-like ATPase domain (pfam02518). In contrast, GvrC contains only a single response regulator receiver domain (pfam00072; summarized in Fig. 4a). Additionally, when performing PSI-BLAST searches, the receiver domains between GvrA and GvrC were sufficiently similar to identify one when searching for the other. When aligning the sequences, we noted that the predicted dimerization interface (amino acid residues KPF) is conserved between both proteins (Fig. S2a). Furthermore, a phylogenetic analysis for each of GvrA, GvrB and GvrC revealed that, while they were conserved across many bacterial species, they were not found in other gas-vesicle-producing organisms such as the archaeon *Hal. salinarum* PHH1. Additionally, in *Burkholderia* sp., only GvrA has been identified so far but not GvrB or GvrC (Fig. S2), although, to date, there have not been any published reports of *Burkholderia* sp. synthesizing gas vesicles naturally.

We were surprised to find that an excess of GvrA/B/C resulted in almost no gas vesicle formation as, in a previous study, we demonstrated that deletion of any one of gvrA, gvrB or gvrC abrogated gas vesicle production. The latter effect was due to a decrease in gvpA1 transcription, suggesting that all three were involved in activating gvpA1 transcription (Tashiro et al., 2016). However, even leaky expression of gvrC from the plasmid alone (without induction) was enough to repress gas vesicle synthesis (Figs 2 and 3). Taken together, these results suggested that even small changes in the transcription levels of the regulatory genes gvrA, gvrB and gvrC altered gas vesicle production. However, we were unsure if excess GvrA, GvrB or GvrC diminished gvpA1 transcription or increased gvpA1 transcription leading to a decrease in gas vesicle formation due to a stoichiometric imbalance of particular gas vesicle proteins, such as GvpF1 (Figs 2 and 3). To determine which hypothesis was correct, we examined activity of a gvpA1::uidA reporter fusion when GvrA, GvrB and GvrC
production was induced. In this strain, transcription of the gvpA1 promoter can be assessed quantitatively by measuring activity of the UidA protein. Under both aerobic (Fig. 4b) and microaerophilic (Fig. 4c) conditions, expression of the gvpA1::uidA fusion decreased when any one of GvrA, GvrB or GvrC was overproduced. In the case of GvrC, even the presence of the uninduced plasmid construct caused a significant decrease in expression of the gvpA1::uidA fusion when compared to the empty vector control (Fig. 4b, c). These results were consistent with our earlier observations of gas vesicles in colonies or in liquid flotation assays, and they confirmed that an excess of any one of the three regulatory proteins decreased gas vesicle production by affecting transcription of the gvpA1 operon.

**Overexpression of GvrA and GvrB increases production of prodigiosin**

While performing experiments examining the impact of an excess of GvrA and GvrB, we noticed that the cultures appeared to produce more of the intracellular pigment, prodigiosin. Prodigiosin production is tightly regulated in S39006 and, as with production of gas vesicles, is regulated in response to cell density (Ramsay et al., 2011). However, no previous studies have identified genes in the gas vesicle operons as regulators of prodigiosin. To determine whether an excess of gas vesicle regulatory proteins impacted prodigiosin synthesis, we examined production under inducing and non-inducing conditions. We found that an increase in GvrA led to a threefold increase in prodigiosin production, while induction of GvrB led to a twofold increase in prodigiosin production. Overexpression of GvrC had no discernible effect on prodigiosin production (Fig. 4d). These results suggest that, in addition to their regulatory role in gas vesicle production, GvrA and GvrB, when in excess, also regulate the production of secondary metabolites such as prodigiosin.

**Excess GvpC does not alter gas vesicle strength**

Previously, many reports have demonstrated the role of GvpC in reinforcing gas vesicle strength (Dunton & Walsby, 2005; Dunton et al., 2006). GvpC is thought to form a reinforcing mesh on the surface of the gas vesicle (Buchholz et al., 1993; Dunton et al., 2006). Furthermore, in *Anabaena*, it has been shown that when gas vesicles, stripped of...
GvpC, were subsequently saturated with GvpC, they were not stronger than when originally isolated (Buchholz et al., 1993). Thus, excess GvpC may not necessarily provide more strength to existing gas vesicles. S39006 mutants of gvpC produced gas vesicles but they collapsed under significantly less pressure than those produced in WT cells, consistent with the view that GvpC was acting as a strengthening protein (Tashiro et al., 2016). Overexpression of GvpC did not appear to alter production of gas vesicles or flotation (Figs 2 and 3). However, we wondered if increasing GvpC might alter the gas vesicle critical collapse pressure. Using pressure nephelometry, we examined the robustness of gas vesicles under increasing pressure but found that there was no difference in critical collapse pressure upon overexpression of GvpC (Fig. 5a).

**Overexpression of GvpV results in cells with large amounts of small bicone vesicles**

A S39006 mutation in either gvpV or gvpN results in cells containing only small bicone vesicles. These observations were interesting as gvpV has not been identified in the well-studied archaeon Hal. salinarum PHH1, while gvpN has been identified in a wide array of other organisms (Englert & Pfeifer, 1993; Englert et al., 1992; Horne et al., 1991; Tashiro et al., 2016). We postulated earlier that GvpV and GvpN might act as molecular chaperones required for the development of mature cylindrical vesicles from the bicones seen in the early stages of the morphogenetic assembly pathway in S39006 (Tashiro et al., 2016). In our initial experiments, increased expression of GvpN had no obvious impacts on gas vesicle formation. In contrast, we noticed a subtle decrease in the amount of gas vesicles present when we overexpressed GvpV. This phenotype became more pronounced on extended incubation and, after 5 days, patches overexpressing GvpV had a bull’s-eye appearance, and PCM analysis revealed a mixture of cells with and without gas vesicles (Fig. S1). To assess cultural heterogeneity, we examined these cells by TEM to see if the apparent lack of gas vesicles (as scored by PCM) was the result of the complete absence of vesicles or perhaps simply the presence of only small bicone vesicles, which do not refract light by PCM. We found that the cells were expressing a heterogeneous mixture of vesicles and with a much greater proportion of smaller bicone vesicles than in WT cells. Additionally, in large numbers of cells, we observed many vesicles, but almost all were small bicones and very few were larger, mature, cylindrical structures (Fig. 5b). The results suggested that the cells lacking gas vesicles (as scored by PCM) still produced copious small bicone vesicles and very few mature vesicles.

**Overexpression of GvpG arrests cell growth and diminishes viable count**

When examining overexpression of individual gas-vesicle-associated proteins on plates, we noted that, after only 1 day, there was less growth when GvpG production was induced. Furthermore, after 2 days, the patches on plates

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**Fig. 3.** Flotation assays of WT S39006 overexpressing individual ORFs within the gas vesicle genetic cluster. WT gas vesicles carrying pQE80-oriT with the indicated ORF (top) were grown in LB with varying concentrations of IPTG (left) in sealed universals at 30 °C for 1 day. Cultures were then left to settle on a stable surface for 48 h and flotation was assessed. From each flotation assay, cells were imaged by PCM (below). Scale bars on the PCM images indicate 1 µm.
appeared punctate (Fig. 2). To examine how cell growth was affected and to quantify the phenotype, we grew cells carrying \textit{gvpG} on a plasmid or an empty vector control (pQE80-\textit{oriT}) under non-inducing conditions. After 4 h of growth, we split the cultures and induced expression in half of each culture by addition of 1 mM IPTG. In cultures carrying the empty vector control (pQE80-\textit{oriT}), irrespective of induction, the optical density (OD\textsubscript{600}) of the culture continued to increase until reaching stationary phase (OD\textsubscript{600} \sim 2.5). However, in strains carrying \textit{gvpG} on a plasmid, when induced, the OD\textsubscript{600} of the cultures levelled off and did not increase any further. In contrast, uninduced cultures carrying the pQE80-\textit{gvpG} plasmid reached the same density as the empty vector control (Fig. 6a).

OD\textsubscript{600} can be a misleading measurement as it records culture turbidity and not cell viability within a culture; therefore, at each time point, we also recorded viable c.f.u. In the cultures with the empty vector control, no changes were observed and the viable cell count reached \sim 4 \times 10^9 cells ml\textsuperscript{-1}, irrespective of induction. In contrast, cultures where GvpG was induced only attained less than 1 \times 10^7 cells ml\textsuperscript{-1} 4 h after induction compared with 1.7 \times 10^9 cells ml\textsuperscript{-1} in the uninduced culture (Fig. 6b).

To assess any impacts on growth and viability due to GvpG overexpression in an alternative host, we examined the effect of excess GvpG in \textit{E. coli} strain W3110. We found no significant difference in either optical density after induction or in viable counts, suggesting that the decrease in cell

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**Fig. 4.** Overexpression analysis of the three regulatory proteins in the gas vesicle genetic cluster. (a) Schematic representation of GvrA, GvrB and GvrC. Conserved domains were determined by analysis for Pfam matches. Receiver domains are indicated by rectangles, the AAA+ ATPase domain is indicated as a black rounded rectangle and the HTH domain is indicated as an oval; the PAS domain is indicated as a star; the histidine kinase domain is in grey and the histidine kinase-like ATPase in GvrB is found in black. Above each domain, the start and ending amino acid numbers are indicated. (b and c) Expression of a \textit{gvpA1::uidA} reporter fusion under aerobic (b) or microaerophilic (c) conditions after 16 h of growth. WT S39006 carrying the pQE80-\textit{oriT}, pQE80-\textit{gvrA}, pQE80-\textit{gvrB} or pQE80-\textit{gvrC} with the indicated concentration of IPTG. Values represent the average of three biological replicates and the error bars indicate \pm SD. (d) Prodigiosin production of WT S39006 carrying the plasmid pQE80-\textit{oriT}, pQE80-\textit{gvrA}, pQE80-\textit{gvrB} or pQE80-\textit{gvrC}. Values represent average prodigiosin production after 16 h of growth in LB for three biological replicates \pm SD.
OD GvpG severely limited cell growth, resulting in a decrease in mids. As in WT S39006, we found that overexpression of above for S39006 was then performed using the same plasmids. The gas vesicle genetic cluster had previously been created (Ramsay et al., 2011). The same experiment described above for S39006 was then performed using the same plasmids. As in WT S39006, we found that overexpression of GvpG severely limited cell growth, resulting in a decrease in OD600 and in viable colony counts. No change in either OD600 or viable colony counts was observed for the empty vector control cultures (with or without induction) or the uninduced culture carrying pQE80-gvpG (Fig. 6c, d). Thus, the change in growth in the presence of excessive GvpG was independent of gas vesicle production.

GvpG contains a single conserved domain and an acidic tail in S39006

Given the effect of GvpG overexpression on cell growth, we examined the structure of this protein further. GvpG is a small 138 amino acid protein and contains a single conserved domain (from amino acid 1 to amino acid 74 in GvpG39006), which has been described in other GvpG-like proteins involved in gas vesicle biosynthesis, but for which no other function has been found (pfam15020). Using PSI-BLAST, we examined NCBI for other proteins similar to GvpG. The most similar proteins were almost all within bacteria, though there is a GvpG protein in the gas vesicle gene cluster from the archaeon Hal. salinarum PHH1 that is more distantly related [see Fig. S1 from Tashiro et al. (2016) for an earlier phylogenetic tree]. We aligned the most closely related amino acid sequences of GvpG-like proteins from different bacterial species (Fig. S3). The alignment revealed that the conserved domain was similar in all GvpG orthologues, though the C-terminal end of the protein varied significantly between different species. For example, GvpG39006 contains a C-terminal tail of 64 amino acids composed largely of acidic amino acids such as aspartic acid (D) and glutamic acid (E). Of the 64 amino acids in the tail of GvpG in S39006, 18 were glutamic acid and 35 were aspartic acid, together comprising 82.8% of the amino acids in the C-terminal tail of GvpG. A pronounced enrichment in acidic amino acids was also observed in the C-terminal regions of GvpG homologues in Psychromonas ingrahamii 37 and Candidatus magnetoglobus multicellularis str. Araruma (Table S1). However, not all GvpG orthologues contained a C-terminal tail enriched for acidic amino acids. For example, the tail of the Desulfoxomirea tiedjei GvpG protein contains only seven amino acids and only one is a glutamic acid or aspartic acid residue. Furthermore, the C-terminal tail of GvpG in Desulfoloba acetoacidans DSM 11109 is not enriched for acidic amino acids, suggesting that this tail region may not be important for the protein’s function.

To explore whether the acidic tail was essential for GvpG function, we engineered a plasmid expressing a variant of GvpG without the C-terminal tail, under an inducible promoter (pQE80-gvpGdomain) and transformed S39006 with this construct. We performed the same experiments as described above, first examining gas vesicle production when the GvpGdomain alone was overexpressed on plates. While growth arrested when the full-length protein was expressed, no obvious difference was observed when the acidic tail was removed (Fig. 7a). Furthermore, overexpression of the GvpGdomain did not affect viable cell counts. Therefore, this implied that the acidic tail of the protein plays an important role in the ability of GvpG to arrest cell growth in S39006 or potentially increases the protein’s stability (Fig. S4).

![Fig. 5](image-url) Physiology of gas vesicles in strains overexpressing GvpC or GvpV. (a) WT S39006 carrying either pQE80-oriT or pQE80-gvpC were grown in LB under inducing (1 mM IPTG) or non-inducing (no IPTG) conditions for 24 h in sealed universals. Cultures were then left to settle for 24 h on the bench and the proportion of gas vesicle present was ascertained over increasing incremental pressure. Data shown are the mean values±SD for three biological replicates. (b) Representative TEM images of S39006 cells overexpressing GvpV. The scale bars indicate 500 nm.
Though the conserved domain of GvpG was unable to arrest cell growth, we also wondered if it was capable of complementing a mutation in gvpG. We transformed cells carrying an in-frame mutation in gvpG with pQE80-gvpGdomain and examined them for gas vesicle expression under different levels of induction. However, the GvpGdomain alone was not capable of complementing gas vesicle formation (Fig. 7b). This suggested that the C-terminal portion of the protein, containing almost exclusively acidic amino acids, may be important both for gas vesicle formation and for maintaining normal cell growth, or it is essential for maintaining protein stability.

**DISCUSSION**

Gas vesicle production in S39006 is a regulated process that facilitates flotation in this pathogen, though the precise physiological role of gas vesicles in the natural environment, or as part of any virulence pathway, is still unclear (Ramsay et al., 2011). From our earlier work, we determined that 11 ORFs were essential for gas vesicle formation in S39006 (gvpA1, gvpA2, gvpA3, gvpF1, gvpF2, gvpF3, gvpG, gvpK, gvrA, gvrB and gvrC). Mutants defective in gvpC produced gas vesicles that exhibited increased pressure sensitivity than those of WT and mutants defective in either gvpN or gvpV produced small diamond-shaped vesicles that failed to develop into the larger, mature bicone structures seen in WT cells. As we were able to complement each of these mutations by expressing the corresponding protein on a plasmid (Tashiro et al., 2016), we knew that the plasmid-based copies of individual proteins were functional. Additionally, because we suspected that stoichiometry of the individual proteins would be important for gas vesicle assembly in S39006, we were able to exploit the existing genetic tools available from our earlier study to probe this question further (Tashiro et al., 2016).

Proteomic studies of gas vesicles in other systems have demonstrated that the relative amounts of individual proteins are
important and that protein–protein interactions play a key role in development of gas vesicles from small diamonds into mature bicones (Chu et al., 2011). Furthermore, a recent paper has attempted to examine the overexpression of several gas vesicle proteins in *Hal. salinarum* to determine how perturbation of the individual proteins affects gas vesicle biosynthesis (Tavlaridou et al., 2013). The latter studies involved reconstitution of gas vesicle production in a non-cognate host by expressing the p-vac region (the two-gene cluster required for gas vesicle formation) in *Haloferax volcanii*, which is not a natural gas vesicle producer strain (Englert et al., 1992; Tavlaridou et al., 2013). The authors supplemented this construct with one that overexpressed individual genes in the *gvpFGHIJKLM* cluster. Using this system, they found that overexpression of GvpG, GvpH and GvpM from *Hal. salinarum* resulted in loss of gas vesicle formation. However, an excess of GvpF, GvpI, GvpJ, GvpK or GvpL had no obvious impacts. Some of these results are similar to those found in this study. For example, GvpM from *Hal. salinarum* is similar to both GvpA2 and GvpA3 from S39006 (see Tashiro et al. (2016), Fig. S1 for a phylogenetic analysis). We also found a slight perturbation in gas vesicle formation when GvpA3S39006 was overexpressed, though not the complete lack of gas vesicle observed in *Hfx. volcanii* (Tavlaridou et al., 2013). Additionally, though we found a dramatic change in cell physiology when we overexpressed GvpG, we did not observe any change in gas vesicle formation or flotation. One possible explanation for this disparity may be due to the acidic tail region of GvpG in S39006 or perhaps the protein is performing a completely different function in gas vesicle assembly. What is particularly interesting is that we were unable to complement gas vesicle formation in a *gvpG* mutant with the conserved domain alone, suggesting that the acidic tail does play a role in both gas vesicle formation.

![Fig. 7. Examination of the GvpG conserved domain. (a) Growth and gas vesicle formation of WT S39006 carrying pQE80-oriT, pQE80-gvpG or pQE80-gvpGdomain was assessed on LBA with the indicated concentration of IPTG (left). Cells were examined after 1 day of growth (top) and 2 days of growth (middle), and PCM images were taken of cells from the plate (bottom). (b) Gas vesicle complementation of a *gvpG* mutant on LBA with the indicated concentration of IPTG. Patch morphology (top image) of a normalized amount of culture from an in-frame *gvpG* mutant carrying pQE80-oriT, pQE80-gvpG or pQE80-gvpGdomain after 2 days of incubation and representative PCM image (bottom image) of cells from the plate. For all PCM images, the scale bar indicates 1 µm.](image-url)
and cell growth or that the tail is essential for protein stability.

We were initially surprised to find that overexpression of GvpG caused a cell growth impact and >100-fold decline in viable colony counts. No previous reports have linked gas vesicle formation to cell division. Furthermore, work examining overexpression of GvpG from Hal. salinarum PHH1 in Hfx. volcanii transformants did not identify this phenotype (Tavlaridou et al., 2013). However, the change in cell growth upon overproduction of GvpG may occur only in native gas vesicle-producing organisms, as we were unable to replicate this phenotype in E. coli, the organism within which we have previously reconstituted S39006 gas vesicles (Ramsay et al., 2011). Thus, it would be interesting to assess the impacts of overexpression of GvpG in the native gas vesicle-producing strain Hal. salinarum PHH1.

It is important to note that overexpression of GvpG was not linked exclusively to gas vesicle formation. Even in an S39006 mutant where the gas vesicle cluster had been completely removed, overexpression of GvpG caused a significant growth defect. This suggests that an S39006-specific system, not present in E. coli, links gas vesicle formation to cell growth or division. The fact that GvpG is both essential for gas vesicle formation and linked with cell division is an interesting observation and requires further study. To our knowledge, this is the first report linking a single gas vesicle protein to cell division.

Some proteins within the S39006 gas vesicle genetic cluster are not found in Hal. salinarum PHH1, e.g. GvpV. As mentioned previously, strains defective in gvpV form small bicone vesicles that do not mature into the larger cylindrical vesicles, and this led us to predict that it may be acting as a chaperone, facilitating the maturation of vesicle formation (Tashiro et al., 2016). Furthermore, Konopka and colleagues reported the isolation of an Ancylobacter aquaticus mutant that produced only bicone gas vesicles, and thus, a mutation disrupting gvpV or a mutation resulting in GvpV overproduction are possible explanations for this phenomenon (Konopka et al., 1975). The relative abundance of individual proteins in a chaperone complex is known to be important. For example, perturbation of GrpE levels, an interacting partner of the chaperone DnaK, impairs DnaK-mediated protein refolding and also leads to changes in cell morphology, resulting in filamentous cells in E. coli (Sugimoto et al., 2008). GrpE is believed to act by impairing FtsZ ring formation, an important stage in cell division (Bi & Lutkenhaus, 1991; Sugimoto et al., 2008). Thus, it is possible an excess of GvpV functions similarly, accumulating within the cell then eventually impairing vesicle formation, perhaps by altering protein turnover or perturbing the protein–protein interactions important in morphogenic assembly. Little is known about protein turnover during S39006 gas vesicle formation and nothing is known about how the entire gas vesicle nanostructure is turned over in this strain.

GvrA, GvrB and GvrC are all required for gas vesicle synthesis (Ramsay et al., 2011; Tashiro et al., 2016). Each of these regulatory proteins shows similarity to two-component systems. These systems are usually composed of a sensor kinase, often located at the cell membrane, and a cytosolic response regulator (Stock et al., 2000). Both GvrA and GvrC contain response regulator receiver domains, though only GvrA contains a putative HTH DNA-binding domain. Additionally, phylogeny analysis showed that GvrA and GvrC are similar to each other (29% identical; 50% similar), but this is almost exclusively within the receiver domains. The dimerization interface within the receiver domains is also perfectly conserved between the two proteins, suggesting that they may form heterodimers. Evolutionarily, duplication is thought to be one route that allowed the expansion of two-component systems within bacteria (Capra & Laub, 2012). However, this would lead to heterodimerization, potentially a seriously problem as the two proteins diverged evolutionarily. Recently, several groups have demonstrated that heterodimerization plays an important role in integrating environmental cues. In Streptomyces venezuelae, the response regulators BldM and WhiL form functional heterodimers capable of integrating cues from their respective developmental pathways (Al-Bassam et al., 2014). In E. coli, two response regulators RcsB and RcsA can form functional heterodimers that upregulate exopolysaccharide capsule production. RcsB is also capable of forming heterodimers with the acid stress response regulator GadE (Castanié-Cornet et al., 2010), as well as with BglI, leading to repression of the aryl-β-D-glucoside (bgl) operon (Venkatesh et al., 2010). Thus, through heterodimer formation, GvrC may titrate away GvrA, leading to repression of gas vesicle formation.

GvrB contains a Per-ARNT-Sim (or PAS) motif, a histidine kinase domain and a histidine kinase-like ATPase. PAS domains can be associated with signal transduction in a variety of eukaryotic and prokaryotic organisms (Henry & Crosson, 2011). They have been associated with cellular response to oxygen, light, small ligands or redox potential. Some proteins that contain PAS domain also bind haem during oxygen sensing. For example, both the oxygen-sensing kinase FixL from rhizobia and the direct oxygen-sensing Dos protein from E. coli contain PAS domains with haem-binding pockets (Delgado-Nixon et al., 2000; Gilles-Gonzalez & Gonzalez, 2004; Monson et al., 1995). The PAS domain in GvrB also contains six residues characteristic of a haem pocket. Furthermore, gas vesicle production is stimulated under reduced oxygen conditions suggesting an important regulatory mechanism for sensing and responding to oxygen concentration in the local environment. The precise biochemical link between dissolved gas in the medium and gas vesicle development is unclear, but it is possible that the haem-binding pocket within GvrB is responsible for sensing and responding to intracellular dissolved O2 levels.

There is interest in developing gas vesicles for biotechnological translation (Altschul et al., 2011; Childs & Webley, 2012; DasSarma et al., 2014), and thus, increasing knowledge of basic gas vesicle development will assist future exploitation of these nanostructures in synthetic biology.
and industrial biotechnology areas. S39006 gas vesicle production has been engineered into E. coli (Li & Cannon, 1998; Ramsay et al., 2011; Tashiro et al., 2016). However, functional reconstitution of vesicle assembly with concomitant flotation appears to be strain dependent in that heterologous host (data not presented). We are currently investigating why reconstitution works in some strains and not in others. Nevertheless, this study has provided the first evidence, to our knowledge, that the relative stoichiometry of individual proteins required for gas vesicle development in S39006 is critical for full and functional morphogenesis. Furthermore, even mild perturbation of the three Gvr regulatory proteins studied here caused almost complete repression of gas vesicle formation. Finally, we have, for the first time, established a link between bacterial growth and binary fission to gas vesicle formation operating through the GvpG protein. This is an interesting observation that warrants further investigation because, conceivably, it could be pertinent to the strain variability seen in functional reconstitution in non-cognate hosts.

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