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RESEARCH ARTICLE

Cysteine Biosynthesis Controls *Serratia marcescens* Phospholipase Activity

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ABSTRACT *Serratia marcescens* causes health care-associated opportunistic infections that can be difficult to treat due to a high incidence of antibiotic resistance. One of the many secreted proteins of *S. marcescens* is the PhlA phospholipase enzyme. Genes involved in the production and secretion of PhlA were identified by screening a transposon insertion library for phospholipase-deficient mutants on phosphatidylcholine-containing medium. Mutations were identified in four genes (*cyaA*, *crp*, *fliJ*, and *fliP*) that are involved in the flagellum-dependent PhlA secretion pathway. An additional phospholipase-deficient isolate harbored a transposon insertion in the *cysE* gene encoding a predicted serine *O*-acetyltransferase required for cysteine biosynthesis. The *cysE* requirement for extracellular phospholipase activity was confirmed using a fluorogenic phospholipase substrate. Phospholipase activity was restored to the *cysE* mutant by the addition of exogenous L-cysteine or *O*-acetylserine to the culture medium and by genetic complementation. Additionally, *phlA* transcript levels were decreased 6-fold in bacteria lacking *cysE* and were restored with added cysteine, indicating a role for cysteine-dependent transcriptional regulation of *S. marcescens* phospholipase activity. *S. marcescens cysE* mutants also exhibited a defect in swarming motility that was correlated with reduced levels of *flhD* and *fliA* flagellar regulator gene transcription. Together, these findings suggest a model in which cysteine is required for the regulation of both extracellular phospholipase activity and surface motility in *S. marcescens*.

IMPORTANCE *Serratia marcescens* is known to secrete multiple extracellular enzymes, but PhlA is unusual in that this protein is thought to be exported by the flagellar transport apparatus. In this study, we demonstrate that both extracellular phospholipase activity and flagellar function are dependent on the cysteine biosynthesis pathway. Furthermore, a disruption of cysteine biosynthesis results in decreased *phlA* and flagellar gene transcription, which can be restored by supplying bacteria with exogenous cysteine. These results identify a previously unrecognized role for CysE and cysteine in the secretion of *S. marcescens* phospholipase and in bacterial motility.

KEYWORDS *Serratia*, cysteine, flagella, phospholipase

Serratia marcescens is a ubiquitous bacterial species that infects organisms ranging from insects to humans (1). In humans, *S. marcescens* is a health care-associated opportunistic pathogen that causes multiple types of infections, including bacteremia and urinary tract infections (UTI). One of the many secreted factors of *S. marcescens* is the phospholipase A (PLA) enzyme, PhlA, that is responsible for the lecithinase activity of this organism (2). PhlA is cytotoxic when added to epithelial cells and exhibits hemolytic activity, likely due to the accumulation lysophospholipid cleavage products that can cause membrane destabilization of target cells (3). PhlA is also known to directly interact with the PhlB protein, which is thought to render PhlA inactive when intracellular to prevent bacterial self-membrane damage (4). The *phlB* gene is located

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directly downstream of the *phlA* open reading frame (ORF) and is expected to be cotranscribed.

The transcriptional regulation of *phlA* in *Serratia liquefaciens* MG1, which was later reclassified as *S. marcescens* (5), has been examined by heterologous expression in *Escherichia coli*. There are two known promoters upstream of *phlA*, pX and pA, that are involved in controlling *phlAB* transcription in response to growth phase, temperature, and glucose and oxygen availability (4, 6). Expression from pA accounts for the majority of *phlA* transcription and is growth-phase dependent, with the highest levels of expression observed during the transition from exponential to stationary phase. The pX promoter controls transcription of the ORF upstream of the *phlAB* locus but has also been shown to contribute to *phlAB* expression, particularly under low-oxygen conditions. A predicted Fnr binding site is present within the pX promoter region, suggesting that anaerobic expression of *phlAB* may also be controlled by this global regulatory protein (6).

A sequence within the pA promoter shares homology with a recognition sequence for binding of the flagellar sigma factor FliA (σ^{28}) (6). Transcription of *fliA* in related *Enterobacteriaceae* species is under the control of the master regulator FlhDC, which controls transcription of flagellar genes in a tiered manner (7, 8). Class 2 genes include those that encode flagellar export proteins (e.g., *fliJ* and *fliP*) as well as *fliA*. In turn, FliA is required for the expression of class 3 genes, including the flagellar filament gene *fliC*. PhlA has been shown to be produced in *E. coli* when the *phlAB* genes are provided on a plasmid, but *E. coli* strains lacking *flhD* no longer produce PhlA (6). PhlA activity was also not observed when *flhD* was mutated in *S. marcescens*; therefore, *flhD* is required for PhlA activity, likely through transcriptional activation via FliA (9). In *S. marcescens* and other bacterial species, *flhDC* is transcriptionally regulated by cyclic AMP (cAMP) receptor protein (CRP) and is subject to catabolite repression in the presence of glucose (8, 10, 11). This paradigm may also hold true for *phlA* transcription, since heterologous expression in *E. coli* required cAMP and the *cyaA* gene encoding adenylate cyclase (6). Taken together, these studies indicate that *phlAB* is likely a member of the flagellar regulon in *S. marcescens* and, furthermore, lead to the hypothesis that PhlA is secreted via the flagella assembly apparatus (4, 9).

Yersinia enterocolitica secretes a phospholipase, YplA, that is also *flhDC* dependent and susceptible to catabolite repression (12, 13). An amino acid alignment of *S. marcescens* PhlA and *Y. enterocolitica* YplA shows 74% identity over 244 residues, suggesting that these proteins may have similar functions. However, YplA is reported to have PLA_2 enzymatic activity whereas PhlA is reported to have PLA_1 activity (14). An additional characterization of YplA revealed that flagellar structural genes were required for extracellular phospholipase activity (12). YplA has also been shown to promote colonization and inflammation in a *Y. enterocolitica* mouse model of gastrointestinal infection (14).

Although transcriptional regulation of *phlA* has been studied primarily using a system of heterologous expression in *E. coli*, the native regulation of *phlA* in *S. marcescens* has yet to be fully characterized. Furthermore, the physiological role of *S. marcescens* PhlA is not known. By screening a transposon mutant library generated in a clinical isolate of *S. marcescens*, we identified *cysE* as a novel gene required for extracellular phospholipase activity. Subsequent experiments were designed to characterize the role of CysE on phospholipase and flagellar gene transcription, as well as on the motility of *S. marcescens*.

RESULTS

Identification of genes required for extracellular phospholipase activity. To identify *S. marcescens* genes that are required for PhlA activity and secretion, a transposon insertion library constructed with clinical bacteremia isolate UMH9 and containing ~32,000 unique mutants (15) was screened for colonies with defects in phospholipase activity on phosphatidylcholine-containing PCY agar. Six mutants having reduced extracellular phospholipase activity were identified from over 15,000

TABLE 1 *S. marcescens* strains and plasmids

Name	Relevant genotype	Description	Reference or source
S. marcescens			
UMH9	Wild type	Bloodstream infection isolate	15
phlAB	Δ*phlAB*::*nptII*	1,519-bp internal deletion of *phlAB* locus, insertion of 1,496-bp *nptII*-containing fragment from pKD4	This study
cysE	*cysE*::Tn	Insertion at position 138 of 822-bp ORF	This study
cyaA	*cyaA*::Tn	Insertion at position 218 of 2,553-bp ORF	This study
cyaA	*cyaA*::Tn	Insertion at position 104 of 2,553-bp ORF	This study
crp	*crp*::Tn	Insertion at position 123 of 633-bp ORF	This study
fliP	*fliP*::Tn	Insertion at position 471 of 762-bp ORF	This study
fliJ	*fliJ*::Tn	Insertion at position 164 of 447-bp ORF	This study
Plasmids			
pKD4		Source of kanamycin resistance gene	40
pSIM19		Recombineering plasmid	41
pBBR1MCS-5		Genetic complementation plasmid, gentamicin resistant	42
pBB1	*phlAB*$^+$	*phlAB* locus with native promoter sequence ligated to pBBR1MCS-5	This study
pBB2	*cysE*$^+$	*cysE* ORF with predicted promoter sequence ligated to pBBR1MCS-5	This study

colonies screened. The chromosomal locations of transposon insertions were determined for each mutant (Table 1), and the phospholipase phenotype of each isolate was compared directly with that of wild-type bacteria. The *fliP* and *fliJ* genes encode flagellar export and chaperone functions, respectively, and the disruption of either resulted in a complete loss of phospholipase activity (Fig. 1). This observation is consistent with the previously hypothesized role of the flagellar export complex in secretion of PhlA. Three additional phospholipase-negative isolates harbored mutations in either the *cyaA* or *crp* gene encoding an adenylate cyclase or cAMP receptor protein, respectively. Both cAMP and CRP are known to modulate the transcription of flagellar genes in *Serratia* and related bacterial species (8, 10, 11) and are necessary for extracellular phospholipase activity when the *S. marcescens phlA* gene is expressed in *E. coli* (6). Lastly, disruption of the BVG96_20000 ORF resulted in a greatly reduced level of extracellular phospholipase activity. BVG96_20000 is predicted to encode a 273-amino-acid protein having serine *O*-acetyltransferase activity (COG1045). The CysE serine acetyltransferase of *E. coli* MG1655 and the UMH9 BVG96_20000 predicted protein product share 86% amino acid identity; therefore, BVG96_20000 will be referred to here as *cysE*. A high level of phospholipase activity, similar to that of the wild-type strain, was restored to the *cysE* mutant upon genetic complementation, demonstrating that the phospholipase phenotype of this mutant was not the result of polar effects on neighboring genes due to the transposon insertion (Fig. 1). It was determined in

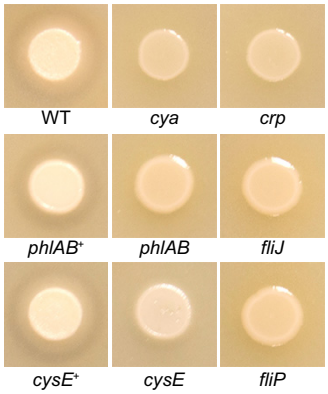

FIG 1 Phospholipase-deficient *S. marcescens* strains. Bacterial suspensions containing *S. marcescens* strains were spotted on PCY agar for examination of phospholipase phenotype. Phospholipase activity can be observed as a zone of clearance in the agar surrounding the wild-type (WT) and complemented mutant strains (*phlAB*$^+$ and *cysE*$^+$).

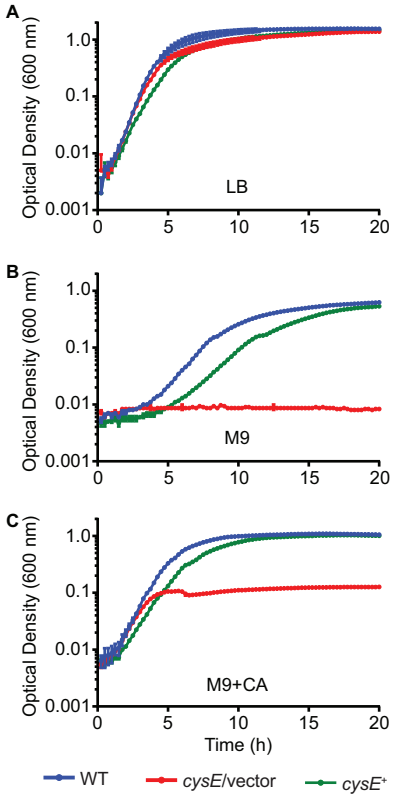

FIG 2 Growth of the *S. marcescens cysE* mutant requires exogenous amino acids. (A) *S. marcescens* wild-type (WT), *cysE* mutant with empty vector (*cysE*/vector), and complemented *cysE* mutant (*cysE*$^+$) strains were inoculated in LB medium and incubated at 37°C. Bacterial growth was measured at an OD_{600} every 15 min, and the means (±standard deviations) from triplicate cultures are shown. (B and C) Bacteria cultured overnight in LB were harvested by centrifugation, washed in M9 medium, and then subcultured in M9 or M9 plus CA as indicated. Bacterial growth was measured as described for panel A.

previous work using high-throughput sequencing that multiple independent *phlA* transposon insertion mutants were present in the tested library (15); however, none were identified in this screen, indicating that the number of screened clones was insufficient to achieve saturation. A *phlAB* deletion mutant was therefore constructed and tested for phospholipase activity in the same assay. As expected, the *phlAB* mutant exhibited a phospholipase-null phenotype on PCY agar, and activity was restored to high levels when *phlAB* was provided on a multicopy plasmid (Fig. 1).

A *Serratia cysE* mutant is auxotrophic for cysteine and *O*-acetyl-L-serine. Due to the previously unrecognized requirement for CysE in phospholipase activity, we sought to further characterize the role of this predicted serine acetyltransferase in *Serratia* PhlA production and secretion. Cysteine biosynthesis from L-serine is accomplished via a two-step reaction involving the production of *O*-acetyl-L-serine (OAS) as an intermediate. In *E. coli*, CysE catalyzes the formation of OAS from acetyl coenzyme A (acetyl-CoA) and serine, and *cysE* mutants are auxotrophic for cysteine (16, 17). The *O*-acetylserine (thiol)-lyase enzyme catalyzes the second step of the pathway, and together with CysE, forms the multifunctional cysteine synthase complex (17, 18). Based on this information, it was hypothesized that *cysE* would be required for *S. marcescens* growth in the absence of exogenous cysteine. The wild-type strain UMH9, the *cysE* transposon mutant, and the complemented *cysE* mutant all exhibited robust growth in LB medium, a rich source of exogenous amino acids (Fig. 2A). Similar high levels of growth were observed for the wild-type and complemented *cysE* mutant strains in defined M9 medium, whereas the *cysE* mutant failed to grow under these conditions (Fig. 2B). The addition of Casamino Acids (CA) to M9 medium (M9 plus CA) resulted in partial restoration of growth for the *cysE* mutant (Fig. 2C).

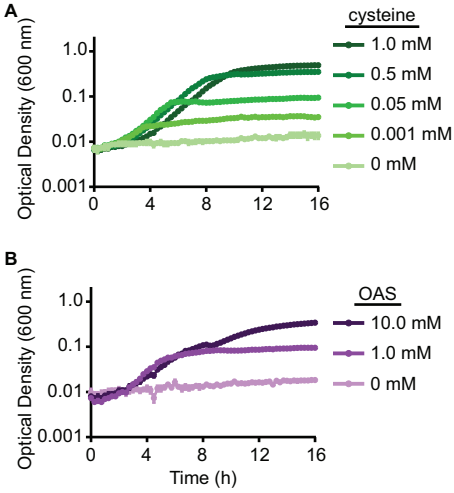

FIG 3 An *S. marcescens cysE* mutant is auxotrophic for cysteine. The *cysE* mutant strain was cultured in M9 medium supplemented with either cysteine (A) or OAS (B) at the indicated concentrations. Bacterial growth was measured at an OD_{600}, and the means (±standard deviations) from triplicate cultures are shown.

To determine whether the *S. marcescens cysE*-dependent growth defect was due to the lack of cysteine, cultures in defined medium were supplemented with increasing concentrations of the amino acid. Maximal growth was observed when bacteria were provided with 1 mM exogenous cysteine, though enhancement of growth was also observed with as little as 0.001 mM cysteine (Fig. 3A). Since the enzymatic activity of CysE results directly in the production of OAS rather than cysteine, growth levels were also measured in the presence of OAS. The supplementation of culture medium with 1 to 10 mM OAS restored the growth of the *cysE* mutant; however, the recovery of growth was less efficient than that observed with cysteine (Fig. 3B). Together, these results demonstrate that *cysE* is required for *S. marcescens* growth due to its role in cysteine biosynthesis.

Phospholipase activity is cysteine dependent. To quantitate the extracellular phospholipase activity of each mutant identified in the genetic screen, a fluorescent substrate specific for PLA enzymes was used to measure the activity from cell-free supernatants of LB cultures. Each of the five mutant strains identified in the screen (*cysE*, *cyaA*, *crp*, *fliP*, and *fliJ* strains) exhibited baseline levels of PLA activity that were similar to that of the *phlAB* null mutant (Fig. 4A). As was observed on PCY agar, genetic complementation of the *phlAB* and *cysE* mutations resulted in significant increases in detected PLA activity compared with that of the respective mutant strain. It is important to note that the phospholipase deficiency of the *cysE* mutant is not simply due to this strain's reduced growth under cysteine limitation, since *cysE* mutants exhibit no growth defect when cultured in LB medium (Fig. 2A). These results confirm the findings of the genetic screen and, importantly, establish that both flagellum-associated genes and *cysE* are required for *Serratia* phospholipase activity.

Based on the well-studied function of the *E. coli* CysE protein, the lack of OAS and/or cysteine was hypothesized to be responsible for the loss of phospholipase activity in the *S. marcescens cysE* mutant. To test this, phospholipase activity from bacterial supernatants cultured in CA-supplemented M9 was measured. Although the tested concentration of CA (0.1%) supplies sufficient amounts of cysteine to partially overcome the auxotrophy of the *cysE* mutant (Fig. 2C), it was not sufficient to restore phospholipase activity to this strain (Fig. 4B). The addition of 1 mM cysteine or 10 mM OAS to *cysE* mutant cultures resulted in a complete restoration of the PLA activity to levels observed with wild-type and complemented mutant strains. Since exogenous OAS is likely converted to cysteine intracellularly through the action of an *O*-acetylserine (thiol)-lyase, and cysteine alone is able to restore PLA activity, it was

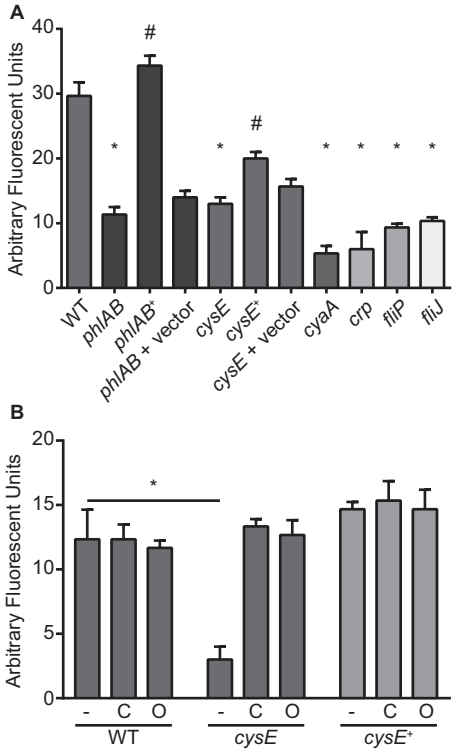

FIG 4 Flagellar and cysteine biosynthesis genes are required for extracellular PLA activity. (A) Cell-free supernatants from *S. marcescens* strains cultured overnight in LB medium were combined with the fluorescent PLA substrate bis-BODIPYFL C_{11}-PC. Fluorescence was measured after 1 h of incubation at 37°C. Bars represent the means (±standard deviations) from triplicate assays. Asterisks denote a significant reduction in PLA activity compared with that of the wild-type (WT) strain, and the pound symbol indicates a significant increase in PLA activity for complemented mutant strains compared with that of the respective uncomplemented mutant (*t* test, $P \leq 0.01$). (B) PLA activity was determined as described for panel A for bacterial strains cultured in M9 plus CA (−) or the same medium supplemented with 1 mM cysteine (C) or 10 mM OAS (O). The asterisk indicates a significant decrease in PLA activity compared with that of the wild-type strain for the unsupplemented condition (*t* test, $P \leq 0.01$).

reasoned that cysteine rather than OAS is the limiting component of extracellular phospholipase activity in these assays. As expected, the supplementation of both the *phlAB* and *fliP* mutant cultures with OAS or cysteine did not restore PLA activity to these strains (data not shown). The addition of cysteine or OAS also did not result in a significant change in wild-type or complemented mutant PLA activity, indicating that the levels of cysteine in these cells are sufficient for maximal activity under the tested conditions.

CysE contributes to *S. marcescens* swarming motility. It is clear that *S. marcescens* extracellular phospholipase activity is dependent on both the production of the PhlA protein and its secretion via the flagellar apparatus; therefore, cysteine deficiency could limit phospholipase activity through either or both of these processes. To determine if bacteria lacking *cysE* were capable of flagellum-mediated motility, *S. marcescens* strains were cultured on LB medium containing 0.6% agar to facilitate surface motility. Wild-type and *phlAB* bacteria swarmed readily over the 16-h incubation period, forming a thin film of growth over the surface of the agar (Fig. 5A). This result is in contrast to that from both the *fliP* and *fliJ* flagellar mutants that failed to migrate beyond the point of inoculation, as expected. Bacteria lacking *cysE* exhibited an intermediate phenotype with an approximately 2-fold decrease in swarm diameter (Fig. 5B). The reduced swarming motility observed with the *cysE* mutant under these conditions was not the result of growth limitation due to a lack of cysteine biosynthesis, since *cysE* bacteria are capable of robust growth in LB medium (Fig. 2A). Interestingly, the *cysE* mutant showed flagellum-dependent swimming motility levels that were equivalent to those of wild-

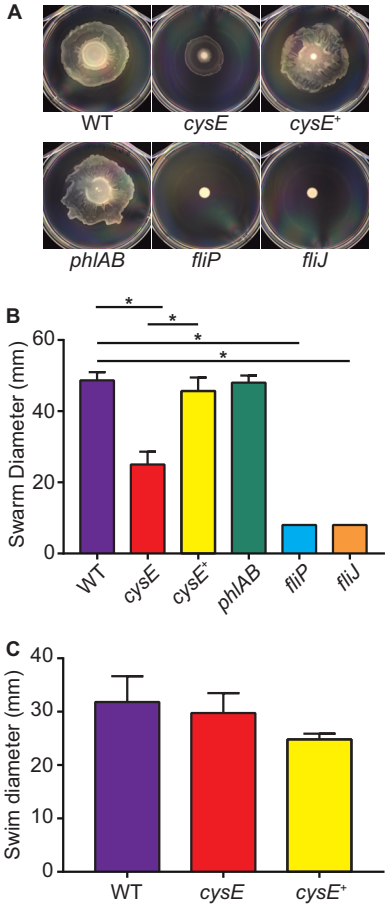

FIG 5 CysE is required for swarming but not swimming motility of *S. marcescens* in rich medium. (A) Suspensions (10 μl) of bacteria were spotted on LB medium containing 0.6% agar and incubated at 30°C for 16 h to facilitate swarming motility. Images of swarm growth in 100-mm petri dishes were captured from a top-down perspective. (B) Bacterial swarming was quantified by measuring the diameter (mm) of growth for each strain. Bars represent the mean (±standard deviations) swarm diameters from three independent experiments, and asterisks identify swarm diameters that were significantly less than those of the wild-type (WT) or complemented *cysE* mutant (*cysE*$^+$) strains as indicated (*t* test, $P < 0.01$). (C) Swimming capability of *S. marcescens* strains was determined as described for panels A and B with the exception that bacteria were cultured on LB medium containing 0.25% agar for 8 h.

type bacteria when tested under similar conditions (LB plus 0.25% agar), demonstrating that the swarming phenotype was not the result of a complete motility defect and that the two forms of flagellum-mediated motility exhibit differential requirements for cysteine (Fig. 5C). Together, these data are consistent with the notion that the loss of secreted PLA activity of *cysE* mutant bacteria is due in part to the modulation of flagellar apparatus function, though at least some functional flagella must remain in bacteria lacking CysE function.

***cysE* mutants have reduced phospholipase and flagellar gene transcription.** The loss of both phospholipase secretion and swarming motility in *cysE* mutant bacteria was hypothesized to be due to a disruption in transcriptional regulation of either *phlA* or flagellum-associated genes. To test this hypothesis, the levels of *phlA* transcripts between wild-type and *cysE* bacteria were compared by reverse transcription-quantitative PCR (qRT-PCR). In cells harboring the *cysE* mutation, an ~6-fold decrease in *phlA* expression levels was observed for bacteria cultured in M9 plus CA (Fig. 6A). This reduced *phlA* expression is consistent with the loss of PLA activity observed under the same growth conditions for bacteria lacking *cysE* (Fig. 4) and indicates that the low level of cysteine supplied by CA is insufficient for optimal *phlA* expression. Complementation of the *cysE* mutation in this experiment restored *phlA* transcription to a level similar to

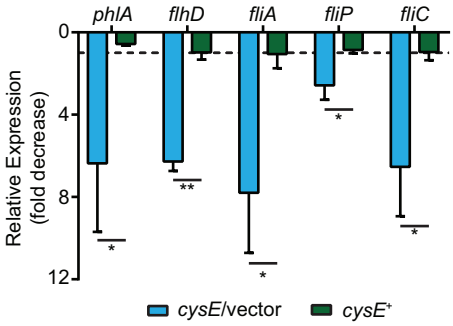

FIG 6 Mutation of *cysE* results in decreased transcription of phospholipase and flagellar genes. cDNA prepared from *S. marcescens* strains cultured in M9 plus CA was used to assess relative levels of phospholipase and flagellar gene transcripts (*x* axis) by qRT-PCR. Transcript levels for the *cysE* mutant harboring an empty vector (*cysE*/vector) and the complemented *cysE* mutant (*cysE*$^+$) are shown as fold decrease relative to that of the wild-type strain. The dotted line at 1.0 on the *y* axis indicates a hypothetical value at which there is no change relative to the wild-type strain. Bars represent the means (±standard deviations) from three independent experiments, and asterisks denote significant differences in relative levels between the *cysE* mutant and complemented *cysE* mutant strains (*t* test). *, $P < 0.05$; **, $P < 0.01$).

that of the wild-type strain and significantly different than that of the *cysE* mutant. To determine whether *phlA* expression was cysteine dependent, transcript abundance was measured from bacteria cultured in M9-plus-CA medium supplemented with either OAS or cysteine, relative to unsupplemented controls. The transcription of *phlA* was increased in these experiments when *cysE* mutant bacteria were provided with cysteine or OAS compared with wild-type *phlA* levels that remained largely unchanged under the same conditions (Fig. 7). These results demonstrate that the *phlA* transcription deficiency of the *cysE* mutant strain is due to the lack of cysteine biosynthesis pathway products and not a secondary function of the serine acetyltransferase protein.

To investigate the possibility that flagellar gene transcription was also dependent on CysE and cysteine levels, *fliP* and *fliC* expression levels were measured. By an extension of the paradigm established for other species of the *Enterobacteriaceae* family, these two genes were chosen as representative examples of class 2 (*fliP*) and class 3 (*fliC*) target genes in the hierarchical flagellar regulatory cascade (8). Similar to *phlA*, both *fliP* and *fliC* were demonstrated to have decreased transcript abundance in *cysE* mutant bacteria compared with that of the wild-type strain (Fig. 6). These flagellar genes also exhibited increased transcription levels relative to unsupplemented control cultures when *cysE* mutant bacteria were provided with cysteine or OAS (Fig. 7). Wild-type bacteria also exhibited minimal increases in *fliP* and *fliC* transcription in the presence of added cysteine or OAS, demonstrating that these products do not stimulate flagellar gene transcription in bacteria competent for serine acetyltransferase.

The transcription of *phlA* in *S. marcescens* (9), as well as *fliP* and *fliC* in related bacterial species (8, 19), is known to require the FlhDC flagellar master regulator. Therefore, it was hypothesized that the OAS and cysteine deficiency of *cysE* mutant bacteria could influence the level of *flhD* transcription, resulting in reduced expression of FlhD-dependent target genes. In bacteria harboring the *cysE* mutation, the transcription of *flhD* was reduced ~6-fold compared with that of wild-type bacteria (Fig. 6). Complementation of the *cysE* mutation restored *flhD* expression to approximately wild-type levels and resulted in a significant difference compared with that of the mutant strain. The alternative sigma factor FliA (σ^{28}) is responsible for the transcription of class 3 flagellar genes (8, 19), and likely *phlA* (6), and the *fliA* gene itself, is also an FlhDC-dependent class 2 gene. Consistent with the observed transcriptional defect of *fhlD* in the *S. marcescens cysE* mutant, *fliA* expression levels were also reduced at a similar level. Furthermore, both *flhD* and *fliA* transcripts increased in abundance when *cysE* mutant bacteria were cultured in medium supplemented with either cysteine or OAS (Fig. 7). Together, these results strongly suggest that the loss of phospholipase

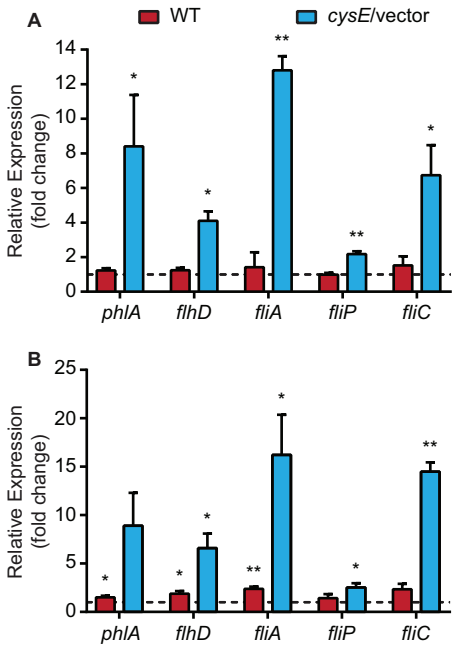

FIG 7 Cysteine and OAS supplementation increase phospholipase and flagellar gene transcription in the *cysE* mutant. (A) cDNA prepared from *S. marcescens* strains cultured in M9 plus CA or M9 plus CA supplemented with 1 mM cysteine was used to assess relative levels of phospholipase and flagellar gene transcripts (*x* axis) by qRT-PCR. Transcript levels for the *cysE* mutant harboring an empty vector (*cysE*/vector) and the wild-type strain (WT) are shown as fold change for bacteria cultured in cysteine-supplemented medium relative to that cultured in M9 plus CA. (B) Transcript levels from bacteria cultured in M9 plus CA supplemented with 10 mM OAS relative to those from bacteria cultured in unsupplemented M9 plus CA were determined by qRT-PCR as described for panel A. Results represent the means (±standard deviations) from three independent experiments, and the asterisks denote relative expression levels that are significantly different from the hypothesized value of 1.0 (dotted line, no change) (one-sample *t* test). *, $P < 0.05$; **, $P < 0.01$.

activity and swarming motility in the *cysE* mutant are due to a role for cysteine at or upstream of the level of *flhD* transcription.

The role of phospholipase *in vivo*. The potential for PhlA to contribute to *S. marcescens* pathogenesis is an attractive hypothesis given the several well-characterized examples of secreted phospholipases with roles in bacterial virulence (20). Although *S. marcescens* is known to cause UTI, primarily in health care environments and in patients with urinary catheters (21), the bacterial factors involved in UTI remain uncharacterized for this organism. A murine model of *S. marcescens* UTI was developed here based on a previously published method (22) to assess the contribution of PhlA to *in vivo* bacterial fitness. When mice were infected with 5×10^7 CFU, bacteria were routinely detected for at least 4 days postinfection (Fig. 8A), indicating that *S. marcescens* is capable of establishing a long-term infection in this environment despite the routine voiding of bladder contents. To determine the contribution of PhlA to *S. marcescens* fitness in this model, a 1:1 mixture of wild-type and *phlAB* mutant bacteria was used to coinfect mice. The competitive index (CI) was used as a measure of relative fitness and determined from the numbers of *phlAB* mutant and wild-type bacteria recovered from homogenized bladder tissue. At all tested time points the *phlAB* mutant exhibited a modest loss of fitness (CI, <1.0) compared with that of the wild-type strain, but only the 24-h time point was statistically significant (Fig. 8B). Although the loss of fitness in the absence of PhlA was minimal, these results warrant further investigation into the potential role of this secreted phospholipase during mammalian infection.

DISCUSSION

Examples of nonflagellar proteins that are secreted via a flagellar export complex are relatively uncommon in the bacteriological literature, yet they are intriguing given the

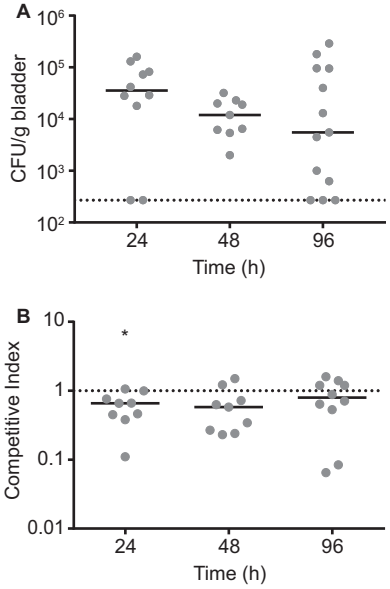

FIG 8 Fitness of the *S. marcescens phlAB* mutant in a murine model of UTI. (A) C57BL/6 mice were infected with a 1:1 mixture of wild-type and *phlAB* mutant bacteria via a urethral catheter at a dose of 5×10^7 CFU. At the indicated times, mice were sacrificed and the total bacterial burden in homogenized bladder tissue was determined by serial dilution and plating. Solid lines represent the median bacterial burdens at each time point, and the dotted line indicates the limit of detection. (B) The relative fitness of the *phlAB* mutant compared with that of the wild type was calculated as a competitive index for the experiment described for panel A. The dotted line represents a CI of 1.0, the level of equivalent fitness between the two strains. Median CI values that were significantly less than a hypothesized value of 1.0 using the Wilcoxon signed rank test are indicated by an asterisk ($P < 0.05$).

structural homology between the flagellar basal body and the dedicated machinery of host-associated type III secretion systems (23, 24). This form of flagellum-mediated protein translocation has been previously described with regard to secretion of the *Y. enterocolitica* YplA phospholipase (12, 25), which shares homology with *S. marcescens* PhlA (14), and has also been predicted for *S. marcescens* based on the genetic requirement for the *flhD* gene when *phlA* is expressed from an inducible and FlhD-independent promoter (9). Unlike those in *Yersinia* species, the *S. marcescens* UMH9 genome does not appear to encode a host-adapted type III secretion system (data not shown). Our data further support flagellum-dependent *Serratia* phospholipase secretion by demonstrating that transposon insertions in the *fliP* and *fliJ* genes, encoding proteins required for flagellar assembly in other species, resulted in the complete loss of extracellular phospholipase activity. This finding is important since it was previously established that the transcription of both *phlA* and flagellar genes was dependent on FlhD, making it difficult to distinguish between a loss of extracellular phospholipase activity due to decreased transcription of *phlA* or a loss of the secretory apparatus (4, 6, 9). A second, and perhaps complementary, mechanism by which *fliP* and *fliJ* mutants may exhibit reduced phospholipase activity is via FlgM-mediated inhibition of FliA activity. The anti-σ^{28} factor FlgM is exported once the hook-basal body structure has been assembled, thereby freeing FliA for the transcription of class 3 genes (19, 26, 27). In the absence of a completed flagellar export structure, as is expected with the *Serratia fliJ* and *fliP* mutants, FlgM remains capable of sequestering FliA and thus inhibiting σ^{28}-dependent transcription. The present study further links the flagellar export system with phospholipase by demonstrating that *cysE* and cysteine are required for the transcription of genes controlling both of these biological functions, most likely by acting at or upstream of the level of *flhD* transcription. By an extension of the *E. coli* paradigm, the finding that transcription of both class 2 (FlhD-dependent) and class 3 (FlhD- and FliA-dependent) flagellar genes was altered by disruption of *cysE* further supports this notion. A similar loss of flagellar function and PhlA secretion has been

previously observed in *S. marcescens* strains that have perturbations in the enterobacterial common antigen synthesis pathway, due to a subsequent repression of the *flhDC* genes via the Rcs phosphorelay (28, 29).

Our finding that the *cysE* mutation resulted in a swarming motility defect but not a swimming motility defect suggests a differential requirement for cysteine during these two forms of flagellum-mediated motility. One potential explanation for this observation is that swarming motility, involving hyperflagellated cells (30), may be more sensitive to a modulation of the flagellar transcriptional hierarchy. This hypothesis is consistent with our observation that the *cysE* mutation results in reduced *flhD* and *fliA* transcript levels yet still allows for swimming motility. It is also possible that swarming-specific products, such as biosurfactants (31), require a functional *cysE* gene for production.

In *E. coli* and other *Enterobacteriaceae*, the CysB transcriptional regulator controls the expression of numerous genes involved in the biosynthesis and transport of cysteine and sulfur (17). CysB-mediated transcriptional activation requires the inducer *N*-acetylserine (NAS), which is derived directly from OAS. Therefore, the addition of OAS to bacteria deficient in CysE could both supply substrate for the production of cysteine and provide a source of NAS inducer. Therefore, it is possible that the lack of phospholipase activity and swarming motility in *S. marcescens cysE* mutants could be due to a loss of CysB activation. Importantly, we have demonstrated that the *phlA* and flagellar gene transcriptional defects associated with the *Serratia cysE* mutation could be reversed with cysteine alone. These results are most consistent with the hypothesis that cysteine is the limiting component of phospholipase activity rather than NAS. However, a role for CysB in the transcriptional activation of flagellar and phospholipase genes has not been formally tested at this point.

The status of PhlA as a *Serratia* virulence factor has long been speculated. *In vitro* experiments previously demonstrated that purified PhlA could induce cytotoxicity in human bladder and cervical epithelial cells (3), and the *Y. enterocolitica* PhlA homolog has been proposed to have a role in pathogenesis during murine gastrointestinal infection (14). The partial loss of fitness for bacteria lacking PhlA observed in this work is intriguing, though not definitive. Since PhlA is secreted from bacterial cells, the production of PhlA by wild-type bacteria may have also benefited *phlA* mutant bacteria during the coinfection experiment, thereby reducing any potential fitness defect of *phlA* mutant bacteria. It may be possible to address this issue of cross-complementation in future experiments by performing monoinfections, though the inherent variability in colonization levels for the UTI model makes this experiment challenging. An additional assessment of the contribution of PhlA to virulence was conducted using a murine bacteremia model established for this strain (15), but no significant differences between the wild-type strain and the *phlAB* mutant were observed (data not shown).

In summary, we have determined that an intact cysteine biosynthesis pathway is required for extracellular phospholipase activity and swarming motility of *S. marcescens*. Cysteine itself appears to be required for achieving high levels of phospholipase and flagellar gene transcription, resulting in defects in FlhDC-dependent processes when levels of this amino acid are insufficient. These results further support a model of coregulation for flagella and phospholipase in *S. marcescens*, and future work will be directed at determining the mechanism by which cysteine controls the transcription of flagellar master regulator genes.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *S. marcescens* strain UMH9 was isolated from a University of Michigan Hospital System patient with bacteremia. *S. marcescens* strains and plasmids used in this study are listed in Table 1. *E. coli* TOP10 (Thermo Fisher) cells were used for routine cloning purposes. *S. marcescens* and *E. coli* were cultured in LB medium (32) unless indicated otherwise. The extracellular phospholipase activity of *S. marcescens* colonies was assayed using PCY agar as described previously (33, 34). Antibiotics were used at the following concentrations: kanamycin, 50 μg/ml; ampicillin, 100 μg/ml; spectinomycin, 100 μg/ml; gentamicin, 6 μg/ml. M9 medium was used to culture *S. marcescens* strains under defined conditions (35). The M9 base medium contained 0.1 mM $CaCl_2$, 1 mM $MgSO_4$, 5 μg/ml

thiamine, and 0.4% glucose and was supplemented with 0.1% Casamino Acids (CA) where indicated. L-Cysteine and OAS were added at the indicated concentrations.

Genetic screen for phospholipase-deficient mutants. A previously generated transposon mutant library using strain UMH9 (15) was used to identify *S. marcescens* genes that contribute to extracellular phospholipase activity. Briefly, 0.1 ml of appropriately diluted mutant pools was spread on a PCY agar plate and incubated at 30°C overnight. Colonies lacking a halo of clearance on PCY agar, indicative of a phospholipase defect, were isolated for further analysis. Genomic DNA from these isolates was extracted using published procedures (36), and the location of the transposon insertion in each strain was determined by sequencing the PCR-amplified fragments of transposon-chromosome insertion site junctions (37).

Construction of a *phlAB* mutant strain. Primers PhlBA-F (5′-ATGCGCCGGCGTTTGCGAGAAATAAAA CTGGAACGCAAAGCCCTGGTGTAGGCTGGAGCTGCTTC-3′) and PhlBA-R (5′-TTAAGTTTTACCTCTGCAGTATC CCCGGTGGCCATCCCTACACATATGAATATCCTCCTTAGT-3′) were used to PCR amplify an *nptII*-containing fragment from pKD4. These primers contain ~50-bp homology at the 5′ end to the *phlAB* locus and ~20-bp homology at the 3′ end to pKD4 (underscored). The PCR product was digested with DpnI and electroporated into UMH9(pSIM19) cells that had been heat treated at 42°C for 20 min prior to transformation. The presence of the Δ*phlAB*::*nptII* allele in kanamycin-resistant transformants was confirmed by PCR and sequencing using primers PhlA-355 (5′-TGCGTCGCGAAACCCTGGAAGA-3′) and PhlB plus 72 (5′-CGGCCGGCGACGCGTAATAACC-3′). The pSIM19 plasmid was cured from the recombinant strain using a previously described method prior to phenotypic analysis (38).

Genetic complementation of the *phlAB* and *cysE* mutations. Primers PhlAB_compF (5′-AAGCTT GACGTTATCGCCCGCACCTTTACC-3′) and PhlAB_compR (5′-GAATTCGCACGGCGGACACCTACCTACA-3′) were used to amplify the *phlAB* ORFs and the 219-bp upstream sequence from UMH9 genomic DNA. Primers CysE_compF (5′-AAGCTTGCGAGCCCGAAAGGACGAAAAACC-3′) and CysE_compR (5′-GAATTCG CTGACCGCCGCCTGAAAAACACA-3′) were used to amplify the *cysE* ORF along with 134-bp upstream sequence. Both PCR products were amplified with Easy A polymerase (Agilent) for incorporation into plasmid pCR2.1-TOPO (Invitrogen). Recombinant plasmids were digested with HindIII and EcoRI, and the *phlAB*- and *cysE*-containing fragments were ligated separately to pBBR1MCS-5. Complementation plasmids were sequenced to verify the proper insertion of *phlAB* and *cysE*. Plasmids were transformed into *S. marcescens* strains via electroporation.

Quantitation of extracellular phospholipase activity. Extracellular phospholipase activity was measured using the fluorogenic PLA substrate bis-BODIPYFL C_{11}-PC (Invitrogen). Bacteria were cultured overnight at 30°C in LB medium, or M9 plus CA with or without supplementation of 1 mM cysteine or 10 mM OAS. Culture aliquots were centrifuged to remove bacteria, and 50 μl of supernatant was transferred into 96-well microplates. The substrate was prepared and mixed with culture supernatants according to the manufacturer's recommendations. Fluorescence (excitation at 480 nm and emission at 350 nm) was measured after 1 h using a fluorescence plate reader. A PLA_2 enzyme from bovine pancreas (Sigma-Aldrich) was used as a positive control in the assay (data not shown).

Motility assays. Swarming and swimming motility were assayed on LB plates containing 0.6% or 0.25% agar, respectively. To assess swarming motility, 10 μl of bacteria from overnight cultures in LB liquid medium were spotted on plates and allowed to dry, and then incubated for 16 h at 30°C. Swimming motility was assayed by stab-inoculating the center of the swim agar and then incubating for 8 h at 30°C. The motility of each strain was determined by measuring the diameters of the resulting growth zones. Plates were imaged using a Q-Count automated colony counter (Spiral Biotech).

Growth of *S. marcescens* strains. For growth analysis in defined medium, bacteria were cultured overnight in LB medium and then collected by centrifugation, washed with M9 base medium, and used to inoculate experimental cultures at an optical density at 600 nm (OD_{600}) of 0.01 in 100-well microplates. M9 medium was supplemented where indicated with 0.1% CA, OAS, or L-cysteine at the given concentrations. For growth analysis in LB medium, bacteria were taken directly from overnight cultures and subcultured to an OD_{600} of 0.01. Bacterial growth from triplicate cultures was measured every 15 min using a BioScreenC instrument during incubation at 37°C with continuous shaking.

RNA purification and qRT-PCR. *S. marcescens* strains were cultured in M9 plus CA overnight and then subcultured in M9 plus CA in the presence or absence of 1 mM cysteine or 10 mM OAS for 4.5 h at 30°C. Cultures were treated with 2 volumes of RNAprotect (Qiagen) at room temperature for 5 min prior to the collection of bacteria by centrifugation. Total RNA was purified using the RNeasy kit (Qiagen) and DNase treated using the Turbo DNA-free kit (Ambion) and repurified.

Each cDNA synthesis reaction mixture consisted of 2 μg of total RNA, random hexamers, 1 U RNaseOUT recombinant RNase inhibitor (Invitrogen), and 200 U Superscript III reverse transcriptase (Invitrogen). Control reaction mixtures for the detection of contaminating genomic DNA were prepared in the same manner except that Superscript III was replaced by diethyl pyrocarbonate (DEPC)-treated water. The remaining RNA template was degraded by the addition of 2 U RNaseH (Invitrogen). The purification of cDNA was accomplished using a QIAquick PCR purification kit (Qiagen).

Purified cDNA was diluted in water 1:5, and 5 μl was aliquoted in triplicates for use as the template for qPCR. The following oligonucleotides were used in the amplification of ~100-bp internal regions of target genes: *gyrB*F (5′-CTCTTCTCAGACCAAGGACAAG-3′) and *gyrB*R (5′-GATTACCTGATGGAGAATCCGG-3′); *phlA*F (5′-GTTTCCAGGCCGGGATTTA-3′) and *phlA*R (5′-TGTACTGCACATCGTCATAGC-3′); *fliP*F (5′-CAA GGTCTATCAGGATGCCTAC-3′) and *fliP*R (5′-GTTTGGCGCAGCATGAAT-3′); *fliC*F (5′-GAGAAAGAAGTGGCTC CTACTG-3′) and *fliC*R (5′-GTCTACGGTCGCCTTGAAATA-3′); *flhD*F (5′-CGATGTTCCGTCTTGGTATTGA-3′)

and *flhD*R (5′-GTTAAAGCGGAAGTGGCAAAC-3′); and *fliA*F (5′-GATGCTGGAAGGGCATGAA-3′) and *fliA*R (5′-ACAGCGTCAGCACCATTT-3′). The *gyrB* transcript was used as an internal control. Brilliant III Ultra-Fast SYBR green (Agilent) was used for monitoring the product amplification on a Stratagene Mx3000P qPCR system. The relative fold change was calculated using the comparative threshold cycle (C_T) method (39). PCR amplification efficiency controls were performed for each primer set and dissociation curves were conducted to verify the amplification of a single product.

Murine UTI model. Overnight cultures of UMH9 and *phlAB* in LB were subcultured at an OD_{600} of 0.1 for ~2 h at 37°C. Bacteria were collected by centrifugation and resuspended in phosphate-buffered saline (PBS) to a density of 10^9 CFU/ml. Competition infections were conducted with a 1:1 mixture of the UMH9 parent strain and the *phlAB* mutant. Female C57BL/6 mice (6 to 8 weeks old) were infected with a 0.05-ml volume containing 5×10^7 total CFU directly into the bladder as described previously (22). Bladder homogenates in PBS were plated on LB agar and in LB containing kanamycin to enumerate CFU. CI values were calculated from the following ratios: $(CFU_{phlAB}/CFU_{wild\text{-}type})^{output}/(CFU_{phlAB}/CFU_{wild\text{-}type})^{input}$. All animal experiments were conducted using protocols approved by the Institutional Animal Care and Use Committee.

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