Inactivation of the Major Hemolysin Gene Influences Expression of the Nonribosomal Peptide Synthetase Gene *swrA* in the Insect Pathogen *Serratia* sp. Strain SCBI

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ABSTRACT Hemolysins are important virulence factors for many bacterial pathogens, including *Serratia marcescens*. The role of the major hemolysin gene in the insect pathogen *Serratia* sp. strain SCBI was investigated using both forward and reverse-genetics approaches. Introduction of the major hemolysin gene into *Escherichia coli* resulted in a gain of both virulence and hemolytic activity. Inactivation of this hemolysin in *Serratia* sp. SCBI resulted in a loss of hemolysis but did not attenuate insecticidal activity. Unexpectedly, inactivation of the hemolysin gene in *Serratia* sp. SCBI resulted in significantly increased motility and increased antimicrobial activity. Reverse transcription-quantitative PCR (qRT-PCR) analysis of mutants with a disrupted hemolysin gene showed a dramatic increase in mRNA levels of a nonribosomal peptide synthetase gene, *swrA*, which produces the surfactant serrawettin W2. Mutation of the *swrA* gene in *Serratia* sp. SCBI resulted in highly varied antibiotic activity, motility, virulence, and hemolysis phenotypes that were dependent on the site of disruption within this 17.75-kb gene. When introduced into *E. coli*, *swrA* increases rates of motility and confers antimicrobial activity. While it is unclear how inactivation of the major hemolysin gene influences the expression of *swrA*, these results suggest that *swrA* plays an important role in motility and antimicrobial activity in *Serratia* sp. SCBI.

IMPORTANCE The opportunistic Gram-negative bacteria of the genus *Serratia* are widespread in the environment and can cause human illness. A comparative genomics analysis between *Serratia marcescens* and a new *Serratia* species from South Africa, termed *Serratia* sp. strain SCBI, shows that these two organisms are closely related but differ in pathogenesis. *S. marcescens* kills *Caenorhabditis* nematodes, while *Serratia* sp. SCBI is not harmful and forms a beneficial association with them. This distinction presented the opportunity to investigate potential differences in regulation of common virulence mechanisms between these two species. With the emergence of antibiotic-resistant microorganisms, there is a widespread need to understand the regulation of pathogenesis. The significance of this study is the presentation of evidence for cross-pathway regulation of virulence factors and how the elimination of one mechanism may be compensated for by the upregulation of others.

KEYWORDS hemolysis, swarming motility, insect pathogenesis, nonribosomal peptide synthetase, serrawettin W2, *Serratia*, hemolysins, nonribosomal synthetase

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Members of the genus *Serratia* are found widespread around the globe and are well known for their roles as insect pathogens (1, 2). A newly recognized *Serratia* species, termed South African *Caenorhabditis briggsae* isolate (SCBI), was identified following its isolation from the nematode *C. briggsae* KT0001 (3). These *C. briggsae* KT0001 nematodes were recovered from soil samples through *Galleria mellonella* (the greater wax moth) larval bait traps in three provinces in South Africa (3). The microbe-nematode complex between *Serratia* sp. strain SCBI and *C. briggsae* KT001 represents a potential emerging entomopathogenic association. Only a few *Serratia* species are known to use a nematode partner to establish an infection in an invertebrate host (4, 5). *Serratia* sp. SCBI demonstrates insect-pathogenic capabilities similar to those of other *Serratia* invertebrate pathogens, with CFU of <1,000 resulting in mortality in *G. mellonella* and *Manduca sexta* (tobacco hornworm) larvae following intrahemocoelic injection (3, 6, 7). Comparative genomic analysis shows that *Serratia* sp. SCBI is closely related to *Serratia marcescens* Db11, a spontaneously streptomycin-resistant mutant of *S. marcescens* Db10 (8). *S. marcescens* Db11 is a broad-host-range pathogen (9–11) and shares many physiological characteristics with *Serratia* sp. SCBI (7). Colonization of *Caenorhabditis* nematodes by these two *Serratia* spp., despite similarity in over 85% of their open reading frames, results in very distinct outcomes, with *Serratia* sp. SCBI exerting no harmful effects and *S. marcescens* Db11 killing the nematode within 7 days (8).

Hemolytic activity is a known virulence factor of *S. marcescens* Db11 that is essential for pathogenesis against *Caenorhabditis elegans* and *Drosophila melanogaster* (10). Hemolysis by *S. marcescens* is due primarily to the pore-forming toxin ShlA, whose gene is transcribed within the same operon as that of its activator and exporter, ShlB (12). Together, ShlA and ShlB act as a two-partner secretion system (13). In comparison, *Serratia* sp. SCBI contains a two-gene operon, containing a hemolysin activator protein gene and a major hemolysin gene, homologous to *shlBA* of *S. marcescens* Db11 (8). The major hemolysin gene of *Serratia* sp. SCBI shares 95% identity with *shlA*, indicating that the two hemolysins likely share the same function. However, given their contrasting effects on *Caenorhabditis* hosts, it is possible that the methods of regulation of hemolytic activity differ between *S. marcescens* Db11 and *Serratia* sp. SCBI.

Temperature is an important environmental factor that can influence the activities of essential virulence factors. A number of virulence-associated genes are downregulated in *S. marcescens* at 37°C, including those for hemolysis. Optimal hemolysis and swarming motility (rapid coordinated translocation of a bacterial population) are observed between 28 and 30°C in *S. marcescens*, with a sharp decrease in both activities at 37°C (14–17). The mechanism behind the temperature regulation of both hemolysis and motility has been elucidated for *S. marcescens* CH-1 (16). At higher temperatures, such as 37°C, a two-component system, termed RssAB, is activated. RssA serves as the sensor kinase and RssB as the response regulator. Once phosphorylated at 37°C, RssB blocks expression of the transcriptional regulator genes *flhDC*. FlhDC regulate the expression of genes involved in flagellar formation, chemotaxis, and cell division (18–20). FlhDC also positively regulate the hemolysin operon, *shlBA* (16). Therefore, with repression of *flhDC* at 37°C by RssB, *shlA* is not expressed, resulting in a loss of both swarming motility and hemolytic activity at 37°C and a switch to biofilm formation. RssB also negatively regulates expression of the mannose-6-phosphate isomerase gene *manA*, which in turn positively regulates the biosurfactant serrawettin W1 (21). Serrawettin W1 is critical for reducing surface tension when *S. marcescens* actively swarms (22), and therefore, its repression at 37°C also contributes to the loss of swarming motility at this temperature.

Serratia spp. can produce one of three types of distinct serrawettins, and these compounds are unique to *Serratia*. Termed serrawettins W1, W2, and W3, these nonionic biosurfactants often play an important role in swarming motility but can also contribute to hemolysis (22–25). Serrawettin W1, also known as serratamolide, contributes to swarming motility, is a broad-spectrum antibiotic, and is hemolytic to sheep and murine red blood cells. Furthermore, serrawettin W1 is cytotoxic to human airway and

corneal limbal epithelial cells (25). *S. marcescens* Db10 produces serrawettin W2, which is encoded by the massive 17,781-bp nonribosomal peptide synthetase (NRPS) gene *swrA* (26). *S. marcescens* Db10 serrawettin W2 is essential for swarming motility, has hemolytic properties, and also acts as a repellent toward *Caenorhabditis* nematodes. Serrawettin W2 also has antimicrobial activity toward *Staphylococcus aureus* (27). The genome of *Serratia* sp. SCBI contains a 17,775-bp gene with over 96% identity to the serrawettin W2 gene found in *S. marcescens* Db10 (8). This similarity in gene sequences suggests that serrawettin W2 produced by *Serratia* sp. SCBI may exhibit the same function, but this has not yet been investigated.

In sharp contrast to *S. marcescens*, *Serratia* sp. SCBI does not exhibit a reduction in the rate of hemolysis or dramatic reductions in swarming or swimming (movement along a nutrient gradient by use of flagella) motility at 37°C (7). This indicates that modes of regulation of hemolysis, and even motility, are likely different between these two species. To try to elucidate the mechanisms involved in the regulation and activity of hemolysis in *Serratia* sp. SCBI, hemolytic activity was investigated by utilizing both forward- and reverse-genetics approaches. The results indicated that while *shlA* confers the ability for *Escherichia coli* to kill *M. sexta*, it is not a critical virulence factor for *Serratia* sp. SCBI. Interestingly, it was observed that hemolysis-deficient *Serratia* sp. SCBI mutants demonstrated hyperswarming and hyperswimming phenotypes and showed an increase in antimicrobial activity. Therefore, the mechanism by which the hemolysis mutants were hypermotile was investigated.

RESULTS

Identification and genetic analysis of *E. coli* fosmid clones with a gain of pathogenicity. To identify virulence factors that cause mortality to *M. sexta* larvae, a *Serratia* sp. SCBI fosmid library was constructed in *E. coli* EPI300 using the pCC1FOS vector (Epicentre). A total of 396 *E. coli* clones were individually injected into 3rd-instar *M. sexta* larvae to identify fosmids carrying virulence genes. A total of 25 clones were selected and confirmed to have a gain of pathogenicity. Compared to the control group injected with wild-type *E. coli* EPI300, the 25 clones caused mortality, delays in development, and/or change of larva color (from green to pink, suggesting that these larvae were unhealthy). All 25 fosmids were end sequenced, and the sequencing results were subjected to a BLAST search against the *Serratia* sp. SCBI genome to identify the genes in each insecticidal *E. coli* clone. Out of the 25 pathogenic *E. coli* clones, one fosmid clone, termed A1-A8, carried the 4,827-bp *Serratia* sp. SCBI major hemolysin gene *shlA* (locus tag SCBI_3479) and its accompanying 1,680-bp exporter, *shlB* (SCBI_3480) (see Table S2 in the supplemental material). Because hemolytic activity is a major virulence factor in *S. marcescens*, fosmid clone A1-A8 was the focus of this study, while the remaining pathogenic clones were stored for future analysis.

Fosmid clone A1-A8 killed an average of 73% of the *M. sexta* larvae injected (Fig. 1A) and demonstrated a gain of hemolytic activity, although the rate of hemolysis was lower than that of wild-type *Serratia* sp. SCBI (Fig. 1B). Lambda Red recombination was utilized to disrupt the *shlA* gene on fosmid A1-A8. Removal of a 3,132-bp portion of the *shlA* gene beginning 42 bp downstream of the start codon resulted in reversion to the nonvirulent, nonhemolytic phenotype of wild-type *E. coli* EPI300. Therefore, *shlA* was responsible for the gain of both hemolytic activity and insecticidal activity in fosmid clone A1-A8.

Inactivation of the *shlA* gene in *Serratia* sp. SCBI results in a significant loss of hemolytic activity yet had no effect on virulence. Because the *Serratia* sp. SCBI *shlA* gene conferred insecticidal activity in *E. coli*, a *Serratia* sp. SCBI miniHimar RB1 transposon mutant library of 2,100 clones was generated and screened for defects in hemolytic activity to determine if a loss of hemolysis would have a significant impact on virulence. The 2,100 transposon mutants were assayed for their ability to lyse sheep red blood cells (SRBCs), and seven mutants with defective hemolytic activity were identified. Of these seven mutants, four mutants (1-C4, 6-E3, 8-E6, and 10-F8) had a complete loss of activity, and the remaining three mutants (18-A11, 22-C11, and 22-H3)

FIG 1 The virulence and hemolytic activity of *E. coli* fosmid clone A1-A8 are due to the major hemolysin gene. (A) Third-instar *M. sexta* larvae were injected with 2×10^5 CFU of either wild-type *Serratia* sp. SCBI (WT SCBI), *E. coli* fosmid clone A1-A8 (A1-A8), *E. coli* fosmid clone A1-A8 with the *shlA* gene deleted (A1-A8 KO), or wild-type *E. coli* EPI300 (EPI300). Larvae were held individually at 37°C for 7 days. Mortality was measured as the percentage of the larval population killed. (B) Hemolytic activity against SRBCs was assessed for wild-type *Serratia* sp. SCBI (WT SCBI), *E. coli* fosmid clone A1-A8 (A1-A8), *E. coli* fosmid clone A1-A8 with the *shlA* gene deleted (A1-A8 KO), and wild-type *E. coli* EPI300 (EPI300). A total of $\geq 4.0 \times 10^6$ CFU was used for each bacterial strain, and the rate of hemolysis was measured over 4 h at 28°C. Error bars represent standard deviations of the results from at least two independent experiments.

demonstrated various reductions in the rate of hemolysis (Fig. 2A). Genetic analysis of these mutants showed that five mutants had the transposon insertion at different points within *shlA* (SCBI_3479). The four mutants with a total loss of hemolytic activity (1-C4, 6-E3, 8-E6, and 10-F8) all had hits within the first 1,500 bp of *shlA* (Fig. 2B). The *shlA* mutant 18-A11 was the only clone with a hit within *shlA* that retained some activity and had the transposon insertion 2,597 bp downstream of the start codon. Mutant 22-C11 had the insertion in a putrescine importer (SCBI_0418), and mutant 22-H3 had the transposon hit a noncoding region between a 5'-nucleosidase (SCBI_1151) and a cytochrome *d* ubiquinol oxidase subunit (SCBI_1152).

All seven hemolysis mutants (1-C4, 6-E3, 8-E6, 10-F8, 18-A11, 22-C11, and 22-H3) were subsequently assayed for virulence in *M. sexta*. Despite the loss or reduction of hemolytic activity, all seven hemolysis mutants killed larvae at a rate similar to that of wild-type *Serratia* sp. SCBI. The 50% lethal time (LT₅₀) values of wild-type *Serratia* sp.

FIG 2 Loss of *shlA* results in a significant reduction or loss of hemolytic activity in *Serratia* sp. SCBI. (A) Hemolytic activity against SRBCs by wild-type *Serratia* sp. SCBI and five hemolysin mutants ($\geq 4.0 \times 10^6$ CFU) was measured over 4 h at 28°C. Error bars represent standard deviations of the results from at least two independent experiments. (B) Representation of the results of rescue cloning the hemolysis mutants; 5 of the 7 hemolysis mutants had the transposon insertion in *shlA*, and all hit in a different location within the gene.

FIG 3 Transposon insertion into *shlA* influenced both swimming and swarming motility. (A) Photographs of wild-type *Serratia* sp. SCBI and the *shlA* mutants following 18 h at 22°C on 0.65% agar PP3 swarm plates; (B) photographs of wild-type *Serratia* sp. SCBI and the *shlA* mutants following 48 h at 28°C on 1.5% agar PP3 swarm plates; (C) photographs of wild-type *Serratia* sp. SCBI and the *shlA* mutants following 8 h at 37°C in 0.2% agar swim plates; (D) summary of swarm and swim ring diameters of wild-type *Serratia* sp. SCBI and the hemolysis mutants. The maximum measurement was 88 mm (the width of the petri dish). Results are shown as the averages ± standard deviations of 9 measurements from 3 independent experiments.

SCBI and mutants 1-C4, 6-E3, 8-E6, 10-F8, 18-A11, 22-C11, and 22-H3 were 2.71, 3.35, 3.30, 3.23, 3.37, 2.56, 3.42, and 3.75 days, respectively. These results indicate that while *shlA* was enough to confer insecticidal activity to *E. coli*, it is not required for insect pathogenesis in *Serratia* sp. SCBI.

Inactivation of the *shlA* gene in *Serratia* sp. SCBI results in hypermotility. To determine if any other physiological functions were affected by alterations in hemolytic activity, all seven *Serratia* sp. SCBI hemolysis mutants were assayed for swarming and swimming motility. All five *shlA* mutants (1-C4, 6-E3, 8-E6, 10-F8, and 18-A11) began swarming earlier and at a higher rate than wild-type *Serratia* sp. SCBI (Fig. 3A). By 18 h, these five mutants had nearly swarmed across the entire 88-mm plate, at which time wild-type *Serratia* sp. SCBI and mutants 22-C11 and 22-H3 had not yet begun to swarm (Fig. 3D). In addition, *shlA* mutants 1-C4, 6-E3, 8-E6, 10-F8, and 18-A11 were able to swarm on hard agar (1.5%), with swarm ring diameters averaging 30.3 ± 3.6 mm following 48 h of incubation at 28°C (Fig. 3B and D). Wild-type *Serratia* sp. SCBI and mutants 22-C11 and 22-H3 were unable to swarm on 1.5% agar. When analyzed for swimming behavior, all five *shlA* mutants showed increased rates of movement (Fig. 3C). On average, these *shlA* mutants had a swim ring diameter between 55.3 and 67.3 mm following 8 h of incubation at 37°C compared to wild-type *Serratia* sp. SCBI, which had an average swim ring diameter of 44.3 ± 0.5 mm (Fig. 3D). Mutants 22-C11 and 22-H3 showed no alterations in swimming behavior from that of wild-type *Serratia* sp. SCBI.

Inactivation of the *shlA* gene in *Serratia* sp. SCBI also results in increased antimicrobial activity. Next, the *shlA* mutants were assayed for antimicrobial activity against *Micrococcus luteus*. These mutants had increased antibiotic activity compared with that of wild-type *Serratia* sp. SCBI (Table 1). The *shlA* mutants 1-C4, 6-E3, 8-E6,

TABLE 1 Antibiotic activity of the *shlA* mutants and *E. coli* fosmid clone A1-F2

Strain	Radius of clearing zone (mm) ^a
Wild-type <i>Serratia</i> sp. SCBI	14.1 ± 1.76
1-C4	16.2 ± 2.36
6-E3	15.9 ± 1.05
8-E6	17.1 ± 1.25
10-F8	16.8 ± 1.89
18-A11	16.1 ± 2.07
Wild-type <i>E. coli</i> EPI300	— ^b
Fosmid clone A1-F2	5.9 ± 1.56

^aRadii of clearing zones were measured following 24 h of incubation at 28°C, with *M. luteus* as the indicator strain. Results are shown as the averages ± standard deviations of 9 measurements from 3 independent experiments.

^b—, no activity.

10-F8, and 18-A11 had average clearing zones of 16.2, 15.9, 17.1, 16.8, and 16.1 mm, with standard deviations (SD) between 1.05 and 2.36 mm. In comparison, wild-type *Serratia* sp. SCBI had an average clearing zone of 14.1 ± 1.76 mm.

Inactivation of the *flhC* gene resulted in loss of motility but had no effect on hemolytic activity. Expression of *shlBA* is under the control of FlhDC in *S. marcescens* CH-1 (16). To determine if FlhDC may be involved in the regulation of *shlBA* in wild-type *Serratia* sp. SCBI, and therefore somehow involved in the hypermotile phenotypes of the five *shlA* mutants, the *Serratia* sp. SCBI miniHimar RB1 transposon mutant library was screened for defects in swimming motility in an attempt to find a mutant with an insertion in the *flhDC* operon. Out of 2,100 mutants screened, 16 mutants with defective swimming were identified. Mutant 12-H4, which had completely abolished swimming motility, was found to contain the transposon insertion in *flhC* (SCBI_2840). Mutant 12-H4 had the transposon insert 354 bp downstream of the start site of *flhC*, which resulted in a total loss of swarming and swimming motility (Fig. 4A and B). In contrast to what is observed in *S. marcescens flhC* mutants, the loss of *flhC* had no effects on hemolytic activity in *Serratia* sp. SCBI (Fig. 4C).

***shlA* mutants showed a significant increase in *swrA* expression.** To further investigate the genes responsible for the hyperswarming phenotype observed in the *Serratia* sp. SCBI *shlA* mutants, qRT-PCR was utilized to measure mRNA levels for genes predicted to be involved in either flagellum or surfactant production. The expression of

FIG 4 Inactivation of *flhC* resulted in loss of swimming and swarming motility but did not influence hemolytic activity. (A) Photographs of wild-type *Serratia* sp. SCBI and *flhC* mutant 12-H4 following 8 h of incubation at 37°C in 0.2% agar plates. (B) Photographs of wild-type *Serratia* sp. SCBI and *flhC* mutant 12-H4 following 42 h of incubation at 28°C on 0.65% agar PP3 swarm plates. (C) Hemolytic activity against SRBCs by wild-type *Serratia* sp. SCBI and *flhC* mutant 12-H4 ($\geq 4.0 \times 10^6$ CFU) was measured over 4 h at 28°C. Error bars represent standard deviations of the results from at least two independent experiments.

FIG 5 Mutants with transposon insertions in *shlA* had significantly increased mRNA levels of the 17,775-bp NRPS *swrA* when swarming. qRT-PCR was performed on RNA extracted from swarmer cells on 0.65% agar PP3 plates. mRNA levels were normalized to the level of the *I21p* housekeeping gene and compared to the calibrator, wild-type *Serratia* sp. SCBI (WT SCBI). Data are presented as the relative changes in gene expression from those of the calibrator obtained under the test conditions. (A) Comparison of *flhD*, *fliC*, and *swrA* mRNA levels when swarm ring diameters were at 40 mm between wild-type *Serratia* sp. SCBI and the five *shlA* mutants; (B) mRNA levels of *flhD*, *fliC*, and *swrA* by wild-type *Serratia* sp. SCBI and *shlA* mutant 6-E3 at swarm ring diameters of 20, 40, and 80 mm. Error bars represent standard deviations of the results from at least two independent experiments. *, $P < 0.05$ (significant differences in relative mRNA levels).

flhD (SCBI_2841), *fliC* (SCBI_2820), and *swrA* (SCBI_4162) in the *Serratia* sp. SCBI hemolysis mutants during swarming was assayed. FlhD is a regulator of flagellar genes, FliC is a flagellar protein, and SwrA is an NRPS hypothesized to catalyze the production of the surfactant serrawettin W2. At a swarm ring diameter of 40 mm, qRT-PCR analysis showed the *shlA* mutants 1-C4, 6-E3, 8-E6, 10-F8, and 18-A11 all had a significant increase in *swrA* mRNA levels (between a 14.7- and 22.4-fold change difference) compared to the level in wild-type *Serratia* sp. SCBI (Fig. 5A). There were no differences in mRNA levels for the *flhD* or *fliC* genes between the mutants and wild-type *Serratia* sp. SCBI. To investigate possible changes in gene expression over different stages of swarming, mRNA levels of the *flhD*, *fliC*, and *swrA* genes from *shlA* mutant 6-E3 and wild-type *Serratia* sp. SCBI were compared at 20 mm, 40 mm, and 80 mm. The level of *swrA* mRNA was highest in mutant 6-E3 at the early stages of swarming (20 mm) and was significantly higher than that in wild-type *Serratia* sp. SCBI at all stages of swarming (Fig. 5B). In *shlA* mutant 6-E3, the level of *swrA* mRNA decreased as the swarming colony expanded. At no point during the swarming process did the levels of *flhD* and *fliC* mRNA differ significantly between *shlA* mutant 6-E3 and wild-type *Serratia* sp. SCBI.

Mutation of different sites within the 17,775-bp *swrA* gene resulted in various changes in antibiotic production. Because the *Serratia* sp. SCBI *shlA* mutants showed increased *swrA* mRNA levels and *swrA* is responsible for the production of the biosurfactant serrawettin W2, which has antimicrobial activity (27), the *Serratia* sp. SCBI miniHimar RB1 transposon mutant library was screened for mutants with altered antibiotic activity in order to try to identify a mutant with a defective *swrA* gene. Mutants 1-A4, 13-G2, and 11-B8 were initially identified as having altered antibiotic activity against *M. luteus*, and rescue cloning showed that all three mutants had the transposon insertion in *swrA* (SCBI_4162). Subsequent confirmation experiments demonstrated that *swrA* mutant 11-B8, which had the transposon insertion at the bp 16198 position, had wild-type levels of antibiotic activity, with an average clearing zone of 13.7 ± 0.5 mm. In contrast, *swrA* mutants 1-A4 and 13-G2 showed no antimicrobial activity against *M. luteus* (Fig. 6C). The *swrA* mutant 1-A4 had the insertion at the bp 8700 position of *swrA*, while *swrA* mutant 13-G2 had the insertion at the bp 10242 position (Fig. 6A). Figure 6B shows the different domains of the NRPS SwrA. The *swrA* mutant 11-B8 hit within an amino acid adenylation domain, while mutants 1-A4 and 13-G2 did not hit any putative domains within *swrA*.

FIG 6 Mutations in *swrA* had various effects on antibiotic activity, and these effects were dependent on the site of transposon insertion in *swrA*. (A) Rescue cloning showed that mutants 1-A4, 13-G2, and 11-B8 had the transposon insertion at different sites within *swrA*. (B) Domain structure of SwrA. (C) Wild-type *Serratia* sp. SCBI and the *swrA* mutants were spot inoculated onto PP3 plates, incubated for 48 h at 28°C, killed by chloroform, and overlaid with warm 0.8% agar containing *Micrococcus luteus*. Clearing zones were observed following 24 h at 28°C. Results are shown as the averages $\pm$ standard deviations of 9 measurements from 3 independent experiments.

The *swrA* mutants displayed various motility phenotypes. Because SwrA is hypothesized to catalyze the production of the surfactant serrawettin W2, *swrA* mutants 1-A4, 13-G2, and 11-B8 were assayed for swimming and swarming motility to determine if there were any defects in motility. It was expected that all three *swrA* mutants would have delayed or abolished swarming motility. However, the results were highly varied and dependent on the site of transposon insertion. On 0.65% agar, *swrA* mutant 1-A4 was defective in swarming motility (Fig. 7A, B, and D). The *swrA* mutant 13-G2 did swarm on 0.65% agar, but the rate of movement was lower than that of wild-type *Serratia* sp. SCBI (Fig. 7A). The *swrA* mutant 13-G2 did not swarm on 1.05% agar (Fig. 7B and D). The *swrA* mutant 11-B8 had slightly decreased rates of swarming motility on 0.65% and 1.05% agar than those of wild-type *Serratia* sp. SCBI (Fig. 7A, B, and D). The three *swrA* mutants demonstrated defects in swimming motility similar to those in swarming motility (Fig. 7C and D). The *swrA* mutant 1-A4 had an average swim ring diameter of only 11.0 ± 0.8 mm compared to wild-type *Serratia* sp. SCBI, which had an average swim ring diameter of 45.7 ± 1.7 mm following 8 h of incubation at 37°C. The *swrA* mutants 13-G2 and 11-B8 had reduced rates of swimming motility, with swim ring diameters of 18.7 ± 2.5 mm and 25.7 ± 3.3 mm, respectively. While these results demonstrate that *swrA* is important in both swarming and swimming motility, this gene is highly complex and appears to have domains important in self-regulation.

The *swrA* mutants demonstrated varied rates of hemolytic activity and virulence. To determine if disruption of the *swrA* gene in *Serratia* sp. SCBI had any impact on virulence, *swrA* mutants 1-A4, 13-G2, and 11-B8 were individually injected into 3rd-instar *M. sexta* larvae and monitored for mortality over a 7-day period. The *swrA* mutant 13-G2 killed *M. sexta* at a rate similar to that of wild-type *Serratia* sp. SCBI, with LT_{50} values of 1.71 and 1.18 days, respectively (Fig. 8A). The *swrA* mutant 11-B8 killed at a slightly reduced rate, with an LT_{50} value of 2.93 days. *swrA* mutant 1-A4 was less virulent than wild-type *Serratia* sp. SCBI, killing only 40% of larvae injected, with a LT_{50} value of 7.28 days. All three *swrA* mutants were also assayed for hemolytic activity. The *swrA* mutants 13-G2 and 11-B8 demonstrated slightly reduced levels of hemolytic activity compared to that of wild-type *Serratia* sp. SCBI (Fig. 8B), but this effect is likely due to a slight decrease in growth rate (Fig. 8C). The *swrA* mutant 1-A4 had completely abolished hemolysis over 4 h against SRBCs.

Complementation of the major hemolysin gene in mutant 1-C4 restored wild-type phenotypes. The major hemolysin gene was cloned and tested for complementation in *shlA* mutant 1-C4. Since inactivation of *shlA* resulted in severely attenuated hemolytic activity, hypermotility, and hyperantimicrobial activity, it was hypothesized

FIG 7 Mutations in *swrA* had various effects on swarming and swimming motility. (A) Photographs of wild-type *Serratia* sp. SCBI and *swrA* mutants 1-A4, 13-G2, and 11-B8 following 24 h and 48 h of incubation at 28°C on 0.65% agar PP3 plates; (B) photographs of wild-type *Serratia* sp. SCBI and *swrA* mutants on 1.05% agar PP3 plates following 24 h and 48 h of incubation at 28°C; (C) photographs of wild-type *Serratia* sp. SCBI and *swrA* mutants in 0.2% agar plates following 8 h at 37°C; (D) summary of swarm and swim ring diameter measurements observed for wild-type *Serratia* sp. SCBI and the *swrA* mutants. Results are shown as the averages ± standard deviations of 9 measurements from 3 independent experiments.

that the introduction of *shlA* on a multicopy plasmid would restore all these phenotypes back to wild-type levels. Since the other *shlA* mutants (6-E3, 8-E6, 10-F8, and 18-A11) demonstrated similar phenotypes, complementation was performed only on one mutant. Plasmid pBAD33 (chloramphenicol resistant [Cm^r]) was introduced into both wild-type *Serratia* sp. SCBI and *shlA* mutant 1-C4 as controls. pBAD33-Hemol was introduced into both wild-type *Serratia* sp. SCBI and *shlA* mutant 1-C4. Wild-type *Serratia* sp. SCBI, *shlA* mutant 1-C4, the control transformants containing pBAD33, the complemented wild-type *Serratia* sp. SCBI, and *shlA* mutant 1-C4 were all assayed for hemolytic activity, swarming and swimming motility, and antimicrobial activity against *M. luteus* (Table 2). The presence of pBAD33 without an insert did not significantly change the phenotypes of wild-type *Serratia* sp. SCBI or *shlA* mutant 1-C4. The presence of pBAD33-Hemol did not significantly change the physiology of wild-type *Serratia* sp. SCBI, even in terms of hemolytic activity. The presence of pBAD33-Hemol did restore the phenotypes of *shlA* mutant 1-C4 back to wild-type levels. The percentage of SRBCs lysed after 4 h of incubation changed from 2.8% to 86.5% between the mutant and the complemented mutant, respectively. Swarming and swimming rates decreased back to wild-type levels in complemented *shlA* mutant 1-C4. Furthermore, antimicrobial activity was restored to the wild-type level in the complemented *shlA* mutant 1-C4, with radii of the clearing zones dropping from an average of 16.9 mm down to 13.2 mm.

Introduction of *swrA* into *E. coli* caused significant increases in both swarming and swimming motility as well as increased antimicrobial activity. An *E. coli* fosmid

FIG 8 The *swrA* mutant 1-A4 was the only *swrA* mutant to display defective virulence and hemolytic activity. (A) Third-instar *M. sexta* larvae were injected with 2×10^4 CFU of either wild-type *Serratia* sp. SCBI or one of the *swrA* mutants (1-A4, 11-B8, or 13-G2) and kept at 37°C for 7 days. Mortality was measured as the percentage of the larval population killed over time. (B) Hemolytic activity against SRBCs by either wild-type *Serratia* sp. SCBI or one of the *swrA* mutants (1-A4, 11-B8, or 13-G2) at $\geq 4.0 \times 10^6$ CFU was measured over 4 h at 28°C. Error bars represent standard deviations of the results from at least two independent experiments. (C) The growth rate of each strain was measured over 24 h at 37°C by determining absorbance at 600 nm.

clone, termed A1-F2, was identified in the *M. sexta* gain-of-pathogenesis screen. Fosmid clone A1-F2 killed approximately 20% of *M. sexta* larva following intrahemocoelic injection. End sequencing of this fosmid showed that it contained *swrA* (Table S3). When assayed for swimming motility, clone A1-F2 had an average swim ring diameter of 55.0 ± 5.0 mm, at which point wild-type *E. coli* EPI300 had not yet begun to swim (Fig. 9A and C). Fosmid clone A1-F2 was also able to swarm on LB plates containing 0.5% agar. Following 18 h of incubation at 28°C, fosmid clone A1-F2 had swarmed across the entire plate (Fig. 9B). Swarming was optimal at 28°C, as fosmid clone A1-F2 had average swarm ring diameters of only 19.5 ± 1.0 mm and 28.5 ± 1.2 mm at 18 h at 22°C and 37°C, respectively. However, *E. coli* fosmid clone A1-F2 had swarmed across the entire plate by 24 h at both 22°C and 37°C. *E. coli* EPI300 was not capable of swarming at any temperature on LB plates containing 0.5% agar. When assayed for hemolysis, fosmid clone A1-F2 showed no hemolytic activity (data not shown). When tested for antimicrobial activity, fosmid clone A1-F2 showed antimicrobial activity against *M. luteus*, but its activity was significantly lower than that of wild-type *Serratia* sp. SCBI (see Table 1). Fosmid clone A1-F2 produced a clearing zone of 5.9 ± 1.56 mm, and wild-type *Serratia* sp. SCBI had a clearing zone of 14.1 ± 1.76 mm. Wild-type *E. coli* EPI300 had no antimicrobial activity.

Physiochemical analysis of serrawettin W2. Matrix-assisted laser desorption ionization mass spectrometry (MALDI MS) analysis of wild-type *Serratia* sp. SCBI cells led to the detection of a compound with an m/z of 754.43697 $[M+Na]^+$, leading to a sum formula of $C_{38}H_{61}O_9N_5$ (calculated m/z of 754.43615 $[M+Na]^+$, Δ ppm of 1.083). This sum formula is in accordance with serrawettin W2 (22). The results showed that m/z

TABLE 2 Complementation of the major hemolysin gene into mutant 1-C4 restored wild-type phenotypes

Strain	Hemolytic activity (%) ^a	Swarming ring diam (avg $\pm$ SD) (mm) ^{b,c}	Swimming ring diam (avg $\pm$ SD) (mm) ^{c,d}	Radius of clearing zone (avg $\pm$ SD) (mm) ^{c,e}
WT <i>Serratia</i> sp. SCBI	93.3	49.3 $\pm$ 11.9	51.3 $\pm$ 2.9	14.5 $\pm$ 1.1
WT <i>Serratia</i> sp. SCBI/pBAD33	88.4	45.0 $\pm$ 8.3	49.7 $\pm$ 1.6	14.3 $\pm$ 1.3
WT <i>Serratia</i> sp. SCBI/pBAD33-Hemol	97.9	53.8 $\pm$ 6.7	56.7 $\pm$ 2.4	13.8 $\pm$ 0.9
1-C4	2.8	88.0 $\pm$ 0	64.3 $\pm$ 1.9	16.9 $\pm$ 1.8
1-C4/pBAD33	2.2	88.0 $\pm$ 0	63.1 $\pm$ 2.3	16.7 $\pm$ 1.5
1-C4/pBAD33-Hemol	86.5	54.7 $\pm$ 15.1	49.3 $\pm$ 2.9	13.2 $\pm$ 1.7

^aHemolytic activity was measured as the percentage of SRBCs lysed following 4 h of incubation at 28°C.

^bSwarm assays were performed at 22°C, with an incubation time of 48 h.

^cThe maximum measurement was 88 mm (the width of the petri dish). Results are shown as the averages $\pm$ SD of 9 measurements from 3 independent experiments.

^dSwim assays were performed at 37°C, with an incubation time of 8 h.

^eAntibiotic activity assays were performed at 28°C, with an incubation time of 24 h. The indicator strain was *M. luteus*. The radii of the clearing zones were measured.

FIG 9 *E. coli* fosmid clone A1-F2, which contains the *swrA* gene, showed increased swimming and swarming motility compared to wild-type *E. coli* EPI300. (A) Photographs of fosmid clone A1-F2 and wild-type *E. coli* EPI300 following 8 h of incubation at 37°C in 0.2% agar swim plates; (B) photographs of fosmid clone A1-F2 and wild-type *E. coli* EPI300 following 18 h at 28°C on 0.5% agar LB swarm plates; (C) summary of swarm and swim ring diameter measurements observed for fosmid clone A1-F2 and wild-type *E. coli* EPI300. Results are shown as the averages ± standard deviations of 9 measurements from 3 independent experiments. N/A, not available (over swarmed plate at 18 h).

754.44 [M+Na]⁺ was also produced by *shlA* mutant 1-C4 and *E. coli* fosmid clone A1-F2 (Fig. S1). MALDI tandem MS (MALDI MS²) of *m/z* 754.44 [M+Na]⁺ (Fig. S2) revealed a neutral loss fragmentation pattern similar to what has been reported for serrawettin W2 (22). Based on these findings, we conclude that *m/z* 754.44 [M+Na]⁺ produced by *Serratia* sp. SCBI, *shlA* mutant 1-C4, *swrA* mutant 11-B8, and *E. coli* fosmid clone A1-F2 is serrawettin W2 or a close derivative thereof.

DISCUSSION

The hemolysin ShlA is an important virulence factor in *S. marcescens* (16, 28–30). A transposon insertion in the *shlBA* operon in *S. marcescens* Db11 results in a significant attenuation of virulence toward *C. elegans*, *D. melanogaster*, and a murine lung infection model (10). Inactivation of *shlA* in *Serratia* sp. SCBI, which shares 95% identity at the nucleic acid sequence level with the *shlA* gene of *S. marcescens*, resulted in a complete loss of hemolytic activity when the transposon hit within the first 1,500 bp of the gene. Abolishment of hemolytic activity did not alter virulence toward *M. sexta*. In contrast to what occurred with *S. marcescens* Db11, this result indicates that hemolysis is not required for insecticidal activity by *Serratia* sp. SCBI. However, *shlA* does have toxic effects. Introduction of the *Serratia* sp. SCBI *shlA* gene, along with its activator *shlB*, into *E. coli* EPI300 on a fosmid resulted in both a gain of hemolytic activity and a gain of pathogenesis. Deletion of *shlA* by lambda Red recombination confirmed that this hemolysin was responsible for the gain of insecticidal activity by *E. coli*. Therefore, while *shlA* does confer virulence in *E. coli*, it is not the driving factor of *Serratia* sp. SCBI pathogenesis.

Interestingly, inactivation of *shlA* in *Serratia* sp. SCBI resulted in significantly increased rates of swarming and swimming motility, as well as increased antimicrobial activity. Furthermore, in contrast to wild-type *Serratia* sp. SCBI, these mutants were capable of swarming on hard agar. This phenomenon has no precedence in the literature, and therefore, several approaches were used to try to pinpoint the cause of increased motility in these mutants. Since FlhDC is a regulator of hemolytic activity in *S. marcescens*, one goal was to determine if FlhDC is a positive regulator of hemolytic activity in *Serratia* sp. SCBI. It was hypothesized that if FlhDC was important in regulating *shlA*, the hypermotile phenotype seen in the hemolysis mutants was result of FlhDC activity. Screening of the miniHimar RB1 transposon library uncovered a mutant (12-H4) with an insertion in *flhC*. The *flhC* mutant 12-H4 had abolished swim-

ming and swarming motility yet demonstrated wild-type hemolysis. This result indicated that hemolysis is regulated differently in *Serratia* sp. SCBI from that in *S. marcescens*, in which inactivation of *flhDC* results in loss of hemolytic activity (16).

Expression analysis by qRT-PCR provided further evidence that FlhDC was not responsible for the hypermotility in the *shlA* mutants. When the expression levels of *flhD* and *flhC* were compared between the hyperswarmer hemolysis mutants and wild-type *Serratia* sp. SCBI, no differences were found. These results provided evidence that the increased rates of motility in *shlA* mutants 1-C4, 6-E3, 8-E6, 10-F8, and 18-A11 were not flagellum driven. Instead, it was the expression of *swrA* that appeared to be responsible. *SwrA* is a large NRPS gene that catalyzes production of the surfactant serrawettin W2 in *S. marcescens* strains Db11 and Db10 (26, 27). Serrawettin W2 is critical for swarming, as it reduces surface tension along the edges of the swarm colony, and it shows antimicrobial activity against *S. aureus*. All five *Serratia* sp. SCBI *shlA* mutants had levels of *swrA* mRNA that were 15 to 22 times higher than in wild-type *Serratia* sp. SCBI. These mutants also had increased antimicrobial activity against *M. luteus* compared to that of wild-type *Serratia* sp. SCBI. When analyzed by mass spectrophotometry, *shlA* mutant 1-C4 was confirmed to overproduce serrawettin W2, further supporting the qRT-PCR data that this biosurfactant accounted for the increased motility and antibiotic activity. Although only *shlA* mutant 1-C4 was analyzed, it is likely that all five *shlA* mutants overproduce serrawettin W2. Further analysis of *shlA* mutant 6-E3 showed that the expression of *swrA* was highest during the early stages of swarming and decreased as the swarm colony moved out across the plate.

Screening of the miniHimar transposon mutant library for altered antibiotic activity against *M. luteus* identified three mutants (1-A4, 13-G2, and 11-B8) with the transposon insertion in *swrA*, providing evidence that serrawettin W2 has antimicrobial properties. However, antibiotic activity was not consistent in all three mutants. The *swrA* mutant 1-A4, which contains the transposon insertion toward the middle of *swrA* at the bp 8700 position, had abolished antimicrobial and hemolytic activities. Furthermore, *swrA* mutant 1-A4 was completely nonmotile, and virulence was severely attenuated. When the transposon was inserted a little further downstream in *swrA* at the bp 10242 position, as seen in mutant 13-G2, there was no antimicrobial activity, and rates of both swimming and swarming motility were significantly reduced. *swrA* mutant 11-B8, which contained the transposon insertion at the very end of *swrA*, did not show significant changes in antimicrobial activity, motility, hemolysis, or virulence from those of wild-type *Serratia* sp. SCBI. Although the presence of wild-type *shlA* appears to regulate the production of SwrA, the results from *swrA* mutant 1-A4, most notably its nonhemolytic phenotype, suggest that SwrA may in turn regulate *shlA* expression. We hypothesize that this regulation occurs in an indirect manner.

While a number of strains of *S. marcescens* carry a version of *swrA*, the *swrA* gene in *S. marcescens* Db10 is similar in both size (17,781 bp) and DNA sequence identity (96%) to the *Serratia* sp. SCBI *swrA* gene (26). Physiochemical analysis of *Serratia* sp. SCBI serrawettin W2 strongly suggests that these two *Serratia* spp. produce very similar cyclodepsipeptides which contain D-3-hydroxydecanoic acid and five amino acids (D-leucine, L-serine, L-threonine, D-phenylalanine, and L-isoleucine), as has also been determined by *in silico* analysis using the antiSMASH program (31). The only difference phenotypically is that serrawettin W2 from *S. marcescens* Db10 shows hemolytic properties, whereas serrawettin W2 from *Serratia* sp. SCBI does not show any hemolysis against SRBCs. It is possible that the serrawettin W2 produced by *Serratia* sp. SCBI is hemolytic toward other red blood cell types, such as those from humans, due to differences in membrane compositions. Nevertheless, the serrawettins from both species are critical for swarming motility and antimicrobial activity.

In *S. marcescens* strain MG1, the gene *swrA* is significantly smaller, approximately 2.7 kb, but the final product is similar in structure to those produced in *S. marcescens* Db10 and *Serratia* sp. SCBI (26, 32, 33). The 2.7-kb *swrA* gene of *S. marcescens* MG1 is homologous to the beginning of the *swrA* gene of both *S. marcescens* Db10 and *Serratia* sp. SCBI. However, the larger size of *swrA* in both *S. marcescens* Db10 and *Serratia* sp.

SCBI and the phenotypic data collected from the three SCBI *swrA* mutants indicate that the entire gene is required for proper synthesis of SwrA. The differences in phenotypes between 1-A4, 13-G2, and 11-B8 are likely due to the production of a truncated form of SwrA, with each mutant having a different version of the lipopeptide. Further investigation into the function of the different modules in *swrA* should provide insight into explaining the phenotypes of *swrA* mutants 1-A4, 13-G2, and 11-B8.

Introduction of the *swrA* gene into *E. coli* demonstrates how important serrawettin W2 is to motility and antimicrobial activity. Without *swrA*, *E. coli* EPI300 is unable to swarm on LB plates containing 0.5% agar. However, with *swrA*, as seen in fosmid clone A1-F2, swarming does occur and is optimal at 28°C. Clone A1-F2 swarmed across an entire plate within 18 h at 28°C, while it took a full 24 h for A1-F2 to swarm across the entire plate at 22°C and 37°C. Though *E. coli* EPI300 swims in LB plates containing 0.2% agar, it occurs at a higher rate when transformed with *swrA*. Fosmid clone A1-F2 displayed swimming rates similar to those of wild-type *Serratia* sp. SCBI and had an average swim ring diameter of 55 mm after 8 h at 37°C. *E. coli* EPI300 did not begin swimming within that time frame; however, it had swim rings of 55 mm or greater following incubation for 24 h at 37°C. Though the *swrA* gene in *S. marcescens* Db10 had hemolytic properties (26), clone A1-F2 showed no hemolysis against SRBCs. Clone A1-F2 did, however, demonstrate antimicrobial activity, indicating that serrawettin W2 serves as both a wetting agent and an antibiotic in *Serratia* sp. SCBI and likely contributes to its survival in polymicrobial environments.

This study has highlighted several important differences between *Serratia* sp. SCBI and *S. marcescens* Db11. First, modes of regulation of hemolytic activity are different between these two organisms. Unlike with *S. marcescens*, the global regulators FlhDC do not appear to have any role as a positive regulator of hemolysis in *Serratia* sp. SCBI. Also, hemolysis is not a requirement for insecticidal activity in *Serratia* sp. SCBI. These results are important for laying the groundwork for understanding how *Serratia* sp. SCBI has evolved as a mutualist of *Caenorhabditis* nematodes, while *S. marcescens* remains a pathogen of these nematodes. *S. marcescens* Db11 relies on its hemolysin for pathogenesis toward both insects and nematodes. Yet, it is clear that *Serratia* sp. SCBI has a different repertoire of essential virulence factors for the killing of insects. The connection between *shlA* and *swrA* in *Serratia* sp. SCBI calls into question why these two genes, which do not reside near each other in the genome, have such strong effects on each other. SwrA clearly plays an important role in the motility of *Serratia* sp. SCBI, and motility is often important in the ability of a bacterium to colonize a host. While further investigation on the mechanisms that *Serratia* sp. SCBI utilizes to colonize *C. briggsae* is needed, it is possible that swarming contributes to successful colonization of the nematode. During this time, the antimicrobial properties of serrawettin W2 would ward off other competitors within the intestine of the nematode. It is also important that during colonization of the nematode, the expression of *shlA* is repressed, as it is likely that ShlA is harmful to the nematode. From the expression data collected from the 5 *Serratia* sp. SCBI *shlA* mutants (1-C4, 6-E3, 8-E6, 10-F8, and 18-A11), disruption of the *shlA* gene clearly affects *swrA* expression, and disruption of *swrA* at a specific location (as seen in *swrA* mutant 1-A4) influences hemolytic activity. Therefore, in order to both successfully colonize the nematode and carry out its role as an insect pathogen, *Serratia* sp. SCBI has evolved a regulatory system that ensures proper gene expression to carry out its dual lifestyle, and this system involves regulating the expression of *shlA* and *swrA*.

In summary, the function of the *Serratia* sp. SCBI *shlA* gene and its role in virulence were elucidated. While ShlA produced by *Serratia* sp. SCBI is solely responsible for hemolytic activity, it appears to play a minor role in insect pathogenesis. Unexpectedly, those *Serratia* sp. SCBI transposon mutants with the major hemolysin gene inactivated were hypermotile and demonstrated increased antimicrobial activity. These phenotypes were due to significantly increased expression of the NRPS *swrA*. Mutants 22-C11 and 22-H3 had reduced hemolysis yet displayed no alterations in motility, suggesting that inactivation of the major hemolysin gene itself is responsible for the effects on

TABLE 3 Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Strains		
<i>Serratia</i> sp. SCBI		
WT	Wild type	3
1-C4	Transposon mutant, disrupted <i>shlA</i>	This study
6-E3	Transposon mutant, disrupted <i>shlA</i>	This study
8-E6	Transposon mutant, disrupted <i>shlA</i>	This study
10-F8	Transposon mutant, disrupted <i>shlA</i>	This study
18-A11	Transposon mutant, disrupted <i>shlA</i>	This study
22-C11	Transposon mutant, disrupted putrescine importer	This study
22-H3	Transposon mutant, disrupted noncoding region	This study
1-A4	Transposon mutant, disrupted <i>swrA</i>	This study
13-G2	Transposon mutant, disrupted <i>swrA</i>	This study
11-B8	Transposon mutant, disrupted <i>swrA</i>	This study
<i>E. coli</i>		
S17-1 λ pir	Donor strain transposon mutagenesis	41
DH5 α λ pir	Host strain for gene complementation	42
EPI300	Host strain for fosmid library	Epicentre Technologies
Fosmid clone A1-A8	Carries <i>Serratia</i> sp. SCBI <i>shlA</i> gene	This study
Fosmid clone A1-F2	Carries <i>Serratia</i> sp. SCBI <i>swrA</i> gene	This study
Plasmids		
pKD46-Gm	Lambda Red recombination plasmid	35
pKD4	Kanamycin resistance cassette template	34
pMiniHimar RB1	Transposon mutagenesis	36
pCC1FOS	Fosmid vector	Epicentre Technologies
pCR2.1-TOPO	TOPO cloning	Invitrogen
pBAD33-Cm	Expression vector for complementation	38
pBAD33-Hemol	Complementation of hemolysin gene	This study

swrA expression. Transposon mutagenesis of the *swrA* gene resulted in various motility, antimicrobial, hemolytic, and virulence phenotypes in *Serratia* sp. SCBI that were dependent on the site of insertion. The introduction of *swrA* in *E. coli* resulted in significant increases in swarming and swimming motility and a gain of antimicrobial activity. How the mechanism of inactivation of *shlA* influences expression of this large NRPS warrants further investigation. Furthermore, investigation into the functions of the different modules of *swrA* should provide evidence as to how inactivation of different portions of this gene results in such varied phenotypes. Finally, this study provides evidence that, as with other *Serratia* spp., both the production of a biosurfactant and flagella are required for swarming motility.

MATERIALS AND METHODS

Bacterial strains and growth conditions. A complete list of all bacterial strains and plasmids used in this study is shown in Table 3. Bacteria were grown overnight at 37°C in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl) with appropriate antibiotics, when required.

Bacterial growth assay. The growth of *Serratia* sp. SCBI mutants was determined at 28°C using methods described previously (7).

Fosmid library construction. The *Serratia* sp. SCBI fosmid library was constructed with the pCC1FOS vector using Epicentre's CopyControl fosmid library production kit, according to the manufacturer's instructions (8).

Gain-of-pathogenicity assay. A total of 396 fosmid clones were assayed for their ability to kill 3rd-instar *M. sexta* larvae using methods described previously (7). Briefly, overnight bacterial cultures were initially diluted to an optical density at 600 nm (OD_{600}) of 1.0 in LB medium. The bacterial culture was further diluted severalfold to obtain an average of 2×10^5 CFU for each fosmid clone. For each diluted sample, 10 μ l was directly injected into the insect hemocoel by use of a sterilized Hamilton syringe. *Serratia* sp. SCBI was used as a positive control, and *E. coli* EPI300 was used as a negative control. For all of the samples, including the controls, 3 larvae were used. The larvae were held individually with food for 7 days at 37°C under a 16-h light/8-h dark cycle. The larvae were monitored for insect mortality or delays in insect development during that time period. Clones displaying a gain of pathogenicity were confirmed by performing 3 biological replicates using 10 *M. sexta* larva for each assay and utilizing the same number of CFU used for the initial screen. Mortality was measured as a percentage of larvae killed by the bacteria relative to the total number of larvae injected per sample.

Sequencing pathogenic fosmids. Fosmids with a gain of function for pathogenicity were isolated using the alkaline lysis method and subsequently end sequenced using Epicentre F-pCC1/pEpiFOS and

R-pCC1/pEpiFOS primers. Fosmid gene location was determined using BLASTN against the *Serratia* sp. SCBI genome. The average size of the fosmids was 40,863 bp (SD, $\pm 2,935$ bp).

Lambda Red recombination in virulent fosmid clones. The major hemolysin gene in fosmid clone A1-A8 was disrupted with lambda Red recombination using methods described previously (34, 35). Briefly, the kanamycin resistance gene from pKD4 was amplified using the primers listed in Table S1 in the supplemental material, which were designed to add 39-bp tails that were homologous to the sequence targeted for inactivation. The thermal cycler conditions were as follows: 94°C for 30 s for initial denaturation, followed by 25 cycles of 94°C for 30 s, 50°C for 30 s, and 68°C for 6 min, followed by 68°C for 10 min for a final extension. Fosmid clone A1-A8 was transformed with pKD46-Gm (35) using a standard chemical complementation protocol and subsequently renamed A1-A8-pKD46. Fosmid clone A1-A8-pKD46 was grown overnight in LB medium containing 10 μ g/ml gentamicin and diluted to an OD₆₀₀ of 0.05 in 5 ml of LB medium. The diluted culture was grown at 28°C until the cells reached an OD₆₀₀ of 0.1, at which time L-arabinose was added to a concentration of 10 mM. The culture was allowed to continue to grow to an OD₆₀₀ of 0.6, at which time it was put on ice for 15 min. At this point, the standard protocol for producing chemically competent cells was followed. For transformation, cells were mixed with 0-, 20-, 50-, or 100-ng PCR products amplified from pKD4 containing the 39-bp tails. Confirmation that homologous recombination had taken place within the major hemolysin gene was done by PCR using the confirmation primers listed in Table S1.

Construction of the *Serratia* sp. SCBI transposon mutant library. *Serratia* sp. strain SCBI was mutagenized by the use of the miniHimar transposon RB1 (36). Both the donor (*E. coli* S17-1 λ pir/pMiniHimar RB1) and recipient (*Serratia* sp. SCBI) were grown overnight in LB medium containing appropriate antibiotics (30 μ g/ml kanamycin for the donor and 10 μ g/ml polymyxin B sulfate for the recipient). One milliliter of each culture was centrifuged at $10,000 \times g$ for 2 min, and the pellet was washed twice with phosphate-buffered saline (PBS) before 20 μ l of a 1:1 cell mixture was spotted onto the center of an LB agar plate and incubated at 37°C for 24 h. Following incubation, the cell mixture was resuspended in 1 ml of PBS, and transconjugants were selected by overnight growth of 100- μ l aliquots from 10^{-5} and 10^{-6} dilutions on LB agar containing 100 μ g/ml kanamycin and 30 μ g/ml polymyxin B sulfate at 37°C. A total of 2,100 isolated transconjugant colonies were selected and grown at 37°C overnight in LB medium containing 40 μ g/ml kanamycin and 10 μ g/ml polymyxin B sulfate in 96-well plates before the addition of an equal volume of 60% glycerol and storage at -80°C for future use.

Screening the miniHimar RB1 transposon mutant library for hemolytic activity. Minor modifications were done on a liquid hemolysis assay described previously (7, 37) in order to screen the *Serratia* sp. SCBI miniHimar RB1 transposon mutant library. Briefly, individual mutants were inoculated into 200 μ l of LB medium containing 25 μ g/ml kanamycin in 96-well plates and incubated for 18 h at 37°C. Twenty microliters of each culture was mixed with 180 μ l of washed sheep red blood cells (SRBCs; 10% [vol/vol] in PBS) in 96-well plates and incubated for 45 min at 37°C with gentle agitation. Plates were centrifuged for 5 min at $3,000 \times g$, and the resulting supernatant was diluted 1:10 in distilled water (dH₂O). The A₄₀₅ was determined on a spectrophotometer to measure the amount of released hemoglobin. As a positive control, 1 ml of SRBCs was lysed by addition of 100 μ l of 20% sodium dodecyl sulfate (SDS) and incubated at 37°C for 15 min. For negative controls, 100 μ l of LB medium was added to 1 ml of washed SRBCs incubated at 37°C, and the samples were treated as described above. Controls were repeated at each temperature in order to calculate the hemolytic units for each sample set at the respective temperature. The following equation was used to calculate hemolytic units (HU): $\text{HU} = [(A_{405} \text{ of sample} - A_{405} \text{ of negative control}) / A_{405} \text{ of positive control}] \times 100$.

Screening the miniHimar RB1 transposon mutant library for antibiotic activity. Individual colonies of transposon-induced mutants of *Serratia* sp. strain SCBI were screened for antibiotic activity by the use of an antibiotic overlay plate assay. PP3 agar (2% proteose peptone 3, 0.5% NaCl, 2% agar) was poured into oversized petri dishes (150 by 15 mm; Fisher Scientific, Canada). Using a 96-well replicator, the transposants stored at -80°C were transferred into new 96-well microtiter plates containing fresh LB medium containing 25 μ g/ml kanamycin and incubated overnight at 37°C. The freshly grown overnight culture was replica plated directly into PP3 medium, and the plates were incubated for 18 h at 28°C. A total of 10 ml of 0.8% agar was mixed with 200 μ l of the indicator strain *Micrococcus luteus* and poured over each inoculated PP3 plate. The mutants were incubated with the overlay for 24 h at 28°C, and zones of clearing were measured. To test individual mutants for antibiotic activity, the same procedure was followed; however, 2 μ l of overnight culture was inoculated onto PP3 plates overlaid with the indicator strain *M. luteus*.

Screening the miniHimar RB1 transposon mutant library for swimming motility. Individual colonies of transposon-induced mutants of *Serratia* sp. strain SCBI were screened for swimming motility by the use of a swim plate assay. Swim agar (0.2% agar) was poured into oversized petri dishes (150 by 15 mm; Fisher Scientific, Canada). Using a 96-well replicator, the transposants stored at -80°C were transferred into new 96-well microtiter plates containing fresh LB medium containing 25 μ g/ml kanamycin and incubated overnight at 37°C. The freshly grown overnight culture was replica plated directly into swim plates using a 96-well metal replicator. The swim plates were incubated for 4 h at 37°C, and the diameters of swim rings were measured. Confirmation of mutants defective in swimming was done using an assay described previously (7).

Molecular analysis of transposon mutants. Genomic DNA (gDNA) from each clone was prepared with the standard chloroform-isoamyl alcohol extraction technique. The gDNA was digested with NsiI (New England BioLabs), which does not cut the transposon, followed by ligation with T4 ligase (New England BioLabs). The ligated DNA was electroporated into *E. coli* DH5 α λ pir cells, and transformants were selected on LB plates containing 25 μ g/ml kanamycin. The transposon-carrying plasmids were

isolated and sequenced using the transposon-specific primers Himar1 (5'-CATTAATACTAGCGACGCCA TCT-3') and 615 (5'-TCGGGTATCGTCTTGAAGGG-3'). Sequences were compared to the *Serratia* sp. SCBI genome using BLASTN.

Gene complementation. For complementation analysis, the major hemolysin open reading frame was amplified with primers F-Hemolysin-Comp (5'-CCCACGGCAATATACGGAGATACA-3') and R-Hemolysin-Comp (5'-TGGCTTACAACGTGTGGATCAGG-3'), a template of 100 ng of *Serratia* sp. SCBI gDNA, and OneTaq Hot Start DNA polymerase (New England BioLabs). The PCR program was 30 s at 94°C for initial denaturation, followed by 30 cycles of 94°C for 30 s, 50°C for 30 s, and 68°C for 6 min, with a final extension time of 10 min at 68°C. PCR products were cleaned up using the QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions. Approximately 100 ng of cleaned-up PCR product was cloned into vector pCR2.1-TOPO using the TOPO-TA cloning kit (Invitrogen) according to the manufacturer's instructions. Invitrogen primers M13 Forward (–20) and M13 Reverse were used to determine the orientation of gene insertion. Depending on the orientation of insertion in pCR2.1-TOPO, plasmid DNA was cut either with XbaI and SacI or with XbaI and HindIII (New England BioLabs). Digested DNA was ligated into pBAD33Cm (38), which had been cut with the same restriction enzymes. The vector was subsequently renamed pBAD33-Hemol and was electroporated into *E. coli* DH5 α *λpir*, and selection was made on LB plates containing 25 μ g/ml chloramphenicol. Plasmid was extracted from successfully transformed *E. coli* DH5 α *λpir* and electroporated into the appropriate strain, and selection was made on LB plates containing 150 μ g/ml chloramphenicol. When we assayed for complementation, all strains were grown in the presence of 10 mM arabinose to induce expression of the cloned gene.

Hemolysis assay. Rates of hemolytic activity for wild-type *Serratia* sp. SCBI, *E. coli* EPI300, fosmid clones, and individual *Serratia* sp. SCBI transposon mutants were measured at 28°C using a liquid assay described previously (7), which measured the rate of SRBC lysis by bacterial culture ($\sim 4.0 \times 10^6$ cells) over 4 h.

Insect viability assay. *E. coli* fosmid clones and *Serratia* sp. SCBI miniHimar RB1 mutants were analyzed for their ability to kill 3rd-instar *M. sexta* larvae using methods described previously (7). Briefly, overnight bacterial cultures were initially diluted to an OD₆₀₀ of 1.0 in LB medium. For the *E. coli* fosmid clones, the bacterial culture was further diluted severalfold to obtain an average of 1×10^5 CFU. For the miniHimar RB1 mutants, the bacterial culture was further diluted severalfold to obtain an average of 2×10^3 CFU. For each diluted sample, 10 μ l was directly injected into the insect hemocoel by use of a sterilized Hamilton syringe. Wild-type *Serratia* sp. SCBI and *E. coli* EPI300 were used as controls in all experiments. For all of the samples, including the controls, 10 larvae were used. The larvae were held individually with food for up to 14 days at 37°C under a 16-h light/8-h dark cycle. The larvae were monitored for insect mortality or delays in insect development during that time period. Mortality was measured as the percentage of larvae killed by the bacteria relative to the total number of larvae injected per sample. From these data, the LT₅₀ values were calculated and defined as the average time required for 50% of the population to die from infection.

Motility assays. Swimming and swarming motility were assayed for wild-type *Serratia* sp. SCBI and all *Serratia* sp. SCBI mutants using methods described previously, with modifications made only to the percentage of agar used (0.65, 1.05, or 1.5%) (7, 39). For *E. coli* and fosmid clone A1-F2, swarming was assayed using LB plates containing 0.5% agar. Briefly, swarm plates were spotted with 2 μ l of overnight culture and incubated for 18 h or longer at 22°C or 28°C. Swim plates containing 0.2% agar were stabbed with overnight culture and incubated for 8 h or longer at 37°C.

RNA extraction from swarmer cells and cDNA synthesis. To obtain cells for RNA extraction, cells were collected from the edges of swarming colonies on 0.65% agar incubated at 28°C. Cells were collected when the swarm colonies were at a diameter of 20 mm, 40 mm, or 80 mm. The swarmer cells were incubated with RNAlater (Qiagen), according to the manufacturer's instructions, followed by storage at -80°C overnight. RNA extraction was performed using the RNeasy minikit (Qiagen) according to the manufacturer's instructions, followed by treatment with DNase (New England BioLabs). Four micrograms of RNA was reverse transcribed into cDNA using GoScript reverse transcriptase (Promega) according to the manufacturer's instructions, quantified with a Qubit 2.0 fluorometer (Invitrogen), and diluted to 10 ng/ μ l.

Analysis of gene expression with quantitative RT-PCR. Amplification and detection of gene expression were performed using the Stratagene Mx3000P qPCR system (Agilent Technologies). The primers used for these experiments are listed in Table S1. The gene *I21p* (40) was used as the normalizer for all qRT-PCR experiments. The RT-PCRs were done using 50 ng of template cDNA, SYBR green PCR master mix (Applied Biosystems), and primer mix (0.3 μ M) in a 25- μ l reaction mixture. The following thermal cycler parameters were used: (i) 15 min at 95°C, (ii) 40 cycles of 95°C for 15 s and 60°C for 30 s, and (iii) a thermal dissociation cycle of 95°C for 1 min, 55°C for 30 s, and incremental increases in temperature to 95°C for 30 s. Reactions were performed in triplicate, and the comparative threshold cycle method was used to quantify gene expression.

Statistical analysis. Data were analyzed by one-way analysis of variance using JMP 10 software (SAS Institute, Inc.). Student's *t* test provided comparisons of means.

Physicochemical analysis of serrawettin W2. For analysis of strains by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS), whole cells were taken from an LB plate and spotted with 1 μ l of a 20 mM 4-chloro- α -cyanocinnamic acid in 70% acetonitrile with 0.1% trifluoroacetic acid on a stainless steel target and air dried. MALDI MS analysis was done with a MALDI LTQ Orbitrap XL (Thermo Fisher Scientific, Inc., Waltham, MA) equipped with a nitrogen laser at 337 nm. For calculation of sum formulas, mass spectra were internally calibrated using calibration mixture 1 (Sequazyme peptide mass

standards kit; Applied Biosystems) and measured in triplicate. Qual Browser (version 2.0.7; Thermo Fisher Scientific, Inc.) was used for spectral analysis and for calculating possible sum formulas.

Accession number(s). The genome sequences and annotations are available at NCBI under the accession numbers [CP003424](#) and [CP003425](#).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JB.00333-17>.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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