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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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Running Title: Hemolysis in Serratia sp. Strain SCBI
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. 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 loss of hemolysis, but did not attenuate insecticidal activity. Unexpectedly, inactivation of the hemolysin gene in *Serratia* sp. SCBI resulted in significantly increased motility as well as increased antimicrobial activity. 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 variable 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 expression of *swrA*, these results suggest *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 found widespread in the environment and can cause human illness. Comparative genomics analysis between *S. marcescens* and a new *Serratia* species from South Africa, termed SCBI, shows that these two organisms are closely related, but differed 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 the 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 elimination of one mechanism may be compensated by up-regulation of others.

Key Words: Serratia, hemolysis, swarming motility, insect pathogenesis, nonribosomal peptide synthetase, serrawettin W2
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) larvae bait traps in three provinces in South Africa (3). The microbe-nematode complex between *Serratia* sp. 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 similar insect-pathogenic capabilities as other *Serratia* invertebrate pathogens with CFUs of <1,000 resulting in mortality in *G. mellonella* and *Manduca sexta* (tobacco horn worm) larvae following intra-hemocoelic injection (3, 6, 7). Comparative genomic analysis shows that *Serratia* sp. SCBI is closely related to *Serratia marcescens* Db11, a spontaneous 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). Despite similarity in over 85% of open reading frames, colonization of *Caenorhabditis* nematodes by these two *Serratia* spp. results in very distinct outcomes, with *Serratia* sp. SCBI exerting no harmful effects while *S. marcescens* Db11 kills the nematode within seven 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 primarily due to the pore-forming toxin ShlA, which is transcribed within the same operon as 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 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 shIA, indicating these two hemolysins likely share the same function. However, given their contrasting effects on Caenorhabditis hosts it is possible that regulation of hemolytic activity differs between S. marcescens Db11 and Serratia sp. SCBI.

Temperature is an important environmental factor that can influence activity of essential virulence factors. A number of virulence-associated genes are downregulated in S. marcescens at 37°C, including hemolysis. Optimal hemolysis and swarming motility (rapid, coordinated translocation of a bacterial population) is observed between 28-30°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 in 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 the response regulator. Once phosphorylated at 37°C, RssB blocks expression of the transcriptional regulators flhDC. FlhDC regulates the expression of genes involved in flagella formation, chemotaxis, and cell division (18-20). FlhDC also positively regulates the hemolysin operon, shlBA (16). Therefore, with repression of flhDC at 37°C by RssB, shIA is not expressed, resulting in 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 manA, which in turn positively regulates the biosurfactant serrawettin W1 (21). Serrawettin W1 is critical for reducing surface tension when S. marcescens is actively swarming (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 serrawettin W1, W2 or 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) *swrA* (26). *S. marcescens* Db10 serrawettin W2 is essential for swarming motility, has hemolytic properties, and also acts as a repellent towards *Caenorhabditis* nematodes. Serrawettin W2 also has antimicrobial activity towards *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 sequence suggests that serrawettin W2 produced by *Serratia* sp. SCBI could exhibit the same function, but it 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 regulation of hemolysis, and even motility, is likely different between these two species. To try and elucidate the mechanisms involved in the regulation and activity of hemolysis in *Serratia* sp. SCBI, hemolytic activity was investigated utilizing both forward and reverse genetics approaches. 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 hyper-swarming and hyper-swimming phenotypes and showed an increase...
in antimicrobial activity. Therefore, the mechanism by which the hemolysis mutants were hyper-motile 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. When compared to the control group injected with wild-type *E. coli* EPI300, the 25 clones caused mortality, delays in development, and/or change of larvae color (from green to pink, suggesting these larvae were unhealthy). All 25 fosmids were end-sequenced and sequencing results were blasted 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* (SCBI_3479) and its accompanying 1,680 bp exporter *shlB* (SCBI_3480) (Table S2 in Supplementary Materials). 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 *M. sexta* larva injected (Figure 1A) and demonstrated a gain of hemolytic activity, though the rate of hemolysis was less than that of wild-type *Serratia* sp. SCBI (Figure 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 non-virulent, non-hemolytic phenotype of wild-type *E. coli* EPI300. Therefore, *shlA* was responsible for both the gain of

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 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) demonstrated variable reductions in the rate of hemolysis (Figure 2A). Genetic analysis of these mutants showed that five 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 (Figure 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 non-coding 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 larva at a similar rate as wild-type Serratia sp. SCBI. The LT_{50} values of wild-type Serratia sp. 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 hyper-motility. 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 faster rate than wild-type Serratia sp. SCBI (Figure 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 (Figure 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 (Figure 3B, 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 (Figure 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 (Figure 3D). Mutants 22-C11 and 22-H3 showed no alterations in swimming behavior compared to 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 when compared with wild-type Serratia sp. SCBI (Table 2). The shlA mutants 1-C4, 6-E3, 8-E6, 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 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 control of FlhDC in *S. marcescens* CH-1(16). To determine if FlhDC may be involved in regulation of *shlBA* in wild-type *Serratia* sp. SCBI, and therefore somehow involved in the hyper-motile 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 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 total loss of swarming and swimming motility (Figure 4A, B). In contrast to what is observed in *S. marcescens* *flhC* mutants, loss of *flhC* had no effects on hemolytic activity in *Serratia* sp. SCBI (Figure 4C).

**shlA** mutants showed a significant increase in *swrA* expression. To further investigate the genes responsible for the hyper-swarming 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 flagella or surfactant production. The expression of *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 flagella genes, FliC is a flagellar protein, and SwrA is a NRPS hypothesized to catalyze 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 wild-type *Serratia* sp. SCBI (Figure 5A). There were no differences in mRNA levels for the *flhD* or *fliC* genes between the mutants and wild-type *Serratia* sp. SCBI.
investigate possible changes in gene expression over different stages of swarming, mRNA levels of 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 wild-type Serratia sp. SCBI at all stages of swarming (Figure 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 and 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 16,198 bp 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* (Figure 6C). The swrA mutant 1-A4 had the insertion at the 8,700 bp position of swrA while swrA mutant 13-G2 had the insertion at the 10,242 bp position (Figure 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...
The *swrA* mutants displayed variable 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 variable and dependent on the site of transposon insertion. On 0.65% agar, *swrA* mutant 1-A4 was defective in swarming motility (Figure 7A, B, D). The *swrA* mutant 13-G2 did swarm on 0.65% agar, but the rate of movement was delayed compared to wild-type *Serratia* sp. SCBI (Figure 7A). The *swrA* mutant 13-G2 did not swarm on 1.05% agar (Figure 7B, D). The *swrA* mutant 11-B8 had slightly decreased rates of swarming motility on 0.65% and 1.05% agar compared to wild-type *Serratia* sp. SCBI (Figure 7A, B, D).

All three *swrA* mutants demonstrated similar defects in swimming motility as was seen in swarming motility (Figure 7C, 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 averaged a 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 variable 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 seven day period. The *swrA* mutant 13-G2 killed *M. sexta* at a
similar rate as wild-type *Serratia* sp. SCBI with LT<sub>50</sub> values of 1.71 and 1.18 days, respectively (Figure 8A). The *swrA* mutant 11-B8 killed at a slightly reduced rate with an LT<sub>50</sub> 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<sub>50</sub> 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 wild-type *Serratia* sp. SCBI (Figure 8B), but this effect is likely due to a slight decrease in growth rate (Figure 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, hyper-motility, and hyper-antimicrobial activity, it was hypothesized that introduction of *shlA* on a multi-copy 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 (Cm<sup>r</sup>) 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, and 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 3). The presence of pBAD33 without 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 wild-type level in 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 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 intra-hemocoelic injection. End sequencing of this fosmid showed that it contained swrA (Table S3 in Supplementary Materials).

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 (Figure 9A, 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 (Figure 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 wild-type Serratia sp. SCBI (see Table 2). 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.** MALDI-MS analysis of wild-type *Serratia* sp. SCBI cells led to the detection of a compound with a mass of \( m/z \) 754.4369 [M+Na]\(^{+}\) leading to a sum formula of C\(_{38}\)H\(_{61}\)O\(_9\)N\(_5\) (calc. \( m/z \) 754.43615 [M+Na]\(^{+}\), ∆ppm 1.083). This sum formula is in accordance with serrawettin W2 (22). Results showed that \( m/z \) 754.44 [M+Na]\(^{+}\) was also produced by *shlA* mutant 1-C4 and *E. coli* fosmid clone A1-F2 (Figure S1). MALDI-MS\(^{2}\) of \( m/z \) 754.44 [M+Na]\(^{+}\) (Figure 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). Transposon insertion in the *shlBA* operon in *S. marcescens* Db11 results in a significant attenuation of virulence towards *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 to the *shlA* gene of *S. marcescens*, resulted in 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 towards *M. sexta*. In contrast to *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 and 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 hyper-motile phenotype seen in the hemolysis mutants was result of FlhDC activity. Screening of the miniHimar RB1 transposon library uncovered a mutant (12-H4) with the insertion in *flhC*. The *flhC* mutant 12-H4 had abolished swimming and swarming motility, yet demonstrated wild-type hemolysis. This result indicated that hemolysis is regulated differently in *Serratia* sp. SCBI compared to *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 hyper-motility in the shlA mutants. When the expression levels of *flhD* and *fliC* were compared between the hyper-swarmer 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 flagella-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.
Serrawettin W2 is critical for swarming, as it reduces surface tension along the edges of the swarm colony, and also shows antimicrobial activity against *S. aureus*. All five *Serratia* sp. SCBI shlA mutants had levels of *swrA* mRNA 15-22X higher than wild-type *Serratia* sp. SCBI. These mutants also had increased antimicrobial activity against *M. luteus* compared to 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. Though 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 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 towards the middle of *swrA* at the 8,700 bp position, had abolished antimicrobial and hemolytic activities. Furthermore, *swrA* mutant 1-A4 was completely non-motile and virulence was severely attenuated. When the transposon was inserted a little further downstream in *swrA* at the 10,242 bp position, as seen in mutant 13-G2, there was no antimicrobial activity and rates of swimming and swarming motility were both 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 when compared to 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 non-
hemolytic phenotype, suggests that SwrA may in turn regulate shlA expression. We hypothesize that this regulation would occur 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 suggest that these two *Serratia* spp. produce a very similar cyclodepsipeptide which contains D-3-hydroxydecanoic acid and five amino acids (D-leucine, L-serine, L-threonine, D-phenylalanine, and L-isoleucine) as it has been also 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, but serrawettin W2 from *Serratia* sp. SCBI did not show any hemolysis against SRBCs. It is possible that the serrawettin W2 produced by *Serratia* sp. SCBI could be hemolytic towards other red blood cell types, such as human, 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 encoding *swrA* is significantly smaller, approximately 2.7 kb, but the final product is similar in structure to that 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 *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 indicates 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, each mutant with 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 will swim in LB plates containing 0.2% agar, it occurs at a faster rate when transformed with swrA. Fosmid clone A1-F2 displayed swimming rates similar to 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 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 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, regulation of hemolytic activity is different between these two organisms. In contrast to *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 into 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 towards 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 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 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 was 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 hyper-motile 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 

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions.** A complete list of all bacterial strains and plasmids 
used in this study are listed in Table 1. 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 following 
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 OD$_{600}$ of 1.0 in LB medium. The bacterial culture was further diluted several-fold to obtain an average CFU value of $2 \times 10^5$ for each fosmid clone. For each diluted sample, 10 µl 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 gain of pathogenicity were confirmed by performing 3 biological replicates using 10 *M. sexta* larva for each assay and utilizing the same CFU value used for the initial screen. Mortality was measured as percentage of larvae killed by the bacteria relative to total number of larvae injected per sample.

**Sequencing Pathogenic Fosmids.** Fosmids with 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 the fosmids was 40,863 bp (SD +/-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 primers listed in Table S1 in Supplementary Materials, which were designed to add 39 bp tails that were homologous to the sequence targeted for inactivation. Thermal cycler conditions were as follows: 94°C for 30 sec. for initial denaturation followed by 25 cycles of 94°C for 30 sec., 50°C for 30 sec. and 68°C for 6 min. followed by 68°C for 10 min. for 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 μg/ml gentamicin and diluted to an OD$_{600}$ of 0.05 in 5 mL LB medium. The diluted culture was grown at 28°C until the cells reached an OD$_{600}$ 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 OD$_{600}$ 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 confirmation primers listed in Table S1 in Supplementary Materials.

**Construction of Serratia sp. SCBI Transposon Mutant Library.** Serratia sp. strain SCBI was mutagenized by the use of the MiniHimar transposon RB1 (36). Both donor (E. coli S17-1 λpir/pMiniHimar RB1) and recipient (Serratia sp. SCBI) were grown overnight in LB medium containing appropriate antibiotics (30 μg/ml kanamycin for donor and 10 μg/ml polymyxin B sulfate for recipient). One milliliter of each culture was centrifuged at 10,000 x g for 2 minutes and the pellet was washed twice with phosphate-buffered saline (PBS) before 20 μl of a 1:1 cell mixture was spotted onto the center an LB agar plate and incubated at 37°C for 24 h. Following incubation, the cell mixture was resuspended in 1 ml PBS and transconjugants were selected by overnight growth of 100 μl aliquots from $10^{-5}$ and $10^{-6}$ dilutions on LB agar containing 100 μg/ml kanamycin and 30 μg/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 μg/ml kanamycin, 10 μg/ml polymyxin B sulfate in 96-well plates before 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 μl of LB medium containing 25 μg/ml kanamycin in 96-well plates and incubated for 18 h at 37°C. Twenty microliters of each culture was mixed with 180 μl washed sheep red blood cells (SRBCs; 10% v/v 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 x g and the resulting supernatant was diluted 1:10 in dH2O. The A405 was determined on a spectrophotometer to measure amount of released hemoglobin. As a positive control, 1 ml of SRBCs was lysed by addition of 100 μl of 20% sodium dodecyl sulfate (SDS) and incubated at 37°C for 15 min. For negative controls, 100 μl of LB medium was added to 1 ml washed SRBCs incubated at 37°C and samples were treated as described above. Controls were repeated at each temperature in order to calculate hemolytic units for each sample set at the respective temperature. The following equation was used to calculate hemolytic units (HU): HU = ((A405 of sample – A405 of negative control)/A405 of positive control) x 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 an antibiotic overlay plate assay. PP3 agar (2% proteose peptone 3, 0.5% NaCl, 2% agar) was poured into oversized petri dishes (150x15 mm; Fisher Scientific, Canada). Using a 96-well replicator, the -80°C stored transposants were transferred into new 96-well microtiter plates containing fresh LB medium containing 25 μg/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 μl 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 μl 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 swim plate assay. Swim agar (0.2% agar) was poured into oversized petri dishes (150x15 mm; Fisher Scientific, Canada). Using a 96-well replicator, the 80°C stored transposants were transferred into new 96-well microtiter plates containing fresh LB medium containing 25 μg/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 from each clone was prepared with the standard chloroform-isoamyl alcohol extraction technique. The gDNA was digested with *NsiI* (New England Biolabs), which doesn’t cut the transposon, followed by ligation with T4 ligase (New England Biolabs). The ligated DNA was electroporated into *E. coli* DH5α λpir cells and transformants were selected on LB plates containing 25 μg/ml kanamycin. The transposon-carrying plasmids were isolated and sequenced using the transposon-specific primers Himar1 (5’-CATTTAATACGTCGACGCTCT-3’) and 615 (5’-
TCGGGTATCGCTCTTGAAGGG-3'). Sequences were compared to the *Serratia* sp. SCBI genome using BlastN.

The genome sequence and its annotations are available at NCBI under the accession numbers CP003424 and CP003425.

**Gene Complementation.** For complementation analysis, the major hemolysin open reading frame was amplified with primers F-Hemolysin-Comp (5’-CCCACGGCAATATA CGGAGATACA-3’) and R-Hemolysin-Comp (5’-TGGCTTACAACGTGTTGGATCAGG-3’), a template of 100 ng *Serratia* sp. SCBI gDNA, and One-Taq Hot Start DNA Polymerase (New England Biolabs). The PCR program was 30 sec. at 94°C for initial denaturation followed by 30 cycles of 94°C for 30 sec., 50°C for 30 sec. 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 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) following manufacturer’s instructions. Invitrogen primers M13 Forward (-20) and M13 Reverse were used to determine the orientation of gene insertion. Depending on orientation of insertion in pCR2.1-TOPO, plasmid DNA was cut with either *Xba*I and *Sac*I or with *Xba*I and *Hind*III (New England Biolabs). Digested DNA was ligated into pBAD33Cm (Guzman *et al.*, 1995), which had been cut with the same restriction enzymes. The vector was subsequently renamed pBAD33-Hemol and was electroporated into *E. coli* DH5α λpir and selection was made on LB plates containing 25 μg/ml chloramphenicol. Plasmid was extracted from successfully transformed *E. coli* DH5α λpir and electroporated into the appropriate strain and selection was made on LB plates containing 150 μg/ml chloramphenicol. When assaying 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 sheep red blood cell (SRBC) lysis by bacterial culture (~4.0 x 10⁶ 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 (Petersen and Tisa, 2012). 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 several-fold to obtain an average CFU value of 1 x 10⁵. For the miniHimar RB1 mutants, the bacterial culture was further diluted several-fold to obtain an average CFU value of 2 x 10³.

For each diluted sample, 10 µl 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 percentage of larvae killed by the bacteria relative to 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 was 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, 38). 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 µl 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 RNA Protect (Qiagen) following manufacturer’s instructions followed by storage at -80°C overnight. RNA extraction was performed using the RNeasy Mini Kit (Qiagen) following manufacturer’s instructions, followed by treatment with DNase (New England Biolabs). Four micrograms RNA was reverse transcribed into cDNA using GoScript™ Reverse Transcriptase (Promega) following manufacturer’s instructions, quantified with a Qubit® 2.0 Fluorometer (Invitrogen), and diluted to 10 ng/μl.

**Analysis of Gene Expression with Quantitative RT-PCR.** Amplification and detection of gene expression was performed using the Stratagene Mx3000P QPCR system (Agilent Technologies). The primers used for these experiments are listed in Table S1 in Supplementary Materials. The gene *l21p* (39) was used as the normalizer for all qRT-PCR experiments. The RT-PCR reactions were done using 50 ng template cDNA, SYBR Green PCR master mix (Applied Biosystems) and primer mix (0.3 μM) in a 25 μl reaction mixture. The following thermal cycler parameters were used: (1) 15 min. at 95°C, (2) 40 cycles of 95°C for 15 s and 60°C for 30 sec, and (3) thermal disassociation cycle of 95°C for 1 min, 55°C for 30 sec and incremental increases in temperature to 95°C for 30 sec. 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 (SDS Institute, Inc.). Student’s t-test provided comparisons of means.
Physiochemical analysis of serrawettin W2. For analysis of strains by Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS) whole cells were taken from a LB-plate and spotted with 1 µl of a 20 mM 4-chloro-α-cyanocinnamic acid in 70% acetonitrile with 0.1% trifluoracetic 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 (Applied Biosystems, Sequazyme peptide mass standards kit) and measured as triplicate. Qual Browser (version 2.0.7; Thermo Fisher Scientific, Inc., Waltham, MA) was used for spectra analysis and to calculate possible sum formulas.

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Reference


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Table 1. Bacterial strains and plasmids used in this study.

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<tr>
<th>Description</th>
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<td><strong>Plasmids:</strong></td>
<td></td>
</tr>
<tr>
<td>pKD46-Gm Lambda Red recombination plasmid</td>
<td>(35)</td>
</tr>
<tr>
<td>pKD4 Kanamycin resistance cassette template</td>
<td>(34)</td>
</tr>
<tr>
<td>pMiniHimar RB1 Transposon mutagenesis</td>
<td>(36)</td>
</tr>
<tr>
<td>pCC1FOS™ Fosmid vector</td>
<td>Epicentre Technologies</td>
</tr>
<tr>
<td>pCR2.1-TOPO™ TOPO Cloning</td>
<td>Invitrogen™</td>
</tr>
<tr>
<td>pBAD33-Cm Expression vector for complementation</td>
<td>(42)</td>
</tr>
<tr>
<td>pBAD33-Hemol Complementation of hemolysin gene</td>
<td>This study</td>
</tr>
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</table>
Table 2. Antibiotic activity of the *shlA* mutants and *E. coli* fosmid clone A1-F2.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Radius of Clearing Zone (mm)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type <em>Serratia</em> sp. SCBI</td>
<td>14.1 ± 1.76</td>
</tr>
<tr>
<td>1-C4</td>
<td>16.2 ± 2.36</td>
</tr>
<tr>
<td>6-E3</td>
<td>15.9 ± 1.05</td>
</tr>
<tr>
<td>8-E6</td>
<td>17.1 ± 1.25</td>
</tr>
<tr>
<td>10-F8</td>
<td>16.8 ± 1.89</td>
</tr>
<tr>
<td>18-A11</td>
<td>16.1 ± 2.07</td>
</tr>
<tr>
<td>Wild-type <em>E. coli</em> EPI300</td>
<td>--</td>
</tr>
<tr>
<td>Fosmid clone A1-F2</td>
<td>5.9 ± 1.56</td>
</tr>
</tbody>
</table>

* Radii of clearing zones were measured in mm following 24 h of incubation at 28°C with *M. luteus* as the indicator strain. Results are shown as the average of 9 measurements from 3 independent experiments, with the standard deviations indicated by +. --, No activity.
Table 3. Complementation of the major hemolysin gene into mutant 1-C4 restored wild-type phenotypes.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Hemolytic Activity&lt;sup&gt;A&lt;/sup&gt;</th>
<th>Swarming&lt;sup&gt;B&lt;/sup&gt;</th>
<th>Swimming&lt;sup&gt;C&lt;/sup&gt;</th>
<th>Antibiotic Activity&lt;sup&gt;D&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT Serratia sp. SCBI</td>
<td>93.3%</td>
<td>49.3 ± 11.9</td>
<td>51.3 ± 2.9</td>
<td>14.5 ± 1.1</td>
</tr>
<tr>
<td>WT Serratia sp. SCBI + pBAD33</td>
<td>88.4%</td>
<td>45.0 ± 8.3</td>
<td>49.7 ± 1.6</td>
<td>14.3 ± 1.3</td>
</tr>
<tr>
<td>WT Serratia sp. SCBI + pBAD33-Hemol 1-C4</td>
<td>97.9%</td>
<td>53.8 ± 6.7</td>
<td>56.7 ± 2.4</td>
<td>13.8 ± 0.9</td>
</tr>
<tr>
<td>1-C4 + pBAD33</td>
<td>2.8%</td>
<td>88.0 ± 0</td>
<td>64.3 ± 1.9</td>
<td>16.9 ± 1.8</td>
</tr>
<tr>
<td>1-C4 + pBAD33-Hemol</td>
<td>2.2%</td>
<td>88.0 ± 0</td>
<td>63.1 ± 2.3</td>
<td>16.7 ± 1.5</td>
</tr>
<tr>
<td>1-C4 + pBAD33-Hemol</td>
<td>86.5%</td>
<td>54.7 ± 15.1</td>
<td>49.3 ± 2.9</td>
<td>13.2 ± 1.7</td>
</tr>
</tbody>
</table>

<sup>A</sup>Hemolytic activity was measured as the percentage of SRBCs lysed following a 4 h incubation at 28°C.

<sup>B</sup>Swarm assays were performed at 22°C with an incubation time of 48 h. The diameters of the swarm rings were measured and these values are expressed as mm.

<sup>C</sup>Swim assays were performed at 37°C with an incubation time of 8 h. The diameters of the swim rings were measured and these values are expressed as mm.

<sup>D</sup>Antibiotic assays were performed at 28°C with an incubation time of 24 h. The indicator strain was *M. luteus*. The radius of the clearing zones were measured and these values are expressed as mm.

* The maximum measurement was 88 mm (the width of the Petri dish). Results are shown as the average of 9 measurements from 3 independent experiments, with the standard deviations indicated by ±.
**FIGURE LEGENDS**

**Figure 1.** The virulence and hemolytic activity of *E. coli* fosmid clone A1-A8 is due to the major hemolysin gene. (A) Third instar *M. sexta* larva were injected with $2 \times 10^5$ 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). Larva were held individually at 37°C for 7 d. Mortality was measured as the percentage of the larva 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 from at least two independent experiments.

**Figure 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 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.

**Figure 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 average of 9 measurements from 3 independent experiments, with the standard deviations indicated by ±.

Figure 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 (≥ 4.0 x 10⁶ CFU) was measured over 4 h at 28°C. Error bars represent standard deviations from at least two independent experiments.

Figure 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 l21p housekeeping gene and compared to the calibrator wild-type Serratia sp. SCBI (WT SCBI). Data are presented as the relative changes in gene expression between the values obtained with the test conditions and the calibrator. (A) 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 from at least two independent experiments. * (p-value <0.05) denotes significant differences in relative mRNA levels.

Figure 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) The 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 average of 9 measurements from 3 independent experiments, with the standard deviations indicated by ±.

Figure 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 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 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 average of 9 measurements from 3 independent experiments, with the standard deviations indicated by ±.

Figure 8. The swrA mutant 1-A4 was the only swrA mutant to display defective virulence and
hemolytic activity. (A) Third instar *M. sexta* larva were injected with $2 \times 10^4$ 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 d. Mortality was measured as the percentage of the larva 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 from at least two independent experiments. (C) The growth rate of each strain was measured over 24 h at 37°C by absorbance at 600 nm.

**Figure 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 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 average of 9 measurements from 3 independent experiments, with the standard deviations indicated by +.
### Table C

<table>
<thead>
<tr>
<th>Condition</th>
<th>E. coli EPI300</th>
<th>Fosmid A1-F2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Swarming</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18 h, 22°C</td>
<td>3.1 ± 0.1</td>
<td>20.0 ± 3.7</td>
</tr>
<tr>
<td>24 h, 22°C</td>
<td>3.1 ± 0.1</td>
<td>76.0 ± 8.0</td>
</tr>
<tr>
<td>18 h, 28°C</td>
<td>3.8 ± 0.2</td>
<td>88.0 ± 0</td>
</tr>
<tr>
<td>24 h, 28°C</td>
<td>3.8 ± 0.2</td>
<td>N/A</td>
</tr>
<tr>
<td>18 h, 37°C</td>
<td>3.8 ± 0.2</td>
<td>27.7 ± 5.4</td>
</tr>
<tr>
<td>24 h, 37°C</td>
<td>4.0 ± 0.1</td>
<td>72.7 ± 9.8</td>
</tr>
<tr>
<td><strong>Swimming</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 h, 37°C</td>
<td>3.5 ± 0.5</td>
<td>60.7 ± 2.5</td>
</tr>
<tr>
<td>24 h, 37°C</td>
<td>70.7 ± 4.5</td>
<td>88.0 ± 0</td>
</tr>
</tbody>
</table>

### Figure A

- **EPI300**
- **A1-F2**

### Figure B

- **EPI300**
- **A1-F2**

- **22°C**
- **28°C**
- **37°C**