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Lauren M. Petersen
University of New Hampshire, Durham

Kaitlyn LaCourse
University of New Hampshire, Durham

Tim A. Schoner
Goethe Universität Frankfurt

Helge Bode
Goethe Universität Frankfurt

Louis S. Tisa
University of New Hampshire, Durham, louis.tisa@unh.edu

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1 **Inactivation of the major hemolysin gene influences expression of the**
2 **nonribosomal peptide synthetase gene *swrA* in the insect pathogen *Serratia* sp.**
3 **strain SCBI.**

4

5 Lauren M. Petersen[1,3], Kaitlyn LaCourse[1,4], Tim A. Schöner[2], Helge Bode[2], and Louis S. Tisa[1]*

6

7 [1]Department of Molecular, Cellular, and Biomedical Sciences, University of New Hampshire,
8 Durham, NH, 03824-2617 USA
9 [2]Merck Stiftungsprofessur für Molekulare Biotechnologie, Fachbereich Biowissenschaften,
10 Goethe Universität Frankfurt, Max-von-Laue-Straße 9 60438 Frankfurt am Main, Germany
11 [3]Present address: The Jackson Laboratory for Genomic Medicine, Farmington, CT 06030-0001,
12 USA
13 [4]Present address: Department of Microbiology, University of Washington, Seattle, WA 98195-
14 5502, USA

15

16 *Corresponding Author. Mailing Address: Department of Cellular, Molecular, and Biomedical
17 Sciences, University of New Hampshire, 46 College Rd., Durham, NH 03824-2617. Telephone:
18 (603) 862-2442. Fax: (603) 862-2621. Email: louis.tisa@unh.edu

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20 Running Title: Hemolysis in *Serratia* sp. Strain SCBI

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22 **ABSTRACT**

23 Hemolysins are important virulence factors for many bacterial pathogens, including 24 *Serratia marcescens*. The role of the major hemolysin gene in the insect pathogen *Serratia* sp. 25 SCBI was investigated using both forward and reverse genetics approaches. Introduction of the 26 major hemolysin gene into *Escherichia coli* resulted in a gain of both virulence and hemolytic 27 activity. Inactivation of this hemolysin in *Serratia* sp. SCBI resulted in loss of hemolysis, but did 28 not attenuate insecticidal activity. Unexpectedly, inactivation of the hemolysin gene in *Serratia* 29 sp. SCBI resulted in significantly increased motility as well as increased antimicrobial activity. 30 qRT-PCR analysis of mutants with a disrupted hemolysin gene showed a dramatic increase in 31 mRNA levels of a nonribosomal peptide synthetase gene, *swrA*, which produces the surfactant 32 serrawettin W2. Mutation of the *swrA* gene in *Serratia* sp. SCBI resulted in highly variable 33 antibiotic activity, motility, virulence and hemolysis phenotypes that were dependent on the site 34 of disruption within this 17.75 KB gene. When introduced into *E. coli, swrA* increases rates of 35 motility and confers antimicrobial activity. While it is unclear how inactivation of the major 36 hemolysin gene influences expression of *swrA*, these results suggest *swrA* plays an important 37 role in motility and antimicrobial activity in *Serratia* sp. SCBI.

38 **IMPORTANCE**

39 The opportunistic gram-negative bacteria of the genus *Serratia* are found widespread in the 40 environment and can cause human illness. Comparative genomics analysis between *S.* 41 *marcescens* and a new *Serratia* species from South Africa, termed SCBI, shows that these two 42 organisms are closely related, but differed in pathogenesis. *S. marcescens* kills *Caenorhabditis* 43 nematodes, while *Serratia* sp. SCBI is not harmful and forms a beneficial association with them. 44 This distinction presented the opportunity to investigate potential differences in the regulation of

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45 common virulence mechanisms between these two species. With the emergence of antibiotic
46 resistant microorganisms, there is a widespread need to understand the regulation of
47 pathogenesis. The significance of this study is the presentation of evidence for cross-pathway
48 regulation of virulence factors and how elimination of one mechanism may be compensated by
49 up-regulation of others.
50
51 **Key Words:** *Serratia*, hemolysis, swarming motility, insect pathogenesis, nonribosomal peptide
52 synthetase, serrawettin W2
53

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54 **INTRODUCTION**

55 Members of the genus *Serratia* are found widespread around the globe and are well
56 known for their roles as insect pathogens (1, 2). A newly recognized *Serratia* species, termed
57 South African *Caenorhabditis briggsae* Isolate (SCBI), was identified following its isolation
58 from the nematode *C. briggsae* KT0001 (3). These *C. briggsae* KT0001 nematodes were
59 recovered from soil samples through *Galleria mellonella* (the greater wax moth) larvae bait traps
60 in three provinces in South Africa (3). The microbe-nematode complex between *Serratia* sp.
61 SCBI and *C. briggsae* KT001 represents a potential emerging entomopathogenic association.
62 Only a few *Serratia* species are known to use a nematode partner to establish an infection in an
63 invertebrate host (4, 5). *Serratia* sp. SCBI demonstrates similar insect-pathogenic capabilities as
64 other *Serratia* invertebrate pathogens with CFUs of <1,000 resulting in mortality in *G.*
65 *mellonella* and *Manduca sexta* (tobacco horn worm) larvae following intra-hemocoelic injection
66 (3, 6, 7). Comparative genomic analysis shows that *Serratia* sp. SCBI is closely related to
67 *Serratia marcescens* Db11, a spontaneous streptomycin resistant mutant of *S. marcescens* Db10
68 (8). *S. marcescens* Db11 is a broad host range pathogen (9-11) and shares many physiological
69 characteristics with *Serratia* sp. SCBI (7). Despite similarity in over 85% of open reading
70 frames, colonization of *Caenorhabditis* nematodes by these two *Serratia* spp. results in very
71 distinct outcomes, with *Serratia* sp. SCBI exerting no harmful effects while *S. marcescens* Db11
72 kills the nematode within seven days (8).

73 Hemolytic activity is a known virulence factor of *S. marcescens* Db11 that is essential for
74 pathogenesis against *Caenorhabditis elegans* and *Drosophila melanogaster* (10). Hemolysis by
75 *S. marcescens* is primarily due to the pore-forming toxin ShlA, which is transcribed within the
76 same operon as its activator and exporter, ShlB (12). Together ShlA and ShlB act as a two-

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77 partner secretion system (13) . In comparison, *Serratia* sp. SCBI contains a two-gene operon,
78 containing a hemolysin activator protein and a major hemolysin gene, homologous to *shlBA* of *S.*
79 *marcescens* Db11 (8). The major hemolysin gene of *Serratia* sp. SCBI shares 95% identity with
80 *shlA,* indicating these two hemolysins likely share the same function. However, given their
81 contrasting effects on *Caenorhabditis* hosts it is possible that regulation of hemolytic activity
82 differs between *S. marcescens* Db11 and *Serratia* sp. SCBI.

83 Temperature is an important environmental factor that can influence activity of essential
84 virulence factors. A number of virulence-associated genes are downregulated in *S. marcescens* at
85 37°C, including hemolysis. Optimal hemolysis and swarming motility (rapid, coordinated
86 translocation of a bacterial population) is observed between 28-30°C in *S. marcescens*, with a
87 sharp decrease in both activities at 37°C (14-17). The mechanism behind the temperature
88 regulation of both hemolysis and motility has been elucidated in *S. marcescens* CH-1(16). At
89 higher temperatures, such as 37°C, a two-component system, termed RssAB, is activated. RssA
90 serves as the sensor kinase and RssB the response regulator. Once phosphorylated at 37°C, RssB
91 block expressions of the transcriptional regulators *flhDC*. FlhDC regulates the expression of
92 genes involved in flagella formation, chemotaxis, and cell division (18-20). FlhDC also
93 positively regulates the hemolysin operon, *shlBA* (16). Therefore, with repression of *flhDC* at
94 37°C by RssB, *shlA* is not expressed, resulting in loss of both swarming motility and hemolytic
95 activity at 37°C and a switch to biofilm formation. RssB also negatively regulates expression of
96 the mannose-6-phosphate isomerase *manA*, which in turn positively regulates the biosurfactant
97 serrawettin W1 (21). Serrawettin W1 is critical for reducing surface tension when *S. marcescens*
98 is actively swarming (22) and therefore its repression at 37°C also contributes to the loss of
99 swarming motility at this temperature.

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100 *Serratia* spp. can produce one of three types of distinct serrawettins and these compounds
101 are unique to *Serratia*. Termed serrawettin W1, W2 or W3, these nonionic biosurfactants often
102 play an important role in swarming motility but can also contribute to hemolysis (22-25).
103 Serrawettin W1, also known as serratamolide, contributes to swarming motility, is a broad-
104 spectrum antibiotic, and is hemolytic to sheep and murine red blood cells. Furthermore,
105 serrawettin W1 is cytotoxic to human airway and corneal limbal epithelial cells (25). *S.*
106 *marcescens* Db10 produces serrawettin W2, which is encoded by the massive 17,781 bp
107 nonribosomal peptide synthetase (NRPS) *swrA* (26). *S. marcescens* Db10 serrawettin W2 is
108 essential for swarming motility, has hemolytic properties, and also acts as a repellent towards
109 *Caenorhabditis* nematodes. Serrawettin W2 also has antimicrobial activity towards
110 *Staphylococcus aureus* (27). The genome of *Serratia* sp. SCBI contains a 17,775 bp gene with
111 over 96% identity to the serrawettin W2 gene found in *S. marcescens* Db10 (8). This similarity in
112 gene sequence suggests that serrawettin W2 produced by *Serratia* sp. SCBI could exhibit the
113 same function, but it has not yet been investigated.

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

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123 in antimicrobial activity. Therefore, the mechanism by which the hemolysis mutants were hyper-
124 motile was investigated.

125 **RESULTS**

126 **Identification and genetic analysis of *E. coli* fosmid clones with a gain of pathogenicity.** To
127 identify virulence factors that cause mortality to *M. sexta* larvae, a *Serratia* sp. SCBI fosmid
128 library was constructed in *E. coli* EPI300 using the pCC1FOS™ vector (Epicentre). A total of
129 396 *E. coli* clones were individually injected into 3rd instar *M. sexta* larvae to identify fosmids
130 carrying virulence genes. A total of 25 clones were selected and confirmed to have a gain of
131 pathogenicity. When compared to the control group injected with wild-type *E. coli* EPI300, the
132 25 clones caused mortality, delays in development, and/or change of larvae color (from green to
133 pink, suggesting these larva were unhealthy). All 25 fosmids were end-sequenced and
134 sequencing results were blasted against the *Serratia* sp. SCBI genome to identify the genes in
135 each insecticidal *E. coli* clone. Out of the 25 pathogenic *E. coli* clones, one fosmid clone, termed
136 A1-A8, carried the 4,827 bp *Serratia* sp. SCBI major hemolysin gene *shlA* (SCBI_3479) and its
137 accompanying 1,680 bp exporter *shlB* (SCBI_3480) (Table S2 in Supplementary Materials).
138 Because hemolytic activity is a major virulence factor in *S. marcescens,* fosmid clone A1-A8
139 was the focus of this study while the remaining pathogenic clones were stored for future analysis.

140 Fosmid clone A1-A8 killed an average of 73% of *M. sexta* larva injected (Figure 1A) and
141 demonstrated a gain of hemolytic activity, though the rate of hemolysis was less than that of
142 wild-type *Serratia* sp. SCBI (Figure 1B). Lambda Red recombination was utilized to disrupt the
143 *shlA* gene on fosmid A1-A8. Removal of a 3,132 bp portion of the *shlA* gene beginning 42 bp
144 downstream of the start codon, resulted in reversion to the non-virulent, non-hemolytic
145 phenotype of wild-type *E. coli* EPI300. Therefore, *shlA* was responsible for both the gain of

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146 hemolytic activity and insecticidal activity in fosmid clone A1-A8.

147 **Inactivation of the *shlA* gene in *Serratia* sp. SCBI results in a significant loss of hemolytic**
148 **activity yet had no effect on virulence.** Because the *Serratia* sp. SCBI *shlA* gene conferred
149 insecticidal activity in *E. coli*, a *Serratia* sp. SCBI miniHimar RB1 transposon mutant library of
150 2,100 clones was generated and screened for defects in hemolytic activity to determine if loss of
151 hemolysis would have a significant impact on virulence. The 2,100 transposon mutants were
152 assayed for their ability to lyse sheep red blood cells (SRBCs) and seven mutants with defective
153 hemolytic activity were identified. Of these seven mutants, four mutants (1-C4, 6-E3, 8-E6, and
154 10-F8) had a complete loss of activity and the remaining three mutants (18-A11, 22-C11 and 22-
155 H3) demonstrated variable reductions in the rate of hemolysis (Figure 2A). Genetic analysis of
156 these mutants showed that five had the transposon insertion at different points within *shlA*
157 (SCBI_3479). The four mutants with a total loss of hemolytic activity (1-C4, 6-E3, 8-E6, and 10-
158 F8) all had hits within the first 1,500 bp of *shlA* (Figure 2B). The *shlA* mutant 18-A11 was the
159 only clone with a hit within *shlA* that retained some activity and had the transposon insertion
160 2,597 bp downstream of the start codon. Mutant 22-C11 had the insertion in a putrescine
161 importer (SCBI_0418) and mutant 22-H3 had the transposon hit a non-coding region between a
162 5'nucleosidase (SCBI_1151) and a cytochrome d ubiquinol oxidase subunit (SCBI_1152).

163 All seven hemolysis mutants (1-C4, 6-E3, 8-E6, 10-F8, 18-A11, 22-C11, and 22-H3)
164 were subsequently assayed for virulence in *M. sexta*. Despite the loss or reduction of hemolytic
165 activity, all seven hemolysis mutants killed larva at a similar rate as wild-type *Serratia* sp. SCBI.
166 The LT_{50} values of wild-type *Serratia* sp. SCBI and mutants 1-C4, 6-E3, 8-E6, 10-F8, 18-A11,
167 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.
168 These results indicate that while *shlA* was enough to confer insecticidal activity to *E. coli*, it is

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169 not required for insect pathogenesis in *Serratia* sp. SCBI.

170 **Inactivation of the *shlA* gene in *Serratia* sp. SCBI results in hyper-motility.** To determine if
171 any other physiological functions were affected by alterations in hemolytic activity, all seven
172 *Serratia* sp. SCBI hemolysis mutants were assayed for swarming and swimming motility. All
173 five *shlA* mutants (1-C4, 6-E3, 8-E6, 10-F8 and 18-A11) began swarming earlier and at a faster
174 rate than wild-type *Serratia* sp. SCBI (Figure 3A). By 18 h, these five mutants had nearly
175 swarmed across the entire 88 mm plate, at which time wild-type *Serratia* sp. SCBI and mutants
176 22-C11 and 22-H3 had not yet begun to swarm (Figure 3D). In addition, *shlA* mutants 1-C4, 6-
177 E3, 8-E6, 10-F8 and 18-A11 were able to swarm on hard agar (1.5%), with swarm ring diameters
178 averaging 30.3 ± 3.6 mm following 48 h of incubation at 28°C (Figure 3B, D). Wild-type
179 *Serratia* sp. SCBI and mutants 22-C11 and 22-H3 were unable to swarm on 1.5% agar. When
180 analyzed for swimming behavior, all five *shlA* mutants showed increased rates of movement
181 (Figure 3C). On average, these *shlA* mutants had a swim ring diameter between 55.3 and 67.3
182 mm following 8 h of incubation at 37°C compared to wild-type *Serratia* sp. SCBI which had an
183 average swim ring diameter of 44.3 ± 0.5 mm (Figure 3D). Mutants 22-C11 and 22-H3 showed
184 no alterations in swimming behavior compared to wild-type *Serratia* sp. SCBI.

185 **Inactivation of the *shlA* gene in *Serratia* sp. SCBI also results in increased antimicrobial**
186 **activity.** Next, the *shlA* mutants were assayed for antimicrobial activity against *Micrococcus*
187 *luteus*. These mutants had increased antibiotic activity when compared with wild-type *Serratia*
188 sp. SCBI (Table 2). The *shlA* mutants 1-C4, 6-E3, 8-E6, 10-F8, and 18-A11 had average clearing
189 zones of 16.2, 15.9, 17.1, 16.8, and 16.1 mm with standard deviations between 1.05 and 2.36
190 mm. In comparison, wild-type *Serratia* sp. SCBI had an average clearing zone of 14.1± 1.76
191 mm.

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192 **Inactivation of the *flhC* gene resulted in loss of motility, but had no effect on hemolytic**
193 **activity.** Expression of *shlBA* is under control of FlhDC in *S. marcescens* CH-1(16). To
194 determine if FlhDC may be involved in regulation of *shlBA* in wild-type *Serratia* sp. SCBI, and
195 therefore somehow involved in the hyper-motile phenotypes of the five *shlA* mutants, the
196 *Serratia* sp. SCBI miniHimar RB1 transposon mutant library was screened for defects in
197 swimming motility in an attempt to find a mutant with an insertion in the *flhDC* operon. Out of
198 2,100 mutants screened, 16 with defective swimming were identified. Mutant 12-H4, which had
199 completely abolished swimming motility, was found to contain the transposon insertion in *flhC*
200 (SCBI_2840). Mutant 12-H4 had the transposon insert 354 bp downstream of the start site of
201 *flhC*, which resulted in total loss of swarming and swimming motility (Figure 4A, B). In contrast
202 to what is observed in *S. marcescens flhC* mutants, loss of *flhC* had no effects on hemolytic
203 activity in *Serratia* sp. SCBI (Figure 4C).
204 ***shlA* mutants showed a significant increase in *swrA* expression.** To further investigate the
205 genes responsible for the hyper-swarming phenotype observed in the *Serratia* sp. SCBI *shlA*
206 mutants, qRT-PCR was utilized to measure mRNA levels for genes predicted to be involved in
207 either flagella or surfactant production. The expression of *flhD* (SCBI_2841), *fliC* (SCBI_2820),
208 and *swrA* (SCBI_4162) in the *Serratia* sp. SCBI hemolysis mutants during swarming was
209 assayed. FlhD is a regulator of flagella genes, FliC is a flagellar protein, and SwrA is a NRPS
210 hypothesized to catalyze production of the surfactant serrawettin W2. At a swarm ring diameter
211 of 40 mm, qRT-PCR analysis showed the *shlA* mutants 1-C4, 6-E3, 8-E6, 10-F8, and 18-A11 all
212 had a significant increase in *swrA* mRNA levels, between a 14.7 and 22.4-fold change difference,
213 compared to wild-type *Serratia* sp. SCBI (Figure 5A). There were no differences in mRNA
214 levels for the *flhD* or *fliC* genes between the mutants and wild-type *Serratia* sp. SCBI. To

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215 investigate possible changes in gene expression over different stages of swarming, mRNA levels
216 of *flhD, fliC,* and *swrA* genes from *shlA* mutant 6-E3 and wild-type *Serratia* sp. SCBI were
217 compared at 20 mm, 40 mm, and 80 mm. The level of *swrA* mRNA was highest in mutant 6-E3
218 at the early stages of swarming (20 mm) and was significantly higher than wild-type *Serratia* sp.
219 SCBI at all stages of swarming (Figure 5B). In *shlA* mutant 6-E3, the level of *swrA* mRNA
220 decreased as the swarming colony expanded. At no point during the swarming process did the
221 levels of *flhD* and *fliC* mRNA differ significantly between *shlA* mutant 6-E3 and wild-type
222 *Serratia* sp. SCBI.

223 **Mutation of different sites within the 17,775 bp *swrA* gene resulted in various changes in**
224 **antibiotic production.** Because the *Serratia* sp. SCBI *shlA* mutants showed increased *swrA*
225 mRNA levels, and *swrA* is responsible for the production of the biosurfactant serrawettin W2,
226 which has antimicrobial activity (27), the *Serratia* sp. SCBI miniHimar RB1 transposon mutant
227 library was screened for mutants with altered antibiotic activity in order to try and identify a
228 mutant with a defective *swrA* gene. Mutants 1-A4, 13-G2 and 11-B8 were initially identified as
229 having altered antibiotic activity against *M. luteus* and rescue cloning showed that all three
230 mutants had the transposon insertion in *swrA* (SCBI_4162). Subsequent confirmation
231 experiments demonstrated that *swrA* mutant 11-B8, which had the transposon insertion at the
232 16,198 bp position, had wild-type levels of antibiotic activity with an average clearing zone of
233 13.7 $\pm$ 0.5 mm. In contrast, *swrA* mutants 1-A4 and 13-G2 showed no antimicrobial activity
234 against *M. luteus* (Figure 6C). The *swrA* mutant 1-A4 had the insertion at the 8,700 bp position
235 of *swrA* while *swrA* mutant 13-G2 had the insertion at the 10,242 bp position (Figure 6A). Figure
236 6B shows the different domains of the NRPS SwrA. The *swrA* mutant 11-B8 hit within an amino
237 acid adenylation domain, while mutants 1-A4 and 13-G2 did not hit any putative domains within

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238 *swrA*.

239 **The *swrA* mutants displayed variable motility phenotypes.** Because SwrA is hypothesized to
240 catalyze the production of the surfactant serrawettin W2, *swrA* mutants 1-A4, 13-G2, and 11-B8
241 were assayed for swimming and swarming motility to determine if there were any defects in
242 motility. It was expected that all three *swrA* mutants would have delayed or abolished swarming
243 motility. However, the results were highly variable and dependent on the site of transposon
244 insertion. On 0.65% agar, *swrA* mutant 1-A4 was defective in swarming motility (Figure 7A, B,
245 D). The *swrA* mutant 13-G2 did swarm on 0.65% agar, but the rate of movement was delayed
246 compared to wild-type *Serratia* sp. SCBI (Figure 7A). The *swrA* mutant 13-G2 did not swarm on
247 1.05% agar (Figure 7B, D). The *swrA* mutant 11-B8 had slightly decreased rates of swarming
248 motility on 0.65% and 1.05% agar compared to wild-type *Serratia* sp. SCBI (Figure 7A, B, D).
249 All three *swrA* mutants demonstrated similar defects in swimming motility as was seen in
250 swarming motility (Figure 7C, D). The *swrA* mutant 1-A4 had an average swim ring diameter of
251 only 11.0 $\pm$ 0.8 mm compared to wild-type *Serratia* sp. SCBI, which averaged a swim ring
252 diameter of 45.7 $\pm$ 1.7 mm following 8 h of incubation at 37°C. The *swrA* mutants 13-G2 and
253 11-B8 had reduced rates of swimming motility with swim ring diameters of 18.7 $\pm$ 2.5 mm and
254 25.7 $\pm$ 3.3 mm, respectively. While these results demonstrate that *swrA* is important in both
255 swarming and swimming motility, this gene is highly complex and appears to have domains
256 important in self-regulation.

257 **The *swrA* mutants demonstrated variable rates of hemolytic activity and virulence.** To
258 determine if disruption of the *swrA* gene in *Serratia* sp. SCBI had any impact on virulence, *swrA*
259 mutants 1-A4, 13-G2, and 11-B8 were individually injected into 3rd instar *M. sexta* larvae and
260 monitored for mortality over a seven day period. The *swrA* mutant 13-G2 killed *M. sexta* at a

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261 similar rate as wild-type *Serratia* sp. SCBI with LT_{50} values of 1.71 and 1.18 days, respectively
262 (Figure 8A). The *swrA* mutant 11-B8 killed at a slightly reduced rate with an LT_{50} value of 2.93
263 days. *swrA* mutant 1-A4 was less virulent than wild-type *Serratia* sp. SCBI, killing only 40% of
264 larvae injected with a LT_{50} value of 7.28 days. All three *swrA* mutants were also assayed for
265 hemolytic activity. The *swrA* mutants 13-G2 and 11-B8 demonstrated slightly reduced levels of
266 hemolytic activity compared to wild-type *Serratia* sp. SCBI (Figure 8B), but this effect is likely
267 due to a slight decrease in growth rate (Figure 8C). The *swrA* mutant 1-A4 had completely
268 abolished hemolysis over 4 h against SRBCs.
269 **Complementation of the major hemolysin gene in mutant 1-C4 restored wild-type**
270 **phenotypes.** The major hemolysin gene was cloned and tested for complementation in *shlA*
271 mutant 1-C4. Since inactivation of *shlA* resulted in severely attenuated hemolytic activity, hyper-
272 motility, and hyper-antimicrobial activity, it was hypothesized that introduction of *shlA* on a
273 multi-copy plasmid would restore all these phenotypes back to wild-type levels. Since the other
274 *shlA* mutants (6-E3, 8-E6, 10-F8, and 18-A11) demonstrated similar phenotypes,
275 complementation was performed only on one mutant. Plasmid pBAD33 (Cm^r) was introduced
276 into both wild-type *Serratia* sp. SCBI and *shlA* mutant 1-C4 as controls. pBAD33-Hemol was
277 introduced into both wild-type *Serratia* sp. SCBI and *shlA* mutant 1-C4. Wild-type *Serratia* sp.
278 SCBI, *shlA* mutant 1-C4, the control transformants containing pBAD33, and the complemented
279 wild-type *Serratia* sp. SCBI and *shlA* mutant 1-C4 were all assayed for hemolytic activity,
280 swarming and swimming motility, and antimicrobial activity against *M. luteus* (Table 3). The
281 presence of pBAD33 without insert did not significantly change the phenotypes of wild-type
282 *Serratia* sp. SCBI or *shlA* mutant 1-C4. The presence of pBAD33-Hemol did not significantly
283 change the physiology of wild-type *Serratia* sp. SCBI, even in terms of hemolytic activity. The

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284 presence of pBAD33-Hemol did restore the phenotypes of *shlA* mutant 1-C4 back to wild-type
285 levels. The percentage of SRBCs lysed after 4 h of incubation changed from 2.8% to 86.5%
286 between the mutant and the complemented mutant, respectively. Swarming and swimming rates
287 decreased back to wild-type levels in complemented *shlA* mutant 1-C4. Furthermore,
288 antimicrobial activity was restored to wild-type level in complemented *shlA* mutant 1-C4, with
289 radii of the clearing zones dropping from an average of 16.9 mm down to 13.2 mm.

290 **Introduction of *swrA* into *E. coli* caused significant increases in both swarming and**
291 **swimming motility as well as increased antimicrobial activity.** An *E. coli* fosmid clone,
292 termed A1-F2, was identified in the *M. sexta* gain of pathogenesis screen. Fosmid clone A1-F2
293 killed approximately 20% of *M. sexta* larva following intra-hemocoelic injection. End
294 sequencing of this fosmid showed that it contained *swrA* (Table S3 in Supplementary Materials).
295 When assayed for swimming motility, clone A1-F2 had an average swim ring diameter of 55.0 $\pm$
296 5.0 mm, at which point wild-type *E. coli* EPI300 had not yet begun to swim (Figure 9A, C).
297 Fosmid clone A1-F2 was also able to swarm on LB plates containing 0.5% agar. Following 18 h
298 of incubation at 28°C, fosmid clone A1-F2 had swarmed across the entire plate (Figure 9B).
299 Swarming was optimal at 28°C as fosmid clone A1-F2 had average swarm ring diameters of only
300 19.5 $\pm$ 1.0 mm and 28.5 $\pm$ 1.2 mm at 18 h at 22°C and 37°C, respectively. However, *E. coli*
301 fosmid clone A1-F2 had swarmed across the entire plate by 24 h at both 22°C and 37°C. *E. coli*
302 EPI300 was not capable of swarming at any temperature on LB plates containing 0.5% agar.
303 When assayed for hemolysis, fosmid clone A1-F2 showed no hemolytic activity (data not
304 shown). When tested for antimicrobial activity, fosmid clone A1-F2 showed antimicrobial
305 activity against *M. luteus*, but its activity was significantly lower than wild-type *Serratia* sp.
306 SCBI (see Table 2). Fosmid clone A1-F2 produced a clearing zone of 5.9 $\pm$ 1.56 mm and wild-

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307 type *Serratia* sp. SCBI had a clearing zone of 14.1 $\pm$ 1.76 mm. Wild-type *E. coli* EPI300 had no

308 antimicrobial activity.

309 **Physiochemical analysis of serrawettin W2.** MALDI-MS analysis of wild-type *Serratia* sp.

310 SCBI cells led to the detection of a compound with a mass of *m/z* 754.43697 $[M+Na]^+$ leading to

311 a sum formula of $C_{38}H_{61}O_9N_5$ (calc. *m/z* 754.43615 $[M+Na]^+$, Δppm 1.083). This sum formula is

312 in accordance with serrawettin W2 (22). Results showed that *m/z* 754.44 $[M+Na]^+$ was also

313 produced by *shlA* mutant 1-C4 and *E. coli* fosmid clone A1-F2 (Figure S1). MALDI-MS^2 of *m/z*

314 754.44 $[M+Na]^+$ (Figure S2) revealed a neutral loss fragmentation pattern similar to what has

315 been reported for serrawettin W2 (22). Based on these findings, we conclude that *m/z* 754.44

316 $[M+Na]^+$ produced by *Serratia* sp. SCBI, *shlA* mutant 1-C4, *swrA* mutant 11-B8, and *E. coli*

317 fosmid clone A1-F2 is serrawettin W2 or a close derivative thereof.

318

319 **DISCUSSION**

320 The hemolysin ShlA is an important virulence factor in *S. marcescens* (16, 28-30). Transposon

321 insertion in the *shlBA* operon in *S. marcescens* Db11 results in a significant attenuation of

322 virulence towards *C. elegans*, *D. melanogaster*, and a murine lung infection model (10).

323 Inactivation of *shlA* in *Serratia* sp. SCBI, which shares 95% identity at the nucleic acid sequence

324 level to the *shlA* gene of *S. marcescens*, resulted in complete loss of hemolytic activity when the

325 transposon hit within the first 1,500 bp of the gene. Abolishment of hemolytic activity did not

326 alter virulence towards *M. sexta*. In contrast to *S. marcescens* Db11, this result indicates that,

327 hemolysis is not required for insecticidal activity by *Serratia* sp. SCBI. However, *shlA* does have

328 toxic effects. Introduction of the *Serratia* sp. SCBI *shlA* gene, along with its activator *shlB*, into

329 *E. coli* EPI300 on a fosmid resulted in both a gain of hemolytic activity and a gain of

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330 pathogenesis. Deletion of *shlA* by lambda Red recombination confirmed that this hemolysin was
331 responsible for the gain of insecticidal activity by *E. coli*. Therefore, while *shlA* does confer
332 virulence in *E. coli*, it is not the driving factor of *Serratia* sp. SCBI pathogenesis.

333 Interestingly, inactivation of *shlA* in *Serratia* sp. SCBI resulted in significantly increased
334 rates of swarming and swimming motility as well as increased antimicrobial activity.
335 Furthermore, in contrast to wild-type *Serratia* sp. SCBI, these mutants were capable of swarming
336 on hard agar. This phenomenon has no precedence in the literature and therefore several
337 approaches were used to try and pinpoint the cause of increased motility in these mutants. Since
338 FlhDC is a regulator of hemolytic activity in *S. marcescens*, one goal was to determine if FlhDC
339 is a positive regulator of hemolytic activity in *Serratia* sp. SCBI. It was hypothesized that if
340 FlhDC was important in regulating *shlA*, the hyper-motile phenotype seen in the hemolysis
341 mutants was result of FlhDC activity. Screening of the miniHimar RB1 transposon library
342 uncovered a mutant (12-H4) with the insertion in *flhC*. The *flhC* mutant 12-H4 had abolished
343 swimming and swarming motility, yet demonstrated wild-type hemolysis. This result indicated
344 that hemolysis is regulated differently in *Serratia* sp. SCBI compared to *S. marcescens*, in which
345 inactivation of *flhDC* results in loss of hemolytic activity (16).

346 Expression analysis by qRT-PCR provided further evidence that FlhDC was not
347 responsible for the hyper-motility in the *shlA* mutants. When the expression levels of *flhD* and
348 *fliC* were compared between the hyper-swarmer hemolysis mutants and wild-type *Serratia* sp.
349 SCBI, no differences were found. These results provided evidence that the increased rates of
350 motility in *shlA* mutants 1-C4, 6-E3, 8-E6, 10-F8, and 18-A11 were not flagella-driven. Instead,
351 it was the expression of *swrA* that appeared to be responsible. SwrA is a large NRPS gene that
352 catalyzes production of the surfactant serrawettin W2 in *S. marcescens* strains Db11 and Db10

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353 (26, 27). Serrawettin W2 is critical for swarming, as it reduces surface tension along the edges of
354 the swarm colony, and also shows antimicrobial activity against *S. aureus.* All five *Serratia* sp.
355 SCBI *shlA* mutants had levels of *swrA* mRNA 15-22X higher than wild-type *Serratia* sp. SCBI.
356 These mutants also had increased antimicrobial activity against *M. luteus* compared to wild-type
357 *Serratia* sp. SCBI. When analyzed by mass spectrophotometry, *shlA* mutant 1-C4 was confirmed
358 to overproduce serrawettin W2, further supporting the qRT-PCR data that this biosurfactant
359 accounted for the increased motility and antibiotic activity. Though only *shlA* mutant 1-C4 was
360 analyzed, it is likely that all five *shlA* mutants overproduce serrawettin W2. Further analysis of
361 *shlA* mutant 6-E3 showed that expression of *swrA* was highest during the early stages of
362 swarming and decreased as the swarm colony moved out across the plate.

363 Screening of the miniHimar transposon mutant library for altered antibiotic activity
364 against *M. luteus* identified three mutants (1-A4, 13-G2, and 11-B8) with the transposon
365 insertion in *swrA,* providing evidence that serrawettin W2 has antimicrobial properties. However,
366 antibiotic activity was not consistent in all three mutants. The *swrA m*utant 1-A4, which contains
367 the transposon insertion towards the middle of *swrA* at the 8,700 bp position, had abolished
368 antimicrobial and hemolytic activities. Furthermore, *swrA* mutant 1-A4 was completely non-
369 motile and virulence was severely attenuated. When the transposon was inserted a little further
370 downstream in *swrA* at the 10,242 bp position, as seen in mutant 13-G2, there was no
371 antimicrobial activity and rates of swimming and swarming motility were both significantly
372 reduced. *swrA* mutant 11-B8, which contained the transposon insertion at the very end of *swrA*,
373 did not show significant changes in antimicrobial activity, motility, hemolysis, or virulence when
374 compared to wild-type *Serratia* sp. SCBI. Although the presence of wild-type *shlA* appears to
375 regulate the production of SwrA, the results from *swrA* mutant 1-A4, most notably its non-

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376 hemolytic phenotype, suggests that SwrA may in turn regulate *shlA* expression. We hypothesize
377 that this regulation would occur in an indirect manner.

378 While a number of strains of *S. marcescens* carry a version of *swrA*, the *swrA* gene in *S.*
379 *marcescens* Db10 is similar in both size (17,781 bp) and DNA sequence identity (96%) to the
380 *Serratia* sp. SCBI *swrA* gene (26). Physiochemical analysis of *Serratia* sp. SCBI serrawettin W2
381 strongly suggest that these two *Serratia* spp. produce a very similar cyclodepsipeptide which
382 contains D-3-hydroxydecanoic acid and five amino acids (D-leucine, L-serine, L-threonine, D-
383 phenylalanine, and L-isoleucine) as it has been also determined by in silico analysis using the
384 antismash program (31). The only difference phenotypically is that serrawettin W2 from *S.*
385 *marcescens* Db10 shows hemolytic properties, but serrawettin W2 from *Serratia* sp. SCBI did
386 not show any hemolysis against SRBCs. It is possible that the serrawettin W2 produced by
387 *Serratia* sp. SCBI could be hemolytic towards other red blood cell types, such as human, due to
388 differences in membrane compositions. Nevertheless, the serrawettins from both species are
389 critical for swarming motility and antimicrobial activity.

390 In *S. marcescens* strain MG1, the gene encoding *swrA* is significantly smaller,
391 approximately 2.7 kb, but the final product is similar in structure to that produced in *S.*
392 *marcescens* Db10 and *Serratia* sp. SCBI (26, 32, 33). The 2.7 kb *swrA* gene of *S. marcescens*
393 MG1 is homologous to the beginning of *swrA* gene of both *S. marcescens* Db10 and *Serratia* sp.
394 SCBI. However the larger size of *swrA* in both *S. marcescens* Db10 and *Serratia* sp. SCBI and
395 the phenotypic data collected from the three SCBI *swrA* mutants indicates the entire gene is
396 required for proper synthesis of SwrA. The differences in phenotypes between 1-A4, 13-G2, and
397 11-B8 are likely due to the production of a truncated form of SwrA, each mutant with a different
398 version of the lipopeptide. Further investigation into the function of the different modules in

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399 *swrA* should provide insight into explaining the phenotypes of *swrA* mutants 1-A4, 13-G2, and
400 11-B8.

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

415 This study has highlighted several important differences between *Serratia* sp. SCBI and
416 *S. marcescens* Db11. First, regulation of hemolytic activity is different between these two
417 organisms. In contrast to *S. marcescens*, the global regulators FlhDC do not appear to have any
418 role as a positive regulator of hemolysis in *Serratia* sp. SCBI. Also, hemolysis is not a
419 requirement for insecticidal activity in *Serratia* sp. SCBI. These results are important for laying
420 the groundwork into understanding how *Serratia* sp. SCBI has evolved as a mutualist of
421 *Caenorhabditis* nematodes, while *S. marcescens* remains a pathogen of these nematodes. *S.*

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422 *marcescens* Db11 relies on its hemolysin for pathogenesis towards both insects and nematodes.
423 Yet, it is clear that *Serratia* sp. SCBI has a different repertoire of essential virulence factors for
424 the killing of insects. The connection between *shlA* and *swrA* in *Serratia* sp. SCBI calls into
425 question why these two genes, which do not reside near each other in the genome, have such
426 strong effects on each other. SwrA clearly plays an important role in the motility of *Serratia* sp.
427 SCBI and motility is often important in the ability of a bacterium to colonize a host. While
428 further investigation on the mechanisms that *Serratia* sp. SCBI utilizes to colonize *C. briggsae* is
429 needed, it is possible that swarming contributes to successful colonization of the nematode.
430 During this time, the antimicrobial properties of serrawettin W2 would ward off other
431 competitors within the intestine of the nematode. It is also important that during colonization of
432 the nematode expression of *shlA* is repressed as it is likely that ShlA is harmful to the nematode.
433 From the expression data collected from the 5 *Serratia* sp. SCBI *shlA* mutants (1-C4, 6-E3, 8-E6,
434 10-F8, and 18-A11), disruption of *shlA* gene clearly affects *swrA* expression, and disruption of
435 *swrA* at a specific location (as seen in *swrA* mutant 1-A4) influences hemolytic activity.
436 Therefore, in order to both successfully colonize the nematode and carry out its role as an insect
437 pathogen, *Serratia* sp. SCBI has evolved a regulatory system that ensures proper gene expression
438 to carry out its dual lifestyle, and this system involves regulating the expression of *shlA* and
439 *swrA*.

440 In summary, the function of the *Serratia* sp. SCBI *shlA* gene and its role in virulence was
441 elucidated. While ShlA produced by *Serratia* sp. SCBI is solely responsible for hemolytic
442 activity, it appears to play a minor role in insect pathogenesis. Unexpectedly, those *Serratia* sp.
443 SCBI transposon mutants with the major hemolysin gene inactivated were hyper-motile and
444 demonstrated increased antimicrobial activity. These phenotypes were due to significantly

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445 increased expression of the NRPS *swrA*. Mutants 22-C11 and 22-H3 had reduced hemolysis yet
446 displayed no alterations in motility, suggesting that inactivation of the major hemolysin gene
447 itself is responsible for the effects on *swrA* expression. Transposon mutagenesis of the *swrA* gene
448 resulted in variable motility, antimicrobial, hemolytic and virulence phenotypes in *Serratia* sp.
449 SCBI that were dependent on site of insertion. Introduction of *swrA* in *E. coli* resulted in
450 significant increases in swarming and swimming motility and a gain of antimicrobial activity.
451 The mechanism for how inactivation of *shlA* influences expression of this large NRPS warrants
452 further investigation. Furthermore, investigation the function of the different modules of *swrA*
453 should provide evidence as to how inactivation of different portions of this gene results in such
454 varying phenotypes. Finally, this study provides evidence that, similar to other *Serratia* spp.,
455 both production of a biosurfactant and flagella are required for swarming motility.
456
457 **EXPERIMENTAL PROCEDURES**
458 **Bacterial Strains and Growth Conditions.** A complete list of all bacterial strains and plasmids
459 used in this study are listed in Table 1. Bacteria were grown overnight at 37°C in LB medium
460 (1% tryptone, 0.5% yeast extract, 1% NaCl) with appropriate antibiotics when required.
461 **Bacterial Growth Assay.** The growth of *Serratia* sp. SCBI mutants was determined at 28°C
462 using methods described previously (7).
463 **Fosmid Library Construction.** The *Serratia* sp. SCBI fosmid library was constructed with the
464 pCC1FOS™ vector using Epicentre's CopyControl™ Fosmid Library Production Kit following
465 manufacturer's instructions (8).
466 **Gain of Pathogenicity Assay.** A total of 396 fosmid clones were assayed for their ability to kill
467 3rd instar *M. sexta* larvae using methods described previously (7). Briefly, overnight bacterial

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468 cultures were initially diluted to an OD_{600} of 1.0 in LB medium. The bacterial culture was further
469 diluted several-fold to obtain an average CFU value of 2 x 10^5 for each fosmid clone. For each
470 diluted sample, 10 µl was directly injected into the insect hemocoel by use of a sterilized
471 Hamilton syringe. *Serratia* sp. SCBI was used as a positive control and *E. coli* EPI300 was used
472 as a negative control. For all of the samples, including the controls, 3 larvae were used. The
473 larvae were held individually with food for 7 days at 37°C under a 16 h light/8 h dark cycle. The
474 larvae were monitored for insect mortality or delays in insect development during that time
475 period. Clones displaying gain of pathogenicity were confirmed by performing 3 biological
476 replicates using 10 *M. sexta* larva for each assay and utilizing the same CFU value used for the
477 initial screen. Mortality was measured as percentage of larvae killed by the bacteria relative to
478 total number of larvae injected per sample.

479 **Sequencing Pathogenic Fosmids.** Fosmids with gain of function for pathogenicity were isolated
480 using the alkaline lysis method and subsequently end-sequenced using Epicentre F-
481 pCC1™/pEpiFOS™ and R-pCC1™/pEpiFOS™ primers. Fosmid gene location was determined
482 using BLASTN against the *Serratia* sp. SCBI genome. The average size the fosmids was 40,863
483 bp (SD +/-2,935 bp).

484 **Lambda Red Recombination in Virulent Fosmid Clones.** The major hemolysin gene in
485 fosmid clone A1-A8 was disrupted with lambda Red recombination using methods described
486 previously (34, 35). Briefly, the kanamycin resistance gene from pKD4 was amplified using
487 primers listed in Table S1 in Supplementary Materials, which were designed to add 39 bp tails
488 that were homologous to the sequence targeted for inactivation. Thermal cycler conditions were
489 as follows: 94°C for 30 sec. for initial denaturation followed by 25 cycles of 94°C for 30 sec.,
490 50°C for 30 sec. and 68°C for 6 min. followed by 68°C for 10 min. for final extension. Fosmid

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491 clone A1-A8 was transformed with pKD46-Gm (35) using a standard chemical complementation
492 protocol and subsequently renamed A1-A8-pKD46. Fosmid clone A1-A8-pKD46 was grown
493 overnight in LB medium containing 10 μg/ml gentamicin and diluted to an OD_{600} of 0.05 in 5
494 mL LB medium. The diluted culture was grown at 28°C until the cells reached an OD_{600} of 0.1 at
495 which time L-arabinose was added to a concentration of 10 mM. The culture was allowed to
496 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
497 standard protocol for producing chemically competent cells was followed. For transformation,
498 cells were mixed with 0, 20, 50, or 100 ng PCR products amplified from pKD4 containing the 39
499 bp tails. Confirmation that homologous recombination had taken place within the major
500 hemolysin gene was done by PCR using confirmation primers listed in Table S1 in
501 Supplementary Materials.

502 **Construction of *Serratia* sp. SCBI Transposon Mutant Library.** *Serratia* sp. strain SCBI was
503 mutagenized by the use of the MiniHimar transposon RB1 (36). Both donor (*E. coli* S17-1
504 λpir/pMiniHimar RB1) and recipient (*Serratia* sp. SCBI) were grown overnight in LB medium
505 containing appropriate antibiotics (30 μg/ml kanamycin for donor and 10 μg/ml polymyxin B
506 sulfate for recipient). One milliliter of each culture was centrifuged at 10,000 x g for 2 minutes
507 and the pellet was washed twice with phosphate-buffered saline (PBS) before 20 μl of a 1:1 cell
508 mixture was spotted onto the center an LB agar plate and incubated at 37˚C for 24 h. Following
509 incubation, the cell mixture was resuspended in 1 ml PBS and transconjugants were selected by
510 overnight growth of 100 μl aliquots from 10^{-5} and 10^{-6} dilutions on LB agar containing 100 μg/ml
511 kanamycin and 30 μg/ml polymyxin B sulfate at 37˚C. A total of 2,100 isolated transconjugant
512 colonies were selected and grown at 37˚C overnight in LB medium containing 40 μg/ml
513 kanamycin, 10 μg/ml polymyxin B sulfate in 96-well plates before addition of an equal volume

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514 of 60% glycerol and storage at -80˚C for future use.

515 **Screening the miniHimar RB1 Transposon Mutant Library for Hemolytic Activity.** Minor
516 modifications were done on a liquid hemolysis assay described previously (7, 37) in order to
517 screen the *Serratia* sp. SCBI miniHimar RB1 transposon mutant library. Briefly, individual
518 mutants were inoculated into 200 µl of LB medium containing 25 µg/ml kanamycin in 96-well
519 plates and incubated for 18 h at 37°C. Twenty microliters of each culture was mixed with 180 µl
520 washed sheep red blood cells (SRBCs; 10% v/v in PBS) in 96-well plates and incubated for 45
521 min. at 37°C with gentle agitation. Plates were centrifuged for 5 min. at 3,000 x g and the
522 resulting supernatant was diluted 1:10 in dH_2O. The A_{405} was determined on a spectrophotometer
523 to measure amount of released hemoglobin. As a positive control, 1 ml of SRBCs was lysed by
524 addition of 100 µl of 20% sodium dodecyl sulfate (SDS) and incubated at 37°C for 15 min. For
525 negative controls, 100 µl of LB medium was added to 1 ml washed SRBCs incubated at 37°C
526 and samples were treated as described above. Controls were repeated at each temperature in
527 order to calculate hemolytic units for each sample set at the respective temperature. The
528 following equation was used to calculate hemolytic units (HU): HU = ((A_{405} of sample – A_{405} of
529 negative control)/A_{405} of positive control) x 100.

530 **Screening the miniHimar RB1 Transposon Mutant Library for Antibiotic Activity.**
531 Individual colonies of transposon-induced mutants of *Serratia* sp. strain SCBI were screened for
532 antibiotic activity by the use an antibiotic overlay plate assay. PP3 agar (2% proteose peptone 3,
533 0.5% NaCl, 2% agar) was poured into oversized petri dishes (150x15 mm; Fisher Scientific,
534 Canada). Using a 96-well replicator, the -80°C stored transposants were transferred into new 96-
535 well microtiter plates containing fresh LB medium containing 25 µg/ml kanamycin and
536 incubated overnight at 37°C. The freshly grown overnight culture was replica plated directly into

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537 PP3 medium and the plates were incubated for 18 h at 28°C. A total of 10 mL of 0.8% agar was
538 mixed with 200 µl of the indicator strain *Micrococcus luteus* and poured over each inoculated
539 PP3 plate. The mutants were incubated with the overlay for 24 h at 28°C and zones of clearing
540 were measured. To test individual mutants for antibiotic activity, the same procedure was
541 followed however 2 µl of overnight culture was inoculated onto PP3 plates overlaid with the
542 indicator strain *M. luteus*.

543 **Screening the miniHimar RB1 Transposon Mutant Library for Swimming Motility.**
544 Individual colonies of transposon-induced mutants of *Serratia* sp. strain SCBI were screened for
545 swimming motility by the use of swim plate assay. Swim agar (0.2% agar) was poured into
546 oversized petri dishes (150x15 mm; Fisher Scientific, Canada). Using a 96-well replicator, the -
547 80°C stored transposants were transferred into new 96-well microtiter plates containing fresh LB
548 medium containing 25 µg/ml kanamycin and incubated overnight at 37°C. The freshly grown
549 overnight culture was replica plated directly into swim plates using a 96-well metal replicator.
550 The swim plates were incubated for 4 h at 37°C and the diameters of swim rings were measured.
551 Confirmation of mutants defective in swimming was done using an assay described previously
552 (7).

553 **Molecular Analysis of Transposon Mutants.** Genomic DNA from each clone was prepared
554 with the standard chloroform-isoamyl alcohol extraction technique. The gDNA was digested
555 with *NsiI* (New England Biolabs), which doesn't cut the transposon, followed by ligation with
556 T4 ligase (New England Biolabs). The ligated DNA was electroporated into *E. coli* DH5α λpir
557 cells and transformants were selected on LB plates containing 25 µg/ml kanamycin. The
558 transposon-carrying plasmids were isolated and sequenced using the transposon-specific primers
559 Himar1 (5'-CATTTAATACTAGCGACGCCATCT-3') and 615 (5'-

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560 TCGGGTATCGCTCTTGAAGGG-3'). Sequences were compared to the *Serratia* sp. SCBI
561 genome using BlastN.
562 The genome sequence and its annotations are available at NCBI under the accession numbers
563 CP003424 and CP003425.
564 **Gene Complementation.** For complementation analysis, the major hemolysin open reading
565 frame was amplified with primers F-Hemolysin-Comp (5'-CCCACGGCAATATA
566 CGGAGATACA-3') and R-Hemolysin-Comp (5'-TGGCTTACAACGTGTTGGATCAGG-3'),
567 a template of 100 ng *Serratia* sp. SCBI gDNA, and One-Taq Hot Start DNA Polymerase (New
568 England Biolabs). The PCR program was 30 sec. at 94°C for initial denaturation followed by 30
569 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
570 min. at 68°C. PCR products were cleaned up using the QIAquick PCR purification kit (Qiagen)
571 according to manufacturer's instructions. Approximately 100 ng of cleaned up PCR product was
572 cloned into vector pCR2.1-TOPO using the TOPO®-TA Cloning kit (Invitrogen) following
573 manufacturer's instructions. Invitrogen primers M13 Forward (-20) and M13 Reverse were used
574 to determine the orientation of gene insertion. Depending on orientation of insertion in pCR2.1-
575 TOPO, plasmid DNA was cut with either *Xba*I and *Sac*I or with *Xba*I and *Hind*III (New England
576 Biolabs). Digested DNA was ligated into pBAD33Cm (Guzman *et al.*, 1995), which had been
577 cut with the same restriction enzymes. The vector was subsequently renamed pBAD33-Hemol
578 and was electroporated into *E. coli* DH5α λpir and selection was made on LB plates containing
579 25 μg/ml chloramphenicol. Plasmid was extracted from successfully transformed *E. coli* DH5α
580 λpir and electroporated into the appropriate strain and selection was made on LB plates
581 containing 150 μg/ml chloramphenicol. When assaying for complementation, all strains were
582 grown in the prescense of 10 mM arabinose to induce expression of the cloned gene.

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583 **Hemolysis Assay.** Rates of hemolytic activity for wild-type *Serratia* sp. SCBI, *E. coli* EPI300,
584 fosmid clones, and individual *Serratia* sp. SCBI transposon mutants were measured at 28°C
585 using a liquid assay described previously (7), which measured the rate of sheep red blood cell
586 (SRBC) lysis by bacterial culture (~4.0 x 10^6 cells) over 4 h.
587 **Insect Viability Assay**. *E. coli* fosmid clones and *Serratia* sp. SCBI miniHimar RB1 mutants
588 were analyzed for their ability to kill 3rd instar *M. sexta* larvae using methods described
589 previously (Petersen and Tisa, 2012). Briefly, overnight bacterial cultures were initially diluted
590 to an OD_{600} of 1.0 in LB medium. For the *E. coli* fosmid clones, the bacterial culture was further
591 diluted several-fold to obtain an average CFU value of 1 x 10^5. For the miniHimar RB1 mutants,
592 the bacterial culture was further diluted several-fold to obtain an average CFU value of 2 x 10^3.
593 For each diluted sample, 10 μl was directly injected into the insect hemocoel by use of a
594 sterilized Hamilton syringe. Wild-type *Serratia* sp. SCBI and *E. coli* EPI300 were used as
595 controls in all experiments. For all of the samples, including the controls, 10 larvae were used.
596 The larvae were held individually with food for up to 14 days at 37°C under a 16 h light/8 h dark
597 cycle. The larvae were monitored for insect mortality or delays in insect development during that
598 time period. Mortality was measured as percentage of larvae killed by the bacteria relative to
599 total number of larvae injected per sample. From these data, the LT_{50} values were calculated and
600 defined as the average time required for 50% of the population to die from infection.
601 **Motility Assays.** Swimming and swarming motility was assayed for wild-type *Serratia* sp. SCBI
602 and all *Serratia* sp. SCBI mutants using methods described previously with modifications made
603 only to the percentage of agar used (0.65, 1.05, or 1.5%) (7, 38). For *E. coli* and fosmid clone
604 A1-F2, swarming was assayed using LB plates containing 0.5% agar. Briefly, swarm plates were
605 spotted with 2 μl of overnight culture and incubated for 18 h or longer at 22°C or 28°C. Swim

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606 plates containing 0.2% agar were stabbed with overnight culture and incubated for 8 h or longer
607 at 37°C.

608 **RNA Extraction from Swarmer Cells and cDNA Synthesis.** To obtain cells for RNA
609 extraction, cells were collected from the edges of swarming colonies on 0.65% agar incubated at
610 28°C. Cells were collected when the swarm colonies were at a diameter of 20 mm, 40 mm or 80
611 mm. The swarmer cells were incubated with RNA Protect (Qiagen) following manufacturer's
612 instructions followed by storage at -80°C overnight. RNA extraction was performed using the
613 RNeasy Mini Kit (Qiagen) following manufacturer's instructions, followed by treatment with
614 DNase (New England Biolabs). Four micrograms RNA was reverse transcribed into cDNA using
615 GoScript™ Reverse Transcriptase (Promega) following manufacturer's instructions, quantified
616 with a Qubit® 2.0 Fluorometer (Invitrogen), and diluted to 10 ng/µl.

617 **Analysis of Gene Expression with Quantitative RT-PCR.** Amplification and detection of gene
618 expression was performed using the Stratagene Mx3000P QPCR system (Agilent Technologies).
619 The primers used for these experiments are listed in Table S1 in Supplementary Materials. The
620 gene *l21p* (39) was used as the normalizer for all qRT-PCR experiments. The RT-PCR reactions
621 were done using 50 ng template cDNA, SYBR Green PCR master mix (Applied Biosystems) and
622 primer mix (0.3 µM) in a 25 µl reaction mixture. The following thermal cycler parameters were
623 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
624 disassociation cycle of 95°C for 1 min, 55°C for 30 sec and incremental increases in temperature
625 to 95°C for 30 sec. Reactions were performed in triplicate and the comparative threshold-cycle
626 method was used to quantify gene expression.

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

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629 **Physiochemical analysis of serrawettin W2.** For analysis of strains by Matrix-assisted laser
630 desorption/ionization (MALDI) mass spectrometry (MS) whole cells were taken from a LB-plate
631 and spotted with 1 µl of a 20 mM 4-chloro-α-cyanocinnamic acid in 70% acetonitrile with 0.1%
632 trifluoracetic acid on a stainless steel target and air-dried. MALDI-MS analysis was done with a
633 MALDI LTQ Orbitrap XL (Thermo Fisher Scientific, Inc., Waltham, MA) equipped with a
634 nitrogen laser at 337 nm. For calculation of sum formulas, mass spectra were internally
635 calibrated using calibration mixture 1 (Applied Biosystems, Sequazyme peptide mass standards
636 kit) and measured as triplicate. Qual Browser (version 2.0.7; Thermo Fisher Scientific, Inc.,
637 Waltham, MA) was used for spectra analysis and to calculate possible sum formulas.
638

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

	Description	**Reference or Source**
***Serratia* sp. SCBI:**		
WT *Serratia* sp. SCBI	Wild-type *Serratia* sp. SCBI	(3)
1-C4	Transposon mutant, disrupted *shlA*	This study
6-E3	Transposon mutant, disrupted *shlA*	This study
8-E6	Transposon mutant, disrupted *shlA*	This study
10-F8	Transposon mutant, disrupted *shlA*	This study
18-A11	Transposon mutant, disrupted *shlA*	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 *swrA*	This study
13-G2	Transposon mutant, disrupted *swrA*	This study
11-B8	Transposon mutant, disrupted *swrA*	This study
***E. coli*:**		
E. coli S17-1 λpir	Donor strain transposon mutagenesis	(40)
E. coli DH5α λpir	Host strain for gene complementation	(41)
E. coli EPI300	Host strain for fosmid library	Epicentre Technologies
Fosmid clone A1-A8	Carries *Serratia* sp. SCBI *shlA* gene	This study
Fosmid clone A1-F2	Carries *Serratia* sp. SCBI *swrA* 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	(42)
pBAD33-Hemol	Complementation of hemolysin gene	This study

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794 **Table 2.** Antibiotic activity of the *shlA* mutants and *E. coli* fosmid clone A1-F2.

Strain	**Radius of Clearing Zone (mm)***
Wild-type *Serratia* 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 *E. coli* EPI300	--
Fosmid clone A1-F2	5.9 ± 1.56

795 * Radii of clearing zones were measured in mm following 24 h of incubation at 28°C with *M.*

796 *luteus* as the indicator strain. Results are shown as the average of 9 measurements from 3

797 independent experiments, with the standard deviations indicated by ±.

798 --, No activity

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799 **Table 3.** Complementation of the major hemolysin gene into mutant 1-C4 restored wild-type
800 phenotypes.

Strain	Hemolytic Activity[A]	Swarming[B *]	Swimming[C *]	Antibiotic Activity[D *]
WT *Serratia* sp. SCBI	93.3%	49.3 ± 11.9	51.3 ± 2.9	14.5 ± 1.1
WT *Serratia* sp. SCBI + pBAD33	88.4%	45.0 ± 8.3	49.7 ± 1.6	14.3 ± 1.3
WT *Serratia* sp. SCBI + pBAD33-Hemol	97.9%	53.8 ± 6.7	56.7 ± 2.4	13.8 ± 0.9
1-C4	2.8%	88.0 ± 0	64.3 ± 1.9	16.9 ± 1.8
1-C4 + pBAD33	2.2%	88.0 ± 0	63.1 ± 2.3	16.7 ± 1.5
1-C4 + pBAD33-Hemol	86.5%	54.7 ± 15.1	49.3 ± 2.9	13.2 ± 1.7

801 [A] Hemolytic activity was measured as the percentage of SRBCs lysed following a 4 h incubation
802 at 28°C.
803 [B] Swarm assays were performed at 22°C with an incubation time of 48 h. The diameters of the
804 swarm rings were measured and these values are expressed as mm.
805 [C] Swim assays were performed at 37°C with an incubation time of 8 h. The diameters of the
806 swim rings were measured and these values are expressed as mm.
807 [D] Antibiotic assays were performed at 28°C with an incubation time of 24 h. The indicator strain
808 was *M. luteus*. The radius of the clearing zones were measured and these values are expressed as
809 mm.
810 * The maximum measurement was 88 mm (the width of the Petri dish). Results are shown as the
811 average of 9 measurements from 3 independent experiments, with the standard deviations
812 indicated by ±.

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813 **FIGURE LEGENDS**

814 **Figure 1.** The virulence and hemolytic activity of *E. coli* fosmid clone A1-A8 is due to the major
815 hemolysin gene. **(A)** Third instar *M. sexta* larva were injected with 2 x 10^5 CFU of either wild-
816 type *Serratia* sp. SCBI (WT SCBI), *E. coli* fosmid clone A1-A8 (A1-A8), *E. coli* fosmid clone
817 A1-A8 with the *shlA* gene deleted (A1-A8 KO), or wild-type *E. coli* EPI300 (EPI300). Larva
818 were held individually at 37°C for 7 d. Mortality was measured as the percentage of the larva
819 population killed. **(B)** Hemolytic activity against SRBCs was assessed for wild-type *Serratia* sp.
820 SCBI (WT SCBI), *E. coli* fosmid clone A1-A8 (A1-A8), *E. coli* fosmid clone A1-A8 with the
821 *shlA* gene deleted (A1-A8 KO), and wild-type *E. coli* EPI300 (EPI300). A total of $\geq$ 4.0 x 10^6
822 CFU was used for each bacterial strain and the rate of hemolysis was measured over 4 h at 28°C.
823 Error bars represent standard deviations from at least two independent experiments.

824

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

831

832 **Figure 3.** Transposon insertion into *shlA* influenced both swimming and swarming motility. **(A)**
833 Photographs of wild-type *Serratia* sp. SCBI and the *shlA* mutants following 18 h at 22°C on
834 0.65% agar PP3 swarm plates. **(B)** Photographs of wild-type *Serratia* sp. SCBI and the *shlA*
835 mutants following 48 h at 28°C on 1.5% agar PP3 swarm plates. **(C)** Photographs of wild-type

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836 *Serratia* sp. SCBI and the *shlA* mutants following 8 h at 37°C in 0.2% agar swim plates. **(D)**
837 Summary of swarm and swim ring diameters of wild-type *Serratia* sp. SCBI and the hemolysis
838 mutants. The maximum measurement was 88 mm (the width of the Petri dish). Results are
839 shown as the average of 9 measurements from 3 independent experiments, with the standard
840 deviations indicated by $\pm$.

841

842 **Figure 4.** Inactivation of *flhC* resulted in loss of swimming and swarming motility but did not
843 influence hemolytic activity. **(A)** Photographs of wild-type *Serratia* sp. SCBI and *flhC* mutant
844 12-H4 following 8 h of incubation at 37°C in 0.2% agar plates. **(B)** Photographs of wild-type
845 *Serratia* sp. SCBI and *flhC* mutant 12-H4 following 42 h of incubation at 28°C on 0.65% agar
846 PP3 swarm plates. **(C)** Hemolytic activity against SRBCs by wild-type *Serratia* sp. SCBI and
847 *flhC* mutant 12-H4 ($\geq$ 4.0 x 10^6 CFU) was measured over 4 h at 28°C. Error bars represent
848 standard deviations from at least two independent experiments.

849

850 **Figure 5.** Mutants with transposon insertions in *shlA* had significantly increased mRNA levels of
851 the 17,775 bp NRPS *swrA* when swarming. qRT-PCR was performed on RNA extracted from
852 swarmer cells on 0.65% agar PP3 plates. mRNA levels were normalized to the *l21p*
853 housekeeping gene and compared to the calibrator wild-type *Serratia* sp. SCBI (WT SCBI). Data
854 are presented as the relative changes in gene expression between the values obtained with the test
855 conditions and the calibrator. **(A)** A comparison of *flhD, fliC,* and *swrA* mRNA levels when
856 swarm ring diameters were at 40 mm between wild-type *Serratia* sp. SCBI and the five *shlA*
857 mutants. **(B)** mRNA levels of *flhD, fliC,* and *swrA* by wild-type *Serratia* sp. SCBI and *shlA*
858 mutant 6-E3 at swarm ring diameters of 20, 40, and 80 mm. Error bars represent standard

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859 deviations from at least two independent experiments. * (p-value <0.05) denotes significant
860 differences in relative mRNA levels.
861
862 **Figure 6.** Mutations in *swrA* had various effects on antibiotic activity and these effects were
863 dependent on the site of transposon insertion in *swrA*. **(A)** Rescue cloning showed that mutants
864 1-A4, 13-G2 and 11-B8 had the transposon insertion at different sites within *swrA*. **(B)** The
865 domain structure of SwrA. **(C)** Wild-type *Serratia* sp. SCBI and the *swrA* mutants were spot
866 inoculated onto PP3 plates, incubated for 48 h at 28°C, killed by chloroform, and overlaid with
867 warm 0.8% agar containing *Micrococcus luteus*. Clearing zones were observed following 24 h at
868 28°C. Results are shown as the average of 9 measurements from 3 independent experiments,
869 with the standard deviations indicated by ±.
870
871 **Figure 7.** Mutations in *swrA* had various effects on swarming and swimming motility. **(A)**
872 Photographs of wild-type *Serratia* sp. SCBI and *swrA* mutants 1-A4, 13-G2, and 11-B8
873 following 24 h and 48 h incubation at 28°C on 0.65% agar PP3 plates. **(B)** Photographs of wild-
874 type *Serratia* sp. SCBI and *swrA* mutants on 1.05% agar PP3 plates following 24 h and 48 h
875 incubation at 28°C. **(C)** Photographs of wild-type *Serratia* sp. SCBI and *swrA* mutants in 0.2%
876 agar plates following 8 h at 37°C. **(D)** Summary of swarm and swim ring diameter measurements
877 observed for wild-type *Serratia* sp. SCBI and the *swrA* mutants. Results are shown as the
878 average of 9 measurements from 3 independent experiments, with the standard deviations
879 indicated by ±.
880
881 **Figure 8.** The *swrA* mutant 1-A4 was the only *swrA* mutant to display defective virulence and

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882 hemolytic activity. **(A)** Third instar *M. sexta* larva were injected with 2 x 10^4 CFU of either wild-
883 type *Serratia* sp. SCBI or one of the *swrA* mutants (1-A4, 11-B8, or 13-G2) and kept at 37°C for
884 7 d. Mortality was measured as the percentage of the larva population killed over time. **(B)**
885 Hemolytic activity against SRBCs by either wild-type *Serratia* sp. SCBI or one of the *swrA*
886 mutants (1-A4, 11-B8, or 13-G2) at $\geq$ 4.0 x 10^6 CFU was measured over 4 h at 28°C. Error bars
887 represent standard deviations from at least two independent experiments. **(C)** The growth rate of
888 each strain was measured over 24 h at 37°C by absorbance at 600 nm.

889

890 **Figure 9.** *E. coli* fosmid clone A1-F2, which contains the *swrA* gene, showed increased
891 swimming and swarming motility compared to wild-type *E. coli* EPI300. **(A)** Photographs of
892 fosmid clone A1-F2 and wild-type *E. coli* EPI300 following 8 h incubation at 37°C in 0.2% agar
893 swim plates. **(B)** Photographs of fosmid clone A1-F2 and wild-type *E. coli* EPI300 following 18
894 h at 28°C on 0.5% agar LB swarm plates. **(C)** Summary of swarm and swim ring diameter
895 measurements observed for fosmid clone A1-F2 and wild-type *E. coli* EPI300. Results are shown
896 as the average of 9 measurements from 3 independent experiments, with the standard deviations
897 indicated by $\pm$.

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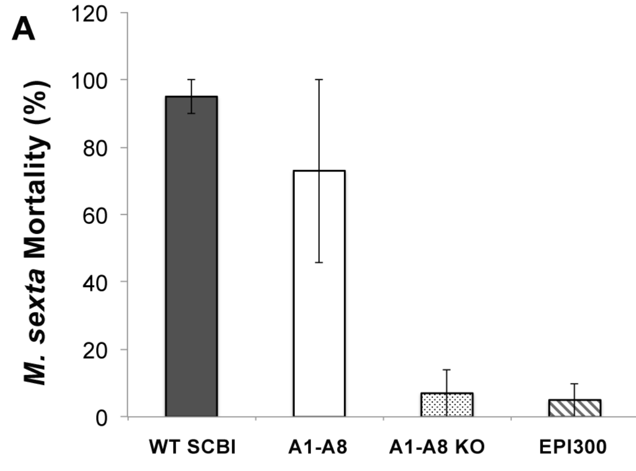

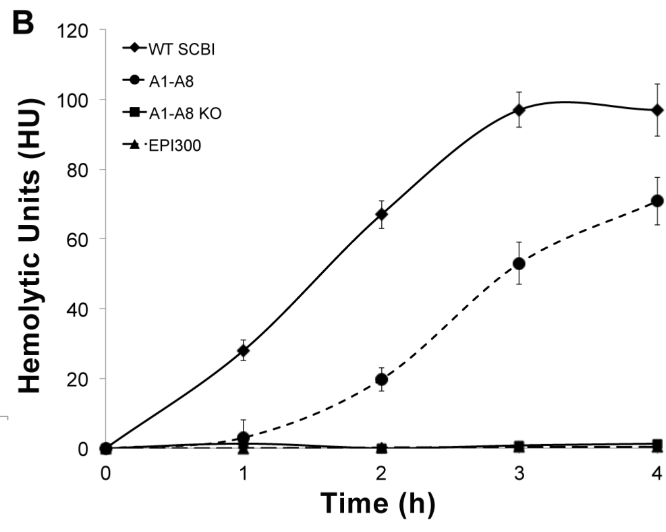

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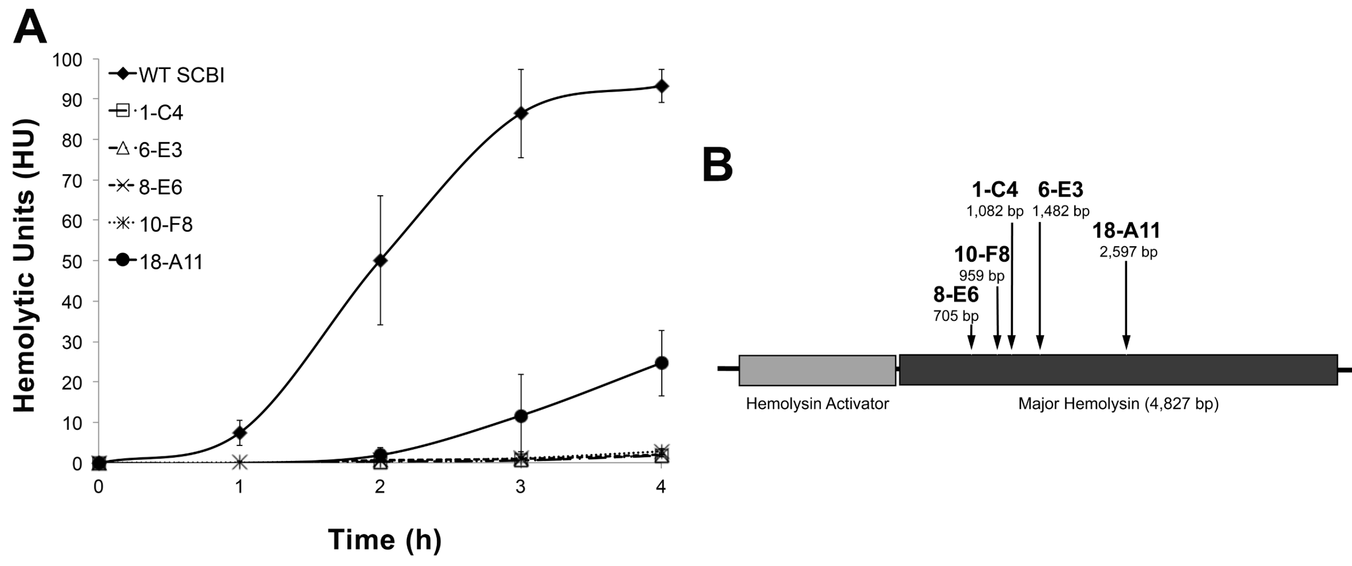

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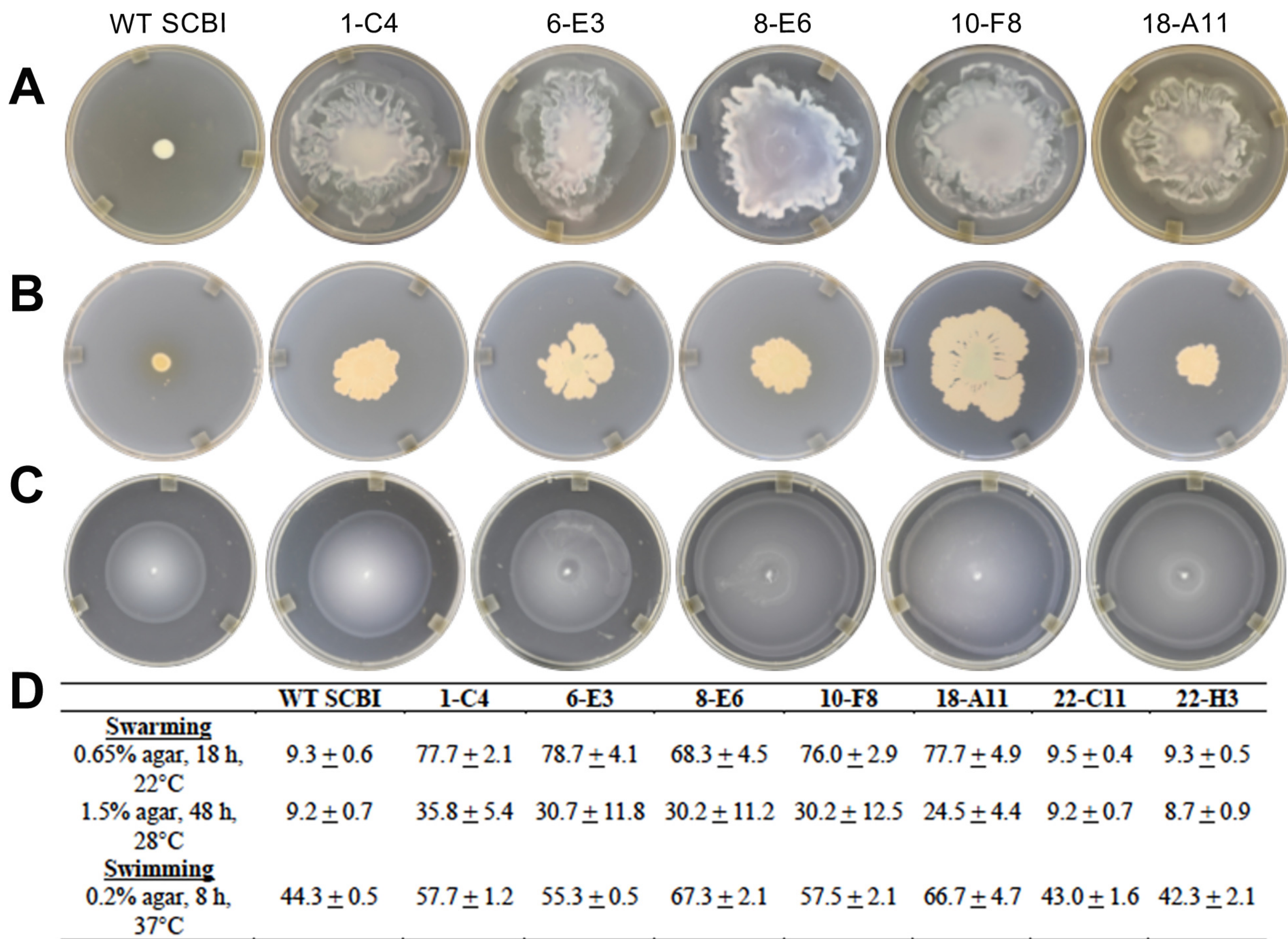

	WT SCBI	1-C4	6-E3	8-E6	10-F8	18-A11	22-C11	22-H3
Swarming								
0.65% agar, 18 h, 22°C	9.3 ± 0.6	77.7 ± 2.1	78.7 ± 4.1	68.3 ± 4.5	76.0 ± 2.9	77.7 ± 4.9	9.5 ± 0.4	9.3 ± 0.5
1.5% agar, 48 h, 28°C	9.2 ± 0.7	35.8 ± 5.4	30.7 ± 11.8	30.2 ± 11.2	30.2 ± 12.5	24.5 ± 4.4	9.2 ± 0.7	8.7 ± 0.9
Swimming								
0.2% agar, 8 h, 37°C	44.3 ± 0.5	57.7 ± 1.2	55.3 ± 0.5	67.3 ± 2.1	57.5 ± 2.1	66.7 ± 4.7	43.0 ± 1.6	42.3 ± 2.1

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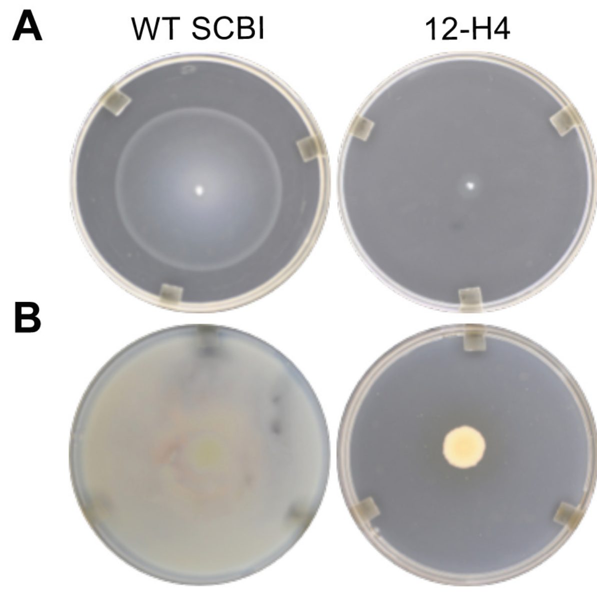

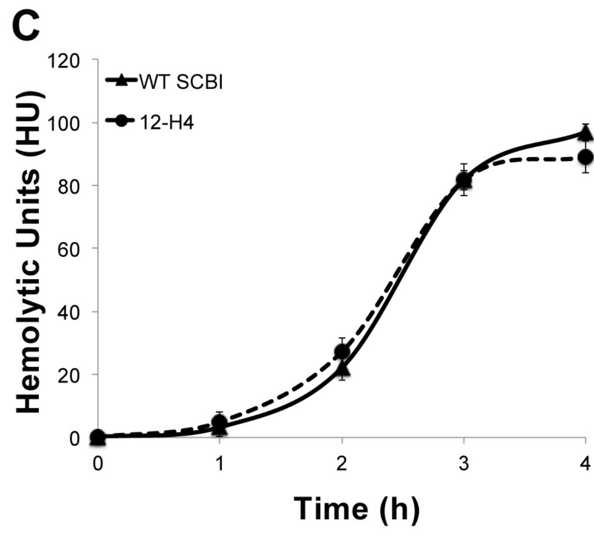

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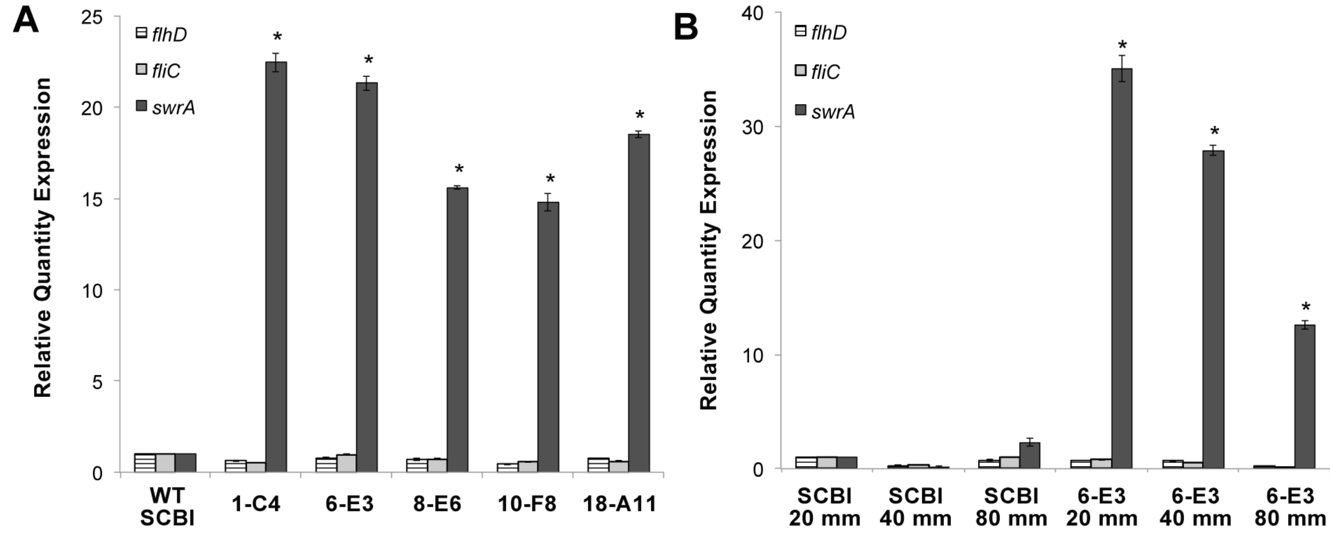

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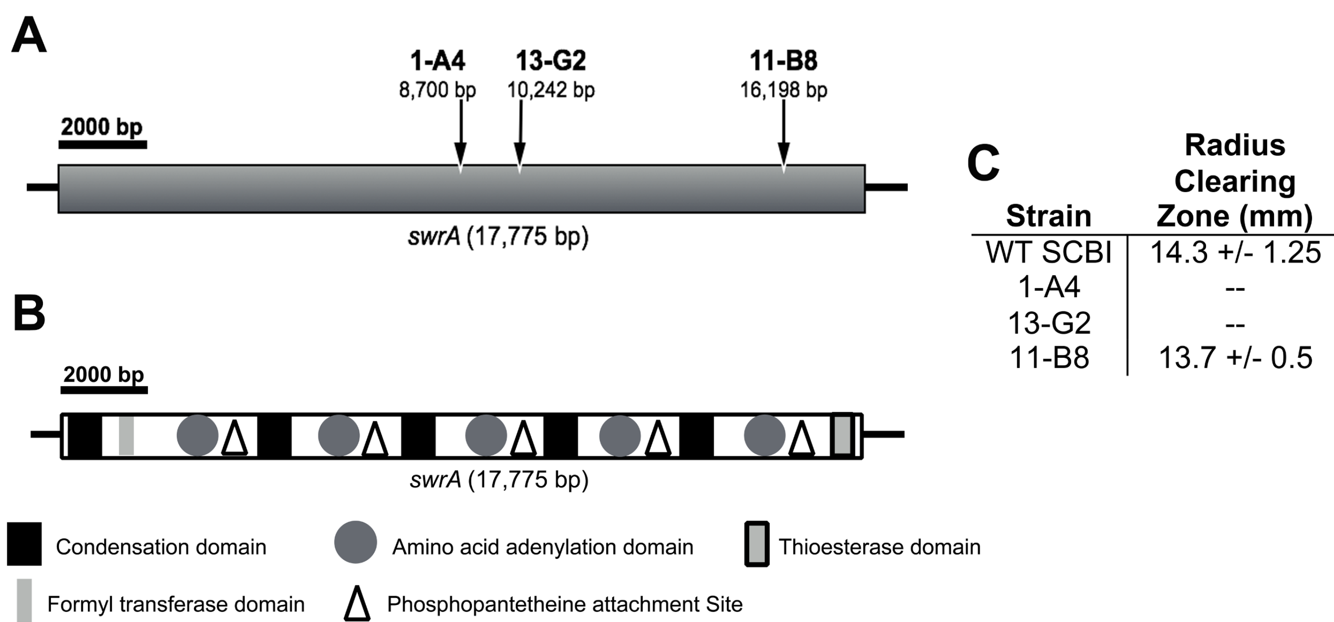

Strain	Radius Clearing Zone (mm)
WT SCBI	14.3 +/- 1.25
1-A4	--
13-G2	--
11-B8	13.7 +/- 0.5

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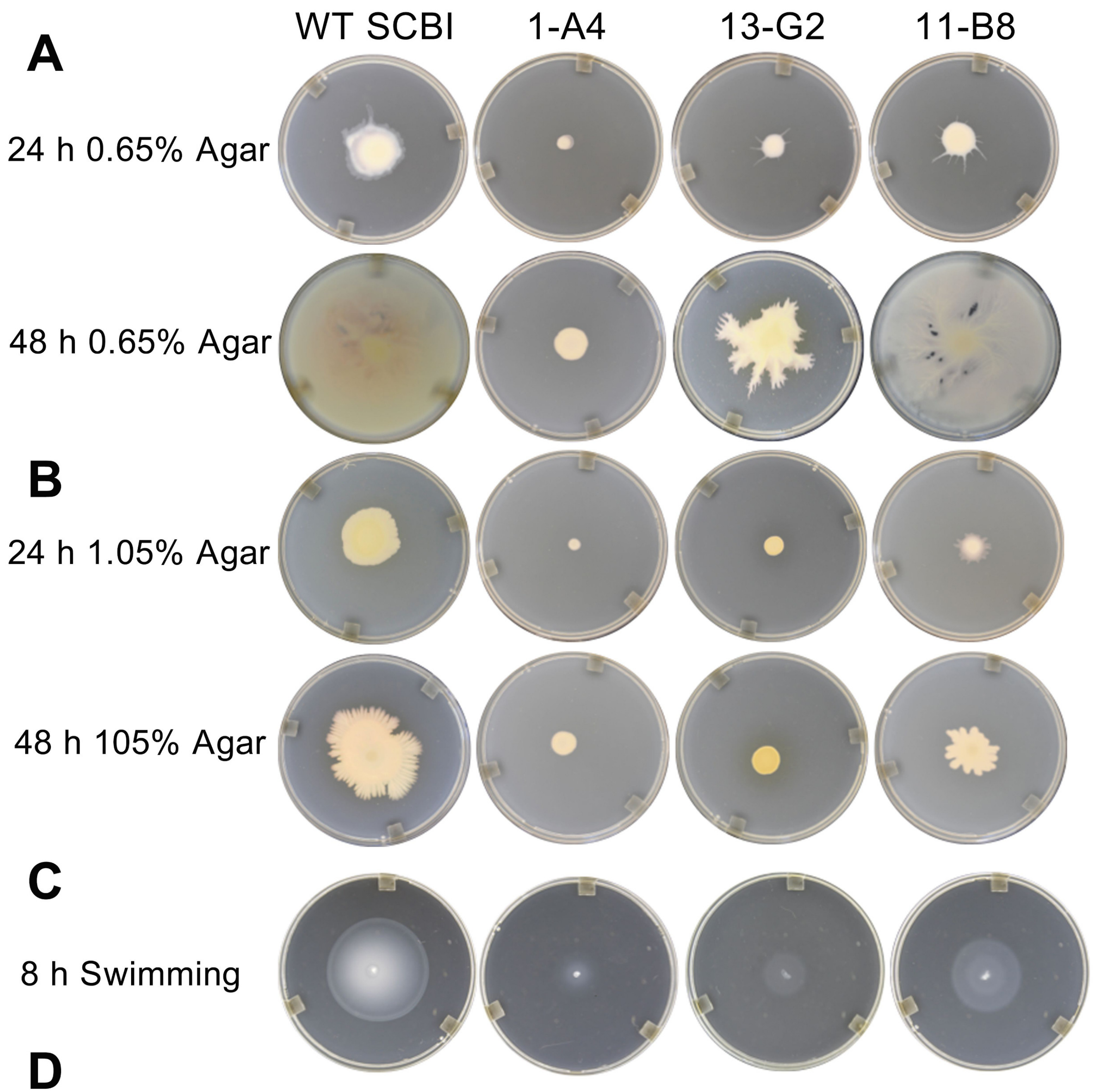

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	WT SCBI	**1-A4**	**13-G2**	**11-B8**
Swarming				
0.65% agar, 24 h	46.33 + 3.7	11.3 + 2.7	14.3 + 4.0	24.0 + 1.6
0.65% agar, 48 h	88.0 + 0	13.0 + 2.2	60.7 + 8.2	88.0 + 0
1.05% agar, 24 h	16.3 + 4.1	8.7 + 1.3	11.0 + 2.2	16.0 + 1.6
1.05% agar, 48 h	43.0 + 5.7	12.3 + 1.9	13.7 + 3.3	22.3 + 3.4
Swimming				
8 h, 37°C	45.7 + 1.7	11.0 + 0.8	18.7 + 2.5	25.7 + 3.3

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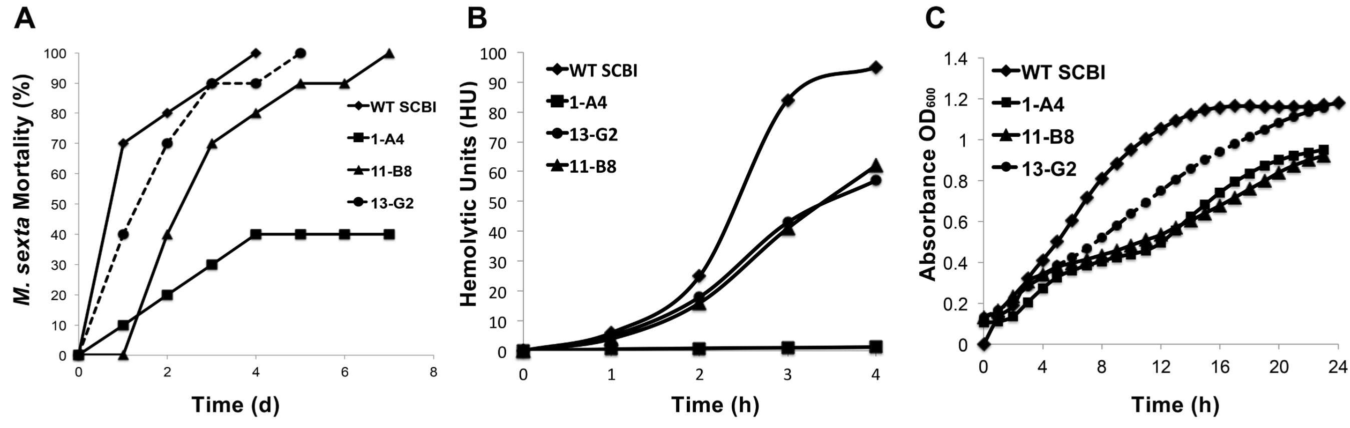

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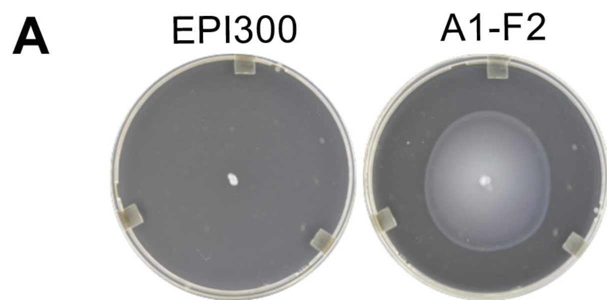

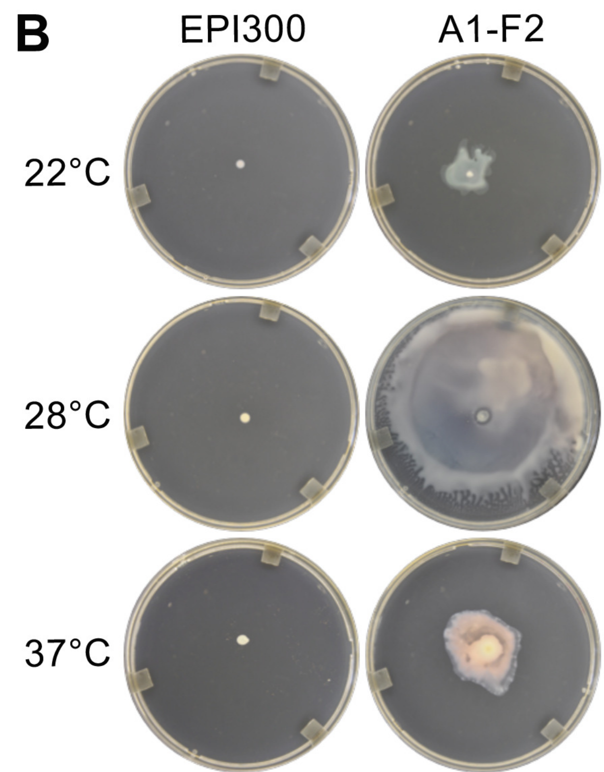

C

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