Serratamolide is a Hemolytic Factor Produced by Serratia marcescens

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Abstract

Serratia marcescens is a common contaminant of contact lens cases and lenses. Hemolytic factors of S. marcescens contribute to the virulence of this opportunistic bacterial pathogen. We took advantage of an observed hyper-hemolytic phenotype of crp mutants to investigate mechanisms of hemolysis. A genetic screen revealed that swrW is necessary for the hyper-hemolysis phenotype of crp mutants. The swrW gene is required for biosynthesis of the biosurfactant serratamolide, previously shown to be a broad-spectrum antibiotic and to contribute to swarming motility. Multicycopy expression of swrW or mutation of the hexS transcription factor gene, a known inhibitor of swrW expression, led to an increase in hemolysis. Surfactant zones and expression from an swrW-transcriptional reporter were elevated in a crp mutant compared to the wild type. Purified serratamolide was hemolytic to sheep and murine red blood cells and cytotoxic to human airway and corneal limbal epithelial cells in vitro. The swrW gene was found in the majority of contact lens isolates tested. Genetic and biochemical analysis implicate the biosurfactant serratamolide as a hemolysin. This novel hemolysin may contribute to irritation and infections associated with contact lens use.

Introduction

Serratia marcescens is a nosocomial pathogen [1,2,3], a common contaminant of contact lens cases and lenses with a number of ocular conditions including keratitis, conjunctivitis, and contact lens acute red eye (CLARE) [4]. Hemolysins are important virulence factors for a wide range of Gram-negative and Gram-positive organisms [5,6,7,8,9]. Known S. marcescens hemolytic exoenzymes are ShlA and PhlA. ShlA is a key virulence factor and a pore-forming hemolysin [10,11], whereas PhlA is phospholipase, one of whose cleavage products is lysoospholipid, a surfactant that can lyse red blood cells [12].

Regulators of the shlA hemolysin gene include the FlhDC flagellar biosynthesis regulator and RssAB, a two component transcriptional regulator [11]. RssAB is a negative regulator of flhDC expression, whereas FlhDC is a positive regulator of the shlA hemolysin operon, shlBA [11]. It was also shown that the cyclic nucleotide cAMP, the adenylate cyclase (CyaA) that generates cAMP, and the cAMP-receptor protein transcription factor (CRP) cAMP-CRP positively regulate FlhDC [13,14]. The phlA gene is also directly regulated by FlhDC and catalyse repression [15,16]. Therefore, it would be predicted that crp mutants should have reduced hemolytic activity through a reduction of both shlA and phlA expression. Unexpectedly, we observed that crp mutants exhibited increased levels of hemolytic activity, suggesting another mechanism of hemolysis. Here we used a genetic approach to gain insight into the mechanism of hemolysis exhibited by crp mutants. Genetic and biochemical analysis in this study support the model that the biosurfactant serratamolide is a hemolysin.

Results

Mutations in crp and cyaA Lead to an Increase in Secreted Hemolytic Activity that is Independent of known Hemolytic Agents ShlA and PhlA

Previously cyaA and crp null mutants were characterized for exhibiting elevated fimbrin and prodigiosin production [17]. Here we describe a novel hemolysis phenotype for these mutants. The cyaA and crp mutant strains exhibited dramatically increased zones of hemolysis on blood agar plates compared to the parental, wild-type (WT) strain CMS376 [18], that produces small zones of hemolysis after several days of incubation at 30°C (Fig 1A). The hyper-hemolytic phenotype could be reversed by returning the wild-type cyaA and crp genes, respectively, on a multicopy plasmid (Figure 1B). From this point onward, we focused on crp mutants, for simplicity.

We tested whether S. marcescens exoenzymes, ShlA and PhlA, were required for increased extracellular hemolysis produced by crp mutants. If one of these enzymes is required for the increased
hemolysis seen in crp mutants, then mutation of shlA or phlA should eliminate the hyper-hemolysis phenotype of the crp mutants. However, disruption of the shlA and phlA genes did not decrease the large hemolytic zones of crp mutant, suggesting that another hemolysis-promoting factor was involved (Fig 1C). Integration of a similar plasmid at fimC was used as a plasmid integration control, and had no impact on hemolysis (Fig 1C).

A potential hemolytic role for serralysin and prodigiosin, a cytotoxic protease [15–20] and membrane-associated pigment respectively, were similarly disproved, as crp phlS and crp pigB double mutants still exhibit high levels of hemolysis (data not shown).

Suppressor Analysis of the crp Hyper-hemolysis Phenotype Implicates Serrawettin as a Hemolytic Factor

To determine the mechanism of hyper-hemolysis, a suppressor analysis approach was taken using random transposon mutagenesis [18]. Multiple mutations that inhibited secreted hemolysis production were identified in a crp mutant background (Fig 2A). The transposon insertion sites from these hemolysis deficient isolates were scattered along the length of the swrW gene (base pair 821, 831, 1396, 2585, and 3078). Strikingly, mutation of the swrW gene led to an unreported metallic gold color on the surface of colonies (Fig 2B). In Figure 2B the image is illuminated from the top to depict the golden coloration, whereas the rest of the images of blood agar plates are illuminated from below to exhibit the zones of hemolysis. The swrW gene was previously implicated in production of serramolide, a dilactone biosurfactant with antimicrobial activity, also known as serrawettin W1 [21,22,23]. Serratamolide was previously shown to be required for surface swarming motility of some strains of S. marcescens [24]. Other surface wetting agents made by S. marcescens are the chemically distinct serrawettin W2 and W3, which are both larger cyclic-peptides, composed of five amino acids and a single acyl side chain [24,25,26].

Zones of surfactant were visible on top of agar plates surrounding colonies. We measured this zone and found that it was significantly larger (p<0.05) around crp mutants (9.0±2.0 mm) compared to the WT (2.2±0.9 mm) (Fig 2C). Mutation of the swrW gene in the WT and crp background completely eliminated this zone (Fig 2C). These data suggest that the surfactant zone is serramolide and that crp mutants produce more of it.

To confirm the above observations, we directly mutagenized the swrW gene. An internal fragment of swrW was cloned into suicide-
promoter probe plasmid, pStvZ3, and introduced into WT and crp mutant strains by conjugation, as previously described [14]. The swrW::pStvZ3 and crp swrW::pStvZ3 mutant strains did not produce zones of surfactant (Fig 2C) or hemolysis (Fig 3C, and data not shown). Mutation of swrW in the laboratory strain Nima and a pigmented clinical isolate K904 led to a similar metallic gold colony color and deficiency in hemolysis on blood agar plates, indicating that the observed phenotypes are not restricted to the CMS376 laboratory strain (Fig 2D and data not shown).

If swrW expression positively impacts hemolysis, then a mutant strain with elevated levels of swrW expression would be expected to be more hemolytic. It has been previously reported that the LysR-family transcription factor, HexS, directly inhibits transcription of swrW [27]. We mutated the hexS gene to test whether it would confer a hyper-hemolysis phenotype, and found that hexS mutants exhibited elevated hemolytic zones similar to the crp mutant (Fig 3A). A hexS swrW double mutant was non-hemolytic, indicating that the hyper-hemolytic phenotype of hexS mutants depends upon swrW (Fig 3A). As with the crp mutant, hexS mutants exhibited significantly larger (p<0.05) surface surfactant zones (13.4±1.1 mm) than the WT, whereas the hexS swrW mutant exhibited no surfactant zones (Fig 2C).

Consistent with serratamalide promoting swarming motility, we tested whether hexS mutants would produce larger zones of swarming. Whereas, the WT strain is competent at swarming, the ΔhexS mutant swarmed earlier and to a greater extent (Figure 3A and data not shown). This swarming phenotype was eliminated in the hexS swrW double mutant indicating that the hexS hyper-swarming phenotype is serratamolide dependent (Figure 3A).

To test the prediction that swrW is more highly expressed in a crp mutant background, the swrW promoter (PswrW) was cloned in front of the tdTomato reporter gene on a pBBR1-based plasmid (Fig 3B). This plasmid was placed in WT and crp mutant strains and fluorescence was measured as a function of culture density. We observed elevated levels of red-fluorescence in the crp mutant compared to the WT strain. Strains bearing a control plasmid without tdTomato exhibited negligible fluorescence (data not shown). A similar result was observed with semi-quantitative

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Figure 3. Genetic evidence that serratamolide mediates hemolysis. A. Hemolysis and swarming by a mutant known to have elevated serratamolide production (hexS) is increased, and these phenotypes require SwrW. B. Elevated expression of a swrW promoter reporter in the crp mutant. Top, expression measured using a plasmid based-tdTomato reporter construct at t = 20 hrs. Asterisk indicates statistical significance (p<0.05) by the Student’s T-test. A representative experiment is shown (n = 4). Error bars indicate one standard deviation. Bottom, semi-quantitative RT-PCR analysis of RNA from WT and Δcrp mutant strains measured relative expression of swrW and internal standard 16S RNA from stationary phase cultures (OD600 = ~3.5). C. Arabinose-inducible expression of the swrW gene in an swrW transposon mutant strain restores hemolysis. D. Swarming motility defect of the swrW mutant is restored by induced expression of the swrW gene.

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RT-PCR analysis of the WT and Δcrp mutant, where swrW transcript was more abundant in Δcrp mutant RNA preparations relative to those from the WT (Figure 3B). These analyses are consistent with a model where cAMP-CRP negatively regulates swrW gene expression in a direct or indirect manner.

Complementation of the swrW Mutant Hemolysis and Swarming Phenotypes

Because the swrW gene is not present in the sequenced Db11 strain of S. marcescens, we do not know its genetic context, so it is possible that some of the mutant phenotypes are due to polar effects on adjacent genes. To ensure that the swrW mutations were responsible for the mutant phenotypes, we cloned the full-length swrW gene and placed it under transcriptional control of the E. coli P_{BAD} promoter in vector pMQ125 [20] to generate the pMQ367 plasmid. The swrW:pStvZ3 mutant bearing the P_{BAD}-swrW plasmid was rescued with respect to swarming motility and exhibited a hyper-hemolysis phenotype when L-arabinose was supplemented in the medium to induce swrW expression, but not in medium without L-arabinose (Fig 3C–D). As expected, the swrW:pStvZ3 mutant expressing the empty vector, pMQ125, did not swarm or exhibit zones of hemolysis consistent with a model where cAMP-CRP negatively regulates swrW expression, but not L-arabinose induction (Fig 3C–D). As a control for the effect of L-arabinose on hemolysis and swarming in general, it was also determined that L-arabinose did not restore swelling or hemolysis to the swrW mutant without the P_{BAD}–swrW construct (data not shown). These data indicate that mutation of swrW rather than a polar effect on an adjacent gene or another mutation is responsible for the swrW mutant phenotypes.

Serratamolide can act as a Hemolysin

Serratamolide, a cyclic and aliphatic aminolipid [21,22] (Fig 4A), was purified to verify its role as a hemolysin. Comparative profiling of secreted metabolites by the S. marcescens WT, and the swrW mutant strain with both the vector and expressing swrW from pswrW (pMQ367), using HPLC and LC-MS, clearly indicated that the key difference lies at a metabolite fraction with m/z at 515.5 that corresponds to serratamolide (Fig 4B and data not shown). To unequivocally assign its molecular identity, we isolated the corresponding fraction by preparative HPLC and confirmed its structural identity by HR-MS and NMR analysis. All spectral data were in accordance with previous reports on serratamolide [29] (data not shown). Furthermore, the purified serratamolide was able to restore swarming activity to an swrW mutant strain, providing biological evidence that the purified compound is serratamolide (Fig 4C).

Purified serratamolide (1 mg/ml) placed in wells in blood agar plates created clear zones of hemolysis, unlike the DMSO mock control (Fig 5A). In addition to rabbit erythrocytes, we observed that serratamolide at a concentration of 20.8 μg/ml completely lysed mouse erythrocytes in solution in less than 10 seconds (Fig 5B).

To test whether the cytotoxic effect of serratamolide is specific to red blood cells, we tested whether serratamolide reduced the viability to immortalized human airway epithelial cell (A549) and human corneal limbal epithelial cell (HCLE) monolayers using a fluorescent viability dye. We observed a dose-dependent cytotoxic effect at a concentration above 12.5 μg/ml (Fig 5C). At the maximum serratamolide concentration of 50 μg/ml the fluorescent viability value of HCLE cells was 1821 ± 432, significantly less than 10483 ± 232 for mock treated cells (p<0.05, Student’s T-test), and similar to 897 ± 5 for detergent lysed cell layers. Significant results were observed for A549 cells (Fig 5C). Fluorescent values for serratamolide treated cells can be compared to those controls values to calculate a % cytotoxicity metric. Serratamolide at 50 μg/ml caused 88.0±2.5% cytotoxicity to A549 lung cells and 95.4±4.0% cytotoxicity to HCLE ocular cells, signifying that serratamolide treatment is toxic to human epithelial cell lines.

Contact Lens Associated Isolates are More Likely to have swrW than Keratitis Isolates

We tested a library of clinical isolates from the Charles T. Campbell laboratory of Ophthalmic Microbiology from keratitis patients for the presence of the swrW gene. PCR was used to probe for the presence of the swrW gene in the chromosome of these strains. Staphylococcus aureus chromosomal DNA was used as a negative control, and the S. marcescens oxyR gene was used as a positive PCR target to ensure DNA quality. Out of all 63 isolates, a total of 22 (34.5%) exhibited PCR amplicons consistent with the swrW gene.

Of the swrW positive clinical isolates, 9 out of 22 (40.1%) tested positive for hemolysis on blood agar plates. To test whether their hemolytic phenotypes were swrW dependent, the swrW gene was mutated in five of the clinical isolates. Mutation of swrW in the five different clinical isolates conferred a loss of swarming motility (data not shown), and three of these five also became deficient in the ability to create zones of hemolysis on blood agar plates (Fig 2D).

Discussion

The data presented here indicate that the bio-surfactant serratamolide can act as a novel S. marcescens hemolysin, and that the non-ribosomal peptide synthetase SwrW is necessary for hemolysis in some clinical and laboratory strains. This conclusion is based upon the genetic data that hemolysis is eliminated in swrW mutants and elevated in crp and hexS mutants that over-express swrW. Biochemical data indicate that purified serratamolide is sufficient to lyse red blood cells and damage epithelial cells in vitro.
red blood cells in solution by serratamolide (TSA) serratamolide (1 mg/ml) to sheep red blood cells. Wells were cut into a hemolysis around serratamolide treated well. White arrow indicates zone of otics [30]. Another role for serratamolide was suggested by Barr-

otics, such as surfactin from Bacillus species and syringomycins from Pseudomonas species can act as hemolysins [32,33,34,35]. Serramic acid, another S. marcescens product was shown to be hemolytic to human and horse red blood cells, but only poorly hemolytic to bovine and sheep red blood cells [36]. This same study tested serratamolide for hemolytic activity against human red blood cells, and the result was negative. The differences between this current study and the previously described study, in which serratamolide was tested for hemolysis [36], may be due to experimental differences, in that the previous authors delivered serratamolide using liposomes composed of several phospholipids, rather than serratamolide alone. Furthermore, the previous study tested serratamolide against human red blood cells but not sheep or murine red blood cells; it is possible that differences in membrane phospholipid composition or surface proteins may result in differential hemolytic activity against red blood cells from different species, as has been shown before for PhIa [12].

Miyazaki and colleagues showed that serratamolide provided protection to S. marcescens against polymorphonuclear leukocyte (PMN) phagocytosis [37]. This is of particular interest because PMNs are the primary leukocyte involved in clearing bacteria corneal infections [38,39,40]. Interestingly, it was shown that Staphylococcus aureus cells coated with serratamolide were also protected from PMN phagocytosis [37]. This leads us to speculate that the presence of S. marcescens-derived serratamolide in contact lens cases or on lenses may better enable other pathogenic bacteria to establish ocular infections.

It was noted that swrW was found in ~35% of the tested ocular clinical isolates, and 40% of the swrW containing isolates were hemolysin positive on blood agar plates, suggesting that hemolytic strains express swrW sufficiently to produce hemolysis. In support of this premise, mutation of swrW in three out of five hemolysis positive strains severely reduced or eliminated hemolysis zones on blood agar plates. Of the swrW negative strains, 42% were hemolysin positive, indicating that other mechanisms of hemolysis are present in ocular clinical isolates. Another gene, swrA [26], is present in some strains of S. marcescens is necessary for production of serrawettin W2, may account for the hemolysin positive phenotype of swrW negative strains. There is genetic evidence that the swrA-dependent product serrawettin W2, a structurally distinct surfactant, can act as a hemolytic agent [41]. Serrawettin W2, consisting of five amino acids with a single acyl chain [25], is detected by Caenorhabditis elegans as a chemical signal to avoid S. marcescens colonies [41]. Transposon mutation of the swrA gene, in strain Db10, led to the loss of hemolysis zones on blood agar plates that was correlated with the loss of serrawettin W2 [41]. Whereas the hemolysis and cytotoxicity data presented here suggest that serratamolide may contribute to bacterial infections, the absence of the swrW gene in many pathogenic and contact lens associated strains indicate that SwrW is not a requirement for colonization of contact lenses or for causing ocular diseases. Serratamolide may be more relevant in environmental settings than for human infections, as the majority of pigmented strains tested (66.7%, n = 9) had the swrW gene, and pigmentation is generally associated with environmental isolates, whereas clinical isolates are almost exclusively non-pigmented [42]. In an environmental setting serratamolide could contribute to the competitiveness of S. marcescens as it is a broad spectrum antibiotic. Furthermore, it

Figure 5. Serratamolide is hemolytic to erythrocytes and cytotoxic to epithelial cells in vitro. A. Hemolysis of DMSO and serratamolide (1 mg/ml) to sheep red blood cells. Wells were cut into a TSA sheep blood agar plate, and DMSO or serratamolide was added to the well and incubated for 24 hours. White arrow indicates zone of hemolysis around serratamolide treated well. B. Hemolysis of murine red blood cells in solution by serratamolide (µg/ml) incubated for 10 seconds. Distilled water was used as a complete lysis control (Lysis). A representative experiment is shown. Error bars indicate one standard deviation. C. Cytotoxicity to immortalized human airway (A549) and corneal (HCLE) epithelial cells was measured using alamar blue fluorescence that provided a positive output for viability of cells. The average of eight independent replicates is shown for each cell line from two separate experiments. Error bars indicate one standard deviation. doi:10.1371/journal.pone.0036398.g005

Genetic data suggests that serratamolide production is regulated by cAMP-CRP in strain CMS376, namely that surfactant zones are increased in a and hemolysis is increased in mutant strains with altered ability to respond to or make cAMP ( and ). Since the cAMP-CRP pathway is well known to regulate genes in response to the nutritional environment of the cell, this may indicate that serratamolide plays a role in a bacterium’s ability to acquire or compete for nutrients. Consistent with the role of serratamolide in competition, it has been shown that serratamolide has antimicrobial activity against both prokaryotes and fungi [22,29], and that swarming motility, which requires biosurfactants such as serratamolide, confers resistance to antibiotics [30]. Another role for serratamolide was suggested by Bar-
was shown that a surfactant produced by *Serratia* sp. ATCC 39006 facilitates the dispersal of the antibiotic pigment prodigiosin [43], and serratamolide may act in an analogous fashion.

Serratamolide has shown promise as an anticancer agent for its proapoptotic effect upon breast cancer and B-cell chronic lymphocytic leukemia cells [44,45]. Therefore, understanding the pathways that control serratamolide production may yield improved ways to generate this cyclodepsipeptide.

Further studies will focus on determining the regulatory pathway by which CRP regulates serratamolide production, and characterizing the role this surfactant plays in host-pathogen interactions.

**Methods**

**Growth Conditions and Strains**

All bacteria were cultured with LB medium (per liter: tryptone – 10 g, yeast extract – 5 g, NaCl – 5 g, with or without agar – 15 g), except when tested for hemolytic zones on blood agar plates (TSA+5% sheep blood, Becton Dickenson BBL TSA II). Swarming plates consisted of LB medium with 0.6% agar (w/v). Antibiotics were supplemented when needed, with kanamycin at 50–100 μg/ml, gentamicin at 10 μg/ml, and tetracycline at 10 μg/ml. The *Escherichia coli* plasmid, SM10 and S17–1, were used for conjugations, and EC100D (Epícentre) was used for plasmid preparations. Strains are listed in Table S1. *S. marcescens* strains used were CMS376 (Presque Isle Cultures strain number 3611) [18], Nima, a strain used by pioneering prodigiosin researcher, Robert Williams and colleagues [46], CHASM a compost heap acquired *S. marcescens* isolate [14], and a number of ocular clinical isolates from keratitis patients from the Charles T. Campbell Laboratory of Ophthalmic Microbiology at the University of Pittsburgh Vision Center.

**Genetic Analysis and Plasmids**

Transposon mutations were introduced into *S. marcescens* using transposon delivery vectors pBT20 [47] and pSC189 [48], and subsequently mapped, as previously described [18]. Transposon mutant strains were collected in 96 well plates, transferred onto blood agar plates, incubated for 2–3 days at 30°C, and screened for colonies with altered hemolysis zones.

For plasmid generation, chromosomal DNA from CMS376 (Table S1) was amplified using Phusion polymerase (NEB). PCR generated amplicons were mixed with linearized vector DNA in an approximately 10:1 ratio and used to transform *Sacharomyces cerevisiae* strain InvSc1 with selection for uracil prototrophy, as previously described [49,50]. Plasmids were isolated from yeast colonies after 3–4 days of growth on uracil-drop out medium [49], and isolated by the smash and grab method [49]. Plasmids were moved into *E. coli* strain EC100D by electroporation, screened by diagnostic PCR, and inserted were verified by sequencing at the University of Pittsburgh Genomics and Proteomics Core facility.

For complementation analysis and overexpression of serratamolide, the *swrW* open reading frame (ORF) was amplified with primers 1630–1631, and cloned using yeast *in vivo* recombining into pMQ125 that had been linearized with EcoRI, using previously described methods [50]. The pMQ125 vector has an arabinose-inducible promoter and a p15a-based replicon [28].

For insertional mutagenesis of *hexS*, *phlA*, *ptsS*, *shlA*, and *swrW*, an internal fragment of each gene was amplified and cloned in either pMQ118 [28] or pStvZ3 [14] that had been linearized with BamH1 using *in vivo* cloning methods as noted above. The primer sets to amplify the internal fragments are listed in Table S2 and were 2014–2015 for *hexS*, 1456–1457 for *phlA*, 996–997 for *ptsS*, 1022–1023 for *shlA*, and 1639–1640 for *swrW*. The resulting plasmids were verified using PCR and sequencing, and introduced into the recipient strain by conjugation and selection for kanamycin (100 μg/ml), as previously described [14]. The *finC* gene was mutated using a pMQ119-based insertion plasmid, pMQ167, as previously described [17].

**Detection of the *swrW* Gene in *S. marcescens* Isolates**

Bacteria from frozen stocks were streaked to single colonies on LB or TSA blood agar plates. DNA was extracted from a single colony using Quick Extract (Epícentre) according to the manufacturer's specifications. PCR was performed using standard Taq polymerase (New England Biolabs), and standard conditions using the primers set 1639 and 1640 (Table S2) to detect the *swrW* gene. *S. marcescens* (CMS376) and *Staphylococcus aureus* (MZ100) chromosomal DNA were used as a positive and negative controls respectively. Analysis was performed twice with each primer set and any reproducibly produced amplicon of the expected size for any strain was considered a positive result. A quality control PCR reaction was also performed on each DNA preparation to eliminate false negative results using previously described primers (736 and 737) that amplify the *syrR* gene [18].

**Analysis of *swrW* Expression**

For analysis of *swrW* expression, a 351 base pair region of DNA immediately upstream of the *swrW* open reading frame was fused with the tettohama derivative of *dovd* [51] in pBBR1-based plasmid, pMQ361, yielding pMQ376. The pMQ361 plasmid was made by digesting pMQ131 with SmaI and mixing with a PCR amplicon containing the *spID* promoter from pMQ118 (Table S1), amplified with primers 2516 and 2517, and an amplicon containing DNA upstream of *swrW* that had been made with primers 2768–9. This region of DNA contains a predicted promoter, as previously noted [21,22,23].

The WT and a Δ*npf* mutant strain [17] bearing pMQ376 were grown overnight at 30°C in LB broth (5 ml) supplemented with kanamycin (100 μg/ml) in 20×150 mm glass test tubes rotated on a TC-7 tissue culture rotor (New Brunswick Scientific), set at speed setting 8. The same strains were grown with the empty vector pMQ131 as a control for background fluorescence. Cultures that had grown to saturation (OD600 = ~5.0), were diluted 1:100 in the same medium and incubated at 30°C with aeration. At designated time points, samples (0.15 ml) were removed to determine culture density (OD = 600 nm) and fluorescence (excitation filter: 545/40, emission filter: 590/20) using a plate reader (Biotek Synergy 2). Background fluorescence was equivalent for both strains (data not shown). Relative fluorescence units (RFU) were determined by dividing fluorescence by culture optical density. The experiment was repeated on two different days with a highly similar result.

To obtain cells for RNA extraction, cultures were first inoculated into 5 ml of LB medium, then vortexed and diluted 1:5000 in LB to reduce the inoculum. The diluted cultures were grown overnight in LB medium, subcultured to OD600 = 0.1, grown to OD600 = 0.8, subcultured to OD600 = 0.1 and allowed to grow. Samples were taken for RNA analysis until the cultures reached OD600 = ~3.5. RNA was isolated from bacteria following the method of Wargo, et al, [52] including the three rounds of DNase treatment. RNA was normalized to 50 ng/μl using DNase-free water and 5 μl was used in each reverse transcription (RT) reaction using SuperScript III RT (Invitrogen) following the manufacturers specifications. A PCR reaction with a 94°C hold for 60 seconds, followed by 30 cycles of 20 seconds at 94°C, 20 seconds at 55°C, and 30 seconds at 72°C, followed by a 72°C hold for 60 seconds was used to detect the amount of transcript from *swrW*.
the 16S rDNA gene as a control to normalize samples and the swrW gene. Other controls included a no-reverse transcriptase and no-RNA reactions, and these showed that there was no contaminating DNA (data not shown). The 16S rDNA gene was amplified by 2639 and 2639 using previously described primers [11]. The swrW gene was amplified with primers 2917 and 2918. Amplicons were run on 1.5% agarose gels and imaged using a Carestream Gel Logic 212Pro device. The experiment was repeated three times with similar results.

Hemolysis, Swarming and Surfactant Zone Assays
Hemolysis on blood agar plates was performed by plating ten microliters of bacteria from a liquid culture onto blood agar plates and incubating at 30°C for 48 hours. For quantitative hemolysis assays, fresh mouse blood from C57BL/6 mice were washed in PBS and suspended in PBS at ~2 x 10^6 red blood cells (RBC) per ml. For the hemolysis assay, 70 µl of RBC suspension was incubated with either 50 µl of DMSO or DMSO containing serratamolide (50 µg/ml in DMSO). RBCs were incubated in sterile µH2O for complete lysis. The cells were incubated for seven minutes in microfuge tubes that were centrifuged for 1 minute (500 g), 100 µl of supernatant was transferred to a microplate and the absorbance was read at 405 nm. The experiment was performed in triplicate on two separate days with similar results.

Swarming assays were performed as previously described [13] using LB agar with reduced agar concentration (0.6% w/v). Bacteria were placed in a small spot on the top of the agar using a sterile toothpick. The plate was then incubated at 30°C for 18–20 hours. The experiment was performed on different days with the same result.

Identification and Purification of Serratamolide
For comparative analysis of secreted metabolites of S. marcescens wild-type and mutants strains, 10 ml of overnight culture was pelleted. The supernatant was extracted with ethyl acetate (5 ml) and the combined ethyl acetate was evaporated in vacuo. The residue was dissolved in 0.5 ml MeOH and an aliquot (20 µl) was evaporated. The purity of the isolated serratamolide was ensured by high resolution mass spec (HR-MS) and 1H NMR analysis in accordance with previously reported data [45] using our previously published methods [53].

Cytotoxicity Analysis
Human lung carcinoma cells ATCC CCL-185, American Type Culture Collection (ATCC), Manassas, VA) were maintained in Gibco Medium 199 with 100 units/ml penicillin G, 0.1 mg/ml streptomycin and 0.5 mg/ml gentamicin, 10% fetal bovine serum (FBS), and 5% sodium bicarbonate.

Human corneal-limbal epithelial cells (HCLE) [54] were obtained from Dr. Jes Klarlund with permission from Ileen Gipson, and were grown in Keratinocyte-SFM (serum free medium) with L-Glutamine, supplemented with 25 µg/ml BPE, 0.2 ng/ml EGF, and 1 mM CaCl2, without any antibiotics.

To measure cytotoxicity, confluent cell layers were exposed to serratamolide in DMSO or DMSO alone, such that the concentration of DMSO in each well was 5% (v/v) for 2 hours at 37°C in 5% CO2. Viability of cell layers was assessed using Alamar Blue (Invitrogen, Camarillo, CA). Cells with only DMSO were used as mock wells to indicate full viability (Mock), while triton X-100 (0.25% v/v) was used to determine the reading for non-viable cells (Lysis). After 2 hours, media was removed from all wells, and 200 µl of a 4% Alamar Blue solution in growth medium was added to each well. The plate was returned to the incubator for 1.5 hours at 37°C in 5% CO2, and relative fluorescence units (RFU) were determined using a plate reader (Biotek Synergy 2) with a 500/27 excitation filter and a 620/40 emission filter.

The percent cytotoxicity value was determined using RFU values from the Alamar Blue analysis. The following formula was used:

\[ \text{Relative Fluorescence Units (RFU)} = \frac{(\text{Mock RFU} - \text{Sample RFU})}{(\text{Mock RFU} - \text{Lysis RFU})} \times 100 \]

Statistical Analysis
All experiments were performed at least twice on different days with reproducible results. Statistical analysis was performed using Prism 5 software and consisted of two-tailed Student’s T-tests of One-way ANOVA with a Tukey post-test, as noted. Significance was set at p<0.05.

Supporting Information
Table S1 Strains and plasmids used in this study.
(DOCX)

Table S2 Oligonucleotide primers used in this study.
(DOCX)

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Author Contributions
Conceived and designed the experiments: RMQS RPK XL. Performed the experiments: RMQS NAS RML SW TIL. Analyzed the data: RMQS SW RFK XL. Contributed reagents/materials/analysis tools: RMQS RPK XL. Wrote the paper: RMQS NAS RML XL.

References
1. Gaynes R, Edwards JR (2005) Overview of nosocomial infections caused by gram-negative bacilli. Clin Infect Dis 41: 848-854.

2. Steppberger K, Walter S, Claros MC, Spenceker FB, Kiess W, et al. (2002) Nosocomial neonatal outbreak of Serratia marcescens–analysis of pathogens by
pulsed field gel electrophoresis and polyacrylamide chain reaction. Infection 30: 277–281.

3. Vonberg RP, Gastmeier P (2007) Hospital-acquired infections related to contaminated substances. J Hosp Infect 65: 15–23.

4. Holden RA, La Hood D, Grant T, Newton-Howe J, Balerdi-Lucas C, et al. (1996) Gram-negative bacteria can induce contact lens related acute red eye (CLARE) responses. Clao J 22: 47–52.

5. Bhaledi S, Bayley E, Valeva A, Balevé I, Walker B, et al. (1996) Staphylocoecal alpha-toxin, alpha-toxin-O, and Escherichia coli hemolysis: prototypes of pore-forming bacterial cytolsins. Arch Microbiol 165: 73–79.

6. Menestrina G, Moser C, Pellet S, Welch R (1994) Secretion of Serratia liquefaciens phospholipase A is growth-phase-dependent, catabolite-repressed and regulated by protein A (PaIPa). Mol Microbiol 15: 562–568.

7. Bibb MJ, DeLong BL, Kieser T, White RL, Klenk HP, et al. (1990) Regulation of antibiotic production by the tetracycline operon of Salmonella typhimurium. Mol Microbiol 4: 355–362.

8. Wu PZ, Zhu H, Stapleton F, Hume E, Aliwarga Y, et al. (2005) Effects of alpha-toxin-deficient Shigella flexneri on the production of peripheral corneal ulceration in an animal model. Curr Eye Res 30: 63–70.

9. Marquet P, Monds KS, McCormick CC, Dixon SN, Sanders ME, et al. (2007) Cholesterol as treatment for pneumococcal keratitis: cholesterol-specific inhibition of pneumolysin in the cornea. Invest Ophthalmol Vis Sci 48: 2661–2666.

10. Hertle R, Schwarz H (2004) Serratia marcescens internalization and replication in human bladder uroepithelial cells. BMC Infect Dis 4: 16.

11. Lin CS, Hsiong JT, Yang CH, Tsai YH, So LH, et al. (2010) ResA-Blf3DC-ShIIBa as a major pathogenesis pathway in Serratia marcescens. Infect Immun 78: 4870–4881.

12. Shimata K, Ohnishi M, Iyoeda S, Gotoh N, Kozumi N, et al. (2009) The hemolytic and cytolytic activities of Serratia marcescens phospholipase A (PmLA) depend on lipoxygenase production by PmLA. Microbiol Med 9: 261.

13. Stella NA, Kalivoda EJ, O’Dee DM, Naji G, Shank RM (2008) Catabolite repression control of flagellum production by Serratia marcescens. Res Microbiol 159: 562–568.

14. Kalivoda EJ, Stella NA, Aston MA, Fender JE, Hume E, O’Dee DM, et al. (2010) AMP-dependent catabolite repression system of Serratia marcescens is growth-phase-dependent, catabolite-repressed and regulated by protein A (PaIPa). Mol Microbiol 77: 306–319.

15. Bhakdi S, Bayley H, Valeva A, Walev I, Walker-Brown K, et al. (1996) Staphylococcal protease from Serratia marcescens which produces surface active exolipids. FEMS Microbiol Lett 145: 35–40.

16. Givskov M, Molin S (1992) Expression of extracellular phospholipase from Serratia liquefaciens. J Bacteriol 175: 2160–2166.

17. Chiang SL, Rubin EJ (2002) Construction of a mariner-based transposon for shuttle vectors: a divergent approach towards a high-mannose type oligosaccharide library. Chem Commun (Camb) 47: 329–349.

18. Menestrina G, Moser C, Pellet S, Welch R (1994) Secretion of Serratia liquefaciens phospholipase A is growth-phase-dependent, catabolite-repressed and regulated by protein A (PaIPa). Mol Microbiol 15: 562–568.

19. Givskov M, Molin S (1993) Secretion of Serratia liquefaciens phospholipase A (PmLA) from Serratia marcescens. Mol Microbiol 7: 229–240.

20. Williams RP, Green JA, Rappo-Port DA (1956) Studies on pigmentation of Serratia marcescens. J Bacteriol 71: 115–120.

21. Kalivoda EJ, Stella NA, O’Dee DM, Naji G, Shank RM (2008) The cyclic AMP-dependent catabolite repression system of Serratia marcescens mediates biofilm formation through regulation of type 1 fimbriae. Appl Environ Microbiol 74: 3461–3470.

22. Wasserman HH, Keggi JJ, Mckeon JE (1962) The structure of serratamolide. J. Am chem Soc 83: 4107–4108.

23. Wasserman HH, Keggi JJ, McKeon JE (1961) Serratamolide, a metabolic produce of Serratia marcescens. J Am Chem Soc 83: 4107–4109.

24. Wasserman HH, Keggi JJ, McKeon JE (1962) The structure of serratamolide. J Am Chem Soc 84: 2978–2982.

25. Li H, Tanaka TF, Tato Y, Nakagawa Y, Matsuoka T (2005) Serratamolide gene required for surfactant sessirawtin W1 production enuates putative ampolilid synthase betaing to nonribosomal peptide synthetase family. Microbiol Immunol 49: 303–310.

26. Matsukawa T, Nagawa M, Nakagawa Y (1989) Fractal spreading growth of Serratia marcescens which produces surface active exolipids. FEMS Microbiol Lett 52: 243–246.

27. Matsuoka Y, Kaneda K, Nakagawa Y, Isu K, Harada-Hotta H, et al. (1992) A novel extracellular cyclic lipopeptide which promotes flagellum-dependent and -independent spreading growth of Serratia marcescens. J Bacteriol 174: 1769–1776.

28. Lindum PW, Anthony U, Christophersen C, Eberl L, Molin S, et al. (1998) N-Acyl-L-homoserine lactone autoinducers control production of an extracellular lipopeptide biosurfactant required for swarming motility of Serratia liquefaciens. Mol Microbiol 27: 635–648.

29. Tanikawa T, Nakagawa Y, Matsuoka T (2006) Transcriptional downregulator HexA controlling prodigiosin and sessirawtin W1 biosynthesis in Serratia marcescens. J Bacteriol 188: 387–396.

30. Shank RM, Kadouri DE, MacEachran DP, O’Toole GA (2009) A new yeast recombineering tool for bacteria. Plasmid 62: 88–97.

31. Dwivedi D, Jansen R, Molinari G, Nimtz M, Johri BN, et al. (2008) Amyloidogenic bacterial lipodepsipeptides and diacyl phosphogluconase derivatives from Serratia. J Nat Prod 71: 637–641.