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Title: The Rcs stress response system modulates *Serratia marcescens* induced inflammation and bacterial proliferation in a rabbit keratitis model

Running title: Rcs system inhibits keratitis

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ABSTRACT

In this study, we tested the hypothesis that the conserved bacterial Rcs stress response system mediates corneal pathogenesis associated with *Serratia marcescens* ocular infections. This was accomplished by modifying Rcs activity using mutant strains. These include a mutant that has a hyper-active Rcs system due to deletion of the IgaA family gene, *gumB*, and a *gumB rcsC* double mutant that is defective for Rcs signaling. The role of the Rcs system and bacterial stress response systems for microbial keratitis is not known. Here we observed that the Rcs-activated *gumB* mutant had a >50-fold reduction in proliferation compared to the wild type within rabbit corneas at 48 h, and demonstrated a notable reduction in inflammation based on inflammatory signs and proinflammatory markers measured at the RNA and protein levels. The *gumB* mutant phenotypes could be complemented by wild-type *gumB* on a plasmid and partially complemented by restoration of *shlA* cytolysin expression and elimination of capsular polysaccharide production. We observed that inactivation of the Rcs stress response system completely restored corneal virulence to the *gumB* mutant. NanoString transcriptional analysis of bacterial genes expressed during microbial keratitis demonstrated expression of *gumB*, *rcsB*, *shlA*, and three metalloprotease genes. Data suggest that the bacterial capsular polysaccharide is not necessary for infection, but capsule overexpression reduces inflammation. Together, these data indicate that GumB regulates virulence factor production through the Rcs system and this overall stress response system is a key mediator of a bacterium's ability to induce vision-threatening keratitis.

INTRODUCTION

The role for bacterial stress systems has been demonstrated as being essential for establishing wild-type levels of microbial pathogenesis in several animal models of infection (1). These include bacterial cell envelope stress response systems in both Gram-negative and Gram-positive bacteria that protect the cell envelope from external and internal host-defense factors (2). The Rcs system is well-studied in *Escherichia coli* and *Salmonella enterica* and conserved in other members of the Enterobacteriaceae (3). In *E. coli* and *S. enterica*, the Rcs system has a role in responding to envelope stress caused by membrane affecting agents including detergents and cell wall damaging compounds such as lysozyme (3). Mutational inactivation of the Rcs system has been reported to increase the virulence potential of several bacteria including *Proteus mirabilis* in a *Galleria mellonella* infection model (4), *Edwardsiella tarda* in a zebrafish model (5), and *S. marcescens* in a murine bacteremia model (6). Similarly, the increased virulence of *Yersinia pestis* compared to other *Yersinia* species is thought to be partially due to an incomplete Rcs system (3). In other models, Rcs system-defective mutants are less virulent. For example, *Citrobacter rodentium* and *E. coli rcsB* mutants were less virulent in rodent intestinal infection models (7, 8). Therefore, it is likely that bacteria tightly regulate the Rcs system during infection because it is important in regulating the expression of genes that balance host-pathogen interactions.

The Rcs system is inhibited by the inner-membrane protein, IgaA, and is essential for growth in *E. coli* and *Salmonella* species (3). The *S. marcescens* IgaA ortholog, GumB, is functionally conserved with other IgaA-family proteins, as *gumB* mutant defects can be complemented in trans by the *E. coli*, *Klebsiella pneumoniae*, and *S.*

enterica IgaA orthologs (9). However, unlike *E. coli* and *S. enterica*, IgaA-proteins are not essential for viability in *P. mirabilis* (10) or *S. marcescens* (9). It is also likely not essential in *Y. pestis*, as it has not turned up in genome wide screens for essential genes (3). Evidence from previous studies suggests that loss of *gumB* locks *S. marcescens* in a “stressed-out” state leading to phenotypes consistent with an activated stress response including loss of flagella production, reduced secretion of toxins, and excess capsular polysaccharide synthesis (9, 11). These phenotypes are reminiscent with those of bacteria in a chronic infection or in biofilms and are associated with a less acute infection (12, 13). Consistently, mice infected with *S. enterica* that coded for partial function IgaA protein were severely defective in systemic mouse infection models (14).

Although mutation of genes for IgaA-family proteins generally reduces virulence, there is reason to suggest that in some circumstances inactivation of IgaA may increase bacterial persistence and resistance to the immune system. For example, in an *in vitro* experiment in which *E. coli* was serially passaged with phagocytic cells, the surviving *E. coli* gained mutations in the *igaA* gene that increased capsule production and resistance to phagocytosis (15). Mutations in *igaA* have also been characterized among multidrug resistant clinical isolates of *E. coli* (16), together suggesting that mutation of *igaA* can aid in pathoadaptation. Consistently, the *igaA* gene was originally found in a screen for mutations that enabled *S. enterica* serovar Typhimurium to proliferate within fibroblast cells (17).

Because of the importance of IgaA-family protein GumB in *S. marcescens* proliferation in an invertebrate infection model, regulation of virulence factors, and cytotoxicity to corneal cells *in vitro* (9, 11), we tested the prediction that GumB and the

Rcs system is crucial for corneal infections. In this study, we analyze the importance of the Rcs system in *S. marcescens* keratitis using a rabbit keratitis model and measure gene expression of known and suspected virulence factor genes *in vivo*. Together, this study indicates that over activation of a bacterial stress response system and the associated shift to a chronic rather than acute-infection lifestyle remarkably reduces pathogenesis without strongly affecting bacterial proliferation.

RESULTS

GumB is necessary for *S. marcescens* proliferation and inflammation in a rabbit corneal infection model.

S. marcescens is a leading cause of contact lens associated corneal infections (18, 19). Previous results using a keratitis isolate of *S. marcescens* indicated that constitutive activation of the Rcs stress response system, through mutation of the *gumB* gene, dramatically reduces the bacterium's ability to cause cytotoxicity *in vitro* using primary human corneal cells and virulence in an invertebrate pathogenesis model (11). However, the influence of the Rcs system on bacterial pathogenesis in a keratitis model is unknown. Cultures of *S. marcescens* wild-type contact lens-associated keratitis isolate K904 (WT) and an isogenic $\Delta gumB$ mutant were grown overnight in LB medium, diluted in PBS, and ~500 CFU in 25 μ l were injected into the corneal stroma. The actual injection was 468 ± 297 for WT and 885 ± 550 for $\Delta gumB$.

At 24 and 48 hours post-inoculation, corneal and conjunctival clinical signs were examined by slit-lamp and evaluated using parameters from a modified MacDonald-Shadduck scoring system (20). At 24 hours post-inoculation, there was little difference in

clinical inflammation scores for the cornea (Figure 1A,B); however, the infiltrates were distinct. Corneas infected with the WT demonstrated a snow-flake like infiltrate in 11 out of 12 eyes, whereas the $\Delta gumB$ mutant infected corneas had globular infiltrate in 11 out of 11 corneas (Figure 1A,C).

At 48 hours, corneal inflammation was higher for eyes infected with the WT than the $\Delta gumB$ mutant (Figure 2). The corresponding corneal inflammatory scores were significantly higher ($p < 0.001$, Kruskal-Wallis with Dunn's posttest) for the WT (median 8.0, 95% CI = 7.13-8.21) compared to the $\Delta gumB$ mutant (median 3.0, 95% CI = 2.44-4.36) (Figure 3A). Corneal scores were the culmination of 0-4 for corneal opacity, area of corneal opacity, corneal vascularization, and corneal staining with fluorescein. Injection of PBS alone as a negative control resulted in a score of 0 ± 0 (Figure 2, 3A). Rabbit eyes infected with WT *S. marcescens* presented with medium to large central corneal ulcers with fluorescein staining indicating loss of the epithelium (Figure 2 and 3B). The $\Delta gumB$ mutant infected eyes either had small ulcers or no ulcer. However, the epithelial defect area did not reach significance between the WT and $\Delta gumB$ mutant.

Other inflammatory signs included as loss of corneal clarity and the presence of hypopyons, a build-up of inflammatory cells in the anterior chamber (Figure 2). Hypopyons are indicative of a high level of ocular inflammation and predictive of worse clinical outcomes (21, 22). Notably, 9 out of 12 eyes infected with the WT demonstrated a hypopyon (Figure 2 arrow) compared to 0 out of 17 for PBS injected eyes ($p = 0.0002$, Fisher's Exact test) and 1 out of 11 for $\Delta gumB$ infected eyes ($p = 0.0028$ compared to WT) (Figure 3C and Table 2). Conversely, eyes infected with the $\Delta gumB$ mutant were generally clear except for globular infiltrates (Figure 1A, 2).

Conjunctival inflammatory scores followed a similar pattern to corneal scores with WT infected eyes having significantly higher scores than the $\Delta gumB$ bacteria ($p < 0.05$, Kruskal-Wallis with Dunn's posttest), although the magnitude of the difference between WT and $\Delta gumB$ infected eyes was less extensive than for corneal inflammation. Scores for both were higher than PBS-injected eyes ($p < 0.0001$ for WT and $p < 0.05$ for $\Delta gumB$) (Figure 3D).

We assessed pro-inflammatory cytokines IL-1 β and Tnf α at the RNA level by NanoString technology in infected corneas at 24 hours post-inoculation (Fig 4A). Relative to the PBS injected eyes, and normalized by GAPDH levels, Tnf α transcript were up 4.9 fold in WT infected eyes compared to PBS infected eyes and 5.7 fold compared to $\Delta gumB$ mutant infected eyes ($p < 0.05$ by Mann-Whitney). The effect was more dramatic for IL-1 β with a 363.8-fold increase in WT injected eyes compared to PBS injected, and 26.2-fold higher in the WT infected eyes than $\Delta gumB$ infected eyes ($p < 0.05$, Mann-Whitney) (Figure 4A). Because the effect was greater for IL-1 β , we evaluated IL-1 β protein levels 48h post-inoculation by ELISA. A >1000-fold increase in IL-1 β was measured in WT infected eyes compared to PBS infected eyes ($p < 0.01$, ANOVA with Tukey's posttest) (Figure 4B). Eyes infected with $\Delta gumB$ had a 56-fold elevation in IL-1 β compared to PBS, but this did not reach significance. The $\Delta gumB$ mutant corneas had 19-fold reduced IL-1 β compared to WT infected corneas ($p < 0.01$) (Figure 4B).

The bacterial burden, at 48 hours post-infection, was 61-fold higher in the wild type infected corneas compared to those infected by the $\Delta gumB$ mutant (Figure 4C), $p < 0.05$, ANOVA with Tukey's posttest. Consistent with higher bacterial numbers and

inflammatory scores, hematoxylin and eosin (H&E) staining of corneal sections showed epithelial defects, edema, and neutrophils in the wild type infected corneas (Figure 5). By comparison $\Delta gumB$ infected corneas had intact epithelial layers and mild edema. Notably, the $\Delta gumB$ mutant infected corneas had globular infiltrates with a biofilm-like morphology (Figure 5).

The $\Delta gumB$ mutant inflammatory defect can be complemented by wild-type *gumB* on a plasmid.

Complementation analysis was performed to ensure that the $\Delta gumB$ defect was due to mutation of *gumB* and not another unknown defect in the $\Delta gumB$ mutant strain or polar effect of the deletion (Figure 6). Similar to the analysis above, the WT with an empty vector negative control injected with an average of 349 ± 11 CFU per cornea induced median corneal inflammatory scores of 7.0 (n=8, 95% CI = 4.89-8.11) at 48 h post-inoculation (Figure 6A). The $\Delta gumB$ mutant with the empty vector negative control injected with 558 ± 90 CFU per cornea caused inflammatory scores of 2.0 (n=8, CI = 1.71-5.04). Eyes were more inflamed, with a score of 7.0 (n=7, CI = 5.70-7.16) when infected with the $\Delta gumB$ mutant with wild-type *gumB* on a plasmid (370 ± 36 CFU injected per cornea) (Figure 6A), and were similar to the WT injected corneas. PBS injected corneas induced corneal scores of 0 (n=8, 95% CI = -0.34-0.84), and the WT + vector and $\Delta gumB$ + *pgumB* (wild-type *gumB* on a plasmid), but not $\Delta gumB$ with vector alone were significantly different than the PBS injection group ($p < 0.001$, Kruskal-Wallis with Dunn's posttest). A similar pattern was observed with conjunctival inflammatory scores where, all groups except the $\Delta gumB$ with empty vector group had significantly higher

conjunctival inflammatory scores compared to PBS injected eyes (Figure 6B). Images of infected eyes confirm the relatively decreased inflammation and increased clarity of corneas infected with the $\Delta gumB$ mutant compared to the other groups (Figure 6C).

The wild-type strain with the empty vector proliferated to 2.2×10^7 CFU per cornea in 48 h compared to 2.7×10^6 CFU for the $\Delta gumB$ mutant with the empty vector, a 8-fold reduction that did not reach significance ($n=8$), ($p>0.05$ by Kruskal-Wallis with Dunn's multiple comparison test). With the *gumB* plasmid in the $\Delta gumB$ mutant CFU per cornea increased to 1.4×10^7 ($n=7$), which was indistinguishable from the wild type (Figure 7A).

Corneal IL-1 β levels were reduced approximately 2-fold from 4032 ± 2308 pg/ml in WT with the empty vector control compared to the $\Delta gumB$ mutant with the vector control (2051 ± 1725 pg/ml). PBS injected eyes had 124 ± 55 pg/ml. The $\Delta gumB$ mutant with *gumB* on a plasmid had 4188 ± 1582 pg/ml. (Figure 7B). A similar trend was observed with Tnf α (Figure 7C).

Since neutrophil mediated damage is a major cause of corneal pathology during microbial keratitis we indirectly measured neutrophil infiltration by biochemically measuring myeloperoxidase (MPO) activity. A clear increase in MPO activity was measured from corneas infected with the wild-type bacteria with vector compared to PBS injected corneas (Figure 7D), $p<0.05$, by ANOVA with Tukey's posttest. The $\Delta gumB$ mutant with the same vector was defective in attracting neutrophils and was not different from the PBS-injected control group, $p>0.05$. Conversely, when the wild-type *gumB* gene was added back to the $\Delta gumB$ mutant, MPO activity was restored and significantly higher than the PBS group ($p<0.01$).

Role of capsule and ShlA cytolysin in GumB mediated corneal infections.

Mutation of IgaA-family proteins like *gumB* is noted to cause a major increase in production of extracellular polysaccharide (9). Here we used previously described isogenic variants of the *gumB* mutant that do not make capsule due to mutations in capsule biosynthetic genes *wecA* and *wza* (9) to test for the importance of over production of capsule in the reduced corneal inflammation phenotype of the $\Delta gumB$ mutant. At 24 hours the *gumB wecA* double mutant produced inflammation indistinguishable from the other groups (Figure 1), but at 48 h $\Delta gumB wza$ and $\Delta gumB wecA$ mutants induced intermediate clinical corneal scores that were elevated as compared to the $\Delta gumB$ mutant and significantly higher corneal inflammatory scores than PBS injected eyes ($p < 0.05$), whereas the $\Delta gumB$ mutant infected eyes were not significantly different from PBS injected eyes ($p > 0.05$, by Kruskal-Wallis with Dunn's posttest) (Figure 3A). Epithelial defects and the frequency of hypopyons were similar to the $\Delta gumB$ mutant (Figure 3B-C). IL-1 β levels for the *gumB wecA* mutant were intermediate compared to the $\Delta gumB$ and wild-type (Figure 4B). CFU were significantly elevated in the $\Delta gumB wza$ double mutant compared to the $\Delta gumB$ mutant (Figure 4C). H&E staining of $\Delta gumB wza$ double mutant eyes were similar to $\Delta gumB$ infected eyes, but showed intermediate levels of neutrophils and a less robust epithelial layer (Figure 5). Together, these suggest that over production of the capsule plays a role in the $\Delta gumB$ mutant keratitis defects, but it is not the only factor involved.

A previous study demonstrated that the $\Delta gumB$ mutant fails to transcribe and produce the ShlA cytolysin (11). The *shlA* cytolysin gene is expressed in an operon with

the *ShlA* transporter gene *shlB*. To test whether lack of *ShlA* biosynthesis was the reason for the $\Delta gumB$ mutant defect, we expressed the *shlBA* operon from a plasmid in the $\Delta gumB$ mutant that was previously shown to complement the $\Delta gumB$ mutant cytotoxicity defective phenotype (11). The corneal inflammatory score went from 2.0 for the $\Delta gumB$ mutant with the vector to 4.5 for the $\Delta gumB$ mutant with the *shlBA* plasmid, compared to 7 for the wild type with vector (n=8) (Figure 6A, C). A similar pattern was observed for conjunctival signs of inflammation (Figure 6B, C). However neither of these changes reached significance. Expression of *shlBA* also partially restored bacterial proliferation in the cornea, from 5×10^6 for the *gumB* mutant with the vector to 1×10^7 for the *gumB* mutant with the *shlBA* plasmid (compared to 3×10^7 for the wild type with the vector, $p > 0.05$, ANOVA with Tukey's posttest), (Figure 7A). IL-1 β and MPO levels were not clearly modified by the *shlBA* plasmids (Figure 7A,C).

The relatively mild impact of the *shlBA* plasmid may come from plasmid instability. Whereas the same plasmid back-bone was used for the vector, *gumB*, and *shlBA* plasmids, there was a notable difference in recovery of bacteria with each of the plasmids after 48 hours in the rabbit cornea. After 48 h, 0% of the bacteria had the *shlBA* plasmid, compared to 76% for the vector and 81% for the *gumB* plasmid. Despite loss of the *shlBA* plasmid in the rabbit by 48 h, ectopic expression of *shlBA* conferred intermediate phenotypes that suggest at a least minor role for *ShlA* in corneal infections.

The $\Delta gumB$ mutant keratitis phenotype is reversed through inactivation of the Rcs pathway.

Data indicates that the $\Delta gumB$ mutant has an overactive Rcs system and that mutation of *rcsB* or *rcsC* genes restore wild-type like phenotypes to the $\Delta gumB$ mutant such as ShlA-dependent cytotoxicity to corneal cells (11). This suggests that over-activation of the Rcs system may be responsible for the reduced virulence of the $\Delta gumB$ mutant. In order to test whether the Rcs system is necessary for the $\Delta gumB$ mutant keratitis phenotype, we used a double mutant defective with a null mutation in the *rcsC* gene, a necessary component of Rcs signaling. We isolated a $\Delta gumB$ mutant with a spontaneous duplication of 49 bp in the *rcsC* gene that creates an out-of-frame mutation. This allele was named *rcsC*-SPV1, for spontaneous pigmented variant number 1, and genetic experiments support that it codes for a null mutation (data not shown).

This $\Delta gumB$ *rcsC* double mutant was introduced into rabbit corneas (503 ± 448 CFU average inocula) to test whether over-activation of the Rcs system due to *gumB* mutation compared to the WT was responsible for the $\Delta gumB$ virulence defect. At 24h post inoculation, the corneas infected with the $\Delta gumB$ *rcsB* double mutant had infiltrates similar to the WT, with 12 out of 12 infiltrates having a snow-flake like morphology (Figure 1A, C). At 48 h the corneal scores were indistinguishable between the $\Delta gumB$ *rcsC* and WT (Figure 2 and 3A), and this was reflected in restored IL-1 β levels ($p < 0.05$, ANOVA with Tukey's posttest), (Figure 4B) the presence of hypopyons/hypopia (Figure 3C), and loss of corneal epithelia and numerous infiltrating neutrophils (Figure 5). CFU were similarly restored to the $\Delta gumB$ mutant by inactivation of the Rcs system ($p < 0.05$ ANOVA with Tukey's posttest, Figure 4C). Together, this data suggests that the Rcs system is a key mediator of *S. marcescens* corneal pathogenesis. Specifically, activation

of the Rcs system reduces virulence and elimination of the Rcs system increases virulence to at least WT levels.

***S. marcescens* gene expression during microbial keratitis is regulated by GumB.**

S. marcescens gene expression in the rabbit cornea was determined 24 hours post-infection using NanoString technology with a target set of 70 bacterial genes based on our previous studies, selected candidate virulence factors, and results of a preliminary RNA-Sequencing experiment performed using cultures grown in LB medium (to be described elsewhere). In two different experiments, eyes were infected with the $\Delta gumB$ mutant (n=4) and the WT (n=5). Of the 70 target genes, 39 were expressed above background levels by the WT while in the rabbit cornea (Table 3). Among the most highly expressed in the cornea included serralyisin metalloproteases *prtS*, *slpB*, and *slpE*, TLR5-ligand flagellin, and importantly for this study genes from the Rcs system (*gumB* and *rcsB*) and the *shlA* cytolysin genes (Table 4).

Twenty-four genes were measured from WT but not $\Delta gumB$ mutant infected eyes: these include motility related genes, a number of transcription factors, the serralyisin protease and the a type I secretion system responsible for secretion of serralyisin-family proteases, hemolysin associated genes including the *shlBA* operon, the *swrW* gene that codes for a nonribosomal peptide synthetase that generates the hemolytic biosurfactant serratamolide (also known as serrawettin W1) (Table 4). Conversely, three bacterial genes were detected from the $\Delta gumB$ infected eyes, but not WT infected eyes, including a capsule biosynthetic gene and a predicted osmotic stress induced lipoprotein (Table 4).

Ten other genes had a ± 2 -fold change in expression between the WT and $\Delta gumB$ mutant. These include two genes with higher expression from the WT: serralyisin-like metalloprotease SlpE and a type I pilus gene. The 8 genes expressed at higher levels from the *gumB* mutant include several stress related genes including *crp*, *oxyR*, and *rcsB* (Table 4). Importantly, these data verify that genes chosen in this study for analysis – those for the capsule, ShlA cytolysin, and Rcs system are expressed during corneal infections.

DISCUSSION

Microbial keratitis is generally treatable using antibiotics, but still leads to vision loss due to rapid growth of the organism before treatment and continued damage to corneal tissue by inflammatory cells, even after the death of the microbes (23-27). In human patients, *S. marcescens* is a leading cause of contact-lens associated keratitis and can lead to vision loss and in rare cases, blindness (18, 19). *S. marcescens* has been shown to readily infect the corneas of rabbits (28-30). It is highly inflammatory, and its lipopolysaccharide and purified proteases are sufficient to induce keratitis (29, 31-34). In a mouse keratitis model, it was demonstrated that the cornea requires TLR4, TLR5, IL-1R and MyD88 to effectively clear *S. marcescens* infections (35), supporting that flagellin and LPS are important pathogen associated molecular patterns (PAMPS) of *S. marcescens* in the cornea. Nevertheless, the only *S. marcescens* gene tested for a role in keratitis using mutant bacterial strains thus far has been the transcription factor *eepR*, which was important for wild-type levels of bacterial proliferation within the rabbit cornea (36).

The purpose of this study was to establish whether the IgaA-family protein GumB from *S. marcescens* and the related Rcs stress response system play a role in microbial pathogenesis during bacterial keratitis. Data from this study demonstrated that a clinical keratitis isolate of *S. marcescens* with constitutively active Rcs system ($\Delta gumB$ mutant) was defective in causing corneal inflammation judged by corneal inflammatory signs, as the *gumB* mutant had a 62.5% reduction in corneal inflammatory scores. These correlate with reduced production of pro-inflammatory cytokines IL-1 β and Tnf α and neutrophil infiltration into the cornea. Similarly, loss of *gumB* reduced the ability of the bacterium to proliferate in the cornea, with a 1.875-Log₁₀ (98%) loss of CFU in *gumB* infected corneas relative to those infected by the WT. Complementation of the *gumB* mutation with wild-type *gumB* reversed the *gumB* mutant defects. Together, these data solidify the role for the Rcs system as having an important role in ocular bacterial host-pathogen interactions.

WT and the $\Delta gumB \Delta rcsC$ strain, with an inactivated Rcs system, induced infiltrates with a snow-flake appearance at 24 h that were reminiscent of the presentation of infectious crystalline keratopathy, in which microbes are thought to form biofilms that spread along the lamellar planes of the corneal stroma that lead to a crystalline or snow-flake like pattern (37). This suggests that WT and Rcs defective strains penetrate into the cornea along the lamellar planes, whereas the Rcs-overexpressing $\Delta gumB$ mutant does not. The difference in spreading may be due to lack of swimming and swarming motility previously demonstrated for the $\Delta gumB$ mutant in *S. marcescens* and other members of the Enterobacteriaceae (9, 38, 39). The *in vivo* expression data presented here support the previous *in vitro* study that flagella and swarming motility-required genes, *fliC* and *swrW* are expressed by the WT but undetectable from the $\Delta gumB$ mutant. Additionally,

expression data suggests highly reduced expression of bacterial proteases by the $\Delta gumB$ mutant. The *slpB* and *slpE* proteins are serralysins, a class of metalloproteases that have been implicated in the spread of *S. marcescens* in the cornea and necrosis and liquefaction of the cornea (28, 29, 40, 41).

Genetic experiments provided insight into the reduced pathogenesis of the $\Delta gumB$ mutant. Our results suggest that overexpression of the *S. marcescens* capsular polysaccharide contributes to the $\Delta gumB$ mutant phenotypes as revealed by intermediate corneal inflammatory phenotypes in the $\Delta gumB$ mutant in which the capsular polysaccharide genes *wecA* and *wza* were mutated (both being required for the extracellular capsule). However, the capsule was clearly not necessary for keratitis as the non-capsulated *gumB wecA* and *gumB wza* mutants both induced ulcers and proliferated in the cornea and caused more inflammation than the hyper-capsulated $\Delta gumB$ mutant. Notably, gene expression of the capsule was not measured in the WT bacteria during infection, further supporting the notion that it is not required for *S. marcescens* to cause keratitis in our model. In fact, the necessity of bacterial capsules during corneal infection has only been studied using isogenic strains with the Gram-positive bacterium *Streptococcus pneumoniae*. Marquart and colleagues demonstrated that *S. pneumoniae* has to be alive to cause ocular inflammation and that a capsule mutant was equally virulent to the capsulated wild type in a rabbit keratitis model (42, 43). In our study, the capsule may prevent the immune system from recognizing the bacteria surface PAMPS or by defending the bacteria against phagocytosis, leading to the reduced inflammation observed with the $\Delta gumB$ mutant.

A role was also evaluated for the ShlA cytolysin. ShlA, a key virulence factor for *S. marcescens* in several models, has not been tested in *in vivo* ocular models. Because expression of *shlA* and its transporter *shlB* is severely reduced in the *gumB* mutant, and *gumB* mutants generated a reduced keratitis relative to the wild type, testing a role for ShlA in the *gumB* keratitis defect was warranted. Here we observed that ectopic expression of *shlBA* in the *gumB* mutant partially restored corneal inflammatory signs of keratitis as well as proinflammatory cytokines. This occurred even though there was a clear selection for loss of the *shlBA* plasmid during the course of infection, relative to the empty vector. This suggests that the role of *shlBA* was underrepresented in this study, but the clear intermediate phenotypes of the *gumB* mutant with multicopy expression of *shlBA* supports the role for ShlA in ocular infections.

Whereas activation of the Rcs system through mutation of *gumB* led to reduced virulence, the inactivation of the Rcs system in the $\Delta gumB$ *rscC* double mutant restored virulence to equal or higher than the WT. Similarly, in a bacteremia model *rscB* mutants caused higher mortality of mice in a capsule-independent manner (6). Anderson and colleagues suggested that elevated *shlBA* expression in the *rscB* mutant may be responsible for the increased virulence (6). This was concluded partially based on previous evidence describing that *rscAB* mutants, which have higher *shlBA* expression, caused higher mortality rates and more severe pathogenesis in a rat lung infection model (44). Together, these suggest that in *S. marcescens* the Rcs system may act to attenuate virulence, perhaps resulting in reduced immune activation and mortality of the host. Interestingly, species of the Enterobacteriaceae reported to have higher virulence in Rcs defective strains, all have ShlA orthologs (*E. tarda*, *P. mirabilis*, and *S. marcescens*),

whereas those species reported to have reduced virulence conferred by *rsc* mutations typically do not have ShlA orthologs (*E. coli*, *C. rodentium* and *S. enterica*).

In a previous study the $\Delta gumB$ mutant was nearly completely avirulent using an invertebrate infection model with an approximately 4-Log₁₀ reduction in CFU compared to the WT (11), whereas in the rabbit cornea they are reduced in proliferation, but just under 2-Log₁₀ CFU. In both cases, however, the $\Delta gumB$ mutant was reduced in microbial pathogenesis, suggesting that the *G. mellonella* model was predictive of the mammalian model. The $\Delta gumB$ mutant has similar growth characteristics in minimal and rich media (9) and *G. mellonella* homogenates (11), suggesting that the observed defect is not due to reduced growth.

The constitutive expression of the Rcs system, through mutation of *gumB*, led to an overall reduction in bacterial CFU per cornea, but also highly reduced neutrophil infiltrates, correlating with reduced PAMP production and presentation. The bacteria developed into globular intrastromal infiltrates that are reminiscent of biofilms, which are typically more tolerant of host defenses and antibiotic therapy. The *in vivo* factors that activate the Rcs system are not fully understood, but include lysozyme and cationic peptides (3). Both of these are key features of the innate defenses of the ocular surface (45). It is likely that after infection is established, it would benefit *S. marcescens* to activate the Rcs system, which would shut off PAMP and toxin production and increase capsule production. However, during early stages, it is probable that some Rcs-inhibited factors contribute to establishing infection such as flagellin, ShlA, and serralysin family proteases. Thus, a well-coordinated balance in activity of the Rcs system is likely required for effective pathogenesis by *S. marcescens*. Consistent with this idea, the key

Rcs system transcription factor, RcsB, is not only modulated in activity by its cognate histidine kinase, it also has numerous binding partners that fine tune its activity, such as RcsA (3). In summary, this study concludes the *S. marcescens* Rcs system is a major regulator of host-pathogen interactions during corneal infections.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Bacterial strains are listed in Table 1 and were maintained at -80°C. Bacteria were streaked to single colonies and individual colonies were used to inoculate LB medium (46). Gentamicin was used at 10 µg/ml to select for plasmid maintenance.

The $\Delta gumB$ *rscC* isolate (strain CMS4141), was isolated as a genetic suppressor of $\Delta gumB$ pigment phenotype (to be described elsewhere). The *rscC* mutant gene was cloned in vector pMQ132 using primers and methods previously described (11). The mutation in *rscC*, noted as *rscC-SPVI*, was determined using Sanger sequencing of the *rscC* gene (University of Pittsburgh Genomics Research Core).

Microbial keratitis studies. The present study conformed to the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research. The study was approved by the University of Pittsburgh Institutional Animal Care and Use Committee (IACUC Protocol 16098925B). For infection experiments, bacteria were grown overnight at 30°C with aeration, normalized by optical density (600 nm), and adjusted to ~500 CFU in 25 µl, and injected into right eye of New Zealand White rabbits. Actual inocula colony counts were determined using the EddyJet 2 spiral plating system (Neutec Group Inc., Farmingdale,

NY) on 5% trypticase soy agar with 5% sheep's blood plates. The plates were incubated overnight at 30°C and the colonies were using the automated Flash and Grow colony counting system (Neutec Group). At 24 and 48 h post-injection, eyes were evaluated for ocular signs of inflammation using a slit-lamp according to a modified McDonald-Shadduck grading system (20). After sacrifice, corneas were harvested with a 10 mm trephine, homogenized with an MP Fast Prep-24 homogenizer using lysing matrix A tubes (MP Biomedicals), and bacteria were enumerated by dilution plating as described above. Homogenates were clarified by centrifugation (10 minutes at 16,000-21000 x g) and stored at -20°C until used for ELISA analysis for pro-inflammatory markers following manufacturers protocols and MPO analysis (47, 48). For IL-1 β analysis, Sigma-Aldrich product RAB1108 was used, and for Tnf α measurement, R&D Systems DuoSet DY5670 was used.

RNA for the nanoString nCounter platform was harvested from corneas following the protocol of Xu, et al. (49) with a few exceptions. Corneas were removed with a trephine and immediately frozen in liquid nitrogen and stored at -80°C until RNA was harvested. Corneas were homogenized as described above. Following phenol chloroform extraction and ethanol precipitation, RNA was run through a ZymoResearch RNA concentrator column, adjusted to 800 ng/ μ l and sent to the University of Pittsburgh Genomics Research Core for nCounter analysis. Results were analyzed using nSolver software and results were uploaded to Gene Expression Omnibus (accession number GSE155486).

For histological analysis, corneas were fixed in 4% paraformaldehyde in phosphate buffered saline (pH 7.4) and embedded in paraffin. Central corneal sections

were generated (5 μ m) and stained with hematoxylin and eosin staining. Slides were imaged using the Olympus Provis AX-70 microscope with MagnaFire 2.1 software.

Statistical analysis. Data was analyzed using GraphPad Prism Software. Non-parametric analysis was used to analyzed inflammatory scores by Mann-Whitney or Kruskal-Wallis analysis, and ANOVA with Tukey's posttest or Student's T-tests were used to analyze other experimental data.

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Table 1. Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
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Strains

K904	contact lens associated keratitis isolate (WT)	(50)
CMS4001	K904 $\Delta gumB$	(9)
CMS4619	K904 $\Delta gumB$ <i>wecA</i> capsule deficient	(9)
CMS4214	K904 $\Delta gumB$ <i>wza</i> capsule deficient	(9)
CMS4141	K904 $\Delta gumB$ <i>rscC</i> -SPV1 Rcs system defective	This study

Plasmids

pMQ132	broad host range shuttle vector <i>ori</i> pBBR1	(51)
pMQ480	pMQ132 with <i>gumB</i> gene under control of P_{lac}	(9)
pMQ541	pMQ132 with <i>shlBA</i> operon under control of P_{nptII}	(11)
pMQ615	pMQ132 with <i>rscC</i> gene under control of P_{lac}	(11)
pMQ616	pMQ132 with <i>rscC</i> -SPV1 allele under control of P_{lac}	This study

Table 2. Hypopyons were less prevalent in $\Delta gumB$ infected eyes.

	Hypopyon ^a	No Hypopyon ^b	P-value vs WT ^c	P-value vs PBS ^c
PBS	0	11	0.0001	NA
WT	10	2	NA	0.0001
$\Delta gumB$	1	10	0.0006	1.0000
$\Delta gumB wecA$	2	7	0.0092	0.1895
$\Delta gumB wza$	1	11	0.0006	1.0000
$\Delta gumB rcsC$	10	2	1.0000	0.0001

^{a-b} Number of eyes with or without hypopyons or extensive fibrin in the anterior chamber.

^c Two tailed Fisher's Exact test, NA = not applicable, significant differences in bold text.

Table 3. *S. marcescens* genes expressed bacterial during keratitis 24 h post-infection

Gene^a	ORF^b	Predicted function	Relative expression^c	SD
<i>hfq</i>	SMADB11_4482	RNA-binding protein	2.52	0.38
<i>hypo.</i>	SMADB11_3915	PapA pilin	2.51	0.78
<i>slpE</i>	not in DB11	metalloprotease	1.42	0.52
<i>hypo.</i>	SMADB11_0909	fimbrial protein	0.90	0.44
<i>fliC</i>	SMADB11_2186	flagellin	0.76	0.42
<i>rscB</i>	SMADB11_2663	stress response	0.63	0.14
<i>hypo.</i>	SMADB11_1627	metallo-beta lactamase	0.62	0.13
<i>slpB</i>	SMADB11_1367	metalloprotease	0.62	0.23
<i>rscA</i>	SMADB11_2329	stress response	0.54	0.16
<i>crp</i>	SMADB11_3828	transcriptional regulator	0.53	0.15
<i>lipB</i>	SMADB11_0865	type 1 secretion	0.50	0.12
<i>lon</i>	SMADB11_1035	protease, SOS response	0.49	0.16
<i>hypo.</i>	SMADB11_1349	CigR-like membrane protein	0.45	0.09
<i>hypo.</i>	SMADB11_1378	sigma-fimbriae	0.39	0.16
<i>hypo.</i>	SMADB11_0362	predicted Hha ortholog	0.38	0.11
<i>oxyR</i>	SMADB11_3997	stress response regulator	0.36	0.10
<i>priS</i>	SMADB11_4311	metalloprotease	0.35	0.03
<i>pigP</i>	SMADB11_3464	2° metabolite regulator	0.34	0.07
<i>hypo.</i>	SMADB11_3973	GGDEF domain protein	0.33	0.07
<i>shlA</i>	SMADB11_3737	ShlA cytolysin	0.32	0.09
<i>flhD</i>	SMADB11_2238	virulence regulator	0.32	0.11

716	<i>mreB</i>	SMADB11_3662	cell division and shape	0.31	0.07
717	hypo.	not in Db11	ShlA-like protein	0.25	0.12
718	<i>pckA</i>	SMADB11_1364	PEP carboxykinase	0.24	0.09
719	<i>chiB</i>	SMADB11_2875	chitinase B	0.23	0.07
720	hypo.	SMADB11_2996	LasB-like peptidase	0.23	0.01
721	<i>gumB</i>	SMADB11_3859	stress response	0.22	0.12
722	<i>gyrA</i>	SMADB11_2665	DNA gyrase	0.22	0.05
723	hypo.	SMADB11_4188	chemotaxis	0.21	0.06
724	<i>shlB</i>	SMADB11_3736	cytolysin transporter	0.21	0.07
725	<i>ssp2</i>	SMADB11_3962	serine protease	0.21	0.15
726	<i>ndpA</i>	SMADB11_2584	nucleoid-associated protein	0.21	0.06
727	<i>srfA</i>	SMADB11_3925	putative virulence regulator	0.20	0.12
728	<i>swrW</i>	not in Db11	serratamolide biosynthesis	0.19	0.10
729	hypo.	SMADB11_3490	GntR-family regulator	0.18	0.08
730	hypo.	SMADB11_1194	UmoD-like protein	0.18	0.10
731	hypo.	SMADB11_1052	fimbrial protein	0.17	0.06
732	<i>chiA</i>	SMADB11_4243	chitinase A	0.15	0.05
733	hypo.	SMADB11_2159	invasin-like protein	0.14	0.07

734 a. gene name based on annotation and similarity to known genes. Hypo. Indicates a
735 hypothetical gene

736 b. ORF number for reference strain Db11 is given.

737 c. Mean expression normalized to housekeeping gene *rpsA*, that was set to 1.0.

738 Genes were only included if expressed in at least 3 of the 5 corneas.

Table 4. Differentially expressed bacterial genes during keratitis 24 h post-infection

Gene	ORF	Predicted function	WT/ $\Delta gumB$	p-value
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Differentially expressed genes:

<i>fimA</i>	SMDB11_3916	pilin	5.1	0.006
<i>slpE</i>	not in Db11	serralyisin metalloprotease	3.1	0.045
hypo.	SMDB11_1194	UmoD-like protein	-9.8	0.001
hypo.	SMADB11_1349	CigR-like membrane protein	-39.7	0.137
hypo.	SMDB11_1627	metallo-beta-lactamase	-42.5	0.044

Gene detected in $\Delta gumB$ infected eyes only:

Gene	ORF	Predicted function	Relative expression	SD
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<i>osmB</i>	SMADB11_1637	osmotically induced lipoprotein	2.38	2.94
<i>wza</i>	not in Db11	capsule biosynthesis	0.61	0.60
hypo	SMADB11_4725	glycosyltransferase	0.51	0.48

Gene detected in WT infected eyes only:

Gene	ORF ^a	Predicted function	Relative expression	SD
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hypo. ^b	SMADB11_0909	fimbrial protein	0.90	0.44
<i>fliC</i>	SMADB11_2186	flagellin	0.76	0.42
<i>recA</i>	SMADB11_0158	recombination protein	0.54	0.16
<i>lipB</i>	SMADB11_0865	type I secretion	0.50	0.12
<i>lon</i>	SMADB11_0344	protease, SOS response	0.49	0.16
hypo.	SMADB11_0362	predicted hemolysin modulator	0.38	0.11

762	<i>prtS</i>	SMADB11_4311	serralysin metalloprotease	0.35	0.03
763	hypo.	SMADB11_3973	GGDEF domain protein	0.33	0.07
764	<i>shlA</i>	SMADB11_3737	ShlA cytolysin	0.32	0.09
765	<i>flhD</i>	SMADB11_2238	virulence regulator	0.32	0.01
766	<i>mreB</i>	SMADB11_3662	cell division and shape	0.31	0.07
767	hypo.	not in Db11	ShlA-like protein	0.25	0.12
768	<i>chiB</i>	SMADB11_2875	chitinase B	0.23	0.07
769	hypo.	SMADB11_2996	LasB-like peptidase	0.23	0.01
770	<i>gumB^c</i>	SMADB11_3859	stress response	0.22	0.12
771	hypo.	SMADB11_4188	chemotaxis	0.21	0.06
772	<i>shlB</i>	SMADB11_3736	cytolysin transporter	0.21	0.07
773	<i>ssp2</i>	SMADB11_3962	serine protease	0.21	0.15
774	<i>ndpA</i>	SMADB11_2584	nucleoid-associated protein	0.21	0.03
775	<i>srfA</i>	SMADB11_3925	putative virulence regulator	0.20	0.12
776	<i>swrW</i>	not in Db11	serratamolide biosynthesis	0.19	0.10
777	hypo.	SMADB11_3490	GntR-family regulator	0.18	0.08
778	hypo.	SMADB11_1052	fimbrial protein	0.17	0.06
779	<i>chiA</i>	SMADB11_4243	chitinase A	0.15	0.05

- 780 a. ORF number for reference strain Db11 is given.
- 781 b. Hypo. Abbreviation for hypothetical
- 782 c. The *gumB* gene is not present in the Δ *gumB* mutant.

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Figure Legends

Figure 1. Rcs status of bacteria influences infiltrate morphology during *S. marcescens*

corneal infections. A. Representative images of infected eyes at 24 h post-infection. **B.**

Median values and interquartile ranges for clinical signs of inflammation 24 h after infection.

C. Representative images of typical infiltrate types.

Figure 2. GumB is required for wild-type inflammation during *S. marcescens* corneal

infections. Representative images of infected eyes at 48 h post-infection. Fluorescein staining

represents epithelial damage. The arrows indicate hypopyons.

Figure 3. GumB and the Rcs system dictate the ability of *S. marcescens* to induce

inflammation during corneal infection. Median values and interquartile ranges for clinical

signs of inflammation 48 h after infection for **A.** cornea and **D.** conjunctiva. The asterisks

indicate significances by Kruskal-Wallis with Dunn's post-test. $\ast = p < 0.05$, $\ast\ast\ast = p < 0.001$,

$\ast\ast\ast\ast = p < 0.0001$. **B.** Average and standard deviation of epithelial defects. $\ast\ast\ast\ast = p < 0.0001$,

ANOVA with Tukey's post-test. **C.** Enumeration of eyes with hypopyon.

Figure 4. GumB mediates inflammation and proliferation within the rabbit cornea. A.

Median values and interquartile ranges are shown of RNA transcripts normalized by GAPDH

RNA levels from corneas 24 hours after infection. The asterisk indicates $p < 0.05$ WT versus

$\Delta gumB$ by Mann-Whitney test ($n = 3-5$ per group). **B.** Mean and standard deviation of IL-1B

protein levels measured by ELISA 48 h post infection ($n = 8-9$ per group). $\ast\ast = p < 0.01$

compared to WT using ANOVA with Tukey's post-test. **C.** Mean and standard deviation of

CFU per cornea at 48 h post infection. Asterisks indicate $p < 0.05$ differences by ANOVA with Tukey's test.

Figure 5. Histological analysis of *S. marcescens* infected corneas at 48 h. Representative images of hemotoxylin and eosin stained corneal sections of eyes at 48 h post-infection. Large neutrophil infiltrates were observed in the WT and $\Delta gumB$ *rscC* infected eyes. The objective magnification is indicated. Black arrows indicate epithelial defects; blue arrows indicate globular infiltrates in $\Delta gumB$ mutant-infected corneas; orange arrows indicate neutrophils.

Figure 6. Complementation of $\Delta gumB$ and multicopy expression of cytolyisin gene *shlA*. Median values and interquartile ranges are shown for corneal (A) and conjunctival (B) inflammatory scores at 48 hours after infection. Significant differences from the $\Delta gumB$ + vector group are indicated by asterisks as determined by Kruskal-Wallis analysis with Dunn's post-test. *= $p < 0.05$, ***= $p < 0.001$, ****= $p < 0.0001$, $n = 7-8$ per group. C. Representative images of infected eyes at 48 h post-infection. Fluorescein staining represents epithelial damage. Black arrows indicate hypopyons.

Figure 7. GumB mediates inflammation proliferation within the rabbit cornea. A. Mean and standard deviation CFU values per cornea for 48 hours after infection. **B-C.** Mean and standard deviation of IL-1B (B) and TNF α (C) protein levels measured by ELISA 48 h post infection ($n = 7-8$ per group). *** = $p < 0.001$ compared to the PBS group by ANOVA with Tukey's post-test for B, and **= $p < 0.01$. **D.** Mean and standard deviation of MPO levels per

831 cornea at 48 h post infection. Asterisks indicate $p < 0.05$ differences by ANOVA with Tukey's
832 test.

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Figure 1. Rcs status of bacteria influences infiltrate morphology during *S. marcescens* corneal infections. **A.** Representative images of infected eyes at 24 h post-infection. **B.** Median values and interquartile ranges for clinical signs of inflammation 24 h after infection. **C.** Representative images of typical infiltrate types.

Figure 2. GumB is required for wild-type inflammation during *S. marcescens* corneal infections. Representative images of infected eyes at 48 h post-infection. Fluorescein staining represents epithelial damage. The arrows indicate hypopyons.

Figure 3. GumB and the Rcs system dictate the ability of *S. marcescens* to induce inflammation during corneal infection. Median values and interquartile ranges for clinical signs of inflammation 48 h after infection for **A.** cornea and **D.** conjunctiva. The asterisks indicate significances by Kruskal-Wallis with Dunn's post-test. $\ast = p < 0.05$, $\ast\ast\ast = p < 0.001$, $\ast\ast\ast\ast = p < 0.0001$. **B.** Average and standard deviation of epithelial defects. $\ast\ast\ast\ast = p < 0.0001$, ANOVA with Tukey's post-test. **C.** Enumeration of eyes with hypopyon.

Figure 4. GumB mediates inflammation and proliferation within the rabbit cornea. A. Median values and interquartile ranges are shown of RNA transcripts normalized by GAPDH RNA levels from corneas 24 hours after infection. The asterisk indicates $p < 0.05$ WT versus $\Delta gumB$ by Mann-Whitney test ($n = 3-5$ per group). **B.** Mean and standard deviation of IL-1 β protein levels measured by ELISA 48 h post infection ($n = 8-9$ per group). ** = $p < 0.01$ compared to WT using ANOVA with Tukey's post-test. **C.** Mean and standard deviation of CFU per cornea at 48 h post infection. Asterisks indicate $p < 0.05$ differences by ANOVA with Tukey's test.

PBS

WT

 $\Delta gumB$ $\Delta gumB wza$ $\Delta gumB rcsC$

10 x

60 x

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