Biologically active pigment and ShlA cytolysin of *Serratia marcescens* induce autophagy in a human ocular surface cell line

Kimberly M. Brothers, Nicholas A. Stella and Robert M. Q. Shanks*

**Abstract**

**Background:** The cellular process of autophagy is essential for maintaining the health of ocular tissue. Dysregulation of autophagy is associated with several ocular diseases including keratoconus and macular degeneration. It is known that autophagy can be used to respond to microbial infections and that certain microbes can exploit the autophagic process to their benefit. In this study, a genetic approach was used to identify surface-associated and secreted products generated by the opportunistic pathogen *Serratia marcescens* involved in activation of autophagy.

**Methods:** A recombinant human corneal limbal epithelial cell line expressing a LC3-GFP fusion protein was challenged with normalized secretomes from wild-type and mutant *S. marcescens* derivatives. LC3-GFP fluorescence patterns were used to assess the ability of wild-type and mutant bacteria to influence autophagy. Purified prodigiosin was obtained from stationary phase bacteria and used to challenge ocular cells.

**Results:** Mutations in the global regulators eepR and gumB genes highly reduced the ability of the bacteria to activate autophagy in corneal cells. This effect was further narrowed down to the secreted cytolysin ShlA and the biologically active pigment prodigiosin. Purified prodigiosin and ShlA from *Escherichia coli* further supported the role of these factors in activating autophagy in human corneal cells. Additional genetic data indicate a role for flagellin and type I pili, but not the nuclease, S-layer protein, or serratamolide biosurfactant in activation of autophagy.

**Conclusions:** This work identifies specific bacterial components that activate autophagy and give insight into potential host-pathogen interactions or compounds that can be used to therapeutically manipulate autophagy.

**Keywords:** Autophagy, Bacteria, Keratitis, Ocular surface, Epithelium, Cornea, Prodigiosin, Cytolysin, Flagella, Fimbriae

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Background
Cells use autophagy to eliminate waste products such as damaged organelles and proteins in order to enhance survival during periods of starvation. Autophagy dysregulation has been linked to many diseases including those of the eye [1–7]. Therefore therapeutic control of autophagy has been suggested for treatment of cancer, metabolic diseases, neurodegenerative disorders, for management of cardiovascular aging, and even for treatment of corneal infections [8–10].

The role of autophagy in the cornea is less well understood, but it is clear that autophagy plays a role in HSV-1 infection and keratoconus [3, 5, 11]. A recent study measured activation of autophagy in mouse corneas following infection with the fungus Aspergillus fumigatus and positively correlated autophagy with the severity of infectious pathology [12]. Similarly, data from a study using the bacterium Pseudomonas aeruginosa, suggest that it benefits from activating autophagy as a means of escaping extracellular killing in macrophages [13]. However, in general, activation of autophagy is thought to protect cells from microbial infection [14, 15]. It is known that a few bacterial proteins such as TlpE from P. aeruginosa, bacterial macrolide, rapamycin, TLR-ligands, and proinflammatory cytokines can activate autophagy [15–17], but knowledge of the scope of infectious components that activate autophagy is limited [15].

Our previous work has demonstrated that sterile culture filtrates (secretomes) of a number of ocular pathogens can activate autophagy in a human corneal limbal epithelial cell line [18], impede cell migration and wound closure [19], and cause cellular death in a bacterial-strain dependent manner [20, 21]. These included secretomes gram positive bacteria such as Staphylococcus aureus and gram negative bacteria including Serratia marcescens [18]. The secreted or shed bacterial components detected by the corneal cells that activate autophagy were not determined. In this study we took advantage of our collection of S. marcescens defined mutants to identify bacterial factors that induce autophagy in corneal cells.

Methods
Analysis of autophagy induced by keratitis isolates
Bacterial stocks (Table 1) were stored at −80 °C and single colonies were obtained on lysogeny broth (LB) agar. Colonies were grown in LB at 30 °C for ~18 h with aeration on tissue culture rollers. Where noted, bacteria were grown with L-arabinose at 1 mM for controlled expression of genes. Secretomes were prepared by normalizing overnight cultures to OD_{600} = 2.0, removal of bacteria by centrifugation at 14,000 rpm and filtration through a 0.22 μm filter (Millex PVDF). Normalized secretomes were added to HCLE cells at a ratio of 500 μl

| Strain or plasmid | Description | Reference or source |
|-------------------|-------------|---------------------|
| Top10             | E. coli laboratory strain | ThermoFisher        |
| PIC3611           | Serratia marcescens wild-type strain | Presque Isle Cultures |
| K904              | S. marcescens keratitis isolate | [22] |
| CMS1722           | PIC3611 with pMQ262 (L-arabinose inducible pig) | [23] |
| CMS2096           | PIC3611 ΔpigA | [24] |
| CMS2097           | PIC3611 ΔeepR | [25] |
| CMS2229           | K904 pigD:tn | This study |
| CMS2232           | K904 swrW:tn | [26] |
| CMS2904           | K904 ΔeepR | [25] |
| CMS3559           | K904 nuCA:tn | This study |
| CMS3900           | K904 filC:pMQ192 | [27] |
| CMS4001           | K904 ΔgumB | [28] |
| CMS4225           | K904 fimC:pMQ167 | [27] |
| CMS4236           | K904 ΔshIB | [20] |
| CMS4334           | K904 shIB:tn | [20] |
| CMS4413           | K904 ΔolaA | [29] |
| CMS4773           | K904 ΔshIB pig:tn | This study |
| Plasmids          | expression vector with L-arabinose inducible promoter | [30] |
| pMQ125            | pMQ492 with shIB operon from S. marcescens | [20] |
per 1 ml of tissue culture medium (KSFM) and incubated at 37 °C + 5% CO₂ for 3 h. In some cases secretomes were further diluted 2-fold (OD₆₀₀ = 1.0) due to excessive cytotoxicity as noted in the text. The autophagy inhibitor 3-methyladenine (3MA) was added to culture media at 5 mM, one hour prior to challenge with secretomes as previously described [18, 31].

To analyze autophagy, LC3-GFP HCLE cells [18] were imaged with 60X magnification on an Olympus IX-81 inverted confocal microscope with Fluoview imaging software. The LC3-GFP cells were generated by lentiviral transduction of the human corneal limbal epithelial cell line from Ilene Gipson [32]. Image J (NIH) was used to quantify images without any image adjustment. Autophagy levels were quantified following recommendations of Klionsky et al. [33] in which the standard deviation of fluorescent pixel intensity of a cell is divided by its mean pixel intensity of the cell. Two to three fields per treatment condition were imaged. The experiment was repeated on at least two different days and at least 50 cells were analyzed per group. The data was averaged from all fields taken per experiment and graphed using GraphPad prism. One way ANOVA with Tukey’s post hoc analysis was used to determine statistical significance at P < 0.05 unless otherwise stated.

**Generation of new mutants strains**

Additional mutants (Table 1) were generated by transposon mutagenesis using the pSC189 mariner transposon delivery system as previously described [34, 35]. Transposons were mapped by using the method of Chiang, et al. [34] To identify nuclease defective mutants, libraries of mutants were transferred from 96-well plates to DNase detection agar (BD Difco) and plates were screened for loss of nuclease zones after 16–20 h of incubation at 30 °C around individual colonies. The transposon was mapped to 89 bp upstream of the nucA open reading frame and results in an almost complete loss of secreted nuclease activity (data not shown). Mutations in the prodigiosin biosynthetic operon were obtained by visually screening mutant libraries of strain K904 and K904 ΔshlB for loss of pigment. Transposon insertions were mapped to base pair 2451 in the K904 strain background and to base pair 1075 of pigE in the ΔshlB strain background.

**Purification of prodigiosin**

Wild-type strain PIC3611 and an isogenic ΔpigA strain were grown overnight in LB broth with aeration. The ΔpigA mutant does not make prodigiosin and served as a negative control. Bacteria were adjusted to OD₆₀₀ = 4, aliquots (5 ml) were pelleted by centrifugation (7000 RPM for 10 min), and supernatants were removed. To extract prodigiosin, bacterial cells were suspended in 100 μl of acidified ethanol (2 ml of 2 M HCl in 98 ml of 95% ethanol) and incubated for one hour with periodic vortexing. Samples were further purified with hydroxyapatite resin. Columns were packed with hydroxyapatite resin (BioRad #16260), equilibrated with acidified ethanol, and samples were run through the columns with acidified ethanol. The mock purification sample from the ΔpigA culture was collected at the same time as the prodigiosin fraction from the wild type was collected. Samples were air-dried and prodigiosin concentration was determined using a standard curve of absorbance at 534 nm using commercial prodigiosin as a standard (Sigma). The same volume of prodigiosin was added from the wild type and ΔpigA-derived samples.

**Results**

*S. marcescens* secretome induction of autophagy is inhibited by 3-methyladenine

Our previous study showed that a subset of ocular bacterial pathogens induced autophagy in a corneal cell line, and that among the strongest induction was observed with *S. marcescens* [18]. In this study, we set out to determine which components of *S. marcescens* using wild-type and genetically manipulated strains induced formation of LC3-GFP puncta. We used two *S. marcescens* strains: strain PIC3611 from Presque Isle Culture collection, a laboratory strain that is likely from an environmental source (biotype TCT), and K904, a contact lens associated keratitis isolate (biotype A6a). Two strains were used to determine whether phenotypes were associated with one particular strain or a more general phenomenon.

Figure 1 demonstrates activation of autophagy following exposure of HCLE LC3-GFP cells to normalized filtered supernatants (secretomes) from strain PIC3611 Fig. 1a, b). The formation of LC3-GFP puncta indicate cells with activated autophagy and can be used in quantification of autophagy [33]. Autophagy stimulation by PIC3611 secretomes could be prevented using autophagy inhibitor 3-methyladenine (3MA), supporting that the observed LC3-GFP phenotype is autophagy dependent (Fig. 1). The same trend was previously shown for strain K904 and with the use of autophagy inhibitor bafilomycin [18]. As an additional control to show that LC3-GFP puncta are not an artifact of fluorescent bacterial components, an HCLE-GFP cell line with no LC3 fusion was used [18]. Following PIC3611 supernatant treatment, no fluorescent focus formation was observed from the GFP control cell line (Fig. 1c).

*S. marcescens* secondary metabolite regulators are necessary for activation of autophagy

The component(s) of the secretome responsible for autophagy induction is unknown. Our previous study

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demonstrated that secretomes could be heated at 95 °C and still induce autophagy [18]. Well-defined S. marcescens strains with deletion mutations in the eepR and gumB genes were tested for loss of autophagy induction because these genes have a conserved and broad impact on S. marcescens biology [20, 24, 25, 28, 36]. The EepR and GumB genes regulate expression of multiple secreted factors including heat-stable secondary metabolites such as the biologically active pigment prodigiosin and the hemolytic and antimicrobial biosurfactant serratamolide. Both the eepR and gumB mutants were defective in activation of autophagy (Fig. 2a).

Additionally, secretomes from strains with mutations in a variety of surface or secreted proteins regulated by EepR and/or GumB were individually tested in the K904 strain background (Figs. 2b and 3). These include the type 1 pilus gene, fimC, the flagellin gene, fliC, the secreted nuclease gene, nucA, the prodigiosin biosynthetic gene, pigD, the cytolsin, shlA, the outer membrane cytolsin transporter gene shlB, the S-layer protein gene, slaA, and the serratamolide biosynthetic gene, swrW.

Mutant analysis suggested that the eepR and gumB mutants share a defect in autophagy induction with a subset of the mutants (Figs. 2b and 3), most notably pigD, which
codes for a gene involved in prodigiosin pigment biosynthesis [37]. Both eepR and gumB are severely defective in prodigiosin production due to reduced transcription of the prodigiosin biosynthetic genes, implicating prodigiosin as a stimulatory factor [25, 28]. Secretomes from mutants unable to make type 1 pili, flagella, and cytolysin / cytolysin transporter (shlA and shlB, respectively) were also defective, but to a lesser extent than the pigment mutants.

**Prodigiosin is necessary and sufficient to induce autophagy in a corneal cell line**

Given that the prodigiosin defective mutants, eepR, gumB, and pigD were defective in inducing autophagy whether they were from the PIC3611 (Fig. 2a) or K904 background (Figs. 2a, b and 3), we analyzed whether prodigiosin played a role in inducing autophagy in the HCLE LC3-GFP cell line in greater depth. First, because the eepR mutation confers pleiotropic effects, we compared the wild-type strain PIC3611 with an isogenic mutant unable to make PigA, which is required for prodigiosin biosynthesis. Similar to the eepR mutant, when only pigment biosynthesis was ablated through deletion of the pigA gene, strain PIC3611 was unable to induce autophagy (Fig. 4a).
We have previously described the use of plasmid pMQ262, which replaces the normal pigment biosynthetic promoter with an arabinose inducible promoter [26, 38], such that prodigiosin pigment biosynthesis is dependent upon arabinose in the growth medium (Fig. 4b). When secretomes from strain PIC3611 with pMQ262 were used to challenge HCLE LC3-GFP cells, the ability to activate autophagy correlated with arabinose induction of pigment production (Fig. 4c). Arabinose, itself, did not induce autophagy (data not shown). These data suggest that prodigiosin is necessary for S. marcescens secretomes to fully activate autophagy in corneal cells.

To test whether prodigiosin was sufficient for activation of autophagy in HCLE LC3-GFP cell line, we purified prodigiosin from strain PIC3611 and mock purified it from the isogenic ΔpigA mutant, and tested these for activation of LC3-GFP puncta formation (Fig. 5a-b). Prodigiosin from PIC3611 (0.9 μM) was able to activate autophagy. The negative control mock purified prodigiosin from the ΔpigA mutant was unable to activate autophagy when added at the same volume. Similarly, commercially available prodigiosin could activate autophagy in a dose dependent manner (Fig. 5a, c).

**Analysis of the ShlA cytolysin in inducing autophagy from corneal cells**

Data from genetic analysis above suggested that the pore forming cytolysin, ShlA, contributes to autophagy induction (Figs. 2b and 3). The ShlB protein is necessary for secretion of ShlA, such that shlB mutants do not secrete ShlA [39]. Consistently, the tested shlB mutant behaved similarly to the shlA mutant (Fig. 2b, and Fig. 3). We generated a double mutant that is unable to make prodigiosin or secrete ShlA (ΔshlB pigD), and this was indistinguishable from the pigD mutant, but had a trend to lower levels of autophagy induction compared to the ΔshlB levels.
Our previous study detected moderate activation of autophagy by a clinical keratitis isolate of \textit{E. coli} in HCLE-GFP cells [18] and that \textit{E. coli} with a \textit{shlBA} plasmid (pMQ492) is able to secrete functional ShlA cytolsin [20]. Here, we observed that ectopic expression of the \textit{S. marcescens} \textit{shlBA} operon increased the ability of \textit{E. coli} secretomes to induce autophagy in HCLE LC3-GFP cells (Fig. 6). Together, these results indicate a role for the ShlA cytolsin in activation of autophagy.

**Discussion**

Several studies have explored the role of ocular autophagy with HSV-1, \textit{Toxoplasma gondii}, and fungal spp. [2, 3, 5] However, the role of ocular autophagy in response to bacterial pathogens remains poorly understood.

This study demonstrated that two strains of \textit{S. marcescens} from different biotypes were capable of activating autophagy in a corneal cell line and identified bacterial factors capable of activating autophagy. Mutations in two different genes that confer major pleiotropic effects on \textit{S. marcescens} behavior, \textit{eepR} and \textit{gumB}, prevented bacterial activation of autophagy. The \textit{eepR} gene is a transcription factor that is required for wild-type levels bacterial proliferation in a rabbit keratitis model as well as positive regulation of secondary metabolites such as prodigiosin and serratamolide [24, 25]. The \textit{gumB} gene codes for a stress response signal transmitting protein that positively regulates prodigiosin and serratamolide, and is necessary for production of the ShlA, ShlB, and flagellin [20, 28]. We therefore tested individual genes controlled by EepR and GumB and identified several bacterial factors that activate autophagy.

Our genetic and biochemical results indicate that prodigiosin can activate autophagy in the tested human corneal cell line. Prodigiosin, 2-methyl-3-pentyl-6-methoxyprodigine, is thought to contribute to bacterial competition, and has antitumor capabilities [37, 40, 41]. Furthermore, prodigiosin was recently shown to activate autophagic cell death in a variety of cancer cell lines and to reduce tumor proliferation in mouse tracheas [42–49]. Many clinical isolates of \textit{S. marcescens} do not synthesize prodigiosin [50], and perhaps this benefits them by reducing activation of the host’s innate immune response.

Beyond prodigiosin, data from this study implicated the ShlA cytolsin in activation of autophagy in corneal cells. Similarly, in an elegant study by the Véscovi group, the pore forming cytolsin ShlA was demonstrated to induce autophagy in Chinese hamster ovary (CHO) cells [51].

In contrast to our work that suggested a role for flagellin as an autophagy inducer, Di Venanzio showed that \textit{S. marcescens} with mutations in \textit{flhA} and \textit{flhD}, which should be defective in flagella production, were able to activate autophagy in CHO cells [51]. These differences may be due to the specific bacterial strain background.
or use of CHO cells versus corneal cells. However, consistent with our finding, data from a recent papers using Salmonella, implicated flagellin as an activator of autophagy in zebrafish and murine RAW cells [52, 53]. To our knowledge there is no previous information on fimbriae / type I pili in activation of autophagy. It is also formally possible that some of the increase in LC3-GFP puncta results from a reduction in autophagic flux leading to the increase in overall autophagosomes. The impact of these bacterial factors on autophagic flux will be tested in subsequent studies.

Conclusions
We have identified S. marcescens activators of autophagy. Whereas prodigiosin and ShlA from S. marcescens have been previously implicated in activating autophagy, this report is the first to demonstrate this with ocular derived cells. The ability of flagellin and fimbria to induce autophagy will need to be further validated using biochemical means, but this report identifies these bacterial factors as potential microbial mediators of autophagy in corneal cells. Since S. marcescens is most commonly associated with the eye as a contact lens associated pathogen, it is possible that corneal cells prime themselves for microbial infection through sensing prodigiosin, flagellin, fimbriae, and ShlA toxins.

Abbreviations
3MA: 3-methyladenine, autophagy inhibitor; ANOVA: Analysis of variance, statistical analysis; CHO: Chinese hamster ovary, cell line; GFP: Green fluorescent protein; HCLE: Human corneal limbal epithelial, cell line; RPM: Rotations per minute

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Authors’ contributions
Writing and editing of manuscript: NAS, KB, RMQS; Study design: KB and RMQS; Data collection NAS, KB; Analysis and interpretation of data KB and RMQS. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used during this current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
The use of recombinant DNA materials was approved by the University of Pittsburgh Institutional Biosafety Committee.

Consent for publication
Not applicable.

Competing interests
Robert MQ Shanks is an Associate Editor of BMC Ophthalmology.

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