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Serratia marcescens ATCC 274 increases production of the red pigment prodigiosin in response to Chi phage infection

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Serratia marcescens **is an opportunistic human pathogen that produces a vibrant red pigment called prodigiosin. Prodigiosin has implications in virulence of *S. marcescens* and promising clinical applications. We discovered that addition of the virulent flagellotropic bacteriophage χ (Chi) to a culture of *S. marcescens* stimulates a greater than fivefold overproduction of prodigiosin. Active phage infection is required for the effect, as a χ-resistant strain lacking flagella does not respond to phage presence. Via a reporter fusion assay, we have determined that the addition of a χ-induced *S. marcescens* cell lysate to an uninfected culture causes a threefold increase in transcription of the *pig* operon, containing genes essential for pigment biosynthesis. Replacement of the *pig* promoter with a constitutive promoter abolished the pigmentation increase, indicating that regulatory elements present in the *pig* promoter likely mediate the phenomenon. We hypothesize that *S. marcescens* detects the threat of phage-mediated cell death and reacts by producing prodigiosin as a stress response. Our findings are of clinical significance for two main reasons: (i) elucidating complex phage-host interactions is crucial for development of therapeutic phage treatments, and (ii) overproduction of prodigiosin in response to phage could be exploited for its biosynthesis and use as a pharmaceutical.**

Keywords Flagella, Gene expression, Genetic transcription, Phage therapy

Serratia marcescens is a Gram-negative opportunistic human pathogen of the family *Yersiniaceae* known for biofilm formation[1], robust swimming and swarming motility[2,3], frequent multi-drug resistance (MDR)[4], and vibrant red pigment, prodigiosin[5,6]. *S. marcescens* is a common cause of nosocomial infections, particularly among immunocompromised individuals[6]. Its robust biofilms and antimicrobial resistance make these infections difficult to eradicate with traditional antibiotics, further complicated by many *S. marcescens* isolates being resistant to tetracycline, beta-lactam, and aminoglycoside-class antibiotics[4]. One promising approach to treating MDR bacterial infections is bacteriophage (phage) therapy[7–9]. This technique makes use of phages, the viruses of bacteria, as antibacterial agents. In addition to circumventing MDR, bacteriophages have been shown to be highly effective against disrupting biofilms, including in a clinical setting[10–14]. Bacteriophage χ is a flagellotropic (flagellum-dependent) *siphoviridae* phage, meaning the infection process begins with attachment to its host's flagellar filament[15–17]. Infection is then postulated to follow the "nut and bolt" model, where rotation of the flagellum brings the phage to the cell surface[18]. As a virulent phage, χ cannot lysogenize[19]. Approximately 200 virions are released after the cell is lysed following a 52–60-min latent period[19,20]. Due to the flagellum's nature as a virulence factor[21,22], χ and other flagellotropic phages may be of particular interest for therapeutic applications[16]. These viruses impose an evolutionary tradeoff that may prove exploitable: a pathogenic bacterium repressing motility to avoid infection by χ would likely be simultaneously attenuating its own virulence. The *S. marcescens* pigment prodigiosin is of clinical significance for several reasons. In addition to its involvement in *Serratia* competition against other bacterial species[23], the compound itself and other similar molecules have been shown to have antimicrobial, anticancer, and immunomodulatory effects in mammals and is currently under investigation for use as a therapeutic agent[5,24–26]. Regulation of pigment production is complex, and dozens of regulatory mechanisms control its production[26,27]. The primary target of transcriptional regulation of pigment production in *S. marcescens* is the *pig* operon *pigABCDEFGHIJKLMN*, with the additional gene *pigO* being present in certain strains of *Serratia* spp. but absent in *S. marcescens* ATCC 274[26,28]. These 14 or 15 genes are co-transcribed as a single polycistronic mRNA of approximately 21 kilobases[28] under the control of

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the P_{pig} promoter, which contains several known and predicted regulatory elements[26,27]. The resulting encoded proteins play various roles in the biosynthesis of prodigiosin through two biosynthetic pathways producing two essential precursor molecules. The monopyrrole 2-methyl-3-n-amyl-pyrrole is synthesized starting with the monounsaturated fatty aldehyde trans-2-octenal, while the dipyrrole 4-methoxy-2,2′-bipyrrole-5-carbaldehyde is synthesized beginning with the amino acid L-proline[26,29]. These two molecules are joined by a condensation reaction to form the tripyrrole prodigiosin[26,29]. Other molecules including malonyl-CoA and pyruvate serve as essential precursors[26,29]. PigP, its gene located elsewhere on the chromosome, seemingly serves as a master regulator of pigmentation in certain *Serratia* strains, such as *Serratia* sp. ATCC 39006[30,31]. A functional homolog of PigP is also present in some strains of *S. marcescens*[32]. Certain regulatory elements may act on the promoter of *pigP* rather than the *pigA–pigN* promoter[32]. While a multi-sequence alignment of ATCC 274 with *Serratia* sp. ATCC 39006 indicated a *pigP* homolog is present in the ATCC 274 genome[33], the gene is not annotated as such and has not been proven to serve as the pigmentation master regulator in this strain.

Factors such as temperature[34], phosphate availability[28,35], oxidative stress[36], envelope stress, denatured proteins[37], cAMP[32], and cell density are known to influence the quantity of prodigiosin produced in *Serratia* spp.[27]. Across *Serratia* species, several quorum sensing (QS) systems influence prodigiosin production, including LuxIR homologs producing and responding to acyl-homoserine lactone (AHL) autoinducers[26], and LuxS homologs, which rely on the QS molecule autoinducer-2 (AI-2)[26], a unique boron-containing compound[38]. There is a remarkable level of diversity among *Serratia* spp. strains regarding which QS systems are present[26], structure and sequence of the P_{pig} promoter and operon[26,28], and the presence or absence of *pigP*. A LuxS homolog is the only characterized QS system influencing pigmentation in *S. marcescens* ATCC 274[39], which lacks the LuxIR homolog SmaIR present in other *Serratia* strains[26]. It is unlikely that any other QS systems are present in ATCC 274, as the genome lacks *luxI* homologs[33]. In addition to QS systems, many conserved regulators such as Fnr[40], CpxRA[37,41], OhrR[36], OmpR[42], and PhoBR[35] influence pigmentation. Dozens of possible regulatory elements have been predicted in the P_{pig} promoter or *pig* operon by sequence analysis, and the corresponding regulators may influence pigmentation as well[27].

In this study, we determined that the addition of bacteriophage χ or a χ phage lysate to a liquid culture of *S. marcescens* ATCC 274 stimulates a significant increase in prodigiosin production. This effect is due, at least in part, to increased transcription of the *pig* operon. While the regulator(s) responsible for this effect remain elusive, it is very likely that they act directly on the P_{pig} promoter, as modification of this promoter abolished the increased pigmentation phenomenon. These findings provide insight into the interactions between *S. marcescens* and χ, a phage that infects pathogenic bacteria and is of interest for clinical applications.

Results

The magnitude of pigment production after χ addition is increased at stationary phase

In this study, we measured the effect on pigmentation of *S. marcescens* ATCC 274 when χ was added at 0.1 multiplicity of infection (MOI) during exponential phase (OD_{600} = 0.5) or stationary phase (OD_{600} = 2.0). We observed a remarkable difference between these conditions both visually and quantitatively (Fig. 1). When χ was added during exponential phase growth at the pigment-permissive temperature of 25 °C, a 1.7-fold increase in pigment concentration occurred, as measured by spectrophotometer absorbance at 535 nm (A_{535}) 18 h after phage was added. In contrast, addition of phage during stationary phase resulted in a 5.5-fold increase in pigment concentration in liquid cultures after 18 h of incubation at 25 °C. This remarkable difference in pigmentation intensity between the two growth conditions is due to both a sizeable reduction in pigmentation of the control culture and a modest but significant increase in pigmentation of the culture that received phage (Fig. 1). Productive phage infection is required for this effect, as a χ-resistant strain lacking flagellin (Δ*fliC*) exhibited pigment production comparable to WT in the absence of phage (average A_{535} = 0.761 vs 0.795 for WT after 18 h of incubation) but did not respond to the addition of χ (Fig. 2).

Mutants lacking *luxS* or the *pigP* homolog still respond to χ phage by increasing pigmentation

To determine the roles of quorum sensing and PigP-mediated regulation on the pigmentation response to χ, we constructed mutants with deletions in either *luxS* or the ATCC 274 *pigP* homolog SMATCC274_40570, encoding a protein essential for the production of the AI-2 QS signal[38] and a putative *pig* operon transcriptional regulator[30], respectively. We found that Δ*luxS* cultures produced less pigment than WT cultures both in the absence and presence of χ (Fig. S1). Cells still responded to χ by increasing pigment production, and the magnitude of increase was similar to WT (a 1.8-fold increase and 5.8-fold increase for exponential and stationary samples, respectively; Fig. 2). When *pigP* was deleted, the pigmentation of the resulting mutant was indistinguishable from WT without χ added (Fig. S1). The magnitude of response to χ was also similar to WT (a 1.7-fold and 5.7-fold increase for exponential and stationary phase samples, respectively; Fig. 2). Both of these deletion mutant strains remain susceptible to χ (Fig. 3) and were found to be highly motile when observed via phase contrast microscopy.

Replacement of the P_{pig} promoter with a constitutive promoter abolishes the effect

To investigate the genetic mechanism of this phenomenon, we replaced the native P_{pig} promoter with the strong constitutive promoter J23119 (iGEM Part:BBa_J23119), generating a mutant strain we have named pig^{q}. This promoter has an *E. coli* sigma-70 consensus sequence and therefore shows a very high transcription level in *E. coli*[43], but has not, to our knowledge, been used previously in *S. marcescens*. When we replaced the entirety of the native *pig* operon 5′-UTR with J23119 and a strong ribosome binding site, we noted a greater overall level of pigmentation (an average A_{535} of 2.09 for pig^{q} versus 0.795 for WT after 18 h of incubation; data not shown). However, pig^{q} cultures showed no statistically significant change in A_{535} values regardless of growth phase in

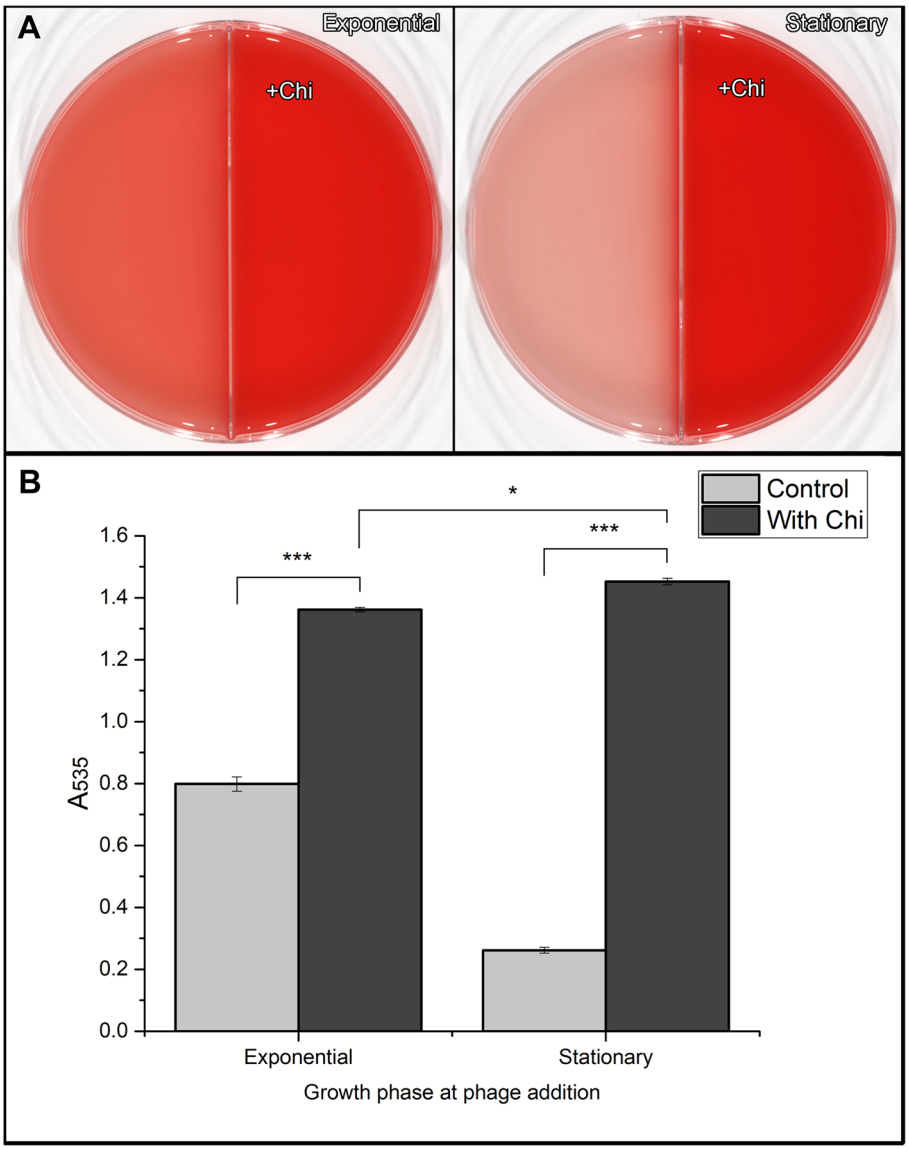

Figure 1. (**A**) Liquid cultures of *S. marcescens* wild type with and without the addition of 0.1 MOI χ phage. After 18 h of incubation at 25 °C with shaking, 10 ml of each culture was pipetted into one half of a split 10 cm petri dish. Plates were imaged with a Cytiva IQ800 instrument. Left, phage was added during exponential phase growth ($OD_{600} = 0.5$); Right, phage was added during stationary phase growth ($OD_{600} = 2.0$). (**B**) Pigmentation in *S. marcescens* wild type cultures as A_{535} quantified via spectrophotometer 18 h after addition of 0.1 MOI of χ phage to cultures in exponential phase ($OD_{600} = 0.5$) or stationary phase ($OD_{600} = 2.0$). Both sets are compared to a control sample without the addition of phage. Data points are mean values consisting of n = 3 replicates. Error bars indicate standard deviation. Student's *t*-test was used to determine statistical significance. P values are represented by asterisks: *P < 0.05; ***P < 0.001.

response to χ (Fig. 2). Phage infection does occur with this strain: the *pig*^{q} mutant exhibited a comparable level of χ susceptibility compared to WT, as demonstrated by a semi-quantitative χ spot assay (Fig. 3).

The pigmentation increase is statistically significant 3 h after the addition of phage

To determine how quickly the pigmentation change becomes significant, we spectrophotometrically measured pigmentation at 1-h timepoints after addition of 0.1 MOI χ phage at stationary phase ($OD_{600} = 2.0$). The difference in pigmentation between the two cultures became significant ($P < 0.05$) 3 h after addition of phage (Fig. 4). Thus, no significant increase in pigmentation was observed before the first phage-mediated lysis occurred after one latent period of approximately 60 min. A_{535} continued to rise in both cultures, but the increase was much larger in the culture with added phage. The difference in A_{535} remained statistically significant throughout the remainder of the experiment. Pigment production began to plateau at around 10 h, but pigmentation still continued to increase slightly for the next 2 h.

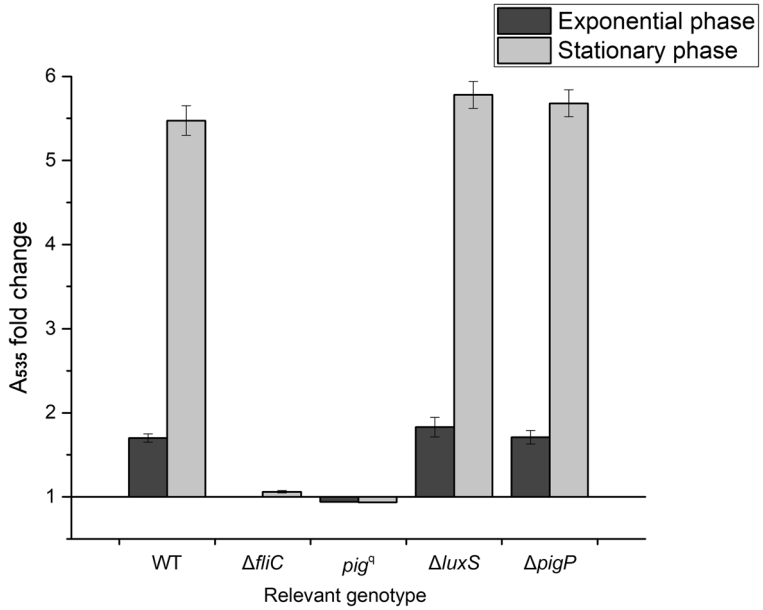

Figure 2. Prodigiosin concentration in *S. marcescens* wild-type or mutant cultures represented as fold change in A_{535} between samples with and without the addition of 0.1 MOI of χ phage in exponential phase ($OD_{600} = 0.5$) or stationary phase ($OD_{600} = 2.0$). All samples were measured via spectrophotometer 18 h after the addition of phage. Data points are mean values consisting of n = 3 replicates. Error bars indicate standard deviation. Student's *t*-test was used to determine statistical significance. All increases in pigmentation were statistically significant apart from the strains Δ*fliC* and *pig*^{q}, which did not show a significant pigmentation change in response to χ.

A χ phage lysate stimulates a pigmentation response in a χ-resistant strain

To investigate whether *S. marcescens* cells were responding to a compound present in a χ phage *S. marcescens* cell lysate, we first generated a lysate by infecting *S. marcescens* Δ*pig* to prevent the presence of prodigiosin in the lysate itself. As removal of phage is not trivial without significantly altering the chemical composition of the lysate, we tested the lysate on a χ resistant strain, namely Δ*fliC*. We found that addition of ultra-purified χ alone had no effect on pigmentation in Δ*fliC* (Fig. 2). Therefore, residual phage in the lysate did not contribute to our observations. Use of a flagellin mutant ensured that no new phage infection occurred, therefore any response observed was due to the presence of lysed cells, and not remaining phage in the lysate. We found that addition of this lysate to an exponential-phase culture resulted in a 1.6-fold increase in A_{535} after 18 h when compared to a control culture, which received fresh LB medium (Fig. 5). This effect is similar to the one obtained when χ phage was added to exponential phase cultures (Fig. 1B). Addition of supernatant from an uninfected *S. marcescens* culture caused a statistically significant 1.2-fold increase, indicating that the effect seen with the χ lysate was to a small degree due to compounds also present in an uninfected culture supernatant. A similar effect was seen when the χ lysate was added to stationary phase cultures as it prompted a 1.5-fold increase in A_{535}. Moreover, addition of a culture supernatant caused no statistically significant effect at stationary phase (Fig. 5). We noted significantly lower overall pigmentation in all cultures at stationary phase compared to exponential-phase cultures. This is different from our findings when χ phage was added, where the effect was much more pronounced in stationary phase cultures (Fig. 1). To confirm whether the increase in pigmentation was specific to the addition of χ lysates, we tested the effect of the supernatant from an *S. marcescens* culture that was mechanically lysed by emulsification, and found that the A_{535} change was the same as previously determined for a non-lysed culture supernatant (Fig. 5B). Overall, the addition of a χ phage lysate prompted a response similar to the addition of the phage itself.

Pigment overproduction correlates with increased *pigA–pigN* operon transcription

To gauge *pig* operon transcription in the presence of a χ phage *S. marcescens* cell lysate, we conducted a transcriptional reporter assay by replacing the first gene in the *pig* operon, *pigA*, with the *lacZ* gene from *E. coli* and performing β-galactosidase assays. Immediately and 1 h after addition of additives, β-galactosidase activity, and thus *pigA–pigN* transcription, was unaffected regardless whether a χ lysate supernatant, uninfected *S. marcescens* cell supernatant, or fresh LB medium was added (Fig. 6). However, 2 h after the addition of the additives, β-galactosidase activity rose in the sample that received the χ lysate, reaching 22 Miller units: 3.3- and 2.6-fold higher values than the cultures that received fresh LB and uninfected culture supernatant, respectively. These trends continued for the next 2 h, with the culture that received χ lysate displaying β-galactosidase activities of 75 and 204 Miller units: 1.8- and 2.0-fold higher than the culture with fresh LB added, respectively. The samples that received an uninfected supernatant showed an intermediate phenotype of 1.3-fold higher than the LB control at both the 3- and 4-h timepoints, more specifically 56 and 130 Miller units, respectively. Overall,

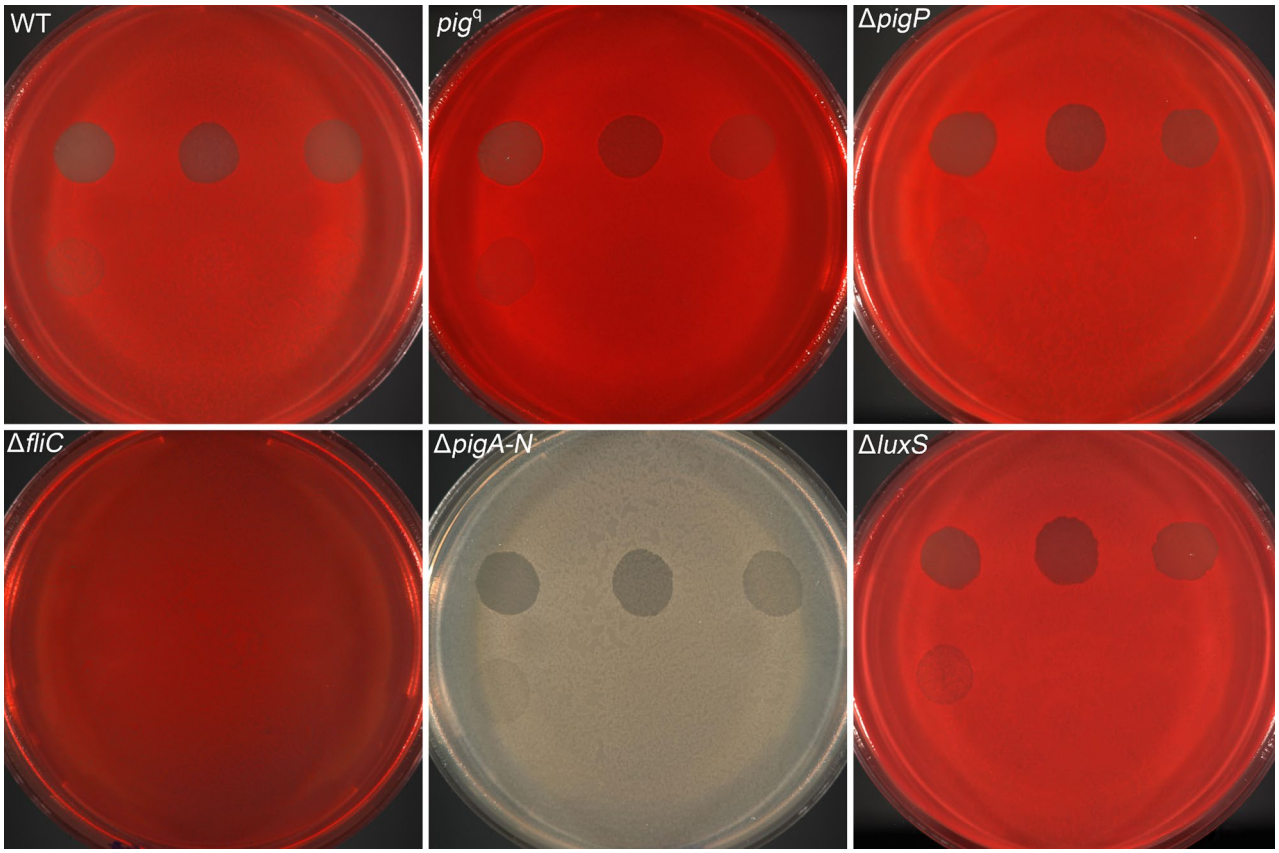

Figure 3. Semi-quantitative phage spot assays for determination of phage susceptibility. *S. marcescens* strains were grown in LB to an OD_{600} of 1.0, mixed with molten LB with 0.5% agar, and poured on an LB 1.5% agar plate. Phage χ dilutions in 0.85% NaCl were spotted on the solidified agar surface, and plates were incubated at 25 °C overnight and imaged using a Cytiva IQ800 instrument. Zones of clearing indicate lysis and thus χ susceptibility. Dilutions are as follows: top row left to right: 10^0, 10^{-1}, 10^{-2}; bottom row left to right: 10^{-4}, 10^{-5}, 10^{-6}.

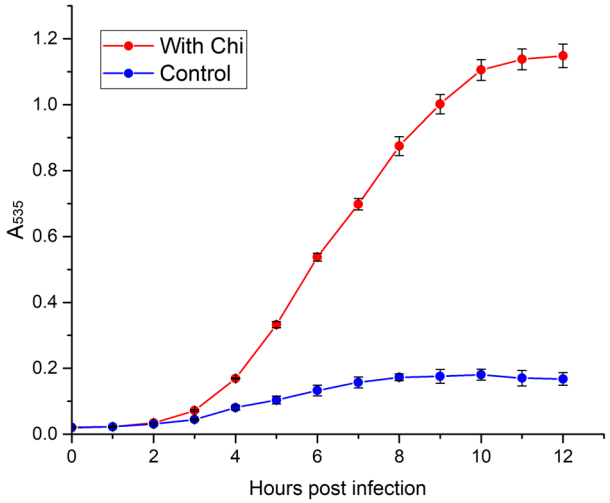

Figure 4. Prodigiosin concentration in *S. marcescens* wild type cultures over time after addition of 0.1 MOI χ phage at stationary phase (OD_{600} = 2.0). Values are given as A_{535} measured using a spectrophotometer, which represents prodigiosin concentration in cultures. No phage was added to the control samples. Data points are mean values consisting of n = 3 replicates. Error bars indicate standard deviation. Student's *t*-test was used to determine statistical significance. The difference in A_{535} between the samples with and without χ is statistically significant ($P < 0.05$) at all timepoints 3–12 h after addition of phage.

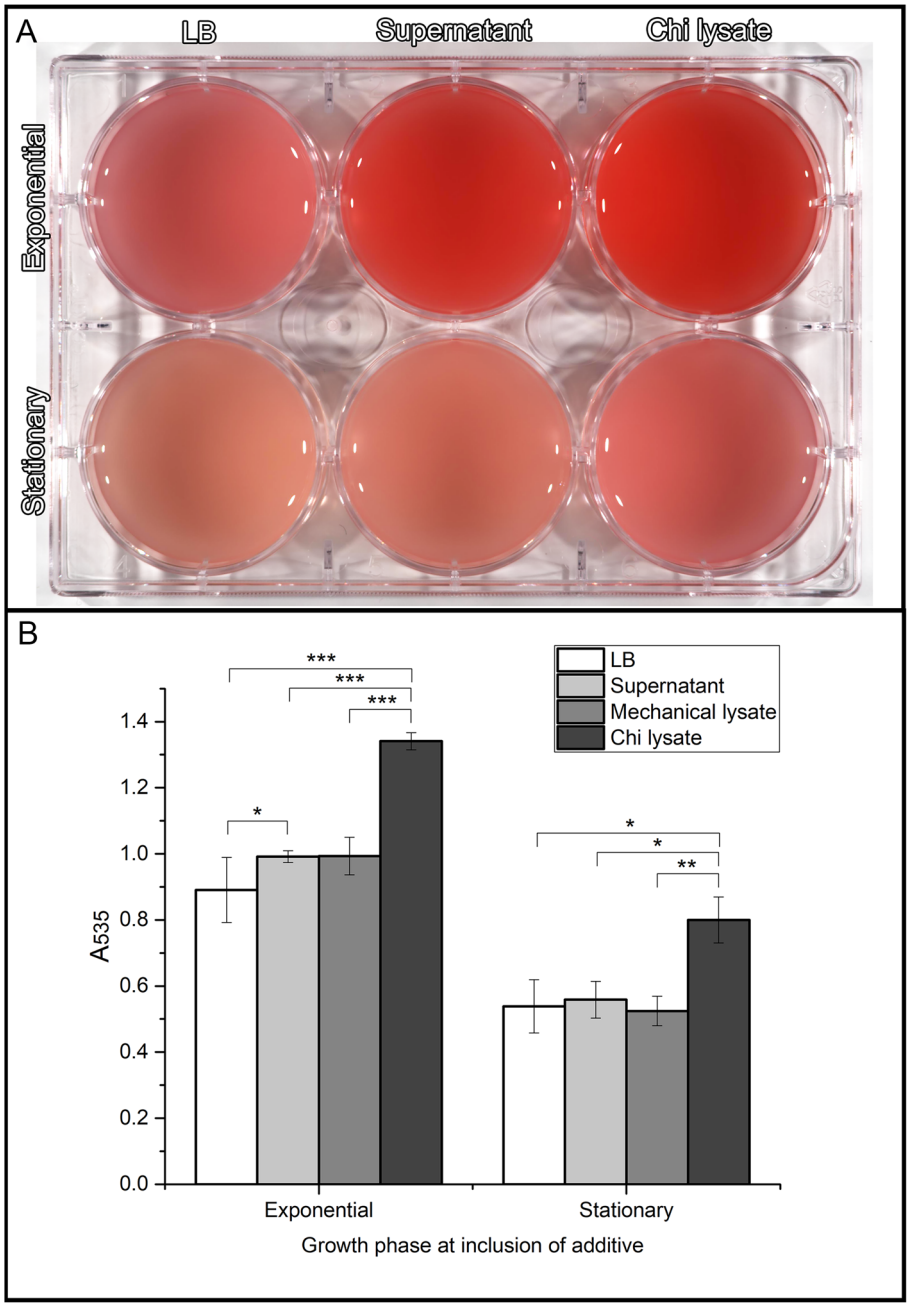

Figure 5. (**A**) Plates of *S. marcescens* Δ*fliC* liquid cultures with added χ phage lysate, Δ*pig* cell supernatant, or fresh LB medium at a ratio of 1:1. After 18 h of incubation at 25 °C with shaking, 5 ml of each culture was pipetted into one well of a 6-well rectangular plate. Plates were imaged with a Cytiva IQ800 instrument. (**B**) Pigmentation in *S. marcescens* Δ*fliC* cultures as A_{535} quantified via spectrophotometer 18 h after addition of a χ phage lysate, Δ*pig* cell supernatant, Δ*pig* culture mechanical lysate, or fresh LB medium to cultures in exponential phase ($OD_{600} = 0.5$) or stationary phase ($OD_{600} = 2.0$). Data points are mean values consisting of three replicates. Error bars indicate standard deviation. Student's *t*-test was used to determine statistical significance. P values are represented by asterisks: *P < 0.05; **P < 0.01; ***P < 0.001.

the time frame of increase in the *pig* operon transcription aligns with the observations from the pigmentation time-course experiment.

Discussion

In this study, we found that the presence of the flagellotropic bacteriophage χ or a cell lysate generated by infecting *S. marcescens* with χ stimulates a significant increase in the production of the red pigment prodigiosin. Prodigiosin is known to be produced as a response to stressful stimuli[26]. There are overlapping regulatory

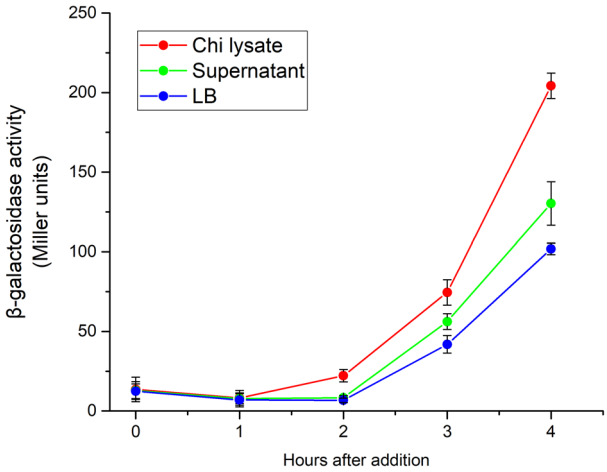

Figure 6. Graph of β-galactosidase activity in Miller units measured at 1-h timepoints after the addition of a χ phage lysate, *S. marcescens* Δ*pig* cell supernatant, or fresh LB medium to an exponential-phase culture of *S. marcescens pig-lacZ* at a ratio of 1:1. Data points are a mean of n = 3 replicates, and error bars represent standard deviation. Student's *t*-test was used to determine statistical significance. The difference between χ phage lysate and LB control samples is statistically significant ($P < 0.05$) 2, 3, and 4 h after addition of lysate. The difference between cell supernatant and LB control samples is statistically significant ($P < 0.05$) 3 and 4 h after addition.

mechanisms between pigmentation and motility[32], and an association between flagellar variation and prodigiosin production[44]. The presence of a flagellum-dependent phage like χ would impart significant selective pressure against motility. Altered motility regulation may inadvertently increase *pig* operon expression as a pleiotropic effect.

Reasons for differences in pigmentation intensity between exponential and stationary phase

We found that the pigmentation effect was manyfold higher when χ was added to cultures in early stationary phase, rather than exponential phase. This result was not anticipated, as cells display higher motility in exponential phase and motility is a requirement for χ infection. The control cultures that did not receive phage were significantly less pigmented when grown overnight at 37 °C, diluted to an OD_{600} of 2.0, and transferred to the pigmentation-permissive temperature of 25 °C. This is due to the fact that these cultures were incubated at the pigment-permissive temperature for a shorter amount of time. Interestingly, cultures that received phage showed an inverse effect, being more pigmented when χ was added at an OD_{600} of 2.0 and transferred to 25 °C, even though these cultures were also incubated at the pigment-permissive temperature for a shorter time than the exponential phase cultures. This indicates that the increased pigmentation response in stationary phase cultures is large enough to overcome the effects of a shorter duration of incubation at 25 °C. Both decreased pigmentation in the control cultures and increased pigmentation in the infected cultures contributed to the large difference in A_{535} between the two growth conditions. Lower motility and higher cell density may result in a smaller proportion of cells being infected by χ at stationary phase. This would result in a greater proportion of viable cells, capable of responding to the cell lysis that occurs in their surroundings. This factor is eliminated when a lysate is added, as new phage infection does not occur, explaining why the difference in A_{535} increase is reversed in the lysate experiment. Finally, differences in overall gene regulation between stationary and exponential phases may also play a role[45–47].

Transcriptional regulators responsible for the phenomenon likely target the *pig* promoter directly

In this study, we presented results of a reporter fusion assay and promoter replacement, both of which led to the conclusion that the regulator(s) responsible for the prodigiosin production increase act on the P_{pig} promoter. While growth conditions and certain stressful stimuli are known to induce prodigiosin overproduction[48], the number of studies on biological interactions that induce a similar effect is limited. Chilczuk et al. discovered that certain compounds produced by a cyanobacterial species stimulate prodigiosin production by *Serratia* sp. ATCC 39006[49]. Interestingly, they found that this increase was largely due to changes in L-proline uptake and fatty acid biosynthesis rather than an effect on *pig* operon transcription or QS systems. This mechanism is very different from our findings in *S. marcescens* ATCC 274, demonstrating the complexity of prodigiosin biosynthesis and the diversity among *Serratia* species. While we have shown that the observed phenomenon is due, at least partially, to *pig* operon transcriptional regulation, it is certainly possible that χ may be influencing precursor availability as well. Connections between phage and QS have been described, and certain phage genomes contain functional QS gene homologs[50]. We constructed a Δ*luxS* mutant, which was incapable of performing AI-2 QS. This strain still responded to χ in a manner similar to WT. Thus, QS does not mediate the pigment response, unless an undiscovered, non-homologous QS system exists in ATCC 274. It is worth noting that while an overall reduction in pigmentation due to *luxS* deletion did occur, it was of a lesser magnitude than previously described in the

literature[39]. We can only speculate that some of our procedures in this study differed from those in Coulthurst et al., including a difference in the incubation temperature.

While we do not know the transcriptional regulators controlling the pigment overproduction during χ infection, we demonstrated that replacement of the P_{pig} promoter abolishes the effect. In addition, deletion of the *pigP* homolog gene did not abolish the effect. This result rules out regulators that bind sequences within the *pig* operon itself, as well as regulators that may control transcription of the *pigP* homolog. In fact, the *pigP* deletion did not have any significant impact on overall pigmentation. As such, we hypothesize that this homolog is nonfunctional. We do not find this to be particularly surprising, as regulation of pigmentation in *Serratia* sp. 39006, the strain in which PigP is most well characterized[30,31], is drastically different than in ATCC 274[26]. The fact that addition of the supernatant of a mechanically lysed culture was statistically indistinguishable from that of a non-lysed culture further indicates that the pigmentation effect is specific to phage-mediated lysis.

A χ-induced cell lysate mimics the effect of χ on pigmentation

To further explore the effect of χ on host transcription, we exposed cultures to a χ-induced cell lysate. This approach was necessary, as very rapidly after phage DNA entry, host gene transcription is often severely reduced[51–54], limiting the effectiveness of gene transcription assays in phage-infected samples. In addition, the χ genome includes the genes CHI_9 and CHI_58, putatively encoding an endonuclease and exonuclease, respectively[55]. It is possible that these nucleases digest the host genetic material, which would also halt host gene transcription. Since assays like RT-qPCR and transcriptional reporter gene fusions traditionally measure transcription levels as an average of a cell population, the reduction in overall gene transcription in infected cells would likely mask the increased *pig* operon transcription in uninfected cells. Through a β-galactosidase assay of cultures grown in the presence of χ, we found that within 1 h of infection, overall transcription decreased to a level that outweighs any inducing effect on the *pig* operon (data not shown). To ameliorate these concerns, we used a phage lysate instead of χ. The results from the lysate experiments aligned well with the pigment concentration (A_{535}) time-course experiment. We found that the difference in β-galactosidase activity becomes significant after 2 h, and subsequently, the difference in pigmentation intensity becomes significant at 3 h.

Clinical and industrial implications of this work

Prodigiosin plays a key role in the lifestyle of *Serratia* species. It is used by *Serratia* species to compete with other bacterial species[23], which may contribute to overall fitness and virulence of *S. marcescens* in certain environments[56]. In contrast, it has been known for several decades that most clinical isolates of *Serratia* are non-pigmented[57,58]. The overall metabolic cost of synthesizing over a dozen different proteins and diverting precursors to produce the pigment appears to be high, as spontaneous pigmentation mutants arise regularly[59]. Therefore, *Serratia* has to balance between the advantages prodigiosin provides and its energetic burden. Our results indicate that the interaction between χ phage and its host is highly complex, and that the bacterium is able to detect phage infection or lysis and to respond by altering transcriptional regulation. It is very likely that χ phage's other hosts are capable of detecting and responding to phage infection as well; *Salmonella enterica* and *Escherichia coli* simply lack a vibrant pigment, thus rendering the response less easily observable. Uncovering more information about phage-host interactions is key to developing phage therapy treatments against pathogenic bacteria.

In addition to the clear applications for phage therapy to treat *S. marcescens* infections, a separate consideration can be made for the implications of this work on the therapeutic applications of prodigiosin itself, as prodiginines have well-demonstrated antibacterial, anticancer, antimalarial, and immunomodulatory activities with strong evidence through in vitro and in vivo studies[5,23,24,60]. We found that addition of a bacteriophage can increase pigment production by more than five times. Additionally, by replacing the promoter of the *pig* operon with a strong constitutive promoter, we generated a strain, pig^{q}, that significantly overproduces pigment without the need of an outside stimulus. Either addition of χ or the utilization of the pig^{q} strain in an industrial setting could be exploited to vastly increase prodigiosin yields in *S. marcescens* cultures for subsequent purification and downstream clinical applications. This would be particularly useful if the effect of χ or the J23119 promoter is found to be additive to other existing strategies for increasing prodigiosin production.

Future study

In the future, identification of the regulator(s) responsible for the pigment overproduction would be desirable. RNA-seq could be used to identify genes with increased or decreased expression in response to χ or a χ lysate. Transcription of the *pig* operon should be measured in response to χ. This is not a trivial experiment, but could be successful by sorting cells via flow cytometry using a fluorescently labelled χ phage to differentiate infected and uninfected cells[61]. Alternatively, a single-cell RNA sequencing method could be employed, as this has recently become feasible in bacteria[62]. Lastly, it needs to be determined whether this phenomenon is specific to χ or flagellotropic phages by exploring other *S. marcescens* phages.

Materials and methods

Strains and plasmids

E. coli K-12 derivatives, *S. marcescens* ATCC 274 mutants, and plasmids used in this study are listed in Table 1.

Construction of mutant strains

To construct *S. marcescens* mutants including those with gene deletions, promoter replacements, and reporter fusions, we developed an optimized targeted allelic exchange approach with the plasmid pKNG101[63] and provide a detailed protocol below. First, the desired allele was amplified by PCR and cloned into pKNG101 following

Strain, plasmid, or phage	Parental strain/backbone	Relevant characteristics	Source
Serratia marcescens ATCC 274 (WT)	ATCC 274	Wild type	Rasika Harshey
Δ*fliC*	ATCC 274	Δ*fliC* (*flaF*)	This study
Δ*luxS*	ATCC 274	Δ*luxS*	This study
Δ*pig*	ATCC 274	Δ*pigABCDEFGHIJKLMN*	This study
Δ*pigP*	ATCC 274	ΔSMATCC274_40570	This study
pig-lacZ	ATCC 274	Δ*fliC*; *pigA*::*lacZ*	This study
pig^{q}	ATCC 274	P_{pig} replaced with J23119	This study
pKNG101	–	MCS, *strAB*, *sacB*, *mobRK2*, *oriV* R6Kγ, *traJK*	Sarah J Coulthurst
pBS1315	pUC18	Cloning vector with J23119 promoter	This study
pBS1355	pKNG101	*fliC* (*flaF*) deletion construct	This study
pBS1359	pKNG101	pig^{q} mutant construct	This study
pBS1361	pKNG101	*pig* operon deletion construct	This study
pBS1362	pKNG101	*pig-lacZ* fusion mutant construct	This study
DH5α-λpir	*Escherichia coli* K-12	F^{-} φ80*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *recA*1 *endA*1 *hsdR*17(r_K^-, m_K^+) *phoA supE*44 λ^{-}*thi*-1 *gyrA*96 *relA*1 *LAMpir*	Howard C. Berg
SM10-λpir	*Escherichia coli* K-12	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu Km*LAMpir*	Clay Fuqua
χ (Chi)	–	Wild type	Saeed Tavazoie

Table 1. Strains, plasmids, and phage used in this study.

standard restriction enzyme cloning procedures. *Escherichia coli* DH5α-λpir was transformed with the resulting ligation mixture and plated on lysogeny broth LB[64] with 50 μg/ml streptomycin (LB str). Deletion constructs were introduced into *E. coli* SM10-λpir by transformation. The plasmid was then mobilized into *S. marcescens* ATCC 274 by biparental mating conjugation. Briefly, ATCC 274 and SM10-λpir containing the deletion plasmid were grown in LB at 37 °C with shaking until an OD_{600} of 0.5 was reached. The two cultures were then mixed at a ratio of 1:1 by optical density, and 100 μl of this mixture was spread onto an LB agar plate without antibiotics. After 24 h of incubation at 37 °C, the bacterial lawn was scraped into a tube containing 1 ml of LB, suspended by vortexing, and spread onto LB containing 50 μg/ml streptomycin and 10 μg/ml tetracycline (LB str/tet) to select against the *E. coli* SM10-λpir strain, taking advantage of the intrinsic tetracycline resistance of ATCC 274. After an overnight incubation step, colonies were streaked on LB str/tet, and a single colony from this plate was inoculated into 10 ml of LB without antibiotics, which was incubated with shaking for 24 h at 37 °C to allow a second homologous recombination event to occur. The culture was then centrifuged at 15,000×*g* for 5 min and washed twice with M9 salts (47.7 mM Na_2HPO_4, 22 mM KH_2PO_4, 8.6 mM NaCl, 18.7 mM NH_4Cl, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$). Dilutions were prepared in M9 salts and spread plated on M9F agar plates containing 10% w/v sucrose as the sole carbon source for SacB counterselection. M9F is a modified M9 minimal medium[65] containing 0.2 mM $FeSO_4$ for improved growth of *S. marcescens*. These plates were incubated at room temperature for three to four days. It is important to note that incubation at higher temperatures or the presence of carbon sources other than sucrose vastly reduced the quality of selection. Large colonies were patched on LB str to verify loss of streptomycin resistance. Genomic DNA was purified by phenol–chloroform extraction, and deletion loci were amplified by PCR followed by confirmation via Sanger sequencing. Note that the DNA purification step is crucial, as *S. marcescens* produces a remarkably robust extracellular nuclease[66] that we found to be capable of retaining activity after colony PCR cycles and subsequently rapidly digesting PCR products. Proteinase K treatment during DNA purification effectively inactivated the nuclease.

Spectrophotometric determination of prodigiosin concentration in cultures and imaging

S. marcescens cultures were routinely grown overnight in LB at 37 °C with 220 RPM shaking. At this temperature, no prodigiosin is produced in the wild-type strain[34]. To test the effect of χ addition at exponential phase, overnight cultures were diluted 1:100 and grown at 25 °C with 220 RPM shaking until an OD_{600} of 0.5 was reached. Phage was then added at an MOI of 0.1, followed by 18 h of incubation at 25 °C with shaking. To test the effect of χ at stationary phase, overnight stationary phase cultures grown at 37 °C were diluted to an OD_{600} of 2.0, χ was immediately added at an MOI of 0.1, and cultures were transferred to a shaking incubator at 25 °C and incubated for 18 h. An MOI of 0.1 was chosen, as a preliminary time course experiment conducted by growing *S. marcescens* with and without χ in a plate reader demonstrated that there was no significant difference in pigmentation between samples with χ added at MOIs of 0.1 and 1.0, while lower MOIs reduced the magnitude of the effect (Fig. S2). Relative prodigiosin concentration was determined by measuring absorbance at 535 nm after lysing cells using a modified acidified ethanol lysis method[67]. Briefly, *S. marcescens* culture in LB was mixed with two volumes of acidified ethanol (100% ethanol with 40 mM HCl) and vortexed vigorously. Cell debris was pelleted in a centrifuge at 5000×*g* for 10 min. One milliliter of supernatant was pipetted into a quartz spectrophotometer cuvette and absorbance at 535 nm was measured. For pigmentation time-courses, 0.5 ml samples of *S. marcescens* cultures were taken at regular intervals and immediately lysed by the addition of 1 ml of acidified ethanol. All samples were assayed via spectrophotometer.

Although A_{535} values are often normalized to OD_{600} in other experiments described in the literature[67], we elected not to normalize, due to the fact that phage-lysed cells and cell debris contribute to OD_{600}. If χ were to cause a large amount of cell death, OD_{600} would likely not decrease proportionally to the reduction in viable cell count. This would cause misrepresentation of results. This is supported by the observation that although χ-mediated cell death occurs in liquid cultures, OD_{600} over time is only slightly reduced, and cultures are not lysed confluently. After addition of χ to an MOI of 0.1, ATCC 274 cultures are not cleared by the phage, OD_{600} takes several hours to slow down, and cultures eventually reach a density comparable to an uninfected control after 16 h (Fig. S3). When endpoint A_{535} readings were taken after overnight incubation, OD_{600} values of all cultures were equal. Additionally, when viable cell count was determined in infected cultures during the production of lysates, we found that cultures with χ had approximately half as many viable cells compared to an uninfected control after 3 h, despite OD_{600} values being equal at this time point. This further supports our reasoning for not normalizing A_{535} to optical density.

A Cytiva IQ800 instrument was used to capture color images of *S. marcescens* cultures. First, 10 ml of culture in LB was pipetted into each half of a split 10 cm circular petri dish. For 6-well rectangular plates, 5 ml of culture was pipetted into each well. Plates were placed into the imager without lids and imaged using epi-illumination with the white tray insert.

Generation of a χ phage cell lysate lacking pigment

To generate the lysate for use in further experiments, a 37 °C overnight liquid culture of *S. marcescens* Δ*pig* was diluted 1:100 and grown in LB at 25 °C with 220 RPM shaking until an OD_{600} of 0.75 was reached. Motility was verified by phase contrast microscopy. Next, χ was added to the culture at an MOI of 0.1, and the culture was returned to the shaking incubator. A second culture was prepared in parallel and did not receive phage. After 3 h, both cultures were centrifuged at 16,000×*g* for 10 min, and supernatants were filtered through 0.2 µm syringe filters. Prior to centrifugation, a small amount of each culture was serially diluted in fresh LB and plated to determine viable cfu/ml, to allow for normalization of the amount of lysate and control supernatant to add in further experiments. The mechanical lysate was prepared similarly to the control supernatant, but cells were lysed using an EmulsiFlex C3 emulsifier (Avestin) prior to centrifugation. After filtration, lysates and culture supernatants were stored at 4 °C, and retained their activity for at least 4 weeks (data not shown).

Spot assays for determining phage susceptibility

Spot assays were used to determine susceptibility to χ phage. Briefly, *S. marcescens* was grown in LB at 25 °C to an OD_{600} of approximately 1.0. Motility was verified by phase contrast microscopy for strains expected to be motile. Next, 100 µl of culture was mixed with 4 ml of LB with 0.5% agar molten at 50 °C, and poured onto an LB agar plate. Fresh 1:10 serial dilutions of χ phage in 0.85% NaCl were prepared. After the top agar solidified, 10 µl of phage dilutions were spotted on the agar surface. Plates were incubated at 25 °C overnight. Zones of lysis indicated phage infection and thus susceptibility. Plates were imaged using a Cytiva IQ800 instrument with trans-illumination.

Beta-galactosidase (LacZ) transcriptional reporter fusion assay

The mutagenesis technique described above was used to generate a mutant strain (*pig-lacZ*) where the *pigA* gene on the chromosome was replaced with the *lacZ* gene PCR-amplified from *E. coli* MG1655, leaving the P_{pig} promoter and the remainder of the *pig* operon intact. To make this strain χ resistant, the flagellin gene *fliC* was deleted additionally. Cultures of *pig-lacZ* were grown overnight at 37 °C in LB. The following day, cultures were diluted 1:100 into fresh LB and grown at 25 °C to an OD_{600} of 0.8. Phage lysate, control supernatant, or fresh LB was added to each set of cultures at a volume ratio of 1:1. Aliquots of each culture were harvested at 0, 1, 2, 3, and 4 h after supplementation with lysate, supernatant, or LB by centrifuging at 16,000×*g* for 10 min and freezing pellets at − 20 °C. A β-galactosidase assay was conducted following an established protocol previously described by Miller[65]. Briefly, pellets were suspended in 800 µl Z-buffer, and permeabilized by adding 25 µl of chloroform and SDS to a concentration of 0.003%. Ortho-Nitrophenyl-β-galactoside (ONPG) was added to a concentration of 0.65 mg/ml. Samples were incubated at 30 °C until a yellow color developed, at which point reactions were stopped by adding Na_2CO_3 to 0.3 M. Cell debris and chloroform were pelleted by centrifugation, and supernatants were assayed via spectrophotometer at 420 nm.

Quantification of culture density over time with and without the addition of χ

To determine the effect χ has on OD_{600} over time, *S. marcescens* cultures were grown in triplicate at 25 °C to an OD_{600} of 0.5, at which point 0.1 MOI χ phage was added to one set of cultures. Every hour after χ addition, OD_{600} was measured. Readings were taken for 16 h.

Statistical analysis

Values given in figures represent mean averages of multiple replicates. Error bars represent standard deviation. Statistical significance between individual data sets was determined using Student's *t*-test, and P values less than 0.05 were considered significant.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Author contributions

N.C.E. and B.E.S. designed experiments. N.C.E. performed experiments. N.C.E. and B.E.S. acquired funding. B.E.S. provided resources. N.C.E. and B.E.S. wrote the manuscript.

Competing interests
The authors declare no competing interests.

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