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Biological cost of pyocin production during the SOS response in *Pseudomonas aeruginosa*

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Abstract

LexA and two structurally related regulators, PrtR and PA0906, coordinate the *P. aeruginosa* SOS response. RecA-mediated autocleavage of LexA induces the expression of a protective set of genes that increase DNA damage repair and tolerance. In contrast, RecA-mediated autocleavage of PrtR induces antimicrobial pyocin production and a program that lyses cells to release the newly synthesized pyocin. Recently, PrtR-regulated genes were shown to sensitize *P. aeruginosa* to quinolones, antibiotics that elicit a strong SOS response. Here, we investigated the mechanisms by which PrtR-regulated genes determine antimicrobial resistance and genotoxic stress survival. We found that induction of PrtR regulated genes lowers resistance to clinically important antibiotics and impairs the survival of bacteria exposed to one of several genotoxic agents. Two distinct mechanisms mediated these effects. Cell lysis genes that are induced following PrtR autocleavage reduced resistance to bactericidal levels of ciprofloxacin, and production of extracellular R2 pyocin was lethal to cells that initially survived UV light treatment. Although typically resistant to R2 pyocin, *P. aeruginosa* becomes transiently sensitive to R2 pyocin following UV light treatment, likely because of the strong downregulation of LPS synthesis genes that are required for resistance to R2 pyocin. Our results demonstrate that pyocin production during the *P. aeruginosa* SOS responses carries both expected and unexpected costs.
Introduction

Bacteria respond to DNA damage and replication fork stress by activating the SOS response. Much of our understanding of the SOS response stems from seminal research in *Escherichia coli* where this response primarily functions to increase the cell’s ability to cope with DNA damage and replication stress (1, 2). In *E. coli* the SOS response of *E. coli* is coordinated by the LexA protein, which directly represses expression of genes that bear a LexA-binding motif. During growth in non-stressed conditions, LexA keeps the expression of target genes at a minimum level. Upon DNA damage and/or replication fork stalling, the nucleoprotein filament formed by RecA binding to single stranded DNA stimulates the latent serine protease of LexA, resulting in LexA autocleavage and derepression of LexA-regulated genes. These LexA-regulated genes code for proteins that enhance nucleotide excision repair, DNA damage tolerance, recombinational repair, and cell cycle control (1, 2).

Autocleavage of LexA during the SOS response can also derepress genes on accessory plasmids, including genes that code for translesion polymerases, antibiotic resistant determinants, and antimicrobial colicins (3-6). Colicins are particularly interesting from the viewpoint of the SOS response because induced colicin production causes cell death, a stark contrast to the protective effects of chromosomal genes regulated by LexA. Colicin synthesis causes death because a co-expressed protein mediates cell lysis (6). Colicins are intraspecies antimicrobial bacteriocins that target non-related individuals. Related individuals are immune to colicin because of an immunity protein coded on plasmid.
The SOS response in *P. aeruginosa* is more complex than that of *E. coli* as it is not only coordinated by LexA, but also by two structurally related regulators, PrtR and PA0906 (7-11). These regulators are coded on the chromosome, and they are found in most *P. aeruginosa* strains and in related *Pseudomonas spp.* (12). As in *E. coli*, DNA damage leads to RecA-mediated autocleavage of LexA and derepression of the protective LexA regulon (8, 11, 13). RecA-single strand DNA nucleoprotein filaments also stimulate the latent serine protease of PrtR and PA0906, resulting in autocleavage and derepression of their target genes (8, 14-16). PrtR directly and indirectly controls the expression of 43 genes involved in the synthesis and release of antimicrobial pyocins, and PA0906 is predicted to control 6 genes whose functions have not yet been elucidated (8, 14).

Pyocins are antimicrobial bacteriocins that target other strains of the same species, although some pyocin types have been found to target other species in addition to *P. aeruginosa* (14). However, pyocin production comes with a cost, because cells that produce pyocin during the SOS response lyse and die. Pyocin has been reported to accumulate to low levels in standard cultures after overnight incubation (17), and the addition of genotoxic agents such as mitomycin C (MMC) during the exponential phase greatly induces pyocin production (14, 17). Pyocin production is initiated during the SOS response when PrtR autocleavage derepresses the expression of *prtN*. In turn, PrtN binds to the P-box in the promoters of R-, F-, and S-type pyocin genes to activate their expression (7, 18). In addition to pyocin biosynthetic genes, PrtN activates expression of
a lysis cassette coding for several proteins including holin-like and chitinase-like enzymes (7). After pyocin synthesis, the lysis cassette mediates cell lysis, killing the cell and releasing pyocin into the environment.

\textit{P. aeruginosa} infections are often treated with fluoroquinolone antibiotics that inhibit DNA gyrase and topoisomerase IV and strongly induce the SOS response and pyocin production. Research has shown that PrtR-regulated genes increase the sensitivity of \textit{P. aeruginosa} to bacteriostatic and bactericidal concentrations of ciprofloxacin (9, 19). It has been hypothesized that in wild type cells, pyocin synthesis makes cells more susceptible to fluoroquinolones because cell lysis enzymes that accompany pyocin production mediate death (9, 19). Whether the production of pyocins influences the sensitivity of \textit{P. aeruginosa} to other genotoxic agents is not known.

In this report, we investigated how induced expression of PrtR regulated genes determines antimicrobial resistance and genotoxic stress survival. We created a strain in which pyocin synthesis genes were no longer induced along with LexA and PA0906-target genes during the SOS response and found that the absence of pyocin production and cell lysis increased resistance to several antibiotics and enhanced the survival of bacteria exposed to one of several genotoxic agents. Further genetic experiments supported the hypothesis that cell death in ciprofloxacin-treated wild type cells was mediated, in part, by cell lysis genes. In addition, we discovered that cell lysis and R2 pyocin were both required to mediate the death of UV-treated cells. Surprisingly, although \textit{P. aeruginosa} is typically resistant to R2 pyocin, we found that UV light-treated
P. aeruginosa transiently loses its R2 pyocin resistance, most likely because of the strong and rapid down-regulation of genetic determinants of R2 pyocin susceptibility during the SOS response. This created a situation where cells that initially survived exposure to UV irradiation were killed by extracellular R2 pyocin that was produced by some cells during their SOS response. Thus, the induced production of pyocins during the P. aeruginosa SOS responses carries both expected and unexpected costs. We discuss the implications of these findings for the SOS response of P. aeruginosa and related bacteria.

MATERIALS AND METHODS

Bacterial Strains and plasmids. All bacterial strains used in this study are listed in Table 1. Constructs used to genetically manipulate strains were generated by PCR-based methods. Unmarked deletions and point mutations were generated in the wild type PAO1 strain by using the pEX18GM vector and previously published methods (20). Strains with prtR\textsuperscript{S162A} and PA0906\textsuperscript{S153A} mutations were created by replacing the codon of the catalytic serine residue with the codon for an alanine residue. The lysis-defective strain Δ0614 Δ0629 was constructed by deleting codons 8-142 and 12-203 of PA0614 and PA0629, respectively. Δ55 (PA0985) was generated by deleting codons 102-466. For complementation of the Δ0614 Δ0629 strain, a construct consisting of 575 basepairs upstream of the PA0614 translation start site, the PA0614 coding region, an in frame fusion of the first 110 codons of PA0615 with the last 87 codons of PA0628, and the PA0629 coding region was cloned into mini-CTX2 and then integrated into the chromosome at the neutral attB according to previously published methods (21). A PA0614-lacZ translational reporter gene was constructed by fusing 575 basepairs
upstream of the PA0614 translation start site plus 3 codons to a promoterless lacZ in mini-CTX2 (22). This reporter gene was then integrated into the chromosome according to previously published methods (21).

**Bacterial growth conditions and treatment.** Bacteria were grown at 37°C in LB on a roller drum or on LB agar unless otherwise noted. Log phase cultures with an optical density at 600 nm of 0.5-0.6 were used for zone of inhibition, assays on ciprofloxacin (2 μg/ml) and mitomycin (15 μg/ml) susceptibility, UV-irradiation experiments, and profiling reporter gene expression during the SOS response. Drugs concentrations for zone of inhibition assays were as follows: ciprofloxacin, 5 μg; paraquat, 10 μl of 100mM solution; H2O2, 10 μl of 6% solution; mitomycin, 20 μg. UV-treatment was done using a germicidal lamp (General Electric) to irradiate serially dilution of cells spotted on agar. In experiments with R2 pyocin and UV light treatment, 1 μl of purified R2 pyocin was mixed with 10 μl of each serial dilution prior to spotting on agar plates. MICs were determined as previously described in Mueller Hinton broth (23).

**Measurement of reporter gene expression.** Expression of PA0614-lacZ was induced by the addition of 1 μg/ml of ciprofloxacin to broth cultures, and LacZ activity was measured with the Galacto-Light™ kit at the indicated times as previously described (22). Expression of recA::GFP was induced by the addition of ciprofloxacin (1 μg/ml) to cultures grown in tryptic soy broth, and GFP fluorescence was quantified at the indicated times.
**Pyocin induction, isolation and assays.** Pyocin was induced in exponential phase cultures as previously described (24). Briefly, 3 µg/ml of MMC was added to a culture with an optical density at 600 nm of 0.25-0.3, and the culture was further incubated for 3-4 h unless noted otherwise. The culture was then treated for 30 m with DNase I (2 U/ml) to reduce the viscosity. Non-lysed cells and debris from lysed cells were pelleted by centrifugation, and the resulting supernatant containing all pyocin types was used to either purify R2 pyocin as previously described (24) or filtered sterilized and spotted onto a lawn of the pyocin indicator strains (24, 25). To generate a lawn of the indicator strain, a 100 µl solution of a 1000-fold dilution of a saturated culture was plated and allowed to dry prior to the addition of pyocin. To detect the activity of pyocin that accumulated in saturated cultures after overnight incubation, cultures were centrifuged to pellet cells and the supernatant was filter sterilized and applied to the indicator strains. Agar plates containing indicator strains were then incubated overnight. Clearing in the lawn that corresponded with the spotted pyocin preparation is indicative of pyocin activity. The indicator strain 13s is sensitive to only R2 pyocin produced by wild type PAO1 (Table 1 and Figure S1A) (24). The indicator strain PML1516d is sensitive to S2 pyocin in addition to one or more other pyocins produced by wild type PAO1 (Table 1 and Figure S1A) (25, 26). The indicator strain NIH5 is sensitive to F2 pyocin in addition to possibly one or more other pyocins produced by wild type PAO1 (Table 1 and Figure S1A) (25, 27).
Quantitative PCR. UV-treated cells were collected from the agar plate at the indicated times and mixed 1:2 (vol/vol) with RNAprotect bacteria reagent (Qiagen). Fixed cells were then pelleted and lysed in TE buffer (pH 8.0) containing 15 mg/ml of lysozyme and 15 μl of Proteinase K (Qiagen). RNA was then isolated using the RNeasy Plus Mini kit (Qiagen).

cDNA for quantitative PCR (qPCR) experiments was generated as previously described (28). qPCR analysis of cDNA of indicated genes and the internal control gene, *proC* (29), was performed with Power SYBR® Green PCR Master Mix (Life Technologies) on a StepOnePlus™real-time PCR machine (Life Technologies). The 2^−ΔΔCT^ method was used to determine the expression level of indicated genes relative to *proC* expression. Relative gene expression data from three biological replicates were normalized, log transformed, mean centered, and autoscaled (30). Oligonucleotide primers are available upon request.

RESULTS

Autoproteolytic activity of PrtR is required for induction of pyocins during standard culture and genotoxic stress. The autoproteolytic cleavage of PrtR that is mediated by RecA during DNA damage or replication stress is likely critical for pyocin production in *P. aeruginosa* (15, 18). To better understand how pyocins affect genotoxic stress resistance, we constructed a *prtR*<sup>S162A</sup> mutant in order to unlink pyocin production from the LexA- and PA0906-regulated components of the RecA-mediated SOS response.
The mutation in \( prtR^{S162A} \) replaces the codon for the catalytic serine residue of the PrtR autprotease domain with a codon for a catalytically inactive alanine residue. This strategy has been widely used to inactivate serine proteases, including the serine autoprotease of LexA (8, 31). We verified that the expression of \( PA0614p::LacZ \), the first gene of the R2 pyocin biosynthetic operon (7), was not induced in \( prtR^{S162A} \) cells exposed to ciprofloxacin (Figure 1A). We also demonstrated that MMC treatment failed to elicit pyocin production in exponential phase cultures of \( prtR^{S162A} \), as MMC treatment does to wild type cultures (Figure 1B and Figure S1A) (14, 17, 18, 24). In contrast, induced expression of the LexA-regulated \( recA::GFP \) reporter gene was normal in \( prtR^{S162A} \) cells exposed to genotoxic stress (Figure 1C), which showed that the \( prtR^{S162A} \) mutation had no effect on the induction of the LexA regulon during the SOS response.

Although pyocin production in exponential phase cultures is strongly induced by genotoxic agents (14, 17, 18, 24), observations by a number of groups have shown that \( P. aeruginosa \) accumulates low levels of R pyocin in cultures incubated overnight under non-stressed conditions (17, 32, 33). However, it has not been clear whether this accumulation of pyocin under non-stressed conditions is due to PrtR autoproteolysis. We used our \( prtR^{S162A} \) strain to determine if the accumulation of pyocin during standard culture under non-stressed conditions was dependent on PrtR autoproteolysis and found no detectable pyocin activity in the supernatant of \( prtR^{S162A} \) cultures (Figure 1B). Importantly, both the wild type and \( prtR^{S162A} \) strains exhibited similar growth dynamics and similar cell yields after overnight incubation (Figure S2). Thus, PrtR autoproteolysis
is required for the production and accumulation of pyocin under standard culture conditions and as well as pyocin production during genotoxic stress.

Pyocin production affects resistance to antibiotics and genotoxic agents.

Previous research showed that transposon-insertion mutations in the *P. aeruginosa* pyocin biosynthetic locus increase resistance to ciprofloxacin (9, 19). These studies have proposed that the sensitivity of a wild type strain is in part due to genotoxic stress eliciting the induction of pyocin production and cell lysis proteins. Consistent with this idea, we found that the $\text{ prtR}^{S162A}$ strain was more resistant than wild type cells to bacteriostatic (Table S1 and Figure S3A) and bactericidal levels (Figure 2A) of ciprofloxacin. To better understand the role of pyocin production in genotoxic stress resistance, we determined how the $\text{ prtR}^{S162A}$ mutation affected resistance to other genotoxic agents. In addition, we explored whether the $\text{ prtR}^{S162A}$ mutation affected resistance to other antimicrobial stresses.

For genotoxic agents, we focused on MMC and UV light because both of these treatments had previously been shown to induce pyocin production (14). We found that the $\text{ prtR}^{S162A}$ strain was slightly more resistant to MMC than the parent strain in zone of inhibition assays (Figure S3B), and that the $\text{ prtR}^{S162A}$ strain exhibited a modest improvement in survival on agar containing bactericidal concentrations of MMC (Figure 2B). To compare the survival of wild type PAO1 and $\text{ prtR}^{S162A}$ cells exposed to UV light, we treated serial dilutions of each strain spotted on agar plates with increasing amounts of UV irradiation and incubated the agar plates overnight to determine the level of survival.
We observed a striking increase in the survival of \textit{prtR}^{S162A} relative to the wild type parent strain (Figure 2C). Treatment of wild type \textit{P. aeruginosa} with UV light (30 J/m$^2$) reduced colony-forming units (CFUs) by five orders of magnitude whereas the CFUs of the \textit{prtR}^{S162A} strain were only reduced by one order of magnitude (Figure 2C). Thus, blocking pyocin production in \textit{P. aeruginosa} improves survival under conditions of genotoxic stress, such as UV irradiation treatment.

The relationship between pyocin production and resistance to other antimicrobial agents proved to be complex. We found that while the \textit{prtR}^{S162A} and wild type strain displayed a similarly sensitivity to growth inhibition by carbenicillin and tetracycline, the \textit{prtR}^{S162A} strain exhibited a 2-fold higher MIC for every aminoglycoside antibiotic tested (Table S1). The \textit{prtR}^{S162A} strain was also more resistant than the wild type strain to paraquat, a superoxide generator (Figure S3C). This increase in paraquat resistance was not due to an enhanced generalized oxidative stress response because \textit{prtR}^{S162A} cells exhibited a normal level of sensitivity to hydrogen peroxide (Figure S3D). It could be that in our conditions, aminoglycoside and paraquat treatment of \textit{P. aeruginosa} elicit an SOS response, as occurs in aminoglycoside-treated \textit{Vibrio cholerae} (34, 35) and paraquat-treated \textit{E. coli} (36), and that pyocin production has a negative effect. However, arguing against this possibility is the observation that \textit{P. aeruginosa} treated with hydrogen peroxide activates an SOS response and induces pyocin gene synthesis (10). An alternative explanation for the role of PrtR in aminoglycoside and paraquat resistance is that basal expression of genes regulated directly and indirectly by PrtR could have cryptic functions that impact aminoglycoside and paraquat resistance.
PrtR-regulated lysis is a determinant of ciprofloxacin resistance and UV irradiation survival. We further pursued a mechanistic understanding on how pyocin production affects *P. aeruginosa* genotoxic stress resistance. Prior work has shown that RecA-mediated PrtR autoproteolysis derepresses *prtN* expression, and PrtN then massively upregulates pyocin genes expression (18, 19). In addition, PrtN activates the expression of a lysis cassette that mediates cellular lysis and releases the newly synthesized pyocin into the environment (7). Previous research hypothesized that induction of this lysis cassette sensitizes *P. aeruginosa* to genotoxic stress (9). Indeed, artificial over-expression of these lysis genes reduces cell viability (30). We tested this hypothesis by assaying the sensitivity of a lysis impaired-strain to UV irradiation and ciprofloxacin.

We constructed a lysis-impaired strain by deleting the holin-like *PA0614* and lysozyme-like *PA0629* genes thought to mediate lysis (7) and confirmed that the Δ0614 Δ0629 strain produced less extracellular pyocin than wild type cells during genotoxic stress (Figure 3A). We found that, relative to wild type, the Δ0614 Δ0629 strain exhibited a striking increase in survival following UV irradiation treatment (Figure 3B). Genetic complementation of the mutations in the Δ0614 Δ0629 strain restored extracellular pyocin production (Figure 3A) and the degree of killing by UV-irradiation to near that of the wild type strain (Figure 3B). In zone of inhibition assays, the level of ciprofloxacin-resistance of the Δ0614 Δ0629 strain was intermediate between wild type cells (more sensitive) and the *prtR*S162A strain (more resistant) (Figure S4). However, Δ0614 Δ0629
and \(\text{prtR}^{S162A}\) cells were equally resistant to bactericidal concentrations of ciprofloxacin (Figure 3C). These results indicate that \(PA0614\) and \(PA0629\) are genetic determinants of genotoxic stress sensitivity and support the hypothesis PrtR-regulated cell lysis sensitizes \(P.\ aeruginosa\) to genotoxic agents.

**R2 pyocin is a strong determinant of UV irradiation survival.** The marked effect of the \(\text{prtR}^{S162A}\) and \(\Delta0614\ \Delta0629\) mutations on UV-irradiation survival led us to wonder whether a particular toxic pyocin might also contribute to the high level of cell death observed for the wild type strain following UV treatment. We tested for this possibility by assaying the UV-irradiation survival of pyocin mutants. Strikingly, we found that a transposon insertion in the R2 pyocin biosynthetic locus, but not in genes that synthesize S2, S4, S5, and F2 pyocins, substantially increased survival to UV irradiation treatment (Figure 3B). An R2 pyocin deletion mutant exposed to UV light also exhibited striking increase in resistance to killing by UV irradiation similar to that of the \(\text{prtR}^{S162A}\) mutant (Figure 4A below). Taken together, these observations indicate that the poor survival of \(P.\ aeruginosa\) treated with UV light requires both R2 pyocin production and cell lysis functions.

We also assayed the ciprofloxacin resistance of the \(\Delta R2\) strain and found that \(\Delta R2\) cells exposed to a bactericidal concentration of ciprofloxacin did not exhibit an increase in survival (Figure 3C). Thus, R2 pyocin synthesis genes have little to no effect on ciprofloxacin resistance in wild type \(P.\ aeruginosa\).
Self-produced R2 pyocin kills *P. aeruginosa* after UV irradiation. How could R2 pyocin synthesis and cell lysis reduce survival of UV-irradiated *P. aeruginosa*? R2 pyocin is an antimicrobial that targets other strains, and typically the producer strain is resistant (14). However, recent research showed that self-produced R2 pyocin can be lethal under certain conditions (32), raising the possibility that R2 pyocin produced by some cells during their SOS response was toxic to the remaining population that survived the UV-light treatment. We tested for this possibility by using two approaches. In one approach, we mixed ΔR2 cells with wild type cells prior to treatment with UV irradiation. We found that addition of wild type cells significantly reduced survival of the ΔR2 strain (Figure 4A), indicating that R2 pyocin produced and released in response to UV irradiation can act in trans between cells to kill other members of the UV-irradiated population. In the second approach, we directly added purified R2 pyocin to the serial dilutions of ΔR2 cells prior to treatment with UV light and found that purified R2 pyocin also reduced survival of ΔR2 cells (Figure 4B). Importantly, purified R2 pyocin had no effect on the growth and survival of non-treated control cells (Figure 4B). These results indicate that self-produced R2 pyocin was lethal to *P. aeruginosa* cells that survived UV light exposure.

Sensitivity to R2 pyocin is a transient phenotype not controlled by PrtR, *PA0906*, and LexA. Colonies that form from ΔR2 and wild type cells that survive UV irradiation are R2 pyocin resistant, indicating the loss of R2 pyocin resistance following UV light treatment was a transient physiological response. We tested whether the induction of LexA, PA0906, and PrtR regulons during the *P. aeruginosa* SOS response
mediate the loss of R2 pyocin resistance. We used the \( PA0906^{S153A} \), \( lexA^{G86V} \), and \( prtR^{S162A} \) mutations to create double and triple mutants in which two or three regulons of the SOS response could not be induced during genotoxic stress. The \( lexA^{G86V} \) protein is resistant to auto-proteolytic cleavage and the LexA regulon cannot be derepressed in \( lexA^{G86V} \) (37). Importantly, none of these mutations had an affect on the R2 pyocin resistance of \( P.\ aeruginosa \) under non-stress conditions (Figure S1B). Following UV light exposure, we observed R2 pyocin sensitive cells in double mutants (\( prtR^{S162A} PA0906^{S153A} \) and \( prtR^{S162A} lexA^{G86V} \)) and in the triple mutant (\( PA0906^{S153A} lexA^{G86V} \) and \( prtR^{S162A} \)), indicating that induction of genes regulated by PrtR, PA0906, and LexA was not required for the loss of R2 pyocin resistance after UV-irradiation treatment in \( P.\ aeruginosa \) cells (Figure 5). Intriguingly, these data suggest that an as-yet unidentified system is activated during the SOS response that regulates pyocin resistance in \( P.\ aeruginosa \).

**Loss of R2 pyocin resistance after UV irradiation correlates with B-band LPS down-regulation.** In \( P.\ aeruginosa \), the B-band O-antigen confers resistance to R2 pyocin by masking a receptor that lies within the core polysaccharide of LPS (32, 33). Mutations that eliminate B-band O-antigen synthesis unmask this receptor have been shown to confer sensitivity to self-produced R2 pyocin (32). We hypothesized that in UV-irradiated \( P.\ aeruginosa \), the R2 pyocin receptor was transiently unmasked and therefore became accessible to self-produced R2 pyocin. We tested this hypothesis by profiling the expression of genes involved in each distinct step of LPS synthesis prior to and after UV treatment of \( \Delta R2 \) cells. Strikingly, we found that the B-band O-antigen
synthesis genes \textit{wbpJ}, \textit{wbpA}, and \textit{wzx} were rapidly and significantly down-regulated 30 minutes after UV treatment (Figure 6A). At 60 minutes post UV-treatment, expression of \textit{wbpJ} and \textit{wzm} was down-regulated by \textasciitilde10-fold and \textit{wbpA} was down-regulated by 5-fold relative to their expression in non-treated control cultures (Figure 6A). In contrast, the A-band O-antigen synthesis genes \textit{wbpZ} and \textit{wzm} genes were unchanged in expression (Figure 6A). Expression of genes involved in lipid A (\textit{lpxA}) and the core polysaccharide (\textit{waaP}) also decreased following UV irradiation (Figure 6B), although not as rapidly as B-band LPS biosynthesis genes. These data clearly show a surprising differential response in the expression of B-band and A-band O-antigen biosynthesis genes following UV treatment.

We assayed the expression of O-antigen biosynthesis genes in the triple \textit{PA0906}\textsuperscript{S153A} \textit{lexA}\textsuperscript{G86V} \textit{prtR}\textsuperscript{S162A} mutant after UV light exposure and found that B-band LPS O-antigen genes were significantly downregulated (Figure 6C). This result indicates that downregulation of B-band LPS O-antigen genes after UV light exposure is not dependent on RecA-mediated autocleavage of LexA, PrtR, or PA0906.

Previous research has shown that mutation of \textit{wbpA} and \textit{wbpJ} eliminates B-band O-antigen synthesis and confers sensitivity to self-produced R2 pyocin (32). Lower expression of \textit{wpbA} and \textit{wpbJ} likely lowers B-band O-antigen synthesis after UV light treatment. In addition, because both B-band and A-band O-antigens compete for the same site on the core polysaccharide during LPS synthesis (38), reduced B-band O-antigen synthesis caused by \textit{wpbA} and \textit{wpbJ} down-regulation was likely compounded by the
relatively high expression of A-band O-antigen synthesis genes. We propose that reduced
B-band LPS synthesis after UV treatment produces patches of LPS where R2 pyocin can
access its receptor.

DISCUSSION

Pyocins are produced by nearly all *P. aeruginosa* strains (14), an indication of their
importance for this species. However, our results clearly show that pyocin biosynthesis
carries a significant cost for *P. aeruginosa*. Induction of PrtR regulated genes impairs
survival during genotoxic stress, lowers resistance to clinically important antibiotics, and
lowers resistance to an agent that causes oxidative stress. Our study has shown that there
are at least two distinct mechanisms by which PrtR regulated pyocin production affects
resistance to genotoxic stress: i) lysis genes induced following PrtR autocleavage reduce
resistance to bactericidal levels of ciprofloxacin and ii) production of extracellular R2
pyocin is lethal to cells that initially survive UV treatment. It is likely that the many
bacterial species that produce bacteriocins as part of their SOS response (39) incur a
biological cost similar to the one we described here during severe genotoxic stress.

LexA and PrtR are conserved in *P. aeruginosa* and it is likely that each regulator
simultaneously interacts with the RecA coprotease during the SOS response. The fact that
genes induced by LexA autocleavage enhance survival and genes induced by PrtR
autocleavage reduce survival raises the question of how *P. aeruginosa* balances the
induction of these two opposing responses during genotoxic stress. Based on research in
E. coli on the autocleavage of LexA and CI phage repressor, we speculate that the rates of LexA and PrtR autocleavage during genotoxic stress differ and that the different rates of LexA and PrtR autocleavage determine which response is induced during a genotoxic event. In E. coli lysogens that harbor lambda prophage, the RecA coprotease also mediates autoproteolysis of two regulators, LexA and CI. However, despite both being targeted by the RecA coprotease, LexA undergoes autoproteolysis at a faster rate than the CI repressor (40). The different rates of autoproteolytic cleavage in vivo reflect the strength of interaction between the site of cleavage and protease active site, and the strength of this interaction is greater in LexA than it is in CI repressor (40, 41). Since the PrtR regulator is orthologous to a phage repressor these findings may have some relevance to the P. aeruginosa SOS response. If PrtR has retained the slower rate of self-cleavage found in its ancestors, then PrtR autocleavage would be slower than LexA autocleavage. With this system, only the LexA regulon would be induced during an acute genotoxic event whereas the PrtR-regulated genes would only be strongly induced if genotoxic stress was sustained. It would be interesting to carry out at study of the relative rates of autocleavage of P. aeruginosa LexA, PrtR, and PA0906 and to analyze the kinetics of the induction of their regulons at different levels of genotoxic stress.

Unexpectedly, we found that downregulation of B-band O-antigen genes after UV light treatment is not dependent on cleavage of LexA, PrtR, and PA0906, suggesting that another pathway that responds to UV light (or the effects of UV light) regulates B-band O-antigen synthesis genes. Interestingly, ciprofloxacin and hydrogen peroxide treatments also cause a modest downregulation of some B-band O-antigen synthesis genes (8-10),
suggesting that this putative pathway responds to other agents that cause DNA damage. Thus, this as yet unidentified regulator could represent an important new DNA damage response pathway in *P. aeruginosa*. Moreover, because B-band O-antigen is required for complement resistance in humans (42, 43), understanding how B-band O-antigen genes are regulated in response to stress could potentially have clinically and therapeutic implications. Determining how UV light treatment affects expression of B-band O-antigen genes will be the subject of future work.

Our surprising discovery that *P. aeruginosa* loses resistance to R2 pyocin following UV treatment indicates that there is a risk for bacteria that produce high molecular weight (HMW) or phage tail-like bacteriocins. B-band O-antigen synthesis confers resistance to R2 pyocin, and loss of B-band O-antigen severely impairs growth (32). Unlike the immunity genes for low molecular weight S-type pyocins, which are genetically linked and co-expressed, the genes coding for the synthesis of B-band O-antigen are genetically distinct from genes that synthesize R2 pyocin (14). Our work demonstrates that these genes are also transcriptionally regulated by distinct mechanisms and that R2 pyocin can be lethal in conditions that down-regulate B-band O-antigen synthesis genes. This raises the possibility that R2 pyocin might restrict the conditions in which *P. aeruginosa* can grow because expression of B-band O-antigen synthesis genes must be maintained to resist R2-pyocin-mediated killing. Many bacteria produce HMW or phage tail-like bacteriocins (44-52) and it is likely that the genes that confer resistance and synthesize the HMW bacteriocin are not genetically linked. These species may also have to balance
the benefits of producing HMW bacteriocins with the risks that may pose under certain conditions.
ACKNOWLEDGMENTS

We thank Colin Manoil for transposon mutant strains, John Govan, Dean Scholl, and Suphan Bakkal for pyocin indicator strains, and the Walker lab for helpful discussion.

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REFERENCES


quantification studies of Pseudomonas aeruginosa by real-time quantitative RT-PCR. Journal of Medical Microbiology 52:403-408.


**Figure Legends**

**Figure 1.** Autoproteolytic activity of PrtR is required for pyocin production. (A) Expression of *PA0614p-lacZ* translational reporter gene in *prtRS162A* and the parent strain treated with 1 μg/ml ciprofloxacin. Error bars indicate SD. (B) Production of pyocin by *prtRS162A* and the parent strain. Filtered supernatant from cultures treated with MMC (3 μg/ml) and from liquid cultures incubated overnight were spotted on lawns of the indicator strains 13s, PML1516d, and NIH5. Clearing of the bacterial lawn is indicative of pyocin-mediated death. See materials and methods and Figure S1A for information on the specificity of indicator strains for different pyocin types. (C) Expression of *recAp::GFP* transcriptional reporter gene in *prtRS162A, lexAG86V*, and the parent strain treated with 1 μg/ml ciprofloxacin. LexA<sup>G86V</sup> protein is resistant to auto-proteolytic cleavage (37). Error bars indicate SD.

**Figure 2.** Autoproteolytic activity of PrtR reduces survival during genotoxic stress. (A) Total CFUs in cultures of wild type PAO1 and *prtRS162A* treated with a bactericidal concentration of ciprofloxacin (2 μg/ml). Error bars indicate SD. (B) Total CFUs of wild type PAO1 and *prtRS162A* on agar plates containing 0 and 15 μg/ml of MMC after overnight incubation. Error bars indicate SD. (C) Surviving CFUs of wild type PAO1 and *prtRS162A* after UV treatment. Cultures were serially diluted 10-fold, spotted on agar, and treated with UV light at the indicated doses. Surviving CFUs determined after overnight incubation.
Figure 3. PrtR-regulated lysis and R2 pyocin genes are determinants of UV-irradiation survival. (A) Production of extracellular pyocin in cultures treated with 3 μg/ml of mitomycin C for 2 h. Clearing of the bacterial lawn of the indicator strains is indicative of pyocin-mediated death. (B) Surviving CFUs of the lysis-defective Δ0614 Δ0629 strain and pyocin mutant strains after UV-treatment (30 J/m²). Cultures were serially diluted 10-fold, spotted on agar, and treated with UV light at the indicated doses. Surviving CFUs were determined after overnight incubation. S2−, PA1150-H12::ISlacZ/hah allele; S4−, PA3866-F01::ISlacZ/hah allele; F2−, PA0633-E12::ISlacZ/hah allele; R1− PA0625-H05::ISlacZ/hah allele (C) Total CFUs in cultures of treated with a bactericidal concentration of ciprofloxacin (2 μg/ml). Error bars indicate SD.

Figure 4. Extracellular R2 pyocin is lethal to cells that initially survive UV treatment. (A) Survival of wild type, ΔR2, and 1:1 mixture of wild type:ΔR2 after UV treatment (30 J/m²). (B) Survival of UV-irradiated ΔR2 cells with or without purified R2 pyocin. Cultures were serially diluted 10-fold, mixed 1:1 with saline or saline with purified R2 pyocin, and spotted on agar prior to UV treatment. Surviving CFUs were determined after overnight incubation. Note ΔR2 cells not treated with UV light were resistant to purified R2 pyocin.

Figure 5. Loss of R2 pyocin resistance after UV treatment is not regulated by RecA-stimulated cleavage of PrtR, LexA, and PA0906 during the SOS response. Cultures were serially diluted 10-fold, mixed 1:1 with saline or saline with purified R2 pyocin, and
spotted on agar prior to treatment with UV (10 J/m²). Surviving CFUs were determined after overnight incubation.

Figure 6. Relative expression of B-band and A-band O-antigen synthesis genes after treatment of wild type cells with UV (15 J/m²) (A) Relative expression of A-band synthesis genes (wbpZ, rmd) and B-band synthesis genes (wbpJ, wbpA, wzx) in wild type cells on agar. Non-treated control cells were incubated on agar for 15 minutes. Error bars indicate the 95% confidence interval. (B) Relative expression of lpxA and waaP in wild type cells on agar. Error bars indicate the 95% confidence interval. (C) Relative expression of A-band synthesis genes (wbpZ, rmd) and B-band synthesis genes (wbpJ, wbpA) in \textit{PA0906}^{S153A} \textit{lexA}^{G86V} \textit{prtR}^{S162A} cells on agar. Non-treated control cells were incubated on agar for 15 minutes. Error bars indicate the 95% confidence interval.
Table 1. Bacterial strains and plasmids used in this study

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<thead>
<tr>
<th>Strain name</th>
<th>Relevant characteristics</th>
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<tr>
<td>Wild type (parent strain)</td>
<td>Wild type <em>P. aeruginosa</em> PAO1</td>
<td>B. Iglewski</td>
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<td><em>prtR</em>&lt;sup&gt;S162A&lt;/sup&gt;</td>
<td>Endogenous <em>prtR</em> has been replaced with <em>prtR</em>&lt;sup&gt;S162A&lt;/sup&gt;, which produces a proteolytically inactive transcriptional regulator</td>
<td>This study</td>
</tr>
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<td><em>prtR</em>&lt;sup&gt;S162A&lt;/sup&gt; <em>PA0906</em>&lt;sup&gt;S153A&lt;/sup&gt;</td>
<td>Endogenous <em>PA0906</em> has been replaced with <em>PA0906</em>&lt;sup&gt;S153A&lt;/sup&gt;, which produces a proteolytically inactive transcriptional regulator</td>
<td>This study</td>
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<td><em>prtR</em>&lt;sup&gt;S162A&lt;/sup&gt; <em>lexA</em>&lt;sup&gt;G86V&lt;/sup&gt;</td>
<td><em>LexA</em>&lt;sup&gt;G86V&lt;/sup&gt; is resistant to autoproteolytic cleavage.</td>
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<td><em>PA0906</em>&lt;sup&gt;S153A&lt;/sup&gt;</td>
<td>Wild type alleles replaced</td>
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<td><strong>S2</strong>-</td>
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<td>(53)</td>
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<td><strong>AR2</strong></td>
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<td><strong>APA0614</strong></td>
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<td><strong>PML1516d</strong></td>
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<td>(25)</td>
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<tr>
<td><strong>NIH5</strong></td>
<td>Indicator for pyocin F1, F2, and F3</td>
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<tr>
<td><strong>13s</strong></td>
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<th>Plasmid name</th>
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<td>PA0614-lacZ</td>
<td><em>PA0614-lacZ</em> translational fusion in CTX2, Tc&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
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<tr>
<td><em>recA</em>::GFP</td>
<td><em>recA</em> promoter in front of a promoterless GFP in pUCP18</td>
<td>(37)</td>
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A table shows the indicator strains and their corresponding producer strains:

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<th>Indicator</th>
<th>Producer Strain</th>
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<tr>
<td>13S (R2 pyocin)</td>
<td>PAO1 Δ0614 Δ0614/+ Δ0629 Δ0629/+</td>
</tr>
<tr>
<td>NIH5 (F-pyocin)</td>
<td>PAO1 Δ0614 Δ0629 Δ0629/+</td>
</tr>
</tbody>
</table>

A graph shows the total CFUs over time for different strains:

- ♣PAO1
- ♣Δ0614
- ♣Δ0629
- ♣ΔR2
- ♣prtR<sub>S162A</sub>

Surviving CFUs for different strains:
Supplementary Figure S1. Pyocin sensitivity of indicator strains and the triple

$PA0906^{S153A}$ lex$A^{G86V}$ prt$R^{S162A}$ mutant. (A) Production of pyocin by wild type and indicated pyocin mutants. Filtered supernatant from cultures treated with MMC (3 μg/ml) was spotted on lawns of an indicator strain. Clearing of the bacterial lawn is indicative of pyocin-mediated death. Note that strain 13S is a specific indicator of R2 pyocin activity. PML1516d and NIH5 are sensitive to more than one pyocin produced by PAO1. (B) Strain $PA0906^{S153A}$ lex$A^{G86V}$ prt$R^{S162A}$ is resistant to R2 pyocin. $PA0906^{S153A}$ lex$A^{G86V}$

prt$R^{S162A}$ cells were mixed 1:1 (V/V) with supernatant containing pyocin produced by MMC-treated PAO1 (+R2) and ΔR2 (negative control) cultures. The mixture was then spotted agar plates. Confluent growth of the spot after overnight incubation indicates resistance to pyocin.
Supplementary Figure S2. Growth curves for the parental PAO1 and \(prtR^{S162A}\) strains. Cultures were inoculated with cells derived from an agar plate and incubated in a baffled flask on a platform shaker. Each data point represents the average of three biological replicates. Error bars indicate SD.
Supplementary Figure S3. Autoproteolytic activity of PrtR reduces resistance to agents that cause or induce DNA damage. (A) Zone of growth inhibition of indicated strains around a filter disk on which 5 μg of ciprofloxacin was applied. Error bars indicate SD. (B) Zone of growth inhibition of indicated strains around a filter disk on which 20 μg of mitomycin C was applied. Error bars indicate SD. (C) Zone of growth inhibition of indicated strains around a filter disk on which 10 μl of 100 mM paraquat was applied. Error bars indicate SD. (D) Zone of growth inhibition of indicated strains around a filter disk on which 10 μl of 6% H₂O₂ solution was applied. Error bars indicate SD.
Supplementary Figure S4. Ciprofloxacin resistance of strains Δ0614 Δ0629. (A) Zone of growth inhibition of indicated strains around a filter disk on which 5 μg of ciprofloxacin was applied. Error bars indicate SD.
Table S1. Minimal inhibitory concentration of several antibiotics for \( \text{prtR}^{S162A} \), \( PA0906^{S153A} \), and PAO1 strains.

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<tr>
<th></th>
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<th>Tobramycin</th>
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<th>Carbenicillin</th>
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<tr>
<td>PAO1</td>
<td>0.75 µg/ml</td>
<td>0.25 µg/ml</td>
<td>0.25 µg/ml</td>
<td>64 µg/ml</td>
<td>16 µg/ml</td>
<td>10 µg/ml</td>
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<tr>
<td>( \text{prtR}^{S162A} )</td>
<td>2.0 µg/ml</td>
<td>0.5 µg/ml</td>
<td>0.5 µg/ml</td>
<td>128 µg/ml</td>
<td>16 µg/ml</td>
<td>10 µg/ml</td>
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<tr>
<td>( PA0906^{S153A} )</td>
<td>0.75 µg/ml</td>
<td>0.5 µg/ml</td>
<td>0.5 µg/ml</td>
<td>128 µg/ml</td>
<td>16 µg/ml</td>
<td>10 µg/ml</td>
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