

# Biological Cost of Pyocin Production during the SOS Response in *Pseudomonas aeruginosa*

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**LexA and two structurally related regulators, PrtR and PA0906, coordinate the *Pseudomonas 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 lipopolysaccharide synthesis genes that are required for resistance to R2 pyocin. Our results demonstrate that pyocin production during the *P. aeruginosa* SOS response carries both expected and unexpected costs.**

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 with *Escherichia coli*, where this response functions primarily to increase the cell's ability to cope with DNA damage and replication stress (1, 2). The SOS response of *E. coli* is coordinated by the LexA protein, which directly represses the expression of genes that bear a LexA-binding motif. During growth under nonstressed 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 resistance 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 coexpressed protein mediates cell lysis (6). Colicins are intraspecies antimicrobial bacteriocins that target nonrelated individuals. Related individuals are immune to colicin because of an immunity protein encoded by a plasmid.

The SOS response in *Pseudomonas aeruginosa* is more complex than that of *E. coli*, as it is coordinated not only by LexA but also by two structurally related regulators, PrtR and PA0906 (7–11). These regulators are encoded by 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-stranded DNA nucleoprotein fila-

ments 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 strongly 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 the 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.

*P. aeruginosa* infections are often treated with fluoroquinolone

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TABLE 1 Bacterial strains and plasmids used in this study

<i>P. aeruginosa</i> strain or plasmid	Relevant characteristic(s)	Source or reference
<b>Strains</b>		
PAO1	Wild type (parent strain)	B. Iglewski
<i>prtR</i> <sup>S162A</sup> mutant	Endogenous <i>prtR</i> replaced with <i>prtR</i> <sup>S162A</sup> , producing proteolytically inactive transcriptional regulator	This study
<i>prtR</i> <sup>S162A</sup> <i>PA0906</i> <sup>S153A</sup> mutant	Endogenous <i>PA0906</i> replaced with <i>PA0906</i> <sup>S153A</sup> , producing proteolytically inactive transcriptional regulator	This study
<i>prtR</i> <sup>S162A</sup> <i>lexA</i> <sup>G86V</sup> mutant	<i>LexA</i> <sup>G86V</sup> resistant to autoproteolytic cleavage	This study
<i>prtR</i> <sup>S162A</sup> <i>lexA</i> <sup>G86V</sup> <i>PA0906</i> <sup>S153A</sup> mutant	Wild-type alleles replaced	This study
S2-mutant	S2 pyocin transposon insertion mutant with PA1150-H12::ISlacZ/hah allele from PW3083, Tc <sup>r</sup>	53
S4-mutant	S4 pyocin transposon insertion mutant with PA3866-F01::ISlacZ/hah allele from PW7522, Tc <sup>r</sup>	53
F2-mutant	F2 pyocin transposon insertion mutant with PA0633-E12::ISlacZ/hah allele from PW2150	53
ΔS5 mutant	Unmarked deletion of <i>PA0985</i>	This study
R2-mutant	R1 pyocin transposon insertion mutant with PA0625-H05::ISlacZ/hah allele from PW2138, Tc <sup>r</sup>	53
ΔR2 mutant	Unmarked deletion of PA0615-PA0628	32
ΔPA0614 ΔPA0629 mutant	Clean deletion of codons 8-142 and 12-203 from PA0614 and PA0629, respectively	This study
ΔPA0614/+ ΔPA0629/+ mutant	Genetic complementation of lysis, PA0614p::PA0614-PA0629 placed at <i>attB</i> site, Tc <sup>r</sup>	This study
PML1516d	Indicator for pyocin S1, S2, and AP41	25
NIH5	Indicator for pyocin F1, F2, and F3	25
13s	Indicator strain for R1 to R5 pyocins	24
<b>Plasmids</b>		
PA0614-lacZ	PA0614- <i>lacZ</i> translational fusion in CTX2, Tc <sup>r</sup>	This study
recAp::GFP	<i>recA</i> promoter in front of promoterless gene for GFP in pUCP18	37

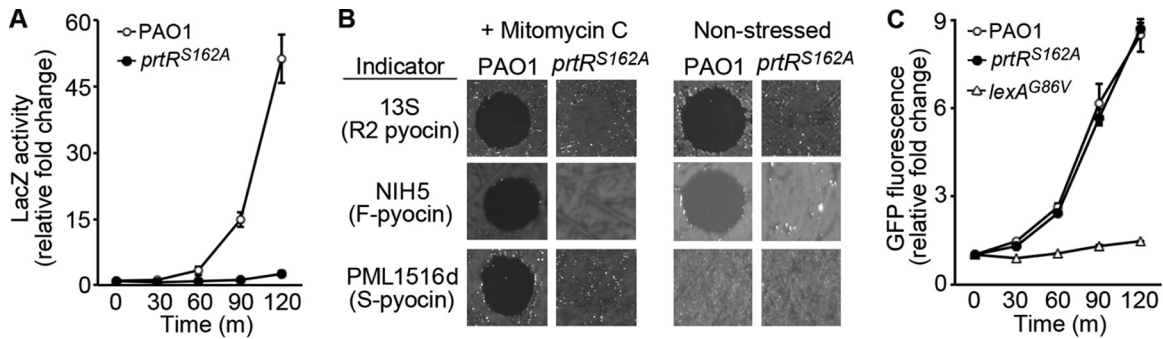
antibiotics that inhibit DNA gyrase and topoisomerase IV and strongly induce the SOS response and pyocin production. Research has shown that PrtR-regulated genes increase the sensitivity of *P. aeruginosa* to bacteriostatic and bactericidal concentrations of ciprofloxacin (9, 19). It has been hypothesized 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 *P. aeruginosa* to other genotoxic agents is not known.

In this study, 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 the death of 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 *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 downregulation 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 of the 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 the *prtR*<sup>S162A</sup> and *PA0906*<sup>S153A</sup> 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 to 142 and 12 to 203 of *PA0614* and *PA0629*, respectively. ΔS5 (*PA0985*) was generated by deleting codons 102 to 466. For complementation of the Δ0614 Δ0629 mutant strain, a construct consisting of the 575 bp 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* site according to previously published methods (21). A *PA0614-lacZ* translational reporter gene was constructed by fusing the 575 bp upstream of the *PA0614* translation start site plus three codons to 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 to 0.6 were used for zone-of-inhibition assays to determine ciprofloxacin (2 μg/ml) and mitomycin (15 μg/ml) susceptibility, UV irradiation experiments, and profiling reporter gene expression during the SOS response. The drug concentrations used for zone-of-inhibition assays were as follows: ciprofloxacin, 5 μg; paraquat, 10 μl of a 100 mM solution; H<sub>2</sub>O<sub>2</sub>, 10 μl of a 6% solution; mitomycin, 20 μg. UV treatment was done by using a germicidal lamp (General Electric) to irradiate serial dilutions of cells spotted onto agar plates. 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 onto agar plates. MICs were determined in Mueller-Hinton broth as previously described (23).



**FIG 1** Autoproteolytic activity of PrtR is required for pyocin production. (A) Expression of the *PA0614p::lacZ* translational reporter gene in the *prtR*<sup>S162A</sup> mutant and the parent strain treated with 1  $\mu$ g/ml ciprofloxacin. Error bars indicate standard deviations. (B) Production of pyocin by the *prtR*<sup>S162A</sup> mutant and the parent strain. Filtered supernatant from cultures treated with MMC (3  $\mu$ g/ml) and from liquid cultures incubated overnight were spotted onto lawns of the indicator strains 13s, PML1516d, and NIH5. Clearing of the bacterial lawn is indicative of pyocin-mediated death (see Materials and Methods; see Fig. S1A in the supplemental material for information on the specificity of indicator strains for different pyocin types). (C) Expression of the *recAp::GFP* transcriptional reporter gene in the *prtR*<sup>S162A</sup> mutant, the *lexA*<sup>G86V</sup> mutant, and the parent strain treated with 1  $\mu$ g/ml ciprofloxacin. LexA<sup>G86V</sup> protein is resistant to autoproteolytic cleavage (37). Error bars indicate standard deviations.

**Measurement of reporter gene expression.** Expression of *PA0614-lacZ* was induced by the addition of 1  $\mu$ g/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 *recAp::GFP* was induced by the addition of ciprofloxacin (1  $\mu$ g/ml) to cultures grown in tryptic soy broth, and GFP fluorescence was quantified at the times indicated.

**Pyocin induction, isolation, and assays.** Pyocin was induced in exponential-phase cultures as previously described (24). Briefly, 3  $\mu$ g/ml of MMC was added to a culture with an optical density at 600 nm of 0.25 to 0.3 and the culture was further incubated for 3 to 4 h unless noted otherwise. The culture was then treated for 30 min with DNase I (2 U/ml) to reduce its viscosity. Nonlysed cells and debris from lysed cells were pelleted by centrifugation, and the resulting supernatant containing all of the pyocin types was either used to purify R2 pyocin as previously described (24) or filter sterilized and spotted onto a lawn of the pyocin indicator strain (24, 25). To generate a lawn of the indicator strain, a 100- $\mu$ l solution of a 1,000-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 corresponding to the spotted pyocin preparation is indicative of pyocin activity. Indicator strain 13s is sensitive to only R2 pyocin produced by wild-type PAO1 (Table 1; see Fig. S1A in the supplemental material) (24). Indicator strain PML1516d is sensitive to S2 pyocin in addition to one or more other pyocins produced by wild-type PAO1 (Table 1; see Fig. S1A) (25, 26). Indicator strain NIH5 is sensitive to F2 pyocin in addition to possibly one or more of the other pyocins produced by wild-type PAO1 (Table 1; see Fig. S1A) (25, 27).

**Quantitative PCR (qPCR).** 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 Tris-EDTA buffer (pH 8.0) containing 15 mg/ml of lysozyme and 15  $\mu$ l of proteinase K (Qiagen). RNA was then isolated with the RNeasy Plus mini-kit (Qiagen).

cDNA for qPCR experiments was generated as previously described (28). qPCR analysis of cDNA of the 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  $2^{-\Delta\Delta CT}$  method was used to determine the expression levels of the indicated genes relative to that of *proC*. Relative gene expression data from three biological replicates were normalized, log trans-

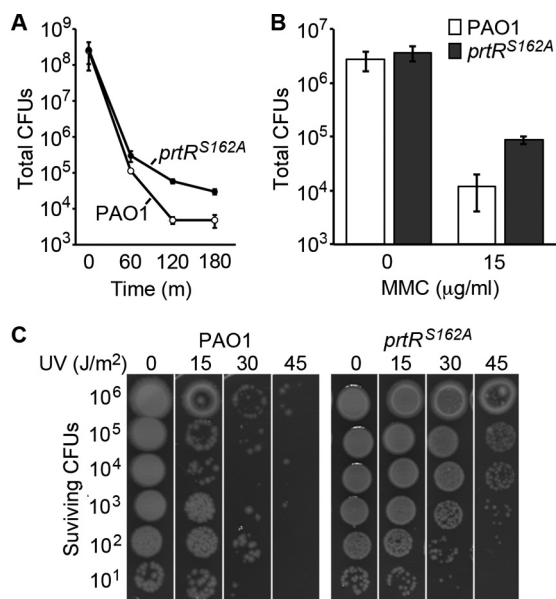
formed, mean centered, and autoscaled (30). The sequences of the oligonucleotide primers used are available upon request.

## 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 *prtR*<sup>S162A</sup> mutation replaces the codon for the catalytic serine residue of the PrtR autoprotease 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*<sup>S162A</sup> mutant cells exposed to ciprofloxacin (Fig. 1A). We also demonstrated that MMC treatment failed to elicit pyocin production in exponential-phase *prtR*<sup>S162A</sup> mutant cultures, as MMC treatment does to wild-type cultures (Fig. 1B; see Fig. S1A in the supplemental material) (14, 17, 18, 24). In contrast, induced expression of the LexA-regulated *recAp::GFP* reporter gene was normal in *prtR*<sup>S162A</sup> mutant cells exposed to genotoxic stress (Fig. 1C), which showed that the *prtR*<sup>S162A</sup> 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 nonstressed conditions (17, 32, 33). However, it has not been clear whether this accumulation of pyocin under nonstressed conditions is due to PrtR autoproteolysis. We used our *prtR*<sup>S162A</sup> mutant strain to determine if the accumulation of pyocin during standard culture under nonstressed conditions is dependent on PrtR autoproteolysis and found no detectable pyocin activity in the supernatant of *prtR*<sup>S162A</sup> mutant cultures (Fig. 1B). Importantly, the wild-type and *prtR*<sup>S162A</sup> mutant strains exhibited similar growth dynamics and similar cell yields after overnight incubation (see





**FIG 2** Autoproteolytic activity of PrtR reduces survival during genotoxic stress. (A) Total CFU counts in cultures of wild-type PAO1 and the *prtR*<sup>S162A</sup> mutant treated with a bactericidal concentration of ciprofloxacin (2 µg/ml). Error bars indicate standard deviations. (B) Total CFU counts of wild-type PAO1 and the *prtR*<sup>S162A</sup> mutant on agar plates containing 0 and 15 µg/ml of MMC after overnight incubation. Error bars indicate standard deviations. (C) Surviving CFU counts of wild-type PAO1 and the *prtR*<sup>S162A</sup> mutant after UV treatment. Cultures were serially diluted 10-fold, spotted onto agar, and treated with UV light at the indicated doses. Surviving CFU counts were determined after overnight incubation.

Fig. S2 in the supplemental material). Thus, PrtR autoproteolysis is required for the production and accumulation of pyocin under standard culture conditions, as well as for 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 due in part to genotoxic stress eliciting the induction of pyocin production and cell lysis proteins. Consistent with this idea, we found that the *prtR*<sup>S162A</sup> mutant strain was more resistant than the wild type to bacteriostatic (see Table S1 and Fig. S3A in the supplemental material) and bactericidal (Fig. 2A) levels of ciprofloxacin. To better understand the role of pyocin production in genotoxic stress resistance, we determined how the *prtR*<sup>S162A</sup> mutation affects resistance to other genotoxic agents. In addition, we explored whether the *prtR*<sup>S162A</sup> mutation affects resistance to other antimicrobial stresses.

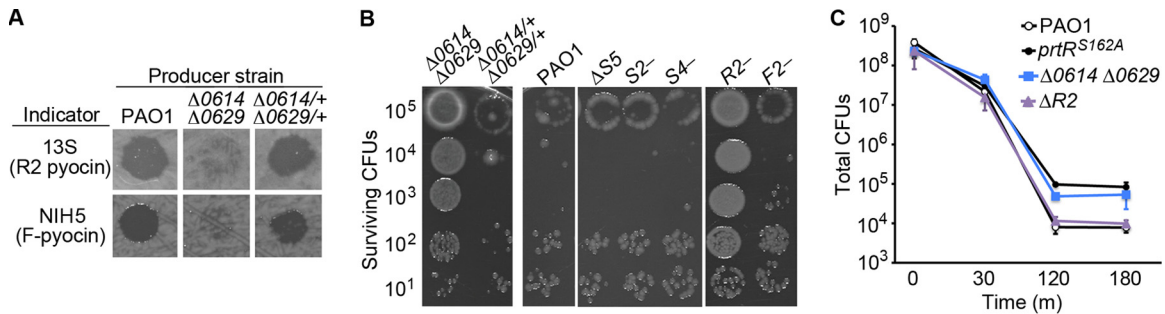
We focused on the genotoxic agents MMC and UV light because both of them had previously been shown to induce pyocin production (14). We found that the *prtR*<sup>S162A</sup> mutant strain was slightly more resistant to MMC than the parent strain in zone-of-inhibition assays (see Fig. S3B in the supplemental material) and that the *prtR*<sup>S162A</sup> mutant strain exhibited a modest improvement in survival on agar containing bactericidal concentrations of MMC (Fig. 2B). To compare the survival of wild-type PAO1 and *prtR*<sup>S162A</sup> mutant cells exposed to UV light, we treated serial dilutions of each strain spotted onto agar plates with increasing amounts of UV radiation and incubated the agar plates overnight

to determine the level of survival. We observed a striking increase in the survival of the *prtR*<sup>S162A</sup> mutant relative to that of the wild-type parent strain (Fig. 2C). Treatment of wild-type *P. aeruginosa* with UV light (30 J/m<sup>2</sup>) reduced the CFU count by 5 orders of magnitude, whereas the CFU count of the *prtR*<sup>S162A</sup> mutant strain was reduced by only 1 order of magnitude (Fig. 2C). Thus, blocking pyocin production in *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 *prtR*<sup>S162A</sup> mutant and the wild-type strain were similarly sensitive to growth inhibition by carbenicillin and tetracycline, the *prtR*<sup>S162A</sup> mutant strain exhibited a 2-fold higher MIC of every aminoglycoside antibiotic tested (see Table S1 in the supplemental material). The *prtR*<sup>S162A</sup> mutant strain was also more resistant than the wild-type strain to paraquat, a superoxide generator (see Fig. S3C in the supplemental material). This increase in paraquat resistance was not due to an enhanced generalized oxidative stress response because *prtR*<sup>S162A</sup> mutant cells exhibited a normal level of sensitivity to hydrogen peroxide (see Fig. S3D). It could be that under our conditions, aminoglycoside and paraquat treatment of *P. aeruginosa* elicited an SOS response, as occurs in aminoglycoside-treated *Vibrio cholerae* (34, 35) and paraquat-treated *E. coli* (36), and that pyocin production has a negative effect. However, arguing against this possibility is the observation that *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 the 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 of 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 gene 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 overexpression 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  $\Delta 0614 \Delta 0629$  mutant strain produced less extracellular pyocin than wild-type cells during genotoxic stress (Fig. 3A). We found that, relative to the wild type, the  $\Delta 0614 \Delta 0629$  mutant strain exhibited a striking increase in survival following UV irradiation treatment (Fig. 3B). Genetic complementation of the mutations in the  $\Delta 0614 \Delta 0629$  mutant strain restored extracellular pyocin production (Fig. 3A) and killing by UV irradiation to levels near those of the wild-type strain (Fig. 3B). In zone-of-inhibition assays, the level of ciprofloxacin resistance of the  $\Delta 0614 \Delta 0629$  mutant strain was intermediate between those of the wild type (more sensitive) and the *prtR*<sup>S162A</sup> mutant strain (more resistant) (see Fig. S4 in the supplemental material). However,  $\Delta 0614 \Delta 0629$  and *prtR*<sup>S162A</sup> mutant cells were equally



**FIG 3** PrtR-regulated lysis and R2 pyocin genes are determinants of UV irradiation survival. (A) Production of extracellular pyocin in cultures treated with 3  $\mu$ g/ml of MMC for 2 h. Clearing of the bacterial lawn of the indicator strains is indicative of pyocin-mediated death. (B) Surviving CFU counts of the lysis-defective  $\Delta 0614 \Delta 0629$  mutant strain and pyocin mutant strains after UV treatment (30 J/m<sup>2</sup>). Cultures were serially diluted 10-fold, spotted onto agar, and treated with UV light. Surviving CFU counts 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 CFU counts in cultures of wild-type PAO1 and  $prtR^{S162A}$ ,  $\Delta 0614 \Delta 0629$ , and  $\Delta R2$  mutant cells treated with a bactericidal concentration of ciprofloxacin (2  $\mu$ g/ml). Error bars indicate standard deviations.

resistant to bactericidal concentrations of ciprofloxacin (Fig. 3C). These results indicate that *PA0614* and *PA0629* are genetic determinants of genotoxic stress sensitivity and support the hypothesis that PrtR-regulated cell lysis sensitizes *P. aeruginosa* to genotoxic agents.

#### R2 pyocin is a strong determinant of UV irradiation survival.

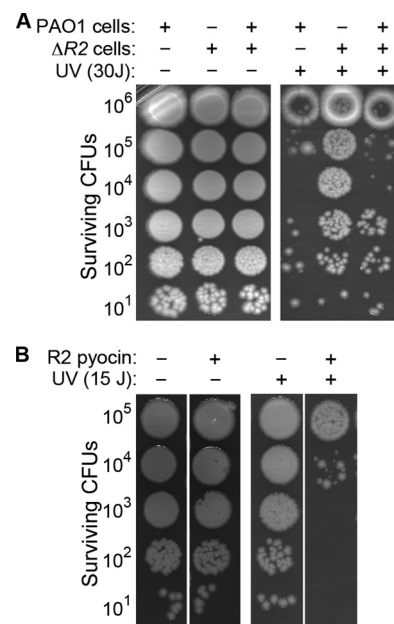
The marked effect of the  $prtR^{S162A}$  and  $\Delta 0614 \Delta 0629$  mutations on UV irradiation survival led us to wonder whether a particular toxic pyocin might also contribute to the high level of cell death observed in the wild-type strain following UV treatment. We tested for this possibility 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 after UV irradiation treatment (Fig. 3B). An R2 pyocin deletion mutant exposed to UV light also exhibited a striking increase in resistance to killing by UV irradiation similar to that of the  $prtR^{S162A}$  mutant (Fig. 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  $\Delta R2$  mutant strain and found that  $\Delta R2$  mutant cells exposed to a bactericidal concentration of ciprofloxacin did not exhibit an increase in survival (Fig. 3C). Thus, R2 pyocin synthesis genes have little to no effect on ciprofloxacin resistance in wild-type *P. aeruginosa*.

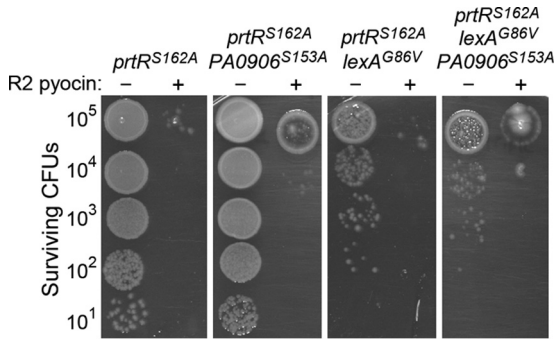
**Self-produced R2 pyocin kills *P. aeruginosa* after UV irradiation.** How could R2 pyocin synthesis and cell lysis reduce the survival of UV-irradiated *P. aeruginosa*? R2 pyocin is an antimicrobial that targets other strains, and typically the producer strain is resistant (14). However, recent research 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 UV light treatment. We tested for this possibility by using two approaches. In one approach, we mixed  $\Delta R2$  mutant cells with wild-type cells prior to treatment with UV irradiation. We found that the addition of wild-type cells significantly reduced the survival of the  $\Delta R2$  mutant strain (Fig. 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 puri-

fied R2 pyocin to the serial dilutions of  $\Delta R2$  mutant cells prior to treatment with UV light and found that purified R2 pyocin also reduced the survival of  $\Delta R2$  mutant cells (Fig. 4B). Importantly, purified R2 pyocin had no effect on the growth and survival of nontreated control cells (Fig. 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  $\Delta R2$  mutant and wild-type cells that survive UV irradiation are R2 pyocin resistant, indicating that the loss of R2 pyocin resistance



**FIG 4** Extracellular R2 pyocin is lethal to cells that initially survive UV treatment. (A) Survival of the wild type, the  $\Delta R2$  mutant, and a 1:1 mixture of the wild type and the  $\Delta R2$  mutant after UV treatment (30 J/m<sup>2</sup>). (B) Survival of UV-irradiated  $\Delta R2$  mutant 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 onto agar prior to UV treatment. Surviving CFU counts were determined after overnight incubation. Note that  $\Delta R2$  mutant cells not treated with UV light were resistant to purified R2 pyocin.



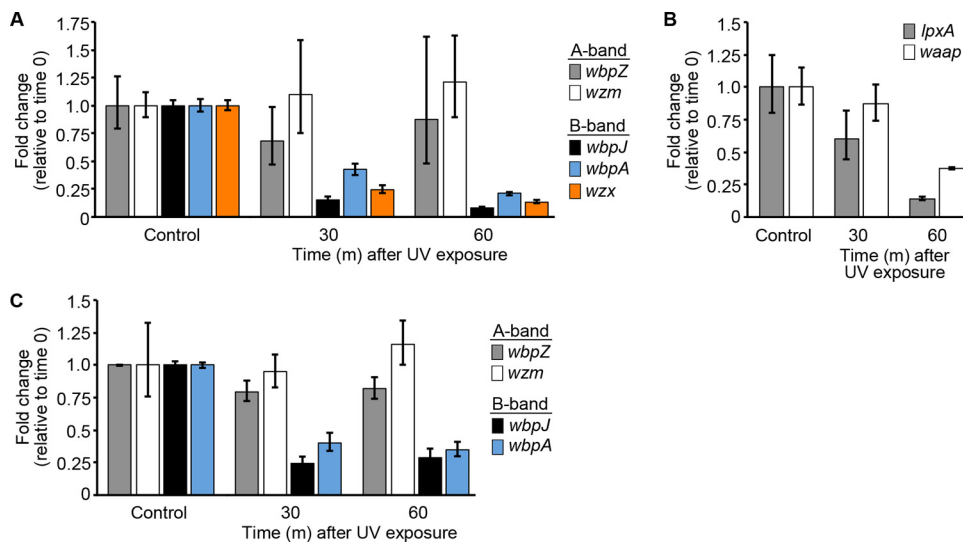
**FIG 5** Loss of R2 pyocin resistance after UV treatment is not regulated by RecA-stimulated cleavage of PrtR, LexA, and PA0906 during the SOS response. Cultures were serially diluted 10-fold, mixed 1:1 with saline or saline with purified R2 pyocin, and spotted onto agar prior to treatment with UV (10 J/m<sup>2</sup>). Surviving CFU counts were determined after overnight incubation.

following UV light treatment is a transient physiological response. We tested whether induction of the LexA, PA0906, and PrtR regulons during the *P. aeruginosa* SOS response mediates the loss of R2 pyocin resistance. We used the *PA0906*<sup>S153A</sup>, *lexA*<sup>G86V</sup>, and *prtR*<sup>S162A</sup> 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<sup>G86V</sup> protein is resistant to autoproteolytic cleavage, and the LexA regulon cannot be derepressed in a *lexA*<sup>G86V</sup> mutant (37). Importantly, none of these mutations had an effect on the R2 pyocin resistance of *P. aeruginosa* under nonstress conditions (see Fig. S1B in the supplemental material). Following UV light exposure, we observed R2 pyocin-sensitive cells in double mutants (*prtR*<sup>S162A</sup> *PA0906*<sup>S153A</sup> and *prtR*<sup>S162A</sup> *lexA*<sup>G86V</sup> mutants) and in a triple mutant (*PA0906*<sup>S153A</sup> *lexA*<sup>G86V</sup> *prtR*<sup>S162A</sup> mutant), indicating that induction of genes regulated by PrtR, PA0906, and LexA is not required for the loss of R2 pyocin resistance after UV irradiation treatment in *P. aeruginosa*.

*nosa* cells (Fig. 5). Intriguingly, these data suggest that an as-yet-unidentified system that regulates pyocin resistance is activated during the SOS response in *P. aeruginosa*.

**Loss of R2 pyocin resistance after UV irradiation correlates with B-band LPS downregulation.** In *P. aeruginosa*, the B-band O-antigen confers resistance to R2 pyocin by masking a receptor that lies within the core polysaccharide of lipopolysaccharide (LPS) (32, 33). Mutations that eliminate B-band O-antigen synthesis and 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 in nontreated and UV light-treated ΔR2 mutant cells. Strikingly, we found that the B-band O-antigen synthesis genes *wbpJ*, *wbpA*, and *wzx* were rapidly and significantly downregulated 30 min after UV treatment (Fig. 6A). At 60 min after UV treatment, the expression of *wbpJ* and *wzm* was downregulated ~10-fold and that of *wbpA* was downregulated 5-fold relative to their expression in nontreated control cultures (Fig. 6A). In contrast, the expression of the A-band O-antigen synthesis genes *wbpZ* and *wzm* was unchanged (Fig. 6A). The expression of genes involved in lipid A (*lpxA*) and the core polysaccharide (*waaP*) also decreased following UV irradiation (Fig. 6B), although not as rapidly as that of 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 *PA0906*<sup>S153A</sup> *lexA*<sup>G86V</sup> *prtR*<sup>S162A</sup> triple mutant after UV light exposure and found that B-band LPS O-antigen genes were significantly downregulated (Fig. 6C). This result indicates that the downregulation of B-band LPS O-antigen genes after UV light exposure is not dependent on RecA-mediated autocleavage of LexA, PrtR, or PA0906.



**FIG 6** Relative expression of B-band and A-band O-antigen synthesis genes after treatment of wild-type cells with UV (15 J/m<sup>2</sup>). (A) Relative expression of A-band synthesis genes (*wbpZ*, *rdm*) and B-band synthesis genes (*wbpJ*, *wbpA*, *wzx*) in wild-type cells on agar. Nontreated control cells were incubated on agar for 15 min. Error bars indicate 95% confidence intervals. (B) Relative expression of *lpxA* and *waaP* in wild-type cells on agar. Error bars indicate 95% confidence intervals. (C) Relative expression of A-band synthesis genes (*wbpZ*, *rdm*) and B-band synthesis genes (*wbpJ*, *wbpA*) in *PA0906*<sup>S153A</sup> *lexA*<sup>G86V</sup> *prtR*<sup>S162A</sup> triple mutant cells on agar. Nontreated control cells were incubated on agar for 15 min. Error bars indicate 95% confidence intervals.



Previous research has shown that mutation of *wbpA* and *wbpJ* eliminates B-band O-antigen synthesis and confers sensitivity to self-produced R2 pyocin (32). Lower expression of *wpbA* and *wpbJ* likely lowers B-band O-antigen synthesis after UV light treatment. In addition, because both B- 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 *wpbA* and *wpbJ* downregulation 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. On the basis of research with *E. coli* on the autocleavage of LexA and the 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 the autoproteolysis of two regulators, LexA and CI. However, despite both being targeted by the RecA coprotease, LexA undergoes autoproteolysis at a higher 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 the protease-active site, and the strength of this interaction is greater in LexA than it is in the 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 lower 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 be strongly induced only if genotoxic stress were sustained. It would be interesting to carry out a 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-an-

tigen genes after UV light treatment is not dependent on the 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 clinical and therapeutic implications. Determining how UV light treatment affects the 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 B-band O-antigen loss severely impairs growth (32). Unlike the immunity genes for low-molecular-weight S-type pyocins, which are genetically linked and coexpressed, the genes that code for B-band O-antigen synthesis are genetically distinct from the genes that code for R2 pyocin synthesis (14). Our work demonstrates that these genes are also transcriptionally regulated by distinct mechanisms and that R2 pyocin can be lethal under conditions that downregulate B-band O-antigen synthesis genes. This raises the possibility that R2 pyocin might restrict the conditions under which *P. aeruginosa* can grow because the 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.

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