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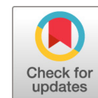
MECHANISMS OF RESISTANCE



Pseudomonas aeruginosa Oligoribonuclease Contributes to Tolerance to Ciprofloxacin by Regulating Pyocin Biosynthesis

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ABSTRACT Bacterial oligoribonuclease (Orn) is a conserved 3′-to-5′ exonuclease. In *Pseudomonas aeruginosa*, it has been demonstrated that Orn plays a major role in the hydrolysis of pGpG, which is required for cyclic-di-GMP homeostasis. Meanwhile, Orn is involved in the degradation of nanoRNAs, which can alter global gene expression by serving as transcription initiation primers. Previously, we found that Orn is required for the type III secretion system and pathogenesis of *P. aeruginosa*, indicating a role of Orn in the bacterial response to environmental stimuli. Here we report that Orn is required for the tolerance of *P. aeruginosa* to ciprofloxacin. Transcriptome analysis of an *orn* mutant revealed the upregulation of pyocin biosynthesis genes. Mutation of genes involved in pyocin biosynthesis in the background of an *orn* mutant restored bacterial tolerance to ciprofloxacin. We further demonstrate that the upregulation of pyocin biosynthesis genes is due to RecA-mediated autoproteolysis of PrtR, which is the major negative regulator of pyocin biosynthesis genes. In addition, the SOS response genes were upregulated in the *orn* mutant, indicating a DNA damage stress. Therefore, our results revealed a novel role of Orn in bacterial tolerance to ciprofloxacin.

KEYWORDS oligoribonuclease, pyocin, *Pseudomonas aeruginosa*, ciprofloxacin

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Pseudomonas aeruginosa is an opportunistic, pathogenic Gram-negative bacterium which causes acute and chronic infections in humans. *P. aeruginosa* is intrinsically resistant to antibiotics largely owing to multiple chromosomally encoded multidrug efflux systems as well as low membrane permeability (1). Interestingly, numerous genes that sensitize bacteria to antibiotics have also been identified in the chromosome of *P. aeruginosa* (2–4). For example, pyocin biosynthesis genes contribute to bacterial susceptibility to quinolones, as mutations in those genes increase bacterial resistance to quinolones. Pyocins are bacteriocins that mainly target other *P. aeruginosa* strains (5). The release of pyocins is mediated by holin-like PA0614 and lysozyme-like PA0629, and pyocin release results in the lysis of the producer cells (6, 7). Expression of pyocin genes is repressed by a DNA binding protein, PrtR. Genotoxic agents, such as mitomycin C and quinolones, induce DNA damage and subsequently activate RecA. The activated RecA then induces the autoproteolysis of PrtR, resulting in the derepression of PrtN, which directly activates the expression of pyocin biosynthesis genes and the subsequent release of pyocins through cell lysis (7–9).

TABLE 1 Bacterial susceptibilities to antibiotics

Strain	MIC (μg/ml)[a]						
	TET	TOB	GM	CAR	MEM	CIP	OFL
PA14	12.5	0.625	1.25	100	2.5	0.25	1
PA14 Δ*orn*	6.25	0.625	1.25	50	2.5	0.03	0.25
PA14 Δ*orn*/*orn*	25	0.625	1.25	100	2.5	0.25	1

[a]TET, tetracycline; TOB, tobramycin; GM, gentamicin; CAR, carbenicillin; MEM, meropenem; CIP, ciprofloxacin; OFL, ofloxacin.

Besides individual genes, bacterial growth modes affect antibiotic resistance levels. Bacteria growing in sessile biofilms are much more resistant to various antibiotics and environmental stresses than planktonic bacteria (10–12). Bacteria inside biofilms are shielded from host phagocytes and antibiotics by the biofilm matrix, which is mainly composed of polysaccharide, DNA, and proteins (13–15). In addition, multidrug efflux systems are upregulated in bacteria embedded in biofilms (16), and the slow growth of bacteria inside biofilms further enhances bacterial resistance to antibiotics (17).

Biofilm formation is regulated by a second messenger, cyclic-di-GMP (c-di-GMP) (18, 19), which is synthesized by enzymes containing glycine-glycine-aspartate-glutamate-phenylalanine (GGDEF) domains and degraded by enzymes containing histidine-aspartate-glycine-tyrosine-proline (HD-GYP) or glutamate-alanine-leucine (EAL) domains (20). EAL domain-containing enzymes hydrolyze c-di-GMP to 5′-phosphoguanylyl-(3′, 5′)-guanosine (pGpG) (21), which is degraded primarily by the oligoribonuclease (Orn). Mutation of *orn* leads to the accumulation of pGpG, which inhibits the function of EAL domain-containing enzymes, resulting in increased levels of c-di-GMP and the consequent hyperbiofilm phenotype (22, 23).

Orn is a highly conserved 3′-to-5′ exonuclease (24). Other than degrading pGpG, Orn plays a major role in the hydrolysis of 2- to 5-nucleotide (nt) RNAs, namely, nanoRNAs (25). NanoRNAs can serve as primers for transcription initiation. The aberrant accumulation of nanoRNAs due to defective Orn alters global gene expression (26). Previously, we found that Orn is required for the expression of the type III secretion system (T3SS) genes in *P. aeruginosa* (27). Whether Orn plays other roles during infection or in the bacterial response to environmental stresses is not known.

In this study, we investigated the role of Orn in bacterial resistance to antibiotics. Mutation of *orn* drastically increased bacterial susceptibility to quinolones but not to tetracycline, aminoglycoside, or β-lactam antibiotics. Gene expression profile analysis and genetic experimentation demonstrated that the upregulation of pyocin biosynthesis genes contributed to the hypersusceptibility to quinolones. We further found that RecA-mediated PrtR autoproteolysis was responsible for the upregulation of pyocin biosynthesis genes. In addition, SOS response genes were found to be upregulated in the *orn* mutant. Thus, our results revealed a novel role of Orn in genome integrity and bacterial resistance to quinolones.

RESULTS

Oligoribonuclease is required for bacterial resistance to fluoroquinolones. In *P. aeruginosa*, oligoribonuclease controls the intracellular levels of c-di-GMP and nanoRNA, both of which affect the expression of multiple genes (22, 26, 28, 29). To test the overall influence of oligoribonuclease on bacterial antibiotic resistance, we examined the MICs of various antibiotics for a Δ*orn* mutant. Mutation of *orn* rendered the bacteria slightly more susceptible to tetracycline and carbenicillin (Table 1). Unexpectedly, the Δ*orn* mutant was much more susceptible to quinolones and showed 8- and 4-fold decreases in the MICs of ciprofloxacin and ofloxacin, respectively. Complementation with an *orn* gene (Δ*orn*/*orn*) restored bacterial resistance (Table 1). These results suggest that oligoribonuclease plays an essential role in bacterial resistance to quinolones.

Upregulation of pyocin genes contributes to increased susceptibility to ciprofloxacin in the *orn* mutant. To understand the mechanism of increased susceptibility

TABLE 2 mRNA levels of pyocin biosynthesis genes in the Δ*orn* mutant compared to those in wild-type strain PA14

PA14 locus tag	PAO1 locus tag	Product	Fold change[a]	*P* value
PA14_07970	PA0612	Repressor, PtrB	3.053	1.892E−03
PA14_07980	PA0613	Hypothetical protein	3.238	6.263E−11
PA14_08000	PA0615	Hypothetical protein	3.585	2.459E−12
PA14_08010	PA0616	Hypothetical protein	3.695	1.222E−08
PA14_08020	PA0617	Probable bacteriophage protein	3.709	2.966E−09
PA14_08030	PA0618	Probable bacteriophage protein	3.664	1.997E−08
PA14_08040	PA0619	Probable bacteriophage protein	3.626	3.419E−13
PA14_08050	PA0620	Probable bacteriophage protein	2.924	2.020E−10
PA14_08060	PA0621	Conserved hypothetical protein	2.376	4.971E−05
PA14_08070	PA0622	Probable bacteriophage protein	3.234	5.329E−12
PA14_08090	PA0623	Probable bacteriophage protein	2.999	1.919E−10
PA14_08100	PA0624	Hypothetical protein	3.342	8.559E−11
PA14_08120	PA0625	Hypothetical protein	3.299	8.352E−09
PA14_08130	PA0626	Hypothetical protein	3.377	3.302E−05
PA14_08140	PA0627	Conserved hypothetical protein	3.108	3.297E−08
PA14_08150	PA0628	Conserved hypothetical protein	3.385	5.495E−08
PA14_08160	PA0629	Conserved hypothetical protein	3.689	6.944E−07
PA14_08180	PA0630	Hypothetical protein	4.223	2.695E−07
PA14_08200	PA0632	Hypothetical protein	3.000	4.495E−06
PA14_08210	PA0633	Hypothetical protein	2.824	4.475E−09
PA14_08220	PA0634	Hypothetical protein	2.740	1.271E−08
PA14_08230	PA0635	Hypothetical protein	2.783	3.407E−06
PA14_08240	PA0636	Hypothetical protein	3.454	8.654E−11
PA14_59220	PA0985	Pyocin S5	2.337	6.858E−07

[a]The data represent the fold change in PA14 Δ*orn* compared with that in PA14, which was given a value of 1.

to ciprofloxacin in the Δ*orn* mutant, we performed transcriptome analyses. No significant decrease in the levels of expression of multidrug efflux system genes in the Δ*orn* mutant compared to those in wild-type strain PA14 was observed (see Table S1 in the supplemental material). Of note, all the genes involved in pyocin biosynthesis were upregulated (Table 2). Real-time PCR assays confirmed the upregulation of genes involved in the production of F-, R-, and S-type pyocins (Fig. 1). Complementation with an *orn* gene restored the expression levels of those genes (Fig. 1).

In *P. aeruginosa*, pyocin biosynthesis is induced by genotoxic agents, such as fluoroquinolones, mitomycin C, and UV light. The production and release of pyocins result in cell lysis (7, 9). Mutation of pyocin biosynthesis genes increases bacterial resistance to ciprofloxacin (2, 3). To test the role of pyocin biosynthesis genes in the

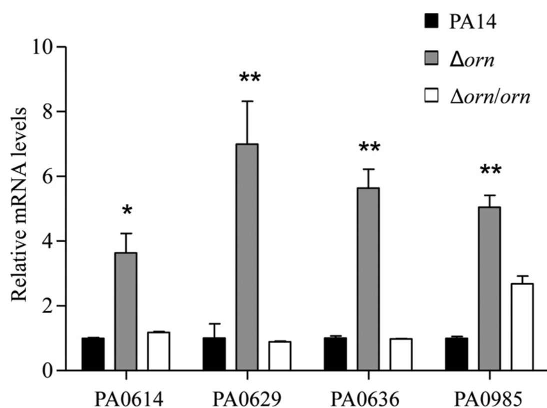

FIG 1 Expression levels of PA0614, PA0629, PA0636, and PA0985. Total RNA was isolated from the indicated strains, and the mRNA levels were determined by real-time PCR by use of PA0668.1 as an internal control. The results shown represent data from three independent experiments with similar results. Error bars represent standard deviations. *, $P < 0.05$, and **, $P < 0.01$, compared to PA14 or the complemented strain by Student's *t* test.

TABLE 3 Bacterial susceptibilities to ciprofloxacin

Strain	Ciprofloxacin MIC (μg/ml)
PA14	0.25
Δ*orn*	0.03
PA14 Δ*prtN*	0.5
PA14 ΔPA0629	0.5
PA14 Δ*orn* Δ*prtN*	0.25
PA14 Δ*orn* ΔPA0629	0.25
PA14/pMMB67EH	0.25
PA14 Δ*orn*/pMMB67EH	0.03
PA14/pMMB67EH-$prtR^{S162A}$-His	0.5
PA14 Δ*orn*/pMMB67EH-$prtR^{S162A}$-His	0.1
PA14 Δ*recA*::Gm	0.03
PA14 Δ*orn* Δ*recA*::Gm	0.01

hypersusceptibility of the Δ*orn* mutant, we knocked out *prtN* and PA0629, which encode a positive regulator of pyocin biosynthesis genes and a putative lysozyme, respectively. Consistent with previous reports, mutation of *prtN* or PA0629 in wild-type strain PA14 increased the level of bacterial resistance to ciprofloxacin by 2-fold (2, 3). However, mutation of *prtN* or PA0629 in the Δ*orn* mutant increased the level of resistance up to 8-fold (Table 3). These results suggest that the upregulation of pyocin biosynthesis genes in the *orn* mutant contributes to the increased susceptibility to ciprofloxacin.

PrtR stability is reduced by the mutation of *orn*. The expression of *prtN* is directly repressed by PrtR (8). Previously, we demonstrated that the PA0612-PA0613 operon is directly regulated by PrtR (30). Real-time PCR assays revealed higher levels of PrtN and PA0613 mRNA in the Δ*orn* mutant (Fig. 2A and B), suggesting a reduced PrtR protein level.

To test the level of the PrtR protein, a *prtR* gene that had a His tag at its C terminus and whose expression was driven by its native promoter (P_{prtR}-*prtR*-His) (31) was transferred into wild-type strain PA14 and the Δ*orn* mutant. Indeed, the PrtR-His level was lower in the Δ*orn* mutant than the PA14 parent strain (Fig. 3A). However, the PrtR mRNA level in the Δ*orn* mutant was 1.3-fold of that in wild-type strain PA14 (Fig. 3B), indicating posttranscriptional regulation. Next, we constructed a *prtR* gene that had a His tag at its C terminus and whose expression was driven by an exogenous *tac* promoter (P_{tac}-*prtR*-His). Transcription of the *prtR*-His was induced by IPTG (isopropyl-β-D-thiogalactopyranoside). To monitor the stability of PrtR, chloramphenicol was added to the culture to block protein translation and the PrtR-His levels were subsequently determined.

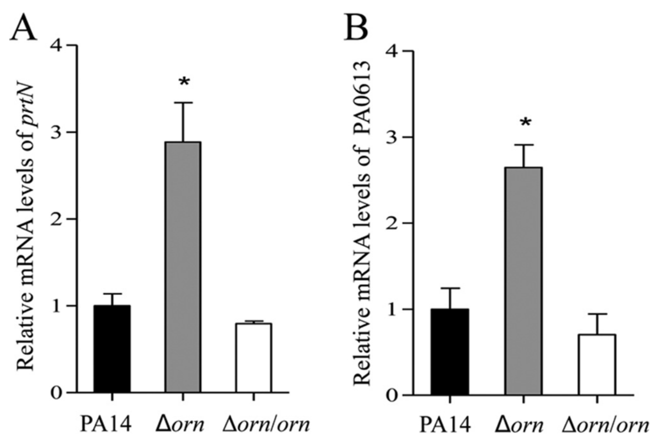

FIG 2 Expression levels of *prtN* and PA0613. The relative levels of *prtN* (A) and PA0613 (B) mRNA in the indicated strains were determined by real-time PCR, with PA0668.1 serving as an internal control. The results shown represent data from three independent experiments with similar results. Error bars represent standard deviations. *, $P < 0.05$ compared to PA14 or the complemented strain by Student's *t* test.

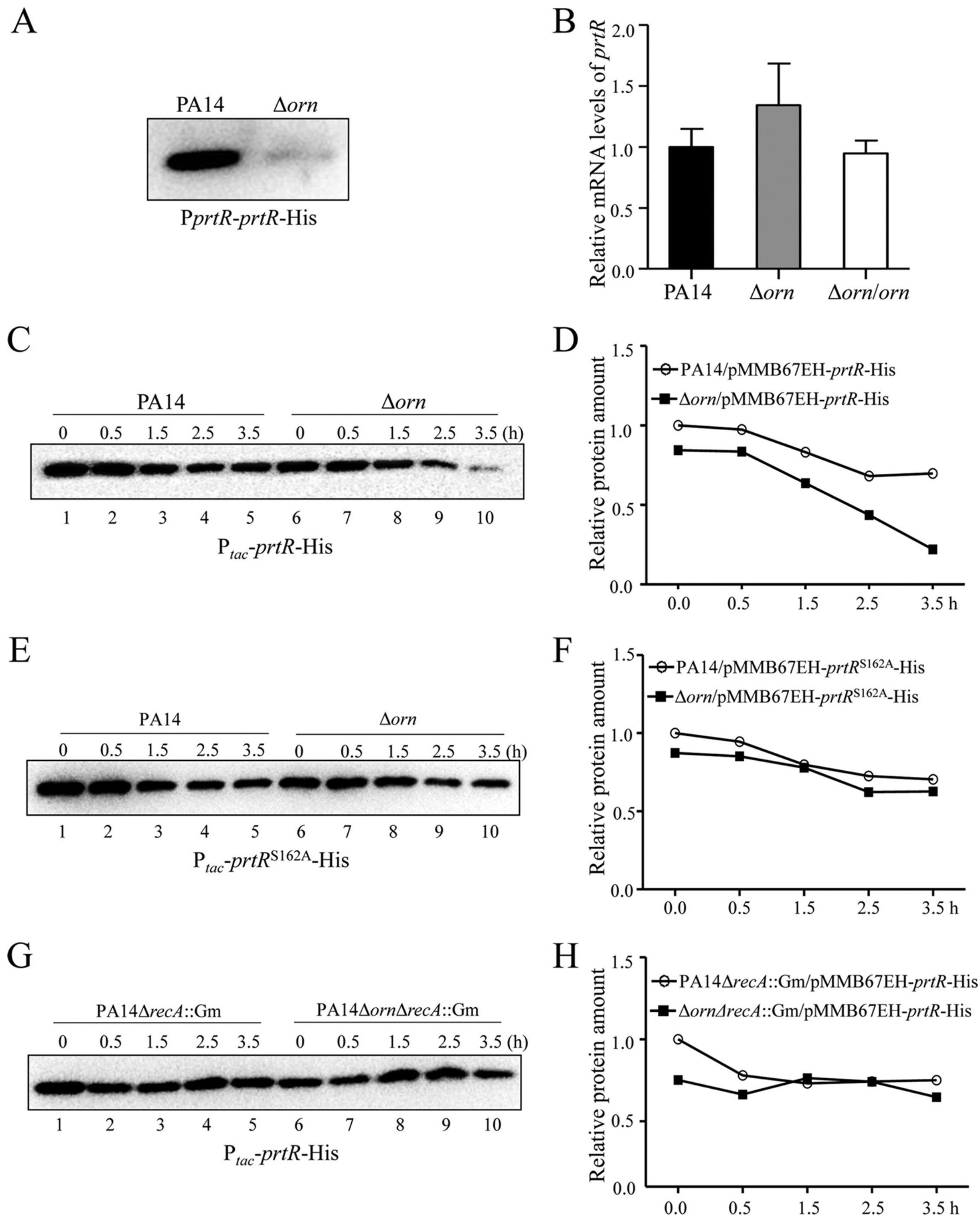

FIG 3 Expression levels of PrtR. (A) Protein levels of PrtR in PA14 or the Δ*orn* mutant with P_{prtR}-*prtR*-His integrated into the chromosome. Bacteria were grown to an OD_{600} of 1.0, and bacterial samples were collected. The levels of PrtR-His were determined by Western blotting. (B) The relative levels of *prtR* mRNA in wild-type strain PA14 and the Δ*orn* mutant were determined by real-time PCR, with PA0668.1 serving as an internal control. Bacteria of the indicated strains carrying pMMB67EH-*prtR*-His (C, D, G, H) or pMMB67EH-*prtR*^{S162A}-His (E, F) were grown to an OD_{600} of 0.4 to 0.6, followed by addition of IPTG to 0.01 mM. After 1 h, 500 μg/ml chloramphenicol was added to the medium. Then, bacteria of each strain were collected at the indicated time points. Samples from equivalent numbers of bacterial cells were loaded onto an SDS-polyacrylamide gel, and the levels of PrtR-His were determined by Western blotting. The band intensities were quantified with ImageJ software.

Right after chloramphenicol was added, the PrtR-His level in the Δ*orn* mutant was slightly lower than that in wild-type strain PA14 (Fig. 3C, lanes 1 and 6, and D). However, the PrtR-His level dropped faster in the Δ*orn* mutant (Fig. 3C and D), indicating decreased stability.

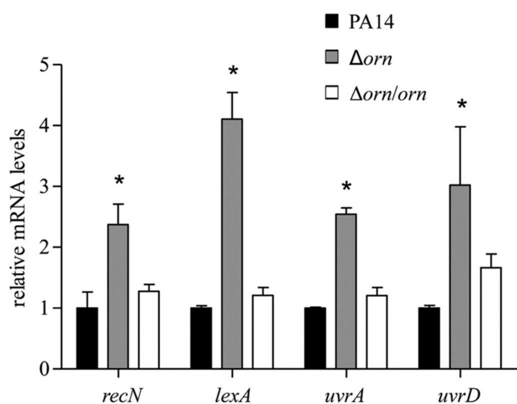

FIG 4 Expression levels of SOS response genes. Total RNA was isolated from wild-type strain PA14, the Δ*orn* mutant, and the complemented strain. The relative levels of *recN*, *lexA*, *uvrA* and *uvrD* mRNA were determined by real-time PCR, with PA0668.1 serving as an internal control. Error bars indicate standard deviations. *, $P < 0.05$ compared to PA14 or the complemented strain by Student's *t* test.

Degradation of PrtR is mediated by its autoproteolytic activity (31). Replacement of the serine residue at codon 162 with an alanine (PrtR^{S162A}) inactivates the autoprotease domain, which increases the stability of the protein (32, 33). To test whether the accelerated degradation of PrtR is due to autoproteolysis, we constructed a *prtR* gene with the S162A mutation (*prtR*^{S162A}) that had a His tag at the C terminus and whose expression was driven by a *tac* promoter (P_{tac}-*prtR*^{S162A}-His) and monitored the stability of the protein. The initial PrtR^{S162A}-His level in the Δ*orn* mutant was slightly lower than that in the wild-type strain PA14. Nevertheless, the PrtR^{S162A}-His proteins exhibited similar stabilities in the two strains (Fig. 3E and F). These results suggest that the *orn* mutation leads to enhanced autoproteolysis of PrtR.

Overexpression of PrtR^{S162A} increases the resistance of the Δ*orn* mutant to ciprofloxacin. If the enhanced autoproteolysis of PrtR plays a major role in the increased susceptibility to ciprofloxacin, the overexpression of stable PrtR^{S162A} should increase bacterial resistance to ciprofloxacin. Indeed, overexpression of PrtR^{S162A} in the Δ*orn* mutant increased the MIC of ciprofloxacin by 4-fold, whereas in the wild-type strain, the MIC was increased by 2-fold (Table 3). These results suggest that overexpression of PrtR^{S162A} has a more profound effect on the Δ*orn* mutant than on wild-type strain PA14.

Overall, our results shown above demonstrate that mutation of *orn* leads to enhanced autoproteolysis of PrtR and, subsequently, the upregulation of pyocin genes, which results in increased bacterial susceptibility to ciprofloxacin.

RecA plays a role in the enhanced autoproteolysis of PrtR in the Δ*orn* mutant. The autoproteolytic activity of PrtR is activated by RecA (31). To assess the role of RecA in the Δ*orn* mutant, we replaced the coding region of *recA* with an *aacC1* cassette (Δ*recA*::Gm) and monitored the stability of PrtR-His. Mutation of *recA* resulted in similar stabilities of PrtR-His in the two strains (Fig. 3G and H). Replacement of the *recA* gene with an *aacC1* cassette (Δ*recA*::Gm) increased bacterial susceptibility to ciprofloxacin. Nevertheless, the difference in the MIC of ciprofloxacin between PA14 and the Δ*orn* mutant was reduced from 8-fold to 2-fold by the mutation of *recA* (Table 3), indicating a role of RecA in the increased susceptibility of the Δ*orn* mutant.

RecA senses DNA damage and initiates an SOS response by inducing the autoproteolysis of LexA (34). Our results so far implied an impairment of DNA integrity in the Δ*orn* mutant. We then measured the expression levels of SOS response genes with a LexA box in the promoter regions. Indeed, the mRNA levels of *recN*, *uvrA*, *uvrD*, and *lexA* were higher in the Δ*orn* mutant, indicating a stronger SOS response (Fig. 4). These results suggest that mutation of *orn* might impair DNA

integrity, which promotes the RecA-mediated autoproteolysis of PrtR and the subsequent upregulation of pyocin biosynthesis genes, resulting in increased bacterial susceptibility to ciprofloxacin.

DISCUSSION

In this study, we found that Orn is required for the stability of PrtR. PrtR is homologous to λ phage CI (7). It binds to and represses the promoter of *prtN*, which encodes the positive regulator for pyocin biosynthesis genes (8). Genotoxic agents initiate the SOS response, during which RecA induces the autoproteolysis of PrtR and the upregulation of pyocin biosynthetic genes (31). Since the release of pyocins is through bacterial cell lysis, the production of the pyocins is under tight regulation. For an individual bacterium, pyocin production and release are detrimental to its survival in response to DNA damage. As a proof, mutations in the pyocin biosynthesis genes increase bacterial resistance to genotoxic agents, such as ciprofloxacin and UV light (2, 3).

However, pyocins are also believed to play a role in bacterial competition by targeting other *P. aeruginosa* strains (35, 36). Therefore, the production and release of pyocins in an unfavorable environment might secure the niche by killing other bacterial strains (37–40). In addition, Oliveira et al. demonstrated that pyocins released by one strain stimulate biofilm formation by a susceptible strain (41). Thus, foreign pyocins might be regarded as a danger signal that induces defense mechanisms for protection and competition.

Besides stimulating biofilm formation in heterologous strains, pyocins have been shown to play important roles in biofilm formation in the isogenic population (42). Turnbull et al. demonstrated that PA0629, which encodes a peptidoglycan-degrading endolysin, is required for the release of extracellular DNA (eDNA), cytosolic contents, and membrane vesicles (MVs) (42). MVs and eDNA play important roles in biofilm formation and integrity (43–47). Interestingly, mutation of *orn* resulted in the upregulation of pyocin biosynthesis genes as well as increased levels of the biofilm-promoting molecule c-di-GMP (22). Consistent with this, the *orn* mutant displays a hyperbiofilm phenotype (23). Overexpression of PA2133 (an EAL domain-containing protein) reduced the intracellular c-di-GMP level and the level of biofilm formation in the *orn* mutant (22). Given the role of pyocin in biofilm formation, it will be interesting to examine whether the upregulated pyocin biosynthesis genes contribute to the hyperbiofilm phenotype of the *orn* mutant.

In this study, we found that the upregulation of pyocin biosynthesis genes in the *orn* mutant was due to the RecA-mediated autoproteolysis of PrtR. Overexpression of PA2133 in the *orn* mutant did not restore the expression levels of those genes or bacterial resistance to ciprofloxacin (data not shown). In addition, we observed higher expression levels of SOS genes in the *orn* mutant. These results indicate that mutation of *orn* might impair the integrity of the genome, which activates RecA. One of the major effects of *orn* mutation is the accumulation of nanoRNAs, which function as primers in transcription initiation, thus altering global gene expression (26). It might be possible that genes involved in DNA replication or repair are aberrantly regulated in the *orn* mutant, and this aberrant regulation leads to activation of the SOS response. Another possibility is that the accumulated nanoRNAs might anneal to single-stranded DNA during DNA replication, interfering with normal DNA synthesis, which induces the SOS response. Further studies are needed to fully elucidate the biological role of Orn.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 4. Bacteria were grown in L broth (5 g of NaCl, 5 g of yeast extract, 10 g of tryptone per liter, pH 7.0) or L agar (L broth with 1.5% [wt/vol] agar) at 37°C. The concentrations of the antibiotics used were as follows: for *Escherichia coli*, ampicillin at 100 μg/ml, gentamicin at 10 μg/ml, and tetracycline at 10 μg/ml; for *P. aeruginosa*, carbenicillin at 150 μg/ml, tetracycline at 100 μg/ml, and gentamicin at 150 μg/ml.

TABLE 4 Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics or function	Source
P. aeruginosa strains		
PA14	Wild-type *P. aeruginosa* strain	David Bradley
PA14 Δ*orn*	*orn* deletion mutant of PA14	This study
PA14 Δ*orn*/*orn*	PA14 Δ*orn* mutant complemented with the *orn* gene inserted into the chromosome	This study
PA14 Δ*prtN*	*prtN* deletion mutant of PA14	This study
PA14 ΔPA0629	PA0629 deletion mutant of PA14	This study
PA14 Δ*orn* Δ*prtN*	*orn* and *prtN* double deletion mutant of PA14	This study
PA14 Δ*orn* ΔPA0629	*orn* and PA0629 double deletion mutant of PA14	This study
PA14 Δ*recA*::Gm	PA14 with *recA* disrupted by insertion of a Gm resistance cassette; Gm^r	This study
PA14 Δ*orn* Δ*recA*::Gm	PA14 Δ*orn* with *recA* disrupted by insertion of a Gm resistance cassette; Gm^r	This study
Plasmids		
pMMB67EH	Shuttle vector between *E. coli* and *P. aeruginosa*; Amp^r	S. Jin lab
pUC18T-Mini-Tn*7*T-Tc	For gene insertion in chromosome; Tc^r	Herbert Schweizer
pEX18Tc	Gene replacement vector; Tc^r *oriT*$^+$ *sacB*$^+$	Herbert Schweizer
pEX18Ap	Gene replacement vector; Ap^r *oriT*$^+$ *sacB*$^+$	Herbert Schweizer
pE1923	pEX18Tc with *prtN* deleted; Tc^r	This study
pE1797	pEX18Ap with *recA* deleted; Amp^r	This study
pE1962	pEX18Tc with PA0629 deleted; Tc^r	This study
pE1777	*prtR* gene of PA14 on pUC18T-Mini-Tn*7*T-Tc with 500-bp upstream regions of *prtR* ORF[a]; Tc^r	This study
pE1990	pMMB67EH-*prtR*-His	This study
pE1986	pMMB67EH-*prtR*^{S162A}-His	This study

[a]ORF, open reading frame.

Plasmid construction. For DNA manipulations, standard protocols or the instructions of the manufacturers of commercial products were followed. Plasmid pE1923 was constructed by cloning a 1,069-bp upstream fragment and a 941-bp downstream fragment of the *prtN* coding region into the EcoRI-HindIII sites of plasmid pEX18Tc. pE1962 was constructed by cloning a 1,245-bp upstream fragment and a 1,208-bp downstream fragment of the PA0629 coding region into the EcoRI-HindIII sites of plasmid pEX18Tc. A 500-bp upstream region of the *prtR* open reading frame was amplified from the chromosomal DNA of wild-type *P. aeruginosa* strain PA14 by PCR and cloned into the KpnI-HindIII sites of pUC18T-Mini-Tn*7*T-Tc, resulting in pE1777. The *prtR* coding region was amplified from PA14 chromosomal DNA and cloned into the KpnI-HindIII sites of pMMB67EH, resulting in pE1990, where the *prtR* gene is under the control of a *tac* promoter and fused with a His tag at the C terminus. A plasmid carrying the *prtR*^{S162A} mutation was constructed by replacing the serine at codon 162 with an alanine codon by overlapping PCR.

RNA sequencing and data analysis. Wild-type strain PA14 and the *orn* mutant were cultured in LB broth at 37°C and grown to log phage (optical density at 600 nm [OD_{600}], 0.8 to 1.0). Total RNA was isolated with an RNeasy Protect Bacteria minikit with on-column DNase I digestion (Qiagen, Shanghai, China). A second round of DNase treatment was performed following a vigorous protocol with a Turbo DNA-free kit (Ambion). 16S, 23S, and 5S rRNAs were removed by using a Ribo-Zero magnetic kit (Bacteria; Epicentre).

Gene expression profiling was carried out by use of the Illumina RNA sequencing (RNA-Seq) technology. Three biological replicates of each sample were performed for RNA-Seq. The rRNA-depleted RNA was fragmented to 150 to 200 bp, and then first- and second-strand cDNAs were synthesized, followed by end repair and adapter ligation. After 12 cycles of PCR enrichment, the libraries were sequenced using an Illumina HiSeq 2500 platform with a paired-end protocol and read lengths of 100 nt.

The sequence data were analyzed using a method described previously (48). Briefly, sequence reads were mapped onto the reference genome of PA14 (GenBank accession number NC_008463) using the CLC Genomics Workbench (version 8.0) program (CLC Bio-Qiagen, Aarhus, Denmark). Then the count data for the expression values were analyzed using the DESeq package of R/Bioconductor software. Genes with different expression levels were identified by performing a negative binomial test using the DESeq package, a cutoff fold change of greater than 2, and a Benjamini-Hochberg (BH)-adjusted P value of less than 0.05. The raw sequence reads were normalized by dividing by size factors and then transformed to the $\log_2(N + 1)$ format, where N is the count number.

RNA extraction and quantitative RT-PCR. Overnight cultures of bacteria were diluted 50-fold into fresh LB medium and grown to an OD_{600} of 2.0. Total RNA was isolated with an RNeasy minikit (Tiangen Biotech, Beijing, China). cDNA was synthesized from total RNA using PrimeScript reverse transcriptase (TaKaRa, Dalian, China) and random primers. For quantitative real-time PCR (RT-PCR), cDNA was mixed with 4 pmol of reverse and forward primers and SYBR Premix Ex TaqTM II (TaKaRa) in a 20-μl reaction system. The primers used in this study are described in Table 5. Quantitative RT-PCR was performed with a CFX Connect real-time system (Bio-Rad, USA). The 16S ribosomal gene PA0668.1 was used as an internal control (49).

TABLE 5 Primers used in this study

Primer	Sequence (5′ → 3′)	Function
KpnI-*prtR*1-S	CAGGAGGGTACCGAGGCGAGCCAGGACCAGTT	*prtR* cloning (500-bp upstream region of *prtR* ORF[a])
HindIII-*prtR*-His-AS	ATTATAAAGCTTTCAGTGGTGGTGGTGGTGGTG ACCTCCCCGCACCAGGGACGGGCCGC	*prtR* cloning
KpnI-*prtR*2-S	ATCGTCGGTACCTAGGCTCTTTACAGAAAATCCATCG	*prtR* cloning (43-bp upstream region of *prtR* ORF)
prtR-PM-S	GGCAACGCGATGGAACCGCTGATCAT	*prtR*^{S162A} mutant cloning
prtR-PM-AS	GTTCCATCGCGTTGCCGGTGAGTTGGG	*prtR*^{S162A} mutant cloning
EcoRI-PA0629up-S	CCGGAATTCCAGCCTCTGCTACCACGTCTATGGC	*prtR* deletion
BamHI-PA0629up-AS	CGCGGATCCCGATCCTCCTGCACTCCGATGGGTT	*prtR* deletion
BamH I-PA0629down-S	CGCGGATCCATGAGCCGGCTCGCTCTGCTCCTGC	*prtR* deletion
HindIII-PA0629down-AS	CCCAAGCTTCGTCTCGCCATCTTTCTCGGACAGC	*prtR* deletion
prtN-RT-S	CGACGATAGCCACAAG	RT-PCR
prtN-RT-AS	GGATGCGATGCTGTC	RT-PCR
prtR-RT-S	GATGCGCAACCTGAAGCA	RT-PCR
prtR-RT-AS	TGAATGGTGTTCTGCGAAACC	RT-PCR
PA0613-RT-S	GTGGTGGTGGAGCACTATCTCA	RT-PCR
PA0613-RT-AS	CCGCAGTGGCGGTACTTC	RT-PCR
PA0614-RT-S	CGCTGCCTGCCAAGGA	RT-PCR
PA0614-RT-AS	ATCAGTACCCAGAGCGGCATT	RT-PCR
PA0629-RT-S	GTGGAGAACCTCAATTACAG	RT-PCR
PA0629-RT-AS	TAGGTGTTGTCGGCAATC	RT-PCR
PA0636-RT-S	TGGAAGACCCGGCAGAAG	RT-PCR
PA0636-RT-AS	CGTTGAGCTTGGACAGATCCT	RT-PCR
PA0985-RT-S	TCAAGCCTCTCCATCTAT	RT-PCR
PA0985-RT-AS	TCCAGTTCATCTCTAACAAG	RT-PCR
recA-RT-S	ATATCAAGAACGCCAACT	RT-PCR
recA-RT-AS	TAGAACTTCAGTGCGTTA	RT-PCR
recN-RT-S	CCACCAGTTGCTCAG	RT-PCR
recN-RT-AS	GTTGCTCAGGGTCTTC	RT-PCR
lexA-RT-S	AATCCCGCCTTCTTCAAT	RT-PCR
lexA-RT-AS	AATGCCGATGTCCTTCAT	RT-PCR
uvrA-RT-S	TCCATCGAACAGAAGTCC	RT-PCR
uvrA-RT-AS	CGCAGGTAGTCGTAGATC	RT-PCR
uvrB-RT-S	TACTTCGTTTCCTACTAC	RT-PCR
uvrB-RT-AS	GAGTCCTTCTCGATATAG	RT-PCR
uvrD-RT-S	GAGCTGATCGAGAATCTT	RT-PCR
uvrD-RT-AS	TTTCTTCCTTGTGGTAGC	RT-PCR
PA0668.1-RT-S	AAGGTCTTCGGATTGTAA	RT-PCR
PA0668.1-RT-AS	GTGCTTATTCTGTTGGTAA	RT-PCR

[a]ORF, open reading frame.

Antibiotic susceptibility assay. The MICs of various antibiotics for *P. aeruginosa* were determined by serial 2-fold dilution in LB medium, as described previously (50, 51). The MIC was recorded as the lowest concentration of antibiotic which inhibited visible growth after incubation at 37°C for 24 h.

PrtR stability assay. To measure the stability of PrtR, overnight bacterial cultures of various *P. aeruginosa* strains were diluted 50-fold in fresh LB with carbenicillin and cultured for 2 h. When the OD_{600} reached 0.4 to 0.6, IPTG was added to the medium to reach 0.01 mM. After 1 h, 500 μg/ml chloramphenicol was added to the medium to block protein synthesis (52). Bacterial cells were collected at the time points indicated above after the addition of chloramphenicol. Samples from equivalent numbers of bacterial cells were separated on SDS-polyacrylamide gels (15% polyacrylamide). The proteins were then transferred onto a polyvinylidene difluoride (PVDF) membrane and incubated with rabbit anti-His antibody (1:1,000; Cell Signaling Technology, USA) at room temperature for 1 h. After washing with 1× phosphate-buffered saline (1× PBS; 274 mM NaCl, 5.4 mM KCl, 20 mM Na_2HPO_4, 4 mM KH_2PO_4, pH 7.4) containing 2% Tween 20 four times, the membrane was incubated with the secondary antibody, horseradish peroxidase-conjugated goat anti-rabbit IgG (1:2,000; Promega, USA), at room temperature for 1 h. Signals were detected with an ECL Plus kit (Millipore), and bands were visualized with a Bio-Rad molecular imager (ChemiDoc XRS+).

Accession number(s). The raw RNA sequencing data has been deposited in the NCBI Short Read Archive (SRA) database under accession no. SRP063080.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.02256-16.

TEXT S1, PDF file, 0.6 MB.

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