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Blocking RpoN reduces virulence of *Pseudomonas aeruginosa* isolated from cystic fibrosis patients and increases antibiotic sensitivity in a laboratory strain

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Multidrug-resistant organisms are increasing in healthcare settings, and there are few antimicrobials available to treat infections from these bacteria. *Pseudomonas aeruginosa* is an opportunistic pathogen in burn patients and individuals with cystic fibrosis (CF), and a leading cause of nosocomial infections. *P. aeruginosa* is inherently resistant to many antibiotics and can develop resistance to others, limiting treatment options. *P. aeruginosa* has multiple sigma factors to regulate transcription. The alternative sigma factor, RpoN (σ^{54}), regulates many virulence genes and is linked to antibiotic resistance. Recently, we described a cis-acting peptide, RpoN*, which is a "molecular roadblock", binding consensus promoters at the -24 site, blocking transcription. RpoN* reduces virulence of *P. aeruginosa* laboratory strains, but its effects in clinical isolates was unknown. We investigated the effects of RpoN* on phenotypically varied *P. aeruginosa* strains isolated from CF patients. RpoN* expression reduced motility, biofilm formation, and pathogenesis in a *P. aeruginosa-C. elegans* infection model. Furthermore, we investigated RpoN* effects on antibiotic susceptibility in a laboratory strain. RpoN* expression increased susceptibility to several beta-lactam-based antibiotics in strain *P. aeruginosa* PA19660 *Xen5*. We show that using a cis-acting peptide to block RpoN consensus promoters has potential clinical implications in reducing virulence and improving antibiotic susceptibility.

Multidrug-resistant organisms (MDROs) are an increasing problem in the healthcare setting. Both Gram-negative and Gram-positive MDROs are prevalent globally[1]. There are few or no antimicrobial agents available for treatment of infections caused by these bacteria[2]. *Pseudomonas aeruginosa*, a Gram-negative, opportunistic pathogen is a leading cause of nosocomial infections and is associated with infections in burn patients[3,4]. *P. aeruginosa* is also responsible for colonizing the respiratory tract and causing chronic infections in individuals with cystic fibrosis (CF)[5]. It is the most common pathogen isolated from individuals with CF and is a major source of morbidity and mortality[6,7].

In CF patients, *P. aeruginosa* undergoes a transformation from a non-mucoid form upon initial colonization of the lungs to a mucoid form as the disease progresses. This results in a chronic debilitating pulmonary infection characterized by the overexpression of alginate. Mucoid strains synthesize large quantities of alginate, enhancing biofilm formation and protecting *P. aeruginosa* from antibiotics or the immune response[8], possibly through formation of microcolonies[9,10]. While aggressive prevention regimens have led to a decline in *P. aeruginosa* prevalence in CF patients, multidrug resistant strains are still prevalent and occurred in 19.4% of CF infections in 2015[11]. *P. aeruginosa* is inherently resistant to a number of antibiotics[12,13]. It can also acquire resistance through exogenous resistance genes via horizontal gene transfer or mutations[14], limiting treatment options. Antimicrobial

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Location	Strain	Source	CF mutations	Reference
Laboratory				
	PAO1-M	—	n/a	C. Manoil (27)
	PAO1-S	—	n/a	D. Haas (15)
	Δ*rpoN*	—	n/a	D. Haas (15)
	PA19660 *Xen5*	Septicemia	n/a	Perkin Elmer
Clinical Isolate				
Seattle Children's Hospital (SCH)				
	SCH0057-7	unknown	ΔF508/ΔF508	This study
	SCH0254-23	unknown	ΔF508/unknown	This study
	SCH0254-116	unknown	ΔF508/unknown	This study
	SCH0254-118	unknown	ΔF508/unknown	This study
	SCH0256-1	sputum	ΔF508/ΔF508	This study
	SCH0338-38	sputum	unknown/unknown	This study
	SCH0354-1	sputum	ΔF508/G551D	This study
	SCH0397-3	unknown	ΔF508/unknown	This study
	SCH03269	sputum	ΔF508/ΔF508	This study
Upstate University Hospital (UUH)				
	UUH0101	sputum	ΔF508/unknown	This study
	UUH0201	sputum	ΔF508/ΔF508	This study
	UUH0202	sputum	ΔF508/ΔF508	This study

Table 1. *P. aeruginosa* strains used in this study.

development is directed toward alternative treatments and novel targets. Promising strategies include enhancing activity of currently available antibiotics and decreasing virulence of the bacteria once an infection occurs[15,16].

P. aeruginosa virulence is caused by many factors, including toxins, proteases, phospholipases, the presence of pili and flagella, and biofilm formation[17]. Virulence is regulated by a network of transcription factors, such as sigma factors RpoS and RpoN, and quorum sensing regulators[18]. The alternative sigma factor, σ^{54} or RpoN, regulates nitrogen assimilation, quorum sensing, motility, biofilm formation and other virulence factors[19–27]. RpoN regulation was recently linked to *P. aeruginosa* tolerance to several antibiotics[28–30]. RpoN binds to specific promoters with conserved −24, −12 sequences upstream of RpoN-regulated genes throughout the genome and is a key virulence regulator[31]. The specific and conserved nature through which RpoN controls its regulon led us to develop the RpoN molecular roadblock, RpoN*. RpoN* is a cis-acting peptide that specifically binds the −24 site of RpoN consensus promoters, blocking transcription by RpoN and other factors[32]. The RpoN* peptide sequence includes the identical amino acids to *A. aeolicus* RpoN Region III that bind with high affinity to the −24 site[33]. We previously described engineering the *rpoN** gene in the broad-host range plasmid pBBR1MCS-5 under control of the ITPG-inducible *trc* promoter[32]. The pBBR1MCS-5 plasmid contains the Gm^R cassette[34] and is present at about 10 copies per cell[35]. When RpoN* is expressed in *P. aeruginosa* laboratory strains, transcription is affected globally and virulence is attenuated[32]. We showed that more than 700 genes are affected, either directly regulated by RpoN or indirectly by other transcription factors under RpoN control[32]. Furthermore, in *P. aeruginosa*, some genes may have promoter binding sites for multiple sigma factors[31]. Thus, loss of RpoN does not always equate to loss of transcription and gene expression. We showed that RpoN* affects virulence in a RpoN-deletion strain of *P. aeruginosa* PAO1. This demonstrates the roadblock's ability to attenuate gene expression by blocking transcription of genes under dual-regulation with RpoN and other sigma factors[32]. This strategy of blocking multiple promoters throughout the *P. aeruginosa* genome may be an effective method to combat virulence and evade development of resistance.

P. aeruginosa isolated from CF patients are phenotypically and genetically varied[36,37]. Many *P. aeruginosa* clinical isolates have mutations, including deletion or loss of function, in the *rpoN* gene[38,39]. It was not known how the cis-acting RpoN* peptide would affect virulence phenotypes in *P. aeruginosa* clinical isolates, particularly in strains that do not express or have low levels of RpoN. In this study, we describe the effects of RpoN* on *in vitro* and *in vivo* virulence of *P. aeruginosa* isolated from CF patients. We also describe RpoN* effects on antibiotic resistance in a laboratory strain. Expression of RpoN* reduced virulence-associated phenotypes in clinical isolates and improved *P. aeruginosa* susceptibility to multiple antibiotics. This study demonstrates that RpoN* has potential clinical applications and represents an effective strategy to combat antibiotic resistance and infections with *P. aeruginosa* in CF patients.

Results

Virulence phenotypes were variable in *P. aeruginosa* isolates from CF patients. *P. aeruginosa* isolated from different CF patients or within the same CF patient have varied phenotypes and genotypes[36,37]. *P. aeruginosa* adapts over time, leading to mutations and changes in expression of genes related to motility, quorum sensing, and overall virulence[38,40]. To determine the virulence-related phenotypic profiles of the strains used in this study (Table 1), each *P. aeruginosa* patient isolate was evaluated for motility and biofilm formation, compared to the virulent positive control strain *P. aeruginosa* PA19660 *Xen5*. Several patient isolates were highly motile

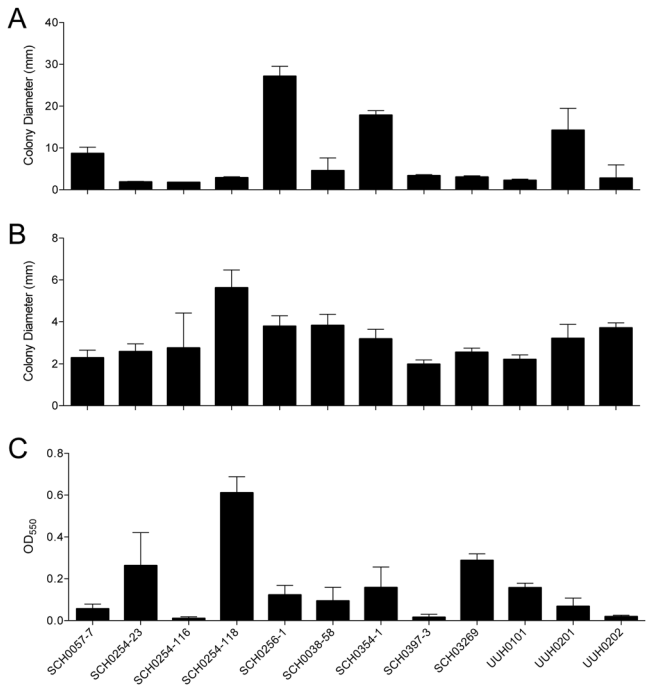

Figure 1. Characterization of virulence phenotypes of *P. aeruginosa* strains isolated from cystic fibrosis patients. *P. aeruginosa* CF patient isolates were compared to laboratory strain PA19660 *Xen5*. All assays were conducted at 37 °C for 24 h. (**A**) Colony diameter of swimming, or flagellar, motility assay conducted on soft (0.3%) agar. (**B**) Twitching, or pili, motility assay conducted on semi-hard (1.3%) agar. Colony diameter was measured across point of inoculation to the edges of bacteria colony. (**C**) Biofilm formation assay was conducted in 96-well microtiter plates. Biofilms were stained with crystal violet (0.1%), solubilized in ethanol (95%), and absorbance measured at OD_{550}. Bars are the mean ± SD; n = 5 to 6 replicates in motility assays and n = 10 in biofilm assay. Each assay was performed at least three separate times and representative results are shown.

in the swimming assay (flagella), including SCH0057-7, SCH0256-1, SCH0354-1 and UUH0201, while others were nonmotile (Fig. 1A, Supplemental Fig. S1A–C). Most strains were motile in the twitching assay (pili) and produced moderate biofilms, with SCH0254-118 migrating the furthest (Fig. 1B, Supplemental Fig. S1A'–C') and forming the most extensive biofilm (Fig. 1C). SCH0254-116, SCH0397-3, and UUH0202 did not form biofilms.

The pathogenesis of patient isolates was evaluated in a *P. aeruginosa* – *C. elegans* infection model. All patient isolates were compared to *E. coli* OP50, an avirulent negative control. SCH0057-7 was the most pathogenic in the paralytic killing assay, which is mediated by hydrogen cyanide production[41,42] (Fig. 2A). Other strains were moderately pathogenic, including SCH0256-1, SCH0354-1, SCH0397-3, and UUH0202. SCH0057-7, SCH0338-58, and UUH0202 were highly pathogenic in the slow killing assay, which mimics establishment and proliferation of an infection and is mediated by the *lasR*, *gacA*, *lemA*, and *ptsP* genes[43], while UUH0201 was moderately pathogenic (Fig. 2B). As expected, the virulence-associated phenotypes of patient isolates varied widely *in vitro* and *in vivo*.

RpoN protein levels varied among patient isolates. Others reported that the *rpoN* gene was mutated or lost in approximately 20% of *P. aeruginosa* isolates from CF patients[38]. Loss or mutation in the *rpoN* gene can result in phenotypes similar to those observed in the patient isolates evaluated here[20,22,23]. Thus, we evaluated relative protein levels of RpoN in these patient isolates by western blot. RpoN levels were moderately high in the positive control *P. aeruginosa* PAO1-S, while low or minimal protein levels were detected in the isogenic Δ*rpoN* mutant negative control (Fig. 3). The faint bands in the Δ*rpoN* strain and several CF patient isolates are background signals due to non-specific antibody binding to another protein or sigma factor with a similar apparent molecular weight. RpoN levels varied in the CF patient isolates, with high levels in SCH0057-7, SCH0397-3, and UUH0201; intermediate levels in SCH0254-116, SCH0338-58, and UUH0202; and background levels in SCH0254-23, SCH0254-118, SCH0256-1, SCH0354-1, SCH03269, and UUH0101.

RpoN* expressed in CF patient isolates reduced virulence-associated phenotypes *in vitro*. The effect of RpoN* expression on motility and biofilm formation in patient isolates was not known. Unfortunately, some patient isolates could not be transformed, and so only four isolates were evaluated for the effects of RpoN* expressed from a plasmid. SCH0057-7, SCH0256-1, SCH0338-58, and SCH0354-1 were transformed with a plasmid expressing RpoN* or the empty vector and selected with gentamicin. If RpoN* affected transcription of virulence-related genes in different genetic backgrounds as previously reported[32], we expected attenuation of virulence-related phenotypes in *P. aeruginosa* CF patient isolates. RpoN* significantly reduced colony diameter in all four patient isolates in the swimming motility assay (Student's t-test, $^{**}p \leq 0.01$, $^{***}p \leq 0.0001$) (Fig. 4A, Supplemental Fig. S3 (top two rows)). RpoN* significantly reduced colony diameter in SCH0057-7,

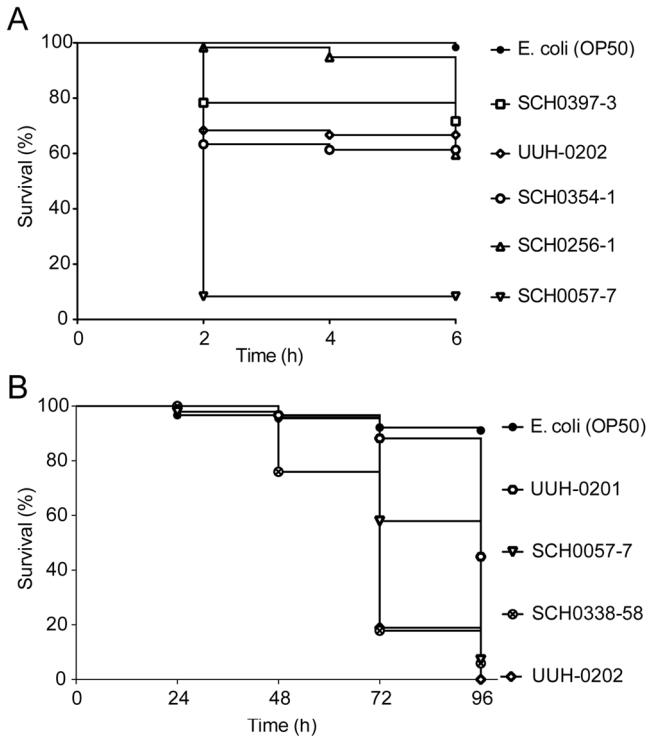

Figure 2. Pathogenesis of *P. aeruginosa* isolated from cystic fibrosis patients in *P. aeruginosa* – *C. elegans* infection model. Kaplan-Meier survival curves for *P. aeruginosa* – *C. elegans* infection assays. (**A**) Paralytic killing assay on BHI agar. Assay was conducted at room temperature and worm status scored every 2 h. (**B**) Slow killing assay on modified NGM agar (0.35% bactopeptone, 2% bactoagar). Assay was conducted at 20 °C and worm status scored every 24 h. Strains used included CF patient isolates, and *E. coli* for reference. n = 48 to 90 worms per strain.

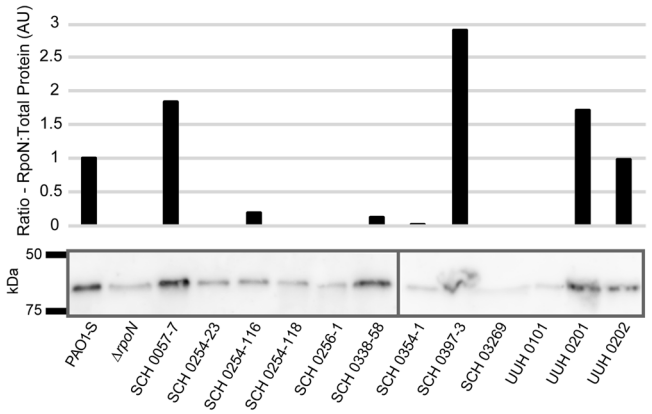

Figure 3. RpoN protein in *P. aeruginosa* isolates is highly variable. Immunoblot (bottom) and analysis (top) of RpoN expression in *P. aeruginosa* CF patient isolates and laboratory strains PAO1-S and Δ*rpoN*. Immunoblots performed on 10% Mini-PROTEAN TGX Stain-Free gels (BioRad). RpoN protein levels were calculated by comparing measured total protein in each lane to the measured RpoN band (presented as arbitrary units (AU)). The background value in Δ*rpoN* was subtracted from all samples and values were normalized against PAO1-S. The graph and immunoblot are representative of immunoblots from multiple bacterial cultures and western blot analyses. Separate immunoblots are indicated by the dividing line. See Supplemental Fig. S2 for full immunoblots.

SCH0256-1, and SCH0338-58 in the twitching motility assay (Student's t-test, $^{**}p \leq 0.01$, $^{***}p \leq 0.0001$) (Fig. 4B, Supplemental Fig. S3 (bottom two rows)). Colony diameter varied widely in SCH0354-1 when RpoN* was expressed and was always smaller than with empty vector, although the difference was not significant. In the biofilm formation assay, RpoN* significantly reduced biofilm formation by SCH0057-7 and SCH0256-1 (Student's t-test, $p \leq 0.0001$) (Fig. 4C). Thus RpoN* reduced virulence-associated phenotypes of *P. aeruginosa* isolated from CF patients.

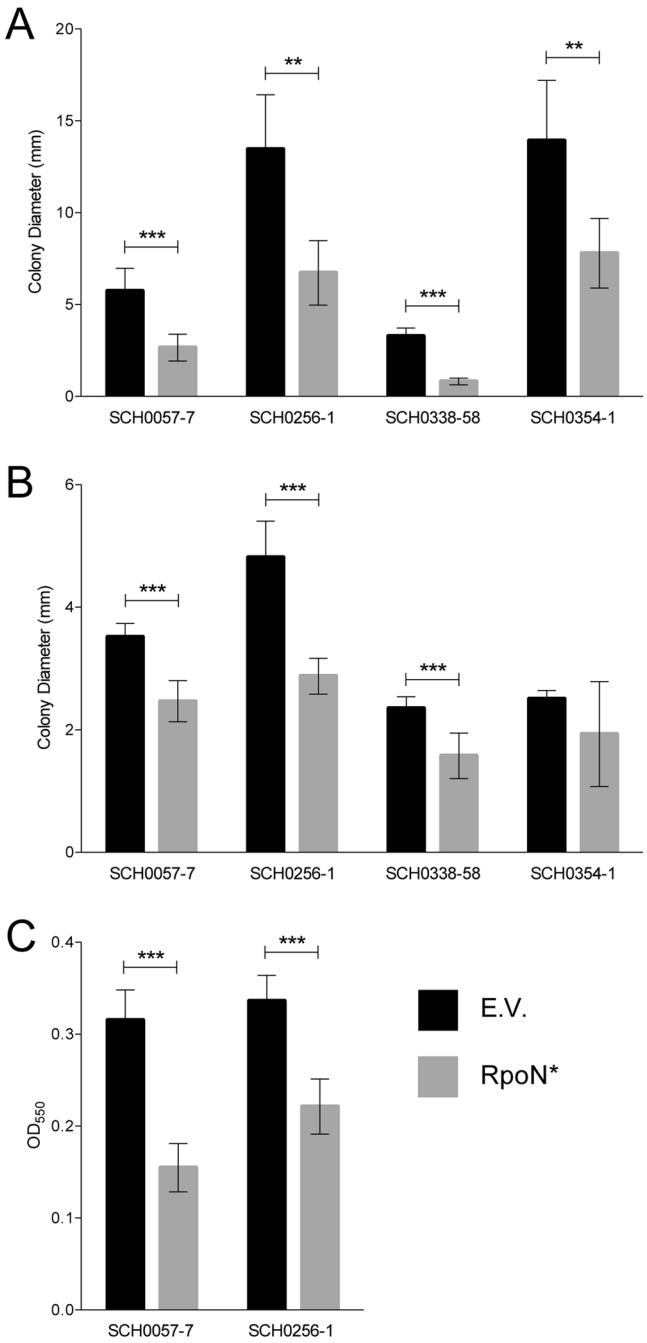

Figure 4. RpoN* expression decreases motility and biofilm formation. *P. aeruginosa* CF patient isolates with empty vector (E.V., black bars), or with RpoN*-expression vector (gray bars). Media was supplemented with gentamicin (30 mg/L), and IPTG (1 mM) when applicable, and all assays were conducted at 37 °C for 24 h. (**A**) Colony diameter of swimming, or flagellar, motility assay conducted on soft (0.3%) agar. (**B**) Colony diameter of twitching, or pili, motility assay conducted on semi-hard (1.3%) agar. (**C**) Biofilm formation assay conducted in 96-well microtiter plates. Bars are the mean ± SD (Student's t-test, ***$p \leq 0.0001$; **$p \leq 0.01$). n = 4 to 5 replicates in motility assays; n = 12 in biofilm assays.

RpoN* expression increased worm survival in *P. aeruginosa* – *C. elegans* infection model. Initial evaluation of patient isolates revealed a single *P. aeruginosa* strain, SCH0057-7, that was both transformable and pathogenic in the *P. aeruginosa* – *C. elegans* infection assay. Therefore, effects of RpoN* on pathogenesis of SCH0057-7 were evaluated using the paralytic killing assay, which is based on *P. aeruginosa* hydrogen cyanide production and mimics conditions in the CF lung[41,42]. Wild-type *P. aeruginosa* SCH0057-7 was the positive, virulent control and *E. coli* was the negative, avirulent control. The test conditions were *P. aeruginosa* SCH0057-7 expressing RpoN* or carrying the empty vector plasmid. If RpoN* affected virulence-related phenotypes in *P. aeruginosa* SCH0057-7, then we expected increased survival of *C. elegans*. Wild type SCH0057-7 and with

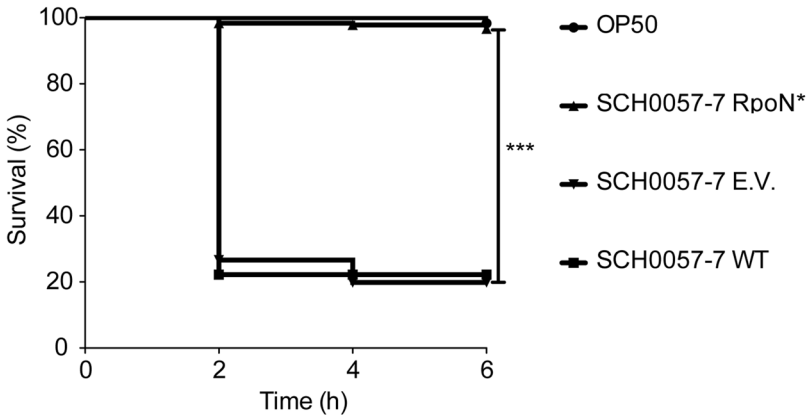

Figure 5. RpoN* promotes *C. elegans* survival in paralytic killing assay. *P. aeruginosa* CF isolate SCH0057-7 wild type, with empty vector (E.V.), or with RpoN*-expression vector, were compared to avirulent *E. coli* OP50. Paralytic killing assay was conducted on BHI agar supplemented with gentamicin (30 mg/L) and IPTG (1 mM), when applicable. Assay was conducted at room temperature, and worm status scored every 2 h. Kaplan-Meier survival curves represent combined survival of three separate assays (exception: *E. coli*). Mantel-Cox log-rank test was used to analyze E.V. and RpoN* curves (***$p \leq 0.0001$). n = 180 worms per SCH0057-7 condition, n = 60 worms for *E. coli*.

the empty vector killed approximately 80% of *C. elegans* (Fig. 5). In contrast, RpoN* expression significantly increased *C. elegans* survival (Mantel-Cox Log-Rank Test, $p \leq 0.0001$). Thus, RpoN* expression reduced pathogenesis of a patient isolate in a *P. aeruginosa* – *C. elegans* infection model.

RpoN* increased susceptibility to select antibiotics *in vitro*. Antibiotic resistance is a problem in CF patients with *P. aeruginosa* infections[44–46]. We previously reported that RpoN* alters transcription of several genes involved in multidrug efflux pumps that confer natural resistance[32]. Additionally, RpoN is implicated in tolerance to various classes of antibiotics[28–30]. We evaluated the effects of RpoN* on antibiotic susceptibility using a MicroScan Neg MIC 43 panel. The test conditions were *P. aeruginosa* PA19660 *Xen5* that was mock-transformed (no vector) or transformed with the empty vector or RpoN* plasmid. We expected that RpoN* would improve antibiotic susceptibility of *P. aeruginosa*. In PA19660 Xen5 mock-transformed or with the empty vector, antibiotic susceptibility profiles were the same, except for gentamicin, which increased in the empty vector strain due to the GM^R selection marker (Table 2). In PA19660 *Xen5* expressing RpoN*, susceptibility to five beta-lactam antibiotics was improved at least 2- to 4-fold (Table 2). Results presented in Table 2 are representative of a single experiment, however these results were highly consistent and repeatable over multiple experiments and biological replicates. The antibiotics with improved susceptibility were cefotaxime, cefepime, and ceftazidime (three cephalosporins), piperacillin (a ureidopenicillin), and imipenem (a carbapenem). Susceptibility to some antibiotics was unchanged (data not shown). For piperacillin, there was at least a 2-fold increase in susceptibility. This improvement is clinically relevant as it changed the status from resistant to sensitive (Table 2). For the other drugs, which there was no change in clinical susceptibility status, presence of RpoN* increased the therapeutic potency of the drugs. The results demonstrate that RpoN* expression increased *P. aeruginosa* susceptibility to several antibiotics.

Discussion

Here, we confirm and expand results of previous studies by showing the ability of RpoN* to abrogate virulence phenotypes in *P. aeruginosa* isolates from CF patients and to improve susceptibility to several antibiotics. Our working model of the mechanism of action of RpoN* is that it binds the -24 promoter consensus sites, blocking transactivation by RpoN and other sigma factors. By altering the transcriptome, RpoN* reduced virulence in well-characterized laboratory strains[32]. Thus, the motivation for this study was to understand the clinical relevance of RpoN*. We demonstrated that RpoN* expressed in CF patient isolates reduced motility and biofilm formation *in vitro*, independently of RpoN protein levels. The RpoN* molecular roadblock protected *C. elegans* from a highly virulent *P. aeruginosa* patient isolate in an *in vivo* infection model. RpoN* also improved *P. aeruginosa* susceptibility to antibiotics.

P. aeruginosa isolated from CF patients are highly variable[36,37], with the *rpoN* gene often mutated or lost[38]. The patient isolates evaluated in this study had a broad range of motility, biofilm formation, RpoN protein levels, and virulence in *C. elegans*. There was no correspondence between most *in vitro* phenotypes, *in vivo* pathogenesis, and RpoN levels (Supplemental Fig. S4). The only correlation observed was between twitching, or pili-associated, motility and biofilm formation (Supplemental Fig. S4F, $p = 0.0357$, $R^2 = 0.37050$). Other studies suggested that *in vitro* phenotypes of *P. aeruginosa* isolates can be related to disease status in CF patients[47]. Patient information and status of *P. aeruginosa* infections is limited for the isolates described here, so a comparison between phenotypes and patient status is not feasible. Interestingly, two isolates, UUH0201 and UUH0202, were obtained five months apart from the same patient, with UUH0201 collected first. The UUH0202 strain was less motile and RpoN protein levels dropped compared to UUH0201, but virulence increased. This supports the concept that *in vitro*

P. aeruginosa PA19660 Xen5				
Antibiotic Class and Name:	Wild Type n = 4	Empty Vector n = 6	RpoN* n = 6	Treatment Guidelines of *P. aeruginosa*†
	Minimal Inhibitory Concentration (MIC)			
Aminoglycosides:				
Gentamicin (Gm)	8 μg/mL (I)	>38 μg/mL (R)	>38 μg/mL (R)	On and off-label in combination with other antimicrobials
Carbapenems:				
Imipenem (Imp)	2 μg/mL (S)	2 μg/mL (S)	≤1 μg/mL (S)	On-label use as an individual drug
Cephalosporins:				
Cefepime (Cpe) [4th generation]	4 μg/mL (S)	4 μg/mL (S)	≤2 μg/mL (S)	On and off-label use individually or in combination with other antimicrobials
Ceftazidime (Caz) [3rd generation]	4 μg/mL (S)	4 μg/mL (S)	≤1 μg/mL (S)	On and off-label use individually or in combination with other antimicrobials
Cefotaxime (Cft) [3rd generation]	16 μg/mL (R)	16 μg/mL (R)	8 μg/mL (R)	On-label use as an individual drug
Penicillins:				
Piperacillin (Pi)	>64 μg/mL (R)	>64 μg/mL (R)	≤16–64 μg/mL (S)	On and off-label use in combination with tazobactam

Table 2. RpoN* expression increases susceptibility to antibiotics. The interpretation as to whether the obtained MIC is resistant (R), intermediate (I), or sensitive (S) is based on therapeutic guidelines for treatment of an infection using a particular antibiotic against a specific organism. †Treatment information and use for each antibiotic obtained from UpToDate in September 2018.

phenotypes reflect *P. aeruginosa* infection status in CF patients[47]. Further work would be needed to fully elucidate such correlations.

The RpoN* molecular roadblock reduced virulence phenotypes in patient isolates with high or low levels of RpoN. Furthermore, there was no substantial difference between CF isolates transformed with the empty vector and in the absence of manipulation (wild type strains). For instance, RpoN protein levels were higher in SCH0057-7 than PAO1-S, and RpoN* reduced flagellar and pili motility, biofilm formation and pathogenesis. In contrast, relative RpoN protein levels were low in SCH0256-1 and SCH0354-1, and yet RpoN* reduced motility. Thus, the roadblock was effective in the presence or absence of the native sigma factor. This is possible since there is a redundancy among *P. aeruginosa* sigma factors, with multiple sigma factors having consensus promoter sites for the same gene[31]. Thus, in the absence of RpoN, other sigma factors promote transcription of certain genes. The results here confirm our previous findings, which show that RpoN* reduced virulence in a laboratory strain that was deleted for *rpoN*[32]. Unfortunately, barriers to transformation precluded evaluating RpoN* in some of the other clinical isolates. However, the strains that were successfully transformed represented much of the diversity across the patient isolates.

The CF patient isolates demonstrated variable pathogenesis in the *C. elegans* paralytic killing model that spans 6 hours. Only one pathogenic isolate, SCH0057-7, was transformable and thus possible to evaluate the effects of RpoN* *in vivo*. This strain and several others were also pathogenic in the slow killing assay. This assay was not used to evaluate RpoN* because of difficulty maintaining the plasmid and RpoN* expression. Gentamicin selection and IPTG induction are not durable, we found[32], because the *C. elegans* cuticle is impermeable and the compounds are poorly absorbed in the intestine[48]. While it is expected that expressing RpoN* in CF isolates would improve *C. elegans* survival in the slow killing assay, it is not feasible with the current vector. If issues with maintaining the plasmid and expression of the roadblock were resolved, it would be interesting to evaluate RpoN* in this assay using patient isolates.

The molecular roadblock, RpoN*, binds numerous promoters in bacterial genomes, altering the transcriptome. RpoN* expression in *P. aeruginosa* greatly reduced transcription of the *mex* family genes[32], which are involved in multidrug efflux pumps[49]. Increased expression of *mex* genes is linked to increased resistance to antibiotics[14]. Therefore, we investigated whether RpoN* alters *P. aeruginosa* susceptibility to antibiotics. We employed a clinical laboratory assay for testing bacterial susceptibility or resistance to antibiotics and found that RpoN* improved antibiotic susceptibility at least two-fold for five different antibiotics, including imipenem. This agrees with previous studies that showed RpoN is involved in *P. aeruginosa* tolerance of carbapenems, quinolones, and tobramycin[28–30]. Unfortunately, the commercial assay uses pre-determined antibiotic concentrations in a 96-well plate, limiting the scope of the roadblock's effects. Additionally, the *P. aeruginosa* strain used here is sensitive to quinolones and tobramycin, so RpoN* effects on resistance to these antibiotics was not evaluated. Unfortunately, testing clinical strains was not feasible for this study. However, it will be important to test such strains, particularly those resistant to quinolones, carbapenems, and tobramycin, to determine the effects of RpoN*. Further studies are needed to uncover the full spectrum of RpoN* effects on antibiotic susceptibility.

MRDOs are increasing worldwide, even those with resistance to entire antibiotic classes. Alarmingly, nearly all antibiotics brought to market in the past 30 years are variations on existing drugs[50]. Research into alternative strategies to treat bacterial infections is a priority, including compounds to enhance activity of existing antibiotics or neutralize virulence factors. The molecular roadblock falls into the latter type. RpoN* binds consensus promoters throughout the *P. aeruginosa* genome, affecting the transcription of numerous virulence factors. Due to

the many binding sites for RpoN*, it is unlikely that resistance to it would develop during treatment. The binding sequence of the RpoN consensus promoter is conserved across gram-negative and gram-positive bacteria (36, 37). The effects of RpoN* on virulence phenotypes of *Pseudomonas putida*, *Burkholderia cepacia*, and *Escherichia coli* have been explored (unpublished data), suggesting that RpoN* may reduce virulence in multiple organisms. More studies are needed to identify the spectrum of RpoN* activity and its resistance frequency. Currently, RpoN* is a tool for antimicrobial development and is not a usable drug. However, a study was recently published using a stapled RpoN-like peptide to reduce transcription of RpoN-related genes in *E. coli*[51]. They found that this stapled peptide, which binds to RpoN consensus promoters, could enter *P. aeruginosa* cells[51]. Finding a small molecule or stapled-peptide that works in the same cis-acting manner as RpoN* would be an effective, clinically relevant strategy to combat *P. aeruginosa* virulence and antibiotic resistance.

Materials and Methods

Bacteria and nematodes. *P. aeruginosa* clinical isolates were provided by Seattle Children's Hospital (SCH strains) and Upstate University Hospital (UUH strains). *P. aeruginosa* PAO1-M was provided by C. Manoil[41], and *P. aeruginosa* PAO1-S and Δ*rpoN* were provided by D. Haas[22]. *P. aeruginosa* PA19660 *Xen5* was purchased from PerkinElmer. *E. coli* OP50 was provided by D. Pruyne (SUNY Upstate Medical University). All strains are listed in Table 1. For long-term storage, bacteria were grown overnight in LB broth at 37 °C with shaking, and frozen in 10% glycerol at −80 °C. *Caenorhabditis elegans* N2 was purchased from the *Caenorhabditis* Genetics Center (University of Minnesota, Minneapolis, MN), and maintained on nematode growth media (NGM) seeded with *E. coli* OP50 at 20 °C[52]. Populations were synchronized via egg lay and grown to the young adult stage at 20 °C[53].

Plasmids. RpoN* and empty vector plasmids were previously described[32]. Plasmids were maintained in *E. coli* INV110 (Invitrogen) with gentamicin selection (30 mg/L). RpoN* expression was induced with isopropyl β-D-1-thiogalactopyranoside (IPTG, 1 mM).

Transformation. Permissive *P. aeruginosa* patient isolates and a lab strain were transformed by electroporation prior to all experiments, per standard protocol[54]. Transformed bacteria were selected on LB agar or BHI agar supplemented with gentamicin (30 mg/L). Individual colonies were picked for each assay.

Western blot analysis. Overnight bacteria cultures were treated with Cell Lytic B Lysis Reagent (Sigma) to generate crude cell lysates. The soluble protein fraction was separated on 10% Mini-PROTEAN TGX Stain-Free protein gels (BioRad), activated for 5 minutes with UV light, imaged and transferred via semi-dry apparatus to a PVDF membrane. Membranes were incubated with primary antibody specific for *E. coli* RNA σ^{54} (1:500, BioLegend) overnight, then with secondary antibody HRP goat anti-mouse (1:10,000, Jackson ImmunoResearch). The chemiluminescent signal was generated with the Pierce SuperSignal West Fempto substrate kit (Thermo Scientific) and detected with ChemiDoc MP Imaging System (Bio-Rad Laboratories). Protein bands and total protein per lane were measured with Image Lab (Version 5.2.1; Bio-Rad Laboratories). RpoN bands were compared to corresponding total detected protein in each lane and the background value in Δ*rpoN* was subtracted from all samples.

Phenotyping assays. Assays to measure swimming, twitching[55], and biofilm formation[56], were conducted according to standard protocols. Transformed *P. aeruginosa* clinical isolates were grown in appropriate media supplemented with gentamicin (30 mg/L), and with or without IPTG (1 mM). Motility and biofilm assays were conducted at 37 °C for 24 h. Images of motility assays were obtained with IVIS-50 (Perkin Elmer) and colony diameter was measured with Living Image software (Perkin Elmer). To study biofilm formation, overnight cultures were diluted 1:3 in LB broth with appropriate conditions, grown for 3 h at 37 °C with shaking. Log-phase cultures were diluted 1:50 into M63 minimal media with 0.4% arginine and 1 mM $MgSO_4$ (30 mg/L gentamicin and 1 mM IPTG included for transformed cultures), and 100 µl added per well to a U-bottom 96-well plate and plates incubated at 37 °C for 24 h. Biofilms were stained with 0.1% crystal violet at RT, extracted in 95% ethanol, and absorbance was measured at 550 nm with a µQuant microplate spectrophotometer (BioTek).

***P. aeruginosa* – *C. elegans* infection assays.** For the paralytic killing assay, laboratory strains, clinical isolates, or transformed *P. aeruginosa* were spread on Brain Heart Infusion (BHI) agar (Difco) with, when applicable, gentamicin (30 mg/L) and with or without IPTG (1 mM). *E. coli* was spread on BHI agar. All plates were grown overnight at 37 °C. Bacteria colonies were swabbed onto BHI agar, supplemented with gentamicin and/or IPTG (1 mM) when applicable, and grown at 37 °C for 24 h[41]. Adult *C. elegans* were added to plates and the assay was conducted at room temperature, per standard protocol[41]. For the slow killing assay, laboratory strains or clinical isolates of *P. aeruginosa* were grown overnight in LB broth at 37 °C with shaking, and cultures were spread on a modified NGM agar (0.35% bactopeptone, 2% bactoagar)[57]. Plates were incubated at 37 °C for 24 h, then at room temperature for an additional 24 h. The assay was conducted at 20 °C, and worms were scored every 24 h per standard protocol[57].

Antibiotic sensitivity testing. Transformed or mock transformed bacteria were grown overnight in LB broth with gentamicin (30 mg/L) and IPTG (1 mM) or only IPTG (1 mM), respectively. MicroScan Neg MIC 43 panels (Beckman Coulter Inc., Brea, CA) were used. Panels were set up per manufacturer's protocol (MicroScan Gram Negative Procedure Manual, version 09/2016) using the RENOX system (Beckman Coulter Inc., Brea, CA) with a final well concentration of $3–7 \times 10^5$ CFU/mL. The following modifications were made to the manufacturer's protocol: LB broth supplemented with IPTG (1 mM) and with or without gentamicin (30 mg/L) was used in place of saline for whole panel. Plates were incubated at 35 °C for 16–20 h and read using a MicroScan

autoSCAN-4 (Beckman Coulter Inc, Brea, CA). MIC values were determined by the MicroScan reader based on optical density. Quality control was performed on the panels per manufacturer's protocol.

Statistics. Data were analyzed using Excel and GraphPad Prism with a significance of $p \leq 0.05$ (Microsoft, Washington; GraphPad Software Inc., California).

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Author Contributions

M.G.L. wrote the manuscript. C.T.N. and J.F.M. conceived the study. M.G.L. and J.L.V. conducted the experiments. M.G.L. generated the figures. All authors reviewed the manuscript.

Additional Information

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