

RpoS Mutation Leads to Prolonged Biofilm Mode of Growth and a Higher Fitness in *Pseudomonas aeruginosa* Biofilms

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Research Article

Keywords: Experimental biofilm evolution, *Pseudomonas aeruginosa*, sigma factor RpoS, biofilms, cyclic-di-GMP, virulence

Posted Date: May 18th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-488211/v1>

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Version of Record: A version of this preprint was published at Cell & Bioscience on July 21st, 2021. See the published version at <https://doi.org/10.1186/s13578-021-00655-9>.

Abstract

Background

Pseudomonas aeruginosa is a notorious opportunistic pathogen causing various biofilm-related infections. Biofilm formation is a unique microbial strategy that allows *P. aeruginosa* to survive adverse conditions such as antibiotic treatment and human immune responses.

Results

In this study, we experimentally evolved *P. aeruginosa* PA01 biofilms for cyclic treatment in the presence of high dose of imipenem, and enriched hyperbiofilm mutants within six cycles in two independent lineages. The competition assay showed the evolved hyperbiofilm mutants can outcompete the ancestral strain within biofilm by prolonging the biofilm mode of growth but not in planktonic cultures. Whole-genome sequencing analysis revealed the hyperbiofilm phenotype is caused by point mutations in *rpoS* gene in all independently evolved mutants and the same mutation was found in *P. aeruginosa* clinical isolates. We further showed that mutation in *rpoS* enhanced biofilm formation by prolonging the biofilm mode of growth and elevating the intracellular c-di-GMP level. Mutation in *rpoS* increased pyocyanin production and virulence in both *P. aeruginosa* laboratory strains and clinical isolates.

Conclusion

Here, our study revealed that antibiotic treatment of biofilm-related *P. aeruginosa* infections might induce a hyperbiofilm phenotype via *rpoS* mutation, which might partially explain antimicrobial treatment failure of many *P. aeruginosa* biofilm-related infections.

Introduction

Microbial cells undergo rapid evolution under stress to adapt to the environment in nature [1] or inside the host [2]. Certain key mutations on genome will greatly help bacterial populations to gain a competitive advantage in adverse environment [3]. To identify these adaptive traits of microbes, experimental evolution experiments are usually conducted to mimic these diverse environmental conditions for accelerating the emerge of well-fitted variants [4]. Employment of next-generation sequencing approaches will facilitate the identification of the mutations of experimental adapted bacterial variants and elucidation of the underlying molecular mechanism of the evolved traits [5]. So far, adaptive experimental evolution has been applied to reveal the molecular basis of drug resistance [6], persistence [7], biofilm formation [8] and etc. An important question for experimental evolution is the relevance of laboratory observation to the evolution in natural conditions. For this point, studies have indicated the mutation derived phenotype which observed in laboratory evolution could be found in clinical isolates [9-12].

Bacterial pathogens are able to form biofilms on both biotic and abiotic surfaces, causing many hospital-acquired and recurrent infections. Biofilms are densely-grown microbial cells embedded in self-secreted

hydrated matrix consisting of polysaccharides, proteins, extracellular DNA and lipids [13]. Previous studies have shown that the biofilm-grown bacteria have distinct phenotypes from planktonic cultures, including gene expression [14, 15] and increased antibiotic resistance [16]. The resistance to antimicrobial agents by biofilm cells can be increased up to 1000-fold compared to planktonic cells [17]. Investigating evolution traits of biofilm cells against antibiotics might provide knowledge about bacterial adaptation during chronic infections.

P. aeruginosa is a notorious opportunistic pathogen, which causes a variety of infections, including wounds, urinary tract and respiratory infections, and is the leading cause of morbidity and death for patients with cystic fibrosis (CF) [18]. Infections caused by *P. aeruginosa* can be very difficult to treat due to its intrinsic resistance to a variety of antibiotics and tends to form biofilms at the site of infection [19, 20]. As an important nosocomial pathogen, *P. aeruginosa* biofilms have been found on various surfaces of indwelling medical devices, including urinary catheters, bone plates, ventricular assist device drivelines and pacemakers [21]. Biofilms provide *P. aeruginosa* an enormous advantage in clinical infection by protecting biofilm cells from the immune system [22] and tolerance to antimicrobial agents [23, 24]. Persisters are subpopulation of isogenic bacteria that tolerance to antibiotics [25], the persister cells were dormant in biofilms and significantly contributes to *P. aeruginosa* biofilm recalcitrance after the cessation of antibiotic therapy [26]. Consideration the issue of drug tolerance and recalcitrance of *P. aeruginosa* biofilm-related infections, urgent, novel antibiofilm therapeutics are needed.

The long-term use of antibiotics in the treatment of *P. aeruginosa* infections in cystic fibrosis patients is well known to drive emergence of diversified drug-resistant variants. Clinical isolates of *P. aeruginosa* has shown distinct biofilm formation capacity and many of them were strong biofilm producers [27]. However, current adaptive experimental evolution studies of *P. aeruginosa* are mainly focusing on planktonic cultures [11, 28, 29]. In this study, we established a clinically relevant model to investigate evolution traits of imipenem-treated biofilm cultures of *P. aeruginosa*. The set-up of the *P. aeruginosa* biofilm experimental evolution model in this study is presented in Figure 1A.

We show here that cyclic exposure of *P. aeruginosa* biofilms to high concentration of imipenem led to emergence of variants with a hyperbiofilm phenotype. After 3 and 6 cycles of treatment, the colony forming units (CFU) of biofilm on glass beads were found to increase 1000-fold. The competition assay showed that the evolved hyperbiofilm variants can outcompete ancestral strain within biofilms but not in planktonic cultures. Genome sequencing analysis revealed that the hyperbiofilm phenotype is caused by single-point mutations in the sigma factor RpoS, which elevated the intracellular c-di-GMP level. Importantly, the mutations on *rpoS* identified in the *in vitro* experimental biofilm model occurred in *P. aeruginosa* clinic isolates with a hyperbiofilm phenotype at a substantial rate. Overall, our data show that under imipenem treatment, mutations in *rpoS* could be selected in *P. aeruginosa* and subsequently lead to enhanced biofilm formation. Thus, caution should be taken when antimicrobial treatment of *P. aeruginosa* infections fails in clinical settings, *rpoS* mutation in cultured isolates may be the root cause and needs to be investigated.

Results

Experimental biofilm evolution selects for *P. aeruginosa* hyperbiofilm mutants

In order to examine the evolutionary traits of *P. aeruginosa* biofilms under antibiotic stress condition, we exposed biofilms of *P. aeruginosa* PAO1 to different concentrations of imipenem (40, 80 and 160 µg/mL), which is 10, 20 and 40 times of the minimum inhibitory concentration (MIC), in a cyclic manner (Figure 1). Imipenem, a widely used last resort antibiotic, has been chosen as the selective pressure for experimental biofilm evolution owing to its' commonly prescribed for treatment of *P. aeruginosa* infections [30].

The biofilms of six independent lineages in different concentrations of imipenem treated group initiated from a common ancestor PAO1 strain were formed on the surface of 5 mm glass beads [31] and treated with imipenem for 24 h. Survivor cells on beads were quantitated by CFU counts. Biofilm survivors were collected and reinoculated in fresh LB medium for the 2nd cycle (Figure 1A). At the first cycle, the CFU counts on bead of each lineage was between $6.30\text{-}6.90 \log_{10}$. After 6 cycles, no hyperbiofilm variants observed in 10× and 20× MIC (Figure S1A and B) treated groups, and two lineages were accumulated hyperbiofilm variants in 40× MIC treated group (Figure S1C). The CFU counts within lineage W1 and W6 biofilms on bead reached 9.82 and $10.01 \log_{10}$ in 40× MIC treated group (Figure 1B). Next, the biofilm formation ability of ancestral, C6W1, C3W6 and C6W6 population were further confirmed by the crystal violet (CV) biofilm assay. Similarly, the CV method revealed that biofilms formed by C6W1, C3W6 and C6W6 population were between 2- and 3-fold higher than the ancestral population (Figures 1C). In order to track when the hyperbiofilm variants have emerged within lineage W1 and W6 population, we picked 6 colonies form each cycle in random and measured the CFU of biofilms on bead. We found that the hyperbiofilm variants of lineage W1 and W6 appeared since cycle 5 and cycle 2 and enriched at cycle 6 (Figure 1D) and cycle 3 (Figure 1E), respectively. These results indicate that the hyperbiofilm variants only accumulated upon higher concentration imipenem treatment, rather than lower concentration of imipenem. The different appearance timing of hyperbiofilm variants in two independent linages - possibly as a result of varying evolutionary rate [32]. More lineages might accumulate hyperbiofilm variants in 40× MIC treated group if we increase the treatment cycles. Based on these results, we chose colonies C6W1C, C3W6F and C6W6F for further study.

Point mutations in *rpoS* lead to hyperbiofilm phenotype of *P. aeruginosa*

In order to elucidate the genetic mechanisms underlying the hyperbiofilm phenotype, we sequenced the C6W1 and C6W6 population, and choose C6W5 population as the negative control. Through comparative genomic analysis, we identified only SNPs in one gene, *PA3622* (encodes sigma factor RpoS), which was mutated in C6W1 and C6W6 population but not in C6W5 population when compared to the ancestral strain. The sigma factor RpoS is well known as a master regulator that controls the expression of genes involved in stress response and virulence factors production in *P. aeruginosa* [33, 34]. A previous study

used transcript profiling to identify that 772 genes were regulated by RpoS in stationary phase and it affects expression of more than 40% quorum-sensing controlled genes [35].

We next re-sequenced of *rpoS* in C6W1C, C3W6F and C6W6F, and identified the nonsynonymous mutations in RpoS of C6W1C (P251L), C3W6F (Q266stop) and C6W6F (Q266stop). To further confirm the causality of *rpoS* mutations for the hyperbiofilm phenotype, we constructed a *de novo* mutant allele with a SNP on the ancestor PAO1 genome to yield RpoS^{P251L} and RpoS^{Q266stop} mutant strains. We found that single point mutation in *rpoS* could produce the hyperbiofilm phenotype (Figure 2A). We also tested the biofilm formation ability of $\Delta rpoS$ strain and confirmed that knockout *rpoS* in *P. aeruginosa* PAO1 indeed increased the biofilm formation (Figure 3A). Complementing the mutation strains with wild type *rpoS* reverted the hyperbiofilm phenotype to the wild-type level (Figure 2A).

Protein domain analysis showed that RpoS consists of 4 regions, and region 4 contains a DNA binding domain. P251L located on the end of region 3, knockout this region increased the biofilm formation. Q266stop mutation leads to RpoS lacking region 4, knockout region 4 has the same phenotype of Q266stop mutation. Moreover, we have constructed region 3 and 4, region 2, 3 and 4 and region 1 deletion strains, all of those region deletion mutants produced the hyperbiofilm phenotype (Figure 2B). Together, these results provide definitive evidence that the SNPs identified are necessary and sufficient to cause hyperbiofilm phenotype in *P. aeruginosa*. Moreover, P251L and Q266stop mutations are very likely to lead to the inactivation of RpoS.

Hyperbiofilm variants outcompete the ancestral strain during biofilm competitions

The convergent emergence of *P. aeruginosa* hyperbiofilm variants from independent lineages suggested a competitive advantage for these variants over the ancestor. We have previously shown that *P. aeruginosa* cells did not share its EPS with its neighboring cells [36] and thus we hypothesized that the evolved hyperbiofilm variants only gain advantage to the ancestor strain under biofilm growth condition. We then tested the competition of hyperbiofilm variants with the ancestral strain in both planktonic cultures and biofilms. The fluorescently tagged hyperbiofilm variants (tagged with mCherry) were mixed with ancestral strain (tagged with GFP) in different ratio and inoculated into the bead-containing 24 well microplate. After 24 h cultivation, the planktonic and biofilm cells were analyzed with flow cytometry. We found that, the planktonic cells of C6W1C was slightly higher than ancestral strain when inoculated at the same ratio (Figure 3A), while no difference was found between C3W6F and C6W6F with ancestral strain when inoculated at the same ratio (Figure 3B and C). Interestingly, the competitions in biofilms confirmed that the hyperbiofilm variants have a significant and predominant selective advantage against the ancestral strain (Figure 3A, B and C). Next, we increased the inoculation ratio of ancestor and hyperbiofilm variants to 5:1, the proportion of ancestor within biofilm was still much less than hyperbiofilm variants (Figure 3D, E and F). These results indicated that the hyperbiofilm variants increased the competition only in biofilms and this phenotype is not related to the change of growth rate, drug resistance and tolerance. Moreover, the competition advantage of hyperbiofilm variants is growth model specific and did not occur in planktonic culture.

Previous studies showed that acquired mutations conferring beneficial traits such as antibiotic resistance will dominate when exposing biofilm bacteria to high concentrations of antibiotic [37]. Therefore, the enrichment of the hyperbiofilm variants could have been achieved by accumulation of mutations that conferred resistance to imipenem. We found, however, that the MIC of imipenem for colonies isolated from the evolved lines (C6W1C, C3W6F and C6W6F) was indistinguishable from that for their ancestor (Figure 3G). One of the most straightforward ways to gain a competitive advantage is increasing the growth rate. To test this point, we measured the growth rates of the evolved hyperbiofilm variants and the ancestor in LB medium. Whereas, there is no significant difference between the hyperbiofilm variants and ancestor (Figure 3H). Next, we measured the biofilm growth curve of PAO1, $\Delta rpoS$ and the complementation strain $\Delta rpoS/p\text{-}rpoS$, the CFU of planktonic cultures were measured at the same time. We found that, the CFU of planktonic culture of all three strains showed no significant difference (Figure 3I). For the biofilm growth, the $\Delta rpoS$ strain indeed produce more biofilm than PAO1 after 6 h incubation and developed 2 log of biofilm CFU after 24 h incubation (Figure 3I). These results indicated that it is not the resistance level and planktonic growth rate select $\Delta rpoS$ mutant; whereas $\Delta rpoS$ has a prolonged biofilm mode of growth which eventually leads to occupation of the biofilm community.

Mutations in *rpoS* lead to an elevated intracellular c-di-GMP levels

Quorum-sensing (QS) [38] and c-di-GMP [39] have been well documented to play important roles in *P. aeruginosa* biofilm formation. To assess whether quorum-sensing and c-di-GMP levels were elevated, we introduced the quorum-sensing and c-di-GMP reporter systems [40-43] into the variants isolated from biofilm evolution experiments and ancestral strain to determine the relative level of corresponding signaling pathways. We found that the fluorescent signal of $P_{lasB}\text{-}gfp$ and $P_{rhlA}\text{-}gfp$ in ancestral strain were higher than that of hyperbiofilm variants (Figure S2A and B), while there were no differences in fluorescent signal of $P_{pqsA}\text{-}gfp$ between the ancestral and hyperbiofilm variants (Figure S2C). For the fluorescent signal of $P_{cdaR}\text{-}gfp$, the hyperbiofilm variants showed 2-fold higher in expression level than the ancestral strain, indicating that the hyperbiofilm variants might have elevated intracellular c-di-GMP levels (Figure S2D). We further showed that the $P_{cdaR}\text{-}gfp$ expression level were increased in $RpoS^{P251L}$, $RpoS^{Q266stop}$ and $\Delta rpoS$ strain compared to the PAO1 wild-type strain (Figure 4A). The second messenger c-di-GMP is a key regulator of *P. aeruginosa* biofilm formation, which is synthesized from two GTP molecules by diguanylate cyclases (DGC) and is degraded into 5'-phosphoguanylyl-(3'-5') guanosine (pGpG) and/or GMP by phosphodiesterases (PDE) [39]. Till now, 43 DGC and PDE proteins have been identified in *P. aeruginosa* [44].

To investigate the mechanism underlying *rpoS* point mutation-induced increasing in intracellular c-di-GMP content, we performed transcriptomic analysis of PAO1, $RpoS^{P251L}$ and $RpoS^{Q266stop}$ strains using RNA-sequencing. Samples were collected after 8.5 h culture owing to the $P_{cdaR}\text{-}gfp$ fluorescent intensity (Figure S3) between mutants and wild type PAO1 strain have the biggest difference at this time point. We found that, 15 DGC and PDE proteins were upregulated at least 2-fold in both $RpoS^{P251L}$ and

RpoS^{Q266stop} strains compared to PAO1 (Table 1). This result indicated that the c-di-GMP metabolism in *rpoS* mutant strains were more active than PAO1.

RpoS regulates the expression of small regulatory RNAs *rsmY* and *rsmZ* in *Legionella pneumophila* [45]. Moreover, *rsmY/Z* participate in the regulation of c-di-GMP production in *P. aeruginosa*, the c-di-GMP levels were strongly reduced in the *rsmY/Z* double deletion mutant [46]. Our transcriptomic analysis showed that the expression of *rsmY* and *rsmZ* was increased 3.84 and 5.04-fold in *RpoS*^{P251L} compared to the PAO1 wild-type, respectively. Next, we measured the expression of *rsmY/Z* in PAO1, *RpoS*^{P251L}, *RpoS*^{Q266stop} and $\Delta rpoS$ strains using reporter fusions [14]. We found the *rsmY/Z* expressions were increased in *RpoS*^{P251L}, *RpoS*^{Q266stop} and $\Delta rpoS$ (Figure 4B and C), which is consistent with the increased level of c-di-GMP of these mutants. These results showed that the mutation of *rpoS* has led to the increase in *rsmY/Z* expression and intracellular c-di-GMP content in *P. aeruginosa*.

***rpoS* mutation associated hyperbiofilm phenotype in clinical isolates**

Our experimental biofilm evolution data has revealed that single-nucleotide mutations on *rpoS* confer *P. aeruginosa* hyperbiofilm phenotype and produce a pronounced competitive advantage within the biofilm microenvironment. In order to analyze the preference of *rpoS* mutation, we downloaded 4000 sequences of *rpoS* from pseudomonas genome database (www.pseudomonas.com). Through comparative analysis, we have identified 241 non-synonymous mutations (6.03% of total sequence), 8 insertion or deletion mutations (0.2% of total sequence) and 5 stop coding mutations (0.13% of total sequence) compared to the PAO1 wild-type strain. Among those mutations, 123 mutations were located on the inter-region of *rpoS* and 131 mutations were within 4 regions (Figure 5A). Moreover, we have identified 2 sequences harbored *RpoS*^{P251L} mutation. We also analyzed the top 5 mutation sites among 4000 sequences, and found L268Q was the top one with 71 sequences (Figure 5B).

Since imipenem has been used for clinical treatment of *P. aeruginosa* infection, we wondered whether *rpoS* mutation caused hyperbiofilm strains exist in clinical isolates. Therefore, we examined the biofilm formation capacity of 288 clinical *P. aeruginosa* isolates obtained from the patients with culture confirmed *P. aeruginosa* infections (Table S1). Through quantitative analysis of biofilm formation by measuring crystal violet staining at OD₅₅₀ nm and total bacterial growth at OD₆₀₀ nm to exclude growth variation, we identified 29 hyperbiofilm isolates (10.07 % of total isolates) in this collection (Figure 5C). Next, we target sequenced *rpoS* of 29 hyperbiofilm isolates and confirmed that #16 isolate harbored non-synonymous mutation in *rpoS*. Interestingly, #16 isolate, which is isolated form the peritoneal drainage fluid, has the same mutation *RpoS*^{P251L} as our experimental evolved variant C6W6F.

The evolved *rpoS* variants are hypervirulent

Pyocyanin production is one of the major virulence factors of *P. aeruginosa*, and plays an important role in *P. aeruginosa* pathogenesis by causes oxidative stress to the host, induces apoptosis in neutrophils and inhibits phagocytosis of macrophages [47, 48]. Previous studies showed that the pyocyanin

production was increased in a *rpoS*-deletion mutant [34]. In order to test the impact of *rpoS* point mutation on our biofilm evolved variants on pyocyanin production, we compared the production of pyocyanin by *P. aeruginosa* PAO1 strain, RpoS^{P251L}, RpoS^{Q266stop} and Δ*rpoS*. As we expected, similar to the Δ*rpoS* mutant, the *P. aeruginosa* RpoS^{P251L} and RpoS^{Q266stop} produced higher amounts of pyocyanin than the wild-type PAO1 strain (Figure 6A). This result suggests that point mutations accumulated in the *rpoS* gene in *P. aeruginosa* clinical isolates have similar effect as *rpoS* gene deletion on its physiology.

Next, we further assessed the impact of evolved *rpoS* point mutations on virulence using the macrophage cytotoxicity model [49]. The RAW264.7 macrophages were infected with *P. aeruginosa* PAO1, RpoS^{P251L}, RpoS^{Q266stop} and Δ*rpoS*, and the release of cytosolic lactate dehydrogenase (LDH) was determined. We found that macrophages infected with RpoS^{P251L}, RpoS^{Q266stop} and Δ*rpoS* released more LDH compared to *P. aeruginosa* PAO1 after 4 h infection (Fig 6B), suggesting mutation on *rpoS* can induce the cell death of macrophage. Altogether, these results suggest that mutations on *rpoS* can enhance the virulence in *P. aeruginosa*.

Discussion

Biofilms represent the predominant lifestyle for most microorganisms in nature. Understanding how microorganisms evolve in biofilms can reveal novel insights of adaptive evolution, especially under stress conditions. For example, small colony variants are enriched in *P. aeruginosa* biofilms after exposure to sodium dodecyl sulfate [50]. The *P. aeruginosa* hyperbiofilm forming variants are often observed from patients who are suffering chronic infections such as CF [51]. We have previously used a planktonic experimental evolution model to demonstrate that oxidative stress drives the evolution of point mutations in the *P. aeruginosawspF* gene, which lead to increase in intracellular c-di-GMP content and exopolysaccharide synthesis [28]. Here, we performed biofilm experimental evolution to examine the adaptive evolution of *P. aeruginosa* biofilms during the treatment of imipenem (160 µg/mL), which case can be reached in some situations in clinical settings. The keratitis infection caused by *P. aeruginosa* usually form corneal biofilms [52]. In a case of the treatment of bacterial keratitis in patients, topical imipenem (50 mg/mL) has been selected as monotherapy for corneal infection [53]. In another study, 1-5 mg/mL imipenem has been used for bacterial keratitis treatment via topical administration[54] . Moreover, we showed that the hyperbiofilm variants could be accumulated in biofilms after cycle antibiotic treatment and these variants were able to outcompete the ancestor strain shortly after appearance. Genome sequencing analysis revealed that the adapted *P. aeruginosa* hyperbiofilm variants in different lineages shared single-point mutations in the same gene, which encodes the sigma factor RpoS.

The *rpoS* gene has been previously well characterized in *P. aeruginosa* for its regulatory role on quorum sensing and virulence. A DNA microarray-based transcriptomic study showed that the expression of *rpoS* in *P. aeruginosa* biofilm cells was downregulated compared to the planktonic cells, inactivation of *rpoS* in *P. aeruginosa* PAO1 increased biofilm formation in flow-cell reactor [15]. In our study, we demonstrated

that *rpoS* point mutations have similar impact to the *rpoS* deletion on biofilm formation in *P. aeruginosa*. Function domain analysis indicates that RpoS contain 4 regions, RpoS as a global regulator, the DNA binding domain was located on region 4. Proline residues are restricts to the first four positions of an α -helix [55], which plays a special role in the stable of protein structure. RpoS^{P251L} and RpoS^{Q266stop} on *P. aeruginosa* genome have shown the same phenotype of hyperbiofilm, pyocyanin production and virulence as $\Delta rpoS$ strain, which means the 251proline to leucine mutation in *rpoS* might results loss of the function on regulation. Moreover, we showed that mutation on *rpoS* increased the production of c-di-GMP and pyocyanin, both of those two molecules were play a very important role in biofilm formation [39] and virulence [56] in *P. aeruginosa*.

Surprisingly, the *rpoS* mutants were found to outcompete the wild-type PAO1 strain under biofilm mode of growth in a short time frame. The *P. aeruginosa* small colony variants (e.g. with *wspF* mutations) are also well known being evolved in *P. aeruginosa* biofilms, which have even higher biofilm formation capacity than the *rpoS* mutants. However, these small colony variants often have a lower planktonic growth rate than the *P. aeruginosa* wild-type strain, and thus can easily be outcompeted by the wild-type in the planktonic phase of growth [28]. Instead, our study showed that the *P. aeruginosa* *rpoS* mutants are able to outcompete the wild-type PAO1 strain by prolonging the biofilm mode of growth while not compromising its planktonic growth rate. Within the microenvironment of biofilm, PAO1 wild-type and *rpoS* mutants live with identical niches and also have identical needs, thus they will compete for precisely the same resources. Our observation that *rpoS* mutants can outcompete the PAO1 could be explained by the competitive exclusion principle [57, 58]. As a sigma factor, RpoS controls a wide range of genes under stationary phase of growth, which shared many characters with biofilm mode of growth, such as lack of nutrients and accumulation of waste products. Further studies should be carried to examining the regulatory roles of RpoS on *P. aeruginosa* physiology and virulence factors under biofilm mode of growth.

Conclusions

Our study showed that imipenem treatment drives rapid evolution of *P. aeruginosa* *rpoS* deficient mutants within biofilms. We provided evidence that *rpoS* mutation not only increase *P. aeruginosa* virulence, but also enhance its intracellular c-di-GMP content. Importantly, the major obstacle for treatment of *P. aeruginosa* infection in clinical is the formation of biofilms. This study raises the possibility that some clinical *P. aeruginosa* strains with *rpoS* mutations could have a selective advantage during imipenem administration, which might have an impact on the antibiotic therapy against *P. aeruginosa* biofilm-associated infections.

Methods

For details see Supplementary Information.

Biofilm experimental evolution

The experimental evolution of *P. aeruginosa* PA01 biofilm was carried out on glass beads formed biofilm [31]. Two autoclaved 5 mm glass beads (Merck KGaA, Darmstadt, Germany) were placed into each well of a 24-well microtiter plate (Nunc, Thermo-Fischer). A LB overnight culture of *P. aeruginosa* was diluted in LB to approx. 1×10^6 bacteria per mL and dispensed into the bead-containing 24 well microplate (1 mL per well). The microplate was then placed in a moisture box and incubated at 37 °C for 24 h at 100 rpm on an orbital shaker. After 24 h, the liquid culture was removed and beads were washed by 0.9 % NaCl for twice to remove loosely attached bacteria. Then transfer one bead into 2 mL microcentrifuge tube containing 1 mL 0.9% NaCl, subjected to 6× 10 s vortex and sonicated in an ultrasonic bath (Worldvicon, Shenzhen, China) at 40 kHz for 5 min. Bacterial suspensions were subsequently serially diluted in 0.9% NaCl before being drop-plated onto lysogeny broth agar plates (Difco). After 24 h of incubation at 37 °C, the residual biofilm was quantified as CFU/bead. Another bead was transferring to a 24 well microplate contain 1 mL LB with 160 µg/mL imipenem. The microplate was then placed in a moisture box and incubated at 37 °C for 24 h without shaking. After 24 h treatment, this bead was washed by 0.9% NaCl for twice and transfer into 2 mL microcentrifuge tube containing 1 mL LB, after vortex and sonicated. 20 µL of bacterial suspensions were subsequently serially diluted in 0.9% NaCl before being drop-plated onto lysogeny broth agar plates (Difco), the rest bacterial suspensions were cultured at 37 °C for 24 h at 200 rpm. After 24 h of cultivation, 100 µL *P. aeruginosa* was diluted in LB to approx. 1×10^6 bacteria per mL and start a new cycle. The rest culture was glycerol stocked at -80 °C. The CFU/bead increased over 100-fold compared to the ancestral strain was defined as hyperbiofilm phenotype variants.

Biofilm competition assay

The biofilm competition assay was carried out on glass beads formed biofilm. The ancestor strain PA01 and mutants were tagged with *gfp* and *mcherry* at the *attB* site to generate the strain PA01 *attB::gfp* and mutant *attB::mcherry* as previously described [59]. Overnight cultures were adjusted OD₆₀₀ to 1.0, cells were mixed 1:1 or 1:5 and confirmed by flow cytometer analysis. The mixed bacteria were diluted in LB to approx. 1×10^6 of per mL and dispensed into the bead-containing 24 well microplate (1 mL per well). The microplate was then placed in a moisture box and incubated at 37 °C for 24 h at 100 rpm on an orbital shaker. After 24 h treatment, the cells in planktonic and biofilm were analyzed by flow cytometer analysis.

DNA extraction, sequencing, and SNP analysis

Genomic DNA of the ancestor and evolved bacterial populations were extracted form glycerol stocked by AxyPerp Bacterial Genomic DNA Miniprep Kit (Corning) and sequenced by Illumina NovaSeq platform. Illumina genomic reads of the isolates were analyzed by CLC Genomics Workbench 20 (Qiagen) using Resequencing analysis module with default parameters for single nucleotide polymorphism (SNP) with *P. aeruginosa* PA01 as reference genome.

RNA extraction, sequencing, and transcriptomic analysis

Samples were collected at the peak of $P_{cdrA^-}gfp$ fluorescence intensity, RNA extraction was performed using the miRNeasy kit (Qiagen) according to the manufacturer's instructions. RNA samples were submitted to Guangdong Magigene Biotechnology Co.,Ltd. (Guangzhou, China) for ribosomal RNA depletion and sequencing. RNA samples were sequenced on an Illumina Hiseq Xten platform and 150 bp paired-end reads were generated.

The quality of raw sequence data was assessed using FastQC (Babraham Bioinformatics). RNA sequence analysis was done using "RNA-seq analysis" module in CLC genomics Workbench 20 (CLC Bio, Aarhus, Denmark) using *P. aeruginosa* PAO1 reference genome downloaded from NCBI database. Adaptor sequences were removed by adaptor trimming function in CLC. Differential gene expression was analyzed using DESeq2 package in R software.

Statistical analysis

Data are presented as mean \pm standard deviation (SD). All other comparisons were made using a one-way analysis of variance (ANOVA) with Student's *t* test. Analyses were performed using GraphPad Prism v.7 (GraphPad Software). Statistical significance was determined using a *P* value of ≤ 0.05 .

Abbreviations

CF: cystic fibrosis; CFU: colony forming units; MIC: minimum inhibitory concentration; CV: crystal violet; QS: Quorum-sensing; DGC:diguanylate cyclases PDE: phosphodiesterases; LDH: lactate dehydrogenase;

Declarations

Acknowledgements

Not applicable.

Authors' contribution

X.D. and Y.L. designed methods and experiments; X.D. carried out the laboratory experiments, analyzed the data and interpreted the results; Y.P. performed DNA manipulation and cytotoxicity assay; Z.C and Y.L. co-designed the DNA sequencing experiments and worked on associated data collection and its interpretation; YM.L. and Y.Z. performed motility and transcriptional fusions reporter assay; K.W. and M.L. collected the clinical strains and performed high biofilm screening; X.D., L.Z. and L.Y. wrote the paper; all authors have contributed to, seen and approved the manuscript.

Funding

This research was supported by the Start-up grant from the Southern University of Science and Technology (Y01416206) Guangdong Natural Science Foundation for Distinguished Young Scholar (2020B1515020003) the Guangdong Natural Science Foundation (2019A1515110640,

2020A1515010316), National Natural Science Foundation of China (81760024), the Guangzhou Municipal Science and Technology Bureau (201607020044).

Availability of data and materials

The DNA and RNA sequence data that support the findings of this study have been deposited in the NCBI Sequence Read Archive (SRA) with the accession number: PRJNA678555. The materials that support the findings of this study are available from the corresponding author upon reasonable request.

Ethics approval and consent participate

Working with the *P. aeruginosa* clinical isolates is approved by the Ethics Committee of the first affiliated hospital of Guangxi Medical University [2017(KY-E-080)].

Consent for publication

Not applicable.

Competing interest

The authors declare that they have no competing interests.

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Figures

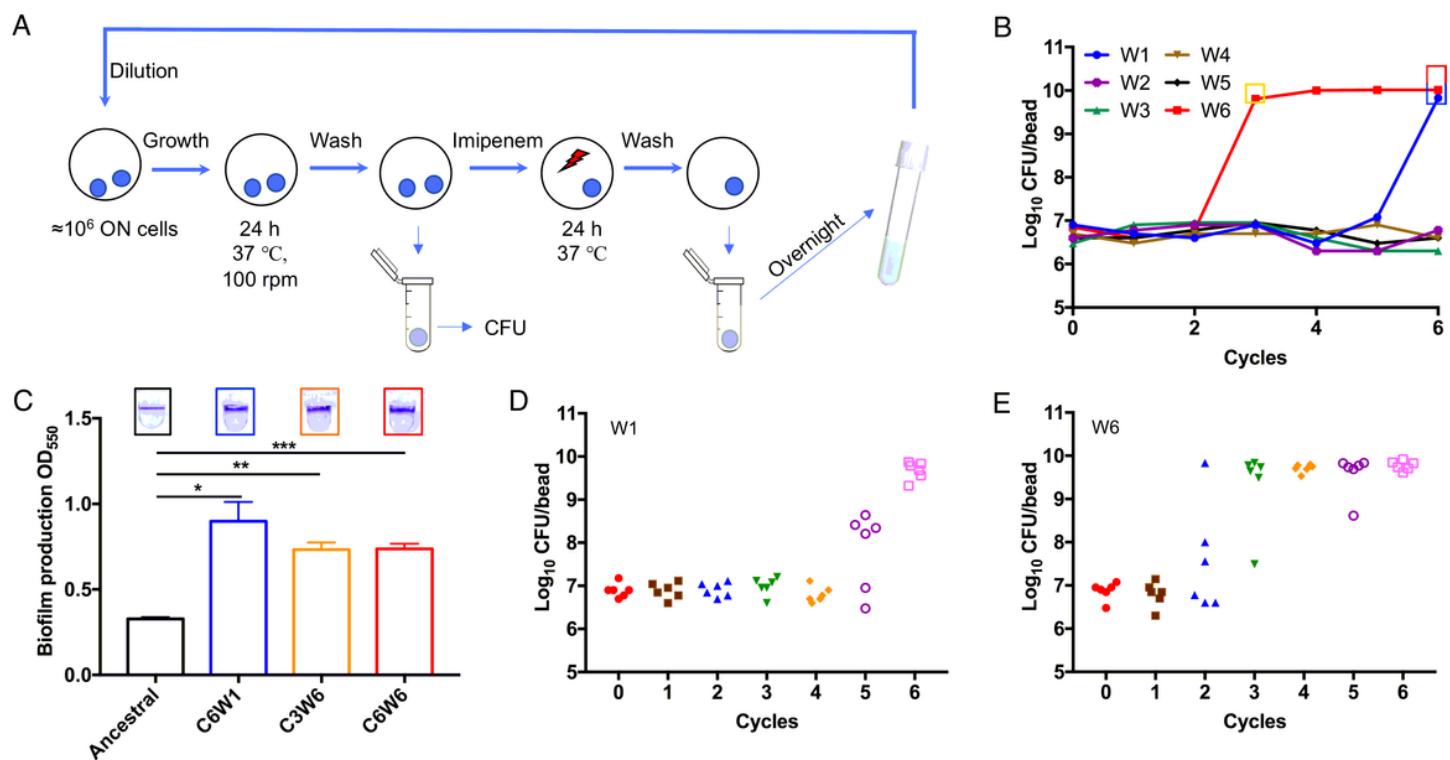


Figure 1

Experimental biofilm evolution of *P. aeruginosa* under antibiotic stress. (A) The setup of experimental biofilm evolution of *P. aeruginosa*. The biofilm of six independent lineages of the *P. aeruginosa* PAO1 were grown on 5 mm glass bead. After 24 h cultivation, one bead was vortex and sonicated for CFU counts, another bead was transferred to a 24 well microplate and treated with 160 µg/mL imipenem. After 24 h treatment, the surviving cells were grown overnight in fresh medium and start another cycle. (B) Evolution of biofilm bacteria exposed to imipenem resulted in a rapid increase in biofilm bacteria CFU on

bead. (C) Crystal violet (CV) staining of biofilms formed by ancestral and hyperbiofilm variant strains on PVC plate. Data are presented as the mean \pm s.d. of five biological replicates. Significance was determined using a Student's t test: *P < 0.05, **P < 0.01 and ***P < 0.001. (D and E) The time frame of emergence of hyperbiofilm variants in lineage W1 (D) and W6 (E). The biofilm formation by the different colonies was displayed with CFU of biofilm cells on 5 mm glass bead.

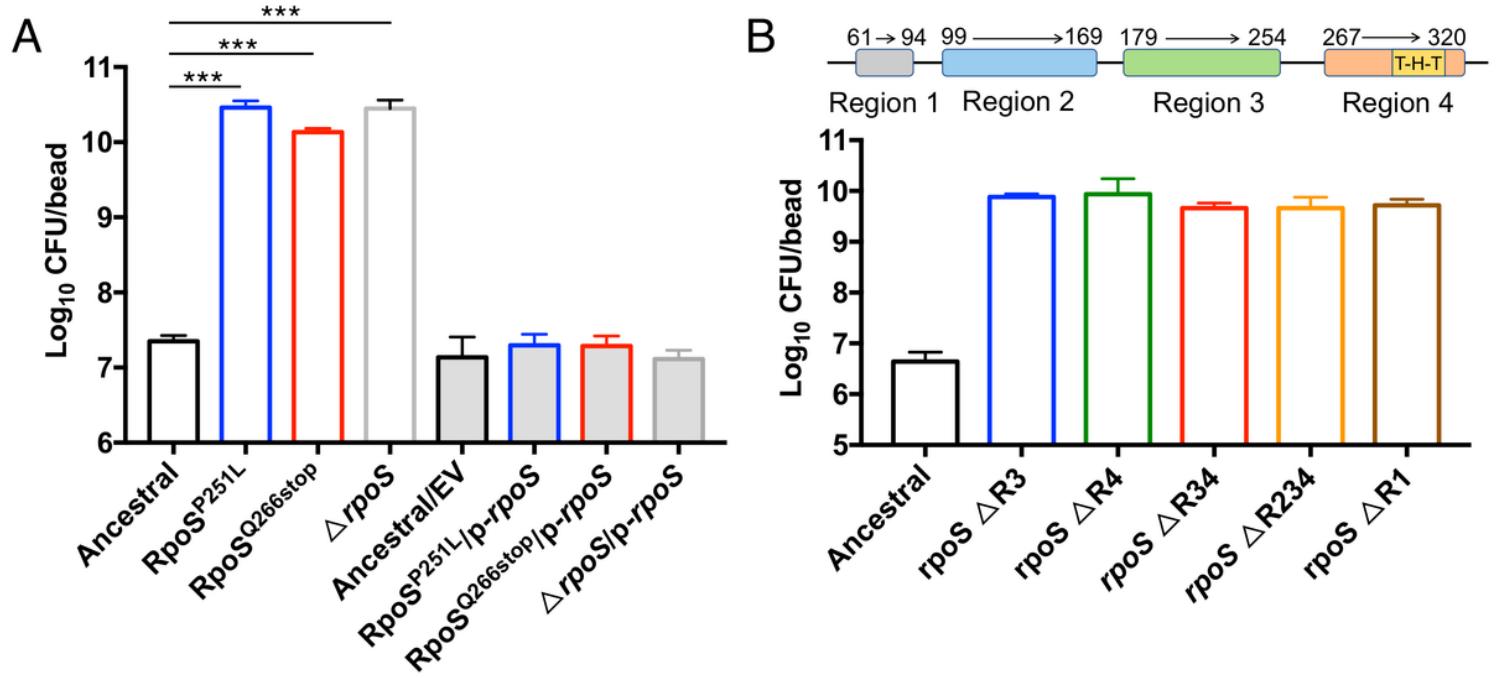


Figure 2

Hyperbiofilm phenotype is caused by *rpoS* mutation. *rpoS* point mutation, full deletion (A) and regions deletion strains (B) were increased the biofilm formation. The biofilm formation by the indicated strains was displayed with CFU of biofilm cells on 5 mm glass bead. Data are presented as the mean \pm s.d. of four biological replicates. Significance was determined using a Student's t test: ***P < 0.001. EV represents the empty vector pHERD20T in this assay.

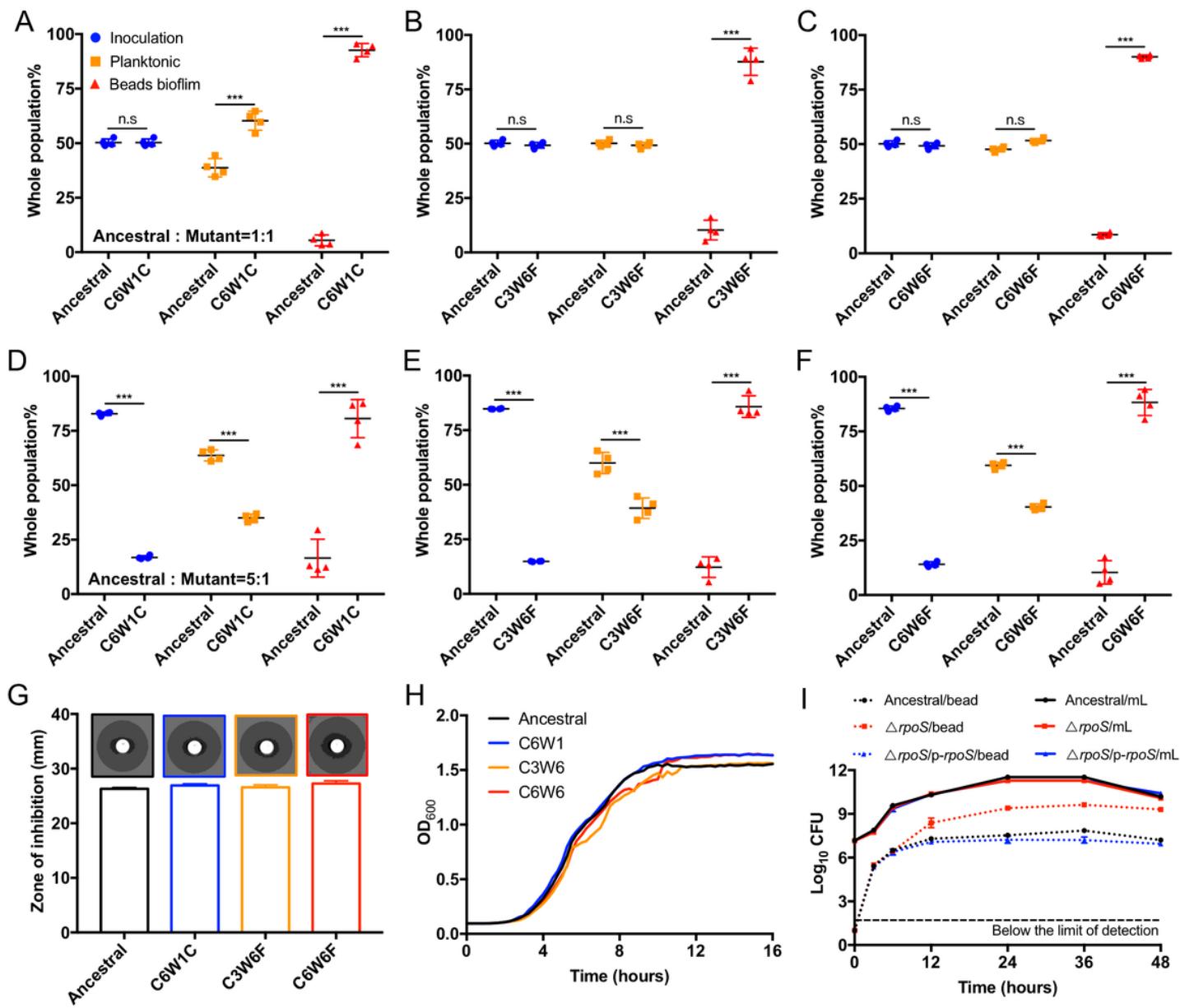


Figure 3

Hyperbiofilm variants prevail in biofilm competitions. (A-F) The competition of ancestor and hyperbiofilm variants in planktonic cultures and biofilms when inoculated at the same ratio (A-C) or ancestral: hyperbiofilm mutant=5:1 (D-F). (G) Disc diffusion antibiotic sensitivity testing, (H) growth curves measurement, and (C) biofilm growth curve were measured. Data are presented as the mean \pm s.d. of four biological replicates. Significance was determined using a Student's t test: n.s indicates no significant difference ($P\geq 0.05$); *** $P < 0.001$.

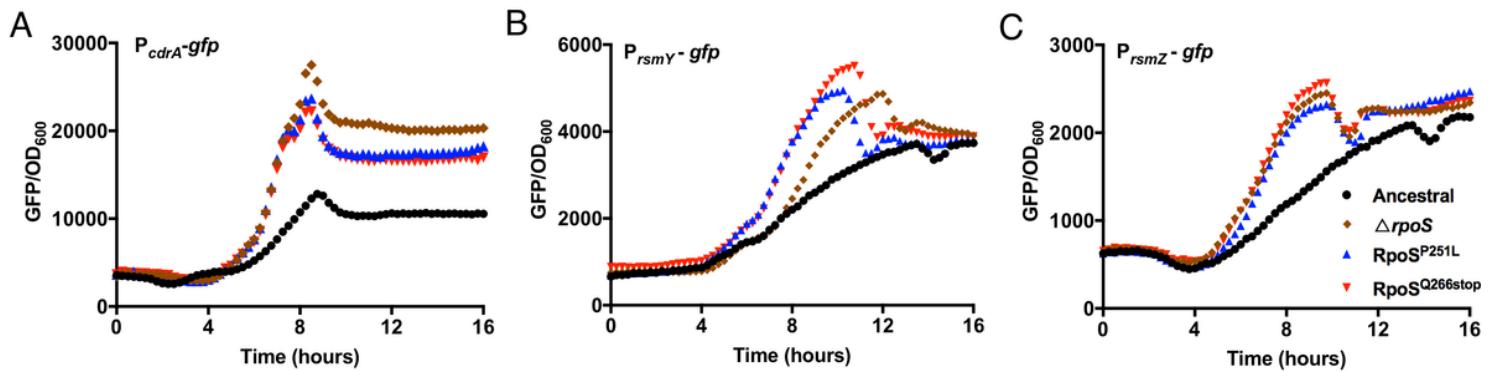


Figure 4

Expression of *PcdrA-gfp*, *PrsmY-gfp* and *PrsmZ-gfp* reporter fusions in *rpoS* mutants and PAO1 wild-type strain. Relative fluorescence intensity (reflected as GFP/OD₆₀₀) was measured in representative strains containing the *PcdrA-gfp* (A), *rsmY* (B) and *rsmZ* (C) reporter fusions. Data are presented as the mean±s.d. of five biological replicates.

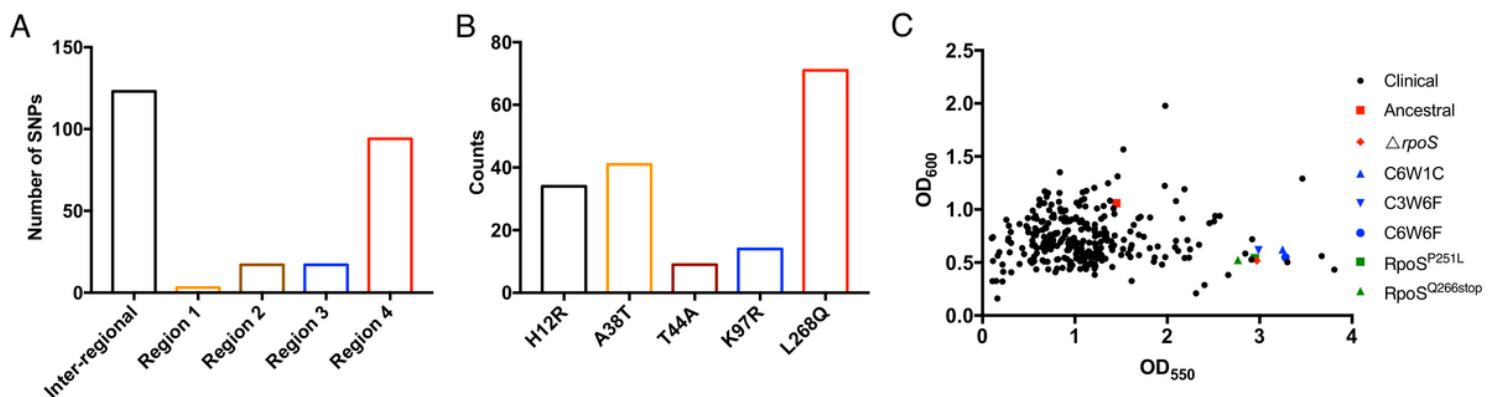


Figure 5

rpoS mutations were existed in *P. aeruginosa* clinical isolates. The distribution of non-synonymous mutation (A) and the top 5 mutations (B) on *RpoS* of *P. aeruginosa*. 4000 sequences of *rpoS* were download form pseudomonas genome database, and the non-synonymous mutations were analyzed by CLC Genomics Workbench. (C) Biofilm formation ability assessment of clinical isolates. A total of 288 clinical isolates from the first affiliated hospital of Guangxi Medical University (shown in black) were analyzed for *rpoS* mutations and biofilm assays on 96 well PVC plates.

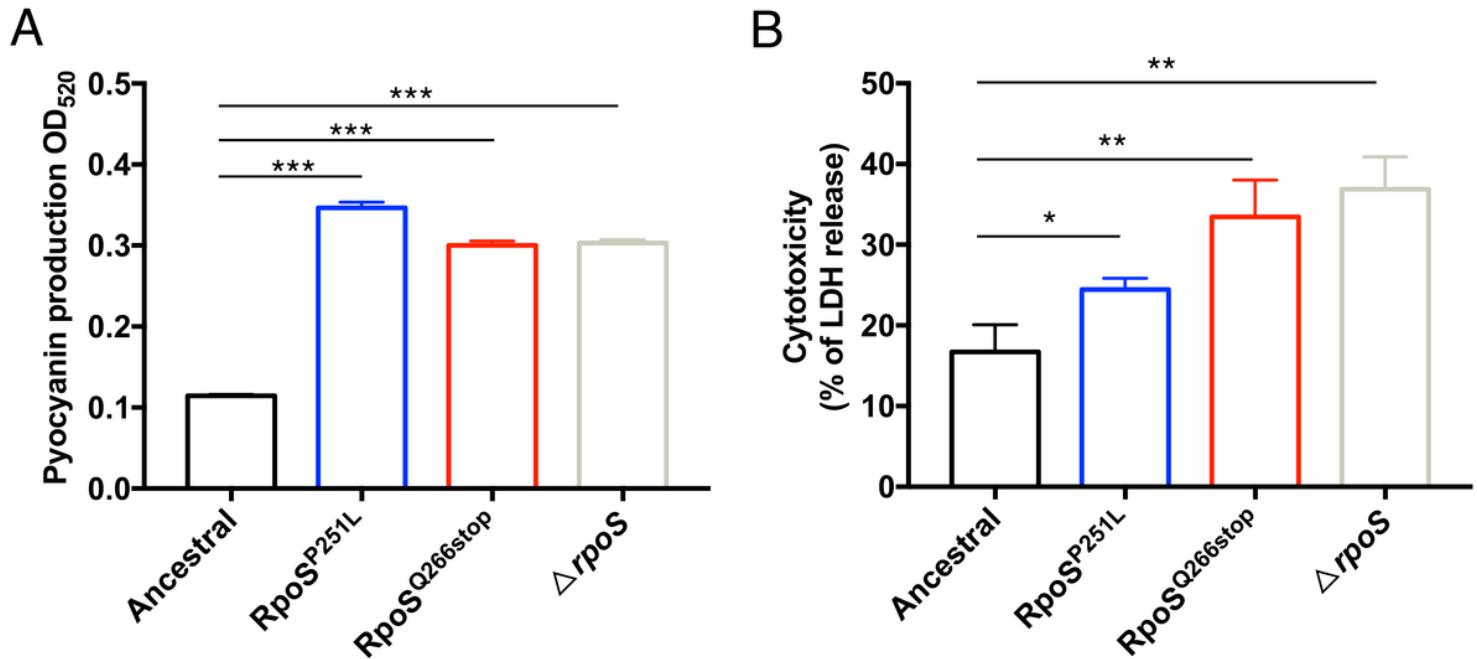


Figure 6

Pyocyanin production and virulence are increased in *rpoS* mutants. The production of pyocyanin (A) and cytotoxicity effect against macrophage cells (B) of *P. aeruginosa* PAO1 wild-type, RpoSP251L, RpoSQ266stop and $\Delta rpoS$. Data are presented as the mean \pm s.d. of four biological replicates. Significance was determined using a Student's t test: * P <0.05, ** P <0.01, ***P <0.001.

Supplementary Files

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