Priority changes between biofilm exopolysaccharides synthesis and rhamnolipids production are mediated by a c-di-GMP-specific phosphodiesterase NbdA in *Pseudomonas aeruginosa*
Priority changes between biofilm exopolysaccharides synthesis and rhamnolipids production are mediated by a c-di-GMP-specific phosphodiesterase NbdA in *Pseudomonas aeruginosa*

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**SUMMARY**

The synthesis of biofilm exopolysaccharides and rhamnolipids (RLs) are two interrelated processes in *Pseudomonas aeruginosa*, but how bacteria coordinate these two processes remains unclear. We collected a *P. aeruginosa* KT1115 with rugose small colony variant (RSCV) phenotype from soil, and used it to study the dynamic regulation mechanism of biofilm polysaccharide and RLs synthesis. The results showed that the overproduction of biofilm exopolysaccharides at biofilm stage ultimately contributed the surge of RLs production at RLs stage. This phenomenon was further verified by comparing PAO1 with its engineered RSCV mutant, PAO1ΔwspF. Further genomic, transcriptomic analyses and gene deletion revealed that downregulation of c-di-GMP level was the key to switch biofilm exopolysaccharides accumulation to RLs surge, by transcriptionally upregulating a c-di-GMP phosphodiesterase NbdA. Overall, this study demonstrates the importance of c-di-GMP in coordinating biofilm exopolysaccharides and RLs synthesis, and provides an inspiration for enhancing RLs production through regulating c-di-GMP level.

**INTRODUCTION**

Rhamnolipids (RLs) are glycolipid biosurfactant which naturally produced by the opportunistic pathogen *Pseudomonas aeruginosa* and by some *Burkholderia* species. RLs have been the subject of increasing attention because of their lower toxicity and higher biodegradability compared with those of chemically synthesized surfactants. These properties are of industrial interest and have been exploited in multiple applications in the pharmaceutical, agriculture, food, detergent, and cosmetic fields.1–3 Despite their broad commercial applications, their large-scale production has not been possible because of low yields and high production costs.

RLs are extracellular metabolites under the control of two interrelated quorum-sensing (QS) systems, *las* and *rhl*.4,5 The acyltransferase RhlA is involved in the synthesis of 3-(3-hydroxyalkanoyloxy) alkanoic acid (HAA).5,6 The rhamnosyltransferase RhlB catalyzes the transfer of dTDP-L-rhamnose to a molecule of HAA to form a mono-RL. The second rhamnosyltransferase, RhlC, sequentially transfers the rhamnosyl group to mono-RL to form di-RL.4 The transcription of both the *rhlA* and *rhlC* is regulated by the Rhl/RhlR QS system.7 Biofilm formation also affects RLs synthesis. Previous studies have found that RLs is critical for maintenance of the biofilm architecture by affecting cell-cell interactions and the attachment of bacterial cells to surfaces.8 However, overproduction of RLs can cause detachment in *P. aeruginosa* biofilms via a central hollowing pattern during the biofilm dispersion process.9,10 In addition to the influence on biofilm structures, RLs also involved in the competition for the common sugar precursor of Psl and Pel exopolysaccharides.11 Hence, the construction of highly efficient methods for RLs production remains an open issue because of the complexity of QS regulation and metabolic competition.

Biofilms can provide beneficial applications in the natural environment as well as in industry. The high cell density and stability, enhanced tolerance toward various adverse environmental conditions are the most important features of biofilms. These properties are commonly used for improving productivity and product yields as a fermentation platform,12 because of biofilm formation was characterized by increased...
tolerance to hazardous environments, such as antimicrobials and alcohols. Several studies have directly compared the performance of suspended culture and biofilm fermentation for bulk chemical products, and found that biofilm-mediated fermentation significantly contributed to the production of succinic acid, and ethanol.

The regulation of biofilm formation and dispersal is the central role of the intracellular second messenger cyclic di-GMP (c-di-GMP). An increased level of c-di-GMP leads to the formation of biofilms, and a decreased level results in the dispersal of biofilm components. The genes encoding the diguanylyl cyclases (DGCs) that synthesize c-di-GMP and the phosphodiesterases (PDEs) that degrade c-di-GMP are redundant, and their expression is not well understood in all cases. Although biofilm development has been extensively studied in P. aeruginosa, how bacteria coordinate biofilm accumulation and RLs production and whether this process has potential applications in RLs production remain unclear.

In this work, we monitored the dynamic changes in exopolysaccharides and RLs production by using the P. aeruginosa RSCV strain KT1115 during a long fermentation process. We found that the formation of RSCV phenotype (overproduction of biofilm exopolysaccharides, Psl and Pel) at the early stage benefits the production of RLs at the late stage. This phenomenon was also verified by comparing PAO1 with its engineered RSCV mutant PAO1ΔwspF. In addition, we systematically analyzed their intracellular c-di-GMP and gene expression patterns using transcriptome data. Our data imply that c-di-GMP is a key player involved in the switch between the biofilm stage and the RLs stage by targeting genes in both exopolysaccharides and RLs synthesis pathways. Finally, our results reveal that the c-di-GMP-related PDE, NbdA, plays a key role in regulating the switch between the biofilm and RLs stages by controlling the c-di-GMP level. Our study sheds new light on the relationship between biofilm exopolysaccharides formation and RLs synthesis and provides strong inspiration for the application of microbial biofilm engineering in fermentation processes as an alternative strategy to improve the yields of RLs and other metabolites.

RESULTS

P. aeruginosa KT1115 exhibited an RSCV phenotype and produced a high yield of RLs

Our previous work showed that P. aeruginosa KT1115 produces a high ratio of di-RLs after fermentation. Here, we examined several other phenotypes of KT1115, including colony morphology, biofilm formation, and total RLs production. KT1115 showed rugose colonies with darker colors on Congo red agar and formed robust biofilms with approximately 1.6-fold higher levels of biofilm exopolysaccharides than PAO1, a model laboratory strain (Figures 1A and 1D). A high level of biofilm exopolysaccharides usually results in bacterial aggregation in planktonic culture. We then used bacterial aggregation to estimate the biofilm formation of KT1115 in liquid culture. The results show that the auto-aggregation of KT1115 cells was visible at a macroscopic level in LB medium, whereas PAO1 did not show any auto-aggregation under the same conditions (Figures 1C and S1C). In rapeseed oil nitrate (RON) medium, when the aggregation was examined under a microscope, it was observed to be aacoacervate containing approximately 100–200 cells (see Figure S1A). These results also confirm that strain KT1115 exhibits a typical RSCV phenotype along with hyperbiofilm formation ability.

It has been reported that biofilm exopolysaccharides and RLs synthesis are competitive processes in P. aeruginosa, and excessive exopolysaccharides production reduces RLs synthesis in vitro after 2 days of incubation. It inspired us to further investigate the effect of exopolysaccharides production on RLs synthesis in fermentation systems. In this work, the RSCV strain KT1115 was incubated in a 1-L shake flask for 7 days. The results from anthrone colorimetric assays showed that KT1115 produced considerably higher levels of RLs than PAO1 after fermentation, which was 31.2% higher than that obtained with PAO1 (Figure 1B). The TLC results also indicated that mono-RLs from KT1115 were significantly higher than those from PAO1 (Figure 1B). Taken together, these data demonstrated that the RSCV strain KT1115 could also produce considerable levels of RLs during fermentation, indicating that there may be other interactions between exopolysaccharides and RLs synthesis in addition to competition.

Exopolysaccharides overproduction extended the production stage and triggered a surge of RLs production

To further explore the RLs synthesis of the RSCV strain KT1115, comparison experiments were conducted with KT1115 and PAO1 in 1-L shake flasks in RON medium. We monitored RLs production, exopolysaccharides production, and oil utilization at 24-h intervals throughout the fermentation process. In biofilm
formation stage, the exopolysaccharides production of KT1115 was significantly higher than that of PAO1 and the relative adsorption of biofilm exopolysaccharides reached a maximal value of 0.53 at 24 h (Figures 2A and 2B), which was consistent with the hyperbiofilm formation ability of KT1115. The exopolysaccharides production was priority while RLs synthesis was maintained at a relatively low level in this stage (Figures 2A and 2B). Of interest, the RLs productivity was entirely different at the end of the biofilm stage, as demonstrated by a sharp increase to $0.508 \pm 0.07 \text{ g/L/h}$, which is 2.4-fold higher than that at the biofilm stage. These phenomena suggested that RLs synthesis is fully activated in this stage, and we thus designated this stage as RLs stage.

Although both KT1115 and PAO1 exhibited two stages during fermentation process, they were different in the duration of the biofilm stage and the final yield of RLs. KT1115 showed a considerably longer period of a higher exopolysaccharides production, lasted from 0 h to 120 h, which was 72 h longer than that found in PAO1 (Figure 2A). Moreover, RLs synthesis in KT1115 was not completely dormant at the biofilm stage, and the total amount of RLs only reached $16.02 \pm 1.3 \text{ g/L}$, whereas the total amount of RLs obtained with PAO1 reached $21.89 \pm 0.4 \text{ g/L}$. To further confirm our results, we compared the transcriptions of genes responsible for RLs synthesis via RNA-seq and RT-qPCR (Figures 2C and S2). The results from the differential expression analysis indicated that the transcriptions of RLs synthesis genes ($rhlA$, $rhlB$ and $rhlC$) were significantly up-regulated (both more than 4-fold change) at 144 h in KT1115. In addition, synthesis genes ($pslA$-$J$) of biofilm Psl exopolysaccharides were significantly down-regulated (3.3-fold change) at 144 h compared with at 24 h in KT1115 (see Table S1). However, similar expression patterns were not observed in PAO1. Moreover, at 24 h, the expression level of $pslA$ in KT1115 was 2.3-fold higher than that in PAO1 (see Figure S3), and these results were consistent with the trend of biofilm exopolysaccharides production (Figure 1A). Taken together, these data revealed that the RSCV strain KT1115 exhibited a longer biofilm stage during fermentation, which ultimately extended the RLs production stage and triggered a surge of RLs production at the late fermentation stage.

**RSCV phenotype promotes high-yield production of RLs**

To further explore this assumption, we used a PAO1-derived RSCV mutant, PAO1ΔwspF\textsuperscript{24}, to assess RLs production during fermentation. Consistent with KT1115, PAO1ΔwspF showed a considerable period of
high biofilm exopolysaccharides production from 0 to 96 h (Figures 3A and S1B). Once entering the RLs stage, the average productivity of RLs was 0.249 ± 0.021 g/L/h, which was 2.6 times higher than that at the biofilm stage (0.093 ± 0.004 g/L/h) and 6.73-fold higher than that of its parental strain PAO1 (0.024 ± 0.003 g/L/h) (Figure 2B). The exopolysaccharides accumulation also contributed to the final yield of RLs, which reached the peak at 192 h, with 39.38 g/L of PAO1ΔwspF, 58.9% higher than that of PAO1.

To further confirm whether biofilm exopolysaccharides overproduction promotes the high-yield production of RLs, we overexpressed the polysaccharide hydrolase PslG in PAO1ΔwspF to reduce its biofilm formation through degrading the exopolysaccharide, PsI.25 Once PslG was induced at 0 h by 1% arabinose, the yield of RLs decreased by 45.5% compared to that of PAO1ΔwspF without arabinose (Figure 3B). Of interest, PslG induced at 24 h slightly recovered RLs production, suggesting that the first 24 h of biofilm formation benefited the yield of RLs at the later stage (Figure 3B). Together, these data demonstrate that the biofilm exopolysaccharides overproduction can contribute to the RLs production at late stage of fermentation.
C-di-GMP is a key player involved in the switch between the biofilm stage and the RLs stage

C-di-GMP is an intracellular messenger molecule with a wide range of implications in biofilm formation and dispersion. We measured the intracellular c-di-GMP level of the two RSCV strains at different stages of fermentation through the fluorescence-based reporter plasmid pCdrA:
gfp
(ASV)s.27 As expected, both KT1115 and PAO1
D
wspF showed significantly higher c-di-GMP levels at the early stage of fermentation, whereas the GFP intensity decreased by 56.1% and 64.3% at the late stage, respectively (Figure 4A). The variation in c-di-GMP indicated that it may play a key role in the switch from biofilm formation to RLs exploitation in the liquid fermentation system.

To further verify the function of c-di-GMP in controlling the switch between the two stages, a c-di-GMP phosphodiesterase (PDE), PA4781, was overexpressed in KT1115 and PAO1
D
wspF to test whether the alteration of the c-di-GMP level would affect the switch phenomenon and the final RLs production. The biofilm exopolysaccharides production was significantly decreased in KT1115 and PAO1
D
wspF after PA4781 overexpressed (Figures 4B–4D). After fermentation, the tendency of RLs production was completely altered in KT1115/pPA4781. KT1115/pPA4781 showed a sharp RLs accumulation at the early stage, which is similar to that of PAO1 (Figures 2B and 4C). Moreover, the total RLs production of KT1115/pPA4781 was 53.5% lower than that of KT1115. A similar phenomenon was also observed in PAO1
D
wspF/pPA4781, which only produced...
approximately 50% RLs compared to PAO1ΔwspF (Figure 4D). These results imply that c-di-GMP is a key player in controlling the switch between the biofilm stage and RLs stage in KT1115 and PAO1ΔwspF, and the formation of RSCV phenotype, in turn, contributed to RL production during fermentation process.

**NbdA regulates the levels of intracellular c-di-GMP in a fermentation-dependent manner**

To further identify genes responsible for the switch between the biofilm and RLs stages in KT1115, we conducted an RNA-seq analysis comparing the expression of PDEs and DGCs (36 in total) between the biofilm stage and the RLs stage, which were reported to play an important role in the regulation of c-di-GMP levels (Figure 5A). Special attention was given to those significant down-regulated DGCs and up-regulated PDEs in the RLs stage. The results showed that there are only three genes met the screening standards (≥2-fold change): siaD, nbdA, and mucR. However, when we further check the expression of the 36 PDEs and DGCs in PAO1, we found that siaD and mucR exhibited a similar expression pattern between the KT1115 and PAO1 (Figures 5D, S4, and S5). Thus, a higher probability was given on nbdA, because the entirely different phenomena in PAO1 and KT1115 is high probably caused by those differential expressed genes between PAO1 and KT1115.

To further confirm whether nbdA can cause the switch in KT1115, we first validated the expression profile of nbdA in KT1115. The qRT-PCR results were consistent with the RNA-seq data, and the expression of nbdA was significantly activated at the RLs stage, which is 10.7-fold higher than that at the biofilm stage (Figure 5B). For further analyses, we generated an in-frame nbdA deletion in KT1115 (KT1115ΔnbdA). Once nbdA was deleted, strain KT1115ΔnbdA was unable to reduce the c-di-GMP level, and the rapid accumulation of RLs also disappeared (Figure 6C).

Given that PAO1ΔwspF exhibited a similar nbdA expression profile as KT1115 (Figure 5B), we also wondered whether nbdA was also responsible for the switch in PAO1ΔwspF. Therefore, we constructed...
a wspF and nbdA double in-frame deletion strain to check its c-di-GMP and RLs productivity in fermentation. As expected, PAO1ΔwspF showed similar results to KT1115 after the deletion of nbdA, including decreased RLs productivity and increased c-di-GMP at the RLs stage (Figures 6B and 6D). Of interest, on the one hand, NbdA has been reported to be an NO sensor, and on the other hand, nitrate metabolism is an important source of NO, so we speculate that NO synthetase may always play an important role in this regulatory process.30,31 Subsequent qPCR results showed that NirS, a nitric oxide synthase, was indeed significantly up-regulated during RLs stage (see Figure S6), which initially confirmed our hypothesis, but more evidence is needed to confirm this hypothesis. Overall, we concluded that NbdA regulates the level of c-di-GMP in a fermentation-dependent manner and therefore controls the shift from the biofilm stage to the RLs stage in RSCV strains during the fermentation process.

**DISCUSSION**

*P. aeruginosa* is widely accepted as a model microorganism in biofilm studies and an ideal chassis in RLs production.5,32 Previous studies reported that biofilm exopolysaccharides and RLs synthesis are competed processes based on in-vitro tube experiments, however, whether this rule is applicable in fermentation is currently unexplored.
unknown. In this work, we observed that *P. aeruginosa* RSCV strains unexpectedly produced considerable levels of RLs at the late stage of fermentation; in contrast, once the PDE gene, PA4781, was overexpressed, the phenomenon of exopolysaccharides overproduction at the early fermentation stage was disappeared, and this step was accompanied by a significantly decreased of RLs production. The data demonstrated that the RSCV strain KT1115 could also produce considerable RLs during fermentation, indicating that there may be other interactions between biofilm exopolysaccharides and RLs synthesis. Previous studies reported that biofilm exopolysaccharides production and RLs synthesis are competitive processes based on *in-vitro* tube experiments; however, whether this rule is applicative in fermentation is currently unknown.9,11,33 When we divided the fermentation process into biofilm stage and RLs stage, we found that RLs synthesis was not completely inhibited in biofilm stage (Figures 2A and 3A), and premature termination of biofilm stage impaired the final amount of RLs accumulation (Figure 3B). These results provide us with an insight into the mechanism of high RLs production from the perspective of biofilm formation and dispersal.34–36 On the one hand, we speculated that the RSCV phenotype in KT1115 and PAO1ΔwspF may promote stable aggregate formation and the threshold for biofilm dispersal (Figures 1 and S1), which led to complete dispersal of the biofilm required more RLs compared to PAO1. On the other hand, the surge of RLs after biofilm stage may benefit greatly from sugar groups provided by the degradation of biofilm exopolysaccharides (Figure 3B).

In *P. aeruginosa*, the c-di-GMP-modulating network consists of 40 genes, including DGCs, PDEs, and some regulator proteins.16 A transcriptome analysis and RT-qPCR validation of the expression of PDEs and DGCs at the biofilm and RLs stages revealed that the *nbdA* gene was significantly upregulated at the RLs stage compared with the biofilm stage in KT1115, and the expression pattern was not observed in PAO1 (Figures 5B–5D). Li and coworkers characterized NbdA (PA3311) as a membrane-bound protein with an identical domain organization consisting of MHYT-GGDEF-EAL and showed that this protein is an NO-induced phosphodiesterase based on its role in NO-induced dispersion.30 The diatomic gas NO can induce dispersal of *P. aeruginosa* and other bacterial biofilms by lowering the c-di-GMP levels.26 It should be noted that nitrate metabolism can produce endogenous NO in *P. aeruginosa*,26,31 and a high concentration of NaNO₃ is

![Figure 6](image-url)

**Figure 6. Comparative analysis between the nbdA-defective strains and RSCV strains**

The effect of *nbdA* defects on the c-di-GMP levels in KT1115 (A) and PAO1 (B) during fermentation was analyzed. The effect of *nbdA* deletion on RLs production in KT1115 (C) and PAO1 (D) during fermentation was analyzed. Significance was determined using Student’s t test, *p < 0.05* **p < 0.01**, ***p < 0.001. **

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beneficial for improving RLs production. Hence, these observations led us to speculate that NaNO₃ in RNO fermentation media (6 g/L) may trigger the overexpression of \textit{nbdA} at the late stage of fermentation.

Cytochrome \textit{cd}₁ nitrite reductase (NirS) is responsible for the reduction of nitrite to NO in \textit{P. aeruginosa}. The transcriptional level of the \textit{nirS} gene in KT1115 was approximately twice that in PAO1, and a similar result was found in strain PAO1\textit{ΔwspF} by RT-qPCR (see Figure S6). The results indicate that nitrate may not only serve as the source of nitrogen but also as the source of a signal molecule to induce upregulation of the \textit{nbdA} gene to decrease the c-di-GMP level at the late stage of fermentation by the correct amount of NO. Studies conducted by Chua et al. demonstrated that a reduced intracellular c-di-GMP content increases the expression of quorum sensing-regulated genes in \textit{P. aeruginosa}, such as \textit{pqs} and \textit{rhl}-regulated pyocyanin and RLs. Taken together, we speculate that in addition to providing a nitrogen source, nitrate metabolites are likely to be signaling molecules that stimulate the dispersal of biofilms and participate in producing a high yield of RLs.

Although some studies have support the notion of biofilm formation in liquid batch cultures regulated by c-di-GMP, and RLs production could be enhanced by blocking biofilm exopolysaccharides synthesis, however, little attention has been paid to the role of c-di-GMP in RLs fermentation process. In this work, we proposed a model for the regulation of the priority changes between the synthesis of biofilm exopolysaccharides and rhamnolipids through a c-di-GMP-specific phosphodiesterase \textit{nbdA} in \textit{P. aeruginosa} RSCV strains, which could be a supplement to the QS regulated RLs synthesis (Figure 7). At the biofilm stage, high intracellular c-di-GMP levels in \textit{P. aeruginosa} could induce the overproduction of exopolysaccharides and decrease RLs production by competing for common sugar precursors. At the RLs stage, upregulation of the PDE gene \textit{nbdA} caused a significant reduce of the c-di-GMP levels, led to transition from the biofilm stage to the RLs stage along with a rapid accumulation of RLs. These findings provide novel insights regarding how RSCV strains coordinate biofilm formation and RLs synthesis and form a theoretical basis for improving the understanding of RLs fermentation behavior, which can be applied for regulating the production cycle of RLs in natural producers and enhancing RLs accumulation in heterologous hosts.

**Limitations of the study**

Although this work attempted to reveal the role of nitrate in the switch from biofilm formation to RLs synthesis, the molecular mechanisms responsible for the regulation of RLs synthesis in nitrate fermentation systems need to be further studied. The hypothesis could potentially be supported by accurate monitoring data of NO₃⁻, NO₂⁻ and NO in fermentation systems or identified by corresponding mutant strains in future studies. In addition, whether the degradation products of biofilm extracellular polysaccharides are directly involved in the synthesis of rhamnolipid also needs further studies.

**STAR METHODS**

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.105531.

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AUTHOR CONTRIBUTIONS

S.L.: Conceptualization, methodology, investigation, formal analysis, writing – original draft, supervision, writing – review and editing. A.X.: Methodology, formal analysis, data curation, resources, writing – original draft, writing – review and editing. Bin Xie: investigation, methodology. Fengxue Xin: resources, writing - review and editing. W.D.: Resources, supervision, writing – review and editing. J.Z.: Conceptualization, methodology, supervision, writing – review and editing. M.J.: Conceptualization, writing – review and editing, supervision, funding acquisition.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

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# STAR★METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Bacterial and virus strains** | | |
| *P. aeruginosa* KT1115 | Isolated from soil | CCTCC M2016686 |
| *P. aeruginosa* KT1115ΔnbdA | This paper | Strain KT1115 with in-frame deletion of nbdA gene (PA3311) |
| *P. aeruginosa* PAO1 | ATCC | ATCC 15692 |
| *P. aeruginosa* PAO1ΔwspF | Xu et al. | Strain PAO1 with in-frame deletion of wspF gene (PA3703) |
| *P. aeruginosa* PAO1ΔnbdA | This paper | Strain PAO1 with in-frame deletion of PA3311 gene, nbdA |
| *P. aeruginosa* PAO1ΔwspFΔnbdA | This paper | Strain PAO1 with in-frame deletion of wspF gene (PA3703) and nbdA gene (PA3311) |
| **E. coli** DH5α | Laboratory stock | E. coli DH5α |
| **E. coli** S17 | Laboratory stock | E. coli S17 |
| **Chemicals, peptides, and recombinant proteins** | | |
| Ampicillin | Aladdin | Cat# A105483-100G |
| Carbenicillin | Aladdin | Cat# C113144-100G |
| Gentamycin | Aladdin | Cat# G100392-25G |
| Congo red | Aladdin | Cat# C100174-25G |
| Trypan blue | Aladdin | Cat# T108743-25G |
| Arabinose | Sigma | Cat# A3256-100G |
| Anthrone | Aladdin | Cat# A108571-25G |
| 1-Naphthol | Aladdin | Cat# N128915-25G |
| MOPS | Aladdin | Cat# M105135-25G |
| Triclosan | Sigma | Cat# 72779-5G-F |
| **Critical commercial assays** | | |
| FastPure Cell/Tissue Total RNA Isolation Kit | Vazyme | Cat# RC101-01 |
| ClonExpress II OneStep Cloning Kit | Vazyme | Cat# C112-01 |
| ClonExpress MultiS OneStep Cloning Kit | Vazyme | Cat# C113-01 |
| HiScript II Q Select RT SuperMix for qPCR | Vazyme | Cat# R232-01 |
| ChamQ SYBR qPCR Master Mix (High ROX Premixed) | Vazyme | Cat# Q341-02 |
| **Deposited data** | | |
| RefSeq file for *P. aeruginosa* KT1115 | Personalbio Corporation | GenBank: GCA_009833435.1_ASM983343v1_protein.faa, https://www.ncbi.nlm.nih.gov/assembly/GCF_009833435.1/ |
| Transcriptome and other analysis data | Science Data Bank | https://doi.org/10.57760/sciencedb.06097, https://www.scidb.cn/s/71BzrQ |
| **Experimental models: Organisms/strains** | | |
| *P. aeruginosa* PAO1 | ATCC | ATCC 15692 |
| *P. aeruginosa* KT1115 | Isolated from soil | CCTCC M2016686 |

(Continued on next page)
**Oligonucleotides**

| REAGENT or RESOURCE                        | SOURCE | IDENTIFIER |
|--------------------------------------------|--------|------------|
| Primers for RT-qPCR, see Table S2         | This paper | N/A        |
| Primer: pJN105/pslG Forward: TTTTTTTGGCTAGCGAATTCA TGGCACGTAAGGGACTCTATCTG | This paper | N/A        |
| Primer: pJN105/pslG Reverse: CTATAGGGCGAATGGAGCTCTC ACTCCCAACCAGCATCTG | This paper | N/A        |
| Primer: pJN105/PA4781 Forward: TTTTTTTGGCTAGCGAATTCA GAGAGCATGCTGGACAGG | This paper | N/A        |
| Primer: pJN105/PA4781 Reverse: CTATAGGGCGAATGGAGCTCTC AGGCCGGTGGCCGGGT | This paper | NA         |
| Primer: Pex18/Up/nbdA Forward: ACGACGCGCATGCCAAGCTTG TGCCGTGGGCCTCGAAG | This paper | NA         |
| Primer: Pex18/Up/nbdA Reverse: AGTGAACCAGCTGAGCTTGTTCCGGCTC | This paper | NA         |
| Primer: Over/Down/nbdA Forward: ACAAGTCAGCTGGTCACCTCGACGGCGGT | This paper | NA         |
| Primer: Pex/Down/nbdA Reverse: TATGACCATGTACCAGAATCCGGCGC GGAAGCGCGCAGCC | This paper | NA         |

**Recombinant DNA**

| REAGENT or RESOURCE                  | SOURCE | IDENTIFIER |
|--------------------------------------|--------|------------|
| pJN105-nbdA (Gm<sup>+</sup>)         | This paper | N/A        |
| pJN105-PA4781 (Gm<sup>+</sup>)       | This paper | N/A        |
| pJN105-PSL (Gm<sup>+</sup>)          | This paper | N/A        |
| pCdrA::gfp (AVS)<sup>+</sup> (Carb<sup>+</sup>) | Rybtke et al. | Fluorescence-based reporter for gauging c-di-GMP levels |
| pEX18Gm-nbdA (Gm<sup>+</sup>)         | This paper | N/A        |

**Software and algorithms**

| REAGENT or RESOURCE                  | SOURCE | IDENTIFIER |
|--------------------------------------|--------|------------|
| Origin 2021                           | OriginLab | https://www.originlab.com/ |
| GraphPad Prism 9                      | GraphPad | https://www.graphpad-prism.cn/ |
| ImageJ                               | Schneider et al. | https://imagej.nih.gov/ij/ |
| Primer Premier 6                      | Premier | http://www.premierbiosoft.com/ |
| HTSeq                                | Anders et al. | https://github.com/htseq/htseq |
| Bowtie2                               | Langmead and Salzberg | http://bowtie-bio.sourceforge.net/bowtie2/index.shtml |

**Other**

| REAGENT or RESOURCE                  | SOURCE | IDENTIFIER |
|--------------------------------------|--------|------------|
| Resource website for data visualization | Hiplot | https://hiplot-academic.com/ |

**RESOURCE AVAILABILITY**

**Lead contact**

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jie Zhou (jayzhou@njtech.edu.cn).
Materials availability

- Plasmids generated in this study are available from the lead contact upon request.
- This study did not generate new unique reagents.

Data and code availability

- The data produced of this paper are publicly available as of the date of publication. These accession numbers for the datasets are listed in the key resources table.
- This study did not generate any unique datasets.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

P. aeruginosa PAO1 (ATCC 15692) has been used in this work as the reference strain. P. aeruginosa PAO1 was cultivated in LB without sodium chloride (LBNS) and aerobically incubated (37°C, 200 rpm). RON medium was inoculated 1:100 using an OD600 of 4 culture of P. aeruginosa PAO1. All other experimental strains were cultured under the same conditions as P. aeruginosa PAO1. When necessary, antibiotics and arabinose were used to induce gene expression.

METHOD DETAILS

Bacterial strains and culture conditions

The bacterial strains and plasmids used in this work are listed in key resources table. Unless otherwise indicated, Escherichia coli was routinely cultivated in Luria-Bertani (LB) medium at 37°C, and P. aeruginosa was cultivated in LB without sodium chloride (LBNS) at 37°C. When necessary, antibiotics were added at the following final concentrations: 300 μg/mL carbenicillin and 30 μg/mL gentamicin for P. aeruginosa and 100 μg/mL ampicillin and 10 μg/mL gentamicin for E. coli. Congo red agar medium was composed of LBNS, 1% agar, 40 mg/mL Congo red, and 15 mg/mL trypan blue. For RLs production, RON medium supplemented with 60 g/L rapeseed oil, 6 g/L NaNO3, 3 g/L yeast extract, 1 g/L KH2PO4, 1 g/L Na2HPO4, 0.1 g/L CaCl2·2H2O, and 0.1 g/L MgSO4 was used for fermentation. The pH of the RON medium was adjusted to 7.0 using a 1 M NaOH solution.

RLs extraction, TLC detection, and quantitation

The sample treatment was determined according to a previously described method with minor modifications. RLs was analyzed by thin-layer chromatography (TLC) on silica gel G plates. The RLs levels produced by different strains were examined using an anthrone colorimetric assay, and the concentration was obtained using a rhamnose standard curve. The RLs productivity indicates the yield of RLs produced per hour, and the specific production rate was calculated in units of g RLs/g dry cell weight (DCW) a/hour.

Exopolysaccharides assays

To quantify the production of the biofilm matrix exopolysaccharides, a Congo red binding assay was performed according to previously described methods with minor modifications. Briefly, 1 mL of P. aeruginosa cells with an OD600 of 0.2 (approximately 1.7 × 10⁸ cells/ml) was collected by centrifugation at 14,000 × g for 5 min and resuspended in 1 mL of MOPS buffer with 15 mg/mL Congo red. After incubation for 60 min at 37°C with vigorous shaking, the bacterial surface-associated exopolysaccharide-bound Congo red was sedimented by centrifugation at 14,000 × g for 10 min, and the unbound free Congo red in the supernatant was measured by the absorbance at 490 nm. The biofilm formation rate was represented by the rate of the binding of Congo red dye to cells. All experiments were carried out at least three times.

Measurement of the intracellular c-di-GMP levels

The c-di-GMP levels of different strains were determined according to a previously described method using pCdrA::gfp(ASV) as a reporter plasmid. The strains containing the pCdrA::gfp(ASV) plasmid were grown in RON medium containing 300 μg/mL carbenicillin, and the green fluorescent signals of cells were measured by a Synergy H2 hybrid reader (BioTek, USA) via the corresponding capture of GFP.
fluorescence acquired at Ex/Em wavelengths of 485/520 nm. The relative fluorescence intensity was calibrated with the OD₆₀₀ and is expressed as the relative fluorescence divided by the OD₆₀₀.

Transcriptomic analysis using RNA-seq data
The transcriptomes of strain KT1115 and model PAO1 at different fermentation stages were compared by RNA-seq. Bacterial cells were collected from fermentation broth after 24 h (representative of the biofilm stage) and 144 h (representative of the RLs stage) of incubation. Total RNA was isolated and purified using a FastPure Cell/Tissue Total RNA Isolation Kit (Vazyme, China). Construction of libraries and sequencing with the Illumina HiSeq platform were performed by Personalgene (China). After paired-end sequencing, clean reads obtained using the NGS QC Toolkit were mapped to the complete genome of KT1115 (NCBI accession: NZ_WUWO01000037) using Bowtie2. Reads that aligned uniquely to the reference sequence were used for gene expression quantification using the RPKM method. Differential expression analysis was performed with DESeq software using an adjusted p value of 0.05 and a fold change greater than or equal to 2 (Benjamini-Hochberg method) as the cutoffs.

Quantitative RT-PCR analysis
Total RNA from the cultivated P. aeruginosa strains in RON medium at 24 h and 144 h was extracted using a FastPure Cell/Tissue Total RNA Isolation Kit (Vazyme, China). After removing DNA, the RNAs were reverse transcribed to cDNAs using a HiScript Q Select RT SuperMix for qPCR (Vazyme, China) according to the instructions. A quantitative real-time PCR (qPCR) assay was performed with cDNA, specific primers (See key resources table and Table S2) and a StepOne Plus instrument (Applied Biosystems, USA). The procedure was as follows: 95°C for 3 minutes, 30 cycles of 95°C for 10 s and 60°C for 30 s, 95°C for 15 s, 60°C for 60 s and 95°C for 15 s. The transcription levels of related genes were calculated using the 2^{-ΔΔCT} method. The gene expression levels were normalized using 16S RNA as an internal control. The stability of 16S rRNA as internal control in the fermentation system was investigated by detecting the CT values of samples at different times (24h, 48h, 96h and 144h), 50 ng cDNA of each samples were used for detection (see Figure S7). At least three technical replicates were performed for each cDNA sample analyzed. The RNA-seq data are available in the Supplementary Data.

Construction of gene deletion and overexpression strains
In-frame deletion strains were constructed using an unmarked, nonpolar deletion strategy. Deletion alleles were amplified from the genome of P. aeruginosa PAO1 and KT1115, and the PCR product was digested with EcoR I and Hind III and then ligated to the EcoR I/Hind III-cut suicide vector pEX18Gm via Gibson assembly. The positive recombinant plasmid was sequenced by Nanjing Tsingke Biological Technology (China). Single recombination mutants were selected on LBNS containing 300 μg/mL carbenicillin and 25 μg/mL triclosan. Double recombination mutants were selected on LBNS plates containing 10% sucrose and confirmed by PCR. The shuttle vector pJN105 was used for the expression of pslG, PA4781, and nbdA (PA3311) in P. aeruginosa. The plasmids and corresponding primers used in this work are listed in key resources table.

QUANTIFICATION AND STATISTICAL ANALYSIS

Quantification
The RLs levels produced by different strains were examined using an anthrone colorimetric assay, and the concentration was obtained using a rhamnose standard curve. For the quantify of biofilm matrix exopolysaccharides, a Congo red binding assay was performed according to previously described methods with minor modifications. The c-di-GMP levels of different strains were determined according to a previously described method using pCdrA::gfp (ASV) as a reporter plasmid by Synergy H2 hybrid reader (BioTek, USA). And quantitative real-time PCR (qPCR) assay was performed with a StepOne Plus instrument (Applied Biosystems, USA). More information please see method details.

Statistical analyses
All statistical analyses were performed using Graphpad Prism software, and significance results of t-tests are indicated by asterisks (p value<0.05). For the stability of 16S rRNA levels, the CT values of 16S rRNA were basically stable in both PAO1, PAO1ΔwspF and KT1115 at different fermentation time (see Figure S7), which proved that 16S rRNA could be used as an internal control for RT-qPCR in the fermentation system.