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ORIGINAL RESEARCH PAPER

Enhanced rhamnolipids production in *Pseudomonas aeruginosa* SG by selectively blocking metabolic bypasses of glycosyl and fatty acid precursors

Liying Lei · Feng Zhao · Siqin Han · Ying Zhang

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Abstract
Objective To enhance rhamnolipids production in *Pseudomonas aeruginosa*, an optimization strategy based on selectively blocking the metabolic bypass that competed precursors with rhamnolipids biosynthesis pathway, containing exopolysaccharide (Psl and Pel) and polyhydroxyalkanoates (PHA) synthesis pathways.
Results Blocking the synthesis of Psl and PHA by genes knockout, both mutants *P. aeruginosa* SG ΔpslAB and *P. aeruginosa* SG ΔphaC1DC2 can grow normally in fermentation medium and increase the production of rhamnolipids by 21% and 25.3%, respectively. While blocking the synthesis of Pel, the cell growth of the mutant strain *P. aeruginosa* SG ΔpelA was inhibited, thus its production yield of rhamnolipids was also decreased by 39.8%. In addition, simultaneously blocking the synthesis of Psl and PHA, a double mutant strain *P. aeruginosa* SG ΔpslAB ΔphaC1DC2 was constructed. Rhamnolipids production was significantly increased in strain SG ΔpslAB ΔphaC1DC2 by 69.7%.
Conclusion Through selectively blocking metabolic bypasses, increasing the amount of glycosyl and fatty acid precursors can significantly enhance rhamnolipids production in *P. aeruginosa*.

Keywords Biosurfactants · Rhamnolipids · Metabolic bypass · *Pseudomonas aeruginosa* · Gene knockout

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10529-020-02838-9) contains supplementary material, which is available to authorized users.

L. Lei · S. Han · Y. Zhang (✉)
Institute of Applied Ecology, Chinese Academy of Sciences, Shenyang 110016, Liaoning, People's Republic of China
e-mail: yzhang207@126.com

L. Lei
e-mail: leily18@163.com

S. Han
e-mail: hansq@iae.ac.cn

F. Zhao (✉)
School of Life Science, Qufu Normal University, Qufu 273165, Shandong, People's Republic of China
e-mail: zhaofeng2019@qfnu.edu.cn; zhao2008569@126.com

Introduction

Rhamnolipids has a broad application prospect and great potential in the field of petroleum industry, environmental remediation, agriculture, food and medicine (Müller et al. 2012). However, the industrial applications of rhamnolipids are currently limited by the low production level and high costs of downstream separation and purification (Shah et al. 2016).

Currently, the studies about improving rhamnolipids production have been focused on the aspect of screening high-yield *P. aeruginosa* strains (Nordin et al. 2013), optimization of fermentation process (Neto et al. 2008; Ma et al. 2016), and regulation of genes related to rhamnolipid synthesis (Gutiérrez-Gómez et al. 2018; He et al. 2017). How to further increase the production of rhamnolipids? Regulating the competitive metabolic bypass of rhamnolipids synthesis pathways would be a breakthrough perspective.

Hydroxyalkanoyloxy-alkanoic acid (HAA) and dTDP-rhamnose are two required precursors in rhamnolipids synthesis. In *P. aeruginosa*, some metabolic pathways compete with the glycosyl precursors and lipid precursors, such as exopolysaccharide (EPS) and polyhydroxyalkanoates (PHA) synthesis pathways, as shown in Fig. 1 (Abdel-Mawgoud et al. 2011; Soberón-Chávez et al. 2005a). EPS mainly includes two polysaccharides, Pel and Psl. As shown in Fig. 1, the dTDP-D-glucose was used as glycosyl precursor for biosynthesis of Pel and Psl. Therefore, knocking out the key genes related to Pel and Psl synthesis to block their pathways will possibly supply more glycosyl precursors to rhamnolipids synthesis pathway. Fatty acids derivatives are used as lipid substrates in both PHA synthesis and rhamnolipids synthesis pathways (Soberón-Chávez et al. 2005b). Previous study showed that the *rhlAB* gene-deficient strain did not produce rhamnolipids, but PHA production was significantly improved (Choi et al. 2011). It suggested that the fatty acids precursors become more available for PHA synthesis when rhamnolipids synthesis is absent. Therefore, if the PHA synthesis pathway is blocked, more fatty acids precursors would flow into the synthesis of rhamnolipids.

In this study, using gene knockout technology, the competitive metabolic bypasses, EPS and PHA pathways, were blocked in *P. aeruginosa* SG. The effect of blocking the Pel, Psl and PHA pathways on the growth and rhamnolipids production was comparatively studied. Based on selectively blocking metabolic bypasses, enhancing rhamnolipids production in *P. aeruginosa* was discussed.

Methods

Bacterial strains, media and culture conditions

The rhamnolipids-producing strain *P. aeruginosa* SG (GenBank accession No. KJ995745) was isolated from the oil reservoir production fluid (Zhao et al. 2015b). LB medium was used to culture *Escherichia coli* DH5α and recombinant strains. During gene manipulation, 100 μg/mL of ampicillin and 50 μg/mL of

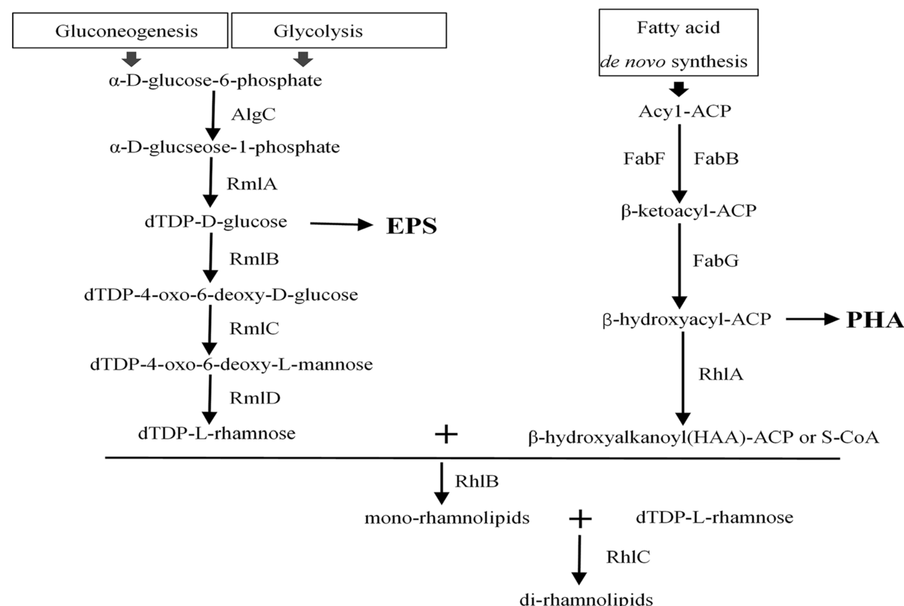

Fig. 1 The recognized rhamnolipids synthesis pathway and metabolic bypass (EPS and PHA)

kanamycin were used for recombinant *E. coli* strains, and 350 μg/mL of kanamycin was used for recombinant *P. aeruginosa* strains. The fermentation medium for rhamnolipids production contained (g/L): glycerol 60, $NaNO_3$ 4.87, $K_2HPO_4 \cdot 3H_2O$ 5.49, KH_2PO_4 6.97, $MgSO_4 \cdot 7H_2O$ 0.8, $CaCl_2$ 0.13, KCl 1.0, NaCl 1.0. The inoculum of the fermentation medium is 3%. Fermentation was performed at 37 °C and 180 rpm for 10 days. The rhamnolipids concentration was determined by oil spreading technique (Zhao et al. 2016). The bacterial biomass of culture liquid was measured by spectrophotometric determination method at 600 nm.

All bacterial strains and plasmids used in this study are listed in Supplementary Table S1. The primer pairs for PCR amplified are listed in Supplementary Table S2.

The *pel* operon (*pelABCDEFG*) and the *psl* operon (*pslABCDFEGHIJKLMNO*) controls the synthesis of Pel polysaccharide and Psl polysaccharide, respectively (Byrd et al. 2009; Friedman and Kolter 2004). The lipid groups of both PHA and rhamnolipids are derived from the de novo synthesis of fatty acids and share β-hydroxy fatty acids as their precursors (Soberón-Chávez et al. 2005b). The genes *phaC1* and *phaC2* are two of key coding genes for polyhydroxyalkanoate synthases (Qi et al. 1997).

To generate the gene fragments *pslAB*, *pelA* and *phaC1DC2*, we use the genomic DNA from *P. aeruginosa* SG as the template and the primer pairs listed in Supplementary Table S2 for PCR process, respectively. The *pslAB* fragment is 2075 bp with a EcoRI and HindIII restriction site at the 5′- and 3′-end. The *pelA* fragment is 2142 bp with a EcoRI and HindIII restriction site at the 5′- and 3′-end. The *phaC1DC2* fragment is 2482 bp with a SalI and a HindIII restriction site at the 5′- and 3′-end.

The *pslAB, pelA* and *phaC1DC2* fragments were respectively cloned into pMD19T(simple) vector, which was confirmed to contain the objective gene by sequencing. The *pslAB* fragment contains two SmaI restriction sites at 1084 bp and 1509 bp. Plasmid pMD19-Δ*pslAB* was purified and recovered to after pMD19T-*pslAB* was digested with SamI. The *pelA* fragment contains two SalI restriction sites at 1009 bp and 1903 bp. Plasmid pMD19T-Δ*pelA* was got by digesting pMD19T-*pelA* with SalI. The *phaC1DC2* fragment contains two Eco52I restriction sites at positions 637 bp and 896 bp and two BamHI restriction sites at positions 1455 bp and 2233 bp. Plasmid pMD19T- Δ*phaC1DC2* was obtained by the digestion of pMD19T- *phaC1DC2* with Eco52I and BamHI.

The Δ*pslAB* fragment was cloned into the EcoRI and HindIII sites of the mobilizable plasmid pK18*mob*SacB to construct recombinant plasmid pK18-Δ*pslAB*. The Δ*pelA* fragment was cloned into the EcoRI and HindIII sites of plasmid pK18*mob*SacB to construct recombinant plasmid pK18-Δ*pelA*. The Δ*phaC1DC2* fragment was inserted into the plasmid pK18*mob*SacB after digesting with SalI and HindIII. The recombinant plasmids pK18-Δ*pslAB*, pK18-Δ*pelA* and pK18-Δ*phaC1DC2* were, respectively, transformed into *E. coli* S17-1 strain. Using conjugation (Schäfer et al. 1994), the plasmids pK18-Δ*pslAB*, pK18-Δ*pelA* and pK18-Δ*phaC1DC2* were, respectively, introduced into the wild-type *P. aeruginosa* SG. Transconjugants were selected on LB plates containing Ampicillin and kanamycin for the first recombination. The second crossover counter-selected event used one-third LB plates containing 20% sucrose and Ampicillin, which may result in restoration of the wild type condition. The single knockout mutants, *P. aeruginosa* SG ΔpslAB, *P. aeruginosa* SG ΔpelA and *P. aeruginosa* SG *ΔphaC1DC2*, were constructed. Plasmid pK18-Δ*pslAB* was introduced into *P. aeruginosa* SG ΔphaC1DC2 to construct double knockout mutant *P. aeruginosa* SG ΔpslAB ΔphaC1DC2.

Results and discussion

Three kinds of single mutant strains SG ΔpslAB, SG ΔpelA and SG ΔphaC1DC2 were cultured in fermentation medium to clarify the effects of these three metabolic pathways on *P. aeruginosa* SG growth and rhamnolipids synthesis. The results were shown in Fig. 2. Cell growth curves indicated that the growth trend of single mutant strain SG ΔpslAB and SG ΔphaC1DC2 was better than of the wild-type strain SG (Fig. 2a). The gene *pelA* deficient strain barely grew during the first 24 of fermentation, and its cell concentration was also lower than the wild-type strain SG during the subsequent fermentation time. These results indicated that *pelA* gene knockout had a negative effect on the growth of SG strain. The yield of rhamnolipids in *pelA* knockout strain was reduced

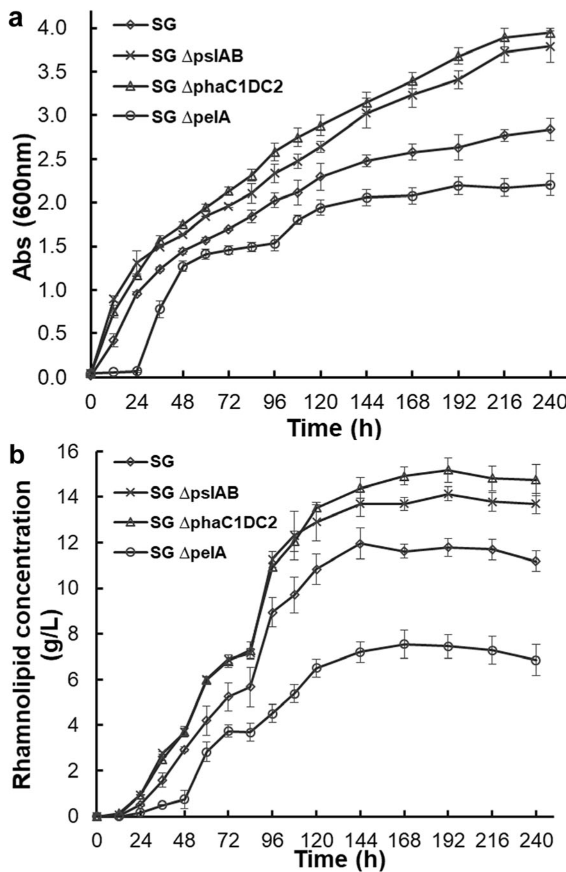

Fig. 2 Effects of knocking out genes *pslAB*, *pelA and phaC1DC2* on cell growth and rhamnolipids production of *P. aeruginosa*. Wild type strain *P. aeruginosa* SG was used as a control. Growth curve was determined by measuring OD_{600} value (**a**); curve of rhamnolipid concentration of the mutant strains SG ΔpslAB, SG ΔpelA, SG ΔphaC1DC2 and wild-type strain SG (**b**). The error bars indicate the standard deviation from the triplicate biological replicates

to 7.198 g/L (3.432 g/L/OD_{600}) (Fig. 2b), which may cause by the growth restriction of the strain. As shown in Fig. 2b, the rhamnolipid yields of strain SG ΔpslAB and SG ΔphaC1DC2 were 14.515 g/L (4.018 g/L/OD_{600}) and 14.994 g/L (4.179 g/L/OD_{600}), respectively. Strain SG ΔpslAB and SG ΔphaC1DC2 produced more rhamnolipids than strain SG 11.996 g/L (4.272 g/L/OD_{600}) with increased values of 21% and 25.3%. In contrast, the rhamnolipids yield of SG ΔpelA is 39.8% lower than wild-type SG strain (Fig. 2b). At the same time, the correlations between the OD_{600} values and rhamnolipids yield of different strains were analyzed by SPSS Statistics software, including wild-type and knockout strains. The analysis results showed that the Pearson correlation coefficient was 0.985, and the *P* value was 0.112, which was not significant at the level of $\alpha = 0.05$. Therefore, there is no correlation between OD_{600} values and rhamnolipids production of the tested strains. Therefore, the increase in rhamnolipids production is not a direct result of differential growth rates. Selectively blocking metabolic bypasses significantly enhanced rhamnolipids production in *P. aeruginosa*.

Although Psl polysaccharides is one of the components of *P. aeruginosa* biofilm, knocking out genes *pslAB* to block its synthesis does not affect the normal growth of strain SG under fermented conditions, but does increase the yield of rhamnolipids. Blocking the synthesis of Psl polysaccharides does allow more glycosyl precursors to flow to the synthesis of rhamnolipids, thereby increasing the yield of rhamnolipids. However, blocking of the synthesis of Pel polysaccharide significantly affect the normal growth of SG strain which may be related to its special role in biofilm of *P. aeruginosa*. The Pel Polysaccharide serves a structural role in the biofilm matrix of *P.aeruginosa* and protects the bacteria by enhancing the resistance to adverse environmental stress (Colvin et al. 2011). Results showed that Pel polysaccharide is more important than Psl polysaccharide in *P. aeruginosa SG*.

Knocking out genes *phaC1DC2*, the mutant strain SG ΔphaC1DC2 increased rhamnolipids production. Results indicated that removing the competition from lipid precursors in metabolic pathways can improve the synthesis of rhamnolipids. As an intracellular energy storage substance (Madison and Huisman 1999), the restriction of PHA synthesis does not affect the normal growth of SG strain. Previous studies showed that compared to the wild-type strains, rhamnolipids production was not enhanced in the PA14 ΔphaC1, PA14 ΔphaC2 and PAO1ΔphaC1, PAO1ΔphaC2 single mutants of *P. aeruginosa* PA14 and PAO1 (Choi et al. 2011). Just knocking out only one *phaC* gene can not completely block the synthesis of PHA. Therefore, we chose to knock out the three genes *phaC1*, *phaD* and *phaC2* at the same time to ensure completely blocking the PHA synthesis.

Blocking the synthesis of Psl polysaccharides and PHA have no inhibition effect on cell growth and can enhance the production of rhamnolipids. Therefore, genes *pslAB* and *phaC1DC2* were knocked out to construct the double mutant strain SG ΔpslAB

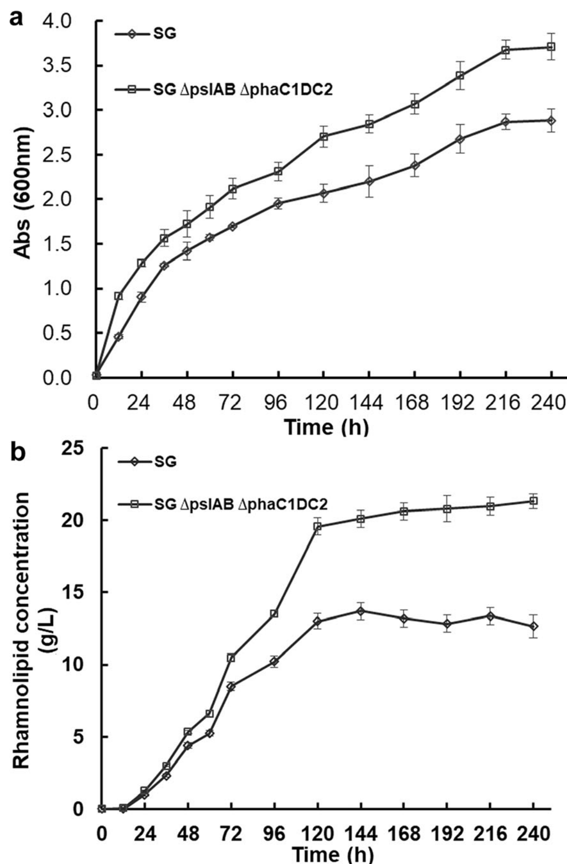

Fig. 3 Effects of simultaneously knocking out genes *pslAB*and *phaC1DC2* on cell growth and rhamnolipids production of *P. aeruginosa*. Wild type strain *P. aeruginosa* SG was used as a control. The cell growth curve was determined by measuring OD_{600} values (**a**); curve of rhamnolipid concentration of the double mutant strain SG ΔpslAB ΔphaC1DC2 and wild-type strain SG (**b**). The error bars indicate the standard deviation from the triplicate biological replicates

ΔphaC1DC2. The results of cell growth and rhamnolipids production were shown in Fig. 3. From the cell growth curve of the strains, the cell concentration and growth trend of the double mutant strain were better than that of the wild-type strain SG (Fig. 3a). Moreover, the rhamnolipids yield of the double mutant strain SG ΔpslAB ΔphaC1DC2 was dramatically increased to 21.496 g/L (5.98 g/L/OD_{600}) (Fig. 3b). Simultaneously blocking the synthesis of Psl polysaccharides and PHA further significantly enhanced rhamnolipids production in *P. aeruginosa*. The rhamnolipids production was significantly increased (59%) in a mutant that completely impaired in PHA synthesis and over expressing *rhlAB-R* genes (Gutiérrez-Gómez et al. 2018). In previous studies on *P. aeruginosa* SG, it was found that the production of rhamnolipids improved 80% by increasing the copy number of *rhlAB* genes with modified promoter (Zhao et al. 2015a). Therefore, compared with other methods for increasing rhamnolipids production, the method of increasing rhamnolipid production by selectively blocking the secondary metabolic bypasses of rhamnolipids synthesis is effective and considerable.

The metabolic engineering strategies of increase flux in precursor- providing pathway is feasible for improve rhamnolipids production in *P. aeruginosa* SG. Using the fermentation medium, the double mutant strain SG ΔpslAB ΔphaC1DC2 produced 21.496 g/L rhamnolipids, 69.7% higher than that of wild-type strain SG. We think the yield of rhamnolipids may be further enhanced after optimization of the medium composition and fermentation process. In further study, we will carry out the related study.

This study further clarified the competitive metabolic pathways and the key genes involved in the synthesis of rhamnolipids. From the aspect of selectively blocking metabolic bypass, we explored a metabolic pathway optimization strategy to increase rhamnolipids production of *P. aeruginosa* SG. Through selectively blocking metabolic bypasses, increasing the amount of glycosyl and fatty acid precursors can significantly enhance rhamnolipids production in *P. aeruginosa*. The results of this study provide data and ideas for enhancing rhamnolipids production by genetic modification and regulation.

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Supporting information Supplementary Table S1—Shows all bacterial strains and plasmids in this study

Supplementary Table S2—Shows the primer pairs used to construct mutants SG ΔpslAB, SG ΔpelA and SG ΔphaC1DC2

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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