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ORIGINAL RESEARCH PAPER

Overproduction of rhamnolipids in *Pseudomonas aeruginosa* PA14 by redirection of the carbon flux from polyhydroxyalkanoate synthesis and overexpression of the *rhlAB-R* operon

Uriel Gutiérrez-Gómez · Martín P. Soto-Aceves · Luis Servín-González · Gloria Soberón-Chávez

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Abstract
Objective To construct *Pseudomonas aeruginosa* PA14 derivatives that overproduce rhamnolipids (RL) by blocking the synthesis of the carbon-storage polymer polyhydroxyalkanoates (PHA) and by overexpressing the *rhlAB-R* operon that encodes for enzymes of RL synthesis and the RhlR transcriptional regulator.
Results In contrast to previous results showing that overexpression of *rhlAB-R* genes in two *P. aeruginosa* strains (PAO1 and ATCC 9027) is sufficient to overproduce RL, we show that a PA14 derivative overexpressing the *rhlAB-R* operon did not increase the synthesis of these biosurfactants. In addition, PA14 mutants deficient in PHA production did not overproduce RL either. However, if the *rhlAB-R* genes were expressed in a mutant that is completely impaired in PHA synthesis, a significant increase in RL production was observed (59%). These results show that RL production in PA14 is limited both by the availability of fatty acid precursors and by the levels of the RhlA and RhlB enzymes that are involved in the synthesis of mono-RL.
Conclusions The limitation of RL production by *P. aeruginosa* PA14 is multifactorial and diverse from the results obtained with other strains. Thus, the factors that limit RL production are particular to each *P. aeruginosa* strain, so strain-specific strategies should be developed to increase their production.

Keywords Rhamnolipids overproduction · Biosurfactants · Metabolic engineering · *Pseudomonas aeruginosa*

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U. Gutiérrez-Gómez · L. Servín-González · G. Soberón-Chávez (✉)
Programa de Producción de Biomoléculas de Interés Biomédico en Bacterias y Hongos, Mexico, Mexico
e-mail: gloria@biomedicas.unam.mx

U. Gutiérrez-Gómez · M. P. Soto-Aceves · L. Servín-González · G. Soberón-Chávez
Departamento de Biología Molecular y Biotecnología, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, Ciudad Universitaria, 04510 Mexico, D.F., Mexico

Introduction

The biosurfactants rhamnolipids (RL) produced by *Pseudomonas aeruginosa* have a high biotechnological potential and have been approved by the US Environmental Protection Agency for use in food products, cosmetics and pharmaceuticals. However, their industrial application is limited by the low level of their production (Banat et al. 2010) and also

because *P. aeruginosa* is an opportunistic pathogen (Gellatly and Hancock 2013).

There have been different attempts to develop strains that overproduce RL, using modified *P. aeruginosa* strains (Grosso-Becerra et al. 2016) or heterologous hosts such as *Pseudomonas putida* KT2440 (Wittgens et al. 2011). The comparison of RL maximum yield and productivity between several strains of *P. aeruginosa*, including the type strain PAO1 and different heterologous hosts, showed that the best yield and productivity was obtained with PAO1 strain cultivated with sunflower oil as carbon source (Dobler et al. 2016).

In *P. aeruginosa* the production of RL is regulated at the level of transcription by a complex regulatory circuit called quorum-sensing (Williams and Cámara 2009). The *rhlAB* operon encodes the enzymes responsible for the synthesis of mono-RL (containing one molecule of rhamnose) whereas *rhlC* encodes the enzyme that synthesizes di-RL (RL with two rhamnose moieties) using mono-RL as substrate. Transcription of *rhlAB* and r*hlC* is activated by RhlR when coupled with the autoinducer butanoyl-homoserine lactone (Soberón-Chávez et al. 2005b). At 37 °C a positive RhlR-dependent regulatory loop is formed, leading to an increased expression of *rhlAB* and the formation of the *rhlAB-R* operon (Croda-García et al. 2011; Grosso-Becerra et al. 2014). It has been shown that the expression from a plasmid of the *rhlAB-R* operon increases RL production in *P. aeruginosa* strains PAO1 and ATCC 9027 (Grosso-Becerra et al. 2016).

The syntheses of RL and of polyhydroxyalkanoates (PHA) in *P. aeruginosa* are interconnected (Nitschke et al. 2011). It has been shown that RhlA, the first enzyme in the RL biosynthetic pathway, uses as substrates CoA-linked fatty acids derivatives (Abdel-Mawgoud et al. 2014) that are also intermediates of PHA synthesis. The synthesis of this polymer is catalyzed by the PhaC1 and PhaC2 synthases (Nitschke et al. 2011) using CoA-linked β-hydroxy fatty acids. When *P. aeruginosa* is cultured in media with either a sugar or glycerol as carbon source, the substrates of PhaC1 and PhaC2 synthases are provided by the PhaG transacylase, that converts ACP-β-hydroxy fatty acids to CoA-β-hydroxy fatty acids (Hoffmann et al. 2000). In addition, it has been reported that the intermediates synthetized by RhlA can be used as substrate for PHA synthesis (Soberón-Chávez et al. 2005a).

The engineered *P. putida* KT2440 strain used for RL production contains a plasmid for expression of the *rhlAB* operon from an inducible promoter and a mutation in *phaC1* that reduces PHA synthesis; this strain produces similar levels of RL as the PAO1 wild type strain of *P. aeruginosa* (Wittgens et al. 2011).

The aim of this work is to construct *P. aeruginosa* PA14 derivatives with increased RL production. This strain is highly virulent (Lee et al. 2006) and produces high RL levels with a similar proportion of mono- and di-RL.

Methods

The concentration of RL was estimated by the orcinol method (Chandrasekaran and Bemiller 1980). PHA was determined by hypochlorite digestion (Berger et al. 1989) and spectophotometric quantification of fatty acids derivatives at 235 nm by a similar procedure to that reported for polyhydroxybutyrate (Slepecky and Law 1960). The conversion of absorbance at 235 nm to PHA weight (expressed as μg/mL) was experimentally determined using a standard curve obtained with a known dry weight of purified PHA resuspended in 1 mL of sulfuric acid as described in Supplementary Fig. S1. Each experiment was done in triplicate and the standard deviation is shown.

Strains were routinely cultured in PPGAS medium (Zhang and Miller 1992) at 37 °C. Samples to measure RL and PHA production were obtained at 16 h of growth; this time point corresponds to the early stationary phase of strain PA14 growth curve (Supplementary Fig. S2), and it has been reported that, at this PA14 growth stage, the RL and PHA maximum yields occur (Gutierrez et al. 2013).

Apramycin and gentamycin were used at a concentration of 100 μg/mL.

The proportion of mono- and di-RL was evaluated by analyzing the image (using the ImageJ software) obtained from a thin-layer chromatography done as previously described (Matsuyama et al. 1987).

The *phaC2*::*MAR2xT7* strain obtained from the PA14 collection of mutants (Liberati et al. 2006) as a transposon insertion in *phaC2* (ID 54085) is completely impaired in PHA production (Supplementary Fig. S3), so we hypothesized that it contained a

mutated *phaC1* gene besides the insertion in *phaC2*. The alignment of the nucleotide sequence of the *phaC1* gene and also of the derived amino acid in the PA14 wild type strain (phaC1wt) and the *phaC2*::*MAR2xT7* mutant (ID 54085) (phaC1m) showed that in the strain obtained from the PA14 mutant collection (ID 54085), the *phaC1* gene has two nucleotide changes: a C to G transversion in nucleotide position 1276 and a G to A transition in nucleotide position 1651, that result in P426A and A551T substitutions in the PhaC1 amino acid sequence.

To further confirm that the *phaC1* gene with two missense mutations (phaC1m) was not functional, we used the *phaC1*::*MAR2xT7* mutant (ID 32531, Liberati et al. 2006) as recipient in complementation tests. We showed that the expression *in trans* from plasmid pUCP20 (West et al. 1994) of the phaC1m gene does not complement the PHA deficient phenotype of the *phaC1*::*MAR2xT7* mutant, while the expression of phaC1wt fully complements PHA production (Supplementary Fig. S3). These results confirms that strain *phaC2*::*MAR2xT7* (ID 54085) is a *phaC1 phaC2* double mutant.

The *aac(3)IV* cassette which confers resistance to apramycin (Gust et al. 2003) was used to construct the *phaG* mutant. The PA14 derived Δ*phaG*::*aac(3)IV* mutant and the Δ*phaG*::*aac(3)IV phaC1 phaC2*::*MAR2xT7* triple mutant used in this work were constructed as reported (Lesic and Rahme 2008). The oligonucleotides used to construct the Δ*phaG*::*aac(3)IV* mutant and the plasmids carrying the phaC1wt and phaC1m genes are shown in Supplementary Table S1.

Results and discussion

To increase RL production in PA14 we introduced plasmid pJGM4-*rhlAB-R* that has been shown to increase production of these biosurfactants in the *P. aeruginosa* strains PAO1 and ATCC 9027 (Grosso-Becerra et al. 2016) but, contrary to our prediction, the production of RL was not increased (Fig. 1). One explanation for this result is that in the PA14 background there is a limitation of the RhlA substrates, which are the CoA-linked fatty acid derivatives produced by RhlY and RhlZ (Abdel-Mawgoud et al. 2014) or of the RhlB substrate TDP-L-rhamnose (Soberón-Chávez et al. 2005b).

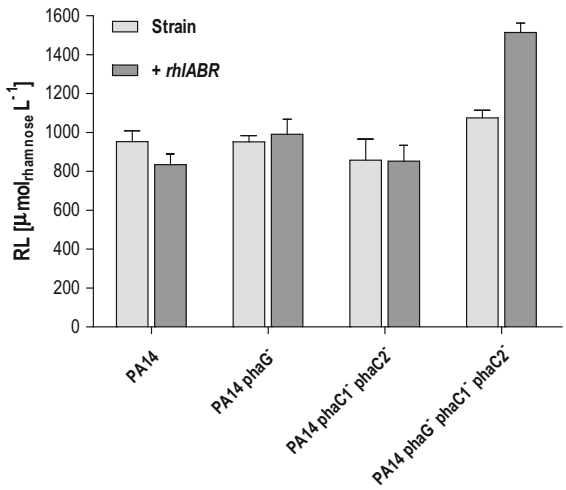

Fig. 1 Evaluation of the effect of mutations in the PA14 genes encoding for the enzymes that participate in the PHA biosynthetic pathway and of the overexpression of the *rhlAB-R* operon in RL production. Overexpression of the *rhlAB-R* operon was achieved by introduction of plasmid pJG4-*rhlAB-R* in these PA14 derivatives. The only strain that showed significant RL overproduction was the Δ*phaG*::*aac(3)IV phaC1 phaC2*::*MAR2xT7* triple mutant carrying pJG4-*rhlAB-R*

To increase the availability of the fatty acid precursor of RL we isolated the following PA14 mutants that are deficient in PHA production: a Δ*phaG*::*aac(3)IV* single mutant, a *phaC1 phaC2*::*MAR2xT7* double mutant, and Δ*phaG*::*aac(3)IV phaC1 phaC2*::*MAR2xT7* triple mutant.

The Δ*phaG*::*aac(3)IV* single mutant showed around a 30% decrease in PHA production, whereas the *phaC1 phaC2*::*MAR2xT7* double mutant was completely impaired in PHA production (Supplementary Fig. S3). Contrary to our predictions, RL production was not increased in either of these mutants (Fig. 1).

These results suggest that the increase in the CoA-fatty acid RhlA substrates in the PHA-deficient mutants is not sufficient to increase the flux of the RL biosynthetic pathway that involves not only RhlA, but also RhlB and RhlC.

To determine whether PA14 RL production was limited both by the availability of the CoA-fatty acids precursors of RhlA and the activity of RhlA and RhlB enzymes, we introduced the pJGM4-*rhlAB-R* plasmid to the PA14 mutants deficient in PHA synthesis.

We found that the level of RL produced by PA14 derivatives is only increased in the Δ*phaG*::*aac(3)IV phaC1 phaC2*::*MAR2xT7* triple mutant carrying

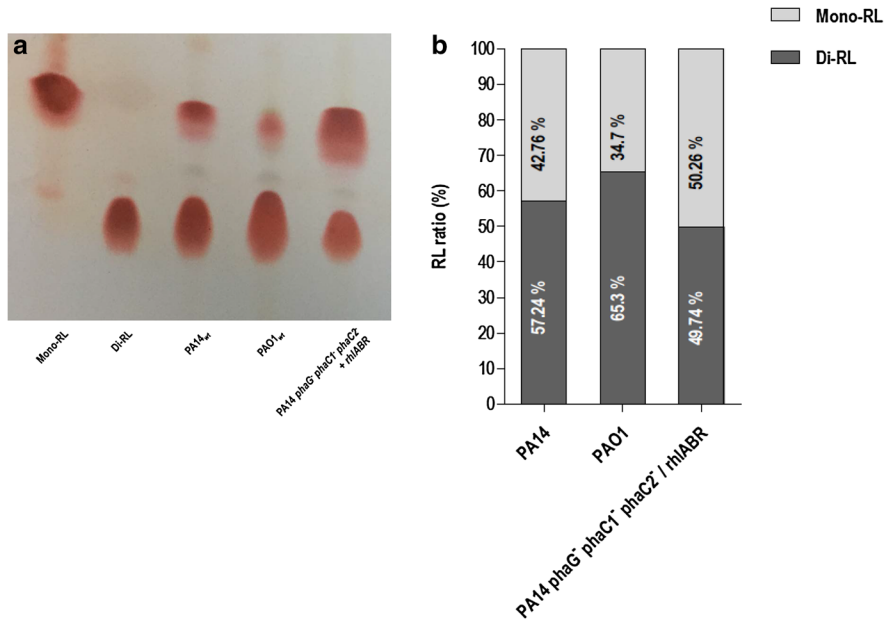

Fig. 2 Production of mono-RL and di-RL observed by thin-layer chromatography (TLC). **a** Chromatogram of culture supernatants of *P. aeruginosa* strains [PA14 wild-type (wt), PAO1 wild-type (wt), and PA14 Δ*phaG*::*aac(3)IV phaC1 phaC2*::*MAR2xT7*/pJG4-*rhlAB-R*]; **b** TLC image analysis using the ImageJ software to determine the ratios of each RL type

plasmid pJGM4-*rhlAB-R* (Fig. 1). This increment in RL production represents a 59% higher level of total RL than the PA14 wild type strain. These results suggest that RL production by PA14 strain is limited not only by the availability of CoA-fatty acids precursors, but also by the RhlA and RhlB enzyme levels. It is also apparent that the factors that limit RL production might be particular for each *P. aeruginosa* isolate, and thus specific metabolic engineering strategies should be developed for each strain.

An unexpected finding was that the *phaC1 phaC2*::*MAR2xT7* double mutant carrying pJGM4-*rhlAB-R* did not show an increase in RL production, even though it is completely impaired in PHA synthesis (Supplementary Fig. S3). This result suggests that the CoA linked (*R*)-β-hydroxy acids synthesized by PhaG cannot be used by RhlA as RL precursors.

The lack of correlation between null PHA production and increased synthesis of RL observed in this work, shows that the strategy for increasing RL production by partial blockage of the PHA biosynthetic pathway, such as the use of a *phaC1* mutant in the *P. putida* KT2440 background (Wittgens et al. 2011) should be evaluated for each strain and not taken for granted.

We measured RL production as the equivalent of rhamnose concentration by the orcinol method after hydrolysis of RL. Thus, if the PA14 RL hyper-producing derivative Δ*phaG*::*aac(3)IV phaC1 phaC2*::*MAR2xT7*/pJG4-*rhlAB-R* presented a lower mono-RL/di-RL ratio, we could overestimate the increment of RL. To determine whether the proportion of mono-RL/di-RL ratio was significant altered in this PA14 derivative we performed a thin-layer chromatography of culture supernatants of this strain compared to PA14 and PAO1 strains (Fig. 2). Strain PAO1 produces a 1:3 ratio of mono-RL:di-RL (Müller et al. 2011), while PA14 has been reported to present a higher 1:2 ratio (Abdel-Mawgoud et al. 2014). It is apparent that the RL hyper-producing strain does not have a lower mono-RL:di-RL ratio, but a slightly higher one, suggesting that RhlC activity is somewhat limiting in this condition. This result shows that the 59% increment in RL production by PA14 Δ*phaG*::*aac(3)IV phaC1*$^{-}$ *phaC2*::*MAR2xT7*/pJG4-*rhlAB-R* was not an overestimation.

The aim of this work was to show that it is feasible to increase RL production even in *P. aeruginosa*

strains that have a high basal level of production of this biosurfactant. The PA14 strain produces around a 55% higher concentration of RL than PAO1 strain in our culture conditions (PAO1 produces 548 μmol of rhamnose in RL per litre of culture), and we were able to further increase their production by 59%. The effect on RL production of increasing the availability of the TDP-L-rhamnose substrate of RhlB and RhlC, and of the overexpression of RhlC remains to be determined.

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Supporting information Supplementary Fig. 1—shows the standard curve use to determine PHA weight from absorbance at 235 nm.

Supplementary Fig. 2—presents the PA14 strain growth curve in PPGAS medium.

Supplementary Fig. 3—shows the PHA production of PA14 derivatives carrying mutations in genes involved in the synthesis of this fatty acid polymer, and the experiments showing that the *phaC1* gene of the *phaC2*::*MAR2xT7* mutant (ID 54085) is not functional since it is unable to complement the *phaC1*::*MAR2xT7* mutant (ID 32531) for PHA synthesis.

Supplementary Table S1—shows the oligonucleotides used to construct the PA14 Δ*phaG*::*aac(3)IV* mutant and the pUCP20 derived plasmids expressing phaC1wt and phaC1m.

Supplementary Table S2—shows the results obtained from the analysis of the image of thin-layer chromatography presented in Fig. 2, using the ImageJ software.

Compliance with ethical standards

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

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