microbial biotechnology

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Minireview

Rhamnolipids produced by *Pseudomonas*: from molecular genetics to the market

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Summary

Rhamnolipids are biosurfactants with a wide range of industrial applications that entered into the market a decade ago. They are naturally produced by *Pseudomonas aeruginosa* and some *Burkholderia* species. Occasionally, some strains of different bacterial species, like *Pseudomonas chlororaphis* NRRL B-30761, which have acquired RL-producing ability by horizontal gene transfer, have been described. *P. aeruginosa*, the ubiquitous opportunistic pathogenic bacterium, is the best rhamnolipids producer, but *Pseudomonas putida* has been used as heterologous host for the production of this biosurfactant with relatively good yields. The molecular genetics of rhamnolipids production by *P. aeruginosa* has been widely studied not only due to the interest in developing overproducing strains, but because it is coordinately regulated with the expression of different virulence-related traits by the quorum-sensing response. Here, we highlight how the research of the molecular mechanisms involved in rhamnolipid production have impacted the development of strains that are suitable for industrial production of this biosurfactant, as well as some perspectives to improve these industrial useful strains.

Received 6 August, 2020; revised 18 October, 2020; accepted 20 October, 2020.
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Microbial Biotechnology (2021) **14**(1), 136–146
doi:10.1111/1751-7915.13700
Funding information The work reviewed here that was done in the authors' laboratory was partially supported by Programa de Apoyo a Proyectos de Investigación e Innovación Tecnológica (PAPIIT) DGAPA, Universidad Nacional Autónoma de México (UNAM), grant number IN201819 and IA203519.

Introduction

Biosurfactants (BS) are surface-active molecules produced by different microorganisms, including bacteria and yeasts, that can minimize the surface and interfacial tension between two immiscible fluids phases. BS have the potential to be used in biomedical, pharmaceutical, cosmetic, food processing, oil and gas industries, as they are highly biodegradable and have low toxicity (Naughton *et al*., 2019). Ramnolipids (RL) are BS that are naturally produced by the opportunistic pathogen *Pseudomonas aeruginosa* and by some *Burholderia* species (Toribio *et al*., 2010). In *P. aeruginosa*, RL synthesis and regulation have been extensively studied since they play a role as a virulence factor. For example, it has been demonstrated that RL reduces mucociliary transport in human respiratory epithelium (Read *et al*., 1992) and that are also involved in biofilm formation (Davey *et al*., 2003) and swarming motility (Caiazza *et al*., 2005; Tremblay *et al*., 2007).

Ramnolipids produced by *P. aeruginosa* have very good physicochemical characteristics to be used in different industrial applications (Nitschke *et al*., 2011; Sekhon Randhawa and Rahman, 2014). They present low toxicity (Johann *et al*., 2016), high biodegradability and are produced at a higher level compared with other bacterial BS (RL are the BS with higher yields, with the only exception of glycolipids produced by yeasts).

This BS reached the market in the last decade; in 2013 nearly 95 000 tons were produced that represented almost 455 million US dollars (Global Market Inc. 2019). However, the industrial applications and commercialization of RL are still limited by the relatively low level of their production and by the pathogenicity of *P. aeruginosa*, which is the best RL producer (Table 1; Chong and Li, 2017). At present, RL that are in the market are used mainly in the petrochemical industry,

bioremediation of different pollutants, household products, agricultural chemicals and personal care products (Sekhon Randhawa and Rahman, 2014). In addition, RL present other activities such as antifungal properties (Borah *et al*., 2016; Sancheti and Ju, 2019), antimicrobial activity, and they show low toxicity (Johann *et al*., 2016) and do not disturb the immune response, so these characteristics could expand their applications to the pharmaceutical industry (Chong and Li, 2017). RL are industrially produced by different companies such as: NatSurFact (USA), AGAE technologies Ltd. (USA), Rhamnolipid, Inc. (USA), GlycoSurf (USA), TensioGreen (USA) and Jeneil biosurfactant (Germany). In addition, Evonik Industries a German company with global presence uses RL in some of its products.

Most *P. aeruginosa* strains produce two forms of RL, mono-RL (containing one rhamnose moiety and a dimer of fatty acids) and di-RL (containing two rhamnose molecules and a fatty acid dimer). The production of mono-RL is catalysed by the coordinate activity of RhlA that produces the fatty acid dimer using as substrate a CoA-linked fatty acid derivative produced by RhlY (enoyl-CoA hydratase) and RhlZ (enoyl-CoA hydratase/isomerase; Abdel-Mawgoud *et al*., 2014, Gutiérrez-Gómez *et al*., 2019) and the rhamnosyl transferase RhlB which uses as substrates dTDP-L-rhamnose and the fatty acid dimer produced by RhlA. In turn, di-RL is produced by the RhlC enzyme, which uses as substrate mono-RL- and dTDP-L-rhamnose.

Some non-pathogenic bacterial isolates belonging to different bacterial species like *P. chlororaphis* (Gunther *et al*., 2006), *P. putida* (Toribio *et al*., 2010) and even *Marinobacter* (Tripathi *et al*., 2019) have been found to naturally produce RL, but their level of production is low compared to *P. aeruginosa* strains. *P. chlororaphis* strain NRRL B-30761 that is able to produce mono-RL (Gunther *et al*., 2005, 2006) has been engineered to produce also di-RL by the expression of *P. aeruginosa rhlC* (Solaiman *et al*., 2015). The non-pathogenic *Marinobacter sp* MCTG107b was reported to produce a mixture of RL, with over 95% of di-RL, being di-RL with a lipidic dimer of C10-C10, the most abundant congener (Tripathi *et al*., 2019). These non-pathogenic RL-producing bacteria are an important resource for the industrial production of RL, but a large of amount of work remains to be done to increase their RL productivity.

Table 1. RL production by recombinant bacterial hosts (modified from Tiso *et al*., 2017), in comparison with *P. aeruginosa* PAO1 and DSM 7108 strains.

Rhamnolipid type	Expression Host	Heterologous gene expressed	Medium/C-source	Maximum yield (g/L)	Reference
mono- and di-RL	Wild-type *P. aeruginosa* PAO1	None	Mineral salts with nitrate/ sunflower oil	36.7 ± 1.2	Müller *et al*. (2011)
	Wild-type *P. aeruginosa* DSM 7108	None	Mineral salts with nitrate/ sunflower oil	35.7 ± 2	Müller *et al*. (2011)
mono- and di-RL	*P. chlororaphis*	*rhlC*	MSM/glucose	0.1	Solaiman *et al*. (2015)
	P. putida KT2440	P_{tac}, *rhlAB/rhlABC/rhlC*	LB/glucose	0.005 (mono-RL) 0.004 (mixture)	Wittgens *et al*. (2017)
	P. aeruginosa	P_{lac}, *estA*	MSP/glycerol	14.6	Dobler *et al*. (2017)
mono-RL	*E. coli*	P_{lac}, *rhlAB*	TY	0.005	Kryachko *et al*. (2013)
	P. fluorescens	P_{tac}, *rhlAB*	GS/glucose	< 0.02	Ochsner *et al*. (1995)
	P. oleovorans	P_{tac}, *rhlAB*	GS/glucose	< 0.02	Ochsner *et al*. (1995)
	Burkhorderia kururiensis	P_{tac}, *rhlAB*	MSP/ glycerol	5.67	Tavares *et al*. (2013)
	P. putida KT2440	P_{tac}, *rhlAB*, *ΔphaC1*	LB/glucose	1.5	Wittgens *et al*. (2011)
	P. putida KT2440	P_{native} (RhlRI), *rhlABRI*	LB	1.68	Cao *et al*. (2012)
	P. putida KT2440	P_{tac}, *rhlAB*	M9/sunflower oil	0.57	Setoodeh *et al*. (2014)
	P. putida KT2440 kT40CZC	$P_{synthetic}$, *rhlAB*	LB/glucose	3.2	Tiso *et al*. (2016)
	P. putida KT2440	$P_{synthetic}$, *rhlAB*	SupM/glucose	14.9	Beuker *et al*. (2016)

An alternative strategy for RL production has been their heterologous production in non-pathogenic bacteria expressing *P. aeruginosa rhlAB* operon from an inducible promoter (Table 1; Wittgens *et al*., 2011; Setoodeh *et al*., 2014). The most successful case of mono-RL heterologous production is the use of *P. putida* KT2440 containing a plasmid encoding the *rhlAB* operon from *P. aeruginosa* PAO1 expressed from an IPTG-inducible promoter (Wittgens *et al*., 2011; Beuker *et al*., 2016).

In this minireview, we will describe some of the molecular aspects of RL synthesis and regulation and their relations with *P. aeruginosa* virulence, highlighting how these research results impact the construction of *Pseudomonas* strains with better characteristics for industrial production of this BS.

RL biosynthesis in *P. aeruginosa*

RhlB and RhlC, the two rhamnosyl-transferases involved in RL biosynthetic pathway (Fig. 1), use dTDP-L-rhamnose as one of their substrates (Ochsner *et al*., 1994; Rahim *et al*., 2001). The first step in the synthesis of this activated sugar, the epimerization of glucose-6-phosphate to glucose-1-phosphate, is catalysed by AlgC, an enzyme that also participates in the biosynthesis of alginate, one of *Pseudomonas* exopolysaccharides (Olvera *et al*., 1999). The conversion of glucose-1-phosphate to dTDP-L-rhamnose is catalysed by the enzymes encoded by the *rmlBDAC* operon (Aguirre-Ramírez *et al*., 2012). Other bacteria like *Escherichia coli* K12 or *P. putida* KT2440 strains have orthologs to the *rml* genes that produce lipopolysaccharide (LPS) containing L-rhamnose, but the level of expression of this operon for LPS synthesis is low in these bacteria, while it is highly induced in *P. aeruginosa* when RL are being synthetized (Aguirre-Ramírez *et al*., 2012).

The lipid RL moiety consists of a dimer of fatty acids (3-(3-hydroxyalkanoyloxy)alkanoic acids or HAA) mainly constituted by 10 carbon chains, but several congeners are present at a lower proportion (Déziel *et al*., 2000). HAA is produced by RhlA (Déziel *et al*., 2003), the first enzyme of the RL biosynthetic pathway and are one of the substrates, together with dTDP-L-rhamnose of RhlB for the synthesis of mono-RL (Fig. 1).

It has been reported that HAA is mainly derived from the fatty acid biosynthesis pathway when this bacterium is cultured with glucose as carbon source (Gutiérrez-Gómez *et al*., 2019), and that the enzymes RhlY (enoyl-CoA hydratase) and RhlZ (enoyl-CoA hydratase/isomerase) play a central role in the synthesis of the Co-A-linked RhlA substrate (Abdel-Mawgoud *et al*., 2014) accounting for 80% of the RL produced (Gutiérrez-Gómez *et al*., 2019). Purified RhlA catalyses *in vitro* HAA biosynthesis from two molecules of (*R*)-3-hydroxyacyl-ACP (Zhu and Rock, 2008), so it is likely that (*R*)-3-hydroxyacyl-ACP coming from *de novo* synthesis is the RhlA substrate that accounts for the 20% of RL synthesis remaining in an *rhlY, rhlZ* double mutant (Gutiérrez-Gómez *et al*., 2019; Fig. 1).

RhlA besides participating in the synthesis of RL also participates in the production of the carbon-storage polymer, polyhydroxyalkanoate (PHA; Soberón-Chávez *et al*., 2005a; Gutiérrez-Gómez *et al*., 2018), since the HAA-CoA produced by RhlA can be used as substrates of the PHA synthases PhaC1 and PhaC2 to produce this polymer. While the (*R*)-3-hydroxyacyl-CoA precursor of PHA, the canonical PhaC1 and PhaC2 substrate (Eggink *et al*., 1992; Langenbach *et al*., 1997), is produced by the coordinate activity of PhaG thioesterase and a CoA ligase (PA3924 in *P. aeruginosa* PAO1 and PA14_13110 in PA14; Hokamura *et al*., 2015), RhlA produces HAA-CoA which is also a PhaC1 and PhaC2 substrate. We reported that *rhlA* and *phaG* single mutants have a decreased PHA production, while the double *rhlA* and *phaG* mutant presents a more drastic PHA deficiency (Gutiérrez-Gómez *et al*., 2019). However, the main evidence of the participation of RhlA in PHA synthesis comes from the partial complementation of a *phaG, rhlA* double mutant that is unable to produce PHA, by the expression of *rhlA* from a plasmid (Gutiérrez-Gómez *et al*., 2019). In addition, *P. aeruginosa* RhlA and PhaG proteins have a 44% amino acid identity (Rehm *et al*., 1998), supporting the finding that they share catalytic characteristics that might be their ability to remove CoA from their fatty acid precursor, since RhlA has to cleave CoA when synthesizing HAA-CoA from two CoA-linked fatty acid precursors. However, a *phaG* mutant is not affected in RL production showing that RhlA does not use as a substrate the (*R*)-3-hydroxyacyl-CoA PHA precursor produced by this thioesterase and the CoA ligase (Gutiérrez-Gómez *et al*., 2019).

Other enzymes that play a key role in RL synthesis are RhlY and RhlZ (Abdel-Mawgoud *et al*., 2014; Gutiérrez-Gómez *et al*., 2019), since the *rhlY*, *rhlZ* double mutant has a severely reduced capacity for RL synthesis, but are also involved in PHA synthesis since this mutant has also reduced PHA production (Abdel-Mawgoud *et al*., 2014; Gutiérrez-Gómez *et al*., 2019). However, the precise role of these enzymes is not known; it has been proposed that (*S*)-3-hydroxyacyl-CoA is the RhlY/RhlZ product (Fig. 1) and that it is the main RhlA substrate (Gutiérrez-Gómez *et al*., 2019), but it is still not completely defined.

The precise knowledge of RL biosynthetic pathway and its relations with PHA synthesis (Fig. 1) are key for the construction of RL hyper-producing strains in which the carbon flux is redirected for the synthesis of this BS.

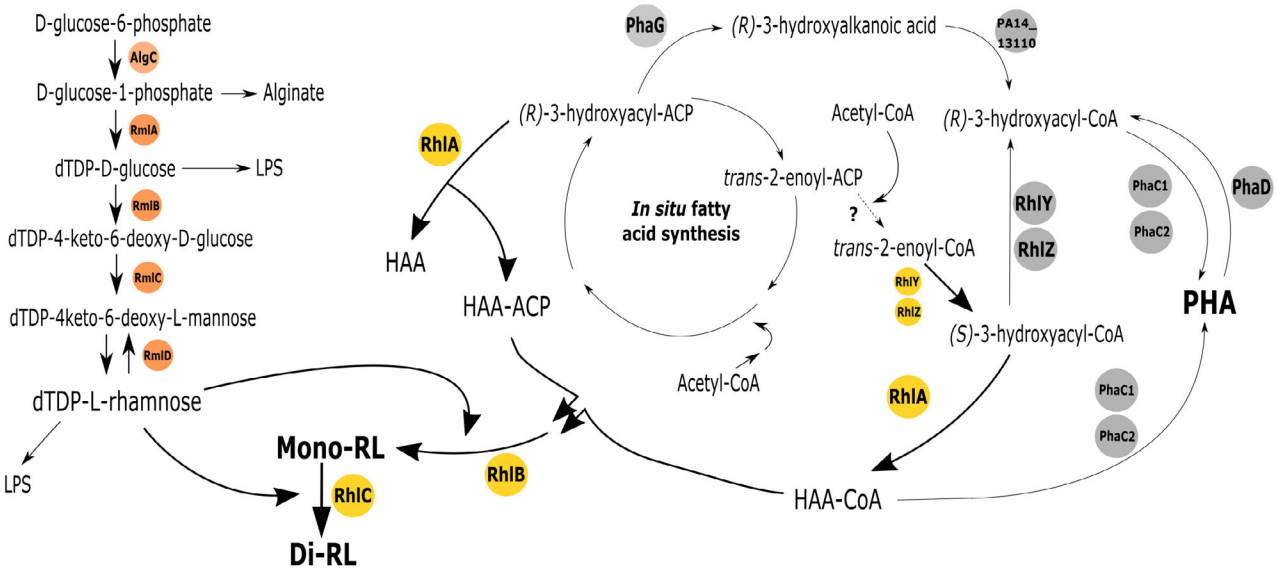

Fig. 1. Mono- and di-RL biosynthetic route and its relations with PHA synthesis. Enzymes inside a yellow circle are those directly involved in RL synthesis, those participating in the synthesis of RL precursor dTDP-L-rhamnose are circled in orange and the enzymes involved in PHA biosynthesis are shown in grey circles (RhlY and RhlZ participate both in RL and in PHA synthesis). HAA and LPS stands for 3-(3-hydroxyalkanoyloxy)alkanoic acids) and lipopolysaccharide respectively.

Gene regulation of rhamnolipid synthesis in *P. aeruginosa*

In *P. aeruginosa,* the expression of the genes involved in RL synthesis is controlled at the transcriptional and post-transcriptional levels (Fig. 2). In the first case, it comprises the quorum-sensing (QS) systems which are a process involving the synthesis and detection of a diffusible signal molecule, called autoinducer (AI), that is accumulated in the medium and allows the bacteria to produce a coordinate behaviour (Williams *et al*., 2007).

P. aeruginosa harbours three QS systems named Las, Rhl and Pqs. In the Las and Rhl systems, the synthases LasI and RhlI produce the AIs *N*-3-oxo-dodecanoyl-homoserine lactone (3O-C12-HSL) and *N*-butyryl-homoserine lactone (C4-HSL) that bind to the regulatory proteins LasR and RhlR respectively (Williams *et al*., 2007). In the Pqs system, PqsR is the regulator protein that binds to 2- heptyl-3-hydroxy-1H-quinolin-4-one (PQS) or 2-heptyl-1H-quinolin-4- one (HHQ), synthesized by the *pqsABCD* and *phnAB* operons, and the *pqsH* gene (in the case of PQS) (Pesci *et al*., 1999; Cao *et al*., 2001; Xiao *et al*., 2006; García-Reyes *et al*., 2020a). When LasR is coupled with 3O-C12-HSL, it activates the expression of several virulence factors and also the expression of *rhlI, rhlR, pqsR* and *pqsH*. Thus, it has been proposed that these three QS systems are arranged hierarchically with the Las system on the top of this regulatory network (Pesci *et al*., 1997; Farrow and Pesci, 2017). The Rhl regulon includes genes involved in virulence factors production as well, but particularly those involved in RL synthesis (Soberón-Chávez *et al*., 2005b). Once *rhlR* and *rhlI* are fully activated by the Las system, the complex RhlR/C4-HSL activates the transcription of the *rhlAB* operon and the *rhlC* gene which forms an operon with a gene (PA1131 in *P. aeruginosa* PAO1) that encodes a protein with no known role in RL synthesis or transport (Wittgens *et al*., 2017). Moreover, at 37 °C, but not at 30 °C, the expression of the *rhlAB* operon can be extended to the *rhlR* and *rhlI* genes creating a positive feedback loop (Croda-García *et al*., 2011; Morales *et al*., 2017). The rise in temperature is detected by the presence of a ROSE-like RNA thermometer at the 5' UTR *rhlA* mRNA (Grosso-Becerra *et al*., 2014). In addition, the expression of the *rmlBDAC* operon also is activated by the Rhl system since one of its three promoters is controlled by the complex RhlR/C4-HSL (Aguirre-Ramírez *et al*., 2012). Thus, inactivation of *rhlR* or *rhlI* abolishes RL synthesis. On the other side, it has been documented that the Pqs system modulates the activity of the Rhl system, particularly affecting the production of pyocyanin (Diggle *et al*., 2003; Farrow *et al*., 2008). The effector involved in this regulation is the enigmatic PqsE protein that is encoded by the *pqsE* gene, which is transcribed within *pqsABCDE* operon (García-Reyes *et al*., 2020a). Inactivation of *pqsE* abolishes pyocyanin production and slightly reduces RL production in PAO1 strain (Farrow *et al*., 2008; Baldelli *et al*., 2020). However, the molecular mechanism by which PqsE affects pyocyanin, but not RL synthesis is not totally

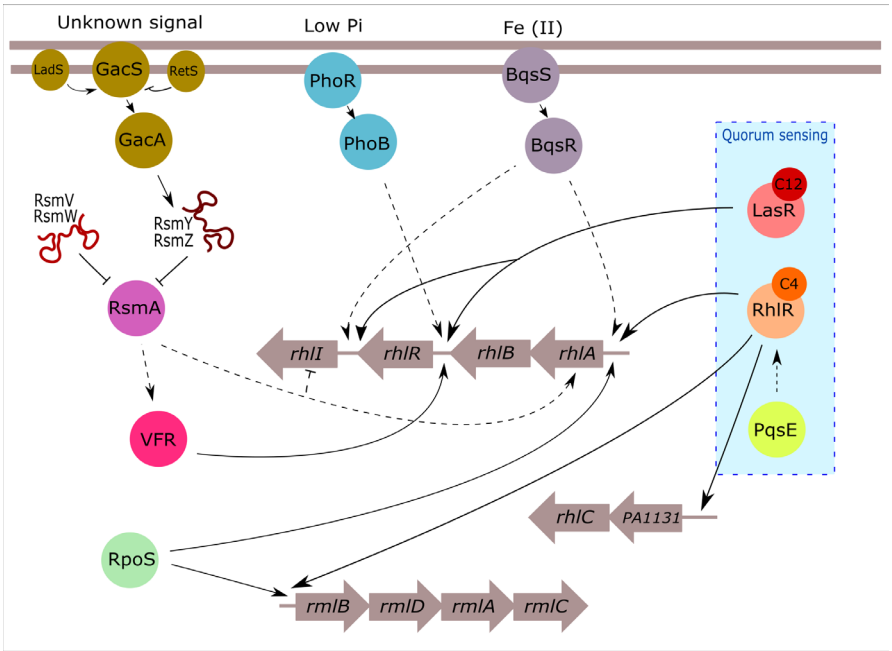

Fig. 2. Transcriptional and post-transcriptional regulation of RL production. Coloured circles show the regulatory proteins involved in the expression of genes encoding the enzymes involved in RL biosynthesis, lines forming stem and loops represent small non-coding RNAs. Activation is shown by an arrow, while a negative regulation is shown by a perpendicular line. Dotted lines represent interactions that have not been fully demonstrated.

understood. It was proposed that PqsE synthesizes an alternative AI that activates RhlR in order to regulate a set of genes, some of them different to those regulated with its canonical AI, C4-HSL (Mukherjee *et al*., 2018). However, a recent study conducted by Groleau *et al*. (2020) suggests that the unknown molecule produced by PqsE is not a diffusible AI, so additional experiments are necessary to determine the molecular mechanism by which PqsE controls the virulence factors production in *P. aeruginosa*.

In addition to QS systems, other regulatory proteins responding to environmental conditions are involved in controlling RL synthesis by regulating the expression of *rhlR* or the *rhlAB* operon. In this regard, *rhlR* transcription is activated not only by the complex Las/C12-HSL but also by Vfr, a *P. aeruginosa* Crp homologue (Croda-García *et al*., 2011), and its expression is dependent on RpoN (Medina *et al*., 2003a). Furthermore, the stationary-phase sigma factor RpoS, responding to stress conditions, partially regulates the expression of the *rhlAB* and *rmlBDAC* operons (Medina *et al*., 2003b; Aguirre-Ramírez *et al*., 2012). Thus, the transcriptional control by these global regulators indicates that different growth and stress conditions, or nutrients availability also can influence the regulation of RL production through the Rhl-QS system. In line with this, in phosphate-limited conditions the PhoB-PhoR system positively regulates *rhlR* expression (Jensen *et al*., 2006), leading to a major activation of the RhlR-dependent genes including *rhlA* transcription (Blus-Kadosh *et al*., 2013). Moreover, the BqsS-BqsR two-component system which responds to the presence of Fe(II) (Kreamer *et al*., 2012) positively regulates C4-HSL production and *rhlA* transcription resulting in increased synthesis of RL (Dong, *et al*., 2008).

The post-transcriptional regulation of RL synthesis is mediated by the Rsm system that is comprised by four non-coding small RNAs named RsmV, RsmW, RsmY and RsmZ which antagonize the activity of the small RNA-binding proteins RsmA and RsmN (Lapouge *et al*., 2008; Miller *et al*., 2016; Janssen *et al*., 2018). These two proteins recognize specific sequences in the untranslated RNA region preventing, in most cases, the translation of the target mRNA (Brencic *et al*., 2009; Morris *et al*., 2013; Vakulskas *et al*., 2015). The transcription of *rsmY* and *rsmZ* is controlled by the two-component system GacS/GacA and by LadS and RetS proteins (Ventre *et al*., 2006; Lapouge *et al*., 2008). It has been shown that the Gac-Rsm pathway modulates RL synthesis at different points (Cocotl-Yañez *et al*., 2020), such as C4-HSL production (Pessi *et al*., 2001), expression of the *rhlAB* operon (Heurlier *et al*., 2004), and indirectly *rhlR* transcription by positively regulating Vfr expression (Burrowes *et al*., 2006). Furthermore, some of these are antagonist effects that cause a positive or a negative effect on RL synthesis, so the whole

picture of Gac-Rsm-dependent RL regulation remains to be completed.

Metabolic engineering strategies to construct *Pseudomonas* RL-hyper-producing strain

As has been briefly described, *P. aeruginosa* RL biosynthesis (Fig. 1) represents a crossroad of central metabolic pathways, such as *de novo* fatty acid synthesis and dTDP-L-rhamnose biosynthesis (connected with alginate biosynthesis and LPS production), with the synthesis of secondary metabolites such as PHA. In addition, the genetic regulation of RL production is intertwined with the production of virulence factors by the QS response and is also subject to modulation by environmental factors (Fig. 2). The complexity of RL biosynthesis and regulation represents a challenge to obtain hyper-producing strains, but at the same time this complexity provides several nodes of the regulatory network and biosynthetic route that can be exploited to achieve higher yields of this BS, as will be further discussed.

In addition, the use of *P. aeruginosa* for the production of RL has the problem of its pathogenicity that has been overcome by the search of non-virulent *P. aeruginosa* strains, or by the heterologous production of RL, mainly in *P. putida* KT2440. Both approaches will be briefly discussed.

Pseudomonas aeruginosa ATCC 9027 is a completely avirulent strain (Grosso-Becerra *et al*., 2016; Soto-Aceves *et al*., 2019) that has a defective QS response (García-Reyes *et al*., 2020b) and produces low amounts of RL. The production of this BS is enhanced reaching similar levels as *P. aeruginosa* PAO1 type strain, when the *rhlAB-R* operon or *rhlR* are expressed from a plasmid, without increasing the virulence of the recombinant strain in a mice model (Grosso-Becerra *et al*., 2016). However, the expression of the *rhlAB* operon from a plasmid, without *rhlR*, only slightly increases RL production (Grosso-Becerra *et al*., 2016), showing that the RhlR-dependent expression of this operon through RhlR positive autoregulatory loop (Croda-García *et al*., 2011; Grosso-Becerra *et al*., 2014) is important for increased RL production in strain ATCC 9027. These results show that ATCC 9027 lack of virulence is not only dependent on QS and that the limiting step for its RL production is the RhlR-dependent expression of *rhlAB* and maybe the *rmlBDAC* operon that is also induced by RhlR (Aguirre-Ramírez *et al*., 2012). It is possible that RL production can be further increased using this strain by selecting mutants that block PHA synthesis, or by overexpressing the *rmlBDAC* operon, a strategy that was used for the heterologous production of RL in *E. coli* (Cabrera-Valladares *et al*., 2006).

The genetically modified *P. aeruginosa* PA14 derivative which expresses a plasmid encoding the *rhlAB-R* operon and has mutations that completely inactivate PHA production (in *phaG*, *phaC1* and *phaC2* genes) has an increased RL production of around 60% compared to PA14 wild type, and is the reported strain with the highest RL production, reaching almost the double of PAO1 (Gutiérrez-Gómez *et al*., 2018). However, this strain is not suitable for the industrial production of RL due to PA14 high virulence (Lee *et al*., 2006). At present, non-virulent derivatives of the PA14 RL hyper-producing derivatives have been isolated (Gutiérrez-Gómez and Soberón-Chávez Mexican patent submission MX/a/2019/006840, June 2019).

The advantage of using genetically engineered *P. aeruginosa* derivatives that overproduce RL is that the operon encoding for the genes involved synthesis of dTDP-L-rhamnose is coordinately induced with the *rhlAB-R-I* operon (Aguirre-Ramírez *et al*., 2012) and at 37 °C is one target of the positive autoregulatory loop of *rhlR* expression (Croda-García *et al*., 2011; Grosso-Becerra *et al*., 2014; Morales *et al*., 2017). The genetic regulation of *rhlY* and *rhlZ* has not been studied, but these genes might also be induced by the QS response since both of them contain in their promoter regions putative RhlR/C4-HSL-binding sequences (Fig. 3). The coordinate induction by QS of *P. aeruginosa rhlAB*, *rhlC*, *rmlBDAC* and possibly of *rhlY* and *rhlZ* enables the construction of RL hyper-producing strains by the expression of RhlR or RhlA, RhlB and RhlR without the need of adding an inducer to the culture medium (Grosso-Becerra *et al*., 2016; Gutiérrez-Gómez *et al*., 2018), such as IPTG that is used in the case of *P. putida* KT2440 (Table 1).

The *P. putida* KT2440 derivative expressing the *rhlAB* operon that was designed to produce mono-RL contains a mutation in *phaC1* that caused a considerable increase in RL production, showing that that PHA synthesis competes for fatty acid derivatives with RL synthesis (Wittgens *et al*., 2011). The contribution of the ROSE-like RNA thermometer to the induction by a rise in temperature of the *rhlAB* operon expressed in *P. putida* KT2440 has been evaluated (Noll *et al*., 2019), but even though at 37 °C a high RL production per cell was achieved, a low amount of biomass was produced, and the increment observed was not directly related to the presence of the RNA thermometer.

As described, the substrates for the synthesis of RL are central metabolic products and their availability is expected to be limited in non-natural RL producers such as *P. putida* KT2440, the strain that has been most successfully used for heterologous RL production (Setoodeh *et al*., 2014; Beuker *et al*., 2016). The *rmlBDAC* operon is only expressed at a low level for LPS synthesis in this

Sequence upstream ***rhlY*** coding sequence:

AAGCCGCTGGCGAC**CT**TCCAGGCCTTGC**AG**TTCAAGCTCGCCGACATGCTCACCGA
ACTCACCGCCAGCCGCCAGATGGTCCGCCTCGGCGCCCATCGGCTGGACCGCGGCG
ACGCCGAGGCGACCCTGTA**CT**GCGCAATGGCCA**AG**CGCTTCGCCACCGACCGCTGC
TTCGATGTCTGCAACGAGGCCTTGCAACTGCACGGCGGCTACGGCTATCTCAACGA
TTATCCGCTGGAGCGCTGGGTACGCGACACCCGCGTGCACCAGATCCTCGAAGGCA
CCAACGAAATCATGCGGGTGATCGTCGCCCGCCGCCTGCTGGAGCAGGGCGGCATG
CTCGATCGCCTGCTGTGAACGCCGCGAACCATCGCATCCGATAGTGCCGGGACGTC
CCGGCCCATGATCCAGTGAGGGACTTC**ATG**

Sequence upstream ***rhlZ*** coding sequence:

GAACAC**CT**GGCTGCCGGAGG**AG**CGCGAGCGCTTCGTCGATCTGTTCGACGCCCAGG
ACACCCGCGAAGGGGTCAACGCCTTCCTCGAGAAGCGCGATCCCAAGTGGCGCAAC
TGCTGAGCCTTCCCATCGAACCCGGCGGGCTGCGCCACAGCCCGCCCGAGCCGAGA
GCCCCGAC**ATG**

PA14_54660 (**RhlZ**) and PP_1412 alignment:

```
PP_1412   1   MTIHCEVLTGVDGARIGIATLDAPKALNALNLPMIEVLGEQLHAWARDPGIVCVLLRGNG  60
              M +  E    + G RIGIATLDA K+LNAL+LPMIE L  +L AWA D GI CVLLRGNG
RhlZ      1   MNVLFEERPSLHGFRIGIATLDAEKSLNALSLPMIEALAAKLDAWAEDAGIACVLLRGNG  60

PP_1412   61  AKAFCAGGDVRALAQACRDHPGSVPPLAASFFAAEYRLDFALHTYPKPLLCWGHGHVLGG  120
              AKAFCAGGDVR L  ACR+ PG VP LA  FFA EYRLD+ +HTYPKP +CW HG+V+GG
RhlZ      61  AKAFCAGGDVRKLVDACREQPGEVPALARRFFADEYRLDYRIHTYPKPFICWAHGYVMGG  120

PP_1412   121 GMGLLQGANVRIVTPSSRLAMPEISIGLYPDVGASWFLARLPGKLGLFFGLTGAPINARD  180
              GMGL+QGA +RIVTPSSRLAMPEI IGLYPDVGASWFLARLPG+LGLF GL+ A +NARD
RhlZ      121 GMGLMQGAGIRIVTPSSRLAMPEIGIGLYPDVGASWFLARLPGRLGLFLGLSAAQMNARD  180

PP_1412   181 ALDLGLADRFLGEHQQEALIEELLQLNWQEQTAIQLNSLLKAEQHRACAELPDAQWLPRR  240
              ALDL LADRFL + QQ+AL+  L+Q+NW E   +QL+SLL+A +H A  ELP+AQ LPRR
RhlZ      181 ALDLDLADRFLLDDQQDALLAGLVQMNWNESPQVQLHSLLRALEHEARGELPEAQLLPRR  240

PP_1412   241 HEIDQLLDVADAASAWRALERLKQHDDPLLADAGHRLHEGCPLTAHLVWEQIRRARYLSL  300
                +D LLD  D ASAW+AL  L+   DPLLA     L EGCP+TAHLVW+QI RARYLSL
RhlZ      241 PRLDALLDQPDLASAWQALVALRDDADPLLARGAKTLAEGCPMTAHLVWQQIERARYLSL  300
```

Fig. 3. *Pseudomonas* RhlY and RhlZ.A. *P. aeruginosa rhlY* and *rhlZ* have putative RhlR/C4-HSL-binding sites in their promoter region (indicated by grey boxes). Nucleotides in bold letters correspond to the invariant binding sequences for LasR or RhlR.B. Amino acid alignment of RhlY and a *P. putida* KT2440 ortholog (PP_1412) that shares 67.3% amino acid identity.

bacterium, thus producing a reduced level of dTDP-L-rhamnose, and it does not have a RhlY ortholog. However, this strain contains a RhlZ ortholog (PP_1412) that shows 67.3% of amino acid identity (Fig. 3) that might produce the RhlA Co-A-linked fatty acid precursor. However, it is likely that the CoA-fatty acid substrate of RhlA is limited in this heterologous hosts due to the lack of RhlY. Thus, the optimization of the production of metabolites used for RL synthesis is a research area that remains to be explored for the construction of *P. putida* KT2440 derivatives with increased RL production.

Table 1 summarizes different strategies to produce RL in heterologous hosts compared with the level of production of this BS by *P. aeruginosa* PAO1 and DSM 7108 wild-type strains.

Future trends

As has been briefly reviewed, understanding the molecular genetics of RL synthesis and regulation has opened a wide variety of strategies to build strains with enhanced RL production that are suitable for the production of this BS at an industrial scale, and there are still many alternatives to explore, some of which have been mentioned in this article. For example, it is important to determine the way that *P. aeruginosa* PqsE modifies RhlR activity and how does this modification cause a marked pyocyanin increment, without causing a similar induction of genes involved in RL synthesis.

Another possibility that remains to be explored to obtain RL hyper-producing non-pathogenic bacteria is the use of *P. chlororaphis* derivatives for the heterologous production of RL, since this non-pathogenic bacterial species possesses a QS response that regulates phenazine production, and which could be genetically engineered for the expression of the *rhlAB* operon, a strategy that has worked in other bacteria to produce or increase RL production.

The industrial production of RL is also limited by the foaming problem of the large-scale BS production, and the design of strategies to control this problem is a field of intense research (Henkel *et al*., 2017; Sodagari and Ju, 2020). The ability of *Pseudomonas* to grow with nitrate as electron donor in microaerophilic or anaerobic conditions and to produce RL has been exploited for *in situ* production of this BS in oil recovery (Zhao *et al*., 2016). Thus, the large-scale production of RL under denitrification conditions is a promising strategy, and *P. aeruginosa* QS-dependent regulation of RL production in this condition is a research area of great importance.

These examples of research perspectives show that the understanding of the molecular mechanisms involved in RL production under different conditions is of great importance for the development of better industrial processes for RL increased share of the surfactant market.

Conflict of interest

The authors declare that they do not have any conflict of interest.

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