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 surfactant, as well as some perspectives to improve these industrial useful strains.

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). Rhamnolipids (RL) are BS that are naturally produced by the opportunistic pathogen *Pseudomonas aeruginosa* and by some *Burkholderia* 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).

Rhamnolipids 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, Rhamnolipid, Inc. (USA), GlycoSurf (USA), TensioGreen (USA), AGAE technologies Ltd. (USA), 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 dTTP-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 (Solaïman 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.

| Rhamnolipids produced by Pseudomonas: an overview | 137 |
|-------------------------------------------------|-----|
| **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) |
| | P. chlororaphis | rhlC | MSM/glucose | 0.1 | Solaïman et al. (2015) |
| | P. putida KT2440 | P tac, rhlAB/rhlABC/rhlC | LB/glucose | 0.005 (mono-RL) | Wittgens et al. (2017) |
| | P. aeruginosa | P tac, estA | MSP/glycerol | 14.6 | Dobler et al. (2017) |
| | E. coli | P tac, 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) |
| | *Burkholderia 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 nativus (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 | P synthetidc, rhlAB | LB/glucose | 3.2 | Tiso et al. (2016) |
| | P. putida KT40C2C | P synthetidc, rhlAB | SupM/glucose | 14.9 | Beuker et al. (2016) |

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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-Ramirez 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 synthesized (Aguirre-Ramirez 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/iso- merase) play a central role in the synthesis of the CoA-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.
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 \( \text{N}-3\)-oxo-dodecanoyl-homoserine lactone (3O-C12-HSL) and \( \text{N}-\text{butyryl-homoserine lactone} \) (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 rhl, 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 rhl 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 rhl 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 rhl 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 understood.

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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 operon (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
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, mliBDAC* 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 *mliBDAC* operon is only expressed at a low level for LPS synthesis in this
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.

### References

Abdel-Mawgoud, A.M., Lépine, F., and Dėziel, E. (2014) A stereospecific pathway diverts β-oxidation intermediates to the biosynthesis of rhamnolipid biosurfactants. *Chem Biol 21*: 156–164.

Aguirre-Ramirez, M., Medina, G., González-Valdez, A., Grosso-Becerra, V., and Soberón-Chavez, G. (2012) *Pseudomonas aeruginosa* miBDC operon, encoding dTDP-L-rhamnose biosynthetic enzymes, is regulated by the quorum-sensing transcriptional regulator RhlR and the alternative sigma S factor. *Microbiology 158*: 908–916.
Rhamnolipids produced by Pseudomonas: an overview

Baldelli, V., D’Angelo, F., Pavoncello, V., Vita Fiscarelli, E., Visca, P., and Rampioni, G. (2020) Identification of FDA-approved antivirulence drugs targeting the Pseudomonas aeruginosa quorum sensing effector protein PqsE. *Virulence* **11**: 652–668.

Beuker, J., Barth, T., Steier, A., Wittgens, A., Rosenau, F., Henkel, M., and Hausmann, R. (2016) High titer heterologous rhamnolipid production. *AMB Expr* **6**: 124.

Blus-Kadosh, I., Zilka, A., Yerushalmi, G., and Banin, E. (2013) The effect of psts and phoB on quorum sensing and swarming motility in *Pseudomonas aeruginosa*. *PLoS One* **8**: e74444.

Borah, S.N., Goswami, D., Sarma, H.K., Cameotra, S.S., and Deka, S. (2016) Rhamnolipid biosurfactant against Fusarium verticillioides to control stalk and ear rot disease of maize. *Front Microbiol* **7**: 1505.

Brench, A., McFarland, K.A., McManus, H.R., Castang, S., Mogno, I., Dove, S.L., and Lory, S. (2009) The GacS/GacA signal transduction system of *Pseudomonas aeruginosa* acts exclusively through its control over the transcription of the RsmY and RsmZ regulatory small RNAs. *Mol Microbiol* **73**: 434–445.

Burrowes, E., Baysse, C., Adams, C., and O’Garra, F. (2006) Influence of the regulatory protein RsmA on cellular functions in *Pseudomonas aeruginosa* PA01, as revealed by transcriptome analysis. *Microbiology* **152**: 405–418.

Cabrera-Valladares, N., Richardson, A.-P., Olvera, C., Trevino, L.G., Dezele, E., Lépine, F., and Soberón-Chávez, G. (2006) Mono-rhamnolipid and 3-(3-hydroxyalkanoyloxy)alkanoic acids (HAAs) production using *Escherichia coli* as a heterologous host. *Appl Microbiol Biotechnol* **73**: 187–194.

Caiazzza, N.C., Shanks, R.M., and O’Toole, G.A. (2005) Rhamnolipids modulate swarming motility patterns in *Pseudomonas aeruginosa*. *J Bacteriol* **187**: 7351–7361.

Cao, H., Krishnan, G., Goumnerov, B., Tsongalis, J., Tompkins, R., and Rahme, L.G. (2001) A quorum sensing-associated virulence gene of *Pseudomonas aeruginosa* encodes a LysR-like transcription regulator with a unique associated virulence gene of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* **98**: 14613–14618.

Cao, L., Wang, Q., Zhang, J., Li, C., Yan, X., Lou, X., et al. (2012) Construction of a stable genetically engineered rhamnolipid-producing microorganism for remediation of pyrene-contaminated soil. *World J Microbiol Biotechnol* **28**: 2783–2790.

Chong, H., and Li, Q. (2017) Microbial production of rhamnolipids: Opportunities, challenges and strategies. *Microb Cell Fact* **16**: 1–12.

Coccoli-Yanez, M., Soto-Aceves, M.P., González-Váidez, A., Servín-González, L., and Soberón-Chávez, G. (2020) Virulence factors regulation by the quorum-sensing and Rsm systems in the marine strain *Pseudomonas aeruginosa* ID4365, a natural mutant in lasR. *FEMS Microbiol Lett* **367**: fnaa092.

Crespo-García, G., Grosso-Becerra, V., González-Valdez, A., Servín-González, L., and Soberón-Chávez, G. (2011) Transcriptional regulation of *Pseudomonas aeruginosa* rhlR: Role of the Crp-ortholog Vfr (virulence factor regulator) and quorum-sensing regulators LasR and RhlR. *Microbiology* **157**: 2545–2555.

Davey, M.E., Caiazza, N.C., and O’Toole, G.A. (2003) Rhamnolipid surfactant production affects biofilm architecture in *Pseudomonas aeruginosa* PAO1. *J Bacteriol* **185**: 1027–1036.

Déziel, E., Lépine, F., Milot, S., and Villermur, R. (2000) Mass spectrometry monitoring of rhamnolipids from growing cultures of *Pseudomonas aeruginosa* 57RP. *Biochim Biophys Acta* **1485**: 145–152.

Déziel, E., Lépine, F., Milot, S., and Villermur, R. (2003) rhlA is required for the production of a novel biosurfactant promoting swarming motility in *Pseudomonas aeruginosa* 3-(3-hydroxyalkanoyloxy)alkanoic acids (HAAs), the precursors of rhamnolipids. *Microbiology* **149**: 2005–2013.

Diggle, S.P., Winzer, K., Chhabra, S.R., Worrall, K.E., Cámara, M., and Williams, P. (2003) The *Pseudomonas aeruginosa* quinolone signal molecule overcomes the cell density-dependency of the quorum sensing hierarchy, regulates rhl-dependent genes at the onset of stationary phase and can be produced in the absence of LasR. *Mol Microbiol* **50**: 29–43.

Dobler, L., de Carvalho, B.R., Alves, Wd S, Neves, B.C., Freire, D.M.G., and Almeida, R.V. (2017) Enhanced rhamnolipid production by *Pseudomonas aeruginosa* overexpressing estA in a simple medium. *PLoS One* **12**: e0183857.

Dong, Y., Zhang, X.F., An, S.W., Xu, J.L., and Zhang, L.H. (2008) A novel two-component system BqsS-BqsR modulates quorum sensing-dependent biofilm decay in *Pseudomonas aeruginosa*. *Commun Integr Biol* **1**: 88–96.

Eggink, G., De Ward, P., and Huijberts, G.N.M. (1992) The role of fatty acid biosynthesis and degradation in the supply of substrates for poly (3-hydroxy-alkanoate) formation in *Pseudomonas putida*. *FEMS Microbiol Rev* **103**: 159–164.

Farrow, J.M., and Pisci, E.C. (2017) Distal and proximal promoters coregulate pqsR expression in *Pseudomonas aeruginosa*. *Mol Microbiol* **104**: 78–91.

Farrow, J.M., Sund, Z.M., Ellison, M.L., Wade, D.S., Coleman, J.P., and Pisci, E.C. (2008) PqsE functions independently of PqsR-*Pseudomonas* quinolone signal and enhances the rhl quorum-sensing system. *J Bacteriol* **190**: 7043–7051.

García-Reyes, S., Soberón-Chávez, G., and Coccoli-Yañez, M. (2020a) The third quorum-sensing system of *Pseudomonas aeruginosa*: *Pseudomonas quinolone signal* and the enigmatic PqsE protein. *J Med Microbiol* **69**: 25–34.

García-Reyes, S., Soto-Aceves, M.P., Coccoli-Yañez, M., González-Váidez, A., Servín-González, L., and Soberón-Chávez, G. (2020b) The outlier *Pseudomonas aeruginosa* strain ATCC 9027 harbors a defective LasR quorum-sensing transcriptional regulator. *FEMS Microbiol Lett* **367**: fnaa122.

Groleau, M.-C., De Oliveira, P.T., Dekime, V., and Deziel, E. (2020) PqsE is essential for RhlR-dependent quorum sensing regulation in *Pseudomonas aeruginosa*. *mSystems* **5**: e00532-20.

Grosso-Becerra, M.V., Croda-García, G., Merino, E., Servín-González, L., Mujica-Espinosa, R., and Soberón-Chávez, G. (2014) Regulation of *Pseudomonas aeruginosa*
virulence factors by two novel RNA-thermometers. *Proc Natl Acad Sci USA* **111**: 15562–15567.

Grosso-Becerra, M.V., González-Valdez, A., Granados-Martínez, M.J., Morales, E., Servín-González, L., Méndez, J.L., *et al.* (2016) *Pseudomonas aeruginosa* ATCC 9027 is a non-virulent strain suitable for mono-rhamnolipids production. *Appl Microbiol Biotechnol* **100**: 9995–10004.

Gunther, W.N., Nuñez, A., Fett, W., and Solaiman, D. (2005) Production of rhamnolipids by *Pseudomonas chlororaphis*, a nonpathogenic bacterium. *Appl Environ Microbiol* **71**: 2288–2293.

Gunther, W.N., Nuñez, A., Fortis, L., and Solaiman, D. (2006) Proteomic based investigation of rhamnolipid production by *Pseudomonas chlororaphis* strain NRRL B-30761. *J Ind Microbiol Biotechnol* **33**: 914–920.

Gutiérrez-Gómez, U., Servín-González, L., and Soberón-Chávez, G. (2019) Role of β-oxidation and de novo fatty acid synthesis in the production of rhamnolipids and poly-hydroxalkanoates by *Pseudomonas aeruginosa*. *Appl Microbiol Biotechnol* **103**: 3753–3760.

Gutiérrez-Gómez, U., Soto-Aceves, M.P., Servín-González, L., and Soberón-Chávez, G. (2018) Overproduction of rhamnolipids in *Pseudomonas aeruginosa* PA14 by redirection of the carbon flux from polyhydroxalkanoate synthesis and overexpression of the rhlAB-R operon. *Biotech Lett.* **40**: 1561–1566.

Henkel, M., Geissler, M., Weggenmann, F., and Hausmann, R. (2017) Production of microbial surfactants: status quo of rhamnolipid and surfactin towards large-scale production. *Biotechnol J.* **12**: 1–10. https://doi.org/10.1002/biot.201600561

Heurlier, K., Williams, F., Heeb, S., Dormond, C., Pessi, G., Singer, D., *et al.* (2004) Positive control of swarming, rhamnolipid synthesis, and lips production by the post transcriptional RsmA/RsmZ system in *Pseudomonas aeruginosa* PAO1. *J Bacteriol* **186**: 2936–2945.

Hokamura, A., Wakida, I., Miyahara, Y., Tsuge, T., Shiratsuchi, H., Tanaka, K., and Matussake, H. (2015) Biosynthesis of poly(3-hydroxybutyrate-co-3-hydroxyalkanoates) by recombinant *Escherichia coli* from glucose. *J. Biosci. Bioeng* **120**: 305–310.

Janssen, K.H., Diaz, M.R., Gode, C.J., Wolfgang, M.C., and Tahr, T.L. (2018) RsmV, a small noncoding regulatory RNA in *Pseudomonas aeruginosa* that sequesters RsmA that control virulence and biofilm formation. *J Bacteriol* **200**: e00277-18.

Jensen, V., Löns, D., Zouai, C., Breidenbruch, F., Meissner, A., Dieterich, G., and Haussler, S. (2006) RhlR expression in *Pseudomonas aeruginosa* is modulated by the Pseudomonas quinolone signal via PhoB-dependent and -independent pathways. *J Bacteriol* **188**: 8601–8609.

Johann, S., Seiler, T.-B., Tiso, T., Bluhm, K., Blank, L.M., and Hollert, H. (2016) Mechanism-specific and whole eco-toxicity of mono-rhamnolipids. *Sci Tot Environ* **549**: 155–163.

Kraemer, N.N., Wilks, J.C., Marlow, J.J., Coleman, M.L., and Newman, D.K. (2012) BqsR/BqsS constitute a two-component system that senses extracellular Fe (II) in *Pseudomonas aeruginosa*. *J Bacteriol* **194**: 1195–1204.

Kryachko, Y., Nalhoo, S., Lai, P., Voordouw, J., Prenner, E.J., and Voordouw, G. (2013) Prospects for using native and recombinant rhamnolipid producers for microbially enhanced oil recovery. *Int Biodeterior Biodegrad* **81**: 133–140.

Langenbach, S., Rehm, B.H.A., and Steinbüchel, A. (1997) Functional expression of the PHA synthase gene *phaC1* from *Pseudomonas aeruginosa* in *Escherichia coli* results in poly(3-hydroxyalkanoate) synthesis. *FEMS Microbiol Lett* **150**: 303–309.

Lapouge, K., Scubert, M., Allain, F.H., and Haas, D. (2008) Gac/Rsm signal transduction pathway of gamma-proteobacteria: from RNA recognition to regulation of social behavior. *Mol Microbiol* **67**: 241–253.

Lee, D.G., Urbach, J.M., Wu, G., Liberati, N.T., Feinbaum, R.L., Miyata, S., *et al.* (2006) Genomic analysis reveals that *Pseudomonas aeruginosa* virulence is combinatorial. *Genome Biol* **7**: R90.

Medina, G., Juarez, K., Diaz, R., and Soberon-Chavez, G. (2003a) Transcriptional regulation of *Pseudomonas aeruginosa* rhlR, encoding a quorum-sensing regulatory protein. *Microbiology* **149**: 3073–3081.

Medina, G., Juarez, K., and Soberon-Chavez, G. (2003b) The *Pseudomonas aeruginosa* rhlAB operon is not expressed during the logarithmic phase of growth even in the presence of its activator RhlR and the autoinducer N-butyryl-homoserine lactone. *J. Bacteriol* **185**: 377–380.

Miller, C.L., Romero, M., Karna, S.L., Chen, T., Heeb, S., and Leung, K.P. (2016) RsmW, *Pseudomonas aeruginosa* small non-coding RsmA-binding RNA upregulated in biofilm versus planktonic growth conditions. *BMC Microbiol* **16**: 155.

Morales, E., González-Valdez, A., Servín-González, L., and Soberón-Chávez, G. (2017) *Pseudomonas aeruginosa* quorum-sensing response in the absence of functional LasR and LasI proteins: The case of strain 148, a virulent dolphin isolate. *FEMS Microbiol Lett.* **364**: 1–9.

Morris, E.R., Hall, G., Li, C., Heeb, S., Kulkarni, R.V., Love-lock, L., *et al.* (2013) Structural rearrangement in an RsmA/CsrA ortholog of *Pseudomonas aeruginosa* creates a dimeric RNA-binding protein, RsmN. *Structure* **21**: 1659–1671.

Mukherjee, S., Moustafa, D.A., Stergioula, V., Smith, C.D., Goldberg, J.B., and Bassler, B. (2018) The PqsE and RhlR proteins are an autoinducer synthase-receptor pair that control virulence and biofilm development in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* **115**: E9411–E9418.

Müller, M.M., Hörmann, B., Kugel, M., Syladt, C., and Hausmann, R. (2011) Evaluation of rhamnolipid production capacity of *Pseudomonas aeruginosa* PAO1 in comparison to the rhamnolipid over-producer strains DSM 7108 and DSM 2875. *Appl Microbiol Biotechnol* **89**: 585–592.

Naughton, P.J., Marchant, R., Naughton, V., and Barat, I.M. (2019) Microbial biosurfactants: current trends and applications in agricultural and biomedicine industries. *J Applied Microbiol* **127**: 12–28.

Nitschke, M., Costa, S., and Contiero, J. (2011) Rhamnolipids and PHAs: recent reports on *Pseudomonas*-derived molecules of increasing industrial interest. *Process Biochem* **46**: 621–630.
Ochsner, U.A., Fiechter, A., and Reiser, J. (1994) Isolation, characterization, and expression in Escherichia coli of the Pseudomonas aeruginosa rhlAB genes encoding a rhamnosyltransferase involved in rhamnolipid regulation of las and rhl quorum sensing in Pseudomonas aeruginosa biofilm formation. J Biol Chem 269: 19787–19795.

Ochsner, U.A., Reiser, J., Fiechter, A., and Witholt, B. (1995) Production of Pseudomonas aeruginosa rhamnolipid biosurfactants in heterologous hosts. Appl Environ Microbiol 61: 3503–3506.

Olvera, C., Goldberg, J.B., Sánchez, R., and Soberón-Chávez, G. (1999) The Pseudomonas aeruginosa algC gene product participates in rhamnolipid biosynthesis. FEMS Microbiol. Lett. 179: 85–90.

Pesci, E.C., Milbank, J.B.J., Pearson, J.P., McKnight, S., Kende, A.S., Greenber, E.P., and Igleswski, B. (1999) Quinolone signaling in the cell-to-cell communication system of Pseudomonas aeruginosa. Proc Natl Acad Sci USA 96: 11229–11234.

Pesci, E.C., Pearson, J.P., Seed, P.C., and Igleswki, B.H. (1997) Regulation of las and rhl quorum sensing in Pseudomonas aeruginosa. J Bacteriol 179: 3127–3132.

Pessi, G., Williams, F., Hindle, Z., Heurler, K., Holden, M.T.G., Câmara, M., et al. (2001) The global posttranscriptional regulator RsmA modulates production of virulence determinants and N-acylhomoserine lactones in Pseudomonas aeruginosa. J Bacteriol 183: 6676–6683.

Rahim, R., Ochsner, U., Olvera, C., Graninger, M., Messner, P., Lam, J.S., and Soberón-Chávez, G. (2001) Cloning and functional characterization of the Pseudomonas aeruginosa rhlC gene that encodes rhamnosyltransferase 2, an enzyme responsible for di-rhamnolipid biosynthesis. Mol Microbiol 40: 708–718.

Read, R.C., Roberts, P., Munro, N., Rutman, A., Hastie, A., Shryock, T., et al. (1992) Effect of Pseudomonas aeruginosa rhamnolipids on mucociliary transport and ciliary beating. J Appl Physiol 72: 2271–2277.

Rehm, B.H.A., Kruger, N., and Steinhübel, A. (1998) A new metabolic link between fatty acid de novo biosynthesis and polyhydroxyalkanoic acid synthesis: the phaG gene from Pseudomonas putida KT2440 encodes a 3-hydroxyacylacyl carrier protein-coenzyme A transferase. J Biol Chem 273: 24044–24051.

Sancheti, A., and Ju, L.-K. (2019) Eco-friendly rhamnolipid based fungicides for protection of soybeans from Phytophthora sojae. Pest Manag Sci 75: 3031–3038.

Sekhon Randhawa, K.K., and Rahman, P.K. (2014) Rhamnolipid biosurfactants—past, present and future scenario of global market. Front Microbiol 5: 454.

Setoodeh, P., Jahmamri, A., Esamloueyan, R., Niazi, A., Ayatollahi, S.S., Aram, F., et al. (2014) Statistical screening of medium components for recombinant production of Pseudomonas aeruginosa ATCC 9027 rhamnolipids by nonpathogenic cell factory Pseudomonas putida KT2440. Mol Biotechnol 56: 175–191.

Soberón-Chávez, G., Aguirre-Ramírez, M., and Sánchez, R. (2005a) The Pseudomonas aeruginosa RhlA enzyme is not only involved in rhamnolipid, but also in polyhydroxyalkanoate production. J Ind Microbiol Biotechnol 32: 675–677.

Soberón-Chávez, G., Lépine, F., and Déziel, E. (2005b) Production of rhamnolipids by Pseudomonas aeruginosa. Appl Microbiol Biotechnol 68: 718–725.

Sodagari, M., and Ju, L.K. (2020) Addressing the critical challenge for rhamnolipid production: discontinued synthesis in extended stationary phase. Process Biochem 91: 83–89.

Soleiman, D.K.Y., Ashby, R.D., Gunther, N.W. IV, and Jerkowska, J.A. (2015) Dirhamnose-lipid production by recombinant non-pathogenic bacterium Pseudomonas chlororaphis. Appl Microbiol Biotechnol 99: 4333–4342.

Soto-Aceves, M.P., Cocoll-Yanez, M., Merino, E., Castillo-Juarez, I., Cortés-López, H., González-Pedrajo, B., et al. (2019) Inactivation of the quorum-sensing transcriptional regulators LasR or RhlR does not suppress the expression of virulence factors and the virulence of Pseudomonas aeruginosa PA01. Microbiology 165: 425–432.

Tavares, L.F., Silva, P.M., Junqueira, M., Mariano, D.C., Nogueira, F.C., Domont, G.B., et al. (2013) Characterization of rhamnolipids produced by wild-type and engineered Burkholderia kururiensis. Appl Microbiol Biotechnol 97: 1909–1921.

Tiso, T., Sabelhaus, P., Behrens, B., Wittgens, A., Rosenau, F., Hayen, H., and Blank, L.M. (2016) Creating metabolic demand as an engineering strategy in Pseudomonas putida rhamnolipid synthesis as an example. Metab Eng Commun 3: 234–244.

Tiso, T., Thies, S., Müller, M., Tsvetanova, L., Carraresi, L., Bröring, S., et al. (2017) Rhamnolipids: production, performance, and application. In Consequences of Microbial Interactions with Hydrocarbons, Oils, and Lipids; Production of Fuels and Chemicals. Handbook of Hydrocarbon and Lipid Microbiology. Lee, S. (ed.). Berlin: Springer International Publishing AG, pp. 587–622.

Toribio, J., Escalante, A.E., and Soberón-Chávez, G. (2010) Production of rhamnolipids in bacteria other than Pseudomonas aeruginosa. Eur J Lipid Sci Technol 112: 1082–1087.

Tremblay, J., Richardson, A.P., Lépine, F., and Déziel, E. (2007) Self-produced extracellular stimuli modulate the Pseudomonas aeruginosa swarming motility behavior. Environ Microbiol 9: 2622–2630.

Tripathi, L., Twigg, M.S., Zompra, A., Salek, K., Irorere, V.U., Gutierrez, T., et al. (2019) Biosynthesis of rhamnolipid by Marinobacter species expands the paradigm of biosurfactant synthesis to a new genus of marine microflora. Microb Cell Fact 18: 164.

Vakulskas, C.A., Potts, A.H., Babitzke, P., Ahmer, B.M.M., and Romeo, T. (2015) Regulation of bacterial virulence by Csr (Rsm) systems. Microbiol Mol Biol Rev 79: 193–224.

Ventre, I., Goodman, A.L., Vallet-Gely, I., Vasseur, P., Soscia, C., Molin, S., et al. (2006) Multiple sensors control reciprocal expression of Pseudomonas aeruginosa regulatory RNA and virulence genes. Proc Natl Acad Sci USA 103: 171–176.
Look who's talking: communication and quorum sensing in the bacterial world. *Phil Trans R Soc B* **362**: 1119–1134.

Wittgens, A., Kovacic, F., Müller, M.M., Gerlitzki, M., Santiago-Schubel, B., Hofmann, D., *et al.* (2017) Novel insights into biosynthesis and uptake of rhamnolipids and their precursors. *Appl Microbiol Biotechnol* **101**: 2865–2878.

Wittgens, A., Tiso, T., Arndt, T.T., Wenk, P., Hemmerich, J., Muller, C., *et al.* (2011) Growth independent rhamnolipid production from glucose using the non-pathogenic *Pseudomonas putida* KT2440. *Microbial Cell Fact* **10**: 80.

Xiao, G., Déziel, E., He, J., Lépine, F., Lesic, B., Castonguay, M.-H., *et al.* (2006) MvfR, a key *Pseudomonas aeruginosa* pathogenicity LTTR-class regulatory protein, has dual ligands. *Mol Microbiol* **62**: 1689–1699.

Zhao, F., Zhou, J., Han, S., Ma, F., Zhang, Y., and Zhang, J. (2016) Medium factors on anaerobic production of rhamnolipids by *Pseudomonas aeruginosa* SG and a simplifying medium for in situ microbial enhanced oil recovery applications. *World J Microbiol Biotechnol* **32**: 54.

Zhu, K., and Rock, C.O. (2008) RhlA converts β-hydroxyacyl-acyl carrier protein intermediates in fatty acid synthesis to the β-hydroxydecanoyl-β-hydroxycaprate component of rhamnolipids in *Pseudomonas aeruginosa*. *J Bacteriol* **190**: 3147–3154.