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Review

Gene regulation of rhamnolipid production in *Pseudomonas aeruginosa* – A review

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ABSTRACT

Pseudomonas aeruginosa produces abundant levels of rhamnolipid biosurfactants which exhibit remarkable chemical and physical characteristics, making these compounds attractive targets for biotechnology research. The complex gene regulation network involved in rhamnolipids' biosynthesis represents a challenge to industrial production, which has been the object of a growing number of studies. This article provides a comprehensive review of the known gene regulatory factors involved in rhamnolipid production within *P. aeruginosa*. The regulatory factors include quorum sensing systems proteins and environmental response, and global regulatory systems within basal bacterial physiology, acting either at transcriptional or post-transcriptional level. The multilayer gene regulation responds to a wide variety of environmental and physiologic signals, and is capable of combining different signals in unique and specific responses.

1. Introduction

Rhamnolipids are glycolipids containing L-rhamnose and β-hydroxy fatty acids moieties. These surfactants have shown outstanding potential as commercial biosurfactants due to their unique chemical characteristics, and in future may serve as replacement for synthetic surfactants. However, several improvements are still required to reach a competitive biotechnological production. This review is the first compilation of the genetic regulation of rhamnolipid production in *Pseudomonas aeruginosa*, and may be used as a reference for further studies focusing on improving the biotechnological production of rhamnolipid using *P. aeruginosa*.

Industrial applications of rhamnolipids include emulsification, detergency, wetting, foaming, dispersing or solubilization, antimicrobial, and antiadhesive activities, in different areas, from bioremediation to food additives (Banat et al., 2000). Biosurfactants are remarkably promising in bioremediation of oil-impacted environments due to their high biodegradability and also to their potential in replacing synthetic surfactants (Sponza and Gök, 2010). Rhamnolipids produced by *P. aeruginosa* are among the most effective surfactants in removing hydrophobic compounds from contaminated soils (Santa Anna et al., 2007). Currently, the estimated cost of biotechnologically produced rhamnolipids, in a 20–100 m^3 scale, ranges from 5 to 20 USD/kg^{-1}, while production costs for chemical surfactants, such as ethyloxylate or alkylpolyglucoside, are between 1 and 3 USD/kg^{-1} (Lang and Wullbrandt, 1999). By 2010, at least one company, Rhamnolipid Inc. in St. Petersburg, USA, was known to commercially produce rhamnolipids and, according to Leitermann et al. (2010), the actual price for rhamnolipids ranges between 200 (20% solution), and 6000 USD/kg^{-1} (98% pure). In order to make rhamnolipid production competitive in the surfactant market, it is essential to reduce production costs and substantially increase production rates.

P. aeruginosa is the third most common nosocomial pathogen and is associated with chronic and eventually fatal lung disease in cystic fibrosis patients (Brencic et al., 2009). This bacterium produces several virulence factors including rhamnolipids, biofilm formation, and antibiotic resistance (Girard and Bloemberg, 2008), which contribute to its great adaptability to a wide range of habitats, such as water, soil, plants and human hosts.

The role of rhamnolipids in bacterial physiology is not yet fully understood. There is some evidence of rhamnolipid participation in physiologic processes, including (1) assimilation of insoluble substrates, especially hydrocarbons, as well as changing the hydrophobicity of the cell surface (Al-Tahhan et al., 2000); (2) antimicrobial activity (Wang et al., 2005); (3) hemolytic activity in human pathogenesis (Fujita et al., 1988); (4) solubilization of *Pseudomonas*

Abbreviations: AHL, acyl homoserine lactones; EC, enzyme commission; EPS, extracellular polysaccharide; FASII, fatty acid synthase-II; HAA, 3-(3-hydroxyalkanoyloxy) alkanoic acid; HAQ, 4-hydroxy-2-alkylquinolone; LPS, lipopolysaccharide; PQS, *Pseudomonas* quinolone signal; QS, quorum sensing; RhlA, transacylase that directly utilizes β-hydroxydecanoyl-ACP intermediates in fatty acid synthesis to generate the HAA portion of rhamnolipids; RhlB, rhamnosyltransferase I; RhlC, rhamnosyltransferase II; RmlA, glucose-1-phosphate thymidylyltransferase (EC 2.7.7.24); RmlB, dTDP-D-glucose 4,6-dehydratase (EC 4.2.1.46); RmlC, dTDP-4-keto-6-deoxy-D-glucose 3,5-epimerase (EC 5.1.3.13); RmlD, dTDP-4-keto-6-deoxy-L-mannose reductase (EC 1.1.1.133).

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quinolone signal (PQS) – a quorum sensing molecule; and (5) promotion of swarming motility (Deziel et al., 2003).

Understanding the mechanisms that control rhamnolipid production in *P. aeruginosa* is a fundamental step towards increasing its production on an industrial scale, and to make it competitive with their synthetic counterparts. In this review we present the current understanding of the genetic regulation of the production of rhamnolipids in *P. aeruginosa*, and highlight the environmental regulatory factors involved in this process.

2. Biosynthesis of rhamnolipids

P. aeruginosa produces two major types of rhamnolipid in liquid cultures: the monorhamnolipid, rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate (Rha-C_{10}-C_{10}) and the dirhamnolipid, rhamnosyl-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate (Rha-Rha-C_{10}-C_{10}) (Deziel et al., 2000). However, 25 rhamnolipid congeners have been described in *P. aeruginosa*, varying in chain length and/or extent of saturation, showing that the addition of a hydrocarbon chain to dTDP-L-rhamnose is not specific to the carbon chains (Deziel et al., 1999). Rhamnolipids biosynthesis occurs through three sequential reactions. RhlA catalyzes the synthesis of the fatty acid dimer moiety of rhamnolipids and free 3-(3-hydroxyalkanoyloxy) alkanoic acid (HAA), while rhamnosyltransferases RhlB and RhlC catalyse the transfer of dTDP-L-rhamnose to either HAA, or a previously generated mono-rhamnolipid, respectively (Deziel et al., 2003). Free HAAs also show surface-tension activities and have been directly related to the promotion of swarming motility (Deziel et al., 2003). Recent studies suggest that RhlA is responsible for diverting the β-hydroxydecanoyl-ACP intermediate from the FASII cycle (bacterial fatty acid synthesis system, reviewed by López-Lara and Geiger (2010)), by directly competing with FabA and FabI for this intermediate (Zhu and Rock, 2008). In addition, RhlA is the only protein required to convert two molecules of β-hydroxyacyl-ACP into an HAA. The diversion of the β-hydroxydecanoyl-ACP intermediate from the FASII cycle providing a substrate for the enzyme RhlAB to produce the hydrocarbon chain in the rhamnolipid molecule is an important step, both biotechnologically and clinically, but is still not fully understood.

2.1. Biosynthesis of L-rhamnose

Rhamnose is a deoxy-hexose sugar widely found in bacteria and plants, but not in humans. The activated L-rhamnose is derived from a glucose scaffold in four sequential steps, yielding deoxythymidine di-phospho (dTDP)-L-rhamnose. The first enzyme in the dTDP-L-rhamnose pathway is glucose-1-phosphate thymidylyltransferase (RmlA, EC 2.7.7.24) which catalyzes the transfer of a thymidylmonophosphate nucleotide to glucose-1-phosphate. The catalytic activity of RmlA is allosterically regulated by the final product of the pathway, dTDP-L-rhamnose (Blankenfeldt et al., 2000b), which makes RmlA the regulatory sensor for the whole downstream pathway. RmlA is a homotetramer with the monomer consisting of three functional domains: one core domain that shares the sequence similarity with nucleotidyltransferases, and two other domains that contain the recognition and binding sites for the nucleotide and sugarphosphate (Blankenfeldt et al., 2000b). The second enzyme, dTDP-D-glucose 4,6-dehydratase (RmlB, EC 4.2.1.46), catalyzes an oxidation of the C4 hydroxyl group of the D-glucose residue, followed by dehydration, leading to the formation of dTDP-4-keto-6-deoxy-D-glucose (Allard et al., 2001). The third enzyme, dTDP-4-keto-6-deoxy-D-glucose 3,5-epimerase (RmlC, EC 5.1.3.13), catalyzes a double epimerization reaction at the C3 and C5 positions of the 4-keto-6-deoxy-D-glucose ring (Graninger et al., 1999). Finally, dTDP-4-keto-6-deoxy-L-mannose reductase (RmlD, EC 1.1.1.133) reduces the C4 keto group of the 4-keto-6-deoxy-L-mannose moiety and leads to the formation of dTDP-L-rhamnose (Graninger et al., 1999). All four enzyme genes are organized as a single operon in *P. aeruginosa*, called *rmlBDAC*.

2.2. dTDP-L-rhamnose and the regulation of rhamnolipids biosynthesis

The dTDP-L-rhamnose pathway plays a pivotal role in the biosynthesis of rhamnolipids, since it represents the reactive molecular form of rhamnose to compose the sugar moiety of these glycolipids. dTDP-L-rhamnose allosterically inhibits the enzyme RmlA upstream in the pathway, therefore reducing its own availability (Blankenfeldt et al., 2000a). In addition, dTDP-L-rhamnose is also channeled to other L-rhamnose-containing extracellular structures such as EPS and LPS. On the other hand, the heterologous production of monorhamnolipids by *Escherichia coli* expressing *rhlAB* operon from *P. aeruginosa* was shown to be limited by dTDP-L-rhamnose availability since only the coexpression of *rhlAB* and *rmlBDAC* operons resulted in increased rhamnolipid production. However, it was lower than its production by *P. aeruginosa* (Cabrera-Valladares et al., 2006). Cabrera-Valladares and colleagues also showed that, in the recombinant *E. coli* expressing *rhlAB* and *rmlBDAC* operons, rhamnolipid production was increased after supplementation with oleic acid, suggesting that fatty acid substrates became a limiting factor for rhamnolipid production in this recombinant strain.

The *rhlAB* operon is transcriptionally and posttranscriptionally regulated by several factors, often related to the quorum sensing system, and seems to be the key point at which rhamnolipid production is controlled in *P. aeruginosa*.

2.3. The role of quorum sensing systems

The quorum sensing (QS) system is a bacterial communication system characterized by the secretion and detection of signal molecules – autoinducers – within a bacterial population, producing coordinated behaviors, after the establishment of a quorum. QS is a global regulatory system found in most bacterial species controlling several and diverse biologic functions, such as virulence, biofilm formation, bioluminescence and bacterial conjugation (Williams and Camara, 2009). The main components of a quorum sensing system are the QS signal synthase, the signal receptor (regulatory protein), and the signal molecule (Williams, 2007). The complex autoinducer/regulatory protein modulates the activity of the QS-regulated genes. There are two known complete conventional QS systems in In *P. aeruginosa*, *las* and *rhl*. The synthases LasI and RhlI produce the homoserine lactones 3OC12-HSL and C4-HSL, respectively, which complex with their correspondent transcriptional regulators, LasR and RhlR, to modulate the transcription of 5–10% of the entire *P. aeruginosa* genome (Dekimpe and Deziel, 2009). A third distinct QS system is formed by the transcriptional factor PqsR (also called MvfR) (Cao et al., 2001), responsible for activating the gene clusters *phnAB* and *pqsABCDE* both required for the production of 4-hydroxy-2-alkylquinolones (HAQs) and *Pseudomonas* quinolone signal (PQS), which is known to influence the production of QS-dependent factors, such as elastase, pyocyanin, PA-1L lectin, and rhamnolipids (Deziel et al., 2005).

It is clear that some QS-regulated genes belong specifically to *rhl* regulon, such as *rhlAB* (rhamnolipids biosynthesis), *lecA* (lectin), *hcnABC* (HCN production) and *phz*ABCDEFG operons (phenazine biosynthesis) (Schuster and Greenberg, 2007). However, there is no evidence of consensus LasR binding site sequence in any promoter region of LasR-regulated gene, and, moreover, most of QS-regulated factors are in some extension influenced by both LasR and RhlR, suggesting that LasR regulation is indirect for some genes (Schuster and Greenberg, 2007). D'Argenio et al. (2007) and colleagues, established that *rhlI* transcription is activated by LasR-3OC12-HSL and that the *rhl* system is *las*-dependent. However,