Rhamnolipids: Detection, Analysis, Biosynthesis, Genetic Regulation, and Bioengineering of Production

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Contents

Abstract As promising biotechnological products, rhamnolipids (RLs) are the most investigated biosurfactants. Over the years, important efforts have been spent and an array of techniques has been developed for the isolation of producing bacterial strains and the characterization of a large variety of RL homologs and congeners. Investigations on RL production by the best known producer, the opportunistic pathogen *Pseudomonas aeruginosa*, have shown that production of RLs proceeds through de novo biosynthesis of precursors. Over the last 15 years, the genetic details underlying RL production in *P. aeruginosa* have been mostly unraveled, revealing a complex regulatory mechanism controlled by quorum sensing pathways of intercellular communication. A number of nutritional and

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cultivation factors affecting RL productivity have also been identified, while the use of many affordable and renewable raw substrates has been described to optimize the production. Multidisciplinary approaches are increasingly adopted to develop methods for the safe, cost-effective, and highly efficient production of RLs at the industrial scale.

1   Introduction and Overview

Rhamnolipids (RLs), the glycolipid biosurfactants produced mainly by *Pseudomonas aeruginosa*, are the most intensively studied biosurfactants. This arises from two contrasting facts. First, they display relatively high surface activities and are produced in relatively high yields after relatively short incubation periods by a well-understood, easy to cultivate microorganism. Second, they are one of the virulence factors contributing to the pathogenesis of *P. aeruginosa* infections, and consequently, many aspects of RL biosynthesis have been investigated, in part, to control their production and effects.

The discovery of RLs dates back to 1946 when Bergström et al. reported an oily glycolipid produced by *Pseudomonas pyocyanea* (now *P. aeruginosa*) grown on glucose. This substance was named pyolipic acid and its structural units were identified as L-rhamnose and β-hydroxydecanoic acid (Bergström et al. 1946a, b; Hauser and Karnovsky 1954; Jarvis and Johnson 1949). The exact chemical nature of these biomolecules was unraveled by Jarvis and Johnson (1949) followed by Edwards and Hayashi (1965). Since then, extensive investigations have been conducted covering various aspects of RL research.

Numerous research teams have contributed to decipher the biosynthetic pathway of RLs (Burger et al. 1963; Déziel et al. 2003; Hauser and Karnovsky 1957, 1958; Rehm et al. 2001; Soberón-Chávez 2004; Zhu and Rock 2008). This was done in conjunction with efforts to identify the genes responsible for RL production, both at the enzymatic (Ochsner et al. 1994a; Rahim et al. 2000, 2001; Rehm et al. 2001; Zhu and Rock 2008) and regulatory (Ochsner et al. 1994b; Pearson et al. 1997; Pesci et al. 1997) levels.

These advancements were made possible largely because of the major efforts conducted on the development of versatile and accurate methods for RL detection and analysis (Déziel et al. 2000; Gartshore et al. 2000; Heyd et al. 2008; Mata-Sandoval et al. 1999; Price et al. 2009; Rendell et al. 1990; Schenk et al. 1995; Siegmund and Wagner 1991). These investigations revealed a large diversity of RL congeners and homologs produced by various *P. aeruginosa* strains under many different culture conditions and also from other bacterial species (Abdel-Mawgoud et al. 2010; Dubeau et al. 2009; Ochsner et al. 1994a, b; Van Gennip et al. 2009).

Another line of research is devoted to understanding the role of these biomolecules for the producing microorganisms as well as their interactions with other biological systems, especially the human body (Abdel-Mawgoud et al. 2010; Van Hamme et al. 2006). One of these roles is to promote the uptake of poorly soluble

hydrocarbons (Koch et al. 1991). Other physiological functions include the control of the bacterial cell surface hydrophobicity for attachment and detachment on different substrates (Al-Tahhan et al. 2000; Arino et al. 1998a; Sotirova et al. 2009; Yuan et al. 2007; Zhong et al. 2007, 2008), and the enhancement and modulation of surface motility (Caiazza et al. 2005; Déziel et al. 2003; Köhler et al. 2000; Tremblay et al. 2007).

Studies about the interactions of RL with other biological systems are numerous. The antibacterial (Abalos et al. 2001; Bergström et al. 1946b; Haba et al. 2003b; Lang et al. 1989; Onbasli and Aslim 2008; Shen et al. 2009; Sotirova et al. 2008; Yilmaz and Sidal 2005), antifungal (Kim et al. 2000; Yoo et al. 2005), antiviral (Cosson et al. 2002; Remichkova et al. 2008), antiphytopathogenic (De Jonghe et al. 2005; Haferburg et al. 1987; Kim et al. 2000; Nielsen et al. 2005, 2006), and algicidal (Wang et al. 2005) properties of RLs have been extensively investigated. RLs released by *P. aeruginosa* have long been known as the heat-stable extracellular hemolysin (Fujita et al. 1988; Johnson and Boese-Marrazzo 1980; Kurioka and Liu 1967; Sierra 1960) and more recently, a RL congener produced by *Burkholderia pseudomallei* was shown to display hemolytic and cytotoxic activities (Häussler et al. 1998, 2003).

Because of their excellent surface activity, the physicochemical properties of RLs have received considerable interest (Abalos et al. 2001; Abdel-Mawgoud et al. 2009; Chen 2004; Cohen and Exerowa 2007; Cohen et al. 2004; Haba et al. 2003b; Hansen et al. 2008; Ochoa-Loza et al. 2001; Ozdemir and Malayoglu 2004; Ozdemir et al. 2004; Pornsunthorntawee et al. 2009). Due to their hydrocarbon-solubilizing properties, they also have been used in the fields of bioremediation and biodegradation (Arino et al. 1998a; Asci et al. 2007, 2008; Avramova et al. 2008; Beal and Betts 2000; Benincasa 2007; Cameotra and Singh 2009; Cho et al. 2004; Churchill et al. 1995).

The potential industrial and biotechnological applications of RLs are thus quite diverse (Singh et al. 2007). RLs have been used for the synthesis and stabilization of nanoparticles (Palanisamy and Raichur 2009; Xie et al. 2006), the preparation of microemulsion (Nguyen and Sabatini 2009; Xie et al. 2007), as an antiagglo-meration agent (York and Firoozabadi 2008), as dispersing agent (Raichur 2007; Tripathy and Raichur 2008), in cleaning soap mixtures (Ecover™ products) and as a source of rhamnose (Linhardt et al. 1989).

Clinical testing of RLs as pharmacoactive compounds has been performed. Some successful trials proved the potential applications of RLs for the treatment of ulcers (Piljac et al. 2008) and of full-thickness wounds (Stipcevic et al. 2006).

These promising properties and potential application of RLs have encouraged researchers to improve the production of RLs, using industrially safe and more affordable processes in order to reduce production costs, which currently restrict the competitiveness of RLs vis-à-vis petroleum-derived surfactants. This goal has been sought through different approaches. First, many attempts have been made to isolate RL producers other than the opportunistic pathogen *P. aeruginosa* (Abouseoud et al. 2008; Celik et al. 2008; Chang et al. 2005; Christova et al. 2004; Gunther et al. 2005; Rooney et al. 2009) or to transfer the genes responsible

for RL production into more industrially safe heterologous hosts, such as *Escherichia coli* (Cabrera-Valladares et al. 2006; Ochsner et al. 1994a). Second, important efforts have been dedicated to the identification of low-cost and renewable raw material as production substrates, such as agroindustrial wastes (Nitschke et al. 2005; Rahman et al. 2002). Finally, an even production of RLs through pure chemical synthesis was also reported (Bauer et al. 2006; Duynstee et al. 1998; Howe et al. 2006).

As highlighted in this brief overview, it would be difficult to present all domains and aspects of RL research in one chapter. Therefore, our aim here is to provide a description of the chemical nature of RLs and to mention the different methods of RL detection and analysis. This chapter aims also to discuss the biosynthetic pathways of different RL precursors and describe the network of genetic regulation controlling their biosynthesis. Finally, different modalities of fermentative production of RLs on large scale are described, with an account of the associated problems and approaches to overcome them.

2 Rhamnolipid Structure, Detection, and Analysis

RLs are among the best studied biosurfactants. As several methods for their detection and analysis have been developed, their structure and characteristics are largely known.

2.1 Structure

RLs are glycosides that are composed, of a glycon part and an aglycon part linked to each other via O-glycosidic linkage.

The glycon part is composed of one (for mono-RLs) or two (for di-RLs) rhamnose moieties linked to each other through α-1,2-glycosidic linkage (Edwards and Hayashi 1965). The 2-hydroxyl group of the distal (relative to the glycosidic bond) rhamnose group remains generally free, although in some rare homologs it can be acylated with a long chain alkenoic acid (Yamaguchi et al. 1976).

The aglycon part, however, is composed of mainly one or two [in few cases, three (Andrä et al. 2006)] β-hydroxyfatty acid chains. These fatty acid chains are most commonly saturated or, less abundantly, mono- or polyunsaturated. Their chain lengths vary from C_8 to C_{16} (Abalos et al. 2001; Déziel et al. 1999a, 2000). These fatty acid chains are linked to each other through an ester bond formed between the β-hydroxyl group of the distal (relative the sugar part) chain with the carboxyl group of the proximal chain (Fig. 1). In most cases, the carboxyl group of the distal β-hydroxyfatty acid chain remains free. However, few homologs have this group esterified with a short alkyl group (Hirayama and Kato 1982). Figure 1 displays the structure of the best known RL congener, α-L-rhamnopyranosyl-α-L-
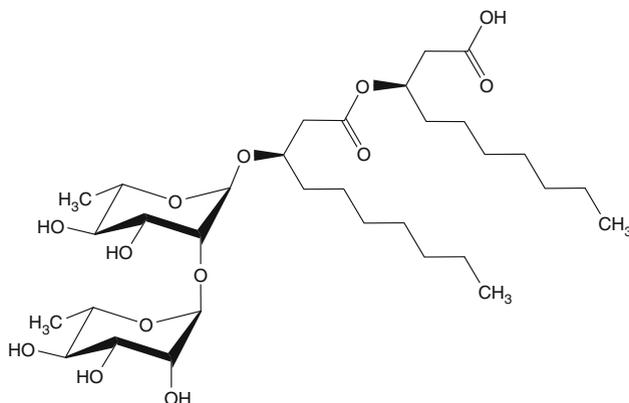
Fig. 1 Chemical structure of the first identified rhamnolipid; known as α-L-rhamnopyranosyl-α-L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate (Rha-Rha-C_{10}-C_{10}). Its full IUPAC name is (R)-3-{(R)-3-[2-O-(α-L-rhamnopyranosyl)-α-L-rhamnopyranosyl]oxydecanoyl} oxydecanoate; Or the synonym name: (R)-3-((R)-3-((2R,3R,4R,5R,6S)-4,5-dihydroxy-6-methyl-3-((2S,3R,4R,5R,6S)-3,4,5-trihydroxy-6-methyltetrahydro-2H-pyran-2-yloxy)tetrahydro-2H-pyran-2-yloxy)decanoyloxy)decanoic acid

rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate, which is typically symbolized as Rha-Rha-C_{10}-C_{10}.

The stereochemical configuration of the β-hydroxy groups of the fatty acid chains is in the R-configuration (Bauer et al. 2006; Schenk et al. 1997).

To date, about 60 different RL congeners and homologs have been reported, as recently reviewed by Abdel-Mawgoud et al. (2010). While *P. aeruginosa* synthesizes a mixture of mono- and di-RLs with hydroxyacyl moieties mostly from C_8 up to C_{12}, species from the *Burkholderia* genus produce principally di-RLs with two rhamnose units and mainly C_{14} hydroxy acyl chains.

2.2 Methods of Detection and Analysis

Several methods with variable precision and purposes are available for the detection and analysis of RLs.

2.2.1 Qualitative Methods

The most widely used method for qualitative, high throughput screening of RL-producing bacterial strains is the cetyltrimethylammonium bromide (CTAB) agar test (Pinzon and Ju 2009a; Siegmund and Wagner 1991). In this method, the anionic RLs form an insoluble complex with this cationic bromide salt, and the complex is revealed using methylene blue present in the agar. The RL-producing strains are

revealed by a dark blue halo around the colony, allowing for facile identification of the presence of RLs.

Another indirect way to detect RLs is based on their hemolytic properties. This approach can be performed in solution, using an erythrocyte suspension to which the RL solution is added. After a predetermined time, the residual erythrocytes are removed by centrifugation and the hemoglobin released is measured at 540 nm (Johnson and Boese-Marrazzo 1980). Alternatively, RL production can be tested using blood agar plates on which the bacteria are directly inoculated. Formation of a halo around the colony can be indicative for the presence of RLs (Carrillo et al. 1996). However, because bacteria can secrete other hemolytic factors such as proteases, this test often leads to false positive results (Siegmund and Wagner 1991) and is less reliable than those based on the tensioactive effects of RLs, such as those described below.

The drop collapsing test (Jain et al. 1991) is a sensitive method for the rapid screening of RL production by various isolates. This assay consists of applying a drop of a bacterial culture supernatant to be tested over a polystyrene plate containing shallow wells covered with oil. The droplet will spread over the oil only if the culture supernatant sample contains RLs. A similar approach is used in the oil spreading test, in which a drop of bacterial supernatant is added on top of an oil/water interface (Morikawa et al. 2000). The presence of a surface-active molecule will cause the oil to be repelled, forming a clearing zone whose diameter can be correlated with the activity of the tensioactive compounds in the supernatant.

A more precise approach based on the tensioactive properties of RLs is the direct measurement of surface tension of culture broths. This method is typically performed with a duNouy-type tensiometer, which measures the force required to pull a thin metal ring out of the surface of the solution. The measurement of the surface tension after sequential dilution of the solution gives the concentration at which the surface tension starts to increase and provides the Critical Micelle Concentration (CMC), which is specific to each surfactant. Thus, the degree of dilution required to attain the CMC allows for the quantification of the surfactant in the initial solution (CMD – Critical Micelle Dilution). However, this method suffers from some drawbacks, as it is time-consuming and not applicable to high-throughput screenings. In addition, as for all the previous indirect tests based on surface tension, it will be affected by the presence of tensioactive compounds other than RLs.

2.2.2 Quantitative Methods

The quantification of RLs can be performed through different strategies.

Spectrophotometric Methods

One of the most widely used methods for RL quantification is the orcinol test. It consists of heating the solvent-obtained extracts of culture supernatants in the

presence of sulfuric acid and orcinol (1,3-dihydroxy-5-methylbenzene). The rhamnose groups of RL are hydrolyzed and transformed into methyl furfural, which then reacts with the orcinol to produce a blue-green color that can be measured spectrophotometrically at 421 nm (Chandrasekaran and BeMiller 1980; Koch et al. 1991). A standard curve is prepared with rhamnose, or preferably with a standard RL mixture, for quantification. When rhamnose is used for building up the calibration curve, a correction factor must be applied to compensate for the extra mass of the lipidic portion of RLs. Déziel et al. (2000) calculated a correction factor of 2.25. One problem with this approach is that the results will vary with the proportion of mono- to di-RLs in the culture to be analyzed. A variation of the orcinol test uses anthrone (9,10-dihydro-9-oxoanthracene) instead of orcinol to create a dye that can be quantified at 625 nm (Helbert and Brown 1957; Hodge and Hofreiter 1962).

A quantitative method based on the interaction of methylene blue, CTAB, and RLs, as illustrated in the CTAB agar test, was described recently. It involves extracting the RLs in chloroform to which is added the two other chemicals, and the complex formed is detected at 638 nm (Pinzon and Ju 2009b).

Chromatographic Methods

The different approaches for RL measurement based on chromatographic procedures are presented.

Thin Layer Chromatography

One of the problems of RL quantification is that these compounds are produced as complex mixtures of congeners (see below), in a medium that may contain many other interfering compounds. RLs can be somewhat purified by simple extraction methods, taking advantage of the fact that they are acidic and thus that they will remain in the aqueous phase in basic medium, while being extractable by relatively nonpolar solvents such as ethyl acetate or ethyl ether after acidification of the aqueous solution. Nevertheless, such crude extracts are seldom pure enough to gravimetrically quantify only RLs present in the broth. Thus, this requires a preliminary separation step prior to quantification.

One such method is thin-layer chromatography (TLC). In normal phase, the polar stationary phase (silica gel) is eluted with a relatively polar mobile phase, for instance, chloroform:methanol:20% aqueous acetic acid (65:15:2) (Koch et al. 1991). This allows for straightforward separation of mono- from the more polar and later eluting di-RLs. Alternatively, a reverse phase TLC method has been developed in which the stationary phase is a hydrophobic C8 matrix eluted with methanol:water:trifluoroacetic acid (90:10:0.25) (deKoster et al. 1994). With this approach, RLs congeners are separated according to the length of their alkyl chains. Once the separation is completed, RLs can be visualized using the orcinol test (Koch et al. 1991), with reagents specific for sugars or fatty acids or with reagents that are used to reveal most organic compounds on TLC such as the "ceric dip" (Mechaly et al. 1997). Densitometric analysis of the revealed spots can be performed for more quantitative data (Matsufuji

et al. 1997), but this approach is not very sensitive compared to those mentioned below. As an alternative, direct mass spectrometric analysis of the eluted TLC plates can be performed using Fast Atom Bombardment (FAB) to ionize the RLs prior to mass analysis (deKoster et al. 1994). Although this method provides good structural information, it is not suitable for quantification purposes.

Gas Chromatography

Because of their relatively high molecular weights, RLs cannot be directly analyzed by gas chromatography (GC). Typically, prior to analysis, RLs are thus hydrolyzed with acid or with a strong base, their acid groups are modified into methyl esters (Van Dyke et al. 1993), and optionally, the hydroxyl groups are further transformed into a trimethylsilyl (TMS) ether (Arino et al. 1996). Rhamnose can be analyzed by GC as a TMS derivative (Arino et al. 1996). The various 3-hydroxyfatty acids are then identified and quantified using flame ionization detection (FID) or mass spectrometry (MS), using standards to determine their retention times and response factors. The main problem with GC analysis is that the relationship between the 3-hydroxyfatty acids in the dilipid portion of the RLs is lost, along with the relationship between the dilipids and their substitution with one (or two) rhamnose moieties.

Liquid Chromatography

High Performance Liquid Chromatography (HPLC) is especially well-suited for RL analysis. It is generally performed using C8 or C18 reverse-phase columns with a water/acetonitrile gradient. However, because they only absorb UV at very short wavelengths, RL detection is problematic. One approach is to derivatize them with *para*-bromoacetophenone in order to produce the corresponding *para*-bromophenacyl esters, which can be detected at 265 nm (Schenk et al. 1995). Alternatively, an Evaporative Light Scattering (ELS) detector, which rapidly evaporates the solvent and monitors the diffraction of a beam of light by the analyte, has been used on occasions (Arino et al. 1996; Noordman et al. 2000). With either type of detection, the main problem is the lack of standards to identify each of the numerous RL congeners present in the culture medium. This can be overcome by using a mass spectrometer as detector.

Liquid Chromatography Coupled to Mass Spectrometry

Direct coupling of reverse phase liquid chromatography to a mass spectrometer provides the advantages of characterizing a given RL congener by its retention time along with its mass spectral signature. This is normally done by splitting the flow coming from the HPLC using a splitter that conveys only a fraction of the eluent into the mass spectrometer. Electrospray Ionization (ESI), and sometimes Atmospheric Pressure Chemical Ionization (APCI), has been mostly used to ionize RLs prior to mass analysis (Benincasa et al. 2004; Déziel et al. 1999b, 2000; Haba et al. 2003a; Monteiro et al. 2007). In negative ESI, the molecular weight of the

pseudomolecular ion [M-H]$^-$ can be directly obtained. This provides some information on the nature of the RL congener eluting from the column at that retention time. In order to improve ionization, ammonium acetate is added to both solvents of the water/acetonitrile gradient (Déziel et al. 1999b, 2000).

Fragmentation of the pseudomolecular ion using MS/MS analysis of the parent ion can provide further structural information, if required. For example, this approach allows the discrimination of Rha-C_{10}-C_8 from the isomeric Rha-C_8-C_{10}, even though they are not chromatographically resolved (Déziel et al. 1999b).

The ability to predict the compound eluting at a given retention time permits quantification, even if the corresponding compound is not available as a pure standard. The response factor of mono-RLs differs from that of the di-RL congeners (Déziel et al. 1999b). But within the same family, the molar response factor is very similar, thus allowing for quantification of all the members of such a family of congeners if one member of the family can be purified. Quantification of a given congener can be performed by integrating the intensity of the peak occurring at the correct retention time in the ion chromatograph of the corresponding pseudomolecular ion. Another alternative is to perform a MS/MS experiment in which a given pseudomolecular ion is fragmented and only one of its fragments is monitored. This approach, called Multiple Reaction Monitoring (MRM), increases the signal-to-noise ratio of the analysis, thus providing a lower limit of detection.

To perform quantification of RLs, an internal standard, such as 16-hydroxyhexadecanoic acid (Déziel et al. 2000), is added to compensate for differences in the ionization efficiencies from sample to sample.

Other Spectroscopic Method

Infrared (IR) has been used mostly to quantify complex mixtures of congeners (Gartshore et al. 2000). This approach is based on the relatively broad IR absorption bands corresponding to various hydroxyl, ester, and carboxylic groups present in RLs. This method has been used for the quantification of complex RL mixtures, but it suffers from interferences by other constituents in the medium and of changes in pH.

Nuclear Magnetic Resonance (NMR) measures the absorption of radio frequencies for various atoms exposed to a magnetic field. It provides very detailed information on the chemical environment of atoms (the proton and ^{13}C) within a molecule. This tool has been used mostly for the structural analysis of purified congeners (Haba et al. 2003a; Monteiro et al. 2007) rather than for quantification of complex RL congener mixtures.

3 Biosynthesis and Genetic Regulation

Details of the pathways involved in RL biosynthesis, including synthesis of the fatty acid and sugar moieties, have been in large part elucidated. Furthermore, a good deal of information is available on the regulation of genes important for RL production.

3.1 Biosynthesis of Rhamnolipids

Following early studies to understand the metabolic pathway of RL biosynthesis (Hauser and Karnovsky 1957, 1958), Burger et al. (1963) reported a putative mechanism of rhamnosylation of fatty acid chains to form RLs according, as an example, to the following reactions for Rha-Rha-C_{10}-C_{10}, (Burger et al. 1963):

$$2\beta-\text{Hydroxydecanoyl}-\text{CoA}$$
$$\rightarrow \beta\text{--hydroxydecanoyl}-\beta\text{--hydroxydecanoate} +2\,\text{CoA--SH} \qquad (1)$$

$$\text{TDP}-1-\text{rhamnose}+\beta-\text{hydroxydecanoyl}-\beta-\text{hydroxydecanoate}$$
$$\rightarrow \text{TDP}+1-\text{rhamnosyl}-\beta\text{--hydroxydecanoyl}-\beta\text{--hydroxydecanoate} \qquad (2)$$

$$\text{TDP}-1-\text{rhamnose}+1-\text{rhamnosyl}-\beta\text{--hydroxydecanoyl}$$
$$-\beta\text{--hydroxydecanoate} \rightarrow \text{TDP}+1\text{--rhamnosyl--1--rhamnosyl} \qquad (3)$$
$$-\beta\text{--hydroxydecanoyl--}\beta\text{--hydroxydecanoate}$$
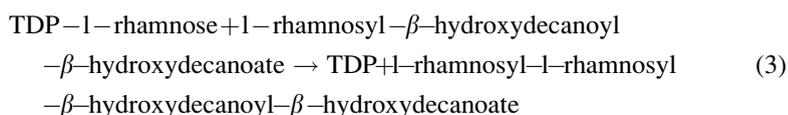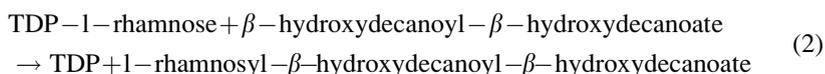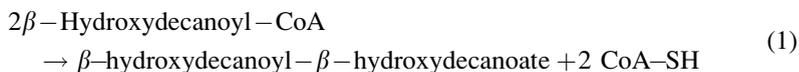
The first reaction involves dimerization of two β-hydroxydecanoic acid chains. The dimer then undergoes two sequential rhamnosylation reactions with two different rhamnosyltransferases: rhamnosyltransferase 1 (Rt-1) in reaction (2) and rhamnosyltransferase 2 (Rt-2) in reaction (3) (Burger et al. 1963).

It was initially hypothesized that the biosynthesis of biosurfactants, in general, and especially of glycolipids, proceeds through one of the three possible pathways:

– Both moieties are synthesized independently of the growth substrate (de novo).
– With a hydrophobic carbon source such as fatty acids and triglycerides, the lipid moieties are directly derived from the carbon source, but the sugar is synthesized de novo.
– The sugar moiety is directly derived from the carbon source, but the lipid component is synthesized de novo.

The biosynthesis of RLs has been largely elucidated. It is present here under three sections, namely: biosynthesis of the lipid moiety, biosynthesis of the sugar moiety, and finally, the enzymatic dimerization and the rhamnosyl transfers, which yield the final products.

3.1.1 Biosynthesis of the Lipid Moiety of Rhamnolipids

A number of reports demonstrate that the biosynthesis of the lipid components of RLs proceeds through the classical pathway of fatty acid synthesis from 2-carbon units. First, Hauser and Karnovsky (1957) reported in vivo experiments where the lipidic component of RLs incorporated radioactivity from various labeled

precursors supplied to the cultures, such as ^{14}C-acetate and ^{14}C-glycerol (Hauser and Karnovsky 1957). Second, the lipidic portion of RL is not substantially altered if different carbon sources are used (Hommel and Ratledge 1993). The observation that the stereochemistry of the β-hydroxyacids in the lipidic portion of RLs matches that of the intermediates in fatty acid biosynthesis, as opposed to that of the intermediates in fatty acid β-oxidation, suggests that fatty acid synthesis is the source for this moiety of RLs (Zhu and Rock 2008). The lipidic moiety of RLs is thus most probably synthesized de novo (Fig. 2).

The classical fatty acid synthetases of type-II (FAS II), which are found in most bacteria and plants, differ from those common to mammals (FAS I), in that these former fatty acid synthetases are not a single multifunctional polypeptide produced from a single gene (White et al. 2005). They are instead part of a dissociated fatty acid synthetase system, in which the individual reactions are catalyzed by separate proteins that are encoded by separate genes (Hoang and Schweizer 1997; White et al. 2005).

The reference system for FAS II biochemistry is *E. coli* (White et al. 2005). In general, however, these enzymes are homologous among bacteria. For example, many isozymes of FabI, an enoyl-acyl carrier protein reductase, have been found in other bacteria such as FabL, FabV, and FabK (Zhu et al. 2009). Yet, the similarities and differences between homologous enzymes in different bacteria remain to be identified (White et al. 2005).

In *E. coli*, fatty acid biosynthesis can be separated into two stages, initiation and cyclic elongation. Each round of elongation requires four chemical reactions (Hoang and Schweizer 1997). Three β-ketoacyl-acyl carrier protein (ACP) synthetases, KAS I (FabB), KAS II (FabF), and KAS III (FabH), the products of *fabB*, *fabF*, and *fabH*, play pivotal roles in fatty acid synthesis. Initiation requires malonyl coenzyme A (CoA) and malonyl-ACP. Malonyl-CoA is synthesized by acetyl-CoA carboxylase, and malonyl-ACP is derived from malonyl-CoA and ACP by the action of malonyl-CoA:ACP transacylase, the product of *fabD*. The first cycle of elongation is initiated by KAS III (FabH), which condenses malonyl-ACP to acetyl-CoA. Subsequent cycles are initiated by condensation of malonyl-ACP with acyl-ACP, catalyzed by KAS I (FabB) and KAS II (FabF). In the second step, the resulting β-ketoester is reduced to a β-hydroxyacyl-ACP by a single, NADPH-dependent β-ketoacyl-ACP reductase (FabG). The third step in the cycle is catalyzed by either the *fabA*- or the *fabZ*-encoded β-hydroxyacyl-ACP dehydratase. The fourth and final step in each cycle involves the conversion of *trans*-2-enoyl-ACP to acyl-ACP, a reaction catalyzed by a single NADH-dependent enoyl-ACP reductase (FabI) (Hoang and Schweizer 1997) (Fig. 2).

For *P. aeruginosa,* a model of FAS II was proposed that is composed of the same enzymatic machinery (Hoang and Schweizer 1999). However, the *fabH* homolog in *P. aeruginosa* has been only tentatively identified. It is therefore only hypothesized that initiation of FAS II is mediated by KAS III (FabH), and subsequently, initiation of elongation cycles are catalyzed by KAS I (FabB) for saturated fatty acid substrates and KAS II (FabF) for unsaturated fatty acid substrates (Hoang and Schweizer 1999). The same team has also characterized FabA, FabB, and FabI proteins in *P. aeruginosa* (Hoang and Schweizer 1997, 1999).
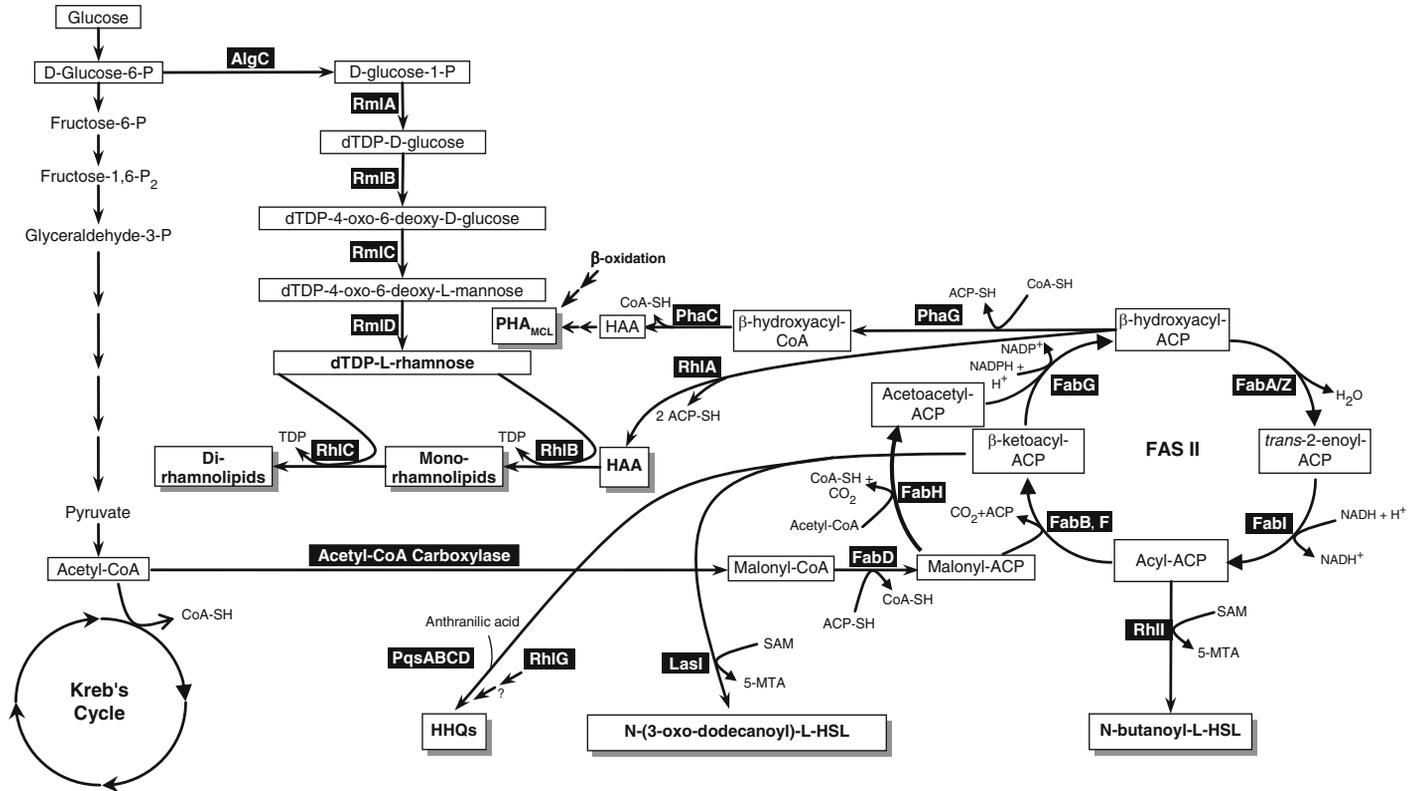
Fig. 2 Metabolic pathway of rhamnolipid biosynthesis and its relation to the biosynthesis of polyhydroxyalkanoates (PHA) and quorum sensing signaling molecules. *AlgC*: phosphomannomutase, *RmlA*: glucose-1-phosphate thymidylyltransferase, *RmlB*: dTDP-D-glucose 4,6-dehydratase, *RmlC*: dTDP-4-dehydrorhamnose 3,5-epimerase, *RmlD*: dTDP-4-dehydrorhamnose reductase, *FabD*: malonyl-CoA:ACP transacylase, *FabH*, *FabB* and *FabF*: β-ketoacyl-ACP synthetases, *FabG*: NADPH-dependent β-ketoacyl-ACP reductase, *FabA*, *FabZ*: β-hydroxyacyl-ACP dehydratases, *FabI*: NADH-dependent enoyl-ACP reductase, *HAA*: 3-(3-hydroxyalkanoyloxy)alkanoic acid, *PqsABCD*: 4-hydroxy-2-heptylquinoline synthesis cluster, *RhlA*: 3-(3-hydroxyalkanoyloxy)alkanoate synthetase, *RhlB*: rhamnosyltransferase 1, *RhlC*: rhamnosyltransferase 2, *RhlI*: *N*-butanoyl-L-HSL synthase, *RhlG*: β-ketoacyl reductase, *PhaG*: (R)-3-hydroxydecanoyl-ACP:CoA transacylase, *PhaC*: PHA synthase, *LasI*: *N*-(3-oxo-dodecanoyl)-L-HSL synthase, *HHQ*: 4-hydroxy-2-heptylquinoline, PHA_{MCL}: Poly(3-hydroxyalkanoates) of medium chain length C_6–C_{14}, *SAM*: S-adenosylmethionine, *5-MTA*: 5′-methylthioadenosine

Interestingly, ACP intermediates in the FAS II pathway are suggested to be contributing to the enormous diversity of bacterial products because they are diffusible entities that can be diverted into other biosynthetic pathways (White et al. 2005) like RL biosynthesis (Déziel et al. 2003; Rehm et al. 2001; Zhu and Rock 2008), polyhydroxyalkanoates (Pham et al. 2004; Rehm et al. 2001), as well as the biosynthesis of quorum sensing signal molecules (Bredenbruch et al. 2005).

The exact link between FAS II intermediates and RL biosynthesis, however, is still a matter of debate. Recently, Zhu and Rock (2008) proved that RhlA directly utilizes β-hydroxydecanoyl-ACP intermediates to generate 3-(3′-hydroxydecanoyloxy) decanoic acid, the ten carbon member of the 3-(3′-hydroxyalkanoyloxy)alkanoic acids (HAAs) portion of RLs. This had been previously suggested (Déziel et al. 2003), based on the amino acid homology of the *rhlA* gene product with PhaG, a 3-hydroxyacyl ACP:CoA transacylase identified in various species of *Pseudomonas* (Déziel et al. 2003; Rehm et al. 1998).

However, these findings contradict previous data suggesting that an enzyme called RhlG is responsible for diverting fatty acid synthesis intermediates into the RL biosynthetic pathway in *P. aeruginosa,* based on its similarity to FabG (Campos-Garcia et al. 1998). However, the work of Zhu and Rock (2008) indicates that there is no enzyme upstream of RhlA for diverting β-hydroxy fatty acid intermediates from FAS II cycle, and that RhlA acts more like as a molecular ruler that preferentially diverts appropriate intermediates from FASII for the synthesis of the HAA moiety of RLs. This finding was based on the biochemical properties of the purified RhlA protein and its products when heterologously expressed in an *E. coli* host (Zhu and Rock 2008). That RhlG has no role in picking up β-hydroxydecanoyl-ACP for HAA synthesis was further supported by the fact that, although the overall structures of the RhlG-NADP$^+$ and FabG-NADP$^+$ complexes are indeed similar, there are key differences related to their function, making RhlG 2,000-fold less active than FabG in carrying out the same reaction. These findings entail that RhlG is indeed a NADPH-dependent β-ketoacyl reductase, but its substrate is not carried by the ACP of fatty acid synthesis (Miller et al. 2006).

Another important issue is whether the substrate of the enzyme dimerizing β-hydroxydecanoyl moieties into HAA is carried by an ACP or by CoA. An earlier experiment performed in vitro with crude cellular extract showed that β-hydroxydecanoyl-CoA is a precursor of HAA (Burger et al. 1963). However, this experiment did not exclude the possibility that the reaction could involve one β-hydroxydecanoyl-CoA and free β-hydroxydecanoyl acid (Burger et al. 1963). A recent experiment performed in vitro with purified RhlA showed that HAAs are produced when β-hydroxydecanoyl-ACP is used as substrate, while no HAAs are obtained when β-hydroxydecanoyl-CoA is used at concentrations up to ten times higher than those of β-hydroxydecanoyl-ACP (Zhu and Rock 2008). Although these results seem in contrast with those of Burger et al. (1963), it remains to be seen whether β-hydroxydecanoyl-CoA could be a precursor of HAAs in vivo.

Remains also to be elucidated is the biosynthesis of some RLs having only one 3-hydroxy-fatty acid instead of the dimmer, attached to one or two rhamnose(s).

Are they degradation products of other RLs or are they formed by direct rhamnosylation of a 3-hydroxy-fatty acid by RhlB and RhlC? The biosynthetic pathway of RLs having one more unsaturation in their HAA chains will also require further investigation.

Biosynthetic Link Between Rhamnolipid-Lipid Moiety and PHA

As many other *Pseudomonas* sp, *P. aeruginosa* is capable of accumulating poly (3-hydroxyalkanoates) (PHAs) granules containing medium chain length (MCL) (C_6 to C_{14}) 3-hydroxyfatty acids (PHA_{MCL}) (Madison and Huisman 1999; Rehm et al. 2001). The biosynthetic pathway of PHA_{MCL} proceeds mainly through FAS-II, when grown on carbon sources metabolized into acetyl-CoA like carbohydrates. On the other hand, when grown on hydrocarbons, it proceeds mainly through β-oxidation (Madison and Huisman 1999; Rehm et al. 1998, 2001; Timm and Steinbuchel 1990). The substrate of the PHA_{MCL} synthases is (R)-3-hydroxydecanoyl-CoA, which is formed from ACP-thioester precursors by the action of the transacylase PhaG (Rehm et al. 2001). Thus, PhaG directly links fatty acid de novo biosynthesis to PHA biosynthesis (Rehm et al. 2001). Based on that, it is understood that RLs and PHA_{MCL} biosynthesis compete with each other for the β- hydroxydecanoyl-ACP precursor, which is an intermediate in FAS-II (Rehm et al. 2001) (Fig. 2). Although that PHA synthase could be responsible for supplying the HAA moieties for RL synthesis has been postulated (Campos-Garcia et al. 1998), another study proved that PHA-synthase negative mutants are still capable of RL production (Pham et al. 2004).

Biosynthetic Link Between the Rhamnolipids Lipidic Moiety and Quorum Sensing Signal Molecules

P. aeruginosa produces two classes of signal molecules, the acyl homoserine lactones (AHLs) and the 4-hydroxy-2-alkylquinolines (HAQs). The most abundant AHLs are *N*-(3-oxododecanoyl)-L-HSL and *N*-butanoyl-L-HSL, while the HAQs include 3,4-dihydroxy-2-heptylquinoline [*Pseudomonas* Quinolone Signal (PQS)] and its precursor 4-hydroxy-2-heptylquinoline (HHQ) (Déziel et al. 2004). Some of these molecules are involved in the regulation of RL synthesis genes expression. The biosynthesis of these signal molecules also requires substrates derived from FAS II by diverting FAS intermediates of a specific fatty acid chain length for their own synthesis (Schaefer et al. 1996).

Using in vitro and in vivo experiments, Hoang and Schweizer (1999) showed that butanoyl-ACP serves as substrate for *N*-butanoyl-L-HSL biosynthesis. They observed that FabI (the enzyme that supplies acyl-ACP like butanoyl-ACP in FAS II) plays a central role in AHL biosynthesis in vivo because a *fabI* mutant of *P. aeruginosa* produced only 50% of the AHL levels found in wild-type cells (Hoang and Schweizer 1999). Moreover, when coupled to FabI, purified *P. aeruginosa* *N*-butanoyl-L-HSL synthase (RhlI) produced *N*-butanoyl-L-HSL from crotonyl-ACP

(the enoyl-ACP precursor of butanoyl-ACP in FAS II) and S-adenosylmethionine (SAM) (Hoang and Schweizer 1999) (Fig. 2). Similarly, N-(3-oxododecanoyl)-L-HSL is synthesized from the β-ketododecanoyl-ACP intermediate of FAS II by the action of N-(3-oxododecanoyl)-L-HSL synthase (LasI) in the presence of SAM (Fuqua and Greenberg 2002; Hoang and Schweizer 1999; Schaefer et al. 1996) (Fig. 2).

That HAQs also obtain some of their biosynthetic precursors from the FAS II cycle was hypothesized (Soberón-Chávez et al. 2005). Ritter and Luckner (1971) and Calfee et al. (2001) proposed a synthetic scheme for HAQs where anthranilate and β-ketodecanoic acid are condensed in a multistep reaction that would produce HHQ, followed by PQS after the release of a one carbon unit as CO_2. This pathway has been verified using labeled substrates (Bredenbruch et al. 2005; Déziel et al. 2004). These reactions are catalyzed by the *pqsABCD* and *pqsH* gene products (Dubern and Diggle 2008). Bredenbruch et al. (2005) suggested that the β-ketoacyl reductase RhlG plays a role, although an intact *rhlG* gene is not required for the production of HAQs. A more detailed description of the biosynthetic pathway of HAQs was recently described (Gross and Loper 2009).

3.1.2 Biosynthesis of Rhamnolipids-Rhamnose Moiety

Rhamnose is a component of the cell wall lipopolysaccharide (LPS) core and of several O-antigen polysaccharides in a variety of gram-negative bacteria, including several strains of *Pseudomonas* (Burger et al. 1963; Rahim et al. 2000).

Early studies on the catabolic pathway of rhamnose were performed using radioactive carbon sources. These showed that the carbons of rhamnose are derived from glycerol and not from acetate, apparently through the condensation of two three-carbon units formed from glycerol without cleavage or rearrangement of its carbon–carbon bonds (Hauser and Karnovsky 1957).

In a later study (Hauser and Karnovsky 1958), both glycerol and propane-1,2-diol were found to provide carbon to the rhamnose of RL and to be equally converted into the precursors of the two halves of the sugar (Hauser and Karnovsky 1958). For fructose as the carbon source, it was suggested that this sugar is cleaved into two triose units that are subsequently recombined to form rhamnose (Hauser and Karnovsky 1958). However, this latter study did not clarify the detailed steps of RL biosynthesis with glucose as the sole carbon source.

The biosynthetic conversion of glucose to rhamnose in *P. aeruginosa* was clarified with the in vivo and in vitro studies of Southard et al. (1959) and of Glaser and Kornfeld (1961). These reports showed that glucose is converted into rhamnose without randomization of the carbon chain and that the carbon at the position 1 of glucose is found at the same position in rhamnose (Glaser and Kornfeld 1961). Glaser and Kornfeld (1961) also explained the previous results of Hauser and Karnovsky (1958) with labeled glycerol, suggesting that two three-carbon compounds initially condense to form glucose. They also showed that in order for D-glucose to be converted into L-rhamnose, the configuration of the carbon 3, 4, and 5 should be inverted and that a reduction at the carbon 6 is required. They also

showed that D-glucose is converted into L-rhamnose through a 4-keto-6-deoxyglucose intermediate and they further postulated that the inversion of the configuration at the carbon 3 and 5 is performed by an isomerization reaction facilitated by enolization of the ketone (Glaser and Kornfeld 1961).

Recently, the biosynthetic link between glucose and rhamnose was found to proceed through the phosphoglucomutase AlgC, as an *algC* mutant does not produce detectable amounts of RLs (Olvera et al. 1999). This phosphoglucomutase converts D-glucose-6-phosphate into D-glucose-1-phosphate, and this compound is then used by RmlA, RmlB, RmlC, and RmlD to produce dTDP-L-rhamnose (Fig. 2) (Olvera et al. 1999; Robertson et al. 1994). dTDP-L-rhamnose is also the precursor for the L-rhamnose present in the outer core oligosaccharide of the lipopolysaccharide (LPS) (Rahim et al. 2000). Furthermore, dTDP-L-rhamnose was recently shown to provide the L-rhamnose as a component of both the flagellin glycan of b-type flagellin (Lindhout et al. 2009) and the *psl*-encoded polysaccharide, which consists of a repeating pentasaccharide containing D-mannose, D-glucose, and L-rhamnose (Byrd et al. 2009).

This pathway only explains how L-rhamnose is probably synthesized when the bacteria are grown with glucose as the carbon source. However, many other carbon sources are more efficient for RL production, such as mannitol (Déziel et al. 1999b), vegetable oils (Trummler et al. 2003), glycerol, or ethanol (Chen et al. 2007a). Under these conditions, the exact biosynthetic pathways remain to be elucidated.

3.1.3 Three Last Enzymatic Reactions in Rhamnolipids Biosynthesis

As mentioned earlier, three enzymatic reactions are required in the final steps of RL biosynthesis in *P. aeruginosa* (Soberón-Chávez et al. 2005): (1) RhlA is involved in the synthesis of the HAAs, the fatty acid dimers, from two 3-hydroxyfatty acid precursors (Déziel et al. 2003; Lépine et al. 2002; Zhu and Rock 2008); (2) the membrane-bound RhlB rhamnosyltransferase uses dTDP-L-rhamnose and an HAA molecule as precursors, yielding mono-RL; (3) these mono-RLs are in turn the substrates, together with dTDP-L-rhamnose, of the RhlC rhamnosyltransferase to produce di-RLs. Unfortunately, few works have characterized these three enzymes (Fig. 2).

Burger et al. (1966) were unable to characterize RhlA because it was relatively labile under their conditions, thus preventing purification. Nonetheless, they showed that β-hydroxydecanoyl-CoA is a precursor of HAAs. Ochsner et al. (1994a) were also unable to purify RhlA and suggested that it is involved in the synthesis or supply of the precursors of subsequent rhamnosyl transferase or that it is necessary for the stabilization or anchoring of RhlB in the cytoplasmic membrane. Based on the analysis of the amino acid sequences derived from the nucleotide sequence of *rhlA*, they deduced that RhlA is 32.5 kDa protein that harbors a putative signal sequence, suggesting that it is located in the periplasm (Ochsner et al. 1994a). Déziel et al. (2003) later proposed that RhlA is responsible for the formation of HAAs, based on the observation that an *rhlB⁻* mutant produce HAAs

but no RLs while a *rhlA*⁻ mutant does not produce neither HAAs nor RLs. Recently however, Zhu and Rock (2008) purified and characterized RhlA. They found that RhlA formed one molecule of β-hydroxydecanoyl-β-hydroxydecanoate (HAA) from two molecules of β-hydroxydecanoyl-ACP. They also showed that RhlA has a greater affinity for ten-carbon substrates (Zhu and Rock 2008). From these findings, they conclude that RhlA uses β-hydroxyacyl-ACP selectively picked from FAS-II cycle. In contrast to the findings of Burger et al. (1966), they did not detect any HAA production using β-hydroxyacyl-CoA, even at a concentration ten times higher than the one used with β-hydroxyacyl-ACP (Zhu and Rock 2008). It remains to be seen whether RhlA can use both substrates in vivo (Rehm et al. 2001), but maybe with different levels of affinity.

Burger et al. (1966) purified RhlB, the enzyme catalyzing the second reaction, and partially purified RhlC, the enzyme catalyzing the third reaction. They showed that they both accepted L-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate, β-hydroxydecanoyl-β-hydroxydecanoate, and β-Hydroxydecanoyl-CoA as glycosyl acceptors, while free β-hydroxydecanoate was not a substrate (Burger et al. 1966). Ochsner et al. (1994a) later described RhlB, based on the analysis of the amino acid sequences deduced from *rhlB* as a protein with at least two putative membrane-spanning domains, which would allow anchoring in the inner membrane. They also partially purified RhlB from the membrane fraction and determined its size to be around 47 kDa (Ochsner et al. 1994a). Rahim et al. (2001) were the first to identify *rhlC*, which encodes for the second rhamnosyl transferase. They found that RhlC contains a transmembrane hydrophobic region, suggesting that it is also an inner membrane bound protein. RhlC specifically converts mono-RL into di-RL (Rahim et al. 2001). They suggested that both mono- and di-RLs are synthesized at the cytoplasmic side of the inner membrane before being transported to the extracellular milieu (Rahim et al. 2001).

Finally, it should be mentioned that the *rhlA*, *rhlB*, and *rhlC* genes were recently identified in the RL-producing species *Burkholderia thailandensis* and *B. pseudomallei* (Dubeau et al. 2009). Interestingly, and in contrast with *P. aeruginosa,* where *rhlC* is separate from *rhlAB*, in these species, they are grouped together in a gene cluster. Furthermore, this RL synthesis gene cluster is duplicated on the chromosome of these bacteria, and both copies are functional and contribute to RL production (Dubeau et al. 2009).

3.2   Regulation of Rhamnolipid Biosynthesis

The control of RL production is complex, since it is influenced by numerous factors at both genetic control and environmental/nutritional levels. As typical secondary metabolites, biosynthesis primarily occurs from the end of the logarithmic or the onset of the stationary growth phases. Two factors concur to explain this: cell density-dependent regulation and limitation of specific nutrients.

3.2.1 Genetic Regulation of Rhamnosyltransferases

At the genetic level, the foundations of our current understanding were established by Urs Ochsner and colleagues in the mid-1990s (Ochsner et al. 1994a, b; Ochsner and Reiser 1995). Using a strategy of random transposon mutagenesis and genetic complementation, they identified the primary biosynthetic and regulatory genes, grouped in an *rhl* gene cluster, responsible for the production of RLs (Fig. 3). RhlA and RhlB are encoded by genes organized in an operon, which is flanked by the regulatory genes *rhlR* and *rhlI*. The main finding was that the expression of *rhlAB* is positively controlled in a cell-density manner by a cell-to-cell communication system called quorum sensing (Ochsner and Reiser 1995; Pearson et al. 1997).

The production of secondary metabolites and virulence factors such as antibiotics and proteases is often controlled by quorum sensing (Miller and Bassler 2001). Gene regulation by quorum sensing implicates that bacteria produce and release chemical signal molecules for which increases in their external concentrations mirrors the cell-population density. Bacteria detect their accumulation and, once a minimal threshold stimulatory concentration is reached, they respond and alter gene expression, and therefore the behavior of the whole population (Fuqua et al. 1994). Gram-negative bacteria typically carry at least one quorum sensing mechanism mediated by a regulator of the LuxR-type and by an AHL synthase of the LuxI-type (Lazdunski et al. 2004).

P. aeruginosa regulates the transcription of an array of genes by quorum sensing. A large proportion of these are directing the production of virulence factors, including proteases, lectins, HCN, phenazines, and RLs (Bjarnsholt and Givskov 2007; Williams and Cámara 2009). In the case of RL biosynthesis, the product of RhlI is the signal butanoyl-homoserine lactone, C_4-HSL, which acts as the activating ligand of the transcriptional regulator RhlR (Fig. 3). The RhlR/C_4-HSL complex then binds to a specific sequence in the *rhlAB* regulatory region to activate the transcription. Interestingly, RhlR was suggested to act as a transcriptional repressor when not bound to its signaling ligand (Medina et al. 2003c). The level of expression of *rhlAB* is thus dependent on the local environmental concentration of this signal. The expression of the second rhamnosyltransferase, encoded by *rhlC*, is coordinately regulated with *rhlAB* by the same quorum sensing regulatory pathway (Rahim et al. 2001).

Besides RhlR/C_4-HSL quorum sensing, two additional cell-to-cell communication systems participate in the quorum sensing circuitry of *P. aeruginosa* and influence *rhlAB* transcription (Fig. 3). First, the *rhl* system is positively upregulated by another LuxR-type regulator called LasR, which is activated by its cognate AHL *N*-3-oxo-dodecanoyl-HSL (3-oxo-C_{12}-HSL) (Latifi et al. 1996; Pearson et al. 1997). Second, besides the LuxR/AHL-type circuits, *P. aeruginosa* carries a distinct quorum sensing system composed of the transcriptional regulator MvfR (PqsR), which directs the biosynthesis of HAQs (Déziel et al. 2004; Gallagher et al. 2002; Pesci et al. 1999) and the activation of many quorum sensing-controlled genes via PqsE (Déziel et al. 2005; Diggle et al. 2003; Farrow et al. 2008). Among

the HAQs, HHQ and PQS act as inducing ligands of MvfR regulator (Xiao et al. 2006). This third level of quorum sensing regulation controls production of RLs by stimulating the RhlR/C$_4$-HSL QS system through MvfR and PqsE: *rhlAB* expression and production of RLs are reduced in PQS-deficient and *pqsE*$^-$ mutants (Déziel et al. 2005; Diggle et al. 2003; Jensen et al. 2007). This is largely explained by the fact that PqsE upregulates *rhlAB* transcription by increasing the activity of
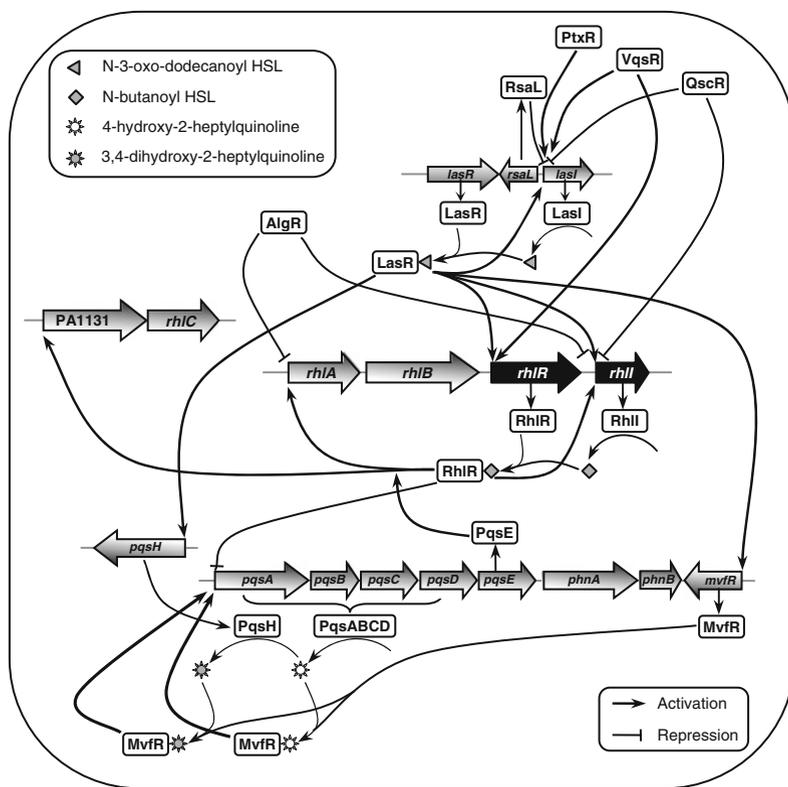
Fig. 3 Genetic regulation of rhamnolipid (RL) biosynthesis in *P. aeruginosa*. Multiple systems of quorum sensing (QS) participate in the control of RL synthesis genes (*rhlA, rhlB, rhlC*). Two QS systems, LasR/I and RhlR/I, depend on acyl homoserine lactones (AHL) ligands, *N*-3-oxo-dodecanoyl-HSL and *N*-butanoyl-HSL, respectively, which bind to their cognate transcriptional regulators, LasR and RhlR, respectively, for regulation of expression of several genes, among which are RL biosynthesis genes. LasR/I and RhlR/I activate the expression of their own auto-inducer synthase genes, *lasI* and *rhlI*, respectively, as a positive feedback. The transcription of *lasI* and *rhlI* is also controlled by other regulators. RhlR/C$_4$-HSL complex is positively regulating expression of the operon *rhlAB* as well as the operon encoding the *rhlC* gene. These last three genes encodes the three enzymes responsible for biosynthesis of RLs. LasR/oxo-C$_{12}$-HSL activates the other QS system in which the transcriptional regulator MvfR (PqsR) binds to its co-inducers 4-hydroxy-2-heptylquinoline (HHQ) and 3,4-dihydroxy-2-heptylquinoline (*Pseudomonas* Quinolone Signal; PQS). LasR/oxo-C$_{12}$-HSL activates the expression of *mvfR*

RhlR (Farrow et al. 2008). Thus both the LasR/3-oxo-C_{12}-HSL and the MvfR/PQS/PqsE quorum sensing signaling pathways end up upregulating the activity of RhlR. Accordingly, while a *rhlR⁻* mutant does not produce any RLs (Ochsner and Reiser 1995), PQS system-deficient (e.g., *mvfR⁻* or *pqsE⁻*) or *lasR⁻* mutants still express *rhlAB* and produce RLs, but at reduced or delayed rates (Dekimpe and Déziel 2009; Déziel et al. 2005; Diggle et al. 2003; Ochsner and Reiser 1995).

Additional regulatory factors modulate the expression of the *rhlAB* operon, all of which acting essentially on some levels of the quorum sensing global circuitry (Fig. 3). Here are some of these factors. The RsaL protein represses the transcription of *lasI*, inducing a large-scale downregulation of quorum sensing-regulated genes, including *rhlAB* (Rampioni et al. 2009). Besides the primary quorum sensing regulators LasR and RhlR, two additional LuxR-type regulators have been reported: QscR represses *rhlAB* transcription (Lequette et al. 2006), while VqsR activates it (Juhas et al. 2004). Additionally, VqsM, largely through modulation of *vqsR* expression, plays a role in regulation of QS signaling in *P. aeruginosa,* incidentally and indirectly upregulating *rhlAB* (Dong et al. 2005). Other regulators known to indirectly affect *rhlAB* transcription include PtxR, which negatively regulates the expression of RhlR target genes through *rhlI* downregulation (Carty et al. 2006) and DksA, which also seems to reduce *rhlI* expression, but nevertheless increases *rhlAB* translation (Jude et al. 2003). On a different level of quorum sensing control, the AlgR regulator was shown to repress RhlR-controlled genes, but in biofilm-growing cells only (Morici et al. 2007); moreover, AlgR directly binds to the *rhlAB* promoter and prevents transcription and RLs production.

At the posttranscriptional level, the production of RLs is positively controlled by the small RNA-binding protein RsmA (Heurlier et al. 2004); however, this control is indirect and the precise mechanism of control has not been identified (Brencic and Lory 2009). GidA, another factor recently reported, primarily activates RhlR-controlled quorum sensing genes also at the posttranscriptional level, and thus controls *rhlAB* transcription and RL production (Gupta et al. 2009).

3.2.2 Genetic Regulation of Biosynthesis of Sugar Moiety

As presented above, the rhamnosyltransferase 1, encoded by *rhlB*, is responsible for catalyzing the coupling of the activated sugar dTDP-L-rhamnose to a β-hydroxyalkanoic acid dimer to yield mono-RLs (Burger et al. 1963). Together with another dTDP-L-rhamnose molecule, mono-RLs are in turn substrates of rhamnosyltransferase 2, encoded by *rhlC*, to produce di-RLs (Rahim et al. 2001). In *P. aeruginosa*, the *rmlBDAC* operon encodes the enzymes catalyzing the conversion of glucose-1-phosphate to dTDP-L-rhamnose (Rahim et al. 2000). The regulation of *rmlBDAC* transcription is not characterized. There is a potential σ^{70}-like promoter sequence upstream of *rmlB* (Rahim et al. 2000). Interestingly, transcriptomic results suggest that this operon is upregulated by the RhlR quorum sensing pathway (Schuster et al. 2003; Wagner et al. 2003), but this has yet to be confirmed.

3.2.3 Regulation of Rhamnolipid Production by Environmental Factors – Links Between Quorum Sensing and the Environment

Restriction in the availability of a number of nutrients, except the carbon source, is known to promote the production of RLs (Guerra-Santos et al. 1986) (see below). For instance, the transcription of *rhlAB* and the production of RLs are inversely proportional with the concentration of iron (Fe) available to the bacterial cells (Déziel et al. 2003; Glick et al. 2010). An explanation is provided by the well-established link between iron availability and quorum sensing in *P. aeruginosa*. Indeed, the expression of *lasIR* (Bollinger et al. 2001; Duan and Surette 2007; Kim et al. 2005) and *rhlIR* (Bredenbruch et al. 2006; Duan and Surette 2007; Jensen et al. 2006) is enhanced by Fe limitation and/or repressed by Fe supplementation.

Production of RLs is inhibited by the presence of NH_4^+, glutamine, asparagine, and arginine as nitrogen source and promoted by NO_3^-, glutamate, and aspartate (Köhler et al. 2000; Mulligan and Gibbs 1989; Van Alst et al. 2007; Venkata Ramana and Karanth 1989). Several reports demonstrate that NO_3^- is the best nitrogen source for RL production (Arino et al. 1996; Manresa et al. 1991; Venkata Ramana and Karanth 1989), and it indeed elicits higher *rhlAB* expression than NH_4^+ (Déziel et al. 2003). The basis for the preference for nitrate in RL production is unknown. On the other hand, high levels of NH_4^+ or glutamine reduce the production of RLs, and this is correlated with a lower glutamine synthase activity (Mulligan and Gibbs 1989). Synthesis of this enzyme, which is upregulated by environmental signals such as nitrogen-limiting conditions, is controlled by the RpoN σ factor (σ^{54}) (Totten et al. 1990). In addition to the major housekeeping σ factor, σ^{70}, many bacteria have alternative σ factors that direct the expression of particular subsets of genes (Potvin et al. 2008). Hence, it is noteworthy that σ^{54} is also required for production of RLs (Ochsner et al. 1994a). While it has been suggested that the *rhlAB* transcription start site contains a σ^{54} promoter (Ochsner et al. 1994a; Pearson et al. 1997), this was later refuted (Medina et al. 2003c). The alternative explanation is provided by the finding that *rhlR* transcription is partially σ^{54}-dependent (Medina et al. 2003a). All this corroborates the frequent observation that production of RLs is increased under nitrogen-limited conditions (Mulligan and Gibbs 1989).

Van Alst et al. (2007) recently contributed intriguing new observations to the connection between utilization of nitrate and production of RLs. Investigating the nitrate sensor-response regulator NarX/NarL, they found that a *narL* mutant strain produces significantly (approximately sixfold) more RLs than the wild type. They proposed that in the absence of its cognate response regulator NarL, NarX may activate an alternative response regulator that, either directly or indirectly, activates *rhlAB* (Van Alst et al. 2007). They also presented some evidence that nitric oxide, the product of nitrite reductase activity in the nitrate dissimilation pathway, activates RL production (Van Alst et al. 2007).

In conclusion, the expression of the *rhlAB* operon and the production of RLs are regulated by both quorum sensing signals and environmental/nutritional factors. However, Déziel et al. (2003) observed that nutritional conditions can supersede

cell-to-cell communication in RL production. Accordingly, exogenously added signals do not modify the onset of induction for genes controlled by both the RpoS σ factor (σ^S) and quorum sensing (Diggle et al. 2002; Medina et al. 2003b; Schuster et al. 2003); now, the *rhlAB* promoter appears partially dependent on σ^S for its expression (Medina et al. 2003b). Indeed, high cell density and/or presence of both RhlR and its ligand signal C_4-HSL do permit upregulation or advancement of *rhlAB* expression before late logarithmic-early stationary phase, when *rpoS* is induced (Medina et al. 2003b; Schuster et al. 2003). Further studies will be required to elucidate the complex interplay between nutrition-based and cell-density-based gene regulation in *P. aeruginosa*.

4 Bioengineering of Rhamnolipid Production

4.1 *Production by* P. aeruginosa

The bioproduction of RLs has been investigated almost exclusively with *P. aeruginosa* strains. In this section, we will present different aspects of such bioprocesses.

4.1.1 Fermentation Strategies

RLs are secondary metabolites and their production coincides with the onset of the stationary phase. Therefore, all cultivation strategies for the microbial production of RLs aim at inducing RL biosynthesis by limiting at least one medium component, for example, the nitrogen or the phosphorous source (Guerra-Santos et al. 1984; Soberón-Chávez et al. 2005).

Corresponding to the growth-limited character of batch cultivations, the growth curve can satisfactorily be fitted to a logistic equation for biomass growth (4) or alternatively to a modified Gompertz equation (5) (Zwietering et al. 1990). The specific growth rate, biomass yield coefficient, and maintenance coefficients can be obtained by this approach (Ramana et al. 1991). Figure 4a illustrates the theoretical time courses of biomass and RL concentration in batch cultivation under growth-limiting conditions. The deviation of the concentrations of biodrymass and RLs based on the current biodrymass results, respectively, in the specific growth rate and specific RL production rate per cell that are shown in Fig. 4b. Actual time courses for growth and RL production in a batch cultivation were recently reported for the sequenced *P. aeruginosa* strain PA01 (Müller et al. 2010).

$$\frac{x}{x_{\max}} = [1 + \exp\{\mu_{\max} \cdot (t_c - t)\}]^{-1} \tag{4}$$
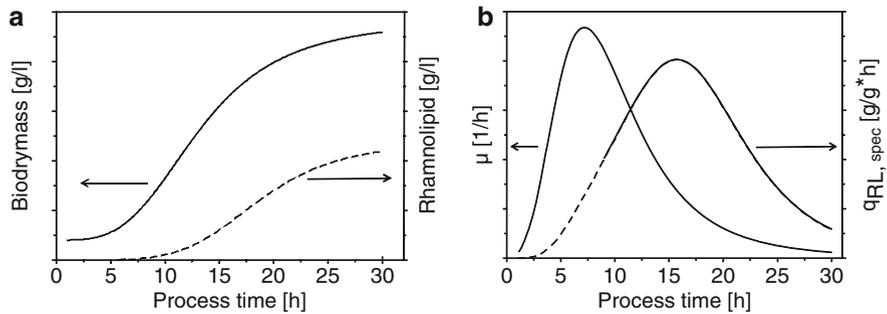
Fig. 4 Schematic representation of (**a**) the time courses of biodrymass and rhamnolipid concentration in a batch cultivation under growth limiting conditions and (**b**) the specific growth rate and specific rhamnolipid production rate per cell in a batch cultivation under growth limiting conditions

$$\frac{x}{x_\infty} = \exp\left\{-\exp\left\{\frac{\mu_{\max}e}{\ln\left(\frac{x_\infty}{x_0}\right)} \cdot \lambda - t) + 1\right\}\right\} \tag{5}$$

Cultivation strategies applied to RL production involve batch, fed-batch, continuous, and integrated microbial/enzymatic processes. Dextrose, glycerol, *n*-alkanes, and triglycerides have been mostly used as carbon sources. Reported nitrogen sources include nitrate, ammonium, urea, corn steep liquor, and complex amino acids containing supplements (Lee et al. 2004; Syldatk and Wagner 1987; Zhang and Miller 1992). The reported biotechnological cultivation strategies applied to the production of RLs are:

- (Fed-)batch cultivations under growth-limiting conditions
- Batch cultivations under resting cells conditions
- Semicontinuous productions with immobilized cells (excluding any nitrogen source)
- Continuous cultivations and production with free cells
- Solid state fermentations

Batch and Fed-Batch Strategies

In general, fed-batch cultivation is the most effective process strategy for achieving high bioproductivities. This is because optimal low concentrations of all substrates can be set and the specific growth rate can be controlled by the feeding. In contrast to continuous cultures there is a limited contamination risk. However, for RL production, this strategy has not been effectively adopted yet. Even though considerable, final RL concentrations in the range of 6–95 g/L have been reported (Chen et al. 2007b; Giani et al. 1997; Hembach 1994; Lee et al. 2004; Trummler et al. 2003), these concentrations are in the same order of magnitude than those reported

for batch cultivations, for which about 5–112 g/L RLs have been achieved (Chen et al. 2007b; Giani et al. 1997; Hembach 1994; Lee et al. 2004; Syldatk et al. 1985). The main difficulties for exploiting the fundamental benefits of fed-batch cultivations are the complex genetic regulation of RL production and the excessive foam formation during aerobic cultivation. Additionally, most of the reported fed-batch strategies just rely on heuristic approaches.

Batch and fed-batch processes with *P. aeruginosa* DSM 7107 and DSM 7108 achieved the best-reported RL production in terms of maximum yield and specific productivity (q_P). In their patent Giani et al. (1997) claimed a production of more than 100 g/L RLs. Unfortunately, insufficient technical details are provided; especially, no information on the applied analytical method for the RL determination is presented (Giani et al. 1997).

Fed-batch cultivation of *P. aeruginosa* BYK-2 with fish oil as carbon source and urea as nitrogen source resulted in the highest specific yield yet reported, ($Y_{P/S}$) of 0.75 g/g (Lee et al. 2004). A final concentration of 17 g/L of RLs was achieved after 216 h of cultivation. In contrast, a batch strategy resulted in a specific yield $Y_{P/S}$ of 0.68 g/g (Lee et al. 2004). A very interesting strategy was proposed by Chen et al. (2007b) who used a pH-stat fed-batch strategy to improve RLs production with 6% glucose in their feed medium. They reported that by excessive feeding of glucose, the accumulation of acidic metabolites occurred, while insufficient supply of glucose lowered RL productivity. Therefore, a pH-stat feeding strategy was applied to control the pH by adjusting the glucose feeding; in that study, a final RL concentration of about 6 g/L was achieved (Chen et al. 2007b).

Resting Cells Cultivations

Trummler et al. (2003) reported an integrated microbial/enzymatic process with resting cells of *Pseudomonas* sp. DSM 2874. By a two-step process, the biomass was first produced and harvested. The resting cells were then suspended in a buffer solution and RL production was induced by addition of the carbon source (rapeseed oil). A volumetric productivity (P_V) of about 0.14 g/L h was achieved by this method. With the same strain, Syldatk et al. (1985) had reported an improvement of RLs yield coefficient $Y_{P/S}$ from 0.16 to 0.23 g/g and $Y_{P/X}$ from 0.61 to 3.30 g/g when cultivated under resting cell conditions compared to growth-limiting conditions with nitrogen limitation.

Continuous and Semicontinuous Cultivations

Because of foaming problems, semicontinuous strategies have been developed with integrated continuous product removal by flotation. Screenings with *Pseudomonas* sp. DSM 2874 revealed that the combination of calcium alginate-immobilized cells with glycerol as the carbon source was the best condition for semicontinuous production of RLs (Syldatk et al. 1984). It is possible to reuse the immobilized

biocatalyst several times after appropriate regeneration of the cells (Siemann and Wagner 1993).

Continuous processes for the production of RLs are very promising in terms of productivity; relatively high specific and volumetric productivities have been reported for such processes. However, few attempts have been made to promote this process strategy, probably because they are more complex in terms of preparation, realization, and control. Cultures under a continuous process also have a higher risk of contamination.

Most of the reported continuous cultivations for RL production have been performed with *P. aeruginosa* DSM 2659 and dextrose as carbon source. The main characteristics of the performed experiments involved carbon and phosphate excess in addition to nitrogen and iron limitation. The peak of specific productivity occurred at relatively low growth rates (Guerra-Santos 1985; Guerra-Santos et al. 1984, 1986) when strain DSM 2659 was cultivated under continuous conditions (33°C, pH 6.25, 20 g/L dextrose). Ochsner et al. (1996) reported volumetric productivities (P_V) of 2 g/L h and a product yield ($Y_{P/S}$) of 0.48 g/g when using corn oil as carbon source for continuous cultivation of *P. aeruginosa* DSM 2659.

Solid State Fermentation

Since they are potent surfactants, foaming is a serious obstacle when producing RLs in an aerated stirred tank bioreactor with a liquid medium. A neat circumvention of this problem is the application of solid state fermentation. Camilios Neto et al. (2008) optimized RL production by *P. aeruginosa* UFPEDA 614 grown on a solid medium impregnated with a solution containing glycerol. On the basis of the volume of impregnating solution added to the solid support, the yield was in the order of 46 g/L of RLs.

4.1.2 Foaming Problems Encountered During Fermentative Production

A serious challenge encountered during RL production under aerobic conditions is the excessive foaming due to the aeration and agitation of the culture broth in the bioreactor (Chayabutra et al. 2001; Reiling et al. 1986; Walter et al. 2010). Conventionally, chemical antifoaming agents are applied, e.g., based on silicone oil, polyethylene glycol, or polypropylene. This applies to the production of RLs as well (Giani et al. 1997). However, the utilization of chemical antifoaming agents may negatively affect the product quality. Mechanical foam control is an alternative to be considered. Without foam control, the generated foam may drain into the exhaust air duct and block the exhaust air filter. This increases the risk of infections, decreases the productivity, and endangers the whole process. Consequently, the working volume of the bioreactor is usually not completely exploited; rather, it has to be reduced substantially to handle foam formation. Typically, about 40% of the nominal reactor capacity is used (Trummler et al. 2003; Walter et al. 2010).

In the future, in situ product removal (ISPR) could represent an interesting option for minimizing foam problems during RL production. While ISPR offers some significant advantages, the most important is mitigation of the pronounced foaming. Until now, all trials on ISPR of RLs by filtration have been ineffective due to rapid fouling of the filtration membranes (Gruber 1991).

Most bioreactors employed in the production of RLs are stirred-tank reactors for general microbial fermentations equipped with conventional radial impellers (Rushton turbine). These reactors may additionally be equipped with a mechanical foam separator (Müller et al. 2010). On a large scale, this may lead to phase separation when utilizing vegetable oils as the carbon source. In such reactors, mixing the highly foamy broth becomes ineffective because of the mode of action of the Rushton turbine. A proposed solution is the combination of an axial propeller and a radial impeller housed in a draft tube, which enhances the dispersion of the hydrophobic substrate by forced vertical circulation (Walas 1997).

4.1.3 Nutritional Factors Affecting Rhamnolipid Production

The effect of different medium culture components, such as carbon and nitrogen sources, and the availability of minerals, on RL production by *P. aeruginosa* are presented.

Carbon Sources

Both water-soluble or water-insoluble carbon sources have been utilized for production of RLs. However, hydrophobic carbon sources such as vegetable oils, are especially effective at promoting the production of RLs. Production processes utilizing a wide range of both natural and petrochemical carbon sources have been published, e.g.,

- Vegetable oils; e.g., (Giani et al. 1997; Trummler et al. 2003)
- Sugars; e.g., (Guerra-Santos et al. 1984; Lee et al. 2004; Reiling et al. 1986)
- Glycerol; e.g.,(Chen et al. 2007a; Syldatk et al. 1985)
- Hydrocarbons; e.g., (Déziel et al. 1996; Syldatk et al. 1985)

Nitrogen, Minerals, and Iron Sources

Nitrate is the best nitrogen source for the induction of RLs production (e.g., Arino et al. 1996; Manresa et al. 1991; Mulligan and Gibbs 1989). For the induction of RL formation in a biotechnological set-up, an appropriate limitation must be achieved. For this purpose, the limitation of nitrogen, phosphorus, or multivalent ions in combination with an excess carbon are employed. Interestingly, nitrate as nitrogen source promotes RL production, while ammonium does not (Arino et al. 1996;

Guerra-Santos et al. 1986). As presented above, this is likely explained by regulatory factors (Déziel et al. 2003). Under anaerobic, denitrifying conditions with *P. aeruginosa* ATCC 10145 (28°C, pH 6.8, 2% (v/v) hexadecane), Chayabutra et al. (2001) showed that phosphorus limitation resulted in a four- to fivefold higher productivity as compared to a nitrogen-limited conditions. For RL production under batch and fed-batch conditions at 25°C and pH 7 with *P. aeruginosa* BYK-2, urea turned out to be the best nitrogen source in combination with fish oil as carbon source (Lee et al. 2004). Not only the type of carbon and nitrogen source but also the respective C/N ratios strongly influence total RL productivity (Guerra-Santos et al. 1984; Santa Anna et al. 2002; Wu et al. 2008). Guerra-Santos et al. showed that for *P. aeruginosa* DSM 2569 (37°C, pH 6.5, glucose, nitrate) C/N ratios between 16/1 and 18/1 lead to the highest RL productivity while no RLs could be observed at C/N ratios lower than 11/1 (Guerra-Santos et al. 1984, 1986). Apart from phosphorus and nitrogen limitations, restricted availability of multivalent ions like Mg, Ca, K, Na and trace element salts also often result in increased RL yields. For instance, highest final RL concentrations (30°C, pH 6.3, sunflower oil) were observed in calcium-free media (Giani et al. 1997).

Abalos et al. (2002) identified the carbon source, the nitrogen source, the phosphate content, and the iron content as critical factors for the medium when producing RLs with *P. aeruginosa* AT10. The maximum biodrymass of 12.06 g/L was obtained, when the medium contained 50 g/L carbon source, 9 g/L $NaNO_3$, 7 g/L phosphate, and 13.7 mg/L $FeSO_4 \cdot 7H_2O$. However, the maximum concentration of RLs, 18.7 g/L, was attained in medium that contained 50 g/L carbon source, 4.6 g/L $NaNO_3$, 1 g/L phosphate, and 7.4 mg/L $FeSO_4 \cdot 7H_2O$.

Low Cost Substrates (Nitrogen and Carbon Sources)

RLs can be considered fine chemicals, for example, for pharmaceuticals or cosmetics, or as bulk surfactants, for example, for cleaning products. For highly pure products, the product costs are mainly determined by the downstream processing. However, if high purity is not required, e.g., for bulk applications, the raw material costs are all-dominant and can amount to 50% of the overall production costs (Mulligan and Gibbs 1993). For example, in batch RL production processes, $Y_{P/S}$ of 0.13 up to 0.69 g/g have been reported, meaning that between 1.5 and 7.7 times more substrate is consumed than product is synthesized. Therefore, low-cost raw materials should be used. In general, less pure materials are less expensive and they are usually tolerated by the microorganisms. Crude materials or waste materials like soap stock, corn steep liquor, molasses, or nonrefined plant oils are promising carbon sources. On the other hand, this is not the case for the nitrogen source; Inorganic sources like ammonia, nitrate, or urea are generally less expensive than complex nitrogen sources like yeast extract, soybean meal, or casein, if comparing the price in terms of elemental nitrogen content. However, in this respect, corn steep liquor is an exception. This by-product of corn wet-milling is an important

constituent of many industrial growth media and is an excellent source of organic nitrogen.

Use of different waste substrates has already been reported, e.g., olive oil mill effluents (Mercade et al. 1993), waste frying oils (Haba et al. 2000), soap stock (Benincasa et al. 2002), or waste free fatty acids (Abalos et al. 2001). Additionally, the production of *P. aeruginosa* biosurfactants, most probably RLs, on whey and distillery waste was reported (Dubey et al. 2005).

4.1.4 Recovery of Rhamnolipids

Downstream processing can represent a significant proportion of the final cost of production of RLs. Most methods of recovery of RLs have been very well reviewed by Heyd et al. (2008). Methods range from those yielding mixtures of different RL congeners to those yielding specific congeners in pure forms. The criteria that govern the selection of a specific recovery method include: (1) the cost associated with the extraction method, which adds to the price of the final product, (2) the proposed purpose of the final product, which influences the level of purity required, and (3) the adaptability of the method to a particular industrial fermentation process. One of the simplest methods of recovery is by acid (Déziel et al. 1999b; Van Dyke et al. 1993; Zhang and Miller 1992) or aluminum sulfate precipitation (Schenk et al. 1995). Acid precipitation depends on acidification of RL to low pH (e.g., around 2), which neutralizes the negative charges on RLs, making them less soluble in the aqueous phase. Aluminum sulfate precipitates RLs by salting out. The precipitated RLs can then be recovered by centrifugation. Another more commonly used method is recovery by solvent extraction (Lépine et al. 2002; Mata-Sandoval et al. 1999; Schenk et al. 1995). In this method, molecules are precipitated by acidification and then extracted with organic solvents such as ether or ethyl acetate. Acidification is not a critical step in this method, but it enhances the net yield (Heyd et al. 2008).

Other methods adapted to downstream processing in continuous fermentative production processes include: adsorption (Dubey et al. 2005), ion exchange chromatography (Abadi et al. 2009; Reiling et al. 1986; Schenk et al. 1995), ultrafiltration (Häussler et al. 1998; Mulligan and Gibbs 1990), and foam fractionation (Gruber 1991; Sarachat et al. 2010). Adsorption methods are based on the use of hydrophobic adsorbent such as amberlite XAD 2 or 16 polystyrene resin that retain hydrophobic (or amphiphilic) substances through hydrophobic interactions. Adsorbed RLs are then released by elution, e.g., with methanol. Ion exchange chromatography exploits the fact that RLs behave as anions at high pHs, which allows their retention on columns of weak anion exchange resins such as (diethylamino)ethyl-sepharose. RLs are released from these resins by adding at least 0.6 M NaCl to the equilibration buffer. Yet, this method has been improved by Abadi et al. (2009), who applied phospholipid-coated colloidal magnetic nanoparticles ion exchange media for the recovery and purification of RLs from culture mixtures. Ultrafiltration with a membrane cutoff of 10 kDa leads to an almost complete

retention of RLs even at neutral pH. Foam fractionation depends on the foaming capabilities of RLs; the foam is directed out of the fermentation vessel to a fractionation column where it collapses in a separate receptacle by the action of acids or shear forces, The water in the film surface, known as the lamella, is then allowed to drain by gravitational force, causing a higher concentration of the surfactant in the collapsed foam (Heyd et al. 2008; Sarachat et al. 2010).

Most of the aforementioned methods result in the recovery of mixtures of different RLs congeners. Alternatively, chromatographic methods are usually the best solutions for separation of specific RL congeners in a pure form. These methods, however, work better after application of one of the extraction methods mentioned above. On the small scale, preparative TLC is a good choice (Monteiro et al. 2007; Sim et al. 1997); however, for large scale downstream processing, preparative column chromatography using silica gel is a better option (Burger et al. 1966; Monteiro et al. 2007). Recrystallization or repurification using TLC can be applied, if necessary (Heyd et al. 2008).

4.2 Alternatives to P. aeruginosa for Rhamnolipid Production

As *P. aeruginosa* is an opportunistic human pathogen, there have been several attempts to address the safety issues when producing RLs on a commercial scale. The two primary strategies are the heterologous production of RLs in nonpathogenic bacteria and the utilization of wild-type RL non-pathogenic producers other than *P. aeruginosa*.

4.2.1 Heterologous Production of Rhamnolipids

The heterologous production of RLs brings along two major advantages as compared to the production with *P. aeruginosa*. The first is the increased safety when handling large amounts of culture broths. The second is the possibility of constitutive RL production, in contrast to the very tightly regulated production in *P. aeruginosa*. Several attempts to produce *Pseudomonas* RLs in heterologous hosts have been reported. Yet, none produces RLs in comparable levels as the best *P. aeruginosa* strains. In view of a commercial production of RLs, there is still a huge potential for genetic optimization. Ochsner et al. (1995) cloned the *rhlAB* rhamnosyltransferase gene into various hosts, *Pseudomonas fluorescens*, *Pseudomonas oleovorans*, *Pseudomonas putida*, and *E. coli*. The best RL production was 60 mg/L and was achieved with *P. putida*, whereas no production was obtained with *E. coli*.

Cabrera-Valladares et al. (2006) succeeded in producing mono-RLs in *E. coli*. They found that the availability in *E. coli* of dTDP-L-rhamnose restricts the production of mono-RLs in this species. By coexpression of the *rhlAB* and the *rmlBDAC* operons, the latter encoding the dTDP-L-rhamnose biosynthesis enzymes, they

generated an RL-producing *E. coli* strain (productivity was 52.2 mg/L). In contrast, Wang et al. (2007) claimed RL production in *E. coli* expressing only *rhlAB*. Cha et al. (2008) reported the heterologous production of RLs in *P. putida*, using recombinant *rhlABRI* genes. A maximum yield of 7.2 g/L of RLs was achieved.

4.2.2 Non-*P. aeruginosa* Rhamnolipid Producers

Conventionally, *P. aeruginosa* is utilized as production strain for the production of RLs. However, RL-producing bacteria have been found in other species and genera as well. This topic was recently reviewed in details by Abdel-Mawgoud et al. (2010). Most RL-producing species belong to the closely related genera *Pseudomonas* and *Burkholderia* in the phylum proteobacteria (Walter et al. 2010). The genus *Burkholderia* arose from the genus *Pseudomonas* and was classified as a new genus in 1992 based on 16S rRNA sequence analysis (Yabuuchi et al. 1992). Consequently, bacteria of this genus have characteristics similar to *Pseudomonas*, and some species indeed produce RLs.

   B. glumae (formerly *Pseudomonas glumae*) (Pajarron et al. 1993), *B. plantarii*, (Andrä et al. 2006), *B. pseudomallei* (Dubeau et al. 2009; Häussler et al. 1998), and *B. thailandensis* (Dubeau et al. 2009) primarily produce one RL species, Rha-Rha-C_{14}-C_{14}. However, a number of other congeners were recently detected in cultures of the two latter (Dubeau et al. 2009), including mono-RLs, mostly Rha-C_{14}-C_{14}. These authors indeed confirmed the very high ratio of di-RLs vs. mono-RLs produced by these species, compared to what is observed in *P. aeruginosa* cultures (Dubeau et al. 2009). This is probably due to the fact that, as stated above, the *Burkholderia rhlC* genes encoding the second rhamnosyltransferase are part of the same operon than the *rhlA* and *RhlB* homologs, in contrast to the situation in *P. aeruginosa* (Dubeau et al. 2009).

   Furthermore, many RL producers belong to *Pseudomonas* species other than *P. aeruginosa* (Gunther et al. 2005, 2006; Oliveira et al. 2009; Onbasli and Aslim 2009). In contrast to the di-RLs of *Burkholderia* species, *Pseudomonas chlororaphis* synthesizes only RLs with one rhamnose unit and two hydroxy acyl moieties (Gunther et al. 2005). The absence of an *rhlC* gene homolog is proposed to explain this finding. RLs have been also detected in cultures of many other genera and species of widely different taxonomical origins. Isolates identified as *Acinetobacter calcoaceticus*, *Enterobacter* sp (Rooney et al. 2009), *Pseudoxanthomonas* sp. (Nayak et al. 2009), *Pantoea* sp. (Rooney et al. 2009; Vasileva-Tonkova and Gesheva 2007), *Renibacterium salmoninarum* (Christova et al. 2004), *Cellulomonas cellulans* (Arino et al. 1998b), *Nocardioides* sp. (Vasileva-Tonkova and Gesheva 2005), and *Tetragenococcus koreensis* (Lee et al. 2005) have been reported to produce RLs. However, for most of these strains, a structure determination of the putative RLs has not been accomplished, and sometimes, the actual identification of the producing strain is not firmly confirmed.

As *P. aeruginosa* is an opportunistic pathogen, another nonpathogenic species (Biosafety level 1) would represent a very interesting alternative – if sufficient RL yields can be obtained. The most prominent nonpathogenic RL producers from the genus *Pseudomonas* are *P. chlororaphis* (Gunther et al. 2005), *P. alcaligenes* (Oliveira et al. 2009), and *P. putida* (Martinez-Toledo et al. 2006; Tuleva et al. 2002), and from the genus *Burkholderia*, they are *B. glumae* (Pajarron et al. 1993), *B. plantarii* (Andrä et al. 2006), and *B. thailandensis* (Dubeau et al. 2009). Despite of the apparent safety advantage of these RL producers, very little is yet known about the biotechnological potential of these species.

5   Conclusion: Prospectives for the Industrial Production of Rhamnolipids

We have gained a wealth of knowledge on rhamnolipidic surfactants of microbial origin. Still, even though over 60 years have passed since their first description (Jarvis and Johnson 1949), RLs have not yet been significantly employed in the industry. Indeed, there is still a long way before achieving widespread bulk bioproduction of RLs, for both technical and economical reasons. Currently, the economic competitiveness of RLs against synthetic surfactants is mainly determined by the low productivity of the bioprocesses employed. However, this is beginning to change, as environmental compatibility becomes an increasingly important factor for the selection of industrial chemicals. Major improvements can be expected if more productive strains can be found and if a better understanding of the underlying regulation can be attained. In view of the complex quorum sensing-regulated induction of RL production in *P. aeruginosa*, further optimization will almost certainly be dependent on a more precise understanding of the mechanisms of regulation. It is expected that significant insights on the regulation and biosynthesis of RLs will be gained from the current systems biology approaches. There are good chances of success in the near future if a more integrated biotechnological approach is effectively adopted for strain and process development. Additionally, the use of new heterologous RL-producing hosts will help to broaden the product spectrum and make it possible to produce single RL congeners.

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