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MINI-REVIEW

On the road towards tailor-made rhamnolipids: current state and perspectives

Andreas Wittgens[1,2] · Frank Rosenau[1,2,3]

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Abstract

Rhamnolipids are biosurfactants with an enormous potential to replace or complement classic surfactants in industrial applications. They consist of one or two L-rhamnose residues linked to one or two 3-hydroxyfatty acids of various chain lengths, which can also contain unsaturated carbon-carbon bonds, yielding a wide variety of different structures each with its specific physicochemical properties. Since different applications of surfactants require specific tenside characteristics related to surface tension reduction, emulsification, and foaming etc., rhamnolipids represent a platform molecule which harbors an enormous potential to adopt tailor-made properties to meet a huge variety of demands of surfactants for food-, healthcare-, and biotechnological applications. We are here giving an overview on current technology to synthesize tailor-made rhamnolipids based on the biotechnological use of different enzymes responsible for rhamnolipid biosynthesis originating from different naturally rhamnolipid-producing microorganism. Furthermore, we present future strategies to determine the number of L-rhamnose and 3-hydroxyfatty acids as well as their specific chain lengths and unsaturations to produce customized rhamnolipids perfectly tuned for every application.

Keywords Rhamnolipids · Biosurfactant · *Pseudomonas* · *Burkholderia* · Biosynthesis pathway

Introduction

Biosurfactants possess outstanding characteristics compared to customary or "conventional" tensides, which are normally synthesized by chemical processes and mainly based on petrochemical and thus fossil resources. In contrast to their conventional counterparts, they are of almost exclusive microbiological origin, they are produced from renewable substrates, show lower toxicity, and are highly or even perfectly biocompatible and biodegradable (Desai and Banat 1997; Van Hamme et al. 2006; Hirata et al. 2009; Lima et al. 2011; Johann et al. 2016). In addition to their eco-friendly and sustainable characteristics, examples exist which provide better foaming properties and—most important for a broad application potential—they especially have remarkable stabilities against extreme pH values, temperatures, and salt concentrations in contrast to many of the traditional surfactants (Pruthi and Cameotra 1997; Makkar and Cameotra 1998; Nitschke and Pastore 2006; Abdel-Mawgoud et al. 2008; Banat et al. 2010).

The term "biosurfactant" denominates a large variety of different molecules with extremely diverse chemical structures particularly produced by different microorganisms, e.g., the lipopeptide surfactin from *Bacillus subtilis* (Arima et al. 1968; Haddad et al. 2009; Dhali et al. 2017), the polymeric emulsan from *Acinetobacter calcoaceticus* (Rosenberg and Ron 1997; Johri et al. 2002; Chamanrokh et al. 2008), or more recently experiencing greater attention the class of glycolipids like mannosylerythritol lipids (MELs) from the genus *Pseudozyma* (Rau et al. 2005; Fukuoka et al. 2007; Saika et al. 2018), trehalose lipids from *Rhodococcus erythropolis* (Peng et al. 2007; Marqués et al. 2009; Luong et al. 2018), or sophorolipids produced by the yeasts of the genus *Candida* (Van Bogaert et al. 2011; Chandran and Das 2012; Konishi et al. 2018).

Furthermore, apart from aforementioned biosurfactants, another particular glycolipid is of considerable biotechnological

✉ Andreas Wittgens
andreas.wittgens@uni-ulm.de

[1] Institute for Pharmaceutical Biotechnology, Ulm-University, Albert-Einstein-Allee 11, 89081 Ulm, Germany

[2] Ulm Center for Peptide Pharmaceuticals, Albert-Einstein-Allee 47, 89081 Ulm, Germany

[3] Synthesis of Macromolecules, Max-Planck-Institute for Polymer Research Mainz, Ackermannweg 10, 45128 Mainz, Germany

interest and has therefore been intensively investigated in the past, which are designated the so-called rhamnolipids. Rhamnolipids (RL) were first described nearly 70 years ago by Jarvis and Johnson (1949) as an oily extract secreted by the human-pathogen *Pseudomonas aeruginosa*. As a molecule with surface-active properties, they possess the typical amphiphilic molecule character represented in this class of either one or two of the name giving L-rhamnose molecules as the hydrophilic part linked through a β-glycosidic bond to one or—in most molecules—two 3-hydroxyfatty acids as the hydrophobic part (Fig. 1; Soberón-Chávez et al. 2005). Since the nomenclature for different rhamnolipid species was inconsistent in the past and different congeners remained unconsidered, we here suggest novel terms and abbreviations to name (new) molecules more precisely but also very easily based on their number of rhamnose residues, their chain lengths, and by the fact whether they contain saturated or unsaturated fatty acids. Based on the number of L-rhamnose sugar residues, the rhamnolipids are classified into mono- and di-rhamnolipids and the number of fatty acids allows a further sub-classification into so-called mono- and di-rhamno-di-lipids (mRdL and dRdL) with two and mono- and di-rhamno-mono-lipids (mRmL and dRmL), respectively, with only one fatty acid chain representing the four major native rhamnolipid species (Syldatk et al. 1985a; Abdel-Mawgoud et al. 2010). Within these species, various congeners have been identified varying in lengths of both 3-hydroxyfatty acid chains typically ranging from C_8 to C_{16} and being a characteristic "fingerprint" mainly depending on the genus of their bacterial producer. Thus, rhamnolipids possess a basic structural diversity, which can then be further expanded by introduction of one or more unsaturated carbon-carbon bonds in one of the fatty acids or in both of them (Abalos et al. 2001; Wittgens et al. 2018). Over the years of rhamnolipid research, more than 60 different congeners have been described featuring fundamentally different physicochemical properties and characteristics, which offers a unique potential to selectively use individual molecules in a variety of biotechnological and industrial applications (Abdel-Mawgoud et al. 2010; Chong and Li 2017).

The biosynthesis of rhamnolipids requires consecutive enzymatic reactions by using only two types of metabolites

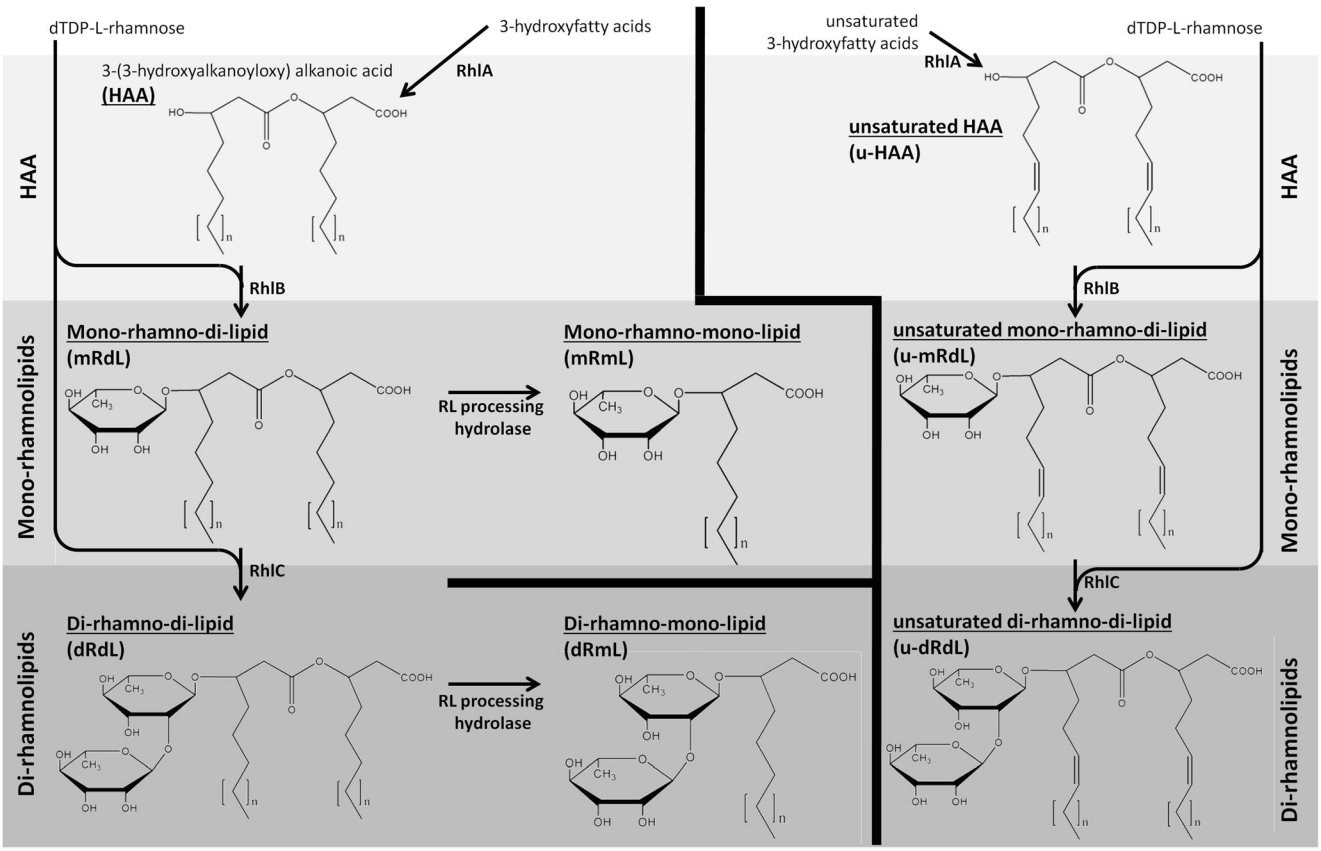

Fig. 1 Chemical structures and biosynthesis of rhamnolipids. The biosynthesis of rhamnolipids occurs in consecutive enzymatic reactions. RhlA synthesizes HAAs by esterification of two 3-hydroxyfatty acids. The rhamnosyltransferases RhlB and RhlC link an HAA molecule first with one and subsequently with a second dTDP-L-rhamnose to generate rhamnolipids. Based on the number of L-rhamnose residues, rhamnolipids are separated into mono- and di-rhamnolipids. Typical rhamnolipid species containing two 3-hydroxyfatty acids (mono-rhamno-di-lipids and di-rhamno-di-lipids) can be processed by hydrolases to create mono-rhamno-mono-lipids and di-rhamno-mono-lipids containing only one fatty acid chain. Unsaturated HAAs and rhamnolipids are most probably synthesized by using unsaturated 3-hydroxyfatty acids. The fatty acids chain lengths of rhamnolipids typically vary between C_8 and C_{16}

originating from the central carbon metabolism (Fig. 1). Initially, the acyltransferase RhlA catalyzes the esterification of two 3-hydroxyacyl molecules and synthesizes 3-(3-hydroxyalkanoyloxy)alcanoic acids (HAAs) as typical precursors. The origin of the 3-hydroxyfatty acids and whether they are bound to the acyl carrier protein (ACP) or to coenzyme A (CoA) are controversially discussed. Zhu and Rock (2008) described an in vitro HAA production using purified RhlA and 3-hydroxydecanoyl-ACP indicating a preference of RhlA for this intermediate from the fatty acid de novo synthesis (Rehm et al. 2001), whereas no HAA formation could be detected using 3-hydroxydecanoyl-CoA as substrate. However, Zhang et al. (2012) described an involvement of the β-oxidation in providing substrates for HAA biosynthesis depending on the used carbon source and they suggested a bypass route to recruit β-oxidation intermediates into the fatty acid de novo synthesis by an unknown β-ketoacyl-ACP synthase. Most recently, Abdel-Mawgoud et al. (2014) characterized 3-hydroxyacyl-CoA as the preferred substrate for HAA production using isotope-labeled carbon sources and *rhlYZ* mutant strains. RhlY and RhlZ are responsible for the conversion of trans-2-enoyl-CoA from the β-oxidation to *R*-3-hydroxyacyl-CoA. They also postulated a linkage between the fatty acid de novo synthesis and the β-oxidation to transfer ACP bound fatty acids to CoA as the central cofactor. The second key enzyme in rhamnolipid biosynthesis, the rhamnosyltransferase I (RhlB), synthesizes mono-rhamnolipids by linking a dTDP-L-rhamnose descending from glucose-6-phosphate (Olvera et al. 1999; Rahim et al. 2000) with an HAA molecule (Ochsner et al. 1994a; Wittgens et al. 2017). Finally, di-rhamnolipids are synthesized by the rhamnosyltransferase II (RhlC) by adding a second molecule of dTDP-L-rhamnose to the preformed mono-rhamnolipids (Rahim et al. 2001). The biosynthesis of mono-rhamno-mono-lipids and di-rhamno-mono-lipids representing a "processed" product lacking one of the fatty acid residues of a complete molecule most probably occurs by two specific but yet unknown α/β-hydrolases, which, taking into account the chain lengths of the substrate esters, reasonably have to be members of the esterase or even lipase family of hydrolases each dedicated to processing of one rhamnolipid species containing two fatty acid chains by removing the second 3-hydroxyacyl (Wittgens et al. 2017).

Different rhamnolipids can already be produced biotechnologically using recombinant genes or biosynthetic operons in the same host organism. Based on their structural diversity and the principle possibility to be targets of enzymatic modifications, rhamnolipids are developing into a platform molecule similar to the biologicals in the pharmaceutical industry. In the near future, processes will be available to produce tailor-made rhamnolipids and to freely choose their number of L-rhamnose sugars in combination with the amount of 3-hydroxyfatty acids, their chain lengths, and the degree of saturation (or unsaturation) allowing to customize properties for any type of specific applications. In this overview, we describe current opportunities for the biosynthesis of specific rhamnolipids using different microbial producer strains and variations of *rhl*-genes and we discuss appearing future strategies including genetic enzyme evolution, optimization of selective purification technologies, and options for chemical and enzymatic modification of rhamnolipids, which may play promising roles to achieve real tailor-made rhamnolipids.

Rhamnolipids in industrial applications

Through the years, numerous applications were investigated and suggested, in which rhamnolipids can replace or complement "conventional" surfactants or which only become possible through the use of rhamnolipids. Traditional applications are especially their use in laundry or dishwashing detergents and cleaning agents (Nguyen and Sabatini 2011). Because of their abilities to form highly stable emulsions, they are also of great interest for applications in cosmetics and food (Klekner and Kosaric 1993; Velikonja and Kosaric 1993; Maier and Soberón-Chávez 2000; Nitschke and Costa 2007; Sinumvayo and Ishimwe 2015). Large-scale applications are the so called microbial enhanced oil recovery (MEOR), where the use of rhamnolipids can significantly increase the amount of recovered oil (Zhang and Zhang 1993; Wang et al. 2007; Al-Sulaimani et al. 2011; Sharma et al. 2018), and the bioremediation to remove crude oil, heavy metals, and other toxic compounds from contaminated soils and waters (Nguyen et al. 2008; Van Hamme and Urban 2009; Wang and Mulligan 2009; Liu et al. 2018). In the agriculture, rhamnolipids can be used as pesticides due to their antimicrobial properties or they increase the plants' nutrient uptake (Stacey et al. 2008; Vatsa et al. 2010; Sha et al. 2011; Chen et al. 2017). Another large field of application is the pharmaceutical industry or biomedicine, where rhamnolipids can be used for example to improve the treatment against bacteria, viruses, and fungi or the wound healing (Irie et al. 2005; Remichkova et al. 2008; Stipcević et al. 2006; Piljac et al. 2008; Chen et al. 2017).

Generally, all these applications pose different requirements to the tenside properties relating to the reduction of surface tension, the ability to form highly stable aggregates, foaming, emulsifying, thickening, and solubilizing etc. These characteristics are strongly dependent on the chemical structure, in the case of rhamnolipids, on the numbers of L-rhamnose and 3-hydroxyfatty acids and the chain lengths, so that every rhamnolipid congener possesses different physicochemical and surface-active properties (Howe et al. 2006; Kłosowska-Chomiczewska et al. 2017).

However, all proposed applications so far are based on the usage of rhamnolipids from *P. aeruginosa* species comprising a mixture of mono- and primary di-rhamnolipids with various

congeners, since only rhamnolipids from this human-pathogen were available in larger quantities in the past. Therefore, an enlargement of the available rhamnolipid portfolio, which means the production of tailor-made rhamnolipids including specific species with defined chain lengths of their fatty acids, will be a great advantage and will open up unexpected fields of industrial applications for these remarkable biosurfactants.

Tailor-made rhamnolipids—today's and tomorrow's challenges

Rhamnolipid-producer strains and their specific chain length

The human-pathogen organism *Pseudomonas aeruginosa* is among the most potent and best characterized native rhamnolipid producer and able to yield titers of 40 g/L rhamnolipid in a bioreactor (Müller et al. 2010). One single publication reported rhamnolipid titers of more than 100 g/L by using the specific strain DSM7108 (Giani et al. 1997), but this record was never reproduced successfully (Müller et al. 2011). While *P. aeruginosa* typically synthesizes a heterogenous mixture of various rhamnolipids species containing fatty acids with a predominant C_{10} short chain length (sc-RL), bacteria from the genus *Burkholderia* produce long-chain rhamnolipids (lc-RL) with a predominant C_{14} species. Among these bacteria are prominent human pathogens like *B. pseudomallei* (Häußler et al. 1998, 2003) or species like *B. plantarii* (Andrä et al. 2006; Hörmann et al. 2010) and *B. glumae* (Manso Pajarron et al. 1993; Costa et al. 2011), which are at least plant pathogens and for example cause wilt in many economically important crops and panicle blight in rice constituting an increasing global important problem (Jeong et al. 2003; Ham et al. 2011). However, there are also examples for naturally non or less pathogenic lc-RL producers like *B. thailandensis* (Dubeau et al. 2009; Funston et al. 2016; Elshikh et al. 2017) or *B. kururiensis* (Tavares et al. 2012). However, all *Burkholderia* species described so far failed to achieve productivities comparable with those of *P. aeruginosa* and the highest titer reported was about 1 to 3 g/L (Costa et al. 2011; Díaz De Rienzo et al. 2016; Funston et al. 2016).

Handling known pathogens or at least bacteria with a considerable but undefined pathogenic potential as it exists for "exotic" isolates represents another principle disadvantage of using wild-type organisms for the production of rhamnolipids at an industrial scale, since safety risks and very limited acceptance by customers prevent healthcare applications and the use in cosmetics and foods (Toribio et al. 2010; Müller and Hausmann 2011). Also, non-pathogenic rhamnolipid producer are a poor choice, since the rhamnolipid biosynthesis in all native producer strains is strongly genetically regulated by a complex regulatory network consisting of the cell-density-dependent *quorum sensing* (Ochsner et al. 1994b; Ochsner and Reiser 1995; Pearson et al. 1997; Nickzad et al. 2015) and probably further signaling mechanisms (Wilhelm et al. 2007; Rosenau et al. 2010; Henkel et al. 2013), which severely hamper an easy and focused overproduction of rhamnolipids in wild-type strains (Toribio et al. 2010). As a consequence, tremendous efforts have been made to genetically optimize native rhamnolipid producers (Grosso-Becerra et al. 2016) or to establish heterologous production of rhamnolipids employing different host organism. The use of *Escherichia coli* proved to be rather limited, because it provides the dTDP-L-rhamnose as precursor for the rhamnolipid biosynthesis only in insufficient trace amounts (Cabrera-Valladares et al. 2006). More successful approaches were based on the use of *Pseudomonas fluorescence* or *Saccharomyces cerevisiae* (Ochsner et al. 1995; Bahia et al. 2018); however, currently, the most promising host is *Pseudomonas putida* (Ochsner et al. 1995; Cha et al. 2008; Wittgens et al. 2011; Cao et al. 2012; Tiso et al. 2016; Beuker et al. 2016a). This non-pathogenic and genetically perfectly accessible strain is meanwhile well established and recombinant strains can produce rhamnolipids at concentrations of up to 15 g/L (Beuker et al. 2016b). Using a heterologous host as a universal genetic and metabolic background further provides the principle opportunity to introduce synthetic and specific biosynthesis pathways for mono-rhamnolipids by expressing the *rhlAB* operon or a mixture of mono- and di-rhamnolipids by expressing a biosynthetic *rhlABC* operon from plasmid constructs allowing to freely choose and optimize appropriate expression levels for example by introducing libraries of synthetic promoters (Wittgens 2013). Furthermore, *P. putida* provides a powerful and flexible metabolic and physiological background allowing the specific biosynthesis of both, sc-RL by expressing genes originating from *P. aeruginosa* as well as of lc-RL using *rhl*-genes from *B. glumae* (Wittgens et al. 2018). Based on the possibility to produce rhamnolipids of different chain lengths in *P. putida* as a platform production strain, it is an attractive option to enlarge the portfolio of rhamnolipids by using synthetic enzymes of other bacterial species (Fig. 2). An interesting class which we want to call very short-chain rhamnolipids (vsc-RL) has been described for *Pseudomonas desmolyticum* containing a predominant species with C_6-C_8 fatty acids (Jadhav et al. 2011), while some thermophilic bacteria of the genus *Thermus* appear to be producers of rhamnolipids which represent extreme counterparts at the opposite scale with very long-chain lengths of rhamnolipids (vlc-RL) with fatty acids up to C_{24} (Řezanka et al. 2011). Moreover, the increasing availability of microbial genome information allows identification of so far only putative rhamnolipid biosynthetic genes, which may easily be characterized for their products in the recombinant *P. putida* system without the need to establish

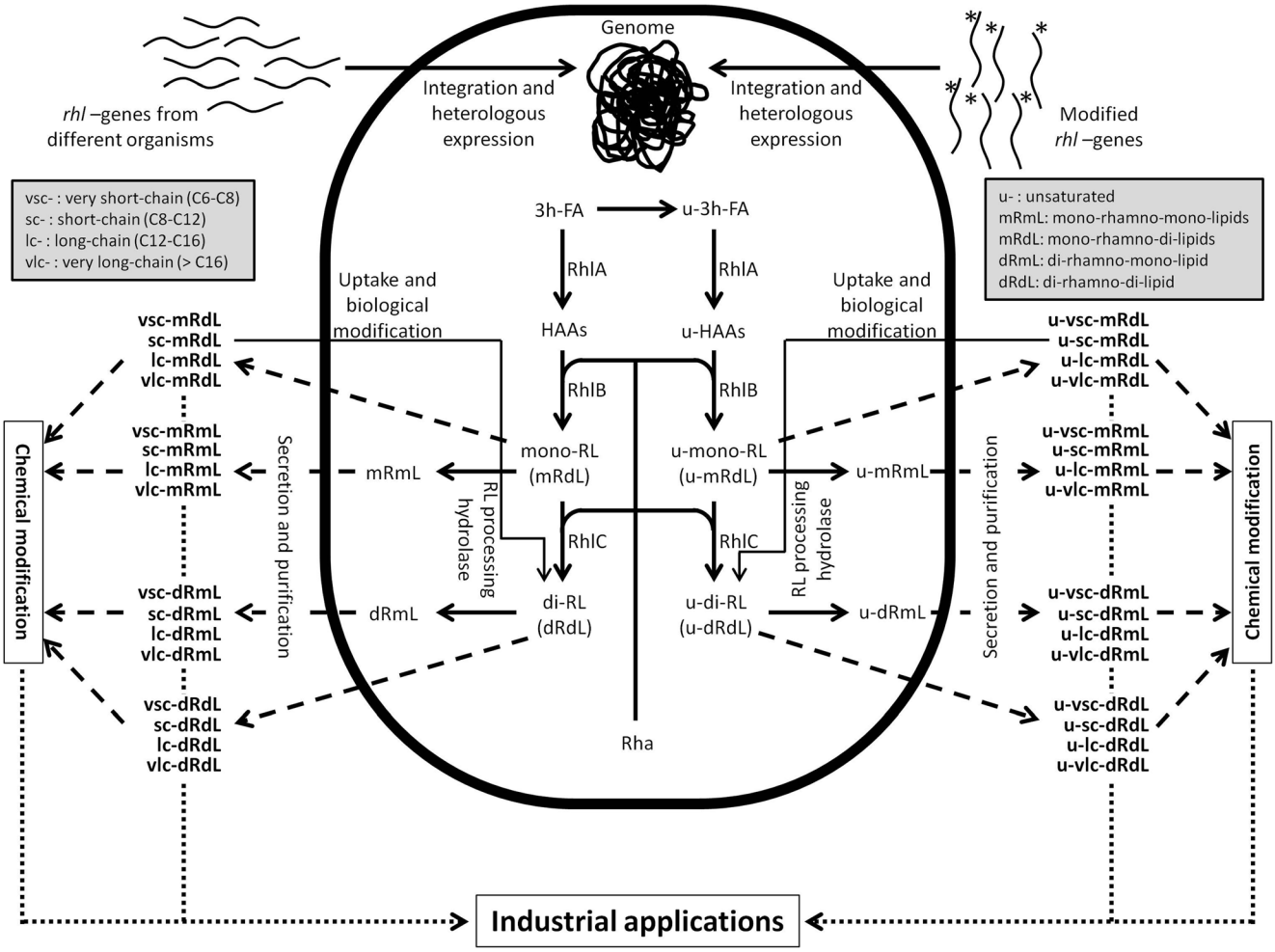

Fig. 2 Strategies for the production of tailor-made rhamnolipids. Native or modified *rhl*-genes from different organisms are introduced in various combinations to determine the numbers of rhamnose residues and the fatty acid chain lengths of the rhamnolipids (RL). Rhamnolipids are synthesized using dTDP-L-rhamnose (Rha) and 3-hydroxyfatty acids (3h-FA) for saturated or u-3h-FA for unsaturated rhamnolipids (u-RL). Mono- and di-rhamno-di-lipids (mRdL and dRdL) can be further processed by specific hydrolases to generate mono- and di-rhamno-mono-lipids (mRmL and dRmL). Single congeners of very-short-chain (vsc-) to very-long-chain (vlc-) rhamnolipids can be obtained using optimized Rhl enzymes or can be enriched by purification after their secretion. All rhamnolipid congeners can be further customized by chemical modifications. The mRdL (native or chemical modified) can be taken up into the cell to undergo a biological modification by a subsequent conversion to di-RL. Therefore, numerous specialized rhamnolipids will be available for every industrial application

cultivation or optimization of growth conditions of the bacterial host to allow biochemical characterization of their products.

Enzyme design for specificity improvement

Current strategies for the production of rhamnolipids are based on the expression of unmodified wild-type *rhl*-genes and operons of them, which result in biosynthesis of heterogenic mixtures of various rhamnolipid species and congeners. A breakthrough, however, and therefore a more than desirable objective to obtain would be to establish tools to optimize rhamnolipid production towards more homogenous preparations already in the process and in the fermenter with ideally only one specific congener of rhamnolipids (i.e., defined chain lengths) being present in the culture medium. A powerful technology to optimize biosynthetic enzymes and pathways is the genetic modification of the respective genes or operons by introducing random or semi-rational alterations into their DNA sequences using methods of the so-called directed or laboratory evolution (Arnold 1996; Martínez and Schwaneberg 2013; Packer and Liu 2015; Reetz 2016). For the biotechnology of rhamnolipids, the logical main target to optimize is RhlA, because this acyltransferase has been shown to be responsible for the chain length selectivity in the biosynthesis of HAA by dimerization of two 3-hydoxyfatty acids and thus RhlA defines the chain length of resulting rhamnolipids. In hybrid *rhlAB* operons with genes from *P. aeruginosa* and *B. glumae* exclusively, the origin of *rhlA* determined if the resulting rhamnolipids contained short-chain or long-chain 3-hydroxyfatty acids (Wittgens et al. 2018).

Optimization of the biosynthetic can in principle be done by mutagenesis in pure random approaches to introduce undirected DNA mutations or as more focused or directed approaches after bioinformatic identification and subsequent manipulation of important enzyme regions or domains, which are responsible for the selection and binding of fatty acids. Both approaches include the alternation of single amino acids or the replacement of entire domains to generate mutant variant or chimeric enzymes with parts originating from different organism. The aim of these efforts is to generate an acyltransferase, which has a much higher specificity for specific fatty acid chain lengths to enrich the desired single predominant rhamnolipid congeners to almost pure preparations already during the fermentation or to shift the specificity of the optimized biosynthetic pathway to a specific but so far uncommon or even unrealized chain length in rhamnolipids.

Rhamnolipid modifying enzymes

One further demand when talking about true tailor-made rhamnolipids and the development of molecules with presumably completely new physicochemical properties is to create an option allowing to freely define the number of fatty acid chains in the rhamnolipid molecule. Apart from the typical rhamnolipids containing two 3-hydroxyfatty acids (mono-rhamno-di-lipids and di-rhamno-di-lipids), also mono-rhamno-mono-lipids and di-rhamno-mono-lipids species have been described containing only a single fatty acid chain (Fig. 1; Syldatk et al. 1985b; Abdel-Mawgoud et al. 2010). Using resting cells of the strain *P. aeruginosa* DSM2874 or with naphthalene as the carbon source, the amount of mono-rhamno-mono-lipids and di-rhamno-mono-lipids could be increased up to almost 80% of total rhamnolipid amount (Syldatk et al. 1985a; Déziel et al. 1999). The formation of these rare rhamnolipid species has been reasoned to occur after the known route of rhamnolipid biosynthesis by the Rhl enzymes through removal of one fatty acid chain by a putative rhamnolipid processing hydrolase most probably belonging to the lipase/esterase enzyme family. The formation of mono-rhamno-mono-lipids and di-rhamno-mono-lipids does not occur as a side reaction by the rhamnosyltransferase I, since RhlB only utilize HAAs, but no single 3-hydroxyfatty acids for the biosynthesis of rhamnolipids, as it was shown in experiments with a single *rhlB* expressing strain and HAAs containing spend media (Wittgens et al. 2017). Due to the fact that this processing step appeared only for mono-rhamno-mono-lipids but not for di-rhamno-mono-lipids which could not be found in *P. putida* after heterologous expression of *rhl*-genes, it is reasonable to suspect that *P. putida* harbors only a mono-RL processing hydrolase, whereas *P. aeruginosa* has at least two of these enzymes, which process either mono-RL or di-RL (Wittgens et al. 2017, 2018). The identification of rhamnolipid processing hydrolases is currently carried out by a systematic co-expression study with more than 40 candidate hydrolases identified in the genomes of *P. putida* and *P. aeruginosa* followed by characterization of their influence on single-chain rhamnolipid formation (Wittgens et al. unpublished). In the case of success, this would be a very easy way to add novel molecules to the portfolio of rhamnolipids and would probably also result in new applications for these novel surfactants (Fig. 2). The identification of further rhamnolipid modifying enzymes is also of importance, which may have similarities to the fungal naringinase from *Aspergillus* sp., which can remove the L-rhamnose residue from di- and mono-rhamnolipids and finally generates HAA and L-rhamnose (Trummler et al. 2003). The L-rhamnose is of special interest as a fine chemical for cosmetics and food (Linhardt et al. 1989; Mixich et al. 1990; Giani et al. 1997), while HAAs as a molecule closely related to the rhamnolipids show surface-active properties itself and thus also could be interesting for industrial applications (Déziel et al. 2003).

Rhamnolipid congener enrichment via purification

Apart from strategies to increase the biosynthesis of specific rhamnolipid congeners, the production of tailor-made rhamnolipids can also be achieved during the downstream processing by enrichment of specific rhamnolipids using special purification conditions (Fig. 2). While common purification processes are targeted to recover as much rhamnolipids as possible depending on the desired purity (Mixich et al. 1997; Witek-Krowiak et al. 2011; Heyd et al. 2008; Müller et al. 2012), some technical opportunities were introduced for more specific rhamnolipid purifications. Manso Pajarron et al. (1993) used a crystallization process using n-hexane for a specific purification of the Rhl-Rhl-C_{10}-C_{10} di-rhamnolipids. Most chromatographic separation processes from thin- or thick-layer chromatography to HPLC are generally used for the identification of single pure rhamnolipid species or congeners (Mata-Sandoval et al. 1999; Déziel et al. 2000; Monteiro et al. 2007; Behrens et al. 2016). However, Tiso et al. (2018) recently present a strategy for the purification of rhamnolipids using a liquid chromatography on a larger scale, which performs excellent especially for mono-rhamnolipids. During the elution process using an increasing amount of ethanol, various fractions containing the mono-rhamnolipid congeners Rha-C_8-C_{10}, Rha-C_{10}-C_{10}, Rha-C_{10}-C_{12}, and Rha-C_{10}-$C_{12:1}$, which successivly exited the chromatography column, were collected. Some fractions contained one mono-rhamnolipid congener in almost pure quality, and others were highly enriched with a specific congener or contained mixtures of up to three of these congeners. Probably, this method can also be adapted for the enrichment of other rhamnolipid species and congeners, e.g., those with longer or single fatty acid chains.

Increased production of unsaturated rhamnolipids

Extending the repertoire of the fatty acids would create an enormous increase in rhamnolipid diversity. This includes mono- or polyunsaturated fatty acids which have been described in one or both fatty acids of the final molecule (Abalos et al. 2001; Abdel-Mawgoud et al. 2010; Wittgens et al. 2018). Since unsaturated fatty acids are known to be already present in the rhamnolipid precursors HAAs (Lépine et al. 2002; Déziel et al. 2003), it can be assumed that the unsaturated 3-hydroxyfatty acids serve as substrate for RhlA, too (Figs. 1 and 2). Subsequently, the resulting unsaturated HAAs (u-HAAs) are then—like their normal counterparts with saturated fatty acids—presumably used by the two rhamnosyltransferases RhlB and RhlC for the biosynthesis of unsaturated mono-rhamnolipids (u-mono-RL) and finally di-rhamnolipids (u-di-RL). Under standard cultivation conditions, unsaturated rhamnolipids represent only a minor fraction of total rhamnolipid congeners (Déziel et al. 1999; Haba et al. 2003; Costa et al. 2011), but it is to be expected that especially unsaturated rhamnolipids offer novel surfactant properties due to their unique structure in comparison to saturated rhamnolipids. Consequently, the intracellular amount of unsaturated 3-hydroxyfatty acids has to be increased to finally earn a higher amount of unsaturated rhamnolipids (u-RL). For realizing this idea, genetic and metabolic modifications could be done to ensure an improved expression of specific desaturases to shift the ratio between saturated and unsaturated 3-hydroxyfatty acids. The implementation of exceptional cultivation conditions and/or special feeding strategies may also contribute to increase the amount of available unsaturated fatty acids for the rhamnolipid production. Other possibilities to enrich the u-RL could be possibly achieved by an extensive enzyme optimization of RhlA to improve its specificity towards unsaturated 3-hydroxyfatty acids or by adapting the purification protocol to yield higher amounts of u-RL.

Chemical modification of rhamnolipids

Rhamnolipids can also be produced by chemical synthesis using the concept of hydrophobically assisted switching phase synthesis followed by further chemical modifications (Howe et al. 2006), which reveals totally artificial rhamnolipids containing a third rhamnose residue (tri-rhamnolipids) or a third 3-hydroxyfatty acid chain (mono- and di-rhamno-tri-lipids), uncommon C_4 or C_{18} fatty acids or an exchange of the carboxy against a hydroxy group at the C_1 atom of the former 3-hydroxyfatty acids. These changes in chemical structures effect a dramatic change in the physicochemical characteristics of the rhamnolipids with respect to the formation of aggregates, bioactivity, and fluidity (Howe et al. 2006). While an exclusive chemical synthesis is disproportionate complex and expensive, biological synthesis of rhamnolipids followed by a chemical modification appears to be very attractive to generate highly customized rhamnolipids for specialized applications (Fig. 2). Except attaching further rhamnose or fatty acid moieties, such modifications could include the insertion or exchange of functional chemical groups to shift the ratio between the hydrophilic and hydrophobic molecule domain and could open up totally unexpected properties. Based on the findings that secreted HAAs and mono-RL can be taken up by the cell again and follow the biosynthesis route towards di-rhamnolipids, which was shown using HAA and mono-RL containing spend media (Wittgens et al. 2017), a chemical modification of these molecules followed by a subsequent biosynthesis of mono- and/or-di-rhamnolipids will be further possible.

Conclusion

Already today, the production of several rhamnolipid species and congeners is possible through the use of different producer strains or more preferable the heterologous expression of their responsible genes to achieve a mixture of either short-chain or long-chain rhamnolipids. By using variations of rhamnolipid synthesizing enzymes, the specific production of mono- and di-rhamnolipids is also possible.

The final steps towards real tailor-made rhamnolipids will be the identification of rhamnolipid processing enzymes for the targeted synthesis of mono- and di-rhamno-mono-lipids as well as an enzyme and purification optimization to enrich specific rhamnolipid congeners containing defined chain length and unsaturations. Further strategies include the enlargement of rhamnolipid portfolio with more chain lengths (vsc- and vlc-RL) and potential chemical modifications.

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Compliance with ethical standards

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

Ethical approval This article does not contain any studies with animals performed by any of the authors.

References

Abalos A, Pinazo A, Infante MR, Casals M, García F, Manresa A (2001) Physicochemical and antimicrobial properties of new rhamnolipids produced by *Pseudomonas aeruginosa* AT10 from soybean oil refinery wastes. Langmuir 17:1367–1371. https://doi.org/10.1021/la0011735

Abdel-Mawgoud AM, Aboulwafa MM, Hassouna NA-H (2008) Characterization of surfactin produced by *Bacillus subtilis* isolate BS5. Appl Biochem Biotechnol 150:289–303. https://doi.org/10.1007/s12010-008-8153-z

Abdel-Mawgoud AM, Lépine F, Déziel E (2010) Rhamnolipids: diversity of structures, microbial origins and roles. Appl Microbiol Biotechnol 86:1323–1336. https://doi.org/10.1007/s00253-010-2498-2

Abdel-Mawgoud AM, Lépine F, Déziel E (2014) A stereospecific pathway diverts β-oxidation intermediates to the biosynthesis of rhamnolipid biosurfactants. Chem Biol 21:156–164. https://doi.org/10.1016/j.chembiol.2013.11.010

Al-Sulaimani H, Joshi S, Al-Wahaibi Y, Al-Bahry S, Elshafie A, Al-Bemani A (2011) Microbial biotechnology for enhancing oil recovery: current developments and future prospects. Biotechnol Bioinf Bioeng 1:147–158

Andrä J, Rademann J, Howe J, Koch MHJ, Heine H, Zähringer U, Brandenburg K (2006) Endotoxin-like properties of a rhamnolipid exotoxin from *Burkholderia* (*Pseudomonas*) *plantarii*: immune cell stimulation and biophysical characterization. Biol Chem 387:301–310. https://doi.org/10.1515/BC.2006.040

Arima K, Kakinuma A, Tamura G (1968) Surfactin, a crystalline peptidelipid surfactant produced by *Bacillus subtilis*: isolation, characterization and its inhibition of fibrin clot formation. Biochem Biophys Res Commun 31:488–494

Arnold FH (1996) Directed evolution: creating biocatalysts for the future. Chem Eng Sci 51:5091–5102. https://doi.org/10.1016/S0009-2509(96)00288-6

Bahia FM, de Almeida GC, de Andrade LP, Campos CG, Queiroz LR, da Silva RLV, Abdelnur PV, Corrêa JR, Bettiga M, Parachin NS (2018) Rhamnolipids production from sucrose by engineered *Saccharomyces cerevisiae*. Sci Rep 8:2905. https://doi.org/10.1038/s41598-018-21230-2

Banat I, Franzetti A, Gandolfi I, Bestetti G, Martinotti M, Fracchia L, Smyth T, Marchant R (2010) Microbial biosurfactants production, applications and future potential. Appl Microbiol Biotechnol 87:427–444. https://doi.org/10.1007/s00253-010-2589-0

Behrens B, Engelen J, Tiso T, Blank LM, Hayen H (2016) Characterization of rhamnolipids by liquid chromatography/mass spectrometry after solid-phase extraction. Anal Bioanal Chem 408:2505–2514. https://doi.org/10.1007/s00216-016-9353-y

Beuker J, Barth T, Steier A, Wittgens A, Rosenau F, Henkel M, Hausmann R (2016a) High titer heterologous rhamnolipid production. AMB Express 6:124. https://doi.org/10.1186/s13568-016-0298-5

Beuker J, Steier A, Wittgens A, Rosenau F, Henkel M, Hausmann R (2016b) Integrated foam fractionation for heterologous rhamnolipid production with recombinant *Pseudomonas putida* in a bioreactor. AMB Express 6:11. https://doi.org/10.1186/s13568-016-0183-2

Cabrera-Valladares N, Richardson AP, Olvera C, Treviño LG, Déziel E, Lépine F, Soberón-Chávez G (2006) Monorhamnolipids and 3-(3-hydroxyalkanoyloxy)alkanoic acids (HAAs) production using *Escherichia coli* as a heterologous host. Appl Microbiol Biotechnol 73:187–194. https://doi.org/10.1007/s00253-006-0468-5

Cao L, Wang Q, Zhang J, Li C, Yan X, Lou X, Xia Y, Hong Q, Li S (2012) Construction of a stable genetically engineered rhamnolipid-producing microorganism for remediation of pyrene-contaminated soil. World J Microbiol Biotechnol 28:2783–2790. https://doi.org/10.1007/s11274-012-1088-0

Cha M, Lee N, Kim M, Kim M, Lee S (2008) Heterologous production of *Pseudomonas aeruginosa* EMS1 biosurfactant in *Pseudomonas putida*. Bioresour Technol 99:2192–2199. https://doi.org/10.1016/j.biortech.2007.05.035

Chamanrokh P, Mazaheri Assadi M, Noohi A, Yahyai S (2008) Emulsan analysis produced by locally isolated bacteria and *Acinetobacter calcoaceticus* RAG-1. Iranian J Environ Health Sci Eng 5:101–108

Chandran P, Das N (2012) Role of sophorolipid biosurfactant in degradation of diesel oil by *Candida tropicalis*. Bioremediat J 16:19–30. https://doi.org/10.1080/10889868.2011.628351

Chen J, Wu Q, Hua Y, Chen J, Zhang H, Wang H (2017) Potential applications of biosurfactant rhamnolipids in agriculture and biomedicine. Appl Microbiol Biotechnol 101:8309–8319. https://doi.org/10.1007/s00253-017-8554-4

Chong H, Li Q (2017) Microbial production of rhamnolipids: opportunities, challenges and strategies. Microb Cell Factories 16:137. https://doi.org/10.1186/s12934-017-0753-2

Costa SGVAO, Déziel E, Lépine F (2011) Characterization of rhamnolipid production by *Burkholderia glumae*. Lett Appl Microbiol 53:620–627. https://doi.org/10.1111/j.1472-765X.2011.03154.x

Desai JD, Banat IM (1997) Microbial production of surfactants and their commercial potential. Microbiol Mol Biol Rev 61:47–64

Déziel E, Lépine F, Dennie D, Boismenu D, Mamer OA, Villemur R (1999) Liquid chromatography/mass spectrometry analysis of mixtures of rhamnolipids produced by *Pseudomonas aeruginosa* strain 57RP grown on mannitol or naphthalene. Biochim Biophys Acta 1440:244–252. https://doi.org/10.1016/S1388-1981(99)00129-8

Déziel E, Lepine F, Milot S, Villemur R (2000) Mass spectrometry monitoring of rhamnolipids from a growing culture of *Pseudomonas aeruginosa* strain 57RP. Biochim Biophys Acta 1485:145–152

Déziel E, Lépine F, Milot S, Villemur R (2003) *rhlA* is required for the production of a novel biosurfactant promoting swarming motility in *Pseudomonas aeruginosa*: 3-(3-hydroxyalkanoyloxy)alkanoic acids (HAAs), the precursors of rhamnolipids. Microbiology 149:2005–2013. https://doi.org/10.1099/mic.0.26154-0

Dhali D, Coutte F, Arias AA, Auger S, Bidnenko V, Chataigné G, Lalk M, Niehren J, de Sousa J, Versari C, Jacques P (2017) Genetic engineering of the branched fatty acid metabolic pathway of *Bacillus subtilis* for the overproduction of surfactin C_{14} isoform. Biotechnol J 12. https://doi.org/10.1002/biot.201600574

Díaz De Rienzo MA, Kamalanathan ID, Martin PJ (2016) Comparative study of the production of rhamnolipid biosurfactants by *B. thailandensis* E264 and *P. aeruginosa* ATCC 9027 using foam fractionation. Process Biochem 51:820–827. https://doi.org/10.1016/j.procbio.2016.04.007

Dubeau D, Déziel E, Woods DE, Lépine F (2009) *Burkholderia thailandensis* harbors two identical *rhl* gene clusters responsible for the biosynthesis of rhamnolipids. BMC Microbiol 9:263–274. https://doi.org/10.1186/1471-2180-9-263

Elshikh M, Funston S, Ahmed S, Marchant R, Banat IB (2017) Rhamnolipids from non-pathogenic *Burkholderia thailandensis* E264: physicochemical characterization, antimicrobial and antibiofilm efficacy against oral hygiene related pathogens. New Biotechnol 36:26–36. https://doi.org/10.1016/j.nbt.2016.12.009

Fukuoka T, Morita T, Konishi M, Imura T, Sakai H, Kitamoto D (2007) Structural characterisation and surface-active properties of a new glycolipid biosurfactant, mono-acylated mannosylerythritol lipid, produced from glucose by *Pseudozyma antarctica*. Appl Microbiol Biotechnol 76:801–810. https://doi.org/10.1007/s00253-007-1051-4

Funston SJ, Tsaousi K, Rudden M, Smyth TJ, Stevenson PS, Marchant R, Banat IM (2016) Characterising rhamnolipid production in *Burkholderia thailandensis* E264, a non-pathogenic producer. Appl Microbiol Biotechnol 100:7945–7956. https://doi.org/10.1007/s00253-016-7564-y

Giani C, Wullbrandt D, Rothert R, Meiwes J (1997) *Pseudomonas aeruginosa* and its use in a process for the biotechnological preparation of L-rhamnose. US005658793A. Hoechst AG, Frankfurt a. M

Grosso-Becerra MV, González-Valdez A, Granados-Martínez MJ, Morales E, Servín-González L, Méndez JL, Delgado G, Morales-Espinosa R, Ponce-Soto GY, Cocotl-Yañez M, Soberón-Chávez G (2016) *Pseudomonas aeruginosa* ATCC 9027 is a non-virulent strain suitable for mono-rhamnolipids production. Appl Microbiol

Biotechnol 100:9995–10004. https://doi.org/10.1007/s00253-016-7789-9

Haba E, Pinazo A, Jauregui O, Espuny MJ, Infante MR, Manresa A (2003) Physicochemical characterization and antimicrobial properties of rhamnolipids produced by *Pseudomonas aeruginosa* 47T2 NCBIM 40044. Biotechnol Bioeng 81:316–322. https://doi.org/10.1002/bit.10474

Haddad NIA, Wang J, Mu B (2009) Identification of a biosurfactant producing strain: *Bacillus subtilis* HOB2. Protein Pept Lett 16:7–13

Ham JH, Melanson RA, Rush MC (2011) *Burkholderia glumae*: next major pathogen of rice? Mol Plant Pathol 12:329–339. https://doi.org/10.1111/j.1364-3703.2010.00676.x

Häußler S, Nimtz M, Domke T, Wray V, Steinmetz I (1998) Purification and characterization of a cytotoxic exolipid of *Burkholderia pseudomallei*. Infect Immun 66:1588–1593

Häußler S, Rohde M, von Neuhoff N, Nimtz M, Steinmetz I (2003) Structural and functional cellular changes induced by *Burkholderia pseudomallei* rhamnolipid. Infect Immun 71:2970–2975

Henkel M, Schmidberger A, Kühnert C, Beuker J, Bernard T, Schwartz T, Syldatk C, Hausmann R (2013) Kinetic modeling of the time course of N-butyryl-homoserine lactone concentration during batch cultivations of *Pseudomonas aeruginosa* PAO1. Appl Microbiol Biotechnol 97:7607–7616. https://doi.org/10.1007/s00253-013-5024-5

Heyd M, Kohnert A, Tan TH, Nusser M, Kirschhofer F, Brenner-Weiss G, Franzreb M, Berensmeier S (2008) Development and trends of biosurfactant analysis and purification using rhamnolipids as an example. Anal Bioanal Chem 391:1579–1590. https://doi.org/10.1007/s00216-007-1828-4

Hirata Y, Ryu M, Oda Y, Igarashi K, Nagatsuka A, Furuta T, Sugiura M (2009) Novel characteristics of sophorolipids, yeast glycolipid biosurfactants, as biodegradable low-foaming surfactants. J Biosci Bioeng 108:142–146. https://doi.org/10.1016/j.jbiosc.2009.03.012

Hörmann B, Müller MM, Syldatk C, Hausmann R (2010) Rhamnolipid production by *Burkholderia plantarii* DSM 9509T. Eur J Lipid Sci Technol 112:674–680. https://doi.org/10.1002/ejlt.201000030

Howe J, Bauer J, Andrä J, Schromm AB, Ernst M, Rössle M, Zähringer U, Rademann J, Brandenburg K (2006) Biophysical characterization of synthetic rhamnolipids. FEBS J 273:5101–5112. https://doi.org/10.1111/j.1742-4658.2006.05507.x

Irie Y, O'toole GA, Yuk MH (2005) *Pseudomonas aeruginosa* rhamnolipids disperse *Bordetella bronchiseptica* biofilms. FEMS Microbiol Lett 250:237–243. https://doi.org/10.1016/j.femsle.2005.07.012

Jadhav M, Kalme S, Tamboli D, Govindwar S (2011) Rhamnolipid from *Pseudomonas desmolyticum* NCIM-2112 and its role in the degradation of Brown 3REL. J Basic Microbiol 51:385–396. https://doi.org/10.1002/jobm.201000364

Jarvis FG, Johnson MJ (1949) A glycolipide produced by *Pseudomonas aeruginosa*. J Am Chem Soc 71:4124–4126. https://doi.org/10.1021/ja01180a073

Jeong Y, Kim J, Kim S, Kang Y, Nagamatsu T, Hwang I (2003) Toxoflavin produced by *Burkholderia glumae* causing rice grain rot is responsible for inducing bacterial wilt in many field crops. Plant Dis 87:890–895. https://doi.org/10.1094/PDIS.2003.87.8.890

Johann S, Seiler TB, Tiso T, Bluhm K, Blank LM, Hollert H (2016) Mechanism-specific and whole-organism ecotoxicity of mono-rhamnolipids. Sci Total Environ 548-549:155–163. https://doi.org/10.1016/j.scitotenv.2016.01.066

Johri A, Blank W, Kaplan D (2002) Bioengineered emulsans from *Acinetobacter calcoaceticus* RAG-1 transposon mutants. Appl Microbiol Biotechnol 59:217–223

Klekner V, Kosaric N (1993) Biosurfactants for cosmetics. In: Kosaric N (ed) Biosurfactants: production—properties—applications. Marcel Dekker, New York, pp 373–390

Kłosowska-Chomiczewska IE, Mędrzycka K, Hallmann E, Karpenko E, Pokynbroda T, Macierzanka A, Jungnickel C (2017) Rhamnolipid CMC prediction. J Colloid Interface Sci 488:10–19. https://doi.org/10.1016/j.jcis.2016.10.055

Konishi M, Morita T, Fukuoka T, Imura T, Uemura S, Iwabuchi H, Kitamoto D (2018) Efficient production of acid-form sophorolipids from waste glycerol and fatty acid methyl esters by *Candida floricola*. J Oleo Sci 67:489–496. https://doi.org/10.5650/jos.ess17219

Lépine F, Déziel E, Milot S, Villemur R (2002) Liquid chromatographic/mass spectrometric detection of the 3-(3-hydroxyalkanoyloxy) alkanoic acid precursors of rhamnolipids in *Pseudomonas aeruginosa* cultures. J Mass Spectrom 37:41–46. https://doi.org/10.1002/jms.244

Lima TMS, Procópio LC, Brandão FD, Carvalho AMX, Tótola MR, Borges AC (2011) Biodegradability of bacterial surfactants. Biodegradation 22:585–592. https://doi.org/10.1007/s10532-010-9431-3

Linhardt RJ, Bakhit R, Daniels L, Mayerl F, Pickenhagen W (1989) Microbially produced rhamnolipid as a source of rhamnose. Biotechnol Bioeng 33:365–368. https://doi.org/10.1002/bit.260330316

Liu G, Zhong H, Yang X, Liu Y, Shao B, Liu Z (2018) Advances in applications of rhamnolipids biosurfactant in environmental remediation: a review. Biotechnol Bioeng 115:796–814. https://doi.org/10.1002/bit.26517

Luong TM, Ponamoreva ON, Nechaeva IA, Petrikov KV, Delegan YA, Surin AK, Linklater D, Filonov AE (2018) Characterization of biosurfactants produced by the oil-degrading bacterium *Rhodococcus erythropolis* S67 at low temperature. World J Microbiol Biotechnol 34:20. https://doi.org/10.1007/s11274-017-2401-8

Maier RM, Soberón-Chávez G (2000) *Pseudomonas aeruginosa* rhamnolipids: biosynthesis and potential applications. Appl Microbiol Biotechnol 54:625–633. https://doi.org/10.1007/s002530000443

Makkar RS, Cameotra SS (1998) Production of biosurfactant at mesophilic and thermophilic conditions by a strain of *Bacillus subtilis*. J Ind Microbiol Biotechnol 20:48–52

Manso Pajarron A, de Koster CG, Heerma W, Schmidt M, Haverkamp J (1993) Structure identification of natural rhamnolipid mixtures by fast atom bombardment tandem mass spectrometry. Glycoconj J 10:219–226. https://doi.org/10.1007/BF00702203

Marqués AM, Pinazo A, Farfan M, Aranda FJ, Teruel JA, Ortiz A, Manresa A, Espuny MJ (2009) The physicochemical properties and chemical composition of trehalose lipids produced by *Rhodococcus erythropolis* 51T7. Chem Phys Lipids 158:110–117. https://doi.org/10.1016/j.chemphyslip.2009.01.001

Martínez R, Schwaneberg U (2013) A roadmap to directed enzyme evolution and screening systems for biotechnological applications. Biol Res 46:395–405. https://doi.org/10.4067/S0716-97602013000400011

Mata-Sandoval JC, Karns J, Torrents A (1999) HPLC method for characterization of rhamnolipids mixtures produced by *Pseudomonas aeruginosa* UG2 on corn oil. J Chromatogr 864:211–220

Mixich J, Rapp KM, Vogel M (1990) Process for producing rhamnose from rhamnolipids. EP0550448. Südzucker AG, Mannheim

Mixich J, Rothert R, Wullbrandt D (1997) Process for the quantitative purification of glycolipids. US5656747A. Hoechst AG, Frankfurt a. M

Monteiro SA, Sassaki GL, de Souza LM, Meira JA, de Araujo JM, Mitchell DA, Ramos LP, Krieger N (2007) Molecular and structural characterization of the biosurfactant produced by *Pseudomonas aeruginosa* DAUPE 614. Chem Phys Lipids 147:1–13. https://doi.org/10.1016/j.chemphyslip.2007.02.001

Müller MM, Hausmann R (2011) Regulatory and metabolic network of rhamnolipid biosynthesis: traditional and advanced engineering

towards biotechnological production. Appl Microbiol Biotechnol 91:251–264. https://doi.org/10.1007/s00253-011-3368-2

Müller MM, Hörmann B, Syldatk C, Hausmann R (2010) *Pseudomonas aeruginosa* PAO1 as a model for rhamnolipid production in bioreactor systems. Appl Microbiol Biotechnol 87:167–174. https://doi.org/10.1007/s00253-010-2513-7

Müller MM, Hörmann B, Kugel M, Syldatk C, Hausmann R (2011) Evaluation of rhamnolipid production capacity of *Pseudomonas aeruginosa* PAO1 in comparison to the rhamnolipid over-producer strains DSM 7108 and DSM 2874. Appl Microbiol Biotechnol 89:585–592. https://doi.org/10.1007/s00253-010-2901-z

Müller MM, Kügler JH, Henkel M, Gerlitzki M, Hörmann B, Pöhnlein M, Syldatk C, Hausmann R (2012) Rhamnolipids—next generation surfactants? J Biotechnol 162:366–380. https://doi.org/10.1016/j.jbiotec.2012.05.022

Nguyen TT, Sabatini DA (2011). Characterization and emulsification properties of rhamnolipid and sophorolipid biosurfactants and their applications. Int J Mol Sci 12:1232–1244. https://doi.org/10.3390/ijms12021232

Nguyen TT, Youssef NH, McInerney MJ, Sabatini DA (2008) Rhamnolipid biosurfactant mixtures for environmental remediation. Water Res 42:1735–1743. https://doi.org/10.1016/j.watres.2007.10.038

Nickzad A, Lépine F, Déziel E (2015) Quorum sensing controls swarming motility of *Burkholderia glumae* through regulation of Rhamnolipids. PLoS One 10:e0128509. https://doi.org/10.1371/journal.pone.0128509

Nitschke M, Costa SGVAO (2007) Biosurfactants in food industry. Trends Food Sci Technol 18:252–259. https://doi.org/10.1016/j.tifs.2007.01.002

Nitschke M, Pastore GM (2006) Production and properties of a surfactant obtained from *Bacillus subtilis* grown on cassava wastewater. Bioresour Technol 97:336–341. https://doi.org/10.1016/j.biortech.2005.02.044

Ochsner UA, Reiser J (1995) Autoinducer-mediated regulation of rhamnolipid biosurfactant synthesis in *Pseudomonas aeruginosa*. Proc Natl Acad Sci U S A 92:6424–6428

Ochsner UA, Fiechter A, Reiser J (1994a) Isolation, characterization, and expression in *Escherichia coli* of the *Pseudomonas aeruginosa rhlAB* genes encoding a rhamnosyltransferase involved in rhamnolipid biosurfactant synthesis. J Biol Chem 269:19787–19795

Ochsner UA, Koch AK, Fiechter A, Reiser J (1994b) Isolation and characterization of a regulatory gene affecting rhamnolipid biosurfactant synthesis in *Pseudomonas aeruginosa*. J Bacteriol 176:2044–2054

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

Olvera C, Goldberg JB, Sánchez R, Soberón-Chávez G (1999) The *Pseudomonas aeruginosa algC* gene product participates in rhamnolipid biosynthesis. FEMS Microbiol Lett 179:85–90. https://doi.org/10.1111/j.1574-6968.1999.tb08712.x

Packer MS, Liu DR (2015) Methods for the directed evolution of proteins. Nat Rev Genet 16:379–394. https://doi.org/10.1038/nrg3927

Pearson JP, Pesci EC, Iglewski BH (1997) Roles of *Pseudomonas aeruginosa las* and *rhl* quorum-sensing systems in control of elastase and rhamnolipid biosynthesis genes. J Bacteriol 179:5756–5767

Peng F, Liu Z, Wang L, Shao Z (2007) An oil-degrading bacterium: *Rhodococcus erythropolis* strain 3C-9 and its biosurfactants. J Appl Microbiol 102:1603–1611. https://doi.org/10.1111/j.1365-2672.2006.03267.x

Piljac A, Stipcević T, Piljac-Zegarac J, Piljac G (2008) Successful treatment of chronic decubitus ulcer with 0.1% dirhamnolipid ointment. J Cutan Med Surg 12:142–146. https://doi.org/10.2310/7750.2008.07052

Pruthi V, Cameotra SS (1997) Production and properties of a biosurfactant synthesized by *Arthrobacter protophormiae*—an antarctic strain. World J Microbiol Biotechnol 13:137–139

Rahim R, Burrows LL, Monteiro MA, Perry MB, Lam JS (2000) Involvement of the *rml* locus in core oligosaccharide and O polysaccharide assembly in *Pseudomonas aeruginosa*. Microbiology 146:2803–2814. https://doi.org/10.1099/00221287-146-11-2803

Rahim R, Ochsner UA, Olvera C, Graninger M, Messner P, Lam JS, Soberón-Chávez G (2001) Cloning and functional characterization of the *Pseudomonas aeruginosa rhlC* gene that encodes rhamnosyltransferase 2, an enzyme responsible for di-rhamnolipid biosynthesis. Mol Microbiol 40:708–718. https://doi.org/10.1046/j.1365-2958.2001.02420.x

Rau U, Nguyen LA, Schulz S, Wray V, Nimtz M, Roeper H, Koch H, Lang S (2005) Formation and analysis of mannosylerythritol lipids secreted by *Pseudozyma aphidis*. Appl Microbiol Biotechnol 66:551–559. https://doi.org/10.1007/s00253-004-1672-9

Reetz MT (2016) Directed evolution of selective enzymes: catalysts for organic chemistry and biotechnology. Wiley-VCH, Weinheim. https://doi.org/10.1002/9783527655465

Rehm BH, Mitsky TA, Steinbüchel A (2001) Role of fatty acid de novo biosynthesis in polyhydroxyalkanoic acid (PHA) and rhamnolipid synthesis by *pseudomonads*: establishment of the transacylase (PhaG)-mediated pathway for PHA biosynthesis in *Escherichia coli*. Appl Environ Microbiol 67:3102–3109. https://doi.org/10.1128/AEM.67.7.3102-3109.2001

Remichkova M, Galabova D, Roeva I, Karpenko E, Shulga A, Galabov AS (2008) Anti-herpesvirus activities of *Pseudomonas* sp. S-17 rhamnolipid and its complex with alginate. Z Naturforsch C 63:75–81

Řezanka T, Siristova L, Sigler K (2011) Rhamnolipid-producing thermophilic bacteria of species *Thermus* and *Meiothermus*. Extremophiles 15:697–709. https://doi.org/10.1007/s00792-011-0400-5

Rosenau F, Isenhardt S, Gdynia A, Tielker D, Schmidt E, Tielen P, Schobert M, Jahn D, Wilhelm S, Jaeger KE (2010) Lipase LipC affects motility, biofilm formation and rhamnolipid production in *Pseudomonas aeruginosa*. FEMS Microbiol Lett 309:25–34. https://doi.org/10.1111/j.1574-6968.2010.02017.x

Rosenberg E, Ron EZ (1997) Bioemulsans: microbial polymeric emulsifiers. Curr Opin Biotechnol 8:313–316

Saika A, Utashima Y, Koike H, Yamamoto S, Kishimoto T, Fukuoka T, Morita T (2018) Biosynthesis of mono-acylated mannosylerythritol lipid in an acyltransferase gene-disrupted mutant of *Pseudozyma tsukubaensis*. Appl Microbiol Biotechnol 102:1759–1767. https://doi.org/10.1007/s00253-017-8698-2

Sha R, Jiang L, Meng Q, Zhang G, Song Z (2011) Producing cell-free culture broth of rhamnolipids as a cost-effective fungicide against plant pathogens. J Basic Microbiol 51:1–9. https://doi.org/10.1002/jobm.201100295

Sharma R, Singh J, Verma N (2018) Optimization of rhamnolipid production from *Pseudomonas aeruginosa* PBS towards application for microbial enhanced oil recovery. 3 Biotech 8:20. https://doi.org/10.1007/s13205-017-1022-0

Sinumvayo JP, Ishimwe N (2015) Agriculture and food applications of Rhamnolipids and its production by *Pseudomonas aeruginosa*. J Chem Eng Process Technol 6:223. https://doi.org/10.4172/2157-7048.1000223

Soberón-Chávez G, Lépine F, Déziel E (2005) Production of rhamnolipids by *Pseudomonas aeruginosa*. Appl Microbiol Biotechnol 68:718–725. https://doi.org/10.1007/s00253-005-0150-3

Stacey SP, McLaughlin MJ, Çakmak I, Hettiarachchi GM, Scheckel KG, Karkkainen M (2008) Root uptake of lipophilic zinc-rhamnolipid complexes. J Agric Food Chem 56:2112–2117. https://doi.org/10.1021/jf0729311

Stipcević T, Pijac A, Pijac G (2006) Enhanced healing of full-thickness burn wounds using di-rhamnolipid. Burns 32:24–34. https://doi.org/10.1016/j.burns.2005.07.004

Syldatk C, Lang S, Matulovic U, Wagner F (1985a) Production of four interfacial active rhamnolipids from n-alkanes or glycerol by resting

cells of *Pseudomonas* species DSM 2874. Z Naturforsch C 40:61–67. https://doi.org/10.1515/znc-1985-1-213

Syldatk C, Lang S, Wagner F, Wray V, Witte L (1985b) Chemical and physical characteritation of four interfacial-active rhamnolipids from *Pseudomonas* spec. DSM 2874 grown on n-alkanes. Z Naturforsch C 40:51–60. https://doi.org/10.1515/znc-1985-1-212

Tavares LFD, Silva PM, Junqueira M, Mariano DCO, Nogueira FCS, Domont GB, Freire DMG Neves BC (2012) Characterization of rhamnolipids produced by wild-type and engineered *Burkholderia kururiensis*. Appl Microbiol Biotechnol 97:1909–1921. https://doi.org/10.1007/s00253-012-4454-9

Tiso T, Sabelhaus A, Behrens B, Wittgens A, Rosenau F, Hayen H, Blank LM (2016) Creating metabolic demand as an engineering strategy in *Pseudomonas putida*—Rhamnolipid synthesis as an example. Metab Eng Commun 3:234–244. https://doi.org/10.1016/j.meteno.2016.08.002

Tiso T, Zauter R, Tulke H, Leuchtle B, Li WJ, Behrens B, Wittgens A, Rosenau F, Hayen H, Blank LM (2018) Designer rhamnolipids by reduction of congener diversity: production and characterization. Microb Cell Factories 16:225. https://doi.org/10.1186/s12934-017-0838-y

Toribio J, Escalante AE, Soberón-Chávez G (2010) Rhamnolipids: production in bacteria other than *Pseudomonas aeruginosa*. Eur J Lipid Sci Technol 112:1082–1087. https://doi.org/10.1002/ejlt.200900256

Trummler K, Effenberger F, Syldatk C (2003) An integrated microbial/enzymatic process for production of rhamnolipids and L-(+)-rhamnose from rapeseed oil with *Pseudomonas* sp. DSM 2874. Eur J Lipid Sci Technol 105:563–571. https://doi.org/10.1002/ejlt.200300816

Van Bogaert I, Fleurackers S, Van Kerrebroeck S, Develter D, Soetaert W (2011) Production of new-to-nature sophorolipids by cultivating the yeast *Candida bombicola* on unconventional hydrophobic substrates. Biotechnol Bioeng 108:734–741. https://doi.org/10.1002/bit.23004

Van Hamme JD, Urban J (2009) Biosurfactants in bioremediation. In: Singh A, Kuhad RC, Ward OP (eds) Advances in applied bioremediation, soil biology, vol 17. Springer-Verlag, Heidelberg, pp 73–89

Van Hamme JD, Singh A, Ward OP (2006) Physiological aspects. Part 1 in a series of papers devoted to surfactants in microbiology and biotechnology. Biotechnol Adv 24:604–620

Vatsa P, Sanchez L, Clement C, Baillieul F, Dorey S (2010) Rhamnolipid biosurfactants as new players in animal and plant defense against microbes. Int J Mol Sci 11:5095–5108. https://doi.org/10.3390/ijms11125095

Velikonja J, Kosaric N (1993) Biosurfactants in food applications. In: Kosaric N (ed) Biosurfactants: production—properties—applications. Marcel Dekker, New York, pp 419–446

Wang S, Mulligan CN (2009) Rhamnolipid biosurfactant-enhanced soil flushing for the removal of arsenic and heavy metals from mine tailings. Process Biochem 44:296–301. https://doi.org/10.1016/j.procbio.2008.11.006

Wang Q, Fang X, Bai B, Liang X, Shuler PJ, Goddard WA 3rd, Tang Y (2007) Engineering bacteria for production of rhamnolipid as an agent for enhanced oil recovery. Biotechnol Bioeng 98:842–853. https://doi.org/10.1002/bit.21462

Wilhelm S, Gdynia A, Tielen P, Rosenau F, Jaeger KE (2007) The autotransporter esterase EstA of *Pseudomonas aeruginosa* is required for rhamnolipid production, cell motility, and biofilm formation. J Bacteriol 189:6695–6703. https://doi.org/10.1128/JB.00023-07

Witek-Krowiak A, Witek J, Gruszczynska A, Szafran RG, Kozlecki T, Modelski S (2011) Ultrafiltrative separation of rhamnolipid from culture medium. World J Microbiol Biotechnol 27:1961–1964. https://doi.org/10.1007/s11274-011-0655-0

Wittgens A (2013) Konstruktion neuer Produktionsstämme für die heterologe Rhamnolipidsynthese in dem nicht-pathogenen Wirt *Pseudomonas putida* KT2440. Dissertation, Ulm University

Wittgens A, Tiso T, Arndt TT, Wenk P, Hemmerich J, Müller C, Wichmann R, Küpper B, Zwick M, Wilhelm S, Hausmann R, Syldatk C, Rosenau F, Blank LM (2011) Growth independent rhamnolipid production from glucose using the non-pathogenic *Pseudomonas putida* KT2440. Microb Cell Factories 10:80. https://doi.org/10.1186/1475-2859-10-80

Wittgens A, Kovacic F, Müller MM, Gerlitzki M, Santiago-Schübel B, Hofmann D, Tiso T, Blank LM, Henkel M, Hausmann R, Syldatk C, Wilhelm S, Rosenau F (2017) Novel insights into biosynthesis and uptake of rhamnolipids and their precursors. Appl Microbiol Biotechnol 101:2865–2878. https://doi.org/10.1007/s00253-016-8041-3

Wittgens A, Santiago-Schuebel B, Henkel M, Tiso T, Blank LM, Hausmann R, Hofmann D, Wilhelm S, Jaeger KE, Rosenau F (2018) Heterologous production of long-chain rhamnolipids from *Burkholderia glumae* in *Pseudomonas putida*-a step forward to tailor-made rhamnolipids. Appl Microbiol Biotechnol 102:1229–1239. https://doi.org/10.1007/s00253-017-8702-x

Zhang CY, Zhang JC (1993) A pilot test of EOR by in situ microorganism fermentation in the Daqing oil field. Dev Pet Sci 39:231–244. https://doi.org/10.1016/S0376-7361(09)70064-0

Zhang L, Veres-Schalnat TA, Somogyi A, Pemberton JE, Maier RM (2012) Fatty acid cosubstrates provide β-oxidation precursors for rhamnolipid biosynthesis in *Pseudomonas aeruginosa*, as evidenced by isotope tracing and gene expression assays. Appl Environ Microbiol 78:8611–8622. https://doi.org/10.1128/AEM.02111-12

Zhu K, Rock CO (2008) RhlA converts β-hydroxyacyl-acyl carrier protein intermediates in fatty acid synthesis to the β-hydroxydecanoyl-β-hydroxydecanoate component of rhamnolipids in *Pseudomonas aeruginosa*. J Bacteriol 190:3147–3154. https://doi.org/10.1128/JB.00080-08