Biodiversity of Biosurfactants and Roles in Enhancing the (Bio)availability of Hydrophobic Substrates

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Contents

Abstract

This chapter focusses on the biodiversity of microbial biosurfactants and the organisms that produce them. Specific attention is given to the low molecular weight glycolipids and lipopeptides produced by bacteria such as *Pseudomonas, Burkholderia, Bacillus, Rhodococcus*, and *Alcanivorax* in addition to other glycolipids synthesized by eukaryotic organisms such as *Starmerella*, *Pseudozyma*, and *Candida* spp. The applications of microbial surfactants utilizing their

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properties for accessing substrates and in microemulsion technology is covered plus reference to potential applications in environmental remediation. Finally a summary of the current state of research and identification of significant areas for further investigation are highlighted.

1 Introduction

Interest in microbial biosurfactants has increased significantly in the last few years largely due to their perceived enormous potential as sustainable replacements for chemical surfactants in a wide range of consumer products. The pressure on companies to use sustainable, green resources to produce their products and consumer interest in "natural" products have effectively fuelled the research interest in these compounds (Banat et al. 2010; Campos et al. 2013; Satpute et al. 2016a; Elshikh et al. 2016; De Almeida et al. 2016). Unsurprisingly the main focus of the research has been directed towards the physicochemical properties of biosurfactants and investigations of how they might be employed as at least partial replacements for chemical surfactants in high turnover consumer products such as laundry detergents, surface cleaners, personal care products, cosmetics and even foodstuffs. There has also been interest in their use in more specialised applications such as pharmaceuticals, exploiting their synergistic potential in combination with antibiotics and other bioactive molecules, and even in the bioactivity of the biosurfactants themselves as antimicrobials and anti-cancer agents (Marchant and Banat 2012a, b; Fracchia et al. 2014, 2015; Díaz de Rienzo et al. 2016b).

Thus far one of the main hurdles to exploitation has been the fermentation yields of biosurfactants, which have often been too low for economic commercial use coupled with the difficulty and cost of downstream processing for the production of defined, pure products. As a result of the current emphasis on the commercial exploitation of biosurfactants less effort has been directed towards investigation into why microorganisms produce these surface active molecules and what role they play in the life history of the organisms. Their function must be significant since many organisms direct a large part of their metabolic energy and resources into their production. Many functions have been ascribed to biosurfactants, including maintenance of biofilm structure, motility, cell adhesion and access to hydrophobic substrates (Fracchia et al. 2012, 2014; Díaz de Rienzo et al. 2016a, c). It is the last of these functions that this chapter will address. In the context of hydrophobic substrate degradation e.g., hydrocarbons biosurfactants have attracted interest as augmentation agents to speed the removal of environmental contaminating fuels and crude oil, both in marine and terrestrial situations. Experiments using biosurfactants in microcosms with hydrocarbonoclastic microorganisms have demonstrated some enhancement of the rate and extent of degradation (Rahman et al. 2003), however, this remains an area of investigation with unexplored potential.

2 Biodiversity of Biosurfactant Producing Microorganisms

Biosurfactants (BSs) are a diverse class of compounds that are synthesised by a wide variety of microorganisms spanning all domains of life (Menezes Bento et al. 2005; Khemili-Talbi et al. 2015; Roelants et al. 2014; Satpute et al. 2016b). The structural diversity of these metabolites is reflected by the diversity of producers and environments from which they can be isolated. Conventionally BS producing microorganisms have been most frequently isolated from hydrocarbon contaminated sites which are typically dominated by a few main microbial consortia including *Pseudomonas, Burkholdeiria, Bacillus, Streptomyces, Sphingomonas* and *Actinobacteria* in soils and sediments, and *Pseudoalteromonas, Halomonas, Alcanivorax* and *Acinetobacter* in marine ecosystems. However, BS producing microorganisms are ubiquitous in nature and have been isolated from a variety of niches including extreme environments such as high salinity (Donio et al. 2013; Pradhan et al. 2013), high temperatures (Elazzazy et al. 2015; Darvishi et al. 2011), and cold environments (Malavenda et al. 2015; Cai et al. 2014).

Pseudomonas and *Bacillus* are the main genera reported for biosurfactant production, however novel biosurfactant producers are continually being reported, recently Hošková et al. 2015 characterised rhamnolipids synthesised by *Acinetobacter calcoaceticus* and *Enterobacter asburiae*. Traditionally efforts to increase the frequency of isolating BS producing microorganisms from hydrocarbon contaminated soils involve enrichment culture techniques, which have been reported to increase recovery of BS producers from 25% to 80% (Thies et al. 2016; Bodour et al. 2003; Steegborn et al. 1999; Rahman et al. 2002; Donio et al. 2013; Pradhan et al. 2013; Walter et al. 2013). Enrichment techniques have been widely applied to members of the genera *Pseudomonas* and *Bacillus* for bioremediation of hydrocarbon contaminates sites (Li et al. 2016). Identification of biosurfactant producing microorganisms still relies on culture dependent techniques and typically qualitative methods for biosurfactant characterisation such as the orcinol assay, drop collapse, oil spreading assay and bath assay (Marchant and Banat 2014). While these methods are applicable for preliminary characterisation of BSs, they are not accurate and often overestimate production yields of biotechnologically important BSs (Marchant and Banat 2014).

In recent years research has focused on metabolic engineering of biosurfactants from a diverse range of microorganisms which has largely been facilitated by the increased number of published genomes of biosurfactant producing organisms (Table 1). Advances in functional metagenomics techniques have enabled the exploitation of these genomes for biosurfactant bio-discovery from diverse environments (Kennedy et al. 2011). Marine environments have proven to be a rich resource for isolating BS producing organisms including from marine sponges (Dhasayan et al. 2015; Kiran et al. 2009, 2010) and hydrocarbon contaminated marine areas (Gutierrez and Banat 2014; Antoniou et al. 2015). Recently Oliveira et al. 2015 have published BioSurfDB, www.biosurfdb.org, a tailored databased specifically for

Table 1 List of published genomes of common biosurfactant producing microorganisms

Species	Biosurfactant	References
Pseudomonas aeruginosa PAO1	Rhamnolipids	(Winsor et al. 2016)
Burkholderia thailandensis E264	Rhamnolipids	(Winsor et al. 2008)
Pseudozyma antarctica T-34	Mannosylerythritol lipids	(Morita et al. 2013a)
Starmerella bombicola NBRC10243	Sophorolipids	(Matsuzawa et al. 2015)
Bacillus amyloliquefaciens RHNK22	Surfactin, Iturin, and Fengycin	(Narendra Kumar et al. 2016)
Alcanivorax borkumensis	Lipopeptide	(Schneiker et al. 2006)
Pseudozyma hubeiensis SY62	Mannosylerythritol lipids	(Konishi et al. 2013)
Bacillus safensis CCMA-560	Pumilacidin	(Domingos et al. 2015)
Pseudozyma aphidis DSM 70725	Mannosylerythritol lipids	(Lorenz et al. 2014)
Rhodococcus sp. PML026	Trehalose lipids	(Sambles and White 2015)
Rhodococcus sp. BS-15	Trehalose lipids	(Konishi et al. 2014)
Gordonia amicalis CCMA-559	Polymeric glycolipids	(Domingos et al. 2013)
Dietzia maris	Polymeric glycolipids	(Ganguly et al. 2016)

analysing biosurfactant production in microorganisms. This database unique to biosurfactant production integrates a range of information including metagenomics, biosurfactant producing organisms, biodegradation relevant genes, proteins and their metabolic pathways, results from bioremediation experiments and a biosurfactant-curated list, grouped by producing organism, surfactant name, class and reference. The database contains 3,736 biosurfactant coding genes and offers a unique platform for recombinant engineering of novel/diverse biosurfactant coding genes identified from a range of microorganisms including uncultivable bacteria (Thies et al. 2016).

Functional metagenomics offers enormous opportunities for recombinant production of novel biosurfactant molecules with tailored applications, however it should be noted that several technical challenges still exist. Biosynthetic clusters for natural products tend to be relatively large and highly regulated especially from yeast and fungal sp., for example the biosynthetic gene cluster from *Ustilago* sp. encoding cellobiose lipids spans 58 Kb (Teichmann et al. 2007). Associated challenges include heterologous expression of large biosynthetic clusters from single fragments, toxicity of associated product to host organism, promoter and transcription factor recognition and extracellular transport of gene products (Jackson et al. 2015). Current research is focussed on improving efficiency of functional metagenomics techniques, for example to overcome dynamic regulation by native promoters within natural biosynthetic gene clusters. Kang et al. (2016) used mCRISTAR, a multiplexed CRISPR and TAR technique which allows for precision promoter engineering by specifically inserting inducible synthetic promoters to replace native promoters. Sophisticated techniques like this could be applied to large biosurfactant biosynthetic gene clusters and with collated resources such as BioSurfdb, efficient heterologous production of tailor made biosurfactants looks promising.

3 Rhamnolipid Biosurfactants in *Pseudomonas* spp. and Related Organisms

Rhamnolipids (RLs) are low molecular glycolipid secondary metabolites predominantly synthesised by the genera *Pseudomonas* and *Burkholderia* spp. RLs are synthesised as a heterogeneous mixture of congeners comprising a hydrophilic rhamnose (Rha) glycosidically linked to one or more β-hydroxy fatty acids (Fig. 1a). They are produced in two main classes, mono- and di-rhamnolipids, and usually vary in alkyl chain length ranging from C_8-C_{16} (Haba et al. 2003a, b; Gunther et al. 2005; Déziel et al. 2000) and degree of saturation, with some polyunsaturated rhamnolipid congeners reported for *P. aeruginosa* (Abalos et al. 2001; Haba et al. 2003a).

The advancement in chromatographic and mass spectrometric techniques in the last few decades has resulted in the identification of up to 60 different RL congeners produced by various microorganisms with *Pseudomonas* spp. and *Burkholderia* spp being the most dominant producers (Abdel-Mawgoud et al. 2010; Mata-Sandoval et al. 1999; Heyd et al. 2008; Dubeau et al. 2009). Among these diverse homologues some more unusual RLs have been detected. Arino et al. (1996) have reported both mono- and di-RL congeners containing mono-lipidic fatty acid only in trace amounts and Andrä et al. (2006) reported a di-RL congener with three C_{14} β-hyroxy fatty acids in *Burkholdieria plantarii*. *P. aeruginosa* preferentially synthesises di-lipidic RLs with alkyl lengths C_{10}-C_{10}, whereas C_{14} β-hydroxy acids are the most abundant fatty acids for the *Burkholderia* spp. (Funston et al. 2016; Hörmann et al. 2010; Häussler et al. 1998; Howe et al. 2006).

In recent years there has been significant emphasis on RL production from non-pathogenic producers as alternatives to the opportunistic pathogen *Pseudomonas aeruginosa* (Marchant et al. 2014). Alternative producers include phylogenetically related species including *Pseudomonas fluorescens* (Vasileva-Tonkova et al. 2011) and the exclusively mono-RL producing strain *Pseudomonas chlororaphis* (Gunther et al. 2005). RL production has also been reported among the more taxonomically distant species such as Acinetobacter and Enterobacter bacteria (Hošková et al. 2015). However production yields are not comparable to RLs produced from *P. aeruginosa*. Care must always be exercised in evaluating claims for new or unusual producers since the methods used to determine the identity of the producer organism and the characterisation of the product are not always sufficiently rigorous. The most promising non-pathogenic biotechnological RL producer is *Burkholdeira thailandensis* E264. It has been shown that *B. thailandensis* produces RL yields comparable to wild-type *P. aeruginosa* making it a suitable candidate for large scale production of RLs. The composition of RLs from *B. thailandensis* differs only in the length of the di-lipid alkyl chains, in *B. thailandensis* C_{14} is the preferred chain length (Funston et al. 2016), whereas in *P. aeruginosa* C_{10} is selectively incorporated to synthesise RLs (Rudden et al. 2015).

The biosynthetic pathway and complex genetic regulation of RL synthesis has been extensively studied in *P. aeruginosa* (Reis et al. 2011; Schmidberger et al.

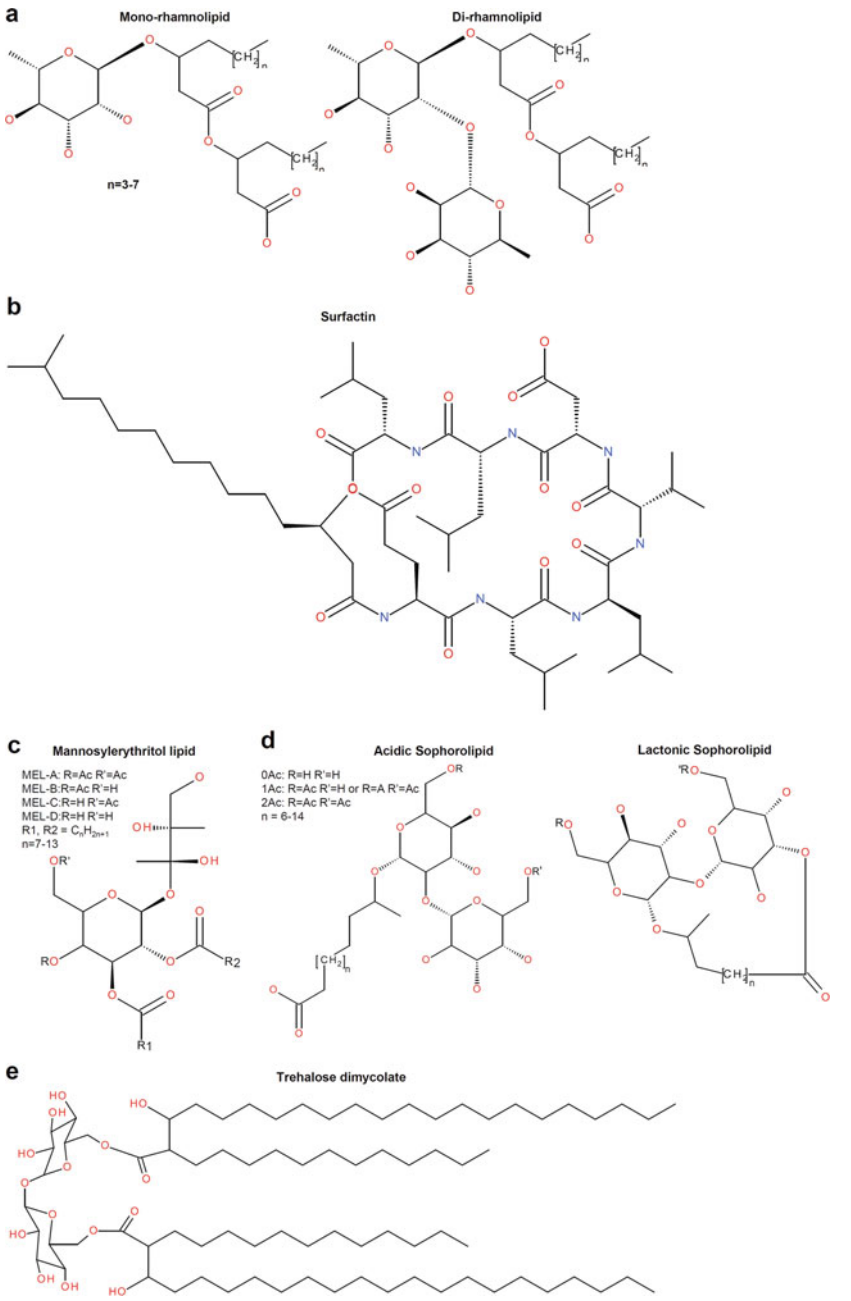

Fig. 1 Molecular structure of common biosurfactants. (**a**) Rhamnolipids from *Pseudomonas* sp. (**b**) Surfactin from *Bacillus* sp. (**c**) Mannosylerythritol lipids from *Pseudozyma* sp. (**d**) Sophorolipids from *Starmerella bombicola* and (**e**) Trehalose dimycolate from *Rhodococcus* sp.

2013). RL precursor molecules are synthesised de novo from central metabolic pathways, dTDP-L-rhamnose is synthesised by the proteins encoded in the *rmlABCD* operon from the rhamnose pathway (Aguirre-Ramirez et al. 2012) and β-hydroxy fatty acids are derived from both β-oxidation and FAS II pathways (Zhang et al. 2014; Zhu and Rock 2008). RL biosynthesis is catalysed by three enzymes, RhlA, RhlB and RhlC. RhlA (rhamnosyltransferase chain A) is responsible for the supply of the lipid precursors for RLs by the dimerisation of two β-hydroxyacyl-acyl carrier proteins ACPs to form one molecule of hydroxyalkanoic acid (HAA). RhlA is selective for C_{10} carbon intermediates and regulates the fatty acid composition of RLs (Zhu and Rock 2008). Mono-RLs are synthesised by the condensation of the precursors derived from the central metabolic pathway by RhlB (rhamnosyltransferase chain B). RhlB transfers one dTDP-L-rhamnose to the HAA producing a mono-RL. Mono-RL together with another dTDP-L-rhamnose is the substrate for rhamnosyltransferase 2 (RhlC) which synthesises di-RLs. RhlA and RhlB form the heterodimer rhamnosyltransferase 1, with RhlB being the catalytic subunit for RL biosynthesis and RhlA responsible for the lipid precursor synthesis. RhlA and RhlB are co-transcribed from *rhlAB* which is clustered together with the regulatory genes *rhlR/I*.

Expression of *rhlAB* is regulated by Quorum Sensing and other regulatory factors in *P. aeruginosa* (Müller and Hausmann 2011). The RhlAB polypeptide is loosely membrane associated, while RhlA is a soluble protein located in the cytoplasm. RhlB has two putative hydrophobic membrane domains associated with the inner membrane (Ochsner et al. 1994). The second rhamnosyltransferase RhlC, encoded by *rhlC,* is co-transcribed from a different bicistronic operon located with a putative major facilitator superfamily transporter (MFS) protein 2.5 Mb away from the *rhlAB* genes. Ochsner and co-workers (1994) also predicted that RhlC is located in the inner membrane based on a hydrophobic region between amino acid residues 257 and 273 (Rahim et al. 2001). This would suggest that RL biosynthesis occurs at the cytoplasmic membrane and RLs are subsequently extracellularly transported. In contrast to *P. aeruginosa, Burkholderia thailandensis* contains the RL biosynthetic *rhlABC* genes clustered together on a single operon which is duplicated in the chromosome; both operons are functional and contribute to RL biosynthesis (Dubeau et al. 2009). For many decades *P. aeruginosa* has been the main focus for RL production, however, in recent years there has been a significant shift toward alternatives for RL production, namely RL production from non-pathogenic producers and heterologous production of RLs in non-pathogenic hosts (Loeschcke and Thies 2015).

The structural diversity of RL congeners from many different producers can be attributed to significant differences in their RL biosynthetic enzymes. For example *P. chlororaphis* is a mono-RL producing only organism (Gunther et al. 2005), which does not contain an RhlC orthologue and cannot synthesise di-RLs from mono-RLs. It has recently been used for recombinant production of di-RLs with *P. aeruginosa rhlC* (Solaiman et al. 2015). RLs from *P. chlororaphis* are synthesised as a heterogeneous mixture of congers with C_{12}-C_{10} or $C_{12:1}$-C_{10} as the predominant β-hydroxy

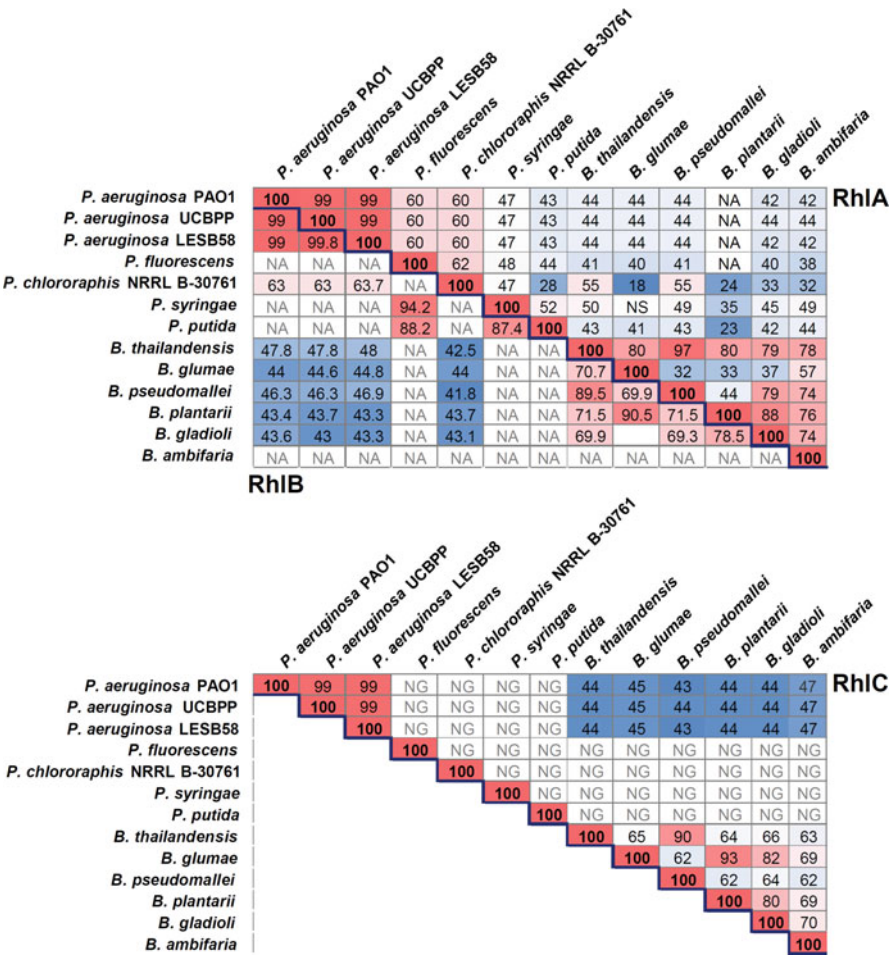

Fig. 2 Rhamnolipid biosynthetic enzymes are highly similar among producing microorganisms. Amino acid (aa) percent identity matrix for rhamnolipid enzymes RhlA, RhlB and RhlC for reported rhamnolipid producing strains. NA – Not aligned, NG – No homologous gene found

moieties, not surprisingly RhlA from *P. chlororaphis* shares only 60% amino acid identity with *P. aeruginosa* (Fig. 2). *Burkholderia* sp. synthesises predominantly di-RLs with C_{14}-C_{14} β-hydroxy fatty acids, both RhlA proteins only share 44% homology with *P. aeruginosa* RhlA (Fig. 2). RhlA functions primarily as an acyltransferase with a conserved α/β-hydrolase domain motif found within the first 100 residues in all RL producing microorganisms. The sequence divergence observed significantly correlates with the specificity of the RhlA enzyme for a certain acyl chain length in the producing microorganism e.g., *P. aeruginosa* RhlA is specific for C_{10} carbon intermediates (Zhu and Rock 2008). Recombinant

production of RLs in non-pathogenic organisms could use for example the specificity of RhlA from selected microorganisms to synthesise tailored compositions of mono- and di-RLs (Wittgens et al. 2011; Dobler et al. 2016).

P. fluorescens and *P. putida* have previously been reported to produce RLs (Tuleva et al. 2002; Martínez-Toledo et al. 2006), however, their production profiles have not been analytically quantified and it seems unlikely, based on the low sequence homology and lack of a RhlC orthologue, that these strains are capable of producing high yields of RLs comparable to *P. aeruginosa*. However, these organisms have been used for heterologous production of *P. aeruginosa* RLs (Ochsner et al. 1995), with *P. putida* KCTC 1067 (pNE2) reported to produce 7.3 g/L using soybean oil as the carbon source. It should be noted that recombinant production of *P. aeruginosa* RLs has extensively been quantified with the orcinol method which significantly overestimates RLs yields (Marchant et al. 2014). Also RLs produced with hydrophobic substrates must be purified before quantification as excess unutilised hydrophobic substrate is always co-extracted and also overestimates actual yields.

RL production is highly conserved among RL producing microorganisms. Fig 3 shows the multiple aligned sequences coloured by conservation with a minimum threshold for >95% identity for both RhlA and RhlC, and 50% for RhlB. RhlA has >20 of the first 110 residues conserved among all sequences which fall with the specific α/β-hydrolase domain. In contrast RhlC has >114 residues conserved among all sequences analysed which span the rhamnosyltransferase/glycosytransferase domains. RhlC is a rhamnosyltransferase enzyme that specifically catalyses the transfer of a second dTDP-L-rhamnose to the mono-RL substrate. dTDP-L-rhamnose is a metabolic precursor for other pathways including lipopolysaccharide (LPS) biosynthesis in *P. aeruginosa* (Rahim et al. 2000). Given the distinct location of *rhlC* on a separate operon to *rhlAB* it is possible that this rhamnosyltransferase could have evolved from conserved rhamnosyltransferase/glycosyltransferase enzymes. Interesting to note is the lack of conservation of RhlB between RL producing microorganisms (Fig. 3), suggesting that RhlB has evolved only for RL biosynthesis. The structural differences in RL congeners is mirrored by phylogenetic analysis of the RL enzymes, for all RL biosynthetic enzymes in *P. aeruginosa* distinct clades are separated from the other reported RL producing strains (Fig. 4). Interestingly, all RL biosynthetic genes from *Burkholderia pseuodomallei* share high sequence homology and are closely related to *Burkholderia thailendesis* (Fig. 2, 4). This is consistent with high reports of RLs from *B. pseuodomallei* which are predominantly C_{14}-C_{14} di-RL congeners (Dubeau et al. 2009).

For next generation RLs it is essential that all the RL biosynthetic enzymes are structurally characterised with regard to specificity and activity. Combining metabolic engineering of RL biosynthesis precursors with precision protein engineering of RL enzymes for enhanced efficiency/activity could offer novel molecular approaches for increased RL production in recombinant hosts.

In *P. aeruginosa* one of the main physiological roles of RLs is hydrocarbon assimilation by increasing the bioavailability of hydrophobic substrates either by

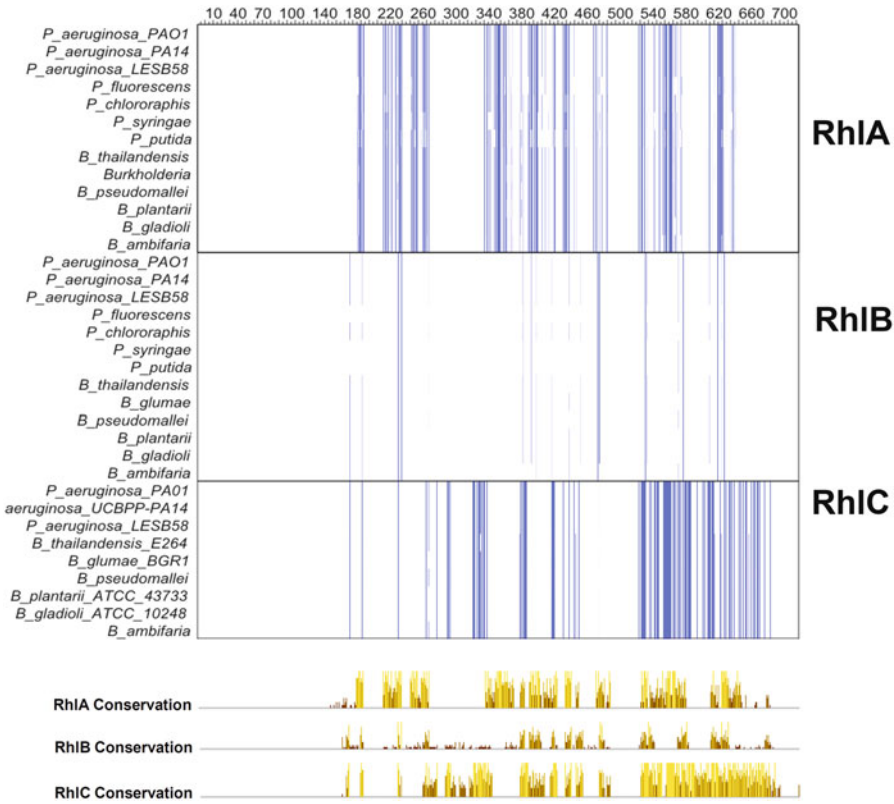

Fig. 3 Multiple sequence alignment showing high conservation in both RhlA and RhlC among all RL producing microorganisms. Sequences were aligned with Mafft-LiNS, edited and visualised in Jalview. Residues are coloured by percent identity with a minimum threshold of 95% conservation shown for RhlA and RhlC and 50% for RhlB

increasing solubility or by increasing cell surface hydrophobicity by inducing the removal of cell associated lipopolysaccharide (LPS). Upon direct contact RLs accumulate as monomers at the aqueous/hydrophobic interface until the concentration reaches and exceeds a critical level known as the critical micelle concentration (CMC), where the RL monomers aggregate into micelles and larger vesicles. Hydrophobic substrates become incorporated within the hydrophobic core of micelles and this effectively enhances their dispersion into the aqueous phase and hence their bioavailability for uptake by cells. This process has been largely studied with alkanes as model substrates and is referred to as "micelle solubilisation" or "pseudosolubilisation" (Fig. 5a, b) (Zhang and Miller 1992). RLs have been also shown to modify the outer cell membrane of *P. aeruginosa* by inducing the removal of the cell associated lipopolysaccharides (LPS) (Al-Tahhan et al. 2000), subsequently increasing cell surface hydrophobicity (CSH) (Fig. 5a). RLs can also induce changes in the composition of outer membrane proteins (OMP) even at

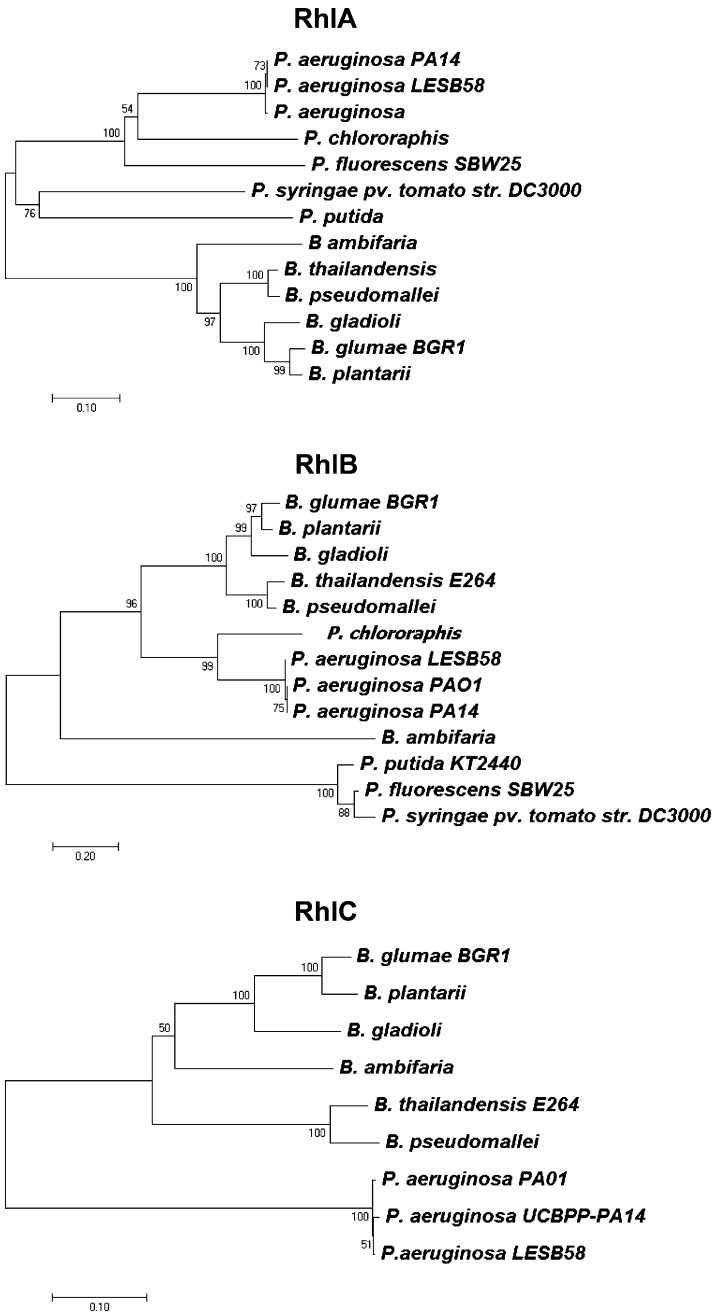

Fig. 4 Phylogenetic analysis of rhamnolipid biosynthetic enzymes. The phylogenetic tree was inferred using the Neighbor-Joining method and the evolutionary distances were computed using the Poisson correction method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are given

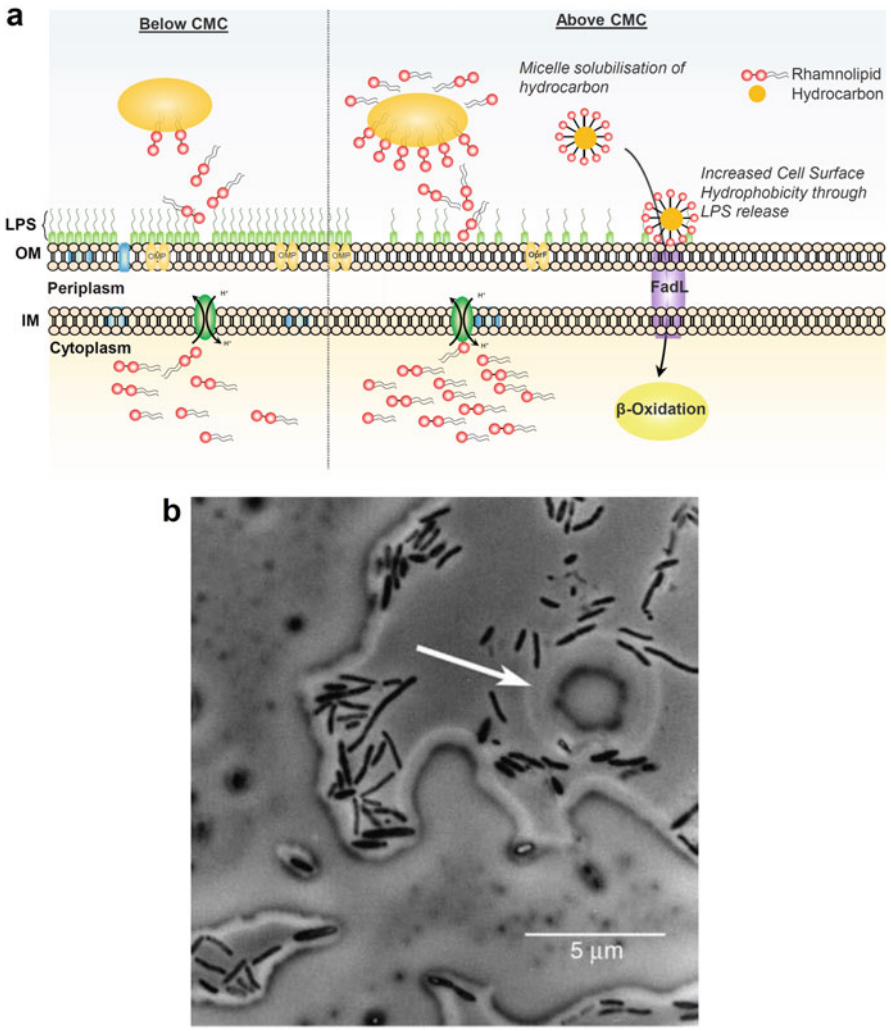

Fig. 5 Solubilisation of hydrophobic substrates by biosurfactant micelles. (**a**) Schematic of hydrocarbon solubilisation by rhamnolipid (RL) biosurfactants. At concentrations below the critical micelle concentration (CMC) monolayers of RL accumulate at the aqueous-hydrocarbon interface. When RL concentrations is above the CMC the hydrocarbon readily partitions into the hydrophobic core of the micelle thus increasing hydrocarbon bioavailability through solubilisation. RLs also induce cell surface changes that increase cell hydrophobicity via the release of cell associated lipopolysaccharides (LPS). (**b**) Photomicrograph of a mixed population of bacteria growing in the presence of hydrocarbons. Cells tend to occur at the interface between the aqueous and hydrophobic phases and a micelle (arrow) can be also observed

concentrations below the CMC. Sotirova et al. 2009 have shown significant decrease in the major OMPs in the presence of RLs below the CMC. They attributed this change in membrane organisation to the binding of RL monomers which may facilitate uptake of hydrophobic compounds in certain regions of the membrane.

4 The Lipopeptide Biosurfactants

Lipopeptide (LPs) are a structurally distinct group of biosurfactants produced by bacteria, mainly *Bacillus* and *Pseudomonas* species but also *Streptomyces* and other Actinobacteria, and fungi such as *Aspergillus* and *Fusarium* (Raaijmakers et al. 2010; Mnif and Ghribi 2015). The diversity of lipopeptide producing organisms and lipopeptide encoding genes has increased remarkably during the last years due to the contribution of molecular approaches such as genome mining and PCR-based detection methods (de Bruijn et al. 2007; Rokni-Zadeh et al. 2011; Domingos et al. 2015). Thus, gene clusters for the biosynthesis of novel lipopeptides were recently discovered in the genome sequences of well-known organisms such as *Bacillus subtilis* (i.e., locillomycins) (Luo et al. 2015a), but also in uncommon organisms such as *Kibdelosporangium* sp., a rare actinobacterium (Ogasawara et al. 2015), and *Janthinobacterium* sp., a soil bacterium (Graupner et al. 2015).

The biosynthetic pathway of lipopeptides, based on non-ribosomal peptide synthetases (NRPSs) – large multi-enzymes that carry out the sequential incorporation of amino acids into the nascent peptide – gives rise to a high diversity of molecular structures, yet built around a conserved template. All lipopeptides consist of a short cyclic or linear oligopeptide – the hydrophilic moiety of the molecule – linked to a fatty acid tail – the hydrophobic moiety, but they can greatly vary in the number, type and order of the amino acid residues as well as the length and branching of the chain (Raaijmakers et al. 2010). Minor changes in the structural organisation can significantly affect the overall physico-chemical properties of the molecule, hence its activity. To add further variability, lipopeptides are often synthesised as mixtures of several congeners and isoforms as, for example, surfactin, bacillomycin L, fengycin and locillomycin that are co-produced in *B. subtilis* and contribute each to a specific trait of the organism phenotype (Luo et al. 2015b).

Lipopeptides have been extensively studied for their broad spectrum of antimicrobial activities, which are due to their exceptional ability to interact with and disrupt cell membranes (Hamley 2015; Cochrane and Vederas 2016). However, lipopeptides are also potent biosurfactants that can stimulate the mobilisation, solubilisation and emulsification of hydrophobic compounds including hydrocarbons, chemicals in general and vegetable or waste oils.

Initially, the use of lipopeptides was limited to microbial enhanced oil recovery (MEOR) and biodegradation. Lipopeptides are able to reduce the interfacial tension (IFT) between crude oil and the water phase to ultra-low values, e.g., below 0.1 mN/m, which is necessary to overcome the capillary forces that trap the oil in porous media, thus enabling its mobilisation and release (Youssef et al. 2007). Both bench-scale and in-situ lipopeptide production by strains of *Bacillus* spp. have proven successful in improving the oil recovery also from wells close to their production limits (Youssef et al. 2013; Al-Wahaibi et al. 2014).

Solubilisation, i.e., the increase in aqueous solubility of hydrophobic compounds, is instead the main mechanism of biodegradation. When present above their critical micelle concentration (CMC), biosurfactants induce a drastic increase in solubilisation of hydrocarbons, heavy metals and chemicals via the micellisation process (Sarubbo et al. 2015), as it was shown to occur for many lipopeptides,

including surfactin and fengycin in *B. subtilis* (Singh and Cameotra 2013) and viscosin, massetolide A, putisolvin, and amphisin in *Pseudomonas* spp. (Bak et al. 2015).

Finally, lipopeptides can promote the formation of emulsions, including micro-emulsions, which hold a tremendous potential for cutting-edge applications such as advanced biofuels and drug-delivery systems. The underlying physico-chemical properties have been studied in particular for surfactin, one of the most powerful biosurfactants known so far, capable of reducing the surface tension of water and air from 72 to 27 mN/m at a concentration as low as 10 μM (Seydlová and Svobodová 2008). The values of Hydrophilic-Lipophilic-Balance (HLB) of 10–12 indicate that surfactin can favour the formation of oil-in-water (O/W) microemulsions, and the Critical Packing Parameter (CPP) ($<1/3$) suggests that spherical micelles is the preferred geometry for surfactin self-assembly (Gudiña et al. 2013). Background knowledge of these parameters is essential to guide the further development of novel applications.

5 Biosurfactants Produced by Actinobacteria

The class of Actinobacteria, comprising 5 subclasses and 9 orders for a total of 54 families, contains a very large diversity of organisms capable of producing biosurfactants of various types (Khan et al. 2012; Kügler et al. 2015). *Rhodococcus*, *Arthrobacter*, *Mycobacterium*, *Nocardia* and *Corynebacterium* genera produce predominantly trehalose-containing glycolipids, where the hydrophilic moiety consists of trehalose – a two α-glucose unit sugar – and the hydrophobic tail can be either mycolic acids or ester fatty acids and can vary greatly for length (up to C_{90}), branching, substitutions and saturation degree. In some strains (e.g., *Arthrobacter parafineous*, *Brevibacterium* sp. and *Nocardia* sp.), the trehalose can be replaced by other sugars such as sucrose and fructose. Most of these trehalose lipids remain cell-bound, making the cell surface highly hydrophobic, while only a minor fraction seems to be released extracellularly (Lang and Philp 1998). Moreover, some Actinobacteria including *Streptomyces* sp., *Actinoplanes* sp. and *Rhodococcus* sp. have also been found capable of synthesising lipopeptide biosurfactants highly variable in both the peptide ring and the hydrophobic tail (Kügler et al. 2015).

Despite such remarkable diversity of producer strains and biosurfactant types, research has only focussed on a few model organisms. *Rhodococcus* sp., for example, has been studied extensively for the ability to degrade hydrocarbons and pollutants in a broad range of situations. Because the cell surface is hydrophobic, the main uptake mode occurs via direct contact with the hydrophobic substrates, while solubilisation mediated by free biosurfactants would have a secondary role (Bouchez-Naïtali and Vandecasteele 2008). It was recently shown that trehalose synthesis through alkane metabolism and gluconeogenesis is an important pathway for biosurfactant synthesis in *Rhodococcus* sp. strain SD-74 that involves three, so far identified, enzymes: the alkane monooxygenase AlkB responsible for the initial step of alkane oxidation, the fructose-biphosphate aldolase Fda for the trehalose

backbone synthesis and finally a putative acyl-coenzyme A transferase TlsA that converts trehalose to trehalose lipids (Inaba et al. 2013). Several other genes involved in the biosynthesis of trehalose lipids have been discovered in the genome of *Rhodococcus ruber* IEGM 231, including a fatty acid synthase I, a cyclopropane mycolic acid synthase, three mycolyltransferases, a maltooligosyl trehalose synthase, a maltooligosyl trehalose hydrolase and a trehalose synthase (Ivshina et al. 2014).

Similarly well studied is *Gordonia* sp., a member of the *Corynebacterium*/*Mycobacterium*/*Nocardia* (CMN) complex. A dual-step uptake of hydrophobic substrates has been suggested for *Gordonia* strains growing on *n*-hexadecane, in which first, during early exponential phase, cells are highly hydrophobic and adhere directly onto the hydrocarbons, then, at a later phase of growth, they become more hydrophilic and access biosurfactant-solubilised droplets of dispersed hydrocarbons (Franzetti et al. 2008). *Gordonia* is known as a potent biodegrader, capable to grow and use as carbon source a large variety of highly hydrophobic and recalcitrant compounds, including solid alkanes (e.g., hexatriacontane with 36 carbon atoms), plastic additives (e.g., phthalate esters) and natural or synthetic isoprene rubber (e.g., *cis*-1,4-polyisoprene) (Lo Piccolo et al. 2011; Drzyzga 2012).

Widespread amongst Actinobacteria is also their ability to produce biosurfactants from agro-industrial wastes or inexpensive oil derivatives. Several substrates have proven suitable to support biosynthesis of trehalose lipid in various organisms, for example in *Rhodococcus* sp. and *Tsukamurella* sp. growing on sunflower frying oil, rapeseed oil or glyceryltrioleate (White et al. 2013; Ruggeri et al. 2009; Kügler et al. 2014) and in the marine *Brachybacterium paraconglomeratum* on oil seed cake, a pressed mixture of the residues after oil extraction (Kiran et al. 2014). It can be anticipated that the use of renewable material for the synthesis of added value products such as biosurfactants is a growing trend in current and near future research.

6 Glycolipids Produced by Eukaryotes

The best known BSs from eukaryotes are the glycolipids from non-pathogenic yeasts, a heterogeneous mixture of structurally diverse compounds that are produced in relatively high yields (claimed to be as high as 400 g/L). Glycolipids from *Starmerella*, *Candida* and *Pseudozyma* sp. are the most extensively studied eukaryotic BSs with a wide range of applications (Roelants et al. 2014) and are currently the most promising for competitive large scale industrial production. Sophorolipids (SLs) are hydrophobic surfactants that comprise a hydrophilic disaccharide sophorose (2-*O*-β-D-glucopyranosyl-D-glucopyranose) β-glycosidically linked to a long chain hydroxyl fatty acid, most commonly $C_{18:1}$. SLs are synthesised in a variety of compounds with >40 structural isomers described in the literature (Van Bogaert et al. 2007). SLs vary by degree of acetylation (mono-, di- or nonacetylated) at C_6' and C_6'', acidic (containing a free carboxylic group) or lactonic (forming a macrocyclic lactone ring at C_4'') form (Fig. 1d), fatty acid length ranging between C_{16} and C_{18} and saturation. All these structural variations can alter the physico-

chemical properties of SLs (e.g., di-acetylated acidic SLs are more hydrophilic compared to nonacetylated) which makes them suitable for different applications.

The complete biosynthetic gene cluster has been described in detail for sophorolipids (SLs) in *S. bombicola* (Saerens et al. 2011a, b, c; Van Bogaert et al. 2013). Similar to the glycolipid produced by *Pseudomonas* sp. and *Burkholderia* the fatty acid composition is to a certain degree regulated by the first enzyme in the SL biosynthetic pathway, cytochrome p450 monooxygenase CYP52M1 which has been shown to preferentially hydroxylate oleic acid ($C_{18:1}$) at the terminal or sub-terminal carbon (Huang et al. 2014; Saerens et al. 2015). The hydroxylated fatty acid is the substrate for two UDP-glucosyltransferases (UGTA1 and UGTB1) which covalently link two glucose molecules to produce acidic SLs (Saerens et al. 2011a, b, 2015).

Recently Ciesielska et al. (2016) characterised the lactonesterase responsible for the esterification of the carboxyl group from the hydroxylated fatty acid to the second glucose molecule producing lactonic sophorolipids. Recent advances in our understanding of SL biosynthesis and regulation has enabled recombinant engineering to produce tailor made compositions of SLs for specific applications (Solaiman et al. 2014) and also facilitated the discovery of novel sophorolipids from the non-pathogenic yeasts *Starmerella* and *Candida* sp. (Kurtzman et al. 2010; Price et al. 2012). Van Bogaert et al. 2016 have produced a novel bolaform SL from engineered *S. boimbicola* that is deficient in both the lactonesterase and acetyltransferase. The bolaform SLs are highly water soluble due to the presence of two hydrophilic moieties compared to their nonacetylated hydrophobic counterparts. They have also produced several recombinant engineered strains that synthesise specific compositions of SLs. The next step for SL production will be fine tuning the production process for specifically engineered strains to produce yields comparable or higher than native producers.

Mannosylerythritol lipids (MELs) are surface active glycolipids synthesised by a variety of phylogenetically related basidiomycetous yeasts with *Pseudozyma* sp. and *Ustilago* sp. being the most common producers. MELs are synthesised as a diverse mixture of congeners differing in length of fatty acid chains, degree of saturation and acetylation. MELs are classified by their acetylation at the C_4 and C_6 positions of the mannose sugar group on the hydrophilic head (Fig. 1c). The number of acetyl groups has a significant impact when considering the physico-chemical and self-assembly properties of MELs, where the more hydrophobic di-acetylated MEL-A has a much lower critical micelle concentration (CMC) compared to the more hydrophilic mono-acetylated counter parts MEL-B and MEL-C (Yu et al. 2015). These significant differences make MELs suitable for a wide range of applications (Morita et al. 2015), for example MEL-A significantly increases the efficiency of gene transfection via membrane fusion while MEL-B/MEL-C have no such effect (Inoh et al. 2010, 2011).

The biosynthetic pathway for mannosylerythritol lipids (MEL) production was originally described in *Ustilago maydis* under nitrogen limiting conditions (Hewald et al. 2006), with five MEL biosynthetic genes clustering together on a single operon. Using comparative genomics Morita et al. (2014) have identified and characterised the MEL biosynthetic gene cluster also in the phylogenetically related yeast *Pseudozyma antarctica*. In contrast to SLs, the first step in MEL biosynthesis

proceeds by the stereospecific mannosylation of erythritol catalysed by Emt1 (erythritol/mannose transferase), this is the substrate for the acyltransferases Mac1 and Mac2, which specifically acylate the mannose at positions C_2 and C_3. Similar to all other biosurfactant biosynthetic pathways discussed here, MELs are regioselectively acylated (at C_2 and C_3 on mannose) by the affinity of Mac1 for short chain fatty acids (C_2–C_8) and Mac2 for medium to long chain fatty acids (C_{10}–C_{18}) (Hewald et al. 2006).

Recently a number of genomes have been published relating to MEL in *Pseudozyma* sp. including *P. antarctica* T-34 and JCM10317 (Morita et al. 2013a; Saika et al. 2014), *P. aphidis* DSM70725 (Lorenz et al. 2014), and *P. hubeiensis* SY62 (Konishi et al. 2013). Similar to SL production, advances in our understanding of the MEL biosynthetic pathway is paving the way for comparative genomics to identify novel taxonomically related producers with unique structural variations (e.g., diastereomer type of MEL-B biosynthesis in *P. tsukubaensis*) (Saika et al. 2016). Morita et al. (2014) have published the transcriptomic profile of the MEL hyper-producer *P. antarctica* providing fundamental insights into regulation of the MEL biosynthetic cluster under specific inducing conditions (i.e., excess of hydrophobic substrates). Combining the transcriptional expression profile of MEL biosynthetic genes with the extracellular composition of MELs (e.g., mass spectrometry) will help elucidate the metabolic state during production, this will be essential for developing a highly efficient and tailored production process. Currently the glycolipids from non-pathogenic yeasts offer the greatest potential for large scale production of biosurfactants at an economic scale that could be competitive with current synthetic production processes. Current advances in the metabolic engineering strategies for these biosurfactants can lead to tailor made production pipelines where biosurfactants with specific compositions and modifications will be produced specifically for the desired application. The future of such tailored pipelines will need to be paralleled by efficient downstream recovery of the desired biosurfactants.

7 Applications of Biosurfactant-Enhanced (Bio)availability of Hydrophobic Substrates

Numerous biotechnologies have been developed that are based on the capability of biosurfactants to interact with hydrophobic substrates and cause phenomena such as decrease of surface and interfacial tensions, solubilisation, dispersion, emulsification, desorption and wetting. Applications of biosurfactants can cover a wide variety of fields, with the main areas being environmental bioremediation, microemulsion-based technologies and conversion of renewable resources.

Environmental Bioremediation Biosurfactants have been traditionally tested for their activity to support and enhance microbial degradation of hydrocarbons and hydrophobic pollutants. Being less toxic and more biodegradable than synthetic surfactants offers important advantages for in-situ applications, thus biosurfactants can be released with less risk in the environment. Moreover, the capability of natural

communities of degrader microorganisms to produce biosurfactants can be exploited directly in the natural environment via biostimulation techniques (Ławniczak et al. 2013). Marine oil spills have been successfully treated in lab-scale or mesocosm experiments where crude oil degradation rates were enhanced through the supply of exogenous rhamnolipids (Chen et al. 2013; Nikolopoulou et al. 2013; Tahseen et al. 2016) or consortia of indigenous microorganisms were stimulated to produce biosurfactants such as rhamnolipids and sophorolipids (Antoniou et al. 2015). Surfactin, because of its long fatty acid chain and hydrophobic amino acid ring, has poorer water solubility, which limits its applications in water systems. However, a surfactin-derivative fatty acyl-glutamate (FA-Glu), consisting of a β-hydroxy fatty acid chain of C_{12}–C_{17} linked to a single glutamic acid, was produced in an engineered *B. subtilis* strain (Reznik et al. 2010) and was positively tested for use in marine oil spills, showing much higher water-solubility and reduced toxicity (Marti et al. 2014).

Being highly hydrophobic, when adsorbed onto soil particles, hydrocarbons, chemicals and heavy metals are very resistant to removal. Enhanced removal of such contaminants can be achieved via soil-washing techniques that more and more often use biosurfactants (Lau et al. 2014). Lipopeptides from both *Bacillus* and *Pseudomonas* strains have shown high efficiency of removal of crude oil (from 60% to 90%) and heavy metals (>40% for cadmium and lead) when used as washing agents (Singh and Cameotra 2013; Xia et al. 2014). A rhamnolipid solution similarly worked well to remove petroleum hydrocarbons (>80%) and was found to further support the biodegradation of organic compounds by a consortium of degrader bacteria (Yan et al. 2011).

Microemulsion-Based Technologies Microemulsions are thermodynamically stable dispersions of oil, water and surfactants, and are highly desirable in applications such as enhanced oil recovery (Elshafie et al. 2015), biodiesel formulation, drug delivery and food and cosmetic products. Rhamnolipid biosurfactants have shown good performance in generating glycerol-in-diesel microemulsions that are stable for over 6 months, thus opening up the possibility to produce greener biofuels that have reduced combustion emissions and also make use of an industrial inexpensive by-product, i.e., glycerol (Leng et al. 2015). In addition, due to their biocompatibility and low toxicity, biosurfactants are ideal candidates for pharmaceutical and cosmetic applications. Both rhamnolipids and sophorolipids have proven to be effective in producing lecithin-based microemulsions – having high affinity for the phospholipids of cell membranes – with various oils. Microemulsions with isopropyl myristate were stable over a wide range of temperatures (10–40 °C) and electrolyte concentrations (0.9–4.0% w/v), which is very attractive for cosmetic and also drug delivery applications (Nguyen et al. 2010; Rodrigues 2015).

Conversion of Renewable Resources Despite the substantial research and huge number of reports about potential applications and benefits from the use of biosurfactants, the commercialisation of these compounds remains at a very early stage. One of the main constraints limiting the diffusion of biosurfactants is their

high production costs. One strategy to overcome this problem is the use of inexpensive waste material as substrate for the growth of biosurfactant-producing microorganisms, with the additional important advantage that renewable resources can be converted into high added value products (Makkar et al. 2011; Banat et al. 2014). In this context a lot of research has been done in the last few years, and various waste oils have been demonstrated to support effectively biosurfactant production. The existing surplus of glycerol, for example, derived as co-product from the oleochemical industry and biodiesel production in particular, makes it one of the preferred substrates. However, it is always necessary to bear in mind that a low value waste material rapidly acquires a commercial value once an application for it has been discovered and a demand developed. The other problem with glycerol produced as a by-product from other processes is its variable purity and contamination by other components. Glycerol has, however, been used to stimulate synthesis of various types of biosurfactants at standard yields, including sophorolipids (over 40 g/L) in the yeast *Starmerella bombicola* ATCC2214, glycolipids (32.1 g/L) in *Ustilago maydis*, mannosylerythritol lipids (16.3 g/L) in *Pseudozyma antarctica* JCM 10317 (Nicol et al. 2012), rhamnolipids (in the range 1.0–8.5 g/L) in strains of *Pseudomonas aeruginosa* (Henkel et al. 2012; Perfumo et al. 2013), and surfactin lipopeptide (in the range 230–440 mg/L) in *B. subtilis* (de Faria et al. 2011; Sousa et al. 2012). Olive oil mill waste is another abundant waste from the extraction of olive oil that is of environmental concern due to the difficulty of further processing. Recent work has shown that oil mill waste can be recycled and used as substrate to produce both rhamnolipid and surfactin biosurfactants from *Pseudomonas* and *Bacillus* respectively, and that considerably higher yields (299 mg/L and 25.5 mg/L respectively) can be obtained when using a hydrolysed form of it (Moya-Ramírez et al. 2015, 2016; Radzuan et al. 2017). A number of other inexpensive materials, including frying oils and fats, and vegetable oils (e.g., rapeseed oil, sunflower oil, soybean oil, canola oil, palm oil) have proven to be an attractive alternative paving the way towards a cost-competitive and environmentally friendly production of biosurfactants (Banat et al. 2014).

8 Current State and Research Needs

The state of our knowledge concerning the production of microbial biosurfactants has now reached the stage where we can identify a number of key areas for future research and development. One area that has already attracted some interest is the expression of biosurfactant synthetic genes in a non-pathogenic heterologous host organism. Achieving expression of the small number of genes directly involved in the synthesis of, for example, the glycolipids from bacteria or yeasts is not particularly difficult to achieve. What is more difficult is to provide in the host organism a metabolic environment able to provide the levels of precursors sufficient to achieve high yields of the target biosurfactants.

Research on the production of biosurfactants is currently advancing strongly into the area of gene expression studies during the growth cycle of the organism and this

is providing important information on the regulation systems operating in these organisms. These data have been valuable in determining how the fermentation production of biosurfactants may be optimised since, although it is often considered that fermentation substrate costs and downstream processing will be cost limiting factors for production, in practice extended fermentation periods require high energy inputs and can be a major cost for the production process. Therefore any reduction in the fermentation duration will be an important step in developing an economic production process. The understanding of how biosurfactant production can be further optimised will depend on the use of metabolomic and systems approaches to the whole problem.

One of the most positive aspects of microbial biosurfactant production is the fact that, in most organisms, the final product is exported from the cells into the growth medium making at least the first steps in separation and purification relatively straightforward. Interestingly, however, we have little information on whether there are specific transporter systems for each biosurfactant or whether general transporter systems are employed. This knowledge will be important as we move to heterologous expression systems so that we can ensure the products are effectively exported from the cells in the same way that they are from the original producers.

The whole field of biosurfactants is an actively expanding one with many new opportunities for both basic research and industrial exploitation.

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