Heterologous production of long-chain rhamnolipids from *Burkholderia glumae* in *Pseudomonas putida* – a step forward to tailor-made rhamnolipids

Andreas Wittgens[1,#,*], Beatrix Santiago-Schuebel[2], Marius Henkel[3], Till Tiso[4], Lars Mathias Blank[4], Rudolf Hausmann[3], Diana Hofmann[5], Susanne Wilhelm[6,#], Karl-Erich Jaeger[7,8], Frank Rosenau[1,#]

[1]Ulm Center for Peptide Pharmaceuticals (U-PEP), Ulm-University, Albert-Einstein-Allee 11, 89081 Ulm, Germany

[2]Central Institute for Engineering, Electronics and Analytics, Section Analytics (ZEA-3), Forschungszentrum Jülich GmbH, Wilhelm-Johnen-Straße, 52428 Jülich, Germany

[3]Institute of Food Science and Biotechnology, Department of Bioprocess Engineering (150k), University of Hohenheim, Fruwirthstraße 12, 70599 Stuttgart, Germany

[4]Institute of Applied Microbiology (iAMB), Aachen Biology and Biotechnology (ABBt), RWTH Aachen University, Worringerweg 1, 52074 Aachen, Germany

[5]Institute for Bio- and Geosciences, IBG-3: Agrosphere, Forschungszentrum Jülich GmbH, Wilhelm-Johnen-Straße, 52428 Jülich, Germany

[6]iQu Collegiate-Didactics, Heinrich-Heine-University Düsseldorf, Universitätsstraße 1, 40225 Düsseldorf, Germany

[7]Institute for Molecular Enzyme Technology (IMET), Heinrich-Heine-University Düsseldorf, Forschungszentrum Jülich GmbH, Wilhelm-Johnen-Straße, 52428 Jülich, Germany

[8]Institute of Bio- and Geosciences, IBG-1: Biotechnology, Forschungszentrum Jülich GmbH, Wilhelm-Johnen-Straße, 52428 Jülich, Germany

[#]former address: Institute for Molecular Enzyme Technology (IMET), Heinrich-Heine-University Düsseldorf, Forschungszentrum Jülich GmbH, Wilhelm-Johnen-Straße, 52428 Jülich, Germany

*corresponding author: Andreas Wittgens

e-mail: andreas.wittgens@uni-ulm.de

phone: +49 731 50-15186

fax: +49 731 50-22719

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Abstract

Rhamnolipids are biosurfactants consisting of rhamnose (Rha) molecules linked through a β-glycosidic bond to 3-hydroxyfatty acids with various chain lengths and they have an enormous potential for various industrial applications. The best known native rhamnolipid producer is the human pathogen *Pseudomonas aeruginosa*, which produces short-chain rhamnolipids mainly consisting of a Rha-Rha-C_{10}-C_{10} congener. Bacteria from the genus *Burkholderia* are also able to produce rhamnolipids, which are characterized by their long-chain 3-hydroxyfatty acids with a predominant Rha-Rha-C_{14}-C_{14} congener. These long-chain rhamnolipids offer different physicochemical properties compared to their counterparts from *P. aeruginosa* making them very interesting to establish novel potential applications. However, widespread applications of rhamnolipids are still hampered by the pathogenicity of producer strains and - even more important - by the complexity of regulatory networks controlling rhamnolipid production, e.g. the so-called *quorum sensing* system.

To overcome encountered challenges of the wild-type the responsible genes for rhamnolipid biosynthesis in *Burkholderia glumae* were heterologously expressed in the non-pathogenic *Pseudomonas putida* KT2440. Our results show that long-chain rhamnolipids from *Burkholderia* spec. can be produced in *P. putida*. Surprisingly, the heterologous expression of the genes *rhlA* and *rhlB* encoding an acyl- and a rhamnosyltransferase, respectively, resulted in the synthesis of two different mono-rhamnolipid species containing one or two 3-hydroxyfatty acid chains in equal amounts. Furthermore, mixed biosynthetic *rhlAB* operons with combined genes from different organisms were created to determine, whether RhlA or RhlB is responsible to define the fatty acid chain lengths in rhamnolipids.

Introduction

During the last years rhamnolipids have attracted increasing interest in industry as they have the potential to replace surfactants of petrochemical origin because of their rapid biodegradability, low toxicity and sustainability of production (Nitschke et al. 2005; Johann et al. 2016; Henkel et al. 2017). The potential applications of rhamnolipids are very broad covering many industries including the pharmaceutical, the chemical and the cosmetic and food industries (Land and Wullbrandt 1999; Maier and Soberón-Chávez 2000; Banat et al. 2010; Tiso et al. 2017).

The production of rhamnolipids was first described nearly seventy years ago for the human pathogen *Pseudomonas aeruginosa* (Jarvis and Johnson 1949). Rhamnolipids are glycolipids composed of either one (mono-rhamnolipid) or two rhamnose molecules (di-rhamnolipid) linked through a β-glycosidic bond to one, or more common, two 3-hydroxyfatty acids with various chain lengths (Fig. 1; Déziel et al. 1999; Abdel-Mawgoud et al. 2010). The biosynthesis of rhamnolipids occurs in three distinct consecutive enzymatic reactions. The first reaction is catalyzed by the acyltransferase (HAA synthase) RhlA (Déziel et al. 2003; Zhu and Rock 2008), which is responsible for the synthesis of the fatty acid dimers 3-(3-hydroxyalkanoyloxy)alkanoic acids (HAAs) by esterification of two 3-hydroxyfatty acids bound to the acyl carrier protein (ACP) descending from the fatty acid *de novo* synthesis (Rehm et al. 2001; Zhu & Rock 2008) or bound to the coenzyme A (CoA) descending from the β-oxidation (Zhang et al. 2012; Abdel-Mawgoud et al. 2014). Mono-rhamnolipids are synthesized by the rhamnosyltransferase I (RhlB) (Ochsner et al. 1994a; Wittgens et al. 2017), which links an HAA molecule with a dTDP-L-rhamnose descending from glucose-6-phosphate (Olvera et al. 1999; Rahim et al. 2000). Finally, the rhamnosyltransferase II (RhlC) generates di-rhamnolipids by adding a second dTDP-L-rhamnose molecule to the mono-rhamnolipids (Rahim et al. 2001). The genes *rhlA* and *rhlB* are organized as a bicistronic operon in *P. aeruginosa* (Ochsner et al. 1994a), while *rhlC* forms an operon with *PA1131*, which is located elsewhere in the chromosome. The gene *PA1131* encodes a transporter of the major facilitator superfamily (MFS), but seems not to be involved in rhamnolipid biosynthesis or secretion (Wittgens et al. 2017).

Apart from *P. aeruginosa* as the best known rhamnolipid producer (Giani et al. 1997; Müller et al. 2011) bacteria from the genus *Burkholderia* produce rhamnolipids as well. Among them are prominent human pathogens like *Burkholderia pseudomallei* (Häußler et al. 1998, 2003)

as well as species like *B. thailandensis* (Dubeau et al. 2009; Funston et al. 2016; Elshikh et al. 2017), *B. kururiensis* (Tavares et al. 2012) or plant pathogens like *B. plantarii* (Andrä et al. 2006; Hörmann et al. 2010) and *B. glumae* (Manso Pajarron et al. 1993; Costa et al. 2011), which 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). In contrast to rhamnolipids from *P. aeruginosa*, which contain short-chain fatty acids with lengths between C_8 and C_{14} and a predominant C_{10}-C_{10} species in mono- and di-rhamnolipids, all investigated *Burkholderia* species almost exclusively synthesize di-rhamnolipids with long-chain fatty acids between C_{10} and C_{16} and a predominant C_{14}-C_{14} species (Manso Pajarron et al. 1993; Häußler et al. 1998; Dubeau et al. 2009; Costa et al. 2011). Due to their longer fatty acid chains rhamnolipids from the genus *Burkholderia* offer novel properties and thus may enlarge the available portfolio of rhamnolipids for potential new industrial applications. All genes responsible for rhamnolipid production are located within a single gene cluster in *Burkholderia* spec., while *P. aeruginosa* harbors two separate *rhl*-operons. Notably, some species like *B. pseudomallei* and *B. thailandensis* carry two identical *rhl* gene clusters on their two chromosomes (Dubeau et al. 2009), while others like *B. glumae* contain only a single gene cluster (Lim et al. 2009; Voget et al. 2015). In *P. aeruginosa*, the rhamnolipid biosynthesis is regulated on the transcriptional level by the *quorum sensing* regulatory network (Ochsner et al. 1994b; Ochsner and Reiser 1995; Pearson et al. 1997), which was recently reported for *B. glumae* as well (Nickzad et al. 2015), and probably by further signaling systems (Wilhelm et al. 2007; Rosenau et al. 2010; Henkel et al. 2013).

The typical complex regulation along with the pathogenicity of producer strains are the main bottlenecks preventing the use of wild-type bacteria for the production of rhamnolipids at an industrial scale, especially if addition to cosmetics or foods is envisaged (Toribio et al. 2010; Müller and Hausmann 2011). For this reason, the heterologous production of rhamnolipids from *P. aeruginosa* was successfully established and optimized in the non-pathogenic *Pseudomonas putida* KT2440 in the past (Ochsner et al. 1995; Wittgens et al. 2011; Beuker et al. 2016a).

In this present study, we used *P. putida* KT2440 as the heterologous host to establish the production of long-chain rhamnolipids. We show that recombinant *P. putida* strains are able to produce the same rhamnolipid species as *Burkholderia*, depending on the specificity of the respective Rhl enzymes. The genes responsible for the production of mono- and di-

126 rhamnolipids from the *B. glumae* strain PG1 were modularly cloned in different combinations
127 and heterologously expressed in *P. putida* KT2440. Furthermore, the ideal simplified
128 metabolic and genetic background of *P. putida* as the heterologous host allowed the
129 expression of hybrid *rhl*-operons containing combined *rhlA* and *rhlB* genes from *P. aeruginosa*
130 and *B. glumae* to get novel insights on the activity of rhamnolipid synthesizing proteins.
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Materials and methods

Bacterial strains and culture conditions

Burkholderia glumae PG1 (CBS 322.89; Urakami et al. 1994) was cultivated in minimal medium E (MME; Vogel and Bonner 1956) supplemented with 20 g/l olive oil as additional carbon source. The MME was prepared as a 50x stock solution in distilled water (10 g/l $MgSO_4 \times 7H_2O$, 100 g/l citrate × H_2O, 175 g/l $Na(NH_4)HPO_4 \times 4H_2O$ and 500 g/l K_2HPO_4; pH 7.0) and diluted for use with distilled water. *Pseudomonas aeruginosa* PAO1 (DSM-22644; Hancock and Carey 1979), *Pseudomonas putida* KT2440 (DSM-6125; Nelson et al. 2002) and *Escherichia coli* DH5α (DSM-6897; Grant et al. 1990) were cultivated in LB medium (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl). *P. aeruginosa* and *E. coli* strains were cultivated at 37 °C while *B. glumae* and *P. putida* strains were cultivated at 30 °C and 150 rpm orbital shaking using 100 ml Erlenmeyer flasks filled with 10 ml medium.

Amplification of *rhl*-genes and plasmid construction

Single *rhl*-genes and the *rhlAB* operon were amplified from the genomic DNA of *B. glumae* PG1 and *P. aeruginosa* PAO1 (isolated with DNeasy Blood and Tissue Kit, QIAGEN, Hilden, Germany) using *Pfu*Turbo DNA polymerase (Agilent Technologies, Waldbronn, Germany) as described by the supplier. The used primers, obtained from Eurofins Genomics (Ebersberg, Germany), and restriction sites used for cloning of the resulting PCR products into the pVLT33 vector (de Lorenzo et al. 1993) are listed in table 1. For maintaining the native ribosomal binding site, all single *rhl*-genes and the *rhlAB* operon were amplified starting 20 bp upstream of the start codon. Restriction enzymes and T4 DNA ligase were obtained from Thermo Fisher Scientific (St. Leon-Rot, Germany) and used as recommended. DNA manipulation was carried out as described in Sambrook and Russell (2001). *E. coli* DH5α cells were transformed with the resulting recombinant plasmids (Tab. 1) using a standard protocol (Hanahan 1983). Positive clones were selected on LB-agar plates containing 50 µg/ml kanamycin.

Production of rhamnolipids in recombinant *P. putida*

P. putida KT2440 cells were transformed with pVLT33 and derivates by electroporation according to Choi et al. (2006) and selected using LB-agar plates or LB liquid medium containing 50 µg/ml kanamycin after incubation at 30 °C.

For the production of rhamnolipids, main cultures of 10 ml LB medium supplemented with 20 g/l glucose and 50 µg/ml kanamycin in 100 ml Erlenmeyer flasks were inoculated to an OD_{580} of 0.05 using overnight cultures and incubated at 30 °C and 150 rpm. The expression of *rhl*-genes was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.4 mM from the beginning of the cultivation.

Extraction of rhamnolipids

Rhamnolipids were extracted from main cultures of *B. glumae* and *P. putida* after cultivation for 24 h. For this purpose, 1 ml of the cultures was centrifuged at 21,500 x *g* for 3 minutes. For analysis of rhamnolipids using thin layer chromatography (TLC) 500 µl supernatant and for the quantification via orcinol assay 100 µl supernatant were transferred to a new reaction tube and each were spiked with 500 µl ethyl acetate. Samples were mixed on a vortex shaker and centrifuged for 30 sec at 21,500 x *g*. Upper phases were removed and the lower phases were used for two further extractions as described above. The three extracts were collected in a new reaction tube. The solvents were evaporated in a vacuum centrifuge.

Thin layer chromatography of rhamnolipids

For rhamnolipid detection using TLC, the dried samples were dissolved in 20 µl ethanol and completely spotted on silica 60 TLC-plates (SIL-G, Macherey-Nagel, Düren, Germany). In addition, 10 µl of a 0.1 % (w/v) commercial rhamnolipid solution (JBR425, Jeneil Biosurfactant Co., LCC, Saukville, USA) containing mono- and di-rhamnolipids were spotted as a positive control. The mobile phase was a mixture of chloroform, methanol and acetic acid at a ratio of 65:15:2 (v/v/v). The staining solution consists of 0.15 g orcinol-monohydrate, 8.2 ml sulfuric acid (60 %) and 42 ml distilled water and was sprayed on the TLC-plates to visualize the rhamnolipids. The dried plates were incubated at 110 °C for 10 min.

Rhamnolipid quantification using orcinol assay

The total amounts of rhamnolipids were determined using the orcinol assay (Chandrasekaran and Bemiller 1980; Ochsner 1993). The evaporated rhamnolipids were dissolved in 100 µL distilled water. Then, 100 µL orcinol solution (16 g/l orcinol-monohydrate) and 800 µL sulphuric acid (60 %) were added. The mixture was incubated for 30 min at 80 °C and 800 rpm and afterwards cooled down to room temperature. The samples were measured at 421 nm

and compared to a rhamnolipid standard (JBR425) with concentrations between 0 and 500 mg/l.

Chemical analysis of rhamnolipids by HPLC-ESI-MS

For the rhamnolipid characterization, high performance liquid chromatography electrospray ionization mass spectrometry (HPLC-ESI-MS) was used. Rhamnolipids were purified from 1 l cultures (5 l Erlenmeyer flasks) of recombinant *P. putida* strains according to Déziel et al. (1999) with small modifications. Cells were removed by centrifugation for 30 min at 6,740 × *g* and 10 °C. The supernatant was acidified with 37% HCL to pH 3 and incubated overnight at 4 °C and 80 rpm. The precipitated rhamnolipids were recovered by centrifugation for 45 min at 8,280 x *g* and 10 °C followed by resuspension in 15 ml acidified water (pH 3, adjusted with 37 % HCl). This suspension was extracted three times with 15 ml ethyl acetate; the organic phases were collected and evaporated under vacuum. The residues were dissolved in 15 ml of 0.05 M sodium bicarbonate, acidified to pH 2 with 37 % HCl and incubated at 4 °C overnight. The rhamnolipids were finally recovered by centrifugation for 60 min at 4,650 x *g* and 4 °C.

For the characterization of rhamnolipids, a binary HPLC system (Agilent 1100 series, Agilent Technologies, Waldbronn, Germany), assembled with a diode array detector (DAD; 190-400 nm) and coupled with a triple quadrupole mass spectrometer (4000 QTRAP™, AB SCIEX, Darmstadt, Germany) assembled with a TurboIon spray source was used.

Rhamnolipid separation was achieved using a ProntoSIL 120-C8-SH (Bischoff Chromatography, Leonberg, Germany) column (2 mm x 150 mm x 3 µm) at 20 °C. The gradient elution was done with deionized water with 0.1 % (v/v) aqueous formic acid (solution A) and acetonitrile with 0.1 % (v/v) formic acid (solution B). The elution started with 60 % solution B isocratic for 4 min, from 4 to 24 min a linear increase from 60 % to 90 % solution B was applied, followed by a second isocratic step (90 % solution B for 10 min) and ended with a return to 60 % solution B in 1 min. The re-equilibration was done with 60% B isocratic for 10 min. All steps were performed at a constant flow rate of 300 µl/min. The injection volume was 20 µL.

The MS was used in the negative enhanced mass spectrum mode scanning from 200 – 1,000 Da. A flow injection analysis (FIA) with a standard was used at first to optimize the following parameter settings: ion spray voltage (IS) -4,500 V, declustering potential (DP) -100 V, curtain gas (N_2) 10 arbitrary units (au), source temperature 500 °C, nebulizer gas (N_2) 50 au and heater gas (N_2) 20 au. Collision energy (CE) and third quadrupole-entry barrier were set to -5 V and 8

V, respectively, to minimize fragmentation entering the LIT in the full scan mode. The negative enhanced product ion (EPI) scan mode was used for structural elucidation MS/MS experiments. Collision energies (CE) ranged from 30 to 70 V.

Separation of mono-rhamnolipid species using preparative thin layer chromatography

Rhamnolipids were purified from culture supernatant as described before. After evaporation of ethyl acetate, the rhamnolipids were dissolved in 2 ml ethanol and spotted in 50 µl aliquots on a preparative TLC-plate with 2 mm thickness of silica 60 (Macherey-Nagel, Düren, Germany). The composition of the mobile phase and the staining solvent is described above. Prior to the detection of rhamnolipid spots by wetting the TLC-plate with the staining solution, all lanes except both outer lanes were covered. After color development, the corresponding areas for both mono-rhamnolipid species were marked and the silica gel was scraped off from the plate. The crushed silica gel was then extracted three times with 10 ml ethyl acetate and the obtained solvent was filtered through a PTFE-membrane filter with 2 µm pore size (VWR, Darmstadt, Germany). After evaporation of the solvent under vacuum, the samples were used for HPLC-ESI-MS analysis as described above.

Results

Biosynthesis of long-chain mono- and di-rhamnolipids in recombinant *P. putida*

The successful heterologous production of rhamnolipids in the non-pathogenic *P. putida* KT2440 expressing *rhl*-genes from *P. aeruginosa* is well-established and qualifies this strain as a suitable host organism to circumvent the complex regulatory system of native rhamnolipid producers (Wittgens et al. 2011; Tiso et al. 2016; Beuker et al. 2016b). It further offers the possibility for the production of both major individual species (mono- and/or di-rhamnolipids) after expression of respective *rhl*-genes or operons (Wittgens et al. 2017). Using *P. putida* as chassis for the expression of *rhl*-genes originating from the genus *Burkholderia* thus allows investigating the function of different Rhl proteins in the same physiological background.

For the establishment of heterologous production of *Burkholderia*-typical long-chain rhamnolipids in *P. putida*, the *rhlAB* operon and the *rhlC* gene from *B. glumae* PG1 were cloned as single elements and as a biosynthetic *rhlABC* operon into the pVLT33 vector (de Lorenzo et al. 1993) yielding the recombinant plasmids pVLT33_$rhlAB_{Bg}$, pVLT33_$rhlC_{Bg}$ and pVLT33_$rhlABC_{Bg}$ (Tab. 1). The genome of *B. glumae* PG1 consisting of two chromosomes has recently been sequenced (Voget et al. 2015) and the *rhl*-genes are available under the accession numbers BGL_2c07470 (*rhlA*), BGL_2c07480 (*rhlB*) and BGL_2c07500 (*rhlC*), respectively. The heterologous expression of the $rhlAB_{Bg}$ operon in *P. putida* resulted in the production of mono-rhamnolipids, which surprisingly form two bands with almost equal intensity on a thin layer chromatography (TLC) plate (Fig. 2). These bands represent two different species of mono-rhamnolipids: the upper band contains typical mono-rhamnolipids containing two 3-hydroxyfatty acids (mono-rhamno-di-lipids), while the lower band with lesser mobility contains mono-rhamnolipids with only a single 3-hydroxyfatty acid chain (mono-rhamno-mono-lipids). For a chemical analysis of the exact composition of each mono-rhamnolipid species by HLPC-ESI-MS, both bands were separately isolated from a preparative TLC-plate. The results confirmed that the upper band contains only long-chain mono-rhamno-di-lipids with chain lengths varying between C_{10}-C_{14} and C_{16}-C_{14} and a predominant Rha-C_{14}-C_{14} congener. In contrast, the lower located band on the TLC-plate contains only mono-rhamno-mono-lipids with fatty acid chains of C_{12} to C_{16} and a predominant Rha-C_{14} congener. Additional data of these analyses exemplarily presenting more detailed information about the identified congeners and positions of specific fatty acids (chain lengths, saturated or

unsaturated) are given in Online Resource 1 (Fig. OR-1 A and B). These chain lengths are typical for rhamnolipids originating from *Burkholderia* species (Abdel-Mawgoud et al. 2010).

Moreover, the heterologous production of *B. glumae* long-chain di-rhamnolipids was observed in recombinant *P*. *putida* upon expression of the biosynthetic *rhlABC*$_{Bg}$ operon (Fig. 2). The single di-rhamnolipid band in this sample showed the same mobility as the sample extracted from the *B. glumae* wild-type strain as a reference (Fig. 2). In contrast to the mono-rhamnolipid sample, no respective di-rhamnolipid species containing a single 3-hydroxyfatty acid (di-rhamno-mono-lipid) was found. In addition, no remaining mono-rhamnolipid bands are visible indicating an entire conversion from mono- to di-rhamnolipids after additional expression of *rhlC.* These results were confirmed by HLPC-ESI-MS, where only di-rhamnolipids with fatty acid chain lengths from C_{12}-C_{10} to C_{16}-C_{14} and a predominant Rha-Rha-C_{14}-C_{14} species were detected. Some rhamnolipids harbor single unsaturated bonds in one of their fatty acids, but we could also identify two di-rhamnolipid congeners with single unsaturations in both alkyl chains (Rha-Rha-$C_{14:1}$-$C_{14:1}$ and Rha-Rha-$C_{14:1}$-$C_{16:1}$) which, at least to our knowledge, has not been described so far. Additional data of these analyses exemplarily presenting most of the identified congeners are given in Online Resource 1 (Fig. OR-1 C).

As expected, *P. putida* cells harboring only the empty vector pVLT33 or expressing the single *rhlC*$_{Bg}$ gene as controls did not produce any kind of rhamnolipids (Fig. 2).

In all cases, the heterologously produced rhamnolipids from *P. putida* as well as the di-rhamnolipids from the *B. glumae* wild-type strain migrated, based on their longer fatty acids, closer to the solvent front compared to the mono- and di-rhamnolipids of the commercial rhamnolipid-standard, which contains rhamnolipids from *P. aeruginosa* with shorter alkyl chains. Furthermore, all samples isolated from *P. putida* showed an additional band located in the range of di-rhamnolipids and stained light violet instead of the brownish color of the rhamnolipid bands. These bands originated from the added IPTG.

After 24 hours of heterologous expression of the *B. glumae rhlAB* operon in the recombinant *P. putida* an amount of 75 mg/l mono-rhamnolipid could routinely be achieved. In comparison, after expression of the biosynthetic *rhlABC*$_{Bg}$ operon, 59 mg/l of di-rhamnolipids were produced (Fig. 2). Without any further implementation of optimization strategies to improve the production of these novel rhamnolipid species, this is already a slight improvement in comparison to 56 mg/l, which could be obtained from *B. glumae* cultures grown with

vegetable oil, in this experiment olive oil, as the best carbon source for a high rhamnolipid titer with this species according to Costa et al. (2011).

Our results reveal that *P. putida* KT2440 is a suitable host for the heterologous production of different rhamnolipid species with various fatty acid chain lengths. Further, the selected expression of *rhl*-genes allows the specific synthesis of mono- and/or di-rhamnolipids.

Construction of functional biosynthetic operons with hybrids of *P. aeruginosa* and *B. glumae* *rhl*-genes

Since *P. putida* is able to produce short-chain as well as long-chain rhamnolipids based on the origin of the heterologously expressed genes, the fatty acid chain lengths of rhamnolipids appear to be determined by the specificity of the Rhl proteins instead of the predominant length of the 3-hydroxyfatty acid species provided by the metabolism of the host organism. However, the precise functions of RhlA or RhlB enzymes for determining the chain lengths of 3-hydroxyfatty acids used for rhamnolipid biosynthesis remain elusive. RhlA may exert specificity for certain chain lengths during the biosynthesis of the HAAs as precursors and RhlB may use all of the molecules provided by RhlA for the synthesis of mono-rhamnolipids. Alternatively, RhlA may synthesize a wide variety of HAAs with different chain lengths and RhlB uses only specific ones for the mono-rhamnolipid biosynthesis, suggesting the specificity for this process being determined by RhlB.

To investigate the influence of RhlA and RhlB in determination of 3-hydroxyfatty acids for the rhamnolipid biosynthesis, the respective genes were amplified each as single from *P. aeruginosa* and *B. glumae* and cloned in all four conceivable combinations as native and hybrid *rhlAB* operons into pVLT33 yielding the recombinant plasmids pVLT33_$rhlA_{Pa}$/$rhlB_{Pa}$, pVLT33_$rhlA_{Pa}$/$rhlB_{Bg}$, pVLT33_$rhlA_{Bg}$/$rhlB_{Pa}$ and pVLT33_$rhlA_{Bg}$/$rhlB_{Bg}$ (Tab. 1, Fig. 3a).

Expression of the operon containing *rhlAB* from *P. aeruginosa* ($rhlA_{Pa}$/$rhlB_{Pa}$) in *P. putida* resulted in the typical mono-rhamnolipid band on a TLC-plate, which showed the same migration behavior as the mono-rhamnolipids of the standard (Fig. 3b). In contrast, if the *rhlAB* operon from *B. glumae* ($rhlA_{Bg}$/$rhlB_{Bg}$) was expressed, two mono-rhamnolipid bands became visible as described earlier (Fig. 3b). However, the heterologous expression of the *rhlAB* operon with *rhlA* from *P. aeruginosa* and *rhlB* from *B. glumae* ($rhlA_{Pa}$/$rhlB_{Bg}$) revealed a single mono-rhamnolipid band like the sample containing the $rhlA_{Pa}$/$rhlB_{Pa}$ operon with both genes from *P. aeruginosa* (Fig. 3b). In contrast, the expression of the $rhlA_{Bg}$/$rhlB_{Pa}$ operon with *rhlA*

from *B. glumae* and *rhlB* from *P. aeruginosa* resulted in two mono-rhamnolipid bands, which show the same pattern as the sample in which both genes originate from *B. glumae* (*rhlA*$_{Bg}$/*rhlB*$_{Bg}$).

These results were confirmed by analyses of the chemical rhamnolipid compositions by HPLC-ESI-MS analysis revealing that RhlA$_{Pa}$ directs the biosynthesis of *P. aeruginosa*-typical short-chain rhamnolipids with a predominant Rha-C_{10}-C_{10} species (Tab. 2). On the contrary, the samples with RhlA$_{Bg}$ contained long-chain mono-rhamno-mono-lipids and mono-rhamno-di-lipids with predominant Rha-C_{14} and Rha-C_{14}-C_{14} species, respectively, which are typical for rhamnolipids from *Burkholderia* spec. (Tab. 2).

However, the expression of the two hybrid operons with combined genes from both organisms resulted in both cases in a two-thirds lower amount of mono-rhamnolipids (66 mg/l with *rhlA*$_{Pa}$/*rhlB*$_{Bg}$ and 31 mg/l with *rhlA*$_{Bg}$/*rhlB*$_{Pa}$) than the expression of the native operons with both genes from the same organism (95 mg/l with *rhlA*$_{Pa}$/*rhlB*$_{Pa}$ and 60 mg/l with *rhlA*$_{Bg}$/*rhlB*$_{Bg}$) (Fig. 3b).

These results confirm that the specificity of RhlA determines the chain lengths of 3-hydroxyfatty acids, which are used for the biosynthesis of HAAs and subsequently for rhamnolipids.

Discussion

All rhamnolipids from *Burkholderia* species contain long-chain fatty acids with a predominant di-rhamnolipid species containing C_{14}-C_{14} chains (Manso Pajarron 1993; Häußler et al. 1998; Dubeau et al. 2009; Costa et al. 2011; Funston et al. 2016). Compared to their counterparts originating from *P. aeruginosa*, which are predominantly composed of fatty acids with shorter chain lengths (C_{10}-C_{10}), rhamnolipids from *Burkholderia* species possess different physicochemical properties and could thus enlarge the portfolio of rhamnolipids available for potential new and valuable industrial applications. For instance, the rhamnolipid mixture from *B. thailandensis* reduced the surface tension of water from 72 to 42 mN/m at a critical micelle concentration (CMC) of 225 mg/l (Dubeau et al. 2009). In comparison, a rhamnolipid mixture from *P. aeruginosa*, with a high content of Rha-Rha-C_{10}-C_{10}, reduce surface tension to 31 mN/m at a CMC of 53 mg/l (Mata-Sandoval et al. 1999).

The non-pathogenic *P. putida* KT2440 is a well-established host organism for the heterologous production of rhamnolipids, which allows to circumvent the complex regulatory networks and pathogenicity of native rhamnolipid producers (Wittgens et al. 2011; Tiso et al. 2016; Beuker et al. 2016a, 2016b), and was used for the first time in this study for the biosynthesis of long-chain rhamnolipids by expression of *rhl*-genes from *B. glumae* PG1. All rhamnolipid species produced by *P. putida* after heterologous expression of *rhl*-genes from *B. glumae* contained the same fatty acids (mainly C_{14} or C_{14}-C_{14}) as those produced by *B. glumae* wild-type which are also known from *B. thailandensis and B. pseudomallei* (Manso Pajarron 1993; Häußler et al. 1998; Dubeau et al. 2009; Costa et al. 2011). Since *P. putida* is able to produce these long-chain rhamnolipids as well as *P. aeruginosa*-typical short-chain rhamnolipids with mainly C_{10} fatty acids, the biosynthesis of specific rhamnolipid species suggests that relevant Rhl enzymes may be responsible rather than the available 3-hydroxyfatty acids provided by the fatty acid *de novo* synthesis in the producing organism.

Surprisingly, the heterologous expression of *rhlAB* from *B. glumae* resulted in the synthesis of two different mono-rhamnolipid species at equal amounts, which were analyzed as the typical mono-rhamnolipids containing a fatty acid dimer and mono-rhamno-mono-lipids containing a single fatty acid. The second species is also known from *P. aeruginosa* (Syldatk et al. 1984a, 1984b; Déziel et al. 1999), but was never described before for *Burkholderia* species. Since it is known that the biosynthesis of mono-rhamno-mono-lipids occurs most probably through hydrolysis of typical mono-rhamnolipids by an unknown enzyme (Wittgens et al. 2017), this

result suggests that this enzyme might be a lipase favoring long-chain fatty acid substrates (>C_{10}) in contrast to esterases (Verger 1997; Rosenau and Jaeger 2000; Wilhelm et al. 2007; Hausmann 2009).

However, the heterologous expression of the biosynthetic *rhlABC* operon from *B. glumae* resulted in the production of long-chain di-rhamnolipids, while no remaining mono-rhamnolipids could be detected, which was also found for some *Burkholderia* species producing rhamnolipids (Monso Pajarron et al. 1993; Häußler et al. 1998). Therefore, the additional expression of *rhlC* in *P. putida* also leads to a complete conversion of mono- to di-rhamnolipids suggesting a high rhamnosyltransferase II activity or a generally low amount of mono-rhamnolipids. In addition, di-rhamno-mono-lipid species could not be found in *P. putida* upon expression of $rhlABC_{Bg}$ demonstrating that the required putative lipase-like enzyme may possess certain specificity for mono-rhamnolipids. Such a di-rhamnolipid species is also known from *P. aeruginosa* (Syldatk et al. 1984a, 1984b) indicating that this organism may harbor two different lipases catalysing the hydrolysis of either mono- or di-rhamnolipids.

Like in other *Burkholderia* species, the *rhl*-genes in *B. glumae* are also arranged within a gene cluster together with the gene *emrB*, which is located between *rhlB* and *rhlC* (Voget et al. 2015). In *P. aeruginosa*, the *rhl*-genes are arranged as two operons, whereby *rhlC* forms an operon together with *PA1131*, which is spatially separated from the *rhlAB* operon. The gene *PA1131* probably encodes a transporter of the major facilitator superfamily (MFS), which was supposed to be involved in rhamnolipid secretion, while *emrB* from *B. glumae* probably encodes a multidrug resistance protein. However, neither *emrB* in this study nor *PA1131* (Wittgens et al. 2017) was necessary for the successful biosynthesis and secretion of rhamnolipids, although *P. putida* harbors no homologous gene. The physiological functions of EmrB and PA1131 remain elusive as well as the secretion mechanism for rhamnolipids in general.

The comparative expression of native and synthetic operons with combined *rhlA* and *rhlB* genes from *P. aeruginosa* and *B. glumae* resulted in the production of either short-chain rhamnolipids with a predominant Rha-C_{10}-C_{10} congener if the used *rhlA* gene originated from *P. aeruginosa* or long-chain rhamnolipids with the two predominant Rha-C_{14} and Rha-C_{14}-C_{14} congeners if *rhlA* from *B. glumae* was used. This result demonstrates that the chain length of fatty acids used for rhamnolipid synthesis is determined by RhlA depending on its origin and not on the availability of 3-hydroxyfatty acids in the identical host. Obviously, RhlA uses

selected 3-hydroxyfatty acids with specific chain length for the biosynthesis of HAAs while RhlB has no or at least an almost neglectable specificity and uses all HAAs provided by the respective RhlA for the biosynthesis of rhamnolipids.

In conclusion, these results demonstrate that the non-pathogenic *P. putida* KT2440 is truly qualified to serve as a platform strain for the production of various rhamnolipid species with different chain lengths coming along with different properties for industrial applications. In contrast to native rhamnolipid producers, it allows the synthesis of different mono- or di-rhamnolipids uncoupled from the complex natural regulation mechanisms and thus without the need for the creation of nutrient limitation. The amount of long-chain rhamnolipids is currently slightly lower compared to those produced by expression of *rhl*-genes from *P. aeruginosa*, but it is already comparable to the amounts, which can be obtained with the *B. glumae* wild-type strain under similar conditions using oil as its favored carbon source. This fact is probably caused by a lower specific activity of the *B. glumae* enzymes or by limited availability of specific 3-hydroxyfatty acids in these bacteria. Undoubtedly, the yield of long-chain rhamnolipids can be further increased and optimization of strains and culture conditions will be part of a further study. Moreover, the identification of rhamnolipid modifying/processing lipase-like enzymes is an interesting topic, since a detailed knowledge of these enzymes will open opportunities to influence the processing of rhamnolipids as a further step forward for the production of tailor-made rhamnolipids.

Conflict of interest

The authors declare that they have no conflict of interest.

Competing interests

The authors declare that they have no competing interests.

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Authors´ contributions

AW planned and executed the experiments, created figures and drafted the manuscript, BSS and DH executed structural analysis of rhamnolipids and critically read the manuscript, MH and TT assisted the plasmid characterization and critically read the manuscript, LMB, RH, SW and KEJ took part in initiating the research and critically read the manuscript, FR initiated the project, supervised the research, coordinated the study and critically read the manuscript. All authors read and approved the final manuscript.

664 **Tab. 1 PCR primers, restriction enzymes and resulting recombinant plasmids**

Gene/ Operon	Primer	Sequence (5´->3´)	Restriction enzymes	Rekombinant plasmids
B. glumae				
rhlA	up	TTGAATTCGGTTACCGCCGGAGTACGCC	*Eco*RI	pVLT33_$rhlA_{Bg}$/$rhlB_{Bg}$
	down	TTTGGTACCCTAGGACAGCAGCGGCATCGG	*Acc*65I	pVLT33_$rhlA_{Bg}$/$rhlB_{Pa}$
rhlB	up	TTTGGTACCGCCCATCAGAGGCGGTATCGATGTC	*Acc*65I	pVLT33_$rhlA_{Bg}$/$rhlB_{Bg}$
	down	TTTTTCTAGATCATGCGCCCGAGGCCTC	*Xba*I	pVLT33_$rhlA_{Pa}$/$rhlB_{Bg}$
rhlAB	up	TTGAATTCGGTTACCGCCGGAGTACGCC	*Eco*RI	pVLT33_$rhlAB_{Bg}$
	down	TTTTTCTAGATCATGCGCCCGAGGCCTC	*Xba*I	pVLT33_$rhlABC_{Bg}$
rhlC	up	TTTTTCTAGAGCCAACCCTGGTGGCACG	*Xba*I	pVLT33_$rhlC_{Bg}$
	down	TTTAAGCTTTCATCCGTGGCGCACCCG	*Hin*dIII	pVLT33_$rhlABC_{Bg}$
P. aeruginosa				
rhlA	up	TTGAATTCAAATTTTTGGGAGGTGTGAAATGCGGCG	*Eco*RI	pVLT33_$rhlA_{Pa}$/$rhlB_{Pa}$
	down	TTTGGTACCTCAGGCGTAGCCGATGGCC	*Acc*65I	pVLT33_$rhlA_{Pa}$/$rhlB_{Bg}$
rhlB	up	TTTGGTACCATAACGCACGGAGTAGCCCCATGC	*Acc*65I	pVLT33_$rhlA_{Pa}$/$rhlB_{Pa}$
	down	TTTTTCTAGATCAGGACGCAGCCTTCAGCC	*Xba*I	pVLT33_$rhlA_{Bg}$/$rhlB_{Pa}$

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Tab. 2 Identified rhamnolipid species from recombinant *P. putida* carrying different hybrid operons with combined *rhlA* and *rhlB* genes from *P. aeruginosa* ($_{Pa}$) and *B. glumae* ($_{Bg}$) using HPLC-ESI-MS analysis. Predominant species are printed in bold.

P. putida KT2440 + pVLT33_			
$rhlA_{Pa}/rhlB_{Pa}$	$rhlA_{Pa}/rhlB_{Bg}$	$rhlABg/rhlB_{Pa}$	$rhlA_{Bg}/rhlB_{Bg}$
Rha-C_{10}	-	-	-
Rha-C_{12}	-	-	-
Rha-C_{14}	-	**Rha-C_{14}**	**Rha-C_{14}**
-	-	Rha-C_{16}	Rha-C_{16}
Rha-C_8-C_{10}	Rha-C_8-C_{10}	-	-
Rha-C_{10}-C_8	Rha-C_{10}-C_8	-	-
Rha-C_{10}-C_{10}	**Rha-C_{10}-C_{10}**	-	-
Rha-C_{10}-C_{12}	Rha-C_{10}-C_{12}	-	-
Rha-C_{12}-C_{10}	Rha-C_{12}-C_{10}	-	-
Rha-C_{12}-C_{12}	Rha-C_{12}-C_{12}	Rha-C_{12}-C_{12}	Rha-C_{12}-C_{12}
Rha-C_{10}-C_{14}	Rha-C_{10}-C_{14}	Rha-C_{10}-C_{14}	Rha-C_{10}-C_{14}
Rha-C_{14}-C_{10}	Rha-C_{14}-C_{10}	Rha-C_{14}-C_{10}	Rha-C_{14}-C_{10}
Rha-C_{12}-C_{14}	Rha-C_{12}-C_{14}	Rha-C_{12}-C_{14}	Rha-C_{12}-C_{14}
Rha-C_{14}-C_{12}	Rha-C_{14}-C_{12}	Rha-C_{14}-C_{12}	Rha-C_{14}-C_{12}
Rha-C_{10}-C_{16}	Rha-C_{10}-C_{16}	Rha-C_{10}-C_{16}	Rha-C_{10}-C_{16}
Rha-C_{16}-C_{10}	Rha-C_{16}-C_{10}	Rha-C_{16}-C_{10}	Rha-C_{16}-C_{10}
-	-	**Rha-C_{14}-C_{14}**	**Rha-C_{14}-C_{14}**
-	-	Rha-C_{12}-C_{16}	Rha-C_{12}-C_{16}
-	-	Rha-C_{16}-C_{12}	Rha-C_{16}-C_{12}
-	-	Rha-C_{14}-C_{16}	Rha-C_{14}-C_{16}
-	-	Rha-C_{16}-C_{14}	Rha-C_{16}-C_{14}

Figures

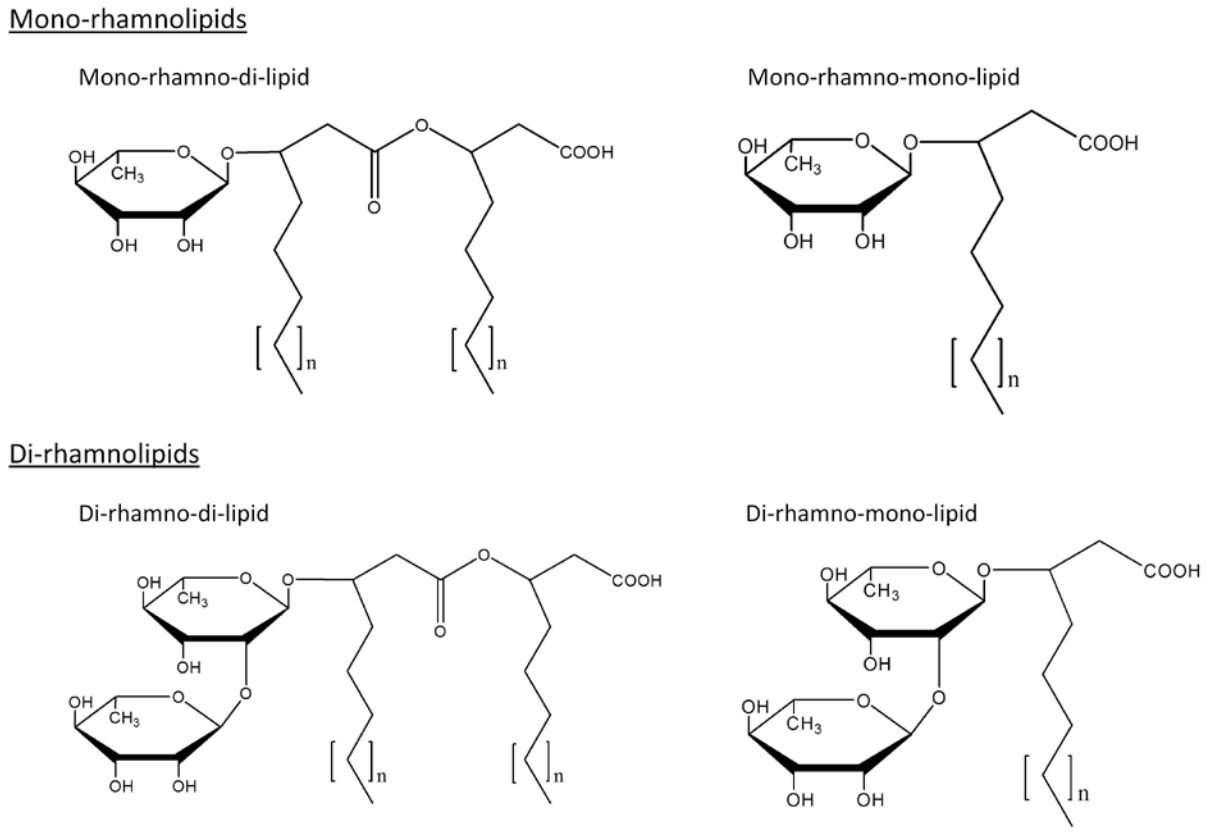

Fig. 1 Chemical structures of rhamnolipids. Rhamnolipids are separated into mono- and di-rhamnolipids based on the number of L-rhamnose residues. Beside typical rhamnolipid species containing two 3-hydroxyfatty acids (mono-rhamno-di-lipid and di-rhamno-di-lipid) there exist species containing only one fatty acid chain (mono-rhamno-mono-lipid and di-rhamno-mono-lipid). Rhamnolipids from *P. aeruginosa* typically contain fatty acids with chain lengths between C_8 and C_{14} (n=1-7) while organisms from the genus *Burkholderia* produce rhamnolipids with longer alkyl chains and typical lengths between C_{10} and C_{16} (n=3-9).

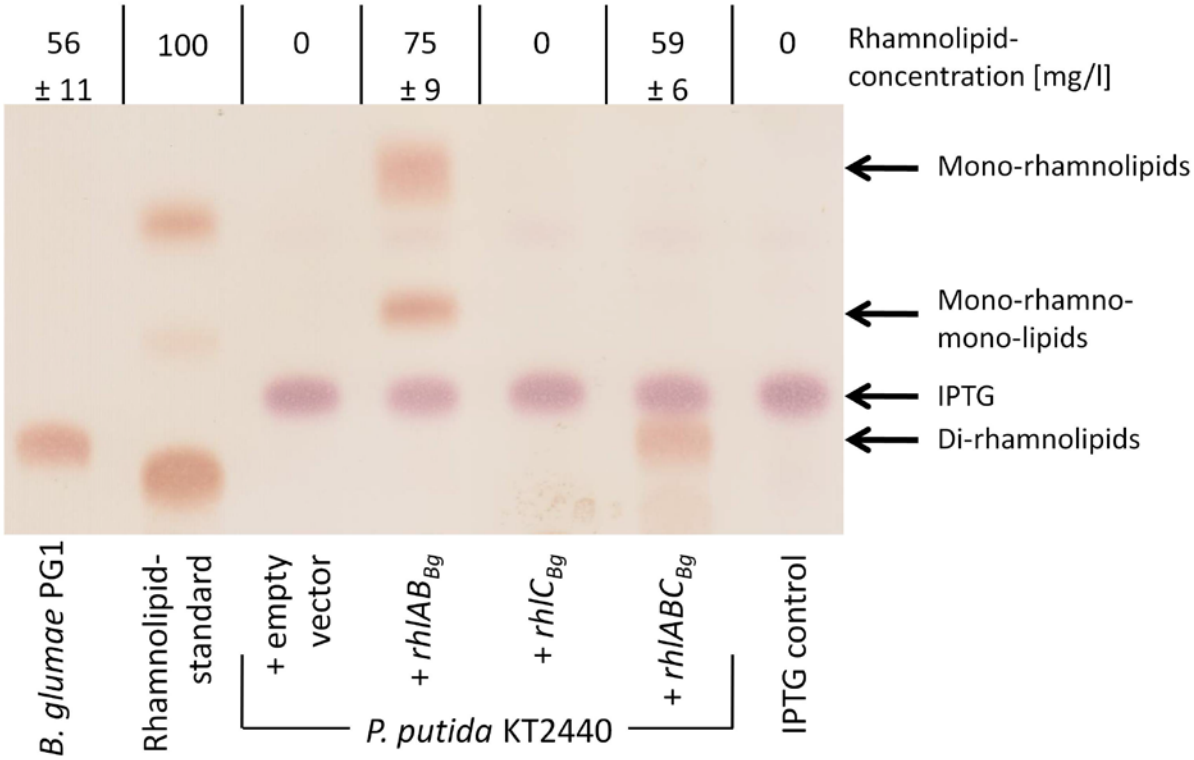

Fig. 2 Rhamnolipid production by *B. glumae* PG1 and recombinant *P. putida* KT2440. Thin layer chromatography (TLC) of extracts from *B. glumae* cultures in comparison to recombinant *P. putida* after heterologous expression of various *rhl*-genes from *B. glumae* ($_{Bg}$). Rhamnolipids are visible as brown bands on TLC-plates as in the rhamnolipid-standard originating from *P. aeruginosa*, which shows a different mobility compared to *Burkholderia*-rhamnolipids due to their shorter fatty acids. Samples extracted from *P. putida* cultures show an additional violet band descending from IPTG as in the extracts of IPTG containing LB media (IPTG control). Rhamnolipid concentrations were determined threefold from independent cultures using the orcinol assay.

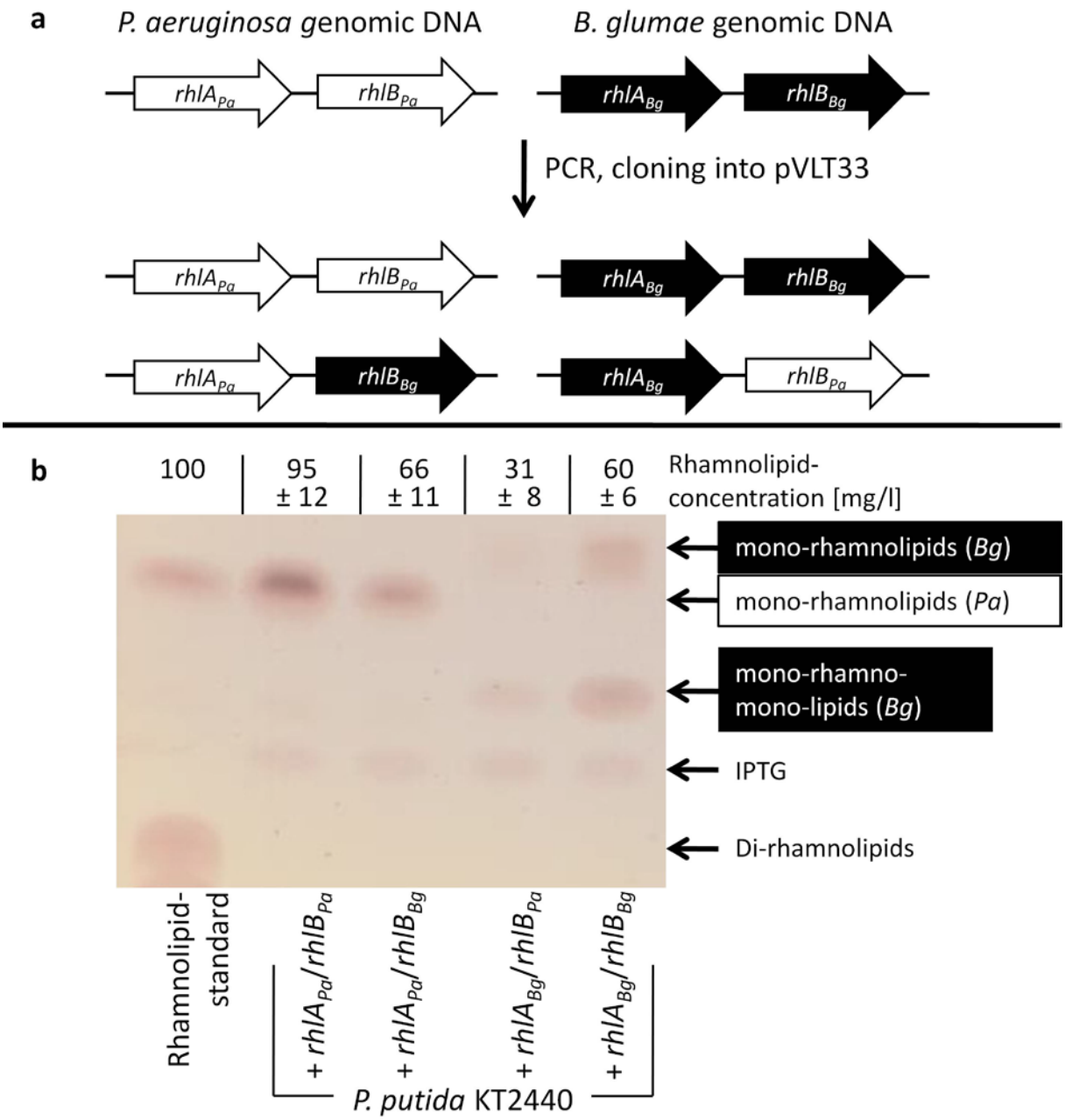

Fig. 3 Construction of synthetic operons with hybrids of *rhlA* and *rhlB* from different organism and their influence on rhamnolipid production after heterologous expression in *P. putida*. (a) The genes *rhlA* and *rhlB* were amplified each as single from the genomes of *B. glumae* PG1 ($_{Bg}$) and *P. aeruginosa* PAO1 ($_{Pa}$). They were subsequently cloned as native or hybrid operons in four different combinations into pVLT33. (b) Thin layer chromatography (TLC) of extracts from recombinant *P. putida* after heterologous expression of hybrid *rhl*-operons. Rhamnolipids are visible as brown bands on TLC-plates as in the rhamnolipid-standard originating from *P. aeruginosa*. Due to their shorter fatty acids *P. aeruginosa*-typical rhamnolipids show a different mobility compared to *Burkholderia*-typical long-chain rhamnolipids. The additional violet band in all *P. putida* samples descends from the added

IPTG. Rhamnolipid concentrations were determined threefold from independent cultures using the orcinol assay.