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Production of *Pseudomonas aeruginosa* Rhamnolipid Biosurfactants in Heterologous Hosts

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The high-level production of rhamnolipid biosurfactants is a unique feature of *Pseudomonas aeruginosa* and is strictly regulated in response to environmental conditions. The final step in rhamnolipid biosynthesis is catalyzed by the *rhlAB* genes encoding a rhamnosyltransferase. The expression of the cloned *rhlAB* genes was studied in heterologous hosts, either under the control of the *rhlR* and *rhlI* rhamnolipid regulatory elements or under the control of the *tac* promoter. A recombinant *P. fluorescens* strain harboring multiple plasmid-encoded copies of the rhamnolipid gene cluster produced rhamnolipids (0.25 g liter^{-1}) when grown under nitrogen-limiting conditions. The highest yields (0.6 g liter^{-1}) and productivities (24 mg liter^{-1} h^{-1}) were obtained in a recombinant *Pseudomonas putida* strain, KT2442, harboring promoterless *rhlAB* genes fused to the *tac* promoter on a plasmid. Active rhamnosyltransferase was synthesized, but no rhamnolipids were produced, by recombinant *Escherichia coli* upon induction of *rhlAB* gene expression.

Biosurfactants are of increasing industrial interest because of their broad range of potential applications, including emulsification, wetting, phase separation, and viscosity reduction. A large variety of microbial surface-active compounds are produced by bacteria, yeasts, and fungi. They are biodegradable and can be produced on renewable substrates and thus have the potential to replace chemically synthesized surfactants, provided that the physiology, genetics, and biochemistry of the biosurfactant-producing organisms are better understood (7).

The rhamnolipids were first described in 1949 (13), and their biosynthesis was studied in vivo by using radioactive precursors (10). In a putative biosynthetic pathway proposed by Burger et al. (2), the synthesis of rhamnolipids proceeds by sequential glycosyl transfer reactions, each catalyzed by a specific rhamnosyltransferase with TDP-rhamnose acting as a rhamnosyl donor and 3-hydroxydecanoyl-3-hydroxydecanoate acting as the acceptor. L-Rhamnosyl-L-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoate and L-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoate, referred to as rhamnolipids 1 and 2, respectively, are the principal glycolipids produced in liquid cultures.

Rhamnosyltransferase 1 is encoded by the *rhlAB* genes, which are organized in an operon. The active enzyme complex is located in the cytoplasmic membrane, with the RhlA protein being localized in the periplasm and the catalytically active RhlB component crossing the membrane (16).

Rhamnolipid formation by *Pseudomonas aeruginosa* occurs under limiting concentrations of nitrogen and iron during the late-exponential and stationary phases of growth (9). A regulatory locus containing the tandemly organized *rhlR* and *rhlI* genes has been identified downstream of the *rhlAB* genes and is ultimately required for their expression. The 28-kDa RhlR protein belongs to the LuxR family of transcriptional activators (17). Its activity is mediated in a cell density-dependent mechanism by *N*-acyl homoserine lactones, which are produced by the RhlI autoinducer synthetase (18).

To overcome the complex environmental regulation of rhamnolipid biosynthesis and to replace the opportunistic pathogen *P. aeruginosa* with a safe industrial strain, we attempted to achieve rhamnolipid formation in heterologous hosts by expressing the *rhlAB* rhamnosyltransferase genes, assuming that a functional rhamnosyltransferase would catalyze the formation of rhamnolipid 2 in these strains (Fig. 1). *P. aeruginosa* PG201 (9), *Pseudomonas fluorescens* ATCC 15453 (22), *Pseudomonas oleovorans* GPo1 (12), *Pseudomonas putida* KT2442 (11), and *Escherichia coli* DH5α (Gibco BRL, Rockville, Md.) were used as foreign hosts in this study.

The plasmid pUO101 contains the complete rhamnolipid *rhlABRI* gene cluster on a 5.8-kb *Eco*RI-*Hin*dIII chromosomal DNA fragment of *P. aeruginosa* PG201 in the broad-host-range cosmid pJRD215 (4). In this construct, the rhamnosyltransferase *rhlAB* operon can only be expressed from its own promoter, which is under the control of the *rhlR* and *rhlI* gene products (Fig. 2). Plasmid pUO98 contains promoterless *rhlAB* genes downstream of the *tac* promoter in the broad-host-range expression vector pVLT35 (5). The construction scheme of pUO98 and the exact fusion of the *tac* promoter to the *rhlAB* genes in pUO98 are shown in Fig. 2. The strong Shine-Dalgarno sequence (GGGAGG) and its preceding 7-bp AT stretch originate from the *P. aeruginosa rhl* sequence. The plasmids pUO101 and pUO98 and the control vectors pJRD215 and pVLT35 were mated into *P. aeruginosa* PG201, *P. fluorescens* ATCC 15453, *P. oleovorans* GPo1, and *P. putida* KT2442 by a triparental mating procedure with *E. coli* HB101 (pRK2013) as the helper strain (6). Transconjugants were isolated on Pseudosel agar (Difco) containing streptomycin (500 μg ml^{-1}). Recombinant strains harboring plasmid pUO101 were grown for 7 days in nitrogen-limiting GS minimal medium (9) containing 2% glycerol as a carbon source. For *E. coli*, M9 minimal medium containing 0.5% glucose was used (15). Rhamnosyltransferase activity in crude lysates and rhamnolipid concentrations in the supernatants were determined as previously described (17) and are shown in Table 1.

In *P. aeruginosa* PG201, the presence of additional plasmid-borne *rhl* gene copies led to a roughly 1.5-fold increase in rhamnosyltransferase activity and rhamnolipids. Among the heterologous hosts containing pUO101, *P. fluorescens* produced rhamnolipids, but the concentration was only 10% of that obtained with the *P. aeruginosa* strain.

The recombinant strains carrying plasmid pUO98 were

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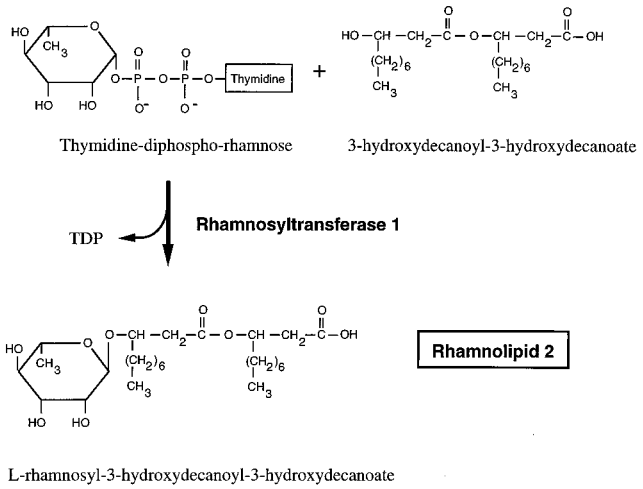

FIG. 1. Formation of rhamnolipid 2 in *P. aeruginosa*. The rhamnosyltransferase has very high substrate specificities for both the sugar nucleotide and the lipid (2).

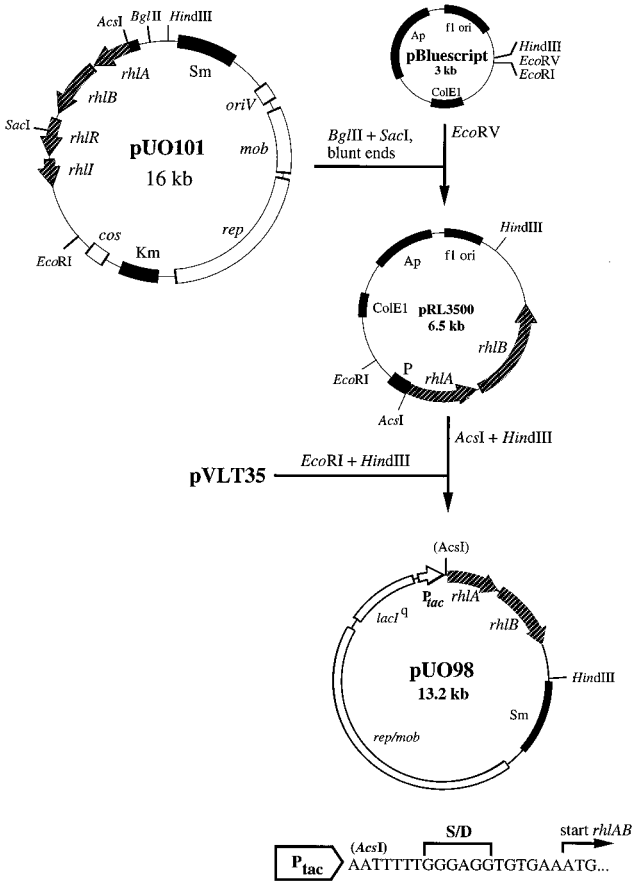

FIG. 2. Construction of the plasmids pUO101 and pUO98 harboring genes relevant for rhamnolipid biosynthesis. A 5.8-kb *Eco*RI-*Hin*dIII DNA fragment containing the *rhlABRI* gene cluster was cloned into the broad-host-range vector pJRD215, resulting in pUO101. Expression of the *rhlAB* genes in pUO101 is driven by the native *rhlA* promoter (shown in black), which is positively regulated by the gene products of *rhlR* and *rhlI*. A 3.5-kb *Bgl*II-*Sac*I fragment was isolated from pUO101, and the ends were blunted with T4 DNA polymerase (15) before ligation of this fragment into the *Eco*RV site in pBluescript SK+ (20), yielding pRL3500. A 3.1-kb *Acs*I-*Hin*dIII DNA fragment from pRL3500 containing the promoterless *rhlAB* rhamnosyltransferase genes was cloned downstream of the *tac* promoter in pVLT35 with the *Eco*RI and *Hin*dIII sites of its polylinker, resulting in pUO98. Note that *Eco*RI and *Acs*I generate ligation-compatible ends, which are lost after ligation; therefore, the *Acs*I site is shown in parentheses. The exact fusion of the *tac* promoter to the *rhlAB* genes is depicted below the pUO98 map. S/D, Shine-Dalgarno sequence.

grown in Luria-Bertani medium supplemented with 1% glucose. The expression of the *rhlAB* genes was induced by the addition of IPTG (isopropyl-β-D-thiogalactopyranoside [3 mM]) during the exponential phase of growth. Uninduced cultures and control strains containing the pVLT35 vector were grown in parallel. The cells were examined for rhamnosyltransferase activity and rhamnolipids 19 h after induction (Table 2). The highest levels of rhamnosyltransferase (45 U ml^{-1}) and rhamnolipids (0.6 g $liter^{-1}$) were obtained in *P. putida* KT2442(pUO98) and were significantly higher than those obtained in *P. aeruginosa* PG201(pUO98) grown under the same conditions. *P. fluorescens*(pUO98) and *P. oleovorans* GPo1 (pUO98) grown in Luria-Bertani medium containing 1% glucose lacked rhamnosyltransferase activity and rhamnolipids. *P. fluorescens*(pUO98) produced rhamnolipids (0.15 g $liter^{-1}$) when grown in minimal medium containing 2% glycerol and when expression of the *rhlAB* genes was induced (data not shown). In *E. coli* DH5α(pUO98), active transferase was detected, but rhamnolipids were absent.

Growth and rhamnolipid production were monitored in detail during cultivation of *P. putida* KT2442(pUO98) in Luria-Bertani medium containing 1% glucose (Fig. 3). Product formation occurred within 1 h after induction, and the biosurfactant volumetric productivity reached maximal levels of 24 mg $liter^{-1}$ h^{-1} during the exponential-phase growth of this recombinant strain.

The rhamnolipids produced by the recombinant strains *P. putida* KT2442(pUO98), grown in Luria-Bertani medium containing 1% glucose, and *P. fluorescens* ATCC 15453(pUO101), grown in GS medium containing 2% glycerol, were extracted from the culture supernatants and analyzed by thin-layer chromatography with purified *P. aeruginosa* rhamnolipids as standards (Fig. 4). Comparison of the R_f values suggested that the induced *P. putida* strain formed rhamnolipid 2, which is composed of 1 rhamnose unit per biosurfactant molecule. Rhamnolipid 2 was also the main type in the recombinant *P. fluorescens* strain, but in addition, small amounts of rhamnolipid 1 containing 2 rhamnose units per molecule were detected. The fatty acid moieties of the rhamnolipids formed by either of these strains were built from two molecules of 3-hydroxydecanoate, as confirmed by methanolyis followed by gas chromatography analysis with 3-hydroxy fatty acids of various chain lengths as standards (data not shown).

It was interesting to note the different efficiencies of rhamnolipid production in the various hosts. When the *rhlAB* genes were under the control of the RhlR-RhlI regulatory system, such as in pUO101, they were poorly expressed in the heterologous hosts, suggesting that the mechanisms governing positive control appear to be quite distinct in these hosts (14). However, even when the expression of the *rhlAB* genes was under the control of the inducible *tac* promoter, active rhamnosyltransferase was not formed in all of the recombinant strains. The *tac* promoter has been shown to act efficiently in a variety of *Pseudomonas* strains (1, 5). The low levels of active rhamnosyltransferase observed in some hosts are therefore rather due to the failure of some hosts to assemble a functional enzyme complex in the cytoplasmic membrane (16). In a comparable study, up to fivefold different levels of protein products

TABLE 1. Rhamnosyltransferase activity and rhamnolipid formation in pseudomonads and in *E. coli* harboring the *P. aeruginosa rhlABRI* gene cluster on plasmid pUO101[a]

Strain	Rhamnosyltransferase activity (U ml^{-1})[b]	Rhamnolipid formation (g liter^{-1})
P. aeruginosa		
PG201(pUO101)	24 ± 4	2.2 ± 0.4
PG201(pJRD215)	18 ± 4	1.6 ± 0.4
P. fluorescens		
ATCC 15453(pUO101)	4.0 ± 0.5	0.25 ± 0.03
ATCC 15453(pJRD215)	1.2 ± 0.5	0.06 ± 0.03
P. oleovorans		
GPo1(pUO101)	0.7 ± 0.2	<0.02
GPo1(pJRD215)	<0.5	<0.02
P. putida		
KT2442(pUO101)	1.9 ± 0.4	<0.02
KT2442(pJRD215)	<0.5	<0.02
E. coli		
DH5α(pUO101)	<0.5	<0.02
DH5α(pJRD215)	<0.5	<0.02

[a] The cells were cultivated for 7 days in nitrogen-limited GS minimal medium with 2% glycerol as the C source. M9 medium containing 0.5% glucose was used for *E. coli*.

[b] One unit of rhamnosyltransferase activity corresponds to the incorporation of 1 nmol of rhamnose from TDP-rhamnose into rhamnolipids h^{-1} (1, 12).

were obtained with the pVLT31 vector for the expression of the *bphC* gene of the polychlorobiphenyl-degrading pathway of *Pseudomonas* sp. strain LB400 in different *Pseudomonas* strains, but these levels were still significantly higher than those obtained with *E. coli* as the host (5).

The capacity to produce rhamnolipids further depends on the availability of the precursor substrates, TDP-rhamnose and 3-hydroxydecanoyl-3-hydroxydecanoate. Obviously, the intracellular concentrations of these precursors are limiting in the *P. aeruginosa* PG201 host when grown in Luria-Bertani medium, explaining why relatively small amounts of rhamnolipids are produced in this rich medium compared with in minimal medium. The donor substrate, TDP-rhamnose, is formed by enzymatic conversion of TDP-glucose; however, this TDP-rhamnose-forming activity is roughly fourfold lower in crude cell extracts of *E. coli* than in those of *P. aeruginosa* (8). TDP-rhamnose serves as the rhamnose donor for lipopolysaccharide biosynthesis in *P. aeruginosa* (19), and rhamnose has recently been shown to be present in the lipopolysaccharide of *P. fluorescens* and *P. putida* as well (21). Thus, it appears that TDP-rhamnose is present in all of the organisms included in this study.

TABLE 2. Rhamnosyltransferase activity and rhamnolipid formation in recombinant strains harboring the *P. aeruginosa rhlAB* genes under *tac* promoter control on plasmid pUO98[a]

Strain	Rhamnosyltransferase activity (U ml^{-1})	Rhamnolipid formation (g liter^{-1})
P. aeruginosa PG201(pUO98)	15 ± 3	0.15 ± 0.05
P. fluorescens ATCC 15453(pUO98)	<0.5	<0.02
P. oleovorans GPo1(pUO98)	<0.5	<0.02
P. putida KT2442(pUO98)	45 ± 5	0.60 ± 0.15
E. coli DH5α(pUO98)	2.5 ± 0.5	<0.02

[a] The cells were grown in Luria-Bertani medium supplemented with 1% glucose. The expression of the *rhlAB* genes was induced during the exponential growth phase (optical density at 450 nm of 0.3 to 0.7) by the addition of 3 mM IPTG, and the cells were cultivated for 19 h after induction.

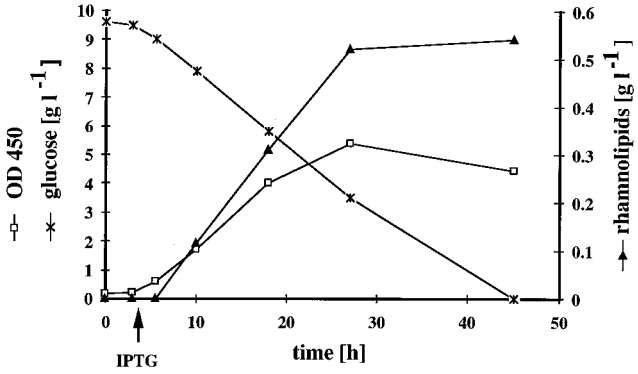

FIG. 3. Growth, rhamnolipid production, and glucose consumption of *P. putida* KT2442(pUO98). The strain was cultivated in Luria-Bertani medium containing 1% glucose in shake flasks at 30°C. The expression of the *rhlAB* genes was induced by the addition of 3 mM IPTG. OD 450, optical density at 450 nm.

The fatty acid precursor 3-hydroxydecanoyl-3-hydroxydecanoate may be missing in *E. coli* but is assumed to be present in fluorescent pseudomonads, which are good producers of poly(3-hydroxyalkanoates) (12). *P. putida* KT2442 has been reported to produce high levels of poly(3-hydroxyalkanoates), even when grown on glucose as the carbon source, whereas other fluorescent pseudomonads were generally less efficient in this respect (11). This suggests that the fatty acid metabolism is regulated differently in this strain, leading to an accumulation of 3-hydroxy fatty acids, which may serve as precursors for poly(3-hydroxyalkanoates) and rhamnolipid synthesis, thereby

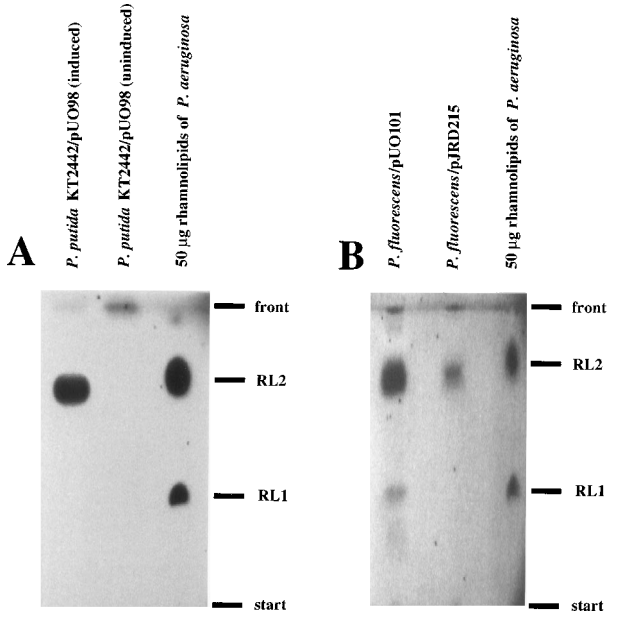

FIG. 4. Thin-layer chromatography analysis of rhamnolipids produced by recombinant strains. (A) *P. putida* KT2442(pUO98) grown in Luria-Bertani medium containing 1% glucose. (B) *P. fluorescens* ATCC 15453 harboring pUO101 or pJRD215 grown in GS medium containing 2% glycerol. Rhamnolipids were visualized with the orcinol reagent (3). The R_f values of purified *P. aeruginosa* rhamnolipids are indicated.

making this strain very useful as a host for heterologous rhamnolipid production.

In this study, we have shown that rhamnolipids can be produced in heterologous strains, provided the rhamnosyltransferase genes are efficiently expressed. Further medium induction and bioprocess optimization should lead to substantially higher biosurfactant productivities with strains which can be applied industrially.

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