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Isolation and Characterization of a Regulatory Gene Affecting Rhamnolipid Biosurfactant Synthesis in *Pseudomonas aeruginosa*

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A mutant strain (65E12) of *Pseudomonas aeruginosa* that is unable to produce rhamnolipid biosurfactants and lacks rhamnosyltransferase activity was genetically complemented by using a *P. aeruginosa* PG201 wild-type gene library. A single complementing cosmid was isolated on the basis of surface tension measurements of subcultures of the transconjugants by using a sib selection strategy. The subcloning of the complementing cosmid clone yielded a 2-kb fragment capable of restoring rhamnolipid biosynthesis, rhamnosyltransferase activity, and utilization of hexadecane as a C source in mutant 65E12. The nucleotide sequence of the complementing 2-kb fragment was determined, and a single open reading frame (*rhlR*) of 723 bp specifying a putative 28-kDa protein (RhlR) was identified. Sequence homologies between the RhlR protein and some regulatory proteins such as LasR of *P. aeruginosa*, LuxR of *Vibrio fischeri*, RhiR of *Rhizobium leguminosarum*, and the putative activator 28-kDa UvrC of *Escherichia coli* suggest that the RhlR protein is a transcriptional activator. A putative target promoter which is regulated by the RhlR protein has been identified 2.5 kb upstream of the *rhlR* gene. Multiple plasmid-based *rhlR* gene copies had a stimulating effect on the growth of the *P. aeruginosa* wild-type strain in hexadecane-containing minimal medium, on rhamnolipid production, and on the production of pyocyanin chromophores. Disruption of the *P. aeruginosa* wild-type *rhlR* locus led to rhamnolipid-deficient mutant strains, thus confirming directly that this gene is necessary for rhamnolipid biosynthesis. Additionally, such PG201::*'rhlR'* mutant strains lacked elastase activity, indicating that the RhlR protein is a pleiotropic regulator.

Biosurfactants are of interest because of their broad range of potential industrial applications, including emulsification, phase separation, wetting, foaming, emulsion stabilization, and viscosity reduction. A variety of microbial surface-active compounds are produced by microorganisms, including bacteria, yeasts, and filamentous fungi. Biosurfactants 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 (22). Biosurfactants include low-molecular-weight glycolipids and lipopeptides and high-molecular-weight polymers such as lipoproteins, lipopolysaccharide-protein complexes, and polysaccharide-protein-fatty acid complexes (reviewed by Reiser et al. [47]). The common lipophilic moiety of a surface-active molecule is the hydrocarbon chain of a fatty acid, whereas the hydrophilic part is formed by ester or alcohol groups of neutral lipids, by the carboxylate group of fatty acids or amino acids, or, in the case of glycolipids, by the carbohydrate part.

The rhamnose-containing glycolipid biosurfactants produced by *Pseudomonas aeruginosa* were first described in 1949 (36). The biosynthesis of these rhamnolipids was subsequently studied in vivo by using various radioactive precursors such as ^{14}C-acetate and ^{14}C-glycerol (28–30), and a putative biosynthetic pathway has been proposed by Burger et al. (9, 10). In this pathway, 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 β-hydroxydecanoyl-β-hydroxydecanoate acting as the acceptor. L-Rhamnosyl-L-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate and L-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate, referred to as rhamnolipids 1 and 2, respectively, are the principal glycolipids produced in liquid cultures. Rhamnolipids 4 and 3, containing only one β-hydroxydecanoyl moiety and one or two rhamnose groups, are synthesized by resting cells only (65). Additional types of rhamnolipids harboring alternative fatty acid chains such as β-hydroxyoctanoyl-β-hydroxydecanoate, β-hydroxydecanoyl-β-hydroxydodecanoate, and β-hydroxydecanoyl-β-hydroxydodec-5-enoate have been purified from the culture supernatants of a clinical isolate of *P. aeruginosa* (49).

The production of extracellular rhamnolipids by *P. aeruginosa* is strictly regulated. Maximal rhamnolipid synthesis is found under conditions of nitrogen limitation during the stationary phase of growth, using either glucose, glycerol, or *n*-paraffins as carbon sources (41). Factors affecting biosurfactant production, such as nutrient limitation and the influence of trace element concentration, have been studied extensively in continuous culture (27). *P. aeruginosa* rhamnolipids play a complex physiological role, as they not only serve for the emulsification of water-insoluble substrates such as alkanes but also are secreted into the medium when glucose or glycerol is used as a C source.

The current knowledge concerning the genetics of biosurfactant production and its regulation has recently been reviewed (48). Koch et al. (38) isolated Tn*5*-Gm^r-induced mutant strains of *P. aeruginosa* affected in the biosynthesis of rhamnolipids. The mutant screening method used was based on the loss of the hexadecane degradation capability. By this method,

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TABLE 1. Strains and plasmids used

Strain or plasmid	Description	Reference or source
Strains		
P. aeruginosa		
DSM2659	Referred to as PG201; prototrophic strain	26, 27
65E12	Tn*5*-Gm^r, rhamnolipid-negative strain; defective in hexadecane utilization	38
65E12	Tn*5*-Gm^r, rhamnolipid-negative strain; defective in hexadecane utilization	38
E. coli		
DH5α	*supE44* Δ*lacU169* (ϕ80 *lacZ*Δ*M15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	BRL
K802	*supE hsdR gal metB*	73
HB101	*supE44 hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*	7
Plasmids		
pSV	Mobilizable broad-host-range cosmid vector	37
pCC70	pSV-derivative harboring DNA sequences affecting rhamnolipid synthesis	This study
pRK2013	Mobilizing helper plasmid	20
pUCP19	pUC19-derived *E. coli-P. aeruginosa* shuttle vector	53
pBluescript SK+	*E. coli* cloning vector	56
pBR322	*E. coli* cloning vector	5
pPZ10	Broad-host-range *lacZ*-based promoter probe vector	54
pSUP201-1	Mobilizable plasmid vector	58
pUO2	pUCP19 containing a 2-kb *Pst*I fragment of pCC70 harboring the PG201 *rhlR* locus	This study
pUO38	pUCP19 containing a 2-kb *Pst*I fragment harboring the 65E12 mutant *rhlR* allele	This study
pRL825	pUCP19 with an 825-bp *Bgl*I-*Bgl*II fragment of pUO2 harboring the functional *rhlR* gene	This study
pSK825	pBluescript SK+ containing the *rhlR* gene	This study
pRL800	pUCP19 with an 800-bp *Ava*I-*Bgl*II fragment of pUO2 harboring the *rhlR* gene without promoter	This study
pIM21	pBluescript SK+ containing an internal fragment of the *rhlR* gene, a tetracycline resistance gene, and a *mob* site	This study
pUO60	pPZ10 containing the *rhlR* gene promoter and part of the *rhlR* coding sequence fused to *lacZ*	This study
pUO61	pPZ10 containing a promoter preceding an ORF upstream of the *rhlR* gene fused to *lacZ*	42

a set of mutants affected in hexadecane utilization was obtained, one of which, mutant 65E12, failed to synthesize rhamnolipids and was unable to grow in minimal media containing hexadecane as the carbon source without the addition of small amounts of purified rhamnolipids, indicating that rhamnolipids play an important role in the efficient solubilization and uptake of water-insoluble substrates. Very low rhamnolipid supplement concentrations in the order of the critical micelle concentration, typically 10 to 50 mg liter^{-1}, were sufficient to trigger the growth of mutant 65E12 in hexadecane-containing minimal medium.

Our central aim is to arrive at a better understanding of rhamnolipid biosynthesis and its regulation by using molecular genetic tools. We report here on the isolation and analysis of a gene involved in the regulation of rhamnolipid biosynthesis.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Bacterial strains and plasmids used in this study are listed in Table 1. LB medium (50) was used for propagating *P. aeruginosa* and *Escherichia coli* strains. A nitrogen-limited minimal medium containing 2% glycerol or 1% hexadecane (27) was used in experiments in which rhamnolipid synthesis in liquid cultures was monitored. For rhamnolipid plate assays, agar plates containing cetyltrimethylammonium bromide (0.02%) and methylene blue (0.0005%) were used (57). The plates will be referred to here as SW blue agar. Antibiotics were applied at the following concentrations: for *E. coli*, 100 μg of ampicillin per ml was used; for *P. aeruginosa*, 100 μg of gentamicin per ml, 150 μg of tetracycline hydrochloride per ml and 500 μg of carbenicillin per ml were used.

Detection and quantitation of rhamnolipids. Rhamnolipids in culture supernatants were detected by determining the surface and interfacial tensions (26) or by direct thin-layer chromatography (37) and quantitated by the orcinol method (11). Rhamnolipid-producing colonies on SW agar plates (57) were identified following the formation of dark blue halos around the colonies on a light blue plate background.

Rhamnosyltransferase assay. *P. aeruginosa* cell extracts were analyzed for rhamnosyltransferase activity by using the assay described by Burger et al. (9, 10), with some modifications. In brief, the following compounds were mixed and incubated in a volume of 200 μl for 30 min at 37°C: 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM EDTA, 0.2 mM β-hydroxydecanoyl-β-hydroxydecanoate, 0.2 mM ^{14}C-TDP-rhamnose (500 cpm nmol^{-1}), and 1 to 50 μg of enzyme. The reaction was stopped by the addition of 300 μg of carrier rhamnolipid in 100 μl of H_2O and 20 μl of 2 N HCl, and the rhamnolipids were extracted twice with 0.5 ml of diethyl ether. The pooled ether fractions were dried by using a Speed Vac for 10 min under reduced pressure, and the rhamnolipids were dissolved in 100 μl of methanol and subsequently counted in Acqua Luma Plus (Lumac, Belgium), using a liquid scintillation counter. One transferase unit corresponds to the incorporation of 1 nmol of rhamnose from TDP-rhamnose into rhamnolipids per h at 37°C.

Elastase assay. A qualitative plate assay for elastase activity was performed as described by Ohman et al. (43). Bacterial cells were plated on nutrient agar (Difco) containing 0.3% insoluble bovine neck ligament elastin (Sigma) and grown for at least 2 days at 37°C. Elastolytic activity was accompanied by the clearance of the medium.

Construction of a *P. aeruginosa* cosmid gene library. *P. aeruginosa* PG201 DNA was isolated by the procedure of Lippke et al. (40) and digested partially with the restriction endonuclease *Sau*3A, and fragments of between 20 to 30 kb were purified by velocity sedimentation in neutral 10 to 30% sucrose gradients (50). A broad-host-range cloning vector, pSV, consisting of pRK290 (20) and pJB8 (35) linked via their

*Eco*RI sites, was constructed (37). pSV cloning vector DNA was purified by equilibrium centrifugation in CsCl density gradients containing ethidium bromide (50), and PG201 DNA fragments to be cloned were ligated into the dephosphorylated single *Bgl*II site of the vector. After in vitro ligation and packaging (52), *E. coli* K802 cells were transduced with the packaged phage particles. Tetracycline-resistant colonies were pooled, yielding the K802/pSV::PG201 cosmid library. The complexity of the library was in the order of 7,000 clones and, with an average insert size of 17.6 kb, therefore covered the *P. aeruginosa* PG201 genome entirely.

Isolation of a complementing cosmid clone. *E. coli* clones harboring recombinant cosmids were mobilized into *P. aeruginosa* mutant strain 65E12 by using a triparental mating procedure with *E. coli* HB101/pRK2013 as the mobilizing helper strain (69). The resulting 65E12 transconjugants were divided into 70 pools of five colonies each and inoculated into a glucose-containing minimal medium. After 48 h of incubation, the surface tension of the culture supernatant was determined. One of the pools yielding a surface tension below 30 mN m^{-1} was further subdivided into subcultures, finally resulting in a single positive clone.

Subcloning of DNA fragments. The DNA from a positive cosmid clone was digested separately with either *Eco*RI, *Sph*I, *Sal*I, or *Pst*I, and the resulting fragment mixtures were ligated with pUCP19 vector DNA, which had previously been treated with the same enzyme followed by alkaline phosphatase. The recombinant plasmids were transformed into mutant strain 65E12 by the magnesium chloride procedure of Potter (46). The transformants were selected on LB agar containing carbenicillin and subsequently replica plated onto SW blue agar to allow detection of rhamnolipid-positive clones. Plasmids were isolated from small cultures of single rhamnolipid-positive clones by using the alkaline lysis method of Ish-Horowicz and Burke (35).

Gene disruption. Plasmid pIM21, used for disrupting the *rhlR* gene, was constructed by ligating an internal 400-bp *Ava*II DNA fragment of the *rhlR* gene to pBluescript SK+ vector DNA previously cut with *Eco*RI. The tetracycline resistance gene from pBR322 (5) and the *mob* fragment from pSUP201-1 (58) were subsequently inserted near the *rhlR* subfragment, yielding the mobilizable plasmid pIM21, which cannot replicate in *Pseudomonas* sp. Tetracycline-resistant *P. aeruginosa* transconjugants were obtained after a triparental mating using *E. coli* DH5α/pIM21 as the donor strain, *P. aeruginosa* PG201 as the recipient, and *E. coli* HB101/pRK2013 as the mobilizing helper strain (69).

Southern blot analysis. Chromosomal DNA was prepared from *P. aeruginosa* as described by Davis et al. (16). After restriction cleavage, the fragments were separated on a 0.8% agarose gel and transferred to a GeneScreen Plus membrane (DuPont), and the blot was hybridized with a ^{32}P-labeled DNA probe (21). The blot was hybridized and washed under the conditions recommended by the manufacturer of the GeneScreen Plus membrane and then exposed to X-ray films.

Determination of promoter activity. A 360-bp *Bam*HI fragment containing the *rhlR* promoter and part of the *rhlR* coding sequence was taken from pSK825 and ligated into the *Bam*HI site of the broad-host-range *lac*-based vector pPZ10 (54), yielding pUO60. In this construct, codon 81 of the *rhlR* coding sequence is fused in frame to the *lacZ* gene. Plasmid pUO61 was constructed by ligating a 1.1-kb *Sal*I-*Pst*I fragment preceding an open reading frame (ORF) about 2.5 kb upstream of the *rhl* gene (42) to pPZ10 vector DNA which had previously been treated with *Sal*I and *Pst*I. Promoter activity was determined indirectly by measuring β-galactosidase activity (50).

TABLE 2. Restoration of rhamnolipid synthesis in mutant strain 65E12 by a single cosmid

Strain[a]	Surface tension[b] (mN m^{-1})	Glycolipids[c] (g $liter^{-1}$)	Rhamnolipid type[d]	
			RL1	RL2
65E12	69.8	<0.01	–	–
65E12/pSV	69.5	<0.01	–	–
65E12/pCC70	29.6	0.43	+	+
PG201	29.8	0.63	+	+

[a] Cells were cultured for 76 h at 30°C in a minimal medium containing 2% glycerol.
[b] Of cell-free supernatants.
[c] Glycolipid levels in the ether-soluble fraction of culture supernatants were determined by the orcinol assay.
[d] The rhamnolipids were distinguished according to their R_f values in the thin-layer chromatography analysis.

DNA sequence analysis. Relevant subfragments were subcloned into the pBluescript SK+ vector (56), and plasmid DNA was prepared (34). The DNA was sequenced by the dideoxy-chain termination method (51), using Sequenase (U.S. Biochemicals Corp.) (66) and the M13 forward and reverse primers. The sequence data were analyzed by using the University of Wisconsin Genetics Computer Group software packages (18, 33).

Nucleotide sequence accession number. The nucleotide sequence reported in this study has been submitted to the GenBank/EMBL data banks and assigned accession number L08962.

RESULTS

Isolation of DNA sequences capable of restoring rhamnolipid biosynthesis in a rhamnolipid-deficient *P. aeruginosa* mutant strain. *P. aeruginosa* mutant strain 65E12 has previously been shown to be unable to produce rhamnolipids (38). Following conjugation of a cosmid library harboring *P. aeruginosa* PG201 wild-type genomic fragments to this mutant strain, a single cosmid clone, pCC70, capable of restoring rhamnolipid synthesis was obtained. This could be shown by surface tension measurements, by using the orcinol assay, which detects rhamnose, and by thin-layer chromatography analysis of the culture supernatants (Table 2).

To localize the DNA sequences relevant for rhamnolipid biosynthesis, subfragments of the pCC70 cosmid clone were subcloned into the *P. aeruginosa-E. coli* shuttle vector pUCP19 (53) and subsequently transformed into mutant strain 65E12. The transformants were grown overnight on LB agar plates containing gentamicin and carbenicillin and then replica plated onto SW blue agar plates. This medium has previously been used to detect bacterial colonies producing rhamnolipids (57). After 5 days of incubation at 30°C, rhamnolipid-positive clones could be identified by the formation of a dark blue halo around the colonies. The mutant strain did not produce such a halo (data not shown). The smallest complementing plasmid, pUO2, was found to harbor a 2-kb *Pst*I DNA insert.

Nucleotide sequence of the complementing DNA region. The DNA sequence of the 2-kb *Pst*I insert capable of restoring rhamnolipid synthesis in mutant strain 65E12 was determined by the sequencing strategy depicted in Fig. 1. A computer analysis using the FRAMES program (18) revealed an ORF consisting of 726 nucleotides (Fig. 2). This region will be referred to here as the *rhlR* protein-coding region. The G+C content of the *rhlR* protein-coding region is 61.7%, which is slightly lower than the published value of 65.2% which is

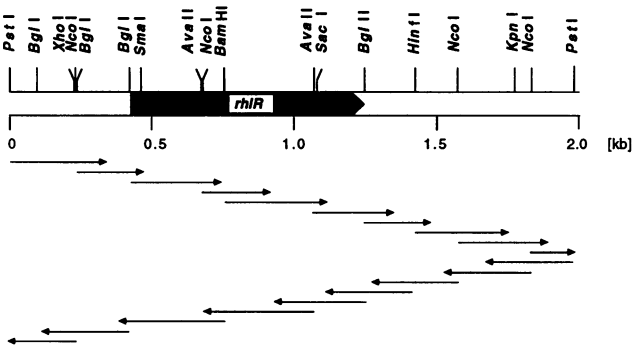

FIG. 1. Restriction map and sequencing strategy of a 2-kb *Pst*I DNA fragment containing the *rhlR* gene. The *rhlR* coding region is shown by the arrow. Additional *Ava*II and *Hin*fI sites are present in the sequence but not shown.

typical for *P. aeruginosa* chromosomal genes (72). The codon usage in the *rhlR* gene agrees with the typical codon preferences found in *P. aeruginosa* genes (72). In the *rhlR* promoter region, −10 and −35 consensus sequences of the σ^{70} type are apparent, and a putative Shine-Dalgarno sequence is present close to the ATG codon. A putative rho-independent termination site is visible 143 nucleotides downstream of the stop codon.

To check the functionality of the *rhlR* gene, the complementing 2-kb *Pst*I fragment was further subcloned. A 825-bp *Bgl*I-*Bgl*II fragment harboring the putative promoter and protein coding sequence was ligated via blunt ends to pUCP19 vector DNA previously cut with *Sma*I and pBluescript SK+ DNA previously cut with *Eco*RV, yielding pRL825 and pSK825, respectively. A 800-bp *Ava*I-*Bgl*II fragment lacking part of the promoter sequence was subcloned into pUCP19, yielding pRL800. Culture supernatants of strains 65E12/pRL825 and 65E12/pRL800 were analyzed for the presence of rhamnolipids. Only pRL825 was capable of restoring rhamnolipid biosynthesis in the rhamnolipid-deficient mutant strain 65E12, whereas pRL800 had no effect (data not shown). These results indicate that pRL825 contains a functional *rhlR* gene.

Determination of the site of mutation in strain 65E12. The rhamnolipid-nonproducing mutant strain 65E12 was originally isolated from a pool of transposon Tn*5*-Gm^r-induced mutant cells (38). Surprisingly, however, the transposon insertion site does not correspond to the *rhlR* locus but affects a gene of unknown function (42). To investigate whether the rhamnolipid deficiency of mutant strain 65E12 was due to the transposon insertion or to a spontaneous mutation event within the *rhlR* gene, the mutant *rhlR* allele was isolated. A Southern blot analysis of the chromosomal DNA from mutant strain 65E12 using the wild-type *rhlR* gene as a probe, indicated that the *rhlR* gene was located on a 2-kb *Pst*I fragment (Fig. 3A). *Pst*I-generated DNA fragments in the range of 1.8 to 2.2 kb were subsequently recovered from a preparative agarose gel and ligated to pUCP19 vector DNA and transformed into *E. coli* DH5α. A plasmid (pUO38) harboring the mutant *rhlR* locus on a 2-kb *Pst*I fragment was identified by colony hybridization. A complementation analysis showed that pUO38 did not restore rhamnolipid biosynthesis in mutant strain 65E12, indicating that the 65E12-derived *rhlR* gene is defective. This was confirmed by analyzing the sequence of a *Bgl*I-*Bgl*II fragment of the mutant *rhlR* coding region, which revealed a deletion of a single nucleotide at the very beginning of the *rhlR* gene,

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PstI ↓
CTGCAGCGCGCCTACGCGCCACTGGGAGCCTTGCTGCCATCGTGCGCCGGGCTGGTCCAT      60
CCGGGCGGTATCGGCGCCATGAGCCTGGCCTTGGCGGCGGGGGTGCCGCAGGTGCTGCTG     120
CCCTGCGCCCACGACCAGTTCGACAATGCCGAACGGCTGGTCCGGCTCGGCTGCGGGATG     180
CGCCTGGGCGTGCCATTGCGCGAGCAGGAGTTGCGCGGGGCGCTGTGGCGCTTGCTCGAG     240
GACCCGGCCATGGCGGCGGCCTGTCGGCGTTTCATGGAATTGTCACAACCGCACAGTATC     300
GCTTGCGGTAAAGCGGCCCAGGTGGTCGAACGTTGTCATAGGGAGGGGGATGCGCGATGG     360
CTGAAGGCTGCGTCCTGAACGGTGCTGGCATAACAGATAGGGTTGCCATGATTTTGCCGT     420
↓BglI          -35          ↓AvaI    -10
ATCGGCAAGGCTGCGCGCTTGACAGCGTCATACCCCGGGCCAATTCTGCTGTGATGCATT     480
      (S/D)
TTATCGATCAGGGCTTACTGCAATGAGGAATGACGGAGGCTTTTTGCTGTGGTGGGACGG     540
                  M  R  N  D  G  G  F  L  L  W  W  D  G
TTTGCGTAGCGAGATGCAGCCGATCCACGACAGCCAGGGCGTGTTCGCCGTCCTGGAAAA     600
 L  R  S  E  M  Q  P  I  H  D  S  Q  G  V  F  A  V  L  E  K
GGAAGTGCGGCGCCTGGGCTTCGATTACTACGCCTATGGCGTGCGCCATACGATTCCCTT     660
 E  V  R  R  L  G  F  D  Y  Y  A  Y  G  V  R  H  T  I  P  F
CACCCGGCCGAAGACCGAGGTCCATGGCACCTATCCCAAGGCCTGGCTGGAGCGATACCA     720
 T  R  P  K  T  E  V  H  G  T  Y  P  K  A  W  L  E  R  Y  Q
GATGCAGAACTACGGGGCCGTGGATCCGGCGATCCTCAACGGCCTGCGCTCCTCGGAAAT     780
 M  Q  N  Y  G  A  V  D  P  A  I  L  N  G  L  R  S  S  E  M
GGTGGTCTGGAGCGACAGCCTGTTCGACCAGAGCCGGATGCTCTGGAACGAGGCTCGCGA     840
 V  V  W  S  D  S  L  F  D  Q  S  R  M  L  W  N  E  A  R  D
TTGGGGCCTCTGTGTCGGCGCGACTTTGCCGATCCGCGCGCCGAACAATTTGCTCAGCGT     900
 W  G  L  C  V  G  A  T  L  P  I  R  A  P  N  N  L  L  S  V
GCTTTCCGTGGCGCGCGACCAGCAGAACATCTCCAGCTTCGAGCGCGAGGAAATACGCCT     960
 L  S  V  A  R  D  Q  Q  N  I  S  S  F  E  R  E  E  I  R  L
GCGGCTGCGTTGCATGATCGAGTTGCTGACCCAGAAGCTGACCGACCTGGAGCATCCGAT    1020
 R  L  R  C  M  I  E  L  L  T  Q  K  L  T  D  L  E  H  P  M
GCTGATGTCCAACCCGGTCTGCCTGAGCCATCGCGAACGCGAGATCCTGCAATGGACCGC    1080
 L  M  S  N  P  V  C  L  S  H  R  E  R  E  I  L  Q  W  T  A
CGACGGCAAGAGCTCCGGGGAAATCGCCATCATCCTGAGCATTTCCGAGAGCACGGTGAA    1140
 D  G  K  S  S  G  E  I  A  I  I  L  S  I  S  E  S  T  V  N
CTTCCACCACAAGAACATCCAGAAGAAGTTCGACGCGCCGAACAAGACGCTGGCTGCCGC    1200
 F  H  H  K  N  I  Q  K  K  F  D  A  P  N  K  T  L  A  A  A
CTACGCCGCGGCGCTGGGCCTCATCTGAAGCGCAGGGCGCGCCGGCCGCCGCGCCCTACC    1260
 Y  A  A  A  L  G  L  I  *
↓BglII
AGATCTGGCAGGTTGCCTGCCGTTCATCCTCCTTTAGTCTTCCCCCTCATGTGTGTGCTG    1320
GTATGTCCTCCGACTGAGAGGGCCCAGGAGTATCAGGGTAGGGATGCCGCCTTTTTTTCT    1380
CGGCCGGCACGACACGGGGACTTGGTCATGATCGAATTGCTCTCTGAATCGCTGGAAGGG    1440
CTTTCCGCCGCCATGATCGCCGAGCTGGGACGCTACCGGCATCAGGTCTTCATCGAGAAG    1500
CTGGGCTGGGACGTGGTCTCCACCTCCAGGGTCCGCGACCAGGAGTTCGACCAGTTCGAC    1560
CATCCGCAAACCCGCTACATCGTCGCCATGGGCCGCCAGGTATCTGCGGTTGTGCCCGCC    1620
TGTTGCCGACGACCGACGCCTACCTGCTCAAGGAAGTCTTCGCCTACCTGTGCAGCGAAA    1680
CCCGCCCAGCGATCCGTCGGTATGGGAGCTTTCGCGCTAGCCCAGCGCGGCGGACGATCC    1740
GCAACTGGCGATGAAGATATTCTGGTCCAGCCTGCAATGCGCCTGGTACCTGGGCGCCAG    1800
TTCGGTGGTGGCGGTGACCACCACGGCCATGGAGCGCTATTTCGTTCGCAACGGCGTGAT    1860
CCTCCAGCGCCTCGGCCCGCCGCAGAAGGTCAAGGGCGAGACGCTGGTCGCGATCAGCTT    1920
                                                        PstI ↓
CCCGGCCTACCAGGAGCGCGGCCTGGAGATGCTGCTGCGCTACCACCCGGAATGGCTGCAG    1981
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FIG. 2. Nucleotide sequence of the 2-kb *Pst*I fragment containing the *rhlR* gene. The deduced amino acid sequence is given in the single-letter code below the nucleotide sequence. The −35 and −10 promoter elements and the Shine-Dalgarno (S/D) sequence are underlined with double and single lines, respectively. A putative rho-independent termination site followed by a run of seven T residues at nucleotides 1340 to 1380 is indicated by a dotted line.

leading to a change in the reading frame after codon seven of the *rhlR* gene (Fig. 3B).

Disruption of the *rhlR* gene. With a view toward elucidating the effects caused by the lack of the RhlR regulatory protein, attempts were made to construct a *P. aeruginosa* strain mutagenized in its *rhlR* locus but having no other genetic defects. Therefore, the *rhlR* gene of *P. aeruginosa* wild-type strain PG201 was disrupted by insertional mutagenesis. For this purpose, plasmid pIM21, harboring an internal *rhlR* subfragment (*'rhlR'*), a tetracycline resistance gene, and a *mob* fragment was constructed. Plasmid pIM21 carries the ColE1 origin and is therefore unable to replicate in *P. aeruginosa*. After a triparental mating using *E. coli* DH5α/pIM21 as the donor strain and *E. coli* HB101/pRK2013 as the helper strain,

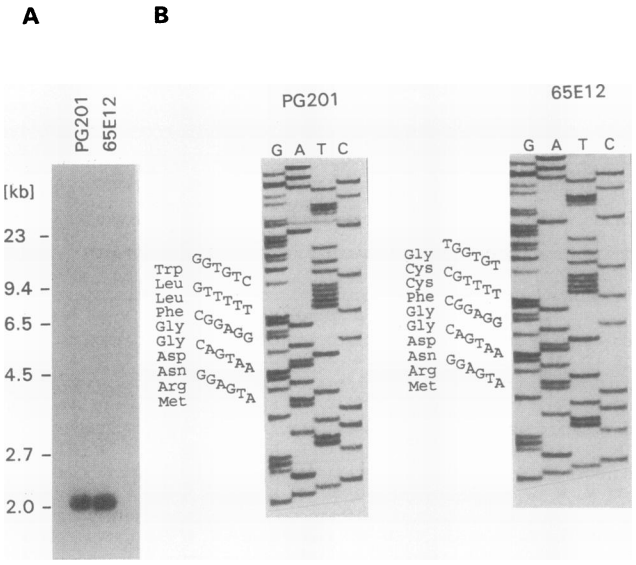

FIG. 3. Determination of the site of mutation within the *rhlR* gene of strain 65E12. (A) Southern blot analysis of *P. aeruginosa* PG201 and mutant 65E12 DNA cut with *Pst*I, using the *rhlR* gene as a labeled probe. It is evident that the *rhlR* gene of mutant strain 65E12 is not interrupted by a transposon insertion. (B) Sequence analysis of the PG201 and 65E12 *rhlR* genes. The protein-coding sequences of the *rhlR* genes starting with ATG are indicated. In the 65E12 *rhlR* allele, a deletion of a single nucleotide (four instead of five T residues) leads to a frameshift mutation after codon 7.

TABLE 3. Effects of the *rhlR* gene on rhamnolipid accumulation and rhamnosyltransferase activity in *P. aeruginosa* strains grown in glycerol-based medium

Strain	Rhamnolipids (g liter^{-1})	Rhamnosyltransferase (U mg^{-1})[a]
PG201	0.88	75 ± 15
PG201/pUO2	0.94	90 ± 15
65E12	<0.1	<5
65E12/pUO2	0.85	40 ± 10
PG201::'*rhlR*'	<0.1	<5

[a] Measured in soluble crude cell extracts. One transferase unit corresponds to the incorporation of 1 nmol of rhamnose into rhamnolipids per h at 37°C.

tetracycline-resistant *P. aeruginosa* transconjugants appeared at a frequency of 10^{-6}. The chromosomal DNA of some of the putative PG201::'*rhlR*' mutants was subjected to Southern blot analysis using the 825-bp *rhlR* gene as a labeled probe (Fig. 4B). The hybridization patterns indicated that plasmid pIM21 had integrated into the homologous *rhlR* locus by a single crossover event (Fig. 4A). The PG201::'*rhlR*' mutants obtained in this way lacked the capacity to produce rhamnolipids (Table 3). These results give further evidence for the involvement of a functional *rhlR* gene in the regulation of the *P. aeruginosa* rhamnolipid biosynthesis.

The *rhlR*-encoded protein is homologous to a number of regulatory proteins. The predicted amino acid sequence of the RhlR protein was found to be homologous to four proteins:

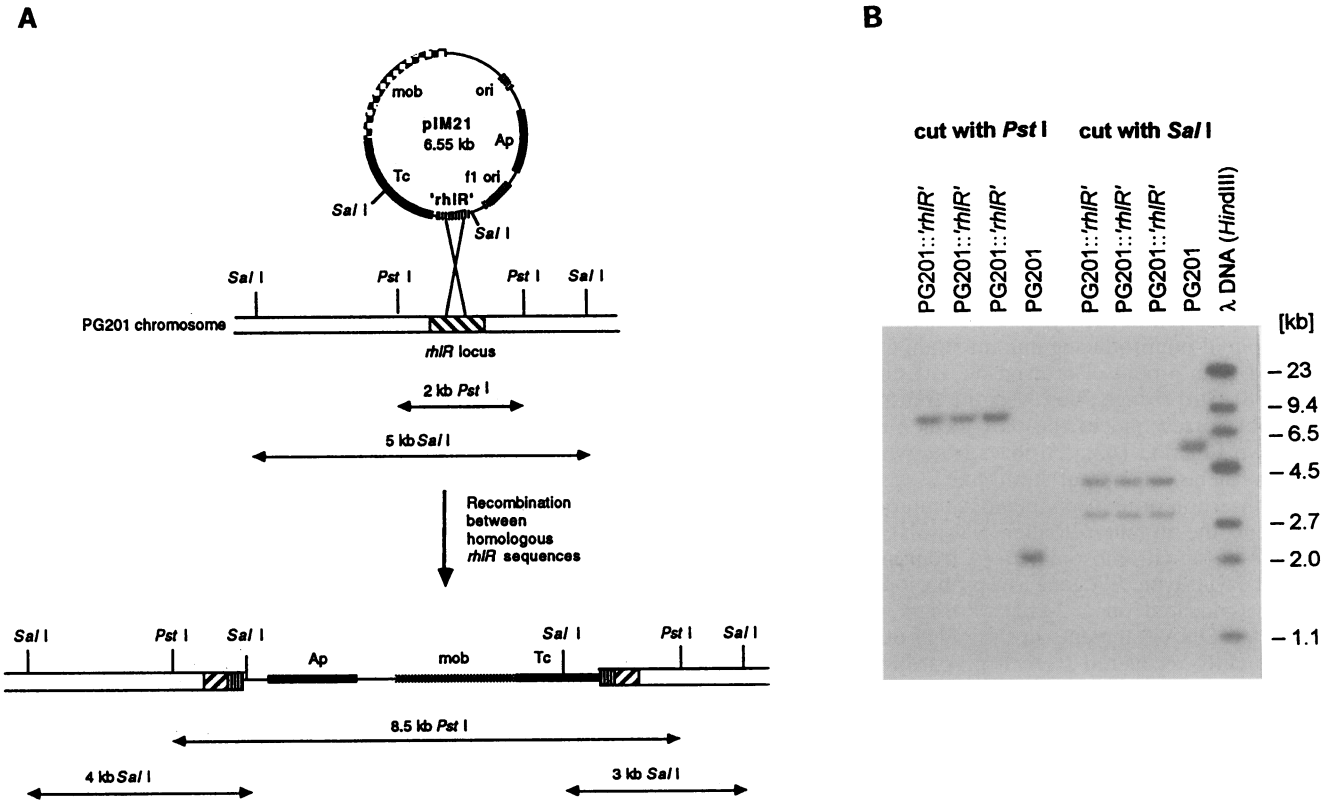

FIG. 4. Disruption of the *rhlR* gene by a plasmid insertion technique. (A) Integration of plasmid pIM21 into the homologous *rhlR* locus of the PG201 chromosome via a single crossover event. Ap, ampicillin resistance gene; Tc, tetracycline resistance gene. (B) Southern blot analysis of PG201::'*rhlR*' transconjugant DNA cut either with *Pst*I or *Sal*I. This result is consistent with the view that the integration of the 6.5-kb plasmid pIM21 into the *rhlR* locus took place by homologous recombination.

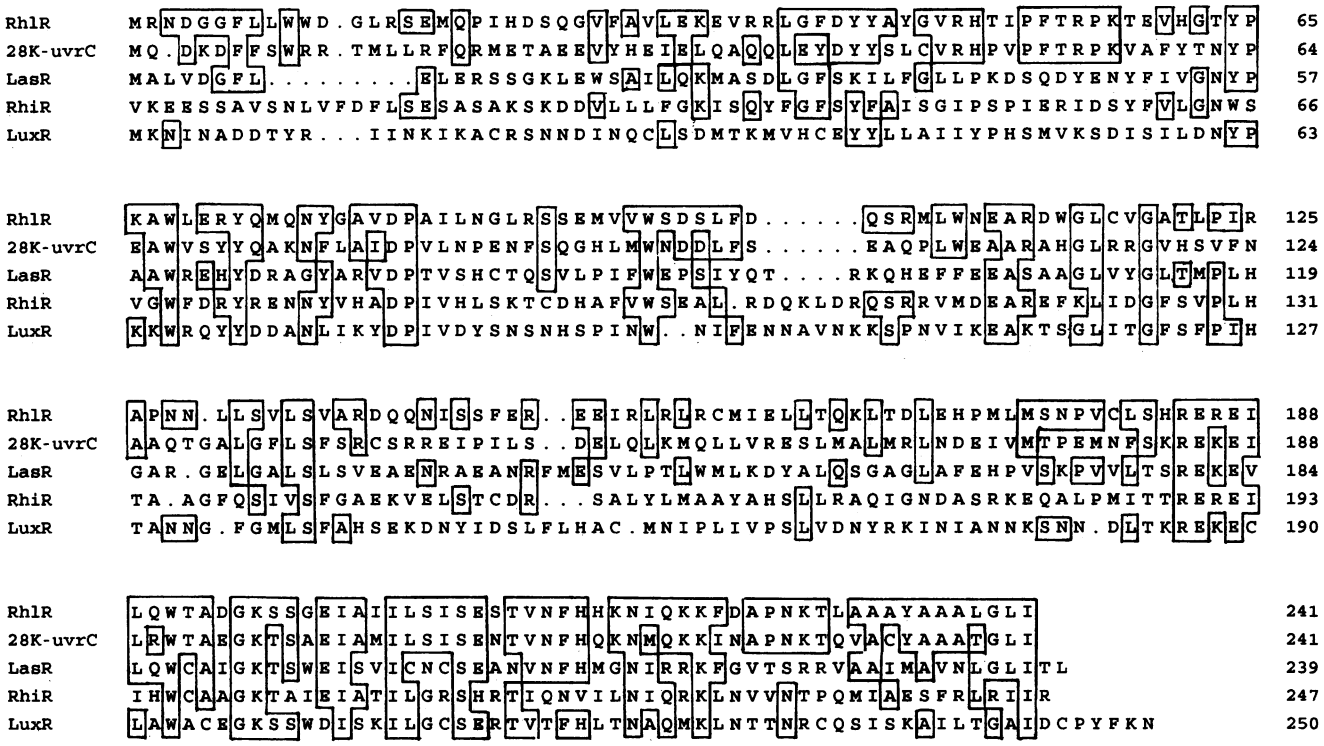

FIG. 5. Alignment of the predicted amino acid sequences of the RhlR sequence with the 28-kDa UvrC (28K-uvrC) (*E. coli*), LasR (*P. aeruginosa*), RhiR (*R. leguminosarum*), and LuxR (*V. fischeri*) sequences.

the 28-kDa protein encoded by the upstream ORF1 of the *E. coli uvrC* gene, the LasR protein of *P. aeruginosa*, the *Rhizobium leguminosarum* RhiR protein, and the *Vibrio fischeri* LuxR protein. The amino acid sequence alignment of the RhlR protein with these four proteins was generated by using the CLUSTAL program (33) and is presented in Fig. 5. The RhlR protein shows overall identities of 40% with ORF1 of *uvrC*, 31% with LasR, 29% with RhiR, and 23% with LuxR, respectively.

A distinct element of homology between the RhlR protein and other DNA-binding and regulatory proteins was found in the C-terminal part of the protein (Fig. 6). This region contains

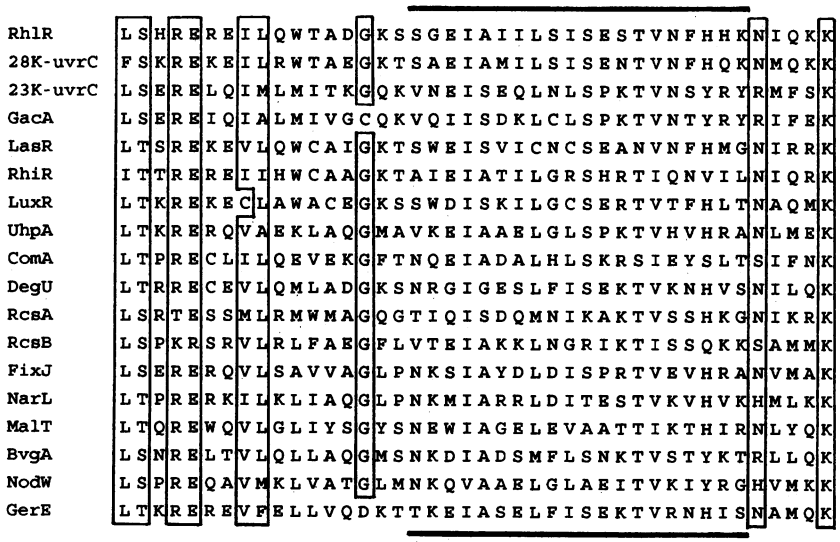

FIG. 6. Alignment of the highly conserved C-terminal domain of the RhlR protein with those of other regulatory proteins. The DNA-binding helix-turn-helix motif is indicated by two lines, and identical or strongly conserved adjacent residues are boxed. The sequences and references are as follows: 28- and 23-kDa UvrC (28K-uvrC and 23K-uvrC) (55), GacA (39), LasR (24), RhiR (13), LuxR (19), UhpA (23), ComA (71), DegU (32), RcsA (64), RcsB (63), FixJ (15), NarL (60), MalT (12), BvgA (2), NodW (25), and GerE (14).

TABLE 4. Expression of the *rhlR* gene

Strain/plasmid[a]	β-Galactosidase activity[b]		
	Complex medium (LB)	Minimal medium	
		Exponential phase	Stationary phase
PG201/pPZ10	<10	<10	<10
PG201/pUO60	147	52	163
PG201/pUO61	175	680	13,600
65E12/pPZ10	<10	<10	<10
65E12/pUO60	82	88	190
65E12/pUO61	<10	310	560
E. coli DH5α/pPZ10	<10	<10	<10
E. coli DH5α/pUO60	57	28	<10
E. coli DH5α/pUO61	86	120	<10

[a] In plasmid pUO60, the *rhlR* promoter is fused to the *lacZ* gene; in pUO61, a promoter upstream of an ORF in the vicinity of the *rhlR* locus is fused to the *lacZ* gene. The cells were cultivated in LB medium and in minimal medium containing 2% glycerol.

[b] Determined in permeabilized cells (50). Values represent the β-galactosidase activity per unit of optical density at 600 nm the cell culture.

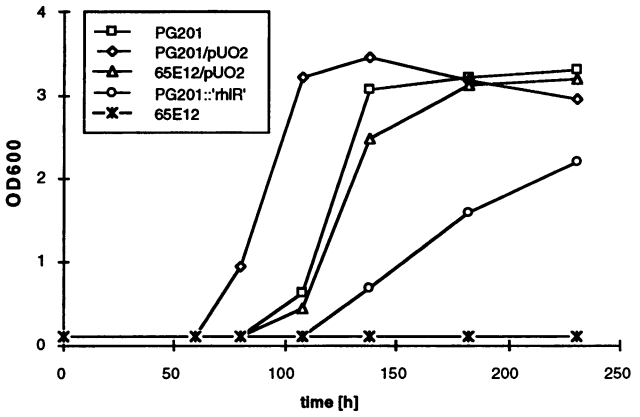

FIG. 7. Effect of the *rhlR* gene on hexadecane utilization capacity. Growth of mutant strain 65E12 in hexadecane-containing minimal medium was restored by the *rhlR* gene present on plasmid pUO2. A growth-stimulating effect was observed in strain PG/pUO2, in which the *rhlR* gene was overexpressed. Strain PG201::'*rhlR*', lacking a functional *rhlR* gene, was found to grow very slowly on hexadecane. OD600, optical density at 600 nm.

a helix-turn-helix DNA-binding motif, which has been reported to be conserved in the so-called response regulators of the two-component regulatory systems (31).

Expression of the *rhlR* regulatory gene. *rhlR* promoter activity was determined by using plasmid pUO60, in which codon 81 of the *rhlR* gene was fused in frame to the *lacZ* gene on the broad-host-range plasmid pPZ10 (54). The *rhlR* gene was found to be constitutively expressed at low levels in *P. aeruginosa* grown in either complex (LB) or minimal medium. During the late exponential and stationary phases of growth in minimal medium, *rhlR* gene expression was slightly enhanced. In *E. coli* DH5α, expression of the *rhlR* gene was detected in complex medium as well as in M9 minimal medium, but only during the exponential phase of growth (Table 4).

Possible targets for the RhlR regulatory protein. Additional rhamnolipid-deficient Tn*5*-Gm^r-induced mutants have recently been isolated and characterized (42). Two of these mutants carry a transposon insertion within an ORF (ORF1) upstream of the *rhlR* locus. To measure ORF1 promoter activity, the ORF1 promoter was fused to the *lacZ* gene of pPZ10, yielding pUO61. This plasmid was subsequently transformed into *P. aeruginosa* wild-type strain PG201 and mutant strain 65E12 to analyze the influence of the RhlR protein on the activity of the ORF1 promoter. β-Galactosidase activity was measured under various growth conditions (Table 4), and the ORF1 promoter was found to be activated during the late exponential and stationary phases of growth under nitrogen-limited conditions in the wild-type strain PG201. In mutant strain 65E12, however, no activation of the ORF1 promoter was found. These data suggest that the RhlR protein plays a direct role in the transcriptional activation of genes involved in rhamnolipid biosynthesis.

Effect of the *rhlR* gene on rhamnolipid accumulation and rhamnosyltransferase activity. Plasmid pUO2 harboring the *rhlR* gene restored rhamnolipid production in mutant strain 65E12. The levels of rhamnolipids found in the culture supernatants of strain 65E12/pUO2 were slightly lower than those of the PG201 wild-type cultures (Table 3). Burger et al. (9, 10) have provided evidence for the involvement of two different transferases in rhamnolipid biosynthesis. The rhamnosyltransferase activity in *P. aeruginosa* crude extracts was determined by a specific assay detecting the rhamnosyl transfer from ^{14}C-TDP-rhamnose to β-OH-decanoyl-β-OH-decanoate (9, 10). The results (Table 3) indicate that pUO2 restored rhamnosyltransferase activity in mutant strain 65E12. However, the specific rhamnosyltransferase activity in 65E12/pUO2 cell extracts was lower than that of PG201 wild-type enzyme preparations.

The effect of the copy number of the *rhlR* gene in *P. aeruginosa* wild-type strain PG201 was investigated by transforming this strain with plasmid pUO2, containing the *rhlR* gene. It has previously been reported that plasmids based on the pUCP19 cloning vector are present in a copy number of 10 to 25 in *P. aeruginosa* PAO1 (53). However, additional copies of the *rhlR* gene in *P. aeruginosa* PG201/pUO2 had a minor effect on the accumulation of rhamnolipids and rhamnosyltransferase activity (Table 3).

Effects of the *rhlR* gene on hexadecane utilization. The efficient utilization of hexadecane as a C source is dependent on the production of rhamnolipids, solubilizing the water-insoluble substrate and facilitating its uptake. The rhamnolipid-deficient mutant strain 65E12 has previously been shown to be unable to grow in minimal medium containing hexadecane (38). Strain 65E12/pUO2, however, utilized hexadecane with an efficiency comparable to that of wild-type strain PG201 (Fig. 7). The PG201::'*rhlR*' strain harboring a truncated *rhlR* gene copy was capable of growing very slowly in media containing hexadecane as a C source (Fig. 7). These findings allow the conclusion that a functional *rhlR* gene facilitates the efficient utilization of hexadecane by activating biosurfactant synthesis. It is also evident from Fig. 7 that the presence of additional *rhlR* gene copies in *P. aeruginosa* PG201/pUO2 considerably shortened the lag phase. However, the final yields of rhamnolipids were not significantly higher than those obtained with wild-type strain PG201 (Table 3).

Effects of the *rhlR* gene on elastase and pyocyanin production. The *rhlR* gene was found to have pleiotropic effects, as shown by the fact that mutant strains 65E12 and PG201::'*rhlR*' lacked elastolytic activity on plates. Elastase production was restored in strain 65E12/pUO2 (Fig. 8A). Also, strains 65E12/pUO2 and PG201/pUO2, harboring multiple copies of the *rhlR* gene on a plasmid, produced higher amounts of chromophores than did the strains containing pUCP19 as a control (Fig. 8B).

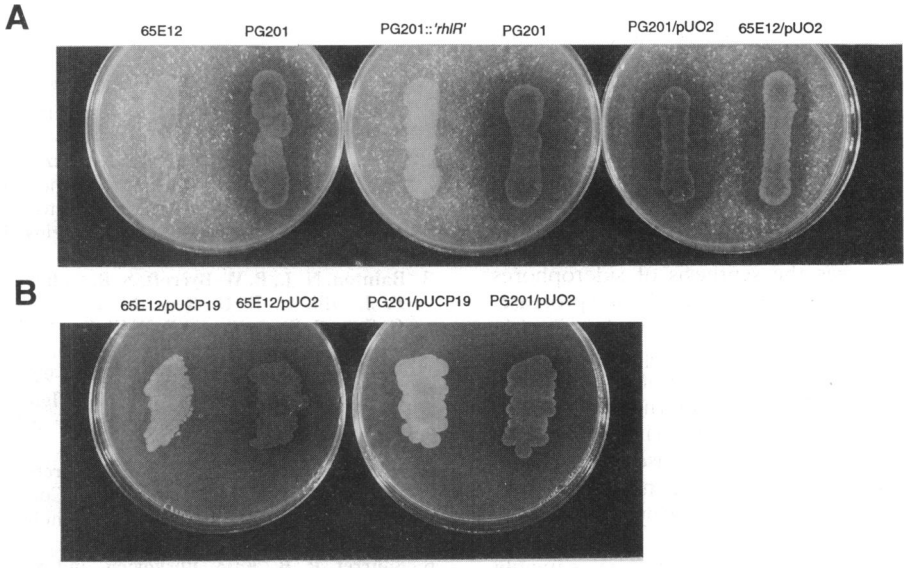

FIG. 8. Pleiotropic effects exerted by the *rhlR* gene. (A) Elastase plate assay using mutant strains 65E12 and the PG201::'*rhlR*', wild-type strain PG201, and strains PG201/pUO2 and 65E12/pUO2. (B) Overproduction of pyocyanin by strains PG201/pUO2 and 65E12/pUO2.

The green color of the culture supernatants was caused by the presence of an extracellular compound identified as pyocyanin (8).

DISCUSSION

The response of bacteria to environmental stimuli has been studied extensively in the past few years, leading to the elucidation of the molecular mechanisms which build up the complex regulatory networks. The stimulus-response coupling involving specific two-component regulatory system has been observed to affect a wide range of bacterial activities (for reviews, see references 1, 6, 61, 62, and 70), such as chemotaxis, nitrogen utilization, phosphate regulation, osmosensitivity, sporulation, virulence, motility, competence, and the production of a variety of secondary metabolites under certain environmental conditions. The production of rhamnolipid biosurfactants by *P. aeruginosa* has previously been shown to depend on nitrogen and iron limitation during the late exponential and stationary phases of growth (68), and rhamnolipid production could be improved in continuous culture using optimized media under carefully controlled conditions (26, 27). These observations suggested early on that rhamnolipid production is controlled by environmental factors possibly involving two-component regulatory systems as well (27). We now present evidence for the existence of a putative transcriptional activator involved in rhamnolipid biosynthesis. The predicted RhlR protein shows a high overall homology to the 28-kDa UvrC, LasR, RhiR, and LuxR proteins, all of which are transcriptional activators. The 28-kDa ORF1 protein of *E. coli* is assumed to enhance the transcription of the *uvrC* repair gene under induced conditions, as a consequence of DNA damage by UV irradiation (51). The *P. aeruginosa* LasR protein has been characterized as a transcriptional activator affecting elastase gene expression (24). The RhiR protein is encoded by a symbiontic plasmid of *R. leguminosarum* controlling the expression of the *rhiABC* operon, thereby contributing to the efficient nodulation of vetch (11). The LuxR protein of the marine bacterium *V. fischeri* is a positive activator of the *lux* regulon for bioluminescence (19). Because of the high degree of homology of the putative RhlR protein with these four transcriptional activators, we assume that RhlR positively regulates the expression of genes involved in rhamnolipid biosurfactant synthesis. A putative target for gene activation by the RhlR protein in the vicinity of an ORF (ORF1) upstream of the *rhlR* locus has recently been identified (42). The transcriptional activation of ORF1 depends on a functional *rhlR* gene and occurs during the late exponential and stationary phases of growth under nitrogen-limited conditions. Since several physical and chemical parameters stimulating rhamnolipid production have been described (27), we assume that the RhlR protein belongs to a signal transduction pathway responsible for activating target genes in response to such environmental stimuli. The RhlR regulator as well as the four most homologous proteins shown in Fig. 5 all lack the typical N-terminal conserved residues but have a C-terminal domain typical for the FixJ family of response regulators of two-component regulatory systems. Considering that no sensors have been found to act in conjunction with RhlR, 28-kDa UvrC, LasR, RhiR, or LuxR, we suggest that these proteins constitute a distinct subgroup of regulators. Recently, two members of this group, LuxR and LasR, have been reported to act in combination with a diffusible signal compound termed autoinducer (45). All autoinducer molecules known to date have been identified as *N*-(3-oxo-alkanoyl) homoserine lactones and are assumed to bind to the N-terminal domains of the regulatory proteins, thereby stimulating the binding of the C-terminal domains to the DNA target sites (3). We assume that the activity of RhlR, the regulator of rhamnolipid biosynthesis, depends on an autoinduction or phosphorylation mechanism, since in vitro phosphorylation of the RhlR protein has not been successful so far (42).

The overexpression of the *rhlR* gene from a multicopy plasmid in *P. aeruginosa* PG201 resulted in a marginal stimulation of rhamnolipid synthesis, suggesting that only a small number of the transactivating RhlR proteins are needed for the maximal activation of rhamnolipid biosynthetic genes.

A widespread feature of response regulators is the activation of genes transcribed by alternative RNA polymerase σ factors

(reviewed by Deretic et al. [17]). Alternative σ factors recognize distinct promoters of genes which are expressed under certain conditions, such as heat shock or nutrient starvation. The *P. aeruginosa rpoN* gene encodes a σ factor (σ^{54}) which is required for the transcription of a number of functionally unrelated genes specifying enzymes of nitrogen assimilation, amino acid uptake, degradation of a variety of organic molecules, and pilin synthesis (67). Iron-regulated promoters from a *Pseudomonas* sp. have been identified and are presumed to be recognized by an alternative σ factor as well (44). Growth in high-iron conditions represses the synthesis of siderophores (iron-chelating agents) as well as exotoxin A, alkaline protease, and elastase in *P. aeruginosa* (4). The *rhlR* gene described in this report has been shown to have pleiotropic effects and might therefore be involved in the regulation of a set of genes whose transcription is dependent on an alternative σ factor. This hypothesis is supported by the facts that (i) mutant strains affected in the *rhlR* locus do not produce elastase on plates and (ii) overexpression of the *rhlR* regulatory gene in wild-type strain PG201 leads to the overproduction of pyocyanin chromophores.

Mutant strain 65E12 carrying transposon Tn*5*-Gm^r is unable to synthesize rhamnolipids and cannot use hexadecane as a C source (38). In addition, as shown in this work, enzyme extracts of this strain lack rhamnosyltransferase activity, indicating that the *rhlR* gene controls rhamnosyltransferase production. A Southern blot analysis of 65E12 DNA by using the labeled *rhlR* gene as a probe revealed that the chromosomal *rhlR* region in the mutant is not interrupted by the transposon (42), and in this work we present evidence that the 65E12 *rhlR* mutant allele carries a single nucleotide deletion within its coding sequence, leading to a frameshift mutation. Mutant strains obtained after disruption of the *rhlR* gene of *P. aeruginosa* wild-type strain PG201 also lacked the capacity to synthesize rhamnolipids, indicating that the *rhlR* locus is essential for rhamnolipid synthesis. However, in contrast to mutant strain 65E12, *P. aeruginosa* deletion strain PG201::*'rhlR'* was capable of growing slowly in hexadecane-based minimal medium without the addition of rhamnolipids, indicating that other factors besides rhamnolipids are lacking in strain 65E12 but not in strain PG201::*'rhlR'*. It has been suggested in the past that strains producing biosurfactants are capable of utilizing water-insoluble substrates by a so-called mediated-uptake mechanism involving solubilized hydrocarbons, whereas strains lacking the capacity to synthesize biosurfactants can utilize these substrates only by direct interaction and uptake of the hydrocarbons dissolved in the aqueous phase or by the direct contact of cells with large hydrocarbon droplets (59). Our results suggest that the rhamnolipid deficiency of strain 65E12 is caused by the mutation in the *rhlR* gene and that the transposon insertion in the 65E12 mutant chromosome is irrelevant for rhamnolipid biosynthesis but may alter the cell surface in a manner which totally impairs growth of mutant strain 65E12 on hexadecane.

In this report, we have presented preliminary evidence for a regulatory protein affecting rhamnolipid biosynthesis in response to environmental stimuli. Further studies will be required to elucidate the targets of the RhlR protein and the regulatory networks leading to rhamnolipid biosurfactant synthesis in *P. aeruginosa*.

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