

The *Pseudomonas aeruginosa* *rhlG* Gene Encodes an NADPH-Dependent β -Ketoacyl Reductase Which Is Specifically Involved in Rhamnolipid Synthesis

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A *Pseudomonas aeruginosa* gene homologous to the *fabG* gene, which encodes the NADPH-dependent β -ketoacyl-acyl carrier protein (ACP) reductase required for fatty acid synthesis, was identified. The insertional mutation of this *fabG* homolog (herein called *rhlG*) produced no apparent effect on the growth rate and total lipid content of *P. aeruginosa* cells, but the production of rhamnolipids was completely abrogated. These results suggest that the synthetic pathway for the fatty acid moiety of rhamnolipids is separate from the general fatty acid synthetic pathway, starting with a specific ketoacyl reduction step catalyzed by the RhlG protein. In addition, the synthesis of poly- β -hydroxyalkanoate (PHA) is delayed in this mutant, suggesting that RhlG participates in PHA synthesis, although it is not the only reductase involved in this pathway. Traits regulated by the quorum-sensing response, other than rhamnolipid production, including production of proteases, pyocyanine, and the autoinducer butanoyl-homoserine lactone (PAI-2), were not affected by the *rhlG* mutation. We conclude that the *P. aeruginosa* *rhlG* gene encodes an NADPH-dependent β -ketoacyl reductase absolutely required for the synthesis of the β -hydroxy acid moiety of rhamnolipids and that it has a minor role in PHA production. Expression of *rhlG* mRNA under different culture conditions is consistent with this conclusion.

Pseudomonas aeruginosa is a bacterium that can be isolated from many different habitats, including water, soil, and plants (5). *P. aeruginosa* is also an opportunistic human pathogen that causes serious nosocomial infections (8). The secretion of numerous toxic compounds and hydrolytic enzymes has been correlated with its pathogenicity (19). These exoproducts include different proteases, such as elastase, LasA protease, and alkaline protease, as well as phospholipase C, exotoxin A, pyocyanine, and rhamnolipids. The production of these compounds is considered to be a virulence-associated trait and is coordinately regulated by a mechanism called “quorum sensing” (11), which depends on the production of N-acylated homoserine lactones harboring acyl substituents of two different lengths; PAI-1 contains a 12-carbon chain (22), while PAI-2 contains a butanoyl moiety (23). These small diffusible signaling molecules activate gene expression at high bacterial densities through interaction with specific transcriptional activators, LasR (22) and RhlR (20), respectively.

The role of these exoproducts in soil or aquatic habitats has not been determined, but it is clear that environmental and clinical *P. aeruginosa* isolates do not represent different populations, since it has been shown that there is a major clone common to pathogenic and environmental isolates of this bacterium (26).

Rhamnolipids are glycolipids produced by *P. aeruginosa* which reduce water surface tension and emulsify oil. These compounds are biodegradable and have potential industrial and environmental applications (14, 17). Recently, rhamnolipids

have been found to have antagonistic effects on economically important zoospore plant pathogens, thus opening up their use as biocontrol agents (29). The rhamnolipids produced by *P. aeruginosa* in liquid cultures (Fig. 1) are mainly rhamnosyl- β -hydroxydecanoyl- β -hydroxydecanoate (monorhamnolipid) and rhamnosyl-rhamnosyl- β -hydroxydecanoyl- β -hydroxydecanoate (dirhamnolipid). Rhamnolipid biosynthesis proceeds through two rhamnose transfers from TDP-L-rhamnose (3). For the synthesis of monorhamnolipid, the enzyme rhamnosyltransferase 1 (Rt 1) catalyzes the rhamnose transfer to β -hydroxydecanoyl- β -hydroxydecanoate, while Rt 2 synthesizes dirhamnolipid from TDP-L-rhamnose and monorhamnolipid. Genes coding for biosynthesis, regulation, and induction of Rt 1 enzyme are organized in tandem in the *rhlABRI* gene cluster around min 38 of the *P. aeruginosa* chromosome (20). The genes encoding Rt 2 have yet to be described.

Polyhydroxyalkanoates (PHAs) are bacterial storage compounds, which are synthesized by the polymerization of β -hydroxyacids by the PHA synthases (PhaC), with the coenzyme A (CoA)-linked fatty acids as substrates (Fig. 1) (31). The NADPH-dependent β -ketoacyl-CoA reductase (PhaB) is responsible for the reduction step in the production of the β -hydroxyacids. These storage compounds are intracellularly deposited as granules in many species. *P. aeruginosa* mainly produces PHAs consisting of medium-chain-length polymers, mainly poly- β -hydroxydecanoate (30).

The fatty acid synthetase system of *Escherichia coli* as well as that of most bacteria and plants is a dissociated fatty acid type of system (i.e., different reactions are catalyzed by separate proteins encoded by separate genes) (7). This biosynthetic pathway has been widely studied at the molecular level in *E. coli* and is encoded by a cluster of genes called *fab* genes which have been cloned and sequenced. As shown in Fig. 1, each round of elongation requires four chemical reactions.

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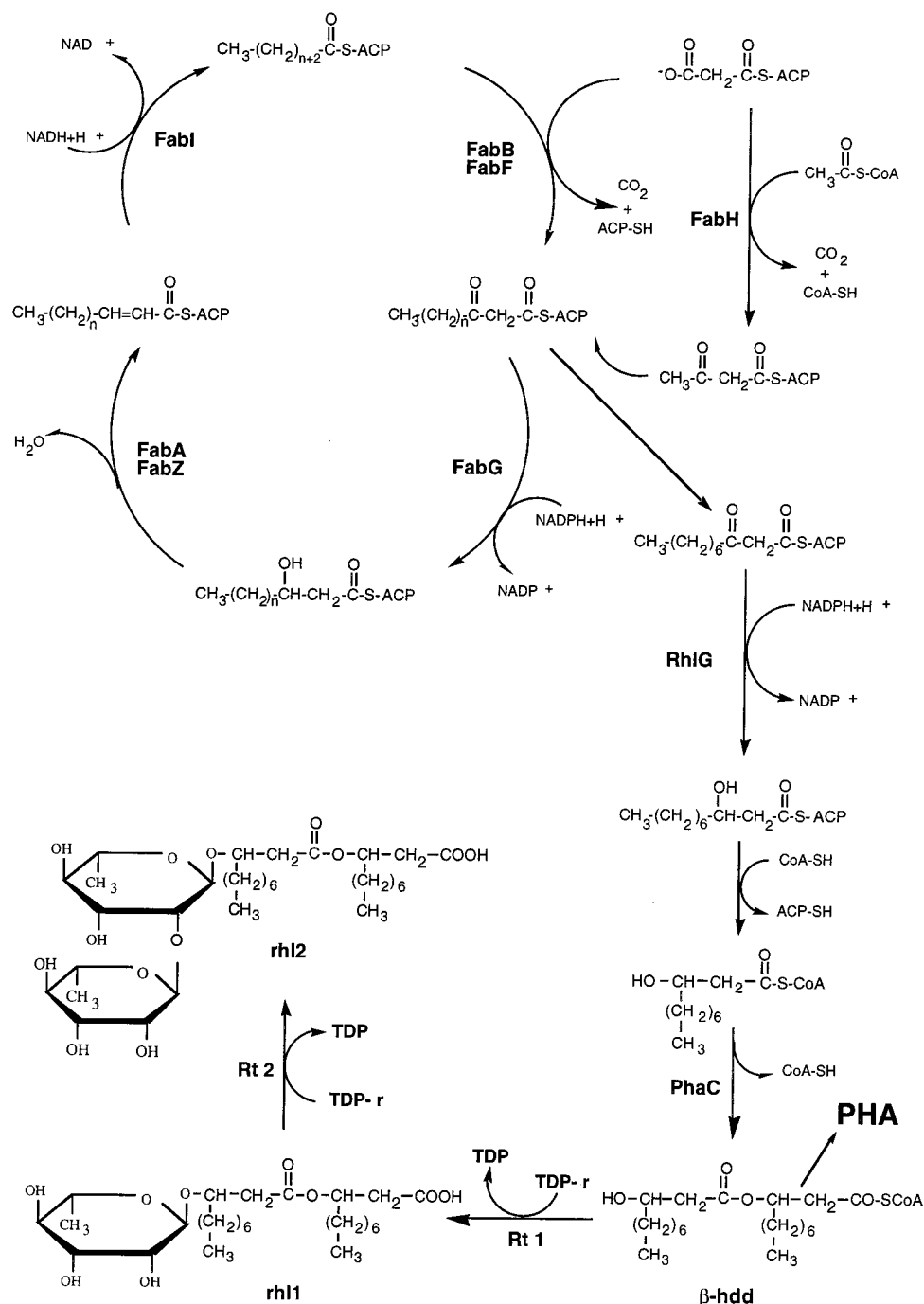


FIG. 1. Schematic representation of the fatty acid biosynthetic pathway showing the deduced role of the RhlG protein in the production of rhamnolipids and PHAs. Initiation of the fatty acid biosynthetic cycle, catalyzed by FabH, requires acetyl-CoA and malonyl-ACP to form aceto-acetyl-ACP. Subsequent cycles are initiated by condensation of malonyl-ACP with acyl-ACP, catalyzed by FabB and FabF. In the second step, the resulting β -ketoester is reduced to a β -hydroxyacyl-ACP by FabG. The third step in the cycle is catalyzed by either FabA or FabZ. The fourth and final step is the conversion of *trans*-2-enoyl-ACP to acyl-ACP, a reaction catalyzed by FabI. TDP-r, thymidine-diphospho-L-rhamnose; PhaC, PHA synthase; rhl 1, monorhamnolipid; rhl 2, dirhamnolipid; β -hdd, β -hydroxydecanoyl- β -hydroxydecanoate.

Initiation requires acetyl-CoA and malonyl-acyl carrier protein (ACP) to form aceto-acetyl-ACP. The first cycle is initiated by Kas III (FabH). Subsequent cycles are initiated by condensation of malonyl-ACP with acyl-ACP, catalyzed by Kas I (FabB) and Kas II (FabF). In the second step, the resulting β -ketoester is reduced to a β -hydroxyacyl-ACP by a single, NADPH-dependent β -ketoacyl-ACP reductase (FabG). The third step

in the cycle is catalyzed by either the *fabA*- or *fabZ*-encoded β -hydroxyacyl-ACP dehydratases. The fourth and final step is the conversion of *trans*-2-enoyl-ACP to acyl-ACP, a reaction catalyzed by a single NADH-dependent enoyl-ACP reductase (FabI).

Recently the complete *P. aeruginosa fab* gene cluster sequence was deposited in the GenBank database (accession no.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s) ^a	Source or reference
Strains		
<i>P. aeruginosa</i>		
W51D	Strain able to degrade surfactants	28
W51D-10	W51D <i>rhlG</i> ::Tc mutant	This work
PAO1	Wild-type strain	B. H. Holloway ^b
ACP5	PAO1 <i>rhlG</i> ::Tc mutant	This work
PAO R1	PAO1 <i>lasR</i> ::Tc mutant	12
<i>C. violaceum</i>		
ATCC 31532	Wild-type strain	16
CV026	ATCC 31532 nonpigmented mutant	16
Plasmids		
pJQ200mp18	Cloning vector Gm ^r , unable to replicate in <i>Pseudomonas</i>	25
pJC1	pJQ200mp18 with a 600-bp <i>rhlG</i> internal fragment	This work
pJC2	pJC1 with a Tc ^r cassette cloned into the single <i>Sma</i> I site of <i>rhlG</i>	This work
pUCP20	pUC19-derived <i>E. coli</i> - <i>Pseudomonas</i> shuttle vector, Cb ^r	33
pJC3	pUCP20 with the PAO1 <i>rhlG</i> gene obtained by PCR	This work
pJC4	pUCP20 with 7 kb of W51D DNA, including the <i>rhlG</i> gene	This work

^a The abbreviations used represent resistance to carbenicillin (Cb^r), gentamicin (Gm^r), and tetracycline (Tc^r).

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U91631). In addition, the *P. aeruginosa* *fabA* and *fabB* genes have been characterized (15). The objective of this work is to present evidence for the existence of a *P. aeruginosa* gene (*rhlG*) encoding a FabG homolog which is specifically involved in the synthesis of the β -hydroxyacid moiety of rhamnolipids. There have been no previous reports on the nature of the enzymes involved in the synthesis of the β -hydroxyacid moiety of rhamnolipids, and it has been assumed that they are the same proteins involved in fatty acid synthesis. Evidence is also presented suggesting that RhlG has a role in PHA synthesis.

MATERIALS AND METHODS

Microbiological procedures. The bacterial strains and plasmids used in this work are shown in Table 1. *P. aeruginosa* strains were routinely grown on Luria-Bertani medium (LB), *Pseudomonas* isolation agar (PIA [Difco]), or PPGAS (the phosphate-limited medium designed for rhamnolipid production) (34) at 29°C. M9 minimal medium supplemented with 0.05% NH₄Cl and gluconate 0.2% (MM + gluconate) was used to induce the production of PHAs. The antibiotic concentrations used for *P. aeruginosa* PAO1 and W51D, respectively, were as follows: carbenicillin, 250 and 50 μ g/ml; gentamicin, 250 and 30 μ g/ml; and tetracycline, 150 and 50 μ g/ml.

Exoproducts and PHA determination. Pyocyanine was extracted with chloroform from the culture supernatant and determined by *A*₆₉₀ as described previously (6). Protease production was measured by halo formation in LB plates containing 1% skim milk and inoculated with 20 μ l of a saturated liquid culture. Total rhamnolipid concentration was determined from culture supernatants of cells grown on PPGAS medium at 29°C for 48 h by measuring the rhamnose concentration after acid hydrolysis by the orcinol method (4). The production of butanoyl-homoserine lactone (PAI-2) by different *P. aeruginosa* strains was determined by using the biosensor developed for the detection of small-chain *N*-acyl-homoserine lactones based on violacein production by *Chromobacterium violaceum* mutant strain CV026 (16). The wild-type *C. violaceum* strain ATCC 31532 produces violacein induced by the autoinducer *N*-hexanoyl-L-homoserine lactone, while mutant CV026 only produces this pigment when given medium supplemented with this autoinducer or related compounds, such as the *P. aeruginosa* PAI-2 autoinducer. PHA was determined after 24 h of growth under nitrogen-deprived conditions (30). Cells were harvested by centrifugation and washed with 100 mM Tris–100 mM NaCl buffer (pH 7). Cells were ruptured by sonication, and the extract was digested with 1.8% sodium hypochlorite for 1 h.

After centrifugation, the pellet was washed twice with ethanol and once with acetone. The PHA concentration is expressed as milligrams of PHA per milligram of protein.

Fatty acid analysis. Total cell lipids were extracted by the method of Folch et al. (10). Briefly, 1 ml of the culture was washed twice and then brought back to the original volume. The following reagents were added with vortexing after each addition: 2 ml of 2:1 methanol-chloroform, 1 ml of 1 N KCl acidified with 0.1 N HCl, and 1 ml of chloroform. In some samples, a white emulsion phase formed between the aqueous and organic phases. In this case, the sample was placed in the refrigerator overnight to allow the emulsion phase to settle. The lower phase (chloroform) was removed and evaporated at 45°C under a nitrogen stream. The fatty acids were analyzed by gas chromatography after methyl esterification (18). Chloroform (0.5 ml) was added, and the sample was vortexed. Two milliliters of BF₃-methanol was added, and the mixture was heated at 80°C for 1 h in an airtight Teflon sealed screw-cap tube (18). The resulting fatty acid methyl esters (FAMES) were extracted three times with 1 ml of hexane, and the three fractions were combined. Finally, the hexane was evaporated at 45°C under a nitrogen stream, and the FAMES were brought to a concentration of 200 μ l with chloroform.

Electron microscopy. PHA production by different *P. aeruginosa* strains was visualized by electron microscopy. Cells were treated for electron microscopic observation as follows. They were washed three times with phosphate buffer at pH 7.2, fixed with 2% glutaraldehyde for 2 h, and washed with phosphate buffer. Further fixation with 2% osmium tetroxide for 2 h was done; all of these procedures were carried out at 4°C. Fixed cells were washed and then dehydrated by passage through a graded ethanol series. After exposure to propylene oxide, samples were placed in L. R. White resin as recommended by the manufacturer. Ultrathin sections were incubated with uranyl acetate, washed with distilled water, treated with lead citrate, washed again, and observed.

Nucleic acid procedures. DNA isolation, cloning and sequencing, Southern and Northern blotting, and nick translation procedures were carried out as described previously (27). RNA was isolated with the RNeasy Lysis kit (BIO101, Inc.). Primer extension analysis was done with two primers (R3 and R4 [Fig. 2]), both located in the 5' region of the *rhlG* gene from *P. aeruginosa* PAO1 (*Pseudomonas* Genome Project contig 1780). The templates used for sequencing reactions were obtained by PCR of total DNA from *P. aeruginosa* PAO1 with the oligonucleotides L1 and R3 or R4 (Fig. 2). The sequencing reactions were done with the Thermo Sequenase radiolabeled terminator cycle sequencing kit (Amersham Life Science, Inc.).

Genetic manipulations. *P. aeruginosa* matings (34) and transformation (21) were done as reported previously. The PAO1 and W51D *rhlG*::Tc mutants (ACP5 and W51D-10, respectively [Table 1]) were constructed by selection of double recombination events with plasmid pJC2. This plasmid is a derivative of plasmid pJC1, which contains a 600-bp *rhlG* internal fragment from *P. aeruginosa* W51D. A 1.4-kb tetracycline resistance gene from plasmid pBSL141Tc (1) was cloned on the unique *Sma*I site of the *rhlG* fragment contained in plasmid pJC1, rendering plasmid pJC2 (Table 1). The W51D *rhlG* internal fragment was obtained by PCR with the oligonucleotides L2' (CGAAGTCTGCAGGTACGGC GAGTGCATCGG) and R2' (GATGCTGCAGATGTTGCCGGTCATGTAG GC) (corresponding to the positions in the PAO1 *rhlG* gene of the L2 and R2 oligonucleotides shown in Fig. 2), with the recognition site for the *Pst*I endonuclease incorporated on the flanking ends of both of them. Plasmid pJC3 is a pUCP20 (33) derivative containing the PAO1 *rhlG* gene obtained by PCR with oligonucleotides L1 and R1 (Fig. 2). Plasmid pJC4 is a pUCP20 derivative containing a 7-kb *Eco*RI W51D DNA fragment which includes the *rhlG* gene. The sequence of the L1 oligonucleotide is not present in the W51D *rhlG* gene region.

Computer analysis of the DNA and protein sequences. Computer analyses of the sequences were carried out by using the GENE WORKS program (IntelliGenetics, Inc.) and the University of Wisconsin Genetics Computer Group (UWCGC) programs. The sequences of different *P. aeruginosa* PAO1 contigs were obtained from the *Pseudomonas* Genome Project web site (<http://www.pseudomonas.com>).

Nucleotide sequence accession number. The sequence of the W51D *rhlG* gene has been deposited in the GenBank database under accession no. AF052586.

RESULTS AND DISCUSSION

Identification and sequencing of the *P. aeruginosa* W51D *rhlG* gene. *P. aeruginosa* W51D is a bacterium which is able to degrade at least 70% of a branched-chain alkylbenzene sulfonate mixture and is resistant to high concentrations of this surfactant (28). In order to study the W51D surfactant catabolic pathway, we have isolated several transposon mutants affected in the degradation of presumed intermediates of the surfactant degradation (unpublished results). During the DNA sequencing of a mutant unable to degrade citronellol, we detected a linked open reading frame (ORF) which was homologous to the *E. coli* *fabG* gene, having 36% amino acid se-

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L 1term.....
 GGCTACAGCGCGGGCTTCGTCGAGCACTACCTGTTGCCGCTGATCTATCCCGCGGCTGACCCCGGCGTGCATGGATCCTTGGCGCCATCGTCTCTGC 100
 TGGTCAACCTGATCGCCTACCTGTATCTGTGGCATTGCCGAGTAGCCGCTGAGCCACGGATGCCACGTCATTCCTGATTAATGCTGCGCCGGCA 200
 GCGAGGCGAATCGTAGGCTGGTCTCCCTGCCAGCCCGGAGTCATTCCCATGCATCCCTATTTTCAGTCTCGCCGGTGCATCGCCCTGGTCACCGGCGG 300
 CAGCCGCGGCATCGGCCAGATGATCGCCCGGGTCTTCTCGAAGCCGGCGCCCGCTCTTCATCTGCGCACGTGACGAGAGGCGCTGCGCCGACACCGCC 400
 S R G I G Q M I A Q G L L E A G A R V F I C A R D A E A C A D T A
 ACACGCTCTCGGCCACTGCCAGCGGATCCCGCCGACCTTTCCAGCGAAGCCGGTGCCTCGGCGCCCTGGCGCAGGCGCTCGGCCGAACCTCAGCG 500
 T R L S A Y G D C Q A I P A D L S S E A G A R R L A Q A L G E L S A
 CGCGGCTGGATATCTGGTGAACAACGCCGGCACCAGTTGGGGCGCAGCGCTGGAAGCTATCCGGTGTCCGGCTGGGAAAGGTATGCAGCTCAATGT 600
 R L D I L V N N A G T S W G A A L E S Y P V S G W E K V M Q L N V
 CACCTCGGTGTTTCTGTCATCCAGCAACTGCTGCCGCTGCTGCCGCTCGGCCAGCGCGGAGAACC CGCGCGGGTGATCAACATCGGCTCGGTGGCG 700
 T S V F S C I Q Q L L P L L R R S A S A E N P A R V I N I G S V A
 L 2
 GGCATCAGCGCCATCGGCCGAACACCTATGCGGCGAGCAAGCCGCGCTGCATCAACTGTGCGGATGCTGGCGAAGGAACCTGGTTCGCGGAGC 800
 G I S A M G E Q A Y A Y G P S K A A L R V F I C A R D A E A C A D T A
 ACATCAATGTCAATGTGATCGCTCCCGACGCTTCCGAGCCGATGACCCGTACATCGCAATGACCCGCGAGGCACTGGAGGCGGACAGCGGCTCGAT 900
 I N V N V I A P G R F P S R M T R H I A N D P Q A L E A D S A S I
 TCCCATGGGCGCTGGGGTGCCTCGGAGAGATGGCGGCTGGCGATCAGCCTGGCCGCGACCGCGCGCGCTACATGACCGGCAACCTGATCCCCATC 1000
 P M G R W G R P E E M A A L A I S L A G T A G A Y M T G N V I P I
 GACGCGGTTTTCATCTCTGAGCGCCCAATCCGGGTAACATGACGCCCCGACCGCTCGCCTCGTCCCGCATGACCCAGACGCTTCTCCCATCGGC 1100
 D G G F H L .
 rcsF M T H S V S P I G
 TACATCCGCTCCTGCTTCATGGAGAAGTTCCGCATCCCGCGCCAGCCGCTGCTCGCGCCGGCGCGGGGGACCTGGAACTGCTGCCGCGCTTCGACC 1200
 Y I R S C F M E K F A I P R Q P L L A P A A R G L E L T L E L P P F D Q
 AGGTGGAAGCGCTGGAAGGCTGGAGCAGGTGATGCTGCTGCTGTTCTCTTCCACCAGGCCCTGGAAGACAAGCCGCGACTGAAGGTGCGCCC 1300
 V E A L E G L E Q V S H V W L L F L F H Q A L E D K P R L K V R P
 GCGCGCCTGGGCGCAACCGCTCGCTGGGAGTCTTCCGACCCCGCGCCACCCTCGGCCGAACGGCATCGGCCAGTCCGTTGGTGCCTGGAGGGCTTC 1400
 P R L G G N R S L G V F A T R A T H R P N G I G Q S V V R L E G F
 R 1
 GAAGCGGGCGCCTGTGGCTGTCCGGAATCGACCTGCTCGACGGCACCCCGTACTCGATATCAAGCCCTACGTACCCCTACGCCGACGCGCTGGCCGACG 1500
 E A G R L W L S G I D L L D G T P V L D I K P Y V P Y A D A V A D A
 CCGCAACCGGCATCGCCGACGCGCGCCGCGCGGCATCGCCGTGGAGTGGAGCGAGCAGGCGCGCGCCAGGCCCATGAGCACGGCCAGCGGTTCGGCA 1600
 R N G I A D A P P P G I A V E W S E Q A R R Q A H E H G C R L R Q
 GCGGTGGCCGAGCTGATCGAGCAATGCTGGCGCAGGACCCACGCGCGCTACCAAGAAGCCAGAGCCCGGCGCGCTACGGGTGCGCCTGTGGGAC 1700
 P V A E L I E Q C L A Q D P R P A Y Q K P E P G R R Y G V R L W D
 CTCGACGTGCATTGGCACTACCCGCGCCCGGACCTGATCCGCTGCTGGACGTGCGCGCGCGCGATTGAGCCTTCTCAGCGCAGCGATTGAGCGCCAGC 1800
 L D V H W H Y P R P D L I R V L D V A G G D .
 GCGTCGCTTTCGAAGACTTCCTGTTTCGAGGATCAGCGGCTCGACCAACGGTCCGCTGTCGCGGAAATGCGCGGTGCGGCTGTCGCTCGTAGGCTGCGT 1900
 CATCGCGATAGACCTCGTAGAGATAGACAGGTCTGTCGGTTCGCGGAAACGTGGAACACAGGACCGCGGCTCGGTGGACACGGAAGCGCG 2000
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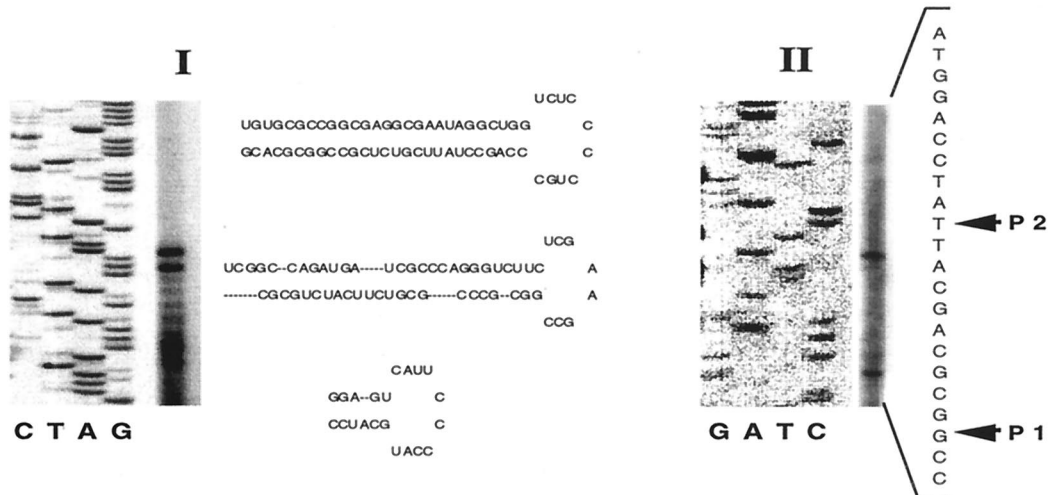


FIG. 2. Characterization of the transcription arrangement of the *P. aeruginosa* PAO1 *rhlG* and *rcsF* genes. (A) Nucleotide sequence of the genes and regulatory sequences. The sequence and position of the oligonucleotides used during this work are shown in the figure and identified as Ln or Rn, depending on their polarity (L oligonucleotides amplify the sequence from 5' to 3', and R oligonucleotides have the opposite polarity). The sequence corresponding to the lux box is double underlined. Arrows indicate the two transcription start sites detected (P1 and P2). SD (Shine-Dalgarno) indicates the ribosome binding site sequence for mRNA translation. The sequences corresponding to putative transcriptional termination sites (term) are shown. (B) Primer extension analysis of the *rhlG* gene with two different oligonucleotides as primers. In panel BI, the primer extension analysis was done with oligonucleotide R3 and revealed the existence of the mRNA secondary structures shown. In panel BII, the oligonucleotide R4 was used, and two transcription start points indicated as P1 and P2 were found.

quence identity along the entire length. This ORF, called *rhlG*, has the characteristic codon usage and bias of its GC composition in the third position of each codon of the *Pseudomonas* genes (32). The alignment of the protein deduced from the sequence of the *rhlG* gene with the proteins deposited in the GenBank database confirmed the presence of the characteristic signature for NADPH binding, as well as the characteristic motifs of dehydrogenases. As shown in Fig. 3, these sequences are conserved in all of the sequenced bacterial and plant FabG proteins. However, the chromosomal region surrounding this *fabG* homolog did not show the presence of other *fab* genes, as has been reported for *P. aeruginosa* PAO1 and *E. coli*.

Another ORF encoding a protein with a sequence 44% identical to that of *E. coli* RcsF, which is involved in regulation of capsular production (13), was detected downstream of *rhlG*. This genetic arrangement and the fact that a *fabG* gene has been already described in *P. aeruginosa* PAO1 led us to the hypothesis that this is a novel gene which encoded a second functional NADPH-dependent β -ketoacyl reductase. In order to test this hypothesis, we constructed an *rhlG::Tc* mutant according to the strategy shown in Fig. 4. The inactivation of the W51D *rhlG* gene did not produce a fatty acid auxotrophy or a decrease in growth rate (data not shown). These results showed that the RhlG protein is not responsible for the total cellular fatty acid synthesis, so a FabG protein should also exist in strain W51D. However, this evidence was not enough to determine the expression and functionality of the *rhlG* gene product.

Identification of the *rhlG* gene in the *P. aeruginosa* PAO1 genome. We decided to study the functionality of the RhlG protein in the *P. aeruginosa* PAO1 strain for two reasons: approximately 95% of its genome has been sequenced (<http://www.pseudomonas.com>), and the existence of the *fabG* gene had already been reported in this strain (GenBank database accession no. U91631). We identified the PAO1 *rhlG* gene in contig 1780 of the *Pseudomonas* Genome Project, showing the characteristic codon usage and bias of GC composition in the third position of each codon of the *Pseudomonas* genes (32). The genetic arrangement of PAO1 is similar to that of strain W51D, in which the *rscF* gene is downstream (Fig. 2). The deduced PAO1 RhlG protein consists of 256 amino acids, with a predicted molecular mass of 26,813 Da and has amino acids 54% identical to those of the W51D RhlG protein (Fig. 3). The great divergence between both *rhlG* genes is mainly due to differences in the sequences at their 5' ends. If the sequences are compared after deletion of the first 52 amino acids of the PAO1 RhlG protein and 112 amino acids of the corresponding protein in W51D, they have 91% identical amino acid sequences. Furthermore, both proteins contain the motifs important for their putative catalytic capabilities (Fig. 3). The difference between the amino-terminal sequences of PAO1 and W51D RhlG proteins is striking, considering that both strains belong to the same species. The significance of this variability is not clear to us. The DNA sequence of the first 300 nucleotides of the PAO1 *rhlG* 5' region (Fig. 2) was confirmed by us, and we found only three differences. This result rules out the possibility that the divergence between the *rhlG* genes is due to major inaccuracies in the reported sequence in contig 1780.

We confirmed that the *rhlG* genes were conserved and that *rscF* was present downstream in PAO1 and W51D strains by PCR amplification of total DNA (Fig. 4C). The following oligonucleotides were used as primers: L2 or L2' (the oligonucleotide corresponding to the W51D *rhlG* gene sequence in the same region) and R2, L2 or L2' and R1, and L3 and R1 (Fig. 2A). The amplified product was a DNA band of the same size from either strain (Fig. 4C), thus validating the high degree of

homology between *rhlG* genes and the conservation of the genetic arrangement inferred from the analysis of the sequence obtained from the *Pseudomonas* Genome Project in contig 1780.

The sequences of the PAO1 *fabG* gene from contig 1761 of the *Pseudomonas* Genome Project and GenBank (accession no. U91631) were compared. The two PAO1 *fabG* gene DNA sequences are not identical. This inconsistency may result from inaccuracies in the sequence of the *Pseudomonas* Genome Project. We compared both PAO1 FabG protein sequences to the deduced protein sequences of the PAO1 RhlG protein and found the amino acids were 33 and 34% identical, respectively (Fig. 3). This is further evidence that RhlG is an NADPH-dependent β -ketoacyl reductase. The PAO1 RhlG protein also aligned with FabG proteins of different origin, as well as with PhaB proteins from *Alcaligenes* sp. strain SH-69 and *Acinetobacter* sp. strain RA3849 (Fig. 3). This result is not surprising, since the PhaB proteins are NADPH-dependent acetoacetyl reductases which participate in PHA synthesis. The significance of the RhlG homology with PhaB proteins is discussed below.

Expression of the *rhlG* gene in *P. aeruginosa* PAO1. We carried out primer extension experiments to determine whether the *rhlG* was expressed in strain PAO1 grown for 48 h on PPGAS, a medium designed to increase rhamnolipid production (34). Two oligonucleotides derived from the DNA sequence reported in contig 1780 corresponding to the 5' end of the *rhlG* gene were used as primers (Fig. 2). These experiments revealed the presence of a specific *rhlG* mRNA, confirming that the gene is expressed under these culture conditions (Fig. 2BI and BII). When the R3 oligonucleotide was used, the extension was aborted very near the putative RhlG protein start codon, suggesting the existence of an mRNA region with a secondary structure that prevented DNA polymerization by reverse transcriptase beyond this point (Fig. 2BI). The DNA sequence within this region predicted the formation of several loops in the mRNA (Fig. 2BI), which could play a role in the regulation of the *rhlG* gene expression at the posttranscriptional level.

Two mRNA start sites were observed when the oligonucleotide R4 was used as a primer in extension experiments (Fig. 2BII). R4 is complementary to the mRNA sequence in which the extension of the primer was aborted with oligonucleotide R3 (Fig. 2A). The most frequent mRNA start site seems to be transcribed from a putative σ^{54} type of promoter, although the $-12/-24$ regions do not present all the elements which have been claimed to be important in these promoters. A similar situation has been found in the *rhlAB* σ^{54} promoter (24). The second, less abundant mRNA start site is a typical σ^{70} type of promoter. These two promoters overlap at their respective -24 and -35 regions. Between nucleotides -43 and -63 (with respect to the putative σ^{54} promoter), the sequence ATCTG TGGCATTGCCGAGTA corresponding to a "lux box" is present (Fig. 2A). The presence of this regulatory sequence strongly suggests that the *rhlG* gene is regulated at the transcriptional level by one of the two LuxR homologs forming part of the quorum-sensing type of response in *P. aeruginosa*, LasR or RhlR. The characteristics of the *rhlG* promoter region (two promoters, one of which is a noncanonical σ^{54} type of promoter, and the presence of a lux box) are very similar to those present in the promoter region of the *rhlAB* operon, which encodes the Rt 1 enzyme (24). In the case of this key enzyme for rhamnolipid biosynthesis, RhlR positively regulates its transcription (20), and the alternative sigma factor σ^{54} is involved in its expression (24). As will be shown later, RhlG protein is involved in the synthesis of one of the rhamnolipid

ConsensusK..ALVTCASRGIG	90
FabGBjap	-----MGLDLPNDNLIRGPLPEAHLDRLDVAVNARVDREGEKPVMLITGASRGIG	49
FabGMsmeg	-----MTVTDNPADTAGEATAGRPAPVSRVSVLVVGGNRCGIG	36
FabGMtub	-----MTATATEGAKPPFVSRVSVLVVGGNRCGIG	28
FabGAact	-----MSETTLLTGSSRGIG	15
FabGatha	MAAAVAA--PRLISLKAIVGKLGFRISQIRQLAPLHSAIPHFGMLRCRSRQPFSTSVVKAQATATEQSPGEVQKVESPVVVITGASRGIG	89
FabGClan	MATATAAGCSGAVALKSLGGRRLLCIPQQLSPVLVAGFGSHAAKSFPILSTRSIATSGIRAQVATAEKVSAGAGQSVESPVVVITGASRGIG	90
FabGBsub	-----MLNDKTAIVITGASRGIG	17
FabGEcoli	-----MNFEGKIALVITGASRGIG	18
FabGVharv	-----MNLLEGKIALVITGASRGIG	18
FabGHinf	-----MQGKIALVITGSTRGIG	16
FabGPaer	-----MSLQGGKVALVITGASRGIG	18
FabGPAO1	-----MSLQGGKVALVITGASRGIG	18
PhaBasp	-----MSQKVAYVITGEMGIG	16
PhaBacsp	-----MSEQKVAYVITGALGIG	17
RhlGPAO1	-----MHPYFSLAGRIALVITGSSRGIG	22
RhlGW51D	-----MRSAGSLVGRPAVSISTGACQRRHSLDQRLARIDQGRHVEDAAQGLDLFLRTDAEAHVHGQDALARAVLQYVITGSSRGIG	82
Consensus	.AIA..L...G.V...A.S...A.....G.....DV.....G.	180
FabGBjap	HATAKLFSEAGWRIISC-ARQPFDERGCPWEAGNDDHFQVDLG-----DHRMLPRAITEVKKRLAG-AP	111
FabGMsmeg	LAIAARRLAADGHKVAVTHRGSGAPDDL-----FGVQC-----DVTDSAGVDFRAFKEVEEHQGP	89
FabGMtub	LAIAAQRLLAADGHKVAVTHRGSGAPKGL-----FGVEC-----DVTDSADVDFRAFTVAEEHQGP	81
FabGAact	KATIALRLAQAGFDIVVHCRSRIIEEAQAVRELQGNARVLQF-----DVSCREAAADKLTADVEAHGA	80
FabGatha	KATIALALGKAGCKVLVNYARSAAKEAEVAKQIEEYGGQAITFGG-----DVSKAIDVDAMMKLTADKWT	154
FabGClan	KATIALSLGKAGCKVLVNYARSSEKAEAEVSKIEAFGGQALITFGG-----DVSKEEDVEAMIKTAVDAWGT	155
FabGBsub	RSIALALAKSGANVVVNYSGNEAKANEVDEIKSMGRKAIKAVKA-----DVSNPDEVDQNMKLTLSVFT	82
FabGEcoli	RAIAETTLAARGAKVIGT-ATSENGAQAIISDYL-----GANGKGLML-----NVTDPASIESVLEKIRAEFFE	79
FabGVharv	RAIAELLVERGATVIGT-ATSEGGAAAISEYL-----GENGKGLAL-----NVTDSIESIEATLKTINDECA	79
FabGHinf	RAIAEELSSKGAFVIGT-ATSEKGAELISAYL-----GDKGKGLVL-----NVTDKESITLLEQIKNDFGD	77
FabGPaer	QATIALELGRIGAVVIGT-ATSASGAEKIAETLKANGVEGAGLVL-----DVSSDESVAATLEHIQOHLGQ	82
FabGPAO1	QATIALELGRIGAVVIGT-ATSASGAEKIAETLKANGVEGAGLVL-----DVSSDESVAATLEHIQOHLGQ	82
PhaBasp	TATQRLHKEGFKVIAG-CGPTRDHAKCWPSKRFWATRFMHPSV-----TWVIGIPPEAFGKTKAEHGT	80
PhaBacsp	SEICRQVLTAGYKI IATVVPREEDREKQWLQSEGFDQSDVRFVL-----TDLNNHBAATAAIIQBAIAEGR	85
RhlGPAO1	QMTAQGLLEAGARVIC-ARDA-----EACADTATRLSAYGDCQAIAPADLSSEAGARLQAALGELSR	83
RhlGW51D	GGLAHAGRADQCDHAPLLRSISGLVIEPARCASSMRQACFGSSRPAMACADTAAELSQYGEICIGLPANLATEGARALAAELSERLEH	172
Consensus	.DIIVVNAGIT-RD..L.RM...W..VI.TNL...F.....M.K---GRIINIGSV..GN.GQA-NY.AAAG.IGF	270
FabGBjap	LHAIVNNAGVSPKTPGDRMTSLTSTDVWVRVPHLNLVAPILLAQGLFDELRAASGSIVNVISLAGSRVHPFAGSAYATSKAALASLTR	201
FabGMsmeg	VEVIVVNAGIS-KDAFLMRMTTEERFEEVINTNLGAFCRAQASRTM-Q---RKRFGRIIFIGSVSGMVGIGNQA-NYAAKAGLIGMAR	173
FabGMtub	VEVIVVNAGIS-ADAFLMRMTTEKFEKVINANLGAFCRAQASRTM-Q---RNKFGRMIFIGSVSGSWGIGNQA-NYAAKAGVIGMAR	165
FabGAact	YGVVVLNAGLT-RDNAPFATDDEDWRVLTETNLGDFNVVLPIMPMIR---RRKAGRIVCITISVGLIGNRGQV-NYASAKAGIIGAAG	165
FabGatha	IDVIVVNAGIT-RDITLIRMQSQWDEVIALNLTGVFLCTQAAVKIMMK---KKR-GRIINISVAGLIGNIGQA-NYPATKGGVIFSE	238
FabGClan	IDVIVVNAGIT-RDGLLIRMKKSQWDEVIDLNLITGVFLCTQAAVKIMMK---KKK-GRIINISVAGLVGNAGQA-NYSAKAGVIGFTK	239
FabGBsub	IDVIVVNAGIT-RDNLIRMKDEWDDVININLKGVENCTKAVTROMMK---QRS-GRIINISVAGVSGNPGQA-NYVAKAGVIGLTK	166
FabGEcoli	IDVIVVNAGIT-RDNLIRMKDEEWNDIETNLSSVFLRSKAVMRAMMK---KRH-GRIITIGSVGTMNGGQA-NYAAKAGLIGFSK	163
FabGVharv	IDVIVVNAGIT-RDNLIRMKDDEWNDIETNLTPYIRMSKAVLRGMKK---KRA-GRIINISVAGTMGNAGQT-NYAAKAGVIGFTK	163
FabGHinf	IDVIVVNAGIT-RDNLIRMKDEEWFIDIMQTNLTSVHLKAMLRSMKK---KRF-GRIINISVAGTSTNPGQT-NYCAKAGVIGFSK	161
FabGPaer	PLIVVNAGIT-RDNLIRMKDDEWFDVNTINENSLRSLKAVLRGMTK---ARW-GRIINISVAGVAMGNAGQT-NYAAKAGLEGFTK	166
FabGPAO1	PLIVVNAGIT-RDNLIRMKDDEWFDVNTINENSLRSLKAVLRGMTK---ARW-GRIINISVAGVAMGNAGQT-NYAAKAGLEGFTK	166
PhaBasp	IDVIVVNAGIT-RDRMFLKMSREDWDAVETINENSMFNTKQVVSMDVE---KGW-GRIINISVNGEKGQAGQT-NYSAAKAGMHGFSM	164
PhaBacsp	VDVIVVNAGIT-RDATFKMSYEQWSQVIDINELKTITVTPVFNKMLE---QKS-GRIINISVNGLKGQFGQA-NYASAKAGIIGFTK	167
RhlGPAO1	LDIIVVNAGIS-WGAALLESYPVSGWEKVMQLNVTSVFSCIQQLPLLRSSASAPNPARVINTGSAIGSAMGEAYAYGFSKALHQLSR	174
RhlGW51D	LDIIVVNAGIT-WGAPLESYPASGWEKVMQLNVTAVTSCIQQLPLLRSSAGQNPARTVINTGSAIGTSGESAYAYGFSKALHQLSR	261
Consensus	.LARE.A.R.ITVN.VAEG.I.TDMT..L..D.....L..IP.GR.G.P.E.A..V.FLA-S..A.V.TG...VNGG..M.-	355 %
FabGBjap	ELAHDYAPHGIRVNAIPGEITDM---LSPDAE-ARVVASIPLRVGTPEVAKVIFFLC-SDAASYVTGAEPVINGOHL--	278 2 8
FabGMsmeg	SISRELDKAGVTANVLPFGYIDETRALDERIQ--GGAIIDFIPDKRVGTVEEVAGAVSFLA-SEDAASYTAGAVIPVIGGMGMGH	255 3 1
FabGMtub	SIARELSKANVTANVVAFGYIDETRALDERIQ--QGALQFIPAKRVGTPEVAGAVSFLA-SEDAASYTAGAVIPVIGGMGMGH	247 3 2
FabGAact	ALAVELAKRKITVNCVAPGLIDTDILDE-NVPID-E-ILKMTIPAGRMGDPEEVAVHVNFLM-GEKAAVITROVIAVNGGLC--	242 2 7
FabGatha	TPAREGASRNINNVVPGFIASDMTAEGLDME--KKILGTIPLGRYGKAEVAGLVEFLALSPAASYITGQAFITIGGTAI--	319 2 6
FabGClan	TVAREYASRNINNVVAPGFISSDMTSLKLGDDIN--KKILETIPLGRYGQPEEVAGLVEFLALNPASSYITGQVFTIGGTM--	320 2 8
FabGBsub	SSAKELASRNITVNAIPGFIIDETMDKLAKDVQ--DEMLKQIPLARFGPESDVSSVVTFLA-SEGARYMTGQTLHIDGMVM--	246 3 1
FabGEcoli	SLAREVASRGITVNVVAPGFIETDMTRALSDQR--AGILAQVPAGRLGGAQELANAVAFLA-SDEAAYITGETLHVNGGMVM--	244 3 3
FabGVharv	SMAREVASRGITVNVVAPGFIETDMTKALNDQR--AATLSNVFAGRLGDPREIASAVVFLA-SPEAAYITGETLHVNGGMVM--	244 3 0
FabGHinf	SLAKEVAARGITVNVVAPGFIATDMTEVLTDEQK--AGILSNVPAGRLGEAKDIKAVAFLA-SDDAGYITGTTLHVNGGLYS--	242 3 2
FabGPaer	ALAREVGSRAITVNAIPGFIIDETMTRELPEAQ--EALLGQIPLGRLGQAEELAKVGFGLA-SDGAYVITGATVPVNGGMYS--	247 3 4
FabGPAO1	ALAREVGSRAITVNAIPGFIIDETMTRELPEAQ--E-LXGPDASAGSPGAGGRDRQGGRLPR-FGRRSYVITGATVPVNGGMYS--	246 3 1
PhaBasp	ALAQLATKGVITNVVPGYIGTDMVKATRPDLV--EKIVATVPVKALGEPSEIASIAWLA-SEENGATGADTSVNGGLHMG--	245 2 6
PhaBacsp	ALAQEGARSNICVNVVPGYTATPMVTAMREDV--KSIEATVPLQRLAAPAEIAAAVMYLV-SEHGAYVITGETLSINGGLYMH--	248 2 9
RhlGPAO1	MLAKELVGEHINNVVAPGRFPSPRMTTRHIANDOALEADSAPMGFRWGRPEEMALASLA-GTAGAYMTGNVPIIDGGFHL--	256 1 0 0
RhlGW51D	MLARELVKDHINNVVAPGRFPSPKMTTRHTANDEQAMAEATVTPMGRWGREEMSAIASLA-SAGAYMTGNITPIIDGGFHLG--	344 5 4

FIG. 3. Multiple alignment of the RhlG deduced amino acid sequence with different NADPH-dependent β-ketoacyl-ACP reductase (FabG) and NADPH-dependent ketoacyl-CoA reductase (PhaB) proteins. Residues within rectangles correspond to identical amino acids, and those shaded are conserved among most of the proteins analyzed. The percentage of identity of the different proteins with PAO1 RhlG is shown in the bottom right column of the figure. Asterisks mark the residues which form the NADPH binding signature, and circles show the amino acids conserved in dehydrogenases. FabGBjap, FabG from *Bradyrhizobium japonicum*; FabGMsmeg, FabG from *Mycobacterium smegmatis*; FabGMtub, FabG from *Mycobacterium tuberculosis*; FabGAact, FabG from *Actinobacillus actinomycetemcomitans*; FabGatha, FabG from *Arabidopsis thaliana*; FabGClan, FabG from *Cuphea lanceolata*; FabGBsub, FabG from *Bacillus subtilis*; FabGEcoli, FabG from *E. coli*; FabGVharv, FabG from *Vibrio harveyi*; FabGHinf, FabG from *Haemophilus influenzae*; FabGPaer, FabG from *P. aeruginosa* (GenBank database accession no. U91631); FabGPAO1, FabG from *P. aeruginosa* PAO1 (contig 1761); PhaBasp, PhaB from *Alcaligenes* sp. strain SH69; PhaBacsp, PhaB from *Acinetobacter* sp. strain RA3849; RhlGPAO1, RhlG from *P. aeruginosa* PAO1 (contig 1780); RhlGW51D, RhlG from *P. aeruginosa* W51D (GenBank database accession no. AF052586).

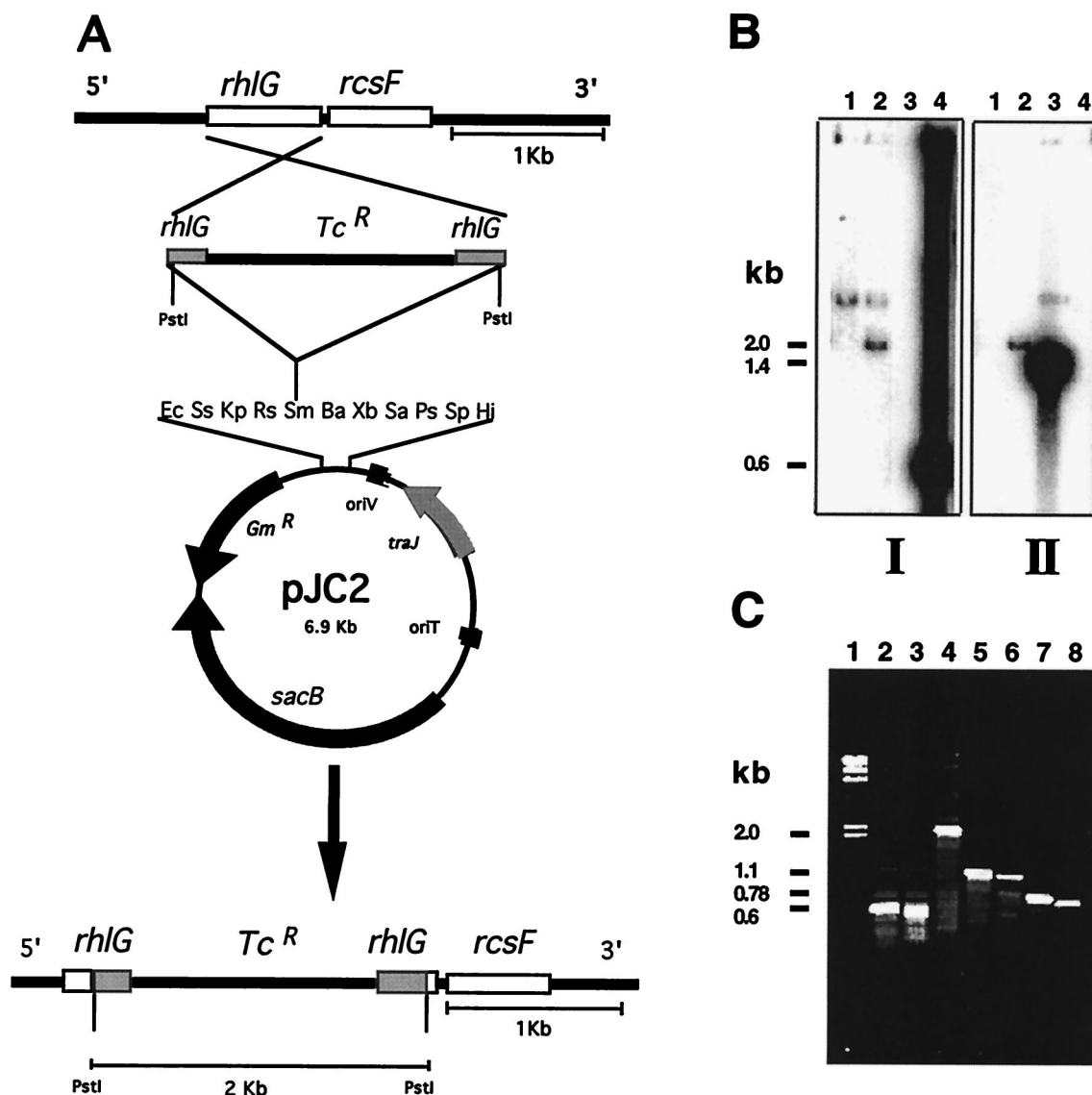


FIG. 4. Molecular characterization of the PAO1 *rhlG* mutant ACP5. (A) Schematic representation of the strategy to construct the *rhlG* mutants (ACP5 and W51D-10). (B) Southern blotting hybridization with the 600-bp insert of plasmid pJC1 (I) and the 1.4-kb *Tc^R* resistance cassette (II) used as probes. Lanes correspond to DNA samples digested with *PstI* endonuclease from the PAO1 genome (lane 1), the ACP5 genome (lane 2), the *Tc^R* cassette (lane 3), and the PCR product of the amplification of the W51D genome with oligonucleotides L2' and R2' (lane 4). (C) Amplification by PCR with different oligonucleotides specific for the *rhlG* and *rcsF* genes. Lanes correspond to the following DNA samples: 1, λ phage genome digested with *HindIII*; 2, PCR product with W51D DNA as a template and the L2' and R2 oligonucleotides as primers; 3, PCR product with PAO1 DNA as a template and the L2 and R2 oligonucleotides as primers; 4, PCR product with ACP5 DNA as a template and the L2 and R2 oligonucleotides as primers; 5, PCR product with W51D DNA as a template and the L2' and R1 oligonucleotides as primers; 6, PCR product with PAO1 DNA as a template and the L2 and R1 oligonucleotides as primers; 7, PCR product with W51D DNA as a template and the L3 and R1 oligonucleotides as primers; and 8, PCR product with PAO1 DNA as a template and the L3 and R1 oligonucleotides as primers.

precursors, so it is likely that the structural similarity between the promoter regions of the *rhlG* gene and the *rhlAB* operon reflects that they are subject to similar genetic regulation. This possibility was examined further (see below).

Construction of a *P. aeruginosa* PAO1 *rhlG::Tc* mutant. The high degree of similarity of the PAO1 and W51D *rhlG* genes, excluding their 5' ends (Fig. 3), enabled us to construct a PAO1 *rhlG::Tc* mutant (ACP5 [Table 1]). Plasmid pJC2, which contains a *rhlG* internal fragment from strain W51D with a *Tc^R* cassette insertion, was transferred by transformation to strain PAO1, and *Tc^R* *Gm^s* transformants which were putative double recombinants carrying an interrupted *rhlG* gene were selected (Fig. 4). One of these transformants is the ACP5

mutant (Table 1), which indeed seems to be the product of a double recombination event in which the *rhlG* gene is interrupted by the *Tc^R* cassette; this conclusion is drawn from the analysis by Southern blot hybridization and PCR amplification as shown in Fig. 4B and C, respectively. The Southern blot hybridization analysis (Fig. 4B) shows that mutant ACP5 contains, as expected, a 2-kb *PstI* fragment with homology with both the *rhlG* gene and the *Tc^R* cassette (lanes 2 in Fig. 4BI and BII) and that this fragment is not present in the PAO1 genome (lanes 1, Fig. 4BI and BII). Unexpectedly, however, the ACP5 DNA retained hybridization with the 3.2-kb *PstI* *rhlG* homologous band. This result can be explained by the presence of heterogeneity in the chromosomes of strain ACP5, in which

TABLE 2. Production of rhamnolipids and pyocyanine by mutant ACP5 and its parental strain, PAO1

Strain	Concn (%) of ^a :		
	Rhamnolipid	Pyocyanine	PHA
PAO1	150 \pm 15 (100)	0.59 (100)	311 \pm 13 (100)
ACP5	<2	0.24 (40.6)	87 \pm 4 (27.9)
ACP5/pJC3	125 \pm 25 (83.3)	0.60 (101.6)	243 \pm 30 (78)
ACP5/pJC4	146 \pm 10 (97.3)	0.64 (108.4)	305 \pm 5 (98)
PAO R1	<2	<0.05	ND ^b

^a Rhamnolipid concentration is expressed as micrograms of rhamnose in rhamnolipids per milliliter of culture. The concentration of pyocyanine is expressed as the A_{690} of the chloroform-extracted culture supernatant. PHA was measured after 24 h of growth on MM + gluconate and is expressed as milligrams of PHA per milligram of protein.

^b ND, not determined.

not all of the *rhlG* copies contain a Tc^r cassette, or by the presence in the PAO1 chromosome of an *rhlG* homolog (probably *fabG*), which gives a hybridization signal of the same size when DNA is digested with *Pst*I. In order to distinguish between these possibilities, PCR was performed in which *rhlG*-specific oligonucleotides (L2 and R2 in Fig. 2) were used to amplify the PAO1 and ACP5 genomes. We found that the expected 600-bp DNA fragment is amplified from PAO1, while a single 2-kb band is amplified from the ACP5 genome (Fig. 4C, lanes 3 and 4), these results clearly show that all of the *rhlG* gene copies in ACP5 contain a 1.4-kb insert (the Tc^r cassette), so the most likely explanation is that we are detecting an *rhlG* homologous gene by Southern blot hybridization, probably *fabG*.

Effect of the *rhlG* inactivation in *P. aeruginosa* PAO1. Mutant ACP5 does not have a fatty acid auxotrophy, grows at the same rate as its PAO1 parental strain, and does not show any significant change in its total lipid profile. Furthermore, the total lipid profiles of the parent and mutant strains were identical (data not shown). This suggests that there must be a functional FabG protein that is responsible for the synthesis of total cellular lipids and other essential products which contain a fatty acid moiety, such as the lipid A molecule (9).

P. aeruginosa produces different secondary metabolites which contain a lipid moiety, such as the autoinducers PAI-1 and PAI-2, as well as rhamnolipids and PHAs. Therefore, we investigated whether the production of some of these compounds was affected by the cassette insertion in the *rhlG* gene (mutant ACP5). Mutants affected in the production of any of the autoinducers are defective in total protease production (2, 22). We used this phenotype as a criterion to evaluate autoinducer production. It was found that mutant ACP5 has the same proteolytic activity as the PAO1 parental strain (data not shown), suggesting that autoinducer production is not affected. Rhamnolipid production in mutant ACP5 is completely abrogated (Table 2), suggesting that the RhlG protein is involved in the reaction leading to the production of the β -hydroxydecanoyl precursor of rhamnolipids (Fig. 1). In order to obtain direct evidence of the involvement of RhlG protein in rhamnolipid production and to rule out that the phenotype of mutant ACP5 was due to a polar effect of the Tc^r cassette insertion in *rhlG* (and not to an inactivation of this gene), we complemented in *trans* the ACP5 mutant with plasmid pJC3, which contains the PAO1 *rhlG* gene (Table 1). The results obtained (Table 2) clearly show that the presence in *trans* of the *rhlG* gene is sufficient to restore the ACP5 capability to produce rhamnolipids.

It was apparent that mutant ACP5 produces lower levels of pigment than strain PAO1 (Table 2). It has been reported that

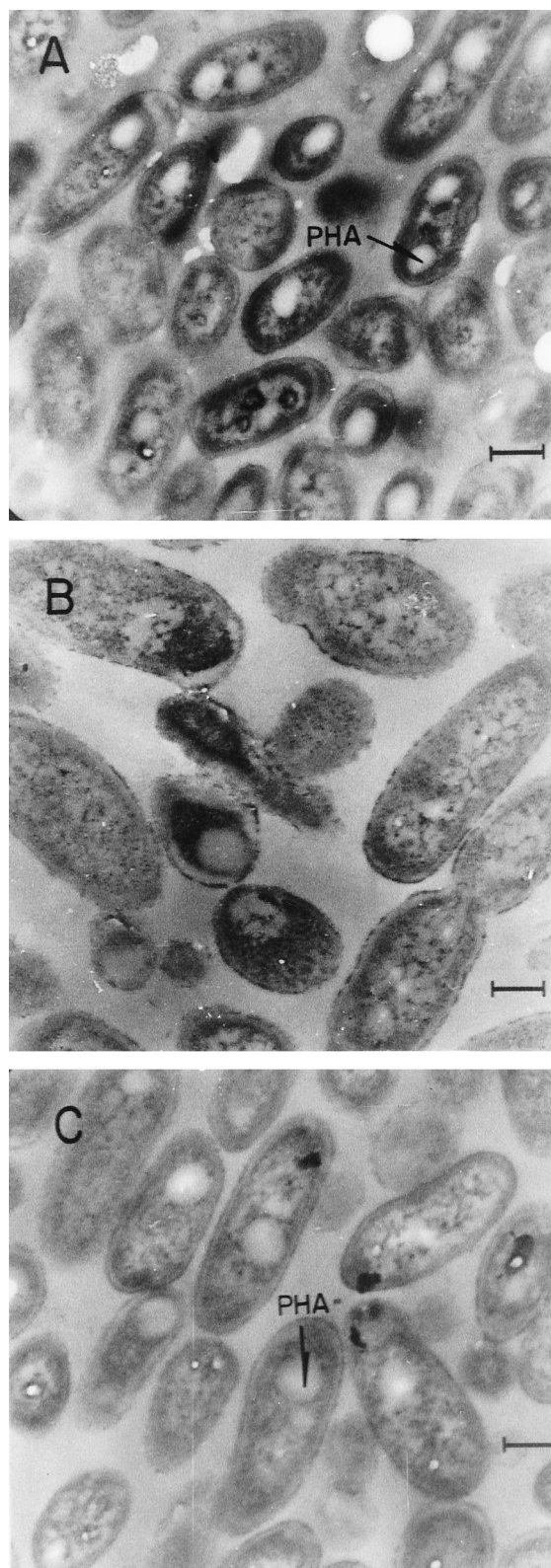


FIG. 5. Electron micrographs of the *P. aeruginosa* strains PAO1 (A), ACP5 (B), and ACP5/pJC4 (C) grown for 24 h on MM + gluconate. Some of the PHA granules are pointed out. Micrographs were taken at a $\times 20,000$ magnification.

production of both rhamnolipids and pyocyanine is induced by PAI-2-mediated activation (20), so we measured PAI-2 production by using the *C. violaceum* CV026 biosensor (16). Mutant ACP5 produced PAI-2 autoinducer at levels similar to those produced by PAO1 (data not shown). At present, we do not have a clear explanation for the reduction in pigment formation by mutant ACP5, but both rhamnolipid production and pyocyanine production are restored upon introduction of a functional *rhlG* gene in plasmid pJC3 (Table 2).

P. aeruginosa is known to produce PHA by using fatty acids from the de novo synthesis as precursors (30). The production of total PHA is reduced in mutant ACP5 at 24 h of growth (Table 2), but reaches the same level as PAO1 after 96 h of growth (data not shown). This defect in PHA synthesis can be observed in the electron micrographs taken after 24 h of growth as a decrease in the number and size of granules in mutant ACP5 (Fig. 5). This deficiency is due to *rhlG* inactivation, since plasmid pJC3 restores PHA production (Table 2). These results suggest that RhlG plays a role in biosynthesis of fatty acids used as substrates for PHA production, but that it is not an absolute requirement.

These findings suggested the existence in PAO1 of other reductases involved in PHA production. As mentioned above, RhlG is homologous to PhaB proteins (Fig. 3), so we decided to search in the *Pseudomonas* Genome Project for PhaB homologs. We found that contig 983 contains an ORF coding for a protein with amino acids 30% identical to those of PhaB from *Acinetobacter* sp. strain RA3849 and 26% identical to those of RhlG from *P. aeruginosa* PAO1. It is very likely that the detected PHA synthesis in mutant ACP5 is due to the presence of an alternative pathway in which the reduction step is catalyzed by the putative acetoacetyl-CoA reductase encoded by the PAO1 *phbB* gene. Since this enzyme is expected to be used in polyhydroxybutanoyl synthesis, it would be interesting to determine whether the lengths of the fatty acid moiety of PHAs produced by mutant ACP5 are different from those of the PHAs produced by the wild-type strain, PAO1.

Plasmid pJC4, which contains 7 kb of the W51D chromosome, including the *rhlG* gene (Table 1), complemented in *trans* mutant ACP5 for rhamnolipid and pigment production and PHA synthesis (Table 2 and Fig. 5), suggesting that this gene has the same function in rhamnolipid and PHA synthesis in both *P. aeruginosa* strains.

Regulation of *rhlG* expression in *P. aeruginosa* PAO1. To obtain additional evidence in support of the involvement of the RhlG protein in rhamnolipid and PHA synthesis, the concentration of *rhlG* mRNA was quantified under different culture conditions. The maximum *rhlG* mRNA concentration is found under conditions in which rhamnolipid production is maximum (that is, the stationary phase of growth on PPGAS medium) (Table 3), but there is also considerable expression when bacteria are grown for 48 h on LB or MM + gluconate medium (Table 3). It is important to point out that in the latter medium, PAO1 also produced rhamnolipids (37 μ g/ml after 24 h of growth). The level of expression of the *rhlG* gene in the exponential phase of growth was low under all culture conditions studied (Table 3). These results provide additional evidence of the involvement of RhlG in the production of secondary metabolites, such as rhamnolipids and PHA.

The DNA sequence of the *rhlG* promoter region suggested that the *rhlG* gene was regulated at the transcriptional level by one of the two LuxR homologs forming part of the quorum-sensing type of response in *P. aeruginosa*, LasR or RhlR. To obtain additional evidence in this respect, we determined the *rhlG* mRNA concentration of the PAO R1 strain (a PAO1 *lasR* mutant) grown on PPGAS medium. We used this mutant be-

TABLE 3. Relative concentration of *rhlG* mRNA on different media and time of growth^a

Strain	mRNA concn with growth time given ^a					
	PPGAS		LB		MM + gluconate	
	6 h	48 h	6 h	48 h	6 h	48 h
PAO1	45	417	35	209	24	248
PAO R1	— ^b	365	ND ^c	ND	ND	ND

^a mRNA concentration is expressed as the ratio of the RNA hybridization detected by autoradiography scanning to total RNA concentration used in the experiment.

^b —, not detected.

^c ND, not determined.

cause it has been reported to be defective in both quorum-sensing regulatory circuits present in *P. aeruginosa* (12, 24). Table 2 shows that in agreement with these observations, PAO R1 lacks rhamnolipid and pyocyanine production when grown on PPGAS medium for 48 h. Unexpectedly, the level of PAO R1 *rhlG* mRNA concentration after 48 h of growth on PPGAS is only slightly lower than that of the wild-type PAO1 strain (Table 3), thus ruling out the direct involvement of LasR as the transcriptional activator of the *rhlG* gene. It is still possible that the RhlR protein activates *rhlG* transcription, since it has been shown that *rhlR* mRNA is expressed at a significant level in the PAO R1 mutant (24).

This is the first report of the existence in *P. aeruginosa* of a ramification of the fatty acid biosynthetic pathway specifically involved in rhamnolipid production. Figure 1 shows the proposed role of RhlG protein in the rhamnolipid biosynthesis pathway. At present, we do not know whether the RhlG substrate is β -ketoacyl linked to ACP or to CoA. Our model (Fig. 1) shows the substrate to be β -ketoacyl-ACP, because most of the RhlG homologs are FabG-like enzymes (Fig. 3). We propose that CoA- β -hydroxyacids are the precursors of rhamnolipids, since the PHA synthases only use as a substrate the CoA-linked fatty acids (31), and the lipid moiety of rhamnolipids (β -hydroxydecanoyl- β -hydroxydecanoate) seems to be the product of these enzymes.

In summary, a new gene, *rhlG*, involved in rhamnolipid biosynthesis has been identified. The deduced RhlG protein shows significant sequence homology with numerous NADPH-dependent ketoacyl reductases. Complementation studies and measurement of the *rhlG* mRNA suggest that the RhlG protein is required for rhamnolipid biosynthesis and can be used in PHA production, but is not necessary for fatty acid synthesis.

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REFERENCES

1. Alexeyev, M. F., I. N. Shokolenko, and T. P. Croughan. 1995. Improved antibiotic resistance gene cassettes and omega elements for *Escherichia coli* vector construction and in vitro deletion/insertion mutagenesis. *Gene* **160**: 63–67.
2. Brint, J. M., and D. E. Ohman. 1995. Synthesis of multiple exoproducts in *Pseudomonas aeruginosa* is under the control of RhlR-RhII, another set of regulators in strain PAO1 with homology to the autoinducer-responsive LuxR-LuxI family. *J. Bacteriol.* **177**:7155–7163.
3. Burger, M. M., L. Glaser, and R. M. Burton. 1963. The enzymatic synthesis of rhamnose-containing glycolipids by extracts of *Pseudomonas aeruginosa*. *J. Biol. Chem.* **238**:2595–2602.

4. Chandrasekaran, E. V., and J. N. Bemiller. 1980. Constituent analyses of glycosaminoglycans. *Methods Carbohydr. Chem.* **8**:89–96.
5. Costerton, J. W. 1980. *Pseudomonas aeruginosa* in nature and disease, p. 15–24. In C. D. Sabath (ed.), *Pseudomonas aeruginosa: the organism, diseases it causes and their treatment*. Hans Huber Publishers, Bern, Switzerland.
6. Cox, C. D. 1986. Role of pyocyanin in the acquisition of iron from transferrin. *Infect. Immun.* **52**:263–270.
7. Cronan, J. E., Jr., and C. O. Rock. 1996. Biosynthesis of membrane lipids, p. 612–636. In F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli and Salmonella: cellular and molecular biology*, 2nd ed. American Society for Microbiology, Washington, D.C.
8. Döring, G., M. Maier, E. Müller, B. Zoubair, B. Tümmler, and A. Kharazmi. 1987. Virulence factors of *Pseudomonas aeruginosa*. *Antibiot. Chemother.* **39**:136–148.
9. Dotson, G. D., I. A. Kaltashov, R. J. Cotter, and C. R. H. Raetz. 1998. Expression cloning of a *Pseudomonas* gene encoding a hydroxydecanoyl-acyl carrier protein-dependent UDP-GlcNAc acyltransferase. *J. Bacteriol.* **180**:330–337.
10. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissue. *J. Biol. Chem.* **226**:497–509.
11. Fuqua, W. C., S. C. Winans, and E. P. Greenberg. 1996. Census and consensus in bacterial ecosystems: the LuxR-LuxI family of quorum sensing transcriptional regulators. *Annu. Rev. Microbiol.* **50**:727–751.
12. Gambello, M. J., and B. H. Iglewski. 1991. Cloning and characterization of the *Pseudomonas aeruginosa lasR* gene, a transcriptional activator of elastase expression. *J. Bacteriol.* **173**:3000–3009.
13. Gervais, F. G., and G. R. Drapeau. 1992. Identification, cloning, and characterization of *rcsF*, a new regulator gene for exopolysaccharide synthesis that suppresses the division mutation *ftsZ84* in *Escherichia coli* K-12. *J. Bacteriol.* **174**:8016–8022.
14. Herman, D. C., Y. Zhang, and R. M. Miller. 1997. Rhamnolipid (biosurfactant) effects on cell aggregation and biodegradation of residual hexadecane under saturated flow conditions. *Appl. Environ. Microbiol.* **63**:3622–3627.
15. Hoang, T. T., and H. P. Schweizer. 1997. Fatty acid biosynthesis in *Pseudomonas aeruginosa*: cloning and characterization of the *fabAB* operon encoding β -hydroxyacyl-acyl carrier protein dehydratase (FabA) and β -ketoacyl-acyl carrier protein synthase I (FabB). *J. Bacteriol.* **179**:5326–5332.
16. McClean, K. H., M. K. Winson, L. Fish, A. Taylor, S. R. Chhabra, M. Camara, M. Daykin, J. H. Lamb, S. Swift, B. W. Bycroft, G. S. A. B. Stewart, and P. Williams. 1997. Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of *N*-acylhomoserine lactones. *Microbiology* **143**:3703–3711.
17. Miller, R. M. 1995. Biosurfactant-facilitated remediation of metal-contaminated soils. *Environ. Health Perspect.* **103**(Suppl.):59–62.
18. Morrison, W. R., and L. M. Smith. 1964. Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron fluoride-methanol. *J. Lipid Res.* **5**:600–608.
19. Nicas, T. I., and B. H. Iglewski. 1985. The contribution of exoproducts to virulence of *Pseudomonas aeruginosa*. *Can. J. Microbiol.* **31**:387–392.
20. Ochsner, U. A., and J. Reiser. 1995. Autoinducer-mediated regulation of rhamnolipid biosurfactant synthesis in *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. USA* **92**:6424–6428.
21. Olsen, R. H., G. DeBusscher, and W. R. McCombie. 1982. Development of broad-host-range vectors and gene banks: self-cloning of the *Pseudomonas aeruginosa* PAO chromosome. *J. Bacteriol.* **150**:60–69.
22. Pearson, J. P., K. M. Gray, L. Passador, K. D. Tucker, A. Eberhard, B. H. Iglewski, and P. Greenberg. 1994. Structure of the autoinducer required for the expression of *Pseudomonas aeruginosa* virulence genes. *Proc. Natl. Acad. Sci. USA* **91**:197–201.
23. Pearson, J. P., L. Passador, B. H. Iglewski, and P. Greenberg. 1995. A second *N*-acylhomoserine lactone signal produced by *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. USA* **92**:1490–1494.
24. Pearson, J. P., E. C. Pesci, and B. H. Iglewski. 1997. Roles of *Pseudomonas aeruginosa las* and *rhl* quorum-sensing systems in control of elastase and rhamnolipid biosynthesis genes. *J. Bacteriol.* **179**:5756–5767.
25. Quandt, J., and M. F. Hynes. 1993. Versatile suicide vectors which allow direct selection for gene replacement in Gram-negative bacteria. *Gene* **127**:15–21.
26. Römling, U., J. Wingender, H. Müller, and B. Tümmler. 1994. A major *Pseudomonas aeruginosa* clone common to patients and aquatic habitats. *Appl. Environ. Microbiol.* **60**:1734–1738.
27. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
28. Soberón-Chávez, G., A. Haidour, J. L. Ramos, J. Campos, and J. Ortigoza. 1996. Selection and preliminary characterization of a *Pseudomonas aeruginosa* strain mineralizing some isomers in a branched-chain dodecylbenzene sulfonate mixture. *World J. Microbiol. Biotechnol.* **12**:367–362.
29. Stanghellini, M. E., and R. M. Miller. 1997. Biosurfactants: their identity and potential efficiency in the biological control of zoospore plant pathogens. *Plant Dis.* **81**:4–12.
30. Timm, A., and A. Steinbüchel. 1990. Formation of polyesters consisting of medium-chain-length 3-hydroxyalkanoates acids from gluconate by *Pseudomonas aeruginosa* and other fluorescent pseudomonads. *Appl. Environ. Microbiol.* **56**:3360–3367.
31. Timm, A., and A. Steinbüchel. 1992. Cloning and molecular analysis of the poly(3-hydroxyalkanoates acid) gene locus of *Pseudomonas aeruginosa* PAO1. *Eur. J. Biochem.* **209**:15–30.
32. West, S. E. H., and B. H. Iglewski. 1988. Codon usage in *Pseudomonas aeruginosa*. *Nucleic Acids Res.* **16**:9323–9329.
33. West, S. E. H., H. P. Schweizer, C. Dall, A. K. Sample, and L. J. Runyen-Janecky. 1994. Construction of improved *Escherichia coli*-*Pseudomonas* shuttle vectors derived from pUC18/19 and sequence of the region required for their replication in *Pseudomonas aeruginosa*. *Gene* **128**:81–86.
34. Wild, M., A. D. Caro, R. M. Miller, and G. Soberón-Chavez. 1997. Selection and partial characterization of a *Pseudomonas aeruginosa* mono-rhamnolipid deficient mutant. *FEMS Microbiol. Lett.* **153**:279–285.