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RhlA Converts β-Hydroxyacyl-Acyl Carrier Protein Intermediates in Fatty Acid Synthesis to the β-Hydroxydecanoyl-β-Hydroxydecanoate Component of Rhamnolipids in *Pseudomonas aeruginosa*▽

Kun Zhu and Charles O. Rock*

Department of Infectious Diseases, St. Jude Children's Research Hospital, Memphis, Tennessee 38105

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Pseudomonas aeruginosa **secretes a rhamnolipid (RL) surfactant that functions in hydrophobic nutrient uptake, swarming motility, and pathogenesis. We show that RhlA supplies the acyl moieties for RL biosynthesis by competing with the enzymes of the type II fatty acid synthase (FASII) cycle for the β-hydroxyacyl-acyl carrier protein (ACP) pathway intermediates. Purified RhlA forms one molecule of β-hydroxydecanoyl-β-hydroxydecanoate from two molecules of β-hydroxydecanoyl-ACP and is the only enzyme required to generate the lipid component of RL. The acyl groups in RL are primarily β-hydroxydecanoyl, and in vitro, RhlA has a greater affinity for 10-carbon substrates, illustrating that RhlA functions as a molecular ruler that selectively extracts 10-carbon intermediates from FASII. Eliminating either FabA or FabI activity in *P. aeruginosa* increases RL production, illustrating that slowing down FASII allows RhlA to more-effectively compete for β-hydroxydecanoyl-ACP. In *Escherichia coli*, the rate of fatty acid synthesis increases 1.3-fold when RhlA is expressed, to ensure the continued formation of fatty acids destined for membrane phospholipid even though 24% of the carbon entering FASII is diverted to RL synthesis. Previous studies have placed a ketoreductase, called RhlG, before RhlA in the RL biosynthetic pathway; however, our experiments show that RhlG has no role in RL biosynthesis. We conclude that RhlA is necessary and sufficient to form the acyl moiety of RL and that the flux of carbon through FASII accelerates to support RL production and maintain a supply of acyl chains for phospholipid synthesis.**

Pseudomonas aeruginosa produces an extracellular glycolipid surfactant composed of β-D-(β-D-hydroxyalkanoyloxy)alkanoic acids (HAA) (primarily β-hydroxydecanoyl-β-hydroxydecanoate) derivatized with one- or two-rhamnose sugars (monorhamnolipid [RL1] and dirhamnolipid [RL2], respectively) (Fig. 1). Rhamnolipid production plays important roles in cell motility (2), the assimilation of hydrophobic carbon sources (22), and biofilm formation (6, 17). Rhamnolipid production is maximal in the stationary phase of growth, is transcriptionally regulated by quorum-sensing circuitry (24), and is posttranscriptionally modulated by the RsmA/RsmZ system (11). Several genes are essential for rhamnolipid biosynthesis, based on the lack of RL1/RL2 formation in mutant strains. The first genes identified were the *rhlAB* operon, and both genes are essential for rhamnolipid formation (23). The idea presented in the first study was that *rhlAB* gene products formed the rhamnosyltransferase I complex involving an RhlAB heterodimer (23). A closer analysis revealed that while both *rhlA* and *rhlB* mutants fail to produce rhamnolipids, *rhlA* mutants did produce HAA, indicating that RhlA is required for the formation of the HAA portion of the molecule and assigning RhlB a role as the rhamnosyl transferase (8). The *rhlC* gene encodes rhamnosyltransferase II, which is responsible for the addition of the second rhamnosyl group to form RL2, based on the observation that *rhlC* knockout strains produce only RL1 (25).

The sequence similarity of RhlA to transacylases, such as PhaG (26), which is involved in poly-β-hydroxyalkanoate (PHA) biosynthesis, led to the proposal that RhlA is a transacylase that catalyzes the transfer of β-hydroxydecanoyl moieties from acyl carrier protein (ACP) to coenzyme A (CoA) (8). This scheme places β-hydroxydecanoyl-CoA as a common intermediate in rhamnolipid and PHA synthesis and would mean that an acyltransferase is required to form HAA. The identification of the *rhlG* gene as essential for rhamnolipid production and the similarity of its product to the FabG of type II fatty acid synthase (FASII) led to the idea that this protein acted upstream of RhlA in reducing a β-ketoacyl-ACP intermediate (3, 21, 30). However, the in vitro activity and crystal structure of RhlG are not consistent with a role for the interaction of this enzyme with the fatty acid biosynthetic pathway (19). In light of the potential utility of rhamnolipids in industrial applications, such as enhanced oil recovery and bioremediation (34), it is important to understand the pathway leading to their biosynthesis in order to genetically engineer efficient rhamnolipid producer strains.

Although RhlA is clearly a necessary step in the formation of the lipid moiety of rhamnolipid, the precise biochemical function of the *rhlA* gene product remains unclear. The stereochemistry of the β-hydroxyacids in HAA matches that of the intermediates in fatty acid biosynthesis, as opposed to that of the intermediates in fatty acid β-oxidation (Fig. 1), suggesting that fatty acid synthesis may be the source for the HAA. However, it is not known if RhlA is responsible for the diversion of the intermediates from the biosynthetic pathway via a transacylation reaction to CoA or the acyltransferase activity that forms HAA. The goal of this work was to assign the role of RhlA in

* Corresponding author. Mailing address: Department of Infectious Diseases, St. Jude Children's Research Hospital, 332 N. Lauderdale, Memphis, TN 38105-2794. Phone: (901) 495-3491. Fax: (901) 495-0399. E-mail: charles.rock@stjude.org.

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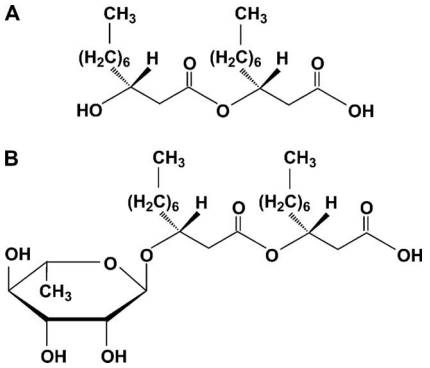

FIG. 1. Chemical structures of HAA and RL1. (A) Structure of HAA illustrated with the most abundant HAA found in extracellular rhamnolipids, β-hydroxydecanoyl-β-hydroxydecanoate. The stereochemistry of the chiral center of the β-hydroxyacids is the same as that found in the β-hydroxy intermediates in fatty acid biosynthesis. (B) Chemical structure of RL1 (L-rhamnosyl-HAA) illustrating the site of sugar attachment to HAA. RL2 has a second rhamnosyl group attached to the rhamnose of RL1 (9).

rhamnolipid biosynthesis based on the biochemical properties of the purified RhlA protein and the products produced by heterologous expression in an *Escherichia coli* host. These experiments show that RhlA directly utilizes β-hydroxydecanoyl-ACP intermediates in fatty acid synthesis to generate the HAA portion of rhamnolipids.

MATERIALS AND METHODS

Materials. [1-^{14}C]acetate (specific activity, 56 mCi/mmol), β-hydroxy[1-^{14}C]decanoyl-CoA (specific activity, 55 Ci/mol), and [1-^{14}C]octanoyl-CoA (specific activity, 50 mCi/mmol) were obtained from American Radiolabeled Chemicals, Inc., and [2-^{14}C]malonyl-CoA (specific activity, 52 mCi/mmol) was obtained from Amersham Pharmacia Biotech. ACP and the His-tagged fatty acid biosynthetic enzymes were purified by affinity chromatography followed by gel filtration chromatography as described previously (5, 10). All other chemicals were reagent grade or better.

Construction of mutants and plasmids. The sources for the strains and plasmids are given in Table 1. The *P. aeruginosa* strain PA14 Δ*rhlG* deletion mutant was created by gene replacement technology described previously (35). Primers p-upf (5′-TACAAAAAAGCAGGCTATGCATCCCTATTTCAGTCTCG) and p-upr (5′-TCAGAGCGCTTTTGAAGCTAATTCGGAGCTGCATGACCTTTTCCCAG) were used to amplify the 345-bp fragment at the 5′ end of *rhlG*. Primers p-dnf (5′-AGGAACTTCAAGATCCCCAATTCGGCGAACAAGCCTATGCCTACGG) and p-dnr (5′-TACAAGAAAGCTGGGTAGAGATGAAAACCGCCGTCGAT) were used to amplify the 302-bp fragment at the 3′ end of *rhlG*. The four primers used for the insertion of the gentamicin resistance cassette between the above two fragments were described previously (4, 35). The genotyping primers were p1L, 5′-GGCTTCGTCGAGCACTACCTGT; p1R, 5′-AGACATGGCTGACCTGCTCCAG; p2L, 5′-ATGCATCCCTATTTCAGCCTC; p2R, 5′-TCAGAGATGAAAACCGCCGT; p3L, GTCTTCATCTGCGCACGTGACG; and p3R, 5′-GCCAGCATCCGCGACAGTTGAT. A fragment harboring Δ*rhlG*::Gm (containing a 120-bp deletion in *rhlG* beginning at nucleotide 346) was constructed by splicing overlap extension PCR and cloned into the suicide vector pEX18ApGW to yield pKZ001. This recombinant plasmid was conjugated from *E. coli* SM10-λpir into strain PA14 with selection on a *Pseudomonas* isolation agar plate (Becton, Dickinson and Company) containing gentamicin (50 μg/ml) and carbenicillin (150 μg/ml). Merodiploids formed via a single crossover event were resolved through 5% sucrose selection in the presence of gentamicin. The Gm marker was subsequently removed by Flp recombinase, and the Flp recombinase target, FRT (85 bp), was left on the chromosome. The *rhlG* deletion in strain KZ1 was verified by PCR utilizing genotyping primers located outside and inside *rhlG*. *P. aeruginosa* PAO1 strain KZ2 (*rhlG*::Gm) was created by using the same method except that the Gm marker was not excised by Flp recombinase. Swarming plates were prepared with M8 minimal medium (6 g Na_2HPO_4, 2 g KH_2PO_4, 0.5 g NaCl per liter) supplemented with $MgSO_4$ (1 mM), glucose (0.2%), and Casamino Acids (0.5%) and solidified with 0.6% agar (2).

The coding region of *rhlA* from strain PA14 was amplified by PCR and cloned into pET28 (Novagen) NdeI/EcoRI restriction sites to yield an inducible *rhlA* expression plasmid, pKZ002. Plasmid pPJ-kana was derived from pBluescript II KS$^+$ by replacing the ampicillin resistance cassette with a kanamycin resistance cassette. The DNA fragment containing the *rhlA* gene was recovered from pET28-*rhlA* by using XbaI/EcoRI and cloned into pPJ-kana to give pKZ003. The coding region of the *rhlB* gene in strain PA14 was amplified by PCR and cloned into pBluescript II KS$^+$ EcoRI/HindIII sites to yield pKZ004.

HAA and rhamnolipid quantitation. The amounts of HAA and rhamnolipid in lipid extracts from cell culture supernatants were determined by thin-layer chromatography coupled with flame ionization detection using an Iatroscan MK-5 instrument. *P. aeruginosa* and *E. coli* strains were grown in M9 medium at 30°C to stationary phase, and cell-free supernatants were collected by centrifugation and filtered through 0.22-μm membranes. Radioactive HAA was prepared from *E. coli* strain BL21(DE3) with pKZ003, grown to 5×10^8 cells/ml and labeled with [1-^{14}C]acetate for 2 h before the cells were harvested. The supernatant was acidified to pH 2.0 and extracted twice with chloroform-methanol (2/1, vol/vol), and the lower organic phase was evaporated to dryness and resuspended in methanol. A 1-μl aliquot of the lipid extract was analyzed on a silica gel-coated rod (Iatron Laboratories) developed with chloroform-methanol-acetic acid (90/10/2, vol/vol/vol) to separate HAA, RL1, and RL2. The amount of each component was determined by comparing the signal from the flame ionization mass detector for each individual component of the lipid extract with a standard curve prepared from β-hydroxydecanoic acid (Matreya, LLC). The fatty acid composition of HAA samples was determined by isolating HAA by thin-layer chromatography and converting them to fatty acid methyl esters by using HCl/methanol. The fatty acid methyl esters were identified and quantitated by using a Hewlett-Packard model 5890 gas chromatograph. The production of rhamnolipid in the culture medium was measured by anthrone colorimetric reaction (33). Briefly, a 100-μl aliquot of the culture supernatant was mixed with 1 ml of anthrone reagent (0.1% in 70% H_2SO_4) and incubated at 80°C for 30 min. After the mixture was cooled to 25°C, the absorbance was read at 625 nm. The rhamnose concentration was obtained by using a rhamnose standard curve.

Detection of HAA and rhamnolipid with MS. Mass spectrometry (MS) of rhamnolipid and HAA was performed by the Hartwell Center for Bioinformatics and Biotechnology at St. Jude Children's Research Hospital. The samples were resuspended in 100% methanol. MS analysis was performed by using a Finnigan TSQ Quantum (Thermo Electron, San Jose, CA) triple quadrupole MS equipped with a nanospray ion source. The instrument was operated in the negative-ion mode using MS (Q1) scanning. The ion source parameters were a spray voltage of 2,000 V, capillary temperature of 100°C, and capillary offset of

TABLE 1. Bacterial strains and plasmids

Strain or plasmid[a]	Relevant property	Source
P. aeruginosa strains		
PA14	Wild type	18
PAO1	Wild type	35
KZ1 (Δ*rhlG*)	*rhlG* deletion in PA14	This study
P32151 (*rhlG*::Tn)	Transposon insertion in PA14	18
KZ4 (*rhlA*::Gm)	*rhlA* disruption in PA14	2
P39482 (*fabI*::Tn)	Transposon insertion in PA14	18
KZ2 (*rhlG*::Gm)	*rhlG* disruption in PAO1	This study
KZ3 (*rhlG*::Tn)	Transposon insertion in PAO1	15
PAO652 (Δ*fabA*)	*fabA* deletion in PAO1	35
E. coli strains		
UB1005	Wild-type laboratory strain	14
BL21(DE3)	Protein expression strain	Novagen
SM10-λpir	DNA mobilizer strain	12
Plasmids		
pEX18ApGW	*P. aeruginosa* suicide vector	4
pKZ001	*rhlG* deletion vector	This study
pKZ002	*rhlA* in pET28	This study
pKZ003	*rhlA* in pBluescript	This study
pKZ004	*rhlB* in pBluescript	This study

[a] Gm, gentamicin; Tn, transposon.

−35 V, and the tube lens offset was set by infusion of polytyrosine tuning and calibration solution (Thermo Electron, San Jose, CA) in electrospray mode. The MS acquisition parameters for Q1 were a scan range of 50 to 800 *m/z*, scan time of 0.65 s, and peak width for Q1 of 0.7 full width at half maximum. Instrument control and data acquisition were performed with Finnigan Xcalibur (version 1.4 SR1) software (Thermo Electron, San Jose, CA).

RhlA purification and assay. The expression of recombinant RhlA protein with an N-terminal His-tag encoded by plasmid pKZ002 was induced with isopropyl-1-thio-β-D-galactopyranoside (IPTG) in *E. coli* BL21(DE3). Cells were collected by centrifugation, resuspended in MCAC buffer (20 mM Tris-HCl, pH 7.9, 500 mM NaCl, 10% glycerol) and lysed with a French press. Soluble proteins were applied to a Ni^{2+}-nitrilotriacetic acid agarose (Qiagen) column and washed with MCAC buffer plus 40 mM imidazole. His-tagged RhlA was eluted with MCAC buffer containing 200 mM imidazole. The fractions containing most of the RhlA protein were pooled, concentrated, and applied to a Superdex S200 column (GE Healthcare) to purify RhlA to homogeneity in a buffer of 20 mM Tris-HCl, pH 7.4, 1 mM dithiothreitol, 50 mM EDTA. Intact MS gave a molecular weight of 34,964, positively identifying the protein as His-tagged RhlA lacking the N-terminal fMet amino acid.

The RhlA activity was determined by measuring the formation of [^{14}C]HAA by using thin-layer chromatography. The standard assay mixture contained 100 μM *E. coli* ACP, 1 mM β-mercaptoethanol, 200 μM malonyl-CoA, 40 μM [1-^{14}C]octanoyl-CoA, 100 μM NADPH, 2 μg *E. coli* FabD, 0.2 μg *Mycobacterium tuberculosis* FabH, 1 μg *E. coli* FabG, 0.1 M sodium phosphate buffer, pH 7.0, and 0.5 μg of RhlA in a final volume of 120 μl. *M. tuberculosis* FabH was used because of its ability to utilize 6- to 12-carbon acyl-CoA thioesters as primers to generate the β-ketoacyl-ACP substrate for FabG. The ACP, β-mercaptoethanol, and buffer were preincubated at 37°C for 30 min to ensure the complete reduction of ACP. The substrate for the RhlA reaction was generated by using FabD to transfer the malonyl group from CoA to *E. coli* ACP to produce malonyl-ACP and *M. tuberculosis* FabH to condense octanoyl-CoA and malonyl-ACP to form β-ketodecanoyl-ACP, followed by its reduction to β-hydroxydecanoyl-ACP by FabG. The reaction was initiated by the addition of RhlA. The RhlA substrate specificity was determined by using the same activity assay containing acyl-CoA primers of different chain lengths, except that the labeled substrate in each case was [2-^{14}C]malonyl-CoA. The reactions were stopped with 2 ml of water, acidified to pH 2.0, and extracted twice with chloroform-methanol (2/1, vol/vol). The lower organic phase was evaporated to dryness and resuspended in 50 μl of methanol. The extract was analyzed by using silica gel H layers (Analtech) developed with chloroform-methanol-acetic acid (90/10/2, vol/vol/vol). The distribution of radioactivity on the plate or gel was determined and quantified by using a Typhoon 9200 PhosphorImager.

RESULTS

Production of HAA and rhamnolipid in *E. coli* cells. The RhlA and RhlB proteins were expressed in *E. coli* cells to confirm that these two gene products were sufficient for RL1 production. The expression of RhlA from plasmid pKZ003 in BL21(DE3) led to the accumulation of an extracellular lipid identified as HAA based on its migration in thin-layer chromatography on silica gel-coated rods (Fig. 2A). Accordingly, base-catalyzed hydrolysis of the HAA gave rise to a more-slowly migrating band that comigrated with β-hydroxydecanoic acid. MS of the HAA sample confirmed this identification, showing a prominent peak at *m/z* 357 corresponding to an HAA consisting of two β-hydroxydecanoates and minor peaks with molecular masses indicating the presence of a dimer between either an 8- and a 10-carbon or a 10- and a 12-carbon hydroxyl fatty acid (not shown). The HAA fraction was purified by thin-layer chromatography and analyzed by gas chromatography of the derived methyl esters (Fig. 2B). The HAA was composed primarily of β-hydroxydecanoic acid (95%) and β-hydroxydodecanoic acid (4%). Thus, HAA was the product of RhlA expression in culture supernatants.

The sugar moiety of rhamnolipid is derived from dTDP-L-rhamnose, and *E. coli* contains an operon that directs dTDP-L-rhamnose synthesis (23, 31). The coexpression of RhlB and RhlA led to the appearance of both HAA and RL1 in the culture supernatants (Fig. 2C) (Table 2). MS confirmed that the new compound formed in the presence of RhlB was RL1 consisting primarily of two β-hydroxydecanoyl moieties and one rhamnose (*m/z* = 503) (not shown). The total amount of HAA produced by *E. coli* strain BL21 expressing RhlA was less than the total HAA (HAA plus RL1 plus RL2) produced by wild-type *P. aeruginosa* strains PA14 and PAO1, even when RhlA expression was induced by using plasmid pKZ002 (Table

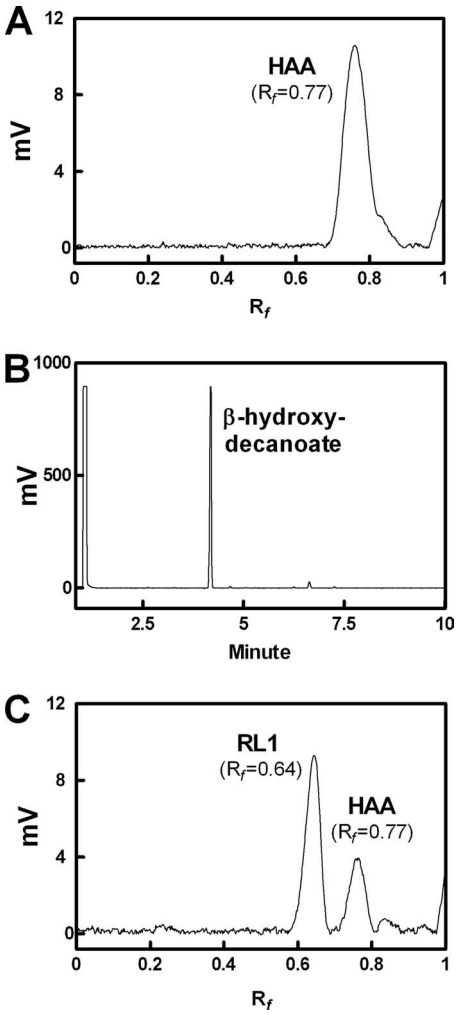

FIG. 2. Extracellular lipids produced by RhlA or RhlA and RhlB expression in *E. coli*. (A) Lipids were extracted from the culture supernatant of strain BL21/pKZ003 expressing RhlA and quantitated by thin-layer chromatography using an Iatroscan MK-5 flame-ionization detector as described in Materials and Methods. A single lipid class (R_f = 0.77) was identified as HAA (see the text). (B) The HAA band from the supernatant of strain BL21/pKZ003 was recovered from the thin-layer plate, and fatty acid methyl esters were prepared and analyzed by gas-liquid chromatography. The methyl ester of β-hydroxydecanoate was the predominant species (95%), with a minor peak of methyl-β-hydroxydodecanoate (4%) also detected at 6.6 min. (C) Lipid composition of the culture supernatant from strain BL21/pKZ003/pKZ004 expressing both RhlA and RhlB. The two lipid classes separated on thin-layer chromatography rods and detected with an Iatroscan MK-5 flame ionization detector were identified as HAA (R_f = 0.77) and RL1 (R_f = 0.64).

TABLE 2. HAA and rhamnolipid production[a]

Strain and/or plasmid(s)	Amt (μg/ml) of:			
	HAA	RL1	RL2	Total
P. aeruginosa strains				
PA14	2.5 ± 0.3	36.3 ± 4.3	144 ± 14	183
P39482 (*fabI*::Tn)	16.4 ± 4.5	45.9 ± 7.1	187 ± 10	249
PAO1	5.9 ± 1.4	18.0 ± 6.6	169 ± 31	193
PAO652 (Δ*fabA*)	4.0 ± 2.7	41.7 ± 4.2	255 ± 18	300
E. coli strain BL21(DE3)	<0.1	<0.1	<0.1	<0.1
Plasmids				
pKZ002	169 ± 11	<0.1	<0.1	169
pKZ002 and pKZ004	122 ± 9	30.8 ± 6.6	<0.1	153
pKZ003	36.1 ± 2.9	<0.1	<0.1	36.1
pKZ003 and pKZ004	8.7 ± 1.4	19.2 ± 3.5	<0.1	27.9
E. coli strain UB1005 (wild type)	<0.1	<0.1	<0.1	<0.1
Plasmids				
pKZ003 and pKZ004	1.2 ± 0.2	0.8 ± 0.2	<0.1	2.0

[a] The indicated strains of *P. aeruginosa* and *E. coli* bacteria were grown in M9 minimal salts medium with 0.4% glycerol as carbon source (20, 28) to a density of 5×10^8 cells/ml, the cell-free medium was extracted, and the concentration of each component was quantitated by flame ionization detection following their separation by thin-layer chromatography as described in Materials and Methods. Tn, transposon.

2). When RhlA and RhlB were expressed from the same promoter, the production of HAA was reduced due to the lower expression of RhlA (pKZ003), but the HAA formed was efficiently converted to RL1 (Table 2). Strain BL21 (*E. coli* B) produced significantly higher levels of HAA and RL1 than strain UB1005 (*E. coli* K-12) (Table 2). The production of RL1 by *E. coli* K-12 strain W3110 expressing RhlAB was reported previously (1), and the reason for this difference between the formation of HAA and RL1 in *E. coli* K-12 and B strains harboring the same plasmids was not apparent.

RhlA enzymatic activity. It was not clear why the *E. coli* B strain permits HAA production while the K-12 derivative does not, but one possibility was that strain BL21 possessed an enzyme, such as an acyl-ACP:CoA transacylase, that was not present in K-12 strains and was required to supply substrates to RhlA. Therefore, an in vitro assay system was designed to determine whether RhlA was responsible for the diversion of fatty acid biosynthetic intermediates to HAA formation. His-tagged RhlA was purified to homogeneity by Ni^{2+}-affinity chromatography followed by Superdex 200 gel filtration chromatography (Fig. 3A). Sodium dodecyl sulfate-gel electrophoresis revealed the presence of a 34-kDa protein, consistent with the subunit molecular mass predicted from the amino acid sequence of the recombinant RhlA (Fig. 3A, right inset). The elution volume of RhlA on the gel filtration column suggested that RhlA is a monomer in solution, based on the calibration of the column with globular protein standards (Fig. 3A, left inset). The intact-mass spectrum of the purified protein gave a molecular weight of 34,964, corresponding to the His-tagged RhlA protein minus the amino-terminal fMet residue (Fig. 3B). A coupled system was used to assess RhlA activity in vitro. First, β-[^{14}C]hydroxydecanoyl-ACP was prepared using [^{14}C]octanoyl-CoA, malonyl-CoA, *E. coli* FabD, *M. tuberculosis* FabH, and *E. coli* FabG. The presence of the β-hydroxydecanoyl-ACP substrate was detected by gel electrophoresis, and this radiolabeled band was reduced when RhlA was added to the reaction mixture, indicating that β-hydroxydecanoyl ACP was a substrate for RhlA (not shown). The formation of [^{14}C]HAA was monitored by thin-layer chromatography (Fig. 4A). HAA synthesis required the presence of RhlA and depended on the formation of β-hydroxydecanoyl-ACP by FabH/FabG. The HAA formed in vitro had the same R_f (0.82) as the ^{14}C-labeled HAA isolated from the medium of strain BL21/pKZ003 (Fig. 4A, lane 5). Thus, purified RhlA was the only

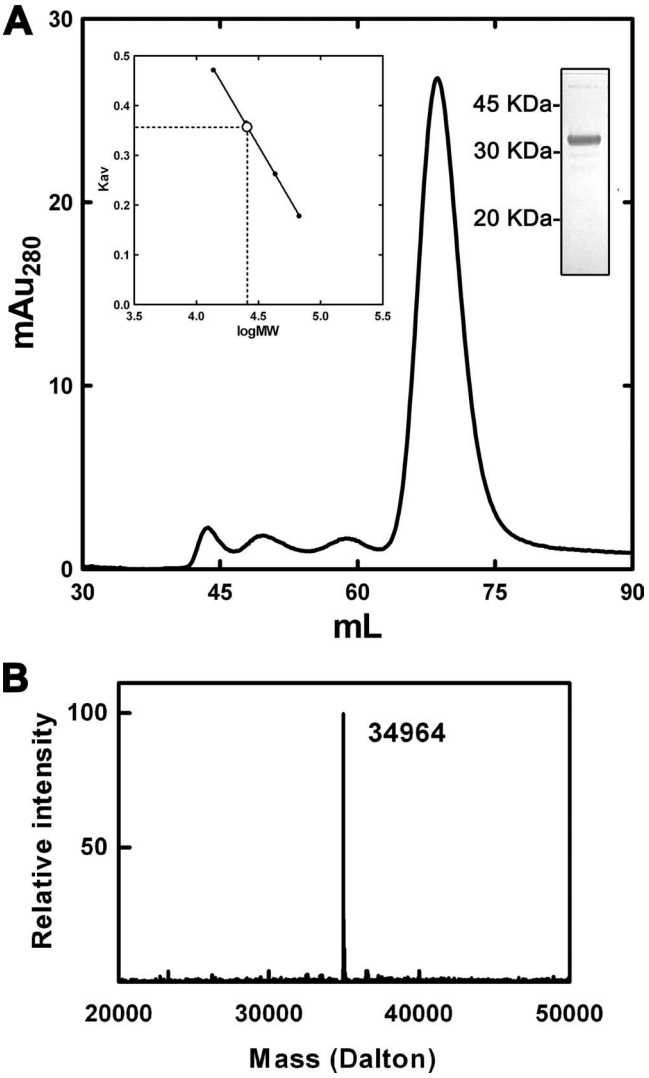

FIG. 3. Purification of N-terminal His-tagged recombinant RhlA. (A) RhlA was purified by affinity chromatography followed by gel filtration on a Superdex 200 column as illustrated in the figure. Purified His-tagged RhlA migrated with an apparent molecular mass of 34 kDa as determined by sodium dodecyl sulfate-gel electrophoresis (right inset). The calculated molecular mass of RhlA in solution based on the calibration of the column with globular protein standards was 26 kDa (left inset), indicating that RhlA is a monomer. mAu_{280}, milli-absorption unit at 280 nm; Kav, distribution coefficient. (B) Intact-mass spectrum of His-tagged RhlA.

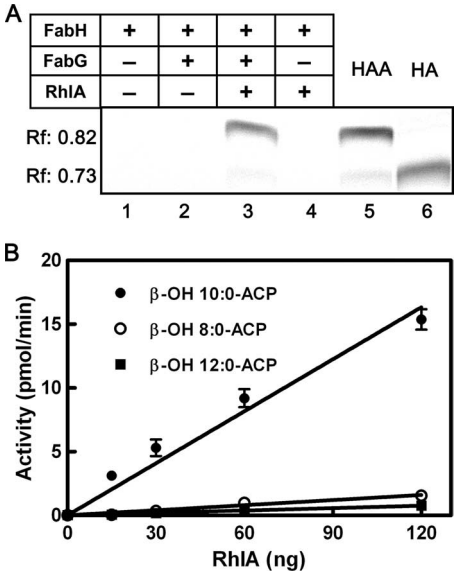

FIG. 4. Enzymatic activity and substrate specificity of RhlA. (A) The substrate β-hydroxydecanoyl-ACP was generated using *M. tuberculosis* FabH, malonyl-ACP, octanoyl-CoA, and FabG as described in Materials and Methods. Results of thin-layer chromatography analysis of the RhlA reaction product in a solvent system of chloroform-methanol-acetic acid (90/10/2, vol/vol/vol) are shown. The solvent migration was from the bottom to the top, and only the cross-section of the autoradiogram that contained labeled material is shown, with the relative motilities of the bands indicated at the left. Lane 5, β-(β-hydroxydecanoyl)decanoic acid, HAA produced by *E. coli* BL21/pKZ003; lane 6, β-hydroxydecanoic acid, HA made from HAA by base-catalyzed hydrolysis comigrated with the β-hydroxydecanoic acid standard (Matreya, LLC). +, present; −, absent. (B) Substrate specificity of RhlA. Substrates β-hydroxyoctanoyl-ACP, β-hydroxydecanoyl-ACP, and β-hydroxydodecanoyl-ACP were synthesized using *M. tuberculosis* FabH, [2-^{14}C]malonyl-ACP, FabG, and hexanoyl-CoA, octanoyl-CoA, or decanoyl-CoA as described in Materials and Methods. The products of the reactions were extracted from the mixture and analyzed by thin-layer chromatography analysis on silica gel G layers developed with chloroform-methanol-acetic acid (90/10/2, vol/vol/vol). The amount of [^{14}C]HAA formed in the reaction was determined by using a PhosphorImager calibrated with a [^{14}C]malonyl-CoA curve. The specific activities were calculated from the slopes. Error bars show standard deviations.

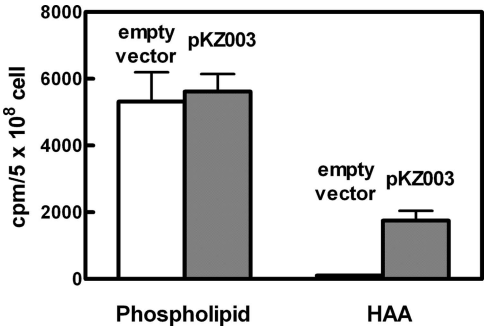

FIG. 5. Acetate incorporation in *E. coli* cells expressing RhlA. Strain BL21 harboring either pKZ003 (*rhlA*) or the empty control vector was grown to a density of 5×10^8 cells/ml and labeled with [^{14}C]acetate for 1 h. The labeled acetate incorporated into phospholipids and the HAA secreted from cells were quantitated by liquid scintillation counting of the organic extracts as described in Materials and Methods. Error bars show standard deviations.

enzyme required to produce HAA from β-hydroxydecanoyl-ACP. We did not detect the formation of HAA by RhlA using β-hydroxydecanoyl-CoA as the substrate at RhlA concentrations up to 10 times higher than we used to detect robust RhlA activity with the β-hydroxydecanoyl-ACP substrate.

The results of our work and that of others (8) showed that 10-carbon fatty acids were the most-abundant constituents in the HAA moiety of rhamnolipids. These data suggested that RhlA functioned as a molecular ruler to divert 10-carbon intermediates to HAA. Accordingly, RhlA exhibited a high degree of substrate selectivity in vitro (Fig. 4B). The highest activity was obtained with β-hydroxydecanoyl-ACP (136.0 ± 5.9 [mean ± standard deviation] pmol/min/μg), with the activity falling off sharply for either two carbons shorter (β-hydroxyoctanoyl-ACP; 9.8% of the level of β-hydroxydecanoyl-ACP) or two carbons longer (β-hydroxydodecanoyl-ACP; 4.7% of the level of β-hydroxydecanoyl-ACP) (Fig. 4B). Thus, the substrate specificity of RhlA accounted for the predominance of 10-carbon β-hydroxyacids characteristic of rhamnolipids produced in vivo (7, 16).

Coupling of fatty acid and rhamnolipid synthesis. The rates of fatty acid synthesis were measured by determining the incorporation of exogenous [^{14}C]acetate into total intracellular and extracellular lipids to determine if the diversion of fatty acids to rhamnolipid formation impacted the production of acyl chains destined for phospholipids. The [^{14}C]acetate incorporation into phospholipid fatty acids in the HAA-producing strain BL21/pKZ003 was not reduced in comparison to that in strain BL21 harboring the empty vector, although 24% of the [^{14}C]acetate was used in the formation of extracellular HAA in the strain expressing RhlA (Fig. 5). Thus, membrane homeostasis in HAA-producing strains was maintained by the acceleration of de novo fatty acid synthesis to compensate for the diversion of acyl chains to HAA. There are two enzymes in the pathway (FabA and FabI) that impact the levels of β-hydroxydecanoyl-ACP and, therefore, the ability of RhlA to compete for this intermediate. FabA is responsible for the conversion of this intermediate to enoyl-ACP, but this enzyme catalyzes the rapid equilibrium between the β-hydroxy and *trans*-2 intermediates, and the activity of FabI is critical to pull the β-hydroxyacyl-ACP to acyl-ACP (10). In *P. aeruginosa*, neither of these genes is essential, but we surmised that their inactivation may slow the rate of β-hydroxydecanoyl-ACP utilization by fatty acid synthesis and allow RhlA greater access to β-hydroxy intermediates. In accord with this idea, strain PAO652 (Δ*fabA*) produced 55% more HAA for rhamnolipid than strain PAO1 and strain P39482 (*fabI*::Tn) produced 36% more HAA for rhamnolipid than strain PA14 (Table 2). Strain PAO1 was more efficient at converting HAA to rhamnolipid than strain PA14. These data indicated that restricting the activity of the enzymes responsible for the conversion of β-hydroxyacyl-ACP to acyl-ACP allowed a higher percentage of the pathway intermediates to be diverted to rhamnolipid production.

RhlG is not involved in rhamnolipid production. Previous work identified the *rhlG* gene as an essential component for rhamnolipid formation based on the lack of extracellular rhamnolipid in *rhlG* deletion strains (3). These data led to the conclusion that RhlG is the enzyme responsible for draining the fatty acid precursors of rhamnolipids away from the central

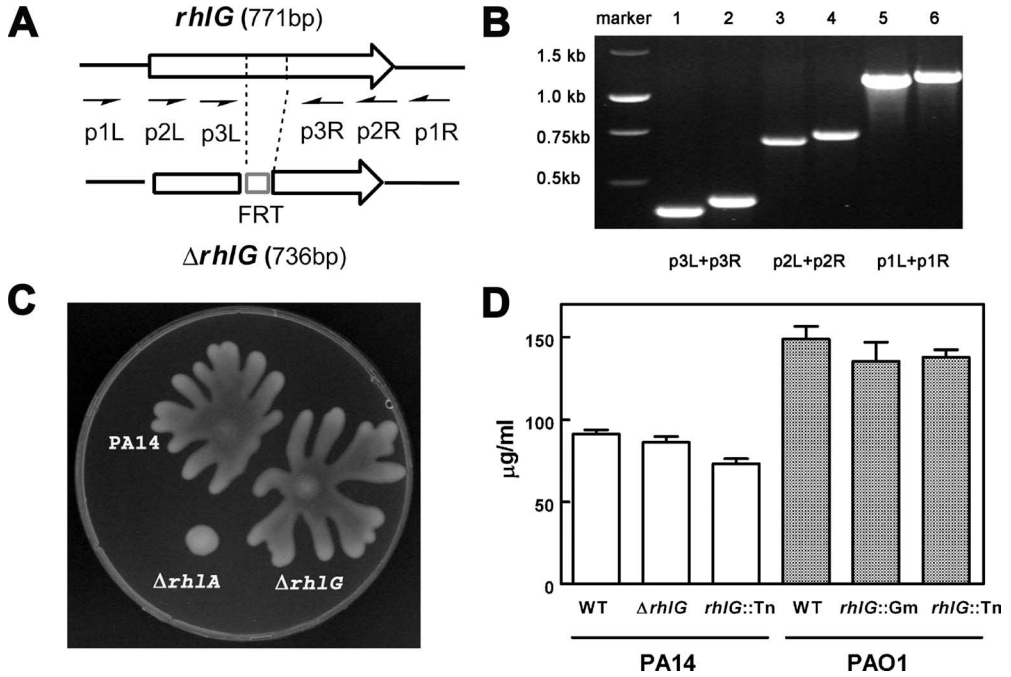

FIG. 6. RhlG does not have a role in rhamnolipid production. (A) The *P. aeruginosa* PA14 *rhlG* mutant had a deletion of 120 bp in the middle of *rhlG*, which was replaced by recombinase target FRT (85 bp). The detailed procedure is described in Materials and Methods. Primers, described for panel B, are shown. (B) PCR verification of the *rhlG* deletion. Lanes 1, 3, and 5, PA14 *rhlG* deletion mutant as the template; lanes 2, 4, and 6, wild-type PA14 as the template. Primers p1L and p1R were used to amplify a fragment of 1,171 bp covering *rhlG* and the flanking 200 bp at both sides. Primers p2L and p2R were used to amplify *rhlG* (771 bp). Primers p3L and p3R were used to amply an *rhlG* internal fragment (400 bp). (C) *P. aeruginosa* strains PA14, KZ4 (Δ*rhlA*), and KZ1 (Δ*rhlG*) were spotted on a swarming plate and grown at 37°C for 24 h. (D) Production of rhamnolipid in *P. aeruginosa* strains PA14 and PAO1 and four *rhlG* mutants. Rhamnolipid concentrations are expressed as concentrations of rhamnose (μg/ml), based on the anthrone colorimetric method for measuring rhamnose in the growth medium. The data were derived from three independent experiments. Strain KZ1 (Δ*rhlG*) generated in this study was used in all above experiments. The same procedure was used to generate PAO1 *rhlG*::Gm strain KZ2, except that the Gm resistance cassette was left on the chromosome. Strains PA14 (*rhlG*::Tn) and PAO1 (*rhlG*::Tn) were obtained from the *P. aeruginosa* transposon insertion libraries (15, 18). Error bars show standard deviations. WT, wild type.

type II fatty acid biosynthetic pathway (3, 21, 30). We investigated this connection by generating strain KZ1 in strain PA14 with the *rhlG* gene deleted as diagramed in Fig. 6A. The presence of the deletion was confirmed by PCR analysis of the genomic DNA (Fig. 6B). The Δ*rhlG* strain exhibited normal swarming ability, a property that is dependent on rhamnolipid secretion (Fig. 6C). Testing the strains on individual swarming plates gave the same result. We also constructed an *rhlG* deletion mutant of strain PAO1 (strain KZ2), and obtained *rhlG* transposon insertion strains in both strains PA14 and PAO1 from the *P. aeruginosa* mutant strain collections (15, 18). All of these knockouts produced a normal amount of extracellular rhamnolipid (Fig. 6D). We obtained strain ACP5, the putative *rhlG* knockout mutant of strain PAO1 that was used to show the connection between RhlG and rhamnolipid production (3). Although strain ACP5 had the expected tetracycline resistance, it had a wild-type *rhlG* gene based on DNA sequencing and secreted normal amounts of extracellular rhamnolipid. In addition, the biochemical analysis of the purified RhlG showed that this enzyme did not prefer ACP thioesters as a substrate (19).

DISCUSSION

Our experiments lead to a simplified model for the production of rhamnolipids in *P. aeruginosa* (Fig. 7). RhlA is responsible for intercepting the β-hydroxydecanoyl-ACPs from the FASII cycle and directly converting them to the HAA moiety of the rhamnolipids without the involvement of an intermediate, such as acyl-CoA, or the participation of another protein. RL1 and RL2 are then formed by the sequential addition of rhamnose moieties to HAA by RhlB and RhlC, respectively. The fact that the expression of RhlA in *E. coli* is necessary and sufficient for the detection of extracellular HAA (Table 2) (1) suggests that RhlA is the only protein required for HAA formation in vivo, but the presence of ancillary proteins that facilitate the diversion of acyl groups from fatty acid synthesis cannot be ruled out. However, purified RhlA catalyzes HAA formation in vitro from β-hydroxydecanoyl-ACP, demonstrating that it is the only enzyme required for HAA synthesis. Furthermore, our in vitro results establish that RhlA is highly selective for 10-carbon acyl-ACP intermediates and thus functions as the molecular ruler that controls the acyl chain composition of rhamnolipids. The activity of fatty acid synthesis accelerates to correct for the drain on the pathway due to the formation of HAA by RhlA and ensure that the supply of fatty acid for membrane phospholipid synthesis is not compromised. The model predicts that RhlA competes with FabA for the β-hydroxydecanoyl-ACP intermediate of fatty acid synthesis, and this conclusion is supported by the increase in rhamnolipid production in *P. aeruginosa* strains with inactivated *fabA* or

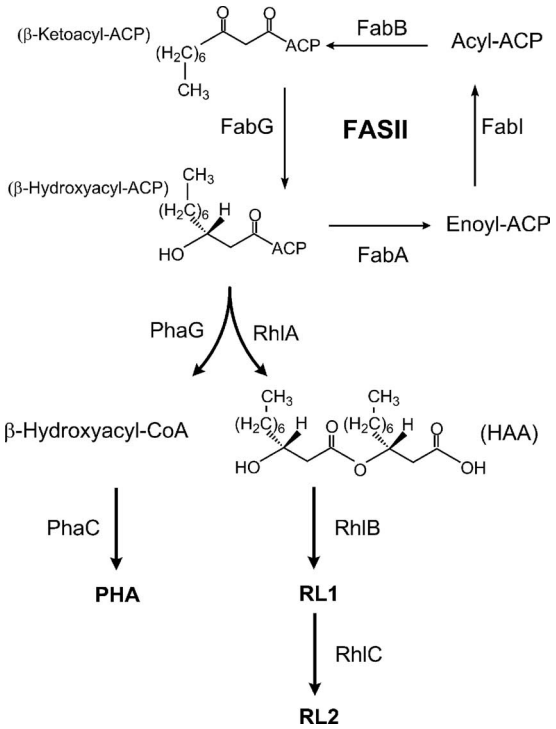

FIG. 7. Proposed rhamnolipid biosynthetic pathway. RhlA is responsible for diverting the β-hydroxydecanoyl-ACP intermediate from the FASII cycle and directly competes with FabA and FabI for this intermediate. RhlA is the only protein required to convert two molecules of β-hydroxyacyl-ACP to HAA. Thus, the amount of HAA formed is governed by the competition between RhlA and FabA/FabI, and then, RhlB and RhlC produce RL1 and RL2, respectively, by the consecutive attachment of rhamnosyl groups. PhaG is a transacylase that converts β-hydroxyacyl-ACP to the corresponding CoA thioester, which is polymerized by PhaC to form PHA.

fabI genes. The formation of RL1 is also recapitulated by the expression of RhlA and RhlB in *E. coli*. The rhamnose biosynthetic pathway is induced as part of the quorum-sensing up-regulation of rhamnolipid gene expression, suggesting that rhamnose formation may be limiting in the *E. coli* model (1, 29). In our experiments, HAA was as efficiently converted to rhamnolipid as it is in *P. aeruginosa*, although the total production of HAA was not as great. Taken together, these observations lead to the conclusion that the genetic regulation of RhlA expression is the only requirement for the initiation of HAA formation for rhamnolipid synthesis in *P. aeruginosa*.

Our data rule out the involvement of two other proteins that have been considered to participate in diverting β-hydroxydecanoyl-ACP to rhamnolipid formation. One idea was that rhamnolipids and PHA are derived from a common β-hydroxydecanoyl-CoA pool (8, 27). It is clear that the PHA synthase (PhaC) utilizes acyl-CoA thioesters and that PhaG is an essential gene in PHA synthesis and functions as a transacylase to convert β-hydroxydecanoyl-ACP to β-hydroxydecanoyl-CoA (13, 26). The utilization of CoA thioesters for PHA synthesis allows carbon from β-oxidation to enter PHA via the (*R*)-specific enoyl-CoA hydratases (32). However, fatty acid synthesis is the only source for rhamnolipid acyl chains in our model. Thus, the balance between rhamnolipid and PHA synthesis from the fatty acid biosynthetic pathway is determined by the competition between PhaG and RhlA for β-hydroxyacyl-ACP intermediates. A notable negative finding was that the *rhlG* gene is not involved in rhamnolipid synthesis and the published metabolic pathways to rhamnolipid involving a step catalyzed by RhlG (3, 8, 30) are not correct. Not only is RhlG not required for rhamnolipid formation in the heterologous host *E. coli*, we were unable to reproduce the published results (3) implicating RhlG in rhamnolipid production using four different *rhlG* knockout strains of *P. aeruginosa* PAO1 and PA14. The fact that RhlG does not efficiently recognize acyl-ACP substrates (19) supports the idea that it is not involved in the metabolism of fatty acid synthase intermediates. Thus, RhlG plays no role in rhamnolipid formation and its physiological substrate remains to be identified.

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