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A Stereospecific Pathway Diverts β-Oxidation Intermediates to the Biosynthesis of Rhamnolipid Biosurfactants

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SUMMARY

Rhamnolipids are multipurpose surface-active molecules produced by the bacterium *Pseudomonas aeruginosa* from L-rhamnose and *R*-3-hydroxyalkanoate ($C_{10\pm2}$) precursors. *R*-3-hydroxyalkanoate precursor is believed to be synthesized de novo. We demonstrate, however, that β-oxidation is the predominant source of this precursor. Inhibition of β-oxidation sharply decreases rhamnolipids production, even when using a nonfatty acid carbon source (glycerol). Isotope tracing shows that β-oxidation intermediates are direct precursors of rhamnolipids. A mutant-based survey revealed an operon coding for enoyl-CoA hydratases/isomerases (ECH/I), named RhlYZ, implicated in rhamnolipids production via an axial role in 3-hydroxyalkanoate synthesis. In vitro, RhlZ is an *R*-ECH/I transforming 2-decenoyl-CoA, a β-oxidation intermediate, into *R*-3-hydroxydecanoyl-CoA, the potential rhamnolipids precursor. Interestingly, polyhydroxyalkanoates share with rhamnolipids the RhlYZ-generated *R*-3-hydroxyalkanoates pool, as demonstrated by the decrease of polyhydroxyalkanoates upon mutation of *rhlYZ* and the increase of rhamnolipids in a polyhydroxyalkanoates-defective mutant.

INTRODUCTION

The bacterium *Pseudomonas aeruginosa* is the best-studied producer of rhamnolipids (RLs), surface-active, multipurpose amphiphilic molecules (Abdel-Mawgoud et al., 2010). RLs are produced as mixtures of congeners composed of one or two rhamnose moieties linked to a dimer of *R*-3-hydroxy fatty acids with chain lengths of $C_{10\pm2}$ (Déziel et al., 2000). Given the biotechnological importance of RLs, much effort is dedicated to maximize the production via optimization of culture conditions (Abdel-Mawgoud et al., 2011). Nonetheless, higher yields are still needed for an economically feasible industrial production. It is becoming obvious that this can only be achieved via metabolic engineering, which requires an exquisite understanding of the metabolic regulation of RLs (Abdel-Mawgoud et al., 2011).

RLs are synthesized from two precursors, *R*-3-hydroxy fatty acids and L-rhamnose, by the actions of three enzymes: RhlA, RhlB, and RhlC. RhlA dimerizes *R*-3-hydroxy fatty acids forming congeners of *R*-3-((*R*-3-hydroxyalkanoyl)oxy)alkanoic acids (HAAs); the most abundant of which in *P. aeruginosa* is C_{10}-C_{10} (Déziel et al., 2003; Zhu and Rock, 2008). The rhamnosyl transferases RhlB and RhlC catalyze the sequential addition of the first and second rhamnose to the C_{10}-C_{10} dimer, forming mono-RL (Rha-C_{10}-C_{10}) and di-RL (Rha-Rha-C_{10}-C_{10}), respectively (Ochsner et al., 1994; Rahim et al., 2001). The focus of the present study is to investigate the in vivo metabolic pathway providing the RLs fatty acid precursors.

The exact lipid precursor of RLs is still unidentified, as the two sole previous in vitro biochemical studies that explored this precursor have reached different conclusions (Burger et al., 1963; Zhu and Rock, 2008). Based on the study performed using crude RhlA extracts, coenzyme A (CoA)-activated 3-hydroxy fatty acids were concluded as precursors of HAAs (Burger et al., 1963). Recently, however, acyl carrier protein (ACP)-activated 3-hydroxy fatty acids were shown, using purified RhlA, to act instead as precursors, although without ruling out their CoA-activated counterparts as possible precursors too (Zhu and Rock, 2008). Because ACP-activated 3-hydroxy fatty acids are intermediates of the de novo fatty acid synthase pathway (FAS-II), it was hypothesized that FAS-II is the pathway providing the lipid precursors of RLs (Zhu and Rock, 2008). Moreover, 3-hydroxy fatty acids of RLs and their corresponding 3-hydroxyacyl-ACP intermediates of FAS-II share an *R*-configuration at the chiral β-carbon bearing the 3-hydroxyl group, whereas the corresponding 3-hydroxyacyl-CoAs intermediates produced from the β-oxidation pathway are in the *S*-form (Zhu and Rock, 2008). However, very recently, the indirect implication of β-oxidation in RLs biosynthesis was evoked when odd chain fatty acid-containing RLs were produced upon feeding *P. aeruginosa* with odd fatty acids (Hori et al., 2011) and when labeled RLs were produced upon feeding with labeled fatty acids (Zhang et al., 2012).

In the present study, we investigated the contribution of β-oxidation as the direct metabolic source of 3-hydroxy fatty acid precursors for RLs biosynthesis using specific inhibitors of β-oxidation along with isotope tracing experiments. We deciphered the channeling route between β-oxidation and RLs biosynthesis using in vivo and in vitro approaches. We also show the contribution of this channeling route on polyhydroxyalkanoates (PHA), an intracellular polymeric product composed also of *R*-3-hydroxy fatty acids building blocks, and the impact of blocking PHA production on RLs biosynthesis.

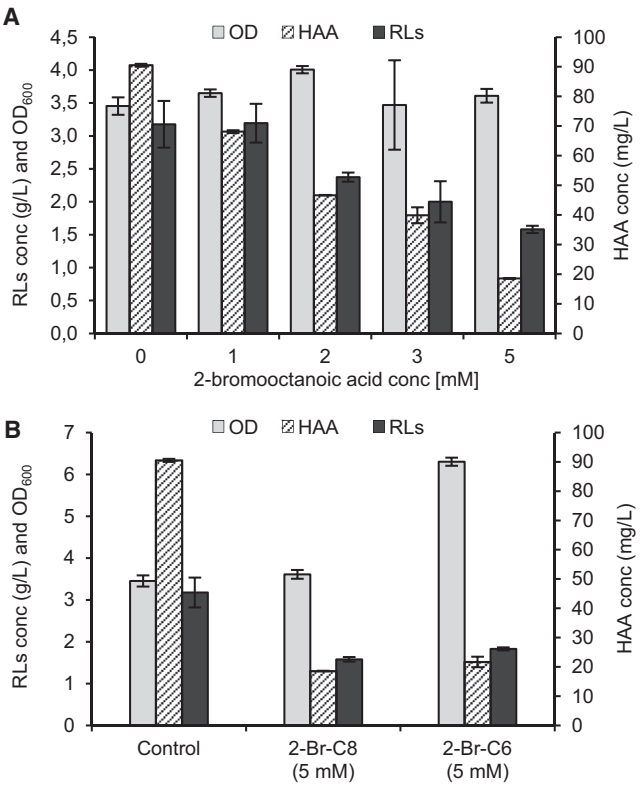

Figure 1. Specific Inhibitors of β-Oxidation Decrease RLs Production

(A) Dose-dependent inhibition of RLs and HAA production by 2-bromooctanoic acid.

(B) 2-Bromohexanoate similarly inhibits RLs production at equivalent molar concentration (5 mM). Strain PA14 was grown in MSM-glycerol for 3 days at 34°C. Data represent the mean of triplicate experiments ± SD.

See also Figures S1 and S2.

RESULTS

Inhibition of β-Oxidation Decreases RLs Production

The implication of β-oxidation on RLs biosynthesis in *P. aeruginosa* was investigated using specific β-oxidation inhibitors. This was conducted in a mineral salts medium (MSM) using glycerol as a sole carbon source and not a fatty acid source, which is naturally demanding β-oxidation for assimilation and growth. The β-oxidation inhibitors tested were acrylic, 4-pentenoic and 2-bromooctanoic acids. Strikingly, at this carbon source condition (glycerol), 2-bromooctanoic acid (2-Br-C_8) reduced RLs production by 50% and the lipid precursor HAA by 80% (Figure S1A available online), whereas the other inhibitors had no effect (Figure S1B).

To explore the specificity of 2-Br-C_8 in RLs inhibition, the dose-dependency of 2-Br-C_8-mediated inhibition of RLs production was examined. Moreover, the chain length specificity of this inhibitory activity was also studied using shorter (2-Br-C_4 and 2-Br-C_6) and longer (2-Br-C_{12}) homologs at equivalent molar concentrations. The results show that 2-Br-C_8 inhibits RLs and HAA production in a dose-dependent manner (Figure 1). Surprisingly, 2-Br-C_6 (5 mM), not previously reported as a β-oxidation inhibitor, also inhibits RLs production to the same extent as 2-Br-C_8 (5 mM) (Figure 1B). The other congeners, 2-Br-C_4 and 2-Br-C_{12}, caused considerable growth inhibition down to 3 mM.

Besides acting as an inhibitor of β-oxidation (Raaka and Lowenstein, 1979), 2-Br-C_8 was previously hypothesized to inhibit PhaG, which transacylates the FAS-II intermediate *R*-3-hydroxyacyl-ACP to the CoA counterpart for PHA biosynthesis (Lee et al., 2001). To rule out the hypothesis that PhaG product could act as precursor for RLs biosynthesis and that the inhibition of RLs production by 2-Br-C_8 is caused by its effect on PhaG (Figure S2A), we tested RLs production in a *phaG* knockout as well as a transposon insertion *phaG* mutant. Neither of the two mutants affects RLs production (Figure S2B), excluding a role of PhaG in the 2-Br-C_8-mediated reduction of RLs production.

β-Oxidation Is the Main Source of Lipid Precursors for RLs

To further evaluate the relative contribution of β-oxidation versus FAS-II in RLs synthesis, strain PA14 was cultivated in MSM using dodecanoic-12,12,12-d_3 acid as the sole carbon source. Implication of either pathway was traced and estimated via examination of the pattern and extent of labeling of the HAA (C_{10}-C_{10}) moiety of RLs produced. If RLs are labeled with three extra Daltons per chain, this would entail that decanoic-12,12,12-d_3 intermediate of β-oxidation is incorporated as the lipid chains of RLs (Figure S3A). Alternatively, if de novo fatty acid synthesis (FAS-II) is predominantly implicated, produced RLs would be mostly unlabeled as their chains would be de novo synthesized from the more abundant unlabeled acetyl-CoA (five unlabeled versus one labeled) units released after complete β-oxidation of dodecanoic-12,12,12-d_3 acid (Figure S3A).

Strikingly, the RLs produced, which consisted solely of mono-RLs, were almost exclusively (>99%) deuterium-labeled with 88% having an extra mass of 6 Da, ($[M-H]^-$ mass-to-change [m/z] 509) and 12% with an extra mass of 3 Da, ($[M-H]^-$ m/z 506). Tandem mass spectrometry (MS/MS) fragmentation of m/z 509 confirmed that the extra 6 Da were distributed equally in the two lipid chains of RLs, 3 Da per chain (Figure S3B) and thus corresponding to Rha-C_{10}(d_3)-C_{10}-(d_3). This clearly demonstrates that β-oxidation is the main supplier of the lipid precursor of RLs when fatty acids are the sole carbon source.

β-Oxidation Is Directly Linked to RLs Pathway

Using perdeuterooctadecanoic acid (C_{18}-d_{35}) and glucose as cosubstrate, Zhang et al. (2012) suggested that the fatty acid substrates of RLs are shortened via β-oxidation to the C_8 chain length after which one C_2-elongation step via FAS-II takes place before diversion of corresponding FAS-II C_{10}-intermediate to RLs biosynthesis (Zhang et al., 2012). However, our isotope tracing experiments using dodecanoic-12,12,12-d_3 acid are suggesting instead a direct link between β-oxidation and RLs biosynthesis. We thus verified the contribution of the hypothesis of Zhang et al. (2012) in the supply of RLs lipid precursors relative to β-oxidation. We fed PA14 with pandeuterododecanoic acid (C_{12}-d_{23}) as sole carbon source. If β-oxidation is solely implicated, each 3-hydroxydecanoate chain of RLs would be labeled with an extra 17 Da (Figure S4A). On the other hand, if FAS-II is implicated according to the model of Zhang et al. (2012), the fatty acid chain of RLs would bear an extra 16 Da (67%) or 15 Da (33%) as the main species (Figure S4B).

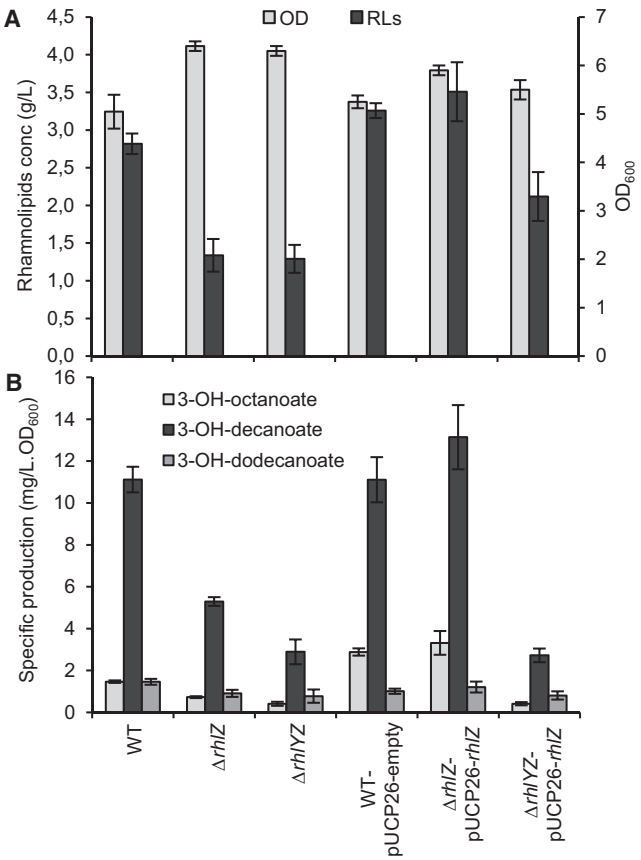

Figure 2. RhlY and RhlZ Are Implicated in RLs Biosynthesis by Controlling the Supply of Lipid Precursors

(A) Deletion mutants, Δ*rhlYZ* and Δ*rhlZ* have similarly reduced RLs production. Complementation of Δ*rhlYZ* with *rhlZ* only partially restores RLs production.

(B) RhlYZ is the main supplier of 3-hydroxyalkanoates ($C_{10\pm2}$) in PA14. WT PA14 strain and mutants were grown for 3 days in MSM-glycerol at 34°C. Lipids were analyzed by GC/MS. Data represent the mean of triplicate experiments ± SD.

See also Figure S6 and Table S3.

Interestingly, using C_{12}-d_{23} as the sole carbon source generated of 3-hydroxydecanoate bearing +17 Da as the species of the highest abundance, whereas those bearing +16 Da and +15 Da only represented 43% and 26% of the intensity of the +17 Da species, respectively. The abundance of the +16 Da and +15 Da species relative to each other were 62% and 38% respectively, which are close to theoretical predictions mentioned above (Figure S5A). These results demonstrate that, when using dodecanoic acid as sole carbon source, the majority (~60%) of the RLs lipidic precursors is directly supplied from β-oxidation without resorting to a bypass through FAS-II.

To further investigate the hypothesis of Zhang et al. (2012), we provided C_{12}-d_{23} (3.75 g/l) together with glucose (10 g/l) as cocarbon sources. Unexpectedly, due to a possible hydrogen-deuterium isotopic negative metabolic effect, glucose was preferentially utilized resulting in the formation of almost exclusively unlabeled 3-hydroxydecanoate (~95%) (Figure S5B). To overcome this effect, we fed the bacterium with an excess of glucose-$^{13}C_6$ (15 g/l) (83 mM) together with dodecanoate-12,12,12-d_3 (30 mM) or tetradecanoate-14,14,14-d_3 (30 mM). Under these conditions, if β-oxidation is solely involved, the lipid chains of RLs would bear an extra 3 Da each. However, if FAS-II is implicated in the C_2-elongation step (mostly with labeled $^{13}C_2$) of the C_8-d_3 intermediate according to the Zhang et al. (2012) hypothesis, the lipid chains of RLs would bear an extra 5 Da instead. The 2 extra Da in this case arises from the FAS-II-mediated condensation of $^{13}C_2$-labeled malonate units mostly originating from glucose-$^{13}C_6$. Strikingly, under these conditions, 3-hydroxydecanoate predominantly bore an extra 3 Da only and minor amounts with extra 4 Da or 5 Da labeling (Figures S5C and S5D), implying its direct β-oxidation origin.

Taken together, our results indicate that, whether fatty acids are provided alone or as cocarbon sources with glucose, β-oxidation remains the main direct supplier of lipid precursors for RLs biosynthesis.

Enoyl-CoA Intermediates of β-Oxidation Are Diverted to RLs Biosynthesis

Involvement of β-oxidation implicates that one of its intermediates, namely, *trans*-2-enoyl-CoA, *S*-3-hydroxyacyl-CoA or 3-ketoacyl-CoA, is converted via an enoyl-CoA hydratase, an epimerase, or a 3-ketoacyl-CoA reductase, respectively, into *R*-3-hydroxy fatty acids, the ultimate lipid precursor of RLs. To figure out this β-oxidation intermediate, RLs production was examined in mutants defective in enzymes carrying out these conversions.

A survey of the *P. aeruginosa* PA14 genome (Winsor et al., 2011) for genes that might code for such enzymes revealed 14 loci predicted to encode enoyl-CoA hydratases (ECH) (Table S3), while no putative epimerases or 3-ketoacyl-CoA reductases were found. We also included in our survey the PHA-related ECH, PhaJ1-4 (Tsuge et al., 2003). RLs productions in transposon (Tn) or knockout (KO) mutants of 12 of these 14 ECH-coding loci were tested. Transposon mutants of *phaJ2* and *phaJ4* had no negative effect on RLs production (Figure S6A). Interestingly, three putative enoyl-CoA hydratases/isomerases (ECH/I) mutants, PA14_40980 (Tn), PA14_54640 (Tn), and PA14_54660 (KO) were associated with remarkable 35%, 56%, and 54% reductions in RLs productions, respectively (Figure S6B). Because of their impact on RLs biosynthesis, they were named *rhlK*, *rhlY*, and *rhlZ*, respectively.

We focused on *rhlY* and *rhlZ*, as their corresponding mutants were the most affected in RLs production. These two genes are arranged in a single computationally predicted operon with *rhlZ* downstream to *rhlY* (Winsor et al., 2011). To exclude the possibility that the RLs phenotype associated with the *rhlY* transposon mutant (Tn) is ultimately caused by a polar effect on its downstream gene *rhlZ*, a double *rhlYZ* knockout mutant (KO) was constructed and its RLs production was compared to that of the simple *rhlZ* knockout mutant. In addition, *rhlZ* was expressed in *trans* from a multicopy plasmid in both mutants. Interestingly, the double knockout did not cause further decrease in RLs production. Complementation with *rhlZ* completely restored RLs production in the simple *rhlZ* mutant, whereas, it only partially complemented RLs production in the double *rhlYZ* mutant (Figure 2A). Moreover, overexpression of *rhlZ* in the wild-type (WT) did not increase RLs production (Figure S6C). Collectively, these results demonstrate a direct role for *rhlZ* in RLs production and, more interestingly, that RhlY is

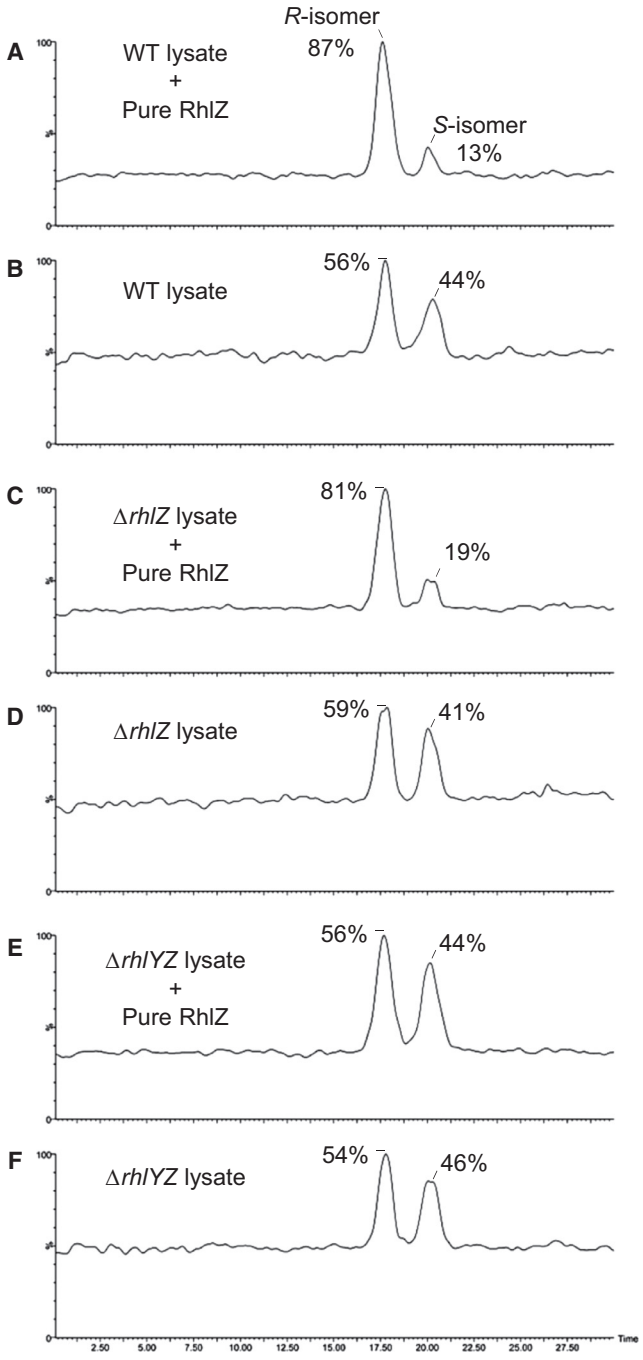

Figure 3. RhlZ Is an *R*-Specific ECH/I Requiring RhlY for Its Activity

(A and B) The purified RhlZ alone did not catalyze any 3-hydroxydecanoate formation from *trans*-2-decenoyl-CoA, therefore (A) total cell lysate of WT was added in conjunction with purified RhlZ (Figure S7) and this caused enrichment of the *R*-3-hydroxydecanoate compared to the total cell lysate of WT alone that produced a nearly racemic mixture of *R*-/*S*-3-hydroxydecanoate as shown in (B).

(C and D) The same was observed with total cell lysate of the mutant Δ*rhlZ*.

(E and F) The factor in lysates of WT or *rhlZ* mutant that is indispensable for RhlZ activity appears to be RhlY as pure RhlZ does not produce any 3-hydroxydecanoate in the presence of total cell lysate of Δ*rhlYZ*. *R*/*S*-3-hydroxydecanoate was analyzed using a chiral HPLC-MS/MS method.

simultaneously required with RhlZ to attain WT level of RLs production.

The ECH/I RhlY and RhlZ Are Main Suppliers of 3-Hydroxydecanoate/Octanoate Pools

To experimentally confirm the putative ECH/I role assigned to RhlY and RhlZ in the production of the 3-hydroxyalkanoate precursors of RLs, we compared the total intra- and extracellular pool of 3-OH-$C_{10\pm2}$ in the mutants and WT (Figure 2B). In agreement with their putative function, both the single Δ*rhlZ* and more markedly the double Δ*rhlYZ* knockouts presented a reduction of 3-OH-$C_{10\pm2}$, more pronouncedly of 3-OH-C_{10} species followed by 3-OH-C_8 then 3-OH-C_{12} (Figure 2B). This remarkable decrease in 3-hydroxy fatty acids precursors is in correlation with the RLs reduction in these mutants (Figure 2A). Moreover, these results demonstrate a pivotal metabolic role for RhlY/RhlZ in the biosynthesis of 3-OH-alkanoates in *P. aeruginosa* in general. Again, *rhlZ* complementation in the double mutant only partially restored the WT level of 3-hydroxy fatty acid, supporting once more the hypothesis that RhlY is simultaneously required with RhlZ to attain the WT level of 3-hydroxy fatty acids precursors and hence RLs.

RhlZ Is an *R*-Specific ECH/I

We hypothesized that RhlZ is an *R*-specific ECH/I (*R*-ECH/I) converting a 2-decenoyl-CoA intermediate of β-oxidation into the corresponding *R*-3-hydroxydecanoyl-CoA precursor of RLs (Figure S2A). This was verified in vitro using purified RhlZ (Figure 3) and *trans*-2-decenoyl-CoA as substrate. The relative proportion of the *R*-stereoisomers and *S*-stereoisomers of 3-hydroxydecanoyl-CoA (3-OH-C_{10}-CoA) produced was estimated using a chiral high-performance liquid chromatography (HPLC)-MS/MS method.

Purified RhlZ alone did not catalyze the formation of 3-hydroxydecanoate from *trans*-2-decenoate. However, clarified cell lysate of WT PA14 strain did convert *trans*-2-decenoate to 3-hydroxydecanoate at 0.53 mg/l (SD: ±0.016) where the *R*-isomer was produced at 0.3 mg/l (SD: ±0.015) and the *S*-isomer at 0.23 mg/l (SD: ±0.012), i.e., at nearly racemic mixture of enantiomers (Figures 4 and 3B). Interestingly, combination of purified RhlZ with a clarified WT cell lysate caused an ~1.5-fold increase in the total yield of 3-hydroxydecanoate (0.75 mg/l, SD: ±0.003) of which the *R*-isomer (0.61 mg/l, SD: ±0.004) was at least 4-fold more abundant than the *S*-isomer (0.14 mg/l, SD: ±0.007) (Figures 4 and 3A). This demonstrates that RhlZ is indeed an *R*-ECH/I, but it requires an unknown factor present in the WT cell lysate. We hypothesized that this factor is a protein because heat treatment of the clarified cell lysate abolished any RhlZ activity with no any 3-hydroxydecanoate produced. According to our previous data (Figure 2), this heat-sensitive factor was likely RhlY. To verify this, we combined purified RhlZ with the clarified cell lysates of Δ*rhlYZ* or Δ*rhlZ*. Consistent with our hypothesis, the *R*-3-OH-C_{10} enrichment was conserved when purified RhlZ was combined with the lysate of Δ*rhlZ* (Figures 3C and 3D), whereas no such enrichment was observed when this purified RhlZ was combined with the lysate of the double mutant Δ*rhlYZ* (Figures S3E and S3F).

The dependency of RhlZ on RhlY suggested a sort of interaction or complex formation. This was verified by a pull down

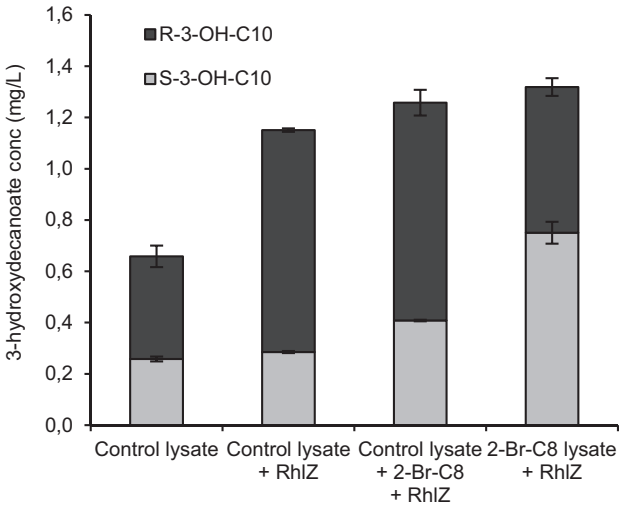

Figure 4. RhlZ Is an *R*-Specific ECH/I and Is Specifically Inhibited by a Metabolite of 2-Br-C_8

In in vitro enzymatic assays in the presence of WT cell lysate, purified RhlZ (Figure S7) causes *R*-specific enrichment of 3-hydroxydecanoate, and this enrichment is blocked when using WT lysate of cells cultivated in the presence of 5 mM 2-Br-C_8. *R*/*S*-3-hydroxydecanoate was analyzed using chiral HPLC-MS/MS. Data represent the mean of triplicate experiments ± SD.

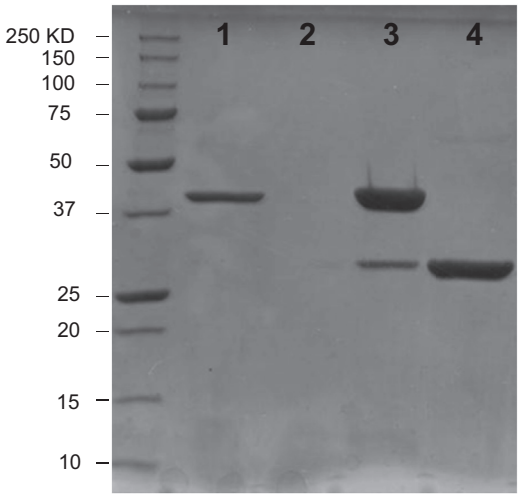

Figure 5. SDS-PAGE of the Pull-Down Assay Showing the Stable Physical Protein-Protein Interaction between RhlY and RhlZ

Purified his_6-tagged RhlZ was mixed with total cell lysate of WT PA14 strain for 1 hr at 4°C, the mixture was then subjected to Nickel affinity chromatographic purification using the same gradient of imidazole as previously done for purification of RhlZ (Figure S7A). Lane 1 is the purified RhlZ-His_6 alone (bait). Lane 2 is the control lysate that was not exposed to the RhlZ-His_6. Lane 3 is the purified protein-RhlZ-His_6 interaction; this protein was shown to be RhlY as shown from its size compared to the purified RhlY-His_6 in lane 4 (see also Figure S7B) as well as by protein sequencing.

experiment using purified hexahistidine-tagged RhlZ (RhlZ-His_6) incubated with the clarified total cell lysate of WT. Strikingly, RhlZ-His_6 pulled RhlY from the lysate (Figure 5), entailing a strong and stable RhlY-RhlZ physical interaction.

Taken together, our results indicate that RhlZ is an *R*-specific ECH/I, whose activity requires the presence of RhlY with which it forms a stable complex.

RhlZ-Associated *R*-Specific Enrichment Is Inhibited by 2-Bromooctanoate

As shown in this study, treating with 2-Br-C_8 or inactivating *rhlZ* decreases RLs production. In order to verify if RhlZ is one of the targets of this β-oxidation inhibitor, we added 3 mM 2-Br-C_8 directly to ECH assay buffer containing RhlZ in combination with clarified lysate of WT. Under these conditions, the RhlZ-associated *R*-specific enrichment was not inhibited (Figure 4). However, because previous reports suggested that the active form of this inhibitor could be one of its metabolites (Raaka and Lowenstein, 1979), we conducted ECH assays using clarified lysate of WT cells that were cultivated in the presence of 2-Br-C_8 (5 mM) and compared this to the control lysate of cells cultivated in the absence of the inhibitor, both in combination with purified RhlZ. Using lysate of cells cultivated in the presence of 2-Br-C_8, supposed to contain the active metabolite of this inhibitor, the RhlZ-mediated enrichment of the *R*-isomer was lost and its abundance approached that when using the control lysate alone (Figure 4). This indicates that RhlZ is at least partially inhibited by a 2-bromooctanoate-derived metabolite.

R-3-Hydroxyacyl-CoA Pool Is Shared between RLs and Polyhydroxyalkanoates Pathways

Because *R*-3-hydroxyacyl-CoA are also the precursors of polyhydroxyalkanoates (PHAs) (Madison and Huisman, 1999; Wang et al., 2012), we tested the hypothesis that the lipid precursors supplied by RhlYZ is shared by PHA and RLs. We first examined the impact of *rhlYZ* double mutation on the production of PHA (Figure 6A). Second, we directly assessed PHA production by transmission electron microscopy using the percentage cross-sectional area of intracellular PHA granules (Figures 6B and 6D). We found a consistent 25% reduction in PHA in the *rhlYZ* mutant (Figures 6A and 6B) entailing a shared precursor pool. Third, we verified if this pool is competitively shared between PHA and RLs by studying the effect of blocking PHA biosynthesis on RLs production. To do so, we knocked out most of PHA-biosynthetic genes, namely Δ*phaC1DC2DFI* (Δ*pha*). This mutation resulted in a remarkable increase in mono-RL (Rha-C_{10}-C_{10}) accompanied with a small decrease in di-RL (Rha-Rha-C_{10}-C_{10}) that together resulted in a 13% increase in total RLs compared to the WT (Figure 6C). This is explained by the accumulation of the shared lipid precursor pool in the PHA-negative mutant that becomes more available for RLs biosynthesis. Assuming a fixed rhamnose pool, the consequent high lipid to rhamnose precursors availability, led to this remarkable doubling in mono-RL congener, of high lipid to rhamnose content, at the expense of di-RL having lower lipid to rhamnose content. We thus propose that the fixed rhamnose precursor pool limited a more striking increase in total RLs.

DISCUSSION

This study unequivocally confirms the implication of β-oxidation as the main direct metabolic source providing lipidic precursors for RLs biosynthesis as evidenced by isotope tracing experiments. A previous report suggested that a β-oxidation

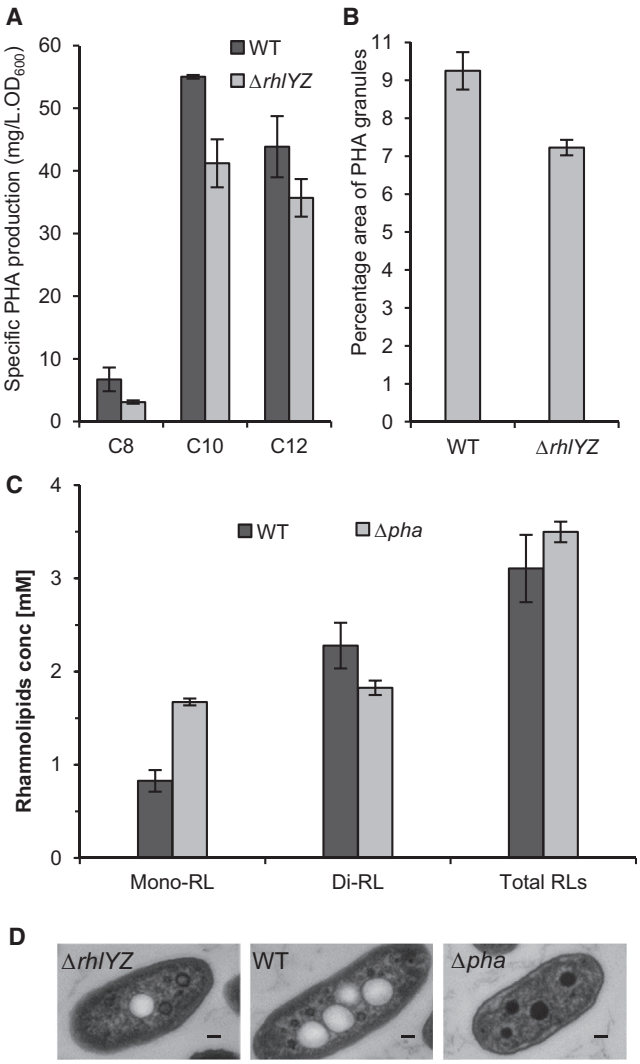

Figure 6. RLs and PHA Share the Same 3-Hydroxyalkanoate Pool

(A and B) Deletion of *rhlYZ* results in a decrease in PHA accumulation, as estimated by (A) GC-MS analysis of hydrolyzed intracellular 3-hydroxy fatty acids (C_8 to C_{12}) as well as by (B) percentage cross-sectional area of PHA granules relative to the whole cell cross-sectional area on images taken by transmission electron microscope, based on an average measurements of more than 400 cells of both WT and mutant.

(C) The PHA-negative mutant (Δ*phaC1phaDphaC2phaD*Δ*phaF*Δ*phaI*) causes a slight increase in RLs, mostly with a 100% enrichment of mono-RLs at the expense of di-RLs.

(D) Thin transmission electron microscope images of PHA granules of *P. aeruginosa* PA14 strains; Δ*rhlYZ*, WT, Δ*pha*. White spheres are PHA granules, whereas black spheres are polyphosphates. Magnification bar represents 100 nm. WT strain and deletion mutants were cultured in MSM-glycerol for 2–2.5 days at 34°C with shaking. Data represent the mean of triplicate experiments ± SD.

intermediate (C_8) undergoes a one-step elongation by FAS-II to the corresponding C_{10} intermediate that is then delivered for RLs biosynthesis (Zhang et al., 2012). This C_2-elongation step is suggested to be mediated via a FabH-like enzyme that condenses acyl-CoA (C_8) with malonyl-ACP (Zhang et al., 2012). Although this model could be contributing, our isotope tracing data actually show that β-oxidation is still the main direct supplier of lipid precursors for RLs biosynthesis, with no bypass via FAS-II. Interestingly, we found that the implication of β-oxidation in RLs production is constitutive and not only when using fatty acid carbon sources. This is corroborated by the striking fact that although a nonfatty acid carbon source is used, specific inhibition of β-oxidation and/or of β-oxidation-related precursor diverting enzymes, via 2-bromooctanoic acid, sharply reduced RLs production. Interestingly, the target inhibited by 2-bromooctanoate seems to be chain length-specific (C_6-C_8), as, except for 2-bromohexanoate that is equally effective, shorter and longer homologs had no remarkable effects on RLs production. In eukaryotes, 2-bromooctanoate is reported to irreversibly inhibit FadA of β-oxidation after conversion to 2-bromo-3-ketooctanoyl-CoA by β-oxidation (Raaka and Lowenstein, 1979).

Our mutant-based survey uncovered the channeling route from β-oxidation toward RLs biosynthesis, in which an enoyl-CoA intermediate of appropriate chain length (C_{10}), is transformed by enoyl-CoA hydratases/isomerases (ECH/I) *rhlK*, *rhlY*, and *rhlZ* to the corresponding 3-hydroxyacyl-CoA, that in turns serves as the lipidic precursors of RLs. This is demonstrated by the sharp reduction in RLs production in the *rhlYZ* mutants, which we attribute to the important reduction (~70%) in the total free 3-hydroxyalkanoates ($C_{10\pm2}$), observed in these mutants. In view of such a decrease, we conclude that RhlYZ constitutes an axial pathway generating the majority of the pool of 3-hydroxyalkanoates (C_8 and C_{10}) in *P. aeruginosa.* Moreover, RhlYZ seems to have a remarkable specificity for the C_{10} chain length, and to lesser extents for C_8 and C_{12}, as these 3-hydroxy fatty acids are the most affected in the *rhlYZ* mutant. This is in agreement with the fact that the fatty acid chains of RLs are predominantly (>80%) C_{10} in *P. aeruginosa* (Déziel et al., 2000).

ECH catalyze the stereospecific hydration of *trans*-2-enoyl-CoA, yielding generally *S*-3-hydroxyacyl-CoA in the second step of β-oxidation (Bell et al., 2002; Koski et al., 2004), while a few *R*-specific-ECH (*R*-ECH), such as PhaJ1-4 of *P. aeruginosa* implicated in PHA synthesis, yield *R*-3-hydroxyacyl-CoA (Tsuge et al., 2003). We found that RhlZ is an *R*-ECH that diverts the enoyl-CoA intermediate of β-oxidation generating the *R*-3-hydroxyalkanaote precursors required for RLs, whose 3-hydroxy fatty acid chains are strictly in the *R*-configuration (Bauer et al., 2006; Schenk et al., 1997). Our results indicate that the RhlY forms a complex with RhlZ and is essential for its activity. We also demonstrated that a metabolite of 2-bromooctanoate abolishes the *R*-stereospecificity of RhlYZ, yet without affecting the net yield of hydration reaction. This selective inhibition suggests that RhlY is exerting a classic *S*-ECH role, while RhlZ has an *R*-isomerase role in the RhlYZ complex and that it is RhlZ, and not RhlY, that is inhibited by the metabolite of 2-bromooctanoate.

The 3-hydroxyalkanoate pool generated by RhlYZ is not only devoted to RLs, but also partly used for PHA synthesis whose precursors are CoA-activated *R*-3-hydroxy fatty acids (Wang et al., 2012). While we expected a more significant increase in RLs production in a PHA-negative mutant, because of the accumulation of lipid precursors, we believe that the limited rhamnose pool hindered a net increase in RLs. As a result, the effect of lipid

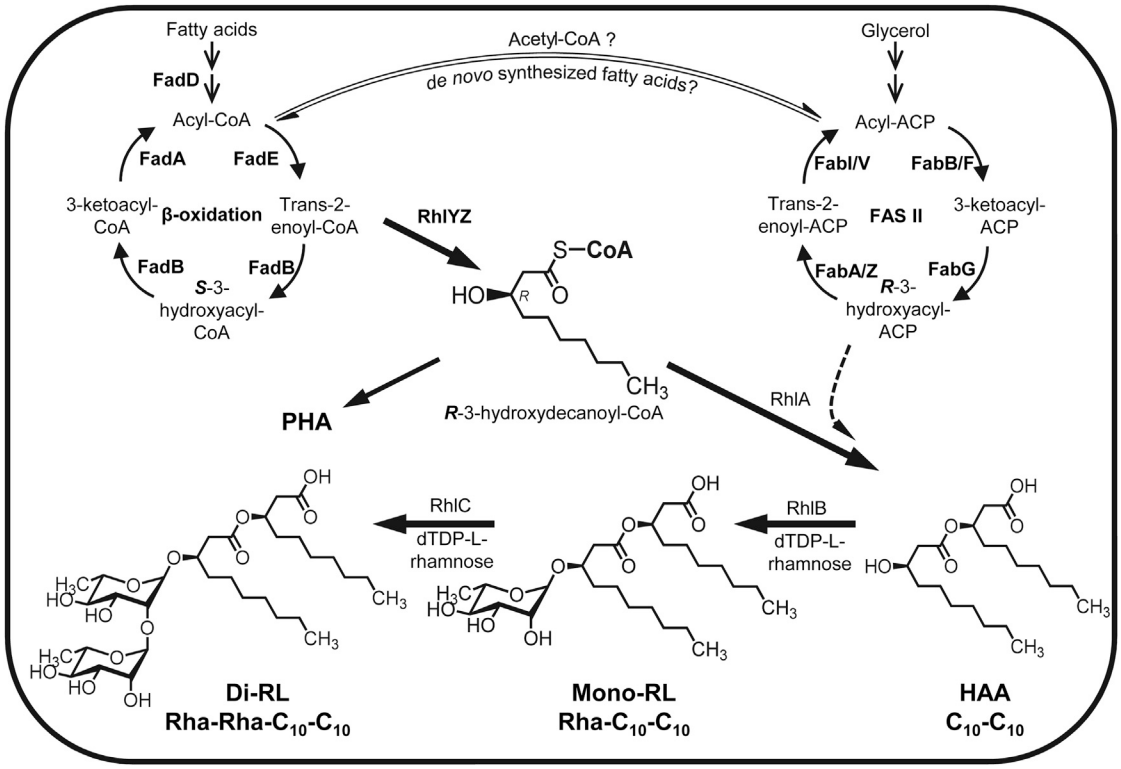

Figure 7. Proposed Model for Metabolic Supply of Lipid Precursors of RLs

With a fatty acid carbon source, shortening of the chain takes place by β-oxidation until the formation of the *trans*-2-decenoyl-CoA intermediate. With a soluble carbon source like glycerol, de novo synthesis of fatty acids via FAS-II forms fatty acids that are proposed to be diverted to β-oxidation to be processed into the *trans*-2-decenoyl-CoA intermediate. In either case, the *trans*-2-decenoyl-CoA is hydrated by the *R*-specific enoyl-CoA hydratase RhlYZ to form *R*-3-hydroxydecanoyl-CoA, which then acts as the direct lipid precursors used by RhlA for the synthesis of HAAs, which is then converted by RhlB and RhlC into RLs. Although clearly less significant, a direct precursor supply from FAS-II could also be contributing in RLs biosynthesis. PHAs share with RLs the same pool of *R*-3-hydroxyacyl-CoA. FabB and FabF, 3-ketoacyl-ACP synthetases; FabG, NADPH-dependent 3-ketoacyl-ACP reductase; FabA, FabZ, 3-hydroxyacyl-ACP dehydratases; FabI, NADH-dependent enoyl-ACP reductase; FadD, acyl-CoA synthetase; FadE, acyl-CoA dehydrogenase; FadB, *trans*-enoyl-CoA hydratase and *S*-3-hydrxoyacyl-CoA dehydrogenase; FadA, 3-ketoacyl-CoA thiolase; RhlA, HAA synthetase; RhlB, rhamnosyltransferase 1; RhlC, rhamnosyltransferase 2. See also Figures S3–S5.

accumulation together with a limited rhamnose pool reflected on the increased ratio of mono-RLs to di-RLs upon inactivation of PHA synthesis. Strictly regulated availability of dTDP-L-rhamnose is expected, as the genes coding for the enzymes responsible for its biosynthesis are regulated by quorum sensing, along with *rhlAB* and *rhlC* (Aguirre-Ramírez et al., 2012). Interestingly, RhlYZ was also reported to be regulated by quorum sensing (Wagner et al., 2007), entailing that RLs biosynthesis is tightly regulated by quorum sensing to synchronize the expression of the terminal-linking enzymes *rhlABC* with precursors supply, rhamnose, and fatty acids.

We propose a model in which acyl-CoA (substrate-derived or de novo synthesized) enters β-oxidation for shortening, and chains of appropriate lengths (C_{10}) are then diverted by the action of RhlYZ to RLs biosynthesis, with PHA biosynthesis pathway sharing the same lipid precursors (Figure 7). Additional work is still required to elucidate the mechanism linking de novo fatty acid synthesis and β-oxidation when using nonfatty acid carbon sources. This link could reveal an important regulatory mechanism of lipid metabolism, where de novo synthesized lipids delivered by FAS-II are reserved for primary cell structures and metabolism, whereas lipid precursors destined for extracellular secondary metabolites like RLs are instead provided via the β-oxidation pathway, such as by R-ECH like *rhlYZ* that is coregulated with *rhlAB* and *rmlBDAC.* This compartmentalization of lipid precursors is thought to be a regulatory mechanism preventing secondary metabolism from competing with primary metabolism for the same central precursors.

These findings will have a great impact on the metabolic engineering strategies aiming at enhancing RLs production. In general terms, our results show that not only *rhlABC* genes are important to be targeted in metabolic engineering strategies but also genes supplying metabolic precursors. Moreover, our findings suggest that fatty acid carbon sources are better than the soluble ones, as the former would be directly incorporated in the lipid chains of RLs after a bypass in β-oxidation, whereas the latter requires a longer de novo metabolic synthesis of lipid precursors. On the other hand, glycerol as carbon source is more metabolically proximal than fatty acids to the rhamnose biosynthetic pathway (Abdel-Mawgoud et al., 2011) as fatty acids require the longer pathway of gluconeogenesis for synthesis of this precursor. Taken together, our observations finally explain

why vegetable oils, which are composed mainly of triglycerides (i.e., glycerol and fatty acids), represent by far the best carbon source for the highest yield of RLs (Müller et al., 2012) as they generate the metabolically optimal cocarbon sources for rhamnose and 3-hydroxyalkanoate precursors biosynthesis.

SIGNIFICANCE

Rhamnolipids (RLs) are bacterial surfactants of biotechnological importance principally produced by *Pseudomonas aeruginosa*. Currently, there is lack of understanding of the definitive metabolic origin of the two precursors of RLs, namely, the sugar, rhamnose, and the lipid chain, 3-hydroxyalkanaote. In this study, we demonstrate that β-oxidation is the main direct suppliers of lipid precursors of RLs. We found that the shunting mechanism of β-oxidation intermediates to RLs biosynthesis is mediated by an *R*-specific enoyl-CoA hydratase complex RhlYZ. This complex diverts enoyl-CoA intermediates of β-oxidation into *R*-3-hydroxyalkanoates that acts as lipid precursors of RLs. It is found that RhlYZ is responsible for ~70% of the total pool of 3-hydroxyalkanoates in *P. aeruginosa* PA14. The RhlYZ-generated pool of 3-hydroxyalkanoates serves as precursors for not only RLs but also for polyhydroxyalkanoates granules synthesis, another secondary metabolite produced by *P. aeruginosa*. Findings of this work decipher an axial pathway, mediated by RhlYZ, linking primary lipid metabolism with RLs biosynthesis and with secondary metabolism in general. These findings enhance the understanding of RLs biosynthesis and provide the foundations for future metabolic engineering strategies aiming at scaling up RLs production.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions

Bacterial strains and plasmids are described in Table S1.

Seed cultures of *P. aeruginosa* PA14 in tryptic soy broth (TSB; Difco) incubated overnight at 37°C under rotation were used to inoculate mineral salts medium (MSM) at a starting OD_{600} of 0.1. MSM has the following composition: Na_2HPO_4 (0.9 g/l), KH_2PO_4 (0.7 g/l), $CaCl_2 \times 2H_2O$ (0.1 g/l), $MgSO_4 \times 7H_2O$ (0.4 g/l), $NaNO_3$ (2 g/l), tryptone (1 g/l), trace element solution (TES, 2 ml/l), pH adjusted to 7. Composition of TES is: $FeSO_4 \times 7H_2O$ (2 g/l), $MnSO_4 \times H_2O$ (1.5 g/l), $(NH_4)_6Mo_7O_{24} \times 4H_2O$ (0.6 g/l). Glycerol (20 g/l) was used as the carbon source unless otherwise stated. Tryptone was omitted when fatty acid carbon sources were tested. Inoculated MSM medium was incubated at 34°C under rotation.

P. aeruginosa was grown on LB agar (NaCl 5 g/l) at 37°C for mating purposes and for selection of transformants after mating or electroporation. It was also grown in LB-no salt (LBNS, with no NaCl) broth at 30°C for resolution of merodiploids in mutant constructions. Antibiotics were added to maintain or select for plasmids as follows: for *Escherichia coli*, carbenicillin (Cb) at 100 μg/ml, gentamicin (Gm) at 30 μg/ml, tetracycline (Tc) at 15 μg/ml, kanamycin (Km) at 50 μg/ml; for *P. aeruginosa*, carbenicillin (Cb) at 300 μg/ml, Gm at 50 μg/ml, and Tc at 125 μg/ml for solid media and at 75 μg/ml for liquid media. Triclosan, at 10 μg/ml, was used to counter select *E. coli*.

Gene Mutation and Cloning

Deletions mutants were generated via homologous allelic replacement using Gateway technology (Choi and Schweizer, 2005). For *rhlZ* overexpression and complementation studies, the coding sequence, including its natural ribosomal binding site, was cloned onto pUCP26. For protein purification, *rhlY* and *rhlZ* were cloned downstream to coding sequence for the His-Tag onto pET28a plasmids. Full details are described in the Supplemental Experimental Procedures.

Protein Expression, Purification, and Preparation of Crude Cell Lysates

The expression of recombinant RhlZ or RhlY proteins with an N-terminal 6xHis-tag encoded by pET28a(+)-*rhlZ* was induced with 0.5 mM isopropyl-1-thio-D-galactopyranoside (IPTG) in a fresh subculture (starting OD_{600} of 0.05) of *E. coli* BL21(DE3) when the OD_{600} = 0.8. Incubation was then continued at 34°C under rotation of 150 rpm for 2–3 hr. Cells were then collected by centrifugation, resuspended in the binding buffer (20 mM $NaPO_4$, pH 7.4, 500 mM NaCl, 25 mM imidazole, 20 mM β-mercaptoethanol) freshly supplemented with Mini Protease Inhibitor Cocktail Tablets (Roche) and 0.5 mM phenylmethanesulfonylfluoride (PMSF), and lysed by sonication for nine cycles each of 30 s interrupted by 30 s of rest on ice. Each cell lysates were clarified by centrifugation at 10,000 × *g* at 4°C for 30–45 min and then applied onto a preconditioned Ni Sepharose High Performance column (HisTrapHP-5ml, GE) using fast protein liquid chromatography (AKTA FPLC, GE). Preconditioning of the column was achieved according to instructions of the manufacturer and flow rate was adjusted at 5 ml/min. The column was washed with five column volumes of binding buffer then a linear gradient was initiated. For RhlZ purification, the gradient was from 0% to 50% elution buffer (20 mM $NaPO_4$, pH 7.4, 500 mM NaCl, 500 mM imidazole, 20 mM β-mercaptoethanol) over 20 column volumes. For RhlY, the gradient was from 0% to 100% elution buffer over 30 column volumes. Fractions of 5 ml were collected throughout the chromatography. RhlZ started to elute out of the column at 130 mM, peaked at 160 mM and tailed at 190 mM imidazole (Figure S7A), RhlY started to elute at 170 mM, peaked at 240 mM and tailed at 335 mM imidazole (Figure S7B). Fractions of these peaks and flanking fractions were applied to 12% (w/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Figure S7). Appearing bands were excised for identification via protein sequencing at the Proteomic services of INRS-Institut Armand Frappier (Canada). Fractions containing pure proteins were pooled together and concentrated with Amicon Ultra-15 (30 kDa, Millipore) for RhlZ and Amicon Ultra-15 (15 kDa) for RhlY, and washed with 900 volumes of buffer (50 mM Tris-Cl, pH 7.7, 1 mM EDTA, 10 mM β-mercaptoethanol, 0.5 mM PMSF). The Amicon-concentrated protein solutions were assayed using Bradford method (protein concentration was 8 mg/ml), then aliquoted and stored at −80°C. Intact protein masses were determined by mass spectrometry (Quattro Premier XE, Micromass) equipped with an electrospray ionization interface at the positive mode using direct infusion of purified proteins at 800 mg/l that were diluted and washed in 50 mM ammonium carbonate solution. The obtained spectra were integrated using the MaxEnt 1 option of MassLynx software. Intact masses of purified proteins were 31,933 and 42,648 Da positively identifying the proteins as His-tagged RhlY and RhlZ, respectively, both lacking their N-terminal fMet amino acid.

Clarified cell lysate of WT PA14 strain and of the mutants Δ*rhlZ* and Δ*rhlYZ* were prepared similarly from cells collected from a 25 ml fresh culture in TSB broth that were then washed and sonicated in lysis buffer (50 mM Tris-Cl, pH 7.7, 1 mM EDTA, 10 mM β-mercaptoethanol, 0.5 mM PMSF) (Davis et al., 2008) to be used fresh or kept at 4°C until use.

Synthesis of *trans*-2-Decenoyl-CoA and Chiral HPLC-MS/MS Assay of Hydratase

trans-2-Decenoyl-Co was synthesized using the mixed anhydride method (Abdel-Mawgoud et al., 2013).

An amount of 2–4 μg of pure enzyme and/or 20 μl of clarified cell lysates was used for the activity assays. Enzymatic reaction set up and enzyme product assay using chiral HPLC-MS/MS were conducted as described by Abdel-Mawgoud et al. (2013).

Chromatographic Analysis of Rhamnolipids, 3-Hydroxy Fatty Acids, and PHA

RLs were analyzed using an HPLC coupled with MS/MS as described previously (Déziel et al., 1999). 3-Hydroxy fatty acids were analyzed using gas chromatography-mass spectrometry (GC/MS). PHA was analyzed by the GC/MS estimation of 3-hydroxy fatty acids pool as well as by estimation of the *trans*-sectional area of PHA granules as visualized by transmission

electron microscope. Full details are described in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2013.11.010.

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