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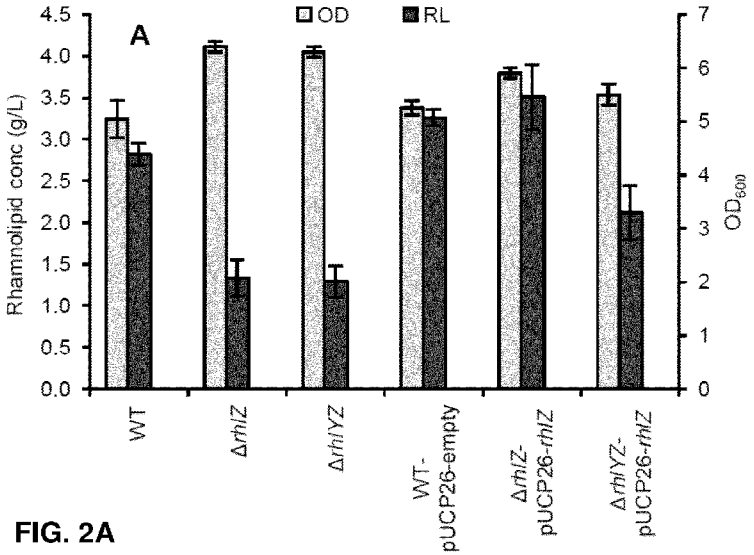

FIG. 2A

(57) Abstract: Novel methods and agents for modulating the production of rhamnolipids (RLs) by a microorganism, through the modulation of the expression and/or activity of R-specific enoyl-CoA hydratases/isomerases (ECH/I), are described. Assays to identify agents that may be useful for modulating the production of RLs by a microorganism are also described.

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MODULATION OF THE BETA-OXIDATION PATHWAY IN THE CONTROL OF RHAMNOLIPID PRODUCTION

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of United States provisional application serial No. 61/915,049 filed December 12, 2013, which is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

[0002] The present invention relates to the production of rhamnolipids (RLs) by microorganisms, and more particularly to the modulation thereof for different applications.

BACKGROUND OF THE INVENTION

[0003] Rhamnolipids are glycolipidic biosurfactants produced by various bacterial species. The bacterium *Pseudomonas aeruginosa* is the best-studied producer of rhamnolipids (RLs), surface-active, multipurpose amphiphilic molecules. RLs are produced as mixtures of congeners composed of 1 or 2 rhamnose moieties linked to a dimer of *R*-3-hydroxyfatty acids with chain lengths of $C_{12\pm4}$ (Déziel *et al.*, 2000).

[0004] RLs are synthesized from two precursors, *R*-3-hydroxyfatty acids and L-rhamnose, by the actions of three enzymes: *RhlA*, *RhlB*, and *RhlC*. *RhlA* dimerizes *R*-3-hydroxyfatty 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- (Rha-C_{10}-C_{10}) and di-RL (Rha-Rha-C_{10}-C_{10}), respectively (Ochsner *et al.*, 1994; Rahim *et al.*, 2001).

[0005] A wide diversity of rhamnolipid congeners and homologues (about 60) that are produced at different concentrations by various *Pseudomonas* species and by bacteria belonging to other families, classes, or even phyla (Abdel-Mawgoud *et al.*, 2010). For example, various *Burkholderia* species have been shown to produce rhamnolipids that have longer alkyl chains than those produced by *P. aeruginosa*.

[0006] Rhamnolipids promote the uptake and biodegradation of poorly soluble substrates, act as immune modulators and virulence factors, have antimicrobial activities, and are involved in surface motility and in bacterial biofilm development. Given the role of rhamnolipids as virulence factors

contributing to pathogenic infection by RL-producing microorganisms such as *Pseudomonas aeruginosa*, the inhibition of the production of RLs constitutes a therapeutic strategy for attenuating the virulence of RL-producing microorganisms.

[0007] 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).

[0008] There is thus a need for a better understanding of the metabolic regulation of RLs, and for novel approaches to modulate the biosynthesis of RLs.

[0009] The present description refers to a number of documents, the content of which is herein incorporated by reference in their entirety.

SUMMARY OF THE INVENTION

[0010] In an aspect, the present invention provides a method for decreasing the production of rhamnolipids (RLs) by a cell, for example a microorganism, said method comprising inhibiting the expression and/or activity of one or more *R*-specific enoyl-CoA hydratases/isomerases (ECH/I) in said cell.

[0011] In another aspect, the present invention provides a method for attenuating the virulence of a rhamnolipid-producing microorganism, said method comprising inhibiting the expression and/or activity of one or more *R*-specific enoyl-CoA hydratases/isomerases (ECH/I) in said rhamnolipid-producing microorganism.

[0012] In another aspect, the present invention provides the use of an inhibitor of *R*-specific enoyl-CoA hydratases/isomerases (ECH/I) for decreasing the production of rhamnolipids (RLs) by a cell, for example a microorganism.

[0013] In another aspect, the present invention provides the use of an inhibitor of *R*-specific enoyl-CoA hydratases/isomerases (ECH/I) for the preparation of a medicament for decreasing the production of rhamnolipids (RLs) by a cell, for example a microorganism.

[0014] In another aspect, the present invention provides the use of an inhibitor of *R*-specific enoyl-CoA hydratases/isomerases (ECH/I) for attenuating the virulence of a rhamnolipid-producing

microorganism.

[0015] In another aspect, the present invention provides the use of an inhibitor of *R*-specific enoyl-CoA hydratases/isomerases (ECH/I) for the preparation of a medicament for attenuating the virulence of a rhamnolipid-producing microorganism.

[0016] In an embodiment, the above-mentioned inhibiting comprises contacting said microorganism with an inhibitor of said one or more *R*-specific ECH/I.

[0017] In an embodiment, the above-mentioned inhibitor is an antibody specifically binding to said one or more *R*-specific ECH/I. In a further embodiment, the above-mentioned antibody is monoclonal.

[0018] In another embodiment, the above-mentioned inhibitor is an inactive or dominant-negative form of said one or more *R*-specific ECH/I.

[0019] In another embodiment, the above-mentioned inhibitor is an RNA interference agent targeting said one or more ECH/I. In a further embodiment, the above-mentioned RNA interference agent is a shRNA or siRNA.

[0020] In an embodiment, the one or more *R*-specific ECH/I comprise an amino acid sequence having at least 50%, 60%, 70%, 80%, 90%, 95% or 100% identity with the amino acid sequence of **FIG. 15A** (SEQ ID NO:2), **16A** (SEQ ID NO:4) and/or **17A** (SEQ ID NO:6).

[0021] In another aspect, the present invention provides a method for increasing the production of rhamnolipids (RLs) by a cell, for example a microorganism, said method comprising increasing the expression and/or activity of one or more *R*-specific enoyl-CoA hydratases/isomerases (ECH/I) in said cell. In an embodiment, the method comprises introducing into said cell one or more nucleic acids encoding said one or more *R*-specific ECH/I. In an embodiment, the method further comprises introducing one or more nucleic acids encoding one or more polypeptides involved in the RL biosynthetic pathway. In a further embodiment, the above-mentioned one or more polypeptides involved in the RL biosynthetic pathway are rhamnosyl-transferases, in yet a further embodiment RhlB and/or RhlC. In an embodiment, the one or more nucleic acids encode the *R*-specific ECH/I defined above. In a further embodiment, the above-mentioned one or more nucleic acids comprise a sequence having at least 50%, 60%, 70%, 80%, 90%, 95% or 100% identity with the nucleotide of **FIG. 15B** (SEQ ID NO:1), **16B** (SEQ ID NO:3) and/or **17B** (SEQ ID NO:5).

[0022] In an embodiment, the above-mentioned method is an *in vivo* method. In an embodiment, the above-mentioned method is an *in vitro* method.

[0023] In another aspect, the present invention provides a method for determining whether an agent may be useful for (i) inhibiting the production of rhamnolipids (RLs) by a cell, for example a microorganism and/or (ii) attenuating the virulence of a rhamnolipid-producing microorganism, said method comprising determining whether said agent inhibits the expression and/or activity of one or more *R*-specific enoyl-CoA hydratases/isomerases (ECH/I). In an embodiment, the method comprises (a) contacting said agent with said one or more *R*-specific ECH/I and (b) determining whether said agent inhibits the expression and/or activity of said one or more *R*-specific ECH/I, wherein the inhibition of said expression and/or activity is indicative that said agent may be useful for (i) inhibiting the production of RLs by a cell and/or (ii) attenuating the virulence of a rhamnolipid-producing microorganism.

[0024] In another aspect, the present invention provides a method for determining whether an agent may be useful for increasing the production of rhamnolipids (RLs) by a cell, for example a microorganism, said method comprising determining whether said agent increases the expression and/or activity of one or more *R*-specific enoyl-CoA hydratases/isomerases (ECH/I). In an embodiment, the method comprises (a) contacting said agent with said one or more *R*-specific ECH/I and (b) determining whether said agent increases the expression and/or activity of said one or more *R*-specific ECH/I, wherein the increase of said expression and/or activity is indicative that said agent may be useful for increasing the production of rhamnolipids (RLs) by a cell.

[0025] In an embodiment, the above method is a cell-free method.

[0026] In another embodiment, the above method is performed in a cell-based system, i.e. the one or more *R*-specific ECH/I are expressed in a cell. In a further embodiment, the one or more *R*-specific ECH/I comprise an amino acid sequence having at least 50%, 60%, 70%, 80%, 90%, 95% or 100% identity with the amino acid sequence of **FIGs. 15A** (SEQ ID NO:2), **16A** (SEQ ID NO:4) and/or **17A** (SEQ ID NO:6).

[0027] In an embodiment, the microorganism is of the *Pseudomonas* genus, in a further embodiment *Pseudomonas aeruginosa*, *e.g.*, *P. aeruginosa* strain PA14.

[0028] Other objects, advantages and features of the present invention will become more apparent upon reading of the following non-restrictive description of specific embodiments thereof, given by way of example only with reference to the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

[0029] In the appended drawings:

[0030] **FIGs. 1A** and **1B** show that specific inhibitors of β-oxidation decrease RLs production in *P. aeruginosa* strain PA14. **FIG. 1A:** Dose-dependent inhibition of RLs and HAA production by 2-bromooctanoic acid. **FIG. 1B**: 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 ± S.D.

[0031] **FIGs. 2A** and **2B** show that *RhlY* and *RhlZ* are implicated in RLs biosynthesis by controlling the supply of lipid precursors. **FIG. 2A**: Deletion mutants, Δ *RhlYZ* and Δ*RhlZ* have similarly reduced RLs production. Complementation of Δ*RhlYZ* with *RhlZ* only partially restores RLs production. **FIG. 2B**: *RhlYZ* is the main supplier of 3-hydroxyalkanoates ($C_{10\pm2}$). Wild-type 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 ± S.D.

[0032] **FIGs. 3A** to **3F** show that *RhlZ* is an *R*-specific enoyl-CoA hydratase/isomerase (ECH/I) whose activity is modulated by *RhlY*. The purified *RhlZ* alone did not catalyze any 3-hydroxydecanoate formation from trans-2-decenoyl-CoA, therefore, **FIG. 3A**: Total cell lysate of WT was added in conjunction with *RhlZ* and this caused enrichment of the *R*-3-hydroxydecanoate compared to the *S*-enantiomer in comparison to the total cell lysate of WT alone as in **FIG. 3B**, which produced a nearly racemic mixture of 3-hydroxydecanoate. **FIG. 3C, 3D**: The same was observed with total cell lysate of the mutant Δ*RhlZ*. **FIG. 3E, 3F**: 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.

[0033] **FIG. 4** shows that *RhlZ* is an *R*-specific ECH/I and is inhibited by a metabolite of 2-bromooctanoate (2-Br-C_8). In *in vitro* enzymatic assays in the presence of WT cell lysate, *RhlZ* 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 ± S.D.

[0034] **FIG. 5** shows an 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 hour at 4°C, the mixture was then subjected to Nickel affinity chromatographic

purification using FPLC using the same gradient of imidazole as previously done for purification of *RhlZ* (**FIG. 14A**). Lane (**1**) is the purified *RhlZ*-His_6 alone (bait). Lane (**2**) is the control lysate which 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**) as well as by protein sequencing.

[0035] **FIGs. 6A** and **6B** show that RLs and PHA share the same 3-hydroxyalkanoate pool. Deletion of *RhlYZ* results in a decrease in PHA accumulation, as estimated by GC-MS hydrolyzed intracellular 3-hydroxyfatty acids (C_8 to C_{12}) (**FIG. 6A**) as well as by 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 (**FIG. 6B**). **FIG. 6C**: 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. **FIG. 6D**: 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 = 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 ± S.D.

[0036] **FIG. 7** is a proposed model of metabolic supply for 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. With a soluble carbon source like glycerol, *de novo* synthesis of fatty acids via FASII forms fatty acids that are then 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 synthesis of HAAs then RLs. Although never demonstrated *in vivo*, a direct precursor supply from FAS II could also be contributing in RL 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.

[0037] **FIGs. 8A** and **8B** show the effect of different inhibitors of β-oxidation on RLs and HAA production. **FIG. 8A**: 2-bromooctanoate (2-Br-C8) inhibits RLs and HAAs production. **FIG. 8B**: Acrylic and 4-pentenoic acid are not inhibiting RLs or HAAs production. Strain PA14 was grown in MSM-

glycerol for 3 days at 34°C.

[0038] **FIGs. 9A** and **9B** show an investigation of a role of PhaG in RLs production. **FIG. 9A**: Role of PhaG in PHA biosynthesis and possible role in RLs biosynthesis. PhaG is a *R*-3-hydroxydecanoyl-ACP:CoA transacylase that converts the *R*-3-hydroxydecanoyl-ACP intermediate of FAS II into the CoA counterpart to enter in the polyhydroxyalkanoates biosynthesis pathway. 2-bromooctanoate-associated inhibition of RLs was suspected to be mediated by PhaG and not by its inhibition of β-oxidation. **FIG. 9B**: PhaG in not implicated in RL production and thus the 2-bromooctanoate-mediated inhibition of RLs. RL production was unaffected in the knock-out mutant, Δ*phaG* (KO), as well as the transposon mutant Δ*phaG* (Tn) compared to the WT PA14 strain. This indicates that PhaG could not be implicated in RL produciton or be the target of the 2-bromooctanoate-mediated inhibition of RLs. Strains were grown in MSM-glycerol for 3 days at 34°C.

[0039] **FIGs 10A** and **10B** show that isotopic tracing demonstrates that lipid precursor of RLs is directly recovered from β-oxidation. **FIG. 10A**: Prediction of isotopic distribution in lipid chains of RLs upon feeding labeled dodecanoate-12,12,12-d3 as sole carbon source. If β-oxidation is involved in supplying the lipidic precursors for RLs pathway, RLs would be labeled at each lipid chain and would carry an extra 3 Da per chain. If *de novo* fatty acid synthesis (FAS II) is responsible for supplying the lipidic precursors for RLs pathway, RLs will be mainly unlabeled because the fatty acid side chains in this case would be constructed from acetate units released from β-oxidation of the dodecanoate-12,12,12-d3 and which are mostly unlabeled. **FIG. 10B**: Fragmentation pattern of the molecular ion of labeled mono-RLd6, m/z 509 confirms a direct link between β-oxidation and RLs. Fragment ions confirm the identity of the parent molecule as mono-RL-d6 and shows the localization of 3 deuterium atoms in each of the two fatty acid chains of labeled mono-RLs Rha-C10-d3-C10-d3. *P. aeruginosa* PA14 was cultivated for 10 days at 34°C in MSM with dodecanoate-12,12,12-d3 (30 mM) as the sole carbon source.

[0040] **FIG. 11** shows a proposed mechanism of incorporation of deuterium-labeled 3-hydroxydecanoate intermediates of dodecanoate-d23 in RLs. (**Box A**) According to a proposed model, 3-hydroxydecanoate will derive directly from β-oxidation, thus would carry the maximum possible deuterium labeling i.e. +17 Da. (**Box B**) According the model of Zhang *et al.* (2012), the octanoate species of β-oxidation will bypass through FASII to get lengthened by one two-carbon unit. Theoretically, resulting decanoate species would carry +16 and +15 at a ratio of 2:1. This ratio arises from the ratio of mono-labeled malonyl-CoA-d1 to unlabeled malonyl-CoA whose abundance is at 2:1 as a result of the carboxylation of mono-labeled acetyl-CoA-d1 replacing a methyl-carried hydrogen atoms

(two) or deuterium atom (one).

[0041] **FIGs. 12A to 12D** shows isotopic tracing of fragment m/z 317 (first column, A1 to D1) and of fragment m/z 201 (second column, A2 to D2) in different labeled carbon source conditions. Dodecanoate-*d*23 (30 mM) (took 7 days to grow) (**FIGs. 12-A1 and 12-A2**), dodecanoate-*d*23 (0.375%) with glucose (10 g/L) (**FIGs. 12-B1 and 12-B2**), dodecanoate-12,12,12-*d*3 (30 mM) with glucose-13C6 (15 g/L) (**FIGs. 12-C1 and 12-C2**) and tetradecanoate-14,14,14-*d*3 (30 mM) with glucose-13C6 (15 g/L) (**FIGs. 12-D1 and 12-D2**). All presented relative abundances of isotopomers are filtered from the nearby isotopic effects. To identify the nature of labeling in the 3-hydroxydecanoate chains of RLs, RLs were acid hydrolyzed, then freed lipid chains were extracted and derivatized using trimethylsilane and analyzed using GC/MS. Two daughters were traced fragments that would help in the identification and localization of labeling particularly in the first two carbon atoms of the lipid chains.

[0042] **FIGs. 13A** to **13C** show the impact of mutation and overexpression of different predicted enoyl-CoA hydratases. **FIG. 13A: *phaJ2* and *phaJ4***. Mutants of the PHA-associated *R*-specific enoyl-CoA hydratases *phaJ2* and *phaJ4* did not show a decrease in RLs production compared to the WT strain of PA14. **FIG. 13B: *RhlK, RhlY, RhlZ.*** The transposon mutants, PA14_40980 (*rhlK*$^-$), PA14_54640 (*rhlY*$^-$), and the deletion mutant PA14_54660 (Δ*rhlZ*) show reduced RLs production. **FIG. 13C: Overexpression of *RhlZ* on pUCP26.** Unexpectedly, overexpression of *RhlZ* in the WT strain PA14 does not increase RLs production; tetracycline was added to maintain plasmid. Cultures were grown for 3 days in MSM–glycerol at 34°C under rotation.

[0043] **FIG. 14A** and **14B** show the purification of N-terminal 6xHis-tagged recombinant *RhlZ*. Both proteins were purified by nickel affinity chromatography using FPLC. (A) *RhlZ* peak elutes at 160 mM imidazole. It migrated with an apparent molecular mass of 40.6 KDa as determined by SDS-PAGE. (B) RhlY peak elutes at 240 mM imidazole. It migrated with an apparent molecular mass of 29.9 KDa as determined by SDS-PAGE.

[0044] **FIG. 15A** and **15B** show the amino acid (SEQ ID NO: 2) and encoding nucleotide sequences (SEQ ID NO: 1), respectively, of *P. aeruginosa* (strain UCBPP-PA14) *RhlK* (PA14_40980, NCBI-GI: 116049771, UniProt: Q02KW4).

[0045] **FIG. 16A** and **16B** show the amino acid (SEQ ID NO: 4) and encoding nucleotide sequences (SEQ ID NO: 3), respectively, of *P. aeruginosa* (strain UCBPP-PA14) *RhlY* (PA14_54640, NCBI-GI: 116048660, UniProt: Q02HP6).

[0046] **FIG. 17A** and **17B** show the amino acid (SEQ ID NO: 6) and encoding nucleotide sequences (SEQ ID NO: 5), of *P. aeruginosa* (strain UCBPP-PA14) *RhlZ* (PA14_54660, NCBI-GI: 116048659, UniProt: Q02R63).

[0047] **FIGs. 18A** and **18B** show the chemical structure of different identified rhamnolipid congeners and homologues (from Abdel-Mawgoud *et al*., 2010).

[0048] **FIGs. 19A** and **19B** show the taxonomical classification of different bacteria reported to produce rhamnolipids (from Abdel-Mawgoud *et al*., 2010).

[0049] **FIGs. 20A and 20B** show the amino acid (SEQ ID NO: 27) and encoding nucleotide sequences (SEQ ID NO: 26), of *P. aeruginosa* (strain UCBPP-PA14) HAA synthase or Rhamnosyltransferase chain A (*RhlA*) (PA14_19110, NCBI-GI: 116051498, UniProt: Q02QW8).

[0050] **FIGs. 21A and 21B** show the amino acid (SEQ ID NO: 29) and encoding nucleotide sequences (SEQ ID NO: 28), of *P. aeruginosa* (strain UCBPP-PA14) Rhamnosyltransferase 1 or Rhamnosyltransferase chain B (*RhlB*) (PA14_19110, NCBI-GI: 116051497, UniProt: Q02QW7).

[0051] **FIGs. 22A and 22B** show the amino acid (SEQ ID NO: 31) and encoding nucleotide sequences (SEQ ID NO: 30), of *P. aeruginosa* (strain UCBPP-PA14) Rhamnosyltransferase 2 (*RhlC*) (PA14_497600, NCBI-GI: 116049062, UniProt: Q02IV0).

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

[0052] In the studies described herein, the present inventors have shown that the β-oxidation pathway is the predominant source of the precursors of RLs. They have also shown that *R*-specific enoyl-CoA hydratases/isomerases (ECH/I) are involved in the production of these precursors, and consequently of RLs, and thus that the modulation of the expression and/or activity of *R*-specific ECH/I has an effect on the production of RLs by RL-producing microorganisms such as *Pseudomonas aeruginosa*. The studies described herein also demonstrate that the pool of precursors synthesized through the activity of *R*-specific ECH/I is shared between RLs and polyhydroxyalkanoates (PHA), and thus that the modulation of the expression and/or activity of *R*-specific ECH/I has also an effect on the production of PHA.

Inhibition of enoyl-CoA hydratases/isomerases (ECH/I)

[0053] Accordingly, in an aspect, the present invention provides a method for decreasing the production of rhamnolipids (RLs) and/or polyhydroxyalkanoates (PHAs) by a microorganism (*e.g.*, a

rhamnolipid- and/or polyhydroxyalkanoate-producing microorganism), said method comprising inhibiting (decreasing, reducing) the expression and/or activity of one or more *R*-specific enoyl-CoA hydratases/isomerases (ECH/I) in said microorganism.

[0054] In another aspect, the present invention provides a method for decreasing the production of rhamnolipids (RLs) by a rhamnolipid-producing microorganism, said method comprising inhibiting the expression and/or activity of one or more *R*-specific enoyl-CoA hydratases/isomerases (ECH/I) in said microorganism.

[0055] In another aspect, the present invention provides a method for decreasing the production of polyhydroxyalkanoates (PHA) by a PHA-producing microorganism, said method comprising inhibiting the expression and/or activity of one or more *R*-specific enoyl-CoA hydratases/isomerases (ECH/I) in said microorganism.

[0056] In an embodiment, the above inhibition comprises contacting the microorganism with an inhibitor of said one or more *R*-specific ECH/I.

[0057] In another aspect, the present invention provides the use of an inhibitor of *R*-specific enoyl-CoA hydratases/isomerases (ECH/I) for decreasing the production of rhamnolipids by a rhamnolipid-producing microorganism.

[0058] In another aspect, the present invention provides the use of an inhibitor of *R*-specific enoyl-CoA hydratases/isomerases (ECH/I) for the preparation of a medicament for decreasing the production of rhamnolipids by a rhamnolipid-producing microorganism.

[0059] In another aspect, the present invention provides the use of an inhibitor of *R*-specific enoyl-CoA hydratases/isomerases (ECH/I) for decreasing the production of PHA by a microorganism.

[0060] In another aspect, the present invention provides the use of an inhibitor of *R*-specific enoyl-CoA hydratases/isomerases (ECH/I) for the preparation of a medicament for decreasing the production of PHA by a microorganism.

[0061] The term “rhamnolipids” as used herein refers to glycolipidic biosurfactants (or surface-active molecules) produced by various bacterial species, and which generally comprises rhamnose moieties (glycon part) and lipid moieties (aglycon part) linked to each other via an O-glycosidic linkage.

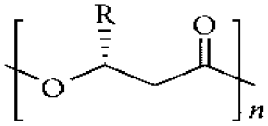
[0062] The glycon part is typically composed of one (mono-RLs) or two (di-RLs) rhamnose moieties linked to each other through an alpha-1,2-glycosidic linkage. The aglycon part, however, is

mainly one or two (in few cases three) β-hydroxy fatty acid chains (saturated, mono-, or polyunsaturated and of different chain length, typically varying from C_8 to C_{16}) linked to each other through an ester bond formed between the β-hydroxyl group of the distal (relative to the glycosidic bond) chain with the carboxyl group of the proximal chain.

[0063] To date, about 60 different RLs have been described (reviewed in Abdel-Mawgoud *et al*., 2010). The structures of these RLs are depicted in **FIGs. 18A-B**.

[0064] The term "rhamnolipid-producing microorganism" as used herein refers to any microorganism, such as bacteria, which has the capacity to synthesize/produce rhamnolipids under suitable conditions. Various bacterial species have been shown to produce RLs, as depicted in **FIGs. 19A-B**. In an embodiment, the rhamnolipid-producing microorganism is of a phylum, class, order, family or genus depicted in **FIGs. 19A-B**. In a further embodiment, the rhamnolipid-producing microorganism is a bacterium of the *Gammaproteobacteria* class. In a further embodiment, the rhamnolipid-producing microorganism is a bacterium of the *Pseudomonadales* order. In a further embodiment, the rhamnolipid-producing microorganism is a bacterium of the Pseudomonadacae family. In a further embodiment, the rhamnolipid-producing microorganism is a bacterium of the *Pseudomonas* genus, such as *P. alcaligenes, P. aeruginosa, P. chlororaphis, P. clemancea, P. collierea, P. fluorescens, P. luteola, P. putida, P. stutzeri and P. teessidea*. In a further embodiment, the rhamnolipid-producing microorganism is *P. aeruginosa*.

[0065] The term polyhydroxyalkanoate (PHA) as used herein refers to biopolyesters composed of hydroxy fatty acids (polyoxoesters). PHAs can consist of short-chain-length hydroxyalkanoic acids (PHA_{SCL}) or medium-chain-length monomers (PHA_{MCL}), depending on strain and culture conditions. More than 150 PHA monomers have been identified. The general structure of polyhydroxyalkanoates (PHAs) and examples of their structural derivatives are depicted below (from Shrivastav *et al*., *Biomed Res Int.* 2013; 2013: 581684).



Poly(3-hydroxyalkanoates) [PHA]

R group		
$-CH_3$	Poly(3-hydroxyalkanoates)	PHA
$-CH_2\text{-}CH_3$	Poly(3-hydroxyvalerate)	PHV
$-(CH_2)_2-CH_3$	Poly(3-hydroxyhexanoate)	PHHex
$-(CH_2)_4-CH_3$	Poly(3-hydroxyoctanoate)	PHO
$-(CH_2)_6-CH_3$	Poly(3-hydroxydecanoate)	PHD
$-CH_2-$ 	Poly(3-hydroxy-5-phenylvalerate)	PHPV

[0066] The term "PHA-producing microorganism" as used herein refers to any microorganism, such as bacteria, which has the capacity to synthesize/produce PHAs under suitable conditions. PHAs are synthesized by a wide range of different Gram-positive (*e.g.*, of the *Bacillus*, *Clostridium*, *Corynebacterium*, *Nocardia*, *Rhodococcus*, *Streptomyces* and *Staphylococcus* genus) and Gram-negative (*Alcaligenes*, *Ralstonia* and *Pseudomonas* genus) bacteria, as well as by some Archaea (notably belonging to the genera *Haloferax*, *Haloarcula*, *Natrialba*, *Haloterrigena*, *Halococcus*, *Haloquadratum, Halorubrum*, *Natronobacterium*, *Natronococcus* and *Halobacterium*) (see Poli *et al.*, Archaea 2011: 2011: 693253; Singh *et al.*, *Microbial Cell Factories* 2009, 8:38), and are deposited as insoluble cytoplasmic inclusions.

[0067] The term "inhibiting" or ("decreasing", "reducing") refers for example to a decrease of at least 10%, in embodiments a decrease of at least 20, 30, 40, 50, 60, 70, 80, 90 or 100% (complete inhibition) relative to a native condition. For example, "decreasing the production of RLs" refers to a lower production of RLs by the microorganism relative to a corresponding "native" microorganism, i.e. in which an *R*-specific enoyl-CoA hydratase/isomerase has not been inhibited.

[0068] The term *R*-specific enoyl-CoA hydratases/isomerases (ECH/I) (also referred to as crotonases) as used herein refers to any enzyme capable of converting a *trans*-enoyl-CoA precursor to an *R*-hydroxyfatty acid (*e.g.*, capable of converting a *trans*-2-enoyl-CoA to an *R*-3-hydroxyfatty acid). "*R*-specific" means that the ECH/I generates a RL/PHA precursor in the *R* configuration (*i.e.*, the β-hydroxyl groups of the fatty acid chains are present in the *R*-configuration). *R*-specific ECH/I are also referred to as ECH type 2 or ECH-2 (whereas *S*-specific ECH/I are referred to as ECH type 1 or ECH-1).

[0069] In an embodiment, the *R*-specific ECH/I comprises the amino acid sequence depicted in **FIGs. 15A** (SEQ ID NO:2), **16A** (SEQ ID NO:4) and **17A** (SEQ ID NO:6) (herein referred to as *RhlK*, *RhlY* and *RhlZ*, respectively), or a sequence having at least 50, 60, 70, 80, 85, 90, 95, 96, 97, 98 or 99% identity with the amino acid sequence depicted in **FIGs. 15A** (SEQ ID NO:2), **16A** (SEQ ID NO:4) and **17A** (SEQ ID NO:6) (and exhibiting hydratase and/or isomerase activity), or with the active domain thereof (i.e. the domain responsible for the hydratase and/or isomerase activity). "Identity" refers to sequence similarity between two polypeptide or nucleic acid molecules (or fragments thereof). Identity can be determined by comparing each position in the aligned sequences. A degree of identity between amino acid or nucleic acid sequences is a function of the number of identical or matching amino acids or nucleotides at positions shared by the sequences. As used herein, a given percentage of identity between sequences denotes the degree of sequence identity in optimally aligned sequences. Optimal alignment of sequences for comparisons of identity may be conducted using a variety of algorithms, such as the local homology algorithm of Smith and Waterman, 1981, *Adv. Appl. Math* **2**: 482, the homology alignment algorithm of Needleman and Wunsch, 1970, *J. Mol. Biol.* **48**: 443, the search for similarity method of Pearson and Lipman, 1988, *Proc. Natl. Acad. Sci. USA* **85**: 2444, and the computerised implementations of these algorithms (such as GAP, BESTFIT, FASTA and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, Madison, WI, U.S.A.). Sequence identity may also be determined using the BLAST algorithm, described in Altschul *et al.*, 1990, *J. Mol. Biol.* **215**:403-10 (using the published default settings). Software for performing BLAST analysis may be available through the National Center for Biotechnology Information. The BLAST algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence that either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighbourhood word score threshold. Initial neighbourhood word hits act as seeds for initiating searches to find longer HSPs. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Extension of the word hits in each direction is halted when the following parameters are met: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T and X determine the sensitivity and speed of the alignment. The BLAST program may use as defaults a word length (W) of 11, the BLOSUM62 scoring matrix (Henikoff and Henikoff, 1992, *Proc. Natl. Acad. Sci. USA* **89**: 10915-10919) alignments (B) of 50, expectation (E) of 10 (or 1 or 0.1 or 0.01 or 0.001 or 0.0001), M=5, N=4, and a comparison of both strands. One measure of the statistical similarity between two sequences using the BLAST algorithm is the smallest sum probability (P(N)), which provides an

indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. In another embodiment, the *R*-specific ECH/I is a homolog of *RhlK*, *RhlY* and/or *RhlZ* from another *Pseudonomas aeruginosa* strain. In another embodiment, the *R*-specific ECH/I is an ortholog of *RhlK*, *RhlY* and/or *RhlZ*. An ortholog is a protein with a sequence that has at least a portion with similarity/identity to a portion of the sequence of another protein, but found in a different species (*e.g.*, in another bacterial species or in another *Pseudonomas* species). An ortholog and the other protein originated by vertical descent from a single protein of a common ancestor. In an embodiment, the ortholog encodes a protein that has at least about 50%, such as at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85, 90, 95, 96, 97, 98 or 99% identity of the total length of the sequence of the protein.

[0070] In an embodiment, the above-mentioned method comprises inhibiting the expression and/or activity of both *RhlY* and *RhlZ*, or orthologs/homologs thereof.

[0071] Other *RhlK* proteins having at least 50% amino acid sequence identity with SEQ ID NO: 2 include *RhlK* from, for example, one or more strains of *Pseudomonas aeruginosa, Pseudomonas putida, Pseudomonas plecoglossicida, Pseudomonas monteilii, Pseudomonas taiwanensis, Pseudomonas entomophila, Pseudomonas stutzeri, Pseudomonas chloritidismutans, Pseudomonas fluorescens, Pseudomonas brassicacearum, Pseudomonas moraviensis, Pseudomonas corrugate,* and *Pseudomonas chlororaphis* (see UNIPROT Cluster ID No. UniRef50_Q88fQ7 - Enoyl-CoA hydratase/isomerase family protein for the list and UniProt reference Nos. of *RhlK* proteins having at least 50% amino acid sequence identity with SEQ ID NO: 2).

[0072] Other *RhlY* proteins having at least 50% amino acid sequence identity with SEQ ID NO: 4 include *RhlY* from, for example, one or more strains of *Pseudomonas aeruginosa, Azotobacter vinelandii*, *Burkholderia caribensis, Burkholderia phenoliruptrix, Burkholderia ambifaria, Burkholderia dolosa , Burkholderia pseudomallei, Burkholderia multivorans, Burkholderia cenocepacia, Vibrio caribbenthicus, Vibrio alginolyticus, Vibrio parahaemolyticus, Herbaspirillum lusitanum, Herbaspirillum frisingense, Pseudomonas stutzeri, Pseudomonas putrefaciens, Pseudomonas cepacia, Shewanella waksmanii, Shewanella oneidensis, Shewanella baltica, Burkholderia graminis, Burkholderia xenovorans, Shewanella decolorationis, Marinobacter santoriniensis, Amphritea japonica, marine gamma proteobacterium, Photobacterium profundum, Pseudoalteromonas lipolytica, Pseudoalteromonas luteoviolacea, Pseudoalteromonas rubra, Pseudoalteromonas piscicida, Pseudoalteromonas citrea, Pseudoalteromonas flavipulchra Lysobacter antibioticus, Lysobacter capsici, Colwellia piezophila, Janthinobacterium agaricidamnosum, Endozoicomonas elysicola, Shewanella*

denitrificans, Oceanospirillum beijerinckii, Ferrimonas kyonanensis, Pseudomonas fulva, Xanthomonas campestris, Xanthomonas hortorum, Xanthomonas cassava, Simiduia agarivorans, Shewanella woodyi, Shewanella sedimini, and *Psychrobacter lutiphocae* (see UNIPROT Cluster ID No. UniRef50_C1DQQ6 - Enoyl-CoA hydratase/isomerase family protein for the list and UniProt reference Nos. of *RhlY* proteins having at least 50% amino acid sequence identity with SEQ ID NO: 4).

[0073] Other *RhlZ* proteins having at least 50% amino acid sequence identity with SEQ ID NO: 6 include *RhlZ* from, for example, one or more strains of *Pseudomonas aeruginosa, Pseudomonas fluorescens, Pseudomonas putida, Pseudomonas taiwanensis, Pseudomonas vranovensis, Pseudomonas mosselii, Pseudomonas denitrificans, Pseudomonas syringae, Pseudomonas chlororaphis, Pseudomonas stutzeri, Marinobacterium litorale, Azoarcus toluclasticus, Afipia felis, Halotalea alkalilenta,* and *Sphaerotilus natans* (see UNIPROT Cluster ID No. UniRef50_V9T1M4 - Enoyl-CoA hydratase for the list and UniProt reference Nos. of *RhlZ* proteins having at least 50% amino acid sequence identity with SEQ ID NO: 6).

[0074] The *R*-specific ECH/I inhibitor includes any compound able to negatively affect the activity of one or more *R*-specific ECH/I by reducing for example its expression (*i.e.*, at the transcriptional and/or translational level), the level of one or more ECH/I mRNA and/or protein, or an activity associated with one or more ECH/I. It includes intracellular as well as extracellular inhibitors. Without being so limited, such inhibitors include RNA interference agents (siRNA, shRNA, miRNA), antisense molecules, ribozymes, proteins (*e.g.*, dominant negative, inactive variants), peptides, small molecules, antibodies, antibody fragments, etc.

[0075] In the studies described herein, the present inventors provide evidence that *RhlZ* and *RhlY* interact, and that this interaction is involved in *RhlZ* activity. Accordingly, in an embodiment, the ECH/I inhibitor blocks or interfere with the interaction between *RhlZ* and *RhlY*, or homologs/orthologs thereof.

[0076] In an embodiment, the *R*-specific ECH/I inhibitor is a neutralizing antibody directed against (or specifically binding to) one or more *R*-specific ECH/I polypeptides. The term “antibody” or “immunoglobulin” is used in the broadest sense, and covers monoclonal antibodies (including full-length monoclonal antibodies), polyclonal antibodies, humanized antibodies, CDR-grafted antibodies, chimeric antibodies, multispecific antibodies, and antibody fragments so long as they exhibit the desired biological activity (*e.g.*, neutralizing an activity of the *R*-specific ECH/I polypeptide). Antibody fragments comprise a portion of a full length antibody, generally an antigen binding or variable region thereof.

Examples of antibody fragments include Fab, Fab', F(ab')2, and Fv fragments, diabodies, linear antibodies, single-chain antibody molecules, single domain antibodies (*e.g.*, from camelids), shark NAR single domain antibodies, and multispecific antibodies formed from antibody fragments. Antibody fragments can also refer to binding moieties comprising CDRs or antigen binding domains including, but not limited to, V_H regions (V_H, V_H-V_H), anticalins, PepBodies, antibody-T-cell epitope fusions (Troybodies) or Peptibodies. In an embodiment, the antibody is a monoclonal antibody. In another embodiment, the antibody is a humanized or CDR-grafted antibody.

[0077] In general, techniques for preparing antibodies (including monoclonal antibodies and hybridomas) and for detecting antigens using antibodies are well known in the art (Campbell, 1984, In "Monoclonal Antibody Technology: Laboratory Techniques in Biochemistry and Molecular Biology", Elsevier Science Publisher, Amsterdam, The Netherlands) and in Harlow et al., 1988 (in: Antibody A Laboratory Manual, CSH Laboratories).

[0078] Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (s.c.), intravenous (i.v.) or intraperitoneal (i.p.) injections of the relevant antigen (e.g., an *R*-specific ECH/I polypeptide, or a fragment thereof) with or without an adjuvant. It may be useful to conjugate the relevant antigen to a protein that is immunogenic in the species to be immunized, *e.g.*, keyhole limpet hemocyanin (KLH), serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, $SOCl_2$, or $R^1N=C=NR$, where R and R^1 are different alkyl groups.

[0079] Animals may be immunized against the antigen (an *R*-specific ECH/I polypeptide or a fragment thereof), immunogenic conjugates, or derivatives by combining the antigen or conjugate (*e.g.*, 100 µg for rabbits or 5 µg for mice) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with the antigen or conjugate (*e.g.*, with 1/5 to 1/10 of the original amount used to immunize) in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, for conjugate immunizations, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

[0080] Monoclonal antibodies may be made using the hybridoma method first described by Kohler et al., Nature, 256: 495 (1975), or may be made by recombinant DNA methods (e.g., U.S. Patent No. 6,204,023). Monoclonal antibodies may also be made using the techniques described in U.S. Patent Nos. 6,025,155 and 6,077,677 as well as U.S. Patent Application Publication Nos. 2002/0160970 and 2003/0083293.

[0081] In the hybridoma method, a mouse or other appropriate host animal, such as a rat, hamster or monkey, is immunized to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the antigen used for immunization. Alternatively, lymphocytes may be immunized *in vitro*. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell. The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

[0082] A human chimeric antibody can be produced in the following manner. cDNA encoding heavy chain variable region (VH) and light chain variable region (VL) obtained from a hybridoma derived from non-human animal cells producing monoclonal antibodies, the cDNA is inserted to each of expression vectors for animal cells having DNA encoding a heavy chain constant region (CH) and light chain constant region (CL) of a human antibody so as to construct a human chimeric antibody expression vector, and this vector is introduced to animal cells to express the human chimeric antibody.

[0083] A humanized antibody refers to an antibody which is obtained by grafting the amino acid sequence of the complementary determining region (CDR) of VH and VL of a non-human animal antibody to CDR corresponding to VH and VL of a human antibody. The region other than CDR of VH and VL is called a framework region (hereinbelow, described as "FR"). A humanized antibody can be produced in the following manner. cDNA encoding an amino acid sequence of VH which consists of an amino acid sequence of CDR of VH of a non-human antibody and an amino acid sequence of FR of VH of any human antibody, and cDNA encoding an amino acid sequence of VL which consists of an amino acid sequence of CDR of VL of a non-human animal antibody and an amino acid sequence of FR of VL of any human antibody are constructed, these cDNAs are inserted respectively into expression vectors for animal cells having DNA encoding CH and CL of a human antibody so as to construct a humanized antibody expression vector, and this vector is inserted into animal cells to express the humanized

antibody.

[0084] Based on the sequences of *R*-specific ECH/I polypeptides (see, *e.g.*, **FIGs. 15A** - SEQ ID NO:2), **16A** - SEQ ID NO:4 and **17A** - SEQ ID NO:6), the skilled person would be able to generate antibodies directed against this polypeptide, which in turn may be used to neutralize its activity.

[0085] In an embodiment, the *R*-specific ECH/I inhibitor is an inactive *R*-specific ECH/I polypeptide (*e.g.*, dominant negative), or a nucleic acid encoding an inactive *R*-specific ECH/I polypeptide. Such an inactive polypeptide may compete with endogenous *R*-specific ECH/I and inhibits its activity.

[0086] In another embodiment, the *R*-specific ECH/I inhibitor is an RNA interference agent targeting an mRNA encoding one or more *R*-specific ECH/I. The term "RNA interference agent" as used herein refers to molecules that specifically binds to a target mRNA and induces its degradation (usually through the RNA-induced silencing complex (RISC) or interferes with its translation, and includes for example microRNA (miRNA) molecules, antisense molecules, small interfering RNA (siRNA) molecules and small/short hairpin RNA (shRNA). Chemically modified nucleosides, such as 2'-substituted arabinonucleosides (*e.g.*, 2'F-ANA) and 2'-substituted RNA (*e.g.*, 2'F-RNA), may be used for incorporation into RNA interference agents to enhance one or more properties, such as nuclease resistance, pharmacokinetics or affinity for a target RNA.

[0087] The RNA interference agent may be expressed from recombinant viral vectors, such as vectors derived from adenoviruses, adeno-associated viruses, lentiviruses, retroviruses, herpesviruses, and the like. Such vectors typically comprise a sequence encoding an RNA interference agent of interest and a suitable promoter operatively linked to the RNA interference agent for expressing the RNA interference agent. The vector may also comprise other sequences, such as regulatory sequences, to allow, for example, expression in a specific cell/tissue/organ, or in a particular intracellular environment/compartment. Methods for generating, selecting and using viral vectors are well known in the art.

[0088] Based on the sequences of nucleic acids encoding *R*-specific ECH/I polypeptides (see, *e.g.*, **FIGs. 15B** - SEQ ID NO:1, **16B** - SEQ ID NO:3 and **17B** - SEQ ID NO:5), the skilled person would be able to generate RNA interference agent directed against *R*-specific ECH/I mRNAs, which in turn may be used to decrease the expression of *R*-specific ECH/I polypeptides.

[0089] Reagents and kits for performing RNA interference are available commercially from for

example Ambion® Inc. (Austin, TX, USA), New England Biolabs® Inc. (Beverly, MA, USA), Sigma-Aldrich® and Invitrogen® (Carlsbad, CA, USA).

[0090] The present inventors have shown that *RhlZ* is at least partially inhibited by a 2-bromooctanoate-derived metabolite. Accordingly, in another embodiment, the *R*-specific ECH/I inhibitor is an analog of 2-bromooctanoate, for example an enoate (*e.g.*, a C_6 to C_{16} enoate) substituted with one or more halogens.

Therapeutic applications

[0091] In view of the evidence that RLs can act as immunumodulators and virulence factors (reviewed in Abdel-Mawgoud *et al*., 2010), the inhibition of *R*-specific ECH/I, and consequently of RLs production, may be useful for the treatment of infection with RL-producing microorganisms. For example, RLs produced by *P. aeruginosa* are believed to be implicated in biofilm initiation (probably via modulation of cell surface polarity and hence initial cell attachment), in biofilm structure maturation, in biofilm dispersion and as a biofilm shield for immune-protection. Biofilm surface-associated RL layer is suggested to be formed in response to contact with immune cells. RL are also reported to slow tracheal ciliary function, and to disrupt the tight junctions of lung epithelial cells as a prelude to invasion. Furthermore, RLs have been shown to induce rapid necrosis of polymorphonuclear (PMN) leukocytes, to be cytolytic to macrophages or at least to inhibit their normal phagocytic response and to act as heat-stable hemolysins. Accordingly, in another aspect, the present invention provides a method for attenuating the virulence of a rhamnolipid-producing microorganism, said method comprising inhibiting (completely or partially) the expression and/or activity of one or more enoyl-CoA hydratases/isomerases (ECH/I) in said rhamnolipid-producing microorganism. In another aspect, the present invention also provides the use of an inhibitor of enoyl-CoA hydratases/isomerases (ECH/I) for attenuating the virulence of a rhamnolipid-producing microorganism.

[0092] For *in vivo* applications, the amount of the *R*-specific ECH/I inhibitor which is effective for decreasing the production of RLs and/or PHA, and/or for attenuating the virulence of a rhamnolipid-producing microorganism, will depend on several factors including the nature and severity of the disease, the chosen prophylactic/therapeutic regimen, the target site of action, the patient's weight, special diets being followed by the patient, concurrent medications being used, the administration route and other factors that will be recognized by those skilled in the art. The dosage will be adapted by the clinician in accordance with conventional factors such as the extent of the disease, the liver function and different parameters from the patient. Typically, 0.001 to 1000 mg/kg of body weight/day will be

administered to the subject. In an embodiment, a daily dose range of about 0.01 mg/kg to about 500 mg/kg, in a further embodiment of about 0.1 mg/kg to about 200 mg/kg, in a further embodiment of about 1 mg/kg to about 100 mg/kg, in a further embodiment of about 10 mg/kg to about 50 mg/kg, may be used. The dose administered to a patient, in the context of the present invention should be sufficient to effect/induce a beneficial prophylactic and/or therapeutic response in the patient over time (decrease in bacterial load, reduction of tissue damage, reduction of one or more symptoms associated with the infection, increased survival time, etc.). The size of the dose also will be determined by the existence, nature, and extent of any adverse side-effects that accompany the administration. Effective doses may be extrapolated from dose response curves derived from in vitro or animal model test systems. For example, in order to obtain an effective mg/kg dose for humans based on data generated from rat studies, the effective mg/kg dosage in rat may be divided by six.

[0093] In an embodiment, the above-mentioned treatment comprises the use/administration of more than one (i.e. a combination of) active/therapeutic agent, including the above-mentioned *R*-specific ECH/I inhibitor. The combination of prophylactic/therapeutic agents and/or compositions of the present invention may be administered or co-administered (*e.g.*, consecutively, simultaneously, at different times) in any conventional dosage form. Co-administration in the context of the present invention refers to the administration of more than one therapeutic in the course of a coordinated treatment to achieve an improved clinical outcome. Such co-administration may also be coextensive, that is, occurring during overlapping periods of time. For example, a first agent may be administered to a patient before, concomitantly, before and after, or after a second active agent is administered. The agents may in an embodiment be combined/formulated in a single composition and thus administered at the same time. In an embodiment, the one or more active agent(s) is used/administered in combination with one or more agent(s) or treatment currently used to prevent or treat the disorder in question (*e.g.*, agents or treatments currently used in the treatment of infections by rhamnolipid-producing microorganisms, such as *P. aeruginosa*.

[0094] The above-mentioned *R*-specific ECH/I inhibitor may be provided in a composition (e.g., a pharmaceutical composition) comprising a carrier, diluent and/or excipient (e.g., a pharmaceutically acceptable carrier, diluent and/or excipient). As used herein “pharmaceutically acceptable carrier” or “excipient” includes any and all solvents, buffers, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible. The carrier can be suitable, for example, for oral, intravenous, parenteral, subcutaneous, intramuscular, intracranial, intraorbital, ophthalmic, intraventricular, intracapsular, intraspinal, intrathecal, epidural, intracisternal, intraperitoneal, intranasal or pulmonary (*e.g.*, aerosol) administration.

[0095] Pharmaceutically acceptable carriers include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. The use of such media and agents for pharmaceutically active substances is well known in the art (Rowe et al., Handbook of pharmaceutical excipients, 2003, 4th edition, Pharmaceutical Press, London UK). Except insofar as any conventional media or agent is incompatible with the active agent, use thereof in the pharmaceutical compositions of the invention is contemplated.

Increasing the production of RLs and/or PHA

[0096] Biosurfactants are in demand for a wide range of industrial applications as they increase solubility, foaming capacity and lower surface tensions. Biosurfactants are attracting much attention because they represent ecological alternatives to their synthetic counterparts: they exhibit lower toxicity, potentially high activities, and stability at extremes of temperature, pH, and salinity. They have a wide variety of structures, and they can be produced from renewable feedstocks by a wide variety of microorganisms. Most importantly, they are biodegradable, making them environmentally friendly, "green" chemicals. In particular, RLs have been used in chemical and pharmaceutical industry or as emulsifier in cosmetics and foods.

[0097] PHAs are linear polyesters that are biodegradeable and are used in the production of bioplastics.

[0098] The demonstration by the present inventors that *R*-specific ECH/I are involved in the biosynthesis of RLs and PHA provides a novel approach for modulating the production of RLs and PHA through metabolic engineering.

[0099] Accordingly, in another aspect, the present invention provides a method for increasing the production of rhamnolipids by a host cell, said method comprising increasing the expression and/or activity of one or more *R*-specific enoyl-CoA hydratases/isomerases (ECH/I) in said host cell.

[00100] In another aspect, the present invention provides a method for increasing the production of PHA by a host cell, said method comprising increasing the expression and/or activity of one or more *R*-specific enoyl-CoA hydratases/isomerases (ECH/I) in said host cell.

[00101] Increasing the expression and/or activity of one or more *R*-specific ECH/I includes inducing the expression of one or more *R*-specific ECH/I in host cell that do not express one or more endogenous *R*-specific ECH/I (or express *R*-specific ECH/I inappropriate for rhamnolipid production), or increasing the expression of one or more *R*-specific ECH/I in host cell that endogenously expressed one

or more *R*-specific ECH/I. The expression may be induced/increased by either introducing a heterologous *R*-specific ECH/I nucleic acid into a host cell, or increasing/inducing the expression and/or activity of one or more endogenous *R*-specific ECH/I.

[00102] A host cell includes any suitable host cell that is capable of producing RLs and/or PHA (either endogenously or heterologously, i.e. following introduction of one or more genes into the host cell). Accordingly, the present invention envisages as a host cell, preferably a non-pathogenic host cell (*e.g.*, non-pathogenic for humans) including a unicellular host cell such as a fungal host cell, for example, a yeast. The host cell may be a bacterial host cell including non-pathogenic bacterial host cells such as bacterial host cells capable of producing rhamnolipids.

[00103] A host cell according to the invention includes an *R*-specific ECH/I gene (*e.g.*, *RhlK*, *RhlY* and/or *RhlZ* or an ortholog/homolog thereof). The *R*-specific ECH/I gene may be under the control of a heterologous promoter. In some embodiments the *R*-specific ECH/I gene is an endogenous gene of the bacterial host cell. In some embodiments the *R*-specific ECH/I gene is a heterologous gene. The term "heterologous" refers to the relationship between two or more nucleic acid or protein sequences that are derived from different sources. For example, a promoter is heterologous with respect to a transcribable polynucleotide sequence if such a combination is not normally found in nature. In addition, a particular sequence may be "heterologous" with respect to a host cell in that it encodes a protein or is included in a protein, for example a recombinant protein, that is not normally expressed by the host cell. Such a heterologous protein accordingly generally is or has been inserted into the respective host cell, tissue, or species. Accordingly, a heterologous promoter is not normally coupled *in vivo* transcriptionally to the coding sequence of the *R*-specific ECH/I gene.

[00104] In an embodiment, the above-mentioned method comprises increasing the expression and/or activity of at least two *R*-specific ECH/I, preferably *RhlY* and *RhlZ* (or orthologs/homologs) thereof, in the cell.

[00105] A wide variety of transcriptional and translational regulatory sequences may be employed, depending upon the nature of the host, as long as RLs and/or PHAs are produced. The transcriptional and translational regulatory signals may be derived from viral sources, such as adenovirus, bovine papilloma virus, cytomegalovirus or simian virus, where the regulatory signals are associated with a particular gene sequence which has a high level of expression. Alternatively, promoters from mammalian expression products, such as actin, collagen and myosin may be employed. Transcriptional initiation regulatory signals may be selected which allow for repression or activation, so

that expression of the gene sequences can be modulated. Of interest may in some embodiments also be regulatory signals which are temperature-sensitive so that by varying the temperature, expression can be repressed or initiated, or are subject to chemical (such as metabolite) regulation. The term "regulatory sequence" includes controllable transcriptional promoters, operators, enhancers, silencers, transcriptional terminators, 5' and 3' untranslated regions which interact with host cellular proteins to carry out transcription and translation and other elements that may control gene expression including initiation and termination codons. The regulatory sequences can be native (homologous), or can be foreign (heterologous) to the cell and/or the nucleotide sequence that is used. The precise nature of the regulatory sequences needed for gene sequence expression may vary from organism to organism, but shall in general include a promoter region which, in prokaryotes, contains both the promoter (which directs the initiation of RNA transcription) as well as the DNA sequences which, when transcribed into RNA, will signal synthesis initiation. Such regions will normally include those 5'-non-coding sequences involved with initiation of transcription and translation, such as the TATA box, capping sequence or CAAT sequence. These regulatory sequences are generally individually selected for a certain embodiment, for example for a certain cell to be used. The skilled artisan will be aware that proper expression in a prokaryotic cell also requires the presence of a ribosome-binding site upstream of the gene sequence-encoding sequence.

[00106] A vector may be employed which is capable of integrating the desired *R*-specific ECH/I gene sequences into the host cell chromosome. Cells which have stably integrated the introduced DNA into their chromosomes can be selected by also introducing one or more markers which allow for selection of host cells which contain the expression vector. The marker may provide for prototrophy to an auxotrophic host, biocide resistance, *e.g.*, antibiotics, or heavy metals, such as copper, or the like. The selectable marker gene sequence can either be directly linked to the DNA gene sequences to be expressed, or introduced into the same cell by co-transformation or co-transfection. Additional elements may also be needed for optimal synthesis of mRNA. These elements may include splice signals, as well as transcription promoters, enhancers, and termination signals.

[00107] The introduced nucleic acid molecule can be incorporated into a plasmid or viral vector capable of autonomous replication in the recipient host. Any of a wide variety of vectors may be employed for this purpose. Factors of importance in selecting a particular plasmid or viral vector include: the ease with which recipient cells that contain the vector may be recognized and selected from those recipient cells which do not contain the vector; the number of copies of the vector which are desired in a particular host; and whether it is desirable to be able to "shuttle" the vector between host cells of different species.

[00108] An expression vector, which may include one or more regulatory sequences and be capable of directing the expression of nucleic acids to which it is operably linked. An operable linkage is a linkage in which a coding nucleotide sequence of interest is linked to one or more regulatory sequence(s) such that expression of the nucleotide sequence sought to be expressed can be allowed. Thus, a regulatory sequence operably linked to a coding sequence is capable of effecting the expression of the coding sequence, for instance in an *in vitro* transcription/ translation system or in a cell when the vector is introduced into the cell. A respective regulatory sequence need not be contiguous with the coding sequence, as long as it functions to direct the expression thereof. Thus, for example, intervening untranslated yet transcribed sequences may be present between a promoter sequence and the coding sequence and the promoter sequence can still be considered "operably linked" to the coding sequence.

[00109] Once the vector or nucleic acid molecule that contains the construct(s) has been prepared for expression, the DNA construct(s) may be introduced into the host cell by any of a variety of suitable means, *i.e.*, transformation, transfection, conjugation, protoplast fusion, electroporation, particle gun technology, calcium phosphate-precipitation, direct microinjection, and the like. After the introduction of the vector, recipient cells are grown in a selective medium, which selects for the growth of vector-containing cells.

[00110] In view of the demonstration that the pool of precursors synthesized through the activity of *R*-specific ECH/I is shared between RLs and PHAs, it might be desirable to reduce the biosynthesis of PHAs to increase the production of RLs, and vice versa.

[00111] *Reduction of the biosynthesis of PHAs to increase the production of RLs*. Thus, in some embodiments the host cell according to the invention is able to produce PHA only to a much lower extent than a corresponding native, wild type host cell. The host cell may for example include a mutation in the gene of an enzyme that catalyzes the formation of PHA from the precursors, or a mutation in a regulatory sequence to which the respective gene is operably linked. The respective mutation may in some embodiments give rise to the formation of a polyhydroxyalkanoic acid synthase that has a reduced activity when compared to a corresponding wild type polyhydroxyalkanoic acid synthase. In some embodiments a capability of the host cell according to the invention to produce PHA is reduced in comparison to a wild type cell, including entirely absent. In some embodiments the host cell according to the invention is incapable of synthesizing PHA. The PHA biosynthetic pathway of the host cell may for example be curtailed, including inactive. The host cell may for example have one or more genes in the PHA synthesis pathway, such as polyhydroxyalkanoic acid synthase gene(s), that is/are disrupted. The

cell may for instance have a knock-out mutation in an endogenous polyhydroxyalkanoic acid synthase gene.

[00112] *Reduction of the biosynthesis of RLs to increase the production of PHAs.* In other embodiments, the host cell according to the invention is able to produce RLs to a lower extent than a corresponding native, wild-type host cell. The host cell may for example include a mutation in the gene of an enzyme that catalyzes the formation of RLs from the precursors (*e.g.*, rhamnosyl-transferases such as *RhlB* and/or *RhlC*), or a mutation in a regulatory sequence to which the respective gene is operably linked. The respective mutation may in some embodiments give rise to the formation of rhamnosyl-transferases that have a reduced activity when compared to corresponding wild type rhamnosyl-transferases. In some embodiments a capability of the host cell according to the invention to produce RLs is reduced in comparison to a wild type cell, including entirely absent. In some embodiments the host cell according to the invention is incapable of synthesizing RLs. The RL biosynthetic pathway of the bacterial host cell may for example be curtailed, including inactive. The host cell may for example have one or more genes in the RL synthesis pathway, such as rhamnosyl-transferase gene(s), that is/are disrupted. The cell may for instance have a knock-out mutation in an endogenous rhamnosyl-transferase gene.

[00113] In embodiments, the method further comprises increasing the expression of one or more genes involved in RL and/or PHA biosynthesis. For example, to increase the production of RLs, the host cell may be modified or treated to produce higher levels of enzymes, *e.g.*, *RhlA*, *RhlB*, and/or *RhlC* (*e.g.*, SEQ ID NOs: 27, 29 and/or 31), or orthologs/homologs thereof (comprising a sequence having at least 50, 60, 70, 80, 85, 90, 95, 96, 97, 98 or 99% identity with the amino acid sequence depicted in SEQ ID NOs: 27, 29 and/or 31, and exhibiting the same enzymatic activity). To increase the production of PHAs, the host cell may be modified or treated to increase higher levels of PHA synthases. In an embodiment, the method further comprises increasing the expression and/or activity of one or more additional proteins/enzymes involves in rhamnolipid production. In an embodiment, the method further comprises increasing the level of one or more substrates/precursors involved in rhamnolipid production.

[00114] A host cell, such as a bacterial host cell, as defined above may be used in a method of producing one or more RLs or PHAs. The host cell is cultured under conditions that allow RL and/or PHA production. A variety of carbon source may be used such as a monosaccharide, *e.g.* glucose, a disaccharide, *e.g.* sucrose, an alcohol, *e.g.* glycerol, an alkane, *e.g.*, *n*-hexadecane, a fatty acid such as caprylic acid (also termed octanoate), vegetable oils (fresh or waste) or mixtures thereof. The bacterial host cell will typically be exposed to a fermentation process. The bacterial host cell may for instance be

in the logarithmic growth phase or in the stationary phase.

[00115] In the method the RL is recovered. Typically the RL is secreted by the bacterial host cell, so that recovering the fermentation/culture medium includes recovering the RLs. Further the method may include enriching, isolating and/or purifying the RLs. The term "enriched" means that the RLs constitute a significantly higher fraction of the total glycolipids, lipids and saccharides present in the solution of interest than in the solution from which it was taken. Enrichment may for instance include membrane filtration, for example for clarification, buffer exchange or concentration purposes. It may also include filtration or dialysis, which may for instance be directed at the removal of molecules below a certain molecular weight, or a precipitation by lowering the pH, or extraction using organic solvents. Chromatography may for example be carried out in the form of a liquid chromatography such as capillary electrochromatography, HPLC (high performance liquid chromatography) or UPLC (ultrahigh pressure liquid chromatography) or as a gas chromatography. The chromatography technique may be a process of column chromatography, of batch chromatography, of centrifugal chromatography or a method of expanded bed chromatography, as well as electrochromatographic, electrokinetic chromatography. It may be based on any underlying separation technique, such as adsorption chromatography, hydrophobic interaction chromatography or hydrophobic charge induction chromatography, size exclusion chromatography (also termed gel-filtration), ion exchange chromatography or affinity chromatography and may also be a method of capillary gas chromatography.

[00116] Methods for extracting RLs are well known in the art and are reviewed in Heyd, M. *et al.* (Development and trends of biosurfactant analysis and purification using rhamnolipids as an example. *Anal Bioanal Chem* **391**, 1579-1590 (2008)) and Abdel-Mawgoud *et al*. (2011). These methods range from those yielding mixtures of different RL congeners to those yielding specific congeners in pure forms. The criteria that govern the selection of a specific recovery method include: (1) the cost associated with the extraction method, which adds to the price of the final product, (2) the proposed purpose of the final product, which influences the level of purity required, and (3) the adaptability of the method to a particular industrial fermentation process. One of the simplest methods of recovery is by acid precipitation (Deziel et al. 1999b; Van Dyke et al. 1993; Zhang and Miller 1992) or aluminum sulfate precipitation (Schenk et al. 1995). Acid precipitation depends on acidification of RL to low pH (*e.g.*, around 2), which neutralizes the negative charges on RLs, making them less soluble in the aqueous phase. Aluminum sulfate precipitates RLs by salting out. The precipitated RLs can then be recovered by centrifugation. Another more commonly used method is recovery by solvent extraction (Lepine et al. 2002; Mata-Sandoval et al. 1999; Schenk et al. 1995). In this method, molecules are precipitated by acidification and then extracted with organic solvents such as ether or ethyl acetate. Acidification is not

a critical step in this method, but it enhances the net yield (Heyd et al. 2008).

[00117] Other methods adapted to downstream processing in continuous fermentative production processes include: adsorption (Dubey et al. 2005), ion exchange chromatography (Abadi et al. 2009; Reiling et al. 1986; Schenk et al. 1995), ultrafiltration (Haussler et al. 1998; Mulligan and Gibbs 1990), and foam fractionation (Gruber 1991; Sarachat et al. 2010). Adsorption methods are based on the use of hydrophobic adsorbent such as Amberlite® XAD®-2 or -16, polystyrene resin that retain hydrophobic (or amphiphilic) substances through hydrophobic interactions. Adsorbed RLs are then released by elution, *e.g.*, with methanol. Ion exchange chromatography exploits the fact that RLs behave as anions at high pHs, which allows their retention on columns of weak anion exchange resins such as (diethylamino)ethyl-Sepharose®. RLs are released from these resins by adding at least 0.6 M NaCl to the equilibration buffer. Yet, this method has been improved by Abadi *et al.* (2009), who applied phospholipid-coated colloidal magnetic nanoparticles ion exchange media for the recovery and purification of RLs from culture mixtures. Ultrafiltration with a membrane cutoff of 10 kDa leads to an almost complete retention of RLs even at neutral pH. Foam fractionation depends on the foaming capabilities of RLs; the foam is directed out of the fermentation vessel to a fractionation column where it collapses in a separate receptacle by the action of acids or shear forces, The water in the film surface, known as the lamella, is then allowed to drain by gravitational force, causing a higher concentration of the surfactant in the collapsed foam (Heyd *et al.* 2008; Sarachat *et al.* 2010).

[00118] Most of the aforementioned methods result in the recovery of mixtures of different RLs congeners. Alternatively, chromatographic methods are usually the best solutions for separation of specific RL congeners in a pure form. These methods, however, work better after application of one of the extraction methods mentioned above. On the small scale, preparative TLC is a good choice (Monteiro et al. 2007; Sim et al. 1997); however, for large scale downstream processing, preparative column chromatography using silica gel is a better option (Burger *et al.* 1966; Monteiro *et al.* 2007). Recrystallization or repurification using TLC can be applied, if necessary (Heyd *et al.* 2008)

[00119] In an embodiment, the method comprises solvent extraction.

[00120] An isolation may include the combination of different methods.

Screening assays for modulators of RL and/or PHA production

[00121] The present inventors have identified *R*-specific enoyl-CoA hydratases/isomerases (ECH/I) as being involved in the biosynthesis of RLs and/or PHAs.

[00122] Accordingly, in another aspect, the present invention also provides a method (screening assay), in an embodiment an *in vitro* method, for determining whether an agent may be useful for (i) inhibiting the production of RLs and/or PHAs by a host cell, such as a rhamnolipid- and/or PHA-producing microorganism and/or (ii) attenuating the virulence of a rhamnolipid-producing microorganism, said method comprising determining whether said agent inhibits the expression and/or activity of one or more *R*-specific ECH/I. In an embodiment, the method comprises (a) contacting said agent with said one or more *R*-specific ECH/I and (b) determining whether said agent inhibits the expression and/or activity of said one or more *R*-specific ECH/I, wherein the inhibition of said expression and/or activity is indicative that said agent may be useful for (i) inhibiting the production of RLs and/or PHAs by the host cell and/or (ii) attenuating the virulence of a rhamnolipid-producing microorganism.

[00123] The present invention also provides a method (screening assay), in an embodiment an *in vitro* method, for determining whether an agent may be useful for increasing the production of RLs and/or PHAs by a host cell, such as a rhamnolipid- and/or PHA-producing microorganism, said method comprising determining whether said agent increases the expression and/or activity of one or more *R*-specific ECH/I. In an embodiment, the method comprises (a) contacting said agent with said one or more *R*-specific ECH/I and (b) determining whether said agent increases the expression and/or activity of said one or more *R*-specific ECH/I, wherein the increase of said expression and/or activity is indicative that said agent may be useful for increasing the production of RLs and/or PHAs by the host cell.

[00124] The determination of the expression and/or activity of *R*-specific ECH/I in the presence (and/or absence) of the test compound may be performed using any known methods to detect nucleic acids or proteins. In embodiments, the expression or activity measured in the presence of the test compound is compared to a control or reference level (*e.g.*, the level obtained in the absence of the test compound) to assess whether the test compound modulates (increases or decreases) the expression or activity of *R*-specific ECH/I.

[00125] The levels of nucleic acid corresponding to an *R*-specific ECH/I can then be evaluated according to the methods disclosed below, *e.g.*, with or without the use of nucleic acid amplification methods. In some embodiments, nucleic acid amplification methods can be used to detect the level of expression of an *R*-specific ECH/I. For example, the oligonucleotide primers and probes may be used in amplification and detection methods that use nucleic acid substrates isolated by any of a variety of well-known and established methodologies (*e.g.*, Sambrook *et al.*, Molecular Cloning, A laboratory Manual, pp. 7.37-7.57 (2nd ed., 1989); Lin *et al.*, in Diagnostic Molecular Microbiology, Principles and

Applications, pp. 605-16 (Persing *et al.*, eds. (1993); Ausubel *et al.*, *Current Protocols in Molecular Biology* (2001 and later updates thereto)). Methods for amplifying nucleic acids include, but are not limited to, for example the polymerase chain reaction (PCR) and reverse transcription PCR (RT-PCR) (see *e.g.*, U.S. Pat. Nos. 4,683,195; 4,683,202; 4,800,159; 4,965,188), ligase chain reaction (LCR) (see, *e.g.*, Weiss, Science 254: 1292-93 (1991)), strand displacement amplification (SDA) (see *e.g.*, Walker *et al*, Proc. Natl. Acad. Sci. USA 89:392-396 (1992); U.S. Pat. Nos. 5,270,184 and 5,455,166), Thermophilic SDA (tSDA) (see *e.g.*, European Pat. No. 0 684 315) and methods described in U.S. Pat. No. 5,130,238; Lizardi *et al.*, *BioTechnol.* **6**:1197-1202 (1988); Kwoh *et al.*, *Proc. Natl. Acad. Sci. USA* 86:1173-77 (1989); Guatelli *et al.*, *Proc. Natl. Acad. Sci.* USA 87:1874-78 (1990); U.S. Pat. Nos. 5,480,784; 5,399,491; U.S. Publication No. 2006/46265. The methods include the use of Transcription Mediated Amplification (TMA), which employs an RNA polymerase to produce multiple RNA transcripts of a target region (see, *e.g.*, U.S. Pat. Nos. 5,480,784; 5,399,491 and US Publication No. 2006/46265).

[00126] The nucleic acid or amplification product may be detected or quantified by hybridizing a labeled probe to a portion of the *R*-specific ECH/I nucleic acid or amplified product. The labeled probe contains a detectable group that may be, for example, a fluorescent moiety, chemiluminescent moiety, radioisotope, biotin, avidin, enzyme, enzyme substrate, or other reactive group. Other well-known detection techniques include, for example, gel filtration, gel electrophoresis and visualization of the amplicons, and High Performance Liquid Chromatography (HPLC). In certain embodiments, for example using real-time TMA or real-time PCR, the level of amplified product is detected as the product accumulates.

[00127] In another embodiment, the expression and/or activity of an *R*-specific ECH/I is measured at the protein level. Methods to measure the amount/level of proteins are well known in the art. Protein levels may be detected directly using a ligand binding specifically to the protein, such as an antibody or a fragment thereof. In embodiments, such a binding molecule or reagent (*e.g.*, antibody) is labeled/conjugated, *e.g.*, radio-labeled, chromophore-labeled, fluorophore-labeled, or enzyme-labeled to facilitate detection and quantification of the complex (direct detection). Alternatively, protein levels may be detected indirectly, using a binding molecule or reagent, followed by the detection of the [protein/ binding molecule or reagent] complex using a second ligand (or second binding molecule) specifically recognizing the binding molecule or reagent (indirect detection). Such a second ligand may be radio-labeled, chromophore-labeled, fluorophore-labeled, or enzyme-labeled to facilitate detection and quantification of the complex. Enzymes used for labeling antibodies for immunoassays are known in the art, and the most widely used are horseradish peroxidase (HRP) and alkaline phosphatase (AP). Examples of binding molecules or reagents include antibodies (monoclonal or polyclonal), natural or

synthetic *R*-specific ECH/I ligands, glycoproteins, monosaccharides (*e.g.*, galactose, galactosamine, lactose) aptamers and the like.

[00128] Examples of methods to measure the amount/level of protein in a sample include, but are not limited to: Western blot, immunoblot, enzyme-linked immunosorbent assay (ELISA), "sandwich" immunoassays, radioimmunoassay (RIA), immunoprecipitation, surface plasmon resonance (SPR), chemiluminescence, fluorescent polarization, phosphorescence, immunohistochemical (IHC) analysis, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry, microcytometry, microarray, antibody array, microscopy (*e.g.*, electron microscopy), flow cytometry, proteomic-based assays, and assays based on a property of the protein including but not limited to ligand binding or interaction with other protein partners, enzymatic activity, fluorescence, etc. For example, the level or activity of *R*-specific ECH/I may be determined by measuring the level of conversion of a substrate of an *R*-specific ECH/I (*e.g.*, a suitable enoyl-CoA substrate such as trans-2-decenoyl-CoA) into a product (an *R*-hydroxyfatty acid such as 3-hydroxydecanoate) (see below).

[00129] In another embodiment, the effect of the test agent on the activity/expression of *R*-specific ECH/I may be assessed using a reporter gene/protein system. For example, a reporter gene may be put under the transcriptional control of a transcriptional control element (*e.g.*, promoter) normally associated with an *R*-specific ECH/I, and the expression of the reporter protein may be measured to determine if the test agent could modulate the transcription of the *R*-specific ECH/I. If in the presence of the test agent the level of the reporter protein is increased, it is an indication that the test agent may be useful to increase the expression of the *R*-specific ECH/I (as the reporter protein is under the transcriptional control of a transcriptional control element normally associated with the the *R*-specific ECH/I. The term “reporter gene” refers to gene encoding a protein (a reporter protein) that can be detected (*e.g.*, by fluorescence, spectroscopy, luminometry, etc.) easily and that is not present normally (endogenously) in the system used. Commonly used reporter proteins include enzymes such as β-galactosidase (encoded by the bacterial gene *lacZ*), luciferase, chloramphenicol acetyltransferase (CAT; from bacteria), GUS (β-glucuronidase), bioluminescent proteins and fluorescent proteins. The method to determine the readout signal from the reporter protein depends from the nature of the reporter protein. For example, for fluorescent reporter proteins, the readout signal corresponds to the intensity of the fluorescent signal. The readout signal may be measured using spectroscopy-, fluorometry-, photometry-, and/or luminometry-based methods and detection systems, for example. Such methods and detection systems are well known in the art.

[00130] In other embodiments, the determination of the effect of the test compound on the

expression and/or activity of *R*-specific ECH/I may be performed indirectly, for example by assessing the production of RLs and/or PHAs by a cell (*e.g.*, using the methods described in the Examples below). In embodiments, the production of RLs and/or PHAs in the presence of the test compound is compared to a control or reference level (*e.g.*, the production measured in the absence of the test compound) to assess whether the test compound modulates (increases or decreases) the expression or activity of *R*-specific ECH/I. Higher levels/production of RLs and/or PHAs in the presence of the test compound being indicative that the test compound increases the expression or activity of *R*-specific ECH/I and vice-versa.

[00131] The method/assay may be a cell-free method/assay or a cell-based method/assay. For the cell-based assay, any cell capable of expressing the *R*-specific ECH/I nucleic acid and protein (or the reporter gene construct described above) may be used in the method/system of the invention. In an embodiment, the above-mentioned cell is a bacterial cell. The cell may be naturally expressing the *R*-specific ECH/I protein, or may be prepared by introducing a nucleic acid encoding the *R*-specific ECH/I or a reporter gene construct (by any transfection, transduction or transformation method), and providing conditions suitable for the expression of the protein. Methods and systems for introducing a nucleic acid into a cell are well known in the art, and include for example conjugation, chemical-based transfection (using calcium phosphate, liposomes, cationic polymers such as DEAE-dextran or polyethylenimine), electroporation, gene gun, viral transduction. Kits for introducing a nucleic acid into a cell are commercially available. The assay may be performed using a native, full-length *R*-specific ECH/I, or an active variant or fragment thereof.

[00132] Test compounds (drug candidates) that may be screened by the method/system of the invention may be obtained from any number of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural or synthetically produced libraries and compounds are readily modified through conventional chemical, physical and biochemical means.

[00133] Screening assay systems may comprise a variety of means to enable and optimize useful assay conditions. Such means may include but are not limited to: suitable buffer solutions, for example, for the control of pH and ionic strength and to provide any necessary components for optimal activity and stability (*e.g.*, protease inhibitors), temperature control means for optimal activity and/or stability of *R*-specific ECH/I, and detection means to enable the detection of its activity. A variety of such

detection means may be used, including but not limited to one or a combination of the following: radiolabelling, antibody-based detection, fluorescence, chemiluminescence, spectroscopic methods (*e.g.*, generation of a product with altered spectroscopic properties), various reporter enzymes or proteins (*e.g.*, horseradish peroxidase, green fluorescent protein), specific binding reagents (*e.g.*, biotin/(strept)avidin), and others.

[00134] The present invention is illustrated in further details by the following non-limiting examples.

Example 1: Materials and Methods

[00135] *Bacterial strains and growth conditions.*

[00136] Bacterial strains and plasmids are described in Table 1.

Table 1: Bacterial strains and plasmids used in the experiments described herein

Strain or plasmid	Description and genotype/Sequence	Source
P. aeruginosa		
PA14 - WT	Wild type strain (WT)	(Lee et al., 2006)
PA14 - Δ*phaG*	Transposon insertion mutant of PA14_54830::*MAR2xT7*	(Liberati et al., 2006)
PA14 - Δ*phaG* (KO)	Knockout markerless mutant of PA14_54830::FRT (623 bp deletion)	This study
PA14 - Δ*rhlK*	Transposon insertion mutant of PA14_40980::*MAR2xT7*	(Liberati et al., 2006)
PA14 - Δ*rhlY*	Transposon insertion mutant of PA14_54640:: *MAR2xT7*	(Liberati et al., 2006)
PA14 - Δ*rhlZ*	Transposon insertion mutant of PA14_54660:: *MAR2xT7*	(Liberati et al., 2006)
PA14 - Δ*rhlZ* (KO)	Knockout markerless mutant of PA14_54660::FRT (961 bp deletion)	This study
PA14 - Δ*rhlYZ* (KO)	Knockout markerless double mutant PA14_54640 -PA14_54660 ::FRT (1855 bp deletion)	This study
PA14 - ΔPHA (KO)	Knockout markerless multiple mutant of Δ*phaC1*, Δ*phaD*, Δ*phaC2*, Δ*phaD*, Δ*phaF*, Δ*phaI*::FRT (6029 bp deletion)	This study
PA14 - Δ*phaC1*	Transposon insertion mutant of PA14_66820::*MAR2xT7*	(Liberati et al., 2006)
PA14 - Δ*phaC2*	Transposon insertion mutant of PA14_66840::*MAR2xT7*	(Liberati et al., 2006)
PA14 - Δ*phaJ2*	Transposon insertion mutant of PA14_51160::*MAR2xT7*	(Liberati et al., 2006)
PA14 - Δ*phaJ4*	Transposon insertion mutant of PA14_11910::*MAR2xT7*	(Liberati et al., 2006)
E.coli		
DH5α-Subcloning efficiency	F⁻ Φ80*lacZ*ΔM15 Δ(*lacZYA-argF*) U169 *recA*1 *endA*1 *hsdR*17(r_k^-, m_k^-) *pho*A *sup*E44 *thi*-1 *gyr*A96*rel*A1 λ⁻, host for complementation cloning vectors.	Invitrogen
SM10 λpir	thi-1, thr, leu, tonA, lacY, supE, recA::RP4-2-Tc::Mu, pir, Km^r, host for complementation conjugative vectors.	(Simon et al., 1983)
BL21 (DE3)	E. coli B F⁻ *dcm ompT hsdS*(r_B^- m_B^-) *gal* λ(DE3), host for complementation expression vectors	Novagen
Plasmids		
pRK2013	Mobilizing vector, oriColE1 RK2-Mob^+ RK2-Tra^+, Km^r	(Figurski and Helinski, 1979)
pDONR221	Entry vector, Km^r	Invitrogen
pEX18ApGW	Suicide gene replacement Gateway compatible (*attL1*, *attL2*) destination vector, Ap^r	(Choi and Schweizer, 2005)
pFLP2	A broad-host-range Flp-*FRT* recombination system, Ap^r	(Hoang et al., 1998)
pEX18ApGW-ΔPA14_54660::Gm-FRT	Gene replacement vector to knockout PA14_54660 (*rhlZ*), Gm^r, Ap^r	This study
pEX18ApGW-ΔPA14_54640-PA14_54660::GmFRT	Gene replacement vector to knockout PA14_54640 (*rhlY*) and PA14_54660 (*rhlZ*), Gm^r, Ap^r	This study
pEX18ApGW-ΔPHA::Gm-FRT	Gene replacement vector to knockout *phaC1*, *phaD*, *phaC2*, *phaD*, *phaF* and *phaI*, Gm^r, Ap^r	This study
pEX18ApGW-ΔPA14_54830::Gm-FRT	Gene replacement vector to knockout PA14_54830 (*phaG*), Gm^r, Ap^r	This study
pEX18T-*fadD2D1*::Gm-FRT	SmaI-Gm::FRT inserted into the deleted SmaI-EcoRV locus of *fadD2D1*	(Kang et al., 2010)
pUCP26	Broad-host-range vector; Tc^r	(West et al., 1994)
pUCP26-*rhlZ*	1245 bp segment encoding RhlZ (PA14_54660) including -57 bp before the start codon to +84 bp after the stop codon of the coding sequence cloned into pUCP26 at EcoRI/HindIII site	This study
pET28a(+)	Overexpression vector for His-tagged fusion proteins, Km^r.	Novagen
pET28a(+)-*rhlZ*	Recombinant vector for expression of His-tagged-rhlZ; the coding sequence from the start codon to +84 bp after the stop codon. The fragment is cloned into pET28a(+) at the NdeI/HindIII site.	This study
pET28(+)-*rhlY*	Recombinant vector for expression of His-tagged-rhlZ; the coding sequence from the start codon to stop codon. The fragment is cloned into pET28a(+) at the NdeI/HindIII site.	This study

[00137] 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), $CaCl_2 \cdot 2H_2O$ (0.1 g/L), $MgSO_4 \cdot 7H_2O$ (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: $FeSO_4 \cdot 7H_2O$ (2 g/L), $MnSO_4 \cdot H_2O$ (1.5 g/L), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (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.

[00138] *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 30ºC for resolution of merodiploids in mutant constructions. Antibiotics were added to maintain or select for plasmids as follows: for *E. 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*.

[00139] *Genes mutation and cloning.*

[00140] The *P. aeruginosa* strain PA14 deletion markerless mutants were constructed by allelic replacement using Gateway technology as described previously (Choi and Schweizer, 2005), with some modifications.

[00141] To generate the ΔrhlZ knockout, the following two pairs of primers, PA14-54660-UpF-GWL and PA14-54660-UpR-Gm with PA14-54660-DnF-Gm and PA14-54660-DnR-GWR, were used to amplify the upstream (5') and the downstream (3') fragments, respectively. After construction of the destination vector pEX18ApGW-ΔPA14_54660::Gm-FRT, it was transformed into *E. coli* DH5α-subcloning efficiency generating *E. coli* DH5α-pEX18ApGW- ΔPA14_54660::Gm-FRT. Plasmids from correct DH5α clones, growing on Gm (30 µg/mL) but neither on Km (50 µg/ml) nor Cb 100 µg/mL, were then transformed by conjugation into strain PA14 on LB agar using triparental mating with the help of DH5α(pRK2013) or diparental mating using E. coli SM10 λpir carrying the plasmid. Conjugation spots were spread onto LB agar containing Gm (50 µg/ml) and triclosan (10 µg/ml) to select for transconjugants of PA14 that had undergone the first homologous allelic replacement. Merodiploids were resolved by growing three transconjugant colonies in LBNS broth overnight, dilutions (usually 10^{-1}, 10^{-2}) of which were plated onto LBNS agar containing Gm (50 µg/ml) and sucrose 10% (w/v). Upon replica plating, positive clones, that underwent the second recombination, were those growing on LB agar Gm (50 µg/ml but not on Cb (300 µg/mL). The Gm resistance cassette was subsequently excised from produced mutants ΔPA14_54660::Gm-FRT (Δ*rhlZ*::Gm-FRT), by Flp recombinase. This was performed by conjugation of Δ*rhlZ*::Gm-FRT mutant with *E. coli* SM10(pFLP2). PA14 mutant clones that had lost the Gm cassette were recovered by plating dilutions, up to 10^{-4}, of suspended conjugation spots onto LB agar containing Cb (300 µg/mL) and Tr (10 µg/ml) to eliminate the *E. coli*. Growing clones were replica plated onto LBNS agar containing sucrose 10% (w/v) to eliminate the pFLP2 plasmid. Growing clones were further confirmed by replica plating onto LB agar containing Cb (250 µg/mL) and on LB agar containing Gm (50 µg/ml) onto which correct clones should not grow and the markerless Δ*rhlZ* (ΔPA14_54660) mutant is generated. The chromosomal deletion was finally confirmed by sequencing after amplification of the mutated region using the two extremities primers, PA14-54660-

UpF-GWL and PA14-54660-DnR-GWR. Similarly, the double knockout mutant Δ*rhlYZ* (ΔPA14_54640-ΔPA14_54660) was constructed using the PA14-54640-UpF-GWL and PA14-54640-UpR-Gm primers for amplification of the upstream fragment in combination with PA14-54660-DnF-Gm and PA14-54660-DnR-GWR for the amplification of the downstream fragment.

[00142] The knockout mutant ΔphaG (ΔPA14_54830, ortholog of phaG of P. putida KT2440 (Rehm et al., 1998)) was constructed using phaG-UpF-GWL and phaG-UpR-Gm primers for the amplification of the upstream fragment in combination with phaG-DnF-Gm and phaG-DnR-GWR primers for the amplification of the downstream fragment.

[00143] The Δ*PHA* multiple knockout mutant (Δ*phaC1*, Δ*phaD*, Δ*phaC2*, Δ*phaD*, Δ*phaF*, Δ*phaI*) was constructed using the PHA-UpF-GWL and PHA-UpR-Gm primers to amplify the upstream fragment and PHA-DnF-Gm and PHA-DnR-GWL primers to amplify the downstream fragment. The plasmid pUCP26-*rhlZ* was constructed by PCR amplification of PA14_54660 from wild type PA14 using *RhlZ*-F-EcoRI and *RhlZ*-R-HindIII primers including the region from -57 bp (with the native RBS) to 84 bp downstream to the stop codon of *RhlZ*. The EcoRI-HindIII digested fragment was cloned into the EcoRI-HindIII-digested pUCP26 vector.

[00144] The expression vector pET28a(+)-*RhlZ* was constructed by PCR amplification of PA14_54660 from the wild-type PA14 using *RhlZ*-F-NdeI and *RhlZ*-R-HindIII primers. The double digested fragment was cloned into the NdeI-HindIII digested pET28a(+) to yield the inducible *RhlZ* expression 56 vector pET28a(+)-*RhlZ* with the His-tag at the N-terminal of *RhlZ*. The plasmid was transformed in thermocompetent *E.coli* BL21(DE3). Similar steps were followed to construct the expression vector pET28a(+)-*RhlY* with an N59 terminal His-tag, by cloning the gene PA14_54640 amplified using *RhlY*-F-NdeI and *RhlY*-R-HindIII primers.

[00145] Plasmid DNA and PCR products were sequenced by the DNA Sequencing and Gene Analysis Center at the Genome Quebec, Montreal, CA. The plasmids and PCR primers used are listed in Tables 1 and 2, respectively.

Table 2: PCR primers* used in the experiments described herein

Name	Sequence (5'-3')	SEQ ID NO:
PA14_54660-UpF-GWL	TACAAAAAAGCAGGCTctggtcgaacaggtggtg	7
PA14_54660-UpR-Gm	TCAGAGCGCTTTTGAAGCTAATTCGtcttcgaaaagcacgttca	8
PA14_54660-DnF-Gm	AGGAACTTCAAGATCCCCAATTCGctgatcgatcgcgacaac	9
PA14_54660-DnR-GWR	TACAAGAAAGCTGGGTgaagacgttcagcaggtagc	10
PA14_54640-UpF-GWL	TACAAAAAAGCAGGCTgacatgctcaccgaactca	11

PA14_54640-UpR-Gm	TCAGAGCGCTTTTGAAGCTAATTCGcatgaagtccctcactggat	12
phaG-UpF-GWL	TACAAAAAAGCAGGCTctttcctgcccggccaat	13
phaG-UpR-Gm	TCAGAGCGCTTTTGAAGCTAATTCGgatgatggtctgctgcgcc	14
phaG-DnF-Gm	AGGAACTTCAAGATCCCCAATTCGcggcgcggaatttcacac	15
phaG-DnR-GWR	TACAAGAAAGCTGGGTccattatacgacgcccgctc	16
PHA-UpF-GWL	TACAAAAAAGCAGGCTtcaaaagaacaataacgagctt	17
PHA-UpR-Gm	TCAGAGCGCTTTTGAAGCTAATTCGgtcagcaggttgatgacg	18
PHA-DnF-Gm	AGGAACTTCAAGATCCCCAATTCGgtatgcctatccggttcaa	19
PHA-DnR-GWR	TACAAGAAAGCTGGGTcgtttccgaatccacttc	20
rhlZ-F-EcoRI	*CGTA*GAATTCATGAACGTGCTTTTCGAA	21
rhlZ-R-HindIII	*ACCC*AAGCTTGAAACAGCTTAGACGCTATG	22
rhlZ-F-NdeI	*CACACA*CATATGAACGTGCTTTTCGAAGAA	23
rhlY-F-NdeI	*CACACA*CATATGAACACTGCCGTCGAAC	24
rhlY-R-HindIII	*ACCC*AAGCTTTCAGCAGTTGCGCCACTT	25

* Small letter nucleotides are the gene specific sequences for generation of knockout mutants. Underlined nucleotides refer to corresponding restriction sites preceded by a number of base pairs for efficient cleavage as recommended by supplier (New England BioLabs®, Inc).

[00146] *Protein expression and purification*

[00147] 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 hours. 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 9 cycles each of 30 seconds interrupted by 30 seconds of rest on ice. Each cell lysates were clarified by centrifugation at 10,000 x *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 twenty column volumes. For *RhlY*, the gradient was from 0% to 100% elution buffer over thirty 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 (**FIG. 14A**), *RhlY* started to elute at 170 mM, peaked at 240 mM and tailed at 335 mM imidazole (**FIG. 14B**). Fractions of these peaks and flanking fractions were applied to 12% (w/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). 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 -80ºC. 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.

[00148] 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 cultures 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 freshly or kept at 4°C until use.

[00149] *Synthesis of trans-2-decenoyl-CoA and chiral HPLC-MS/MS assay of hydratase.*

[00150] 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).

[00151] *Chromatographic analysis of rhamnolipids, 3-hydroxy fatty acids and PHA.*

[00152] RL were analyzed using a high-performance liquid chromatography (HPLC) coupled with tandem mass spectrometry (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.

[00153] *HPLC-MS analysis and identification of rhamnolipids*. HPLC/MS analysis of RL was performed as reported previously (Déziel *et al.*, 1999) with some modifications. RL containing culture broths were centrifuged at 10,000 *x* g for 10 min to prepare cell free supernatants. An aliquot of 250 µL of such supernatant was kept at -20ºC until the day of analysis. In HPLC vials, 25 µl of such supernatant

was diluted to 500 µl of acetonitrile:water (70:30) to which 2-heptyl-3-hydroxyquinoline-d4 (HHQ-d4) was added at 5.6 mg/L as internal standard (Dubeau *et al.*, 2009). Samples were analyzed using an HPLC (Waters® 2795, Alliance® HT) equipped with a C8 reversed phase column (Eclipse® XDB-C8, particle size of 5 µm, and of 4.6 x 150 mm dimensions, Agilent®, USA). The detector was a triple tandem quadrupole mass spectrometer (qMS/MS, Quattro Premier® XE, Micromass®) equipped with an electrospray ionization interface. All rhamnolipids were detected and quantified in the negative ion mode generating $[M-H]^-$ pseudomolecular ions. Data were processed by MassLynx® software with its QuanLynx® function (version 4.1, Waters®).

[00154] HPLC separation of RLs was performed with a sample injection volume of 20 µL and mobile phase at a flow rate of 0.4 ml/min with a gradient of water (A):acetonitrile (B), both containing 2 mM ammonium acetate and adjusted to pH 8. The gradient was set according to the following profile: 0 min, 30% A + 70% B; 1 min, 25% A + 75% B; 10 min, 100% B; 16 min, 100% B; 17 min, 30% A + 70% B; 20 min, 30% A + 70% B. The mobile phase flow was split through a Valco® Tee with 10% of the flow introduced into the mass spectrometer. The mass spectrometer was in the negative ESI mode with capillary voltage set at 3.0 kV, cone voltage at 20 V, source and desolvation temperatures of 120 and 150ºC respectively, cone and desolvation gas (N2) flow of 15 and 100 L/Hr, respectively. The scanning mass range was from 130 to 800 Da. Quantification was performed by comparing integration data of the pseudomolecular ion $[M-H]^-$ peak areas of different RLs homologues with those of the internal standard using response factors previously determined through calibration curves made with pure mono-RL and pure di-RL. Collision-induced dissociation (CID) experiments for fragmentation of parent molecular ion were performed using argon as the collision gas at a pressure of 3.28 X 10-3 87 mBar, corresponding to a flow of 0.35 mL/min. The scanning mass range for daughter ions was from 100 to 530 Da for mono-RL and from 100 to 650 Da for di-RL. Collision energy was adjusted at 20 eV for both molecules.

[00155] *GC/MS analysis of 3-hydroxy fatty acids and PHA.* For the analysis of total 3-hydroxy fatty acids, 3 ml of whole culture broths grown in MSM-glycerol were collected in screw-capped glass tubes to which nonanoic acid (Sigma®, USA) was added as the internal standard at 15 mg/L. These broths were then extracted with 3 ml ethyl acetate and vortexed for 10 seconds. Two ml of the organic phase was collected in small glass vials and evaporated to dryness under a stream of nitrogen gas. Residues were derivatized by silylation using BSTFA (*N,O*-bis(trimethylsilyl) trifluoroacetamide); Supelco®, PA, USA) in a total reaction volume of 100 µl, composed of 25 µl BSTFA and 75 µl acetonitrile, each added freshly. The reaction was incubated at 70°C for 30 min, then cooled to room temperature and diluted to 1000 µl with acetonitrile prior to injection into the gas chromatograph (see below). This way, the final concentrations of 3-hydroxy fatty acids as well as internal standard are twice

higher than their original concentrations in the culture broths.

[00156] Similar steps were followed for the estimation of PHA (Costa *et al.*, 2009), with some modifications. A triplicate of two batches of washed cell pellets recovered from 4 ml of 50-60 hours old cultures of *P. aeruginosa* in MSM-glycerol medium grown under rotation at 34°C were prepared. Cell pellets of first batch, intended for free intracellular fatty acid analysis, were suspended in 1 ml dH_2O and extracted with 1 ml $CHCl_3$ while vortexing for 15 min. Cell pellets of the second batch, intended for analysis of total (free and hydrolyzed) fatty acids including PHA, were suspended in 1 ml 15% H_2SO_4 and similarly extracted with $CHCl_3$ while heating at 100°C for 140 min to hydrolyze PHA. $CHCl_3$ layers were collected and evaporated to dryness before derivatization by silylation as above. The values of free intracellular 3-hydroxyfatty acids obtained from the first batch (were 10-fold lower) were subtracted from those of the second batch to obtain the concentration of PHA. Analysis of the trimethylsilyl derivatives of the 3-hydroxyfatty acid was performed by gas chromatography/mass spectrometry (GC/MS) (Trace GC Ultra® - Polaris® Q, Thermo Fisher Scientific®, Waltham, MA) equipped with a ZB-5MS (ZebronTM113) capillary column (30 m X 0.25 mm internal diameter, 0.25 µM film thickness, Phenomenex®) using the electron impact ionization mode. The GC program started at an oven temperature of 70°C that was increased at 30°C/min to 110°C and held at 110°C for 5 min, then increased at 20°C/min to 165°C and held at 165°C for 5 min, then increased at 30°C/min to 310°C and held for 0.5 min with an overall oven run time of 19.42 min. The sample injection volume was 1 µL. The Polaris Q MS was operated in full scan positive ion mode covering the mass range of 70-400 with a total scan time of 0.41 sec. MS scan start time was at 6.6 min and the ion source temperature was at 250°C. The injector and detector (ion source) temperatures were 200°C and 250°C, respectively. Helium was used as the carrier gas at a constant flow rate of 1.1 ml/min. Quantification was performed by comparing integration data of M•+ 122 fragment ions resulting from the electron impact of the trimethylsilyl esters of 3-hydroxy fatty acids with those of the internal standard using response factors previously determined through calibration curves made with pure standards. The experiments were conducted in triplicate.

[00157] *Electron microscopy for PHA granules visualization and measurement.* A volume of 1 ml of culture of cells grown in MSM-glycerol was sampled after 50 hours (OD_{600} = 3.0) of incubation at 34°C under rotation. Cells, recovered by centrifugation at 5,000 x g for 5 minutes, were fixed using 2.5% gluteraldehyde in phosphate buffer saline (PBS) for 1 hour then washed three times with 3% sucrose in PBS and kept at 4°C overnight. Staining was performed with 1.3% osmium tetroxide in collidine buffer. Fixed stained material was dehydrated in solutions with progressively increasing concentrations of acetone (25-100%). The material was kept overnight in a 1:1 volume mixture of Spurr resin and acetone, then immersed twice, each 1 hour, in a bath of Spurr mixtures. The block containing fixed cells

was cut into small pieces, placed in BEEM capsules, filled with Spurr resin and held at room temperature for 16 hours and then polymerized at 60ºC for 20-30 hours. Ultrathin sections (70-100 nm thick) were examined by a Hitachi H-7100 electron microscope with an accelerating voltage of 75 kV. Estimation of PHA production was made using the photos obtained from the electron microscope and the area measurement tools of Adobe Photoshop® (version CS3 extended).

Example 2: Inhibition of β-oxidation decreases RLs production

[00158] 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 sole carbon source and not a fatty acid source 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% (**FIG. 8A**), whereas the other inhibitors had no effect (**FIG. 8B**).

[00159] 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}) homologues at equivalent molar concentrations. The results show that 2-Br-C_8 inhibits RLs and HAA production in a dose-dependent manner (**FIG. 1A**). 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) (**FIG. 1B**). The other congeners, 2-Br-C_4 and 2-Br-C_{12}, caused considerable growth inhibition down to 3 mM.

[00160] 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 polyhydroxyalkanoates (PHA) biosynthesis (Lee *et al.*, 2001). To rule out the hypothesis that PhaG product could act as precursor for RL biosynthesis and that the inhibition of RLs production by 2-Br-C_8 is caused by its effect on PhaG (**FIG. 9A**), RL production was tested in a *phaG* knockout as well as a transposon insertion *phaG* mutant. Neither of the two mutants affects RL production (**FIG. 9B**), excluding a role of PhaG in the 2-Br-C_8-mediated reduction in RL production.

Example 3: Contribution of β-oxidation as source of lipid precursors for RLs

[00161] To further evaluate the relative contribution of β-oxidation versus FASII in RL synthesis, PA14 were cultivated in MSM using dodecanoic-12,12,12-d_3 acid as 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 3 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 (**FIG. 10A**). Alternatively, if *de novo* fatty acid synthesis (FASII) is predominantly implicated, produced RLs would be mostly unlabeled as its 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 (**FIG. 10A**).

[00162] 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, ($[M-H]^-$ *m/z* 509) and 12% with an extra mass of 3 Da, ($[M-H]^-$ *m/z* 506). Tandem MS/MS fragmentation of *m/z* 509 confirmed that the extra 6 Da were distributed equally, 3 Da per chain (**FIG. 10B**) 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.

Example 4: β-oxidation is directly linked to RLs pathway

[00163] Using perdeuterooctadecanoic acid (C_{18}-d_{35}) and glucose as co-substrate, 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 FASII 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 RL biosynthesis. We thus verified the contribution of the hypothesis Zhang *et al.* 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 (**FIG. 11A**). On the other hand, if FAS II is implicated according to the model of Zhang *et al.*, the fatty acid chain of RL would bear extra 16 Da (67%) or 15 Da (33%) as the main species (**FIG. 11B**).

[00164] 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, while those bearing +16 Da and +15 Da only represented 43% and 26% of the intensity of the +17 Da species, respectively. The abundances of the +16 Da and +15 Da species relative to each other were 62% and 38% respectively, close to theoretical prediction mentioned above (**FIG. 12A**). 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 FASII.

[00165] To further investigate the hypothesis of Zhang *et al.*, C_{12}-d_{23} (3.75 g/L) was provided together with glucose (10 g/L) as co-carbon 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%) (**FIG. 12B**). To overcome this effect, the bacterium were fed 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 FASII 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*. hypothesis, the lipid chains of RLs would bear an extra 5 Da instead. The 2 extra Da in this case arises from the FASII-mediated condensation of $^{13}C_2$-labelled 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 (**FIG. 12C, D**).

[00166] Taken together, these results indicate that, whether fatty acids are provided alone or as co-carbon sources with glucose, β-oxidation remains the main direct supplier of lipid precursors for RLs biosynthesis.

Example 5: Enoyl-CoA intermediates of β-oxidation are diverted to RLs biosynthesis

[00167] 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-hydroxyfatty 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.

[00168] A survey of the *P. aeruginosa* PA14 genome (Winsor *et al.*, 2011) for genes that might code for such enzymes revealed fourteen loci predicted to encode enoyl-CoA hydratases (ECH) (Table 3), while no predicted epimerases or 3-ketoacyl-CoA reductases were found. The polyhydroxyalkanoate (PHA)-related ECH, PhaJ1-4 (Tsuge *et al.*, 2003), was also included in the survey. 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 (**FIG. 13A**). 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 (**FIG. 13B**). Because of their impact on RL synthesis, they were respectively named *RhlK,*

RhlY and *RhlZ*.

Table 3: Predicted enoyl-CoA hydratases (crotonases) expressing genes and orthologues of *phaJ* genes having crotonoase like activity in *P. aeruginosa* PA14 and the availability of their transposon mutants.

PA14 locus tag	PAO1 locus tag	Transposon mutant available (Liberati et al., 2006)
PA14_17850	PA3591	No
PA14_19740	PA3426	Yes
PA14_26690	PA2890	Yes
PA14_27360	PA2841	Yes
PA14_28310	PA2767	Yes
PA14_40980	PA1821	Yes
PA14_41950	PA1748	No
PA14_43440	PA1629	Yes
PA14_48200	PA1240	Yes
PA14_51110	PA1021	Yes
PA14_54640	PA0745	Yes
PA14_54660	PA0744	No**
PA14_56250	PA4330	Yes
PA14_65840	PA4980	No
PA14_21310	PA3302*	No
PA14_51160	PA1018*	Yes
PA14_63290	PA4788*	No
PA14_11910	PA4015*	Yes

* PA3302, PA1018, PA4788 and PA4015 are reported in *Pseudomonas aeruginosa* as PHA-related hydratases; PhaJ1, PhaJ2, PhaJ3 and PhaJ4, respectively.
** A knockout deletion mutant of this gene was prepared in this study.

[00169] *RhlY* and *RhlZ* were selected for further studies, as their corresponding mutants were the most affected in RL 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 RL 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, and complementation with *RhlZ* only partially complemented RLs production in this double mutant (**FIG. 2A**). Moreover, overexpression of *RhlZ* in the WT did not increase RLs production (**FIG. 13C**). Collectively, these results demonstrate a direct role for *RhlZ* in RLs production and, also that *RhlY* cooperates with *RhlZ* to attain native level of RLs production.

Example 6: The ECH/I *RhlY* and *RhlZ* are main suppliers of 3-hydroxydecanoate/octanoate pools

[00170] To experimentally confirm the putative ECH/I role assigned to *RhlY* and *RhlZ* in the

production of the 3-hydroxyalkanoate precursors of RLs, the total intra- and extracellular pool of 3-OH-$C_{10\pm2}$ was compared in the mutants and WT (**FIG. 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} (**FIG. 2B**). This significant decrease in 3-hydroxy fatty acids precursors correlates with the RLs reduction in these mutants (**FIG. 2A**). Moreover, these results demonstrate a 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-hydroxyfatty acid, supporting the hypothesis that *RhlY* cooperates with *RhlZ* to attain the WT level of 3-hydroxyfatty acids precursors and hence RLs.

Example 7: *RhlZ* is an *R*-specific ECH/I

[00171] It was 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 RL (**FIG. 9A**). This was verified *in vitro* using purified *RhlZ* (**FIG. 3**) and trans-2-decenoyl-CoA as substrate. The relative proportion of the *R*- and *S*-stereoisomers of 3-hydroxydecanoyl-CoA (3-OH-C_{10}-CoA) produced was estimated using a chiral HPLC-MS/MS method.

[00172] 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 (**FIGs. 4 and 3B**). Interestingly, combination of purified *RhlZ* with a clarified WT cell lysate caused a nearly 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) (**FIGs. 4 and 3A**). This demonstrates that *RhlZ* is indeed an *R*-ECH/I, but that a factor present in the WT cell lysate is involved in its activity. It was hypothesized that this factor is a protein since heat treatment of the clarified cell lysate abolished *RhlZ* activity with no any 3-hydroxydecanoate produced. According to the previous data (**FIG. 2**), this heat-sensitive factor is likely *RhlY*. To verify this, purified *RhlZ* was combined with the clarified cell lysates of Δ*RhlYZ* or Δ*RhlZ*. Consistent with the hypothesis that the heat-sensitive factor is *RhlY*, the *R*-3-OH-C_{10} enrichment was conserved when purified *RhlZ* was combined with the lysate of Δ*RhlZ* (**FIG. 3C, D**), whereas, no such enrichment was observed when this purified *RhlZ* was combined with the lysate of the double mutant Δ*RhlYZ* (**FIG. 10E, F**).

[00173] The dependency of *RhlZ* on *RhlY* suggested a sort of interaction or complex formation. This was verified by a pull-down experiment using purified hexahistidine-tagged *RhlZ* (*RhlZ*-His_6) incubated with the clarified total cell lysate of WT. *RhlZ*-His_6 pulled *RhlY* from the lysate (**FIG. 5**), entailing a stable *RhlY-RhlZ* physical interaction between these two proteins.

[00174] Taken together, these results indicate that *RhlZ* is an *R*-specific ECH/I that is functional *in vivo* in the presence of *RhlY* with which it forms a stable complex.

Example 8: *RhlZ*-associated *R*-specific enrichment is inhibited by 2-bromooctanoate

[00175] As shown above, treatment with 2-Br-C_8 or inactivation of *RhlZ* decreases RLs production. In order to verify if *RhlZ* is one of the targets of this β-oxidation inhibitor, 3 mM of 2-Br-C_8 was directly added 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 (**FIG. 4**). However, because previous reports suggested that the active form of this inhibitor could be one of its metabolites (Raaka and Lowenstein, 1979), ECH assays were conducted using clarified lysate of WT cells 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 (**FIG. 4**). This indicates that *RhlZ* is at least partially inhibited by a 2-bromooctanoate-derived metabolite.

Example 9: *R*-3-hydroxyacyl-CoA pool is shared between RLs and polyhydroxyalkanoates pathways

[00176] Because *R*-3-hydroxyacyl-CoA are also the precursors of polyhydroxyalkanoates (PHA) (Madison and Huisman, 1999; Wang *et al.*, 2012), the hypothesis that the lipid precursors supplied by *RhlYZ* is shared by PHA and RLs was tested. The impact of *RhlYZ* double mutation on the production of PHA was first examined (**FIG. 6A**). Second, PHA production was directly assessed by transmission electron microscopy using the percentage cross-sectional area of intracellular PHA granules (**FIGs. 6B, D**). A consistent 25% reduction in PHA was found in the *RhlYZ* mutant (**FIGs. 6A, B**) entailing a shared precursor pool. Third, it was verified whether this pool is competitively shared between PHA and RLs by studying the effect of blocking PHA biosynthesis on RLs production. To do so, most of PHA-biosynthetic genes, namely Δ*phaC1DC2DFI* (Δ*pha*), were knocked out. This mutation resulted in an 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 (**FIG. 6C**). This is explained by the accumulation of the shared lipid precursor pool in the PHA-negative mutant that becomes more available for RL 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. It thus appears that the fixed rhamnose precursor pool limited a more striking increase in total RL.

[00177] The scope of the claims should not be limited by the preferred embodiments set forth in the examples, but should be given the broadest interpretation consistent with the description as a whole.

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CLAIMS

1. A method for decreasing the production of rhamnolipids (RLs) by a microorganism, said method comprising inhibiting the expression and/or activity of one or more *R*-specific enoyl-CoA hydratases/isomerases (ECH/I) in said microorganism.

2. A method for attenuating the virulence of a rhamnolipid-producing microorganism, said method comprising inhibiting the expression and/or activity of one or more *R*-specific enoyl-CoA hydratases/isomerases (ECH/I) in said rhamnolipid-producing microorganism.

3. The method of claim 1 or 2, wherein said inhibiting comprises contacting said microorganism with an inhibitor of said one or more *R*-specific ECH/I.

4. The method of claim 1 or 2, wherein said inhibitor is an inactive or dominant-negative form of said one or more *R*-specific ECH/I.

5. The method of claim 1 or 2, wherein said inhibitor is an RNA interference agent targeting a nucleic acid encoding said one or more ECH/I.

6. The method of claim 5, wherein said RNA interference agent is a shRNA or siRNA.

7. The method of any one of claims 1 to 6, wherein said one or more *R*-specific ECH/I comprise a sequence having at least 50% identity with the amino acid sequence of SEQ ID NO:2, SEQ ID NO:4 and/or SEQ ID NO:6.

8. The method of claim 7, wherein said one or more *R*-specific ECH/I comprise a sequence having at least 90% identity with the amino acid sequence of SEQ ID NO:2, SEQ ID NO:4 and/or SEQ ID NO:6.

9. The method of claim 8, wherein said one or more *R*-specific ECH/I comprise the amino acid sequence of SEQ ID NO:2, SEQ ID NO:4 and/or SEQ ID NO:6.

10. The method of any one of claims 1 to 9, wherein said method is an *in vitro* method.

11. The method of any one of claims 1 to 9, wherein said method is an *in vivo* method.

12. A method for increasing the production of rhamnolipids (RLs) by a microorganism, said method comprising increasing the expression and/or activity of one or more *R*-specific enoyl-CoA hydratases/isomerases (ECH/I) in said microorganism.

13. The method of claim 12, wherein said method comprises introducing into said microorganism one or more nucleic acids encoding said one or more *R*-specific ECH/I.

14. The method of claim 12 or 13, wherein said method further comprises introducing one or more nucleic acids encoding one or more polypeptides involved in the RL biosynthetic pathway.

15. The method of claim 14, wherein said one or more polypeptides involved in the RL biosynthetic pathway are rhamnosyl-transferases.

16. The method of claim 15, wherein said encoding one or more rhamnosyl-transferases are *RhlB* and/or *RhlC*.

17. The method of any one of claims 13 to 16, wherein said one or more nucleic acids encodes the *R*-specific ECH/I defined in any one of claims 7 to 9.

18. The method of claim 17, wherein said one or more nucleic acids comprise a sequence having at least 50% identity with the nucleotide sequence of SEQ ID NO:1, SEQ ID NO:3 and/or SEQ ID NO:5.

19. The method of claim 18, wherein said one or more nucleic acids comprise a sequence having at least 90% identity with the nucleotide sequence of SEQ ID NO:1, SEQ ID NO:3 and/or SEQ ID NO:5.

20. The method of claim 19, wherein said one or more nucleic acids comprise the nucleotide sequence of SEQ ID NO:1, SEQ ID NO:3 and/or SEQ ID NO:5.

21. A method for determining whether an agent may be useful for (i) inhibiting the production of rhamnolipids (RLs) by a microorganism and/or (ii) attenuating the virulence of a rhamnolipid-producing microorganism, said method comprising determining whether said agent inhibits the expression and/or activity of one or more *R*-specific enoyl-CoA hydratases/isomerases (ECH/I).

22. The method of claim 21, comprising (a) contacting said agent with said one or more *R*-specific ECH/I and (b) determining whether said agent inhibits the expression and/or activity of said one or more *R*-specific ECH/I, wherein the inhibition of said expression and/or activity is indicative that said agent may be useful for (i) inhibiting the production of RLs by a microorganism and/or (ii) attenuating the virulence of a rhamnolipid-producing microorganism.

23. A method for determining whether an agent may be useful for increasing the production of rhamnolipids (RLs) by a microorganism, said method comprising determining whether said agent

increases the expression and/or activity of one or more *R*-specific enoyl-CoA hydratases/isomerases (ECH/I).

24. The method of claim 23, comprising (a) contacting said agent with said one or more *R*-specific ECH/I and (b) determining whether said agent increases the expression and/or activity of said one or more *R*-specific ECH/I, wherein the increase of said expression and/or activity is indicative that said agent may be useful for increasing the production of rhamnolipids (RLs) by a microorganism.

25. The method of any one of claims 21 to 24, wherein said method is a cell-free method.

26. The method of any one of claims 21 to 24, wherein said one or more *R*-specific ECH/I are expressed in a cell.

27. The method of any one of claims 21 to 26, wherein said one or more *R*-specific ECH/I comprise a sequence having at least 50% identity with the amino acid sequence of SEQ ID NO:2, SEQ ID NO:4 and/or SEQ ID NO:6.

28. The method of claim 27, wherein said one or more *R*-specific ECH/I comprise a sequence having at least 90% identity with the amino acid sequence of SEQ ID NO:2, SEQ ID NO:4 and/or SEQ ID NO:6.

29. The method of claim 28, wherein said one or more *R*-specific ECH/I comprise the amino acid sequence of SEQ ID NO:2, SEQ ID NO:4 and/or SEQ ID NO:6.

30. The method of any one of claims 1 to 29, wherein said microorganism is of the *Pseudomonas* genus.

31. The method of claim 30, wherein said microorganism is *Pseudomonas aeruginosa*.

32. Use of an inhibitor of *R*-specific enoyl-CoA hydratases/isomerases (ECH/I) for decreasing the production of rhamnolipids (RLs) by a microorganism.

33. Use of an inhibitor of *R*-specific enoyl-CoA hydratases/isomerases (ECH/I) for the preparation of a medicament for decreasing the production of rhamnolipids (RLs) by a microorganism.

34. Use of an inhibitor of *R*-specific enoyl-CoA hydratases/isomerases (ECH/I) for attenuating the virulence of a rhamnolipid-producing microorganism.

35. Use of an inhibitor of *R*-specific enoyl-CoA hydratases/isomerases (ECH/I) for the preparation of a medicament attenuating the virulence of a rhamnolipid-producing microorganism.

36. The use of any one of claims 31 to 35, wherein said inhibitor is the inhibitor defined in any one of claims 4 to 6.

37. The use of any one of claims 32 to 36, wherein said one or more *R*-specific ECH/I comprise a sequence having at least 50% identity with the amino acid sequence of SEQ ID NO:2, SEQ ID NO:4 and/or SEQ ID NO:6.

38. The use of claim 37, wherein said one or more *R*-specific ECH/I comprise a sequence having at least 90% identity with the amino acid sequence of SEQ ID NO:2, SEQ ID NO:4 and/or SEQ ID NO:6.

39. The use of claim 38, wherein said one or more *R*-specific ECH/I comprise the amino acid sequence of SEQ ID NO:2, SEQ ID NO:4 and/or SEQ ID NO:6.

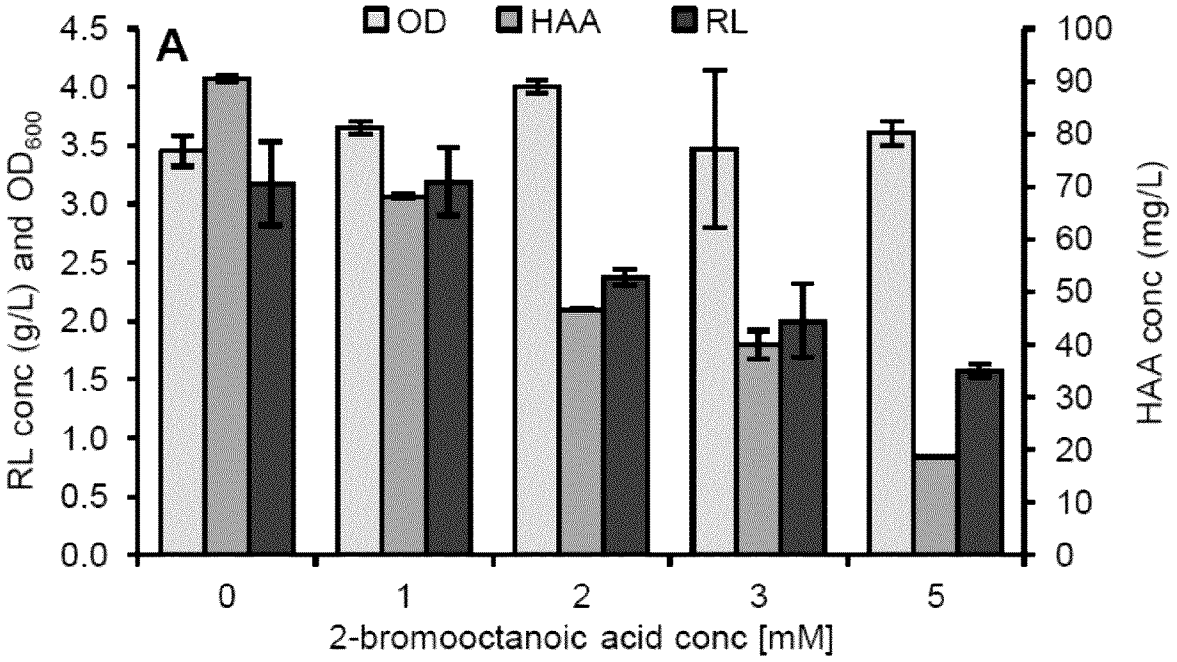

A
OD
HAA
RL
RL conc (g/L) and OD_{600}
HAA conc (mg/L)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
5
2-bromooctanoic acid conc [mM]

FIG. 1A

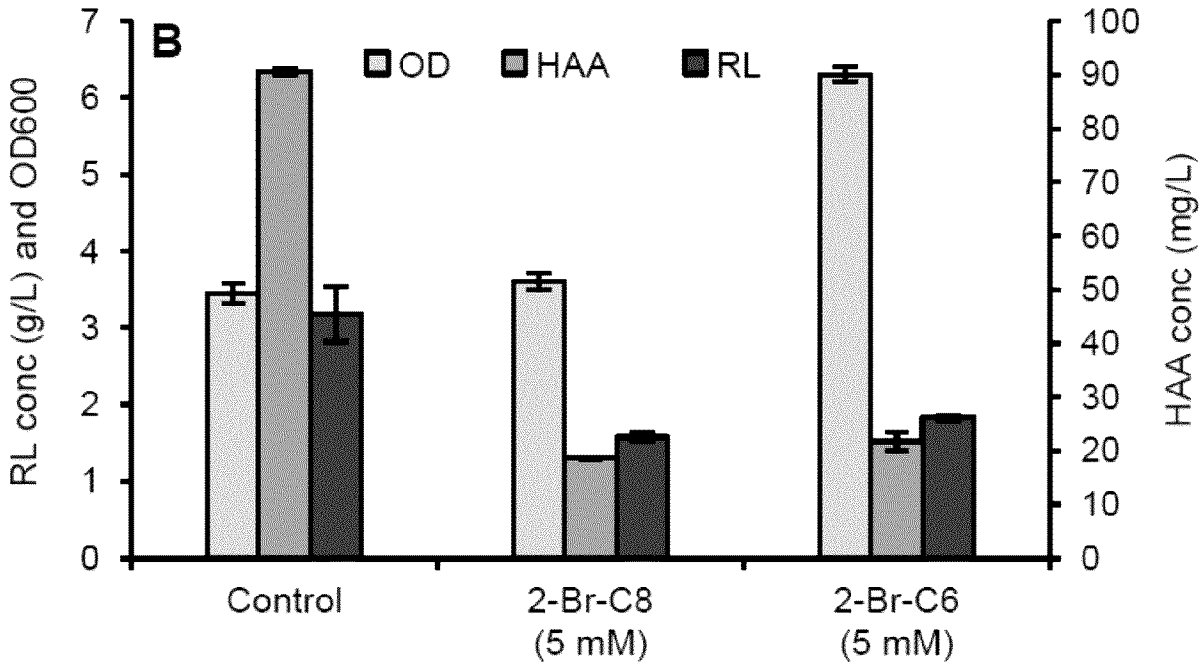

B
OD
HAA
RL
RL conc (g/L) and OD600
HAA conc (mg/L)
7
6
5
4
3
2
1
0
100
90
80
70
60
50
40
30
20
10
0
Control
2-Br-C8
(5 mM)
2-Br-C6
(5 mM)

FIG. 1B

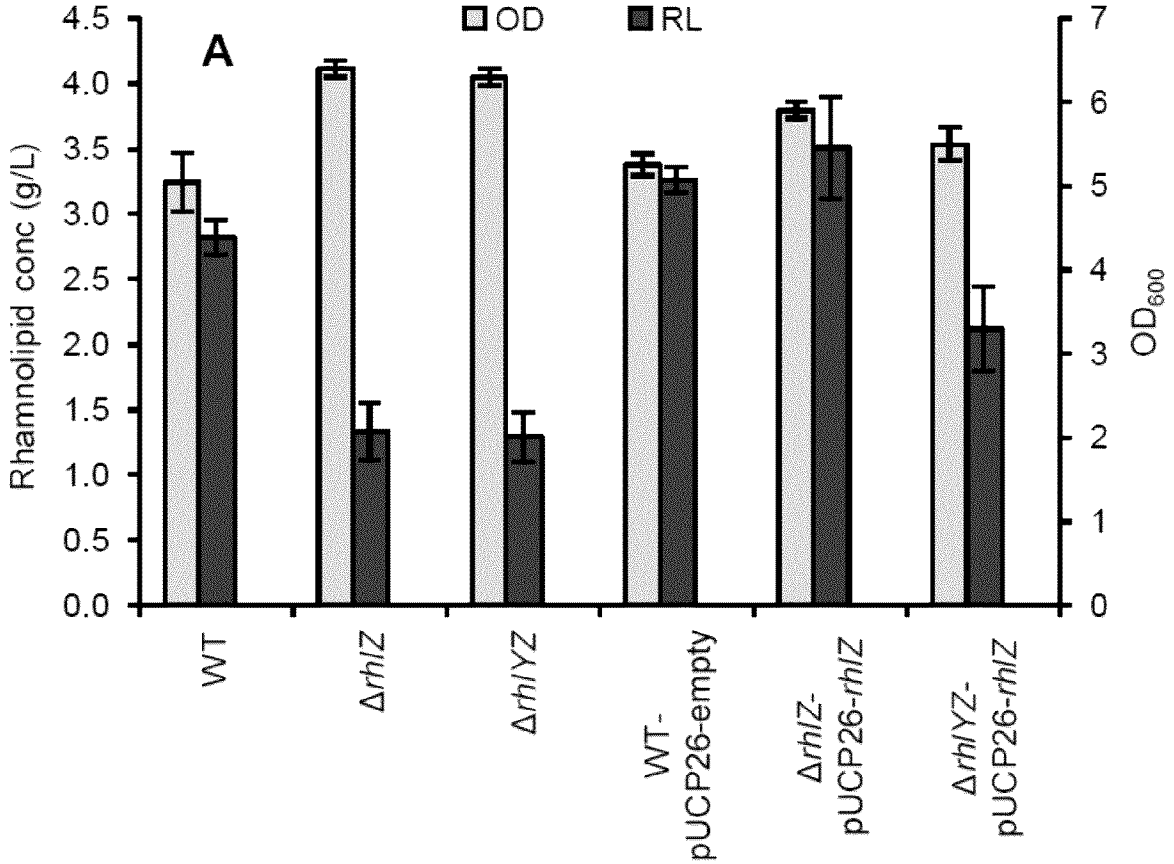

A
OD
RL
Rhamnolipid conc (g/L)
OD_{600}
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
7
6
5
4
3
2
1
0
WT
ΔrhlZ
ΔrhlYZ
WT-pUCP26-empty
ΔrhlZ-pUCP26-rhlZ
ΔrhlYZ-pUCP26-rhlZ

FIG. 2A

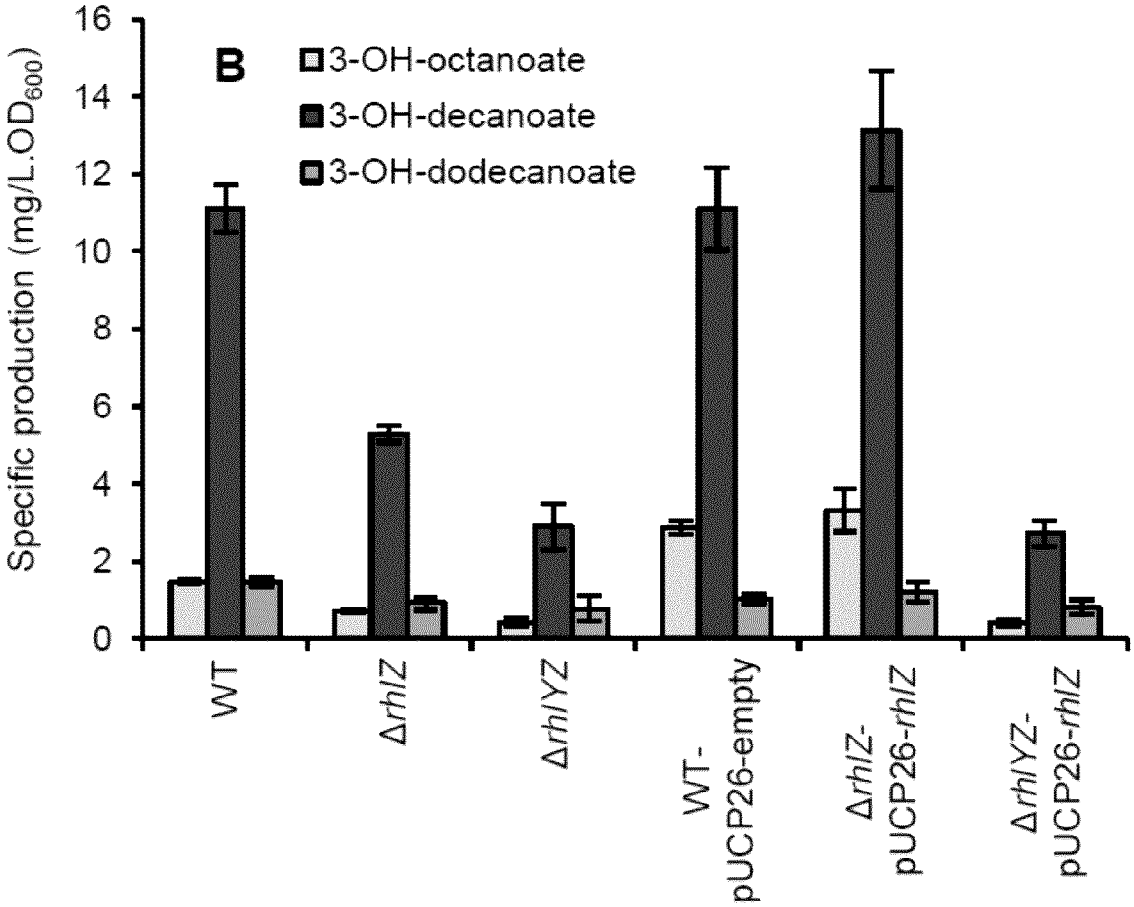

B
3-OH-octanoate
3-OH-decanoate
3-OH-dodecanoate
Specific production (mg/L.OD600)
16
14
12
10
8
6
4
2
0
WT
ΔrhlZ
ΔrhlYZ
WT-pUCP26-empty
ΔrhlZ-pUCP26-rhlZ
ΔrhlYZ-pUCP26-rhlZ

FIG. 2B

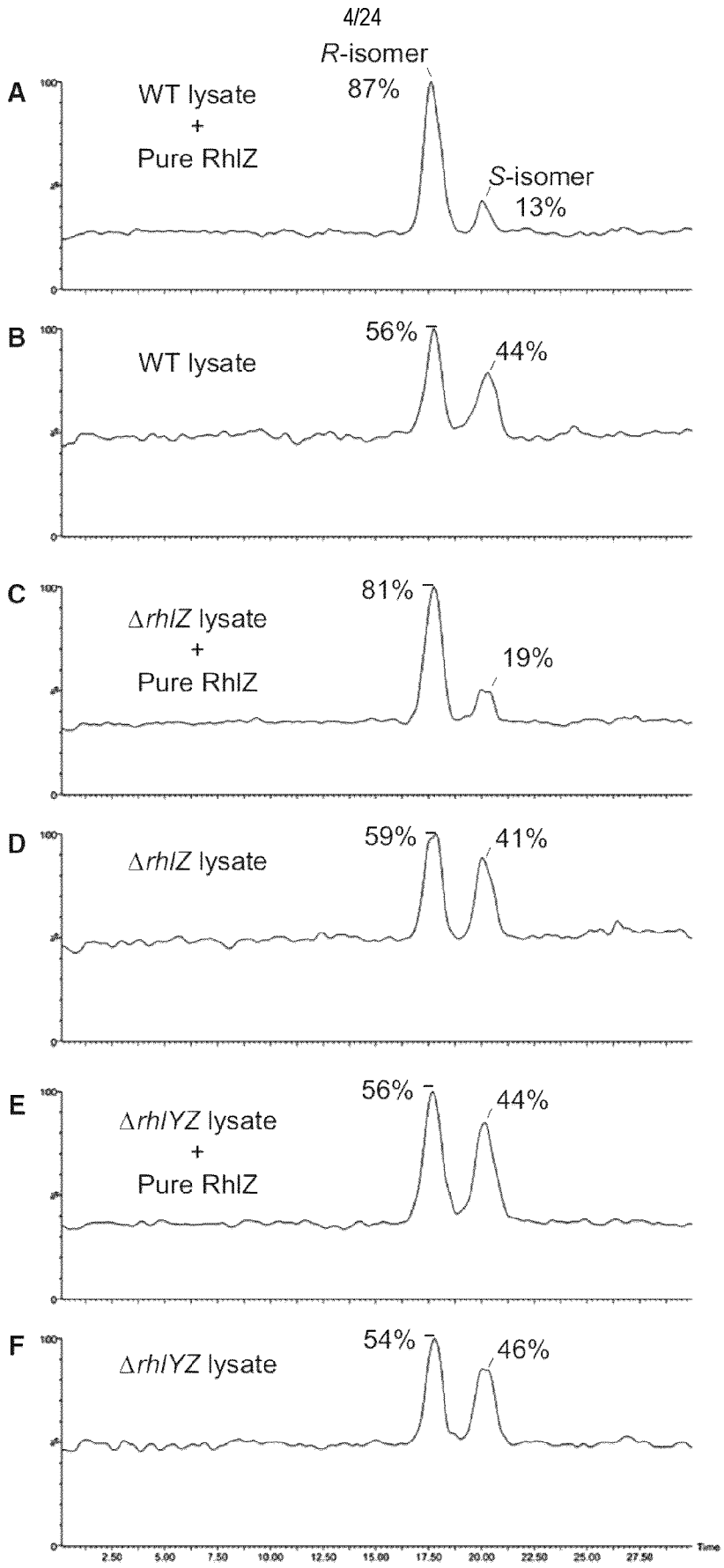

A
WT lysate
+
Pure RhlZ
R-isomer
87%
S-isomer
13%
B
WT lysate
56%
44%
C
ΔrhlZ lysate
+
Pure RhlZ
81%
19%
D
ΔrhlZ lysate
59%
41%
E
ΔrhlYZ lysate
+
Pure RhlZ
56%
44%
F
ΔrhlYZ lysate
54%
46%
2.50
5.00
7.50
10.00
12.50
15.00
17.50
20.00
22.50
25.00
27.50
Time

FIG. 3

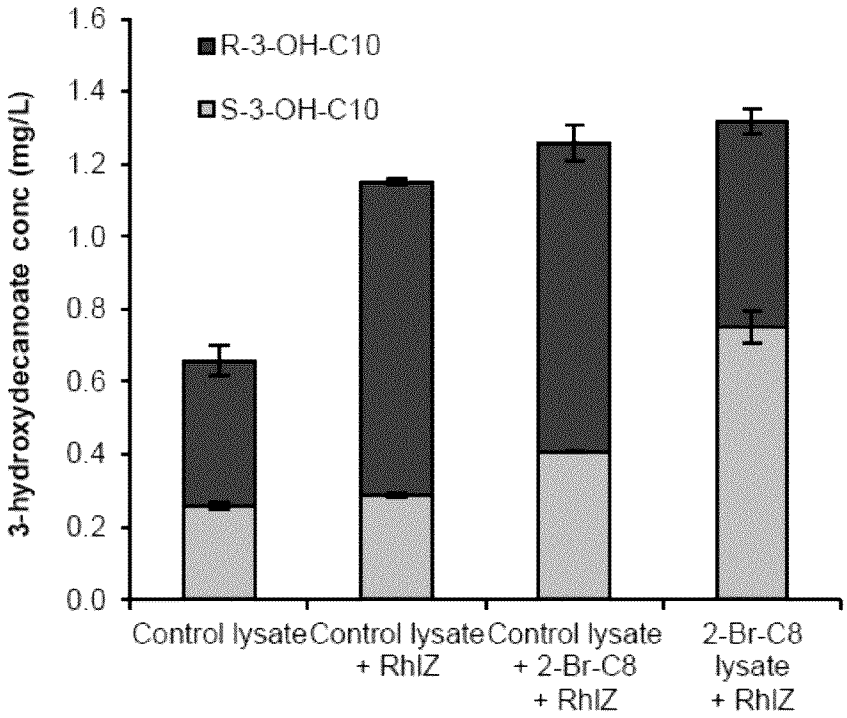

1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
R-3-OH-C10
S-3-OH-C10
3-hydroxydecanoate conc (mg/L)
Control lysate
Control lysate + RhlZ
Control lysate + 2-Br-C8 + RhlZ
2-Br-C8 lysate + RhlZ

FIG. 4

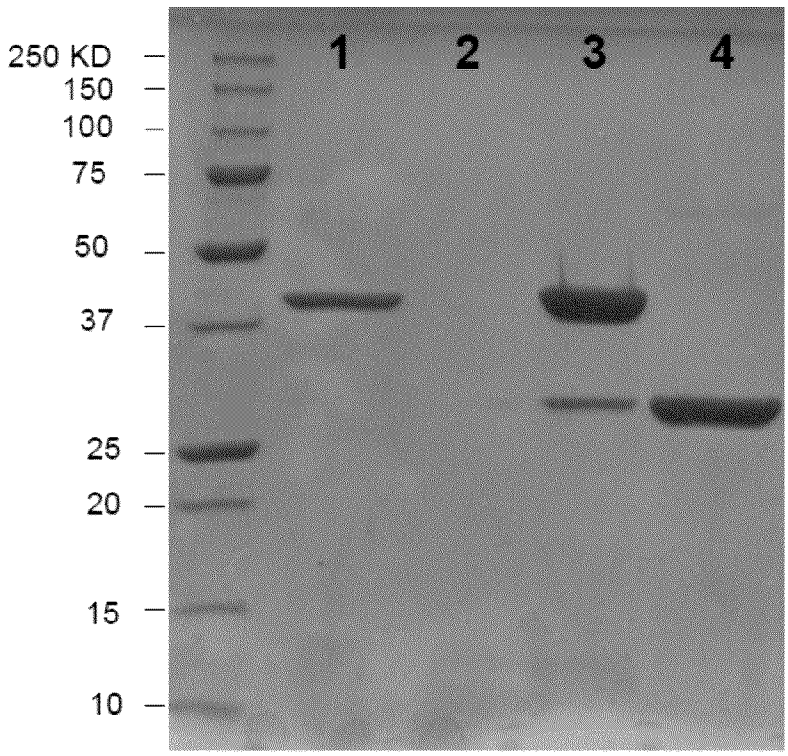

250 KD
150
100
75
50
37
25
20
15
10
1
2
3
4

FIG. 5

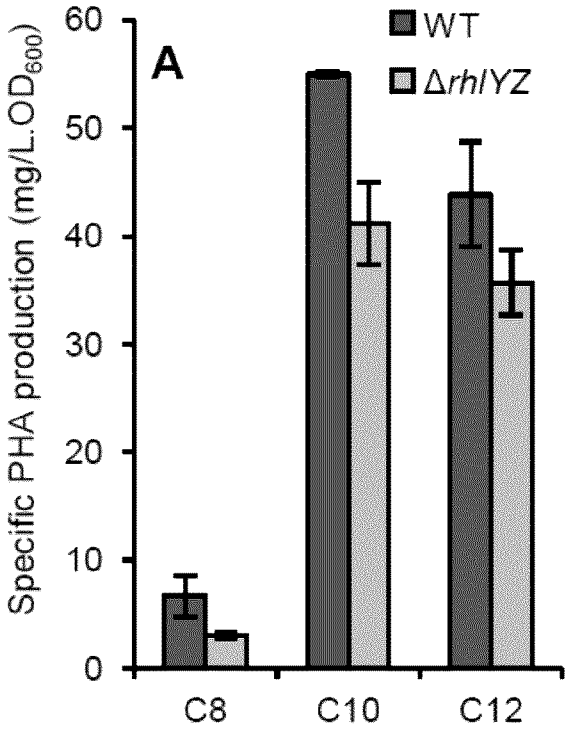

A
WT
ΔrhlYZ
Specific PHA production (mg/L.OD600)
60
50
40
30
20
10
0
C8
C10
C12

FIG. 6A

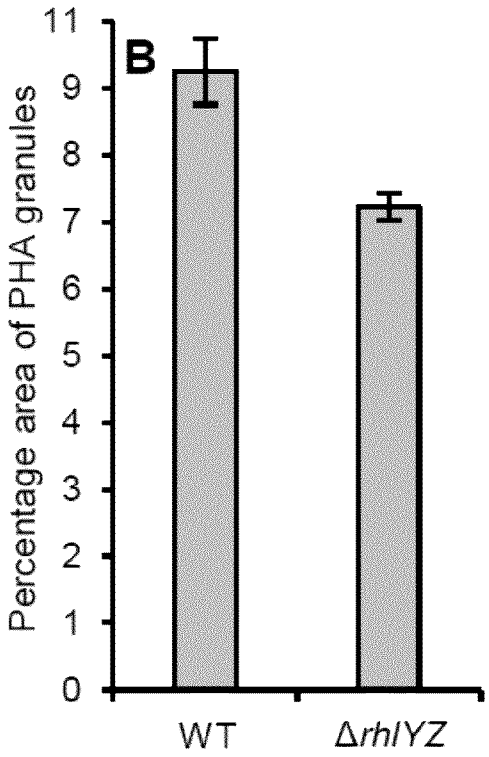

B
Percentage area of PHA granules
11
9
8
7
6
5
4
3
2
1
0
WT
ΔrhlYZ

FIG. 6B

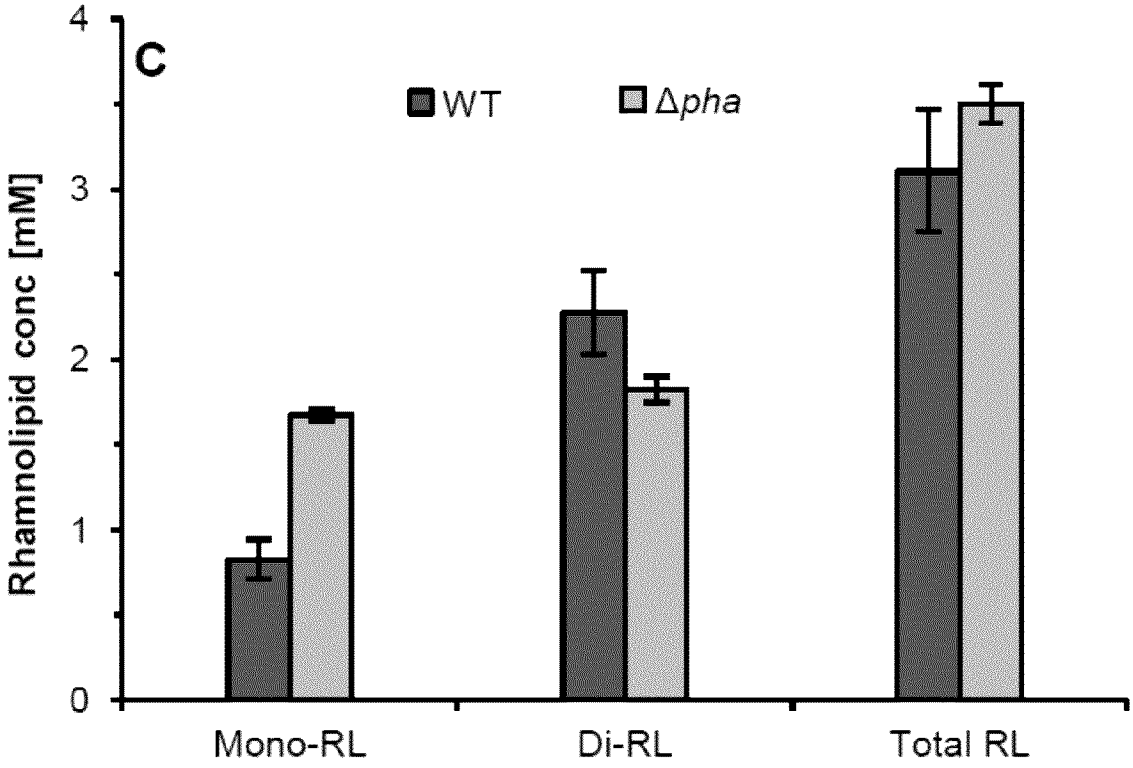

C
WT
Δpha
Rhamnolipid conc [mM]
0
1
2
3
4
Mono-RL
Di-RL
Total RL

FIG. 6C

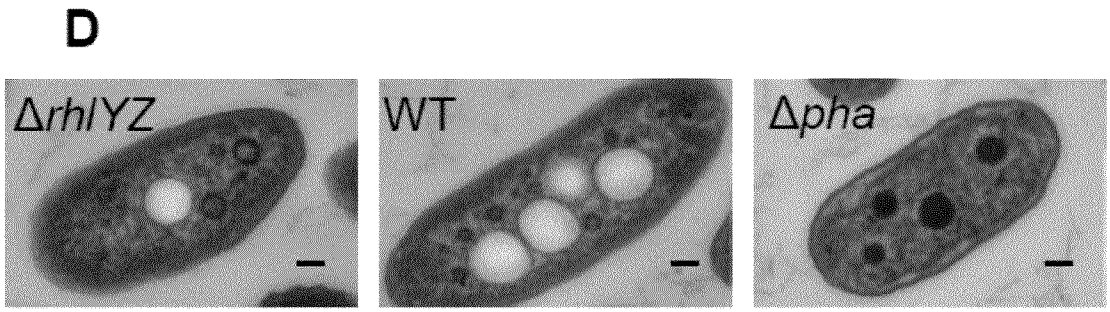

D
ΔrhlYZ
WT
Δpha

FIG. 6D

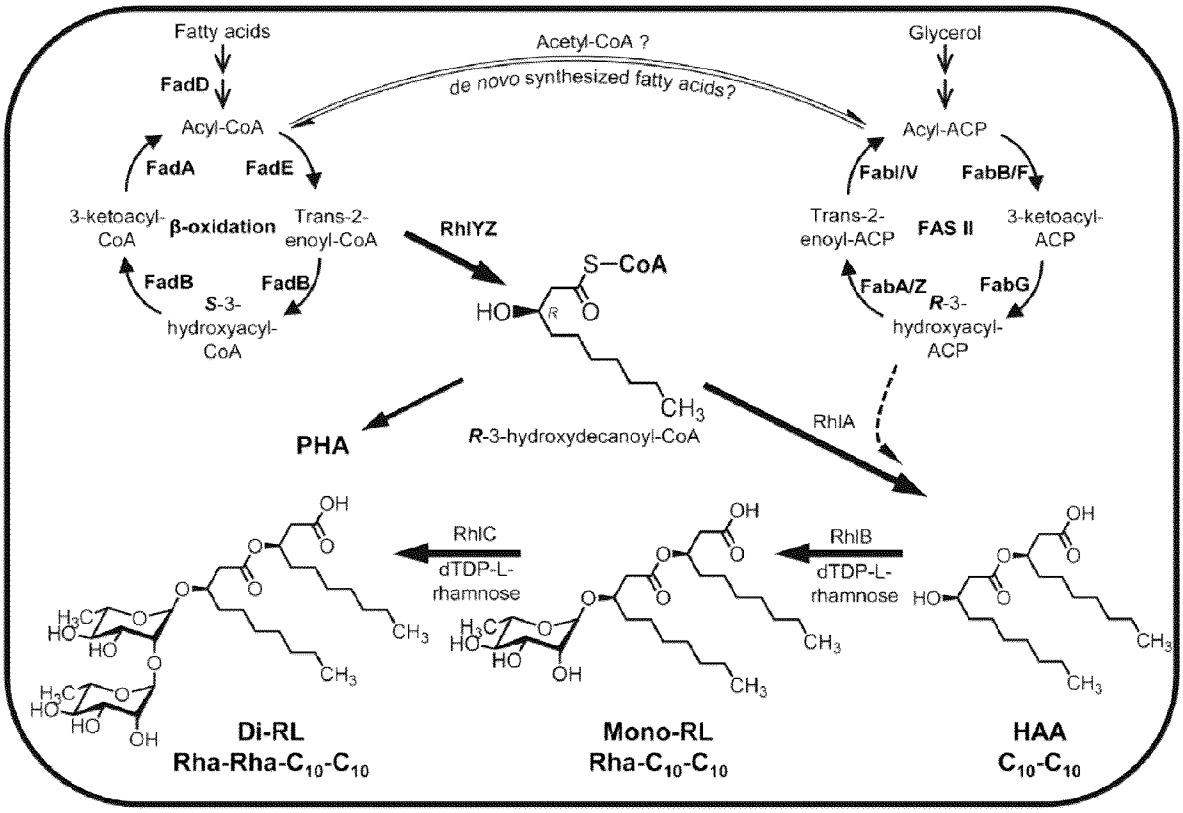

Fatty acids
FadD
Acyl-CoA
FadA
FadE
3-ketoacyl-CoA
β-oxidation
Trans-2-enoyl-CoA
FadB
FadB
S-3-hydroxyacyl-CoA
Acetyl-CoA ?
de novo synthesized fatty acids?
Glycerol
Acyl-ACP
FabI/V
FabB/F
Trans-2-enoyl-ACP
FAS II
3-ketoacyl-ACP
FabA/Z
FabG
R-3-hydroxyacyl-ACP
RhlYZ
S—CoA
HO
R
O
CH3
R-3-hydroxydecanoyl-CoA
PHA
RhlA
RhlC
dTDP-L-rhamnose
RhlB
dTDP-L-rhamnose
Di-RL
Rha-Rha-C10-C10
Mono-RL
Rha-C10-C10
HAA
C10-C10

FIG. 7

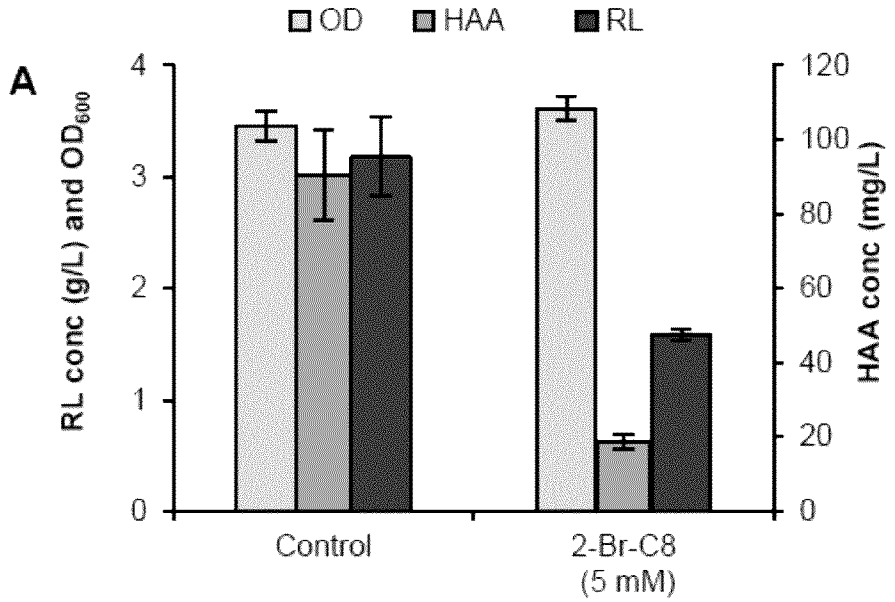

OD
HAA
RL
A
RL conc (g/L) and OD600
HAA conc (mg/L)
0
1
2
3
4
0
20
40
60
80
100
120
Control
2-Br-C8
(5 mM)

FIG. 8A

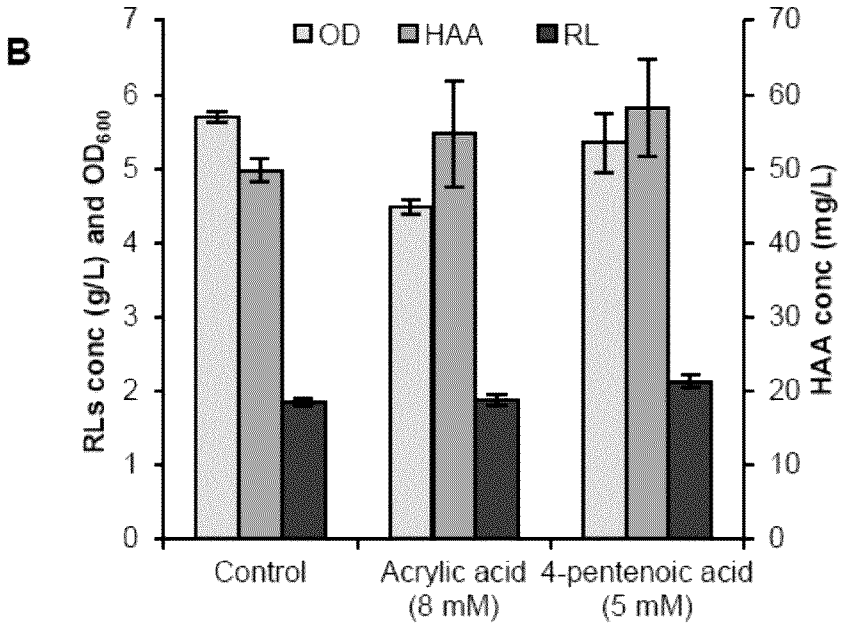

B
OD
HAA
RL
RLs conc (g/L) and OD_{600}
HAA conc (mg/L)
0
1
2
3
4
5
6
7
0
10
20
30
40
50
60
70
Control
Acrylic acid (8 mM)
4-pentenoic acid (5 mM)

FIG. 8B

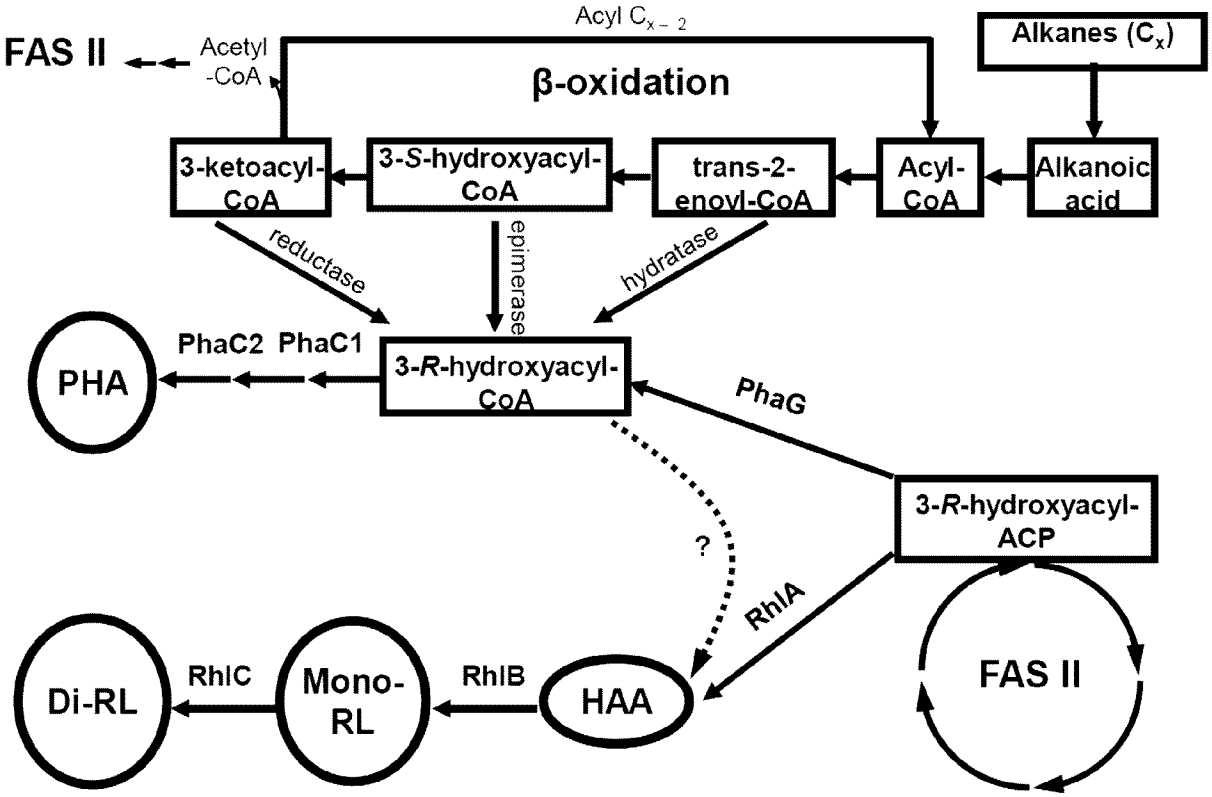

FAS II
Acetyl -CoA
Acyl C_{x-2}
β-oxidation
Alkanes (C_x)
3-ketoacyl-CoA
3-S-hydroxyacyl-CoA
trans-2-enoyl-CoA
Acyl-CoA
Alkanoic acid
reductase
epimerase
hydratase
PHA
PhaC2 PhaC1
3-R-hydroxyacyl-CoA
PhaG
3-R-hydroxyacyl-ACP
?
RhlA
Di-RL
RhlC
Mono-RL
RhlB
HAA
FAS II

FIG. 9A

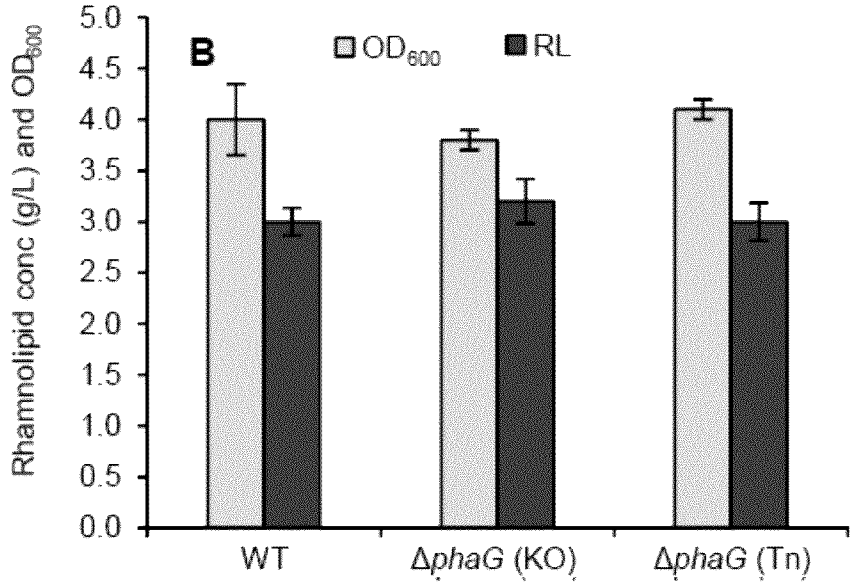


FIG. 9B

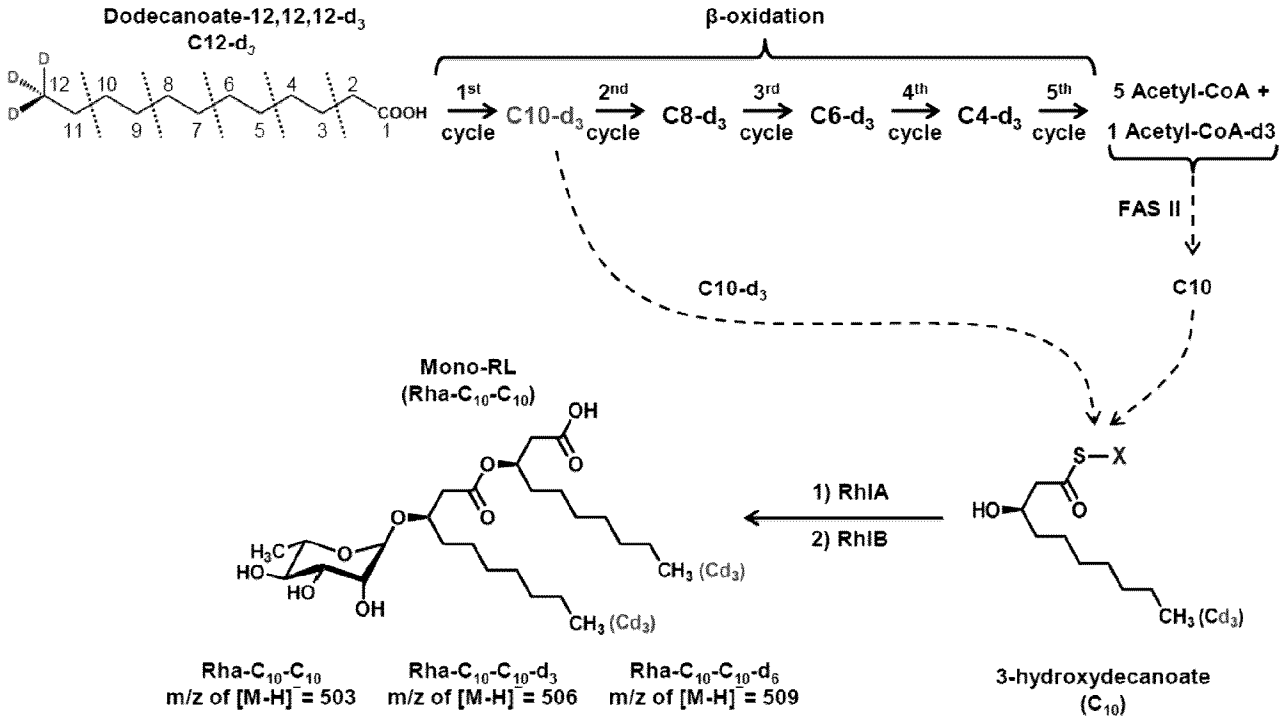


FIG. 10A

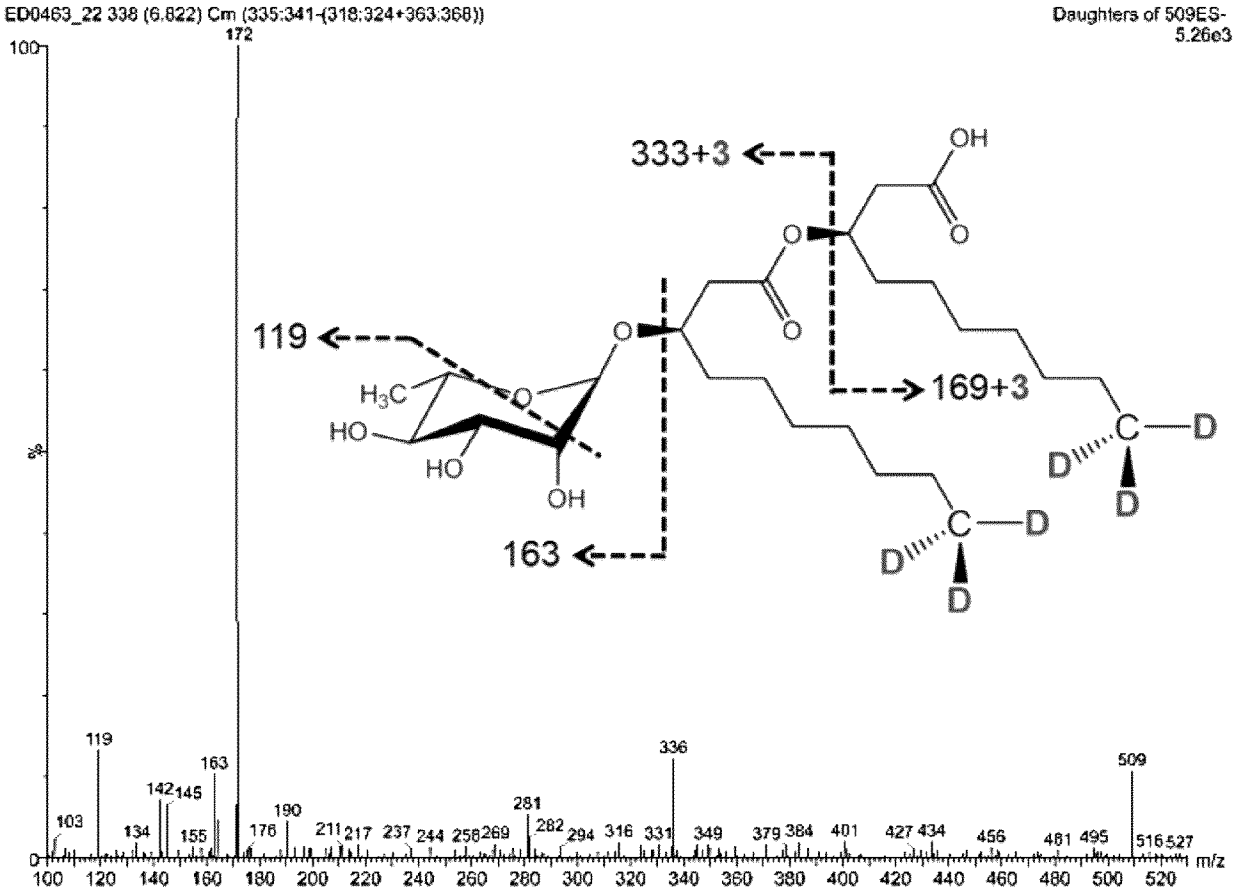

ED0463_22 338 (6.822) Cm (335:341-(318:324+363:368))
Daughters of 509ES-
5.26e3
100
%
0
172
119
163
142
145
103
134
155
176
190
211
217
237
244
258
269
281
282
294
316
331
336
349
379
384
401
427
434
456
481
495
509
516
527
100 120 140 160 180 200 220 240 260 280 300 320 340 360 380 400 420 440 460 480 500 520
m/z
333+3
169+3
119
163
OH
O
H3C
HO
HO
OH
C
D

FIG. 10B

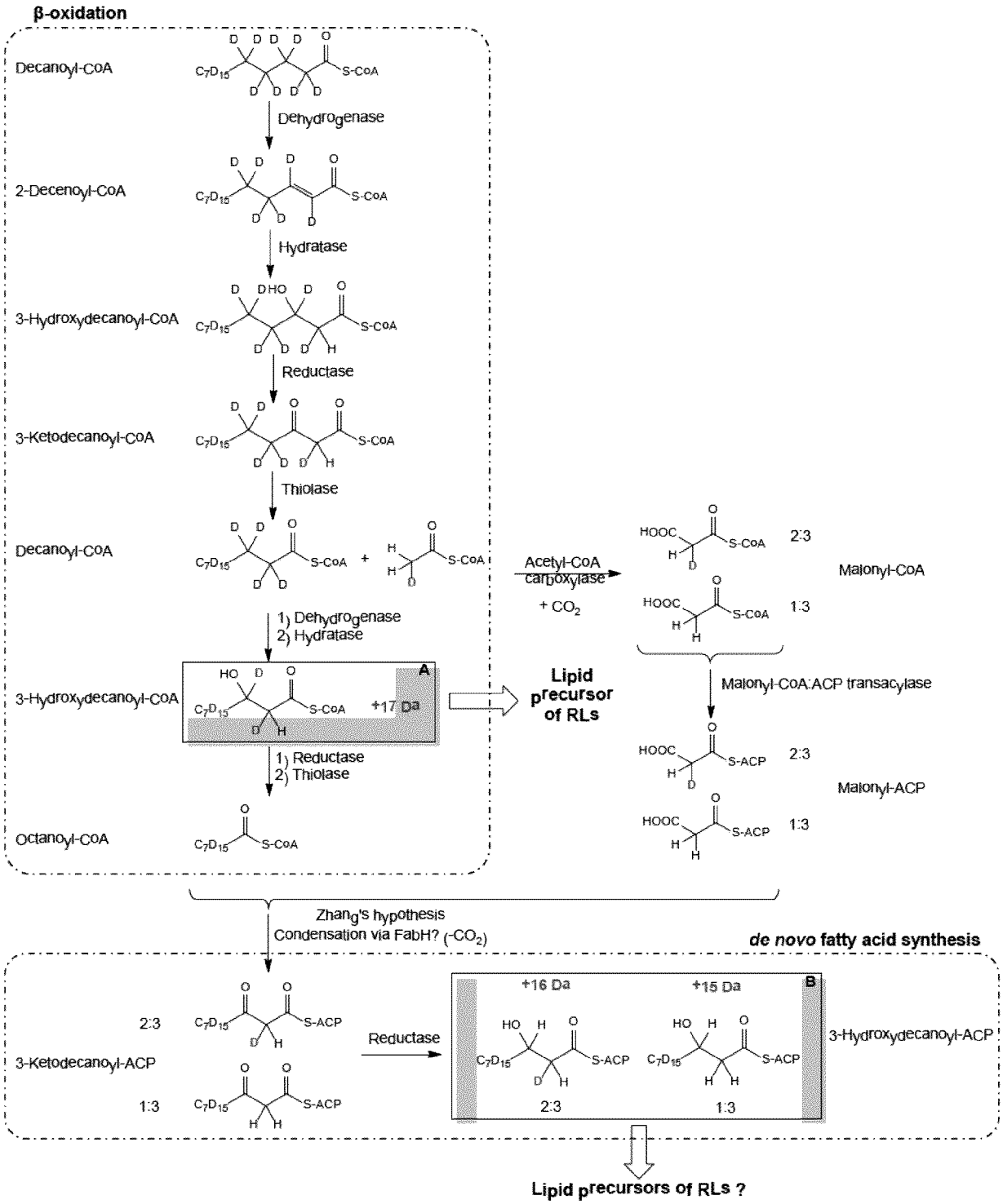

β-oxidation
Decanoyl-CoA
Dehydrogenase
2-Decenoyl-CoA
Hydratase
3-Hydroxydecanoyl-CoA
Reductase
3-Ketodecanoyl-CoA
Thiolase
Decanoyl-CoA
1) Dehydrogenase
2) Hydratase
3-Hydroxydecanoyl-CoA
+17 Da
A
1) Reductase
2) Thiolase
Octanoyl-CoA
Acetyl-CoA carboxylase
+ CO2
2:3
Malonyl-CoA
1:3
Malonyl-CoA:ACP transacylase
Lipid precursor of RLs
2:3
Malonyl-ACP
1:3
Zhang's hypothesis
Condensation via FabH? (-CO2)
de novo fatty acid synthesis
2:3
3-Ketodecanoyl-ACP
1:3
Reductase
+16 Da
+15 Da
B
2:3
1:3
3-Hydroxydecanoyl-ACP
Lipid precursors of RLs ?

FIG. 11

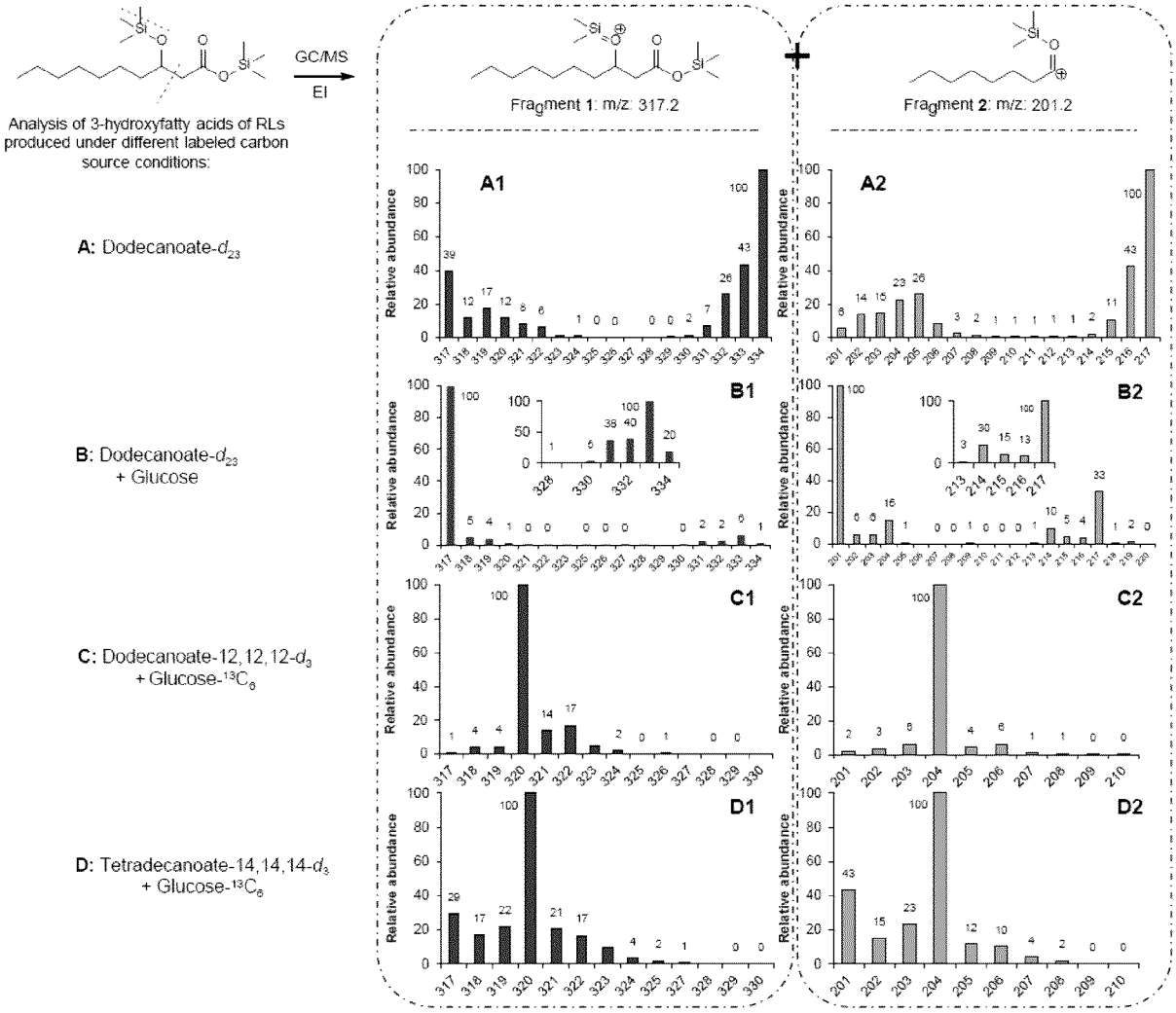


FIG. 12

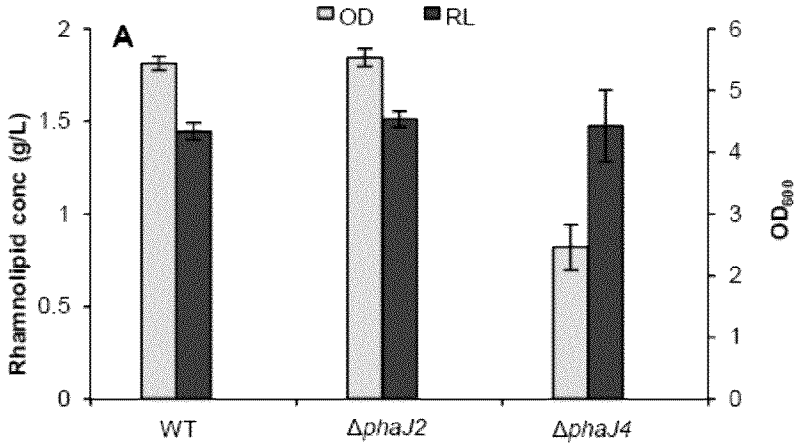


FIG. 13A

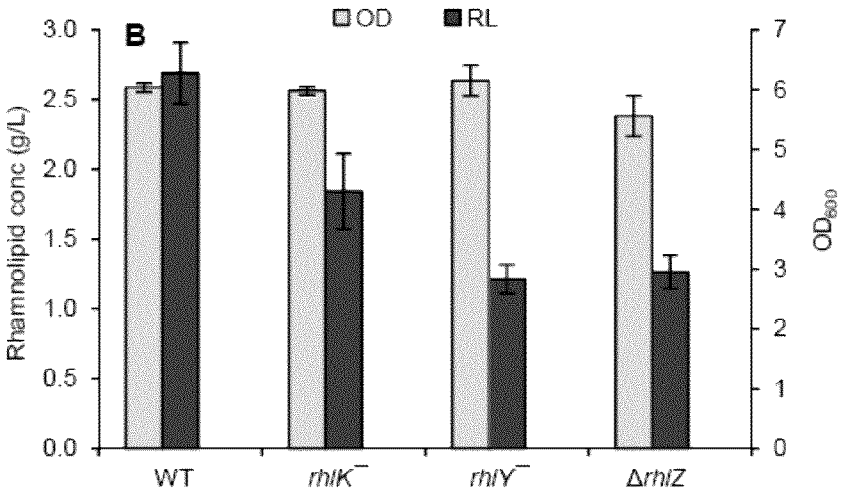


FIG. 13B

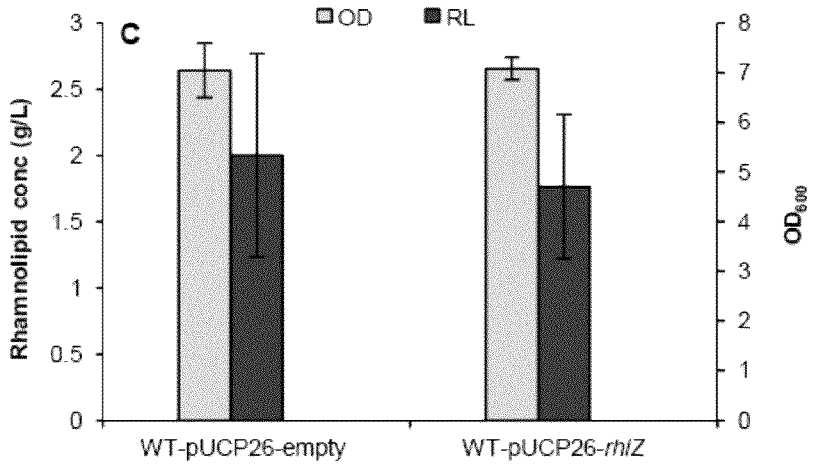


FIG. 13C

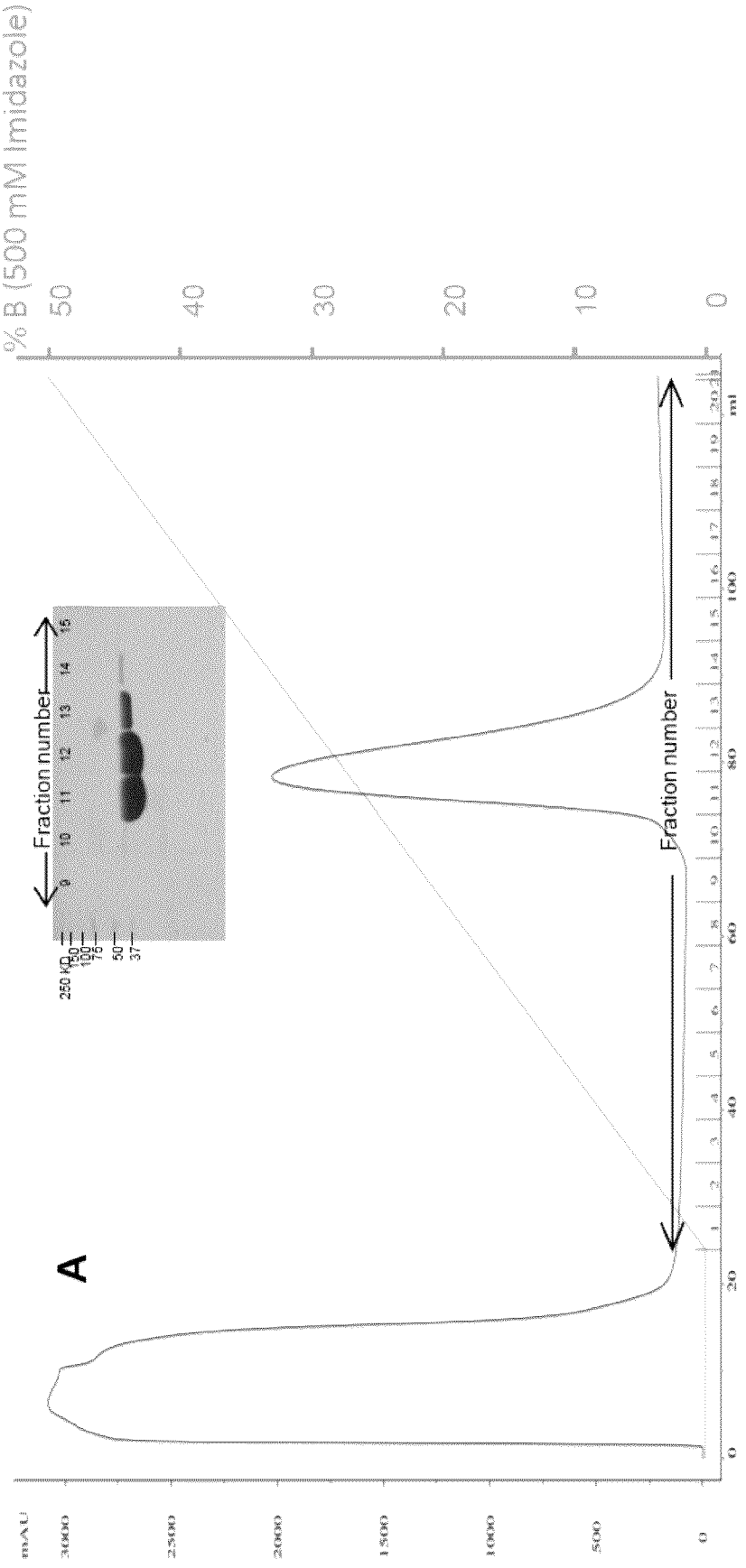

% B (500 mM Imidazole)
50
40
30
20
10
0
mAU
3000
2500
2000
1500
1000
500
0
ml
Fraction number
Fraction number
A
250 KD
150
100
75
50
37

FIG. 14A

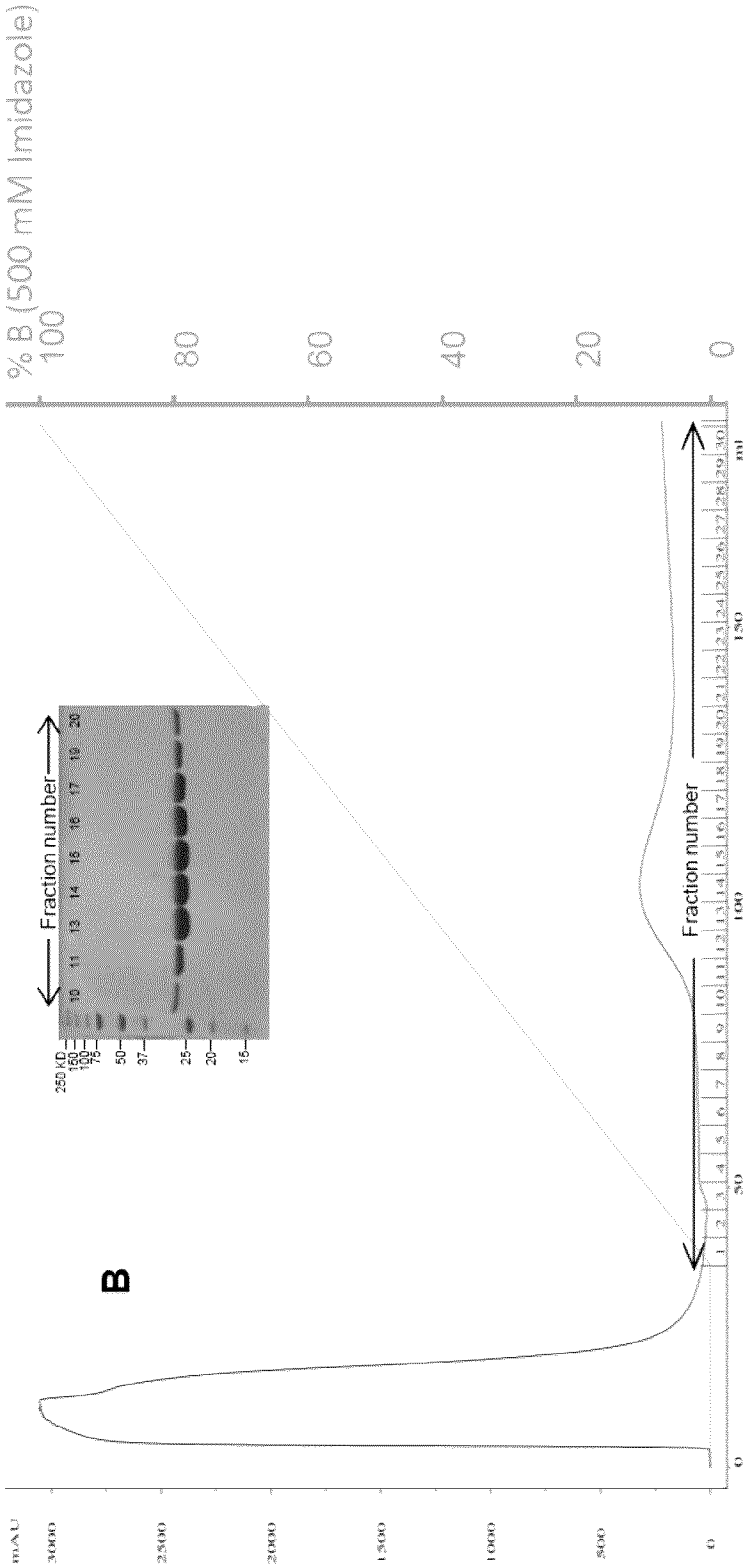

% B (500 mM Imidazole)
100
80
60
40
20
0
mAU
3000
2500
2000
1500
1000
500
0
Fraction number
Fraction number
B
250 kD
150
100
75
50
37
25
20
15

FIG. 14B

MTEYNAFRVELADKIAHVQINRPDKINAMNQDFWREIIEIFRWVDDTDEVRVVVLSGAGK
HFSSGIDLMLLAQVGSQLGKDVGRNADALRRKILELQASFNAVDNCRKPVLAAIQGYCLG
GAIDLVSACDMRYSTADAQFSIKEIDIGMAADVGTLQRLPRIIGDGMMRELAYTGRMVDG
EEARSIGLVNRTYADQAALMDGVFELARQIAAKSPIAIRGTKEMIRYMRDHRVDDGLEYV
ATWNAAMLQSADLRVAMAAHMAKQKPEFAD

FIG. 15A

gtgaccgagtacaacgcattccgtgtggagctggcagacaagatcgcccacgtccagatcaaccgtccggacaagatcaatgccatgaacca
ggacttctggcgcgagatcatcgagatcttccgctgggtcgacgataccgacgaagtgcgggtggtcgtgctgtccggggcgggcaagcattt
ttcctcggggatcgacctgatgctgctggcccaggtcggcagccaactgggcaaggacgtcggccgcaacgccgacgccctgcgtcgcaag
atcctcgagctgcaggcctcgttcaacgcggtcgacaactgccgcaagccggtgctcgcggcgatccagggctactgcctgggcggcgccat
cgacctggtctcggcctgcgacatgcgctactcgaccgccgacgcgcagttctcgatcaaggagatcgacatcggcatggccgccgacgtcg
gcaccctgcagcgcctgccgcggatcatcggcgatggcatgatgcgcgagctggcctacaccgggcgcatggtcgacggcgaagaggcac
ggagcatcggcctggtcaatcgtacctacgcggaccaggccgcattgatggatggggtcttcgaactggcgcggcagatcgccgcgaagtcg
ccgatcgccatccgtggtaccaaggaaatgatccgctatatgcgcgaccaccgggtcgacgacggcctcgagtacgtcgctacctggaacgc
cgccatgctccagtccgccgacctgcgggtggcgatggccgcgcatatggccaagcagaagccggaattcgctgattga

FIG. 15B

MNTAVEPYKASSFDLTHKLTVEKHGHTALITINHPPANTWDRDSLIGLRQLIEHLNRDDD
IYALVVTGQGPKFFSAGADLNMFADGDKARAREMARRFGEAFEALRDFRGVSIAAINGYA
MGGGLECALACDIRIAERQAQMALPEAAVGLLPCAGGTQALPWLVGEGWAKRMILCNER
VDAETALRIGLVEQVVDSGEARGAALLLAAKVARQSPVAIRTIKPLIQGARERAPNTWLPE
ERERFVDLFDAQDTREGVNAFLEKRDPKWRNC

FIG. 16A

atgaacactgccgtcgaaccctacaaggcttcctccttcgacctgacccacaagctcaccgtggaaaagcacgggcacaccgcgctgatcacc
atcaaccacccgccggccaacacctgggaccgcgactcgctgatcggcctgcgccaactgatcgagcacctcaaccgcgacgacgatatcta
cgccctggtagtgaccggccaggggccgaagttcttctccgccggcgccgacctgaacatgttcgccgacggcgacaaggcccgcgctcgc
gagatggcccgccgcttcggcgaggccttcgaggcgctgcgcgatttccgtggggtgtcgatcgcggcgatcaacggctacgccatgggcg
gcggcctggagtgcgccctcgcctgcgacatccgcatcgccgagcgccaggcgcagatggccctcccggaggccgcggtgggcctgctgc
cctgcgccggcgggacccaggcgctgccctggctggtgggcgaaggctgggccaagcggatgatcctctgcaacgagcgggtggatgcgg
aaaccgccctgcgcatcggcctggtcgaacaggtggtggacagcggcgaggcgcgcggcgccgccctgctgctggcggccaaggtggca
cgccagagcccggtggcgatccgcaccatcaagccgctgatccagggtgcccgcgaacgcgcgccgaacacctggctgccggaggagcg
cgagcgcttcgtcgatctgttcgacgcccaggacacccgcgaaggggtcaacgccttcctcgagaagcgcgatcccaagtggcgcaactgct
ga

FIG. 16B

MNVLFEERPSLHGFRIGIATLDAEKSLNALSLPMIEALAAKLDAWAEDAGIACVLLRGNGA
KAFCAGGDVRKLVDACREQPGEVPALARRFFADEYRLDYRIHTYPKPFICWAHGYVMGG
GMGLMQGAGIRIVTPSSRLAMPEIGIGLYPDVGASWFLARLPGRLGLFLGLSAAQMNARD
ALDLDLADRFLLDDQQDALLAGLVQMNWNESPQVQLHSLLRALEHEARGELPEAQLLPR
RPRLDALLDQPDLASAWQALVALRDDADPLLARGAKTLAEGCPMTAHLVWQQIERARYL
SLAEVFRLEYAMSLNCTRHPDFAEGVRARLIDRDNAPNWHWPQVESIPQTVIEAHFEPTWE
GEHPLAGL

FIG. 17A

atgaacgtgcttttcgaagaacgcccgagcctgcatggcttccgcatcggcattgccaccctggatgcggaaaagagcctgaacgccctgtccc
tgccgatgatcgaggccctggcggcgaaactcgacgcctgggccgaggacgccggcatcgcctgcgtgctgttgcgcggcaacggcgcca
aggccttttgcgccggcggcgacgtgcgcaagctggtggacgcctgccgtgagcaacccggcgaggtaccggccctggcgcgacgcttctt
cgccgatgaataccgcctcgactaccgcatccacacctacccgaaaccccttcatctgctgggcccacggctatgtaatgggcggcggcatggg
gctgatgcagggtgccgggattcgcatcgtcaccccgtccagccgcctggcgatgccggagatcggcatcggcctgtacccggacgtcggc
gccagctggttcctcgcccgcctgcccggcaggctcggcctgttcctcggcctgagcgcggcgcagatgaacgcccgcgacgctctcgacct
cgacctggccgaccgcttccttctcgacgaccagcaggacgccctgctcgccggcctggtgcagatgaactggaacgagtcgccccaagtgc
aacttcacagcctgcttcgcgccctcgaacacgaggcccgcggcgaactgccggaggcccagttgctcccgcgccggccgcgcctggacgc
actgctcgaccagccggacctggcgagcgcctggcaggcgctggtggcgctgcgcgacgacgccgacccgctgcttgcgcgtggcgcgaa
gaccctggccgagggctgcccgatgaccgcgcatctggtctggcagcagatcgagcgtgcgcgctacctgtcgctggccgaggtgttccgcc
tggaatacgccatgagcctgaactgcacgcgccatccggacttcgccgaggggtacgcgcaaggctgatcgatcgcgacaacgcaccgaa
ctggcattggccgcaggtggaatcgatcccgcagacggtgatcgaggcgcacttcgagccgacctgggagggcgagcatccgctggcggg
gctctga

FIG. 17B

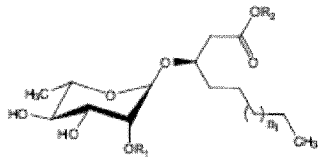
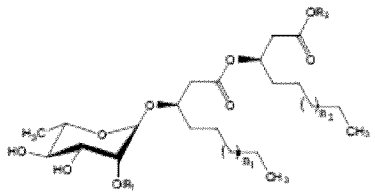
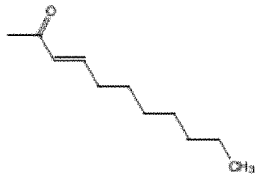
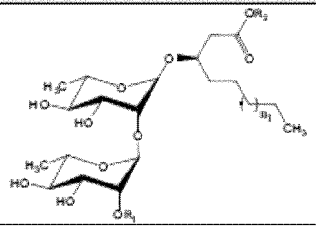
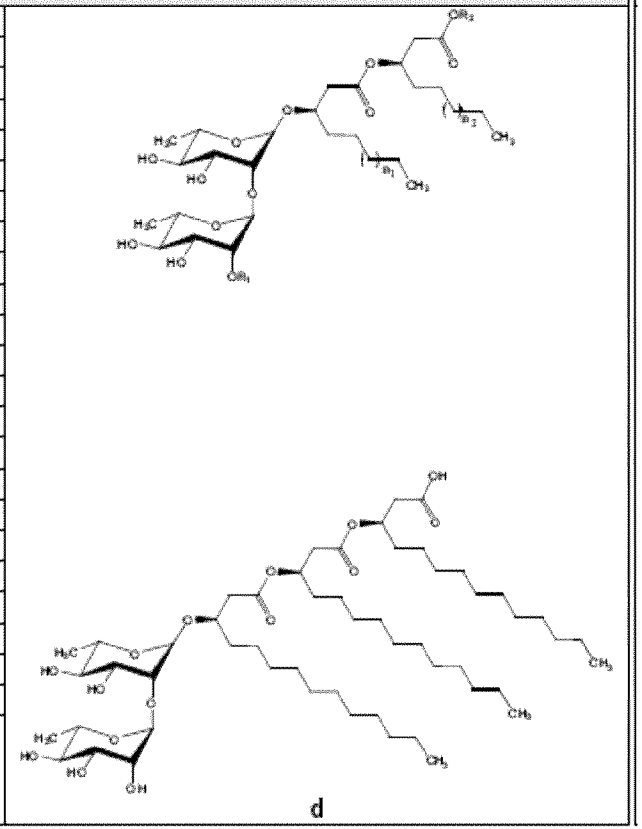
No.	Symbol	M. Form.	MW	R_1	n_1	n_2	R_2	Structure
Mono-rhamno-mono-lipidic congeners								
1	Rha-$C_{8:2}$	$C_{14}H_{22}O_7$	302.32	H	1(−4H)	–	H	
2	Rha-C_8	$C_{14}H_{26}O_7$	306.35	H	1	–	H	
3	Rha-C_{10}	$C_{16}H_{30}O_7$	334.41	H	3	–	H	
4	Rha-$C_{12:2}$	$C_{18}H_{30}O_7$	358.43	H	5(−4H)	–	H	
5	Rha-C_{12}	$C_{18}H_{34}O_7$	362.46	H	5		H	
6	Rha-$C_{14:2}$	$C_{20}H_{34}O_7$	386.48	H	7(−4H)	–	H	
Mono-rhamno-di-lipidic congeners								
7	Rha-C_8-C_8	$C_{22}H_{40}O_9$	448.55	H	1	1	H	
8	Rha-C_8-$C_{10:1}$	$C_{24}H_{42}O_9$	474.58	H	1	3(−2H)	H	
9	Rha-$C_{10:1}$-C_8	$C_{24}H_{42}O_9$	474.58	H	3(−2H)	1	H	
10	Rha-C_8-C_{10}	$C_{24}H_{44}O_9$	476.60	H	1	3	H	
11	Rha-C_{10}-C_8	$C_{24}H_{44}O_9$	476.60	H	3	1	H	
12	Rha-C_{10}-$C_{10:1}$	$C_{26}H_{46}O_9$	502.64	H	3	3(−2H)	H	
13	Rha-C_{10}-C_{10}	$C_{26}H_{48}O_9$	504.65	H	3	3	H	
14	Rha-C_8-C_{12}	$C_{26}H_{48}O_9$	504.65	H	1	5	H	
15	Rha-C_{12}-C_8	$C_{26}H_{48}O_9$	504.65	H	5	1	H	
16	Rha-C_{10}-$C_{12:1}$	$C_{28}H_{50}O_9$	530.69	H	3	5(−2H)	H	
17	Rha-$C_{12:1}$-C_{10}	$C_{28}H_{50}O_9$	530.69	H	5(−2H)	3	H	
18	Rha-C_{10}-C_{12}	$C_{28}H_{52}O_9$	532.71	H	3	5	H	
19	Rha-C_{12}-C_{10}	$C_{28}H_{52}O_9$	532.71	H	5	3	H	
20	Rha-C_{10}-$C_{14:1}$[a]	$C_{30}H_{54}O_9$	558.74	H	3	7(−2H)	H	
21	Rha-C_{12}-$C_{12:1}$[a]	$C_{30}H_{54}O_9$	558.74	H	5	5(−2H)	H	
22	Rha-C_{10}-C_{14}[a]	$C_{30}H_{56}O_9$	560.76	H	3	7	H	
23	Rha-C_{12}-C_{12}	$C_{30}H_{56}O_9$	560.76	H	5	5	H	
24	Rha-C_{12}-C_{14}	$C_{32}H_{60}O_9$	588.81	H	5	7	H	
25	Rha-C_{14}-C_{14}	$C_{34}H_{64}O_9$	616.87	H	7	7	H	
26	Rha-C_{14}-C_{16}	$C_{36}H_{68}O_9$	644.92	H	7	9	H	
27	Rha-C_{16}-C_{16}	$C_{38}H_{72}O_9$	672.97	H	9	9	H	
28	Rha-C_{10}-C_{10}-CH_3	$C_{27}H_{50}O_9$	518.68	H	3	3	CH_3	
29	Decenoyl-Rha-C_{10}-C_{10}	$C_{36}H_{64}O_{10}$	656.89	b	3	3	H	 b: α-decenoyl group
Di-rhamno-mono-lipidic congeners								
30	Rha-Rha-C_8	$C_{20}H_{36}O_{11}$	452.49	H	1	–	H	
31	Rha-Rha-C_{10}	$C_{22}H_{40}O_{11}$	480.55	H	3	–	H	
32	Rha-Rha-$C_{12:1}$	$C_{24}H_{42}O_{11}$	506.58	H	5(−2H)	–	H	
33	Rha-Rha-C_{12}	$C_{24}H_{44}O_{11}$	508.60	H	5	–	H	
34	Rha-Rha-C_{14}	$C_{26}H_{48}O_{11}$	536.65	H	7	–	H	

FIG. 18A

Di-rhamno-di-lipidic congeners								
35	Rha-Rha-C_8-C_8	$C_{28}H_{50}O_{13}$	594.69	H	1	1	H	
36	Rha-Rha-C_8-C_{10}	$C_{30}H_{54}O_{13}$	622.74	H	1	3	H	
37	Rha-Rha-C_{10}-C_8	$C_{30}H_{54}O_{13}$	622.74	H	3	1	H	
38	Rha-Rha-C_{10}-$C_{10:1}$	$C_{32}H_{56}O_{13}$	648.78	H	3	3(--2H)	H	
39	Rha-Rha-C_{10}-C_{10}	$C_{32}H_{58}O_{13}$	650.79	H	3	3	H	
40	Rha-Rha-C_8-$C_{12:1}$	$C_{32}H_{56}O_{13}$	648.78	H	1	5(--2H)	H	
41	Rha-Rha-$C_{12:1}$-C_8	$C_{32}H_{56}O_{13}$	648.78	H	5(–2H)	1	H	
42	Rha-Rha-C_{10}-$C_{12:1}$	$C_{34}H_{60}O_{13}$	676.83	H	3	5(--2H)	H	
43	Rha-Rha-$C_{12:1}$-C_{10}	$C_{34}H_{60}O_{13}$	676.83	H	5(–2H)	3	H	
44	Rha-Rha-C_{10}-C_{12}	$C_{34}H_{62}O_{13}$	678.84	H	3	5	H	
45	Rha-Rha-C_{12}-C_{10}	$C_{34}H_{62}O_{13}$	678.84	H	5	3	H	
46	Rha-Rha-C_{10}-$C_{14:1}$	$C_{36}H_{64}O_{13}$	704.89	H	3	7(--2H)	H	
47	Rha-Rha-C_{12}-$C_{12:1}$	$C_{36}H_{64}O_{13}$	704.89	H	5	5(--2H)	H	
48	Rha-Rha-$C_{12:1}$-C_{12}	$C_{36}H_{64}O_{13}$	704.89	H	5(–2H)	5	H	
49	Rha-Rha-C_{12}-C_{12}	$C_{36}H_{66}O_{13}$	706.90	H	5	5	H	
50	Rha-Rha-C_{12}-C_{14}	$C_{38}H_{70}O_{13}$	734.95	H	5	7	H	
51	Rha-Rha-C_{14}-C_{12}	$C_{38}H_{70}O_{13}$	734.95	H	7	5	H	
52	Rha-Rha-C_{14}-C_{14}	$C_{40}H_{74}O_{13}$	763.00	H	7	7	H	
53	Rha-Rha-C_{14}-C_{16}	$C_{42}H_{78}O_{13}$	791.06	H	7	9	H	
54	Rha-Rha-C_{16}-C_{14}	$C_{42}H_{78}O_{13}$	791.06	H	9	7	H	
55	Rha-Rha-C_{16}-C_{16}	$C_{44}H_{82}O_{13}$	819.11	H	9	9	H	
56	Rha-Rha-C_{14}-C_{14}-C_{14} [d]	$C_{54}H_{100}O_{15}$	989.36	Structure drawn separately [d]				
57	Rha-Rha-C_{10}-C_{10}-CH_3	$C_{39}H_{80}O_{13}$	664.82	H	3	3	CH_3	
58	Decenoyl-Rha-Rha-C_{10}-C_{10}	$C_{42}H_{74}O_{14}$	803.03	b	3	3	H	

d

FIG. 18B

Name of bacterial sp.	Culture medium	RL composition	Reference
Phylum: Actinobacteria, class: Actinobacteria			
Renibacterium salmoninarum	Mineral salts medium (MSM)+2% *n*-hexadecane or *n*-paraffin	Mono- and di-RL	(Christova et al. 2004)
Cellulomonas cellulans	Mineral salts+yeast extract+3% glycerol or 2% *n*-hexadecane	Novel rhamnose-containing glycolipid (gluco-rhamno-ribo-lipid)	(Arino et al. 1998b)
Nocardioides sp.	MSM+2% *n*-paraffin	RL[a] (congeners unidentified)	(Vasileva-Tonkova and Gesheva 2005)
Phylum: Firmicutes, class: Bacilli			
Tetragenococcus koreensis	Glucose yeast peptone/sodium acetate/mineral salts +2–5% (*w*/*v*) NaCl	RL (congeners unidentified)	(Lee et al. 2005)
Phylum: Proteobacteria, class: Betaproteobacteria			
B. glumae	Not-mentioned	Rha-Rha-C_{14}-C_{14}, C_{12}-C_{14}, and C_{14}-C_{16} (beside their isomers)	(Pajarron et al. 1993)
B. pseudomallei	Vogel–Bonner medium, glycerol medium	Di-RL congeners with C_{12}-C_{12}, C_{12}-C_{14}, C_{14}-C_{14}, C_{14}-C_{16}, and C_{16}-C_{16}	(Häussler et al. 1998)
	Nutrient broth (NB)+4% glycerol		(Dubeau et al. 2009)
B. plantarii	Mineral salts+yeast ext+soy bean oil	Rha-Rha-C_{14}, Rha-Rha-C_{14}-C_{14}, and Rha-Rha-C_{14}-C_{14}-C_{14}	(Andrä et al. 2006)
B. thailandensis	NB+4% glycerol or canola oil	Mono- and di-RL congeners with C_{12}-C_{14}, C_{14}-C_{14}, C_{14}-C_{16}, and C_{16}-C_{16}	(Dubeau et al. 2009)
Phylum: Proteobacteria, subphylum: delta/epsilon subdivision, class: Deltaproteobacteria			
Myxococcus sp.	Peptone medium+0.2% starch+0.2% glucose	Myxotyrosides A and B which are rhamno-amino-lipids	(Ohlendorf et al. 2008)
Phylum: Proteobacteria, class: Gammaproteobacteria			
Acinetobacter calcoaceticus	MSM+10% glycerol	Mono- and di-RL with C_{10}-C_{10}	(Rooney et al. 2009)
Enterobacter asburiae	MSM+10% glycerol	Mono- and di-RL with C_{10}-C_{10}	(Rooney et al. 2009)
Enterobacter hormaechei	MSM+10% glycerol	Mono- and di-RL with C_{10}-C_{10}	(Rooney et al. 2009)
Pantoea stewartii	MSM+10% glycerol	Mono- and di-RL with C_{10}-C_{10}	(Rooney et al. 2009)
Pantoea sp.	MSM+2% *n*-paraffin or kerosene	RL[a] (congeners unidentified)	(Vasileva-Tonkova and Gesheva 2007)
P. alcaligenes	Nutrient agar (NA), mineral medium+0.5% (*w*/*v*) of palm oil	Mono- and di-RL with C_8-C_{10}, C_{10}-C_{10}, and C_{10}-C_{12}	(Oliveira et al. 2009)
P. aeruginosa	MSM+soluble or insoluble carbon sources	Refer to text	Refer to text

FIG. 19A

Name of bacterial sp.	Culture medium	RL composition	Reference
P. cepacia	Nutrient broth	RL[a] (congeners unidentified)	(Onbasli and Aslim 2009)
P. chlororaphis	Kay's minimal medium, MSM+2% glucose, Siegmund Wagner (SW) agar	Mono-RL with C_{10}-C_8, C_{10}-C_{10}, C_{12}-C_{10}, $C_{12:1}$-C_{10}, C_{12}-C_{12}, $C_{12:1}$-C_{10}, C_{14}-C_{10}, and $C_{14:1}$-C_{10}	(Gunther et al. 2005, 2006)
P. clemancea	MSM+3% glycerol+high phosphate+no trace elements	Mono- and di-RL with C_{10}-C_{10}	(Rahman et al. 2009)
P. collierea	MSM+3% glycerol+high phosphate+no trace elements	Mono- and di-RL with C_{10}-C_{10}	(Rahman et al. 2009)
P. fluorescens	Bushnell–Haas Broth+0.1 mg yeast, 0.1 X NB+2,000 IU penicillin	RL[a] (congeners unidentified)	(Husain 2008)
P. fluorescens	NA, NB, MSM+2% (*v/v*) olive oil and 1 g/L ammonium nitrate	RL[a] (congeners unidentified)	(Abouseoud et al. 2008a, b)
P. fluorescens	NB, tryptic soy broth (TSB), minimal medium+petrol 0.1% *v/v*	RL[a] (congeners unidentified)	(Wilson and Bradley 1996)
P. fluorescens	MSM+2% *n*-paraffin or kerosene	RL[a] (congeners unidentified)	(Vasileva-Tonkova et al. 2006)
P. fluorescens	Nutrient broth	RL[a] (congeners unidentified)	(Onbasli and Aslim 2009)
P. luteola	Sugar beet molasses at 5% *w/v*	RL[a] (congeners unidentified)	(Onbasli and Aslim 2009)
P. putida	Mineral salts+yeast extract+glucose+corn oil	RL, the exact structures were not determined	(Martinez-Toledo et al. 2006)
P. putida	Sugar beet molasses at 5% *w/v*	RL[a] (congeners unidentified)	(Onbasli and Aslim 2009)
P. putida	Mineral salt agar+2% hexadecane	RL[a] (congeners unidentified)	(Tuleva et al. 2002)
P. putida	Mineral salts medium+phenanthrene	Suggested to be RL[a]	(Cuny et al. 2004)
P. stutzeri	MSM+crude oil 1%	RL[a] (congeners unidentified)	(Celik et al. 2008)
P. stutzeri	MSM+glucose 10%	RL[a] (congeners unidentified)	(Janiyani et al. 1992)
P. stutzeri	Nutrient broth	RL[a] (congeners unidentified)	(Onbasli and Aslim 2009)
P. teessidea	MSM+3% glycerol+high phosphate+no trace elements	Mono- and di-RL with C_{10}-C_{10}	(Rahman et al. 2009)
Pseudoxanthomonas sp.	MSM+2% mannitol	Not definitely confirmed	(Nayak et al. 2009)

[a] Identity of RL has not been definitively confirmed

FIG. 19B

```
   1 atgcggcgcg aaagtctgtt ggtatcggtt tgcaagggcc tgcgggtaca tgtcgagcgc
  61 gttgggcagg atcccgggcg cagcacggtg atgctggtca acggcgcgat ggcgaccacc
 121 gcctcgttcg cccggacctg caagtgcctg gccgaacatt tcaacgtggt gctgttcgac
 181 ctgcccttcg ccgggcagtc gcggcagcac aacccgcagc gcgggttgat caccaaggac
 241 gacgaggtgg aaatcctcct ggcgctgatc gagcgcttcg aggtcaatca cctggtctcc
 301 gcgtcctggg gcggtatctc cacgctgctg gcgctgtcgc gcaatccgcg cggtatccgc
 361 agctcggtgg tgatggcatt cgcccctgga ctgaaccagg cgatgctcga ctacgtcggg
 421 cgggcgcagg cgctgatcga gctggacgac aagtcggcga tcggccatct gctcaacgag
 481 accgtcggca aatacctgcc gccgcgcctg aaagccagca accatcagca catggcttcg
 541 ctggccaccg gcgaatacga gcaggcgcgc tttcacatcg accaggtgct ggcgctcaac
 601 gatcggggct acctggcttg cctggagcgg atccagagcc acgtgcattt catcaacggc
 661 agctgggacg agtacaccac cgccgaggac gcccgccagt tccgcgacta cctgccgcac
 721 tgcagtttct cgcgggtgga gggcaccggg catttcctcg acctggagtc caagctggcc
 781 gcggtacgcg tgcaccgcgc cctgctcgag cacctgctga agcaaccgga gccgcagcgg
 841 gcggaacgcg cggcgggatt ccacgagatg gccatcggct acgcctga
```

FIG. 20A

```
MRRESLLVSV CKGLRVHVER VGQDPGRSTV MLVNGAMATT ASFARTCKCL     50
AEHFNVVLFD LPFAGQSRQH NPQRGLITKD DEVEILLALI ERFEVNHLVS    100
ASWGGISTLL ALSRNPRGIR SSVVMAFAPG LNQAMLDYVG RAQALIELDD    150
KSAIGHLLNE TVGKYLPPRL KASNHQHMAS LATGEYEQAR FHIDQVLALN    200
DRGYLACLER IQSHVHFING SWDEYTTAED ARQFRDYLPH CSFSRVEGTG    250
HFLDLESKLA AVRVHRALLE HLLKQPEPQR AERAAGFHEM AIGYA         295
```

FIG. 20B

```
   1 atgcacgcca tcctcatcgc catcggctcg gccggcgacg tatttccctt catcggcctg
  61 gcccggaccc tgaaactgcg cgggcaccgc gtgagcctct gcaccatccc ggtgtttcgc
 121 gacgcggtgg agcagcacgg catcgcgttt gtcccgctga gcgacgaact gacctaccgc
 181 cggaccatgg gcgatccgcg cctgtgggac cccaagacgt ccttcggcgt gctctggcaa
 241 gccatcgccg ggatgatcga gccggtctac gagtacgtct cggcgcagcg ccatgacgac
 301 atcgtggtgg tcggctcgct atgggcgctg ggcgcacgca tcgctcacga gaagtacggg
 361 attccctacc tgtccgcgca ggtctcgcca tcgaccctgt tgtcggcgca cctgccgccg
 421 gtacacccca agttcaacgt gcccgagcag atgccgctgg cgatgcgcaa gctgctctgg
 481 cgctgcatcg agcgcttcaa gctggatcgc acctgcgcgc cggagatcaa cgcggtgcgc
 541 cgcaaggtcg gcctggaaac gccggtgaag cgcatcttca cccaatggat gcattcgccg
 601 cagggcgtgg tctgcctgtt cccggcctgg ttcgcgccgc cccagcagga ttggccgcaa
 661 cccctgcaca tgaccggctt cccgctgttc gacggcagta tcccggggac cccgctcgac
 721 gacgaactgc aacgctttct cgatcagggc agccggccgc tggtgttcac ccagggctcg
 781 accgaacacc tgcagggcga cttctacgcc atggccctgc gcgcgctgga acgcctcggc
 841 gcgcgtggga tcttcctcac cggcgccggc caggaaccgc tgcgcggctt gccgaaccac
 901 gtgctgcagc gcgcctacgc gccactggga gccttgctgc catcgtgcgc cgggctggtc
 961 catccgggcg gtatcggcgc catgagccta gccttggcgg cgggggtgcc gcaggtgctg
1021 ctgccctgtg cccacgacca gttcgacaat gccgaacggc tggtccggct cggctgcggg
1081 atgcgcctgg gcgtgccgtt gcgcgagcag gagttgcgcg gggcgctgtg gcgcttgctc
1141 gaggacccgg ccatggcggc ggcctgtcgg cgtttcatgg aattgtcaca accgcacagt
1201 atcgcttgcg gtaaagcggc ccaggtggtc gaacgttgtc atagggaggg ggatgctcga
1261 tggctgaagg ctgcgtcctg a
```

FIG. 21A

```
   1 mhailiaigs agdvfpfigl artlklrghr vslctipvfr daveqhgiaf vplsdeltyr
  61 rtmgdprlwd pktsfgvlwq aiagmiepvy eyvsaqrhdd ivvvgslwal gariahekyg
 121 ipylsaqvsp stllsahlpp vhpkfnvpeq mplamrkllw rcierfkldr tcapeinavr
 181 rkvgletpvk riftqwmhsp qgvvclfpaw fappqqdwpq plhmtgfplf dgsipgtpld
 241 delqrfldqg srplvftqgs tehlqgdfya malralerlg argifltgag qeplrglpnh
 301 vlqrayaplg allpscaglv hpggigamsl alaagvpqvl lpcahdqfdn aerlvrlgcg
 361 mrlgvplreq elrgalwrll edpamaaacr rfmelsqphs iacgkaaqvv erchregdar
 421 wlkaas
```

FIG. 21B

```
   1 atggaccgga tagacatggg cgtgctggtg gtgctgttca atcctggcga cgacgacctg
  61 gaacaccttg gcgaactggc ggcggcgttt ccgcaactgc gcttcctcgc cgtcgacaac
 121 tcaccgcaca gcgatccgca gcgcaatgcc cggctgcgcg ggcaaggcat cgccgtgctg
 181 caccacggca accggcaggg catcgccggc gccttcaacc aggggctcga cgcgctgttc
 241 cggcgtggcg tgcagggtgt gctgctgctc gaccaggact cccgtcccgg cggcgccttc
 301 ctcgccgccc agtggcgcaa cctgcaggcg cgcaacggtc aggcctgcct gctcggccca
 361 cggatcttcg accggggcga ccggcgcttc ctgccggcca tccacctcga cgggctggcg
 421 ctcaggcaac tgtccctgga cggcctgacg atcccacagc gcacctcgtt cctgatctcc
 481 tccggctgcc tgctgacccg cgaggcctac cagcgcctcg gccacttcga cgaggaactg
 541 ttcatcgacc atgtggacac cgagtacagc ctgcgcgccc aggcgctgga cgtgcccctg
 601 tacgtcgacc cgcggctggt cctcgagcac cgcatcggca cgcgcaagac ccgccgcctc
 661 ggcggtctca gcctcagcgc gatgaaccac gccccgctgc gccgctacta cctggcgcgc
 721 aacggcctgc tggtcctgcg ccgctacgcc cggtcctcgc cgctggccct gctggcgaac
 781 ctgccgaccc tgacccaggg cctcgcggtg ctcctgctcg aacgcgacaa gctgctcaag
 841 ctgcgctgcc tgggctgggg cctgtgggac ggcctgcggg ggcgcggcgg cgcactggag
 901 cgcaaccgcc cgcgcctgct gaagcgcctc gccggtccgg ccgtggcgtc cgtagcttcc
 961 ggcaaggcca aggcctag
```

FIG. 22A

```
   1 mdridmgvlv vlfnpgdddl ehlgelaaaf pqlrflavdn sphsdpqrna rlrgqgiavl
  61 hhgnrqgiag afnqgldalf rrgvqgvlll dqdsrpggaf laaqwrnlqa rngqacllgp
 121 rifdrgdrrf lpaihldgla lrqlsldglt ipqrtsflis sgclltreay qrlghfdeel
 181 fidhvdteys lraqaldvpl yvdprlvleh rigtrktrrl gglslsamnh aplrryylar
 241 ngllvlrrya rssplallan lptltqglav lllerdkllk lrclgwglwd glrgrggale
 301 rnrprllkrl agpavasvas gkaka
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FIG. 22B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2014/051060

A. CLASSIFICATION OF SUBJECT MATTER
IPC: ***A61K 38/46*** (2006.01), ***A61K 31/7088*** (2006.01), ***A61K 31/713*** (2006.01), ***A61P 31/04*** (2006.01), ***C12N 1/20*** (2006.01), ***C12N 1/36*** (2006.01) (more IPCs on the last page)

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC: ***A61K 38/46*** (2006.01), ***A61K 31/7088*** (2006.01), ***A61K 31/713*** (2006.01), ***A61P 31/04*** (2006.01), ***C12N 1/20*** (2006.01), ***C12N 1/36*** (2006.01) (more IPCs on the last page)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)
TotalPatent, STN (Biosis, CaPlus, Medline, Agricola), Canadian Patent Database
Pseudomonas aeruginosa, Pseudomonas, rhamnolipid, enoyl-CoA hydratase/isomerase, enoyl, enoyl coenzyme a, crotonase, virulence, PhaJ, Rhl

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	ABDEL-MAWGOUD, A.M. *et al.* "A stereospecific pathway diverts β-oxidation intermediates to the biosynthesis of rhamnolipid biosurfactants". Chemistry & Biology. 16 January 2014 (16-01-2014). Vol. 21, pages 156-164, ISSN 1074-5521 *the whole document*	1-39
A	ABDEL-MAWGOUD, A.M. *et al.* "Rhamnolipids: diversity of structures, microbial origins and roles". Applied Microbiology and Biotechnology. 2010. Vol. 86, pages 1323-1336, ISSN 0175-7598	1-39

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:
"A"	document defining the general state of the art which is not considered to be of particular relevance
"E"	earlier application or patent but published on or after the international filing date
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O"	document referring to an oral disclosure, use, exhibition or other means
"P"	document published prior to the international filing date but later than the priority date claimed
"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&"	document member of the same patent family

Date of the actual completion of the international search 4 February 2015 (04-02-2015)	Date of mailing of the international search report 09 February 2015 (09-02-2015)
Name and mailing address of the ISA/CA Canadian Intellectual Property Office Place du Portage I, C114 - 1st Floor, Box PCT 50 Victoria Street Gatineau, Quebec K1A 0C9 Facsimile No.: 001-819-953-2476	Authorized officer Julie Treadwell (819) 994-8909

C12N 15/113 (2010.01) , ***C12N 15/60*** (2006.01) , ***C12N 15/61*** (2006.01) , ***C12N 9/88*** (2006.01) , ***C12N 9/90*** (2006.01) , ***C12P 19/44*** (2006.01) , ***C12Q 1/02*** (2006.01) , ***C12Q 1/527*** (2006.01) , ***C12Q 1/533*** (2006.01) , ***C12Q 1/68*** (2006.01) , ***G01N 33/48*** (2006.01)