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Enhanced rhamnolipid production in Burkholderia thailandensis transposon knockout strains deficient in polyhydroxyalkanoate (PHA) synthesis

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	residual PHA production in the phb mutant strains suggests B. thailandensis possesses a secondary unelucidated PHA synthesis pathway.
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Dear Professor Steinbuchel,

Please find attached an original paper title "**Enhanced rhamnolipid production in *Burkholderia thailandensis* transposon knockout strains deficient in polyhydroxyalkanoate (PHA) synthesis**" for consideration for publication in AMB.

In this paper we report the effects on rhamnolipid production through the knockout of PHA synthetic genes in *Burkholderia thailandensis*.

This manuscript follows up on our previous AMB publication " Funston SJ, Tsaousi K, Rudden M, Smyth TJ, Stevenson PS, Marchant R, Banat IM (2016) Characterising rhamnolipid production in *Burkholderia thailandensis* E264, a non-pathogenic producer. Appl Microbiol Biotechnol 100: 7945-7956".

This manuscript and its findings have not been previously published or under consideration for publication in any other journal. All named authors have approved this manuscript and its submission to AMB.

Yours sincerely,

Ibrahim Banat

Enhanced rhamnolipid production in *Burkholderia thailandensis* transposon knockout strains deficient in polyhydroxyalkanoate (PHA) synthesis

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Abstract

Microbially produced rhamnolipids have significant commercial potential however, the main bacterial producer, *Pseudomonas aeruginosa,* is an opportunistic human pathogen, which limits biotechnological exploitation. The non-pathogenic species *Burkholderia thailandensis* produces rhamnolipids, however yield is relatively low. The aim of this study was to determine whether rhamnolipid production could be increased in *Burkholderia thailandensis* through mutation of genes responsible for the synthesis of the storage material polyhydroxyalkanoate (PHA), thereby increasing cellular resources for the production of rhamnolipids. Potential PHA target genes were identified in *B. thailandensis* through comparison with known function genes in *Pseudomonas aeruginosa*. Multiple knockout strains for the *phbA*, *phbB* and *phbC* genes were obtained and their growth characteristics, rhamnolipid and PHA production determined. The wild type strain and an RL-deficient strain were used as controls. Three knockout strains (Δ*phbA*1, Δ*phbB*1 and Δ*phbC*1) with the best enhancement of rhamnolipid production were selected for detailed study. Δ*phbB*1 produced the highest level of purified RL (3.78 g/l) compared to the wild type strain (1.28 g/l). In Δ*phbB*1 the proportion of mono-rhamnolipid was also increased compared to the wild type strain. The production of PHA was reduced by at least 80% in all three *phb* mutant strains, although never completely eliminated. These results suggest that, in contrast to *Pseudomonas aeruginosa*, knockout of the PHA synthesis pathway in *Burkholderia thailandensis* could be used to increase rhamnolipid production. The evidence of residual PHA production in the *phb* mutant strains suggests *B. thailandensis* possesses a secondary unelucidated PHA synthesis pathway.

Keywords Rhamnolipid – *Burkholderia thailandensis* – PHA – knockout strains

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Introduction

There is considerable current industrial interest in microbial biosurfactants as replacements for chemical surfactants in a wide range of commercial products including, food (Campos *et al.* 2013), pharmaceutical (Fracchia *et al*. 2015), health (Elshikh *et al*. 2016), petroleum (Darne *et al.* 2016) and as general replacement to chemical surfactants (Marchant & Banat 2012b), however the route to exploitation has so far not been without difficulties (Marchant & Banat, 2012a). The main issues have been focussed around obtaining sufficient yield from microbial fermentation coupled with the need to find cost effective downstream processing strategies to produce a commercially viable final product competitive with the chemical surfactants in current use. Although there is a wide range of different biosurfactants produced by bacteria, fungi and yeasts, the low molecular weight glycolipid biosurfactants have attracted most attention and within this group the rhamnolipids have been investigated

extensively. The first identified and most widely studied rhamnolipid producer is *Pseudomonas aeruginosa*, however this bacterium is a known opportunistic pathogen which precludes industrial exploitation as a biosurfactant producing organism. A potential solution to this problem has been to seek alternative naturally occurring non-pathogenic microorganisms that could be used in place of *P. aeruginosa* (Marchant *et al.* 2014). One such organism is *Burkholderia thailandensis* which produces predominantly di-rhamnolipid with $C_{14}C_{14}$ alkyl chains (Funston *et al.* 2016).

A review published by Müller & Hausmann (2011) proposed a systems biology approach to genetically 'enhance' the bacterial metabolome to maximise rhamnolipid production to an industrially viable scale using methods such as genetic alteration of existing RL producers and recombinant production using heterologous hosts (Müller & Hausmann 2011). Several studies have been carried out on *P. aeruginosa* and other potential RL producing strains in an attempt to produce a strain that is highly efficient in RL production. A study by Wang *et al.* (2007) showed that cloning the RL synthesis genes *rhlA* and *rhlB* into the non-RL producing strains *P. aeruginosa* PAO1 Δ*rhlA* and *Escherichia coli* BL21(DE3) using transposon mediated chromosome integration resulted in both newly engineered strains producing RL. The engineered *E. coli* BL21(DE3) strain was shown to produce only mono-RL demonstrating that genetic engineering can also be used to produce specific RL structures reducing downstream processing and purification stages (Wang *et al.* 2007). Zhao *et al.* (2015) showed the successful production of RL from *Pseudomonas stutzeri* DQ1 under anaerobic conditions after insertion of the *rhlABRI* operon. These studies show that heterologous production of RL is possible however RL production rates by these mutants (1.61 gl^{-1}) are still too low to be regarded as industrially viable where an approximate yield of 4 gl^{-1} is required (Zhao *et al.* 2015). Finally, Grosso-Becerra *et al.* (2016) have attempted to address both the problem of the pathogenicity of *P. aeruginosa* and the yield of rhamnolipids by cloning some of the RL synthesis genes *rhlA* and *rhlB* into an apparently non-pathogenic strain *P. aeruginosa* ATCC 9027. This study showed that the recombinant strain was able to produce mono-rhamnolipid with a yield comparable to *P. aeruginosa* PAO1 and that the recombinant ATCC 9027 strain was non-pathogenic in a murine model (Grosso-Becerra *et al.* 2016).

A different approach to increase RL yield is to genetically modify the metabolic processes within the bacterial cell to streamline RL production and therefore to

artificially drive more resources towards RL production at the expense of other non-essential secondary metabolites. One such method of achieving this would be to abrogate the biosynthetic pathways of other secondary metabolites that are in direct competition for resources or precursors used in RL production. An example pathway is that of polyhydroxyalkanoic acid (PHA) synthesis. PHAs are linear polyesters produced by bacteria as intercellular carbon storage granules when carbon is present in excess. Pham *et al.* (2004) showed that the biosynthetic pathways of RLs and PHAs in *P. aeruginosa* are highly similar and compete for the same 3-hydroxy fatty acid precursors (Pham *et al.* 2004). PHAs produced by *P. aeruginosa* primarily comprise medium chain length (MCL) monomers of between 6 – 14 carbon atoms. This diverse range in PHA monomer structure is thought to be dependent on carbon source, specific PHA synthase enzymes and the metabolic pathways involved (Madison & Huisman 1999; Lee *et al.* 2004). When MCL fatty acids are available as the sole source of carbon for bacteria, β-oxidation is the main pathway used whereas when unrelated carbon sources are present the *de novo* fatty acid biosynthesis pathway is used (Rehm *et al.* 1998). It was shown that the acyl carrier protein (ACP) intermediates produced by the *de novo* fatty acid biosynthesis pathway are used as precursors for the MCL monomers and the specific substrate for the MCL-PHA synthase enzyme is (R)-3-hydroxyacyl-coenzyme A (CoA) (Rehm *et al.* 1998; Fiedler *et al.* 2000). Therefore, to serve as a substrate for PHA synthesis, (R)-3-hydroxyacyl-ACP must first be converted to its corresponding CoA derivative.

Studies by Rehm *et al.* (1998) and Fiedler *et al.* (2000) showed that in *P. aeruginosa* the *phaG* gene coding for (R)-3-hydroxyacyl-ACP:CoA transacylase plays an important role in linking the PHA and fatty acid synthesis pathways (Rehm *et al.* 1998; Fiedler *et al.* 2000). The *rhlA* gene coding for rhamnosyltransferase I in *P. aeruginosa* was also shown to directly compete with *phaG* for (R)-β-hydroxyalkanoyl-ACP and in addition *rhlA* was also shown to be able to produce CoA-linked fatty acid dimers using ACP-linked fatty acids (Soberón-Chávez *et al.* 2005; Cabrera-Valladares *et al.* 2006). This means that *rhlA* may also play a role in PHA synthesis in *P. aeruginosa*. This appears to be evident as *P. aeruginosa phaG* mutants are still capable of PHA production at a low level whereas in other RL- producing *Pseudomonas* spp. *phaG* mutants are completely deficient in PHA production (Gutierrez *et al.* 2013). This close relationship is evident in the structure of the RhlA

and PhaG enzymes as there is a 57% DNA sequence homology between the two corresponding genes in *P. aeruginosa* PA14. In addition both enzyme functions can be silenced using the same inhibitor compound, 2-bromohexanoic acid (2-BrHA) resulting in the inhibition of both RL and PHA simultaneously (Gutierrez *et al.* 2013).

It is clear from recent research that in *P. aeruginosa* the synthesis of PHA and RLs are closely linked with both synthetic processes competing for the same precursors. Although this presents a promising point for metabolic manipulation towards RL overproduction in *P. aeruginosa* recent work has shown that this may prove difficult. A study by Choi *et al.* (2011) reported a range of mutant strains each with different PHA or RL synthesis genes knocked out. Results from this study showed that whilst the knockout of RL synthesis genes led to a significant increase in PHA production, this was not the case for RL production in strains where PHA synthesis genes had been knocked out (Choi *et al.* 2011). The lack of increased RL production in PHA synthesis mutants may be connected with the stringent transcriptional control of RL synthesis that exists in *P. aeruginosa* mediated through various quorum sensing and environmental regulatory systems which have been widely elucidated in previous research (Perfumo *et al.* 2013). In contrast to this however, Torrego-Solana *et al.* (2013) showed that when the *phaC*1, *phaZ* and *phaC*2 genes were all knocked out together using site directed mutagenesis in the same mutant, *P. aeruginosa* 47T2 ΔAD, a 28% increase in RL production was observed when grown using waste frying oils (2%) as a carbon source. This mutant also showed a higher rate of carbon consumption and an increase in the conversion efficiency of oleic acid to (E)-10-hydroxy-8-octadecanic acid highlighting the rearrangement of metabolic processes resulting from the PHA knockout (Torrego-Solana *et al.* 2012).

In this paper we report the effects on rhamnolipid production through the knockout of PHA synthetic genes in *Burkholderia thailandensis*.

Materials and Methods

Microorganism and culture conditions

The wild type organism used for this study was *Burkholderia thailandensis* E264 (ATCC 700388). The *B. thailandensis* transposon mutants were obtained from the

University of Washington (Gallagher *et al.* 2013) and were maintained on either NB agar (*Oxoid*) or NB broth (*Oxoid*) (Supplementary Table S1). Rhamnolipid and PHA production studies were carried out on a small scale. Seed cultures of *B. thailandensis* E264 were grown in 1 L Erlenmeyer flasks with 100 ml NB + 4% glycerol (v/v) at 30°C with 200 rpm rotary shaking for 24 h. The $OD_{600\ nm}$ of the seed culture was adjusted to ~2.0 with sterile NB + 4% glycerol (v/v) before inoculation of batch fermentation flasks. Ten ml of this seed culture was then added to 90 ml sterile NB + 4% glycerol in a 1 L Erlenmeyer flask and cultures were incubated at 30°C with 200 rpm rotary shaking.

Polymerase Chain Reaction (PCR)

PCR reactions were carried out in 0.2 ml PCR tubes and contained a final concentration of 1 X PCR Buffer (*Invitrogen*) 1.5 mM $MgCl_2$, 0.2 mM of each d.NTP (*Invitrogen*), 0.5 µM forward and reverse primers, 50 ng template DNA and 1 U recombinant *Taq* DNA Polymerase (*Invitrogen*). All PCR amplification was performed using a TC5000 Thermo cycler (*Bibby Scientific Ltd.*, UK) and run under the following conditions; 1 X initial denaturation cycle of 5 min at 95°C, 30 X cycles of denaturation at 95°C for 30 sec, annealing at 55-65°C for 30 sec and extension at 72°C for 90 sec followed by a final extension cycle at 72°C for 10 min. Following amplification PCR products were routinely held at 4°C.

Extraction, purification and quantification of rhamnolipid

Extraction of rhamnolipids from cell free supernatant was carried out using acid precipitation followed by solvent extraction described by Smyth *et al.* (2010). Samples taken from fermentations were first centrifuged at 10,500 x g for 15min to remove the cells. Hydrochloric acid (*Sigma-Aldrich*) was used to adjust the pH of the cell free supernatant to ~2.0. The supernatant was then extracted three times with an equal volume of HPLC grade ethyl acetate (*Sigma-Aldrich*) and the aqueous phase was discarded. Anhydrous $MgSO_4$ (*Sigma-Aldrich*) was then added at a concentration of 0.01 g/ml and mixed through the ethyl acetate to remove any residual aqueous phase. Ethyl acetate recovered from the extractions was filtered using grade 1 filter paper (*Whatman*) then dried under vacuum using a rotary evaporator (*Buchi*, Switzerland) leaving a crude honey like RL extract. The sample was then dried again using nitrogen gas and weighed to give the RL crude extract yield.

Solid phase extraction (SPE) was used to remove any impurities from the RL crude extract. Strata SI-1 Silica (55 µm, 70 Å) Giga tubes (*Phenomenex*) were used to separate and clean up the rhamnolipid containing crude extracts. HPLC grade chloroform (*Sigma-Aldrich*) was passed through the SPE column until the silica was fully conditioned, the sample was then dissolved in a small amount of chloroform and applied to the column. Chloroform was then passed through the column until any contaminants had been washed out. A solvent solution of chloroform/methanol at a ratio of 5:0.3 was then used to elute the mono-rhamnolipids. A solvent solution of chloroform/methanol (ratio 5:0.5) was used to elute the di-rhamnolipids.

Purified samples for analysis by ESI-MS were first diluted to a concentration of 0.1 mg/ml in HPLC grade methanol (*Sigma-Aldrich*). Direct infusions were carried out on a Thermo Spectra LCQ™ mass spectrometer fitted with a quadrupole ion trap. Rhamnolipid samples were analysed in ion negative mode with an acquisition range varying between 50-1000 Da. A spray voltage of 3.5 kV was used with a capillary temperature of 250˚C.

HPLC-QToF-MS of rhamnolipid samples was carried out after SPE purification. For HPLC separation the following parameters were used, Static phase, Aglient poroshell SB-C3, 2.1x100 mm, particle size 2.7 µm. Mobile phase 1, H_2O (4 mM ammonium acetate) and mobile phase 2, MeCN were used for chromatographic separation as follows; 0-17 min 50% - 70% mobile phase 2, 17.0-17.5 min 70% mobile phase 2, 17.5-18.0 min: 70% - 50% mobile phase 2, 18 – 20 min: 50% mobile phase 2.

Polyhydroxyalkanoic acid (PHA) extraction and quantification using GC-MS

PHA was extracted from *B. thailandensis* E264 cells using a method described by Guo *et al.* (2011) with some adjustments. Cells were collected by centrifugation of 50 ml liquid culture at 13,000 x g for 15 min. The cell supernatant was discarded and the pellet lyophilised for 48 h. The cell dry weight was recorded and the lyophilised material extracted with chloroform and sodium hypochlorite (30 ml chloroform/g dry cell biomass with 3 ml/g 20% sodium hypochlorite solution v/v in d.H_2O). Extraction took place over 24 h before centrifugation at 3000 x g for 10min. The chloroform phase was carefully removed and filtered using grade 1 filter paper (*Whatman*). The PHA was then precipitated using ice cold methanol and samples were dried completely using

compressed nitrogen gas. The PHA extracts were weighed to obtain a crude extract yield before preparation for GC-MS analysis.

Methanolysis was carried out to prepare samples for GC-MS analysis as described by Wang *et al.* (2009). Chloroform was added at 400 µl/mg dry weight to dissolve the PHA and an equal volume of sulphuric acid: methanol solution (1.7 : 0.3) was added to it and the solution was incubated at 100°C for 140 min. For the recovery and clean-up of methyl esters of PHA monomers samples were cooled to room temperature and 2 ml 25% ammonia (*Sigma Aldrich*) was added in a drop wise fashion until the sample could be safely vortexed for 2 min. Samples were then centrifuged at 3000 x g for 5 min and 1.5 ml of the chloroform phase was carefully collected and 500 µl d.H_2O was added, the sample was then vortexed vigorously and centrifuged at 3000 x g for 5 min. The chloroform phase was used for analysis.

GC-MS analysis was performed on an Agilent triple quadrupole equipped with an MS detector and fitted with an Agilent HP-5 ms column (30 m length, 0.25 mm internal diameter, 0.25 mm film) (*Agilent Technologies*, USA). PHA monomer samples in the form of hydroxyalkanoic acid methyl esters and PHB as hydroxybutyric acid methyl esters were analysed. Methanolyzed samples (2 µl) were automatically injected into the GC at a split ratio of 1:50. Hexadecanoic acid was used as internal standard and was added before the methanolysis. The injection temperature was set at 280°C while the oven and column temperatures were programmed as 60°C for 1 min then increased to 120°C at 20°C /min, and then increased to 250°C at 15°C/min and held for 5 min. Compressed helium was used as carrier gas. Mass spectra were acquired at 1250 scan speed using electron impact energy of 70 eV at 200°C ion-source and 280°C interface temperatures respectively. The PHAs were identified using the NIST database.

Quantification of glycerol consumption during fermentations using GC-MS

Samples were taken for glycerol analysis by aseptically removing 1000µl culture from the fermentation vessel. Cells were removed by centrifugation for 2min at 10500xg and the cell supernatant was carefully transferred to a clean, sterile 1.5ml Eppendorf tube and stored at -20°C for further analysis. Prior to GC-MS analysis glycerol was first derivatised to glycerol triacetate and separated from the culture supernatant (Wu, et al. 2011). This was done by adding 10µl NMIM (N-Methylmidazole) to 10µl culture

supernatant in a 1.5ml Eppendorf tube. A volume of 75µl acetic anhydride was then added and samples were incubated at room temperature for 5min. After incubation 100µl dH2O was added and the sample was vortexed for 10sec. Dichloromethane was then added at a volume of (100µl) and 10µl hexadecane was also added as an internal standard. The sample was vortexed briefly and left to separate at room temperature. The organic phase was then collected and 0.1g Na_2SO4 (anhydrous) was added to remove any residual aqueous phase from the sample. Samples were then filtered using grade 1 filter paper (Whatman® qualitative filter paper, Grade 1) and added to clean, labelled HPLC vials (Sigma-Aldrich).

GC analysis was performed on an Agilent system equipped with an MS detector (Agilent Technologies, CA, USA). An HP-5 silica based capillary column (30m×0.25mm ×0.25 µm) was used for the separation of the glycerol derivative with a split ratio of 50:1. Compressed helium was used as the carrier gas at a flow rate of 1ml min^{-1} in constant flow mode. The initial column temperature was 140°C for 2min which then increased to 250°C at a rate of 20°C/min and maintained for 2min. The inlet temperature was set at 210°C and the detector temperature was 250°C.

Results

Quantitative analysis of PHAs produced by *B. thailandensis* E264 using GC-MS

There have been no previous reports of PHA production within *B. thailandensis*, therefore chemical analysis of *B. thailandensis* cultures was carried out to determine if this bacterium produces PHAs. Any PHAs present in the supernatants of *B. thailandensis* E264 shake flask cultureswere extracted and analysed by GC-MS using protocols previously developed for PHA analysis in *P. aeruginosa*. GC-MS analysis of *B. thailandensis* E264 culture supernatant extracts showed the presence of PHA monomers corresponding to polyhydroxybutyrate, polyhydroxyhexanoate (C6), polyhydroxyoctanoate (C8), polyhydroxydecanoate (C10) and polyhydroxydodecanoate (C12) (Figure 1).

Identification of genes associated with PHA production in *B. thailandensis* E264

Due to the novel identification of PHA monomers in *B. thailandensis* supernatant extracts the identification of putative PHA synthesis genes was considered paramount in establishing a potential mechanism for PHA synthesis in this species. There have been reports of PHA production in *Burkholderia sacchari* and other un-characterised strains such as *Burkholderia* sp. USM (JCM15050) (Chee *et al.* 2010; Mendonça *et al.* 2014). Unfortunately the genomic information and gene annotations for these organisms are limited and therefore un-reliable to use for identification of PHA synthesis genes in *B. thailandensis*. *P. aeruginosa* is a known producer of PHA and fully annotated genomic and proteomic sequence data for various strains are readily available (Hoffmann 2004; Pham *et al.* 2004). As such it was decided to use *P. aeruginosa* as a model organism for PHA analysis in *B. thailandensis*.

In *P. aeruginosa* the primary genes associated with PHA synthesis are *phaC1* and *phaC2* which code for poly(3-hydroxyalkanoic acid) synthase 1 and poly(3-hydroxyalkanoic acid) synthase 2 respectively **(**Hoffmann *et al.* 2000). In addition *phaG* which codes for the enzyme (R)-3-hydroxydecanoyl-ACP:CoA transacylase, was also found to play a major role in PHA synthesis (Hoffmann *et al.* 2000). The peptide sequences expressed from *phaC1*, *phaC2* and *phaG* in *P. aeruginosa* PAO1 were obtained from the NCBI. To determine putative PHA synthesis within *B. thailandensis* a BLASTp analysis was carried out using these sequences against the *B. thailandensis* E264 genome (taxid:271848). Results from the BLAST search showed that PhaC1 and PhaC2 possessed sequence similarity with a poly-beta-hydroxybutyrate polymerase found in *B. thailandensis* (Accession No: WP_019254714.1) with a percentage identity of 40% and 39% respectively and an 86% and 39% query coverage. The RhlA homolog in *B. thailandensis* proved to have the highest sequence identity with PhaG with a percentage identity of 43% and a 91% query coverage. Further *in silico* analysis showed that the poly-beta-hydroxybutyrate polymerase within *B. thailandensis* to which both PhaC1 and PhaC2 showed similarity is encoded by a gene annotated as *phbC* and that within the *B. thailandensis* genome this gene is given the locus tag BTH_I2255. Following closer examination of *phbC* within the *B. thailandensis* E264 genome it became clear that this gene may be part of a small gene operon or gene cluster containing other genes potentially involved in PHA synthesis (Figure 2A). These additional genes were identified as *phbA*, *phbB* and *phaR* and the function of their products were computationally predicted using COGs

(Clusters of Orthologous Groups of proteins). The *phbA* gene was found to code for acetyl CoA-acetyltransferase, the *phbB* gene was found to code for acetyacetyl-CoA reductase while the *phaR* gene was found to code for an unknown protein, however using BLASTx alignments its function was predicted to be as a DNA binding polyhydroxyalkanoate synthesis repressor.

It was clear from these results that the gene cluster identified could be potentially involved with the production of PHA in *B. thailandensis* E264. To investigate any specific metabolic pathways within which these genes played a major role the gene products were analysed using the Kyoto Encyclopedia of Genes and Genomes (KEGG). KEGG analysis showed that *phbA*, *phbB* and *phbC* have a role in the butyrate biosynthesis pathway in *B. thailandensis* E264. Specifically, the combination of these three gene products results in the formation of poly-β-hydroxybutyrate from Acetyl CoA (Figure 2B). The *phaR* gene was not found to be part of any metabolic KEGG pathways, this is understandable however if its main function is to act as a DNA binding-transcriptional repressor. In addition, *phbA*, coding for acetyl CoA-acetyltransferase, was also found to be involved in many other important metabolic pathways including different carbon metabolism pathways.

Selection of *B. thailandensis* transposon mutants

Gallagher *et al.* (2013) reported the creation of a sequence defined transposon mutant library within *B. thailandensis* providing a wide range of single insertion transposon mutants for research purposes (Gallagher et al. 2013). Using this library multiple transposon insertion mutants for *phbA*, *phbB* and *phbC* were identified and acquired (Supplementary Table S1). Transposon insertion into the gene of interest within each mutant was confirmed via PCR using primers external to the respective gene, (Data not shown).

Fermentation analysis of *B. thailandensis* transposon mutants and initial screening of RL synthesis

The 10 transposon mutant strains, WT *B. thailandensis* E264 and *B. thailandensis* Δ*rhlAD* (which is deficient in RL production) were cultured in shake flasks. Samples were taken every 24h throughout the fermentation period to measure bacterial growth

and every 48h to measure glycerol depletion. End-point samples were taken after 264h fermentation to quantify RL and PHA production.

B. thailandensis strains with transposon insertions in the same gene produced almost identical growth curve patterns and values (Supplementary Figure S1). There was however a wide variation in the growth of the mutant strains with all transposon mutants producing OD_{600} values less than the WT strain throughout the fermentation period. The *phbC* mutants produced the least amount of biomass after 264h fermentation ~1.82 g, with the *phbB* and *phbA* mutants producing ~3.06 g and ~5.49 g respectively in comparison to the WT which produced 6.66 g. The Δ*rhlAD* strain produced 7.85 g.

GC-MS was used to quantify glycerol concentrations in the medium throughout the fermentation period. There was no significant difference in glycerol depletion pattern between any of the *B. thailandensis* phb mutant strains and the WT strain. The Δ*rhlAD* strain however showed a slower decline in glycerol concentration and had the highest residual concentration value of 2.04% (w/v) after 264h. All phb mutants and the WT strain had a final glycerol concentration within the range of 1.28% - 1.94% (w/v) (Supplementary Figure S2).

Gravimetric analysis of crude extracts obtained from each culture showed a variation in the range of RL production (g/l) among the *B. thailandensis* mutant strains for each of the three PHA synthesis genes (Figure 3). There was a significant increase in crude RL yield in all three *phbA* and *phbB* mutants when compared with the WT (Figure 4). This pattern was less conclusive in the *phbC* mutant strains, with two strains showing a trend toward increased RL production and two strains showing a trend toward reduced RL production when compared with the WT, these trends did not however reach statistical significance (Figure 3). The transposon mutant that produced the highest crude RL yield was Δ*phbB*1 with a yield of 3.99 g/l compared to 1.49 g/l in the WT, this represented a 2.68 fold increase in RL production compared to that of the WT. As expected the Δ*rhlAD* mutant was shown to produce 0.71 g/l crude RL extract however this represents mostly contaminants and other extracellular material that had been carried through in the initial solvent extraction. Based on these data it was decided that the mutants with the highest crude RL yield for each gene would be further investigated; therefore the *phbA*1, *phbB*1 and *phbC*1 mutants were used for all

further analysis in this study. Interestingly when crude RL yield for each of these three mutants was normalised to dry cellular biomass it was found that all three mutant strains possessed a higher RL production yield than that of the WT (Figure 4).

Quantitative analysis of RL and PHA production by *B. thailandensis* transposon mutants

To obtain a more accurate determination of RL production by the selected transposon mutant strains, solid phase extractions (SPE) were carried out on the crude RL extracts to remove any contaminants or other cellular products that had been initially co-extracted with the RLs. The *phbB*1 mutant still showed the highest level of RL production with a yield of 3.78 g/l purified RL, while the WT strain had a yield of 1.28 g/l. Results from the other mutant strains showed that *phbA*1 produced 2.33 g/l purified RL and the *phbC*1 produced 1.43g/l. Both the phbA1 and the *phbB*1 mutants had a yield of purified RL that was significantly higher than that of the WT strain E264. The RL negative strain Δ*rhlAD* showed barely detected levels of RLs (Figure 5A). GC-MS analysis of the WT strain, the transposon mutant strains *phbA*1, *phbB*1, *phbC*1 and the Δ*rhlAD* strains alongside a hexadecanoic acid standard allowed for direct quantification of the amount of PHA present in each sample (Figure 5B). All of the transposon mutants produced significantly less PHA than both the WT and the RL negative strain. Interestingly PHA production although significantly reduced was not completely eliminated in the three transposon mutant strains.

Qualitative Analysis of RL production in *B. thailandensis* transposon mutants

As the *phbB*1 mutant was shown to have the greatest increase in RL production compared to the WT strain. LC-MS analysis was carried out to examine if there were any differences in specific RL congeners produced (Figure 6). Results showed that there was a significant shift in the ratio of mono-RL:di-RL produced by the *phbB*1 mutant compared to the WT strain (Table 1).

Discussion

The main aims of this study were to establish the presence of a PHA synthesis system in *B. thailandensis*, and to determine if the disruption of this system would lead to an

increase in RL production by driving more carbon towards RL synthesis. Both PHAs and RLs are secondary metabolite compounds that are produced using similar biosynthetic pathways. In addition they share some of the same pre-cursor molecules meaning that, from a systems biology perspective, there is potential for process manipulation to increase RL production. A recent study by Choi *et al.* (2011) was highly effective in demonstrating this relationship in *P. aeruginosa* showing that the knockout of RL synthesis genes led to an increase in PHA production by freeing up more of the shared (R)-3-hydroxyfatty acid precursor molecules. They were unable however to show the converse of this as the knockout of PHA synthesis genes did not result in increased RL production (Choi *et al.* 2011). This is where increasing RL production in *P. aeruginosa* becomes problematic, as a number of other studies have shown that RL synthesis is stringently regulated in *P. aeruginosa* by a complex cell density dependant QS system (Perfumo *et al.* 2013).

This study has shown for the first time that *B. thailandensis* has a functional PHA synthesis system and is capable of producing a range of PHA monomers. These data have also predicted one possible pathway and precursor molecules used by *B. thailandensis* for PHA synthesis (Figure 2B). Similar to RL synthesis Acetyl CoA is a major precursor for PHA synthesis. This is converted to acetoacetyl CoA by acetyl CoA – acetyltransferase which is encoded by *phbA*. Acetyl CoA – acetyltransferase is then reduced to R - 3 – hydroxybutanol – CoA by the product of the *phbB* which encodes for acetoacetyl – CoA – reductase. The product of the *phbC*, poly(R) – hydroxyalkanoic acid synthase, class I, then converts R - 3 – hydroxybutanol – CoA to poly – β – hydroxybutyrate. At present this is the only PHA biosynthesis system that has been identified in *B. thailandensis* and shown to be functional in a laboratory study. There is, however a possibility that there may be other genes or gene operons that could contribute to PHA accumulation (as is the case with RL production) such as potential *phaC* homologs found on chromosome 2 (WP_009895308.1) and chromosome 1 (WP_009890861.1). The presence of such genes would account for the residual PHA production observed by this study in transposon mutant of *phbA*, *phbB* and *phbC*.

To fully analyse the functionality of this PHA synthesis system a number of transposon mutants were selected for each gene in the operon with the exception of *phaR,* which is a PHA synthesis repressor and on knockout would only lead to an increase in PHA

production which was not the aim of this study. Growth kinetics of each strain showed a wide variation between the mutant strains, the WT *B. thailandensis* E264 strain and the RL negative Δ*rhlAD* indicating that the change in metabolic flux or increase/decrease in specific metabolic precursors has a big effect on how carbon is used within the cell, specifically for cell growth.

Observations made during this study showed that the increase in acetyl CoA availability through *phbA* knockout resulted in a significant decrease in PHA production coupled with a slight increase in RL synthesis and slight reduction in cell replication/growth rate. This indicates that although there was a significant reduction in PHA the resulting free acetyl CoA was not efficiently driven towards RL production. The fundamental reason for this is due to the fact that acetyl CoA is used for a wide range of cellular processes within the cell. Oh *et al.* (2014) demonstrated that in *Burkholderia* acetyl Co-A is used with oxaloacetate for the synthesis of citrate in the tricarboxylic acid (TCA) cycle, which is subsequently used as a precursor for oxalic acid production (Oh *et al.* 2014). Oxalic acid plays a very important role in the survival of *Burkholderia* upon entry to stationary phase. Oxalic acid is produced by *Burkholderia* species through cell density dependant QS systems which predict the onset of stationary phase and subsequently counteract increasing pH levels caused by environmental ammonia accumulation and preventing significant population crashes (Goo *et al.* 2012). As this system plays such a significant role in *B. thailandensis* it is possible that the majority of free acetyl co-A created by the mutation of *phbA* is utilised in oxalic acid synthesis and therefore there was no significant increase in RL production.

In contrast to this the *phbC* mutant strains were hypothesised to have the greatest effect on increasing RL production. This was due to the product of the *phbC* gene, poly(R) – hydroxyalkanoic acid synthase, class I, being highly specific to PHA production with no evidence that it is involved in any other metabolic pathways in *B. thailandensis*. The mutation of this gene resulted in a significant reduction in cell growth and biomass accumulation coupled with a significant decrease in PHA,however there was no significant increase in RL production yield after SPE clean up. The RL production yield of the best performing *phbC* mutant, *phbC*1, was on a similar level to that observed in the WT strain meaning that the hypothesis was invalid. There was however a significant increase in specific productivity of RL in the *phbC*1

mutant as the cell produced significantly more RL per g of DCB than the wild type (1.03 gRL/gDCB compared to 0.22 gRL/gDCB). Therefore although the overall level of RL produced was similar to that of the WT strain, the Δ*phbC*1 strains cells were producing RL at a level 4.68 fold higher than the WT strain.

Knockout of the *phbB* gene proved to be the most optimal mutation for increasing RL production in *B. thailandensis* resulting in a 2.95 fold increase in RL yield for the *phbB*1 mutant (3.78 g/l purified RL compared to the WT strain 1.28 g/l purified RL). In addition the Δ*phbB*1 strain showed a significant decrease in PHA production and a decrease in biomass. Transposon mutation of the *phbB* gene in *B. thailandensis*, which codes for acetoacetyl CoA reductase, leads to an increase of acetoacetyl CoA within the cells. This resulted in successfully driving more carbon towards RL production. One explanation for this is that it could have been caused by high amounts of acetoacetyl CoA reaching a threshold and possibly initiating down regulation of PHA production through the *phaR* gene, there is however limited evidence to support this in other bacteria as expression of the *phaR* gene seems to be initiated directly by poly (R)-3-hydroxybutyrate (Maehara *et al.* 2002).

Another possible explanation is that unused acetoacetyl coA was recycled through the fatty acid synthesis pathway and its subsequent components redirected towards RL production. It is clear from this study that the knockout of the *phbB* gene produces the most significant improvement in RL production in *B. thailandensis*. As the production of PHA was not completely knocked out it may indicate the presence of additional PHA synthesis genes in the *B. thailandensis* genome which may be functional and contribute to overall PHA accumulation. Studies of PHA synthesis in *P. aeruginosa* have demonstrated that RhlA can aid in PHA synthesis (Soberón-Chávez *et al.* 2005; Cabrera-Valladares *et al.* 2006). *B. thailandensis* also possesses a RhlA homolog therefore a similar process may be occurring here, however the previously mentioned potential of *B. thailandensis* possessing additional and as yet un-elucidated PHA synthesis genes is also a valid hypothesis. Research by Choi *et al.* (2011) showed that RL production in *P. aeruginosa* could not be enhanced in this way due to the stringent transcriptional regulation systems which control RL production in *P. aeruginosa* (Choi *et al.* 2011).

It is at this point where the findings of this study become more significant as this was not the effect seen in *B. thailandensis*, instead when PHA production was knocked out in *B. thailandensis* there was a significant increase in RL production meaning that unlike *P. aeruginosa*, RL production in *B. thailandensis* does not seem to be as stringently regulated. This finding could have significant implications in further process optimisation of *B. thailandensis* for maximal RL production. Furthermore, this study has shown that when PHA production was abrogated in *B. thailandensis* not only was there a significant increase in overall RL production but there was also a large shift in the ratio of mono-RL:di-RL produced, resulting in an almost equal ratio of mono-RL:di-RL in the *phbB*1 mutant strain compared to the predominantly di-RL producing WT strain. This increase in mono-RL may have been caused by the increase in free C based precursors allowing for more HAA production. However, as the cells are still producing rhamnose at the same rate as the WT there may not be enough for the efficient conversion of mono-RL to di-RL that is seen in the WT strain where less overall RL is produced.

These findings show that the flux of closely related metabolic pathways can be manipulated to re-route specific resources towards a desired product. Another interesting observation from this study was that the glycerol consumption of the PHA transposon mutant strains was not significantly different from that of the WT strain or the RL negative Δ*rhlAD* strain. Confirming that although the growth kinetics and metabolite production differ between strains, similar amounts of glycerol are consumed during the fermentation process indicating that a different ratio of metabolic products are formed. What is clear is that metabolic flux may be a key area in successfully creating a *B. thailandensis* mutant strain that can produce high levels of RL. Further work in this area could be to attempt to knockout the oxalic acid synthesis pathway in addition to the PHA synthesis pathway to free up more fatty acid precursor for RL synthesis. While this would however require culture pH to be closely maintained externally it could potentially lead to a further increase in RL production.

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Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals.

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Figure Legends

Figure 1. GC-MS chromatogram showing specific PHA monomers produced by WT *B. thailandensis* E264 (peaks 1-4, 6) and a hexadecanoic acid internal standard (peak 5) used for direct quantification of each PHA monomer.

Figure 2. A. Putative PHA synthesis gene cluster within the genome *B. thailandensis* E264, identified using the Burkholderia genome database (Winsor *et al.* 2008). BTH_I2255 (*phbC)* encodes a poly-β-hydroxybutyrate polymerase, BTH_I2256 (*phbA*) encodes an acetyl-CoA-acetyltransferase, BTH_I2257 (*phbB*) encodes a acetyl-CoA-reductase and BTH_I2258 (*phbR*) encodes a hypothetical protein predicted to be involved with the regulation of PHA synthesis. **B.** The proposed biochemical pathway for the synthesis of PHA in *B. thailandensis*.

Figure 3. RL production in *B. thailandensis* transposon mutants after 264 h fermentation in shake flasks. Mean weights of crude RL extracts from three replicate cultures with error bars representing SD in weight from the mean. Data analysed using a one-way ANOVA with *post hoc.* Dunnett's multiple comparisons tests, (**** $p < 0.0001$, *** $p < 0.001$).

Figure 4. Comparison of RL production rate of selected *B. thailandensis* transposon mutants against the WT strain and the RL deficient Δ*rhlAD* strain. Mean weights of crude RL extracts normalised to dry cellular biomass (gl^{-1}/gDCB) from three replicate cultures with error bars representing SD in production rate from the mean. Data analysed using a one-way ANOVA with *post hoc.* Dunnett's multiple comparisons tests, (**** $p < 0.0001$, *** $p < 0.001$).

Figure 5. A. Comparison of purified RL produced by *B. thailandensis* transposon mutants after SPE of crude RL extracts compared with the WT and the RL deficient Δ*rhlAD* strain. Mean weights of purified RL extracts from three replicate cultures with error bars representing SD in weight from the mean. **B.** Quantification of total PHA using GC-MS in *B. thailandensis* transposon mutant strains compared compared with the WT and the RL deficient Δ*rhlAD* strain. Mean weights of total PHA produced by three replicate cultures with error bars representing SD in weight from the mean. Data analysed using a one-way ANOVA with *post hoc.* Dunnett's multiple comparisons tests, (**** $p < 0.0001$, *** $p < 0.001$).

Figure 6. A. HPLC-MS analysis of RLs produced by WT *B. thailandensis* E264. **B.** HPLC-MS analysis of RLs produced the *B. thailandensis phbB*1 mutant strain.

Table Legends

Table 1. Comparison of specific RL congener production between WT *B. thailandensis* and the PHA deficient *B. thailandensis phbB*1 transposon mutant after 264 h fermentation in NB + 4% glycerol.

Supplementary Figure Legends

Figure S1. Growth analysis of *B. thailandensis* PHA transposon mutant strains **A.** transposon mutants of the *phbA* gene, **B.** transposon mutants of the *phbB* gene & **C.** transposon mutants of the *phbC* gene. Mean values with error bars representing standard deviation from the mean (n=3).

Figure S2. Glycerol concentrations of *B. thailandensis phb* transposon mutants, *B. thailandensis* E264 WT and *B. thailandensis rhlAD* throughout a 264 h fermentation period. Values were obtained using GC-MS. Mean values with error bars representing standard deviation from the mean (n=3).

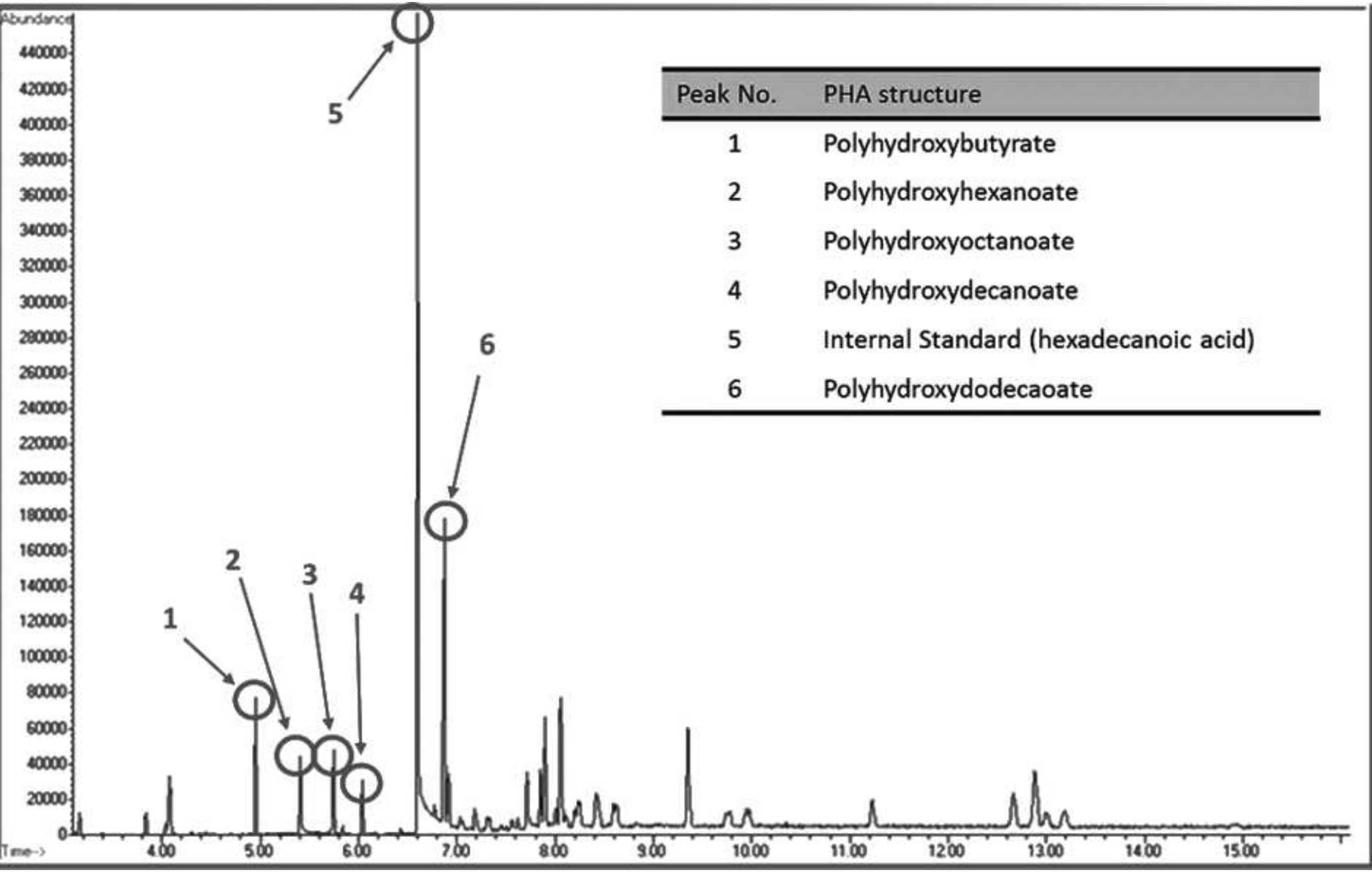
Supplementary Table Legends

Table S1. List and description of *B. thailandensis* transposon mutants in PHA synthesis genes used in this study.

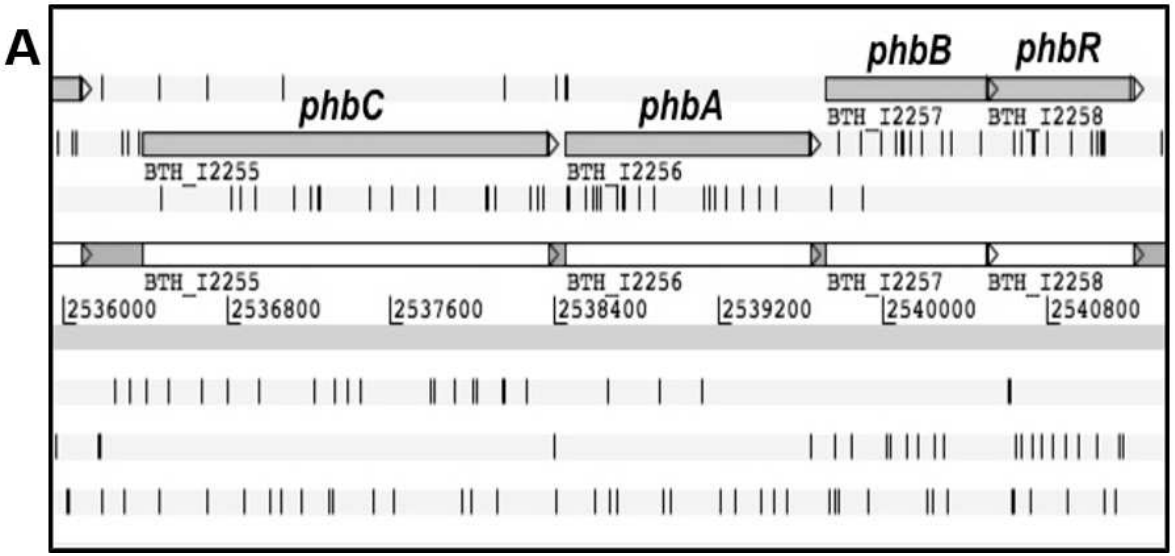
Table 1

Rhamnolipid Congener	Pseudomolecular Ion (m/z)	Retention time (min)	Relative abundance (%)	
			E264	*phbB*1
Rha-C12-C12	559.4	6.5	**0.27**	**1.96**
Rha-C12-C14/C14-C12	587.4	8.8	**7.7**	**19.68**
Rha-C14-C14	615.5	11	**19.11**	**27.94**
Rha-Rha-C12-C12	705.4	5.3	**4.88**	**3.84**
Rha-Rha-C12-C14/C14-C12	733.5	7.5	**25.69**	**19.93**
Rha-Rha-C14-C14	761.5	9.5	**39.88**	**26.15**
Rha-Rha-C14-C16/C16-C14	789.5	11.5	**2.46**	**0.51**

Figure 1



Peak No.	PHA structure
1	Polyhydroxybutyrate
2	Polyhydroxyhexanoate
3	Polyhydroxyoctanoate
4	Polyhydroxydecanoate
5	Internal Standard (hexadecanoic acid)
6	Polyhydroxydodecaoate


A
phbB
phbR
phbC
phbA
BTH_I2257
BTH_I2258
BTH_I2255
BTH_I2256
BTH_I2255
BTH_I2256
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2536800
2537600
2538400
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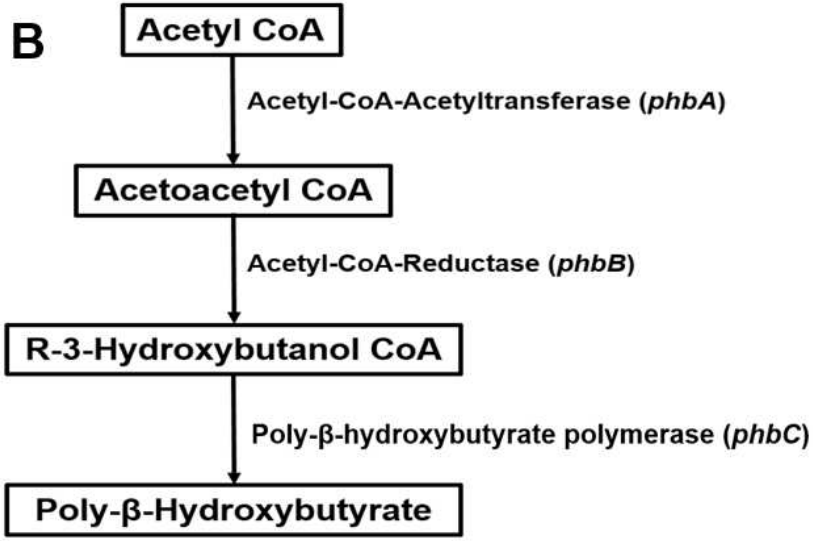
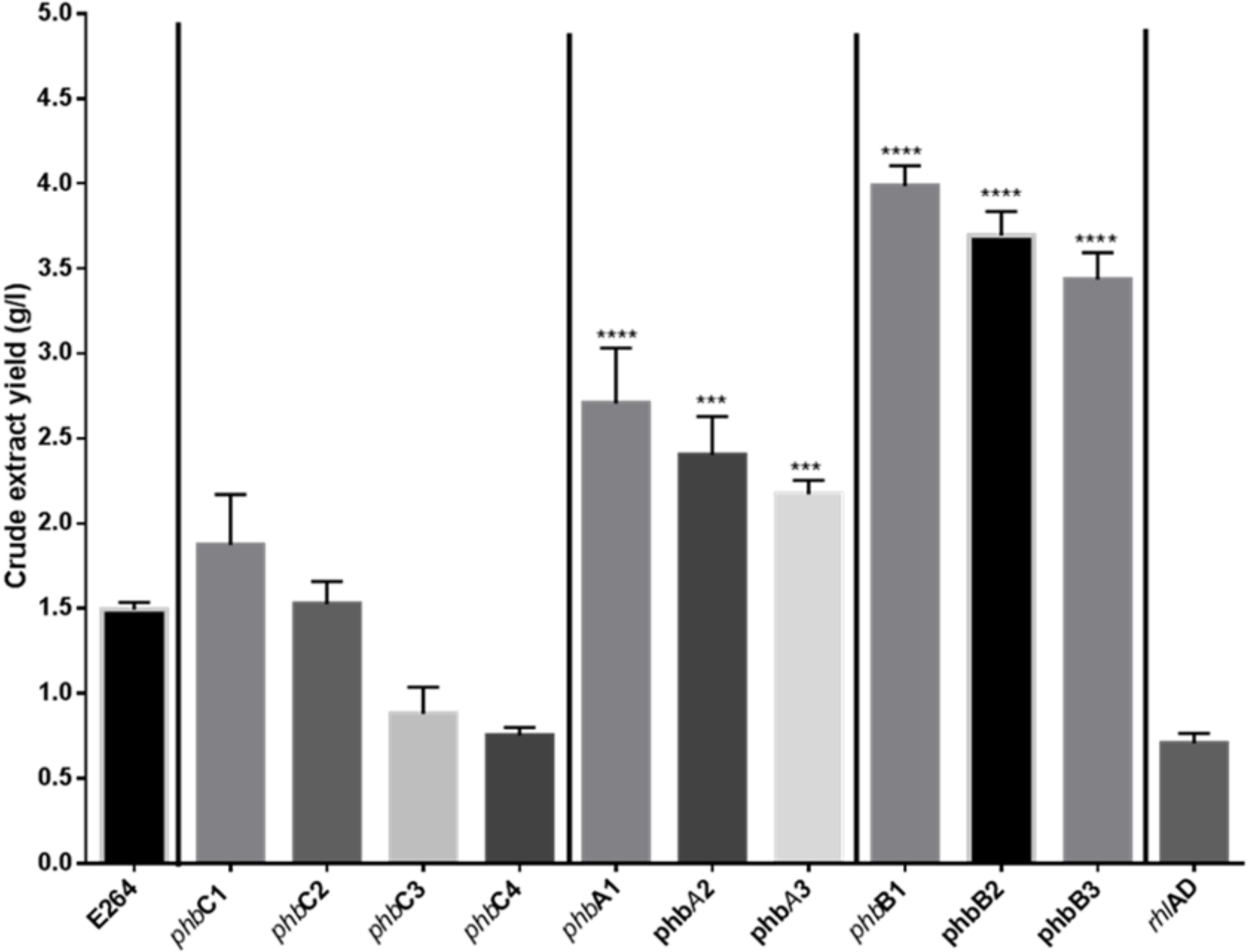
2540000
2540800


B
Acetyl CoA
Acetyl-CoA-Acetyltransferase (phbA)
Acetoacetyl CoA
Acetyl-CoA-Reductase (phbB)
R-3-Hydroxybutanol CoA
Poly-β-hydroxybutyrate polymerase (phbC)
Poly-β-Hydroxybutyrate


Crude extract yield (g/l)
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
****
***
***
****
****
****
E264
phbC1
phbC2
phbC3
phbC4
phbA1
phbA2
phbA3
phbB1
phbB2
phbB3
rhlAD

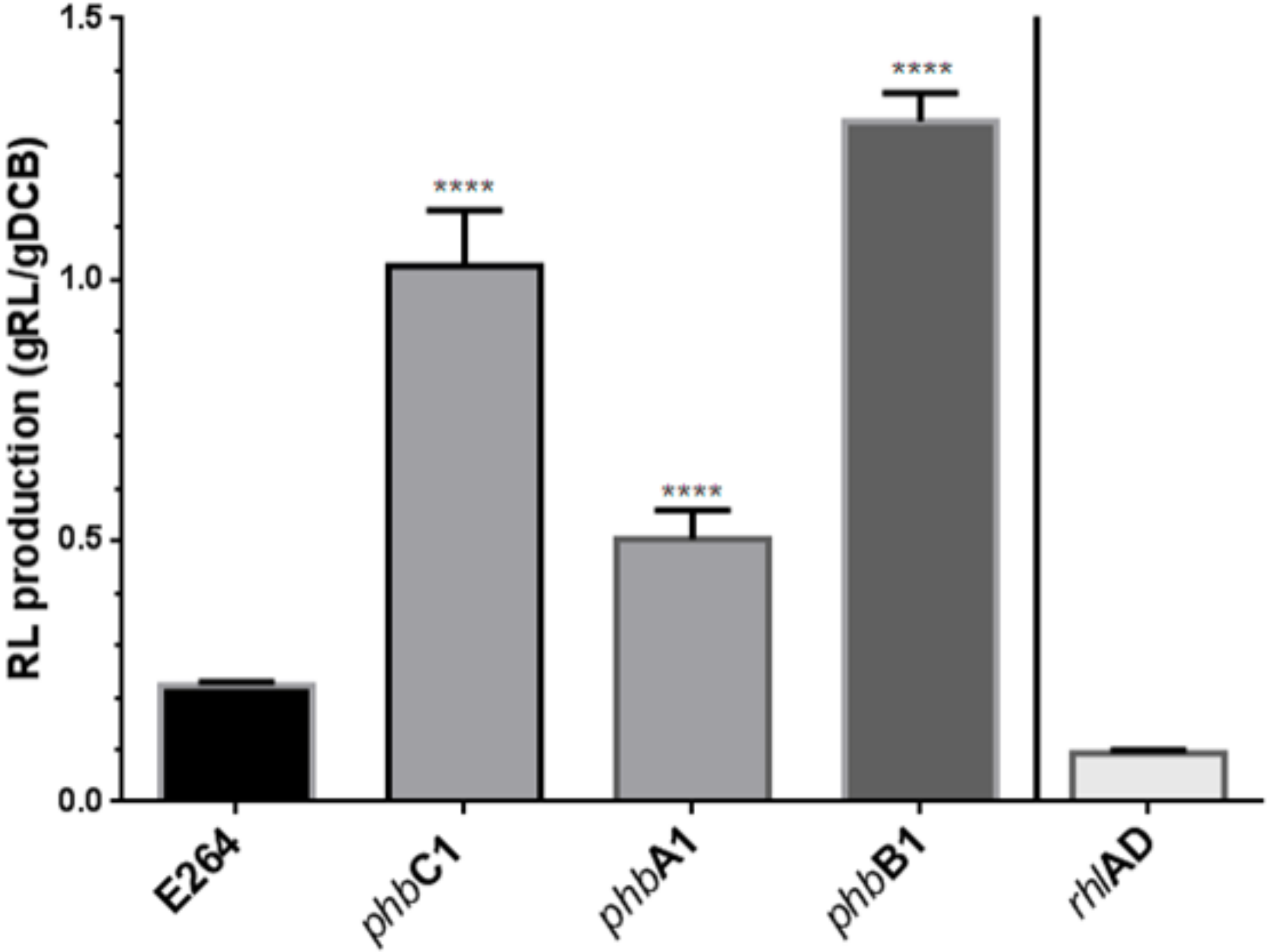
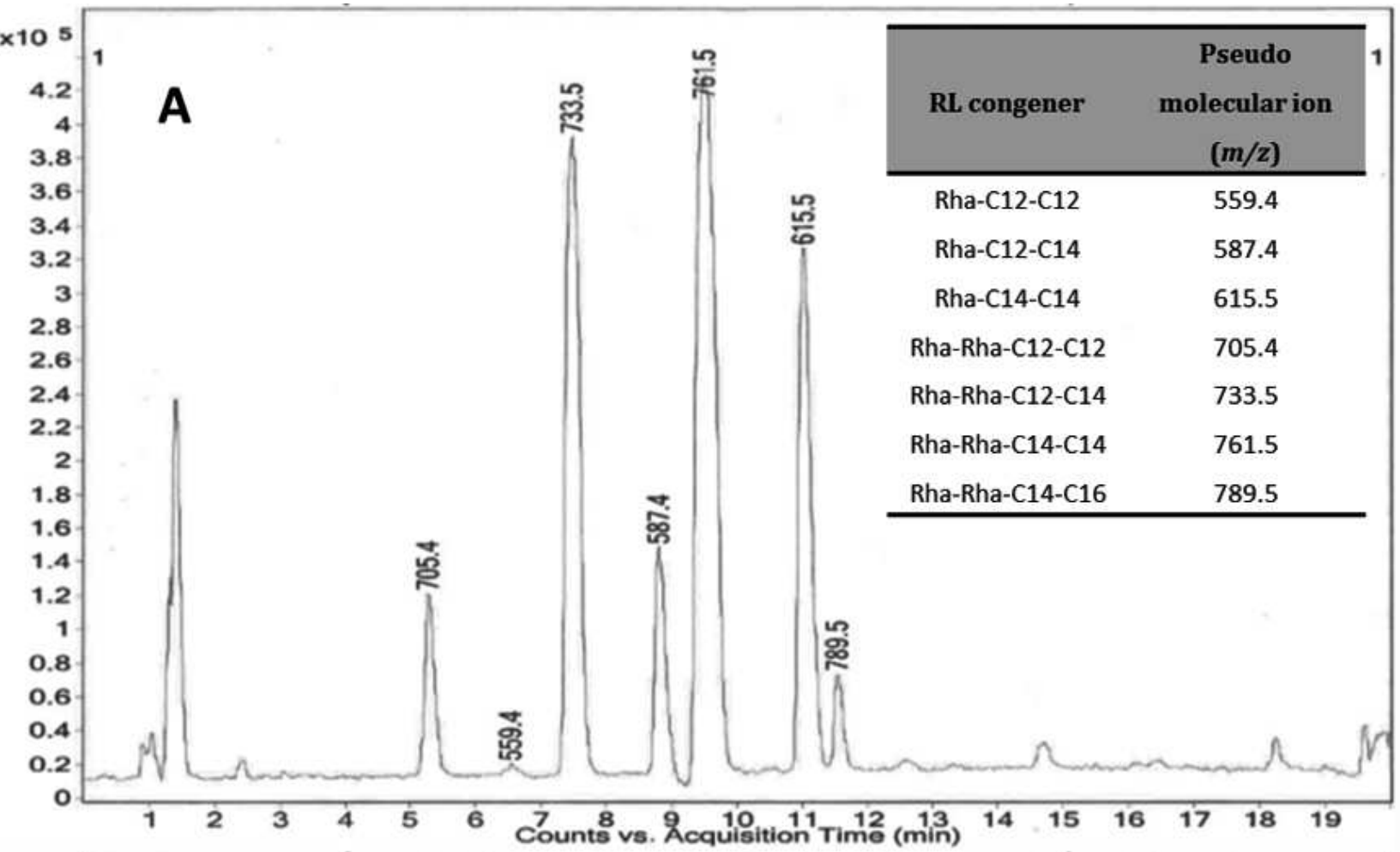

RL production (gRL/gDCB)
1.5
1.0
0.5
0.0
****
****
****
E264
phbC1
phbA1
phbB1
rhlAD

Figure 4

Figure 5

A
Purified RL (g/l)
0
1
2
3
4
5
E264
phbC1
phbA1
phbB1
rhlAD
****
****
****
B
Total PHA (g/l)
0.0
0.1
0.2
0.3
E264
phbC1
phbA1
phbB1
rhlAD
****
****
****

Figure 6



RL congener	Pseudo molecular ion (m/z)
Rha-C12-C12	559.4
Rha-C12-C14	587.4
Rha-C14-C14	615.5
Rha-Rha-C12-C12	705.4
Rha-Rha-C12-C14	733.5
Rha-Rha-C14-C14	761.5
Rha-Rha-C14-C16	789.5