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# Polyhydroxyalkanoate Synthesis Affects Biosurfactant Production and Cell Attachment to Hydrocarbons in *Pseudomonas* sp. KA-08

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**Abstract** Stressful conditions prevailing in hydrocarbon-contaminated sites influence the diversity, distribution, and activities of microorganisms. Oil bioremediation agents should develop special characteristics to cope with these environments like surfactant production and cellular affinity to hydrocarbons. Additionally, polyhydroxyalkanoate (PHA) accumulation was proven to improve tolerance to stressful conditions. *Pseudomonas* sp. KA-08 was isolated from a chronic oil-contaminated environment, it is highly tolerant to xylene, and it is able to accumulate PHA and to produce surfactant compounds that lower the water surface tension (ST) as well as bioemulsifiers. In this work, we studied the effect of the capability to accumulate PHAs on biosurfactant production and microbial attachment to hydrocarbons (MATH). Our results showed that PHA synthesis capability has a favorable effect in the production of compounds which affect the ST but not on the production of bioemulsifiers. On the other hand, PHA accumulation affects cellular affinity to xylene. MATH analysis showed that a PHA-negative mutant increased its affinity to xylene compared with the wild-type strain. This result was also observed in *Pseudomonas putida* GPp104 (a $PHA^-$ mutant), suggesting that this effect could be generalized to other *Pseudomonas* strains.

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## Introduction

Hydrocarbons are one of the contaminants more distributed worldwide. Remediation of oil-contaminated environments could be done by several ways, but bioremediation is one of the cheapest and environmental-friendly techniques. Sometimes, bioremediation includes the seeding of selected bacteria to enhance xenobiotic biodegradation. In this case, microorganisms that should cope with hydrophobic substrates need to develop some special characteristics to survive on those environments. These characteristics involve changes in the cell membranes' composition [8, 31] and/or secretion of surface-active compounds to allow hydrophobic substrate bioavailability. These surfactants are of different chemical nature and can be soluble like glycolipids, lipopeptides, fatty acids, and polymers or particulated, in which extracellular vesicles or the whole cell acts as a biosurfactant [13].

On the other hand, seeded microorganisms should be able to adapt efficiently to the new environment. It has been described that polyhydroxyalkanoate (PHA) accumulation enhances bacterial environmental fitness especially under environmental stress conditions such as UV irradiation, osmotic, thermal, and oxidative stress [1, 20, 30, 36]. PHAs are energy and carbon storage compounds accumulated as intracellular granules that can be mobilized and used under unfavorable conditions. Many bacterial species are able to accumulate PHAs. Most of PHA-producing *Pseudomonas* spp. are able to produce medium chain length PHAs (mclPHA) composed of C6 to C14 monomer units. PHA metabolism is related with several metabolic pathways. Soberón-Chávez et al. [34] described that in *P. aeruginosa* metabolic pathways of PHA and a biosurfactant rhamnolipid are related by one of their precursors: 3-hydroxyacids. Nevertheless, the relationship

between PHA synthesis and biosurfactant production has been only partially studied [5, 23, 24, 37].

*Pseudomonas* sp. KA-08, isolated from activated sludge, is able to grow using diesel and high concentration of xylene as unique carbon source and to synthesize PHA and glycolipidic biosurfactants [10]. In this work, we analyzed the relevance of PHA accumulation capabilities on biosurfactant production, emulsification capability, and cell membrane hydrophobicity, as a strategy to improve the performance of oil bioremediation agents.

## Materials and Methods

### Bacterial Strain and Growth Conditions

Bacteria, plasmids, and primers used in this work are listed in Table 1. For PHA-accumulating conditions, cultures were grown in 100 ml flasks with 10 ml of modified E medium [38] supplemented with 1 % MT microelements [17], 1 mM $MgSO_4 \cdot 7H_2O$, and 0.25 % w/v sodium octanoate (Sigma). For non-PHA-accumulating growth conditions, Luria–Bertani medium (LB) was used. For both conditions, cultures were incubated at 32 °C and 300 rpm. *E. coli* S17-1 was grown in Luria–Bertani medium (LB) at 37° and 180 rpm. When necessary, tetracycline (10 μg/ml) and kanamycin (25 μg/ml) were added. Viable cell counts were performed in LB medium, and results are expressed as colony forming units per ml (CFU/ml).

### Screening of Genes Involved in PHA Metabolism

The search of genes responsible for mclPHA production in *Pseudomonas* sp. KA-08 was performed using PCR amplification strategies. Degenerate primers (hipo248up, C1low, Z720up, and D526low) were designed to amplify the mclPHA genes using the sequences of mclPHA genes of other *Pseudomonas* species available in the databases (Table 1). PCR amplification reactions were performed in a final volume of 50 μl containing 0.3 μM of each primer, 0.2 mM of dNTPs, and 2.5U of Go *Taq* polymerase (Promega). The reaction mixtures were subjected to 30 cycles of amplification in a Techne Thermocycler with the following conditions, 94 °C for 1 min, 50/60 °C for 30 s, 72 °C for 1 min, and a final extension of 10 min at 72 °C. Amplification products were sequenced by Macrogen Inc. (Korea). Sequences were aligned, assembled, and analyzed using the following programs available online: BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), CAP3 sequence assembly program (http://pbil.univ-lyon1.fr/cap3.php),

**Table 1** Bacterial strains, plasmids, and primers used in this study

| | Relevant genotype | References |
|---|---|---|
| *Pseudomonas* sp. KA-08 | PHA + xylene degrader | [10] |
| *Pseudomonas* sp. KA-mut | $PHA^-$ mutant $Km^R$ | This work |
| *P. putida* KT2440 | mclPHA producer | [12] |
| *P. putida* GPp104 | PHA negative mutant of KT2442 | [15] |
| *P. putida* GPp-PHA | PHA negative mutant of KT2442/pBBR1MCS5 containing a *phaC1* | [3] |
| *E. coli* S17-1 λ*pir* | ($F^-$) RP4-2-Tc::Mu *aphA*::Tn*7recA*λ*pir*lysogen; $Sm^rTp^r$ | [33] |
| *E. coli* Top 10 | F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL($Str^R$) endA1 $\lambda^-$ | Invitrogen |
| Plasmids | | |
| pGEM-T easy | PCR cloning vector | Promega |
| pEX18Tc | $Tc^R$; *ori*$T^+$*sacB*$^+$, gene replacement vector with MCS from pUC18 | [14] |
| pEXΔ*phaC1ZC2* | $Tc^R$; *ori*$T^+$*sacB*$^+$Δ*phaC1ZC2::km*$^r$ | This work |
| Primers | Sequences 5′–3′ | |
| hipo248up | TGTTCACYTGGGAVTACC | [3] |
| C11536low | CACCAGGAATCGGTGTGCTTG | [3] |
| Z720up | TGATCAACATGCGCMTGCT | [3] |
| D526low | GCYACCAGCATCATSAYCTGRTA | [3] |
| C1 | CAGTGAAGGCGCGGTGGTGT | This work |
| C1rec | TCAGCTTGGG**GGATCC**CCAGCAGGGTCAGGGCGTTG | This work |
| C2rec | ACCCTGCTGG**GGATCC**CCCCAAGCTGAGCAGCGACCC | This work |
| D1 | ATCCCCACATCCCCCTCGCG-3 | This work |

ORFfinder (http://www.ncbi.nlm.nih.gov/projects/gorf/), and ClustalW (http://www.ebi.ac.uk/Tools/clustalw/).

### Mutant and Recombinant Strains' Construction

The PHA$^-$ mutant *Pseudomonas* KA-mut was obtained using two steps of PCR crossover deletion method described by Link et al. [19] to delete *phaC1ZC2*. Briefly, in the first step, two independent PCR reactions were performed using *Pseudomonas* KA-08 chromosomal DNA as template and two pairs of primers: primers C1 and C1Rec to amplify a *phaC1* fragment of 404 bp (PCR1), and primers C2Rec and D1 to amplify a *phaC2D* fragment of 719 bp (PCR2) (Table 1). PCR conditions were: initial denaturation of 5 min at 94 °C, 35 cycles of 45 s at 94 °C, 30 s at 63 °C (for PCR 1) or 62 °C (for PCR 2), and a final extension of 10 min at 72 °C. The second step was performed using the two fragments obtained on the first step PCR as template and C1 and D1 primers. The resulted amplicon of this second step was cloned into the cloning vector pGEM-T easy (Promega), and a kanamycin resistance gene was cloned into a new-generated BamHI site. The construction was sub-cloned into pEX18Tc vector resulting in pEXΔ*phaC1ZC2*. This construction *w*as transferred to *Pseudomonas* sp. KA-08 by conjugation using *E. coli* S171 λ pir. Transconjugant strains were selected by plating on LB agar containing Km and sucrose (5 %). Km$^R$ colonies were then screened for plasmid loss in LB agar plus Tet and sucrose. Km$^R$-Tet$^S$-Sac$^R$ colonies were grown in E medium supplemented with 0.25 % sodium octanoate for testing PHA accumulation. Clones without the capability to accumulate PHA were selected.

PHA accumulation was qualitatively analyzed by Nile blue staining [22]. PHA content was determined from lyophilized cells subjected to methanolysis and hot chloroform extraction. Methyl ester derivatives were chromatographically analyzed as previously described [2].

### Biosurfactant Production

Anionic nature of the produced surfactant was observed by bacterial cultivation in CTAB methylene blue agar plates [32].

For surface tension measurements, cultures were grown under PHA-accumulating conditions during 72 h. Free-cell supernatants were obtained by centrifugation, and surface tension was measured using a Du Nouy tensiometer (Cenco-DuNouy 70545). Non-inoculated medium was used as control, and distilled water was used for tensiometer calibration.

Emulsification activity was measured in cultures grown under PHA-accumulating or -not accumulating conditions according to Cooper and Goldenberg [6]. Briefly, 5 ml of hexane was added to 5 ml free-cell supernatants and it was strongly vortexed at high speed for 2 min. The mixture was then allowed to stand still for 24 h (EA24) prior to measurement. Emulsification activity was defined as the ratio between the heights of the emulsion layer relative to the original volume and expressed as percentage.

### Microbial Attachment to Hydrocarbons (MATH test)

Adherence of bacteria to hydrocarbons was used as a measure of cell-surface hydrophobicity according to Rosenberg et al. [29]. Briefly, cells of cultures grown under PHA-accumulation or -not accumulation conditions were collected by centrifugation, rinsed with sterile deionized water, and then resuspended in PUM buffer ($K_2HPO_4{\cdot}3H_2O$ 22.2 g/l; $KH_2PO_4$ 7.26 g/l; urea 1.8 g/l; $MgSO_4{\cdot}7H_2O$ 0.2 g/l). $OD_{600nm}$ was measured and adjusted to $OD_{600nm} = 1$. Xylene was added to the cell suspension and left to stand for 10 min at 30 °C; after this, the mixture was vortexed for 2 min and left to stand for 15 min to allow phases' separation. Aqueous phase was taken, and $OD_{600nm}$ was measured. The percentage adhesion was calculated as follows:

$$(1 - OD_{600nm} \text{ after mixing}/OD_{600nm} \text{ before mixing}) \times 100.$$

### Statistical Analysis

For statistical analysis, three parallel experiments were conducted and data represent mean ± SD. Student's two-tailed *t* test with confidence level >95 % ($P < 0.05$) was performed to determine the significance of differences.

### Nucleotide Sequence Accession Number

The nucleotide sequences obtained here have been deposited in the EMBL nucleotide sequence database accession #: FR691074 (corresponding to the *Pseudomonas* sp. KA08 *phaC1ZC2D* genes).

## Results and Discussion

### Analysis of PHA Gene Cluster

*Pseudomonas* strains are able to synthesize mclPHA by classII PHA polymerases [25]. In most of the *Pseudomonas* species, the mclPHA cluster (*phaC1ZC2DFI*) encodes two synthase genes (*phaC1* and *phaC2*) separated by a gene coding for a PHA depolymerase (*phaZ*), a transcriptional activator (*phaD*), and two genes (*phaFI*) encoding phasin-like proteins [9]. In *Pseudomonas* sp. KA-08, 4 ORF

encoding *phaC1ZC2D* genes were found using PCR techniques. Bioinformatic analysis showed that PhaC1, PhaZ, and PhaC2 presented a 99, 95, and 95 % similarity with *Pseudomonas putida* F1, respectively, while the TetR regulator PhaD presented 100 % similarity with *P. putida* DOT-T1E.

The activity of this cluster was tested by measuring PHA production. Polymer quantification showed that KA-08 strain produced mclPHA containing 1.40 ± 0.15 % CDW of C6 monomers and 16.85 ± 1.70 % CDW of C8 monomers when grown using sodium octanoate as carbon source.

In silico analysis of the aminoacid sequences of the proteins involved in PHA synthesis and degradation showed that PhaC1 presented the conserved aminoacids necessary for class II synthases' catalytic activity (Cys 296, Asp 452, and His 480) and other conserved amino acids like Trp 398, which participates in synthases' dimerization, and Cys 431 involved in transesterification reaction [27, 35]. PhaC2 presented a nonsense mutation on aminoacid 203, suggesting that this protein should be inactive. A catalytic aminoacid triad (Ser–His–Asp) as active site is described in most of the PHA depolymerases [16]; the catalytic Ser is embedded in a GxSxG sequence motif, known as ''lipase box,'' and is also found in other hydrolases. This lipase box was also found in the aminoacid sequence of the putative depolymerase PhaZ of *Pseudomonas* sp. KA-08.

The high percentage of similarity of the ORFs found with the other studied PhaC1 and PhaZ, plus the presence of conserved aminoacid required for the catalytic domain of those proteins, could indicate that this cluster is responsible for the synthesis and degradation of mclPHA in *Pseudomonas* sp. KA-08.

## Characterization of a *Pseudomonas* sp. KA-08 PHA-Negative Mutant (KA-mut)

To analyze the effect of PHA accumulation on surfactant production and bacterial adherence to hydrocarbons, a PHA mutant was constructed using PCR deletion methods. This strain contains a 3300-bp deletion and a Km cassette insertion in the mclPHA cluster. Mutation was verified by PCR analysis (data not shown). As expected, the mutant was unable to produce PHA, showing no detectable amounts of C6 monomers and only 0.061 ± 0.006 % CDW of C8 monomers by GC measurements in 24-h cultures performed under PHA-accumulation conditions (sodium octanoate as carbon source).

The mutant was also analyzed in order to determine if this mutation affects growth capabilities. In LB, a rich medium in which PHA accumulation is not detected (Fig. 1a), the growth of both strains was similar, but under

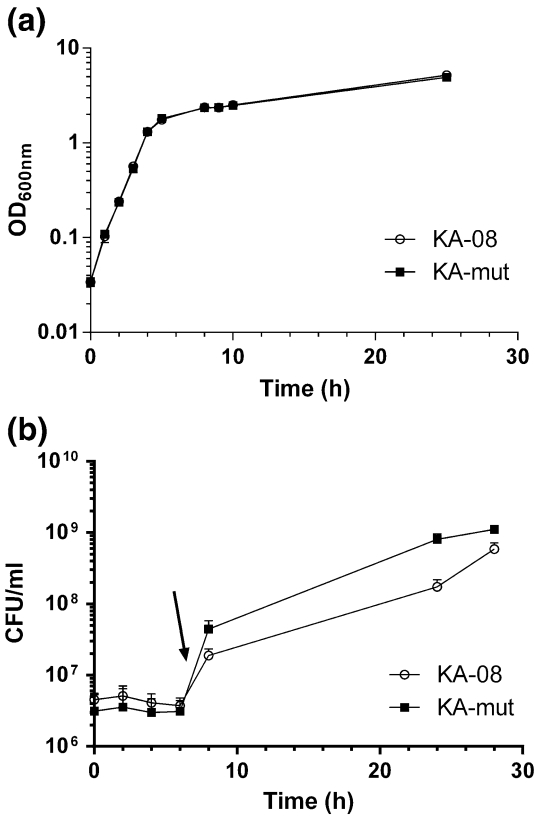


**Fig. 1** Growth curves of the wild-type and the PHA-negative strain of *Pseudomonas* sp. KA-08 in different culture media. **a** Optical density ($OD_{600nm}$) in LB medium **b** viable counts (CFU/ml) in E medium supplemented with sodium octanoate. The *arrow* shows the time when intracellular PHA granules become detectable by Nile blue staining

PHA-accumulating conditions (E medium + sodium octanoate) the mutant strain showed a slight increase in the number of CFU/ml compared to the wild-type strain (Fig. 1b). This slight growth increase in the mutant is in concordance with the time that the wild-type *Pseudomonas* sp. KA-08 begins to accumulate PHA (Fig. 1b), indicating that carbon metabolism was different in both strains.

## Effect of PHA in Surfactant Production and Bacterial Adherence to Hydrocarbons

PHA metabolism is related with other metabolic pathways as biosurfactant production and fatty acid biosynthesis and degradation [21]. The relationship between PHA and biosurfactant (rhamnolipid) production was previously described in *P. aeruginosa* where both metabolic pathways are related by 3-hydroxyacid precursors [26, 34]. It is described that other biosurfactants with different chemical nature, such as other glycolipids or lipopeptides, also use 3-hydroxyacid precursors for their biosynthesis [18, 39].

The effect of PHA on surfactant production was analyzed in different strains of *P. aeruginosa,* showing that the

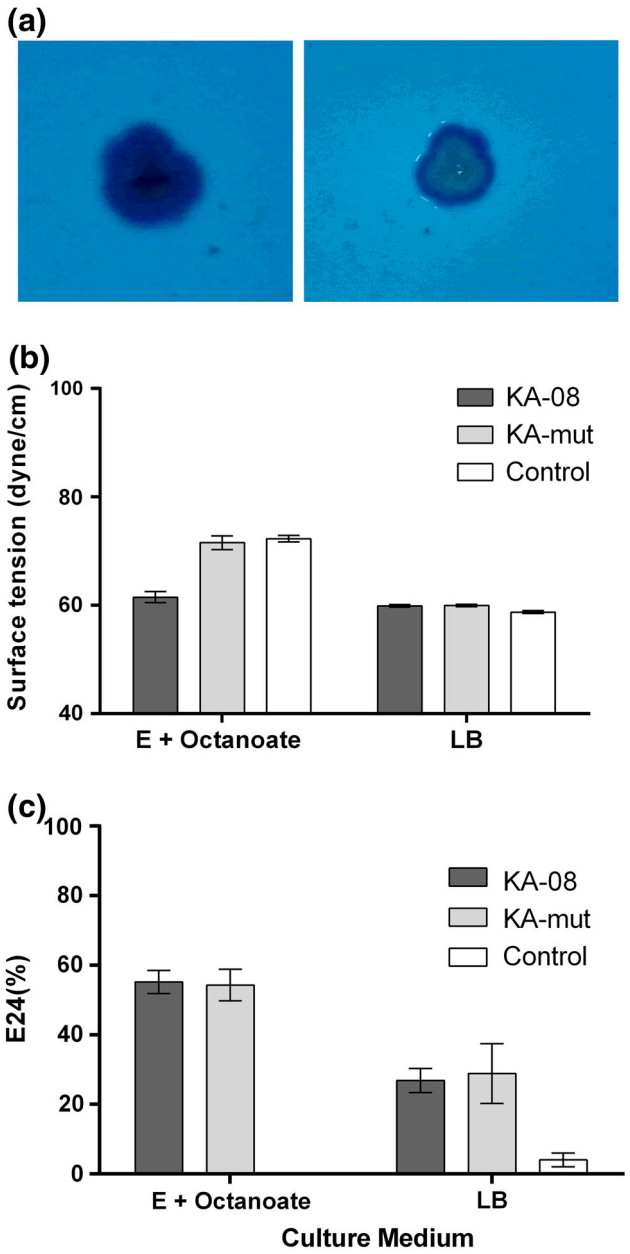


**Fig. 2** Surfactant production in *Pseudomonas* sp. KA-08 and its PHA-negative mutant. **a** CTAB methylene *blue* assay. Surface tension **b** and emulsification activity **c** of free-cell supernatants of cultures grown under PHA-accumulation or -not accumulation conditions

deficiency in PHA biosynthesis had opposite effects on the amount of rhamnolipid produced [24]. On the other hand, in *P. extremaustralis* biofilms, the presence of PHA had a positive influence on a lipopeptide surfactant production [37].

Due to the scarce information about the effect of PHA accumulation in biosurfactant production, our results could contribute to enlighten this subject. Previous analysis of biosurfactant production in KA-08 strain performed by TLC revealed the presence of glycolipids and other lipidic moieties [10]. Surfactant production assays in CTAB methylene blue agar plates showed a blue staining of the bacterial colonies and a dark blue halo surrounding the colonies denoting the presence of cell-attached anionic biosurfactant moieties (Fig. 2a).

Biosurfactants are compounds with different properties. The most obvious property of biosurfactant compounds is their ability to effectively lower water surface tension [11], but another function of microbial surfactants is the ability to form stable emulsions; those surface-active compounds are called bioemulsifiers.

In general, high-molecular-mass biosurfactants are better emulsifiers than low-molecular-mass bioemulsifiers, but are not necessarily efficient in lowering surface tension [7]. Because of that, it is important to analyze both parameters. To analyze the effect of PHA accumulation capability on biosurfactant production, surface tension (ST) and emulsification activity (E24) were conducted in both strains, KA-08 and KA-mut.

The wild-type strain showed a higher ST reduction than that observed in the mutant strain when growing in PHA-accumulating conditions, while both strains presented similar ST when growing in LB medium (Fig. 2b). It has been described that the carbon source affects the biosurfactant production [28]; this is in concordance with our results, where the wild-type strain was able to secrete compounds that reduce the ST when grown on E medium plus octanoate but not in LB medium. Interestingly, in E medium plus octanoate there were no differences in the ST between the control and KA-mut, indicating that the mutant was not able to produce this surfactant (Fig. 2b). These results indicate that besides the carbon source, the capability to accumulate PHA has influence in tensoactive compound production.

Bioemulsifier production was analyzed by measuring E24 index. This test revealed that both strains showed a high emulsification activity, near to 60 %, in E medium plus octanoate, and around 20 % when they grew in LB medium. These results indicate that only the carbon source affected the E24 but not the capability to accumulate PHA (Fig. 2c). A significant presence of bioemulsifier was obtained in LB, where no lowering of the ST was observed.

This could indicate that more than one biosurfactant compound is secreted by *Pseudomonas* sp. KA-08. This is supported by the observation that ST reduction was affected by PHA accumulation capabilities, but emulsion stabilization was not. On the other hand, TLC assays showed the presence of glycolipids and other compounds of lipidic nature in surfactant crude extracts [10].

Finally, another interesting characteristic in oil remediation agents is their capability to attach to the hydrocarbons [8]. As *Pseudomonas* sp. KA-08 is highly tolerant to xylene and is able to degrade this compound [10], microbial attach

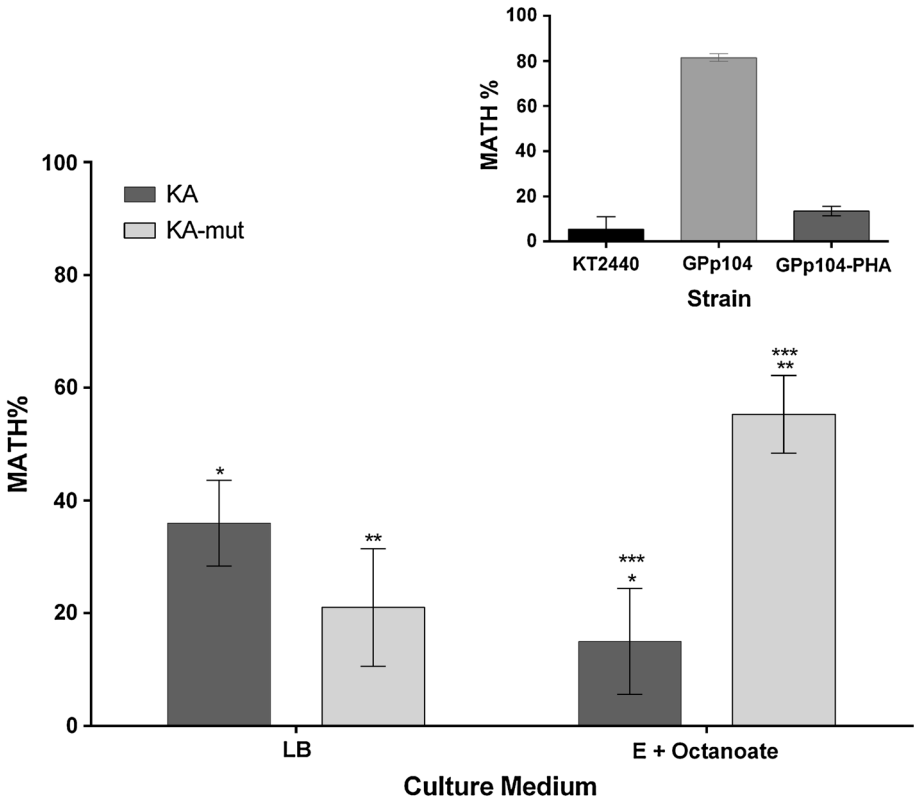


**Fig. 3** Microbial attachment to hydrocarbons (MATH) of *Pseudomonas* sp. KA-08 wild-type and KA-mut strains under PHA-accumulation or -not accumulation conditions and *P. putida* KT2440, Gpp104, and GPp-PHA (*inbox*) grown under PHA-accumulation conditions

to hydrocarbons (MATH) assays were performed using xylene as hydrocarbon to test bacterial adherence. In LB medium, both strains presented no significant differences in their affinity to xylene ($P > 0.05$, Fig. 3). Wild-type strain showed a significant lower affinity to xylene when it grew under PHA-accumulating conditions compared with cultures grown in LB medium ($P = 0.0049$). By contrast, the mutant strain presented higher affinity to xylene when it grew in E medium plus octanoate than in LB ($P = 0.0026$) (Fig. 3). Under PHA-accumulation conditions, the wild-type strain showed a significant lower affinity to xylene than the mutant strain ($P < 0.0001$). The increased affinity to xylene shown by the PHA-mutant strain could be related to the redirection of carbon flux to fatty acid biosynthesis. Chang et al. [4] described that a higher fatty acid accumulation is related with an enhanced cell-surface hydrophobicity. Due to KA-mut being unable to use acyl-CoA to synthesize PHA, this precursor could be diverted to de novo fatty acid biosynthesis.

The same MATH analysis was performed with *P. putida* KT2440 and a PHA-negative mutant *P. putida* GPp104, showing similar results under PHA-accumulation conditions, where the MATH of the wild-type strain was significantly lower than the MATH of the PHA-negative mutant. When the capability to accumulate PHA was restored in *P. putida* GPp104 strain by complementation with a plasmid containing a class II polymerase, its affinity to xylene was similar to the WT (Fig. 3 inbox, $P = 0.27$). These results show that the effect of PHA accumulation capability on cell-surface hydrophobicity could be extended to other *Pseudomonas* strains.

In conclusion, PHA metabolism affects different characteristics of *Pseudomonas* sp. KA-08 that are relevant for its fitness in hydrocarbon-contaminated environments. On the one hand, the capability to accumulate and degrade this polymer enhances the production of surface tension-lowering compounds like biosurfactants, but has no effect on bioemulsifier compounds. On the other hand, PHA-accumulation capabilities affect bacterial outer membrane characteristics. This effect was also observed in other *Pseudomonas* strains (*P. putida* KT2240 and *P. putida* GPp104) suggesting that this is not strain dependent.

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