When metabolic prowess is too much of a good thing: how carbon catabolite repression and metabolic versatility impede production of esterified α,ω-diols in Pseudomonas putida KT2440

Chunzhe Lu1, Christos Batianis2, Edward Ofori Akwafo1, Rene H. Wijffels1,4, Vitor A. P. Martins dos Santos1,2,3 and Ruud A. Weusthuis1*

Abstract

Background: Medium-chain-length α,ω-diols (mcl-diols) are important building blocks in polymer production. Recently, microbial mcl-diol production from alkanes was achieved in E. coli (albeit at low rates) using the alkane monoxygenase system AlkBGTL and esterification module Atf1. Owing to its remarkable versatility and conversion capabilities and hence potential for enabling an economically viable process, we assessed whether the industrially robust P. putida can be a suitable production organism of mcl-diols.

Results: AlkBGTL and Atf1 were successfully expressed as was shown by oxidation of alkanes to alkanols, and esterification to alkyl acetates. However, the conversion rate was lower than that by E. coli, and not fully to diols. The conversion was improved by using citrate instead of glucose as energy source, indicating that carbon catabolite repression plays a role. By overexpressing the activator of AlkBGTL-Atf1, AlkS and deleting Crc or CyoB, key genes in carbon catabolite repression of P. putida increased diacetoxyhexane production by 76% and 65%, respectively. Removing Crc/Hfq attachment sites of mRNAs resulted in the highest diacetoxyhexane production. When the intermediate hexyl acetate was used as substrate, hexanol was detected. This indicated that P. putida expressed esterases, hampering accumulation of the corresponding esters and diesters. Sixteen putative esterase genes present in P. putida were screened and tested. Among them, Est12/K was proven to be the dominant one. Deletion of Est12/K halted hydrolysis of hexyl acetate and diacetoxyhexane. As a result of relieving catabolite repression and preventing the hydrolysis of ester, the optimal strain produced 3.7 mM hexyl acetate from hexane and 6.9 mM 6-hydroxy hexyl acetate and diacetoxyhexane from hexyl acetate, increased by 12.7- and 4.2-fold, respectively, as compared to the starting strain.

Conclusions: This study shows that the metabolic versatility of P. putida, and the associated carbon catabolite repression, can hinder production of diols and related esters. Growth on mcl-alcohol and diol esters could be prevented by deleting the dominant esterase. Carbon catabolite repression could be relieved by removing the Crc/Hfq attachment.
Introduction

*Pseudomonas putida*: its versatile metabolism and carbon catabolite repression system

*Pseudomonas putida* KT2440 is a well-known Gram-negative bacterium, increasingly attracting industrial attention. Its tolerance to chemical solvents [1], its ability to accumulate large amounts of mcl-PHA [2] and its increasingly available genetic tools [3] make *P. putida* KT2440 a promising chassis for the production of bulk chemicals [4–7].

*P. putida* KT2440 has a versatile metabolism, which is illustrated by its ability to grow on dozens of different carbon sources, including amino acids, alcohols and a wide range of lignin derivatives [8]. In its natural environment, soil, multiple carbon sources are available. To maximize its growth rate, *P. putida* KT2440 needs to select for a preferred substrate. This selection is precisely regulated by carbon catabolite repression (CCR) of essential genes for uptake and catabolism of non-preferred carbon sources [5].

The molecular mechanisms for carbon catabolite repression are primarily controlled by the Crc protein in *Pseudomonas*. Crc regulates the expression of over 130 genes in *P. putida* [9, 10] at the translation level, including those for assimilation of hydrocarbons, such as n-alkanes, benzoate and toluene. Crc forms a complex with the RNA-binding protein Hfq on its distal part in presence of an attachment site (A-rich motifs, AANAANAA) which is located near the translation initiation region of target mRNAs to inhibit translation (Fig. 1a). Except for Crc, the cytochrome o ubiquinol oxidase (Cyo), a terminal oxidase of the electron transport chain, also conduces to catabolite repression. Cyo is considered to play an active role in cell growth under sufficient oxygen supply. Although the mechanism behind Cyo is still unknown, deleting *CyoB*—coding the key gene of Cyo—relieves catabolite repression [11]. Knocking out Crc and *CyoB* at the same time showed a superimposed effect on relieving repression in rich medium [11].

These types of CCR systems are necessary for the robustness of microbes in their natural environments when facing multiple substrates, but may impede their performance in industrial biotransformations, in which desired products may act as preferred substrates over the applied substrate [5, 6].

**Microbial synthesis of medium-chain-length α,ω-diols**

Medium-chain-length α,ω-diols (mcl-diols) are versatile chemicals used as fuels, detergents and precursors of polyesters and polyurethanes, which are the two primary sources for the production of biobased plastics [12–15]. 1,6-Hexanediol is one of the common α,ω-diols, traditionally produced by hydrogenation of adipic acid [16].
This process requires a high energy input and expensive catalysts, and it usually results in significant byproduct formation. Due to these unsustainable traits, microbial synthesis is attracting more and more attention as a promising alternative solution [17, 18].

Van Nuland and coworkers investigated the conversion of medium-chain alkanes and alkanols into mcl-diols by E. coli, by expressing the monoxygenase system AlkBGT from Pseudomonas putida GPO1 [19]. In E. coli these enzymes act in an orthogonal fashion, as E. coli does not employ CCR by the Crc protein. Van Nuland et al. encountered two challenges: AlkBGT overoxidized hydroxy groups and was unable to oxidize the terminal methyl group of alkanols. They solved the first challenge by protecting the hydroxy groups by esterifying them with acetyl-CoA using the alcohol acetyltransferase Atf1 from Saccharomyces cerevisiae. Glucose was used to provide energy for maintenance and acetyl-CoA for esterification. The esterification of the first hydroxyl group also solved the second challenge as it enabled the ω-oxidation of medium chain alkanol esters. The final products were diacetoxyalkanes: α,ω-diols esterified at both ends with acetate.

In P. putida GPO1, the alkBGT system is part of the alkane degradation pathway, encoded on the OCT plasmid [20]. AlkB, an integral membrane protein, is an alkane monoxygenase, inserting a single oxygen atom from molecular oxygen into a terminal carbon–hydrogen bond of n-alkanes. The soluble rubredoxin AlkG, which is reduced by the rubredoxin reductase AlkT, is responsible for delivering electrons to AlkB. AlkL is a transporter located in the periplasmic membrane, facilitating uptake of hydrophobic compounds [21, 22]. The alkane degradation genes are split into two clusters, AlkST and AlkBFGHJKL (Fig. 1b). The whole pathway is activated by AlkS controlling the AlkST cluster by the promoter PalkS2 and the AlkBFGHJKL cluster by the promoter PalkB. In P. putida GPO1 the Crc protein inhibits translation of AlkS [20], AlkB and AlkG mRNAs to repress AlkBGT activity when cells grow on a minimal medium with preferred substrates such as succinate and lactate [23, 24]. P. putida KT2440 is relatively close to P. putida GPO1, and both share the carbon catabolite repression system mediated by Crc and Hfq in Pseudomonas and related species.

Results and discussion
Introducing AlkBGT-Atf1 into P. putida KT2440
AlkBGT alone and in combination with Atf1 was introduced into P. putida KT2440, resulting in strains P-BGTL and P-BGTLA, respectively. To test the proper functioning of the enzymes, n-hexane and hexyl acetate were separately used as substrates in 1-mL resting-cell suspensions with OD_{600} of 3. When 1% v/v n-hexane (76 mM) was fed in the resting-cell suspensions, P-BGTL produced 0.3 mM 1-hexanol while P-BGTLA produced 2.0 mM 1-hexanol and 0.3 mM hexyl acetate (Fig. 3a) after 20 h. Strain P-L, harboring the substrate transporter alkL, was used as a negative control strain, and did not produce any of these compounds. These results show that both AlkBGT and Atf1 were successfully expressed in P. putida. It was observed that the presence of Atf1 facilitated the production of oxidized products (hexanol and
hexyl acetate). P-BGTLA produced far more 1-hexanol than hexyl acetate indicating that either Atf1 is the rate-limiting step or that hexyl acetate produced by Atf1 was hydrolyzed back to 1-hexanol by esterases present in \textit{P. putida} KT2440. Similar experiments have been performed in \textit{E. coli} by van Nuland et al. [19]. When 1% v/v n-hexane was fed to \textit{E. coli} harboring AlkBGT, over 95% of the total products were overoxidized (hexanoic acid) and minor amounts of 1-hexanol were detected. \textit{E. coli} harboring AlkBGT-Atf1 produced 9.20 mM 1,6-diacetoxyhexane as the major product and 2.23 mM 6-hydroxy hexyl acetate under same conditions described above. However, hexanoic acid, 6-hydroxy hexyl acetate and 1,6-diacetoxyhexane were not found in our conversions. This may be attributed to a low activity of AlkBGT in \textit{P. putida} KT2440 resulting from carbon

\begin{figure}[h]
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\includegraphics[width=\textwidth]{fig2.png}
\caption{Proposed \textit{ω}-oxyfunctionalization pathway of medium-chain-length alkyl acetate in \textit{P. putida} KT2440. Orange arrows represent desired pathways, while blue dashed arrows represent breakdown pathways for which esterases are responsible. CCR carbon catabolite repression, AlkB alkane monooxygenase, AlkG rubredoxin, AlkT rubredoxin reductase, Atf1 alcohol acetyl transferase, EST esterases, ADH alcohol dehydrogenase, ALDH aldehyde dehydrogenase, ALR aldehyde reductase, CAR carboxylic acid reductase, PPT phosphopantetheinyl transferase.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig3.png}
\caption{a Hexane and b hexyl acetate conversion by AlkBGT-Atf1 in \textit{P. putida} KT2440 in 20 h. 1% v/v hexane and hexyl acetate were added as substrates. Glucose was used for cell maintenance and acetyl-CoA supply. 6HHA 6-hydroxy hexyl acetate, DAH 1,6-diacetoxyhexane, P-L \textit{P. putida} KT2440 with alkL expression, P-BGTL \textit{P. putida} KT2440 with alkBGTL expression, P-BGTLA \textit{P. putida} KT2440 with alkBGTL-Atf1 expression. Bars with different letters indicate significant difference from each other (comparisons were implemented among bars with the same color) (p < 0.05).}
\end{figure}
catabolite repression. Similarly, when n-hexane was fed, 1,6-diacetoxyhexane and 6-hydroxy hexyl acetate were not detected in P-BGTLA. We questioned that AlkB-GTL-Atf1 could not convert hexyl acetate into 6-hydroxy hexyl acetate and 1,6-diacetoxyhexane in P. putida.

To test if hexyl acetate can be converted by AlkB-GTL-Atf1, 1% v/v of hexyl acetate (60 mM) was fed to P-L, P-BGTL and P-BGTLA. In a 20-h conversion, 0.77 mM 6-hydroxy hexyl acetate and 0.55 mM 1,6-diacetoxyhexane were detected in P-BGTLA (Fig. 3b). This proves that AlkB-GTL-Atf1 is able to ω-oxidize hexyl acetate in P. putida KT2440, however, more alcohol products were produced compared to the diester production. 1-Hexanol was found in all strains when hexyl acetate was fed, indicating that esterases were functioning, resulting in the hydrolysis of hexyl acetate to hexanol and acetate.

Catabolite repression on AlkB-GTL-Atf1

In all experiments, glucose was added to provide energy for maintenance and acetyl-CoA for ester bond formation by Atf1 under resting-cell conditions. n-Hexane or hexyl acetate was fed as substrate to produce hexyl acetate and diacetoxyhexane. However, P. putida KT2440 prefers glucose over n-hexane and hexyl acetate, triggering activation of carbon catabolite repression to repress expression and translation of AlkB-GTL [10]. To test this hypothesis, we tried citrate instead of glucose as it was reported that citrate does not activate carbon catabolite repression of the alkane degradation pathway [10]. Compared to glucose, the production of 6-hydroxy hexyl acetate from hexyl-acetate was increased by 66.9% on citrate reaching 1.5 mM (Fig. 4a). This is in agreement with our hypothesis that glucose represses AlkB-GTL function in P. putida KT2440.

To tackle glucose repression on a molecular level, we used three approaches: (i) knocking out the carbon catabolite repression genes Crc and/or CyoB; (ii) increasing the expression of the AlkS activator; (iii) removing the Crc/Hfq attachment sites from the AlkB-GTL-Atf1 genes.

We deleted Crc and CyoB, either separately or together, and the mutants were transformed with AlkL, AlkB-GTL and AlkBGLT-Atf1 to investigate the effects of the regulators on the activity of AlkB-GTL and AlkB-GTL-Atf1. It was shown that all mutants, especially the
double-knockout strain ΔCrcΔCyoB, grew significantly slower than the wild-type strain (results not shown). The results with hexyl acetate as substrate are shown in Fig. 4b when only AlkBGTL was expressed. It shows that the Crc knockout had no effect on 6-hydroxy hexyl acetate production and that the CyoB knockout increased 6-hydroxy hexyl acetate by 25.6%. When both Crc and CyoB were deleted, 6-hydroxy hexyl acetate production reduced with 88.0%. This conflicts with previous research performed by Dinamarca and his colleagues [11] who showed that Crc and Cyo have an additive influence on the alkane degradation pathway. However, they used the lacZ gene as an orthogonal reporter to replace alkane degradation genes to test how knockouts affect gene expression under the control of the promoter PalkB. In our case, AlkBGTL was directly used to produce 6-hydroxy hexyl acetate, and was connected to the rest of metabolism, e.g. due to NADH requirement. Because deletion of Crc and CyoB will affect the expression of many genes [31], the whole metabolism will be significantly changed, which may have led to poor availability of NADH. This may be the reason why strain ΔCrcΔCyoB-BGTL only produced trace amounts of 6-hydroxy hexyl acetate.

To test the performance of AlkBGTL-Atf1 in the knockouts strains, the plasmid pBGTL-Atf1 was separately introduced, generating strains P-BGTLA, ΔCrc-BGTLA, and ΔCyoB-BGTLA. Compared to P-BGTLA, ΔCrc-BGTLA produced the same amount of 6-hydroxy hexyl acetate and diacetoxyhexane (Fig. 4c), indicating that the Crc knockout cannot increase AlkBGTL-Atf1 activity. The diacetoxyhexane production by strain ΔCyoB-BGTLA increased by 36.5% in comparison with the P-BGTLA strain, indicating that esterification was improved. The knockout of CyoB might release the repression on Atf1 or limit electron transport chain to provide more acetyl-CoA to Atf1. In AlkBGTL-Atf1 case, not only AlkS, AlkB, and AlkG, but also Atf1 were repressed because Atf1 was controlled by PalkB too. However, because Crc represses the alkane degradation pathway mainly through reducing the expression level of AlkS, which is the activator of the whole pathway, AlkS was reportedly overexpressed to counter repression of Crc on the pathway [24]. In this work, pSEVA658-AlkS was, respectively, transformed into strains ΔCrc-BGTLA and ΔCyoB-BGTLA, giving rise to strains ΔCrc-BGTLAS and ΔCyoB-BGTLAS to over-express AlkS. Strain ΔCrc-BGTLAS produced 14% more 6-hydroxy hexyl acetate and 76% more diacetoxyhexane, while strain ΔCyoB-BGTLAS produced 60% more 6-hydroxy hexyl acetate and 65% more diacetoxyhexane (Fig. 4c). But, because Crc and Cyo are global regulators, knocking them out affects the whole metabolism of P. putida, as indicated by the reduced growth rates.

To establish less pleiotropy, we decided to remove the sequences encoding the mRNA attachment sites of the Crc/Hfq complex from the overexpressed genes. The attachment sites in front of AlkB, AlkG and Atf1 were removed, generating a plasmid pSEVA658-alkB’G’T-Atf1’ (Fig. 5), which was subsequently transformed into P. putida KT2440 resulting in strain P-B’G’T’LA’. Compared with P-BGTLA, the total production of P-B’G’T’LA’ was increased by 82%. Among all five tested strains (ΔCrc-BGTLA, ΔCyoB-BGTLA, ΔCrc-BGTLAS, ΔCyoB-BGTLAS, and P-B’G’T’LA’), ΔCyoB-BGTLAS and P-B’G’T’LA’ were the two best-performing strains, producing 3.3 and 3.2 mM total products (6-hydroxy hexyl acetate and diacetoxyhexane), respectively (Fig. 4c). The difference between ΔCyoB-BGTLAS and P-B’G’T’LA’ was the ratio of 6-hydroxy hexyl acetate and diacetoxyhexane. P-B’G’T’LA’ showed the highest diacetoxyhexane concentration (2.52 mM) while ΔCyoB-BGTLAS generated the highest 6-hydroxy hexyl acetate concentration (1.2 mM). AlkS was overexpressed in ΔCyoB-BGTLAS to ensure high expression of AlkBGTL-Atf1 to counter carbon catabolite repression, leading to the highest total products. But 6-hydroxy hexyl acetate is not fully converted to diacetoxyhexane, indicating esterification of 6HHA is still limited. P-B’G’T’LA’ significantly released the inhibition of Atf1, facilitating higher production of diacetoxyhexane. The plasmid pSEVA658-B’G’T’-Atf1’ was used for further tests, given its high production and lower pleiotropy.

**Growth test of P. putida KT2440 on hexyl acetate and DAH**

*P. putida* KT2440, known for its versatile metabolism, can grow on dozens of different carbon sources [5–8]. To date, it is unknown whether *P. putida* KT2440 can utilize aliphatic acetate esters and diesters for growth. In our study, hexyl acetate was hydrolyzed to hexanol (Fig. 3b), which can be easily further utilized via β-oxidation. Thus, we assumed *P. putida* KT2440 is able to use hexyl acetate and diacetoxyhexane as the only carbon source for growth, in which esterases perform the first step, the hydrolysis of esters. If so, this is a potential threat for *P. putida* KT2440 to be engineered for hexyl acetate and diacetoxyhexane production.

Wild-type *P. putida* KT2440 was used for growth tests on M9 medium with 10 mM hexyl acetate or diacetoxyhexane as the only carbon source. The hexyl acetate case (Fig. 6a) showed a 48 h lag phase, after which the biomass concentration increased quickly and the hexyl acetate concentration decreased. In the end, 95% of the initially added hexyl acetate was consumed and the optical density reached its maximum at 1.15 with an OD₆₀₀
In the diacetoxyhexane case, the biomass concentration increased and the diacetoxyhexane concentration reduced over time (Fig. 6b). After a 24-h lag phase, the diacetoxyhexane concentration rapidly decreased until it was completely consumed at 72 h. Nevertheless, the maximum optical density was only 0.72 with an OD$_{600}$ increase of 0.52, half of the OD increase of the hexyl acetate case. The difference between these two cases is that 1-hexanol was hydrolyzed from hexyl acetate and 1,6-hexanediol was obtained from diacetoxyhexane breakdown. According to Li et al. [30], 1,6-hexanediol cannot be utilized by the native metabolism of *P. putida* KT2440.
*P. putida*, while 1-hexanol can be easily assimilated. So in the diacetoxyhexane case, only the released acetate could be used as carbon source for cell growth. Therefore, a higher biomass concentration was obtained when hexyl acetate was fed. In summary, *P. putida* KT2440 is able to use hexyl acetate or diacetoxyhexane as the only carbon source to grow. We hypothesized that hydrolysis of hexyl acetate into 1-hexanol and acetate or hydrolysis of diacetoxyhexane into 1,6-hexanediol and acetate is the first and essential step for cell growth. Esterases are supposed to be responsible for this hydrolysis [32–34]. To prevent their action, we verified the presence and activity of relevant esterases.

**Screening of esterases in *P. putida* KT2440**

In *P. putida* KT2440, several esterase genes have been reported, such as *EstZ* and *EstA* [35]. However, it is unclear how many esterases were active in the above cases. To check for potential esterases, searches were performed based on the genome of *P. putida* KT2440 using the NCBI and CAZymes databases. As a result, 16 putative esterases were selected (Table 2).

To evaluate the performance of these 16 esterases, *E. coli* NEBT7 was used as a host for quick screening due to its relatively clear esterase background compared with *P. putida* KT2440. *E. coli* NEBT7 transformed with an empty pET26b plasmid was used as control strain. pET26b plasmids harboring the (putative) esterase genes were introduced into *E. coli* NEBT7. The transformed strains were used for monoester and diester hydrolysis. Induced resting *E. coli* cells (1 g<sub>cdw</sub>/L) were fed with 20 mM alkyl (C6–C10) acetate ester or diacetoxyhexane in tightly capped tubes. Production of mcl-n-alcohols (C6–C10), 6-hydroxy hexyl acetate and 1,6-hexanediol was used as an indicator to confirm esterase activity.

According to these criteria, three esterases (EstC, EstZ, Est12/K) were active in alkyl acetate ester hydrolysis (Fig. 7a). The rest of esterases showed no alcohol production (results not shown). Est12/K was always the best-performing esterase, no matter which alkyl acetate ester was fed. Of the 16 candidates seven esterases—Est1, EstB, EstC, EstP, EstZ, Est11, Est12/K—showed diacetoxyhexane hydrolysis activity, either to 6-hydroxy hexyl acetate or 1,6-hexanediol (Fig. 7b). Among these seven esterases, Est12/K showed the best hydrolysis performance producing 7.7 mM 1,6-hexanediol, followed by EstB, EstC and Est11 exhibiting almost the same ability to hydrolyze diacetoxyhexane. Est1 only produced trace amounts of 6-hydroxy hexyl acetate and 1,6-hexanediol.

**Evaluation of the deletion of esterase in *P. putida* KT2440**

In the above analysis, esterases were highly expressed in *E. coli*. However, they might show different activity in *P. putida* KT2440 due to different expression levels of the esterase and diverse metabolism. To investigate their performance in *P. putida*, esterase knockout (KO) mutants were generated. Single KO and multiple KO mutants of *EstB*, *EstC*, *EstP*, *EstZ* and *Est12/K* in *P. putida* KT2440 were made and evaluated in hydrolysis tests. In addition to esterases, β-oxidation was assumed to be involved in consumption of alcohols from ester hydrolysis, facilitating this hydrolysis. Thus, key genes (*FadA*: acetyl-CoA acetyltransferase (PP_2137, PP_2215), *FadB*: 3-hydroxyacyl-CoA dehydrogenase/enoyl-CoA hydratase (PP_2047, PP_2136, PP_2214, PP_2217), *FadE*: acyl-CoA dehydrogenase (PP_2048, PP_2216) [36, 37] of β-oxidation were deleted, generating *P. putida* ΔBOX as a control strain.

For alkyl acetate ester hydrolysis in *P. putida* and esterase KO mutants, hexyl acetate (20 mM) was taken as example. The control strains, *P. putida* KT2440 and *P.
**putida** ΔBOX, formed around 5.5 mM 1-hexanol after 48 h of incubation (Fig. 8a). This shows that under the incubation conditions applied, β-oxidation did not play a significant role in alcohol consumption. The ΔEstBCPZ strain showed similar results, indicating that EstB, EstC, EstP and EstZ are not involved in hexyl acetate hydrolysis in *P. putida* cultivated under these conditions. When Est12/K was deleted, 1-hexanol was not detected anymore. For diacetoxyhexane hydrolysis, the data showed a similar trend (Fig. 8b). The intermediate ester, 6-hydroxy hexyl acetate, the product of a single hydrolysis, was not observed. In brief, Est12/K is the most dominant esterase responsible for the breakdown of alkyl acetate and diacetoxyhexane in *P. putida* KT2440.

### Diacetoxyhexane production in engineered *P. putida* KT2440

In the next step, we combined the esterase knockout strain with the expression of Crc-independent AlkB-GTL-Atf1. pSEVA658-B‘G‘T-L-Atf1* was transferred to *P. putida* ΔEst12/K giving rise to ΔEst12/K-B‘G‘TLA*. When n-hexane was used as substrate in resting-cell suspension of strain ΔEst12/K-B‘G‘TLA*, 0.4 mM hexanol and 3.7 mM hexyl acetate were produced. The production of hexyl acetate was increased by 12.7-fold compared with that of P-BGTLA. This mainly resulted from the deletion of Est12/K, preventing the hydrolysis of hexyl acetate. Besides, optimized AlkBGT also provides more hexanol for hexyl acetate formation. However, 6-hydroxy hexyl acetate and 1,6-diacetoxyhexane were still not detected (data not shown). In a similar setup, using hexyl acetate as substrate, ΔEst12/K-B‘G‘TLA* produced 6.9 mM total products (Fig. 9b). Compared with P-B‘G‘TLA*, ΔEst12/K-B‘G‘TLA* produced 1.1-fold more total products. The production of 6-hydroxy hexyl acetate increased by 3.8-fold, indicating that 6-hydroxy hexyl acetate might be hydrolyzed when Est12/K is present. In the end, the total amount of products was increased by 4.2-fold in ΔEst12/K-B‘G‘TLA* compared with the starting strain P-BGTLA. 11.5% of the substrate (hexyl acetate) was o-oxidized by ΔEst12/K-B‘G‘TLA*, 4.2-fold of that of P-BGTLA.

Both interventions, preventing hydrolysis of esters and uncoupling ester production from carbon catabolite repression, resulted in improved ester production. However, ester production is still lower in *P. putida* compared with that in *E. coli*. There are three main differences between the two hosts for ester synthesis. First, in a similar setup *E. coli* showed a threefold higher production rate when hexane was used as starting substrate [19]. The lower production rate of *P. putida* may be attributed to the availability of NADH—required for AlkBGT activity—and acetyl-CoA—required for Atf1 activity. Whereas *E. coli*’s dissimilation is based on NADH, *P. putida*’s dissimilation has a stronger focus on NADP+ reduction, mediated by the ED-EMP pathway [38]. Although this is generally an advantage in *P. putida* with regard to its metabolic prowess, in this specific case, this may have resulted in a low availability of NADH and hence a lower AlkBGT activity. Glucose was added to provide the acetyl-CoA. However, when glucose was fed to *P. putida*, the majority of glucose is not directly assimilated but is channelled to periplasmic gluconate and 2-ketogluconate pathways, and secreted in the presence of abundant

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**Fig. 8** Esterase deletion mutants of *P. putida* KT2440 for a 20 mM hexyl acetate hydrolysis test in 48 h and b 20 mM diacetoxyhexane hydrolysis test in 24 h. Glucose was used for cell maintenance. 6HHA 6-hydroxy hexyl acetate, DAH 1,6-diacetoxyhexane, ΔBOX *P. putida* KT2440ΔPP_2014-18ΔPP_2047-48ΔPP_2136-37, ΔEstBCPZ *P. putida* KT2440ΔEstBΔEstCΔEstPΔEstZ, ΔEst12/K *P. putida* KT2440ΔEst12/K, ΔEstBCPZ12/K *P. putida* KT2440ΔEstBΔEstCΔEstPΔEstZΔEst12/K
glucose [39], limiting the supply of acetyl-CoA. In addition, glucose is the preferred substrate for *E. coli*, but not for *P. putida* [10]. The conversion of hexane results in the accumulation and excretion of many intermediate molecules. Some of these molecules may be preferred as substrate over glucose, resulting in a lower availability of acetyl-CoA and hence a lower Atf1 activity. The same reasoning, based on the difference in substrate preference between *E. coli* and *P. putida* may also be applied to the second difference found. In *E. coli* AlkBGT overoxidizes a part of the hexanol into hexanoic acid. Esterification of hexanol and 6-hydroxy hexyl acetate with acetyl-CoA mediated by Atf1 was not able to prevent this completely. None of hexanoic acid, 6-hydroxyhexanoate, and adipic acid were observed as byproduct using *P. putida*. This can however not be attributed to a high activity of Atf1, because both hexanol and 6-hydroxy hexyl acetate accumulated. It seems more likely that overoxidation did occur, but that the overoxidized product hexanoic acid was possibly preferred as substrate over glucose and hexane. The third difference is that in *E. coli* hexane can be directly converted into diacetoxyhexane. This direct conversion does not occur in *P. putida*, although it is able to convert hexane into hexyl acetate and added hexyl acetate in diacetoxyhexane. The ability of AlkBGT to oxidize both hexane and hexyl acetate simultaneously in *E. coli* and not in *P. putida* suggests that in the latter microorganism the ratio of intracellular concentrations of hexane and hexyl acetate is larger, favoring hexane oxidation by substrate competition.

**Conclusions**

*P. putida* KT2440 is known for its robustness, versatile metabolism and high oxidative capacity, all of which render it a preferred platform for a range of industrial applications [6–8, 40, 41]. This study shows that this metabolic prowess also has its down side. We found that metabolic versatility and associated carbon catabolite repression hinder oxyfunctionalization of alkanes by alkBGTL-Atf1 in *P. putida* KT2440. Both could be overcome by 1) deleting the committed step of diacetoxy alkane degradation performed by esterase Est12/K and 2) by deleting the Crc/Hfq attachment site of relevant genes involved in oxyfunctionalization to avoid catabolite repression. Nevertheless, the realized level of diacetoxy alkane production is still low, and more research is required to make the process industrially applicable.

Although the role of catabolite repression in direct product formation was diminished, the results also indicate that the formed (by)products may still affect other parts of the metabolic network by carbon catabolite repression. Biotechnological process development is largely about engineering the most (cost-)effective conversion of a chosen substrate into a chosen product. The
substrate preference of *P. putida* may conflict with these choices, if the product is preferred as substrate. This phenomenon is already known as the Kluyver effect for some yeasts, which are not able to convert some disaccharides into ethanol, because ethanol is preferred as substrate [42]. Sugars are often the main substrates used in biotechnological processes. Sugars are, however, not the preferred substrates of *P. putida*. This ultimately hampers the ability to use *P. putida* for the production of certain compounds, e.g. organic acids and amino acids. The strategy we used to delete mRNA Crc/Hfq attachment sites may alleviate this when applied to genes involved in sugar catabolism for other applications. Moreover, the ability to regulate many enzyme activities at the same time with Crc/Hfq, as long as the genes involved contain an (introduced) mRNA attachment site, could be a promising tool in metabolic engineering.

**Materials and methods**

**Strains, plasmids and medium**

Strains and plasmids used in this study are listed in Table 1. *E. coli* 5α was used for cloning purposes. *E. coli* NEBT7 was used for ester hydrolysis tests. The construction of plasmids is described in the supplementary file. *P. putida* KT2440 as the parental strain was engineered for different study aims. According to Nicolas and Daniel's method [43], a suicide plasmid pGNW2 and a self-curing plasmid pQURE6 bearing the I-SceI gene were used to delete genes in *P. putida* KT2440. The M9 medium consisted of 1 × M9 minimal salts, 0.2 mM MgSO₄·7H₂O, 55 mM glucose and 1 mL/L trace elements USFe [44]. M9 medium was used for cell preculture and induction of genes of interest. It consisted of M9 medium, 0.1 mM CaCl₂·2H₂O, 100 mM 3-(N-morpholino)propanesulfonic acid sodium salt buffer (MOPS), and 1% v/v glucose [45]. The media were adjusted to pH 7.0 with 1 M hydrochloric acid or sodium hydroxide and subsequently filter-sterilized using a 0.22 μm Nalgene® polyethersulfone (PES) filter (ThermoFisher). Kanamycin (Km) and gentamicin (Gm) were separately added at 50 μg/mL and 10 μg/mL, respectively, when needed.

**Cultivation and gene expression**

Strains harboring plasmids pCOM_alkL, pBTL10 or pBGTL-Atfl were inoculated in LB overnight. The overnight culture (1% v/v) was used to inoculate M9 medium and cultured overnight again. OD₆₀₀ was always determined spectrophotometrically with 4 mm cuvette. The day after, this overnight culture was used to inoculate 50 mL of M9 medium at an initial OD₆₀₀ of 0.25, induced by 0.025% v/v dicyclopropylketone (DCPK) for gene expression. After 5-h induction at 30 °C and 250 rpm agitation in a Kuhner shaker incubator, cells were harvested by centrifugation at 4200 × g for 10 min. Cell pellets were suspended at 1 g₆₉₀/L in resting cell buffer, containing 50 mM KPi pH 7.4, 2 mM MgSO₄, and 1% v/v glucose. Strains harboring pSEVAb plasmids were cultivated under the same conditions as described above [46, 47], except for induction with 1 mM 3-methylbenzoate.

**Ester hydrolase screening and strain construction for tests**

For the ester hydrolase screening, two main databases, NCBI and the Carbohydrate-Active enZymes [48, 49], were used to search putative hydrolase-like esterase or lipase in *P. putida* KT2440. Each putative ester hydrolase gene (*EstX*) with a Strep-tag II at the C-terminal end was inserted into a pET26b plasmid to generate pET26b-EstX by HiFi assembly. All primers for these constructions are listed in Table S1. *E. coli* NEBT7 was transformed with the pET26b-EstX to give rise to *E. coli*-EstX for ester hydrolysis tests.

**Resting-cell hydrolysis assay**

*E. coli*-EstX was inoculated in LB medium and grown overnight. An aliquot of the overnight culture (300 μL) was inoculated in 30 mL M9F medium and grown overnight. This culture was used to inoculate 50 mL M9 medium at an initial OD₆₀₀ of 0.15. After 5 h of cultivation (OD₆₀₀=0.6–0.8), 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to induce ester hydrolase gene expression at 25 °C, at 250 rpm agitation in a Kuhner incubator for 16 h. Once induction finished, the cells were harvested by centrifugation at 4200 × g.

Cell pellets were suspended in the resting-cell buffer to a density of 1 g₆₉₀/L. Aliquots of 1 mL resting cells were taken for each reaction in a Pyrex tube. Alkyl (C6-C10) acetate and diacetoxyhexane (20 mM) were added to each tube, and then the tube was cultivated for 20 h at 30 °C, 250 rpm. All reactions were performed in triplicate. Afterwards, 1% v/v of phosphoric acid was immediately added to stop reactions and all tubes were placed on ice. Samples were extracted by diethyl ether with 0.2 mM dodecane as an internal standard for GC analysis.

**Growth test on hexyl acetate and diacetoxyhexane medium**

*P. putida* KT2440 was cultivated in LB overnight. The overnight culture (1% v/v) was used to inoculate 50 mL M9 medium in a 250-mL shake flask and cultivated overnight again. The day after, this overnight culture was used to inoculate 10 mL M9 medium in 50-mL tubes (10 mM hexyl acetate or diacetoxyhexane as the only carbon source) with an initial OD₆₀₀ of 0.15. Three tubes were taken out every 24 h for optical density and substrate concentration assays. Samples for GC analysis were prepared as mentioned above.
Table 1: Plasmids and strains used in this work

| Name                      | Description                                                                 | References |
|---------------------------|-----------------------------------------------------------------------------|------------|
| **Plasmids**              |                                                                             |            |
| pCOM10-AlkL               | pCOM10 vector containing AlkL gene; Km<sup>R</sup>                           | [22]       |
| pBTL10                    | pCOM10 vector containing AlkBGTL genes; Km<sup>R</sup>                      | [50]       |
| pBGTL-Atf1                | pCOM10 vector containing AlkBGTL-Atf1 genes; Km<sup>R</sup>                 | [19]       |
| pSEVAb628                 | Expression vector; oriV(RK2); XylS, Pm; Gm<sup>R</sup>                      | [47]       |
| pSEVAb658                 | Expression vector; oriV(RSF1010); XylS, Pm; Gm<sup>R</sup>                  | [47]       |
| pSEVAb628-AlkS            | pSEVAb628 vector containing Pm → AlkS; Gm<sup>R</sup>                      | This work  |
| pSEVAb658-8’G’TLA<sup>*</sup> | pSEVAb658 vector containing Pm → AlkB*G*TL-Atf1*; Gm<sup>R</sup>           | This work  |
| pET26b-EstX               | pET26b vector containing T7 → EstX, X represents each putative esterase gene; Km<sup>R</sup> | This work  |
| pGNW2                     | Suicide vector used for gene deletion in P. putida KT2440, oriV(R6K) containing P14g → msfGFP; Km<sup>R</sup> | [43]       |
| pGNW2-ΔCrc                | Derivative of vector pGNW2 containing HRs to delete Crc (PP_5292)           | This work  |
| pGNW2-ΔCyoB               | Derivative of vector pGNW2 containing HRs to delete CyoB (PP_0813)          | This work  |
| pGNW2-ΔEstX               | Derivative of vector pGNW2 containing HRs to delete EsteraseX (Table 2)     | This work  |
| pGNW2-PP_2047-48          | Derivative of vector pGNW2 containing HRs to delete PP_2047-48              | This work  |
| pGNW2-PP_2136-37          | Derivative of vector pGNW2 containing HRs to delete PP_2136-37              | This work  |
| pGNW2-PP_2214-18          | Derivative of vector pGNW2 containing HRs to delete PP_2214-18              | This work  |
| **Strains**               |                                                                             |            |
| P. putida KT2440          | Wild-type strain, derived from P. putida mt-2                                 | [51]       |
| ΔCrc                     | P. putida KT2440ΔCrc                                                        | This work  |
| ΔCyoB                    | P. putida KT2440ΔCyoB                                                        | This work  |
| ΔCrcΔCyoB                | P. putida KT2440ΔCrcΔCyoB                                                    | This work  |
| ΔEstBCPZ                 | P. putida KT2440ΔEstBCPZ                                                    | This work  |
| ΔEst12/K                 | P. putida KT2440ΔEst12/K                                                    | This work  |
| ΔEstBCPZ12/K             | P. putida KT2440ΔEstBCPZΔEst12/K                                            | This work  |
| P-GNWX-ΔBOX              | P. putida KT2440ΔPP_2047-48ΔPP_2136-37ΔPP_2214-18                           | This work  |
| P-L                      | P. putida KT2440 harboring pCOM10-AlkL                                       | This work  |
| P-BGTL                   | P. putida KT2440 harboring pBTL10                                            | This work  |
| P-BGTLA                  | P. putida KT2440 harboring pBGTL-Atf1                                        | This work  |
| ΔCrc-BGTL                | P. putida KT2440ΔCrc harboring pBTL10                                        | This work  |
| ΔCyoB-BGTL               | P. putida KT2440ΔCyoB harboring pBTL10                                       | This work  |
| ΔCrcΔCyoB-BGTL           | P. putida KT2440ΔCrcΔCyoB harboring pBTL10                                   | This work  |
| ΔCycB-BGTLA              | P. putida KT2440ΔCycB harboring pBTL10                                       | This work  |
| ΔCrcΔCycB-BGTLA          | P. putida KT2440ΔCrcΔCycB harboring pBTL10                                   | This work  |
| ΔCycB-BGTLAS             | P. putida KT2440ΔCycB harboring pBTL10                                       | This work  |
| P-B’G’TLA<sup>*</sup>     | P. putida KT2440 harboring pSEVAb658-B’G’TLA<sup>*</sup>                    | This work  |
| ΔEst12/K- B’G’TLA<sup>*</sup> | P. putida KT2440ΔEst12/K harboring pSEVAb658-B’G’TLA<sup>*</sup>           | This work  |
| E. coli blank             | E. coli NEBT7 harboring pET26b                                              | This work  |
| E. coli -EstX             | E. coli NEBT7 harboring pET26b                                              | This work  |

HRs = homologous regions

*Represents removal of Crc/Hfq mRNA attachment sites in the gene
### Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13068-021-02066-x.

### Additional file 1: Table S1 Primers used in this study.

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### Authors’ contributions

All authors designed the work. CL and EA conducted, analysed and interpreted the experiments. CL drafted and wrote the manuscript. All authors read and approved the final manuscript.

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### Availability of data and materials

All data generated or analysed during this study are included in this published article and its additional information files.

### Declarations

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

### Author details

1. Bioprocess Engineering, Wageningen University and Research, Wageningen, The Netherlands. 2. Laboratory of Systems and Synthetic Biology, Wageningen University and Research, Wageningen, The Netherlands. 3. Lifeglimmer GmbH, Berlin, Germany. 4. Faculty of Biosciences and Aquaculture, Nord University, Bodø, Norway.

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