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Metabolic engineering of *Pseudomonas putida* KT2440 for medium-chain-length fatty alcohol and ester production from fatty acids

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ABSTRACT

Medium-chain-length fatty alcohols have broad applications in the surfactant, lubricant, and cosmetic industries. Their acetate esters are widely used as flavoring and fragrance substances. *Pseudomonas putida* KT2440 is a promising chassis for fatty alcohol and ester production at the industrial scale due to its robustness, versatility, and high oxidative capacity. However, *P. putida* has also numerous native alcohol dehydrogenases, which lead to the degradation of these alcohols and thereby hinder its use as an effective biocatalyst. Therefore, to harness its capacity as a producer, we constructed two engineered strains (WTΔ*pedF*Δ*adhP*, GN346Δ*adhP*) incapable of growing on mcl-fatty alcohols by deleting either a cytochrome *c* oxidase PedF and a short-chain alcohol dehydrogenase AdhP in *P. putida* or AdhP in *P. putida* GN346. Carboxylic acid reductase, phosphopantetheinyl transferase, and alcohol acetyltransferase were expressed in the engineered *P. putida* strains to produce hexyl acetate. Overexpression of transporters further increased 1-hexanol and hexyl acetate production. The optimal strain G23E-MPAscTP produced 93.8 mg/L 1-hexanol and 160.5 mg/L hexyl acetate, with a yield of 63.1%. The engineered strain is applicable for C_6–C_{10} fatty alcohols and their acetate ester production. This study lays a foundation for *P. putida* being used as a microbial cell factory for sustainable synthesis of a broad range of products based on medium-chain-length fatty alcohols.

1. Introduction

1.1. Medium-chain-length fatty alcohols

Medium-chain-length (Mcl) fatty alcohols (C_6–C_{10}) hold the potential to be substitutes for diesel and jet fuels, because of their high energy density, high cetane number, and low vapor pressure (He et al., 2018; Rajesh Kumar et al., 2016). Mcl-fatty alcohols are also broadly applied in cosmetics, surfactants, and plasticizers (Pfleger et al., 2015; Xu et al., 2016). The global market volume is estimated to reach more than USD 10 billion by 2023 (Krishnan et al., 2020). Their acetate esters are widely present in fruits and alcoholic beverages as aroma substances (Dennis et al., 2012; Gómez and Ledbetter, 1994). These esters are used as flavors and fragrances due to their fruity odor (Ji et al., 2006; Mahattanatawee et al., 2018). However, fatty alcohols and their esters are now primarily derived from petrochemical resources (Rupilius and Ahmad, 2007), which is not sustainable and environmentally friendly. Meanwhile, medium-chain fatty acids (MCFAs) have been commercially produced from acidified organic wastes by ChainCraft and Earth Energy Renewables via a process named chain elongation (Angenent et al., 2016; Grootscholten et al., 2013; Roghair et al., 2016). Besides, the microbial production of MCFAs from glucose was also well-investigated (Deng et al., 2020; Grisewood et al., 2017; Wu et al., 2019). Therefore, they are considered as renewable substrates. Biosynthesis of mcl-fatty alcohols and their acetate esters from renewable sources is gaining traction (Akhtar et al., 2015; Gudiminchi et al., 2012; Hernández Lozada et al., 2020; Hu et al., 2020a). In this paper, we describe the efficient conversion of fatty acids into fatty alcohols and their acetate esters through a robust microbial catalyst.

The first step in the conversion of fatty acids into fatty alcohols is the formation of aldehydes. Two options are possible. One option is that fatty acids are converted into acyl-CoA or acyl-ACP by ATP-dependent acyl-CoA synthase or acyl-ACP synthase respectively, which are subsequently reduced to the corresponding aldehydes by fatty acyl-CoA

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reductase (ACR) or fatty acyl-ACP reductase (AAR) (Hu et al., 2020a). So far, ACR and AAR have shown poor activity with long-chain molecules as substrate (Nicolaas A Buijs, Yongjin J. Zhou, Verena Siewers, 2015; Zhou et al., 2016), and even lower with medium-chain molecules (Fatma et al., 2016; Youngquist et al., 2013). The other option is to use carboxylic acid reductase (CAR) (Yunus and Jones, 2018), which uses both ATP and NADPH as cofactors. A modified CAR (RF1+303) from *Mycobacterium marinum* (MmCAR) has been specifically constructed for medium-chain fatty acids (Hu et al., 2020a). Considering the feasibility of the aforementioned options, we therefore choose to investigate the latter one. The formed aldehydes can be reduced to alcohols by aldehyde reductases (ALRs). Many NADPH-dependent ALRs such as *E. coli*'s YjgB (Pick et al., 2013) and YqhD (Jarboe, 2011) have been used for microbial production of fatty alcohols (Akhtar et al., 2013; Cao et al., 2015; Liu et al., 2014). Fatty alcohols can be further converted into fatty alcohol acetate esters by the alcohol acetyltransferase Atf1(Kruis et al., 2019; Lu et al., 2022; van Nuland et al., 2017).

1.2. *Pseudomonas putida as a robust biocatalyst*

Pseudomonas putida KT2440 is known for its tolerance to high concentrations of organic solvents, flexible metabolism, and excellent capability for mcl-polyhydroxyalkanoates (PHA) production (Martin--Pascual et al., 2021; Yadav et al., 2021). Nevertheless, its versatile metabolism has actually hindered *P. putida* from being efficiently used because it has, among others, various esterases and alcohol dehydrogenases that can break down the alcohols and esters to be produced. Acetate esters were reported to be hydrolyzed by seven esterases, among which Est12 was the primary one (Lu et al., 2021). Fourteen genes were annotated as alcohol dehydrogenase (ADH) genes in *P. putida* (Karp et al., 2018). Some of these genes have been reported to play important roles in alcohol metabolism. For example, four genes (*pedE*, *pedH*, *pedI*, *aldB1*) were deleted to impair n-butanol metabolism in strain GN346 (Vallon et al., 2015). PedE and PedH are extensively investigated pyrroloquinoline quinone (PQQ)-dependent alcohol dehydrogenases (Wehrmann et al., 2020; Wehrmann and Patrick Billard, Audrey Martin-Meriadec, Asfaw Zegeye, 2017). PedF (PP_2675) and AdhP (PP_3839) were recently proven to be the key enzymes for short-chain alcohol metabolism (Thompson et al., 2020). As a cytochrome *c* oxidase, PedF is responsible for PQQ cofactor regeneration which is essential for the activity of PedE and PedH. Besides, it was pointed out that PedF was more important than PedE and PedH (Thompson et al., 2020). AdhP is a NAD-dependent alcohol dehydrogenase for short-chain alcohols (Demling et al., 2021; Thompson et al., 2020). It is not clear yet which ADHs are responsible for medium-chain alcohol consumption. Thus, investigations on these ADHs and evaluation of their effects on medium-chain alcohol metabolism are required to remove obstacles for *P. putida* to become a feasible chassis for the production of mcl-fatty alcohols and their acetate esters.

1.3. *Import-export*

Substrate import and product export are crucial for a variety of biotechnological applications, including biosynthesis using fatty acids as starting materials. Introducing transporter proteins has been the focus of numerous investigations (He et al., 2019; Kim et al., 2019; Patil et al., 2017; van Nuland et al., 2017). The import of fatty acids and derivatives has been intensively studied in many microorganisms. FadL from *E. coli* was the first discovered microbial fatty acid transporter, responsible for the import of exogenous fatty acids (He et al., 2019; Salvador López and Van Bogaert, 2021; Yoo et al., 2019). FadL, as a well-known long-chain fatty acid transporter, was successfully applied for multiple hydrophobic compounds uptake (He et al., 2019; Yoo et al., 2019). In addition, FadL was used to transport medium-chain fatty acids but with less affinity than long-chain substrates (Salvador López and Van Bogaert, 2021). AlkL from *Pseudomonas putida* GPo1 was used to transport n-alkanes and

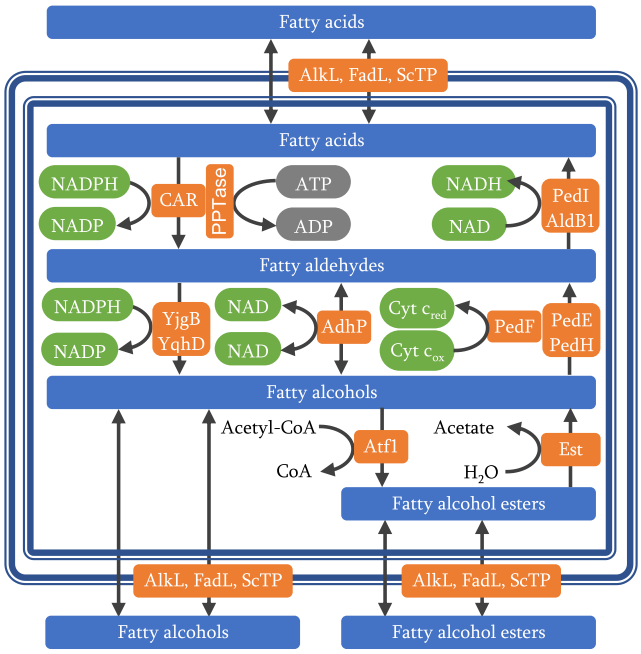

Fig. 1. Synthesis pathway of fatty alcohols and alkyl acetates from medium-chain fatty acids (C_6–C_{10}) in the engineered *Pseudomonas putida* KT2440. AlkL, n-alkane transporter; FadL, fatty acid transporter; ScTP: putative short-chain fatty acids transporter; CAR, carboxylic acid reductase from *Mycobacterium marinum*; PPTase, phosphopantetheinyl transferase; AldB1 and PedI, cytoplasmic aldehyde dehydrogenases; YjgB and YqhD, NADPH-dependent aldehyde reductases; AdhP, NAD-dependent alcohol dehydrogenase; PedF, a cytochrome *c* oxidase; PedE and PedH, pyrroloquinoline quinone (PQQ)-dependent alcohol dehydrogenases; Atf1, alcohol acetyl transferase; Est, esterase.

fatty acid methyl esters (Nuland et al., 2016; van Nuland et al., 2017). ScTP was annotated as a short-chain fatty acid transporter in *P. putida* KT2440. It may be active for medium-chain molecules too. Therefore, it is necessary to assess the impact of transporters on the production of medium-chain alcohols and esters.

1.4. *Rendering P. putida a strong alcohol and ester producer*

In this study, we aimed to block the consumption of medium-chain-length fatty alcohols through the deletion of the above-described genes. We subsequently assessed the growth dynamics of the resulting *P. putida* strains. As a proof of concept for the production of medium-chain n-alcohol and alkyl acetate in *P. putida*, we introduced the genes coding for carboxylic acid reductase, phosphopantetheinyl transferase, and alcohol acetyltransferase (Fig. 1). Furthermore, fatty acid transporters were overexpressed to boost product titers. The optimally engineered strain was applied to convert C_6–C_{10} substrates into the corresponding alcohols and esters.

2. Materials and methods

2.1. *Chemicals and medium*

DNA polymerase, T4 DNA ligase and restriction enzymes were purchased from New England Biolabs, ThermoFisher. Fatty acids, aldehydes, alcohols, esters, and m-toluic acid (3-methylbenzoate, 3-MB) were purchased from Sigma-Aldrich, Alfa Aesar, Avantor. The M9 medium, composed of 55 mM glucose, 1x M9 minimal salts, 0.2 mM $MgSO_4{\cdot}7H_2O$ and 1 mL/L trace elements US^{Fe} (Bühler et al., 2002), was used for cell growth and induction of genes of interest. The M9* medium was prepared the same as the M9 medium except for using 2.5 mM or 10

mM medium-chain fatty alcohols (C_5–C_{10}) substituting glucose as carbon source. A resting-cell buffer, consisting of 1% v/v glucose, 2 mM $MgSO_4$ and 50 mM KPi (pH 7.4), was prepared to resuspend induced cells for alcohol and ester production tests.

2.2. Construction of plasmids and engineered strains

E. coli DH 5α was used for molecular cloning purposes. *Pseudomonas putida* KT2440 and *Pseudomonas putida* GN346 were used as starting strains for alcohol dehydrogenase deletion. The target genes that needed to be deleted were amplified by PCR and inserted into pGNW2 vector via Golden Gate assembly. The obtained suicide plasmid pGNW2 and a self-curing plasmid pQURE6·H bearing the I-SceI gene were used to delete genes in *P. putida* KT2440 (Volke et al., 2020). The deletion of genes in the obtained knockout strains was validated by colony PCR. MmCAR and Atf1 were codon optimized for *P. putida* and synthesized by Twist Bioscience. The genes of interest for ester production were amplified and inserted into pSEVAb238 plasmid. The resulting plasmids were validated by sequencing. These plasmids were introduced into the engineered *P. putida* for alcohol and ester production. The presence of the plasmids in all strains was verified by colony PCR. All plasmids and engineered strains are listed in Table 1. The primers and gene sequences are listed in Table S1.

2.3. Microplate-based growth tests

Growth studies of *P. putida* and its ADH deletion mutants were performed using microplate reader kinetic assays (Thompson et al., 2020). The strains were cultivated in LB medium overnight. This preculture (1% v/v) was used to inoculate 50 mL M9 medium and cultivated overnight at 30 C°. After 16 h of incubation, the overnight culture was washed twice with M9 medium without glucose and was used to inoculate 200 μL M9* medium in 96-well plates (Costar; 3595) at an initial OD600 of 0.15. The optical density was measured at 600 nm (OD_{600}) every 30 min for 60 h in a BioTek Synergy Mx plate reader at 30 C° with medium continuous shaking.

2.4. Resting-cell conversion

Engineered *P. putida* strains were inoculated in LB medium and cultivated overnight. This overnight preculture (1% v/v) was used to inoculate 50 mL M9 medium which was incubated overnight. The day after, the culture was used to inoculate 50 mL M9 medium at an initial OD_{600} of 0.2. After 4–5 h of incubation (when OD_{600} reached 0.6–0.8), 1 mM 3-MB was added to induce gene expression at 30 °C, 250 rpm for 18 h. After induction, the induced cells were harvested by centrifugation at 4200 x g.

Cell pellets were washed once with M9 medium and suspended in the resting-cell buffer to a density of 1.5 g_{cdw}/L. Aliquots of 1 mL resting cells were taken for each reaction and placed in a Pyrex tube. Fatty acids (C_6–C_{10}) were added to each tube at a concentration of 8.6 mM. Afterwards, these tubes were incubated in a Kühner incubator for 20 h at 30 °C, 250 rpm. Each reaction was performed in triplicate. Once the reaction was completed, 1% v/v of phosphoric acid was instantly added to stop reactions and the tubes were placed on ice. Aliquots of 1 mL diethyl ether with 0.2 mM dodecane as an internal standard were added to extract products. After 1 h of extraction with a rotator (Labinco, The Netherlands), the tubes were centrifuged for 20 min at 4200 x g. The supernatant was collected for GC analysis.

2.5. GC analysis

GC analysis was conducted on an Agilent 7890A gas chromatograph equipped with a flame ionization detector (FID) using an HP-5 column (30 m × 30 m × 0.25 μm). 1 μL sample was injected in the split-less mode under the following temperature program: 50 °C for 3 min,

Table 1
Plasmids and strains used in this work.

Name	Description	Reference
Plasmids		
pGNW2	Suicide vector used for gene deletion in *P. putida* KT2440; *oriV (R6K)* containing *P14g→msfGFP*; Km^R	Volke et al. (2020)
pGNW2-Δ*PP_2675*	Derivative of vector pGNW2 containing HRs to delete *PP_2675*	This work
pGNW2-Δ*PP_3839*	Derivative of vector pGNW2 containing HRs to delete *PP_3839*	This work
pGNW2-Δ*est12*	Derivative of vector pGNW2 containing HRs to delete *est12* (*PP_3812*)	Lu et al. (2021)
pGNW2-Δ*scTP*	Derivative of vector pGNW2 containing HRs to delete *scTP* (*PP_3124*)	This work
pQURE6·H	Conditionally-replicating vector; oriV(RK2), XylS/Pm→*I-SceI* and P14g(BCD2)→*mRFP*; Gm^R	Volke et al. (2020)
pSEVAb238	Expression vector; *oriV(RK2)*; *XylS, Pm*; Km^R	(Damalas et al., 2020; Martin-Pascual et al., 2021)
pSEVAb238-Atf1	pSEVAb628 vector containing *Pm→Atf1*; Km^R	This work
pSEVAb238-MA	pSEVAb628 vector containing *Pm→MmCAR-Atf1*; Km^R	This work
pSEVAb238-MEA	pSEVAb628 vector containing *Pm→MmCAR-EcPPTase-Atf1*; Km^R	This work
pSEVAb238-MPA	pSEVAb628 vector containing *Pm→MmCAR-PpPPTase-Atf1*; Km^R	This work
pSEVAb238-MEAB	pSEVAb628 vector containing *Pm→MmCAR-EcPPTase-Atf1-YjgB*; Km^R	This work
pSEVAb238-MEAD	pSEVAb628 vector containing *Pm→MmCAR-EcPPTase-Atf1-YqhD*; Km^R	This work
pSEVAb238-MPAB	pSEVAb628 vector containing *Pm→MmCAR-PpPPTase-Atf1-YjgB*; Km^R	This work
pSEVAb238-MPAD	pSEVAb628 vector containing *Pm→MmCAR-PpPPTase-Atf1-YqhD*; Km^R	This work
pSEVAb658	pSEVAb658 vector containing *Pm*; Gm^R	(Damalas et al., 2020; Martin-Pascual et al., 2021)
pSEVAb658-fadL	pSEVAb658 vector containing *Pm→FadL*; Gm^R	This work
pSEVAb658-alkL	pSEVAb658 vector containing *Pm→AlkL*; Gm^R	This work
pSEVAb658-scTP	pSEVAb658 vector containing *Pm→ScTP*; Gm^R	This work
Strains		
WT	Wild-type *P. putida* KT2440 strain, derived from *P. putida* mt-2(Worsey and Williams, 1975)	Bagdasarian et al. (1981)
WT-b658	*P. putida* KT2440 harboring pSEVAb658	This work
GN346	*P. putida* KT2440 Δ*upp* Δ*pedE* Δ*pedH* Δ*pedI* Δ*PP_0545*	Simon et al. (2015)
Δ*est12*	*P. putida* KT2440 Δ*est12*	This work
Δest12ΔscTP	*P. putida* KT2440 Δ*est12* Δ*scTP*	This work
WTΔ*pedF*	*P. putida* KT2440 Δ*PP_2675*	This work
WTΔ*adhP*	*P. putida* KT2440 Δ*PP_3839*	This work
WTΔ*pedF*Δ*adhP*	*P. putida* KT2440 Δ*PP_2675* Δ*PP_3839*	This work
GN346Δ*pedF*	*P. putida* GN346 Δ*PP_2675*	This work
GN346Δ*adhP*	*P. putida* GN346 Δ*PP_3839*	This work
WT-alkL	*P. putida* KT2440 harboring pSEVAb658-alkL	This work
WT-fadL	*P. putida* KT2440 harboring pSEVAb658-fadL	This work
WT-scTP	*P. putida* KT2440 harboring pSEVAb658-scTP	This work
G23	*P. putida* GN346 Δ*PP_3839*	This work
G23E	GN346 Δ*PP_3839* Δ*Est12*	This work

(*continued on next page*)

Table 1 (*continued*)

Name	Description	Reference
G23E-A	G23E harboring pSEVAb238-Atf1	This work
G23E-MA	G23E harboring pSEVAb238-MA	This work
G23E-MEA	G23E harboring pSEVAb238-MEA	This work
G23E-MPA	G23E harboring pSEVAb238-MPA	This work
G23E-MEAB	G23E harboring pSEVAb238-MEAB	This work
G23E-MEAD	G23E harboring pSEVAb238-MEAD	This work
G23E-MPAB	G23E harboring pSEVAb238-MPAB	This work
G23E-MPAD	G23E harboring pSEVAb238-MPAD	This work
G23E-MPAfadL	G23E harboring pSEVAb238-MEA, and pSEVAb658-fadL	This work
G23E-MPAalkL	G23E harboring pSEVAb238-MEA, and pSEVAb658-alkL	This work
G23E-MPAscTP	G23E harboring pSEVAb238-MEA, and pSEVAb658-scTP	This work

15 °C/min increase to 180 °C, 7 °C/min increase to 230 °C, 30 °C/min increase to 350 °C, which was held for 3 min. Quantification was carried out by using the corresponding standards.

3. Results and discussion

3.1. Growth of *P. putida* strains KT2440 and GN346 on fatty alcohols

P. putida KT2440 is regarded as a promising host for medium-chain fatty alcohol production (Malca et al., 2013; Simon et al., 2015). However, it has been reported that *P. putida* can also utilize alcohols such as n-butanol and n-hexanol for cell growth because of its versatile metabolism (Fig. 2a) (Lu et al., 2021; Rühl et al., 2009). Here, C_5–C_{10} fatty alcohols were separately used as the sole carbon source for growth tests. 10 mM n-Pentanol, n-hexanol, n-nonanol and n-decanol were fed in the minimal medium. Only 2.5 mM n-heptanol and n-octanol were used given their severe toxicity to *P. putida* and many other microorganisms (Neumann et al., 2005). The wild-type *P. putida* KT2440 strain grew on all tested alcohols (Fig. 2b). The strain GN346, lacking the alcohol dehydrogenases PedE and PedH and the aldehyde dehydrogenases PedI and AldB1, has been reported as a strain incapable of growing on n-butanol (Simon et al., 2015). Thus, we hypothesized that it might not be able to grow on other medium-chain alcohols either. We conducted growth tests on minimal medium using GN346 and noticed that it can still grow on every single tested alcohol (Fig. 2g), although at lower growth rates than those of the wild-type strain. In all cases, GN346 also showed a longer lag phase compared with the wild-type strain. Overall, it shows that the deletion of *pedE*, *pedH*, *pedI* and *aldB1* was not enough

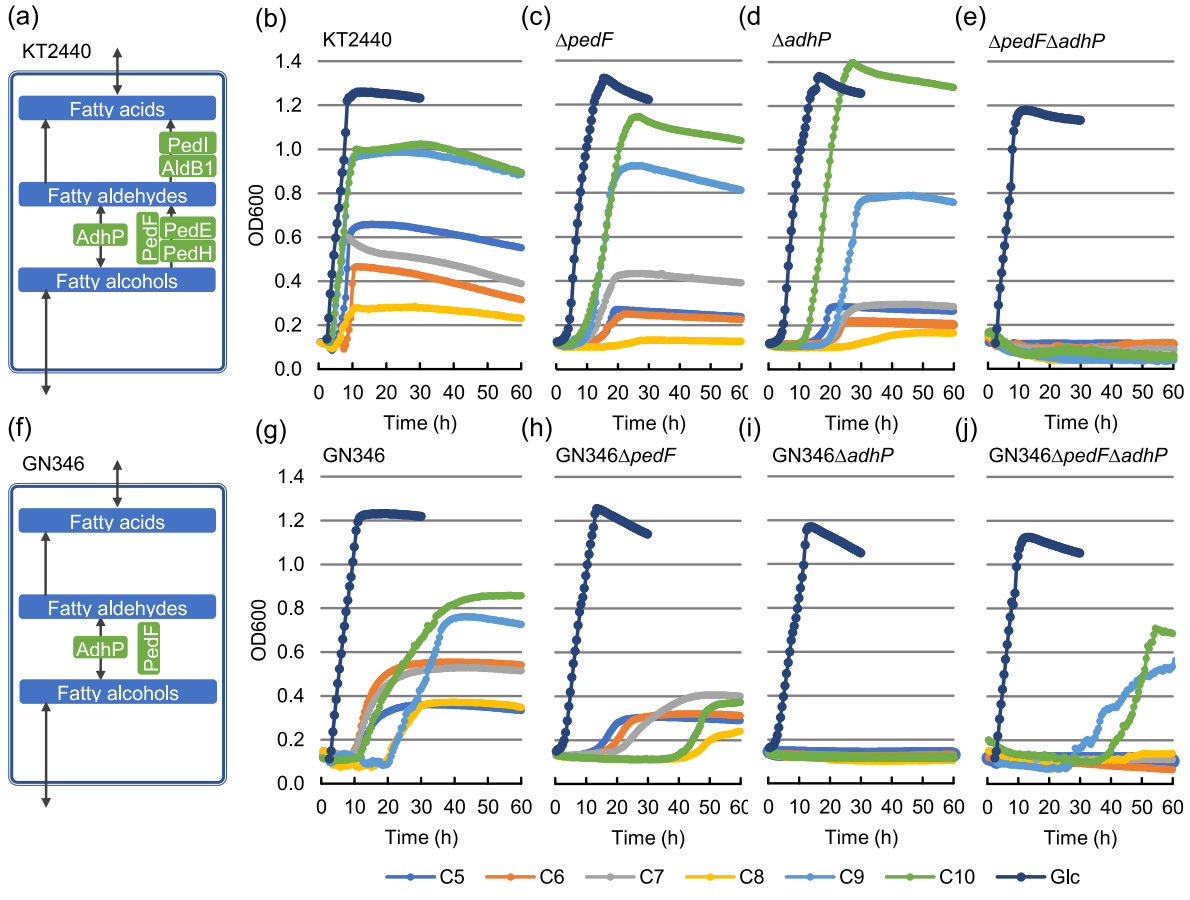

Fig. 2. Medium-chain fatty alcohols metabolism in *P. putida* KT2440, GN346 and their derivatives. (a) Oxidation of fatty alcohol in *P. putida* KT2440. The growth dynamics of (b) *P. putida* KT2440, (c) Δ*pedF*, (d) Δ*adhP*, (e) Δ*pedF*Δ*adhP* on minimal medium using medium-chain fatty alcohol as the sole carbon source in a 96 well plate. (f) Oxidation of fatty alcohol in *P. putida* GN346. The growth dynamics of (g) *P. putida* KT2440, (h) GN346Δ*pedF*, (i) GN346Δ*adhP*, (j) GN346Δ*pedF*Δ*adhP* on minimal medium using medium-chain fatty alcohol as the sole carbon source in a 96 well plate. The concentration of n-pentanol, n-hexanol, n-nonanol and n-decanol were 10 mM, while that of n-heptanol and n-octanol were 2.5 mM C5: n-pentanol; C6: n-hexanol; C7: n-heptanol; C8: n-octanol; C9: n-nonanol; C10: n-decanol; Glc: 27.7 mM glucose.

to halt alcohol breakdown and, thus, that other enzymes responsible for medium-chain alcohol metabolism were still active.

3.2. Restricting growth of P. putida on fatty alcohols

Building upon the previous research (Thompson et al., 2020), the cytochrome *c* oxidase PedF (PP_2675) and the short-chain alcohol dehydrogenase AdhP (PP_3839) were selected as the main knock-out candidates to diminish cell growth on medium-chain fatty alcohols. The single knockouts (Δ*pedF*, Δ*adhP*) and double knockout (Δ*pedF*Δ*adhP*) were constructed based on the wild-type strain KT2440 and GN346, giving rise to Δ*pedF*, Δ*adhP*, Δ*pedF*Δ*adhP*, GN346Δ*pedF*, GN346Δ*adhP* and GN346Δ*pedF*Δ*adhP*. The growth dynamics of these knockout strains were monitored over time. Each fatty alcohol (C_5–C_{10}) was separately used as the sole carbon source in the growth assay. The strains Δ*pedF* and Δ*adhP* showed minor growth defects when cells grew on the M9 medium using C_5–C_9 fatty alcohol as a sole carbon source compared to wild-type *P. putida* (Fig. 2b, c and 2d). The strain Δ*adhP* had a longer lag phase in comparison to Δ*pedF*. The double knock-out strain Δ*pedF*Δ*adhP* showed no growth for all tested alcohols (Fig. 2e), suggesting that *pedF* and *adhP* are essential genes for the oxidation of medium-chain alcohols in *P. putida*. Although GN346Δ*pedF* had a longer lag phase on all tested alcohols compared to GN346, it still grew on all substrates, reaching a lower biomass (Fig. 2h). Similarly to Δ*pedF*Δ*adhP*, GN346Δ*adhP* displayed no growth on the tested alcohols. Surprisingly, GN346Δ*pedF*Δ*adhP* was observed to grow on n-nonanol and n-decanol after a long lag phase. This indicates that still unknown enzymes for C_9 and C_{10} alcohol metabolism were activated when the genes (*pedE*, *pedH*, *pedI*, *aldB1*, *adhP*, and *pedF*) were deleted at the same time. In summary, deletion of either the genes *pedE*, *pedH*, and *adhP* or *pedF* and *adhP* can block *P. putida* from utilizing medium-chain alcohols for cell growth. It is the first time that two engineered *P. putida* strains (Δ*pedF*Δ*adhP*, GN346Δ*adhP*) were constructed to completely disable cell growth on medium-chain alcohols as the sole carbon source. All strains grew well on glucose (Fig. 2), excluding that *pedF* or *adhP* is an essential gene and indicating the deleted genes are only essential for the tested alcohol metabolism. For C_5–C_8 alcohols alcohol metabolism, the PQQ-cofactor-dependent ADHs (PedE, PedH) and AdhP are essential dehydrogenases. In the case of C_9–C_{10} alcohols, further investigations are required to elaborate on it. Overall, WTΔ*pedF*Δ*adhP* and GN346Δ*adhP* can be used for further conversion tests. This work paves the way for *P. putida* to become a feasible chassis for not only medium-chain alcohol production but also for the biosynthesis of alcohol-derived compounds.

3.3. Alcohol and alkyl acetate production by expression of MmCAR

To produce fatty alcohols from fatty acids, carboxylic acid reductase (CAR) and a phosphopantetheinyl transferase (PPTase) were expressed to reduce acid to aldehyde. Here, a codon-optimized MmCAR (MmCAR RF1+303, from now on defined as just MmCAR), for *P. putida* was used for alcohol and alkyl acetate production. Besides, the esterase Est12 was previously reported to be a hurdle for ester production in *P. putida* (Lu et al., 2021). Thus, *est12* was deleted in GN346Δ*adhP*, resulting in GN346Δ*adhP*Δ*est12* (briefly named G23E) (Fig. 3a), for alcohol production tests. Hexanoic acid was used as a starting material in this section because Hu and colleagues reported that MmCAR had the highest activity on C6 molecules (Hu et al., 2020b).

MmCAR alone, MmCAR together with either PPTase from *E. coli* (EcPPTase) or PPTase from *P. putida* (PpPPTase) were introduced into strain G23E, resulting in G23E-M, G23E-ME, G23E-MP, respectively. However, when hexanoic acid was fed into the induced resting-cell suspensions of these three strains, no hexanal and n-hexanol were observed (data not shown). This indicates that either these enzymes were not active properly, that hexanal and n-hexanol were converted into other compounds, such as storage materials; or that both were formed but not exported. It is known that *P. putida* is able to transport acetate esters of mcl-alcohols (Lu et al., 2021, 2022). To investigate whether n-hexanol was produced intracellularly, alcohol acetyltransferase Atf1 was therefore adopted to capture n-hexanol into hexyl acetate. Atf1 either with MmCAR-EcPPTase or MmCAR-PpPPTase was introduced to G23E, resulting in G23E-MEA and G23E-MPA. Atf1 alone was introduced into G23E giving rise to G23E-Atf1 as a negative control. When 8.6 mM hexanoic acid was fed, G23E-Atf1 produced 0.04 mM hexyl acetate in 4 h (Fig. 3b). This indicates that there are native gene products that enable the reduction of hexanoic acid to n-hexanol. With co-expression of MmCAR and Atf1, G23E-MA produced 0.1 mM hexyl acetate which is 250% of that of G23E-Atf1, indicating that MmCAR

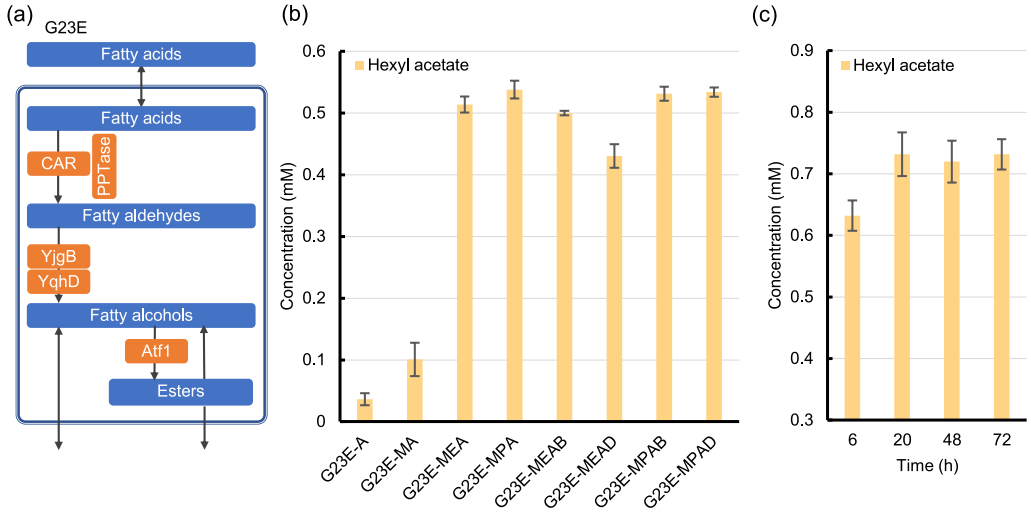

Fig. 3. The production of acetate ester of fatty alcohol in the engineered *P. putida* strains under resting-cell conditions. (a) The synthesis pathway of fatty alcohol and its acetate ester in the strain G23E. (b) Conversion from 8.6 mM hexanoic acid and 10 g/L glucose to hexyl acetate in 4 h. A, Atf1: alcohol acetyl transferase; M, MmCAR: MmCAR, carboxylic acid reductase from *Mycobacterium marinum*; E, phosphopantetheinyl transferase from *Escherichia coli*; P, phosphopantetheinyl transferase from *Pseudomonas putida*; B, YjgB: NADPH-dependent aldehyde reductase; D, YqhD: NADPH-dependent aldehyde reductase. (c) Hexyl acetate production in G23E-MPA over time with 8.6 mM hexanoic acid and 10 g/L glucose.

indeed contributes to hexyl acetate production. When PPTases were overexpressed, G23E-MEA and G23E-MPA produced 0.51 and 0.54 mM hexyl acetate, respectively, indicating that both PPTases were active and facilitated hexyl acetate production. PPTase from *P. putida* was confirmed to be compatible with MmCAR for the first time.

When MmCAR and PPTase were expressed, n-hexanol was not detected, while it was trapped in the form of hexyl acetate only in the presence of Atf1. Moreover, overexpression of MmCAR, PPTase, and Atf1 resulted in an increased hexyl acetate production, indicating that the flux to n-hexanol was improved and the esterification was not a limiting step. n-Hexanol was produced but not excreted, or may have been utilized by other competing pathways which requires further investigations.

As previously mentioned, hexanol might be metabolized. This could be the case for hexanal too. Given that Atf1 helps to trap hexanol, the expression of aldehydes reductases might be beneficial for boosting the capacity of the pathway. Two aldehyde reductases, YjgB and YqhD, were separately introduced into G23E-MEA and G23E-MPA, resulting in G23E-MEAB, G23E-MEAD, G23E-MPAB and G23E-MPAD. However, these four strains failed to produce more hexyl acetate (Fig. 3b). This is reasonable compared to the results of parental strains because no hexanal or n-hexanol was observed in all cases, suggesting that the reduction from hexanal to n-hexanol is not the limiting step. We then explored the production of hexyl acetate with G23E-MPA in a time-lapse experiment. Hexyl acetate accumulated over time, reaching its peak at 20 h at 0.73 mM with a yield of 80.3% and remaining stable (Fig. 3c). Meanwhile, 7.7 mM hexanoic acid was not consumed, indicating that its transport is likely to be a limiting step.

3.4. Introduction of transporters boosts production of alcohols and esters

Extracellular n-hexanol was not found, but its intracellular presence was proven by using Atf1-induced hexyl acetate production as a reporter. To improve hexyl acetate and/or n-hexanol production, we introduced transporters that shuttle these compounds out of the cell and facilitate the transport of hexanoic acid. Three transporters - FadL, AlkL, and ScTP - were chosen to assess whether their presence increases the conversion of hexanoic acid into n-hexanol and hexyl acetate in *P. putida* (Fig. 4a). Genes - *fadL*, *alkL*, and *scTP* - were separately inserted into pSEVAb658 plasmid, giving rise to plasmids pSEVAb658-fadL, pSEVAb658-alkL and pSEVAb658-scTP. These plasmids are low copy number (10–12) plasmids harboring an RFS1010 origin of replication (Damalas et al., 2020; Meyer, 2009). These three plasmids were separately transferred into strain G23E-MPA, resulting in G23E-MPAfadL, G23E-MPAalkL and G23E-MPAscTP. When 8.6 mM hexanoic acid was fed, all three strains produced more hexyl acetate than the parent strains (Fig. 4b). Hexyl acetate production was increased by 15.7% in G23E-MPAfadL compared to G23E-MPA. This indicates that FadL facilitates the conversion of hexanoic acid into hexyl acetate, but with limited improvement, which may be caused by the low affinity of FadL to medium-chain fatty acids (Salvador López and Van Bogaert, 2021). G23E-MPAalkL produced 150% more total products, including 0.27 mM hexanol. Compared to FadL, AlkL performed better in the production of n-hexanol and hexyl acetate. When ScTP was overexpressed, the total production increased by 240% reaching a maximum of 1.73 mM compared to G23E-MPA (Fig. 4b). The three transporters are reported to have a broad substrate specificity (He et al., 2019; Julsing et al., 2012; Tan et al., 2017; Yoo et al., 2019). It is likely that the enhanced production was caused by the export of fatty alcohols, fatty aldehydes, and esters, but it cannot be ruled out that this resulted from a higher import of fatty acids by the same transporters.

We therefore tested the effect of transporters on cell growth in M9 minimal medium using fatty acids (C_6, C_8, C_{10}) or alcohols (C_6, C_8, C_{10}) as the sole carbon source. The plasmids harboring a transporter - pSEVAb658-alkL, pSEVAb658-fadL, and pSEVAb658-scTP - were separately introduced into wild-type *P. putida* KT2440, giving rising to WT-alkL, WT-fadL, and WT-scTP. The empty plasmid pSEVAb658 was introduced, resulting in WT-b658 as a negative control strain. Glucose was fed as a negative control. To induce transporter expression, 1 mM 3-methylbenzoate was added at 0 h. The maximum specific growth rate (μ_{max}) of each strain on substrates was calculated (Table 2). When transporters were present, growth rates on glucose dropped compared to the rate of WT-b658, indicating that transporter expression has a negative effect on cell growth. In the case of hexanoate and octanoate, the growth rate increased when a transporter was introduced compared to that of WT-b658. For the decanoate case, none of the three transporters had a positive effect on the growth rate compared to WT-b658. Growth rates of WT-alkL and WT-scTP on n-hexanol and n-octanol were

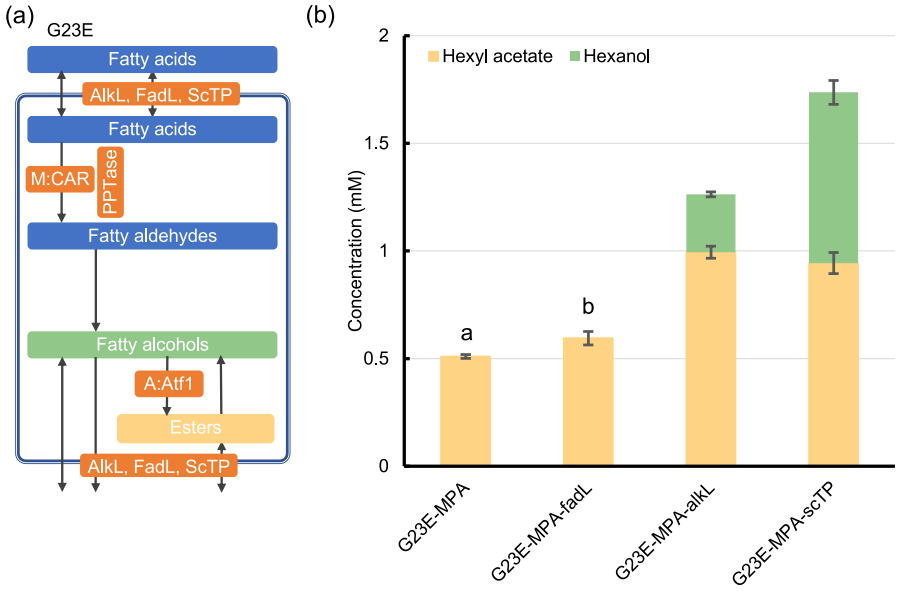

Fig. 4. The effect of transporters on hexyl acetate production feeding with 8.6 mM hexanoic acid and 10 g/L glucose. (a) The synthesis pathway of fatty alcohol and its acetate ester in the strain G23E in presence of transporters. (b) Production of n-hexanol and hexyl acetate with overexpression of transporters. A, Atf1: alcohol acetyl transferase; M, MmCAR: MmCAR, carboxylic acid reductase from *Mycobacterium marinum*; P, phosphopantetheinyl transferase from *Pseudomonas putida*; fadL, the long-chain fatty acids transporter from *Escherichia coli*; alkL, alkylic substrates transporter from *Pseudomonas oleovorans*; ScTP, putative short-chain fatty acids transporter from *Pseudomonas putida*. Bars with different letters indicate significant difference from each other ($p < 0.05$).

Table 2
Growth rates of the wild-type *P. putida* strain and transporter-overexpressed strains.

Strains	Growth rate μ_{max} (h^{-1})						
	Glucose	Hexanoate	Octanoate	Decanoate	n-Hexanol	n-Octanol	n-Decanol
WT-b658	0.49 ± 0.04	0.17 ± 0.01	0.11 ± 0.02	0.46 ± 0.04	0.11 ± 0.02	0.11 ± 0.05	0.40 ± 0.02
WT-alkL	0.34 ± 0.02	0.25 ± 0.04	0.22 ± 0.02	0.32 ± 0.02	0.20 ± 0.00	0.16 ± 0.01	0.26 ± 0.02
WT-fadL	0.29 ± 0.04	0.26 ± 0.08	0.30 ± 0.07	0.30 ± 0.14	N.G.[a]	N.G.[a]	N.G.[a]
WT-scTP	0.26 ± 0.1	0.27 ± 0.02	0.40 ± 0.03	0.49 ± 0.01	0.19 ± 0.10	0.19 ± 0.04	0.28 ± 0.03

[a] N.G.: no growth. Growth rates were calculated from cultures of M9 minimal medium containing 55.4 mM glucose, 10 mM hexanoate, octanoate, decanoate, hexanol, decanol, and 2.5 mM octanol in 96-well plate. 1 mM 3-Methylbenzoate was added to induce alkL, fadL, scTP expression.

raised while no growth was observed for WT-fadL. This might be because the improved transport of n-hexanol and n-octanol and the overexpressed FadL are detrimental to cell growth under these conditions. In summary, ScTP performs best among three transporters for tested fatty acids and alcohols. Therefore, it was used for the conversion of other fatty acids.

Given its excellent results on the production of hexanol and hexyl acetate (Fig. 4b), we assumed ScTP is a primary transporter for medium-chain fatty acids and is involved in their metabolism in *P. putida*. Thus, a knockout strain (Δ*scTP*) was constructed to assess its effect on cell growth using hexanoic acid or octanoic acid as the sole carbon source. Nevertheless, no significant growth differences were observed between the wild-type strain and Δ*scTP* (Fig. S2), suggesting that ScTP is only one of the transporters for medium-chain fatty acids and not the predominant transporter.

We introduced three transporters to assess their influence on hexyl acetate production. All of them can facilitate the production of n-hexanol and hexyl acetate, this is possibly attributed to the product export and substrate import. Usually, compounds harboring a $logP_{o/w}$ value between 2 and 4 can passively diffuse through bacteria membranes (De Bont, 1998; Inoue and Horikoshi, 1991). This is the case for C_6–C_{10} fatty acids. We accordingly assumed that product export plays a major role in the increase of product formation.

3.5. From medium-chain fatty acids to esters

To evaluate the substrate specificity of the engineered strain G23E-MPAscTP, C_6–C_{10} fatty acids were used as substrates for bioconversion (Fig. 5a). As a result, alkyl acetate, alcohol, and aldehyde (C_9 and C_{10}) were observed under resting-cell conditions when 8.6 mM substrate was added (Fig. 5b). Using C_6 and C_7 acids as substrates, G23E-MPAscTP produced a similar level of total products - 2 mM and 2.14 mM, respectively - while the total production from C_8, C_9, and C_{10} fatty acids were 1.4 mM, 0.5 mM, and 0.2 mM respectively, suggesting that MmCAR has a high activity to hexanoic acid and heptanoic acid. When the number of carbon atoms increases above C_7, the production of total products (aldehyde, alcohol, ester) decreased. This is in line with previous research (Hu et al., 2020a), in which MmCAR was modified specifically for C_6 molecules and exhibited much higher activity towards hexanoic acid than octanoic acid and decanoic acid (Hu et al., 2020a). Despite that, the second-highest octanol production (182.3 mg/L) was reached. The reported productions were usually between 15 and 100 mg/L (Akhtar et al., 2015; Lonsdale et al., 2015). The highest production of octanol (1.3 g/L) was produced at a >90% C_8 specificity in an optimal engineered *E. coli* (Hernández Lozada et al., 2020). Our strain G23E-MPAscTP is applicable for all C_6–C_{10} alcohols. For C_9 and C_{10} fatty acids, the corresponding aldehyde was detected, indicating that aldehyde reductase activity for longer chain aldehydes was a limiting step. Besides, the low production of C_9 and C_{10} products can be further improved by tailor-designing the carboxylic acid reductase through protein engineering. The engineered strain G23E-MPAscTP was applicable for C_6–C_{10} fatty acids, indicating this opens the door to the bioproduction of a broad range of alcohols and esters.

4. Conclusions

In this work, we achieved bioproduction of C_6–C_{10} alcohols and their acetate esters from the corresponding fatty acids in engineered *P. putida* strains. Based on the previous research (Thompson et al., 2020), we unraveled the essential genes underpinning the catabolism of medium-chain fatty alcohol in this bacterium. This hurdle has thus far hindered *P. putida* from being an efficient chassis for the production of

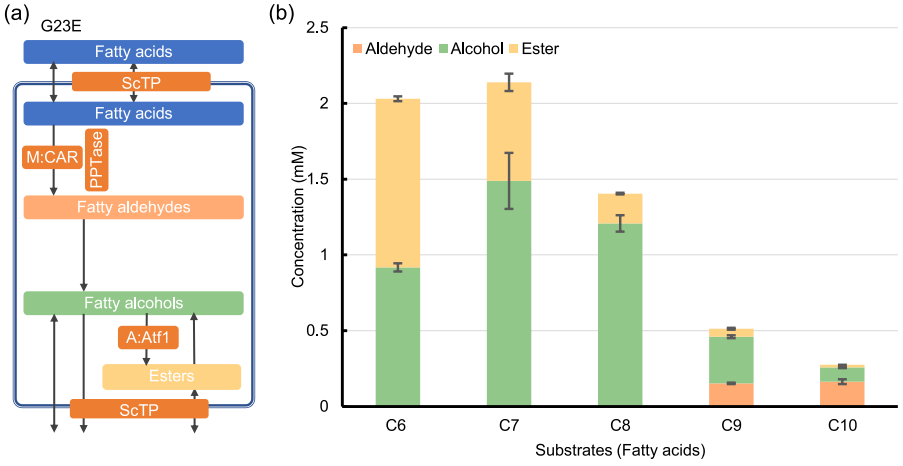

Fig. 5. The production of alkyl acetates and fatty alcohols from 8.6 mM medium-chain fatty acids ($C_{6\text{-}10}$) and 10 g/L glucose in the engineered strain G23E-MPAscTP. (a) The synthesis pathway of medium-chain alkyl acetate from fatty acids. (b) The production of aldehydes, alcohols, and alkyl acetate under resting-cell conditions in 20 h.

medium-chain fatty alcohols and esters. We successfully removed this major bottleneck. Furthermore, we enhanced production by introducing transporters that facilitate the synthesis process, presumably by their role as exporters. Altogether, this body of work lays a solid foundation for the use of the industrial workhorse *P. putida* for the bioproduction of medium-chain fatty alcohols and esters.

Notes

The authors declare that they have no competing interests.

CRediT authorship contribution statement

Chunzhe Lu: Conceptualization, Investigation, Methodology, Formal analysis, Data curation, Software, Visualization, Writing - original draft, Writing - review & editing. **Edward Ofori Akwafo:** Investigation, Methodology, Data curation. **Rene H. Wijffels:** Project administration, Formal analysis, Supervision, Writing -review & editing. **Vitor A.P. Martins dos Santos:** Project administration, Supervision, Writing - review & editing. **Ruud A. Weusthuis:** Project administration, Conceptualization, Funding acquisition, Supervision, Writing - review & editing.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymben.2022.11.006.

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