

Application of AlkBGT and AlkL from *Pseudomonas putida* GPo1 for Selective Alkyl Ester ω -Oxyfunctionalization in *Escherichia coli*

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ABSTRACT

The enzyme system AlkBGT from *Pseudomonas putida* GPo1 can efficiently ω -functionalize fatty acid methyl esters. Outer membrane protein AlkL boosts this ω -functionalization. In this report, it is shown that whole cells of *Escherichia coli* expressing the AlkBGT system can also ω -oxidize ethyl nonanoate (NAEE). Coexpression of AlkBGT and AlkL resulted in 1.7-fold-higher ω -oxidation activity on NAEE. With this strain, initial activity on NAEE was 70 U/g (dry weight) of cells (g_{cdw}), 67% of the initial activity on methyl nonanoate. In time-lapse conversions with 5 mM NAEE the main product was 9-hydroxy NAEE (3.6 mM), but also 9-oxo NAEE (0.1 mM) and 9-carboxy NAEE (0.6 mM) were formed. AlkBGT also ω -oxidized ethyl, propyl, and butyl esters of fatty acids ranging from C_6 to C_{10} . Increasing the length of the alkyl chain improved the ω -oxidation activity of AlkBGT on esters of C_6 and C_7 fatty acids. From these esters, application of butyl hexanoate resulted in the highest ω -oxidation activity, 82 U/ g_{cdw} . Coexpression of AlkL only had a positive effect on ω -functionalization of substrates with a total length of C_{11} or longer. These findings indicate that AlkBGT(L) can be applied as a biocatalyst for ω -functionalization of ethyl, propyl, and butyl esters of medium-chain fatty acids.

IMPORTANCE

Fatty acid esters are promising renewable starting materials for the production of ω -hydroxy fatty acid esters (ω -HFAEs). ω -HFAEs can be used to produce sustainable polymers. Chemical conversion of the fatty acid esters to ω -HFAEs is challenging, as it generates by-products and needs harsh reaction conditions. Biocatalytic production is a promising alternative. In this study, biocatalytic conversion of fatty acid esters toward ω -HFAEs was investigated using whole cells. This was achieved with recombinant *Escherichia coli* cells that produce the AlkBGT enzymes. These enzymes can produce ω -HFAEs from a wide variety of fatty acid esters. Medium-chain-length acids (C_6 to C_{10}) esterified with ethanol, propanol, or butanol were applied. This is a promising production platform for polymer building blocks that uses renewable substrates and mild reaction conditions.

The global demand for polymers is expected to grow in the coming years (1) and thus also the need for sustainable polymer production processes. ω -Hydroxy fatty acids (ω -HFAs) and dicarboxylic acids (DCAs) are building blocks of polymers such as polyesters and polyamides (1–3). These compounds can be produced from medium-chain-length fatty acids (MCFAs) by oxidation of the terminal methyl group, a reaction called ω -oxidation (4).

A recent development is the production of fatty acids by processes using microbial chain elongation from organic waste material, yielding both odd- and even-chain-length fatty acids ranging from C_4 to C_9 (5, 6).

Chemical ω -oxidation of nonactivated terminal methyl groups remains challenging due to the inert nature of these bonds. This results in poor selectivity (7, 8). Biocatalytic ω -oxidation can be a solution for the terminal activation of fatty acid (esters). Several classes of enzymes can ω -oxidize terminal methyl groups (9). A key advantage is that some of these enzymes only oxidize the terminal methyl group. CYP52 enzymes have been applied in whole-cell approaches to yield high titers of ω -oxidized product from (esterified) fatty acids, but this approach is limited to a chain length of $\geq C_{12}$ (10, 11).

The alkane hydroxylase system AlkBGT from *Pseudomonas putida* GPo1 could be a more promising approach for ω -oxidation of medium-chain-length compounds. This system is composed of a monooxygenase (AlkB), a rubredoxin (AlkG), and a rubredoxin

reductase (AlkT), all located on the *alk* operon (12). AlkB was found to oxidize methyl groups of medium-chain alkanes and medium-chain fatty acids (13). AlkB has a remarkably relaxed substrate specificity. This enzyme can oxidize C_5 to C_{16} alkanes, cycloalkanes, alkenes, and thioethers (14–16).

Recently, the AlkBGT system has also been shown to efficiently catalyze the ω -oxidation of methyl esterified fatty acids. Methyl pentanoate to methyl dodecanoate were ω -oxidized by this system when expressed in *Escherichia coli*. The activity of AlkB was highest on methyl nonanoate (NAME), even higher than on *n*-octane and *n*-nonane. This activity declined strongly for methyl esters of fatty acids with a chain length shorter than 8 (17). Outer membrane protein AlkL was successfully applied to further increase the activity of *E. coli* expressing AlkBGT on larger, more hydrophobic molecules. The ω -oxidation activity of cells harboring AlkL on

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TABLE 1 Plasmids used in this study

Plasmid	Characteristics	Reference
pCOM10_alkL	pCOM10 vector containing <i>alkL</i>	18
pGec47	<i>alk</i> operon on pLAFR1	26
pSTL	<i>alkTL</i> on pCOM10	This study
pBT10	<i>alkBGT</i> on pCOM10	This study; created as described in reference 17
pBTL10	<i>alkBGT</i> on pCOM10	This study; created as described in reference 18

methyl dodecanoate was improved 28-fold in small-scale assays and 62-fold in a two-liquid-phase setup (18, 19).

Using an esterified fatty acid as a substrate has several advantages over the use of free fatty acids. First, the substrate does not dissolve well in water and forms a separate phase. The ω -oxidized product accumulates in the organic phase, which eliminates the need of water removal for downstream processing (18). Second, esterified MCFAs might pose fewer toxicity problems to the cell than free MCFAs. Free fatty acids inhibit growth of a large variety of organisms (20). Medium-chain fatty acids can freely diffuse through the membrane of *E. coli* (21), inhibit its growth, and cause leakage of the membrane (22, 23). But when methyl nonanoate or methyl dodecanoate was added to growing *E. coli* W3110 cultures, the growth rate declined only slightly (18). This suggests that esterified MCFAs are less toxic than free MCFAs. Third, the broth does not need to be acidified for product recovery when acids are esterified, reducing the amount of chemicals needed for downstream processing.

Considering the broad substrate specificity of AlkB and the above-mentioned advantages for the use of esterified fatty acids, it is also interesting to assess whether AlkBGT facilitates ω -oxidation of alkyl esters with a longer alkyl chain. By increasing the length of the alkyl chain, the total length of the substrate increases. This might boost activity of AlkBGT on shorter-chain fatty acid esters.

In this study, it was investigated in whole-cell experiments whether the AlkBGT system can also ω -oxidize ethyl, propyl, and butyl esters of C_6 to C_{10} fatty acids. Also, AlkL was applied to evaluate whether this could increase the ω -oxidation activity of whole cells.

MATERIALS AND METHODS

Plasmids, strains, and chemicals. The plasmids used in this study are listed in Table 1. pCOM10_alkL and pGec47 were kindly provided by Bruno Bühler. Plasmids pBT10 and pBTL10 were constructed as described in references 17 and 18, respectively. *E. coli* TOP10 (Invitrogen) was used for cloning purposes. *E. coli* NEB7 (New England BioLabs) was used for conversion and toxicity studies.

Chemicals were ordered with the highest purity available from the following vendors: Sigma-Aldrich (dodecane, nonanoic acid [NA], ethyl hexanoate, propyl hexanoate, butyl hexanoate, butyl octanoate, methyl nonanoate, ethyl nonanoate [NAEE], butyl decanoate, 6-hydroxy ethyl hexanoate, and 7-hydroxy ethyl heptanoate), Alfa Aesar (ethyl octanoate, ethyl decanoate, and propyl decanoate), Merck (ethyl heptanoate), Pfaltz and Bauer (propyl heptanoate), TCI (propyl octanoate and 9-hydroxy nonanoic acid methyl ester), LGC Standards (butyl heptanoate), Santa Cruz Biotechnology (propyl nonanoate), and TRC (9-hydroxy nonanoic acid ethyl ester, 9-oxo methyl nonanoate, and azelaic acid mono-ethyl ester).

TABLE 2 Growth rate of *E. coli* pSTL in the presence of NA or NAEE^a

Initial concn (mM)	NA		NAEE	
	μ (h ⁻¹)	Remaining concn (mM)	μ (h ⁻¹)	Remaining concn (mM)
0	0.52 $\pm$ 0		0.52 $\pm$ 0	
1	0.55 $\pm$ 0	0.47 $\pm$ 0.03	0.37 $\pm$ 0.02 ^b	0.18 $\pm$ 0.06
10	-0.24 $\pm$ 0	7.14 $\pm$ 0.56	0.30 $\pm$ 0	8.77 $\pm$ 0.50

^a Remaining NA and NAEE were measured at the end of the culture phase.

^b A lag phase of 2 h was observed.

Cultivation and gene expression for conversion studies. Cultivation was done in a rotary shaker at 250 rpm and 37°C. All strains used for conversion studies were grown overnight in 5 ml of LB plus 50 μ g/ml of kanamycin. The next day, 500 μ l of the overnight culture was transferred to 50 ml of mineral medium in a 300-ml shake flask. The mineral medium contained 1 $\times$ M9 salts, 0.5% glucose, 2 mM MgSO₄, 1 ml/liter of US^{Fe} trace elements (24), and kanamycin. Cells were again cultured overnight and then inoculated into the same mineral medium to 0.05 g (dry weight) of cells (g_{cdw})/liter in 300- or 500-ml shake flasks. The culture was directly induced with 0.025% DCPK (dicyclopropyl ketone) and incubated for 4 h.

Toxicity studies. *E. coli*(pSTL) was grown as described above, except that the cells were not induced with DCPK. The overnight mineral medium culture was used to inoculate 250 ml of mineral medium to a density of 0.05 g_{cdw}/liter. This culture was grown to a density of 0.18 g_{cdw}/liter and then divided into 20-ml cultures. Nonanoic acid or ethyl nonanoate was added, and growth rates were determined by measuring the optical density at 600 nm. Experiments were done in duplicate.

Whole-cell conversions. DCPK-induced cells were harvested by centrifugation at 4,255 $\times$ g for 10 min and resuspended in 50 mM pH 7.4 phosphate buffer containing 1% of glucose and 2 mM MgSO₄. Biomass concentration was set to 1 g_{cdw}/liter. One-milliliter conversion tests were done in triplicate at 37°C in a closed vessel at 250 rpm. Prior to the conversion, cells were incubated in the shaker for 5 to 10 min. The conversion was started by addition of 5 mM substrate from a concentrated stock in ethanol; the final concentration of ethanol in the reaction was 2.5% (vol/vol). Initial activities in units per gram of cells (dry weight) were determined by quantification of oxidized product after 5 min of incubation, in which 1 U = 1 μ mol/min. Time-lapse tests were done similarly to the determination of the initial activities, except that 300 μ l of resting cell suspension was used and different incubation times were applied.

GC analysis. The whole reactions were extracted with 1:1 CHCl₃-MeOH containing 0.2 mM dodecane as an internal standard. The time-lapse samples were extracted by adding 200 μ l of the reaction to 800 μ l of CHCl₃ containing 0.2 mM dodecane. For qualitative analysis, samples were analyzed with a Thermo Scientific TRACE Ultra gas chromatograph (GC) coupled to a DSQII mass spectrometer. Quantitative analysis was done with an Agilent HP 6890 GC coupled to a flame ionization detector (FID). Response factors of chemicals that were not commercially available were based on structurally similar chemicals.

RESULTS AND DISCUSSION

Toxicity tests. First it was tested if ethyl nonanoate (NAEE) was indeed a better substrate than nonanoic acid (NA) by testing their effect on the growth rate. In Table 2, results of the toxicity study are shown.

In the presence of 1 mM NA, the growth rate of *E. coli* was similar to that of the control, but growth ceased at a concentration of 10 mM. The growth rate was negatively affected by the presence of 1 mM NAEE, but cells still grew in the presence of concentrations up to 10 mM. Because the growth rate of *E. coli* in the pres-

TABLE 3 Specific activities for ω -oxidation of NAME and NAEE with different *E. coli* strains in a whole-cell bioconversion^a

Strain	Substrate	Sp act (U/g _{cdw})	Ratio of activity, NAEE/NAME
<i>E. coli</i> (pSTL)	NAME	No product	
	NAEE	No product	
<i>E. coli</i> (pBT10)	NAME	84 $\pm$ 10	0.49
	NAEE	41 $\pm$ 6	
<i>E. coli</i> (pBTL10)	NAME	105 $\pm$ 6	0.67
	NAEE	70 $\pm$ 3	

^a Applied biomass concentrations: pSTL strain, 0.98 g_{cdw}/liter; pBT10 strain, 1.04 g_{cdw}/liter; and pBTL10 strain, 0.96 g_{cdw}/liter.

ence of NAEE at 10 mM was higher, this was considered a more suitable substrate.

Whole-cell conversions with AlkBGT(L). To verify functionality of the AlkBGT enzyme system with methyl nonanoate (NAME), resting-cell conversions were done. *E. coli* was used carrying either the pBT10 vector or the pBTL10 vector for the positive control. *E. coli* pSTL was used as a negative control (Table 3).

Cells expressing AlkBGT yielded the product 9-hydroxy methyl nonanoate (9HNAME) from the substrate NAME, confirming the functionality of the AlkBGT enzyme system. The specific activity was 84 U/g_{cdw}. This activity was lower than the 104 U/g_{cdw} reported by Schrewe and colleagues (17) but in the same order of magnitude. The presence of outer membrane protein AlkL improved the ω -oxidation activity from 84 U/g_{cdw} to 105 U/g_{cdw}, or 1.3-fold. This is in line with earlier findings, where the ω -oxidation activity on NAME increased from 104 U/g_{cdw} to 128 U/g_{cdw}, a 1.2-fold improvement (18).

The same strains were tested with nonanoic *ethyl* ester as the

substrate, to investigate the effect of using a longer ester length. In this case 9-hydroxy ethyl nonanoate was detected, indicating that AlkB is also active on NAEE in both absence and presence of AlkL. The effect of increasing the alkyl chain length only led to a decrease in activity of 33% in cells coexpressing AlkL. As expected, the presence of AlkL does seem to increase the substrate availability of NAEE. The ω -oxidation activity of the pBTL10 strain was 1.7-fold higher than the activity of the pBT10 strain. The effect of AlkL is more evident for NAEE than NAME. The more apolar character of NAEE compared with NAME likely decreases transport into the cell, and therefore the presence of AlkL has a more profound influence.

Whole-cell conversion time-lapse with pBT10 and pBTL10.

We then explored production of ω -functionalized odd-chain esters with *E. coli* strains carrying pBT10 and pBTL10 in a time-lapse experiment. These tests were done with NAEE as a substrate. The same experiment was done with NAME to compare ω -oxidation activity and the effect of a longer alkyl chain on this activity. Also, both pBT10 and pBTL10 strains were used to assess whether AlkL has a positive influence on yield.

The results of these conversions are shown in Fig. 1. For all samples, the presence of NAME was confirmed, except the samples at 120 min. This suggests that the substrate became limiting after 60 min. The highest yields were reached with the pBTL10 strain, which converted 4.4 mM NAME, nearly 90%, into ω -oxidized product. The pBT10 strain converted 3.9 mM NAME into ω -oxidized product, close to the amount converted by the pBTL10 strain. The presence of AlkL improved the whole-cell activity slightly, as tests with the pBTL10 strain yielded higher product titers at 30 min than did tests with the pBT10 strain.

The predominant product that was detected was 9HNAME, representing more than 85% of the ω -oxidized products. Under

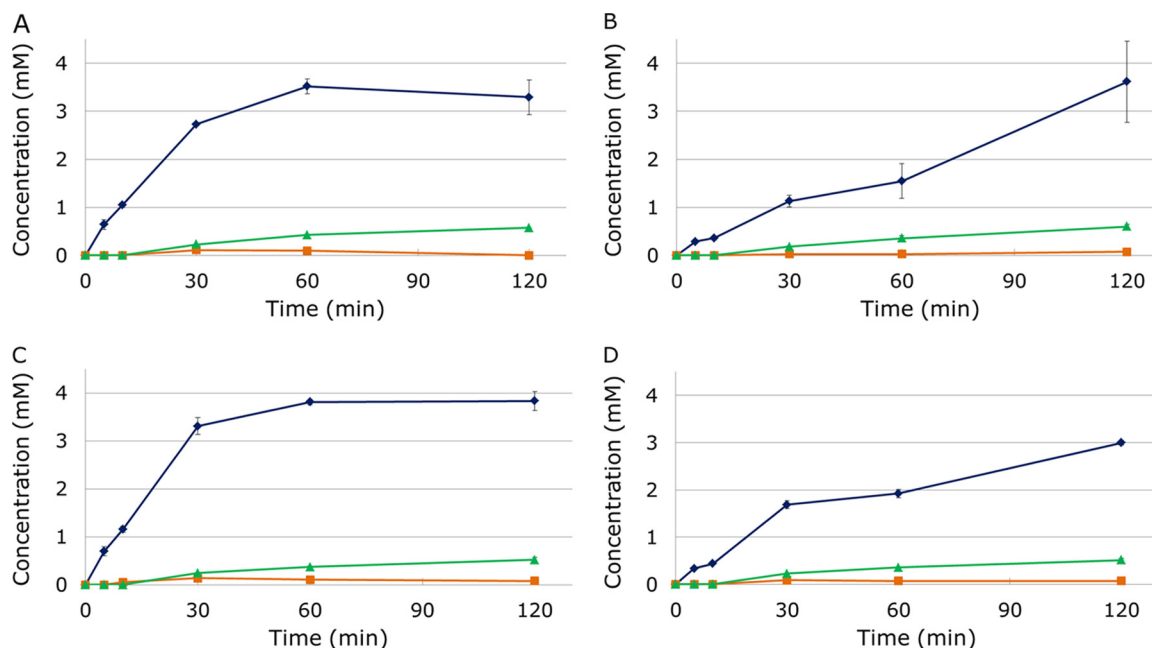


FIG 1 Resting whole-cell conversion time-lapse. (A) pBT10 strain plus 5 mM NAME; (B) pBT10 strain plus 5 mM NAEE; (C) pBTL10 strain plus 5 mM NAME; (D) pBTL10 strain plus 5 mM NAEE. Diamonds, 9-hydroxy product; triangles, 9-carboxy product; squares, 9-oxo product. The applied biomass concentration was 1.1 g_{cdw}/liter.

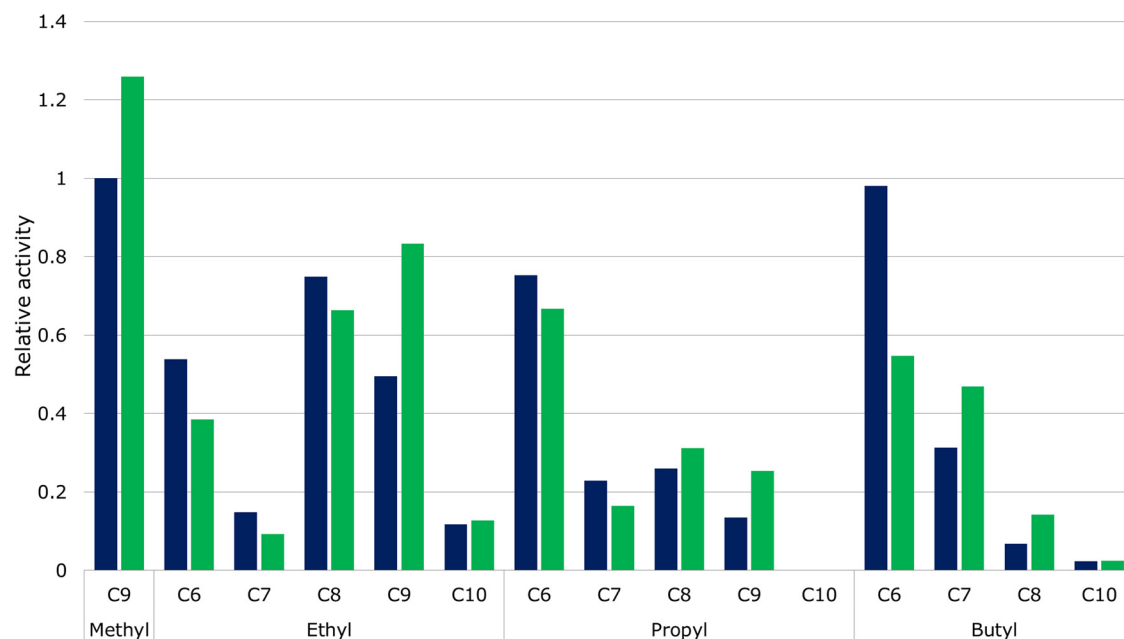


FIG 2 ω -Oxidation activities of pBT10 (blue bars) or pBTL10 (green bars) strains on methyl, ethyl, propyl, and butyl esters of C_6 to C_{10} fatty acids, relative to the activity of the pBT10 strain on NAME. The tests with propyl heptanoate yielded multiple products, of which only ω -hydroxy propyl heptanoate was identified and used for activity calculations.

these conditions, the ω -aldehyde hardly accumulates (the highest concentration measured was 0.14 mM). But AlkB does overoxidize the substrate, as about 0.5 mM ω -acid of methyl nonanoate (9CNAME) was detected. This was comparable with findings in an earlier work (17). A recent publication by the same group reported the apparent K_s values for C_{12} oxygenation products (19), and the apparent K_s for the aldehyde was lower than for the alcohol. This might apply to C_9 as well, resulting in higher

activity for aldehyde oxidation and thus 9CNAME accumulation. Still, the predominant product was the alcohol (alcohol/acid ratio of 100:17).

In the tests in which NAE was used as the substrate, the initial activity was lower, corresponding with findings presented in Table 3. After 120 min, however, the conversion with pBT10 yielded 4.3 mM oxidized product, nearly as much as the conversions with NAME. The ω -aldehyde did not accumulate above 0.1 mM. The

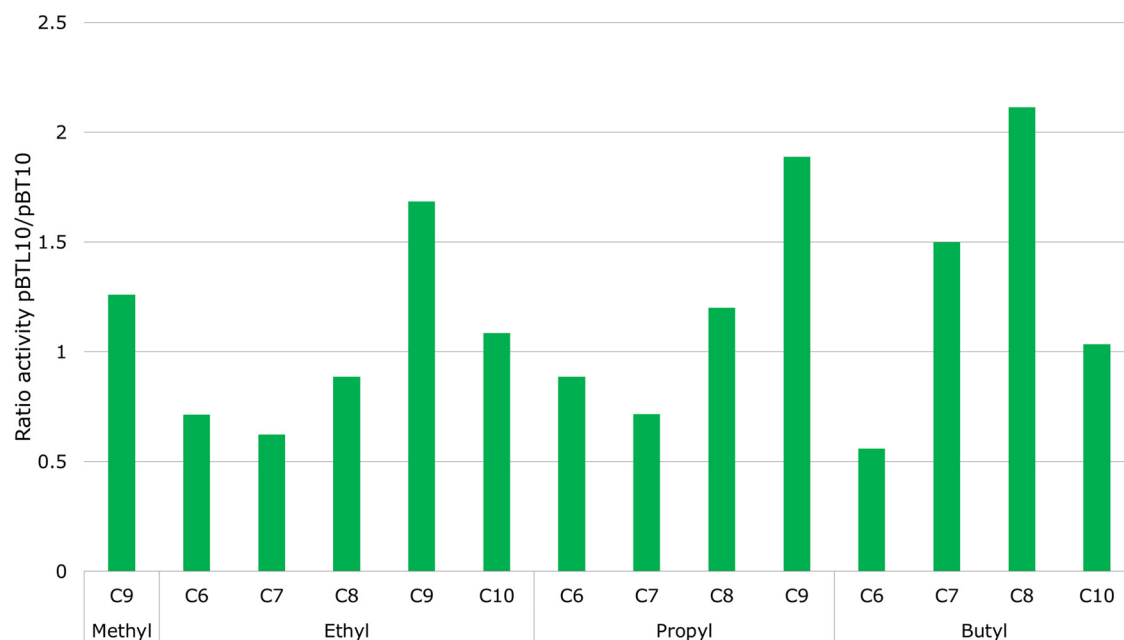
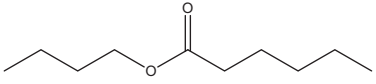
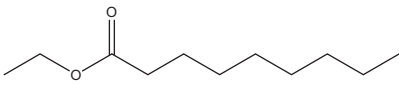
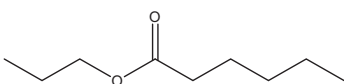
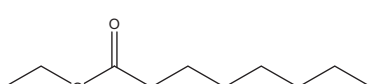
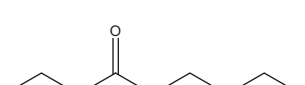
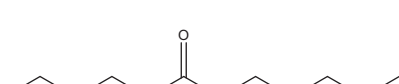
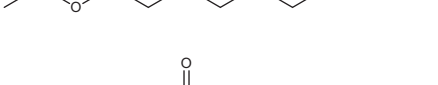
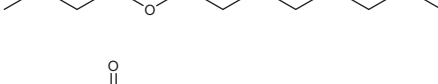
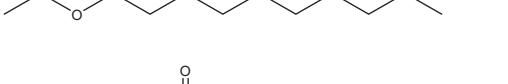


FIG 3 Ratio of activities of pBTL10 and pBT10 strains per substrate.

TABLE 4 Overview of tested substrates with confirmed activity of AlkBGT, arranged by initial activity (5 min)

Rank	Name	Structure	Presence of AlkL ^a	Initial activity (U/g _{cdw})
1	Butyl hexanoate		—	82 ± 7
2	Ethyl nonanoate		+	70 ± 3
3	Propyl hexanoate		—	63 ± 7
4	Ethyl octanoate		—	63 ± 3
5	Ethyl hexanoate		—	45 ± 4
6	Butyl heptanoate		+	39 ± 4
7	Propyl octanoate		+	30 ± 0
8	Propyl nonanoate		+	21 ± 1
9	Propyl heptanoate		—	19 ± 1
10	Ethyl heptanoate		—	12 ± 0
11	Butyl octanoate		+	12 ± 4
12	Ethyl decanoate		+	11 ± 2
13	Butyl decanoate		+	2 ± 0

^a For each substrate, tests with and without AlkL were done, and the setup that yielded the highest activity was selected. —, the strain carried pBT10; +, the strain carried pBTL10.

alcohol/acid ratios after 120 min were 100:17 for pBT10 and pBTL10 tests, similar to conversions with NAME.

AlkB ω -oxidation of medium-chain esters with various alkyl ester chain length. Knowing that AlkB has a very broad substrate specificity (14) and that NAEE was also accepted, activity assays were done to see if this was also the case for other acyl and alcohol chain lengths. Ethyl, propyl, and butyl esters of C_6 to C_{10} fatty acids were applied as substrates, with the exception of butyl nonanoate, as this chemical was not available. The results are shown in Fig. 2.

ω -Oxidation activity was detected for nearly all esters tested. Hydroxylation was found to occur only on the ω -position. These results indicate that the position of the ester group within the molecule does not strongly affect activity. Negative-control experiments with the pSTL strain on the different substrates did not yield products (data not shown). Activity on hexanoate and heptanoate esters could be greatly enhanced by increasing the alcohol length. Ethyl hexanoate and butyl hexanoate were ω -oxidized by the pBT10 strain at 45 U/g_{cdw} and 82 U/g_{cdw}, respectively. The activity on butyl hexanoate was nearly as high as for the positive control, NAME. These are both C_{10} molecules, but the positions of the ester moieties are different. The comparable activities indicate that the total length of the ester substrate is more important than the position of the ester group. Apparently the polar nature of the ester bond hardly influences the substrate binding in the hydrophobic binding pocket (25). Among the ethyl esters, a rather low activity was found for ethyl heptanoate. This phenomenon was also observed among methyl esters for methyl heptanoate (17). Still, the activity on this chain length could be enhanced by increasing the length of the alcohol group, as ethyl heptanoate was ω -oxidized by the pBT10 strain at 12 U/g_{cdw} and butyl heptanoate by the pBTL10 strain at 39 U/g_{cdw}. The tests with propyl heptanoate clearly yielded two products, but only the ω -hydroxy product could be identified. The two products gave similar peak areas in the chromatogram, suggesting that the by-product represents about half of the formed product. Only the ω -hydroxy product was used in activity determination.

For C_8 and higher lengths, the activity decreased with increasing ester length. This was also the case in tests with the pBTL10-carrying strain. So either AlkL does not efficiently transport these molecules into the periplasmic space or AlkB is not very active on these esters. If the first explanation applies, this effect would be contributed to the presence of the ester group and not to the length of the molecule, as AlkL was shown to transport even hexadecane (16). Esters were also shown to be transported before, so this does not seem a likely explanation (18). When we analyzed the ratio of activities of pBTL10 and pBT10 strains per substrate, this explanation also did not seem to apply (Fig. 3). Therefore, it is likely that AlkB activity decreased with these longer esters. In Table 4, the tested substrates with their structures are arranged based on the initial activities. It is also shown if AlkL improved the initial activity.

When does AlkL have a beneficial effect? It became clear that the tested substrates in this study were more efficiently ω -oxidized by the pBTL10 strain than by the pBT10 strain when the total chain length (meaning the sum of carbon atoms in the acyl and the alkyl chain) exceeded 10. Only methyl nonanoate did not seem to follow this trend. This was also found to be the case in earlier work (17). Considering that the total length of the molecule was the most important factor, the ratios of pBTL10 and pBT10 activities

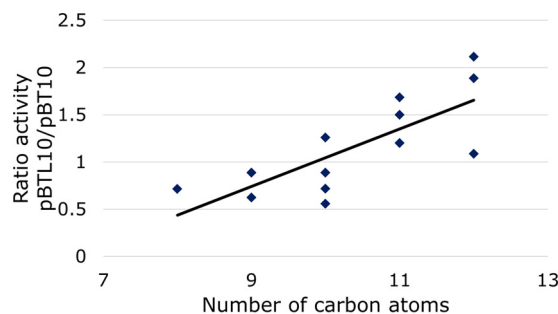


FIG 4 Ratio of activities of the pBTL10 and pBT10 strains plotted against the number of carbon atoms of the tested substrate. Butyl decanoate data were not used for this experiment, as the absolute activity was very low on this substrate.

were plotted against the number of carbon atoms of the different substrates (Fig. 4). For most substrates, the effect of AlkL was beneficial only when the number of carbon atoms exceeded 10. These substrates have a logarithm of the partition coefficient for *n*-octanol and water ($\log P_{o/w}$) above 4. Substrates with a $\log P_{o/w}$ between 1 and 4 reach high concentrations within the membrane, but when the $\log P_{o/w}$ exceeded 4 this is not the case, owing to low solubility (26). These data confirm that conversions with substrates having a $\log P_{o/w}$ higher than 4 suffer from poor mass transfer if AlkL is not present.

Conclusions. The AlkBGT enzyme system was successfully applied for ω -oxidation of NAEE, and an activity of 67% compared to when NAME was used as the substrate when AlkL was also expressed. It has been shown before that AlkB overoxidizes the ω -hydroxy product of methyl esters (17–19). AlkB also overoxidizes NAEE to the acid product, after conversion of the hydroxy derivative toward the aldehyde. The aldehyde did not accumulate above 0.1 mM.

Also, propyl and butyl esters of medium-chain fatty acids (C_6 to C_{10}) were accepted as a substrate by AlkB. Furthermore, the activity of AlkB on esters of shorter fatty acids could be enhanced by using a longer alcohol donor for the ester substrate.

AlkL was shown to have a positive effect on initial activity only when the total number of carbon atoms was 11 or higher, with the exception of methyl nonanoate. This seems to correspond well with earlier findings that solvents with a $\log P_{o/w}$ below 4 easily diffuse into the membrane and solvents with a $\log P_{o/w}$ above 4 do not (18, 27).

These findings demonstrate the possibility of sustainable production of medium-chain ω -hydroxy esters, i.e., with a chain length of C_6 to C_{10} . Substrates with a longer alkyl chain are also efficiently ω -oxidized, and increasing the alkyl chain resulted in high ω -oxidation activities on C_6 and C_7 esters.

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We declare that there are no conflicts of interest.

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REFERENCES

- Schaffer S, Haas T. 2014. Biocatalytic and fermentative production of α,ω -bifunctional polymer precursors. *Org Process Res Dev* 18:752–766. <http://dx.doi.org/10.1021/op5000418>.
- Metzger JO. 2009. Fats and oils as renewable feedstock for chemistry. *Eur J Lipid Sci Technol* 111:865–876. <http://dx.doi.org/10.1002/ejlt.200900130>.
- Bitto NJ, Graichen FHM, Monahan BJ. 2009. Functionality at the end of a fatty acid chain—chemical and biological routes to ω -hydroxylated fatty acids. *Lipid Technol* 21:216–219. <http://dx.doi.org/10.1002/lite.200900055>.
- Kusunose M, Kusunose E, Coon MJ. 1964. Enzymatic ω -oxidation of fatty acids: I. Products of octanoate, decanoate and laurate oxidation. *J Biol Chem* 239:1374–1380.
- Steinbusch KJJ, Hamelers HVM, Plugge CM, Buisman CJN. 2011. Biological formation of caproate and caprylate from acetate: fuel and chemical production from low grade biomass. *Energ Environ Sci* 4:216–224.
- Grootsholten TIM, Steinbusch KJJ, Hamelers HVM, Buisman CJN. 2013. Chain elongation of acetate and ethanol in an upflow anaerobic filter for high rate MCFA production. *Bioresour Technol* 135:440–445. <http://dx.doi.org/10.1016/j.biortech.2012.10.165>.
- Labinger JA, Bercaw JE. 2002. Understanding and exploiting C—H bond activation. *Nature* 417:507–514. <http://dx.doi.org/10.1038/417507a>.
- Crabtree RH. 2004. Organometallic alkane CH activation. *J Organomet Chem* 689:4083–4091. <http://dx.doi.org/10.1016/j.jorganchem.2004.07.034>.
- van Beilen JB, Funhoff EG. 2007. Alkane hydroxylases involved in microbial alkane degradation. *Appl Microbiol Biotechnol* 74:13–21. <http://dx.doi.org/10.1007/s00253-006-0748-0>.
- Picataggio S, Rohrer T, Deanda K, Lanning D, Reynolds R, Mielenz J, Eirich LD. 1992. Metabolic engineering of *Candida tropicalis* for the production of long-chain dicarboxylic acids. *Nat Biotechnol* 10:894–898. <http://dx.doi.org/10.1038/nbt0892-894>.
- Lu W, Ness JE, Xie W, Zhang X, Minshull J, Gross RA. 2010. Biosynthesis of monomers for plastics from renewable oils. *J Am Chem Soc* 132:15451–15455. <http://dx.doi.org/10.1021/ja107707v>.
- Eggink G, van Lelyveld PH, Arnberg A, Arfman N, Witteveen C, Witholt B. 1987. Structure of the *Pseudomonas putida* alkBAC operon. Identification of transcription and translation products. *J Biol Chem* 262:6400–6406.
- McKenna EJ, Coon MJ. 1970. Enzymatic ω -oxidation: IV. Purification and properties of the ω -hydroxylase of *Pseudomonas oleovorans*. *J Biol Chem* 245:3882–3889.
- van Beilen JB, Kingma J, Witholt B. 1994. Substrate specificity of the alkane hydroxylase system of *Pseudomonas oleovorans* GPo1. *Enzyme Microb Technol* 16:904–911. [http://dx.doi.org/10.1016/0141-0229\(94\)90066-3](http://dx.doi.org/10.1016/0141-0229(94)90066-3).
- Rojo F. 2005. Specificity at the end of the tunnel: understanding substrate length discrimination by the AlkB alkane hydroxylase. *J Bacteriol* 187:19–22. <http://dx.doi.org/10.1128/JB.187.1.19-22.2005>.
- Grant C, Deszcz D, Wei Y-C, Martínez-Torres RJ, Morris P, Folliard T, Sreenivasan R, Ward J, Dalby P, Woodley JM, Baganz F. 2014. Identification and use of an alkane transporter plug-in for applications in biocatalysis and whole-cell biosensing of alkanes. *Sci Rep* 4:5844.
- Schrewe M, Magnusson AO, Willrodt C, Bühler B, Schmid A. 2011. Kinetic analysis of terminal and unactivated C-H bond oxyfunctionalization in fatty acid methyl esters by monooxygenase-based whole-cell biocatalysis. *Adv Synth Catal* 353:3485–3495. <http://dx.doi.org/10.1002/adsc.201100440>.
- Julsing MK, Schrewe M, Cornelissen S, Hermann I, Schmid A, Bühler B. 2012. Outer membrane protein AlkL boosts biocatalytic oxyfunctionalization of hydrophobic substrates in *Escherichia coli*. *Appl Environ Microbiol* 78:5724–5733. <http://dx.doi.org/10.1128/AEM.00949-12>.
- Schrewe M, Julsing MK, Lange K, Czarnotta E, Schmid A, Bühler B. 2014. Reaction and catalyst engineering to exploit kinetically controlled whole-cell multistep biocatalysis for terminal FAME oxyfunctionalization. *Biotechnol Bioeng* 111:1820–1830. <http://dx.doi.org/10.1002/bit.25248>.
- Desbois A, Smith V. 2010. Antibacterial free fatty acids: activities, mechanisms of action and biotechnological potential. *Appl Microbiol Biotechnol* 85:1629–1642. <http://dx.doi.org/10.1007/s00253-009-2355-3>.
- Maloy SR, Ginsburgh CL, Simons RW, Nunn WD. 1981. Transport of long and medium chain fatty acids by *Escherichia coli* K12. *J Biol Chem* 256:3735–3742.
- Fay JP, Farias RN. 1975. The inhibitory action of fatty acids on the growth of *Escherichia coli*. *Microbiology* 91:233–240.
- Royce L, Liu P, Stebbins M, Hanson B, Jarboe L. 2013. The damaging effects of short chain fatty acids on *Escherichia coli* membranes. *Appl Microbiol Biotechnol* 97:8317–8327. <http://dx.doi.org/10.1007/s00253-013-5113-5>.
- Bühler B, Bollhalder I, Hauer B, Witholt B, Schmid A. 2003. Use of the two-liquid phase concept to exploit kinetically controlled multistep biocatalysis. *Biotechnol Bioeng* 81:683–694. <http://dx.doi.org/10.1002/bit.10512>.
- Alonso H, Kleifeld O, Yeheskel A, Ong Poh C, Liu Yu C, Stok Jeanette E, De Voss James J, Roujeinikova A. 2014. Structural and mechanistic insight into alkane hydroxylation by *Pseudomonas putida* AlkB. *Biochem J* 460:283–293. <http://dx.doi.org/10.1042/BJ20131648>.
- Eggink G, Lageveen RG, Altenburg B, Witholt B. 1987. Controlled and functional expression of the *Pseudomonas oleovorans* alkane utilizing system in *Pseudomonas putida* and *Escherichia coli*. *J Biol Chem* 262:17712–17718.
- de Bont JAM. 1998. Solvent-tolerant bacteria in biocatalysis. *Trends Biotechnol* 16:493–499. [http://dx.doi.org/10.1016/S0167-7799\(98\)01234-7](http://dx.doi.org/10.1016/S0167-7799(98)01234-7).