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Acyltransferases

Bacterial Acyltransferases as an Alternative for Lipase-Catalyzed Acylation for the Production of Oleochemicals and Fuels

*Tim Stöveken and Alexander Steinbüchel**

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Bacterial acyltransferases are a new class of enzymes, and the first member was identified as WS/DGAT in Acinetobacter baylyi *ADP1. Their unspecificity have been used in several biotechnological applications for lipid modification, a field that has been dominated by the use of lipases. Examples are the biosynthesis of jojoba-like wax esters and fatty-acid ethyl esters. In addition, these enzymes are also capable of synthesizing acylthioesters. Acyloxoesters and acylthioesters can thus be produced in vivo by whole-cell fermentations rather than in vitro in an enzyme reactor. In this Minireview, we focus on the biotechnological utilization of acyltransferases for the production of modified lipids from renewable resources.*

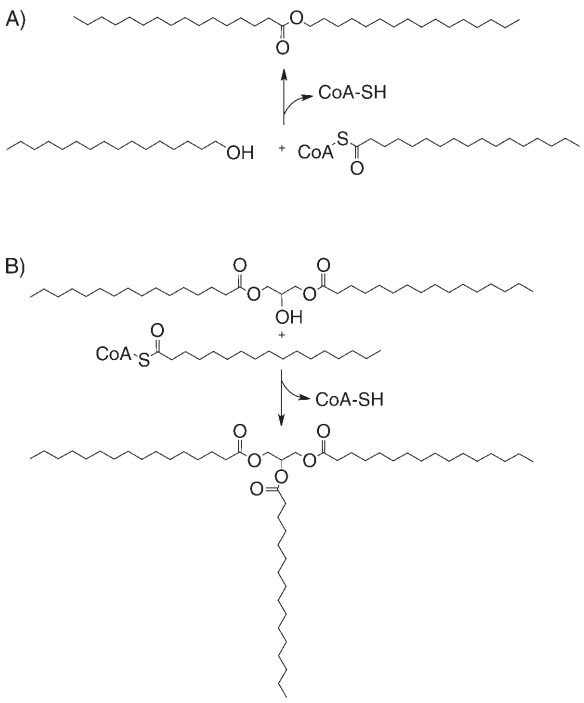
1. Introduction

In prokaryotes, the by far most dominant and best investigated storage compounds are polyhydroxyalkanoic acids (PHA).[1,2] They represent a very diverse class of storage polyesters comprising more than 150 different hydroxyalkanoic acids. Recently, the scientific and biotechnological interest has grown for other intracellular storage compounds, such as cyanophycin, triacylglycerols (TAGs), and wax esters (WEs).[3] TAGs and WEs occur as carbon- and energy-storage compounds in bacteria. WEs in particular are frequently found in the genus *Acinetobacter*.[4–7] Sporadically, WE accumulation has also been described for members of the genera *Moraxella* and *Micrococcus*.[8,9] Quite recently, more and more examples for WE-storing bacteria were found in the group of hydrocarbonoclastic bacteria, such as *Marinobacter hydrocarbonoclasticus*, *Alcanivorax jadensis*, *Alkanivorax borkumensis*, and *Thalassolituus oleivorans*.[10,11] These oil-degrading bacteria are specialized in hydrocarbon degradation.[10–12] One peculiarity of these bacteria, and especially of *A. borkumensis*, is their ability to withstand extended starvation periods, and to multiply very rapidly in oil-polluted water. It is believed that the accumulation of storage lipids is one possible adaptation to withstand such unfavorable conditions.[10,13] TAG accumulation is frequently described for the group of actinomycetes, such as *Mycobacterium, Streptomyces, Rhodococcus*, and *Nocardia.* The stored TAGs serve mainly as reserve compounds, but an involvement in the regulation of membrane fluidity, or their function as a sink for reducing equivalents is also proposed.[14] Another interesting aspect of TAG metabolism is the ability of *M. tuberculosis* to accumulate TAGs, either under dormancy and virulence conditions. The probable influence of TAG metabolism in the pathogenesis of this bacterium has been discussed.[15,16]

In eukaryotes, different specialized enzyme classes are responsible for synthesis of TAGs and WEs.[17] Only recently, a new class of acyltransferases was identified in bacteria. They catalyze the last step of either TAG or WE biosynthesis (Scheme 1). Because of their physiological reactions, they were termed WE synthase/acyl coenzymeA:diacylglycerol acyltransferase (WS/DGAT; CoA = coenzyme A).[18,19]

Oleochemicals are of relevance to the chemical industry. In particular, WEs have many possible applications, for example as lubricants and food additives, but they are also of interest in medical and cosmetic applications. The first biotechnological processes utilizing the WS/DGAT have been developed, and will be discussed in the following sections. Today, WEs are mainly produced chemically or by employing immobilized lipases.[20] However, lipase catalysis requires the chemical production of fatty alcohols as substrates. In contrast, employing WS/DGAT allows WE synthesis from

[*] Dipl.-Biol. T. Stöveken, Prof. Dr. A. Steinbüchel
Institut für Molekulare Mikrobiologie und Biotechnologie
Westfälische Wilhelms-Universität Münster
Corrensstr. 3, 48149 Münster (Germany)
Fax: (+49) 251-833-8388
E-mail: steinbu@uni-muenster.de
Homepage: http://mibi1.uni-muenster.de/



Scheme 1. In vivo reactions catalyzed by bacterial acyltransferases of the WS/DGAT familiy. A) WE synthase reaction; B) diacylglycerol acyltransferase reaction.

cheap renewable resources, such as fatty acids or carbohydrates.[21–23]

2. WS/DGAT: A New Class of Acyltransferases

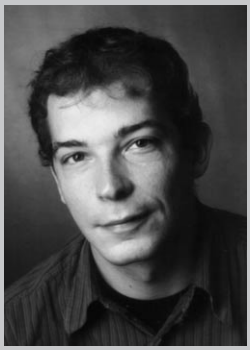
In eukaryotic organisms, several acyltransferases are known which catalyze the transfer of long-chain acyl residues. These are members of DGAT1 and DGAT2 family, the phospholipid:DAG acyltransferases, which mediate TAG synthesis, or the coenzyme A-dependent WE synthase from jojoba (*Simmondsia chinensis*).[17,24,25] Detailed characterizations have only been carried out for the WS from jojoba and a diacylglycerol acyltransferase from *Mortierella ramanniana* var. *angulispora*.[25,26]

Several bacteria that store lipids have been described, but little is known about the participating enzymes. The first bacterial long-chain acyltransferase that catalyses the last step of TAG and WE metabolism was detected in 2003 in *A. baylyi* ADP1 (formerly known as *A. calcoaceticus* ADP1), and is encoded by the *atfA* gene.[18] It was described as a bifunctional enzyme, catalyzing both the acyl coenzyme A-dependent acylation of diacylglycerides and of fatty alcohols. This novel enzyme did not show homologies to any of the known eukaryotic DAG acyltransferases or to the jojoba WE synthase. Furthermore, no homology to known genes in the databases could be observed.[18] In contrast to eukaryotic WS and DGAT enzymes, which are exclusively membrane proteins, the WS/DGAT is rather amphiphilic, and is only loosely attached to the membrane by electrostatic interactions.[19,25–29]

At about the same time, another family of long-chain acyltransferases was described. These so called polyketide-associated proteins (Pap) were almost exclusively found in mycobacteria, in addition to the above-mentioned WS/DGAT, and only one homologue was described in *Streptomyces coelicolor*.[30] Although these two enzyme families possess some physiological similarities (discussed in Section 4), they do not share sequence homologies to each other. The occurrence of two independent acyltransferases in mycobacteria further underlines the importance of lipid-synthesizing or modifying enzymes for these bacteria.

3. Catalytic Potential of Bacterial Acyltransferases

Interest in acyltransferases has increased because of its extraordinarily broad substrate range.[19] The WS/DGAT from *A. baylyi* ADP1 turned out to be an almost general acyltransferase. WS/DGAT accepts linear alcohols ranging from ethanol to triacontanol. The highest activities were measured for medium-chain-length fatty alcohols, from tetradecanol to octadecanol. Activities with ethanol or butanol were low, but these alcohols were also accepted as substrates. For long-chain alcohols larger than dodecanol, activity decreases with increasing chain length, but even triacontanol was accepted as substrate. The specificity for the other substrate of the WS reaction, the acyl-CoA thioesters, was only analyzed for thioesters having carbon chain lengths

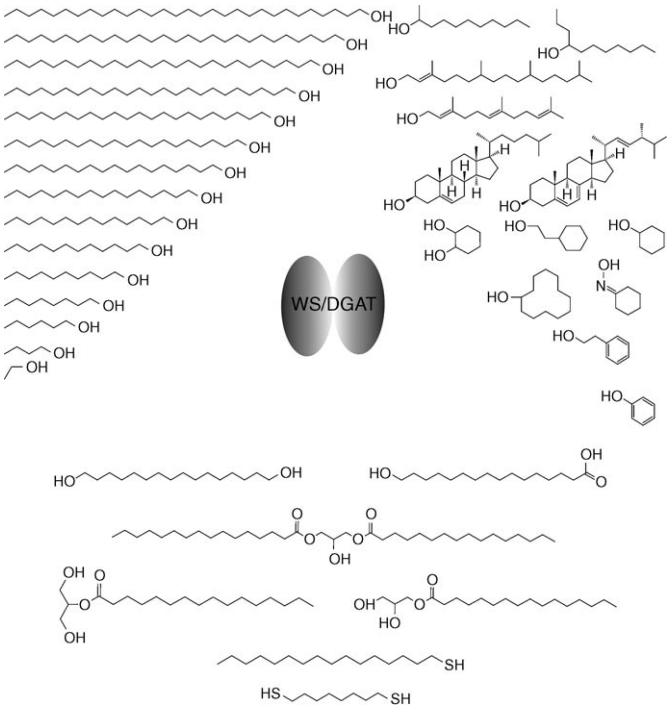


Tim Stöveken, born in 1975, studied biology at the University of Münster, and completed his diploma research project in Prof. Steinbüchel's group in 2003. He was working on his PhD under Prof. Steinbüchel, and investigated the biochemical characteristics and biotechnological applicability of bacterial acyltransferases.

Alexander Steinbüchel was born in 1953, and studied Biology at the University of Göttingen. After receiving his PhD (Prof. Schlegel, 1983), he spent one year at the Rockefeller University (New York) with Prof. DeDuve. Returning to Göttingen, he completed his habilitation in 1991. He has been Professor of Microbiology and Director of the Institute for Microbiology at the University of Münster since 1994. He was awarded the Philip Morris research prize in 1992. His research interests include the metabolism and biotechnological production of polyhydroxyalkanoates, and the biosynthesis and biotechnological production of polyamides.

for the acyl moieties from C_2 up to C_{20}. All these investigated acyl-CoA substrates served efficiently as donors for acyl moieties, with the highest activities obtained for carbon chain lengths in a range of C_{14} to C_{18}. Comparing 1-, 2-, and 3-decanol, the primary alcohol is preferred, but the secondary alcohols are also accepted as substrates.

Not only wax monoesters can be synthesized by WS/DGAT. The formation of wax diesters was demonstrated by WS/DGAT-mediated double acylation of hexadecanediol. Besides the natural linear alcohol substrates, the enzyme also acylates cyclic, aliphatic, and even aromatic alcohols. Even enzyme-catalyzed acylation of cyclohexanone oxime was demonstrated. WS/DGAT-mediated acylation of steroids was demonstrated for cholesterol and for ergosterol if expressed in *E. coli*. Expression of *atfA*, in a quadruple mutant of *Saccharomyces cerevisiae* which was no longer capable of producing any TAG or steryl esters, restored the capability of TAG accumulation, whereas formation of steryl esters could not be restored.[31] Furthermore, not only the formation of oxoesters of long-chain fatty alcohols and long-chain fatty acids is catalyzed by the enzyme, but also the formation of corresponding thio WEs.[22] The other reaction catalyzed by WS/DGAT in vivo is the acylation of diacylglycerols. For these substrates, a clear preference for terminal primary hydroxy groups, especially at the *sn*-3 position, was also observed. Interestingly, monoacylglycerols also served as substrates, indicating that DAG synthesis may not exclusively occur via the Kennedy pathway by sequential acylation of glycerol-3-phosphate, but also by a possible involvement of WS/DGAT in the formation of diacylglycerols in vivo as well. Besides these substrates, various mono-, di-, and oligosaccharides, such as glucose, fructose, galactose, cellulose, and agarose, were tested as acyl acceptors. Under reaction conditions used for the previously described substrates, no WS/DGAT-mediated acylation of these molecules was detectable. Further examples for compounds for which no acylation could be demonstrated were L-tyrosin, D/L-lactic acid, ferulic acid, and 1-naphthol.[49] This extraordinarily broad substrate spectrum leads to the presumption that only the provision of hydrophobic substrates by the host limits the products synthesized by this enzyme (Scheme 2).



Scheme 2. Overview of hydroxy-group-containing substrates for bacterial acyltransferases belonging to the WS/DGAT family.

Further bacterial acyltransferases of this type have been identified after the description of the *A. baylyi* ADP1 WS/DGAT. Most of the 15 WS/DGAT homologous proteins from *M. tuberculosis* H37Rv showed both DGAT and WS activity. As anticipated, all enzymes showed clearly higher DGAT activity than WS activity; this is consistent with the occurrence of TAGs in these bacteria, whereas WEs are not reported as storage compounds in mycobacteria.[16]

In the genome of *A. borkumensis* SK2, two acyltransferase homologous genes were identified (*atfA1* and *atfA2*). These acyltransferases are also active over a broad substrate range, as it was previously demonstrated for the WS/DGAT from *A. baylii* ADP1. AtfA1 accepted many linear alcohols with a range of chain lengths; the highest activity was found for decanol. Butanol was also very effectively acylated, whereas tetracosanol reacted only at a very low rate. This enzyme was highly active with phenolic or cyclic alcohols, such as cyclohexanol or cyclohexylethanol. AtfA2 conversion of linear alcohols is comparable to for AtfA1. The highest activity was found with decanol and butanol, whereas tetracosanol was poorly accepted. The specificity of AtfA2 towards cyclic and aromatic alcohols was still higher than that determined for AtfA1. Dramatic differences were observed for different palmitoylglycerol substrates. Noticeably, DGAT activity was only demonstrated for AtfA1, whereas AtfA2 did not show any significant activity with dipalmitoylglycerol (DPG). AtfA2 acylated 1-, 2-, and 3-monopalmitoylglycerol very efficiently, whereas AtfA1 showed a clear preference for 1-monopalmitoylglycerol. The accumulation of WEs and TAGs in vivo in *A. borkumensis* SK2 is assumed to be mostly due to AtfA1 activity, as inactivation of *atfA2* did not lead to a change in the quantity or composition of the storage lipids.[10]

In *Marinobacter hydrocarbonoclasticus* DSM8798, four WS/DGAT homologous genes, *ws1*, *ws2*, *ws3*, and *ws4* were identified. For two, WS1 and WS2, substrate profiles were described; TAG formation was only observed for WS1. As for other acyltransferases investigated, the specificity of WS2 towards linear primary alcohols of different chain length is low, whereas a preference for shorter alcohols like decanol and dodecanol was observed. Highest activity was reported with palmitoyl-CoA as acyl-CoA component; however, acyl-CoAs with shorter or longer chain length of the acyl moiety are accepted to a smaller extent. The activity level and substrate selectivity of WS1 from *M. hydrocarbonoclasticus* were comparable to those of the *A. baylyi* enzyme, to which it has an amino acid sequence identity of 45%. One peculiarity of *M. hydrocarbonoclasticus* is the accumulation of isoprenoid WEs during growth on phytol; both WS1 and WS2 are capable of synthesizing isorenoid WEs.[11,32]

A comparison of the substrate preferences of the acyltransferases that have been characterized and described revealed that all of them are potent acyltransferases, exhibiting very broad, but also substantially different substrate ranges. Therefore, investigation of further bacterial acyltransferases will most probably also lead to enzymes with biotechnologically interesting substrate selectivities. Examples are mono- and diacylglycerols, which are used as high valuable emulsifiers. Another example would be the formation of sugar esters, leading to biocompatible surfactants. Furthermore, the esterification of bioactive molecules, such as vitamins, steroids, or secondary metabolites of plants, may be performed employing bacterial acyltransferases if these compounds possess hydroxy or thiol groups. Such an approach could be used in the production of molecules which retain their bioactivity but have altered properties, such as diffusion, solubility, or stability. Taking into account the diverse environments and carbon sources of the previously presented bacteria, the differences in substrate selectivity of the acyltransferases described so far is probably a result of adaptation towards the available carbon sources, as they determine the composition of the WE.[19,10,11]

4. The Proposed Motif of the Active Site

Multiple sequence alignments identified the highly conserved domain HHXXXDG in WS/DGATs as part of the catalytic center. Almost all WS/DGAT homologues investigated possess this motif. In most of the acyltransferases that are known to be active, this motif was not or only marginally modified.[10,11,16,18,19] The HHXXXDG motif (NCBI Conserved Domain Data Base accession pfam00668) is the catalytic active site in nonribosomal peptide synthases (NRPS).[33]

Another group of enzymes possessing this motif as their active site are the polyketide-associated proteins (Pap). The reactions catalyzed by these enzymes are very similar to that of the WS/DGATs. The substrate range for PapA5 from *M. tuberculosis* comprises alcohols of different chain length as well as secondary or tertiary alcohols.[30] Two further Pap enzymes, PapA2 and PapA1 from *M. tuberculosis*, catalyze the subsequent palmitoylation of trehalose-2-sulfate.[34] Although structural information for some of these enzymes is available, low sequence identity between them prevents a comparison of structural homology.[35,36] Another related and well characterized enzyme is chloramphenicol acetyltransferase. As for the Pap enzymes, no structure comparisons can be carried out owing to a lack of similarity between sequences. Although the sequence are different, it can be assumed that the catalytic mechanism of this enzyme class is closely related.

Replacement of His132 or His133 by leucine of the *A. baylyi* ADP1 acyltransferase by site-specific mutagenesis lowered WS activity dramatically, whereas mutations of Asp137 and Gly138 had no significant effect on activity. Complete loss of activity of a mutant in which both histidine residues are replaced by leucine indicated that the loss of one histidine is complemented to some extent by the adjacent histidine residue.[49] Histidine acts as a base, deprotonating the hydroxy group of the fatty alcohol or the DAG. The resulting oxoanion acts as a nucleophile, with the carbon atom of the thioester bond of acyl-CoA, resulting in the formation of the oxoester bond of the WE or TAG. The protonated histidine is then regenerated by transferring the proton to CoA-S^-.

5. In-Vivo Production of Fatty-Acid Derivatives in Recombinant Bacteria

In industrial processes, lipases and starch-hydrolyzing enzymes are among the most important and most frequently used enzymes. They are used as biocatalyst for hydrolysis, esterification, transesterification, and alcoholysis.[37,38] Most of the lipase-catalyzed processes are performed in organic solvents in the absence of water. In contrast, bacterial acyltransferases perform the biotechnologically relevant synthesis reactions in aqueous systems. However, the enzymes are only active with CoA-activated fatty acids. Therefore, in-vitro applications as biocatalyst in biotechnological processes in analogy to lipase catalyzed esterifications will not be economic. However, this dependence on CoA esters may provide an advantage as well: In contrast to lipases, acylation is the native reaction catalyzed by WS/DGAT under physiological conditions; it can therefore be carried out in vivo. The only prerequisite is the provision of substrates in the respective host.

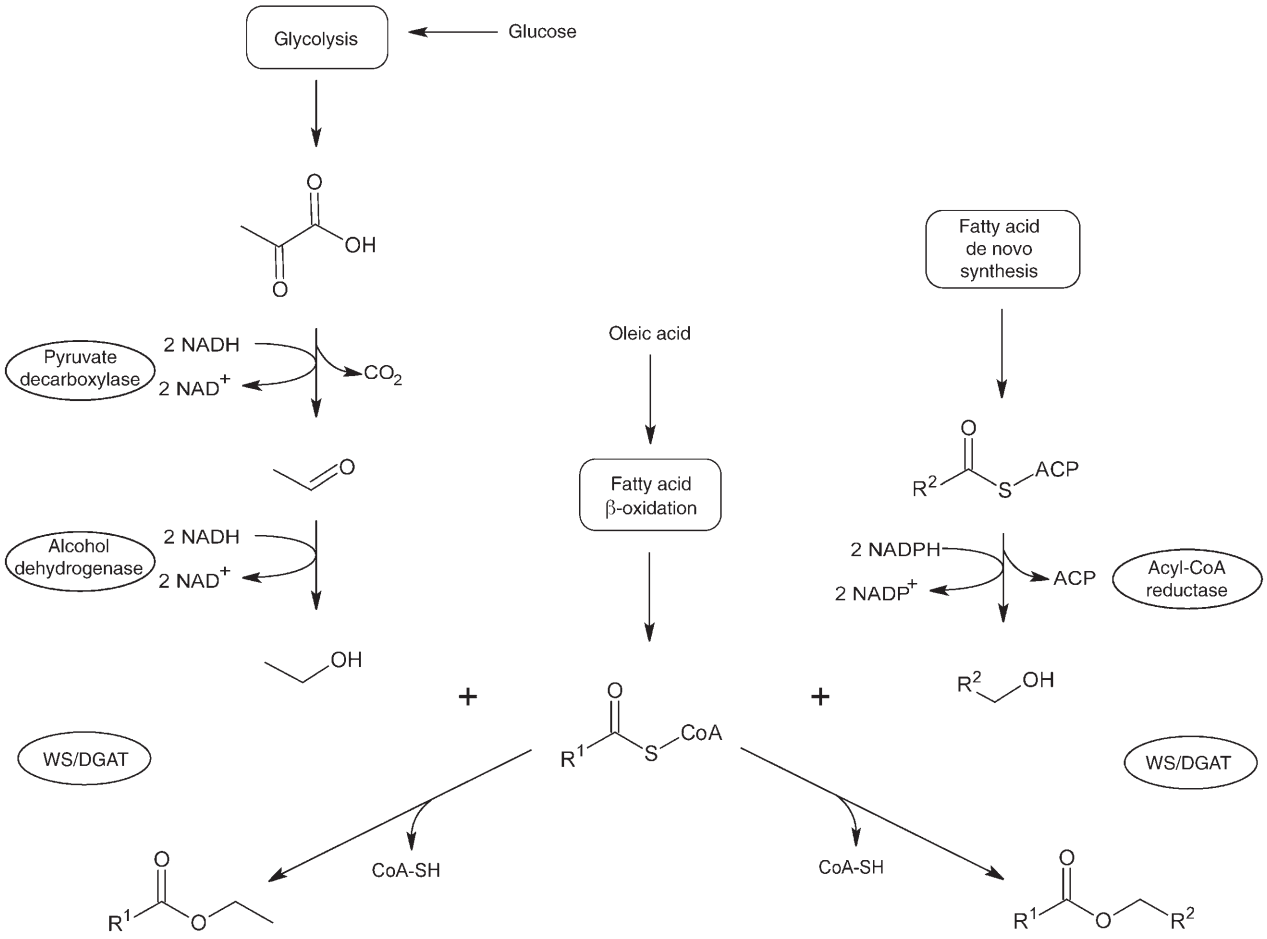
The first production of WE derivatives in vivo was described for an *A. baylyi* ADP1 mutant that was lacking an acyl-CoA reductase (Acr1), and catalyzed the first step in WE biosynthesis.[18,39,40] Thus, this strain was not able to produce long-chain aldehydes that would normally be reduced to fatty alcohols. Therefore, the desired alcohol constituent can be provided externally to the cells together with the medium, and is then acylated with endogenous acyl-CoA to the corresponding WE. This attempt was first used for in-vivo production of wax diesters of 1,16-hexadecanediol esterified with palmitic and oleic acid, but also for synthesis of thio and dithio WEs, such as palmitic acid hexadecyl thioester or 1,8-*S*-dipalmitoyloctadecanedithiol, respectively.[22,41] Thioesters are of interest as they can be used as activated esters for synthesis of peptides or macrocyclic antibiotics.[42–44] Further optimization of these processes will be necessary for technical applications, however, the feasibility of WS/DGAT catalysis has been demonstrated. Furthermore, owing to the low substrate specificity of the WS/DGATs, it is likely that this biocatalyst can be used for the generation of a multitude of tailormade WEs.

Another approach for the synthesis in vivo was in recombinant *E. coli*. This bacterium is not able to produce WEs naturally. Heterologous expression of WS/DGAT alone does not result in WE formation. The bottleneck in this case is the lack of a fatty aldehyde reductase to catalyze the second step in WE synthesis. As no bacterial fatty aldehyde reductase has been described in literature, Kalscheuer et al. employed an acyl-CoA reductase from *Simmondsia chinensis*, that also shows fatty aldehyde reductase activity,[45] and WEs formed (Scheme 3).[21] Besides accumulation of long chain WEs, such as palmitoyl oleate, oleyl oleate, or palmitoyl palmitate,

formation of fatty acid butyl esters was observed. The occurrence of the latter was ascribed to the presence of trace amounts of 1-butanol in one component of the medium. This further underlines the high degree of unspecificity of this enzyme.[21]

Fatty acid methyl esters (FAMEs) and fatty acid ethyl esters (FAEEs) are possible substitutes for petroleum-based diesel. The demand for alternative energy sources has rapidly grown during the last few years. FAMEs are usually derived from oilseed crops by alkali-catalyzed transesterification of TAG, and are therefore referred to as biodiesel. The major advantage of biodiesel is that it originates primarily from renewable resources and that its CO_2 cycle is balanced, although the methanol used for transesterification is mainly produced from syngas and natural gas. Furthermore, biodiesel can replace conventional diesel without the need to modify machinery extensively. A disadvantage is that the acreage for oil crops is limited and almost exhausted.

As described above, the WS/DGAT also has the capability to accept ethanol as a substrate, resulting in the formation of FAEE. In 2006, we presented the "microdiesel" process, which employs the WS/DGAT.[23] For this process, a recombinant *E. coli* strain was engineered that contains the genes *pdc* (pyruvate decarboxylase) and *adhB* (alcohol dehydrogenase) for ethanol production from *Zymomonas mobilis*, and the *atfA* gene from *A. baylyi* ADP1 on one plasmid (pMicrodiesel). Biosynthesis of FAEE by recombinant *E. coli* is summarized in Scheme 3. In this first description of a biotechnological process for FAEE production in bacteria, intracellular accumulation of biodiesel, referred to as microdiesel, was achieved (Scheme 3). One of the major bottlenecks for this process is the relatively low reaction rate of the acyltransferase with ethanol as substrate.[19] Keeping in mind the large number of recently discovered bacterial acyltransferases, it is very likely that an enzyme better suited for this process will be found among them. Furthermore, directed evolution of acyltransferases may result in selection of enzymes with higher specificity towards ethanol.[39] Another important aspect of microdiesel fuel production is the possible development of processes that are not dependant on oilseed crops, but can utilize bulk materials, such as sugars, or better starch, cellulose, and hemicellulose. This will not only significantly extend the substrate range that can be used for fuel production, but will also use resources that do not compete with food and feed production. Microdiesel will certainly not substitute other biofuels in the near future, but it has the



Scheme 3. Recombinant WE and FAEE biosynthesis in *E. coli*. Modular system for the biosynthesis of tailored WEs. The choice of alcohol-generating reaction leads to the biosynthesis of the designated ester in vivo from renewable resources. ACP = acyl carrier protein, NADPH = nicotinamide adenine dinucleotide phosphate.

perspective to become part of a new sustainable energy mix, replacing fossil resources.

6. Future Applications

Since the first description of a bacterial acyltransferase, much effort was undertaken to establish in-vivo production of WEs and other oleochemicals from renewable resources using this enzyme. The principal applicability has been demonstrated for several products.[21–23,41] The extraordinarily broad substrate range of the enzyme makes it very likely that the biotechnological production of further esters will be achieved. For all future applications it will be essential to identify the enzymes that are best adapted to the process in terms of substrate selectivity and physical properties. The large number of bacterial acyltransferases that are found in the databases can be evaluated to find the ideal candidate. Another possibility is the methodologies of directed protein evolution.[46–48] For this purpose, efficient screening systems, for example those based on Nile red, must be applied.[39]

As described in Section 5, *E. coli* is not the ideal host for either FAEE or WE production. Provision of CoA-activated fatty acids appears to be a bottleneck in the process and must be improved. The use of another expression host may help to solve this problem. TAG- or WE-accumulating strains, such as *R. opacus* or *A. baylyi* ADP1, have a naturally high acyl CoA content. In this regard, *R. opacus* may be of interest, as this bacterium is able to store amounts of TAG up to 90% of its cellular dry weight, and can be grown to high cell densities.[14]

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