View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connector

FEBS Letters 585 (2011) 3538–3543

FEBS Letters
journal homepage: www.FEBSLetters.org

A prokaryotic acyl-CoA reductase performing reduction of fatty acyl-CoA to fatty alcohol

Per Hofvander [a,*], Thuy T.P. Doan [a,b], Mats Hamberg [c]

[a] Department of Plant Breeding and Biotechnology, Swedish University of Agricultural Sciences, P.O. Box 101, SE-230 53 Alnarp, Sweden
[b] Department of Biology, Nong Lam University, Ho Chi Minh City, Viet Nam
[c] Division of Physiological Chemistry II, Department of Medical Biochemistry and Biophysics, Karolinska Institutet, 171 77 Stockholm, Sweden

ARTICLE INFO

Article history:
Received 8 September 2011
Revised 30 September 2011
Accepted 10 October 2011
Available online 19 October 2011

Edited by Peter Brzezinski

Keywords:
Acyl-CoA reductase
Aldehyde reductase
Fatty alcohol
Fatty aldehyde
Marinobacter aquaeolei

ABSTRACT

The reduction of acyl-CoA or acyl-ACP to fatty alcohol occurs via a fatty aldehyde intermediate. In prokaryotes this reaction is thought to be performed by separate enzymes for each reduction step while in eukaryotes these reactions are performed by a single enzyme without the release of the intermediate fatty aldehyde. However, here we report that a purified fatty acyl reductase from *Marinobacter aquaeolei* VT8, evolutionarily related to the fatty acyl reductases in eukaryotes, catalysed both reduction steps. Thus, there are at least two pathways existing among prokaryotes for the reduction of activated acyl substrates to fatty alcohol. The *Marinobacter* fatty acyl reductase studied has a wide substrate range in comparison to what can be found among enzymes so far studied in eukaryotes.
© 2011 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

1. Introduction

Long-chain primary fatty alcohols are commonly found in their free form or as a component of wax esters in plants, insects and mammals. Often these compounds are building blocks of boundaries as epicuticular wax layers towards the outside environment. Fatty alcohols are synthesised via reduction of acyl-CoA or acyl-ACP and can be further esterified to acyl-CoA resulting in wax esters [1–4]. Wax esters are neutral lipids that are also produced in a few bacterial genera [5–7]. In bacteria, wax esters are most likely serving as energy reserves and are often found in marine environments and associated with species that can degrade crude oil or in marine sediments [8–10]. It can be anticipated that medium and long-chain hydrocarbons from oil spills and decaying organic matter in this way can be utilized for accumulating dense energy reserves.

The bacterial pathway leading to wax ester production has mainly been studied in the genus *Acinetobacter* which synthesise and store wax esters under nitrogen-limiting conditions [11–14]. The medium and long-chain fatty alcohols used for the biosynthesis of wax esters in prokaryotes are believed to be produced via a two-step reduction of either acyl-CoA or acyl-ACP to the corresponding fatty alcohol via an intermediate fatty aldehyde [15,16]. This is contrasting to eukaryotes where a single enzyme can perform both reduction steps without releasing the intermediate fatty aldehyde [4]. A study of mutants of *Acinetobacter calcoaceticus* deficient in wax ester biosynthesis revealed a mutant impaired in the first reduction step leading to fatty aldehyde [14]. Expression of the corresponding gene, *acr1*, in *Escherichia coli* and assays of extracts resulted in aldehyde production. Although production of alcohol could also be observed this was attributed to endogenous activity of *E. coli* that could further reduce the aldehyde to fatty alcohol. A mutation in the second reduction step from aldehyde to alcohol was not identified.

In a recent paper a putative gene sequence was identified in *Marinobacter aquaeolei* VT8 which corresponding protein product was shown to reduce added aldehyde substrates to fatty alcohol and this enzyme was termed FALDR (Fatty Aldehyde Reductase) [17].

We have expressed and purified hypothetical protein Maqu_2220 of *M. aquaeolei* VT8, which is identical with the *M. aquaeolei* VT8 FALDR enzyme. In this report we show that the *Marinobacter* enzyme can, in addition to reduce fatty aldehydes, also reduce acyl-CoAs and acyl-ACP to fatty alcohols and thus perform both reduction steps, thereby establishing that also in prokaryotes both these reductions can be performed in an NADPH-dependent manner by a single enzyme. Thus, contrary to what previously has been anticipated in the literature, there are at least two pathways available among prokaryotes for the reduction of activated acyl chains to fatty alcohols. The *M. aquaeolei* VT8 fatty acyl reductase

* Corresponding author. Fax: +46 40 415519.
E-mail address: per.hofvander@slu.se (P. Hofvander).

0014-5793/$36.00 © 2011 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.febslet.2011.10.016

was furthermore shown to be active with acyl-CoA as well as acyl-ACP and with substrates of different chain lengths and modifications.

2. Materials and methods

2.1. Vector construction

DNA with sequence corresponding to GenBank accession YP_959486 (hypothetical protein Maqu_2220 [*M. aquaeolei* VT8]) was ordered synthetically (Eurofins). The synthetic gene sequence was optimised for *E. coli* expression at ordering. The synthetic gene was amplified in a two-step procedure using Phusion™ High-Fidelity PCR (Finnzymes) essentially according to the manufacturer's instructions. Primers for amplification were designed to introduce *att*B-sites for Gateway® cloning as well as a sequence which upon fusion with the gene sequence introduced a TEV protease cleavage site into a resulting expressed protein (Appendix A). The purified PCR fragment was introduced into pDONR™221 using BP Clonase™ II (Invitrogen) resulting in plasmid, pEntry-MarFAR. After purification and sequence verification the modified gene was further introduced into pDEST-HisMBP [18] using LR Clonase™ II Plus (Invitrogen) resulting in plasmid pHisMBPMarFAR.

2.2. Heterologous protein expression and purification

Plasmid pHisMBPMarFAR was transformed to *E. coli* strain Rosetta (DE3) (Novagen) for heterologous protein expression.

A single colony was used to inoculate 40 ml of Luria-Bertani broth (LB) supplemented with 50 μg/ml carbenicillin and 34 μg/ml chloramphenicol. The culture was incubated overnight at 37 °C with agitation. A volume of the overnight culture was added to 800 ml antibiotic supplemented LB media to a final OD 600 of 0.1 and then incubated at 37 °C with 240 rpm until OD 600 was 0.5–0.8 (90–100 min). The culture was transferred to room temperature for 30 min and then protein expression was induced by the addition of Isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM. The induced culture was allowed to grow for another 4–6 h at room temperature under agitation, 240 rpm. Bacteria were pelleted by centrifugation at 5000×*g* for 20 min at 4 °C. The bacterial pellet was flash frozen in liquid nitrogen and stored at −80 °C for subsequent processing.

The bacterial pellet was resuspended in 16 ml ice cold 100 mM phosphate buffer, pH 7.0 containing 40 mM imidazole, 10% (v/v) glycerol, 1× Complete Protease Inhibitor (Roche Applied Bioscience) and 16 μl Lysonase (Novagen). Ice cold 0.1 mm glass beads were added to the bacterial suspension at a ratio of 1:1 (w/v). The suspension was homogenized using a FastPrep™-24 (MP Biomedicals) at 4 m/s, using 3 pulses of 30 seconds with 2 min rest at 4 °C between pulses. The cell lysate was clarified by centrifugation at 4000×*g* for 10 min at 4 °C. MgCl2, ATP and KCl were added to 5 mM, 2 mM and 150 mM respectively. Subsequently the lysate was mixed and incubated on ice for 10 min. The lysate was then further clarified by centrifugation at 10 000×*g* for 10 min at 4 °C.

The supernatant was loaded on a pre-equilibrated 1 ml HisTrap™ HP column (GE Healthcare) with a flow rate of 1 ml/min using a BioLogic LP liquid chromatography system (Bio-Rad). The column was then washed with 100 mM phosphate buffer, pH 7.0 containing 40 mM imidazole and 10% (v/v) glycerol at a flow rate of 1 ml/min. Bound protein was eluted using a 20 ml linear gradient of 40–500 mM imidazole in 100 mM phosphate buffer, pH 7.0 with 10% (v/v) glycerol at a flow rate of 1 ml/min. Eluted proteins were collected in 0.5 ml fractions and subsequently aliquoted, flash frozen in liquid nitrogen and stored at −80 °C until further analysis and enzyme assays. Protein concentration of individual fractions was determined using BCA Protein Assay (Pierce). Protein integrity of individual fractions was determined using polyacrylamide gel electrophoresis (PAGE).

2.3. Substrate synthesis

[^{14}C]-labelled fatty acyl-chains were either obtained from commercial sources or synthesised.

[1-^{14}C]ricinoleic (12-hydroxy-octadec-9-enoic) acid was synthesized biochemically from [1-^{14}C]oleic acid by castor bean microsomes according to Bafor et al. [19].

[1-^{14}C]-labelled straight-chain fatty acids were prepared starting with the next lower fatty alcohols, which were brominated by refluxing with 48% HBr in the presence of tetrabutylammonium bromide as phase transfer catalyst. The bromides were dissolved in dry dimethyl sulfoxide and treated with [^{14}C]-sodium cyanide at 65 °C for 22 h. Following alkaline hydrolysis and purification by reversed-phase HPLC, materials of >98% purity were obtained. The specific radioactivity as determined by mass spectrometry and liquid scintillation counting was 389 kBq/μmol.

Acyl-CoA subtrates were synthesised essentially according Sànchez et al. [20]. [^{14}C]palmitoyl-ACP was synthesised enzymatically from free acid and ACP using *E. coli* acyl-ACP synthetase (provided by Prof. John Ohlrogge, East Lansing, USA) according to Kuo and Ohlrogge [21].

2.4. Enzyme assay

Assays were performed in 50 μl of 100 mM phosphate buffer, pH 7.0 containing 10 mM NADPH, 50 μM [^{14}C]fatty acyl-CoA or –ACP and 3 mg/ml BSA using 0.45 μg of purified protein. Reactions were incubated at 30 °C for 30 min and then stopped by the addition of 10 μl glacial acetic acid (HAc) and immediately extracted with thorough vortexing using 375 μl methanol:chloroform 1:1 (v/v), 125 μl chloroform and 250 μl H_2O. After centrifugation at 6000×*g* for 2 min the chloroform phase was transferred to a fresh vial.

Extracted lipids were separated on TLC Silica gel 60 (Merck) using hexane:diethyl ether:HAc at 55:45:0.5 (v/v/v/) as a mobile phase. Radioactive emission was measured for up to 15 hours using electronic radiography (Instant Imager, Canberra Packard) with [^{14}C]oleyl alcohol (kindly provided by Bayer CropScience) of known specific activity applied on the TLC plate as a standard.

The identity of enzyme reaction products were determined by GC–MS (Appendix C).

2.5. Sequence analysis

Bacterial protein sequences homologous to *M. aquaeolei* VT8 accession YP_959486 were collected by conducting BLASTP against all assembled RefSeq genomes in the Microbes section at NCBI.

A phylogenetic tree of selected fatty acyl-CoA reductase amino acid sequences was assembled by using ClustalW-alignment and then the Neighbour-Joining algorithm with Bootstrapping of CLC DNA Workbench (CLC bio).

3. Results and discussion

3.1. Expression and purification of hypothetical protein Maqu_2220

Hypothetical protein Maqu_2220 of *M. aquaeolei* VT8 has the same amino acid sequence as NCBI reference sequence YP_959486. The corresponding ORF was produced synthetically and inserted into an *E. coli* expression system using Gateway™ cloning. In the chosen expression system Maqu_2220 was expressed as a fusion protein with the maltose binding protein (MBP) which has

been shown to enhance protein solubility in *E. coli* [22]. Further the MBP fusion tag has been shown beneficial for the solubility of Maqu_2220 and not influence activity of the resulting enzyme [17]. MBP can be used as an affinity tag but the used expression vector, pDEST-HisMBP, also provide an N-terminal His-tag fusion [18]. The His-tag was utilized for purification of the expressed protein which after elution using an imidazole gradient resulted in an essentially pure enzyme preparation (Appendix B).

3.2. Maqu_2220 reduces fatty acyl-CoA substrates to fatty alcohol

In a recent publication the protein corresponding to Maqu_2220 was determined to be an aldehyde reducing protein (FALDR) and thus presented as a 'missing link' in prokaryotic biosynthesis of wax esters [17]. However, we wanted to investigate whether this protein also could perform both reduction steps from fatty acyl-CoA to fatty alcohol as a fatty acyl-CoA reductase (FAR), thus eliminating the need for a separate acyl-CoA reduction to aldehyde performing step in *M. aquaeolei* VT8 (Fig. 1).

It was shown that acyl groups from various acyl-CoA substrates were efficiently converted into fatty alcohols by the purified protein. Saturated acyl-CoA substrates of carbon chain length 10–20 as well as oleoyl, erucoyl and ricinoleoyl groups were all, to various extents, converted to fatty alcohols (Figs. 2 and 3). Purified fractions of *E. coli* containing control plasmid, pET15b, did not display any activity with acyl-CoA substrate (results not shown). Of the tested substrates, Maqu_2220 had the highest activity in production of fatty alcohols with 18:1-CoA with a specific activity of 108.2 nmol/mg protein∗min followed by 20:0-CoA at 77.4 nmol/mg protein∗min, 18:0-CoA at 67.7 and 16:0-CoA at 43.0 nmol/mg protein∗min (Fig. 2). The addition of a C12-hydroxyl group to 18:cis-9 yielding ricinoleoyl-CoA, reduced the fatty alcohol production rate to 27.3 nmol/mg protein∗min. Shorter acyl chains than 16 carbon were less effectively utilized and with these substrates substantial amounts of both free fatty acids and fatty aldehyde were formed (Fig. 3). Free fatty acid and aldehyde is most likely released into the medium and not bound to the enzyme as enzyme activity is not reduced by intermediate product accumulation (results not shown). This suggests that although accepted as substrates, the full reaction does not take place efficiently but intermediate products are formed and released from the initial cleavage of the acyl-CoA substrate and after the first reduction step.

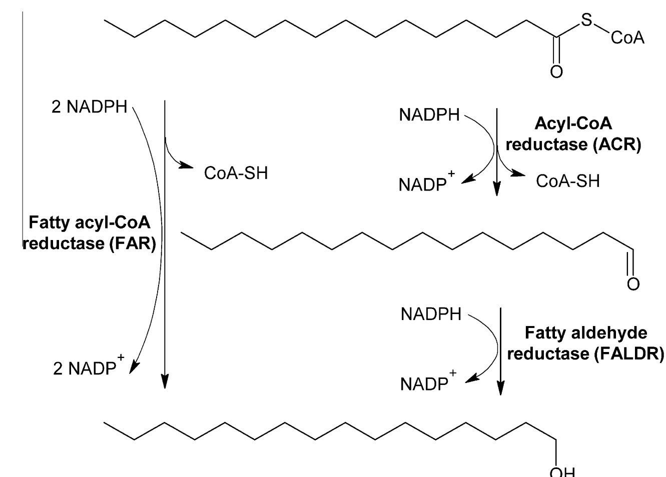

Fig. 1. Schematic view of fatty alcohol biosynthesis in eukaryotes via fatty acyl-CoA reductase (FAR) and in prokaryotes via the consecutive reduction reactions of acyl-CoA reductase (ACR) and fatty aldehyde reductase (FALDR).

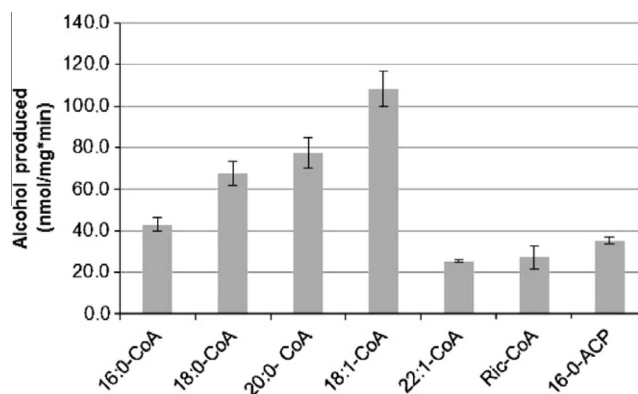

Fig. 2. Activity of the Maqu_2220 protein with different activated [^{14}C]-fatty acyl substrates. Bars represent rate of alcohol production in nmol/mg protein∗min over 30 min at 30 °C and are means of two independent experiments. Ric-CoA (ricinoleoyl-CoA)

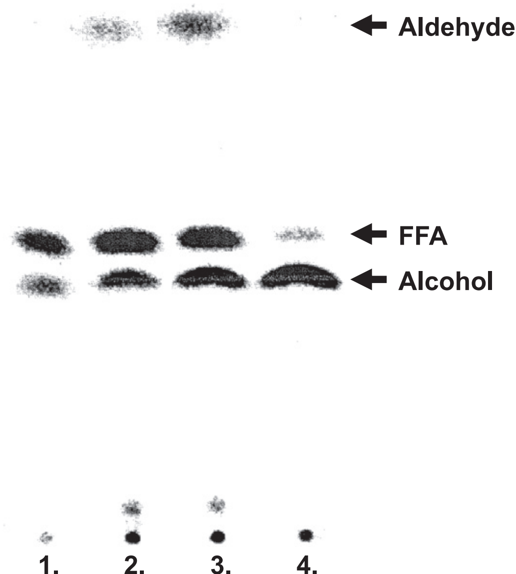

Fig. 3. Distribution of radioactive products on TLC-plate after incubation of the Maqu_2220 protein with [^{14}C]-fatty acyl-CoA substrates and subsequent separation. The identity of reaction products were established by GC–MS (Appendix C). Lane 1: 10:0, Lane 2: 12:0, Lane 3: 14:0, Lane 4: 16:0. FFA (free fatty acid)

Prokaryotic de novo fatty acid biosynthesis leads to formation of 16:0 and 18:0-ACP whereas the CoA activated acyl chains mainly are derived from oxidative reactions with medium and long chain hydrocarbons. Therefore 16:0-ACP was also tested as a substrate for Maqu_2220 and found to be well accepted, albeit with somewhat lower rate of alcohol production than the acyl-CoA substrate at the same concentration (Fig. 2). The difference in activity of Maqu_2220 with 16:0 activated by CoA or ACP was in fact much less than the difference in activity of 16:0-CoA to 14:0 and 18:0-CoA substrates. The minor difference in activity between 16:0-ACP and 16:0-CoA indicate that *M. aquaeolei* VT8 is capable of fatty alcohol production (with subsequent conversion to wax esters) from both de novo fatty synthesis as well as from exogenous carbon chains. In this context, it is interesting to note that *M. aquaeolei* VT8 was isolated from a sample acquired at the head of an offshore oil well in southern Vietnam and is a member of a genus which has been shown to degrade oil [23,24].

Thus it is shown that a prokaryotic genome can encode an enzyme with the same catalytic activity as eukaryotic fatty acyl-

Table 1
Accessions with a significant homology and similar size as Maqu_2220.

Accession	Strain	aa	Identity (%)
ZP_01892457	*Marinobacter algicola* DG893	512	78
ADP96574	*Marinobacter adhaerens* HP15	511	78
YP_436183	*Hahella chejunensis* KCTC 2396	505	55
ZP_01305629	*Oceanobacter* sp. RED65	514	44
YP_002433039	*Desulfatibacillum alkenivorans* AK-01	535	30

CoA reductases establishing this enzyme among FARs involved in primary alcohol and wax ester production.

3.3. Maqu_2220 homology to other putative prokaryotic proteins

Maqu_2220 represents a protein of 513 aa. An additional five putative bacterial FARs which are of a similar size (480-550 aa) and containing the same conserved domains as proven eukaryotic FARs can be found via BLAST of proteins in the fully sequenced Microbes section of NCBI. Accessions listed in Table 1, could be candidates for additional prokaryotic FARs catalysing both reduction steps of fatty acids to fatty alcohols. The bacterial species listed, mainly assigned to gammaproteobacteria, are quite closely related, which could mean that this type of genes and enzymes is very rare and confined to a small section of bacterial genera. Interestingly, among all the more than 1800 microbial genomes which can be subjected to BLASTP, all five genomes containing an ORF with significant homology are found in marine environments. There are other amino acid sequences found with homology to FAR which fall into two classes of 750–820 aa and 1470–1600 aa. They are essentially FAR enzymes with C-terminal extensions, although extensions are not related between the two classes indicating diverging functions of these two groups.

3.4. Maqu_2220 homology to eukaryotic proteins

The first gene encoding a FAR isolated and enzymatically proven was from jojoba (*Simmondsia chinensis*) [4]. Since that time several other genes coding for FARs have been isolated and biochemically characterised in heterologous systems [1,25–30]. Common to the now defined *M. aquaeolei* VT8 FAR and FARs of eukaryotic origin is a conserved domain (I/V/F)-X-(I/L/V)-T-G-X-T-G-F-L-(G/A) as part of a predicted Rossmann-fold which is suggested to be the NAD(P)H binding site [31]. In addition there is a motif, Y-X-X-X-K, corresponding to an active site of NADP-dependent enzymes and shown to be present in characterised FARs [32]. The *Marinobacter* FAR also contains a C-terminal domain, sometimes referred to as the Male Sterile 2 domain, which is common among all characterised FARs but is as yet of unknown function. Fig. 4 comprises a phylogenetic tree of the now defined *Marinobacter* FAR with FARs from eukaryotes where biochemical data of substrate or product specificity are available. A multitude of other sequences with a similar primary structure could also be found which so far are uncharacterized. On a wider search through eukaryotes related to plants, homologous sequences can be found in the moss *Physcomitrella patens* (Acc. XP_001771307 and XP_001758118). Homologous sequences can also be found among protista such as *Euglena gracilis* which is included in the tree (Fig. 4) but also from *Trypanosoma cruzi* (Acc. XP_809421) and incomplete sequences of *Phytophthora infestans*. Interestingly translated ORFs with the C-terminal domain common to all determined FARs connected to an NAD(P)H binding Rossmann-fold could not be found among algae and fungi. Comprehensive and exhaustive sequencings efforts may reveal such algal or fungal sequences though.

3.5. Maqu_2220 is a FAR with a wide substrate range

While *Marinobacter* FAR has a considerable activity with quite diverse substrates of different carbon chain lengths, unsaturation and other substitutions, most FARs close to *Marinobact*er FAR in the tree of Fig. 4 have a narrow range. Recently a purified FAR from rice, called DPW and that was shown to be important for normal pollen wall development was characterised and found quite specific for 16:0 carbon substrates [27]. Likewise, purified *Arabidopsis* AtFAR6 and AtFAR2 have similar specificities with a large preference for 16:0 carbon chains (Hofvander et al., unpublished results). *E. gracilis* store wax esters as energy reserves and an heterologously expressed *Euglena* FAR mainly yielded saturated 14 and 16 carbon alcohols which correspond to the major alcohol components shown in characterised storage wax esters [28,33]. Insect FARs involved in pheromone synthesis are also quite specific with a preference for 14 or 16 carbon substrates [34,35]. In the phylogenetic tree displayed in Fig. 4, exceptions are the honey bee FAR (AmFAR) and mouse FAR1 (MmFAR1) which seem to have wider substrate ranges [1,29]. Specificities observed are most likely a function of which requirements are placed on the end product, i.e. protective surface waxes or signalling pheromones, but will also depend on which substrates spatially and temporally are available in the system studied.

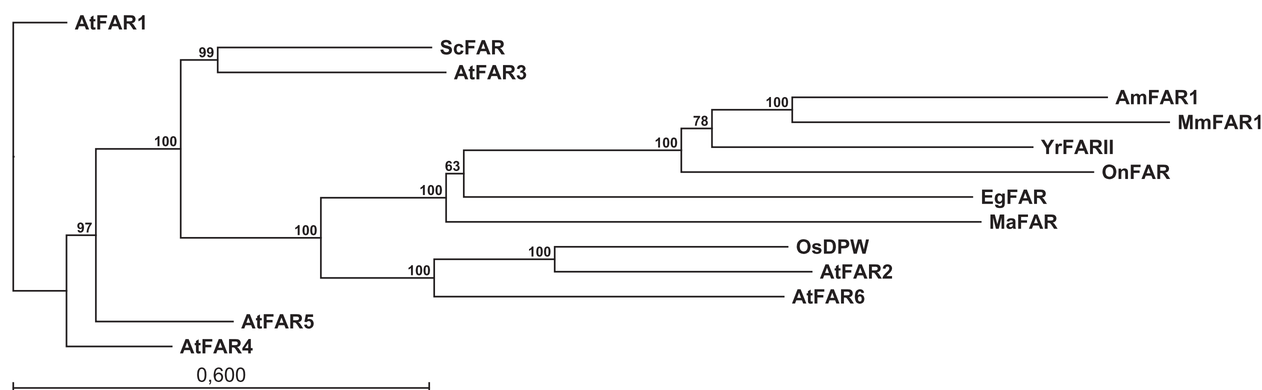

Fig. 4. Neighbor-joining, unrooted phylogenetic tree of selected FAR enzymes where biochemical or product data are available in the literature. FAR encoding genes are ubiquitous among eukaryotic genomes. The proteins correspond to NCBI accession numbers: YP_959486 (MaFAR), ADI60057 (EgFAR), (NP_197642 (AtFAR1), NP_187805 (AtFAR2), NP_567936 (AtFAR3), NP_190040 (AtFAR4), NP_190041 (AtFAR5), B9TSP7 (AtFAR6), NP_001049083 (OsDPW), AAD38039 (ScFAR), ADD62441 (YrFARII), ACY07546 (OnFAR), NP_001180219 (AmFAR), NP_081655 (MmFAR1). Species abbreviations are: Ma (*Marinobacter aquaeolei*), Eg (*Euglena gracilis*), At (*Arabidopsis thaliana*), Os (*Oryza sativa*), Sc (*Simmondsia chinensis*), Yr (*Yponomeuta rorrellus*), On (*Ostrinia nubilalis*), Am (*Apis mellifera*), Mm (*Mus musculus*).

In plants and animals the fatty alcohols and wax esters commonly serves very specialised functions where physical properties are of importance. A higher stringency FAR enables the organism to fine tune the contribution to the specific properties of compound or mixture of compounds where the fatty alcohol is utilized. In contrast, wax esters in the marine bacterial species serve primarily as energy storage. For this purpose it is most probably an advantage if a wider range of substrates can be utilized, either from internally synthesised resources or from hydrocarbons available in the environment. Interestingly, in one plant, jojoba (*S. chinensis*) wax esters are accumulated in seeds as energy reserves [36]. Here a seemingly another evolutionary route has been taken where seed wax ester biosynthesis genes probably has been recruited from genes involved in protective wax biosynthesis and thus returning to the older role of wax esters forming enzymes for energy reserve synthesis, as found in *Marinobacter* species.

In conclusion, Maqu_2220 of *M. aquaeolei* VT8 has now been proven to have the same type of activity as eukaryotic FARs and may, together with homologous sequences from marine bacteria of Table 1, be the prototypical versions of this enzyme. Specialisation of enzyme function may have occurred upon migration to land and evolution of more complex organisms. Wax esters have been suggested as important for survival during dehydration in the water air boundary facilitating terrestrial life [37]. Waxes and wax esters are furthermore found as a protective layer towards the outside environment in plants as well as insects and mammals. Future research on additional FARs from various organisms and wax ester biosynthesis could lead to interesting evolutionary perspectives. If this type of enzyme is not found among i.e. algae, the question arise how these marine bacteria or genes of these prokaryotes fit in with the accepted evolutionary tree of eukaryotes such as terrestrial plants. Finally we suggest that Maqu_2220 is renamed fatty acid reductase (FAR) from the previously suggested name fatty aldehyde reductase (FALDR) [17].

Acknowledgements

We thank Professor Sten Stymne for valuable advice on experimental approaches and helpful comments on the manuscript. This work was funded by the European Commission and is part of the European Commission Framework 7 project ICON (Industrial Crops Producing Added Value Oils for Novel Chemicals).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2011.10.016.

References

[1] Cheng, J.B. and Russell, D.W. (2004) Mammalian wax biosynthesis – I. Identification of two fatty acyl-coenzyme A reductases with different substrate specificities and tissue distributions. Journal of Biological Chemistry 279, 37789–37797.

[2] Cheng, J.B. and Russell, D.W. (2004) Mammalian wax biosynthesis - II. Expression cloning of wax synthase cDNAs encoding a member of the acyltransferase enzyme family. Journal of Biological Chemistry 279, 37798–37807.

[3] Lardizabal, K.D., Metz, J.G., Sakamoto, T., Hutton, W.C., Pollard, M.R. and Lassner, M.W. (2000) Purification of a jojoba embryo wax synthase, cloning of its cDNA, and production of high levels of wax in seeds of transgenic Arabidopsis. Plant Physiology 122, 645–655.

[4] Metz, J.G., Pollard, M.R., Anderson, L., Hayes, T.R. and Lassner, M.W. (2000) Purification of a jojoba embryo fatty acyl-coenzyme A reductase and expression of its cDNA in high erucic acid rapeseed. Plant Physiology 122, 635–644.

[5] Holtzapple, E. and Schmidt-Dannert, C. (2007) Biosynthesis of isoprenoid wax ester in Marinobacter hydrocarbonoclasticus DSM 8798: Identification and characterization of isoprenoid coenzyme A synthetase and wax ester synthases. Journal of Bacteriology 189, 3804–3812.

[6] Kalscheuer, R. et al. (2007) Analysis of storage livid accumulation in Alcanivorax borkumensis: Evidence for alternative triacylglycerol biosynthesis routes in bacteria. Journal of Bacteriology 189, 918–928.

[7] Waeltermann, M. and Steinbuechel, A. (2005) Neutral lipid bodies in prokaryotes: Recent insights into structure, formation, and relationship to eukaryotic lipid depots. Journal of Bacteriology 187, 3607–3619.

[8] Alvarez, H.M., Pucci, O.H. and Steinbuchel, A. (1997) Lipid storage compounds in marine bacteria. Applied Microbiology and Biotechnology 47, 132–139.

[9] Klein, B., Grossi, V., Bouriat, P., Goulas, P. and Grimaud, R. (2008) Cytoplasmic wax ester accumulation during biofilm-driven substrate assimilation at the alkane - water interface by Marinobacter hydrocarbonoclasticus SP17. Research in Microbiology 159, 137–144.

[10] Rontani, J.F., Bonin, P.C. and Volkman, J.K. (1999) Production of wax esters during aerobic growth of marine bacteria on isoprenoid compounds. Applied and Environmental Microbiology 65, 221–230.

[11] Fixter, L.M., Nagi, M.N., McCormack, J.G. and Fewson, C.A. (1986) Structure, distribution and function of wax esters in Acinetobacter-calcoaceticus. Journal of General Microbiology 132, 3147–3157.

[12] Ishige, T., Tani, A., Takabe, K., Kawasaki, K., Sakai, Y. and Kato, N. (2002) Wax ester production from n-alkanes by Acinetobacter sp strain M-1: Ultrastructure of cellular inclusions and role of acyl coenzyme A reductase. Applied and Environmental Microbiology 68, 1192–1195.

[13] Kalscheuer, R. and Steinbuchel, A. (2003) A novel bifunctional wax ester synthase/acyl-CoA : diacylglycerol acyltransferase mediates wax ester and triacylglycerol biosynthesis in Acinetobacter calcoaceticus ADP1. Journal of Biological Chemistry 278, 8075–8082.

[14] Reiser, S. and Somerville, C. (1997) Isolation of mutants of Acinetobacter calcoaceticus deficient in wax ester synthesis and complementation of one mutation with a gene encoding a fatty acyl coenzyme a reductase. Journal of Bacteriology 179, 2969–2975.

[15] Kalscheuer, R. (2010) Genetics of wax ester and triacylglycerol biosynthesis in bacteria in: Handbook of Hydrocarbon and Lipid Microbiology (Timmis, K.N., Ed.), pp. 527–535, Springer, Berlin Heidelberg.

[16] Waeltermann, M., Stoeveken, T. and Steinbuechel, A. (2007) Key enzymes for biosynthesis of neutral lipid storage compounds in prokaryotes: Properties, function and occurrence of wax ester synthases/acyl-CoA : diacylglycerol acyltransferases. Biochimie 89, 230–242.

[17] Wahlen, B.D., Oswald, W.S., Seefeldt, L.C. and Barney, B.M. (2009) Purification, Characterization, and Potential Bacterial Wax Production Role of an NADPH-Dependent Fatty Aldehyde Reductase from *Marinobacter aquaeolei* VT8. Applied and Environmental Microbiology 75, 2758–2764.

[18] Nallamsetty, S., Austin, B.P., Penrose, K.J. and Waugh, D.S. (2005) Gateway vectors for the production of combinatorially-tagged His(6)-MBP fusion proteins in the cytoplasm and periplasm of *Escherichia Coli*. Protein Science 14, 2964–2971.

[19] Bafor, M., Smith, M.A., Jonsson, L., Stobart, K. and Stymne, S. (1991) Ricinoleic acid biosynthesis and triacylglycerol assemblt in microsomal preparations from developing castor bean (Ricinus communis) endosperm. Biochemical Journal 280, 507–514.

[20] Sanchez, M., Nicholls, D.G. and Brindley, D.N. (1973) The relationship between palmitoyl-coenzyme A synthetase activity and strefication of sn-glycerol 3-phosphate in rat liver mitochondria. Biochemical Journal 132, 697–706.

[21] Kuo, T.M. and Ohlrogge, J.B. (1984) A novel, general radioimmunoassay for acyl carrier proteins. Analytical Biochemistry 136, 497–502.

[22] Kapust, R.B. and Waugh, D.S. (1999) *Escherichia coli* maltose-binding protein is uncommonly effective at promoting the solubility of polypeptides to which it is fused. Protein Science 8, 1668–1674.

[23] Huu, N.B., Denner, E.B.M., Ha, D.T.C., Wanner, G. and Stan-Lotter, H. (1999) *Marinobacter aquaeolei* sp., a halophilic bacterium isolated from a Vietnamese oil-producing well. International Journal of Systematic Bacteriology 49, 367–375.

[24] Yakimov, M.M., Timmis, K.N. and Golyshin, P.N. (2007) Obligate oil-degrading marine bacteria. Current Opinion in Biotechnology 18, 257–266.

[25] Domergue, F. et al. (2010) Three Arabidopsis Fatty Acyl-Coenzyme A Reductases, FAR1, FAR4, and FAR5, Generate Primary Fatty Alcohols Associated with Suberin Deposition. Plant Physiology 153, 1539–1554.

[26] Rowland, O., Zheng, H.Q., Hepworth, S.R., Lam, P., Jetter, R. and Kunst, L. (2006) CER4 encodes an alcohol-forming fatty acyl-coenzyme A reductase involved in cuticular wax production in Arabidopsis. Plant Physiology 142, 866–877.

[27] Shi, J. et al. (2011) Defective Pollen Wall Is Required for Anther and Microspore Development in Rice and Encodes a Fatty Acyl Carrier Protein Reductase. Plant Cell 23, 2225–2246.

[28] Teerawanichpan, P. and Qiu, X. (2010) Fatty Acyl-CoA Reductase and Wax Synthase from *Euglena gracilis* in the Biosynthesis of Medium-Chain Wax Esters. Lipids 45, 263–273.

[29] Teerawanichpan, P., Robertson, A.J. and Qiu, X.A. (2010) A fatty acyl-CoA reductase highly expressed in the head of honey bee (*Apis mellifera*) involves biosynthesis of a wide range of aliphatic fatty alcohols. Insect Biochemistry and Molecular Biology 40, 641–649.

[30] Wang, A.M., Xia, Q., Xie, W.S., Dumonceaux, T., Zou, J.T., Datla, R. and Selvaraj, G. (2002) Male gametophyte development in bread wheat (Triticum aestivum L.): molecular, cellular, and biochemical analyses of a sporophytic contribution to pollen wall ontogeny. Plant Journal 30, 613–623.

[31] Aarts, M.G.M. et al. (1997) The Arabidopsis MALE STERILITY 2 protein shares similarity with reductases in elongation/condensation complexes. Plant Journal 12, 615–623.

[32] Doan, T.T.P., Carlsson, A.S., Hamberg, M., Bulow, L., Stymne, S. and Olsson, P. (2009) Functional expression of five Arabidopsis fatty acyl-CoA reductase genes in *Escherichia coli*. Journal of Plant Physiology 166, 787–796.
[33] Tucci, S., Vacula, R., Krajcovic, J., Proksch, P. and Martin, W. (2010) Variability of Wax Ester Fermentation in Natural and Bleached *Euglena gracilis* Strains in Response to Oxygen and the Elongase Inhibitor Flufenacet. Journal of Eukaryotic Microbiology 57, 63–69.
[34] Lassance, J.-M. et al. (2010) Allelic variation in a fatty-acyl reductase gene causes divergence in moth sex pheromones. Nature 466, 486–489.
[35] Lienard, M.A., Hagstrom, A.K., Lassance, J.-M. and Lofstedt, C. (2010) Evolution of multicomponent pheromone signals in small ermine moths involves a single fatty-acyl reductase gene. Proceedings of the National Academy of Sciences of the United States of America 107, 10955–10960.
[36] Miwa, T.K. (1971) Jojoba oil wax esters and derived fatty acids and alcohols: gas chromatographic analyses. Journal of the American Oil Chemists Society 48, 259–264.
[37] Finkelstein, D.B., Brassell, S.C. and Pratt, L.M. (2010) Microbial biosynthesis of wax esters during desiccation: Adaptation for colonization of the earliest terrestrial environments? Geology 38, 247–250.