



## Microbial fatty acid transport proteins and their biotechnological potential

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# Microbial fatty acid transport proteins and their biotechnological potential

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## Abstract

Fatty acid metabolism has been widely studied in various organisms. However, fatty acid transport has received less attention even though it plays vital roles for the cells, such as export of toxic free fatty acids or uptake of exogenous fatty acids. Hence, there are important knowledge gaps in how fatty acids cross biological membranes and many mechanisms and proteins involved in these processes still need to be determined. The lack of information is more predominant in microorganisms, even though the identification of fatty acids transporters in these cells could lead to new drug targets or improvements in microbial cell factories.

This review provides a thorough analysis of the current information on fatty acid transporters in microorganisms, including bacteria, yeasts and microalgae species. Most information available relates to the model organisms *Escherichia coli* and *Saccharomyces cerevisiae*, but transport systems of other species are also discussed. Intracellular trafficking of fatty acids and their transport through organelle membranes in eukaryotic organisms is described as well. Finally, applied studies and engineering efforts using fatty acids transporters are presented to show the applied potential of these transporters and to stress the need for further identification of new transporters and their engineering.

### **Keywords:**

Fatty acid

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Yeast

Microalgae

Introduction

Transport processes are essential to living organisms and the control of transport across lipid membranes was one of the milestones to the generation of life on Earth (Lancet, Zidovetzki, & Markovitch, 2018; Mansy, 2010). Transport proteins play a big role in cell physiology, such as nutrient uptake and export of toxic compounds, and are predicted to make up 13.7% and 5.8% of the *Escherichia coli* and *Saccharomyces cerevisiae* proteome, respectively (Claus, Jezierska, & Bogaert, 2019).

However, research on transport processes has always be limited due to technical difficulties in comparison with enzymes. Most transport proteins are membrane integrated or membrane associated proteins. The membrane environment necessary for the correct folding of membrane proteins complicates their purification and the following *in vitro* studies or crystallizations for structural studies. This results in an underrepresentation of structures of membrane proteins in databases, as they represent less than 3% of protein structures in the Protein Data Bank while they comprise around 25% of all known proteins (Newport, Sansom, & Stansfeld, 2019). Therefore, when studying membrane proteins, it is necessary to rely on other techniques, such as homology modelling or mutagenesis (Futagi, Kobayashi, Narumi, Furugen, & Iseki, 2019). Besides this, heterologous expression or overexpression of membrane proteins can easily lead to membrane stress in cells, requiring careful strategies to ensure proper expression and activity (Kang & Tullman-Ercek, 2018). Another disadvantage related to transport processes is the lack of chemical change in their activity, which renders transport activity measurement more laborious and less straight-forward than enzymatic activity (Brouwer et al., 2013).

Despite all these limitations, transport proteins have been studied since the last century, when a lactate transporter was first identified in *E. coli* (Jones & Kennedy, 1969). Since then, many transporters have been identified for a wide diversity of molecular structures. However, the transport of hydrophobic substances has been traditionally associated with passive diffusion.

During the last years, extensive evidence of protein assisted transport for hydrophobic molecules has led to a paradigm shift in the scientific community (Claus et al., 2019). Although the transport of hydrophobic compounds requires different mechanisms than soluble compounds, the employment of transport proteins allows cells to regulate this process and it opens opportunities for engineering and improving these transport processes.

Among the different hydrophobic cellular compounds, the most prevalent ones in metabolism are fatty acids, serving both as energy storage and membrane constituents. Transport of fatty acids in animals is tightly regulated, both intra and extracellularly. Fatty acids are transported across different organs and cellular compartments via soluble fatty acid binding proteins as well as membrane bound transporters (Glatz, 2015; Glatz, Luiken, & Bonen, 2010). Perturbation in the transport of fatty acids and lipids give place to pathologies, such as adrenoleukodystrophy, which is associated to mutations in the peroxisomal fatty acid transporter (Tarling, Vallim, & Edwards, 2013). This shows the importance of these mechanisms for correct physiological functions. Also in plants, lipid transport plays indispensable roles in processes such as seed development and cutin synthesis. In plants, fatty acids are synthesized in plastids, modified in the endoplasmic reticulum, stored in cytosolic lipid bodies and degraded in peroxisomes. This high compartmentalization of fatty acid metabolism in plants needs the coordinated participation of membrane transporters and carrier proteins for the intracellular trafficking of fatty acids. The transport of lipids and fatty acids in plants has been reviewed elsewhere (N. Li, Xu, Li-Beisson, & Philippar, 2016).

Fatty acid metabolism has been extensively studied in microbial model organisms such as *E. coli* and *S. cerevisiae*, but also in oleaginous bacteria, yeasts and microalgae (Hu, Zhu, Nielsen, & Siewers, 2019; Magnuson, Jackowski, Rock, & Cronan, 1993). Oleaginous organisms, such as *Rhodococcus jostii* or *Yarrowia lipolytica*, are those able to store lipids as 20% or more of their dry cell weight, reaching 80% in some cases (Alvarez et al., 2019). While microbial fatty acid

metabolism has been highly studied and engineered, fatty acid transport in microorganisms still present knowledge gaps. The study of fatty acids in microorganisms is relevant because the scientific evidence acquired from the model organisms *E. coli* and *S. cerevisiae* lays the biochemical basis for research in higher organisms. In addition, their easy manipulation allows a more thorough and flexible study of the transport systems and their components. Moreover, microbial production of fatty acids is gaining attention due to their wide range of applications, mainly as biofuels and nutrition supplements, but also in animal feed, cosmetics and lubricants (Vasconcelos, Teixeira, Dragone, & Teixeira, 2019).

Increasing export rates for fatty acids can be highly beneficial for microbial cell factories for two reasons. On the one hand, overproduction of a metabolite causes feedback inhibition as well as toxic effects in the cell that severely affect productivity. The removal of product from the cellular space allows for a continuous production and a higher yield per cell mass (Kell, Swainston, Pir, & Oliver, 2015). On the other hand, the downstream processing of fermentation processes has a significant contribution to the final cost-effectiveness of the production process, due to the need to break open cells to extract the desired product, as well as the subsequent separation process from all cell constituents (Jezierska & Van Bogaert, 2017). The export of fatty acids into the extracellular space allows for a simpler downstream processing, where cells are removed, and the product can be directly obtained from a media that contains much less by-products (Borodina, 2019).

Despite the limitations associated with the study of membrane proteins and transport, several fatty acid import and export systems have been identified in microorganisms. This review presents the current scientific knowledge on fatty acid transport across biological membranes, as well as examples of engineering fatty acid transport to improve microbial cell factories.

## Fatty acid transport in bacteria

### Fatty acid uptake systems

#### Import of fatty acids in *E. coli*: the FadL-FACS system

The first discovered microbial transport systems for fatty acids was the FadL-FACS system from *E. coli* for the import of exogenous long-chain fatty acids. It was described in 1969 that the ability of *E. coli* to degrade exogenous fatty acids was associated to the presence of a fatty acid-CoA synthetase (FACS). Characterization of this enzyme suggested the presence of another transport protein which was discovered later in 1978 (Nunn & Simons, 1978). The gene encoding this protein, named *fadL*, belongs to the fatty acid degrading (*fad*) regulon containing eight catabolic genes for fatty acids (Feng & Cronan, 2009), including *fadD* which codes for the FACS. The regulon is controlled by the *fadR* gene, whose product activates the *fad* genes upon binding to acyl-CoA. Both *fadL* and *fadD* are expressed in a basal level to allow detection of exogenous fatty acids by *fadR* (Nils Joakim Færgeman & Knudsen, 1997).

FadL is a long-chain fatty acid-specific transporter with a  $\beta$ -barrel structure present in the outer membrane of *E. coli*. The presence of lipopolysaccharides in the outer membrane renders the cell impermeable to hydrophobic molecules such as fatty acids. FadL is necessary for the uptake of exogenous fatty acids so that they can be used both as a source of energy and as a constituent of phospholipids and triacylglycerols, relieving the bacteria from spending energy and resources into synthesizing new fatty acids (Van Den Berg, 2005). The structure and mechanism of FadL has been studied by Van der Berg et al (Van Den Berg, Black, Clemons, & Rapoport, 2004). FadL is a long 14-strand  $\beta$ -barrel that contains special features: in the extracellular region two loops containing  $\alpha$ -helices form a hydrophobic groove; in the intracellular region a hatch domain with three  $\alpha$ -helices blocks the channel; the N-terminus extends through the barrel towards the

extracellular regions; finally, the strand S3 shows a peculiar bend or kink that disrupts the  $\beta$ -sheet formation and forms a lateral opening (Figure 1A). Concerning the mechanism of transport, the authors initially considered at a first stage that the fatty acids would bind first to the hydrophobic groove, then they would diffuse to an internal high-affinity binding pocket where they would lead to conformation changes both in the N terminus and the hatch domain opening a path for their liberation in the periplasmic space (Van Den Berg, 2005). Nonetheless, this model was refuted after showing the rigidity of the hatch domain (Hearn, Patel, Lepore, Indic, & Van Den Berg, 2009). The importance of the lateral opening in the  $\beta$ -barrel caused by the S3 kink led to support the hypothesis of lateral diffusion, in which the fatty acids would not be liberated to the periplasmic space but rather through the opening space in the  $\beta$ -barrel to the outer membrane (Lepore et al., 2011; Figure 1B). A summary of studied residues from FadL can be found in Table 1, all of them conserved in homologue fatty acids transporters from other species.

Once the fatty acids have been transported by FadL through the outer membrane, they move to the inner membrane. Even though there are reports that proteins may be involved in the process (A. Azizan & Black, 1994), it is likely that this step happens spontaneously. A piece of evidence that supports passive diffusion from the outer membrane to the inner membrane, as well as a flip-flop movement inside each membrane, is the necessity of a proton motive force that can generate acidic conditions in the periplasm leading to the protonation of fatty acid and the subsequent increase in liposolubility (Azliyati Azizan, Sherin, DiRusso, & Black, 1999). Once in the inner membrane, these fatty acids are catalysed by FACS to form acyl-CoA molecules, which are destined to further catabolic or anabolic processes. FACS is a soluble protein that is recruited to the membrane to assist in the transport of fatty acids (Overath, Pauli, & Schaire, 1969). The mechanism of this recruitment is not known and it possibly happens due to conformational changes after binding of ATP and it has been observed to be assisted by the presence of D-lactate, among other conditions (Mangroo & Gerber, 1993). The activity of FACS is necessary for



the metabolism of exogenous fatty acids and it is speculated that it promotes the transport of fatty acids through vectorial acylation analogous to the vectorial phosphorylation of sugars (Black & Dirusso, 2003).

### Engineering the fatty acid import system of *E. coli* for biotechnological applications

The import of fatty acid into cells is important for several industrial processes, such as fermentation processes using fatty acids and waste oils as carbon source. The cellular catabolism of fatty acids leads to the formation of acetyl-CoA, which delivers the acetyl group to the citric acid cycle providing energy and intermediates to the cell. Furthermore, acetyl-CoA is a precursor for important metabolites, such as branched-amino acids (Amorim Franco & Blanchard, 2017).

Engineering efforts to produce 3-hydroxypropionate (bioplastic precursor) from fatty acids showed the importance of controlling the expression of the FadL-FACS system (B. Liu et al., 2019). While using a strong promoter for FACS resulted in an increase in productivity, it led to an important decrease when used for FadL. However, the use of medium or weak constitutive promoters for FadL allowed to increase the productivity. This exemplifies the common problem associated to overexpression of membrane proteins, which can increase membrane stress and decrease cell growth and overall productivity (Kang & Tullman-Ercek, 2018). The constitutive expression of FACS and FadL, next to other metabolic modifications, led to a 3-hydroxypropionate yield of 1.56 g/g when grown in a 5L bioreactor using palmitic acid as substrate. In another study where *E. coli* was engineered for the production of lycopene (N. Liu et al., 2020), overexpression of FACS led to a small increase in the lycopene titre (from 29 to 33 mg/g DCW). Nevertheless, growth of the engineered strain in a mix of glucose, waste oil and yeast extract allowed for a total yield of 94 mg/g DCW.

Another important set of applications for the import of fatty acids are whole cell biotransformations. In these, bacteria grown previously to reach a certain biomass density act as catalysers to modify fatty acids or derived compounds through a limited number of enzymatic

steps in order to produce a compound of higher value. In such process, cells act as capsules containing enzymes and are not grown during the biotransformation process. Deletion of FACS and overexpression of FadL showed an increase on fatty acid hydroxylation when expressing the cytochrome P450 CYP153A from *Marinobacter aquaeolei*, a heterologous cytosolic enzyme in *E. coli*, suggesting that other proteins can replace the role of FACS in recruiting free fatty acids from the inner membrane and modifying them (Bae, Park, Jung, Lee, & Kim, 2014). Another study involving both fatty acids and hydroxy-fatty acids determined the impact of FadL expression on the biotransformation of these compounds to hydroxy-fatty acids and keto-fatty acids, respectively (Jeon et al., 2018). Enhanced expression of FadL led to a five-fold increase in the single step transformation of oleic acid and 10-hydroxyoctadecanoic acid to 10-hydroxyoctadecanoic acid and 10-keto-octadecanoic, respectively, as well as a two-fold increase in the multistep transformation of ricinoleic acid to the ester molecule ((Z)-11-(heptanoyloxy)undec-9-enoic acid). This study also showed the negative effects that excessive overexpression of FadL can have on overall productivity. Finally, in another study FadL was overexpressed in a strain expressing human Cav1 proteins (Shin et al., 2019). Cav1 proteins stimulate the formation of endosomes that excise from the inner membrane. The formation of endosomes increased the uptake rates of ricinoleic acid two-fold and caused a decrease of fatty acid toxicity in the inner membrane. However, overexpression of FadL in this strain did not lead to a further increase in fatty acid uptake.

### Import of fatty acids in other bacteria

Besides the fadL-FACS system from *E. coli*, other systems for the import and assimilation of exogenous fatty acids have been studied in other bacteria. All bacterial fatty acid import systems were found to be dependent on energy supply whether in the form of ATP or in the form of a proton gradient (Calmes & Deal, 1976). One of the first systems studied was that of other gram-negative bacteria such as *Pseudomonas oleovorans* and *Caulobacter crescentus*, which showed

similar characteristics to the fadL-FACS system (Toscano & Hartline, 1973; Zalatan & Black, 2011). Due to the different plasma membrane structure, it has been observed that import of fatty acids in gram positive bacteria occurs differently than in gram-negative bacteria (Figure 2A and 2B). Although fatty acid metabolism and transport has not extensively been studied in gram positive model bacteria, such as *B. subtilis*, some studies have been performed on other gram-positive bacteria. In the lipophilic gram-positive bacteria *Nocardia asteroides* (Calmes & Deal, 1976), the fatty acid uptake system was, in contrast to the previously discussed systems, found to be constitutively expressed and able to import fatty acids as free fatty acids, which would be activated by a soluble FACS at a later and independent stage. With a  $K_M$  of 870  $\mu M$ , the transport process from *N. asteroides* was found to have much less affinity than that of *E. coli* ( $K_M$  between 15 and 34  $\mu M$ ). The import of fatty acids in another gram-positive bacterium, *Streptomyces coelicolor* (Banchio & Gramajo, 1997), also showed a constitutive expression, as well as a dependency on pH levels in the environment, decreasing the uptake of long-chain fatty acids with increasing pH. The import system of this bacterium was found to be specific for ionized fatty acids (at pH 7 or higher), while protonated ones were claimed to use a passive process rather than an active one (at a pH lower than 7).

In the fadL-FACS system, FACS is associated to the membrane, linking the formation of acyl-CoA to transport. Nevertheless, the production of acyl-CoA from exogenous fatty acids is not conserved in all bacteria, and other ways of activating and incorporating exogenous fatty acids have been studied, such as acyl-ACP synthase in *Vibrio harveyi*, or fatty acid kinases in gram positive bacteria (Cronan, 2014). The different ways to in which exogenous fatty acids are activated and metabolized has been reviewed elsewhere (Yao & Rock, 2017).

The study of fatty acid import in bacteria can serve different interests, such as improving the fatty acid import capacities of organisms of industrial importance or identifying new drug targets for pathogenic bacteria able to import fatty acids from the host. Among the different bacteria

that are of interest for industry, the gram-positive genus *Rhodococcus* has attracted special attention for their ability to withstand and degrade a wide variety of pollutant, including hydrocarbons and lignin-derived compounds (Kim, Choi, Yoo, Zylstra, & Kim, 2018). Some species of this genus, such as *Rhodococcus opacus* and *Rhodococcus jostii*, can be considered oleaginous, being able to accumulate high amounts of triacylglycerol when growing in carbon-excess conditions (Alvarez et al., 2019). A fatty acid importer from *R. jostii* was found in a cluster with genes involved in lipid metabolism after a homology search using known ACB-transporters for hydrophobic compounds. The function of this gene (ro05645 or *ltp1*) was confirmed after its overexpression, which led to an increased growth in media containing fatty acids as substrate, as well as the uptake of fluorescently labelled fatty acids (Villalba & Alvarez, 2014). Furthermore, the overexpression of *ltp1* was used to increase both the growth (2.2-fold) and the lipid accumulation (3.5-fold) of *R. jostii* when growing in olive mill waste (Herrero, Villalba, Lanfranconi, & Alvarez, 2018).

*Mycobacterium tuberculosis* is the causing agent of tuberculosis, one of the deadliest diseases in the world. This bacterium is adapted to survive for long periods of time inside the human host, partially thanks to its ability to metabolize host-derived fatty acids. This ability is especially important, as these cells tend to reside within lipid-rich sites, such as inside foamy macrophages (Lovewell, Sassetti, & VanderVen, 2016). *M. tuberculosis* presents a cell envelope containing mycolic acid and glycolipids which acts as a barrier for hydrophobic molecules. A multiprotein complex was observed to be involved in the uptake of fatty acids during macrophage infection as well as in monocultures (Figure 2C). The first member of this complex to be identified was LucA, a protein also involved in the uptake of cholesterol (Nazarova et al., 2017). It was observed that a mutant lacking LucA was unable to incorporate fatty acids, and a transcriptomic analysis of such mutant revealed the involvement of genes from the Mce1 locus in the uptake of fatty acids. From this locus, gene *rv0167* coding for the putative permease YrbE1A was found to be directly involved in fatty acid import. A further screening of mutants lacking the ability to

assimilate fatty acids when infecting macrophages revealed the participation of other proteins (Nazarova et al., 2019), such as MceD1, MceG and OmamB. While the function of OmamB is not known, a similar protein OmamA was found to interact with LucA and stabilize the fatty acid import complex. MceG is a putative ATPase that may be involved in providing the energy for the transport of the fatty acids. Finally, MceD1 and other proteins from the same locus such as MceA1 and MceC1, may have structural roles in the formation of the fatty acid import complex. In addition to the complex Mce1, another gene (*rv1272*) from *M. tuberculosis* showing homology to the *ltp1* gene from *R. jostii* as well as to the lipid A export gene *msbA* from *E. coli* was confirmed to import fatty acids when expressed in *E. coli* (Martin & Daniel, 2018). A Blast search of *rv1272* against the SwissProt database showed that this gene is homologous (39.39% identity) to the uncharacterized transporter YfiC from *Bacillus subtilis*, suggesting that the role of this transporter might be associated to the transport of fatty acids or other hydrophobic compounds.

## Fatty acid export systems

### The fatty acid export system in *E. coli* and its engineering

In its natural environment, *E. coli* must face high concentrations of fatty acids and other hydrophobic compounds and therefore it must contain mechanisms to prevent toxic effects, such as export proteins. At the same time, secretion of endogenous fatty acids is not detected for wild type strains under normal conditions. Nevertheless, fatty acid secretion in *E. coli* was observed for the first time when expressing a thioesterase from the plant *Umbellaria californica* (Voelker & Davies, 1994). This enzyme was expressed to generate free fatty acids inside the cell. In normal conditions, free fatty acids do not accumulate intracellularly in bacteria, as they are directly transferred from acyl-ACP to glycerol-3-phosphate (Magnuson et al., 1993). On the other hand, plants possess thioesterases to liberate fatty acids from acyl-ACP and these free fatty acids are used for anabolic processes (Gerhardt, 1992). The oilseed from *U. californica*

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2  
3 accumulates medium-chain fatty acids, mainly lauric acid (C<sub>12:0</sub>) (Davies, Anderson, Fan, &  
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5 Hawkins, 1991), and the expression of its thioesterase in *E. coli* leads to the same fatty acid  
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7 profile (Voelker & Davies, 1994). However, the accumulation of fatty acids was only observed  
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9 when the  $\beta$ -oxidation pathway was disabled. Secretion of lauric acid was shown by the  
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11 observation of extracellular laurate crystals when growing on solid media. Later engineering of  
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13 *E. coli* showed that saturated and monounsaturated fatty acids with 12 till 18 carbon atoms can  
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15 be secreted in an efficient way reaching 40 mg/L of extracellular fatty acids, but no specific efflux  
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17 protein for fatty acids was identified (H. Liu et al., 2012).  
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22 The accumulation of medium-chain fatty acids is toxic to bacteria due to their ability to  
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24 destabilize the membrane and interfere with essential activities, such as creation of proton  
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26 gradients (Lennen et al., 2011). This toxic activity was used to identify proteins involved in fatty  
27  
28 acid export that would protect the cells from the accumulation of medium-chain fatty acids  
29  
30 when expressing the thioesterase from *U. californica* (Lennen, Politz, Kruziki, & Pfleger, 2013).  
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32 The genes whose knock out led to important effects on cell viability under these conditions were  
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34 *rob*, *acrAB*, *tolC* and, at a lower extent, *emrAB*. The activity of the first three genes is tightly  
35  
36 linked, as *rob* is a regulatory gene that induced *acrAB* expression among other genes, and TolC  
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38 is an unspecific porin from the outer membrane that forms a complex with *acrAB*. The AcrAB-  
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40 TolC complex is a transporter from the Resistance-Nodule-Division (RND) superfamily that spans  
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42 from the intracellular space until the outer side of the bacterial membrane, forming a channel  
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44 whose mechanism is regulated by allosteric changes and a proton gradient (Wang et al., 2017;  
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46 Zgurskaya & Nikaido, 1999). The complex is formed by three TolC components, six AcrA  
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48 components and three AcrB components (Figure 3). The mechanism behind the binding of  
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50 ligands to AcrB is not precisely known, but due to the large number of different substrates that  
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52 the AcrAB-TolC complex can transport it is thought that there might be several binding sites in  
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54 the same binding pocket (Nakashima, Sakurai, Yamasaki, Nishino, & Yamaguchi, 2011). In any  
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56 case, the binding of a substrate changes the conformation of the AcrB monomer from the  
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3 relaxed or access state to a binding state, which induces conformational changes in the rest of  
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5 the complex. Through several interactions with AcrB, AcrA continues the conformational  
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7 changes to TolC, so that it can change from the closed state to the open state, opening the  
8  
9 channel. Finally, the AcrB subunit bound to the ligand changes to another conformation (open  
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11 state), liberating the substrate into the open channel for its liberation to the extracellular  
12  
13 medium (Wang et al., 2017).  
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17 Due to the broad specificity of the AcrAB-TolC system, the AcrB component has been engineered  
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19 through directed evolution to improve the export rate of different hydrophobic molecules, such  
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21 as medium-chain alcohols, alkanes and alkenes (Chen, Ling, & Chang, 2013; Fisher et al., 2014;  
22  
23 Mingardon et al., 2015). However, no protein engineering of AcrB to improve fatty acid export  
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25 has been documented. Genetic engineering to improve medium-chain fatty acid export in *E. coli*  
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27 was performed by overexpressing several potential transporters (J. Wu et al., 2019).  
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29 Overexpression of either of three transport proteins, namely AcrE, MdtC and MdtE, was found  
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31 to increase medium-chain fatty acid extracellular concentrations from 600 mg/L to values  
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33 between 800 and 1100 mg/L. One must realize that these proteins are part of larger protein  
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35 complexes whose other components were not overexpressed in the study.  
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41 The AcrEF complex displays homology to the well-studied AcrAB-TolC complex. Yet, the AcrEF  
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43 complex has been observed to be expressed at lower levels than the AcrAB complex and it has  
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45 shown an important function in cell division and chromosome segregation (Lau & Zgurskaya,  
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47 2005). Nevertheless, the AcrAB and AcrEF complexes are from a structural point of view highly  
48  
49 related: the amino acid sequence of acrF shares 87% similarity with acrB, and acrE is homologous  
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51 to acrA (80% AA similarity). Therefore, it is likely that acrE fulfils the same role as acrA in the  
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53 acrEF-TolC complex as the channel that connects acrF and TolC. Yet, when overexpressing acrA  
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55 and acrB from the acrAB-TolC complex separately, no increased fatty acid export was observed.  
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57 Hence, the mechanism behind the overexpression leading to increased export activity of a  
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channel protein that connects the inner membrane pump (which contains the substrate binding) and the outer membrane porin remains unsolved. Furthermore, in the same study three-fold higher extracellular medium-chain fatty acid concentrations were achieved when overexpressing the three transport proteins (acrE, mdtC and mdtE) simultaneously, while observing only a 10% reduction in the OD (J. Wu et al., 2019).

### Export of fatty acids in other bacteria

As observed in the previous section, fatty acid export in *E. coli* seems to be mainly associated to protection against membrane-related toxic effects caused by large concentrations of medium and long-chain fatty acids (Desbois & Smith, 2010). Medium-chain fatty acids have antimicrobial effects affecting a wide range of bacteria (Huang, Alimova, Myers, & Ebersole, 2011). Therefore, it is expected that many bacteria present export systems similar to AcrAB-TolC to export toxic fatty acids, although the specific systems have not been studied to date.

Besides medium chain fatty acids, polyunsaturated fatty acids display antimicrobial properties and in reaction to this, several pathogenic bacteria have developed systems to prevent their toxicity. Some of these systems are the degradation of exogenous fatty acids or their incorporation into phospholipids (Jiang et al., 2019). However, the environments where some pathogenic bacteria must thrive in, such as the skin or the mouth, contain unsaturated fatty acids and therefore they need more advanced protection mechanisms such as fatty acid export systems (Choi et al., 2013; Parsons, Yao, Frank, Jackson, & Rock, 2012). In the gram-positive bacterium *Staphylococcus aureus*, an opportunistic pathogen found on the skin, a fatty acid export system was found when screening for strains resistant to linoleic acid (Alnaseri et al., 2019). The strain *S. aureus* FAR7 showed a mutation in the transcription factor *farR*, which led to the upregulation of the *farE* gene, encoding an efflux pump from the RND superfamily. Also the physiological response to PUFAs of the gram-negative pathogenic bacteria *Acinetobacter baumannii*, was studied and revealed the upregulation of the *adeJ* gene, a component of the



multidrug efflux pump *adeIJK* (Jiang et al., 2019). It was observed that the deletion of this gene increased the susceptibility to PUFAs, leading to a six-fold increased growth delay in the presence of docosahexaenoic acid. However, the mutation did not lead to an accumulation of PUFAs in the cell. Nevertheless, growth experiments showed the ability of *adeIJK* to affect membrane lipid homeostasis and to export lipids to the extracellular medium. These results suggested that the *adeIJK* pump fulfils a similar role as the *emhABC* pump from *Pseudomonas fluorescens*, which controls lipid homeostasis through the efflux of endogenous long-chain fatty acids, both saturated and monounsaturated, in response to temperature changes (Adebusuyi & Foght, 2011). Another studied fatty acid export system from a pathogenic bacterium is the *farAB* system from *Neisseria gonorrhoeae*, which shows high similarity to the *emrAB* system from *E. coli*, and whose deletion leads to susceptibility to the long-chain fatty acids oleic acid, linoleic acid and palmitic acid. The minimal inhibitory concentration decreased from 1600 µg/ml to 50 µg/ml in the case of unsaturated fatty acids and from 100 µg/ml to 12.5 µg/ml in the case of palmitic acid (Lee & Shafer, 1999).

Photosynthetic microorganisms are of special interest due to their ability to fix carbon from the atmosphere to produce industrially relevant compounds in a more efficient way than plants. *Synechocystis* sp. PCC 6803 is the model organism for cyanobacteria and it has been observed to secrete long-chain fatty acids, up to 13% of the cellular biomass, without any genetic modifications (X. Liu, Sheng, & Curtiss, 2011). The deletion of *Synechocystis* sp. PCC 6803 genes *slr0180* and *slr2131*, homologous to the respective *E. coli* genes *acrA* and *acrB*, showed an effect in the fatty acid secretion of an engineered strain of *Synechocystis* sp. PCC 6803 specialized in the extracellular production of fatty acids (Bellefleur, Wanda, & Curtiss, 2019). While the complementation with *acrA* did not lead to a recovery of the fatty acid secretion rates, the complementation of *slr2131* with *acrB* allowed for an increase in both extracellular and intracellular fatty acid concentrations.

Another fatty acid export mechanism has been identified in another cyanobacteria, *Synechococcus elongatus* a. This system was found through the genomic and transcriptomic analysis of a mutant able to produce free fatty acids but resistant to their toxicity. Inactivation of this export system, composed by the genes *rndA1* and *rndB1*, led to susceptibility to exogenous saturated medium-chain fatty acids and unsaturated long-chain fatty acids. Orthologs of *rndB1* are found in most genomes from cyanobacteria, but not in those of *Synechocystis* sp. PCC 6803. The RndA1B1 system from *S. elongatus* allows for efficient secretion of oleic acid, but palmitate is not transported and therefore it accumulates in the cells (Kato et al., 2015).

### Fatty acid transport in yeast

### Uptake of fatty acids in yeast

Fatty acid import in yeast follows the same principle of vectorial acylation described for *E. coli*. In *Saccharomyces cerevisiae*, the import system is composed of Fat1 and Faa1 or Faa4. Mutations in these genes show hampered growth under fatty acid auxotrophic conditions (Black & Dirusso, 2003). Although *S. cerevisiae* cannot use fatty acids as growth substrate, it needs to incorporate exogenous fatty acids in case of inhibition of fatty acid synthesis by cerulenin or during anaerobic growth as desaturases require molecular oxygen to produce unsaturated fatty acids.

Fat1 is an ortholog of the mammal fatty acid transport proteins identified in murine species (Nils J. Færgeman, DiRusso, Elberger, Knudsen, & Black, 1997), and it has been described to be involved both in the import of fatty acids and in the activation of very-long-chain fatty acids (Zou, Dirusso, Ctrnacta, & Black, 2002). Fat1 is an integral membrane protein with two transmembrane domains (Obermeyer, Fraisl, DiRusso, & Black, 2007; Figure 4). The conserved

ATP/AMP binding region characteristic of acyl-CoA synthetases is separated by a portion of the protein that is inserted into the membrane. The intracellular C-terminus contains a region conserved among other fatty acid transport proteins with very-long-chain acyl-CoA synthetase activity (VLACS). Finally, the soluble Faa1 has been observed to interact with the C-terminus of this protein to activate the imported fatty acids. The transport mechanism of Fat1 is unknown but several residues from Fat1 have been mutated to study their effects (Zou et al., 2002). These experiments have shown that although most residues affect both transport and acyl-CoA synthetase activity, the mutation of certain residues separates these two activities, suggesting that they follow different mechanisms. On one hand, F528A and L669R mutations abolish transport function, but retain some acyl-CoA synthetase activity. On the other hand, S258A and D508A mutations abolish acyl-CoA synthetase activity while retaining some transport activity. Fat1 has been proposed to be situated not only in the plasma membrane but also in lipid bodies, endoplasmic reticulum and peroxisomes (Van Roermund et al., 2012).

*S. cerevisiae* contains two main acyl-CoA synthetases for long-chain fatty acids, Faa1 and Faa4. These proteins interact with Fat1 to form a complex that combines transport and activation of fatty acids. Faa1 is responsible for most of the acyl-CoA synthetase activity observed in *S. cerevisiae* and it has been observed to interact with the carboxy terminal of Fat1 as described in several studies, including yeast two-hybrid experiments (Zou et al., 2003). Although the activity of Faa4 is lower than Faa1, it is the only acyl-CoA synthetase gene that can rescue fatty acid import activity in a  $\Delta$ Faa1 mutant, suggesting that its mode of action is identical (Johnson, Knoll, Levin, & Gordon, 1994). Next to vectorial acylation, endocytosis plays a significant role in the uptake of exogenous fatty acids (Jacquier & Schneider, 2010). The deletion of Ypk1, a protein-kinase involved in endocytosis was found to affect fatty acid import by reducing it by half compared to the wild-type. These results led to investigate the involvement of other proteins associated to endocytosis End3, Vrp1 and Srv2, whose deletion also hampered fatty acid import

at the same extent. The involvement of all these genes stresses the importance of endocytosis for fatty acid import, probably due to the internalization of fatty acid-rich membrane domains.

Besides *S. cerevisiae*, the uptake of exogenous fatty has been studied in other yeasts. *Cryptococcus neoformans* is an important fungal pathogen that infects alveolar macrophages and it is responsible for increasing deaths in immunosuppressed individuals. This yeast has been observed to import exogenous fatty acids for the formation of lipid droplets; and the presence of oleic acid stimulates its replication, both in extracellular form and during macrophage infection (Nolan, Fu, Coppens, & Casadevall, 2017). However, no molecular mechanism or components has been described for this process. The acquisition of exogenous fatty acids in *Candida albicans*, another important fungal pathogen, has been studied at the molecular level. CaFaa4, the ortholog gene for Faa4 and Faa1 from *S. cerevisiae*, was characterized and observed to be essential for fatty acid import (Tejima, Ishiai, Murayama, Iwatani, & Kajiwara, 2018). Note that in contrast to *S. cerevisiae*, in *C. albicans* only one Faa gene seems to be involved. The same holds true for *Y. lipolytica*. Moreover, the mechanism behind fatty acid transport is not conserved across yeasts. Although *Y. lipolytica* possesses an ortholog of Fat1 from *S. cerevisiae*, this protein is not associated to fatty acid import and it has been suggested to be involved in fatty acid export from lipid bodies (R. Dulermo, Gamboa-Meléndez, Dulermo, Thevenieau, & Nicaud, 2014). Furthermore, the acyl-CoA synthetase from *Y. lipolytica* (YIFaa1), while being the only gene involved in fatty acid activation, it is not essential for growth on fatty acids (R. Dulermo, Gamboa-meléndez, & Ledesma-amaro, 2015).

### Intracellular trafficking of fatty acids in yeast

Fatty acids must be activated to acyl-CoA before they can enter a specific metabolic pathway. As described in the previous section, exogenous fatty acids are imported and converted to acyl-CoA nearly simultaneously. Acyl-CoA can also be derived from *de novo* synthesised fatty acids and from fatty acids contained in the neutral lipids stored in lipid bodies. The joint action of

lipases and acyl-CoA synthetases, such as the Fat1 from *S. cerevisiae* and its ortholog in *Y. lipolytica*, leads to the mobilisation of fatty acids from lipid bodies (T. Dulermo, Thevenieau, & Nicaud, 2014). Acyl-CoA molecules must reach the destination inside the cell where they will be degraded, stored or used to build other molecules (DiRusso & Black, 1999). The intracellular trafficking routes for fatty acids and acyl-CoA molecules are shown in Figure 5. In *S. cerevisiae*, this intracellular transport is facilitated by an acyl-CoA binding protein coded by the gene *acb1*. Although it has been observed that this protein facilitates transport of acyl-CoA to lipid bodies for the formation of triacylglycerol, it is not necessary for the survival of the cell (Schjerling et al., 1996). Therefore, either there are other transport mechanisms, or they are not needed for the diffusion of acyl-CoA molecules across the cytosol.

In yeast, fatty acids are catabolized by  $\beta$ -oxidation in peroxisomes. The transport of fatty acids in the peroxisome of *S. cerevisiae* has been studied, revealing the involvement of several proteins. Free fatty acids, mainly medium-chain fatty acids, can be imported into the peroxisome by passive diffusion or an unidentified system (Hettema et al., 1996). Long-chain fatty acids, in the form of acyl-CoA, are transported into the peroxisome through the heterodimeric transporter formed by Pxa1 and Pxa2 (Shani, Sapag, Watkins, & Valle, 1996). It has been proposed that the Pxa1/Pxa2 transporter would cleave the fatty acyl-CoA prior to transport and it would introduce only the free fatty acid portion into the peroxisome (Van Roermund et al., 2012). This mechanism prevents accumulation of CoA in the peroxisome and it has already been described in plants (Fulda, Schnurr, Abbadi, Heinz, & Browse, 2004). Fatty acids transported by Pxa1/Pxa2 need to be activated by re-conversion to acyl-CoA to enter the  $\beta$ -oxidation cycle. Two acyl-CoA synthetases have been associated to the peroxisomal activation of fatty acids: Fat1 and Faa2. Substitution of the Pxa1/Pxa2 transporter with the human orthologue showed that Fat1 must interact with the yeast Pxa1/Pxa2 transporter to be active (Van Roermund et al., 2012). While Fat1 is also found in other regions of the cell, such as the plasma membrane, Faa2 is found exclusively in peroxisomes. Faa2 accepts a wide range of fatty acids as substrate, but it has a

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3 preference for medium-chain fatty acids (Knoll, Johnson, & Gordon, 1994). The general model  
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5 of peroxisomal fatty acid transport can be observed in Figure 5. The peroxisomal transport of  
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7 fatty acids in *Y. lipolytica* has also been studied (R. Dulermo et al., 2015). The overall system  
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9 seems similar to that of *S. cerevisiae*, with distinct routes for long and medium-chain fatty acids.  
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11 Both Pxa1/Pxa2 and Fat1 are involved in the peroxisomal transport. However, *Y. lipolytica* lacks  
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13 the Faa2 protein for the activation of medium-chain fatty acids, and it has been proposed that a  
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15 coumarate ligase-like protein might fulfil this role instead (R. Dulermo et al., 2015).  
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## 20 Export of fatty acids in yeast

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23 Just as for prokaryotic organisms, secretion of free fatty acids is no common natural process in  
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25 yeasts or fungi. Yet, upon the engineered intracellular accumulation of free fatty acids, yeasts  
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27 are able to secrete them to the extracellular medium (Arhar & Natter, 2019; Scharnewski,  
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29 Pongdontri, Mora, Hoppert, & Fulda, 2008). Some proteins involved in the export of fatty acids  
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31 in *S. cerevisiae* have been described. An omics analysis of fatty acid secreting mutants of *S.*  
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33 *cerevisiae* identified potential fatty acid export protein Mrp8, though no experimental results  
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35 are available for this transporter (Fang et al., 2016). An experimentally proven fatty acid export  
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37 protein is Tpo1, from the MFS superfamily, that was initially identified as a polyamine  
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39 transporter involved in the resistance of yeast towards spermidine (Albertsen, Bellahn, Krämer,  
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41 & Waffenschmidt, 2003; Tomitori, Kashiwagi, Sakata, Kakinuma, & Igarashi, 1999). Further  
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43 studies identified different substrates for this transporter, including medium-chain fatty acids  
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45 (Legras et al., 2010). Other proteins involved in fatty acid export are the pathogen-related yeast  
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47 (Pry) proteins. *S. cerevisiae* contains three Pry proteins, two of which are involved in the export  
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49 of sterol molecules (Darwiche, El Atab, Cottier, & Schneiter, 2018). One of these proteins, Pry1,  
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51 is also able to bind fatty acids and its deletion hampers the secretion of fatty acids in a fatty acid  
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53 accumulating mutant strain (Darwiche, Mène-Saffrané, Gfeller, Asojo, & Schneiter, 2017).  
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Fatty acid export in yeast has been engineered to improve productivity of microbial cell factories. The heterologous expression of human transporter FATP1 in *Y. lipolytica* increased extracellular fatty acid titre from 60mg/L to 190 mg/L. Furthermore, this transporter also showed activity towards fatty alcohols, rising the percentage of extracellular fatty alcohols from 9% to 29% of total fatty alcohols produced (Hu, Zhu, Nielsen, & Siewers, 2018). In *S. cerevisiae*, Tpo1 has been engineered through directed evolution to increase medium-chain fatty acid export rate (Zhu et al., 2020). The mutants were obtained by selecting for increased resistance against medium-chain fatty acids through two rounds of enrichment in selective media containing decanoate of a library of Tpo1 mutants obtained by error-prone PCR. The mutations F322L, T45S, and I432N increased resistance against both decanoate and octanoate and F322L was identified as the mutation with the highest impact. Integration of two copies of this engineered Tpo1 in *S. cerevisiae* increased the extracellular fatty acids with a chain length of 6 to 10 carbon atoms about 2-fold and those with 12 and 14 carbon atoms about 4-fold.

## Fatty acid transport in microalgae

### Export of fatty acids in microalgae

Microalgae release fatty acids to the extracellular medium in their natural habitat, proposedly as allelopathic compounds (Allen, Ten-hage, & Leflaive, 2018; Sushchik, Kalacheva, Zhila, Gladyshev, & Volova, 2003). Although allelopathic compounds can have a wide chemical diversity, fatty acid and derivatives are common in water ecosystems and their production is increased under conditions that do not allow for optimal growth (such as nitrogen or phosphate limitation) but allow for efficient photosynthesis (Allen et al., 2018). Microalgae fatty acid production has been linked to allelopathic effects on competitor organisms due to the capacity of certain fatty acids to alter membrane permeability (J. T. Wu, Chiang, Huang, & Jane, 2006).

For example, the green algae *Uronema confervicolum* can secrete 1.45 µg/L of free fatty acids, 77% of which correspond to linoileic and linolenic acid (Allen et al., 2018). In the same study, these fatty acids were observed to inhibit growth of the diatom *Fistulifera saprophila*, although at higher concentrations than those produced by *U. confervicolum*. The green alga *Chlorella vulgaris* has also been observed to produce fatty acids at a concentration of 0.85 mg/L/10<sup>6</sup> cells under phosphate limiting conditions (DellaGreca et al., 2010). The mixture of fatty acids changes drastically when comparing two scientific reports and is reflected in the production of palmitic acid, which can be absent from the fatty acid mixture or be the most abundant depending on the growth conditions, possibly due to the mode of CO<sub>2</sub> supply or different medium compositions (DellaGreca et al., 2010; Sushchik et al., 2003). The fatty acid mixture produced by *C. vulgaris* has been observed to be toxic to the alga *Raphidocelis subcapitata*, resulting in the extinction of this alga when grown in a coculture with *C. vulgaris* (DellaGreca et al., 2010). While microalgae are observed to secrete fatty acids, no uptake has been described, probably due to the autotrophic nature of microalgae.

Fatty acid secretion in microalgae can be increased by means of genetic engineering. Knockdown of long-chain acyl-CoA synthetase genes *cracs1* and *cracs2* in *Chlamydomonas reinhardtii* cc849 (a cell wall deficient strain) increased extracellular fatty acid production from 2.93 mg/10<sup>9</sup> cells to 8.19 mg/10<sup>9</sup> cells and 9.66 mg/10<sup>9</sup> cells, respectively (Jia et al., 2016). Overexpression of transcription factor NobZIP1 in *Nannochloropsis oceanica* increased the extracellular fatty acid content by 40% (D. Li et al., 2019). The study of the effects of NobZIP1 revealed that one of the negatively regulated targets, UDP-glucose dehydrogenase, an enzyme involved in cell wall polymer metabolism, is linked to lipid metabolism. Silencing this enzyme through interference RNA increased the extracellular fatty acid content by 20% (D. Li et al., 2019). Although the secretion of fatty acids in algae has been widely studied, no export system has been identified to date.



## Intracellular trafficking of fatty acids in microalgae

Microalgae, analogous to plants, synthesize fatty acids in plastids. However, synthesis of triacylglycerol and other lipids takes place in the endoplasmic reticulum and therefore the newly synthesized fatty acids must be export from the plastid to the cytosol. A plastid fatty acid exporter acting on free fatty acids, AtFAX1, has been identified and studied in *Arabidopsis thaliana* (N. Li et al., 2015). Orthologues of this protein can be found in microalgae, and their function has been studied in some of them. The green-alga model *Chlamydomonas reinhardtii* contains two orthologues, CrFAX1 and CrFAX2, whose overexpression respectively increased neutral lipid content 15% and 17% under nitrogen limiting conditions (N. Li et al., 2019). Overexpression of these genes produced more and larger lipid droplets, increasing the content of intracellular triacylglycerol by 38%. The fatty acid composition did not show significant variation, showing that these transporters are involved in the transport of both saturated and unsaturated fatty acids. A fatty acid plastid exporter, CmFAX1, has also been identified in an extremophilic red microalgae, *Cyanidioschyzon merolae*, that inhabits sulfuric acid hot springs (Takemura, Imamura, & Tanaka, 2019). This exporter was confirmed to be located in the plastid membrane through targeted immunofluorescence and its fatty acid transport activity was verified through complementation experiments in yeast cells lacking the FAT1 transporter. Deletion of CmFAX1 in *C. merolae* increased free fatty acid content in the total cell extract 2.5-fold and CmFAX1 overexpression increased lipid droplet formation 2.4-fold. In contrast to the CrFAX transporters, the overexpression of CmFAX1 introduced significant changes in the fatty acid composition of the triacylglycerol molecules, incorporating C14:0, C14:1 and C20:0 fatty acids and increasing the content of C18:2 (Takemura et al., 2019).

After the free fatty acids have abandoned the plastid, they are incorporated into lipid droplets in the form of glycerolipids. For this, they need to be activated to acyl-CoA by a long-chain acyl-CoA synthetase. *C. reinhardtii* encodes three putative long-chain acyl-CoA synthetase and two

of them were found to be associated to lipid droplets in a proteomic study (Nguyen et al., 2011). The deletion of one of them, LCS2, led to a 2-fold increase in triacylglycerol rich in polyunsaturated fatty acids (e.g. 52:10 or 54:9) showing that this enzyme is mainly associated to the activation of saturated fatty acids produced *de novo* in the plastids (X. Li et al., 2015).

Once the growth conditions change and the lipid reserves are needed for survival and growth, fatty acids get mobilised from lipid droplets by lipases and long-chain acyl-CoA synthetases. The resulting acyl-CoA molecules must be incorporated into peroxisomes for their degradation (Kong et al., 2017; Figure 6). This is speculated to happen in similar way to plants and yeast, via a transporter with thioesterase activity and the later participation of a peroxisomal long-chain acyl-CoA synthetase. While there have been no studies to date on microalgae to support this hypothesis, *C. reinhardtii* possesses a putative fatty acid peroxisomal transporter, ABCD1 (Kong, Romero, Warakanont, & Li-Beisson, 2018). An overview of the intracellular trafficking of fatty acids and acyl-CoA molecules in *C. reinhardtii* can be observed in Figure 6.

Conclusion and future perspectives

Even though the role of passive diffusion of fatty acids through membrane is only lately being put into question, the study of fatty acid transport systems during the last years has shed light on several of these systems and their mechanism (Table 2). Differences in membrane structure and divergence of physiological needs cause a wide diversity of fatty acid transport systems. This diversity is reflected on the existence of both single protein systems and multiprotein complexes as well as transport systems with or without acyl-CoA synthetase activity. Furthermore, the higher intracellular complexity of eukaryotic organisms needs the presence of transporters for the organelle membranes and trafficking systems between different hotspots for fatty acid metabolism and storage. Although many proteins involved in the microbial transport of fatty acids have already been identified, there are still important knowledge gaps, such as the

transport mechanism of most of these proteins, the identification of fatty acid export proteins for microalgae or the study of fatty acid transport systems in filamentous fungi, such as *Aspergillus niger* or other model microorganisms such as *B. subtilis*. Finally, the engineering examples showed in this review are expected to keep increasing in number as more fatty acid transport proteins are identified and their mechanisms are elucidated. This will be an essential step in the optimization of microbial cell factories that consume or produce fatty acids and related compounds.

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Conflict of Interest

The authors declare no conflict of interest

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Tables

Table 1: FadL residues which have been shown or suggested to be involved in transport of fatty acids

Residue	Region	Effect on transport	Reference
F3	Binding Pocket	Abolished	(Lepore et al., 2011)
N33/P34/A35	Hatch Domain	Reduced	(Hearn et al., 2009)
S100/N101/Y102/G103	S3 kink	Abolished	(Hearn et al., 2009)
G143	Hatch Domain	-	(Van Den Berg, 2005)
R157	Binding Pocket	-	(Van Den Berg, 2005)
G212	Hatch Domain	No effect	(Hearn et al., 2009)
D348	Binding Pocket	Abolished	(Lepore et al., 2011)

Table 2: Proteins involved in microbial fatty acid transport processes

Protein Name	Organism	UniProt identifier	Activity	Localization	Reference
FadL	<i>E. coli</i>	P10384	Fatty acid import	Outer plasma membrane	(Nunn & Simons, 1978) (Lepore et al., 2011)
FACS	<i>E. coli</i>	P69451	Long-chain acyl-CoA ligase	Inner plasma membrane	(Overath, Pauli, & Schaire, 1969)
Ltp1	<i>R. jostii</i>	Q0S4W2	Fatty acid import	Plasma membrane	(Villalba & Alvarez, 2014) (Herrero et al., 2018)
Mce1 complex	<i>M. tuberculosis</i>	O07412 (YrbE1A)	Fatty acid import	Plasma membrane	(Nazarova et al., 2019)
Rv1272c	<i>M. tuberculosis</i>	P9WQJ3	Fatty acid import	Plasma membrane	(Martin & Daniel, 2018)
AcrAB-TolC Complex	<i>E. coli</i>	P0AE06 (AcrA) P31224 (AcrB) P02930 (TolC)	Fatty acid export	Double membrane	(Lennen et al., 2013) (Wang et al., 2017)
AcrEF-TolC complex	<i>E. coli</i>	P24180 (AcrE) P24181 (AcrF) P02930 (TolC)	Fatty acid export	Double membrane	(Wu et al., 2019)
FarE	<i>S. aureus</i>	A0A114B8E3†	Fatty acid export	Plasma membrane	(Alnaseri et al., 2019)
AdelJK complex	<i>A. baumannii</i>	Q2FD95† (Adel) Q2FD94† (Adel) Q24LT6 (AdeK)	Fatty acid export	Double membrane	(Jiang et al., 2019)
EmhABC complex	<i>P. fluorescens</i>	Q4KH22† (EmhA) Q4KH23†	Fatty acid export	Double membrane	(Adebusuyi & Foght, 2011)

		(EmhB) A0A4P7I039 (EmhC)			
<b>FarAB complex</b>	<i>N. gonorrhoeae</i>	Q9RQ30 (FarA) Q9RQ29 (FarB)	Fatty acid export	Double membrane	(Lee & Shafer, 1999)
<b>Slr2131</b>	<i>Synechocystis</i> sp. PCC 6803	P73998	Fatty acid export	Inner plasma membrane	(Bellefleur et al., 2019)
<b>RndA1B1 complex</b>	<i>S. elongatus</i>	Q31KM1 (RndA1) Q31KM0 (RndB1)	Fatty acid export	Inner plasma membrane	(Kato et al., 2015)
<b>Fat1</b>	<i>S. cerevisiae</i>	P38225	Fatty acid import and very long-chain acyl-CoA ligase	Plasma membrane, lipid bodies, ER and peroxisome	(Zou et al., 2002)
<b>Faa1</b>	<i>S. cerevisiae</i>	P30624	Long-chain acyl-CoA ligase	Plasma membrane, mitochondria, ER, lipid droplets	(Zou et al., 2003)
<b>Faa4</b>	<i>S. cerevisiae</i>	P47912	Long-chain acyl-CoA ligase	Plasma membrane, cytoplasm, lipid droplets	(Johnson et al., 1994)
<b>Ypk1</b>	<i>S. cerevisiae</i>	P12688	Protein kinase (Endocytosis)	Cytoplasm and plasma membrane	(Jacquier & Schneider, 2010)
<b>End3</b>	<i>S. cerevisiae</i>	P39013	Actin and calcium binding (Endocytosis)	Plasma membrane, cytoskeleton and endosomes	(Jacquier & Schneider, 2010)
<b>Vrp1</b>	<i>S. cerevisiae</i>	P37370	Actin binding (Endocytosis)	Cytoskeleton	(Jacquier & Schneider, 2010)
<b>Srv2</b>	<i>S. cerevisiae</i>	P17555	Actin and adenylate cyclase binding (Endocytosis)	Cytoskeleton	(Jacquier & Schneider, 2010)
<b>CaFaa4</b>	<i>C. albicans</i>	A0A1D8PU56	Long-chain acyl-CoA ligase	Cytoplasm	(Tejima et al., 2018)
<b>YIFat1</b>	<i>Y. lipolytica</i>	Q6C5Q8	Fatty acid transport and long-chain acyl-CoA ligase	Lipid bodies and peroxisomes	(R. Dulerio et al., 2014)
<b>YIFaa1</b>	<i>Y. lipolytica</i>	Q6C8Q3	Long-chain acyl-CoA ligase	Cytoplasm	(R. Dulerio et al., 2015)
<b>Acb1</b>	<i>S. cerevisiae</i>	P31787	Acyl-CoA-binding	Cytoplasm, nucleus and extracellular region	(Schjerling et al., 1996)
<b>Pxa Complex</b>	<i>S. cerevisiae</i>	P41909 (Pxa1) P34230 (Pxa2)	Fatty acid import and acyl-CoA thioesterase	Peroxisome	(Shani et al., 1996)

<b>Faa2</b>	<i>S. cerevisiae</i>	P39518	Acyl-CoA ligase	Peroxisome	(Knoll et al., 1994)
<b>Tpo1</b>	<i>S. cerevisiae</i>	Q07824	Fatty acid export	Plasma membrane	(Legras et al., 2010)
<b>Cracs1</b>	<i>C. reinhardtii</i>	A8JH58	Long-chain acyl-CoA ligase	Unknown	(Jia et al., 2016)
<b>Cracs2</b>	<i>C. reinhardtii</i>	A0A3S6D659	Long-chain acyl-CoA ligase	Unknown	(Jia et al., 2016)
<b>NobZIP1</b>	<i>N. oceanica</i>	A0A3S7YS51	Transcription factor	Nucleus	(D. Li et al., 2019)
<b>CrFAX1</b>	<i>C. reinhardtii</i>	Cre10.g421750†	Fatty acid export	Plastid	(N. Li et al., 2019)
<b>CrFAX2</b>	<i>C. reinhardtii</i>	Cre08.g366000†	Fatty acid export	Plastid	(N. Li et al., 2019)
<b>CmFAX1</b>	<i>C. merolae</i>	M1V3K0	Fatty acid export	Plastid	(Takemura et al., 2019)
<b>LCS2</b>	<i>C. reinhardtii</i>	Cre13.g566650‡	Long-chain acyl-CoA ligase	Lipid bodies	(Nguyen et al., 2011) (X. Li et al., 2015)
<b>ABCD1</b>	<i>C. reinhardtii</i>	Cre15.g637761‡	Putative peroxisome transporter	Peroxisome	(Kong et al., 2018)

† Multiple Entries available in UniProt

‡ AlgaePath identifier (no uniprot identifier available)

<http://algaepath.itps.ncku.edu.tw/index.html>



## List of Figures with captions

**Figure 1 A) Structure of FadL:** The  $\alpha$ -helices of the extracellular region forming the opening of a hydrophobic groove are shown in orange. Inside the protein, several residues that can interact from the binding pocket are shown in red. The hatch domain of the intracellular region is shown in blue. Finally, one of the  $\beta$ -strands (strand 3 or S3), presents a bend or a kink, shown in green, that leads to the formation of an opening in the intramembrane surface of the protein. **B) Transport mechanism of FadL:** Schematic representation of the transport mechanism of FadL proposed by Lepore et al., 2011. The yellow box represents the bilipid layer and the red hexagons the lipopolysaccharide layer.

**Figure 2 Bacterial fatty acid import systems:** **A)** In certain Gram-negative bacteria a membrane-associated acyl-CoA synthetase imports the fatty acids to the cytosol in the form of acyl-CoA, a process known as vectorial acylation. **B)** In Gram-positive bacteria, single proteins have been identified in organisms such as *R. jostii* and *M. tuberculosis* which may be able to import free fatty acids into the cells, which will be later activated for their metabolization. **C)** In *M. tuberculosis*, the multiprotein complex Mce has been identified. The figure shows a hypothetical model adapted from Nazarova et al., 2019.

**Figure 3 Structure of the AcrAB-TolC complex:** The multiprotein complex AcrAB-TolC spans from the inner to the outer membrane.

**Figure 4 Structure of Fat1:** Fat1 presents two transmembrane domains and two domains which are partially integrated in the membrane. ATP/AMP binding domains are found near the two latter domains. A VLACS domain is found further in the C-terminal region. Finally, the soluble Faa1 has been observed to interact with the C-terminus of this protein to activate the imported fatty acids.

**Figure 5 Intracellular trafficking of fatty acids in yeast cells:** Simplified fatty acid transport routes in *S. cerevisiae* based on current knowledge. Both plasma membrane transport processes and intracellular trafficking routes are showed.

**Figure 6 Intracellular trafficking of fatty acids in microalgae cells:** Fatty acid intracellular trafficking routes in *C. reinhardtii*. No transport system has been described for the outer plastid membrane and for the export of free fatty acids to the extracellular media.

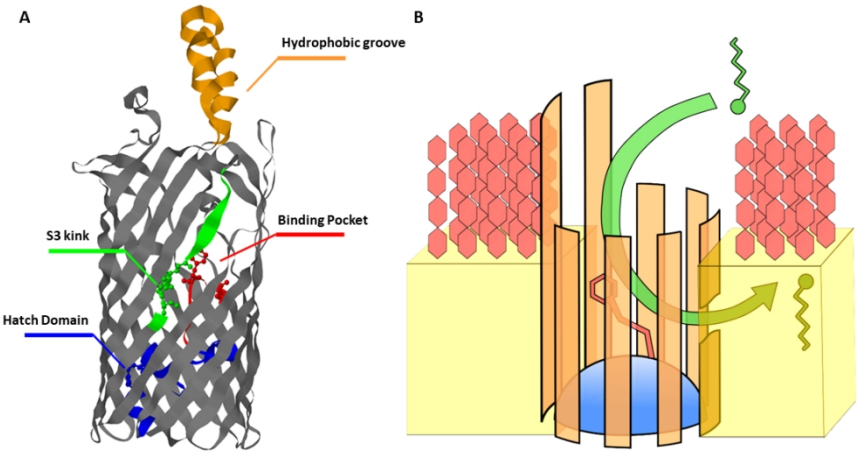


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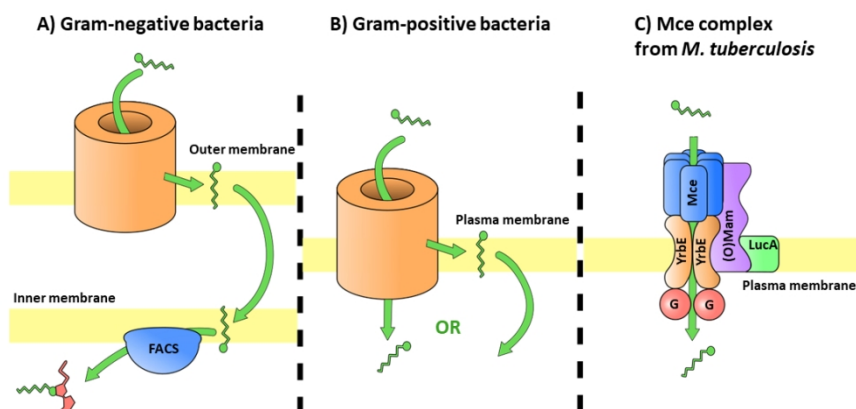


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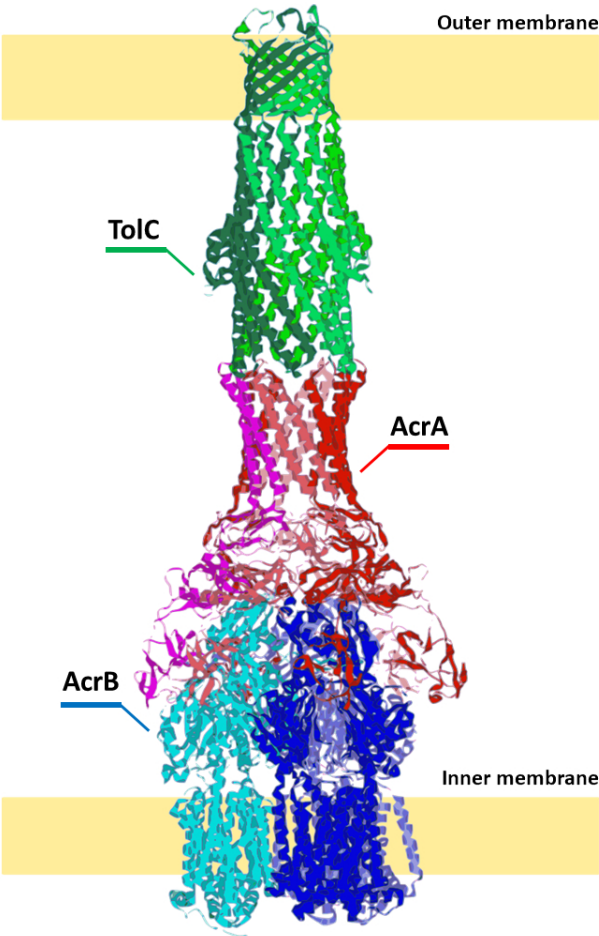


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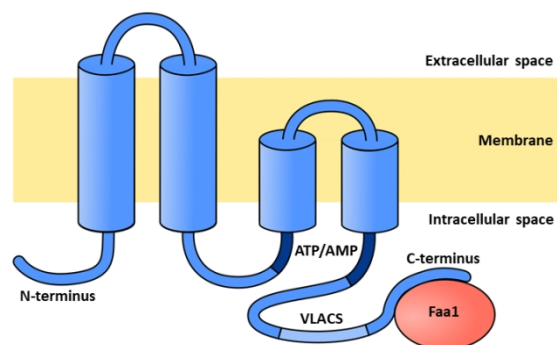


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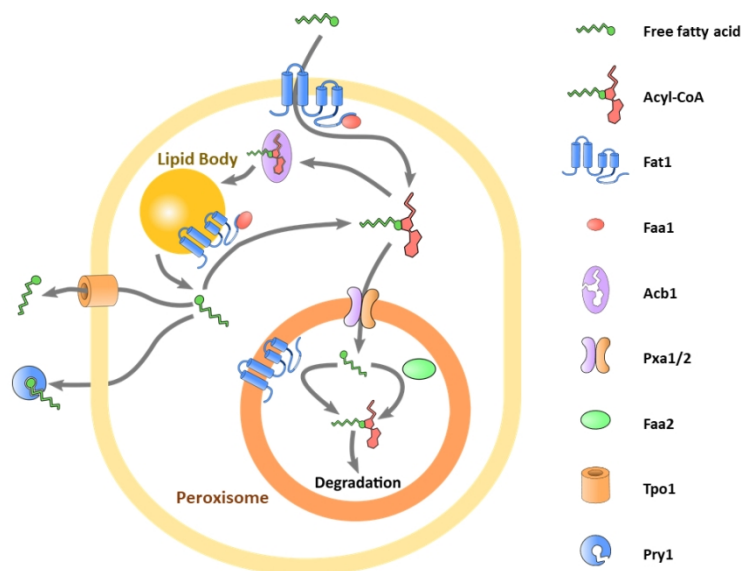


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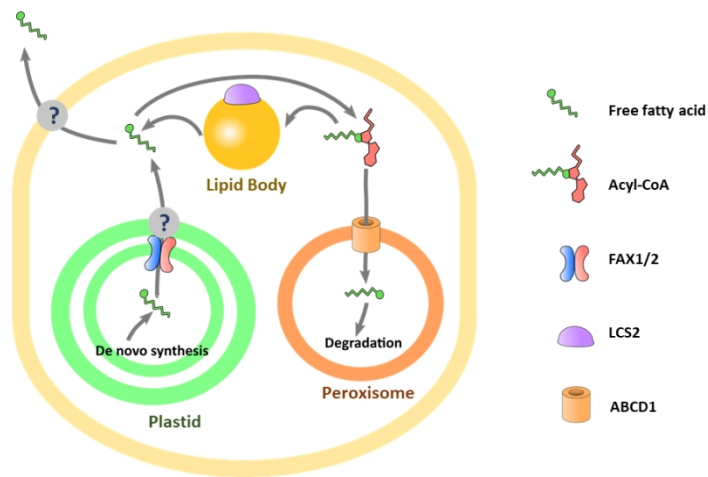


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