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"Click Chemistry" for the Simple Determination of Fatty-Acid Uptake and Degradation: Revising the Role of Fatty-Acid Transporters

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Fatty acids (FAs) have numerous functions in all living organisms, ranging from structural roles and energy production to the biosynthesis of secondary metabolites. Because of the high energy content of exogenous FAs, their acquisition is central of metabolism, and several biological systems are known, although their precise roles are not yet entirely clear. We investigated the roles of FadD (CoA ligase) and FadL (FA transporter) in different bacterial strains by using an improved version of click-chemistry-assisted labelling of azido-FAs. The high sensitivity of this method allows a direct and precise assessment of FA metabolism, and is thus far better suited than growth experiments. Our results show that although FA activation is indeed essential for FA degradation, their transport can be independent of transporters like FadL.

Fatty acids (FAs) have several roles in all living organisms, far beyond energy production and assembly of the well-known and vital lipid bilayer. One of the most recent observations is that FAs are the major carbon source for dNTP biosynthesis in endothelial cells during angiogenesis (and thus also tumour growth) in mammals.[1] Additionally, many bacteria incorporate FAs into various secondary metabolites, such as acylated nonribosomal peptides, pyrones and stilbenes.[2] In all of these cases, transport of exogenous FA into the cell and activation of the rather unreactive carboxylic head group are important, if not vital, for example, when FAs constitute the sole carbon source. In *Escherichia coli* two proteins are involved in FA acquisition (Figure 1):[3] FadL, a passive and unselective FA transporter in the outer membrane, and FadD, a coenzyme A (CoA) ligase that activates fatty acids with concomitant ATP hydrolysis. The CoA thioesters thus formed then enter several biochemical pathways, including oxidative degradation and polyketide or nonribosomal peptide synthesis.[4]

By using our reported method for azide labelling, it is possible to follow these pathways in a very direct fashion.[5] The method employed in this work involves the derivatisation of azido fatty acids (AFAs) from hydrolysed cell-pellet extracts with a bicyclononyne (BCN, **1**, Scheme 1) and subsequent analysis by HPLC-MS. The use of BCN,[6] rather than the previously

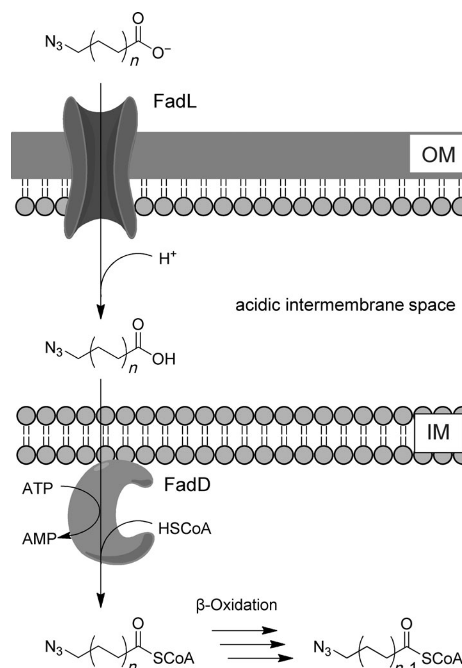
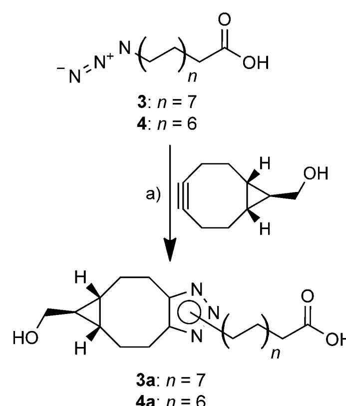


Figure 1. Established model of fatty acid transport through the outer membrane (OM) by FadL and subsequent activation by inner membrane (IM)-associated FadD in Gram-negative bacteria[8] shown for the azido fatty acids used in this work. Oxidative degradation by C_2H_4 steps is shown for the activated CoA-thioesters that are formed in vivo.



Scheme 1. In vitro derivatisation of AFAs with BCN. a) ACN, RT, 24 h.

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used dibenzoazacyclooctyne derivative (TDAC, **2**, Figure S1 in the Supporting Information)[7] not only leads to better peak shapes, but also has a more straightforward synthesis as well as easier purification and isolation of single diastereomers, whose click adducts form physically identical enantiomers (Scheme S1).

First, BCN was tested as a suitable derivatisation agent for AFAs. 16-Azidohexadecanoic acid (**3,** C_{16}-AFA) was treated with BCN in acetonitrile at room temperature for 24 h, leading to the expected click adduct **3a**. Next, a chromatographic analysis of the reaction products of **3** with pure *exo*-BCN or a mixture of *exo*- and *endo*-BCN was performed: there was little notable difference in retention time, and no by-products were visible (Figure S2). This shows that the synthetic procedure can be simplified if desired, although in this work pure *exo*-BCN was used. (*Endo*-BCN can also be obtained from Sigma–Aldrich.)

We then investigated the role of FadD and FadL in the uptake and subsequent degradation of AFAs with our model organism *E. coli* strain MG1655. Mutants of MG1655 lacking either *fadD* (BW25113Δ*fadD*) or *fadL* (BW25113Δ*fadL*) were compared their abilities to produce C_{14}-AFA (**4**) from externally provided C_{16}-AFA (**3**) by the β-oxidative degradation pathway (Figure S3).[4] If FAs cannot easily diffuse across the bacterial membrane and their activation is crucial for β-oxidation, no (or at least a decreased) production of AFA degradation products would be expected in the deletion strains,[9,10] whereas it should be detectable in the wild-type strain, as shown in our previous work.[5]

The feeding experiment clearly showed that FadD is essential for processing long-chain FAs (LCFAs), as no degradation product was observed in the Δ*fadD* mutant 4 h after addition of **3** (Figure 2). This is in accordance with previous findings showing that *Pseudomonas aeruginosa* depends on FadD homologues for survival in medium with FAs as the sole carbon source (β-oxidation was shown only indirectly, by growth experiments).[8] The influence of FadL on the process is more subtle, as a delay (~1 h) in FA degradation was observed for the Δ*fadL* mutant compared to wild type. This suggests that AFA transport into the cell does not necessarily require the FadL transporter, but might occur by diffusion. This is especially likely given the low pH in the intermembrane space of Gram-negative bacteria;[11] the low pH facilitates protonation of FA carboxylate moieties caught in the outer membrane and subsequent diffusion to the inner membrane, where no FA transporter has been identified.[12] Furthermore the findings of Hearn et al.[13] strongly reinforce the notion that FAs generally are more free to move within and between membranes than earlier models would suggest; this is in accordance with our results.

To get a clearer picture of the influence of FadL on FA uptake, AFA degradation was monitored in various *Sinorhizobium meliloti* mutants lacking genomic *fadL*.[14] Instead, the mutants expressed FadL from *Agrobacterium tumefaciens* (At), *Mesorhizobium loti* (Ml), or *S. meliloti* (Sm); the FadL-deletion mutant (SmΔ*FadL*) was used as a control. The growth of these mutants on minimal medium with oleic or palmitic acid as the sole carbon source was previously investigated: wild-type *S. meliloti* was unable to grow under these conditions, so its FadL is most likely not involved in the transport of these FAs.[14] The mutants were fed with **3** under normal growth conditions, and the amount of oxidation product **4** was measured (by derivatisation to **4a**) 6 h after feeding (to account for the slower growth rate of *S. meliloti* compared to *E. coli*; Figure 3). As expected from the FadL-deletion experiment in *E. coli*, all tested strains showed degradation activity, including the deletion mutant, although certain differences in efficacy were observed. The greatest amounts of **4** were detected with variants expressing At and Ml, closely followed by Sm; as expected, the lowest production was observed with SmΔ*FadL*. This suggests different degrees of contribution of FadL to the FA degradation process; previously merely a broad chain-length tolerance was reported.[15]

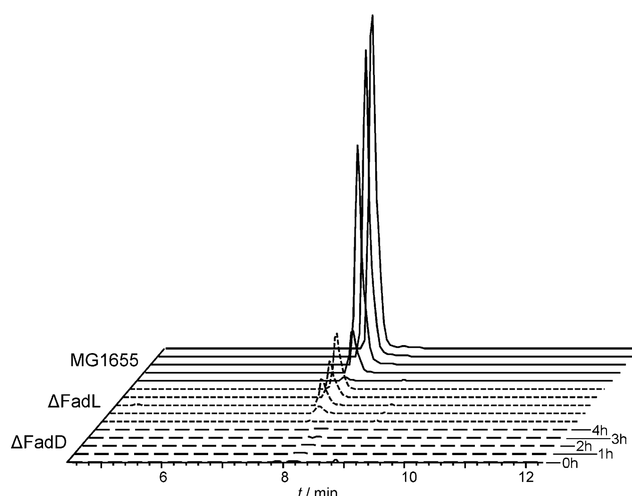

Figure 2. Extracted ion chromatograms of derivatised AFA degradation product **4a** (*m/z* 420) at different time points after feeding of **3** at 0.1 mM to the wild type (MG1655; ——) and ΔFadL mutant (····). The ΔFadD (-----) mutant was grown with the higher concentration of 1 mM of **3**. Whereas deletion of *fadL* significantly slows degradation of external AFA in comparison to the wild type, deletion of *fadD* completely prevents AFA degradation even at higher substrate concentration.

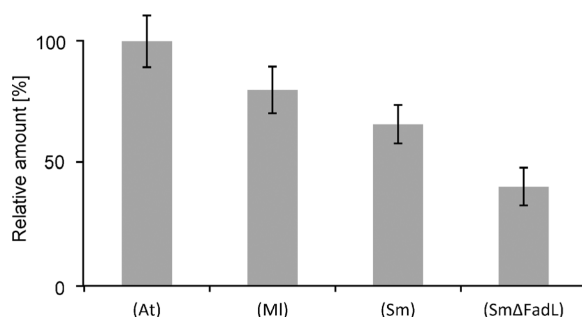

Figure 3. Amounts of **4a**, normalised to the strongest producer, after 5 h of feeding 1 mM **3** to various *S. meliloti* FadL strains. Although there is a much greater amount of oxidation product with the At mutant, even in the complete absence of FadL (SmΔFadL), significant amounts of oxidation product can still be observed (Figure S4).

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After confirming that AFA uptake and degradation are possible in a complete medium, the *S. meliloti* mutants were tested for growth in minimal medium with FAs as the sole carbon source. As it has been shown that growth on palmitic acid minimal medium is possible for at least some of the strains,[14] growth was compared under the same conditions but with **3** or stearic acid as carbon source (stearic acid has about the same length as **3**). As the solubility of both **3** and stearic acid are very low (compared to the more soluble oleic acid tested previously), the experiment was conducted on an opaque agar emulsion, and the resulting clearing of the growing bacteria by FA consumption was observed 8 days after plating. As predicted by the cycloaddition-based assay, all strains, including the FadL deletion mutant, were able to grow on AFA minimal medium with only little observable differences. Interestingly, no growth at all was observed on stearic acid minimal medium (Figure S5), although it was still observable to some degree with palmitic acid for At and Ml, as has been shown previously.[14]

It thus appears that not only might FadL from different strains exhibit different chain-length specificities, but that the permeability of the different FAs greatly differs, as concluded from the differences in growth of the FadL deletion mutants on different carbon sources. An AFA with slightly polar end group (instead of a nonpolar alkyl chain) might interact better with the charged outer membrane,[8] thereby facilitating uptake compared to standard FAs. Although this can be regarded as a disadvantage of AFAs as tools for studying FA transport, our data (Figures 2 and 3) show that they can still be used to determine the FadL contribution to FA uptake. Even more subtle differences in FA metabolism, such as depicted in Figure 3, can be accessed.

Our experiments confirmed that CoA ligation (FadD) is essential in the FA degradation pathway in Gram-negative bacteria. It is known that some organisms express more than one FadD homologue, with differences in substrate specificity, and thus are less prone to compromised FA degradation by a single knockout,[16] as is known for *P. aeruginosa*.[8] The role of FadL, however, is less clear cut: FA degradation occurred irrespectively of the type (or even presence) of FadL; rather, it seems to depend on the FA type. We thus suggest that FadL merely facilitates AFA migration through the outer membrane, thus boosting AFA consumption within the cell. It has been shown that FadL also plays a role in the transport of signalling molecules such as acylhomoserine lactones.[14] Thus, the general role of FadL might be the facilitation of lipophilic signals, as there is no chemical driving force to direct their transport into the cell. For FAs, FadD fulfils this role by their conversion into FA-CoA thioesters.

As FadL mutants generally grow very slowly or not at all on FA-containing minimal medium, the effect of an *fadL* mutation is difficult to analyse by growth experiments and is time consuming. The principal advantage of our approach is the very fast execution (5 h compared to 8 days) and precise determination of differences in FA uptake and degradation when compared to an analysis of cell growth alone, as the latter fails to detect FA degradation directly under normal growth conditions.[17] Even radioisotope feeding experiments, to the best of our knowledge, have been conducted only under unnatural growth conditions.[18]

Experimental Section

General experimental procedures: Solvents and reagents were obtained from Sigma–Aldrich. Silica gel chromatographic purification was performed on an SP1 Flash Purification System (Biotage, Uppsala, Sweden) with 40M KP-Sil cartridges (Biotage) and a UV detector. ^{1}H and ^{13}C NMR spectra for the synthesis products were recorded on AV500 (500 MHz; Bruker) and AV400 (400 MHz) spectrometers with $CDCl_3$ as solvent and internal standard (^{1}H NMR: $CHCl_3$: $\delta = 7.24$ ppm; ^{13}C NMR: $CDCl_3$: $\delta = 77.00$ ppm). ESI HPLC MS analysis was performed with a UltiMate 3000 system (Dionex) coupled to a AmaZon X mass spectrometer (Bruker) in MeCN/0.1% formic acid in H_2O (5:95→95:5% over 22 min, flow rate 0.6 mL min^{-1}).

Analytical procedure: For determination of **3** degradation the extracted ion chromatogram (EIC) peak of its first degradation product C_{14}-AFA-BCN adduct **4a** ($[M+H]^{+} = 420$) was monitored. Excess **3a** was not monitored, although its presence could always be detected.

Strains and cultivation conditions: *E. coli* MG1655 (wild type) and mutants BW25113Δ*fadD* and BW25113Δ*fadL* were obtained from the Keio collection,[19] and grown on solid and liquid lysogeny broth (LB, pH 7.0) at 30 °C on a rotary shaker (180 rpm). For plasmid selection kanamycin (40 μg mL^{-1}) and/or chloramphenicol (34 μg mL^{-1}) were added. The *S. meliloti* strains (described previously)[14] were grown in tryptone yeast (TY, pH 7.0) medium with kanamycin (200 μg mL^{-1}), gentamicin (40 μg mL^{-1}) and IPTG (100 μM) at 30 °C on a rotary shaker (180 rpm).

Azido fatty acid degradation experiments: The FA degradation experiments were conducted by addition of **3** (1.0 or 0.1 mM) to culture. Inoculation was performed from an overnight preculture. The optical density (OD_{600}) was set to 0.1, and the cultures were grown in 100 mL Erlenmeyer flasks containing LB (15 mL, pH 7.0) or tryptone yeast (TY, pH 7.0) medium at 30 °C on a rotary shaker (180 rpm) for 2 h before feeding. At the indicated times, culture samples (1 mL) were centrifuged (17 000 *g*, 5 min). The cell pellets were then lysed by incubation with NaOH (500 μL, 1 M) at 90 °C for 60 min. The lysate was acidified by addition of HCl (90 μL, 6 M), and hexane (800 μL) was added. The sample was mixed, centrifuged as before and the organic layer (500 μL) was placed in a 1.5 mL tube. After evaporation of the solvent the extract was dissolved in acetonitrile (200 μL) containing **1** (1 mM) and stirred for 24 h at 30 °C. An aliquot (100 μL) was subjected to HPLC-MS analysis.

Growth on MOPS-buffered minimal medium plates: For growth experiments of *S. meliloti fadL* mutants the strains were grown on carbon-free agar solutions based on MOPS minimal medium[20] supplemented with IPTG (500 μM) for *fadL* induction and Brij 58 (0.5%) for better solubility of fatty acids. Stearic acid or 16-azidopalmitic acid (5 mM; carbon source) was added with mannitol (3 g L^{-1}). A bacterial suspension (2 μL, $OD_{600} = 1$, in NaCl (0.9%)) was spotted on each plate and photographed after 2 or 8 days.

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Keywords: azido fatty acids · fatty acid transport · fatty acids · labeling · metabolism

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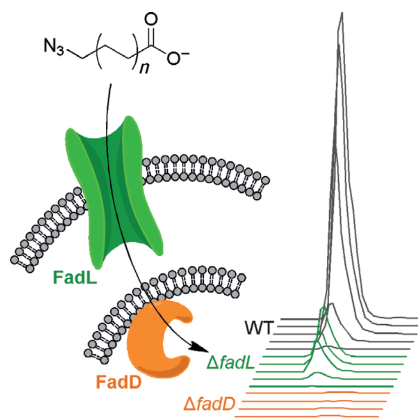
No way to hide: Click-chemistry-based fatty-acid analysis helps shed light on the behaviour of the bacterial proteins FadD and FadL for fatty-acid transport under natural growth conditions, and casts doubt upon the essentiality of FadL for fatty-acid transport through the membrane.



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