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Heterologous overexpression of a monotopic glucosyltransferase (MGS) induces fatty acid remodeling in *Escherichia coli* membranes

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ABSTRACT

The membrane protein monoglucosyldiacylglycerol synthase (MGS) from *Acholeplasma laidlawii* is responsible for the creation of intracellular membranes when overexpressed in *Escherichia coli* (*E. coli*). The present study investigates time dependent changes in composition and properties of *E. coli* membranes during 22 h of MGS induction. The lipid/protein ratio increased by 38% in MGS-expressing cells compared to control cells. Time-dependent screening of lipids during this period indicated differences in fatty acid modeling. (1) Unsaturation levels remained constant for MGS cells (~62%) but significantly decreased in control cells (from 61% to 36%). (2) Cyclopropanated fatty acid content was lower in MGS producing cells while control cells had an increased cyclopropanation activity. Among all lipids, phosphatidylethanolamine (PE) was detected to be the most affected species in terms of cyclopropanation. Higher levels of unsaturation, lowered cyclopropanation levels and decreased transcription of the gene for cyclopropane fatty acid synthase (CFA) all indicate the tendency of the MGS protein to force *E. coli* membranes to alter its usual fatty acid composition.

1. Introduction

Establishing a stable cell membrane is known to be essential for the regulation of most channels and transporters [1–4]. The stability and permeability of bacterial membranes are regulated by altering the chemical properties of membrane lipids, thus enabling the organism to adapt to changes in the extracellular milieu [5,6]. Fatty acids of lipids can easily be modified after their synthesis in a process known as *homoviscous adaptation* [7–9], which allows the cells to maintain an optimum fluidity (or viscosity) where a stable and highly selective cellular membrane can be created [10–12]. Various strategies exist for these modifications in different types of bacteria [13,14], such as (i) variation in acyl chain length distribution and (ii) introduction of branches via the β-ketoacyl-acyl carrier protein synthase III (FabH) controlled elongation pathway [9], (iii) incorporation of double bonds (unsaturation/desaturation) [14] and (iv) cyclopropanation of fatty acids [13].

Incorporation of double bonds creates a pronounced kink in the acyl chains of lipids and therefore tends to induce a disorder when all lipids are aligned in a bilayer. Thus, membranes with high unsaturated fatty acid (UFA) content have lower transition temperatures and a higher permeability compared to membranes with high saturated fatty acid (SFA) content [7,15]. Another type of modification is the conversion of a pre-existing double bonds (C═C) into their cyclic forms of methylated fatty acids (referred as *cyclopropanation*) [7,16]. This transformation is usually observed in bacteria entering the stationary phase of growth and is catalyzed by a membrane-associated enzyme called the cyclopropane fatty acid synthase (CFA), which is transcribed by the *cfa* gene [7,17]. In comparison with unsaturated fatty acids, cyclopropanated fatty acids create membranes with even lower phase-transition temperatures, increased fluidity and higher permeability to solutes (less ordered membranes) [7]. The modifications described above occur during cellular growth in order to maintain a stable and a highly selective cellular membrane.

Lipid and fatty acid modifications have been well-studied in the Gram-negative bacterium, *Escherichia coli* (*E. coli*) [18–20]. Under physiological conditions, *E. coli* has a mixture of acyl chains linked to a glycerol backbone, one saturated at the sn-1 position and the other unsaturated at the sn-2 position [21]. It can modify these fatty acids under various stress conditions [9,22–25] Changes in the saturated/unsaturated fatty acid ratio affect the fatty acid ordering of the membrane and have

Abbreviations: MGS, monoglucosyldiacylglycerol synthase; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin; GlcDAG, α-monoglucosyldiacylglycerol; CFAs, cyclopropanated fatty acids; UFAs, unsaturated fatty acids; SFAs, saturated fatty acids; CFA, cyclopropane fatty acid synthase; FAME, fatty acid methyl ester; RpoE, RNA polymerase subunit sigma 24 (σE) factor; PlsB, glycerol-3-phosphate acyltransferase; PSD, phosphatidylserine decarboxylase; FabH, β-ketoacyl-acyl carrier protein synthase; IM, inner membrane; OM, outer membrane, FT-IR, Fourier transform infrared spectroscopy; Cryo-TEM, cryo-transmission electron microscopy; FCM, flow cytometry; FSC-H, forward scatter (cell size); SSC-H, side scatter (granularity)

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an influence on the mobility of membrane proteins [26,27]. Motional freedom of membrane proteins in a bilayer can also be affected both by the lipid/protein ratio and the type of proteins present in the membrane [26,28,29].

Excessive production of a membrane protein is considered as a stressful condition for the cell [30,31], but it does not normally affect the intricate lipid–protein balance, since it is usually accompanied by an upregulated phospholipid synthesis so that the lipid/protein ratio remains constant [32,33]. However, some external or internal stress conditions, for example overexpression of a membrane protein affecting lipid metabolism can disturb this balance [9,34], as also found in this work. The unbalanced lipid and protein production might lead to the formation of intracellular membranes (sacks, vesicles or tubules etc.) [35–38]. A monotopic membrane protein, monoglucosyldiacylglycerol synthase (MGS) from *Acholeplasma laidlawii* was reported to result in the formation of such intracellular vesicles in *E. coli* when overexpressed [39]. Moreover, MGS overexpression has been previously shown to result in an upregulated phospholipid synthesis [40] but its connection to *homoviscous adaptation* and effects on the membrane order in *E. coli* has never been investigated previously.

Vesicle formation is an interesting but still not well-understood process. Thus understanding how MGS influences the bilayer during its induction can perhaps help us to gain further insight into the vesiculation phenomenon. Since the MGS protein is membrane-associated [41,42] and has the ability to stimulate phospholipid synthesis [40], it might also have the ability to affect fatty acid metabolism and its regulation on the packing properties of membranes. However, this possibility has not been investigated so far. We therefore investigated how *E. coli* membranes change during the induction/expression of the MGS protein by monitoring changes in lipid composition, fatty acid species and the protein/lipid ratio. Comparisons with data from investigations of regular *E. coli* membranes revealed several interesting types of modifications in membranes where the MGS protein was overexpressed. Here we report that MGS has a dramatic influence on the regulation of fatty acid synthesis, which in turn results in an alternative path for achieving membrane homeostasis.

2. Materials and methods

2.1. Growth and overexpression

The original gene for MGS protein from *A. laidlawii* was cloned into an *E. coli* strain BL21-AI™ (Invitrogen) as described previously [39,40]. During all assays BL21-AI *E. coli* strain with an empty vector served as a negative control. All transformants were selected with 100 μg/ml carbenicillin.

An overnight culture was prepared in 2× Luria–Bertani Broth (2× LB; 20 g/l Tryptone, 10 g/l yeast extract, 10 g/l NaCl) medium supplemented with 100 μg/ml carbenicillin and grown at 37 °C with 200 rpm for 15–16 h. On the next day, the overnight culture was inoculated as 1% into 2× LB fresh medium in the presence of 100 μg/ml carbenicillin. Cultures were grown at 37 °C with 200 rpm shaking until $OD_{600\ nm}$ values ~0.3–0.4 were obtained, then the temperature was decreased to 22 °C and gene expression was induced with 0.2% (w/v) L-arabinose and 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG). Control (BL21 AI) cells not containing the MGS gene were treated equally in parallel to the cells containing the MGS gene (BL21 AI-MGS).

Vesiculation was monitored at different time points (0, 0.25, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 10 and 22 h after IPTG addition) by sampling from cultures or completely harvesting cells (if indicated).

2.2. Cryogenic transmission electron microscopy (cryo-TEM)

Cell cultures (1 ml for each time point) were harvested (13,000 rpm/4 °C/10 min.) at different time points and flash frozen in 1× phosphate-buffered saline (PBS) buffer until they were investigated by cryo-TEM [39]. The cryo-TEM measurements [43] were carried out using a Zeiss Libra 120 Transmission Electron Microscope (Carl Zeiss NTS, Oberkochen, Germany). Analysis was performed under cryo-conditions and the microscope was operating at 80 kV and in zero loss bright-field mode. Digital images were recorded under low dose conditions with a BioVision Pro-SM Slow Scan CCD camera (Proscan GmbH, Scheuring, Germany) and iTEM software (Olympus Soft Imaging System, GmbH, Münster, Germany). In order to visualize as many details as possible, an underfocus of approximately 2 μm was used to enhance the image contrast.

Prior to imaging, the samples were treated by means of placing a drop of the sample solution on a grid with a holey polymer film (hole size ~2–6 μm) and then thinned by blotting it with some filter paper. This was done in an environmental chamber at 25 °C and close to 100% humidity in order to avoid dehydration of the sample. Once blotted the sample was quickly vitrified in liquid ethane held at a temperature just above its freezing point (−183 °C). After vitrification the sample was transferred to the microscope while maintaining it cold with liquid nitrogen and avoiding air to get in contact with the sample.

2.3. Flow cytometry (FCM) analysis

Vesiculation was screened using cells (500 μl culture) harvested at different stages of induction. Pelleted cells were resuspended in 500 μl BD FACS Flow™ buffer (BD Biosciences) and further diluted ×20 times. Cell membranes were stained with 1 μM FM® 4–64 membrane stain (Molecular Probes) and incubated on ice for 30 min at dark. During the flow cytometry analysis no gating was performed and 15 event/s were attained. Each run was made with 10,000 total events for each sample and the Cell Quest software was used during all measurements. Graphs were prepared with the FlowJo (Version 10.0.6) analysis software (Figure S2, Supporting Information).

2.4. Western blot analysis

Cell cultures (1 ml) were harvested as described above and were solubilised with 1 ml solubilisation buffer (100 mM HEPES, 20 mM $MgCl_2$ and 1% DDM). Samples were clarified by centrifugation at 13,000 rpm, 4 °C for 10 min. Western blots were performed with 20 μl of the clarified supernatants and proteins were detected with Penta-His™ antibody (BSA-free, QIAGEN) and Goat anti-mouse IgG HRP conjugate. The blots were visualized with ECLPlus Western Blotting Detection kit (GE-Healthcare) and recorded with CCD camera. Quantification of bands was performed with ImageGauge 4.0 software (FujiFilm Science Lab). A calibrated western blotting was performed to calculate the number of MGS molecules per cell for each time point. A purified MGS sample was applied in parallel as a standard and developed together with MGS-induction series on the same blot and all band intensities were normalized against previously measured OD_{600} values.

2.5. Lipid extraction and thin-layer chromatography (TLC)

Radioactive lipid analysis was performed with cell cultures (10 ml) grown as described previously. 0.1 μCi/ml of [1-^{14}C] acetate (Perkin Elmer, 55.3 mCi/mmole) was included in the growth media in order to obtain fatty acid labeling. Cells were harvested completely by centrifugation at 13,000 rpm at 4 °C for 20 min. Pellets were washed with 100 mM HEPES (pH 8) buffer and frozen at −20 °C overnight. Lipids were extracted from harvested cell pellets using the Bligh & Dyer extraction protocol [44] and samples were applied onto standard Silica gel 60 TLC plates (Merck), which was then developed in a chloroform/methanol/acetic acid 85:25:10 (v/v) solvent system in one dimension. TLC plates were dried after the run at room temperature and incubated with a PhosphoImager (FujiFilm) screen for 20 h; lipid bands were then visualized and quantified by electronic autoradiography (FLA3000) using