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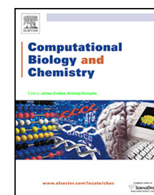

Research article

Enzyme function is regulated by its localization

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ABSTRACT

To better understand how enzyme localization affects enzyme activity we studied the cellular localization of the glycosyltransferase MurG, an enzyme necessary for cell wall synthesis at the spore during sporulation in the bacterium *Bacillus subtilis*. During sporulation MurG was gradually enriched to the membrane at the forespore and point mutations in a MurG helical domain disrupting its localization to the membrane caused severe sporulation defects, but did not affect localization nor caused detectable defects during exponential growth. We found that this localization is dependent on the phospholipid cardiolipin, as in strains where the cardiolipin-synthesizing genes were deleted, MurG levels were diminished at the forespore. Furthermore, in this cardiolipin-less strain, MurG localization during sporulation was rescued by external addition of purified cardiolipin. These results support localization as a critical factor in the regulation of proper enzyme function and catalysis.

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1. Introduction

The study of the mechanisms of protein localization in bacteria is simplified by their lack of multiple sub-cellular structures. *Bacillus subtilis* is a particularly tractable model, as during sporulation it generates a specialized intracellular compartment, the forespore. Sporulation occurs in exponential cells undergoing starvation and begins with asymmetrical division producing two sister cells of dramatically different size with a specialized spore septum. The smaller of the two cells, the forespore, is engulfed by the larger mother cell generating a double membrane around the forespore. The cortex is synthesized between the two membranes while the spore coat is generated on the outside of the spore. Finally, the mature spore is released upon lysis of the mother cell (Errington, 1993).

Several proteins localize specifically to the forespore through a diverse array of mechanisms such as diffusion and capture, targeted insertion, or selective degradation (Rudner et al., 2002; Shapiro et al., 2002, 2009). The caveat of such mechanisms is that they assume a pre-existing asymmetric distribution of the target molecule that is then transmitted to other proteins without explaining the origin of the asymmetry.

A recent study discovered that the basis for the localization during sporulation in *B. subtilis* of the membrane bound protein SpoVM was in part due to hydrophobic residues on an amphipathic helix (Ramamurthi et al., 2006; Ramamurthi and Losick, 2009). SpoVM can discriminate between the different curvatures of membrane surfaces and localize specifically to the forespore. Conversely, the protein DivIVA involved in cell growth and sporulation recognizes zones of negative curvature in the cell septum and poles and binds to membranes through a specific phenylalanine residue in its N-terminal coiled-coil domain (Lenarcic et al., 2009; Oliva et al., 2010; Ramamurthi and Losick, 2009)

A third example invoking geometric cues during sporulation, involves the anionic phospholipid cardiolipin (diphosphatidylglycerol) that has an uncommon dimeric structure in which two phosphatidyl moieties are linked by a glycerol generating a highly curved conic shape (Lecocq and Ballou, 1964). In eukaryotic cells, cardiolipin is known to organize mitochondrial topology and stabilize protein complexes (Schlattner et al., 2009), but also determines the distribution of essential mitochondrial proteins such as the pro-apoptotic factor Bid (Kim et al., 2004; Lutter et al., 2000; Petit et al., 2009), cytochrome c (Demel et al., 1989; Kagan et al., 2005) and the nucleoside diphosphate kinase NDPK (Tokarska-Schlattner et al., 2008). In the bacterium *Escherichia coli*, cardiolipin determines the localization of the osmosensor ProP, the mechanosensitive channel of small conductance MscS (Romantsov et al., 2009), the cell division protein MinD (Mileykovskaya and Dowhan, 2000; Mileykovskaya et al., 2003) and the protease HtrA (DegP) (Romantsov et al., 2007; Skorko-Glonek et al., 1997).

In *B. subtilis* cardiolipin is synthesized mainly by a membrane-bound enzyme encoded by the *clsA* gene (Kawai et al., 2004). Although cardiolipin represents only 1.4% of the total lipids of cells growing exponentially, at 4 h after initiation of sporulation (T4) its levels go up to 5.4% (Kawai et al., 2004) and to 26.5% in spores (Kawai

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et al., 2006). Cardiolipin-deficient cells sporulate with 50% less frequency than wild type cells (Kawai et al., 2004). Additionally, cardiolipin-deficient spores show increased sensitivity to wet heat and oxidizing agents (Griffiths and Setlow, 2009a). Cardiolipin can specifically accumulate at the spore although a GFP fusion to its synthase ClsA did not show a preferred distribution (Kawai et al., 2006). How then, does cardiolipin mainly localize to the spore? One model states that the preference of cardiolipin for the spore is due to its self-organization into domains that intrinsically prefer zones of high curvature which can be found at the poles and forespore membrane (Huang et al., 2006). An alternate possibility is based on the fact that lipids with overall conical shapes and wide hydrophobic tails such as cardiolipin prefer structures with negative curvature (Antonny, 2006; van den Brink-van der Laan et al., 2004). Although the forespore presents a globally positive curvature to the mother cell compartment (Ramamurthi et al., 2009), during engulfment the curvature at the migrating septal junction becomes extremely negative in the mother cell and could thus be a zone for cardiolipin accumulation

During sporulation in *B. subtilis*, cell wall synthesis becomes restricted to the forespore (Meyer, 2010). Cell wall gives shape to the bacterial cell and is composed of peptidoglycan, a rigid molecule made of repeated subunits of disaccharide peptide monomers (van Heijenoort, 2001). Studying cell wall synthesis during sporulation is tractable system for the study of enzyme activity, as once engulfment is finished, spore cell wall synthesis is only obligatory for its resistance to high temperature. Therefore, inactive enzyme mutants essential for sporulation are generally not otherwise lethal. The glycosyltransferase MurG is the last cytoplasmic enzyme of the peptidoglycan synthesis pathway, which is critical for sporulation, and although it is not an integral membrane protein, it transforms the membrane bound lipid I into lipid II by adding soluble UDP-GlcNAc. MurG localizes to zones of peptidoglycan synthesis and in *E.coli*, lipids that co-purify with MurG are enriched in cardiolipin (van den Brink-van der Laan et al., 2003).

Given recent interest in understanding how changes in cellular localization could regulate enzymatic activity (Meyer et al., 2014; Scheu et al., 2014), we investigated the mechanism of MurG localization to the forespore during sporulation. We found that localization of MurG directly impacts its function. During sporulation, MurG initially distributed diffusely throughout the cell and then gradually localized to the membrane patch where cell wall synthesis is active, the forespore. We show that MurG localization is dependent on an amphipathic N-terminal helical domain through which it inserts into membrane. Conversely, we show that the phospholipid cardiolipin is necessary to achieve this particular distribution of MurG. Finally, sporulation efficiency is severely affected in both cardiolipin-deficient and MurG-delocalized mutants. Thus the specific localization of the enzyme MurG through the mechanism that we here uncover is essential to perform effectively its catalytic functions.

2. Methods

2.1. Reagents

Purified *E. coli* cardiolipin was obtained from Avanti Polar Lipids, Inc., FM4-64 from molecular probes.

2.2. Fluorescence microscopy

For cultures in resuspension medium at 30 °C, 100 μL of sporulating cells were taken at designated times after resuspension. To each sample, 0.5 mL of FM4-64 (100 mg/mL) was added just before the cells were collected by centrifugation. The pellet was resuspended in 10 mL PBS, and added to a poly-L-lysine pre-treated coverslip. All microscopy was performed on a Nikon Eclipse 90i with a 100× objective using phase contrast and captured by a Hamamatsu Orca-ER camera using Nikon Elements BR software. Exposure for YFP, FITC and TRITC was 400 ms for all pictures taken.

2.3. Image analysis

We determined the forespore-specific localization of fluorescent signal from MurG-YFP as in (Real et al., 2008). In brief for each cell, we measured the maximum YFP fluorescence across a 10-pixel-wide window along the middle of the forespore and the maximum YFP fluorescence across across a 10-pixel-wide window in the middle of the mother cell while subtracting background noise for both. We took the average YFP fluorescence of the window for the forespore and mother cell for each cell and used these numbers to calculate the ratio of forespore to mother cell fluorescence. We repeated this for 100 cells from at least 3 experiments for each strain tested. Sporulating cells selected at random represented both engulfing and engulfed forespores. All operations were performed using software programmed with Matlab.

2.4. Time-lapse microscopy

Imaging with 1.2% agarose pads were prepared as in (Becker and Pogliano, 2007). FM 4-64 (Molecular Probes) was added to a final concentration of 0.5 μg/mL in a 1.2% solution of molten agar/media (CH or LB diluted 1:5 in water) and added to the well of a culture slide and covered with a glass slide. After cooling, the slide was removed and two air pockets were cut out of the agar leaving a 3 to 5 mm agar bridge in the center of the well. Sporulating cells resuspended in 25% LB with 0.5 μg/mL FM4-64 at 30 °C were added at T2 to the agar bridge and covered by a glass cover slip. To prevent drying during the experiment, 50% glycerol was applied to the region of contact between the slide and the coverslip. The slide was then allowed to equilibrate in an environmentally controlled chamber at 30 °C (Precision Control Weather Station) for at least 30 min prior to visualization. Images were acquired using an Applied Precision Spectris microscope.

2.5. Engulfment assay

Percentages of cell engulfment in a culture of sporulating cells in CH resuspension media was measured as in (Sharp and Pogliano, 1999). Briefly at different time-points during sporulation, a sample of cells is incubated briefly with the red membrane dye FM4-64 and Mitotracker-green. Forespores from cells that have finished engulfment will be stained with Mitotracker-green but not FM4-64 as this dye does not cross membranes. At each timepoint we calculate the ratio of engulfed vs total sporulating cells.

2.6. Addition of cardiolipin

10 mg of cardiolipin (Avanti Polar Lipids) were resuspended in DMSO to a 100 mM concentration and aliquoted for storage at −20 °C in glass tubes. Strains JDB1689 and JDB2238 (see Supplemental methods) were grown and resuspended in parallel. At T3 after the start of sporulation 30 μL of 100 mM cardiolipin were added to 3 mL of JDB2238 cells in resuspension to have a final concentration of 1 mM cardiolipin, while 30 μL of DMSO were added in parallel to 3 mL of JDB2238 cells in resuspension.

3. Results

We constructed a C-terminal YFP fusion and imaged the localization of the glycosyltransferase MurG to study its sub-

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