Membrane Topology Analysis of the *Escherichia coli* Aromatic Amino Acid Efflux Protein YddG

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Key Words

β-Lactamase • Fluorescence microscopy • Inner membrane protein • Poles of the cell • Reporter protein • Translation fusion • Transmembrane segment • ZsGreen

Abstract

YddG is an inner membrane protein (IMP) that exports aromatic amino acids in *Escherichia coli*. Topology models of YddG produced by sequence-based analysis in silico have predicted the presence of 9 or 10 potential transmembrane segments. To experimentally analyze the membrane topology of YddG, we used randomly created fusions to β-lactamase (BlaM) as a reporter. The selection of such fusions under 50 μg/ml of ampicillin had to fit with the periplasmic location of the BlaM domain. Five periplasmic loops of YddG predicted by the 10-transmembrane (TM) helices model were identified via the characterization of 12 unique in-frame fusions distributed along the *yddG* coding region. To confirm the 10-TM helices model further, cytoplasmic regions of YddG were identified with the help of ZsGreen fluorescent protein as a reporter. The presence of four cytoplasmic regions and the cytoplasmic localization of the C-terminus were revealed. Therefore, a 10-TM helices topology with cytoplasmic locations of the N- and C-termini is supported. The present data confirm the 'positive-inside rule' for IMPs

and the early results of other workers regarding the cytoplasmic location of the C-terminus of YddG. The pole-specific localization of YddG-ZsGreen in *E. coli* cells was detected by fluorescence microscopy.

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Introduction

Bacterial inner membrane proteins (IMPs) represent an important class of polypeptides that are involved in a wide variety of cellular functions. Among the ~4,500 open reading frames in the *Escherichia coli* genome (<http://www.ncbi.nlm.nih.gov>), approximately 900 (20%) are predicted to encode IMPs that belong to the helical bundle class of proteins [Díaz-Mejía et al., 2009]. These proteins span the membrane with different numbers of transmembrane (TM) helices. Because of their hydrophobic and amphiphilic nature, IMPs are difficult to study and to obtain high-resolution three-dimensional structures. Therefore, an accurate topology model describing the number of TM spans and their orientations relative to the lipid bilayer is an important characteristic of an IMP. Topology models are usually produced by sequence-based prediction, which is better to confirm by experimental approaches [Yuan et al., 2010]. A construc-

tion of translation fusions of the studied IMP with a topological reporter molecule is usually used for this purpose. β -Lactamase (BlaM), green fluorescent protein (GFP) and alkaline phosphatase are able to act as topological reporter molecules when fused to different portions of an IMP [Broome-Smith et al., 1990; Manoil, 1991; Rapp et al., 2004].

YddG is an IMP that participates in the export of aromatic amino acids in *E. coli* [Doroshenko et al., 2007] and paraquat in *Salmonella typhimurium* [Santiviago et al., 2002]. Therefore, YddG is classified as belonging to the aromatic amino acid/paraquat exporter (ArAA/P-E) family (2.A.7.17) of the drug/metabolite transporter (DMT) (2.A.7) superfamily of proteins (M.H. Saier, www.tcd.org). The size (293 aa) and primary structure of YddG, as the only protein of the ArAA/P-E family, most resembles the proteins of the drug/metabolite exporter (DME) family (2.A.7.3) within the DMT. These proteins have 10 putative TM helices, but this has been experimentally shown only for PecM, a DME member [Rouanet and Nasser, 2001].

The location of the YddG C-terminus was earlier determined in topology mapping studies [Rapp et al., 2004]. Further experimental investigations of the structure of YddG are crucial for understanding its biological functions and properties and its possible applications in the metabolically engineered *E. coli* strains overproducing various aromatic compounds. In the present study, we investigated the general topology of the YddG membrane protein by constructing translational fusions of the C-terminal-truncated YddG with β -lactamase or *Zoanthus* sp. green fluorescent protein (ZsGreen) [Matz et al., 1999] reporters. The YddG-BlaM hybrids increased β -lactamase activity when the BlaM domain was located in the periplasmic space. In contrast, YddG-ZsGreen produced intensive fluorescence when the ZsGreen domain was located in the cytoplasmic compartment.

The obtained data unequivocally confirm the 10-TM helices-based membrane topology of YddG. Localization studies of YddG-ZsGreen showed enhanced fluorescence at the poles of *E. coli* cells.

Results and Discussion

Construction and Analysis of the *yddG'*-*blaM* Gene Fusions

In silico topological analysis of YddG has predicted the existence of 9- or 10-TM helices in this protein, depending on the software used: 9 according to TMPRED [Hof-

mann and Stoffel, 1993], TM FINDER [Deber et al., 2001] and TopPred II [Claros and von Heijne, 1994], and 10 by SOSUI [Hirokawa et al., 1998] and TMHMM [Krogh et al., 2001] (fig. 1).

To clarify the membrane topological organization of YddG, *yddG'*-*blaM* fusions were created between exonuclease III-truncated *yddG* derivatives and the *blaM* gene encoding the leaderless β -lactamase. First, the recombinant plasmid pBRyddG-*blaM* was constructed based on pBR322 (Ap^R, Tc^R) (GenBank accession number J01749.1 [Sutcliffe, 1979]) as a vector. The *yddG* gene was inserted downstream of the P_{lac} promoter and upstream of *blaM*. In the presence of excess IPTG (1 mM and up to 3 mM) in the culture medium, this recombinant plasmid conferred ampicillin resistance (Ap^R) to ~10 μ g/ml and a high resistance to phenylalanine (up to 20 mg/ml) to TG1 cells. Such a low level of Ap^R was consistent with a cytoplasmic location for the C-terminal portion of the hybrid YddG-BlaM protein [Rouanet and Nasser, 2001]. The resistance to phenylalanine was indicative of YddG functioning as an aromatic amino acid exporter [Doroshenko et al., 2007]. Therefore, the hybrid protein was integrated into the inner membrane with its C-terminal portion located in the cytoplasm. The decrease of the IPTG concentration to 0.1 mM resulted in decreases of Ap^R and phenylalanine resistance. According to these results, it could also be proposed that overexpression of the YddG-BlaM fusion protein was not toxic to the bacterial cell even under conditions of a fully induced P_{lac} promoter.

A set of internal deletions in pBRyddG-*blaM* was obtained by random partial digestion of the 3'-terminus of the *yddG* gene (see 'Experimental Procedures'). The recombinant plasmids of interest were obtained by transforming TG1 cells and selecting tetracycline-resistant (Tc^R) clones. Approximately 100 Ap^R clones from 500 tested Tc^R clones were selected by growing on Luria-Bertani (LB) agar plates containing 1 mM IPTG and 50 μ g/ml of ampicillin. This ampicillin concentration permitted the selection of only those clones with β -lactamase exported to the periplasm [Rouanet and Nasser, 2001]. The junctions of 12 randomly chosen *yddG'*-*blaM* fusions were sequenced. All of these fusions were located within the *yddG* coding region but were not evenly distributed. They were grouped into five regions corresponding to their periplasmic loops or their surrounding regions in the 10-TM helices topology model proposed by SOSUI and TMHMM (fig. 1b). No truncated variants of YddG conferred a high resistance to phenylalanine to *E. coli* cells. The level of ampicillin resistance for these 12 fu-

Fig. 1. a Hydropathy plot of YddG. The algorithm of Kyte and Doolittle [1982] was used with a window of nine residues. **b** YddG topology prediction from several computer programs. The gray rectangles correspond to putative TM helices. The positions of the designed YddG-BlaM and YddG-ZsGreen fusions are shown by solid and dashed lines, respectively.

sions was determined more precisely, and it was in the range of 75–200 $\mu\text{g/ml}$ (fig. 2). The low ampicillin resistance, 75 $\mu\text{g/ml}$, corresponded to the junctions of Val₂₁, Tyr₈₅, and Lys₂₁₈ located in TMs I, III, and VIII. The higher ampicillin resistances, 200 and 150 $\mu\text{g/ml}$, corre-

sponded to the junctions of Gly₃₀ and Ser₂₆₅ located in the predicted periplasmic loops between TM helices I and II as well as between TM helices IX and X. The detection of these periplasmic loops confirmed the location of the N- and C-termini of YddG in the cytoplasm.

Fig. 2. Model for the topological organization of YddG based on the properties of the YddG-BlaM fusions, the hydrophathy profile and the positive-inside rule [Von Heijne, 1986]. The positions at which BlaM and ZsGreen were fused to YddG are indicated by arrows and rectangles, respectively. The numerals inside the figures

correspond to the number of the YddG amino acid residues and the level of ampicillin (Ap) resistance ($\mu\text{g/ml}$) for YddG-BlaM and to the level of fluorescence (%) for YddG-ZsGreen. The positions of the charged Asp and Glu residues are shown by '-'; Arg and Lys residues are indicated by '+'.

Immunodetection of the YddG-BlaM Fusions

To ensure that different levels of fusion protein expression did not affect the topological analysis, the accumulation of YddG-BlaM fusions in membrane extracts was semiquantitatively evaluated by Western blotting experiments using commercially available (Abcam) mouse antibodies to β -lactamase, as described in 'Experimental Procedures'. As can be seen in figure 3a, all tested fusion proteins were expressed at similar levels. As compared with the standard set of reference soluble proteins, all fusions had greater mobilities in SDS-PAGE than those predicted from the calculated molecular masses, as usually reported for TM helices-based proteins [Rouanet and Nasser, 2001]. Nevertheless, the sizes of the hybrid proteins were compatible with the distribution of the fusion junctions along the *yddG* coding region. So, the detected difference in Ap^R level for the strains with YddG-BlaM fusions manifested the features of the tested protein topologies.

Construction and Analysis of the YddG-ZsGreen Fusions

To confirm the orientation of YddG in the inner membrane determined using the YddG-BlaM fusions, we

made YddG-ZsGreen fusions, including a fusion consisting of a full-size YddG domain. All YddG-ZsGreen fusions were constructed by overlap extension PCR (see 'Experimental Procedures'). The plasmid pZsGreen (Clontech) was used as a template for amplification of the reporter gene. The fusion junctions were chosen in such a way as to test the presence of all four cytoplasmic loops predicted above. Three fusion points (Gln₅₉, Pro₆₁ and Leu₇₁) were chosen between TM helices II and III, fusion point Lys₁₁₇ was between TM helices IV and V, and fusion point Arg₁₈₁ was between TM helices VI and VII. Three fusion points (Val₂₄₇, Tyr₂₅₀ and Pro₂₅₃) were in TM helix IX because of the complicated hydrophobic plot in this region; thus, the cytoplasmic location of several amino acid residues could not be excluded (fig. 1a). The fusions Glu₉₄ and Gln₂₀₆ had the same points of YddG' junction with the reporter, serving as two 'periplasmic-positive' YddG-BlaM fusions and 'cytoplasmic-negative' YddG-ZsGreen controls. The strong fluorescence signal accepted as 100% was generated by the Gly₂₉₃ fusion containing a full-size YddG (fig. 2). The plasmid with this fusion also conferred resistance to phenylalanine to *E. coli* cells, in contrast to truncated YddG-ZsGreen fusion variants. The cytoplasmic-negative controls, the fusions Glu₉₄ and

Fig. 3. Western blots of YddG-BlaM (**a**) and YddG-ZsGreen (**b**) fusions. Equal amounts of solubilized membrane fractions were separated by SDS-PAGE, and fusion proteins were visualized by immunoblotting with monoclonal antibodies against corresponding reporter proteins. The amino acids at which fusions oc-

curred were as follows: **a** 1 = Ser₂₇₁; 2 = Ser₂₆₅; 3 = Lys₂₁₈; 4 = Gln₂₀₆; 5 = Ile₁₄₈; **c** (control) = cell extract of TG1 pBR322, expressing β -lactamase. **b** 1 = Gln₂₀₆; 2 = Arg₁₈₁; 2 = Lys₁₁₇; 3 = Glu₉₄; 4 = Pro₆₁; 5 = Gln₅₉; **c** (control) = ZsGreen.

Gln₂₀₆ with predicted periplasmic locations of the ZsGreen domain, showed the lowest signal, ~3%. Two fusions, Leu₇₁ and Tyr₂₃₀, generating strong signals of ~100%, appeared to be false positives because they were cleaved from the YddG protein and the released ZsGreen accumulated in the cytoplasm, as determined by Western blotting (data not shown) and by fluorescence microscopy (see below). The other YddG-ZsGreen fusions generated signals with fluorescence intensities of ~20–30%.

According to immunodetection of YddG-ZsGreen by Western blotting of the membrane protein fraction, all fusions were of the predicted sizes based on the locations of the junctions along the *yddG* gene. Fusion proteins with a cytoplasmic location of the ZsGreen domain were expressed at nearly the same levels (fig. 3b). Fusions Glu₉₄ and Gln₂₀₆, with ZsGreen domain in the periplasmic space, were weakly detectable in the membrane fraction. ZsGreen protein, as has been shown for GFP, was probably improperly folded when localized outside of the cytoplasm and was cleaved by proteases [Feilmeier et al., 2000]. Therefore, the obtained data concerning YddG-ZsGreen fusion proteins' fluorescence correlated well with the general 10-TM helices YddG topology predicted above using BlaM-tagged fusions.

The fluorescent signals of fusions Val₂₄₇, Tyr₂₅₀ and Pro₂₅₃ within TM helix IX were typical of a cytoplasmic location of YddG' termini tagged by ZsGreen (these signals varied from 17 to 37%). (Significant variations in the fluorescent signals of ~10% were observed for two closer fusions, Gln₅₉ and Pro₆₁, localized in the first cytoplasmic loop as well; see fig. 1b.) Keeping in mind that in these

fusions, ZsGreen is linked to rather short portions of TM helix IX, the fluorescence data could not exclude the possible complex topology of the native YddG in this region. To detail the YddG topology, more sophisticated methods are probably necessary.

Intracellular Localization of the YddG-ZsGreen Hybrids as Assessed by Fluorescence Microscopy

To visualize the intracellular localization of YddG-ZsGreen, we inspected TG1 cells containing full-length YddG-ZsGreen fusion, fusions Lys₁₁₇, Arg₁₈₁, Gln₂₀₆, and Leu₂₃₈, as well as false fusion Leu₇₁ using fluorescence microscopy. For the controls, cells of strains TG1 (pZsGreen) and TG1 (pBR322) were used. All of these plasmid-carrier strains were pregrown in LB medium in the presence of IPTG. The fluorescence signals were located at the poles of the cells containing full-length YddG-ZsGreen (Gly₂₉₃) and the fusions Lys₁₁₇, Arg₁₈₁, and Leu₂₃₈. As can be seen from figure 4a, d, where the results for Gly₂₉₃ and Arg₁₈₁ fusions are shown, the fluorescence was usually stronger at one (perhaps, 'old' [Brandon et al., 2003]) pole of the cells. Cells with practically equal intensities of fluorescence on both poles were also seen (fig. 4d). This is probably caused by a lag of YddG appearance at new poles after cell division.

Cells of the control strains, TG1 (pZsGreen) and TG1 (pBR322) (which carried the plasmid for native ZsGreen and the initial expression vector without any reporter gene), demonstrated either uniformly distributed fluorescence over the entire intracellular space (fig. 4b) or no fluorescence (fig. 4c), respectively. Cells containing the

Fig. 4. Distribution of YddG-ZsGreen fusions in live *E. coli* TG1 cells. Confocal fluorescent (left panels) and bright-field (right panels) micrographs are shown. The plasmids in TG1 cells were as follows: **a** pBR-YddG(Gly₂₉₃)-ZsGreen. **b** pZsGreen. **c** pBR322. **d** pBR-YddG(Arg₁₈₁)-ZsGreen. **e** pBR-YddG(Leu₇₁)-ZsGreen. **f** pBR-YddG(Gln₂₀₆)-ZsGreen. The images were ob-

tained using an LSM510 META microscope that was equipped with a water immersion 63×/1.2 NA C-Apochromat objective. ZsGreen was excited at 488 nm and the emission light was detected using an HFT UV/488/543/633 MBS and an LP505 filter. Bars = 5 μm.

false-positive fusion Leu₇₁ also demonstrated uniform fluorescence throughout the cell. Fluorescence was absent in the case of fusion Gln₂₀₆, which had a periplasmic localization of the ZsGreen domain (fig. 4f).

Therefore, the fluorescence microscopy data confirm the membrane localization of YddG-ZsGreen fusions detected via Western blotting and indicate a polar localization of YddG. In the case of the false fusion Leu₇₁, where the ZsGreen domain was cut off, fluorescence was distributed throughout the whole cell. The polar localization of YddG may be connected with a probable YidC-mediated

pathway of YddG biogenesis and insertion into the internal membrane [Kiefer and Kuhn, 2007; Xie and Dalbey, 2008]. In spite of the fact that the mechanism by which multispanning IMPs are inserted into the target membrane is not completely understood, two main machines of the Sec/YidC-translocon and the YidC insertase have been identified [reviewed in Kuhn, 2009; Lührink et al., 2005]. In fact, YidC insertase localizes predominantly to the poles of *E. coli* [Urbanus et al., 2002], whereas Sec-based complexes are distributed throughout the circumference of the inner membrane [Brandon et al., 2003].

Conclusions

In this study, we investigated the topological organization of YddG in the *E. coli* inner membrane. YddG is the first *E. coli* amino acid exporter for which the topology was experimentally determined. The identification of this protein as a paralogue of RhtA and YdeD, which were formerly characterized as threonine and cysteine exporters, was followed by the finding of YddG's ability to export aromatic amino acids under conditions of their overproduction in cells [Doroshenko et al., 2007]. RhtA and YdeD are related to the DME family (2.A.7.3). The topology of YddG experimentally coincides with the previously determined 10-TM helices topology with cytoplasmic locations of N- and C-termini of the DME-family protein PecM. Moreover, global topological analysis of *E. coli* IMPs has revealed cytoplasmic locations of both termini as being the predominant topology of *E. coli* proteins [Daley et al., 2005].

Based on the information obtained from our study using YddG-ZsGreen fusion proteins, it may be possible to design new strategies to enhance YddG accumulation in the inner cell membrane. These strategies will most likely require the optimization of exporter gene expression, the targeting of the nascent IMP and its insertion into the *E. coli* inner membrane. These approaches will be available for many other exporters with a YddG-like type of biogenesis and will greatly improve the performance of metabolically engineered strains producing intracellular metabolites of commercial interest.

Experimental Procedures

Strains, Plasmids and Growth Conditions

The TG1 strain [K12 $\Delta(lac-pro)$, *supE*, *thi*, *hsd* Δ 5 F'(*tra* Δ 36, *proAB*⁺, *lacI*^q, *lacZ* Δ M15)] was used as a recipient for plasmid constructions. All recombinant plasmids were constructed based on pBR322 [Bolivar et al., 1977; Sutcliffe, 1979]. The media used were LB, SOB or M9 minimal medium, with 0.4% glucose as the carbon source [Sambrook and Russell, 2001]. Solid media were prepared by adding agar (15 g/l) to the liquid media. Antibiotics were used at the following concentrations: ampicillin at 100 μ g/ml and tetracycline at 12.5 μ g/ml.

The resistance to phenylalanine (minimal inhibitory concentration) was determined as described previously [Doroshenko et al., 2007].

Plasmid pZsGreen encoding a variant of wild-type ZsGreen was purchased from Clontech. ZsGreen is an analog of the GFP from *Aequorea victoria* and a member of the reef coral fluorescent protein (RCFP) family. The ZsGreen monomer shares structural homology with GFP but, unlike GFP, can self-associate in solution to form dimers and tetramers. In spite of this feature, published

studies have shown that ZsGreen may be used as a fusion tag [Heddle and Mazaleyrat, 2007].

Construction of Plasmids

Construction and restriction analysis of the recombinant DNA, agarose gel electrophoresis, Ca²⁺-dependent transformation and electroporation of *E. coli* cells were all carried out using standard experimental protocols [Sambrook and Russell, 2001]. Restriction endonucleases, T4 DNA ligase, exonuclease III, S1 nuclease and the Klenow fragment of *E. coli* DNA polymerase (Fermentas) were used according to the manufacturer's instructions. The sequences of all primers are available upon request.

In pBR322, *blaM* was inactivated by deleting the *DraI* restriction fragment (711 bp). Re-ligation yielded the plasmid pBR322 Δ *blaM*. To construct the *yddG-blaM* fusion, a DNA fragment containing the *yddG* gene was amplified from TG1 chromosomal DNA, and the *P*_{lac} promoter was amplified by PCR from the ZsGreen plasmid. These two fragments were joined using overlapping PCR to create the *yddG* gene cassette downstream of the *P*_{lac} promoter. The DNA fragment containing a truncated version of *blaM* was amplified from the pBR322 plasmid and then linked with the *yddG* gene under the control of the *P*_{lac} promoter by overlapping PCR. The primers introduced *AatII* and *DraI* sites to the 5'- and 3'-ends, respectively, of the final DNA amplification product. *NcoI* and *SacI* restriction sites were also introduced in the *yddG-blaM* fusion region for the construction of the truncated variants by exonuclease III treatment (see the following section). After restriction digestion by *AatII* and *DraI*, the obtained DNA fragment was purified and ligated into the *AatII-DraI* sites of the pBR322 Δ *blaM* plasmid to yield pBRy*yddG-blaM*.

Thirteen specific PCR products containing truncated *yddG* genes after the *P*_{lac} promoter fused in frame with ZsGreen were obtained by overlapping PCR of two sets of fragments (the *yddG*' fragments amplified from pBRy*yddG-blaM*, and the ZsGreen fragment from pZsGreen). After restriction digestion by *AatII* and *DraI*, the fragments were cloned into the pBR322 Δ *blaM* plasmid, which yielded a set of pBRy*yddG-ZsGreen* plasmids. The structures of the obtained plasmids were confirmed by sequencing the YddG-ZsGreen fusion regions.

Deletions with Exonuclease III and the *yddG-blaM* Gene Fusions

The plasmid pBRy*yddG-blaM* was used to construct a series of gene fusions by nested deletions from the 3'-end of *yddG*. Exonuclease III digests linear DNA possessing 5' overhangs or blunt ends, but not 3' overhangs. The *NcoI*-produced DNA end in pBRy*yddG-blaM* was sensitive to this nuclease, whereas the *SacI*-driven end was resistant. After linearization of the plasmid by *NcoI* and *SacI* treatment, 12 mg of the plasmid DNA was resuspended in 50 μ l of exonuclease III buffer and incubated at 30°C. Exonuclease III (500 U in 2.5 μ l) was then added. At this temperature, the exonuclease III digestion reaction proceeded at 200 bases/min. Aliquots (2 μ l) were removed at 45-second intervals, immediately mixed with 7.5 μ l of S1 nuclease (2 U of S1 nuclease) and incubated on ice. After all the aliquots were collected, the tubes were removed from the ice and incubated at room temperature for 30 min. The tubes were then incubated at 70°C for 10 min to deactivate the S1 nuclease. The samples were analyzed on a 1% agarose gel. The digestion products were purified, end-filled with deoxyribonucleotides (10 mM) by the Klenow fragment of *E. coli*

DNA polymerase I (1 U/ μ g of DNA) and then ligated to the pBR322 cassette. The ligation products were used to transform *E. coli* TG1 cells, and the transformants were selected on LB plates containing tetracycline.

Measurement of Ampicillin Resistance of Cells Expressing the β -Lactamase Fusion Proteins

The ampicillin resistance of individual *E. coli* TG1 cells containing various fusion plasmids was determined by growing the cells in LB to mid-log phase (3×10^8 cells/ml). The cells were then washed in an equal volume of fresh medium and plated onto the same medium (solidified using 1.5% agar) containing 0, 50, 75, 100, 125, 150, 175, 200 and 225 μ g/ml of ampicillin and 1 mM IPTG (100–400 cells per plate). The colony numbers were determined from three independent experiments. The obtained plasmids were analyzed by DNA sequencing to identify the fusion junction sites.

Measurement of the Fluorescence of the YddG-ZsGreen Fusions

To measure the fluorescence of the YddG-ZsGreen fusions, *E. coli* TG1 cells containing one of the obtained plasmids were grown in LB to mid-log phase (3×10^8 cells/ml) in the presence of 1 mM IPTG. To measure the ZsGreen fluorescence spectrum, we used a Synergy 2 multidetection microplate reader (BioTek Instruments, Inc., USA) with a 485-nm excitation filter and a 528-nm emission filter. The OD₆₀₀ was quantified simultaneously using the same instrument. The fluorescence values were normalized to the cell density in the cultures. The fluorescence signal-to-noise ratio (arbitrary units) was calculated using the formula [(signal – noise)/noise]; where ‘signal’ is the fluorescence signal from the induced cells and the ‘noise’ corresponds to the fluorescence signal from noninduced cells. Finally, arbitrary units were indicated as a percentage, where the fluorescence of Gly₂₉₃ YddG-ZsGreen was set at 100%.

Preparation of Membrane Extracts

Membrane fractions were prepared according to the protocol of Rouanet and Nasser [2001]. To solubilize the obtained membrane extracts, 20% Triton X-100 was added to a final concentration of 2% and incubated at 4°C for 1 h. Protein concentration was determined as described by Bradford [1976].

Western Blotting

Samples were prepared by heating at 37°C for 5 min in Laemmli SDS sample buffer [Laemmli, 1970]. Proteins were separated using 0.1% SDS-12% PAGE and were electrotransferred onto a polyvinylidene difluoride membrane using a Trans-Blot SD semidry electrophoretic transfer cell (Bio-Rad). Mouse anti-*E. coli* β -lactamase (Abcam) and rabbit anti-RCFP sera (Clontech) were used as the primary antibodies, and goat anti-mouse and anti-rabbit alkaline phosphatase conjugates (Sigma) were used as the respective secondary antibodies. The blot was developed using 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (Fermentas), as described by Sambrook and Russell [2001].

Confocal Laser Scanning Microscopy

To observe ZsGreen fluorescence, we diluted overnight cultures 1:50 in fresh LB media containing the appropriate antibiotic and 1 mM IPTG to an OD₆₀₀ of 0.5. To immobilize the bacteria, we mixed the live culture with an equal volume of 1% low-melting-point agarose (Panreac) in phosphate-buffered saline and immediately poured the mixture onto a 0.17-mm glass slide for jellyfication. The agarose was melted and cooled to 40°C before mixing.

The cells were imaged using an LSM510 META inverted microscope (Carl Zeiss) with a water immersion 63 $\times$ /1.2 NA C-Apochromat objective. ZsGreen fluorescence was excited at 488 nm. The emission was detected using a HFT UV/488/543/633 main beam splitter through a LP505 long-pass filter. The typical voxel size and pixel times were 0.15 $\times$ 0.15 $\times$ 1.0 μ m and 1.6 μ s, respectively.

References

- Bolivar F, Rodriguez RL, Greene PJ, Betlach MC, Heynecker HL, Boyer HW, Crosa JH, Falkow S: Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. *Gene* 1977;2:95–113.
- Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;72:248–254.
- Brandon LD, Goehring N, Janakimaran A, Yan AW, Wu T, Beckwith J, Goldberg MB: IcsA, a polarly localized autotransporter with an atypical signal peptide, uses the Sec apparatus for secretion, although the Sec apparatus is circumferentially distributed. *Mol Microbiol* 2003;50:45–60.
- Broome-Smith JK, Tadayyon M, Zhang Y: β -lactamase as a probe of membrane protein assembly and protein export. *Mol Microbiol* 1990;4:1637–1644.
- Claros MG, von Heijne G: TopPred II: an improved software for membrane protein structure predictions. *Comput Appl Biosci* 1994;10:685–686.
- Daley DO, Rapp M, Granseth E, Melen K, Drew D, von Heijne G: Global topology analysis of the *Escherichia coli* inner membrane proteome. *Science* 2005;308:1321–1323.
- Deber CM, Wang C, Liu LP, Prior AS, Agrawal S, Muskat BL, Cuticchia AJ: TM Finder: a prediction program for transmembrane protein segments using a combination of hydrophobicity and nonpolar phase helicity scales. *Protein Sci* 2001;10:212–219.
- Díaz-Mejía JJ, Babu M, Emili A: Computational and experimental approaches to chart the *Escherichia coli* cell-envelope-associated proteome and interactome. *FEMS Microbiol Rev* 2009;33:66–97.
- Doroshenko V, Airich L, Vitushkina M, Kolokolova A, Livshits V, Mashko S: YddG from *Escherichia coli* promotes export of aromatic amino acids. *FEMS Microbiol Lett* 2007;275:312–318.
- Feilmeier BJ, Iseminger G, Schroeder D, Webber H, Phillips GJ: Green fluorescent protein functions as a reporter for protein localization in *Escherichia coli*. *J Bacteriol* 2000;182:4068–4076.
- Hedde C, Mazaleyat SL: Development of a screening platform for directed evolution using the reef coral fluorescent protein ZsGreen as a solubility reporter. *Prot Eng Des Sel* 2007;20:327–337.
- Hirokawa T, Boon-Chieng S, Mitaku S: SOSUI: classification and secondary structure prediction system for membrane proteins. *Bioinformatics* 1998;14:378–379.

- Hofmann K, Stoffel W: Tmbase – a database of membrane spanning proteins segments. *Biol Chem Hoppe-Seyler* 1993;347:166.
- Kiefer D, Kuhn A: YidC as an essential and multifunctional component in membrane protein assembly. *Int Rev Cytol* 2007;259:113–138.
- Krogh A, Larsson B, von Heijne G, Sonnhammer EL: Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *J Mol Biol* 2001;305:567–580.
- Kuhn A: From the Sec complex to the membrane insertase YidC. *Biol Chem* 2009;390:701–706.
- Kyte J, Doolittle RF: A simple method for displaying the hydropathic character of a protein. *J Mol Biol* 1982;157:105–132.
- Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680–685.
- Luirink J, von Heijne G, Houben E, de Gier J-W: Biogenesis of inner membrane proteins in *Escherichia coli*. *Annu Rev Microbiol* 2005;59:329–355.
- Manoil C: Analysis of membrane protein topology using alkaline phosphatase and β -galactosidase gene fusions. *Methods Cell Biol* 1991;34:61–75.
- Matz MV, Fradkov AF, Labas YA, Savitsky AP, Zaraisky AG, Markelov ML, Lukyanov SA: Fluorescent proteins from nonbioluminescent *Anthozoa* species. *Nat Biotechnol* 1999;17:969–973.
- Rapp M, Drew D, Daley DO, Nilsson J, Carvalho T, Melén K, de Gier J-W, von Heijne G: Experimentally based topology models for *E. coli* inner membrane proteins. *Protein Sci* 2004;13:937–945.
- Rouanet C, Nasser W: The PecM protein of the phytopathogenic bacterium *Erwinia chrysanthemi*, membrane topology and possible involvement in the efflux of the blue pigment indigoidine. *J Mol Microbiol Biotechnol* 2001;3:309–318.
- Sambrook J, Russell DW: *Molecular Cloning: A Laboratory Manual*, ed 3. Cold Spring Harbor, Cold Spring Harbor Laboratory Press, 2001.
- Santiviago CA, Fuentes JA, Bueno SM, Trombert AN, Hidalgo AA, Socias LT, Youderian P, Mora GC: The *Salmonella enterica* sv. Typhimurium *smvA*, *yddG* and *ompD* porin genes required for the efficient efflux of methyl viologen. *Mol Microbiol* 2002;46:687–698.
- Sutcliffe JG: Complete nucleotide sequence of the *Escherichia coli* plasmid pBR322. *Cold Spring Harb Symp Quant Biol* 1979;43:77–90.
- Urbanus ML, Fröderberg L, Drew D, Bjork P, de Gier JWL, Brunner J, Oudega B, Luirink J: Targeting, insertion, and localization of *Escherichia coli* YidC. *J Biol Chem* 2002;277:12718–12723.
- Von Heijne G: The distribution of positively charged residues in bacterial inner membrane proteins correlates with the transmembrane topology. *EMBO J* 1986;5:3021–3027.
- Xie K, Dalbey RE: Inserting proteins into the bacterial cytoplasmic membrane using the Sec and YidC translocases. *Nat Rev Microbiol* 2008;6:234–244.
- Yuan J, Zweers JC, van Dijk JM, Dalbey RE: Protein transport across and into cell membranes in bacteria and archaea. *Cell Mol Life Sci* 2010;67:179–199.