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

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### Key Words
\(\beta\)-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 \(\beta\)-lactamase (BlaM) as a reporter. The selection of such fusions under 50 \(\mu\)g/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.

### Introduction
Bacterial inner membrane proteins (IMPs) represent an important class of polypeptides that are involved in a wide variety of cellular functions. Among the \(\sim 4,500\) 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 reporter molecules of the studied IMP with a to-

positional reporter molecule is usually used for this pur-

pose. β-Lactamase (BlaM), green fluorescent protein
(GFP) and alkaline phosphatase are able to act as topo-

logical reporter molecules when fused to different por-

tions of an IMP [Broome-Smith et al., 1990; Manoil, 1991; 

Rapp et al., 2004].

YddG is an IMP that participates in the export of aro-

matic 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


tcdb.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 experi-

mentally shown only for PecM, a DME member [Rouanet 

and Nasser, 2001].

The location of the YddG C-terminus was earlier de-

termined in topology mapping studies [Rapp et al., 2004].

Further experimental investigations of the structure of 

YddG are crucial for understanding its biological func-

tions and properties and its possible applications in the 

biological reporter molecules when fused to different por-

tions of an IMP [Broome-Smith et al., 1990; Manoil, 1991; 

Rapp et al., 2004].

To clarify the membrane topological organization of 

YddG, yddG’-blaM fusions were created between exonu-

clease III-truncated yddG derivatives and the blaM gene 

encoding the leaderless β-lactamase. First, the recombi-

nant plasmid pBryddG-blaM was constructed based on 

pBR322 (ApR, TcR) (GenBank accession number J01749.1 

[Sutcliffe, 1979]) as a vector. The yddG gene was inserted 

downstream of the Plac 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 con-

ferred ampicillin resistance (ApR) to ~10 μg/ml and a 

high resistance to phenylalanine (up to 20 mg/ml) to 

TG1 cells. Such a low level of ApR was consistent with a 

cyttoplasmic 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 [Do-

rosenko 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 

ApR 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 bac-

terial cell even under conditions of a fully induced Plac 

promoter.

A set of internal deletions in pBryddG-blaM was ob-

tained by random partial digestion of the 3’-terminus of 

the yddG gene (see ‘Experimental Procedures’). The re-

combinant plasmids of interest were obtained by trans-

forming TG1 cells and selecting tetracycline-resistant 

(TcR) clones. Approximately 100 ApR clones from 500 

tested TcR clones were selected by growing on Luria-Ber-

tani (LB) agar plates containing 1 mM IPTG and 50 μg/ 

ml of ampicillin. This ampicillin concentration permit-

ted 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-

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, depend-

ing 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 determine the general topology of the YddG mem-

brane, 10-TM helices-based membrane topology of YddG 

was constructed by constructing translatable 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 lo-

cated in the cytoplasm. The decrease of the 

IPTG concentration to 0.1 mM resulted in decreases of 

ApR 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 bac-

terial cell even under conditions of a fully induced Plac 

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cells. The level of ampicillin resistance for these 12 fu-
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Amino acid residue number

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.

ersions was determined more precisely, and it was in the range of 75–200 μg/ml (fig. 2). The low ampicillin resistance, 75 μg/ml, corresponded to the junctions of Val31, Tyr85, and Lys218 located in TMs I, III, and VIII. The higher ampicillin resistances, 200 and 150 μg/ml, corresponded 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-termi of YddG in the cytoplasm.
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 ApR 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...
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Gln\textsubscript{206} with predicted periplasmic locations of the ZsGreen domain, showed the lowest signal, \(\sim 3\%\). Two fusions, Leu\textsubscript{71} and Tyr\textsubscript{230}, generating strong signals of \(\sim 100\%\), 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 \(20\text{-}30\%\).

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 Glu\textsubscript{94} and Gln\textsubscript{206}, 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 Val\textsubscript{247}, Tyr\textsubscript{230} and Pro\textsubscript{253} 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 \(\sim 10\%\) were observed for two closer fusions, Gln\textsubscript{293} and Pro\textsubscript{61}, 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 Lys\textsubscript{117}, Arg\textsubscript{181}, Gln\textsubscript{206}, and Leu\textsubscript{238}, as well as false fusion Leu\textsubscript{71} 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 (Gly\textsubscript{293}) and the fusions Lys\textsubscript{117}, Arg\textsubscript{181}, and Leu\textsubscript{238}. As can be seen from figure 4a, d, where the results for Gly\textsubscript{293} and Arg\textsubscript{181} 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

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**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 occurred were as follows: a 1 = Ser\textsubscript{271}; 2 = Ser\textsubscript{265}; 3 = Lys\textsubscript{218}; 4 = Gln\textsubscript{206}; 5 = Ile\textsubscript{148}; c (control) = cell extract of TG1 pBR322, expressing \(\beta\)-lactamase. b 1 = Gln\textsubscript{206}; 2 = Arg\textsubscript{181}; 2 = Lys\textsubscript{117}; 3 = Glu\textsubscript{94}; 4 = Pro\textsubscript{61}; 5 = Glu\textsubscript{59}; c (control) = ZsGreen.
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 multispansing 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; Luirink 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].

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 obtained 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.
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 parologue 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 terminus as being the predominant topology of previously characterized amino acid 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 Δ(lac-pro), supE, thi, hsdS Δ5 F’(tra)D36, proAB, lacP, lacZΔM15]) 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 from LB, SOB or M9 minimal medium, with 0.4% glucose as the carbon source [Sambrook and Russell, 2001].

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 [Hedde 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 (71 bp). Re-ligation yielded the plasmid pBR322ΔblaM. To construct the yddG-blaM fusion, a DNA fragment containing the yddG gene was amplified from TGI 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 AartI 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 AartI and DraI, the obtained DNA fragment was purified and ligated into the AartI-DraI sites of the pBR322ΔblaM plasmid to yield pBRyddG-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 pBRyddG-blaM, and the ZsGreen fragment from pZsGreen). After restriction digestion by AartI and DraI, the fragments were cloned into the pBR322ΔblaM plasmid, which yielded a series of pBRyddG-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 pBRyddG-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 pBRyddG-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 μl of exonuclease III buffer and incubated at 30°C. Exonuclease III (500 U in 2.5 μl) was then added. At this temperature, the exonuclease III digestion reaction proceeded at 200 bases/min. Aliquots (2 μl) were removed at 45-second intervals, immediately mixed with 7.5 μl 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.
DNA polymerase I (1 U/µg 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 \((3 \times 10^8 \text{cells/ml})\). 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 µg/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 \((3 \times 10^8 \text{cells/ml})\) 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 OD_{600} was quantified simultaneously for jellification. The agarose was melted and cooled to 40 °C before jellification.

**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 Laemmlli SDS sample buffer [Laemmlli, 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 OD_{600} 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 jellification. 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×/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 × 0.15 × 1.0 µm and 1.6 µs, respectively.

**References**


