New *Escherichia coli* outer membrane proteins identified through prediction and experimental verification

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Abstract

Many new *Escherichia coli* outer membrane proteins have recently been identified by proteomics techniques. However, poorly expressed proteins and proteins expressed only under certain conditions may escape detection when wild-type cells are grown under standard conditions. Here, we have taken a complementary approach where candidate outer membrane proteins have been identified by bioinformatics prediction, cloned and overexpressed, and finally localized by cell fractionation experiments. Out of eight predicted outer membrane proteins, we have confirmed the outer membrane localization for five—YftM, YaiO, YfaZ, CsgF, and YliI—and also provide preliminary data indicating that a sixth—YfaL—may be an outer membrane autotransporter.

Keywords: outer membrane protein; bioinformatics; SecB; autotransporter

From the known high-resolution structures of transmembrane proteins, only two basic architectures have been identified so far: the helix bundle and the β-barrel (von Heijne 1999). Helix bundle proteins have been extensively studied, both from an experimental and from a bioinformatics perspective, and rather reliable prediction methods exist for their identification from sequence data alone (Chen et al. 2002; Melén et al. 2003). β-Barrel proteins have received comparatively less attention, and only a few methods have been proposed for identification and topology prediction of such proteins (Casadio et al. 2003; Bagos et al. 2004; Berven et al. 2004; Bigelow et al. 2004).

An important use of bioinformatics prediction schemes is to guide the experimentalist toward targets that are highly likely to correspond to true instances of the particular kind of gene or protein of interest. Here, we have used the recently developed Hunter predictor (Casadio et al. 2003) to select likely outer membrane proteins among the nonannotated part of the *Escherichia coli* proteome, and have experimentally verified the predicted outer membrane localization of five hitherto uncharacterized proteins: YftM, YaiO, YfaZ, CsgF, and YliI. We further provide data indicating that a sixth protein, YfaL, is an outer membrane autotransporter.

Results

Selection of target proteins

From the list of 18 new outer membrane proteins predicted by the Hunter predictor in the *E. coli* proteome (see Table 3 in Casadio et al. 2003), we initially chose 11

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proteins, characterized by different lengths and different numbers of predicted β-strands, for further analysis. Despite repeated attempts, only eight of these genes could be cloned in our vector system. Therefore, we focused our experimental analysis on this set of putative outer membrane proteins (Table 1).

Cloning and expression of target proteins

The eight target genes were cloned into the pING vector (Johnston et al. 1985), and a hemagglutinin (HA) tag was added to the C terminus of the gene products for immunodetection. Induction with arabinose and labeling with [^{35}S]-Met in all cases gave rise to a protein product that could be immunoprecipitated by an HA antibody and was of the expected molecular weight (data not shown).

In initial [^{35}S]-Met labeling experiments, we noted that seven of the eight proteins appeared as doublets (Fig. 1), possibly reflecting inefficient removal of the signal peptide (because of its large size, small molecular weight differences could not be detected in pulse-chase experiments with YfaL). To study this possibility further, we blocked SecA-dependent translocation through the inner membrane SecYEG translocon by adding sodium azide 30 sec prior to the addition of [^{35}S]-Met (Oliver et al. 1990). As seen in Figure 1, after a 1-min pulse, significantly more of the higher molecular-weight form was seen in the presence than in the absence of azide for all seven proteins, strongly suggesting that these proteins are translocated across the inner membrane in a SecA- and translocon-dependent process.

Outer membrane localization of target proteins

To assay the possible outer membrane localization of the target proteins, we separated outer and inner membranes by successive two- and six-step sucrose gradient centrifugation. The purity of the inner and outer membrane fractions was determined by Western blotting against the inner and outer membrane marker proteins Lep and OmpA, respectively.

Table 1. *Proteins included in the study*

UniProt code	Molecular weight (processed, HA tagged)	Urea pellet	SecB dependence	UniProt annotation
CSGF_ECOLI	14.2	+	+	Biogenesis of curli organelles
YHJY_ECOLI	25.1	–	+	Lipase 1
YTFM_ECOLI	63.8	+ +	+	Hypothetical protein
YFAL_ECOLI	129.7	N.D.	N.D.	Putative autotransporter
YAIO_ECOLI	28.3	+ +	+	–
YLII_ECOLI	40.0	+	+	Putative glucose dehydrogenase
YFAZ_ECOLI	17.9	+	+	–
YAGZ_ECOLI	19.1	–	+	Fimbrillin

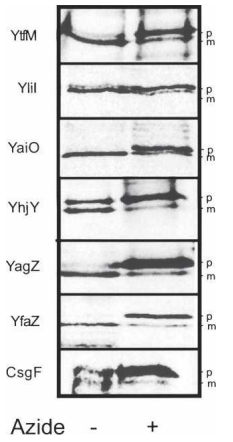

Figure 1. Translocation of the target proteins across the inner membrane is SecA dependent. Protein expression was induced for 5 min with arabinose, followed by labeling for 1 min with [^{35}S]-Met (YliI was labeled for 3 min). Sodium azide was added to a final concentration of 2 mM (+ lanes) 30 sec prior to radio-labeling. Proteins were immunoprecipitated with antisera against the HA-tag. Precursor (*p*) and mature (*m*) forms of the proteins are indicated.

All target proteins were found in the outer membrane fraction, together with the control outer membrane protein OmpA (Fig. 2). A higher molecular-weight form migrating slightly slower than the 150-kDa standard was seen for YtfM, possibly representing an SDS-resistant dimeric form of the protein. For the relatively strongly expressed YaiO and YliI proteins, trace amounts were also present in the inner membrane fraction, most likely due to cross-contamination.

It was recently shown that cytosolic aggregates of misfolded proteins cosediment with the outer membrane fraction upon sucrose density gradient centrifugation, and that the inclusion-body binding proteins IbpA and IbpB can be used as a marker for these aggregates (Laskowska et al. 2004). To evaluate if our overexpressed target proteins are inserted into the outer membrane and do not simply copurify in aggregates, we developed a protocol in which the purified outer membrane fraction is washed with 5 M urea to dissolve potential aggregates but leave membranes intact. Similar procedures are often used to demonstrate the correct insertion of helix bundle proteins into membranes (Chen et al. 2003). Western blots against IbpA,B showed that aggregates are solubilized by urea treatment, whereas the major outer membrane protein OmpA remains in the membrane pellet (Fig. 3A).

As shown in Figure 3B, only YaiO and YftM remained totally in the urea-resistant outer membrane fraction (the latter gave rise to two additional lower molecular weight

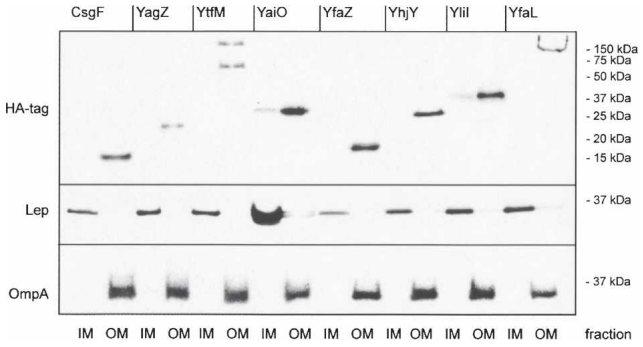

Figure 2. Membrane fractionation. Cells were grown at 37°C, and expression of HA-tagged target proteins was induced with arabinose at an OD_{600} of 0.4–0.6. Cells were harvested 45 min after induction and lysed by French pressing. Inner and outer membrane fractions were prepared by sucrose density gradient centrifugation, separated by SDS-PAGE, and probed by immunodecoration of the HA-tagged proteins. Western blots of the outer membrane marker OmpA and the inner membrane marker Lep are also shown.

bands upon urea treatment; the identity of these bands is unknown). CsgF and YfaZ were also largely urea-resistant, while YfaL, YliI, YhjY, and YagZ were to a greater or lesser extent removed from the outer membrane fraction.

Since the formation of inclusion bodies is often reduced at lower temperature, YfaL, YliL, YhjY, and CsgF were expressed also at 30°C (Fig. 3C). The amounts of CsgF and YliI in the outer membrane fraction increased, whereas YhjY was still mainly extracted. Expression of YfaL was too low for detection under these conditions. Our results strongly suggest that at least five of the eight proteins (YftM, YaiO, YfaZ, CsgF, YliI) are localized to the outer membrane.

The 130-kDa protein YfaL has been predicted to be an autotransporter (Yen et al. 2002). Supporting this, the C-terminal part shows considerable homology with the AIDA-I autotransporter of *E. coli* O126:H27, which mediates binding to an integral membrane glycoprotein on HeLa cells (Laarmann and Schmidt 2003). In general, autotransporters consist of an outer membrane C-terminal translocator domain and a globular N-terminal passenger domain that mediates the ultimate function of the protein. After translocation through the outer membrane, the passenger domain is (often autolytically) cleaved off (Henderson et al. 1998).

As noted above, we could not detect YfaL in the outer membrane after cell fractionation and urea extraction. A substantial amount of the expressed protein seems to end up in aggregates, which is in concert with the fact that the inclusion body binding protein IbpB is up-regulated upon overexpression of YfaL (data not shown). Interestingly, when whole cells were subjected to SDS-PAGE and Western blotting against the C-terminal HA-tag, an additional band at 55 kDa appeared (Fig. 4).

We considered the possibility that the 55-kDa fragment is the cleaved translocator domain of the autotransporter, which would be similar in size to the AIDA-I translocator domain (47.5 kDa). As many autotransporters are serine/threonine proteases, we tested if the cleavage of the putative translocator domain could be inhibited by the serine/threonine protease inhibitor Pefabloc SC. Indeed, when YfaL was expressed in the presence of Pefabloc SC, the 55-kDa band disappeared (Fig. 4). The putative cleaved 75-kDa N-terminal passenger domain does not contain a HA-tag and thus cannot be detected by Western blotting.

SecB dependence

It is generally assumed that the cytoplasmic chaperone SecB facilitates the export of precursor polypeptides by

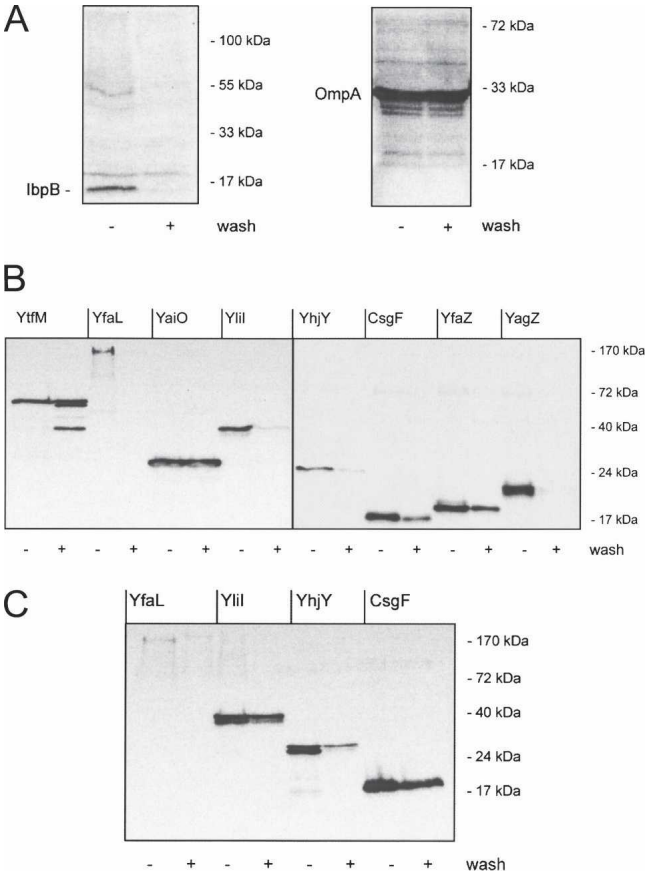

Figure 3. Urea wash of outer membrane fractions. Outer membrane fractions from MC1061 cells overexpressing the different target proteins were prepared as described in Figure 2. After fractionation, membranes were washed with PBS plus 5 M urea for 1 h in the cold. Urea-treated membranes (+ wash) and untreated controls (– wash) were analyzed by Western blotting. (*A*) Western blots of the inclusion body binding protein B (IbpB; the cells in this experiment were induced for expression of YfaL) and the major outer membrane protein OmpA. (*B*) Western blots of the indicated HA-tagged target proteins expressed at 37°C. (*C*) Western blots of the indicated HA-tagged target proteins expressed at 30°C.

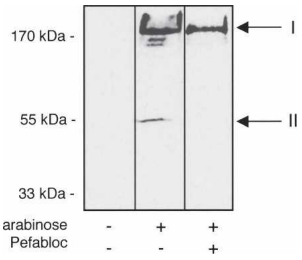

Figure 4. Analysis of the putative outer membrane autotransporter YfaL. Cells were grown in LB medium at 30°C, and expression of HA-tagged YfaL was induced with arabinose at an OD_{600} of 0.4–0.6 for 2 h. Induced cells were grown in the presence or the absence of Pefabloc SC. Cells were harvested and subsequently analyzed by Western blotting against the HA-tag. Full-length YfaL (I) and the putative 55-kDa translocator domain (II) are indicated.

maintaining them in a translocation competent conformation and by delivering them to SecA (Randall and Hardy 2002). However, up until recently, it has been shown for only six proteins (PhoE, LamB, MBP, GBP, OmpF, and OmpA) that their export is facilitated by SecB (Kumamoto and Beckwith 1985; de Cock et al. 1992; Powers and Randall 1995), whereas four proteins (PhoA, Lpp, RbsB, and AmpC) do not seem to require SecB (Knoblauch et al. 1999; Xu et al. 2000; Randall and Hardy 2002; Dekker et al. 2003). Twelve additional proteins were recently identified as SecB substrates in a proteomics screen (Baars et al. 2006).

In an attempt to expand the list even further, we expressed HA-tagged CsgF, YfaZ, YagZ, YhjY, YaiO, YliI, and YftM in the *secB* null strain MC4100Δ*secB* (no expression was seen for YfaL) and the control strain MC4100 (Fig. 5). With the possible exception of YhjY, the relative amount of precursor was clearly increased in the *secB* null strain compared with wild type for all seven proteins, indicating that SecB facilitates their targeting. As expected, the uncleaved precursor form pro-OmpA accumulated in the transformed *secB* null strains but not in the transformed control strains (data not shown).

Discussion

Until recently, identification of bacterial outer membrane proteins by computational approaches (other than standard sequence similarity searches) has been a neglected field in bioinformatics. Here, we have experimentally verified that at least five of the top candidate outer membrane proteins identified by the Hunter predictor among the unannotated portion of the *E. coli* proteome (Casadio et al. 2003)—YftM, YaiO, YfaZ, CsgF, and YliI—are in fact localized in the outer membrane. Target protein selection based on bioinformatics predictions followed by experimental verification is thus a viable alternative to large-scale proteomics approaches (Molloy et al. 2000) for the identification of bacterial outer membrane proteins, and may be particularly effective for low-abundance proteins or proteins that are expressed only under certain conditions.

The outer membrane localization of the five proteins was experimentally verified by sucrose density gradient centrifugation and urea treatment of the outer membranes. Only YhjY gave ambiguous results: We could not determine if it is located in the outer membrane as it is difficult to overexpress and ends up mostly in inclusion bodies. YagZ could be extracted completely from the outer membrane fraction with 5 M urea and is thus not embedded in the outer membrane. YagZ is identical with the protein MatB (meningitis-associated and temperature-regulated), which has been shown recently to be the major fimbrillin of the Mat fimbria (and thus not directly inserted in the outer membrane) (Pouttu et al. 2001). MatB is expressed in some pathogenic strains (MENEC) but not in the laboratory strain K12. Finally, our results indicate that YfaL is an autotransporter with a cleavable 55-kDa translocator domain.

In common with previously studied outer membrane proteins, we find that targeting of the outer membrane proteins identified here is facilitated by the SecB chaperone, suggesting that SecB dependence may be a common characteristic of outer membrane proteins.

Materials and methods

Enzymes and chemicals

Unless otherwise stated, all enzymes were from Promega or New England Biolabs. [^{35}S]-Met and [^{14}C]-methylated marker

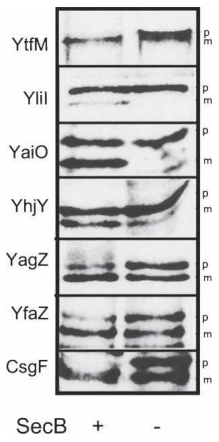

Figure 5. Analysis of SecB dependence. The indicated HA-tagged target proteins were expressed in the *secB* null mutant MC4100Δ*secB* (– SecB) and the wild-type strain MC4100 (+ SecB). Protein expression was induced with arabinose for 5 min, followed by labeling for 1 min with [^{35}S]-Met. Proteins were immunoprecipitated with antisera against the HA-tag. Precursor (*p*) and mature (*m*) forms of the proteins are indicated. Note the slow cleavage of YliI, which is complete only after a 3-min pulse in wild-type cells (Fig. 1).

proteins were from Amersham-Pharmacia Biotech. Protein A–Sepharose and sodium azide were from Sigma Chemical. Pansorbin was from Calbiochem Biochemicals &Immunochemicals. BigDye Terminator v1.1 Cycle Sequencing Kit was from AB Applied Biosystems,, and oligonucleotides were from CyberGene AB. The QuikChange site-directed mutagenesis kit was from Stratagene. The Expand Long Template PCR System was from Roche Diagnostics GmbH, and the QIAquick PCR purification kit was from Qiagen. All mutants were confirmed by sequencing of plasmid DNA at BM Labbet AB. Rabbit polyclonal anti-HA-tag (influenza HA-epitope) antibody was from Abcam Limited. BCA protein concentration assay was from Pierce, and Pefabloc was from Biomol.

DNA techniques

The genes encoding the eight *E. coli* target proteins were amplified from *E. coli* strains MG1655 (Blattner et al. 1997) or MC1061 using Expand Long Polymerase. For cloning into and in vivo expression from the pING1 plasmid (see Whitley et al. 1994), both ends of the gene were modified during PCR amplification by introducing a XhoI site and an initiator ATG codon encoded by the 5′ primer, and by changing the 3′ end of the gene by a reverse primer encoding a HA-tag, YPYDVPDYA, two stop codons (TAA TAG), and a SmaI site. Thus, the 5′ region of the gene was modified to …CTCGAGTATG… (XhoI site and initiator codon underlined). The resulting fragment was cloned into the pING vector behind the *ara* promoter using an XhoI site and a SmaI site introduced by site-specific mutagenesis.

Strains, plasmids, culture conditions, and pulse experiments

Experiments were performed in *E. coli* strain MC1061 (Dalbey and Wickner 1986), MC4100 (Casadaban and Cohen 1979), and MC4100Δ*secB* (R.S. Ullers., F. Schwager, D. Ang, C. Georgopoulos, and P. Genevaux, in prep.). Constructs were expressed from the pING plasmid (Johnston et al. 1985) by induction with L-arabinose.

E. coli strains were transformed with the pING vector carrying the relevant constructs under control of the arabinose promoter were grown at 37°C in M9 minimal medium supplemented with 100 μg/mL ampicillin, 0.5% (w/v) fructose, 100 mg/mL thiamine, and all amino acids (50 μg/mL each) except methionine. An overnight culture was diluted 1:25 in fresh medium, shaken for 3.5 h at 37°C, induced with arabinose (0.2% [w/v]) for 5 min, labeled with [^{35}S]-Met (75 μCi/mL) for 1 min, and put on ice. Sodium azide (final concentration 2 mM) was added 30 sec before radiolabeling. Samples were acid-precipitated with trichloroacetic acid (TCA) (10% [v/v] final concentration), resuspended, and then analyzed by immunoprecipitation with HA-antiserum combined with SDS-PAGE as described previously (Fröderberg et al. 2004). Proteins were visualized in a Fuji FLA-3000 PhosphorImager using the Image Reader V1.8J/Image Gauge V 3.45 software.

Separation of outer and inner membrane fractions

Cell fractionation was carried out essentially as described in Laskowska et al. (2004) using two subsequent sets of sucrose density gradients. Samples (1000 mL) of strain MC1061 transformed with a pING vector harboring the different OMPs were grown at 37°C and 30°C, respectively. Expression of the outer membrane proteins was induced by the addition of 0.1% arabinose at an OD_{600} of 0.4–0.6. Cells were harvested 45 min after induction at 6000*g* using a Beckman 8.1000 rotor.

The 1000 OD_{600} units of cells were resuspended in 6 mL buffer K (50 mM triethanolamine [TEA], 250 mM sucrose, 1 mM EDTA, 1 mM dithiothreitol [DTT], 0.1 mg/mL Pefabloc at pH 7.5) and lysed by two cycles of French pressing (18,000 psi). The lysate was clarified of unbroken cells by 20-min centrifugation at 8,000*g*. The supernatant was transferred on top of a two-step sucrose gradient: bottom to top, 1 mL 55% (w/w), 5.5 mL 9% (w/w). All sucrose gradients were prepared in buffer M: 50 mM TEA, 1 mM EDTA, and 1 mM DTT (pH 7.5). The gradients were spun for 2.5 h at 210,000*g* in a Beckman SW 40 rotor, and the membrane fraction was collected from the top of the 55% sucrose step. This fraction, which contains the entire membranes, was diluted 1:1 with buffer M and subjected to a six-step sucrose gradient to obtain pure inner and outer membrane fractions. The assembly of this second gradient was as follows (from bottom to top): 0.8 mL at 55%; 2.0 mL at 50%, 45%, 40%, and 35%; 0.8 mL at 30% (all w/w) and 3.3 mL of the sample. The gradients were spun for 15 h at 210,000*g* in a Beckman SW 40 rotor, and the inner and outer membrane fractions were collected from the top of the 40% and 50% sucrose steps, respectively.

The purity of the fractions was confirmed by Western blotting against Lep and OmpA as inner and outer membrane markers, respectively. The protein concentration of the fractions was determined by a BCA assay according to the instructions of the manufacturer (Pierce).

Aggregate removal

Outer membranes containing 50 μg of protein were resuspended in PBS/5 M urea and washed by rotating for 1 h in the cold room. Membranes were collected in Beckman TLA 100.3 at 194,000*g* for 20 min. Urea-washed membranes and unwashed control membranes were analyzed by Western blotting against the HA-tag. Blotting against OmpA was used as a control for a protein that is properly inserted into the outer membrane and that cannot be washed away. Blotting against IbpB was used to show that the aggregates could be washed away by the 5 M urea treatment.

Immunoblot analysis

The expression of the target proteins (with HA-tag fused to the C terminus), Lep, OmpA, and IbpA,B (the IbpB antiserum cross-reacts with IbpA) in the inner/outer membranes and aggregates was monitored by immunoblot analysis. Cells were cultured as described above. Purified inner/outer membranes or aggregates (5 μg protein) were solubilized in Laemmli solubilization buffer and were separated by SDS-PAGE. Proteins were transferred from the polyacrylamide gel to a polyvinylidene fluoride (PVDF) membrane (Millipore). Subsequently, membranes were blocked with 5% milk and decorated with antisera to the components listed above. Proteins were visualized with secondary HRP-conjugated antibodies (Bio-Rad) using the ECL system according to the instructions of the manufacturer (Amersham Pharmacia) and a Fuji LAS 1000-Plus CCD camera. Blots were quantified using the Image Gauge software (version 3.4). Experiments were repeated at least twice. If the membrane had to be tested with more than

one antibody, it was washed with 5 M urea and 10 mM DTT overnight at 37°C, blocked, and reused as before (Terzi et al. 2004).

Protease inhibition assay for YfaL

MC1061 transformed with a pING vector harboring the *yfaL* gene fused to a C-terminal HA-tag was grown at 30°C in LB medium supplemented with 100 μg/mL ampicillin. Expression was induced by the addition of 0.1% arabinose at an OD_{600} of 0.4–0.6 in the presence or absence of 1 mg/mL Pefabloc serine protease inhibitor. Cells were harvested 2 h after induction, and 0.15 OD_{600} units of whole cells/well was run on an SDS-PAGE and analyzed by Western blotting as described above.

Acknowledgments

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