
A scalable, GFP-based pipeline for membrane protein overexpression screening and purification

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Abstract

We describe a generic, GFP-based pipeline for membrane protein overexpression and purification in *Escherichia coli*. We exemplify the use of the pipeline by the identification and characterization of *E. coli* YedZ, a new, membrane-integral flavocytochrome. The approach is scalable and suitable for high-throughput applications. The GFP-based pipeline will facilitate the characterization of the *E. coli* membrane proteome and serves as an important reference for the characterization of other membrane proteomes.

Keywords: *Escherichia coli*; membrane protein overexpression; membrane protein isolation; membrane protein characterization; GFP

Membrane proteins (MPs) account for 20%–25% of all open reading frames in sequenced genomes, and fulfill a wide range of central functions in the cell (Wallin and von Heijne 1998). However, our knowledge of this important class of proteins is still poor, mainly because of a lack of generally applicable approaches to the overexpression and purification steps that precede functional and structural analysis. Novel approaches in these areas are required to facilitate and speed up MP research.

The bacterium *Escherichia coli* is still the most widely used vehicle for MP overexpression. Overexpression in the cytoplasmic membrane is preferred to overexpression in inclusion bodies, since the isolation of functional MPs from the membrane is usually more successful than

refolding from inclusion bodies (Drew et al. 2003). Green fluorescent protein (GFP) fusions can be used to facilitate the monitoring of MP overexpression in the cytoplasmic membrane (Drew et al. 2001). If the fusion protein ends up in inclusion bodies, GFP does not fold and is therefore not fluorescent; in contrast, if the fusion is expressed in the cytoplasmic membrane, GFP folds properly and is fluorescent. GFP is only fluorescent in the cytoplasm of *Escherichia coli* (Drew et al. 2002), which means that GFP-based screens work only for MPs that have their C terminus located in the cytoplasm. Recently, nearly all *E. coli* cytoplasmic MPs were fused to GFP for a membrane proteome topology screen (Daley et al. 2005). Approximately 80% of all *E. coli* cytoplasmic MPs have a cytoplasmic C terminus, and thus GFP can be used to monitor the overexpression levels of the majority of *E. coli* MPs (Daley et al. 2005).

Here, we present a generic pipeline for rapid overexpression screening, detergent extraction, and purification of MPs based on a simple MP-GFP fusion approach. We show that milligram amounts of pure functional MP can

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be obtained for *E. coli* cytoplasmic MPs from liter-scale cultures. The approach is scalable and thus suitable for high-throughput applications.

Results

GFP-based MP overexpression screen in E. coli

Since fluorescence is one of the most convenient ways to follow a protein overexpression and purification procedure (see Waldo et al. 1999), we sought to establish a generally applicable and easily scalable pipeline for MP overexpression and purification based on His₈-tagged GFP fusions. To facilitate the removal of the GFP moiety, we also included a tobacco etch virus (TEV) protease site between the MP and GFP-His₈ (see Materials and Methods).

The first step in the pipeline development was to establish a generally applicable way to rank MPs according to their overexpression levels. To this end, 48 genes coding for *E. coli* MPs were selected based on their C_{in} topology from a library of MP-GFP fusions covering almost the whole *E. coli* membrane proteome (Fig. 1; Daley et al. 2005). For

enhanced expression, cells were cultured at 25°C after induction, and expression was tested in 1 mL and 1 L culture volumes. We did not observe significant differences in expression levels due to the different culture volumes (data not shown); i.e., 1 mL was found to be a convenient culture volume for rapid and reliable overexpression screening. Using a standard protocol, nine MP-GFP fusions were isolated from 1 L cultures (Fig. 1; see “GFP-based purification scheme,” below). There is a good correlation between GFP fluorescence measured in whole cells and the amount of MP-GFP fusion that can be purified, indicating that whole-cell fluorescence is indeed a useful indicator for the overexpression of MP-GFP fusions.

GFP-based solubilization screen

The GFP moiety in the MP-GFP fusion makes it possible to rapidly monitor the ability of different detergents to extract the overexpressed fusion protein from the membrane. Table 1 shows results for the YbaT-GFP fusion. Although the ultimate choice of detergent will depend also on the ability to preserve the MP in a fully functional

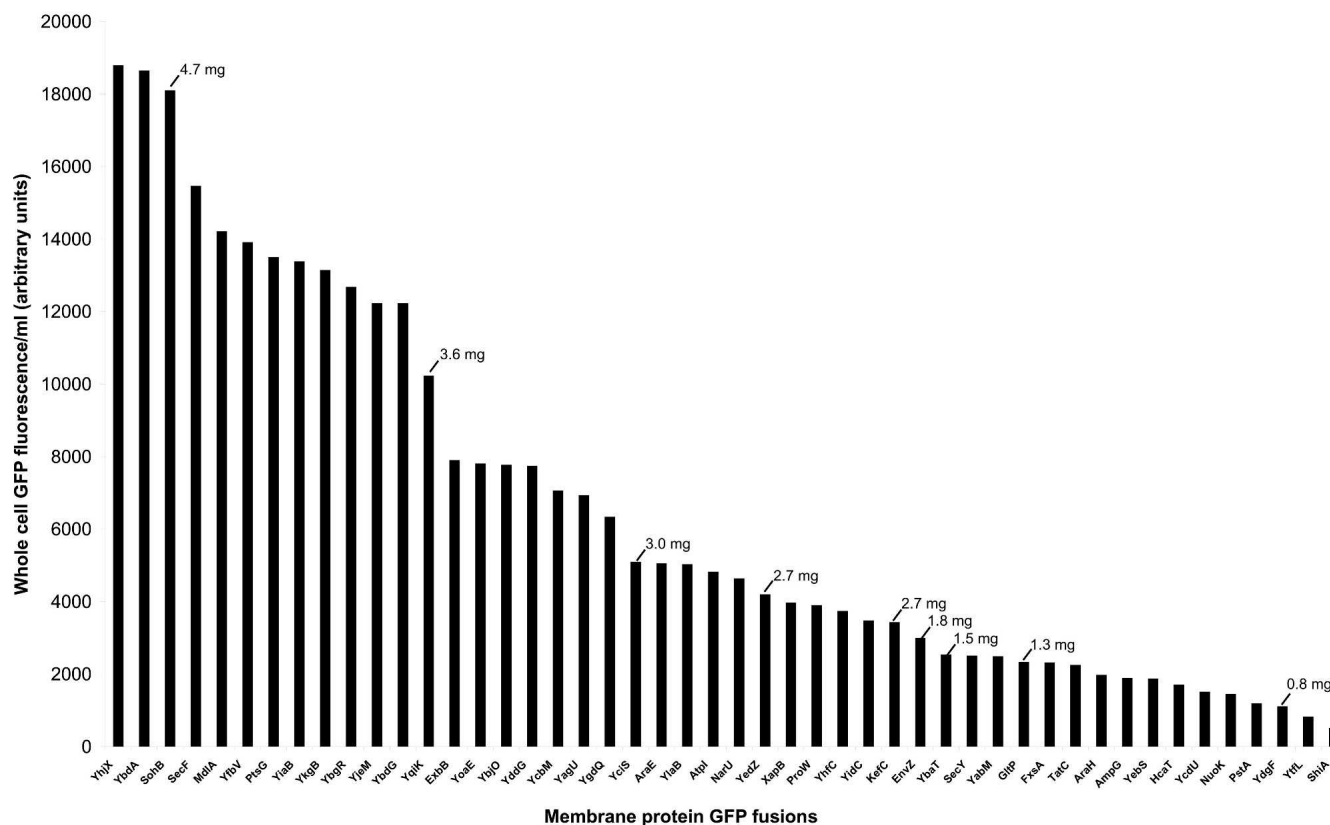


Figure 1. GFP-based MP overexpression screen. Forty-eight *E. coli* MPs were overexpressed as GFP fusions in *E. coli* as described in Materials and Methods. Whole-cell GFP fluorescence is expressed in arbitrary units. Nine MP-GFP fusions were isolated by using a standard protocol as described in Materials and Methods. The amount of purified MP-GFP fusion that can be isolated from 1 L of culture is shown in milligrams.

Table 1. GFP-based detergent screen for the YbaT-GFP fusion

% Detergent (w/v)	% Solubilized
1% n-dodecyl- β -D-maltopyranoside	88
1% Cymal 7	87
2% Cymal 6	84
1% n-undecyl- β -D-maltoside	72
1% n-decyl- β -D-maltopyranoside	56
2% n-octyl- β -D-glycopyranoside	44

GFP fluorescence was used to monitor the extraction of the membrane protein-GFP fusion from the membrane. The sum of the fluorescence of the nonsolubilized material and solubilized material equaled the fluorescence of the starting material in all cases. For details, see Materials and Methods.

state, poorly extracting detergents can be eliminated quickly in this step. GFP fluorescence is a good and time-saving alternative for the gel electrophoresis/Western blotting experiments usually used in detergent screens.

GFP-based purification scheme

To optimize the final step in the pipeline, we purified nine MP-GFP fusions (Fig. 1). These fusions differ widely in

size and are, as inferred from the whole-cell GFP fluorescence levels, overexpressed to different levels. Fusion proteins were purified using a combination of IMAC and size-exclusion chromatography (see Materials and Methods). The GFP moiety of the MP-GFP fusion allows the purification to be followed visually; e.g., binding efficiency of a fusion to a column or precipitation can be seen directly. The GFP moiety of the MP-GFP fusion makes it also possible to quickly and accurately determine protein concentrations.

Recovery of functional MP-GFP fusions

The TEV protease is functional in the presence of many detergents (Mohanty et al. 2003), and we reasoned that inclusion of a TEV site between the MP and the GFP-His₈ moiety should make it possible to recover intact, full-length MP from MP-GFP fusions. To test this final step in the pipeline, purified YbaT-GFP (a putative amino acid transporter), GltP-GFP (a glutamate transporter) (Wallace et al. 1990), and YedZ-GFP (a protein of unknown function) were digested with His-tagged TEV protease (Fig. 2A). The digests were almost complete and pure MP could be

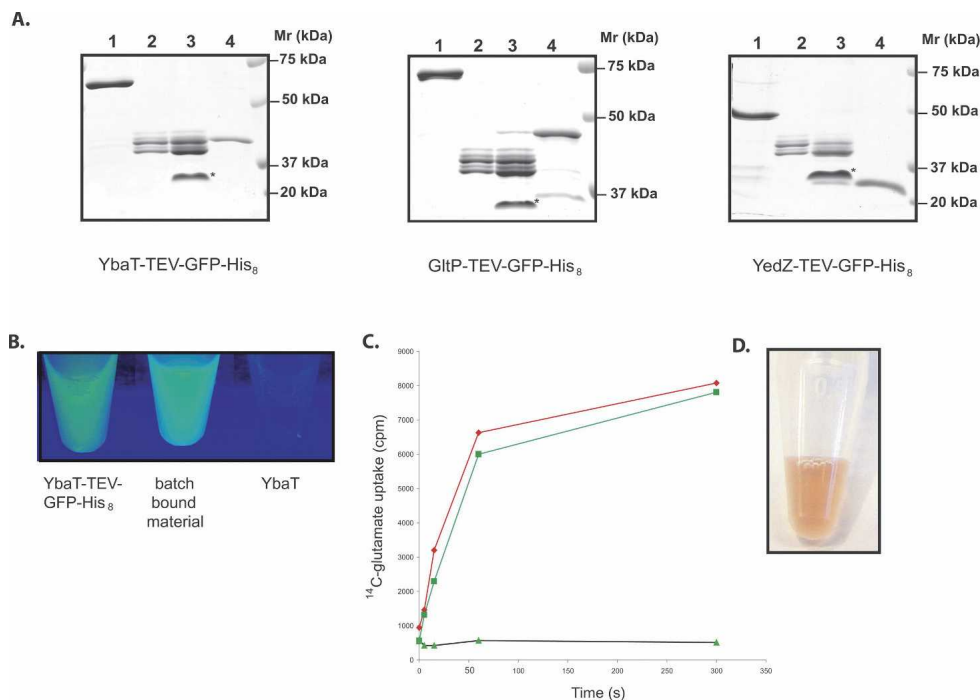


Figure 2. Digestion of MP-GFP fusions and characterization of recovered proteins. (A) SDS-PAGE of purified and TEV-digested YbaT- (left), GltP- (middle), and YedZ- (right) GFP fusions as described in Materials and Methods: purified fusion (lane 1), TEV protease (lane 2), eluted batch bound material (lane 3; GFP is marked with *), and recovered membrane protein (lane 4). The gel was stained with Coomassie brilliant blue R-250. (B) Cleavage of YbaT-GFP with TEV protease monitored under UV light: YbaT-GFP (lane 1), batch bound material (lane 2), and recovered YbaT (lane 3). (C) Glutamate uptake activity of proteoliposomes containing purified GltP recovered from a GltP-GFP fusion (diamonds), purified GltP-His₈ (squares), and control liposomes containing no protein (triangles). (D) Purified YedZ under ambient light.

obtained by removal of undigested MP-GFP fusion, clipped-off GFP-His₈, and His-tagged TEV protease by batch-binding to a Co-Talon resin (Fig. 2A). GFP fluorescence can be used to monitor both the effectiveness of the TEV digestion and the purity of the recovered MP (Fig. 2B).

To test whether the isolated MP is functional, purified GltP was reconstituted into lipid vesicles, and its activity was compared to purified GltP-His₈ (Fig. 2C). There was no difference in the glutamate uptake activity between GltP recovered from GltP-GFP and purified GltP-His₈.

YedZ attracted our attention since cells overexpressing YedZ-GFP were not green but orange, suggesting the presence of some kind of cofactor. Topology studies have shown that YedZ consists of six transmembrane segments connected by very short loops, with both the N- and C-terminal ends in the cytoplasm (Drew et al. 2002). YedZ belongs to a bacterial protein family of unknown function, UPF0191 (<http://www.sanger.ac.uk>), and is coded in the same operon as YedY, a periplasmic molybdoenzyme (Loschi et al. 2004). Its orange color suggests the presence of some kind of cofactor, although none of the Web-based prediction tools we used to analyze its sequence identified any potential cofactor binding motifs. To test the pipeline also on a potentially cofactor binding and previously uncharacterized protein, we decided to study YedZ further.

The recovered YedZ protein was, just as cells overexpressing YedZ-GFP, orange (Fig. 2D). Optical spectra of the purified YedZ protein were recorded under oxidizing and reducing conditions (Fig. 3A). Under reducing

conditions, the optical spectrum shows a maximum at 558 nm, indicating that YedZ contains a cytochrome *b*. Moreover, mass spectrometry (MS) showed that YedZ contains a cofactor with a molecular weight of 617 Da, which corresponds with the mass of heme *b* (Fig. 3B). YedZ contains a single heme, as determined by the pyridine hemechromogen assay (Zhu et al. 1999; Barber et al. 2002).

The absorption spectra of YedZ are typical of that of a cytochrome *b*, except for the broad and poorly resolved peak in the 450–500 nm region, most apparent under oxidizing conditions. This peak is an indication that YedZ also has a bound flavin (Barber et al. 2002). The flavin content of YedZ was determined by reverse-phase liquid chromatography after extraction of the chromophore (Fig. 3C). YedZ contains FMN rather than FAD, with a molar ratio of 0.7 FMN per YedZ molecule. The FMN content was further confirmed by a fluorescence assay (Burch et al. 1957). The determined FMN/protein ratio of 0.7, and the fact that FMN can be extracted from YedZ, shows that the flavin is not covalently attached to the protein. The FMN was not detected in MS, most likely because of the rather harsh porous R1 micropurification, that was required to clean up the YedZ sample for MS (see Materials and Methods).

We conclude that the GFP-based pipeline is compatible with the purification of proteins containing non-covalently bound cofactors; our simple purification procedure will facilitate the further characterization of the YedZ protein, which together with the periplasmic molybdoenzyme YedY forms a novel kind of nitrate reductase (D. Drew and J.W. de Gier, unpubl.).

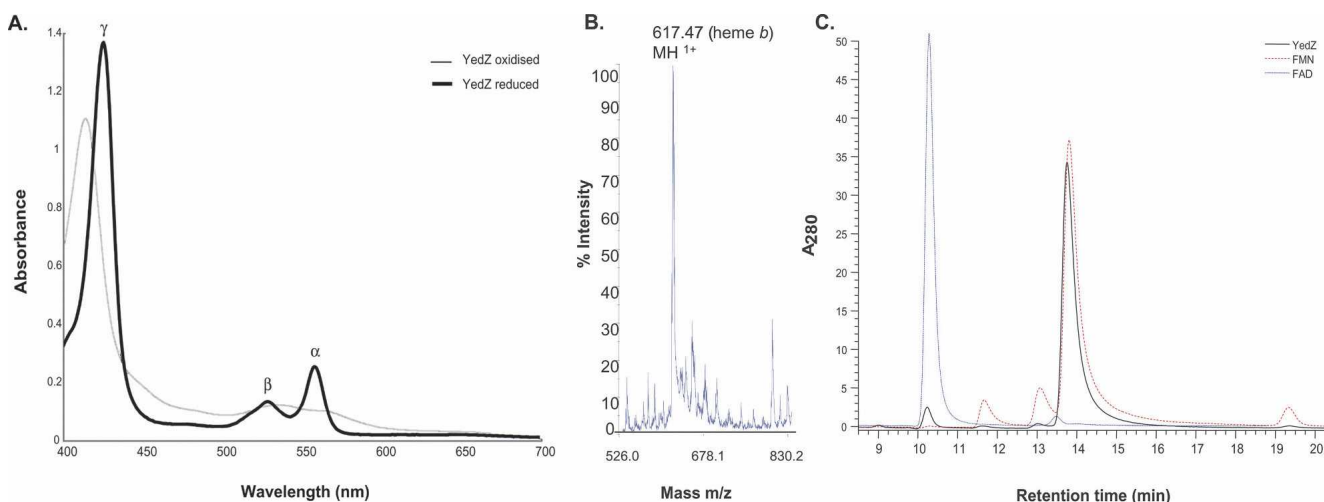


Figure 3. Optical absorption spectra of YedZ and identification of its cofactors. (A) Optical spectra of purified YedZ (concentration of ~ 0.01 mg/ml) after oxidation with ferricyanide and reduction with sodium dithionite. The absorption maxima of the reduced form of YedZ are $\alpha = 558$ nm, $\beta = 528$ nm, and $\gamma = 425$ nm. (B) MALDI-TOF MS spectrum of purified YedZ in reflectron mode. Measurements were in the m/z range of 500–1000 mass units. The mass of 617,47 represents the singly charged ion of heme *b*. (C) Identification by reverse-phase liquid chromatography of the flavin FMN in the supernatant from TCA-precipitated YedZ.

Overexpression of MP-GFP fusions in Lactococcus lactis

Lactococcus lactis, a promising new host for the overexpression of eukaryotic MPs, was used to explore if the GFP-based pipeline can be transferred to other systems (Kunji et al. 2003). The overexpression of eukaryotic MPs in the commonly used prokaryotic overexpression systems, such as *E. coli*, is notoriously difficult. It has been shown recently that the bacterium *L. lactis* may be an attractive alternative for the overexpression of MPs (Kunji et al. 2003). *L. lactis* is an easy-to-handle and low-cost system with little tendency to produce inclusion bodies. To establish if the GFP-based pipeline can be used also in *L. lactis*, we chose the human KDEL-receptor (KDELr) as a test case. It has been reported that 6 μg of functional KDELr can be expressed per liter of *L. lactis* culture (Kunji et al. 2003).

KDELr-GFP was, just as KDELr, expressed in the *L. lactis* membrane (Fig. 4A). Specific binding of tritium labeled YTSEHDEL peptide (a KDELr ligand) to membranes isolated from cells overexpressing KDELr-GFP was >10 times higher than for membranes isolated of cells expressing the KDEL-receptor without GFP (Fig. 4B). Based upon GFP fluorescence, good estimates of the overexpression level could be obtained even from a 200 μL culture (data not shown), which means that overexpression screening can be done in a 96-well format for *L. lactis*.

We conclude that GFP can be used to monitor MP-GFP overexpression in *L. lactis* and that GFP seems to stabilize overexpressed KDELr, resulting in the

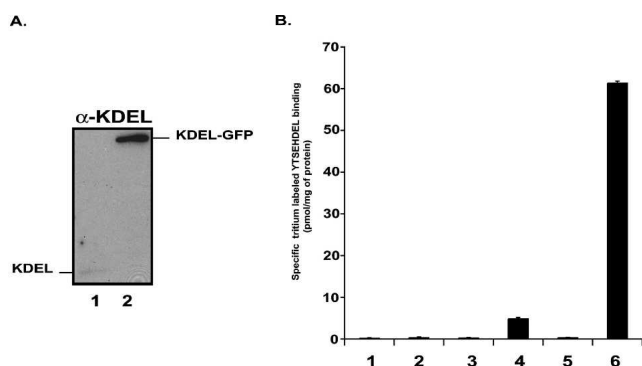


Figure 4. Functional overexpression of the human KDEL-receptor as a GFP fusion in *L. lactis*. (A) Western blot probed with antibodies raised against peptide 192–212 of the KDEL-receptor: membranes of *L. lactis* cells overexpressing the KDEL-receptor (lane 1) and membranes of *L. lactis* cells overexpressing a KDEL-GFP fusion (lane 2). (B) KDELr ligand binding studies carried out with isolated membranes of the uninduced (lane 1) and induced (lane 2) control strain (harboring an empty expression vector), the uninduced (lane 3) and induced (lane 4) strain with a KDELr expression vector (Kunji et al. 2003), and the uninduced (lane 5) and induced (lane 6) strain with the KDELr-GFP fusion expression vector. Binding assays were carried out in duplicate.

production of more functional protein. We have previously made a similar observation of increased overexpression of some GFP-tagged MPs in *E. coli* (Drew et al. 2003).

Conclusion

We have established a generic, GFP-based pipeline for rapid overexpression screening, detergent selection, and purification of functional MPs in *E. coli*. The use of the pipeline is exemplified by the identification and characterization of *E. coli* YedZ, the first membrane-integral flavocytochrome found to date. The GFP-based approach makes it possible to monitor all steps in the pipeline in a very easy manner. The approach is scalable and thus suitable for high-throughput applications. The GFP-based pipeline will facilitate the characterization of the *E. coli* membrane proteome and serves as an important reference for the characterization of other membrane proteomes.

*Materials and methods**Expression of MP-GFP fusions in E. coli*

Genes encoding MPs were amplified by conventional PCR, and cloned into a modified pET28(a+) vector that harbors the TEV protease recognition sequence (ENLYFQ↓G) followed by a C-terminally 8-histidine tagged GFP. Vectors harboring the MP-GFP fusions were transformed freshly for each experiment into BL21(DE3)pLysS, and cells were grown at 37°C on Luria broth (LB) medium containing 50 $\mu\text{g}/\text{mL}$ kanamycin and 30 $\mu\text{g}/\text{mL}$ chloramphenicol. We used plasmids encoding cytoplasmic resistance markers rather than periplasmic ones (e.g., β -lactamase) to avoid an extra workload for the Sec-translocon, which is involved in both the translocation of proteins across the cytoplasmic membrane and assembly of (overexpressed) MPs into the cytoplasmic membrane (Drew et al. 2003). One milliliter cultures were grown in 2-mL tubes in a thermomixer (Eppendorf) at 900 rpm, and 1 L cultures were grown in 3-L baffled conical flasks in an Innova 4330 (New Brunswick Scientific) shaker at 250 rpm. When the cultures had reached an OD_{600} 0.4–0.5, the temperature was switched to 25°C and MP-GFP expression was induced for 4 h with 0.4 mM isopropyl- β -D-thiogalactoside (IPTG). For 1 mL cultures, cells were harvested by centrifugation at 20,100g for 3 min; 1 L cultures, at 6200g for 10 min. Cells were washed once in an equal volume of PBS buffer. Cells taken from 1 mL of culture were resuspended in 200 μL PBS and transferred to a 96-well plate, and GFP emission was measured at 510 nm, with an excitation wavelength of 485 nm, on a SpectraMax Gemini (Molecular Devices).

GFP-based detergent screen

The detergents *n*-dodecyl- β -D-maltopyranoside (DDM) (1% w/v), *n*-undecyl- β -D-maltoside (1% w/v), *n*-decyl- β -D-maltopyranoside (1% w/v), cymal 7 (1% w/v) or cymal 6 (2% w/v),

and *n*-octyl- β -D-glucopyranoside (2% w/v) (all from Anatrache, Inc.) were added to membrane suspensions (3 mg of protein/mL in $1\times$ PBS), and samples were incubated for 1 h at 4°C under mild agitation. Nonsolubilized material was removed by centrifugation at 140,000g for 1 h at 4°C, and GFP emission was subsequently measured in both the resuspended pellet (nonsolubilized material) and solubilized material as described above.

Purification of MP-GFP fusions

Cells overexpressing MP-GFP fusions from a 1 L culture were broken by means of French pressing. Membranes were isolated by means of ultracentrifugation and subsequently solubilized in PBS with 1% (w/v) DDM. The suspension contained \sim 3 mg of protein/mL and was cleared by ultracentrifugation (30 min, 150,000g). The solubilize, to which 10 mM imidazole was added, was loaded onto a newly packed 5 mL Ni-NTA superflow resin column (Qiagen) at a flowrate of 0.5 mL/min. The column was washed with PBS containing 0.1% DDM and 20 mM imidazole for 20 CVs (column volumes). The column was subsequently washed with another 20 CVs in the same buffer containing 40 mM imidazole. MP-GFP fusions were eluted with PBS with 0.1% DDM and 250 mM imidazole. Eluted fractions were subsequently concentrated and loaded onto a Superdex 200 gel filtration column (Amersham Pharmacia) in PBS with 0.1% (w/v) DDM. The amount of purified MP-GFP fusion was measured using the BCA assay (Pierce) and GFP emission as described above using a GFP standard. The two different ways of measuring the amount of purified MP-GFP fusion were consistent.

Recovery of MPs from MP-GFP fusions

One milligram of MP-GFP fusion was incubated overnight at 10°C with 1 mg of His₁₀-TEV protease. The incubation mixture was then batch-bound to 0.5 mL Co-Talon resin (Clontech). After 1-h incubation at 4°C under mild agitation, the resin was spun down by centrifugation at 4000g for 5 min, and unbound material, i.e., the MP that had been clipped off from the MP-GFP fusion, was collected from the supernatant. Pelleted resin was washed several times, and the wash solution together with the original supernatant was passed through a filter to exclude any loose resin grains. Material bound to the resin, i.e., His₁₀-TEV, cleaved GFP-His₈, and uncleaved MP-GFP fusion, was eluted with 250 mM imidazole. Five micrograms of protein (as determined with the BCA assay, Pierce) of all fractions was assayed by SDS-PAGE/Coomassie brilliant blue R-250 staining.

Reconstitution of GltP in proteoliposomes and transport assays

Equal amounts of GltP recovered from a GltP-GFP fusion and purified GltP-His₈ were reconstituted in proteoliposomes. For assays of L-glutamate uptake driven by artificial gradients, the proteoliposomes were washed twice with 20 mM morpholine-ethanesulfonic acid (Mes) (pH 6) and 100 mM potassium acetate and concentrated. Proton motive force driven uptake was initiated by diluting the proteoliposomes 75-fold into 120 mM Mes, 100 mM methylglucamine, 0.7 mM valinomycin, and 1.3 mM L-[¹⁴C]-glutamate prewarmed at 30°C. Both a proton motive force and sodium ion motive force was created by dilution into the same buffer containing

100 mM NaOH instead of methylglucamine. Control experiments were performed by diluting the proteoliposomes into the buffer with which they were loaded (Gaillard et al. 1996).

Characterization of YedZ

Recovered YedZ was oxidized in 2 mM [Fe(CN)₆]³⁻. Subsequently, absorption spectra were measured with a light spectrophotometer (SHIMADZU, UV-1061), before and after reduction with sodium dithionite (final concentration, 10 mM).

For MALDI-TOF MS identification of cofactors, YedZ was micropurified using poros R1 (C4 like material) microcolumns (Gobom et al. 1999). A saturated solution of sinapinic acid (20 mg/mL) in 75% acetonitrile, 1% formic acid was used as an elution buffer. After deposition onto the MALDI target, the sample was washed with 10 mM diammonium citrate buffer to remove matrix adducts. The protein/heme ratio of YedZ was determined with a pyridine hemechromogen assay (Zhu et al. 1999; Barber et al. 2002).

To distinguish FMN from FAD and estimate the amount of flavin per YedZ molecule, YedZ was heated at 80°C for 45 min, and diluted in 50 mM ammonium acetate (pH 5.5). No degradation of FAD was observed during similar treatment of control samples. Samples were sonicated for 60 sec, and debris was removed by centrifugation. YedZ supernatant, FAD and FMN were adjusted to a concentration of 19.4 μ M and injected into a HPLC on a C18 column with mobile phase 66% 50 mM ammonium acetate pH 5.5/34% methanol. The flavin content of YedZ was also monitored using the method described by Burch (1957).

Expression of MP-GFP fusions in *L. lactis*

The Nisin A expression system (Kunji et al. 2003) was used to express MP-GFP fusions in *L. lactis*. GFP fluorescence was monitored as described above. The isolation of membrane vesicles, KDELr Western-blotting experiments, and ligand binding assays in the presence of CHAPS (0.5%) were performed as described (Kunji et al. 2003).

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