Preparative Scale Cell-free Production and Quality Optimization of MraY Homologues in Different Expression Modes

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Yi Ma, Daniela Münch, Tanja Schneider, Hans-Georg Sahl, Ahmed Bouhss, Umesh Ghoshdastider, Jufang Wang, Volker Dötsch, Xiaoning Wang, and Frank Bernhard

From the School of Bioscience and Bioengineering, South China University of Technology, Guangzhou 510006, China, the Institute of Biophysical Chemistry, Centre for Biomolecular Magnetic Resonance, J.W. Goethe-University, Frankfurt-am-Main 60438, Germany, the Institute of Medical Microbiology, Immunology, and Parasitology, Pharmaceutical Microbiology Section, University of Bonn, Bonn 53105, Germany, and the Institute of Biochemistry and Molecular and Cellular Biophysics, University Paris-Sud, UMR 8619 Orsay, France

Background: Functional MraY translocases can be cell-free expressed in high levels.
Results: Bacillus subtilis MraY activity is stable and robust, whereas Escherichia coli MraY depends on lipids.
Conclusion: Activity of MraY can be modulated by cell-free expression modes. Artificial hydrophobic environments have a strong impact on the MraY sample quality.
Significance: New strategy for the efficient production and analysis of drug targets.

MraY translocase catalyzes the first committed membrane-bound step of bacterial peptidoglycan synthesis leading to the formation of lipid I. The essential membrane protein therefore has a high potential as target for drug screening approaches to develop antibiotics against Gram-positive as well as Gram-negative bacteria. However, the production of large integral membrane proteins in conventional cellular expression systems is still very challenging. Cell-free expression technologies have been optimized in recent times for the production of membrane proteins in the presence of detergents (D-CF), lipids (L-CF), or as precipitates (P-CF). We report the development of preparative scale production protocols for the MraY homologues of E. coli and Bacillus subtilis in all three cell-free expression modes followed by their subsequent quality evaluation. Although both proteins can be cell-free produced at comparable high levels, their requirements for optimal expression conditions differ markedly. B. subtilis MraY was stably folded in all three expression modes and showed highest translocase activities after P-CF production followed by defined treatment with detergents. In contrast, the E. coli MraY appears to be unstable after post- or cotranslational solubilization in detergent micelles. Expression kinetics and reducing conditions were identified as optimization parameters for the quality improvement of E. coli MraY. Most remarkably, in contrast to B. subtilis MraY the E. coli MraY has to be stabilized by lipids and only the production in the L-CF mode in the presence of preformed liposomes resulted in stable and translocase active protein samples.

Bacterial cell wall biosynthesis represents an important pathway for antibiotic intervention, providing numerous individual target sites both on the level of enzyme inhibition and on the level of substrate sequestration. Enzymes involved in synthesis of the bacterial peptidoglycan layer are prevalent targets for antibiotic and drug developments, and the exploitation of their potential is continuously increasing in view of the growing emergence of multiresistant bacteria (1). The phospho-N-acetyl-muramoyl-pentapeptide translocase MraY is an integral membrane protein with 10 predicted transmembrane segments catalyzing the first membrane bound step of peptidoglycan biosynthesis by leading to the formation of lipid I upon transfer of uridine monophosphate-N-acetyl-muramoyl-pentapeptide onto the lipid carrier undecaprenyl phosphate (C55-P) (2-4). The MraY protein of Bacillus subtilis (Bs-MraY) has a calculated molecular mass of 36 kDa, whereas its homologue of Escherichia coli (Ec-MraY) has a higher molecular mass of 40 kDa. The proteins have a similar predicted topology with both termini located in the periplasmic space (2, 5). Their difference in size is mainly due to an enlarged N-terminal domain and an insertion into periplasmic loop III in the Ec-MraY protein (supplemental Fig. S1).

The enzymatic characterization and evaluation of MraY and similar membrane proteins as drug targets is still problematic.

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1 To whom correspondence may be addressed: Institute of Life Science, General Hospital of the People’s Liberation Army, Beijing, China.
2 To whom correspondence may be addressed: Max-von-laue Str. 9, Frankfurt-am-Main 60438, Germany. Tel.: 49-69-798-29620; Fax: 49-69-798-29632; E-mail: ibern@bpc.uni-frankfurt.de.
3 The abbreviations used are: C55-P, undecaprenyl phosphate; D, detergent; L, lipid; P, precipitate; CF, cell-free; LS, n-lauroyl sarcosine; CECF, continuous exchange cell-free; RM, reaction mixtures; Ni2+ -NTA, nickel-nitritoltriacid acid; SEC, size exclusion chromatography; Bistris, 1,3-bis(tris(hydroxymethyl)methylamino)propane; PC, phosphocholine; MP, membrane protein; DPC, n-dodecylphosphocholine; DHPD, dihexanoyl-phosphatidylcholine; LMPG, 1-myristoyl-2-hydroxy-sn-glycero-3-phospho-rac-(1-glycerol); β-OG, n-octyl β-D-glucopyranoside; DDM, n-dodecyl β-D-maltoside; CMC, critical micelle concentration; PL, polar lipid.
due to their unfavorable properties. Although structural details could provide important information for directed drug development, corresponding approaches are restricted mainly by the limited amounts of available protein. Expression of membrane proteins in conventional in vivo systems is notoriously difficult due to a number of intrinsic problems such as high hydrophobicity, inefficient translocation, toxicity, or degradation (6). Despite several recent improvements, the production of membrane proteins such as MraY therefore remains critical.

Cell-free (CF) expression techniques have emerged as an efficient approach to produce even complex membrane proteins that are otherwise very difficult to obtain (7). The evident advantage of this technology for the production of membrane proteins is the largely reduced complexity of the expression environment that minimizes toxic effects and that can even eliminate requirements for targeting and translocation machineries. A further valuable and unique feature of CF expression systems is their open accessibility and their high tolerance for numerous additives such as detergents or lipids. This allows completely new approaches to synthesize membrane proteins in the presence of detergents (D-CF mode) or supplied artificial lipids (L-CF mode). If no hydrophobic environments are provided, the synthesized membrane proteins precipitate after translation (P-CF mode). However, those P-CF-generated precipitates appear to form predominantly type I aggregates that can be relatively easily resolubilized without extensive refolding processes (8). Examples of polytropic and multidomain transporters or G-protein-coupled receptors from eukaryotic origin have already been reported to become synthesized in a functionally folded condition in CF extracts of E. coli (9–14). However, systematic studies to directly correlate the quality of CF-expressed membrane proteins with the applied expression conditions have been difficult to perform as additional procedures such as in vitro reconstitution were required for functional analysis.

We describe the high level production of two MraY proteins in defined CF expression systems. The established protocols yield milligram amounts of purified MraY proteins out of a few milliliters of reaction volumes in less than 2 days. We could modulate the specific activity of the CF-produced MraY proteins by modification of expression conditions and by choosing different CF expression modes. The CF production in optimized conditions resulted in MraY sample quality with enzymatic properties comparable with samples isolated from conventional cellular expression systems. Despite their homology, the two MraY proteins showed different characteristics upon CF expression. Although Bs-MraY was produced as a stable and functionally folded enzyme in an array of different conditions, the Ec-MraY protein was highly susceptible to aggregation and functionally folded enzyme in an array of different conditions, CF expression. Although Bs-MraY was produced as a stable and

**EXPERIMENTAL PROCEDURES**

**Detergents and Lipids**—Polyethylene glycol P-1,1,3,3-tetramethyl-butyldimethyl-ether (Triton X-100) was obtained from Merck Biosciences, Darmstadt, Germany; polyoxyethylene-(23)-lauryl-ether (Brij35), polyoxyethylene-(20)-cetyl-ether (Brij58), polyoxyethylene-(20)-stearyl-ether (Brij78), polyoxyethylene-(20)-oleyl-ether (Brij98), digitonin, n-lauroyl sarcosine (LS), and soybean phosphocholine mixture (PC) were obtained from Sigma; 1-myristoyl-2-hydroxy-sn-glycero-3-phospho-rac-(1-glycerol) (LMPG) and E. coli polar lipid (PL) mixtures were obtained from Avanti Polar Lipids, Alabaster, AL; n-octyl β-D-glucopyranoside (β-OG), dihexanoyl-phosphatidylcholine (DHPC), and SDS were obtained from Ana trace, High Wycombe, United Kingdom; n-dodecyl β-D-maltoside (DDM) was obtained from AppliChem, Darmstadt, Germany.

Lipids were dissolved in chloroform and thoroughly dried in a glass vial under a continuous stream of nitrogen using a rotary evaporator. The lipid film was resolubilized in 20 mM Tris, pH 8.2, 5 mM Mg(OAc)2, and 160 mM KOAc, and shaken and vortexed to obtain a homogenous suspension. The suspension was extruded with a Mini-Extruder (Avanti Polar Lipids) through a membrane with a 0.2-μm mesh size according to the manufacturer’s instructions. The produced liposomes were stored at 4 °C for a few days or at −80 °C for longer periods. The extrusion procedure was repeated prior to use after long term storage.

**Cell-free Expression Technology**—CF expression was performed in self-prepared extracts of E. coli strain A19. S30 extracts, T7 RNA polymerase, and basic reaction compounds were prepared as previously described (15, 16). Reactions were performed in the continuous exchange cell-free (CECF) configuration and incubated for ~16 h at 30 °C with gentle shaking, if not otherwise stated. Reaction mixtures (RM) and feeding mixtures were composed and prepared according to published protocols (17). The volume ratio of reaction:feeding mixtures was generally kept at 1:15 for analytical scale reactions and 1:17 for preparative scale reactions. For 55-μl analytical scale reactions, self-made Mini-CECF reactors were used with regenerated cellulose membranes with 14 kDa cut-off (17). The Mini-CECF reactors were incubated in standard 24-well microplates and the cavities of the microplates were used as a feeding mixtures compartment. Preparative scale reactions between 1 and 3 ml were performed with self-made Maxi-CECF reactors and with commercial Slide-A-Lyzer units (Pierce) as a RM container (17). Membranes and Slide-A-Lyzer were replaced for each new reaction.

**Strains, Plasmids, and DNA Manipulations**—The 972-bp Bs-MraY and 1080-bp Ec-MraY coding regions were cloned from B. subtilis strain 168 and E. coli strain MG1655, respectively. The coding regions were amplified from purified chromosomal DNA with the following primers: Bs-MraY-H10 for, 5′-CGC-GGATCCATGCTT GAGCAAGTCTCTTG-3′, and rev, 5′-CCGCTCGAGTAACCAACCTCGATGTAATTC-3′; Ec-MraY-H10 for, 5′-CGCGGATCCATGCTTCTTGCCTGCGGGAAC-3′, and rev, 5′-CCGCTCGAGCCTACCTTCAGCGTGT-GCCA-3′; Ec-H6-MraY for, 5′-ATAAGAATTCG-
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GGCCGCATGCTTGAGCAAGTCATTG-3’ and rev, 5’-CGC-GGATCCCTAAGCTTACCTTAGTTGCC-3’. Restriction sites used for subsequent cloning are shown in bold. The restricted PCR products were ligated into modified vectors pIVEX 2.4c (Roche Diagnostics, Penzberg, Germany) or pET21a(+)(Novagen, Darmstadt, Germany). To ensure fast purification, the Ec-MraY and Bs-MraY proteins were expressed from the resulting plasmids pET21a-Bs-MraY-H10 and pET21a-Ec-MraY-H10 with a C-terminal poly(His)$_10$ tag. The plasmid pIVEX2.4c-Ec-H6-MraY was used to produce Ec-MraY with a N-terminal poly(His)$_6$ tag. DNA templates used for CF expression were purified with commercial kits (Qiagen, Hilden, Germany) and used at stock concentrations of 0.2 μg/μl.

Protein Sample Preparation and Purification—After incubation, P-CF reactions were centrifuged at 18,000 × g for 10 min to pellet the expressed membrane proteins together with co-precipitating impurities. The pellet was washed twice with buffer (20 mM sodium phosphate, pH 7.2, 150 mM NaCl, 2 mM β-mercaptoethanol, and 30% glycerol) and finally suspended in appropriate detergent solutions in a volume equal to the initial RM volume. The suspensions were incubated at 25 °C for 2 h on a shaker to solubilize the membrane proteins. For the resolution of P-CF-produced MraY proteins, the following detergents dissolved in Milli-Q water (Millipore) were used: 2% (w/v) Triton X-100, 2% (w/v) β-OG, 2% (w/v) Fos12, 2% (w/v) DHPC, 2% (w/v) DDM, 0.8% (w/v) LS, 0.75% (w/v) LMPG, or 2% (w/v) SDS. After incubation, the residual precipitate was pelleted by centrifugation at 18,000 × g for 10 min and the supernatant was used for further purification.

For the D-CF expression mode, detergents were supplied into the RM and fedding mixtures at the following final concentrations: Brij35 (50 × CMC), Brij58 (75 × CMC), Brij78 (75 × CMC), Brij98 (50 × CMC), digitonin (8 × CMC), DDM (16 × CMC), and Triton X-100 (15 × CMC). For the L-CF expression mode, preformed extruded liposomes composed of E. coli polar lipids or phosphocholines were added to the RM at final concentrations of 4.6 mg/ml.

The CF-expressed MraY proteins were purified by immobilized metal-chelated affinity chromatography. Volumes of 1 ml of either D-CF soluble expressed MraY or P-CF-resolubilized MraY samples were mixed with 300 μl of Ni$^{2+}$-loaded NTA resin slurry (Qiagen, Hilden, Germany). The mixtures were supplemented with 10-fold column buffer (20 mM Na$_2$PO$_4$, pH 7.2, 300 mM NaCl, 20 mM imidazole, 0.2% DDM) and incubated for ~10 h at 4 °C with gentle shaking. The mixture was poured into an empty column and washed with 10 column volumes each of 20, 40, 60, and 80 mM imidazole dissolved in column buffer. Bound MraY proteins were then eluted with column buffer supplemented with 300 mM imidazole. For analysis by size exclusion chromatography (SEC), samples obtained by immobilized metal-chelated affinity chromatography were loaded on a Superdex 200 3.2/30 column (GE Healthcare, Europe, Munich, Germany) pre-equilibrated with SEC buffer (150 mM NaCl, 20 mM HEPES, pH 7.6, and 2 mM β-mercaptoethanol) supplemented with the appropriate detergent. SEC was performed with an AKTA purifier system (GE Healthcare) at flow rates of 50 μl/min. Staphylococcus aureus MraY used as control was expressed and purified as previously described (18). Purified proteins were stored at 4 °C or shock-frozen in liquid nitrogen in buffer (150 mM NaCl, 20 mM Tris, pH 8.0, 2 mM β-mercaptoethanol, and 30% glycerol) and then stored at −80 °C.

**SDS-PAGE and Immunoblotting**—For SDS gel analysis, protein samples supplemented with SDS sample buffer were loaded on 16% (w/v) Tris glycine-SDS gels and stained with Coomassie Blue. For Western blot analysis, the gels were transferred on a 0.45-μm polyvinylidene difluoride (PVDF) membrane (Millipore) in a wet Western blot apparatus for 35 min at 340 mA. Membranes were blocked for 1 h at room temperature (RT) in blocking buffer containing 1 × PBST buffer (0.137 mM NaCl, 0.003 mM KCl, 8 mM Na$_2$PO$_4$, and 1.5 mM KH$_2$PO$_4$ in H$_2$O, pH 7.4 and 0.05% Tween 20) and 4% skim milk powder (Fluka, Sigma). The membrane was blocked in 25 ml of PBST buffer with 1.25 g of milk powder overnight at 4 °C, washed three times at RT with 25 ml of PBST buffer, and incubated with the primary mouse anti-His antibody (Qiagen) at 1:2000 dilution. The membrane was washed three times at RT with 25 ml of PBST buffer. The membrane was further incubated for 1.5 h at RT with the secondary antibody goat anti-mouse IgG horseradish peroxidase conjugate (Sigma) at a 1:5000 dilution in PBST buffer. The membrane was subsequently washed three times at RT with PBST buffer. Finally, the blots were analyzed by chemiluminescence in a Lumi-imager F1™ (Roche Diagnostics, Penzberg, Germany).

**Enzymatic Synthesis of Radiolabeled UDP-MurNAc-pentapeptide**—The reaction mixture contained 100 nmol of purified UDP-MurNac-tripeptide (+-Ala--Glu-L-Lys, University of Warwick), 50 mM Bistris propane, pH 8, 5 mM MgCl$_2$, 3 mM ATP, 25 mM NH$_4$SO$_4$, 5 mM KCl, 0.5 mM DTT, 200 nmol of d-[14C]Ala (3.7 kBq/μl), and 3 μg each of purified enzymes MurF and DdlA from S. aureus (19) in a final volume of 130 μl. After incubating for 90 min at 30 °C the mixture was analyzed by analytic HPLC and quantitation was obtained by spectral absorbance (20). The radiolabeled product was diluted 10-fold with purified nonradiolabeled UDP-MurNac-pentapeptide.

**MraY in Vitro Activity Assay**—The standard MraY reaction contained 5 nmol of C$_{55}$-P, 10 mM N-lauroyl sarcosine, 100 mM Tris-HCl, pH 8, 40 mM MgCl$_2$, 50 nmol of UDP-MurNac-pentapeptide from Staphylococcus simulans (20), and 0.1–0.5 μg of MraY from B. subtilis in a total volume of 50 μl. The reaction was incubated at 30 °C for 90 min. Products were extracted with 1-butanol/pyridine acetate, pH 4.2 (1:1; v/v), and separated by thin layer chromatography (TLC; silica plates, 60F254; Merck) and visualized by phosphomolybdic acid staining and quantified by densitometry. For determination of $K_m$ values the MraY activity was assayed as described above except that the reaction contained 2.5 nmol of C$_{55}$-P (50 μM), 0.2 μg of purified MraY from B. subtilis, and various concentrations of the radiolabeled substrate UDP-MurNAc-pentapeptide (0.5 to 500 μM).

**Kinetic Analysis**—For determination of $K_m$ values, MraY activity was assayed as described above with various concentrations of UDP-MurNAc-pentapeptide (0.5 to 500 μM) while
maintaining the concentration of C\textsubscript{55}^\textsuperscript{+}P at a fixed value (50 \(\mu\text{M}\)). For calculation of kinetic parameters, Michaelis-Menten model and nonlinear regression were used (GraphPad Prism version 5.01). Detection and quantification of radiolabeled spots was carried out using a Storm PhosphorImager (GE Healthcare).

The inhibition of MraY activity by tunicamycin was assayed in the range of 5–200 \(\mu\text{M}\). All values represent the average of three independent experiments.

**Circular Dichroism (CD) Spectroscopy**—CD spectroscopy was performed with a Jasco J-810 spectropolarimeter (Jasco Labortechnik, Gross-Umstadt, Germany) using a quartz cuvette with a path length of 0.1 cm. Protein was dissolved in 10 mM sodium phosphate and 2 mM dithiothreitol, pH 7.2, supplemented with either 2% (w/v) DDM or 0.2% (w/v) Brij35. Assays were carried out at a standard sensitivity with a band width of 3 nm and a response of 1 s. The data pitch was 0.2 nm and the scanning rate 50 nm min\(^{-1}\). Spectra were recorded from 190 to 260 nm and the presented data are the average of five scans.

**RESULTS**

**CF Expression Protocol Development for MraY Production**—First, efficient basic CF expression protocols for the preparative scale production of Bs-MraY and Ec-MraY had to be established. Both proteins were expressed with a C-terminal poly-(His)\(_{10}\) tag to facilitate subsequent detection and purification. Purified plasmid DNA was added as template into the RM and the proteins were first produced in the CECF configuration in Germany) using a quartz cuvette with a path length of 0.1 cm. Protein was dissolved in 10 mM sodium phosphate and 2 mM dithiothreitol, pH 7.2, supplemented with either 2% (w/v) DDM or 0.2% (w/v) Brij35. Assays were carried out at a standard sensitivity with a band width of 3 nm and a response of 1 s. The data pitch was 0.2 nm and the scanning rate 50 nm min\(^{-1}\). Spectra were recorded from 190 to 260 nm and the presented data are the average of five scans.

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**FIGURE 1.** P-CF expression of MraY with different Mg\(^{2+}\) concentrations (12–22 mM) followed by Western blotting. A, Bs-MraY; B, Ec-MraY. P-CF-produced precipitated MraY and co-precipitated impurities were washed and suspended in S30-C buffer. Sample volumes of 2 \(\mu\text{l}\) were separated by 16% SDS-PAGE, stained with Coomassie Blue, and identified by Western blotting with anti-His antibodies. Arrows indicate the synthesized MraY proteins. Mg\(^{2+}\) concentrations are indicated in millimolar above the lanes, marker proteins (M) are in kDa.

**FIGURE 2.** Resolubilization screening of P-CF-produced MraY proteins. P-CF-generated precipitates were suspended in either 2% (w/v) Triton X-100, 2% (w/v) β-OG, 2% (w/v) DPC, 2% (w/v) DHPC, 2% (w/v) DDM, 0.8% (w/v) lauroyl sarcosine (LS), 0.75% (w/v) LMPG or 2% (w/v) SDS and solubilized fractions were separated from the residual precipitate by centrifugation. Sample volumes of 5 \(\mu\text{l}\) were analyzed by 16% SDS-PAGE. The solubilization efficiencies were determined by densitometry after immunoblotting using anti-His antibodies. Control is P-CF-expressed Bs-MraY or Ec-MraY without detergent solubilization. A, Bs-MraY; B, Ec-MraY.

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![Graph showing solubility and yield percentages for different detergents.]

**FIGURE 3.** D-CF expression analysis of MraY proteins. A, RM samples with the final volume of 2 μl were separated by 16% (w/v) SDS-PAGE and after Western blotting immobilized using anti-His antibodies. Lanes B1–B5, Bs-MraY; lanes E1–E5, Ec-MraY. Supplied detergents in the D-CF reactions are 8× CMC digitonin (lanes B1 and E1), 50× CMC Brij35 (lanes B2 and E2), 75× CMC Brij58 (lanes B3 and E3), 75× CMC Brij78 (lanes B4 and E4), and 50× CMC Brij98 (lanes B5 and E5). Marker proteins (M) are indicated in kDa. B, solubilization and production efficiency of D-CF-expressed Bs-MraY in the presence of DDM (16× CMC), Triton X-100 (7× CMC), digitonin (8× CMC), Brij35 (75× CMC), and Brij98 (50× CMC). Controls are P-CF-expressed Bs-MraY and Ec-MraY protein for the D-CF produced Bs-MraY and Ec-MraY proteins, respectively. 2-μl RM samples were separated by 16% SDS-PAGE, immobilized using anti-His antibodies after Western blotting, and the identified MraY signals were quantified by densitometry.

LS, DPC, and SDS resulted in almost 100% resolubilization of Ec-MraY.

As an alternative option that prevents the initial precipitation of MraY after translation, the proteins were synthesized in the D-CF mode in the presence of the detergents DDM, Triton X-100, digitonin, Brij35, Brij78, and Brij98. These detergents are known to be tolerated by the CF expression system in the supplied concentrations. Of the analyzed detergents, digitonin, Brij35, Brij78, and Brij98 were able to solubilize the synthesized Bs-MraY completely (Fig. 3). The solubilization efficiencies of DDM and Triton X-100 were estimated to be less than 20%. In addition, the detergents DDM, Triton X-100, and digitonin in the analyzed concentrations significantly reduced the production yield of Bs-MraY if compared with its P-CF expression. For Ec-MraY, quantification was not possible after D-CF expression with most detergents as the protein remained highly aggregated and did not migrate as a clear and resolved band upon analysis by SDS-PAGE (Fig. 3A). The only exception was D-CF expression in the presence of digitonin, which resulted in a well resolved band at an apparent molecular mass of 35 kDa. Again, the production yield of Ec-MraY in the presence of digitonin was reduced similar to the results obtained with Bs-MraY (Fig. 3B).

Only a minor fraction of the Bs-MraY protein remained aggregated by SDS-PAGE analysis after its D-CF expression in Brij78 and Brij98 and no residual aggregation was detectable after its D-CF expression in Brij35. After screening concentration ranges of the supplied detergents, the optimal CMCs for best production and solubilization of Bs-MraY were determined to 25× CMC for Brij35, 75× CMC for Brij78, and 50× CMC for Brij98.

The resolubilized P-CF precipitates as well as the soluble D-CF-expressed proteins could be purified by Ni²⁺-NTA affinity chromatography to apparent homogeneity (supplemental Fig. S2). Furthermore, the immobilization of MraY proteins to the Ni²⁺-NTA material was used to systematically exchange the primary detergents initially used either as supplements in the D-CF mode or for resolubilization of P-CF precipitates against different secondary detergents. By that strategy, a variety of MraY samples solubilized in different micelles was generated for systematic quality analysis.

**Homogeneity and Stability of P-CF- and D-CF-expressed MraY Proteins**—Purified P-CF-produced Bs-MraY was either solubilized in 0.75% (w/v) LMPG and exchanged into 0.1% (w/v) Brij35 upon affinity purification, or solubilized in 2% (w/v) DDM and exchanged into the lower concentrated 0.2% (w/v) DDM. The samples were then analyzed by CD spectroscopy for the content of secondary structural elements (supplemental Fig. S3). The amount of α-helical structural elements was determined to be 61% for samples in Brij35 and 68% for samples in DDM. These data are comparable with the predicted α-helical content of 70% for Bs-MraY.

The homogeneity of MraY samples purified by Ni²⁺-NTA affinity chromatography was evaluated by SEC elution profiling to detect potential residual protein aggregates (Fig. 4). Proteomicelles of P-CF-produced Bs-MraY solubilized in DDM showed symmetric elution peaks at a volume corresponding to ~140 kDa. Considering that the DDM micelle contributes within a range of 80–120 kDa to the determined mass of the proteomicelles, this result then gives evidence of monomeric Bs-MraY in the elution fractions. The purified Bs-MraY remained stable in DDM for at least 1 week at 4 °C as judged by repeated SEC analysis. The quality of P-CF-produced Bs-MraY was dependent on the nature of the detergent micelle. Bs-MraY samples resolubilized in LMPG showed an additional second SEC elution peak close to the void volume, indicating residual aggregates (data not shown). The SEC elution profiles of detergent-solubilized Ec-MraY samples were generally much more heterogeneous and in best cases, only a broad elution peak presumably containing larger aggregates were detected after P-CF expression and resolubilization in detergent DPC (Fig. 4).

**Modulation of Enzymatic Activity of Bs-MraY by the CF Expression Mode**—By taking advantage of the P-CF, D-CF, or L-CF expression modes, the MraY proteins could be synthesized as precipitates, as proteomicelles, or as a lipid-associated complex. The different CF expression modes could affect the yield and in particular the functional folding of the synthesized membrane proteins and are thus valuable tools to modulate the sample quality. First we analyzed the activity of Bs-MraY alternatively synthesized in the P-CF or D-CF modes and by using a variety of detergents for post-translational or cotranslational solubilization. The functional activity of the synthesized MraY samples was evaluated by the specific lipid I formation after transfer of the UDP-MurNac-pentapeptide to C₅₅-P in vitro.
Evaluation of the samples was done by detection of synthesized lipid I by TLC after extraction from the reaction mixture (Fig. 5). Quantification was carried out by monitoring lipid I synthesized with radiolabeled UDP-MurNAc-pentapeptide.

Translocase activity of Bs-MraY samples could be detected after production in almost any of the analyzed CF reaction conditions, regardless whether the protein was precipitated after translation (P-CF mode) or kept soluble by addition of detergents (D-CF mode). To quantify the effects of the selected detergent on the specific activity of the CF-produced protein samples, we systematically analyzed the impact of the three detergents, DPC, DDM, and LMPG, on P-CF-produced Bs-MraY. The three detergents have been used in any combination as the primary detergent to initially solubilize the precipitated MraY samples as well as secondary detergents for micelle exchange upon immobilized metal-chelated affinity chromatography purification (Table 1). Translocase activity was quantified using the radiolabeled UDP-MurNAc-pentapeptide and the results were normalized according to the corresponding protein concentrations for obtaining the specific activities. The selection of the primary detergent for the initial solubilization of P-CF-produced Bs-MraY had a clear impact on the final sample quality. Solubilization in DDM resulted in the highest specific activities with all three secondary detergents, DPC, DDM, and LMPG, on P-CF-produced Bs-MraY. However, the activity was highest if the protein remained in DDM during purification. The solubilization of P-CF-produced Bs-MraY precipitates in LMPG or DPC resulted in less than half of the activity if compared with the sample solubilized and remaining in DDM (Table 1). Again the activity was best if the sample was analyzed in DDM as a secondary detergent. DPC and LMPG appear to irreversibly inhibit part of the enzyme fraction as the subsequent exchange into DDM did not restore the full activity. In contrast, once folded in DDM, the protein stays relatively stable even in LMPG or DPC. All CF produced samples of Bs-MraY had higher specific activities if compared with the control protein isolated from S. aureus.

Bs-MraY samples expressed in the D-CF mode in the presence of Brij35 and with Brij35, DDM, DPC, or LMPG as secondary detergents showed only moderate activities (Table 1). Similar to the resolubilization in LMPG or DPC, the cotranslational solubilization in the Brij35 resulted in an irreversible reduction of the specific activity of Bs-MraY. DDM could not be used for the D-CF expression as it already inhibits the CF reaction at lower concentrations. Also D-CF expression in Brij35 resulted in a notable reduction of Bs-MraY production if compared with the P-CF expression.

In the L-CF mode, preformed liposomes composed of phosphocholines (PC) were added into the RM and the synthesized proteins have the possibility to insert cotranslationally into the membranes. The liposomes fuse during the incubation and precipitate together with nonincorporated synthesized MraY protein. After the reaction, the precipitate therefore potentially consists of a mixture of MraY precipitate, empty liposomes, and lipid-protein complexes. The relative activities of these L-CF sample mixtures was measured. Moderate activity was detected with the Bs-MraY L-CF (PC) sample, comparable with the values obtained after D-CF expression.

Stabilization of Ec-MraY by Lipids and Modulation of the Reaction Conditions—In contrast to Bs-MraY, samples of the Ec-MraY protein were mostly completely inactive after production in P-CF and D-CF modes (Table 1). No activities were detected after P-CF expression and solubilization in DPC or LMPG. As DDM is much more inefficient for the solubilization of P-CF-generated Ec-MraY precipitates if compared with Bs-MraY, a significantly higher concentration had to be used to finally solubilize an estimated fraction of 20% of the precipitated protein. Spurious activity of the DDM-solubilized sam-

![FIGURE 4. SEC elution profiles of P-CF-produced MraY samples loaded on a Superdex 200 3.2/300. Samples were resolubilized in 2% (w/v) DPC and exchanged to 0.2% (w/v) DDM upon affinity purification. SEC was performed in 150 mM NaCl, 20 mM HEPES, and 2 mM β-mercaptoethanol. V₀, void volume; A, Bs-MraY; B, Ec-MraY.](image)

![FIGURE 5. Synthesis of lipid I by recombinant Bs-MraY and Ec-MraY proteins. In vitro activity assays for MraY were performed with 0.5 μg of enzyme incubated with 5 nmol of C55 and 50 nmol of UDP-MurNAc-pentapeptide. Reaction products were separated by TLC and subsequently quantified by storage phosphorscreen technology. Lane 1, S. aureus MraY control; lane 2, Bs-MraY P-CF produced and resolubilized in 0.75% (w/v) LMPG; lane 3, Bs-MraY P-CF produced and resolubilized in 2% (w/v) DDM; lane 4, Bs-MraY P-CF produced and resolubilized in 2% (w/v) DPC; lane 5, Bs-MraY D-CF produced in the presence of Brij35 (50× CMC). All samples were purified by Ni²⁺-NTA chromatography prior to enzymatic activity assay. Lane 6, Bs-MraY L-CF produced in the presence of phosphocholines (4.56 mg/ml); lane 7, Ec-MraY L-CF produced in the presence of polar lipid (4.64 mg/ml); lane 8, Ec-MraY L-CF produced in the presence of polar lipid (4.64 mg/ml) and 5 mM DTT; lane 9, Ec-MraY L-CF produced in the presence of phosphocholines (4.64 mg/ml); lane 10, assay without addition of enzyme; lane 11, assay without enzyme and addition of purified lipid I (1 nmol).](image)
Cell-free Expression of MraY Proteins

TABLE 1

| Protein | CF mode | Primary detergent | Secondary detergent | Yield | Activity |
|---------|---------|------------------|---------------------|-------|----------|
| Bs-MraY | P-CF    | 0.75% LMPG       | 0.2% DDM            | ++    | 5.0      |
|         |         |                  | 0.1% DPC            | ++    | 4.8      |
|         |         |                  | 0.05% LMPG          | ++    | 4.6      |
|         |         |                  | 2% DDM              | ++    | 21.7     |
|         |         |                  | 0.05% LMPG          | ++    | 10.9     |
|         |         |                  | 0.1% DPC            | ++    | 16.3     |
|         |         |                  | 0.1% DPC            | ++    | 5.3      |
|         |         |                  | 0.2% DDM            | ++    | 8.4      |
|         |         |                  | 0.05% LMPG          | ++    | 7.9      |
|         |         |                  | 0.2% DDM            | ++    | 4.6      |
|         |         |                  | 0.05% LMPG          | ++    | 4.0      |
|         |         |                  | 0.1% DPC            | ++    | 4.3      |
|         |         |                  | 0.5% Brij35         | ++    | 4.2      |
|         |         |                  | 0.5% Brij35         | ++    | 4.2      |
| Ec-MraY | P-CF    | 0.75% LMPG       | 0.2% DDM            | ++    | 0        |
|         |         |                  | 5% DDM              | ++    | 0        |
|         |         |                  | 5% DDM              | ++    | (3.0)*   |
|         |         |                  | 25 °C, 5 mM DTT     | 6 mM β-ME | +      |
|         |         |                  | 5% DDM              | ++    | 0        |
|         |         |                  | 22 °C, 5 mM DTT     | 6 mM β-ME | +      |
|         |         |                  | 5% DDM, 1 mg/ml PC  | ++    | 0        |
|         |         |                  | 2% DPC              | ++    | 0        |
|         |         |                  | 2% DPC              | ++    | 0        |
|         |         |                  | 0.5% Brij35         | ++    | 0        |
|         |         |                  | 0.5% Brij35         | ++    | 0        |
|         |         |                  | 22 °C               | ++    | 0        |
|         |         |                  | 0.5% Brij35         | ++    | 0        |
|         |         |                  | 5 mM DTT            | 6 mM β-mercaptoethanol | +      |
|         |         |                  | 5 mM DTT            | 6 mM β-mercaptoethanol | +      |
|         |         |                  | 22 °C               | 0.7% Digitonin | +      |
|         |         |                  | 0.7% Digitonin      | +      |
|         |         |                  | 22 °C, 5 mM DTT     | 0.7% Digitonin | +      |
|         |         |                  | 22 °C               | 0.7% Digitonin | +      |
| L-CF    | PL      | ++               | 4.3                 |
|         | PL, 5 mM DTT | ++         | 5.5                 |
|         | PL, 22 °C, 5 mM DTT | +      | 2.3                 |
|         | PC      | ++               | 3.0                 |

* MraY yields after solubilization/purification are rated according to Western blot signal intensities: ++ = 100 μg/ml; +++ = 500 μg/ml; ++++ = >500 μg/ml.

* Activity analysed by lipid I formation after 90 min of incubation. *S. aureus* activity as a control accounts for approximately 3 nmol/μg of protein in that assay.

Samples are unstable and need to be analyzed instantly after expression.

...whereas Bs-MraY is completely devoid of cysteines (supplementary Fig. S1). Therefore, the reducing conditions of the CF reactions were additionally modified by increasing the DTT concentration from 2 to 5 mM. As a result of these modifications, the total production yields of Ec-MraY were reduced down to 60% by incubation at 22 °C. A further temperature reduction to 20 °C resulted in a significant decrease of the expression efficiency down to only 35% if compared with the incubation at 30 °C. Moderate translocase activity of Ec-MraY samples could only be measured if expression at lower temperatures and increased DTT concentrations were combined and if reducing conditions were maintained during the purification procedure (Table 1). However, yields were very low with only ~50 μg/ml of RM and the stability of the samples at 4 °C was limited to a few days in the best cases.

In contrast, the L-CF expression appeared to be a reliable approach for the production of functional Ec-MraY. The expression in the presence of supplied PC liposomes resulted in stable activity already at standard conditions at 30 °C incubation (Table 1). A slight improvement was obtained after replacing the PC liposomes with liposomes prepared of *E. coli* PL mixtures. Reduced expression temperatures and/or increased DTT concentrations did not result in any further significant...
improvements of the Ec-MraY specific activity. The stability of the L-CF-produced Ec-MraY samples was much higher and the samples could reliably be measured during several days. Based on that finding, ~2 mg of functional and stable Ec-MraY protein can be produced in the L-CF mode in 1 ml of RM. Adding lipids post-translationally by solubilization of P-CF produced Ec-MraY precipitates with a mixture of DDM, and lipids did not restore the translocase activity (Table 1).

Enzymatic Characteristics of Bs-MraY—To define the enzymatic characteristics of CF-produced MraY protein, a sample of the highly active P-CF-produced Bs-MraY solubilized in 0.2% (w/v) DDM was prepared as described above. The kinetics for lipid I formation of the protein sample was analyzed in the translocase assay and typical Michaelis-Menten kinetics were observed for its specific substrate UDP-MurNAc-pentapeptide in the 0.5 to 500 μM concentration range. The $K_m$ value of the purified Bs-MraY translocase for this substrate was determined to 36.2 ± 3.6 μM. Tunicamycin inhibits enzymes catalyzing the transfer of UDP-GlcNAc or UDP-MurNAc-pentapeptide onto polyprenyl-phosphate carrier lipids (22), thereby interfering with cell envelope biosynthesis processes. We investigated the effect of tunicamycin on the transfer of the precursor UDP-MurNAc-pentapeptide onto the undecaprenyl phosphate carrier $C_{55}$-P as catalyzed by the P-CF-produced Bs-MraY sample. The translocase activity was clearly inhibited by tunicamycin and an IC$_{50}$ of ~22 μM was determined (Fig. 7).

DISCUSSION

Bacterial cell wall biosynthesis represents one of the most important target pathways for antibacterial chemotherapy and is still a priority area for pharmaceutical research (1, 23, 24). Lipid I is assembled on the inner surface of the cytoplasmic membrane as the first lipid-linked intermediate of peptidoglycan synthesis by transfer of the phospho-MurNAc-pentapeptide moiety of UDP-MurNAc-pentapeptide to the membrane anchor $C_{55}$-P (25–27). This first membrane-bound step is catalyzed by the integral translocase MraY (3, 28). MraY is essential for bacterial viability as well as a ubiquitous presence in the eubacterial kingdom. The protein could therefore become a prime drug target, but despite the numerous inhibitors of MraY described, no one has yet entered clinical use as antibiotic (29, 30). The difficult production of MraY and other MPs involved in peptidoglycan biosynthesis so far have largely retarded the detailed study of their biosynthetic mechanisms and the search of novel antibacterials. Nevertheless, the substrate specificity of MraY was intensively studied and a number of enzymatic assays designed for throughput screening of inhibitors have been developed (31–33).

The overproduction of Ec-MraY as well as Bs-MraY has been attempted in conventional in vivo expression systems before. The expression of Bs-MraY in E. coli cells could be optimized up to final yields of ~1.2 mg/liter of cell culture (3). The production of Ec-MraY in similar systems appeared to be much more difficult and was accompanied by limited success, giving only modest efficiencies of some 30-fold increases in expression (2, 34). In our CF system based on E. coli extracts we made the opposite observation. The production of Ec-MraY with routine efficiencies of 2 mg/ml of RM was twice as high as that of Bs-MraY. Both proteins were produced at preparative scales and their full-length synthesis was verified by immunodetection of their C-termini. The observed putative dimeric complexes upon SDS-PAGE analysis are common to many MPs at higher concentrations. It is not yet clear whether they represent residual native MP dimers that resisted SDS denaturation or whether
they are artifacts induced by SDS treatment. The CF system is devoid of post-translational control systems that scrutinize, e.g. reconstituted MPs, and degrade misfolded proteins. The high instability of Ec-MraY associated with increased degradation might therefore contribute to the observed low expression in *E. coli* cells.

The characteristics of the purified CF-expressed Bs-MraY can be compared with published data of the protein isolated from *E. coli* cells (3). Both samples show faster migration upon SDS-PAGE analysis probably due to incomplete SDS denaturation, a feature that appears to be common for membrane proteins (35). The CF-expressed Bs-MraY showed specific translocase activity under all tested conditions. The protein was active if either cotranslationally solubilized with supplemented detergents in the D-CF mode or if initially produced as a precipitate in the P-CF mode and then post-translationally solubilized. The recovered activity of P-CF samples indicates that reversible type I aggregates are formed upon precipitation of Bs-MraY, which can be resolved without extensive denaturation and refolding procedures (8). Similar observations were made previously with other P-CF-expressed MPs and it can be considered as a frequent, although not general, feature of the P-CF mode (9, 10). The specific activity of Bs-MraY was clearly highest if the protein was primarily solubilized and further kept in DDM. All other analyzed detergents reduced the specific activity to ~20–30%. The positive effect of DDM seems to result rather from an optimized folding of the protein than from an improved access of ligands as higher specific Bs-MraY activity could hardly be restored after exchange from other primary detergents into DDM. DDM together with LS were also described as the best detergents for the in vivo expressed Bs-MraY (3). LS could not be analyzed as a primary detergent in our system as it is not tolerated by the CF reaction and thus cannot be used in the D-CF mode, and it was also not efficient in the solubilization of the P-CF-generated precipitates. The *Km* value of P-CF-produced Bs-MraY in DDM for its substrate UDP-MurNAc-pentapeptide was found 27-fold lower than the value obtained with in vivo expressed Bs-MraY (3). Also the IC50 value for the inhibitor tunicamycin was ~40% lower.

Translocase activity of Ec-MraY was hard to detect with protein samples generated by P-CF or D-CF expression modes and the protein was generally highly unstable in detergent, in contrast to Bs-MraY. Ec-MraY and Bs-MraY share ~45% sequence similarity. Many conserved residues are located in the cytoplasmatic loops and are responsible for substrate recognition and catalytic processes (2). The periplasmatic loops are more variable and in particular interesting for antibiotic targeting as they are more readily accessible by antibiotic treatments. The cysteine residues appeared to affect the stability of Ec-MraY in detergent micelles as increased concentrations of reducing agents in combination with reduced expression temperatures had positive effects on the activity of Ec-MraY. The involvement of the two cysteine residues in intermolecular cross-links is likely as strong aggregation was observed upon SEC or SDS-PAGE analysis. The cysteines are conserved in MraY proteins of other enterobacteria and similar characteristics of those proteins can be speculated. The aggregation of Ec-MraY appears to be additionally promoted by its fast expression kinetics in the CF system.

Stable translocase activity of Ec-MraY could only be measured if the protein was produced in the L-CF mode in the presence of supplemented lipids. Lipids can play important roles as chaperones in the folding and stabilization of proteins as well as in their activation (36, 37). The cotranslational presence of lipids seems to be important as their re-addition to P-CF generated Ec-MraY precipitates that did not have positive effects on translocase activity. The cotranslational association of Ec-MraY with the provided liposomes appears to stabilize protein folding and probably prevents its aggregation. The activity of several MraY homologous has been reported to depend on lipids. Membrane-extracted and detergent-solubilized MraY proteins of *S. aureus* and *Micrococcus luteus* could be reactivated to some extent by the addition of lipids (38, 39). Accordingly, the translocase activity of Ec-MraY isolated from crude membranes was found to depend on the presence of phospholipids and only activity associated with liposomes was detected (40). The nature of this lipid activation is not yet clear and the presented strategy for Ec-MraY production might represent a perspective for its analysis. We could obtain similar translocase activities of Ec-MraY with supplemented complex lipid mixtures or with single types of lipids. The L-CF expression mode of MPs is still an emerging technique and many details require further evaluation. So far it is difficult to analyze whether the synthesized MPs associate with the lipids or whether they partially or completely integrate into the bilayer. Ionic exchange or affinity chromatography resulted in almost complete loss of MraY translocase activity, indicating that a potential associated co-isolated compound stabilizing Ec-MraY might get lost during too harsh purification procedures (34, 38, 40). Activation by phosphatidylglycerol and other lipids was speculated before, and those compounds may act in the stabilization of the structural integrity of the protein solubilized in detergent micelles (38). Several parameters appear therefore to affect the quality of Ec-MraY. Reduced expression temperature and increased DTT concentrations resulted in moderate but unstable translocase activity also in the absence of lipids. Once aggregated, the activity of Ec-Mray cannot be recovered by addition of lipids and extensive denaturation and refolding procedures may be applied. Although lipids seem therefore not to be absolutely essential for translocase activity, the cotranslational presence of lipids appears to significantly stabilize the folding of nascent Ec-MraY, resulting in reduced aggregation and improved sample quality. Despite their eminent role for Ec-MraY, lipids did not show detectable effects on the quality of Bs-MraY.

The presented work demonstrates new approaches for the characterization and comparison of pharmaceutically important MraY homologues. Efficient CF expression protocols have been established for the fast and preparative scale production of functional MraY proteins representing homologues of Gram-positive and Gram-negative bacteria. The proteins are now accessible for pharmaceutical studies and screening approaches by in vitro analysis in defined environments and the work also opens new perspectives for structural characterization. We further could provide a systematic quality optimization of the analyzed proteins by variation of the hydrophobic
environment in the CF reactions, which could serve as a guideline for similar approaches. We show for the first time marked differences in the characteristics of MraY proteins and the detected properties may provide a basis for the development of specific inhibitors or general antibiotics. The reported production protocols will furthermore, enable approaches to study the interaction of MraY proteins in well defined conditions without any undesired background of lipids or any other membrane proteins.

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