The MraY translocase catalyzes the first membrane step of bacterial cell wall peptidoglycan synthesis (i.e. the transfer of the phospho-N-acetylmuramyl-pentapeptide motif onto the undecaprenyl phosphate carrier lipid), a reversible reaction yielding undecaprenylpyrophosphoryl-N-acetylmuramyl-pentapeptide (lipid intermediate 1). This essential integral membrane protein, which is considered as a very promising target for the search of new antibacterial compounds, has thus far been clearly underexploited due to its intrinsic refractory nature to overexpression and purification. We here report conditions for the high level overproduction and for the first time the purification to homogeneity of milligram quantities of MraY protein. The kinetic parameters and effects of pH, salts, cations, and detergents on enzyme activity are described, taking the Bacillus subtilis MraY translocase as a model.

The growing evidence of multiresistance of pathogenic bacteria to currently used antibiotics requires the development of new therapeutic compounds and the identification and exploitation of novel targets (1). The enzymes of the pathway for cell wall peptidoglycan biosynthesis that are essential for bacterial growth and specific to eubacteria constitute such a set of interesting potential targets that should be explored in detail. Indeed, peptidoglycan, the heteropolymetric mesh of the bacterial cell wall, plays a critical role in protecting bacteria against osmotic lysis. It is also responsible for the maintenance of a defined cell shape and is intimately involved in the cell division process (2). The peptidoglycan monomer unit, N-acetylglucosaminyl-β-1,4-N-acetylmuramyl-pentapeptide (GlcNAc-MurNAcβ-pentapeptide), is synthesized by enzymes located in the cytoplasm or at the inner side of the cytoplasmic membrane, and its polymerization, occurring at the outer side of the cytoplasmic membrane, is catalyzed by the penicillin-binding proteins, the targets of the β-lactam antibiotics (3). This implies the passage of the monomer unit from the cytoplasm to the periplasm through the hydrophobic environment of the membrane, a process involving the transfer of this hydrophilic unit onto a lipid carrier, undecaprenyl phosphate (C55-P) (3, 4). The first membrane step is the transfer of the phospho-MurNAc-pentapeptide moiety onto C55-P, yielding C55-PP-MurNAc-pentapeptide (lipid 1), a reaction catalyzed by the MraY enzyme (Scheme 1). Since this reaction consists of the translocation of a peptidoglycan precursor moiety from the cytoplasm to the membrane, the latter enzyme has been named MraY “translocase” (5). This reaction is reversible, but in vivo it is drawn by coupling to the subsequent reaction catalyzed by the MurG transferase (5–8). The MraY translocase is an integral membrane protein whose topology has been recently determined (9); it is composed of 10 transmembrane segments, five cytoplasmic domains, and six periplasmic domains, including the N- and C-terminal ends. The latter model has been established with MraY proteins from Escherichia coli and Staphylococcus aureus and thus appears to be conserved in both Gram-negative and Gram-positive bacteria. Alignment of bacterial MraY sequences shows that the five cytoplasmic domains comprise many highly conserved amino acid residues. The presence of the MraY translocase exclusively in bacteria, the fact that it is essential for viability (which has been demonstrated in E. coli and Streptococcus pneumoniae (10, 11)), its accessibility from the periplasmic space, and the recent identification of some natural inhibitors explain the renewed interest for this target. MraY is inhibited by nonclinically used antibiotics such as tunicamycin, amphotericin, mureidomycin, liposidomycin, and muralmycin (12–15). Recently, simplified analogues of liposidomycin, named riburamycins, have been shown to be powerful MraY inhibitors and to possess antibacterial activities against Gram-positive organisms (16, 17). Moreover, this protein has been shown to be the target of the lytic protein LysE of phage dX174 (18). However, all studies on MraY reported to date only involved crude membrane preparations as the source of this bacterial enzyme. We here describe the significant overexpression and for the first time the purification to homogeneity of the MraY enzyme as well as detailed investigations of its biochemical properties.

**EXPERIMENTAL PROCEDURES**

**Chemicals**

N-Lauroyl sarcosine was purchased from USB Corporation; Triton X-100, n-octyl-β-D-glucopyranoside, tunicamycin, UDP-GlcNAc, and ATP were from Sigma; CHAPS was from ICN; n-dodecyl-β-D-maltoside (DDM) was from Fluka; Tween 20 was from VWR; and isopropyl-β-D-thiogalactopyranoside (IPTG) was from Eurogentec. C55-P was provided by the Institute of Biochemistry and Biophysics of the Polish Academy of Sciences. UDP-MurNAc was prepared as described by Blanot et al.
an N-terminal His6 extension. The same procedure was used to clone
In these constructs, the BamHI, generating plasmids pET28b plasmid vectors (Novagen) opened by compatible sites NcoI and
BglII and inserted into the p99A (Amersham Biosciences) and
Trc (Roche Applied Science). The PCR fragment was cut by BspLU11I and
primers (see Table I) and the Expand high fidelity polymerase system
strain 168 chromosome using BS1 and BS2 oligonucleotides as PCR
or radioactivity measurement, were pooled and lyophilized. The purifi-
ations were freed from imidazole and concentrated using
A similar procedure was used for extraction with other detergents. In
that case, two successive treatments were performed, and the final
concentration of detergent was 61, 41, 32, and 27.3 mM for n-octyl-β-D-
glucopyranoside, CHAPS, Triton X-100, and N-lauroyl sarcosine,
respectively.

Purification of Histidine-tagged MraY
Solubilized membrane proteins were mixed and incubated for 2 h at
4 °C with Ni²⁺-NTA-agarose (Qiagen) (15 mg of proteins/ml of resin)
pre-equilibrated in buffer B (20 mM sodium phosphate, pH 7.2, 300 mM
NaCl, 30% glycerol, 3.9 mM DDM, 2 mM 2-mercaptoethanol). After
incubation, the resin was transferred to an Econo-Pac chromatography
column (Bio-Rad) and washed first with 5 column volumes of buffer B.
Further washings and protein elution were performed with increasing
concentrations of imidazole, from 5 to 300 mM, in buffer B. After assays
for translocase activity and SDS-PAGE analysis, pure MraY protein-
containing fractions were freed from imidazololate and concentrated using
a Vivaspin concentrator (Vivasience) in 30 mM Tris-HCl buffer, pH 7.5,
containing 150 mM NaCl, 10% glycerol, and 3.9 mM DDM.

Protein Monitoring
Protein concentrations were determined using the QuantiProBCA
assay kit (Sigma) and bovine serum albumin as the standard and/or by
quantitative amino acid analysis with a Hitachi model L8800 analyzer
(ScienceTec) after hydrolysis of samples in 6 N HCl for 24 h at 105 °C.

Enzymatic Synthesis of unlabeled and Radiolabeled
UDP-MurNAc-pentapeptide
The reaction mixtures contained 100 mM Tris-HCl, pH 8.6, 2 mM
dithiothreitol, 30 mM MgCl₂, 1 mM UDP-MurNAc, 20 mM ATP, 1.2 mM
L-Ala (or 1 mM L-[^14]C]Ala), 1.2 mM each D-Glu, meso-D2pm, and D-Ala-
and 200 units each of enzymes MurC, MurD, MurE, and MurF. After
2 h at 37 °C, the formation of UDP-MurNAc-pentapeptide was
followed by analytical HPLC on a column of Nucleosil SC18 (250 × 4.6
mm; Alltech France) using elution with 50 mM ammonium formate, pH
4.3, at a flow rate of 0.6 ml/min (28). A first purification of UDP-
MurNAc-pentapeptide from the reaction mixtures was performed by gel
filtration on a column of Sephadex G-25, as previously described (29).
Fractions containing these products, as judged by absorbance at 262 nm
or radioactivity measurement, were pooled and lyophilized. The purifi-
cation was completed by HPLC on a column of Vydac 218TP11022 (250 ×
22 mm; Touzart & Matignon) using elution with 50 mM ammonium formate,
4.3, at a flow rate of 7 ml/min. The purity of UDP-MurNAc-
pentapeptide was checked by analytical HPLC and spectral absorbance
analysis, and quantitation was obtained by amino acid analysis of a
sample after hydrolysis in 6 N HCl for 16 h at 95 °C.

Assays for Translocase Activity
Standard MraY Assay—The assay was performed in a final volume of
10 µl containing 100 mM Tris-HCl, pH 7.5, 40 mM MgCl₂, 1.1 mM
C₅₅-P, 250 mM NaCl, 0.25 mM UDP-MurNAc-[³²P]pentapeptide (337
Bq), and 8.4 mM N-lauroyl sarcosine. The reaction was initiated by the
denaturation of the Kₕ values, the MraY activity as assayed as described
above with various concentrations of one substrate (60 µM to 3.7 mM for
UDP-MurNAc-pentapeptide; 70 µM to 1.1 mM for C₅₅-P) while main-
taining the other at a fixed value (1.1 mM for C₅₅-P, 0.25 mM for
UDP-MurNAc-pentapeptide). Data were fitted to the equation v =

Bacterial Strains and Growth Conditions
The E. coli strains DH5α (Invitrogen), BL21(DE3) (Promega), and
C43(DE3) (Avidia) were used as hosts for plasmids and for the overpro-
duction of the MraY enzyme. 2YT (25) was used as a rich medium, and
4. For strains carrying drug resistance genes, ampicillin and
50 µg/ml, respectively.

Purification and Characterization of MraY Translocase
Preparation of Crude Enzyme
E. coli strain C43(DE3) harboring recombinant plasmid pETYBS62
was grown at 37 °C in 2YT-kanamycin medium (5-liter culture). At an
A₅₀₀ of ~0.7, IPTG was added at a final concentration of 1 mM, and
incubation was continued for 16 h at 25 °C with shaking. Cells were
harvested by centrifugation (8,000 × g for 20 min at 4 °C), washed in
100 ml of 25 mM Tris-HCl, pH 7.5, and resuspended in 10 ml of the same
buffer containing 2 mM 2-mercaptoethanol, 150 mM NaCl, 30% glycerol,
and 1 mM MgCl₂ (buffer A). Bacteria were broken by sonication (Bio-
block Vibrapell sonicator model 72412). The resulting suspension was
centrifuged at 200,000 × g for 30 min at 4 °C in a Beckman TL100
centrifuge. The pellet consisting of membranes and associated proteins
(14 g wet weight, 1.2 g of proteins) was washed three times with buffer
A and then subjected to solubilization by detergents as described below.

Solubilization of MraY
Membrane vesicles containing the overexpressed MraY protein were
resuspended in 20 ml of buffer A. DDM was added at a final concen-
tration of 17.8 mM, and the mixture was incubated at 4 °C for 2 h
under shaking. After centrifugation (200,000 × g, 30 min at 4 °C), a first
supernatant (DM1) was recovered. The insoluble material was then
subjected to a new cycle of solubilization in buffer A containing 21.5 mM
DDM. Supernatant DM2 was recovered after centrifugation. Two fur-
ther rounds of solubilization/centrifugation were performed in the same
conditions (21.5 mM DDM), generating supernatants DM3 and DM4,
respectively.
VA/K + A) using the MDFitt software developed by M. Desmadrill (UMR 8619 CNRS, Orsay, France).

Coupled Assay with MurG—The coupled MraY-MurG assay was carried out in a volume of 10 μl containing 100 mM Tris-HCl, pH 7.5, 40 mM MgCl₂, 1.1 mM C55-P, 150 mM NaCl, 0.25 mM UDP-MurNAc-pentapeptide, 0.02 mM UDP-[14C]GlcNAc, 10% Me₂SO, 0.6 mM N-lauroyl sarcosine (or 1.95 mM DDM). The reaction was initiated by the addition of 0.2 μg of MraY, and the mixture was incubated for 25 min at 37°C under shaking.

In all cases, the reaction was stopped by heating at 100°C for 1 min, and the radiolabeled substrates (UDP-MurNAc-pentapeptide and UDP-GlcNAc) and reaction products (C₅₅-P-P-MurNAc-pentapeptide (lipid I) or C₅₅-P-MurNAc(-pentapeptide)-GlcNAc (lipid II)) were separated by TLC on silica gel plates LK6D (Whatman) using 2-propanol/ammonium hydroxide/water (6:3:1; v/v/v) as a mobile phase. The radioactive spots were located and quantified with a radioactivity scanner (model Multi-gamma 29976VA (Applied Biosystems) equipped with a 337-nm nitrogen laser. Delayed extraction on a PerSeptive Voyager-DE STR instrument (Applied Biosystems) was used as an external calibrant.

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) Mass Spectrometry Analysis

MALDI-TOF mass spectra were recorded in the linear mode with delayed extraction on a PerSeptive Voyager-DE STR instrument (Applied Biosystems) equipped with a 337-nm nitrogen laser.

MraY—The samples were prepared according to Gruber et al. (30). 0.5 μl of MraY preparation was deposited on the plate and allowed to dry. Subsequently, 0.5 μl of matrix solution (10 mg/ml α-cyano-4-hydroxy-cinnamic acid in 50% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid) was applied to the dried sample and again allowed to dry. Spectra were recorded in the positive ion mode at an acceleration voltage of 20 kV and an extraction delay time of 300 ns. The MraY protein was used as an external calibrator.

Lipid I and UDP-MurNAc-pentapeptide—One μl of matrix solution (10 mg/ml 8-aza-2-thiobenzamide in 20 mM diammonium citrate) was deposited on the plate, followed by 0.5 μl of sample (dissolved in 2-propanol/methanol (1:1; v/v) for lipid I and water for UDP-MurNAc-pentapeptide). After evaporation of the solvents, spectra were recorded in the negative ion mode at an acceleration voltage of −20 kV and an extraction delay time of 100 ns. A mixture of UDP-MurNAc, UDP-MurNAc-α-L-alanine-Glu, and UDP-MurNAc-pentapeptide was used as an external calibrator.

RESULTS AND DISCUSSION

The MraY translocase catalyzes the first membrane step of peptidoglycan synthesis, an essential step considered as a very promising target for the search of new antibacterial compounds. It has been clearly underexploited to date, most probably because of the refractory nature of this protein to overexpression and purification. Indeed, all previous attempts to overproduce to high levels the MraY translocase, to purify it, or at least to detect it by SDS-polyacrylamide gel electrophoresis were unsuccessful (31, 32). In fact, only a radiolabeled form of this protein has been detected to date using in vitro translation experiments (9, 10). We recently investigated the membrane topology of the E. coli MraY protein and showed that its expression as a fusion with β-lactamase was an advantage for its stability and/or production. A significant but moderate 30-fold increase of MraY activity was detected in cells in which the MraY-BlaM fusion was expressed (9). The solubilized fusion protein was subsequently used for the development of a high throughput screening assay based on fluorescence detection (17). A similar (28-fold) overproduction factor was reported by Brandish et al. (32) when the E. coli mraY gene was cloned in the expression vector pTrc99A. Why the levels of overexpression were in all cases so modest was unclear given the well documented strength of the promoters used. We thus decided to revisit this question by performing a complete set of experiments using mraY genes from various bacterial species, different plasmid vectors, and different host strains and conditions for expression.

The mraY genes from E. coli, S. aureus, T. maritima, and B. subtilis were amplified by PCR and cloned into the pTrc99A and pET28b expression vectors. In all of these constructs, the mraY gene product was expressed with a Hisᵩ tag extension to allow its easy purification on Ni²⁺-NTA-agarose. Strain C43(DE3), which is particularly well adapted for high level expression of membrane proteins (33), was chosen as the host strain, since the extent of MraY overexpression in that strain was systematically about 5-fold higher than that observed in the other E. coli strains tested (DH5α and BL21(DE3)). In the case of B. subtilis MraY, expression from pTrc99A and pET28b vectors resulted in 25- and 145-fold increased levels of translocase activity, respectively, when cells were grown and induced at 37°C and 5- and 330-fold, respectively, when cells were first grown at 37°C and then induced at 25°C. Similar results were obtained with MraY from E. coli, S. aureus, and T. maritima (Table II). We therefore essentially used pET28b-derivative plasmids and induction at 25°C in all subsequent experiments.

Small scale cultures (50 ml) were then performed to identify the best MraY candidate to purify (i.e. the one, among the aforementioned species, exhibiting the best compromise between overexpression level and enzyme stability during the extraction and purification steps). Membrane proteins of IPTG-induced and noninduced cells were solubilized with DDM and tested for MraY activity. Cells carrying the different mraY-overexpressing plasmids all contained a similar 100–500-fold increased level of MraY activity in membranes when induced with IPTG. At this step, however, no significant increase of a protein band that could correspond to MraY (calculated mass of about 36 kDa) was detected by SDS-PAGE analysis of the extracts (data not shown). Various detergents (N-lauroyl sarcosine, DDM, Triton X-100, n-octyl-β-D-glucopyranoside, and CHAPS) were then tested for their efficiency to extract the
MraY proteins from the membranes and to maintain them in an enzymatically active form (Table III). N-Lauroyl sarcosine and DDM appeared more efficient than the other detergents for MraY extraction. However, the ionic detergent N-lauroyl sarcosine did not allow a sufficient binding of MraY on Ni\(^2\)+-NTA-agarose, the resin used for the final step of purification. Most of the MraY protein was eluted at very low concentrations of imidazole (5–20 mM) and was thus only poorly purified in these conditions. In the case of DDM, the affinity of MraY for the resin was much better, and the purification of a protein eluted at higher concentrations of imidazole, ranging from 20 to 300 mM, was observed. Since the protein yield and purification state appeared significantly higher in the case of the *B. subtilis* MraY protein coded by the pETYBS62 plasmid (N-terminal His\(_6\)-tagged form), the latter was chosen for large scale purification and enzyme characterization experiments.

**Overproduction and Purification of *B. subtilis* MraY**—The sequence of the *B. subtilis mraY* gene amplified from strain 168 and cloned into pETYBS62 plasmid showed some minor differences from that found in databases (Pasteur data base, available on the World Wide Web at genolist.pasteur.fr/SubtiList/); the sequence 5\(^{80}\)CGTGAT\(^{94}\) (coding for RD) repeatedly appeared as GCTCAT (coding for AH) in the products from several independent PCRs using high fidelity polymerase. The molecular mass calculated for the *B. subtilis* MraY protein was 36,568 Da, taking into account the N-terminal extension tag consisting in Met-Ser-His\(_6\).

A 5-liter culture of *E. coli* C43DE3(pETYBS62) was induced with IPTG, and membranes were prepared and tested for MraY activity. As previously observed with 50-ml cultures, the specific activity in this crude membrane extract was about 300-fold higher than that detected in control cells carrying the pET28b vector (66 *versus* 0.2 units/mg of protein, respectively). The extraction of the MraY protein was achieved by four successive treatments of membranes with DDM: one with 17.8 mM DDM (extract DM1) followed by three with 21.5 mM DDM (extracts DM2–DM4). Table IV recapitulates the levels of MraY activity detected in these different extracts. The differential extraction with DDM allowed us to remove a large amount of proteins but only little of the MraY activity in DM1. About one-third of the total MraY activity was recovered in DM2, with a 20% increase in specific activity. Protein amounts in DM3 and DM4 were lower, with a specific activity of ~50 units/mg of protein as compared with 80 units/mg of protein in DM2. The DM2 extract was thus chosen for the purification of the MraY protein.

The purification was carried out in a single affinity chromatography step (see “Experimental Procedures”). Solubilized membranes (DM2 extract, 280 mg of proteins) were mixed with Ni\(^2\)+-NTA-agarose and incubated for 2 h at 4 °C under shaking. The resin was transferred to a chromatography column, and washing and elution steps were carried out with a discontinuous gradient of imidazole (0, 5, 10, 20, 35, 45, 60, and 300 mM). Fractions were collected and analyzed for protein content and translocase activity (Table VI). In the absence of imidazole and up to a 5 mM concentration of this compound, almost all of the MraY activity remained bound to the column. It started to be released from the Ni\(^2\)+-NTA resin at 10 mM, but the specific activity was shown to be maximal in the 60 and 300 mM imidazole-containing fractions. About 6 mg of pure MraY protein were recovered in the latter fractions, with a specific activity of ~1,900 units/mg of protein, 9,500-fold higher than the basal activity detected in wild-type *E. coli* membranes (~0.2 units/mg of protein).

Analysis of the latter purified fractions by SDS-PAGE showed a unique protein band migrating as a protein of 31 kDa (Fig. 1). It was also detected by Western blot using monoclonal antibodies directed against the histidine tag (data not shown). The molecular mass of the protein as deduced from gel migra-
on the column; lanes 2–4, purification of the MraY protein on Ni2+/NTA-agarose. After extensive washing of the resin with buffer B, elution of proteins was performed with a discontinuous gradient of imidazole (Im; 0, 5, 10, 20, 35, 45, 60, and 300 mM). The protein content and MraY activity in these fractions were determined. ND, not detectable.

| Fraction     | Protein | Activity | Specific activity | Yield |
|--------------|---------|----------|------------------|-------|
| DM2 extract  | 283     | 22,900   | 81               | 100   |
| Flow-through | 152     | 2,945    | ND               | 13    |
| Im 0 mM      | 41      | ND       | ND               |       |
| Im 5 mM      | 31      | ND       | ND               |       |
| Im 10 mM     | 18      | 3,430    | 190              | 15    |
| Im 20 mM     | 22      | 12,290   | 560              | 54    |
| Im 35 mM     | 9       | 6,860    | 760              | 30    |
| Im 45 mM     | 5       | 6,880    | 1,380            | 30    |
| Im 60 mM     | 2       | 3,850    | 1,925            | 17    |
| Im 300 mM    | 4       | 7,815    | 1,955            | 34    |

**Table IV** Purification of the *B. subtilis* MraY protein from membranes with DDM detergent

C43(DE3) cells harboring the pETYBS62 plasmid were grown and induced with IPTG. The membranes were prepared and subjected to four successive treatments with DDM, generating extracts DM1, DM2, DM3, and DM4, respectively, after high speed centrifugation. The protein content and MraY activity in these fractions were determined.

| Extract | Protein | Specific activity | Total activity |
|---------|---------|------------------|---------------|
| Membranes | 1,200 | 66 | 79,200 |
| DM1 | 154 | 13 | 2,000 |
| DM2 | 283 | 81 | 22,900 |
| DM3 | 224 | 50 | 11,200 |
| DM4 | 94 | 52 | 4,890 |

* Membrane suspension resulting from the first incubation with the detergent (not centrifuged).

**Table V** Purification of the *B. subtilis* MraY protein from the DM2 extract on Ni2+/NTA-agarose

The total DM2 extract was loaded onto the column, and washing and elution were carried out with a discontinuous gradient of imidazole (Im; 0, 5, 10, 20, 35, 45, 60, and 300 mM). The protein content and MraY activity of the different fractions were determined. ND, not detectable.

**Fig. 1. Purification of the *B. subtilis* MraY enzyme, as judged by SDS-PAGE analysis.** Membrane vesicles from *E. coli* C43(DE3)pETYBS62) cells were prepared, and the MraY protein was extracted with DDM as described under “Experimental Procedures.” The second DDM extract (DM2) was used as starting material for purification of the MraY protein on Ni2+/NTA-agarose. After extensive washing of the resin with buffer B, elution of proteins was performed with a discontinuous gradient of imidazole, from 5 to 300 mM. Fractions were analyzed by SDS-PAGE, and staining was performed with Coo-phenylenediamine and Coomassie Blue R250 (Merck). Lane 1, crude DM2 extract loaded on the column; lanes 2–4, 45, 60, and 300 mM imidazole-containing fractions, respectively. Molecular mass standards (M) indicated on the right are as follows: phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14 kDa).

**Fig. 2. MALDI-TOF mass spectrometry analysis of purified MraY showing peaks of *m/z* 36,551.0, 18,262.4, 12,177.6, and 9,142.8 that were assigned to be the [M + H]+, [M + 2H]2+, [M + 3H]3+, and [M + 4H]4+ ions, respectively. The molecular mass of MraY calculated from the gene sequence is 36,568 Da (N-terminal Met-Ser-His6 tag included).

**Fig. 3. Thin layer chromatography separation of substrate (UDP-MurNAc-pentapeptide) and product (lipid I) of MraY.** The MraY assay was carried out in the presence of UDP-MurNAc-[14C]pentapeptide, C55-P, and purified MraY. The substrate (spot A) and synthesized product (spot B) were separated by TLC and detected with a radioactivity scanner, as detailed under “Experimental Procedures” (Rf values of 0.25 and 0.58, respectively).
Coupling of Purified MraY and MurG Activities—Radiolabeled C_{55}-PP-MurNAc-pentapeptide-GlcNAc (lipid II) was formed when both MurG enzyme and UDP-[\textsuperscript{14}C]GlcNAc were incorporated in the MraY reaction mixture, further indicating that coupling MraY translocase/MurG transferase reactions could be successfully performed with purified preparations of the two enzymes. In our conditions, in which UDP-GlcNAc was in limited quantity (see "Experimental Procedures"), a complete incorporation of GlcNAc was observed. No lipid II was formed when MurG was omitted, demonstrating the absence of MurG in the MraY preparation. In addition, we showed that no MraY activity could be detected when either of the two substrates, UDP-MurNAc-pentapeptide or C_{55}-P, was omitted in the reaction mixture, demonstrating the absence of either compound (and in particular of any traces of the C_{55}-P carrier lipid) in the preparation. No radiolabeled lipid was formed when UDP-MurNAc-[\textsuperscript{14}C]pentapeptide was replaced by UDP-[\textsuperscript{14}C]GlcNAc in the standard translocase assay, indicating that the purified MraY preparation was also not contaminated by WecA enzyme (36), a membrane activity catalyzing the transfer of [\textsuperscript{14}C]GlcNAc from UDP-GlcNAc onto the C_{55}-P carrier lipid.

Biochemical Properties of the Pure MraY Enzyme—The translocase activity of the pure enzyme preparation was characterized in more detail. First, the effects of pH, salts, metal ions, and detergent concentrations were tested. The effect of pH was determined in the range 5.9–10.5 by using three different buffers. A plateau of maximal activity was observed between 7.2 and 8.0, as shown in Fig. 4A. It should be noted that this optimal value corresponds approximately to the internal bacterial pH that ranges between 7.6 and 7.8 (37). The MraY enzyme exhibited almost no activity at pH values below 6.5. All subsequent experiments were performed at pH 7.6. The effect of salts was investigated with NaCl and KCl in the range 0–1.7 M. MraY behaved similarly with respect to these two salts (Fig. 4B). The enzyme activity greatly increased (by a factor of about 5) between 0 and 150 mM and then reached a plateau value that did not significantly vary above 250 mM and up to the maximal salt concentration tested. The MraY activity showed an abso-

![Fig. 4. Biochemical properties of the pure MraY enzyme. Shown are the effects of pH (A); salts (NaCl (■) and KCl (●)) (B); cations (MgCl\textsubscript{2} (■) and MnCl\textsubscript{2} (●)) (C); and detergents (N-lauroyl sarcosine (■), Tween 20 (△), Triton X-100 (●), DDM (○), n-octyl-\textbeta-p-glucopyranoside (△), and CHAPS (•)) (D) on the MraY translocase activity, expressed in nmol/min/mg of protein. The standard assay was carried out as described under "Experimental Procedures" with the following modifications: in A, bis-Tris-HCl buffer was used for the pH range 5.9–7.0, Tris-HCl buffer was used for pH 7.2–9.4, and glycine buffer was used for pH 10.5.

![Fig. 5. Inhibition of MraY activity by tunicamycin. Incubation was as described under "Experimental Procedures" except that various amounts of tunicamycin were added in the reaction mixture.](https://example.com/f5.png)
lute requirement for a divalent cation (Fig. 4C). Optimal enzyme activity was observed with Mg\(_{2+}\) for concentrations ranging from 40 to 75 mM. Although Mn\(^{2+}\) could replace Mg\(^{2+}\), the translocase activity detected in the presence of this cation was much lower, representing only \(-1\%\) of that observed with Mg\(^{2+}\). As compared with Mg\(^{2+}\), the effect of Mn\(^{2+}\) concentration on enzyme activity showed a curve shifted toward lower concentrations, and the optimal concentration range estimated in that case was 15–30 mM (Fig. 4C).

We also tested the effects of various detergents on MraY translocase activity (Fig. 4D). N-Lauroyl sarcosine clearly appeared as the most efficient one, with an optimal concentration ranging between 7 and 16 mM. At a 32 mM concentration of this detergent, the residual activity was \(-50\%\) of the optimum. In the presence of Tween 20 or Triton X-100 (optimal concentrations of 7.7 and 15 mM, respectively), the MraY activity represented about 30% of that observed in the presence of N-lauroyl sarcosine. The activity in the presence of DDM was weaker by an order of magnitude but surprisingly remained intact for sarcosine. The activity in the presence of DDM was weaker by an order of magnitude but surprisingly remained intact for sarcosine. The activity in the presence of DDM was weaker by an order of magnitude but surprisingly remained intact for sarcosine. The activity in the presence of DDM was weaker by an order of magnitude but surprisingly remained intact for sarcosine.

In the optimal conditions for MraY activity determined ranging between 7 and 16 mM. A ta3 2m M concentration of this detergent, the residual activity was 50% of the optimum. In the absence of any contaminating protein or substrate (in particular C55-P) originating from membranes. Its apparent substrate requirement for a divalent cation (Fig. 4)

Acknowledgments—We thank G. Auger and C. Parquet for helpful discussions and S. Back for assistance in the cloning experiments.

REFERENCES

1. Walsh, C. (2000) Nature 406, 775–781
2. Nanninga, N. (1998) Microbiol. Mol. Biol. Rev. 62, 110–129
3. van Heijenoort, J. (2001) Nat. Prod. Rep. 18, 503–519
4. Rogers, H. J., Perkins, H. B., and Ward, J. B. (1980) Microbial Cell Walls and Membranes, pp. 239–297, Chapman and Hall, London
5. Neuhauß, F. C. (1971) Acc. Chem. Res. 4, 297–303
6. Umbreit, J. N., and Strominger, J. L. (1971) J. Bacteriol. 112, 1306–1309
7. Fess, D. D., and Neuhauß, F. C. (1973) J. Biol. Chem. 248, 1556–1576
8. Mjeng-Lecreux, D., Texier, L., and van Heijenoort, J. (1990) Nucleic Acids Res. 18, 2810
9. Bouhass, A., Mjeng-Lecreux, D., Le Beller, D., and van Heijenoort, J. (1999) Mol. Microbiol. 34, 576–585
10. Boyle, D. S., and Dunachie, W. D. (1998) J. Bacteriol. 180, 6429–6432
11. Thanassi, J. A., Hartman-Neumann, S. L., Dougherty, T. J., Dougherty, B. A., and Pucci, M. J. (2002) Nucleic Acids Res. 30, 3152–3162
12. Bugg, T. D., and Brandish, P. E. (1994) FEBS Microbiol. Lett. 119, 255–262
13. Brandish, P. E., Kimura, K. I., Inoue, M., Southgate, R., Lonsdale, J. T., and Bugg, T. D. (1996) Antimicrob. Agents Chemother. 40, 1640–1644
14. McDonald, I. A., Barbieri, L. R., Carter, G. T., Lenyo, E., Lotvin, J., Petersen, P. J., Siegel, M. M., Singh, G., and Williamson, R. T. (2002) J. Am. Chem. Soc. 124, 10260–10261
15. Ikeeda, M., Wachi, M., Jung, H. K., Ishino, F., and Matsushashi, M. (1991) J. Bacteriol. 173, 1021–1026
16. Dini, C., Collette, P., Drochon, N., Guillot, J. C., Lemoine, G., Mauvais, P., and Aszodi, A. (2000) Bioorg. Med. Chem. Lett. 10, 1839–1843
17. Stachyra, T., Dini, C., Ferrari, P., Bouhass, A., van Heijenoort, J., Mjeng-Lecreux, D., Blanot, D., Biton, J., and Le Beller, D. (2004) Antimicrob. Agents Chemother. 48, 897–902
18. Bernhardt, T. G., Roof, W. D., and Young, R. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 4297–4302
19. Blanot, D., Auger, G., Liger, D., and van Heijenoort, J. (1994) Carbohydr. Res. 252, 107–115
20. Liger, D., Masson, A., Blanot, D., van Heijenoort, J., and Parquet, C. (1995) Eur. J. Biochem. 230, 80–87
21. Auger, G., Martin, B., Bertrand, J., Ferrari, P., Fanchon, E., Vaganay, S., Petiot, Y., van Heijenoort, J., Blanot, D., and Dideberg, O. (1998) Protein Expression Purif. 13, 23–29
22. Gordon, E., Flouret, B., Chantalat, L., van Heijenoort, J., Mjeng-Lecreux, D., and Dideberg, O. (2001) J. Biol. Chem. 276, 10999–11006
23. Dementin, S., Bouhass, A., Auger, G., Parquet, C., Mjeng-Lecreux, D., Dideberg, O., van Heijenoort, J., and Blanot, D. (2001) Eur. J. Biochem. 268, 5800–5807
24. Crouvoisier, M., Mjeng-Lecreux, D., and van Heijenoort, J. (1999) FEBS Lett. 449, 289–292
25. Miller, J. H. (1972) Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
26. Sambruck, J., Pritzsch, E. F., and Mannatis, T. (1989) Molecular Cloning: a Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
27. Dugat, M., and Ehrlich, S. D. (1979) Gene (Amst.) 6, 23–28
28. Flouret, B., Mjeng-Lecreux, D., and van Heijenoort, J. (1981) Anal. Biochem. 114, 59–63
29. Mjeng-Lecreux, D., Flouret, B., and van Heijenoort, J. (1982) J. Bacteriol. 151, 1109–1117
30. Gruber, G., Godovac-Zimmermann, J., Link, T. A., Coskun, U., Rizzo, V. F., Betz, C., and Baier, S. M. (2002) Biochem. Biophys. Res. Commun. 298, 383–391
31. Mjeng-Lecreux, D., Parquet, C., Desvait, L. R., Pla, J., Flouret, B., Ayala, J. A., and van Heijenoort, J. (1989) J. Bacteriol. 171, 6126–6134
32. Brandish, P. E., Burnham, M. K., Lonsdale, J. T., Southgate, R., Inoue, M., and Bugg, T. D. (1996) J. Biol. Chem. 271, 7609–7614
33. Miroux, B., and Walker, J. E. (1996) J. Mol. Biol. 260, 289–298
34. Pigeon, R. P., and Silver, R. P. (1994) Mol. Microbiol. 14, 871–881
35. Cadene, M., and Chait, B. T. (2000) Anal. Chem. 72, 5655–5658
36. Hyland, S. A., and Anderson, M. S. (2003) Anal. Biochem. 317, 156–165
37. Willford, F. M., and Maloney, P. C. (1996) in Escherichia coli and Salmonella: Cellular and Molecular Biology (Neidhardt, F. C., Curtiss, R., III, Ingraham, J. L., Lin, E. C. C., Low, K. B., Magasanik, B., Reznikoff, W. S., Riley, M., Schaechter, M., and Umbarger, H. E., eds) Vol. 1, pp. 283–306, American Society for Microbiology, Washington, D.C.
38. Elbein, A. D., Gafford, J., and Kang, M. S. (1979) Arch. Biochem. Biophys. 196, 311–318
39. Hedefelt, A., Keenan, R. W., and Elbein, A. D. (1979) Biochemistry 18, 2186–2192
Purification and Characterization of the Bacterial MraY Translocase Catalyzing the First Membrane Step of Peptidoglycan Biosynthesis
Ahmed Bouhss, Muriel Crouvoisier, Didier Blanot and Dominique Mengin-Lecreulx

J. Biol. Chem. 2004, 279:29974-29980.
doi: 10.1074/jbc.M314165200 originally published online May 6, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M314165200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 34 references, 12 of which can be accessed free at http://www.jbc.org/content/279/29/29974.full.html#ref-list-1