Active Site Mapping of MraY, a Member of the Polyprenyl-phosphate *N*-Acetylhexosamine 1-Phosphate Transferase Superfamily, Catalyzing the First Membrane Step of Peptidoglycan Biosynthesis[†]

Bayan Al-Dabbagh,[‡,§] Xavier Henry,[‡,||] Meriem El Ghachi,[‡,⊥] Geneviève Auger,[‡] Didier Blanot,[‡,#] Claudine Parquet,[‡] Dominique Mengin-Lecreulx,[‡,#] and Ahmed Bouhss*[,‡,#]

Univ Paris-Sud, UMR 8619, Institut de Biochimie et de Biophysique Moléculaire et Cellulaire, Bât. 430, Orsay, France, and CNRS, UMR 8619, Laboratoire des Enveloppes Bactériennes et Antibiotiques, Orsay, France

Received April 9, 2008; Revised Manuscript Received June 23, 2008

ABSTRACT: The MraY transferase is an integral membrane protein that catalyzes an essential step of peptidoglycan biosynthesis, namely the transfer of the phospho-*N*-acetylmuramoyl-pentapeptide motif onto the undecaprenyl phosphate carrier lipid. It belongs to a large superfamily of eukaryotic and prokaryotic prenyl sugar transferases. No 3D structure has been reported for any member of this superfamily, and to date MraY is the only protein that has been successfully purified to homogeneity. Nineteen polar residues located in the five cytoplasmic segments of MraY appeared as invariants in the sequences of MraY orthologues. A certain number of these invariant residues were found to be conserved in the whole superfamily. To assess the importance of these residues in the catalytic process, site-directed mutagenesis was performed using the *Bacillus subtilis* MraY as a model. Fourteen residues were shown to be essential for MraY activity by an *in vivo* functional complementation assay using a constructed conditional *mraY* mutant strain. The corresponding mutant proteins were purified and biochemically characterized. None of these mutations did significantly affect the binding of the nucleotidic and lipidic substrates, but the k_{cat} was dramatically reduced in almost all cases. The important residues for activity therefore appeared to be distributed in all the cytoplasmic segments, indicating that these five regions contribute to the structure of the catalytic site. Our data show that the D98 residue that is invariant in the whole superfamily should be involved in the deprotonation of the lipid substrate during the catalytic process.

The rapid emergence of multiresistant pathogenic bacteria to commonly used antibiotics is a considerable threat for public health (*1*). Thus, research on new therapeutic compounds has become a necessity. The enzymes of the peptidoglycan biosynthesis pathway (*2, 3*) which are essential for bacterial life represent important targets for antibacterial chemotherapy. The MraY transferase catalyzes the first membrane step of this pathway; it consists of the transfer of the phospho-*N*-acetylmuramoyl-pentapeptide motif from the nucleotide precursor UDP-MurNAc-pentapeptide[1] onto the undecaprenyl phosphate carrier lipid (C_{55}-P), yielding C_{55}-PP-MurNAc-pentapeptide (lipid I) (Figure 1). The corresponding *mraY* gene was previously demonstrated to be essential for bacterial growth (*4, 5*). MraY is an integral membrane protein composed of 10 transmembrane segments joining four periplasmic loops and five cytoplasmic sequences that contain many invariant residues (*6*). Both the N- and C-terminal ends are located in the periplasm. Recently, for the first time, the purification to near homogeneity of a MraY protein (from *Bacillus subtilis*) was reported, allowing further detailed biochemical characterization of this enzyme (*7*).

A hypothetical two-step catalytic mechanism for the MraY reaction had been earlier proposed (*8*) (Figure 2). It consisted of the attack, by a nonidentified nucleophile residue of the enzyme, of the β-phosphate of UDP-MurNAc-pentapeptide, leading to the formation of a covalent enzyme-phospho-MurNAc-pentapeptide intermediate and the release of UMP. The second step then corresponded to the attack, by an

[†] This work was supported by the Centre National de la Recherche Scientifique (UMR 8619) and by the European Community (EURINTAFAR, LSHM-CT-2004−512138).

* Corresponding author. Mailing address: Laboratoire des Enveloppes Bactériennes et Antibiotiques, Institut de Biochimie et Biophysique Moléculaire et Cellulaire, UMR 8619 CNRS, Université Paris-Sud, Bât. 430, 91405 Orsay Cedex, France. Phone: 33-1-69-15-61-34. Fax: 33-1-69-85-37-15. E-mail: ahmed.bouhss@u-psud.fr.

[‡] Univ Paris-Sud, UMR 8619, Institut de Biochimie et de Biophysique Moléculaire et Cellulaire.

[§] Recipient of a scholarship from the CROUS de Versailles, Service des Relations Internationales, Antony, France.

[||] Present address: Centre d'Ingénierie des Protéines, Université de Liège, Institut de Chimie B6a, B-4000, Liège, Belgium.

[⊥] Present address: Unité de Pathogénie Bactérienne des Muqueuses, Département de Microbiologie, Institut Pasteur, 28 rue du Dr. Roux, 75724 Paris cedex 15, France.

[#] CNRS, UMR 8619, Laboratoire des Enveloppes Bactériennes et Antibiotiques.

[1] Abbreviations: GlcNAc, *N*-acetylglucosamine; MurNAc, *N*-acetylmuramic acid; UDP-MurNAc-pentapeptide, UDP-*N*-acetylmuramoyl-L-Ala-D-iGlu-*meso*-diaminopimeloyl-D-Ala-D-Ala; C_{55}-P, undecaprenyl phosphate; DDM, *n*-dodecyl-β-D-maltoside; IPTG, isopropyl-β-D-thiogalactopyranoside; Ni^{2+}-NTA-agarose, nickel-nitrilotriacetate-agarose; lipid I, C_{55}-PP-MurNAc-pentapeptide; lipid II, C_{55}-PP-MurNAc-(pentapeptide)-GlcNAc.

10.1021/bi8006274 CCC: $40.75
Published on Web 08/02/2008

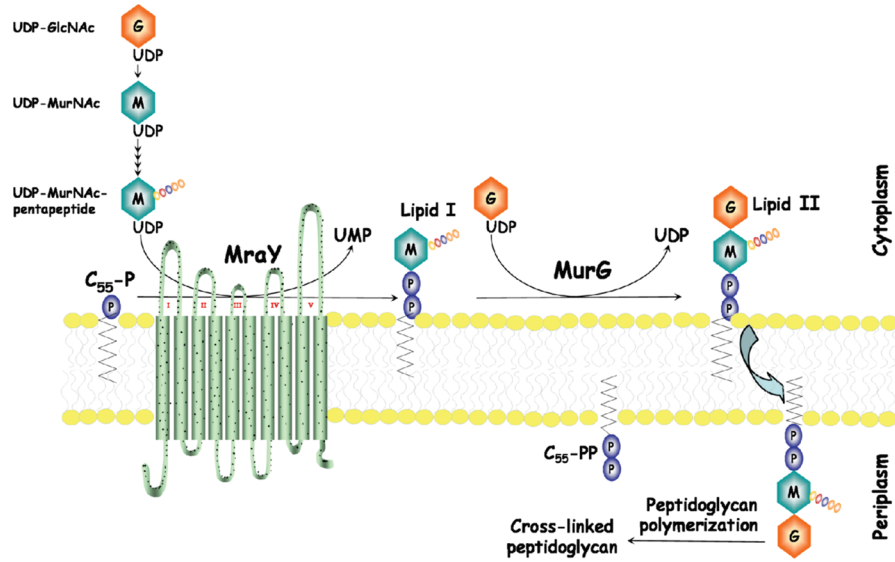

FIGURE 1: Peptidoglycan biosynthesis pathway. The first stage located in the cytoplasm consists of the biosynthesis of the nucleotide precursors UDP-GlcNAc and UDP-MurNAc-pentapeptide by a set of highly specific enzymes. Two membrane enzymes, MraY and MurG, then catalyze the subsequent steps of transfer of the phospho-MurNAc-pentapeptide and GlcNAc motifs to the undecaprenyl phosphate carrier lipid, generating the undecaprenyl-pyrophosphoryl-MurNAc-(pentapeptide)-GlcNAc (termed lipid II). The final stage corresponds to the polymerization reactions of the disaccharide pentapeptide motif that are catalyzed by glycosyltransferases and transpeptidases (penicillin-binding proteins) at the outer side of the cytoplasmic membrane. M, G and the five colored beads linked to M represent MurNAc, GlcNAc and the pentapeptide, respectively. C_{55}-P and C_{55}-PP are for undecaprenyl phosphate and undecaprenyl pyrophosphate, respectively.

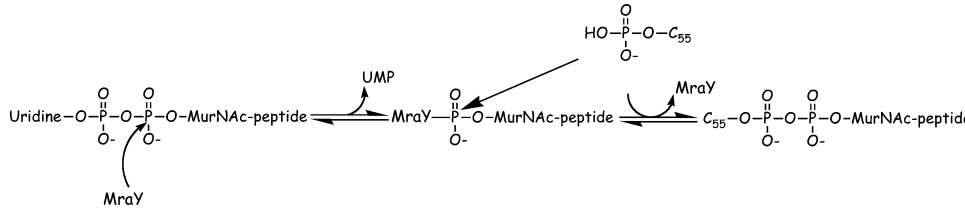

FIGURE 2: Hypothetical two-step catalytic mechanism of MraY proposed by Heydanek et al. (*8*).

oxyanion from C_{55}-P, of the phosphate of the covalent intermediate, resulting in the formation of lipid I and regeneration of the native enzyme form.

Several classes of natural product inhibitors of MraY have been described: tunicamycins, mureidomycins, liposidomycins, muraymycins and the bacteriolytic E protein from bacteriophage ϕX174 (*9, 10*). The riburamycins, synthetic analogues of liposidomycins, have been shown to be powerful inhibitors of this enzyme (*11, 12*). Recently, it was demonstrated that colicin M, an enzyme excreted by some *Escherichia coli* strains, degraded the lipid I and lipid II products of MraY and MurG, respectively (*13*).

The elucidation of the membrane topology of MraY and the alignment of sequences of its orthologues from both Gram-negative and Gram-positive species revealed a set of five well-conserved hydrophilic sequences (I to V) located at the inner side of the membrane and containing 34 invariant amino acid residues (Figure 3) (*6*). Many of these residues were shown to be invariant or highly conserved in the whole polyprenyl-phosphate *N*-acetylhexosamine 1-phosphate transferase superfamily that contains other prokaryotic and eukaryotic proteins catalyzing the same type of reaction (*14–19*). Only a few of these residues were recently subjected to site-directed mutagenesis experiments in the aim to identify their functional roles (*14, 20*). These analyses were focused on three aspartate residues of MraY (*20*) and WecA (*14*) from *E. coli*, a choice based on the assumption that these residues have nucleophilic side chains that could potentially be involved in binding of divalent metal ions or in catalysis, as shown previously for other glycosyl and prenyl transferases. A weakness of these interesting but limited studies, however, was the use of crude membrane extracts with low levels of expression of these proteins that rendered the interpretation of the results quite difficult. In the present work, MraY, the only member of the superfamily which has been purified to homogeneity (*7, 21*), was used as a model for mapping the residues of the catalytic site. All the invariant residues were taken into consideration, and nineteen polar and/or charged ones, exposed toward the cytoplasm and possibly involved in the catalytic process, were subjected to site-directed mutagenesis experiments. The corresponding mutant proteins were purified and analyzed in terms of functional complementation and enzymatic properties.

EXPERIMENTAL PROCEDURES

Materials. *N*-Lauroyl sarcosine was purchased from USB, *n*-dodecyl-β-D-maltoside (DDM) from Anatrace and isopropyl-β-D-thiogalactopyranoside (IPTG) from Eurogentec. Oligonucleotide synthesis and DNA sequencing were performed by MWG-Biotech. Undecaprenyl phosphate (C_{55}-P) was provided by the Institute of Biochemistry and Biophysics of the Polish Academy of Sciences, and UDP-MurNAc-pentapeptide was prepared as described by Bouhss et al. (*7*).

Bacterial Strains, Plasmids, and Growth Conditions. The *E. coli* strains DH5α (Life Science Technologies, Inc.),

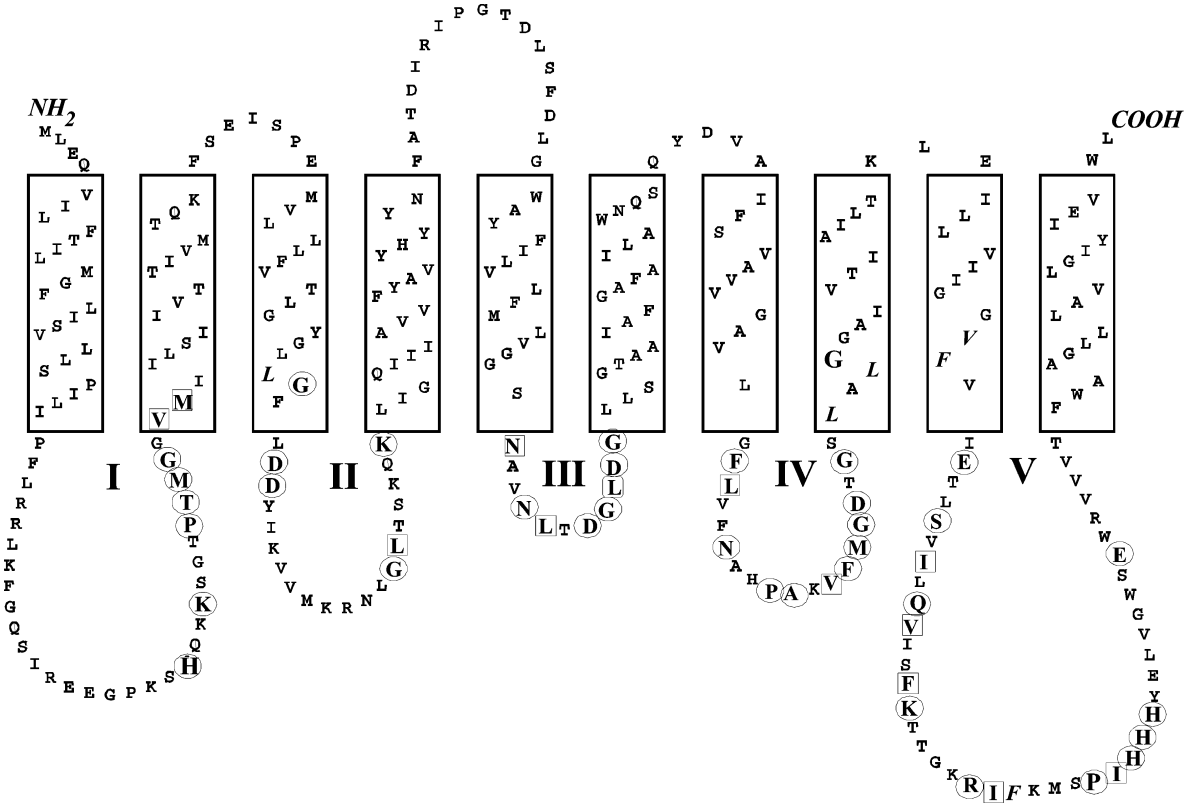

FIGURE 3: Membrane topology of the *B. subtilis* MraY protein. The model consists of ten transmembrane segments, four periplasmic loops and five cytoplasmic sequences corresponding to the conserved hydrophilic patterns I−V (*6*). Conserved residues by identity (circle) and by similarity (square) are indicated.

XL10-Gold (Stratagene), BW25113 (*22*) and C43(DE3) (Avidis-France) were used as hosts for plasmids and for the overproduction of the MraY enzyme. The plasmid vector p*Trc*99A was obtained from Amersham Biosciences, and the pMAK705 plasmid bearing a thermosensitive replicon was a kind gift from S. R. Kushner (*23*). The pETYBS62 plasmid, a pET28b derivative plasmid expressing the *B. subtilis mraY* gene product with an N-terminal 6×His-tag, has been previously described (*7*). The p*Trc*YTM plasmid, a pTrc99A derivative plasmid expressing the *Thermotoga maritima mraY* gene product, has been previously described (*7*).The BW25113 strain and the pKD3, pKD46, and pCP20 plasmids used for gene disruption experiments (*22*) were kindly provided by B. Wanner via the *E. coli* Genetic Stock Center (Yale University). 2YT (*24*) was used as a rich medium, and bacterial growth was monitored at 600 nm with a Shimadzu UV-1601 spectrophotometer. For strains carrying drug resistance genes, ampicillin, kanamycin and chloramphenicol were used at 100, 60 and 25 $\mu g \cdot mL^{-1}$, respectively.

General DNA Techniques and E. coli Cell Transformation. PCR amplification of genes from the *E. coli* chromosome was performed in a Thermocycler 60 apparatus (Biomed) using the Expand-Fidelity polymerase from Roche. The DNA fragments were purified using the Wizard PCR Preps DNA purification kit (Promega). Small and large scale plasmid isolations were carried out by the alkaline lysis method, and standard techniques for endonuclease digestions, ligation and agarose gel electrophoresis were performed as previously described (*25*). Transformation of *E. coli* cells with plasmid DNA was performed as described by Dagert and Ehrlich (*26*) or by electroporation.

Site-Directed Mutagenesis. Site-directed mutagenesis of the *B. subtilis* His_6-tagged MraY enzyme was performed directly on the pETYBS62 expression plasmid (*7*) by using the "Quikchange II XL site-directed mutagenesis kit" from Stratagene and the oligonucleotides shown in Table 1s, Supporting Information. Mutations introduced in the *mraY* gene sequence were checked by DNA sequencing.

Generation of a Chromosomal mraY Gene Deletion. A thermosensitive *mraY* mutant strain (derived from *E. coli* strain BW25113) was constructed in several steps by using the procedure of Datsenko and Wanner (*22*). As *mraY* is essential for growth, its inactivation in the chromosome was performed in the BW25113 strain carrying a plasmid, p*Trc*YTM, that expresses the *mraY* gene from *T. maritima* (*7*). The two oligonucleotides used for the PCR amplification of the antibiotic resistance (Cm^R) gene from pKD3 flanked by sequences designed for specific disruption of the *mraY* gene in the chromosome were 5′-CCGCGCTGTTCATCT-CATTGTGGATGGGCCCGCGTATGATTGCT-CATTTCATATGAATATCCTCCTTAG-3′ and 5′-TTCAT-AGTGGTGATGAATCGGTGCCATGCG-GAAAATACGTTGTCCGCGCGTGTAGGCTG-GAGCTGCTTC-3′. The deletion of the *mraY* gene and its replacement in the chromosome by the chloramphenicol resistance gene was verified by PCR. The antibiotic resistance gene was then excised by transformation with the pCP20 plasmid expressing the Flp recombinase (*22*). Finally, the p*Trc*YTM plasmid was cured and replaced by a plasmid whose replication is thermosensitive, derived from pMAK705 (*23*), harboring the *mraY* gene from *Staphylococcus aureus* (pMAKYSA). The resulting strain, BW25113 Δ*mraY*

(pMAKYSA), which was subsequently demonstrated to be thermosensitive for growth (see below), was named *Ecoli*TsMraY.

Complementation Assay. Competent cells of the thermosensitive mutant strain *Ecoli*TsMraY were transformed by the various plasmids to be tested. The cell suspension mixed with plasmid DNA was kept on ice for 4 h before heating for 3 min at 42 °C. Then, 2YT medium was added and cells were incubated at 30 °C for 2 h. Aliquots from the final suspensions were plated onto two 2YT-kanamycin plates, one incubated at 30 °C and the other at 42 °C. Growth was observed after 24 h of incubation.

Pool Levels of Peptidoglycan Precursors. The wild-type strain BW25113 and the thermosensitive mutant strain *Ecoli*TsMraY were grown exponentially at 30 °C in 2YT broth (0.8 L cultures). At the appropriate cell density (OD = 0.1, about 10^8 cells/mL), the temperature of the culture was either maintained at 30 °C or increased to 42 °C. Incubation was continued until the first effects on the growth of the mutant strain at 42 °C were observed, *ca.* 90 min later. At this time, cells were rapidly chilled to 0 °C and harvested in the cold, and the extraction and quantitation of the peptidoglycan nucleotide precursors were performed as previously described (*27*).

Expression and Purification of Histidine-Tagged Wild-Type and Mutant MraY Proteins. E. coli C43(DE3) cells carrying either the pETYBS62 plasmid (His-tagged wild-type *B. subtilis* MraY) or one of the derivative plasmids listed in Table 1s (His-tagged mutated MraY) were grown as described by Bouhss et al. (*7*). The overexpression and purification of the His-tagged wild-type and mutant MraY proteins were performed following the method previously described for wild-type MraY (*7*).

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 M HCl at 105 °C for 24 h.

MraY Transferase Assay. The MraY activity was assayed as described by Bouhss et al. (*7*). The K_m values for UDP-MurNAc-pentapeptide and C_{55}-P were determined by varying the concentration of one substrate while maintaining the other fixed (UDP-MurNAc-pentapeptide, 3 mM, or 6 mM when the K_m was higher than 2 mM; C_{55}-P, 1.1 mM). Data were fitted to the equation $v = VS/(K + S)$ using the MDFitt software developed by M. Desmadril (UMR 8619 CNRS, Orsay, France). All experiments were performed in triplicate.

pK_a Determination. The pK_a values were determined with a pH-meter (Schott) by titration with 0.1 M HCl of C_{55}-P (3.1 mM) dissolved in 10% Triton X-100. Initially, the pH of the C_{55}-P solution had been brought to 12.4 with NaOH. As a control, the two pK_a values of histidine were determined under the same conditions, and values similar to those reported in the literature (*28*) were obtained. The experiments were performed at room temperature (25 °C).

RESULTS

Nineteen of the MraY invariant residues located within the five cytoplasmic segments of MraY were polar and/or charged residues that could potentially be involved in substrate binding or in the catalytic process (Figure 3). To assess their importance for enzyme activity, site-directed mutagenesis was carried out followed by a detailed characterization of the mutant proteins.

Site-Directed Mutagenesis of the Conserved Residues. The mutagenesis of the nineteen invariant or highly conserved polar residues was performed on plasmid pETYBS62, a pET28b derivative harboring the *B. subtilis mraY* gene (*7*). This plasmid allowed the expression of the MraY protein with an N-terminal His_6 extension under the control of an IPTG-dependent promoter. The residues of interest were H45 and K48 (the numbering corresponds to the position in the wild-type sequence) located in the first cytoplasmic segment; D98, D99 and K116 located in cytoplasmic segment II; N168, N171, D174 and D177 located in cytoplasmic segment III; N221 and D231 located in cytoplasmic segment IV; and E264, S267, K276, R281, H289, H290, H291 and E299 located in cytoplasmic segment V. His and Asp residues were replaced by Arg and Asn, respectively; Asn and Ser residues were replaced by Ala while Glu, Lys and Arg residues were substituted by Gln. These specific substitutions were chosen to abolish the charge and/or the polarity of the residues with a minimal sterical change.

Construction of a Thermosensitive mraY Mutant. A thermosensitive *mraY* mutant strain, *Ecoli*TsMraY, derived from the *E. coli* strain BW25113, was constructed in several steps as described in Experimental Procedures. Briefly, the chromosomal *mraY* gene was deleted in this strain, which harbored a pMAKYSA plasmid carrying the *mraY* gene from *S. aureus* and whose replication is thermosensitive.

Since the pMAKYSA plasmid bore a thermosensitive replicon, the effects of the specific inactivation of the *mraY* gene were observed by shifting exponentially growing cells of *Ecoli*TsMraY and BW25113 from 30 to 42 °C. Both strains showed an identical growth rate when cultured at 30 °C. However, after *ca.* 90 min at 42 °C, mutant cells stopped growing and rapidly lysed, as judged by a net decrease of turbidity of the culture, thereby confirming that the *mraY* gene is essential for growth (Figure 4). The fact that growth inhibition occurred only after a few hours was explained by the time required for the plasmid and the preexisting MraY enzyme molecules to be progressively diluted and cured from the cells. The extraction and quantification of the pools of peptidoglycan nucleotide precursors from these strains was performed as previously described (*27*). Both wild-type and mutant strains contained similar levels of UDP-MurNAc-pentapeptide, the MraY nucleotide substrate, when grown at 30 °C: 719 ± 54 and 675 ± 62 nmol/g of bacteria (dry cell weight), respectively. However, following a temperature shift to 42 °C, the pool of UDP-MurNAc-pentapeptide was accumulated by *ca.* 2-fold (1704 ± 96 nmol/g of bacteria, dry cell weight) in mutant cells, but not in wild-type cells (890 ± 73 nmol/g of bacteria, dry cell weight). These findings were in agreement with the arrest of expression of *mraY* and the depletion of its product, lipid I, in mutant cells.

Functional Complementation Assays. The thermosensitive *Ecoli*TsMraY mutant was used to assay the mutated plasmids for functional complementation. Although this strain did not express the T7 RNA polymerase required for the expression of genes under control of the T7 promoter, its transformation with the pETYBS62 plasmid resulted in an increase of MraY activity in cell membranes that was estimated to 2.5- and

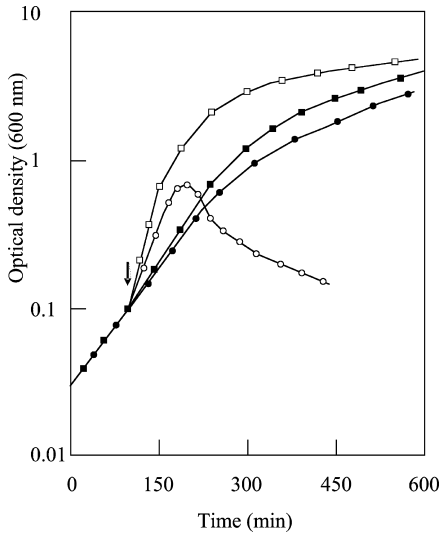

FIGURE 4: Lytic phenotype of the thermosensitive *mraY* mutant strain. Cells were grown exponentially at 30 °C in 2YT medium. At an optical density of *ca.* 0.1 (arrow), the temperature of the culture was either maintained at 30 °C or shifted to 42 °C. Symbols: (■, □) BW25113 strain grown at 30 °C and at 42 °C, respectively; (●, ○) *Ecoli*TsMraY strain grown at 30 °C and at 42 °C, respectively.

9.2-fold in the absence and presence of IPTG inducer, respectively. Thus, the pETYBS62 harboring the wild-type *mraY* gene allowed the thermosensitive mutant to grow under restrictive conditions, even in the absence of IPTG. The complementation assays were performed with and without IPTG at 1 mM, a concentration that did not result in a toxic effect on cell growth. Three categories of mutations (plasmids) were obtained (Figure 5): (i) mutations that complemented the thermosensitive *mraY* mutant strain in the presence or not of IPTG (N221, E264, S267, K276, R281) as it is the case for the wild-type plasmid; (ii) mutations that complemented the Ts mutant only in the presence of IPTG (H45, K48, N168, D177, H291, E299); (iii) mutations that did not complement the mutant even in the presence of inducer (D98, D99, K116, N171, D174, D231, H289, H290). If we assume that the cell levels of expression of the mutated proteins were more or less equivalent, the *in vivo* complementation assays indicated that at least the residues of the third category should be very important for activity and that the catalytic properties of the corresponding mutated proteins were presumably greatly altered.

In Vitro Activity of the Mutant MraY Proteins. Fourteen mutant proteins of the second and third categories (H45R, K48Q, D98N, D99N, K116Q, N168A, N171A, D174N, D177N, D231N, H289R, H290R, H291R and E299Q) were overproduced in the C43(DE3) strain, extracted from membranes by the DDM detergent and purified to homogeneity, as described for the wild-type enzyme (*7*). All mutant proteins were produced and purified at a similar level compared to the wild-type protein (data not shown). The specific activities of the wild-type and mutant MraY proteins were first determined using the standard MraY assay (*7*). The activities of all the mutants appeared to be greatly affected when compared to the wild-type protein. A clear correlation was observed between the *in vitro* activity of the proteins and the ability of the corresponding expression plasmids to complement the conditional thermosensitive *mraY* mutant (Table 1). Plasmids allowing functional complementation were those producing MraY mutant proteins which exhibit an activity higher than 0.5% of the wild-type level. Three mutants (D98N, D99N and H289R) presented a highly affected activity (decreased by 3 to 5 orders of magnitude). In order to rule out a possible contamination of these purified preparations of MraY by the chromosomally expressed MraY protein, membranes of C43(DE3) cells harboring the empty plasmid vector pET28b were prepared and proteins were extracted and purified on Ni^{2+}-NTA-agarose as described above. In these conditions, the MraY activity was exclusively recovered in the flow-through fraction (data not shown), confirming that the untagged MraY protein did not exhibit significant affinity for the Ni^{2+}-NTA-agarose.

Enzymatic Properties of the MraY Mutants. K_m values for UDP-MurNAc-pentapeptide and C_{55}-P and k_{cat} values were determined for each MraY mutant protein (Table 2). Mutants H45R, K48Q and H291R had a 2- to 3-fold increased K_m for UDP-MurNAc-pentapeptide relative to the wild-type enzyme. The other mutants presented a K_m for UDP-MurNAc-pentapeptide similar to that of the wild-type protein, except the D98N protein, which had a *ca.* 3-fold decreased K_m. Concerning the lipid substrate, wild-type and mutant proteins had a similar K_m for C_{55}-P except D98N and D99N, for which this constant was increased by 2-fold. However, the k_{cat} was dramatically reduced in all cases by 2 to 5 orders of magnitude except for the E299Q protein (10-fold decrease). Therefore, according to k_{cat} values, we can define three types of conserved residues: (i) residues D98, D99 and K116 of the cytoplasmic segment II and H289 of the cytoplasmic segment V for which the mutation led to a dramatic decrease of k_{cat} (10^4- to 10^5-fold); (ii) residues H45, K48, N168, N171, D174, D177, D231, H290 and H291 for which the corresponding mutant proteins presented a catalytic activity reduced by 2 to 3 orders of magnitude; (iii) the E299 residue of the cytoplasmic segment V, whose replacement resulted in a less affected k_{cat} (10-fold decrease). Owing to the modest variations of the K_m values observed, this classification is valid when the specificity constants (k_{cat}/K_m) are considered (Figure 6). It is worth noting that residues important for activity were not clustered but distributed in the five cytoplasmic segments. This suggests the involvement of all the cytoplasmic segments in the formation of the catalytic site.

Effect of $MgCl_2$ and pH on Activity of MraY Mutants. The effects of magnesium (essential to MraY activity) and pH on the activity of the wild-type and mutant proteins were tested. In the case of the wild-type enzyme, the optimal Mg^{2+} concentration was between 30 and 60 mM while higher concentrations (> 100 mM) abolished the activity. However, all mutant proteins showed an activity profile different from that of the wild-type enzyme (Table 2s, Supporting Information). The wild-type protein had only 2% of its optimal activity at 250 mM of Mg^{2+} while all mutant proteins, except E299Q, showed an activity higher than 30% of their optimal activities. At the latter concentration, the H45R, D174N and D177N mutants conserved a higher residual activity (more than 70%). This observation could suggest the involvement of these histidine and aspartate residues in the binding of the metal ion.

The activity of the different MraY proteins was determined at pH 9.4 and 7.6. The activity ratio between these two pH

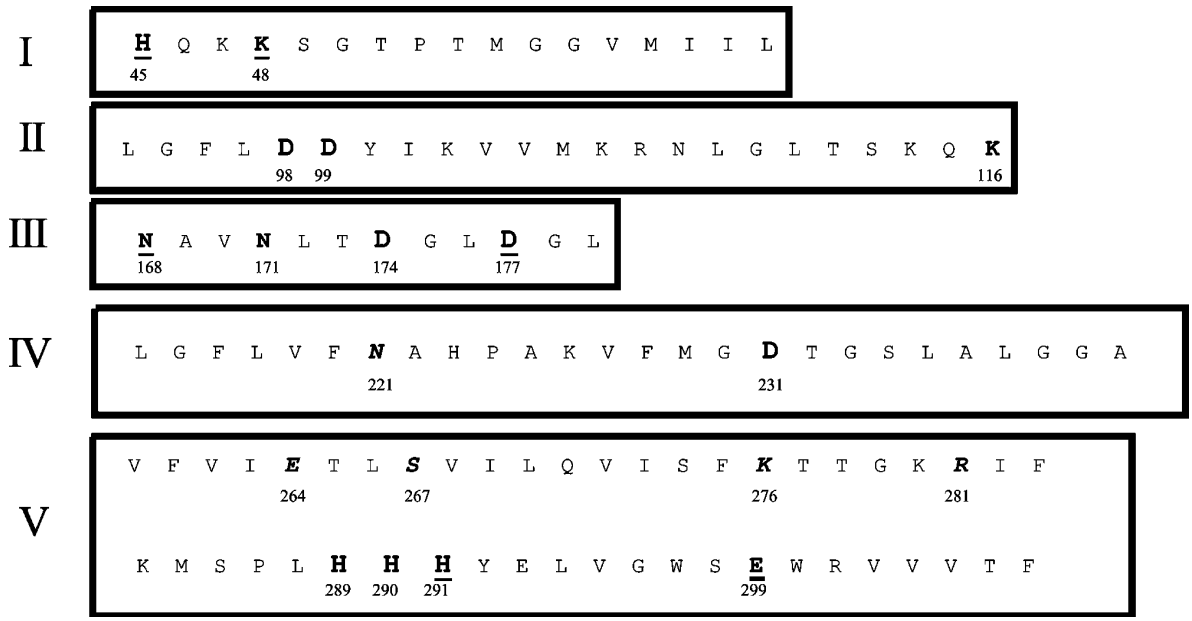

FIGURE 5: Functional complementation of the thermosensitive *Ecoli*TsMraY strain by mutated plasmids. The residues concerned by site-directed mutagenesis (bold) were located within the five cytoplasmic segments of MraY. We observed three categories of mutations: (i) plasmids which complemented the thermosensitive strain in the presence or not of IPTG (italics); (ii) plasmids which complemented only in the presence of IPTG (underlined); (iii) plasmids which did not complement at all (Roman type).

Table 1: Enzymatic Activity of Wild-Type (WT) and Mutant MraY Proteins[a]

MraY protein	activity (units/mg of protein)	functional complementation
WT	1700 ± 120 (100)	+
E299Q	180 ± 40 (11)	+
D177N	28 ± 5 (1.6)	+
N168A	26 ± 5 (1.5)	+
H45R	9.2 ± 0.6 (0.54)	+
K48Q	8.7 ± 1.4 (0.51)	+
H291R	8.6 ± 1.3 (0.50)	+
D231N	7.2 ± 1.9 (0.42)	−
D174N	6.6 ± 0.6 (0.39)	−
N171A	5.8 ± 0.8 (0.34)	−
H290R	2.4 ± 0.4 (0.14)	−
K116Q	0.78 ± 0.13 (0.046)	−
D98N	0.24 ± 0.06 (0.014)	−
D99N	0.13 ± 0.02 (0.0076)	−
H289R	0.02 ± 0.008 (0.0012)	−

[a] The numbers in parentheses are relative values, the enzymatic activity of the wild-type protein being considered as 100%. The assay was performed under standard conditions as described in Experimental Procedures. One unit of MraY activity corresponds to 1 nmol of lipid I produced per min. The functional complementation experiments were carried out in the presence of the IPTG inducer. Results are expressed as mean ± standard deviation of three independent experiments.

Table 2: Kinetic Parameters of Wild-Type (WT) and Mutant MraY Proteins[a]

	protein	k_{cat} (min^{-1})	K_m (mM) UM5	K_m (mM) C_{55}-P
	WT	320 ± 25 (100)	0.94 ± 0.15	0.16 ± 0.04
I	H45R	1.4 ± 0.1 (0.44)	3.4 ± 0.2	0.22 ± 0.03
	K48Q	1.6 ± 0.2 (0.50)	1.3 ± 0.1	0.26 ± 0.03
II	D98N	0.07 ± 0.005 (0.022)	0.34 ± 0.05	0.32 ± 0.03
	D99N	0.05 ± 0.008 (0.016)	0.76 ± 0.11	0.35 ± 0.03
	K116Q	0.09 ± 0.035 (0.028)	2.0 ± 0.3	0.22 ± 0.02
III	N168A	4.3 ± 0.3 (1.3)	0.95 ± 0.07	0.21 ± 0.02
	N171A	0.81 ± 0.13 (0.25)	0.73 ± 0.04	0.24 ± 0.01
	D174N	0.74 ± 0.11 (0.23)	0.99 ± 0.10	0.17 ± 0.02
	D177N	3.9 ± 0.4 (1.2)	0.86 ± 0.09	0.23 ± 0.02
IV	D231N	0.98 ± 0.16 (0.31)	1.0 ± 0.1	0.17 ± 0.02
V	H289R	0.003 ± 0.001 (0.00094)	1.1 ± 0.1	0.25 ± 0.04
	H290R	0.23 ± 0.04 (0.072)	1.3 ± 0.2	0.15 ± 0.02
	H291R	1.9 ± 0.8 (0.59)	3.4 ± 0.3	0.27 ± 0.03
	E299Q	32 ± 4 (10)	1.4 ± 0.3	0.22 ± 0.03

[a] Roman numerals indicate the cytoplasmic segments of MraY in which the mutated residues are located. The K_m values for UDP-MurNAc-pentapeptide (UM5) and C_{55}-P were determined by varying the concentration of one of the substrates while maintaining the other fixed (UM5, 3 mM, or 6 mM when the K_m was higher than 2 mM; C_{55}-P, 1.1 mM). The numbers in parentheses are relative values compared to that of the wild-type (100%). Results are expressed as mean ± standard deviation of three independent experiments.

values (pH 9.4/pH 7.6) was less than 0.8 for all analyzed proteins except the D98N mutant for which it was 2.5 (Table 3). The effect of pH was examined in more detail in the range 6.8−9.4 for the wild-type and D98N proteins. The D98N protein showed a pH profile different from that of the wild-type enzyme, with a maximal activity at pH 9.0−9.4 (Figure 7). Moreover, the specificity constant of the D98N protein was determined at different pH values for both substrates UDP-MurNAc-pentapeptide and C_{55}-P. As shown in Figure 8, the K_m for the nucleotide substrate did not vary with pH while that for C_{55}-P increased by 3-fold between pH 7.2 and 9.4. In this pH range, the specificity constant increased by factors of 11 and 2.5 for UDP-MurNAc-pentapeptide and C_{55}-P, respectively (data not shown), whereas the k_{cat} augmented by 11-fold (Figure 8).

These results suggest the involvement of the D98 aspartate residue in a process of deprotonation essential for catalysis. A likely hypothesis would be the deprotonation of the phosphate from C_{55}-P; this would be required to allow the nucleophilic attack of the phosphate oxyanion of C_{55}-P onto the β-phosphate of UDP-MurNAc-pentapeptide. In order to validate or at least support this hypothesis we carried out measurements of the pK_a values of C_{55}-P, as described in Experimental Procedures. Two pK_a values of 6.6 ± 0.1 and 9.2 ± 0.2, corresponding to the ionization of the two hydroxyl groups of the phosphate, were determined.

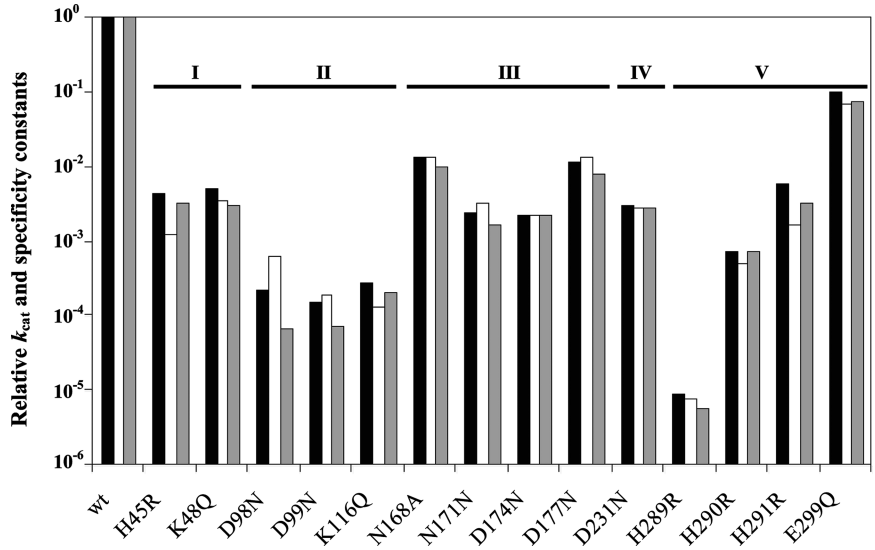

FIGURE 6: Catalytic constant (black) and specificity constants of the wild-type and mutant MraY enzymes for both substrates: UDP-MurNAc-pentapeptide (white) and C_{55}-P (gray). Values calculated from the data in Table 2 were normalized relative to the k_{cat} and the specificity constants of the wild-type enzyme, respectively, taken as 1. The roman numerals indicate the cytoplasmic segments.

Table 3: Enzymatic Activity Ratio of the Wild-Type MraY Enzyme (WT) and Its Mutated Forms at pH 9.4 and pH 7.6[a]

protein	pH 9.4/pH 7.6
WT	0.36 ± 0.04
H45R	0.75 ± 0.16
K48Q	0.64 ± 0.14
D98N	2.5 ± 0.4
D99N	0.32 ± 0.08
K116Q	0.69 ± 0.15
N168A	0.68 ± 0.23
N171A	0.77 ± 0.2
D174N	0.67 ± 0.25
D177N	0.22 ± 0.09
D231N	0.83 ± 0.14
H289R	0.42 ± 0.13
H290R	0.35 ± 0.19
H291R	0.42 ± 0.11
E299Q	0.63 ± 0.28

[a] The values for the activities at pH 7.6 are indicated in Table 1. Results are expressed as mean ± standard deviation of three independent experiments.

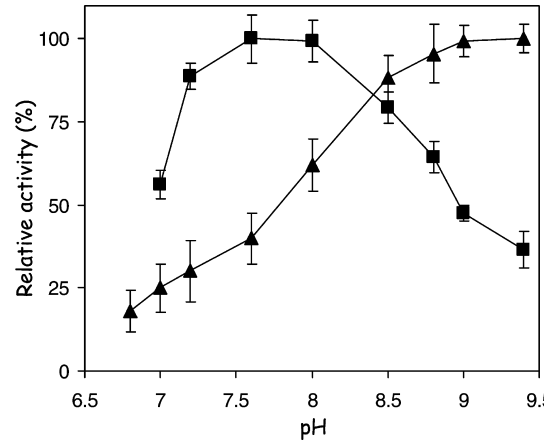

FIGURE 7: Effect of pH on the enzymatic activity of wild-type (square) and D98N (triangle) MraY proteins. For each protein the activity at pH 7.6 is indicated in Table 1. Each data point represents the mean ± standard deviation of three independent experiments.

DISCUSSION

MraY is a membrane protein essential for bacterial growth; therefore, it constitutes an interesting target for combating the resistance of bacterial pathogens to antibiotics. This enzyme is a member of the polyprenyl-phosphate *N*-acetylhexosamine 1-phosphate transferase superfamily (*9, 19*). To date, no protein of this superfamily has been obtained in a purified form, except MraY (*7, 21*). No crystal structure has been reported for any member of this superfamily, and only the topologies of the integral membrane proteins MraY, GPT and WecA were determined recently (*6, 14, 29*). Therefore, to get some insights into the catalytic mechanism and the active site structure of these enzymes, the roles of the invariant residues of this protein superfamily were investigated by using a site-directed mutagenesis approach, taking the MraY enzyme from *B. subtilis* as a model. The results obtained could thus be generalized to orthologues and paralogues of this enzyme superfamily.

A thermosensitive conditional *mraY* mutant strain (*Ecoli*TsMraY) was constructed in the present work. When grown under restrictive conditions, this mutant accumulated UDP-MurNAc-pentapeptide, the MraY nucleotide substrate, and then rapidly lysed, as expected for an arrest of the synthesis of the peptidoglycan lipid intermediates. Similar effects were observed when this or the subsequent membrane steps of peptidoglycan synthesis were inhibited by using either antibiotics (moenomycin, vancomycin) (*30*) or mutations (*31*), or when the supply of C_{55}-P, the lipid substrate of MraY, was specifically blocked (*32*).

Nineteen *mraY* mutants were generated by site-directed mutagenesis of polar invariant residues and the MraY activity of the corresponding mutant proteins was first tested *in vivo* for functional complementation using the thermosensitive mutant strain. The N221A, E264Q, S267A, K276Q and R281Q mutants allowed functional complementation without need for IPTG, suggesting that the corresponding residues, which are not conserved among the members of the superfamily, were not crucial for MraY activity. Two additional categories of mutations were observed: those that did not complement the thermosensitive strain even in the presence of IPTG (D98N, D99N, K116Q, N171A, D174N, D231N, H289R and H290R) and those requiring IPTG for functional

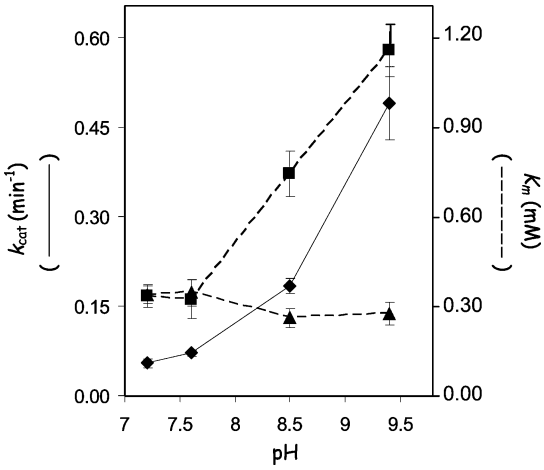

FIGURE 8: The k_{cat} (continuous line) and K_m (dashed line) values of the D98N protein as a function of pH. The parameters were determined for the nucleotide substrate (triangle) and C_{55}-P (square). The K_m for the nucleotide substrate was globally pH independent while the K_m for C_{55}-P was increased by *ca*. 3-fold at pH 9.4 compared to pH 7.2. However, for the two substrates the specificity constant increased with the pH. Each data point represents the mean ± standard deviation of three independent experiments.

complementation (H45R, K48Q, N168A, D177N, H291R and E299Q). The corresponding residues were thus expected to play an important role in the catalytic process and/or binding of the substrates. The fourteen mutant proteins of the two latter categories were purified to near homogeneity, and all of them exhibited a greatly altered enzymatic activity. It is noteworthy that a good correlation was observed between the enzymatic activity of the mutants and their capability to complement the *mraY* mutant strain. An enzyme activity representing at least 0.5% of the activity of the wild-type enzyme was apparently required to allow complementation of the mutant strain. The three mutations corresponding to the D98 and D99 residues and to the H289 residue were those yielding the most dramatic losses of activity.

The availability of pure MraY mutant proteins free of any traces of endogenous C_{55}-P substrate (*7*) allowed us to determine the kinetic properties for both the nucleotide and the lipid substrate. This is the first time such a detailed analysis has been performed for a member of this enzyme superfamily. The K_m for UDP-MurNAc-pentapeptide was increased in the case of the substitution of the invariant residues of cytoplasmic segment I (H45R and K48Q) and the third invariant histidine residue of cytoplasmic segment V (H291R). This might suggest the involvement of the two cytoplasmic segments I and V, which are specific of the MraY family, in the recognition of the nucleotide substrate and probably of the lactoyl-peptide moiety that is characteristic of this nucleotide substrate.

The mutation of single invariant residues of MraY did not significantly affect the ability of the enzyme to bind its substrates, suggesting that these interactions would rather require the contribution of many residues of the catalytic site. This could be explained by the size of the substrates and by the various potential interactions with the enzyme.

The kinetic constant of the fourteen mutants analyzed was highly affected (2−5 orders of magnitude relative to wild-type MraY) except for E299Q, which exhibited 10% of wild-type activity. This implies that the E299 residue is not essential for the catalytic process. On the contrary, the greatly affected k_{cat} of the remaining thirteen mutant proteins implies that the corresponding residues would be involved in the catalytic process and/or maintaining of the substrate in an appropriate configuration for an efficient catalysis. The mutation of these residues would affect the spatial positioning of the substrates in the catalytic site. The efficient orientation of the reactive groups of both substrates required for the establishment of the productive molecular attack would be highly affected. These important residues for activity were distributed in all the cytoplasmic segments, thereby forming the catalytic site.

A catalytic nucleophile was theoretically required for the proposed two-step catalytic mechanism for the MraY reaction (*8*). Recently, Lloyd et al. (*20*) suggested that the D267 residue of *E. coli* MraY (corresponding to D231 in *B. subtilis* MraY) that is located in the conserved hydrophilic sequence III could play this specific role. However, the recent topological analysis of *E. coli* WecA showed that the corresponding residue in this protein (D217) was predicted to be buried in the eighth transmembrane segment (*14*). These authors then proposed D156 (corresponding to D174 of *B. subtilis* MraY) as an alternative candidate for the catalytic nucleophile, an assumption based on the lack of detectable enzymatic activity for the D156E and D156N WecA mutants (*14*). In fact, we here observed that the mutation of either of these two residues D231 and D174 of MraY did not lead to a complete loss of activity, contrary to what was expected for the inactivation of a critical residue that is absolutely required for activity. Indeed, the purified D231N and D174N proteins both exhibited an activity representing *ca*. 0.3% of that of the wild-type enzyme, a value 300-fold higher than that observed for another mutant (H289R).

It should be noted that the two-step mechanism hypothesis of Heydanek et al. (*8*) was based on experiments performed with a nonpurified MraY enzyme. Therefore, the observations they made might be attributed to the presence of contaminating enzymes and C_{55}-P lipid substrate in their preparation. An alternative mechanism consists of a direct attack of the phosphate oxyanion of C_{55}-P onto the β-phosphate of UDP-MurNAc-pentapeptide. This would lead to the formation of lipid I and UMP in only one step (Figure 9). The invariant polar residues are likely candidates to be involved in the formation of a covalent "enzyme-phospho-MurNAc-pen-

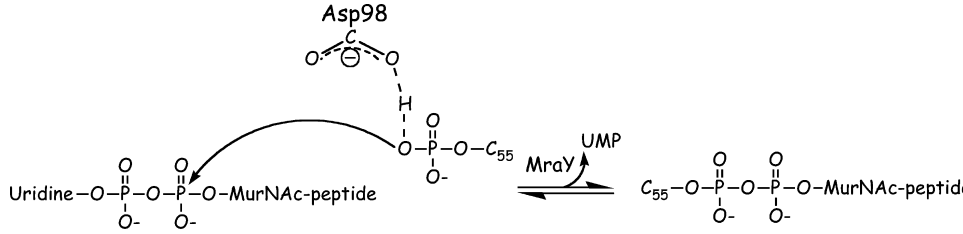

FIGURE 9: Hypothetical one-step catalytic mechanism of MraY and the role proposed for the D98 invariant residue.

tapeptide" reaction intermediate. The finding that the mutation of these residues severely altered but did not totally abolish the enzyme activity would be more consistent with a direct attack mechanism.

Another aspect of this work concerned the effect of Mg^{2+} and pH on the activity of the mutant proteins. Mg^{2+} (or Mn^{2+}, with a lower efficiency) is absolutely required for the enzyme activity (*7*). All the mutants except E299Q behaved differently from the wild-type protein with respect to the binding of the metal ion, i.e. they all exhibited a high residual activity at concentrations of Mg^{2+} above 100 mM which otherwise nearly abolished the activity of the wild-type enzyme. Clearly, a direct interaction of all thirteen residues with the metal ion is unlikely. The fact that three mutants, H45R, D174N and D177N, remained particularly active at the highest concentrations of Mg^{2+} tested (250 mM) strongly suggests the involvement of the corresponding residues in the binding of the metal ion. Lloyd et al. (*20*) previously speculated that the D115 and D116 residues of *E. coli* MraY (corresponding to D98 and D99 of *B. subtilis* MraY) should be involved in the binding of the metal ion. The authors based this hypothesis in view of the similarity to the DDXD/N motif of the farnesyl diphosphate synthetases where an Asp-Asp pair is involved in the chelation of Mg^{2+} (*33, 34*). However, no experimental data supporting this hypothesis was obtained. Based on the existence of the above pattern, Lehrer and co-workers (*14*) suggested the involvement of the aspartate residue D91 of *E. coli* WecA (corresponding to D99 of *B. subtilis* MraY) located in the second cytoplasmic segment in the binding of the metal ion. Our data do not exclude the involvement of the second residue of the Asp-Asp pair (D99 of *B. subtilis* MraY) in metal binding. However, as discussed below, the present work now suggests another role for the first aspartate residue (D98 of *B. subtilis* MraY).

The effect of pH on the activity of the purified mutant MraY proteins showed that only mutant D98N displayed a totally different pH profile compared to that of the wild-type enzyme. Interestingly, the latter mutant exhibited a progressive increase of activity in the whole range of pH considered. This effect was due to an increase of k_{cat} (by 11-fold) between pH 7.2 and 9.4. This pH effect thus appears to be related to the catalytic process rather than to enzyme/substrate interactions. The D98 residue would thus be involved in a deprotonation process of a substrate that is required for catalysis. Only the C_{55}-P substrate is a potential target for the deprotonation process since one of the two hydroxyls of its phosphate group is involved in the nucleophilic attack on the β-phosphate of the UDP-MurNAc-pentapeptide (Figure 9). The two pK_a values of C_{55}-P are 6.6 and 9.2, corresponding to the ionization of the two hydroxyl groups of the phosphate. These pK_a values are different from those, 4 and 8, determined for phosphatidic acid (*35*). If the hydroxyl having the higher pK_a (9.2) were located in a strategic position for catalysis and were involved in the enzymatic attack, its deprotonation would be required because the physiological pH is about 7.4. Such a deprotonation would be ensured by D98. In fact, at the physiological pH the major form would be spontaneously protonated (70-fold more than the deprotonated form) while at pH 9.4 the ratio of the deprotonated form would be significantly higher (1.4-fold more than the protonated form). The D98 residue is invariant among all the members of the polyprenyl-phosphate *N*-acetylhexosamine 1-phosphate transferase superfamily. Thus, its role in the deprotonation of an hydroxyl of the phosphate of the lipid substrate would be conserved among all the members of the superfamily.

SUPPORTING INFORMATION AVAILABLE

Table 1s shows the sequences of oligonucleotides used for site-directed mutagenesis of *mraY* gene, and Table 2s shows the effect of the metal ion Mg^{2+} on the activity of wild-type and mutant MraY proteins. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Walsh, C. (2000) Molecular mechanisms that confer antibacterial drug resistance. *Nature 406*, 775–781.
2. van Heijenoort, J. (2001) Recent advances in the formation of the bacterial peptidoglycan monomer unit. *Nat. Prod. Rep. 18*, 503–519.
3. Vollmer, W., Blanot, D., and de Pedro, M. A. (2008) Peptidoglycan structure and architecture. *FEMS Microbiol. Rev. 32*, 149–167.
4. Thanassi, J. A., Hartman-Neumann, S. L., Dougherty, T. J., Dougherty, B. A., and Pucci, M. J. (2002) Identification of 113 conserved essential genes using a high-throughput gene disruption system in *Streptococcus pneumoniae*. *Nucleic Acids Res. 30*, 3152–3162.
5. Boyle, D. S., and Donachie, W. D. (1998) *mraY* is an essential gene for cell growth in *Escherichia coli*. *J. Bacteriol. 180*, 6429–6432.
6. Bouhss, A., Mengin-Lecreulx, D., Le Beller, D., and Van Heijenoort, J. (1999) Topological analysis of the MraY protein catalysing the first membrane step of peptidoglycan synthesis. *Mol. Microbiol. 34*, 576–585.
7. Bouhss, A., Crouvoisier, M., Blanot, D., and Mengin-Lecreulx, D. (2004) Purification and characterization of the bacterial MraY translocase catalyzing the first membrane step of peptidoglycan biosynthesis. *J. Biol. Chem. 279*, 29974–29980.
8. Heydanek, M. G., Jr., Struve, W. G., and Neuhaus, F. C. (1969) On the initial stage in peptidoglycan synthesis. 3. Kinetics and uncoupling of phospho-N-acetylmuramyl-pentapeptide translocase (uridine 5′-phosphate). *Biochemistry 8,* 1214–1221.
9. Bugg, T. D., Lloyd, A. J., and Roper, D. I. (2006) Phospho-MurNAc-pentapeptide translocase (MraY) as a target for antibacterial agents and antibacterial proteins. *Infect. Disord. Drug Targets 6*, 85–106.
10. Dini, C. (2005) MraY Inhibitors as Novel Antibacterial Agents. *Curr. Top. Med. Chem. 5*, 1221–1236.
11. Stachyra, T., Dini, C., Ferrari, P., Bouhss, A., van Heijenoort, J., Mengin-Lecreulx, D., Blanot, D., Biton, J., and Le Beller, D. (2004) Fluorescence detection-based functional assay for high-throughput screening for MraY. *Antimicrob. Agents Chemother. 48*, 897–902.
12. Dini, C., Didier-Laurent, S., Drochon, N., Feteanu, S., Guillot, J. C., Monti, F., Uridat, E., Zhang, J., and Aszodi, J. (2002) Synthesis of sub-micromolar inhibitors of MraY by exploring the region originally occupied by the diazepanone ring in the liposidomycin structure. *Bioorg. Med. Chem. Lett. 12*, 1209–1213.
13. El Ghachi, M., Bouhss, A., Barreteau, H., Touzé, T., Auger, G., Blanot, D., and Mengin-Lecreulx, D. (2006) Colicin M exerts its bacteriolytic effect via enzymatic degradation of undecaprenyl phosphate-linked peptidoglycan precursors. *J. Biol. Chem. 281*, 22761–22772.
14. Lehrer, J., Vigeant, K. A., Tatar, L. D., and Valvano, M. A. (2007) Functional characterization and membrane topology of *Escherichia coli* WecA, a sugar-phosphate transferase initiating the biosynthesis of enterobacterial common antigen and O-antigen lipopolysaccharide. *J. Bacteriol. 189*, 2618–2628.
15. Soldo, B., Lazarevic, V., and Karamata, D. (2002) *tagO* is involved in the synthesis of all anionic cell-wall polymers in *Bacillus subtilis* 168. *Microbiology 148*, 2079–2087.
16. Burda, P., and Aebi, M. (1999) The dolichol pathway of N-linked glycosylation. *Biochim. Biophys. Acta 1426*, 239–257.
17. Dal Nogare, A. R., Dan, N., and Lehrman, M. A. (1998) Conserved sequences in enzymes of the UDP-GlcNAc/MurNAc family are

essential in hamster UDP-GlcNAc:dolichol-P GlcNAc-1-P transferase. *Glycobiology 8*, 625–632.

18. Lehrman, M. A. (1994) A family of UDP-GlcNAc/MurNAc: polyisoprenol-P GlcNAc/MurNAc-1-P transferases. *Glycobiology 4*, 768–771.
19. Bouhss, A., Trunkfield, A. E., Bugg, T. D., and Mengin-Lecreulx, D. (2008) The biosynthesis of peptidoglycan lipid-linked intermediates. *FEMS Microbiol. Rev. 32*, 208–233.
20. Lloyd, A. J., Brandish, P. E., Gilbey, A. M., and Bugg, T. D. (2004) Phospho-*N*-acetyl-muramyl-pentapeptide translocase from *Escherichia coli*: catalytic role of conserved aspartic acid residues. *J. Bacteriol. 186*, 1747–1757.
21. Boniface, A., Bouhss, A., Mengin-Lecreulx, D., and Blanot, D. (2006) The MurE synthetase from *Thermotoga maritima* is endowed with an unusual D-lysine adding activity. *J. Biol. Chem. 281*, 15680–15686.
22. Datsenko, K. A., and Wanner, B. L. (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. U.S.A. 97*, 6640–6645.
23. Hamilton, C. M., Aldea, M., Washburn, B. K., Babitzke, P., and Kushner, S. R. (1989) New method for generating deletions and gene replacements in *Escherichia coli*. *J. Bacteriol. 171*, 4617–4622.
24. Miller, J. H. (1972) *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
25. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
26. Dagert, M., and Ehrlich, S. D. (1979) Prolonged incubation in calcium chloride improves the competence of *Escherichia coli* cells. *Gene 6*, 23–28.
27. Mengin-Lecreulx, D., Flouret, B., and van Heijenoort, J. (1982) Cytoplasmic steps of peptidoglycan synthesis in *Escherichia coli*. *J. Bacteriol. 151*, 1109–1117.
28. Greenstein, J. P., and Winitz, M. (1961) *Chemistry of the Amino Acids*, Vol. 1, John Wiley & Sons, Inc., New York, London.
29. Dan, N., Middleton, R. B., and Lehrman, M. A. (1996) Hamster UDP-*N*-acetylglucosamine:dolichol-P *N*-acetylglucosamine-1-P transferase has multiple transmembrane spans and a critical cytosolic loop. *J. Biol. Chem. 271*, 30717–30724.
30. Lara, B., Mengin-Lecreulx, D., Ayala, J. A., and van Heijenoort, J. (2005) Peptidoglycan precursor pools associated with MraY and FtsW deficiencies or antibiotic treatments. *FEMS Microbiol. Lett. 250*, 195–200.
31. Mengin-Lecreulx, D., Texier, L., Rousseau, M., and van Heijenoort, J. (1991) The *murG* gene of *Escherichia coli* codes for the UDP-*N*-acetylglucosamine: *N*-acetylmuramyl-(pentapeptide) pyrophosphoryl-undecaprenol *N*-acetylglucosamine transferase involved in the membrane steps of peptidoglycan synthesis. *J. Bacteriol. 173*, 4625–4636.
32. El Ghachi, M., Derbise, A., Bouhss, A., and Mengin-Lecreulx, D. (2005) Identification of multiple genes encoding membrane proteins with undecaprenyl pyrophosphate phosphatase (UppP) activity in *Escherichia coli*. *J. Biol. Chem. 280*, 18689–18695.
33. Tarshis, L. C., Yan, M., Poulter, C. D., and Sacchettini, J. C. (1994) Crystal structure of recombinant farnesyl diphosphate synthase at 2.6-Å resolution. *Biochemistry 33*, 10871–10877.
34. Marrero, P. F., Poulter, C. D., and Edwards, P. A. (1992) Effects of site-directed mutagenesis of the highly conserved aspartate residues in domain II of farnesyl diphosphate synthase activity. *J. Biol. Chem. 267*, 21873–21878.
35. Moncelli, M. R., and Becucci, L. (1995) The intrinsic pK_a values for phosphatidic acid in monolayers deposited on mercury electrodes. *J. Electroanal. Chem. 385*, 183–189.

BI8006274