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Topological Analysis of the Membrane-Bound Glucosyltransferase, MdoH, Required for Osmoregulated Periplasmic Glucan Synthesis in *Escherichia coli*

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The MdoH protein is essential for synthesis of the osmoregulated periplasmic glucans, known as membrane-derived oligosaccharides (MDOs), in *Escherichia coli*. Mutants lacking MdoH are deficient in a glucosyltransferase activity assayed in vitro. The MdoH protein is the product of the second gene of an operon, and it has been shown to span the cytoplasmic membrane. The MdoH protein comprises 847 amino acids and is poorly expressed as observed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. We have experimentally measured the topological organization of MdoH within the membrane by construction of fusions to β-lactamase as a reporter. Analysis of 51 different MdoH–β-lactamase fusions suggested that the MdoH protein crosses the cytoplasmic membrane eight times, with the N and C termini in the cytoplasm. Moreover, a 310-amino-acid domain is present in the cytoplasm between the second and third transmembrane segments. It was deduced from the measurement of the MDO biosynthetic activity of truncated or fused MdoH proteins that almost all the C-terminal residues are necessary for this activity. The model of the MdoH protein in the membrane suggests that this protein could be directly involved in the translocation of nascent polyglucose chains to the periplasmic space.

Membrane-derived oligosaccharides (MDOs) are a class of soluble glucans located in the periplasmic space of *Escherichia coli* (23). Glucose is the sole monosaccharide: 5 to 12 units form a highly branched structure, where β-1,2-linked glucose constitutes a linear backbone to which branches are attached by β-1,6 linkages. MDOs are substituted by *sn*-1-phosphoglycerol, phosphoethanolamine, and succinic acid *O* ester (11). Kennedy (10) discovered that MDO synthesis is at a maximum when cells are grown in a medium with low osmolarity. Osmoregulated periplasmic glucans (OPGs) analogous to MDOs are found in the periplasm of various gram-negative bacteria (23).

In *E. coli*, the *mdoGH* operon encodes two proteins (MdoG and MdoH) necessary for the assembly of the polyglucose backbone. Its expression is osmotically controlled (15). MdoG is a 56-kDa periplasmic protein whose function remains to be determined (17). MdoH is a 97-kDa protein spanning the cytoplasmic membrane (17). MdoH is essential to the in vitro glucosyltransferase activity described by Weissborn and Kennedy (35). This glucosyltransferase system catalyzes the elongation of β-1,2 polyglucose chains requiring a β-glucoside as a primer, UDP-glucose as a substrate, the acyl carrier protein, and a trypsin-sensitive membrane fraction (30, 31, 35). MdoG and MdoH are probably the only proteins specifically involved in the biosynthesis of the glucan backbone. Several attempts were made to select new mutants after mutagenesis with nitrous acid (36) or with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (14). In both cases, all the selected mutations mapped in two previously described genetic loci: the *mdoGH* operon and the glucose-1-phosphate uridylyltransferase gene, *galU*.

The nucleotide sequence of the *mdoGH* operon has revealed a strong similarity (69% of 3,264 nucleotides are identical) with two genes present at the *hrpM* locus of *Pseudomonas syringae* pv. syringae (17). These two genes are known to be required for both the expression of disease symptoms on host plants and the development of the hypersensitive reaction on nonhost plants (19). Actually, *P. syringae* produces OPGs with structural features very similar to those of OPGs of *E. coli*, apart from their neutral character (27). In contrast, no significant sequence similarity was found between the *mdoGH* operon and the genes governing the synthesis of the cyclic β-glucans in the *Rhizobiaceae* family: *ndvA*/*chvA* or *ndvB*/*chvB* (17, 34). Thus, functionally homologous membrane-bound proteins which catalyze the synthesis of β-linked OPGs exhibit divergent amino acid primary sequences. Nevertheless, one should expect some conservation of the structural organization of these proteins with respect to the membrane.

We wished to elucidate the topology of MdoH within the cytoplasmic membrane by fusion protein experiments. Thus far, no topological analysis of an OPG biosynthetic enzyme has been reported. However, a topological model of the NdvA/ChvA protein has been suggested by analogy with the topological model of HlyB established experimentally (34). Thus, we have investigated the membrane organization of the MdoH protein by using the β-lactamase fusion system developed by Broome-Smith et al. (2). This system has the advantage of positively selecting both cytoplasmic and periplasmic fusions. We propose a topological model of MdoH which includes eight membrane-spanning segments, three large regions located in the cytoplasm, and two small regions exposed to the periplasmic compartment.

MATERIALS AND METHODS

Strains and media. The *E. coli* K-12 strains used in this study are listed in Table 1. Bacteria were grown at 30°C with vigorous shaking. Media used were Luria broth (LB), minimal M63 medium (18), or the low-osmolarity medium previously described (15). Solid media were prepared by adding agar (15 g/liter) to liquid media. The following antibiotics were used in media at the concentrations indicated: ampicillin at 50 μg/ml and kanamycin at 50 μg/ml.

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TABLE 1. *E. coli* K-12 derivatives and plasmids used

Strain or plasmid	Genotype[a] or characteristics	Source or reference
Strains		
JM83	Δ(*lac-pro*) *ara rpsL thi* (ϕ80 Δ*lacZ*-M15)	32
NFB213	*his pgi*::Mu Δ(*zwf-edd*)*1 eda-1 mdoH200*::Tn*10 rpsL*	15
NFB216	JM83; *mdoH200*::Tn*10 pyrC46*	16
K38	HfrC	26 via T. Pugsley
Plasmids		
pT7-6	Amp^r, expression vector T7*10*p	26
pGP1-2	Kan^r, Φ(λpL-T7*1*) λcI857	26
pYZ4	Kan^r, cloning vector	2
pYZ5	Tet^r, carrying the mature β-lactamase coding region	2
pNF150	Kan^r, pYZ4 carrying the mature β-lactamase coding region from pYZ5	17
pNF244	Amp^r, pUC8 carrying *mdoG*$^+$ *H*$^+$	16
pNF309	Kan^r, pYZ4 carrying *mdoH*$^+$	17
pNF318	Kan^r, pNF150 carrying *mdoH*$^+$	This work
pNF401	Kan^r, pNF150 carrying *mdoH* with a terminal *Bcl*I deletion	This work
pNF441	Amp^r, pT7-6 carrying *mdoH*$^+$	This work
pNF450	Amp^r, pT7-6 carrying *mdoH* with an internal *Eco*RV deletion	This work
pNF458	Amp^r, pT7-6 carrying *mdoH* with a terminal *Sma*I deletion	This work
pNF461	Amp^r, pT7-6 carrying *mdoG*$^+$ *H*$^+$	This work
pNF468	Amp^r, pT7-6 carrying *mdoH* with a terminal *Kpn*I deletion	This work

[a] Genetic nomenclature according to Bachmann (1).

DNA purification and transformation. Standard procedures (21, 25) were used for large-scale plasmid DNA isolation, rapid analysis of recombinant plasmids, and transformation of *E. coli* cells.

Restriction enzymes and ligase. Restriction endonucleases (New England Biolabs, Beverly, Mass.; Boehringer, Mannheim, Germany; Eurogentec, Seraing, Belgium) were used according to manufacturers' recommendations. Restriction fragments were ligated with T4 DNA ligase (Boehringer) in 66 mM Tris-HCl (pH 7.5)–7 mM $MgCl_2$–6 mM dithiothreitol–1.5 mM ATP. Incubations were done at 30°C for 2 h.

In vitro construction of plasmids. All the plasmids used in this work are listed in Table 1. Construction of pNF150 and pNF309 is described in Loubens et al. (17). The 3.1-kb *Bam*HI fragment of pNF309 was inserted into the *Bam*HI site of pNF150 to give pNF318. In this plasmid, *mdoH* expression is governed by the p*lacUV5* promoter present in the vector.

The 1.0-kb *Bam*HI-*Bcl*I fragment of pNF309 was purified and ligated into pNF150, digested with *Bam*HI, to give pNF401.

Deletions with exonuclease III and *blaM-mdoH* gene fusions. Plasmids pNF318 and pNF401 were used to construct a series of gene fusions by nested deletions from the 3′ end of *mdoH*. Exonuclease III digests linear DNA having 5′ overhanging ends or blunt ends, whereas 3′ overhanging ends are resistant to nuclease action. In the polylinker of pNF318 and pNF401, the *Hin*dIII site is sensitive to the nuclease action while the *Sst*I site is resistant.

After linearization, 12 μg of DNA was resuspended in 34 μl of exonuclease III buffer (according to the manufacturer's recommendations) and kept at 30°C. At this temperature, the exonuclease III digestion reaction proceeded at 100 bases per min. Exonuclease III (100 U in 1 μl; Boehringer) was added, and aliquots (5-μl) were removed at 30-s intervals and added to tubes on ice containing 4 volumes of water. When all time points had been taken, the tubes were removed from ice and incubated at 70°C for 10 min. The buffers were adjusted, and S1 nuclease (40 U; Boehringer) was added. The tubes were incubated at 37°C for 15 min, and the samples were analyzed on 0.8% agarose gel. Exonuclease III-digested DNAs were purified and filled in with deoxynucleotides (10 mM) by using the Klenow fragment of *E. coli* DNA polymerase I (1 U per μg of DNA; Boehringer). After ligation DNAs were transformed into NFB216 cells and transformants were selected on LB plates containing kanamycin at 50 μg/ml. On average, 20 to 30% of the colonies were resistant to ampicillin.

DNA sequencing and computer analyses. Double-stranded *mdoH-blaM* fusion plasmid DNAs were sequenced according to the Sequenase version 2.0 sequencing protocol (United States Biochemicals). The sequencing primer hybridizing at the 5′ end of *blaM* (5′-CCCACTCGTGCACCCAACT-3′) was produced by Eurogentec.

Deduced amino acid sequences were analyzed by using computer programs made available by BISANCE (5) and molecular biology services accessible on the World Wide Web: ExPaSy (http://expasy.hcuge.ch) and Infobiogen (http://www.infobiogen.fr).

Measurement of ampicillin resistance of cells expressing β-lactamase fusion proteins. The ampicillin resistance of individual cells of *E. coli* NFB216 containing various fusion plasmids was determined by growing the cells in LB to mid-log phase (3×10^8 cells/ml), washing the cells in an equal volume of fresh medium, and plating 200 to 400 of them onto plates of the same medium containing 0, 10, 50, 100, or 200 μg of ampicillin/ml. Colony numbers were determined in duplicates of three independent experiments. For each plasmid-harboring strain, the resistance was measured as the ratio of colony numbers on ampicillin plates to colony numbers on the control (no ampicillin) plates.

MDO analysis. To measure MDO synthesis, cultures were done overnight in the low-osmolarity medium containing 0.45 mM 2-[^{3}H]glycerol (296 MBq/mmol; New England Nuclear) and labeled MDOs were extracted by the charcoal adsorption procedure of Kennedy (10). When necessary, 1.5 ml of the pyridine extract obtained by this procedure was chromatographed on a column of Sephadex G-25 medium (Pharmacia Co.). The column was 2 cm^2 in cross section and 45 cm high and had been equilibrated with 0.15 M ammonium acetate in 7% (vol/vol) aqueous propanol. The column was eluted with the same buffer at the rate of 17 ml/h. Fractions (1.5-ml) were collected and counted.

For the analysis of MDO synthesis in strain NFB213 and its derivatives, the low-osmolarity medium was complemented with 0.24 mM D-[U-^{14}C]-glucose (125 MBq/mmol) and the cells were harvested in mid-log phase.

Radioactive labeling and PAGE of proteins. pT7-6 and its derivatives were introduced into strain K38(pGP1-2), which was then grown at 30°C and harvested in the logarithmic phase of growth. Cells were suspended in minimal M63 medium containing 1% methionine assay medium (Difco Laboratories), shaken at 30°C for 1 h, and then shifted to 42°C to induce the expression of the T7 RNA polymerase gene. A fresh solution of rifampin was added (0.2 mg/ml), and incubation was continued for 30 min before the culture was shifted back to 30°C. After a further 20 min of incubation, labeling was performed with Tran^{35}S-label (a mixture of 85% [^{35}S]methionine and 15% [^{35}S]cysteine) (37.0 TBq/mmol; ICN Biomedicals) for 5 min. The proteins were separated by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS–10% PAGE) after the samples had been boiled for 5 min in loading buffer. The labeled proteins were identified by autoradiography, and their molecular masses were estimated by comparison with protein size markers.

Proteolytic digestion of proteins in spheroplasts. Strain K38 harboring plasmids pNF441 (MdoH$^+$) or pNF458 (carboxy-terminal deletion) was grown in 15 ml of LB at 30°C and harvested in mid-log phase (3×10^8 cells/ml). Radioactive labeling was performed as described above. Chloramphenicol (0.2 mg/ml) was added, and the cells were collected by centrifugation and resuspended in 10 ml of a solution containing 10 mM Tris (pH 8.0), 20% sucrose, 5 mM EDTA, and 20 μg of lysozyme per ml. The cells were then incubated for 30 min on ice to allow the formation of spheroplasts, which was monitored microscopically. After spheroplast formation, proteinase K (Appligene, Illkirch, France) was added to a final concentration of 0.3 mg/ml and the suspension was incubated for 30 min on ice. Phenylmethylsulfonyl fluoride (3 mM) was added, the spheroplasts were centrifuged, and the proteins were analyzed as described above.

RESULTS

Visualization of the MdoH protein. The open reading frame corresponding to the *mdoH* gene starts with ATG at position 1951 and slightly overlaps the *mdoG* open reading frame, ending with TAA at position 1956 (the sequence is $_{1930}$GGAGC TACCAGTTACCTGCCAA**ATG**AATAAGA$_{1960}$ [17]). But neither this ATG nor another potential start codon (ATG at position 1981) is preceded by a consensus ribosome-binding sequence. However, since an active protein fusion was obtained by joining the *mdoGH* DNA up to the *Eco*RV site found at the beginning of *mdoH* (position 2025) to a truncated *lacZ* gene (Fig. 1) (15), the translation of *mdoH* should not begin after this site.

When the envelope proteins of cells harboring plasmids containing the *mdoGH* operon (like pNF244, a derivative of the high-copy-number vector pUC8) were analyzed by SDS-PAGE, we could not observe the overproduction of a protein with an apparent molecular mass corresponding to that expected for MdoH (97 kDa) (17, 29). In an attempt to visualize MdoH, a 4.7-kb *Ssp*I DNA fragment containing *mdoGH* was cloned into plasmid pT7-6 under the control of a bacteriophage T7 RNA polymerase promoter (26) to give pNF461. The proteins specifically expressed from this plasmid were labeled

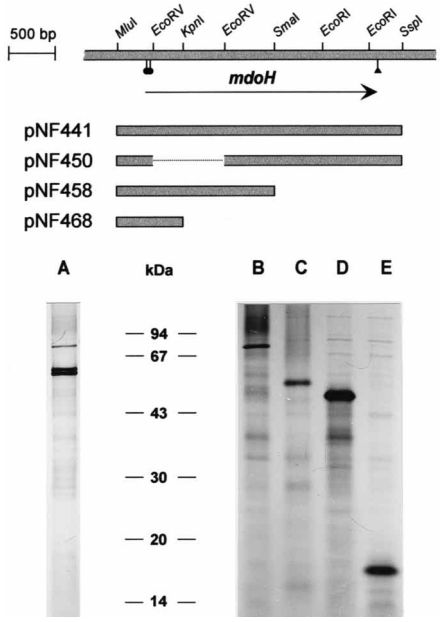

FIG. 1. Autoradiography of SDS-PAGE of various derivatives of the MdoH protein expressed under the control of a T7 promoter and labeled with ^{35}S (see Materials and Methods). (Top) Restriction map, plasmid constructs, and genetic organization of the *mdoH* gene. DNA segments present in the various derivatives of plasmid pT7-6 are indicated by boxes. The arrow indicates the location and orientation of the *mdoH* gene. Black circles indicate the two potential initiation codons; the black triangle indicates the stop codon. (Bottom) Lane A, proteins expressed from plasmid pNF461 (*mdoG*$^+$*H*$^+$, not shown in the top panel); lanes B to E, proteins expressed from plasmids pNF441, pNF450, pNF458, and pNF468, respectively.

with Tran^{35}S-label and analyzed by SDS-PAGE (see Materials and Methods). Autoradiography of the gels showed the presence of two thick protein bands, corresponding to MdoG and its precursor form, with the sizes expected from the nucleotide sequence (17) and one thin band, corresponding to MdoH (Fig. 1, lane A). However, this latter band had an apparent molecular mass smaller than that predicted from the DNA sequence (80 versus 97.0 kDa). The wild-type sequence and three deletions of *mdoH* were cloned into plasmid pT7-6, and the corresponding proteins were analyzed by SDS-PAGE (Fig. 1, lanes B to E). First, the in-frame *Eco*RV deletion (pNF450) gave a protein with an apparent molecular mass of 55 kDa, while 66.5 kDa was expected. The two other constructions (pNF458 and pNF468) were C-terminal deletions expected to encode proteins of 55.3 and 17.4 kDa; they gave proteins of 48 and 17 kDa, respectively. As the shortest band runs exactly at the predicted position, this result supports the possibility that the ATG at position 1951 is the initiation codon. On the other hand, since MdoH is a transmembrane protein, it is possible that its hydrophobic character causes its abnormal migration on SDS-PAGE, as was reported previously for several membrane proteins. In fact, the underestimation of the molecular mass of MdoH (80 versus 97.0 kDa) is very similar to those of LacY (30 versus 46.5 kDa [3]), TrkG (35 versus 53.5 kDa [22]), and MalF (40 versus 56.9 kDa [24]).

Prediction of the topology of the glucosyltransferase MdoH. The hydropathic profile was determined by the Kyte and Doolittle algorithm (13), with windows of 7 to 19 residues. The profile obtained with a window of seven residues (17) revealed two large hydrophobic regions. We can predict that the first region contains two or three membrane-spanning segments and the second region contains six or seven such segments. The three remaining parts of the protein—the amino-terminal (138 amino acids), central (302 amino acids), and carboxy-terminal (146 amino acids) regions—exhibit a high degree of hydrophilicity and should represent water-soluble domains.

The prediction of the secondary structure of the polypeptide chain made by the Chou and Fasman (4) and Gibrat et al. (8) algorithms confirmed that the most hydrophobic regions have a highly α-helical nature. We have also used two computer programs available on the World Wide Web (see Materials and Methods). TMpred, from the Swiss Institute for Experimental Cancer Research (Epalinges sur Lausanne, Switzerland), is based on the statistical analysis of TMbase, a database of naturally occurring transmembrane proteins (9). TMAP, from the European Molecular Biology Laboratory (Heidelberg, Germany), is based on multiple aligned sequences, and we used two homologous proteins: HrpM of *P. syringae* (19) and OpgB of *Erwinia chrysanthemi* (20). Both programs gave the same result (two membrane-spanning segments in the first hydrophobic region and four membrane-spanning segments in the second) with only slight differences concerning the limits of the transmembrane segments (Fig. 2A). To test the validity of this model, we used β-lactamase as a topological reporter.

Construction of *mdoH-blaM* gene fusions. In a previous report (17), we have described the construction by direct cloning into restriction sites of three gene fusions between *mdoH* and a truncated version of the β-lactamase gene, *blaM*. From the observed phenotypes it was possible to ascertain that MdoH is a transmembrane protein. These fusions were at amino acids 25, 470, and 542 from the start codon of MdoH (17). The first hybrid protein was water soluble, while the second and the third were membrane bound; the third MdoH–β-lactamase hybrid conferred ampicillin resistance on isolated bacteria, but the other two conferred resistance to ampicillin only when the cells were patched on the plates.

Random β-lactamase fusions throughout the MdoH protein were obtained by digesting the *mdoH* gene from the 3′ end with exonuclease III and ligating the blunt-ended DNA with the *′blaM* gene. For this procedure, plasmid pNF318 was constructed; this placed the *mdoH* gene (2,541 bp, preceded by 315 bp from the 3′ end of *mdoG* and followed by a 250-bp untranslated region) downstream of the p*lacUV5* promoter and upstream of a truncated version of *blaM* (see Materials and Methods). Thus, partial exonuclease III digestions produced random-sized internal deletions which fused *mdoH* to *blaM*. The fusion point between the *mdoH′* and *′blaM* genes was determined by DNA sequencing (see Materials and Methods). Because it appeared that this procedure produced very few fusions in the N-terminal part of MdoH, we substituted for pNF318 a new plasmid, in which 2.2 kb of the 3′ end of the insert had been deleted (pNF401) (see Materials and Methods). From the two selections, 48 unique in-frame fusions were discovered. Together with the three site-specific fusions previously obtained, these fusions gave a fairly even coverage of the MdoH protein (Fig. 3).

Cellular location of the β-lactamase moiety of the hybrid proteins. The position of the β-lactamase moiety, i.e., cytoplasmic or periplasmic, for each of the 51 unique MdoH-BlaM fusion proteins was determined by streaking the colonies, up to individual cells, with sterile toothpicks onto LB agar plates containing 50 μg of ampicillin/ml. The results were confirmed for a representative set of fusions by determining the ampicillin resistance more precisely (see Materials and Methods). Under these conditions, only the fusions at amino acids 166, 175, and 542 conferred resistance on isolated cells and allowed the formation of isolated colonies on plates containing 200 μg of ampicillin/ml (Table 2). These strains were considered to have

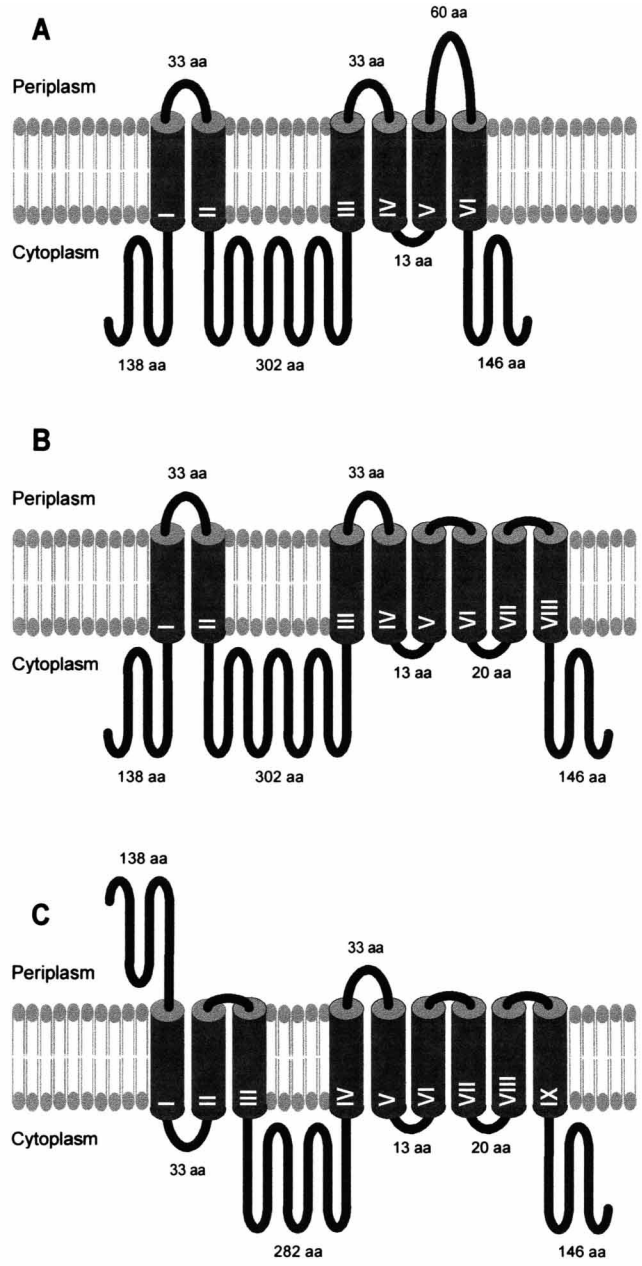

FIG. 2. Alternative models of the orientation of the MdoH protein in the membrane. Computer programs predicted one model (A), whose validity was tested by using β-lactamase as a topological reporter. From this study, two alternative models (B and C) were obtained, which were then tested by determining the protease susceptibilities of the amino-terminal regions. aa, amino acid; I to IX, membrane-spanning segments.

the β-lactamase moiety in the periplasm. However, the fusions at amino acids 525, 549, 563, 574, 579, 584, 645, 694, and 709 conferred only a partial resistance, and these strains were considered to have the β-lactamase moiety unsteadily or partly exposed to the periplasm. Those strains with an ampicillin sensitivity identical to that of the control strains bearing pNF318 or pNF401 were considered to have the β-lactamase moiety in the cytoplasm.

Topological model of MdoH. From the location of the β-lactamase moiety of the hybrid proteins we obtained direct confirmation of the positions in the cytoplasm of the three hydrophilic domains and of the transmembrane character of segments I, II, III, and VI (Fig. 2A). However, the ampicillin-sensitive phenotype of the fusions at amino acids 623, 661, and 670 indicated that the predicted third periplasmic domain does not exist and that two additional transmembrane segments should be present (Fig. 2B and 3 show a precise localization of every amino acid residue). The weak resistance observed for the fusions at amino acids 645, 694, and 709 could be explained by the fact that hybrid proteins have lost a series of positively charged amino acids which should act as strong topological determinants to direct the downstream regions to the cytoplasm (33).

Finally, on the basis of MdoH–β-lactamase hybrid analysis, we could not exclude the alternative model shown in Fig. 2C, in which the amino terminus of MdoH is in the periplasmic space. Indeed, if that was the case, the hybrid proteins corresponding to fusions at amino acids 25 to 166 could have an inverted localization due to the loss of efficient topological signals. This alternative model implied a third membrane-spanning segment in the N-terminal region.

Protease susceptibility of the amino-terminal region of MdoH in spheroplasts. To distinguish between these topological models, we used proteinase K susceptibility to probe for the presence of the MdoH amino terminus in the periplasm (see Materials and Methods). To monitor the action of the protease, we used cells harboring plasmids pNF441 and pNF458 (Fig. 1), which specifically express MdoH and a carboxy-terminal deletion occurring at the end of the second cytoplasmic domain of MdoH, respectively. Plasmid pNF458 was chosen because the radioactive labeling was much more efficient for the truncated form than for the complete form of MdoH (Fig. 1). If the model with the amino terminus present in the cytoplasm is correct (Fig. 2B), then a polypeptide corresponding to a cleavage(s) in the first periplasmic domain, but protected by an intact cytoplasmic membrane, is expected, with a molecular mass between 18 and 21 kDa. If the alternative model with the amino terminus exposed to the periplasm is correct (Fig. 2C), then this polypeptide would be completely degraded by the proteinase K. Moreover, a second polypeptide, with a molecular mass between 34 and 37 kDa, corresponding to the carboxy terminus, is expected in the case of pNF458. After the proteinase K treatment of the truncated form of MdoH, two polypeptides with apparent molecular masses of 33 and 19 kDa were actually observed (Fig. 4, lanes D and F, bands 1 and 4), two additional polypeptides with apparent molecular masses of 30 and 26 kDa (Fig. 4, lanes D and F, bands 2 and 3) would correspond to the digestion of degradation products observed before the treatment (Fig. 4, lane C). The experimentally observed pattern of resistance to proteinase K in spheroplasts thus followed the predictions from the MdoH topology model shown in Fig. 3.

MDO biosynthetic activity of C-terminal MdoH-BlaM fusions and MdoH deletions. When plasmid pNF244 (the complete *mdoGH* operon cloned into pUC8) was introduced into strain NFB216 (*mdoH200*::Tn*10*), the MDO synthesis, measured by 2-[^{3}H]glycerol incorporation, was increased to three to four times that of the wild-type strain, JM83 (16). When the same experiment was done with plasmid pNF309 (*mdoH*$^+$ cloned into pYZ4, downstream from the p*lacUV5* promoter), the MDO synthesis was only increased by a factor of 1.5. The same was true when pNF309 was introduced into strain NFB213 (*pgi zwf*) and the MDO synthesis was measured by [^{14}C]glucose incorporation as previously described (15). This result provides confirmation that MdoG has the main limiting activity in MDO biosynthesis (15).

Various fusion proteins were tested for their MDO biosyn-

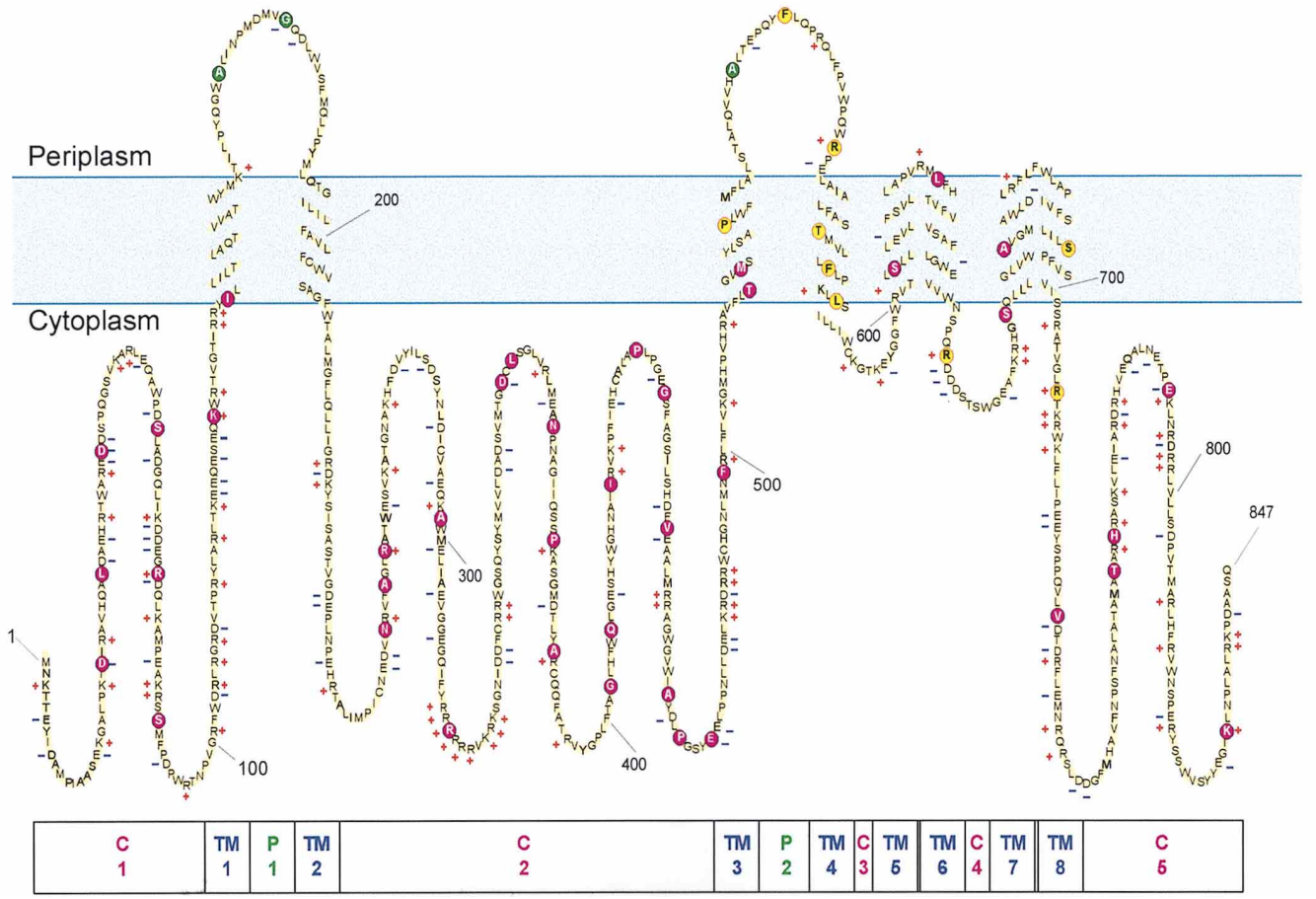

FIG. 3. Positions of β-lactamase fusions superimposed on the model of the MdoH protein in the membrane. The positions of the β-lactamase fusions are indicated by green circles when they conferred resistance to 200 μg of ampicillin/ml, by yellow circles when they conferred partial resistance (Table 2), and by red circles when they conferred no resistance. Plus and minus signs indicate positively and negatively charged amino acids, respectively. C1 to C5, cytoplasmic segments; TM1 to TM8, TMS; P1 and P2, periplasmic segments.

thetic activity in both the *mdoH200*::Tn*10* and the *mdoH*$^+$ backgrounds. All but one of them (at amino acid 833) were inactive, and in no case was this defective phenotype negatively dominant over the wild-type phenotype. By contrast, the fusion at amino acid 833 gave a fully active protein. During the preliminary screening of MdoH-BlaM fusions, one cytoplasmic hybrid protein was characterized. However, nucleotide sequencing revealed that this fusion (at amino acid 825) was not in frame and that β-lactamase was expressed through translation reinitiation. As a consequence of this anomalous fusion, an 833-amino-acid variant of MdoH was produced (MdoH . . . SSW_{825}PCVTQKRW_{833}); this protein was slightly active, since a strain bearing the mutant multicopy plasmid produced only 7.6% of the MDOs synthesized by the control (JM83). The stability of the protein was tested by using the pT7-6 expression vector as described above. Analysis by SDS-PAGE showed a protein band very similar in intensity to that observed with an *mdoH*$^+$ plasmid, indicating that the mutant protein was as stable as the wild type.

These results confirm the previously reported observation that deletion from the last *Eco*RI site present in *mdoH* (Fig. 1) completely abolished the MDO synthesis (16). Nucleotide sequencing revealed that, by chance, the deletion brought a stop codon just after the 818th codon of *mdoH*. Thus, it appeared that amino acids between 818 and 833 are essential to the protein activity.

TABLE 2. Ampicillin resistance of 22 *mdoH-blaM* translational fusions

Fusion designation	% Resistance at ampicillin concn (μg/ml) of:			
	10	50	100	200
AA 88	0	0	0	0
AA 140	0	0	0	0
AA 166	100	100	100	100
AA 175	100	100	100	100
AA 256	0	0	0	0
AA 516	0	0	0	0
AA 519	0	0	0	0
AA 525	100	100	0	0
AA 542	100	100	100	100
AA 549	100	100	40	15
AA 563	100	100	100	0
AA 574	100	0	0	0
AA 579	100	0	0	0
AA 584	100	0	0	0
AA 606	0	0	0	0
AA 623	0	0	0	0
AA 645	100	0	0	0
AA 661	0	0	0	0
AA 670	0	0	0	0
AA 694	80	0	0	0
AA 709	100	0	0	0
AA 729	0	0	0	0

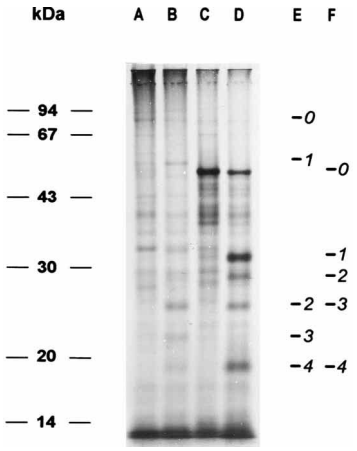

FIG. 4. Proteinase K susceptibility of MdoH in spheroplasts. Cells expressing *mdoH*$^+$ (pNF441; lanes A and B) or a truncated form (pNF458; lanes C and D), under the control of a T7 promoter, were labeled with ^{35}S and transformed in spheroplasts. Aliquots of the spheroplast suspensions were treated with proteinase K. Then, the proteins from the treated (lanes B and D) or the control (lanes A and C) spheroplasts were subjected to SDS-PAGE and autoradiography. Lane E and F show the positions of the major protein bands obtained for MdoH and its truncated form, respectively.

DISCUSSION

Expression of the *mdoGH* operon from a multicopy plasmid leads to a three- to fourfold increase in MDO biosynthesis (16). SDS-PAGE analysis of the proteins synthesized under these conditions failed to reveal the overproduction of any protein (29). Expression of *mdoG* isolated from *mdoH* leads to the same increase in MDO biosynthesis and to the overproduction of a 56-kDa periplasmic protein: the product of *mdoG* (17). In this study, we have observed that when the *mdoGH* operon is expressed from a multicopy plasmid under the control of a T7 promoter, MdoG is overproduced, its precursor is slowly processed, and much less MdoH is produced than MdoG (Fig. 1, lane A). Moreover, when *mdoH* was isolated on a multicopy plasmid, the MDO synthesis was only increased by a factor of 1.5. We can propose several explanations of these phenomena. MdoG is the only limiting protein in the MDO biosynthetic system, and when MdoG is expressed together with MdoH, the former is produced in a slight excess. If we consider the fact that translation of *mdoH* starts 5 bases before the end of *mdoG*, we can imagine interference between ribosomes translating the two genes (7). This phenomenon would be accentuated by the absence of a consensus ribosome binding site in front of *mdoH*. Moreover, 24% of the 847 amino acids of MdoH are hydrophobic and the location of this protein in the membrane seems to be a limitation on its synthesis. Accumulation of a large amount of this protein in the membrane could become toxic for the cells. One can see in Fig. 1 that as soon as hydrophobic residues were removed from MdoH, the amount of the truncated protein was increased. Finally, attempts to detect specific mRNAs or to measure chromosomal gene fusion activities have indicated that the *mdoGH* promoter is very weak (14, 15). Thus, the number of MdoH proteins in the membrane is probably very low.

In this study, we used β-lactamase hybrids and protease susceptibility to probe the topological organization of the MdoH protein in the membrane. From these data, we now propose a topological model. Obviously, details of the exact limits of each domain could be slightly different, but the general topological organization of MdoH, with eight transmembrane segments, seems sound.

The topological model of MdoH has several interesting features which can be related to known or suspected functions of the protein. The three large cytoplasmic domains (C1, C2, and C5) are probably involved in the polymerization of glucose units from UDP-glucose. One can also expect that at least one of these domains interacts with the acyl carrier protein, whose exact function in the polymerization remains obscure (11, 30, 31), even though a recent study has shed light on the domains of this protein that are probably implicated in the interaction with MdoH (28).

In this study, we took the opportunity of checking the implication of the C-terminal residues in the MdoH activity. In fact, this activity decreased abruptly when the residues between 818 and 833 were removed. Even if we cannot exclude the possibility that these deletions induced a misfolding of the protein, these data clearly indicate that only a very few C-terminal residues (15 to 25) of MdoH are unnecessary to the MDO synthesis.

Two short segments (P1 and P2) of about 30 residues each are facing the periplasmic space. This is much less than the number of residues present at the cytoplasmic face of the membrane. However, one can imagine that these periplasmic domains are essential for some interaction of MdoH with another component(s) of the biosynthetic machinery, such as MdoG. Actually, preliminary experiments indicated that the insertion of four residues into P2 induced a severe reduction of MDO synthesis (12).

Multiple transmembrane segments (TMS) were previously found in bacterial proteins involved in various carbohydrate transports. Moreover, six TMS were predicted in the NdvA/ChvA protein involved in β-glucan translocation in *Rhizobiaceae* (34). Similarly, the FKS1 component of the multisubunit fungal 1,3-β-D-glucan synthase exhibits 12 potential TMS and was speculated to be involved in transporting the growing glucan polymer across the membrane (6). The presence of eight TMS in MdoH suggests that this protein could be directly involved in the translocation of the nascent polyglucose chain to the periplasmic space.

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