Membrane Topology of the *Escherichia coli* AmpG Permease Required for Recycling of Cell Wall Anhydromuropeptides and AmpC β-Lactamase Induction

Aicha Chahboune,[1] Marc Decaffmeyer,[2] Robert Brasseur,[2] and Bernard Joris[3]*

Département de Biologie, Faculté des Sciences, Université My Ismail, Meknès, Morocco,[1] and Centre de Biophysique Moléculaire Numérique, Faculté Universitaire des Sciences Agronomiques de Gembloux, Gembloux,[2] and Centre d'Ingénierie des Protéines, Institut de Chimie, Université de Liège, Sart Tilman (Liège),[3] Belgium

Escherichia coli, **and presumably most other gram-negative bacteria, possesses an efficient protein machinery for recycling its peptidoglycan during cell growth. The major recycled peptidoglycan product is *N*-acetylglucosamine-1,6-anhydro-*N*-acetylmuramic acid-tetrapeptide. Its uptake from the periplasm into the cytoplasm is carried out via the AmpG protein, an intrinsic membrane protein. In gram-negative bacteria carrying an *ampC* β-lactamase-inducible gene on their chromosomes, the induction mechanism is directly linked to peptidoglycan recycling. After identification of the different putative hydrophobic segments by computing, the AmpG topology was experimentally determined by using β-lactamase fusion. In the proposed model, AmpG contains 10 transmembrane segments and two large cytoplasmic loops.**

The bacterial cell wall contains a cross-linked heteropolymer named peptidoglycan or murein that protects the cell from osmotic lysis and determines its shape. Although this highly cross-linked network forms a rigid and insoluble envelope, the peptidoglycan sacculus is nevertheless continuously remodeled throughout the bacterial cell cycle. As the bacterium grows and divides, extracellular peptidoglycan hydrolases cleave covalent bonds within the peptidoglycan sacculus to allow insertion of new glycan chains during enlargement of the cell and to perform the separation of daughter cells (11, 19). In contrast to *Bacillus subtilis*, which loses as much as 30% of its peptidoglycan per generation (1), *Escherichia coli*, and presumably most other gram-negative bacteria, releases only around 5 to 8% of peptidoglycan fragments into the medium per generation (9). This different percentage of released peptidoglycan is the consequence of the presence of a very efficient protein machinery for peptidoglycan recycling in *E. coli* (8, 23). The major recycled peptidoglycan product is *N*-acetylglucosamine-1,6-anhydro-*N*-acetylmuramic acid-tetrapeptide (GlucNAc-anhydro-MurNAc-tetrapeptide or anhydromuropeptide) (13, 25). Its uptake, from the periplasm into the cytoplasm, is carried out via the AmpG permease specific for GlucNAc-anhydro-MurNAc or GlucNAc-anhydro-MurNAc-peptide transport (4, 13). In the cytoplasm, the anhydromuropeptide is degraded and the tripeptide reenters the peptidoglycan biosynthesis pathway.

In many gram-negative bacteria, the presence of penicillin or other β-lactam antibiotics outside the cell induces the synthesis of the chromosomal AmpC β-lactamase (20). The inducible *ampC* β-lactamase gene is transcriptionally controlled by the divergently transcribed regulator gene *ampR* (15). AmpR is a DNA-binding protein belonging to the LysR superfamily (10),

and it has two regulatory properties: (i) in the absence of β-lactam inducer, AmpR is complexed by UDP-MurNAc-pentapeptide and acts as a repressor; (ii) in the presence of a β-lactam antibiotic, the anhydro-MurNAc-oligopeptide (penta-, tetra-, and tripeptides) (5) accumulates in the cytoplasm by concomitant inhibition of membrane D,D-peptidases and the activation of the autolytic system, displaces the UDP-MurNAc-pentapeptide ligand, and converts AmpR into an activator, so that the AmpC β-lactamase is produced (12). The inactivation of *ampG* by mutation or deletion confers noninducible and microconstitutive β-lactamase phenotypes to the bacterial cell (14, 16). These results clearly demonstrate the links between β-lactamase induction and peptidoglycan recycling. The *E. coli ampG* gene has been cloned (16) and encodes a 491-amino-acid protein with a molecular mass of 53 kDa. Along the AmpG primary structure, several segments have been identified, and the AmpG protein was predicted to be an integral membrane protein, with 10 transmembrane segments (16).

Our interest in AmpG was stimulated by the fact that this peptidoglycan-specific permease could be used to transport new drugs mimicking the murein recycled compounds into the cytoplasm. These new compounds could directly or indirectly inhibit an enzyme involved in the peptidoglycan biosynthesis. In this way, and to better understand the AmpG transport mechanism, we determined the membrane topology of the AmpG protein by using the β-lactamase fusion procedure described by Broome-Smith et al. (2).

MATERIALS AND METHODS

Prediction of the membrane-spanning segments. Putative transmembrane α helices in AmpG were identified by a hydrophobic moment plot (7). In this approach, the prediction is achieved by computing the mean hydrophobic moment ($<\mu>$) and the mean hydrophobicity index ($<H>$) for each possible peptide segments of 11 residues along the AmpG sequence. Following these values, each undecapeptide segment can be predicted to be (i) a monomeric transmembrane segment (T), (ii) a transmembrane segment which could oligomerize (multimeric transmembrane segment or M), or (iii) an amphiphilic segment (S) (6, 7).

* Corresponding author. Mailing address: Centre d'Ingénierie des Protéines, Institut de Chimie, B6, Université de Liège, B-4000 Sart Tilman (Liège), Belgium. Phone: 32 4 366 29 54. Fax: 32 4 366 3364. E-mail: bjoris@ulg.ac.be.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
E. coli XLI-BlueMRF′	Δ(*mcrA*)183 Δ(*mcrCB-hsdSMR-mrr*)173 *endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* (F′*proAB lacIqZ* ΔM15 Tn*10* [Tet^r])	Stratagene
E. coli XLI-Blue MR	Δ(*mcrA*)183 Δ(*mcrCB-hsdSMR-mrr*)173 *endA1 supE44 thi-1 recA1 gyrA96 relA1 lac*)	Stratagene
PCRscript SK	Cloning vector for PCR fragments; Cm^r	Stratagene
PCRscript SK	Cloning vector for PCR fragments; Amp^r	Stratagene
pJBS633	Source of the *blaM* gene encoding TEM β-lactamase without its signal peptide (mature TEM β-lactamase); Km^r	2
pGKS171-3	Source of *E. coli ampG* gene; Cm^r	14
pYZ4	pBR322 derivative carrying a modified LacZα peptide under the control of the *lacUV5* promoter; Km^r	27
pCIPblaM	pYZ4 derivative carrying *blaM* gene under the control of the *lacUV5* promoter; for details, see text	This study
pCIPFus1 to pCIPFus16	pCIPblaM derivatives carrying *ampG-blaM* hybrid; for details, see text	This study

Bacterial strains, plasmids, DNA methods, and growth conditions. The strains and plasmids used in this study are listed in Table 1. For cell selection, kanamycin and chloramphenicol were used at 50 and 30 μg ml⁻¹, respectively. Ampicillin was used at 100 μg ml⁻¹ for PCR-Script SK selection.

Standard procedures for DNA manipulations and cell transformation were used (24). Small- and medium-scale preparations of plasmids from *E. coli* were performed by using a GFX Micro Plasmid Prep kit (Amersham Pharmacia Biotech) and NucleoBond AX (Macherey-Nagel), respectively. PCR fragments were purified by agarose gel electrophoresis. The fragments of interest were recovered from agarose gel by using a GFX-PCR kit (Amersham Pharmacia Biotech). The integrity of the fragment generated by PCR was verified by nucleotide sequencing by the dideoxy chain termination method and with the help of an ALFexpress DNA sequencer (Amersham Pharmacia Biotech).

A 1,130-kb DNA fragment containing the *blaM* coding region was PCR amplified with the pJBS633 plasmid as template and two primers, blaM_Up (5′TAA **GGA TCC** TGA GAG CTC CGT CAC CCA GAA ACG3′) and blaM_Down (5′CTA **GAA TTC** GGT ATC TGC GCT CTG CTG AA3′) (the first codon of the *blaM* coding region is underlined, and BamHI, SacI, and EcoRI sites are in boldface), designed to introduce flanking BamHI-SacI and EcoRI sites. The amplified fragment was cloned into PCR-Script SK-Cm plasmid. After the amplified sequence was checked by DNA sequencing, the BamHI- and EcoRI-digested fragment was subcloned into the corresponding sites of the pYZ4 plasmid, generating pCIPblaM. In this construct, the *blaM* gene is not expressed but its 5′ end is flanked by NcoI, BamHI, and SacI unique restriction sites (Fig. 1).

The various forms of the *ampG* gene truncated at the 3′ end were amplified by PCR from pGKS171-3 with the oligonucleotides listed in Table 2 as lower primers. The AmpG-UP oligonucleotide (5′-TAA**CCATGG**CCAGTCAATATT TACGTATTTTTCAA-3′), used as the upper primer for the 5′ end of the gene in all amplifications, introduced an NcoI site (shown in boldface type) containing the initiation codon of *ampG*. The Fus₁ampG to Fus₁₄ampG oligonucleotides, used for lower primers, introduced a SacI site at the 3′ end of the amplified fragment. The generated DNA fragments were purified and then cloned with PCR-Script SK-Amp. After its DNA sequence was checked, the recombinant plasmid was cut with both NcoI and SacI enzymes and then inserted between the same sites of the pCIPblaM vector. For fusions 2 to 14 (pCIPFus2 to pCIP-Fus14), the in-frame insertion resulted in the expression of the truncated form of *ampG* fused to the mature form of the *blaM* β-lactamase via short intermediate peptide linkers originating from the creation of SacI site. For fusion 1 (pCIP-Fus1), the full length of the *ampG* gene was fused in frame to the *blaM* gene. In

each case, *E. coli* XL1 Blue MR transformants containing the recombinant plasmids were selected for growth in the presence of kanamycin (50 μg ml⁻¹). The constructs were confirmed by restriction analysis and DNA sequencing of the in-frame fusions after isolation of the plasmids. The *ampG-blaM* junctions were sequenced with the fluorescent primer BlaMfus, 5′-CY5-TACCGCTGTT GAGATCCAGTTCGAT-3′.

Topological analysis and ampicillin resistance of cells expressing β-lactamase fusion proteins. To probe the presence of the β-lactamase moiety outside or inside the cell, the method of Broome-Smith et al. (2) was used. In this method, a single cell producing a periplasmic β-lactamase fusion can survive and form a colony on an agar plate containing 10 μg of ampicillin ml⁻¹, 50 μg of kanamycin ml⁻¹, and 1 mM IPTG (isopropyl-β-ᴅ-thiogalactopyranoside), whereas a cell producing a cytoplasmic β-lactamase fusion is ampicillin sensitive (single cell screening). On the contrary, when a clone producing a cytoplasmic β-lactamase moiety is plated at high density (i.e., "toothpicked"), it does grow (patch screening). Indeed, in the latter case, ampicillin-induced lysis releases β-lactamase into the medium, where it hydrolyses the ampicillin and thus enables the remaining cells to survive. In patch screening, colony growth is independent of the location, cytosolic or periplasmic, of the β-lactamase moiety and indicates the production of functional AmpG-β-lactamase hybrid.

The ampicillin resistance of individual cells of *E. coli* XL1 Blue MR harboring the pCIPFusx plasmids was determined by plating appropriate dilutions of exponential phase cultures onto Luria-Bertani (LB) agar plates containing 0, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, and 200 μg of ampicillin ml⁻¹. The MIC of ampicillin was estimated as the lowest inhibitory concentration that inhibits cell growth.

Cellular compartments and β-lactamase activity. The periplasmic, cytoplasmic, and membrane fractions of the cells were obtained by lysozyme treatment as described by Lindström et al. (17). β-Lactamase activity was determined by using nitrocefin as substrate (21).

RESULTS AND DISCUSSION

Prediction of transmembrane segments and construction and analysis of *ampG-x-blaM* fusions. A hydrophobic moment plot analysis of the AmpG polypeptide chain by the method of Eisenberg et al. (7) highlighted 14 hydrophobic segments (TS₁

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           NcoI    BamHI    SacI
5′ GGTTTTTACCATGGGGGATCCTGAGAGCTCCGTCACCCAGAAACG3′
                             E  L  R  H  P  E  T
                        Mature TEM β-lactamase
```
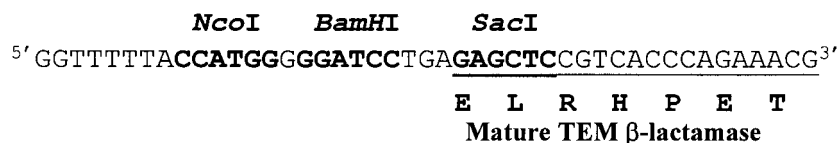
FIG. 1. Sequence data related to plasmid pCIPblaM. A DNA fragment containing the *blaM* coding region was PCR amplified with pJBS633 as template and with two primers designed to introduce flanking BamHI-SacI and EcoRI sites. The amplified fragment was cloned into PCR-Script SK-Cm plasmid. After the amplified sequence was checked by DNA sequencing, the BamHI- and EcoRI-digested fragment was subcloned into the corresponding sites of the pYZ4 plasmid, generating pCIPblaM. The *blaM* gene is not expressed in this construct, but its 5′ end is flanked by NcoI, BamHI, and SacI unique restriction sites, shown in the sequence in boldface type. For more information, see the text.

TABLE 2. Oligonucleotides used for AmpG-TEM hybrid production[a]

Fusion name	Nucleotide position	Fusion position	Sequence
Fus1ampG	C1473	T^{491} ↓	5′TTA **GAG CTC** CGT CAG ATG CGT TTT TCG TAG C3′
Fus2ampG	A1371	S^{457} ↓ FLP	5′ AA**G AGC TC**A GAG AAG TGC GTC AAA TCC AGC G3′
Fus3ampG	T1260	A^{420} ↓ YPA	5′AA**G AGC TC**G GTA CGG GAG ATA AAG TTG TCA TTT ACT CG3′
Fus4ampG	G1140	G^{380} ↓ WST	5′AA**G AGC TC**G CCG TGT GCT TCA ACA AAC CAA CCC3′
Fus5ampG	A1056	T^{352} ↓ QFA	5′TTA **GAG CTC** AGT AGC GGA AAA TGA CTT A TT ACA TAG CG3′
Fus6ampG	G966	A^{322} ↓ VFF	5′TTA **GAG CTC** GGC TGC GCCCAT GCT GTA GAG3′
Fus7ampG	G861	S^{287} ↓ LFR	5′TAG **AGC TC**T GAC AGG CGC TGC ATC AAA ATC3′
Fus8ampG	A774	G^{258} ↓ EVG	5′TTA **GAG CTC** ACC CGC ATC AAA CCC GAC GCC3′
Fus9ampG	G600	T^{200} ↓ IPV	5′TTA **GAG CTC** GGT GTC GGT TGG TTC TGG TGC3′
Fus10ampG	G516	G^{172} ↓ WQG	5′TTA **GAG CTC** GCC CAG CCA TTT ATC TGC CAG3′
Fus11ampG	G516	T^{133} ↓ DVL	5′TTA **GAG CTC** GGT TTT CCA CGC ATC GAA G3′
Fus12ampG	T318	Q^{106} ↓ LRW	5′TTA **GAG CTC** TTG GGT GCC TGG TTC GAG AAA3′
Fus13ampG	A222	P^{74} ↓ PFF	5′TTA **GAG CTC** AGG CGT GTA GCG GTC CAT CAG3′
Fus14ampG	C126	E^{42} ↓ NID	5′TTA **GAG CTC** GAC CGT CAT CAA GGC CTG3′
Fus15ampG	A459	R^{153} ↓ LGM	5′TTA **GAG CTC** ACG GTA ACC CAG CAC GCT GAT3′
Fus16ampG	G558	P^{186} ↓ CII	5′TTA **GAG CTC** GGG GAT CAA CAG TGC CGC CAT3′

[a] The SacI restriction sites are shown in boldface type.

to TS$_{14}$), which might be 14 putative transmembrane segments (for details, see Fig. 2 and Materials and Methods).

To determine the membrane topology of AmpG deduced from the hydrophobic properties of the polypeptide chain (see above), 14 directed fusions, *ampG*-x-*blaM*, were created between a truncated *ampG* gene generated by PCR and the *blaM* gene encoding a leaderless TEM β-lactamase. To obtain *ampG*-x-*blaM* hybrids, the pCIPblaM expression plasmid (a derivative of pYZ4) (27) was constructed. It allows an in-frame *blaM* cloning of a PCR-amplified *ampG*-x fragment containing NcoI and SacI restriction sites at its 5′ and 3′ ends, respectively (for details, see Materials and Methods).

Fusion sites (Fig. 2) were chosen to be located in the middle of the putative cytoplasmic or periplasmic loops (F2 to F14) (Fig. 2) connecting hydrophobic segments deduced by hydrophobic moment plot analysis or at the C-terminal end of AmpG (F1). *E. coli* XL1 Blue MR harboring pCIPFusx-derivative plasmids (pCIPFus1 to pCIPFus14, Table 1) and carrying *ampG*-x-*blaM* hybrids was selected for its ability to grow on LB agar plates containing 50 μg of kanamycin ml^{-1}. The expression of the *ampG*-x-*blaM* hybrids in these recombinant *E. coli* cells was highlighted by their ability to grow when patched onto LB agar containing 10 μg of ampicillin ml^{-1}, 50 μg of kanamycin ml^{-1}, and 1 mM IPTG. Indeed, under these conditions, ampicillin resistance was independent of the localization (cytosolic or periplasmic) of the β-lactamase moiety (2). All of the

fusions constructed conferred ampicillin resistance in patch screening, thereby clearly indicating that they all expressed *ampG*-x-*blaM* fusions.

To assess the cytoplasmic or periplasmic location of the β-lactamase moiety, the MIC of ampicillin for single cells (single cell screening) was determined by plating appropriate dilutions of exponential cell cultures on LB agar plates containing 10 μg of ampicillin ml^{-1}, 50 μg of kanamycin ml^{-1}, and 1 mM IPTG. Under these conditions, the failure to confer ampicillin resistance indicates that the BlaM moieties of the fusions are cytoplasmic. In contrast, the BlaM moieties of the fusion proteins that conferred ampicillin resistance are expected to be translocated across the bacterial cytoplasmic membrane and exposed in the periplasm. This was the case for AmpG-x-BlaM fusions at amino acids S^{457}, A^{322}, G^{258}, Q^{106}, and E^{42}, which allowed growth on plates containing an ampicillin concentration of ≥40 μg ml^{-1} (Table 2). The fusions at amino acids T^{491}, A^{420}, G^{380}, T^{352}, S^{287}, T^{200}, G^{172}, T^{133}, and P^{74} did not confer ampicillin resistance and were considered to have a cytoplasmic β-lactamase moiety (Table 2). In these cases, the ratio of the cytoplasmic to periplasmic β-lactamase activity ranged from 5/1 to 50/1 and the periplasmic activity was indeed much too small to confer sufficient ampicillin resistance. In addition, part of the so-called periplasmic activity might be due to a small proportion of the cell lysis during the separation of the various cellular compartments.

TABLE 3. AmpG-TEM hybrid characterization for antibiotic single cell resistance and determination of AmpG topology[a]

Characteristic	Fusion															
	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12	F13	F14	F15	F16
Amino acid position	T^{491}	S^{457}	A^{420}	G^{380}	T^{352}	A^{322}	S^{287}	G^{258}	T^{200}	G^{172}	T^{133}	Q^{106}	P^{74}	E^{42}	R^{153}	P^{186}
Km^R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Km^r + Amp^r + IPTG	S	R	S	S	S	R	S	R	S	S	S	R	S	R	S	S
MIC (μg/ml)	10	60	10	10	10	50	10	90	10	10	10	40	10	>200	10	10
β-lactamase moiety location	In	Out	In	In	In	Out	In	Out	In	In	In	Out	In	Out	In	In

[a] All AmpG-TEM fusions grew on LB agar plates supplemented with kanamycin, ampicillin, and IPTG when they were replicated in patch. This result indicates that the recombinant *E. coli* cells express the *ampG*-x-*blaM* in-frame fusion (for details, see text). The final concentration of IPTG was 1 mM. Km^r, kanamycin resistant (50 μg/ml); Amp^r, ampicillin resistant (10 μg/ml); R, resistant; S, sensitive; MIC, ampicillin MIC for *E. coli* recombinant cells induced by IPTG; In, β-lactamase domain exposed in the cytoplasm; Out, β-lactamase domain exposed in the periplasm.

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        10              30          F14      50
                      TS1                 ↓
MSSQYLRIFQQPRSAILLILGFASGLPLALTSGTLQAWMTVENIDLKTIGFFSLVGQAYV          TS2
    ├MMMMMMMMMM — M ■┤                              ┤M ■ MMMM — M ■

TS2      70       F13                F12    110   TS4
       ↓                 90  TS3        ↓
FKFLWSPLMDRYTPFFFGRRRGWLLATQILLLVAIAAMGFLEPGTQLRWMAALAVVIAFC
■┤ MM              ├MMMMMMTTTTMMMM┤          ┤MMTTTMTM ■

TS4  130    F11              F15          F10    TS6
       ↓                 150        ↓           ↓
SASQDIVFDAWKTDVLPAEERGAGAAISVLGYRLGMLVSGGLALWLADKWLGWQGMYWLM
                          M    *      ├MMMMM┤        ┤■MM ■ MMT

    F16          F9
TS6 ↓ 190              210              230 TS7
AALLIPCIIATLLAPEPTDTIPVPKTLEQAVVAPLRDFFGRNNAWLILLLIVLYKLGDAF
├TTTTMMMMMMMMMM ■┤        S              ├MTTMMMMMMM ■┤

             F8                      F7
     250          270  TS8        ↓ 290  TS9
AMSLTTTFLIRGVGFDAGEVGVVNKTLGLLATIVGALYGGILMQRLSLFRALLIFGILQG
    MM              M         *├MMMMMMMMM┤    ┤MMMM ■ MMM ■┤

             F6
TS9  310              330  TS10        350  F5
ASNAGYWLLSITDKHLYSMGAAVFFENLCGGMGTSAFVALLMTLCNKSFSATQFALLSAL
├┤                  MM  M    ┤TMMMMMMM ■┤            MMS  ↓ F3
                         F4
     370 TS11             390 TS12          410
SAVGRVYVGPVAGWFVEAHGWSTFYLFSVAAAVPGLILLLVCRQTLEYTRVNDNFISRTA
S              ├MMM ■ M ■┤  ┤MMMMMMMMM — TMM ■┤    F2

     430 TS13        450              470 TS14
YPAGYAFAMWTLAAGVSLLAVWLLLLTMDALDLTHFSFLPALLEVGVLVALSGVVLGGLL
├M — TMTT — MMMMMMMTTTMTTMMMM — ┤  ┤■M ■ MMMMMMTMMMTTTTTMMMMM

          F1
TS14  490  ↓
DYLALRKTHLT
├────┤
```
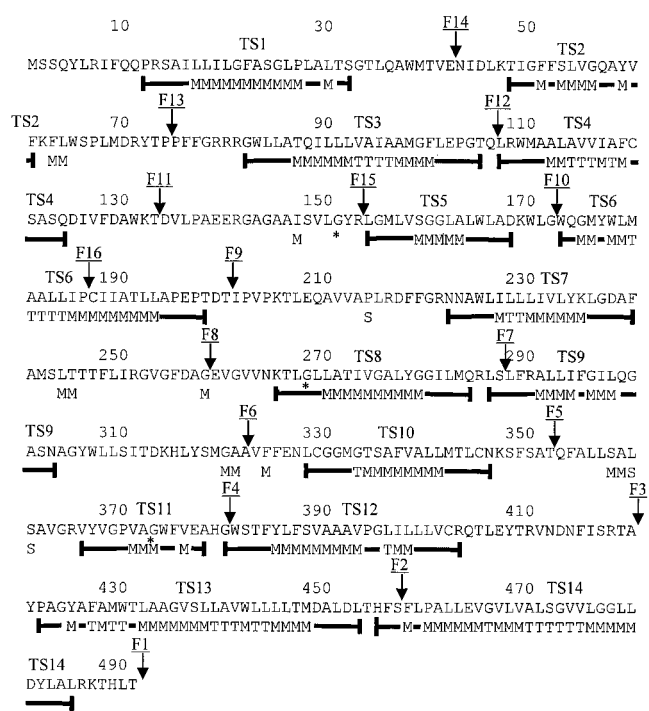
FIG. 2. Prediction of *E. coli* AmpG transmembrane segments. Eisenberg prediction symbols T, M, and S represent monomeric transmembrane segments, multimeric transmembrane segments, and amphiphilic α helices, respectively. These symbols are placed at the positions corresponding to the centers of the sliding spans of 11 residues used to perform the prediction. The segments corresponding to the thick lines under the amino acid sequence and including the Eisenberg symbols represent the length of the predicted transmembrane segment by the following rule: three consecutive M or T symbols are required for a nucleus of transmembrane segment. TSx represents hydrophobic segments deduced from the Eisenberg analysis. Arrows indicate the positions of AmpG-Bla M fusions (for details, see Table 2 and the text). The Gly-Asp substitutions in the three characterized AmpG mutants yielding a noninducible β-lactamase phenotype are marked by asterisks (for details, see the text and reference 19).

Topology of AmpG. The AmpG topological model derived from ampicillin resistance data contains 10 transmembrane hydrophobic segments: TS_1, TS_2, TS_3, TS_4, TS_7, TS_8, TS_9, TS_{10}, TS_{13}, and TS_{14} (Fig. 3) which delimit six cytoplasmic

hydrophilic domains, including the N- and C-terminal ends, and five periplasmic hydrophilic domains. In this model, the putative transmembrane segments TS_5, TS_6, TS_{11} and TS_{12} are not included in the membrane and are located in the cytoplasm. Except for BlaM fusions F5 (T^{352}) and F10 (G^{172}), the BlaM fusions were in agreement with the transmembrane segments predicted by Eisenberg analysis (see above). The presence of prolyl residues in the middle of the hydrophobic segments TS_6 and TS_{12} (Pro^{186} and Pro^{394}, Fig. 2) suggests the existence of a truncated α helix or of a turn in these hydrophobic segments. By inserting a β turn in these hydrophobic segments, another possibility for the polypeptide chain to cross the membrane would be expected if it adopts a β-stranded structure. In this case, a hydrophobic segment would give rise to two hydrophobic β strands. To probe this hypothesis, we have included the transmembrane segments TS_5 and TS_{11} in this study, although they do not contain a prolyl residue in their sequence, because the four hydrophobic segments, TS_5, TS_6, TS_{11}, and TS_{12}, would generate eight hydrophobic β strands which could be associated in the membrane to form a β-barrel structure. To rule out the hypothesis of the presence of β-stranded segments, two additional *blaM* fusions were generated at the positions of the putative β turns in TS_5 and TS_6 (fusions F15 and F16, Table 2). The resulting fusions were ampicillin sensitive when they were grown as single cells, indicating that the fusion points (R^{153} and P^{186}) were located in the cytoplasm. No additional fusions have been performed with TS_{11} and TS_{12}, because they share similar hydrophobic properties with TS_5 and TS_6. In conclusion, the final model for AmpG topology is that derived from ampicillin resistance data (Fig. 3).

The transmembrane topology deduced from the TEM β-lactamase fusions shows that the AmpG N and C termini are exposed in the cytoplasm and that the number of transmembrane segments must be an even number. F_3, F_4, F_9, and F_{10} fusions exclude the TS_5, TS_6, TS_{11}, and TS_{12} segments as transmembrane α helices. These results are in contradiction with those obtained by the Eisenberg analysis for the prediction of the transmembrane segments. Before we discuss the reasons that these four hydrophobic segments are not embedded in the cytoplasmic membrane, we note that it was unexpected that these four segments could be grouped by pairs according to their length and their hydrophobicity. Indeed, TS_5 and TS_{11} contain 14 and 13 residues, respectively, whereas TS_6 and TS_{12}
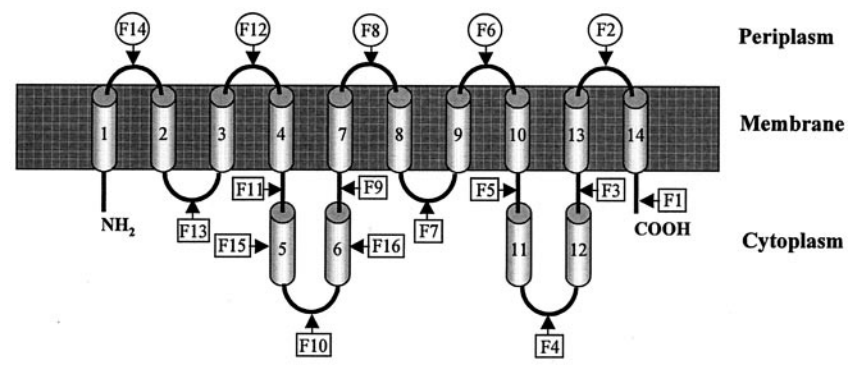

FIG. 3. Proposed topological model of *E. coli* AmpG protein. Arrows indicate the positions of AmpG-BlaM fusions that have been constructed (for details, see Table 2 and Fig. 2). Digits represent hydrophobic segments deduced from the Eisenberg analysis.

contain 26 and 24 residues, respectively. For the first pair, the length of the α helices is probably too short to cross the cytoplasmic membrane if we accept that a minimum length of 18 residues is required to do so. In our prediction, TS_2 also contains 14 residues. However, the hydrophobicity of TS_2 is higher than that of TS_5 and of TS_{11}. The two hydrophobic segments for the second pair, TS_6 and TS_{12}, contain a central prolyl residue. Though prolyl residues are frequently found in transmembrane segments, in the case of AmpG these prolyl residues could act as α-helix breakers and give rise to α helices too short to span the membrane. On the other hand, if TS_5 and TS_{11} are not in the membrane and if TS_6 is a transmembrane segment, TS_{11} and points of fusion F4 and F5 would be exposed in the periplasm, which contradicts results of the TEM-β-lactamase fusion experiments. Finally, in the topological model proposed in Fig. 3, the positive-inside rule of von Heijne (26) is respected for the orientation of transmembrane α helices (data not shown). Out of the 10 transmembrane segments previously predicted by Lindquist et al. (16), 8 are in agreement with our topological model and correspond to TS_1, TS_3, TS_4, TS_6, TS_7, TS_8, TS_{10}, TS_{12}, TS_{13}, and TS_{14}. The two remaining transmembrane segments predicted by these authors were TS_6 and TS_{12} and have been eliminated in our topological model and replaced by the TS_2 and TS_9 transmembrane segments. The same authors also reported three AmpG mutants yielding a noninducible phenotype. The mutations are identical Gly-Asp substitutions located at positions 151, 268, and 373, respectively (Fig. 2). Interestingly, the G151D and G373D mutations are located in two large cytoplasmic domains of AmpG and probably highlight the importance of these cytoplasmic domains in the AmpG transport mechanism. The last mutation, G268D, is located at the beginning of the transmembrane segment TS8 and probably destabilizes the transmembrane scaffolding.

A recent study of the AmpG permease substrate specificity and mechanism of transport shows that carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) prevents the uptake of GlucNAc-anhydro-MurNAc or GlucNAc-anhydro-MurNAc-tetrapeptide by AmpG (4). This inhibition by CCCP suggests that the AmpG permease is a single component permease dependent on the proton motive force as demonstrated for some members of the sugar transport system belonging to the oligosaccharide-H^+ symporter family (22). With respect to the transport mechanism of AmpG, the two large cytoplasmic loops containing TS_5, TS_6, TS_{11}, and TS_{12} may be involved in a scissors-type mechanism similar to that envisioned for lipid flipping by the *E. coli* MsbA ABC transporter (3) or in a swinging rearrangement as proposed for the *E. coli* BtucD ABC transporter (18).

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