Topology of the Membrane Protein LamB by Epitope Tagging and a Comparison with the X-Ray Model

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We previously developed a genetic approach to study, with a single antibody, the topology of the outer membrane protein LamB, an Escherichia coli porin with specificity towards maltodextrins and a receptor for bacteriophage lambda. Our initial procedure consisted of inserting at random the same reporter epitope (the C3 neutralization epitope from poliovirus) into permissive sites of LamB (i.e., sites which tolerate insertions without deleterious effects on the protein activities or the cell). A specific monoclonal antibody was then used to examine the position of the inserted epitope with respect to the protein and the membrane. In the present work, we set up a site-directed procedure to insert the C3 epitope at new sites in order to distinguish between two-dimensional folding models. This allowed us to identify two new surface loops of LamB and to predict another periplasmic exposed region. The results obtained by random and directed epitope tagging are analyzed in light of the recently published X-ray structure of the LamB protein. Study of 23 hybrid LamB-C3 proteins led to the direct identification of five of the nine external loops (L4, L5, L6, L7, and L9) and led to the prediction of four periplasmic loops (I1, I4, I5, and I8) of LamB. Nine of the hybrid proteins did not lead to topological conclusions, and none led to the wrong predictions or conclusions. The comparison indicates that parts of models based on secondary structure predictions alone are not reliable and points to the importance of experimental data in the establishment of outer membrane protein topological models. The advantages and limitations of genetic foreign epitope insertion for the study of integral membrane proteins are discussed.

Determination of the structure of a protein by X-ray crystallography is an essential step in the study of its functional organization. In the case of membrane proteins, this determination proves especially difficult, and only a few structures have been established (reviewed in reference 31). In the absence of direct structural data, topological models for membrane proteins may be derived from a combination of approaches, including sequence-based predictions and genetic, biochemical, or immunological methods (reviewed in reference 19).

In our laboratory, we developed a genetic approach which relies on a simple principle. The method consists of insertion of a short reporter peptide corresponding to a linear epitope into permissive sites of the protein. By definition, such sites tolerate insertions without loss of all biological properties of the protein and therefore without major changes in its structure and cellular location (5, 8) (reviewed in reference 21). The hybrid proteins generated are then probed with a monoclonal antibody (MAb) specific for the reporter epitope (8, 11). In addition to providing topological information, this method gives indications about the regions responsible—or irrelevant—for the functions of the protein. The method has now been used for the study of a number of other bacterial outer membrane proteins (1, 26, 37).

In gram-negative bacteria, the outer membrane serves as an efficient permeability barrier that protects the cell against harmful compounds, such as antibiotics, disinfectants, and detergents (30). It contains a number of transport proteins that mediate the passage of a limited range of solutes. One important class is constituted by porins which facilitate and regulate the entry of small (<600 Da) hydrophilic molecules (29). In the last few years, the X-ray structures of several such general porins have been established. They are integral membrane proteins organized as homotrimers. Each subunit consists of a barrel of 16 consecutive antiparallel β-strands. This organization was found in the crystal structures of the general porins of Rhodobacter capsulatus (40), Rhodopseudomonas blasica (24), and Escherichia coli K-12 (14).

More recently, the three-dimensional structure of LamB was solved at a 3.1-Å (1 Å = 0.1 nm) resolution by X-ray crystallography (34). LamB, like general porins, allows passive diffusion of small hydrophilic molecules across the outer membrane. In addition, LamB constitutes a substrate-specific porin which facilitates the diffusion of maltose and maltodextrins, hence its name, maltoporin (16, 17). LamB also serves as a specific cell surface receptor for a number of bacteriophages, including phage lambda, hence its other name, lambda receptor (see references in reference 12). The active form of LamB is a homotrimer (25, 27, 28), with each monomer containing 421 amino acids (13).

Genetic, immunological, biochemical, and functional studies led to a two-dimensional (2D) folding model in which the LamB monomer spans the membrane through β-strands with loops or turns protruding on either side of the lipid bilayer (named here as model 1 [Fig. 1A]) (9). More recently, a 2D folding model was proposed on the basis of the X-ray structure of the porins OmpF and PhoE (named here as model 2 [Fig. 1A]) (33). All of these models predicted 16 transmembranous

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β-strands. The X-ray analysis confirmed that, although LamB is not homologous in amino acid sequence to the general porins, it shares their common overall structural organization of three water-filled β-barrels. However, unlike all of the general porins, the LamB barrels are constituted of 18 antiparallel β-strands instead of 16 (Fig. 1B) (20, 34).

We previously used a random two-step foreign epitope insertion approach to probe the topology and functions of LamB (10, 12). In the first step, we looked for potential permissive sites by random insertion of a BamHI linker into the lamB gene and by screening for stable, nontoxic mutated LamB proteins with remaining biological activities. In a second step, we determined which mutant proteins could accept further genetic insertion of a peptide of 11 residues encoding a well-studied linear epitope, the C3 epitope from poliovirus, for which a neutralizing antibody was available (22, 42). Most mutant proteins could accept this further insert without loss of both LamB activities, showing that the corresponding sites were indeed permissive and that the hybrid proteins were reflecting the structure of wild-type LamB. The localization of the C3 epitope with respect to the LamB-C3 hybrid and with respect to the outer membrane was then examined.

In the present work, we pursued this analysis by using site-directed epitope insertion based on the differences between 2D models 1 and 2. We chose 10 new sites in LamB, located in regions of the protein for which little or no genetic, biochemical, or immunological information was available and which had been positioned with respect to the membrane essentially.

**FIG. 1.** Localization of C3 epitope insertions. (A) Localization on the 2D model (model 1) proposed in reference 11. The 421 amino acid residues are oriented from the left (amino terminus) to the right (carboxyl terminus). Rectangles correspond to residues predicted in β-strands (the boundaries of the β-strands are indicated by small numbers); thin lines correspond to loops and turns. C3 epitope insertion sites are indicated by large numbers. Starting from the N terminus, the thick lines below the 2D model indicate the surface (dark grey) and periplasmic (light grey) loops or turn regions according to the X-ray model. The external loops of the X-ray model are indicated by an L followed by a number corresponding to the nomenclature of reference 34. The thick lines above the 2D model indicate the predicted surface (dark grey) and periplasmic (light grey) loops or turn regions on the 2D model proposed by Schirmer and Cowan (33) (model 2) on the basis of the general porin structure. The sites identified by random insertion (8, 11) are boxed; the sites chosen in the site-directed insertion approach are circled. Amino acid sites 153, 204, 253, 300, and 374, which identified external loops L4, L5, L6, L7, and L9, respectively, are framed in boxes or circles. (B) Localization on the 2D model derived from the crystal structure of LamB (34). The 421 amino acid residues are oriented from the left (amino terminus) to the right (carboxyl terminus). The barrel corresponding to a monomer has been flattened on the page. Thin rectangles correspond to residues in a transmembrane β-strand; thin lines correspond to loops and turns (the boundaries of the external loops and periplasmic loops or turns are indicated by small numbers). C3 epitope insertion sites are indicated by large numbers. As in panel A above, the sites identified by random insertion (8, 11) are boxed; the sites chosen in the site-directed insertion approach are circled. The external loops are indicated by an L followed by a number corresponding to the nomenclature of reference 34. Amino acid sites 153, 204, 253, 300, and 374, which identified external loops L4, L5, L6, L7, and L9, respectively, are framed in boxes or circles.
according to sequence-based predictions. The results obtained here and those obtained previously (10, 12) are discussed in the light of the X-ray structure of LamB, and the procedure of foreign epitope insertion for membrane protein topology determination is evaluated. LamB is one of the few membrane proteins—the most documented case to our knowledge—for which it becomes possible to compare the results and predictions of genetic epitope tagging with those of X-ray analysis.

**MATERIALS AND METHODS**

**Strains, media, and chemicals.** The LamB-negative strain pop6510 (?) (thr leu tonB thi lacY1 recA dx5 metA supE) was used as a final recipient for the lamB recombinants. Strain JM501 was used for infection with the recombinant M13mp18-lamB phages, after in vitro site-directed mutagenesis (see references in reference 12). Plasmid pAC1 is a pBR322 derivative harboring the wild-type lamB gene under p-tac-promoter control. *E. coli* AC1 is pop6510 harboring plasmid pAC1 (?) .

Phage λ with a wild-type host range (λh+) and its extended host range derivatives (λh0 and λhh+) were described earlier (see references in reference 12). The approximate efficiency of plating with respect to that on a lamB-positive strain was determined by spot tests on lawns of bacteria with 10⁻² to 10⁻⁸ dilutions of each phage stock. When a strain was sensitive to only host range mutants of λ, it was declared partially sensitive.

The media and chemicals used in this study were described previously (reference 11 and references therein). Utilization of maltodextrins by the LamB mutant strains was assessed on MacConkey solid medium, with 0.4% maltodextrins as a carbon source. Red colonies are able to utilize dextrins (Dex⁺ phenotype); colonies impaired in utilization of dextrins are white (Dex⁻ phenotype). Oligonucleotides were synthesized by Genset (Paris, France).

**Directed two-step insertion of the C3 epitope.** The 11-residue-long C3 neutralization epitope from poliovirus (residues 93 to 103 of VP1 capsid protein) was inserted into 10 sites of the LamB protein (corresponding to two natural and eight newly created unique restriction sites in lamB).

(i) Creation of unique BamHI restriction sites by in vitro mutagenesis. At each of the eight sites (after amino acids 27, 56, 77, 100, 204, 210, 221, and 300), a unique BamHI restriction site was first created by site-directed mutagenesis on a recombinant M13mp18-lamB phage (phase 12), so that the cohesive ends generated after cleavage by the restriction enzyme BamHI would all occur in the same phase with respect to the lamB sequence (phase 0). We designed eight oligonucleotides corresponding to specific sites of lamB incorporating a central BamHI site (underlined) in the same reading frame as follows:

- site 27,5': CCAGACTACGGTTCGGATCCTGCTCAAAGTAAATAC-3
- site 56,5': AAGAGGGCGATAAGTCGGATCCTAGCTTCTATTTCGAC-3
- site 77,5': ACTGGGAAGCTACCTCGGATCCTGATCCGGCCTTCC-3
- site 100,5': AGGCTCCACCATCTCGGATCCTTGGGCAGGTAAGC-3
- site 204,5': GATAACTATCGTCTGTCGGATCCTGTTGATGGCGCATC-3
- site 210,5': ATGGCGCATCGAAATCGGATCCTGACGGCTGGTTATT-3
- site 221,5': CTGAACATACTCAGTCGGATCCTAGTGTCCTGAAGG-3
- site 300,5': CTGGGATAACGACTCGGATCCTAACGGCACCAAGT-3

After in vitro mutagenesis, the linker-modified region of the lamB gene was transferred to the plasmid vector pAC1. For this purpose, DNA fragments were exchanged between replicative forms of the M13 recombinant phages and the pAC1 vector with FspI-NcoI double digestions for sites 27 to 124, ClaI-NcoI double digestions for sites 204 to 279, and NcoI-SmaI double digestions for sites 300 and 304, as previously described (12, 23). The recombinant pAC1 derivatives were finally introduced into pop6510 by electroporation.

(ii) In frame insertion of the C3 epitope into the unique BamHI restriction sites. The same double-stranded oligonucleotide encoding the C3 epitope was then independently inserted into each of the newly created BamHI sites. The two complementary oligonucleotides encoding the C3 epitope were 5'-GAT CCG GAT AAG CCG GCC TCG ACC ACT AAC GAT AAA AAG TAC CCC-3' (coding strand) and 5'-GAT TTT ATT CGG TGCC ACG TCG ACC ACT AAC GAT AAA AAG TAC CCC-3' (complementary strand). Each recombinant plasmid was first digested with BamHI, and then the linearized plasmid was purified from 5%
acrylamide gels by electrophoresis in Tris-borate-EDTA buffer (7). After cloning of the C3 double-stranded oligonucleotides and transformation into pop610, plasmid DNAs extracted from the recombinant clones obtained were screened by double digestions with the appropriate restriction enzymes. The selected constructions were further checked by DNA sequencing with the Sequenase kit (U.S. Biochemical Corp.) according to the manufacturer’s recommendations. For insertion of the C3 epitope at amino acid sites 279 and 304, we used naturally existing unique restriction sites (NcoI and DraIII, respectively) in the lamB gene. Two sets of double-stranded oligonucleotides encoding the C3 epitope with compatible protruding extremities were especially devised to allow in-frame insertion of C3 at these two sites. At the NcoI site, the two oligonucleotides encoding the C3 epitope were 5′-ATG GAT AAC CGC CGG TCG AGC AAC AAG GAT AAG CCT-3′ and 5′-CAT GAG GAT TTC TTC ATG GTG CTA CGA CGC CGG GTT ATC-3′. At the DraIII site, the two oligonucleotides encoding the C3 epitope were 5′-GTG TGA TAA CCC GGC GTG GAC CAC TAA CAA GGA TAA GTT CAA-3′ and 5′-AAC TTA TCC TTG TTA GTG GTC GAC GCC GGG GTG TTA CAC CAC TTG-3′. Immunological characterization of the LamB proteins. (i) Sera. Four different anti-LamB sera were used in this study: a mouse polyclonal antipeptide serum directed against residues 69 to 83 of the mature LamB monomer (41), a rabbit polyclonal anti-LamB trimer serum raised against purified LamB trimers (7), and two anti-LamB MAb’s (18) raised in mice against purified native LamB protein. The determinants recognized by the two MAb’s (surface exposed for E72 and periplasmic for I141) are conformational and belong, at least in part, to the COOH-terminal region of LamB (15). Two anti-poliovirus sera were used: a mouse polyclonal anti-C3 peptide antibody directed against residues 93 to 103 of the VP1 capsid protein from poliovirus type 1 and an anti-C3 MAb raised in mice against heat-denatured poliovirus particles and specifically directed against position 97 to 103 of the C3 epitope (8). (ii) Immunoblotting experiments. Linker insertion mutant proteins and LamB-C3 hybrid proteins were tested by Western blotting (immunoblotting) under denaturing conditions, with either anti-LamB peptide or anti-C3 peptide polyclonal antibodies. Heat-denatured bacterial extracts were prepared as described previously (12). The equivalent of 2.5 × 10^7 cells was applied per well in a sodium dodecyl sulfate (SDS)-10% polyacrylamide gel. (iii) Immunoprecipitations. Solubilized LamB trimers were detected with either polyclonal anti-LamB trimer or the anti-C3 MAb. Immunoprecipitations were performed under native conditions (70°C). Samples were prepared and tested as previously described (11). (iv) Colony blotting. Exposition of the C3 epitope at the surface of intact cells was tested by colony blotting with anti-C3 peptide serum. Single colonies were inoculated into ampicillin-containing plates and incubated overnight. Cells were transferred to a nitrocellulose filter, which was washed by vigorous agitation in phosphate-buffered saline (PBS) plus 1% gelatin (PBSG). The washed filter was incubated for 1 h with polyclonal anti-C3 antibody in PBSG at a final dilution of 1:2,000. The filter was washed five times with 50 ml of PBS-0.05% Tween 20, and anti-mouse immunoglobulin G-alkaline phosphatase (Bio-Rad, Richmond, Calif.) was added at a 1:1,000 final dilution in PBSG. After 1 h, the filter was washed as described above, and nitroblue tetrazolium-bromochlorindophenol phosphate substrate solution (Bio-Rad) was added. (v) ELISA. Enzyme-linked immunosorbent assays were performed with intact cells and with sonicated samples as described previously (7). Wells were coated with a bacterial cell suspension at an optical density at 600 nm of 0.5, either as whole cells or after sonication (three pulses of 15 s each). Anti-LamB MAb E72 (intact cells) or MAb 1141 (sonicated samples) was added at a final dilution of 1:2,000; anti-C3 MAb was added at a final dilution of 1:1,000. Strains AC1 (LamB-negative) and AC3 (LamB-positive) were included as controls for detection with anti-LamB MAb’s. Strain AIC264-VP1 (8) was used as a positive control for detection with the anti-C3 MAb. (vi) Cytosfluorometric analysis. Exposition of the C3 epitope at the surface of intact cells was further tested by cytofluorometry with the anti-C3 MAb Isopropy-β-D-thiogalactopyranoside (IPTG)-induced cells (10^7 M final dilution, 1 generation) were incubated (5 × 10^8 bacteria) with anti-C3 MAb (final dilution, 1:250) in 100 μl of PBS plus 1% bovine serum albumin. After 45 min, 1 ml of PBS was added, cells were centrifuged briefly (60 s) in the microcentrifuge, and fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (Diagnostik Pasteur, Paris, France) was added (at a final dilution of 1:50), and then the mixture was incubated for 45 min. After dilution of 1 ml of PBS cells were pelleted by centrifugation as described before, resuspended in 5 ml of PBS, and analyzed in a cytofluorometer (Cytotoolsorgraf IIS; Orthodiagnostic System, Inc.). Mean fluorescence for the whole population was recorded. Strain AC1 (LamB-negative) was used as a negative control, and AIC264-VP1 was used as a positive control (insertion of C3 after amino acid 153 [8]) for anti-C3 labelling.

RESULTS

In our previous publications, permissive and nonpermissive sites in LamB were identified by random two-step insertions of the C3 epitope (8, 10, 11). As recalled above, at permissive sites, the foreign epitope insertion preserves at least some of the biological activities of the protein, namely lambda receptor and malfactorol activities, ensuring at least some structural conservation, while at nonpermissive sites, the insertion leads to the complete loss of both LamB functions.

In the present paper, we used site-directed mutagenesis to insert the C3 epitope from poliovirus into 10 new sites of the LamB protein. These sites are located in regions of the protein for which little or no genetic, biochemical, or immunological information was available and/or for which 2D models (9) and 2 (33) gave conflicting predictions. Table 1 and Fig. 1 give details about the choice of the new insertion sites.

In a first step, a BamHI linker was inserted into eight sites in the lamB gene, leading to the in-frame addition of three amino acids (SDP) to the LamB sequence. (Sites were designated by the position of the preceding LamB residue in the amino acid sequence. The LamB mutants corresponding to linker insertions were named “SPA,” followed by the number corresponding to the site of insertion; the LamB-C3 hybrids were named “SPA,” followed by the site number and by C3). Insertion of the linker preserved both LamB activities in six cases (sites 57, 77, 204, 210, 221, and 300), which were thus potentially permissive, impaired both activities at site 27, and abolished both activities at site 100, which was thus nonpermissive (Tables 2 and 3). Western blotting experiments with anti-LamB peptide serum showed comparable levels of expression and the expected molecular weights for all of the mutant proteins (data not shown).

The next step consisted of genetic insertion of the C3 epitope into these eight sites, as well as into two natural restriction sites (sites 279 and 304). We will first describe the changes in functions and antigenic properties of LamB which give an idea of the structural modifications provoked by the insertion, and then we will present the data on the localization of the C3 reporter epitope with respect to the protein and with respect to the membrane, which directly reflect the topological organization of the LamB-C3 hybrids.

Characteristics of the LamB-C3 proteins. (i) Functionality of the proteins. The effects of C3 epitope insertions on the phage receptor and malfactoror function of LamB are summarized in Table 3. The six potential permissive sites indeed proved permissive, as did the two sites corresponding to natural restriction sites. More precisely, sites 56, 221, 300, and 304 maintained both activities; sites 204 and 210 specifically affected phage receptor activity; and site 279 was specific for

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<th>Table 1. Choice of new insertion sites in LamB in this study</th>
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<td>Amino acid site in LamB</td>
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<td>300</td>
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<td>304</td>
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* Prediction 1 according to the 2D model of LamB folding in reference 9.
* Prediction 2 according to the 2D model of LamB folding presented in reference 33.

New prediction according to site-directed insertions (this work). ?, not determined; n.p., nonpermissive site.
maltoporin function. Insertion at site 77 affected both functions, but only partially, so that it was considered partially permissive. Insertion at site 100 led to a complete loss of phage receptor activity but the protein maintained some ability to ferment maltodextrins, so that the site was labelled as weakly permissive. Insertions at site 100 led to a complete loss of both functions, so that it was considered nonpermissive.

(ii) Antigenic properties of the hybrid proteins. The expression of the LamB-C3 hybrid proteins was visualized by Western blotting of heat-denatured whole-cell extracts with two mouse polyclonal antibodies raised against synthetic peptides corresponding to LamB (peptides 63 to 83 of LamB) and to C3 (peptides 93 to 103 of the VP1 protein from poliovirus). (Sera are described in Materials and Methods.) The intensities of the bands detected with the anti-LamB peptide serum (Fig. 2A) were roughly comparable for all constructs, indicating overall similar amounts of LamB proteins present in the different mutants. Under the same conditions, with the anti-C3 peptide antiserum (Fig. 2B), all of the LamB-C3 hybrid proteins were also detected, but the intensities of the corresponding bands varied from very strong (for SPA279-C3 and SPA27-C3, for example) to very weak (for SPA100-C3 and SPA304-C3), indicating differences in anti-C3 peptide antibody binding even in denaturing conditions.

Immunoprecipitations of the solubilized LamB-C3 hybrid proteins were performed under native conditions with a rabbit polyclonal anti-LamB trimer antibody. Trimeric forms of the LamB hybrid proteins were detected in 8 out of 10 cases, that is, for all permissive sites (sites 56, 77, 204, 210, 221, 279, 300, and 304) (data not shown). In the case of hybrid SPA279-C3, detection was weak, and several discrete faster-migrating bands were apparent, suggesting degradation. Trimers were not detected by this technique for the two hybrids corresponding to insertions at site 27 (weakly permissive) and site 100 (nonpermissive).

Further tests of the effects of the C3 epitope insertion on the tertiary and quaternary structure of LamB were performed with two anti-LamB conformational MAbs by ELISA. MAb E72, directed against surface-exposed determinants of LamB, was used with intact cells, while MAb I141, directed against periplasmic determinants of LamB, was used with sonicated bacterial lysates.

For five permissive sites, hybrids SPA77-C3, SPA204-C3, SPA210-C3, SPA221-C3, and SPA304-C3, recognition by both MAbs was slightly affected or not affected (between 68 and 94% of wild-type recognition by E72 and between 64 and 100% of wild-type recognition by I141 [Table 3]), confirming that the insertions did not appreciably distort the structure of LamB.

C3 insertion at the other three permissive sites provoked some distortion detectable by changes in the conformational epitopes. At site 56, internal epitopes were more affected than the external ones. At site 279 (hybrid SPA279-C3), where the insert affected maltodextrin transport but not phage receptor activity, both external and internal epitopes were strongly modified, suggesting that this region might be part of the MAb target or indicating possible long-range effects with distortion of the protein. This explanation would also account for the degradation observed with this construct (described above). At site 300, both types of determinants were significantly altered (Table 3).

As expected, for hybrid SPA100-C3 (nonpermissive), there was essentially no recognition by either external or internal MAbs (8% of wild-type recognition by E72 and 17% of wild-type recognition by I141, while for the LamB-negative strain, these values were 5% and 13%, respectively [Table 3]). Surprisingly, hybrid SPA27-C3 (the weakly permissive site) was still, although poorly, detected by both the external and internal MAbs, indicating some conservation of the epitopes recognized by the MAbs and therefore a less drastic perturbation than that for SPA100-C3.

Localization of the C3 epitope. To determine the accessibility of the C3 epitope on the hybrids and its localization with respect to the membrane, we used the specific anti-C3 MAb. The accessibility of the C3 epitope on solubilized LamB-C3 was examined by immunoprecipitation under native conditions (Fig. 3) and on sonicated bacterial lysates by ELISA (Table 4).

Immunoprecipitation experiments allowed the detection of trimeric forms of LamB for the 10 LamB-C3 hybrids, but with various efficacies; the intensities of the bands corresponding to the LamB-C3 trimers detected were variable. Hybrids SPA56-C3, SPA77-C3, SPA204-C3, SPA210-C3, and SPA221-C3 were well recognized, while weak recognition was observed for hybrids SPA27-C3, SPA279-C3, and SPA304-C3, and very weak recognition was observed for SPA100-C3 and SPA300-C3 (Fig. 3). This disparity could likely be due to differences in the accessibility of or in the constraints exerted on the insert (3, 11).

For the inserts at site 100 (nonpermissive), a fast-migrating

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**TABLE 2. Localization of the 23 insertion sites**

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<tr>
<th>Amino acid site in LamB&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Deduced location&lt;sup&gt;b&lt;/sup&gt;</th>
<th>3D structure&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Loop/turn identified&lt;sup&gt;d&lt;/sup&gt;</th>
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<td>Direct</td>
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<td>27</td>
<td>?</td>
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<tr>
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<td>β-Strand (57–68)</td>
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<td>352</td>
<td>Pred. periplasm</td>
<td>Periplasm (352–361)</td>
<td>I8</td>
</tr>
<tr>
<td>368</td>
<td>? (n.p.)</td>
<td>β-Strand (352–374)</td>
<td></td>
</tr>
<tr>
<td>374&lt;sup&gt;*&lt;/sup&gt;</td>
<td>External</td>
<td>β-Strand (362–374)</td>
<td>L9</td>
</tr>
<tr>
<td>399</td>
<td>Pred. external</td>
<td>External (375–405)</td>
<td>L9</td>
</tr>
</tbody>
</table>

<sup>a</sup> Sites chosen for site-directed insertions (Direct [this work]) or identified by random insertions (Random [8, 11]). Asterisks indicate sites located in the crystal structure very close to the correctly predicted region as follows: site 146, upstream surface loop L4 (149 to 166); site 221, upstream periplasmic loop I5 (224 to 225); site 236, upstream surface loop L6 (239 to 265); and site 374, upstream surface loop L9 (375 to 405).

<sup>b</sup> ?, not determined; n.p., nonpermissive site; External, cell surface exposed as shown by cell surface detection of C3; Pred. external, predicted to be cell surface exposed according to phenotypic characteristics; Pred. periplasm, predicted to be in the periplasm according to phenotypic characteristics of the mutant, including C3 antigenicity.

<sup>c</sup> Loop or turn identified according to the 3D structure (34). L, external loop; I, periplasmic turn or loop. The number after L or I indicates the order of the regions as they appear on the primary sequence from the N terminus to the C terminus (Fig. 1).

<sup>d</sup> Location of the insertion sites on the crystal structure of LamB (34). Numbers in parentheses indicate the boundaries of the secondary structure region comprising the site.
form of the SPA100-C3 protein was detected, suggesting proteolysis or abnormal oligomerization (Fig. 3). For weakly permissive site 27, the fact that trimers were detected with the anti-C3 MAb is in agreement with the observation that the conformational LamB antibodies still bind to the SPA27-C3 protein (Table 3) and with the fact that the corresponding strain was still able to weakly ferment maltodextrins. With SPA100-C3 and SPA27-C3, trimers were not detected with the anti-LamB trimeric serum, suggesting a lower level of sensitivity of this assay (unpublished results).

Detection of the C3 epitope on intact cells was assayed in three ways: by ELISA with the anti-C3 MAb, by cytofluorometry, and by colony blotting with the anti-C3 peptide polyclonal antibody (Table 4). Significant signals with all three methods were obtained with C3 inserted at sites 204 and 300, the strongest signals being obtained with site 204. ELISA and cytofluorometry appeared more sensitive than colony blotting and also yielded positive signals at sites 210 and 304 (Table 4).

These data demonstrated unambiguously that the regions of residues 204 and 300 are facing outside the cell. The data also indicated that the insertions at sites 210 (downstream of 204) and 304 (downstream of 300) allow cell surface expression of the C3 epitope.

We expected that by ELISA with sonicated bacterial lysates, in addition to the sites which allowed detection of the C3 epitope on intact cells, sites corresponding to periplasmic loops or turns of the protein would allow detection of the C3 epitope. This was the case for sites 27, 56, and 221 (Table 4). The two cell surface-exposed sites at positions 204 and 300 gave a positive signal in this assay (63 and 34%, respectively). Unexpectedly, the two other sites at positions 210 and 304, although they gave a positive signal by ELISA on intact cells, did not yield a significant signal with sonicated bacterial lysates. This may be due to changes in the conformation of the hybrid proteins upon fragmentation of the envelope.

The fact that at site 27, as well as at sites 56 and 221, the epitope was recognized by the anti-C3 antibody on sonicated bacterial lysates but not on intact cells is compatible with the idea that these three sites correspond to surface regions of the LamB trimer, either facing the periplasm or partially hindered by the membrane on intact bacteria (see Discussion).

**DISCUSSION**

**Site-directed insertion of the C3 epitope.** Site-directed insertion of the C3 epitope allowed us to identify two new sur-
faceloops of LamB (L5 and L7) and to predict a new periplasmic region (I1) and provided indirect topological and functional information about other regions. We will discuss the results obtained at each new insertion site going from the NH2 terminus to the COOH terminus of LamB. Among the 10 insertion sites, only one proved strictly nonpermissive. Upon C3 insertion at site 27, both LamB activities were affected (phage receptor function was abolished, and utilization of maltodextrins was impaired), so the site was declared weakly permissive. The C3 insertion still allowed the detection of trimers (Fig. 3 and Table 4), the C3 epitope was clearly detected on membrane fragments, and there was residual recognition by the conformational MAbs (Table 3). These properties show that the insert does not abolish folding and assembly of the hybrid protein and confirm that this site is weakly permissive. At any rate, these results do not discriminate between model 1 and model 2 (Fig. 1A). On the X-ray structure, site 27 lies in the middle of the first cell surface-exposed loop, L1 (residues 16 to 39 [34]).

At site 56, both LamB activities were maintained and the C3 epitope was detected on the surface of the hybrid trimers both by immunoprecipitation of solubilized LamB hybrids (Fig. 3)

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**TABLE 4. Antigenicity of the C3 epitope in the LamB hybrids**

<table>
<thead>
<tr>
<th>Strains</th>
<th>Western blottinga</th>
<th>Immuno-precipitationb</th>
<th>Colony blottingc</th>
<th>ELISAd</th>
<th>Mean % fluorescence by cell sortere</th>
</tr>
</thead>
<tbody>
<tr>
<td>pop6510</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>44</td>
<td>15</td>
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<tr>
<td>ACl</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>42</td>
<td>19</td>
</tr>
<tr>
<td>AIC264-VP1</td>
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<td>+</td>
<td>+</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>SPA27.C3</td>
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<td>−</td>
<td>47</td>
<td>70</td>
</tr>
<tr>
<td>SPA56.C3</td>
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<td>−</td>
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<td>41</td>
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<td>SPA77.C3</td>
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<td>−</td>
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<td>−</td>
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<td>32</td>
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<td>−</td>
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<td>25</td>
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<td>+/−</td>
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<td>57</td>
<td>34</td>
</tr>
<tr>
<td>SPA304.C3</td>
<td>+/−</td>
<td>+/−</td>
<td>−</td>
<td>54</td>
<td>21</td>
</tr>
</tbody>
</table>

a Western blotting of denatured samples was revealed by polyclonal anti-C3 peptide antibody (see Materials and Methods for the characteristics of the sera and see the legend to Fig. 2B). b Immunoprecipitations were performed with the anti-C3 MAb under native conditions (see Fig. 3). c Colony blotting was performed with the polyclonal anti-C3 peptide antibody. d For ELISA with intact cells and sonicated lysates, we used anti-C3 MAb. The values are indicated as percentages of the optical density at 405 nm (OD405) for the positive control strain AIC264-VP1 (8). The OD405 recorded for AIC264-VP1 on intact cells was 0.850; that on sonicated lysates was 0.610. Each assay was repeated at least twice and in independent experiments. The results are averages. The estimated relative error for intact cells is 15%, and that for sonicated lysates is 10%. e Cytofluorometric analysis with the anti-C3 MAb. Values indicate the mean fluorescence recorded for the whole population and are given as percentages of the value recorded for the positive control strain AIC264-VP1 (41%). The estimated relative error is 15%. ND, not done.
and by ELISA of sonicated bacterial lysates (Table 4), but not at the surface of intact cells. The C3 insertion maintained at least some recognition by the conformational anti-LamB MAbs, confirming that deformations of the hybrids were limited. Because SPA56-C3 displays wild-type lambda receptor activity, it is tempting to propose that the corresponding region of LamB is facing the periplasm (Fig. 1A). This would suit model 2 but not model 1 and agrees with the X-ray model in which this site belongs to the first periplasmic turn (I1, residues 51 to 56).

At permissive site 77, the inserted C3 epitope was well detected on the surface of solubilized trimers, and recognition of the SPA77-C3 protein by the MAbs was only mildly affected. This suggested that the site is not buried in the trimer. However, because the C3 epitope was detected neither on membrane fragments nor on intact cells, no other topological conclusions could be made. On the X-ray structure, this site lies within the distal portion of the second external loop (L2, residues 69 to 79) of LamB. Since L2 is supposed to play a critical role in monomer-monomer association, it is surprising that trimers are formed by the hybrid protein.

At nonpermissive site 100, the C3 insertion distorted the protein and prevented detection of LamB trimers in all of the assays. This is well explained by the X-ray model, in which this site lies within a transmembrane β-strand preceding the transverse loop L3 and located at the interface between monomers.

At permissive site 204, the C3 epitope was detected on the surface of intact cells and in extracts by all of the techniques used. Moreover, recognition by anti-LamB MAbs was only very moderately affected. Deformations of the hybrids are thus limited, and the corresponding region should be at the bacterial surface. These results fit well with model 2 and not with model 1. In agreement with this conclusion, on the X-ray model, site 204 is located in the middle of the fifth external loop of LamB (L5, residues 198 to 209).

At site 210, the C3 epitope was weakly recognized on intact cells by ELISA and cytofluorometry but not by colony blotting. Unexpectedly, it was poorly recognized or not recognized on membrane fragments. The fact that at this site the C3 insertion affected specifically phage receptor activity and did not appreciably affect recognition by the conformational MAbs suggests that changes are local and point to a cell surface location. This conclusion is reinforced by the fact that the nearby site 204 is at the cell surface (described above). The 3D structure confirms this assumption, showing that residue 210 lies just next to external loop L5 (resides 198 to 209).

Site 221 behaves essentially like site 56 and is thus predicted to face the periplasm. The periplasmic location of site 221 is also in agreement with our previous data on C3 insertion at site 219 (proposed periplasmic) (11). This fits well with model 2 and is compatible with model 1, in which this site would be near the periplasmic end of a β-strand. On the X-ray structure, residue 221 is located three residues upstream of a periplasmic turn (residues 224 to 225). The data suggest that the C3 peptide can recreate the end of the truncated β-strand compatible with LamB functions and expose the C3 epitope in the periplasm.

At site 279, the C3 epitope was poorly detected on the solubilized native hybrid proteins (Table 4) and was not detected on membrane fragments (Table 4). Moreover, recognition by the conformational MAbs was strongly impaired, indicating possible deformations of the hybrid. All of this prevented safe topological conclusions from being drawn.

At permissive site 300, the C3 epitope was detected on intact cells, while both LamB activities were preserved. This site should thus be at the cell surface. The result contradicted the prediction of model 1 and agreed with that of model 2. On the X-ray structure, site 300 is located in the middle of the seventh external loop of LamB (L7, residues 299 to 301).

At permissive site 304, the C3 epitope was poorly detected on the solubilized native hybrid proteins (Table 4) and was not detected on membrane fragments (Table 4). The C3 epitope was weakly detected at the surface of intact cells in ELISA and by cytofluorometry, suggesting that the site could be at or near the cell surface (Table 4). This fits with the cell surface location of site 300 and agrees with model 2 but not with model 1. On the X-ray structure, site 304 is located three residues downstream of external loop L7 (residues 299 to 301).

**Topological conclusions.** The results obtained here by site-directed insertion of C3 (10 hybrid proteins) and previous experiments involving random insertion (13 hybrid proteins) (8, 11) led us to study 23 hybrid proteins altogether (Fig. 1 and Table 2).

Fourteen sites led to topological conclusions: 5 sites demonstrated cell surface exposure of five different external loops of LamB (L4, L6, and L9 and the two newly identified loops L5 and L7) out of the nine loops observed on the X-ray structure. Nine sites did not reveal obvious information about the location of the C3 epitope with respect to the outer membrane: at six permissive sites, the C3 epitope was not detected, and the other three sites were nonpermissive. (Remarkably, these three sites were all located in β-strand regions of the X-ray model.)

Four sites (146, 210, 236, and 399) specifically affecting phage adsorption were also predicted at the cell surface. None of these four sites allowed cell surface detection of the C3 epitope. Strikingly, three of them are located just next to three cell surface loops of LamB (sites 146, L4, 210, L5, and 236, L6). The fourth one lies within a loop (site 399, L9). Conversely, C3 insertions which had no effect on phage receptor activity were compatible with a periplasmic location. Thus, on the basis of the recognition of the C3 epitope by the anti-C3 MAb in different assays, we proposed the periplasmic locations of five sites (sites 56, 183, 219, 221, and 352), which were confirmed by the X-ray structure (loops I1, I4, I5, and I8).

In terms of 2D models, the C3-directed insertion procedure described here allowed, in 6 cases out of 10, discrimination between models 1 and 2. The conclusions for the six sites were in favor of model 2 and agreed with the X-ray model, showing that predictions based on alignments with proteins of known structure are far better than secondary structure predictions based on properties of the individual amino acid residues.

Strikingly, topology based only on secondary structure predictions led to the wrong conclusions about the folding of large portions of LamB. Model 1 differed from the X-ray model in the regions between residues 18 and 136, 202 and 236, and 273 and 319 (i.e., about 45% of LamB length), which correspond to all of the regions for which no experimental data were available. In contrast, all of the experimental data about epitope insertion fit with the X-ray model, and this accounts for about 55% of LamB length. Model 2, based on sequence alignments with porins of known structure, was more secure (94% of correctly predicted regions) but still predicted 16 instead of 18 transmembranous β-strands, essentially because of mispredictions in region 123 to 148 (a structurally important region of LamB which folds deeply within the barrel [34]). Also in model 2, the sizes of the predicted loops were generally larger than the size actually shown by the structure and, in particular, L7 (13 residues instead of 3), L8 (14 residues instead of 3), and I6 (11 residues instead of 2).
Limits to the approach. The two major types of limitations to the approach are due to (i) the properties of the insert and (ii) the nature of the insertion site.

(i) Properties of the insert. Detection of the epitope by a specific antibody depends on a number of factors. One is the size of the reporter peptide. Since the length of the part of the β-strands crossing the membrane is about 11 residues, there is a possibility that the C3 peptide inserted at a site which is not surface located could span the outer membrane and so could point enough to the exterior so as to be detected on intact cells. For example, when the insertion site is located within the outer membrane but close to the periplasm, the C3 recognition site within the C3 peptide (residues 97 to 103) (42) could point into the periplasm and therefore lead to detection by the anti-C3 MAb at the surface of the solubilized trimers. This was likely to be the case for C3 insertion at site 221. The resolution of the approach, which is limited by the size of the inserted peptide, could perhaps be improved with smaller inserts. Linear epitopes usually include at least six residues (4), a fact which sets the theoretical resolution limit of the method.

The accessibility and the conformation(s) of the peptide carrying the epitope after insertion may also be a limitation. Constraints exerted on the insert may disfavor the conformation(s) recognized by the specific MAb, so that the affinity towards the peptide may be decreased or abolished. This difficulty can be overcome either with a set of different MABS specific for the same antigenic region, so that different conformations of the peptide could be assessed (3), or with an anti-peptide polyclonal antibody able to recognize several epitopes on the inserted peptide (25a).

The affinity of the MAB towards the original epitope may also be critical for the sensitivity of the detection of the inserts.

(ii) Nature of the insertion site. The C3 insert must be compatible with productive interactions between monomers and between trimers and the membrane (permissive sites). Thus, regions essential for the structure of the protein, like transmembrane regions, cannot be probed directly by this approach. Finally, in some cases, the inserted foreign sequence may have long-range effects on the structure of the protein, preventing any safe topological interpretation.

Concluding remarks. The epitope tagging approach (random or site directed) was developed to avoid some of the problems raised by widely used gene fusion techniques in which an active enzyme moiety (β-galactosidase, alkaline phosphatase, or β-lactamase) is fused to the protein under study (reviewed in references 19, 32, and 39). First, these techniques involve large probes which may interfere with the folding and localization of the protein under study. Second, they usually involve deletion of increasing portions of the COOH-terminal end of the target protein, resulting in loss of activities and therefore doubts about the relationship between the structure of the hybrid protein and that of the target protein. Probably, one or both of these reasons explain why such fusions have not been successfully used for determining outer membrane protein topology; it is indeed known that the COOH-terminal end of the outer membrane proteins is critical for their assembly (7, 35). The epitope tagging approach constitutes a powerful and convenient way to probe the topology of outer membrane proteins. It has now been successfully used for several other bacterial outer membrane proteins (1, 26, 36–38) and for eukaryotic cytoplasmic membrane proteins (2, 6). However, in the case of the bacterial inner membrane protein MalG from E. coli, the C3 epitope was not detectable by the specific MAB on any of the hybrids generated (14a). We do not know if the use of another epitope-MAb couple would solve the problem.

The present work represents the first extensive comparison of topological information drawn from epitope tagging with the 3D structure of an integral membrane protein.

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