Antibodies against Synthetic Peptides and the Topology of LamB, an Outer Membrane Protein from *Escherichia coli* K12†

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ABSTRACT: LamB, an outer membrane protein from *Escherichia coli* K12, is involved in the transport of maltose and maltodextrins across the outer membrane and constitutes a receptor for a number of bacteriophages. A recent folding model proposes that LamB spans the outer membrane through a number of transmembranous segments separated by regions exposed either to the cell exterior or to the periplasm. This model is essentially based on predictions of structure and genetic arguments relying on the hypothesis that the mutations studied did not alter the folding of the protein. In order to obtain direct evidence with the unaltered protein, we elicited polyclonal antibodies against synthetic peptides corresponding to several LamB sequences. We chose four regions. Three of them [aa 147–161 (peptide 2), aa 371–385 (peptide 3), and aa 399–413 (peptide 4)] are predicted to face the outside of the cell, and the fourth [aa 19–33 (peptide 1)] is predicted to be periplasmic. By immunoblotting against extracts of various mutants, these antibodies were shown to be specific for LamB and targeted to the selected regions. In some cases, the recognition sites for antibodies were narrowed down to parts of a region. In vivo, on intact cells, anti-peptides 2, 3, and 4 reacted with LamB in an ELISA; this confirmed that regions of peptide 2 and 3 are located, at least in part, at the cell exterior and provided the first proof for a similar situation of the region of peptide 4. Under the same conditions, anti-peptide 1 did not react with LamB. However, this antiserum recognized LamB in *Escherichia coli* membrane fragments, providing the first experimental support for the periplasmic localization of peptide 1. The location of peptide 2 at the cell surface was confirmed by immunoelectron microscopy and was also supported by the effects of anti-peptide 2 antibodies on phage inactivation in vitro and in vivo.

LamB is an integral outer membrane protein from *Escherichia coli* K12 with two biological functions: it behaves as a pore involved in the permeation of maltose and maltodextrins (hence, its name maltoporin) (Nakae & Ishui, 1980; Luckey & Nikaido, 1980) and as a cell surface receptor for several bacteriophages including phage λ (hence, its alternative name λ receptor) (Charbit & Hofnung, 1985). The active form is a trimer. The protein is synthesized as a precursor with an NH_2-terminal signal peptide, which is cleaved upon export to yield monomers of 421 residues. Upon induction by maltose or maltodextrins, LamB becomes a major component of the outer membrane. The amino acid sequence of LamB was deduced from the nucleotide sequence of its structural gene (Clément & Hofnung, 1981).

The precise structures of very few integral membrane proteins have been established. Most of them are hydrophobic in nature. The best known, bacteriorhodopsin (Ovchinnikov, 1987) and the photosynthetic center of *Rhodopseudomonas viridis* (Deisennhoffer et al., 1985), have essentially α-helical transmembranous regions.

In contrast, LamB is rather hydrophilic, contains very few α-helical ordered regions, and is rich (over 50%) in β-strand structures as indicated by circular dichroism and infrared data (Vogel & Jähnig, 1986). Therefore, LamB, like OmpF, another *E. coli* outer membrane protein rich in β-sheets, is a suitable model to elucidate the organization of β-strands with respect to the membrane (Kleffel et al., 1985).

A model for the folding of the protein was proposed on the basis of a combination of structural predictions from the amino acid sequence, and of genetic and immunological data (Charbit et al., 1988; Figure 1). In brief, residues affecting the interaction of LamB with bacteriophages or with monoclonal antibodies directed toward the protein were placed on the outside of the cell. The number of membrane-spanning fragments was maximized. The model predicted that the regions around residues 153 and 374 were external. This view was enforced by genetic insertion of a foreign epitope in these regions and the detection of the epitope on the cell surface (Charbit et al., 1986).

It is important to notice that, although coherent, this model relies upon predictions of structure and indirect evidence from mutated LamB proteins. Interpretation of this evidence, including those derived from the detection of an inserted foreign epitope at the cell surface, is based on the assumption that no reorganization of the protein occurred upon genetic modification.

To obtain direct tests for the validity of this model, we have used antibodies against synthetic peptides corresponding to LamB sequences as probes. The present paper reports the production and the characterization of such antibodies and their use to study the organization of the LamB protein.

Materials and Methods

Bacterial Strains, Chemicals, and Plasmids. All strains were derivatives of pop 6510 (*thr leu tonB thi lacY recA*

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Table I

peptide	sequence[a]	coupling function	carrier protein[b]	ELISA titer[c] peptide[d]	ELISA titer[c] LamB in vitro	ELISA titer[c] LamB in vivo	hydrophobicity average[e]
1	EQQCFQTTGAQSKYR	amino groups	KLH	ND[f]	1500	<100	−0.89
2	SEAGGSSSFASNNIY	amino groups	BSA	7600	600	820	−0.36
3	(C)KWDEKWGYDYTGNAD	cysteine	BSA	15000	2000	2000	−1.44
4	FNGGSFGRGDSDEWTF	amino groups	KLH	5000	500	900	−0.85

[a] The sequence of amino acid residues used is shown in the one-letter code. Residues between parentheses were added for convenience of coupling. [b] The carrier protein was either keyhole limpet hemocyanin (KLH) or bovine serum albumine (BSA). [c] ELISA titers were determined as the dilution for which the signal was half the maximum absorbance recorded. In vitro, the negative control is the nonimmune serum and in vivo the LamB-negative strain (pop6510). [d] Anti-peptide ELISA were conducted essentially as described under Materials and Methods except that the plates were coated with 3 μg of peptide conjugates per well. Conjugates are the covalent coupling of the peptide to thyroglobulin through free amino groups. [e] The hydrophobicity of a peptide segment was defined as the average of the hydrophobicity values of its residues using the normalized consensus scale defined by Eisenberg et al. (1982). [f] Not determined.

dex5metA supE); due to the *dex5* mutation in gene *lamB*, no LamB protein is detectable in this strain (Bouges-Bocquet et al., 1984).

Plasmid pAC1 carries the wild-type *lamB* gene under *ptac*12 promoter control, as well as the *lac* I^q gene. The expression of LamB is thus inducible with IPTG. In all except four mutants (see following paragraph), the modified LamB proteins were expressed on plasmids derived from pAC1. The wild-type LamB control was strain pop6510 (pAC1), which was renamed AC1. Details of these different strains, which are recalled in the text, are already described (Boulain et al., 1986).

In four cases, the mutant LamB proteins (strains BB81, 90, 91, and 97) were expressed under p*tac*12 promoter control from plasmids derived from plasmid pBB° (Bouges-Bocuet et al., 1984) which does not carry *lac* I^q. In these four strains, the *lac* I^q gene, which represses LamB expression in the absence of IPTG, was carried on an F′ episome. In these four mutants, modifications in the *lamB* gene was generated by linearization of the corresponding plasmids after partial digestion with the restriction enzyme *Fnu4H1*. Deletions and substitutions around the *Fnu4H1* site were obtained by partial digestion with exonuclease *Bal*31, followed by the filling of the resulting DNA extremities using Klenow DNA polymerase, before ligation (Bernadette Bouges-Bocquet, unpublished results). Strains carrying point mutations in *lamB* gene are described previously (Charbit et al., 1988).

Media and chemicals were as in Boulain et al. (1986).

Peptides (Arg-Gly-Asp-Ser and Gly-Asp-Ser) were purchased from Sigma, and peptide Gly-Arg-Gly-Asp-Ser-Pro-Lys was a gift from Dr. M. Pierschbacher (La Jolla Cancer Research Foundation, La Jolla, CA).

Miscellaneous Methods. LamB was purified by the method of Gabay (1982). Purified LamB was suspended in 5 mM EDTA, 2% Triton X-100, and 10 mM Tris-HCl, pH 7.4, at a final concentration of 4 mg/mL. Rabbit antisera, kindly provided by D. Perrin, against the native LamB protein recognized the LamB monomers as well as the trimeric and multimeric forms of the protein. Monoclonal antibodies (MABs)[1] E302 and I436 were kindly provided by J. Gabay. E302 is directed against a region of LamB located at the cell exterior while I436 recognizes an internal determinant (Gabay et al., 1986; Desaymard et al., 1986).

Synthesis of Peptides and Conjugation to Carrier Proteins. Peptides, derived from the sequence of the *lamB* gene from *E. coli* K12 (Clément & Hofnung, 1981), were synthesized according to Merrifield (1963). The composition of all peptides was confirmed by amino acid analysis, and the purity of the product was checked by HPLC. The positions of the four peptides along the LamB protein, as well as their sequences, are given in Table I and shown in Figure 1. A cysteine residue was added to the amino terminus of peptide 3 in order to couple it to bovine serum albumin, using *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP, Pharmacia) (Pain & Surolia, 1981). Peptides 1, 2, and 4 were coupled to the carrier through free amino groups using 0.07% glutaraldehyde (Baron & Baltimore, 1982).

Immunization and Purification of Antibodies. Peptide conjugates in PBS (0.3 mg per animal) were mixed in an equal volume of complete Freund's adjuvant and injected intradermally at multiple sites into two rabbits. Booster injections of the conjugate in incomplete Freund's adjuvant were given on days 14 and 60. Blood samples were weekly taken after day 18. Sera were adsorbed with bacterial cells of a LamB-negative strain (pop6510) as described (Schenkman et al., 1983). Immunoglobulins were then purified by ammonium sulfate precipitation (33% saturation at 0 °C). Samples were dialyzed against PBS and then concentrated 10-fold by saccharose dialysis.

ELISA. Microtiter plates (Nunc) were coated by overnight incubation at 37 °C with antigen diluted in PBS (100 μL per well). Excess of antigen was discarded, and wells were blocked with 250 μL of PBS containing 0.5% gelatin, for 1 h at 37 °C. Then, 100-μL serial dilutions of sera (in PBS containing 0.5% gelatin) added per well and incubated for a further 2 h at 37 °C. After extensive washes, the antigen–antibody complex was developed by peroxidase-labeled anti-rabbit (or anti-mouse) antibodies (1 h, 37 °C) and ABTS [2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid), 20 min at room temperature]. Each assay was run in duplicate simultaneously with immune serum and the corresponding preimmune serum.

(*A*) *With Immobilized Peptide.* Peptide coupled to the carrier (thyroglobulin) was coated onto the plate (60 ng of peptide per well), and the ELISA was performed as described above. When competition experiments were performed, the sera were first incubated for 1 h at 37 °C in the presence of various concentrations of antigen and then loaded into the wells.

(*B*) *With Purified LamB Protein.* Wells were coated with 100 ng of LamB protein in PBS. Controls for LamB coating into the wells were conducted with monoclonal anti-LamB antibodies E302 and I436 (ascite fluid diluted 1:5000), and proteins were extracted as described by Gabay (1982), from either the LamB-positive strain ACl or the LamB-negative strain pop6510. When MABs E302 and I436 were used, a positive signal was detected with strain PACl while no labeling

[1] Abbreviations: aa, amino acid(s); BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; KLH, keyhole limpet hemocyanin; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; MAB, monoclonal antibody.

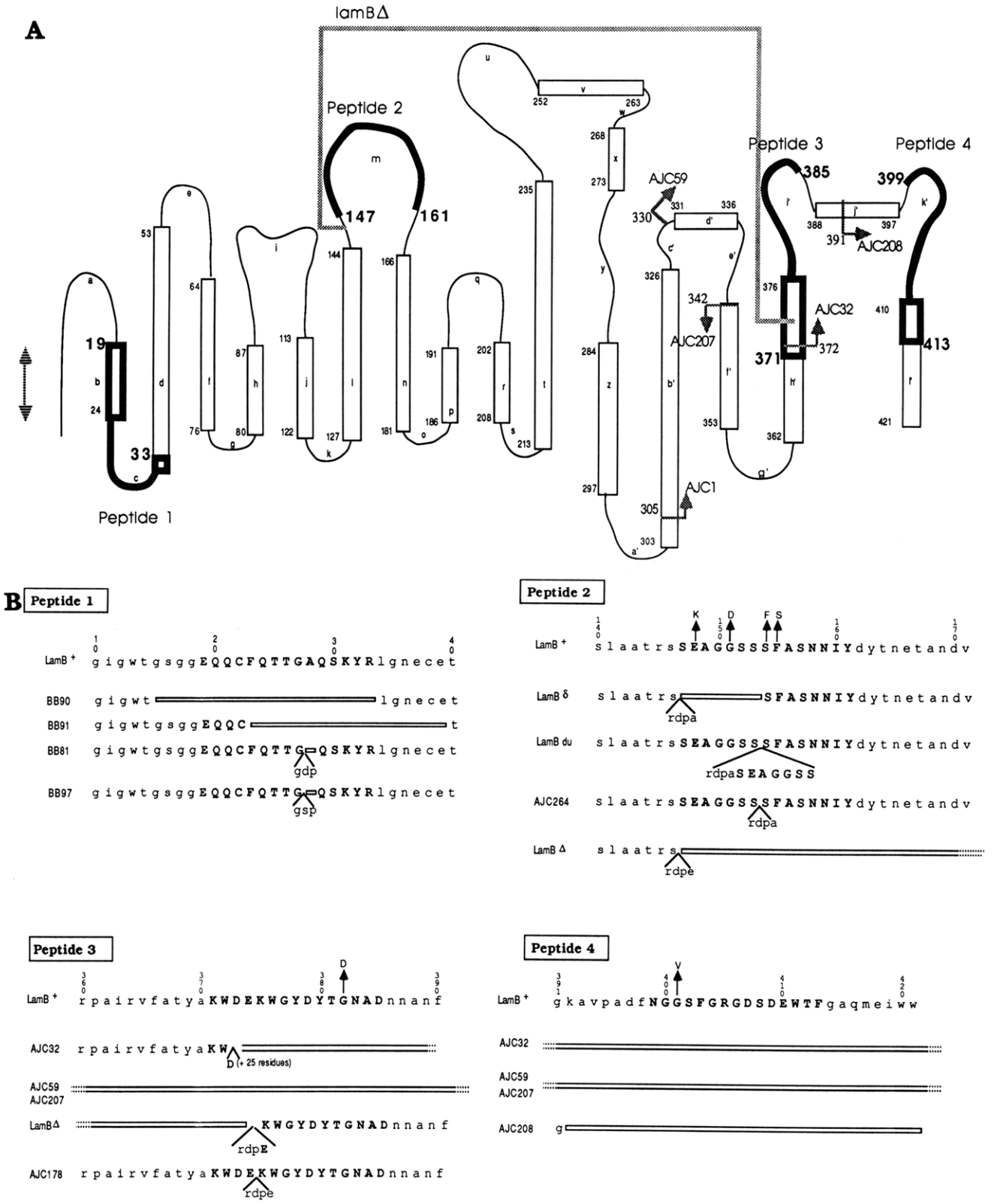

FIGURE 1: Position of the four peptides and of relevant mutations on the model for LamB folding. (A) Folding model (Charbit et al., 1988). The regions predicted as β-strands are represented as rectangles. The vertical lines are perpendicular to the plane of the membrane. Peptides 1–4 are indicated with thick black lines as well as their initial and last residue numbers. Relevant deletions are represented by grey lines. *lamB*Δ is a large internal deletion from residue 147 to residue 372 (limits included) together with an insertion of three residues (RDP). All the others are COOH-terminal deletions: the number of the first residue deleted as well as their name (AJC...) is indicated. Numbering starts at aa residue 1 of the mature protein. (B) Details of the sequence. The sequences of the regions corresponding to the four peptides are represented in black capital letters. The neighboring sequences are in normal letters. Deletions are indicated by horizontal double bars. Insertions are indicated below the sequence, together with the exact changes due to relevant mutations. AJC32 corresponds to a frame-shift mutation. Point mutations leading to substitutions are represented by vertical arrows above the LamB sequence (Charbit et al., 1988).

was detected with strain pop6510.

(*C*) *With Intact Bacteria.* Microtiter plates were coated with 5×10^6 intact bacteria (100 μL per well of bacteria at OD_{600} = 0.1 in PBS). Controls were performed in triplicate with anti-LamB MABs E302 and I436 at dilutions of 1:5000 and 1:2000. Assays were performed simultaneously on

Table II

mutant[a]	modification[b] site	modification[b] type, nature	length of protein in aa	immunoblotting results with antibodies directed against peptide 1 (19–33)	peptide 2 (147–161)	peptide 3 (371–385)	peptide 4 (399–413)
(1) pAC1	wild-type	LamB	421	+[d]	+	+	+
(2) 352–264 du	153	I	432	ND[c]	+	+	+
(3) LamBδ	146	Di	418	ND	–	+	+
(4) LamBΔ	146	Di	198	ND	–	–	+
(5) AJC264	153	I	425	+	+	+	+
(6) AJC208	391	Dc	439	+	+	+	–
(7) AJC207	342	Dc	478	+	+	–	–
(8) AJC78	374	I	425	ND	+	+	+
(9) AJC32	372	Dc	398	ND	+	–	–
(10) AJC1	305	Dc	331	ND	+	–	–
(11) AJC59	330	Di	379	ND	+	–	–
(12) BB81	28	S + I	423	+	ND	ND	ND
(13) BB90	15	Di	405	–[e]	ND	ND	ND
(14) BB91	23	Di	405	–	ND	ND	ND
(15) BB97	28	S + I	423	+	ND	ND	ND

[a] All mutants are described in Figure 1. [b] Mutations are noted as follows: I = insertion; Di = internal deletion; Dc = deletion of C-terminus; S = substitution. [c] ND, not determined. [d] (+) indicates that the mutant LamB protein was clearly detected. [e] (–) indicates that no LamB protein could be detected.

LamB-positive and LamB-negative strains.

(D) With Membrane Fragments. Cultures of pop6510 and ACl were harvested by centrifugation, washed twice with PBS, and suspended in PBS at OD_{600} = 1. Cells were then lysed by sonication on ice (four pulses of 20 s in an MSE sonicator). The suspension was centrifuged, and the pellet, homogenized in PBS, was used for coating the plates (100 μL per well corresponding to 2×10^7 bacteria). Controls were performed with anti-LamB MABs E302 and I436 at a dilution of 1:5000. Assays were run in triplicate.

Immunoblotting of Crude Bacterial Extracts. Bacterial strains were grown at 37 C in liquid minimal medium supplemented with casamino acids and glucose. At OD_{600} = 1, cells were harvested by centrifugation, suspended in $^1/_{20}$th volume of water. Total cellular extracts were diluted in an equal volume of loading buffer (10% glycerol, 2% SDS, 0.05% bromophenol blue, and 15 mM β-mercaptoethanol in 60 mM Tris-HCl, pH 6.8) and then boiled for 5 min. Samples were separated on 10% SDS–polyacrylamide slab gels according to Laemmli (1970). Proteins were transferred electrophoretically onto a nitrocellulose filter. After extensive washing and blocking with PBS/3% gelatin, the nitrocellulose sheet was incubated with anti-peptide antisera and diluted 1:100 in PBS containing 0.5% gelatin. Then antigen–antibody complexes were revealed by using peroxidase-conjugated goat anti-rabbit IgG (Biosys, diluted 1:1000) as indicator antibody and diaminobenzidine as peroxidase substrate (Guesdon et al., 1984).

Electron Microscopy. Bacterial cell immunolabeling and detection by electron microscopy were performed as described (Charbit et al., 1986). Briefly, 1.5 mL of bacteria cells was grown in L broth (Davis et al., 1980) to OD_{600} = 0.6, washed with PBS, and then adsorbed on polylysine-coated grids and fixed with 2.5% paraformaldehyde for 10 min. After the grids were washed, then were incubated with immune serum (diluted 1:10) for 60 min at room temperature. The grids were then washed, and antibodies were revealed with 20-nm protein A–gold particles. Micrographs were obtained with a Siemens Elmiskop 101 electron microscope.

Ability of Anti-Peptide Antibodies to Inhibit Phage λ Inactivation by LamB. (A) In Vitro Assay. Purified LamB was incubated at 37 °C for 2 h either with partially purified anti-peptide antibodies (dilution 1:20) or in the absence of antibody. LamB concentrations in the assay varied from 3.7 μM to 37 pM (corresponding respectively to 8.5×10^{14} and 8.5×10^9 molecules of LamB trimer/mL). The phage suspension (5×10^3 plaque-forming units per assay in a dilution medium of 10 mM Tris-HCl, pH 7.5, and 4 mM $MgSO_4$) was then added and incubated for 30 min at 37 °C. Half (0.1 mL) of the incubation mixture was taken and plated with indicator bacteria (ACl) onto solid medium. All assays were done in duplicate, and controls were performed with preimmune sera.

(B) In Vivo Assay. Assays were also performed with intact bacteria cells under conditions described in (A), except that the incubation mixture was centrifuged in order to eliminate the bacterial cells before plating indicator cells (ACl) onto solid medium. The bacterial concentration varied from 2.5×10^7 bacteria/mL to 2.5×10^5 bacteria/mL, again corresponding to LamB concentrations varying from 3.7 nM to 37 pM.

Results

Our goal was to study the localization of defined regions of the LamB protein with respect to the outer membrane. We proceeded in three steps. First, we raised antibodies against the corresponding synthetic peptides. Second, we followed the appearance of anti-LamB antibodies in the sera and checked their specificity with cellular extracts from mutant strains. Third, we examined the ability of the antibodies to recognize LamB in vivo on intact cells.

Raising and Characterization of Antibodies. Four different regions were chosen (Figure 1). Three were predicted to face outside the cell:peptide 2 (147–161); peptide 3 (371–385); peptide 4 (399–413). The fourth region, pepide 1 (19–33), was predicted to be hidden from the cell surface. Four synthetic peptides (15 amino acids long), with sequences corresponding to the relevant LamB regions, were coupled to a carrier protein and injected into rabbits (Table I). Sera were collected and screened by ELISA for the presence of antibodies reactive against the LamB protein. Details of immunogens and antibody titers are listed in Table I. The specificity of antisera for LamB was further checked by immunoblotting against an extract from a LamB-positive strain. The sequence specificity of the anti-peptide antibodies was then established by immunoblotting experiments with extracts of various LamB mutants (see Table II for details). The results for each anti-peptide antibody are described successively below. For simplicity, the serum elicited with peptide 1 will be called serum 1, with peptide 2 serum 2, and so on.

Peptide 1 (19–33). Peptide 1, coupled to KLH through free amino groups, was injected into two rabbits (see Materials and

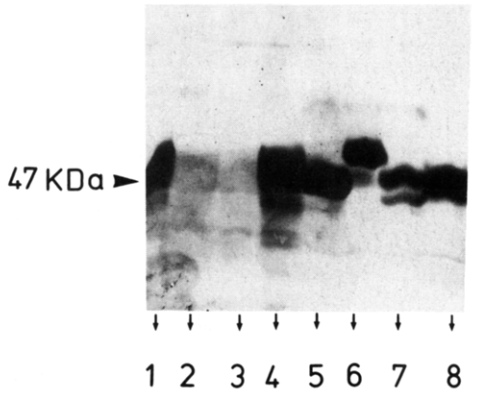

FIGURE 2: Detection of LamB proteins with serum 1. Extracts of eight different *E. coli* strains were heat denatured and separated on an SDS–10% polyacrylamide slab gel. LamB proteins were immunodetected by anti-peptide 1 antibodies as described under Materials and Methods. Lanes 1, 2, 3, and 4 correspond to mutants BB81, BB90, BB91, and BB97, respectively. Lane 5, wild-type LamB strain PACl. Lanes 6, 7, and 8 correspond to mutants AJC207, AJC208, and AJC 264, respectively. The arrow indicates the migration of denatured wild-type LamB protein. Characteristics of LamB mutations are described on Figure 1 and Table II.

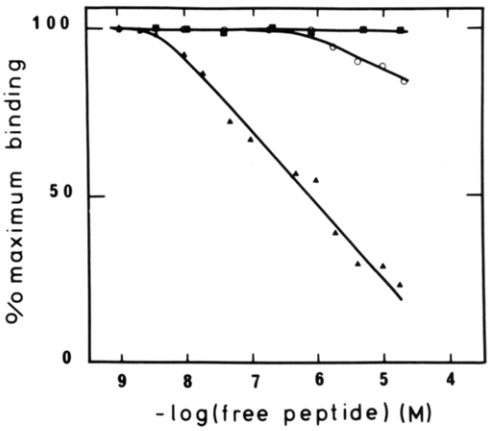

FIGURE 3: Competition of serum 2 for various peptides. The binding of serum 2 to peptide 2 immobilized in ELISA wells was monitored as described under Materials and Methods. The inhibition of this binding was followed in the presence of various concentrations of either the C-terminal peptide [FASNNIY (▲)] or the modified related peptide called "I-minus" [SEAGGSSSFASN*N-Y* (○)] or an unrelated peptide [peptide 4 (■)]. The percentage of maximum binding was plotted versus the concentration of free peptide.

Methods). One animal was found to be a poor anti-LamB responder (titer inferior to 1:250), while the second gave an anti-LamB titer of 1:1500 (serum 1). The maximal response was reached at day 33 and did not increase after boosting.

Serum 1 recognized only one protein, migrating with the apparent molecular weight of LamB, by immunoblotting against an extract from a strain with a wild-type LamB (Figure 2, lane 5). This serum failed to detect LamB in two mutants in which all or most of the region of peptide 1 was deleted (Figure 2, lanes 2 and 3); Mutants BB90 and BB91 were normally detected by the polyclonal anti-LamB serum (data not shown). In contrast, serum 1 recognized the LamB protein expressed in two mutants in which Ala-28, located near the middle of peptide 1, was substituted by three residues (Figure 2, lanes 1 and 4). Genetic modifications in regions of LamB distant from peptide 1 (Figure 2, lanes 6, 7, and 8; after residues 342 and 391 and near residue 153, respectively) did not prevent detection by this serum.

Peptide 2 (7–161). Peptide 2 was coupled to BSA through free amino groups, and thus essentially by its N-terminus. The resulting complex was injected into two rabbits. Both animals produced anti-peptide antibodies recognizing LamB (anti-LamB titers 1:600 and 1:400) (Table I). In both cases, anti-LamB antibodies appeared at day 40 and reached a plateau at day 75. The serum with the highest titer (named hereafter serum 2) was further characterized. Its anti-peptide titer was 1:7600 (see Table I). This serum was found to react in an ELISA with the parent peptide (aa 147–161) and with a short peptide corresponding to the C-terminus part of the peptide (F-A-S-N-N-I-Y). Serum 2 failed to recognize a related peptide, named "I-minus", in which the penultimate Ile residue is missing (S-E-A-G-G-S-S-S-F-A-S-N-*N-Y*). These data were also confirmed by competition experiments. The short peptide was able to inhibit the binding of antibodies to peptide 2 while an unrelated peptide (peptide 4) and the related "I-minus" peptide had no effect on this interaction (Figure 3).

Serum 2 recognized only one protein, migrating with the apparent molecular weight of LamB, by immunoblotting of an extract of ACl (Figure 4A). Analysis of the reactivity of serum 2 with various mutants, presented below, showed that serum 2 was specific for the region of peptide 2. Mutant *lamB*Δ, which carries a large deletion including peptide 2

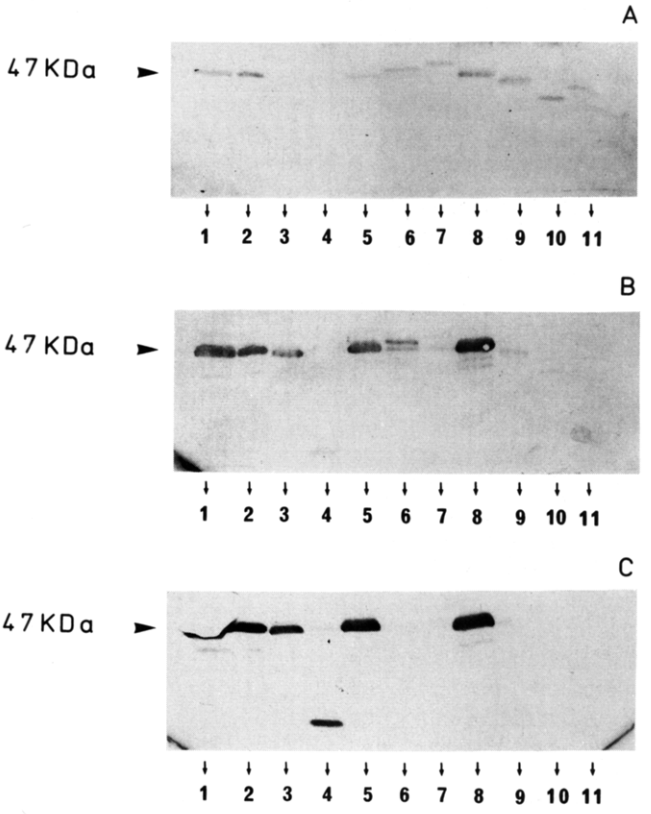

FIGURE 4: Detection of LamB proteins with sera 2–4. Extracts of various strains were heat denatured and separated on an SDS–10% polyacrylamide slab gel. LamB proteins were immunodetected as described under Materials and Methods, with either serum 2 (part A) or serum 3 (part B) or serum 4 (part C). Lane 1, wild-type LamB strain AC1; lane 2, *lamB* du; lane 3, *lamB δ*; lane 4, deletion *lamB*Δ; lane 5, mutant AJC264; lane 6, AJC208; lane 7, AJC207; lane 8, AJC178; lane 9, AJC32; lane 10, AJC1; lane 11, AJC 59. Arrows indicate the migration of denatured wild-type LamB protein. It should be noted that the apparent migration of the LamB mutant proteins is in agreement with their respective sizes reported in Table II.

(residues 146–374), was not recognized (Figure 4A, lane 4). A mutant protein containing a duplication of the first half of peptide 2 (residues 146–153) gave a normal signal (Figure 4A, lane 2; mutation *lamB* du). Insertion of four residues after the middle part of peptide 2 (position 153) affected only slightly the recognition of the protein (Figure 4A, lane 5;

mutant AJC 264) while a protein which carries both a deletion of the first half of peptide 2 (residues 147–153) and the insertion of 4 aa (R-D-P-A) was poorly detected (Figure 4A, lane 3). Point mutations at positions 148 (Glu → Lys), 151 (Gly → Asp), and 154 (Ser → Phe) were normally detected by serum 2 in an immunoblotting assay while mutation of residue 155 (Phe → Ser) prevented the recognition of the protein (data not shown). Finally, as expected, mutations occurring in parts of the protein distant from peptide 2 did not affect detection by serum 2 (Figure 4A, lanes 8–11).

Peptide 3 (371–385). Peptide 3 was coupled to BSA by its N-terminus through an added cysteine residue. Both rabbits immunized with this immunogen elicited anti-LamB antibodies. Maximum anti-LamB titers (1:2000 for both animals) were obtained at day 44 and were persistent for at least 2 months. These sera were named serum 3a and serum 3b (serum 3a being used in the following study). Serum 3a has an anti-peptide titer of 1:15 000 (see Table 1).

These sera proved specific for LamB and essentially the region of peptide 3 as tested by immunoblots (Figure 4B). However, LamB mutants lacking this region were faintly detected (Figure 4B, lanes 7, 9, and 10), suggesting that another region of LamB cross-reacted weakly with serum 3. The large deletion, *lamB*Δ, removing residues 146–374 in LamB, including the first three residues of peptide 3, was not detected by the same technique (Figure 4B, lane 4). In contrast, an insertion of four residues after position 374 and the substitution of residue 382 (Gly → Asp) did not prevent detection (Figure 4B, lane 8 and data not shown).

Peptide 4 (399–413). Peptide 4, like peptide 1, was coupled to KLH. The maximum response was recorded at day 75. Anti-LamB titers were 1:500 and 1:250 for the two immunized rabbits. The serum with the highest titer (serum 4) had an anti-peptide titer 10-fold higher than its anti-LamB titer (Table I). The shorter peptides R-G-D-S, G-D-S, and G-R-G-D-S-P-K were not able to compete with the binding of serum 4 to peptide 4, even at 5000-fold excess (data not shown).

Serum 4 was shown by immunoblotting to be specific for LamB and for the region of peptide 4. It recognized the wild-type LamB protein (Figure 4C, lane 1) as well as mutant *lamB*Δ with a large internal deletion of the protein (Figure 4C, lane 4). It failed to detect protein mutants in which the C-terminus of LamB, including the region of peptide 4, was deleted (Figure 4B lanes 6, 7, 9, and 10). A mutant in which residue 401 was substituted (Gly → Val) was poorly recognized by serum 4 (data not shown).

Anti-Peptide Antibodies and LamB Topology. The anti-peptide antibodies were then used to prove in vivo the topology of LamB with respect to the outer membrane. This was done by using two different approaches. First, we studied the binding of antibodies to bacterial cells. Second, we examined the ability of the antibodies to compete with phage adsorption on LamB.

Antibody Binding. Antibody binding to intact cells was detected either by ELISA (Table I) or by electron microscopy (Figure 5).

(*A*) *ELISA.* Sera 2–4 gave a positive response in the ELISA assay with intact cells. In these conditions, the anti-LamB titers were 800, greater than 2000, and about 900, respectively. Peptides 2–4 are thus likely to be on the outer side of the outer membrane. For these three sera, the anti-LamB titers were in the same range in vitro and in vivo (see Table I). In contrast, serum 1 failed to detect LamB on intact cells, suggesting that peptide 1 is not accessible on the cell surface (Table I). As a control, we performed an ELISA with serum 1 on membrane fragments prepared by sonication (Materials and Methods). Under these conditions, serum 1 detected LamB with a titer of 1:1800.

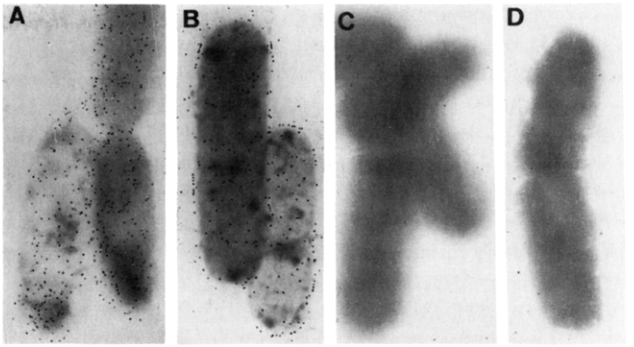

FIGURE 5: Electron micrographs of bacterial cells after immunolabeling. Bacteria producing the wild-type LamB protein (strain ACl) were labeled with either anti-LamB antibodies (A) or anti-peptide 2 antibodies (B). Controls with anti-LamB antibodies (C) and anti-peptide 2 antibodies (D) were performed on the corresponding LamB-negative strain (pop6510).

As a supplementary control, to ensure that the ELISA performed with intact cells allowed a clear distinction between cell surface exposed and internal regions of LamB, we used two previously characterized MABs. MAB E302, recognizing a cell surface exposed determinant of LamB, was indeed able to label the protein on intact cells. In contrast, MAB I436, which is directed against a LamB region facing the periplasm, was unable to label the protein on the intact cell. However, MAB I436 did allow the detection of LamB in membrane fragments.

(*B*) *Electron Microscopy.* Antibody binding was revealed with gold particles coupled to protein A. ACl was labeled with gold particles when the polyclonal anti-LamB serum (positive control) and when serum 2 were used (Figure 5, panels A and B). The label appeared a little weaker with serum 2 (Figure 5, panel B). The LamB-negative strain was not labeled under the same conditions (Figure 5C,D). Incubation of neither $LamB^+$ nor $LamB^-$ strains with sera 1, 3, and 4 allowed labeling by protein A–gold.

Phage Inactivation. Binding to phage λ to LamB can be affected by both polyclonal and certain monoclonal sera directed against the native LamB protein (Gabay et al., 1986). We examined whether the sera against the four peptides could prevent the inactivation of phage λ by LamB. In vitro, under our assay conditions, LamB at 2 ng/mL inactivated 83% of phage. Addition of preimmune serum did not affect this inactivation. The addition of serum 2 reduced phage inhibition by LamB from 83% to 41%. Sera 1, 3, and 4 had no effect. In vivo, LamB at a concentration of 3.5 ng/mL inactivated 56% of phage. Serum 2 reduced this inactivation to 23%. Again, sera 1, 3, and 4 had no effect.

Discussion

Immunological approaches are now widely used to study the structure of membrane proteins (Ovchinnikov, 1987). In the case of the outer membrane protein LamB, monoclonal antibodies raised against the native protein were shown to recognize determinants facing either the bacterial surface or the periplasm (Gabay et al., 1986). However, this approach presents two problems. First, it is difficult to identify precisely the amino acid residues which constitutes the antigenic determinants; these determinants may even be discontinuous. Second, this procedure does not yield antibodies directed against selected regions of the protein.

In order to circumvent these difficulties, we decided to elicit antibodies directed against synthetic peptides representing four distinct selected regions of LamB.

Choice of the Peptides and of the Mode of Coupling. According to the model for the folding of LamB, three of the chosen regions face the outside of the cell (peptides 2–4), and one faces the periplasm (peptide 1) (see Figure 1). For two of these regions (peptides 2 and 3), experimental data supported already the proposed location; (i) mutations at amino acid positions 148, 151, and 15 (in peptide 2) and 382 (in peptide 3) prevented absorption of phage λ (Charbit et al., 1984); (ii) mutations at residues 333, 382, 386, 389, and 394 yielded resistance to monoclonal antibodies (Desaymard et al., 1986), (iii) a foreign epitope, when inserted into sites 153 (whithin peptide 2) and 374 (within peptide 3), was detected on the surface of intact cells with specific monoclonal antibodies (Charbit et al., 1986). However, the proposed location required also the assumption that the various mutations did not affect the folding of the protein. For the two other regions (peptides 1 and 4), there is no experimental data: their proposed location is simply a logical consequence of the hypotheses on which the model is based.

We used synthetic peptides of 15 residues corresponding to rather hydrophilic regions and including segments of high predicted flexibility (Eisenberger et al., 1982). We used them as immunogens in rabbits. The choices of coupling reagents and carrier proteins were empirical. It is sometimes suggested that linking a synthetic peptide by its terminal amino acid allows it to adopt a "native" conformation, thus yielding antibodies against the parental protein. In first attempts, peptide 1 was coupled to the carrier by its C-terminus, but this complex failed to elicit anti-LamB antibodies in two animals (data not shown), whereas anti-LamB antibodies were obtained when this peptide was coupled by both terminal amino acid residues simultaneously. We may therefore suggest that, at least in this case, the best coupling is the one that restores constraints existing in the natural environment. Only one coupling procedure was tested since the anti-LamB responses were satisfactory. We do not, however, exclude that different presentation of these peptides could have yielded sera showing a better recognition of the LamB protein.

Antigenicity and Specificity. The next step was to set up a simple procedure for the detection of anti-LamB antibodies. It has been reported that ELISA assays are not suitable to monitor antibodies against LamB because the presence of detergent in the protein solubilization buffer prevents binding of LamB to plastic (Gabay et al., 1986). We did not encounter such a problem. By using an anti-LamB monoclonal antibody (E302), we found that, under our assay conditions, LamB was coated onto the microtiter plates and was readily detectable in the ELISA assay.

Both the anti-LamB and the anti-peptide responses were monitored in sera 2–4. We found that the anti-peptide titers were approximately 8–12-fold higher than the anti-LamB titer, indicating that only a small fraction of the antibody was able to bind to the native protein.

Interestingly, peptide 3 was found to be the most immunogenic in terms of anti-LamB and anti-peptides. Such a result is not surprising since (i) it is the most hydrophilic peptide, with a mean hydropathy of −1.44 (Table I), (ii) a polyclonal antiserum directed against denatured LamB reacted strongly with peptide 3 but poorly with peptide 2 and undetectably with peptide 1 and 4 in a dot assay (data not shown), and (iii) epitopes recognized by two monoclonal antibodies (E177 and E302) were mapped in part to the peptide 3 region (Desaymard et al., 1986). Thus, peptide 3 corresponds to an immunodominant region of LamB.

Analysis of the sequence specificity of each serum (see Results) provides indications on the major antigenic determinant of the corresponding peptide.

Results obtained with serum 1 and the lack of immunogenicity of peptide 1, when coupled by its C-terminus to the carrier, suggest that the major antigenic determinants of peptide 1 are located toward one or both extremities.

Serum 2, elicited against region 147–161 of LamB, was directed essentially against the C-terminus of the sequence.

In particular, insertion of four residues after the fourth amino acid of peptide 3 does not modify recognition. The major antigenic determinant of peptide 3 involved at least some of its four N-terminal residues.

The RGDS sequence is not the antigenic site of peptide 4. It should be recalled that RGDS is the active site on fibronectin, a protein cell adhesion factor, and that LamB is able to promote cell adhesion (Ruoslahti & Pierschbacher, 1986). We could not define any further the antigenic domain of peptide 4.

Interestingly, serum 4 allowed detection of the LamBΔ mutant protein which carries a large deletion from residues 147–373, and had never been detected by immunoblotting or by immunoprecipitation with the previously available anti-LamB sera (Boulain et al., 1986). This lack of detection was therefore not due, as initially hypothesized, to instability of the protein but rather to the loss of immunogenic determinants. Serum 4 should provide a useful tool to study the exportation of this mutant protein.

The use of mutant proteins thus appeared useful for characterization of anti-peptide sera. Studies with more LamB mutants should allow us to define more precisely the critical antigenic residues.

Topology and Phage Receptor Site. The four sera were then used to localize the corresponding LamB regions with respect to the outer membrane. This was done by examining the binding of antibodies to intact cells and by studying the potential of the sera to compete with phage adsorption.

Serum 2 displayed comparable ELISA titers with respect to the extracted LamB protein and to LamB on intact cells. This shows that, in the outer membrane, peptide 2 is accessible to the antibodies. This was confirmed by immunoelectron microscopy. Since the immunogenic part of peptide 2 is contained within C-terminus half, we may further propose that at least part of the sequence from residues 155–161 is accessible to antibodies in vivo.

Results obtained with serum 3 indicate that peptide 3 is exposed at the cell surface. However, serum 3 did not allow detection of LamB by immunoelectron microscopy, suggesting that the accessibility of peptide 3 in vivo was less than that of peptide 2. It is interesting to mention that topological studies, including immunoelectron microscopy, with a foreign epitope inserted within peptides 2 and 3 have already suggested a better accessibility of the region of peptide 2.

Serum 4 also reacted with intact cells in ELISA. This provides the first experimental evidence that peptide 4 (or at least part of it) is exposed at the cell surface. Mutations at site 401 (within peptide 4) affected the phage λ ability to infect *E. coli*. However, this LamB region has been found not to be involved in the primary reversible interaction between phage and receptor but only in a later irreversible step (Braun-Breton & Hofnung, 1981). Before this study, there was thus no compelling reason to propose that this region was exposed, and alternative models where this region was periplasmic were not

discounted (Charbit et al., 1984). It should be noted that serum 4 did not allow detection of LamB by immunoelectron microscopy.

Serum 1 failed to detect LamB on intact cells by ELISA as well as by immunoelectron microscopy, suggesting that peptide 1 is not accessible on the cell surface. The internal localization of this region was confirmed by showing that peptide 1 is detected on membrane fragments Such a result is compatible with the present model of LamB folding where the first part of peptide 1 is embedded in the outer membrane and the terminal part constitutes a loop in the periplasm.

Only serum 2 showed ability to inhibit phage inactivation by the LamB protein in vivo as well as in vivo. This confirmed that peptide 2 is exposed and plays a direct role in phage adsorption. The absence of competition by serum 3 may be due to various reasons such as low affinity of the serum or poor accessibility of the peptide to the serum. Serum 4 did not compete with phage adsorption in vivo and in vitro, but, as recalled above, this region is probably not involved in the primary binding of the phage on its receptor (Braun-Breton & Hofnung, 1981).

The different approaches used in this study yield a coherent view of the accessibility of the four regions of the LamB protein studied as well as of the sensitivity of the methods used to probe the topology. Peptide 2 was shown to be exposed by all criteria: ELISA, immunoelectron microscopy, and inhibition of phage adsorption. Peptides 3 and 4 were detected by ELISA on intact cells but not by immunoelectron microscopy. We propose that they are also expressed in a less protruding way. With the protocols used by us, immunoelectron microscopy appeared to be less sensitive than ELISA for detecting these peptides.

By comparing the ability of anti-peptide antibodies to bind LamB in vitro, in cellular extracts, and in vivo, on intact cells, we have thus been able to bring direct experimental evidence for the location of three regions of LamB at the cell surface. In the case of region 1, the absence of detection of LamB on intact cells, but its detection in membrane fragments, suggests strongly that it is exposed to the periplasm.

Finally, since two-dimensional crystals of LamB have recently been obtained (Lepault et al., 1988), one may expect that they could be used to probe directly the topology of the periplasmic region at the corresponding face of the crystal with the relevant anti-peptide antibodies.

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