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Mutations affecting antigenic determinants of an outer membrane protein of *Escherichia coli*

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The *Escherichia coli* LamB protein is located in the outer membrane. It is both a component of the maltose and maltodextrin transport system, and the receptor for phages λ and K10. It is a trimer composed of three identical polypeptide chains, each containing 421 residues. Six independent mutants have been isolated, in which the LamB protein is altered in its interaction with one or more monoclonal antibodies specific for regions of the protein that are exposed at the cell surface. Some of the mutations also altered the binding site for phage λ. All of the mutations were clustered in the same region of the *lamB* gene, corresponding to residues 333–394 in the polypeptide. This and previous results strongly suggest that a rather large segment of the LamB polypeptide, extending from residue 315 to 401, is exposed at the outer face of the outer membrane. This segment would bear the epitopes for the four available anti-LamB monoclonal antibodies that react with the cell surface, and part of the binding site for phage λ.

Key words: LamB protein/phage λ receptor/epitopes/monoclonal antibodies/structure of membrane protein

Introduction

Our knowledge of the secondary and tertiary structure of integral membrane proteins is still very limited (Henderson and Unwin, 1975; Deisenhofer *et al.*, 1984, 1985). In most cases, models for the structure of membrane proteins are based on their amino acid sequences and on spectroscopic data. Experimental data concerning the topography of membrane proteins, and in particular the identification of exposed regions of these proteins, are of importance to validate the theoretical models. In the present work, we have used a new approach to obtain such topographical information on the outer membrane protein encoded by the *lamB* gene in *Escherichia coli*.

The LamB protein is a porin involved in the transport of maltose and maltodextrins and also functions as the receptor for phages like λ and K10 (reviewed in Schwartz, 1983). In its native state, the LamB protein is an oligomer composed of three identical subunits (Palva and Westerman, 1979; Neuhaus, 1982) and is exposed at both faces of the membrane (reviewed in Gabay *et al.*, 1985). Spectroscopic data indicate that its secondary structure consists predominantly of β-pleated sheets (Ishii *et al.*, 1981; Neuhaus, 1982). The amino acid sequence, as deduced from the nucleotide sequence of the *lamB* gene (Clement and Hofnung, 1981), indicated that the polypeptide is composed of 421 amino acid residues. Examination of the sequence failed to reveal long hydrophobic portions constituting obvious candidates for membrane-spanning segments (Clement and Hofnung, 1981). However, secondary structure predictions, and an analysis of the hydropathic profile, suggest that the polypeptide may cross the membrane as many as 13–17 times (Charbit *et al.*, 1984). A combination of approaches has led to the identification of four regions of the protein which seem to be exposed at the outer face of the membrane (reviewed in Gabay *et al.*, 1985). The location of these regions would be compatible with the above-mentioned structure predictions.

In a new attempt to identify surface-exposed portions of the LamB polypeptide we made use of a set of previously characterized anti-LamB protein monoclonal antibodies (mAbs). Four of these mAbs are specific for antigenic determinants exposed at the cell surface (Gabay and Schwartz, 1982; Schenkman *et al.*, 1983). Mutants bearing specific alterations in one or more of the epitopes recognized by these mAbs have now been isolated, and the corresponding mutations are mapped by DNA sequencing. The results, together with those of previous studies, strongly suggest that a large domain of the LamB polypeptide, located rather close to the carboxy-terminal end, is exposed at the cell surface.

Results

Selection of mutants with altered LamB epitopes

It was previously observed that *E. coli* K12, which contains LamB protein in its outer membrane, is specifically killed by anti-LamB protein serum in the presence of complement (Gabay, 1977). This is also the case when anti-LamB mAbs are used instead of serum, provided that these mAbs are (i) specific for epitopes exposed at the cell surface and (ii) of isotypes which fix complement. Of the six available anti-LamB mAbs, four are specific for surface-exposed epitopes, and have been called E-mAbs, the others having been called I-mAbs (Schenkman *et al.*, 1983). Three of the four E-mAbs, numbered 72, 177 and 302 fixed complement (they are of the γ2 isotype) whereas the fourth, mAb347, did not (γ1 isotype) (Gabay *et al.*, 1985). A mutant with an alteration in the epitope recognized by a given mAb should survive the cytotoxic effect of this mAb in the presence of complement. However, most of the mutants found to survive such a selection either lacked the LamB protein or had greatly reduced amounts of it (data not shown). To obtain mutants which produced normal amounts of a structurally altered protein, we combined the selection procedure described above with a cycle of growth in the presence of 10^{-5} M maltose, i.e. under conditions where cells with reduced amounts of LamB protein are at a severe growth disadvantage (Szmelcman and Hofnung, 1975). Colonies of bacteria which survived several cycles of this double selection were then screened by immunoblotting using both the mAb and a polyclonal anti-LamB serum. Colonies which were negative with the mAb and positive with the anti-LamB protein serum were considered potential mutants. Six independent mutants were studied. Four were obtained from a selection with mAb302, and two from a selection with mAb72. No mutant has yet been obtained with mAb177. When P1 phage grown on these six mutants was used to transduce strain pop3350, (deleted for the *lamB* gene), all of the resulting Mal^+ transductants had the same antigenic pheno-

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Table I. Antigenic phenotype of the mutants

Bacterial strains	Relevant genotype	Mutagen	mAb used for selection	Immunological reactivity of the monoclonal antibodies							
				Whole bacteria				Bacterial extracts			
				302	72	347	177	302	72	347	177
pop3	lamB$^+$			++	++	++	++	++	++	++	++
pop3205	lamB102	EMS		−	−	−	−	−	−	−	−
pop2517	lamB2517	EMS	302	±	+	−	++	±	++	−	++
pop2518	lamB2518	EMS	302	±	+	−	++	±	++	−	++
pop2519	lamB2519	u.v.	302	−	+	+	++	−	++	++	++
pop2520	lamB2520	u.v.	302	±	+	−	++	±	++	−	++
pop2521	lamB2521	u.v.	72	−	−	±	++	−	−	++	++
pop2522	lamB2522	u.v.	72	+	−	++	++	++	+	++	++
pop1081	lamB103	EMS		−	++	++	++	−	++	++	++
pop1084	lamB106	EMS		++	++	++	++	++	++	++	++

Strains pop2517−2522 are derivatives of pop3 (*lamB*$^+$) carrying the different mutations isolated during the course of this work. Strain pop3205 carries an early nonsense mutation in *lamB*. Strains pop1081 and pop1084 are previously described λ-resistant mutants with alterations at residues 382 and 401 in the LamB protein, respectively. (The other λ-resistant mutants described by Charbit *et al.*, 1984, were also tested, and all reacted like wild-type with the mAbs). ELISA tests were performed both with whole bacteria and with bacterial extracts. In the first case the bacteria were adsorbed onto microtiter plates by incubating at 37°C cells suspended at 5×10^8 cells/ml in 0.1 M sodium bicarbonate buffer, pH 9.6. In the second case the cell extracts were tested on plates which had been coated with anti-LamB polyclonal IgG, as previously described (Bloch and Desaymard, 1985). The notations ++, +, ± and − correspond to 100−80%, 80−40%, 40−10% and <10% of the values found with pop3, respectively.

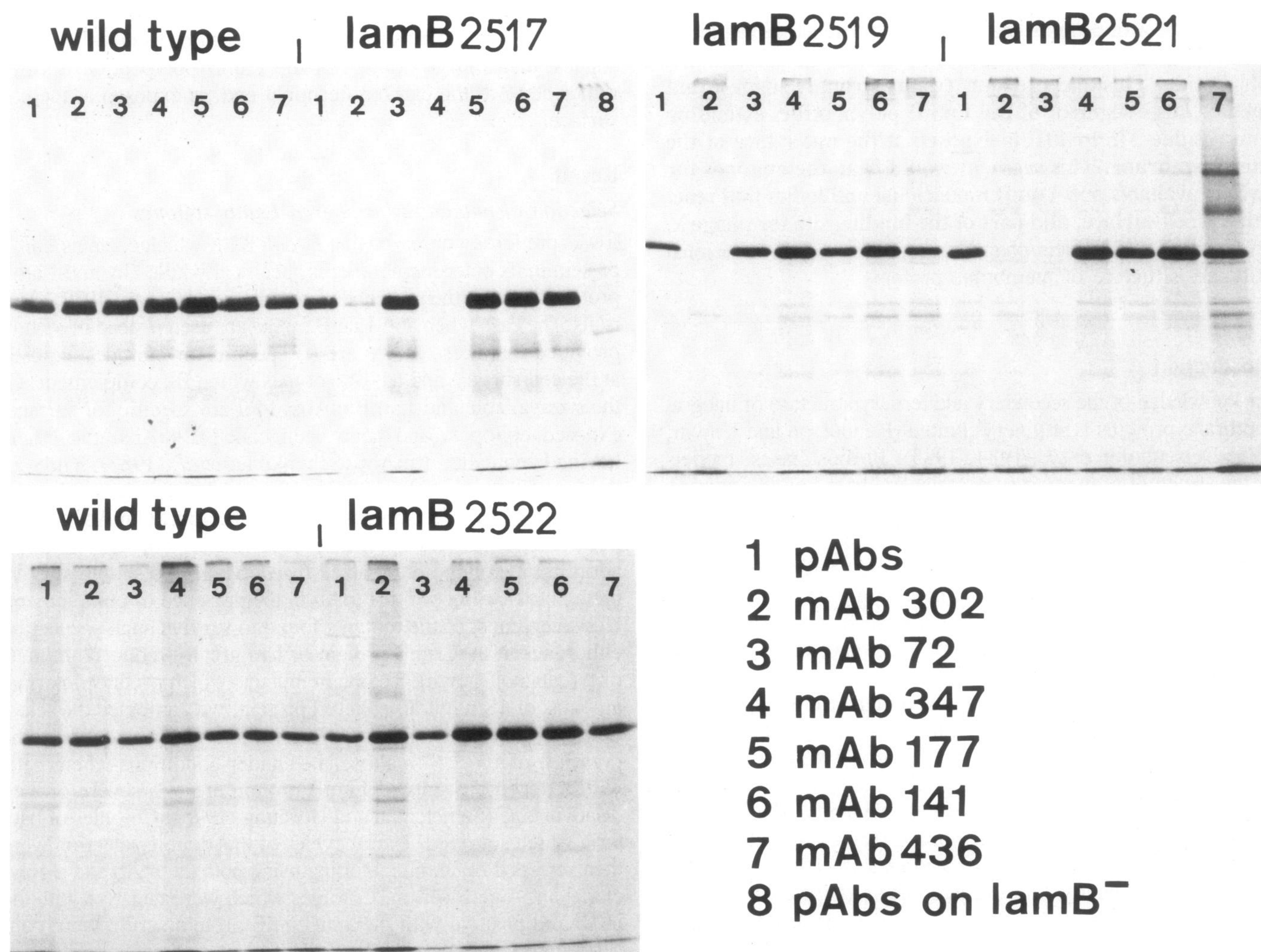

Fig. 1. Characterization of the mutant LamB proteins by immunoprecipitation. Ascites fluid containing the mAbs, or purified anti-LamB protein rabbit IgG (pAbs) were incubated with extracts from cells labelled *in vivo* with [^{35}S]methionine. Protein A−Sepharose was used for precipitation of the immune complexes. The pellets were dissolved, and subjected to polyacrylamide gel electrophoresis followed by autoradiography. **Lane 8** (pAbs on LamB$^-$) is a control where purified anti-LamB protein IgG was incubated with extracts from a strain (pop3205) devoid of LamB protein.

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         333              382             386 387     389          394              401
...Glu Ser Gln ... Thr Gly Asn ... Asp Asn Asn Ala Asn Phe ... Ala Val Pro ... Gly Gly Ser
...GAA TCC GAC ... ACC GGT GCT ... GAT AAC AAC GCG AAC TTC ... GCC GTT CTT ... GGC GGC AGC

         TTC          GAT          TAC     GAC GCG TAC             TTT              GAC
         Phe          Asp          Tyr     Asp     Tyr             Phe              Asp
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lamB mutation	2517,2518, 2520	103	2522	2519	2521	106
mAb302	±	-	+	-	-	+
mAb72	+	+	-	+	-	+
mAb347	-	+	+	+	+	+
phage λV	S	R	R	R	S	R
phage λVho	S	S	S	S	S	R

Fig. 2. Mutations altering surface-exposed epitopes and their position with regard to λ-resistant mutations. Portions of the wild-type amino acid and nucleotide sequences are shown on the first lines, the numbers corresponding to the position of the amino acid residues in mature LamB protein. Under these lines are indicated: the nature of the different mutations, their denomination (*lamB2517* etc.), their effect on the structure of the epitopes recognized by mAbs 302, 72 and 347 (+, wild-type epitope; − or ±, altered epitope) and their effect on the sensitivity of the cells to phages λV and λVho (S, sensitive; R, resistant). Uninterrupted arrows correspond to mutations described in this work. Interrupted arrows correspond to previously sequenced λ-resistant mutations (Clement *et al.*, 1983).

type as the original mutants. The transductants, rather than the original mutants, were used for the rest of this work (pop2517–2522).

Immunochemical characterization of mutant LamB proteins

The reactivity of the mutant LamB proteins with the mAbs was studied by three different tests. Enzyme-linked immunosorbent assays (ELISAs) were performed with whole cells and with cell extracts containing detergent (octyl POE), and immunoprecipitations were performed with ^{35}S-labeled cell extracts. The ELISA performed with whole cells from mutants selected with mAb302 or mAb72 were negative with these same mAbs, as expected (Table I). The results of the two tests performed on cell extracts (ELISA and immunoprecipitations) were perfectly consistent with one another (Table I and Figure 1). Unexpectedly, however, the tests performed with whole cells and the tests performed with cell extracts did not always yield identical results. For instance a clear discrepancy is seen for strain pop2522. This strain failed to react *in vivo* with mAb72, the antibody used for the selection, but it displayed a quasi-normal reactivity with this antibody *in vitro*. This result indicates a conformational change of the epitope upon solubilization of the protein.

In two of the mutants, pop2519 and 2522, the only altered epitope corresponded to the mAb used for the selection. In the other mutants, however, the epitope for another mAb was also altered (for mAb347 in three mutants, and for mAb302 in one mutant). The epitope for mAb177 was unaltered in the six mutants, as were the epitopes for the two I-mAbs, numbered 141 and 436.

ELISA analysis, with whole cells and with cell extracts, was also applied to strains bearing the phage λ resistance mutations described by Charbit *et al.* (1984). All but one reacted normally with the six mAbs, the exception being a strain bearing mutation *lamB103* known to correspond to a change of residue 382 from Gly to Asp (Table I).

Effects of the mutations on maltose transport and phage adsorption

The mutants were all unimpaired in their ability to transport maltose at 10^{-6} M, consistent with the fact that they were selected for rapid growth in the presence of low concentrations of this sugar (data not shown). They were also unimpaired in their sensitivity to phage K10. With respect to phage λ, not all mutants behaved identically. For four of them, the efficiency of plating and the adsorption of phage λ were normal, whereas for two, pop2519 and pop2522, the efficiency of plating of this phage was 10^{-3} or less, and they failed to adsorb the phage at a detectable rate. Therefore, these two strains could be classified as λ-resistant mutants. Like several previously isolated λ-resistant mutants, including the *lamB103* mutant (Hofnung *et al.*, 1976; Clement *et al.*, 1983; Charbit *et al.*, 1984), these two strains were still sensitive to a host range mutant of λ called λho.

Mapping of the mutations by DNA sequencing

A new technique was used to clone the mutant *lamB* genes *in vivo* (see Materials and methods). DNA sequencing was then performed using the dideoxy technique of Sanger *et al.* (1977) (Figure 2).

Three of the strains, pop2517, 2518 and 2520, which were phenotypically indistinguishable, bore the same mutation resulting in a change of amino acid residue 333 from Ser to Phe. At the DNA level this change happened to convert a *Hin*fI restriction site into an *Eco*RI restriction site, as was verified directly. The mutations present in the three other mutants were all different. Noteworthy is the case of strain pop2519 which bears two closely linked mutations. In this case, we do not know whether the alteration of the epitope recognized by mAb302 requires the two mutations to be present together for the inactivation of a functional epitope. The nucleotide change corresponding to one of the mutations present in pop2519 resulted in the creation of a new restriction site for *Mlu*I. The presence of the site was also verified directly.

Discussion

From the limited number of mAb-resistant mutants analyzed during the course of this work, it is already possible to draw several conclusions concerning the structure of the LamB protein. The first is that the epitopes recognized by the four E-mAbs are

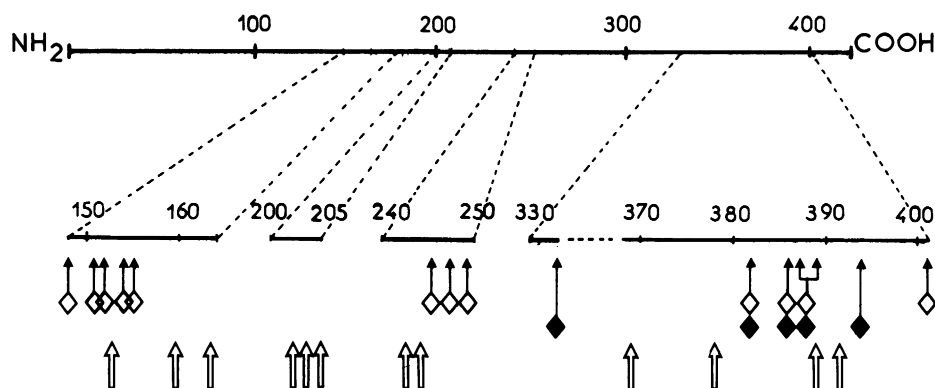

Fig. 3. Surface-exposed regions in the LamB polypeptide. The figure summarizes available information on amino acid residues and peptide bonds assumed to be exposed at the cell surface. Open vertical arrows indicate the points of cleavage by subtilisin or trypsin *in vitro* (Schenkman *et al.*, 1984). (Cleavage at these points is prevented by E-mAbs, but not by I-mAbs). The positions of amino acid alterations rendering the cells resistant to phage λ and/or K10 (Roa and Clement, 1980; Clement *et al.*, 1983; Charbit *et al.*, 1984; and this work) are shown by open diamonds. The positions of alterations inactivating at least one of the epitopes for E-mAbs (this work) are shown by closed diamonds.

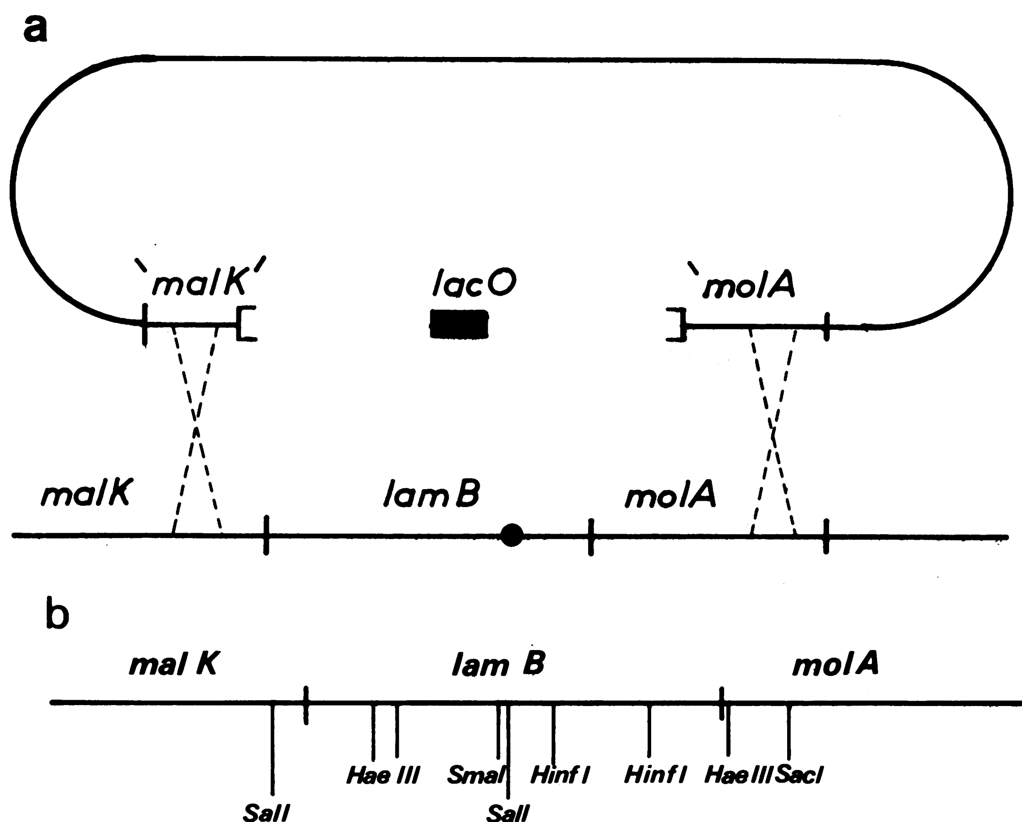

Fig. 4. Procedure used for cloning the mutant *lamB* genes. Plasmid pSM4 *malB*Δ*501* (**A**) was constructed by deleting a 2.8-kb DNA fragment containing the entire *lamB* gene from the 5.4-kb *Eco*RI fragment of bacterial DNA present in pSM4. A 95-bp fragment containing the *lac* operator (black box) was ligated into the plasmid in place of the missing DNA. Cloning of the mutant *lamB* genes (the mutation is shown as a dot) was obtained by selecting for the reciprocal recombination events shown in the figure (see text). The positions of the relevant restriction sites in *lamB* and the adjacent genes are shown in **b**.

necessarily all different. Indeed if two of them had been identical they would have been systematically altered together in all of the mutants. For the same reason, the epitopes and the binding sites for phages λ or K10 are distinct. However, the results indicate that the epitopes corresponding to three of the E-mAbs (72, 302 and 347), as well as the binding site for phage λ, do overlap or are at least located in close proximity to one another. Hence, a single amino acid change can alter two or three of these epitopes, or one epitope and the binding site for phage λ (Table I and Figure 2).

Are the amino acid changes described here located within the epitopes or do they induce conformational changes leading to an alteration of these epitopes? Biochemical and biophysical evidence indicates that the LamB protein is a rigid, highly structured molecule, firmly embedded in the membrane (Ishii *et al.*, 1981; Neuhaus, 1982; Schwartz, 1983). Genetic data show that missense mutations affecting the various functions of the protein are both extremely rare and generally located in a few very discrete areas of the gene (Roa and Clement, 1980; Wandersman and Schwartz, 1982; Clement *et al.*, 1983; Charbit *et al.*, 1984). This latter result would not be expected if single amino acid changes could easily alter the overall structure of the protein. Therefore, we

think it unlikely that the amino acid changes studied here would affect the epitopes through long-range conformational changes. However we do not exclude the possibility of short-range effects. Such effects could in fact explain the two cases where an amino acid change led to the disappearance of an epitope *in vivo*, and where this epitope reappeared upon solubilization of the protein (epitope 72 in pop2522 and epitope 347 in pop2521).

The location of the mutations described here (Figure 2) is perfectly consistent with previous studies indicating that a region essential to the structure of the epitopes for the four presently available E-mAbs is located in the immediate vicinity of residue 391, or downstream from it (Gabay *et al.*, 1983; Schenkman *et al.*, 1984). One point should be mentioned regarding the epitope for mAb302. We see here that it is altered by changes in amino acid residues which are located rather far apart, the extremes being at positions 333 and 394. This would seem to imply that the epitope for mAb302 is discontinuous, and that in the native protein residues 333, 382, 387 and/or 389, and 394 might be close to each other. This might not have been anticipated since mAb302 was reported to still react with LamB protein which had been denatured by boiling in the presence of SDS (Gabay *et al.*, 1983). However this latter conclusion was based on the observation that mAb302, in the presence of protein A, induced the precipitation of LamB protein which had first been boiled in the presence of SDS, and then diluted in a non-ionic detergent. Quantitative experiments (S.Schenkman and M.Schwartz, unpublished) have later shown that only ~20% of the LamB protein was precipitated under these conditions, even at saturating mAb concentrations, whereas essentially 100% was precipitated when native LamB protein was used. This result would be consistent with the notion that the mAb302 epitope is discontinuous, and that a fraction only of the SDS-denatured protein molecules undergo a partial renaturation which enables them to be recognized by mAb302.

It now seems extremely likely that the polypeptide segment extending from residue 369 to 401 is exposed at the cell surface. Indeed this segment is involved in the binding of three different E-mAbs, and of phages λV and λVho (Figures 2 and 3). In addition, it is cleaved in four different places by proteolytic enzymes acting on the LamB protein *in vitro*, this action being prevented by E-mAbs. We already mentioned that the exposure of this segment and three other previously defined segments at the cell surface (see Figure 3), would be compatible with secondary structure predictions and with the hydropathic profile of the LamB polypeptide protein.

One result obtained in the present study does not appear to be immediately compatible with the published models. The change of amino acid at position 333, obtained three times independently, resulted in an alteration of the epitopes for two E-mAbs, namely mAbs302 and 347. Residue 333, according to published models, would be located at the inner face of the outer membrane (Charbit *et al.*, 1984). It seems unlikely that an amino acid change in a segment located at the inner face of the membrane could induce a conformational change in epitopes located on the other face of the membrane. This change would have to be very subtle indeed since the binding sites for mAb72, and for phage λ, which seem to overlap with the mAb302 epitope, were not detectably altered. When one examines the published models for LamB protein structure there is no compelling reason to assume that the polypeptide crosses the membrane between residues 315 and 401. Therefore we favor the idea that this whole region, including region 333, is exposed at the cell surface. This rather large C-terminal domain bears part or all of the binding sites for the four available E-mAbs as well as for phage λ. Different wild strains of *E. coli* and *Shigella* were recently shown to vary very much in the ability of their LamB protein to bind the different E-mAbs (Block and Desaymard, 1985). This variability may reflect the fact that constant selective pressures are exerted to alter the structure of exposed regions of the polypeptide, which would tend to serve as receptors for substances that are toxic to the bacterium. Sequencing of the *lamB* gene from several strains of *E. coli* and *Shigella* may provide additional information on the structure of the epitopes for the E-mAbs.

In a previous study (Schenkman *et al.*, 1984) we showed that four short segments of the LamB polypeptide were protected by E-mAbs against proteolysis *in vitro*, and concluded that these segments were probably exposed on the cell surface *in vivo*. Now, we have shown that specific amino acid residues located in one of these segments are involved in the binding of these mAbs. This work with the LamB protein demonstrates that monoclonal antibodies can be used as additional tools to probe the structure of membrane proteins.

Materials and methods

Strains, media and miscellaneous techniques

Most bacterial strains were derived from *E. coli* K12 strain pop3 (F^- *Δlac U169*, *araD139*, *rpsL*, *thi*, *relA*) described as MC4100 by Casadaban (1976). Strain pop3205 carries the *lamB102* nonsense mutation (Hofnung *et al.*, 1976) and pop-3350 carries deletion *malBΔ15* which covers most of the *lamB* and part of the *malK* genes (Raibaud *et al.*, 1979). Strain pop2502 is a derivative of MM294 (Talmadge and Gilbert, 1980) and carries deletion *malBΔ101* which covers the entire *malB* region, including the *lamB* gene (Raibaud *et al.*, 1979).

Phage strains (λV, λVho, K10 and P1), monoclonal antibodies, growth media, and most genetical as well as immunological techniques, including the preparation of mono- and polyclonal antibodies were described in previous publications (Miller, 1972; Wandersman and Schwartz, 1982; Bloch and Desaymard, 1985).

Mutant selection

Several cultures of strain pop3 were mutagenized with ethyl methanesulfonate (EMS) or u.v. light. After growth in M63 maltose medium, the bacterial cells were washed, and $\sim 5 \times 10^7$ cells were incubated at 0°C for 1 h with mAb diluted in 200 μl of veronal buffer (5 mM Veronal, 0.15 M NaCl, 0.15 mM $CaCl_2$, 0.15 mM $MgCl_2$, pH 7.5). The cells were pelleted, resuspended in 200 μl of veronal buffer containing pre-adsorbed guinea pig serum as a source of complement, and incubated at 37°C for 30 min. Optimal concentrations of both mAb and complement were determined in order to obtain a maximal killing (80−99%) of pop3 (*lamB*$^+$) and a minimal killing (<20%) of pop3205 (*lamB*102). Cells which survived the treatment with mAb plus complement were first grown in M63 medium containing 10^{-2} M maltose. Then, 10^6 cells were diluted into 100 ml of M63 supplemented with 10^{-5} M maltose. After overnight growth, maltose was added to a concentration of 10^{-2} M to allow bacterial growth to saturation. Two to four additional cycles of selection were performed. After the last treatment, the cells were plated on minimal maltose agar. Mutant colonies were transferred to two nitrocellulose filters (Millipore). The filters were saturated wth 1% bovine serum albumin (BSA) and one of them was incubated with the mAb used for the selection and the other with the polyclonal anti-LamB protein IgG fraction. The binding of the antibodies to bacteria was revealed by using anti-mouse or anti-rabbit immunoglobulin antibodies labelled with horseradish peroxidase (Biosys France) and diaminobenzidine (Sigma) as substrate.

Cloning of the mutant lamB genes in vivo

Plasmid pSM4, a gift from S.Michaelis, is a derivative of pBR322 carrying a 5.4-kb *Eco*RI DNA fragment containing the *lamB* gene of *E. coli* (Figure 4). This plasmid was digested with the restriction endonucleases *Sma*I and *Sac*I and then with nuclease *Bal*31, in such a way that the *lamB* gene was entirely deleted (the *Sal*I site at the end of the *malK* gene was eliminated) while extensive bacterial sequences were retained on both sides of the deletion. Ligation of the plasmid was performed in the presence of a 10-fold molar excess of a 95-bp *Alu*I fragment containing the *lac* operator (a gift from H.Buc's laboratory). A new plasmid was thus obtained, pSM4 *malBΔ501*, in which 2.8 kb of bacterial DNA, including the *lamB* gene, had been replaced by a 95-bp DNA fragment containing the *lac* operator. When this plasmid was introduced into a *lac*$^+$ strain such as pop2502, titration of the *lac* repressor resulted in the constitutive synthesis of β-galactosidase (Sadler *et al.*, 1977) and, therefore, in the formation of dark blue colonies on

solid media containing 10 μg/ml of tetracycline and 40 μg/ml of 5-bromo-4-chloro-3-indolyl β-D-galactoside (Xgal). This provided a means to identify recombinants in which reciprocal events had occurred such that the chromosomal *lamB* gene had been transferred onto the plasmid (Figure 4). Such recombinants, which did not carry multiple copies of *lac* operator, gave pale blue colonies on Xgal-containing agar. The cloning of the mutant *lamB* genes was then performed as follows: using phage P1, the mutant genes were transduced into the pop2502 background (Mal^+ selection), the transductants were transformed with pSM4*malB 501* DNA which had been irradiated with u.v. light to increase the frequency of recombination, as described by Chattoraj *et al.* (1984), and the transformants were plated onto complete medium agar (L agar) containing 10 μg/ml tetracycline and 40 μg/ml Xgal. Pale blue colonies, which appeared at a frequency of ~2%, were purified, and the presence of a plasmid containing the *lamB* gene was confirmed.

DNA sequencing

Sequencing of the DNA corresponding to the first 80 residues of LamB was not performed for the five mutants altered in the mAb302 epitope since deletion analysis has previously shown (Gabay *et al.*, 1983) that all of the determinants involved in the constitution of this epitope are located in the second half of the polypeptide. In the case of *lamB*2417, 2518, 2519, 2520, 2521, the 1-kb *Hae*III fragment containing the sequence encoding from residue 80 to the carboxy-terminal of the LamB protein was purified from the plasmid. The *Hae*III fragment was digested with *Hin*fI or *Alu*I. All of the resulting fragments were subcloned into the *Sma*I site of phage M13 mp11 and sequenced. In the case of mutant *lamB*2522, which was not affected in epitope 302, the entire *lamB* gene was sequenced. The 1-kb *Hae*III fragment was digested and sequenced as above. In addition, the 750-bp *Sal*I fragment encoding the NH_2-terminal end of the LamB protein was purified from the plasmid digested with *Hae*III. The resulting fragments were subcloned in the *Sma*I site of phage M13 mp11 and sequenced. DNA sequencing was performed according to the dideoxy technique of Sanger *et al.* (1977). With all mutants the only nucleotide changes found in the sequenced fragments were those shown in Figure 2.

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