The EMBO Journal vol.5 no.11 pp.3029-3037, 1986

Probing the topology of a bacterial membrane protein by genetic insertion of a foreign epitope; expression at the cell surface

Alain Charbit, Jean Claude Boulain[2], Antoinette Ryter[1] and Maurice Hofnung

Unité de Programmation Moléculaire et Toxicologie Génétique, (CNRSUA271, INSERMU163) and [1]Unité de Microscopie éléctronique, Institut Pasteur, 25 rue du Dr Roux, 75015 Paris, France

[2]Present address: Service de Biochimie, Département de Biologie, Centre d'Etude Nucléaire de Saclay, Saclay, France

Communicated by M.Hofnung

The LamB protein is a trimeric integral outer membrane protein from *Escherichia coli* K12 which functions as a pore for maltodextrins and a receptor for bacteriophages. When inserted into two selected sites of LamB, a foreign antigen, the C3 epitope from poliovirus, was exposed at the cell surface with its normal antigenic properties. Since these genetic insertions did not affect in any essential way the routing, activity and folding of the LamB protein, we conclude that the two corresponding LamB sites are at the cell surface as predicted by our recent model. We discuss the implications of our results for the study of protein topology with a single epitope and the direct cloning and cell surface expression of epitopes of interest as well as the development of live vaccines or diagnostic tests.

Key words: protein structure/epitope insertion/bacterial surface/vaccines

Introduction

The LamB protein is a trimeric integral outer membrane protein from *Escherichia coli*. It serves two main biological functions. Firstly, LamB is the 'maltoporin'; it is involved in the permeation of maltose and maltotriose, and required for the entry of higher dextrins (review in Nikaido and Vaara, 1985). Secondly, LamB is the 'λ receptor' (Thirion and Hofnung, 1972; Randall-Hazelbauer and Schwartz, 1973) used as cell surface receptor by a number of bacteriophages including phage λ (Charbit and Hofnung, 1985 and references therein). We recently proposed a first model for the folding of the protein in the outer membrane (Figure 1; Charbit *et al.*, 1984). This model was based on the sequence of the protein deduced from DNA sequence data (Clément and Hofnung, 1981) and on the assumption that regions where mutations led specifically to resistance to phage λ were located at the outside of the cell.

To provide a direct test of our model we inserted a foreign epitope into three sites of the LamB protein located in two regions that were predicted to be facing the outside of the cell. We show that the insertions did not affect in any essential way the stability, location and functions of LamB. We demonstrate that in two cases the epitope is recognized on whole cells by specific antibodies, and thus the two corresponding LamB regions are indeed at the cell surface. Since these constructions result in stable bacterial strains with a new surface antigen, they are also of potential utility to devise 'exposition vectors' able to present a given epitope at the cell surface (Charbit *et al.*, 1986).

We discuss the perspectives opened by our results for the study of protein topology, for the development of live vaccines, diagnostic tests and the direct cloning at the bacterial cell surface of epitopes of interest.

Results

Insertion of the C3 epitope

We had previously determined sites in the LamB protein where it is possible to insert several amino acids (in one case up to 11) without affecting most of its properties (Boulain *et al.*, 1986). The modified proteins were present as trimers in the outer membrane, and conferred the capacity to grow on dextrins and to adsorb phages requiring the LamB protein. We started with three different modified LamB proteins. The modifications had been made by insertion of a *Bam*HI linker after amino acid 146 (plasmid pAJC352), amino acid 153 (plasmid pAJC264) and amino acid 374 (plasmid pAJC178) of the mature LamB protein (Boulain *et al.*, 1986). These residues were located in segments of the protein predicted to be exposed on the outside of the cell (Charbit *et al.*, 1984) (Figure 1).

As a passenger we chose the well characterized C3 epitope from the VP1 coat protein of type 1 poliovirus. The three-dimensional structure of poliovirus has been determined at 2.9 Å resolution by X-ray crystallography (Hogle *et al.*, 1985). The C3 epitope is a hydrophilic peptide which corresponds to a turn located at amino acid residues 93-103 of the VP1 protein, a major immunogenic protein of the virus (Wimmer *et al.*, 1984). C3 is a neutralization epitope against which a monoclonal antibody raised against heat-inactivated virions was available (called hereafter anti-C3 monoclonal antibody) and a polyclonal serum raised against the corresponding synthetic peptide (called hereafter anti-VP1 serum).

An oligonucleotide encoding the 11 amino acid residues of the C3 epitope and flanked by *Bam*HI cohesive ends was inserted into the selected sites of gene *lamB* (Figure 2; Materials and methods). Gene *lamB* was carried on the multicopy plasmid pAC1 under *tac* promoter control (Boulain *et al.*, 1986). The length of the coding strand of the insert corresponded to an exact number of codons (13 codons) to maintain the reading frame in *lamB*. We had previously shown that this was an essential condition to obtain non-toxic constructions (Boulain *et al.*, 1986). The insert-carrying clones were tested by 'immunoblotting' after heat denaturation with anti-LamB monomer serum and with the anti-VPI serum (Figure 3). About half of the clones encoded a protein reacting with both sera and presenting a slower migration than the wild-type LamB protein, as expected for the in-phase addition of 13 residues including the C3 epitope. These hybrid proteins were named according to the site of insertion of the C3 epitope: 146-C3, 153-C3 and 374-C3.

Properties of the modified LamB proteins

The amounts of hybrid proteins detected by immunoblotting were comparable to those of wild-type LamB protein, indicating that

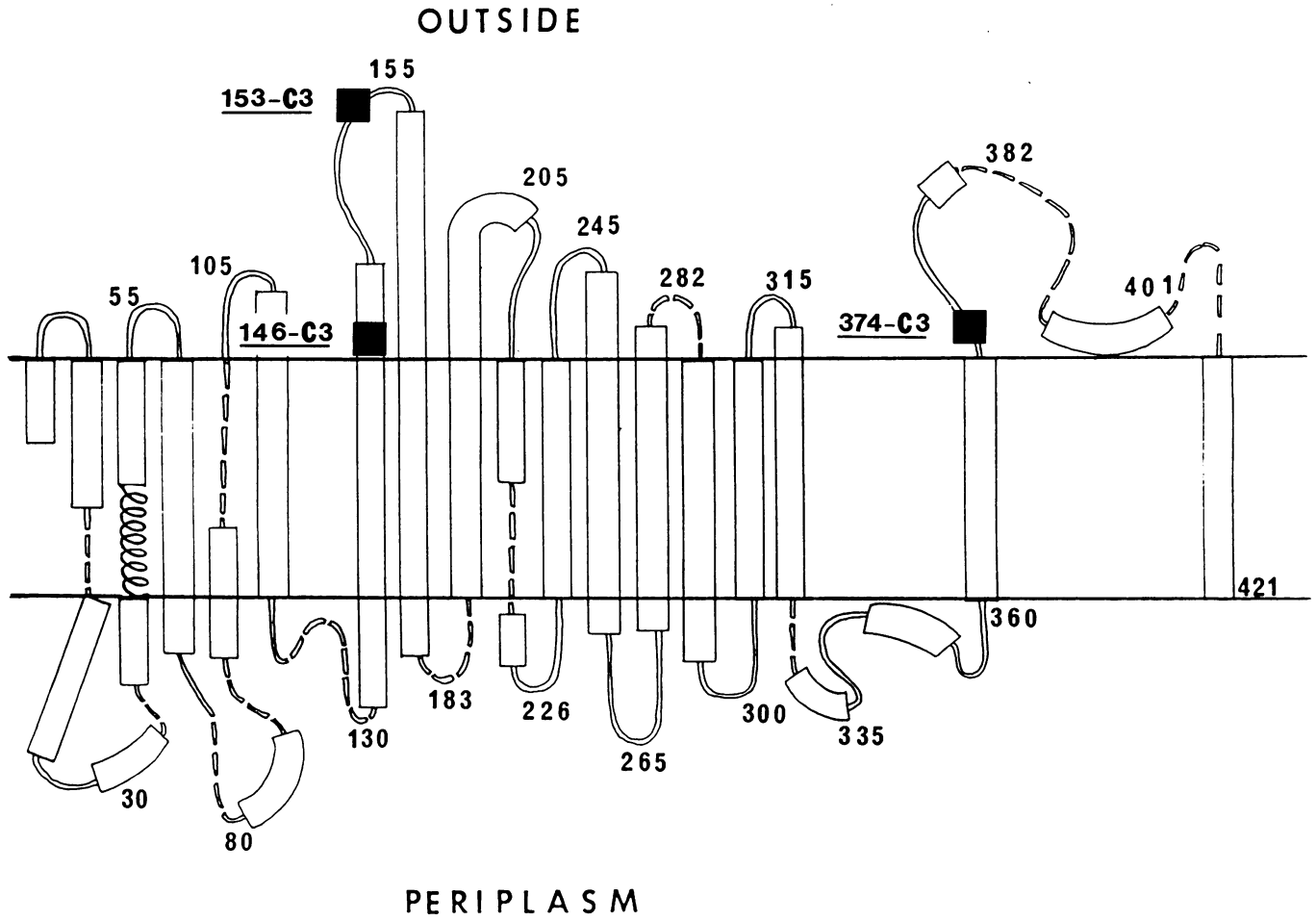

Fig. 1. Model for the folding of the LamB protein in the outer membrane. This model was based on DNA sequence data and on the localization of a missense mutation yielding phage resistance (Charbit *et al.*, 1984). The symbols for secondary structure are the following: || beta extended, — turn, λλλλλ alpha-helix, ----- coil. Amino acid residues are numbered from the N-terminus of the mature protein. The inserts at residues 146, 153 and 374 are indicated by dark squares; they correspond respectively to plasmids pAJC352−C3, pAJC264−C3 and pAJC178−C3.

the hybrid proteins were stable (Figure 3). On induction of synthesis of the hybrid proteins with isopropyl β-D-thiogalactoside (IPTG) during exponential growth, no inhibitory effect could be detected (see details in Materials and methods). Since overproduction of mutant proteins unable to yield trimers is usually toxic (Bouges-Bocquet *et al.*, 1984a, 1984b; Boulain *et al.*, 1986), this result suggested that the hybrid proteins were normally assembled in the outer membrane.

We then examined more precisely whether the insertion of the epitope had modified the properties of the initial LamB mutant proteins. Two immunological techniques were used: (i) immunoprecipitation of non-heat-denatured extracts with an anti-LamB trimer antibody; (ii) *in situ* immunodetection with the same serum.

As mentioned above, the active form of the LamB protein is a trimer. Upon SDS−PAGE, in the absence of heat-denaturation, trimers correspond to several bands (mainly two) with different migrations. (At least two factors may result in such trimer subspecies: binding of different amounts of detergent and binding of another envelope component). Also higher multimers, most probably aggregated trimers, can be detected; we refer to these as multimers (Vos-Sheperkeuter and Witholt, 1984; Boulain *et al.*, 1986).

For the three LamB−C3 hybrid proteins, trimers and multimers could be detected by immunoprecipitation with the anti-LamB trimer serum (Figure 4). *In situ* immunodetection revealed clearly the hybrid proteins 146−C3 and 153−C3 even in the absence of lysis with chloroform (data not shown). The 374−C3 hybrid was very poorly detected and only after lysis with chloroform, presumably because, in this case, insertion of the C3 epitope inactivates a major antigenic determinant (Boulain *et al.*, 1986; Gabay *et al.*, 1985; Desaymard *et al.*, 1986).

The biological properties of the hybrid proteins were then examined (Table I). The 153−C3 hybrid protein conferred both the capacity to grow on dextrins and the sensitivity to certain phages requiring the LamB protein. The 374−C3 protein was comparable to the 153−C3 protein, except that it allowed only growth of the phage with the most extended host-range. Hybrid protein 146−C3 was able to confer the ability to grow on dextrins but no sensitivity to any of the phages which use the LamB protein.

In conclusion, the three hybrid proteins were able to yield trimers (and multimers) and to confer to *E. coli* at least some of the biological properties due to the LamB protein. This suggested strongly that the hybrid proteins were normally localized in the outer membrane and that their folding and organization did not differ in any essential way from those of the wild-type LamB protein. *In situ* immunodetection confirmed this in the cases of hybrids 146−C3 and 153−C3.

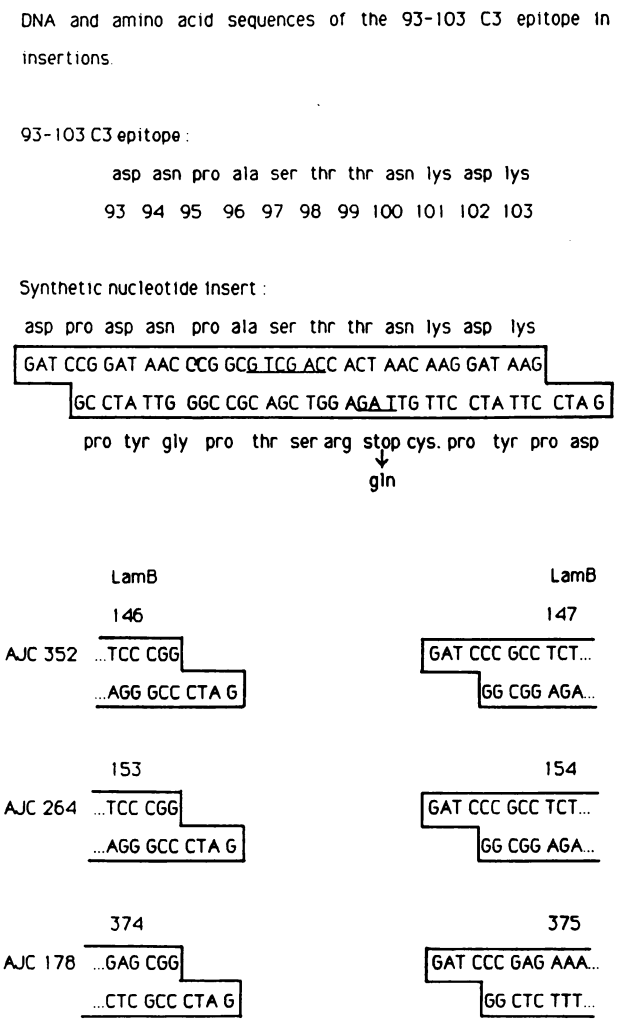

Fig. 2. DNA and amino acid sequence of the C3 epitope inserts into the LamB protein. The upper part = amino acid sequence of the C3 epitope corresponding to amino acid residues 93–103 from the VP1 protein of poliovirus serotype 1. The middle part = the DNA sequence of the double-stranded synthetic oligomer encoding the C3 epitope (39 mer). The codons chosen were the most frequently used in bacterial outer membrane proteins. The extremities correspond to *Bam*HI sticky ends. For easier detection of the insert a *Sal*I site has been incorporated in the sequence insert (underlined on the coding strand). The peptides encoded in the frame expected upon precise insertion into the *Bam*HI sites of the linker-modified *lamB* genes are indicated. The same oligonucleotide could be used with the three modified LamB proteins, because the *Bam*HI linkers in *lamB* were inserted in the same phase: the cutting sites for *Bam*HI are exactly between two codons. The upper strand encodes the C3 epitope. The other strand encodes four proline and one glycine residue. It contains a TAG translation stop codon (amber, underlined); since the recipient strain for all the constructions (pop6510) contained a strong nonsense suppressor (*supE*) the stop codon was translated as a glutamine. The lower part shows DNA sequences at the *Bam*HI linker insertion sites used to insert the VP1 sequence. The extremities generated after digestion of the DNA with restriction enzyme *Bam*HI are represented. The numbers correspond to the amino acid positions on the wild-type LamB protein.

Reactivity and position of the C3 epitope

We then investigated whether the epitope could be recognized on the non-denatured hybrids. Immunoprecipitation experiments were performed on non-heat-denatured extracts containing each of the hybrid proteins. We used the two different sera available against the C3 epitope: the anti-VP1 polyclonal serum raised against the corresponding peptide and the anti-C3 monoclonal antibody raised against the virion (Figure 4).

Hybrid proteins 153–C3 and 374–C3 were recognized by the two sera. Bands corresponding to trimeric and multimeric forms of the protein as well as a band corresponding to the denatured monomers were detected. (Under the conditions used, a fraction of the wild-type LamB protein and of the parental LamB-*Bam*HI modified proteins encoded by plasmids pAJC352, pAJC264, pAJC178 is also detected as monomers). A particularly strong response was observed with the anti-C3 serum and a trimeric form of the 153–C3 hybrid (apparent mol. wt of 80 kd), whereas for hybrid 374–C3, two trimeric forms were detected equally (apparent mol. wt of 80 kd and 100 kd). Interestingly, slow migrating forms of the hybrid proteins (apparent mol. wt 200 kd corresponding to multimers), which can also be observed with the wild-type LamB protein, could be detected with the anti-LamB trimer serum, but not with the anti-VP1 or anti-C3 sera. It appears that the C3 epitope is not accessible in these multimeric aggregates.

In contrast to 153–C3 and 374–C3, the 146–C3 hybrid could be detected very weakly and only at the level of the denatured monomer with the anti-VP1 and anti-C3 sera. This suggested that in the 146–C3 hybrid, the C3 epitope is either not accessible to the antibodies, or not recognizable because of conformational constraints (see Discussion).

On intact bacterial cells with the *in situ* assay, the 153–C3 hybrid could be detected by both antisera (and *a fortiori* following chloroform lysis) (data not shown). The best signal was obtained with the anti-C3 antibody. The 374–C3 hybrid was detected only with the anti-C3 antibody and with a lower signal than the 153–C3 hybrid. The 146–C3 hybrid was not revealed in any of the conditions used.

In the case of hybrids 153–C3 and 374–C3, we showed directly that the C3 epitope was exposed at the surface of the bacterial cells by treating whole cells with the anti-C3 (mouse) antibody. Two different techniques were used. (i) Immunofluorescence experiments were performed using fluorescein linked to anti-mouse antibodies (Gabay and Schwartz, 1982). Strains producing hybrid proteins 153–C3 and 374–C3 respectively were labelled, showing that the C3 epitope was accessible at their surface (data not shown). (ii) Electron microscopic observations were carried out with gold-immunolabelling (Figures 5 and 6). The surfaces of the same strains, examined on thin sections, were covered with gold particles, with the anti-C3 as well as with the anti-LamB serum (Figure 5a, b, c and d). The labelling appeared a little weaker with hybrid protein 374–C3 than with hybrid 153–C3, when the anti-C3 serum was used. A similar result had already been observed with *in situ* labelling (see above), suggesting that in hybrid 374–C3 the presentation of the C3 epitope is less favourable than in hybrid 153–C3 for its interaction with the anti-C3 serum. However, the number of labelled hybrid proteins 374–C3 at the cell surface was significant as shown by whole cell labelling (Figure 6a). Control experiments performed with the same strains under the same conditions did not detect β-galactosidase (a cytoplasmic protein) and maltose-binding protein (a periplasmic protein) showing that the technique was indeed specific for cell surface epitopes. In particular, these controls excluded the possibility that detection of the C3 epitope could be an artefact due to disruption of the envelope (Figure 6b and c).

Taken together, these results show that in the 153–C3 and the 374–C3 hybrids, the C3 epitope was exposed on the trimer and at the surface of the bacteria. Exposure or reactivity of the epitope towards the anti-C3 serum was better in 153–C3 than in 374–C3. In the case of hybrid protein 146–C3, it appeared that the C3 epitope was either hidden or conformationally constrained.

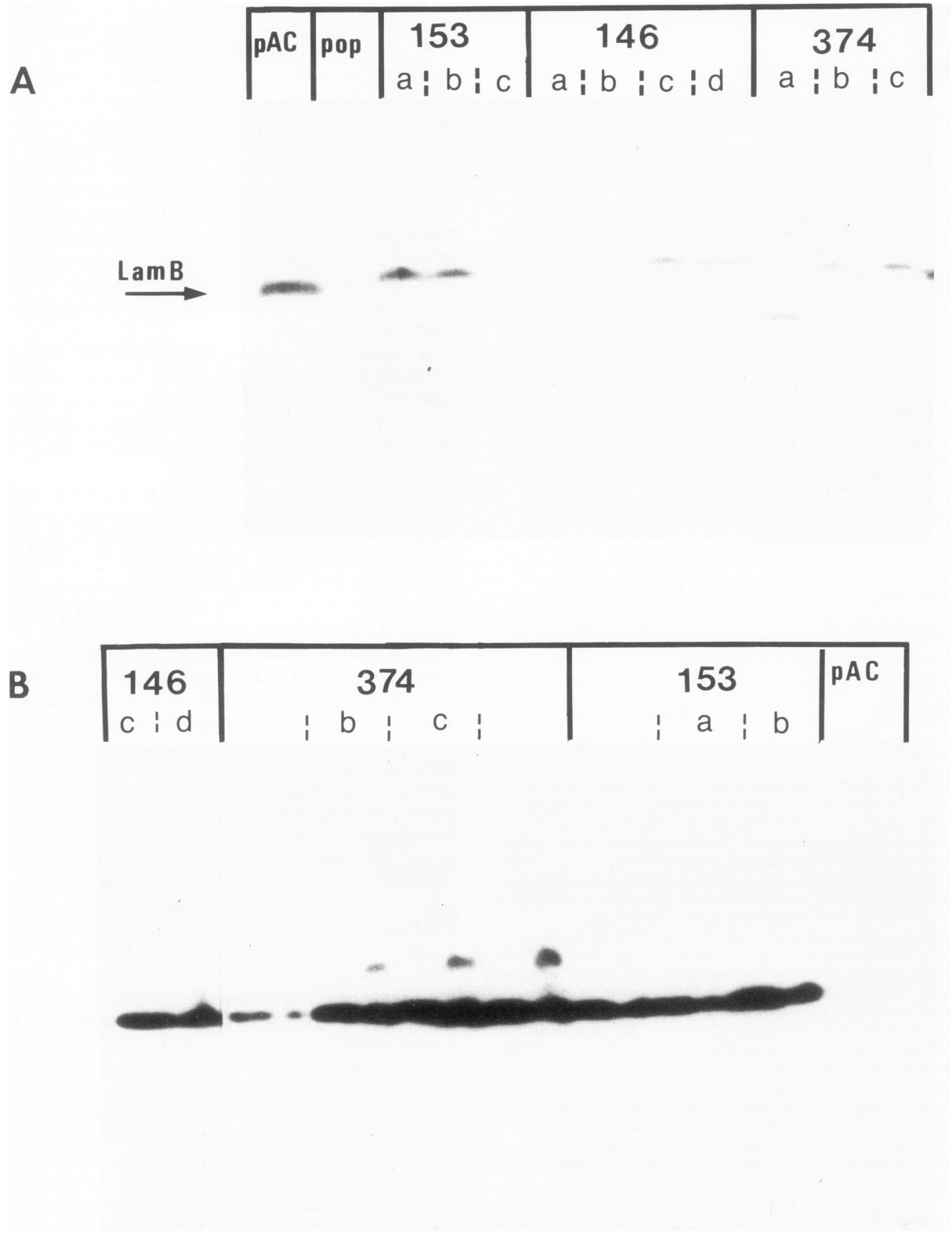

Fig. 3. Immunoblot experiments. Immunoblots were performed as described (Boulain *et al.*, 1986) In all cases the strain was pop6510 or derivatives with the relevant plasmid. pop: pop6510; pAC: plasmid pAC1 encoding the wild-type LamB protein; 146, 153, 374 indicate the amino acid position in LamB where insertion occurred. (**A**) Revelation with the anti-LamB monomer serum. **a**,**b**,**c** and **d** designate different isolates. In about half of the clones insertion resulted in a slight increase in apparent mol. wt of the protein detected, as expected upon insertion with the correct orientation for translation of the C3 epitope: 143a, b; 146c, d; 374b, c. In other cases (opposite orientation), no protein was detected or, in the case of 374a, a protein with a lower apparent mol. wt.
(**B**) Revelation with the anti-VP1 serum. 153a, b; 146c, d; 374b, c correspond to plasmids presumed to express the C3 epitope (see above). They indeed react with the anti-VP1 serum. The columns with no letters correspond to clones that were not tested first with the anti-LamB serum.

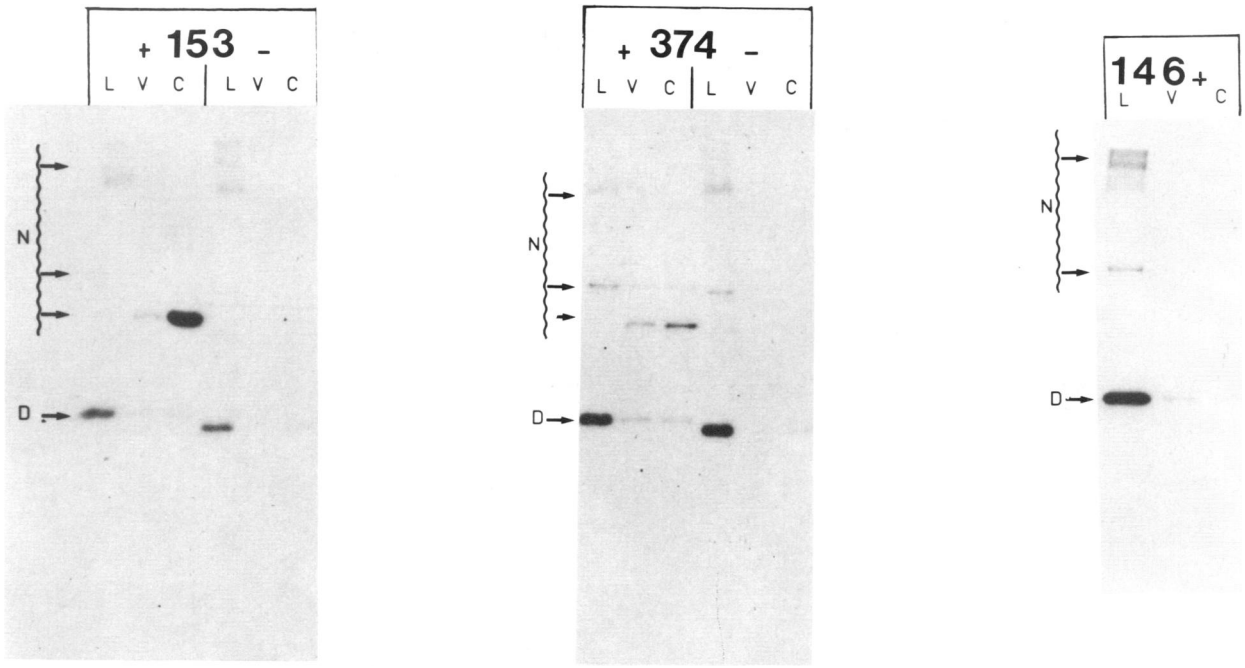

Fig. 4. Immunoprecipitation of non-heat-denatured extracts using either anti-LamB trimer or anti-VP1 or anti-C3 serum. For insertions at sites 153 and 374, the left part of the picture (indicated by +) represents the results with the insert, and the right part (indicated by −) the results with the initial vehicle. For the insertion at site 146, only the results with the C3 insert are presented. Columns L, V, C correspond respectively to anti-LamB, anti-VP1, anti-C3 sera. D (for denatured) followed by an arrow, indicates the migration of the monomeric form of the hybrid; N (for native) followed by an arrow indicates the trimeric or multimeric forms of the hybrid. With the anti-LamB trimer serum, multimeric forms (apparent mol. wt >200 kd) of the hybrid proteins 146−C3, 153−C3, 374−C3, could be revealed. These forms were not revealed with the two other sera. The strain was always pop6510 with the relevant plasmids.

Table I. Summary of the characteristics of the LamB−C3 hybrid proteins and the initial LamB-*Bam*HI modified vehicle proteins

Hybrid LamB−C3	146−C3		153−C3		374−C3		(wild-type LamB)
Plasmid	pAJC352−C3	pAJC352	pAJC264−C3	pAJC264	pAJC178−C3	pAJC178	pAC1
Length of protein	438	425	438	425	438	425	421
Immunoprecipitation							
anti-VP1 serum	+*	−	+	−	+	−	−
anti-C3 serum	+*	−	+++	−	+	−	−
(no heat denaturation)							
In situ detection							
anti-VP1 serum	−	−	+	−	−	−	−
anti-C3 serum	−	−	+++	−	+	−	−
Sensitivity to IPTG	R	R	R	R	R*	R	R
Sensitivity to phages							
λh⁺	R	R	R	I	R	R	S
λho	R	R	S	S	R	I	S
λhh*	R	S	S	S	S	S	S
K10	R	R	R	R	R	R	S

The three hybrid proteins were detected by immunoblotting with anti-LamB monomer and anti-VP1 serum (Figure 3) and by immunoprecipitation with anti-LamB trimer serum (Figure 4). They all conferred the capacity to grow on dextrins. **Line 1**. Names of the hybrid proteins. The number indicates the site of insertion of the C3 epitope on the LamB mature protein (see details on Figure 1). **Line 2**. Names of the plasmids. The properties of the plasmids without the C3 insert and of the wild-type plasmid (pAC1) are recalled for comparison (Boulain *et al.*, 1986). **Line 3**. Length of the protein in amino acid residues as predicted from the DNA sequence. **Line 4**. Immunoprecipitation with anti-VP1 serum and anti-C3 antibody (Figure 4). + indicates that trimeric or multimeric forms of the hybrid protein were detected; +++ indicates a particularly strong response; +* indicates detection, but only at the level of the monomeric form of the hybrid; − indicates no detection at all. **Line 5**. *In situ* detection on colonies transferred on nitrocellulose filters. Assays were performed with or without lysis by chloroform with qualitatively similar results, using either anti-VP1 serum or anti-C3 antibody; + means detection with or without lysis; +++ indicates particularly strong response with and without lysis; − means no detection in the two conditions. **Line 6**. Sensitivity to IPTG. R means no sensitivity; R* means sensitive in certain conditions (see Materials and methods for details). **Line 7**. Sensitivity to phages. This was determined by examining the efficiency of plating (e.o.p.) of the phages by spot test of various dilutions of phage stock on a lawn of bacteria of ML medium. S indicates sensitive (e.o.p. = 1); R indicates resistant (e.o.p. $<10^{-5}$); I indicates intermediate (10^{-3} >e.o.p. $>10^{-1}$). λh⁺ is the wild-type host range phage; λh and λhh* are respectively one step and two step mutants with extended host range. K10 is another phage using the lambda receptor for adsorption (Charbit and Hofnung, 1985 and references therein).

Discussion

The results provide information on the topology of the LamB protein and open interesting perspectives for the immunochemical study of proteins and the cell surface presentation of epitopes. These three aspects will be examined.

We have shown here that it is possible to insert a foreign

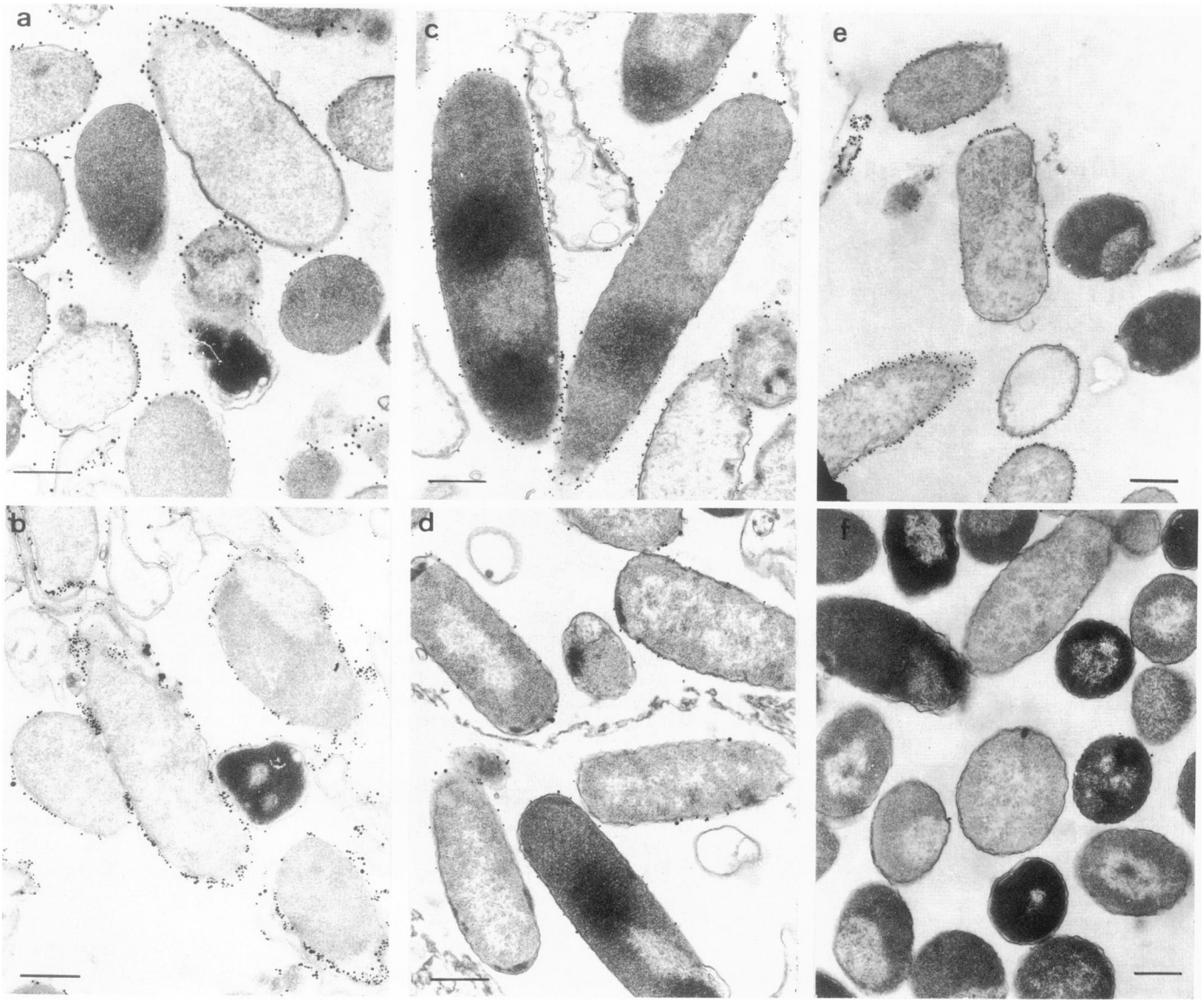

Fig. 5. Thin sections of bacteria after immunolabelling. **a** and **b**: bacteria producing the 153−C3 hybrid protein, labelled after incubation with anti-LamB (**a**) or anti-C3 serum (**b**). **c** and **d**: bacteria producing the 374−C3 hybrid protein, using anti-LamB (**c**) or anti-C3 serum (**d**). **e** and **f**: bacteria producing the wild-type LamB protein after incubation with anti-LamB (**e**) or anti-C3 serum (**f**). The strain was always pop6510 with the relevant plasmids. Bar = 0.5 μm.

epitope, the C3 epitope from the VP1 protein of poliovirus, into certain sites of the LamB protein without affecting most of the properties of this protein. The hybrid proteins are stable. They are non-toxic and can be produced at a high level without effect on the exponential growth rate. They are able to yield trimers which appear to be correctly inserted into the outer membrane. They confer the ability to grow on dextrins and, except in one case (site 146), the sensitivity to at least one LamB-specific phage.

In two cases (sites 153 and 374) the C3 epitope is recognized on the LamB trimers by the anti-C3 monoclonal antibody. Furthermore, in these two cases, the epitope is exposed and recognized by the antibody at the cell surface. By contrast, insertion into site 146 affects the ability of the epitope to be recognized in the absence of protein denaturation. This was attributed either to non-accessibility or to conformational constraints on the epitope. Since in this case the trimers are not recognized by the anti-VP1 polyclonal serum, non-accessibility seems the most likely. However we cannot exclude the possibility that the epitope would be constrained in a conformation that prevents recognition by the sera.

Previous attempts to expose at the cell surface a protein or a peptide genetically fused to LamB led to a loss of all the biological functions of LamB. In most cases the hybrids were toxic when produced at high levels, so that demonstration of exposure was not done or was subject to possible artefacts (Silhavy *et al.*, 1977; Hall *et al.*, 1982; Bouges-Bocquet *et al.*, 1984a, 1984b; review in Silhavy and Beckwith, 1985; Guesdon *et al.*, 1985).

The results presented here constitute the first direct argument for the cell surface location of the region of amino acids 153 and 374 and thus for two predictions of our model (Figure 1) (Charbit *et al.*, 1984). Two lines of results were already compatible with our model, but did not constitute direct arguments. Monoclonal antibodies raised against the LamB protein indicated that a C-terminal region was exposed at the cell surface (Gabay *et al.*, 1985; Desaymard *et al.*, 1986). Protease digestion experiments also indicated several potential surface exposed regions.

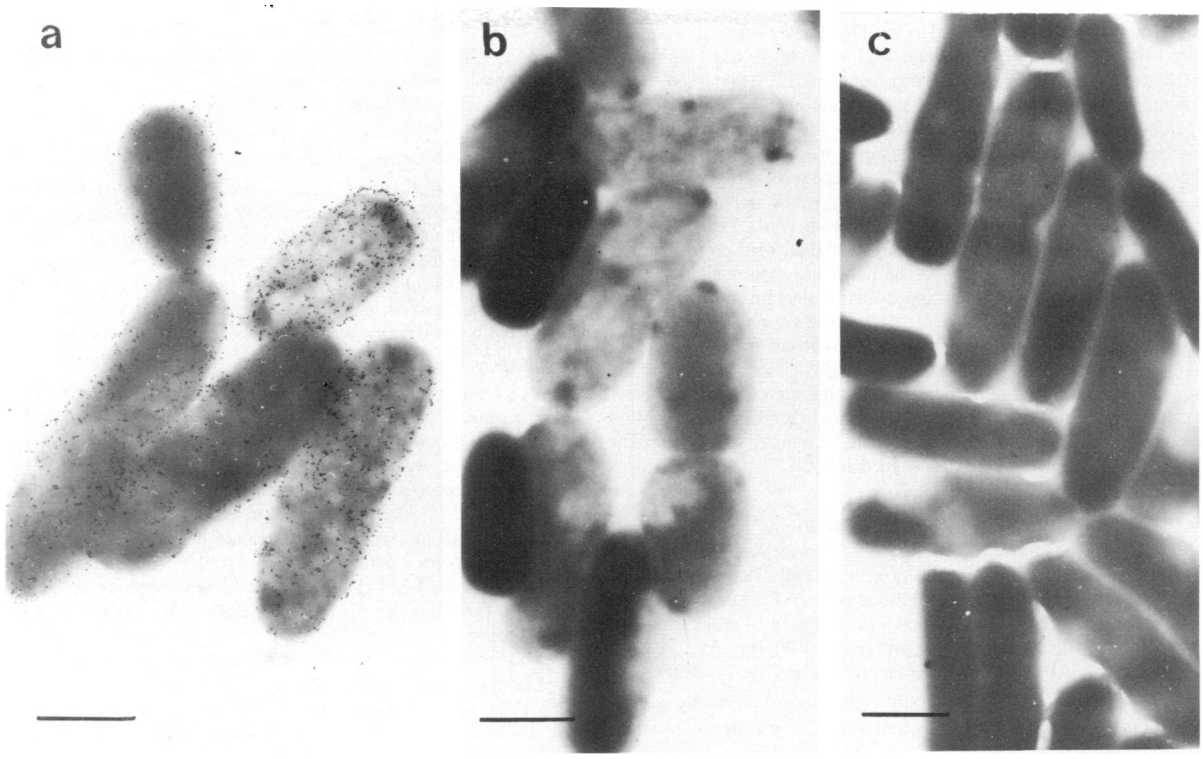

Fig. 6. Electron micrographs of whole cells after immunolabelling. **a**: bacteria producing 374−C3 hybrid protein, with anti-C3 serum (as in Figure 5d, thin section). **b** and **c**: bacteria producing the 153−C3 hybrid protein. **b**: with anti-β-galactosidase serum; **c**: with anti-MBP (maltose-binding protein) serum. These experiments were performed as controls to check that under the conditions used a cytoplasmic protein (β-galactosidase) and a periplasmic protein (MBP) were not detected. The strain was always pop6510 with the relevant plasmids.

However, digestion was obtained with LamB protein extracted from the outer membrane and not on whole cells, so that cell surface exposure could only be hypothesized on the basis of competition between protease and monoclonal antibodies (Shenkman *et al.*, 1984; Gabay *et al.*, 1985).

For OmpA, another outer membrane protein, a comparable model was proposed and insertion of a foreign peptide could be detected with protease digestion experiments (Freudl *et al.*, 1986 and references therein).

The fact that there are sites in the LamB protein which can accept the insertion of a peptide without important alterations of the properties of the LamB protein or of the antigenic properties of the peptide suggests a general way to study proteins by 'scanning' them with a well defined epitope against which antibodies are readily available. A single serum could then suffice to study different regions of a protein. This also raises interesting questions. What are the conditions to be fulfilled by the peptide for such an insertion to be possible (e.g. maximal length, amino acid composition, secondary structure, hydrophilicity)? [The total length of the insert including the adaptor parts is 17 amino acid residues, among which 11 correspond to the C3 epitope]. How many such sites do exist in the LamB protein? In other proteins? [It has already been shown that a foreign sequence could be inserted between the two domains of the pIII capsid protein from bacteriophage f1 without disrupting pIII function. The foreign sequence, within the hybrid protein, was accessible to specific antibodies on the infectious particle (Smith, 1985)]. In which cases do the constraints prevent recognition of the epitope? How do the sites detected depend on the passenger peptide? The results presented suggest that a direct search for insertion sites by a single step procedure is possible and may help answer these questions.

The type of 'cell surface exposure vector' we have developed could also be used for the identification and direct cell surface cloning of (unknown) epitopes from a protein. Other applications such as simple diagnostic tests based on bacterial agglutination, or immunopurification of different proteins 'tagged' with a foreign epitope can be envisaged.

Finally, the construction of bacterial strains with a new surface epitope opens the way to the development of live oral vaccines with non-pathogenic strains exposing antigenic determinants from a pathogen. Such constructions have already been made by cloning bacterial proteins carrying various surface antigens into *E. coli*. (Engleberg *et al.*, 1984) or into the attenuated strain *Salmonella typhi* Ty21a (Formal *et al.*, 1981; Clements and El-Morshidy, 1984; Yamamoto *et al.*, 1985). The procedure we have developed should allow the extension of this approach to peptide epitopes from almost any origin. Several different epitopes could be exposed on the same cell to provide multivalent immunogens. The next steps are to find out whether the constructions are effective in other strains more suitable for vaccination purposes (such as invasive enterobacteriae) and if the recombinant strains elicit an immune response against the antigen. In particular, it will be important to explore whether (and when) the bacteria behave as adjuvants or as suppressors of immunogenesis. These aspects are now being investigated.

Materials and methods

Strains, media, chemicals, oligonucleotide insertion

Strain pop6510 (*thr leu tonB thi laeY1 recA dex5 metA supE*) was used as a recipient for all transformations. *dex5* is a mutation in gene *lamB* which prevents adsorption of all known phages using the LamB protein as well as growth on dextrins (Bouges-Bocquet *et al.*, 1984). Plasmid pAC1 and its three derivatives containing the *Bam*HI linker insertions at amino acid 146 (plasmid mutant pAJC352),

amino acid 153 (plasmid mutant pAJC264), and amino acid 374 (plasmid mutant pAJC178) of the mature LamB protein used to insert the C3 sequence, have already been described (Boulain *et al.*, 1986). Strain AC1 is strain pop6510 harboring plasmid pAC1, strain AJC352 is pop6510 with plasmid pAJC352 and so on. Gene *lacI*^{q} overproducing the *lac* repressor was also present on the plasmid so that *lamB* expression could be induced with IPTG.

Media and chemicals were as described (Boulain *et al.*, 1986). The two single-stranded oligonucleotides corresponding to the C3 epitope sequence were prepared by J. Igolen (Institut Pasteur, Paris) and purified on a 20% acrylamide–urea denaturing gel according to standard techniques (Maniatis *et al.*, 1982). Plasmid DNAs were cut with the *Bam*HI restriction enzyme and purified on acrylamide gel as described in Maniatis *et al.* (1982). The linker insertion method already described (Boulain *et al.*, 1986) was then applied. After ligation and transformation into pop6510, restriction analysis on 1.5% agarose gel revealed that 75% of the clones selected as resistant to ampicillin had inserted a fragment containing a *Sal*I restriction site.

Total extracts of these clones were then tested by immunoblotting with anti-LamB monomer serum and anti-VP1 serum (Figure 3) (Boulain *et al.*, 1986). The constructions corresponding to modified proteins recognized by both antisera (anti-LamB and anti-VP1) were kept and studied further.

Assays for toxicity of the hybrid proteins

Addition of IPTG (10^{-3} M) to a culture of strain pop6510(pAC1) induces a high level of expression of gene *lamB*. The basal level of expression in ML medium corresponds to ~1500 monomers of LamB protein per cell. The increase upon induction is 30- to 40-fold (A.Charbit, unpublished results). For certain modified proteins, this results in a toxic effect, which was attributed to an overloading of the outer membrane with an abnormal protein (Bouges-Bocquet *et al.*, 1984a, b; Boulain *et al.*, 1986), in particular for mutant proteins unable to yield trimers.

IPTG was added at OD_{600} = 0.2 to cultures growing exponentially in ML medium and optical density was recorded up to 1.5. The growth rate of strain pop6510 (pAC1) was not affected under these conditions. In the case of derivatives producing the three hybrid proteins 146–C3, 153–C3, 374–C3, growth of the culture was identical to the growth of the parental strain, indicating no toxic effect. However, for hybrid 374–C3 addition of IPTG upon inoculation of a colony from solid medium to the liquid resulted in a strong growth inhibition. Thus, in contrast to what was observed with 146–C3 and 153–C3, overproduction of hybrid protein 374–C3 appeared to be toxic to the cell under certain conditions.

Sera

Anti-LamB sera. Two rabbit antisera were used in this study (kindly provided by Dr Greetje Vos-Sherpekeuter, Groningen, The Netherlands). (i) A serum raised against denatured LamB protein. This serum, called here 'anti-monomer serum', recognizes only the denatured monomers. It was used for the immunoblotting experiments. (ii) A serum raised against native LamB protein. This serum, called here 'anti-trimer serum' recognizes the LamB monomers as well as the trimeric and multimeric forms of the protein. It was used for all immunoprecipitation experiments under non-denaturing conditions as well as for *in situ* immunodetection, and optical and electron microscopy.

Anti-polio sera. Two sera were used. (i) One polyclonal serum raised against a synthetic peptide corresponding to residues 93–104 of the VP1 protein of type 1 poliovirus. Immunization had been done in rabbit with peptide coupled to keyhole limpet hemocyanin (KLH). This serum is called here anti-VP1 serum (van der Werf *et al.*, 1983). (ii) One monoclonal (mouse) serum raised against purified C-particles (heat-inactivated virions) (Wychowski *et al.*, 1983). This serum, called here anti-C3 antibody, is able to neutralize the virus, to react with low affinity with the free peptide and with high affinity with the peptide bound to KLH or fused to β-lactamase.

Anti β-galactosidase rabbit serum. This was kindly provided by Nicole Guizot and anti-maltose-binding protein (anti-MBP) by Winfried Boos (University of Konstanz).

Immunoprecipitation

Immunoprecipitations were performed essentially as described in Vos-Sheperkeuter and Witholt (1984). Cells were grown at 37°C in liquid minimal medium supplemented with casamino acids and ampicillin and with glucose as a carbon source. At OD_{600} = 0.6 they were induced for 10 min with IPTG 10^{-3}M, then labelled with [^{35}S]methionine (10 μCi/ml of cell culture) for 10 min followed by 5 min chase with cold methionine 0.01% final concentration. Bacteria were harvested by centrifugation, washed and the pellet resuspended in 50 mM glucose, 10 mM EDTA, 25 mM Tris pH 8.0, 4 mg/ml lysozyme. After 5 min at room temperature, spheroplasts were lysed by addition of a solution of SDS (2% final concentration) followed by 10 min incubation at 70°C. These extracts were then diluted 10-fold in Triton X-100 0.9%, EDTA 5 mM, Tris 50 mM pH 8, ovalbumin 0.1%, mixed with antiserum (5–10 μl of undiluted serum) and incubated at room temperature for 30 min. After addition of protein A–Sepharose gel (at 10% final concentration), incubation was pursued for 15 min and the purified mutant LamB proteins were recovered in the centrifugation pellet. After three washes, the pellet was resuspended in sample buffer (60 mM Tris pH 6.8, 10% glycerol, 2% SDS, 0.05% bromophenol blue), heated at 70°C for 5 min and run on SDS–PAGE. The hybrid LamB proteins were revealed by autoradiography of the dried gel. The three sera (anti-LamB trimer, anti-VP1, anti-C3) were used separately for each hybrid protein.

In situ detection of the hybrid proteins

This method allows detection of proteins *in situ* on bacterial colonies transferred onto a nitrocellulose filter (Guesdon *et al.*, 1985; Boulain *et al.*, 1986). In the absence of chloroform a cell surface protein such as LamB or periplasmic proteins are detected, a cytoplasmic protein such as β-galactosidase is not. In the presence of chloroform even cytoplasmic proteins can be detected. Detection of a cell surface protein is usually better in the presence than in the absence of chloroform presumably because its accessibility to antibodies is increased.

Immunoelectron microscopy

Cells were grown in ML medium+ampicillin and induced with IPTG 10^{-3}M [and maltose 2% when maltose-binding protein was examined (Figure 6c) at OD_{600} = 0.2 until OD_{600} = 0.6. For each preparation, 1.5 ml of cell culture were collected, washed twice in PBS and resuspended in 50 μl PBS. 5–10 μl of serum adsorbed as described below were then added and the mixture incubated for 1 h at 37°C. After two washes in PBS, the pellet was resuspended and incubated for 15 min at room temperature in 250 μl of a suspension of protein A–gold particles (12 nm) prepared according to Horrisberg (1979). Then the mixture was washed twice in PBS and fixed overnight in 1 ml glutaraldehyde 2.5% in veronal buffer pH 6.8. After one wash, cells were concentrated in agar C (Whitehouse *et al.*, 1977), post-fixed for 1 h with 1% osmium tetroxide, then treated for 1 h in 1% uranyl acetate and finally embedded in Epar.

Electron microscope preparations of whole bacteria were performed by adsorbing glutaraldehyde-fixed labelled cells on to polylysine-coated grids. The antisera were purified according to the technique described in Vos-Sheperkeuter and Witholt (1984). The anti-LamB trimer serum was adsorbed with whole cells from strain pop6510, the anti-C3 serum with strain AC1.

Acknowledgements

We thank Sylvie van der Werf, Radou Crainic, Marc Girard and Florian Horaud for the anti-polio sera and Greetje Vos-Sherpekeuter for the anti-LamB sera. We are grateful to Hugues Bédouelle and Julian Davies for critical reading of the manuscript. This work was supported by grants from the Association pour le Développement de la Recherche sur le Cancer, the Ligue Nationale contre le Cancer and the Fondation pour la Recherche Médicale.

References

Bouges-Bocquet,B., Guesdon,J.L., Marchal,C. and Hofnung,M. (1984a) *Modern Approach to Vaccines*. Cold Spring Harbor Laboratory Press, NY.

Bouges-Bocquet,B., Villarroya,H. and Hofnung,M. (1984b) *J. Cell. Biochem.*, **24**, 217–228.

Boulain,J.C., Charbit,A. and Hofnung,M. (1986) *Mol. Gen. Genet.*, in press.

Charbit,A. and Hofnung,M. (1985) *J. Virol.*, **53**, 667–671.

Charbit,A., Clément,J.M. and Hofnung,M. (1984) *J. Mol. Biol.*, **175**, 395–401.

Charbit,A., Boulain,J.C. and Hofnung,M. (1986) *C.R. Acad. Sci. Paris*, **302**, 617–620.

Clément,J.M. and Hofnung,M. (1981) *Cell*, **27**, 507–514.

Clements,J.D. and El-Morshidy,S. (1984) *Infect. Immun.*, **46**, 564–569.

Davis,R.W., Botstein,D. and Roth,J.R. (1980) *Advanced Bacterial Genetics*. Cold Spring Harbor Laboratory Press, NY.

Desaymard,C., Débarbouillé,M., Jolit,M. and Schwartz,M. (1986) *EMBO J.*, **5**, 1383–1388.

Engleberg,N.C., Pearlman,E. and Eisenstein,B.I. (1984) *J. Bacteriol.*, **160**, 199–203.

Formal,S.B., Baron,L.S., Kopecko,D.J., Washington,O., Powell,C. and Life,C.A. (1981) *Infect. Immun.*, **34**, 746–750.

Freudl,R., MacIntyre,S., Degen,M. and Henning,U. (1986) *J. Mol. Biol.*, **188**, 491–494.

Gabay,J. and Schwartz,M. (1982) *J. Biol. Chem.*, **257**, 6627–6630.

Gabay,J., Shenkman,S., Desaymard,C. and Schwartz,M. (1985) In Macario,A.J.L. and de Macario,C. (eds), *Monoclonal Antibodies Against Bacteria*. Academic Press Inc., NY.

Guesdon,J.L., Bouges-Bocquet,B., Debarbouillé,M. and Hofnung,M. (1985) *J. Immunol. Methods*, **84**, 53–63.

Hall,M., Schwartz,M. and Silhavy,T.J. (1982) *J. Mol. Biol.*, **156**, 93–112.

Hogle,J.M., Chow,M. and Filman,D.J. (1985) *Science*, **229**, 1358–1365.

Horrisberg,M. (1979) *Biol. Cell.*, **36**, 253–258.

Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, NY.

Nikaido,H. and Vaara,M. (1985) *Microbiol. Rev.*, **49**, 1–32.
Randall-Hazelbauer,L. and Schwartz,M. (1973) *J. Bacteriol.*, **116**, 1436–1446.
Schenkman,S., Tsugita,A., Schwartz,M. and Rosenbush,J.P. (1984) *J. Biol. Chem.*, **259**, 7570–7576.
Silhavy,T.J., Shuman,H.A., Beckwith,J. and Schwartz,M. (1977) *Proc. Natl. Acad. Sci. USA.*, **74**, 5411–5415.
Silhavy,T.J. and Beckwith,J.R. (1985) *Microbiol. Rev.*, **49**, 308–318.
Smith,G.P. (1985) *Science*, **228**, 1315–1317.
Thirion,J.P. and Hofnung,M. (1972) *Genetics*, **71**, 207–216.
van der Werf,S., Wychowski,C., Bruneau,P., Blondel,B., Crainic,R., Horodniceanu,F. and Girard,M. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 5080–5084.
Vos-Sheperkeuter,G.H. and Witholt,B. (1984) *J. Mol. Biol.*, **175**, 511–528.
Whitehouse,R.L.S., Benichou,J.C. and Ryter,A. (1977) *Biol. Cell.*, **30**, 155–178.
Wimmer,E., Jameson,B.A. and Emini,E. (1984) *Nature*, **308**, 19.
Wychowski,C., Van der Werf,S., Siffert,O., Crainic,R., Bruneau,P. and Girard,M. (1983) *EMBO J.*, **2**, 2019–2024.
Yamamoto,T., Tamura,Y. and Yokota,T. (1985) *Infect. Immun.*, **50**, 925–928.

Received on 14 July 1986; revised on 15 August 1986