Immunological relatedness of the LamB proteins among members of Enterobacteriaceae

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We have studied the immunological relatedness of LamB proteins from a wide range of enterobacterial species, using antibodies directed against denatured *Escherichia coli* K12 and *Klebsiella pneumoniae* LamB proteins (LamBE.c. and LamBK.p., respectively), and anti-peptide antibodies directed against 10 distinct loops of LamB from *E. coli* K12 predicted to protrude either side of the outer membrane. We have shown that a protein immunologically related to LamBE.c. and LamBK.p. was present in all members of Enterobacteriaceae tested. A protein recognized by several anti-peptide antibodies was identified in *E. coli*, *Shigella sonnei*, *Salmonella typhimurium* and *Kleb. pneumoniae*, as well as in two *Citrobacter* species, two *Enterobacter* species and *Kluyvera ascorbata*. The recognition patterns obtained with the anti-peptide antibodies were in agreement with the LamB protein sequence data available. They indicated that the cell surface and also the periplasmic loops of LamB are subject to great antigenic variability.

Introduction

In *Escherichia coli* K12, the *lamB* gene encodes the maltose-inducible integral outer-membrane protein LamB. LamB constitutes a pore specifically involved in the diffusion of maltose and maltodextrins (for reviews, see Hengge & Boos, 1983; Naikaido & Vaara, 1985). It also serves as a cell-surface receptor for a number of phages, including λ (Charbit & Hofnung, 1985). The presence of a protein immunologically related to the *E. coli* LamB protein has been reported in several members of the family Enterobacteriaceae (Pick & Wober, 1979; Bloch & Desaynard, 1985). Comparison of the antigenic determinants of LamB from different enterobacteria, using monoclonal antibodies (mAb) directed against surface-exposed or periplasmic portions of the *E. coli* LamB protein (LamBE.c.), indicated great variability of the external epitopes, and better conservation of the internal ones. Similar studies using mAbs or anti-peptide antibodies have been carried out on other outer-membrane proteins such as FepA (Rutz *et al.*, 1991) and OmpC, OmpD (Singh *et al.*, 1992) and P2 porins (Hamel *et al.*, 1992). They allowed the identification of both

conserved and highly variable epitopes, providing information on the structural organization of these proteins in the outer membrane.

The *lamB* genes from four enterobacteria have now been sequenced: *E. coli* (Clément & Hofnung, 1981), *Shigella sonnei* (Roessner & Ihler, 1987), *Salmonella typhimurium* (Francoz *et al.*, 1990), and *Klebsiella pneumoniae* (Werts *et al.*, 1992). Comparison of their deduced amino acid sequences revealed an overall high degree of similarity. The data showed that the N-terminal third of LamB was the most conserved part of the molecule and that the variability occurred mainly within surface-exposed regions of the protein (Charbit *et al.*, 1988; Werts *et al.*, 1992).

In the present work, we have studied the immunological relatedness of the LamB proteins from 15 enterobacterial species (Table 1), with either polyclonal antibodies directed against denatured *E. coli* LamB and *Kleb. pneumoniae* LamB (LamBK.p.), or anti-peptide antibodies directed against 10 different portions of the LamBE.c. protein scattered along the polypeptide chain. Four peptide sequences correspond to regions predicted to be facing the periplasm, and the six others to regions exposed at the cell surface (Fig. 1a). The 15 species chosen were all able to utilize maltose as carbon source. Two of them were unable to use dextrins as carbon

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source. They represent a large panel of Enterobacteriaceae, all more or less related to *E. coli*.

Methods

Strains, media, growth on dextrins and phage sensitivity patterns. The strains used in this study are listed in Table 1. The ability of the different enterobacteria to utilize maltose and maltodextrins as carbon source was tested at 30 °C on MacConkey agar supplemented with maltose (0.5%) or dextrins (0.5%). On these media, bacteria able to utilize the sugar as carbon source form dark red colonies (Mal⁺ or Dex⁺ phenotype), whereas those which are unable to do so form pink or white colonies (Mal⁺ or Dex⁻ phenotype). All the strains were tested for their *in vivo* sensitivity to phage λ, as well as to 11 other LamBE.c.-specific phages by the spot-test procedure (Charbit & Hofnung, 1985).

Immunization procedures and antibodies. Two polyclonal antibodies raised against denatured (monomeric) LamB protein were used: (i) a rabbit antibody directed against the denatured LamB protein from *E. coli* K12 (called here anti-LamBE.c. antibody; Boulain *et al.*, 1986) and (ii) a rabbit antibody directed against the denatured LamB protein from *Kleb. pneumoniae* (anti-LamBK.p. antibody). This serum was obtained after intradermal immunization of rabbits with 50 µg denatured LamBK.p. The first injection was performed in Freund's complete adjuvant (FCA) with denatured LamBK.p. obtained after electroelution of the monomeric form of the protein from a 10% (w/v) SDS-polyacrylamide gel. Two booster injections were performed at 29 and 88 days with 50 µg of the affinity-purified (Werts *et al.*, 1992) heat-denatured protein in Freund's incomplete adjuvant (FIA).

Ten polyclonal anti-LamBE.c. peptide antibodies were used in this study. For simplification, all the antibodies directed against cell-surface-predicted regions of LamB will be preceded by E (for external), and those directed against regions facing the periplasm by I (for internal). Four of these anti-peptide antibodies (anti-I 1, anti-E 2, anti-E 3 and anti-E 4) have been described previously (Molla *et al.*, 1989). Anti-peptide I 1 was able to react with native purified LamBE.c., but not on the surface of intact bacteria, whereas anti-E 2, E 3 and E 4 were able to react with LamBE.c. at the surface of intact cells. Six new anti-

LamBE.c. peptide antibodies (see location on the LamBE.c. folding model, Fig. 1) were obtained: anti-I 12, anti-I 15, anti-I 16, and anti-E 5, anti-E 7 and anti-E 13. Peptides were synthesized according to Merrifield (1963) and were coupled to ovalbumin using 1% (v/v) glutaraldehyde (Harlow & Lane, 1988). For each peptide, three female BALB/c mice were immunized subcutaneously with 100 µg peptide conjugate, in FCA. Two subsequent injections were done intraperitoneally with 100 µg peptide conjugate in FIA at 21 and 50 days. Blood samples were taken at 30 and 59 days. Animals were sacrificed on day 66.

Fig. 1. (a) Position of peptides on the two-dimensional folding model of LamB (adapted from Charbit *et al.*, 1991). Previously described peptides are stippled, new ones are black. Boxed residues represent β-strands, simple lines represent either turns or non-ordered regions of LamB. The approximate extent of the transmembrane region is represented by the arrow on the left. (b) Alignment of the four known LamB amino acid sequences. Positions of peptides are boxed.

Table 1. Enterobacterial strains used in this study

All strains come from the Service des Entérobactéries (Pasteur Institute) except *E. coli* strains (laboratory collection), *Kleb. pneumoniae* KAY 2026 (Springer & Lengeler) and *Ent. cloacae* which is a clinical isolate from the Villeneuve-Saint-Georges Hospital, France (A. Dublanchet).

Species	Strain
<i>Escherichia coli</i>	P4X8 (LamB ⁺)
<i>Escherichia coli</i>	pop6510 (LamB ⁻)
<i>Shigella sonnei</i>	S60-80
<i>Salmonella typhimurium</i>	LT2
<i>Citrobacter freundii</i>	CDC 22-76
<i>Citrobacter diversus</i>	CDC 1066-71
<i>Klebsiella pneumoniae</i>	KAY 2026
<i>Enterobacter cloacae</i>	
<i>Enterobacter aerogenes</i>	A1
<i>Erwinia raphontici</i>	1025
<i>Erwinia herbicola</i>	E20
<i>Hafnia alvei</i>	I 563272
<i>Serratia marcescens</i>	T 504 (ATCC 13880)
<i>Edwardsiella tarda</i>	T 10396
<i>Kluyvera ascorbata</i>	T 648-74
<i>Providencia heimbachae</i>	T 8025-83

Table 2. Recognition of enterobacterial LamB proteins by Western blotting with different sera

The four strains in the upper part are those of known lamB gene sequence. The strains in the lower part have been classified arbitrarily, except that strains of the same genus are placed consecutively.

Species	Mc dex*	Anti-LamB antibody†		Anti-peptide antibody†									
		E.c.	K.p.	I 1 (19-33)	I 12 (69-83)	I 15 (121-134)	I 16 (292-304)	E 13 (85-99)	E 2 (147-161)	E 5 (247-261)	E 7 (327-340)	E 3 (371-386)	E 4 (399-413)
<i>Escherichia coli</i> P4X8	+	++	++	++	+++	+	++	+++	+++	+	++	++	++
<i>Shigella sonnei</i>	+	+++	+++	++	+++	+	++	+++	+++	+	++	++	++
<i>Salmonella typhimurium</i>	+	+++	+++	+/	+++	+	-	++	+/	-	-	+/	++
<i>Klebsiella pneumoniae</i>	+	++	++	-	-	+	+	-	-	-	-	+/	-
<i>Citrobacter freundii</i>	+	++	++	-	+++	+	-	++	-	+/	-	-	+/
<i>Citrobacter diversus</i>	+	+++	+++	-	+++	+	+	-	-	+/	+/	-	+
<i>Enterobacter cloacae</i>	+	+++	+++	-	-	+	+	+/	-	+	+/	-	+
<i>Enterobacter aerogenes</i>	+	+++	+++	-	-	+/	+/	-	-	-	-	-	+
<i>Erwinia rhapontici</i>	+	++	++	-	+	-	+/	+	+/	-	-	+	-
<i>Erwinia herbicola</i>	+	+	+	-	-	-	+/	-	-	+/	+/	-	-
<i>Hafnia alvei</i>	-	++	+	-	+	-	+	-	-	-	-	-	-
<i>Serratia marcescens</i>	-	+++	++	-	-	-	-	+	-	-	-	-	-
<i>Edwardsiella tarda</i>	+	+/	+	-	-	-	-	-	-	-	-	-	-
<i>Kluyvera ascorbata</i>	+	++	+	-	+++	+	+	+	-	+/	+	-	+/
<i>Providencia heimbachae</i>	+	+	-	-	-	-	-	-	-	-	-	-	-

* Phenotype on MacConkey dextrin medium.

† -, No recognition; +/ -, weak positive recognition, often masked by high background; + to + + +, positive recognition, increasing intensity of detection.

All the anti-peptide antibodies were able to react specifically with LamBE.c. in Western-blots with an extract of the LamB-positive *E. coli* strain AC1 (Boulain *et al.*, 1986), at a dilution of 1/3000. There was no detection with the LamB-negative strain pop6510.

Immunodetection experiments. Bacteria were grown at 30 °C in liquid minimal medium supplemented with casamino acids (0.02%; Difco), and maltose (0.02%) as carbon source. At OD₆₀₀ = 1, cells were harvested by centrifugation and concentrated 25-fold in water. Samples were then collected, heat-denatured for 5 min at 100 °C in loading buffer and run on 10% SDS-polyacrylamide gels (approximately 5×10^7 bacteria per well). After electrophoretic transfer to a nitrocellulose filter, the extracts were analysed with the different anti-LamB sera. In the Western blotting experiments, the anti-peptide sera were used at a final dilution of 1/1000 (except anti-I 12, at 1/30000) and the polyclonal anti-LamBE.c. and anti-LamBK.p. at 1/10000 and 1/4000, respectively.

Results

The presence of a protein immunologically related to the *E. coli* K12 LamB protein was assayed by Western blotting on total cellular extracts from 15 enterobacterial strains with anti-LamBE.c. or anti-LamBK.p. antibodies raised against the denatured proteins (see Methods). It was also tested with six anti-LamBE.c. peptide antibodies directed against regions predicted to be at least partly cell-surface-exposed, and four antibodies directed against regions predicted to face, at least partly, the periplasm (Fig 1*a*). These 15 strains were chosen because they were all able to utilize maltose as carbon source (see Methods).

Polyclonal antibodies directed against LamBE.c. and LamBK.p. proteins

In all the members of the family Enterobacteriaceae studied, a maltose-inducible protein with an apparent molecular mass comparable to that of LamBE.c. (approximately 47 kDa) was detected by both anti-LamBE.c. and anti-LamBK.p. antibodies (Fig. 2*a, b*). *Providencia heimbachae* was the only strain for which there was a weak recognition by anti-LamBE.c. antibody, and where specific recognition by anti-LamBK.p. antibody could not be ascertained because of important non-specific cross-reactions with other bacterial components. A good overall correlation was observed between the recognition patterns obtained with the two antibodies, indicating that the immunogenic epitopes of these two LamB proteins were at least partly conserved in different enterobacteria.

Anti-peptide antibodies directed against internal regions of LamBE.c.

Anti-I 1 antibody [I 1: amino acids (aa) 19–33 of LamBE.c.] recognized equally the *E. coli* and the *Shigella sonnei* (LamBS.s.) LamB proteins, but only poorly LamB

from *Sal. typhimurium* (LamBS.t.), and not at all LamB from *Kleb. pneumoniae* (LamBK.p.). Changes were found in the sequence of this region in LamBS.t.) and LamBK.p., but not in LamBS.s. (Fig. 1), suggesting that the anti-I 1 recognition site lies within this central part of peptide I 1. In all the other enterobacteria, no LamB-like protein was recognized by anti-I 1 antibody, indicating that region I 1 was subject to great antigenic polymorphism.

Anti-I 12 antibody (I 12: aa 69–83 of LamBE.c.) recognized LamBE.c., LamBS.s. and LamBS.t., but not LamBK.p. Two changes are found in this region in LamBS.t. (at the N terminus and in the middle of peptide 12). Since these changes did not affect detection by anti-I 12, the recognition site of this antibody is probably located toward the C-terminal end of peptide 12. This assumption is supported by the fact that the same two changes are present also in LamBK.p. The lack of recognition of LamBK.p. is likely to be due to the three additional changes found in the distal part of the peptide at positions 71, 72 and 78. Anti-I 12 antibody also recognized a LamB protein in the two members of the genus *Citrobacter*, *Kluyvera ascorbata* (a clinical isolate which is classified between *Citrobacter* and *Enterobacter*; Brenner, 1978), *Hafnia alvei* and *Erwinia rhapontici* (Fig. 2*b*).

Anti-I 15 antibody (I 15: aa 121–134 of LamBE.c.) recognized LamBE.c., LamBS.s., LamBS.t. and LamBK.p. proteins. It also recognized LamB from the two *Citrobacter* strains, *Kluy. ascorbata* and *Enterobacter*.

Anti-I 16 antibody (I 16: aa 292–304 of LamBE.c.) recognized LamBE.c. and LamBS.s., LamBK.p. poorly, but not LamBS.t. There are five changes in this region of LamBS.t. Anti-I 16 antibody also reacted with LamB from *Kluy. ascorbata*, *C. diversus*, *Ent. cloacae* and poorly with *Ent. Aerogenes*, *Erw. rhapontici* and *Erw. herbicola*.

Anti-peptide antibodies directed against external regions of LamBE.c.

Anti-E 13 antibody (E 13: aa 85–99 of LamBE.c.) recognized the LamB proteins from *E. coli*, *Shig. sonnei* and *Sal. typhimurium*, but not from *Kleb. pneumoniae*. The two changes in this region of LamBK.p. are probably responsible for the loss of recognition. Anti-E 13 antibody also showed specific cross-reaction with *C. freundii*, *Ent. cloacae*, *Kluy. ascorbata* and *Serratia marcescens*.

Anti-E 2 antibody (E 2: aa 147–161 of LamBE.c.) recognized LamBE.c. and LamBS.s., LamBS.t. weakly, but not LamBK.p. This region is highly variable in LamBS.t. (five changes out of 15 residues) and LamBK.p.

Fig. 2. Recognition of enterobacterial LamB proteins by Western blotting with different sera. (a) Recognition of LamB-like proteins in extracts from maltose-induced (+) and non-induced (-) enterobacterial cultures by anti-LamBE.c. serum. (b) Recognition of LamB-like protein in extracts from maltose-induced enterobacterial cultures by anti-I 12 (upper part) or by anti-LamBK.p. (lower part) antibodies. The negative control *E. coli* pop6510 is not shown. To avoid the diffuse smear seen in *E. coli* P4X8 recognized by anti-I 12, the extract was diluted tenfold for the blot labelled with anti-LamBK.p. C, *E. coli* pop6510; 1, *E. coli* P4X8; 2, *Sal. typhimurium*; 3, *Serr. marcescens*; 4, *H. alvei*; 5, *Kleb. pneumoniae*; 6, *Edw. tarda*; 7, *P. heimbachae*; 8, *Kluy. ascorbata*; 9, *Erw. rhapsontici*; 10, *C. freundii*; 11, *C. diversus*; 12, *Ent. cloacae*; 13, *Shig. sonnei*; 14, *Erw. herbicola*; 15, *Ent. aerogenes*.

(eight changes, with three changes identical to those present in LamBS.t.) (Fig. 1). Anti-E 2 did not react with any of the 12 other enterobacterial species (except weakly with *Erw. rhapsontici*).

In addition to *E. coli* and *Shig. sonnei*, anti-E 5 antibody (E 5: aa 247–261 of LamBE.c.) reacted with *Ent. cloacae* and weakly with the two *Citrobacter* species, *Erw. herbicola* and *Kluy. ascorbata*. This antibody did not recognize LamBS.t. and LamBK.p. either, for which many changes (substitutions, deletions and insertions) were found in this region.

Anti-E 7 antibody (E 7: aa 327–340 of LamBE.c.) was able to recognize LamBE.c. and LamBS.s., but not LamBS.t. and LamBK.p., for which three and four changes were found in this region, respectively. Anti-E 7 reacted positively with *Kluy. ascorbata*, and only weakly with *C. diversus*, *Erw. herbicola*, and *Ent. cloacae*.

Anti-E 3 antibody (E 3: 371–386 of LamBE.c.) recognized LamBS.s. well, and LamBS.t. and LamBK.p. poorly. Among the 11 other enterobacteria tested, anti-E 3 antibody reacted only with *Erw. rhapsontici*.

Anti-E 4 antibody (E 4: aa 399–413 of LamBE.c.)

recognized LamBE.c., LamBS.s. and LamBS.t., but not LamBK.p. Comparison of LamBS.t. and LamBK.p. amino acid sequences suggest that the anti-E 4 antibody recognition site (which was previously shown to be cell-surface-exposed; Molla *et al.*, 1987) lies within the distal part of peptide E 4. Anti-E 4 antibody reacted also with the two *Enterobacter* species and *C. diversus* (and only very poorly with *C. freundii* and *Kluy. ascorbata*).

Discussion

A maltose-inducible protein, immunologically related to the *E. coli* maltoporin was detected by an anti-LamBE.c. serum in all the Mal⁺ enterobacteria tested. Proteins in all but one were also recognized by an anti-LamBK.p. serum. However, recognition of the various proteins by anti-peptide sera raised against 10 loop regions of LamBE.c. was very variable, showing that these regions of the protein present high antigenic variability.

The regions of peptides E 2, E 5, E 3 and E 4, have been demonstrated previously to be cell-surface-exposed (Molla *et al.*, 1987; Charbit *et al.*, 1991). Amino acid

comparison of the four LamB proteins sequenced showed that these four regions were highly variable (Werts *et al.*, 1992; Fig. 1b). Our results showed that these regions were also antigenetically highly polymorphic.

There was no correlation between the location of the peptides chosen for the induction of anti-peptide antibodies, within the LamB protein (cell surface or periplasmic), and the degree of conservation of the antigenic determinants. For example anti-peptide I 1, directed against a region of LamBE.c. previously shown to face the periplasm (Molla *et al.*, 1987), recognized *E. coli*, *Shig. sonnei* and *Sal. typhimurium* but none of the other species. Similarly, anti-E 2 and anti-E 3, directed against cell surface loops, recognized few species. In contrast, anti-peptide I 12, I 15, I 16 (predicted internal loops) and E 13 (predicted external loops) were able to recognize a LamB protein in 8 to 10 of the 15 enterobacteria tested. Region E 7 (aa 327–340) was antigenically variable among the enterobacteria. This is in agreement with previous genetic data (Desaymard *et al.*, 1986) which suggested that residue 333 of LamBE.c. was exposed at the cell surface. Region E 13 toward the N terminus of the protein (aa 85–99), although more conserved, also appeared to be subject to antigenic variability, which suggests that it is exposed on the surface of the protein.

The LamB proteins from three strains (*P. heimbachae*, *Edwardsiella tarda* and *Serr. marcescens*) were only poorly or not recognized by the anti-peptide sera. In addition, the LamB proteins of *P. heimbachae* and *Edw. tarda* were only poorly or not recognized by the anti-LamBE.c. or anti-LamBK.p. antibodies. Interestingly, these two strains could still use dextrans as carbon source, suggesting that in spite of important antigenic variability in the surface loops of the protein, the function and thus the overall structure might be conserved. Similar results were observed with the ferric enterobactin receptor FepA, an outer-membrane protein for which it was found that distantly related bacteria contained an outer-membrane protein that transported the native *E. coli* siderophore but possessed only slight structural similarity to *E. coli* FepA (Rutz *et al.*, 1991). The other strain (*Serr. marcescens*) was unable to use dextrans as carbon source (Table 1 and Methods). However, it possessed a protein still immunologically related to LamBE.c., since it was recognized by both the anti-LamBE.c. and anti-LamBK.p. sera. Thus, in spite of the loss of the two main known functions of LamBE.c. (phage reception and dextrin utilization), this protein still shares antigenic determinants with LamBE.c.

The *Shig. sonnei* LamB protein is the most related to the *E. coli* K12 LamB protein. It was the only protein to be recognized by all the sera, and the only one to have

conserved the phage receptor function of LamB from *E. coli* K12 (see Methods). None of the other bacterial species were sensitive to any of the LamB-specific phages tested (data not shown). Other than the species least related to *E. coli* (*Edw. tarda*, *P. heimbachae* and *H. alvei*), which were either poorly or not recognized by the anti-peptide antibodies, there was no correlation between the pattern of antibody recognition and the phylogenetic relationships between the strains tested. In fact, closely related strains (for example *C. freundii* and *C. diversus*) often had different recognition patterns.

In the future, it will be interesting to extend this study by choosing new regions of the protein, in particular predicted conserved regions.

In terms of the topology and folding of the LamB protein, DNA sequence analysis of the *lamB* genes from *P. heimbachae*, and *Edw. tarda*, should provide particularly interesting information on the regions of the protein that are critical for the conservation of the sugar transport function of LamB, and thus for its overall structure.

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