Journal of Cellular Biochemistry 24:217–228 (1984)
Protein Transport and Secretion 215–226

Linker Mutagenesis in the Gene of an Outer Membrane Protein of Escherichia coli, LamB

B. Bouges-Bocquet,[+] H. Villarroya, and M. Hofnung

Unité de Programmation Moléculaire et Toxicologie Génétique, CNRS LA 271, INSERM U.163, Institut Pasteur, 75015 Paris, France

In order to identify sequences involved in the localization of LamB, an outer membrane protein from E coli K12, mutagenesis by linker insertion has been performed on a *lamB* gene copy carried on a plasmid devised for this purpose. An analysis of the first set of 16 clones constructed by this technique shows that, in these clones, the *lamB* protein is altered either by frameshift mutations leading to abnormal COOH terminal (usually premature termination) or by in-phase deletions or small insertions. Except for two in-phase linker insertions, which only slightly changed the behavior of the protein, the modified proteins are either toxic to cell growth or unstable. In all cases examined so far, the modified proteins were in the outer membrane. We suggest that toxicity is due to incorrect folding, which leads to disruption of the outer membrane. The nature of the genetic alterations leads to the hypothesis that the first 183 amino acids of the LamB mature protein contain, together with the signal sequence, all the instructions needed for proper localization.

Key words: linker mutagenesis, outer membrane, lambda receptor

The Gram-negative bacterium E coli K12 is composed of four compartments: the cytoplasm, the inner membrane, the periplasm, and the outer membrane. The exportation of proteins in either the periplasm or the outer membrane requires an NH_2-terminal extension, the signal sequence, which is cleaved off during protein positioning (for review, see [1]).

Construction of protein fusions [1] showed that the signal sequence was not sufficient to allow translocation of hybrid proteins to one of these two compartments [2,3]. In the case of the outer membrane protein LamB, it was proposed that determinants for localization were present within the sequence of the mature protein [4–6].

However, the folding and/or the abnormal hydrophobicity of the hybrid proteins, in particular the fused cytoplasmic protein beta-galactosidase, interfere with

[+]Deceased February 27, 1984.

Received June 13, 1983; revised and accepted November 18, 1983.

their localization. In order to identify the determinants for localization and to avoid the artifacts due to the fused protein, we have undertaken modification of the LamB protein [7,8] by linker mutagenesis. By this technique, it has been possible to obtain rapidly a set of proteins with abnormal termination (usually premature) or containing in-phase deletions or small insertions (four amino acids).

This paper reports preliminary results on the properties and location of these modified proteins.

MATERIALS AND METHODS

Linker Mutagenesis

The plasmid pBBO used for the constructions reported in this paper was obtained by insertion of a tac_{12} promotor [9] between the *Eco*RI and *Bam*HI sites of plasmid pHSF1 [10], just before the Shine and Dalgarno sequence of gene *lamB*. The resulting plasmid (Fig. 1, top) expresses the LamB protein upon induction with lactose or Isopropyl-β-D-thiogalactoside (IPTG).

For linker mutagenesis (Fig. 1), pBBO was first linearized with a restriction endonuclease. The extremities of the linearized vector were filled in with Klenow polymerase and modified plasmids were then obtained after ligation in the presence of *Xho*I linker (CCTCGAGG). The letter in the plasmid name refers to the enzyme used to linearize the vector, respectively a for *Ava*II, h for *Hgi*AI, e for *Eco*RV, n for *Nco*I, s for *Sal*I, m for *Sma*I, and c for *Cla*I.

For each construction, further studies showed that the majority of the clones had a phenotype corresponding to linker insertion with the predicted translation phase. Other phenotypes were obtained with low frequencies. They presumably corresponded to plasmids where a few nucleotides have been deleted by Klenow polymerase. Some of these clones were selected when they presented phenotypes of interest, even if they did not contain linkers.

In all cases, the restriction site that was opened to linearize the vector disappeared in the final construction. It was thus an indication that mutagenesis had indeed occurred around this site. In addition, in the cases where *Xho*I linker insertions happened, we checked to make sure that the site of cleavage by the *Xho*I enzyme corresponded to the mutagenesis site (Fig. 2).

Some deletions also were isolated. A *Sal*I cleavage site in the middle of gene *lamB* [17] generates the same cohesive ends as those generated by *Xho*I at the linker site, and thus, deletions between these two points could easily form. pBBc2 results from such a deletion between the *Sal*I site and a linker inserted at the *Cla*I site, and pBBs1 between the *Sal*I site and a linker at the *Eco*RV site. pBBa6 carries an *Ava*II-*Ava*II deletion. One additional deletion (in pBBm1) occurred spontaneously in the process of plasmid construction after linearization with *Sma*I endonuclease. According to restriction analysis with several restriction enzymes, pBBm1 contains approximately a 240-base pair (bp) deletion, which includes the *Sma*I and *Sal*I sites and three *Taq*I sites located upstream from *Sma*I, and excludes the *Cla*I site and the *Taq*I site downstream from *Sma*I. This deletion thus removes at least amino acids 183 to 250 in the LamB protein.

Strains

In order to obtain a low level of expression of LamB, an F′ episome, kindly provided by J.H. Miller, carrying the $lac1^{Q1}$ mutation, which leads to an overproduc-

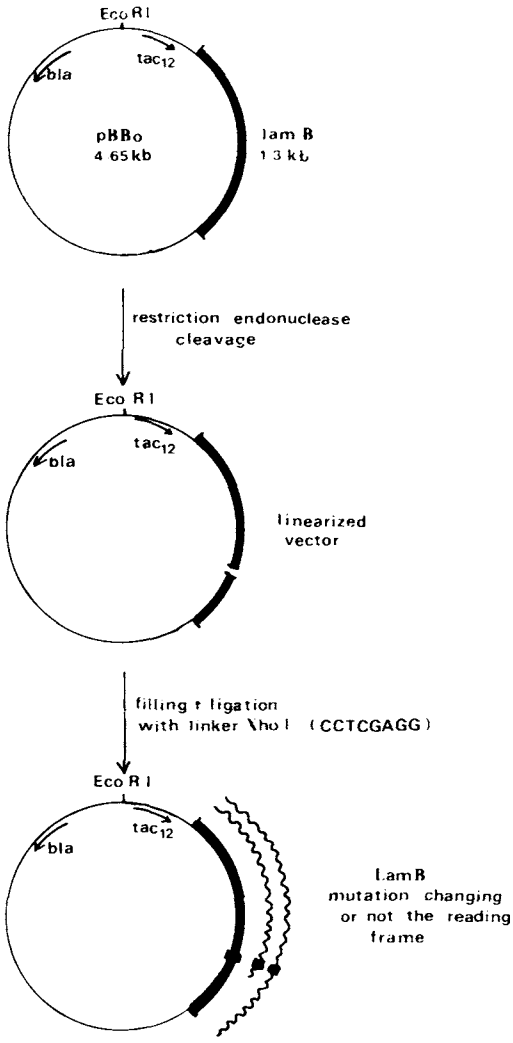

Fig. 1. Construction of modified proteins by linker insertion (see text for details).

tion of the *lac* repressor, was present in all the strains before introduction of the multicopy plasmid carrying *lamB*.

LamB is the receptor of phage lambda [8]. It also acts as a pore for maltodextrins [11]. LamB mutants are therefore usually selected when resistance to phage lambda or inability to grow on maltodextrins is desired. Two strains have been used in this paper. BB_1 (*thr leu ton*B *thi lac*Y1 *rec*A *dex*5 *met*A *sup*E, F' *lac*I^{Q1} Z+Y+ *pro*AB+) is a derivative of pop 6510, which contains an episome. This strain bears a very stable mutation *dex*5 in gene *lamB*, preventing phage absorption and growth on maltodextrins (E. Lepouce, personal communication). Because of its *rec*A phenotype, it was used for the constructions and conservation of plasmids. BB_2 (*thr leu ton*B *thi lac*Y1 *mal*B Δ 15, F'lacI^{Q1} Z+Y+ *pro*AB+) is a derivative of pop 728 [12] with addition

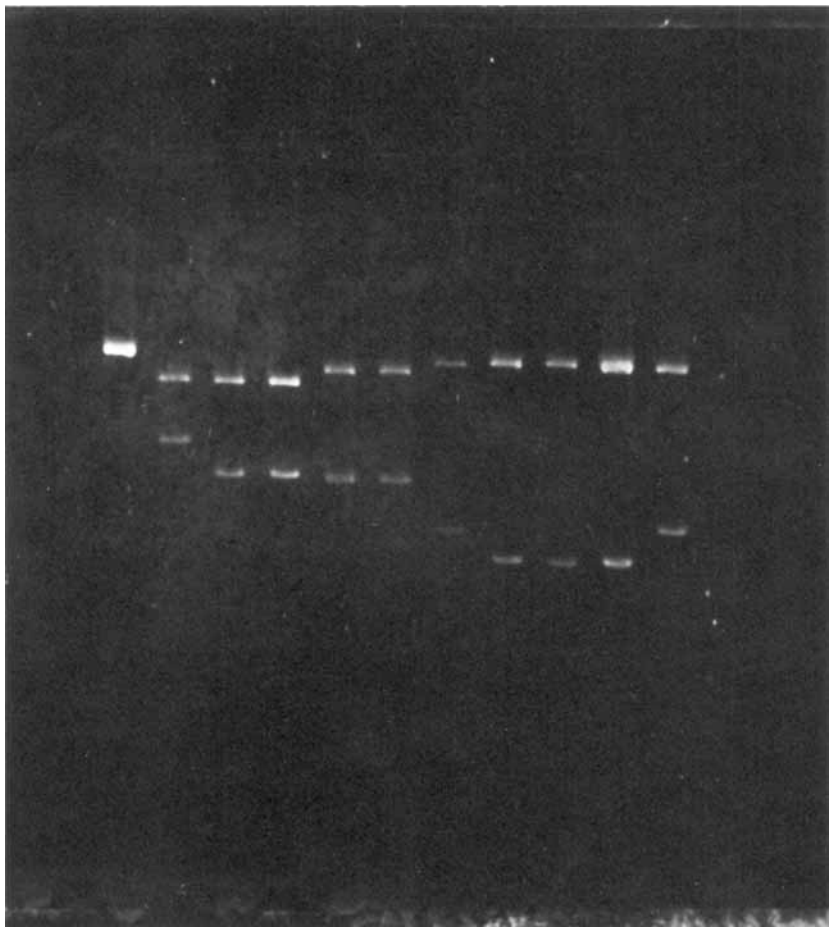

Fig. 2. DNA fragments of plasmids, on 1.5% agarose gel, after hydrolysis with *Eco*RI + *Xho*I (except for the extreme right lane, where *Xho*I was replaced by *Sal*I). The plasmids are (from the left to right) pBBo, pBBa8, pBBh1, pBBa4, pBBe2, pBBe3, pBBm5, pBBc1, pBBc5, pBBc7, and pBBo.

of the episome. This strain, because of its *mal*B Δ 15 deletion, expresses no chromosomal LamB protein. It was chosen for studying the modified LamB proteins encoded by the plasmids.

Mutants Characterization

BB_1 was transformed with modified plasmids and the phenotype of the resultant clones was characterized by two criteria: (1) their resistance to phage lambda and (2) their sensitivity to IPTG, the inducer of the *tac* promotor.

Induction with 10^{-3} M IPTG results in an overproduction of modified LamB. Four classes were identified based on their sensitivity and growth characteristics on solid Luria medium with 10^{-3} M IPTG: $IPTG^R$, no inhibition; $IPTG^S$, colonies

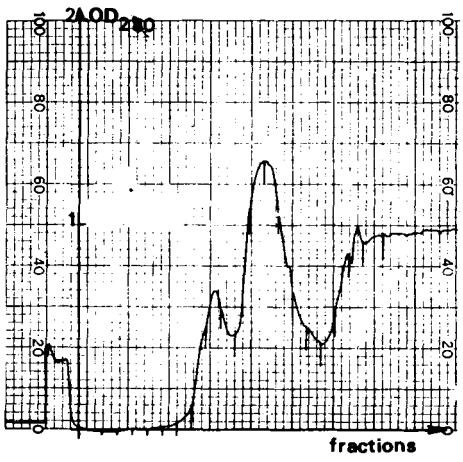

Fig. 3. Optical density at 280 nm from top to bottom of a sucrose density gradient (see ref [14] and Materials and Methods).

appear translucent; $IPTG^{SS}$, the strain forms small colonies; $IPTG^{SSS}$, almost no growth is observed. The plasmid pBBO with wild-type *lamB* gene results in an $IPTG^{S}$ phenotype.

Immunoprecipitation

Immunoprecipitation was performed according to Ito et al [13]. Strain BB_2 and its derivatives containing plasmids were grown in minimal medium with phenylmethylsulfonylfluoride (PMSF) 5×10^{-4} M. At $DO_{600} = 0.8$, 10^{-3} M IPTG was added, and after 10 min, cells were labeled for 2 min with 10 μCi/ml of ^{35}S-methionine. The reactions were stopped by trichloroacetic acid (TCA) precipitation, and the protocol of Ito et al [13] was used for immunoprecipitation. The samples were run on a 10% SDS-acrylamide gel prepared according to Laemmli [16].

Protein Localization

Protein localization was performed according to the method of Crowlesmith et al [14]. Strain BB_2 and its derivatives containing plasmids were grown in minimal medium with 5×10^{-4} M PMSF. At $DO_{600} = 0.7$, IPTG(10^{-3} M) was added, and after 40 min the cells were washed in 5 ml HEPES buffer (10 mM HEPES, pH 7.4, with PMSF and 25% sucrose) and lysed by passage through a French pressure cell at 15,000 lb/in^2. The lysate was layered onto 1 ml of 25% sucrose over a 1-ml cushion of 60% sucrose and subjected to a preliminary centrifugation (3 hr at 50,000 rpm). The soluble fraction was separated from the membranes, which were then layered onto a second sucrose gradient (60–30% sucrose in steps of 5%). A typical membrane profile obtained from such a gradient is presented on Figure 3. The location of the two peaks in the gradients corresponds to those usually found for inner and outer membrane fragments, respectively. Fractions collected from these two bands of the gradient were TCA-concentrated, boiled for 3 min in SDS, and run on a 10% SDS-acrylamide gel [16].

TABLE I. Derivatives of Plasmid pBBO Carrying Modified *lamB* genes: The Properties of the Original Plasmid and of Sixteen Such Derivatives Are Reported

	Phase shift								
	pBBo	pBBa8	pBBh1	pBBa4	pBBe2	pBBn6	pBBm5	pBBc1	pBBh7
Mutagenesis site (in amino acids)		411	321	306	294	279	183	132	28
Predicted length	421	432	~326	323	~302	299	189	140	32
Sensitivity to λ[a]	S	R	R	R	R	R	R	R	R
Sensitivity to IPTG[b]	S	SSS	SS	SS	S	S	S	R	R
Localisation according to Osborn grad.	OM[c]	OM	OM	OM	OM	—	—	—	—
Apparent MW on gel (in kD)[d]	47.8	48.9	38	38	36.3	34.3	25.1	—	—
In amino acids	421	431	335	335	320	302	221	—	—

Phase shift = Mutations that affect the reading frame, ordered with decreasing length of LamB peptides. Unchanged reading frame = Mutations that do not affect the reading frame, in frame deletions or insertions.

[a]Sensitivity to lambda: R, resistant; S, sensitive; —, not tested.

[b]Degree of sensitivity to IPTG (see text).

[c]Outer membrane.

[d]Apparent molecular weights (MW) were calculated according to the major LamB peptide in Figure 6.

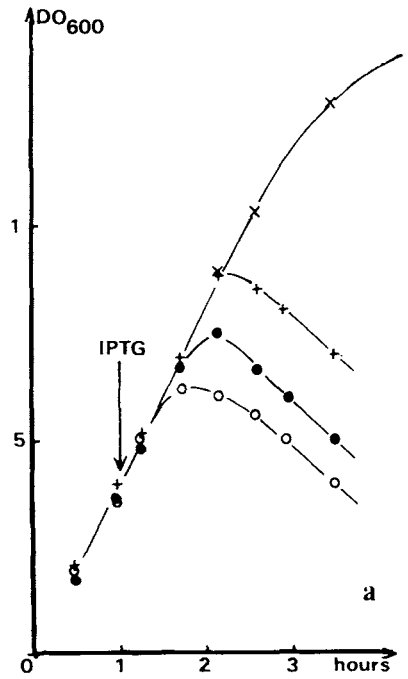

Fig. 4. Growth curve in liquid Luria medium of strain BB_1 containing no plasmid (×), plasmids pBBO (+), pBBa4 (●), pBBa3 (○). Ampicillin (100 μg/ml) was added to the growth medium of strains with plasmids as well as 10^{-3} M IPTG at $DO_{600} = 0.35$.

Unchanged reading frame							
pBBa3	pBBa6	pBBe3	pBBs1	pBBm1	pBBc5	pBBx7	pBBc2
411	306	294	192	183	132	132	132
~422	316	425	309	~340	~425	~425	350
S	R	S	R	R	R	S	R
SSS	SSS	SS	SSS	SSS	SSS	SS	SSS
OM	OM	OM	OM	OM	OM	OM	—
47.8	35.5	47.8	38	37.1	47.8	47.8	—
421	313	421	335	327	421	421	—

RESULTS

Strain Characterization

This paper describes in some detail a set of 16 plasmids bearing modified *lamB* genes (see construction in Materials and Methods) that belong to two categories (Table I). In the first category, the insertion of linkers results in a frameshift and abnormal termination of the protein. The second category contains insertions and deletions that do not modify the reading frame and thus code for proteins with unchanged COOH termini.

The mutagenesis site reported on the first line corresponds to the cleavage site of the restriction endonuclease used for the construction. It represents approximately the length of unchanged peptide at the NH_2 terminal of the protein. The length reported on the second line is only approximate, since small deletions may have occurred.

A lambda-resistant strain becomes lambda-sensitive when transformed with the pBBO plasmid containing the wild-type *lamB* gene. Most of the time, the modified LamB proteins do not retain their ability to yield lambda-sensitive strains (Table I, line 3). The protein coded by pBBa8, in which only the last 10 amino acids at the COOH terminal have been changed, has lost this property. Strains pBBe3 and pBBc7, however, are representative of plasmids in which linker insertion has only slightly modified the behaviour of the protein: it renders the clones lambda-sensitive and $IPTG^{SS}$. Strain pBBa3, which has a small deletion at the COOH terminal region and contains no linker insertion, also retains the capacity to confer sensitivity to lambda, but the protein expression inhibits growth even when produced at moderate level (Table I, lines 3 and 4).

Transformation of strain BB_1 or BB_2 with one of the plasmids causes a sensitivity to IPTG (Table I, line 4). This sensitivity also is shown by growth curves in liquid Luria medium (Fig. 4). Hypersensitive strains ($IPTG^{SSS}$) lyse after one generation or so when 10^{-3} M IPTG is added. Other sensitive strains also lyse but after a longer period of growth.

Electron microscopy reveals no differences between strain BB_1 with or without plasmid pBBO when induced for 40 min with IPTG, except that a higher level of lysed bacteria is observed. When transformed with plasmid pBBa3, strain BB_1, however, exhibits some anomalies under the same conditions. Septum formation seems to be inhibited (Fig. 5a,b) and membrane budding with the release of numerous vesicles is observed (Fig. 5c,d,e).

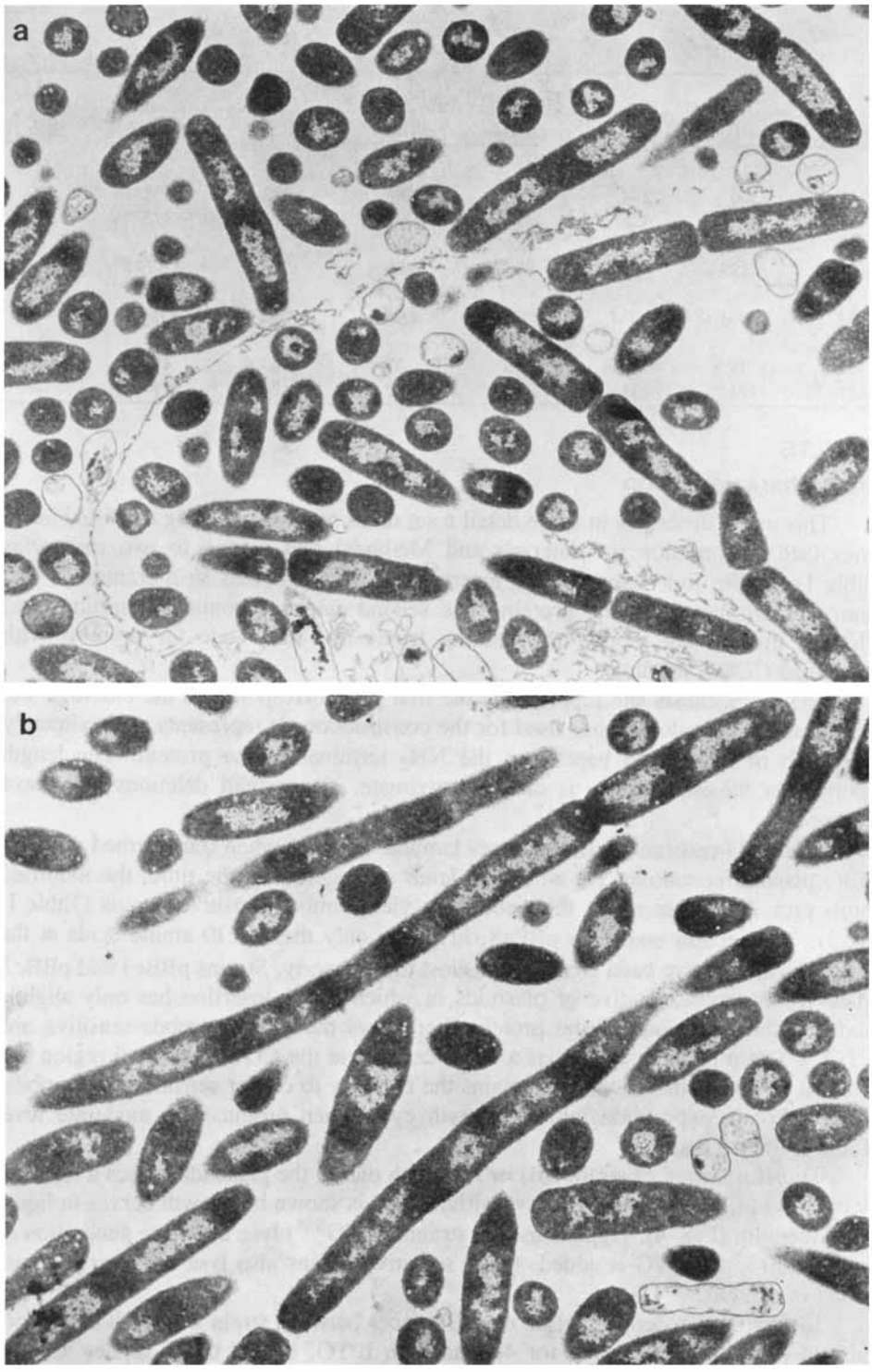

Fig. 5. Electron microscopy of strain BB_1 containing plasmid pBBo (a,c) or plasmic pBBa3 (b,d,e). Cells were grown in liquid Luria medium with ampicillin (100μg/ml) to DO_{600} = 0.5, then induced during 40 min with 10^{-3} M IPTG before fixation, (a,b) × 14,000. (c,d,e) × 73,500 (photo Jean-Claude Bénichou).

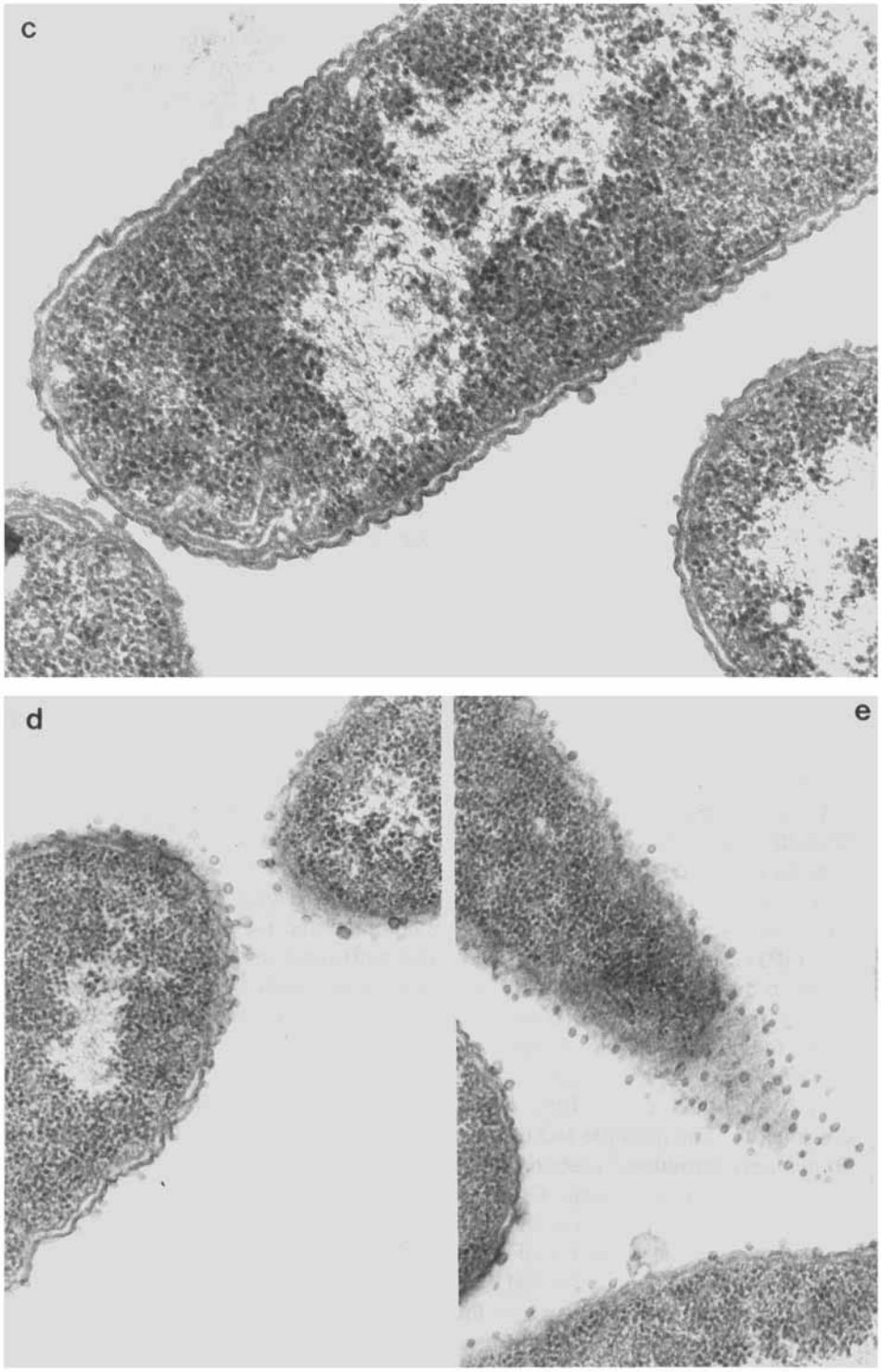

Figure 5 (c–e)

Protein Characterization

Strain BB_2, which has the *lamB* gene deleted, has been transformed by the set of plasmids. The whole cell extracts of the resulting strains were immunoprecipitated after induction with IPTG and labeling for 2 min (Fig. 6). Except for pBBc1 and pBBh7, all the proteins encoded by the plasmids could be detected and the molecular weight of the heaviest bands observed on the gel (Table I, lines 5 and 6) were in close agreement with those predicted.

The presence of two bands in several strains is not yet understood. The stability properties of these two bands during at least 20 min do not suggest that one of them represents a precursor form. Experiments are underway to study this phenomenon.

For proteins with premature termination, stability decreases with length. Proteins, encoded by plasmids pBBO and pBBa8 are stable in whole cells. Those encoded by plasmids pBBh1 and pBBa4 exhibit halflives of about 30 min in whole cells. After a 2-min labelling period followed by cold TCA precipitation, degradation products could already be detected. It is of interest to note that these degradation products appear as discrete bands corresponding to differences of about 15 amino acids.

Using the membrane separation techniques described by Crowlesmith et al [14], it appears that the modified LamB proteins thus far tested are totally localized in the outer membrane, as found for the wild-type LamB protein.

DISCUSSION

Toxicity of Modified Proteins

Contrary to what was observed for fusions with beta-galactosidase [3,15], lethality of the modified proteins studied in this paper does not seem to originate from a block in the secretion pathway. Indeed, membrane-separation experiments indicate that all of the modified LamB proteins are localized in the outer membrane.

A likely explanation could be that the lysis that is observed when wild-type LamB protein is overproduced results from overloading of the outer membrane with this protein (overproduction of periplasmic proteins do not induce lysis, P. Duplay, personal communication). Incorrect folding of modified proteins in the membrane may also result in disruption of the outer membrane and increase the sensitivity of strains to IPTG. Such a hypothesis is consistent with electron microscopy pictures. In the frame of this hypothesis, lethality of proteins where only the last ten amino acids at the COOH terminal have been changed, suggest that these amino acids are important functionally in interacting with the membrane.

For proteins of premature termination, sensitivity to IPTG decreases as the molecular weight decreases (Table I). This decrease is likely due to the decrease of protein stability. The multiple and discrete bands observed when immunoprecipitating the prematurely terminated proteins encoded by plasmids pBBh1, pBBa1, pBBe2, and pBBn6 (Fig. 6) are intriguing. One could speculate that the existence of domains containing about 15 amino acids that are relatively protected from degradation would be consistent with the presence of transmembraneous stretches of about 15 amino acids in the portion of LamB located before amino acid 321.

In conclusion, one can observe that all modified LamB proteins except those with small insertions, such as the proteins encoded by pBBe3 and pBBc7, are either toxic or unstable. This result should be kept in mind during attempts to construct secretion vectors.

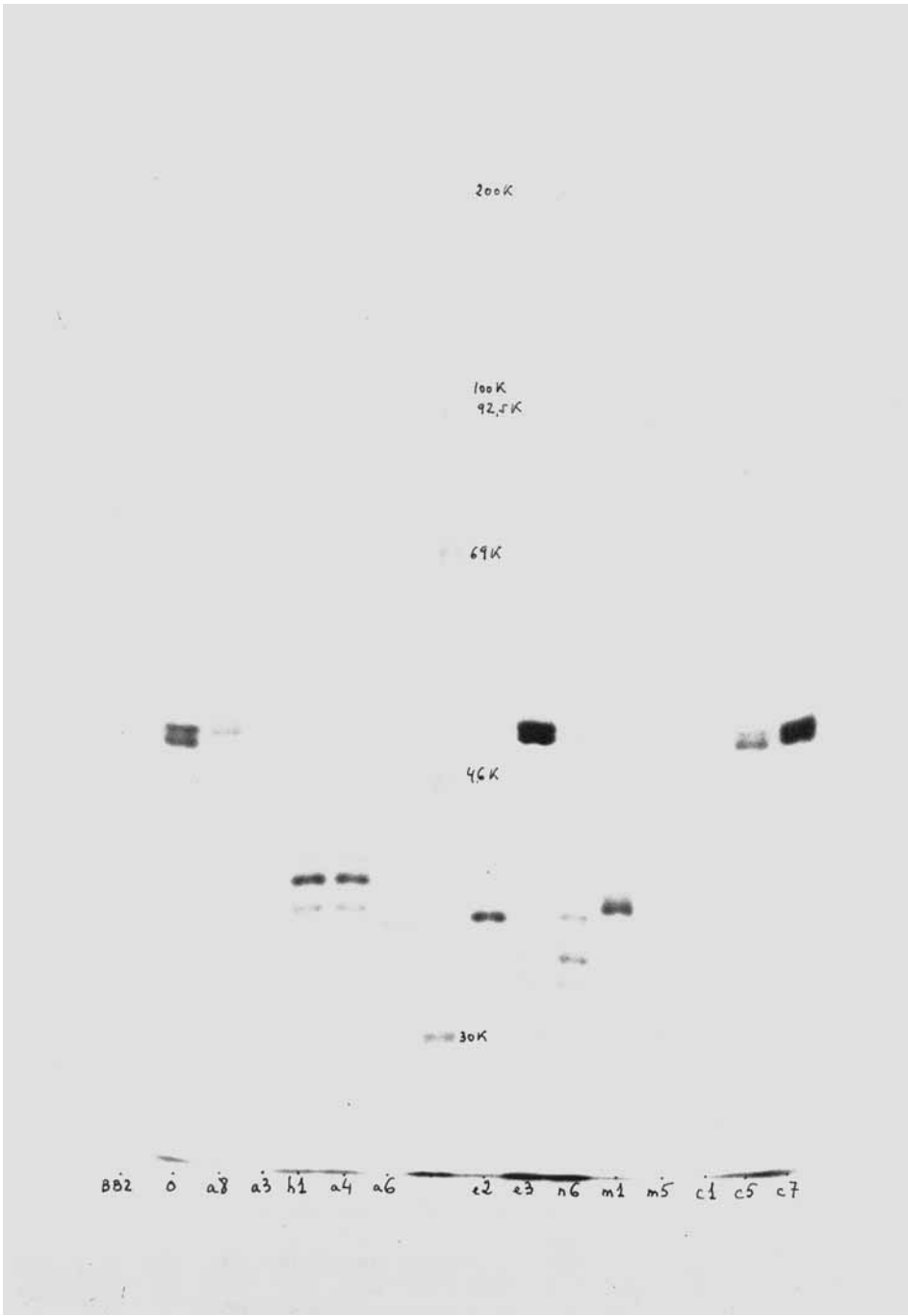

Fig. 6. Immunoprecipitation of whole cellular extracts. Cells at DO_{600} = 0.8 were induced for 10 min with 10^{-3} M IPTG, then labeled for 2 min with 35S-Methionine. Extreme left lane = strain BB_2, without plasmid; other lanes, with plasmid as indicated (see Table I). Note molecular weight marker in the center lane (8th lane starting from the left).

Sequences Affecting Localization of LamB Protein

As indicated above, the modified proteins thus far studied appear to be totally localized in the outer membrane (Table I). Therefore, none of the determinants involved in proper location in the outer membrane have been changed in these proteins. The aberrant foldings apparently do not affect location. Plasmids pBBml, pBBs1, and pBBe2 contain deletions that cover the C-terminal part of LamB after amino-acid 183. The location of the corresponding proteins in the outer membrane indicate that the first 183 amino acids of the mature LamB protein, contain, together with the signal sequence, all the information required for proper localization of the protein (Table I).

These data appear to indicate that there are no determinants for the efficiency of localization after amino acid 183, in contrast to the conclusions based on the properties of hybrid LamB-LacZ proteins [5]. These latter conclusions were the poor efficiency of transfer to the outer membrane of a LamB-LacZ hybrid protein containing 170 amino acids from LamB and the greater efficiency of transfer of the hybrid containing 41 amino acids from LamB. These results might be due to partially blocked transfer of the hybrid protein for energetic reasons. The folding of a longer portion of LamB protein into the outer membrane could liberate more free energy, enabling a higher fraction of beta-galactosidase to cross the inner membrane.

ACKNOWLEDGMENTS

The authors wish to thank Jean-Claude Bénichou, who performed the electron microscopy, and Hugues Bedouelle, Elie Dassa, and Eric Gilson for technical advice. This work was supported by grants from the DGRST and CNRS (C.P. 960002), the NATO (grant 1297), the Fondation pour la Recherche Médicale, the Association pour la Recherche sur le Cancer, and the Ligue Nationale contre le Cancer.

REFERENCES

1. Michaelis S, Beckwith J: Annu Rev Microbiol 36:435-465, 1982.
2. Moreno F, Fowler AV, Hall MN, Silhavy TJ, Zabin I, Schwartz M: Nature 286:356-359, 1980.
3. Bassford PJ, Silhavy TJ, Beckwith JR: Bacteriol 139:19-31, 1979.
4. Hall MN, Silhavy TJ: Annu Rev Genet 15:91-142, 1981.
5. Hall MN, Schwartz M, Silhavy TJ: J Mol Biol 156:93-112.
6. Benson SA, Silhavy TJ: Cell 32:135-1335, 1983.
7. Thirion JP, Hofnung M: Genetics 71:207-16, 1972.
8. Randall-Hazelbauer LL, Schwartz M: J Bacteriol 116:1436-1446, 1973.
9. de Boer HA, Comstock LJ, Vasser M: Proc Natl Acad Sci USA 80:21-5, 1983.
10. Clément JM, Perrin D, Hedgpeth J: Mol Genet G 185:302-310, 1982.
11. Szmelcman S, Hofnung M: J Bacteriol 124:112-118, 1975.
12. Hofnung M, Lepouce E, Braun-Breton C: J Bacteriol 148:853-860, 1981.
13. Ito K, Bassford PJ, Beckwith JR: Cell 24:707-717, 1981.
14. Crowlesmith I, Schindler M, Osborn MJ: J Bacteriol 135:259-269, 1978.
15. Silhavy TJ, Shuman HA, Beckwith J, Schwartz M: Proc Natl Acad Sci USA 74:5411-5415, 1977.
16. Laemmli UK: Nature 227:680-685, 1970.
17. Clément JM, Hofnung M: Cell 27:507-514, 1981.