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High-Sensitivity Detection of Newly Induced LamB Protein on the *Escherichia coli* Cell Surface

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The kinetics of the appearance at the cell surface of the outer membrane LamB protein after induction were determined by using specific antibodies and radioiodinated protein A as a probe. This was done in two different induction systems. First, LamB protein was induced in a wild-type strain by the simultaneous addition of cyclic AMP and maltose. Second, an operon fusion strain in which the *lamB* gene is expressed under *lac* promoter control was used; in this system, LamB protein can be induced by isopropyl-β-D-thiogalactopyranoside. When uninduced cells were grown in glucose minimal medium, background expression of the *lamB* gene was found to be ca. 10-fold lower in *lac-lamB* cells than in wild-type cells. The level of LamB protein present in uninduced wild-type cells could, however, be reduced by supplementing the growth medium with Casamino Acids. After induction, the LamB protein appeared at the cell surface of both strains within a few minutes, and then the LamB level per cell increased linearly. The time lag in cell surface exposure of LamB protein differed slightly under both induction conditions: the LamB protein appeared at the surface of *lac-lamB* cells within 3 min of induction, whereas in wild-type cells it could not be detected earlier than after 4 to 5 min of induction.

During or after their synthesis in the cytoplasm, outer membrane proteins are transported through the cytoplasmic membrane and the peptidoglycan layer. They insert into the outer membrane (for reviews, see references 5, 14, and 16) and in some cases are exposed at the cell surface. To follow the appearance of newly synthesized and inserted outer membrane proteins at the cell surface, one needs, first, an adequate immunoprobe and, second, a well-controlled induction system with a short time lag between synthesis and surface exposure.

As a model for studying the appearance of a newly synthesized outer membrane protein, we have chosen the maltose-inducible LamB protein (phage λ receptor). One approach is to follow the expression of the *lamB* gene after induction of glucose-grown *Escherichia coli* wild-type cells with maltose. However, there is a detectable amount of LamB protein present in uninduced wild-type cells (21, 22), and since this background of "old" LamB protein reduces the sensitivity with which newly synthesized LamB protein can be detected, we have also followed the induction of LamB protein in a *lac-lamB* fusion strain. In such cells, the *lamB* gene is expressed under *lac* promoter control, independent of the maltose regulon and of the cyclic AMP-dependent control systems, whereas the protein is exported to the outer membrane normally (13). The *lac-lamB* strain used in this study carries the fusion on the bacterial chromosome.

In this paper, we describe the conditions necessary to, first, maximize the sensitivity with which newly synthesized LamB protein can be detected at the cell surface and, second, minimize the time which elapses between protein synthesis and exposure at the cell surface.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* K-12 TK24 (18) lacking the major outer membrane proteins OmpC, OmpF, and OmpA was a kind gift of L. L. Randall; it was grown at 37°C in LB medium (15) supplemented with 0.5% (wt/vol) maltose.

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E. coli K-12 pop5217 [genotype: HfrC Δ(*gal-att*$^{\lambda}$-*bio*) *rpoB500 metA*] is a Rif^{r} *metA* derivative of strain RW592 (pop5216; 12). It has a wild-type *malB* region in which *lamB* expression is maltose inducible. Derived from this wild-type strain by P1 transduction for *metA*$^{+}$ Δ*malB107* is the *lamB* deletion strain pop5218 [genotype; HfrC Δ(*gal-att*$^{\lambda}$-*bio*) *rpoB500* Δ*malB107*). Δ*malB107* deletes all of the *malB* region. The operon fusion strain was derived from the deletion strain and is called pop5228 [genotype: HfrC Δ(*gal-att*$^{\lambda}$-*bio*) *rpoB500* Δ*malB107 lacL8UV5p lacZ'*:: *mal'K lamB*::*lac'Z*]. In this fusion strain, gene *lamB* is expressed from the *lacL8UV5p* promoter (19, 23), which is independent of the cap cyclic AMP control. pop5228 was constructed in two steps. In the first step, pop5218 was lyogenized with phage λ σ_3*h*434 (9), which carries the operon fusion and is thermoinducible. Because pop5218 has no *att*$^{\lambda}$ or *malB* region, the phage integrates in the chromosomal *lac* operon. The lysogens are Lac^{+} λ^{s} and thermosensitive. In the second step, the lysogens were cured from the phage by a heat-shock treatment. pop5228 is a Lac^{-} λ^{s} thermoresistant derivative of such a lysogen. It was checked that *lamB* expression is isopropyl-β-D-thiogalactopyranoside (IPTG; Calbiochem-Behring, La Jolla, Calif.) inducible in this strain and that introduction of a Δ*cap-5* deletion did not prevent *lamB* induction (presence of the L8UV5 mutation).

Bacteria were grown at 37°C in minimal salts medium (26) with 0.5% (wt/vol) glucose and supplemented with thiamine (2 μg/ml) and biotin (20 ng/ml). In the case of the wild-type strain, L-methionine (100 μg/ml) or Casamino Acids (0.2% [wt/vol] was also added. LamB protein was induced by adding either 4 mM cyclic AMP (Sigma Chemical Co., St. Louis, Mo.) and 0.4% (wt/vol) maltose or 1 mM IPTG to the wild-type and fusion strains, respectively. Inducers were added to exponentially growing cultures at a cell density of 0.2 to 0.25 mg (dry weight) of cells per ml (26).

Preparation of LamB-specific antiserum. LamB antigen was isolated from envelopes of maltose-grown cells of *E. coli* TK24 by extraction with sodium dodecyl sulfate (20) and subsequent release of the protein from the peptidoglycan complex by a salt treatment (2, 6). The resulting crude LamB

preparation was further purified by chromatography on a quaternary aminoethyl-Sephadex A-50 column, essentially by the method of Gabay and Schwartz (1). Based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the preparation contained 95% pure LamB protein in its oligomeric (i.e., trimeric) form.

After some preimmune serum had been collected from the rabbits, immunization was started by the injection of 0.2 mg of the purified protein in Freund complete adjuvant, both subcutaneously and intramuscularly. Booster injections of 0.2 to 1.0 mg of antigen were administered every 10 days, and antiserum was collected 60 and 75 days after the first injection.

The specificity of the antiserum was tested in an Ouchterlony double-diffusion test in the presence of 1.0% (vol/vol) Triton X-100 and in immunoprecipitations with L-[^{35}S]methionine-labeled, detergent-solubilized cell lysates. The antiserum was cleared by absorption with intact OsO_4-fixed cells for 1 h at 37°C as described below.

Radioiodination of protein A. Protein A (Pharmacia Fine Chemicals, Uppsala, Sweden) was labeled with ^{125}I by the chloramine-T method (11) and separated from free ^{125}I by chromatography on a Sephadex G-50 column, which had been prerun with phosphate-buffered saline (PBS) containing 1 mg of bovine serum albumin per ml. Based on full recovery of protein A in the void volume peak, protein A was usually labeled to an initial specific activity of ca. 2 μCi/μg. Subsequent ^{125}I decay was corrected for in all experiments.

Quantitation of LamB protein at the cell surface. After induction of LamB protein in an exponentially growing culture, samples were withdrawn and diluted twofold into ice-cold fixation medium, i.e., minimal salts medium with OsO_4 (osmium tetroxide; 0.1% final concentration, [wt/vol]; Serva, Heidelberg, Federal Republic of Germany) (24). Cells were left overnight in the fixation medium at 0 to 4°C. Fixed cells were washed twice with PBS (pH 7.2) and resuspended in this buffer. Based on measurements of the optical density at 450 nm, each sample was divided into a number of aliquots containing 0.2 mg (dry weight) of cell (ca. 0.6×10^9 cells) each. The aliquots were stored at −20°C.

Fixed cells were precoated with normal rabbit serum from which the immunoglobulin G had been removed by passage over a Sepharose-protein A (Pharmacia) column to reduce aspecific binding of serum proteins to the cell surface (3). Precoated cells were collected by centrifugation (3 min in an Eppendorf centrifuge), resuspended in PBS, and incubated with LamB-specific antiserum. The same amount of antiserum was added to all samples of an induction experiment, and it was determined in a separate experiment that this amount was sufficient to detect all cell surface-exposed LamB protein present in the cells; during the antiserum incubation (30 min at room temperature), the serum concentration did not exceed 1% (vol/vol). Antibody-labeled cells were collected, washed once with PBS, and labeled with ^{125}I-protein A; a constant and saturating amount of radioiodinated protein A was added to all samples, and incubation was performed for 15 min at room temperature. Cells were collected, washed twice with PBS, and resuspended in a total volume of 300 μl. Samples were counted as gammaemitters in a heterogeneous system with tetrabutyltin-loaded scintillator (Gammavial C; Koch-Light, Colnbrook, England). By taking decay into account, the amount of cell-bound radioactivity could be expressed as the number of protein A molecules bound per cell, thus allowing a direct comparison of individual experiments.

Cell losses which occurred during the series of incubations and washings in Eppendorf vials could largely be prevented by the addition of tryptone (Difco Laboratories, Detroit, Mich.; C. L. Woldringh, personal communication): a drop of 5% (wt/vol) tryptone was therefore added to the samples after each resuspension step.

RESULTS

Optimalization of the detection procedure. To reliably follow the appearance of newly synthesized LamB molecules at the cell surface, it was necessary to maximize the specificity of the probe and to stop insertion of the newly induced protein effectively upon sampling.

Insertion of LamB protein was stopped by direct fixation of the cells to ensure that the newly inserted protein did not leave its initial insertion site during sample preparation. Various fixation procedures did not prevent subsequent immunological detection; LamB antigenic determinants were best preserved after fixation with either OsO_4 or formaldehyde (Table 1).

Of the two successful fixatives, we chose to work with OsO_4 for two reasons. First, in contrast to formaldehyde, OsO_4 reacts primarily with lipids and only to a limited extent with proteins (7). Second, OsO_4 fixation is stable, whereas formaldehyde fixation can be reversed by extensive washings (7). The main disadvantage of OsO_4, i.e., the low penetration rate (4), should not be a problem when the primary aim is to fix the surface of a bacterial cell. This is especially true for *E. coli* K-12 strains which have no O-antigen chains.

It is clear that fixation of the cells with OsO_4 stopped LamB insertion effectively, since cells sampled 1 to 2 min after induction showed the same low level of LamB protein as did cells sampled just before induction (see Fig. 2).

Cell surface exposed LamB protein was probed with a specific antiserum. To test the specificity of the probe, we compared the binding of antibodies to uninduced cells with that to cells induced for 11 or 22 min (Fig. 1). The LamB-specific antiserum recognized cell surface exposed LamB protein in OsO_4-fixed cells, as shown by the fact that antibody binding was induction dependent and increased linearly with induction time. The LamB-specific antiserum also contained antibodies directed against other cell surface components, since there was considerable antibody binding to cells deleted for the *lamB* gene (Fig. 1, triangles), which could not be accounted for by aspecific antibody binding (Fig. 1, squares). The contaminating antibodies were probably directed against lipopolysaccharide rather than other outer membrane proteins since anti-LamB antibodies precipitated essentially pure LamB protein from L-[^{35}S]methionine-labeled cell lysates, and an Ouchterlony test of the antiserum against lipoprotein, which is a regular contaminant in porin preparations (10, 17), showed no immunoprecipitate.

The contaminating antibodies were absorbed with various amounts of fixed *lamB* deletion cells or fixed uninduced *lac-lamB* cells. The antiserum was best cleared by a single absorption step with 100 mg of OsO_4-fixed, uninduced *lac-lamB* cells per ml of antiserum (Fig. 1, open circles); repeating this absorption step did not improve antiserum specificity any further.

By using the cleared anti-LamB antiserum as a probe, the level of LamB protein detected in uninduced *lac-lamB* cells was found to be extremely low and to approach the level of aspecific binding (Fig. 1, open circles and squares, respectively). Since a similar low LamB level was detected when a cell lysate of uninduced ^{35}S-labeled *lac-lamB* cells was

TABLE 1. Antigenic preservation of LamB protein in variously fixed *E. coli lac-lamB* cells

Induction time (min)	Molecules of protein A bound per cell via LamB-specific antibodies[a] after fixation of the cells with:		
	OsO_4	Formaldehyde[b]	Formaldehyde + glutaraldehyde[b]
−1[c]	82	88	61
4	164	224	134
10	820	893	496
20	1,886	2,050	1,090

[a] Cell surface exposed LamB protein was detected with cleared anti-LamB antiserum (see the text).
[b] Fixation was by the methods in reference 7, using 2% (vol/vol) formaldehyde and 0.2% (wt/vol) glutaraldehyde.
[c] This sample was withdrawn from the culture 1 min before induction of LamB protein was started.

immunoprecipitated, it is clear that the cleared antiserum probes the level of LamB protein reliably.

Kinetics of LamB insertion. LamB protein was followed at the surface of *lac-lamB* cells (Fig. 2A) or wild-type cells (Fig. 2B) as a function of induction time. When the two strains were grown under similar conditions, the lag time in the appearance of newly induced LamB molecules at the cell surface was found to be 2.5 to 3 min in *lac-lamB* cells (closed and open circles) and about 6 min in wild-type cells (squares). After these lag times, the LamB level increased at a higher rate in wild-type cells than in *lac-lamB* cells. As a consequence, the LamB protein reached a much higher level in induced wild-type cells than in induced *lac-lamB* cells: after induction for one doubling time (i.e., 60 min for wild-type cells and 65 min for *lac-lamB* cells, when cells were grown in glucose minimal medium at 37°C), we detected 15,000 to 20,000 and 4,000 to 6,000 molecules of protein A per cell for wild-type and *lac-lamB* cells, respectively. These relative amounts were confirmed when outer membranes of the two strains were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Based on the dimensions of immunoglobulin G relative to the cell surface exposed part of LamB protein, it is likely that the above numbers correspond to the number of LamB trimers per cell.

Figure 2 also reveals that the LamB level in uninduced *lac-lamB* cells is 10-fold lower than that in similarly grown uninduced wild-type cells. Since a high background level of old LamB protein decreases the sensitivity with which newly induced LamB protein can be detected, we searched for growth conditions which would result in a minimal background expression of the *lamB* gene in wild-type cells. Supplementing the growth medium with Casamino Acids was found to be effective for this purpose (Fig. 2B, open and closed triangles): in three independent experiments, the LamB background level in cells grown in the presence of Casamino Acids was found to vary between 100 and 300 molecules of protein A per cell, whereas growth of wild-type cells in the presence of methionine as the sole amino acid resulted in a background level ranging from 700 to 1,000 molecules of protein A per cell.

The lag time in the appearance of LamB protein at the cell surface was also found to be shortened by Casamino Acids: newly induced LamB protein could now first be detected after 4 to 5 min of induction (Fig. 2B, open and closed triangles). After this lag time, the LamB level increased at a very high rate, corresponding to the high growth rate (doubling time, 45 min) of wild-type cells in Casamino Acids-supplemented medium.

Comparing the induction kinetics of cell surface exposed LamB protein in *lac-lamB* cells to those in wild-type cells grown under optimal induction conditions, i.e., in medium supplemented with Casamino Acids, two important differences were observed. First, the level of LamB protein in uninduced cells was two- to threefold lower in *lac-lamB* cells than in wild-type cells, and second, the lag time preceding the appearance of LamB protein at the cell surface was 1 to 2 min shorter in *lac-lamB* cells than in wild-type cells. A third difference, i.e., the higher rate of newly induced LamB insertion in wild-type cells, most likely resulted from a higher level of overall protein synthesis in these rapidly growing cells, which accounts for a factor of 1.3 in the relative rates.

DISCUSSION

In this paper, we have optimized conditions to monitor the appearance of a newly synthesized outer membrane protein at the cell surface as fast as possible. We have done this by using two different LamB induction systems.

The first requirement is a very low background of LamB protein at the cell surface before induction. The background level of LamB protein in uninduced cells was found to be

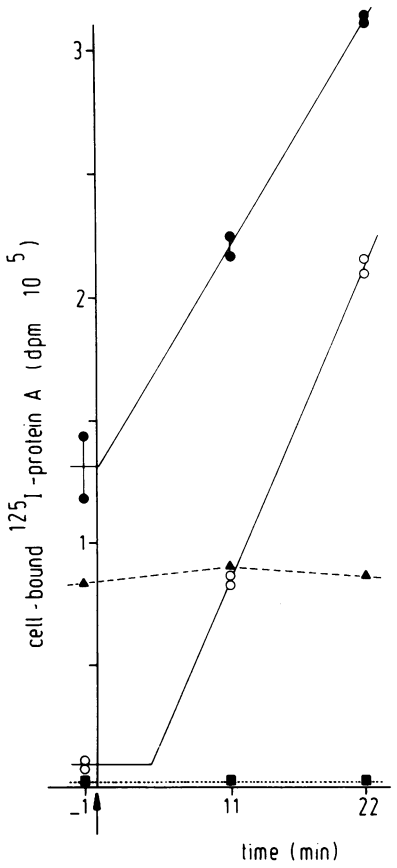

FIG. 1. Binding of LamB-specific antibodies to the surface of cells with various LamB contents. *E. coli* pop5228 *lac-lamB* cells (●, ○, and ■) or pop5218 Δ*lamB* cells (▲) were induced with IPTG at time zero (arrow). Uninduced cells sampled 1 min before induction and cells induced for 11 or 22 min were fixed with OsO_4 and probed for LamB protein; 2 μl of antiserum and 10^6 dpm of ^{125}I-protein A were added to 0.2 mg of fixed cells. The following antisera were used: anti-LamB antiserum (● and ▲), anti-LamB antiserum absorbed with 100 mg of fixed uninduced *lac-lamB* cells per ml of antiserum (○) or preimmune serum (■).

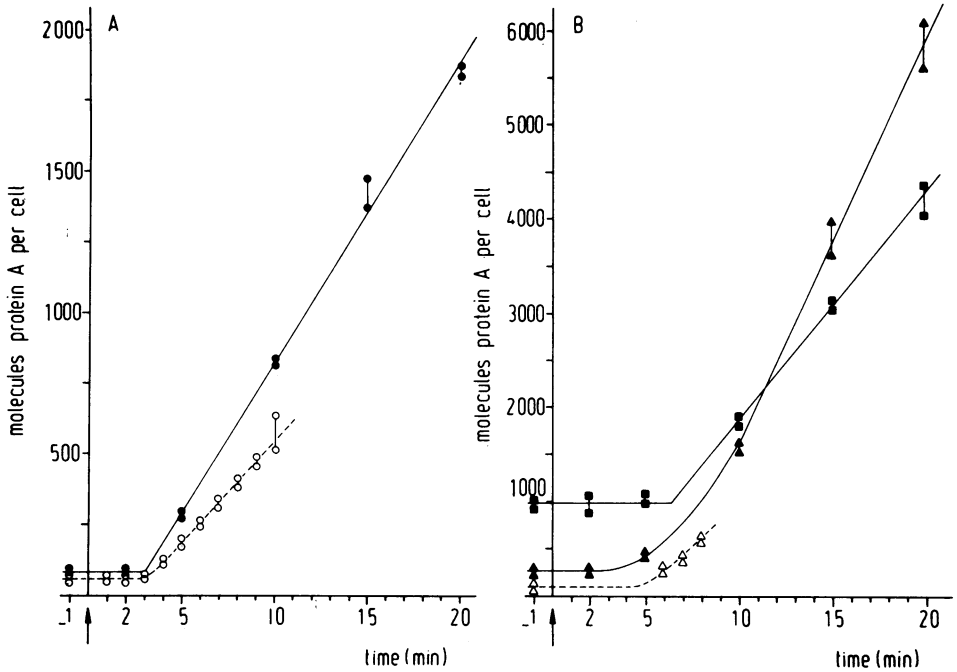

FIG. 2. Kinetics of LamB insertion in *lac-lamB* (A) and wild-type (B) cells. (A) *E. coli* pop5228 *lac-lamB* cells were grown in glucose minimal medium (●) and induced with IPTG at time zero (arrow). (B) *E. coli* pop5217 wild-type cells were grown in glucose minimal medium supplemented with L-methionine (■) or Casamino Acids (▲ and △) and induced with cyclic AMP and maltose at time zero (arrow). Duplicate samples containing 0.2 mg of OsO_4-fixed cells were incubated with 1.5 μl of cleared anti-LamB antiserum and 10^6 dpm of ^{125}I-protein A. Cell-bound radioactivity is expressed as the number of protein A molecules bound per cell, which was calculated from the radioactivity bound to 0.2 mg (dry weight) of cells (ca. 0.6×10^9 cells) and the specific activity of ^{125}-protein A. The onset of LamB insertion in *lac-lamB* or in wild-type cells grown in the presence of Casamino Acids was determined more precisely in separate experiments, represented by the open symbols (○ in A and △ in B, respectively).

lowest in a *lac-lamB* fusion strain, ranging from 40 to 90 molecules of protein A (most likely equivalent to LamB trimers) per cell. This approaches the aspecific binding of 30 to 60 molecules of protein A per cell. Although *lamB* background expression in wild-type cells could be manipulated by changing the growth conditions, the LamB background in uninduced wild-type cells remained at least two- to threefold higher than that found in *lac-lamB* cells.

The second requirement is the rapid appearance of detectable LamB protein after induction. The shortest lag time, in the order of 3 min, was found for the fusion strain. Lag times in the appearance of LamB protein at the cell surface varied with different growth and induction conditions. The observed differences could have several causes. First, entrance of the inducers, IPTG or cyclic AMP and maltose, into the cells and the action they exert on the different regulating systems may not follow the same time course. Second, the sensitivity with which newly induced LamB protein can be detected may itself affect the observed lag times. In general, LamB was detected earlier when the LamB background level was lowered. In particular, in wild-type cells (Fig. 2B), where induction was performed with cyclic AMP and maltose in all cases, the lag in LamB insertion decreased as the background level of old LamB protein was lowered. The converse was also true: an extremely long lag time (15 to 30 min) was observed in glycerol-grown wild-type cells, which have a high LamB background level (8; unpublished data).

To summarize our results, it is clear that optimal surface detection after induction requires a measuring system with good sensitivity and maximum contrast between pre- and postinduction labeling. The *lac-lamB* fusion strain provides us with such a system, and we have therefore used it to follow the topography of LamB insertion (25).

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