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Role of Lipopolysaccharide in Assembly of *Escherichia coli* Outer Membrane Proteins OmpA, OmpC, and OmpF

GEORG RIED, INGRID HINDENNACH, AND ULF HENNING*

Max-Planck-Institut für Biologie, D-7400 Tübingen, Federal Republic of Germany

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Selection was performed for resistance to a phage, Ox2, specific for the *Escherichia coli* outer membrane protein OmpA, under conditions which excluded recovery of *ompA* mutants. All mutants analyzed produced normal quantities of OmpA, which was also normally assembled in the outer membrane. They had become essentially resistant to OmpC and OmpF-specific phages and synthesized these outer membrane porins at much reduced rates. The inhibition of synthesis acted at the level of translation. This was due to the presence of lipopolysaccharides (LPS) with defective core oligosaccharides. Cerulenin blocks fatty acid synthesis and therefore that of LPS. It also inhibits synthesis of OmpC and OmpF but not of OmpA (C. Bocquet-Pagès, C. Lazdunski, and A. Lazdunski, Eur. J. Biochem. 118:105–111, 1981). In the presence of the antibiotic, OmpA synthesis and membrane incorporation remained unaffected at a time when OmpC and OmpF synthesis had almost ceased. The similarity of these results with those obtained with the mutants suggests that normal porin synthesis is not only interfered with by production of mutant LPS but also requires de novo synthesis of LPS. Since synthesis and assembly of OmpA into the outer membrane was not affected in the mutants or in the presence of cerulenin, association of this protein with LPS appears to occur with outer membrane-located LPS.

It remains unknown how proteins of the outer membrane of gram-negative bacteria are sorted to this membrane. We are studying this question with the 325-residue OmpA protein (7) of *Escherichia coli* (18, 19, 25, 26) and have asked whether non-*ompA* mutants exist which might no longer incorporate the OmpA protein, and possibly others, into the outer membrane. Toward this end we have performed selections for resistance to an OmpA-specific phage under conditions which exclude the appearance of mutations in the *ompA* gene. Paradoxically, almost all mutants recovered so far possessed an unaltered OmpA protein, present in the outer membrane, and greatly reduced quantities of the outer membrane porins OmpC and OmpF, which have nothing to do with infection by this phage. Here we show that these mutants produced defective lipopolysaccharides (LPS). These and some other results shed some light on the role of LPS in the membrane assembly of these three proteins.

MATERIALS AND METHODS

Bacterial strains, phages, and growth conditions. The *ompA*$^+$ derivative of strain UH203 (19) is *lac supF recA proA* (or *proB*) *rpsL*(F′ *lacI*^{q} *lacZΔM15 proAB*$^+$). Its *secA* derivative, strain UH204, has the same characteristics but, in addition, is *leu*::Tn*10* and *secA*(Ts51) (17, 44). The construction of a *non* mutant (49) in strain UH203 *ompA*$^+$ was started with a precursor of this strain, JC6650 *pro his* (15). Phage P1 was grown on strain P400 (which is *his*$^+$ *non* [57]) and used to transduce the former strain to *his*$^+$; the *non* allele is cotransduced at a frequency of ~30% and was identified by the inability of strains carrying it to give rise to mutants resistant to phage T7 (49). The *his*$^+$ *non* strain was made *recA* by transduction with phage T4GT7 (63) grown on strain JC10240 (10) which is *recA srl*-300::Tn*10*. Transductants resistant to tetracycline were screened for *recA* by their sensitivity to UV irradiation. Finally, the F′*lacI*^{q} was introduced as described for UH203 (19). Strain CS1389 (9) was used as a control in the RNA-DNA hybridization experiments. It is Δ*ompC* and *ompRI* and hence does not transcribe *ompF*, which is highly homologous to *ompC* (36). The OmpA-specific phages Ox2 (24, 37, 38) and K3 (22, 37, 38), as well as those specific for OmpC and OmpF, TuIb and TuIa (11), respectively, have been described. Cells were grown in L broth (35) at 37°C if not specified otherwise. Supplements were glucose (0.5%), isopropylthiogalactoside (IPTG, 1 mM), ampicillin (50 μg/ml), chloramphenicol (30 μg/ml), tetracycline (15 μg/ml), kanamycin (20 μg/ml), and cerulenin (100 μg/ml). For the experiments with cerulenin, cells were grown in a medium as previously described (29). For labeling of RNA, cells were grown at 37°C in the MOPS (morpholinepropanesulfonic acid)-AHC medium specified previously (1). Induction of OmpA synthesis in cells harboring pRD87 (see below) is lethal because of overproduction of OmpA. When grown in the presence of glucose plus IPTG, only slightly more OmpA is produced than in chromosomally *ompA*$^+$ strains. Therefore, selection for resistance to phage Ox2 was performed on solid L broth containing both compounds.

Plasmids. In plasmid pRD87 (18) the *ompA* gene is under the control of the *lac* regulatory elements. The operon fusion *ompA-cat* (pRD87-cat) was constructed as follows. The *cat* gene is located on a 0.85-kilobase *Hin*dIII-*Xba*I fragment of plasmid pDS5/3 (58). Downstream from the *Hin*dIII site, a ribosome binding site but no promoter is present. In pRD87, a unique *Hin*dIII site exists immediately downstream from the *ompA* terminator. The isolated *Hin*dIII-*Xba*I fragment was made blunt ended by filling in with the Klenow fragment of DNA polymerase I. After restricting pRD87 with *Hin*dIII, it was filled in in the same way. Upon ligation of the *Hin*dIII-*Xba*I fragment into the blunt-ended pRD87, strain UH203 *ompA*$^+$ *non* was transformed and plated onto LB medium containing glucose, IPTG, and ampicillin. Replica plating (30) onto medium containing chloramphenicol instead of ampicillin identified cells with *cat* in the correct orientation in pRD87-cat. Plasmid pTUompC-65, a derivative of pUC18 (64), carries the *ompC* gene under the control

* Corresponding author.

of the *lac* regulatory elements. This *ompC* gene was derived from pMY111 (36). Its construction will be described elsewhere. To remove pRD87-cat from cells, the strain was transformed with pK18 (47), specifying resistance to kanamycin. Transformants were grown for about 40 generations in the presence of this antibiotic and plated on medium containing it. Replica plating on medium with ampicillin identified colonies missing pRD87-cat (surprisingly, only 1%).

RNA-DNA hybridization. Log-phase cells (10 ml; optical density at 600 nm, 0.7) were labeled for 1 min with 0.17 mCi of [^{3}H]uridine (Amersham-Buchler) and killed as detailed earlier (9). RNA was extracted with guanidine thiocyanate-sarcosyl and isolated by sedimentation through CsCl (8). The *ompC* gene was isolated (as a 1.3-kilobase *Kpn*I-*Hin*dIII fragment) from pTUompC-65 by agarose gel electrophoresis followed by electroelution and further purification using an ELUTIP column (Schleicher & Schuell, Inc.). The gene was applied to nitrocellulose filters (1.4 pmol per dot), denatured (0.5 M NaOH), and immobilized by UV irradiation. Hybridization with labeled RNA (1.5×10^6 cpm per filter) was performed as previously described (9). Labeled mRNA from strain CS1389 was used to determine nonspecific background radioactivity.

Other techniques. Most of the other techniques used have been described previously, such as preparation of cell envelopes (26), pulse-chase experiments with [^{35}S]methionine, trypsin treatment, immunoprecipitation, and immunoblotting (17, 32). LPS was isolated as detailed earlier (20). Both protein and LPS were electrophoresed on Laemmli-type (28) sodium dodecyl sulfate (SDS)-polyacrylamide (12.5%) gels. To separate the OmpC and OmpF proteins, an SDS-polyacrylamide (11%) gel containing 4 M urea in the running gel was used (39). Silver staining was used for visualization of LPS (59), and protein was stained with Coomassie brilliant blue.

RESULTS

System used for mutant selection. Non-*ompA* mutants unable to insert the OmpA protein into the outer membrane would most likely be conditional, since it is unlikely that a cellular component exists specifically for insertion of this protein. Such mutants should include the export machinery, such as the temperature-sensitive *secA* (44) or *prlA*/*secY* (13, 56) mutants. We, therefore, first tested if we could distinguish *secA*(Ts) and *secA*$^+$ with the OmpA-specific phage Ox2. For successful infection, this phage, in contrast to several other OmpA-specific virions, such as K3, requires almost the full wild-type complement of OmpA. The *secA*(Ts) strain still grew, albeit slowly, at 35°C. When this strain was grown at 35°C and tested with phage at this temperature, it was resistant not only to Ox2 but also to the OmpA-specific phage K3 and phage T4, which, in *E. coli* K-12, uses the OmpC protein as a receptor (D. Montag, S. Hashemolhosseini, and U. Henning, J. Mol. Biol., in press). Even at the permissive temperature (28°C), the strain accepted the three phages with a reduced efficiency of plating (10^{-2} to 10^{-3}) and they produced turbid plaques. Apparently, these T-even-type phages require a wild-type *secA* gene for efficient growth. (This would be understandable if their proteins 20 and 40, which have recently been shown to be membrane proteins [34], use the Sec pathway for membrane incorporation.) Nevertheless, the *secA*(Ts) and *secA*$^+$ strains were grown at 35°C, mixed at various ratios, and plated with Ox2 at this temperature. *secA*(Ts) cells could easily be found in the presence of up to a 10^4-fold excess of the *secA*$^+$ cells.

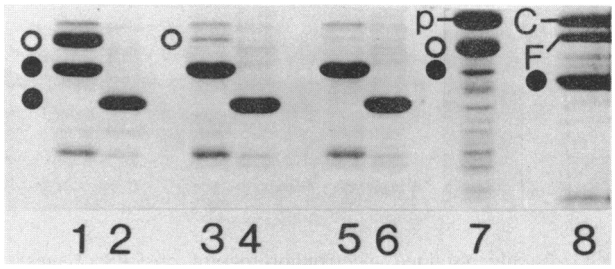

FIG. 1. Stained SDS-polyacrylamide gel electrophoretogram. Envelopes from cells grown in the presence of glucose were solubilized at 100 (lanes 1, 3, and 5) or at 50°C (lanes 2, 4, and 6). Lanes: 1 and 2, parental strain; 3 and 4, mutant II-4; 5 and 6, mutant III-9; 7, parental strain grown for 3 h in the presence of IPTG (note the accumulation of the OmpC precursor [p] and the reduction of the concentration of OmpA [9]); 8, gel containing 4 M urea (39) used to separate OmpC (C) from OmpF (F) (both are present in about equal quantities in the glucose-grown parental strain). The gel system used for lanes 1 through 7 does not separate OmpC from OmpF. Symbols: ●, OmpA; ○, mixture of OmpC and OmpF.

To completely exclude selection for *ompA* mutants, a strain was used which is chromosomally *ompA*$^+$ and carries a plasmid harboring, under the control of the *lac* regulatory elements, the wild-type *ompA* gene coupled to the *cat* gene via an operon fusion (pRD87-cat). Selection for resistance to chloramphenicol avoids loss of or decreased rates of transcription of *ompA*. Growth in the presence of IPTG is lethal because of overproduction of OmpA. However, growth in the presence of glucose plus IPTG will induce expression of the plasmid-borne *ompA* gene to a degree not deleterious to cells (16) and still cause resistance to chloramphenicol, up to 30 μg/ml. Selection was then made with this strain for resistance to Ox2 at 35°C, in the presence of glucose, IPTG, and chloramphenicol. Preliminary experiments revealed that this selection, however, yielded mainly mucoid colonies. Therefore, strain UH203 *ompA*$^+$ *non* was used as a host for plasmid pRD87-cat.

Mutants. Mutants resistant to phage Ox2 appeared at frequencies of 10^{-6} to 10^{-7}. With a few exceptions which have not yet been clarified, these mutants (8, of independent origin, analyzed) were not conditional, i.e., would grow at 28 or 42°C. Either they were resistant to phage K3 or this phage infected with a reduced efficiency of plating. All had also become resistant to phages TuIa and TuIb (receptors: OmpC and OmpF protein, respectively), or these phages infected with much reduced efficiency of plating, i.e., the phages caused lysis when applied at high concentration to a lawn of cells but could not form plaques. They had also become resistant to T4, requiring the OmpC protein for adsorption. As expected, the mutants were chromosomal; transformation of a parental strain with their resident plasmid pRD87-cat showed that the phage resistance-sensitivity pattern was not associated with the plasmid.

An electrophoretic analysis revealed that all mutants possessed more or less severely reduced concentrations of the OmpC and OmpF proteins; examples are shown in Fig. 1. By using an immunoblot, it could be shown that the faintly stained band (Fig. 1, lane 3) represents the porins (not shown). Furthermore, the porins of the mutants are apparently present in the outer membrane as trimers. It requires boiling in SDS to dissociate them into monomers (51); Fig. 1 demonstrates that the band in question is absent in extracts

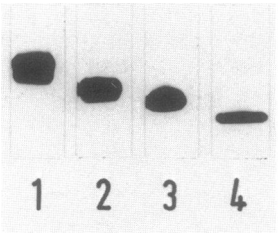

FIG. 2. Silver-stained electrophoretogram of LPS. Lanes: 1, parent strain; 2, *E. coli* B; 3, mutant II-4; 4, mutant III-9. The core oligosaccharide of *E. coli* B is smaller, by about 4 sugar residues, than that of *E. coli* K-12 (45, 46).

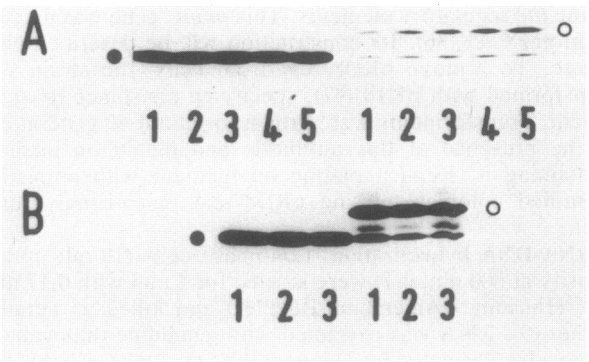

FIG. 3. Autoradiogram of an SDS-polyacrylamide gel electrophoretogram of immunoprecipitates. (A) Mutant II-4; (B) parent strain. Left halves, Precipitates with anti-OmpA serum; right halves, precipitates with anti-OmpC and -OmpF serum (with this serum some OmpA always coprecipitated). Cells were labeled with [^{35}S]methionine for 1 min and chased with methionine for 2 (lane 1), 20 (lane 2), 60 (lane 3), 120 (lane 4), and 180 (lane 5) min (A) and for 2 (lane 1), 120 (lane 2), and 180 (lane 3) min (B). Symbols: ○, OmpC and OmpF; ●, OmpA.

obtained at 50°C. There was no reduction in the concentration of OmpA. Two properties of this polypeptide, aside from its activity as a phage receptor, can be used to assess whether or not it is assembled correctly in the outer membrane (for a more detailed description and relevant references, see reference 26). The protein consists of two moieties, a membrane domain encompassing residues 1 to about 170 and a periplasmic domain consisting of the COOH-terminal part (7). The former is thought to exist in the membrane in an antiparallel β-sheet conformation (38, 60). Treatment of isolated cell envelopes with trypsin will leave the membrane domain intact and lead to a loss of the periplasmic part. Breaking the β-structure requires boiling in SDS; OmpA not assembled in the outer membrane does not possess the final β-structure (19). The boiled protein migrates electrophoretically as a 36-kilodalton species; when it is dissolved in the detergent at 50°C, the molecular weight determined in this way is 32,000. This behavior has been called heat modifiability. In all cases, OmpA was heat modifiable and yielded a tryptic fragment of the same size as that obtained from parental cell envelopes. Thus, nothing detectable was wrong with the OmpA protein of the mutants.

It is long known that, in *E. coli* or *Salmonella typhimurium*, LPS mutants may possess reduced amounts of several outer membrane proteins (14, 27, 31). We, therefore, asked whether we may have been dealing with such mutants. Phage U3 will infect *E. coli* K-12 only when it produces an LPS with a complete core oligosaccharide (62), while phage C21 can only infect cells with an LPS of the *E. coli* B type, i.e., lacking galactose residues (52, 55). Most mutants were resistant to phage U3 and sensitive to C21, and the inverse was found for the parent. To demonstrate the LPS defect directly, LPS was isolated from a few mutants and analyzed electrophoretically (Fig. 2). Clearly, we had obtained LPS mutants. Two mutants, III-9 and II-4, were used to select for resistance to phage C21. In both cases, resistant clones were recovered which had become sensitive to phage U3 and had lost the mutant phenotype,including production of wild-type amounts of OmpC and OmpF proteins. Hence, their mutant phenotype was caused by the defects in LPS synthesis. These two strains were studied further.

Overproduction of the OmpC protein. Why does a defective LPS cause a decrease of the two porins in the outer membrane? We have first asked whether this barrier can be overcome by increasing the production of the OmpC protein. The results were the same for both mutants. Plasmid pRD87-cat was removed from the mutants and replaced by pTUompC-65 carrying the *ompC* gene under the control of the *lac* regulatory elements. When grown in the presence of IPTG, the cells possessed much more OmpC protein, as much as that shown in Fig. 1 (lane 1). This is considerably less than was found when the parental strain, carrying pTUompC-65, was grown in the presence of the inducer (Fig. 1, lane 7). Also, the parent was killed (lysis) by such overproduction of OmpC, whereas the mutants under this condition grew up normally and remained viable. However, the mutant cells possessing pTUompC-65 remained just as resistant to phages T4 and TuIb as the mutant without pTUompC-65. We therefore determined whether the overproduced OmpC is assembled in the outer membrane. If so, the protein would be completely insoluble in SDS at 50°C and resistant to trypsin in isolated cell envelopes (51) and would expose a site cleavable with proteinase K at the cell surface (40). All these criteria were met by the OmpC protein present in the two mutants and expressed from pTUompC-65 (data not shown). Thus, when overproduced, OmpC can be inserted into the outer membrane but remains unable to serve as phage receptor.

Porin synthesis is inhibited at the level of translation. Mutants II-4 and III-9 were pulse-labeled for 60 s with [^{35}S]methionine and chased with methionine for the times indicated in Fig. 3. The results were the same for both strains. Porin was synthesized at only low rates, and the labeled protein was stable, consistent with the conclusion that it is present in the membrane as trimers (see above).

To assess at which level of gene expression the inhibitory mechanism acts, we measured *ompC* mRNA, labeled with [^{3}H]uridine, by hybridization to *ompC* DNA. Hybridization (in counts per minute) to filter-bound DNA for [^{3}H]mRNA from various strains was as follows: UH203 *ompA*$^{+}$ *non*, 350; mutant II-4, 450; mutant III-9, 750; and CS 1389 (Δ*ompC ompRI*), 20. There was no inhibition of transcription; in fact, in mutant III-9, the level of *ompC* mRNA was reproducibly found to be about twice that of the parental strain; the cause for this increase remained unknown.

The effect of the fatty acid analog cerulenin. Cerulenin blocks fatty acid synthesis (5, 21). It has been reported that it also inhibits synthesis of OmpC and OmpF but not that of OmpA (2). The effect on OmpF synthesis in *E. coli* B was recently investigated in more detail (3). It was found that, in the presence of cerulenin, the protein failed to trimerize, followed by inhibition of OmpF synthesis; the accumulated monomer was degraded. The authors suggested that it was

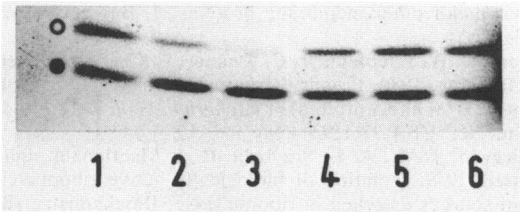

FIG. 4. Action of cerulenin. To cells grown to an optical density at 600 nm of 0.5, cerulenin was added, and after 6 (lane 1), 18 (lane 2), and 30 (lane 3) min they were labeled with [^{35}S]methionine followed by a 2-min chase with methionine. The same pulse-chases were performed in the absence of the drug (lanes 4 to 6). OmpA (●) and OmpC and OmpF (○) were immunoprecipitated by the addition of both antisera against OmpA and OmpC and OmpF. An autoradiogram of an SDS-polyacrylamide gel electrophoretogram is shown.

most likely inhibition of synthesis of LPS which caused these effects. Since their results are similar to those we obtained with our mutants, we have asked whether the OmpA protein produced in the presence of the drug was normally incorporated into the outer membrane. Figure 4 shows that cerulenin acted with our K-12 strain as previously described (2, 3). Figure 5 demonstrates that OmpA, produced in the presence of cerulenin, gave rise to the same tryptic fragment as the protein synthesized in the absence of the antibiotic. Also, all OmpA synthesized in the presence of cerulenin was as heat modifiable as is normally observed with this protein (not shown). Hence, OmpA was normally assembled in the membrane.

DISCUSSION

Our data show that production of an LPS with a defective core oligosaccharide had no influence on synthesis and normal membrane insertion of OmpA, while the rate of OmpC and OmpF synthesis was greatly reduced. (For unknown reasons, these results differ somewhat from those obtained earlier [31]. A drastic decrease of the concentration of OmpF in *E. coli* LPS mutants, and also in a heptose-less strain, was observed, but no effect on the concentration of the OmpC protein, designated proteins b and c at the time,

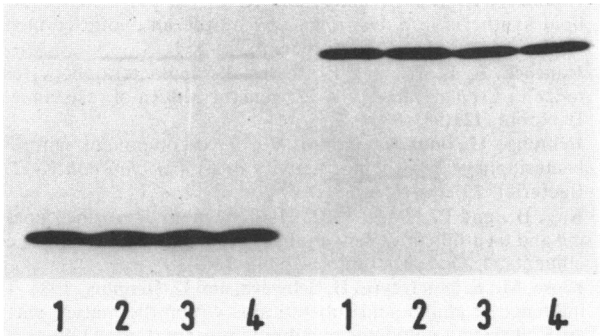

FIG. 5. Autoradiogram of an SDS-polyacrylamide gel electrophoretogram of immunoprecipitates. Cells in lanes 1 and 2 were grown in the presence of cerulenin for 18 and 30 min, respectively, and then labeled with [^{35}S]methionine for 1 min, followed by a 2-min chase with methionine; the same procedure was done in lanes 3 and 4, but in the absence of cerulenin. Cell envelopes were treated with trypsin (left half) or left without the protease (right half).

respectively, was seen). The residual porin was apparently assembled normally in the outer membrane. OmpC, when overproduced in the mutants, could be inserted into the membrane at a much higher concentration. This would be understandable if trimerization of OmpC, which appears to be a prerequisite for membrane assembly (3, 4), required LPS. The mutant would then show that the affinity of the protein to a defective LPS was not null. However, it must have caused a build up of monomers which probably feedback inhibited their own synthesis. If in the sequence monomer → dimer → trimer (42, 50) the monomer concentration was much increased, the reactions, both of which may require LPS, could be driven to the trimer state. It should be noted, however, that the results do not tell which part(s) of the LPS is required for normal synthesis and membrane incorporation of the porins. While it is clear that the mutants produced LPS with defective core oligosaccharide, this need not mean that it was the missing sugars which are required; rather, their absence could lead to an LPS which interfered with, e.g., trimerization, by an incorrect association with the protein. In other words, the mutant LPS may have acted as an inhibitor. Again, such inhibition could partially be overcome by an increase of monomer concentration.

It certainly appears that the action of cerulenin has the same basis as that causing the phenotype of our mutants. The drug blocked LPS synthesis and led to a failure of the OmpF porin to trimerize, the monomer accumulated, OmpF synthesis was inhibited, and the monomer was slowly degraded; significantly, the accumulated monomer could no longer trimerize even after lipid synthesis was restored (3). It is rather likely, therefore, that trimerization and membrane assembly of the porin require newly synthesized LPS. In contrast, OmpA was produced at normal rates and was also quantitatively incorporated into the outer membrane in the presence of the drug. Cerulenin completely abolishes de novo synthesis of LPS but not the conversion of a preformed lipid A precursor to an underacylated LPS which is translocated to the outer membrane (61). When the results of Walenga and Osborn (61) are compared with those of Bolla et al. (3), it appears that this underacylated LPS is able to promote porin trimerization. While the incorporation of [^{14}C]acetate into LPS fatty acids was already completely blocked after 5 min in the presence of cerulenin, the preformed lipid A precursor became exhausted, i.e., converted to underacylated LPS, after only 15 to 30 min (61). Similarly, trimerization was inhibited by only about 50% after 6 min and reached near complete inhibition between 16 and 30 min action of cerulenin (3). OmpA synthesis and membrane incorporation were still perfectly normal after a 30-min incubation of cells in the presence of the drug. We conclude that this protein can associate with LPS preexisting in the membrane. This fact practically excludes earlier proposals, suggesting a transporting role for LPS, and creates a new problem concerning sorting of OmpA to the outer membrane.

It had been shown that LPS is able to structurally renature a denatured OmpA and that this renaturation can be achieved with the lipid A moiety alone (53). On the basis of these and other results (12, 19), a role of LPS in the cellular sorting of this protein had been proposed (11, 12, 19, 53). However, LPS essentially occurs only in the outer leaflet of the outer membrane (for a review, see reference 43). How does OmpA find this LPS? Since the protein is not guided to the membrane together with LPS, it is difficult to envision how the primary recognition of the membrane occurs via an

LPS-OmpA interaction, unless some sort of local discontinuity in the inner leaflet exists.

A feedback mechanism which operates at the translational level has been described for a number of outer membrane proteins. Expression of a mutant *ompC* gene was found to inhibit synthesis of the outer membrane proteins OmpA, OmpF, OmpC, LamB, and Lc (6). Similarly, synthesis of some mutant OmpA proteins inhibited production of OmpA, OmpC, OmpF, and LamB (unpublished results). It is therefore particularly intriguing that in our mutants, when translation of *ompC* mRNA was inhibited, OmpA synthesis was not affected, demonstrating the existence of yet another, more specific inhibitory mechanism. It is unfortunate that in the parental strain used, neither the maltoporin LamB nor the PhoE porin could be identified on electrophoretograms by staining after growth in the presence of maltose or upon phosphate starvation, respectively. Mutants will have to be selected in another strain in order to find out whether the inhibition is generally porin specific (which all trimers are) or acts only on the *ompC* and *ompF* translation.

Finally, it is not surprising that the mutants possessing wild-type OmpA were resistant to OmpA-specific phages. It has been shown previously that correct interaction of LPS core sugars with the protein is required for phage receptor activity (23, 41, 48). As demonstrated by the mutants overexpressing the OmpC protein but still remaining resistant to phages T4 and TuIb, the same conclusion can be drawn for this polypeptide.

When the manuscript was completed, a very interesting paper (54) came to our attention. The OmpF porin is secreted from spheroplasts into the medium (33). Sen and Nikaido demonstrated that this soluble porin is in all probability a monomer and that it can trimerize and become incorporated into cell envelope preparations, provided that Triton X-100 is present (54). We suggest that this detergent may substitute for the newly synthesized LPS which appears to be required for porin trimerization and its assembly into the outer membrane.

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