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Quantitation of the Loss of the Bacteriophage Lambda Receptor Protein from the Outer Membrane of Lipopolysaccharide-Deficient Strains of *Escherichia coli*

LINDA L. RANDALL

Wallenberg Laboratory, University of Uppsala, Uppsala, Sweden

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The receptor for the phage lambda, a protein component of the outer membrane, is present at decreased levels in strains of *Escherichia coli* that are deficient in lipopolysaccharide. Loss of the protein was quantitated both by an assay of the phage receptor function and by an assay of antiserum-blocking ability to detect inactive protein. The loss of protein was correlated with the loss of sugar residues and phosphate from the core region of the lipopolysaccharide. Implications for the importance of ionic interactions in the stabilization of the outer membrane are discussed.

The outer membrane of gram-negative bacteria is exterior to both the cytoplasmic membrane and the rigid peptidoglycan layer. It differs from the cytoplasmic membrane in two important ways: (i) the assembly of the outer membrane involves a translocation step from the site of synthesis of the components, in the cytoplasm or at the cytoplasmic membrane, to the final site exterior to the peptidoglycan; and (ii) the outer membrane contains not only lipid and protein, as does the cytoplasmic membrane, but in addition lipopolysaccharide (LPS). Elucidation of the interactions of these membrane components is critical to the understanding of the structure and assembly of the outer membrane. Recent work with both *Escherichia coli* and *Salmonella typhimurium* has shown that defects in LPS are accompanied by decreased levels of the major proteins in the outer membrane (1, 12). The present investigation utilizes a protein with an assayable function, the receptor for the phage lambda, to quantitate the loss of a minor protein species and correlate that loss with defects in LPS. Implications for the importance of ionic interactions in the stabilization of the structure of the outer membrane are discussed.

MATERIALS AND METHODS

Abbreviations. Abbreviations used are: Tris buffer, tris(hydroxymethyl)aminomethane (pH was adjusted with HCl); EDTA, ethylenediaminetetraacetic acid; LPS, lipopolysaccharide.

Materials. Triton X-100 was purchased from Rohm and Haas. Tryptone was obtained from Difco. All other chemicals were of the highest quality commercially available. Antisera raised against the purified lambda receptor were the generous gift of M. Schwartz.

Bacteria and growth conditons. All bacterial strains used were *E. coli* K-12. CR34 (Thy$^-$ Leu$^-$ Thr$^-$ Thi$^-$ Lac$^-$ Str^r) and its heptose-deficient derivative GR467 were obtained from J. Koplow. JE 1011 (F$^-$ Thr$^-$ Leu$^-$ Trp$^-$ His$^-$ Thy$^-$ Thi$^-$ Ara$^-$ Lac$^-$ Gal$^-$ Xyl$^-$ Mtl$^-$ Str^r) and its novobiocin supersensitive derivatives NS1 through NS6 were obtained from M. Matsuhashi. D21 (Pro$^-$ Trp$^-$ His$^-$ Amp A) and its LPS-deficient derivatives D21e7, D21f1, D21f2 were obtained from H. Boman. The composition of the LPS of the mutants is given in Table 1. Bacteria were grown with shaking at 37 C in tryptone broth medium. *E. coli* K-12 Hfr G6 is a lambda-sensitive strain that was used as the indicator strain for plating phage in the receptor assay.

Assay of lambda receptor activity. Receptor activity was assayed as an inactivation of phage lambda (λ) as described previously (17). One milliliter of a suspension containing between 3×10^8 and 6×10^8 plaque-forming units of phage λvh (a host range mutant of the virulent phage λv) in 10^{-2} M $MgSO_4$ was mixed with 0.5 ml of the cell fraction to be assayed diluted between 200- and 500-fold in 10^{-2} M Tris buffer, pH 7.6. The cell fraction was replaced by Tris buffer in the control mixture. Mixtures were incubated at 37 C, and at times as indicated 0.1-ml samples were taken and added to tubes containing 10^9 indicator bacteria (Hfr G6) in 0.1 ml of 10^{-2} M $MgSO_4$, also at 37 C. After 5 additional min to allow adsorption, soft agar was added (2.5 ml) to each tube and the counts were plated.

Assay of antiserum-blocking ability. The antiserum-blocking ability of extracts was used as an assay for inactive receptor protein (i.e., protein that had lost the ability to inactivate phage and therefore would not be detected by the receptor activity assay described above). The logic of the assay is described at length in Results. Extracts to be tested are sub-

TABLE 1. *Quantitation of the lambda receptor protein in lipopolysaccharide-deficient strains of E. coli K-12*

Strain	Present data		Data from literature				
	% of parental level		Composition of LPS (% of parental level)				Reference
	Receptor activity[a]	Antiserum-blocking activity[b]	Galactose	Glucose	Heptose	Phosphate	
CR34	100	ND[c]	100	100	100	100	18
GR467	51	ND	35	36	34	61	
D21	100	100	100	100	100	ND	3, 5
D21e7	34	ND	<2	60	100	ND	
D21f1	31	ND	<2	<2	100	ND	
D21f2	37	37	<1	<1	<1	ND	
JE1011	100	100	100	100	100	100	
NS1	19	ND	7	0	0	16	21
NS2	<10	<10	14	0	<5	11	
NS3	33	36	14	0	13	8	
NS4	<10	ND	14	0	33	11	
NS5	41	52	14	0	60	15	
NS6	100	ND	100	100	100	100	

[a] Values are an average of three or more determinations.
[b] Values are single determinations.
[c] ND, Not determined.

jected to three incubations in series. (i) A 0.05-ml amount of antisera, diluted 10^{-3} in saline, was mixed with 0.05 ml of the extract diluted 10^{-1} in 10^{-2} M Tris buffer, pH 7.6, and incubated for 15 min at 37 C. (ii) A 0.02-ml amount of incubation mixture (i) was mixed with 0.01 ml of outer membrane extract containing a known amount of lambda receptor activity. After incubation for 15 min at 37 C, 1.0 ml of 10^{-2} M Tris buffer, pH 7.6, was added to the mixture. (iii) The diluted incubation mixture (ii) was assayed for receptor activity as given in the preceding section.

Controls were done to be certain that (i) the inactivation of the receptor by antisera was specific; (ii) incubating for 15 min at 37 C was sufficient time for the reaction to be complete; (ii) the dilutions of antisera and extract in the first incubation were such that all the antisera would be blocked if parental extracts were used and; (iv) the amount of receptor in the second incubation was such that 70 to 80% would be inactivated if the antisera remained completely unblocked by the first incubation. The antiserum-blocking ability of extracts from the mutant strains was calculated relative to the antiserum-blocking ability of the outer membrane extract from the parent determined in the same experiment.

Fractionation of the cells. Fifty-milliliter cultures were harvested when the cell density reached approximately 5×10^{8} cells/ml (cell density was assessed turbidimetrically with a Beckman photometer). Volumes of each culture containing 2×10^{10} cells were centrifuged and the cell pellet was washed once with 4 ml of 10^{-2} M Tris buffer, pH 7.6. The washed cells were resuspended in 2 ml of 10^{-2} M Tris buffer, pH 7.6, 10^{-2} M $MgSO_4$ and broken by sonication. Soluble and envelope fractions were separated by centrifugation at 20,000 × *g* for 40 min.

The envelope proteins were further fractionated by differential solubilization as described by Schnaitman (19). The envelopes were suspended in 4 ml of 2% Triton X-100, 10^{-2} M $MgCl_2$, 10^{-2} M Tris buffer, pH 7.6, and incubated for 20 min at room temperature (24 C). This procedure solubilizes the cytoplasmic membrane proteins but not the outer membrane, which is pelleted by centrifugation for 60 min at 100,000 × *g*. The outer membrane was solubilized by resuspension in 4 ml of 2% Triton X-100, 5×10^{-3} M EDTA, 10^{-2} M Tris buffer, pH 7.6, and the fractions were diluted 500-fold in 10^{-2} M Tris buffer, pH 7.6, and assayed as described above.

Preparation of LPS. LPS was prepared by the method of Galanos et al. (8) from cells grown in tryptone broth medium, washed, and acetone dried. 2-Keto-3-deoxyoctonic acid was determined by the thiobarbituric acid method described by Dröge et al. (7) as method A.

RESULTS

The receptor for the bacteriophage lambda is a protein component of the outer membrane of *E. coli* (17). The amount of lambda receptor protein in each strain of three series of LPS-defective mutants was determined by assaying the extracts for the phage receptor activity and for the ability to block antisera raised against purified receptor. The mutants and the respective parent of each series were harvested from exponential-phase cultures in tryptone broth,

the cells were fractionated, and the following fractions were assayed for receptor activity: cell wash, soluble cytoplasm, solubilized cytoplasmic membrane, solubilized outer membrane, and media in which cells were grown to stationary phase overnight, In all cases the only significant receptor activity found was that in the solubilized outer membrane fraction.

An example of the relative receptor activity found in the outer membrane fraction of a series of LPS-defective mutants is shown in Fig. 1. The rate of inactivation of the phage lambda by the cell extract has been shown to be proportional to the concentration of receptor in the extract (17). Thus, the greater the slope in Fig. 1, the more receptor is present. It can be seen that the outer membrane fraction of the parent JE1011 contained about twice as much receptor as the mutant NS5 and 10-fold more than NS2.

Tamaki et al. (21) selected the NS series of mutants as supersensitive to the antibiotic novobiocin and characterized the LPS from each strain. NS2 and NS5 were shown to be deficient in heptose in the core region of the LPS. Another novobiocin-supersensitive mutant, NS6, was shown to have parental-type LPS. The solubilized outer membrane fraction from NS6 had the same amount of receptor activity as the parental outer membrane fraction (Fig. 1). Therefore, the decrease in receptor activity was not correlated to the novobiocin-supersensitive phenotype but to the LPS defect.

To test the possiblity that there might be an inactivator present in the mutant, an outer membrane extract from NS2 that shows almost no receptor activity was incubated for 15 min at 37 C with the solubilized outer membrane from the parent, JE1011. There was no observable effect on the activity of receptor from JE1011 (Fig. 1). JE1011 outer membrane extract was also incubated with the soluble cytoplasmic fraction and the solubilized cytoplasmic membrane of NS2. Again, no effect on receptor activity was seen.

The possibility that the receptor protein was present in an inactive form in one of these fractions was tested. The soluble cytoplasm and solubilized cytoplasmic membrane from mutant NS3 were assayed for antiserum-blocking ability as an indicator of the presence of inactive receptor protein. No blocking ability was found. The antiserum-blocking assay will be discussed in detail below.

The receptor activity in the solubilized outer membrane of each strain was determined and the activity relative to the parental level was calculated from the half-time of inactivation of the phage (Table 1). Table 1 also includes data from the literature concerning the chemical composition of the LPS from the various strains. It can be seen that the LPS-deficient strains all showed reductions in the receptor activity found in the outer membrane. The activity present varied from 10% in NS2, which had less than 5% of the parental level of heptose, to 50% in GR467, which is a leaky mutant have approximately 35% of the parental levels of galactose and glucose, as well as heptose (18).

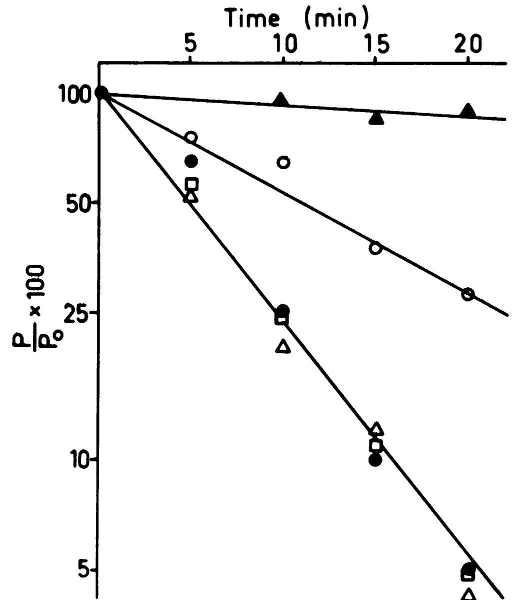

FIG. 1. *Receptor activity in parent JE1011 and NS mutants. Receptor was assayed as an inactivation of phage lambda as described in Materials and Methods. The number of plaques (P) obtained at time t is plotted as a percentage of that (P_0) obtained at time t = 0. The solubilized outer membrane fractions assayed were: JE1011 (●), NS 5 (○), NS 2 (▲), and NS 6 (△). Solubilized outer membrane of JE1011 was also assayed after an incubation with outer membrane of NS 2 (□).*

The series of mutants derived from D21 show a successive loss of sugar residues from the LPS core (3). The amount of receptor is decreased in the mutant that lacks only the galactose residue (D21e7) as well as in the strains that are deficient in glucose (D21f1) and heptose (D21f2). The data from the literature compiled in Table 1 show that loss of the sugar residues in the core region of LPS is accompanied by a loss of phosphate. In the case of the D21 series there are no quantitative data available, but Boman

and Monner (5) have observed that the level of phosphate in the core region of D21e7, D21f1, D21f2 is greatly decreased relative to D21.

The receptor for the phage lambda has been shown to be a protein (17), but the purified receptor has not been directly assayed for LPS. A trivial explanation for the loss of receptor activity in the LPS-deficient mutants would be that an LPS component in addition to the receptor protein is necessary for an active receptor complex. In that case, extracts from LPS-deficient mutants would contain the receptor protein in an inactive form. To demonstrate the absence of the receptor protein in these mutants, I have used antisera raised against purified receptor. The logic is as follows. The lambda receptor is inactivated by incubation with the antisera (Fig. 2). Therefore the assays of receptor activity after such an incubation can be used as an indicator of antiserum activity. If the antisera are not blocked, they can inactive the receptor; if the antisera are blocked, they have no effect on receptor activity (Fig. 3). If extracts from the LPS-deficient strains contain receptor in an inactive form (i.e., no ability to inactivate the phage), that protein should retain enough antigenic determinants to block the antisera, assuming the antisera contain a spectrum of different antibodies and not only one class of antibody that is specific for the active conformation of the receptor. The extracts are tested by a series of incubations: (i) the extract in question is incubated with antisera; (ii) the treated antisera are then incubated with active lambda receptor; (iii) finally the receptor activity is assayed to see whether the antisera were blocked by the first incubation, thereby indicating the presence of inactive receptor protein in the extract. Determinations of the antiserum-blocking ability were carried out for several of the strains and the results are given in Table 1 as blocking ability relative to the parental strains. It is seen that the amount of receptor activity correlated well with the antiserum-blocking ability.

Antiserum-blocking ability may be a direct indicator of the amount of protein present, but there remains one serious criticism of the conclusion. Again consider the possibility that the active receptor contains an LPS component; then it follows that the antisera would contain anti-LPS. Even if the mutant extracts contained an inactive receptor protein that would block the anti-protein components, the anti-LPS component would remain unblocked and could thus inactivate added receptor. To eliminate this possbility, LPS was prepared from the parent of the D21 series and added to the extract of D21f2, the heptoseless mutant. There was no increase in the receptor activity of the D21f2 extract when assayed directly as inactivation of phage, nor was there any increase in antiserum-blocking ability. In the first case, one can argue the protein and LPS cannot reassociate in vitro to yield an active receptor complex. Nevertheless, the LPS was added in excess (final concentration in the heptoseless extract was 0.086 mg/ml relative to 0.01 mg/ml in D21 extracts) and should have blocked any anti-LPS components in the antisera was efficiently as a D21 extract. Therefore, we can conclude that LPS is not directly involved in the receptor activity.

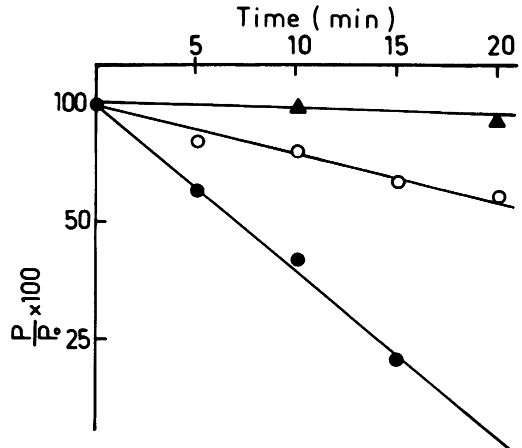

FIG. 2. *Inactivation of lambda receptor by antisera. Dilutions of antisera were mixed with solubilized outer membrane from D21 (diluted 10^{-1}), incubated 15 min at 37 C, and subsequently assayed for receptor activity as given in Materials and Methods. The number of plaques (P) at time t is plotted as percentage of that (P_0) at time t = 0. Saline control, no antisera added (●); antisera diluted 1:400 (▲); antisera diluted 1:1,000 (○).*

DISCUSSION

After elimination of the trivial explanation that LPS is part of the active site of the lambda receptor (see above), there remain several hypotheses to explain the simultaneous decrease of LPS and receptor activity. These possibilities were previously discussed by Koplow and Golfine (12) and Ames et al. (1) in their discussion of the absence of the major proteins from the outer membrane of heptoseless bacteria. In the following discussion it must be remembered that a primary effect of the LPS deficiency may be the loss of the major proteins for any of the reasons discussed below, and this loss in turn could cause a secondary loss of minor protein species.

First, Koplow and Goldfine (12) suggest the

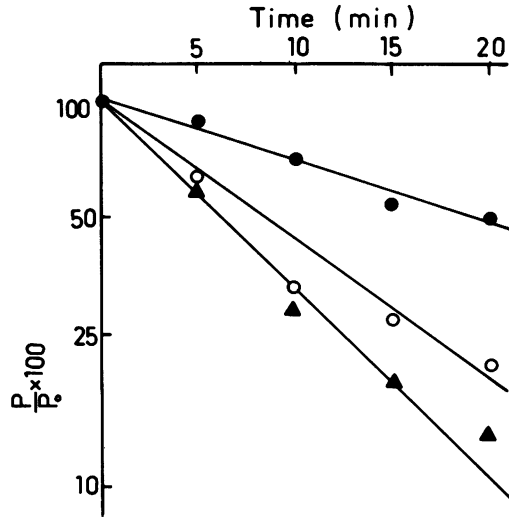

FIG. 3. *Assay of antiserum-blocking ability of solubilized outer membrane. The ability of antisera to inactivate the lambda receptor is assayed after (i) preincubation of antisera with control buffer, i.e., unblocked antisera (●), and (ii) preincubation of antisera with solubilized outer membrane from D21, i.e., blocked antisera (▲). The control (○), receptor incubated with buffer, gives a lower activity than receptor treated with blocked antisera because of this residual receptor activity from the preincubation of antisera with D21 outer membrane.*

possibility that genes for the synthesis of the major protein components are in the same operon as LPS genes and thus a mutation in a regulatory locus or a polar mutation would result in a concomitant loss of LPS and protein. There are no genetic data for the major proteins, but we can definitely eliminate this possbility as the direct cause of the loss of the receptor protein since the gene for the receptor, *lamB*, is well characterized as the second gene of a maltose operon in the *mal B* region (80 min on the genetic map of *E. coli*). The operon is induced by maltose and is under the control of the regulatory product of *mal T* (11). The LPS mutations have been mapped at *lpc B*, between 55 and 60 min for the NS mutants derived from JE1011, and at *lps A*, 72 min, for the series derived from D21.

We can eliminate common regulation at the level of transcription of the *lam B* and LPS genes; however, there might be a common regulatory mechanism at the level of synthesis of the components of the outer membrane. Results from Inouye's laboratory indicate that the outer membrane proteins are synthesized in a somewhat different manner from that of cytoplasmic proteins (10, 13). It is possible that there is a specialized protein synthetic system regulated in conjunction with LPS synthesis, but at present there is no evidence for such regulation.

Second, assembly of the outer membrane might involve translocation of LPS and protein as a complex. Thus, protein components could not appear in the outer membrane if LPS were altered in such a way as to eliminate those interactions. This might be the case for the major proteins, but it is not likely that the lambda receptor protein is translocated with the LPS. Mühlradt et al. (16) showed that the LPS of *S. typhimurium* is exported to the outer membrane at about 200 sites, which correspond to the adhesion sites observed by Bayer (2). In contrast to LPS, the lambda receptor enters the outer membrane exclusively at the septum region (A. Ryter, H. Shuman, and M. Schwartz, J. Bacteriol, in press). Assuming that LPS is exported by the same mechanism in both *S. typhimurium* and *E. coli*, we can conclude that the receptor is not translocated with the bulk of the LPS.

A third, more probably interpretation is that the LPS is important in stabilizing the structure of the outer membrane. The charged groups associated with the core may be involved in ionic interactions with the protein components. The lambda receptor may be held in position by direct ionic bonds with the LPS or by interaction with the major proteins, which in turn form ionic bonds with LPS. Evidence for protein-protein interactions in the outer membrane is found in the demonstration by Haller and Henning (9) that the protein network of the entire outer membrane can be cross-linked by a reagent spanning only about 0.3 nm.

There are several other indications that ionic interactions play an important role in the stabilization of the outer membrane structure. Levy and Leive observed that the addition of EDTA to *E. coli* results in the release of approximately 50% of the LPS (14). Schnaitman (19) and DePamphilis (6) pointed out the importance of divalent cations in the outer membrane when they developed a differential extraction technique for the cell envelope. The cytoplasmic membrane is solublized by Triton X-100 alone, but solubilization of the outer membrane is effected only in the presence of both Triton X-100 and EDTA. Solubilization of the lambda receptor from the outer membrane requires EDTA as well as detergent (17).

Many investigations concerning the cell surface of gram-negative bacteria have implicated alterations of LPS as a major factor in increased penetration of antibiotics and lysozyme through

the outer membrane (4, 15, 20, 21). Koplow and Goldfine (12) and Ames et al. (1) have demonstrated that the outer membranes of LPS-deficient strains are also deficient in the major protein components and have pointed out the difficulties in attributing the increase in permeability directly to the mutation in LPS.

Ambiguities arise in the interpretation of the simultaneous loss of LPS and the major proteins because no genetic data are available. In this work the loss of a minor protein species, the receptor for phage lambda, is quantitated and correlated with LPS deficiences. Since the genetics of the receptor is well established, the smilutaneous decrease of receptor protein and LPS cannot be due to a polar or regulatory mutation. The data suggest that the loss of the core region of the LPS and its associated phosphate groups leads to a general destabilization of the outer membrane and subsequent loss of receptor protein.

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