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ISOLATION AND CHARACTERIZATION OF AN *ESCHERICHIA COLI* MUTANT WITH ALTERATION IN THE OUTER MEMBRANE PROTEINS OF THE CELL ENVELOPE

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SUMMARY

A mutant was isolated from *Escherichia coli* K-12 which became more sensitive towards dyes and detergents. This mutant, which was isolated following nitrosoguanidine mutagenesis, was shown to carry at least two distinct mutations The first mutation resulted in the reduction of an acidic protein in the outer membrane fraction of the cell envelope. The second mutation affected the activity of phosphoglucoisomerase. The alteration of the membrane proteins in this mutant was demonstrated by polyacrylamide gel electrophoresis in 2 % sodium dodecyl sulfate, as well as by anion-exchange column chromatography of membrane proteins rendered soluble in 6 M urea by the procedure of Moldow *et al.* (C. Moldow, J. Robertson and L. I. Rothfield, *J. Membrane Biol.*, 1972, in the press).

INTRODUCTION

Electron microscopic studies have shown that the cell envelope of *Escherichia coli* and other Gram-negative enteric bacteria is a complex multilayered structure, in which the rigid layer of peptidoglycan and its associated components is sandwiched between the inner cytoplasmic membrane and the outer membrane-like layer[1,2] Separation of cytoplasmic and outer membrane fractions was first achieved by Miura and Mizushima[3,4], using sucrose density gradient centrifugation of EDTA–lysozyme spheroplast membranes. Osborn *et al.*[5] have demonstrated several differences in the chemical constituents and enzymatic complements of these two membrane fractions. In agreement with the immunological data of Miura and Mizushima in *E. coli* K-12, lipopolysaccharide was found to be almost exclusively in the outer membrane of the cell envelope in *Salmonella typhimurium*. The protein components of these two membrane fractions were also found to differ in size distribution as revealed by polyacrylamide gel electrophoresis in 2 % sodium dodecyl sulfate, and a predominant polypeptide band with an approximate molecular weight of 40000 was found to reside in the outer membrane fraction of the cell envelopes of *E. coli*, *S. typhimurium* and other Gram-negative bacteria[5,6–8]. Recent work of Moldow *et al.*[9] suggests that this major outer membrane protein component is composed of a number of polypeptides

of similar sizes which could be fractionated by ion-exchange column chromatography and by polyacrylamide gel electrophoresis in 6 M urea at various pH values. The present communication is concerned with the isolation and characterization of an *E. coli* mutant with alteration in this major component of the outer membrane proteins.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The strains of *E. coli* K-12 used in the present study are listed in Table I. Strain EIII required glucosamine for growth due to a genetic defect in the activity of glucosamine-6-phosphate synthetase[10]. Media and growth conditions were the same as previously described[10].

TABLE I

CHARACTERISTICS OF BACTERIAL STRAINS

Strain	*Mating type*	*Relevant genotypes*	*Source and reference*
AB1157	F⁻	*arg⁻pro⁻leu⁻thr⁻his⁻thi⁻gal*K⁻*str*A^r	E. A. Adelberg
EIII	F⁻	*arg⁻pro⁻leu⁺thr⁺his⁺thi⁻gal*E⁻*str*A^r*glm*S⁻	Wu[10]
E127	F⁻	*arg⁻pro⁻leu⁺thr⁺his⁺thi⁻gal*E⁻*str*A^r*pgi*⁻	EIII (NTG), this paper
E134	F⁻	*arg⁻pro⁻leu⁺thr⁺his⁺thi⁻gal*E⁻*str*A^{r}T4^r	EIII (NTG), this paper
HfrC	Hfr	*str*A^s	
DF2000	Hfr	*pgi-2 zwf-2 str*A^s	D Fraenkel[32]
EIII *pgi*⁻	F⁻	*arg⁺glm*S⁻*pgi*⁻ (other markers not tested)	DF2000 × EIII, this paper
E127 *pgi*⁺	F⁻	*arg⁺pgi*⁺ (other markers not tested)	HfrC × E127, this paper

Isolation of the mutant strain E127

As shown previously, there was a very rapid loss of viability of cells of this mutant strain EIII upon removal of glucosamine from the media even in the presence of sucrose. The loss of viability of glucosamine starved cells was accompanied by an extensive degradation of preexisting peptidoglycan. We have taken advantage of this strong selection pressure to isolate mutants resistant to this killing. An exponentially growing culture of strain EIII was harvested, washed and treated with nitrosoguanidine at 100 μg/ml for 20 min at 37 °C at pH 6.0 (ref. 11). The cells were washed once with normal saline and grown overnight at 30 °C in proteose peptone beef extract broth containing 200 μg/ml glucosamine–HCl Cells were washed again and resuspended in proteose peptone beef extract broth containing 10 % sucrose but no glucosamine. The cells were then incubated at 42 °C for 90 min to enrich non-autolytic mutants. The survivors of this starvation of glucosamine for a limited duration were plated on proteose peptone beef extract media containing 0.05 % glucosamine–HCl and the plates were incubated at 30 °C. Mutant strain E127 was isolated as one of these glucosamine-independent "revertants".

Genetic techniques

Conjugation was performed as described previously[10] To construct a *pgi*⁻ derivative of strain EIII, *arg*⁺ *atrA*^{r} recombinants were selected in a mating ex-

periment between strain E111 and DF2000. Likewise, to isolate *pgi*$^+$ derivative of strain E127, *arg*$^+$*strA*^{r} recombinants were selected in a cross between E127 and HfrC. These *arg*$^+$*strA*^{r} recombinants were grown and assayed individually for phosphoglucoisomerase activity and the final assignment of *pgi* marker was based on direct biochemical assays of this enzyme

Enzyme assays

Preparation of cell free extracts and assays for the activities of glucosamine-6-phosphate synthetase, glucosamine-6-phosphate deaminase, UDPG:α-D-galactose-1-phosphate uridylyltransferase and UDPG 4-epimerase were described previously[10,12]

Activities of phosphoglucoisomerase, glucose-6-phosphate dehydrogenase, phosphoglucomutase and UDPG pyrophosphorylase were measured spectrophotometrically by coupling these reactions to the reduction of $NADP^+$ by glucose 6-phosphate. The incubation mixtures contained, in a final volume of 1 ml, 50 μmoles of Tris–HCl (pH 7.8), 10 μmoles of $MgCl_2$, 0.3 μmole $NADP^+$, crude extract and 1 μmole of glucose 6-phosphate for the assay of glucose-6-phosphate dehydrogenase.

For the assay of phosphoglucomutase, 50 μmoles Tris–HCl (pH 7.8), 7.5 μmoles $MgCl_2$, 1 μmole dithiothreitol, 0.3 μmole $NADP^+$, 10 nmoles glucose 1,6-diphosphate, excess glucose-6-phosphate dehydrogenase, crude extract and 1 μmole of glucose 1-phosphate were used.

50 μmoles of Tris–HCl (pH 7.8), 7.5 μmoles $MgCl_2$, 0.3 μmole $NADP^+$, excess glucose-6-phosphate dehydrogenase, crude extract and 1 μmole of fructose 6-phosphate were used for the assay of phosphoglucoisomerase; 50 μmoles of Tris–HCl (pH 7.8), 1 μmole of $MgCl_2$, 1 μmole of dithiothreitol, 1 μmole of $NADP^+$, 0.5 μmole of UDPG, 10 nmoles of glucose 1,6-diphosphate, excess of glucose-6-phosphate dehydrogenase and of phosphoglucomutase, crude extract and 5 μmoles of inorganic pyrophosphate for the assay of UDPG pyrophosphorylase activity.

Activities of these enzymes were expressed as μmoles of product formed per mg of protein per hour.

Preparation of cell envelope fractions

Bacteria were grown aerobically with vigorous shaking in 500 ml proteose peptone beef extract broth in a 2-l Erlenmeyer flask and harvested at mid-logarithmic phase of growth. Cells were washed with saline and resuspended in 10 mM Tris–HCl (pH 7.8). The cells were then sonicated at 4 °C and centrifuged at 1000 × *g* for 10 min in a refrigerated Sorvall centrifuge for the removal of unbroken cells. The supernatant solution was centrifuged at 270000 × *g* for 2 h at 4 °C. The pellet was resuspended in 1 ml of 10 mM Tris–HCl (pH 7.8). Lysozyme was added to a final concentration of 100 μg/ml and the suspension was incubated at room temperature for 30 min. The lysozyme-treated sample was brought up to 12 ml with 10 mM Tris–HCl (pH 7.8) containing 5 mM EDTA and 0.2 M KCl, and centrifuged again at 270000 × *g* for 2 h at 4 °C. The pellet was washed once more with the same buffer containing EDTA and KCl. The final pellet was taken up in 1 ml of water and the protein concentration was determined according to Lowry *et al.*[13].

Separation of inner and outer membranes of E. coli cell envelope

Fractionation of *E coli* cell envelope into inner cytoplasmic membrane and outer membrane was accomplished with the procedure of Osborn *et al.*[5]. Cultures in

proteose peptone beef extract broth were grown with vigorous aeration at 37 °C to an absorbance of 0.6 at 600 nm. Cells were harvested by centrifugation and resuspended in cold 0.75 M sucrose–10 mM Tris–HCl (pH 7.8) at a final absorbance of the suspension of 10. Lysozyme was added to a final concentration of 100–150 μg per ml of cell suspension. About 2 min later, the lysozyme treated cells were diluted slowly with two volumes of 1.5 mM EDTA (Na^+), pH 7.5. The spheroplasts formed by the combined action of EDTA and lysozyme were lysed by brief sonic oscillation. The membranes were collected by centrifugation at 270000 × *g* for 2 h at 2–6 °C, washed with 0.25 M sucrose–3.3 mM Tris–1 mM EDTA by ultracentrifugation, and the washed membrane was suspended in small volume of 25 % sucrose (w/w) containing 5 mM EDTA (pH 7.5). The membrane suspension was then subject to isopycnic sucrose density gradient centrifugation at 41000 rev /min for 12–16 h at 2–6 °C in a step-wise sucrose gradient of 30 to 60 % sucrose. Centrifugation to equilibrium resulted in separation of discrete membrane bands.

Polyacrylamide gel electrophoresis

All gels were run with circulating tap water for cooling. Sodium dodecyl sulfate gel electrophoresis was performed on 7.5 % polyacrylamide gels in the presence of 2 % sodium dodecyl sulfate according to Fairbanks[14,15]. Samples (150–200 μg protein per gel) were heated to 100 °C for 20 min in 2 % sodium dodecyl sulfate, 25 mM dithiothreitol, 25 mM Tris–HCl (pH 7.8) before being applied to the gels (0.6 cm × 10 cm). Electrophoresis was run for 7–9 h at 3 mA per gel with pyronin Y as the tracking dye. The gels were fixed, the sodium dodecyl sulfate removed by soaking in 25 % isopropanol in 10 % acetic acid and stained with Coomassie blue as described by Fairbanks[14,15]. For radioactive samples, ethylene diacrylate was used as the cross-linking agent at a final concentration of 0.2 %. Gels were sliced to 1 mm thickness each, and each slice was dissolved in 1 ml of 1 M NH_4OH and counted in 10 ml of Patterson–Greene's scintillation solution[16].

7.5 % polyacrylamide gel electrophoresis was also performed in the presence of 6 M urea in potassium acetate buffer (pH 4.5) as described by Reisfeld *et al.*[17]. Samples were applied in 10 mM sodium acetate buffer (pH 4.5) containing 6 M urea, 5 mM β-mercaptoethanol and 10 % sucrose. Electrophoresis was run at 3 mA per gel for 12–14 h, and were stained for 2 h with 0.25 % Amido Black in 10 % acetic acid and destained electrophoretically. For alkaline urea gels, 7.5 % polyacrylamide gels were prepared in 6 M urea and 0.1 M Tris–HCl buffer (pH 8.5). The running buffer was 25 mM Tris–glycine buffer at pH 9.2. Electrophoresis was run at 3 mA per gel for 4–5 h, stained with Amido Black and destained electrophoretically as described above.

DEAE-cellulose column chromatography of membrane proteins in 6 M urea

Solubilization and fractionation of membrane proteins by DEAE-cellulose column chromatography were carried out by the method of Moldow, *et al.*[9]. DEAE-cellulose (Cellex D, Biorad) was extensively washed with 0.5 M NaOH, 0.5 M HCl and then water. The washed DEAE-cellulose was equilibrated with 5 mM Tris–HCl (pH 7 8) containing 6 M urea.

The pellet obtained after centrifugation of the cell envelope fraction at 270000 × *g* for 2 h at 4 °C, was resuspended and extracted with 1 ml of 6 M guanidinium thiocyanate buffered with 10 mM Tris–HCl at pH 7.8, and centrifuged at 175000

$\times g$ for 2 h at room temperature. The extraction and centrifugation were repeated and the supernatant solutions were combined, and dialyzed against 250 ml of 5 mM Tris–HCl buffer (pH 7.8) containing 6 M urea at 4°C overnight with two changes of dialysate. The solubilized membrane proteins were applied to a 1 cm $\times$ 22 cm column. The column was washed with 100 ml of 5 mM Tris–HCl (pH 7.8) buffer containing 6 M urea, followed by a linear gradient of 0 to 0.2 M NaCl in the same Tris–HCl–urea buffer (200 ml each). The column was further eluted with 0.5 to 1 M NaCl in the same buffer, followed by 100 ml of 6 M guanidinium thiocyanate in 5 mM Tris–HCl buffer (pH 7.8).

Isolation of lipopolysaccharide and analysis of its chemical composition

For the isolation of lipopolysaccharide, bacterial cells were grown in proteose peptone beef extract broth in a 12-l fermentor. The cells at the early stationary phase of growth were collected by continuous-flow centrifugation in a refrigerated Sorvall centrifuge. Lipopolysaccharide was isolated using the phenol–water procedure of Westphal *et al.*[18]. Heptose and 2-keto-3-deoxyoctonate were assayed as described by Osborn[19]. Total phosphate was determined by the method of Ames and Dubin[20]. Glucose was determined with hexokinase and ATP coupled with the reduction of $NADP^+$ in the presence of excess glucose-6-phosphate dehydrogenase, following the hydrolysis of the lipopolysaccharide in 1 M HCl at 100 °C for 5 h in sealed evacuated tubes. Internal standards were included and the values given have been corrected for 10–20 % loss of added glucose during hydrolysis. Glucosamine was measured by the Elson–Morgan reaction[21], after hydrolysis of the lipopolysaccharide in 4 M HCl at 100 °C for 6 h in vacuo. 20–30 % loss of added glucosamine during hydrolysis was also corrected for.

For the preparation of lipid A-free polysaccharide, lipopolysaccharide solution was hydrolyzed in 1 % acetic acid at 100 °C for 30 min. The insoluble lipid A was removed by centrifugation at 20000 $\times g$ for 10 min at 4 °C. The supernatant solution which contained lipid A-free polysaccharide, was assayed for total phosphate by the method of Ames and Dubin[20].

Chemicals and other assays

All chemicals used were reagent grade from commercial sources. ^{14}C- and ^{3}H-labeled amino acid mixtures (NEC-445, 100 μCi/ml and NET-250, 1 mCi/ml, respectively) were purchased from New England Nuclear Corp. Radioactivities were determined in a Beckman scintillation counter with toluene-based scintillation solution.

RESULTS

General characteristics of strain E127

Strain E127 was obtained as a survivor of glucosamine starvation following nitrosoguanidine mutagenesis of strain E111. In addition to the ability to grow in the absence of glucosamine, other phenotypic properties in this mutant which were suggestive of alterations in its cell envelope, are listed in Table II. Thus strain E127 was found to be resistant to T4 phage, sensitive to C21 phage and its growth was poor on EMB and MacConkey media, suggesting increased sensitivity towards dyes and

detergents. As described below, there were at least two mutational events in strain E127, either or both of which may be responsible for these phenotypic differences between the mutant and the parental strain.

TABLE II

PHENOTYPIC PROPERTIES OF MUTANT STRAIN E127 AS COMPARED TO THOSE OF THE PARENTAL STRAIN E111

Growth	*Strain E111*	*Strain E127*
Nutrient agar (no NaCl)	−	−
Nutrient agar + 0.05% glucosamine	+	−
Nutrient agar containing 0.5% NaCl	−	+
Nutrient agar containing 0.5% NaCl + 0.05% glucosamine	+	+
L broth (containing 0.2% glucose)	−	±
L broth + 0.05% glucosamine	±	+
EMB + 1% *N*-acetylglucosamine	+	−*
MacConkey + 1% *N*-acetylglucosamine	+	−**
Phage sensitivity		
T_4	S	Resistant in minimal media, partially resistant in rich media
C21	S	S

* Plating efficiency of strain E127 on EMB–GlcNAc plate was about $5 \cdot 10^{-4}$

** Plating efficiency of strain E127 on MacConkey–GlcNAc plates was about $5 \cdot 10^{-4}$

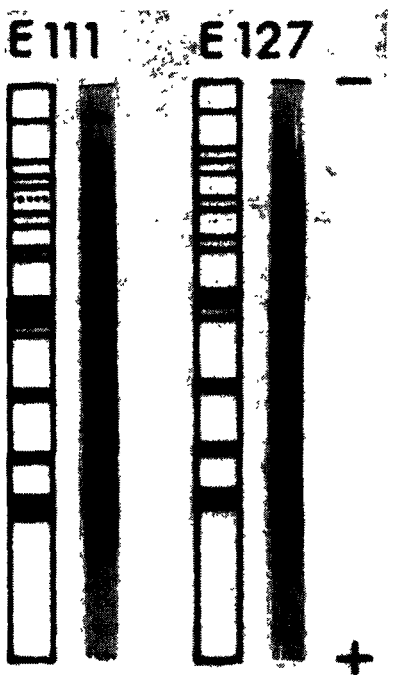

Fig 1 Polyacrylamide gel electrophoresis in the presence of 2% sodium dodecyl sulfate of total cell envelope proteins isolated from the wild-type strain (E111, left gel) and from the mutant strain (E127, right gel). The details for the preparation of samples and for the sodium dodecyl sulfate gel electrophoresis were given in the Materials and Methods.

Alterations of the envelope proteins in strain E127

When the total envelope proteins of strain E127 and strain E111 were analyzed by polyacrylamide gel electrophoresis in the presence of 2 % sodium dodecyl sulfate, there was a significant reduction in the staining of a protein band with a molecular weight of approx. 40000 (Fig. 1). This protein band represents the major component seen by sodium dodecyl sulfate gel electrophoresis and is largely derived from proteins located in the outer membrane of the cell envelope[5,7].

The diminution of the major band in strain E127 as compared to that of E111 was confirmed by a double-labeling experiment. E111 and E127 were labeled with ^{3}H- and ^{14}C-labeled amino acid mixtures, respectively. The cells were then mixed and treated as a single sample during isolation of the cell envelope and the subsequent analysis. When the isolated envelope preparation was solubilized with 2 % sodium dodecyl sulfate at 100 °C for 20 min and analyzed by polyacrylamide gel electrophoresis in 2 % sodium dodecyl sulfate, there was a significant reduction in ^{14}C counts in the region of the major band as compared to ^{3}H counts (Fig. 2). The profiles of soluble proteins (270000 × *g* supernatant) analyzed by sodium dodecyl sulfate gel electrophoresis in the same double-labeling experiment showed no difference between

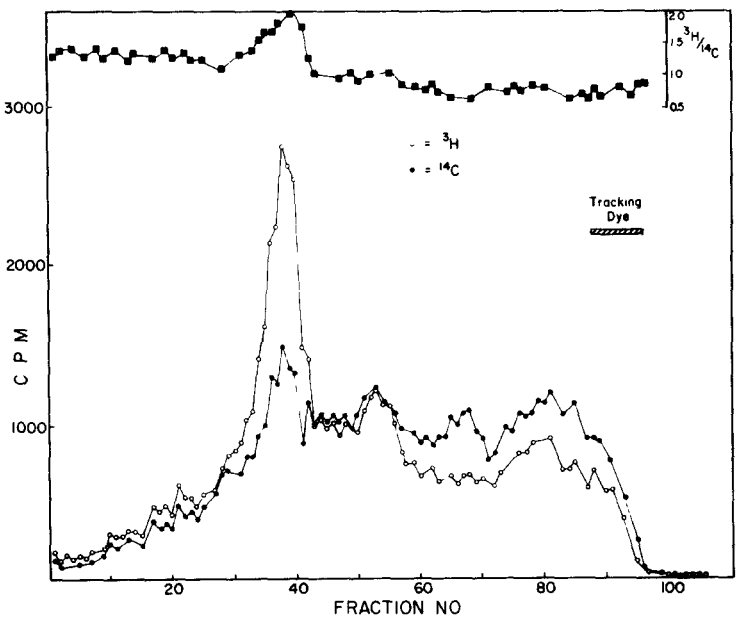

Fig 2. Polyacrylamide (7.5 %) gel electrophoresis in 2 % sodium dodecyl sulfate of mixed membrane proteins from wild-type strain E111 (labeled with [^{3}H]amino acids) and mutant strain E127 (labeled with [^{14}C]amino acids) E111 and E127 cells were grown in 200 ml of minimal media (M9) supplemented with 20 μg/ml each of proline, arginine, leucine, threonine and histidine, 5 μg/ml of thiamine, 200 μg/ml of glucosamine–HCl and 1 % glycerol The cells were labeled with radioactive amino acid mixtures (200 μCi [^{3}H]amino acids for E111 cells and 100 μCi [^{14}C]amino acids for E127 cells) for 3 h at 37 °C These two cultures were then mixed upon harvest and thereafter treated as a single sample. The doubly labeled cell mixture was sonicated and mixed membrane fraction was prepared. Samples were treated with 2 % sodium dodecyl sulfate at 100 °C for 20 min before electrophoresis in 7.5 % polyacrylamide gel with ethylene diacrylate as the cross-linking agent The gel was sliced into 1 mm thickness each and each slice was dissolved in 1 ml of 1 M NH_4OH and counted in 10 ml of toluene-based scintillation solution containing 33 % Triton X-100.

E111 and E127 (data not shown). Comparable results were obtained when the labels were reversed (^{3}H-labeled E127 and ^{14}C-labeled E111), namely there was a decrease in the major component of membrane proteins from E127 as compared to E111.

When the solubilization of membrane proteins from *E. coli* or *S. typhimurium* with sodium dodecyl sulfate was carried out at 37 °C or 70 °C, instead of 100 °C, the polyacrylamide gel electropherogram became quite different; instead of a major component with a molecular weight of 40000, two bands were seen with apparent molecular weights of 60000–70000 and 30000–35000, respectively[5,22]. When E127/E111 doubly labeled membrane proteins were solubilized at 70 °C with 2% sodium dodecyl sulfate, it was obvious that the protein components reduced in E127, corresponded to both the 59000 and the 28000 mol. wt species (Fig. 3a). When the same sample was solubilized at 100 °C, the resultant gel showed a reduction of the protein component with molecular weight of 38000 (Fig. 3b).

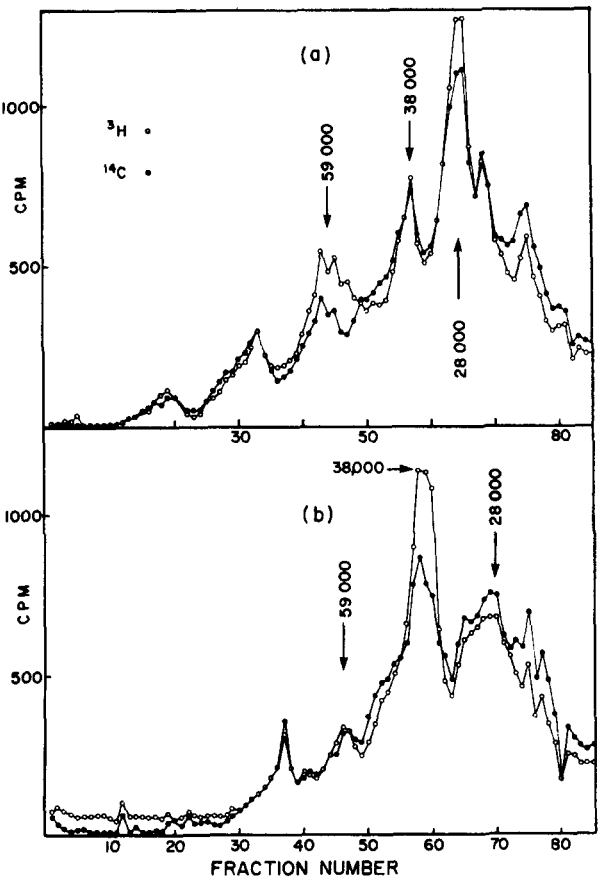

Fig 3 Polyacrylamide gel electrophoresis of doubly labeled membrane proteins from strain E111 (^{3}H-labeled wild type) and strain E127 (^{14}C-labeled mutant) (a) Membrane proteins were solubilized at 70 °C with 2% sodium dodecyl sulfate for 30 min (b) Same preparation of mixed membranes was solubilized with 2% sodium dodecyl sulfate at 100 °C for 20 min The details of labeling of the cells, preparation of membrane fractions, sodium dodecyl sulfate gel electrophoresis and counting of the gel slices were the same as described in the legend to Fig 2

As mentioned above, this major protein component seen in polyacrylamide gel electrophoresis after solubilization of membrane proteins at 100 °C with 2 % sodium dodecyl sulfate is probably a mixture of a number of polypeptides of similar molecular weight[9]. To examine the membrane protein missing in strain E127 in greater detail, we took advantage of a new procedure developed by Moldow *et al.*[9] for the solubilization and fractionation of membrane proteins based on differences in charge/mass ratio. The doubly labeled ([^{3}H]E111 and [^{14}C]E127) membrane proteins were solubilized by guanidinium thiocyanate, followed by dialysis against 6 M urea in 10 mM Tris–HCl (pH 7.8). The urea-soluble membrane protein mixture was then fractionated by DEAE-cellulose column chromatography in the presence of 6 M urea. The results are shown in Figs 4 and 5. A mildly acidic protein with a net negative charge at pH 7.8, was greatly reduced in the membrane of strain E127 as compared to strain E111. This conclusion based on results obtained by anion-exchange

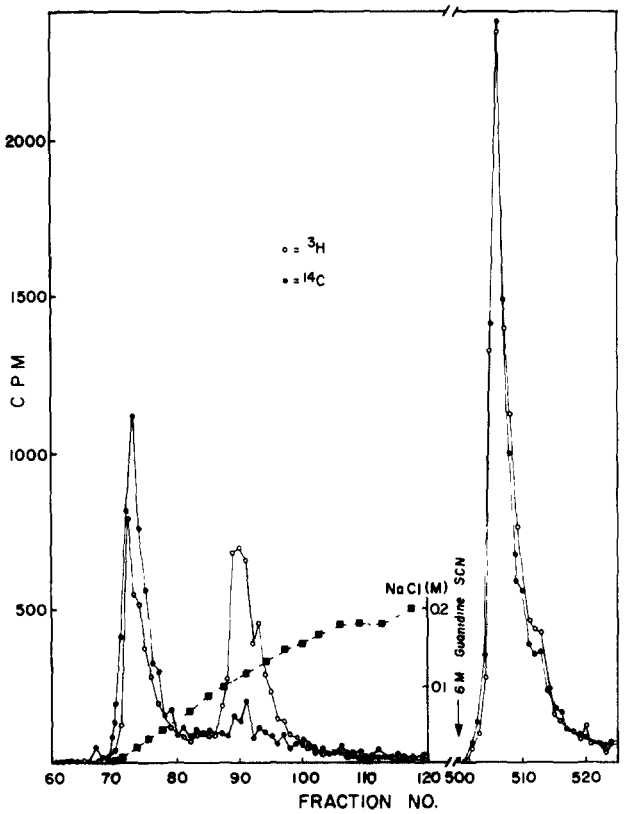

Fig. 4. DEAE-cellulose column chromatography of membrane proteins from wild type and mutant cells in 5 mM Tris–HCl (pH 7.8) and 6 M urea. Doubly labeled membrane proteins (^{3}H-labeled wild type strain E111 and ^{14}C-labeled mutant strain E127) were solubilized in 6 M guanidinium thiocyanate, dialyzed against 6 M urea in 5 mM Tris–HCl (pH 7.8) and fractionated on a DEAE-cellulose column in 6 M urea according to Moldow *et al.*[9]. After washing the column with 5 mM Tris–HCl (pH 7.8) containing 6 M urea, a linear gradient of 0 to 0.2 M NaCl in the same buffer was used to elute the adsorbed proteins. Very little radioactive proteins were eluted between 0.2 and 1 M NaCl gradient. The final elution was accomplished with 6 M guanidinium thiocyanate. For details, see Materials and Methods.

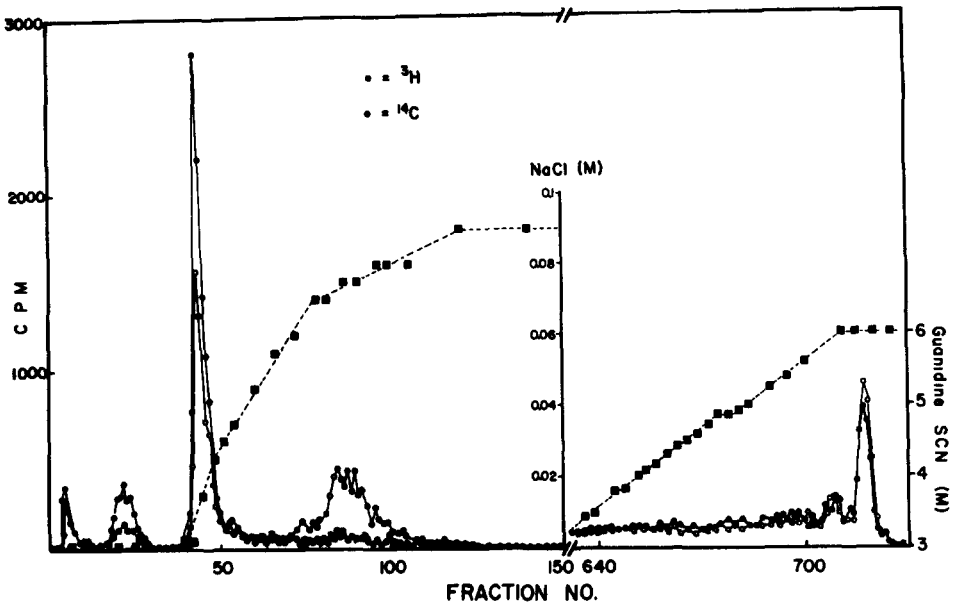

Fig 5 Same as Fig 4 except the sample applied to DEAE cellulose column was [³H]amino acids labeled mutant membrane (E127) and [¹⁴C]amino acids labeled wild-type strain (E111)

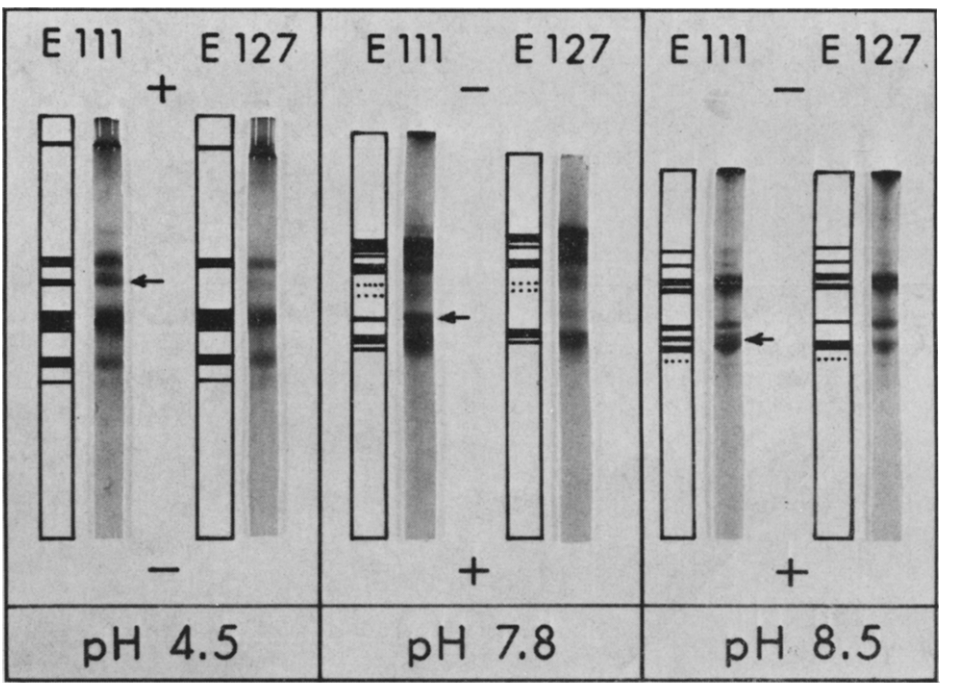

Fig 6 Polyacrylamide gel electrophoresis of membrane proteins from wild-type strain (E111) and mutant strain (E127) in 6 M urea at pH 4 5, 7 8, and 8 5, respectively The procedures used to solubilize the membrane proteins in 6 M urea and those for gel electrophoresis in 6 M urea at acidic, neutral and alkaline pH were given in detail in Materials and Methods

column chromatography was supported by polyacrylamide gel electrophoresis in 6 M urea at pH 4.5, 7 8, and 8.5, respectively (Fig. 6). At pH 4 5, the protein greatly reduced in strain E127 migrated slowly towards cathode, whereas in gels run at pH 7.8 and 8.5, a fast moving protein (towards anode) was greatly reduced in E127. These results with urea gels supported the data of DEAE-cellulose fractionation of membrane proteins that an acidic protein is present in greatly reduced amount in strain E127

Using the procedure of separation of inner and outer membranes developed by Osborn *et al.*[5], it was found that the protein greatly reduced in strain E127 was a component of the outer membrane proteins (Figs 7 and 8). Proteins from inner cytoplasmic membrane and those from outer membrane are distinctly different in polyacrylamide gel electrophoresis in 2 % sodium dodecyl sulfate. Inner membrane proteins contained more slow-moving protein bands (apparent mol. wt 50000 or above) whereas outer membrane contains a major protein band with an apparent molecular weight of approx. 40000 The protein band in the inner membrane at the position corresponding to the major outer membrane protein is probably due to contamination of this fraction by outer membrane. In the urea gel at pH 8.5, there seemed to be a few minor and fast moving bands towards anode in the mutant extract which were not readily seen in the extract prepared from the wild type. The relationship between these fast moving bands and the protein missing in this mutant is not clear.

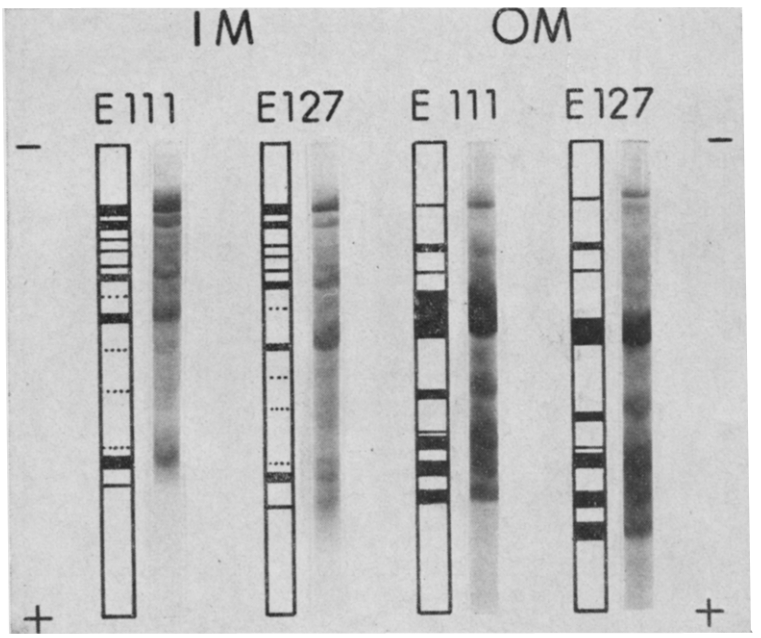

Fig 7. Polyacrylamide gel electrophoresis of proteins from outer and cytoplasmic membrane fractions of wild-type strain (E111) and the mutant strain (E127) in 2% sodium dodecyl sulfate Separation of spheroplast membrane into inner (IM) and outer membrane (OM) fractions was achieved, using the procedure of Osborn *et al* [5]

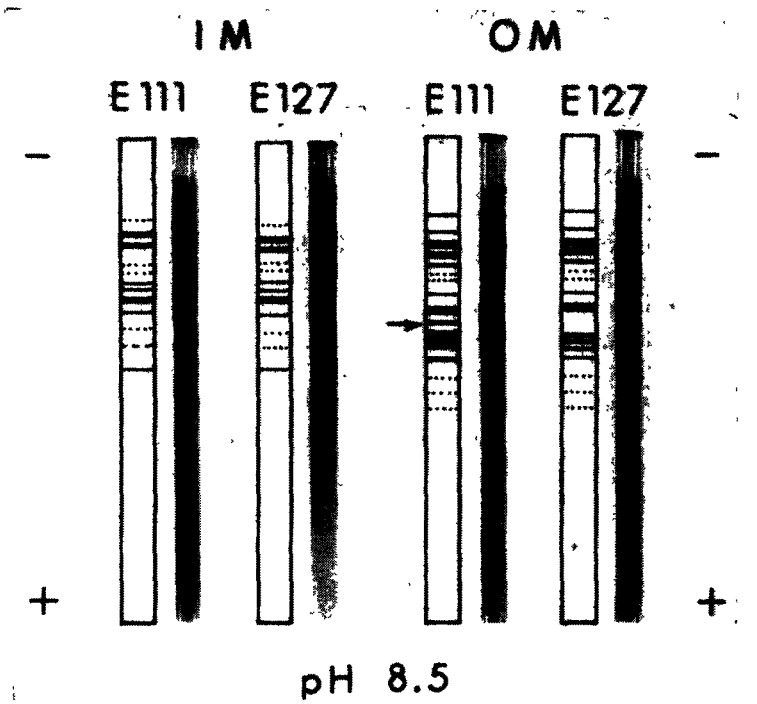

Fig 8 Polyacrylamide gel electrophoresis of proteins from outer (OM) and cytoplasmic membrane (IM) fraction of wild-type strain (E111) and the mutant strain (E127) in 6 M urea at pH 8.5. Other details, same as those given in the legends to Figs 6 and 7

Defective phosphoglucoisomerase (Table III)

The presence of this biochemical defect in strain E127 was accidentally uncovered and would account for both the altered phage sensitivity and the growth inhibition on EMB and MacConkey media. The apparent resistance of E127 cells to T4 phage could have resulted from the restriction, during subsequent infections, of non-glucosylated T4* phage produced in a phosphoglucoisomeraseless mutant grown

TABLE III

ACTIVITIES OF ENZYMES INVOLVED IN THE INTERCONVERSIONS OF SUGAR PHOSPHATES IN THE CRUDE EXTRACTS OF AB1157, E111 AND E127

*Enzymes**	*Specific activity (μmoles/mg protein per h)*		
	AB1157	*E111*	*E127*
Phosphoglucoisomerase	65 9	71 6	0 75
Glucose-6-phosphate dehydrogenase	n d ***	3 3	3 9
Phosphoglucomutase	n d	10 9	7 7
UDPG pyrophosphorylase	n d	1 26	3 14
Galactose-1-phosphate uridylyltransferase	5 0	4 4	2 5
UDPgalactose 4-epimerase	34	0 06	0 04

* Washed cells were sonicated with a Branson sonifier and the sonicated crude mixture was centrifuged at 20000 × *g* for 20 min The 20000 × *g* supernatant fluid was used for enzyme assays

** n d = not determined

in the absence of exogenous glucose[23]. However, no adsorption of T4 phage by E127 cells was detectable under conditions which effected irreversible adsorption of 90% of T4 phage by E111 cells. The data given in Table IV strongly suggested that the altered phage susceptibility of strain E127 resulted from changes in the lipopolysaccharide structure due to a defective phosphoglucoisomerase. Both the restoration of T4 sensitivity in the presence of glucose, and the fact that resistance to phage C21 required the addition of both glucose and galactose to the plating media, were to be expected of a phosphoglucoisomerase-deficient mutant. When lipopolysaccharide was isolated by the phenol–water procedure[18], the glucose content in the purified lipopolysaccharide from E127 was about one-third of that present in E111 (Table V). Moreover, the lipopolysaccharide from E127 was a good acceptor of purified UDPG: lipopolysaccharide glucosyltransferase (a gift from Dr E. Müller) but not an acceptor of UDPgalactose:lipopolysaccharide galactosyltransferase (a gift of Dr E. Müller) unless it was preincubated with UDPG and glucosyltransferase (data not shown).

The second observation related to the *pgi* defect was the alteration in the buoyant density of the outer membrane fraction isolated from this mutant The outer

TABLE IV

PHAGE SUSCEPTIBILITY OF MUTANT STRAIN E127 IN DIFFERENT PLATING MEDIA

*Additions**	*Strain E111*		*Strain E127 pgi⁻*		*Strain E127 pgi⁺*	
	T4	*C21*	*T4*	*C21*	*T4*	*C21*
None	S	S	R	S	S	S
Galactose	S	R	R	S	S	R
Glucose	S	S	S	S	S	S
Glucose and galactose	S	R	S	R	S	R

* Plating media were proteose peptone beef extract agar and proteose peptone beef extract soft agar, containing 0.1 ml of 20% glucosamine–HCl 0.1 ml of 20% galactose or 20% glucose was added as indicated

TABLE V

CHEMICAL COMPOSITION OF LIPOPOLYSACCHARIDE FROM E111, E127 AND E134

Chemical constituent	*μmoles/mg lipopolysaccharide*		
	Strain E111	*E127*	*E134*
Heptose	0.68	0.61	0.49
2-Keto-3-deoxyoctonate	0.53	0.60	0.66
Total phosphate	1.08	0.91	0.76
Glucose	0.28	0.10	0.02
Glucosamine	0.50	0.58	0.63
Galactose*	—	—	—
P_i in polysaccharide**	0.64	0.41	0.32

* Galactose content in lipopolysaccharide was not determined However, paper chromatography of lipopolysaccharide hydrolysate (1 M HCl, 5 h at 100 °C) in *n*-butanol–pyridine–water (6:4.3, v/v/v) showed no trace of galactose in any of these three lipopolysaccharide preparations

** Lipid A-free polysaccharide was prepared as described in Materials and Methods

membrane fraction of strain E127 was significantly less dense than that of strain E111 and this could be correlated to the glucose deficiency in the lipopolysaccharide of this strain (Table VI). The restoration of normal buoyant density of E111 *pgi*⁻ outer membrane by prior growth in media supplemented with glucose provided further evidence that the structure of lipopolysaccharide in the outer membrane of the cell envelope affected its buoyant density. Further confirmation of these results was obtained with another *E. coli* mutant, strain E134. Strain E134 was totally resistant to T4 E134 cells were not killed by T4 infection and under conditions in which more than 95 % of T4 were adsorbed by E111 cells, there was no detectable adsorption by E134 cells. The lipopolysaccharide isolated from this mutant contained no detectable glucose (Table V) When spheroplast membrane from this mutant was fractionated, the two membrane fractions were almost identical in buoyant densities (1.17 g/ml).

TABLE VI

EFFECT OF *pgi*⁻ DEFECT ON THE BUOYANT DENSITY OF OUTER MEMBRANE

Strain	*Buoyant density of membrane fraction (g/ml)*	
	Light fraction (inner membrane)	*Heavy fraction (outer membrane)*
E111 *pgi*⁺	1 170	1 206
E127 *pgi*⁻	1 166	1 186
E111 *pgi*⁻	1 174	1 186
E127 *pgi*⁺	1 165	1 208
E111 *pgi*⁻ grown on glucose	1 172	1 208

The deficiency in phosphoglucoisomerase activity in strain E127 and its consequent alteration in the lipopolysaccharide structure, is unrelated to the defect in the outer membrane protein of the cell envelope in the same mutant. This conclusion is based on the fact that strain E111 *pgi*⁻ (Table I) showed no alteration in membrane proteins whereas strain E127 *pgi*⁺ (Table I) retained its membrane protein defect as examined by sodium dodecyl sulfate gel electrophoresis (data not shown).

Independence of growth of E127 on glucosamine

As mentioned previously, strain E127 was isolated as a mutant of strain E111 resistant to killing by glucosamine starvation. It grew well in proteose peptone beef extract media without added glucosamine. Addition of glucose to proteose peptone beef extract broth inhibited the growth of E127 cells, presumably because of an accumulation of glucose 6-phosphate[24]. When E127 cells were grown in proteose peptone beef extract broth containing 0.05 % glucosamine–HCl, very low activity of glucosamine-6-phosphate synthetase was detected. However, this was found to be due to repression of the synthesis of this enzyme by glucosamine, rather than to a genuine loss of this activity in E127. Therefore, it would appear that there was a third mutation in E127 which caused direct or indirect reversion of the defect in glucosamine-6-phosphate synthetase.

DISCUSSION

The work of Leive, Heppel and others on the effects of EDTA or osmotic shock on the permeability of Gram-negative bacterial cells have suggested an important and unique function of the outer layer of the cell envelope in these bacteria[25,26]. These previous studies have suggested that the outer membrane constitutes a permeability barrier, in addition to that imposed by the cytoplasmic membrane, against entry and/or exit of a variety of molecules. Many recent observations concerning the susceptibility of bacterial cells towards a number of unrelated antibiotics, dyes and detergents[27–30] and those concerning the release of periplasmic proteins by osmotic shock or as a result of mutational events[31], are all consistent with this view.

The data presented in this paper provide further evidence that alterations in the chemical composition of the components of outer membrane of the cell envelope are accompanied by increased sensitivity of the mutant cells towards dyes and detergents. The observation that the buoyant density of the outer membrane of a *pgi*⁻ mutant of *E. coli* was significantly lower than that of the wild type strain was surprising. The data does not distinguish among various possible explanations. It remains to be seen whether there is a decrease in the absolute amounts of lipopolysaccharide per mg of outer membrane protein or per cell, as compared to that in the parental strain. It is worth noting that Osborn *et al.*[5] have observed a similar reduction in the buoyant density of outer membrane from a heptoseless mutant of *Salmonella typhimurium*.

The mutant described in this paper showed a significant difference in outer membrane proteins. However, the physiological consequence of this loss of an outer membrane protein is probably minor, since the mutant cells grow and divide at a normal rate. Occasionally, a mild tendency of E127 cells to form chains of four or more cells was observed. The lack of a distinct phenotype of this mutant makes the genetic studies of this mutation extremely difficult. The present study does provide independent genetic evidence that the major component of the membrane proteins seen in sodium dodecyl sulfate gel electrophoresis is a mixture of a number of proteins of similar molecular weights but different charge/mass ratios, a conclusion arrived at by the recent work of Moldow *et al*[9]. In addition, the results shown in Figs 4 and 5 would indicate that it would be feasible to purify the protein missing in mutant E127 cells since the radiochemical purity of the peaks in Figs 4 and 5 seems high. From the data in these two figures, the amount of the protein missing in mutant E127 appears to account for approximately 2 % of the total membrane proteins Since outer membrane proteins constitute two thirds of the total membrane proteins, the protein absent in strain E127 represent about 3 % of outer membrane proteins. The physiological function of this outer membrane protein remains unknown

Recent studies on antibiotic-sensitive mutants of *E. coli* have shown that alterations in the core region of the lipopolysaccharide of these mutants are accompanied by changes in their susceptibility towards certain bacteriophages including T4[27,29]. The present work on strains E127 and E134 strongly suggest that the glucose moiety rather than the phosphate in the polysaccharide portion of *E coli* lipopolysaccharide is an important part of the T4 receptor

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