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Alterations in the Outer Membrane of the Cell Envelope of Heptose-Deficient Mutants of *Escherichia coli*

JANE KOPLOW AND HOWARD GOLDFINE

Department of Microbiology, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19174

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The composition of the cell envelope of a heptose-deficient lipopolysaccharide mutant of *Escherichia coli*, GR467, was studied after fractionation into its outer and cytoplasmic membrane components by means of sucrose density gradient centrifugation. The outer membrane of GR467 had a lower density than that of its parent strain, CR34. Analysis of the fractionated membranes of GR467 indicated that the phospholipid-to-protein ratio had increased 2.4-fold in the outer membrane. The ratio in the mutant cytoplasmic membrane was also increased, although to a lesser extent. By employing a third parameter, the lipid A content of the outer membrane, it was found that the observed phospholipid-to-protein change in the outer membrane was due predominantly to a decrease in the relative amount of protein. This decrease in protein was particularly significant, since it was concomitant with a 68% decrease in the lipid A recovered in the outer membrane of GR467 relative to the lipid A recovered in the outer membrane of CR34. Similar findings were observed in a second heptose-deficient mutant of *E. coli*, RC-59. The apparent protein deficiency in GR467 was further studied by subjecting solubilized envelope proteins to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. It was found that major envelope proteins which were localized in the outer membrane were greatly diminished in GR467. Two revertants of GR467 with the wild-type amounts of heptose had wild-type relative levels of protein in their outer membranes. A partial heptose revertant had a relative level of protein in its outer membrane between those of the mutant and wild type.

The organization of the gram-negative bacterial cell envelope may be considered on a number of levels: (i) the chemical composition and linear structure of the major macromolecules, e.g., lipopolysaccharide, lipoprotein, protein, phospholipid, and peptidoglycan; (ii) the stereochemistry of these macromolecules; (iii) the cross-linking of repeating units of a given macromolecule; and (iv) the bonds linking different macromolecules. These factors all contribute to the formation of a multilayered structure which supports such phenomena as active transport of small molecules, enzymatic reactions, and, indeed, the growth of existing envelope structures involving translocation of preformed subunits or in situ synthesis. The cell envelope is generally thought to consist of three distinct layers—the outermost layer containing lipopolysaccharide (LPS), protein, and phospholipid, a rigid peptidoglycan layer, and the cytoplasmic membrane consisting mainly of protein and phospholipid (7, 22, 24, 28, 37, 44).

Extensive literature exists describing the chemical composition and linear structure of the LPS in gram-negative bacteria. A large portion of the information available has been derived from analyses of *Salmonella* strains (19). These analyses indicate that the LPS structure can be divided into three regions, designated as O-antigen polysaccharide, core polysaccharide, and lipid A. The O-antigen sugars, when present, vary from species to species and provide a basis for classifying *Salmonella* and *Escherichia coli* smooth strains. Analyses of the core composition of *Salmonella* and *E. coli* strains have revealed many similarities between the two groups. In *Salmonella* rough mutants (i.e., lacking O antigen), the most extensive core structure includes the distal sugars *N*-acetylglucosamine, glucose, and galactose with an inner core of heptose (usually L-glycero-D-*manno*heptose) and KDO (2-keto-3-deoxyoctanoic acid). Phosphorus and ethanolamine may also be present in the inner core region (Fig. 1). The differences between *E. coli*

D-GlcNAc | α-1,2 ↓ D-Glc —α-1,2→ D-Gal —α-1,3→ D-Glc (← α-1,6 D-Gal) —1,3→ L-α-D-Hep —1,3→ L-α-D-Hep —1,5→ KDO —2,(7/8)→ KDO → Lipid A

P-P-EtN, P (on the two heptoses)

KDO ↓ 2,(4/5) KDO —7→ P → EtN

FIG. 1. *Inner core structure of Salmonella LPS. Abbreviations:* D-*GlcNAc, N-acetyl-*D-*glucosamine;* D-*Gal,* D-*galactose;* D-*Glc,* D-*glucose; Hep, heptose; EtN, ethanolamine; KDO, 2-keto-3-deoxyoctanoic acid.*

and *Salmonella* core regions are in part due to differences in the proportions of the constituents. KDO is linked by a ketosidic bond to the lipid A component, which consists primarily of glucosamine disaccharide units in which the available hydroxyl groups are substituted by phosphorus and long-chain fatty acids. The major lipid A fatty acid is β-OH myristate. In *Salmonella* it is principally linked to the amino group of glucosamine, but some β-OH myristate is present on hydroxyl groups as 3-D-myristoxymyristic acid (31).

We have investigated the relationship between some of the major macromolecules in the cell envelope of a heptose-deficient *E. coli* mutant, GR467 (32). The heptose deficiency of strain GR467 was previously found to be correlated to the presence of a species of KDO-lipid A glycolipid which could be extracted with lipid solvents (32). We sought to determine whether the presence of this extractable glycolipid was associated with other changes in the composition or organization of the cell envelope. After separation of the cell envelope into outer and cytoplasmic fractions, we noted a correlation between the heptose deficiency of the mutant and a marked change in the relative amounts of protein, phospholipid, and lipid A in the outer membrane fraction, and a possible change in the distribution of outer membrane and inner membrane materials.

(This investigation was presented by J. K. in partial fulfillment of the M. Sc. degree from the University of Pennsylvania.)

MATERIALS AND METHODS

Bacterial strains and bacteriophage. Strain GR467 is a temperature-sensitive, heptose-deficient mutant (32) derived from *E. coli* CR34 (Thy^-,Leu^-,Thr^-,Thi^-,Lac^-,Str^r) by ethyl methane sulfonate mutagenesis (10). The mutant retains the same markers as given for the parent strain. Strain CR34 itself is a derivative of *E. coli* K-12. Strains R11 and R16 are spontaneous revertants of strain GR467 which have heptose levels comparable to that of strain CR34 but which retain the temperature sensitivity of the mutant as well as the markers given above. Strains R2 and R17 are, respectively, a spontaneous revertant of intermediate characteristics and a presumptive revertant which resembles strain GR467, both to be described further in Results. *E. coli* 0111: B_4 and its heptose-deficient derivate, RC-59, were obtained from E. C. Heath. The bacteriophage C21 was obtained from M. J. Osborn.

Growth conditions and media. Cells to be used for membrane preparations were grown with shaking in 1-liter flasks containing 250 ml of medium or in 4-liter flasks containing 1 liter of medium at 30 or 42 C. For this purpose, either a New Brunswick reciprocating or gyrorotary shaker, set at a rate of 100 rpm, was used. The medium used was Trypticase soy broth (BBL) supplemented with 10 μg of thymine per ml (TSB + thy). Growth was monitored by measuring turbidity with a Klett-Summerson colorimeter equipped with a no. 66 filter.

The minimal-sodium dodecyl sulfate (SDS) agar used for testing revertants contained a salt composition essentially as described by Davis and Mingioli (6) except that NaH_2PO_4 and Na_2HPO_4 were substituted for KH_2PO_4 and K_2HPO_4 in order to dissolve the SDS supplement. The basic salts medium contained per liter: Na_2HPO_4 (10 g); NaH_2PO_4 (4.2 g); KCl (0.075 g); $MgSO_4$ (0.1 g); and NH_4SO_4 (1.0 g). For agar plates, the salts medium was supplemented with 0.6% SDS, 0.2% glucose, and 20 μg each of thymine, leucine, and threonine per ml and 2 μg of thiamine per ml.

Isolation and screening of GR467 heptose-positive revertants. Isolated colonies were obtained by the streak plate method from an overnight culture of strain GR467 grown at 30 C in TSB + thy. Eight colonies were selected and tested for growth on MacConkey-lactose agar (Difco) and for growth at 42 C on TSB + thy agar. None of the eight grew on either medium, whereas the parent strain, CR34, could grow on both media. An overnight culture (grown in TSB + thy) was obtained from one of these colonies, and approximately 10^8 cells were spread on each of several MacConkey-lactose agar plates. After incubation overnight at 30 C, the plates contained 100 to 200 colonies, of which 32 were directly streaked onto MacConkey-lactose agar. When restreaked, all of the colonies continued to give growth on this medium. Sixteen of these colonies were further tested for (i) sensitivity to bacteriophage C21 by the use of the soft-agar overlay method (5), (ii) ability to grow in the presence of 0.6% SDS, and (iii) presence or absence of an extractable KDO-lipid A material (32) which is found in strain GR467 but not in strain CR34 (see below).

Revertants were screened for the presence or ab-

sence of the extractable KDO-lipid A as described previously (32, 39) by use of ion-exchange paper chromatography of phospholipids obtained from ^{32}P-labeled cells. The molar ratios of heptose to KDO, in the revertants, were determined after direct hydrolysis of total membranes. For the estimation of heptose, 0.2-ml portions of total membranes (1.8 to 2.6 mg of protein) were hydrolyzed in 1 ml of 3.5 N HCl (final concentration equal to 3.0 N) for 3 h at 110 C. The hydrolysates were extracted with an equal volume of diethylether, and the aqueous phases were retained for measurement of heptose by the cysteine-H_2SO_4 method of Osborn (26). For the estimation of KDO, 0.2-ml portions of total membranes were hydrolyzed as described by Osborn et al. (28). The hydrolysates were centrifuged at 3,000 rpm for 15 min, and the supernatant solutions were retained for measurement of KDO by the thiobarbiturate method of Weissbach and Hurwitz (43). Since standards were not available, the molar extinction coefficients as given by Osborn (26) were used to quantitate both compounds.

Extraction of phospholipids. Total phospholipids which included phosphatidylethanolamine (PE), phosphatidylglycerol (PG), cardiolipin (CL), and KDO-lipid A were extracted from membrane preparations by the method of Bligh and Dyer (2). In some experiments, KDO-lipid A was separated from the remaining phospholipids by selective elution from silicic acid columns as described previously (32) but in a scaled-down fashion. Eluants containing PE + PG + CL or KDO-lipid A were evaporated to dryness, and their phosphorus content was determined.

Preparation of fatty-acid methyl esters. The total fatty acids in each membrane fraction and the fatty acids in Bligh-Dyer extracts from the same fractions were obtained by saponification as described by Nesbitt and Lennarz (25). Samples containing pentadecanoic acid (Applied Science Laboratories) as an internal standard were hydrolyzed in sealed test tubes at 83 to 85 C for 14.5 h. After extraction with glass-distilled diethylether (Burdick and Jackson Laboratories), fatty acids were methylated with diazomethane. Separation of fatty acids by gas-liquid chromatography was carried out on a column (0.125 in by 6 ft [about 0.3 cm by 1.8 m]) of 10% EGSS-X on Gas Chrom P (100/200 mesh, Applied Science Laboratories) at 180 C. Peak areas were measured by triangulation and were identified in part by comparison of retention times to those of 14:0, 16:0, 16:1, 18:0, and 18:1 methyl esters contained in a standard mixture (type NIH-D, Applied Science Laboratories). 17-Cyclopropane and 19-cyclopropane methyl ester standards were obtained from Analabs, Inc. A β-OH myristate standard was obtained from N. C. Johnston.

Quantitative determinations. Protein content was determined by the method of Lowry et al. (18) with bovine serum albumin (fraction V, Sigma Chemical Co.) as the standard. Total phosphorus was determined by the method of Bartlett (1), with K_2HPO_4 as the standard.

The deoxyribonucleic acid (DNA) content in CR34 and GR467 log-phase cells was determined by using the diphenylamine reaction as given in the Giles and Myers modification of the Burton method (4, 11). Washed cell pellets were suspended in 10% trichloroacetic acid in Corex centrifuge tubes, kept on ice for 30 min, and then centrifuged at 10,000 rpm in a Sorvall SS-34 rotor for 30 min. The pellets were suspended in 5% trichloroacetic acid and placed in a boiling water bath for 30 min. After heating, the samples were chilled and centrifuged at 10,000 rpm for 20 min. Portions of the supernatant solution were taken for measurement of DNA content as indicated. Salmon sperm DNA (Sigma Chemical Co.) was used as the standard.

Enzymatic assays. Reduced nicotinamide adenine dinucleotide (NADH) oxidase acitivity was assayed as described by Osborn et al. (28). Glucose-6-phosphate dehydrogenase activity was assayed as described by Josephson and Fraenkel (13) except that $MgCl_2$ was omitted from the incubation mixture.

Preparation of cell envelope membrane fractions. Outer and cytoplasmic membranes were prepared essentially as described by Schnaitman (36, 37). One-liter cultures of cells, grown in TSB + thy to a turbidity reading of 140 to 180 $Klett_{66}$ units, were harvested by centrifugation at 6,000 rpm in a Sorvall GS-3 rotor for 40 to 50 min. The cells were washed 2 times by suspension in 30 ml of 50 mM tris(hydroxymethyl)aminoethane (Tris) buffer containing 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.8, and centrifugation at 12,000 rpm in a Sorvall SS-34 rotor for 30 min. Pellets were stored frozen; thawed pellets were suspended in the Tris-EDTA buffer and homogenized for 1 min with a Virtis-23 homogenizer set for the "high" speed in order to remove flagellar and capsular material. The cells were sedimented and again suspended in the Tris-EDTA buffer (25 to 30 ml). One milligram each of deoxyribonuclease I and pancreatic ribonuclease A (both from Sigma Chemical Co.) was added to each 25- to 30-ml suspension. The suspensions were passed through a French pressure cell (Aminco) three to four times at 14,000 to 16,000 lb/in^2, followed by the addition of $MgCl_2$ to each broken cell suspension to a final concentration of 2 mM. Unbroken cells were removed by centrifugation at 12,000 rpm for 15 min. The extract thus obtained was centrifuged at 50,000 rpm in a Spinco type 65 fixed-angle rotor for 90 min. The pellets obtained were suspended in 3 to 5 ml of 10 mM *N*-2-hydroxyethylpiperazine-*N*'-2'-ethanesulfonic acid (HEPES; Sigma Chemical Co.) buffer, pH 7.4, with the aid of a glass tissue homogenizer. Suspensions thus prepared will be referred to as total membranes.

To separate outer membranes from cytoplasmic membranes, total membranes (10 to 40 μg of protein) were layered onto a discontinuous gradient modified from the gradients described by Schnaitman (37). The gradient contained 4 ml of 2.02 M, 14 ml of 1.44 M, and 10 ml of 0.77 M sucrose (enzyme grade, Schwarz/Mann) made in 10 mM HEPES buffer, pH 7.4. Loaded gradients were centrifuged at 24,000 rpm in a Spinco SW25.1 rotor for 19 h. In some cases, when a SW27 rotor was used, the volume of each step of the gradient was increased by 20% and centrifugation was carried out at 26,000 rpm for 15 h. Fractions

were collected by piercing the bottom of the centrifuge tubes with an 18-gauge syringe needle and dripping the contents of the tube into test tubes. The absorbance of each fraction was read at 280 and 260 nm with a correction then made for the presence of nucleic acids by multiplying (absorbance 280 nm/absorbance 260 nm) × absorbance 280 nm. Peak fractions were pooled, diluted approximately fourfold with HEPES buffer, and centrifuged at 50,000 rpm in a type 65 fixed-angle rotor for 90 min. The pellets were washed by suspension in the HEPES buffer followed by centrifugation at 50,000 rpm for 90 min. The washed pellets were suspended in a small volume of distilled water with the aid of a glass tissue homogenizer.

All materials, equipment, and membrane fractions were maintained at 4 C.

Polyacrylamide gel electrophoresis. Acrylamide (no. X5521, Eastman)-bis acrylamide (Eastman) slab gels were prepared by using the buffer system described by Laemmli (16) except that the gel buffer concentrations were cut in half. The upper spacing gel contained 4% acrylamide in the presence of 0.0625 M Tris-hydrochloride, pH 6.8, and 0.1% SDS, whereas the lower resolving gel contained 10% acrylamide in the presence of 0.187 M Tris-hydrochloride, pH 8.8, and 0.1% SDS. Ammonium persulfate and *N,N,N',N'*-tetramethylethylenediamine (Eastman) were used as the polymerizing agents. The running buffer contained 6.0 g of Trizma (Tris) base and 28.8 g of glycine per liter of distilled water (20). Gels were run at 7 mA for about 5.5 h and then stained with 0.2% Coomassie Brilliant Blue (Sigma Chemical Co.) dissolved in a solvent composed of 7% glacial acetic acid and 50% methanol. Gels were destained with a 7% glacial acetic acid-plus-5% methanol solvent mixture.

Proteins in the membrane preparations were solubilized at either 100 C for 1 min or at 70 C for 20 min by using a solution, essentially as described by Laemmli (16), which contained 0.0625 M Tris-hydrochloride, pH 6.8, 2.0% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.001% phenol red. Ten to 20 μg of protein was added to a given well. The marker proteins used were myoglobin (molecular weight = 17,500), ovalbumin (molecular weight = 45,000), and bovine serum albumin (molecular weight = 67,000). These were dissolved in the solution given above. All three markers were obtained from Sigma Chemical Co. On some gels, a mixture of 30*S* and 50*S* ribosomal proteins were run as a control. A ribosome subunit mixture (a gift from K. Ogawa, Dept. of Microbiology, Univ. of Pennsylvania) was solubilized at 70 C for 20 min or at 100 C for 1 min in the solubilizing solution given above.

Proteins present in the following materials were also solubilized as described above and subjected to polyacrylamide gel electrophoresis: (i) total cell extracts obtained by a 1.5-min sonication of washed cells suspended in the Tris-EDTA buffer used to prepare total membranes; (ii) the supernatant fraction obtained after homogenization of cells with the Virtis-23 homogenizer; (iii) the supernatant fraction obtained in the step which yielded total membranes; and (iv) concentrated spent medium. Spent TSB + thy medium was concentrated by passage through an Amicon ultrafiltration cell equipped with an Amicon PM-10 Diaflo membrane.

Solubilized electrophoresis samples were stored frozen.

RESULTS

Fractionation of cell envelopes. Total cell envelope membranes of strains CR34, GR467, 0111:B_4, and RC-59 were prepared, as indicated in Materials and Methods, from log-phase cells. Subsequent separation of outer and cytoplasmic membranes was achieved by sucrose density centrifugation. The protein profiles of typical separations are given in Fig. 2A and B for strains CR34 and GR467 and in Fig. 2C and D for strains 0111:B_4 and RC-59. In both cases two distinct bands were visible, the upper band (ρ = 1.184) being designated as cytoplasmic membranes and the lower one (ρ = 1.241) as outer membranes. Enzymatic and chemical markers were used to confirm the origins of these two fractions. The positions of the cytoplasmic membranes coincided for both strains, but the position of the outer membrane of strain GR467 appeared at a lower density (ρ = 1.212) than those derived from strain CR34. Similar patterns were found when total membranes were prepared from cells of overnight cultures or from cells grown for 2 h at 30 C and then shifted to 42 C for an additional 2 h. It should be noted that, in early experiments, the discontinuous sucrose gradient described by Schnaitman (37) was used. However, the resolution of membrane fractions from strain GR467 was not as pronounced with that gradient as with the one described in this study.

Relative amounts of phospholipid and protein in membrane fractions. An indication of the nature of the alteration in the heptose-deficient mutants was noted when the ratio of phospholipid to protein was determined for the fractionated membranes. Average values from a series of CR34 and GR467 preparations are given in Table 1. A pronounced difference is noted in the outer membrane preparations in that the ratio for strain GR467 is about 2.4 times that for strain CR34. Likewise, when this ratio was determined for strains 0111:B_4 and RC-59 (Table 2), an approximately twofold increase was found for the mutant. Variations among preparations of outer and cytoplasmic membranes could be partly the consequence of unseparated cell envelope fragments. The preparations in question appear to have significant trailing on the leading side of the cytoplasmic membrane peaks which may represent material that never separated or became reaggregated.

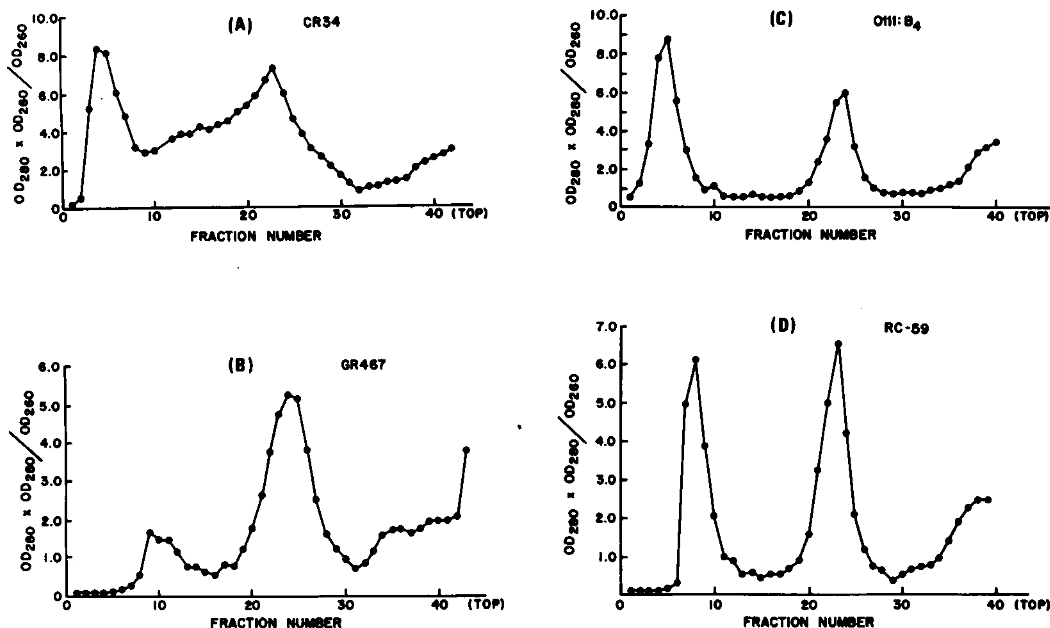

FIG. 2. *Protein profiles of fractionated total membranes of heptose-deficient and parent strains obtained by sucrose density gradient centrifugation (see Materials and Methods).*

TABLE 1. *Phospholipid-to-protein ratio in total and fractionated membranes of CR34 and GR467*

Membrane prepn	Avg μg of phospholipid[a]/mg of protein ± standard deviation[b]
CR34	
total	233 ± 24
outer	263 ± 49
cytoplasmic	362 ± 108
GR467	
total	356 ± 70
outer	626 ± 87
cytoplasmic	543 ± 83

[a] Not corrected for KDO-lipid A phosphorus contribution.

[b] By using Student's *t*-test to compare corresponding preparations, values of $\alpha \ll 0.005$ were obtained for total membranes and outer membranes; cytoplasmic membrane preparations yielded a value of α between 0.005 and 0.01.

Schnaitman (37), White et al. (44), and Osborn et al. (28) observed material of a density intermediate between that of cytoplasmic and outer membranes which was thought to be fused or unseparated membrane fragments. In some of the cytoplasmic membrane preparations, ribosomal contamination also contributes to the variability found for the phospholipid-to-protein ratio. Among the series of CR34 preparations, it was generally found that the phospholipid-to-protein ratio was greater for the cytoplasmic membrane than for the outer membrane. This is in agreement with findings using

TABLE 2. *Comparison of relative phospholipid and fatty acid content in heptose-deficient and parental strains*

Membrane prepn	μg of phospholipid/mg protein	μg of Bligh-Dyer fatty acid/mg of protein		μg of total fatty acid/mg of protein
		Estimated	Experimental	
CR34				
Outer	243[a]	169[b]	ND[c]	247
Cytoplasmic	255[a]	179[b]	ND	191
Total	203[a]	142[b]	ND	169
GR467				
Outer	622[a]	497[d]	444	476
Cytoplasmic	394[a]	278[d]	332	353
Total	275[a]	194[d]	184	194
0111:B4				
Outer	218[a]	153[b]	188	215
Cytoplasmic	306[a]	214[b]	270	263
Total	203	142[b]	153	189
RC-59				
Outer	470[a]	329[b]	399	392
Cytoplasmic	368[a]	258[b]	309	296
Total	253	177[b]	148	182

[a] Corrected for KDO-lipid A phosphorus + methanol-eluted phosphorus contribution (see Results).

[b] Calculated by multiplying μg of phospholipid/mg of protein × 0.7.

[c] ND, not done.

[d] Corrected for KDO-lipid A fatty acid contribution.

E. coli J-5 (a uridine diphosphate-galactose-4-epimerase⁻ strain derived from 0111:B_4) (37, 44) and with the observations of Osborn et al. (28) using *Salmonella typhimurium* G-30.

A previous study of strain GR467 (32) showed that a KDO-lipid A-like material which contains phosphorus could be extracted with a chloroform-methanol (2:1, vol/vol) solvent mixture. Since the phospholipids obtained here were extracted with the chloroform-methanol system described by Bligh and Dyer (2), it was necessary to correct for KDO-lipid A phosphorus. The major phospholipids, PE, PG, and CL, can be separated from KDO-lipid A by selective elution from silicic acid (32) using chloroform-methanol (7:3, vol/vol, and 1:9, vol/vol) in that order. Any additional phosphorus-containing material more polar than KDO-lipid A was eluted with methanol and combined with the chloroform-methanol (1:9) eluant. As is noted in the footnotes to Table 1, the average phospholipid-to-protein ratios did not include the above correction. When the correction was made for several individual experiments included in these averages (as well as for the 0111:B_4 and RC-59 preparations), the KDO-lipid A phosphorus plus methanol-eluted phosphorus contribution ranged from 3.7 to 14.0% of the total lipid phosphorus.

Quantitation of total fatty acids and those in chloroform-methanol extracts. The demonstration of increased phospholipid-to-protein ratios in the mutants found by measuring lipid phosphorus was also reflected by the quantitation of the total and chloroform-methanol-extractable (32) fatty acids in the various membranes (Table 2). Predicted values for the GR467 ratios of chloroform-methanol-extractable fatty acids to protein included corrections for KDO-lipid A fatty acid contributions. The corrections were made by employing determinations of KDO-lipid A phosphorus (see above) and the weight ratio of phosphorus to fatty acids found in KDO-lipid A as determined in the previous study (32). The comparable weight ratio data were not available for strain RC-59.

The total fatty acid-to-protein ratios for strains CR34 and GR467 showed an approximately twofold increase for the mutant versus parent strain in the outer membrane. This is consistent with the data in Table 1. As would be expected, there was a significant increase in the amount of total fatty acids in strain CR34 over the amount of chloroform-methanol-extractable fatty acids (estimated). This was due largely to the inclusion of β-OH myristate upon direct saponification. It should be noted that β-OH myristate is associated uniquely with lipid A. Other long-chain fatty acids such as lauric and myristic acids would also contribute to this increase. Total fatty acids in the cytoplasmic membrane and total membranes of strain CR34 also show increases over the estimates, which are roughly proportional to the β-OH myristate contribution. Likewise, in strain GR467, for which experimental values for the chloroform-methanol extracts already include some β-OH myristate, the increase in the total is about proportional to the increase in the amount of β-OH myristate. Although examination of the corresponding data for strains 0111:B_4 and RC-59 does not reveal the above pattern in all cases, no unique experimental errors were apparent. It should be noted that a primary source of error in the analyses involved here is the fact that determinations were done on different days by using portions of a particulate material whose homogeneity may have varied from day to day, for instance, due to aggregation and the presence of degradative enzymes.

Distribution and recovery of protein and phospholipid. A further indication of changes in the heptose-deficient mutants is provided by examining the distribution of protein and phospholipid between the membrane fractions. This data is given in Table 3 for membranes obtained from both gradient types (see above) in the case of strains CR34 and GR467, and from only the modified gradient in the case of strains 0111:B_4 and RC-59. Although the CR34 and GR467 averages are derived from two gradient types, comparable results were obtained with the two gradient types. The distribution of protein for both pairs of strains indicated a marked decrease in the protein content of the outer membrane of each heptose-deficient strain and an increase in the cytoplasmic membrane. The protein recovery in outer plus cytoplasmic membranes remained the same. Whereas the recovery for outer plus cytoplasmic membranes was approximately 40%, a total recovery of about 70% was found when protein in fractions not pooled as either outer or cytoplasmic membranes was included. Most of this additional protein was found at the top of the gradient and may have included membrane protein solubilized during the course of centrifugation, protein of ribosomal origin, and/or soluble cytoplasmic proteins. The last probably contribute little to this fraction since, in measuring the soluble enzyme, glucose-6-phosphate dehydrogenase, approximately 95 to 99% of the total units remained with the supernatant fraction when total membranes were sedimented at

TABLE 3. *Recovery of total membrane protein and phospholipid after fractionation*

Strain[b]	Recovery of protein and phospholipid (%) from[a]					
	OM		CM		OM + CM	
	Protein	P-Lipid	Protein	P-Lipid	Protein	P-Lipid
CR34	21.0 (18.0-26.7)	39.6 (25.9-47.5)	18.6 (15.0-22.8)	22.2 (15.9-30.4)	39.6 (33.0-44.4)	62.7 (56.3-68.0)
GR467	7.3 (5.0-9.6)	15.6 (9.9-19.5)	29.4 (25.0-35.3)	47.2 (32.9-57.8)	36.5 (34.0-40.7)	61.6 (52.2-67.7)
0111:B_4	22.9	26.0	22.6	39.5	45.6	65.4
RC-59	5.5	20.1	27.6	44.5	37.0	69.6

[a] Percentages are relative to the amount (in milligrams) of phospholipid layered onto the gradient. Numbers in parentheses represent range of recovery. Abbreviations: OM, outer membrane; CM, cytoplasmic membrane; PR, protein; PH, phospholipid.
[b] Data for strains CR34 and GR467 are averages based on four or five experiments. Data for strains 0111:B_4 and RC-59 are based on a single experiment.

166,000 × *g* before being placed on sucrose gradients. We have not attempted to characterize this top material.

The distribution of phospholipid between the cytoplasmic and outer membranes of strains GR467 and CR34 indicates a pattern similar to that found with protein. A factor contributing to the variability in the distribution of phospholipid (and protein) has already been suggested in relation to the data in Table 1. It should be noted that, although the recovery in outer plus cytoplasmic membranes was approximately the same for both pairs of strains, there did not appear to be as great a shift for strain RC-59 phospholipids relative to the distribution pattern for strain 0111:B_4. Since this pair of strains has not been examined as extensively as strains CR34 and GR467, we cannot ascertain whether experimental variability or a species difference is involved.

Correlation between phospholipid, protein, and lipid A in the outer membranes. The distribution of protein and phospholipid between the membranes of strain GR467 suggested that the predominant change with respect to the outer membrane phospholipid-to-protein ratio was due to a decrease in the protein content. We sought, therefore, to further characterize the relationships between the principal macromolecules in the mutant outer membrane by quantitating β-OH myristate in order to obtain the relative amounts of protein to lipid A and of phospholipid to lipid A. The results (Table 4) support our preliminary conclusion.

Purity of the membrane fractions. The extent of contamination of the outer membrane by a cytoplasmic membrane component was judged by the distribution of NADH oxidase, one of a number of enzymes which have been reported to be localized in the cytoplasmic membranes of both *E. coli* (22) and *Salmonella typhimurium* (27, 28). The outer membrane fractions of strains CR34 and 0111:B_4 contained relatively little of the cytoplasmic membrane bound NADH oxidase (Table 5). The outer membranes of the two mutant strains were somewhat more contaminated by NADH oxidase.

TABLE 4. *Correlation between protein, phospholipid, and lipid A*

Membrane prepn	mg of protein/μg of β-OH myristate[a]	μg of phospholipid/μg of β-OH myristate[a, b]
CR34		
Outer ...	0.029	7.1
Total ...	0.070	13.4
GR467		
Outer ...	0.011	7.0
Total ...	0.047	12.9
0111:B_4		
Outer ...	0.038	8.2
Total ...	0.054	10.9
RC-59		
Outer ...	0.017	7.4
Total ...	0.047	12.0

[a] Includes *trans*-2-tetradecenoic acid.
[b] Except for values for 0111:B_4 and RC-59 total membranes, all values of phospholipid/β-OH myristate are corrected for KDO-lipid A phosphorus contribution.

Cross-contamination in the other direction was judged by measuring the β-OH myristate content of each fraction. It was necessary here and with the previous data (Table 2) to consider the amount of β-OH myristate as including *trans*-2-tetradecenoic acid, a degradation prod-

TABLE 5. *Distribution of NADH oxidase between fractionated membranes*

Strain[a]	Sp act[b]		
	Outer membrane	Cytoplasmic membrane	Total membranes
CR34	0.120	1.429	1.430
	0.358	3.026	0.872
GR467	0.070	1.703	1.362
	0.379	1.665	1.206
0111:B_4	0.226	1.740	1.250
RC-59	0.402	1.263	0.907

[a] For strains CR34 and GR467, two separate experiments were performed.
[b] Expressed as micromoles per minute per milligram of protein.

TABLE 6. *Distribution of β-OH myristate and KDO between fractionated membranes*[a]

Strain	Percentage of total in outer + cytoplasmic membranes recovered in			
	Outer membrane		Cytoplasmic membrane	
	β-OH myristate[b]	KDO	β-OH myristate[b]	KDO
CR34	80	90	20	10
GR467	26	29	74	71
0111:B_4	70	ND[c]	30	ND
RC-59	49	ND	51	ND

[a] KDO and β-OH myristate determinations were done on separate preparations.
[b] Includes *trans*-2-tetradecenoic acid.
[c] ND, not done.

uct derived from β-OH myristate under alkaline hydrolysis conditions (31, 33). The two membrane fractions as well as total membranes were saponified overnight (14.5 h) to insure hydrolysis of amide-linked fatty acids. In one case, for strain CR34, cytoplasmic membrane saponification was carried out for only 2 h. Controls showed that this shorter period of hydrolysis yielded approximately 65% of the β-OH myristate obtained when hydrolysis was carried out overnight. Total fatty acid recovery minus β-OH myristate, however, did appear to be complete with the shorter hydrolysis time. Also, the relative proportions of the major nonpolar fatty acids were the same whether hydrolysis was for 2 h or overnight.

Distribution of β-OH myristate between the outer and cytoplasmic membranes of the four strains is given in Table 6. The outer membranes of strains CR34 and 0111:B_4 contained 80 and 70%, respectively, of the total β-OH myristate recovered in the outer plus cytoplasmic membrane fractions. In sharp contrast to strain CR34, strain GR467 contained only 26% of the β-OH myristate in its outer membrane. Likewise for strain RC-59, the distribution appeared to be altered in that 49% of the β-OH myristate was with the outer membrane. This finding was paralleled, in strain GR467, by the distribution of KDO (Table 6).

Fatty acid composition. An analysis of the total and chloroform-methanol-extractable fatty acids in the various membrane fractions is given in Table 7. Except for β-OH myristate, a comparison of the relative proportions of the individual fatty acids of corresponding membrane fractions does not reveal any noteworthy differences. β-OH myristate does appear to comprise a greater percentage of the total fatty acids in the membranes of strain GR467 than in CR34. As noted in preceding sections, the hydrolysates of the chloroform-methanol-extractable lipids of strain GR467 contained β-OH myristate. Although the comparable chloroform-methanol-extractable fatty acid analyses were not done for strain CR34 in the experiment presented, other analyses indicated that only trace amounts of β-OH myristate would be found. Similar results were found for strains 0111:B_4 and RC-59 (Table 7) concerning both total β-OH myristate and chloroform-methanol-extractable β-OH.

It is of particular interest that the β-OH myristate found in the cytoplasmic membranes of strains GR467 and RC-59 was not extracted to as large an extent by chloroform-methanol as was the β-OH myristate in the outer membranes. In strain GR467, the chloroform-methanol-extractable portion constituted 62% of the outer membrane and 31% of the cytoplasmic membrane β-OH myristate. In strain RC-59, the corresponding values were 56% in the outer membrane and 28% in the cytoplasmic membrane.

The total nonpolar fatty acids in the membrane fractions were compared by calculating the ratio of unsaturated plus cyclopropane to saturated fatty acids (Table 8). No differences were found between mutant versus parent outer, cytoplasmic, or total membranes. However, in both mutant and parent strains, the cytoplasmic membranes were found to have relatively more unsaturated plus cyclopropane fatty acids than did their corresponding outer membranes. This observation is similar to that made by White et al. (44) in their analysis of the fatty acid composition of total phospholipids of these

TABLE 7. *Fatty acid composition of fractionated membranes*

Strain[b]	Amt of fatty acid (%) in[a]																			
	12:0		14:0		16:0		16:1		17cyc		18:0		18:1		19cyc		β-OH myristate[c]		Unknowns	
	OM	CM	OM	CM	OM	CM	OM	CM	OM	CM	OM	CM	OM	CM	OM	CM	OM	CM	OM	CM
CR34																				
A	1.4	1.7	4.5	2.6	33.3	32.0	9.9	12.6	3.5	4.9	1.7	2.0	29.7	36.1	1.9	2.2	13.7	4.0	0.46	1.4
B	ND																			
GR467																				
A	2.5	2.0	5.4	3.3	27.1	26.3	9.9	15.6	1.6	3.2	2.4	1.4	24.7	33.2	1.1	0.8	18.5	11.6	6.5	3.5
B	0.9	0.5	4.1	1.8	31.6	32.8	10.2	15.0	2.0	3.8	2.3	1.4	30.5	38.2	1.0	0.7	11.6	3.6	5.9	2.5
0111:B_4																				
A	2.4	1.0	8.3	4.0	27.0	26.0	18.7	24.3	2.6	3.6	1.0	1.1	22.0	32.8	0.3	0.4	12.3	4.0	3.5	2.9
B	0.6	0.4	3.4	2.1	29.6	25.0	23.0	29.3	3.8	4.5	1.5	0.8	33.8	36.0	0.8	0.5	—	—	3.7	1.4
RC-59																				
A	2.2	1.2	6.9	4.1	25.6	24.6	16.5	21.5	1.9	2.8	1.8	1.2	23.9	30.3	—[d]	0.4	14.7	7.0	6.5	3.6
B	1.6	0.5	5.8	2.9	26.3	28.1	17.4	23.6	2.5	3.3	2.0	1.3	28.2	35.6	—	0.4	8.4	2.0	8.0	2.8

[a] Number before colon represents number of carbon atoms; number after colon represents number of double bonds. Abbreviations: cyc, cyclopropane; OM, outer membrane; CM, cytoplasmic membrane.

[b] (A) Direct saponification; (B) saponification of Bligh-Dyer extracts. All analyses were carried out after 14.5 h of saponification except for that of CR34 (CM), which was carried out after 2 h of saponification.

[c] Includes *trans*-2-tetradecenoic acid.

[d] —, Absent or trace amounts.

TABLE 8. *Comparison of membrane fatty acids*[a]

Strain	Total membranes	Outer membrane	Cytoplasmic membrane
CR34	1.25	1.10	1.46
GR467	1.27	1.00	1.60
0111:B$_4$	1.41	1.13	1.91
RC-59	1.49	1.16	1.81

[a] Based on direct saponification data. Expressed unsaturated + cyclopropane fatty acids/saturated fatty acids.

membrane fractions from *E. coli*.

Protein composition of total and fractionated membranes. The apparent protein deficiency in the cell envelope of strain GR467 was further examined by solubilization of membrane proteins in the presence of 2% SDS and subsequent polyacrylamide gel electrophoresis of the resulting polypeptides. Figure 3 shows the polypeptide patterns derived from total, outer, and cytoplasmic membranes which were solubilized at 70 C for 20 min or at 100 C for 1 min as indicated in the figure legend. The two conditions for solubilization were selected in light of previous studies with *E. coli* (12, 40, 45) and *S. typhimurium* (28) which reported the anomalous behavior of major cell envelope proteins depending on the temperature of solubilization (see Discussion). In the CR34 total membranes pattern, a prominent band was found at a molecular weight position estimated to be 30,000 (band D) after solubilization at 70 C (column 7), whereas solubilization at 100 C (column 13) produced two prominent bands designated B and C (molecular weights estimated to be 38,500 and 37,500, respectively). These three bands and band A (molecular weight = 40,500) were found to be localized in the outer membrane (columns 3 and 9). It is clear in Fig. 3 that, with either temperature of solubilization, the outer membrane polypeptides were considerably diminished in the appropriate GR467 samples (columns 4, 8, 10, 14).

Figure 3 also shows polypeptide patterns derived from CR34 and GR467 cytoplasmic membranes (columns 5, 6, 11, and 12). Although there appear to be differences in the GR467 patterns, especially as concerns a number of bands having molecular weights less than 30,000 and a band in the region of bands B and C, we have not consistently found these differences. Moreover, it is unlikely that the 38,000-molecular-weight band (columns 6 and 12) represents outer membrane contamination since the band is equally prominent with both temperatures of solubilization, but only a trace

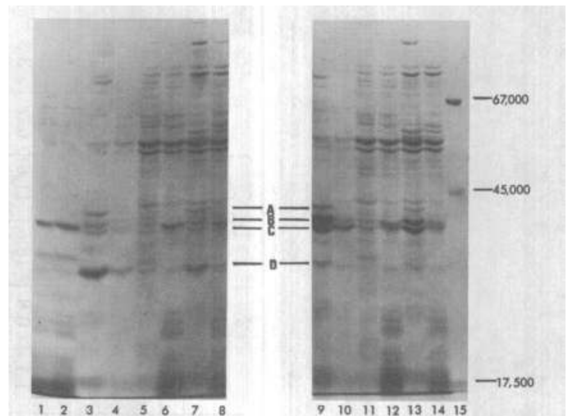

FIG. 3. *SDS-polyacrylamide gel electrophoresis of total and fractionated cell envelope proteins from CR34 and GR467. (1 and 2) Mixture of 50S and 30S ribosomal proteins; (3) CR34 outer membrane (OM) proteins; (4) GR467 OM; (5) CR34 cytoplasmic membrane (CM) proteins; (6) GR467 CM; (7) CR34 total membrane (TM) proteins; (8) GR467 TM; samples 9–14 follow the same order as 3–8; samples 1 and 3–8 were solubilized at 70 C for 20 min, and samples 2 and 9–14 were solubilized at 100 C for 1 min. In each case, 10 µg of protein was electrophoresed. Sample 15 contained the molecular weight standards given in Materials and Methods. Additional details concerning solubilization of samples and the conditions of electrophoresis are also given in Materials and Methods.*

amount of material appears at the position of band D with either temperature of solubilization.

The existence of the temperature-dependent phenomenon described above was particularly useful when attempting to detect the outer membrane polypeptides in preparations presumed to have relatively small amounts of those polypeptides. Proteins found in the following preparations were examined by polyacrylamide gel electrophoresis (Fig. 4): (i) total cell extracts obtained by sonication; (ii) supernatant fractions obtained after homogenization of cells with a Virtis-23 homogenizer; (iii) supernatant fractions obtained from the centrifugation step yielding total membranes; and (iv) spent growth medium. In the total cell extracts patterns (columns 1 through 4), it was possible to detect the temperature-dependent phenomenon discussed above and the diminution in strain GR467 (columns 3 and 4) of the outer membrane polypeptides exhibiting this phenomenon despite relatively small quantities of those polypeptides. Outer membrane polypeptides could not be detected in the remaining preparations represented in Fig. 4.

Isolation and characterization of

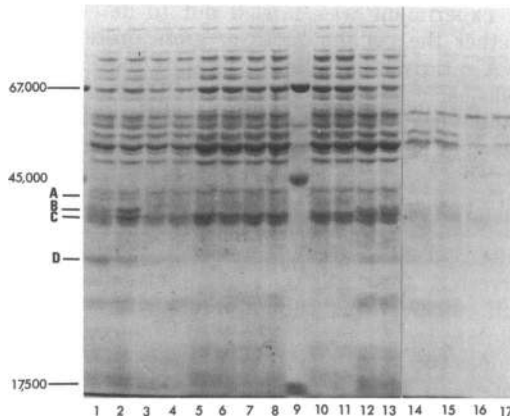

FIG. 4. *SDS-polyacrylamide gel electrophoresis of proteins found in: CR34 total cell sonicates (1 and 2); GR467 total cell sonicates (3 and 4); CR34 soluble cytoplasmic materials (5 and 6); GR467 soluble cytoplasmic materials (7 and 8); CR34 Virtis-23 homogenates (10 and 11); GR467 Virtis-23 homogenates (12 and 13); CR34 spent medium (14 and 15); GR467 spent medium (16 and 17). Samples 1, 3, 5, 7, 10, 12, 14, and 16 were solubilized at 70 C for 20 min, and samples 2, 4, 6, 8, 11, 13, 15, and 17 were solubilized at 100 C for 1 min. In each case except for samples 14–17, 10 μg of protein was electrophoresed. For samples 14–17, approximately 40 μg of protein was electrophoresed. Sample 9 contained the molecular weight standards given in Materials and Methods. Additional details concerning solubilization of samples and conditions of electrophoresis are also given in Materials and Methods.*

revertants. In the course of this study, we have characterized the mutant GR467 in terms of a protein deficiency in the outer membrane which occurs in addition to the heptose deficiency. It was thus necessary to determine whether the two alterations were coordinated or independent. Towards this end, a number of spontaneous revertants, presumably containing normal levels of heptose, were isolated by plating out samples of dense suspensions of strain GR467 onto the bile salts-containing medium, MacConkey-lactose agar. Heptose-deficient strains are more sensitive to bile-salts than are their parent strains (9, 21).

Sixteen colonies which grew on MacConkey agar were further tested for their sensitivity to the LPS core-specific phage, C21. Phage C21 has been used, together with other core-specific phages, to classify rough mutants of both *Salmonella* (29) and *E. coli* (34, 35) by correlating phage susceptibility to deletions in the core constituents. The susceptibility of *E. coli* rough mutants to C21 has been shown to depend on the absence of or a reduction in the relative amount of core galactose (14, 30, 35); in addition, the presence of heptose was found to be important (35). The precise nature of the receptor site is not known. Strain CR34, which has little galactose in its LPS core and a substantial complement of heptose, is susceptible to C21, whereas strain GR467, which has only 30% of the heptose present in strain CR34, is not (32). Two of the sixteen presumptive revertants, R11 and R16, gave clear plaques with the soft-agar overlay technique (5), whereas the remaining ones, including R2 and R17, gave turbid plaques.

A division in the characteristics of the presumptive revertants was also seen with respect to the presence of extractable KDO-lipid A (32), sensitivity towards SDS, the phospholipid-to-protein ratio in their outer membranes, and the ratio of heptose to KDO in total membranes as summarized in Table 9 for strains R11, R16, R2, and R17.

The proteins of the total membranes of strains R11, R16, and R2 were compared by means of polyacrylamide gel electrophoresis with those from strains CR34 and GR467 (Fig. 5). With the revertants, R11 and R16, solubilization at 70 C for 20 min (columns 2 and 3, respectively) showed that band D had regained its prominence relative to the other major bands. When the proteins in these same preparations were solubilized at 100 C for 1 min (columns 8 and 9), the patterns obtained were similar but not identical to those seen in Fig. 3 for strain CR34 under these conditions of solubilization. The revertant patterns showed bands B and C to be prominent, but there was also a substantial amount of material at the position of

TABLE 9. *Partial characterization of spontaneous GR467 revertants*

Strain	^{32}P (%) in KDO-lipid A band[a]	Growth on SDS-minimal agar[b]	μg of phospholipid/mg of protein in outer membrane	μmol of heptose/μmol of KDO in total membranes
CR34	3.3	+	263[c]	1.19
GR467	19.0	–	626[c]	0.28
R11	5.6	+	267	1.27
R16	4.0	+	253	1.08
R2	13.2	–	340	0.63
R17	17.7	–	ND[d]	0.24

[a] Total phospholipids were chromatographed on Amberlite WB-2 paper with diisobutylketone-acetic acid-water (8:5:1, vol/vol/vol) as the solvent as described previously (32).
[b] Modified Davis-Mingioli medium supplemented with 0.6% SDS (see Materials and Methods).
[c] Taken from Table 1.
[d] ND, not done.

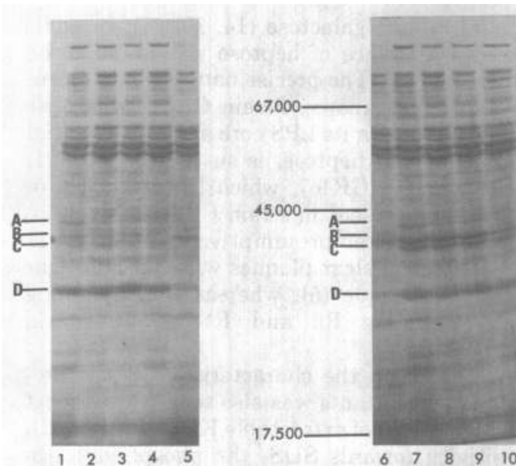

FIG. 5. *SDS-polyacrylamide gel electrophoresis of total membrane proteins from CR34, GR467, R11, R16, and R2. Samples 1 (CR34), 2 (R11), 3 (R16), 4 (R2), and 5 (GR467) were solubilized at 70 C for 20 min, and samples 6 (GR467), 7 (CR34), 8 (R11), 9 (R16), and 10 (R2) were solubilized at 100 C for 1 min. The total membrane preparations for CR34 and GR-467 were different from those used for Figure 3. In each case above, 10 μg of protein was electrophoresed. The numbers 67,000, 45,000, and 17,500 represent the molecular weights of protein standards (see Materials and Methods) run simultaneously with the above samples. Additional details concerning solubilization and conditions of electrophoresis are given in Materials and Methods.*

band D. When R2 total membranes were solubilized at 70 C (column 4), it was not easily discernible whether the outer membrane polypeptides in question were intermediate in prominence, although this did appear to be so in the pattern obtained after solubilization at 100 C (column 10). As was mentioned above, some of the presumptive revertants had levels of the extractable KDO-lipid A comparable with the level in strain GR467. When the total membranes of one of these, R17, were electrophoresed (70 and 100 C solubilization), the patterns obtained (not shown) resembled those of GR467 membranes.

The 16 strains selected as presumptive revertants did not grow on TSB + thy agar at 42 C, indicating that the heptose and protein deficiencies in strain GR467 are independent of the temperature-sensitive mutation in that strain. Furthermore, in the previous study (32) revertants were found which were no longer temperature sensitive but which still produced the extractable KDO-lipid A glycolipid.

Cellular mass and protein-to-DNA ratio in strains CR34 and GR467. In light of the marked alterations discussed above, the following experiment was carried out to determine whether the cellular mass or gross protein-to-DNA ratio was affected in strain GR467. Two flasks each for strains CR34 and GR467 (250 ml of medium in each case) were inoculated with overnight cultures such that their starting optical densities were the same. For each strain, one culture was allowed to grow for 4 h at 30 C while the other was grown at 30 C for 2 h and then shifted to 42 C for an additional 2 h. When harvested, the total wet weight of cells from each culture, total cellular protein, and total cellular DNA were measured. The results (Table 10) show that in all cases the same protein to DNA ratio is found. However, the mutant cells possessed strikingly less mass than the parental cells although the cultures were harvested at the same optical density.

DISCUSSION

In this study, we initially observed that the outer membrane of an *E. coli* heptose-deficient strain, GR467, had a lower density than the outer membrane of its parent strain as shown by sucrose density gradient centrifugation (Fig. 2). A similar observation has been noted by Osborn et al. (28) in a study which included *Salmonella typhimurium* strains, one of which, G-30A, is heptose deficient. It was stated that, upon fractionation of the envelope membranes of G-30A, the heaviest membrane band was essentially missing in a sucrose density gradient and that in the mutant all of the LPS was found at the position of the usual M band (intermediate between the heavy and two light bands).

The outer membrane of strain GR467 was characterized in terms of the relative amounts of protein, phospholipid, and lipid A. In stain GR467, the protein content was reduced approximately 60% relative to lipid A, whereas the ratio of phospholipid to lipid A remained the same as that of the corresponding fraction from

TABLE 10. *Comparison of cellular mass and total protein-to-total DNA ratio in strains CR34 and GR467*[a]

Strain	Growth temp (C)	Final OD[b]	Total wet wt (g)	Total mg of protein/total mg of DNA
CR34	30	286	1.36	23.2
	30 + 42	302	1.46	21.9
GR467	30	299	1.07	21.5
	30 + 42	292	1.00	22.5

[a] Conditions of experiments are described in Results.

[b] Optical density measured with a 660-nm filter.

strain CR34. The distribution of protein recovered in fractionated mutant membranes provided supportive evidence. There was a marked decrease in the proportion of protein recovered in the outer membranes accompanied by an increase in the proportion of protein recovered in the cytoplasmic membranes. In strain GR467, there also appeared to be a parallel, although less pronounced, change with respect to the distribution of phospholipid. However, the ratios of protein to lipid A and phospholipid to lipid A clearly indicate that the dominant change was in the protein component. Similar findings were obtained with another heptose-deficient strain, RC-59, as concerns the relative ratios just discussed and the distribution of protein between outer and cytoplasmic membranes. There was no substantial difference in the distribution of phospholipids in this case. The distribution of the cytoplasmic membrane marker, NADH oxidase (Table 5), showed that it contaminated the outer membranes of strains GR467 and RC-59 more than the outer membranes of the respective parent strains. Despite this increased cytoplasmic membrane protein contamination, the phospholipid-to-protein ratio in the mutant outer membranes was significantly higher than in the parent strains.

Additional evidence for the apparent decrease of outer membrane proteins was obtained by analyzing the SDS-solubilized proteins by polyacrylamide gel electrophoresis. In GR467 total membranes (Fig. 3 and 5), there was a significant decrease in the relative amounts of major polypeptides that exhibited changes in their apparent molecular weights depending on the temperature of solubilization. After separation of total membranes by sucrose density gradient centrifugation, these diminished polypeptides were found to be localized in the outer membrane (Fig. 3). The diminution in certain polypeptides in GR467 outer membrane samples is particularly striking since the same amount of protein was subjected to electrophoresis for the CR34 and GR467 outer membrane samples. It is possible that portions of the polypeptides A, B, C, and D exist as fragments having a molecular weight too low to be resolved under the conditions used here (i.e., they may migrate off the gel) or that there are fragments and aggregates, derived from the polypeptides in question, encompassing a wide range of molecular weights with no one polypeptide being abundant enough to produce prominent bands. We have not investigated these possibilities. Based on the results shown in Fig. 4, it does not appear that the outer membrane polypeptides being considered have been transferred to the cytoplasmic membrane, preferentially lost when cells are homogenized with a Virtis-23 homogenizer, preferentially excreted into the growth medium, or lost with the soluble cytoplasmic materials during membrane fractionation. SDS-polyacrylamide gel electrophoresis of solubilized cytoplasmic membrane proteins of strain GR467 showed a polypeptide that was not seen in the same fraction from strain CR34. This band appeared to migrate between bands B and C in the 100 C-solubilized total membrane fraction (Fig. 3). In view of its presence in strain GR467 and not strain CR34, it is tempting to speculate that it is related to the missing outer membrane proteins, but no further evidence for this relationship is available at present.

It is of particular interest that the major outer membrane polypeptides (bands B, C, and D) exhibit changes in their apparent molecular weights. The gel patterns obtained for strains CR34, GR467, and the revertants suggest that both bands B and C, which are predominant when solubilization is carried out at 100 C, contribute to band D, which is found when solubilization is carried out at 70 C. These changes resemble those in other recent reports. Wu (45), investigating the envelope proteins of *E. coli* K-12-derived strains, found a prominent 38,000-molecular-weight polypeptide band on SDS-polyacrylamide gels when solubilization was carried out at 100 C in the presence of SDS. This band was diminished and two other bands corresponding to molecular weights of approximately 59,000 and 28,000 were augmented when solubilization was carried out at 70 C. Furthermore, when membrane samples and alkaline gels prepared in the presence of 6 M urea were used, several bands appeared in the 38,000-molecular-weight region. With either SDS or urea gels, the polypeptide bands in question were found to be localized in the outer membranes. Inouye and Yee (12) have also studied the effects of temperature on the dissociation of these proteins. Their results parallel those presented by Wu (45). In addition, they presented evidence that the three 70 C-induced peaks are biochemically distinct (12).

Restoration of the major outer membrane polypeptides was found in the spontaneous revertants R11 and R16, and apparent partial restoration was found in revertant R2 (gel patterns in Fig. 5 and phospholipid/protein ratios in Table 9). Together with the analyses of the relative heptose levels (Table 9), these data suggest that the protein and heptose deficiencies are the result of a single mutation. The possibility that strain GR467 is a double mutant with respect to its heptose and protein deficien-

cies is unlikely, since it exhibited a relatively high reversion frequency which is more characteristic of a single mutation. Furthermore, we have examined and found characteristics similar to those described for strain GR467 in a second heptose-deficient strain, RC-59. This strain was isolated independently of strain GR467 from a different parent strain. It is unlikely that strains GR467 and RC-59 would each have two independent mutations for identical characteristics.

The possibility exists that the genes for the synthesis of the polypeptides in question and the genes involved with the synthesis or incorporation of heptose are part of the same operon. The phenomena we have studied could then be the result of a mutation at a regulatory locus or possibly the result of a polar mutation in one of the genes alluded to above. There are presently no genetic data to support or refute these possibilities.

Alternatively, these genes may be in different loci and the heptose lesion may cause the outer membrane protein deficiency. The heptose deficiency could be the result of a mutation in the synthesis of sedopheptulose-7-PO_4, in line with the observations of Eidels and Osborn (9) using *Salmonella typhimurium*. However, strain GR467 is not a transketolase mutant (unpublished results). The mutation may also involve one of a number of steps in the conversion of sedoheptulose-7-PO_4 to L-glycero-D-*manno*heptose such as those proposed by Lehmann et al. (17). The work of Osborn et al. (27) suggests that the core constituents are assembled in the cytoplasmic membrane and are subsequently translocated by an unknown mechanism to the outer membrane. It is not known where the synthesis of outer membrane proteins is completed nor how they are translocated. It is possible that translocation or complete synthesis of these proteins is dependent on the extent of completion of LPS core residues or on the configuration of KDO-lipid A residues (possibly altered in a heptose-deficient strain). A third general possibility is that the primary lesion is concerned with the synthesis of the major outer membrane proteins, and the concomitant heptose-deficiency results from the initial protein deficiency.

It should be noted that White et al. (44), Kulpa and Leive (15), and Wu (45) described conditions wherein the absence of LPS core galactose gave rise to "light" outer membranes. Neither White et al. (44) nor Kulpa and Leive (15) presented data on the biochemical nature of these light outer membranes. Wu, however, has described this phenomenon in a mutant ostensibly selected as a glucosamine-independent revertant of a glucosamine-requiring *E. coli* mutant (45). The revertant had a phosphoglucoisomerase (Pgi) mutation not present in the original mutant and also exhibited a diminution of a major outer membrane polypeptide as shown by polyacrylamide gel electrophoresis. Wu found that when the Pgi^- mutation in the revertant was corrected by transduction, the density of its outer membrane was the same as for the outer membrane of the original Pgi^+ strain, but that the polypeptide deficiency remained. These observations imply that changes in the density of the outer membrane need not accompany changes in outer membrane proteins, but may be related to changes in the core polysaccharide in an unknown manner.

An unexpected finding in both of the heptose-deficient strains we have examined, but most strikingly in strain GR467, was that a substantial portion of β-OH myristate was recovered with the cytoplasmic membrane. It should be pointed out that the present results do not verify the corresponding data presented in the previous study by Rooney and Goldfine (32), wherein it appeared that 90% of the β-OH myristate recovered in the outer plus cytoplasmic membranes of both strains CR34 and GR467 remained with the outer membranes. Since the earlier data were derived from a single experiment, we feel that our more extensive use of the Schnaitman procedure and its present reproducibility concerning the distribution of β-OH myristate indicate that the earlier data was not representative. In strain GR467, the distribution of a second LPS constituent, KDO, also paralleled the change found with β-OH myristate (Table 6). The differences noted here could be related, at least for strain GR467, to the previous observation (32) that some of the LPS moieties in that mutant are more lipophilic than the LPS moieties of its parent stain. The heterogeneity of LPS in strain GR467 may give rise to conditions where KDO-lipid A-containing molecules are intercalated between cytoplasmic membrane components. It is also possible that the differences noted above reflect an accumulation of KDO-lipid A precursors in the cytoplasmic membrane.

Detailed analyses of the fatty acids of the fractionated membranes did not indicate any noteworthy differences among the nonpolar fatty acids (Table 7). However, the proportion of β-OH myristate seemed to be greater in the mutants. Our analyses of the total fatty acids in the 0111:B_4 outer membrane are not in agree-

ment with those of White et al. (44). Those authors, using *E. coli* J-5 (a uridine diphosphate-galactose-4-epimerase$^-$-derivative of 0111:B_4 grown in galactose-supplemented minimal medium), reported that 60% of the total outer membrane fatty acids consisted of β-OH myristate as opposed to 12% in our study. It is not clear why this large difference exists between our results. One major factor, the conditions of hydrolysis, was the same in both studies (25, 44). Differences in our analyses could have arisen due to the fact that White et al. (44) separated and purified their total membranes by a combination of particle electrophoresis and sucrose density gradient centrifugation, whereas we used only sucrose density gradient centrifugation towards this end. Another possibility is that differences in the growth conditions employed resulted in the synthesis of different proportions of lipid A. For our studies, a rich medium was used throughout with growth occurring at 30 C, whereas White et al. (44) used a minimal medium and grew their cells at 37 C.

We have thus far described the changes in the cell envelope of strain GR467 by quantitating and localizing selected marcomolecules. It is obvious that our description is still relatively crude and that any attempt to define changes in the physical dimensions of the envelope would be speculative. However, along these lines it is worthwhile to note some electron microscope investigations by Shands et al. (38) using purified LPS from smooth and R_a (complete core but no O antigen) *Salmonella* strains. That work revealed ribbon-like structures having a trilaminar appearance when viewed on edge. The thickness of the ribbons from both strains was essentially the same. In contrast to this, purified LPS from a heptose-deficient strain appeared as vesicular lamellae or diffuse trilaminar structures. Furthermore, the thickness of the heptose-deficient structures was less than that of the other two types of LPS.

Our data are consistent with changes in the physical dimensions of the outer or total membranes from strain GR467. The marked decrease in the outer membrane proteins and the fact that the phospholipid-to-lipid A ratio remained the same despite the apparent redistribution of both phospholipid and KDO-lipid A material suggest that the thickness or surface area of the outer membrane or total membranes may have changed. Another possible change is suggested by a reconsideration of the earlier data presented by Rooney and Goldfine (32). It was found that LPS from strain GR467 contained one-third as much heptose and about one-half as much phosphorus as LPS from strain CR34. In work with *Salmonella*, it has been suggested that some of the LPS phosphorus serves to cross-link heptose units by formation of phosphodiester bonds (8). Thus, it is possible that the core region of GR467 LPS is less extensively cross-linked than the same region in strain CR34. This, in turn, could affect the spatial distribution of LPS. It should be noted that, although we have observed a depletion of outer membrane proteins in strain GR467, the total protein-to-DNA ratio does not appear to be different from that in strain CR34 (Table 10).

The structure of the gram-negative cell envelope has been correlated in a variety of studies with phage susceptibility (3, 23, 29, 34, 35), resistance or sensitivity to antibiotics (3, 23, 41, 42), ability to form mating pairs during bacterial conjugation (3, 23), and detergent sensitivity (9, 19, 21, 45). Of particular interest is work by Boman et al. (3) and Monner et al. (23) in which the basis of ampicillin resistance was studies in *E. coli*. These studies implicated cell surface alterations, among them alterations in the composition of the LPS moiety, as factors that can be involved in the expression of ampicillin resistance.

Other studies, presented by Tamaki et al. (41, 42), have linked sensitivity to novobiocin, spiramycin, coumermycin and actinomycin D to alterations in the LPS composition of mutants they have isolated. In addition, Tamaki and Matsuhashi (41) have correlated LPS alterations with increased sensitivity towards lysozyme. These studies and those of Boman et al. (3) and Monner et al. (23) clearly indicate the importance of LPS for the penetration of low-molecular-weight molecules through the outer layers of the cell envelope. The work presented here raises the question of the importance of outer membrane proteins in these phenomena.

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ADDENDUM

After completion of this work, we learned of similar studies on heptose-deficient mutants of *Salmonella typhimurium* by G. Ferro-Luzzi Ames, E. N. Spudich, and H. Nikaido (J. Bacteriol. **117**:406–416) which have revealed similar depletions of outer membrane proteins in these mutants. We thank Dr.

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