Biochimica et Biophysica Acta, 466 (1977) 160—175

BBA 77642

BIOSYNTHESIS AND STRUCTURE OF LIPOPOLYSACCHARIDE IN AN OUTER MEMBRANE-DEFECTIVE MUTANT OF *ESCHERICHIA COLI* J5

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(Received September 6th, 1976)

Summary

Membrane-defective mutants of *Escherichia coli* J5 were isolated on the basis of supersensitivity to the antibiotic novobiocin. These mutants display an increased sensitivity to a wide range of antibiotics and to several dyes and detergents. In addition, several mutants leak the periplasmic enzymes, alkaline phosphatase and ribonuclease. This evidence indicates an outer membrane defect in these mutants. The inner and outer membranes of one mutant were separated and subjected to compositional analysis. A deficiency in galactose-containing lipopolysaccharide in the outer membrane of the mutant was observed. Two possible causes of this deficiency were examined and discounted: defective galactose uptake into the cell, and defective translocation of lipopolysaccharide from the inner membrane. Extraction and chemical analysis of mutant and wild type lipopolysaccharides suggests that the mutant is defective in the enzyme which transfers glucose to the growing lipopolysaccharide core, UDPglucose transferase. Thus, the mutant's deficiency in galactose-containing lipopolysaccharide can be ascribed to the fact that adddition of glucose to the lipopolysaccharide core is a prerequisite for galactose addition. The physiological implications of this alteration are discussed.

Introduction

A central question in membrane biology is still largely unanswered: how do membrane components interact to result in specific membrane functions? One approach to this problem has been to analyze the membrane composition of bacterial mutants which display membrane-related physiological defects [1—5]. In this way the involvement of specific membrane components in various membrane functions can be assessed.

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This paper describes the isolation and physiological characterization of outer membrane-defective mutants derived from *Escherichia coli* J5. One mutant was extensively characterized with regard to lipopolysaccharide synthesis and structure. *E. coli* J5 is a UDPgalactose-4-epimerase-negative mutant of *E. coli* O111: B4 [6]. As a result, J5 cannot utilize galactose as a carbon source and, in its absence, synthesizes incomplete lipopolysaccharide molecules. These lipopolysaccharide molecules lack the O-antigen polysaccharide side chains as well as the last two sugars of the core region [6,7]. Thus, the lipopolysaccharide composition of J5, when grown in the absence of galactose, is similar to *rfa* mutants of other *E. coli* strains [8]. Such mutants are defective in the synthesis of the core region of lipopolysaccharide [9]. This ability to manipulate the structure of a major outer membrane component lends added versatility to the study of membrane mutants.

Materials and Methods

Organisms and media. *E. coli* J5 was obtained from L. Leive (N.I.H., Bethesda, Md.). Bacteriophage CP13 was isolated in this laboratory and is described elsewhere [10]. For membrane prepartion, 1.0% protease peptone No. 3 Difco), 0.1% beef extract (Difco), and 0.5% NaCl was used [11]. In some cases, this medium was supplemented with D-galactose at various concentrations. For chemical sensitivity studies, nutrient broth (Difco) supplemented with 0.1% glucose and nutrient broth (Difco) supplemented with 0.5% glucose and 0.5% galactose were used. Solid medium was prepared by adding Bacto-agar (Difco) at 1.5%. Carbon source utilization was carried out in Vogel and Bonner [12] basic minimal medium supplemented with glucose or galactose at 0.5%.

Isolation of mutants. Memnbrane mutants of *E. coli* J5 were induced with NTG and selected for using novobiocin (200 μg/ml) in the presence of pencillin G as described by Tamaki et al. [4].

Sensitivity to chemical agents. Sensitivity to chemical agents was determined by spreading mid-exponential phase cells onto nutrient agar plates ($1 \cdot 10^7$—$3 \cdot 10^7$ cells/plate). For tests of dyes and detergents, appropriate dilutions of these chemicals were made to the agar medium before inoculation. Antibiotic-impregnated discs were laid onto the agar after inoculation to test their inhibitory effects on growth.

Enzyme leakage studies. Alkaline phosphatase was assayed as described by Garen and Levinthal [13]. Ribonuclease was assayed as described by Lopes et al. [1]. β-Galactosidase was assayed by measuring *o*-nitrophenyl-β-D-galactopyranoside hydrolysis after disruption of fully induced cells as described by Kepes [14].

Leakiness of the outer membrane was determined by measuring enzyme levels in cells and culture fluid as described by Lopes et al. [1].

Isolation of inner and outer membranes. The basic technique employed for the preparation and separation of membranes was that of Osborn et al. [15] with the following minor modifications. Cells were grown in protease peptone medium in the presence of [2-^{3}H]glycerol (1.0 mM, 0.2 Ci/mol) and in the presence or absence of D-[1-^{14}C]galactose (0.5 mM, 0.1 Ci/mol for J5 and mutant M19 or 0.05 Ci/mol for mutant M2) at 30°C to an absorbance of 0.4—0.5 at

530 nm. Lysozyme was used at 200 μg/ml of cell suspension, incubation on ice was for 4 min before the addition of EDTA and the resultant spheroplasts were lysed by osmotic shock.

The protein content of membrane suspensions was determined by the method of Lowry et al. [16].

Kinetics of galactoside uptake. Overnight cultures were subcultured into protease peptone medium to yield a starting absorbance at 530 nm of 0.1. Isopropylthiogalactoside, a gratuitous inducer of the lactose operon, was added to 0.2 mM. The cultures were grown at 30°C with aeration and 5-ml samples were taken at 20—40-min intervals. At each sampling time, the A_{530nm} of the culture was measured and galactoside uptake, using *o*-nitrophenyl-β-D-galactopyranoside, was assayed by the method of Kepes [14]. With intact cells, the rate of *o*-nitrophenyl-β-D-galactopyranoside hydrolysis is limited by its uptake, which can occur in two ways: (1) through the action of the inducible, membrane-bound galactoside permease; and (2) by passive diffusion into the cell. These processes can be dissociated by the use of an inhibitor of permease activity such as *N*-ethylmalemide [14].

Pulse-labeling experiments. Preliminary experiments were performed to determine D-[1-^{14}C]galactose pulse-chase conditions. Cells were grown in 100 ml of protease peptone medium at 30°C with aeration. When cultures reached an A_{530nm} = 0.4—0.5 they were centrifuged (10 000 × *g* for 5 min at 25°C), resuspended in 1 ml of medium (30°C), and held at 30°C for 3 min. The cell suspension was transferred to a serological test tube containing 10 μl D-[1-^{14}C]-galactose (5 μmol/ml, spec. act. = 10 Ci/mol) and held at 30°C for a 1 min pulse. The suspension was then diluted rapidly into 100 ml of medium (30°C) containing unlabeled galactose (0.05 mM). Incubation at 30°C with aeration was continued and samples were removed at various times and assayed by the trichloroacetic acid-filter paper technique of Mans and Novelli [17]. For preparation of membrane following [^{14}C]galactose pulse, the chase with unlabeled galactose was performed for 0, 5, or 15 min after which cell metabolism was stopped by rapid immersion of the culture in a solid CO_2/acetone bath. Membranes were then prepared as described above.

Lipopolysaccharide extraction. Cells were grown at 30°C in 15—25-l batches in protease peptone medium with and without galactose (0.5 mM). The cells were harvested at mid-exponential phase (A_{530nm} = 0.5—0.6). Lipopolysaccharide was extracted by the phenol/chloroform/light petroleum method of Galanos et al. [18]. This method extracts only rough (incomplete) lipopolysaccharide molecules. The bacterial residue from the extraction of M2 cells grown with galactose was further subjected to another lipopolysaccharide extraction employing the hot phenol/water method of Westphal and Jann [19] as described by Leive et al. [20]. This procedure extracts smooth (complete) lipopolysaccharide, O-antigen chains, and, to a small extent, rough (incomplete) lipopolysaccharide.

Compositional analysis of extracted lipopolysaccharides. Colitose and 2-keto-3-deoxyoxtonate were measured by the colorimetric method of Burtseva et al. [21]. This procedure allows the quantitative determination of 2-keto-3-deoxyoctonate and 3,6-dideoxyhexoses in mixtures. The procedure of Osborn [22] was used to assay the heptose content of extracted lipopoly-

saccharide. Sedoheptulose anhydride monohydrate was used to standardize the reaction. Phosphate was determined by the method of Bartlett [23].

Gas-liquid chromatography was used to determine lipopolysaccharide sugar composition. The alditol acetates of the sugars were prepared by the method of Porter [24] which allows the simultaneous determination of hexosamines and neutral sugars. A Hewlett-Packard Model 5750B chromatograph equipped with a flame detector and a 1/8 inch × 6 ft stainless steel column was used for the analysis. The column was packed with 3% ECNSS-M on 100—200 mesh Gaschrom Q. The column temperature was maintained at 185°C and N_2 was utilized as the carrier gas. Standards were prepared by the above procedure from glucose, galactose and glucosamine.

Results

16 stable, novobiocin-supersensitive mutants were isolated. Several mutants grew with an increased generation time relative to that of J5 (30 min), the longest being that of M2 (45 min). Eight of the 16 isolates, including the isolate on which this study is centered (M2), can be defined as possessing defective permeability barriers on the basis of an increased sensitivity to several antibiotics, dyes and detergents. These included ampicillin, chloramphenicol, colistin, neomycin, polymyxin B, dihydrostreptomycin, oxytetracycline, tetracycline, chlortetracycline, rifampicin, novobiocin, taurochloric acid, deoxycholic acid, and acridine orange. While the presence or absence of galactose in the growth medium did not affect the sensitivity of J5 to these chemicals, the mutant strains were more sensitive when grown in its absence.

Five of the eight permeability-defective mutants were found to leak periplasmic enzymes. The leakage of these enzymes has been used as the main criterion for the identification of outer membrane-defective mutants [1] since the outer membrane is the only barrier between these enzymes and the external environment [25].

The mutant strain M2 leaked ribonuclease and alkaline phosphatase during the exponential phase of growth. Total cellular enzyme levels for the two enzymes and the extent of ribonuclease leakage (6.8% of total cellular ribonuclease) agreed well with those reported for other *E. coli* "periplasmic-leaky" mutants [1]. Values obtained here for alkaline phosphatase leakage (8.2% of total cellular alkaline phosphatase) are somewhat lower than reported for these other mutants. Growth in the presence or absence of galactose had little effect on enzyme leakage by either strain.

No leakage of the cytoplasmic enzyme β-galactosidase could be detected in either strain during mid-exponential phase growth (Fig. 1). As M2 entered the stationary phase, however, an increase in the β-galactosidase level of the culture fluid is observed. This increase could be due to leakage during stationary phase as has been reported for other membrane mutants [26]. Such a conclusion is supported by the finding that in M2, the increase in extracellular enzyme is correlated with an increase in the "accessibility" [26] of intracellular β-galactosidase.

These results indicate that, during exponential growth, the permeability defect displayed by mutant M2 is the result of an outer membrane defect while

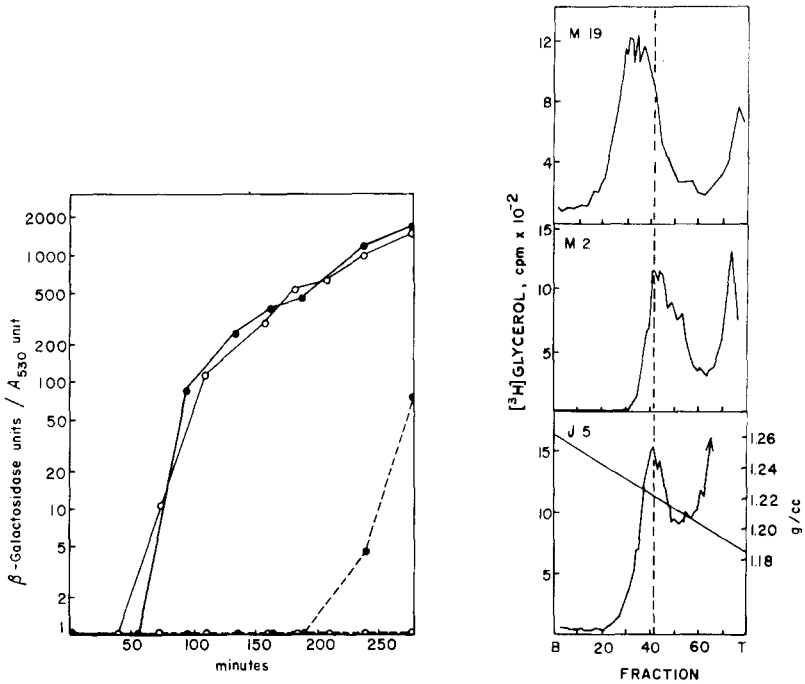

Fig. 1. β-Galactosidase activity as a function of time in culture for J5 and M2. β-Galactosidase was assayed as described in Materials and Methods. This assay, as applied here, is capable of detecting 0.03 enzyme unit. •———•, M2 intracellular; ○———○, J5 intracellular; •------•, M2 extracellular; ○------○, J5 extracellular. At 280 min, the level of enzyme leaked into the medium by J5 is measurable (0.3 units/ $A_{530\ nm}$ unit) but does not appear on the graph due to the logarithmic nature of the abscissa. All other values graphed on the ordinate represent no detectable enzyme activity.

Fig. 2. Isopycnic centrifugation in sucrose gradients (40—55%) of membrane preparations from strains J5, M2 and M19 grown in the presence of [^{3}H]glycerol.

the inner membrane appears to retain its integrity with regard to leakage of proteins.

Preparation and separation of inner and outer membranes

In all density profiles presented, a comparison of peak heights between different graphs is of little value since the quantity of cells used and the efficiency of recovery of radioactive tracer molecules varied from experiment to experiment.

Fig. 2 shows the membrane density profiles on 40—55% sucrose density gradients for J5, M2, and M19 grown in the absence of galactose but in the presence of [2-^{3}H]glycerol which serves as a convenient marker of total membrane phospholipid. The J5 profile agrees well with that previously reported [27]. At these concentrations of sucrose, the inner membrane remains at the top of the gradient while the lipopolysaccharide-containing outer membrane moves into the gradient. While J5 and M2 outer membranes banded at the same density (ρ = 1.22), M19 outer membrane banded at a higher density (ρ = 1.23).

Fig. 3 shows the membrane density profiles on 40—55% sucrose density gradients for J5, M2, and M19 grown in the presence of D-[1-^{14}C]galactose and

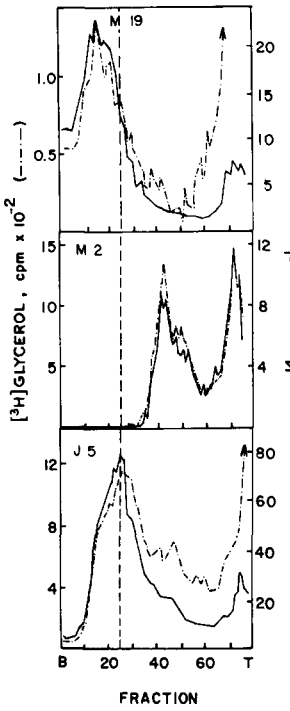

Fig. 3. Isopycnic centrifugation in sucrose gradients (40—55%) of mebrane preparations from strains J5, M2 and M19 grown in the presence of [^{3}H]glycerol and [^{14}C]galactose. The gradients used had the same linear density profile as those in Fig. 2.

[2-^{3}H]glycerol. Again, the J5 profile agrees well with previous studies [27], and demonstrated a density shift of the outer membrane (cf. Fig. 2) from ρ = 1.22 to ρ = 1.24. This increase in outer membrane density is due to the presence of complete rather than incomplete lipopolysaccharide molecules, since galactose is used exclusively to complete lipopolysaccharide molecules [27]. The outer membrane of strain M19 underwent a density shift of the same magnitude as that of J5 (from ρ = 1.23 to ρ = 1.25). Its final position, however, was again at a greater density than the outer membrane of J5. This result suggests that the compositional change in M19 may not involve lipopolysaccharide, and that the protein and/or phospholipid content may be altered instead.

The density profile of mutant M2 shows that its outer membrane did not change density at all when cells were grown in the presence of galatose. This suggests that M2 may be either incapable of synthesizing complete lipopolysaccharide and/or does not possess quantitatively as much lipolysaccharide as does J5. Fig. 3 also shows that the distribution of galactose between the inner membrane and outer membrane was changed in M2. There was a much greater proportion of the total galactose in the inner membrane of M2 as compared to the proportion found in J5 or M19. Density profiles of all three strains on 25—55% sucrose density gradients reflected the outer membrane differences for the three strains, but revelaed no differences in the density of the inner membrane.

Preparation of isolated outer membrane and inner membrane for compositional analysis required the use of 35—55 and 25—55% sucrose density gradients for M2 and J5, respectively. In both cases, separation of inner membrane and outer membrane on these gradients was sufficient to reduce membrane cross-contamination to less than 10% on the basis of membrane-associated enzyme activities (NADH-oxidase and phospholipase A) [15]. Isolated inner and outer membranes of J5 and M2 were assayed for acid-insoluble [2-^{3}H]-glycerol, D-[1-^{14}C]galactose and protein content relative to cell mass. The most significant difference found was that the outer membrane of J5 contains nearly nine times the acid-insoluble galactose of the outer membrane of M2.

A lipopolysaccharide alteration in M2 is also suggested by the finding that when grown in the presence of galactose this strain, but not J5, is sensitive to bacteriophage CP13 whereas both are sensitive in the absence of galactose. Bacteriophage CP13 is known to discriminate between these strains at the adsorption stage [10].

Kinetics of galactose uptake

Preliminary experiments showed that the rate of incorporation of [^{14}C]-galactose into acid-insoluble cell components was independent of galactose concentration over the range of 0.2—80 mM in the case of J5, but was dependent on galactose concentration over this range in M2. In addition, at each concentration of galactose and at each point in the growth curve tested, M2 showed much less galactose incorporation than did J5. A possible explanation for the failure of M2 to incorporate wild type levels of galactose into lipopolysaccharide is that the mutant is defective in galactose uptake. At galactose concentrations greater than 0.1 mM the MeGal transport system accounts for little galactose uptake [28,29]. Under these conditions, other transport systems are responsible for galactose uptake. Since one of these is the lactose system, the galactoside *o*-nitrophenyl-β-D-galactopyranoside was used to evaluate galactose uptake in M2. The rate of in vivo hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside by β-galactosidase is limited by the rate of *o*-nitrophenyl-β-D-galactopyranoside transport by β-galactoside permease [14]. Fig. 1 shows that the levels of intracellular β-galactosidase in M2 and J5 were nearly identical over 280 min of growth in protease peptone medium. The time course of galactoside permease activity in J5 and M2 is shown in Fig. 4. J5 and M2 displayed equal levels of permease activity throughout exponential growth. As M2 entered stationary phase, permease activity leveled off and fell behind that of J5. After 100 min of growth, *o*-nitrophenyl-β-D-galactopyranoside uptake due to permease activity in J5 was equal to or greater than uptake due to passive diffusion. Passive diffusion in M2, however, accounted for much more *o*-nitrophenyl-β-D-galactopyranoside uptake than in J5, both in absolute terms and in relation to the levels of permease activity. This result supports the contention that M2 possesses a defective permeability barrier.

Lineweaver-Burk plots were constructed from these data. Permease concentration was varied by using different concentrations of cells. The K_m ± S.D. for galactose in J5 was 1.10 ± 0.01 mM and in M2 was 1.77 ± 0.06 mM. The V at various permease concentrations in the two strains never varied by more than 30%. These differences between the kinetic parameters of M2 and J5 galacto-

side permease are not considered to be significant. It is, therefore, unlikely that the inability of M2 to incorporate wild type levels of galactose into lipopolysaccharide was based on an inability to transport galactose into the cell. The high level of passive diffusion of *o*-nitrophenyl-β-D-galactopyranoside into M2 could, however, explain the concentration dependence of galactose incorporation into lipopolysaccharide macromolecules in this mutant.

Kinetics of lipopolysaccharide translocation

Since galactose uptake is not the cause of M2's inability to incorporate wild type amounts of galactose into outer membrane lipopolysaccharide, there remained two other possibilities: (1) a defect in the synthesis of galactose-containing lipopolysaccharide, or (2) a defect in the translocation of galactose-containing lipopolysaccharide to the outer membrane. In either case, the defect cannot be absolute since M2 does possess some galactose-containing lipopolysaccharide in its outer membrane.

Lipopolysaccharide is known to be synthesized on the inner membrane, and

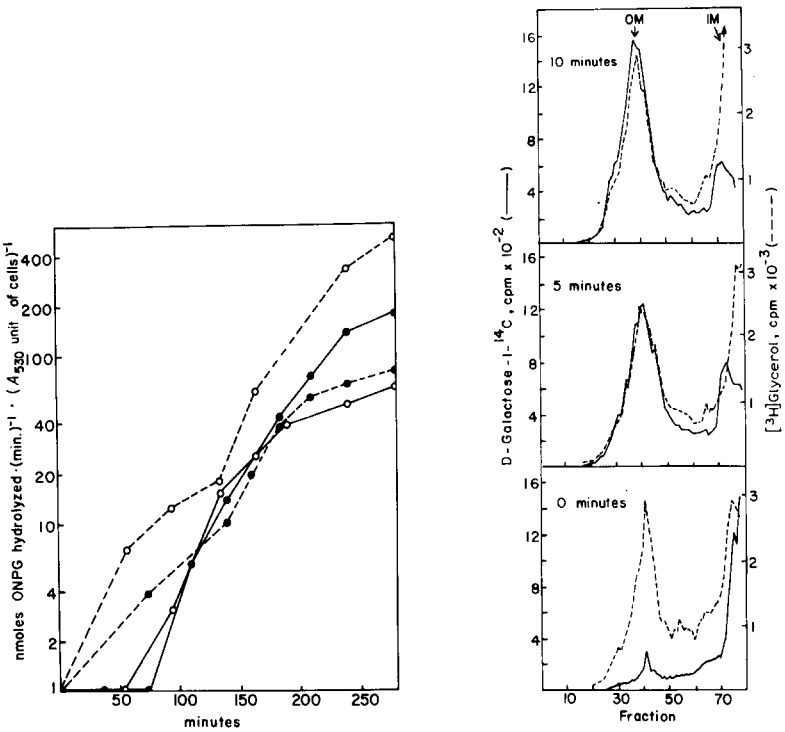

Fig. 4. Time course of galactoside permease activity and galactoside diffusion as measured by *o*-nitrophenyl-β-D-galactopyranoside hydrolysis. Dashed lines depict hydrolysis limited by galactoside diffusion in the presence of *N*-ethylmaleimide, a galactoside permease inhibitor. Solid lines represent hydrolysis limited by permease activity which was calculated by subtracting the hydrolysis rate in the presence of the permease inhibitor from that which was measured in its absence . •, J5; ○, M2.

Fig. 5. Isopycnic centrifugation in sucrose gradients (40—55%) of membranes isolated from J5 cells after a 1 min [^{14}C]galactose pulse and a [^{12}C]galactose chase of 0, 5 and 10 min.

then irreversibly translocated, by an unknown mechanism, to the outer membrane [30]. Cells were grown in the presence of galactose for varying lengths of time to study the possibility of a translocation defect in M2. The membranes of these cells were isolated and separated on sucrose density gradients. If translocation proceeds normally, the amount of outer membrane galactose should increase continually with time. These experiments showed that translocation of galactose-containing lipopolysaccharide from the inner membrane to outer membrane in M2 proceeds at a rapid rate for a short time (less than 6 min) and then decreases.

These anomalies of lipopolysaccharide translocation in M2 were further investigated by studying the fate of an increment of galactose in the growth medium as it is taken up by the cell, incorporated into lipopolysaccharide and translocated to the outer membrane. Preliminary experiments determined that after several generations of growth without galactose, both M2 and J5 could be effectively labeled by a 1 min pulse of [^{14}C]galactose and that labeling was effectively terminated within 1 min by a [^{12}C]galactose chase. It was not possible to pulse-label M2 with [^{14}C]galactose if it was previously grown for several generations in the presence of galactose.

Fig. 5 shows the density profiles of membranes isolated from J5 cells after a 1 min ^{14}C pulse and a [^{12}C]galactose chase of varying duration. Immediately following the pulse, nearly all the [^{14}C]galactose was associated with the inner membrane. After 5 min much of the [^{14}C]galactose had been chased into the outer membrane, and by 10 min nearly all had been translocated to the outer membrane. In contrast, when this experiment was performed with M2 cells (Fig. 6) a large fraction of [^{14}C]galactose remained associated with the inner

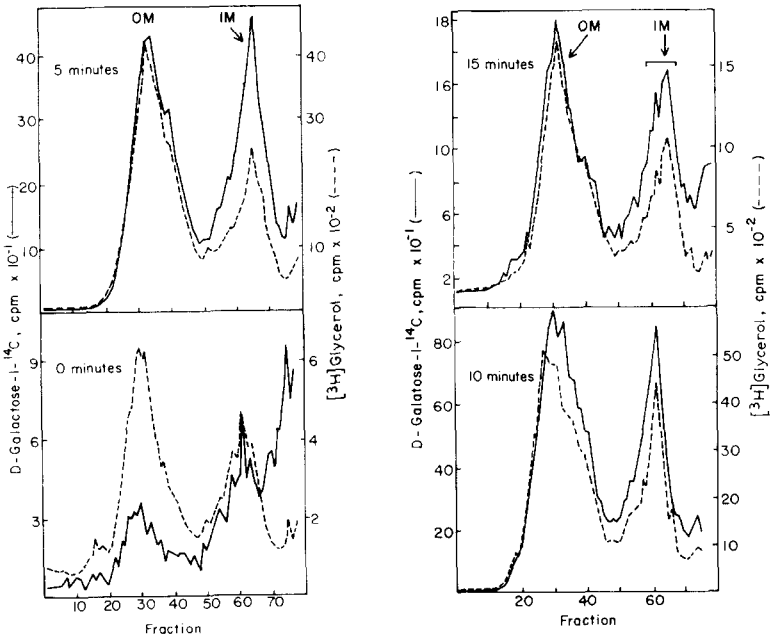

Fig. 6. Isopycnic centrifugation in sucrose gradients (35—55%) of membranes isolated from M2 cells after 1 min [^{14}C]galactose pulse and a [^{12}C]galactose chase of 0, 5, 10 and 15 min.

membrane even after 15 min of [^{12}C]galactose chase.

These experiments confirmed the observation that translocation of galactose-containing lipopolysaccharide from the inner membrane to the outer membrane in M2 proceeds for a short time, after which it rapidly decreases.

Assay of 2-keto-3-deoxyoctonate and colitose in whole cells and in acid-soluble cell components

To assess the relative amounts of lipopolysaccharide in M2 and J5, a sugar found in the core region of lipopolysaccharide, 2-keto-3-deoxyoctonate was assayed. In addition, a saccharide, colitose, present in the O-antigen portion of the complete J5 lipopolysaccharide was quantitated. The results of these measurements using whole cells and acid-insoluble cell material are shown in Table I. Acid-insoluble 2-keto-3-deoxyoctonate levels in both strains grown without galactose and in M2 grown with galactose were approximately the same, indicating that they possess the same amount of lipopolysaccharide per cell and that if M2 is defective in lipopolysaccharide synthesis, the defect occurs subsequent to 2-keto-3-deoxyoctonate. The finding that J5, when grown with galactose, gave slightly higher 2-keto-3-deoxyoctonate values probably reflects a failure to totally eliminate the cross-reactivity of colitose in the 2-keto-3-deoxyoctonate assay. The amount of acid-soluble 2-keto-3-depxyoctonate (calculated by subtracting the acid-insoluble value from the whole cell value) was nearly the same in all cases, demonstrating that M2 does not accumulate large amounts of 2-keto-3-deoxyoctonate in some intermediate form.

In contrast to the similarity in 2-keto-3-deoxyoctonate levels, M2 grown with and without galactose possessed little colitose and none was detected in acid-insoluble form. J5 contained substantial amounts of colitose when grown with galactose as expected. The presence of small amounts of colitose in the lipopolysaccharide of J5 when grown without galactose has also been observed by Elbein and Heath [6]. The failure to detect colitose in M2 grown with galactose strongly suggests the existence of a lipopolysaccharide biosynthetic defect in this strain, possibly responsible for the altered membrane characteristics.

Compositional analysis of extracted lipopolysaccharide

A lipopolysaccharide biosynthetic defect(s) in M2 would be reflected in the

TABLE I

ASSAY OF 2-KETO-3-DEOXYOCTONATE AND COLITOSE IN WHOLE CELLS AND IN ACID-INSOLUBLE CELL COMPONENTS

Strain	Galactose grown	nmol/$A_{530\ nm}$ unit cells			
		Whole Cells		Acid-Insoluble cell material	
		Colitose	2-Keto-3-deoxyoctonate	Colitose	2-Keto-3-deoxyoctonate
J5	—	2.3	43.3	1.7	30.8
J5	+	31.5	52.6	24.0	42.1
M2	—	0.8	41.0	0	27.5
M2	+	0.3	40.5	0	28.7

TABLE II

CHEMICAL COMPOSITION OF LIPOPOLYSACCHARIDE EXTRACTED FROM *E. COLI* J5 AND M2 GROWN IN THE PRESENCE AND ABSENCE OF GALACTOSE

Strain	Galactose grown	Molar ratio						μmol/mg lipopolysaccharide		
		Heptose	Gal	Glc	GlcN	2-keto-3-deoxy-octonate	Phosphorus	Heptose	2-keto-3-deoxy-octonate	Phosphorus
J5 [a]	—	1	0	0.55	1.11	1.38	0.78	1.1	1.52	0.86
M2 [a]	—	1	0	0.08	0.94	1.50	0.55	1.3	1.95	0.72
M2 [a]	+	1	0	0.08	0.92	1.50	0.42	1.3	1.59	0.69
M2 [b]	+	1	0.42	0.83	1.42	1.33	0.49	1.4	1.86	0.69

[a] Extraction procedure: chloroform/phenol/light petroleum

[b] Extraction procedure: hot phenol/water using as starting material the residue from extraction by procedure a of M2 cells grown with galactose.

composition of the lipopolysaccharide. Thus, lipopolysaccharide was extracted from M2 cells grown in the presence or absence of galactose. These extracted lipopolysaccharide species will be referred to as M2(+)LPS 1 and M2(−)LPS, repsectively. As a control, lipopolysaccharide was also extracted from J5 cells grown without galactose and is designated J5(−)LPS. The procedure employed extracted only rough lipopolysaccharide (lacking O antigen). The residue from the extraction of M2 cells grown with galactose was also subjected to a procedure capable of extracting smooth lipopolysaccharide (containing O antigen) as well as free O-antigen polysaccharides. The resulting lipopolysaccharide is termed M2(+)LPS 2. The yields of these various extracted lipopolysaccharide species relative to the starting dry weight of cells were: M2(−)LPS = 0.7%, M2(+)LPS 1 = 0.95%, M2(+)LPS 2 = 0.50%, and J5(−)LPS = 1.8%.

Gas-liquid chromatography was used to identify the alditol acetate derivatives of galactose, glucosamine, and heptose. Heptose, 2-keto-3-deoxyoctonate, and phosphorus were quantitatively determined by colorimetric assays of samples of the extracted lipopolysaccharide species. The composition of each extracted lipopolysaccharide is shown in Table II. The molar ratio of heptose was arbitrarily set as one and all other ratios are calculated relative to heptose concentration. In addition, quantities of heptose, 2-keto-3-deoxyoctonate, and phosphorus are given in μmol/mg lipopolysaccharide. It is difficult to compare the results shown here for J5(−)LPS with those of Elbein and Heath [6] or Leive et al. [20] since they used the hot phenol-water extraction method exclusively and removed the Lipid A moiety by mild-acid hydrolysis before performing the compositional analysis. Such hydrolysis is known to cause loss of lipopolysaccharide components other than Lipid A (e.g. 2-keto-3-deoxyoctonate) [6]. Our results indicate that core backbone regions of the extracted lipopolysaccharide have the same heptose and 2-keto-3-deoxyoctonate content but that M2 lipopolysaccharide has somewhat less phosphate associated with its core. The most striking differences were seen in the core sugars distal to the heptose. When grown without galactose, M2 lipopolysaccharide had decreased levels of glucosamine and glucose relative to J5. When grown with galactose, it appeared that M2 synthesized two types of lipopolysaccharide, one (M2(+)LPS 1) similar to M2(−)LPS and another (M2(+)LPS 2) which contained galactose and a greater amount of glucosamine than J5(−)LPS. Gas-liquid chromatographic analysis of M2(+)LPS 2 failed to reveal any additional peaks which could represent colitose, suggesting that this material contains no O-antigen polysaccharides. This data supports the hypothesis that M2 is defective in lipopolysaccharide biosynthesis even in the presence of galactose.

Discussion

The central finding of our study was that changes in outer membrane-mediated processes can be effected by relatively minor alterations in lipopolysaccharide structure. This was evidenced by the finding that while J5 and its outer membrane defective derivative, M2, possessed equal amounts of outer membrane lipopolysaccharide as measured by 2-keto-3-deoxyoctonate content, M2 outer membrane contained nine times less galactose-containing lipopolysaccharide than did J5. This lack of outer membrane galactose in M2 was not due

TABLE III

INCOMPLETE LIPOPOLYSACCHARIDE STRUCTURES AND THEIR COMPOSITIONS BASED ON FIG. 7

Lipopolysaccharide structure	mol/mol heptose				
	Heptose	Gal	Glc	GlcN	2-Keto-3-deoxy-octonate
(A) Lipid A-(KDO)$_3$-Hep(R_1, R_2 or R_3 [a])-Hep-Hep-Glc (J5 incomplete LPS)	1	0	0.56	1.0	1.28
(B) Lipid A-(KDO)$_3$-Hep-Hep-Glc	1	0	0.50	1.0	1.50
(C) Lipid A-(KDO)$_3$-Hep(R_1, R_2 or R_3 [a])-Hep	1	0	0.14	1.0	1.28
(D) Lipid A-(KDO)$_3$-Hep(R_1, R_2 or R_3 [b])-Hep-(Glc) [c]	1	0	0.08	0.88	1.25
(E) Lipid A-(KDO)$_3$-Hep(R_1, R_2 or R_3 [a])-Hep-Glc(GlcN)-Gal-Glc (J5 complete core)	1	0.43	1.0	1.43	1.29

[a] R_1 (= Hep), R_2 (= GlcN-Glc) and R_3 (= 0) present in equal porportions
[b] Side group content: R_1, 50%; R_2, 10%; R_3, 40%
[c] This glucose is present on 7% of the lipopolysaccharide molecules.

to the inability of galactose to enter the cell since the galactoside permease activity of M2 was normal. Studies of the kinetics of lipopolysaccharide translocation to the outer membrane suggested that M2 was either defective in translocating galactose-containing lipopolysaccharide from the inner membrane to the outer membrane or that M2 synthesized two types of lipopolysaccharide when grown in the presence of galactose (one which contains galactose and one which does not) resulting in an apparent translocation defect. This last conclusion was borne out by the compositional analysis of lipopolysaccharide extracted from M2 and J5.

The compositional data in Table II can be compared with the composition of the various hypothetical lipopolysaccharide structures in Table III. These hypothetical structures and their compositions are based on the wild type lipopolysaccharide structure shown in Fig. 7. In this way, the structures of the

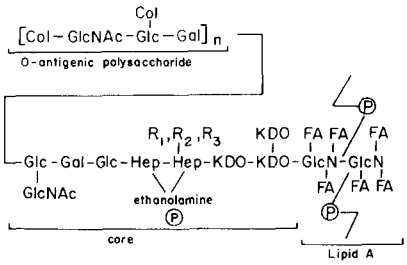

Fig. 7. Lipopolysaccharide structure of *E. coli* J5 when grown in the presence of galactose; after Morrison and Leive [35]. Col, colitose; GlcNac, *N*-acetylglucosamine; Glc, glucose; Gal, galactose; Hep, heptose; KDO, 2-keto-3-deoxyoctonate; GlcN, glucosamine; FA, fatty acid; R_1, heptose; R_2, GlcN-Glc; R_3, nothing. R_1, R_2 and R_3 are present in equal overall proportions [31].

extracted lipopolysaccharides could be deduced. As anticipated, the composition of J5(—)LPS correlates well with that of J5 incomplete lipopolysaccharide (structure A). The main change in composition seen in M2(—)LPS was a large reduction in glucose. The compositions of structures B and C in Table III reflect the loss of side group and backbone glucose, respectively. Neither change is sufficient to account for the decrease in glucose seen for M2(—)LPS in Table II. Its structure, therefore, must include loss of glucose in both the core backbone and side groups. Further, since some lipopolysaccharide in M2 can be elongated to include galactose, and since this elongation requires the presence of the first glucose residue in the core backbone, there must be some M2(—)LPS which has these glucose residues. This situation is represented by structure D in which 7% of the lipopolysaccharide molecules contain the first glucose residue in the core backbone. Other combinations of side group composition and backbone glucose are, of course, possible but structure D correlates well with the finding of a 9-fold decrease in outer membrane galactose in M2. Since in J5, only 66.6% (2/3) of the lipopolysaccharide molecules are capable of being elongated with galactose [31], a 9-fold decrease in outer membrane galactose would be reflected by 7—8% of the lipopolysaccharide molecules possessing core glucose.

Two types of lipopolysaccharide were extracted from M2 cells grown in the presence of galactose. M2(+)LPS 1 seems to be identical with M2(—)LPS and can also be represented by structure D. M2(+)LPS 2 has a composition which correlates fairly well with that of the complete lipopolysaccharide core of J5 (structure E).

These structures lead to the conclusion that in M2 the enzyme activity which adds glucose to the growing lipopolysaccharide core backbone, UDPglucose-lipopolysaccharide transferase (glucosyltransferase) [11,32] is greatly reduced. Such a reduction explains the observation that M2 displayed its membrane defectiveness both in the presence and absence of galactose since the activity of this enzyme is presumably not dependent on galactose. A glucosyltransferase defect also explains why, in the presence of galactose, M2 incorporated less of this saccharide into its lipopolysaccharide: the addition of glucose to the lipopolysaccharide core is a prerequisite for the addition of galactose and the former may occur at a rate much slower than the rate at which translocation proceeds. Thus, lipopolysaccharide molecules of structure D were translocated to the outer membrane before the glucose and galactose residues could be added. This mechanism could also explain the deceleration in translocation of galactose-containing lipopolysaccharide which was observed with M2 if first galactose addition and later glucosylation were rate limiting for translocation of this form of lipopolysaccharide to the outer membrane. Our results also suggest that translocation may not require complete lipopolysaccharide. Instead, whatever lipopolysaccharide form is available to the mechanism(s) will be translocated to the outer membrane.

While the difference in lipopolysaccharide structure between J5 and M2 is seemingly minor, especially when grown in the absence of galactose (structures A and D in Table III) this small change could be of physiological significance. In this regard, Inouye [33] has proposed that lipopolysaccharide may control the specificity and extent of outer membrane permeability by regulating the

passage of molecules through channels in the outer membrane formed by complexes of lipoprotein.

The lipopolysaccharide analyses also showed that M2 lipopolysaccharide is less phosphorylated than J5 lipopolysaccharide. This could result in less cross-linking of lipopolysaccharide molecules [34] and hence a destabilized outer membrane.

In view of the co-operative nature of membrane structure and function the possibility exists that the membranes of M2 may also be altered in lipid or protein composition. Analysis of the total phospholipids of M2 and J5 revealed that these strains possess essentially the same phospholipid composition. Thus, a gross change in this membrane component is unlikely although the analysis performed in this study would not detect minor changes which may, nonetheless, be of physiological significance. Analysis of the membrane proteins of M2 and J5 is currently underway in our laboratory. Preliminary experiments indicate no major qualtitative changes but we have not eliminated the possibility of quantitative differences.

In summary, we conclude that the structure of lipopolysaccharide molecules is of central importance to the function of the gram-negative outer membrane as permeability barrier. A small alteration in structure, such as the loss of one core sugar residue from 60% of the lipopolysaccharide molecules apparently causes a breakdown of this barrier. While possible alterations in the outer membrane proteins of M2 could be of additional physiological significance, this study has illustrated the degree of structural specificity upon which membrane-mediated cellular functions rely.

Acknowledgements

The authors would like to thank Drs. R.J. Erickson, R. Bretthauer, and J.M. Shively for helpful discussions. C.J.P. gratefully acknowleges support from the Alfred P. Schmitt Foundation of the University of Notre Dame. The work described here was in partial fulfillment of the requirements for the Ph.D degree at the University of Notre Dame for C.J. Pazoles. This work was supported in part by Biomedial Sciences Support Grant FR/RR-0733-08 from General Research Resources, Bureau of Health Professions and Manpower Training, National Institutes of Health.

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