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Isolation and Properties of Complement-Resistant Strains of *Escherichia coli* K-12

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Several strains that were resistant to the bactericidal action of antibody and complement were isolated from *Escherichia coli* K-12 W3110/SM by selecting them through the medium containing antiserum and complement. They can be agglutinated by antiserum against the parent strain and showed similar immune adherence reactivity to the parent when sensitized with this antiserum. Few differences were found in the compositions of phospholipids and proteins between both inner and outer membranes of these strains and those of the parent. However, there were fewer short-chain and more long-chain fatty acids in these strains than in the parent. It was also found that unsaturated fatty acids decreased and saturated and cyclopropanoic acids increased in phosphatidylethanolamine and phosphatidylglycerol in both inner and outer membranes of one of these strains when compared with those from the parent. Therefore, the resistance of these strains to the complement-mediated bactericidal action was considered to be due to the rigidity of their membrane structures, which might repel the insertion of membrane-attack complement complex C5b-9, although they could fix the earlier complement components up to the step of the formation of $\overline{C4b,2a,3b}$ complex enzyme.

When the sensitized *Escherichia coli* or liposomal model membrane is attacked by complement, membrane phospholipids are liberated into the surrounding medium in an amount proportional to the number of lesions formed (7, 8, 10, 12, 13). This release of membrane phospholipids is considered to be due to the displacement by the membrane-attack complement complex C5b-9, when this is inserted into the phospholipid bilayer, as proposed by Mayer as the doughnut-model hypothesis (16).

In this report we will show that the complement-resistant strains isolated from *E. coli* K-12 W3110/SM have a less-fluid membrane structure due to a shift of fatty acid composition than that of the parent, although they can react with early acting complement components up to the C3b fixation step similar to the parent.

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MATERIALS AND METHODS

Bacteria. *E. coli* K-12 strain W3110/SM was obtained by selecting spontaneous mutants of *E. coli* K-12 W3110 (21). It can grow in medium containing streptomycin up to a concentration of 500 μg/ml. Bacteria were grown in Trypticase soy broth (TSB) (Baltimore Biological Laboratory, Cockeysville, Md.) or tris(hydroxymethyl)aminomethane (Tris) glucose medium (4) containing 0.1% glucose (TGM) instead of the 0.2% glucose described originally. They were labelled by growing them in Tris glucose medium containing 0.03% cold glucose plus 0.5 μCi [*U*-^{14}C]-glucose (specific activity, 101 mCi/mmol) per ml. The bacterial strains were maintained on TSB agar slants. The complement-resistant strains isolated were maintained in a frozen state in 50% glycerol in TSB at −70°C.

E. coli K-12 W3110 (λ) was used for the production of bacteriophage λ. For the induction and assay of λ phage, tryptone broth, consisting of 10 g of tryptone (Difco Laboratories, Detroit, Mich.) and 2.5 g of NaCl per liter of distilled water, pH 7.0, and tryptone agar were used.

Proteus vulgaris HX 19 was kindly supplied from the Laboratory of Culture Collections of the Institute for Microbial Diseases, Osaka University, Suita, Osaka.

Buffer. Physiological saline containing 0.005 M Tris-hydrochloride buffer, pH 7.3, 0.15 mM $CaCl_2$, and 1.0 mM $MgCl_2$ (TBS^{2+}) was used for washing and suspending bacteria and for diluting reagents.

Antiserum and complement. Rabbit antiserum against heat-killed (60 min at 56°C) *E. coli* K-12 W3110 was prepared and inactivated by heating it for 60 min at 56°C. It was frozen and stored at −20°C without any preservative.

Guinea pig complement serum was collected from more than 100 animals. The sera were pooled and

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centrifuged at 30,000 rpm for 60 min, and the floating lipid layer was removed (10). The serum was absorbed in the cold with packed *E. coli* K-12 W3110/SM, corresponding to 2.0×10^{10} bacteria per ml of serum, which had been killed by heating for 60 min at 56°C and then washing thoroughly with TBS^{2+}. The complement and the absorbed complement (C_{abs}) were frozen and stored at −70°C. The hemolytic activity of the complement in the original serum was 230 50% hemolytic complements per ml, which dropped to 204 50% hemolytic complements per ml after absorption, when estimated by the standard method of Mayer (15). The complement was inactivated by heating it for 60 min at 56°C (Δcomplement or ΔC_{abs}). The sterilization of the antiserum and complement was performed by filtering the diluted mixture of antiserum and complement through a detergent-free Micro Filter (FM030; Fuji Photo Film Co., Ltd., Tokyo).

Immune bactericidal assay. For screening the resistant strains isolated, a modification of the immune bactericidal microassay (12, 21) was used. Briefly, a 4-drop (4× 0.025 ml) reaction mixture in TBS^{2+} was put in each well of a microtiter plate (Cooke Engineering Co., Alexandria, Va.). In the reaction mixture the concentration of one or two (for obtaining a checkerboard pattern) out of the three reactants, bacteria, complement, and antiserum, was varied serially. After the plate was incubated for 60 min at 37°C on a vibrator (Micro Mixer; Taiyo Bussan Co., Ltd., Tokyo), each reaction mixture received 1 drop of 1 mg of streptomycin per ml dissolved in fivefold-concentrated TSB. The plate was incubated further overnight at 37°C without vibration. The growth of bacteria was graded from 4 (full growth) to 0 (no growth).

IA assay. For the immune adherence (IA) assay, a 4-drop reaction mixture containing a serial dilution of C_{abs}, a dilution of antiserum, and 5×10^6 bacteria was put in each well of a microtiter plate. A control series containing no antiserum was also included for each bacterial strain for the control on C_{abs} only. After the plate was incubated for 60 min at 37°C on a vibrator, the reaction mixture received 1 drop of the suspension containing 2.5×10^6 washed human erythrocytes (type O). The plate was vibrated for 15 min at 37°C and left stationary for 60 min at 37°C before reading. IA hemagglutination was graded from 4 (strongest agglutination, covering the entire bottom of the well) to 0 (no agglutination).

Phage assay. A logarithmic-phase culture of *E. coli* K-12 W3110(λ) grown in tryptone broth at about 2×10^8 bacteria per ml was induced for λ phage by the addition of mitomycin C (Sigma Chemical Co., St. Louis, Mo.) at a concentration of 1.0 μg/ml. The culture was incubated further for 120 min at 37°C and then diluted serially. A 0.1-ml amount of the 10^{-6} or 10^{-8} dilution was added to 5 ml of melted soft tryptone agar containing *E. coli* K-12 W3110/SM or each of the complement-resistant strains isolated on a tryptone agar plate containing 250 μg of streptomycin per ml. After solidification, the plates were incubated overnight at 37°C before counting the plaques.

Preparation of outer and inner membranes. The membranes were prepared from ^{14}C-labeled bacteria by spheroplasting them with lysozyme-ethylenediaminetetraacetic acid followed by hypoosmotic burst and sonic treatment and separated by a linear or stepwise sucrose density gradient centrifugation (18–20). The preparations obtained were confirmed by electron microscopy.

SDS-PAGE. For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the discontinuous SDS buffer system of Laemmli (14) was used. The separating gel consisted of 9% acrylamide on 0.375 M Tris-hydrochloride buffer, pH 8.8, containing 0.1% SDS (Pierce Chemicals Co., Rockford, Ill.), and the stacking gel contained 5% acrylamide in 0.125 M Tris-hydrochloride buffer, pH 6.8, containing 0.1% SDS. The slab gel (1 or 2 mm thick) was polymerized by adding 0.025% (vol/vol) *N,N,N′,N′*-tetramethylethylenediamine (Nakarai Chemicals, Ltd., Kyoto) and 0.06% (wt/vol) ammonium persulfate (Nakarai Chemicals, Ltd., Kyoto) to acrylamide-*N,N′*-methylenebisacrylamide (30:0.8). The samples were treated with 1% SDS and 1% β-mercaptoethanol overnight at room temperature, followed by boiling for 2 min. A 15-μl amount of the treated samples was applied to each of the wells. The electrode buffer was 0.025 M Tris-hydrochloride buffer containing 0.192 M glycine and 0.1% SDS, pH 8.3. Electrophoresis was carried out at room temperature for 2 to 3 h at a constant current of 25 mA (1). After electrophoresis, the gel slab was removed from the sandwich, fixed, and stained with 0.2% Coomassie brilliant blue (Nakarai Chemicals, Ltd., Kyoto) in 50% methanol-7% acetic acid. It was then swollen in 7% acetic acid and dried on a piece of paper. Autoradiography was carried out by exposing the dried gel stuck on the filter paper to Fuji X-ray film (Medical Kx; Fuji Photo Film Co., Ltd., Tokyo) for 5 to 7 days.

TLC of lipid. For thin-layer chromatography (TLC), the lipids were extracted by the method of Bligh and Dyer (3). They were chromatographed on aluminum-sheet silica gel (no. 5553; E. Merck AG, Darmstadt, Germany) with a chloroform-methanol-water mixture (65:25:4, by volume) or on Silica Gel G (E. Merck AG) on a glass plate with a chloroform-methanol-acetic acid-water mixture (100:20:12:5, by volume). The autoradiogram was prepared similarly to that described above. The separated phospholipids were obtained by detection with iodine vapor. After the iodine was removed by sublimation from the marked spots, the gel in each spot was scraped off and then extracted with a chloroform-methanol mixture (1:2, by volume, for phosphatidylethanolamine [PE] and 1:4 for phosphatidylglycerol [PG]).

Gas-liquid chromatography of fatty acids. The fatty acids of lipid fractions extracted from the membrane preparations or of each lipid separated by TLC were transmethylated with benzene-methanol-H_2SO_4 (10:20:1, by volume) under reflux for 60 min. The fatty acid methyl esters were then extracted with *n*-hexane (17).

Gas-liquid chromatography of the methyl esters was performed with a Hitachi gas chromatograph (063 FID). A stainless-steel column tube (2 m by 3 mm) packed with 15% ethylene glycol succinate polymer on Chromosorb W (80 to 100 mesh) was used. The column temperature was 190°C. N_2 (0.7 kg/cm^2) was used as the carrier gas.

Detection of bacterial phospholipase A. The

detection of bacterial phospholipase A was performed by indirect hemolysis in which sheep erythrocytes were incubated with lecithin and the bacteria were treated with colistin lysozyme as described previously (6).

Materials. [U-^{14}C]glucose was purchased from Daiichi Pure Chemicals, Tokyo. Acrylamide was obtained from Seikagaku Kogyo Co., Ltd., Tokyo, and N,N'-methylenebisacrylamide was from Wako Pure Chemicals Co., Ltd., Osaka.

RESULTS

Isolation of the complement-resistant strains from *E. coli* K-12 W3110/SM. *E. coli* K-12 W3110/SM, grown in TSB overnight at 37°C, was diluted with TGM to 10^{-3} or 10^{-4}. One volume of the dilution was mixed with 4 volumes of the mixture in TGM containing a 1:600 dilution of antiserum and a 1:12 dilution of complement, which had been sterilized by passage through a detergent-free membrane filter as described above. After incubation at 37°C for 1 to 3 days, tubes showing bacterial growth were selected. The bacteria were grown in TSB and treated again with antiserum and complement as described above. After a few blind passages in antiserum and complement, the bacteria grown were streaked on TSB agar plates containing 250 μg of streptomycin per ml. The isolated strains were again treated serially with antiserum and complement, and the strains isolated from them were selected by the immune bactericidal microassay as described above. Five strains were finally selected after the 5 to 15 passages in antiserum and complement (Table 1). Theoretically, the last wells of series B in this table received only about seven bacteria, which could survive and grow in cases of the resistant strains.

These strains have shown the agglutination by the antiserum against the parent strain to give the same or a similar titer as that of the parent strain. They are all sensitive to bacteriophage λ and gave plating efficiencies similar to that of the parent.

It has been shown that phospholipase A, either serum derived or bacterial, does not participate in the formation of complement-mediated lesions on the bacterial surface (6, 10, 12). It also has not been observed that these complement-resistant strains are deficient in their phospholipase A.

IA reactivity of the complement-resistant strains sensitized with the antibody against the parent strain. These strains were resistant to the killing action of the complement. Nevertheless, they reacted with the complement and gave a similar IA reactivity when they were sensitized with the antiserum against the parent strain. This reactivity was not due to the natural antibody to the new or revealed antigen(s) on the individual strain, which might have left after absorbing the complement serum with the parent strain (C_{abs}), because they gave almost no IA reactivity and showed similar patterns as the unrelated *Proteus vulgaris* when they reacted with C_{abs} without antiserum to the parent (Table 2). Therefore, the resistant strains isolated were still reacting with complement, at least to the step of C3b fixation on their cell surface similar to the parent strain.

Phospholipid composition in the inner and outer membranes of the complement-resistant strain. Inner and outer membranes were isolated from ^{14}C-labeled bacteria of each strain by sucrose density gradient centrifugation of the disrupted spheroplasts (18–20). Lipid frac-

TABLE 1. *Immune bactericidal microassay for E. coli K-12 W3110/SM and its complement-resistant strains*[a]

Series	Strain	Bactericidal activity at dilution of bacteria of:											
		2^1	2^2	2^3	2^4	2^5	2^6	2^7	2^8	2^9	2^{10}	2^{11}	2^{12}
A	W3110/SM	4	4	3	0	0	0	0	0	0	0	0	0
	CR 1	4	4	4	4	4	4	4	4	4	4	4	4
	CR 2	4	4	4	4	4	4	4	4	4	4	4	4
	CR 3	4	4	4	4	4	4	4	4	4	4	4	4
	CR 4	4	4	4	4	4	4	4	4	4	4	4	4
	CR 5	4	4	4	4	4	4	4	4	4	4	4	4
B	W3110/SM	0	0	0	0	0	0	0	0	0	0	0	0
	CR 1	4	4	4	4	4	4	3	3	3	3	2	2
	CR 2	4	4	4	4	4	4	3	3	3	3	2	2
	CR 3	4	4	4	4	4	4	3	3	3	3	2	2
	CR 4	4	4	4	4	4	4	3	3	3	3	2	2
	CR 5	4	4	4	4	4	4	3	3	3	3	2	2

[a] The 4-drop reaction mixture in each well contained a 1:12 dilution of complement, a dilution of antiserum, and 1 drop of the bacterial suspension diluted serially from 2×10^9 bacteria per ml (A) or from 1×10^6 bacteria per ml (B). See the text for details.

TABLE 2. *IA reaction of complement-resistant strains from E. coli K-12 W3110/SM*[a]

Strain	Anti-*E. coli* K-12 antiserum	IA at concn of C_{abs} (×1/8) of:							
		2^0	2^{-1}	2^{-2}	2^{-3}	2^{-4}	2^{-5}	2^{-6}	2^{-7}
E. coli K-12 W3110/SM	1:360	2	3	3	4	4	3	1	0
	1:720	2	3	3	4	4	3	1	0
	0	1	1	1	1	0	0	0	0
CR 1	1:360	2	3	3	4	4	3	1	0
	1:720	2	3	3	4	4	3	1	0
	0	1	1	1	1	0	0	0	0
CR 3	1:360	2	3	3	4	4	3	1	0
	1:720	2	3	3	4	4	3	1	0
	0	1	1	1	1	0	0	0	0
P. vulgaris HX 19	1:360	1	1	1	1	0	0	0	0
	1:720	1	1	1	1	0	0	0	0
	0	1	1	1	1	0	0	0	0

[a] The 4-drop reaction mixture in each well contained 5×10^6 bacteria, C_{abs}, and antiserum. The concentrations of the latter two are indicated. The plate was incubated for 60 min at 37°C before adding the human erythrocytes. See the text for details.

tions were extracted from them and analyzed by TLC (Fig. 1). It was found, however, that there is little difference in lipid compositions of both membranes between the complement-resistant strains and the parent. Apparent differences of the intensities of the spots between these strains shown in Fig. 1 might be due to the difficulty in measuring the lipid extracted from each membrane fraction. The ratios of the intensities of the spots in each fraction were similar in the same kind of membrane fraction.

Membrane proteins of the complement-resistant strains. Membrane fractions of the complement-resistant strains were treated with β-mercaptoethanol and SDS and analyzed by SDS-PAGE (Fig. 2). Under the conditions employed, both membrane fractions from each strain were resolved into 30 to 40 protein bands. However, it was again found that there was little difference in the membrane protein composition between the complement-resistant strains and the parent.

Fatty acid composition of the membrane phospholipids. Fatty acid compositions in the lipid fraction extracted from both the inner and outer membrane preparations of these strains were analyzed by gas-liquid chromatography (Table 3). Both membranes of the complement-resistant strains possessed fewer short-chain acids and more long-chain acids than the parent strain. The membrane consisting of the longer fatty acids could be expected to be harder than that consisting of the shorter ones.

The difference of the fatty acid composition was more obvious when those in PE and PG, main membrane phospholipids, were compared (Table 4). In those phospholipids of a complement-resistant strain, CR 3, unsaturated fatty acids (especially 18:1), which made the membrane more fluid, decreased and converted to the saturated fatty acids (16:0, 18:0) or cyclopropanoic acids (17:0▽, 19:0▽), both of which made the membrane more rigid.

These results suggest that the complement-resistant strains have more rigid membranes than the parent strain and repel the insertion of the membrane-attack complex C5b-9 into them.

DISCUSSION

Sensitivity of a gram-negative bacterium to complement-mediated bactericidal action is considered to be governed by various factors, e.g., capsular material or K antigen, which blocks the access of complement components to the membrane; the variation of antigens on the membrane, which requires the different antibodies to fix complement; and the distribution or number of antigenic sites on the membrane, which might cause the different efficiency of antibody to fix complement. The complement-resistant strains described in this paper can be agglutinated by the antiserum against the parent strain and even give the same immune adherence reactivity as the parent strain when they are sensitized with this antiserum. Therefore, these strains can fix early reacting complement components up to the C3b step similar to the parent. They are, nevertheless, refractory to the killing action of the complement and are considered to resist the insertion of C5b-9 complexes into their surface membrane.

When the lipid compositions of both inner

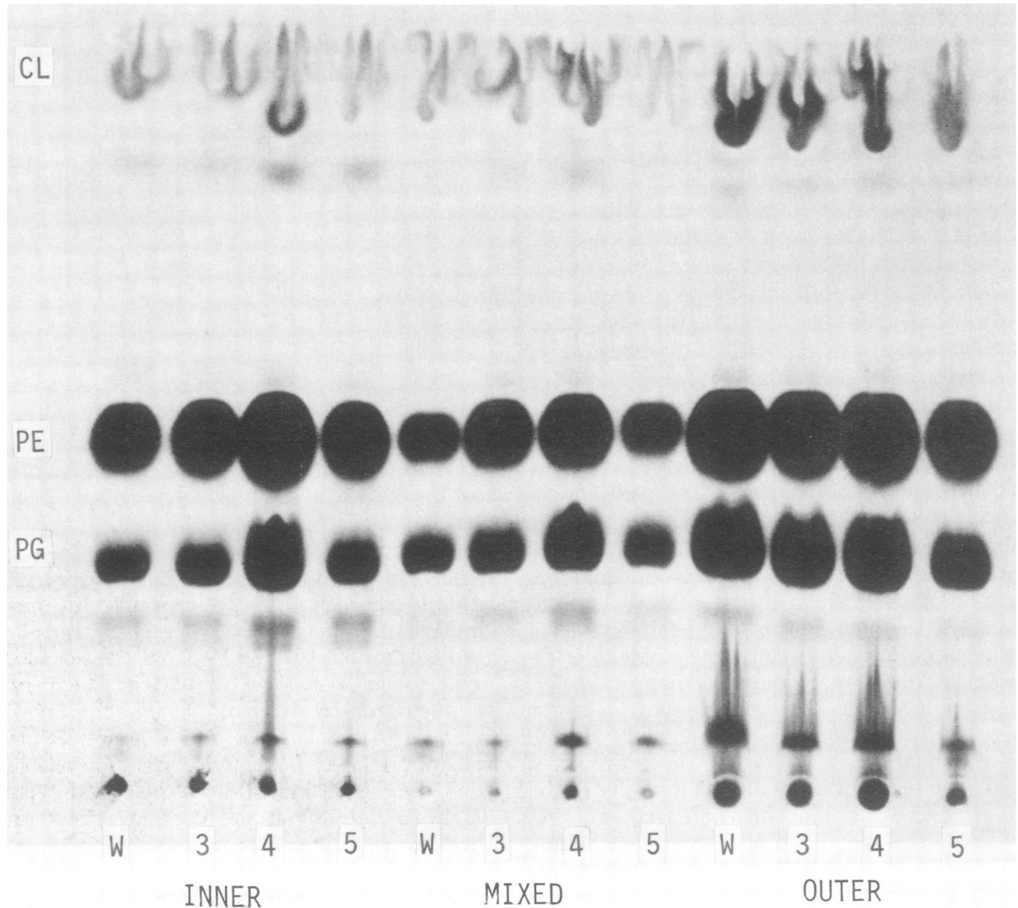

FIG. 1. *Autoradiogram of TLC of lipid fractions extracted from inner and outer membrane fractions and from the fraction containing both membranes of E. coli K-12 W3110/SM and its complement-resistant strains. Abbreviations: INNER, inner membrane fraction; OUTER, outer membrane fraction; MIXED, fraction obtained as a band between inner and outer membrane bands by sucrose density gradient centrifugation, corresponding to the band M described by Osborn et al. (18); W, parent strain; 3, CR 3; 4, CR 4; 5, CR 5; CL, cardiolipin. Chromatography was performed on aluminum sheet silica gel with chloroform-methanol-water (65:25:4, by volume).*

and outer membrane preparations of the complement-resistant strains were compared with those of the parent strain by TLC, there was little difference between them (Fig. 1). There was also little difference between them in the protein compositions of both the membranes when they were compared by SDS-PAGE (Fig. 2).

However, it was found that there were significant differences in the fatty acid composition of these strains. The complement-resistant strains possess fewer short-chain and more long-chain fatty acids. Moreover, when the fatty acid composition in individual phospholipids was compared, less in unsaturated fatty acids and more in saturated and cyclopropanoic acids were found in PE and PG of the resistant strain. These shifts in fatty acid composition make the membrane more rigid. Therefore, the resistance of the isolated strains to complement-mediated bactericidal action is considered to be due to the rigidity of their membrane structures, which might repel the insertion of the membrane-attack unit complex C5b-9 into the membrane phospholipid bilayer (8, 13, 16). Similar correlation has recently been observed between the fatty acid composition of *E. coli* strain B, which has grown under various conditions and its susceptibility to the bactericidal action of the complement (M. Okada and K. Inoue, in preparation).

We have shown that antibody and complement can kill *E. coli* in the absence of lysozyme, which is necessary to convert the bacteria into

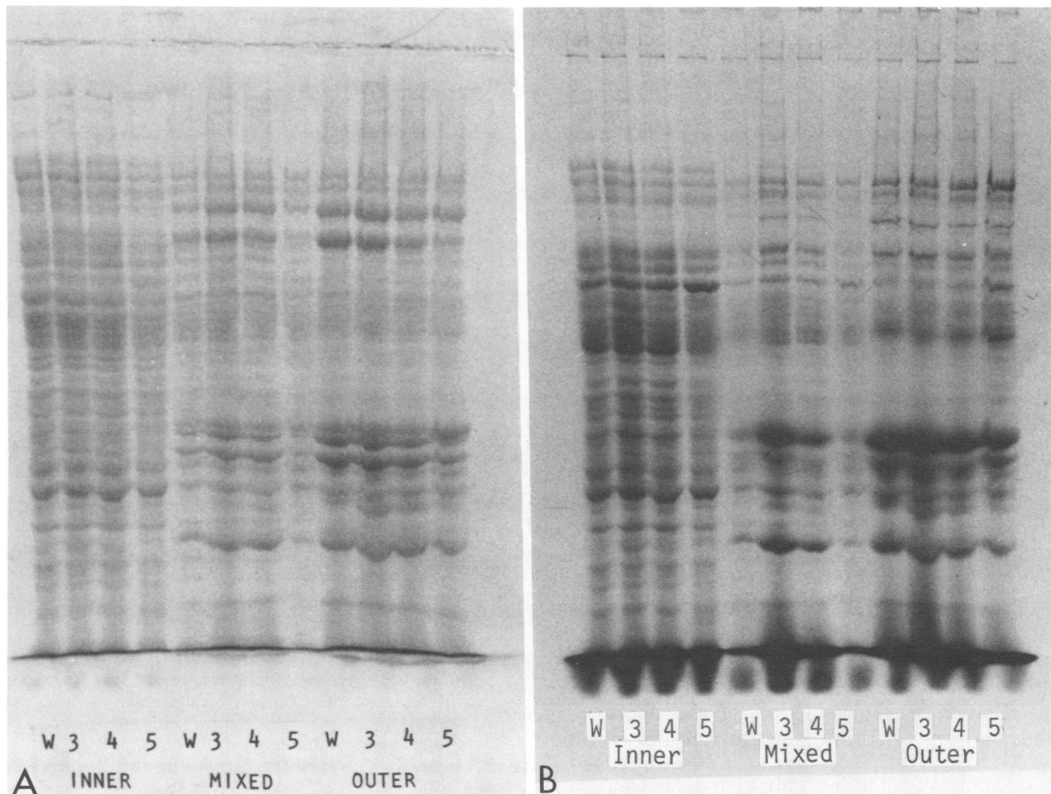

FIG. 2. *SDS-PAGE and its autoradiogram of the inner and outer membrane fractions and the fraction containing both membranes of E. coli K-12 W3110/SM and its complement-resistant strains. (A) Fixed, stained, and dried gel slab; (B) its autoradiogram. See the legend of Fig. 1 for abbreviations.*

TABLE 3. *Percent fatty acid compositions of inner and outer membranes of E. coli K-12 W3110/SM and its complement-resistant strains*

Acid	Fatty acid composition (%)							
	Inner membrane				Outer membrane			
	W[a]	CR 3	CR 4	CR 5	W	CR 3	CR 4	CR 5
14:0	4.4	2.8	2.5	2.6	5.7	3.9	3.7	4.5
16:0	53.7	47.3	45.0	44.9	50.0	44.0	48.6	48.2
16:1	1.8	2.9	3.1	3.4	2.3	2.3	2.2	2.3
17:0▽	30.9	29.2	28.0	28.8	28.2	27.7	30.2	27.6
18:0	TR[b]	0.7	1.1	1.0	0.3	1.2	1.0	0.8
18:1	2.2	7.0	8.9	5.5	3.2	6.9	6.4	3.4
19:0▽	6.2	6.6	7.9	9.7	5.2	7.9	4.5	8.1

[a] W, Parent strain, W3110/SM.
[b] TR, Trace.

spheroplasts (5, 11). We have also demonstrated that antibody and complement make "channels" or "holes" in the cell wall but not in the cytoplasmic membrane in the absence of lysozyme. Periplasmic enzymes, such as alkaline phosphatase, leak out through these channels, but intracellular enzymes, such as β-D-galactosidase, are not released from the bacteria unless lysozyme or plakin (β-lysin) is present in the reaction mixture (5, 9). The present experiments show that the shift in the fatty acid composition of the complement-resistant strains to that of a more rigid membrane structure has occurred not only in the outer membrane but also in the inner (cytoplasmic) membrane. The enzymes of phospholipid synthesis have been shown to lo-

TABLE 4. *Percent fatty acid compositions of PE and PG in inner and outer membranes of E. coli K-12 W3110/SM and its complement-resistant strain, CR 3*

Acid		PE (%)				PG (%)			
		Inner		Outer		Inner		Outer	
		W[a]	CR 3	W	CR 3	W	CR 3	W	CR 3
Myristic	14:0	3.4	2.0	2.7	3.2	1.6	0.6	1.3	3.9
Palmitic	16:0	44.9	44.9	38.3	45.6	31.1	44.3	31.2	45.7
Palmitoleic	16:1	6.5	0.9	1.1	1.1	1.3	1.3	1.3	2.7
cis-9,10-Methylene hexadecanoic	17:0▽	17.7	31.8	19.8	26.3	5.7	15.0	7.7	16.5
Stearic	18:0	0.9	1.2	2.1	3.6	5.0	0.7	3.5	5.5
cis-Vaccenic	18:1	23.7	11.4	32.2	15.5	53.5	32.0	51.4	20.0
Lactobacillic	19:0▽	2.9	7.7	3.9	4.7	1.8	6.2	3.7	6.3

[a] W, Parent strain, W3110/SM.

calize in the cytoplasmic membrane (2, 22). The incorporation of phospholipids into the outer membrane should require the translocation from the sites of synthesis. The shift in the fatty acid composition of both membranes might be due to the general shift of the fatty acid synthesis in these resistant strains, although the compositions in phospholipids and fatty acids of these membranes are different.

These complement-resistant strains were isolated by repeated selection of the bacteria in the medium containing antiserum and complement. These strains might be spontaneously occurring mutants and could be kept in the usual culture medium for several passages. However, their properties of resistance to complement action were gradually lost when they were maintained repeatedly in the usual medium. Back mutants might easily occur that would overgrow and replace the mutants themselves after several passages in the usual culture medium.

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