Analysis of Outer Membrane Components of *Escherichia coli* ML308 225 and of a Serum-Resistant Mutant

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Escherichia coli ML308 225 in the early exponential phase of batch cultures was killed by human serum in a complement-dependent reaction. Under the same conditions, a mutant derived from this strain by multiple subcultures in human serum was resistant to killing by serum. The components of the outer membrane of both the parent and mutant strains were analyzed. The protein and phospholipid analyses were identical, but the mutant strain produced twice as much lipopolysaccharide as the parent strain. These results suggest that the extent of bacterial surface coverage by lipopolysaccharide determines the degree of sensitivity to serum.

The outer membrane is the peripheral component of the gram-negative bacterial cell envelope and as such might be expected to strongly influence the degree of bacterial sensitivity to bactericidal and bacteriolytic agents such as serum complement. The role of one constituent of the outer membrane is already well known; there is a positive correlation between the lipopolysaccharide (LPS) content and sensitivity to serum (23). In addition, defects in LPS synthesis are responsible for so-called "rough" mutants which are more susceptible to serum (4, 14). Outer membrane proteins have been shown to be antigenic (7) and as such would interact with specific antibodies and indirectly stimulate serum complement activation. The phospholipid composition of membranes determines their fluidity and their susceptibility to complement action, although both positive and negative correlations between membrane fluidity and the bactericidal action of complement have been reported in *Escherichia coli* and *Acholeplasma laidlawii*, respectively (6, 12).

E. coli ML308 225 is sensitive to serum complement in the early and midexponential growth phases. We have isolated a complement-resistant mutant of this strain which is morphologically indistinguishable from the parent strain and which shows similar antigenic specificity. On this basis the mutation is apparently not the reversion of a rough serum-sensitive strain to a "smooth" serum-resistant strain with a complete polysaccharide structure in the outer membrane LPS. We have quantitatively and qualitatively examined the components of the outer membrane of both the mutant and parent strains in an attempt to account for the complement resistance of the mutant.

MATERIALS AND METHODS

Growth of bacteria. *E. coli* ML308 225 was a gift from W. A. Hamilton of the Department of Biochemistry, University of Aberdeen. It was maintained on nutrient agar slopes. Liquid cultures were grown at 37°C in minimal salts medium containing 0.5% (wt/vol) glucose. Growth was monitored by reading the absorbance at a wavelength of 650 nm, and cultures were harvested by centrifugation.

Sera. Human sera were obtained fresh from healthy volunteers and from outdated blood supplied by the Auckland Regional Blood Transfusion Center. Bactericidally active sera were pooled and stored frozen at -80°C. This material was used without further treatment as a source of human serum complement. Antibacterial antiserum was prepared by multiple subcutaneous injection of rabbits with heat-killed cultures of *E. coli* ML308 225, followed by bleeding from the marginal ear vein. The serum was tested for its ability to agglutinate bacterial suspensions. It was serially diluted in samples of 0.05 ml, and 0.05-ml samples containing approximately 10^8 bacteria were added.

Antiserum to human serum complement component C9 was purchased from Hoechst, Auckland, New Zealand, and was heated to 56°C for 30 min. Human serum (1 ml) was treated with 0.1 ml of the antiserum at 4°C for 2 h and was then centrifuged. This treatment was repeated once. Control samples were incubated with two samples of heated rabbit serum. Serum depleted of C9 did not form a precipitin line with this antiserum in double-diffusion analysis.

Assay of bacterial killing and lysis. Freshly harvested bacteria were suspended in the assay buffer, which was 30 mM Tris-hydrochloride (pH 7.5) containing 0.15 mM calcium chloride and 0.02 mM magnesium sulphate. Assay mixtures contained up to 0.2 ml of serum. The total volume was 1.0 ml, and the bacterial suspension was adjusted to yield approximately 2.5×10^8 bacteria in each assay mixture. Assay mixtures were incubated at 37°C for up to 60 min. Viable counts were made with serially diluted samples spread on nutrient agar plates, followed by overnight

incubation at 37°C. Results are expressed as the percentage of bacteria surviving after a given time.

Isolation of a serum-resistant mutant. A sample (0.1 ml) from an overnight batch culture in minimal glucose medium was used to inoculate 5.0 ml of sterile human serum. This culture was incubated overnight at 37°C and was subcultured by transferring 0.1 ml to 5.0 ml of fresh, sterile human serum; this procedure was repeated 40 times in total. The final subculture was used to inoculate a minimal glucose batch culture. When the organisms were in the early exponential growth phase (absorbance at 650 nm [A_{650}] of 0.15), serum sensitivity was tested as described above. This culture was also plated out on nutrient agar, and 12 single-colony isolates were tested for serum sensitivity.

A serum-resistant culture of one of the single-colony isolates was used to prepare an antiserum as previously described.

Purification and characterization of outer membrane components. Outer membrane proteins were extracted, measured, and electrophoretically analyzed as previously described (3).

LPS was extracted and purified from freshly harvested or cold acetone-dried bacteria as previously described (3a).

Samples of 2.0 ml of the pools of purified LPS were each mixed with an equal volume of 2% (vol/vol) acetic acid and incubated at 100°C for 4 h. The resulting precipitates were washed twice with distilled water and dried in vacuo. They were then used to prepare fatty acid methyl esters (1). These samples were subjected to gas-liquid chromatography with the Pye Unicam 104 apparatus. The column measured 1.2 m $\times$ 6 mm and was packed with Chromosorb G containing 0.5% (vol/wt) diethylene glycol succinate. The carrier gas flow was 40 ml of nitrogen per min, and the temperature program was 135°C for 1 min, followed by heating at 7°C per min to 205°C. Fatty acid methyl esters were detected in the effluent with a flame ionization detector. Standards were purchased from Sigma Chemical Co., St. Louis, Mo. The methyl ester of β -hydroxymyristic acid was identified by its relative retention time (16).

LPS samples (1 mg) were also hydrolyzed for 4 h in 0.5 ml of 6 N HCl at 100°C. Monosaccharides in the hydrolysate were qualitatively identified by silica gel thin-layer chromatography with ethyl acetate-2-propanol-water-pyridine (25:14:7:2; vol/vol) as the solvent and by descending paper chromatography with 1-butanol-water-ethanol (10:2:1; vol/vol) as the solvent (22).

Purified LPS was used to prepare a rabbit antiserum. A solution of 440 μ g of LPS in 1.0 ml of water was mixed with 0.5 ml of complete Freund adjuvant (Difco Laboratories, Detroit, Mich.). Multiple subcutaneous injections (0.3 ml total) were carried out at intervals of 2 weeks, and serum was obtained after bleeding from the marginal ear vein.

Phospholipid was extracted from whole bacteria. The cells harvested from 101 (A_{650} of 0.15) and 51 (A_{650} of 0.9) minimal glucose batch cultures were extracted, and the phospholipids were purified as described by Osborn and Rothfield (17). Samples of the purified phospholipids were prepared for analysis of fatty acid methyl esters by the same procedures as those used for lipids derived from LPS (1).

RESULTS

Bacterial growth and serum sensitivity.

A series of bactericidal experiments was performed at different stages of growth of a single bacterial culture. The initial rate of bacterial killing was measured in each case and was converted to a percentage of the initial rate of killing at an A_{650} of 0.15. These results are shown in Fig. 1. It can be seen that bacterial killing was very slow for organisms in the initial lag before growth commenced. The rate of killing rose rapidly to a maximum in the early exponential growth phase and then declined more slowly as growth proceeded. Stationary-phase bacteria were completely resistant to the action of serum complement under the assay conditions used. For the remainder of the bactericidal experiments reported here, bacteria were harvested at an A_{650} of 0.15. The depletion of complement component C9 completely inhibited bacterial killing, and in fact there was a slight increase in the viable count of bacteria after 60 min, indicating that the organisms utilized the serum for growth.

The minimal glucose batch culture inoculated from the serum subcultures was found to retain more than 98% viability under the same conditions used for the bactericidal assay. Of 12 single-colony isolates from this culture, 10 were similarly resistant to serum when tested at an A_{650} of 0.15. One of these isolates was used for all subsequent experiments.

Immunological comparison of parent and mutant strains. Antisera were prepared against both *E. coli* ML308 225 and the mutant strains and against LPS purified from *E. coli* ML308 225. Both strains yielded identical agglutination titers of between 1:32 and 1:64 with all three antisera. After cross-absorption experiments in which 0.5 ml of each antibacterial antiserum was incubated at 4°C for 30 min with a suspension of specific bacteria (10^9 cells), the sera, upon centrifugation, both failed to agglutinate either bacterial strain.

The mutant strain was provisionally designated *E. coli* ML308 GLT. It was maintained on nutrient agar slopes and stored at -80°C. Resistance to serum in the early exponential phase of batch culture has proved stable over 18 months.

Analysis of outer membrane components of parent and mutant strains. The electrophoretic profiles of the outer membrane proteins of both strains are shown in Fig. 2. Outer membrane protein extracts were prepared from bacteria in the early exponential (A_{650} of 0.15) and late exponential (A_{650} of 0.9) growth phases of batch cultures. It is apparent that the profiles for the outer membrane proteins of the parent

FIG. 1. Variation in serum sensitivity throughout the bacterial growth cycle. Symbols: $\circ$ — $\circ$, A_{650} values for the culture; $\bullet$ — $\bullet$, initial rate of bacterial killing expressed as a percentage of the initial rate of killing at an A_{650} of 0.15.

FIG. 2. Densitometric scanning of discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis of outer membrane proteins. The anode is to the left in each case. (A) *E. coli* ML308 225 harvested in the early exponential growth phase. (B) *E. coli* ML308 225 harvested in the later exponential growth phase. (C) *E. coli* ML308 GLT harvested in the early exponential growth phase. (D) *E. coli* ML308 GLT harvested in the late exponential growth phase.

strain. The central triplet in these profiles (3, 20) has been resolved into four bands by some workers (15). It is thus possible that the parent and mutant strains vary with respect to the additional minor component.

Total outer membrane protein from bacteria from the early exponential growth phase represented 5.4% of dry weight in the ML308 225 strain, whereas for organisms from the late exponential growth phase this figure was 2.4%. The corresponding figures for the ML308 GLT mutant strain were 5.1 and 2.0%, respectively.

A significant difference was found when the quantitative recoveries of LPS from the two strains were compared (Table 1). In both strains the LPS concentration fell as growth proceeded through the exponential phase of batch culture. However, the mutant strain contained twice as much LPS as the parent strain in the early exponential (serum-sensitive) growth phase.

Purified LPS samples from the two strains were qualitatively indistinguishable (Table 1). In each case, the fatty acids of lipid A represented about 11% of the total carbohydrate. The relative proportions of lauric, myristic, and β -hydroxymyristic acids were similar to those reported for other species of *Enterobacteriaceae* (14). However, there was little palmitic acid even in samples from bacteria harvested in the late exponential growth phase. In the corresponding samples from bacteria harvested in the early exponential growth phase, only trace amounts of palmitic acid were detected. Monosaccharides in LPS hydrolysates were determined qualitatively. Mannose, glucose, galactose, fucose, and *N*-acetylglucosamine were positively identified in hydrolysates of LPS from both parent and

strain are similar to those previously reported (3, 20) and that there are no obvious differences between these and the profiles for the mutant

mutant strains. Standards corresponding to the rare monosaccharides often found in LPS (14) were not available. It is possible that some monosaccharides present in the LPS samples were destroyed by the relatively severe hydrolysis conditions used (2).

Phospholipid was extracted from whole bacteria, and only the fatty acids were determined. The relative proportions of fatty acids in total phospholipid extracts may not exactly correspond to those of the phospholipids in the outer membrane but the variation is likely to be small (13). There were no significant qualitative or quantitative differences between the two strains (Table 2). Similar results have been reported for *E. coli* ML30 (16).

DISCUSSION

E. coli ML308 225 in the early exponential growth phase of batch culture was readily killed by human serum. This reaction was clearly complement dependent, as serum depleted of complement component C9 was not bactericidal. The reaction occurred without the addition of antibacterial antiserum. Previous results showed that natural antibodies were not necessary to the reaction (Allen and Scott, in press). As bacterial growth proceeded to the late exponential phase, sensitivity to serum declined, as has been reported for other strains of *E. coli* (8, 19).

Repeated subculturing of *E. coli* ML308 225 in sterile human serum permitted the isolation of a strain which was immunologically indistinguishable from *E. coli* ML308 225 but which was not sensitive to serum under the assay conditions used at any point in its growth cycle.

Analyses of the outer membrane proteins and of the phospholipid fatty acyl chains of both the ML308 225 and ML308 GLT strains showed no significant qualitative or quantitative differences. The LPS fractions of the two strains were also apparently qualitatively similar, but there was a significant quantitative difference. In the early exponential growth phase, the serum-resistant ML308 GLT strain contained about 7.0% LPS, whereas the serum-sensitive parent strain contained only 3.5% LPS. Although the measurements of LPS in these experiments were done by carbohydrate determinations, it is important to note that the relative proportions of fatty acids in each sample were similar. Thus, the mutation present in the ML308 GLT strain did not result in the synthesis of longer polysaccharide chains attached to normal amounts of lipid A, but caused increased synthesis of the whole LPS molecule.

At first sight, these results are at variance with accepted ideas of membrane structure. It is difficult to see how increased amounts of LPS can be incorporated into the membrane without the displacement of other membrane components. However, consistent results have been presented by Gmeiner and Schlecht (10), who have shown that rough mutants of *Salmonella typhimurium* incorporate more LPS, on a molar basis, into their outer membranes. This change was accompanied in some cases by increased phospholipid concentrations, but changes in outer membrane protein concentrations were small. These workers suggest that the outer membrane proteins may undergo a conformational change which permits the packing of more

TABLE 1. Analysis of LPSs extracted from *E. coli* ML308 225 and ML308 GLT

Bacterial strain	Exponential growth phase	A ₆₅₀ when harvested	Total LPS (% dry wt)	Lipid A (% of total LPS)	Fatty acid composition (% of total fatty acids)			
					Lauric	Myristic	Palmitic	β -Hydroxy-myristic
ML308 225	Early	0.15	3.5	10.5	18	22	1	59
	Late	0.9	2.0	11.7	16	20	9	55
ML308 GLT	Early	0.15	7.0	11.0	17	22	2	59
	Late	0.9	3.6	11.3	17	21	7	55

TABLE 2. Analysis of phospholipids from *E. coli* ML308 225 and ML308 GLT

Bacterial strain	Exponential growth phase	A ₆₅₀ when harvested	Total phospholipid (% dry wt)	Fatty acid composition (% of total fatty acids)				
				Myristic	Palmitic	Hexadecenoic	Methylene hexadecenoic	Octadecenoic
ML308 225	Early	0.15	0.9	2	30	33	6	29
	Late	0.9	1.1	2	32	34	7	25
ML308 GLT	Early	0.15	0.9	3	34	36	4	23
	Late	0.9	1.0	3	33	35	4	25

lipid chains into the partial bilayer of the outer membrane.

Other experiments with *E. coli* ML308 225 also suggest that increased coverage of the cell surface with LPS is responsible for resistance to serum. Bacteria in the early exponential growth phase can bind added LPS so as to become serum resistant. Of the bound LPS, only about half can be removed by chemical extraction. This firmly bound LPS increases the total LPS concentration in the bacteria by about 75% (3a).

A cell-surface protein of molecular weight 15,000 which can bind LPS has been recently reported in *Salmonella minnesota* R595 (9). There is no evidence for increased concentrations of such a protein in the outer membrane extract of the resistant mutant strain, but another protein may fill the same role in *E. coli*. This comparison raises the question of how widespread among the *Enterobacteriaceae* is the phenomenon of LPS binding. In a preliminary study, we have shown that *E. coli* K-12 can be protected from serum by LPS from *E. coli* ML308 225 (R. J. Allen and G. K. Scott, unpublished data).

It seems clear that the amount of LPS in the outer membrane determines the sensitivity to serum of *E. coli* ML308 225 in the early exponential growth phase. However, we have shown that at a later stage in the growth cycle, the relatively serum-resistant bacteria have a lower LPS concentration. The value falls from 3.5 to 2.0%, and a similar decline (7.0 to 3.5%) is observed in the mutant strain. These results are comparable with the fall in outer membrane protein levels (3) and probably relate to the reported release of outer membrane fragments from *E. coli* (18). We may conclude that other factors are involved in the resistance to serum of bacteria in the late exponential growth phase of batch cultures.

There is considerable evidence for a link between metabolic activity and serum sensitivity. The killing of *Pasteurella septica* by serum is inhibited by respiratory inhibitors or uncouplers (11). Antibody and complement inhibit the uptake of iron by *E. coli* O111, and cell death follows, probably as a result of the absence of a species of transfer ribonucleic acid which is synthesized via an iron-dependent enzyme. This effect of complement can be overcome by the addition of excess ferric ammonium citrate (5). Neither of these mechanisms appears important in the serum killing of *E. coli* ML308 225. Inhibition of a wide range of metabolic and biosynthetic processes, including oxidative phosphorylation, does not inhibit complement action on *E. coli* ML308 225. Similarly, the presence of 1 mM ferric ammonium citrate does not inhibit

this bactericidal action (21). However, there may be other metabolic factors which affect serum sensitivity and which have not yet been explored. It is thus possible that *E. coli* ML308 GLT differs from its parent strain in some aspect(s) of metabolism which contributes to its relative resistance to serum.

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