Relation Between Excreted Lipopolysaccharide Complexes and Surface Structures of a Lysine-Limited Culture of *Escherichia coli*

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ABSTRACT

KNOX, K. W. (Twyford Laboratories, London, England), MARET VESK, AND ELIZABETH WORK. Relation between excreted lipopolysaccharide complexes and surface structures of a lysine-limited culture of *Escherichia coli*. *J. Bacteriol.* **92**:1206–1217. 1966.—The lysine-requiring mutant *Escherichia coli* 12408, when grown in 15 liters of defined medium containing a suboptimal amount of lysine, showed a biphasic type of growth. During a long stationary phase of 15 hr, there was a steady accumulation of diaminopimelic acid (DAP) and an antigenic complex of lipopolysaccharide (LPS) and lipoprotein; the accumulation continued unchanged until the end of the second growth phase. The rapid rate of DAP excretion suggested that it was the result of a derepressed state of a biosynthetic pathway. LPS excretion was such that the amount in the culture fluid was doubled during a period corresponding to the normal generation time for the organism; this suggested that the LPS-lipoprotein complex was a product of unbalanced growth. Surface defects were suggested by the action of lysozyme, which, in low concentrations (10 μ g/ml), lysed the lysine-limited cells even in the absence of ethylenediaminetetraacetic acid, but had no effect at 10 μ g/ml on cells grown with adequate lysine. Electron microscopy of cells excreting the LPS complex showed them to be surrounded by a mass of stacked leaflets and globules, some of which were bounded by triple membranes. Sections showed no lysis but changes in cell surfaces; outer layers of the walls had numerous blebs whose outer membranes were sometimes continuous with the outer triple membrane of the wall. LPS-lipoprotein probably originates from these blebs.

Lysine-requiring mutants of *Escherichia coli* which lack *meso*-2,6-diaminopimelic acid decarboxylase show a biphasic type of growth and excrete a number of compounds when grown under lysine-limiting conditions (16, 19, 36). Lipid-containing polymers were isolated from the culture filtrates by Bishop and Work (4), Meadow (18), and Municio et al. (19). The material isolated by Bishop and Work (4), then designated "lipoglycopeptide," was found to be related to cell walls; on further examination (28, 37), it was shown to contain a lipopolysaccharide component which was indistinguishable chemically and serologically from the lipopolysaccharide extracted from cells.

A modification of the method of Bishop and Work (4) has resulted in the isolation of a water-soluble complex which differs from lipoglycopeptide in containing greater amounts of phosphatidylethanolamine, and also a larger number of amino acids in a protein component (Knox and Work, *J. Gen. Microbiol. in press*; Knox, Cullen, and Work, *unpublished data*). Accordingly, the product present in the culture fluid will now be designated "lipopolysaccharide complex," rather than "lipoglycopeptide."

Since lipopolysaccharide is known to be a component of the cell walls of gram-negative bacteria, an electron microscopic examination was made of the cells during the various phases of growth; this showed marked changes in surface structure, which could be related to accumulation of lipopolysaccharide complex in the culture fluid. The properties of this complex were ex-

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aminated by use of immunological methods, chemical estimation, and electron microscopy.

E. coli is known to be resistant to lysozyme unless the surface structures are disorganized, for instance, by ethylenediaminetetraacetic acid (EDTA) (23). In the present case, the defects in surface structure brought about by lysine limitation appeared to lead to changes in susceptibility to lysozyme.

MATERIALS AND METHODS

Microorganism and methods of culture. The strain of *E. coli* ATCC 12408 was that used in earlier studies (4, 28). Cultures were grown at 37 C in a basal medium containing (grams per liter): $(\text{NH}_4)_2\text{HPO}_4$, 15; KH_2PO_4 , 2; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05; $\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$, 0.1; glycerol, 20; the pH was adjusted to 7.0. "Lysine-limiting" medium contained 0.06 g of L-lysine hydrochloride per liter, and "control medium" contained 0.20 g of L-lysine hydrochloride per liter. Cultures were grown at 37 C on a rotary shaker in 2-liter flasks containing 80 ml of medium, and these were used as inocula (960 ml) for cultures in a stirred fermentor (30-liter) containing 14 liters of medium with an aeration rate of 5 liters per minute. Samples were removed at appropriate intervals. Cell walls were made by the method of Allsop and Work (1). Total viable counts were made by plating 0.1 ml of appropriate dilutions of cultures onto agar containing salts, glucose, and L-lysine (0.2 g per liter); revertants were counted on the same medium without lysine. The differences between the two counts gave figures for lysine-requiring organisms.

Analytical methods. Measurements of the optical density of culture samples were made on an EEL portable colorimeter with filter OB10. Cells were removed by centrifuging at $14,000 \times g$ for 20 min, and diaminopimelic acid was determined on 0.1 ml of culture fluid by the acid-ninhydrin method (35). The amount of lipopolysaccharide complex in culture fluid was estimated after passing a suitable volume of culture fluid, which had been concentrated by vacuum distillation at 35 C, through a column of Sephadex G200 and eluting with water. The first carbohydrate-containing fraction, as determined by the phenol- H_2SO_4 method (10), contained the lipopolysaccharide complex; its total carbohydrate content was determined. The complex previously isolated by this procedure contained 18% carbohydrate, and provided a standard for calculating the amount of lipopolysaccharide complex in eluates and, therefore, in the samples of culture fluid.

The relative amounts of lipopolysaccharide complex in small samples of culture fluid were compared immunologically by use of the quantitative precipitin method with 0.05 to 0.20 ml of culture fluid and 0.5 ml of homologous antiserum; precipitated antibody protein was determined by the method of Lowry et al. (17). Heptose was determined by a modification (22) of the method of Dische (9). Amino acids, including diaminopimelic acid, were determined in hydrolysates (6 N HCl, 18 hr at 100 C) of cell walls in a Technicon amino acid analyzer.

Lipopolysaccharide complex and lipopolysaccharides. Cells were grown under lysine-limiting conditions for 26 hr, and the lipopolysaccharide complex was isolated from the culture fluid by the method of Bishop and Work (4). Extracellular lipopolysaccharide was isolated from this preparation by extraction with aqueous phenol, followed by precipitation with ethyl alcohol and magnesium ions (28).

Cellular lipopolysaccharide was extracted from cell suspensions [25 mg (dry weight) per ml] by heating in 45% aqueous phenol at 65 C for 10 min (33). After cooling and centrifuging, the aqueous phase was extracted with ether and centrifuged at $100,000 \times g$ for 2 hr. The lipopolysaccharide pellet was resuspended in water, and the heptose content was estimated. By comparison with the heptose content of a preparation of purified lipopolysaccharide (28), the amount of lipopolysaccharide extracted from the cells was calculated.

Immunology. Rabbit antisera to extracellular lipopolysaccharide were prepared by repeated intraperitoneal injections of 10- μg amounts of material in 0.9% NaCl. Conjugated antibody was made by coupling the globulin fraction of antiserum (37% ammonium sulfate precipitate) to ferritin (General Biochemicals, Chagrin Falls, Ohio), by the method of Sri Ram et al. (26). Conjugated antibody (0.5 ml) was added to 10 ml of a cell suspension of *E. coli* grown in lysine-limiting medium for 26 hr, and the mixture was kept at 37 C for 2 hr. The agglutinated cells were washed three times with 0.9% NaCl, and examined by electron microscopy.

Electron microscopy. For negative staining, one drop of the whole culture was placed on a grid (covered with a carbon-coated nitrocellulose film), and mixed with an equal volume of a 1% (w/v) aqueous solution of phosphotungstic acid, which had been adjusted to pH 7 with 1 N KOH. After 5 to 30 sec, the liquid was withdrawn with filter paper, leaving a thin film which was allowed to dry at room temperature.

For the examination of sections of cells, the unwashed cells were fixed with osmium tetroxide and uranyl acetate according to the procedure described by Kellenberger, Ryter, and Séchaud (12), dehydrated in ethyl alcohol, and embedded in Epon. Sections were cut with a Cambridge Huxley microtome with glass knives, and were usually stained with uranyl acetate for 1 or 2 hr.

Specimens were examined in a Philips EM 100 electron microscope operating at 60 kv, and fitted with a 0.25- μ objective aperture.

Action of lysozyme on cells and walls. Cells from 18-hr cultures grown in lysine-limited or control medium were centrifuged and suspended in 0.03 M tris(hydroxymethyl)aminomethane (Tris)-HCl buffer, pH 8.0. Tubes were set up with the cells in a final volume of 10 ml, containing 10 to 100 $\mu\text{g}/\text{ml}$ of lysozyme or 10^{-4} M EDTA, or both (23). Control tubes contained only buffer. The suspensions were incubated at 37 C, and the rate of decrease in optical density was followed. Suspensions of walls in buffer were examined in the same way.

RESULTS

Production of diaminopimelic acid and lipopolysaccharide complex during the growth cycle. To relate the formation of extracellular products to the structure of the cells throughout the cycle of biphasic growth, a lysine-limited culture of *E. coli* 12408 was examined at intervals over 26 hr, and compared with a control culture in adequate lysine. Samples (50 ml), removed at appropriate intervals, had their optical density measured and were cooled in an ice bath and used for electron microscopy and for estimation of diaminopimelic acid and lipopolysaccharide complex.

The growth rates in both media were comparable for the first 6 hr (mean generation time, 60 min); growth then ceased abruptly in lysine-limited medium, but continued for 6 hr more in the control medium (Fig. 1). After 12 hr, the yield of lysine-limited cells was 1.09 mg (dry weight) per ml; control cells yielded 2.52 mg (dry weight) per ml. Soon after the end of growth in the lysine-limited medium, measurable amounts of diaminopimelic acid appeared in the culture fluid, the amount increasing linearly on continued incubation (Fig. 2); a similar increase in the amount of lipopolysaccharide complex in the culture fluid, as determined by the quantitative precipitin method, was also observed (Fig. 2). The accumulation of both products occurred mainly during a period (from 6 to 21 hr) when there was only a slight increase in the optical density of the culture or the dry weight of the cells (dry weight at 7 hr, 1.01 mg/ml; at 18 hr, 1.05 mg/ml). The respective values for lipopolysaccharide complex after 6 and 21 hr were 0.11 and 0.55 mg/ml; the corresponding values for diaminopimelic acid were 0.01 and 1.64 mg/ml.

On continued incubation beyond 21 hr in lysine-limited medium, a second phase of growth started, in which diaminopimelic acid and lipoglycopeptide were produced at the same rate as in the stationary phase. This second phase of growth ceased at about 26 hr, after which there was no further extraction of these components into the culture fluid. The final levels of diaminopimelic acid and lipopolysaccharide complex were 2.14 and 0.70 mg/ml, respectively.

Viable counts were made during several other similar experiments. At the end of the first phase of growth, 1 ml of culture fluid contained 2×10^8 to 9×10^8 lysine-requiring cells, and less than 100 revertants with no requirement for lysine. Subsequently the numbers of revertants increased logarithmically until the end of secondary growth. During the stationary phase, the lysine-requiring cell count sometimes fell slightly; growth resumed

FIG. 1. Effect of lysine limitation on the growth of *Escherichia coli* ATCC 12408. Symbols: ●, 200 mg of L-lysine HCl per liter of culture fluid; □, 60 mg of L-lysine HCl per liter.

FIG. 2. Appearance of extracellular products after depletion of lysine. Symbols: ○, optical density of culture; □, extracellular lipopolysaccharide complex; △, diaminopimelic acid.

during the secondary phase. At the end of growth, the numbers of lysine-requiring cells were fairly consistent in different experiments (10^8 to 9×10^8 per ml). The final figures for revertants, although always lower than those of the lysine-requiring strain, were much more variable (8×10^5 to 3×10^6 per ml), and probably depended on the numbers in the inoculum. It was concluded that secondary growth was not necessarily due to concomitant increases in numbers of original strain and revertant, although sometimes the increases in revertant numbers were sufficient significantly to affect total population figures. At other times, when the revertant counts were

low throughout, the observed increases in optical density could only have originated from growth of the lysine-requiring strain.

When a portion of the culture fluid after 26 hr of growth was centrifuged at $144,000 \times g$ for 1 hr, a gelatinous pellet was deposited. A portion of this was retained for examination in the electron microscope. The remainder on resuspension in water gave an opalescent solution precipitating strongly with antiserum to lipopolysaccharide complex. The conclusion that the pellet was predominantly lipopolysaccharide complex was supported by the fact that it contained heptose and phosphatidylethanolamine. The total heptose content of the pellet enabled an estimate of the amount of material to be made by comparing this value with the known lipopolysaccharide-complex content of the culture filtrate; it was concluded that 10% of the complex in the culture fluid had been deposited by ultracentrifugation.

In the control medium, the amounts of diaminopimelic acid and lipopolysaccharide complex present after 26 hr represented only 1 and 10%, respectively, of the amounts produced in this time by lysine-limited cells.

Extraction of lipopolysaccharides from cells. To find out whether lysine-depleted cells contained abnormal amounts of lipopolysaccharide, another similar experiment was carried out, in which approximately 4 liters of samples of culture fluid was withdrawn after 12 and 18 hr. The yield of cells from lysine-limited medium was 1.0 mg (dry weight) per ml after both 12 and 18 hr; in control medium, the yields were 3.1 and 3.4 mg (dry weight) per ml after 12 and 18 hr, respectively. The amount of lipopolysaccharide extracted from lysine-limited cells represented 1.64 and 1.69%, respectively, of the dry weight of the cells after 12 and 18 hr of growth; for cells grown in control medium, the corresponding values were 1.86 and 1.83%. These values suggest that there was little difference between the lipopolysaccharide contents of cells grown in limited or sufficient lysine.

Effect of lysozyme and EDTA on fresh cells. The lysozyme susceptibility of lysine-limited cells was compared with that of control cells by examining the lytic effects of low concentrations (10 $\mu\text{g}/\text{ml}$) of lysozyme and EDTA (Fig. 3). The control cells were hardly affected by lysozyme and EDTA, either alone or in combination, whereas lysozyme caused some lysis of lysine-limited cells even without EDTA. Higher concentrations of lysozyme attacked the control cells in the presence of EDTA. There was no difference in susceptibility of walls prepared from the two types of cells.

FIG. 3. Effect of lysozyme on cells of *Escherichia coli* grown for 18 hr in minimal medium. Black points, control cells grown with 300 mg per liter of L-lysine hydrochloride; open points, lysine-limited cells grown with 60 mg per liter of L-lysine hydrochloride. Cells were harvested and suspended in 10 ml of 0.03 M Tris buffer (pH 8.0) containing 5 μmoles of EDTA and 100 μg of lysozyme in the appropriate tubes as indicated. Tubes were incubated at 18 C.

Composition of cell walls. Cell walls prepared from lysine-limited and control cells showed almost identical amino acid compositions; the results differed very little from the analysis given by Bishop and Work (4) for the walls of lysine-limited cells, except for higher lysine contents in both of the present preparations (1.7 moles per 10^4 g of walls).

Changes in cell ultrastructure during growth. Sampling of the whole culture at intervals during growth revealed the state of the culture. Negatively stained preparations of control cells grown in excess lysine rarely showed any extracellular material during the 26-hr growth period. Preparations of lysine-limited cultures at early stages of growth resembled control cultures, but later could be distinguished by the presence of extracellular material. The amount of material increased with age; after 18 hr the cells were surrounded by packed masses of globules and hollow spheres, ranging in size from 12 to 200 μm in diameter, and there were also some structures resembling stacked leaflets (Fig. 4). The material deposited from the final lysine-limited culture medium by centrifugation at $144,000 \times g$ for 1 hr, and subsequently negatively stained, consisted of spheres, globules, and stacked leaflets resembling those surrounding the lysine-limited cells (Fig. 5). Sections of the centrifuged pellet showed that the globules were bounded by triple-layered limiting membranes.

FIG. 4. Negatively stained preparation of unwashed lysine-limited cells after 26 hr of growth. The cells have a convoluted surface with attached pili, and are surrounded by numerous extracellular globules and stacked leaflets. $\times 47,000$.

FIG. 5. Negatively stained preparation of material deposited from the culture fluid after 26 hr of growth by centrifugation at $144,000 \times g$ for 1 hr. Globules of varying dimensions and stacked leaflets are present. $\times 47,000$.

Thin sections of the cells examined during lysine limitation showed that, when grown for up to 6 hr, the cells were nearly normal and were hardly distinguishable from the control cells (Fig. 7). Both types of cells had a dense cytoplasm, a fibrillar nuclear region and a convoluted, multilayered cell wall separated from the cytoplasmic membrane by an electron-transparent zone. The transparent zone was more noticeable in the lysine-limited cells.

After 8 hr of growth, there were noticeable differences between the control and lysine-limited cells. On the outer side of the convoluted cell wall of the lysine-limited cells, small blebs were noticeable, and extracellular globules were also present. After 12 hr, lysine-limited cells were easily recognized by the numerous blebs attached to the cell walls and by the presence of extracellular globules (Fig. 8). At the same time, circular areas of low electron density began to appear in the dense cytoplasm, especially in the polar regions (Fig. 8 and 9). In sections stained with phosphotungstic acid, the peripheries of these light areas were electron-dense.

Intracytoplasmic membranous organelles (mesosomes) were observed in both control and lysine-limited cells, but they were more numerous in the lysine-limited cells examined between 12 and 21 hr of growth (Fig. 9). Occasionally, the electron-transparent space separating the membranes of these organelles was seen to be continuous with the space between the cytoplasmic membrane and the outer layers of the cell wall.

The amount of extracellular material increased with age, as did the number of blebs on the cell walls; after 18 to 20 hr of growth, many blebs were present (Fig. 10 and 12). Several layers, including an outer triple-layered membrane and a wide inner electron-transparent zone, can be recognized in the cell wall outside the cytoplasmic membrane (Fig. 11). The transparent zone was also present in material fixed in glutaraldehyde and osmium tetroxide, but not in material fixed in potassium permanganate. Occasionally, phosphotungstic acid will also stain this region. Of these layers, the outer triple-layered membrane was consistently found to be involved in the formation of blebs (Fig. 11 and 12). The extracellular globules also had triple-layered limiting membranes, suggesting that they were derived from the blebs, and therefore originated from the outer triple-layered membrane of the cell wall.

The cells were agglutinated by antiserum to the lipopolysaccharide complex. Sections of lysine-limited cells which had been agglutinated with antibody coupled with ferritin showed the ferritin

to be distributed in clumps around the cells, indicating that the extracellular material contained lipopolysaccharide (Fig. 6). Very little ferritin was observed inside the globules or outside control cells grown with adequate lysine.

DISCUSSION

The extent of accumulation of products in the culture fluid when lysine-requiring mutants of *E. coli* are grown under lysine-limiting conditions is known to be dependent on the energy source and amount of aeration, as well as on the concentration of lysine (4, 16, 19). Growth ceases when lysine is exhausted, to be followed subsequently by a secondary growth phase. In the present investigation, the first and second phases of rapid growth were separated by a long stationary period of about 15 hr, during which extracellular products increased at a steady rate; this allowed the rate of accumulation of products to be studied over a period when there was no increase in cell mass. The growth curves previously published (4, 16, 19) did not show such a pronounced stationary phase, probably because of different cultural conditions.

It has been suggested (2, 16) that the secondary growth is due to reversion of the culture to lysine independence. This was not the case with our findings, which showed that during secondary growth lysine-dependent cells increased by at least 2×10^9 , whereas increases in the revertant varied from 4×10^5 to 10^9 per ml. During many previous experiments on small scales (*unpublished data*), reversion did not always occur. No explanation for secondary growth has been found.

The total amount of lipopolysaccharide complex present in the culture fluid, as estimated by column chromatography, was 700 mg per liter, an amount which is considerably greater than the yield of "mucopeptide" (20 mg per liter) reported by Municio et al. (19) with a different strain of *E. coli*, and of "lipoglycopeptide" (150 mg per liter) obtained by Bishop and Work (4) under similar growth conditions. It is probable that the earlier low yields reflect ineffective recoveries from solution rather than low production of material, since the amount of diaminopimelic acid accumulating in the culture fluid (2.1 g per liter) was comparable with that obtained by Bishop and Work (4), but less than that reported by Municio et al. (19).

The rates of appearance of diaminopimelic acid and lipopolysaccharide complex in the culture fluid can be compared with the amounts of these components present in the cells (Table 1). In the stationary period between the first and second phases of growth (from 6 to 21 hr of incubation), the culture contained approximately

FIG. 6. Section of lysine-limited cells grown for 26 hr which have been agglutinated by ferritin-conjugated lipopolysaccharide antibody, showing ferritin grouped around the organisms. $\times 80,000$.

FIG. 7. Cells grown in excess lysine for 9 hr. Typical control cell with convoluted cell wall (w) separated by a narrow space from the cytoplasmic membrane (cm), dense cytoplasm, and a fibrillar nuclear region. Some of the cells are slightly plasmolysed (arrow). $\times 47,000$.

FIG. 8. Lysine-limited cells after 12 hr of growth, with extracellular globules (g) and small blebs attached to the cell wall (arrows). The cytoplasm contains circular areas of low electron density (e). $\times 52,000$.

FIG. 9. Detail of lysine-limited cells after 12 hr of growth. The multilayered cell wall is forming blebs (arrows), and an extracellular globule (g) is present. An intracytoplasmic membranous organelle (m) can be seen. $\times 135,000$.

FIG. 10. Detail of a lysine-limited cell after 18 hr of growth. Numerous blebs are present on the cell wall. A wide and relatively electron-transparent space (arrow) separates the outer layers of the cell wall from the cytoplasmic membrane (cm). $\times 108,000$.

FIG. 11. Detail of a lysine-limited cell after 18 hr of growth. The blebs are formed by the outer triple-layered membrane of the cell wall (arrows). $\times 180,000$.

FIG. 12. Transverse section of a lysine-limited cell after 20 hr of growth showing numerous blebs formed by the outer layers of the cell wall. $\times 162,500$.

1 g (dry weight) of cells per liter. Our results show that nearly 2% of the weight of the cell consists of lipopolysaccharide, whereas about 0.4% is wall diaminopimelic acid (4). During this stationary period, the amounts of diaminopimelic acid and lipopolysaccharide complex in 1 liter of culture fluid increased by 1.63 and 0.44 g, respectively. Thus, in 15 hr, the amount of diaminopimelic acid accumulating in the culture fluid is approximately 400 times the amount present in the cell walls. The amounts of lipopolysaccharide complex in the culture fluid and lipopolysaccharide in the cell wall may be related through their heptose contents (28): the cells from 1 liter of culture contain 3.2 mg of heptose, whereas 1 liter of culture filtrate contains 70 mg. Thus, the amount of heptose-containing material accumulating in the culture fluid is 22 times that in the cell wall.

The rapid rate of formation of diaminopimelic acid could result from exhaustion of lysine by release from feed-back control by lysine of one or more of the enzymes synthesizing diaminopimelic acid, since several of these enzymes are known to be inhibited by lysine (27, 38). The rate of accumulation of lipopolysaccharide complex is considerably lower than that of diaminopimelic acid. From the values for heptose contents given above, it may be concluded that, during each 40-min period of the stationary-growth phase, the cells synthesize and excrete an amount of lipopolysaccharide equal to the amount already present in the cell. Thus, it would appear that the enzymatic reactions leading to the formation of extracellular lipopolysaccharide are proceeding at rates comparable with those in exponentially growing cells, which double their mass in 1 hr.

Among the products accumulating in the culture fluid are nucleotides, the presence of which Lilly et al. (16) took to indicate cell lysis in cul-

tures of *E. coli* 26-26. However, electron microscopy of cells actively excreting many products over the whole growth cycle has not provided evidence for such lysis. Certainly, *E. coli* cells are not normally permeable to either diaminopimelic acid (15, 34) or nucleotides; but the susceptibility of lysine-limited cells to lysozyme in the absence of EDTA suggests that the cells had abnormal permeability properties. Treatment with EDTA has been shown to cause a nonspecific increase in permeability of *E. coli*, and to release half of the cell wall lipopolysaccharide into solution (13, 14). This suggests that an altered permeability to lysozyme of lysine-limited cells may have its origin in a defect in the outer lipopolysaccharide-containing layers of the wall.

Changes in surface structure might also account for the accumulation of a soluble, extracellular, high-molecular weight complex of components normally present in the wall. The observed changes suggested that the lipopolysaccharide complex originated from the outer layers of the wall. The blebs were bounded by triple-layered membranes which were sometimes seen to be continuous with the outer triple-layered membrane of the wall. The innumerable particles surrounding the cells were often also bounded by triple-layered membranes, suggesting that they originated from blebs broken off from the walls. Since lipopolysaccharide synthesis appears to have continued at a normal rate, although division of the lysine-requiring strain was arrested during the stationary phase between the two growth periods, it is evident that an unbalanced type of growth occurred. The extension of the outer layers of the wall in the form of blebs might be regarded as a natural consequence of the unbalanced growth of this portion of the cell, owing to restricted protein synthesis elsewhere imposed by lysine limitation.

TABLE 1. Comparison of the amounts of cellular and extracellular components produced between the 6th and 21st hr of culture

Source	Component ^a	Amt of component (g)		
		After 6 hr	After 21 hr	Increase
Whole cells (from 1 liter)	Dried cells	1.01	1.05	0.04
	Diaminopimelic acid	0.004	0.004	* _b
	LPS	0.017	0.017	*
	Heptose in LPS	0.0032	0.0032	*
Culture fluid (1 liter)	Diaminopimelic acid	0.01	1.64	1.63
	LPS complex	0.11	0.55	0.44
	Heptose in LPS complex	0.018	0.088	0.070

^a LPS, lipopolysaccharide.

^b Not significant.

An abnormally wide electron-transparent zone immediately outside the cytoplasmic membrane is another characteristic feature of the lysine-limited cells; the material in this zone has not been identified, so it is not possible to say whether it, too, was subject to unbalanced growth. The regular width of the zone and the fact that it can be stained suggest that it is not an artefact caused by shrinkage of the cytoplasm, but is part of the wall, which, as in other gram-negative bacteria (5, 8, 21, 31), is very complex.

Although the main differences between the control and lysine-limited cells were seen in the wall structure, some changes were also observed in the internal features. Intracytoplasmic membranous organelles (mesosomes; 29) were seen more often in the lysine-limited cells, which also contained numerous circular electron-transparent areas in the cytoplasm. Murray (20) suggested that such areas may represent accumulations of a polysaccharide, possibly glycogen, surrounded by a layer of protein.

The outer triple-layered membrane of walls of gram-negative bacteria may be a composite structure containing both lipoprotein and lipopolysaccharide components (3, 5). Thin sections of cells of *E. coli* 12408 agglutinated with ferritin-labeled antibody to lipopolysaccharide indicated that the antigen was on and beyond the outer surface, a finding in agreement with that of Shands (25). A surface structure in which both lipopolysaccharide and lipoprotein are exposed would be in accord with observations on the availability of phage receptor sites (32) and the susceptibility of smooth strains to the lethal action of complement (30). Interpretations of the results of electron microscopy have usually derived from the premise that triple-layered membranes contain distinct layers of protein, phospholipid, and sometimes polysaccharide (7, 24). However, Green and Perdue (11) now propose that the trilaminar structure is a composite of two entirely different membrane layers.

The repeating units of mitochondrial membranes, studied by Green and his colleagues (11), generally contain 20 to 30% phospholipid; analyses of other membranes, summarized by Brown (6), show the presence of 15 to 30% lipid, 25 to 70% protein, and up to 20% carbohydrate. The complex isolated from the culture fluid of *E. coli* 12408 contains approximately 60% lipopolysaccharide, 26% lipid (mostly phosphatidylethanolamine), and 11% protein (Knox and Work, J. Gen. Microbiol., *in press*; Knox, Cullen, and Work, *unpublished data*). If the complex is, indeed, derived directly from the outer membrane, the results suggest that this

membrane may be composed of repeating units of lipopolysaccharide and lipoprotein, with more polysaccharide and less protein than is found in most membranes. Since neither of the triple-layered membranes of *E. coli* have been separated intact, it has not been possible to prove this supposition directly. Analyses of whole walls did not suggest that there was any abnormality in the protein content of the walls of lysine-limited cells; it is therefore unlikely that the surface defect in these cells is due to a deficiency of protein.

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