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Citrate-Tris(hydroxymethyl)aminomethane-Mediated Release of Outer Membrane Sections from the Cell Envelope of a Deep-Rough (Heptose-Deficient Lipopolysaccharide) Strain of *Escherichia coli* O8

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A heptose-deficient lipopolysaccharide strain of *Escherichia coli* O8, strain F515, was found to release portions of its outer membrane when cells were exposed to 10 mM citrate buffer (pH 2.75) for 30 min and subsequently exposed to 100 mM tris(hydroxymethyl)aminomethane buffer (pH 8.00). The outer membrane component released was found to be composed of protein, lipopolysaccharide, phospholipid (cardiolipin, phosphatidylethanolamine, and phosphatidylglycerol), and alkaline phosphatase. The outer membrane component was released from the cell envelope in the absence of cell lysis, as no glucose-6-phosphate dehydrogenase activity or succinic dehydrogenase activity was detected. Morphologically, the outer membrane component appeared to consist of laminar fragments and vesicles which had an associated alkaline phosphatase activity.

The gram-negative cell envelope is a complex multilayered structure which consists of the cytoplasmic membrane, the periplasmic space (containing the murein and, in some instances, a specialized lipoprotein, numerous degradative enzymes, and binding proteins), and the outer membrane (7). Although the various components of the gram-negative cell envelope each have their own properties, they are extensively interrelated (30).

The complex interactions of the components of the gram-negative cell envelope have made isolation of the various cell envelope components difficult. Isolation of the outer membrane in particular has proven to be a complex procedure resulting in only a partially purified preparation of a somewhat modified form of outer membrane (28, 29, 34, 30). Uncontaminated outer membrane of a marine pseudomonad has been isolated by washing whole cells with 0.5 M NaCl and subsequently suspending the cells in 0.5 M sucrose (12). This isolation procedure has been effective for relatively few marine organisms (9; R. A. Laddaga and R. A. MacLeod, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, J26, p. 186), presumably indicating that these organisms have rather novel outer membrane structures.

In the course of investigating the effect of low pH on cell-associated alkaline phosphatase activity, we noted that upon resuspension of the cells in Tris buffer, a considerable amount of the alkaline phosphatase of a deep-rough strain (heptose-deficient lipopolysaccharide [LPS]) no longer remained associated with the cells (27). Subsequent investigation indicated that exposure of a deep-rough strain of *Escherichia coli* O8 to 10 mM citrate buffer (pH 2.75) for 30 min and subsequent exposure to 100 mM Tris buffer (pH 8.00) resulted in the release of sections of the outer membrane from the cell envelope.

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MATERIALS AND METHODS

The bacterial strain utilized in this study was *E. coli* O8 strain F515 (39), which possesses heptose-deficient LPS and bears the genetic markers *pro his met mtl* Str^r F^-. The bacterial strain was the generous gift of Günter Schmidt, Max-Planck Institut-für Immunbiologie, Freiburg, West Germany.

Cells were grown in phosphate-limiting medium that consisted of 0.05 M Tris-hydrochloride buffer, 0.08 M NaCl, 0.02 M KCl, 0.02 M NH_4Cl, 0.003 M Na_2SO_4, 0.001 M $MgCl_2$, 2×10^{-4} M $CaCl_2$, 2×10^{-6} M $ZnCl_2$, and 0.5% (wt/vol) peptone (Difco Laboratories), adjusted to pH 7.5 and supplemented with 50 μg each of L-proline, L-methionine, and L-histidine per ml, 10^{-5} M inosine, and 0.5% glucose as a carbon source.

Single colonies of the strains under investigation were used to inoculate 10 ml of phosphate-limiting medium in an Erlenmeyer flask (50 ml) and were grown at 35°C in a G25 controlled-environment rotary shaker-incubator (New Brunswick Scientific Co.) at 150 rpm for 12 h. This culture provided a 2% inoculum for the experimental culture, which was grown under identical conditions (with the same lot of all medium components throughout the study) for 8.5 h before harvesting.

Preparation of outer membrane. The outer membrane component was routinely prepared by har-

vesting two 500-ml volumes of an 8.5-h culture of *E. coli* O8 strain F515, grown on phosphate-limiting medium, by centrifugation (5,000 × *g* for 10 min), suspending the cell pellet in 10 ml of 100 mM Tris buffer (pH 8.00), pelleting the cells by centrifugation (12,000 × *g* for 10 min at 4°C), suspending the cell pellet in 10 ml of 10 mM citrate buffer (pH 2.75), incubating the cell suspension for 30 min at room temperature, pelleting the cells by centrifugation (12,000 × *g* for 10 min at 4°C), suspending the cells in 10 ml of 100 mM Tris buffer (pH 8.00), incubating the cells for 10 min at room temperature, pelleting most of the cells by low-speed centrifugation (5,000 × *g* for 20 min at 4°C), and removing all of the remaining whole cells from the outer membrane preparation by centrifugation (48,000 × *g* for 20 min at 4°C). The outer membrane component is devoid of whole cells, the treatment does not result in cell lysis (27), and the yield of outer membrane is consistent.

Electron microscopy. Samples for routine embedding were prefixed for 20 min at room temperature by the addition of 0.10 volume of 5% (vol/vol) glutaraldehyde in 67 mM cacodylate buffer (pH 6.7). The prefixed cells were then pelleted by centrifugation (12,000 × *g* for 10 min at 4°C), resuspended in 5% (vol/vol) glutaraldehyde in 67 mM cacodylate buffer (pH 6.7), and fixed for 1.5 h at room temperature. Fixed cells were then pelleted by centrifugation (12,000 × *g* for 10 min at 4°C) and enrobed in 4% Noble agar (Difco) as described by Murray et al. (31). The enrobed samples were washed five times for 10 min with 67 mM cacodylate buffer (pH 6.7). The samples were then postfixed with 2% OsO_4 (Polysciences Inc.) for 2 h at room temperature, washed and dehydrated as previously described (16), and subsequently embedded in Vestapol W (Polysciences Inc.). Thin sections were cut by using an LKB Ultratome III 8800 and stained with uranyl acetate (1% aqueous solution, pH 4.8) and Reynolds lead citrate (36).

Alkaline phosphatase was localized by a modified Gomori end-product localization method (10). This incubation mixture contained 0.2 mg of *p*-nitrophenyl phosphate per ml, 0.5% (wt/vol) sodium barbital, 0.02 M $Ca(NO_3)_2$, and 0.01 M $MgCl_2$ in 100 mM Tris buffer (pH 8.00) or in 67 mM cacodylate buffer (pH 8.00). Samples were incubated in the incubation mixture (or in the incubation mixture lacking *p*-nitrophenyl phosphate as a control) for 10 min at room temperature, pelleted by centrifugation (12,000 × *g* for 10 min at 4°C), suspended in fresh 2% aqueous $Pb(NO_3)_2$, and incubated for 10 min at room temperature. The samples were then washed twice with 67 mM cacodylate buffer (pH 6.7) and subsequently fixed and embedded as previously described.

Samples for critical-point drying were placed on Formvar-coated 400-mesh copper grids and critical-point dried by the method of Cohen et al. (6). After critical-point drying, samples were shadowed with platinum by using a Balzers BA 360M apparatus operating at room temperature as previously described (35).

All specimens were examined with an AE1 801 electron microscope operating with an accelerating potential of 60 kV.

Light microscopy. Specimens were examined in wet mounts by differential interference microscopy with a Leitz Dialux microscope. Specimens were photographed with Panatomic X film (Eastman Kodak).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Sodium dodecyl sulfate-discontinuous polyacrylamide gel electrophoresis system J4179 of Neville and Gossman (32) was utilized for analysis of protein components. Protein samples (~2 mg/ml) were solubilized in 1 ml of water containing 10 mg of Na_2CO_3, 40 mg of sodium dodecyl sulfate, and 100 μl of 2-mercaptoethanol by boiling for 10 min. Solubilized protein samples (50 μl) were applied to a 10-cm slab gel consisting of 11% acrylamide and 0.1% bis-acrylamide and were electrophoresed with a constant current of 50 mA. Gels were subsequently stained with Coomassie brilliant blue overnight and destained electrophoretically. Molecular weight assignments were made as described by Weber and Osborn (44), utilizing a Dalton Mark VI sodium dodecyl sulfate molecular weight marker kit (Sigma Chemical Co.).

Phospholipid extraction and analysis. Lipid was extracted from the outer membrane component by the method of Bligh and Dyer (4). Extracted lipid was analyzed by chromatography on Silica gel 13179 thin-layer chromatography plates (Eastman Kodak), using solvent system IV of Ames (1) (chloroform-methanol-glacial acetic acid [65:25:8, vol/vol/vol].) Phospholipids were identified by the use of phospholipid standards (Supelco, Inc.). Thin-layer chromatograms were visualized with iodine vapors, ninhydrin spray (3% [wt/vol] in acetone), and a phosphate-specific spray (Phospray; Supelco, Inc.).

Assays utilized. Alkaline phosphatase activity was determined at room temperature by following the production of *p*-nitrophenol, due to the hydrolysis of *p*-nitrophenyl phosphate, at 420 nm by using a Unicam SP 1800 double-beam spectrophotometer equipped with a Unicam AR 25 recorder. The assay mixture utilized was that of Garen and Levinthal (14), except that the Tris buffer concentration was 100 mM rather than 1 M.

Glucose-6-phosphate dehydrogenase activity was determined by the method of Langdon (21).

Succinic dehydrogenase was assayed according to the method of Khouw and McCurdy (19) by following the reduction of 2,4-dichloroindophenol by the decreasing optical density at 600 nm.

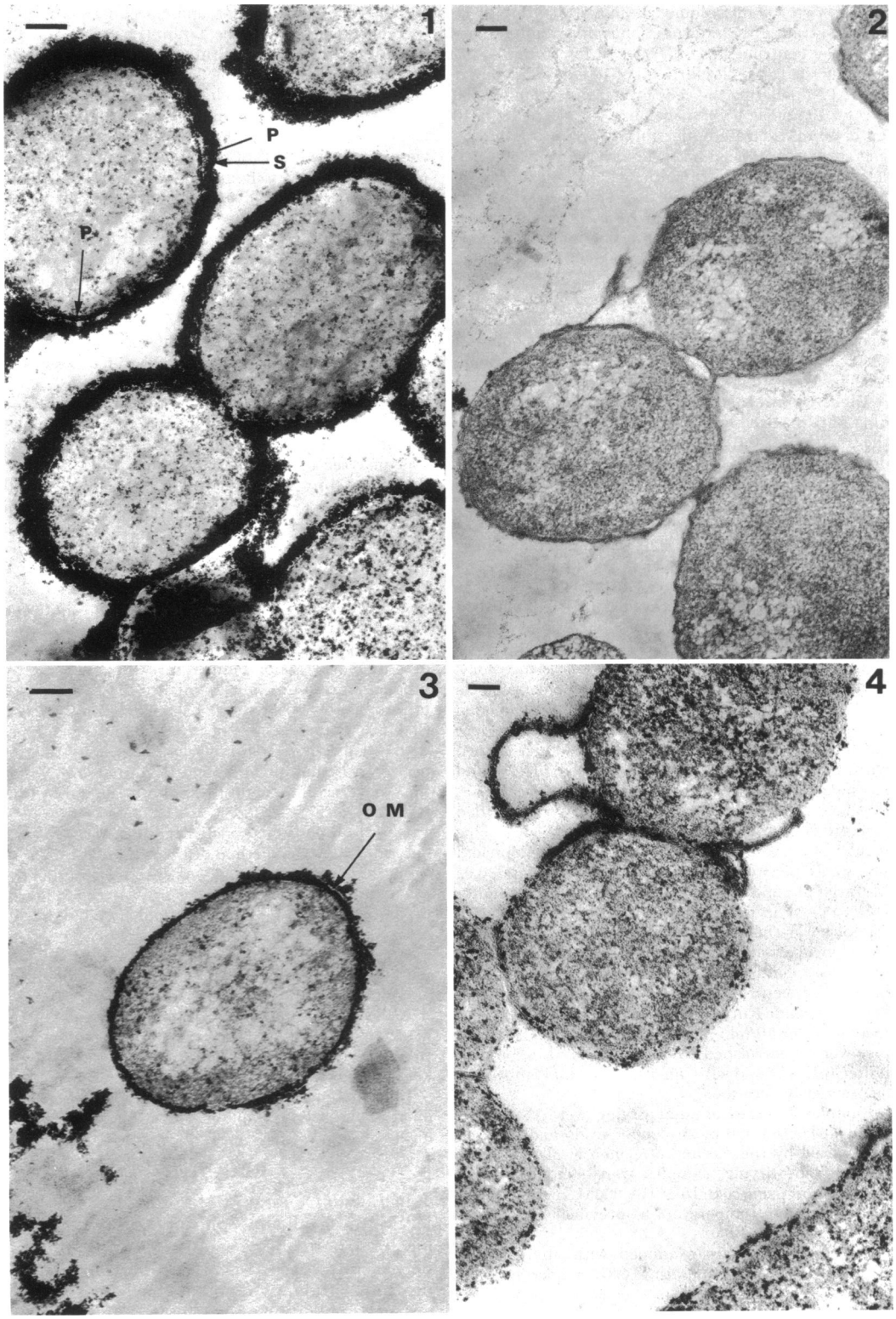
Protein concentration was determined by the method of Lowry et al. (23), employing bovine serum albumin as a standard.

LPS was determined spectrophotometrically by the determining 2-keto-3-deoxyoctonate concentration by the method of Weissbach and Hurwitz (45), using 2-keto-3-deoxyoctonate (Sigma) as a standard.

Phosphate was determined by the method of Fiske and Subbarow (11), employing inorganic phosphate as a standard.

RESULTS

In *E. coli* O8 strain F515 (a heptose-deficient LPS strain), alkaline phosphatase is normally localized, both on the cell surface and within the periplasmic space (Fig. 1 and 2) (25, 26). In a previous study, we noted that an extensive amount of alkaline phosphatase activity was released from cells by first exposing them to 10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mM citrate buffer (pH 2.75) and subsequently to 100 mM Tris buffer (pH 8.00) (27). The cytochemical localization of alkaline phosphatase after the described treatment was examined to determine whether the treatment had modified the localization of alkaline phosphatase within the cell envelope.

E. coli O8 strain F515 treated with citrate buffer and Tris buffer (as described in Materials and Methods) had a rather unusual cellular morphology. Citrate-Tris-treated cells appeared to have lost portions of their outer membrane, and the remaining cell-associated alkaline phosphatase activity was found to be localized on the surface of the cytoplasmic membrane and on the surface of remaining outer membrane (Fig. 3 and 4). Gomori end-product localization of alkaline phosphatase in the 100 mM Tris buffer (pH 8.00) supernatant indicated that the alkaline phosphatase activity was associated with "membrane-like" structures (Fig. 5 and 6). To ensure that citrate-Tris treatment of the strain resulted in the release of portions of the outer membrane to the Tris buffer supernatant and was not an artifact of the Gomori localization technique, a number of different morphological techniques were utilized.

Thin sections of cells treated with citrate and Tris buffers revealed that portions of their outer membrane were missing and that other sections of the outer membrane were only loosely associated with the cell envelope, whereas untreated cells had a normal morphology (Fig. 7 and 8). Examination of thin sections of the Tris buffer supernatant revealed the presence of vesicles and laminar fragments with a "double-track" profile (data not shown).

Interference contrast microscopy confirmed the presence of small vesicles in the Tris supernatant after cells had been pretreated with citrate buffer (Fig. 9), and examination of cells pretreated with citrate buffer and suspended in Tris buffer revealed that the vesicles originated from the bacterial cells as blebs of the cell envelope. The process of outer membrane release by citrate-Tris treatment was further examined by critical-point drying and shadowing citrate-treated cells suspended in Tris buffer. Large cell wall blebs were noted on the surface of treated cells (B in Fig. 10 and 11), and free vesicles of roughly the same dimensions as the cell wall blebs and having the same texture as the cell surface were noted in close proximity to treated cells (Fig. 11). Neither cell wall blebs nor free vesicles were observed in cells that were untreated or exposed to Tris buffer without pretreatment with citrate buffer (Fig. 12).

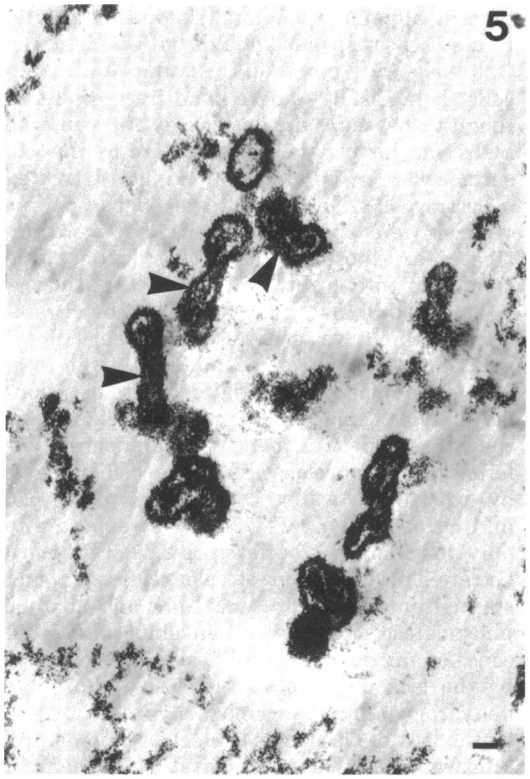
The yield of material released by the citrate-Tris treatment was generally consistent (10% of the total cellular protein and 7.3% of the total LPS), although minor variations were occasionally noted (Table 1). The material released by the citrate-Tris treatment was significantly different from that released from the cell envelope by exposure to water, by exposure to only 10 mM citrate buffer (pH 2.75), or by exposure to only 100 mM Tris buffer (pH 2.75) (Table 1). Citrate-Tris treatment of cells released a substantial amount of protein, LPS, and alkaline phosphatase from the cell envelope in the absence of any detectable cell lysis (as determined by the lack of detectable glucose-6-phosphate dehydrogenase and succinic dehydrogenase and by the lack of lysed cells in electron micrograph preparations) (Table 1). Citrate treatment of cells released smaller amounts of protein and LPS and almost no alkaline phosphatase from the cell envelope in the absence of cell lysis (Table 1). Tris treatment of cells released a smaller amount of protein, a large amount of LPS, and a small amount of alkaline phosphatase. Exposure of cells to water resulted in the release of a small amount of protein, a small amount of LPS, and a small amount of alkaline phosphatase from the cell envelope in the absence of cell lysis (Table 1). The citrate-Tris-released material contained 0.47 mg of chloroform-methanol-extractable material per ml, of which the majority was LPS, although 20 to 30% was phospholipid. The phospholipid component consisted of roughly equivalent amounts of cardiolipin, phosphatidylglycerol, and phosphati-

FIG. 1. *Gomori end-product localization of alkaline phosphatase in untreated cells of E. coli O8 strain F515. Note the cell surface (S) and periplasmic (P) distribution of the alkaline phosphatase. The bar in this and subsequent micrographs represents 100 nm.*

FIG. 2. *E. coli O8 strain F515 cells treated identically to those in Fig. 1, except that p-nitrophenyl phosphate (substrate for alkaline phosphatase) was deleted as a control. Note the lack of lead salts.*

FIG. 3. *Gomori end-product localization of alkaline phosphatase in E. coli O8 strain F515 after exposure of cells to 10 mM citrate buffer (pH 2.75) and subsequently to 100 mM Tris buffer (pH 8.00). Note that a substantial portion of the outer membrane (CM) is absent.*

FIG. 4. *E. coli O8 strain F515 cells sequentially exposed to 10 mM citrate buffer (pH 2.75) and 100 mM Tris buffer (pH 8.00) and treated identically to those cells in Fig. 3, except that p-nitrophenyl phosphate was deleted from the reaction mixture to serve as a control preparation for Fig. 3. Note the absence of lead salts, the loss of portions of the outer membrane, and the presence of loosely associated outer membrane in the form of a large bleb.*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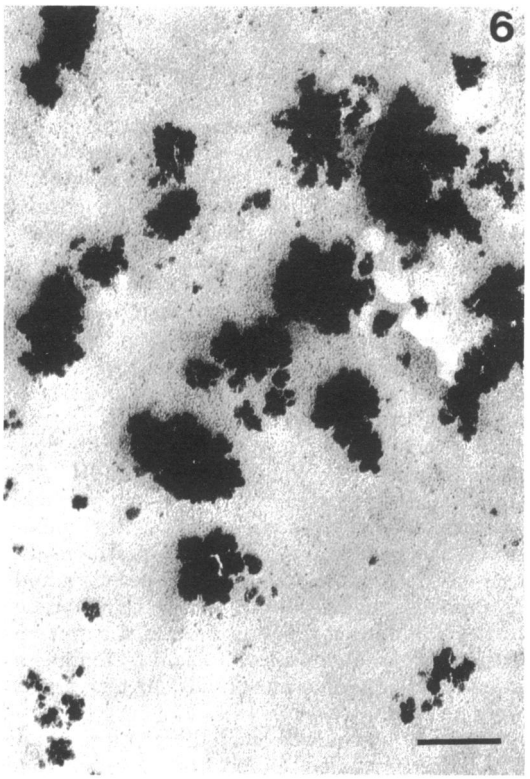
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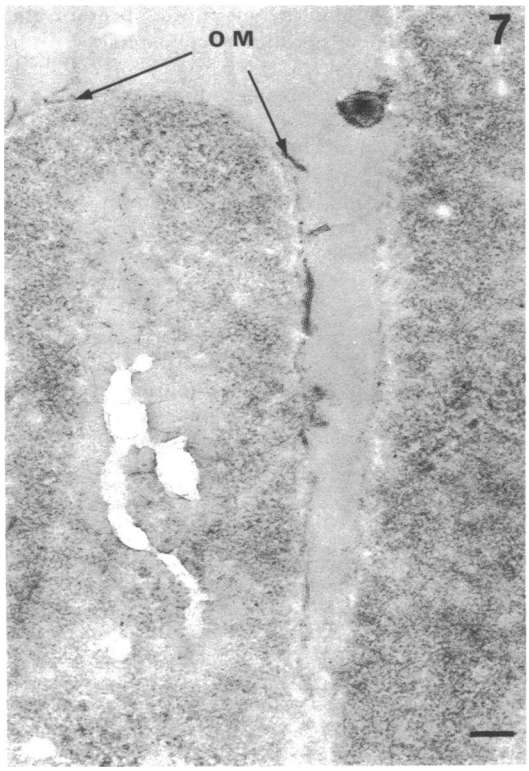
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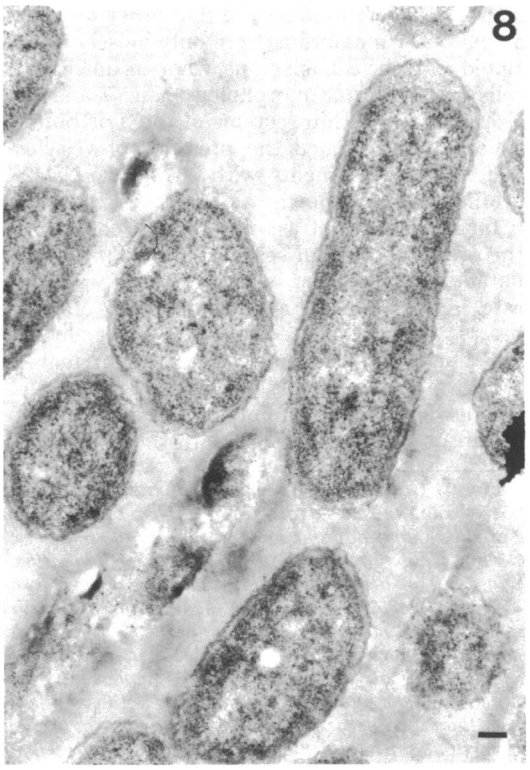
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dylethanolamine as determined by thin-layer chromatography using phospholipids as standards (Table 2).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis indicated that the cell envelope material released by exposure to 10 mM citrate buffer contained at least six proteins with apparent molecular weights of 42,000, 36,000, 35,000, 32,000, 31,000, and 27,000 (Fig. 13). The citrate-Tris-released outer membrane material contained at least 39 proteins including those with apparent molecular weights of 37,500, 32,500, 17,000, and 11,500 (Fig. 13). The material released from the cell envelope of cells exposed solely to 100 mM Tris buffer (pH 8.00) contained at least 33 proteins, with the majority of these being present in the citrate-Tris-released material, although the relative amounts of each protein released were substantially different (Fig. 13). The citrate-released proteins were found as components of both the Tris-released material and the citrate-Tris-released material (Fig. 13). The Tris-released material contained four proteins with apparent molecular weights of 50,500, 30,000, 23,000, and 22,000 which were not detectable in the citrate-Tris-released material (Fig. 13). The citrate-Tris-released material contained nine proteins with apparent molecular weights of 46,000, 38,000, 26,000, 24,000, 20,000, 19,000, 13,000, 12,000, and 11,500 which were not detectable in the Tris-released material (Fig. 13). The material released by the citrate-Tris treatment had a higher portion of lower-molecular-weight components compared with the material released by exposure of cells solely to Tris buffer.

DISCUSSION

E. coli O8 strain F515 is a deep-rough (heptose-deficient LPS) strain which was indirectly obtained from a wild-type strain through successive mutagenesis (39; Günter Schmidt, personal communication). Strain F515 is typical of deep-rough strains in that, compared with its wild-type parent, it is more sensitive to antibiotics (25), releases a significant portion of its periplasmic enzymes to the culture medium (25), has an altered enzyme localization pattern (25, 26), has an altered outer membrane morphology (3, 41; R. T. Irvin, Ph.D thesis, University of Calgary, Calgary, Alberta, Canada, 1977), and has decreased levels of and an altered composition of outer membrane protein (2, 17, 20; Irvin, Ph.D. thesis).

After exposure of strain F515 to 10 mM citrate buffer (pH 2.75) for 30 min and subsequently to 100 mM Tris buffer (pH 8.00) for 10 min, the cells were found to have lost portions of their outer membrane (Fig. 1 to 4, 7, and 8). The outer membrane appears to be released from the cell envelope by first dissociating at a single point to produce either a bleb (evagination of the outer membrane) which enlarges as more and more outer membrane dissociates from the cell envelope until eventually the bleb dissociates from the cell envelope to form a free vesicle, or the outer membrane dissociates from the cell envelope in a linear fashion to produce a "flap" of outer membrane which, if it dissociates from the cell envelope below a certain critical size, remains as a laminar fragment of outer membrane rather than forming an outer membrane vesicle.

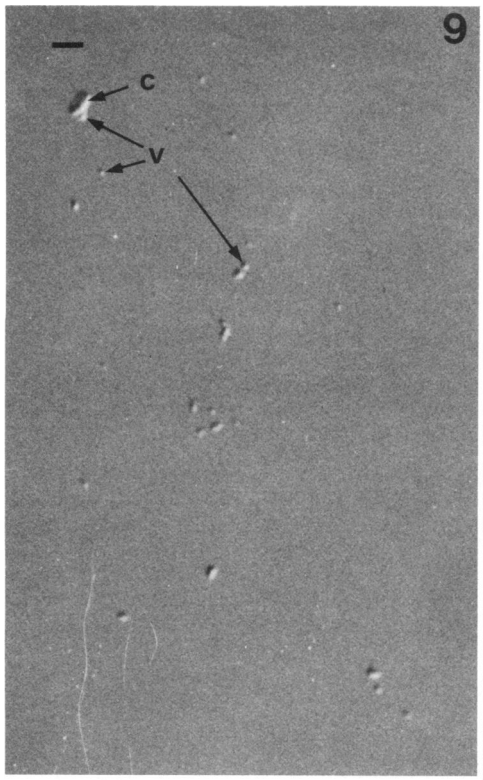
Alkaline phosphatase is normally localized within the periplasmic space and on the cell surface in strain F515 (Fig. 1 and 2) (25, 26). When strain F515 is sequentially exposed to 10 mM citrate buffer (pH 2.75) and 100 mM Tris buffer (pH 8.00), a considerable amount of the cell-associated alkaline phosphatase is released (Table 1) (27) from the cell envelope in addition to the portions of outer membrane. The residual cell-associated alkaline phosphatase of citrate-Tris-treated cells was found to be localized on the surface of the outer membrane and within the periplasmic space in regions of the cell envelope where the outer membrane was retained and on the surface of the cytoplasmic membrane in regions of the cell envelope where the outer membrane was not retained (Fig. 3). The citrate-Tris-released alkaline phosphatase was found to be associated with outer membrane vesicles and laminar fragments (Fig. 5 and 6). It seems likely that alkaline phosphatase is retained on the surface of the outer membrane and the surface

FIG. 5. *Gomori end-product localization of alkaline phosphatase released into 100 mM Tris buffer (pH 8.00) from E. coli O8 strain F515 previously treated with 10 mM citrate buffer (pH 2.75). Note the presence of a membrane-like structure (arrowheads) with associated alkaline phosphatase activity.*

FIG. 6. *Thin section of lead salts formed by Gomori end-product localization of soluble alkaline phosphatase (i.e., pure alkaline phosphatase and incubation mixture) to serve as a control for Fig. 5. Note that the lead crystals formed are distinctly different from those deposited on the outer membrane as seen in Fig. 1 and 5.*

FIG. 7. *Thin section of E. coli O8 strain exposed to 10 mM citrate buffer (pH 2.75) and 100 mM Tris buffer (pH 8.00). Note that portions of the outer membrane (CM) are absent and that some portions of the outer membrane are only marginally associated with the remaining cell envelope.*

FIG. 8. *Thin section of E. coli O8 strain F515 not exposed to citrate and Tris buffer.*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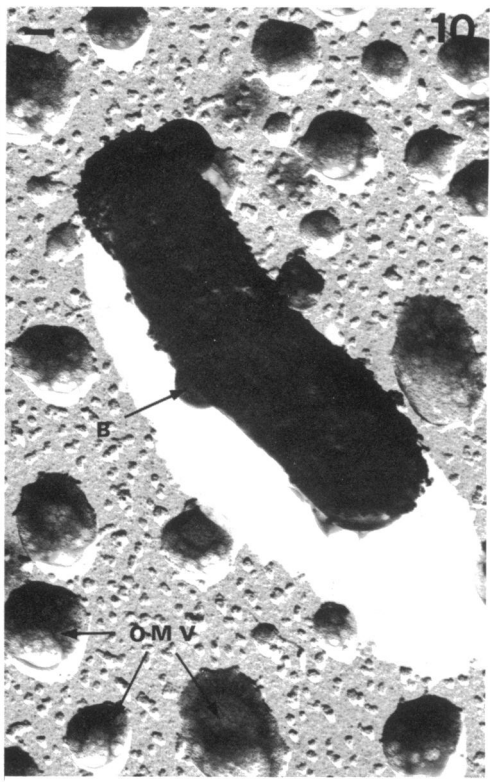
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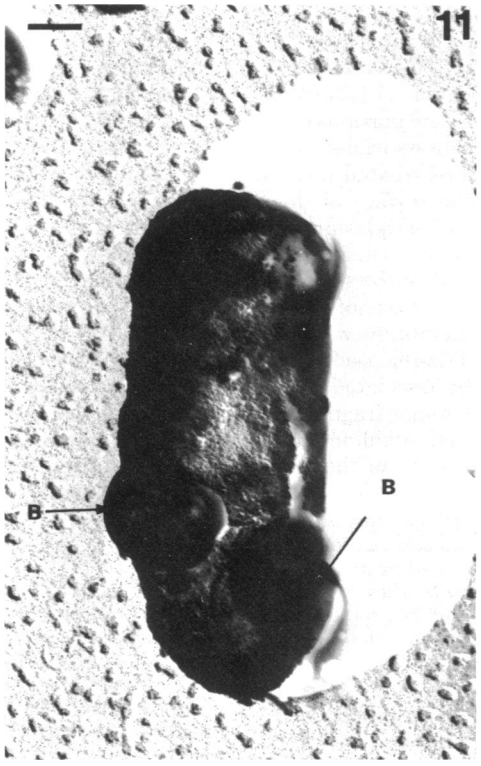
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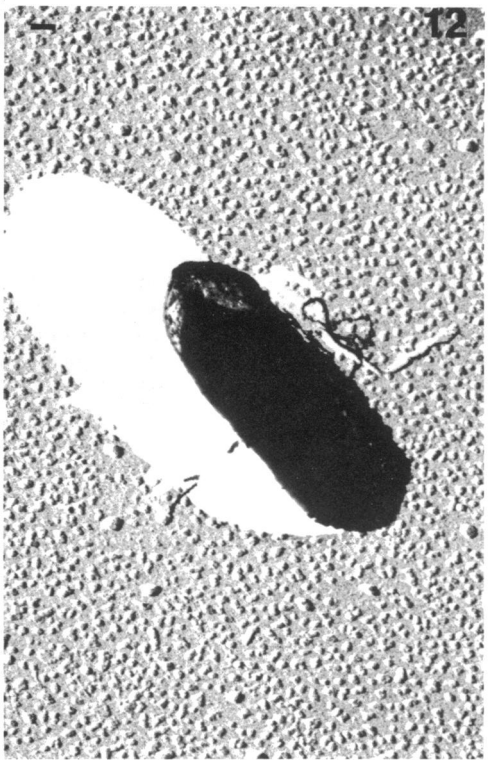
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of the cytoplasmic membrane through either hydrophobic interactions with phospholipid, as the deep-rough outer membrane is quite hydrophobic (2, 17, 20, 33, 41, 42) and the compositions of cytoplasmic and outer membrane are substantially different (2, 20, 40, 41), or through protein-protein interactions with peptidoglycan-associated proteins which are also found within the outer membrane proper (5, 24, 37).

Biochemical analysis of the material released from the cell envelope of strain F515 by sequential exposure to 10 mM citrate buffer (pH 2.75) and 100 mM Tris buffer (pH 8.00) was consistent with the morphological observations indicating that the material was outer membrane. The material was found to contain protein (including all major outer membrane proteins [Fig. 13] [10]), LPS, and phospholipid (Tables 1 and 2). The phospholipid contained in the material was identified as cardiolipin, phosphatidylethanolamine, and phosphatidylglycerol (Table 2), normal constituents of the outer membrane (34, 46). The material also contained a significant amount of alkaline phosphatase (Table 1) which has previously been shown to be associated with the outer membrane in this strain (25–27). The citrate-Tris treatment consistently released ~7% of the outer membrane from the cell envelope (based on the extent of LPS released), although occasional fluctuations in yield were noted. The material released from the cell envelope by citrate-Tris treatment was substantially different from the material released due to exposure either to citrate buffer or to Tris buffer, both in terms of composition and individual proteins released (Table 1; Fig. 13).

The proteins released by exposure of cells to citrate and Tris buffer included proteins with apparent molecular weights of 37,500, 32,500, 17,000, and 11,500, values which are in good agreement with those reported for the major outer membrane proteins of *E. coli* (15). The relative quantity of these proteins was fairly low and presumably was due to an extensive release of periplasmic components and the presence of outer membrane components which are generally not retained in outer membrane preparations (most outer membrane purification proce-

TABLE 1. *Compositional analysis of material released from E. coli O8 strain F515 by exposure to water, 100 mM Tris buffer (pH 8.00), 10 mM citrate buffer (pH 2.75), or 100 mM Tris buffer (pH 8.00) after cells were exposed to 10 mM citrate buffer (pH 2.75)*

Sample	Protein (μg/ml)	LPS (nmol of KDO[a]/ml)	Alkaline phosphatase	
			U/ml	U/mg of protein
Water wash[b]	725	14	3.48[c]	4.80
Tris wash[d]	791	97	6.18	7.83
Citrate wash[d]	835	5	0.02	0.03
Tris wash of citrate[d]-washed cells	1,423	63	18.75	13.18
Water-citrate-Tris-washed cells	9,670	785	11.41	1.18
CM[e] extract of Tris wash of citrate-washed cells	ND[f]	14	ND	ND

[a] KDO, 2-Keto-3-deoxyoctonate.
[b] Cells (1 liter) were harvested and concentrated to 20 ml, and the sample was split and treated as described.
[c] U, 1 μmol of *p*-nitrophenol hydrolyzed per min.
[d] Glucose-6-phosphate dehydrogenase and succinic dehydrogenase were not detectable, but sonic extracts of whole cells gave good activities for both enzymes.
[e] CM, Chloroform-methanol extraction as described by Bligh and Dyer (4).
[f] ND, No detectable material.

TABLE 2. *Thin-layer chromatography analysis of lipid extracted from the outer membrane component of E. coli O8 strain F515 released by treating cells with 10 mM citrate (pH 2.75) and 100 mM Tris (pH 8.00)*

Sample	R_f[a]	Ninhydrin reaction	Phosphate reaction[b]
Phosphatidylethanolamine	0.64	+	+
Phosphatidylserine	0.45	+	+
Phosphatidylglycerol	0.76	−	+
Phosphatidic acid	0.96	−	+
Cardiolipin	0.90	−	+
A[c]	0.93	−	+
B[d]	0.77	−	+
C[e]	0.64	+	+

[a] Using solving system IV of Ames (1).
[b] Phospray (Supelco, Inc.).
[c] A, Identified as cardiolipin.
[d] B, Identified as phosphatidylglycerol.
[e] C, Identified as phosphatidylethanolamine.

FIG. 9. *Interference contrast micrograph of material released from E. coli O8 strain F515, previously treated with 10 mM citrate buffer (pH 2.75), into 100 mM Tris buffer (pH 8.00). Compare a single whole cell (C) to the vesicles (V). Bar represents 1 μm.*

FIG. 10 and 11. *E. coli O8 strain F515 exposed to 10 mM citrate buffer (pH 2.75) and 100 mM Tris buffer (pH 8.00) and subsequently critical point dried and shadowed. Note the evaginations of the outer membrane (B), which eventually dissociate from the cell to form outer membrane vesicles (OMV).*

FIG. 12. *Untreated cells of E. coli O8 strain F515 which were critical point dried and shadowed with platinum. Note the absence of outer membrane blebs and the absence of free outer membrane vesicles.*

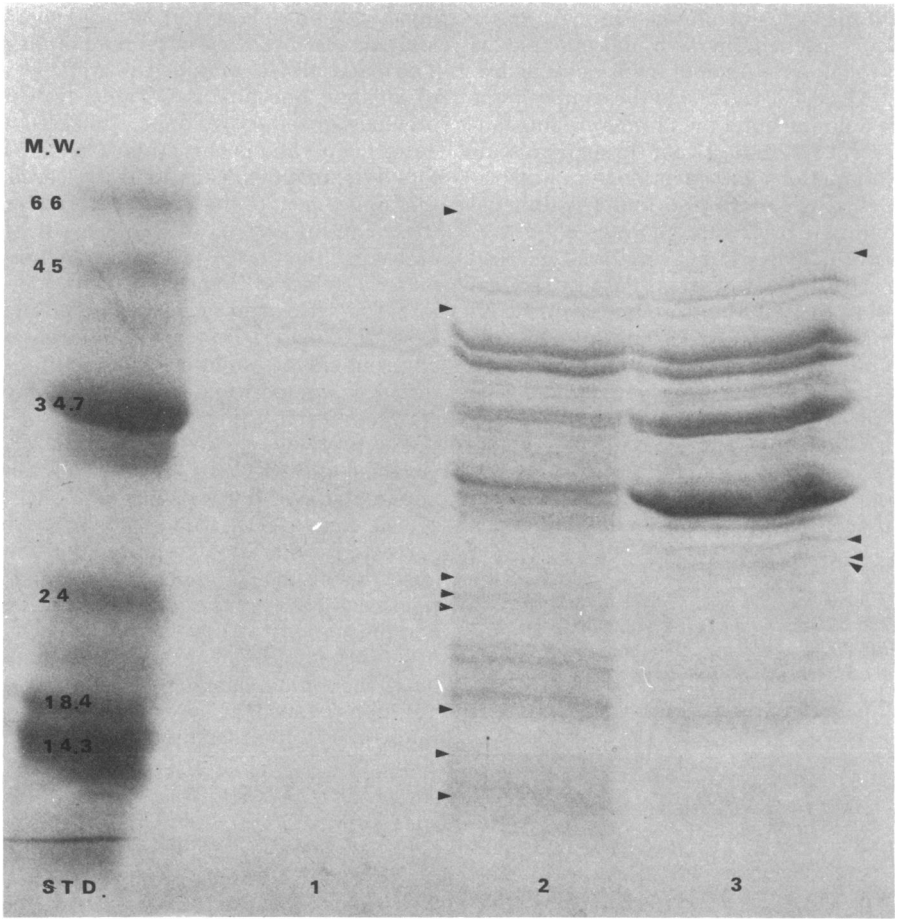

FIG. 13. *Sodium dodecyl sulfate gel of the cell envelope material released from E. coli O8 strain F515 by exposure to 10 mM citrate buffer (pH 2.75) (lane 1), sequential exposure to 10 mM citrate buffer (pH 2.75) and 100 mM Tris buffer (pH 8.00) (lane 2), and exposure to 100 mM Tris buffer (pH 8.00) (lane 3). The citrate-released material (lane 1) and the Tris buffer-released material (lane 3) were concentrated twofold before being applied to the gel. Bands which are present in the citrate-Tris-released material or in the Tris-released material but not in the other are marked with arrowheads (note that some of the bands did not photograph well).*

dures utilize Tris buffer or EDTA or both, which releases a significant amount of outer membrane proteins from the outer membrane). That most of the proteins present in the outer membrane released by citrate-Tris treatment were present (although greatly reduced in quantity [Table 1]) in the material released by exposure solely to Tris buffer is not disturbing, as outer membrane is normally continually overproduced and sloughed off the cell, and with any washing procedure a certain amount of outer membrane is released to the wash. That exposure of cells solely to Tris buffer resulted in substantially more LPS, less protein, and less alkaline phosphatase being released from the cell envelope compared with citrate-Tris exposure supports the supposition that citrate and Tris act concertively to release sections of outer membrane (Table 1). The citrate-Tris treatment released outer membrane from cells in the mid-log phase as readily as from the late log phase (the phase of growth used primarily in these studies) but, owing to the convenience of having maximal levels of alkaline phosphatase (25) and of having a higher total yield, late-log-phase cells were utilized (unpublished data, this laboratory). The release of outer membrane from the cell envelope of *E. coli* O8 strain F515 required pretreatment of cells with citrate or EDTA and subsequent exposure to Tris buffer and could not be accomplished by manipulations of pH or ionic strength (unpublished data, this laboratory).

The release of outer membrane appeared to require Tris buffer, as neither *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), cacodylic acid, nor boric acid could replace the Tris buffer (unpublished data, this laboratory).

The mechanism of citrate-Tris-mediated release of outer membrane from the cell envelope of this deep-rough strain of *E. coli* O8 is elusive but appears to imply perturbation of the interaction of the outer membrane with either lipoprotein which is covalently associated with the peptidoglycan (5) or with peptidoglycan-associated proteins (13, 24, 37, 43) (to allow for separation of the outer membrane from the cell envelope) as well as perturbation of interactions within the outer membrane proper (i.e., to produce a free end). It is likely that these interactions are at least partially mediated by LPS or LPS-associated proteins, as wild-type strains will not release outer membrane when sequentially exposed to citrate and Tris buffers. Furthermore, it seems likely that these interactions are basically ionic in character and may be disrupted by the introduction of positive changes within the outer membrane, as suggested by Schindler and Teuber (38).

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