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Identification and Partial Characterization of a Novel Bipartite Protein Antigen Associated with the Outer Membrane of *Escherichia coli*

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A study by crossed immunoelectrophoresis performed in conjunction with precipitate excision and polypeptide analysis identified a new antigen complex in the envelope of *Escherichia coli* ML308-225. This antigen corresponds to antigen 43 in the crossed immunoelectrophoresis profile of membrane vesicles (P. Owen and H. R. Kaback, Proc. Natl. Acad. Sci. USA 75:3148–3152, 1978). Immunoprecipitation experiments conducted with specific antiserum revealed that the complex was expressed on the cell surface and that it contained, in equal stoichiometry, two chemically distinct polypeptides termed α and β (M_rs of 60,000 and 53,000, respectively). The β polypeptide was heat modifiable, displaying an apparent M_r of 37,000 when solubilized at temperatures below 70°C. Analysis of fractions obtained following cell disruption, isopycnic centrifugation, and detergent extraction indicated that both α and β polypeptides were components of the outer membrane. The two polypeptides were not linked by disulfide bonds, and neither was peptidoglycan associated. The complex contained no detectable lipopolysaccharide, enzyme activity, fatty acyl groups, or other cofactors. Neither correlated with *E. coli* proteins of similar molecular weight which had previously been shown to be associated with the outer membrane. Antibodies were raised to individual α and β polypeptides. Each of these sera was shown to be subunit specific when tested against denatured membrane proteins. In contrast, each immunoglobulin preparation coprecipitated both α and β polypeptides when tested against undenatured proteins derived from Triton X-100-treated membranes. The results reveal the presence of a novel bipartite protein antigen in the outer membrane of *E. coli*.

The outer membrane of *Escherichia coli* is known to contain a number of major protein species (1, 15). Several of these are porins involved in the passive accumulation of small hydrophilic solutes (2, 19). Others, such as the iron-regulated outer membrane proteins and the vitamin B_{12} receptor, are involved in the TonB-dependent uptake of more specific solutes (4). Other major proteins of the *E. coli* outer membrane include the Braun lipoprotein (the Lpp protein), peptidoglycan-associated lipoprotein, and the *ompA* gene product. In part at least, these appear to play a structural role for the cell envelope (1, 15). Several other minor proteins and enzymes have also been identified and studied (10, 17, 20, 35). In addition to the above components, there exist in the outer membrane several additional proteins about which considerably less is known, e.g., protein III, an 83-kilodalton iron-regulated protein, and several proteins in the 60- to 40-kilodalton range (15, 26).

In an attempt to identify and characterize the major antigens of the cell envelope of aerobically grown *E. coli*, a crossed immunoelectrophoresis (CIE) reference profile has been established in which over half of the 50 or so resolved antigens have been identified in functional terms or partially characterized (for reviews, see references 21 to 23). The most dominant antigens of the outer membrane include lipopolysaccharide (LPS), the Braun lipoprotein, the OmpF and OmpC porins, the OmpA protein, and two unidentified antigens termed antigens 35 and 43. In the present communication, we focus on antigen 43 and show that it displays properties which clearly distinguish it from other protein antigens described to date for the *E. coli* outer membrane.

(Preliminary accounts of this work have been presented [P. Caffrey and P. Owen, 103rd Ordinary Meeting, Society for General Microbiology, Warwick, United Kingdom, 1985, P20; P. Caffrey and P. Owen, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, K10, p. 195]).

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* strains used in this study are listed, together with sources and relevant genotypes, in Table 1. *E. coli* ML308-225 was used in all experiments except when indicated.

Cells were normally grown aerobically at 37°C on minimal medium A containing 1% (wt/vol) disodium succinate as the sole carbon source (5) and supplemented when appropriate with one of the following isotopes (per liter): ^{14}C-labeled protein hydrolysate, 250 μCi; [1,4-^{14}C]succinic acid, 83 μCi; $H_2{}^{35}SO_4$, 0.7 to 5 mCi; L-[^{35}S]methionine, 2.5 to 5 mCi; $^{59}FeSO_4$, 1 mCi; and D-[2-^{14}C]riboflavin, 42 μCi. For the preparation of membranes labeled with [^{3}H]palmitate or [^{3}H]glycerol, cells were grown on M63 medium (8). Changes in the conditions of growth on minimal medium A were only made in experiments designed to elucidate the regulation of antigen 43 in *E. coli* ML308-225. Such alterations included (i) changes in the carbon source (by using glucose, maltose, galactose, lactose, or melibiose, each at a final concentration of 0.4% [wt/vol]); degree of aeration (involving the use of baffled or unbaffled shake flasks and different shake speeds [0, 100, or 200 rpm] and involving anaerobic growth on glucose); pH (pH 6, 7, or 8); temperature (22 or 37°C); osmolarity (by the addition of 15 or 20% [wt/vol] sucrose);

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TABLE 1. *E. coli* strains

E. coli strain	Relevant genotype	Source[a]
ML308-225	*lacI lacZ lacY*$^+$ *lacA*$^+$	H. R. Kaback
W3110	*thy*	T. J. Foster
RK4936	F$^-$ *araD* Δ(*lac*)*U169 flbB btuB*::Tn*10 gyrA relA rpsL metE* λ$^-$	CGSC 6405
C600	*thi thr leu fhuA lacY supE* λ$^-$	CGSC 3004
HB261	*thi thr leu his rpsL flaI*	G. Hazelbauer
BRE69	*araD* Δ(*lac*)U169 *flbB relA rpsL deoC ptsF* Δ*ompA*	E. Bremer
MRE600	Wild type	NCIMB 8270

[a] CGSC, *E. coli* Genetic Stock Center, Yale University, New Haven, Conn. NCIMB, National Collection of Industrial and Marine Bacteria, Torry Research Station, Aberdeen, Scotland.

(ii) changes in the concentrations of Mg^{2+} (from 400 to 0 μM), SO_4^{2-} (400, 80, and 8 μM), and K^+ (100 mM, 5 mM, 50 μM, and 0 mM); and (iii) various supplements of the growth medium, including $FeSO_4$ (10 μM), $FeCl_3$ (10 and 100 μM), citrate (1 mM) with or without $FeCl_3$ (100 μM), hemoglobin (10 μM), vitamin B_{12} (10 μg to 1 ng/ml), riboflavin (0.1 mM), molybdate (1 mM), nitrate (10 mM), fumarate (10 mM), and the amino acids tryptophan, phenylalanine, tyrosine, and lysine (each at 0.2% [wt/vol]).

Cells, grown to an A_{560} of 1.0, were harvested by centrifugation at 10,000 × *g* at 4°C for 5 min and washed once in distilled water.

Serotyping. O and H serotyping was performed at the International *Escherichia* and *Klebsiella* Center, Statens Seruminstitut, Copenhagen, Denmark.

Preparation of membrane fractions. Bacterial envelopes were harvested by centrifugation following French pressure cell lysis of spheroplasts in the presence of lysozyme and EDTA (26, 34). Lysozyme was omitted from this procedure only in the experiments relating to peptidoglycan association (26). Inner and outer membranes were separated by subjecting washed envelopes to isopycnic centrifugation (200,000 × *g* for 48 h at 4°C) on 30 to 55% (wt/wt) sucrose gradients as previously described (8).

To establish the solubility of membrane proteins in detergent solution, ^{14}C-amino acid-labeled envelopes (50 μl; 2 mg of protein per ml) in 50 mM Tris hydrochloride buffer (pH 7.5) were treated at either 25 or 0°C with an equal volume of detergent solution (2% [vol/vol] for Triton X-100 and Nonidet P-40 and 1% [wt/vol] for sodium deoxycholate, cetyl trimethylammonium bromide, and sodium *N*-lauryl sarcosinate) in buffered Tris (pH 7.5). Pellets and supernatant material obtained by centrifugation (150,000 × *g* for 1.5 h at 25°C) in an airfuge (Beckman Instruments, Inc., Fullerton, Calif.) were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. For analysis by CIE, membrane fractions were solubilized as previously described (24).

All membrane fractions were prepared and extracted in the presence of a cocktail of protease inhibitors containing N^{α}-*p*-tosyl-L-lysinechloromethylketone (1 mM), phenylmethylsulfonyl fluoride (1 mM), and benzamidine hydrochloride (1 mM) and were stored at −70°C (26).

Purification of outer membrane antigens. LPS was isolated from lyophilized envelopes of *E. coli* ML308-225 by phenol-water extraction (33). Flagellar filaments were purified from *E. coli* W3110 as detailed by dePamphilis and Adler (6).

Preparation of immunoglobulins. Antisera to envelopes of *E. coli* ML308-225 were raised in rabbits by intradermal injection as previously described (24). Serum specific for antigen 43 (anti-43 serum) was raised in a similar manner by using precipitates excised from 40 wet CIE gels (24). For the preparation of immunoglobulins to individual polypeptides (i.e., anti-α and -β sera), immunoprecipitated antigen 43 was dissociated at 100°C in Laemmli sample buffer (14) and subjected to SDS-polyacrylamide gel electrophoresis by using 10% (wt/vol) polyacrylamide separating gels. Strips of acrylamide containing either the α or β polypeptide were excised from gels which had been stained and dried. These were rehydrated in 0.1 M NaCl and macerated in a tissue homogenizer with adjuvant appropriate for the immunization schedule (24). Serum directed against the β polypeptide and found to contain significant titers of antibody to LPS was exhaustively adsorbed by two rounds of incubation (2 h at 25°C) with LPS purified from *E. coli* ML308-225 (final concentration, 260 μg/ml of serum). The isolation and concentration of immunoglobulins have been described in detail elsewhere (24).

Progressive immunoadsorption experiments (22) involving adsorption of antienvelope and anti-43 immunoglobulins with washed cells of *E. coli* ML308-225 were performed essentially as outlined by Owen and Kaback (27).

Immunoprecipitation of antigen 43. Pilot experiments conducted in the presence of a cocktail of protease inhibitors (8) established the following conditions for optimum immunoprecipitation: viz., incubation at 25°C for 18 h of mixtures containing purified immunoglobulin and Triton X-100-solubilized membranes at relative protein concentrations of 47:1 (anti-43), 240:1 (anti-α), and 310:1 (anti-β).

Electrophoretic techniques. Procedures for performing all immunoelectrophoretic procedures have been previously documented in detail (24). In experiments involving excision of CIE immunoprecipitates combined with polypeptide analysis, all antibody preparations and buffers were pretreated with a cocktail of four protease inhibitors (8). The concentration of inhibitors in the gels and buffers was 1/40 of that used in the pretreatment of the serum (8).

SDS-polyacrylamide gel electrophoresis (14) was performed by using 7.5, 10.0, 12.5, or 15% (wt/vol) acrylamide separating gels, 4.5% (wt/vol) acrylamide stacking gels, and up to 16 molecular weight standards (5). Samples were routinely heated for 5 min at 100°C in Laemmli sample buffer (14) containing 2-mercaptoethanol except when indicated below. Proteins were detected by autoradiography or fluorography or by staining with Coomassie brilliant blue. Carbohydrates (LPS) were detected by the sensitive silver-staining method (32). The procedure for Western blotting (immunoblotting) has been described previously (8). Limited proteolysis by using staphylococcal V8 protease was performed on ([^{35}S]methionine-labeled) proteins excised from SDS-polyacrylamide gels. The products of proteolysis were resolved by electrophoresis in SDS by using 15% (wt/vol) polyacrylamide separating gels and were detected by protein staining and by autoradiography (28).

Electron microscopy. Samples were negatively stained with 1% (wt/vol) ammonium molybdate containing 0.1% (wt/vol) bovine serum albumin (pH 6.8) and examined in a Philips EM300 electron microscope.

Analytical procedures. Protein was estimated by a modification (9) of the Lowry procedure which eliminates interference from Triton X-100. Bovine serum albumin was used as the standard. Lipid extraction was performed essentially as described previously (8). Stoichiometry measurements were computed from densitometer scans performed by a Joyce Loebl (Gateshead, England) densitometer on autoradi-

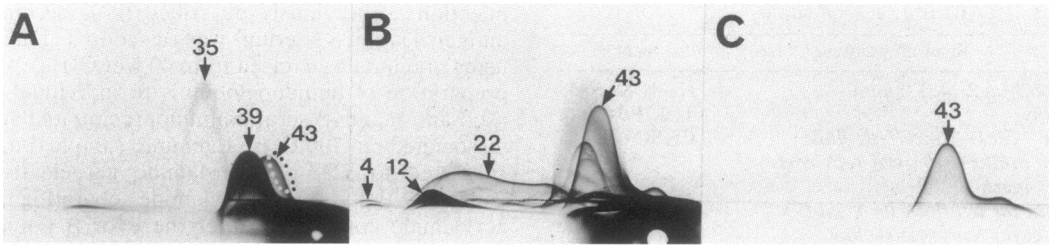

FIG. 1. Resolution of antigen 43 by CIE and generation of specific antiserum. Triton X-100–EDTA extracts (A to C, 12, 40, and 30 μg of protein, respectively) of outer membranes (A) or envelopes (B and C) of *E. coli* ML308-225 were analyzed by CIE against antienvelope immunoglobulins (A and B, 3.4 and 3.6 mg of protein per ml of gel, respectively) or against anti-43 immunoglobulins (C, 3.3 mg of protein per ml of gel). Several of the salient antigens, including antigens 4 (common protein antigen), 12 (ATPase), 22 (succinate dehydrogenase), 35, 43, and 39 (OmpF and OmpC proteins), are identified by number by the system of Owen and Kaback (27). Indicated by dots in panel A is the region of immunoprecipitate 43 chosen for excision and subsequent immunization to generate anti-43 serum (C). The single major immunoprecipitate observed in panel C contrasts with the complex profile observed following analysis of similar preparations against polyvalent antibody (B). The anode is to the left and top of all immunoplates.

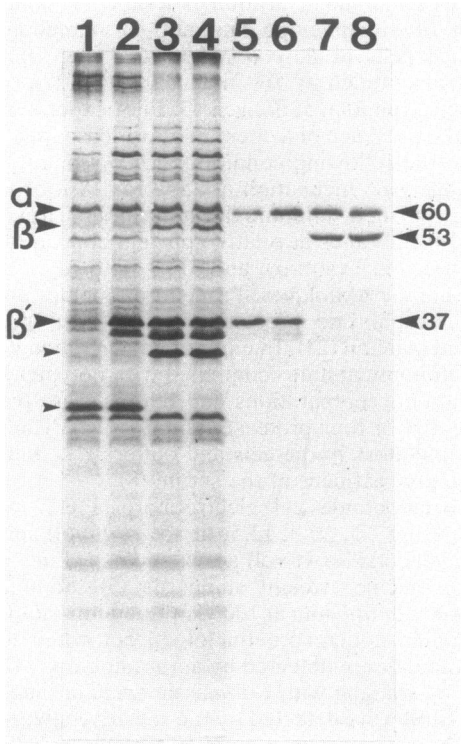

FIG. 2. Subunit composition of antigen 43. A Triton X-100–EDTA extract of envelopes prepared from *E. coli* ML308-225 grown in the presence of ^{14}C-amino acids was incubated with anti-43 immunoglobulins (Materials and Methods), and the resultant immunoprecipitate was analyzed by SDS-polyacrylamide gel electrophoresis by using separating gels containing 12.5% (wt/vol) acrylamide. Lanes: 1 to 4, Triton X-100–EDTA extract (20 μg of protein) of ^{14}C-labeled envelopes; 5 to 8, immunoprecipitated antigen 43. Samples were heated prior to electrophoresis in Laemmli sample buffer (14) containing 2-mercaptoethanol at 50°C for 5 min (lanes 1 and 5), 70°C for 5 min (lanes 2 and 6), 100°C for 5 min (lanes 3 and 7), and 100°C for 15 min (lanes 4 and 8). The apparent molecular masses (in kilodaltons) of the α and β (β′) polypeptides of antigen 43, together with their counterparts in the profile of envelope proteins, are indicated at the side of the autoradiogram. Note that at 70°C (lane 2), the β′ polypeptide is in part masked by the OmpF protein. Indicated by small arrowheads are the identities of both molecular-weight forms of the OmpA protein. The R_f values of the 60-, 53-, and 37-kilodalton proteins with respect to the 33-kilodalton form of the OmpA protein were 0.53, 0.59, and 0.90, respectively.

ograms and fluorograms of dried SDS-polyacrylamide slab gels resolving ^{14}C-protein hydrolysate-labeled polypeptides.

Radiochemicals. [9,10(*n*)-^{3}H]palmitic acid (50 Ci/mmol), [2-^{3}H]glycerol (1 Ci/mmol), U-^{14}C-protein hydrolysate (56 mCi/mg of atom), [1,4-^{14}C]succinic acid (117 mCi/mmol), D-[2-^{14}C]riboflavin (60.6 mCi/mmol), L-[^{35}S]methionine (1,390 Ci/mmol), and ^{125}I-labeled protein A (30 mCi/mg) were obtained from Amersham International, Amersham, United Kingdom, and $^{59}FeSO_4$ (32.5 mCi/mg) was from New England Nuclear Corp., Boston, Mass.

RESULTS

Preparation of specific antiserum. Antiserum specific for antigen 43 was raised by immunization with immunoprecipitates excised from CIE immunoplates resolving outer membrane antigens (Materials and Methods and Fig. 1). The resultant pool of anti-43 immunoglobulins generated a single intense immunoprecipitate when tested against cell envelopes of *E. coli* ML308-225 by CIE (Fig. 1C), by CIE with intermediate gel, or by immunodiffusion (data not shown). As anticipated (23, 25), anti-43 antibody, as well as preimmune serum, also contained trace levels of immunoglobulins directed against common protein antigen (4 in Fig. 1B; see Fig. 4A).

Polypeptide composition. Analysis on SDS-polyacrylamide gels of the immune complex obtained following incubation of anti-43 antibody with Triton X-100 extracts of ^{14}C-amino-acid-labeled envelopes of *E. coli* ML308-225 revealed the presence of two bacterial polypeptides (Fig. 2). One (termed α), with an apparent M_r of 60,000, had an electrophoretic mobility which was unaffected by the temperature (50 to 100°C) used to solubilize the complex. The other (termed β or β′) was heat modifiable in a manner similar to that of other outer membrane proteins (3, 15, 20) and migrated with an apparent M_r of 37,000 (β′) if heated to a temperature of 70°C or below and 53,000 (β) if heated at higher temperatures (Fig. 2, lanes 5 to 8). The α and β polypeptides did not appear to be linked by disulfide bonds, since omission of 2-mercaptoethanol did not result in the appearance of any additional molecular species (data not shown). Western blotting experiments confirmed these observations and also indicated that anti-43 immunoglobulins possessed antibodies capable of recognizing the α polypeptide and both M_r forms of the β polypeptide (see Fig. 4A, lanes 3 and 4).

None of the above observations appeared to be artifacts caused by the presence of immunoglobulin, since identical

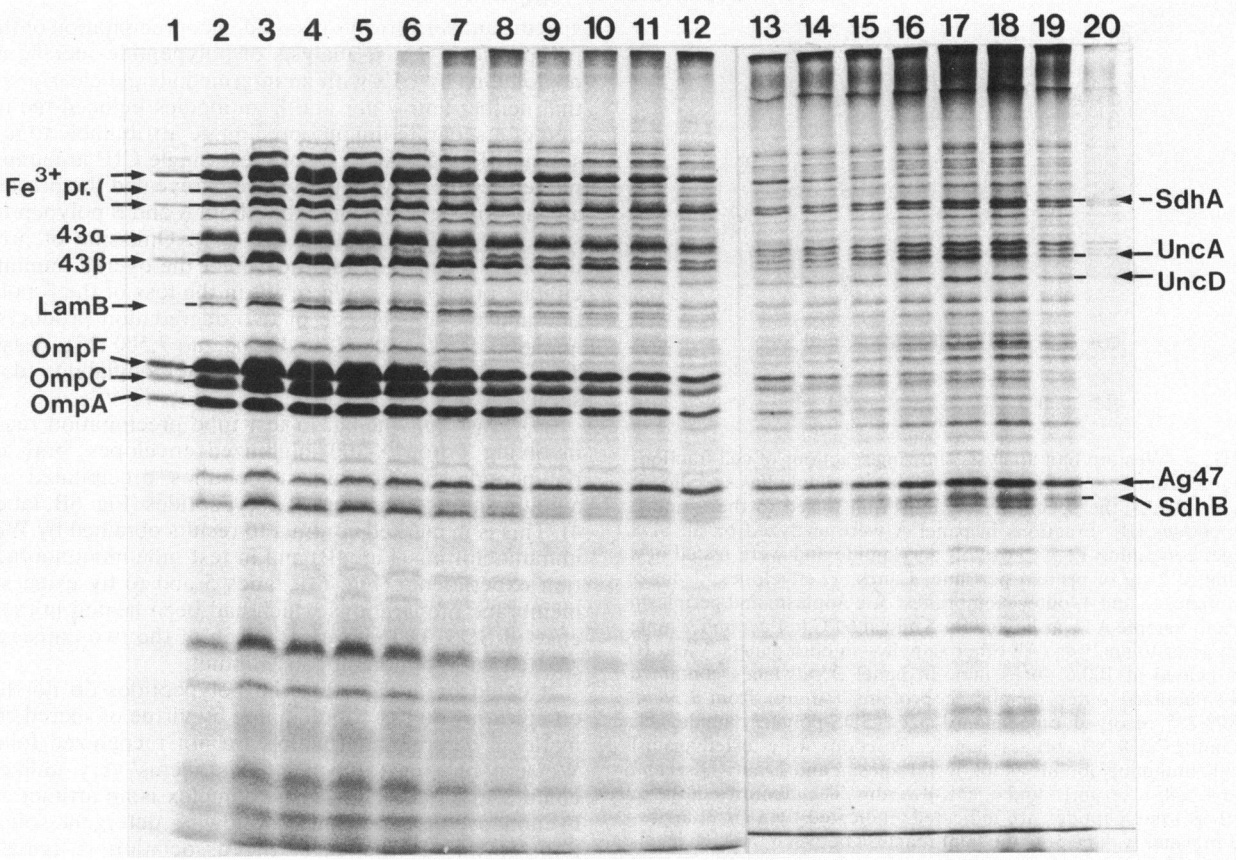

FIG. 3. Cellular location of antigen 43. ^{14}C-labeled envelopes of *E. coli* ML308-225 were subjected to isopycnic centrifugation, and the membranes in each of the resultant 0.5-ml fractions were harvested (8). Equivalent volumes of all fractions were analyzed by SDS-polyacrylamide gel electrophoresis. Fraction 1 represents material harvested from the bottom of the gradient. The identities of the α and β subunits of antigen 43 together with those of known marker proteins for both the outer membrane (the OmpA, OmpF, OmpC, LamB, and iron-regulated proteins [Fe^{3+} pr.]) and inner membrane (antigen 47 [Ag47] and the SdhA, SdhB, UncA, and UncD proteins) are indicated at the sides of the autoradiogram.

behavior was observed for the corresponding polypeptides in isolated envelopes (Fig. 2, lanes 1 to 4). Furthermore, other molecules whose apparent M_rs are temperature dependent (e.g., the OmpA, OmpF, and OmpC proteins [15]) or are known to be affected by 2-mercaptoethanol (immunoglobulin G) displayed the anticipated behavior (Fig. 2).

To establish whether the protein complex contained LPS, similar immunoprecipitation experiments were performed on envelopes prepared from *E. coli* ML308-225 grown in the presence of [^{14}C]succinate. Profiles identical to that shown in Fig. 2, lane 8, were obtained for immunoprecipitated antigen 43 following fluorography. Even on prolonged exposures or following silver staining for carbohydrate (32), there was no trace of the ladder profiles characteristic of the heterogeneously sized smooth LPS of this organism (data not shown). A 1% (wt/wt) concentration of LPS would have been readily detected by these procedures.

Location. To establish convincingly the location of antigen 43 in the cell envelope, inner and outer membranes were separated by isopycnic centrifugation and the sedimentable material in all fractions from the sucrose gradient was analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 3) and by fused rocket immunoelectrophoresis (data not shown). Both polypeptides of antigen 43 partitioned in a manner identical to that observed for known marker proteins of the outer membrane (15, 26), being most prevalent in membranous material of buoyant density (ρ) of 1.24 g/ml (Fig. 3, lanes 3 to 5). None of these proteins featured in membranous material (ρ, 1.18 to 1.16 g/ml) containing established (5, 8, 26) marker polypeptides for the plasma membrane (Fig. 3, lanes 15 to 20).

These results were confirmed and extended in Western blotting experiments (Fig. 4A) and by rocket immunoelectrophoresis (data not shown) by using various isolated cellular fractions. In contrast to outer membranes and envelopes, the isolated inner membranes, soluble (cytoplasm and periplasm) fractions of the cell, and concentrated cell-free supernatants (data not shown) did not possess polypeptides reactive with anti-43 antibody (Fig. 4A).

More recent immunogold-labeling experiments performed on thin sections of *E. coli* (P. Caffrey, J. Beesley, and P. Owen, unpublished data) also clearly locate antigen 43 to the outer membrane. From all these data, there seems little doubt that antigen 43 is a component of the outer membrane.

Surface expression of antigen 43. Cells of *E. coli* ML308-225 were readily agglutinated by specific anti-43 antibodies, and analysis of the resultant adsorbed sera by CIE indicated that significant removal of anti-43 immunoglobulins had occurred (data not shown). This confirms the results of earlier progressive immunoadsorption experiments which established that antigen 43, together with a limited number of other components (the OmpF and OmpC pro-

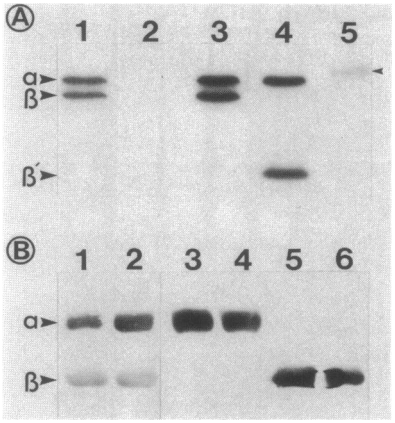

FIG. 4. Western blot analysis showing reactions of cell fractions prepared from *E. coli* ML308-225 with immunoglobulins specific for antigen 43 (A), the specificity of antiserum raised to the α and β polypeptides (B). Fractions in panel A were analyzed on an SDS minigel containing 12.5% (wt/vol) acrylamide and were tested at a loading of 2 μg of protein per lane. Lanes: 1, envelopes; 2, inner membranes; 3 and 4, outer membranes; 5, cytoplasm and periplasm fraction. Sample A4 was heated for 5 min at 60°C in Laemmli sample buffer prior to analysis. All other samples (in both panels A and B) were heated at 100°C for 5 min. In panel B, all lanes contained SDS-solubilized outer membrane proteins (40 μg) from *E. coli* ML308-225 resolved on standard-sized SDS-polyacrylamide gels containing 7.5% (wt/vol) acrylamide. Fractions were tested against specific immunoglobulins as follows. Lanes: 1 and 2, anti-43 serum; 3 and 4, anti-α serum; 5 and 6, anti-β serum. The identities of the α, and β (β') polypeptides are indicated. Indicated by a small arrowhead in panel A, lane 5, is the faint reaction between the high levels of enterobacterial common protein antigen (monomer M_r, 62,000) present in the cytoplasm fraction and low-titer homologous antibody found in serum. Only the relevant parts of the immunoblots are shown.

teins, LPS, and antigen 38), was accessible to antibody on the surface of *E. coli* ML308-225 (23).

Distinct α and β polypeptides. The presence of a cocktail of protease inhibitors during most manipulations (see Materials and Methods) and different properties of heat modifiability strongly suggest that the α and β polypeptides are distinct entities. This was confirmed by the observation that limited (V8) proteolysis of the two proteins generated radically different peptide profiles (data not shown). Furthermore, the two proteins were immunologically distinct since polypeptide-specific antibodies could be raised by immunization with bands excised from SDS-polyacrylamide gels (see Materials and Methods and Fig. 4B).

Complex formed by α and β polypeptides. Several lines of evidence lend support to the hypothesis that the α and β polypeptides represent subunits of a novel (bipartite) protein antigen. (i) Densitometer measurements indicated that the α and β polypeptides were present in equal stoichiometry (0.98:1.0). (ii) The α and β polypeptides copartitioned and maintained this stoichiometry following a range of nondenaturing treatments. These included extraction of envelopes with a variety of detergents (see Materials and Methods) and test tube immunoprecipitation experiments conducted for different time periods up to 24 h and over a 10-fold range of antibody concentration (data not shown).

(iii) The two proteins in question were chemically and immunologically distinct. Yet reactions conducted under nondenaturing conditions with anti-43 or with subunit-specific antisera always resulted in coprecipitation of the two polypeptides. Thus, analysis of polypeptide-specific immunoglobulins by CIE with an intermediate gel clearly showed that neither anti-α nor anti-β antibodies induced the formation of a separate immunoprecipitate attributable to a single subunit (Fig. 5). Analysis of these single CIE immunoprecipitate arcs for polypeptides always revealed the presence in all parts of the arc examined of both α and β polypeptides in similar stoichiometry (see Fig. 6A). Omission of protease inhibitors during CIE did not affect the overall immunoprecipitate profile but could result in the loss of the β polypeptide and the appearance of two degradation products with apparent M_rs (at 100°C) of 43,500 and 7,500 (Fig. 6, lane 3). The former retained properties of heat modifiability (data not shown).

(iv) Most convincing, in test tube precipitation reactions involving Triton X-100-solubilized envelopes, both of the polypeptide-specific immunoglobulins precipitated similar amounts of both the α and β polypeptides (Fig. 6B, lanes 1 to 4). This is in marked contrast to results obtained by Western immunoblotting (Fig. 4B) and in test tube immunoprecipitation experiments (Fig. 6B, lanes 5 and 6) by using similar membrane preparations which had been heated in the presence of SDS. In these cases, each of the two antisera only reacted with its homologous subunit.

It could be argued that the polypeptides do not form a complex in situ but coprecipitate by virtue of shared conformational determinants which are not recognized following Western blotting. However, this seems very unlikely. It could also be argued that the complex is an artifact caused by noncovalent association following detergent solubilization. If this is the case, the association is remarkably specific, since a comprehensive analysis of membrane antigens resolved by CIE for *E. coli* (23, 25) has failed to detect any evidence for random association of polypeptides following detergent solubilization. Cross-linking experiments are presently being undertaken to resolve this issue.

Properties of antigen 43. CIE performed in conjunction with zymogram stains or analysis for specific (radiolabeled) cofactors provides an elegant method for assessing biochemical properties of membrane antigens (21, 23). By applying these techniques to the analysis of unlabeled membranes or membranes prepared from cells grown in the presence of $^{59}Fe^{3+}$, [^{14}C]riboflavin, [^{3}H]palmitate, or [^{3}H]glycerol, it was shown that antigen 43 neither expressed any of a wide variety of enzyme activities (listed in reference 21) nor possessed iron or flavin cofactors. Neither polypeptide was

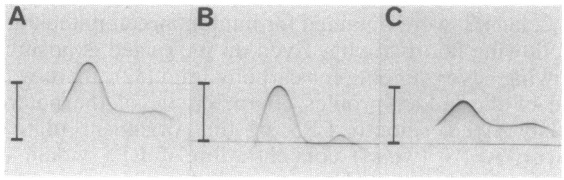

FIG. 5. Analysis of subunit-specific sera by CIE with an intermediate gel. Triton X-100–EDTA extracts (20 μg of protein) of outer membranes were analyzed by CIE by using intermediate gels (bracketed) containing no immunoglobulins (A), anti-α antibody (B; 14 mg of protein per ml of gel), and anti-β antibody (C; 18 mg of protein per ml of gel). In all cases, the main gel contained anti-43 immunoglobulins (2.5 mg of protein per ml of gel). Note that both anti-α and anti-β antibodies caused depression of immunoprecipitate 43 but did not induce the formation of separate immunoprecipitates. The anode is to the left and top of all protein-stained immunoplates.

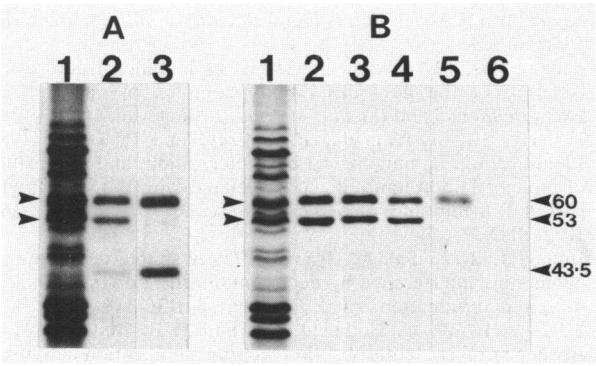

FIG. 6. Coprecipitation of both α and β polypeptides from Triton X-100-treated membranes of *E. coli* ML308-225 during CIE (A) and test tube immunoprecipitation reactions (B). (A) Triton X-100–EDTA extracts of ^{14}C-amino acid-labeled outer membranes were analyzed by CIE against anti-43 immunoglobulins as described in the legend to Fig. 1. Immunoprecipitate 43 was excised, and antigens were extracted (25) for analysis by SDS-polyacrylamide gel electrophoresis. Lanes: 1, Triton X-100–EDTA extract of ^{14}C-labeled envelopes (26 μg of protein); 2 and 3, immunoprecipitate 43 excised from CIE plates run in the presence or absence of protease inhibitors, respectively (see Materials and Methods). (B) Lanes: 1, Triton X-100–EDTA extract (15 μg of protein) of ^{14}C-labeled outer membranes; 2 to 4, immune complexes precipitated from Triton X-100–EDTA extracts of ^{14}C-labeled outer membranes by anti-43, -α, and -β immunoglobulins, respectively; 5 and 6, immune complexes precipitated by anti-α and -β immunoglobulins, respectively, from identical detergent extracts which were preheated at 100°C for 15 min in the presence of 1% (wt/vol) SDS. Samples in both panels were analyzed on SDS-polyacrylamide separating gels containing 12.5% (wt/vol) acrylamide. The apparent molecular masses (in kilodaltons) of the polypeptides, together with their counterparts in the profile of membrane proteins, are indicated at the side of the fluorograms, only the relevant parts of which are shown. Note that although anti-β antibodies reacted strongly in Western blots (Fig. 4B, lanes 5 and 6), they appear to be poorly precipitating for the SDS-treated β polypeptide (lane B6). A faint β band could be detected on prolonged exposure.

soluble in aqueous organic solvents nor labeled with [^{3}H]palmitate or [^{3}H]glycerol in a manner typical of envelope-associated lipoproteins (8). Other experiments involving the treatment of envelopes with a variety of detergents (see Materials and Methods) indicated that antigen 43 had a solubility akin to that of the major outer membrane proteins. Neither polypeptide appeared to be peptidoglycan associated since, unlike the OmpF and OmpC proteins (15), both could be fully solubilized from peptidoglycan-containing envelopes by SDS at 37°C (data not shown).

Polypeptides not related to known envelope-associated *E. coli* proteins. To establish that antigen 43 is indeed a novel protein complex, it is necessary to rule out a relationship between the constituent polypeptides and other documented *E. coli* protein antigens which possess similar molecular weights and properties and which are capable of an association with outer membrane fractions, e.g., flagellin and soluble common protein antigen (monomer M_r, about 60,000 in both instances [1, 25]), the BtuB protein (M_r, 60,000 [29]), the TolC protein (M_r, 55,000 [17]), and the heat-modifiable OmpA protein (15). Although data are not shown, all such candidates were eliminated on the basis of the following experimental evidence.

(i) Flagella. *E. coli* ML308-225 is nonmotile and was serotyped as O13:O68:H$^-$. Flagellar filaments could not be isolated from this strain by the method of dePamphilis and Adler (6). Although both α- and β-polypeptides were detected in outer membranes from motile *E. coli* W3110 cells, flagellar filaments purified from this strain failed to blot with anti-43 immunoglobulins. Furthermore, electron microscopy of negatively stained preparations of cells, purified outer membranes, or immunoprecipitated antigen 43 failed to detect structures characteristic of flagellar filaments or hook and basal bodies (7) or, indeed, of other surface features, such as S layers or fimbriae (11, 30). Finally, both α and β polypeptides were detected by Western blotting in certain *flaI* and *flaB* mutants (Table 1) which do not produce flagellar proteins.

(ii) Common protein antigen. This contaminating antigen generated a CIE immunoprecipitate (4 in Fig. 1B) which was clearly distinct from that formed by antigen 43. Furthermore, antiserum specific for common protein antigen did not react with antigen 43 as judged by CIE with an intermediate gel or by Western blotting.

(iii) BtuB protein. Antigen 43 was not repressed by the addition of vitamin B_{12} to the growth medium (13) and was expressed in a mutant (*E. coli* RK4936) containing an insertion in the *btuB* gene.

(iv) TolC protein. Neither the α nor the β polypeptide displayed the properties of heat modifiability described for the TolC protein (17).

(v) OmpA protein. The β polypeptide was present in an *E. coli* mutant (BRE69) containing a deletion of the *ompA* gene and did not react with anti-OmpA serum in Western blotting experiments.

Finally, the α polypeptide has recently been purified and displays an amino acid composition that is distinctly different from compositions reported for any of the above proteins (P. Caffrey and P. Owen, manuscript in preparation).

Expression of antigen 43. From a consideration of numerous carefully controlled experiments in which only one variable was changed at a time, it was clear that the expression of either protein was not dramatically affected by changes in the growth conditions (Materials and Methods). This is in marked contrast to the behavior of other outer membrane proteins of *E. coli* ML308-225, such as the OmpF, OmpC, and LamB proteins and iron chelate receptors, for example, which respond to changes in osmolarity and maltose and iron concentrations in the anticipated fashions (15, 19).

It soon became clear, however, that the yield of the protein fluctuated widely but did so in a manner that did not correlate with conditions of growth. These fluctuations were most convincingly established when cells were grown at different times under identical conditions in the same batch of growth medium. Again this was in sharp contrast to the behavior of most other outer membrane proteins. The different yields of antigen 43 could not be attributed to loss of the antigen into the growth medium or wash buffers or to loss by proteolysis.

Antigen 43 has been detected by Western blotting in a wide variety of *E. coli* strains, both rough and smooth. In some, notably ML308-225, C600, and BRE69 (Table 1), the two polypeptides were readily detected in the Coomassie blue-stained profile of outer membranes resolved by SDS-polyacrylamide gel electrophoresis. In others, the polypeptides could only be discerned by Western blotting. Analysis of over 20 miscellaneous K-12 derivatives (not listed in Table 1) has failed to indicate any relationship between genotype and antigen 43 production.

DISCUSSION

The balance of the available data strongly supports the hypothesis that antigen 43 is an outer membrane component composed of two chemically and immunologically distinct protein subunits in a 1:1 ratio, i.e., $(\alpha\beta)_x$. The detection of such a bipartite (heterooligomeric) protein antigen is interesting since it implies a complexity of organization not normally associated with outer membrane components. Most outer membrane components described to date appear to exist as monomers or as homooligomers (e.g., homotrimers). There is some evidence from cross-linking experiments for interactions between the OmpA protein and the Braun lipoprotein (15), but a complex of defined stoichiometry has not been isolated. There is also some evidence that the closely related OmpF and OmpC proteins can form mixed (hetero)trimers in situ (12), but this is controversial (15). Perhaps the strongest evidence for a heteropolymeric complex of two outer membrane proteins occurs in *Neisseria gonorrhoeae*, in which several groups have reported an association between the major porin (protein I) and the 2-mercaptoethanol-modifiable protein, protein III (16, 18, 31).

Despite considerable effort, we are still unable to ascribe a function to antigen 43. The protein complex possessed no obvious enzyme activity or common cofactors. A detailed study of its expression has also failed to give positive indications of function. However, in its variability of yield, antigen 43 does differ significantly from the other major *E. coli* outer membrane proteins. We suspect that a form of phase variation may account for these observations. It is possible that antigen 43 represents for *E. coli* a novel type of S-layer (30) or surface appendage (36). If this is the case, it does not possess the distinctive morphology which normally characterizes such structures (11, 30). Purification and characterization of the individual subunits, together with careful topographical studies (presently in progress), should help to assess these possibilities.

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