STUDY OF ENVELOPE PROTEINS IN *E. COLI cet* AND *recA* MUTANTS BY SDS ACRYLAMIDE GEL ELECTROPHORESIS

I.B. Holland† and Susan Tuckett

Division of Biology
The University of Texas at Dallas
P.O. Box 30365, Dallas, Texas 75230

(Received March 16, 1972)

Disc-electrophoresis of *E. coli* envelope proteins on SDS acrylamide gels reproducibly revealed up to 50 distinct polypeptide bands. Corresponding molecular weights ranged from 105,000 to 20,000 daltons or less. Major bands corresponded to molecular weights of 73,000, 48,000, 36,000 and 30,000 with the latter constituting up to 20% of the total envelope protein depending upon the method of isolation. Minimum levels of detection using stained gels equaled 0.25 µg protein or 1% of total sample analyzed; for a polypeptide of molecular weight 40,000 daltons this was calculated to be equivalent to 1,200 molecules per cell envelope. In envelopes from a *cetB*⁻ mutant strain (refractory to colicin E2), an additional band, constituting up to 5% of the total envelope protein was present. The molecular weight of this protein, which was maximally present in wild type envelopes in only trace amounts, is 44,000 daltons, indicating a cellular concentration of approximately 6×10^3 molecules per envelope. This new band was not affected by heating envelope preparations to 100° prior to electrophoresis, but was largely eliminated by washing isolated envelopes in low ionic strength buffer, or by pre-incubating cells with trypsin prior to preparation of envelopes. Treatment of isolated envelopes with Triton X-100, which preferentially releases inner membrane proteins from the envelope (18), resulted in the extraction of a preponderance of the high molecular weight polypeptides, including the 44,000 dalton protein from envelopes of the mutant. The major polypeptides of the envelope and the low molecular weight components were not extracted by Triton X-100. The properties of the 44,000 dalton protein indicated that it is relatively loosely associated with the surface envelope and may be exposed on the external surface of the cytoplasmic membrane. Possible explanations for the appearance of this protein in mutant strains and its relationship to the inability of these to respond, specifically to surface bound colicin E2, will be discussed. Extensive analysis of envelopes from *recA*⁻ mutants was also carried out and revealed an unusual amount of variation in polypeptide profiles obtained from different preparations. However, no consistent quantitative or qualitative difference between *recA* and *rec*⁺ strains was obtained. In *recA*,*cetB* double mutants, the increased level of the 44,000 dalton polypeptide was identical to that found in the *rec*⁺,*cetB* mutant.

† Present address: Department of Genetics, University of Leicester, Leicester LE1 7RH, England; to whom reprint requests should be sent.

1. INTRODUCTION

Previous studies of the polypeptide content of *Escherichia coli* envelopes and membrane fractions (1-5) have been based upon the sodium dodecyl sulphate (SDS) polyacrylamide gel systems first described by Shaprio, Vinũela and Maizel (6) and Webber and Osborn (7). Recently Laemmli (8) has described a method of disc-electrophoresis with SDS-acrylamide gels in which greatly improved resolution of bacteriophage T4 proteins was observed. Using this technique we now show that greatly increased resolution of *E. coli* envelope proteins can also be obtained. In addition, in an attempt to maximize reproducibility of profiles and to establish some physico-chemical characters of the major polypeptides present, several factors involved in preparations or disaggregation of the envelope were examined. With these techniques we have initiated a study of the functional organization of the proteins of the *E. coli* envelope by examining mutant strains thought to have altered membranes. We have previously isolated a series of pleiotropic mutants of this type (9) which adsorb colicin E2, but fail to show the rapid degradation of DNA found in wild type strains, and the cells survive. In a preliminary study we have shown that envelopes isolated from these cet^- mutants have increased levels of a specific polypeptide compared to the wild type. We have now confirmed the presence of this polypeptide in the cell surface of one such mutant *in vivo* and in isolated envelopes prepared under a variety of conditions. This protein, molecular weight 44,000, appears to be relatively loosely associated with the cell envelope and is probably located close to the external surface of the cytoplasmic membrane. A previous report by Inouye (10) has indicated that *recA* mutants may also have altered cell membranes. Extensive analysis of a *recA* mutant was therefore carried out and revealed considerable variation in envelope proteins obtained from different preparations but no consistent difference between envelopes from rec^+ and *recA* strains was observed.

2. METHODS

(a) *Organisms*

ASH102 (*cetB3*) is a colicin E2 refractory mutant derived from the E2-sensitive strain ASH10 (cet^+ *thi met thyA leu lac* $str^r\lambda^+$, F^-) and has been described previously (9). ASH10 (thy^+ *recA*) and ASH102 (thy^+ *recA*) were isolated as recombinants after crossing with the *recA1* strain Hfr KL16 and were kindly provided by R.S. Buxton. Organisms were grown in Nutrient broth (Oxoid No. 2) or minimal glucose medium (M9) supplemented with thymine (80 µg per ml) and amino acids as described previously (11).

(b) *Preparation of envelopes*

Strains grown in nutrient broth to stationary phase, or in M9-glucose medium, to late exponential phase (3×10^8 cells per ml), were washed twice in 40 ml 10^{-2} M potassium phosphate buffer, pH 7.1 (K-buffer) and resuspended in K-buffer to approximately 10^{10} cells per ml. Samples, 5 to 10 ml, were sonicated at 4° with a Branson Sonifier at full deflection for four 30 sec periods, with 60 sec rest intervals. Sonicated samples were then centrifuged at 40,000 rpm (110,00 x g) in a Model L Spinco for 30 min at 4°. The pellet was resuspended in K-buffer and again centrifuged at 110,000 x g. This washing procedure was repeated twice more and the final pellet (envelope) suspended in a small volume of sodium phosphate buffer, pH 7.1 (Na-buffer) to a final concentration of 2 to 7 mg protein per ml. It is essential to use a sodium based buffer in order to avoid subsequent precipitation of sodium dodecyl sulphate (SDS) and for the same reason SDS must be used after recrystallization from absolute ethanol. Envelope material was then dissolved by the addition of

SDS and 2-mercaptoethanol, each to a final concentration of 1%. Starting from 10^{11} cells this procedure usually yielded 2 mg protein in the final envelope pellet. This represents 8% of the total protein of the clarified sonicate as determined by the Lowry method, or 5% as determined by radioactive content when envelopes were isolated from cells previously labeled with [^{14}C]amino acids. Envelope fractions contained less than 0.03% sonicate RNA and less than 0.5% sonicate β-galactosidase. Dissolved envelopes were stored, prior to analysis, at -20° and were quite stable. For Triton X-100 treatment of envelopes, samples suspended in Na-buffer were adjusted to 2% (w/v) with respect to the detergent, incubated 30 min at 20° and centrifuged 30 min at 160,000 x g. Pellets and supernatants after addition of SDS and mercaptoethanol were then stored at -20° as above. In control experiments comparable analyses of envelope and cytoplasmic proteins from a cell sonicate showed no major contamination of the envelope with cytoplasmic proteins.

(c) *Gel electrophoresis*

The basic electrophoretic procedure and gel constituents followed that of Laemmli (8). To glass tubes (14 cm x 0.6 cm), previously rinsed with 0.5% Photoflow (Kodak), was added 9 cm (2.6 ml) of the separating gel containing 10 or 8.0% acrylamide, 0.1% SDS and 0.3 M Tris·HCl, pH 8.8. A 2.3 cm (0.7 ml) layer of stacking gel, containing 3% acrylamide, 0.1% SDS and 0.125 M Tris·HCl, pH 6.8, was then added. The electrode buffer, pH 8.3, contained 0.025 M Tris, 0.192 M glycine and 0.1% SDS. Electrophoresis was carried out with a Canalco electrophoresis apparatus at room temperature at 4 mA per gel for approximately 6 hr. Electrophoresis was terminated when the bromophenol blue marker reached approximately 5 mm from the end of the tube. Gels were removed from tubes, rinsed in distilled water and fixed and stained with Coomassie blue as described previously (2). Finally gels were destained by continuous washing with 7.5% acetic acid in a Canalco Destainer. The relative protein content of different bands was estimated from the areas under the peaks.

(d) *Sample application*

The envelope samples dissociated in 1% SDS and containing 50 to 400 μg of protein (1.3×10^9 to 1×10^{10} cell equivalents) were usually diluted with an approximately equal volume of buffer-D, containing 0.025 M Tris·HCl, pH 6.8; 4% SDS; 20% glycerol; 10% 2-mercaptoethanol and 0.001% bromophenol blue. Samples were then heated to 70° for 2 min or as indicated in the text, cooled and 0.2 to 0.4 ml (25 to 200 μg protein) carefully layered onto the gels. With labeled preparations samples containing 20 to 40,000 cpm were applied to gels. With dilute samples protein was first concentrated by placing in dialysis tubing and immersing in polyethylene glycol for about 30 min at room temperature.

(e) *Autoradiography and gel scanning*

For autoradiographic studies, cultures (50 to 100 ml) growing exponentially (3×10^7 cells) in M9-glucose medium were labeled for 3 to 4 generations with [^{14}C]amino acid mixtures (Amersham, CFB104 S.A. 50 mCi per mmole). Final concentration of isotope was 0.05 μCi per ml. Incorporation of radioactive label into hot TCA insoluble material was determined as described previously (11). Stained gels were scanned directly using a Canalco microdensitometer. With labeled preparations gels were first fixed and stained in the usual way and then sliced longitudinally into 4 slices for autoradiography as described by McCorquodale and Buchanan (12). These slices were mounted on Whatman #1 filter paper and then dried down to infinite thinness under vacuum with an infrared lamp. Dried slices were placed over x-ray film (Blue sensitive SB54) tightly clamped and exposed for 2 to 4 weeks before developing. Finally the developed films were scanned with a Joyce-Loebel microdensitometer.

(f) *Materials*

Components for preparation of acrylamide gels were obtained from Canalco (Rockville, Md.); SDS (Eastman) was re-crystallized from ethanol, SDS (Schwarz/Mann) was used without re-crystallization. Crystalline Coomassie blue was obtained from Colab Laboratories, Inc., Ill. Bovine pancreas trypsin (type III) and soybean trypsin inhibitor (type 1-5) were obtained from Sigma; lysozyme (salt free) from Worthington Biochemical Corp.; BSA (bovine serum albumin, fraction V) from Armour; egg albumin (ovalbumin, 1 x crystallized, salt free, grade V) from Sigma; β-lactoglobulin (3 x crystallized) and fibrinogen (bovine, fraction I) from Pentex Inc., Ill.

3. RESULTS

(a) *Separation of envelope proteins by disc-electrophoresis*

In our earlier studies using the procedure of Webber and Osborn for the fractionation of envelope proteins, we usually obtained 13 to 15 distinct bands on 10% SDS-acrylamide gels (2). When similar preparations were analyzed on 10% gels with a 3% stacking gel using the Laemmli procedure, up to 50 distinct bands can be regularly obtained (Fig. 1). Fig. 2 shows a distribution of some standard proteins on 10% gels and with this data and on the basis of 15 to 20 repeated analyses of different envelope preparations, molecular weights (± 5%) have been assigned to various individual or groups of bands which routinely appear. Group A, constitutes 4 to 5 usually minor bands corresponding to polypeptide molecular weights of 90,000 to 105,000 daltons. Groups B and D are quite major bands, each containing one predominant polypeptide with corresponding molecular weights of 76,000 and 87,000 daltons respectively. These bands are usually well defined as shown here, with the size of band B greatly expanded in a few preparations. Group C, constitutes at least 3 minor bands appearing between group B and D and with molecular weights around 80,000. Group E, contains between 20 and 24 intermediate-sized and minor bands with molecular weights between 52,000 to 75,000 daltons. Envelope preparation differences are most often seen in this latter group in which somewhat variable individual peak maxima appear in different samples. Group F, is a complex of at least 3 polypeptides with the most distinct band corresponding to a molecular weight of 48,000. This band is a major chatacteristic of virtually all preparations examined. Group G, constitutes about 3 minor polypeptides, molecular weights around 45,000 daltons which in *cet* mutants (see below) is dominated by an apparently homogeneous band with a molecular weight equivalent to 44,000 daltons. Group H, is a complex of at least 3 major polypeptides (36,000 to 38,000 daltons) which are characteristic of all preparations although not always as well resolved as shown in Fig. 1. Group I, is a major almost invariable feature of envelope preparations and has a molecular weight equivalent to 30,000 daltons. In occasional preparations this band was seen to be resolved into 2 distinct peaks. Group J, variously contains 3 to 6 intermediate sized bands with molecular weights ranging from 21,000 to 29,000 daltons. Group K, constitutes a minimum of 3 major polypeptides variably resolved from the dye front and having molecular weights of 20,000 daltons or less. Finally it may be noted that occasional preparations may show very reduced levels of either Group F or I and that this is associated with large amounts of poorly resolved polypeptide in the regions of Groups B and D (e.g. Fig. 3). This effect is probably due, at least in part, to incomplete disaggregation of the envelope preparations since the same effect can be reproduced by omission of mercaptoethanol from the suspending medium.

As indicated above, some variation between the profiles obtained from individual preparations of the same strain is observed, although repeated analysis of the same material is perfectly reproducible. The basic pattern shown in Fig. 1 is nevertheless observed in most preparations which have in-

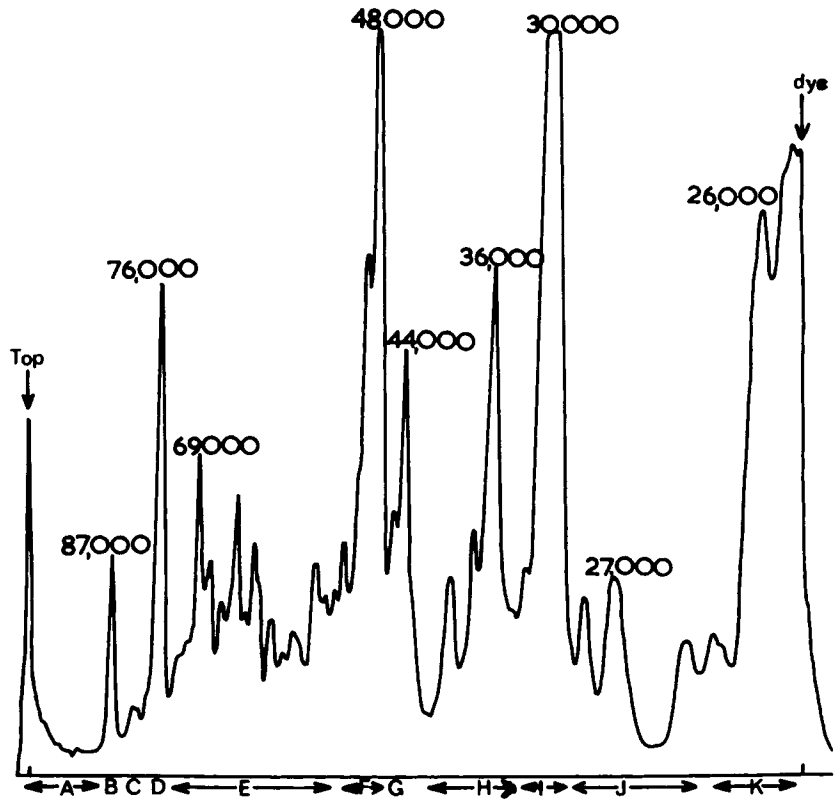

Fig. 1. Separation of envelope polypeptides on 10% acrylamide gels. Strain ASH 102 was grown in nutrient broth to stationary phase and envelopes isolated as described in Methods. Envelope proteins dissolved in 2% SDS and 5% mercaptoethanol were heated to 70° for 2 min; approximately 100 µg protein was then applied to the top of the stacking gel and electrophoresed as described in Methods. The figure shows Canalco microdensitometer tracing of gels stained with Coomassie blue. The first peak on the profile marks the top of the gel and is not stained material. The molecular weights of the major polypeptides are averages of analyses of at least 15 separate preparations.

cluded those isolated from several different K12 strains or those from the same strain grown in M9 glucose medium or nutrient broth. Moreover, as shown in Fig. 3, very similar profiles are obtained whether stained bands or amino acid labeled bands after autoradiography are scanned.

(b) *Analysis of envelopes from* Cet$^+$ *and* CetB *strains*

Cet mutants adsorb colicin E2 to the cell surface, but the subsequent rapid degradation of DNA observed in wild type strains is blocked (9). Previous studies (2) have shown that envelopes isolated from *cet*$^-$ mutants display a prominent band on acrylamide gels which is either absent or present in only minor amounts in the wild type. From the electrophoretic mobility of the band the molecular weight was estimated to be about 40,000 daltons. Envelopes obtained from *cetB* mutants have now been re-investigated using the disc-continuous gel electrophoresis system. *Cet*$^+$ and *cetB* bacteria were first grown in the presence

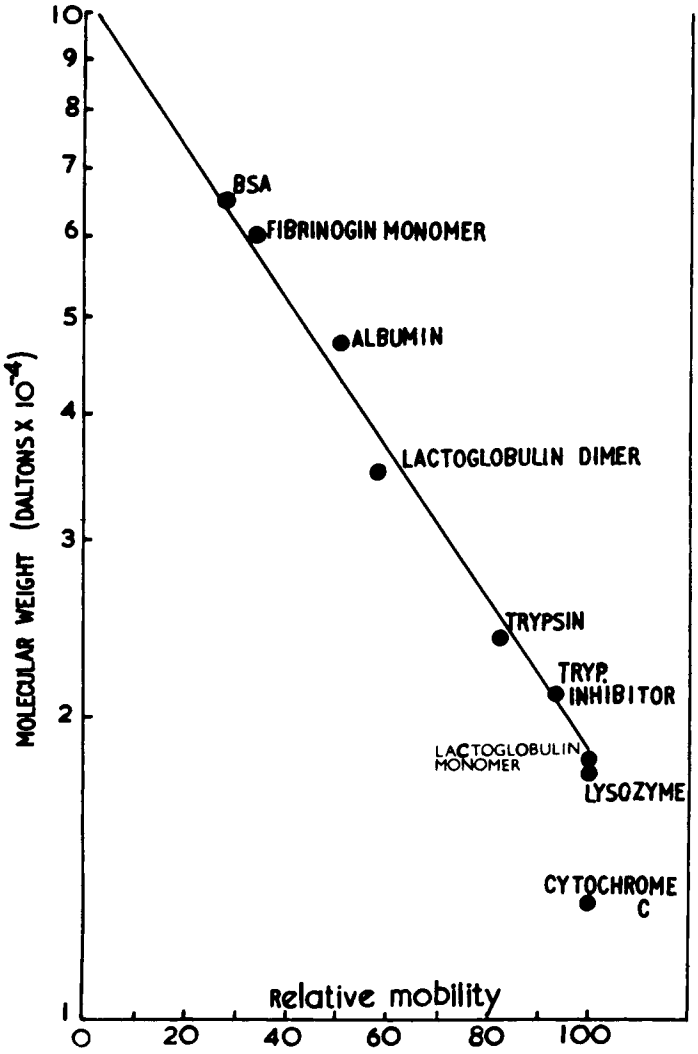

Fig. 2. Relationship of polypeptide molecular weight to relative mobility on 10% SDS acrylamide gels. Standard proteins in 2% SDS and 5% mercaptoethanol were heated to 70°, electrophoresed and gels stained as in Fig. 1 and in Methods.

of [^{14}C]labeled amino acids and envelopes isolated by the usual procedure. These preparations were then analyzed by gel autoradiography and the results are shown in Fig. 4. Envelopes obtained from *cetB* strains clearly contained an extra band whose corresponding molecular weight was calculated to be 44,000 daltons. A band with an apparently identical electrophoretic mobility was present in the wild type profile (see also Fig. 11) but constituted only about 20% the amount found in the mutant profile. The properties of the extra polypeptide present in mutant strains was now investigated in several ways.

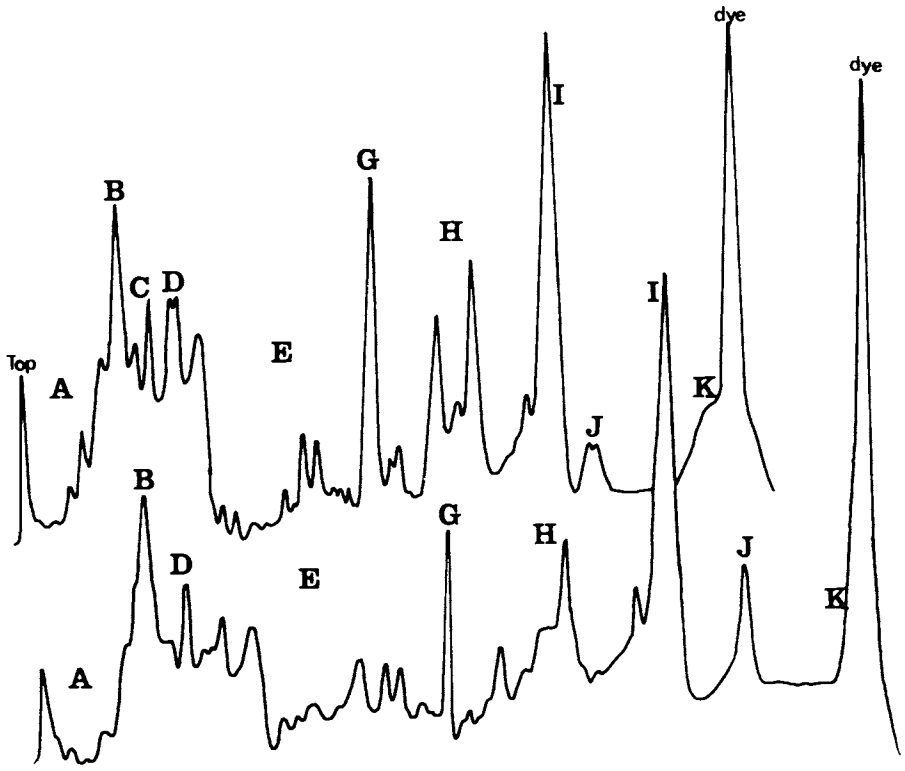

Fig. 3. Comparison of radioactive and stained polypeptide profiles. Strain ASH 102 was grown in M9-glucose medium for 4 generations at 37° in the presence of [^{14}C]amino acids; envelopes were prepared and analyzed as in Fig. 1. About 25 µg protein and 20,000 cpm were applied to the gels. The lower profile was obtained by scanning Coomassie blue stained gels. The same gel was then autoradiographed as described in Methods and the upper profile was obtained by scanning the exposed film with a Joyce-Loebel microdensitometer.

(c) *Influence of preparation conditions and disaggregation methods on envelope protein profiles*

Effect of heating envelope preparations prior to analysis. Prior workers have emphasized heating preparations to different temperatures, in the mercaptoethanol-SDS mixture, in order to obtain complete disaggregation of the envelope proteins. We have, therefore, compared gel proteins obtained from unheated envelope preparations in 2.5% SDS with similar preparations heated to 70° or to 100°. The results are shown in Fig. 5. Some loss of minor high molecular weight components was occasionally observed in preparations heated to 70° compared to the unheated samples, but the differences were small. In both cases disaggregation of the envelope appeared to be complete since all the protein entered the gel. Heating preparations to 100° under these conditions had little effect upon the general profile but did lead to a great reduction in the 30,000 dalton band with a correspondingly large increase in material banding around 34,000 daltons indicating aggregation of breakdown products (Fig. 5 (d)). Heating to 70° was therefore chosen as a convenient standard procedure for all subsequent analyses.

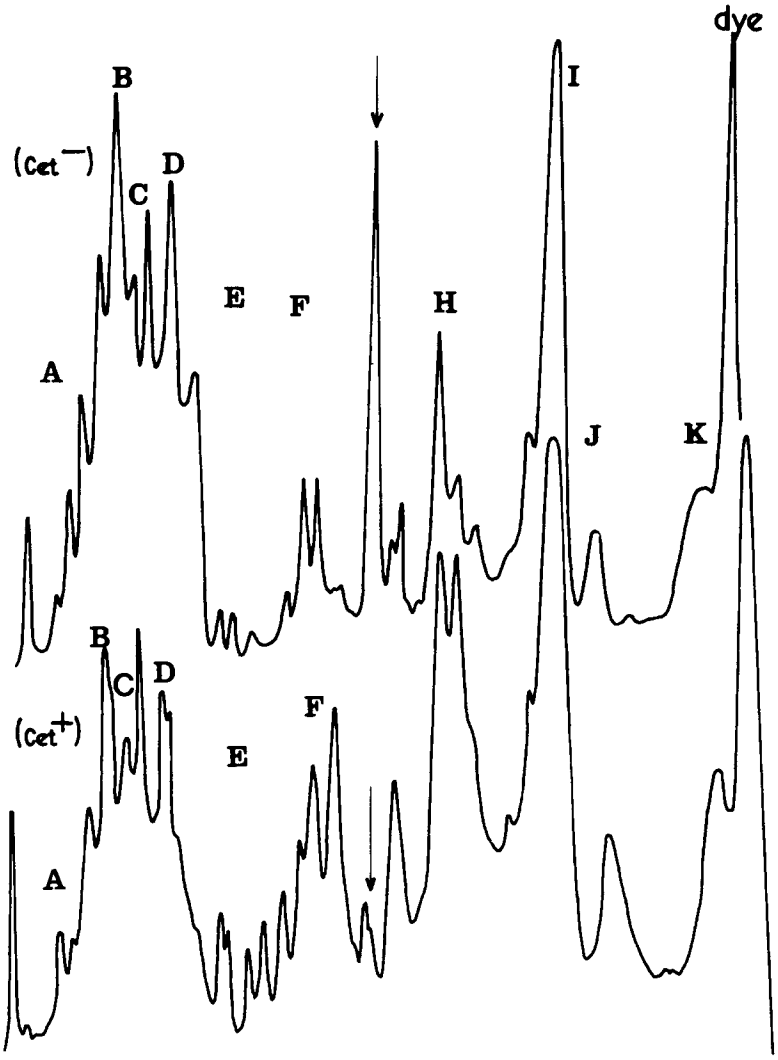

Fig. 4. Gel autoradiographic profiles of ASH 10 (*cet*$^+$) and ASH 102 (*cetB*) envelope proteins. Both strains were grown in the presence of [^{14}C]amino acids and envelopes (100 µg, 40,000 cpm) were analyzed on 10% gels as in Fig. 3. Autoradiograms were prepared and the exposed film scanned with a Joyce-Loebel microdensitometer. (a) Upper profile, ASH 102 envelope proteins with the arrow indicating the 44,000 dalton band. Lower profile, envelope from the wild type ASH 10. (b) Appearance of gels scanned in (a). ASH 10 profile is the right hand gel.

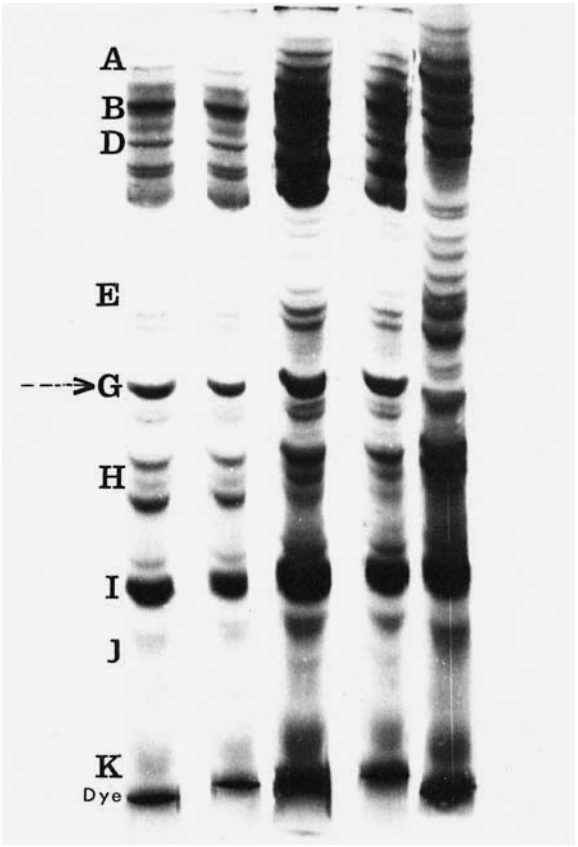

Fig. 4 (b).

In some studies (2,13) membrane preparations were dialized overnight at 37° against 0.1% SDS to prevent precipitation of SDS during subsequent electrophoresis. When such samples were analyzed after heating to 70° and 100°, identical results to those shown in Fig. 5 were obtained. In any event, in the current study re-crystallization of the SDS was found to prevent subsequent precipitation and hence dialysis was no longer necessary.

The *cetB* mutant Ash 102 was used in these analyses and as shown in Fig. 5 the 44,000 dalton band is not dispersed by high temperature, indicating that the enhanced peak does not arise from poor disaggregation of some smaller molecular weight species in the mutant envelope. Such an effect has previously been observed for DnaB$^-$ mutants (14).

Effect of ionic strength of wash buffer used for purification of envelopes. Salton and co-workers (15) demonstrated that the retention of membrane-bound enzymes in *Micrococcus lysodeicticus* membrane particles is very dependent on the ionic strength of the wash buffer and also the particular regime of washing procedures used. Envelopes from *cet*$^+$ and *cetB* strains were therefore isolated using 10^{-1} M, 10^{-2} M or 5 x 10^{-4} M phosphate buffer as the washing agent. Alternatively, envelopes prepared by the usual procedure using 10^{-2} M phosphate buffer, were subsequently subjected to 3 additional washes with 5 x 10^{-4} M buffer. The results of the latter experiment are shown in Fig. 6. The additional washing procedure in the case of either strain clearly leads to the preferential loss of a few specific proteins into the wash fluid. Polypeptides from group D (76,000 daltons) and group F (50,000 daltons) in particular are affected, whereas the bulk of the protein remains in the particulate fraction. Protein estimations showed that about 25% of the initial envelope material is eluted by this

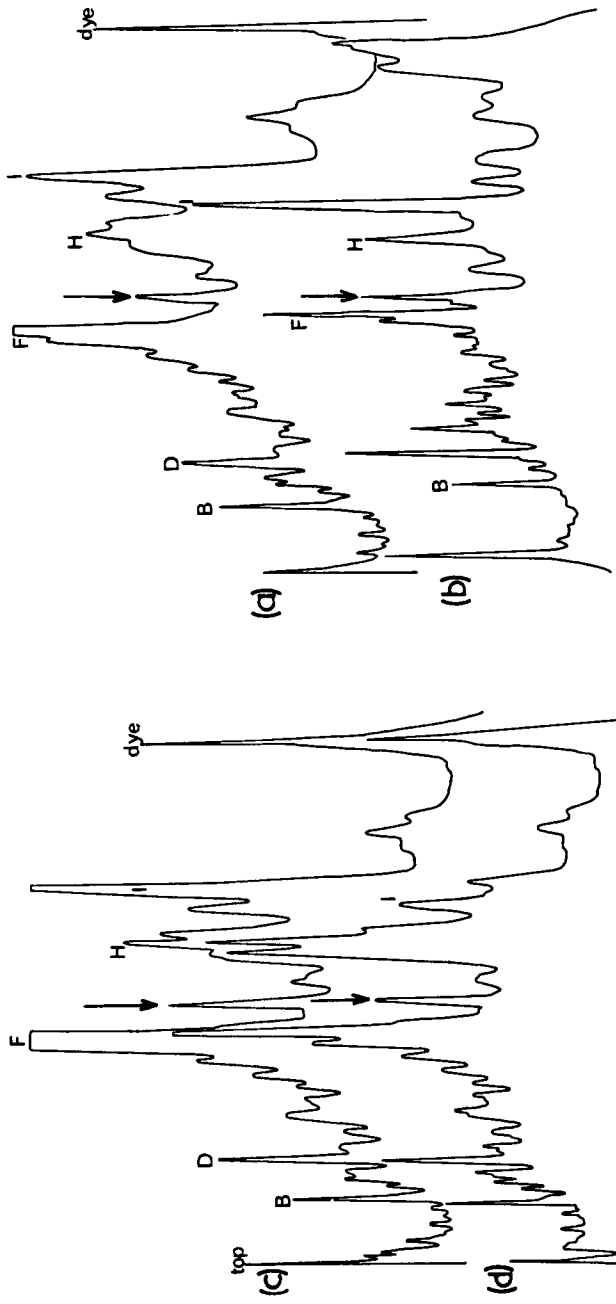

Fig. 5. Effect of preheating isolated envelopes prior to electrophoresis. Strain ASH 102 was grown in nutrient broth to stationary phase and envelopes prepared by the standard procedure, mixed with an equal volume of buffer D (see Methods) and heated to various temperatures prior to electrophoresis on 10% gels. Profiles are microdensitometer scans of stained gels, each loaded with approximately 100 µg protein. (a) Envelope protein dialyzed against buffer D at 37° for 14 hr., then heated to 70° for 2 min prior to electrophoresis. (b) Envelope proteins prepared without dialysis and no preheating. (c) Envelope proteins prepared without dialysis and heated to 70° for 2 min in buffer D. (d) Envelope proteins prepared without dialysis and heated to 100° for 2 min in buffer D. The arrow marks the 44,000 dalton band present in the mutant.

procedure. Reference to Fig. 6B shows that the 44,000 band present in mutant envelopes is also eluted by the low ionic strength buffer. Fig. 7 shows the result obtained when envelopes were purified using a single buffer of either low or high ionic strength. Independently of the wash buffer the general gel pattern was again similar, but some significant differences are evident. In particular, purification of envelopes using 0.1 M buffer resulted in reduced levels of the 30,000 dalton band in gel profiles, whereas several proteins from groups A and E are absent or reduced in envelopes prepared by washing with low ionic strength buffer. Comparison with Fig. 6 shows that the missing polypeptides do not, in most cases, coincide with those obtained by successive washing with 10^{-2} and 5×10^{-4} M buffer. Nevertheless, most of the material banding at the 44,000 dalton position was again lost from mutant envelopes at low ionic strength indicating that this protein is in a relatively loose association with the envelope. In contrast, the minor polypeptide of corresponding molecular weight in wild type envelopes was not, apparently, eluted at low ionic strength.

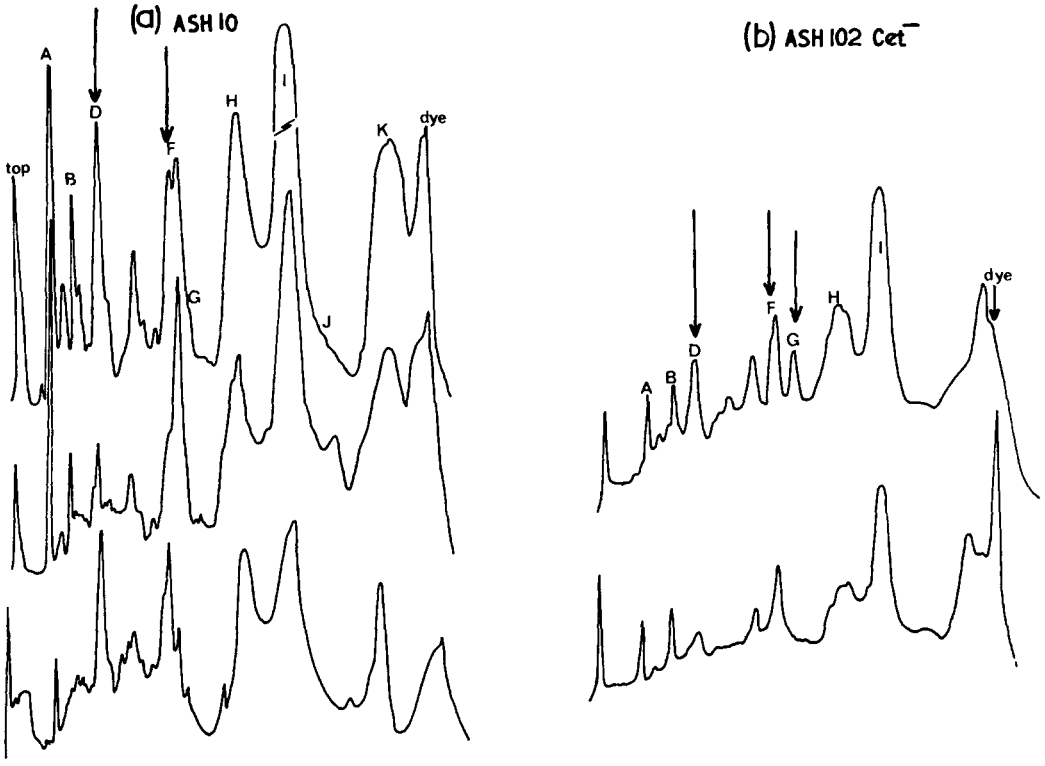

Fig. 6. Elution of specific polypeptides from envelopes by washing at low ionic strength buffer. Envelopes were first prepared from ASH 102 and ASH 10 grown in nutrient broth and then washed, in the absence of SDS, an additional 3 times with 5×10^{-4} M K-buffer and the final pellet dissolved in SDS and mercaptoethanol in the usual way. Supernatant fractions were combined, concentrated and mixed with buffer D. All samples were then heated to 70° and analyzed on the 10% gels prior to staining and scanning as in Fig. 1. (a) Upper profile, ASH 10 envelopes prepared by standard procedure by washing in 10^{-2} M K-buffer; middle profile, pellet remaining after additional wash at low ionic strength; lower profile, supernatant from the low ionic strength wash. (b) Upper profile, ASH 102 envelopes prepared by standard procedure; lower profile, pellet from ASH 102 remaining after low ionic strength wash. Arrows mark the polypeptides preferentially eluted.

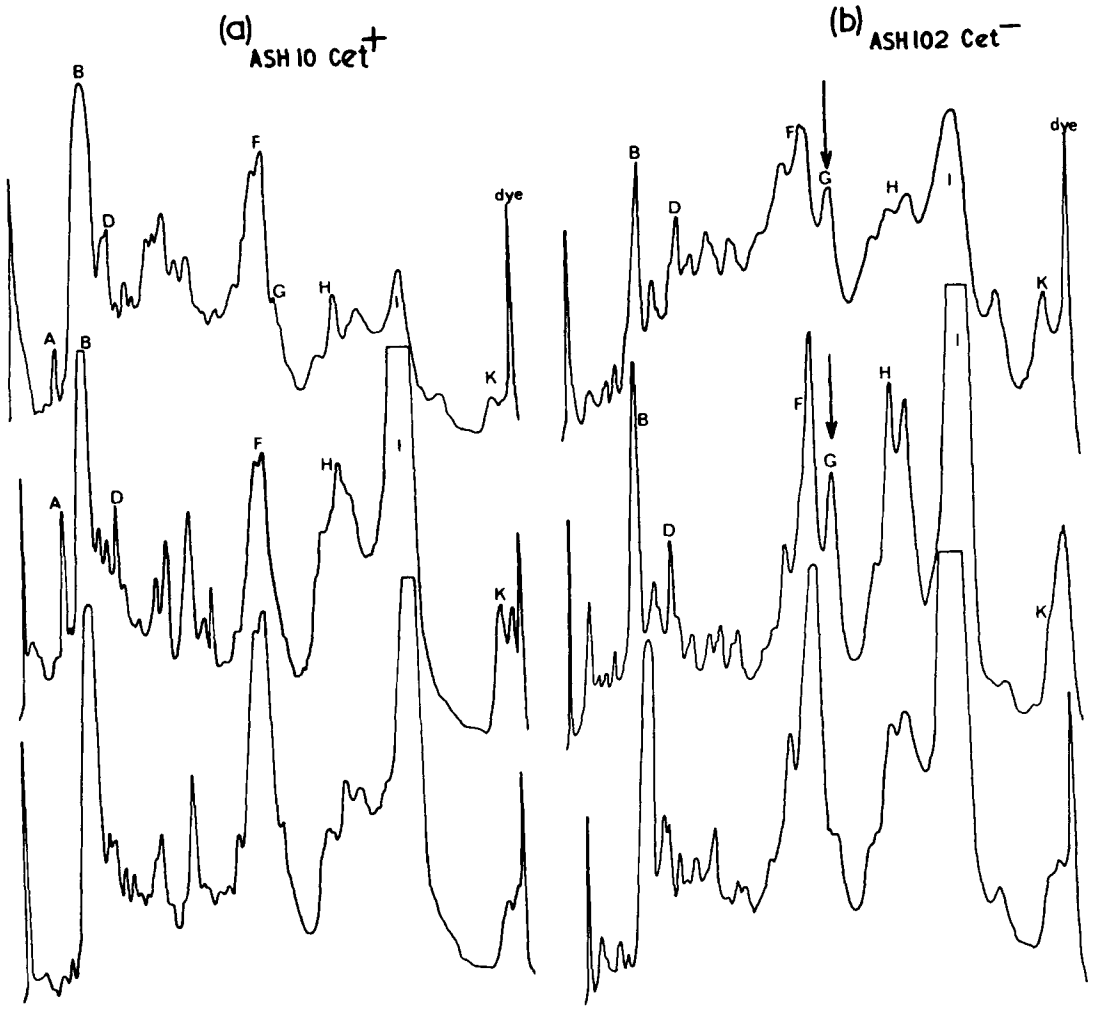

Fig. 7. Preparation of envelopes using K-buffer of different ionic strengths. (a) ASH 10 envelopes. Upper profile, envelopes prepared with 10^{-1} M K-buffer; middle profile, envelopes prepared with 10^{-2} M K-buffer; lower profile, envelopes prepared with 5×10^{-4} M K-buffer. (b) Profiles as in (a) but using strain ASH 102 as source of envelopes. Arrow marks the 44,000 dalton polypeptide. Profiles are microdensitometer scans of Coomassie blue stained gels. Other experimental details as in Fig. 1.

Envelopes isolated from cells pre-treated with trypsin. Bacterial cells incubated with up to 200 µg per ml of trypsin do not lose colony-forming ability and growth is not, apparently, inhibited. However, a major protein constituent (molecular weight 7,500 daltons) of the cell wall (murein layer) is removed by incubation of cell envelopes with trypsin (16) and surface-bound colicin can also be readily digested by addition of the enzyme to treated cells (17). Trypsin treatment, therefore, may be used as a tool to detect protein in the outermost layers of the cell surface or proteins exposed on the surface of membranes. Late logarithmic phase cultures of strain Ash 102 (*cetB*) were incubated with trypsin, thoroughly washed, and trypsin-inhibitor added prior to

breakage and isolation of cell envelopes. Envelopes were purified and analyzed on 10% acrylamide gels by the standard procedure. The results shown in Fig. 8 demonstrate that several polypeptides present in envelopes from control cells are absent or reduced in amount in envelopes from trypsin-treated cells; in particular, the 44,000 dalton protein is largely eliminated as is a constituent of group H (37,000 daltons). Material banding at 30,000 daltons (group I) is also greatly reduced and a new band, presumably a trypsin induced breakdown fragment, is observed having a molecular weight of approximately 28,000. Finally, a major peak corresponding to group B appears to be absent, together with

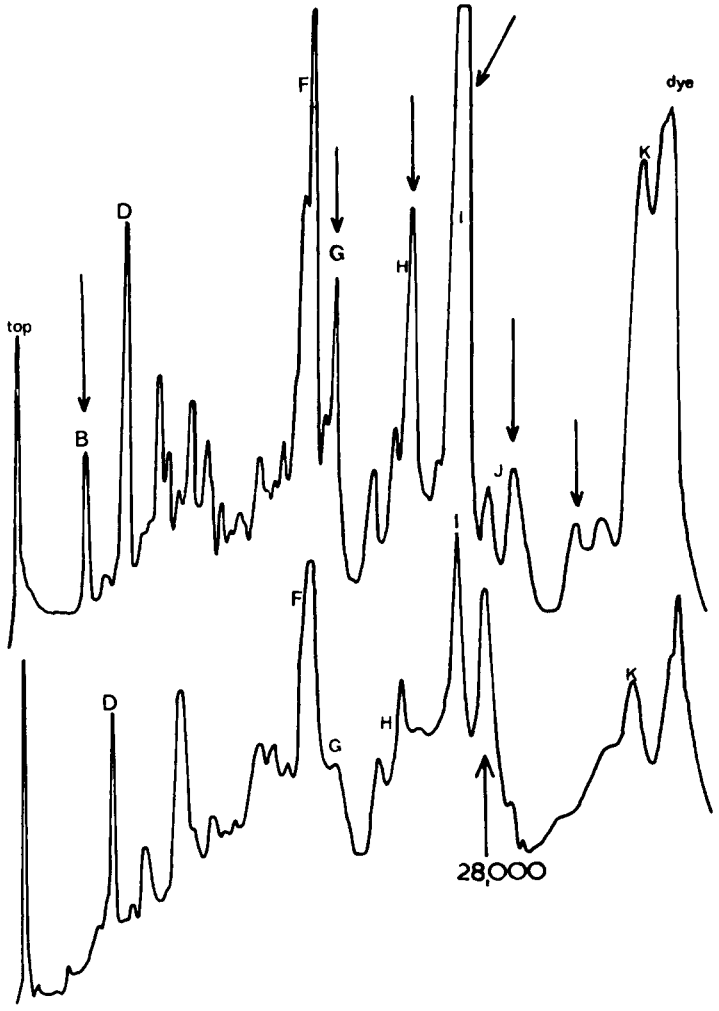

Fig. 8. Envelopes from trypsin pretreated cells. Stationary phase cultures of ASH 102 (*cetB*) washed and suspended in Na-buffer pH 8.0 were first pretreated with trypsin 100 µg per ml per 10^{10} cells for 30 min at 37°. Trypsin was removed by washing 3 times with K-buffer and trypsin inhibitor (50 µg per ml) was then added and envelopes isolated and analyzed as in Fig. 1. Profiles are from 10% acrylamide gels stained with Coomassie blue. Upper profile, envelopes from normal cells; lower profile, envelopes isolated from trypsin pretreated cells. Arrows mark major polypeptides removed or reduced by trypsin treatment.

2 or more bands from group J. The loss of these latter low molecular weight polypeptides is particularly interesting since Schnaitman (4) has produced evidence that similar proteins are predominantly present in the outer, rather than the inner membrane of the envelope. The finding that the mutant band is lost under these conditions suggests that this protein is specifically present in the envelope *in vivo* and is located either in the outermost layers of the cell surface or is exposed on the external surface of the cytoplasmic membrane.

(d) *Analysis of Triton X-100 soluble envelope proteins*

Schnaitman (4) reported that fractionation of the total envelope into wall-enriched (including outer membrane) and a cytoplasmic membrane enriched fraction could be obtained by sucrose gradient centrifugation. This procedure was carried out in an attempt to locate the surface layer containing the mutant protein, but the 2 fractions obtained by sucrose gradient centrifugation failed to show any significant differences when the protein content of each was analyzed on 10% acrylamide gels (A.C.R. Samson, personal communication). More recently Schnaitman (18) has shown that many cytoplasmic membrane proteins can apparently be solubilized from whole envelope preparations by extraction with Triton X-100. Cultures of the *cet*$^-$ mutant, ASH 102, were therefore sonicated in the presence of 20% sucrose to minimize hybridization of lipoprotein fragments derived from different surface membranes (19). Envelopes were isolated in the usual way and Triton X-100 soluble and insoluble proteins were then analyzed on 10% acrylamide gels. Triton X-100 treatment released about 30% of the envelope protein and as shown in Fig. 9 many high molecular weight polypeptides were selectively solubilized in preference to smaller polypeptides by this procedure. Thus, polypeptides from groups A, C, D and E appear in the soluble fraction whereas the major envelope proteins of groups F, H and I largely remain in the Triton X-100 insoluble particles. Fig. 9 clearly shows that the "mutant" protein is readily solubilized by Triton X-100 although the corresponding band in profiles of envelopes from the wild type strain ASH 10 was not, apparently, released (see also Fig. 11). These results indicate that the 44,000 dalton polypeptide may be closely associated with the cytoplasmic membrane in the envelope of mutant strains.

(e) *Analysis of envelope preparations from* recA *mutants*

Inouye and Pardee (1) reported that inhibition of cell division in *E. coli*, for example by thymine starvation, was accompanied by the appearance of increased amounts of a specific polypeptide (55,000 daltons) in cell envelope preparations. Mutants carrying a *recA* allele, however, failed to show this increase and moreover, under conditions of thymine starvation, the cells continued to divide (10). This indicated that the product of the *recA*$^+$ gene may participate in some way in the regulation of DNA metabolism and cell division at the membrane level. Envelopes were therefore isolated from *rec*$^+$ and *recA1* derivatives of ASH 10 and analyzed in the usual way. In addition, since *cetB*, *recA* double mutants have reduced refractivity to colicin E2 (Buxton and Holland, unpublished results), envelopes were also prepared from a *recA1* derivative of ASH 102 and analyzed on 10% and 8% SDS-acrylamide gels. As shown in Fig. 10 the levels of the 44,000 dalton band were virtually identical in both *recA* and *rec*$^+$ derivatives of ASH 102. Increased levels of this polypeptide were not therefore suppressed in the presence of the *recA* mutation. Further examination of Fig. 10 shows that a polypeptide, molecular weight of about 40,000 daltons, from group H is greatly reduced in envelopes from strains carrying the *recA1* mutation whether or not the *cetB* allele is present. Other differences between the envelopes of *rec*$^+$ and *recA* strains were also evident, particularly the decrease of the 76,000 and 48,000 dalton bands. However, none of these differences, including the reduction of the 40,000 band shown in Fig. 10 was

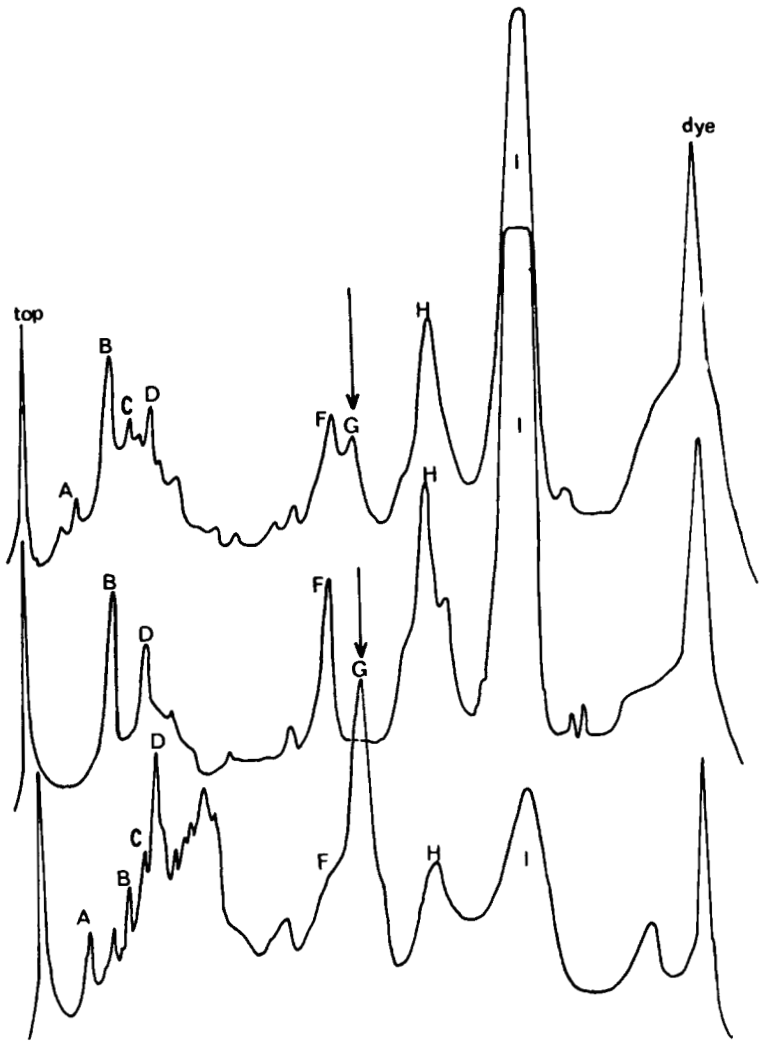

Fig. 9. Analysis of Triton X-100 soluble envelope protein. Envelopes were isolated from strain ASH 102 (*cetB*) by the standard procedure. The final pellets, before dissolving in SDS, were extracted with Triton X-100 as described in Methods. Triton X-100 non-extractable proteins and Triton X-100 extractable proteins were then dissolved in SDS and analyzed on 10% gels as in Fig. 1. Upper profile shows normal ASH 102 envelopes; middle profile, envelope protein remaining after Triton X-100 extraction; lower profile, protein in Triton X-100 extract. Arrows mark the 44,000 dalton polypeptide.

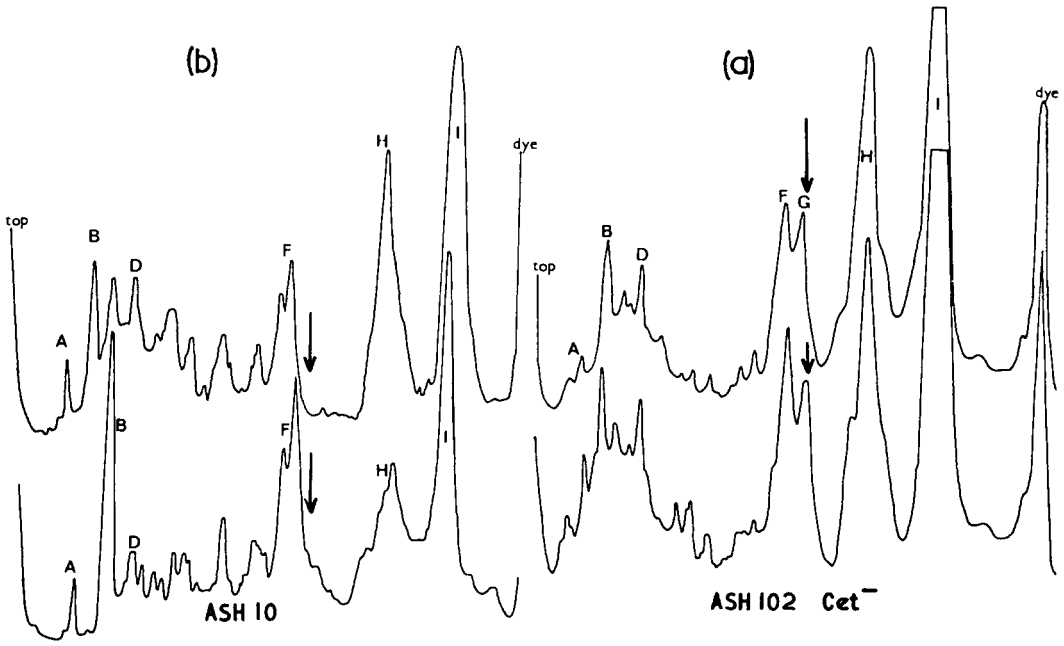

Fig. 10. Comparison of envelope proteins from ASH 102, *cetB* and ASH 102, *cetB*,*recA1* strains on 10% SDS gels. (a) Upper profile, envelopes from ASH 102 *cetB*,*recA1*; lower profile, ASH 102 *cetB*,*rec*$^+$. (b) Upper profile, ASH 10, *recA*; lower profile, ASH 10 *rec*$^+$. Arrows mark the position of 44,000 dalton protein. Experimental details as in Fig. 1.

obtained consistently. This is illustrated in Fig. 11 where other preparations were analyzed on 8% acrylamide gels in order to increase the resolution of the higher molecular weight polypeptides. In this case no significant differences between *rec*$^+$ and *recA* strains were seen. This figure confirms the presence of high levels of the 44,000 dalton protein in envelopes from both *rec*$^+$ and *recA* derivatives of the *cetB* strain ASH 102 and also clearly shows the presence of a minor band of similar mobility in the wild type ASH 10 profile (Fig. 11a).

4. Discussion

There is now a growing interest in determining how the multiplicity of functions known to be associated with the bacterial membrane, are organized and assembled. The method described here provides an efficient method for analyzing the polypeptide content of bacterial envelopes and membranes. No significant variation in gel profiles, inherent to the electrophoresis system, was detected. Considerable quantitative variation in some polypeptide peaks was, however, attributed to the method of isolation of envelopes and the preparation of the sample prior to electrophoresis. As found by Fairbanks *et al.* (20) with erythrocyte ghosts, particular polypeptides were eluted from envelopes by washing with either high or low ionic strength buffer and 10^{-2} M phosphate buffer was chosen quite arbitrarily as the standard washing agent. As also found by Fairbanks *et al.*, heating dissociated envelopes to high temperature in the presence of SDS induced some aggregation of specific polypeptides; in this study the major envelope polypeptide, molecular weight 30,000 daltons, appeared to readily undergo aggregation under these conditions. This polypep-

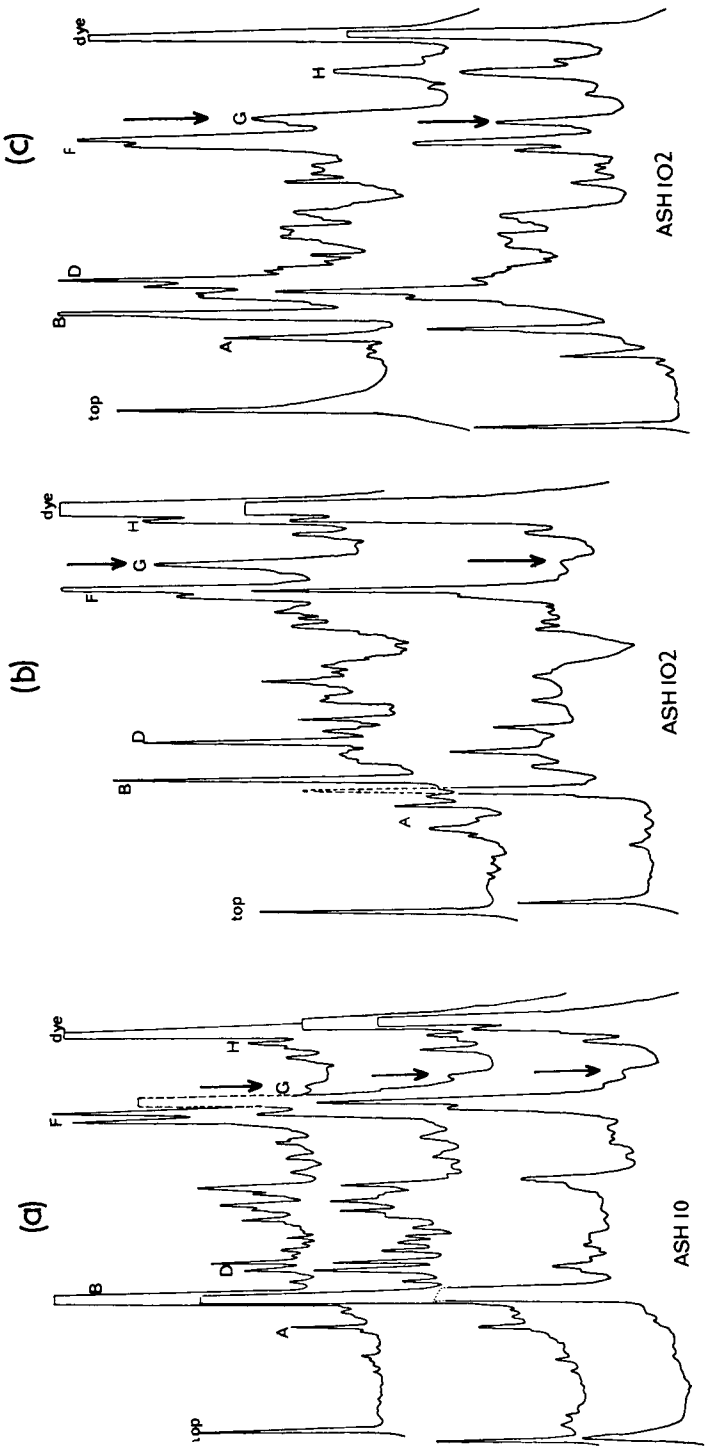

Fig. 11. Analysis of *cetB* and *recA* envelopes on 8% acrylamide gels. Experimental details as in Fig. 1 except that stacking gel contained 8% acrylamide. (a) ASH 10 envelopes. Upper, ASH 10 rec^+; middle, ASH 10 *recA*; lower, ASH 10 rec^+ after removal of Triton X-100 soluble protein. (b) ASH 102. Upper, ASH 102 rec^+, normal envelope; lower, after removal of Triton X-100 soluble protein. (c) Upper, ASH 102 rec^+; lower, ASH 102 *recA*. The arrows mark position of 44,000 dalton band.

tide was also the major protein lost from envelope preparations washed with high ionic strength buffers, conditions corresponding to those used by several workers to prepare membranes from Kaback vesicles (21). Another likely source of variation in these analyses is the possibility that proteases are present in the envelope preparations and that these seriously distort the polypeptide profile. This possibility was not excluded but was minimized by immediately dissolving isolated envelopes in SDS and storing at -20° until analyzed. Samples stored for several weeks in this way gave perfectly reproducible profiles whereas samples kept at 4° even in the presence of SDS showed accumulation of small molecular weight material after a few days. To further reduce or eliminate variable factors, we are now investigating different methods of cell breakage and the possible effects of cell age on envelope profiles.

The sensitivity obtained by this electrophoretic technique indicated that a single band, constituting approximately 0.25 μg of protein, i.e. 1% of the total protein analyzed, can be detected with either stained or radioactively labeled profiles. This amount of protein is equal to 3×10^9 cell equivalents which, assuming a reasonably quantitative yield of envelope protein by the isolation procedure, corresponds to 1,200 polypeptide chains per envelope for a 40,00 dalton protein. The lower limits of detection for polypeptides of 20,000 and 105,000 daltons is 500 and 2,500 molecules per cell, respectively. From the data of Cox *et al.* (22), the amount of cytochrome-B_1, molecular weight 62,000 daltons (23), present in *E. coli* is approximately 600 molecules per cell, i.e., just below the minimum level of detection when whole envelopes are analyzed. However, since extraction of envelopes with Triton X-100 appears to give a 2- to 3-fold enrichment for cytoplasmic membrane proteins (18) and since we find this fraction to contain predominantly polypeptides over 50,000 daltons, the minimum level of detection may fall as low as an equivalent of 200 to 300 molecules per cell. Thus, at least some typical membrane proteins constituting, for example, part of the electron-transport chain or membrane-bound permeases, could be detected as single bands under these conditions. Some future studies will be directed toward determining this.

The data obtained so far in our analysis of envelope proteins of *E. coli* do not permit any firm conclusions regarding the presence of structural proteins in bacterial membranes similar to those found in erythrocytes and other eukaryote cell membranes (20,24). Although major polypeptide bands were consistently observed in envelope preparations with molecular weights 48,000, 36,000, 30,000 and 20,000 daltons and equal to approximately 40% of the total protein, we have observed that with increasing resolution of electrophoretic techniques, the heterogeneity of membrane protein also increases. Thus the major bands observed in the present study may ultimately prove to be complex. The presence of large amounts of structural protein may in any case reflect functional specialization in different membranes and Schnaitman (4) has suggested that the outer membrane in the *E. coli* envelope may be composed of a few major polypeptides whereas the inner (cytoplasmic) membrane is extremely complex. In support of this we find that Triton X-100 extraction of whole envelopes, which largely separates the inner membrane from the outer membrane-wall complex (18), leaves the 3 or 4 major polypeptides in the insoluble wall fraction, whereas a very heterogeneous mixture of minor components was released with the cytoplasmic membrane fraction. It is of considerable interest that the latter fraction contains a preponderance of high molecular weight polypeptides (over 50,000 daltons), whereas the insoluble, wall-outer membrane fraction, is composed of mainly low molecular weight material (25). This may reflect a restricted capacity of the cell to export large molecular weight proteins for incorporation into extracellular layers.

In this study we have confirmed that isolated envelopes from *cetB* mutants contain a major polypeptide constituting about 5% of the total protein or 6,000 molecules per envelope. The molecular weight of this protein was calculated to be 44,000 daltons. A corresponding band was always observed in profiles of wild type envelopes, but at levels of 1/4 to 1/5 that found in the mutant. The wild type polypeptide, in contrast to the mutant protein, was not, however,

released from envelopes by Triton X-100 treatment or by washing in low ionic strength buffers and its relationship to the mutant protein is therefore not clear. The presence of the 44,000 dalton protein in the cell surface *in vivo* was demonstrated by the finding that it could be removed from the cell surface, together with several other polypeptides, by pre-incubation of mutant cells with trypsin. This procedure, as might be expected, only affected those envelope polypeptides apparently associated with the outer layers of the surface. This result strongly suggests that the 44,000 dalton protein, at least in the mutant, is not an integral part of the cytoplasmic membrane, although the possibility that it is loosely associated with the external surface of this membrane is not excluded. The relatively loose association of the mutant polypeptide with the cell surface was in fact verified by the finding that it could be readily removed from isolated envelopes by washing with low ionic strength buffer. Preparation of envelopes from either wild type or mutant strains in high ionic strength buffer did not, however, lead to greater yields of the 44,000 dalton protein. This indicated that the high levels of this protein in the mutant strain was not due to its greater retention in isolated envelopes compared to the wild type, but reflected a true increase in amount of this polypeptide in the cell surface.

The finding that the 44,000 dalton protein was wholly released from envelopes of the mutant by Triton X-100 treatment is difficult to interpret at the moment. It is not clear whether this reflects separation of this protein from the rest of the envelope in physical association with the cytoplasmic membrane, or whether the protein is specifically leached from the wall-outer membrane complex independently of the release of cytoplasmic membrane proteins. The picture is further complicated by the fact that at several hundred sites in the *E. coli* surface, the wall and outer membrane may be in close physicochemical association with the inner membrane (26). Location of the mutant protein in such sites could explain its susceptibility to trypsin and its release by Triton X-100 treatment. Of considerable interest now is how increased levels of the mutant protein arise and how this relates to the insensitivity of mutant strains to colicin E2. The most likely reason for increased amounts of the protein is defective regulation of synthesis or assembly into some surface structure, including the malfunctioning of a polypeptide cleavage enzyme possibly involved in normal maturation of membrane proteins. Labeling of envelope proteins in wild type and mutant strains in pulse chase experiments should test the validity of the latter explanation.

We have so far been unable to determine whether the increased levels of the 44,000 dalton protein in mutant envelopes is a direct cause or an effect of the cell's inability to promote DNA degradation after fixation of colicin E2. However, the *cetB3* mutation is dominant in partial diploids under certain conditions (Buxton and Holland, unpublished results), and it seems possible that "mutant" protein directly blocks interaction of colicin molecules with the cytoplasmic membrane, subsequent to the initial binding to the E2 surface receptor, which Sabet and Schnaitman (27) report to be present in the cell wall (murein) fraction. We have previously shown that some *cet* mutants display a wide range of pleiotropic effects including UV sensitivity, recombination deficiency, abnormal cell division and reduced growth of bacterio-phage λ (9), in varying degrees in different isolates. Nevertheless, of 3 different mutants initially analyzed, all showed similar increases in the 44,000 dalton protein in the cell envelope (2). Correlation between phenotype and the structural gene changes which give rise to increased amounts of this polypeptide are thus not likely to be simple in this system. We anticipate in any event that further characterization of the properties and regulation of assembly of this polypeptide into the cell surface in different mutants should facilitate some understanding of the functional organization of the cell membranes.

Inouye (10) has previously suggested that the *recA*$^+$ gene product may be involved in some way in the regulation of cell division. In addition, Gross *et al.* (28) reported that *recA*,*polA* double mutants of *E. coli* appear to be lethal and suggested that the *recA*$^+$ gene product may somehow be involved in DNA

replication, i.e., a membrane-associated function. An extensive analysis of envelopes prepared in a variety of ways from *recA* mutants, however, failed to reveal any significant differences from the wild type. Profiles obtained from separate preparations were, however, rather more variable than those from the wild type parent. A specific polypeptide, molecular weight about 40,000 daltons, was often reduced in these preparations, but this particular band showed some variation in *cet* mutants and this change could not, therefore, be considered to be specific. If *recA* mutants do have an altered membrane protein, the normal cellular concentration must be very small (less than a few hundred molecules per cell) or the change is undetectable by SDS electrophoresis which will not detect altered proteins in membranes unless the alteration has a radical quantitative effect. In other experiments, we have also so far failed to detect any differences in envelope proteins from isogenic R^+ (resistant transfer factor) and R^- strains or in minicell envelopes compared to normal cells (unpublished results). These studies are now being extended to the analysis of enriched cytoplasmic membrane fractions rather than whole envelopes in a continued investigation of membrane proteins with particular reference to DNA metabolism.

ACKNOWLEDGMENTS

We are extremely grateful to Dr. R.C. Clowes for making available the facilities of his laboratory during the course of this work. The study was supported by U.S. Public Health Service grants GM-14394 and AI-10468 to Dr. Clowes, GM-13234, a divisional grant, and by a travel grant to I.B.H. from the Wellcome Trust. We are also indebted to Dr. D. Beckman and Dr. D.J. McCorquodale for advice on gel autoradiography.

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