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RELEASE OF A SPECIAL FRACTION OF THE OUTER MEMBRANE FROM BOTH GROWING AND PHAGE T4-INFECTED *ESCHERICHIA COLI* B

MARILYN R. LOEB * and JAN KILNER

Department of Microbiology, The George Washington University Medical Center, 2300 Eye Street, N.W., Washington, D.C. 20037 (U.S.A.)

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Summary

Growing *Escherichia coli* release envelope material into the medium. Upon infection with T4 phage increased amounts of this material are released and at a greater rate. In order to determine whether both inner and outer membranes are present in this material, and whether the material released by growing cells differs from that released by infected cells, we have examined the protein composition of envelope released by growing and T4-infected *E. coli* B. Our results show: (a) the protein composition of envelope released from growing or infected cells is similar, (b) the proteins present are representative of the outer membrane, (c) the major outer membrane protein of *E. coli* B, protein II*, is deficient in the released material. We therefore conclude that the envelope material released from growing or infected *E. coli* represents a special fraction of the outer membrane. This finding is discussed in relation to outer membrane structure and function. In addition, data are presented on the differing outer membrane protein composition of substrains of *E. coli* B obtained from different laboratories.

Introduction

Growing *E. coli* release envelope material into the medium [1]. Similar material is also released immediately upon adsorption of T4 phage to the cell, but at a greatly increased rate (20 to 30-fold) [2]. Since the material released by uninfected or infected cells contains lipopolysaccharide, in addition to phospholipid and protein [1—3], it must be derived, at least in part, from the outer membrane.

In order to clarify the origin of the released material and its relationship to cellular envelope, we have examined the polypeptide composition of envelope material released by both log phase-uninfected and phage T4-infected *E. coli*

* To whom reprint requests should be addressed at the present address: Dept. of Pediatrics, University of Rochester School of Medicine and Dentistry, Rochester, N.Y. 14642, U.S.A.

B. The results, reported below, demonstrate: (a) the polypeptides present in released envelope material are the same for uninfected and infected cells, (b) these polypeptides are derived from the outer membrane of the cell, (c) this released outer membrane material is not representative of cellular outer membrane since it is deficient in the major outer membrane polypeptide, protein II* *.

Material and Methods

Microorganisms. The strains of *E. coli* and phage used in these experiments, their source and their pertinent characteristics are listed in Table I. The method of preparing stocks of phage T4BeG59S and the rationale for using this phage to obtain released material from infected cells have been described previously [2].

Media. Cells were grown at 37°C with aeration in M9-salts medium [6] containing 0.12% glucose plus other additions as noted below.

Preparation of total membrane and outer membrane. Cells were grown in 500 ml M9-glucose containing 1 mg/ml of casamino acids and 1 μCi/ml ^{3}H-labeled amino acid mixture (0.27—58 Ci/mmol). The rate of isotope incorporation paralleled the rate of cell growth. The cells were collected in the cold by centrifugation at 8000 $\times g$ for 15 min, washed twice with 30 ml 0.05 M Tris/1 mM EDTA (pH 7.8), and stored at —20°C for later use. The frozen cells were thawed to 4°C in 25 ml of the same buffer and broken either by passage through a French press (Ribi cell fractionator; Dupont-Sorvall) 2—3 times at a pressure of 12 000—15 000 psi after addition of 10 μg deoxyribonuclease and ribonuclease, or by sonication for 2 min in 15-s intervals with the temperature maintained between 4 and 10°C. $MgCl_2$ was added to a final concentration of 2 mM, the cells were centrifuged at 7000 $\times g$ for 10 min and the supernatant obtained was centrifuged at 225 000 $\times g$ for 45 min to yield the pellet of total membrane. Outer membrane was obtained by centrifugation of total membrane on a discontinuous sucrose gradient in 0.10 M *N*-2-hydroethylpiperazine-*N'*-2-ethane sulfonic acid (HEPES), pH 7.4 [7], or by twice repeated treatment with 2% Triton X-100 at room temperature [8].

Isolation of radioactively-labeled envelope material released by uninfected and infected cells. 8 ml of *E. coli* were grown with aeration for 5 generations to a cell density of $5 \cdot 10^8$ cells/ml in M9 salts-glucose containing 1 mg/ml casamino acids and 50 μCi U-^{14}C-labeled protein hydrolysate. The cells were chilled, centrifuged at 8000 $\times g$ for 10 min and washed 3 times with M9 salts to remove accumulated released envelope. They were then resuspended in medium of the same composition as that in which they had been grown, including isotope, and aerated at 37°C for 10 min. 0.2 mg L-tryptophan was added, the culture was divided into 2 equal portions and one these was infected with phage T4Bes at a multiplicity of 10; the other portion served as the uninfected sample. After continued incubation for 2 min, each culture was poured into one-half vol. ice-cold M9-salts and centrifuged at 8000 $\times g$ for 15 min to remove

* The nomenclature used for outer membrane proteins I*a*, I*b* and II* is that of Schmitges and Henning [4].

TABLE I

BACTERIAL AND PHAGE STRAINS USED

Bacteria	Source	Pertinent phage resistant properties
E. coli B	L.D. Simon, 1972	resistant to TuI*b*
E. coli B_R *	L.D. Simon, 1975	resistant to TuI*b*
E. coli B_G *	G. Weinbaum	resistant to TuI*b*
E. coli B_M *	M. Bayer	resistant to TuI*b*
E. coli B_p *	M.L. Robbins	resistant to TuI*b*
E. coli B; Ia^-	J. Foulds	resistant to TuI*a*, TuI*b*
E. coli CR63	L.D. Simon	
E. coli KL16	Yale Genetics Center	
E. coli JF568	J. Foulds	
E. coli JF688	J. Foulds	resistant to TuI*a*
E. coli JF701	J. Foulds	resistant to TuI*b*
E. coli JF699	J. Foulds	resistant to TuII*

Phage	Source	Pertinent properties
T4BeG59s	G. Steisinger	e^-(gene deleted), s^-, ac^-, q^-
T2, T3, T4, T5, T6, T7	M. Bayer	wild type
TuI*a*	J. Foulds	receptor is protein I*a*
TuI*b*	J. Foulds	receptor is protein I*b*
TuII *	J. Foulds	receptor is protein II*
$\lambda b_2 c$	M. Gottesman	virulent phenotype

* Note that the subscripts used to identify these substrains refer only to the source of the substrains and not known genotypic or phenotypic differences.

cells. The supernatant fluid was centrifuged again at this speed, and then at 12 000 × *g* for 1 h. The supernatant fraction, now free of cells and containing released material, was dialyzed against 2 l of 0.01 M Tris/2 mM EDTA (pH 7.5) for 48 h with 4 changes of buffer. 2 ml dialyzed supernatant was layered on the top of a 35 ml linear gradient of 20—55% sucrose in 0.01 M Tris/2 mM EDTA (pH 7.5) and centrifuged for 65 h in an SW 27 rotor at 27 000 rev./min. 23—26 fractions were collected and the radioactivity of each fraction was determined. Typical results appear in Fig. 1 and will be discussed below. The released envelope material, found in fractions 10, 11, and 12, was dialyzed against distilled water and then concentrated by lyophilization or by precipition with cold trichloroacetic acid. In the latter case this was performed as follows: 100 μg purified RNA were added to each ml of dialysate to act as carrier in the precipitation. Cold trichloroacetic was added to a concentration of 10%. After 30 min the precipitate was collected by centrifugation and washed three times with acetone to remove trichloroacetic acid. The precipitate was dissolved in 0.5 ml of the sample buffer used in electrophoresis. Since the sample buffer is made with 0.05 M Tris at a pH (pH 6.8) which is near the end of the buffering range of Tris, the residuum of trichloroacetic acid in the precipitate usually resulted in a lowering of the pH to about pH 3—4, as determined with pH paper. Therefore the pH was adjusted to approximately pH 7 by careful addition of 1.5 M Tris (pH 8.0).

SDS-polyacrylamide gel electrophoresis. Discontinuous gels of 10% acrylamide/0.27% *N,N'*-bis methyleneacrylamide were used. The formulae for sample buffer, stacking gel, separating gel and electrode buffer were taken from

Laemmli [9] with the following differences. (a), 2 mM EDTA was present in electrode buffer, sample preparation buffer, and the gel itself; (b), the concentration of Tris in the sample buffer was 0.05 M; (c), catalyst concentrations for the slab gels were 0.05% *N,N,N',N'*-tetramethylethylenediamine (TEMED) and 0.1% ammonium persulfate; for the tube gels, 0.04% TEMED and 0.06% ammonium persulfate; (d), slab gels (1.5 mm thick, 10—15 cm long) were run at 80 V or 25 mA per slab; tube gels (0.5 cm diameter, 10 cm long) at 2 mA per tube.

Slab gels were either fixed and stained with Coomassie Brilliant Blue R [10] to reveal protein bands or with periodate-Schiff reagent to reveal lipopolysaccharide [11]. For autoradiography, slab gels were dried and exposed to Kodak RP Royal X-Omat film. Tube gels were either fixed and stained, or else frozen, and sliced into 1-mm discs with a Mickle gel slicer (Brinkmann Instruments). The gel slices were soaked in 0.5 ml NCS tissue solubilizer/water (9 : 1, v/v) for 2 h at 50°C; 10 ml Liquiflour were added and radioactivity determined. At least 90% of the radioactivity added was recovered.

Other procedures. Lipopolysaccharide was isolated from *E. coli* B by the method of Galanos et al. [12]. Phospholipids were isolated from labeled cells by the procedure of Bligh and Dyer [13]. The protein content of the membrane preparations was determined by the Lowry method [14]. This was performed either on membranes suspended in Tris buffer or on membranes suspended in sample buffer before the addition of mercaptoethanol (which interferes with the determination). It was advantageous to have the membranes in sample buffer, since this yielded a very homogeneous preparation. RNA, used as carrier in the trichloroacetic acid-precipitation of released material, was purified from yeast RNA by four extractious with an equal volume of phenol; the RNA was precipitated by the addition of 2.5 vols. ethanol and collected after storage at −20°C for 24 h. The incorporation of radioactive amino acids into cells was determined by precipitation of cells with cold trichloroacetic acid [2]. Valine sensitivity of *E. coli* was measured following the procedure described by Quay and Oxender for determining leucine-sensitivity [15].

Chemicals. Radioactive compounds and NCS solubilizer, Amersham Searle; Liquifluor, New England Nuclear; molecular weight markers, Triton X-100, Sigma; gel reagents, Bio-Rad, except for sodium dodecyl sulfate which was obtained from BDH Chemicals, Ltd., Catalogue number 30176; enzymes, Worthington Biochemical Corp.

Results

Effect of membrane isolation procedure on polypeptide composition. Before comparing the polypeptide composition of released envelope material with that of cellular envelope, it was necessary to determine the extent to which the composition of the latter depends on the method of envelope isolation. Total membrane and outer membranes were obtained as described in Material and Methods and also by variations of these procedures, as noted below. In addition, membranes were also prepared after breakage of cells by alternate cycles of freezing and thawing in the presence of lysozyme, EDTA (MacGregor, C.H., personal communication). In all, 5 different total membrane preparations and

19 outer membrane preparations were examined. Typical results appear in Fig. 2 for cells broken by sonication. The total membrane preparations show few differences in the relative amounts of polypeptides except for those with molecular weights below 30 000. The outer membrane preparations show variations in their minor polypeptides at all molecular weights; however, the relative amounts of the major outer membrane proteins, II*, I*a* and *a*, are not affected by the isolation procedure. In all cases the ratio of II* to I*a*+ a, as determined by scanning of stained gels, was about 2. Thus, these experiments indicate that except for the major outer membrane proteins and the total membrane proteins greater than 30 000 daltons, a comparison of the protein composition of outer membrane and released envelope material requires caution.

The proteins labeled *a*, I*a* and II* in Fig. 2 have been identified as follows. *E. coli* B, does not contain I*b* [4,16]. Although at first we thought that our strain might be different and that the doublet we now identify as *a* and I*a* was actually I*a* and I*b*, respectively, this is not the case. First, our strain of *E. coli* B is sensitive to phages TuIa and TuII* but not TuIb; these phages require proteins I*a*, II* and I*b*, respectively, as receptors [17]. Secondly, membranes from a mutant of our *E. coli* B, resistant to TuIa, show no band in the position we now identify as I*a* (data not shown). Third, while our gel system should [4], but does not, separate I*a* and I*b*, (as determined by a comparison of membrane preparations from *E. coli* K12 strains JF688, JF701 and JF568), our membrane preparations lack I*b* when run on gels that do separate I*a* and I*b* (Foulds, J. and Chai, T.-J., personal communication) (data not shown). Hence, the lower molecular weight member of the doublet is I*a*. The upper member, a polypeptide we call *a*, may be identical to the *a* of Lugtenberg [18]. The band we call II*, 33 000 daltons, is identified as such because it has the same electrophoretic mobility as the well-characterized II* from other strains, notably *E. coli* JF568 (data not shown). In addition this band shows the well-documented property of heat modification of mobility, as does our band I*a* [19,20].

Polypeptide composition of released material. The material released from uninfected or infected cells was dialyzed and centrifuged through a sucrose gradient. Fig. 1 shows the distribution of radioactivity in the gradient after centrifugation. The envelope fraction of released material appears in one peak (fractions 9—13). The non-envelope fraction, so designated because it lacks lipids [2], appears in two peaks (fractions 17—23 and 24—26). Although both uninfected and infected cells contain all 3 fractions, infected cells release both more envelope material than uninfected cells, and considerably more non-envelope material. This latter, probably due to leakage of material from infected cells, has not been examined further by us, although it has been previously discussed [2].

One feature of the experimental design should be clarified. The material released by infected cells contains both material released in the 10 min before infection as well as material released as a result of infection (see Material and Methods). Using the data obtained in Fig. 1, it can be calculated that uninfected cells release 5860 cpm of envelope in 12 min or 488 cpm/min. Infected cells release a total of 13 420 cpm in 12 min; however 4880 of this is due to envelope released in the 10 min prior to infection. Therefore, the amount of envelope material released as a result of infection is 13420—4880/2 min or

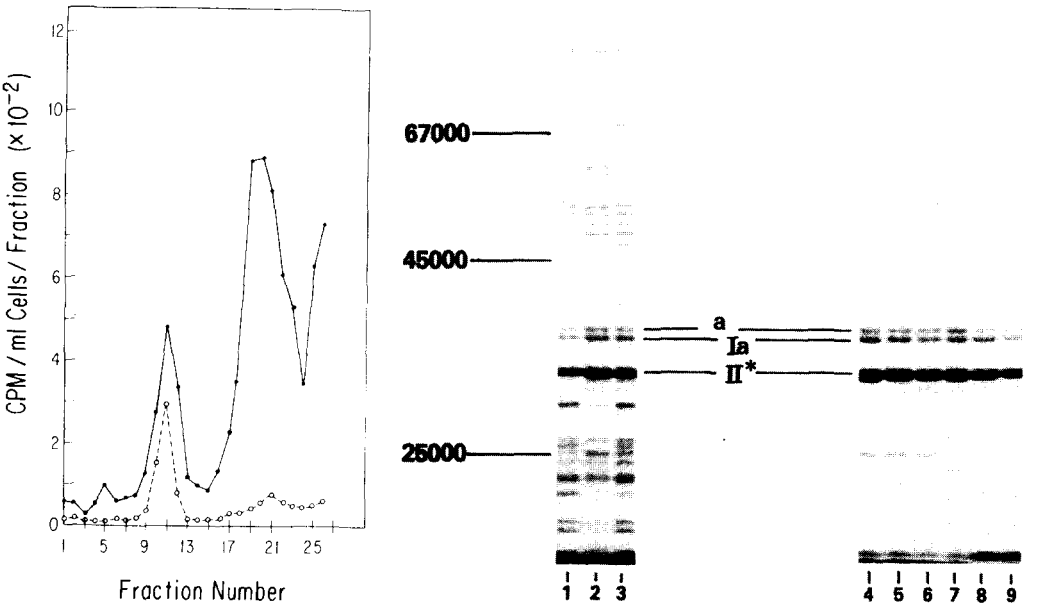

Fig. 1. Sucrose gradient centrifugation of non-dialyzable material released by uninfected (○), and infected (●) cells. The top of the gradient is on the right.

Fig. 2. Effect of isolation procedure on the polypeptide composition of total membranes and outer membranes. Total membranes and outer membranes were prepared from cells disrupted by sonication, as described in Materials and Methods. Columns 1—3, total membranes; columns 4—6, outer membrane separated on a sucrose gradient; columns 7—9, outer membranes obtained by Triton X-100 extraction. Variations were as follows: 2, 5, 8, none; 1, 7, cells were washed and resuspended in 0.01 M sodium phosphate (pH 7.0). $MgCl_2$ was not added until the Triton X-100 extraction; 3, 6, 9, EDTA was omitted; 4, EDTA was present throughout.

4270 cpm/min. Hence, in this experiment the rate of release of envelope from infected cells is nine times faster than from uninfected cells.

After sucrose centrifugation, the polypeptide composition of the envelope fraction and of the fractions near and at the top of the gradient were examined (Fig. 3). The total non-dialyzable material released from uninfected cells (column 6) has considerably fewer polypeptides than that obtained from infected cells (column 4). However, the polypeptide composition of the envelope fraction of each of these is similar (columns 5 and 3, uninfected and infected cells, respectively), showing 2 major bands and several faint minor bands. By comparing the electrophoretic mobility of these bands with those found in total and outer membrane preparations electrophoresed on the same slab (not shown), the 2 major bands can be identified as proteins *a*/*Ia* (as sometimes happens, *a* and I*a* were not resolved during this gel run) and protein II*, indicating that released envelope from infected or uninfected cells is derived from the outer membrane.

In order to obtain more quantitative data on the polypeptides present in

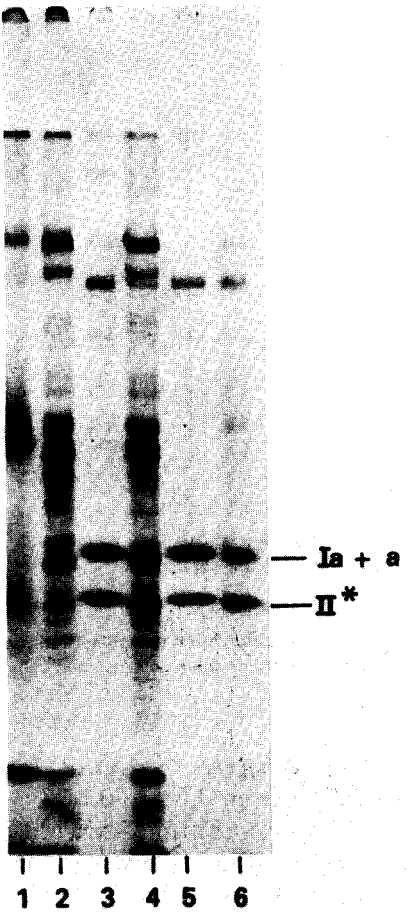

Fig. 3. Autoradiogram obtained after SDS polyacrylamide gel electrophoresis of various fractions released from uninfected and infected cells. This gel was exposed to film for three months. Columns 1—4, material from infected cells; 5 and 6, material from uninfected cells. 1, fractions 24—26, from Fig. 1, 4900 cpm; 2, fractions 18—22, (Fig. 1) 4500 cpm; 3, fractions 10—12 (Fig. 1) 2500 cpm; 4, total non-dialyzable released material, 5800 cpm; 5, fractions 10—12 (Fig. 1) 2200 cpm; 6, total non-dialyzable released material, 2400 cpm.

released envelope, this material, labeled with ^{14}C-labeled amino acids, was co-electrophoresed with outer membrane labeled with ^{3}H-labeled amino acids. Fig. 4 shows the distribution of dpm across the gel, in the case where the released envelope was obtained from infected cells. Similar data was obtained with the envelope fraction released from uninfected cells. Material appearing in fractions 31—35 represents protein II*; that appearing in fractions 36—41 represents proteins I*a* and *a*. The large amount of radioactivity at the bottom of the gel (fractions 1—12) amounting to 50% of the total dpm is discussed below.

The number of different polypeptides in released material is quite small; those which are present migrate with outer membrane polypeptides indicating that the envelope fraction of released material is derived from the outer membrane. However, it is notable that released material, compared to outer membrane, is deficient in II*. In four separate experiments, ratios of II*:I*a*+*a* for

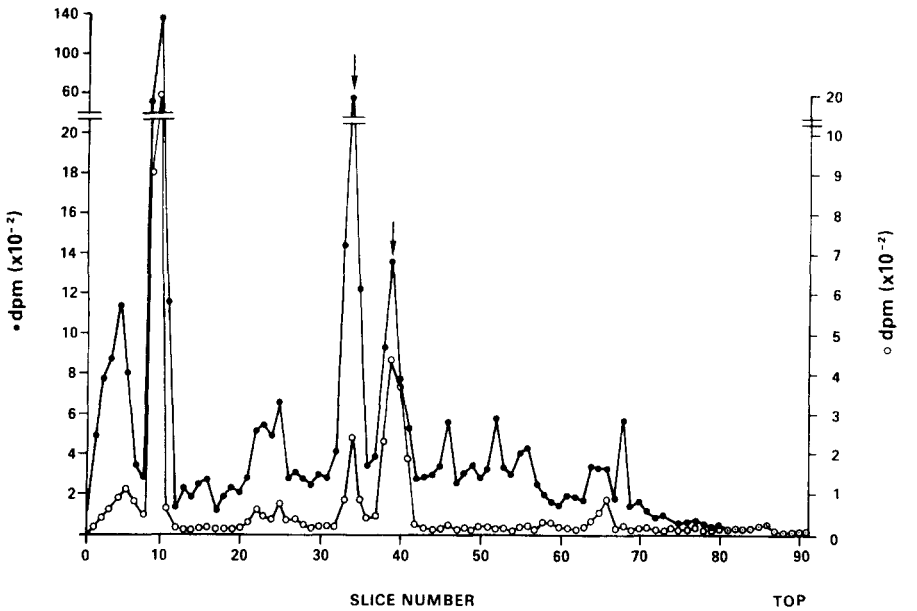

Fig. 4. Comparison of the polypeptide composition of cellular outer membrane from ^{3}H-labeled amino acid-labeled cells and of the envelope fraction released from ^{14}C-labeled amino acid-labeled infected cells. Arrows point to II* and I*a*+*a*. ○, ^{14}C; ●, ^{3}H.

cellular outer membrane varied from 2.24 to 2.59, whereas the ratios for released material from uninfected or infected cells varied from 0.34 to 0.55.

Control experiments. Several control experiments were performed to verify the initial finding that released outer membrane is deficient in protein II*. The results were the same if the isotopes were reversed, or if released outer membrane was concentrated by lyophilization or trichloroacetic acid precipitation. Also, when outer membrane preparations were subjected to some of the procedures used to isolate released outer membrane, such as extensive dialysis, no loss of protein II* occurred, nor did outer membrane lose II* upon incubation at 37°C for 3 h. In addition, total released material from uninfected cells, precipitated with trichloroacetic acid immediately after centrifugation, also showed a low proportion of II*. (Data is not shown for any of the above.) These experiments indicate that the initial result is not due to an isotope effect, the method of isolation of released material, the presence of a protease in outer membrane specific for II*, or the contamination of the I*a*+*a* region of the gel with non-envelope material.

Another set of control experiments was necessitated by our finding that 2% of the amino acid label incorporated into the cells was dispersed into fatty acids and hence into phospholipid and lipopolysaccharide. These components would not be expected to affect the ratio of II*:I*a*+*a* since they migrate to the bottom of the gel (fractions 1—12, Fig. 4) and are, in fact, partly responsible for the large amount of radioactivity found in that region (data not shown). Nevertheless, the possibility that a labeled non-protein envelope component was migrating with proteins II*, I*a* or *a* was eliminated by labeling cells with radioactive leucine or arginine, less than 0.02% of whose label is dispersed into

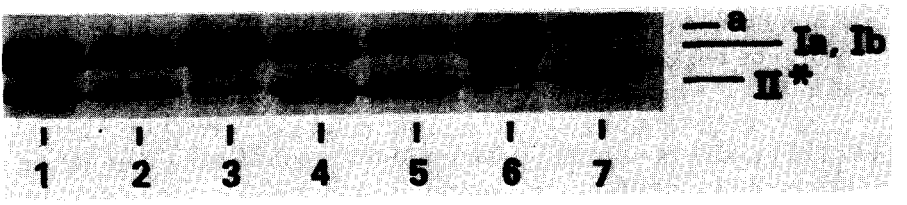

Fig. 5. Comparison of the proteins in outer membrane of different strains and substrains of *E. coli*. The bacteria were grown in M9-glucose and broken by sonication. Outer membranes were prepared by Triton X-100 extraction. Only that part of the stained gel containing the proteins in question is shown. 1, *E. coli* KL16; 2, *E. coli* CR63; 3, *E. coli* Bp; 4, *E. coli* B_G; 5, *E. coli* B_M; 6, *E. coli* B_R; 7, *E. coli* B used in these experiments.

fatty acids (data not shown). In a typical experiment the ratios of proteins II*: I*a*+*a* found in [4,5-^{3}H]leucine-labeled cellular outer membrane and released outer membrane obtained from uninfected cells were 2.14 and 0.47, respectively.

Variation of outer membrane composition among substrains of E. coli B. While II* is the major outer membrane protein in our strain of *E. coli* B, other investigators found I*a* to be the major outer membrane protein of *E. coli* B [4,20]. It is possible that differences in culture conditions are responsible for the disparate finding [21]. Therefore, in order to determine if our strain of *E. coli* B is indeed different, we obtained four strains of *E. coli* B and also two *E. coli* K12 strains from different laboratories cultured them under the same conditions, and compared the polypeptide composition of their outer membranes. Fig. 5 shows the result of this experiment.

It is clear that for the five *E. coli* B strains, B, B_R, B_M, B_G, and B_P, the II*:I*a* ratios vary. B_R and B_P have much more I*a* than II*, and thus are more like the *E. coli* B strains used by other workers. It is interesting to note that two substrains which differ widely, namely the *E. coli* B strain used in the experiments described in this communication and *E. coli* B_R, were obtained from the same laboratory but at different times.

We also tested all of the *E. coli* B substrains for properties characteristic of *E. coli* B. As expected, they all (a) were valine resistant, (b) plated all T phage tested, namely T2, T3, T4, T5, T6, T7 and (c) did not plate λ phage. On the other hand, the two K12 strains shown in Fig. 5, plated λ phage as well as the T phage, and were valine sensitive.

Discussion

These experiments, which were performed in order to gain information on the function, organization and structure of the *E. coli* cell surface, have shown that released outer membrane material from either growing or infected *E. coli* is deficient in protein II*. To interpret this finding we can envisage two patterns of outer membrane structure. In one, the major outer membrane proteins are distributed in a homogeneous manner around the cell, but the particular mechanism responsible for the release of outer membrane material results in protein II* being left behind. In the other, the distribution of proteins II*, I*a*

and *a* is not homogeneous and II* is greatly reduced in that part of the membrane destined to be released.

The latter model is supported by results from other laboratories. Protein II* and I*a* have different functions, II* being needed for the formation of stable mating pairs [22] and I*a* being used in the transport of small molecules through the outer membrane barrier [23,24]. Protein I*a*, in fact, appears to be part of a pore structure from which protein II* is excluded [25]. Although II* and I*a* are both on the exposed surface of the cell [26], as expected for phage receptors, I*a* is tightly bound to the peptidoglycan [20]. In addition, studies with cross-linking agents have shown that protein I (i.e., I*a* and/or I*b*) becomes cross-linked only to itself, whereas protein II* becomes cross-linked to itself and also to some extent to free lipoprotein [27]. The inability of proteins I and II* to cross-link with each other may be due to lack of mutually neighboring amino groups or to actual spatial separation of I and II* in the membrane. These results [27] and those presented here indicate that there is a place on the outer membrane from which II* is either functionally and/or physically excluded. If indeed, as results from some laboratories show (refs. 1 and 2 and our own unpublished data), released outer membrane is relatively deficient in protein, then there must be a portion of the outer membrane enriched in phospholipid (which occurs only on the inner surface of the outer membrane [26]) and in lipopolysaccharide (which occurs only on the outer surface of the outer membrane [28]); and conversely there must be other regions of the outer membrane enriched in protein. Furthermore, the fraction of the outer membrane which we describe in this communication represents a sizable portion of the membrane since it has been estimated that T4 phage, at a multiplicity of 30, release 30% of the outer membrane in 4 min [2].

The actual mechanism of release from growing or infected cells is unknown. Perhaps release from growing cells is related to septation since electron micrographs of dividing *E. coli* show blebs of outer membrane in the region of septum formation [29]. Although the outer membrane material released by phage has the same protein composition as that released by growing cells, phage at sufficient multiplicities cause the release of as much outer membrane in 4 min as growing cells release in 45 min [2]. It is difficult at this time to envisage how this is done. Perhaps the outer membrane fraction released by phage is derived from those areas of the membranes to which the phage irreversibly adsorb, the membrane adhesion zones [30]. Release in this case would be a result of the series of steps leading up to the injection of virus DNA. However, since these adhesion zones comprise only 6% of the surface of *E. coli*, it is difficult to see how viral adsorption can cause up to 30% of the surface to be released unless some kind of spreading reaction takes place. Another possibility is that material is released in the first, or reversible, step of virus adsorption. In this step the virus randomly hits the cell surface. It then either rapidly moves two-dimensionally in search of a membrane adhesion zone or it continues to bounce off and on the cell until it chances to hit such a zone [31].

Mention should also be made of the recent results of Hoekstra et al. [5], who characterized released outer membrane material obtained from *E. coli* in late stationary phase under limiting oxygen conditions. They found, unlike earlier workers [1,3], no difference in the absolute protein content between

cellular outer membrane and released outer membrane and, unlike the data presented here, little difference in the relative polypeptide composition. It is difficult to comment on the reasons for these disparate results, except to note that different strains of *E. coli* were used, and more importantly, the bacteria were in quite different physiological states at the time released envelope was collected. We have recently found that certain conditions, which do not result in a change of the protein composition of the outer membrane, do increase the amounts of II* present in material released by growing cells (manuscript in preparation).

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