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PROTEIN ANALYSIS OF OUTER MEMBRANES PREPARED FROM *ESCHERICHIA COLI* K 12 BY DIFFERENT PROCEDURES

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SUMMARY : The protein content of outer membranes prepared according to four different procedures from *Escherichia coli* strain CR 34 were comparatively analyzed by sodium-dodecyl-sulfate-polyacrylamide gel electrophoresis. This paper outlines that those materials were not identical with respect to the protein distribution. The possible origins of this non-identity are discussed.

INTRODUCTION

The question of the topological repartition of the various protein species in the outer membrane of Gram-negative bacteria is still open. This membrane is believed to be simpler than the cytoplasmic membrane with respect to the functions assumed (1). The number of protein species associated with the outer membrane is supposed to be more restricted than in the cytoplasmic membrane and thus more susceptible to analysis.

The presence of tightly bound "major proteins", some with possible structural role (2) and other with well defined physiological functions (3) has been reported. Other quantitatively important proteins are released at various steps during outer membrane preparation. Some of those proteins are thought to lie in the periplasmic space (4) or to be loosely bound to various constituants of the envelope (5). Their precise location in the intact envelope is however not known.

The most usual techniques which have been developed to isolate the outer membrane of *Escherichia coli* fall in two classes. The first involves either a mechanical breakage of the cells as reported by KOPLOW and GOLDFINE (6) or the burst of lysozyme-EDTA spheroplasts according to OSBORN et al. (7). In those disruptive methods, further steps are needed to separate the outer membrane from the rest of the cell material. The second class consists in recovering the small outer membrane fragments which are released in the course of lysozyme-EDTA spheroplast formation

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(8) as described by WOLF-WATZ et al. (9) and by MIZUSHIMA and YAMADA (10). Those non disruptive preparations have been reported to yield highly purified outer membranes. In addition they have the advantage of being achieved rapidly and easily adapted to large scale preparation.

The aim of the present study was to compare the protein content of outer membrane preparations obtained from one strain of *Escherichia coli* K 12 by the four procedures cited above.

The heterogeneity of the results led to compare the different profiles with the proteins released after treatments known to remove specific elements of the cell envelope such as the procedures according to LEIVE et al. (11) and to NEU and HEPPEL (12).

MATERIALS AND METHODS

Strain and culture conditions : The strain CR 34, used throughout this work, was a gift of J. KOPLOW. Its relevant phenotypic characters are Thr^-, Leu^-, Lac^-, Thy^- and T_1^R.

The bacteria were grown to late logarithmic phase (A 540 = 0.7) in MLT complete medium, which contained per liter : Difco-Bacto Tryptone (10 g) ; Difco-Bacto Yeast extract (5 g) ; Sodium chloride (5 g) and Thymine (250 mg).

Membrane isolation : The methods of OSBORN et al. (7), WOLF-WATZ et al. (9), and MIZUSHIMA and YAMADA (10) were employed without any modification. In the method of KOPLOW and GOLDFINE (6) an Eaton Press was used instead of a French Press to disrupt the bacteria.

Bacterial fractions : Periplasmic proteins were prepared by recovering the osmotic shock-fluid of bacteria according to NEU and HEPPEL (12). EDTA-extractable material was prepared according to LEIVE et al. (11).

Polyacrylamide gel electrophoresis : The proteins were analyzed with the sodium-dodecyl-sulfate-poltacrylamide gel electrophoresis system described by LUGTENBERG et al. (13). Acrylamide and methylene-bis-acrylamide were obtained from Serva, and sodium-dodecyl-sulfate from Serlabo (France). Molecular weight standards (Bovine serum albumine, Ovalbumine, Chymotrypsinogen A and Cytochrom C) were purchased from Boehringer. Protein samples were heated 3 min. at 100°C in sample buffer before electrophoresis. The protein concentration was estimated by the method of LOWRY et al. (14).

RESULTS AND DISCUSSION

The protein content of the outer membranes prepared according to the four procedures mentioned in the Materials and Methods section was analyzed by sodium-dodecyl-sulfate-polyacrylamide gel electrophoresis (Fig. 1). Both methods of KOPLOW and GOLDFINE and of OSBORN et al. gave relatively similar profiles, showing mainly two major polypeptides of

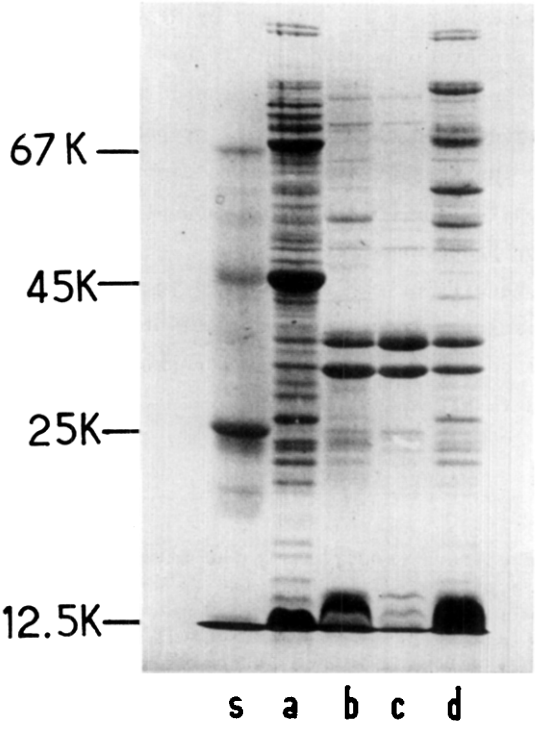

Figure 1 : Sodium-dodecyl-sulfate-polyacrylamide gel electrophoresis of the proteins from outer membrane preparations according to : WOLF-WATZ et al. (a), OSBORN et al. (b), KOPLOW and GOLDFINE (c) and MIZUSHIMA and YAMADA (d).
(S) : molecular weight standards (see Materials and Methods).

MW 33.000 and 36.000, which both constituted about 50 % of the total protein content of the extracts. These findings can be compared with those of KOPLOW and GOLDFINE (6) who found for the same strain two major protein species of MW 37.500 and 38.500. The little differences in the apparent molecular weights might be due to the gel system.

The material extracted according to MIZUSHIMA and YAMADA contained the same 33.000 and 36.000 dalton proteins but in a smaller relative proportion (15 %) whereas additional proteins were present, particularly in the high molecular weight range (over 45.000).

The protein content of the material isolated by the procedure of WOLF-WATZ et al. was very different. First, the number of distinct proteins was greater and second, there were few correspondences between their migrations and those of the polypeptides obtained by the other methods. The preparation displayed two "major" proteins of MW 44.000 and

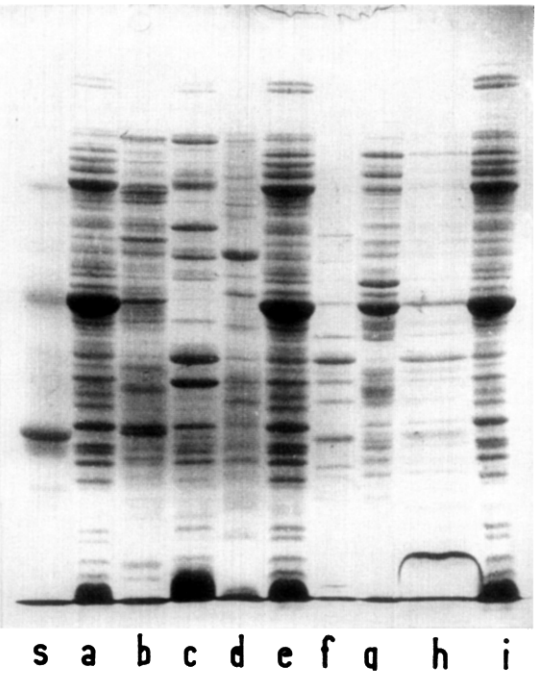

Figure 2 : Comparison of the materials extracted by the procedures of WOLF-WATZ et al. and MIZUSHIMA and YAMADA with different bacterial fractions.
a, e, i : outer membrane (WOLF-WATZ et al.), b : cytoplasmic material, c : outer membrane (MIZUSHIMA and YAMADA), d : cytoplasmic membrane (KOPLOW and GOLDFINE), f : plasmolysis fluid (NEU and HEPPEL, Stage I), g : shock fluid (NEU and HEPPEL, Stage II), h : EDTA extractable material (LEIVE et al.), S : molecular weight standards.

70.000. Such species were absent from extracts made by the three other procedures. Conversely, the two proteins of MW 33.000 and 36.000 were relatively less represented (6 % of the total proteins).

The discrepancies might be related to the process of recovery : the starting material consisted in the surpernatant of lysozyme-EDTA spheroplasts both in the WOLF-WATZ et al. (9) and in the MIZUSHIMA and YAMADA (10) procedures. However, the outer-membrane fragments released were first aggregated at pH 5 and recovered by low speed-centrifugation in the former case while directly collected by high speed centrifugation in the latter. A part of the material precipitated at pH 5 could consist in free proteins released during spheroplast formation. Such free proteins might either originate from the cytoplasm of accidentally ruptured spheroplasts or from the envelope itself (i.e. weakly bound envelope proteins or free periplasmic proteins).

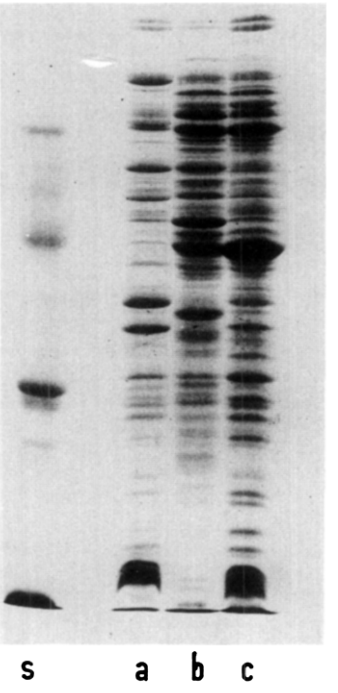

Figure 3 : Occurence of non-sedimentable proteins in the material extracted by the procedure of WOLF-WATZ et al.
S : molecular weight standards, a : outer membrane (MIZUSHIMA and YAMADA), b : outer membrane supernatant (MIZUSHIMA and YAMADA), c : outer membrane (WOLF-WATZ et al.).

The contamination by β-galactosidase activity of the material aggregated at pH 5 was found less than 0.1 % of that in the cytoplasm of induced bacteria (data not shown). Moreover, most protein species present in the cytoplasmic material of ruptured bacteria were absent from the preparation of WOLF-WATZ et al. (Fig. 2 a, b) as judged by comparative electrophoresis. Thus, in spite of some proteins of probably fortuitous similar migration properties in the cytoplasm, the supernatant of lysozyme-EDTA spheroplasts was not appreciably contaminated by intracellular material. This conclusion prompted us to compare outer and cytoplasmic membrane preparations.

The major proteins present in the outer membrane prepared according to WOLF-WATZ et al. were absent in the cytoplasmic membrane obtained as described by KOPLOW and GOLDFINE (Fig. 2 d). They were partly released in the supernatant of bacteria treated with Tris-EDTA according to LEIVE

et al. (11). They were detectable in the hypertonic suspending medium of bacteria which constituted the "stage I" of the procedure of NEU and HEPPEL (12) but were very abundant in the so-called "shock-fluid" (Stage II in the same method) (Fig. 2 f, g, h).

That most of the material aggregated pH 5 in the procedure of WOLF-WATZ was non-sedimentable by conventional means is illustrated by figure 3. A spheroplast supernatant was first submitted to a high speed centrifugation according to MIZUSHIMA and YAMADA. The high speed centrifugation supernatant contained most of the protein species recovered in the WOLF-WATZ procedure, particularly the major constituents of MW 48.000 and 70.000.

It is known that periplasmic proteins are released during the formation of EDTA-lysozyme spheroplasts (12). Hence, the supernatant of spheroplast did contain those proteins in addition to the "peeled-off" outer membrane fragments (8). The acidification of this supernatant at pH 5, was assumed by the authors (9) to promote the aggregation of the outer membrane fragments. The present work shows that it also favored the precipitation of periplasmic proteins, both fraction acting possibly as mutual carriers.

The primitive location of those so-called "periplasmic" proteins is not known accurately. They are generally thought to be retained in the periplasmic space by the outer membrane functionning as a barrier (4). Alternately, they have been considered to be loosely associated to components of the cell envelope (15). Our results seem to favor the second hypothesis since at least some of the proteins present in the shock-fluid were found sedimenting with the outer membrane prepared according to MIZUSHIMA and YAMADA (see Figures 2 c and g).

We are thus led to conclude :

i) that the material prepared according to WOLF-WATZ et al. is unsuitable for the isolation of the so-called major proteins of *Escherichia coli* outer membrane, and

ii) that the definition of an outer membrane protein is ambiguous since its depends on methodological criteria, each procedure leading to a different protein pattern.

The repartition of proteins accessible to non penetrating labelling reagents in outer membranes obtained by both the disruptive (6, 7) and the non-disruptive (9, 10) methods would help the understanding of the architecture of intact outer membrane.

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