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MUTATIONAL CHANGE OF MEMBRANE ARCHITECTURE*

MUTANTS OF *ESCHERICHIA COLI* K12 MISSING MAJOR PROTEINS OF THE OUTER CELL ENVELOPE MEMBRANE

MARGARETE SCHWEIZER, HEINZ SCHWARZ, INGEBORG SONNTAG and ULF HENNING

Max-Planck-Institut für Biologie, 7400 Tübingen (G.F.R.)

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SUMMARY

Mutants of *Escherichia coli* have been analyzed which miss two of the major proteins of the outer cell envelope membrane. The two proteins I and II*, normally are present at high concentrations (about 10^5 copies per cell).

In such mutants, as compared with wild type, the phospholipid-to-protein ratio in the outer membrane has increased by a factor of 2.3 causing a considerable difference in density between wild type and mutant membranes. The concentrations of two other major components of the outer membrane, lipopolysaccharide and Braun's lipoprotein, did not change.

The protein-deficient mutants do not exhibit gross functional defects in vitro. An increased sensitivity to EDTA and a slight such increase to dodecyl sulfate (but not to deoxycholate or Triton X-100) was observed, loss of so-called periplasmic enzymes was not found, and other differences to wild type are marginal. The mutants can grow with normal morphology. It is not possible, however, to prepare "ghosts" (particles of size and shape of the cell without murein, surrounded by a derivative of the outer membrane, and possessing the major proteins of this membrane) from them. This fact confirms our earlier suggestion that the proteins in question are required for the shape maintenance phenomenon in ghosts, and the mutants reject the speculation that these proteins are involved in the expression of the genetic information specifying cellular shape.

Freeze-fracturing showed that in mutant cells, and in sharp contrast to wild type, the far predominant fracture plane is within the outer membrane. The concentration of the well known densely packed particles at the outer, concave leaflet of this fracture plane is greatly reduced. It was not possible, however, to clearly establish that one or the other protein is part of these particles because these ultrastructural differences were not apparent in mutants missing either one of the proteins only. The biochemical and ultrastructural data allow the conclusion that the loss of two major proteins and the concomitant increase of phospholipid concentration has changed the architecture of the outer membrane from a highly oriented structure, with a large fraction of protein-protein interaction, to one predominantly exhibiting planar lipid bilayer characteristics. *E. coli* thus can assemble rather different outer

* Dedicated to Professor Feodor Lynen on the occasion of his 65th birthday.

membranes, a fact excluding that outer membrane formation constitutes a highly ordered or strictly sequential assembly-line process.

INTRODUCTION

The outer cell envelope membrane of *Escherichia coli*, and very likely of Gram-negative bacteria in general, contains a set of major (major regarding their cellular concentrations) proteins in a molecular weight range between about 10 000 and 37 000. Much of the present knowledge on these proteins has recently been summarized [1, 2] and several relevant such facts will be taken up again in Discussion. We have shown previously [1, 3, 4] that in *E. coli* B/r and K12 there are three such proteins that we have named protein I (approx. 37 000 daltons, identical with Rosenbusch's [5] matrix protein, and very likely with Schnaitman's [6] protein 1 and Bragg's [7] protein A_1), protein II* (approx. 33 000 daltons, very likely identical with Schnaitman's [6] protein 3a or 3b and Bragg's [7, 8] protein B), and protein IV (identical with Braun's [2, 9] lipoprotein). A fourth protein, protein III, may also belong to this class [4]. Proteins I, II*, and IV can be produced in rather large quantities. It has been calculated that about 10^5 copies each of protein I [5, 10] and II* [10], and about $5 \cdot 10^5$ copies of protein IV [11, 12] can be present per cell.

We have described isolation and briefly some properties of mutants that miss proteins I and II*, and such mutants did not exhibit any gross functional defects [13]. There are thus several obvious questions: (i) How and to what degree has the outer membrane of the mutants changed; (ii) are there recognizable alterations in cellular physiology, and (iii) for what purpose does the cell produce the two proteins in such large quantities and has evolved for them structures that allow the formation of a net-like [14], and at least in case of protein I also a highly regular arrangement [5] in the cell envelope?

In this communication we present some data concerning the former two questions. In regard to the second question a very large number of parameters can be tested (e.g. many details regarding the outer membranes, properties as a diffusion barrier [15] including the uptake of macromolecules, action of colicins and phages, ultrastructure of the outer membrane, retention of periplasmic substances, topology of phospholipid and of the remaining outer membrane proteins, etc.). Some of these have not been determined and others only superficially; the first main objective of our studies with these mutants was to see which physiological cellular functions are compatible with what degree of alteration of the outer membrane.

MATERIALS AND METHODS

Strains and growth conditions. Most of the strains used are derivatives of *E. coli* K12 P400 [16]. Their relevant characteristics are shown in Table III. Strains F2464 (a wild type K12) and its heptose-deficient derivative F2754 were the kind gifts of Dr. K. Jann. If not stated otherwise cells were grown with forced aeration in a complete medium (Antibiotic Medium No. 3, Difco) at 30 °C. Growth for the question on release of periplasmic enzymes was at 37 °C in a low phosphate (0.064 mM) minimal medium essentially according to Brockman and Heppel [17]; instead of

Bactopeptone glucose (0.2 %) and vitamin-free casamino acids (0.05 %) were used.

Analytical procedures. Lipopolysaccharide was quantitated by determination of ketodeoxyoctonate [18]. We have isolated [19] lipopolysaccharide from various K12 strains, such preparations were found to be free of protein (amino acid analysis) and phospholipid (fatty acid analysis) and always to contain 8–8.6 % ketodeoxyoctonate. Lipopolysaccharide concentrations in membrane preparations were calculated on the basis of this value. All protein concentrations were determined by amino acid analyses of acid hydrolyzates (6 M HCl, 24 h, 110 °C). Fatty acids were quantitated as detailed by Overath et al. [20], and the phospholipid concentration in membrane fractions was calculated on the basis of the fatty acid analyses. Alkaline phosphatase and cyclic phosphodiesterase were measured according to Brockman and Heppel [17], the latter enzyme with bis(nitrophenyl)phosphate as substrate. Two methods for dodecyl sulfate-polyacrylamide gel electrophoresis were used. The pattern of Fig. 1 was obtained by slab gel electrophoresis (Tris · HCl buffer system) according to Studier [21] as detailed by Ames [22]. This method leads to losses of lipoprotein [23] during staining and destaining; therefore, for the data of Table II another procedure was employed (ref. 24, in tubes with a phosphate buffer system) that is inferior in separation but does not cause such losses. Samples were always boiled (5 min) before application to the gel.

Sensitivity to detergents and EDTA. Sensitivity to surfactants and EDTA was tested by overlaying an Antibiotic No. 3 agar plate with 2.5 ml of soft agar containing about 10^7 cells of a given strain. Circular filter papers were laid onto the plate and 0.06 ml of the substance to be tested (concentrations see Table III) was placed onto the papers. The zone of growth inhibition was recorded after 14 h growth at 37 °C.

Electron microscopy. For freeze-fracturing glycerol was added to cells growing at 30 °C at a final concentration of 30 %. The cells were pelleted (10 min at $6000 \times g$), filled in gold holders and quickly frozen in liquid Freon 12. The specimens were freeze-fractured at −100 °C in a Balzers BAE301 or BA500M instrument and shadowed with platinum-carbon before carbon replication. Replicas were viewed in a Philips 201 electron microscope at 60 kV. For etching cells were suspended in 0.1 M phosphate buffer, pH 7.0, containing 3 % glutaraldehyde and fixed for 1 h at room temperature. Glycerol was omitted and the etch time was 2–3 min.

All negative copies are printed so that in the final figures (Figs. 2–6) the shadows are black.

RESULTS

The mutants

They have been described before [13] and the main facts will only briefly be summarized here. Mutants missing protein I (P692 and P530) were the kind gift of Dr. P. Reeves. They were isolated as being tolerant to colicins (among others) E2 and E3 [25]; the nature of the mutation(s) is unknown. In P692 and P530 mutants resistant to phage TuII* were selected, and this selection very frequently leads to loss of protein II* [1, 26]. The genetic locus (tut) of the latter mutants represents the structural gene of protein II* [26]. Fig. 1 gives an example for the electrophoretic profiles of cell envelopes from such strains. It should be pointed out that we do not know if the loss of these proteins from the outer membrane is truly complete. Their presence in amounts

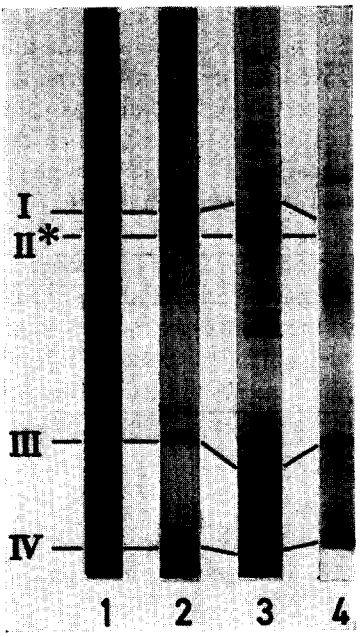

Fig. 1. Dodecyl sulfate-polyacrylamide gel electrophoreses of cell envelopes. 1, strain P400; 2, strain P400 tut2; 3, strain P692; 4, strain P692 tut2bI. The photograph shows gels from different runs with non-identical times of electrophoresis, therefore, the differing band positions are by no means strain specific. Sample 3 represents 100 μg and samples 1, 2, and 4, 50 μg dry material.

below about 5 % of their normal concentration would probably no longer be detectable with the methods presently used.

Outer cell envelope membrane

Separation of outer and plasma membranes was performed according to Osborn et al. [18]. The densities of the bands L_1 and L_2 that are enriched for plasma membrane did not differ between mutant P692 tut2dI (missing proteins I and II*) and wild type P400. Both M-band, an unresolved mixture of outer and plasma membranes, and H-band, the fraction most enriched for outer membrane, showed a rather drastically decreased density in the mutant. The densities for M and H from strain P400 were 1.223 and 1.239, respectively, while these densities for the mutant were 1.208 and 1.228, respectively. The technique to separate the membranes requires EDTA and, in addition, the studies by Mühlradt and Golecki [27] have shown that outer membrane prepared this way is topologically an artifact. It was therefore possible that the altered mutant densities were artificial in that due to the mutationally caused loss of the proteins other membrane components could have been lost during purification. We have therefore prepared cell envelopes according to Braun et al. [28] and subjected these to isopycnic sucrose density gradient centrifugation. Mutant cell envelopes differed in their density just as much from wild type envelopes as the fractions containing outer membrane did. Preparation of cell envelopes involves only shaking of cells with glass beads and centrifugations (all operations carried out in the cold), and the experiment shows that the artifact considered does not apply. Another

artifact could have been that mutant outer membrane is less well separated from the plasma membrane and that mutant H- and M-bands simply contain more of this membrane than the corresponding wild type fractions. We have therefore determined the specific activities of succinate and D-lactate dehydrogenases in all fractions from the sucrose gradients. In all cases the specific activities of the two enzymes in the H-band was near 6 % of those in the L_1-fraction (indicating less purer outer membrane fractions than those obtained by Osborn et al. [18] with *Salmonella* but of about the same quality as reported by Overath et al. [20] for *E. coli*).

Lyophilized H-band material was analyzed for its composition concerning protein, lipopolysaccharide and phospholipid. It is of some experimental interest to note that when initially we determined the protein content with the Lowry et al. method the sum of all components exceeded the input dry weight by about 20 %, and values near 100 % were only obtained after the protein content was established by amino acid analyses of H-band acid hydrolyzates. The results are shown in Table I.

TABLE I

COMPOSITION OF OUTER MEMBRANE FRACTIONS

The data (all expressed as percent dry weight) are average values from analyses of three different preparations of each strain.

	E. coli strain	
	P400	P692 tut2dI
Protein	36.5	22.6
Lipopolysaccharide	30.8	32.7
Phospholipid	35	50
Phospholipid/protein	0.96	2.2

It can be seen that the lipopolysaccharide concentration per dry weight very nearly is the same in both strains while the phospholipid-to-protein ratio has increased in the mutant by a factor of 2.3. The phospholipid-to-protein ratio in the plasma membrane fractions from both wild type and mutant P692 tut2dI was found to be near 2, thus, in this respect the mutant outer membrane has become similar to the plasma membrane. It should be mentioned that these ratios can show considerable variations if one compares data published for different strains and organisms grown under different conditions (e.g. refs. 18, 20, 29 and 30), also, it is not clear to what degree the fractions isolated (by different methods) may be partial artifacts of isolation and to what degree the protein content may have been overestimated (see above). We do not, therefore, put much emphasis on the absolute values but rather on the difference between wild type and mutant.

Phospholipid extracted with chloroform/methanol was analyzed for its fatty acid composition by gas chromatography. The values (data not shown) for the fatty acids $C_{12:0}$, $C_{14:0}$, $C_{16:0}$, $C_{16:1}$, $C_{18:0}$, and $C_{18:1}$ did not differ between mutant and wild type; the C_{17} and C_{19} cyclopropane fatty acids were present in both cases in amounts too low as to be reliably determinable.

As mentioned in Introduction one of the major outer membrane proteins is

protein IV, Braun's lipoprotein. About one-third of the total lipoprotein present is linked covalently to the murein layer of the envelope while about two-third are present not covalently linked to this heteropolymer [12, 31]. The amount of bound lipoprotein was determined by amino acid analysis of murein isolated by the "hot sodium dodecyl sulfate" method [11]. Wild type and mutant did not differ in this respect, wild type murein contained 32.6 % and mutant murein 34.5 % lipoprotein per dry weight murein. Free lipoprotein was determined by microdensitometric measurements of stained electrophoretograms from cell envelopes. With this method bound lipoprotein with murein cannot penetrate the polyacrylamide gel and in the position of free lipoprotein no other protein is present in significant amounts [10]. As can be seen from the data (numbers in brackets) in Table II the microdensitometric determination is not very accurate, and in order to obtain more comparable data we have included strain P692 (parent of P692 tut2dI missing only protein I). The areas measured are expressed relative to the number of envelopes and relative to the dry weight of envelopes. Clearly, both calculations did not reveal indications for any remarkable changes in the concentrations of free lipoprotein.

TABLE II

AMOUNT OF FREE LIPOPROTEIN IN CELL ENVELOPES

The data are arbitrary units of microdensitometrically determined areas. The numbers in brackets show the values of two different sample volumes (n and $2n$) applied to a gel. A, densitometric areas calculated for the same number of envelopes per strain; B, those areas calculated for the same dry weight of each envelope preparation.

	E. coli strain		
	P400	P692	P692 tut2dI
Densitometric area A	7 (7.8, 14)	8 (8, 15)	7 (6, 17.5)
Densitometric area B	110 (80, 240)	128 (151, 212)	85 (90, 160)

Behavior of cells

For most parameters tested no dramatic differences between wild type, mutants missing protein I, II* or both have been found and only some of these results will be presented briefly.

Lipopolysaccharide-defective mutants of the deep rough type of *E. coli* and *Salmonella typhimurium* show a marked reduction in the concentration of major outer membrane proteins in the 30 000–40 000 daltons range (for further details and references see Discussion). It has been shown that such mutants during growth loose large quantities of so-called periplasmic enzymes such as alkaline phosphatase or 2',3'-cyclic phosphodiesterase into the medium [32, 33]. With strains P400, P692, P692 tut2dI and tut2bI (cf. Table III) growing in a low phosphate minimal medium at 37 °C differences in the extracellular concentrations of the enzymes mentioned were not found.

Earlier [13] we have stated that the mutants in question are resistant to detergents and EDTA as their parents are. For testing this behavior we had chosen concentrations of these compounds that are typically growth inhibiting for certain colicin

TABLE III

(±), very turbid zone of growth inhibition of 0.25–0.5 cm; ±, turbid zone of 0.5 cm; +, clear zone of 0.5 cm; ++, clear zone of 0.8–1 cm. Strains P530 tut1cII and P692 tut2eI possess a mutationally altered protein II* [1]. *E. coli* F2464 is the parent of F2754.

Strain	Protein affected	Detergent and EDTA sensitivities					
		EDTA		Deoxycholate	Dodecyl sulfate		Triton X-100
		0.5 M	0.05 M	0.25 M	0.5 M	0.05 M	10 %
P400		(±)	–	–	–	–	–
P530	missing I	±	–	–	–	–	–
P692	missing I	±	–	–	–	–	–
P400 tut2	missing II*	(±)	–	–	–	–	–
P530 tut1fII	missing I, II*	+	–	–	±	–	–
P692 tut2dI	missing I, II*	+	–	–	±	–	–
P530 tut1cII	missing I; II* altered	±	–	–	–	–	–
P692 tut2eI	missing I; II* altered	±	–	–	–	–	–
E. coli F2464		–	–	–	–	–	–
E. coli F2754	Lipopolysaccharide defective	±	–	++	++	+	++

tolerant mutants (e.g. ref. 34). Using 10–100-fold higher concentrations a clear difference regarding EDTA and a slight difference concerning dodecyl sulfate sensitivities became apparent. Table III summarizes the results. The increase in sensitivity to EDTA is specific for the loss of both proteins I and II* and such mutants are also somewhat sensitive to dodecyl sulfate. The pattern of resistance clearly is very different from that of an *E. coli* mutant of the deep rough type (Table III).

All strains of the P530 and P692 series listed in Table III were tested on McConkey-maltose agar. All grew well (in contrast to the deep rough mutant F2754 which, expectedly, did not grow at all on these media containing bile salts and crystal violet) and scored positive for maltose. This fact does not, of course, exclude more subtle changes of the outer membrane properties as a molecular sieve.

It may finally be mentioned that all types of mutants tolerate, for growth, the same extremes of pH (5.0–8.5) as their parent and that there is also no difference regarding the sucrose concentration that is growth inhibiting (0.9–1 M).

Cellular shape

Rod-shaped ghosts have been purified from *E. coli* and other Gram-negative organisms [10, 24]. Ghosts are structures which are surrounded by a derivative of the outer membrane which maintains their shape, they are devoid of murein and free from all cytoplasmic material. The protein of ghosts consists almost exclusively of proteins I, II*, III, and IV (see Introduction). We had suggested that one or more of these proteins are required for shape maintenance of ghosts and we had speculated that there may be a causal connection between this shape maintenance phenomenon and shape determination, i.e. that the proteins may be involved in a self assembly process that participates in the expression of the genetic information specifying cellular shape.

Ghosts can be prepared from mutants missing protein I or protein II* but not from strains missing both these proteins. Regardless as to the stage of ghost purification the addition of lysozyme causes a rapid and complete disintegration of the rod-shaped structures into small vesicular particles. Obviously, and similar to a situation we had found with *Spirillum serpens* [35], the two proteins are required for shape maintenance in ghosts.

As stated earlier [13], all strains as observed with the phase contrast microscope are of normal rod-shaped morphology when grown in complete medium at 30 °C. This is also true for growth at lower temperatures but not for mutants missing both proteins when grown at 42 °C. The populations constitute a mixture of almost spherical and more ovoid cells; also, lysed cells are found in such cultures. Alteration of cellular shape is specific for the loss of both proteins, mutants lacking protein I or II* only do not differ much morphologically from their parent wild type strains at the higher temperature.

Ultrastructure of the outer membrane: freeze-fracture faces

Comparison of the freeze-fracture faces from wild type (P400), strain P400 tut2 (missing protein II*), P692 (missing protein I), and P692 tut2bI (missing proteins I and II*; the same results have been obtained with another such mutant, P692 tut2dI) showed the following. The inner (convex) and outer (concave) plasma membrane faces did not exhibit strain-specific differences and their appearance was identical to that seen by other authors with *E. coli* [36–39] or other organisms (e.g. refs. 29, 40 and 41). These facts are therefore not specifically documented here.

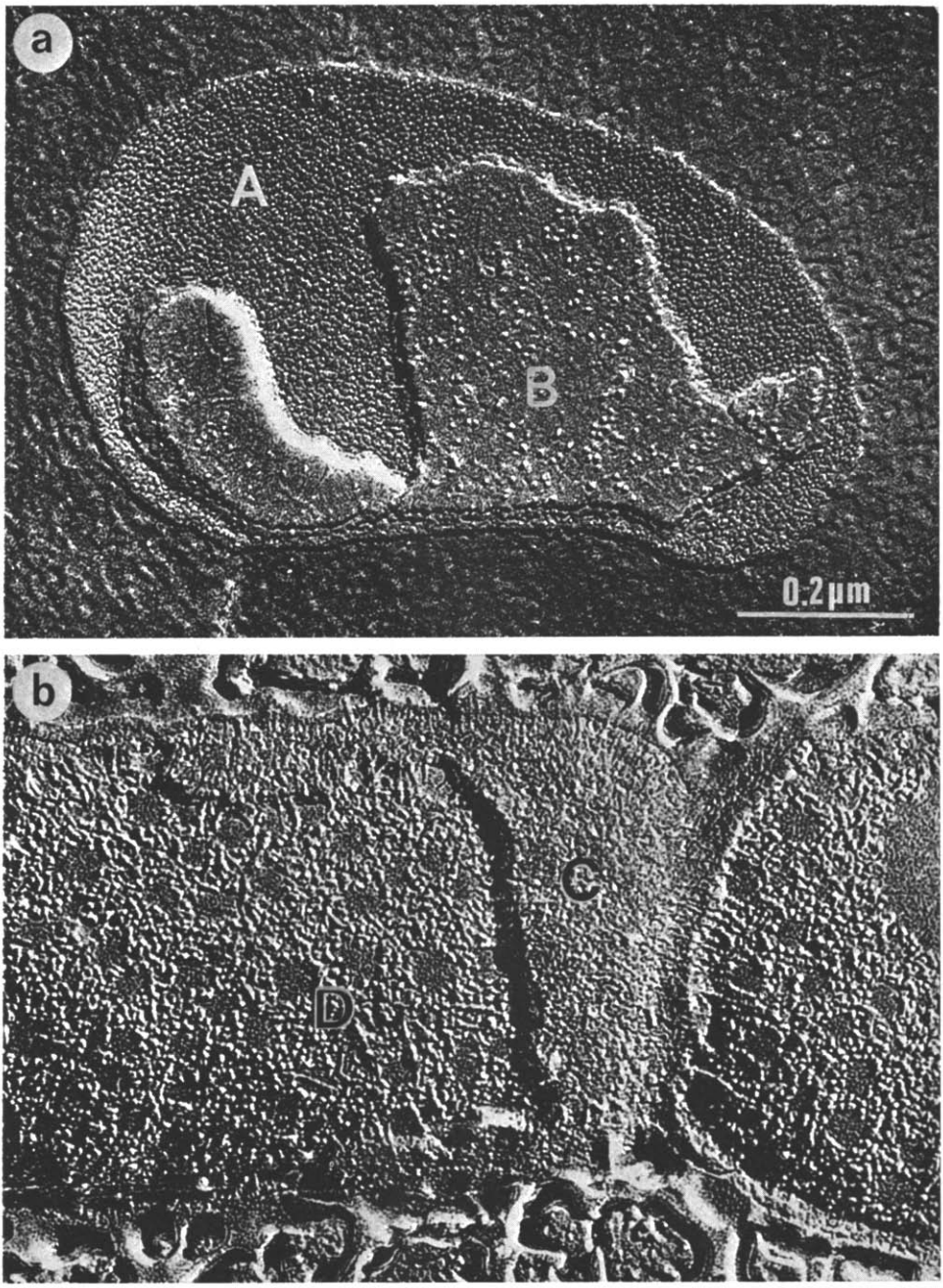

Fig. 2. Fracture faces of wild type (P400). (a), concave, outer faces; (b), convex, inner faces. A, outer membrane; B, plasma membrane; C, outer membrane; D, plasma membrane. The view (a) is unusual in that a fairly large part of the outer membrane is exposed.

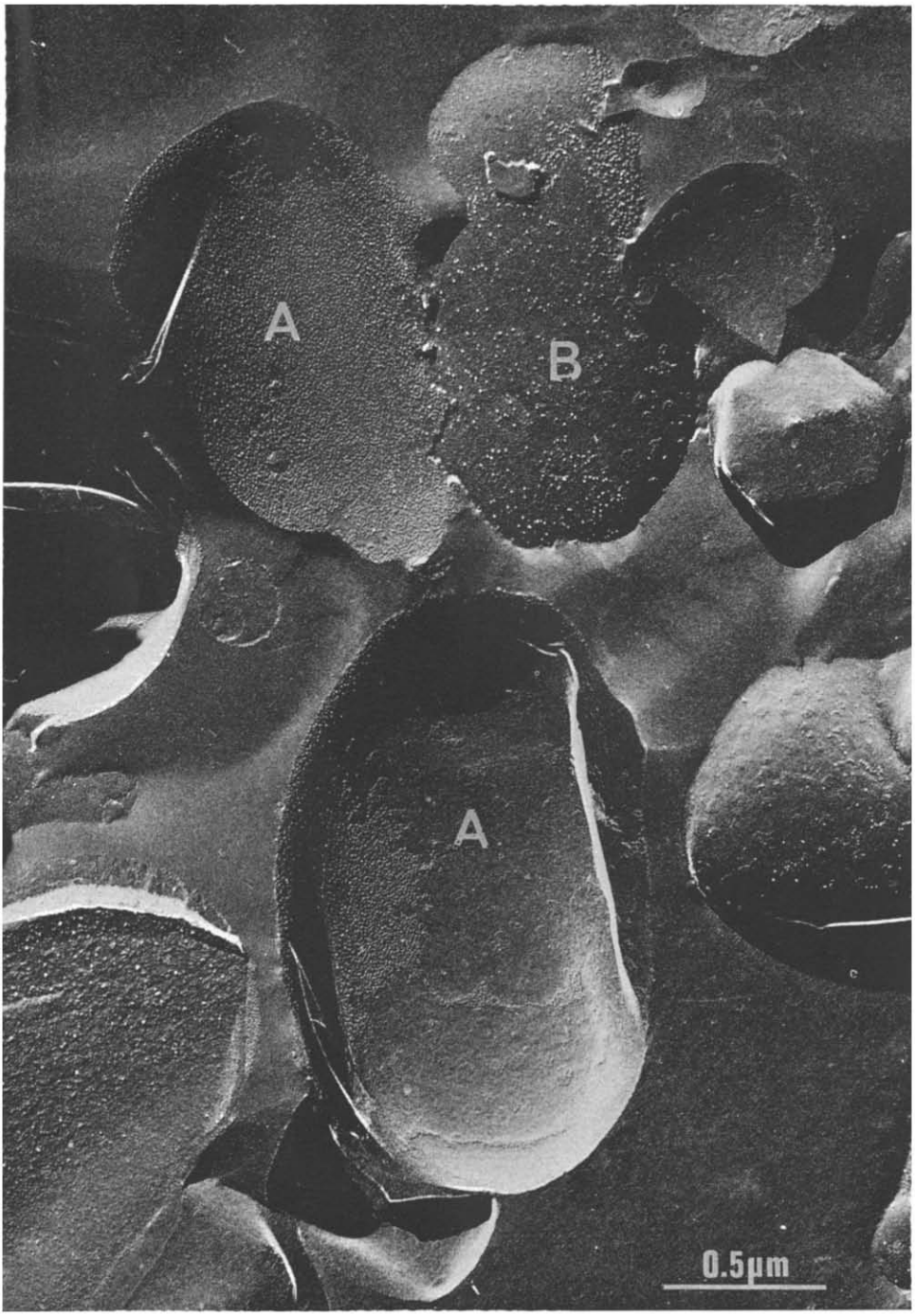

Fig. 3. Typical fracture faces of mutant P692 tut2bI missing protein I and II*. The mutant fractures almost exclusively within the outer membrane. A, outer faces; B, inner face of this membrane. Note the very inhomogeneous distribution of the concave leaflet particles.

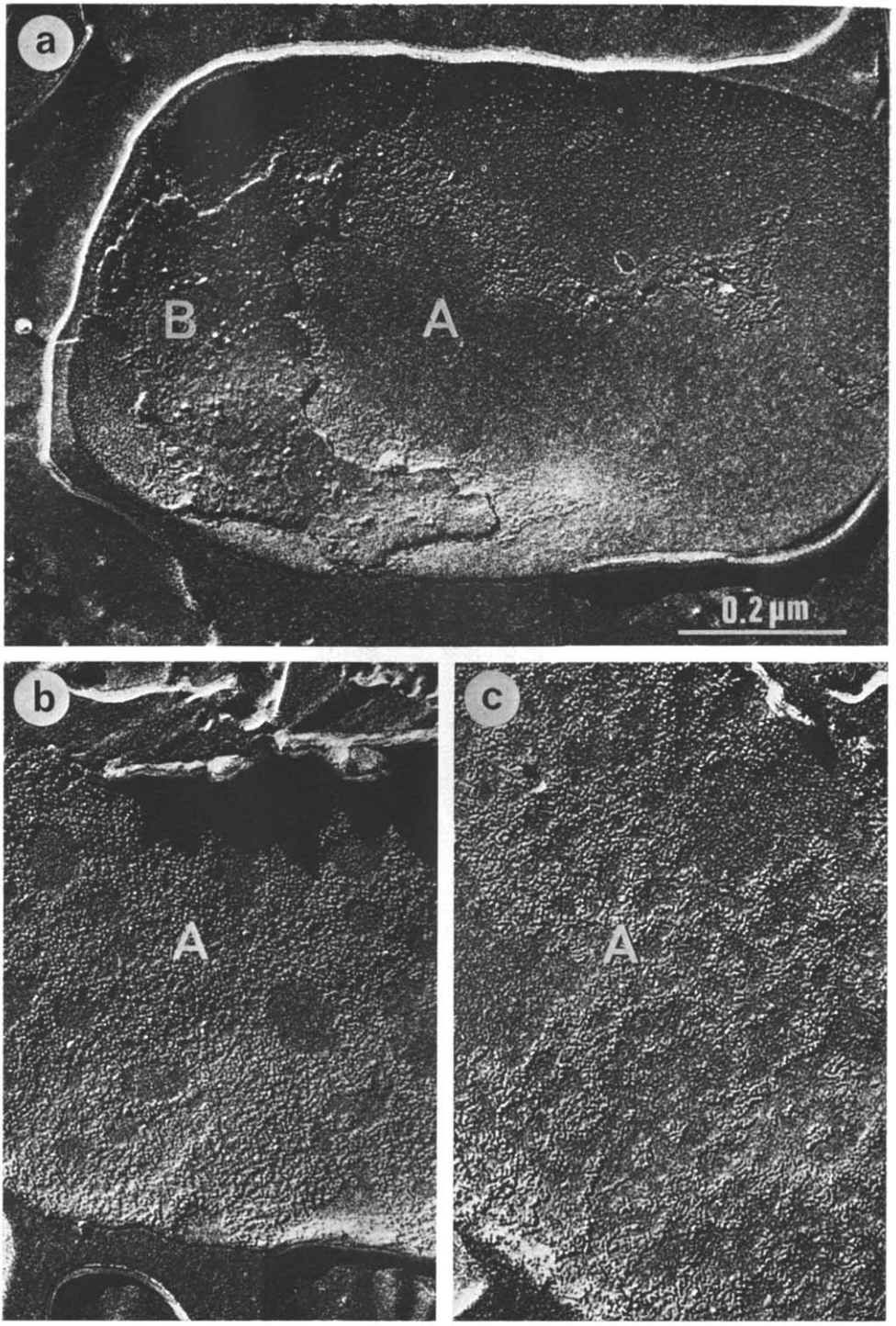

Fig. 4. Variation of particle distribution at the outer face (A) of the outer membrane from mutant P692 tut2bI. In a, part of the outer plasma membrane face (B) is visible. Faces as (b) and (c) were more frequent than that of (a).

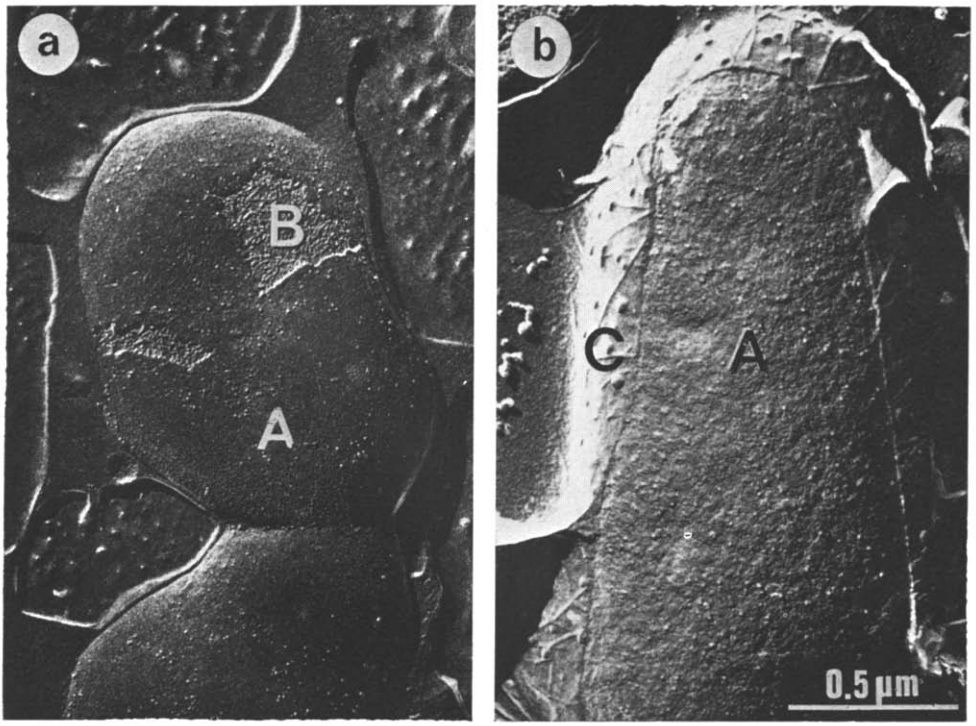

Fig. 5. Inner faces of mutant P692 tut2bI. A, outer membrane; B, plasma membrane; C, cell surface with attached pili. (b), represents a deeply etched preparation. Compare the rather smooth outer membrane face of (a) (or that of B in Fig. 3) with this from wild type (C in Fig. 2).

Rather drastic differences, however, are apparent concerning the major outer membrane fracture faces ($\widehat{CW2}$ and $\widetilde{CW2}$, respectively, of van Gool and Nanninga [38]) between wild type and mutant P692 tut2bI. In wild type these faces were seen only rarely and very rarely extending over a larger part of the cell, the predominant fracture plane being located within the plasma membrane. Fig. 2a shows the characteristic pattern of the outer face of the wild type outer membrane with its very dense packing of particles having a diameter of about 10 nm [38]. Similarily, the corresponding inner leaflet (Fig. 2b) was found only as relatively small areas, its irregular and rough appearance closely resembling that published earlier for *E. coli* [38]. Mutant P692 tut2bI was fractured almost exclusively within the outer membrane and in most cases the fracture face extended over the whole cell (Figs. 3–5). It thus at first was difficult to be sure that the face exposed indeed represents the fracture plane within the outer membrane. The following evidence demonstrates that this fracture plane cannot be anything else than that within the outer membrane. Its appearance excludes that it is a plasma membrane face (see also Fig. 2b). Fig. 5b shows that what we identify as inner leaflet does not represent the outer cell surface: on this photograph clearly both this outer surface (note the attached pili) and the inner leaflet are exposed. Furthermore, this inner leaflet and corresponding outer, concave leaflet are seen at about equal frequencies, and since more than 80 % of all fracture faces seen consist in these two leaflets, the outer leaflet cannot consist of anything else but the outer fracture

face of the outer membrane unless mutant cells should fracture in most cases between outer membrane and the murein layer of the envelope. We have therefore in addition used etching and found that the appearance of these faces did not change thus proving that one is looking at the interior of a membrane and not at a space between outer and plasma membrane [29, 38, 42].

Both these leaflets differ substantially from those of wild type cells. The inner mutant face has become rather smooth but has retained the sparsely and irregularly distributed particles. The outer mutant face does not exhibit a homogeneous appearance and two types were seen at about equal frequencies. In the first (Figs. 3 and 4a) the densely packed particles of this face from wild type have almost completely disappeared. In the second (Figs. 4b and 4c) such particles are still seen, however, more or less large areas of this face are not covered with particles.

To our disappointment we have not found truly unequivocal differences between wild type and mutants missing protein I or II*. In particular, size and density of packing of the particles characteristic for the outer membranes' outer fracture face were not clearly distinguishable between such strains. The possibility exists that the surface density of these particles differs between wild type and mutant P400 tut2 (without protein II*). In several cases small areas of the outer leaflet of the mutant were not covered with the particles in question (Fig. 6b), and such areas have not been

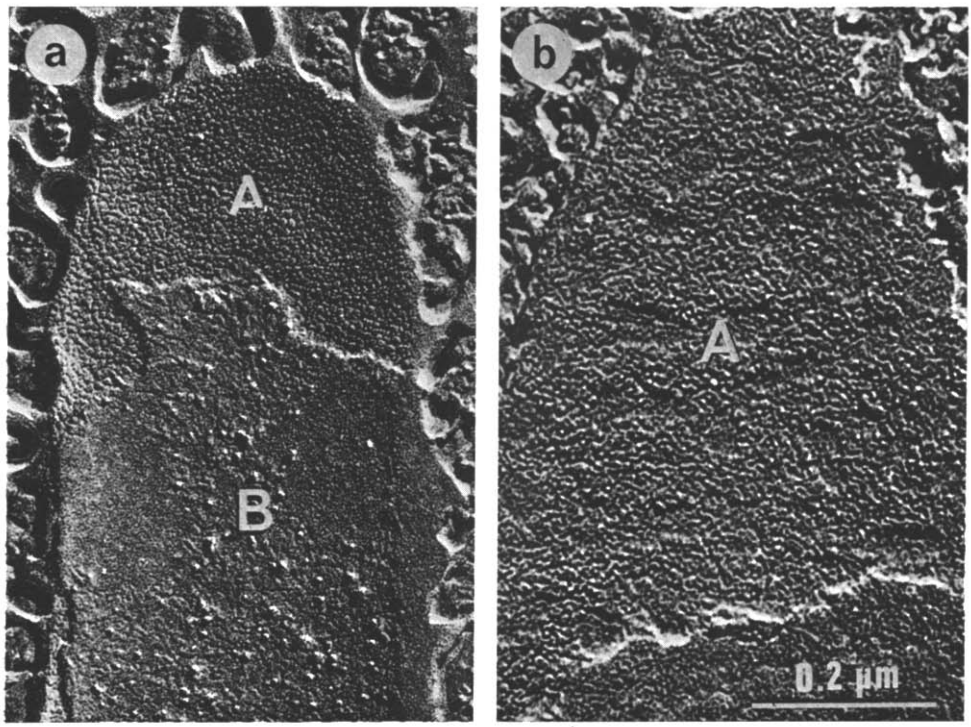

Fig. 6. Comparison of outer fracture faces of wild type (P400; (a)) and mutant P400 tut2 (b) missing protein I* only. A, outer membrane; B, plasma membrane. As detailed in the text the mutant face A did not always show this altered distribution of particles.

found in wild type cells. The problem regarding an interpretation, however, consists in the fact that (i) such areas were not always seen on the mutant fracture face, and (ii) as pointed out before this fracture face is not exposed very often at all in such mutants or in wild type and only very rarely extends over large areas of the cell.

DISCUSSION

Mutant outer membrane and cellular functions

The data on the compositions of wild type and mutant outer membranes have been obtained as weight percent of a given substance in dry membrane material and thus per se do not tell us anything about the surface distribution of the main components, i.e. the biochemical data alone do not give an answer to the question if there are changes in composition per unit surface or volume of the membrane. Since, as seen by electron microscopy, the thickness of the mutant outer membrane did not change drastically it can be calculated from the difference in densities that the surface per dry weight mutant membrane should not be larger than that of wild type membrane by more than one or a few percent. In other words, if the mutant membrane does not have holes (and there is no reason to assume this, see e.g. Table III) it must have considerably more phospholipid and less protein per unit surface than wild type. Quantitation of this surface density would await measurement of cellular surface in the straines used.

The gross change in outer membrane composition certainly is not accompanied by such changes in the cellular functions tested, and under most normal laboratory conditions the mutants would not easily be recognizable. All basic cellular functions are essentially unaffected (see section on Cellular shape for the mutants behavior at 42 °C) and one should probably wonder just how significant for membrane structure and assembly a number of "major" proteins of other biological membranes (that are not amenable to the coli type of experimentation) may be. It has also become obvious that any single model of the architecture of the outer membrane cannot describe it completely, and basic alterations in this architecture have recently also been suggested for mutants producing defective lipopolysaccharide [29, 43, 44].

The mutants and their properties have one implication regarding the assembly of the outer membrane. This assembly could be visualized as a highly ordered assembly-line process, (cf. ref. 45) with the formation, in a definite sequence, of various complexes (protein-protein, protein-phospholipid, etc.) already in the cytoplasm or plasma membrane. If so, such a process obviously need not be highly ordered. There appears to be a protein-phospholipid feedback system but another coupling has not been observed. Proteins I and II* are incorporated whether proteins II* and I, respectively, are present, and the absence of both does neither affect the formation of an outer membrane nor the amounts of incorporation of two other major components: lipopolysaccharide and Braun's lipoprotein.

Cellular shape

Since the demonstration of the shape maintenance phenomenon in ghosts [24] and the very dense, net-like arrangement of proteins I, II*, III and IV in or at the outer cell envelope membrane [5, 14] we had considered the possibility that these proteins belong to a self assembly system which participates in the expression of cellular shape,

i.e. in shape determination. Doubts to this view have already been put forward [46] and the isolation of morphologically normal mutants missing proteins I and II* showed that at least their assembly is not required for the cell to express cylindrical shape [13]. We have reported here that from such mutants ghosts cannot be obtained, and this fact demonstrates that shape maintenance in ghosts is not, or at least not directly, related to the expression of the genetic information for rod shape.

However, the mechanisms of this expression are not entirely indifferent to the structure of the outer cell envelope membrane as is evidenced by the behavior of the mutants at 42 °C. Growth of mutants without proteins I and II* at 42 °C not only leads to an aberrant morphology but also to lysis of a fraction of the culture. Therefore, cellular functions must become defective which are performed normally at lower temperatures (30–37 °C). We have not studied the mutant behavior at 42 °C in any detail because (i) of the unsurmountable problem of causality, and (ii) a defectiveness at 42 °C does not invalidate any of the conclusions drawn in this communication.

Ultrastructure and comparison with lipopolysaccharide-defective mutants

Two clear differences between mutants missing proteins I and II* and wild type were found. In sharp contrast to wild type the predominant fracture plane in the mutant is within the outer membrane and there is a very marked reduction in surface density of the particles typically associated with the outer membranes' outer fracture face. The former alteration will be discussed below. Obviously, the most straightforward explanation for the latter change would consist in the assumption that the particles in question at least partially consist of proteins I and II*. The situation seems to be somewhat more complicated, however. Because of their inhomogeneous distribution (cf. Figs. 3 and 4) we have not attempted a precise quantitation of their surface density; from inspecting a larger number of photographs we can estimate that the number of particles remaining is about 20–50 % of the wild type density. It is out of question that the mutants would still possess 20–50 % of the wild type concentrations of protein I or II*. The majority of the remaining particles therefore either is not of proteinaceous nature or contains other proteins. This observation may indicate that also in wild type the composition of these particles is not homogeneous.

We believe that a somewhat puzzling observation consists in the absence of clear differences between wild type and the mutants missing one protein only. It yet may well be that the following arguments are valid. Protein I has been shown to be tightly associated with the murein layer of the envelope [5] and it is rather likely that that part of the protein which may be buried in the lipid phase of the outer membrane is not large at all [47]. Protein I, therefore, may not be part of the particles characteristic for the outer fracture face. It has, in addition, been shown by Schnaitman [48] that mutants missing protein I can overproduce protein II* and we have confirmed this observation for strain P692. If then protein II* is part of the particles a difference to wild type may become apparent only in mutants missing protein II* but not in those missing protein I, and Fig. 6 may demonstrate this difference.

It is well known that so-called deep rough mutants (their lipopolysaccharide usually lacks heptose and except lipid A contains only 1–3 ketodeoxyoctonate residues as the sole remainder of its polysaccharide moiety) of *E. coli* and *S. typhimurium* are rather pleiotropic. Their outer membrane contains much reduced amounts

of the major proteins and an increased amount of phospholipid while the concentration of defective lipopolysaccharide did not change [29, 49, 50]. These mutants show a greatly increased sensitivity to detergents, certain dyes and mutagens (e.g. refs. 30, 49, 50–52) and they can loose substantial amounts of periplasmic proteins during growth [32, 33, 43].

The changes in density of the outer membrane, degree of total phospholipid increase and protein decrease in such mutants are very similar to the changes reported here for the protein-deficient mutants. Yet, the behavior towards surfactants is very different between the two types of outer membrane mutants (cf. Table III).

The conclusions that can be drawn from these facts are only few. A causal connection seems now to be applicable: incorporation into the outer membrane of defective lipopolysaccharide causes a decrease in protein concentration which in turn can cause an increase in phospholipid concentration.

The reason for the different behavior towards detergents is not clearly deducible last not least because it is not clear just how many proteins are affected in deep rough mutants; inspection of the electrophoretograms published for *Salmonella* by Ames et al. [49] suggests that the number may not be small. Deep rough mutants of *Salmonella* lose fairly large amounts of lipopolysaccharide into the medium [49]. It may thus be that such mutants indeed have, if only transiently, holes in their outer membrane (see Fig. 4 of ref. 29), a possibility that might explain their various sensitivities including that towards lysozyme [53, 54].

Recently, deep rough mutants of *Salmonella* and *E. coli* have also been subjected to ultrastructural analyses by freeze-fracturing and etching [29, 43, 44]. The outcome may briefly be summarized as follows. The outer membrane cleavage was much stronger in mutant than in wild type cells; "indicating a loss of orientation of the outer membrane, resulting in a more planar structure" [43]. Smit et al. [29] were able to establish a quantitative correlation between the surface density of particles at the outer fracture face and the degree of losses of major outer membrane proteins in their mutants; an increasing degree was paralleled by a decreasing particle density. The authors concluded that the particles are at least protein-containing entities. Our data are practically in complete agreement with the findings of these authors and we show in addition that the same general situation can be found in the presence of *E. coli* K12 lipopolysaccharide that definitely is not greatly altered.

On the basis of other data we have suggested repeatedly [10, 14, 24] that the *E. coli* outer cell envelope membrane does not strictly follow a lipid bilayer-protein mosaic model [55]. As has been pointed out by Bayer et al. [44] the very fact alone that *E. coli* only rarely exhibits a tendency to cleave within the outer membrane makes it difficult to apply this model. Apparently, loss of two major proteins and the concomitant increase of phospholipid concentration in our mutants has changed the architecture of this membrane from a highly oriented structure (very likely with protein-protein interactions predominant) to one predominantly exhibiting planar lipid bilayer characteristics.

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It has recently been found accidentally that mutants missing proteins I and II* exhibit one pronounced defect. Cultures left at room temperature for several days

lyse, and preliminary experiments indicate that this is due to the action of a cellular phospholipase. Also, these mutants are killed by repeated freezing (−50 °C) and thawing in the presence of 30 % glycerol.

After submission of this manuscript a paper on a similar subject by Verkleij et al. [56] appeared. Their electronmicroscopic analysis in essence agree well with ours.

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