

Bacteriophage-Resistant Mutants of *Salmonella typhimurium* Deficient in Two Major Outer Membrane Proteins

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Mutants resistant to bacteriophages (P221 and PH105 or PH51) were isolated from a *rfa* strain of *Salmonella typhimurium*. They were found deficient in separate 33,000- to 36,000-dalton band proteins (major band proteins). Double mutants derived from both types of mutants were deficient in both of the bands. The growth behavior of all the mutants was normal. The outer membrane of the mutants appeared to be more wrinkled than normal and formed vesicles in many of the mutants. In freeze-fractured cells, changes were seen in the outer membrane (particleless patches in the concave fracture face, the particles themselves being smaller than normal). These changes were more marked in the double mutants.

Several studies on the outer membrane (OM) proteins of gram-negative bacteria have recently been published by many laboratories (5, 8, 10, 16). Sodium dodecyl sulfate (SDS)-gel electrophoresis of the OM of *Escherichia coli* B/r reveals four major polypeptides (8, 10): protein I, apparent molecular weight 38,000; protein II*, molecular weight 33,000; protein III, molecular weight 17,000; and protein IV (also called the lipoprotein or Braun protein), molecular weight 7,000. Part of protein IV is covalently linked to peptidoglycan. The structure and properties of this very interesting protein are now well known (5). Protein III is often contaminated heavily with lipopolysaccharide (LPS) (8). Protein I is closely associated with peptidoglycan and cannot be solubilized from the membrane by SDS at temperatures below 60°C (16).

The 33,000- to 38,000-dalton "major bands" of SDS-gels (proteins I and II*) of *E. coli* can be separated into subbands by some SDS-gel electrophoresis methods (24). Gels of OM preparations of *E. coli* O111 have four distinct bands in this region. The proportions of these bands vary with the growth condition of the bacteria (23). Lysogenicity to bacteriophage PA-2 was also found to change the proportions of these polypeptides (24).

The protein pattern of the OM of *Salmonella typhimurium* differs to some extent from that of *E. coli* (23) but has, as a common feature, "major bands" in the region of 33,000 to 36,000 daltons (1, 15).

Mutants of *E. coli* missing either protein I (7) or II* (6, 25) or both (9) were isolated recently on the basis of their resistance to certain colicins and/or bacteriophages. Even the double mutants missing both bands appeared normal in their shape and behavior in culture (9).

Bacteriophages have been used extensively for the isolation and characterization of another OM component, LPS (27). We have been looking for suitable phages to isolate mutants of OM proteins in *Salmonella*. We hoped to find them from among phages that do not use LPS as their receptor. In preliminary experiments we found that the receptors of the bacteriophages P221 (29), PH51, and PH105 are localized in SDS-gels in the 33,000- to 36,000-dalton major band region. We describe here mutants of two major band proteins in *S. typhimurium* isolated on the basis of their resistance to these phages.

MATERIALS AND METHODS

Bacterial strains. Strain SH5014 of *S. typhimurium* LT2, a *rfa* mutant derived from strain SL1027 (27), was used as the parent of the phage-resistant mutants. SH5014 is sensitive to the LPS phages (see below) Br60, Ffm, and Br2 and resistant to P22C2, FO, 6SR, and C21, suggesting that it has a defective LPS typical of *rfaK* or *rfaJ* mutants (27). On the basis of its normal resistance to detergents, it is clear that it is not the "deep rough" type reported to have an altered OM structure (2). The structure of its LPS has not been studied in more detail.

LPS phages. Bacteriophages, which need a certain structure of LPS to be able to attack the bacteria, were used as indicators of LPS structure. The phages used were P22C2, 9NA, FO, 6SR, C21, Br60, Br2, and Ffm (27).

"Major band phages." P221 (29) is a phage much used for studies of LPS mutants. *S. typhimurium* strains with complete, smooth LPS are not sensitive to it presumably because of steric hindrance (27) by the O polysaccharide, whereas all rough mutants with defective LPS are sensitive. P221 is probably a recombinant between phages P22 and Fels 1, a pro-phage of *S. typhimurium* LT2 (28).

PH51 and PH105 were isolated in our laboratory (to be published). PH51 is probably a recombinant between ES18 (11) and Fels 1. PH105 was isolated from *S. typhimurium*-positive feces. These phages attack all rough (*rfa*) mutants of *S. typhimurium*. P221- and PH51-lysogenic bacteria are coimmune but not immune to PH105.

Isolation of mutants. Portions (0.1 ml) of an overnight broth culture of bacteria treated with the mutagen diethyl sulfate (13) were spread on nutrient agar plates, and drops of phage suspension, about 10⁸ plaque-forming units (PFU) per ml, were added. After overnight incubation at 37°C, phage-resistant colonies were picked and purified by two successive single-colony isolations on nutrient agar. Mutants were checked for sensitivity and lysogenicity to the three major band phages and for sensitivity to the LPS phages to detect possible changes in their LPS. Only mutants that could not adsorb the particular phage used for selection and that had the same LPS phage sensitivity pattern as the parent strain (only few isolates were altered in this respect) were chosen for further work.

Phage adsorption on whole bacteria. In preliminary experiments, P221 was shown to adsorb best on bacteria at a low salt concentration, which was therefore adopted for the standard assay. The other two phages adsorbed at this concentration as well as in ordinary phage buffer.

The bacteria from 5 ml of an overnight broth culture were collected by centrifugation. Chloramphenicol (0.1 ml, 1 mg/ml), sterile, distilled water (0.8 ml), and a standard phage dilution (0.1 ml, about 30,000 PFU/ml) were added. After incubation for 1 h at 37°C, the bacteria were lysed by adding a drop of chloroform, and the number of PFU in the supernatant after centrifugation was counted. A 90% reduction of PFU compared to a control treated similarly but with the bacteria omitted was taken as evidence of adsorption. In these circumstances, SH5014 adsorbed about 90% of P221, 95% of PH51, and 100% of PH105.

Assay of phage receptor activity. To 50 µg of the preparation to be tested, 0.1 ml of 2% Triton X-100 in 0.1 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 7.8) (TX buffer), 0.1 ml of 15 mM MgCl₂, 0.5 ml of distilled water, and 0.1 ml of the phage dilution (about 30,000 PFU/ml) were added. After incubation for 1 h at 37°C, the number of PFU was counted. A control without "receptor" preparation was treated identically. A reduction of PFU was used as measure of receptor activity and expressed

as follows: 0 to 10% inactivation (-), 10 to 50% inactivation (±), 50 to 90% inactivation (+), 91 to 97% inactivation (++), and >97% inactivation (+++).

Membrane preparations and SDS-acrylamide gel electrophoresis. **Method I** (1). Cells were harvested at the stationary growth phase in L broth and were suspended in 0.05 M Tris-hydrochloride buffer (pH 6.8) after being washed once with the same buffer. The cell suspension was subjected to sonic oscillation, for a total of 2.5 min and intermittent cooling for 30 s in every 30 s. The sonic extract was centrifuged at 30,000 × g for 30 min and washed twice with 0.05 M Tris-hydrochloride buffer (pH 6.8). Large membrane fragments and unbroken, intact cells were removed by low-speed centrifugation at 750 × g for 10 min.

Method II (17). The bacteria were grown on enriched nutrient agar plates (19) at 37°C. They were collected with 0.01 M Tris-hydrochloride buffer (pH 7.8) and washed twice with the same buffer. To prepare envelopes, 1 g of bacteria (wet weight) was suspended in 10 ml of 0.01 M Tris-hydrochloride (pH 7.8) containing 0.01 M ethylenediaminetetraacetate (EDTA) and 1.3 mg of lysozyme. After 10 min, 0.4 ml of 1 M MgCl₂, 0.3 mg of deoxyribonuclease, and 0.3 mg of ribonuclease were added. After 5 min the mixture was centrifuged for 20 min at 1,600 × g. The pellet was washed once with 10 ml of 0.01 M Tris-hydrochloride (pH 7.8) containing 0.01 M EDTA and then once with 10 ml of 0.01 M Tris-hydrochloride (pH 7.8) containing 5 mM MgCl₂.

The cell envelopes prepared from 1 g of bacteria were extracted with 10 ml of TX buffer followed by centrifugation for 20 min at 1,600 × g at room temperature. The supernatant (TX extract; rough protein content, 1 mg/ml) was collected and stored at -20°C. The pellet was washed once with the same buffer (20 ml). The wash was discarded.

The pellet was then extracted with 10 ml of TX buffer containing 5 mM EDTA, followed by centrifugation as described above. This extraction procedure was repeated with 20 ml of the TX-EDTA buffer. The supernatants (TX-EDTA extracts 1 and 2) were collected and stored at -20°C. TX-EDTA extract 1 had a rough protein content of 1 mg/ml. The insoluble residue was suspended in 10 ml of TX-EDTA buffer (rough protein content of TX-EDTA-insoluble fraction, 3 mg/ml) and stored at -20°C.

SDS-gel electrophoresis. SDS-gel electrophoresis was carried out in 10 or 9% acrylamide (thickness, 0.8 mm) essentially by the method of Laemmli (12). Each well received 25 µg of protein that had been heated at 100°C for 5 min in the presence of SDS and β-mercaptoethanol. Reference proteins used for molecular weight markers are described elsewhere (14).

Electron microscopy. Bacteria were grown at 37°C in broth and harvested at the logarithmic phase of growth by centrifugation.

For thin sections, the pellets were suspended for prefixation in 3% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2) and washed three times in the buffer.

Postfixation took place for 2 h in the same buffer

containing 1% osmium tetroxide. The sections were obtained from Epon 812-embedded samples and stained with uranyl acetate and lead citrate.

For freeze fracturing, the bacteria were frozen in the presence of 30% (vol/vol) glycerol and fractured at -120°C in a Balzers apparatus (Lichtenstein). Micrographs were taken with a JEOL-100B electron microscope operating at 80 kV.

RESULTS

Phage-resistant mutants. Phages PH51, PH105, and P221 were chosen for this work because they attack bacteria with the most defective LPS ("heptoseless" mutants) and they are not inactivated by isolated LPS of various *rfa* strains. Therefore, it was considered probable that their receptors were not in LPS but, rather, in other—perhaps protein—components of the OM. In preliminary experiments, their receptors could be localized in the 35,000-dalton region in SDS-gels of isolated OM.

The isolation of PH51- and PH105-resistant bacterial mutants was relatively easy. Most of the mutants isolated no longer adsorbed the phage used for the selection of the particular mutant. The sensitivity of these mutants to the LPS phages had not changed, indicating that the mutation had not affected their LPS.

When P221 was used for isolating resistant mutants, only a few were found. None of these was fully satisfactory: they adsorbed the phage, at least to some extent, and TX-EDTA extracts prepared from them, as well as the extract from the parent strain, inactivated P221.

All PH51-resistant (not lysogenic) mutants remained sensitive to PH105 and P221. All PH105-resistant mutants were sensitive to PH51 but also turned out to be resistant to P221. On the basis of results presented below, it is probable that PH105 and P221 use the same receptor.

The PH51-resistant mutant used for most experiments was SH5551; the PH105- and P221-resistant mutant was SH6017. Double mutants were isolated from these after mutagenic treatment by selecting for PH105 or PH51 resistance, respectively. SH6260 and SH6261, double mutants derived from SH6017, are resistant to PH51, whereas SH6263 and SH6264, double mutants derived from SH5551, are resistant to PH105 (Table 1).

SDS-gel electrophoresis of the membrane proteins. The PH51- and PH105/P221-resistant mutants and the double mutants selected from them were compared with the parent, SH5014, with respect to their membrane proteins, by subjecting membrane fragments to SDS-gel electrophoresis (method I). The parent, SH5014, and all of its derivatives seem to differ from the *S. typhimurium* LT2 strains described by Ames (1) in having a diminished amount of the 35K protein. (See reference 2 for use of a number followed by the letter K to designate molecular weights of proteins.) This difference, the basis of which has not been investigated, should not affect this study because all mutants are derivatives of the same strain, SH5014, and are compared with it.

The mutants are lacking one or both of two major polypeptide bands in the 33K to 36K region, which have been correlated with the outer membrane of *S. typhimurium* (1, 2) (Fig. 1). The 34K band is missing in mutant SH5551, which is resistant to PH51 only, whereas the 36K band is missing in SH6017, which is resistant to PH105/P221. The double mutants resistant to all of these phages are lacking both the 34K and the 36K bands. Several other mutants resistant to either PH51 or PH105/P221 or both consistently showed these same changes. The third strong band, 33K, is unaltered in all of the mutants. Some changes

TABLE 1. Sensitivity of *S. typhimurium* SH5014 and its phage-resistant single and double mutants and lysogenic derivatives to the three major band phages^a

No.	Strains	Derived from	Sensitivity to:		
			PH51	PH105	P221
SH5014	Parent		+	+	+
SH5551	PH51-resistant mutant	SH5014	-	+	+
SH6263	PH105-resistant mutant	SH5551	-	-	-
SH6264	PH105-resistant mutant	SH5551	-	-	-
SH6017	PH105-resistant mutant	SH5014	+	-	-
SH6260	PH51-resistant mutant	SH6017	-	-	-
SH6261	PH51-resistant mutant	SH6017	-	-	-
SH5554	PH51 lysogenic mutant	SH5014	-	+	-
SH6191	P221 lysogenic mutant	SH5014	-	+	-

^a All sensitive (+) bacteria also adsorbed the phage in question, whereas the resistant (-) ones did not. Adsorption tests were not performed with the lysogenic strains.

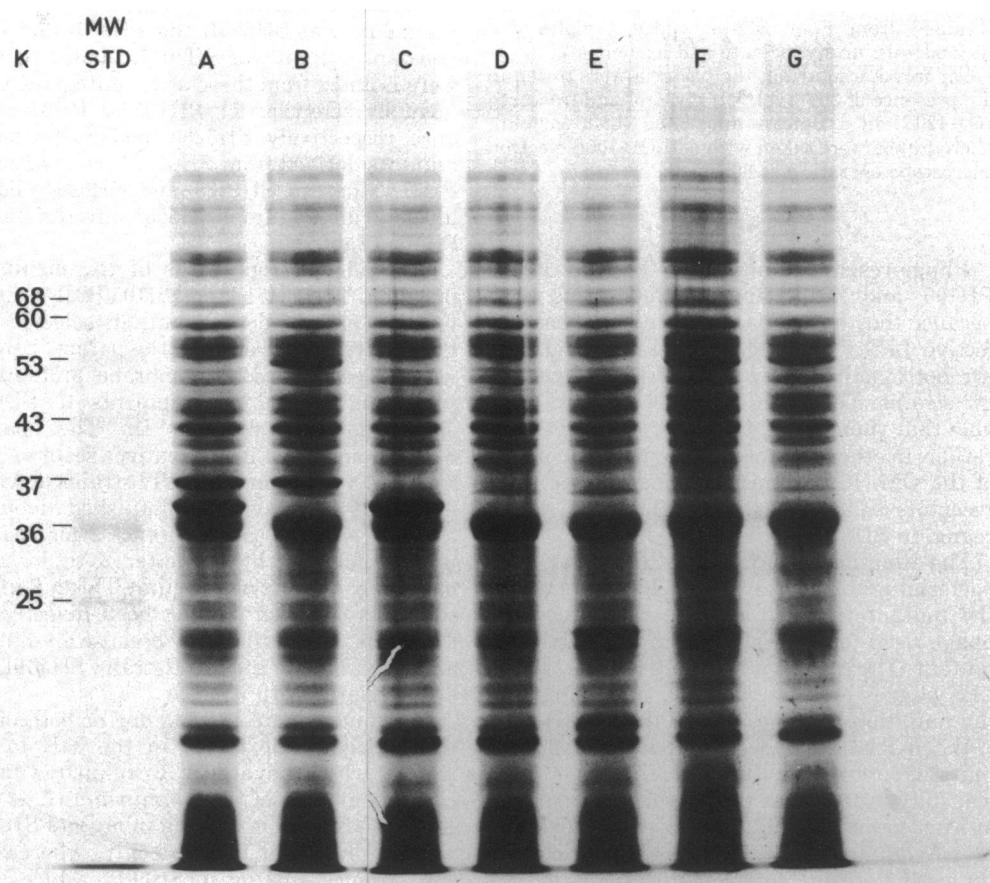


FIG. 1. SDS-gel electrophoresis (10% gel) of total membrane fraction of the parent SH5014 (A); the PH51-resistant mutant SH5551 (B); the PH105/P221-resistant mutant SH6017 (C); and the double mutants (D) SH6260 and (E) SH6261 derived from SH6017, and (F) SH6263 and (G) SH6264 derived from SH5551. MW, Molecular weight standards.

are also seen (Fig. 1) in the apparent amounts of some minor bands, but these changes are not consistent with sensitivity or resistance to the phages. These results suggest that the 34K polypeptide may be the receptor of PH51 and that the 36K polypeptide may be the receptor of PH105/P221.

The protein pattern obtained from PH51 and P221 lysogenic strains did not differ from that of the parent strain (not shown).

Solubilization of the phage receptor. To study the phage receptors more closely, we looked for a way to solubilize them in a form in which they would still be active and testable with phage inactivation. The Triton X-100-EDTA extraction (17) seemed to be a gentle enough method; to improve the yield, we started with lysozyme-EDTA-prepared envelopes (method II). The TX extract is expected to

contain mainly proteins of the cytoplasmic membrane (CM), whereas about half of the OM proteins are found in the TX-EDTA extracts, and the other half remain in the insoluble residue (21).

The ability of these extracts to inactivate the major band phages is shown in Table 2. Very little phage-inactivating capacity was found in TX extracts (CM); the first TX-EDTA extract (OM) and the TX-EDTA-insoluble residue were the fractions containing most of the phage receptor activity. The fractions from the phage-resistant mutants were lacking the corresponding phage inactivation capacity, whereas the lysogenic strains behaved like the parent.

TX extracts of all strains gave a number of bands corresponding to CM proteins and were lacking in the 33K to 36K bands typical of OM. These bands were prominent in both the TX-

EDTA extracts (Fig. 2) and the TX-EDTA-insoluble residue (not shown), corresponding to the absence of phage-inactivating capacity in the TX extracts and its presence in the other fractions.

In the phage-resistant mutants, the protein pattern of the 33K to 36K region was the same as that seen in Fig. 1. In the single mutants, either the 34K band (in SH5551, PH51 resistant) or the 36K band (in SH6017, PH105/P221 resistant) was missing. The double mutants were lacking both of these bands.

In addition, a prominent band with an apparent molecular weight of approximately 30,000 was seen in the TX-EDTA extracts of all the strains (Fig. 2). Probably this band was also present in the total membrane fractions (Fig. 1) but was partially hidden under the strong 33K protein band.

Electron microscopy. In thin section (Fig. 3) as well as in freeze-fractured replicas (Fig. 4 and 5), the cell envelope of strain SH5014 is typical of wild-type *S. typhimurium*, as de-

scribed earlier (26; K. Lounatmaa, P. H. Mäkelä, and M. Sarvas, *J. Bacteriol.*, in press; K. Lounatmaa and N. Nanninga, manuscript in preparation). In short, the concave fracture face of the outer membrane (OM) is characterized by densely packed particles of homogeneous size (8 to 10 nm in diameter). The basic appearance of the convex fracture face of the outer membrane (OM) is that of a smooth surface with few pits and particles varying in size.

All mutants when compared with the parent strain showed morphological changes of the OM. These changes were fairly small but were found consistently in several experiments and in a number of mutants. No alterations of other components of the cell were detected.

PH51-resistant mutant SH5551. Mutant SH5551 looks very similar to the parent; however, the OM appears to be more wrinkled in the mutant (Fig. 6) than in the parent (Fig. 3), and a few vesicles are seen in freeze-fractured preparations (arrowheads, Fig. 12). In addition,

TABLE 2. Ability of the TX and TX-EDTA extracts and the remaining insoluble residue to inactivate the three major band phages^a

Strain	Preparation tested	Inactivation of phages:		
		PH51	PH105	P221
Parent SH5014	TX extract	-	+	±
	TX-EDTA extract 1	++	+++	++
	TX-EDTA extract 2	±	+	±
	TX-EDTA-insoluble residue	+	+++	++
PH51 resistant SH5551	TX extract	-	+	±
	TX-EDTA extract 1	-	+++	++
	TX-EDTA extract 2	-	+	±
	TX-EDTA-insoluble residue	-	+++	++
PH105 resistant SH6017	TX extract	-	-	-
	TX-EDTA extract 1	++	-	-
	TX-EDTA extract 2	±	-	-
	TX-EDTA-insoluble residue	+	-	-
Double mutants	TX extract	-	-	-
	TX-EDTA extracts	-	-	-
	TX-EDTA-insoluble residue	-	-	-
Lysogenic strains SH5554 SH6191	TX extract	NM ^b	NM	NM
	TX-EDTA extract 1	++	+++	++
	TX-EDTA-insoluble residue	±	+++	+

^a -, No inactivation; ±, 10 to 50% inactivation; +, 50 to 90% inactivation; ++, 91 to 97% inactivation; and +++, >97% inactivation. The extracts were prepared from the same amount of bacteria in each case, and their protein contents were not grossly different.

^b NM, Not measured.

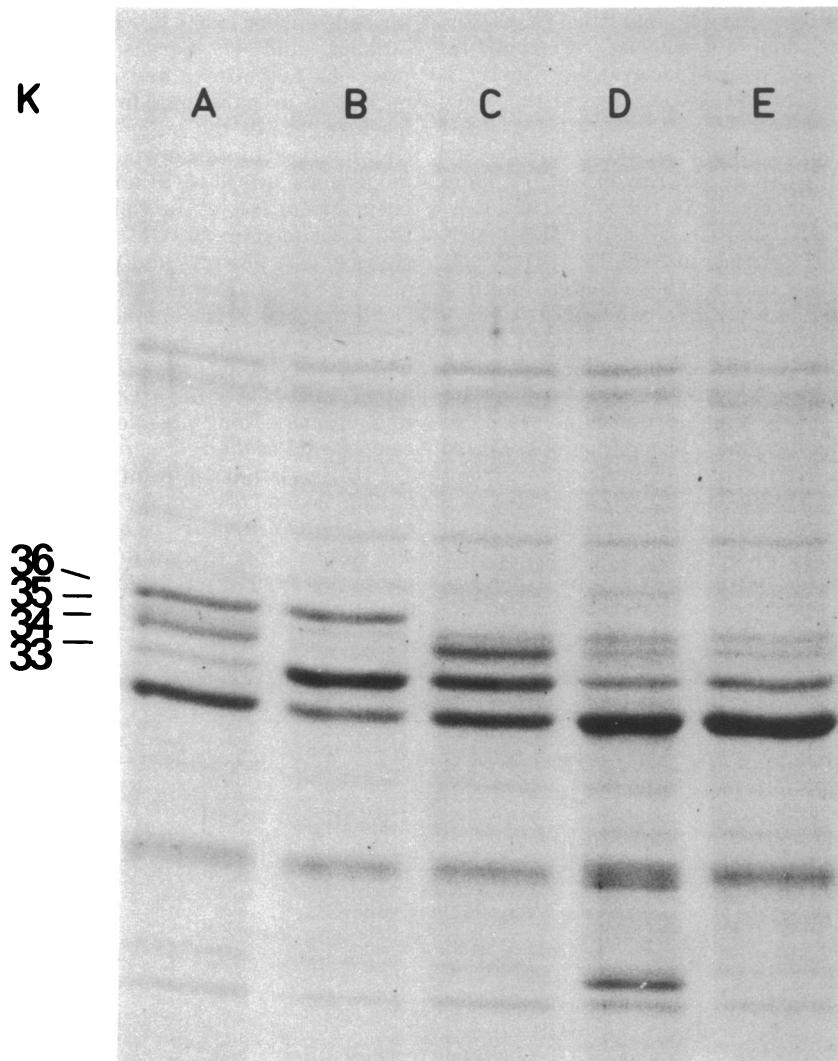


FIG. 2. SDS-gel electrophoresis (9% gel) of TX-EDTA extract of the parent SH5014 (A); the PH51-resistant mutant SH5551 (B); the PH105/P221-resistant mutant SH6017 (C); and double mutants (D) SH6261 derived from SH6017 and (E) SH6263 derived from SH5551.

the OM (Fig. 13) appears to contain more numerous particles than that of the parent (cf. Fig. 5).

PH105- and P221-resistant mutant SH6017. Mutant SH6017 is also very similar to the parent, but some small vesicles are seen outside the OM, both in thin section (Fig. 9) and in freeze-fractured cells (arrowheads, Fig. 17). Also, this mutant seems to have exceptionally numerous particles in the OM (Fig. 18).

One new fracture face can be observed (Fig. 19). The vesicles on this face indicate that it is the fractured surface (FS) of the bacterial cell. This is an unusual plane of fracture (4), and we wish to point out that there was no etching in

the preparation of the micrographs. The interesting feature of this fracture face is that it is covered by densely packed 8- to 10-nm particles like the OM. Vesicles are also seen. Other well-known fracture faces, described earlier, can be seen in this micrograph for comparison.

Double mutants SH6260, SH6261, SH6263, and SH6264. Morphological changes are more marked in the double mutants SH6260 (Fig. 10, 20, 21), SH6261 (Fig. 11, 22), SH6263 (Fig. 7, 14), and SH6264 (Fig. 8, 15, 16) than in the parents. All of these mutants look very similar to each other irrespective of whether the parent was SH6017 (as was the case for double mutants SH6260 and SH6261) or SH5551 (double

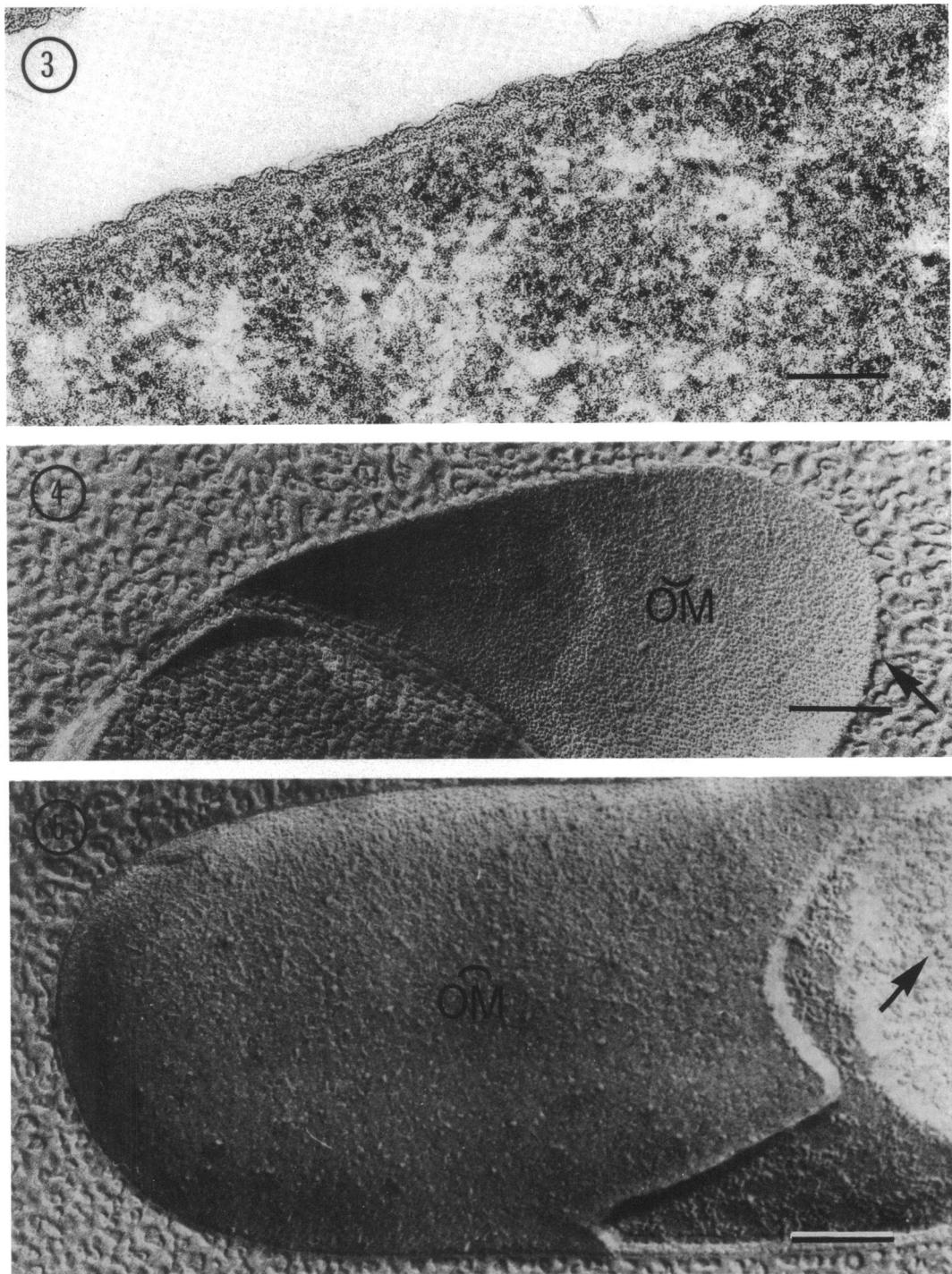


FIG. 3-5. Ultrastructure of the OM of the parent strain SH5014. Bar, 0.1 μm . Arrows in freeze-fracture micrographs indicate the direction of shadowing.

FIG. 3. Thin section showing typical bilayer structure of the OM.

FIG. 4. Outer concave (OM) fracture face covered by typical 8- to 10-nm particles.

FIG. 5. Outer convex (OM) fracture face with pits and particles.

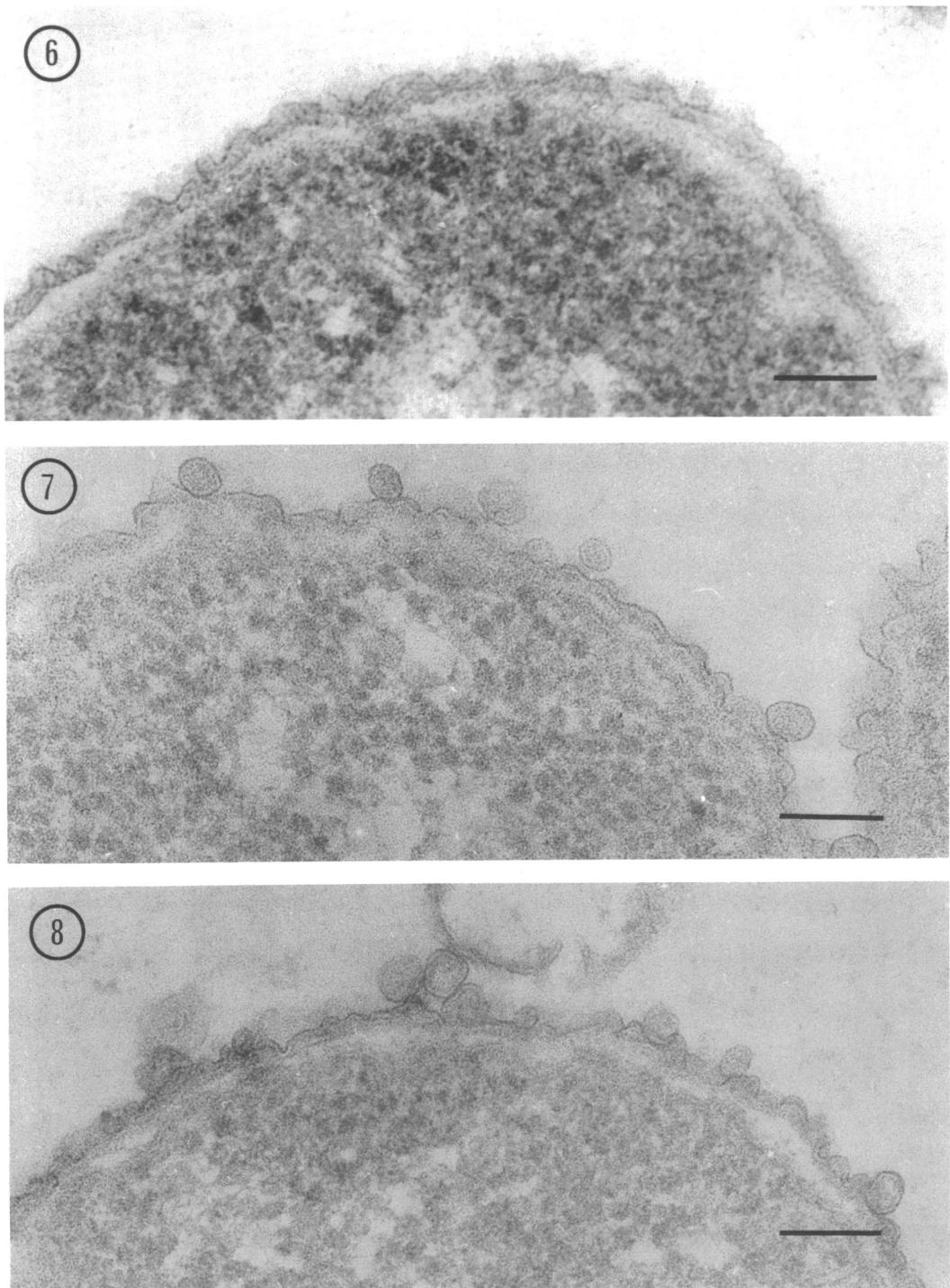


FIG. 6-8. Ultrastructure of the OM of phage-resistant mutants in thin section.

FIG. 6. Wrinkled OM of the PH51-resistant mutant SH5551.

FIG. 7 and 8. Double mutants SH6263 and SH6264 (resistant to all phages, selected from the PH51-resistant mutant SH5551) covered by numerous vesicles.

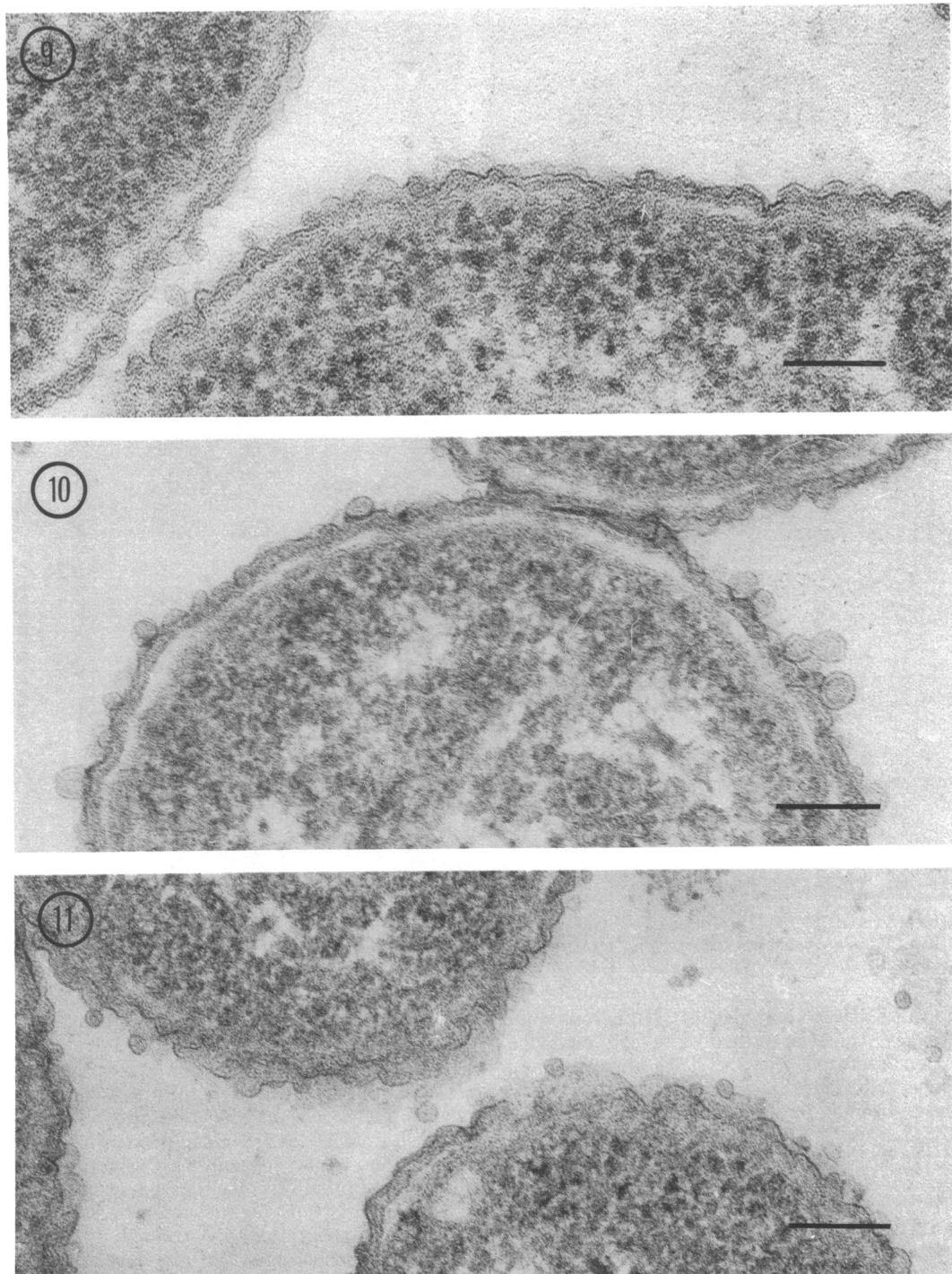


FIG. 9-11. Ultrastructure of the OM of phage-resistant mutants in thin section.

FIG. 9. PH105/P221-resistant mutant SH6017 showing numerous vesicles.

FIG. 10 and 11. Double mutants SH6260 and SH6261 (resistant to all phages, selected from the PH105/P221-resistant mutant SH6017) also with a number of vesicles.

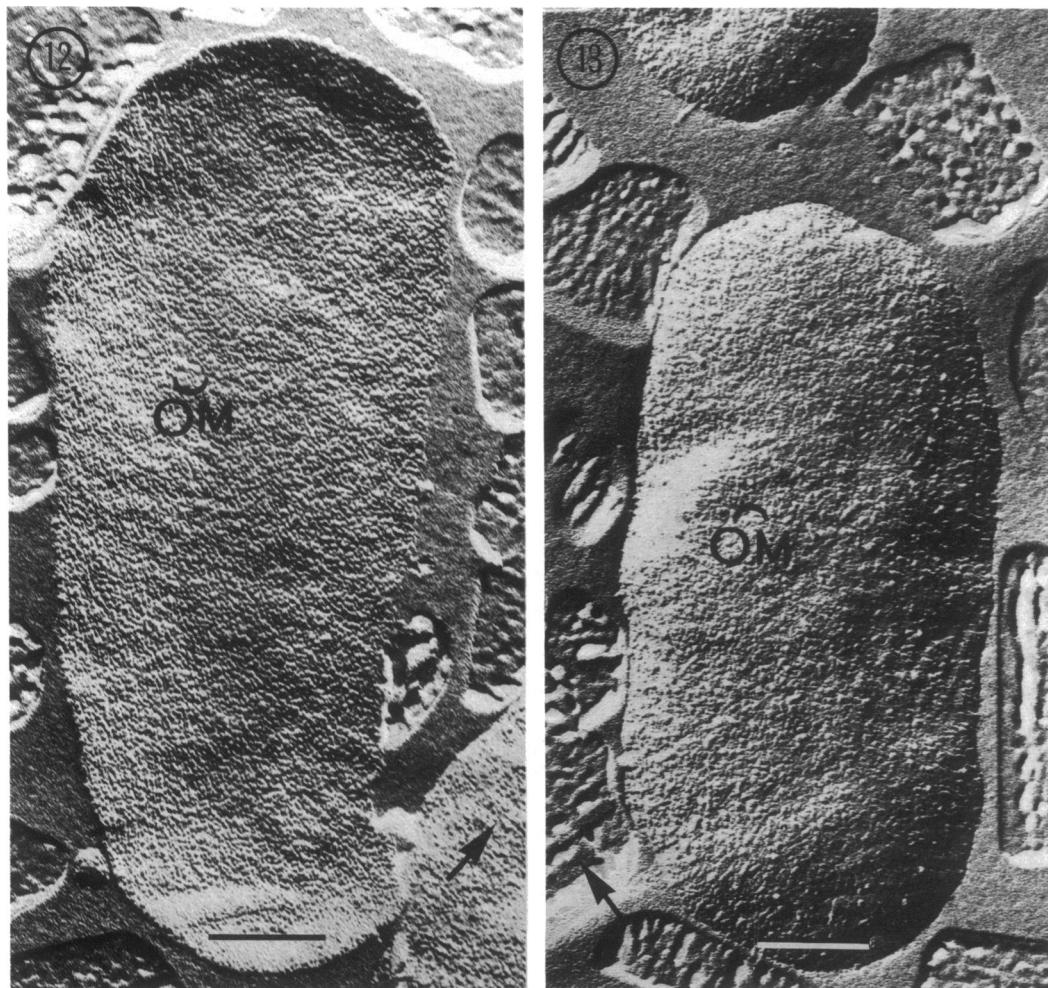


FIG. 12 and 13. Fracture faces of the OM of the phage PH51-resistant mutant SH5551.

FIG. 12. Outer concave fracture face (OM) showing normal pattern.

FIG. 13. Outer convex fracture face (OM) covered by numerous particles.

mutants SH6263 and SH6264). Numerous small vesicles are seen outside the cell envelope. When fractured, these vesicles always show a concave fracture face, suggesting that they were, in fact, cross-fractured (this situation would arise if their wall was not a lipid bilayer). In Fig. 14 (SH6263), two particles are seen on the inside face of a vesicle (arrowhead). The form and size of these particles look similar to those on the neighboring concave fracture face of the outer membrane (OM).

A prominent feature of all these mutants is numerous particleless patches in the outer concave fracture face (OM). The particles of this face also seem to be smaller than the particles of the parent strain (Fig. 4).

DISCUSSION

We isolated from *S. typhimurium* mutants resistant to either one or both of two bacteriophages. The LPS of the mutants was unchanged. Instead, the single mutants were deficient in one of two separate OM proteins, and double mutants were lacking both of them. These proteins were identified as the 34K and the 36K polypeptides as described by Ames (1, 2). In bacteriophage PH51-resistant mutants the 34K band and in PH105/P221-resistant mutants the 36K band was missing, suggesting that the 36K band would be the receptor for PH105 and P221 and the 34K band would be the receptor for PH51. The third strong OM

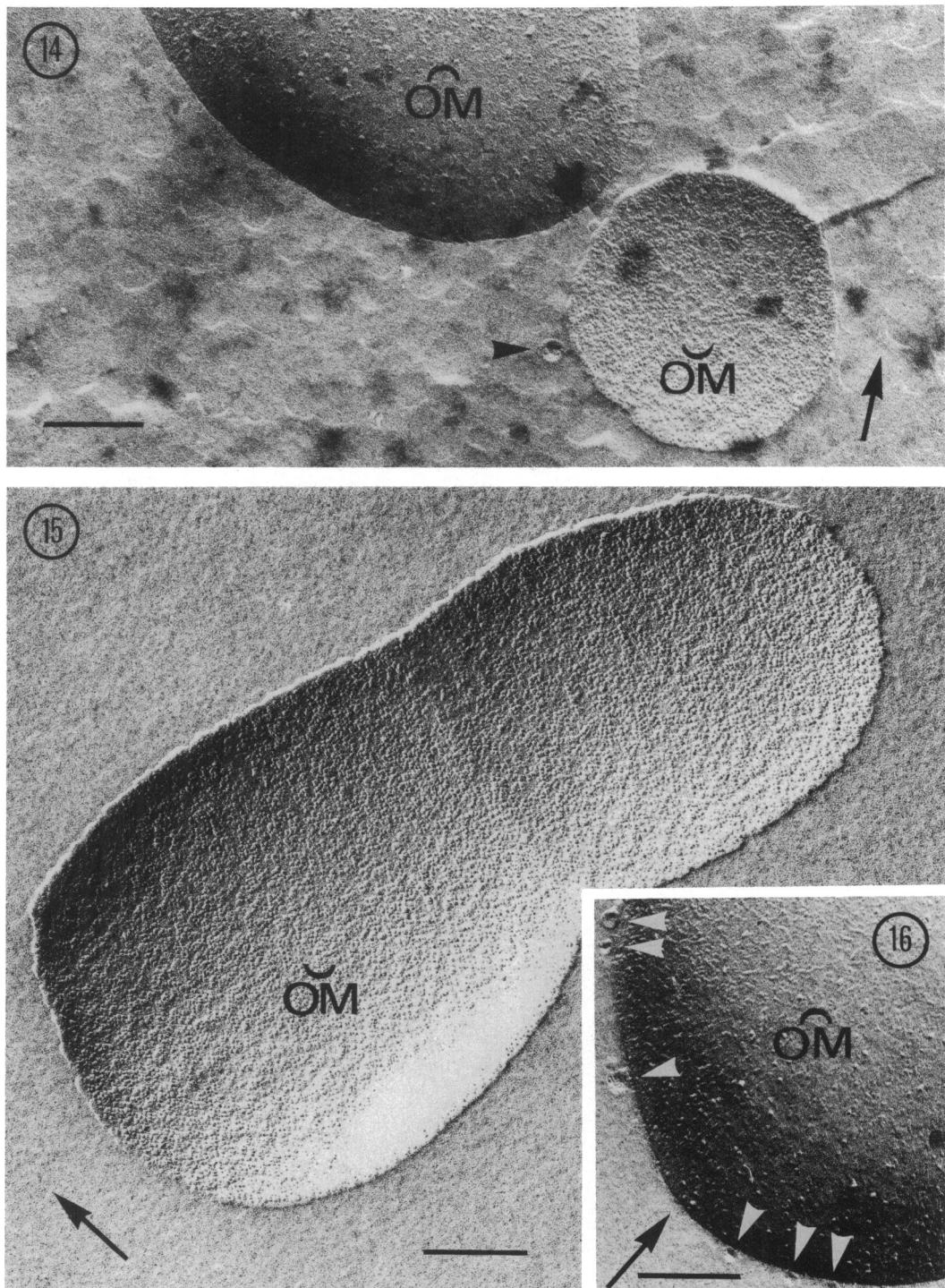


FIG. 14-16. Fracture faces of the OM of the double mutants SH6263 and SH6264 (resistant to all phages) selected from the PH51-resistant mutant SH5551. Bar, 0.2 μ m.

FIG. 14. Double mutant SH6263. In the concave face ($\widehat{\text{OM}}$), there are numerous particleless patches; the particles look smaller than those of the parent strain. Note two particles in the wall of a fractured vesicle (arrowhead).

FIG. 15. Outer concave fracture face ($\widehat{\text{OM}}$) of SH6264 with smooth particleless patches and small particles.

FIG. 16. Corresponding outer convex fracture face ($\widehat{\text{OM}}$) of SH6264 surrounded by numerous vesicles (arrowheads).

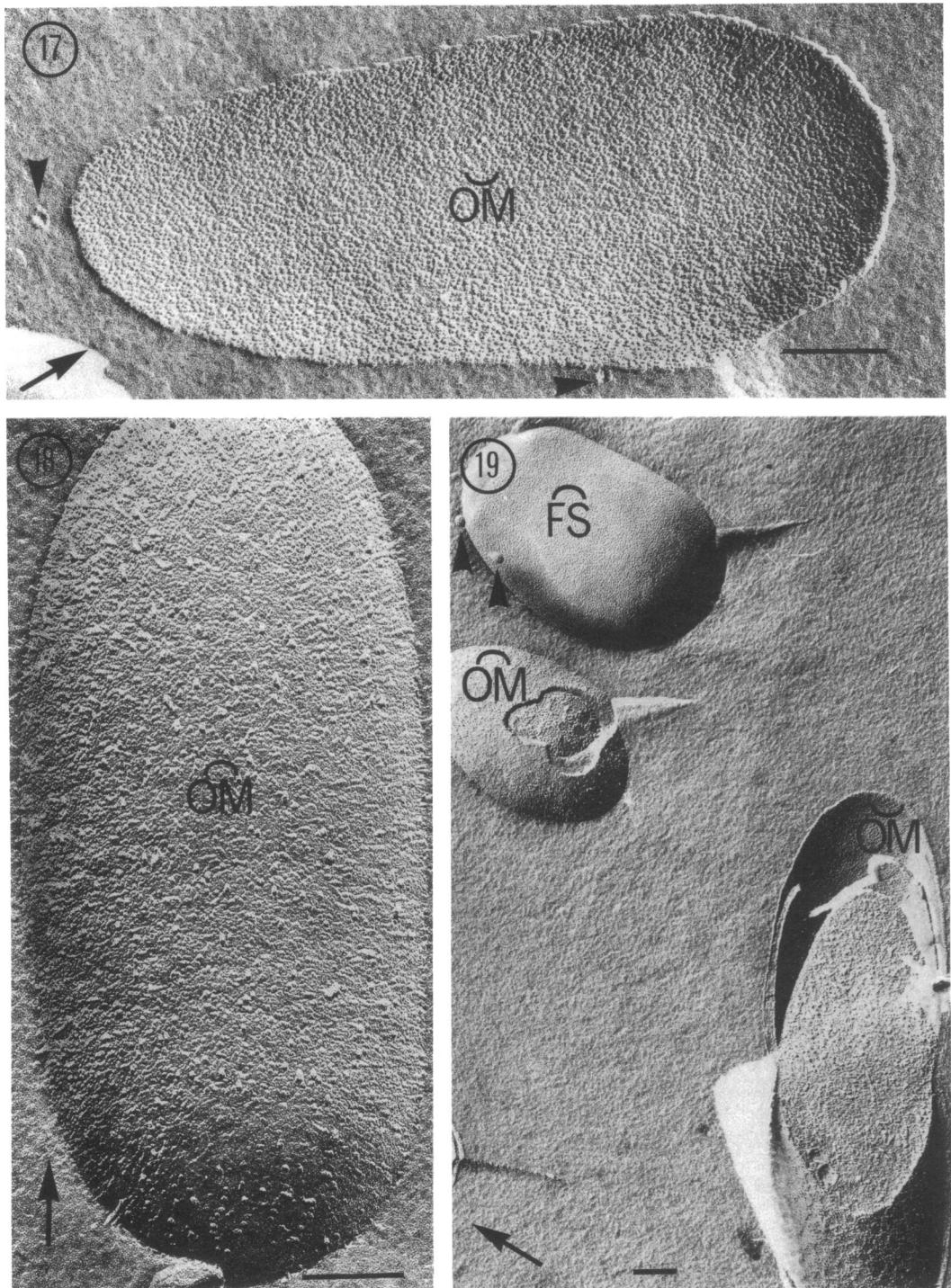


FIG. 17-19. Fracture faces of the OM of the PH105/P221-resistant mutant SH6017.

FIG. 17. This concave face (\widehat{OM}) looks identical to the corresponding fracture face of the parent strain SH5014.

FIG. 18. Convex face (\widehat{OM}) is covered by numerous particles.

FIG. 19. Fractured surface (\widehat{FS}) with two vesicles (arrowheads) is tightly covered by particles. Compare with other fracture faces.

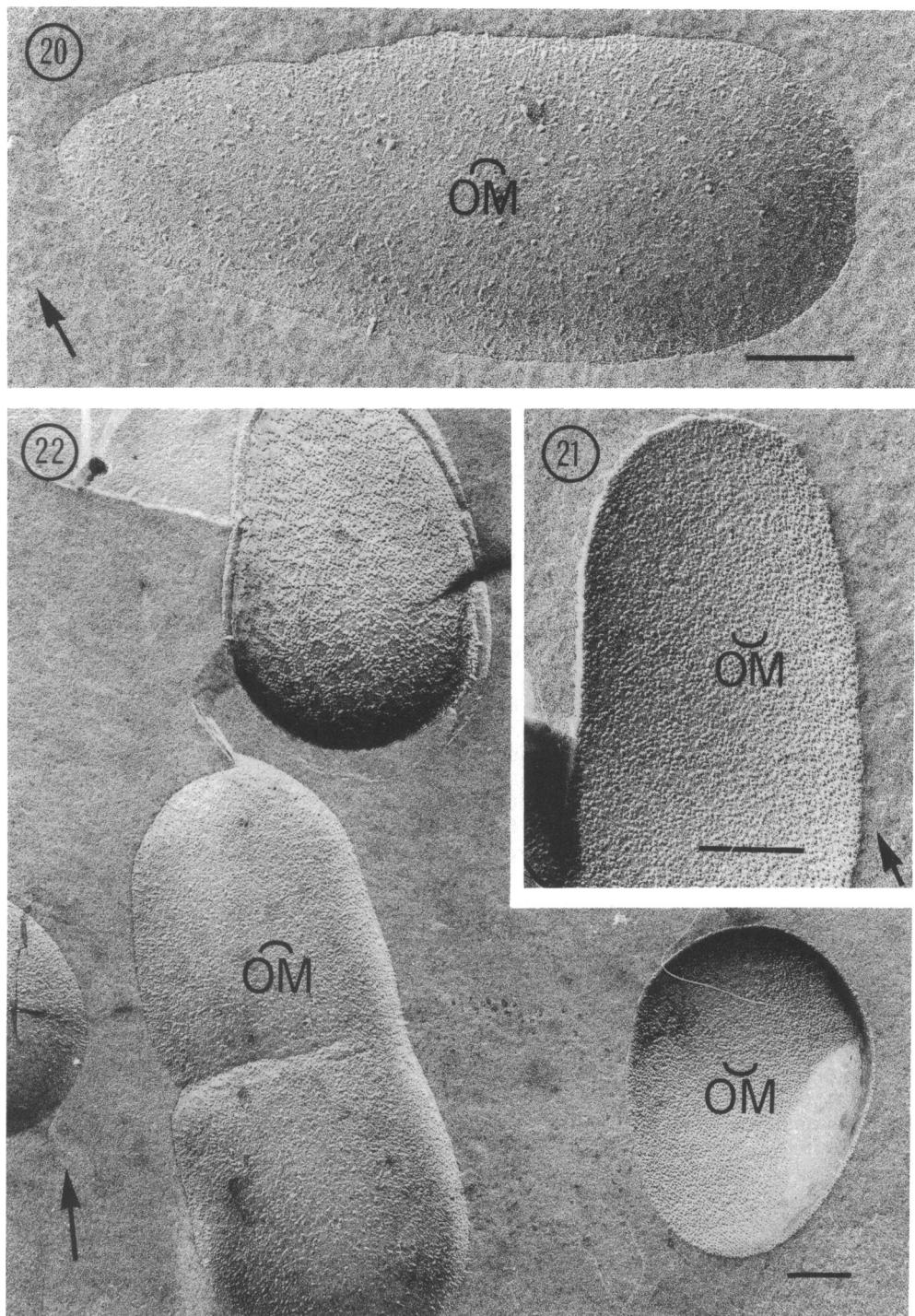


FIG. 20-22. Fracture faces of the OM of the double mutants SH6260 and SH6261 (resistant to all phages) selected from the PH105/P221-resistant mutant SH6017.

FIG. 20. Convex face (OM) of SH6260 is covered by numerous particles.

FIG. 21. Concave face (OM) of SH6260 with smooth particleless patches and small 5- to 7-nm particles.

FIG. 22. Concave (OM) and convex (OM) faces of SH6261 look identical to those of SH6260.

band, 33K, was unchanged in all of the mutants and no new bands appeared.

We could solubilize the phage receptors by Triton X-100-EDTA extraction (17). Phage-inactivating capacity was absent from the TX extract containing CM components and present in fractions containing OM components. SDS-gel electrophoresis of the TX-EDTA extract showed again one or the other of two major band polypeptides missing in mutants resistant to either PH51 or PH105/P221.

Because changes in the OM protein pattern of *E. coli* caused by lysogenization with a bacteriophage, whose receptor is one of the proteins, have been described (15), we also looked for a possible effect of lysogenization, with negative results. Lysogenicity for PH51 or P221 did not affect the outer membrane protein pattern, and the extracts of the lysogenic bacteria inactivated all the three major band phages as well as did extracts of the parent strain.

The ultrastructure of the OM was affected by the mutations. The changes were small in both single-step mutants SH5551 and SH6017. We think that they are real because they were seen in the majority of individual bacteria examined and also in several preparations. The alterations were more marked in the double mutants (lacking both the PH51 and the PH105/P221 receptor).

Two major kinds of changes were seen in all of the double mutants: numerous vesicles outside the OM, particleless patches in the OM. Many fractured vesicles show a concave fracture face (arrowheads, Fig. 16), which could correspond to the inside face of the outer leaflet of the OM. In one vesicle some particles are seen that look similar to those of OM (Fig. 14).

The outer concave fracture face (OM) of all the double mutants shows patches devoid of the 8- to 10-nm particles, which in the parent strain are tightly packed on this surface. The remaining particles of this fracture face (OM) of the double mutant also appear to be smaller than the corresponding particles of normal cells. It is tempting to correlate this reduction in number and size of particles with the absence of two OM proteins in these mutants. The outer concave fracture face (OM) with particleless areas resembles the corresponding face of "deep rough" (Re) mutants, which also have reduced amounts of OM proteins (24, 26).

A cell exceptionally fractured at the cell surface allows us to view the outer leaflet of the OM from both sides (OM and FS). Interestingly, both faces are structurally identical. The probably proteinaceous particles typical of the outer concave fracture face (OM) also cover the

FS, suggesting that they extend across the distance of the outer leaflet.

We would also like to point out that the frequent fracturing of the cells through the OM differs from that found by other workers (3, 26) and is caused by the high concentration of glycerol (30%, vol/vol) used in their preparation (Lounatmaa and Nanninga, in preparation). With this method, the cell wall of smooth *S. typhimurium* fractures through the OM much more easily than when lower concentrations of glycerol are used. Bayer et al. (3) and Smit et al. (26) found that deep rough Re mutants are fractured through the OM more easily than smooth or less-rough strains and conclude that this is due to a profound alteration of the structure of the OM in these mutants. The double mutants studied by us behave in a similar manner: fracturing through the OM happened so easily that fracture faces deriving from the plasma membrane were seen only rarely.

Although a large amount of protein is missing from the OM of these phage-resistant mutants, both the double and the single mutants grow normally, do not have a tendency to revert, and do not have increased sensitivity to detergents (e.g., deoxycholate), dyes, or antibiotics (P. H. Mäkelä and M. Nurminen, manuscript in preparation). This is rather surprising, compared with the deep rough mutants mentioned above, which are characteristically very sensitive to these chemicals (18, 20).

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