JOURNAL OF BACTERIOLOGY, Mar. 1973, p. 1491-1504
Vol. 113, No. 3

A Polypeptide Bacteriophage Receptor: Modified Cell Wall Protein Subunits in Bacteriophage-Resistant Mutants of *Bacillus sphaericus* Strain P-1

LAWRENCE HOWARD AND DONALD J. TIPPER
Department of Microbiology, University of Massachusetts Medical School, Worcester, Massachusetts 01604

Received for publication 2 October 1972

Bacillus sphaericus strain P-1 has previously been shown to have a tetragonally arrayed (T layer) protein which forms the outer layer of the cell wall. The T layer was quantitatively extracted from whole cells by 6 M urea, and the T layer subunits were purified by electrophoresis of the extracts on acrylamide gels containing 0.1% sodium dodecyl sulfate or 6 M urea. Using ethylene diacrylate cross-linked gels, the T layer was found to make up 16% of the total cellular protein. A virulent bacteriophage which is inactivated by purified T layer was isolated from soil. Twenty-four phage-resistant mutants were isolated, of which 17 had T layer subunits of increased mobility on sodium dodecyl sulfate acrylamide gels. No mutants devoid of T layer were found. Mutants were grouped into six classes according to the molecular weight of their T layer subunits. These ranged from that of the wild type, 150,000 down to 86,000. Two mutants from different classes were examined in detail. Cells of the mutant strains did not adsorb phage nor did cell walls isolated from these mutants inactivate phage. The amino acid composition of the T layers from mutants differed little from that of the wild-type T layer.

The presence of a regularly arrayed surface layer with tetragonal symmetry (T layer) on cell walls of a gram-positive bacillus was first reported in *Bacillus polymyxa* by Baddiley (4). Subsequent investigation revealed that this structure was sensitive to proteolytic enzymes and had an amino acid composition indicative of protein rather than peptidoglycan (12). In a more detailed study, Nermut and Murray (20) confirmed that the structural array was proteinaceous in nature by demonstrating that its integrity was lost upon treatment with hydrogen bond disrupting agents, proteases, and sodium dodecyl sulfate (SDS). This protein structure was concluded to be the outermost component of the cell wall and was hypothesized to be linked by noncovalent bonds to an underlying polysaccharide layer. In the genus *Bacillus*, regularly arrayed globular layers have been seen in several species, in some of which (*B. cereus*, *B. anthracis*, and *B. macroides* strain P) it closely resembled that of *B. polymyxa* (8, 14). Ordered structures on bacterial surfaces have been described in a number of gram-positive and gram-negative organisms (9); however, in most cases the chemical nature of the observed structures has not been completely defined.

Our long-term objective is to study the physiological role of T layer and its biosynthesis, commencing with subunit synthesis and proceeding with excretion and assembly on the cell surface. As part of this program, we have investigated the potential role of T layer as a phage receptor. It was hypothesized that its location was well suited to such a role and that selection for phage resistance might yield mutants defective in receptor because of alteration in or complete lack of T layer. Such mutants would probably be defective in late or early steps in T layer synthesis, respectively; and comparison of these mutants with the parent would yield information on the function of T layer as well as on its synthesis.

The existence of specific cell wall receptors required for phage attachment has been well documented for a variety of microorganisms (22). Modification of the teichoic acid polymer has been accompanied by phage resistance in *B. subtilis* (28), *Lactobacillus plantarum* (7), and *Staphylococcus aureus* (24). Loss of F pili in *Escherichia coli* (5) and FP pili in *Pseudomonas aeruginosa* (27), resulting in the failure of specific phages to adsorb, has been shown to be a mechanism of phage resistance in these organisms. Likewise, numerous workers have demonstrated that phage-resistant mutants in a variety of gram-negative organisms have specific alterations in their cell wall lipopolysaccharide component (17, 22).

Bacillus sphaericus strain P-1 was chosen for these studies because its T layer has been well characterized by Brinton and co-workers (C. Brinton, J. E. McNary, and J. Carnahan, Bacteriol. Proc., p. 48, 1969). It consists of a single polypeptide with a molecular weight of about 140,000 which can be solubilized from cell walls at low pH and which reaggregates on neutralization to give sheets with the configuration of native T layer (C. Brinton, personal communication). A very similar T layer makes up more than 50% of the dry mass of cell walls of *B. sphaericus* strain 9602 (16; D. J. Tipper, unpublished observations).

B. sphaericus strain P-1, tentatively identified by Brinton as a *B. brevis* species, has been identified as a *B. sphaericus* species. Its T layer was found to constitute 16% of the total cell protein, and it is almost entirely localized in the cell walls. T layer has been identified as a binding site for a virulent phage, M, isolated from soil. Twenty-four phage-resistant mutants have been isolated, and their T layers have been examined for evidence of alteration.

This paper was presented in part at the 72nd annual meeting of the American Society for Microbiology, Philadelphia, Pa., April 1972.

MATERIALS AND METHODS

Bacterial strains and phages. *Bacillus* strain P-1 and its phage, designated CP-1, were obtained from C. Brinton. Phage M was isolated from soil by standard techniques (13) by using *Bacillus* strain P-1 as host. *Bacillus* strain P-1 has oval to round subterminal spores within a locally swollen sporangium. *Bacillus* strain P-1 was unable to dissolve crystals of tryosine or to produce acid from glucose as sole carbon source. It was able to grow on nutrient broth containing 5% NaCl. All of these properties differentiate a *B. sphaericus* species from a *B. brevis* species, and Ruth Gordon has confirmed these observations and identified strain P-1 as a *B. sphaericus* species.

Cell wall peptidoglycan of *B. sphaericus* strain P-1 contained lysine, alanine, glutamic acid, and aspartic acid, but no diaminopimelic acid. On hydrolysis, it gave rise to N$^{\epsilon}$ (aminosuccinoyl) lysine, characteristic of an N$^{\epsilon}$-aspartyl-lysine linkage. These are also characteristics of the peptidoglycan of *B. sphaericus* 9602 (16).

Medium and growth conditions. Cells were grown in a medium consisting of 0.5% yeast extract (Difco), 0.1% sodium glutamate, 0.1% sodium acetate, and 0.8% sodium chloride (YE medium). Cells were grown at 37 C with continuous shaking at 200 rpm in a New Brunswick shaker.

Phages M and CP-1 were propagated by adding a sample of a phage suspension at a multiplicity of one to a growing culture of *B. sphaericus* strain P-1 at approximately 10^8 colony-forming units per ml. After 2 h of incubation at 37 C, the lysate was filtered through a 0.45-μm membrane filter (Millipore Corp.) and stored at 4 C.

Isolation of mutants resistant to phage M. For isolation of mutants resistant to phage M, 0.2 to 0.3 ml of a log-phase culture of *B. sphaericus* and 0.1 ml of a sterile, fresh phage M lysate were added to 2.5 ml of molten YE medium containing 0.75% agar. The mixture was overlaid on a YE plate containing 2% agar and, after incubation for 48 h, colonies were picked and streaked on another plate. A single colony was picked from the second plate, and this subculture was tested for sensitivity to phages M and CP-1. All of the phage M-resistant mutants described in this paper remained sensitive to phage CP-1 and retained colonial morphology, microscopic appearance, and position and shape of spores characteristic of the parent strain.

Mutants resistant to phage M were designated MBR (M bacteriophage resistant). Mutants MBR-1 through MBR-12 were spontaneous mutants, whereas mutants with higher numbers were isolated after mutagenesis of *B. sphaericus* with *N*-methyl-*N'*-nitro-nitrosoguanidine (2). For mutagenesis, cells from the surface of an agar plate were suspended in 2 ml of 0.05 M sodium cintrate, pH 6.0, containing 100 μg of nitrosoguanidine per ml and incubated for 20 min at 37 C. After one washing in YE medium, cells were incubated for 4 h in YE medium before being exposed to phage as for isolation of spontaneous mutants. Subcultures were tested for sensitivity to phage M by spotting a sterile lysate of phage M on a lawn of confluent bacterial growth and for sensitivity to phage CP-1 by the soft agar assay method (1).

Preparation of purified T layer from cell walls. Cell walls of wild-type and phage-resistant mutants were isolated after sonication of whole cells as previously described (16). Tetragonal-layer protein was purified through the acid dissociation and subsequent reassociation steps (C. Brinton, personal communication). Radioactively labeled T layer was purified from cells grown in YE medium containing 0.027 μCi of ^{14}C-valine per ml. The ^{14}C-valine had a specific activity of 260 mCi/mmol and was added without unlabeled carrier valine. More than 50% of the added radioactivity was incorporated into cells.

Neutralization of phage M by T layer, cell walls, and whole cells. Phage neutralization by purified T layer and unfractionated cell walls was carried out in YE broth at 37 C. Phage M was

incubated with at least two dilutions of cell walls or purified T layer, and the uncentrifuged samples were assayed for infective phage (1). Adsorption of phage M to whole cells was measured by using log-phase cells which had been washed once in cold medium and suspended to approximately 5×10^7 colony-forming units per ml in fresh medium containing 0.001 M KCN. Phage was added to give a multiplicity of infection of about 0.1. The mixture was incubated at 37 C with shaking. Periodically, samples were removed and centrifuged for 2 min at 6,000 $\times$ *g*, and the supernatant fluid was assayed for infectious phage.

Extraction of T layer from whole cells. T layer protein was extracted from cells which had previously been harvested by centrifugation and washed once in cold 0.15 M NaCl. Routinely, 5×10^9 cells were suspended in 1.0 ml of 8 M urea buffered with 0.01 M Na_2HPO_4, pH 7.0. The cell suspensions were incubated at 37 C with gentle shaking for 30 min and centrifuged at 17,000 $\times$ *g* for 10 min. The cells in the pellet were suspended at 37 C in 1.0 ml of 6 M urea and centrifuged, and supernatant fluid was pooled with the original 8 M urea supernatant fraction. SDS was added to give a final concentration of 0.1%, and a 1.0-ml sample was dialyzed against SDS buffer at 25 C overnight in preparation for gel electrophoresis. The volume of the dialyzed sample was recorded.

Acrylamide gel electrophoresis. For quantitative determination of T layer protein, 10% acrylamide gels, cross-linked with ethylene diacrylate, were prepared and run according to the procedure of Alpers and Glickman (3). Urea at 6 M was included in all gels. Urea extracts of whole cells were applied directly above the gel surface, and gel buffer was carefully layered on top of the sample. After electrophoresis, gels were placed in cold 12.5% trichloroacetic acid and stored overnight at 4 C before being sliced into 3-mm sections. When varying concentrations of purified T layer were subjected to electrophoresis, recovery of protein in the T layer band of gels was 80% $\pm$ 5%.

SDS polyacrylamide gels were prepared, run, and stained with Coomassie blue according to the procedure of Osborn and Weber (26). One-half the normal amount of cross-linker was used, and the molarity of the phosphate in the gel buffer was reduced by one-half to decrease the running time.

For quantitative assay of ^{14}C-labeled T layer protein in SDS gels, samples were run in triplicate. One gel was stained with Coomassie blue, and the other two were placed in 12.5% trichloroacetic acid overnight. The T layer band, located by comparison of stained and unstained gels, was sliced from the gel, and the gel slices were solubilized by 0.1 ml of 30% H_2O_2 in scintillation vials at 45 to 50 C overnight. The samples were counted after the addition of 10 ml of Aquasol. Under these conditions the efficiency of counting of internal standards of ^{14}C-toluene was 82%.

Purification of T layer protein from MBR mutants. Cells of phage-resistant mutants from 400 ml of late-log phase culture were harvested and washed once with 0.15 M NaCl. Each batch of cells was extracted once only with 20 ml of 8 M urea as described above. SDS was added to a final concentration of 0.1% to the urea-soluble fraction, and the samples were heated at 100 C for 5 min to ensure inactivation of proteolytic enzymes. The samples were dialyzed against SDS dialysis buffer (0.01 M Na_2HPO_4, pH 7.0, containing 0.1% SDS and 0.1% 2-mercaptoethanol) overnight, reduced to 0.1 their original volume in vacuo, and applied to a Sephadex G-100 column (2.5 $\times$ 65 cm) which had been equilibrated with SDS dialysis buffer. Fractions of 2 ml were collected, and the optical density of each was measured at 280 nm. The fractions forming the main peak eluted at or near the void volume. They were pooled and dialyzed extensively against distilled H_2O. Gel electrophoresis established the near purity of these protein fractions. They had a mobility on SDS gels identical to the putative T layer band in the original urea extracts.

Amino acid analysis of T layer protein and peptidoglycan. Protein samples were hydrolyzed in 6 N HCl at 110 C for 18 h, and the hydrolysates were analyzed on a Beckman 120C amino acid analyzer.

To determine the amino acid composition of the peptidoglycan, cell walls were heated at 100 C for 15 min in 4% SDS to remove T layer protein. The insoluble material was washed five times with distilled water and hydrolyzed in 6 N HCl at 105 C for 12 h. The products of hydrolysis were examined by high-voltage paper electrophoresis by using a pH 1.9 buffer (15).

Protein determinations. Protein was determined by the method of Lowry et al. (19) by using bovine serum albumin as a standard. Values for protein in the presence of urea were determined by using a blank with urea at a comparable concentration. Protein determinations on whole cells were done after incubation of the cells in 1.0 N NaOH at 37 C for 1 h.

Turbidity measurements. Culture growth was determined by measuring turbidity increase on a Klett-Summerson colorimeter with a red filter.

Chemicals and protein standards. Uniformly labeled ^{14}C-valine at 260 mCi/mmol was purchased from Schwartz-Mann, New York, N.Y. Additional Chemicals used were as follows: *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, Mo.), and *p*-toluenesulfonyl fluoride (Aldrich Chemical Co., Milwaukee, Wis.). Proteins for molecular weight standards on SDS acrylamide gels were β-galactosidase (Sigma), catalase (Sigma), and bovine serum albumin (Calbiochem, Los Angeles, Calif.). Phosphorylase a was a gift from G. Fairbanks.

Electron microscopy. Phage M and cell walls were treated with 2% sodium phosphotungstate (pH 6.8) and examined in a Hitachi HU-11E electron microscope.

RESULTS

Definition and morphology of T layers. Negatively stained preparations of partially fragmented cell walls of *B. sphaericus* strain P-1 have a tetragonally arrayed structure which is definitive of T layer (Fig. 1). This structure can also be seen on the exterior of

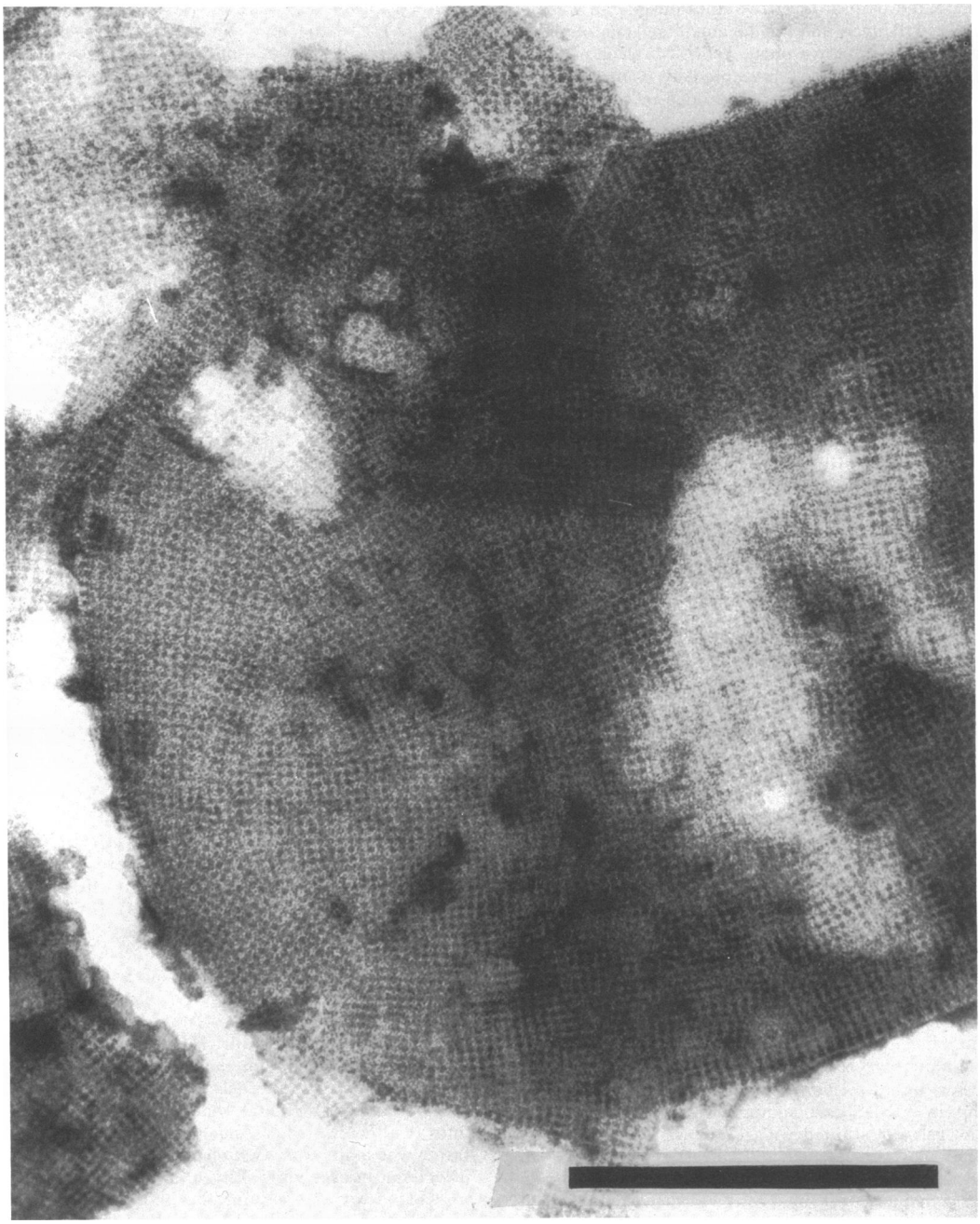

FIG. 1. *Electron micrograph of negatively stained cell walls isolated from Bacillus sphaericus strain P-1. Bar equals 500 nm.*

negatively stained whole cells of this strain, and T layer, purified from its cell walls by dissociation and reassembly, has the same morphology. The purified T layer migrates as a single band on SDS gel electrophoresis (C. Brinton, personal communication). Thin sections of cells and walls of *B. sphaericus* show an outer cell wall layer that stains heavily with osmium, which is missing from cells or walls extracted with 8 M urea (S.C. Holt, unpub-

lished observations), and which is probably T layer.

Extraction of T layer from whole cells by urea. Log-phase cells were harvested, washed once in saline, and extracted with urea as described in Materials and Methods. The extracted cells were suspended in distilled water and sonicated to give greater than 90% breakage. The sonicated cells were centrifuged at 12,000 × *g* for 10 min to separate the soluble (cytoplasmic) fraction from the insoluble (cell wall) fraction. The cytoplasm was dialyzed against a solution of 8 M urea, 1% SDS, and 1% 2-mercaptoethanol overnight, and the cell wall fraction was suspended in an identical solution and incubated at 37 C for 2 h. The culture medium, after removal of the cells, was concentrated to dryness in vacuo, and the residue was treated in the same manner as the cell wall fraction. The SDS gel patterns of the proteins in the urea extract, cytoplasmic and cell wall fractions, and culture medium are presented in Fig. 2. T layer, purified by dissociation and reassembly, gave a single band, as previously reported by Brinton (Fig. 5). The predominant and slowest migrating protein seen in the urea wash of whole cells has the same mobility and is presumably T layer. In mutants in which the T layer band has a lower molecular weight (see below), no band remained in the region of the gel occupied by wild-type T layer which, therefore, is the only component of the urea wash of whole cells with this mobility. The cytoplasmic fraction contained three bands of about equal staining intensity in the T layer region of the gel, whereas the cell walls from urea-extracted cells gave only a few faint bands in this region. The culture medium contained one predominant protein, which coincided in mobility with the T layer subunits.

Quantitative data on the distribution of T layer in the four fractions was obtained by growing cells from a 10% inoculum to late log phase in YE broth containing 0.067 μCi of ^{14}C-valine per ml. Fractions were derived and run on SDS gels as described in the previous experiment. Radioactivity in the T layer region of each gel was determined. Distribution among cellular fractions and culture medium of ^{14}C label migrating in the T layer position on SDS acrylamide gels was, in counts per minute per milliliter of culture, as follows: urea wash of whole cells, 5,600; cell wall fraction, 60; cytoplasmic fraction, 650; culture medium, 425. The band in the cytoplasmic gel with the mobility of T layer was associated with two other bands of the same staining intensity. These bands were not cleanly separated by slicing and together comprised 9% of the total counts in the T layer region of the gels. T layer in this fraction (if any) is thus roughly one-third of 9%, or 3%. On this basis the cell wall fraction from the extracted cells contained 1%, the medium 7%, and the urea extract 89% of the total T layer in the culture. Thus, 93% of the T layer in the culture was associated with cells, and 96% of this was extracted by urea.

Estimation of total T layer protein. Growing cells were examined to determine what percentage of their total protein was T layer. Culture samples were removed at various times, and the cells were extracted with urea. The resultant fractions were subjected to electrophoresis on ethylene diacrylate cross-linked acrylamide gels. The protein content of the urea extracts, urea-insoluble fractions, and the T layer band of the gels was determined (Fig. 3). Total cellular protein was the sum of values obtained for urea-extractable and nonextractable material. T layer, corrected for 80% recovery from the gels, constituted 16% of the total cellular protein at all times. T layer comprised about 30% of the total protein extracted by urea from whole cells.

Morphological characteristics of phage M. Phage M (Fig. 4) had nearly spherical, apparently polyhedral, heads averaging 52 nm in diameter, with a tail of about 115 nm in length. The tail had a sheath of helical subunits that started about 10 nm from the head, was about 11 nm in diameter, and had two base plates, separated by about 12 nm, which were sometimes visible at the distal end. Isolated core and contracted sheath segments, 7 and 15 nm in diameter, respectively, were also visible in many preparations. Frequently phage particles with empty heads and contracted tails were seen in phage preparations. Phage M gave clear plaques, 1 to 2 mm in diameter at 24 h, on *B. sphaerius* strain P-1.

Inactivation pf phage M by purified T layer. Serial dilutions (10-fold) of purified T layer were incubated with 10^6 plaque-forming units per ml of phage M in 1.0 ml of YE medium. After 1 h at 37 C, residual infectious phage was determined. At 2.2 μg of T layer protein per ml there was a 50% reduction in phage titer (Table 1).

SDS acrylamide gel patterns of T layer protein from wild-type, MBR-9 and MBR-10 cells. Inactivation of phage M by purified T layer suggests that phage M uses T layer, which appears to cover both the cylindrical and end surface of *B. sphaerius* P-1 cells (Fig. 1) as a receptor. One class of phage M-resistant mutants might, therefore, result from

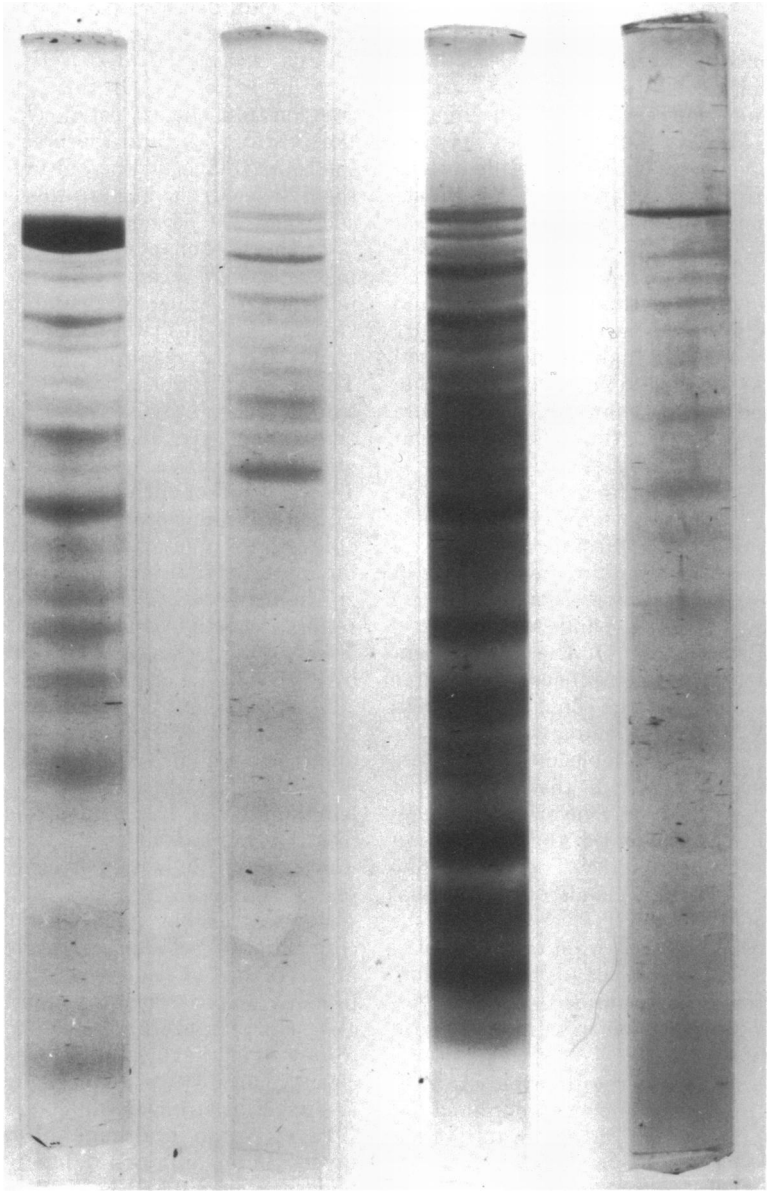

FIG. 2. *SDS acrylamide gel electrophoresis of proteins in cellular fractions and medium from a log-phase culture. Gels from left to right are urea extract of whole cells, cell wall fraction of urea-extracted cells, cytoplasm from urea-extracted cells, and culture medium; samples were derived from the following relative volumes of culture: 1.0, 2.5, 1.5, and 2.5, respectively.*

loss or modification of the T layer protein. The urea extracts of several phage-resistant mutants were examined on SDS acrylamide gels. Figure 5 presents the gel pattern of purified wild-type T layer, the urea extract of wild-type cells, and the urea extract of MBR-10 and MBR-9 cells. In urea washes of wild-type cells, T layer formed the slowest-moving and predominant band. The urea extract of MBR-10 cells had no band with the mobility of wild-type T layer, but contained a new, predominant band of greater mobility. That this band is not identical to wild-type T layer subunits is clearly demonstrable when urea extracts, or purified T layer from wild-type cells, are run as a mixture with the mutant urea extract (Fig. 5). The remaining major bands in wild-type and MBR-10 urea extracts, visible in the heav-

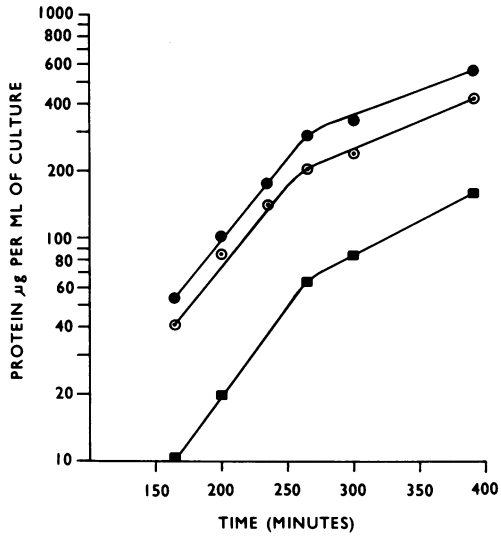

FIG. 3. *T layer protein and urea-extractable and -nonextractable protein present during growth of a bacterial culture.* ■, *T layer protein;* ⊙, *urea-extractable protein; and* ●, *urea-nonextractable protein. The values for T layer protein have not been corrected for the 80% recovery of protein from gels.*

ily loaded gels shown in Fig. 5, are identical, and serve as mobility references. The new band of MBR-10 cells actually consisted of two bands, as can be more clearly demonstrated when lower concentrations of protein are run (see below). A second mutant, MBR-9, was also found to lack a major band in the T layer band area (Fig. 5). Again, a new, predominant band of greater mobility was visible.

Urea extracts of whole cells and of freshly prepared cell walls from unextracted cells were compared on SDS gels. The cell walls were treated with 8 M urea overnight before being prepared for electrophoresis. The patterns of T layer proteins obtained from the wild-type, MBR-9, and MBR-10 strains can be seen in Fig. 6. The position of the major band derived from the wild type was the same when extracts of whole cells were compared with extracts of isolated cell walls. This was also true for extracts of MBR-10 and MBR-9 strains. Smaller quantities of total protein were used on these gels than were used on the gels in Fig. 5, and it can now be clearly seen how the T layer band predominates in the urea extracts and that the extracts of MBR-10 contain two new major bands.

In collaboration with Stanley Holt and Jack Pate, we have examined whole cells and cell walls of MBR mutants by electron microscopy. A tetragonal array resembling that seen on wild-type cells was seen on purified cell walls of mutants MBR-9 and MBR-10 in preparations negatively stained with phosphotungstate. Thin sections of glutaraldehyde-fixed cells showed a layer on the outer surface of their cell walls closely resembling that seen on wild-type cells, and this layer is therefore T layer by morphological definition. Extraction of cells or cell walls of these mutants with urea removes this layer, and the protein that comprises most of the protein extracted from these cell walls is, therefore, assumed to be the T layer protein of these mutants. Details of these studies will be reported later. Storage of unextracted cell walls of these mutants, even at 4 C, resulted in a decrease in band intensity on SDS gels, and in some instances the appearance of protein bands of increased mobility was noticed. Concomitantly, T layer morphology disappeared in negatively stained wall preparations of mutants MBR-9 and MBR-10.

Electrophoresis on SDS gels of cytoplasmic fractions of MBR-9 and MBR-10 yielded 25 to 30 protein bands which were identical in migration and relative staining intensity to bands derived from the wild-type cytoplasmic fraction. Acid hydrolysates of the peptidoglycan of the wild-type, MBR-9, and MBR-10 strains were subjected to high-voltage electrophoresis, and all were found to contain alanine, glutamic acid, lysine, aspartic acid, and N^{ϵ} (aminosuccinoyl)-lysine. No diaminopimelic acid was found in the walls of either mutant or wild-type strains.

Inactivation of phage M by cell walls and whole cells of mutants MBR-9 and MBR-10. Cell walls of strain MBR-9 and MBR-10 were tested for their ability to neutralize phage M. Phage at 3.0×10^5 plaque-forming units per ml were incubated with walls of wild-type, MBR-9, and MBR-10 at 5 and 50 μg of protein per ml in YE broth. Residual phage was determined after 45 and 120 min. Cell walls from MBR-9 and MBR-10 cells failed to neutralize phage. Wild-type walls were an effective inhibitor of plaque-forming ability (Table 2).

Intact cells of wild-type, MBR-9, and MBR-10 were tested for the ability to adsorb phage. The titer of unadsorbed phage present in the supernatant fluid after centrifugation was determined at various time intervals after the addition of phage to cells (Fig. 7). In contrast to wild-type cells, cells of neither mutant adsorbed phage M.

Experiments to detect protease activity in mutants MBR-9 and MBR-10. The possibility that mutant cells contained protease activity which converted wild-type, T layer subunits to proteins of decreased molecular weight was

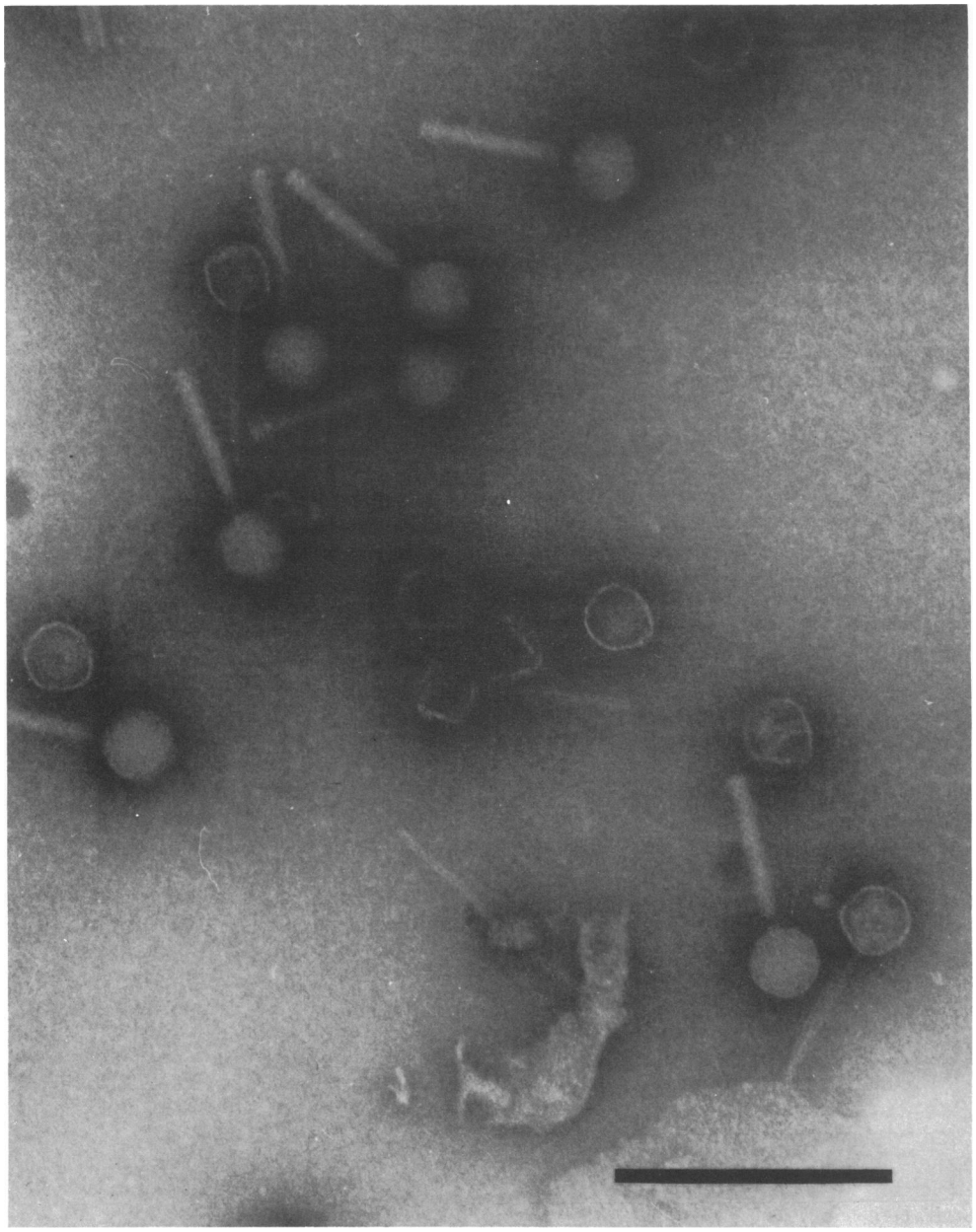

FIG. 4. *Electron micrograph of a negatively stained preparation of phage M. Bar equals 200 nm.*

investigated. Log-phase cells of wild-type, MBR-9, and MBR-10 were washed once in saline, suspended at one-third the volume of the original culture in 0.01 M Na_2HPO_4 (pH 7.0) containing 0.15 M NaCl, and sonically treated at 4 C to more than 90% breakage. Unfractionated sonic extracts with 0.6 mg of protein were incubated in a total volume of 1.0 ml of buffer used for sonication with 1.0 mg of wild-type, T layer labeled with 2.1×10^4 counts/min of ^{14}C-valine. After 2 h at 37 C, the incubation mixture was dialyzed against a solution of 8 M urea, 1% SDS, and 1% 2-mercaptoethanol to disaggregate T layer. Samples of each mixture were subjected to acrylamide gel electrophoresis, and radioactivity was determined in 3- to 5-mm gel slices. In each case, more than 90% of the radioactivity originally present in the incubation remained with the band of wild-type, T layer mobility (Table 3),

so no endopeptidase or extensive exopeptidase activity was detected.

Inhibitors of proteases, *p*-toluensulfonyl fluoride and phenylmethene-sulfonyl fluoride (10, 11), were tested for their ability to prevent the appearance of mutant-type T layer on cells of the MBR-9 and MBR-10 strains. Cells of these two mutants were grown in the presence of 1 mM concentrations of these inhibitors for 1.5 and 2.5 h, but SDS gel patterns indicated that this resulted in no alteration in the molecular weight of the mutant T layers.

Classification of phage M-resistant mutants by T layer subunit size. A total of 24 phage M-resistant mutants has been examined for altered T layer subunits, and 17 of these contained no protein migrating on SDS gels in the position of wild-type, T layer subunits. The use of urea washes of whole cells, instead of isolated cell walls, in screening of mutants for altered T layer subunits, was chosen for convenience and to reduce the possibility of proteolytic modification of T layer proteins. The T layer proteins obtained by urea extraction of phage M-resistant mutants were run on SDS acrylamide gels, and their molecular weights were determined with reference to a standard

TABLE 1. *Inactivation of phage M by purified T layer protein*

T layer protein concn (μg/ml)	Phage titer (10^{-4} × PFU/ml)[a]
0	90
0.022	93
0.22	77
2.2	45
22	6
280	0.015

[a] PFU, Plaque-forming units.

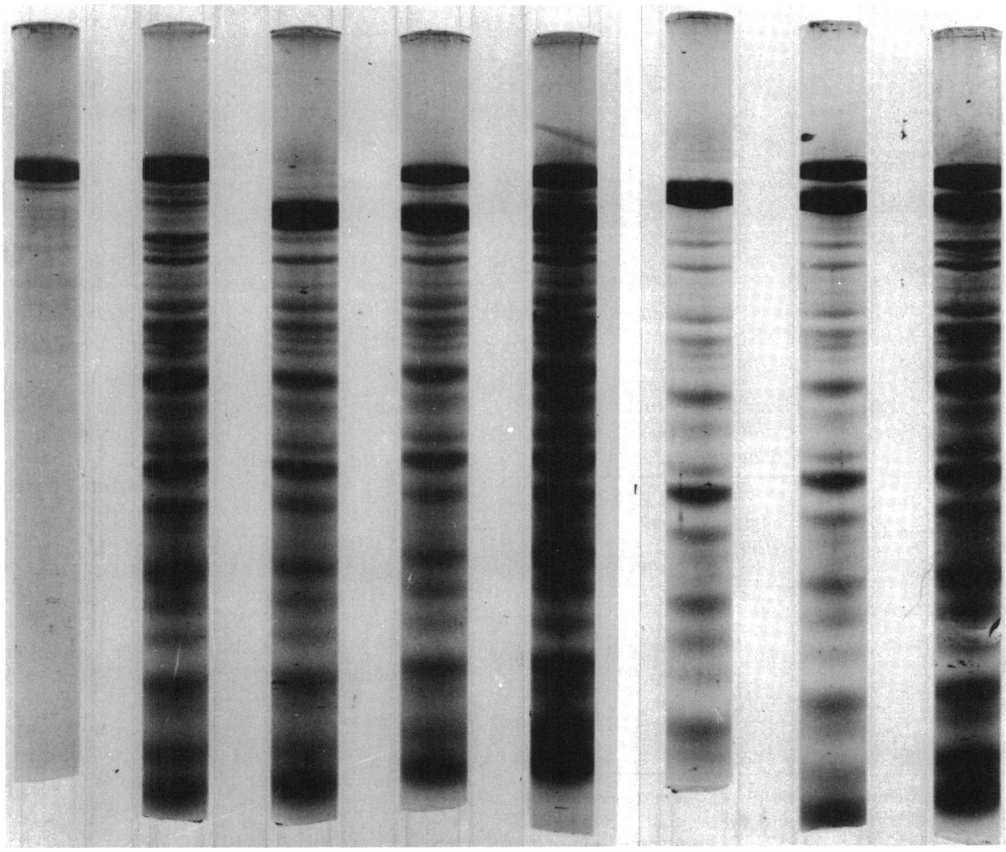

FIG. 5. *SDS acrylamide gel electrophoresis of purified T layer and of urea extracts of wild-type, MBR-10, and MBR-9 cells. Gels from left to right are: purified T layer from wild-type cells; urea extract, wild-type cells; urea extract, MBR-10 cells; purified T layer of wild-type cells and urea extract, MBR-10 cells; urea extract of wild-type and MBR-10 cells; urea extract, MBR-9 cells; purified T layer of wild-type cells and urea extract, MBR-9 cells; and urea extract of wild-type and MBR-9 cells.*

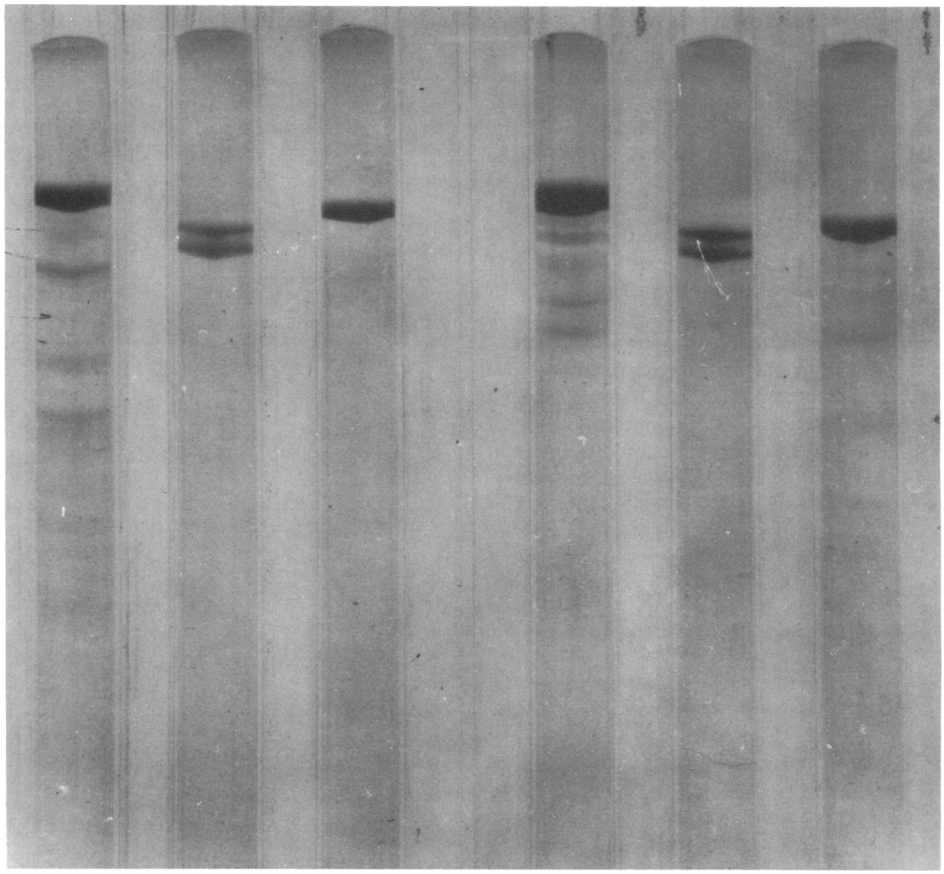

FIG. 6. *Comparison on SDS acrylamide gels by urea extracts of whole cells and isolated cell walls of the wild type, MBR-10, and MBR-9 strains. Gels of urea extracts from left to right are: wild-type cells; MBR-10 cells; MBR-9 cells; wild-type walls; MBR-10 walls; and MBR-9 walls.*

TABLE 2. *Effect on phage titer of incubation of wild-type, MBR-9, and MBR-10 cell walls with phage M*[a]

Strain	Protein (μg/ml)	Phage titer (10^{-4} PFU/ml)	
		(45 min)	(120 min)
Wild type	50	1.2	1.2
	5	6.9	7.6
MBR-9	50	31	27
	5	29	24
MBR-10	50	34	29
	5	27	20
Broth alone	—	29	33

[a] Protein contents of cell walls were determined by the Lowry procedure and were almost entirely T layer protein (Fig. 6). PFU, Plaque-forming units.

curve (Fig. 8). In common with other investigators (21), we found a hyperbolic relationship between molecular weight and relative mobility in the molecular weight range above 100,000. Only small amounts of the urea extracts were run, thereby improving resolution and ensuring that T layer subunits were the only visible protein band.

Table 4 presents a classification of phage-resistant mutants based on the molecular weight of the T layer subunits extracted from each strain. Wild-type T layer subunits have been assigned a molecular weight value of 150,000. Mutants in group A contained T layer subunits indistinguishable from those of the wild-type on SDS gels, whereas the remainder contained T layer subunits of decreased molecular weight. The T layer subunits of group B could only be distinguished from those of the wild-type by running low concentrations of mutant protein in admixture with wild-type, T layer. Although the mutant subunits above 100,000 in molecular weight varied only slightly in mobility on SDS gels, members of different groups were seen as two distinct bands when run as mixtures, whereas mutant proteins within a group gave a single band when run as mixtures. T layer proteins from mutants in

group C comigrated with β-galactosidase. Members of group E contained two protein bands, the faster of which comigrated with the T layer protein from group F. The position of a given mutant protein on SDS gels was reproducible when derived from cultures on different occasions.

Proteolytic cleavage of T layer subunits in the 6 M urea extracts of mutants might account for the lower molecular weight of these subunits. Log-phase cells of the wild-type strain and a representative from each of groups B, C, D, and E were washed in saline, and the cells from each culture were divided into equal portions. One-half of the cell population was extracted with urea in the routine manner, whereas the other half was extracted twice with 5.5 M guanidine hydrochloride at 100 C for 5 min (23). The acrylamide gel mobility of the mutant T layer subunits was the same whether extraction was with urea or with hot guanidine hydrochloride, indicating that the altered T layer subunits are present as such before extraction.

Purification and amino acid analysis of mutant T layer proteins. Attempted purification of mutant T layer from cell wall preparations obtained from MBR-9 and MBR-10 cells by disaggregation and reassembly (see Materials and Methods) was unsuccessful, because the preparations would not reassemble in vitro. Because the T layer subunits still constitute the highest-molecular-weight protein component of the urea extracts of these mutants (Fig. 6, 7) and 30% of the total protein in this fraction, it was possible to purify these subunits by Sephadex G-100 column chromatography in 0.1% SDS. Purification, as indicated by SDS gel electrophoresis, was at least 90% complete.

The amino acid composition of the purified T layers of MBR-4, MBR-9, MBR-21 (each from a different molecular weight class; Table 4), and wild type is presented in Table 5. There is little difference in the molar ratio of the amino acids of the mutant T layers, and percent recovery indicates that they are at least 85% protein. No hexosamines or phosphate were detectable. Native T layer is entirely composed of protein (C. Brinton, personal communication). The similarity in the amino acid composition of mutants and wild-type T layer proteins is consistent with the hypothesis that the new proteins extracted from mutants are derivates of wild-type T layer. Neither glucosamine nor muramic acid was present in the preparations which were, therefore, free of peptidoglycan contamination.

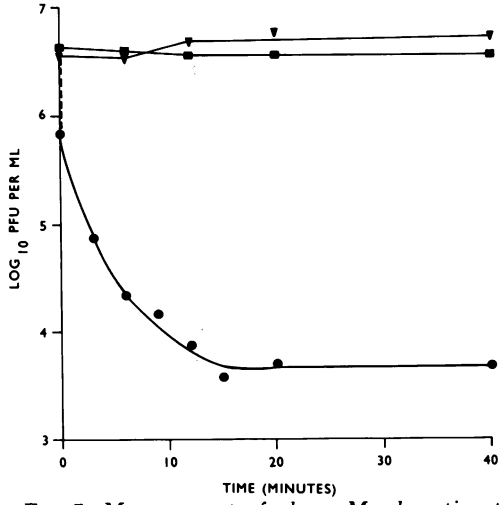

FIG. 7. *Measurement of phage M adsorption to whole cells of wild-type, MBR-9, and MBR-10 strains.* ●, *Wild-type;* ■, *MBR-10;* ▼, *MBR-9.*

TABLE 3. *Distribution of ^{14}C label in SDS acrylamide gels after incubation of labeled T layer with extracts from wild-type, MBR-9, and MBR-10 cells*

Cell extract	Total radioactivity in gel (%)		
	Wild-type T layer region	MBR-9 and MBR-10 T layer region	Remainder of gel
Wild type	95	5	0
MBR-9	91	5	4
MBR-10	93	6	1
None	91	4	5

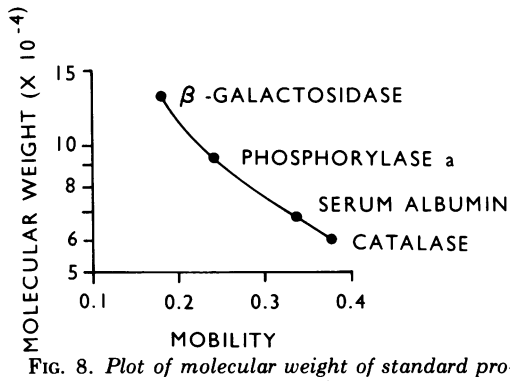

FIG. 8. *Plot of molecular weight of standard proteins against mobility on SDS acrylamide gels. Mobility is measured relative to bromophenol blue. Standards were β-galactosidase, phosphorylase a, bovine serum albumin, and catalase with molecular weights of 130,000, 94,000, 86,000, and 80,000, respectively.*

TABLE 4. *Grouping of phage-resistant mutants on the basis of T layer molecular weight*[a]

Mutants	Group	Mol wt
MBR-1, 5, 6, 7, 8, 31, 84	A	150,000
MBR-2, 20, 21, 27	B	140,000
MBR-18, 23, 65	C	130,000
MBR-9	D	105,000
MBR-10, 11, 12	E	93,000 and 86,000
MBR-3, 4, 14, 22, 38, 68	F	86,000

[a] Wild-type T layer has a molecular weight of 150,000.

TABLE 5. *Molar ratios of amino acids in T layer protein of MBR-4, MBR-9, MBR-21, and wild-type strains*[a]

Amino acid	MBR-4	MBR-9	MBR-21	Wild type
Lysine	.53	.64	.59	.60
Histidine	0	0	.04	0
Arginine	.09	.09	.14	.06
Aspartate	1.00	1.00	1.00	1.00
Threonine	.80	.91	.94	1.07
Serine	.44	.46	.45	.48
Glutamate	.76	.65	.78	.60
Proline	.18	.18	.20	.23
Glycine	.55	.60	.61	.62
Alanine	.94	1.01	1.11	1.11
Valine	.60	.56	.63	.62
Isoleucine	.26	.26	.29	.24
Leucine	.36	.31	.45	.33
Tyrosine	.21	.19	.18	.21
Phenylalanine	.24	.25	.25	.26

[a] All values are relative to aspartic acid which was set at 1.00. The amount of aspartic acid in μmol per 100 mg of protein for each sample was: MBR-4, 96; MBR-9, 97; MBR-21, 103; wild-type, 96. Cysteine, tryptophan and methionine were not determined. 0, Not detected. Percent recovery is the proportion of the dry weight accounted for by the amino acids listed. The percent recovery for MBR-4, MBR-9, MBR-21, and wild type was 84, 85, 98, and 87, respectively.

DISCUSSION

The T layer of *B. sphaericus* strain P-1 comprises 16% of the total cell protein. The surface of the cell appears to be completely covered with T layer (Fig. 1), and 96% of the cellular T layer is extractable by 6 M urea, a procedure which fails to extract high-molecular-weight cytoplasmic proteins, suggesting the absence of a large pool of cytoplasmic precursor of T layer. Because only 7% of the total T layer of the culture is found in the culture medium, the synthesis of this polymer seems to be strictly controlled.

The wild-type T layer is probably the receptor for phage M, and the T layers of MBR mutants are apparently modified so that they no longer function as phage M receptors. Of the 24 MBR mutants investigated, 17 have T layer subunits of molecular weight sufficiently reduced to be clearly separated from wild-type T layer on gel electrophoresis. It is estimated that differences of up to 5% in molecular weight would not have been detected. The seven mutants whose T layer is inseparable from wild-type T layer on electrophoresis may have lost a portion of their polypeptide smaller than 5%, or they may simply be the products of missense mutations. The reduced molecular weight of the T layer subunits in the other 17 MBR mutants was seen whether the T layer was obtained from purified cell walls or from urea extracts of whole cells. Direct extraction of T layer from viable cells by hot guanidine hydrochloride gave the same pattern, making it unlikely that the reduced molecular weight was a result of proteolysis following extraction. These 17 mutants were divided into five discrete groups on the basis of the mobility of their T layer subunits, but again it is possible that members of a single group may differ from one another by up to 5% of their molecular weight.

The dependence of mobility in SDS acrylamide gels on molecular weight has been well established (21, 26). The paucity of protein markers of more than 100,000 in molecular weight precludes the assignment of precise molecular weight values to the larger T layer subunits from mutant cells. However, T layer subunits from mutants in group C were inseparable from β-galactosidase, and the molecular weight value of 130,000 for this group is probably fairly accurate. The β and β′ components of *B. subtilis* ribonucleic acid polymerase both have molecular weights close to 155,000 and are not separated by SDS gel electrophoresis under standard conditions (18). Wild-type T layer is inseparable from these polypeptides under these conditions (T. Lind, personal communication). Extracts of MBR mutants contained no residual protein band with the mobility of wild-type T layer, suggesting that the wild-type subunit contains a single polypeptide which has been modified in these mutants.

The parental and mutant T layers contain at least 85% protein (Table 5), and even on this basis, mutants MBR-4 and MBR-9 must have lost 45 and 32%, respectively, of their protein content. Changes in the content of potential minor nonprotein components cannot be evaluated, but could not account for the marked changes in molecular weight observed in the

majority of the MBR mutants.

The reduced size of these subunits may be due to nonsense mutations at appropriate sites in the structural gene for the T layer protein. Alternatively, missense mutations in this gene, rendering the completed polypeptide sensitive to normal protease components of the cell at unique sites, might result in T layer subunits having the molecular sizes detected in the cell walls of the mutants. Either mechanism suggests that the mutation resides in the structural gene for the T layer subunit.

The two bands seen in class E mutants could be explained by the missense mechanism, because a mutation resulting in a protein subunit which is sensitive to proteolysis at two alternative sites might result in two high-molecular-weight products which are themselves resistant to further proteolysis. A second possibility is that the 93,000 molecular weight subunit derived from an appropriate nonsense mutation might be uniquely sensitive to proteolysis, resulting in partial degradation to a subunit of 83,000 daltons. It is unlikely that the wild-type strain contains two different polypeptides of molecular weight 150,000 which are both mutated simultaneously to products of similar or dissimilar molecular weight. The amount of protein in the two bands from mutants MBR-10, judging from the staining intensity of gels, appears to be approximately equal when log-phase cultures are examined. However, in older cultures the lower-molecular-weight protein predominates, suggesting that it is derived from the 93,000 daltons protein by a relatively inefficient proteolysis. Mutants in group E were restreaked from isolated colonies several times without a change in their double-banded gel pattern, so it is unlikely that this pattern is derived from a heterogeneous cell population.

It was predicted that a major class of phage-resistant mutants would result from nonsense mutations giving rise to truncated polypeptide incapable of self-assembly, resulting in cells devoid of T layer. The absence of such mutants among our 24 isolates was surprising and suggests that T layer is essential for growth of this strain of bacillus. The hexagonally arrayed cell wall protein of *Spirillum serpens* is not present when calcium is omitted from the growth medium (6), but at present we know no means of phenotypically preventing the appearance of T layer on the cell surface of *B. sphaericus*. The function of this layer remains undetermined, but because T layer with a subunit molecular weight as small as 86,000 is found on the cell surface, almost one-half of the wild-type subunit is dispensable for self-assembly and also for function. Shoer and Rappaport (25) reported that a mutant of *B. subtilis* with greatly reduced extracellular protease activity secreted a protein which was demonstrated by fingerprint analysis to be a fragment of the wild-type protease. Because the molecular weight of the protease from the mutant was only 55% of that of the wild-type enzyme, the authors suggested that the tertiary structure of the enzyme was not the property determining its excretion. Likewise, our phage-resistant mutants with drastically altered T layer subunit molecular weight still excrete T layer protein. We are currently attempting to find intracellular precursors of cell wall T layer. If we are successful, it will be possible to determine whether these precursors in mutants are of wild-type or of mutant molecular weight.

ACKNOWLEDGMENTS

We are grateful to J. J. Gauthier and Ruth Gordon for examining the biochemical properties of *Bacillus sphaericus* strain P-1 and to Jack Pate for the electron microscopy.

This investigation was supported by National Science Foundation research grants GB 30567 and Public Health Service research grant HD 06518-01 from the National Institute of Child Health and Human Development.

LITERATURE CITED

1. Adams, M. H. 1959. Bacteriophages. Interscience Publishers, Inc., New York.
2. Adelberg. E. A., M. Mandel, and G. C. C. Chen. 1965. Optimal conditions for mutagenesis by N-methyl-N^1-nitro-N-nitrosoguanidine in *Escherichia coli* K 12. Biochem. Biophys. Res. Commun. **18**:788–795.
3. Alpers, D. H., and R. Glickman. 1970. Method for determination of specific activity of proteins in polyacrylamide gels. Anal. Biochem. **35**:314–320.
4. Baddiley, J. 1964. Teichoic acids and the bacterial cell wall. Endeavour **23**:33–37.
5. Brinton, C. C., Jr., and H. Beer. 1967. The interaction of male-specific bacteriophages with F-pili, p. 251–289. *In* J. S. Colter and W. Paranchych (ed.), The molecular biology of viruses. Academic Press Inc., New York.
6. Buckmire, F. L. A., and R. G. E. Murray. 1970. Studies on the cell wall of *Spirillum serpens*. 1. Isolation and partial purification of the outermost cell wall layer. Can. J. Microbiol. **16**:1011–1022.
7. Douglas, L. J., and M. J. Wolin. 1971. Cell wall polymers and phage lysis of *Lactobacillus plantarum*. Biochemistry **10**:1551–1555.
8. Ellar, D. J., and D. G. Lundgen. 1967. Ordered substructure in the cell wall of *Bacillus cereus*. J. Bacteriol. **94**:1778–1780.
9. Glauert, A. M., and M. J. Thornley. 1969. The topography of the bacterial cell wall. Annu. Rev. Microbiol. **23**:159–198.
10. Gold, A. M. 1967. Sulfonylation with sulfonyl halides, p. 706–711. *In* C. H. W. Hirs (ed.) Methods in enzymology, vol. 11 Academic Press Inc., New York.
11. Goldberg, A. L. 1971. Effect of protease inhibitors on protein breakdown and enzyme induction in starving *Escherichia coli*. Nature N. Biol. **234**:51–52.
12. Goundry, J., A. L. Davison, A. R. Archibald, and J. Baddiley. 1967. The structure of the cell wall of *Bacillus polymyxa* (N.C.I.B. 4747). Biochem. J. **104**:1c.

13. Harrigan, W. F., and M. E. McCance. 1966. Laboratory methods in microbiology. Academic Press Inc., New York.
14. Holt, S. C., and E. R. Leadbetter, 1969. Comparative ultrastructure of selected aerobic spore-forming bacteria: a freeze-etching study. Bacteriol. Rev. **33**: 346-378.
15. Hungerer, K. D., J. Fleck, and D. J. Tipper. 1969. Structure of the cell wall peptidoglycan of *Lactobacillus casei* R094. Biochemistry **8**:3567-3577.
16. Hungerer, K. D., and D. J. Tipper. 1969. Cell wall polymers of *Bacillus sphaericus* 9602. I. Structure of vegetative cell wall peptidoglycan. Biochemistry **8**:3577-3587.
17. Lindberg, A. A., and C. G. Hellerqvist. 1971. Bacteriophage attachment sites, serological specificity, and chemical composition of the lipopolysaccharide of semirough and rough mutants of *Salmonella typhimurium*. J. Bacteriol. **105**:57-64.
18. Losick, R., R. G. Shorenstein, and A. L. Sonenshein. 1970. Structural alteration of RNA polymerase during sporulation. Nature (London) **227**:910-913.
19. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurements with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
20. Nermut, M. V., and R. G, E. Murray. 1967. Ultrastructure of the cell wall of *Bacillus polymyxa*. J. Bacteriol. **93**:1949-1965.
21. Neville, D. M. 1971. Molecular weight determination of protein dodecyl sulfate complexes by gel electrophoresis in a discontinuous buffer system. J. Biol. Chem. **246**:6328-6334.
22. Osborn, M. J. 1969. Structure and biosynthesis of the bacterial cell wall. Annu. Rev. Biochem. **38**:501-538.
23. Pringle, J. R. 1970. The molecular weight of the undergraded polypeptide chain of yeast hexokinase. 1970. Biochem. Biophys. Res. Commun. **39**:46-52.
24. Shaw, D. R. D., D. Mirelman, A. N. Chatterjee, and J. T. Park. 1970. Ribitol teichoic acid synthesis in bacteriophage-resistant mutants of *Staphylococcus aureus* H. J. Biol. Chem. **245**:5101-5106.
25. Shoer, R., and H. P. Rappaport. 1972. Analysis of a *Bacillus subtilis* proteinase mutant. J. Bacteriol. **109**:575-583.
26. Weber, K., and M. Osborn. 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. **244**:4406-4412.
27. Weppelman, R. M., and C. C. Brinton, Jr. 1971. The infection of *Pseudomonas aeruginosa* by RNA pilus phage PP7: the adsorption organelle and the relationship between phage sensitivity and the division cycle. Virology **44**:1-17.
28. Young, F. E. 1967. Requirement of glucosylated teichoic acid for adsorption of phage in *Bacillus subtilis* 168. Proc. Nat. Acad. Sci. U.S.A. **58**:2377-2384.