JOURNAL OF BACTERIOLOGY, May 1974, p. 654-662

Vol. 118, No. 2
Printed in U.S.A.

Detachment and Chemical Characterization of the Regularly Arranged Subunits from the Surface of an *Acinetobacter*

MARGARET J. THORNLEY,[1] KAREEN J. I. THORNE, AND AUDREY M. GLAUERT

Strangeways Research Laboratory and Dunn Nutritional Laboratory, University of Cambridge and Medical Research Council, Cambridge, England

Received for publication 9 October 1973

Acinetobacter sp. strain MJT/F5/199A carries an array of tetragonally arranged subunits on its outer surface. The subunits can be detached from isolated cell walls by incubation with 1 M urea or by washing with water after treatment with 10 mM ethylenediaminetetraacetic acid or ethyleneglycol-bis(β-aminoethylether)N,N'-tetraacetic acid. After removal of the urea, they reaggregate into the same ordered array at air-water interfaces in the presence of $MgCl_2$. The detached subunits were characterized as an acidic protein of molecular weight 65,000. They represent one-fifth of the total cell wall protein.

The envelope of the gram-negative bacterium *Acinetobacter* sp. strain MJT/F5/199A has already been extensively studied by electron microscopy. (The problems involved in determining the correct taxonomic position of this strain are discussed by Thornley et al. [10].) The envelope can be fractionated into different layers by relatively simple techniques (10), and the chemical composition of the fractions has been determined (9). Outside the plasma membrane, the layers of the cell wall consist of the peptidoglycan-containing dense layer, the intermediate layer, and the outer membrane, which carries an array of tetragonally arranged subunits on its outer surface. The appearance and arrangement of these subunits on the intact organism have been studied by freeze-etching (8), and detached subunits were found to have the ability to reassemble in vitro to form the same pattern as that seen on the intact cell (4).

The present paper describes the results of a study of the detachment and characterization of the subunits. The effects of various methods of detachment were compared by using electron microscopy and disc electrophoresis. These experiments made it possible to identify the protein band containing the material of the subunits on polyacrylamide gels after electrophoresis, and led to the purification and chemical characterization of the material of the subunits.

[1] Present address: Immunology Division, Department of Pathology, University of Cambridge, Addenbrookes Hospital, Cambridge, England.

MATERIALS AND METHODS

Acinetobacter sp. strain MJT/F5/199A (NCIB 10885) was grown in an FE 007 fermentor (Biotec Ltd., Selsdon, South Croydon CR2 8YD, England) by using maximum aeration at a temperature of 28 to 30 C. The fermentor contained 700 ml of heart infusion broth (Difco) with 0.01% of $CaCl_2$ and 0.1 ml of silicone MS antifoam A (Hopkin & Williams Ltd., Chadwell Heath, Essex, England). Cultures were harvested in the mid-logarithmic phase. Some additional preparations were grown in the same medium in conical flasks, aerated by shaking at 28 C, and harvested in the late logarithmic phase.

The method of preparation of the fragments of the cell wall has already been described (10); briefly, it consists of the breakage of the cells in the French press, treatment with deoxyribonuclease and ribonuclease, and four washes in 20 mosmol of phosphate buffer, pH 7.4 (10). The extent of contamination of the cell wall preparation with material derived from the plasma membrane was less than 2% (9). These fragments of cell wall were the starting material for most of the experiments to be described. In addition, some comparisons were made with the other preparations derived from the cell wall fragments (10): these were the outer membrane (OM), obtained from the cell wall preparation by treatment with lysozyme, and the papain-treated outer membrane (PTOM), from which the surface subunits had been removed.

Studies of the detachment of subunits from cell wall fragments are described in this paper. In most experiments, a portion of cell walls containing about 1 mg of protein was suspended in 10 ml of each of the solutions to be studied and incubated under the conditions shown in Table 1. Each suspension was then centrifuged at 37,000 $\times$ *g* for 20 min, and the supernatant fluid was retained and called SN1. The

sediment was washed with 10 ml of distilled water and centrifuged, giving the final sediment and the supernatant fraction SN2. A portion of each final sediment was negatively stained and examined in the electron microscope, while the SN1 solutions were dialyzed for 24 h against two changes of distilled water. After this, both supernatant fractions from each treatment were freeze-dried and dissolved in a small volume of distilled water. Protein determinations were made on samples of each preparation by the Folin Lowry method (7), and the proteins present were studied by disc electrophoresis, using for comparison quantities of the pellet and SN1 and SN2 preparations, which had been obtained from the same amount of the original cell wall preparation.

Disc electrophoresis was performed on 10% polyacrylamide gels in 0.1% sodium dodecyl sulfate (SDS) as described by Weber and Osborn (11), with bovine serum albumin, ovalbumin, and lysozyme as standards. Before electrophoresis, samples (10 to 50 μg of protein) were incubated with 0.1% SDS in 0.1 M sodium phosphate, pH 7.2, and 5% mercaptoethanol in a total volume of 50 to 100 μliters for 20 min at 70 C. These conditions were chosen because incubation at 37 C for 18 h did not completely solubilize all the pellet protein; incubation at 100 C for 5 min resulted in an altered electrophoretic pattern as reported by Inouye and Yee (6) for *Escherichia coli*. Proteins were detected by staining with 1% amido black in 7% acetic acid overnight, followed by destaining with 7% acetic acid. Glyco-compounds were detected by the periodate-Schiff method (12). Samples were also analyzed by isoelectric focusing in wide-range (pH 3 to 10) and narrow-range (pH 3 to 6) Ampholine in polyacrylamide gels by A. J. Barrett.

Molecular weights were determined by gel filtration (1) of the dialyzed and concentrated urea extracts through Sephadex G-150 (Pharmacia, Uppsala, Sweden) in 20 mM sodium phosphate, pH 6.8, containing 1 M urea, 0.02% mercaptoethanol, and 0.2% SDS, to prevent aggregation. Protein was detected by its absorbance at 280 nm. Bovine serum albumin, ovalbumin, and lysozyme were used as standards.

The amino acid composition of the protein, purified by gel filtration, dialyzed against water, and hydrolyzed with 6 N HCl at 115 C for 67 h, was determined with a Technicon Autoanalyser by W. J. Branch.

The properties of the protein were also investigated by chromatography on a 10-ml column of diethylaminoethyl (DEAE)-cellulose (Whatman Biochemicals Ltd., Maidstone, Kent, England) in 0.1 M tris-(hydromethyl)aminomethane (Tris)-hydrochloride, pH 8, containing 1 M urea. The column was eluted with a gradient of increasing molarity. Initially, the mixing chamber contained 40 ml of 0.1 M Tris-hydrochloride, pH 8 with 1 M urea, and the reservoir contained 40 ml of 1.0 M Tris-hydrochloride, pH 8, with 1 M urea. Next, 3-ml fractions were collected and monitored for protein by measurement of the absorbance at 280 nm.

The technique used for negative staining was to invert a grid coated with collodion and carbon onto the surface of the sample to be studied. The grid was allowed to float for a few seconds, and then it was transferred to the surface of the negative staining solution for 2 to 3 min, followed by draining on filter-paper. The negative staining solution used was 1% ammonium molybdate with the pH adjusted to 7.0. The grids were examined in an AEI EM6B electron microscope operating at 60 kV with a 50-μm objective aperture.

RESULTS

Preparations of fragments of cell wall were subjected to various treatments intended to remove the surface subunits with the least possible change in the underlying layers. Negatively stained preparations of the treated fragments after one wash in distilled water were examined, and the results for some of the more useful treatments are summarized in Table 1 and illustrated in Fig. 1 to 4.

The untreated portions of the cell wall resemble those illustrated in reference 10 (Fig. 6 to 8). They are often folded or rolled, and the subunits may be seen on the surface as rows of white dots but are most clearly visible where they project from the folded edges of the fragment (Fig. 1). Removal of the subunits sometimes leaves large smooth areas of the underlying outer "unit" membrane, as with 1 M urea treatment (Fig. 2), or may be accompanied by breakdown of the outer membrane into much smaller vesicles, as with 8 M urea or 1.5 M guanidine hydrochloride (Table 1). The most complete removal of subunits with the minimum damage to the outer membrane was obtained by treatment for 2 h at 37 C with 1, 1.5, or 2 M urea. Almost complete removal of the subunits was observed after treatment for 30 min with either of the chelating agents, ethylenediaminetetraacetic acid (EDTA) or ethyleneglycol-bis(β-aminoethylether)N,N'-tetraacetic acid (EGTA), followed by a wash in distilled water. However, a few traces of granular material remain at the folded edges of the fragments (Fig. 3 and 4, arrows), and these probably represent residual material from the layer of surface subunits. After both of these treatments, the surface of the outer membrane, on some fragments of cell wall, appears to be interrupted by "channels" that retain the negative stain (Fig. 4).

The results of disc electrophoresis, in the presence of SDS, of samples from the preparations described, each derived from the same quantity of the original cell wall preparation, are compared in Fig. 5. The initial cell wall preparation gave rise to at least 12 bands, and the second largest of these, at position A, was the main site of modifications produced by treatments that removed the subunits. After treatment with 2 M urea, the pellet produced a

TABLE 1. *Conditions of treatment of fragments of cell wall and the effects observed by electron microscopy of negatively stained preparations of pellets after one wash in water*

Treatment	Temp (C)	Time (min)	Effects observed on:	
			Outer "unit membrane"	Subunits
Guanidine-hydrochloride, 6 M	37	120	Degraded to vesicles	Removed
Guanidine-hydrochloride, 1.5 M	37	120	Partially degraded	Some removed
Urea, 8 M	37	120	Some degraded	Removed
Urea, 2 M	37	120	Intact	Removed
Urea, 1.5 M	37	120	Intact	Removed
Urea, 1.0 M	37	120	Intact	Removed
Urea, 0.5 M	37	120	Intact	Some removed
Urea, 1.0 M	37	30	Intact	Present
EDTA[a], 10 mM, pH 8.0	24	30	Intact, few "channels"	Removed
EGTA[a], 10 mM, pH 8.0	24	30	Intact, few "channels"	Removed
EGTA, 10 mM, pH 8.0	24	10	Intact	Removed

[a] EDTA, ethylenediaminetetraacetate, disodium salt; EGTA, ethyleneglycol-bis(β-aminoethyl ether)N,N'-tetraacetic acid. Both EDTA and EGTA were used in 0.1 M ammonium bicarbonate.

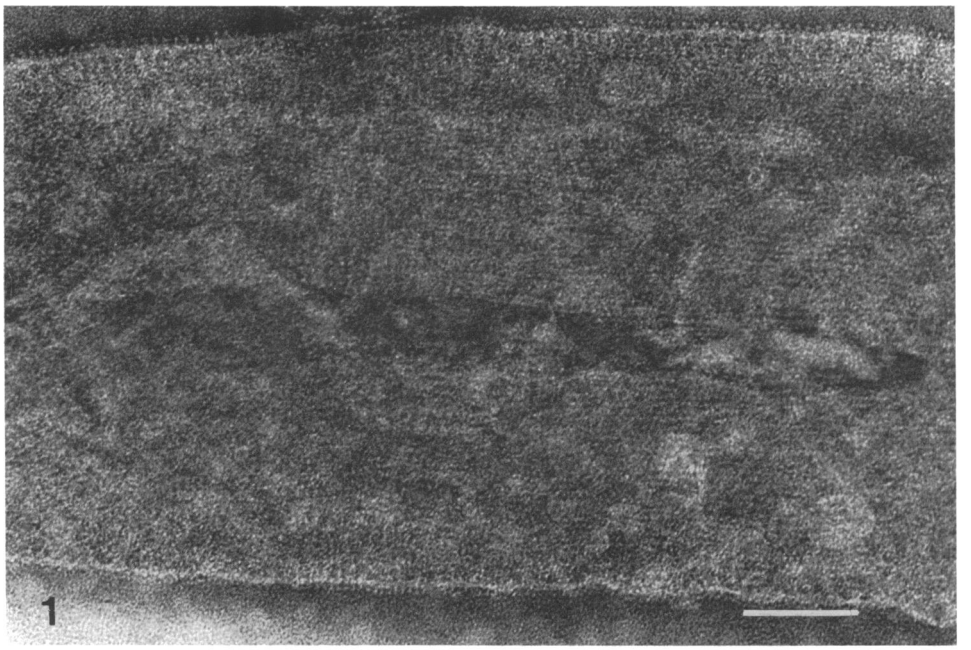
pattern of bands almost the same as that of the control, except that only a minor band was now present at position A. The supernatant fraction, SN1, showed one major band at this position, together with only faint traces of other components, whereas the water wash, SN2, gave only a very faint band at position A. This indicates that the main part of the material represented by this band was present in the urea solution. The same result was obtained with 1 M urea.

The residual wall material after treatment with 10 mM EDTA at pH 8.0, followed by one wash in distilled water, also showed a very similar pattern of bands to that of the control, with some reduction in intensity of the band at position A. The first supernatant fluid from this treatment gave only a faint band at position A, but the water wash, SN2, contained a major band at A and only one other very faint band. A considerable part of the material of band A was therefore removed by the distilled water wash after the EDTA treatment. Very similar results were obtained by EGTA treatment, and these are also illustrated in Fig. 5.

Further evidence that band A did indeed represent the surface subunit proteins was obtained by disc electrophoresis of the fractions derived from the cell wall preparations and previously studied by electron microscopy (10) and by chemical methods (9). These were the OM preparation, obtained from the cell walls by treatment with lysozyme, which contained the outer "unit" membrane itself, together with the surface subunits and some material of the intermediate layer, and the PTOM, which had lost the surface subunits but retained the "unit" membrane structure. Gels for electrophoresis were prepared from portions containing 50 μg of protein of the cell wall, OM, and PTOM preparations (Fig. 6). The pattern derived from the OM resembled that of the cell wall in its major bands but showed the loss of several polypeptides of high molecular weight; this corresponds to the loss of 25% of the protein initially present in the cell wall at this stage in the preparation (9). Greater differences are seen between the patterns of the OM and PTOM preparations; these differences include the complete lack of band A in the PTOM preparation. The relative amounts of bands B and C also differ, since band B is the largest band in the wall and OM preparations, whereas, in the PTOM, band C is larger. This change probably reflects the breakdown by papain of the material of band B. The percentage of protein lost at this stage is 46% (9).

Comparison of the patterns obtained by disc electrophoresis (Fig. 5, 6) with the presence or absence of the surface subunits, as detected by electron microscopy, indicates that band A contains the material derived from the subunits. Preparations from which the subunits have been removed, such as the PTOM and the urea-, EDTA-, or EGTA-treated walls, show either a disappearance or a reduction in intensity of this band, whereas the urea extract and the water wash after EDTA or EGTA treatment contain this band as the main component. Other minor bands in the urea-SN1 and EDTA-SN2 preparations differ in position, and therefore band A is the only common constituent in these preparations.

The detached subunits may be recognized by their ability to reassemble to form the same pattern as that seen on the intact cell wall (4; M. J. Thornley, A. M. Glauert, and U. B.



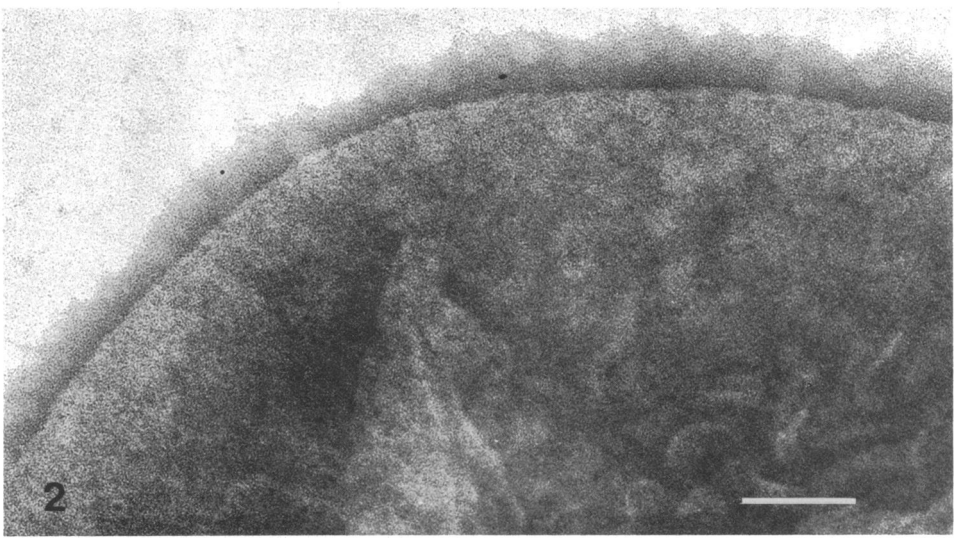
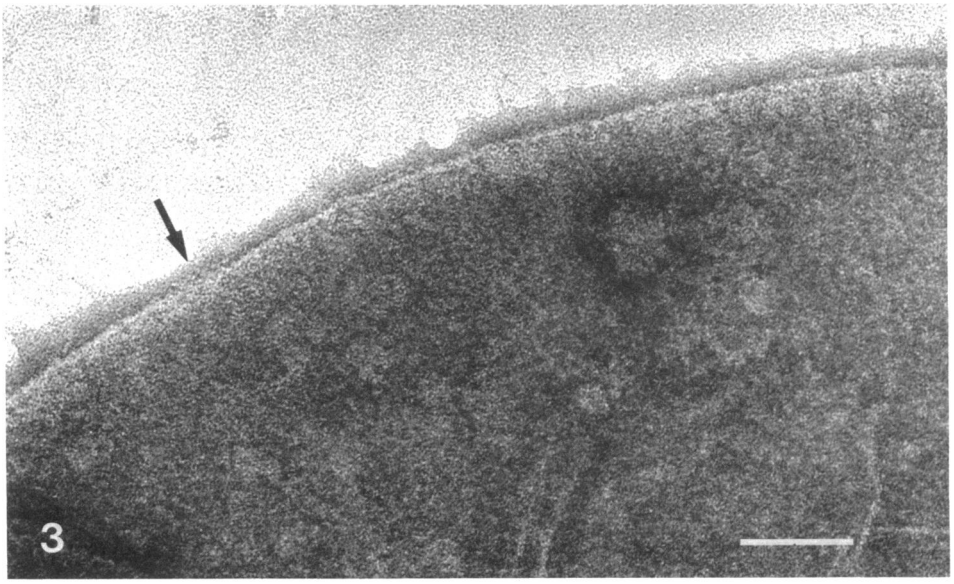


FIG. 1 and 2. *Electron micrographs of negatively stained preparations of cell walls isolated from Acinetobacter strain MJT/F5/199A. The scale marks represent 0.1 μm. Fig. 1. Surface subunits are visible projecting from the surface of a folded fragment of untreated cell wall. Fig. 2. Subunits are no longer visible on the surface of a cell wall treated with 1 M urea for 2 h at 37 C and then washed with distilled water.*

Sleytr, Phil. Trans. Roy. Soc., in press), and this property was examined in extracts. Samples of material extracted with urea and then dialyzed and concentrated were placed in small vials, with and without the addition of 20 mM $MgCl_2$. After 30 min at room temperature (24 C), surface samples examined by negative staining showed only amorphous material in vials without $MgCl_2$, but, in the presence of $MgCl_2$, arrays of subunits were present (Fig. 7). At protein concentrations of about 150 μg/ml or greater, these arrays were numerous, but they were detectable in preparations containing 70 μg of protein per ml. The water wash after



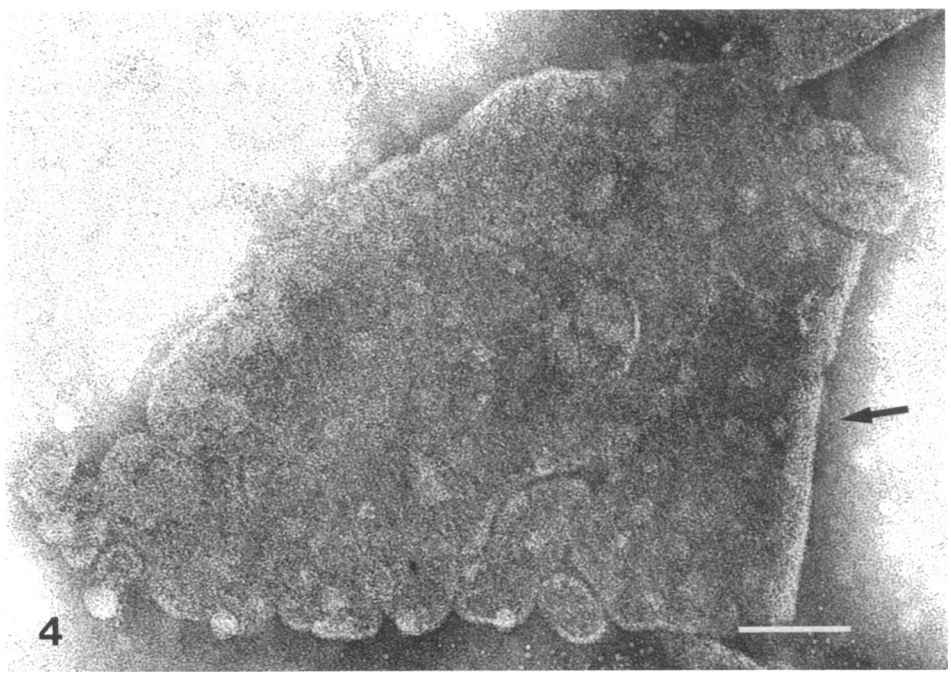


FIG. 3 and 4. *Negatively stained preparations of cell walls. The scale marks represent 0.1 μm. Fig. 3. A few subunits (arrow) are still present after the cell walls have been treated with 10 mM EDTA, pH 8, for 30 min at 24 C, followed by a wash in distilled water. Fig. 4. After treatment with 10 mM EGTA, pH 8, for 30 min at 24 C, followed by a wash in distilled water, a few subunits are still present (arrow). Channels are visible on the surface of the outer membrane of the cell wall.*

EGTA treatment was examined in the same way, and arrays were found in preparations containing 80 μg of protein per ml after incubation with $MgCl_2$ or $CaCl_2$. These observations confirm the identification of band A as the location of the material of the subunits.

The amount of protein released from the cell walls by treatment with 1 M urea followed by

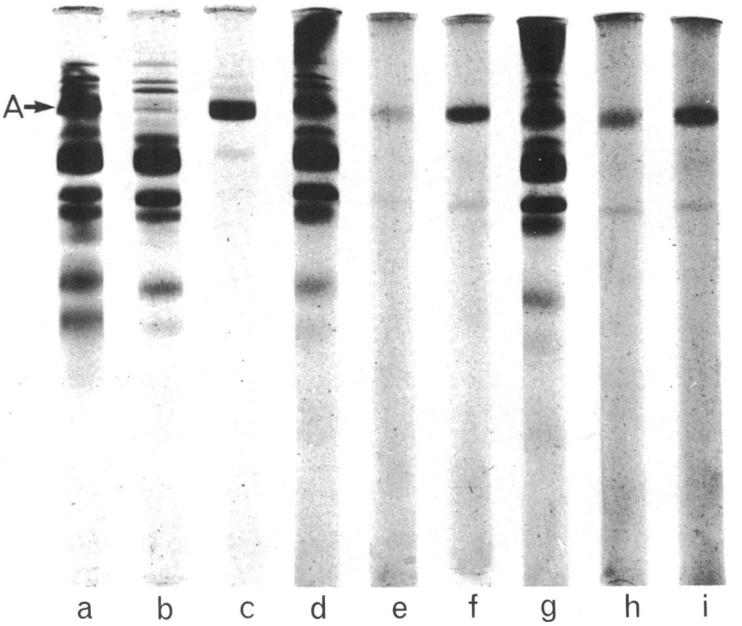


FIG. 5. *SDS acrylamide gel electrophoresis of chemically treated cell walls. (a) Intact wall; (b) wall treated with 2 M urea; (c) 2 M urea extract; (d) wall treated with 10 mM EDTA and washed with distilled water; (e) 10 mM EDTA extract; (f) water wash after EDTA treatment; (g) wall treated with 10 mM EGTA and washed with distilled water; (h) 10 mM EGTA extract; (i) water wash after EGTA treatment. The band solubilized by urea, EDTA, and EGTA is labeled A.*

washing with water, as determined chemically, represented 26% of the total cell wall protein. Densitometer tracings of polyacrylamide gels of this material showed that 82% of the protein was in band A. Densitometer tracings of the polyacrylamide gels of the whole cell wall showed that band A contained 19% of the protein. It may therefore be concluded that the surface subunits comprise 20% of the cell wall protein.

When the surface subunits were released with 1 M urea, some carbohydrate was also solubilized, at a level of 4% of the protein. The possibility that the surface subunits are therefore glycoprotein was tested by staining the polyacrylamide gels of this material by the periodate-Schiff method (12). Although faint traces of glyco-positive material were seen in gels after electrophoresis in SDS, it is believed that this was an artifact due to incomplete removal of the SDS. After electrophoresis in the absence of SDS, at pH 9.5 in Tris-glycine (15% acrylamide), no glyco-positive material was found. It is concluded that the surface subunits are not glycoprotein.

The molecular weight, as determined by disc electrophoresis, was 62,000. Gel filtration on Sephadex was used as an alternative method for obtaining the molecular weight (Fig. 8). When the preparation of subunit protein was chromatographed on Sephadex G-150 in 20 mM phosphate buffer containing 1 M urea and 0.02% mercaptoethanol, two peaks of protein were obtained (Fig. 8a), one an aggregate at the exclusion volume (peak I) and the other of molecular weight 66,000 (peak II). Aggregation could be avoided by including 0.2% SDS in the eluting solvent (Fig. 8b). Under these conditions the main peak was eluted like a protein with molecular weight 66,000 together with a minor peak of molecular weight 50,000. Inspection of Fig. 5 shows that urea extracts are contaminated with traces of protein of this size. When peaks I and II, produced in the absence of SDS, were rechromatographed in the presence of SDS they both behaved like a protein of molecular weight 66,000.

The amino acid composition of the surface subunit protein is given in Table 2. Levels of proline, sulfur-containing amino acids, and tyrosine were too low to be detected. The protein is predominantly acidic, as shown by its content of 26.5% acidic amino acid residues compared with 7.4% basic. Histidine was the least abundant amino acid. If the protein contains two histidine residues per molecule, its theoretical molecular weight would be 67,500.

The acidity of the protein was confirmed in

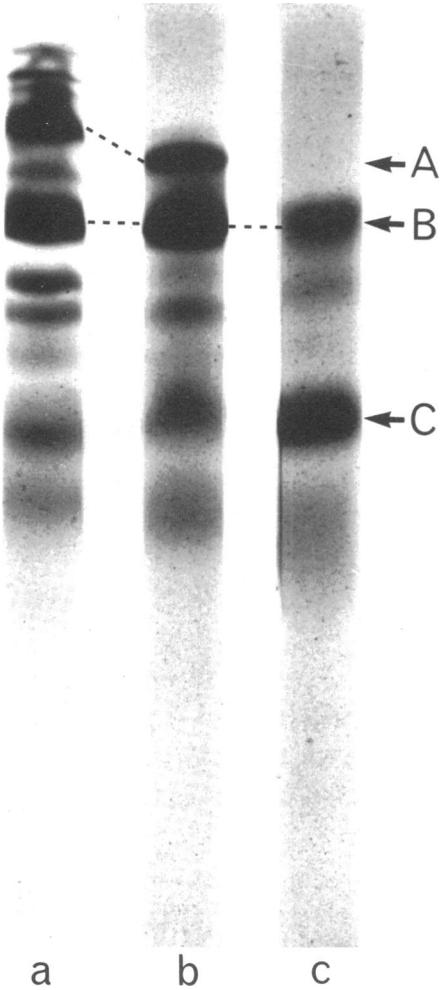


FIG. 6. *SDS acrylamide gel electrophoresis of (a) cell walls, (b) outer membranes, and (c) papain-treated outer membranes. Gel a was obtained in a separate experiment from gels b and c; dotted lines connect the positions of bands A and B in gels a and b.*

two ways. After isoelectric focusing in polyacrylamide gel, it appeared as a single, negatively charged band, It was eluted as a single peak from DEAE-cellulose only when the concentration of eluting solvent reached 0.4 M.

DISCUSSION

The subunits on the outer surface of this strain of *Acinetobacter* are composed of acidic proteins, apparently without glyco-residues, and are thus chemically similar to the hexagonally ordered surface subunits of *Spirillum serpens* (3). The protein from *S. serpens* has a much higher molecular weight (140,000) than that from *Acinetobacter* (65,000), but there is some evidence that the *S. serpens* protein is a trimer.

The ability of EDTA and EGTA to disrupt the attachment of the *Acinetobacter* subunits to the surface of the cell wall suggests that the protein molecules interact with the bacterial surface through polyvalent cations. The acidity of the protein would facilitate this. Buckmire and Murray (2) investigated the detachment of the subunits from the surface of intact cells of *S. serpens* and found that the most effective agent was 1 M guanidine hydrochloride. Urea was active but only at concentrations above 4 M, and EDTA was ineffective. The attachment of this protein to the bacterial surface therefore differs somewhat from that of *Acinetobacter*, where guanidine hydrochloride was only effective at 6 M (at which level it destroyed the integrity of the outer membrane), whereas 1 M urea removed the protein efficiently and EDTA was fairly active. The removal by urea is probably due to a change in conformation of the protein which disturbs its interaction both with cell surface and with the other protein subunits in the tetragonal array. This change is apparently reversible, as after removal of the urea the proteins can reassemble into the ordered array (4; Thornley et al., in press). The released subunits have a strong tendency to aggregate, and urea alone was not enough to prevent aggregation during gel filtration, so that the repulsion between the proteins had to be increased with SDS.

The reassembly of the isolated subunits from *Acinetobacter* does not require the presence of a template or of specific cations. Although Mg^{2+} or Ca^{2+} was used in this study, the presence of a wide range of monovalent or divalent cations would allow reassembly (4; Thornley et al., in press). In both of these respects, the subunits of *Acinetobacter* differ from those of *S. serpens*, which require fragments of the cell wall and divalent cations for reassembly (3). It would therefore appear that the proteins of the two species have gross similarities in their mode of attachment, but that they differ in detail.

Regularly arranged surface subunit proteins have also been detached from gram-positive bacteria. The tetragonally arranged protein of *Bacillus polymyxa* has been shown to have a high content of acidic amino acids and some carbohydrate is present (J. Goundrey, A. L. Davison, A. R. Archibald, and J. Baddiley, Biochem J. **104**:1C-2C, 1967), whereas the subunits that can be removed from the surface of *Bacillus sphaericus* with 6 M urea constitute 16% of the total cell protein and have a molecular weight of 150,000 (5).

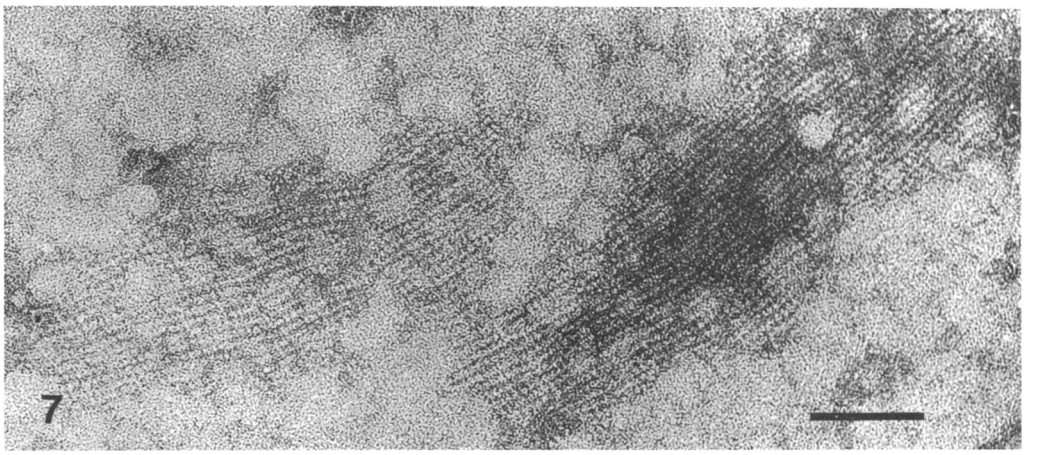


FIG. 7. *Arrays of subunits are visible in an electron micrograph of a negatively stained preparation of the material extracted from cell walls by urea, dialyzed, and then allowed to stand at room temperature for 30 min in the presence of 20 mM $MgCl_2$. The scale mark represents 0.1 μm.*

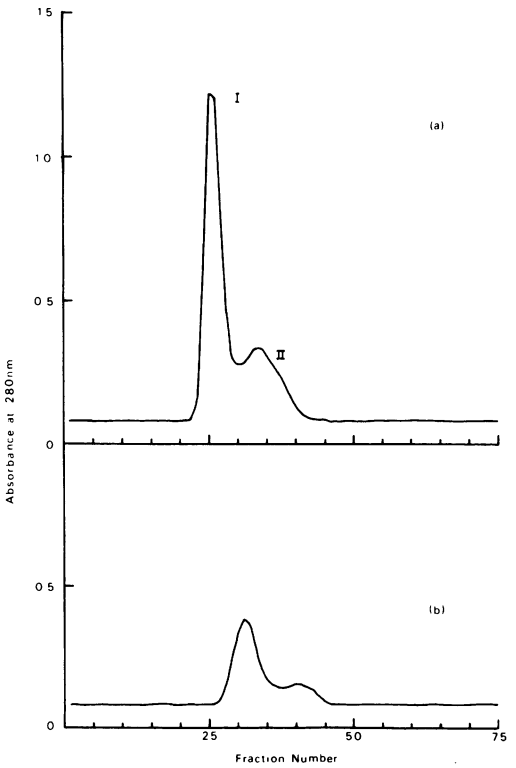


FIG. 8. *Gel filtration of surface subunit protein on Sephadex G-150 (2.5 by 57 cm; void volume 72 ml; 3-ml fractions). (a) Eluting solvent: 20 mM sodium phosphate (pH 6.8), 1 M urea, 0.02% mercaptoethanol. Bovine serum albumin eluted in fraction 35. (b) Eluting solvent: 20 mM sodium phosphate (pH 6.8), 1 M urea, 0.02% mercaptoethanol, 0.2% SDS. Bovine serum albumin eluted in fraction 30.*

TABLE 2. *Amino acid composition of subunit protein of Acinetobacter sp. strain MJT/F5/199A*

Amino acid	Percentage composition (mol/100 mol)
Aspartic acid	17.41
Threonine	8.45
Serine	3.68
Glutamic acid	9.06
Glycine	21.10
Alanine	12.67
Valine	9.31
Isoleucine	2.98
Leucine	5.13
Phenylalanine	2.86
Lysine	5.84
Histidine	0.34
Arginine	1.17
Tryptophan	Not determined

The biological functions of these regularly arranged subunits on bacterial surfaces are not yet known, but it is possible that they have a protective role. Certainly the protein of *S. serpens* protects the cell from infection by *Bdellovibrio bacteriovorus* (F. L. A. Buckmire, Bacteriol. Proc., p. 43, 1971). Conversely, the protein of the gram-positive *B. sphaericus* has been shown to be a receptor for bacteriophages (5). It seems likely that similar specific functions will be found to be associated with these arrays of subunits when the physiology and chemistry of a greater range of bacteria has been studied.

ACKNOWLEDGMENTS

We are grateful to K. C. Day, R. A. Parker, and M. Neal for skillful technical assistance.

LITERATURE CITED

1. Andrews, P. 1965. The gel-filtration behaviour of proteins related to their molecular weights over a wide range. Biochem. J. **96**:595-606.
2. Buckmire, F. L. A., and R. G. E. Murray. 1970. Studies on the cell wall of *Spirillum serpens*. I. Isolation and partial purification of the outermost cell wall layer. Can. J. Microbiol. **16**:1011-1022.
3. Buckmire, F. L. A., and R. G. E. Murray. 1973. Studies on the cell wall of *Spirillum serpens*. II. Chemical characterization of the outer structured layer. Can. J. Microbiol. **19**:59-66.
4. Glauert, A. M., and M. J. Thornley. 1973. Self-assembly of a surface component of a bacterial outer membrane. John Innes Symp. **1**:297-305.
5. Howard, L., and D. J. Tipper. 1973. A polypeptide bacteriophage receptor: modified cell wall protein subunits in bacteriophage-resistant mutants of *Bacillus sphaericus* strain P-1. J. Bacteriol. **113**:1491-1504.
6. Inouye, M., and M. L. Yee. 1973. Homogeneity of envelope proteins of *Escherichia coli* separated by gel electrophoresis in sodium dodecyl sulfate. J. Bacteriol. **113**:304-312.
7. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
8. Sleytr, U. B., and M. J. Thornley. 1973. Freeze-etching of the cell envelope of an *Acinetobacter* species which carries a regular array of surface subunits. J. Bacteriol. **116**:1383-1397.
9. Thorne, K. J. I., M. J. Thornley, and A. M. Glauert. 1973. Chemical analysis of the outer membrane and other layers of the cell envelope of *Acinetobacter* sp. J. Bacteriol. **116**:410-417.
10. Thornley, M. J., A. M. Glauert, and U. B. Sleytr. 1973. Isolation of outer membranes with an ordered array of surface subunits from *Acinetobacter*. J. Bacteriol. **114**:1294-1308.
11. Weber, K., and M. J. Osborn. 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. **244**:4406-4412.
12. Zacharius, R. M., T. E. Zell, J. H. Morrison, and J. J. Woodlock. 1969. Glycoprotein staining following electrophoresis on acrylamide gels. Anal. Biochem. **30**:148-152.