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HEXAGONAL SURFACE ARRAY IN A PROTEIN-SECRETING BACTERIUM, *BACILLUS BREVIS* 47

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***Bacillus brevis* 47, a protein-secreting bacterium, contained two major proteins with approximate molecular weights of 150000 and 130000 in the cell wall. The cell surface was covered with a hexagonally arranged array of six structural units about 4 nm in diameter with a lattice constant of 14.5 nm. The regular array structure as well as the chemical composition of cell envelopes remained the same regardless of the growth conditions. A mutant, strain 47-57, which was isolated as a phage resistant colony, contained only the 150000 protein as a major cell wall protein. Although the mutant had hexagonally arranged arrays with the same lattice constant as that of wild-type cells, the distribution of mass in the unit cell differed considerably from that of the wild-type cells. The number of structural units in the unit cell of the mutant was reduced from six to three. Taking these results together with filtered images of the wild-type and mutant envelopes, two possible models for the surface array of *B. brevis* 47 are discussed.**

Introduction

The presence of ordered structures on bacterial surfaces has been demonstrated in a number of Gram-positive and Gram-negative bacteria [1–3]. Most of the regular surface patterns have been shown to consist of arrays of subunits with hexagonal or tetragonal symmetry [1,3]. The surface subunits of Gram-positive bacteria predominantly show a tetragonal order, whereas Gram-negative bacteria have mostly hexagonal arrays. In the *Bacillus* species examined to date, only three species, *B. alvei, B. anthracis* and *Bacillus* species C.I.P. 76-111, have been shown to have hexagonal arrays of subunits [3–6].

B. brevis 47, which was isolated from soil as a protein producing bacterium, secreted up to 12 mg/ml of protein under optimal growth conditions [7]. This bacterium has morphologically a unique cell wall structure consisting of three layers (two proteinaceous layers and a peptidoglycan layer) and sheds the outer two proteinaceous cell wall layers concomitantly with a prominent increase in protein secretion [8]. In order to correlate alterations in the cell wall structure with protein secretion, we analyzed by a combination of biochemical and physical techniques the cell envelopes prepared from cells grown under protein- and nonprotein-secreting conditions, namely in 1.5 and 0.5% phosphate medium [9]. Regardless of the growth conditions, the cell envelopes had a similar chemical composition and a hexagonal array with the same lattice constant of 14.5 nm. Based on the optically filtered images of the cell envelopes prepared from the wild-type cells and a cell wall altered mutant, we propose two possible models for the surface array of *B. brevis* 47.

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Materials and Methods

Organisms and Media

Bacillus brevis 47 was grown at 37°C with vigorous rotary shaking in synthetic media containing 0.5 or 1.5% phosphate. The synthetic media contained 10 g of glucose, 10 g of ammonium sulfate, 0.1 g of $MgSO_4 \cdot 7\ H_2O$, 0.1 g of $CaCl_2 \cdot 2\ H_2O$, 0.01 g of $MnSO_4 \cdot 6\ H_2O$, 0.01 g of $FeSO_4 \cdot 7\ H_2O$ and 15 g or 5 g of a KH_2PO_4-K_2HPO_4 mixture per liter of distilled water. The ratio of KH_2PO_4 to K_2HPO_4 was maintained constant by weight at 3 to 7. In the case of strain 47-57, 0.01% of uracil was added to the medium.

A virulent bacteriophage, C-7, was isolated from soil using *B. brevis* 47-5 (a uracil auxotroph of *B. brevis* 47) as an indicator. Strain 47-57 was selected from phage C-7 resistant colonies by determining the protein composition of each cell by SDS-polyacrylamide gel electrophoresis as described below. Strain 47-57 required uracil for growth and lacked a protein with an approximate molecular weight of 130000, one of the major cell wall proteins.

B. brevis 47 secreted about 0.3 mg/ml and about 1.1 mg/ml of protein in 0.5% and 1.5% phosphate medium, respectively [9].

Cell growth was monitored turbidimetrically at 660 nm.

Isolation of cell envelopes

Cells grown to the late-logarithmic phase were harvested at 4°C by centrifugation and washed once with 50 mM Tris-HCl buffer (pH 7.5). For a 600 ml culture, the washed cell pellet was suspended in 20 ml of 50 mM Tris-HCl buffer (pH 7.5), mixed with 20 ml of glass beads (0.1–0.11 mm, B. Braun, Melsungen) and disrupted four times for 30 s each in a Braun homogenizer fitted with a cooling device. Crude cell envelopes were centrifuged down (48000 × *g*, 4°C, 30 min) after unbroken cells had been removed by centrifugation (10000 × *g*, 4°C, 5 min). The pellet was washed three times by suspension in 50 mM Tris-HCl buffer (pH 7.5). The washed cell envelopes were suspended in 9 ml of the same buffer. 1.5 ml of the envelope suspension was layered on each of six discontinuous sucrose gradients consisting of 1.5 ml of 70% sucrose, 1 ml of 65% sucrose and 1 ml of 60% sucrose in 50 mM Tris-HCl buffer (pH 7.5), and then centrifuged (140000 × *g*, 4°C, 3 h, Hitachi RPS 40-2 rotor). Three bands were visible, one at the top of the 60% sucrose layer, one at the 60–65% interface and one at the 65–70% interface. The major band at the interface between the 65 and 70% sucrose layers was recovered, diluted with 50 mM Tris-HCl buffer (pH 7.5) and centrifuged at 105000 × *g* for 1 h to remove the sucrose. Finally the envelope fraction was suspended in 50 mM Tris-HCl buffer with or without 10 mM $MgSO_4 \cdot 7\ H_2O$ and stored at 4°C. In some cases the major band was diffused entirely in the 70% sucrose layer. This diffused band, however, had the same protein composition as the major band at the 65–70% interface. Two other lighter bands were not used in this study.

Chemical and enzymic treatment of cell envelopes

(1)Triton X-100 treatment. Cell envelopes in 50 mM Tris-HCl buffer (pH 7.5) were mixed with 1% Triton X-100 in the presence or absence of 10 mM $MgSO_4 \cdot 7\ H_2O$, incubated at 37°C for 30 min with slow shaking, and centrifuged (165000 × *g*, 4°C, 60 min) to separate the soluble and insoluble fractions. The insoluble fraction was washed once with 50 mM Tris-HCl buffer (pH 7.5) containing 10 mM $MgSO_4 \cdot 7\ H_2O$ and suspended in the same buffer to analyze protein, lipid and hexosamine as described below.

(2) DNAase and RNAase treatment. Cell envelopes in 50 mM Tris-HCl buffer (pH 7.5) containing 10 mM $MgSO_4 \cdot 7\ H_2O$ were incubated at 37°C for 30 min with a final concentration of 200 μg/ml of each enzyme, centrifuged at 165000 × *g* for 60 min, and suspended in the same buffer for chemical analysis.

Polyacrylamide gel electrophoresis

Sample proteins were prepared for SDS- and 8 M urea-polyacrylamide gel electrophoresis by heating samples at 100°C for 5 min with 1% SDS containing 1% β-mercaptoethanol, and by incubating cell envelopes at 37°C for 10 min with 8 M urea in 0.05 M Tris-HCl buffer (pH 7.5), followed by centrifugation (10000 × *g*, 10 min, 4°C) to remove insoluble materials, respectively. SDS-polyacrylamide gel (10%) electrophoresis was performed as described by Laemmli [10]. In the case of the urea system, polyacrylamide gels (5%) as

described by Laemmli [10] were used substituting 8 M urea for 0.1% SDS. After electrophoresis, gels with SDS and with urea were stained at room temperature overnight and for 30 min, respectively, in a staining solution consisting of 0.05% Coomassie brilliant blue, 25% isopropylalcohol and 10% trichloroacetic acid, and destained in several changes of 10% acetic acid.

Electron microscopy

Negative staining was performed on 200-mesh copper grids with a Formvar support film. Grids were floated on a droplet of the cell envelope suspension diluted appropriately with 5 mM Mg^{2+} and moved onto another droplet of 1% uranyl acetate, and excess fluid was removed with filter paper. A Hitachi HU 12 A electron microscope, operating at 75 kV, was used. Polystyrene latex particles (0.109 μm, Nissin EM Co. Ltd., Tokyo) were used to calibrate the microscope magnification.

Optical diffraction and filtering

Optical diffraction patterns were recorded on a diffractometer (Nihon Bunko Ltd.) of standard design with Tropel lenses and a Spectra-Physics helium-neon laser as the light source.

Optical filtering was performed as described by Katsura [11].

Analytical procedures

Protein was determined by the method of Lowry et al. [12] with bovine serum albumin as a standard. Hexosamine content of cell envelopes was determined by the Elson-Morgan method [13] using glucosamine as a standard after hydrolysis in sealed tubes with 6 M HCl at 105°C for 24 h. Lipids were extracted as described by Bligh and Dyer [14]. Phosphorus contents in the lipid fraction and in various fractions of cell envelopes were determined as described by Chen et al. [15] after hydrolysis in sealed tubes with 6 M HCl at 105°C for 24 h.

Reagents

DNAase and RNAase were purchased from Sigma Chemical Co. RNA polymerase B from *B. stearothermophilus* was purchased from Seikagaku Kogyo Ltd.

Results

Chemical characterization of cell envelopes

The cell envelopes prepared from cells grown with either 0.5 or 1.5% phosphate contained two major proteins with approximate molecular weights of 150000 and 130000 (Fig. 1, slots 1 and 2). About 20% of the total cellular protein was recovered in the cell envelope fraction after treatment with DNAase and RNAase, followed by Triton X-100 extraction in the presence of Mg^{2+} (Table I). The DNAase and RNAase treatment significantly reduced the phosphorus content of the cell envelopes, indicating that nucleic acids still

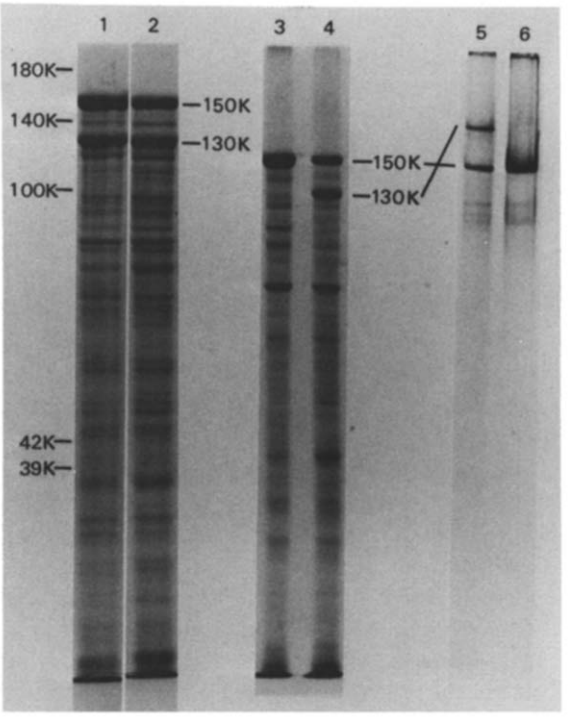

Fig. 1. Composition of cell envelope proteins. Cell envelopes prepared from *B. brevis* 47 grown with 0.5% (slot 1) or 1.5% phosphate (slots 2, 4, 5) and those from a phage-resistant mutant, *B. brevis* 47-57, grown with 1.5% phosphate (slots 3, 6) were analyzed for the protein composition by electrophoresis. Slots 1 to 4 were electrophoresed in the presence of SDS using 20 μg protein and a molecular weight marker, RNA polymerase B. Slots 5 and 6 were electrophoresed in the presence of urea using 10 μg protein. 150 kDa and 130 kDa proteins isolated by preparative SDS-polyacrylamide gel electrophoresis were electrophoresed as bands with a lower and a higher mobility in the urea buffer system, respectively.

TABLE I

EFFECTS OF VARIOUS TREATMENTS ON THE CHEMICAL COMPOSITION OF CELL ENVELOPES

Cell envelopes were prepared from *B. brevis* 47 grown with 0.5 and 1.5% phosphate and treated as described in Materials and Methods. Procedures for chemical analysis are also described under Materials and Methods.

	nmol/mg protein					
	0.5% P_i medium			1.5% P_i medium		
	Cell envelopes	Treatment with		Cell envelopes	Treatment with	
		DNAase + RNAase	Triton X-100 + Mg^{2+}		DNAase + RNAase	Triton X-100 + Mg^{2+}
Protein recovery (%)	24.6	23.6	20.7	24.2	22.7	21.2
Total P_i	416	243	233	264	181	130
P_i in lipid fraction	82	72	59	61	65	28
P_i in nonlipid fraction	303	158	161	184	104	100
Total hexosamine	109	105	110	83	73	71
Hexosamine in nonlipid fraction	105	97	98	72	68	63

remained associated with the cell envelope fraction. Triton X-100 solubilized lipids without affecting the phosphorus content of the nonlipid fraction. Hexosamine, a typical component of the peptidoglycan layer, remained associated with the cell envelopes even after the Triton X-100 extraction. Although there was some difference in the quantities of components of the cell envelopes prepared from cells grown with 0.5 and 1.5% phosphate, the significance of such a difference was not appreciated at the time.

When the composition of protein solubilized with Triton X-100 from the cell envelopes was compared with that of the insoluble cell envelopes, none of the two major proteins was solubilized by Triton X-100 in the presence of Mg^{2+}, whereas they were solubilized to a large extent by Triton X-100 when Mg^{2+} was absent from the solubilization buffer (data not shown). However, sodium dodecyl sulfate (SDS) at 0.25% solubilized nearly all cell envelope proteins. The SDS-insoluble envelopes were composed of the peptidoglycan constituents such as glucosamine, muramic acid, glutamic acid, diaminopimelic acid, alanine, and galactosamine (data not shown).

Electron microscopy

Negatively stained cell envelopes prepared from cells grown in either 0.5 or 1.5% phosphate medium had regular hexagonal arrays over the entire surface with a lattice constant of about 14.5 nm when measured directly on negatively stained images (Fig. 2A and B).

Optical diffraction of these two negatively stained images also demonstrated clearly that the array was of the hexagonal packing type. The diffraction spots extended to some of the fourth order spots, (2, 2) and possibly (3, 1), which showed that the resolution of the micrographs was about 3 nm (Fig. 2A inset and 3B). (See Ref. 16 for the indexing and the orders of the diffraction spots.) Furthermore, the diffractograms indicated that the negatively stained images were composed of two overlapping layers corresponding to both sides of the flattened envelope. When calculated from the diffractograms, the hexagonal lattice constant for the envelopes prepared from either 0.5 or 1.5% phosphate medium was 14.5 nm.

Combined with the protein composition results of the cell envelopes, the fine structure of the regular array of macromolecules seems to be the same regardless of the growth conditions.

Optical filtering

Although a highly enlarged micrograph showed ringlike structures in some part, most of it showed

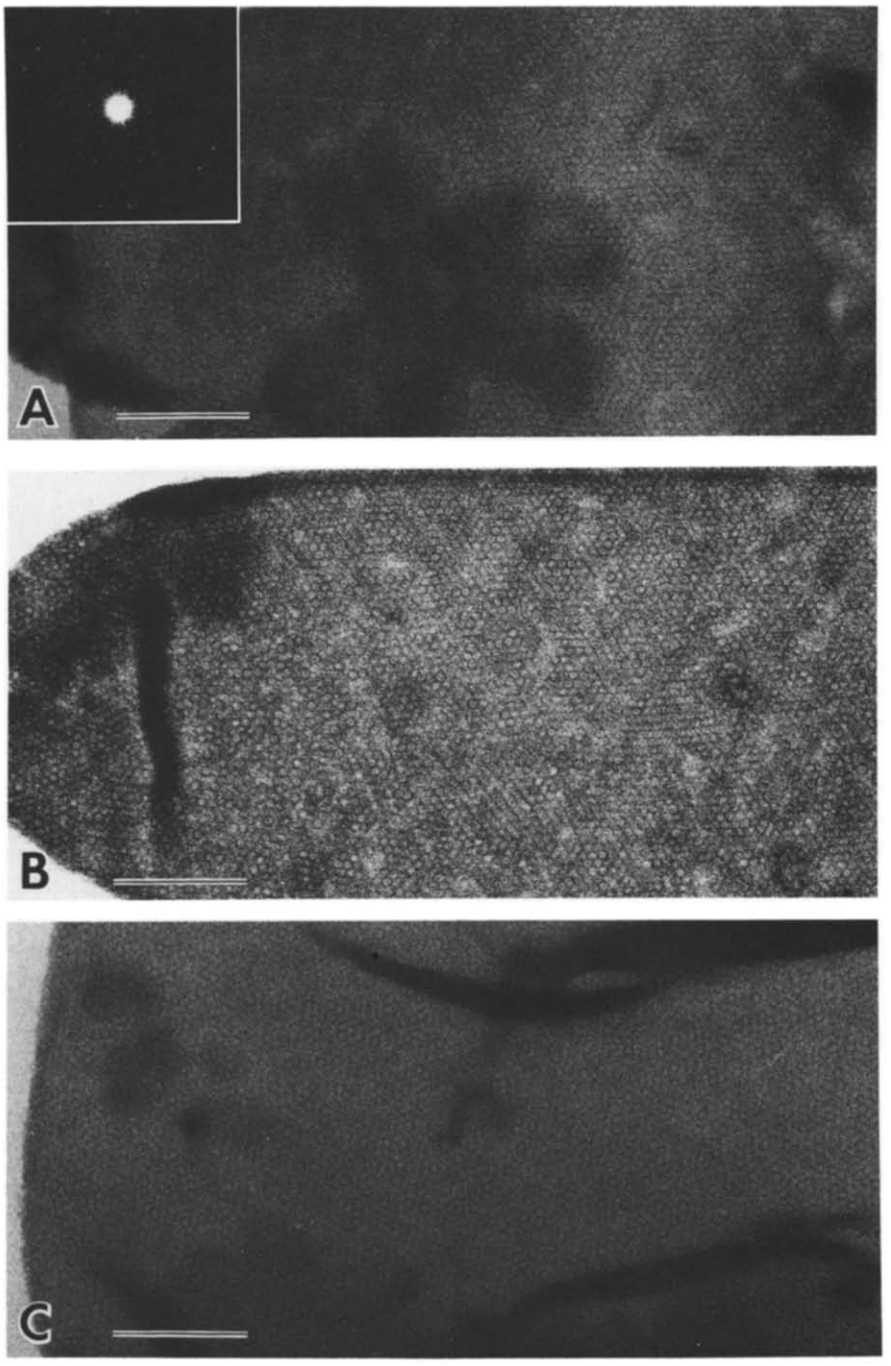

Fig. 2. Negatively stained images of cell envelopes. Cell envelopes were prepared from wild-type cells grown with 0.5% (A) and 1.5% (B) phosphate and from a phage-resistant mutant, strain 47-57, grown with 1.5% (C) phosphate, and examined with an electron microscope. The optical diffractogram corresponding to the negative image of (A) is shown in the inset of (A). Bars represent 200 nm.

only a confused image, since there was superposition of both sides of the flattened envelope. Therefore, a one-sided image was obtained by optical filtering of the original micrograph. Up to the fourth order diffraction spots were used in the filtering to get a resolution of about 3 nm. The one-sided filtered image (Fig. 3A) showed that the envelope consisted of a regular hexagonal array of rings made of six structural units. The hexamer rings in this figure do not necessarily represent the whole protein molecules but the stain-excluding region where the protein is thicker than the other part. That is, part of the protein molecules may be present also outside the rings.

Optical diffraction and filtering of envelopes prepared from a phage resistant mutant, strain 47-57

The cell envelopes of the mutant were composed mainly of the 150 kDa protein and appeared to be free of the 130 kDa protein (Fig. 1). Negatively stained cell envelopes showed the same hexagonal arrays as those of wild-type cell envelopes (Fig. 2C). Although the reciprocal lattice structure of the mutant and wild-type cell envelopes was the same based on the optical diffractograms shown in Fig. 3B and C, the relative intensity of the diffraction spots of the mutant cell envelopes differed from that of wild-type cell envelopes. The absence of the third and fourth order spots in the mutant diffractogram is probably due to the structure factor, i.e. the distribution of mass in the unit cell, as well as to a small disorder in the mutant cell envelopes. The optically filtered image of the mutant cell envelopes (Fig. 3D) revealed the difference between the wild-type and mutant cell envelopes much more

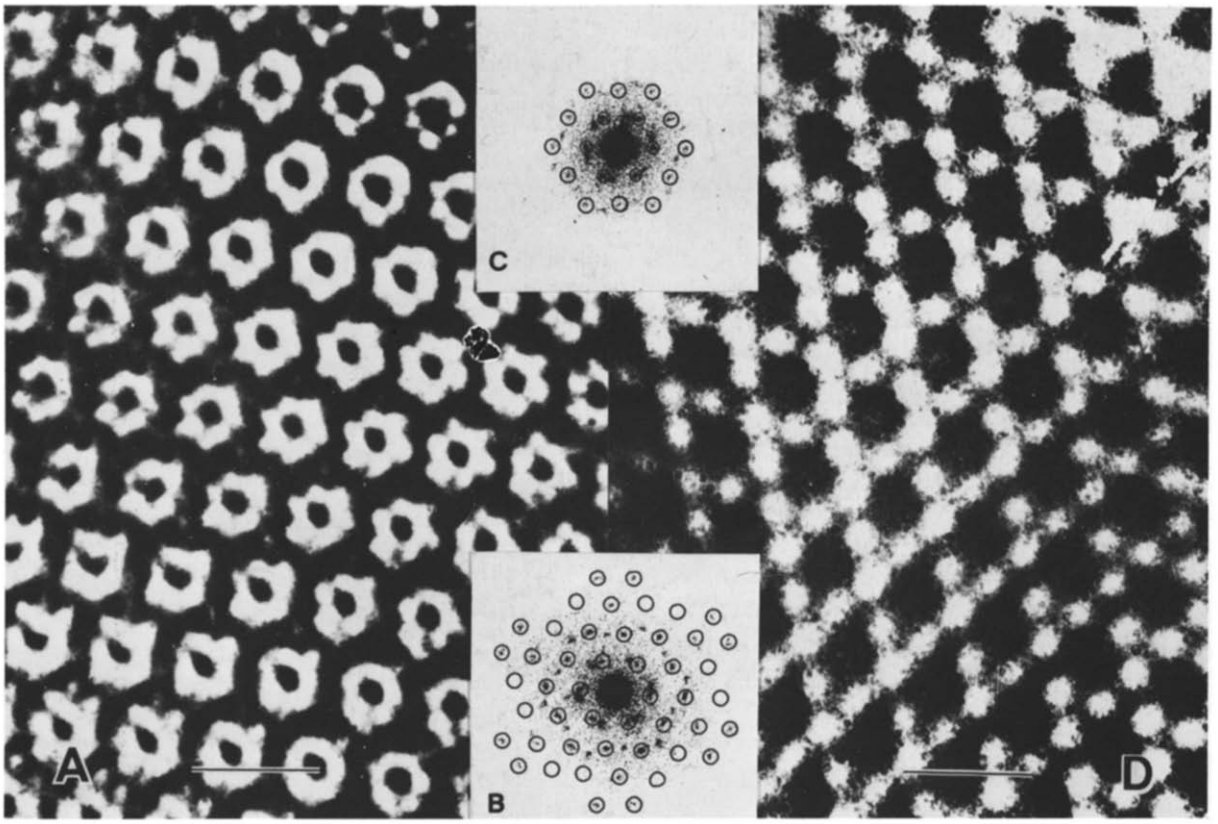

Fig. 3. Optical diffractograms and one-sided filtered images of the wild type and mutant cell envelopes. The optical diffractograms, B and C, were from the micrographs shown in Fig. 2 B and C, respectively. Circles in the optical diffractograms show the diffraction spots used to obtain the one-sided filtered images (A and D). A and B: The cell envelopes of *B. brevis*47 grown with 1.5% phosphate. C and D: The cell envelopes of a phage-resistant mutant grown with 1.5% phosphate. Bars represent 20 nm.

clearly than the diffractograms alone. The hexamer rings in the wild-type cell envelopes were replaced by trimers in the mutant cell envelopes, and accordingly the number of structural units in the unit cell was reduced from six to three.

Discussion

B. brevis 47 secretes very large amounts of protein into the medium and is quite different in cell wall structure from most Gram-positive bacteria where the cell wall appears to be a 15 to 80 nm thick, fairly homogenous, electron dense layer [8]. *B. brevis* 47 has a characteristic three layered cell wall, which resembles in some respects the multilayered cell wall structure observed in thin sections of vegetative cells of *Bacillus* species C.I.P. 76-111 and on partially plasmolyzed or autolyzed cells of *B. polymyxa* and *C. nigrificans* [6,8,17,18]. As part of our work to characterize the roles of the cell surface structure in protein secretion, we prepared and examined the cell envelopes from cells grown under protein- and nonprotein-secreting conditions. Biochemical analyses (Fig. 1 and Table I) as well as physical analyses (Figs. 2 and 3) showed essentially the same chemical and structural properties for the cell envelopes regardless of the growth conditions.

The cell envelopes of *B. brevis* 47 contained two major proteins with approximate molecular weights of 150000 and 130000 (Fig. 1). These major proteins were resistant to solubilization by Triton X-100 when Mg^{2+} was present. This is similar to the resistance of outer membrane proteins in *E. coli* to solubilization by Triton X-100 [19]. These results together with those of previous morphological studies [8,9] indicate that these two major proteins should be components of the cell wall.

Close examination of the cell envelopes with an electron microscope and an optical diffractometer revealed a hexagonal lattice structure on the surface regardless of the growth conditions (Figs. 2 A and B). This was further confirmed by the technique of optical filtering (Fig. 3A). *B. brevis* 47 has hexagonal arrays of hexamer rings with a lattice constant of 14.5 nm over the entire cell surface (Figs. 2 and 3A). A similar hexagonal lattice structure has been shown in three *Bacillus* species; *B. alvei* strain 183, *B. anthracis*, and *Bacillus* species C.I.P. 76-111 with lattice constants of 10–12, 8, and 11 nm, respectively [4–6].

All subunits composing regular arrays seem to be proteinaceous in nature although isolation and chemical characterization of the regular array were carried out in only a few species such as the T layer of *B. brevis* P-1 (identified later as *B. sphaericus*) [20]. The cell envelopes of a mutant of *B. brevis* 47, strain 47-57, which was isolated from phage resistant colonies, were composed mainly of the 150 kDa protein (Fig. 1) and had hexagonal lattice arrays similar to those of the wild-type cell envelopes on the surface when examined under an electron microscope (Fig. 2C). The reciprocal lattice structure including the lattice constant (14.5 nm) was the same in the mutant and wild-type cells. However, the distribution of mass in the unit cell of the mutant cells differed considerably from that of the wild type cells. The optically filtered image of the mutant (Fig. 3D) revealed a unit cell consisting of three structural units instead of six. This is an interesting example in which the 3-fold

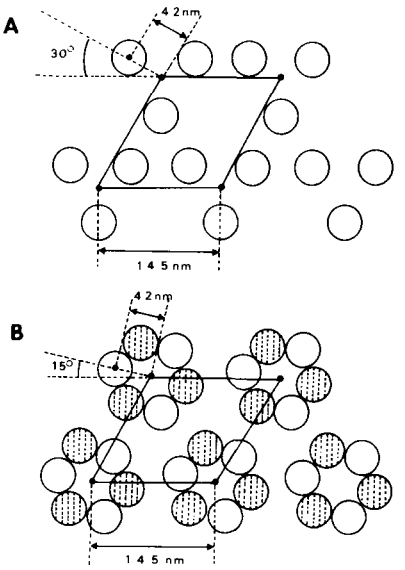

Fig. 4. Models of the arrangement of protein molecules in the wild-type and mutant cell envelopes. (A) The mutant cell envelopes. (B) A model of the wild-type cell envelopes. The hexagonal unit cell is depicted by parallelograms with solid lines. ○, 150 kDa protein, ◎: 130 kDa protein. Note that the stain-excluding regions in the filtered images may represent only relatively thick parts of the proteins and not the whole protein molecules.

symmetry of the unit cell gives the 6-fold symmetry of the diffraction pattern according to Friedel's law.

The way of interpreting the filtered images in terms of the protein components was not unique, but the simplest way would be as follows (Fig. 4). The mutant cell envelopes consist of a *p3* lattice [21] made of trimers of the 150 kDa protein at the lattice points. The distance from the center of the trimer to the center of the structural unit (= the thickest part of the 150 kDa protein) is about 4.2 nm, and it makes an angle of about 30° from the lattice line. One can obtain a hexamer ring by inserting the 130 kDa protein molecules at places located between each pair of the three structural units of the trimer in the two-dimensional projection of the cell envelopes. This can be done either by inserting the 130 kDa protein molecules between the 150 kDa protein molecules in the same plane or by putting a layer of the 130 kDa protein molecules on the layer of the 150 kDa protein molecules. However, rotation of the hexamer by about 15° may be necessary to adjust the structural units to their position in the wild-type cell envelopes. Thus, according to this interpretation, both the wild-type and the mutant cell envelopes have only the *p3* symmetry (having only a 3-fold rotational axis at the lattice point), although the former may appear to have the *p6* symmetry (having a 6-fold rotational axis at the lattice point) at first glance.

The following alternative interpretation is also feasible. The 'hexamer' of the 130 kDa protein is inserted into the cavities on the layer of the 150 kDa protein as shown in Fig. 5. In this case the trimers of the 150 kDa protein cannot be seen in the filtered image of the wild-type cell envelopes, because the 130 kDa protein molecules are protruding to a much larger extent than the 150 kDa protein molecules. According to this interpretation, the 'hexamer' should be made of three (rather than six) polypeptide chains of the 130 kDa protein, or the 'trimer' should be made of six polypeptide chains of the 150 kDa protein, since we could assume based on the electrophoretogram shown in Fig. 1 that there are roughly equal numbers of the 130 kDa and 150 kDa protein molecules in the cell envelopes.

Both the models described above are consistent with our previous results [22] obtained by reconstituting the cell wall from the 130 kDa and 150 kDa proteins and peptidoglycan. The two outer protein layers, outer and middle wall layers, of the cell wall were composed of the 130 kDa and 150 kDa protein, respectively. Furthermore, a mixture of the 130 kDa and 150 kDa proteins, when dialyzed against an Mg^{2+} containing buffer, led to the formation of cylindrical structure composed of two layers, indicating that there are some specific interactions between these proteins. Currently we are trying to reconstitute a regular structure from the 130 kDa protein to confirm our models for the surface structure.

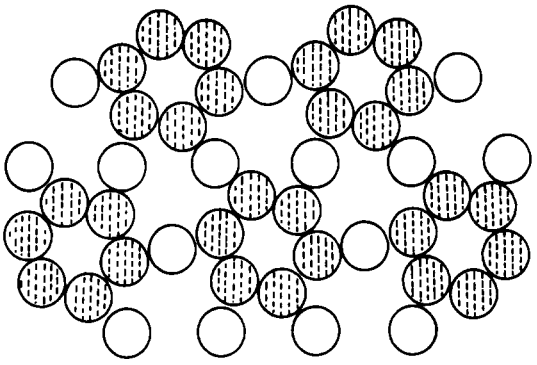

Fig. 5. An alternative model of the arrangement of protein molecules in the wild-type cell envelopes. ○, 150 kDa protein, ⊜, 130 kDa protein. Note that the 150 kDa protein molecules may not be seen in the filtered image of the wild-type cell envelopes, since the 130 kDa protein molecules are protruding to a much greater extent than the 150 kDa protein molecules.

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