Ultrastructure of the Cell Wall of *Bacillus polymyxa*

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The macromolecular arrangement on the surface of *Bacillus polymyxa* was revealed by metal shadowing of whole cells and wall fragments; it consisted of a rectangular array of 70-A globules with a repeating interval of 100 A. The substructure was studied in plan with phosphotungstic acid (pH 6) or uranyl acetate as negative stains of fragments and was studied also in profile with sections of embedded material. Staining of sections of cells fixed with glutaraldehyde showed that layering (approx. 80-A dense, 40-A light, and 120-A dense layers, outermost layer first) could be demonstrated in the cell wall with lead or uranyl acetate, used together or separately. The outer "dense" layer corresponded to the regularly arrayed structure (RS); it was removed by guanidine hydrochloride, sodium lauryl sulfate, cold formamide, and by trypsin. The RS layer (isolated by a hydrogen bond breaking reagent, guanidine hydrochloride) was disrupted by agents such as sodium lauryl sulfate or damaged by 3 M sodium chloride. Qualitative chemical tests, ultraviolet absorption, and removal by trypsin indicated that the structured layer consisted mainly of protein, but exact characterization was not attempted. The globular units making up the layer consisted of a small number of subunits, imperfectly resolved by negative staining. The underlying polysaccharide appeared to be covalently bound to the deepest (probably mucopeptide) layer since it required "hot" formamide for its removal. A survey of species was not made.

The cell walls of gram-positive organisms have been considered both homogeneous and relatively thick in comparison with the cell walls of gram-negative bacteria. Certainly, the profile view of gram-positive cell walls in sections has only shown occasional layering, e.g., in *Micrococcus radiodurans* (39). The surface view has shown little evidence of ordered arrangements of macromolecules, with the exception again of *M. radiodurans* which has a surface resembling in some aspects that shown by some gram-negative organisms (23), *Bacillus polymyxa* (1), and *Clostridium tetani* and *C. botulinum* (37). It was hoped, but not realized, that some orderly structure would be apparent in those cell walls consisting mainly of mucopeptide, as had been suggested by Baddiley (1) for high-quality cell wall preparations of *B. polymyxa*. We now know (Baddiley, *personal communications*, and confirmed by our own observations reported herein) that this latter example is a protein-containing structure.

The magnificently regular rectangular array displayed by *B. polymyxa*, which is unusual in close-packing structures, impelled us to try to resolve the ultrastructure and to determine its

location in the cell wall. Some information about the types of linkages holding the structural units together and to the cell wall was obtained. We wished to relate our observations with those of Baddiley, which are yet to be published, and we used a combination of electron microscopy and chemical dissection similar to that in previous investigations (25, 27).

MATERIALS AND METHODS

Strain and cultivation. The strain of *B. polymyxa* used by Baddiley (received from him in 1964 as no. 4747) was used throughout the experiments. It was cultivated on yeast-glucose-agar [0.5% yeast extract (Difco), 2.0% glucose, and 1.5% agar] at 20 or 30 C, or in yeast-glucose broth at 20 C on a reciprocating shaker. The organism lost its gram-positive character as the culture aged, and, on yeast-glucose plates, it sporulated after 7 days at room temperature. A copious and glutinous slime was produced, especially when grown on plates at lower temperatures. The cultures for some experiments were grown overnight in broth on a shaker.

Preparation of cells for electron microscopy. Cells were harvested from broth or washed off plates. The cells were suspended and were washed with 0.85% sodium chloride (referred to as "saline") to remove the slime layer and then were washed twice in distilled water before suspension at a suitable density for deposition on grids for electron microscopy. We also

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tried a 30-min wash with chloroform-methanol (2:1) for the same purpose as suggested by Baddiley (*personal communication*), followed by washes in distilled water. However, saline was more effective in our experience.

Preparation of cell walls. The harvested, washed cells were suspended in 0.001 M phosphate buffer (pH 6.7) in an ice bath and were treated in an ultrasonic generator (MSE, 20 kc, with an operating output of 1.7 amp) for 1 to 2 min. The fragmented cells were immediately washed with cold 0.03 M phosphate buffer (pH 6.7) or in distilled water. The cell-wall fragments were obtained by differential centrifugation, the requirements for which changed according to the degree of disintegration. In a few instances, the cell walls were separated from ribosomes and other cytoplasmic portions of the cell by differential centrifugation in a Ficoll density gradient (Pharmacia Fine Chemicals, Uppsala, Sweden) from 2 to 20% by use of a Spinco L-2 ultracentrifuge at 40,000 $\times g$.

Shadowing and staining. Cell-wall fractions were shadowed lightly (3:1 slope) with tungsten oxide in an Edward's Evaporator (model 13E6). Any excess deposition of metal decreased the visibility of the structure. Negative staining was successful only when 1% uranyl acetate (pH 4 to 5) or 1% phosphotungstic acid at a pH below 6.0 was used. The best results were obtained with uranyl acetate. A drop containing the material was put on the grid, and the excess was sucked off with a capillary after 2 min, immediately after which, and before drying, the negative stain was applied as a droplet; excess was removed with a filter paper after 20 sec, and the grid was allowed to dry. Deposition of an enveloping carbon layer (to stabilize the preparation and minimize charge effects) was often added.

Sections. Cells were fixed according to the procedure of Ryter and Kellenberger (15, 32), hereafter called R-F fixation, or with 3% glutaraldehyde (Eastman Kodak, Co., Rochester, N.Y.) in 0.05 M phosphate buffer (pH 7.2) for 3 hr or more at room temperature (6). After glutaraldehyde fixation, the cells were washed once with distilled water or with the same buffer before being coated with 2% Special Agar (Noble) from Difco. Small cubes of agar were then washed several times with distilled water or phosphate buffer and, after dehydration through an acetone series, were embedded in Vestopal W. An overnight washing with phosphate buffer was also used. The Vestopal was polymerized at 60 C for 3 days; the blocks were then trimmed and the sections were cut with a Porter-Blum ultramicrotome.

The glutaraldehyde-fixed sections were poststained by: (i) 1 or 4% osmium tetroxide in the R-K buffer for 60 and 30 min, respectively; (ii) 1% uranyl acetate, pH 4.5, for 50 to 60 min; (iii) 1.0 or 0.5% potassium permanganate for 30 min; (iv) 2% phosphotungstic acid (pH 7.2 with KOH) for 20 min; (v) 1% phosphomolybdic acid (pH 7.2) for 30 min; (vi) lead citrate, according to Reynolds (30), diluted 1:2, for 10 min; and (vii) combinations of uranyl acetate, osmium, and lead as indicated in the text and in the legends.

Chemical methods. The effect of enzymes and of hydrogen-bond breaking agents were observed after floating the grids with cell wall preparations face down

on the surface of the given solution in a glass depression plate, followed by washing with distilled water. The use of a heating block enabled treatment of grids at 35 C for enzyme action. Formamide extraction was performed after the method described (7, 25); "cold" formamide refers to extraction at 20 C, and "hot" formamide, to extraction at 200 C.

Proteins were determined by the Lowry method (21); sugars were determined by use of the anthrone test (35), and limited information on the nucleic acid or protein content was achieved by obtaining an ultraviolet absorption curve from 230 to 290 m μ (Beckman DU spectrophotometer). Phosphorus was determined by the method of King (16).

The dry weight estimations were obtained by pipetting known volumes onto small aluminum taring dishes and drying for 6 hr at 105 C.

The enzymes used were lysozyme (three times crystallized), trypsin (crystallized, salt free), pepsin (3x crystallized), and lipase (no. 448); all were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio.

Microscopy. Preliminary electron microscopy was undertaken with a Philips EM-100 (modified by the addition of small-bore pole pieces and a Ladd anode, by mechanical astigmatism correction, and by additional stabilization of the input); other micrographs for comparison and for special measurement were taken with the Philips EM-200; the initial magnification on the film was usually 15,000 to 20,000.

Measurements were made either directly on the print or, for more precise and comparative purposes, directly on the film with a projection comparator (Profile Projector; Nippon Kogaku K. K., Tokyo, Japan).

RESULTS

Observations on intact cells. Initial observations of shadowed preparations made from all the cultures grown on agar plates gave clear indication that the outer surface of the cell wall consisted of a regular, rectangular array of globular elements (Fig. 1). Some difficulty in the revelation of structure on cells from cultures less than 24-hr-old was largely solved by washing the cells, whatever the growth medium, in one or more changes of saline; the structure became apparent in cells throughout the growth cycle, including in those from the logarithmic phase. Although the structure was found in all specimens examined, we have no quantitative data about the possession of this superficial structure in the population of cells; it is probable that it occurs on all cells.

The basic observations were made on shaken cultures in 200 ml of yeast-glucose broth (30 C for 18 hr); samples were removed at hourly intervals. The optical density changes (Bausch & Lomb Spectronic 20 at 520 m μ) showed that the lag phase lasted for up to 3 hr, the log phase, for 3 to 12 hr, and the stationary phase occurred 12 hr after growth.

FIG. 1. *Bacillus polymyxa* from 7-day-old culture, washed with saline and distilled water before tungsten oxide shadowing (3:1). Note the transverse orientation of structure. Marker represents 0.1 μ .

FIG. 2. Cell from 5-hr old culture, mostly empty, showing longitudinal orientation of structure. Tungsten oxide shadowed. Marker represents 0.1 μ .

The profile of the cell wall as observed in sections of embeddings of cells grown for 5, 9, and 18 hr in the above experiments did not show any significant differences. It was observed regularly that there were two moderately dense layers separated by a light interspace (cf., Fig. 10 and later text). These early observations indicated not only that there was a regular structure present as an outer component of the cell wall, but also that the profile of the cell wall was more complex than we had expected. We made further attempts to resolve the structure and identify the layers in the profile. Fortunately, the whole cell wall, including the outer structured layer, seemed to survive cell rupture, suspension in buffers (see the data on effect of buffers in Table 1), and the differential centrifugation required for clean preparations; this stability facilitated additional observations.

Structure of the outer layer. A carefully cleaned preparation of the cell walls of early stationary-phase organisms was lightly shadowed or negatively stained with uranyl acetate (Fig. 2-5); both preparations showed a regular structure (RS) in the outer surface. The globules forming the surface were approximately 70 Å in all dimensions, with an average interspace of 30 Å. These measurements were for both the shadowed and the uranyl acetate stained specimens. Thus, center-to-center spacing was essentially 100 Å. On some cells, the globules were in transverse rows (Fig. 1); on others, they appeared to be in longitudinal rows (Fig. 2 and 3). The latter type of orientation was the more frequent, and spacing was measured mostly on cells with such orientation.

It was not clear from high-resolution microscopy of negatively stained preparations whether or not the globules themselves were square, as might have been assumed from our earlier preparations. They seemed to contain a small number, two to five, of subunits arranged in a ring (Fig. 4 and 5). However, the number observable within a given unit was uncertain; when the number was small, the subunits gave the appearance of being rodlets. Disturbances of the "rings" were observed which could have been caused by forces in the preparation and negative staining or by electron beam bombardment. It is probable that further detail can be resolved with careful microscopy. A more detailed physical study of the RS will be presented elsewhere (Nermut and Murray, *unpublished data*).

We tested the sensitivity of this (RS) layer to various enzymes and chemicals, especially to those that are known to be capable of breaking hydrogen or electrovalent bonds. The general results of these experiments are shown in Table 1;

some results are documented with electron micrographs (Fig. 6-8).

Guanidine hydrochloride was used because it was known to attack the hydrogen bonds of proteins when used in 3 M concentration (38). The RS layer of *B. polymyxa* was readily removed by the action of 2 or 3 M guanidine hydrochloride in 10 min. Lower concentrations (less than 0.1 M) were not effective in this amount of time, but shaking for 3 to 5 hr in 0.01 M guanidine hydrochloride removed a large proportion of the RS layer in the form of sheets without any change in the regularity of the arrangement of globules (Fig. 4). These "sheets" served as starting material for other experiments (Table 1). It is likely that the low concentration of guanidine hydrochloride was breaking bonds between the RS layer and the underlying part of the cell wall, which we later deduced to be polysaccharide in nature. This reagent did not affect the globules as such, which were dispersed by higher concentrations (5 M). A similar effect was observed when lipase was used and the globules were liberated; however, no further work was done with this system because it was difficult to know what substrates might be involved.

The sensitivity of the RS layer to trypsin, pepsin, sodium hydroxide, and trichloroacetic acid testified to the presence of proteins. This was supported by the very destructive effect of sodium lauryl sulfate, which is known to attack the protein moiety of the cellular lipoproteins (2, 11, 29). A treatment of isolated RS layer for the minimal possible time (about 1 sec) with 0.1% sodium lauryl sulfate abolished the regularity of surface structure, and a treatment of 60 sec disintegrated the sheets into small irregular pieces.

The action of sodium chloride was quite different. In repeated experiments involving cell wall preparations on grids, the RS layer was damaged by any treatment with 3 M NaCl. The intact cell was much less sensitive because even 3 M NaCl did not remove the RS layer, but, to judge from the sections of whole cells thusly treated, the actual structure was damaged or denatured so that only a very irregular agglomeration of material was attached to the surface (Fig. 13). Therefore, it appeared that 3 M sodium chloride attacked the structural units themselves and not the linkages between the RS layer and the underlying layer.

Hydroxylamine (e.g., 0.1% for 1 min) produced great changes, but the nature of the effect on the RS layer was not clear. RS globules were replaced by much larger, irregular aggregates more than 200 Å in size (Fig. 7). It was assumed that a rearrangement of the macromolecular

FIG. 3. Fragment of a cell wall from 5-day-old culture. Tungsten oxide shadowed. Marker represents 0.1μ .
FIG. 4. Part of the RS layer removed from the cells by shaking them for 5 hr with 0.01% guanidine hydrochloride. Tungsten oxide shadowed. Marker represents 0.1μ .

FIG. 5. Cell wall stained negatively with 1% uranyl acetate. Marker represents 0.1 μ .

complex must have taken place. Ether had a curious effect on isolated sheets of the RS layer, which was damaged by exposure of the shortest possible duration; the sheets did not disintegrate, but the globules were distorted.

The choices of time, pH, and the concentration of the phosphate buffer must have played an

important role, because removal of the globules by 1 M NaCl was more effective at pH 8 than at pH 6 (Fig. 8). Sometimes only the globules were removed, leaving behind a much thinner support layer with slight regularities of structure. These rather crude experiments made it clear that the structured layer was removable, was itself com-

TABLE 1. *Effect of enzymes, various solutions, and solvents on the RS layer*

Agent ^a	Concn	Duration of treatment	Sensitivity of RS layer ^b
<i>Enzymes</i>			
Trypsin, 0.06 M phosphate buffer (pH 8.0), at 36 C	0.1%	60 min	—
Pepsin, glycine-HCl buffer (pH 2.2), at 36 C	0.5%	60 min	—
Lipase, 0.06 M phosphate buffer (pH 7.2), at 36 C	0.5%	60 min	±
Lysozyme, 0.1 M Tris buffer (pH 7.0)	0.02%	30 and 60 min	+
<i>Buffer solutions</i>			
Phosphate buffer (pH 6.0), at 20 and 36 C	0.06 M	60 min	+
Phosphate buffer (pH 7.0), at 20 and 36 C	0.06 M	60 min	+D
Phosphate buffer (pH 8.0), at 20 and 36 C	0.06 M	60 min	+D
Tris buffer (pH 7.0), at 20 and 36 C	0.1 M	60 min	+D
Glycine-HCl buffer (pH 2.2), at 20 and 36 C	0.1 M	60 min	+D
<i>Various solutions</i>			
NaCl, pH 6.8	0.17 M	60 min	+
NaCl, 0.06 M phosphate buffer (pH 8.0)	0.2 M	20 min	+D
NaCl	3 M	10 min	— or +D
NaOH	0.01 N	20 min	—
Trichloroacetic acid at 4 C	5%	30 min	— or +D
EDTA ^c , pH 7.0	2%	20 min	+
Distilled water	—	60 min and 6 weeks, respectively	+
<i>Organic solvents</i>			
Acetone	90%	1 min	+
Alcohol-acetone	4:1	1 min	±
Chloroform-ethyl alcohol	2:1	20 and 60 min, respectively	+
Ether (effect on sheets)	—	2 sec	—
<i>Hydrogen (hydrophobic) bond breakers</i>			
SLS ^d (effect on whole cells)	0.05%	2 hr	+D
SLS (effect on whole cells)	0.25%	3 hr	—
SLS (effect on whole cells)	1.0%	5 min	—
SLS (effect on cell walls dried on grids)	1.0%	1, 5, 10, 30 min	—
SLS (effect on isolated RS layer)	0.1%	10 and 30 sec	No sheets, no RS
SLS (effect on isolated RS layer)	0.1%	60 sec	Disintegration
Guanidine hydrochloride	0.01–1.0%	20 min	Removed in sheets
Guanidine hydrochloride	3 M	5 min	—
Hydroxylamine (effect on cell walls)	0.5, 1.0, 2.0%	1, 5, 10, 30 min	+D, changed
Hydroxylamine (effect on sheets of regular structure)	0.1%	1, 2, 10 sec	Sheets disintegrated
Urea	6 M	30 min	—
Formamide, at 20 C	100%	5 min	—

^a Used at room temperature, unless stated otherwise.^b Symbols: +, present; —, absent; and +D, present but partially damaged.^c EDTA = ethylenediaminetetraacetic acid.^d SLS = sodium laurylsulfate.

plex (Fig. 9), and was only a portion of the total cell wall. Furthermore, it was probable that the structure consisted of protein, in whole or in large part. This was later supported by ultraviolet absorption and protein determinations after collection of the particles from a sodium lauryl sulfate or "cold" formamide extraction of the whole cells or purified cell walls. This opinion on the chemical nature of the surface structure substantiates the view of Baddiley

(*personal communication*, 1966). The layers appeared to be held together by hydrogen bonds, especially the linkage of RS to the substrate layer; other linkages may be involved in the adherence of globules to each other (Nermut and Murray, *unpublished data*).

Detailed study of the cell wall profile. The thickness and the character of the RS layer was established by comparison of the profile of normal cell walls with those after treatment with agents

FIG. 6. Cell walls treated with 1% sodium lauryl sulfate for 30 min. Tungsten oxide shadowed. Marker represents 0.1 μ .

FIG. 7. Cell wall after treatment with 2% hydroxylamine for 30 min. Tungsten oxide shadowed. Marker represents 0.1 μ .

FIG. 8. Cell wall after treatment with 1 M NaCl in 0.1 M phosphate buffer (pH 8.0) for 20 min. Tungsten oxide shadowed. Shows partial removal of RS. Marker represents 0.1 μ .

FIG. 9. RS layer stripped off the cells by shaking with 0.01% guanidine hydrochloride for 5 hr and stained negatively by 1% uranyl acetate. The preparation was carbon-coated before examination. This piece is convoluted and thus exposes the inner side of the layer (arrow), which is not structured and may be the support for the globules. Marker represents 0.1 μ .

FIG. 10 and 10a. Cells from an overnight, yeast-glucose broth shake culture, sectioned after R-K fixation and after embedding in Vestopal. Uranyl acetate staining. Note (arrow) the darker inner part of the cell wall, and the underlying plasma membrane. Marker represents 0.1 μ .

FIG. 11. Section of *Bacillus polymyxa* shaken for 5 hr in 0.067 M phosphate buffer (pH 8.0) at room temperature. Note the very clear-cut structure of the cell wall and the thin "layer" (track) adhering to the cell wall and separated from the cytoplasm. Preparation as in Fig. 10. The cell has been damaged by the buffer. Attention should be focused on the cell wall structure; poor preservation of other elements of cell structure must be tolerated.

that removed the RS layer. Cells from overnight cultures in yeast-glucose broth were washed in saline and distilled water to remove the obscuring slime layer and were then treated separately with: (i) 0.25% sodium lauryl sulfate for 5 hr (on a shaker) or 1% sodium lauryl sulfate for 5 min; (ii) formamide at room temperature for 5 min (referred to as "cold" formamide); (iii) 3 M NaCl for 10 min; and (iv) 0.066 M phosphate buffer (pH 8.0) on a shaker for 5 hr (Table 1).

Sodium lauryl sulfate and formamide caused under these conditions the removal of the superficial dense layer shown in the profile of normal cells (Fig. 10 and 10a and Table 2). As a result, the cells were surrounded by one single dense layer of about 100- to 120-A thickness (Fig. 12 and 13). Both ultraviolet absorption and application of the Lowry method showed that protein was removed from the cells and that it appeared in the supernatant fluids in both cases. Treatment with 3 M NaCl damaged the RS layer (Fig. 14), but did not cause its removal.

Shaking (up to 5 hr) with phosphate buffer (pH 8.0) did not remove the RS layer from intact cells, as might have been expected on the basis of our results with isolated cell walls. The sections of cells so treated (Fig. 11) showed a clear-cut and contrasting dense layer immediately inside what we had considered to be the mucopeptide layer of the cell wall, which was otherwise of typical profile. In this case, the cytoplasm had separated from the cell wall and from the dense track noted above. Under the usual conditions of sectioning, one would have expected this component to be a part of the plasma membrane. However, the exact anatomical affiliation and nature of this line remained obscure.

An attempt was made to gain general information about the remaining cell wall layer after treatment with sodium lauryl sulfate or cold formamide. Hot formamide was known to extract the polysaccharides of streptococci (7, 18) and teichoic acid from *B. megaterium* (10). Cells were first extracted with formamide at room temperature for 5 min to remove the superficial RS layer, and then they were boiled in formamide

TABLE 2. Thickness of the layers of *Bacillus polymyxa* cell wall from sectioned material, fixed and embedded by R-K procedure

Treatment of cells	Layers ^a				Total thickness	No. of measurements
	a	b	c	d		
Intact cells from 5-, 9-, and 18-hr cultures	80	65	105	—	250	80
Cells extracted with sodium lauryl sulfate	—	—	110	—	110	20
Cells treated with formamide at room temperature	—	—	100	—	100	17
Cells treated with formamide at 205 C	—	—	—	60	60	21

^a Abbreviations: a = outer dark layer; b = light intermediate layer; c = inner dark layer; and d = inner layer after hot formamide.

for 3 min. The remaining insoluble material was centrifuged at $15,000 \times g$ for 25 min (for better results at $30,000 \times g$ for 10 min), and washed once with distilled water before resuspension and placing on grids or before embedding the pellet in Vestopal (according to the usual R-K procedure). The shadowed preparations revealed thin membranes of the same general shape as the cells and a smooth surface (Fig. 15). No structure was found even with negative staining and high primary magnification in the electron microscope. Sections showed a single, thin, cell-shaped sac resembling a wall component and having an average thickness of 60 A (Fig. 16). This was about 50% of the thickness of the dense layer that remained after extraction with sodium lauryl sulfate or formamide at room temperature. The weight of cell wall material removed by hot formamide was estimated by determining the dry weight of a sample of isolated and purified cell walls treated with "cold" formamide and washed twice, and determining the dry weight of an identically treated sample of cell walls further

FIG. 12-16. Figures represent preparations of cells or walls submitted to the damaging effects of different agents. Poor preservation of other elements of cell structure must be tolerated; attention should be focused on the cell wall structure.

FIG. 12. Cell wall profile obtained after shaking the culture with 0.25% sodium lauryl sulfate for 5 hr. Preparation as in Fig. 10 (R-K). Note that there is one layer instead of two, as shown in Fig. 10 and 11.

FIG. 13. Cells treated with formamide at room temperature for 5 min. Preparation as in Fig. 10 (R-K).

FIG. 14. Section through a cell treated 10 min with 3 M NaCl. Preparation as in Fig. 10 (R-K). The outer layer shows swelling and distortion. Same magnification as in Fig. 12 and 13; marker represents 0.1 μ .

FIG. 15. The rigid membrane remaining after boiling the cells in formamide for 3 min. Tungsten oxide shadowed.

FIG. 16. Section through the membranes obtained as in Fig. 15. Postfixed and embedded as in Fig. 10. Marker represents 0.1 μ .

extracted with hot formamide. In another type of experiment, we precipitated (7) both proteins (with acid alcohol) and carbohydrates (with acetone) from the hot formamide extract, and we established the dry weight and the presence of proteins, carbohydrates, and phosphorus. The dry weight of the extracted material amounted to 52% of the cell wall deprived of RS layer. Tests for proteins and phosphorus gave negative results; carbohydrates were always present. "Cold" formamide removed protein and only a very small amount of polysaccharide from whole cells. These results emphasized the complexity of a gram-positive cell wall and also indicated covalent bonding of the polysaccharide component to the underlying layer, which was assumed to be mucopeptide. The latter was not sensitive to lysozyme. This was strikingly different from the findings of Krause and McCarty (18), Perkins (28), and Nermut (25) with a variety of gram-positive bacteria. No lysis could be observed, either in tubes (followed by measurement of turbidity) or on grids (followed by electron microscopy). The concentration of lysozyme was 200 to 2,000 $\mu\text{g/ml}$ in tris(hydroxymethyl)-aminomethane (Tris) buffer (pH 7.0), with or without calcium or magnesium addition; the exposure time was up to 12 hr.

Revelation of wall layers by staining. The staining experiments were undertaken on sections of cells that had been fixed with glutaraldehyde alone. These sections were poststained with uranyl acetate, osmium tetroxide, potassium permanganate, lead, or various combinations of these which have been mentioned in Materials and Methods.

Uranyl acetate staining revealed a cell-wall profile that resembled that of cells fixed by the standard R-K method in which uranyl acetate was used. Two dense layers were observed and were usually separated by a space of lower density and variable thickness (Fig. 17). The whole cell wall was about 250-A thick; the inner dense layer measured about 120 A, the superficial layer, about 80 A, and the interspace, although definite, and approximately 50 A thick, was not usually sharp enough for exact measurement. This can be compared with data (Table 2) obtained after R-K fixation. Sections stained also by lead revealed the same profile with more contrast (Fig. 18).

With osmium tetroxide staining, the site of the cell wall was defined by two very thin lines encompassing an overall thickness of about 160 A. These stained layers measured approximately 40 to 50 A, and the innermost layer was usually the thicker (Fig. 19). Additional poststaining with lead citrate only served to emphasize the same general picture (Fig. 19a and 20). Cells fixed

primarily by osmium tetroxide and the sections doubly stained with uranyl acetate and lead citrate revealed essentially the same profile observed after uranyl acetate staining alone (Fig. 21 and 22).

The concentration of potassium permanganate was 0.5%, because concentrations of 1% or greater usually damaged the cells or caused precipitation on or near the cells. The profile of the cell wall was very similar to that observed after staining with osmium tetroxide alone and consisted of two very thin lines about 140 A apart (Fig. 23). The thickness of the total profile shown in osmium tetroxide and potassium permanganate stained sections was about 80 to 100 A less than that observed with uranyl acetate. Since neither permanganate nor osmium tetroxide can stain proteins, the logical explanation was that the superficial RS (protein) layer was not stained; however, no proof was attempted.

Lead citrate was the only poststaining agent not found suitable. The cell wall was seen as a thin double track and was barely outlined. Yet, in the cytoplasm, the ribosomal particles appeared to be very heavily stained.

We compared the profiles of the cell wall observed in sections of intact cells; uranyl acetate staining (alone or in combination with osmium tetroxide or with lead) seemed to encompass all components and thus reflect more faithfully the actual wall structure. The seemingly different modes of staining of the agents used were surprisingly informative.

DISCUSSION

Our observations confirm the finding of Baddiley (1) that the cell walls of gram-positive *Bacillus* species can possess an orderly array of macromolecular components and an unusual fine structure. This component of the cell wall does not consist of mucopeptide as had at first been considered and thus conforms to the findings of Baddiley (*personal communication*). Our approach has involved an electron microscopic analysis of structure, rather than a formal chemical study, and stems from the work of Weidel, Martin, and Frank (22, 41), who studied the arrangement of components of the walls of *Escherichia coli*.

Macromolecular arrays on the outer surface of bacterial cell walls were first observed by Houwink (12) with a *Spirillum* species. These have since been observed on a number of gram-negative organisms but particularly on spirilla of diverse physiological types (34). These arrays usually exhibit hexagonal packing and have a complex fine structure (23). A similar external layer on *M. radiodurans*, which is undoubtedly a gram-positive organism (39), shows a hexagonal array, but it is more complicated in its anatomical rela-

FIG. 17-23. Figures are intended to show only cell wall profiles after various stainings. Fixation with glutaraldehyde alone followed by dehydration appears to cause damage and deformation in the plasma membrane region. For this reason, the plasma membrane is not resolved. The lack of other fixation components contributes to the unusual cytoplasmic appearance and variance in Fig. 10. In these figures, the extent of the wall structure is delimited by arrows.

FIG. 17. Cell-wall profile after fixation with glutaraldehyde and poststaining with 1% uranyl acetate for 60 min. Note the broad light interspace between the cell wall and the visible cytoplasmic substance. No plasma membrane is visible.

FIG. 18. Cell-wall profile after fixation with glutaraldehyde. The section was poststained with uranyl acetate and lead citrate.

FIG. 19. Cells fixed as in Fig. 17; section poststained with 1% osmium tetroxide.

FIG. 19a. Cells fixed as in Fig. 17; section poststained with uranyl acetate, followed by lead citrate.

FIG. 20. Cells fixed as in Fig. 17; section poststained with 1% osmium tetroxide, followed by lead citrate.

FIG. 21. Cells fixed as in Fig. 17; section poststained with osmium tetroxide and uranyl acetate.

FIG. 22. Cell fixed as in Fig. 17; section poststained with 4% osmium tetroxide for 30 min, then 1% uranyl acetate for 40 min, followed by lead citrate. The cell wall is of uranyl acetate type; no light interspace is seen between the dark inner layer and the lighter outer one as seen in Fig. 18-21. Marker represents 0.1 μ .

FIG. 23. Cell fixed by glutaraldehyde, poststained with 0.5% potassium permanganate for 30 min. The profile is similar to that in Fig. 19 and 20.

tions than the usual layers exemplified by species of *Spirillum*. The only observations of a rectangular assay with approximately 100-Å spacing that we are aware of is that made by Labaw and Moseley (19) on an unidentified organism, which might well have been a gram-positive *Bacillus* species.

The distinct linear arrangement of surface fine structure has been observed by Houwink (13) on *C. nigrificans* and by Takagi et al. (37) on *C. tetani* and *C. botulinum*. It is reminiscent of the pattern observed on a photosynthetic bacterium by deBoer and Spit (4), but there was no suspicion of a mosaic of variant arrangements on the surface of *B. polymyxa*. In none of these studies was there sufficient resolution to show the unit arrangement.

A superficial regular structure on *Bacillus* species has not been reported, despite the frequency with which certain ones of them are used as model systems. Structure has been more often observed as part of the spore membrane or the exosporium (8), which have little or no relation to the superficial structure that we have been considering here. The infrequency of the observation may not be too surprising because of the initial difficulty that we experienced (as well as Baddiley, *personal communication*) in demonstrating the structure on the surface of young cells. The difficulty was due to masking by superficial capsular (or "microcapsular") material. Certainly the structure was always present in this strain when the cell wall surface was suitably washed. Those investigators preparing cell walls for the customary analysis of structure and utilizing the common methods of cleaning the preparation (e.g., with trypsin) would be likely to strip off or destroy the structure. They not only would miss this considerable component of the cell wall in any morphological examination, but also would leave it out of chemical considerations as well (3, 34, 42).

The profile shown in sections of *B. polymyxa* stained with metal salts is certainly less homogeneous than our preconceptions had allowed us to believe, and the different methods of poststaining revealed the differentiation of layers that would not otherwise have been evident. A diagrammatic summary of these positive effects and the dimensions of the layers concerned are shown in Fig. 24 and 25. The cell wall profiles revealed in preparations that included treatment with uranyl acetate definitely showed a considerable outer component, which had not been visible in comparable experiments with the cell walls of *B. megaterium* (26). There was no doubt that the removal of the structured layer with sodium lauryl sulfate or formamide left behind a rela-

FIG. 24. Schematic representation of the cell-wall profile of cells fixed by glutaraldehyde and poststained with different metals. UA = uranyl acetate.

FIG. 25. Tentative scheme for the structure of *Bacillus polymyxa* cell wall. Pr = protein; PS = polysaccharide; and MP = mucopolysaccharide. Weak bonds are represented by narrowest cross-hatched zone.

tively smooth and much thinner cell wall. The corresponding profile showed the loss of the distinct outer component. The nature of the profile in sections and the appearance and staining reactions of the outer component in particular might serve to distinguish those organisms possessing this type of surface from those that do not.

The plan and profile of walls after treatment with a wide range of reagents give us information about the linkage between the various structural components (31). The capability of agents to disrupt hydrogen bonds and to remove sheets of the RS layer indicates that those linkages binding the external layer to the layers underneath are weak and probably consist of hydrogen bonds. The integrity of these sheets indicates that different linkages are involved in holding the globules of the structure in the array. The globules can be released by appropriate treatment with low concentrations of sodium lauryl sulfate, which would be indicative of hydrophobic bonds.

The effect of sodium chloride solutions is indicative of ionic or salt linkages within the RS layer; yet, the damage to the RS layer by various hydrogen bond breaking reagents testifies to the presence of hydrogen bonds as well. It is possible that the globular elements are attached to or embedded in a wall component, which acts as a support and allows the removal of the structured layer as a unit. A suggestion of this arrangement is observable in Fig. 9. The actual roles of these bonds in the maintenance of structure cannot yet be decided. The presence of some salt linkages seems probable, and we can only conclude that they are not between the RS layer and the polysaccharide layer below it.

An incidental finding in this morphological study is that the cell wall of this organism did contain considerable protein structural elements in addition to carbohydrates, despite an earlier opinion that gram-positive cell walls usually possessed a limited selection of amino acids (34). It has been shown that the M protein of Group A streptococci can be removed with trypsin without affecting the integrity of the cells (20), and that the M protein could be detected in isolated cell-wall preparations (33). The type-specific M protein, along with some other protein fractions, overlies the polysaccharide layer containing the group-specific carbohydrates, which in turn overlies the mucopeptide (17). The anatomical relations here are very similar to what can be deduced of the profile of *B. polymyxa* cell walls (Fig. 25). The cell walls of *B. subtilis* have been found to contain 7 to 10% of protein material (some strains have revealed a similar superficial structure in our work), even though trypsin digestion was used in the preparation of the isolated walls (14). Strain differences may account for presence or absence of protein and for protein resistance to enzymatic digestion.

The profile of *B. megaterium* is somewhat different (26). There are only two distinguishable layers, set very close together without a clear interspace, and they are both stained after treatment with osmium tetroxide, lead citrate, and potassium permanganate (which was not the case for *B. polymyxa*); the external layer is not stained. Furthermore, the layers in this case seemed to be covalently bonded and more strongly held together than with the RS layer that we have studied. A further distinction lies in the lack of sensitivity to lysozyme exhibited by intact cells of *B. polymyxa* and by the layers of isolated cell wall, contrasting with the highly sensitive *B. megaterium*.

Routine procedures designed to take advantage of embedding and sectioning often involve double staining (or even triple when cells are fixed in

osmium tetroxide). There is evidence of specific interaction of strains; when the cells are first treated with uranyl acetate, the general contrast is increased by lead poststaining, not vice versa. What little is known of the mechanisms of staining (5, 36) does not yet help in the biochemical dissection of the cell wall. We are certain that we can not directly identify the elements of any wall profile.

Cells that had been washed in phosphate buffer up to 5 hr (the time made little difference) showed cytoplasmic retraction, and a layer appeared within what we had held to be the inner boundary of the cell wall (Fig. 14). One possibility is that the outer layer of the plasma membrane delaminates some portion of its structure. On the other hand, it might be an unrecognized portion of the cell wall. The plasma membrane in these particular *Bacillus* species is difficult to define; in sections, it often seems to consist of dense outer track with a fine space of low density on its inside. These profiles (24, 40) suggest the possibility of a substance that fills the "gap" between the plasma membrane and the cell wall and that behaves differently according to the conditions of treatment.

We have tried to distinguish the components of the electron microscopic profile of the wall. Uranyl acetate provided the best routine differentiation of the layers and was best, on sections of glutaraldehyde-fixed cells, for the differentiation of the cell-wall types exemplified by *B. megaterium* and *B. polymyxa*. The proteinaceous, superficial RS layer was distinctive in structure and was present only in some species of *Bacillus* and in a few other gram-positive cells. An adequate comparative study has not been made, even within the genus *Bacillus*. The presence of a similar layer on *Clostridium* species has been indicated (13, 37), and we can confirm the observation on *C. tetani*. The differentiation of the gram-positive cell wall profile into layers may not be uncommon; it must represent some separation of cell wall components, even when a superficial protein array is not present. We have noted such a differentiation in the case of *Listeria monocytogenes* (9), but we have not yet identified an RS layer. It is not known whether strains of *B. polymyxa* can lose this layer; the strains that we have worked with, no. 4747 and ATCC 10401, are essentially identical. The underlying polysaccharide layer was composed of at least two different fractions; one was soluble in hot formamide (corresponding probably to the "plastic" layer of *B. megaterium*), and the other corresponded to the mucopeptide component. These two fractions seem to be covalently linked.

We have not yet attempted a refined chemical analysis of these layers.

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