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Arrangement of Peptidoglycan in the Cell Wall of *Staphylococcus* spp.

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The arrangement of peptidoglycan in the cell wall of *Staphylococcus* was observed with the newly developed freeze-fracture technique, using *n*-octanol instead of water as the freezing medium. The replica of the trichloroacetic acid-extracted cell wall (TCA-wall) showed two areas. One of them has a concentric circular structure, a characteristic surface structure of the staphylococcal cell wall, and the other showed an irregular and rough surface. The chemical analysis of the wall revealed that the TCA-wall consisted of mostly peptidoglycan. By digesting the TCA-wall with lysozyme, the circular structures were greatly disturbed, and they disappeared after 60 min of treatment. From these observations it can be expected that the peptidoglycan is arranged in a concentric circular manner in the newly generated cell wall of *Staphylococcus*.

Information on the arrangement of peptidoglycan and the other wall polymers in the bacterial cell wall is of primary importance for understanding the mechanisms of cell wall synthesis and of the action of antibiotics and the unusual structure of morphological mutants. However, the arrangement and the distribution of these macromolecules in the cell wall are not fully understood. In gram-negative bacteria macromolecules form morphologically identifiable distinct layers, an outer membrane and a murein sacculus (14). No such layered structures have been detected in gram-positive bacteria. The cell wall forms a thick electron-dense layer outside of the cytoplasmic membrane (9). Concerning the arrangement of macromolecules, Verwer and Nanninga (16) reported that the peptidoglycan in the cell wall of *Bacillus subtilis* is arranged tangentially to the long axis of this rod-shaped bacterium. Such an arrangement was also suggested in *Escherichia coli* (17) and *Spirillum serpens* (11). We have presented evidence that in *Staphylococcus* the macromolecules are arranged in a concentric circular fashion in the newly generated cross wall (3). A similar observation has been reported by Giesbrecht and Wecke (8).

To observe the finer structure of the arrangement of the peptidoglycan in the cell wall, it is necessary to reveal the surface of the cell wall, which consists mainly of peptidoglycan. By extracting the cell wall with sodium dodecyl sulfate and trichloroacetic acid, the cell wall consisting mainly of peptidoglycan could be obtained. However, this wall was highly hydrophilic and showed low electron density even after staining with uranyl acetate or lead compounds. Therefore, the fine structure of this cell wall has not been observed with ordinary electron microscopic techniques, such as freeze fracturing, negative staining, and thin-sectioning.

In this paper we present a new technique in which specimens are frozen and fractured in an organic solvent, octanol. Electron microscopic observation of the replica of the fracture face revealed a circular arrangement of the peptidoglycan on the cell wall of *Staphylococcus*.

MATERIALS AND METHODS

Bacterial strains. Strain 209P of *Staphylococcus aureus* and strain KD of *S. epidermidis* were obtained from our culture stocks and grown on nutrient agar or PYK broth at 37°C (2). PYK broth consists of 5 g of polypeptone, 5 g of yeast extract, 3 g of K_2HOP_4, 2 g of glucose, and 1,000 ml of distilled water. The pH was adjusted to 7.2.

Isolation of the cell wall. The organisms were cultured overnight in PYK broth with continuous shaking at 37°C. To 450 ml of fresh PYK broth in a 1,000-ml flask 50 ml of the overnight culture was added, and incubation was continued for 6 h at 37°C with continuous shaking (Gyrotory Shaker, G10; New Brunswick Scientific Co., New Brunswick, N.J.). The cells grown in 10 such flasks were collected by centrifugation at 8,000 × *g* for 30 min. After being washed once with distilled water by centrifugation, the cells were suspended in distilled water. To prepare the cell wall fraction, the cells were disintegrated by mixing them with small glass beads (0.1 mm in diameter; Willy A Bachofen Maschinenfabrik, Basel, Switzerland) in a Dyno-Mill cell disintegrator (Willy A. Bachofen Maschinenfabrik). The cell wall fraction was collected by centrifugation (15,000 × *g*, 20 min) after nondisrupted cells and glass beads were separated by low-speed

centrifugation (2,000 × *g*, 10 min) and suspended in distilled water.

Chemical extraction of the cell wall. The cell wall fraction was treated with a 2% solution of sodium dodecyl sulfate (SDS) at 37°C for 30 min. This fraction was termed the SDS wall. After being washed four times with distilled water, the SDS wall was suspended in 0.05 M phosphate buffer (pH 7.8) containing 200 μg of trypsin (bovine pancreas; P-L Biochemicals, Inc., Milwaukee, Wis.) per ml and incubated at 37°C for 15 h and then with 100 μg of pepsin (ICN Pharmaceuticals Inc, Cleveland, Ohio; 3X crystallized) per ml in 0.01 M HCl solution at 37°C for 3 h. This fraction was termed the pepsin wall and was further extracted with 5% trichloroacetic acid (TCA) at 4°C for 45 h and then with 10% TCA at 90°C for 10 min. This fraction, termed the TCA wall, was suspended and stored in distilled water.

Chemical analysis of the cell wall fraction. The protein content was determined by Lowry's method (10). The amount of teichoic acid was estimated from the phosphorus content by the method of Allen (1). The amount of peptidoglycan in the cell wall fraction was estimated from the amount of *N*-acetylglucosamine measured by a modification of the Morgan-Elson procedure (13). The amounts of these wall components were expressed on the basis of milligrams (dry weight) of each lyophilized fraction.

Freeze-etching method. Specimens for freeze-etching were fixed for 30 min at 4°C by 1% glutaraldehyde prepared in 0.05 M cacodylate buffer, suspended in a 0.1 M solution of sodium chloride, quickly frozen in Freon 22 precooled in liquid nitrogen, and fractured in a Balzers freeze fracture unit (Balzers type BAF 301; Balzers, Lichtenstein) at −110°C and at a pressure of 7×10^{-6} torr or less. The fracture plane was then etched for 10 min at −100°C. The replica of the freeze-etched surface was made by shadowing with platinum-carbon (2 nm thick) at an angle of 45° followed by evaporating carbon (25 nm thick) in a vertical position. The replica was cleaned by the ordinary method using sodium hypochloride and examined with a Hitachi HU-12A electron microscope at 75 kV.

Freeze-fracture method using octanol as freezing medium. To obtain a surface view of the cell wall by the freeze-fracture technique, we tried to fracture the specimens frozen in organic solvents. The solvents tested in this experiment were *n*-octanol, *n*-octane, and Freon 113. These solvents were all reagent grade. *n*-Octanol and *n*-octane were obtained from Ishizu Pharmaceutical Co., Ltd., Osaka, Japan, and Freon 113 was a product of Daikin Co., Ltd., Osaka, Japan. Among these solvents, we chose octanol as the most suitable one for this purpose. The cell wall surface frozen and fractured in this solvent was exposed better than in the other solvents. The problems encountered with the other solvents were that a drop of the specimen could not be formed on the specimen holder because of their low surface tension, and fracture planes in these solvents were not as clear as those obtained in octanol.

The freeze-fracture procedure was as follows. The specimens were fixed with 1% glutaraldehyde, dehydrated in an ethyl alcohol series, and finally suspended in 100% octanol. The specimens immersed in octanol were frozen in Freon 22 precooled with liquid nitrogen and fractured in a Balzers freeze-etching unit. Immediately the replica of the fracture face was made in the usual manner. Before the replica was cleaned, the specimen stage was dipped into octanol in a small petri dish. The replica with the specimen still beneath it was dipped up on a specimen grid in octanol. The grid now containing the replica was placed on a filter paper saturated with ethanol to soak up excess octanol and then dipped into ethanol. The grid was finally dipped into sodium hypochloride to clean the replica. When the specimen was directly dipped into water without removing the octanol, a strong reaction occurred between water and the solvent, and the replica was broken into small fragments.

Ferritin-conjugated antibody. Anti-staphylococcal antiserum was prepared by injecting strain 209P, killed with 1% formaldehyde and treated with a 0.1% solution of chrome alum, intravenously into rabbits three times at 5- to 7-day intervals. The IgG fraction was separated from the immune serum by the ammonium sulfate fractionation method. Ferritin from horse spleen was purchased from ICN Pharmaceutical Inc. and further purified by the cadmium sulfate recrystallization method. Conjugation of IgG and ferritin was done with *pp*-difluoro-*m*-*m*-dinitrodiphenyl sulfone as a coupling agent (12).

Thin-section technique. Staphylococci were fixed with 1% glutaraldehyde in 0.15 M sodium cacodylate solution and then with 1% osmium tetroxide buffered with Veronal acetate solution. After dehydration with ethanol the specimens were embedded in Epon 812. Thin sections were cut with a diamond knife attached to a Porter-Blum Ultramicrotome MT-2 and examined in a Hitachi HU-12A electron microscope after being stained with uranyl acetate and lead citrate.

Lysozyme digestion. The TCA wall was suspended in 0.15 M phosphate-buffered saline (pH 7.2) containing 50 μg of lysozyme (6× crystallized; Seikagaku Kogyo Co., Ltd., Tokyo, Japan) per ml and incubated at 37°C. At various times after incubation, samples of the suspension were withdrawn and processed for freeze-fracturing by the octanol method. The degree of digestion of the TCA wall by lysozyme was estimated from the decrease in optical density of the suspension measured at 660-nm wave length (Klett unit; Fuji Photometer Co., Ltd., Tokyo, Japan).

RESULTS

Chemical analysis of the cell wall fractions. Table 1 shows the results of the chemical analysis of the fractions of the cell walls of strains KD and 209P. Most of the cell-wall-associated proteins were removed by the treatment with SDS. Further treatment with proteinases seemed not to be effective in removing more proteins from the SDS wall. The phosphorus content of the wall, indicating the amount of teichoic acid or teichuronic acid in the wall, markedly decreased after the treatment with TCA, suggesting that these polysaccharides could effectively be removed by this treatment. However, the amount of *N*-acetylglucosamine, which represents the amount of the peptidoglycan in the cell wall, did not significantly change during the extraction processes. We can say that the TCA wall is the

TABLE 1. Chemical analysis of the cell wall fraction

Fraction	*S. aureus* 209P			*S. epidermidis* KD		
	Protein	Phosphorus	NAcGl[a]	Protein	Phosphorus	NAcGl
Crude wall	165[b]	11.5	156	120	18.5	188
SDS wall	5	12.0	240	2.5	21.0	210
Pepsin wall	5	12.5	273	2.5	21.0	216
TCA wall	5	0	195	2.5	0	264

[a] *N*-Acetylglucosamine.
[b] Micrograms per milligram (dry weight) of each fraction.

wall fraction consisting mainly of the peptidoglycan.

Freeze-etching studies of the cell wall fraction. Figure 1 is an electron micrograph of freeze-etched whole cells of strain 209P. The surface structure of the cell wall exposed after etching is similar to that observed by scanning electron microscopy (SEM) (3). Two parts separated by a thick line were seen on the cell wall surface (arrows in Fig. 1); the concentric circular structures were visible on one part but not on the other. The lines of the individual circular structures were seen to be finer and more delicate than those observed by SEM and were shown to consist not of a single long circular line but of many rows of circularly arranged short lines. This circular structure was thought to be derived from complex structures of teichoic acid and peptidoglycan.

Since the substantial structure of the cell wall was determined by the arrangement of glycan chains in the wall, the arrangement of glycan chains in the wall should be investigated. Observation of the surface of the TCA wall might be expected to bring us information on the arrangement of glycan chains in the cell wall. From the chemical analysis the TCA wall was thought to consist mostly of the peptidoglycan. However, the TCA wall processed by the ordinary freeze-etching method has rarely revealed its surface structure. Only a cross-sectioned profile of the wall fragments was disclosed. This was mainly because of the highly hydrophilic and less rigid nature of the TCA wall. To reveal the surface structure of such a soft and hydrophilic wall fraction we had to employ a new technique in which the surface of such a cell wall structure was exposed more efficiently. For this purpose we tried to use an organic solvent as a freezing medium for freeze-fracture of the TCA wall.

Freeze-fractured surface of the TCA wall in octanol. Initially it is important to show that the fracture plane of *Staphylococcus* revealed by the octanol method of freeze-fracturing represented the outer surface of this bacterium. For this purpose strain 209P was treated with ferri-

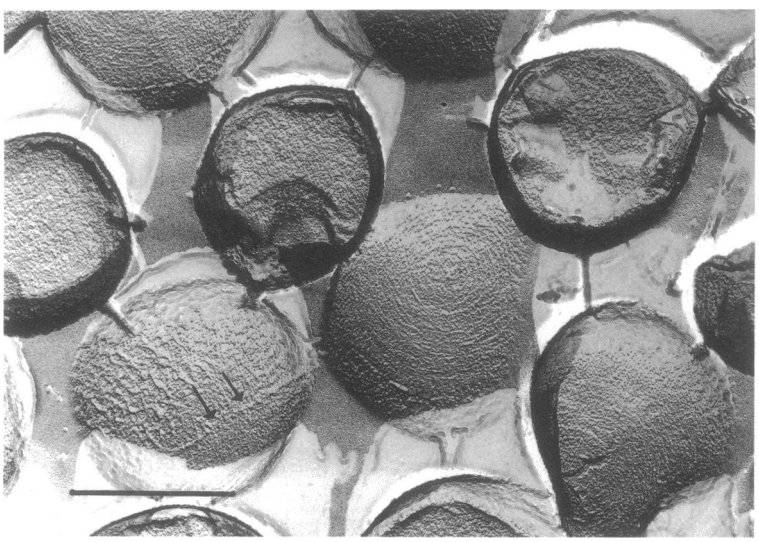

FIG. 1. Electron micrograph of the replica of the freeze-etched surface of whole cells of *Staphylococcus aureus* strain 209P. Arrows indicate the thick line on the cell wall separating the surface into two areas. Scale bar represents 0.5 μm.

FIG. 2. Freeze-fractured replica of *S. aureus* strain 209P treated with ferritin-IgG prepared by the octanol method.

tin-immunoglobulin G (IgG) conjugate, dehydrated, immersed in octanol, and freeze-fractured. The fracture plane created by the octanol method was seen not as a flat and smooth surface but as a rugged and rough surface. Many convex hemispheres having many small particles of the size of ferritin molecules were seen on the surface (Fig. 2). Since specific labeling of ferritin particles on the outermost surface of *Staphylococcus* was shown by the thin-sectioning technique (Fig. 3), these hemispheres were the surface of ferritin-treated staphylococcal cells. This indicated that the fracture plane of this bacterium produced by the octanol method represents the outermost surface. Furthermore, the surfaces of strains 209P and KD not treated with ferritin-IgG and exposed by this technique showed a concentric circular structure, the unique and characteristic structure of staphylococcal cells (Fig. 4). The image of the circular structure revealed by the octanol method was somewhat different from that revealed by the ordinary freeze-fracture technique. The lines of the circles were thick and slightly elevated from the cell wall surface in the octanol method. These differences might be due to the differences in the medium used for immersing the specimens for freezing (octanol versus water).

Using this freeze-fracture technique, we tried to reveal the surface structure of the TCA wall. When the TCA wall was fractured in octanol, the fracture plane showed an irregular and rough surface as mentioned above and on this surface not frequently but definitely the surface of the wall was exposed (Fig. 5). It was seen as a slightly convex round area that was smoother than the other part of the fracture plane. The concentric circular structure was seen in some of these areas. The area showing this circular structure (areas indicated by "C" in Fig. 5) was thought to be the newly exposed surface of the cell wall. The surface adjacent to the area showing the circular structure was rough, and this

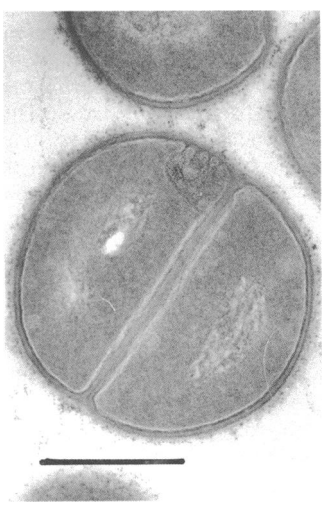

FIG. 3. Thin-sectioned profile of ferritin-IgG-treated *S. aureus* strain 209P.

FIG. 4. Freeze-fractured replica of whole cells of *S. aureus* strain 209P prepared by the octanol method. The surface of the cell is very similar to that observed by scanning electron microscopy (15).

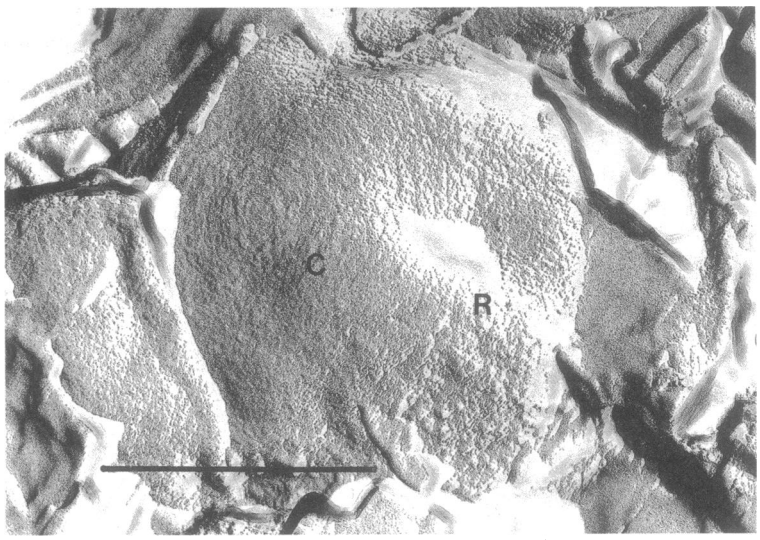

FIG. 5. Freeze-fractured replica of the TCA wall of *S. aureus* strain 209P prepared by the octanol method. The surface was composed of two areas, one of which showed the circular structure (areas indicated by C) and the other an irregular surface (indicated by R).

area might represent the old surface of the cell wall (area indicated by R in Fig. 5).

Effect of lysozyme digestion on the cell wall structure. The TCA wall was sensitive to digestion by lysozyme. The density of the TCA wall suspension treated with lysozyme decreased in one experiment from 161 to 101 during 35 min of incubation. Electron microscopy of the freeze-fractured surface exposed by the octanol method (Fig. 6) showed that the circular structure of the cell wall gradually became obscure and by 60 min of incubation, the circular structures had disappeared from most of the cell surfaces observed.

DISCUSSION

Electron microscopy of the TCA-extracted cell wall of *Staphylococcus* showed the presence of a concentric circular structure (ccs), a characteristic structure of the staphylococcal cell, on a part of the cell wall surface. The ccs has been revealed on the newly generated cell wall surface by the scanning electron microscope and on the cross wall by staining with uranyl acetate (3). This structure was shown to disappear after the cells or isolated cell walls were treated with TCA, which removes most of the teichoic acid (3). Teichoic acid has been shown to be covalently linked to the peptidoglycan (6), so it could be expected that the peptidoglycan would also be arranged circularly on the cell wall surface.

The method used in this experiment to observe the surface structure of the TCA-wall was a freeze-fracture technique utilizing octanol as a freezing medium. *N*-Octanol is an alcohol practically insoluble in water and has a polar region in the molecules. As the cell wall surfaces, especially that of the TCA wall, were hydrophilic when the cell walls or cells were immersed in octanol, a hydrophilic interface is assumed to be formed between the cell wall surface and the polar region of the octanol molecules. After freezing and fracturing, a fracture plane is presumed to be created at this interface.

The surface of the bacterial cell is composed not only of hydrophilic materials but also of some hydrophobic materials, and some of these materials might be removed during the dehydration or freeze-fracturing process. The morphological differences in the surface structures obtained by the ordinary method and the octanol method of freeze-fracturing might be due to the removal of some wall materials or structural distortion caused by the interaction of hydrophilic cell wall materials or both and the hydrophobic region of the octanol molecules.

However, as shown by the ferritin-IgG method, materials firmly attached to the wall such as ferritin molecules conjugated with antibody, are not removed by freeze-fracturing. This indicated that though some loosely associated components might be removed after freeze-fracturing, the substantial structures of the cell wall are presumably not altered by the octanol method. Therefore, it can be stated that the ccs on the TCA wall represents the circular arrangement of

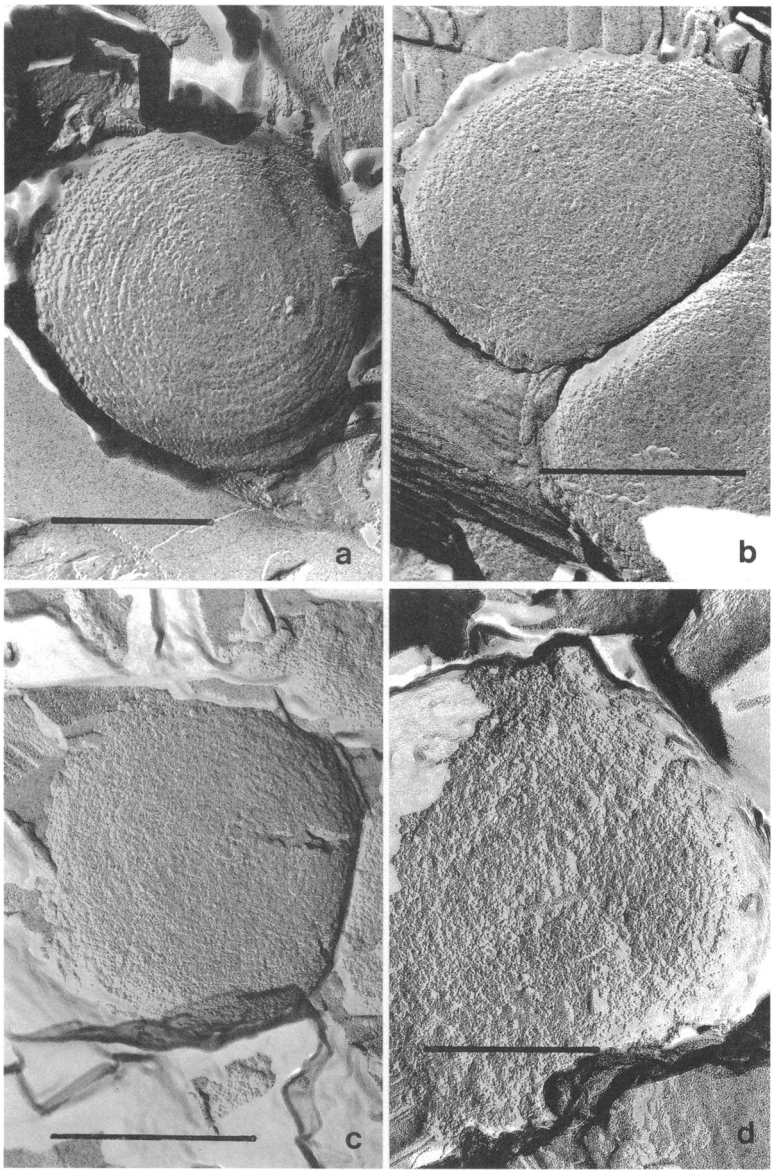

FIG. 6. Replicas of the TCA wall of *S. epidermidis* strain KD exposed by the octanol method and treated with 50 μg of lysozyme per ml. (a) Control not treated with lysozyme. (b) Treated for 15 min at 37°C. (c) Treated for 30 min at 37°C. (d) Treated for 60 min at 37°C. The circular structure was clearly visible on the untreated wall and the 15-min-treated wall, but it was not clear on the 30- and 60-min-treated walls.

peptidoglycan in the cell wall. The enzyme digestion experiment supported this observation. Lysozyme, an enzyme that specifically attacks the peptidoglycan, digested out the circular structures.

The glycan chains in the bacterial cell wall are thought to have a length of 14 to 16 disaccharide units (4). As the length of one unit is approximately 1 nm (5), the length of glycan chains does not exceed 16 nm. Since the distance between two glycan chains was deduced to be 1.25 nm (5), and the circular lines observed on the TCA-wall were wider than this value, the line might represent not a single chain of glycan but a bundle of several glycan chains.

The ccs was visible only on the cross wall or on the newly generated cell wall surface, and after one or two generations this structure disap-

peared, and the surface of the cell wall becomes rough and irregular (3). The TCA-wall surface also showed the same changes, indicating a loss of regularity in the arrangement of the peptidoglycan. These changes were thought to occur as a result of the enzymatic digestion of the wall surface and its repairing reaction mediated by autolytic enzymes (7). These changes might be related to the expansion of the cell wall to fit the surface increase after cell separation.

The synthesis of the cross wall proceeds as a closing iris, and after completion the wall thickness increases (8). It still remains to be shown whether the synthesis of the thickening wall also occurs in the regular circular way.

ACKNOWLEDGMENT

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