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Ultrastructural Localization of Carbohydrates on Thin Sections of *Staphylococcus aureus* with Silver Methenamine and Wheat Germ Agglutinin-Gold Complex

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Postembedding staining of intracellular carbohydrates on thin sections of *Staphylococcus aureus* was studied by the silver methenamine and the wheat germ agglutinin-gold techniques. Staining of silver grains was observed on both the cell wall and the cross wall. The staining was interpreted to be due to teichoic acid. Labeling by wheat germ agglutinin-gold particles was observed on both the cell wall and the cross wall, and the staining pattern resembled that of silver methenamine staining. Therefore, the labeling was considered to be due to *N*-acetylglucosamine of teichoic acid. The combination of two types of cytochemical techniques was useful to localize and characterize the carbohydrates of the bacterial cell.

The silver proteinate and methenamine techniques have been used for the ultrastructural localization of carbohydrates in various tissues (3, 5) and bacteria (7, 8, 19, 22) by postembedding staining. These techniques are based on oxidation of 1-2-α-glycol bonds released by periodic acid (7, 8).

Recently, cytochemical techniques using lectins were employed for the localization of carbohydrates in mammalian tissues (6, 12, 18, 20, 21). The lectin-gold techniques particularly allows a high-resolution view of ultrastructural localization of carbohydrates on thin sections by postembedding staining (18, 21). This technique is based on the characteristic features of lectins, glycoproteins of nonimmune origin that bind specifically to certain sugar residues.

The combination of these two techniques may be useful to localize and characterize the carbohydrates, since each technique relies on distinctively different mechanisms to detect carbohydrates. We examined the localization and characterization of the carbohydrates on thin sections of *Staphylococcus aureus* with silver methenamine and with colloidal gold coated with wheat germ agglutinin (WGA).

Preparation of WGA-gold complex. Colloidal gold particles (15 nm in diameter) were prepared by the method of Frens (9). The WGA-gold complex was prepared by the method of Geoghegan and Ackerman (10). Briefly, the optimal amount of WGA (E. Y. Laboratories, San Mateo, Calif.) was determined to be 100 μg for stabilization of 10 ml of colloidal gold, as estimated by the salt flocculation test. After the WGA-gold solution was mixed for 5 min, 1 ml of 1% (wt/vol) polyethylene glycol (molecular weight, 20,000) in 0.01 M phosphate-buffered saline (pH 7.4) was added. The mixture was centrifuged twice at 16,000 × *g* for 1 h and resuspended in 0.5 ml of phosphate-buffered saline containing 0.02% polyethylene glycol.

Processing of bacteria. *S. aureus* 209P was grown in heart infusion broth at 37°C for 18 h. Cells were harvested by centrifugation and washed with 0.1 M phosphate buffer. The pellets were fixed with 1% glutaraldehyde in the buffer at 4°C for 2 h and embedded in 2% agar. After the cells were washed with the buffer at 4°C overnight, they were dehydrated by a graded series of ethanol and embedded in Spurr resin. Thin sections were cut on a Porter-Blum ultramicrotome, collected on uncoated 200-mesh nickel grids, and processed for cytochemical labeling.

Cytochemical labeling. For the cytochemical labeling with silver methenamine, silver staining was performed by the method of Walker and Short (22). Briefly, the sections were put in a solution with or without 0.5% periodic acid for 20 min, washed twice with distilled water, and transferred to the silver solution. After incubation at 60°C for 30 min, the sections were washed twice with distilled water, incubated for 2 min at room temperature with 0.5% sodium thiosulfate, washed with distilled water, and dried.

For the cytochemical labeling with WGA-gold, the grids were first incubated for 5 min in 1 drop of 0.01 M phosphate-buffered saline. They were transferred to 1 drop of WGA-gold solution (50 μg/ml) in the presence or absence of 0.1 M *N*-acetylglucosamine (Sigma Chemical Corp., St. Louis, Mo.) and incubated for 30 min at room temperature. The sections were washed with phosphate-buffered saline, rinsed with distilled water, and dried. They were stained with uranyl acetate and lead citrate and examined with a Hitachi H-500 H electron microscope.

Figure 1 shows the staining of sections with silver methenamine. Silver deposits were arranged along the cell wall and the cross wall. The deposits of silver were present as two layers on the cell wall and as three layers on the cross wall. No silver grains were observed on the cell when the oxidation with periodic acid was omitted (Fig. 2).

After incubation of the sections with the WGA-gold complex, the labeling was mainly observed on the cell wall (Fig. 3, 4, and 6) and the cross wall (Fig. 5). Two layers of gold particles were found on the cell wall, and three layers were found on the cross wall (Fig. 4 and 5). The staining pattern

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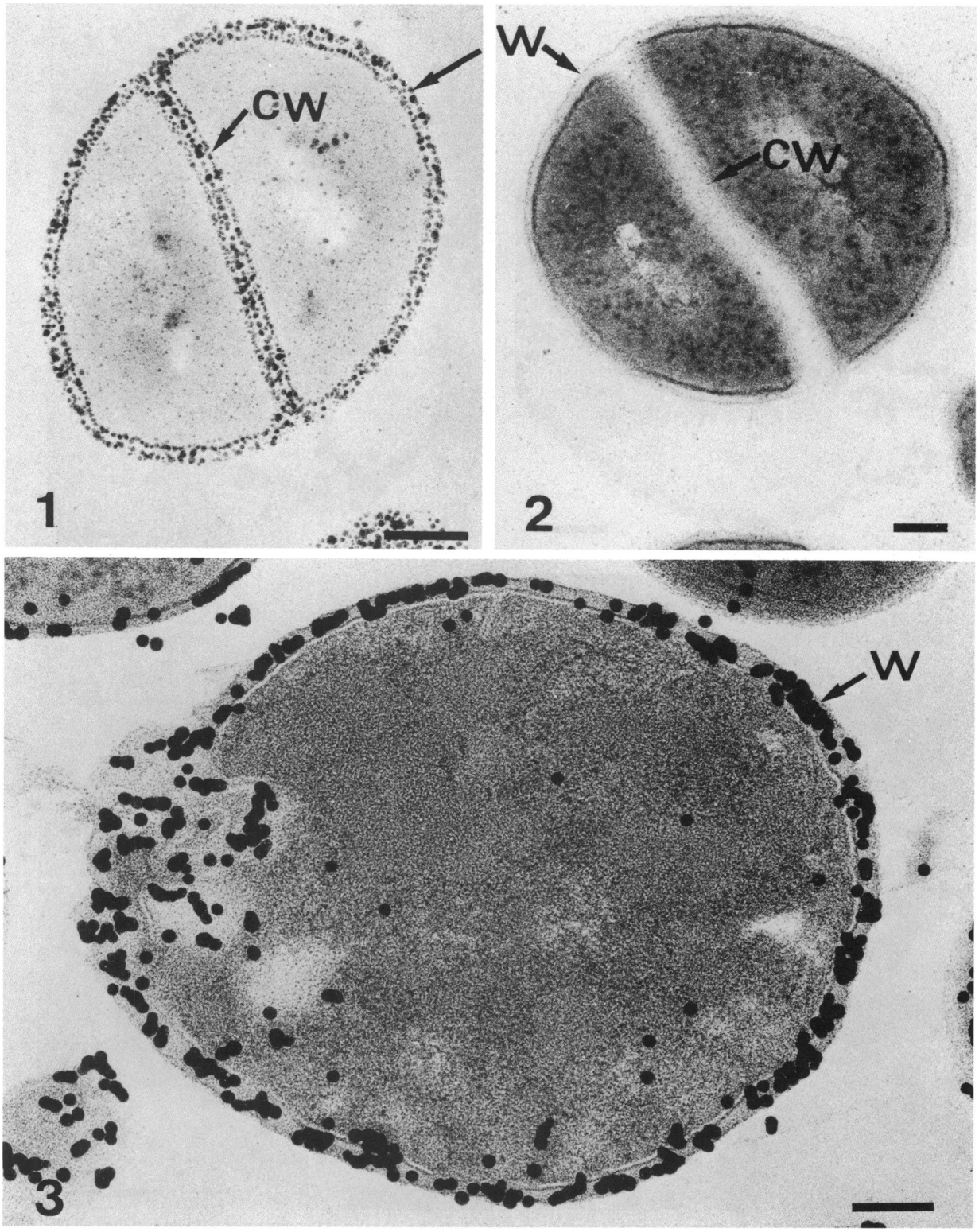

FIG. 1. Thin section of *S. aureus* treated with periodic acid and then labeled with silver methenamine. Note the two layers of silver deposits on the cell wall (W) and three layers on the cross wall (CW). Bar = 0.1 μm.

FIG. 2. Thin section of *S. aureus* labeled with silver methenamine alone and then stained with uranyl acetate and lead citrate. No staining of silver grains is observed on the cell wall (W) or on the cross wall (CW). Bar = 0.1 μm.

FIG. 3. Thin section of *S. aureus* incubated with WGA-gold complex. Gold particles are present on the cell wall (W). Bar = 0.1 μm.

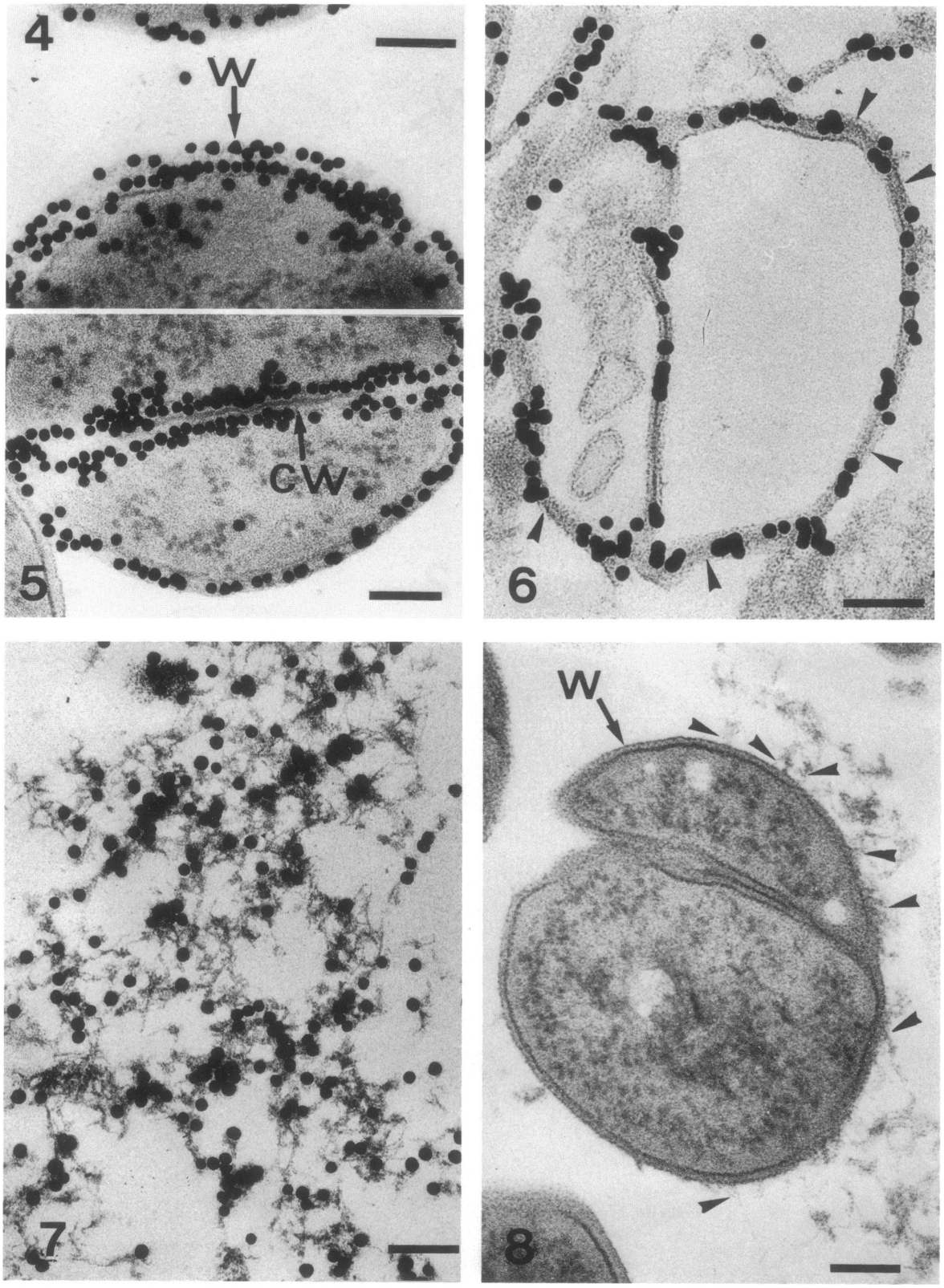

FIG. 4. Thin section of *S. aureus* incubated with WGA-gold complex. Note the labeling of two layers of particles on the cell wall (W). Bar = 0.1 μm.

FIG. 5. Thin section of *S. aureus* incubated with WGA-gold complex. Note the three layers of particles on the cross wall (CW). Bar = 0.1 μm.

FIG. 6. Thin section of *S. aureus* incubated with WGA-gold complex. The cell wall (arrowheads) of the autolytic cell is densely labeled with gold particles. Bar = 0.1 μm.

FIG. 7. Thin section of *S. aureus* incubated with WGA-gold complex. Note the particles on the soluble fragments of the cell wall. Bar = 0.1 μm.

FIG. 8. Cytochemical control, with sections incubated with WGA-gold in the presence of *N*-acetylglucosamine. Very few particles are observed on the cell. Note the continuity (arrowheads) of the soluble fragments with the cell wall (W). Bar = 0.1 μm.

with WGA-gold closely resembled that with silver methenamine. Gold particles were also observed on the soluble fragments of the cell wall (Fig. 7). The width of the fragments was very similar to that of the outer layer of the cell wall. In some specimens, fragments were found to be attached to the cell wall (Fig. 8, arrowheads). No particles were seen on the cells incubated with WGA-gold in the presence of *N*-acetylglucosamine (Fig. 8).

The cell wall of the gram-positive bacteria is composed of two kinds of polymers, peptidoglycans and teichoic acid. Staining procedures for carbohydrates probably stain one or both of these molecules. The cell wall teichoic acid of nonmutant *S. aureus* consists of a ribitol phosphate polymer and *N*-acetylglucosamine (1, 4, 14, 15), whereas that of *S. epidermidis* consists of a glycerol phosphate polymer and *N*-acetylglucosamine or glucose (4, 16).

Postembedding staining of the sections of *S. aureus* with silver methenamine revealed two layers of silver grains on the cell wall and three layers on the cross wall. The negative staining of silver grains when the periodic acid treatment was omitted suggests that the deposit of silver grains is not an artifact caused by the aldehyde group of glutaraldehyde. A similar staining pattern was found by silver proteinate staining in the other gram-positive bacteria (7, 8). Frehel and Ryter (8) found that silver proteinate stained the nonpeptidoglycan polymers of the cell wall, and they interpreted the silver deposit found on the cell wall of *Bacillus subtilis* as teichoic acid for the following reasons. (i) The silver deposit disappeared after NaOH treatments. (ii) The silver deposit had the same location as concanavalin A, which binds to teichoic acid. (iii) In *B. subtilis*, the main nonpeptidoglycan polymer containing 1-2-α-glycol linkages is teichoic acid. Therefore, the silver deposit on the *S. aureus* cell wall found in the present study may also be due to teichoic acid, which is composed of ribitol phosphate and *N*-acetylglucosamine (1, 14, 15).

WGA is a lectin that binds specifically with *N*-acetylneuraminic acid, *N*-acetylgalactosamine, and *N*-acetylglucosamine (11, 17). Lotan et al. (13) found that WGA agglutinates the cells of *S. aureus* H and *S. aureus* 52A5, a mutant that does not have teichoic acid, but not cells of *S. aureus* 52A2, a mutant whose teichoic acid does not have *N*-acetylglucosamine. They also found, by techniques such as agar gel diffusion, quantitative precipitation, and inhibition of hemagglutination of trypsinized rabbit erythrocytes, that WGA interacts with the teichoic acid from *S. aureus* H but not with that from *S. aureus* 52A2. These data suggest that WGA-gold labeling on *S. aureus* in the present study is due to *N*-acetylglucosamine of both teichoic acid and peptidoglycans. However, the close similarity of the staining pattern of silver methenamine and WGA-gold suggests that the labeling of gold particles may be due to *N*-acetylglucosamine in teichoic acid, although we cannot exclude the possibility that the gold particles are also associated with *N*-acetylglucosamine in the peptidoglycans. We are now trying to clarify this point cytochemically in conjunction with pretreatment of bacteria with a number of chemicals, including trichloroacetic acid.

The labeling of WGA-gold particles was localized over the soluble fragments of staphylococcal cells. Similar fragments were also observed in degraded *S. aureus* and were found to contain *N*-acetylglucosamine and muramic acid (2). The fragments labeled with WGA-gold in the present study were probably released from the cell wall of *S. aureus* (Fig. 8).

In conclusion, the combination of two different kinds of cytochemical techniques, such as silver methenamine and the lectin-gold technique, was useful to localize and characterize the carbohydrates in the bacterial cells.

LITERATURE CITED

1. **Baddiley, J., J. G. Buchann, U. L. Rajbhandary, and A. R. Sanderson.** 1962. Teichoic acids for the walls of *Staphylococcus aureus* H. Biochem. J. **82**:439–448.
2. **Blümel, P., B. Reinicke, M. Lahv, and P. Giesbrecht.** 1983. Cell wall degradation of *Staphylococcus aureus* by autolysins and lysozyme, p. 323–326. *In* R. Hakenbeck, J.-V. Höltje, and H. Labishinski (ed.), Target of penicillin. Walter de Gruyter & Co., Berlin.
3. **Chung, J., W. Mautner, and E. Grisham.** 1958. Silver impregnation for electron microscopy. J. Biophys. Biochem. Cytol. **4**: 841–842.
4. **Davison, A. L., and J. Baddiley.** 1964. Teichoic acids in the walls of *Staphylococci*. Nature (London) **2**:872–874.
5. **De Martino, C., and L. Zamboni.** 1967. Silver methenamine for electron microscopy. J. Ultrastruct. Res. **19**:273–282.
6. **Fisher, J., P. J. Klein, M. Vierbuchen, B. Skutta, G. Uhlenbruck, and R. Fischer.** 1984. Characterization of glycoconjugates of human gastrointestinal mucosa by lectins. Histochemical distribution of lectin binding sites in normal alimentary tract as well as in benign and malignant gastric neoplasm. J. Histochem. Cytochem. **32**:681–689.
7. **Frehel, C., P. Robbe, R. Tonelli, and A. Ryter.** 1982. Relationship between biochemical and cytochemical results obtained on *Bacillus megaterium* and *Bacillus subtilis* cell wall polysaccharides. J. Ultrastruct. Res. **81**:78–87.
8. **Frehel, C., and A. Ryter.** 1982. Electron microscopic cytochemical study of cell wall polysaccharides in *Bacillus subtilis* and two strains of *Bacillus megaterium*. J. Ultrastruct. Res. **81**:66–77.
9. **Frens, G.** 1973. Controlled nucleation for the regulation at the particle size in monodisperse gold solution. Nature (London) Phys. Sci. **241**:20–22.
10. **Geoghegan, W. D., and G. A. Ackerman.** 1977. Adsorption of horseradish peroxidase, ovomucoid and anti-immunoglobulin to colloidal gold for the indirect detection of Concanavalin A, wheat germ agglutinin and goat antihuman immunoglobulin G on cell surfaces at the electron level: a new method, theory and application. J. Histochem. Cytochem. **25**:1187–1200.
11. **Horisberger, M., and M. Vontanthem.** 1979. Multiple marking of cell surface receptors of gold granules: simultaneous localization of 3 lectin receptors of human erythrocytes. J. Microsc. (Paris) **115**:97–102.
12. **Katsuyama, T., and S. S. Spicers.** 1978. Histochemical differentiation of complex carbohydrates with variants of the Concanavalin A-horseradish peroxidase method. J. Histochem. Cytochem. **26**:233–250.
13. **Lotan, R., N. Sharon, and D. Mirelman.** 1975. Interaction of wheat-germ agglutinin with bacterial cells and cell-wall polymers. Eur. J. Biochem. **55**:257–262.
14. **Morse, S. J.** 1962. Studies on the chemistry and immunochemistry of cell walls of *Staphylococcus aureus*. J. Exp. Med. **116**: 229–245.
15. **Nathenson, S. J., N. Ishimoto, J. S. Anderson, and J. L. Strominger.** 1966. Enzymic synthesis and immunochemistry of α- and β-N-acetyl-glucosaminylribitol linkages in the teichoic acids from *Staphylococcus aureus*. J. Biol. Chem. **241**:651–658.
16. **Oshima, Y., T. Ohtomo, Y. Ichiman, M. Chromarat, and K. Yoshida.** 1984. Comparison of cell wall teichoic acid fraction isolated from three different encapsulated strains of *Staphylococcus epidermidis*. Ann. Microbiol. **135**:353–365.
17. **Peter, B. O., S. Ebisu, I. Goldstein, and M. Flashner.** 1973. Interaction of wheat germ agglutinin with sialic acid. Biochemistry **18**:5505–5511.
18. **Roth, J.** 1983. Application of lectin-gold complex for electron microscopic localization of glycoconjugates on thin section. J. Histochem. Cytochem. **31**:987–999.
19. **Rousseau, M., and J. Hermier.** 1975. Localisation en microscopie electronique des polysaccharides de la paroi chez les

bacteries en sporulation. J. Microsc. Biol. Cell. **23**:237–248.
20. **Sato, A., and S. S. Spicer.** 1982. Ultrastructural visualization of galactose in the glycoprotein of gastric surface cells with a peanut lectin conjugate. Histochem. J. **14**:25–38.
21. **Suganuma, T., S. Tsuyama, and F. Murata.** 1985. Glycoconjugate cytochemistry of the rat fundic gland using lectin/colloidal gold conjugate and Lowicryl K 4M. Helix pomatia lectin is a specific for mucosa neck cells in fundic glands of the rat gastric mucosa. Histochemistry **38**:489–495.
22. **Walker, P. D., and J. Short.** 1968. The location of mucopolysaccharides on ultrathin section of bacteria by the silver methenamine staining technique. J. Gen. Microbiol. **52**:467–471.