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Asymmetric Distribution of Charge on the Cell Wall of *Bacillus subtilis*

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The cell wall of *Bacillus subtilis* is capable of binding different kinds of metal ions. The wall-ion complex appears to be dependent on both phosphoryl from teichoic acid and carboxylate from peptidoglycan. In the present study, cationized ferritin (CF) was used as a probe for charge distribution on the wall of *B. subtilis* 168. Detergent-extracted cell walls bound CF only on the outer wall face. Completed cell poles bound CF, but septa did not. When the walls were permitted to autolyze briefly, binding of CF occurred on both faces. In contrast, limited hydrolysis of the walls by egg white lysozyme resulted in the penetration of CF into the wall matrix. When walls were made teichoic acid-free, CF-binding asymmetry was preserved, suggesting that carboxyl groups were oriented toward the surface. Walls with carboxylates chemically neutralized also retained charge asymmetry. Phosphate-free and carboxyl-modified walls bound CF only poorly or not at all. These results indicate that negative charges contributed by both phosphate and carboxyl are responsible for the binding of CF and that the observed asymmetry in the distribution of the label is due to the orientation of teichoic acid and muramyl peptides toward the outside of the cell wall, above the plane of the glycan strands.

Although the cell walls of gram-positive bacteria appear to be amphoteric (18, 21), they seem to have a marked selectivity toward the binding of cations (3, 19). Several laboratories have provided evidence to suggest that phosphate groups of teichoic acids (TAs) serve as ligands for metal ions (3, 7, 11, 19). Other negative charges are contributed by unsubstituted carboxyl groups of muramyl peptides, which have also been shown to interact with metal ions (3, 19). Previous work (4) revealed that TA molecules of *Bacillus subtilis* were oriented perpendicularly from the external cell wall surface. Similarly, Archibald et al. (1) have suggested that the wall of *Staphylococcus lactis* is arranged with TAs protruding away from the plasma membrane. This type of favored positioning could facilitate phage attachment or the binding of positively charged groups.

Cationized ferritin (CF) has been reported to be a valuable tool for detection of negatively charged groups on the surface of *B. subtilis* (10) and *Streptococcus faecalis* (22). In the present study, CF was used as a probe to locate the distribution of accessible negatively charged groups on isolated cell walls of *B. subtilis*. Detergent-extracted cell walls could bind CF only on the outer surface. Cell wall poles and cylinders could bind CF on their external surfaces, but septa did not. After cell walls were briefly autolyzed, they were able to bind CF on both their inner and outer surfaces. Lysozyme-treated cell walls could bind CF throughout the entire wall matrix. When phosphate groups were removed or carboxyl groups were modified, asymmetric binding of CF toward the outside was retained. The results suggest that both TAs and muramyl peptides are situated above the plane of the glycan strands in isolated cell walls of *B. subtilis*.

MATERIALS AND METHODS

Cell wall preparation. Cell walls of *B. subtilis* 168 (*trpC2*) were prepared as described previously (7, 20), except that only 1% (wt/vol) hot sodium dodecyl sulfate (SDS) was used to extract autolysin, loosely bound proteins, and nucleic acids. Isolated cell walls were washed twice with boiling SDS and then several times with hot distilled water to remove the detergent. The extracted walls contained 1.6 μmol of phosphorus, 1.5 μmol of hexose, and 90.5 μg of protein per mg of wall, and no DNA or RNA (5). The Lowry-positive value was due largely to peptide bonds in the peptidoglycan and not to actual protein (5). Further extraction did not reduce these values. Cell walls that had not been washed with SDS were permitted to autolyze in 20 mM ammonium carbonate buffer (pH 9.5) at 37°C. The decrease in absorbance at 500 nm was monitored until approximately 50% reduction in turbidity was achieved. The walls were then extracted with SDS and water as described above. All other treatments were performed on cell walls that had been extracted with boiling SDS. Cell walls were digested by lysozyme (10 μg/ml in phosphate-buffered saline [40 mM phosphate-0.15 M sodium chloride]; pH 7.3) during incubation at 37°C for 1 h. TA was removed from cell walls by treatment with 0.1 N NaOH and N_2 gas overnight (13). The procedure used for blocking carboxyl groups with carbodiimide and ethylenediamine was described previously (7). Treated or modified cell walls were washed extensively with distilled water and then freeze-dried before examination by electron microscopy (EM).

EM. Whole mounts and thin sections of cell walls were labeled and prepared for EM by the following procedure. CF (11.5 mg of ferritin per ml; Miles-Yeda) was diluted 1:1 with 0.05 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 6.8) and dialyzed against HEPES for 6 h at 4°C. *B. subtilis* cell walls were suspended in the same buffer to a concentration of 1.0 mg (dry weight) per ml. CF was diluted 1:10 (saturating conditions) in buffer, and the final reaction mixture consisted of 100 μl of CF and 100 μl of cell wall suspension, which was incubated at 22°C for 15 min. Walls were then pelleted by centrifugation and washed five times with 1.5-ml volumes of buffer. For whole mounts, 200-mesh Formvar carbon-coated EM grids were touched to the washed suspension and air dried before

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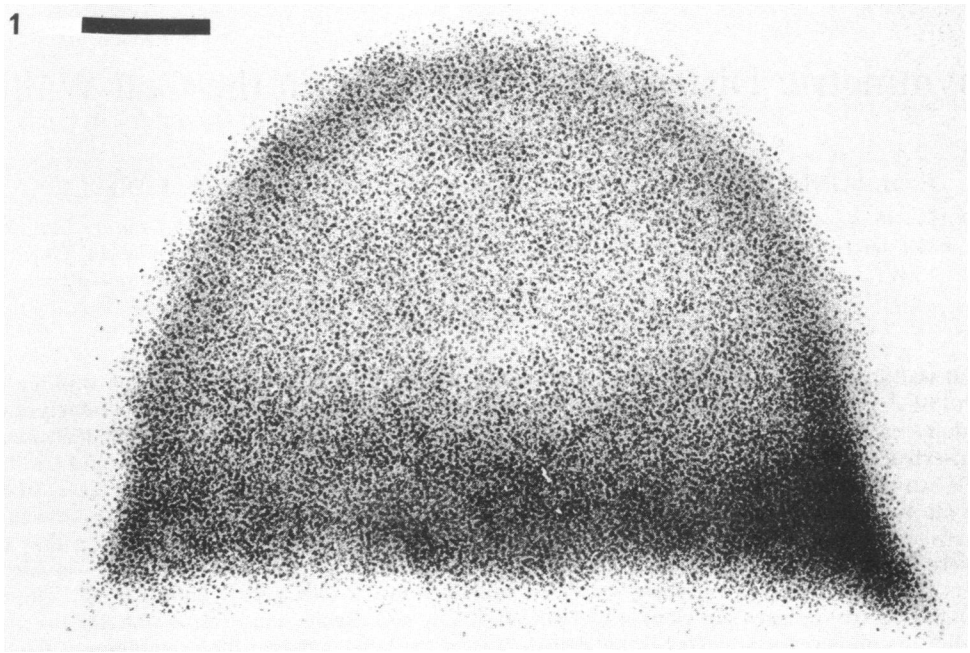


FIG. 1. A whole mount of a polar cap of a *Bacillus subtilis* wall which was stained with CF as the only electron-scattering agent. The entire cap adsorbed the cationic probe, indicating that anionic sites are available over the surface. Bar, 100 nm.

viewing by EM. For thin sections, washed walls were fixed for 60 min at 22°C in 5% glutaraldehyde in 0.05 M HEPES buffer (pH 6.8), washed in buffer, dehydrated through an ethanol-polypropylene oxide series, and embedded in Epon 812. EM was performed with either a Philips EM300 or EM400T electron microscope operating under standard conditions at 60 kV with the cold trap in place. Energy-dispersive X-ray spectroscopy verified that iron, and therefore CF, was the only contrasting agent present in cell wall preparations. In some cases, cell walls were additionally stained with 2% uranyl acetate for 5 min.

RESULTS AND DISCUSSION

The purpose of this study was to examine the distribution of charge on the cell wall of *B. subtilis*. CF, an electron-dense agent capable of complexing with negatively charged molecules, was used as a marker for localizing anionic centers. The results are seen in Fig. 1 through 9. In all cases in which intact, unmodified, or undigested cylindrical or polar wall was seen, CF bound only to the outer surface. Penetration or accessibility of CF to the inner surface was not a problem, since the cavities within the wall fragments were 0.5 μm or greater. This indicated that available anionic sites must be distributed over the entire surface of the wall and that the inner surface was unable to bind the cationic probe. Septa could not bind CF because the anionic groups present are hidden or protected until cell separation occurs, a process which requires autolysis to expose outer pole faces. When carboxylate groups were blocked with ethylenediamine, or when TA was removed with dilute alkali, the wall retained its ability to bind CF in an asymmetric manner. If both carboxyl and phosphate groups were removed, CF did not bind well at all. This indicated that the anionic nature of the wall must be due mainly to TAs and muramyl peptides, although it may be that carboxylate is contributed from some other source, such as a tenaciously bound protein (9, 20) or uronic acid (17). In undigested wall, only surface groups bound CF, because intact cell wall is not permeable to the probe (14). Therefore, negatively charged groups within the wall were not detected. However, lysozyme treatment, which causes hydrolysis of β1-4 linkages in glycan strands, resulted in penetration of the cationic label throughout the entire wall matrix. In contrast, autolysis resulted in CF binding to both inner and outer surfaces, but not within the wall matrix. These results can be interpreted as evidence of outward orientation, away from the cytoplasmic membrane, of negative charges in the cell wall of *B. subtilis*. The outward alignment of negatively charged cell wall groups may represent a favored orientation with respect to biological function. Exposed phosphate and carboxyl groups could act to scavenge cations necessary for the action of autolytic enzymes (12) and the proper maintenance of other necessary cellular processes. Peptidoglycan is synthesized in the membrane, and it is known that TA is inserted together with peptidoglycan as the wall is being assembled (2). The negatively charged membrane may repel the newly synthesized and negatively charged peptidoglycan-TA conjugates. This repulsion of like charges may be involved in pushing peptidoglycan oligomers, with their attached TA and muramyl peptides directed above the plane of the sugar moieties, from the membrane to the inner wall surface. Likewise, the outward orientation of negative charges dictates that TAs and muramyl peptides of each daughter cell would face each other at the newly formed septum. Repulsion of negative charges could create an impetus for cells with newly completed septa to separate.

Lysozyme treatment caused hydrolysis of glycosidic bonds, allowing CF to penetrate and bind throughout the wall matrix. This observation is in agreement with the past finding that subsurface TA is exposed when the wall is partially digested (6, 8), although previously unexposed peptidoglycan carboxyl groups would also be available.

In a previous report (E. M. Sonnenfeld, T. J. Beveridge, and R. J. Doyle, Can. J. Microbiol., in press), we observed that when very dilute solutions of CF were used to complex with cell walls of *B. subtilis*, the label was localized in a

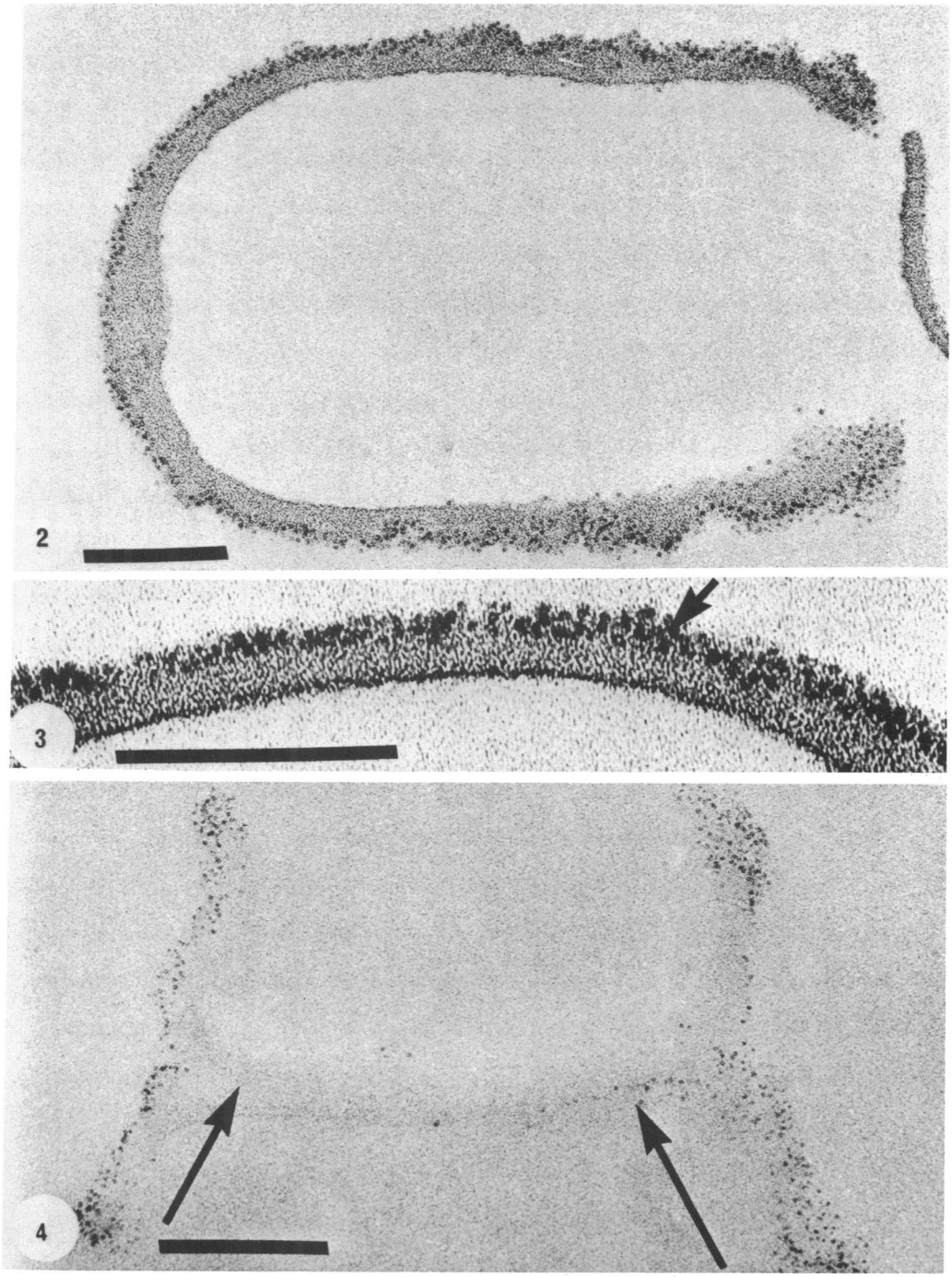


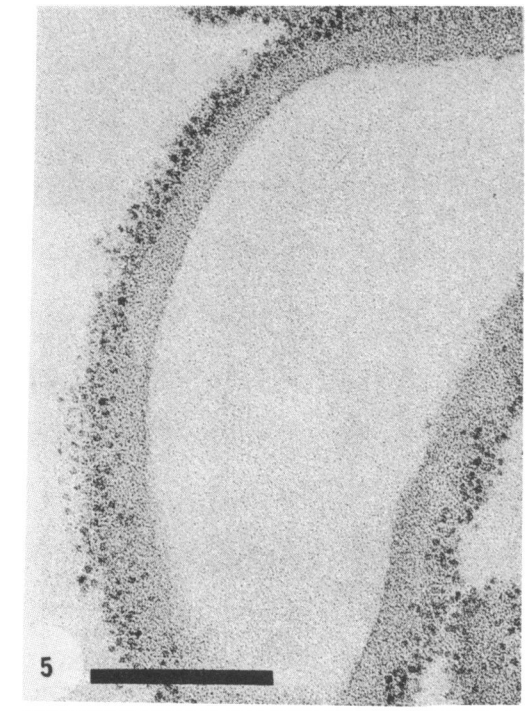
FIG. 2. Thin section of a polar cap which is joined to cylindrical wall stained with cationized ferritin. Only the outer surface of the wall adsorbed the cationic probe. Bar, 100 nm.

FIG. 3. High magnification of a thin section of cylindrical wall, which clearly shows asymmetrical distribution of CF. The arrow points to CF. Bar, 100 nm.

FIG. 4. Thin section of cylindrical wall containing a septum (arrows) which has been treated with CF. Unlike the previous thin sections, this sample was not contrasted with uranyl or lead salts, so that the CF probe is more apparent. Very little CF was adsorbed to the septum, whereas the outer surface of the cylindrical wall was labeled. Bar, 100 nm.

highly electronegative area on cell poles. In the present study, higher concentrations of CF were used, resulting in a loss of resolution of the electronegative area on the cell pole. The CF label did, however, bind only on outer wall faces (Fig. 2, 3, and 4) unless the walls had been partially degraded by lysozyme or autolysis (Fig. 8 and 9).

The results reported here do not agree with those of Frehel et al. (10). Those workers found that CF could bind equally well to the outer and inner surfaces of isolated *B. subtilis* cell walls. These differences may be a reflection of the methods involved in the isolation of cell walls. If walls are permitted to autolyze during the extraction procedure, then, according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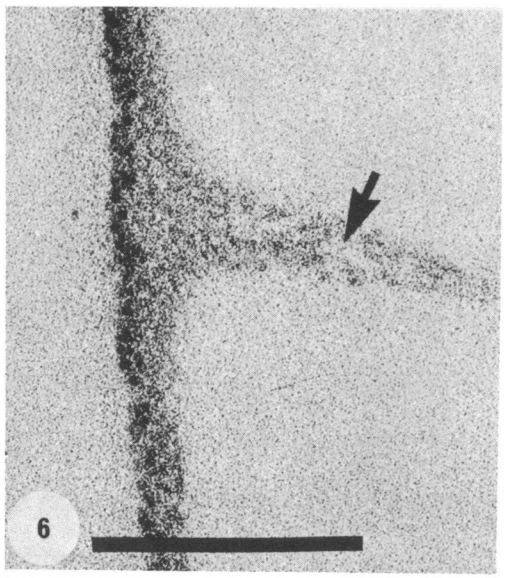
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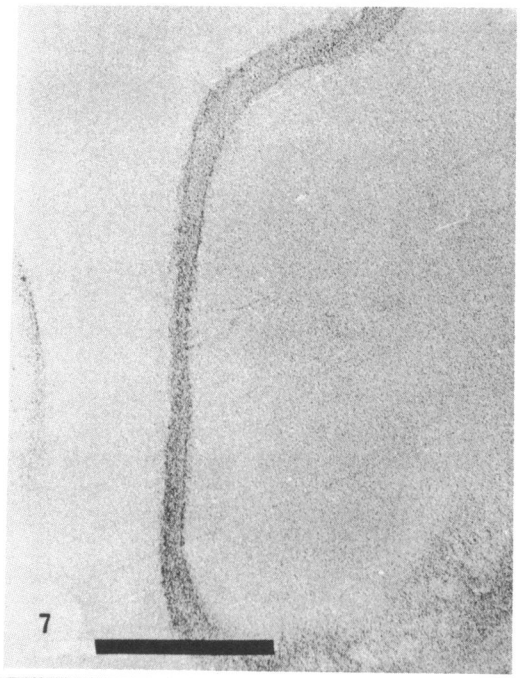
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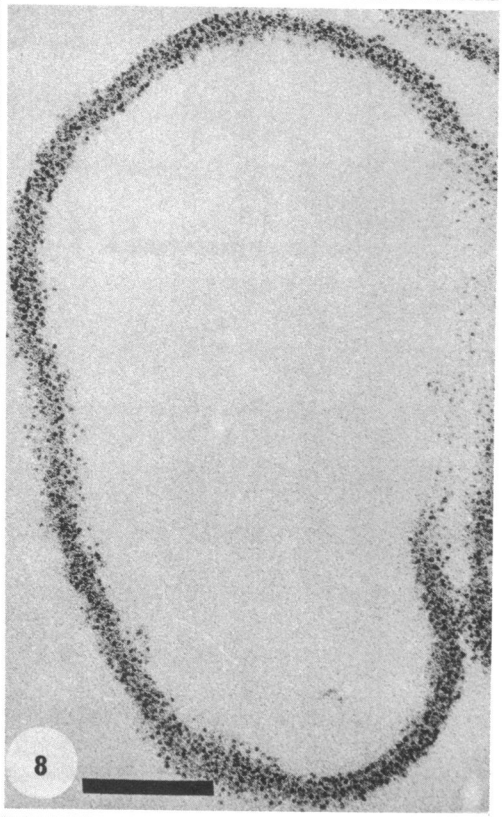
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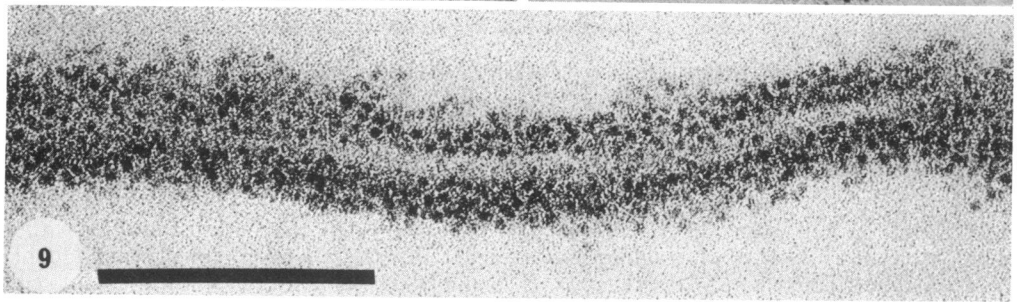
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to our results, CF would bind to both inner and outer surfaces. This observation indicates that cell wall closest to the membrane is susceptible to autolytic attack, even though it appears to be insensitive to turnover in living cells (15). Under normal conditions, the membrane may regulate autolysins of the inner wall surface in such a way that turnover is impossible. Since our isolated walls were devoid of membrane, this stringent autolysin control was absent.

In EM, many bacilli demonstrate surface irregularities. This irregularity arises as the result of inside-to-outside growth, such that an intact layer is deposited outside the cytoplasmic membrane. With further additions, this layer moves outward and is elongated as the cell grows. Eventually it is subject to rupture, mainly owing to autolysins. These endopeptidic or glycosidic cuts open the structure and render it more porous (16). Evidently, the material becomes so porous that large particles like CF can find many more sites to bind on the external face, both to carboxyl groups of exposed peptidoglycan and also to phosphate groups of TAs.

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FIG. 5. Thin section of a cell wall that was chemically modified with carbodiimide and ethylenediamine to reverse anionic carboxylate sites on peptidoglycan before treatment with CF. TA should have been the only anionic polymer in these walls, and the asymmetric distribution of the cationic probe was still retained. Bar, 100 nm.

FIG. 6. The binding of CF to TA-free cell wall. This wall had TA extracted by mild alkali treatment. The peptidoglycan should have been the only anionic polymer left, yet the asymmetric distribution of CF was retained. Bar, 100 nm.

FIG. 7. Binding of CF to TA-free and carboxyl-modified cell walls. This wall had TA extracted and the carboxylate groups of the peptidoglycan reversed by ethylenediamine treatment. CF was not adsorbed to these walls. Bar, 100 nm.

FIG. 8. Interaction between CF and lysozyme-treated cell walls. This wall was treated with lysozyme to partially hydrolyze the β1-4 linkages in the glycan strands of peptidoglycan. The wall fabric was so broken up that CF penetrated and was adsorbed throughout the wall matrix. Bar, 100 nm.

FIG. 9. The effect of autolysis on the uptake of CF by cell walls. This wall underwent partial autolysis. This treatment disrupted the natural charge asymmetry seen in native walls, and both surfaces (inner and outer) were labeled by the probe. Bar, 100 nm.