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Sites of Metal Deposition in the Cell Wall of *Bacillus subtilis*

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Amine and carboxyl groups of the cell wall of *Bacillus subtilis* were chemically modified individually to neutralize their electrochemical charge for determination of their contribution to the metal uptake process. Mild alkali treatment removed ca. 94% of the constituent teichoic acid (expressed as inorganic phosphorus) and allowed estimation of metal interaction with phosphodiester bonds. Chemical modifications of amine functions did not reduce the metal uptake values as compared to native walls, whereas extraction of teichoic acid caused a stoichiometric reduction in levels. In contrast, alteration of carboxyl groups severely limited metal deposition of most of the metals tested. X-ray diffraction and electron microscopy suggested, in this case, that the form and structure of the metal deposit could be different from that found in native walls. The observations suggest that carboxyl groups provide the major site of metal deposition in the *B. subtilis* wall.

The cell wall of *Bacillus subtilis* and many other gram-positive bacteria provides the bacterium with a rigid and protective sacculus interposed between the cell and its environment. It consists of a highly organized collection (19, 39) of anionic hetero- and homopolymers which are mostly polysaccharides. When grown in the presence of phosphate and reduced magnesium, the bacteria produce walls consisting primarily of teichoic acid and peptidoglycan (1, 36, 38), although a galactosamine polymer (37) and a bound protein (14) may be present.

All soluble and colloidal material, such as organic nutrients and essential metals, must contact and percolate through the wall substance before gaining access to the plasma membrane and, ultimately, the cytoplasm. Likewise, metabolic wastes must be transferred through the wall meshwork before extracellular liberation. It is to be expected that the polyanions of the wall would interact with and bind the cations of the aqueous environment. This has been demonstrated (3, 5, 9, 12, 18, 21, 32, 35), and, in the case of *B. subtilis*, the amounts can be substantial (3-5). The metal binding appears to be at least a two-step process, in which the first event is a stoichiometric interaction between metal and reactive chemical groups in the wall fabric. The next event is an inorganic deposition of increased amounts of metal, which can be readily discerned by electron microscopy (4-7) because of the electron scattering by the walls.

Little is known about the active sites of metal deposition within the wall, although the phosphodiester groups of teichoic acid have been implicated in the binding of magnesium (23, 30). This study attempts to locate the active sites by comparing the metal uptake values of native *B. subtilis* walls with those in which the electrochemical charges (reactive groups) of the various wall constituents have been chemically modified or removed. In addition, the nature of the metal deposit has been studied by X-ray diffraction, and its electron-scattering ability has been studied in thin sections.

MATERIALS AND METHODS

Cell wall preparation. Cell walls of *B. subtilis* Marburg (University of Western Ontario collection no. 1032) were prepared from an exponentially growing culture as previously described (5). The purity of the wall fragments was ascertained by electron microscopy (negative stains and thin sections) and by testing for NADH oxidase as a marker for plasma membrane enzymes.

Chemical modification of reactive groups within the wall fabric. (i) Amine groups. Amine groups were chemically neutralized by the addition of *s*-acetylmercaptosuccinic anhydride (Eastman Organic Chemicals, Rochester, N.Y.) so that both a carboxyl and sulfhydryl group were introduced (28; see Fig. 1). The anhydride was dissolved in ethanol to make a 20 mM solution. Five milliliters of this solution was added to a suspension of bacterial walls (50 mg [dry weight] of walls in 45 ml of deionized and double-distilled water) to produce a final anhydride concentration of 2 mM. The reaction was carried out under nitrogen gas at a constant pH of 6.8 with constant stirring for 6 h at 22°C. At this time the walls were pelleted by centrifugation, washed three times with 50-ml volumes of deionized and double-distilled water by centrifugation, and dialyzed against 6 liters of deionized, double-distilled water for 12 h at 4°C.

(ii) Detection of number of amine groups modified. Two separate methods were used to determine

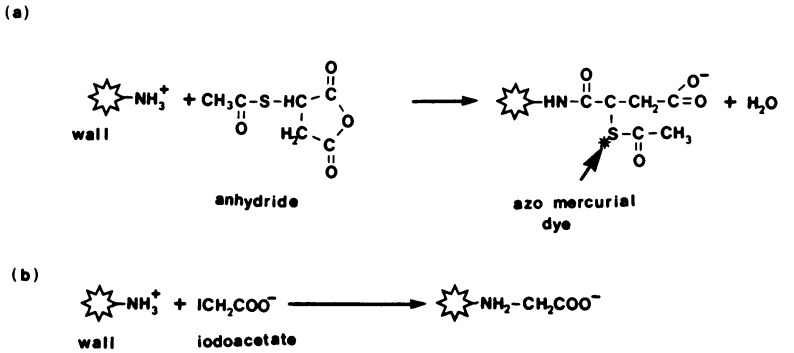

FIG. 1. *(a) The outline of the reaction between B. subtilis cell walls and s-acetylmercaptosuccinic anhydride. The large arrow points to the binding site of the azo-mercurial dye used to stain the walls after the reaction. (b) The reaction between the walls and sodium iodoacetate.*

the number of amine groups affected by the anhydride reaction: the ninhydrin method of Moore and Stein (34) and the azo-mercurial colorimetric assay of Horowitz and Klotz (24). In the first instance, a reduction of the free amine groups as compared to those in native walls was used to estimate the blocked sites; in the second method, the uptake of 4-(*p*-dimethylaminobenzene-azo) phenyl mercuric acetate by the sulfhydryl group of the wall-bound anhydride was monitored at 460 nm. In this case, 2.5 mg of anhydride-wall was suspended in 48 ml of heptanol-saturated 0.1 M glycine buffer (pH 10.5; Sigma Chemical Co.) containing 1 mM $MgCl_2$, and 2 ml of 6×10^{-6} M dye dissolved in heptanol was added. The reaction proceeded for 6 h at 22°C in the dark (to inhibit *trans-to-cis* conversion of the dye), and at this time the organic phase was carefully separated from the aqueous phase. The organic phase was monitored for a decrease in absorbance at 414 nm as compared to the original stock solution, whereas the aqueous phase, which contained the wall fragments, was monitored for an increase in absorbance at 460 nm. Native walls were also treated with the dye in the same manner and served as the control. In both cases the natural light-scattering ability of the wall fragments was reduced by the addition of equal volumes of glycerol.

(iii) Amine and hydroxyl groups. Fifty milligrams of walls was treated in 50 ml of aqueous 0.05 M sodium iodoacetate (Eastman Organic Chemicals) maintained at pH 8.0 for 6 h at 22°C, and then washed and dialyzed as outlined above under amine groups. This reagent typically attached to amine functions at low or neutral pH (22; see Fig. 1), but may bind to phenolic or hydroxyl groups at the higher pH (29).

(iv) Detection of carboxymethylated groups. Two methods were used to detect carboxymethylated groups: the ninhydrin method (34), using leucine as a standard, estimated the reduction in free amino groups, whereas titration for free iodide (2) in the reaction supernatant estimated total carboxymethylation.

(v) Carboxyl groups. Three chemical ligands of different electrochemical charge were attached by carbodiimide reaction (16): glycine ethyl ester (Eastman Organic Chemicals) neutralized the carboxyl charges of the wall; glycinamide (Aldrich Chemical Co.) made the walls slightly electropositive; and ethylenediamine (Fisher Scientific Co.) made them distinctly electropositive (Fig. 2). In each case, a 50-ml aqueous suspension of wall fragments was made 0.5 M with the chemical ligand, and enough 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (Storey Chemical Corp.) was added to make the carbodiimide concentration 0.2 M. The reaction mixture was continuously stirred for 6 h at 22°C at a constant pH of 4.75, and after this period of time it was washed and dialyzed as previously described.

(vi) Detection of modified carboxyl groups. For both the glycine ethyl ester and glycinamide situations, 5 mg of the walls was hydrolyzed in 6 M HCl at 110°C for 72 h, and the quantity of glycine, as compared to native walls, was detected by a Beckman model 102C Amino Acid Analyzer. For the ethylenediamine situation, the increase in free amino groups was detected by the ninhydrin reaction (34).

(vii) Extraction of teichoic acid. Fifty milligrams of walls was suspended in 50 ml of aqueous 0.1 N NaOH and agitated by a stream of nitrogen gas at 35°C for 24 h according to the method of Hughes and Tanner (27). The walls were washed and dialyzed as previously described. The supernatant of the reaction mixture was pooled with the washing fluids and freeze-dried. Both this concentrate and a 5-mg sample of extracted walls were used for determination of phosphorus (15) to estimate the efficiency of the extraction. Therefore, throughout the paper, teichoic acid will be expressed as micromoles of inorganic phosphorus.

Reaction of walls with metal solutions. One milligram (dry weight) of either native or chemically altered walls was incubated in 2 ml of a 5 mM solution of metal salt, washed, and processed for inorganic analysis as outlined by Beveridge and Murray (4, 5). Since the extraction of teichoic acid removed 54.3% of the dry weight of the walls (4), the metal uptakes of teichoic acid-less walls used 0.457 mg per 2 ml of the reaction mixture. This allowed easy comparison between these walls and the chemically modified walls. The reactivity of the chloride salts of Na, K, Mg, Ca, Mn, FeIII, Cu, and AuIII, and of $Ni(NO_3)_2$, on the altered walls was extensively studied for comparison

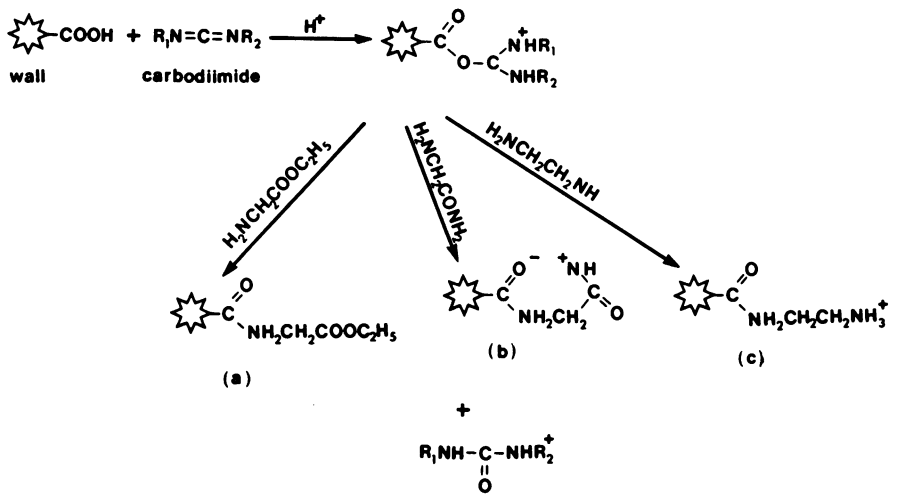

FIG. 2. *The outline of the reaction between walls activated by carbodiimide linkage of: (a) glycine ethyl ester; (b) glycinamide; and (c) ethylenediamine.*

with the uptake values of native walls, but select heavy-metal salts [e.g., $PdSO_4$, $ScCl_3$, $In_2(SO_4)_3 \cdot 9H_2O$, $LaCl_3 \cdot 6H_2O$, $PrCl_3$, $Sm_2(SO_4)_3 \cdot 8H_2O$, and $Ce(OH)(NO_3)_3 \cdot 3H_2O$ (all ICN Canada Ltd.)] were also used.

Most metals were detected by atomic absorption analysis using a Perkin-Elmer model 403 atomic absorption unit in either the flame or graphite furnace mode as previously described (5). The heavy-metal analyses were done by the X-ray fluorescence technique, using a Philips PW1450 Atomic Sequential X-ray Spectrophotometer (4).

Electron microscopy and X-ray diffraction. The walls for electron microscopy were processed as described above. After unbound metal was washed from the walls, unfixed whole mounts were made by floating grids on drops of wall suspensions for 15 s and blotting them dry. No staining reagents other than the original metal solutions were used. For thin-sectioned material, both unfixed and fixed walls were used. In the former case, washed walls were immediately dehydrated through an ethanol-propylene oxide series to Epon 812. Other walls were fixed in aqueous 4% glutaraldehyde (Polysciences, Inc.) for 30 min, well washed with deionized and double-distilled water, and dehydrated through an ethanol-propylene oxide series to Epon 812. No metal but the metal bound during uptake reaction was used as an electron-scattering agent (4–7), and all the reagents were tested to eliminate stray contamination. Thin sections were examined with a Philips EM300 electron microscope operating under standard conditions at 60 kV.

For X-ray diffraction, small samples of the plastic embeddings used for electron microscopy were mounted on glass fibers, and each was suspended in the center of a Gandoffi powder camera (diameter, 57.3 mm). Each sample was analyzed using suitable filtered radiation (e.g., FeK alpha radiation for gold), and identification was obtained by comparison with the Joint Committee on Powder Diffraction file (1974) for each element.

Preparation of [^{3}H]diaminopimelic acid-labeled walls. Cell walls labeled with [^{3}H]diaminopimelic acid were prepared according to reference 5 and were used to determine deleterious effects exerted on the walls during either the teichoic acid extraction or the various chemical modifications.

RESULTS

Modification of amine groups. Nearly 0.3 μmol of $-NH_3^+$ (96 to 100% of that available) was affected by the *s*-acetylmercaptosuccinic anhydride treatment as determined by both the ninhydrin and azo-mercurial methods (Table 1). Since the ninhydrin reaction is not a high-resolving assay for substituted amino groups, one other assay system for each chemical modification (e.g., azo-mercurial test for the anhydride and free I^- test for the iodoacetate modifications) was performed for correlation of the data. It was interesting that the mercury of the azo-mercurial dye could be detected by its electron-scattering ability in the thin sections of the anhydride-modified walls (A-walls), and that the dye was equally distributed in the wall, indicating that amines were affected throughout the wall fabric by the treatment (Fig. 3A). Native walls treated with the dye did not show any scattering power, which confirmed the absence of the endogenous sulfhydryl groups in the *B. subtilis* wall (Fig. 3B).

Approximately 0.3 μmol of $-NH_3^+$ (98% of those available) was detected in iodoacetate-treated walls (I-walls) by the ninhydrin reaction, which compared favorably with the A-wall values (Table 1). On the other hand, detection of free iodide indicated a greater degree of carboxymethylation in the I-walls (127%) than that

attributable to amine functions alone (i.e., 0.380 versus 0.294 μmol/mg of walls). Possibly this could indicate high pH-carboxymethylation of exposed hydroxyl groups in the wall (29).

Table 2 compares the metal uptake values of native walls, A-walls, and I-walls for nine metals. In all cases but Fe and Cu, the uptake values were approximately equal to or greater than the native wall situation. Both the A-walls and the I-walls contained between 75 and 80% of the normal iron content, and electron microscopy revealed that these walls possessed a reduced number of typical iron crystalloids (5). The Cu content, on the other hand, dropped by ca. 72% of the normal uptake value, and these walls (both A- and I-walls) possessed little electron-scattering power.

TABLE 1. *Determination of modified amine groups*[a]

Treatment	No. of amine groups affected (μmol/mg of walls)	
s-Acetylmercaptosuccinic anhydride (A-walls)	0.288[b]	0.302[c]
Sodium iodoacetate (I-walls)	0.380[d]	0.294[c]

[a] Each assay system was repeated four times, and the values are expressed as the mean. α_s^b = 1%; α_s^d = 6%; α_s^c = 7 and 9%.
[b] As determined by the azo-mercurial dye.
[c] As determined by the ninhydrin method.
[d] As determined by titration for free iodide.

Modification of carboxyl groups. Amino acid analysis of the native walls revealed that the Glu:Ala:Dpm:Gly ratio was 1:2:1:0 (Table 3). The increased glycine content of glycine–ethyl ester-modified walls (GE-walls) and of glycinamide walls (G-walls) allowed the determination of altered carboxyl groups (Table 4). Those groups affected by ethylenediamine (E-walls) were detected by the ninhydrin method (Table 4). In all three cases, the amounts detected compared favorably with the glutamic acid content of native walls (compare Table 3 with Table 4).

The metal uptake values were reduced in all three cases as compared to the native wall situation (Table 5). In general, the reduction corresponded directly to the increase in electroposi-

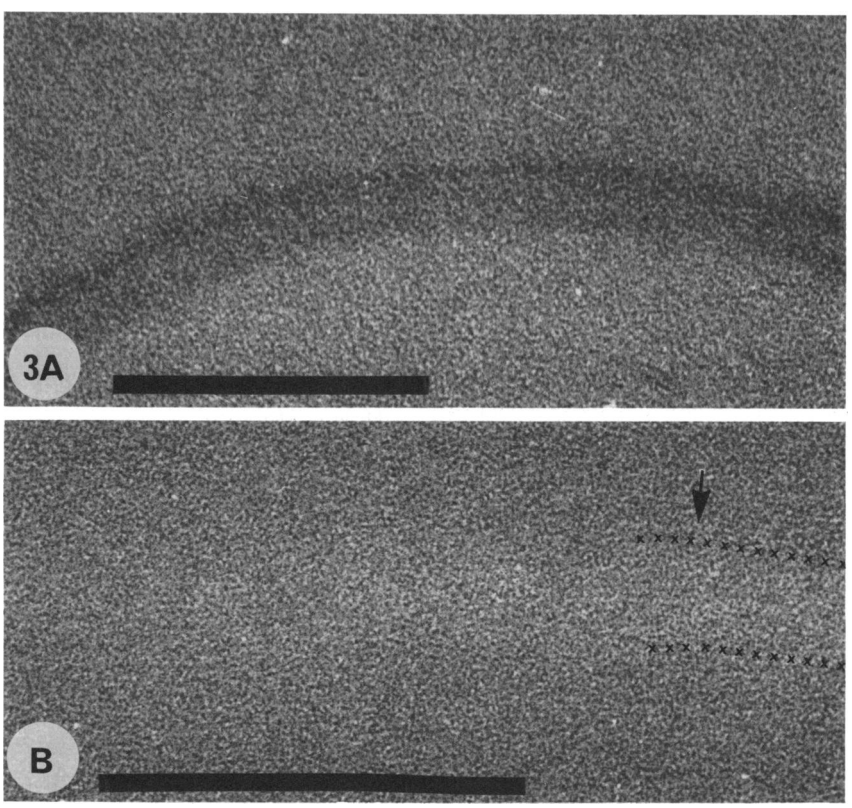

FIG. 3. *(A) Portion of a thin-sectioned wall which has had a sulfhydryl introduced to the constituent amine groups by s-acetylmercaptosuccinic anhydride reaction and has been stained by the azo-mercurial dye. (B) A native wall (no sulfhydryl groups) which has been stained with the azo-mercurial dye. The wall is the light area (arrow) outlined in part by the ×'s. Bar = 100 nm.*

TABLE 2. *Metal uptake values for B. subtilis walls with chemically modified amine groups*[a]

Metal	Modification to amine groups		Native walls (μmol/mg of wall)
	s-Acetylmercaptosuccinic anhydride (A-walls) (μmol/mg of wall)	Sodium iodoacetate[b] (I-walls) (μmol/mg of wall)	
Na	2.680	2.206	2.697
K	1.600	2.000	1.944
Mg	7.664	8.806	8.226
Ca	0.851	1.082	0.399
Mn	0.820	0.880	0.801
FeIII	2.680	2.860	3.581
Ni	0.320	0.341	0.107
Cu	0.760	0.860	2.990
AuIII	0.391	0.427	0.363

[a] The values for the modified walls are the mean from four separate uptake experiments. α_s = 0.1 - 4.0%. The values for the native walls are from reference 4.

[b] Hydroxyl groups may also be modified at the high pH of this reaction.

TABLE 3. *Amino acid analysis of B. subtilis walls*

Amino acid	μmol/mg of wall	Molar ratio
Glu	0.254	1
Ala	0.568	2
Dpm	0.233	1
Gly	0.014	0

tivity of the bound ligand (i.e., GE →→→ E-walls). The reduction was most noticeable with the monovalent alkali metals (Na and K), where there was a complete absence of detectable metal in all three types of walls. In all other cases, GE-walls contained more metal than E-walls.

The reduction in metal deposition was so pronounced that it could easily be detected by electron microscopy (compare Fig. 4A and B). In fact, the nature of the metal deposit was often altered from that found in native walls (compare Fig. 5A and B). For example, E-walls were devoid of typical iron crystalloids, the size and granularity of cerium or palladium deposits were greatly reduced in G- and E-walls, and several of the lanthanides (notably La, Pr, and Sm) formed aggregates of oxide hydrate on the surfaces of G- and E-walls. The effect was more complicated with walls exposed to a gold chloride solution. Native walls accumulated elemental gold (Fig. 7; reference 5), but as the electropositivity increased (GE → E-walls), there was a progressive accumulation of the hydroxide (Fig. 8 and 9), which is more typical of this unstable aquo-ion (11).

Extraction of teichoic acid. The native walls contained 54.3% of their dry weight as teichoic acid when grown under the described conditions (4). Treatment with 0.1 N NaOH extracted 93.8% (1.04 μmol of P_i per mg of wall) as determined by phosphorus analyses, and the treatment did not disturb the typical ultrastructure of the wall in thin section (Fig. 6).

The metal uptake values for these walls can be found in Table 6; in all instances there was a reduced metal deposition. In the case of Na and K, the reduction was approximately equal to the amount of teichoic acid (as phosphorus) extracted. For Mg and Cu, the uptake was ca. 50% of the teichoic acid, whereas almost all Ca binding was removed.

Detection of leached [^{3}H]diaminopimelic acid during chemical modification and extraction of walls. Walls with a tritium label in the diaminopimelic acid of the peptidoglycan (5)

TABLE 4. *Determination of modified carboxyl groups*

Treatment[a]	No. of carboxyl groups affected (μmol/mg of walls)
Glycine ethyl ester (GE-walls)	0.278[b]
Glycinamide (G-walls)	0.266[b]
Ethylenediamine (E-walls)	0.292[c]

[a] Ligands linked by carbodiimide reaction.

[b] As determined by amount of additional glycine in the walls.

[c] As determined by the ninhydrin method; mean of four observations. α_s = 8%.

TABLE 5. *Metal uptake values for walls with chemically modified carboxyl groups*[a]

Metal	Modification to carboxyl groups			Native walls (μmol/mg of wall)
	Glycine ethyl ester[b] (GE-walls) (μmol/mg of wall)	Glycinamide[c] (G-walls) (μmol/mg of wall)	Ethylenediamine[d] (E-walls) (μmol/mg of wall)	
Na	0	0	0	2.697
K	0	0	0	1.944
Mg	0.520	0.300	0.160	8.226
Ca	0.380	0.360	0.300	0.399
Mn	0.732	0.680	0.100	0.801
FeIII	2.260	0.240	0.240	3.581
Ni	0.024	0.004	0.004	0.107
Cu	0.993	0.506	0.260	2.990
AuIII	0.214	0.103	0.018	0.363

[a] The values for the modified walls are the mean of four separate uptake experiments. α_s = 0.1 - 3.0%. The values for the native walls are from reference 4.

[b] COO^- is neutralized.

[c] COO^- is slightly electropositive.

[d] COO^- is electropositive.

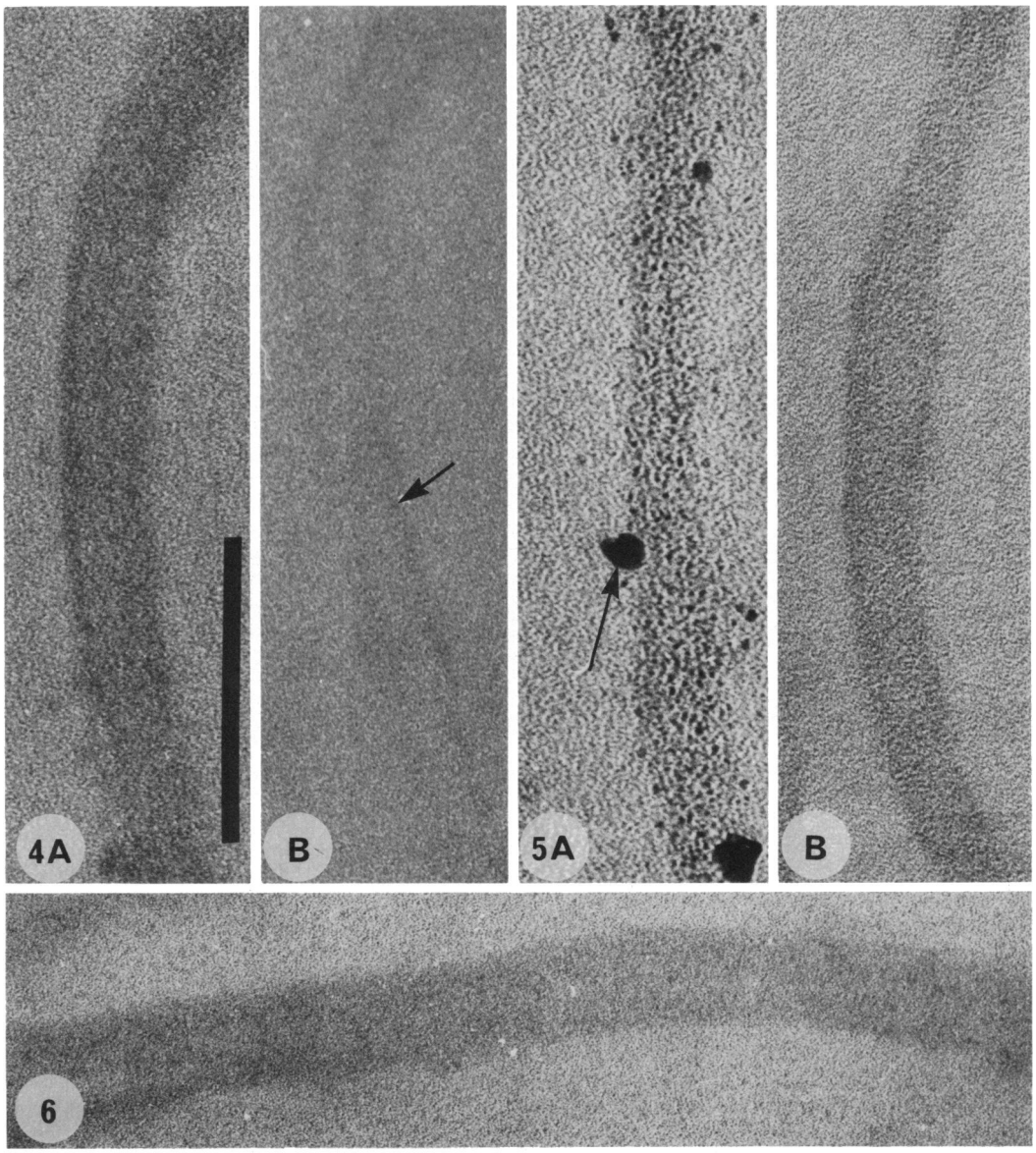

FIG. 4. *(A) Portion of a native wall which has been stained with indium as the only electron-scattering agent. (B) A wall which has had the carboxyl groups neutralized by carbodiimide linkage of glycinamide and has then been stained with indium. Arrow points to the wall. E-walls had no discernible scattering properties.*

FIG. 5. *(A) Portion of a native wall which has been stained with palladium as the only electron-scattering agent. Arrow points to a large staining deposit. (B) A wall which has been neutralized with glycinamide. The palladium aggregates are smaller, and the wall has less electron-scattering power.*

FIG. 6. *Portion of a wall which has had the teichoic acid removed by dilute alkali. It has been saturated with uranyl acetate as the only electron-scattering agent before fixation (see reference 4). Bar = 100 nm.*

were monitored for leached [*G*-^{3}H]diaminopimelic acid during each of the chemical modifications and the teichoic acid extraction to obtain an index of murein integrity. In no instance did this amount account for more than 0.01% of the labeled diaminopimelic acid.

DISCUSSION

The cell wall of *B. subtilis* Marburg 168 consists primarily of two polymeric constituents: teichoic acid, which is an anionic polymer of α-D-glycopyranosyl glycerol phosphate (1), and

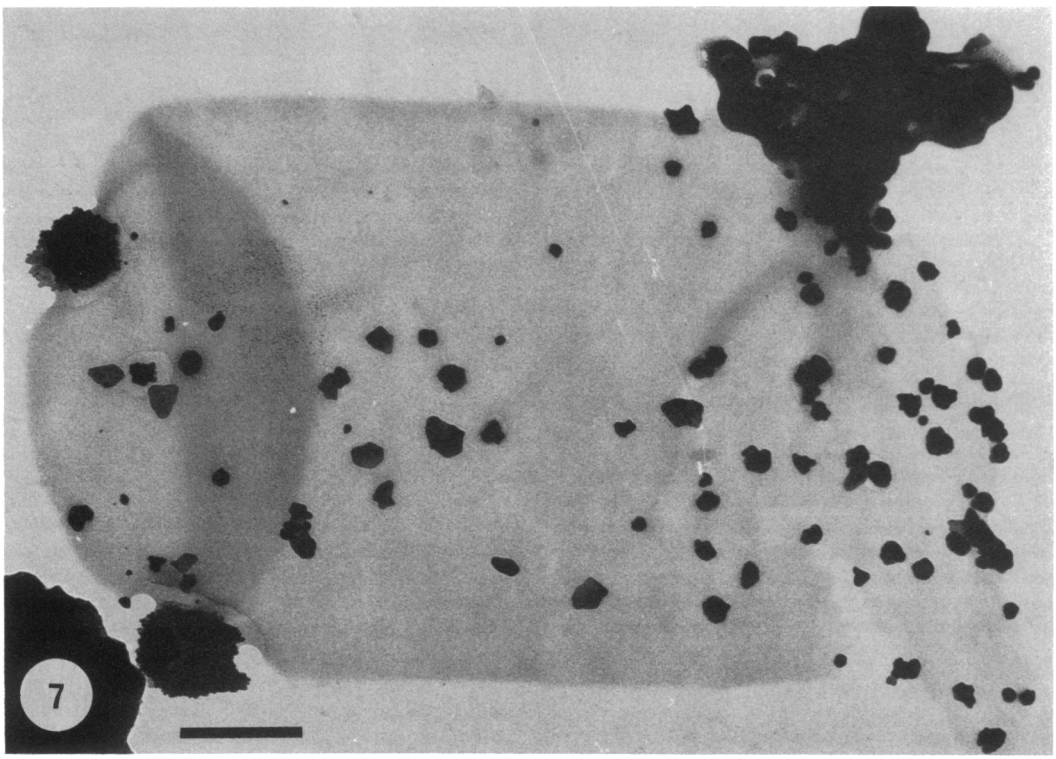

FIG. 7. *Whole mount of an unfixed wall which was suspended in a 5 mM gold chloride solution for 10 min and was well washed before electron microscopy. No staining reagent other than the gold was used. Bar = 100 nm.*

peptidoglycan, which is an alternating polymer of β-(1,4)-linked *N*-acetylglucosamine and *N*-acetylmuramic acid residues. Approximately 35% of the polysaccharide strands of the peptidoglycan are linked to one another through the D-alanyl-(L)-*meso*-diaminopimelyl bonds of its constituent short peptide chains [L-Ala-D-Glu-(L)-*meso*-Dpm-D-Ala] (26, 40). Therefore, these walls consist of sugar, phosphate, and amino acid residues which give the fabric an anionic charge density and make them an appropriate model system for studying the interaction of metals in solution with biological polymers.

Using this system, we have demonstrated that relatively large amounts of metal can bind with the wall, and we have suggested that a two-step mechanism is possible (5): i.e., the initial binding reaction occurred between stoichiometric amounts of soluble metal and reactive sites within the wall. This metal then nucleated an inorganic deposition, which accumulated non-stoichiometric amounts. That this metal is strongly bound has been demonstrated by the failure of hydrated amorphous silica to leach the walls under geochemical conditions (3), although partial digestion of the wall enhances metal replacement (5).

The wall offers a number of potential sites for metal binding (Fig. 10). Discrete reactive sites within the wall can be modified by specific chemical probes; e.g., amino functions react specifically with *s*-acetylmercaptosuccinic anhydride, which converts the electropositive charge to negative. Likewise, carboxymethylation will render these groups electronegative, but, at higher pH, may neutralize hydroxyl functions (29). Distinctly anionic sites, such as glutamic carboxyl and teichoic phosphodiester groups, should be of paramount importance in the metal-binding process. Carbodiimide linkage of specific ligands can neutralize (e.g., glycine ethyl ester) or reverse (e.g., ethylenediamine) carboxyl charges (16), whereas mild alkaline conditions extract teichoic acid (phosphodiester groups) from the wall (27), although a galactosamine polymer may also be liberated (37).

Amine groups, being electropositive, are not considered to be potent chelators for most metals, but they cannot be entirely discounted. Some metals form anionic complexes in aqueous

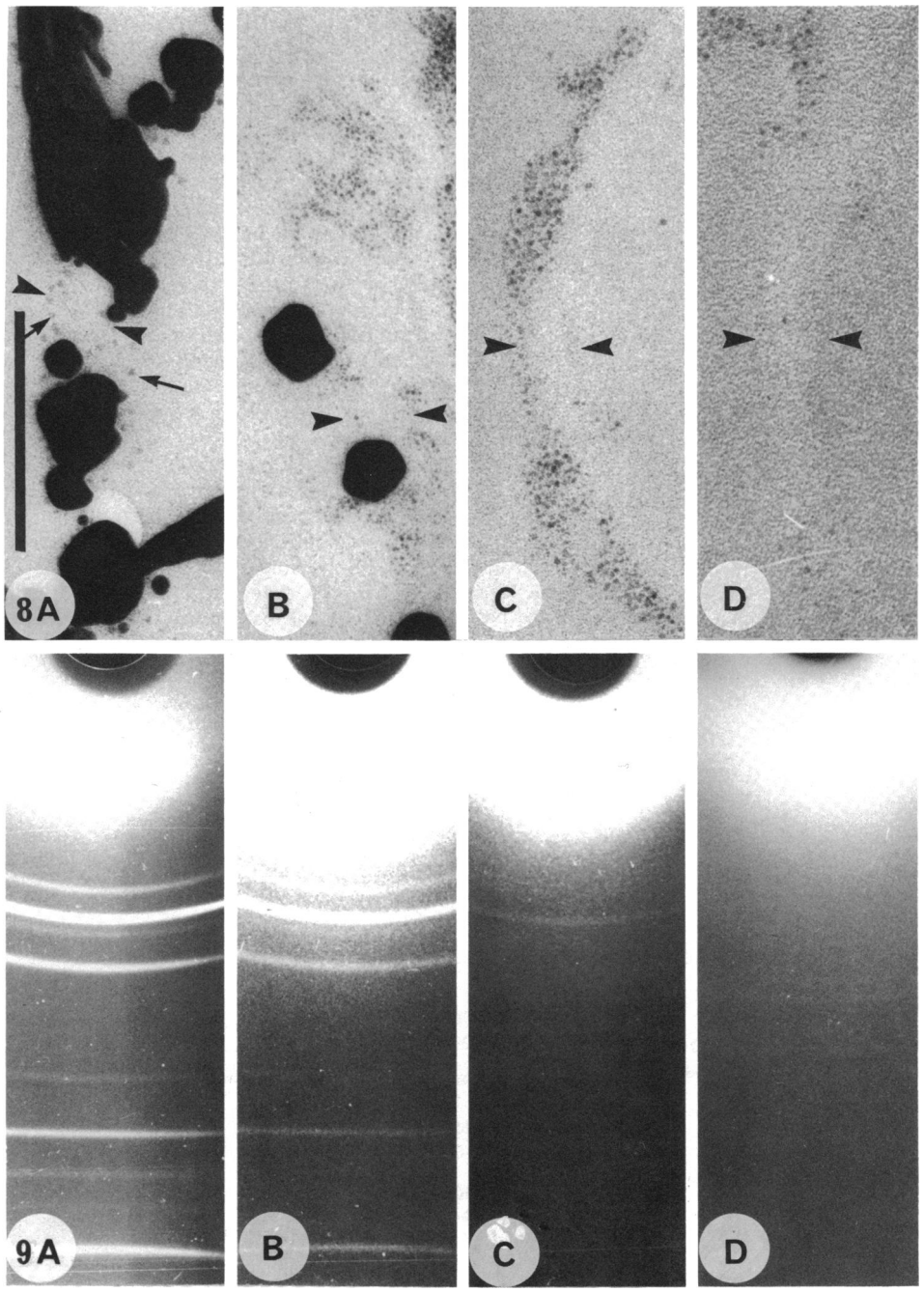

FIG. 8. *A sequence of thin sections of walls which were treated with gold chloride, as in Fig. 7, before they were fixed and embedded. (A) Native wall; (B) GE-wall; (C) G-wall; and (D) E-wall. The small arrows in (A) point to small ferritin-concanavalin A conjugates (5) which delimit the wall surfaces. This staining reagent was not used in (B), (C), and (D). The larger arrows in all thin sections point to the wall boundaries. All micrographs are the same magnification, and the bar in (A) = 100 nm.*

FIG. 9. *A sequence of X-ray diffractograms produced by the walls shown in Fig. 8. (A), (B), (C), and (D) correspond directly with their counterparts in Fig. 8.*

TABLE 6. *Uptake values of walls devoid of teichoic acid (inorganic phosphorus)*[a]

Metal	Extracted walls (μmol of P_i per 0.457 mg of walls[b])	Native walls (μmol of P_i per mg of walls[b])	Difference (μmol)	Stoichiometry based on teichoic extract[c]
Na	1.497	2.697	1.200	1.0
K	0.782	1.944	1.162	1.0
Mg	7.683	8.226	0.543	0.5
Ca	0.012	0.399	0.387	0.4
Mn	0.656	0.801	0.145	0.1
FeIII	1.720	3.581	1.861	2.0
Ni	0.021	0.107	0.086	0.1
Cu	2.488	2.990	0.502	0.5
AuIII	0.265	0.363	0.098	0.1

[a] The values for the extracted walls are the mean of four separate uptake experiments. α_s = 0.1 - 1.0%. The values for the native walls are from reference 4.

[b] Since 54.3% of the wall was teichoic acid, the uptake values of the extracted walls are per 0.457 mg for easy comparison with the native wall results.

[c] 1.04 μmol of inorganic phosphorus extracted per mg of walls.

solution [e.g., $Pb(OH)_3^{4-}$, $Pb(OH)_4^{2-}$, etc.] which can bind to these groups. In fact, Cu^{2+} preferentially binds to amines over carboxylates (25), and it is noted that with both A- and I-walls the copper-bonding capacity is drastically reduced (Table 2). In general, most metals are bound in equal or greater amounts as compared to the native wall results (Table 2). It is not surprising that some had increased binding, since the chemical alteration introduced exogenous carboxyl groups to the wall (Fig. 1).

It was of interest that free iodide detection during the iodoacetate reaction indicated wider carboxymethylation than attributable to the amine groups alone (Table 1). Also, I-walls bound more metal than A-walls, presumably due to the increased number of exogenous COO^- available. Possibly, some hydroxyls of the muramic acid residues were altered (Fig. 10b), although we cannot entirely discount other possibilities (e.g., efficiency of methods for I^- versus $-NH_3^+$ detection, etc.).

The iron uptake of these modified walls was also reduced. Electron microscopy of thin sections of both types of wall revealed a reduction

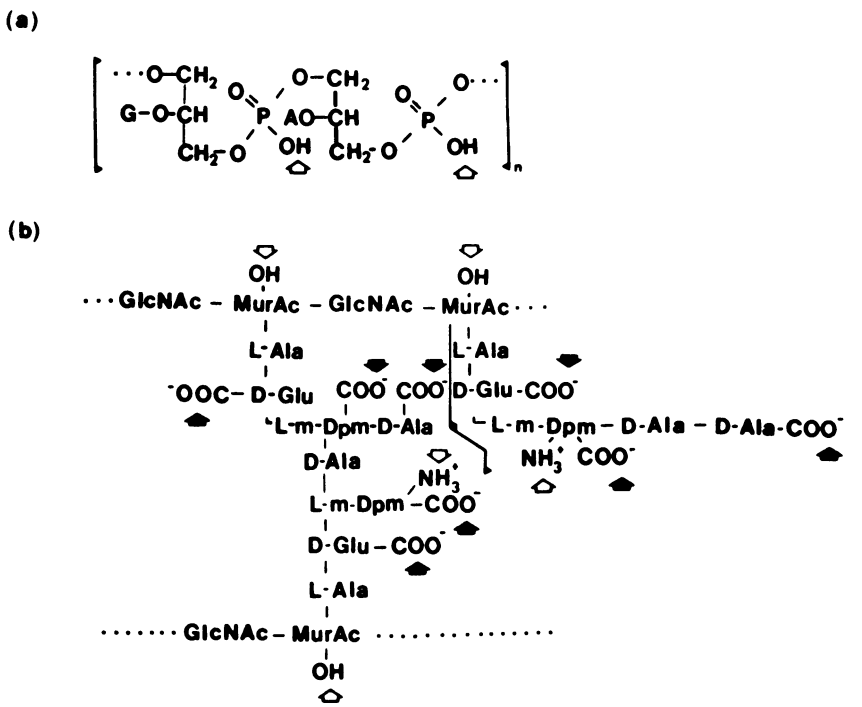

FIG. 10. *(a) Structure of the teichoic acid in the B. subtilis wall which may be partially substituted with ester-linked* D*-alanine (D) and which contains* D*-glucose (G) attached to the glycerol backbone (36). Arrows point to the ionizable groups of the phosphodiester bonds. n = ca. 20 to 30 residues. (b) Structure of the peptidoglycan which shows both the transpeptide-linked and unlinked conditions. About 35% of the glycan strands are cross-linked, and, in this case, five carboxyl and one amine group should be available. Each unlinked pentapeptide has three carboxyl and one amine groups. Some ionizable groups are indicated; the solid arrows indicate the carboxyl groups, and the open arrows point to other available groups. All abbreviations are as usual except* L*-m-Dpm = (*L*)-meso-diaminopimelic acid.*

in the number of "crystalloids" when compared to native walls. Possibly the changes in the charge density in the walls due to the chemical modifications subtly altered the complex set of events involved in crystalloid formation (11) and lowered the uptake.

The phosphodiester groups of teichoic acid are potent magnesium chelators (23, 30), but should also mediate the binding of other metals (13, 25). Our teichoic acid-less walls bound less metal than native walls. Since the extraction reduced the wall substance by 54.3%, our results have been adjusted for easy comparison with the other binding results. The native walls contained 1.11 μmol of teichoic acid (expressed as P_i) per mg of walls, and 93.8% or 1.04 μmol/mg of walls was extracted under the described conditions. Based on these results, it was apparent that the reduction of metal-binding capacity had close stoichiometry with the extracted teichoic acid (Table 6). The monovalent alkali metals appear to bind in equimolar amounts, whereas Mg, Ca, and Cu required at least two teichoic (phosphodiester?) residues, presumably due to their divalent nature. These results agree well with the Mg binding of teichoic acid from *Lactobacillus buchneri* (30) and *Staphylococcus aureus* (alanine ester residues converted by hydroxylamine; 23).

Chemical modification of the carboxyl groups of the wall had the most profound effect on metal deposition (Table 5). In all instances the uptake values were reduced, and, in the case of E-walls, the reduction was the most severe of any of the alterations to the wall. The most apparent site for these chemical modifications is the $-COO^-$ of the glutamic acid of the peptidoglycan (Fig. 10b), and the number of altered $-COO^-$ agrees well with the amount of glutamic acid detected (compare Tables 3 and 4). We therefore suggest that it may be the constituent glutamic acid of the *B. subtilis* peptidoglycan that is the most potent metal scavenger in the wall. Recently, Matthews and Doyle, using equilibrium dialysis for metal binding in chemically altered *B. subtilis* walls, have confirmed some of these results (T. H. Matthews and R. J. Doyle, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, K86, p. 159).

Amide groups are only protonated at low pH and are therefore considered neutral (i.e., G-walls should have the same charge density as GE-walls). In general, a small decrease in metal deposition was noted for G-walls, which could be due to a slight decrease in electronegativity (see dipolar ion form, Fig. 2b). On the other hand, this chemical modification could have subtly altered the wall architecture and restricted the exposure of available groups.

Electron microscopy demonstrated that the size of the metal deposit was reduced as the electronegative charge of these groups became increasingly electropositive (i.e., GE → [G?] → E-walls; Fig. 5A and B are representative). This was not apparent in thin sections of teichoic acid-less walls, and, therefore, we suggest that it is through carboxyl groups that the inorganic deposition of metals occurs. Possibly these reactive sites are more readily accessible to soluble metal than are the phosphodiester groups of teichoic acid. Although the peptidoglycan of bacterial walls is a highly ordered, quasi-crystalline structure (19, 20, 39), defects large enough to allow access of lysozyme must occur (17). Nuclear magnetic resonance spectra indicate that, even though the glycan strands are firmly rigid, their constituent cross-linking groups have a high degree of motional freedom (31), which would allow growth of metal aggregates. Therefore, it seems reasonable that soluble metal could easily penetrate the peptidoglycan, come in contact with the highly reactive $-COO^-$ of glutamic acid, and form large deposits in the vicinity of the cross-linking chains.

It is remarkable, too, that the alteration of COO^- can change the nature of the reaction deposit so profoundly, as was found with AuIII (Fig. 7 through 9). In this instance the elemental form of the deposit was converted to the hydroxide, which is more typical of an inorganic nonreducing process (11).

Control experiments using unfixed native walls in thin sections or whole mounts (Fig. 7) revealed that the elemental form was not due to glutaraldehyde reduction of the metal during the fixation process. In fact, GE-, G-, and E-walls that were fixed with the aldehyde contained a noncrystalline form of gold (compare Fig. 8A with Fig. 8B, C, and D).

X-ray crystallography can differentiate between high (crystalline) and low (hydroxide, etc.) ordered structure, since the atomic lattices of the former produce distinct diffraction lines. The size of the deposit is also important, since large crystalline aggregates give sharp, clear lines, whereas small aggregates produce diffuse, broad lines.

The sequence of X-ray diffractograms (Fig. 9) and thin sections (Fig. 8) indicate that, as the $-COO^-$'s of the wall were neutralized or made electropositive, the crystallinity of the gold deposition product decreased. Neutralized walls (GE-walls) produced two distinct deposition products; one was a large crystalline form which occurred within the wall fabric and was similar to that found in native walls (compare Fig. 8B with Fig. 7 and 8A), and the other was found at the wall surfaces and was a small aggregate with

diffuse, noncrystalline boundaries (Fig. 8B). Although distinct $Au(OH)_3$ lines were absent in diffractograms (Fig. 9B, C, and D), presumably due to the small size and decreased amounts of the metal present (Table 5), we assume this aggregate to be gold hydroxide because of its noncrystalline form and its predilection for growth on the wall surface.

The larger crystalline gold deposits were rare in G-walls and absent in E-walls, but the smaller aggregate was found in both (Fig. 8C and D). In each case, the deposition was a wall surface phenomenon. This would indicate that, although gold is able to penetrate into the fabric of chemically altered walls, it nucleates and grows aggregates externally as on an inert surface. Native walls, on the other hand, possess internal reactive $—COO^-$ groups which mediate the crystalline deposition process, another indication that the $—COO^-$'s of the wall are important as metal-binding sites.

Little information is available concerning the structural organization of teichoic acid in the *B. subtilis* wall. It is covalently bound to the glycan strands of the peptidoglycan (1) and is inserted into preexisting wall as a teichoic-peptidoglycan complex, presumably at the inner surface of the wall (10, 26, 33, 41). That it does extend to the outer wall surface can be demonstrated by concanavalin A binding (8). Possibly, since it is in intimate association with the firmly rigid glycan backbone of the peptidoglycan, it possesses little lateral flexibility to accommodate growth of large metal deposits.

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