JOURNAL OF BACTERIOLOGY, Feb. 1991, p. 961–967
0021-9193/91/030961-07$02.00/0

Vol. 173, No. 3

Intracellular Location of the Autolytic *N*-Acetylmuramyl-L-Alanine Amidase in *Bacillus subtilis* 168 and in an Autolysis-Deficient Mutant by Immunoelectron Microscopy†

JAN A. HOBOT[1]* AND HOWARD J. ROGERS[2]

Electron Microscopy Unit, University of Wales College of Medicine, Heath Park, Cardiff CF4 4XN,[1] and The Biological Laboratory, University of Kent, Canterbury, Kent CT2 7NJ,[2] United Kingdom

Received 11 June 1990/Accepted 1 November 1990

Antisera against purified autolytic *N*-acetylmuramyl-L-alanine amidase from *Bacillus subtilis* 168 were prepared in rabbits. They neutralized the enzymatic action of the purified amidase acting on isolated sodium dodecyl sulfate (SDS)-treated walls from the same organism. They also inhibited the lysis of native walls, but only after the walls lysed partially. Amidase adsorbed to insoluble walls still combined with antibody. Antisera did not stop the lysis of whole cells. Lowicryl HM20 sections of both strain 168 and its autolytic mutant strain FJ6 were prepared by the progressive-lowering-of-temperature technique, immunolabeled with the antisera, and visualized with colloidal gold particles as markers. The highest concentration of gold particles seemed to be in the septa of dividing cells, followed by the side walls. There was some labeling of the cytoplasm. Adsorption of sera with SDS-treated walls reduced the overall labeling of sections considerably but did not alter the relative intracellular distribution of particles. The results for strains 168 and FJ6 were similar. Labeling of SDS-treated walls unexpectedly revealed the presence of a wall-bound amidase fraction.

Despite many years of study of the autolytic enzymes of bacteria, there are aspects of their function, regulation, and intracellular location that have not yet been elucidated unambiguously. *Bacillus subtilis* 168 produces two peptidoglycan hydrolases that act as autolysins. Both have been purified and characterized (9, 23). The much more active one is the *N*-acetylmuramyl-L-alanine amidase (the amidase). The second autolysin is an endo-β-*N*-acetylglucosaminidase (the glucosaminidase). Previous work on the intracellular location of autolysins in bacteria (1, 11, 12) has relied on studying local changes in the appearance of cell walls of bacteria taken from suspensions incubated under optimum conditions for autolysis. Deductions about the intracellular location have been drawn from the positions of bulges which develop when wall synthesis is inhibited in gram-negative bacteria (18, 19) and from the circumferential bursting of staphylococci after incubation followed by osmotic shock (20). The relatively slow turnover of old poles of rod-shaped cells relative to their side walls has also been used to deduce the distribution of autolysins (3, 4). None of this work can distinguish local differences in wall susceptibility from local concentrations of autolysins. Activation and intracellular migration of autolysin molecules may also confuse the picture.

Antisera to the purified amidase have now been used to immunogold-label bacterial sections prepared by the progressive-lowering-of-temperature (PLT) technique (13–15). These techniques together should allow unambiguous determination of the location of the autolysins.

* Corresponding author.

† This paper is dedicated to Howard Rogers, who passed away during the final revision of this work. His tremendous contribution to microbiology, in particular to our understanding of the structure and function of bacterial cell walls, cannot be underestimated. His enthusiasm and never-ending search for fresh avenues to explore will be greatly missed and should serve as an example to all in the quest for scientific knowledge.

MATERIALS AND METHODS

Microorganisms. *B. subtilis* 168 (*trpC*) and *B. subtilis* FJ6 (*metC3/lyt-2*) were used. The strains were kept as frozen spore suspensions.

Media and growth conditions. The casein hydrolysate medium (16) CHSC was that used previously (24) but supplemented with tryptophan (1 μg/ml) for growth of strain 168. Volumes (100 ml) were inoculated with 4 μl of a spore suspension and incubated overnight at 35°C with aeration. A broth medium (LB) containing 10 g of tryptone (Difco), 5 g of yeast extract (Difco), 10 g of NaCl, and 1 g of glucose made up to 1 liter was also used sometimes. Cells were harvested at the exponential (1.5 h) or late-exponential/stationary phase (4 h), as judged from the rate of increase in cell numbers by hemacytometer cell counts. Cells from overnight cultures were also harvested.

Preparation of antisera. Young adult Sandy Lop rabbits were injected intramuscularly with 2 mg of enzyme protein suspended in complete Freund's adjuvant. Three such injections were given at monthly intervals, followed after 3 months by a booster dose given subcutaneously. The rabbits were bled 2 or 3 weeks after the booster dose, and the separated serum was fractionated by $(NH_4)_2SO_4$ precipitation followed by chromatography on DEAE-Sephadex A50; the columns were eluted with 0.05 M sodium acetate buffer, pH 5.0. The sera thus fractionated were used in the immunolabeling studies. An immunoglobulin G (IgG) fraction was also separated from a pooled fractionated sample by isoelectric focusing.

Preparation of SDS-treated and "native" cell walls. The procedures used for sodium dodecyl sulfate (SDS) treatment and cell wall preparation were those of Fein and Rogers (7).

Purified amidase preparation. The amidase was isolated as described previously (23).

Estimation of amidase. The method for amidase estimation was described previously (23). When inhibition by antisera was studied, suitable dilutions of enzyme and antibody were mixed in 0.05 M Tris-HCl buffer, pH 7.2, and kept on ice for

30 min. Volumes of borate buffer, pH 9.5, and $MgSO_4$ solutions were then added to give the required concentrations (23); the solutions were warmed to 37°C, and a sufficient amount of SDS-treated wall suspension was added to give a concentration of 0.5 mg/ml, which was mixed and incubated at 37°C. The OD_{450} was measured at intervals. The units of enzyme activity were defined as the percentage drop in optical density per minute.

Preparation of material for electron microscopy. Bacteria were fixed directly in culture medium with glutaraldehyde (final concentration, 1% [vol/vol]) for 45 min. They were then harvested by filtration onto a filter (Millipore; diameter, 45 mm; pore size, 0.22 μm), washed once with CHSC or LB medium, and taken up into agar blocks by adding 4% (wt/vol) agar made up in either CHSC or LB medium (14, 15). Isolated, freeze-dried, SDS-treated cell walls (<1 mg) were rehydrated in 200 μl of sterile distilled water at 20°C, fixed in 1% (vol/vol) glutaraldehyde (in distilled water) for 1 h, microfuged (11,300 × *g*) for 2 min, and taken up into agar blocks by adding 4% (wt/vol) agar in distilled water to the pellet.

These blocks were then processed immediately by dehydration in ethanol by the PLT technique. The infiltration, embedding, and polymerization with the acrylic resin Lowicryl HM20 were done at −35°C (13–15). Briefly, the dehydration protocol with ethanol was as follows: 30%, 0°C, 30 min; 50%, −20°C, 1 h; 70%, −35°C, 1 h; 100%, −35°C, 2 h. Infiltration was at −35°C with increasing concentrations of Lowicryl HM20 in ethanol as follows: resin–100% ethanol (1:1), 1 h; resin–100% ethanol (2:1), 1 h; pure resin, 1 h; fresh resin, overnight; the next day, fresh resin, 2 h. After infiltration, the resin was polymerized in gelatin capsules (size 0) by indirect UV irradiation (360 nm) for 24 h at −35°C, followed by further hardening with direct UV irradiation at room temperature for 3 days (13–15). Thin sections were cut on an LKB Ultramicrotome III and picked up on the shiny surfaces of naked nickel grids (300 mesh, hexagonal, high transmission). The grids were air dried without the aid of filter paper.

Immunolabeling protocols. The grids bearing sections were completely immersed in drops (30 to 50 μl) of solution at room temperature. Excess fluid at each step was removed by gently shaking the grids once, but not so as to dry them out. The labeling protocol was 0.5% ovalbumin (no. 32467; Calbiochem-Behring, La Jolla, Calif.) in 0.01 M phosphate-buffered saline (PBS), pH 7.4, for 5 min; antiamidase serum, diluted 1:50 in PBS containing 0.6% bovine serum albumin (BSA) (no. A-8022; Sigma Chemical Co., St. Louis, Mo.), pH 7.4, for 1 h; PBS containing 0.6% BSA for 1 min; 0.02 M Tris-HCl buffer, pH 8.2, twice for 1 min; 10-nm protein A-colloidal gold complex (Bioclinical Services Ltd., St. Mellons, Cardiff, U.K.) diluted 1:5 in 0.02 M Tris-HCl buffer, pH 8.2, for 1 h; Tris-HCl buffer for 1 min; double-distilled water, twice for 1 min; counterstained with 4% (wt/vol) aqueous uranyl acetate for 20 min; final wash with 0.5 ml of double-distilled water dispensed slowly over both sides of the grid from an Eppendorf micropipette (Eppendorf, Hamburg, Federal Republic of Germany). The grids were then air dried without the aid of filter paper. Control treatments were protein A-colloidal gold alone, with the antibody serum replaced by PBS containing 0.6% BSA or by pooled, normal rabbit serum (no. S-2632; Sigma) diluted 1:50 in PBS containing 0.6% BSA for 1 h.

Gold particle counts. Counts were made over three areas of the cells: cytoplasm, cell walls, and septa. Septa were defined as the region between cell compartments; areas of cell wall on either side, continuous with the cylindrical portion of the wall, were not included. Surface areas were calculated directly on the micrographs by an eyepiece magnifier (×10) fitted with a micrometer scale graticule. Cells (approximately 16) were sampled from the field of view of four micrographs per treatment, results coming from three experiments. The results, expressed as gold particles per square micrometer, are estimates, with variations in label distribution over the areas counted represented by standard deviations of the sample.

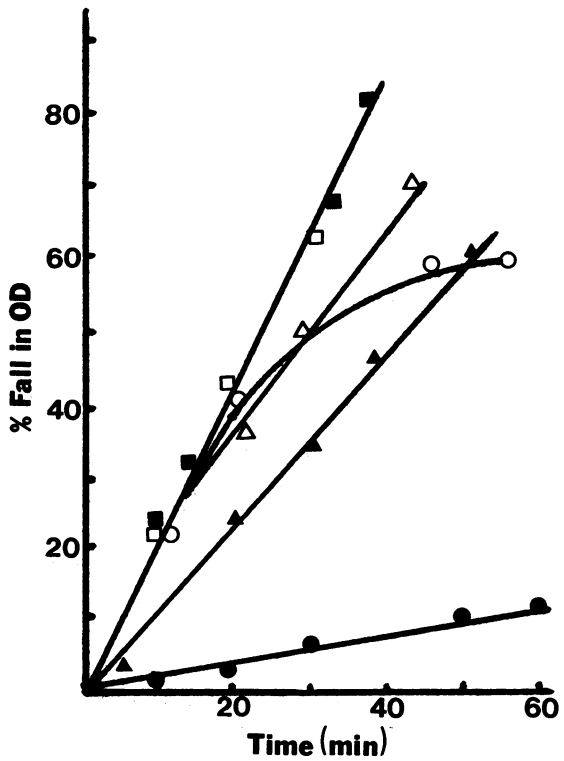

FIG. 1. Neutralization of soluble purified amidase acting on SDS-treated cell walls by 10 and 50 μl of an antiserum (△, ○) and of amidase present in native cell walls (▲, ●). Control rate of lysis of SDS-treated walls with purified amidase (■) and native walls (□), neither with antiserum added.

Electron microscopy. All sections were examined in a Philips 300 electron microscope operated at 80 kV.

RESULTS

Neutralization of purified amidase by antiserum. Figure 1 shows the effect of antiserum dilutions mixed with purified enzyme on the rate of lysis of SDS-treated walls. For 75% inhibition of the action of 2.5 U of enzyme, 10 μl of antiserum was required. This antiserum was used for immunogold labeling. Several preparations of antiserum were tested, and all inhibited the action of the enzyme. The IgG fraction was also very active, but unfortunately not enough was available for immunolabeling studies.

Action of antiserum on autolysis of native walls. Native walls retain autolysins, and the batch used autolysed at about the same rate as SDS-treated walls mixed with 2.5 U of the purified amidase per ml. When sufficient antiserum was added to have 50% neutralized, soluble, purified enzyme acting on SDS-treated walls, two phases to the lysis of native

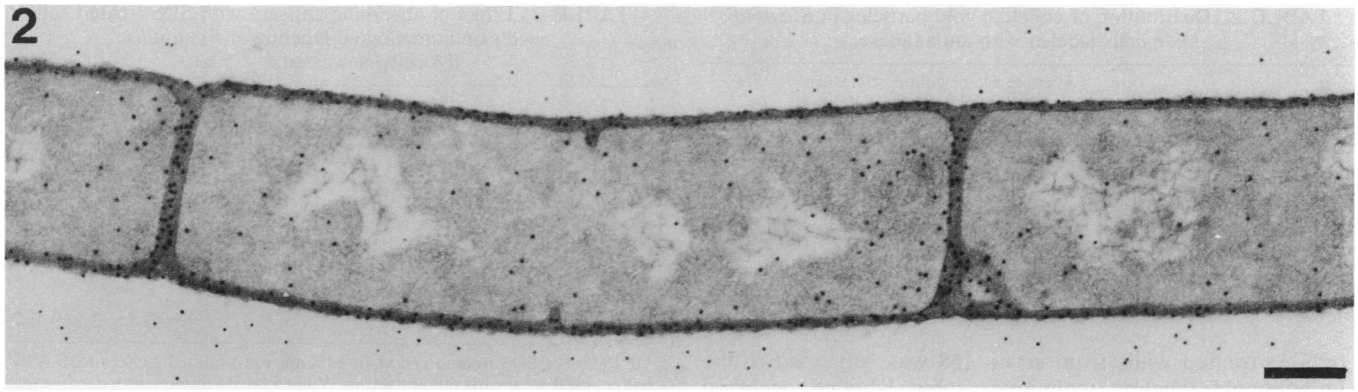

FIG. 2. Immunogold labeling of sections of strain 168 taken from cultures grown for 1.5 h. Bar, 0.2 μm.

walls could be seen (Fig. 1). The first, during which the antiserum had little or no effect on the rate of autolysis, lasted until the OD_{450} of the suspension had been reduced by 30 to 35%. The rate then decelerated to about that expected of an antiserum-purified enzyme mixture. Either enzyme buried in the wall was not accessible to antibody, or the active domain of the enzyme was inaccessible to antibody when it was combined with its insoluble peptidoglycan substrate.

For the subsequent immunolabeling experiments, it was important to know whether the antibody-combining sites of such a wall-associated enzyme were still accessible. To test for this, antibody dilutions were mixed with native walls at 0°C and left for 30 min. The mixture was then centrifuged at <5°C for 5 min at maximum speed in a cooled microfuge (M.S.E. Co.). The supernatant was removed and tested for its ability to neutralize the action of purified enzyme. Samples of native walls without antibody and antisera without walls were subjected to the same treatment. The native walls were found to remove the neutralizing ability of the antiserum; 0.75 mg removed about 50% of the neutralizing ability from a dose of antiserum which would have neutralized by 95% 2.5 U of soluble purified enzyme. Control experiments with SDS-treated walls in place of the native walls showed that only small amounts of the neutralizing ability of the sera were removed (0 to 10%). In similar control experiments, larger amounts were removed from the IgG antiserum fraction (20 to 25%).

Accessibility of antibody to cell wall-bound enzyme. If the active domains of enzyme molecules associated with peptidoglycan are not available to the antibody whereas the antibody-combining sites of these active domains are still functional, it should be possible to show this by using SDS-treated walls. Amidase is strongly and specifically adsorbed by walls of *B. subtilis* (10), and its functional groups are known to be engaged with the substrate (23). The enzyme should behave similarly to that in native walls. Accordingly, SDS-treated walls were mixed with purified enzyme at 0°C and allowed to stand for 30 min; the supernatant was removed by centrifugation, the pellet was washed once with cold 0.05 M Tris-HCl buffer at 0°C, and dilutions of antiserum were added. The dilutions of antiserum were such that when mixed with purified enzyme in solution, they inhibited its ability to hydrolyze SDS-treated walls by 80%. The lysis of walls with bound enzyme was inhibited by only 44%. The time course for the lysis of the walls bearing bound enzyme was similar to that for the native walls, although the period during which the antisera had no effect was shorter. The addition of large volumes of antisera to suspensions of whole cells did not prevent lysis of the cell wall at the control rate, as measured by native wall preparations (Fig. 1), suggesting that the added antiserum had little or no effect on the rate of autolysis occurring in the cell wall of whole cells.

Intracellular location of the amidase. In strain 168, gold particles were distributed between the walls, septa, and cytoplasm (Fig. 2). This distribution was not changed by altering either the age of the culture from which the bacteria were harvested (Table 1) or the medium in which they were grown (CHSC or LB; data not shown for the latter). Gold counts were greatest for the septa and the walls; the very low background counts were noted (Tables 1 to 3). There was no specific label in the nucleiod, the gold counts being the same as for background (data not presented; see Fig. 1 and 2). Surprisingly, the labeling pattern in the autolysis-deficient mutant strain FJ6 was very similar to that for strain 168 (Fig. 3 and Table 2). An area within the cell wall-septal junction boundary region appears to have less label in strain 168 than in strain FJ6 (Fig. 2 and 3). Whether this has any significance for the distribution or action of the amidase is not clear and requires further investigation.

Labeling of SDS-treated walls and absorption of serum with SDS-treated walls. The presence of active amidase in SDS-treated walls would not be expected. Indeed, one of the original purposes of treatment with dodecyl or decyl sulfate (SDS) was to remove and inactivate autolytic enzymes from cell wall preparations (2, 25). The decrease in the OD_{450} of SDS-treated walls from strain 168, when incubated at 37°C for 2 h in borate-$MgSO_4$ solution used for amidase estimation, was about 3%, compared with a loss of 68% in 15 min by a suspension of walls not treated with SDS (i.e., native walls). It was therefore unexpected that when a preparation

TABLE 1. Distribution of colloidal gold particles in *B. subtilis* 168 cells labeled with antiamidase

Cell compartment	Average no. of gold particles/μm² ± SD in cells harvested at:		
	Overnight	1.5 h	4 h
Cytoplasm	79 ± 16	70 ± 19	62 ± 24
Septa	451 ± 173	401 ± 94	295 ± 83
Cell wall	325 ± 47	367 ± 99	411 ± 119
Background resin	3.7 ± 1.2	1.4 ± 0.5	3.3 ± 1.8

TABLE 2. Distribution of colloidal gold particles in *B. subtilis* FJ6 cells labeled with antiamidase

Cell compartment	Average no. of gold particles/μm^2 ± SD in cells harvested at:		
	Overnight	1.5 h	4 h
Cytoplasm	82 ± 22	84 ± 25	66 ± 17
Septa	583 ± 107	439 ± 49	591 ± 104
Cell wall	571 ± 83	588 ± 136	506 ± 145
Background resin	0.8 ± 0.3	0.9 ± 0.3	1.4 ± 0.7

TABLE 3. Effect of absorbing antisera with SDS-treated cell walls on immunogold labeling of *B. subtilis* 168 cells harvested at 1.5 h

Cell compartment	Average no. of gold particles/μm^2 ± SD at cell wall/antiserum ratio[a]:			
	0:10	5:12.5	5:25	5:50
Cytoplasm	84 ± 19	34 ± 12	18 ± 4	21 ± 4
Septa	743 ± 178	244 ± 79	251 ± 87	323 ± 65
Cell wall	553 ± 114	103 ± 47	99 ± 50	142 ± 41
Isolated SDS-treated walls	71 ± 17	11 ± 7	14 ± 9	23 ± 9
Background resin	2.0 ± 0.5	5.8 ± 0.3	2.6 ± 0.5	2.2 ± 0.7

[a] Ratios of SDS-treated cell walls to antiserum, diluted 1:50 in PBS-BSA buffer, used to absorb the antiserum. Values based on amounts used in the biochemical neutralizing tests.

of SDS-treated walls from strain 168 was subjected to the immunogold labeling technique, some labeling occurred (Fig. 4a and Table 3).

When the antiserum was mixed with SDS-treated cell walls and the mixture was centrifuged, with the supernatants being used in the immunogold technique, a large reduction in the overall labeling of sections occurred (Fig. 5a and b). Labeling of the SDS-treated cell wall preparations was also greatly reduced (Fig. 4b and Table 3). The reduction in gold counts affected all three cell compartments about equally, and the relative labeling distribution of cell walls, septa, and cytoplasm by the treated antisera remained about the same as for the untreated samples (Table 3). However, a comparison of the ratio of gold particle counts between different cell compartments in strain 168 seemed to suggest that overall there may be slightly more label in the septa than in the cell walls (from 6:54:40 for cytoplasm, septa, and cell walls of untreated samples in column one of Table 3 to 6:66:28 for treated samples in columns two to four in Table 3).

Electron microscope methods. Until recently, it was considered that for immunolabeling studies involving colloidal gold, the resin of choice was the polar acrylic Lowicryl K4M. Recently (5, 6) it has been shown that the high background counts generally associated with the nonpolar resins, among which the less-polar acrylic Lowicryl HM20 was classed, can be overcome by coupling antibody to protein A-colloidal gold directly. However, this can lead to aggregation of the gold conjugates, reducing the specificity of localization. Background counts can be lowered by simply adding 0.6% BSA to the PBS buffer in which antibody or serum is diluted (13). Lowicryl HM20 has a much lower viscosity than Lowicryl K4M at −35°C, allowing better infiltration of specimens. It also gives as good an ultrastructural preservation of bacterial cell walls (15) and the same distribution and intensity of label as Lowicryl K4M (results for *B. subtilis* not included [13]).

The strategy of preparing cells by PLT (see Materials and Methods) to investigate a cell wall-associated enzyme was based on the previous findings that this procedure preserves bacterial cell walls very well (14), as evidenced by work on the periplasmic gel of *Escherichia coli* (15). Also, this method can retain high levels of immunoreactivity within cells during their processing for electron microscopy (13, 22).

The immersion method of immunolabeling sections on naked grids that has been used here has the advantage that it labels both surfaces of the section. This gives a sensitive labeling response without superimposition effects (Fig. 2 and 3). The washing procedure avoids the irregularities of variable jet washes and gives clean, well-labeled sections (13).

DISCUSSION

A possibly higher concentration of amidase in the septa and perhaps somewhat lower concentration in the side walls of *B. subtilis* 168 would explain the results obtained by Burdett (1). This author found small gaps bored around the cross-walls during the first 5 min of autolysis of strain 168 at pH 8.5, the optimum for amidase at 37°C. Only later did similar holes appear along the side walls. Our results for strain FJ6, however, are quite different. Burdett (1) found no evidence of wall breakdown during the first 3 h of incubation at 37°C and pH 8.5, yet by immunogold labeling of the

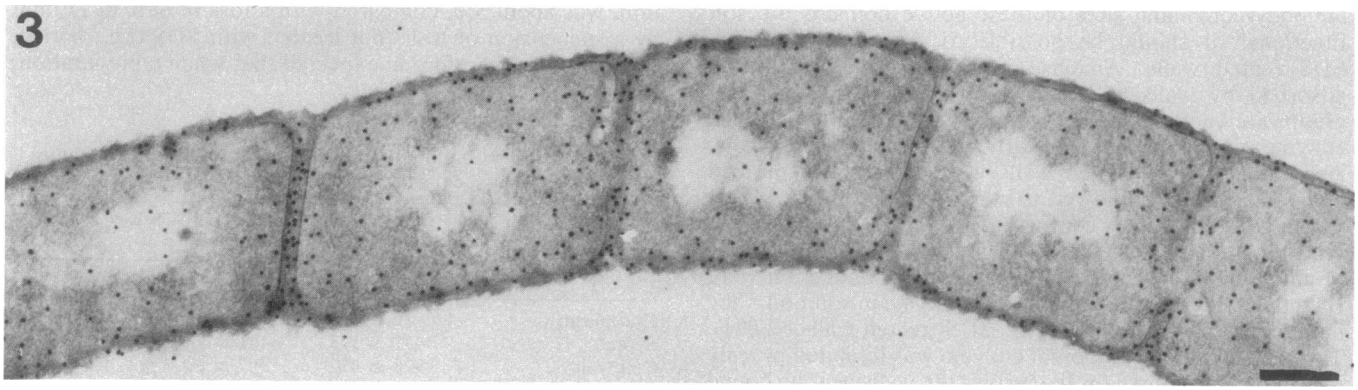

FIG. 3. Immunogold labeling of sections of strain FJ6 taken from cultures grown for 1.5 h. Bar, 0.2 μm.

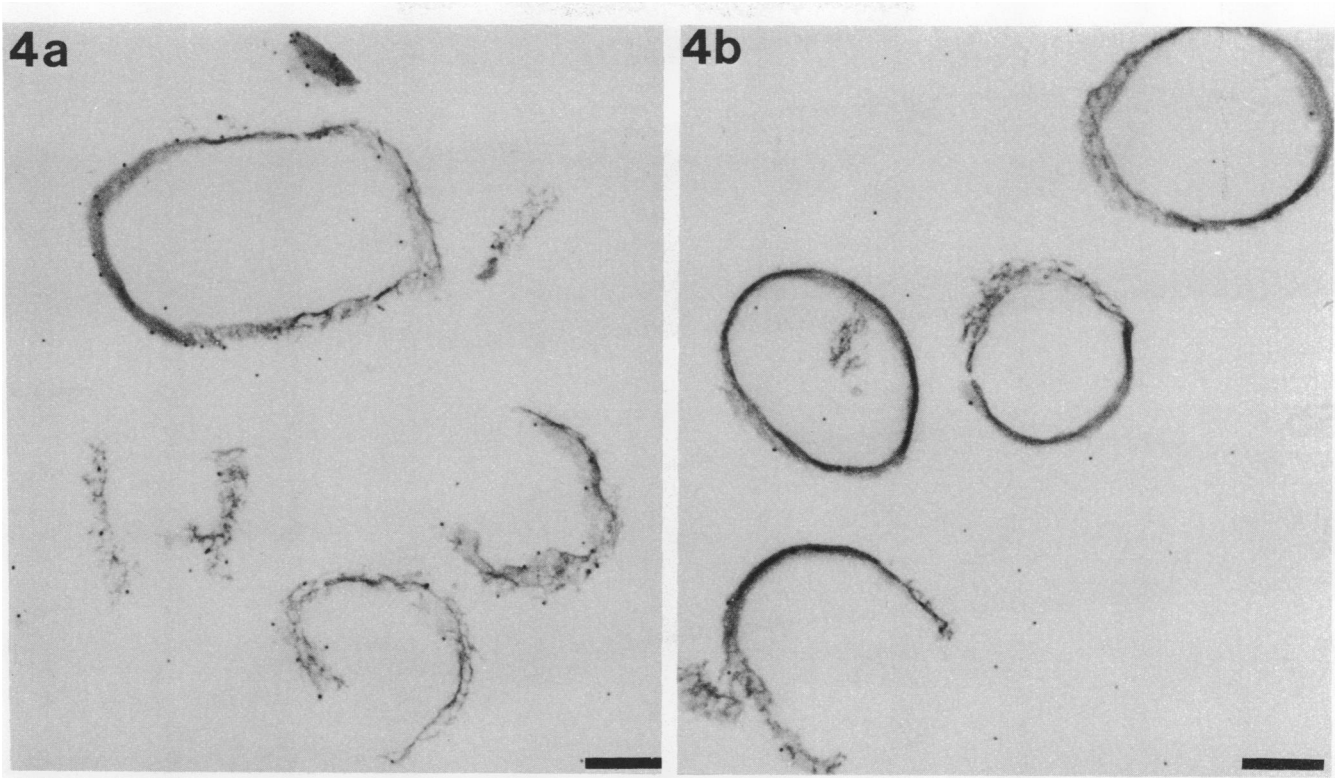

FIG. 4. Immunogold labeling of sections of isolated SDS-treated walls (a) with untreated serum and (b) with antiserum after absorption with an SDS-treated cell wall preparation. Bars, 0.2 μm.

amidase, there is little difference in its location and distribution between strains 168 and FJ6. That there are less active autolysins in strain FJ6 than in strain 168 there can be no reasonable doubt, especially when the microorganism has been grown in casein hydrolysate medium (24). Moreover, cell suspensions, native wall preparations, and 5 M LiCl extracts from the strain all behave as though only about 10 to 15% of the wild-type strain autolysin is active at pH 8.5, the pH optimum for the amidase (7). The simplest explanations for the immunolabeling results are the presence in strain FJ6 of either enzymatically inactive, immunologically cross-reactive protein or enzymatically active protein combined with a powerful inhibitor. We cannot decide between these possibilities at present other than to say that the inhibitor cannot be lipoteichoic acid, which does not inhibit the amidase, although it strongly inhibits the glucosaminidase (23).

There is an apparent discrepancy between the amount of antibody removed by SDS-treated cell walls, as measured by neutralization of isolated enzyme and when compared with the reduction in the gold labeling of sections. One possible explanation of this discrepancy is that with postembedding immunoelectron microscopic techniques, all available antigenic sites are generally not labeled (13, 21). As a rough approximation, the efficiency of labeling (EL) can be estimated if the total number of antigen molecules (e.g., enzyme molecules per cell) is known. For Lowicryl K4M, Griffiths and Hoppeler (8) found that the EL varied from 1.4 to 18.4%, depending on the cellular compartment counted. The EL may provide an upper figure for detecting antigenic sites by immunoelectron microscopy in a particular system. There is also probably a lower threshold figure below which no antigen can be detected (17). Both upper and lower figures are to a great extent dependent upon the electron microscopic preparation procedures used (21, 22), this being one of the reasons why PLT processing was used in this study (see Results, Electron microscope methods). In the present study with the autolytic enzyme amidase, we calculate a very approximate EL of 12% with untreated serum, which fell to 3% after absorption of serum with SDS-treated walls. The fall of 9%, due most probably to a reduction of antibody titer in the absorbed antiserum, is therefore more comparable to the small loss of 0 to 10% of antiserum or antibody neutralizing power determined biochemically. Being able now to label, with the absorbed serum, only 3% of the total amidase molecules available would give rise to the lower gold counts in Table 3.

The removal from the antisera by SDS-treated walls of antibody reacting in the immunogold labeling system, without removal of significant amidase neutralizing power, could equally suggest the presence of a separate antibody. The purified enzyme used as an antigen to produce the sera showed only one band staining with Coomassie blue in polyacrylamide gel electrophoresis (PAGE) examinations. However, Western immunoblots made from PAGE examinations of the enzyme showed a very faint band at 30 kDa as well as a strong band at 40 to 50 kDa, the molecular mass of the amidase. Western blots from PAGE gels of lysozyme digests of SDS-treated walls showed a band at 40 to 50 kDa, but also a very strong band at the lower molecular mass (7a), confirming the presence of amidase in SDS-treated walls as revealed by immunoelectron microscopy (Fig. 4a).

These results, taken together with the experiments on the neutralizing ability of the serum and also the accessibility of antibody to amidase bound to SDS-treated walls, suggest (i) that the untreated serum has an antibody(ies) that recognizes

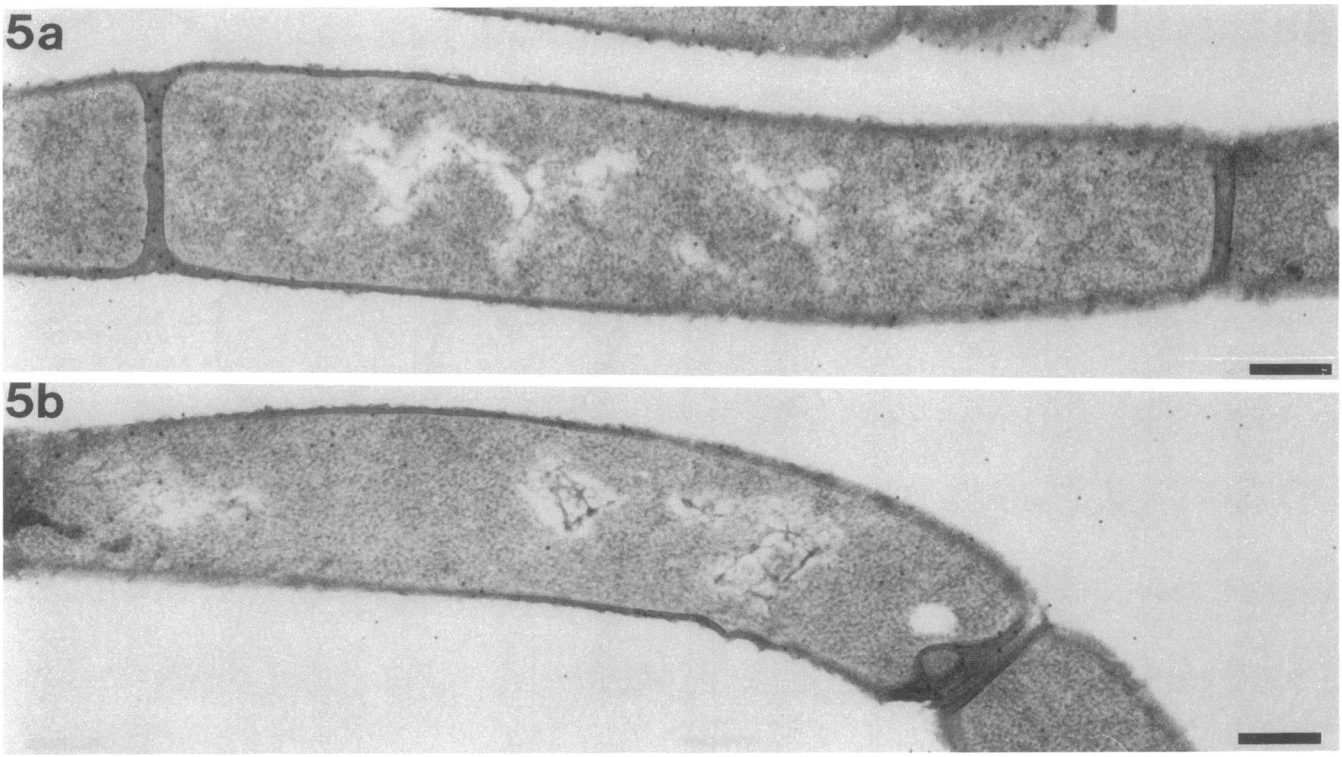

FIG. 5. Immunogold labeling of sections of (a) strain 168 and (b) strain FJ6 with antisera previously absorbed with an SDS-treated cell wall preparation. Bars, 0.2 μm.

both the amidase of 40 to 50 kDa and also a fraction from purified amidase preparations at 30 kDa, the latter probably tightly bound to SDS-treated cell walls and (ii) that after incubation of serum with SDS-treated walls and possible depletion from the serum of an antibody(ies) reactive as in i, the cellular distribution after immunogold labeling is still similar to that obtained with untreated serum. Interestingly, enzyme found to be bound to SDS-treated walls was to some extent able to promote lysis of cell walls, being at the same time somewhat inaccessible to the neutralizing effect of the antiserum. It is possible that a tightly bound fraction of the amidase molecule (the active domain; 30 kDa?) is relatively inaccessible to antibody in whole or isolated native and SDS-treated cell walls (i.e., nonlysozyme digests). In the light of these results, we are currently investigating the immunological properties of this serum in relation to amidase distribution within the cell.

REFERENCES

1. **Burdett, I. D. J.** 1980. Analysis of sites of autolysis in *Bacillus subtilis* by electron microscopy. J. Gen. Microbiol. **120**:35–49.
2. **Connover, M. J., J. S. Thompson, and G. D. Shockman.** 1966. Autolytic enzyme of *Streptococcus faecalis*: release of soluble enzyme from cell walls. Biochem. Biophys. Res. Commun. **23**:713–719.
3. **Doyle, R. J., J. Chaloupka, and V. Vintner.** 1988. Turnover of cell walls in microorganisms. Microbiol. Rev. **52**:554–567.
4. **Doyle, R. J., and A. L. Koch.** 1987. The functions of autolysins in the growth and division of *Bacillus subtilis*. Crit. Rev. Microbiol. **5**:19–22.
5. **Durrenberger, M.** 1989. Removal of background label in immunocytochemistry with the apolar Lowicryls by using washed protein A-gold pre-coupled antibodies in a one-step procedure. J. Electron Microsc. Techn. **11**:109–116.
6. **Durrenberger, M., M. A. Bjornsti, T. Uetz, J. A. Hobot, and E. Kellenberger.** 1988. The intracellular location of the histonelike protein HU in *Escherichia coli*. J. Bacteriol. **170**:4757–4768.
7. **Fein, J. E., and H. J. Rogers.** 1976. Autolytic enzyme-deficient mutants of *Bacillus subtilis* 168. J. Bacteriol. **127**:1427–1442.

7a. **Foster, S. J. (University of Sheffield).** Personal communication.

8. **Griffiths, G., and H. Hoppeler.** 1986. Quantitation in immunocytochemistry: correlation of immunogold labeling to absolute number of membrane antigens. J. Histochem. Cytochem. **34**: 1389–1398.
9. **Herbold, D. R., and L. Glaser.** 1975. The purification of *Bacillus subtilis* autolytic amidase. J. Biol. Chem. **250**:1676–1683.
10. **Herbold, D. R., and L. Glaser.** 1975. Interaction of *N*-acetylmuramic acid L-alanine amidase with cell wall polymers. J. Biol. Chem. **250**:7231–7238.
11. **Higgens, M. L., J. Coyette, and G. D. Shockman.** 1973. Sites of cellular autolysis in *Lactobacillus acidophilus*. J. Bacteriol. **111**:1375–1382.
12. **Higgens, M. L., H. M. Pooley, and G. D. Shockman.** 1970. Sites of cellular autolysis in *Streptococcus faecalis* as seen by electron microscopy. J. Bacteriol. **103**:504–512.
13. **Hobot, J. A.** 1989. The Lowicryls and low temperature embedding for colloidal gold methods, p. 75–115. *In* M. A. Hayat (ed.), Colloidal gold: principles, methods, and applications, vol. 2. Academic Press, San Diego.
14. **Hobot, J. A.** *In* N. Mozes, P. S. Handley, H. J. Busscher, and P. Rouxhet (ed.), Microbial cell surface analysis: structural and physico-chemical methods, in press. VCH Publishers, New York.
15. **Hobot, J. A., E. Carlemalm, W. Villiger, and E. Kellenberger.** 1984. Periplasmic gel: new concept resulting from the reinvestigation of bacterial cell envelope ultrastructure by new methods. J. Bacteriol. **160**:143–152.
16. **Janczura, E., H. R. Perkins, and H. J. Rogers.** 1961. Teichuronic acid: a mucopolysaccharide in wall preparations from vegetative cells of *Bacillus subtilis*. Biochem. J. **80**:82–93.
17. **Kellenberger, E., M. Durrenberger, W. Villiger, E. Carlemalm, and M. Wurtz.** 1987. The efficiency of immunolabel on Lowicryl

sections compared to theoretical predictions. J. Histochem. Cytochem. **35**:959–969.
18. **Lederberg, J.** 1956. Bacterial protoplasts induced by penicillin. Proc. Natl. Acad. Sci. USA **42**:574–577.
19. **Lederberg, J., and J. St. Clair.** 1958. Protoplasts and L-type growth of *Escherichia coli*. J. Bacteriol. **75**:143–160.
20. **Mitchell, P., and J. Moyle.** 1957. Autolytic release and osmotic properties of "protoplasts" from *Staphylococcus aureus*. J. Gen. Microbiol. **16**:184–194.
21. **Newman, G. R., and J. A. Hobot.** 1987. Modern acrylics for post-embedding immunostaining techniques. J. Histochem. Cytochem. **35**:971–981.
22. **Newman, G. R., and J. A. Hobot.** 1989. Role of tissue processing in colloidal gold methods, p. 33–45. *In* M. A. Hayat (ed.), Colloidal gold: principles, methods, and applications, vol. 2. Academic Press, San Diego.
23. **Rogers, H. J., C. Taylor, S. Rayter, and J. B. Ward.** 1984. Purification and properties of autolytic endo-β-*N*-acetylglucosaminidase and the *N*-acetylmuramyl-L-alanine amidase from *Bacillus subtilis* strain 168. J. Gen. Microbiol. **130**:2395–2402.
24. **Rogers, H. J., and G. Wright.** 1987. Early changes in bacterial cell envelopes after inhibition of peptidoglycan synthesis, as shown by the use of a fluorescent probe. J. Gen. Microbiol. **133**:2567–2572.
25. **Shockman, G. D., J. S. Thompson, and M. J. Connover.** 1967. The autolytic enzyme system of *Streptococcus faecalis*. II. Partial characterization of the autolysin and its substrate. Biochemistry **6**:1054–1065.