Immunocytochemical Identification and Localization of Active and Inactive α-Amylase and Pullulanase in Cells of Clostridium thermosulfurogenes EM1

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Clostridium thermosulfurogenes EM1 formed blebs, i.e., protrusions still in contact with the cytoplasmic membrane, that originated from the cytoplasmic membrane during growth in batch culture and continuous culture. They could be observed squeezed between the cell wall and cytoplasmic membrane in cells with seemingly intact wall layers (surface layer and peptidoglycan layer) as well as in cells with wall layers in different states of degradation caused by phosphate limitation or high dilution rates. Blebs were found to turn into membrane vesicles by constriction in cases when the cell wall was heavily degraded. Bleb and vesicle formation was also observed in the absence of substrates that induce α-amylase and pullulanase synthesis. No correlations existed between bleb formation and the presence of active enzyme. Similar blebs could also be observed in a number of other gram-positive bacteria not producing these enzymes, but they were not observed in gram-negative bacteria. For immuno-electron-microscopic localization of α-amylase and pullulanase in C. thermosulfurogenes EM1, two different antisera were applied. One was raised against the enzymes isolated from the culture fluid; the other was produced against a peptide synthesized, as a defined epitope, in analogy to the N-terminal amino acid sequence (21 amino acids) of the native extracellular α-amylase. By using these antisera, α-amylase and pullulanase were localized at the cell periphery in samples taken from continuous culture or batch culture. In samples prepared for electron microscopy by freeze substitution followed by ultrathin sectioning, blebs could be seen, and the immunolabel pinpointing α-amylase enzyme particles was seen not only randomly distributed in the cell periphery, but also lining the surface of the cytoplasmic membrane and the blebs. Cells exhibiting high or virtually no enzyme activity were labeled similarly with both antisera. This finding strongly suggests that α-amylase and pullulanase may occur in both active and inactive forms, depending on growth conditions.

Clostridium thermosulfurogenes EM1 is a thermophilic, anaerobic bacterium that is capable of utilizing starch as its carbon and energy source (14). This organism produces and secretes thermostable α-amylase and pullulanase (1, 14, 26). Cultivation of bacteria in continuous culture under phosphate and substrate limitation causes overproduction and secretion of amylolytic enzymes, and various states of wall degradation (1). In negatively stained cells with seemingly intact wall layers (surface layer and peptidoglycan layer), blebs, i.e., protrusions still in contact with the cytoplasmic membrane, that originated from the cytoplasmic membrane during growth in batch culture and in continuous culture, have been observed squeezed between wall layers and cytoplasmic membrane (2). In cultures containing cells with wall layers heavily degraded by cell growth under phosphate limitation or at high dilution rates, not only blebs but also membrane vesicles originating from blebs by constriction have been found (2). Blebs, vesicles, and the cytoplasmic membrane are covered by a monolayer of small particles (2). These particles were assumed to be amylolytic enzymes exposed at the surface of the membrane prior to secretion into the culture medium.

To investigate whether correlations exist between these cytological features, enzyme location, and α-amylase and pullulanase activity, a detailed electron-microscopic study was conducted with C. thermosulfurogenes EM1. Besides conventional methods such as negative staining and low-temperature embedding, we used freeze substitution, which is known to permit good structural preservation of the cell (17, 18) and which allows identification and localization of α-amylase and pullulanase in well-preserved cells by immuno-electron microscopy (15, 16). To obtain high specificity when applying our immunolabeling techniques, we used antisera directed against a peptide synthesized as a defined epitope in analogy to the N-terminal amino acid sequence (21 amino acids) of the native extracellular α-amylase, in addition to antisera raised against the enzymes enriched from the cell-free culture fluid. Within the scope of these investigations, we also tried to find whether α-amylase and pullulanase may occur in different states, i.e., active and inactive, depending on growth conditions.

MATERIALS AND METHODS

Organisms and growth conditions. C. thermosulfurogenes EM1 (DSM 3896), C. thermocaeticum (DSM 2955), C. acetobutylicum (DSM 1733), Bacillus stearothermophilus (DSM 22), B. subtilis (DSM 10), B. licheniformis (DSM 603), Lactobacillus helveticus (DSM 20075), Escherichia coli (DSM 498), and Alcaligenes eutrophus (DSM 428) were obtained from the German Culture Collection, Brunswick, Federal Republic of Germany.

Experiments in batch culture were conducted on complex medium or mineral medium with different carbon sources. Clostridia and L. helveticus grew anaerobically in Hungate
tubes filled with 10 ml of the respective medium or in 250- or 500-ml glass bottles containing 100 ml of medium. *C. thermosulfurogenes* EM1, *C. thermoaceticum*, and *B. stearothermophilus* were cultivated at 60°C, and all other strains were cultivated at 37°C. The initial pH in batch culture was 6.6; the concentration of the substrate was 1% (wt/vol). The complex medium was prepared as described previously (14). Experiments with *C. thermosulfurogenes* EM1 in continuous culture were performed in 1-liter fermentors with 0.5 liter of culture medium at 60°C. The mineral medium used contained the following components (percentages, wt/vol): KH₂PO₄, 0.68; (NH₄)₂SO₄, 0.25; MgCl₂, 0.016; cysteine HCl, 0.05; CoCl₂, 0.0012. Vitamin and trace element solutions were added at 1 ml/liter of medium. The concentration of the added substrate was 1% (wt/vol). For growth on low-phosphate concentrations the medium contained 0.003% (wt/vol) KH₂PO₄. Strictly anaerobic conditions were maintained by continuous gassing with sterile nitrogen. The chemostat was agitated at 150 rpm. The pH value of the culture was kept at a level of 5.9 by automatic addition of 2 N KOH solution. Samples were taken after steady-state conditions had been reached (at least six volume changes).

**Enzyme assays.** Enzyme activities were assayed by measuring the amount of reducing sugars liberated during incubation with starch or pullulan by the dinitrosalicylic acid method (4). Sodium acetate buffer at 20 mM (pH 5.0) (pullulanase) or phosphate buffer at 20 mM (pH 6.0) (α-amylase) were used for the enzyme test at an incubation temperature of 65°C. α-Amylase and pullulanase activities were measured after culture broth containing cells, as well as in the cell-free supernatant, prepared by centrifugation of the culture broth at 10,000 × g for 5 min. Total enzyme activity was defined as the sum of cell-bound and extracellular activities. One unit of α-amylase or pullulanase activity is defined as the amount of sugar released per minute under the specified conditions (maltose as standard).

**Enzyme purification and preparation of antisera.** α-Amylase and pullulanase were isolated from the culture broth, purified, and characterized as described previously (26, 26a). Anti-α-amylase and anti-pullulanase antisera were prepared as previously described (5, 26). Anti-α-amylase peptide antisera was obtained as follows: a peptide was synthesized in analogy to the known sequence of α-amylase of *B. eutrophus* (3, 26a) by the method of Sheppard (7, 24). The peptide was coupled to hemocyanin (8), and the complex was used for immunization of rabbits by established techniques (5). The obtained serum was purified on a protein A-Sepharose Cl-4B column. The column was equilibrated against 0.1 M KPO₄ buffer (pH 7.0) containing 0.15 M NaCl at a rate of 1.5 ml/min. The antibodies were eluted in 1-ml fractions with 0.1 M glycine HCl buffer (pH 3.0) and immediately buffered with 125 µl of 1 M KPO₄ buffer (pH 8.0) per fraction.

**Characterization of antisera.** Immunoreactivity of α-amylase and pullulanase antisera were investigated by the Ouchterlony technique by using the supernatant of cells grown on starch (19). The immunospecificities of anti-α-amylase, pullulanase, and α-amylase peptide antisera were tested by Western immunoblotting (28) with a semidry electroblotter (Biometra) for 15 min and 5 mA/cm² of nitrocellulose. The background was covered with 1% (wt/vol) bovine serum albumin and after incubation with the specific antisera (20 µg of anti-α-amylase peptide antiserum per ml, 24 µg of α-amylase antiserum per ml, or 24 µg of pullulanase antiserum per ml), the reaction between the antisera and the isolated α-amylase or the cell extract, respectively, was shown by using peroxidase-conjugated secondary antibodies (1:500).

**Negative staining.** Cells were negatively stained with 0.5% (wt/vol) neutralized phosphotungstic acid as described previously (29).

**Freeze substitution.** *C. thermosulfurogenes* EM1 cells grown on 1% glucose or 1% starch were removed from continuous culture and washed twice with 50 mM phosphate-buffered saline (0.9% NaCl; pH 6.9). Afterwards, small droplets of the cell suspension were put between two electron microscopic copper grids and rapidly frozen in liquid propane at −170°C. The ice formed in the samples was replaced by 100% methanol during a slow warming procedure (from −170 to −35°C). Afterwards, the methanol was replaced by low-temperature embedding resin (Lowicryl K4M); all further steps of preparation were performed as described (22).

**Low-temperature embedding.** Cell samples removed from batch cultures or continuous cultures were washed with phosphate-buffered saline. Chemical fixation and all further preparation steps were performed as described (22).

**Electron microscopic immunolabeling experiments.** Solutions of protein A-gold for electron-microscopic immunocytochemistry were prepared by established procedures (23, 25).

Labeling experiments (10) aimed at the localization of α-amylase and pullulanase were performed with cells of *C. thermosulfurogenes* EM1 prepared by two different low-temperature techniques. Immunocytochemical studies with anti-α-amylase peptide antiserum and single- and double-labeling experiments with antisera directed against α-amylase or pullulanase were performed with cells of *C. thermosulfurogenes* EM1 prepared for electron microscopy according to the low-temperature embedding procedure (22). The presence of α-amylase at the cell periphery, lining the cytoplasmic membrane and the blebs, was documented for *C. thermosulfurogenes* EM1 samples prepared by freeze substitution. Anti-α-amylase antiserum was applied for this purpose.

To reveal the possible occurrence of nonspecific adsorption of protein A-gold to sections, we directly incubated ultrathin sections with protein A-gold, omitting the antibody step. The absence of nonspecific binding of immunoglobulin G to the ultrathin sections was verified by application of preimmune serum followed by incubation on protein A-gold. To make sure that there was no nonspecific binding between the sections of clostridia and antisera prepared in rabbits, ultrathin sections were labeled with a nonspecific antiserum, also raised in rabbits (antiserum directed against membrane-bound hydrogenase from *A. eutrophus* H16).

Electron micrographs were taken with a Philips EM 301 transmission electron microscope at calibrated magnifications. Evaluations of electron micrographs were performed on enlarged prints at calibrated magnifications.

**Enzymes and chemicals.** Pullulan from *Aureobasidium pullulans*, maltose, hemocyanin from keyhole limpets, glutaraldehyde, and chemicals were purchased from Sigma Chemical Co., St. Louis, Mo. Soluble starch was obtained from Fluka, Neu-Ulm, Federal Republic of Germany. Biochemicals and enzymes were from Boehringer GmbH, Mannheim, Federal Republic of Germany.

**RESULTS**

**Bleb formation.** Formation of blebs and vesicles originating from the cytoplasmic membrane has been observed...
previously in negatively stained *C. thermosulfurogenes* EM1 cells (2) cultivated both in batch culture and in continuous culture. Preparation of cells for ultrathin sectioning by conventional chemical fixation and embedding in Spurr low-viscosity resin (27) was not suitable for documenting the occurrence of blebs (data not shown). However, blebs could be clearly shown by freeze substitution, i.e., replacement of chemical fixation by freezing, followed by substitution of ice by methanol, and finally embedding in low-temperature resin (Lowicryl K4M), polymerized at −35°C by UV light (Fig. 1A).

We used negative staining to investigate bleb formation in a number of other gram-positive bacteria, grown in batch culture, with seemingly intact cell walls. Blebs could be found in all these gram-positive bacteria and in those which are not known to produce α-amylase or pullulanase (Table 1). No blebs were seen in gram-negative bacteria.

**Relationships between bleb formation, cell wall degradation, and enzyme activity.** Bleb formation and cell wall degradation in *C. thermosulfurogenes* EM1 were systematically investigated by negative staining of cells grown in continuous culture under a variety of conditions (Table 2). From these results it was evident that blebs were formed under all conditions investigated, i.e., not only in cells with partially or totally degraded wall layers, but also in cells with seemingly intact wall layers (Fig. 1B). On average, 90% of the individual cells of a sample exhibited blebs. The various ratios of extracellular α-amylase and pullulanase activities to the total activity of the culture (i.e., cell-bound plus extracellular activity), obtained by variation of the substrate, dilution rate, and phosphate concentration (Table 2), did not significantly vary the observable rate of bleb formation.

Blebs were also formed in cells which were grown in batch culture on starch or on glucose. No significant difference was observed between samples taken from the exponential or stationary phase of growth. In batch culture, cell wall degradation was visible only when cultivation was performed in a medium with low phosphate concentration (1 mM phosphate instead of 50 mM). When cells were grown in batch culture on 1% starch in mineral medium, values of 310 U/liter for α-amylase and 230 U/liter for pullulanase activity were measured. Only about 10% of these total enzyme activities were found to be extracellular (i.e., in the culture fluid), and 90% were cell bound. Growth in batch culture on glucose instead of starch in mineral medium was paralleled by a significant decrease in the activities of the amylytic enzymes. Under these conditions, 30 U of α-amylase per liter and 50 U of pullulanase per liter were detected as cell-bound enzymes; no enzymatic activities could be detected in the culture fluid.

**Immunological reactivity.** When the Ouchterlony technique was applied to the analysis of immunoreactivity (Fig. 2), a distinct reaction between α-amylase or pullulanase antiserum and the supernatant of cells grown on starch could

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**TABLE 1. Analysis of bleb formation in gram-positive and gram-negative bacteria**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Gram-staining behaviour</th>
<th>Growth conditions</th>
<th>Growth temp (°C)</th>
<th>Bleb formation</th>
<th>α-Amylase activity (U/liter)</th>
<th>OD₅₇₈*</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. subtilis</em></td>
<td>+</td>
<td>Aerobic</td>
<td>37</td>
<td>+</td>
<td>250</td>
<td>266*</td>
</tr>
<tr>
<td><em>B. licheniformis</em></td>
<td>+</td>
<td>Aerobic</td>
<td>37</td>
<td>+</td>
<td>50</td>
<td>451*</td>
</tr>
<tr>
<td><em>B. stearothermophilus</em></td>
<td>+</td>
<td>Aerobic</td>
<td>60</td>
<td>+</td>
<td>167</td>
<td>77*</td>
</tr>
<tr>
<td><em>C. acetobutylicum</em></td>
<td>+</td>
<td>Aerobic</td>
<td>37</td>
<td>+</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td><em>C. thermosaceticum</em></td>
<td>+</td>
<td>Anaerobic</td>
<td>60</td>
<td>+</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td><em>A. eutrophus</em></td>
<td>−</td>
<td>Aerobic</td>
<td>30</td>
<td>−</td>
<td>600*</td>
<td></td>
</tr>
<tr>
<td><em>L. helveticus</em></td>
<td>+</td>
<td>Anaerobic</td>
<td>37</td>
<td>+</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>−</td>
<td>Aerobic</td>
<td>37</td>
<td>−</td>
<td>310*</td>
<td></td>
</tr>
</tbody>
</table>

* Samples were taken at the end of the exponential phase of growth; blebs were visualized by negative staining. All organisms were cultivated on complex medium in batch culture. The medium for *C. thermosaceticum* was supplemented with 1% glucose; *L. helveticus* was grown with 1% lactose as the carbon source. 

* OD₅₇₈, Optical density at 578 nm. Asterisks denote Klett units.
be observed. The same finding was obtained in experiments using these antisera and a cell extract prepared from these cells. A positive immunoreaction was also observed when these antisera were incubated with cell extracts prepared from glucose-grown cells. However, no reaction was detectable when the antisera were used for this kind of analysis as applied to the supernatant of cells grown on glucose.

Further immunological investigations were performed by using anti-α-amylase peptide antiserum. Interestingly, no positive reaction was obtained when native α-amylase isolated from the cell-free culture fluid of C. thermosulfurogenes EM1 was used. However, immunoreactivity could clearly be seen in Western blotting experiments when this

**TABLE 2. Influence of various growth conditions on enzyme activities, bleb formation, and cell wall preservation**

<table>
<thead>
<tr>
<th>Physiologic conditions</th>
<th>OD$_{578}$</th>
<th>Enzyme activity (U/liter)</th>
<th>Bleb formation</th>
<th>Cell wall preservation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total</td>
<td>Extracellular</td>
<td>%</td>
</tr>
<tr>
<td>1% starch, 50 mM P, D = 0.075/h</td>
<td>2.2</td>
<td>3,000</td>
<td>1,980</td>
<td>66</td>
</tr>
<tr>
<td>1% starch, 0.2 mM P, D = 0.075/h</td>
<td>1.9</td>
<td>1,100</td>
<td>1,045</td>
<td>95</td>
</tr>
<tr>
<td>1% starch, 50 mM P, D = 0.19/h</td>
<td>2.0</td>
<td>470</td>
<td>330</td>
<td>70</td>
</tr>
<tr>
<td>1% starch, 50 mM P, D = 0.03/h</td>
<td>2.0</td>
<td>2,638</td>
<td>1,366</td>
<td>51</td>
</tr>
<tr>
<td>1% glucose, 50 mM P, D = 0.075/h</td>
<td>1.8</td>
<td>40</td>
<td>60</td>
<td>+++</td>
</tr>
<tr>
<td>1% glucose, 0.3 mM P, D = 0.075/h</td>
<td>1.7</td>
<td>83</td>
<td>140</td>
<td>+++</td>
</tr>
<tr>
<td>2% starch, 50 mM P, D = 0.075/h</td>
<td>2.0</td>
<td>172</td>
<td>58</td>
<td>34</td>
</tr>
<tr>
<td>2% starch, 0.2 mM P, D = 0.075/h</td>
<td>2.3</td>
<td>550</td>
<td>392</td>
<td>71</td>
</tr>
<tr>
<td>1% lactose, 50 mM P, D = 0.075/h</td>
<td>1.5</td>
<td>34</td>
<td>100</td>
<td>+++</td>
</tr>
<tr>
<td>1% galactose, 50 mM P, D = 0.075/h</td>
<td>1.5</td>
<td>90</td>
<td>156</td>
<td>+++</td>
</tr>
</tbody>
</table>

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*a C. thermosulfurogenes EM1 was cultivated anaerobically in continuous culture under various physiological conditions (P, phosphate; D, dilution rate) at pH 5.9 and 60°C.

*b OD$_{578}$, Optical density at 578 nm.

*c Total, Cell-bound and extracellular enzyme activity; extracellular, extracellular enzyme activity; %, percentage of total activity that is extracellular.

*d ++++, 90% of the cells show blebs or good preservation of cell wall; ++++, 50% of the cells show blebs, 70% of the cells show good preservation of the cell wall.

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**FIG. 2. Ouchterlony immunodiffusion test with antisera against native α-amylase and pullulanase.** Samples were prepared after growth of bacteria on 1% (wt/vol) starch (A and B) or 1% (wt/vol) glucose (C and D). The central wells (wells no. 4) contained 450 μg of α-amylase antiserum (A and C) or 270 μg of pullulanase antiserum (B and D). Growth conditions are described in the legend to Fig. 1. (A and B) Well 1, cell extract not centrifuged (190 μg of protein); well 2, cell extract centrifuged at 10,000 × g (177 μg of protein); well 3, supernatant (62 μg of protein). (C and D) Well 1, cell extract not centrifuged (216 μg of protein); well 2, cell extract centrifuged at 10,000 × g (204 μg of protein); well 3, supernatant (89 μg of protein).

**FIG. 3. Western blotting of enriched denatured α-amylase (68 kDa) and nondenatured cell extracts of C. thermosulfurogenes EM1 grown on 1% (wt/vol) starch in continuous culture.** Growth conditions are described in the legend to Fig. 1. Lanes: 1, 8.8 μg of enriched denatured α-amylase incubated with 24 μg of α-amylase antiserum per ml; 2, 8.8 μg of enriched denatured α-amylase incubated with 20 μg of α-amylase peptide antiserum per ml; 3, 30 μg of nondenatured cell extract incubated with 24 μg of α-amylase antiserum per ml; 4, 36 μg of nondenatured cell extract incubated with 24 μg of pullulanase antiserum per ml. All antibody incubation steps were conducted overnight at 4°C. The arrows in lanes 3 and 4 indicate protein bands which correspond to the activity staining of these enzymes.
enzyme sample was used (Fig. 3). The prepared peptide antiserum could recognize only the denatured α-amylase (Fig. 3).

These data, taken together, indicate that the amino acids making up the N-terminal sequence (the peptide antiserum had been raised against a peptide of this sequence) may not be exposed in the native enzyme and that this sequence is accessible for binding of sequence-specific immunoglobulin G antibodies after denaturation.

Western blotting of nondenatured cell extracts of C. thermosulfurogenes EM1 by using the pullulanase or the α-amylase antiserum showed the specificity of the antisera. One band for pullulanase and three bands for α-amylase (Fig. 3, lanes 3 and 4) correspond to the activity staining of these enzymes (26, 26a).

Identification and localization of α-amylase and pullulanase in C. thermosulfurogenes EM1 by immunoelectron microscopy of cells grown in continuous culture. Immunolabeling experiments with the antiserum raised against α-amylase isolated from the bacteria, performed with cells prepared by freeze substitution, exhibited labeling specific for α-amylase in the immediate vicinity of the blebs (Fig. 4) and generally at the cell periphery (results not shown). No significant amount of label was present in the interior of the cells, i.e., in the cytoplasm or the nuclear region. The respective experiments for pullulanase (results not shown) revealed a similar situation. Very similar labeling was also observed when the cells were prepared by the Lowicryl technique, in both single- and double-labeling experiments (Fig. 5 and 6). However, because these cells had to be chemically fixed (in contrast to those prepared by freeze substitution), no formation of blebs could be seen.

The antiserum produced against a peptide synthesized, as a defined epitope, in analogy to the 21-amino-acid N-terminal sequence of the native extracellular α-amylase, was used for labeling experiments performed by the Lowicryl technique, with cells grown on starch or on glucose in continuous culture. The label was seen primarily along the cell periphery when the cells had been grown on starch (Fig. 7A). Growth of the cells on glucose resulted in a different labeling pattern; the label was not concentrated along the cell periphery, but occurred mainly in the cytoplasm (Fig. 7B); the nucleoid region was not labeled.

Figure 5G shows an immunolabeling control experiment which was representative of all controls (see Materials and Methods). The testing of nonspecific binding of protein A-gold was carried out for all single- and double-labeling experiments with all kinds of antisera and all different embedding techniques. The application of preimmune serum which was conducted with chemically fixed and freeze-substituted cells showed no nonspecific labeling. The incubation with a nonspecific antiserum (membrane-bound hydrogenase from A. eutrophus H16) also showed no labeling.

DISCUSSION

Cytological aspects. Application of an electron-microscopic preparation technique aimed at conserving the natural cytological features of living cells, i.e., freeze substitution followed by low-temperature embedding and UV polymerization, revealed that bleb formation occurs as previously described (2). Conventional preparation methods, involving chemical fixation, failed to demonstrate the presence of blebs. Obviously, the cytoplasmic membrane is very sensitive to alterations of the chemical moieties and concentrations in its surroundings. Bleb formation was found not only in C. thermosulfurogenes EM1 producing and secreting active α-amylase and pullulanase, but also in bacteria that are not active in this respect, independent of growth conditions (Table 1). In addition, other gram-positive bacteria showed formation of blebs derived from the cytoplasmic membrane. It can be speculated that gram-positive bacteria, in general, show formation of blebs. An explanation or characterization of a physiological role of this phenomenon is not yet possible. In contrast, typical gram-negative bacteria obviously lack this characteristic. Bleb formation was observed in Neisseria gonorrhoeae; however, the blebs originated from the outer membrane, as also occurs in several other gram-negative bacteria (6, 9, 20, 30).

As observed and described previously (2), cell wall degradation took place, under phosphate limitation, in cells grown both in continuous culture and in batch culture. It is known that in gram-positive bacteria, cell wall assembly is coupled with turnover of cell wall components (13). Newly formed peptidoglycan moves from its site of synthesis at the cell membrane to the periphery of the cell, where it is sloughed off. It may be assumed that, under phosphate limitation, C. thermosulfurogenes EM1 is no longer able to synthesize new cell wall components and that the process of sloughing off the outermost wall layer continues. The result would then be a net loss of cell wall density and stability, visible by electron microscopy as cell wall "degradation." Intact cells, i.e., cells grown under conditions where no wall degradation occurred, exhibited as many blebs as cells
LOCALIZATION OF α-AMYLASE AND PULLULANASE

A

B

C

D

E

F

G

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FIG. 6. Double label of α-amylase and pullulanase with antisera raised against the native enzymes and two different sizes of protein A-gold. Growth conditions are described in the legend to Fig. 1. Cells were prepared by low-temperature embedding. Pullulanase was labeled with small protein A-gold (3.6 nm); α-amylase was labeled with 11-nm protein A-gold. Bar, 0.2 μm.

which showed cell wall degradation. This finding indicates that blebs are not a consequence of the loss of cell wall integrity. We stress this point because, without this finding, one could speculate that the loss of integrity of the surface layer might be the cause of bleb formation.

Enzyme localization. As revealed by application of immunoelectron microscopy, α-amylase and pullulanase are located at the periphery, i.e., in the immediate vicinity of the cytoplasmic membrane, of cells harvested from continuous culture or batch culture. The technique is not suitable for discriminating between active and inactive enzymes, as long as inactivation does not completely destroy all the various epitopes of the enzymes which had given rise to the many immunoglobulin G types present in the polyclonal antisera applied. By using an antiserum produced against the enzyme-specific artificially synthesized peptide, we could exclude possible nonspecific immunological reactions. The results obtained by this approach confirmed the findings derived from the application of the other antiserum, raised against the native enzyme. The kind of immunoreactivity observed for the antiserum directed against the artificially synthesized enzyme-specific peptide, i.e., reactivity with enzyme in ultrathin sections, but lack of reactivity with the native enzyme enriched from the cell-free culture fluid, may be explained by the assumption that the respective stretch of amino acids is buried inside the native enzyme. This implies that only partially denatured enzyme (16, 21), as is present in samples prepared for electron-microscopic ultrathin sectioning, can be recognized by the antibodies. It has been reported in the literature that it may be difficult to obtain polyclonal antisera that react with the respective antigen in its non-denatured state (11, 12).

Active and inactive enzymes. In experiments aimed at localizing α-amylase and pullulanase by immunoelectron microscopic techniques, cells exhibiting high enzyme activity and those exhibiting virtually no enzyme activity were both labeled. This finding strongly suggests that α-amylase and pullulanase occur, in C. thermosulfurogenes EM1, in an active or an inactive form, depending on growth conditions. As the antiserum developed against the synthesized peptide gave positive labeling results in immunoelectron-microscopic experiments for cells containing active as well as inactive α-amylase (glucose-grown cells), we conclude that the inactive form of the enzyme also contains the respective amino acid sequence.

Although cells containing active enzyme and those containing inactive enzyme were both labeled, there were differences in their labeling patterns. In glucose-grown cells containing virtually no active enzyme, the immunolabel (antiserum produced against the synthesized peptide) was distributed all over the cytoplasm (not in the nucleoid region) and, in small amounts, along the cell periphery. Thus, it seems likely that active α-amylase is located predominantly at the cell periphery, whereas inactive α-amylase might be located in the cytoplasm. As a possible explanation of this distribution of enzymes, it could be speculated that the cytoplasmic forms of the enzymes have a conformation that is different from that of the active enzymes. In this case, the difference would have to be great enough to cause inactivity, but small enough to allow at least partial preservation of immunoreactivity. A different explanation might be that the cytoplasmic form of the enzyme is not yet processed. In this case, the α-amylase of C. thermosulfurogenes EM1 could provide a system for further studies on the mechanism of secretion.

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