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Antigenic Properties and Immunoelectron Microscopic Localization of *Mycobacterium fortuitum* β-Lactamase

BARBARA WAGNER,[1] LANFRANCO FATTORINI,[2] MANFRED WAGNER,[1] SHAO-HONG JIN,[2]†
ROLAND STRACKE,[3] GIANFRANCO AMICOSANTE,[4] NICOLA FRANCESCHINI,[4]
AND GRAZIELLA OREFICI[2]*

Institute of Experimental Microbiology, University of Jena,[1] and Institute of Molecular Biotechnology,[3] 0-7745 Jena, Germany, and Laboratory of Bacteriology and Medical Mycology, Istituto Superiore di Sanità, 00161, Rome,[2] and Departments of Sciences and Biomedical Technology and of Biometrics, University of L'Aquila, 67100 L'Aquila,[4] Italy

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***Mycobacterium fortuitum* is a fast-growing *Mycobacterium* species which produces a β-lactamase involved in the intrinsic resistance of the microorganism to β-lactam antibiotics. An anti-β-lactamase serum against the purified enzyme was raised in rabbits. Antibody binding was specific for native β-lactamase, and enzyme activity was partially inhibited by the serum; furthermore, cross-reactions with denatured class A β-lactamases were observed. This serum was used as a probe in immunogold labeling for the localization of the cell-bound β-lactamase in both the low-level producer ATCC 19542 (parental strain) and the overproducer mutant D316. By the combination of preembedding immunogold labeling and replica technique, it was shown that the β-lactamase was uniformly distributed on the whole external cell surface, where it appeared to be associated with a Tween 80-removable capsule-like material. Compared with the parental strain, a much higher level of expression of surface enzyme was observed in strain D316. Surface labeling was more intense in the stationary phase of growth than in exponentially growing cells. The data obtained are interpreted in the context of the intrinsic resistance of *M. fortuitum* to β-lactam antibiotics.**

Mycobacterium fortuitum is an environmental fast-growing *Mycobacterium* species which has been recognized as a cause of sporadic cutaneous abscesses and postoperative infections in patients undergoing dialysis, mammoplasty, transplant of heart valves, and cardiac bypass (37, 38). Clinical isolates are resistant in vitro to many antimicrobial agents including the conventional antituberculous drugs and most β-lactam antibiotics with the exception of cefoxitin, cefmetazole, imipenem, and clavulanic acid-amoxicillin combinations (2, 9, 10, 28).

The high levels of intrinsic resistance to β-lactams shown by fast-growing mycobacteria may depend on interacting mechanisms such as β-lactamase production, permeability of their membranes to these drugs, and affinity for penicillin-binding proteins (11, 23). We previously showed that *M. fortuitum* produces both cell-bound and exocellular β-lactamases (12). The released enzyme has been purified and characterized as a broad-spectrum class A β-lactamase with a molecular weight of 29,000 (1, 15). Furthermore, the gene encoding the enzyme has recently been cloned and the nucleotide sequence has been determined (33).

Because β-lactamases in gram-negative bacteria are found in the periplasmic space (4, 21, 34), those of mycobacteria have been assumed to be similarly located (24). In this report an anti-β-lactamase serum against the purified enzyme of *M. fortuitum* was raised in rabbits and was used to study some antigenic properties of the β-lactamase as well as the localization of the enzyme by immunogold labeling in electron microscopy.

* Corresponding author. Mailing address: Laboratory of Bacteriology and Medical Mycology, Istituto Superiore di Sanità, Viale Regina Elena, 299, 00161 Rome, Italy. Phone: 39-6-49902333. Fax: 39-6-491723.

† Present address: National Institute for the Control of Pharmaceutical and Biological Products, Temple of Heaven, 100050, Beijing, People's Republic of China.

MATERIALS AND METHODS

Organisms. *M. fortuitum* ATCC 19542 (parental strain) and the high-level β-lactamase producer mutant D316, which was obtained from the parental strain by nitrosoguanidine-induced mutagenesis (12), were used throughout the study. Both strains were grown for 8 days at 37°C in Mueller Hinton broth (Oxoid, Basingstoke, England) containing 1 μg of malachite green per ml under agitation (180 rpm). At various times, growth of the cultures was monitored by CFU determinations.

Kinetics of cell-bound β-lactamase production. Cell-bound β-lactamase production and CFU were determined as described previously (12). Briefly, after centrifugation of the cultures, the cellular pellet was washed with phosphate-buffered saline (PBS; pH 7.2). An aliquot of the pellet was used for immunogold labeling studies, while other aliquots were dried to a constant weight or were ultrasonicated. The supernatant of the ultrasonicate was used to determine the cell-bound β-lactamase activity by a spectrophotometric assay with cephaloridine as the substrate (12).

Purification of the β-lactamase. The enzyme was purified from the culture supernatants of the mutant strain D316 by gel filtration on a Sephadex G-75 column (Pharmacia, Uppsala, Sweden); this was followed by chromatofocusing on a Mono P HR5/20 column (Pharmacia) (1).

Preparation of the anti-β-lactamase serum. New Zealand White rabbits (Charles River, Calco, Italy) were inoculated intravenously on days 0, 7, 14, and 21 with a mixture containing 1 mg of the purified enzyme in 1 ml of PBS; the mixture was emulsified with 1 ml of incomplete Freund's adjuvant (Difco, Detroit, Mich.) and 2 ml of 2% (vol/vol) Tween 80. The animals were exsanguinated on day 28; the blood was allowed to clot at room temperature, and sera were stored in aliquots at −80°C. Preimmune sera were also collected from all the animals.

ELISA. The antibody titer of the anti-β-lactamase serum was determined by enzyme-linked immunosorbent assay (ELISA). Flat-bottom plates (Microtiter; Dynatech, Alexandria, Va.) were coated overnight at room temperature (RT) with 1 μg of β-lactamase suspended in 100 μl of 0.1 M carbonate buffer (pH 9.6). Plates were washed with PBS–Tween 20 (0.05%; vol/vol) and serum dilutions in PBS plus 0.01% gelatin were added. After 2 h of incubation at RT and three washings, 200 μl of goat anti-rabbit immunoglobulin G (IgG) alkaline phosphatase-conjugated antiserum (Sigma Chemical Co., St. Louis, Mo.) diluted 1:1,000 in PBS-gelatin was added and the plates were incubated for 2 h at RT. After washing, 200 μl of 1 mg of *p*-nitrophenylphosphate (Sigma) per ml diluted in diethanolamine buffer was added. After 1 h at RT, the optical density at 405 nm was read with a Titertek Multiscan instrument (Skatron, Oslo, Norway). The results were calculated by subtracting the absorbance of the anti-β-lactamase serum in the absence of the coating antigen.

A well was considered positive for the presence of anti-β-lactamase antibodies

when its absorbance was at least twice the absorbance of the well coated only with the antigen.

Neutralization of β-lactamase activity by anti-β-lactamase serum. The effect of the anti-β-lactamase serum on the enzyme activity was investigated by a neutralization test as described previously (31). Purified enzyme (final concentration, 33 μg/ml) was incubated with various amounts of serum in 0.05 M sodium phosphate buffer (pH 7) at 30°C, and then portions of the reaction mixtures were assayed for β-lactamase activity after 2, 10, or 60 min. The reaction mixtures were centrifuged at 14,000 × *g* for 15 min to separate immunoprecipitates, and the β-lactamase activities in the supernatants were assayed.

Gel electrophoresis and immunoblotting. The purified *M. fortuitum* enzyme, the crude supernatant from the D316 ultrasonicate, as well as class A or C nonmycobacterial β-lactamases (6, 7) were run in a denaturing sodium dodecyl sulfate (SDS)-polyacrylamide gel (26). They were also run in a nondenaturing polyacrylamide gel with the same reagents and conditions used for SDS-polyacrylamide gel electrophoresis (PAGE) but without SDS (20). Serum specificity and the immunological characterization of the enzyme were assessed by immunoblotting (35). The following β-lactamases were compared: type I from *Bacillus cereus* (class A, partially purified; purchased from Sigma), *Enterobacter cloacae* Q908R β-lactamase (class C; purified as described previously [16]), and form I from *Citrobacter diversus* ULA-27 (class A; purified as described previously [13]).

The proteins were separated by either SDS–12% PAGE or 12% PAGE in a mini-Protean II cell (Bio-Rad Laboratories, Richmond, Calif.) by using two identical gels carrying the same samples. One of the gels was stained with 0.1% Coomassie brilliant blue in 10% acetic acid–40% methanol and was destained with 10% acetic acid–40% methanol. The proteins of the second gel were transferred onto nitrocellulose by a Mini Trans-Blot module (Bio-Rad) by using Tris-glycine buffer (0.025 M Tris-0.192 M glycine) with 20% methanol at 14 V overnight. Nitrocellulose strips were soaked for 1 h at 37°C in Tris HCl-buffered saline (0.9% NaCl, 0.01 M Tris [pH 7.4]) containing 3% bovine serum albumin (Tris-BSA) and were incubated for 1 h with anti-β-lactamase serum diluted 1:2,000 in Tris-BSA containing 0.05% Tween 20 (Tris-BSA-Tween). After washing in 0.05% Tween 20–saline, the strips were incubated for 1 h with goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma) diluted 1:1,000 with Tris-BSA-Tween and were washed in 0.05% Tween 20–saline. All incubations and washings were performed at RT. Binding of the enzyme-labeled antibodies was detected by using a freshly prepared mixture of equal volumes of the AP color development reagent naphthol phosphate (0.4 mg/ml in water) and fast red (6 mg/ml in 0.2 M Tris HCl buffer [pH 8.2]; Bio-Rad).

Protein content was determined by the method of Bradford (5), with BSA used as the standard (5).

Electron microscopy. (i) General procedures. Cells from the logarithmic and the stationary growth phases of both strains were harvested by centrifugation at 3,000 × *g* for 10 min and were washed with PBS. Cells were vigorously passaged several times through a tuberculin needle to decrease bacterial clumping. For ultrastructural observations of the cell envelope, an aliquot of the bacteria was fixed with lysine-glutaraldehyde and was stained with ruthenium red (22); this was followed by fixation with osmium tetroxide and conventional embedding in Epon. Another aliquot was pretreated with 5% Tween 80 in PBS for 60 min at 37°C under agitation (27).

Colloidal gold suspensions with mean particle sizes of 16 and 10 nm were prepared as described previously (14, 32).

Swine anti-rabbit IgG (Seravac, Prague, Czech Republic), twice absorbed with bacillus Calmette-Guérin (BCG), was absorbed to colloidal gold in borate buffer (pH 9) (17). Protein A (Sigma) from *Staphylococcus aureus* was adsorbed to colloidal gold at pH 7.2. Another anti-rabbit IgG–gold preparation with a mean particle size of 10 nm was purchased from Sigma.

(ii) Preembedding labeling and embedding technique. The sedimented bacteria were mixed with an equal volume of anti-β-lactamase serum and were incubated for 1 h at RT. After three washings with PBS, the samples were incubated with protein A-gold at RT for 1 h and were then washed with PBS. Controls were performed by mixing cell suspensions with an equal volume of either rabbit preimmune serum or anti-β-lactamase serum absorbed overnight with excess β-lactamase or by omitting the anti-β-lactamase serum and incubating with gold reagent alone. An aliquot of the labeled bacteria was further processed by the replica technique (see below), while another aliquot was prepared for observation on ultrathin sections. The bacteria were first embedded in agar and fixed in a mixture of 0.5% formaldehyde (freshly prepared from paraformaldehyde) and 0.05% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) containing 0.2% NaCl and 0.02% $MgCl_2$. The samples were then treated with 0.05 M NH_4Cl in cacodylate buffer for 15 min and were washed with the same buffer. Dehydration in graded ethanol (30, 50, 70, and 90% and then twice in 100%) at progressively lower temperatures down to −20°C and infiltration with Lowicryl K4M or Lowicryl HM20 (Chemische Werke, Waldkraiburg, Germany) at −20 to −35°C were performed as described previously (3, 8). Embedding in Lowicryl HM20 included the treatment of bacteria before dehydration with 5% uranyl acetate for 30 min. The resins were polymerized with UV light with LKB Polylite 2114 equipment at a distance of 40 cm. During polimerization the gelatin capsules were dipped into an ethanol bath cooled to −35°C (18).

(iii) Postembedding labeling. Ultrathin sections were mounted on nickel grids. All steps of the labeling procedures were performed at RT unless indicated otherwise.

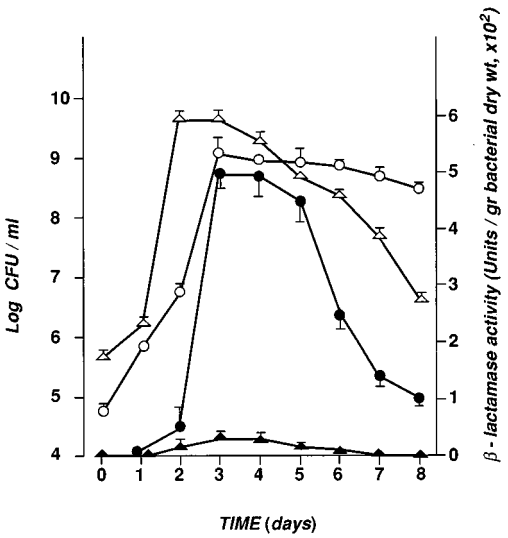

FIG. 1. Kinetics of cell-bound β-lactamase production in *M. fortuitum*. ▲, mean ± standard deviation β-lactamase activity of strain ATCC 19542; △, mean ± standard deviation CFU of strain ATCC 19542 per milliliter; ●, mean ± standard deviation β-lactamase activity of strain D316; ○, mean ± standard deviation CFU of strain D316 per milliliter.

Prewashings were done for 20 min each in 0.1 M Tris HCl buffer (pH 8.3) containing either 0.1 M lysin, 2% gelatin, or 0.25% BSA. The rabbit anti-β-lactamase serum was applied at a dilution of 1:20 at 4°C overnight. After 10 washings with buffer, the sections were incubated with anti-rabbit IgG–gold (diluted 1:50 in buffer) for 30 min. After five washings in buffer and then in double distilled water, the sections were poststained with 2% uranyl acetate in methanol; this was followed by staining with lead citrate.

(iv) Preparation of replicas. Gold-labeled and washed bacteria were spread onto small pieces of glass previously coated with polylysin. The bacteria were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 30 min. The cells were dehydrated in graded acetone (30, 50, 70, and 90% and then twice in 100%) and were dried at the critical point in CO_2 (31°C, 78 × 10^5 Pa) to preserve the delicate bacterial ultrastructures. Coating of the samples with carbon was performed by glow discharge in an acetylene atmosphere (pressure, 13.33 Pa; electrode gap, 30 mm; diameter, 90 mm; electrode potential, 1 kV; time, 15 s). The thickness of the carbon coat was 15 to 20 nm. The coated samples were detached from the glass support with vapors of hydrofluoric acid and were transferred to distilled water. After cleaning with sodium hypochlorite, the replicas were washed several times with distilled water and were mounted on copper grids with carbon-coated supporting films.

Ultrathin sections and replicas were observed under a Siemens Elmiscope 1 transmission electron microscope at an acceleration voltage of 80 kV. Representative photos were obtained by an observer blinded to the experimental groups.

RESULTS

Kinetics of cell-bound β-lactamase production. The kinetics of cell growth and bound β-lactamase activity for both ATCC 19542 and D316 strains are shown in Fig. 1. After a 48-h logarithmic phase of growth, the parental strain showed a short stationary phase of growth (24 h) that was followed by a rapid decrease in colony count. The production of cell-bound β-lactamase in this strain was low and did not correlate with bacterial growth.

Mutant strain D316 initially grew more slowly than the parental strain, but its stationary phase had a longer duration, and the final growth yield at 8 days was higher than that of the parental strain. A marked cell-bound β-lactamase activity was detected in this strain (approximately 14-fold higher than that in the parental strain), reaching the maximum early during the stationary phase.

Antigenic properties of the *M. fortuitum* β-lactamase. A highly purified β-lactamase preparation from *M. fortuitum*

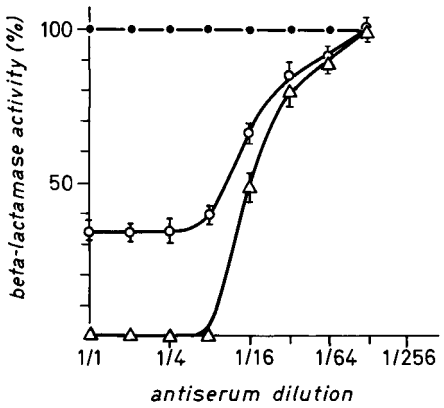

FIG. 2. Neutralization curve obtained for titration of β-lactamase from *M. fortuitum* D316 with anti-D316 β-lactamase serum. Purified enzyme (33 μg/ml) was incubated with various serum dilutions at 37°C for 2 min, and then portions of the reaction mixtures were assayed for their β-lactamase activities (○). The remaining reaction mixture was centrifuged to separate the immunoprecipitates, and the β-lactamase activities in the supernatants were determined (△). A dose-response curve obtained for titration of the β-lactamase with preimmune sera is also shown (●). Shown are means ± standard deviations of triplicate determinations.

D316 was used to raise a polyclonal serum in rabbits with an ELISA titer higher than 1:256,000. Figure 2 shows that when this serum was used in an assay of neutralization of β-lactamase activity, the maximum degree of neutralization after 2 min of incubation was approximately 65% of the initial activity even when excess antibody was used. Similar results were also obtained after 10 or 60 min of incubation (data not shown). The remaining enzyme activity (35% of the initial activity) was not due to the presence of antibody-unreactive β-lactamase, because it was removed by centrifugation of the samples.

The enzyme activity was not affected by incubation with a nonimmune rabbit serum.

By electrophoresis under denaturing conditions and immunoblotting with the rabbit hyperimmune serum, *M. fortuitum* β-lactamase appeared to be a homogeneous protein band strongly reactive with anti-β-lactamase serum (Fig. 3a and b, lanes 1). The ultrasonicates of whole cells of *M. fortuitum* D316 exhibited in immunoblots (Fig. 3b, lane 2) one major band with a reaction corresponding to that of the purified β-lactamase and some less reactive bands with higher molecular weights. Class A β-lactamases from *B. cereus* (Fig. 3a and b, lanes 3) and *C. diversus* (Fig. 3a and b, lanes 5) cross-reacted with the *M. fortuitum* enzyme, while the class C β-lactamase from *E. cloacae* (Fig. 3a and b, lanes 4) did not. A contaminant protein with an apparent molecular weight of 25,000 present in the commercial *B. cereus* β-lactamase preparation showed no reaction with the serum. When the materials were electrophoresed under nondenaturing conditions, the *M. fortuitum* β-lactamase reacted with the serum (Fig. 4a and b, lanes 1) and the supernatant of the D316 ultrasonicate showed a faint band corresponding to that of the β-lactamase (Fig. 4a and b, lanes 2). Among the β-lactamases tested for comparison, no cross-reaction was observed with the *C. diversus* enzyme (Fig. 4a and b, lanes 3); as for the *B. cereus* and *E. cloacae* β-lactamases, no blotting data could be obtained because they did not penetrate into the gel under our experimental conditions.

Cellular distribution of β-lactamase. After fixation with lysine-glutaraldehyde and staining with ruthenium red, the cell envelope of the parental strain was found to be well preserved and consisted of three layers separated from the cytoplasmic membrane by an electron-transparent zone (Fig. 5). The tripartite structure consisted of an inner layer (13 to 20 nm) with a medium electron density, an electron-transparent layer (6.5 to 16 nm), and an irregularly shaped outer layer with some ribbon-like structures that protruded outward. After embedding the cells in Lowicryl, the outer layer was not well preserved.

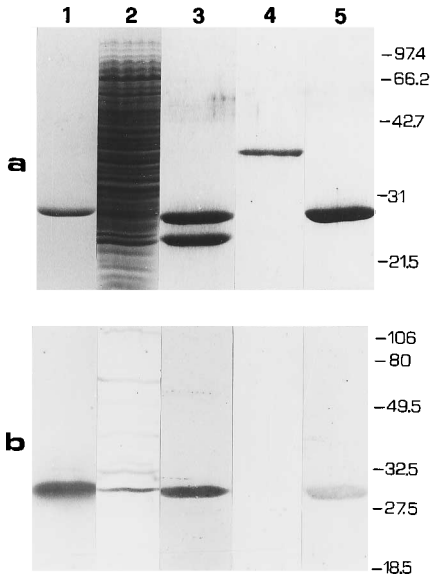

FIG. 3. SDS-PAGE (a) and immunoblotting (b) of β-lactamases from *M. fortuitum* D316 (4 μg of protein) (lanes 1), D316 ultrasonicate (160 μg of protein) (lanes 2); *B. cereus*, type I (30 μg of protein) (lanes 3), *E. cloacae* Q908R (5 μg of protein) (lanes 4), and *C. diversus*, form I (15 μg of protein) (lanes 5). Numbers on the right are molecular weights (in thousands).

Exponentially growing cells of mutant strain D316 incubated with the anti-β-lactamase serum and the gold reagent before embedding showed a weak surface labeling (Fig. 6A); in contrast, cells at the stationary phase of growth exhibited a heavy surface labeling (Fig. 6B) which decreased with time (Fig. 6C).

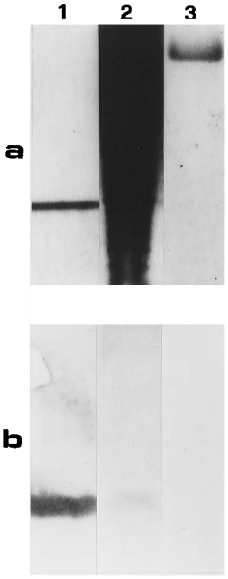

FIG. 4. PAGE (a) and immunoblotting (b) of β-lactamases from *M. fortuitum* D316 (4 μg of protein) (lanes 1), D316 ultrasonicate (160 μg of protein) (lanes 2), and *C. diversus*, form I (15 μg of protein) (lanes 3).

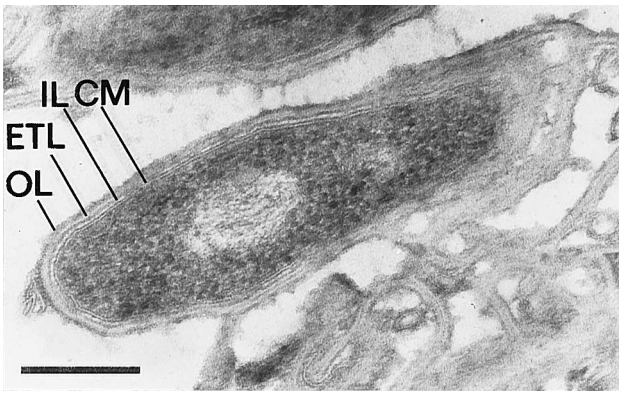

FIG. 5. Electron photomicrograph of an ultrathin section of *M. fortuitum* ATCC 19542 after fixation with lysin-glutaraldehyde and staining with ruthenium red. The outer layer (OL), electron-transparent layer (ETL), inner layer (IL), and cytoplasmic membrane (CM) are indicated. Bar, 0.25 μm.

No labeling was seen in cells incubated with protein A-gold only (Fig. 6D).

Replicas from labeled cells of both parental and D316 strains showed that the *M. fortuitum* β-lactamase was distributed on the whole external surface and that the labeling of logarithmic-phase cells (Fig. 7A and B) was lower than that of the stationary-phase cells (Fig. 7C and D). The cells of the D316 mutant appeared to be more heavily and uniformly labeled than the cells of the parental strain. When cells of the D316 mutant (stationary phase) were treated with 5% Tween 80 for 1 h before labeling, immunogold particles were detected only in association with the stripped off, amorphous material, thus suggesting that the β-lactamase was bound to a Tween 80-soluble surface matrix (Fig. 7F, arrow).

Control experiments in which the anti-β-lactamase serum was replaced by preimmune serum and then the gold reagent or experiments with gold reagent alone showed no immunolabeling (Fig. 7E).

The intracellular distribution of β-lactamase was studied by postembedding labeling of ultrathin sections from bacteria which were already incubated with the same antiserum and gold reagent of a different particle size before embedding and fixation. The enzyme was clearly seen to be located within different areas of the cytoplasm, often connected with the cytoplasmic membrane and the cell wall (Fig. 8A). On some sites a continuity of the intracellular label (gold particles of 10 nm) with the label of the cell surface (gold particles of 16 nm) could be observed (Fig. 8A, arrow). It should be noted that the surface-localized enzyme was preserved only in bacteria which were already labeled before fixation and embedding. Without such stabilization by antibodies, no superficial enzyme could be demonstrated by postembedding labeling. Sections which were incubated only with the gold reagent showed no labeling (Fig. 8B). Sections incubated with β-lactamase-absorbed antiserum and anti-rabbit IgG–gold also showed no labeling (Fig. 8C).

DISCUSSION

In general, the β-lactamases of mycobacteria have been reported to be constitutive intracellular enzymes which are released into the medium mainly in aged cultures following cellular lysis (25). Among fast-growing mycobacteria, β-lactamase from *M. fortuitum* was recovered from both culture superna-

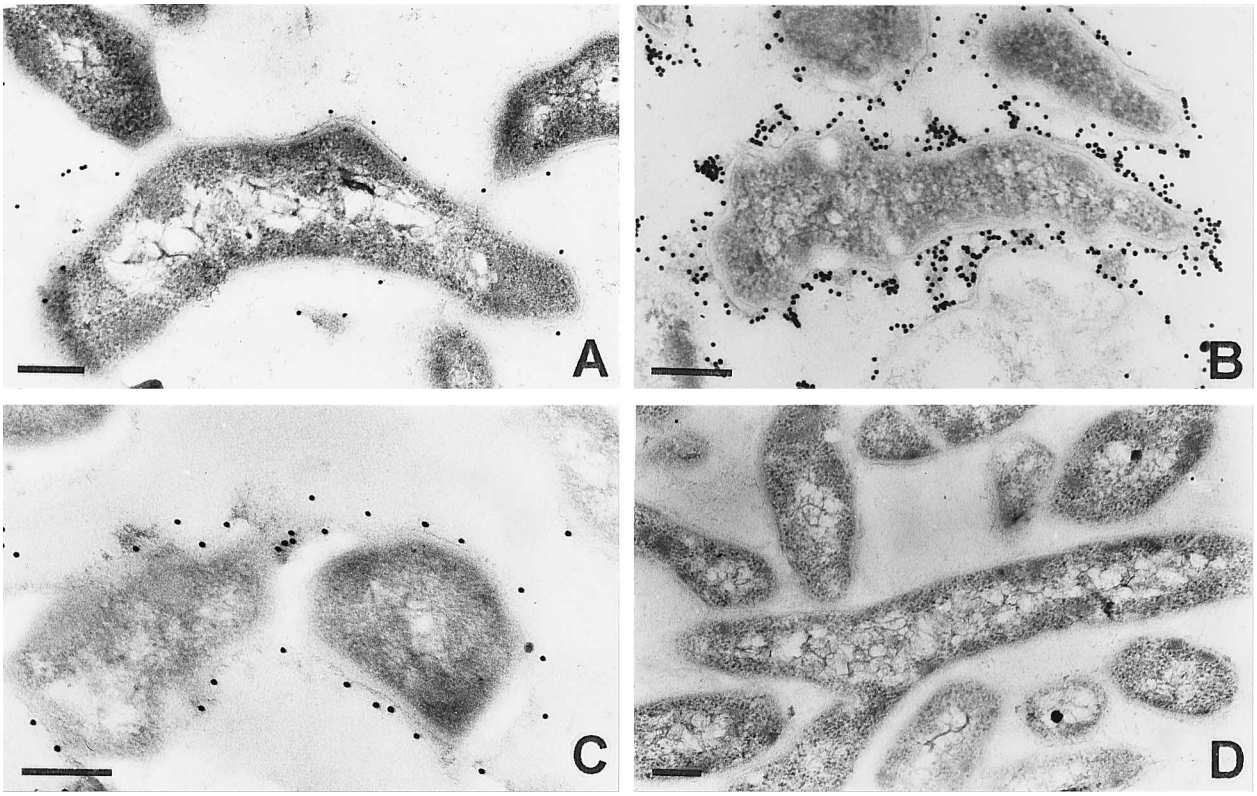

FIG. 6. Preembedding labeling showing the localization of β-lactamase on the cell surface of *M. fortuitum* D316 at different times of growth. (A) Log phase (63 h); (B) early stationary phase (100 h); (C) late stationary phase (163 h); (D) control section; early-stationary-phase cells incubated with protein-A gold only. Bars, 0.25 μm.

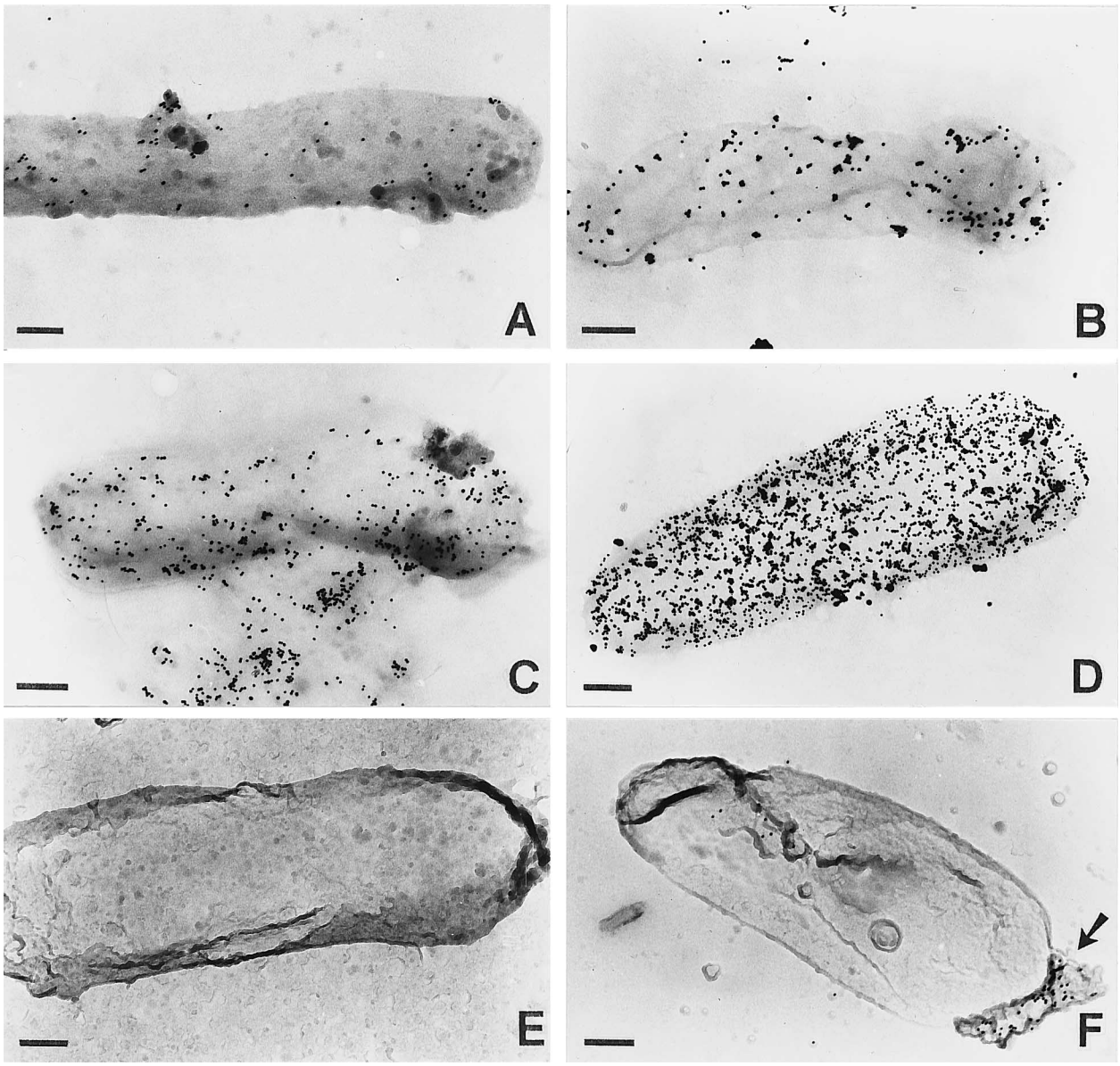

FIG. 7. Cell replicas showing the localization of β-lactamase on the cell surface of *M. fortuitum* after preembedding labeling. (A) Strain ATCC 19542, log phase; (B) strain D316, log phase; (C) strain ATCC 19542, stationary phase; (D) strain D316, stationary phase; (E) control section of strain ATCC 19542 incubated with protein A-gold only or with preimmune serum and protein A-gold; (F) strain D316, stationary-phase cells, pretreated with 5% Tween 80 for 1 h before labeling. Bars, 0.25 μm.

tants and extracts of cells grown in submerged cultures (28, 36). In previous studies by our group, the β-lactamase released by the overproducer mutant *M. fortuitum* D316 was purified and characterized (1), and the role played by the enzyme in the resistance of the microorganism to β-lactam antibiotics was studied (12). The present work was undertaken to investigate some antigenic properties and the cellular distribution of the enzyme.

By the use of a specific anti-β-lactamase serum it was shown that the interaction between antibodies and β-lactamase was very efficient since immunocomplexes formed rapidly and no remaining β-lactamase activity could be found in the supernatants. The lack of cross-reaction with native class A β-lactamase from *C. diversus* indicates that the antibodies are possibly directed against a conformational surface epitope(s) specific for *M. fortuitum* β-lactamase. However, cross-reactions of the mycobacterial enzyme with denatured class A β-lactamases, inclusive of that of *C. diversus*, suggests that our serum also contained cross-reactive antibodies that recognized linear epitope(s) common to the family of class A β-lactamase.

The specificity of the anti-β-lactamase serum and its suitability for use in immunolocalization studies were also demonstrated by the observation that nondenatured whole-cell lysates gave only one band in immunoblots; the band corresponded in molecular weight to the β-lactamase protein. The detection of extra bands with molecular weights of greater than 29,000 under denaturing conditions was probably due to the formation of aggregates of the β-lactamase itself or with other cellular components.

By preembedding immunogold labeling and electron microscopy, it was shown that the enzyme was located in the external bacterial surface and that labeling was particularly evident in

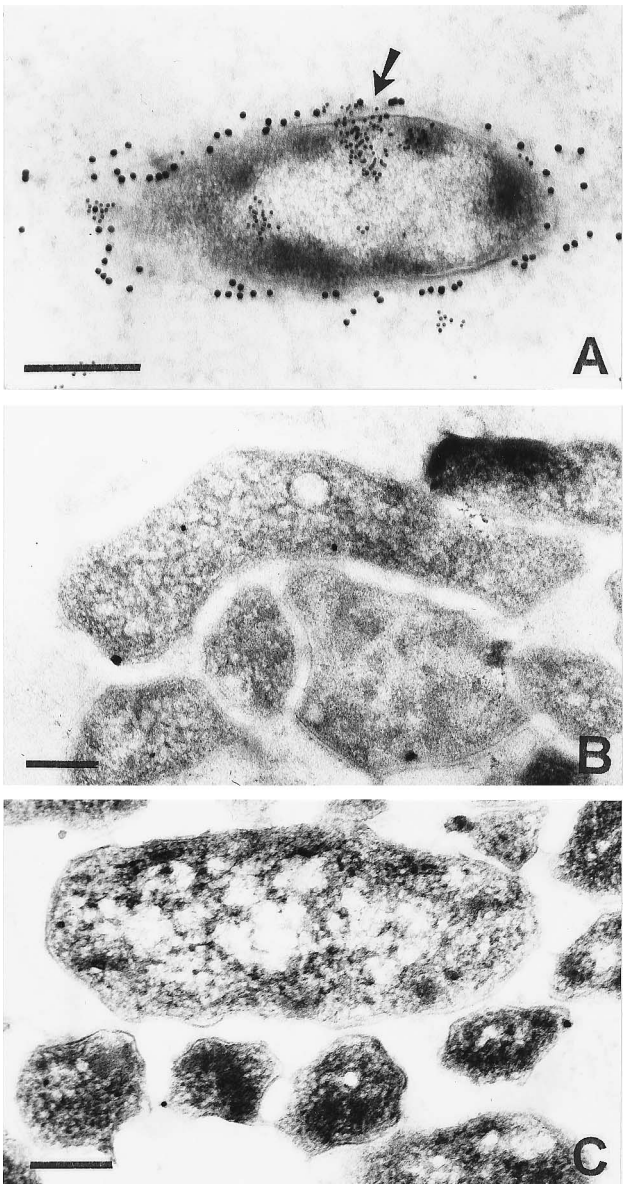

FIG. 8. Pre- and postembedding labeling showing the localization of β-lactamase on the cell surface and in the cytoplasm of *M. fortuitum* D316 (stationary phase). (A) Preembedding labeling performed with anti-β-lactamase serum followed by anti-rabbit IgG–gold (particle size, 16 nm). After fixation with glutaraldehyde and embedding in Lowicryl, the sections were incubated again with anti-β-lactamase serum and then anti-rabbit IgG–gold (particle size, 10 nm). The arrow shows a site in which a continuity between intracellular and cell surface label can be observed. (B) Control section, incubation with anti-rabbit IgG–gold only. (C) Control section, incubation with β-lactamase-absorbed antiserum and anti-rabbit IgG–gold. Bars, 0.25 μm.

stationary-phase cells, in keeping with the kinetics of cell-bound enzyme activity (Fig. 1). Replicas from labeled cells showed that the β-lactamase was rather uniformly distributed on the whole bacterial surface, where it appeared to be intimately associated with a thick capsule-like layer. The nature of this material was not investigated; however, its removal by the nonionic detergent Tween 80 suggests its similarity to other capsule-like structures of mycobacteria (29, 30).

The labeling of the cell-bound β-lactamase by the same antiserum raised against the exocellular form of the enzyme demonstrated the homology of the two forms. It could be speculated that, similar to *Bacillus licheniformis* (19, 39), the exocellular β-lactamase is derived from the cell-bound enzyme by peptide cleavage. Some support for this hypothesis also comes from the abundant release into the medium of the exocellular enzyme observed in both the D316 and the parental strains (12).

To the best of our knowledge this is the first report on the cellular and subcellular localization of a mycobacterial β-lactamase; the results presented here seem to indicate that the enzyme is synthesized within the cytoplasm and is subsequently exported to the cell surface.

The intrinsic resistance of *M. fortuitum* to β-lactams is probably determined by the interplay of cell wall barrier and β-lactamase activity, as reported for *Mycobacterium chelonei* (23). Surface-associated β-lactamase may efficiently act in concert with the low permeability of the mycobacterial cell wall (24) to provide a high degree of resistance to β-lactams. These mechanisms appear to be intermediate between those developed by gram-negative bacteria (which have relatively permeable cell walls and periplasmic β-lactamases) and gram-positive bacteria (which have cell-bound plus exocellular β-lactamase and penicillin-binding protein alterations) (34).

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