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Morphological Changes Induced by β-Lactam Antibiotics in *Mycobacterium avium-intracellulare* Complex

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In vitro activity of seven β-lactam antibiotics against strains of *Mycobacterium avium-intracellulare* was evaluated by the agar dilution method. The activity was influenced by the presence or absence of Tween 80 in Dubos medium, and cephazolin and cefotaxime were effective against most strains in the presence of Tween 80. β-Lactam antibiotics at low concentrations induced long filamentous cells with branching. In contrast to the filaments induced by ampicillin, in which septation was rarely observed, filaments induced by cephazolin had many septa, suggesting that the mechanisms of filament induction were different from the drugs used. At high concentrations, ampicillin and cephazolin induced osmotically sensitive cells with bulging at polar end of the cells. Analysis of penicillin binding proteins (PBPs) of the organism showed that there were at least nine PBPs with molecular weights between 32,000 and 94,000 in the cytoplasmic membrane. Ampicillin showed the highest affinity for PBPs 1a or 1b, or both, and also PBPs 3a or 3b, or both. In contrast, there was very little specificity of binding of cephazolin for any of the PBPs.

Mycobacterium avium-intracellulare complex is known to be resistant to most antituberculous drugs (4, 7, 11). Recently, however, certain β-lactam antibiotics, especially some cephalosporins, have been reported to be effective against the organism both in vitro and in vivo (6, 14, 19). It is well known that β-lactam antibiotics induce spheroplasts, filaments, or round cells in gram-negative bacteria, and the morphological responses elicited by the antibiotics are correlated with the affinities of the various drugs for the penicillin-binding proteins (PBPs) (17). For instance, in *Escherichia coli*, mezlocillin at a low concentration binds preferentially to PBP3 and as a result induces filaments (3), and mecillinum binds to PBP2, inducing osmotically stable round cells (16). In contrast, β-lactam antibiotics do not usually show such effects on gram-positive bacteria. However, benzylpenicillin is reported to induce filamentous forms in *Clostridium perfringes* (20) and in *Streptococcus bovis* (9) by inhibiting septation.

The present studies were performed to investigate the interaction of β-lactam antibiotics with *M. avium-intracellulare* in terms of binding to PBPs and morphological responses.

MATERIALS AND METHODS

Bacteria. *M. avium* Kirchberg, ATCC 3717, and ATCC 15769 and *M. intracellulare* TMC 1406 and TMC 1411 were from the stock culture in our laboratory. *M. intracellulare* 64, 101, 102, 103, 104, 105, 107, 108, and 135 were clinical isolates obtained from two different hospitals in Japan. They were stored in a −20°C freezer.

Media. Dubos agar medium (Difco Laboratories) with or without Tween 80 (0.06%) and Dubos liquid medium with Tween 80 were used throughout the experiments.

Antibiotics. Ampicillin, sulbenicillin, piperacillin, cephaloridine, cephazolin, cefotaxime, and moxalactam were obtained commercially.

Determination of MIC. MICs were determined by the agar dilution method by using Dubos agar medium with or without Tween 80. Inoculum sizes were adjusted to ca. 10^4 cells per drop. All plates were incubated at 37°C for 3 weeks.

Light microscopy. The slide culture method was used for observation of morphological changes induced by the antibiotics. Ten milliliters of Dubos agar medium (without Tween 80) containing various amounts of antibiotics was poured into a standard petri dish (9 cm in diameter) and allowed to harden. Blocks of agar ca. 1 by 1 cm were cut out with a blade and transferred to the surface of sterile microscope slides. After inoculation of *M. intracellulare* on the block, a sterile coverslip was placed on the top of the agar block. The slide was kept in a petri dish, into which a small amount of water was poured. The preparation was incubated at 37°C and checked under a phase-contrast microscope once or twice a day.

Preparation for electron microscopy. The tannic acid fixation method (18) was used to prepare specimens for electron microscopy. Cells were inoculated on Dubos agar medium (without Tween 80) containing various amounts of antibiotic. After 2 to 7 days of incubation at 37°, the cells were harvested and pre-fixed with 0.15 M cacodylate buffer containing 1% osmium tetraoxide for 1 h at room temperature. These pre-fixed cells were then washed with 0.15 M cacodylate buffer and post-fixed with 0.15 M cacodylate buffer containing 2% tannic acid and 2% glutaraldehyde for 2 h at room temperature. After being washed with cacodylate buffer, they were again fixed with 1% osmium tetraoxide overnight at 4°C. Cells were dehydrated in a graded series of ethanol and propyrene oxide and embedded in Epon 812. Samples were cut with a diamond knife, stained with uranyl acetate and lead citrate, and observed with a JEOL 100CX electron microscope. For scanning electron microscopy, fixed cells were dehydrated in a graded series of ethanol and amylacetate, dried by the critical-point method, and observed with a Hitachi S-700 scanning electron microscope.

Preparation of membranes. Cells were grown in a 1.5-liter batch culture for 5 days at 37°C with rotary shaking. After cooling on ice, the cells were harvested by centrifugation at 6,000 × *g* for 10 min and washed twice in 0.05 M Tris-hydrochloride buffer (pH 7.5) containing 1 mM $MgCl_2$ and 1

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TABLE 1. Comparative activities of seven β-lactam antibiotics against *M. avium-intracellulare*

Bacterium[a]	Antibiotic	Tween 80[b]	MICs for % of strains:		
			50	90	Range
M. avium (3)	Ampicillin	−	>160	>160	20–>160
		+	>160	>160	5–>160
	Piperacillin	−	>160	>160	40–>160
		+	>160	>160	20–>160
	Cephazolin	−	160	>160	40–>160
		+	40	40	5–40
	Cefotaxime	−	80	80	40–80
		+	40	40	2.5–40
M. intracellulare (11)	Ampicillin	−	10	>160	2.5–>160
		+	5	>160	1.25–>160
	Sulbenicillin	−	>160	>160	80–>160
		+	5	160	5–160
	Piperacillin	−	>160	>160	10–>160
		+	40	160	1.25–160
	Cephaloridine	−	40	>160	10–>160
		+	20	80	10–80
	Cephazolin	−	20	>160	2.5–>160
		+	5	10	1.25–10
	Cefotaxime	−	40	160	2.5–160
		+	5	20	1.25–20
	Moxalactam	−	160	>160	40–>160
		+	40	160	1.25–160

[a] Numbers in parentheses indicate the number of strains tested.
[b] MICs were determined on Dubos agar medium without (−) or with (+) Tween 80.

mM 2-mercaptoethanol. The washed cells were disrupted by grinding with twice the amount (wt/wt) of acid-washed glass beads (90 to 140 μm) for 40 to 60 min on ice. A 10-fold volume of 0.05 M Tris-hydrochloride buffer was added to the paste. Cell debris and glass beads were removed by centrifugation for 20 min at 8,000 × *g*, and the membranes were pelleted out from the supernatant by centrifugation at 100,000 × *g* for 60 min at 4°C. The membranes were resuspended in ice-cold Tris-hydrochloride buffer, washed twice by centrifugation, and finally dispersed into the above buffer with a Teflon homogenizer. The membrane suspension was stored in a −80°C freezer until use.

Analysis of PBPs. For the standard assay of PBPs, 10 μl of dilutions of [^{14}C]benzylpenicillin (Amersham Corp; specific activity, 54 Ci/mol) was bound to 100 μl of membranes for 10 min at 30°C, and the reaction was terminated by the addition of 2.4 μl of nonradioactive benzylpenicillin (120 mg/ml) and 5 μl of 20% sodium lauroyl sarcosinate. After the solution was allowed to stand for 20 min at room temperature, 60 μl of gel sample buffer (0.125 M Tris-hydrochloride [pH 6.8], 2% sodium dodecyl sufate, 10% glycerol, and 0.001% bromophenol blue) was added, boiled immediately for 5 min, and applied to gel slots of a 10% sodium dodecyl sulfate-polyacrylamide slab gel which had been previously subjected to electrophoresis for 60 min to remove ammonium persulfate. The discontinuous buffer system and the electrophoresis has been described in detail by Laemmli (8). The gel was stained for 1 h with 0.1% Coomassie brilliant blue in 50% methanol–10% acetic acid, diffusion-destained with several changes of 5% methanol–10% acetic acid, and prepared for fluorography (Kodak RP Royal X-Omat X-ray film) as described by Bonner and Lasky (1).

For the competition experiments, 100 μl of membranes was preincubated with 5 μl of either distilled water or dilutions of the nonradioactive β-lactams for 10 min at 30°C. Then 10 μl of 50 μCi of [^{14}C]benzylpenicillin per ml was added for a further 10 min at 30°C, and the binding was terminated by the addition of 2.4 μl of nonradioactive benzylpenicillin and 5 μl of 20% Sarkosyl as described above. Samples were fractionated by electrophoresis, and the level of [^{14}C]benzylpenicillin bound to each protein was measured as described above.

RESULTS

Susceptibility of *M. avium-intracellulare* to β-lactam antibiotics. The results of susceptibility tests on Dubos agar medium with or without Tween 80 are shown in Table 1. The susceptibility of individual strains to these drugs varied, and the presence of Tween 80 increased the susceptibility of the strains 2- to 10-fold. Cephazolin and cefotaxime appeared to be the most effective drugs when Tween 80 was added to Dubos agar medium, and other drugs were effective against only some of the strains. Two out of three *M. avium* strains were highly resistant to these drugs, whereas both laboratory strains of *M. intracellulare* belonged to the most susceptible group. β-Lactamase-resistant drugs (cefotaxime and moxalactam) did not necessarily show higher activity than β-lactamase-sensitive drugs.

Microscopic observation. *M. avium* ATCC 15769 and *M. intracellulare* 64, 102, 103, and 104, either susceptible or resistant to the drugs tested, were inoculated on Dubos agar medium (without Tween 80) containing various amounts of ampicillin, piperacillin, cephaloridine, cephazolin, or cefotaxime and examined for their morphological responses. Since the morphological responses induced by each of the antibiotics were similar irrespective of the strains used, the results obtained from strain 103 only are reported.

β-Lactam antibiotics (ampicillin, piperacillin, cephaloridine, cephazolin, and cefotaxime) at low concentrations (one-half to one-fifth of the MIC) induced long filamentous cells with branching. Figure 1 shows phase-contrast micrographs of normal control cells incubated for 3 days (Fig. 1a) and cells incubated with 3 μg of ampicillin per ml (MIC = 10 μg) for 3 days (Fig. 1b). All of the β-lactams induced filaments, but the filaments induced by penicillins were generally much longer than those induced by cephalosporins and had many

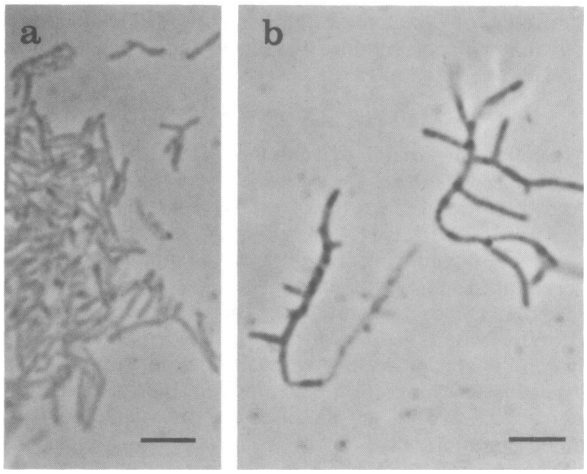

FIG. 1. Phase-contrast micrographs of *M. intracellulare* 103. (a) Control cells incubated on Dubos agar medium for 3 days without drug. (b) Cells treated with 3 μg of ampicillin per ml for 3 days. Bars, 5 μm.

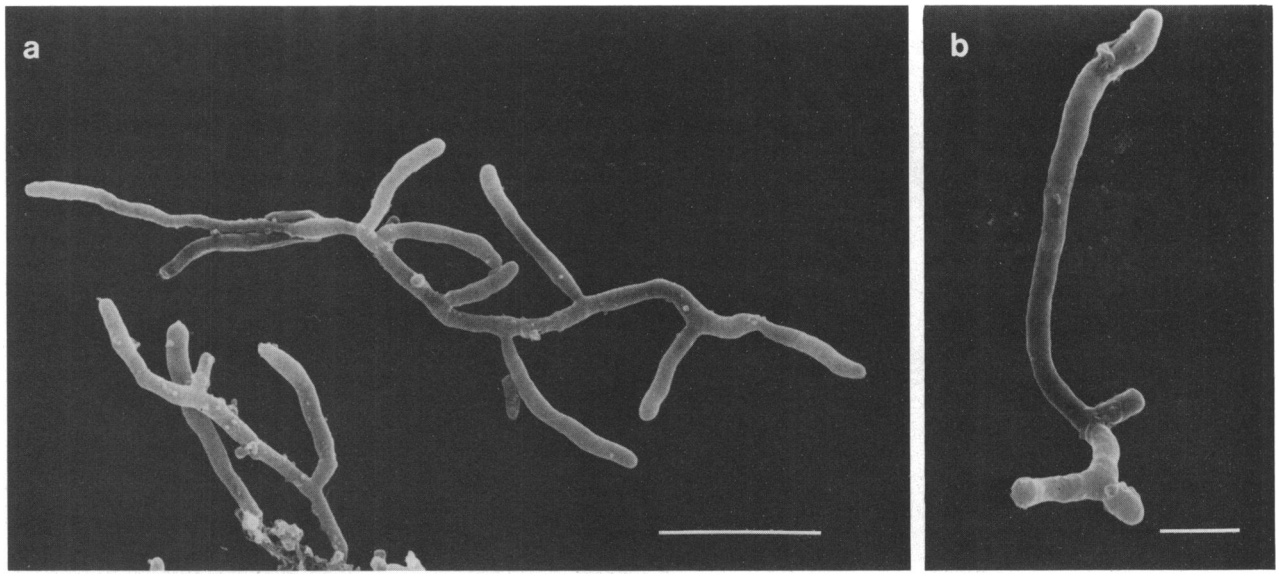

FIG. 2. Scanning electron micrographs of *M. intracellulare* 103 exposed to 3 μg of ampicillin per ml (a) and 2 μg of cephazolin per ml (b). Bars, 5 μm (a) and 1 μm (b).

branches. There appeared to be no difference in the effect of drugs at three different concentrations tested (one-half, one-third, and one-fifth of the MICs). Figure 2 shows scanning electron micrographs of branching filaments induced by 2 μg of cephazolin per ml (MIC = 10 μg) (Fig. 2b). The length of the cells grown in the presence of ampicillin often exceeded 30 μm (Fig. 2a). When stained with Ziehl-Neelsen's acid-fast stain, these cells retained acid fastness. In a liquid medium, the cells treated with the same concentration of the drugs were less elongated than those observed in agar medium. It might simply be due to the poor growth of the cells in the medium.

Control cells cultured on Dubos agar medium were also elongated to a certain extent in the initial stage of growth. However, they became short bacilli after 2 to 3 days of incubation.

Occasionally, partial ghost cells were observed in the filaments induced by both ampicillin and cephazolin. However, they were morphologically different (Fig. 3). In the case of ampicillin-induced filaments, the ghost part often extended to the whole branch (Fig. 3a), whereas that observed in the cephazolin-induced filaments was limited to only a part of the filament (Fig. 3b). This was due to the difference in septation. Ampicillin-induced filaments had no or only a few septa (Fig. 4a and b). In contrast, filaments induced by cephazolin had many septa within the filaments (Fig. 4c and d). However, the shape of these septa was abnormal; they were wider than normal and irregular in shape. Figure 4e shows a normal control cell with a septum in the middle.

The cell wall of β-lactam antibiotic-treated cells underwent some structural changes. When fixed with tannic acid, the cell wall of the normal cell was seen to consist of three distinct zones: a tannic acid-stained outermost zone, a transparent zone, and an innermost zone (Fig. 5a). However, after exposure to β-lactams, this profile was changed

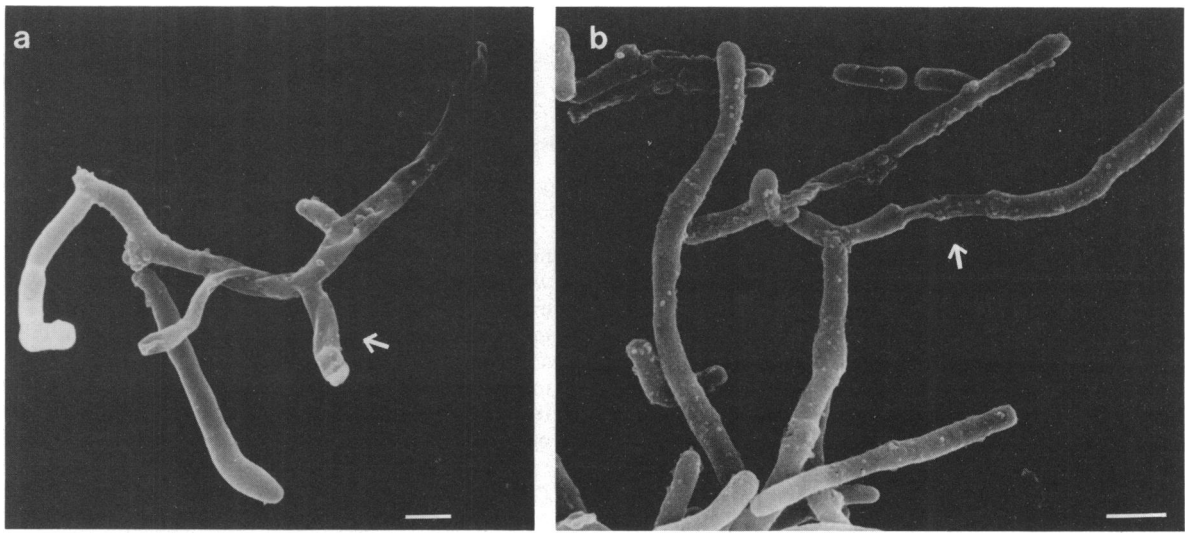

FIG. 3. Scanning electron micrographs of branching filaments of *M. intracellulare* 103. (a) Partial ghost cell induced by 3 μg of ampicillin per ml. (b) Cells exposed to 2 μg of cephazolin per ml. Arrows indicate the ghost part. Bars, 1 μm.

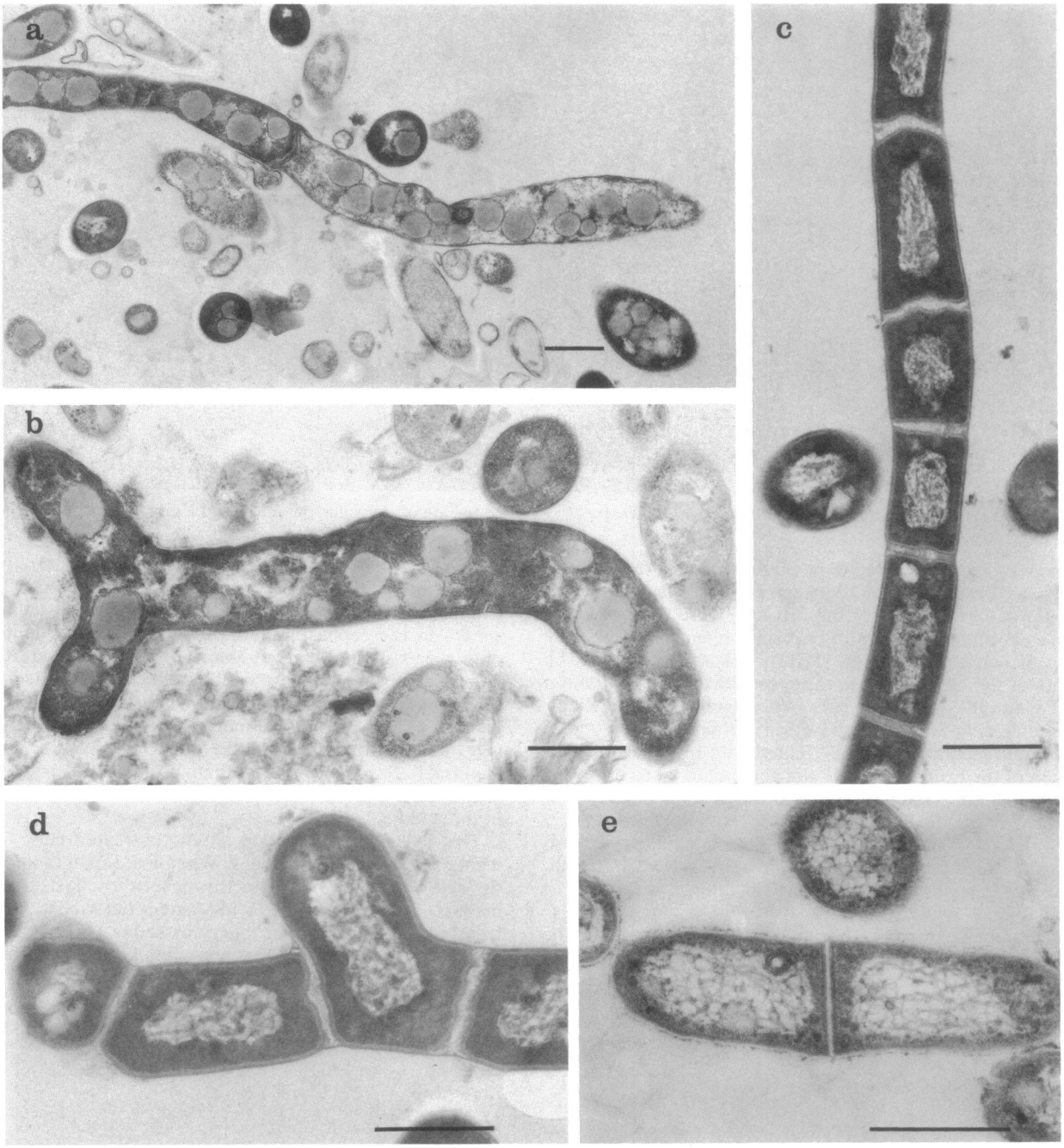

FIG 4. Ultrathin section of branching filaments of *M. intracellulare*. (a and b) Cells exposed to 3 μg of ampicillin per ml. (c and d) Cells exposed to 2 μg of cephazolin per ml. (e) Control cells incubated on Dubos agar. Bars, 0.5 μm.

considerably. The outermost and transparent zones became obscure, or very often completely disappeared, and the innermost zone became very thick (Fig. 5b).

At high concentrations (MIC or more), β-lactam antibiotics induced completely different forms of the organism. Figure 6a shows the cells treated with 100 μg of ampicillin per ml. Since at these concentrations, most cells were converted to ghosts, sucrose was added to the medium at a concentration of 0.3 M to maintain the shape of the cell. Swelling at the terminal end of the cells was the most commonly observed change. Cephazolin at higher concentrations (10 μg/ml or more) also caused bulging at the polar end of the cells (Fig. 6b). Since addition of sucrose was necessary to induce bulging, it was presumed that this part was osmotically fragile, presumably spheroplastic. However, the transmission electron micrograph shown in Fig. 7 indicates that the cell wall was still present in this area, and some material can be seen outside the cell wall. The cell wall in this area had the same thickness (about 27 mm) as that of the cylindrical part, and a partial defect of the cell wall was only occasionally observed.

PBPs of *M. intracellulare* and affinity of β-lactams for the PBPs. Since the effects of β-lactams on the morphogenesis of *M. intracellulare* varied depending on the drugs and their concentrations, we analyzed the PBPs of *M. intracellulare* 103 and the affinities of ampicillin and cephazolin for the

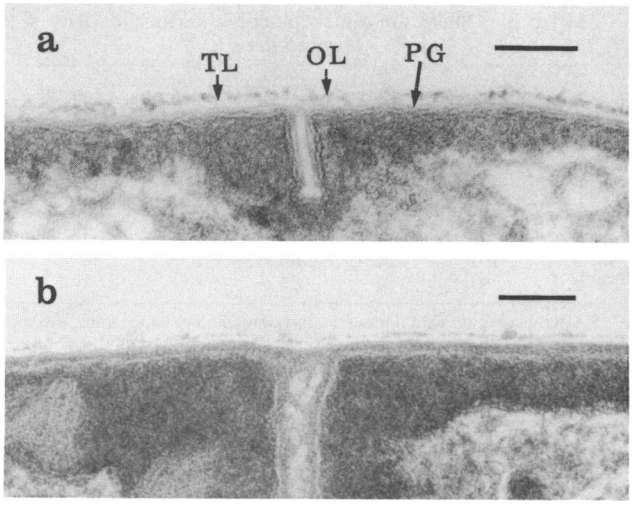

FIG. 5. High magnification of cell surface structure. (a) A control cell. (b) A cell treated with 2 μg of cephazolin per ml. OL, tannic acid-stained outermost zone; TL, transparent zone; PG, innermost zone. Bars, 0.1 μm.

PBPs. At least nine PBPs with molecular weights between 32,000 and 94,000 were observed in the cytoplasmic membrane of *M. intracellulare* (Fig. 8 and Table 2).

The affinity of β-lactams for these PBPs was determined by scanning of the fluorograph with a microdensitometer. The peaks obtained were carefully cut out and weighed. The results are shown in Table 3. Ampicillin showed the highest affinity for PBPs 1a or 1b, or both, and for 3a or 3b, or both. In contrast, cephazolin appeared to bind all of the PBPs rather uniformly.

DISCUSSION

It is generally known that most mycobacterial species are resistant to β-lactam antibiotics. However, there are some species of mycobacteria susceptible to the drug. One such species is the *M. avium-intracellulare* complex (6, 14, 19),

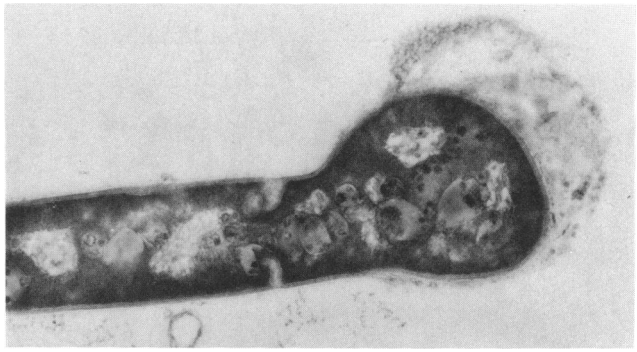

FIG. 7. Ultrathin section of a cell treated with 100 μg of ampicillin per ml. Bar, 0.5 μm.

and the reason appears to be due to the absence of β-lactamase activity of the organism (6). We found, however, that the susceptibility was influenced by the composition of the medium, and addition of Tween 80 greatly increased the susceptibility of the organism to the drug. Presumably Tween 80 decreases the permeability barrier of the organism, which is thought to be the general resistance mechanism in this organism (5, 11).

β-Lactam antibiotics at low concentrations induced filamentous cells in the *M. avium-intracellulare* complex. However, the mechanisms of induction of filamentous cells appeared to be different according to the drugs used. Ampicillin induces filaments by inhibiting the septation in a manner similar to its effect on *E. coli*, whereas cephazolin induces filaments but does not inhibit septation. Presumably, cephazolin inhibits cell separation, which under normal conditions occurs after the completion of septation. So far as we know, there have been no reports showing that a particular β-lactam antibiotic induces filamentation by inhibiting cell separation.

At high concentrations, β-lactam antibiotics induced different forms of the organism. Such a dose-dependent effect has already been reported for *E. coli*. Ampicillin and peni-

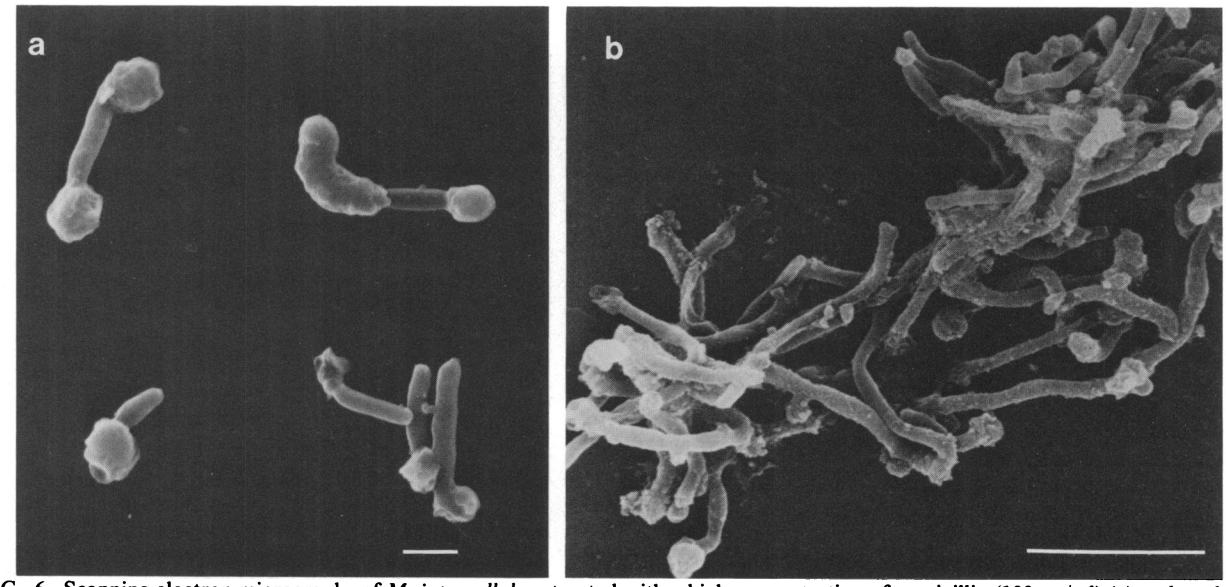

FIG. 6. Scanning electron micrographs of *M. intracellulare* treated with a high concentration of ampicillin (100 μg/ml) (a) and cephazolin (10 μg/ml) (b). Bars, 1 μm (a) and 5 μm (b).

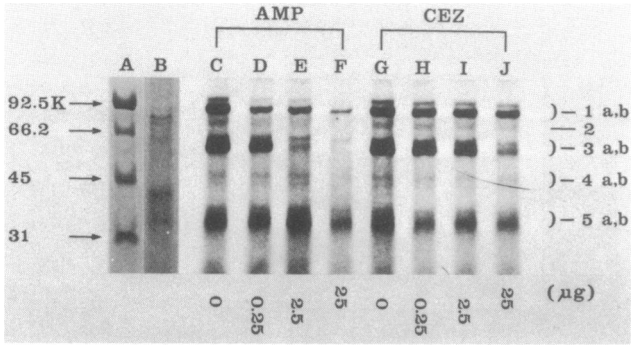

FIG. 8. PBPs of *M. intracellulare* 103 and affinities of ampicillin and cephazolin for the PBPs. Shown are Coomassie-blue stained marker proteins (lane A) and membrane proteins (lane B) of *M. intracellulare* after fractionation on a 10% sodium dodecyl sulfate-polyacrylamide gel. The PBPs were labeled with [^{14}C]benzylpenicillin. Lanes C and G, Without competition. Amounts of 0.25 (lane D), 2.5 (lane E), and 25 μg (lane F) of nonradioactive ampicillin and 0.25 (lane H), 2.5 (lane I), and 25 μg (lane J) of cephazolin were used for the competition.

cillin G have highest affinity for PBP2 and PBP3 of *E. coli*. Consequently, these drugs cause bulging and elongated cells at their lowest effective concentrations. However, at highest concentrations, these drugs also bind to PBP 1a and 1b and cause spheroplasting (3). Ampicillin and cephazolin at higher concentrations also produced osmotically susceptible cells from *M. avium-intracellulare*, and when sucrose was added to the agar medium, these compounds induced bulging at the polar end of the cells. Furthermore, the swollen part was encapsulated with some substance (Fig. 7). We do not know the nature of the substance. Presumably, it was the cell wall material excreted from the cell, but because of the inhibition of transpeptidation by the antibiotics, incorporation of the material into the cell wall was inhibited. Also, it could have been from the inhibition of peptidoglycan synthesis that occurs at high drug concentrations in many organisms.

Analysis of PBPs of *M. intracellulare* revealed that at least nine PBPs were present in the cytoplasmic membrane. The function of each PBP is not yet clear. However, our data indicated that ampicillin, which inhibited septation at low concentrations, had the highest affinities for PBPs 1a or 1b, or both, and PBPs 3a or 3b, or both, suggesting that one of the PBPs is involved in septum formation. In contrast, cephazolin bind to the mycobacterial PBPs rather uniformly: there is very little specificity of binding of cephazolin to any PBP. Possibly assays of another drug with a higher binding specificity could elucidate the functions of each mycobacterial PBP.

TABLE 2. Molecular weights and relative abundance of PBPs of *M. intracellulare*

PBP	Estimated mol wt	% Total binding[a]
1a	94,000	27.9
1b	90,000	
2	75,000	2.8
3a	60,000	39.5
3b	55,000	
4a	46,000	3.0
4b	44,000	
5a	37,000	26.8
5b	32,000	

[a] The fluorograph shown in Fig. 8 was scanned with a densitometer, and the peaks obtained were cut out and weighed.

TABLE 3. Affinities of β-lactam antibiotics for the PBPs of *M. intracellulare*

PBP	% Binding after competition[a] with:					
	Ampicillin (μg)			Cephazolin (μg)		
	0.25	2.5	25	0.25	2.5	25
1a, b	44	31	21	50	49	47
2	69	70	26	39	26	25
3a, b	57	22	10	63	40	29
4a, b	101	82	60	55	45	35
5a, b	92	95	74	65	70	71

[a] After incubation with nonradioactive ampicillin or cephazolin, PBPs were labeled with [^{14}C]benzylpenicillin. Results are expressed as a percentage of the image density of each PBP measured with a densitometer.

It is known that *M. avium-intracellulare* elongates into a long filament at the initial stage of growth (10, 12). We also observed such elongated forms during the first 2 to 3 days of incubation in the control culture. However, they never showed branching and became short bacilli after that. In contrast, filaments exposed to β-lactams were much longer, were branched, and never divided into coccobacillary forms.

In the early studies of the growth of mycobacteria phagocytized by HeLa cells, Shepard (15) and Brosbe and coworkers (2) reported that *M. avium* and Batty strains grow as branching filaments in the cells. It is not known whether the induction of branching filament is due to intracellular growth or to penicillin G added to the tissue culture medium. Presumably, penicillin G plays an important role in the induction of branching filaments under these conditions.

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