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Morphological Response of *Bilophila wadsworthia* to Imipenem: Correlation with Properties of Penicillin-Binding Proteins

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The penicillin-binding protein (PBP) patterns of six strains of *Bilophila wadsworthia* were investigated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis and subsequent fluorography of membrane preparations labelled with [^{3}H]benzylpenicillin. The PBP profiles among the strains were similar; generally, seven to nine PBP-reactive bands could be visualized; their molecular weights ranged from 31 to 137 kDa. The relative affinities of the PBPs of four strains of *B. wadsworthia* for imipenem were examined and correlated with the morphological responses of the cells to imipenem. Morphological changes were examined by light and electron microscopies. Light microscopy revealed that at low concentrations (less than the MIC), imipenem induced the formation of rounded and bulging cells; rarely, elongation without filamentation was observed. In the presence of imipenem at the MIC, spheroplast formation was observed. Scanning and transmission electron microscopies revealed round forms together with larger, multilobate cells in the presence of subinhibitory concentrations of imipenem, suggesting that new growth sites were initiated while cell division was inhibited. Peeling of the outer membrane was also seen. Spheroplasts were very large (up to 30 μm in diameter) and stable in aqueous solution. Inhibition of the PBPs could be seen in the presence of low imipenem concentrations.

Bilophila wadsworthia is a recently described anaerobic gram-negative bacillus commonly encountered in intra-abdominal infections (3); it has also been described in other infectious processes (2). While *B. wadsworthia* is found in the stool in mean counts of only 10^5 to 10^6/g (where total bacterial counts are $\sim 10^{12}$/g), it was the third most common anaerobe isolated during a study of infection related to gangrenous and perforated appendices (1, 5). Most strains are β-lactamase positive (24). Our early studies (27) indicated significant resistance to β-lactamase-stable β-lactams, such as imipenem and cefoxitin, among strains of *B. wadsworthia*. However, susceptibility results were difficult to interpret because many strains of *B. wadsworthia* produced a heavy haze on agar dilution plates, even in the presence of high concentrations of antimicrobial agents. Studies that used viability staining (triphenyltetrazolium chloride [TTC]), viability counts, and microscopy of the hazy areas showed that no net increase in viable cells occurred and that the haze consisted of spheroplasts (24).

Penicillin-binding proteins (PBPs) are the targets of binding for β-lactam antibiotics; upon binding, interference in normal cell wall synthesis ensues and results in disrupted cells and eventual cell death. The action of β-lactam drugs on certain PBPs governs the morphological changes in bacteria seen in the presence of these antibiotics (6, 7, 18, 20). Also, alteration in the binding of β-lactam antibiotics to the PBPs is a well-established resistance mechanism reported both in aerobic and in anaerobic bacteria (4, 10, 19, 28). The purpose of the study described here was to define the PBP patterns of *B. wadsworthia*, to describe the morphological changes that occur in the transition of the conventional growth to hazy growth upon exposure to imipenem, and to determine the role of PBPs in these changes.

MATERIALS AND METHODS

Bacterial cultures. The following six Wadsworth Anaerobe Laboratory (WAL) clinical isolates of *B. wadsworthia* were studied: WAL 7781, WAL 7813, WAL 7849, WAL 8144, WAL 8283, and WAL 9077. Strains were identified by standard methods (3, 12, 23). Isolates were maintained at −70°C in double-strength skim milk and were passed on brucella agar (Difco Laboratories, Detroit, Mich.) supplemented with 5% sheep blood, 5 μg of hemin per ml, 1 μg of vitamin K_1 per ml, and 1% pyruvic acid (Sigma Chemical Co., St. Louis, Mo.). All tests were performed with organisms from subcultures on brucella agar.

Chemicals. All chemicals used were obtained from Sigma Chemical Co. unless otherwise noted.

Antimicrobial agents. Imipenem (Merck Sharp & Dohme, West Point, Pa.) and penicillin G (Eli Lilly & Company, Indianapolis, Ind.) were obtained from the manufacturers and were prepared by the methods outlined in the reference method of the National Committee for Clinical Laboratory Standards (15). [^{3}H]penicillin was prepared and kindly donated by Avery Rosegay (Merck Sharp & Dohme, Rahway, N.J.).

Susceptibility studies. The antimicrobial susceptibility studies were performed by the Wadsworth agar dilution

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(AD) technique outlined in National Committee for Clinical Laboratory Standards document M11-A2 (15) and the *Wadsworth Anaerobic Bacteriology Manual* (23). *Bacteroides fragilis* ATCC 25285 and *Bacteroides thetaiotaomicron* ATCC 29741 were included as quality control organisms. MICs were determined on supplemented brucella agar which included 1% pyruvic acid to enhance the growth of *B. wadsworthia*. Plates were incubated for 48 h inside an anaerobic chamber (Anaerobe Systems, San Jose, Calif.); MICs were determined with the aid of TTC as described previously (24).

Broth macrodilution was performed by the methods described by the National Committee for Clinical Laboratory Standards (15) and in the *Wadsworth Anaerobic Bacteriology Manual* (23). Brucella broth (Difco) supplemented with 5 μg of hemin per ml, 1 μg of vitamin K_1 per ml, and 1% pyruvic acid was used as the base medium. MICs were recorded as the lowest concentration of each antimicrobial agent that did not permit visible growth.

β-Lactamase production. β-Lactamase production was assessed by the chromogenic cephalosporin nitrocefin disk test (Cefinase; BBL Microbiology Systems, Cockeysville, Md.) with 48-h cultures grown on brucella agar (supplemented with pyruvate). Growth was smeared onto a moistened disk, and the color change (from yellow to red for a positive reaction) was recorded within 10 min. Cell envelope preparations (20 μl) were tested similarly.

Subculture of the hazy area. Cells were grown on AD plates as described above. The growth from the hazy area in the presence of imipenem at concentrations of 32 to 64 μg/ml was transferred both to brucella agar plates without imipenem and to a hypertonic medium (to support the growth of cell wall-deficient forms). The hypertonic medium (MGSA) consisted of mycoplasma broth base (Difco), 1.0% glucose, 10% yeast extract (Difco), 10% mycoplasma-free fetal calf serum (Flow Laboratories, McLean, Va.), 1% pyruvic acid, and 1.5% agar. Cultures were incubated anaerobically, and growth was subsequently subcultured onto the same medium.

Light microscopy of the morphological changes on antibiotic-containing medium. Cells were grown on AD plates, and the MIC was determined at 48 h. Conventional growth, growth from the hazy areas (beyond the MIC), and colonies from subcultures of hazy areas (Brucella and MGSA) were removed and smeared onto microscope slides, fixed for 1 min by flooding with methanol (Mallinckrodt, St. Louis, Mo.), and stained for 5 min with Gram's crystal violet. Stains were observed by light microscopy at ×1,000 magnification. Also, cells grown in broth macrodilution tubes were observed at timed intervals (7, 24, and 48 h) after inoculation by dark-field microscopy at ×400 magnification. Growth from the hazy areas on AD plates was transferred to a drop of distilled water on a slide, covered with a coverslip, and examined to determine the stability of the spheroplasts in an aqueous (i.e., hypotonic) solution.

EM. Strains WAL 8144 and WAL 8283 were prepared for electron microscopy (EM). Organisms were grown for 48 h on imipenem-containing AD plates. Growth from (i) the control plate, (ii) AD plates with imipenem at concentrations less than the MICs, and (iii) the hazy area (i.e., agar dilution plates with imipenem at levels greater than the MIC) were further processed for EM. The growth was fixed by flooding the plates with 2.5% (vol/vol) glutaraldehyde (Polysciences, Inc., Warrington, Pa.) in 0.1 M potassium phosphate buffer (KPB; pH 7.4) for 1.5 h at room temperature. The growth was then scraped off gently with bent glass rods and was transferred to a centrifuge tube with a Pasteur pipette. The resulting suspension was centrifuged (5,000 × *g*, 10 min) and washed once with KPB. The pellet was resuspended in 2% (vol/vol) osmium tetroxide in 0.1 M KPB (pH 7.4) and the mixture was prepared for transmission EM (13) and scanning EM (9).

Cell envelope preparation. Cells were grown for 72 h in brucella broth (Difco Laboratories) supplemented with 5 μg of hemin per ml, 1 μg of vitamin K_1 per ml, and 1% pyruvic acid. The cell preparations were maintained at 4°C for all subsequent procedures. Growth was harvested by centrifugation (12,000 × *g*, 35 min) and was washed once with 0.05 M KPB (pH 7.0). The pellet was then resuspended in a 5- to 10-ml aliquot of 0.05 M Tris-HCl buffer (pH 7.5) containing 10 mM $MgCl_2$ and 5 μg of DNase per ml and was broken by three passages through a French pressure cell (SLM Instruments Inc., Urbana, Ill.) at 12,000 lb/in^2 (~83 MPa). The cell lysate was centrifuged (1,000 to 1,200 × *g*, 10 min) to remove unbroken cells; the supernatant was again centrifuged (45,000 × *g*, 45 min) to pellet the envelope material. The pellet (containing cell envelope) was washed once with 0.05 M Tris-HCl (pH 7.5) and was stored at −20°C or was used immediately. The pellet was extracted with a 400- to 500-μl volume of 0.05 M KPB (pH 7.0) containing 1 M NaCl, 2% Triton X-100, and 1 mM β-mercaptoethanol. The mixture was incubated at 4°C for 30 min and was then centrifuged at 45,000 × *g* for 30 min. The supernatant (containing the inner membrane) was stored at −70°C and was used for PBP assays within 24 h. Protein was assayed with the Pierce Protein Assay Kit (Pierce, Rockford, Ill.). The β-lactamase activity in the supernatant was measured by the nitrocefin disk assay; no β-lactamase activity was detected in the envelope preparations used for the PBP or competition assays.

PBP assays. *Escherichia coli* K-12 (ATCC 23716) was included in the assays as a reference standard and was also used as an internal molecular mass reference. A total of 300 μg of envelope protein in a total volume of 30 μl was mixed with 5-μl aliquots of [^{3}H]benzylpenicillin (10 μg/ml; specific activity, 60.28 mCi/mg). The mixtures were incubated at 37°C for 10 min; the reaction was then stopped by adding 5 μl of nonradioactive penicillin (12.5 mg/ml) and 5 μl of Sarkosyl (20%). The volume was adjusted to 80 μl with 0.2 M KPB (pH 7.0), and the samples were maintained at room temperature for 20 min before adding the electrophoresis sample buffer (20 μl). Samples were boiled for 5 min and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (14) containing 10% acrylamide at a ratio of acrylamide to methylene bisacrylamide of 30:0.8, and the gels were stained conventionally with Coomassie blue stain. Molecular mass standards (Bio-Rad Laboratories, Richmond, Calif.) were included with each gel, and standard curves were constructed. Molecular masses were calculated by regression analyses (Symphony; Lotus Development Corp., Cambridge, Mass.). For fluorography, destained gels were impregnated with En^{3}Hance (NEN Research Products, Boston, Mass.) for 1 h and were then rinsed with water for 0.5 h and dried onto filter paper. Dried gels were incubated with prefogged X-OMAT AR 5 Film (Eastman Kodak) for 5 days to 4 weeks, and the densities of the PBP bands were measured on a videodensitometer (model 620; Bio-Rad).

Affinities of PBPs for imipenem. The affinities of the PBPs for imipenem were assayed in four selected strains, one β-lactamase-negative (WAL 7813) and three β-lactamase-positive (WAL 7781, WAL 8144, and WAL 8283) strains. Appropriate concentrations of imipenem were mixed with

TABLE 1. Molecular masses of PBPs from *B. wadsworthia*

PBP	Molecular mass (kDa) of PBP from[a]:					
	WAL 7781	WAL 7813	WAL 7849	WAL 8144	WAL 8283	WAL 9077
1	89–99	92–119	97–123	101–137	86–95	86–95
2	88–91	87–91	ND[b]	(86)	(73–87)	ND
3	83–85	73–78	74–77	70–73	ND	75
4	66–74	62–68	64–68	61–64	60–73	63
5	ND	62	ND	ND	ND	ND
6	(62)	(60)	(63)	(60)	(66)	ND
7	(51–55)	50–55	52–54	47–53	(51)	ND
8	41–46	39–42	38–42	38–41	39–42	42
9	(37)	35	(32–35)	31–35	32–35	(33)

[a] Two values represent a range from two or more assays. Parentheses indicate a weak band(s).
[b] ND, not detected.

300 μg of envelope protein to yield final imipenem concentrations of 0.00125 to 512 μg/ml in a total volume of 30 μl. These mixtures were first incubated at 37°C for 10 min, and then 5-μl aliquots of [^{3}H]benzylpenicillin were added and the samples were thereafter processed as described above for PBP assays.

RESULTS

PBP patterns of *B. wadsworthia*. The PBP patterns of the strains tested are summarized in Table 1. The PBP profiles of the different strains were fairly similar; generally seven to nine PBP-reactive bands could be visualized. The molecular masses of the PBP-reactive bands ranged from 31 to 137 kDa; these PBPs were assigned numbers 1 to 9. PBP 1 was always the most prominent band observed; it was typically wide and diffuse and was possibly composed of multiple bands. PBPs 6 and 7 could not readily be detected; when visualized, they were generally light.

Susceptibility to imipenem. The MICs of imipenem were 0.125 μg/ml for strains WAL 7781, WAL 7813, WAL 8144, WAL 8283, and WAL 9077 and 0.0625 μg/ml for strain WAL 7849. The MICs by the broth macrodilution test were 0.25 μg/ml for all the strains. β-Lactamase was produced by all of the strains except for WAL 7813.

Affinities of PBPs for imipenem. The fluorographs of the competition assays were examined visually and with a densitometer, and the concentrations of imipenem at which the density of the band was decreased by 50% (IC_{50}s) were obtained. A fluorograph of a PBP competition assay with imipenem (WAL 7781) is shown in Fig. 1. The IC_{50}s for the various PBPs are listed in Table 2. Imipenem had a strong affinity for the low-molecular-mass PBPs (PBPs 7, 8, and 9) and PBP 2, which were usually inhibited at concentrations less than the MICs. Fifty percent of PBPs 1, 3, and 4 were generally inhibited at concentrations near the MICs; an additional band with a very high affinity for imipenem (IC_{50}, <0.03 μg/ml) was seen at the PBP 5 region only with strain WAL 7813. PBP 4 was fully inhibited by 1.0 μg of imipenem per ml for all the strains tested, whereas total inhibition of PBP 1 was not usually achieved except with higher concentrations (up to 256 μg/ml). A weak band (PBP 6) was occasionally visualized, with IC_{50}s for PBP 6 being less than the MICs.

Morphological response. The normal cell morphology of *B. wadsworthia*, as seen by transmission EM, is shown in Fig. 2. The cells were approximately 1-μm wide and 2- to 4-μm long. The cell wall was characteristic by having a tight arrangement of various layers, and the periplasmic space was not readily evident.

(i) Light microscopy. At concentrations near the MIC, the initial (7-h) response to imipenem in broth was rounding and slight elongation of the cells (about two times the regular cell length) with bizarre, abnormal morphology; this was followed by the formation of spheroplasts (22 h). The spheroplasts were formed either from the end or from the middle of the cells. Some elongation, but no filamentation, was observed at subinhibitory concentrations; at concentrations greater than the MIC, the formation of round forms was followed by the formation of spheroplasts and then lysis.

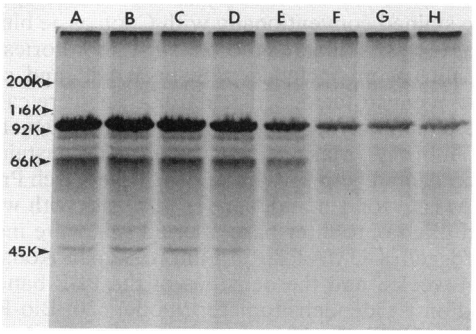

FIG. 1. Competition of imipenem for the PBPs of *B. wadsworthia* WAL 7781. Molecular mass standards are indicated on the left (in thousands). Lane A, control (no imipenem); lanes B to H, imipenem at concentrations of 0.00125, 0.005, 0.03, 0.06, 0.125, 0.5, and 2.0 μg/ml, respectively.

TABLE 2. Competition of imipenem for the PBPs of *B. wadsworthia*

PBP	IC_{50} (μg/ml)			
	WAL 7781	WAL 7813	WAL 8144	WAL 8283
1	0.125	0.125	1.0	0.5
2	0.03	0.03	ND[a]	0.5
3	0.125	0.125	0.06	ND
4	0.125	0.125	0.25	0.125
5	ND	<0.03	ND	ND
6	ND	0.03	ND	0.03
7	0.03	0.125	0.06	ND
8	0.03	0.03	0.06	0.12
9	0.03	ND	0.06	0.03

[a] ND, not detected.

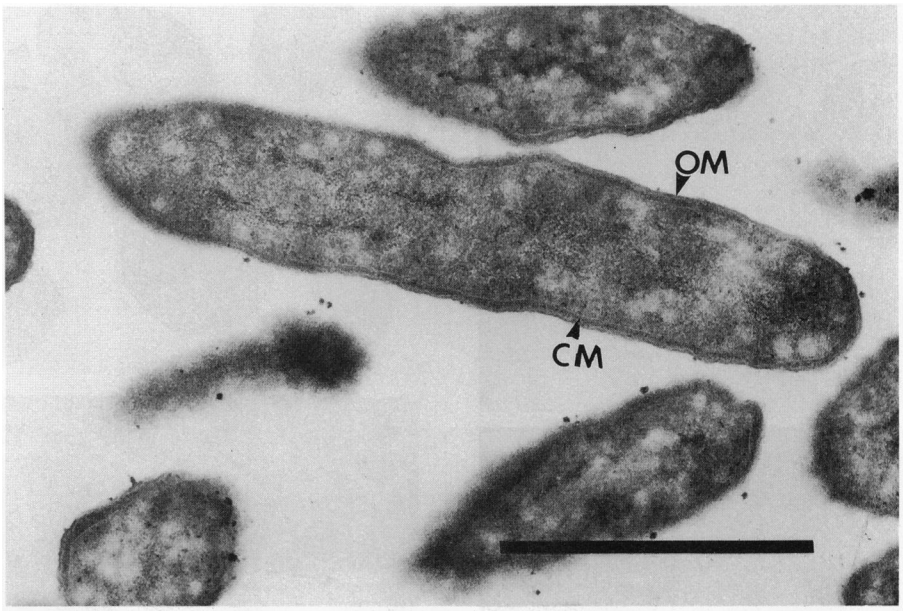

FIG. 2. Thin-section electron micrograph of *B. wadsworthia* WAL 8283 grown on brucella agar with 1% pyruvate, without imipenem. OM, outer membrane; CM, cytoplasmic membrane. The peptidoglycan layer is not readily visible in *B. wadsworthia*. Bar, 1 μm.

The results obtained from AD plates at 48 h correlated with those from the broth macrodilution tubes. At low concentrations (~0.1× the MIC), imipenem induced the formation of rounded and bulging cells, and at increasing imipenem concentrations, the rounded forms were enlarged. Rare elongation, but no filamentation, was observed, and at concentrations approximating the MIC, spheroplast formation was noted. The spheroplasts were readily stained with crystal violet and were up to 30 μm in diameter (24).

(ii) Electron microscopy. Scanning EM of cells grown in the presence of imipenem at less than and at the MICs revealed round forms and larger, multilobate cells (Fig. 3A and B). However, cell division seemed inhibited and no filamentation was seen. Also, at concentrations at and less than the MIC, doughnut-shaped cells were observed (Fig. 3C) (the center of the cells collapsed). By transmission EM, imipenem was noted to induce peeling of the outer membrane, leading to an increased irregularity of the cell surface (Fig. 4A) and, eventually, to spheroplast formation (Fig. 4B). Multilobate cells were observed by scanning EM (Fig. 4C). The spheroplasts (Fig. 5) were uncommonly large (up to 30 μm in diameter, compared with the 2- to 4-μm length of the intact cells) and were stable in aqueous solution.

The first subculture of the spheroplasts on the hypertonic medium (MGSA) yielded a few fried-egg-like colonies, and cells appeared as spherical and bulging forms upon crystal violet staining. Reversion to parent cellular and colonial morphologies took place by the second subculture on MGSA. Colonies that were recovered from a subculture on regular brucella agar had mostly reverted to a normal morphology upon the first subculture.

DISCUSSION

The PBP profiles found in the six *B. wadsworthia* strains tested were comparable to each other, and the molecular masses of the PBPs were fairly similar to those described in *E. coli* (21). Generally, seven to nine PBPs could be visualized; their molecular masses ranged from 31 to 137 kDa. The involvement of the high-molecular-mass PBPs of *E. coli* (PBPs 1, 2, and 3) in the synthesis of the cell wall is well established (20). Inhibition of certain PBPs by β-lactam antibiotics has specific effects on the morphology of *E. coli*. Inhibition of PBP 1 (which is important in cell division) leads to spheroplasting and lysis, inhibition of PBP 2 (which is needed for cell shape maintenance) results in spherical forms, and inhibition of PBP 3 causes inhibition of septum formation and initiation of new sites, resulting in filamentation of the cells (6, 20, 25). Subsequent studies with other organisms have yielded similar results, and the enzyme activities of the different PBPs have been assumed to be comparable to those of the PBPs of *E. coli* (7, 8, 17, 18).

In *E. coli*, the primary target for imipenem is PBP 2; binding causes cell rounding and lethality is mediated by binding to PBPs 2 and 1 (11, 22). In *B. wadsworthia*, binding to the higher-molecular-mass PBPs, PBPs 1, 3, and 4, occurred at concentrations approximately equal to the MIC, and the low-molecular-mass PBPs were generally inhibited at concentrations less than the MICs. The effect of imipenem on the cell wall was clearly seen at sub-MICs, with EM showing rounding of the cells and peeling of the outer membrane leading to an irregular cell surface. Furthermore, the formation of the multilobate cells, seen both in scanning EM and in transmission EM, indicates that new growth sites were initiated, while cell division was inhibited. Elliott and Greenwood (8) investigated the effect of imipenem on the morphology of *Pseudomonas aeruginosa* and found that, initially, spheroplasts were formed and abnormal cell morphology with evidence of cross-wall formation was seen. Also, they found that the spheroplasts emerged at the midzone or at the end of the cells and that they swelled to several times their initial diameter before lysis (similar to the results reported here). However, they tested imipenem only at concentrations greater than the MIC; this might explain why they did not observe cell rounding.

We cannot readily explain the cause of the doughnut-shaped cells observed by scanning EM; possibly, an uneven

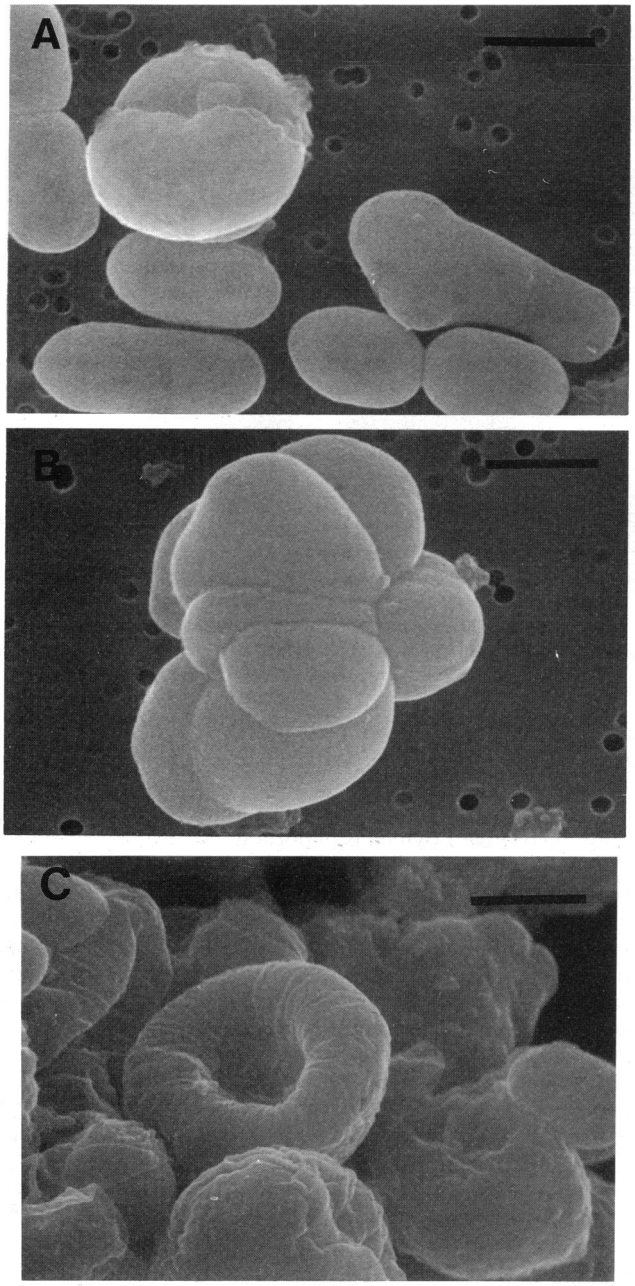

FIG. 3. Scanning electron micrographs of *B. wadsworthia* WAL 8283 grown in the presence of imipenem showing rounding of the cells and peeling of the cell wall as the initial response (0.312 μg/ml) (A), large, multilobate cells with irregular division sites demonstrating that the final stage of cell division is inhibited (0.25 μg/ml) (B), and abnormal, doughnut-shaped cells in the presence of imipenem at the MIC (0.25 μg/ml). Bars, 1 μm.

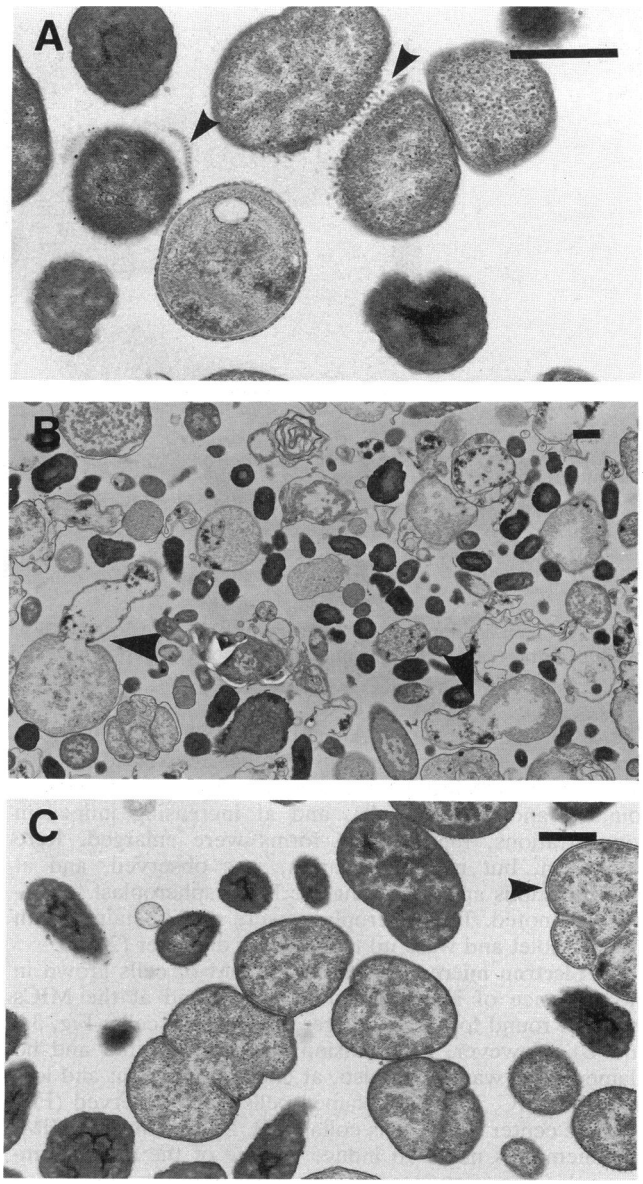

FIG. 4. Transmission electron micrographs of *B. wadsworthia* WAL 8283 grown in the presence of imipenem showing the initial response of peeling of the outer membrane together with lipopolysaccharide shedding (arrowheads) leading to an irregular cell surface (0.312 μg/ml), round and abnormally shaped cells and early spheroplast formation (arrowhead) (0.125 μg/ml) (B), and aberrant growth sites initiated as seen in scanning EM (C); note the detaching outer membrane (arrowed) (0.312 μg/ml). Bars, 1 μm.

tensile strength of the cell wall results from imipenem binding to the PBP responsible for cell shape maintenance (PBP 2 in *E. coli*). These forms may correspond to the bulging cells seen by light microscopy. Similar doughnut-shaped cells have been observed as intermediate morphological forms between viable and nonviable *Campylobacter* spp. (16). The mechanism for this is also not known, but the authors (16) suggested that it is associated with progressive degeneration of the cell wall.

Inhibition of PBP 3 has been associated with filament formation in *E. coli*. Imipenem does not significantly bind to PBP 3 in *E. coli*, nor does it cause filamentation (22). Similarly, no filamentation of *B. wadsworthia* was observed in the presence of any imipenem concentrations tested. Some elongation of the cells together with bulging, bizarre forms was seen. Spratt (20) proposed that bulging filament formation in *E. coli* could be due to inhibition of both PBPs 2 and 3; this might have contributed to the bulging effect seen

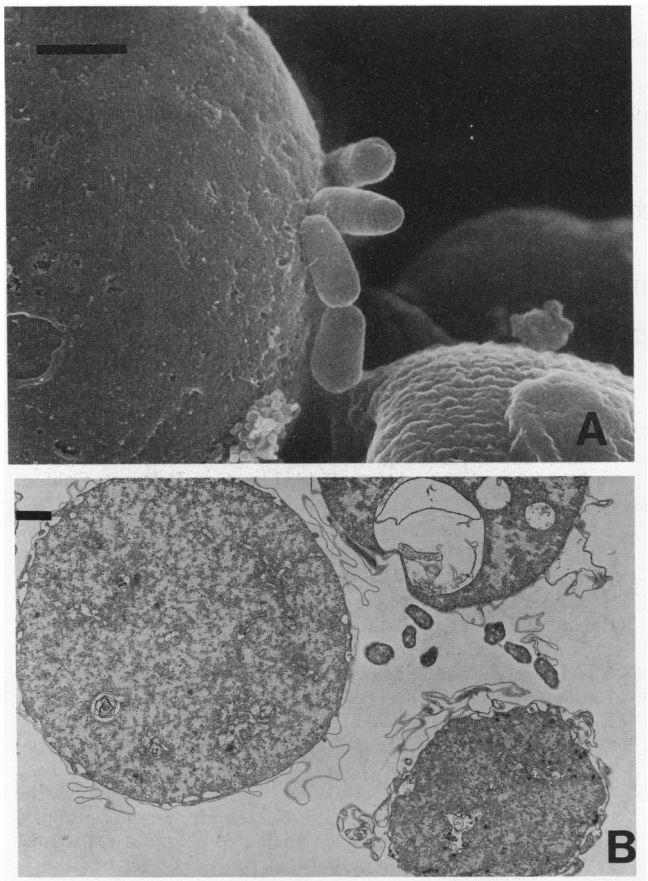

FIG. 5. *B. wadsworthia* WAL 8283 spheroplasts on imipenem-containing (64 μg/ml) plates as seen by scanning EM (A) and transmission EM (B). Note the peeling of the cell wall and the magnitude of the spheroplasts compared with those of cells of the regular size. Bars, 1 μm.

during the present study, implying that some inhibition of these enzymes may have occurred. However, the multilobate cells and septum formation seen by EM suggest that the enzyme responsible for the new growth sites (corresponding to PBP 3 in *E. coli*) is not totally inhibited by imipenem. The PBP of *B. wadsworthia* (PBP 6) corresponding in molecular mass to PBP 3 of *E. coli* was not readily detectable. On those occasions when PBP 6 was visualized during competition assays, labelling was inhibited at low imipenem concentrations; further studies are required to establish whether this PBP has the same enzymatic activity as PBP 3 in *E. coli*. Filamentation caused by exposure to imipenem has been described in *B. fragilis* by Piddock and Wise (18). They studied PBPs of the *B. fragilis* group and found that imipenem bound preferentially to PBP 3, which they thought correlated with PBP 2 in *E. coli*. They noted an unexpected response of filamentation at low concentrations, and they saw bulging and distorted filaments before lysis. Similarly, Onoe et al. (17) reported that mecillinam, an agent associated with the production of round forms in *E. coli*, caused filament formation but no rounding effect on *Fusobacterium nucleatum*.

The lower-molecular-mass PBPs (i.e., PBPs 4, 5, and 6) in *E. coli* have been associated with carboxypeptidase reactions and are considered to be nonessential to cell growth or cell morphology; PBP 7 has been implicated as the lethal target in the lysis of nongrowing *E. coli* cells (26). As seen in *E. coli* (22), we found that the low-molecular-mass PBPs in *B. wadsworthia* are bound at low imipenem concentrations.

The results of the present study demonstrate that the persisting hazy growth of *B. wadsworthia* on imipenem-containing AD plates consists mostly of spheroplasts. Intact cells were only rarely seen by light microscopy of the hazy area; the high proportion of intact cells seen by EM most likely is due to the extensive cell mixing (because of centrifugation) during the fixing procedure, which favors intact cells. Furthermore, previous studies that used viability counts from AD plates showed no net growth in this area (24). Strain WAL 7813 exhibited less haze and yielded fewer spheroplasts than the other three strains tested. There does not seem to be any correlation between β-lactamase and haze production; however, some β-lactamase-negative strains produce a haze, and some β-lactamase-positive strains do not (unpublished data).

In conclusion, seven to nine PBPs could be visualized in *B. wadsworthia*. The morphological responses to imipenem and the affinity of imipenem for certain PBPs correlated with data for other organisms. Further studies are needed to establish the specific enzymatic activities of the PBPs of *B. wadsworthia* and their role in morphology. The hazy growth seen on imipenem-containing plates consists of spheroplasts which are capable of reverting back to the morphology of the parent upon removal of the antibiotic. The clinical significance of the spheroplasts, if any, and their viability and reversion potential in vivo are unknown.

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