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Properties of the Penicillin-Binding Proteins of Four Species of the Genus *Bacteroides*

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The penicillin-binding proteins (PBPs) of four species of the genus *Bacteroides* were examined in cell envelope preparations from exponentially growing cultures and intact cells. Upon examination by sodium dodecyl sulfate-polyacrylamide electrophoresis, three major high-molecular-weight PBPs (molecular weight, 58,000 to 82,000) were resolved, and low-molecular-weight PBPs were seen in all strains except *Bacteroides fragilis*. The sporadic appearance of PBP 4 in *B. fragilis* (molecular weight, approximately 45,000) was shown not to be influenced by the concentration of free iron available in the medium or by the stage of growth at which the batch culture was harvested. No PBP that was inhibited by an aerobic environment was demonstrated. The affinity of 35 β-lactam antibiotics for the PBPs from envelope preparations was examined and correlated with the morphological response. Most compounds bound initially to PBP 2 and then PBP 1, correlating with a primary response of filamentation and then spheroplasting and lysis. Compounds such as clavulanic acid bound to PBP 3 at concentrations causing round cells. Based on the data from this study, it is proposed that the three high-molecular-weight PBPs of *Bacteroides fragilis*, *Bacteroides vulgatus*, *Bacteroides thetaiotaomicron*, and *Bacteroides ovatus* correlate to the three high-molecular-weight PBPs of *Escherichia coli* and that the PBPs of *Bacteroides* species perform the same enzymic role in cell wall biosynthesis as their counterparts in *E. coli*. Therefore, the components of PBP 1 are involved in cell elongation, PBP 2 is involved in septum formation, and PBP 3 is involved in maintenance of cell shape (i.e., PBP 2 in *Bacteroides* spp. = PBP 3 in *E. coli*, and PBP 3 in *Bacteroides* spp. = PBP 2 in *E. coli*).

Species of the genus *Bacteroides* are commonly encountered in infection, and certain of the species are the anaerobes most resistant to antimicrobial agents (8) and in particular the β-lactam class of antibiotics, owing to the presence of potent β-lactamases (17, 18). The development of β-lactamase-stable β-lactams has not led to greatly enhanced activity for *Bacteroides* species, and the recent emergence of resistance to cefoxitin, which is not attributable to classical β-lactamase activity (2, 6), has renewed interest in the mechanism of action of β-lactams in *Bacteroides* species.

The mechanism of action of β-lactam antibiotics has been extensively studied in aerobic bacteria (25) and the anaerobe *Clostridium perfringens* (27). The gram-negative bacteria examined include species such as *Escherichia coli*, *Pseudomonas aeruginosa*, and *Salmonella typhimurium* (13, 21, 23). The identification of the killing targets of β-lactam agents by a convenient electrophoretic and fluorographic method has led to the detection of a group of proteins located in the cell envelope called the penicillin-binding proteins (PBPs). In gram-negative bacilli, between six and eight PBPs are routinely detected, with the high-molecular-weight PBPs 1, 2, and 3 being essential for normal cell growth. Correlation between the morphological response of the bacterial cell (when grown in the presence of a β-lactam) and the affinity of that same β-lactam for certain PBPs and the study of mutants (spontaneous or induced) with defective PBPs under certain conditions such as temperature have led to the proposal of certain metabolic roles for the essential high-molecular-weight PBPs. The components of PBP 1 are thought to be involved in cell division, PBP 2 is thought to be involved in the maintenance of cell shape, and PBP 3 is thought to be involved in septum formation.

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PBPs from *Bacteroides fragilis* have been examined by several groups (1, 10; M. Otsuki, A. Sugiura, A. I. Takenada, T. Nishino, and T. Tamino, Proc. 13th Int. Congr. Chemother., p. 2.11/3-12, 1983; L. J. V. Piddock and R. Wise, Proc. 13th Int. Congr. Chemother. p. 2.11/3-7, 1983), and similar profiles of the high-molecular-weight PBPs have been obtained. In this study, we provide a detailed account of the properties of the PBPs of four species of the genus *Bacteroides*—*B. fragilis*, *B. vulgatus*, *B. thetaiotaomicron*, and *B. ovatus*—and from affinity data and morphological response to a wide range of β-lactam antibiotics, we propose that certain PBPs are analogous to PBPs with similar molecular weights detected in envelopes from aerobic gram-negative bacilli. The role of PBPs in the susceptibility to β-lactams of species of the genus *Bacteroides* is discussed.

MATERIALS AND METHODS

Bacterial strains and media. *B. fragilis* B1 (a clinical isolate), *B. fragilis* B108 (NCTC 9343, ATCC 25285), *B. vulgatus* B4 (a clinical isolate), *B. thetaiotaomicron* B116 (NCTC 10582, ATCC 29148), and *B. ovatus* B120 (NCTC 11135, ATCC 8483) were chosen as representative strains of the four selected species of the genus *Bacteroides* for which MICs of benzylpenicillin are 8 to 16 μg/ml (moderately susceptible). Cultures were maintained in liquid nitrogen. They were subcultured from storage onto blood agar plates (5% horse blood in blood agar base no. 2; Oxoid Ltd., Basingstoke, United Kingdom) and incubated at 37°C for 24 to 48 h in an anaerobic work station (Don Whitley Scientific Ltd., Shipley, United Kingdom) with an atmosphere of 80% nitrogen–10% carbon dioxide–10% hydrogen. Growth in the liquid medium took place in Wilkins-Chalgren anaerobe broth (Oxoid).

Chemicals. Unless otherwise stated, all chemicals were

obtained from BDH, Poole, United Kingdom. Antibiotic powders were obtained as gifts from their respective pharmaceutical companies: ampicillin, amoxycillin, carbenicillin, flucloxacillin, methicillin, benzylpenicillin, ticarcillin, temocillin, and clavulanic acid from Beecham Pharmaceuticals, Brentford, United Kingdom; azlocillin and mezlocillin from Bayer, Leverkusen, Federal Republic of Germany; mecillinam from Leo Laboratories Ltd., Aylesbury, United Kingdom; piperacillin from Lederle Laboratories, Gosport, United Kingdom; cephaloridine, cephalexin, cefuroxime, and ceftazidime from Glaxo Group Research Ltd., Greenford, United Kingdom; cefamandole, cefaclor, and moxalactam from Eli Lilly & Co. Ltd., Basingstoke, United Kingdom; cefoxitin and imipenem from Merck Sharp & Dohme Ltd., Hoddesdon, United Kingdom; cefsulodin and a novel cephalosporin, CGP 31523A, from CIBA-GEIGY Ltd., Horsham, United Kingdom; cefotaxime from Roussel Laboratories Ltd., Uxbridge, United Kingdom; cefotetan from Imperial Chemical Industries PLC Macclesfield, United Kingdom; ceftriaxone from Roche Products Ltd., Welwyn Garden City, United Kingdom; aztreonam from the Squibb Institute for Medical Research, Princeton, N.J.; a novel penem, Sch 34343, from Schering Corp., Bloomfield, N.J.; a novel cephalosporin, HR 810, from Roussel UCLAF, Paris, France; and a new monobactam, Ro 17-2301, from Roche Products.

Antibiotic susceptibility determination. The MIC of each compound was determined by the agar dilution method and a microtiter tray broth method. For agar dilution MIC estimation, inocula of 10^4 and 10^6 CFU were used to test all strains. The inocula were obtained by transferring 1 μl of an appropriate dilution of overnight culture to the surface of the antibiotic-containing Wilkins-Chalgren anaerobe agar by a multipoint inoculating device (Denley-Tech Ltd., Billingshurst, United Kingdom). All plates were incubated anaerobically at 37°C for 24 h. The MIC of the antibiotic was defined as the concentration (in micrograms per milliliter) at which there was an estimated 99% reduction in the original inoculum.

For microtiter tray broth MIC estimation, one inoculum of 10^5 CFU/ml was used to test all strains; it was obtained by adding 50 μl of a suitable dilution of overnight culture in Wilkins-Chalgren anaerobe broth to 50 μl of an appropriate dilution of antibiotic in Wilkins-Chalgren anaerobe broth. The microtiter trays were sealed with an adhesive clear plastic seal and carefully taken into the anaerobic work station, and the plate seal was then carefully punctured over each well to allow anaerobic incubation at 37°C for 48 h. The MIC was taken as the concentration of antibiotic at which no visible growth occurred. When MICs were examined in the presence of 0.2 mM sodium EDTA or 0.75 μg of clavulanic acid per ml, each compound was added to the sterile Wilkins-Chalgren anaerobe broth before the addition and dilution of test antibiotic.

Bacterial envelope preparation. Static cultures were grown at 37°C in Wilkins-Chalgren anaerobe broth in the anaerobic work station, and growth was monitored spectrophotometrically at 675 nm. Envelope preparations were essentially as described by Spratt (23), except that the temperature was maintained between 0 and 4°C at all times and the preparation was frozen quickly by using liquid nitrogen. Glycerol (10% [wt/vol]) was also used at all times in the buffers. Envelope preparations were stored at a protein concentration of not less than 10 mg/ml.

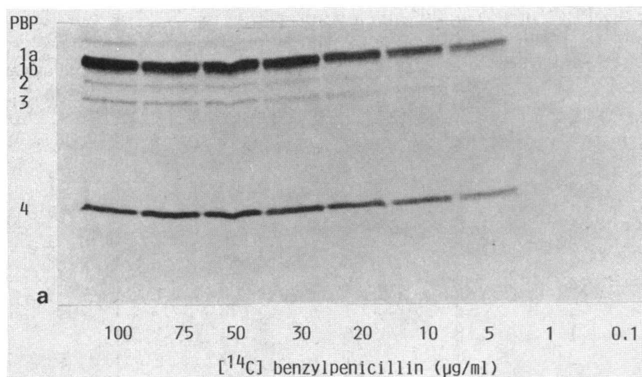
Assay of PBPs: sodium dodecyl sulfate-polyacrylamide electrophoresis and fluorography. Envelope preparations were thawed and adjusted to a protein concentration of 5 mg/ml and equilibrated at 30°C before use, and they were tested before the PBP assay for the presence of β-lactamase by using the chromogenic cephalosporin nitrocefin (15). All preparations used in this study were nitrocefin negative. The PBP assay and PBP competition assay for nonradiolabeled β-lactams were performed essentially as described by Spratt (23) and Curtis et al. (3).

The PBPs in intact cells were examined by two methods. (i) A cell suspension in 50 mM phosphate buffer (pH 7; 37°C) containing between 10^8 and 10^9 CFU/ml was used instead of an envelope preparation in the PBP assay, and for the duration of the assay a nitrocefin test for β-lactamase production was monitored. (ii) In growing cells, a 20-ml culture of each strain in Wilkins-Chalgren anaerobe broth was grown to mid-logarithmic phase, and 1 ml of culture was withdrawn and added to 100 μl of [^{14}C]benzylpenicillin (400 μg/ml), mixed, and incubated for 1 h in the anaerobic work station. The reaction was terminated by the addition of 5 μl of unlabeled penicillin (120 mg/ml). The tube was closed and carefully withdrawn from the anaerobic work station. The suspension was centrifuged, the cell pellet was suspended in 50 μl of 50 mM sodium phosphate buffer (pH 7; 25°C), 20 μl of 20% (wt/vol) sarcosyl was added, mixed, and incubated at room temperature for 20 min. The samples were then ultracentrifuged, and sample buffer and 2-mercaptoethanol were added as in the assay of PBPs from bacterial envelopes.

The kinetics of [^{14}C]benzylpenicillin binding to PBPs and the rate of acylation and deacylation of PBPs by [^{14}C]benzylpenicillin were examined essentially as described by Spratt (23).

PBPs were detected on sodium dodecyl sulfate-polyacrylamide gels containing 10% acrylamide; the ratio of acrylamide to methylene bis-acrylamide was 30:0.3. All electrophoresis was performed on a vertical system (20 by 20 cm; Raven Scientific, Haverhill, United Kingdom) with a discontinuous buffer system using a sodium dodecyl sulfate-Tris-glycine buffer; a constant current of 40 mA per gel was applied for 3 to 4 h with a sample size of 50 μl. After electrophoresis, all gels were removed and stained with PAGE Blue 83 (BDH). On most gels, molecular size standards (range, 12,300 to 78,000; BDH) were electrophoresed for quality control. The mobility of the molecular weight standards was used to estimate the molecular weights of the PBPs. Destained gels were impregnated with the fluor 2,5-diphenyloxazole and dried onto thick filter paper. The dried gels were exposed to prefogged Kodak X-Omat S film and sandwiched between two 1/16-in. (ca. 0.16-cm) aluminium sheets, wrapped in two light-proof plastic bags, and stored at −70°C for 8 to 12 weeks. The films were developed in an automatic Kodak RP developer, and the dark bands were quantitated by scanning on a laser densitometer (Ultroscan Laser Densitometer; LKB, Croydon, United Kingdom).

Morphological response of bacteria growing in the presence of β-lactam antibiotics. Doubling dilutions of test antibiotic were added to prewarmed (but not prereduced) Wilkins-Chalgren anaerobe broth and taken to the anaerobic work station. A 20% inoculum from an overnight culture was used to inoculate the antibiotic-containing broth which was then incubated statically at 37°C. At timed intervals, samples were withdrawn and placed on a 0.75% (wt/vol) agarose-overlaid slide and examined by differential interference microscopy (Photomicroscope III with DIC; Carl Zeiss [Oberkochen] Ltd., Welwyn Garden City, United Kingdom), and the results were photographed on Kodak Panatomic X black-and-white film (50 ASA).



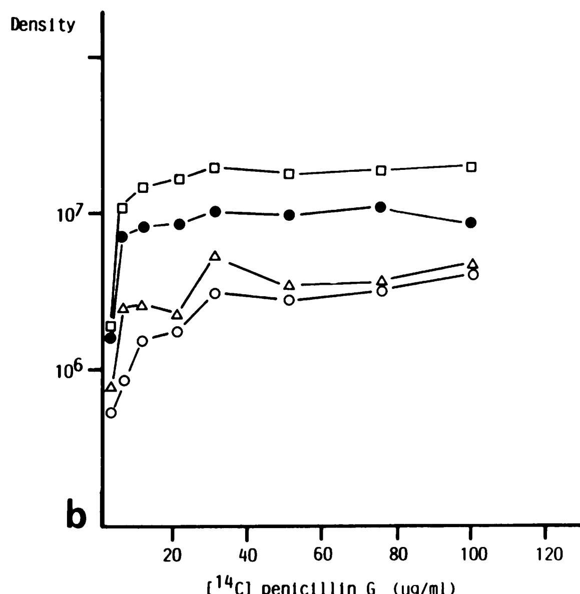


FIG. 1. (a) Fluorograph of the kinetics of binding of [^{14}C]benzylpenicillin to the PBPs of *B. thetaiotaomicron*. (b) Densitometer data from panel a presented graphically. □, PBP 1; ○, PBP 2; △, PBP 3; ●, PBP 4. The density units are arbitrary.

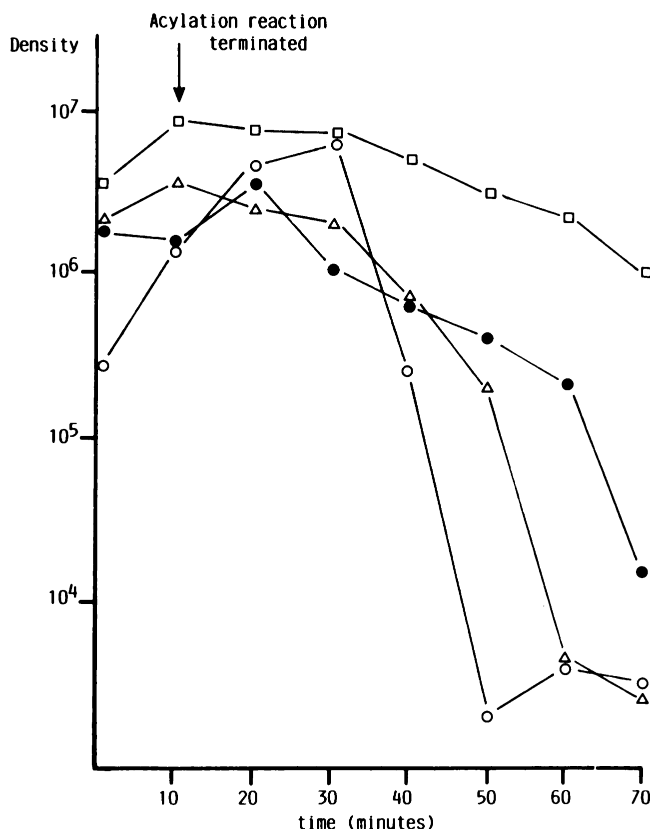


FIG. 2. The deacylation of the PBPs of *B. vulgatus*. □, PBP 1; ○, PBP 2; △, PBP 3; ●, PBP 4. The density units are arbitrary.

RESULTS

PBPs from *Bacteroides* spp. To examine the PBPs in species of the genus *Bacteroides* in the first instance, the conditions of the PBP assay for *E. coli* were used (3, 23). However, to obtain optimum elucidation of PBPs from *Bacteroides* spp., various methodological experiments were performed. It was found that 10% acrylamide gels with 30:0.3 acrylamide:methylene bis-acrylamide gave clear banding with all PBPs clearly visualized, that all PBPs were saturated after 10 min of incubation and no PBPs with slow kinetics of binding (<70 min) were seen, that the PBPs from *Bacteroides* spp. required a higher concentration (compared with *E. coli*) of [^{14}C]benzylpenicillin for saturation (Fig. 1), that 30°C was the optimum incubation temperature for the assay, and that the deacylation of the PBPs from *Bacteroides* spp. was fairly rapid for PBPs 2 and 3, with the most stable PBP being PBP 1 (Fig. 2) and the most unstable being PBP 2, with no binding detected after 50 min.

It can be seen that species of the genus *Bacteroides* contain PBPs in the sarcosyl-soluble fraction (presumably cytoplasmic membrane) with molecular weights between 38,000 and 82,000. *B. fragilis* has three major PBPs, PBP 1, 2, and 3; PBP 1 can be resolved into a doublet and occasionally a triplet, PBP 1a, 1b, (and 1c). *B. vulgatus*, *B. thetaiotaomicron*, and *B. ovatus* have a PBP profile similar to that of *B. fragilis* and an additional PBP (PBP 4) with a molecular weight of 45,000 to 47,000 (Table 1).

The PBPs in species of the genus *Bacteroides* were examined in cultures growing in the anaerobic work station (i.e., in vivo); the PBPs were therefore not exposed to an aerobic environment until the PBP assay had been terminated. The PBP profile obtained for each species was the same as that obtained from envelope preparations; however, the autoradiographs obtained from the electrophoresed gels were not as "clean" as those obtained from envelope

TABLE 1. Molecular weights of PBPs from envelope preparations of species of the genus *Bacteroides*

PBP	Mol wt in species				
	B. fragilis	*B. vulgatus*	*B. thetaiotaomicron*	*B. ovatus*	*E. coli*[a]
1a	82,000	75,000	78,000	75,000	91,000
1b	80,000	74,000	75,000	70,000	(86,000)[b]
1c	78,000	ND[c]	ND[c]	ND	
2	75,000	70,000	70,000	63,000	66,000
3	70,000	62,000	65,000	58,000	60,000
4	ND	47,000	44,000	45,000	49,000
5	ND	38,000	ND	ND	42,000

[a] According to Spratt (23).
[b] According to Spratt et al. (24).
[c] ND, Not detected.

TABLE 2. Competition of penicillin derivatives for the PBPs of *B. fragilis* envelope preparations

Antibiotic	MIC (μg/ml) in broth (10^5 CFU/ml)	I_{50} (μg/ml) for PBP						Morphological response[a]
		1a	1b	1c	2	3	4	
Ampicillin	16	4	4	4	4	1	>32	F + R + S
Azlocillin	8	<2	<2	<2	<2	<2	4	F + S
Amoxycillin	16	8	8	8	128	16	ND[b]	R + S
Carbenicillin	4	1	1	1	1	16	>64	F + S
Methicillin	64	32	32	32	8	4	16	BF
Mezlocillin	8	1	1	1	1	8	ND	F
Mecillinam	>128	>1,024	>1,024	>1,024	256	128	ND	BF + R
Penicillin G	16	2	2	2	2	1	>128	F + R + S
Piperacillin	2	1	1	1	8	16	>64	F
Ticarcillin	16	2	2	2	1	1	ND	F + S
Temocillin	32	16	16	16	16	32	ND	F

[a] At the MIC. F, filamentation; R, round forms; S, spheroplasting; BF, bulging filaments.
[b] ND, Not detected.

preparations, and two proteins with high molecular weights (above that of PBP 1, designated 1′ and 1″) were consistently labeled. These proteins, however, did not participate in the competition assays with nonlabeled β-lactams. PBPs were also examined in suspensions of whole cells; it was found that the labeled PBPs were identical to those obtained in the in vivo assays, with proteins 1′ and 1″ being labeled. The PBP profiles obtained from either whole cell assay were comparable to those obtained from envelope preparations. It was therefore assumed that no PBP was inhibited in an aerobic environment and that the PBP affinity data obtained from envelope preparations was an estimation of the in vivo PBPs. However, data obtained from experiments with whole cells were essential to estimate the interplay between the accessibility of PBPs and the role of β-lactamase and permeability, which are parameters absent in the envelope preparation PBP assay.

The *B. fragilis* PBPs were sensitive to temperature, and PBP 2 was difficult to elucidate. To stabilize PBP 2, 10% (vol/vol) glycerol was added to all buffers, and strict maintenance of temperature (<4°C) during envelope preparation was observed. Under these conditions, PBP 4 was sometimes detected. The occasional difficulty in PBP 2 elucidation and the unpredictable appearance of PBP 4 were not due to the time (in the bacterial growth cycle) of cell harvest for envelope preparation (data not shown) and was also shown not to be affected by the concentration of available iron in the culture medium.

Morphological response of *Bacteroides* spp. to β-lactams. Most β-lactam compounds examined showed a primary morphological response of filamentation (Tables 2 to 5). A second response of lysis was observed at higher concentrations or after prolonged incubation. Compounds normally associated with round forms, such as mecillinam, clavulanic acid, and imipenem, caused filamentation at subinhibitory concentrations, and at concentrations approximating the MIC, bulging and distorted filaments were seen. For mecillinam and clavulanic acid, high concentrations caused classical round forms; imipenem caused spheroplasting and lysis. Some compounds, such as ampicillin, induced a range of morphological changes which were antibiotic concentration dependent, and at a specific concentration it was possible to observe a specific response. However, some responses, especially in *B. thetaiotaomicron*, were extremely transient. Some compounds caused only one morphological response; e.g., cephaloridine, cefoxitin, and ceftazidime almost exclusively caused spheroplasting and lysis, and cefotaxime almost exclusively caused filamentation. For penicillin derivatives and cephalosporins, most responses were consistent with the observations made for the same compounds in aerobic gram-negative rod-shaped bacteria (3, 7, 11, 12, 14, 23).

Affinity of PBPs for β-lactam compounds. The affinity of the PBPs from the four selected species of the genus *Bacteroides* was examined primarily in envelope preparations; some of the data are shown in Tables 2 to 5. The gels

TABLE 3. Competition of cephalosporins for the PBPs of *B. vulgatus* envelope preparations

Antibiotic	MIC (μg/ml) in broth (10^5 CFU/ml)	I_{50} (μg/ml) for PBP							Morphological response[a]
		1a	1b	1c	2	3	4	5	
Cephaloridine	32	64	64	ND[b]	16	16	64	>512	S
Cephalexin	32	32	32	ND	2	4	4	>256	F
Cefamandole	128	4	4	ND	4	>512	>512	>512	BF
Cefoxitin	8	2	2	ND	16	64	0.5	>256	S + L
Cefuroxime	4	>256	>256	ND	4	8	8	4	F + S + L
Cefaclor	16	128	128	ND	16	32	64	32	F + S
Cefsulodin	64	32	32	ND	128	64	1,024	ND	(F)[c] + S
Cefotaxime	4	4	4	ND	>512	4	>512	>512	F
Ceftriaxone	4	8	8	ND	16	32	64	>256	F
Ceftazidime	32	<4	<4	ND	>512	32	32	>512	S

[a] At the MIC. S, Spheroplasting; F, filamentation; BF, bulging filaments; L, lysis.
[b] ND, Not detected.
[c] Transient initial response.

TABLE 4. Competition of cephalosporins for the PBPs of *B. thetaiotaomicron* envelope preparations

Antibiotic	MIC (μg/ml) in broth (10^5 CFU/ml)	I_{50} (μg/ml) for PBP							Morphological response[a]
		1a	1b	1c	2	3	4	5	
Cephalexin	64	>256	>256	ND[b]	2	32	>256	ND	F + S
Cefamandole	128	64	64	ND	>256	16	128	>256	BF
Cefoxitin	64	4	4	ND	16	64	32	>256	S (+ L)[c]
Cefuroxime	64	128	128	ND	32	>512	128	>512	F
Cefaclor	128	64	64	ND	<16	16	64	>1,024	F + S
Cefsulodin	>128	128	128	ND	128	256	>256	>256	(F) + S
Ceftazidime	>128	16	16	ND	>256	8	>256	ND	S

[a] At the MIC. F, Filamentation; S, spheroplasting; BF, bulging filaments; L, lysis.
[b] ND, Not detected.
[c] Transient initial response.

from competition assays were examined visually and by densitometer, and the I_{50} values (concentrations at which the density of the band on the autoradiograph was decreased by 50%) were adjusted to equate with the nearest value used in an MIC scheme (e.g., 6.25 would become 8 μg/ml, 17 would become 16 μg/ml). The higher-molecular-weight PBPs were involved in binding at concentrations approximating the MIC for all compounds tested.

Most penicillin derivatives had no affinity for a specific PBP in *B. fragilis*, *B. vulgatus*, and *B. thetaiotaomicron*, but in most instances PBP 2 was the primary target followed by PBP 1. Some compounds, e.g., amoxycillin (in *B. fragilis*), bound to PBP 1 and 3 at approximately the same concentration with little binding to PBP 2, and some compounds bound to PBP 1 and 2 at the same concentration. The cephalosporins were shown to have affinity for the same high-molecular-weight PBPs as the penicillin derivatives. Several cephalosporins showed more specificity to certain PBPs than the pencillin derivatives, with ceftazidime binding to PBP 1 only in *B. vulgatus*. As with the penicillin derivatives, several cephalosporins bound to PBP 1 and 2 at the same concentration (cefaclor in *B. fragilis*, cefamandole in *B. vulgatus*). Compounds that showed specificity for one PBP in one species did not always show the same specificity in the other three species examined; e.g., cephalexin was specific for PBP 2 in *B. thetaiotaomicron* but not in *B. fragilis* or *B. vulgatus*, although the primary target was PBP 2 with these species. Compounds such as imipenem, mecillinam, and clavulanic acid bound to PBP 3 initially and then PBP 2, and imipenem bound to the components of PBP 1 at concentrations correlating with the MIC.

The binding of selected β-lactam agents to the PBPs in intact cells was examined by using both methods for *B. fragilis* and the whole cell suspension method only for the remaining three species. The data obtained did not indicate any major discrepancies between the affinity data obtained for PBPs in envelope preparations and that of the target PBPs for each respective β-lactam, in that the binding of the PBPs in growing cells and cell suspensions indicated that for most compounds the primary target was PBP 2 followed by PBP 1. A different bias of affinity was shown for some compounds, which may demonstrate the availability of particular PBPs in the in vivo environment.

DISCUSSION

The profiles of PBPs from envelope preparations of the four species of the genus *Bacteroides* were not dissimilar to the profiles of PBPs obtained from envelope preparations of aerobic gram-negative rods such as *E. coli* and *P. aeruginosa*. The PBPs from *Bacteroides* spp. were given numbers and letters so that they correlated to PBPs with molecular weights similar to those in *E. coli* for ease of comparison. From the data obtained from PBP assays of intact cells, it is evident that no resolved PBP that had affinity for any of the unradiolabeled β-lactam compounds studied was inhibited by an aerobic environment. It was therefore assumed that the PBPs detected from envelope preparations were representative of those in the in vivo environment. The envelope preparations were prepared from bacterial cultures in the late logarithmic phase, which is the stage of the growth cycle at which the production of β-lactamase decreases (19), and it was found that contamination of the envelope preparations by β-lactamase was rare. Production of β-lactamase during experiments with intact cells was, however, a problem, but it was resolved by using cells that were entering the stationary phase. The consistent presence of the high-molecular-

TABLE 5. Competition of penicillin derivatives for the PBPs of *B. ovatus* envelope preparations

Antibiotic	MIC (μg/ml) in broth (10^5 CFU/ml)	I_{50} (μg/ml) for PBP							Morphological response[a]
		1a	1b	1c	2	3	4	5	
Azlocillin	32	8	8	ND[b]	128	16	>256	>256	
Amoxycillin	32	>1,024	>1,024	ND	8	8	>1,024	>1,024	S + L
Flucloxacillin	32	4	4	ND	<4	16	>1,024	>1,024	(F)[c] + S
Methicillin	>128	32	2	ND	16	16	>1,024	>1,024	BF
Mezlocillin	16	2	2	ND	<2	16	>512	>512	F + S
Penicillin G	8	16	32	ND	>1,024	>1,024	>1,024	>1,024	F + R + L
Ticarcillin	32	8	8	ND	8	16	ND	ND	F
Temocillin	64	>1,024	>1,024	ND	>1,024	>1,024	256	ND	

[a] At the MIC. S, Spheroplasting; L, lysis; F, filamentation; BF, bulging filaments; R, round forms.
[b] ND, Not detected.
[c] Transient initial response.

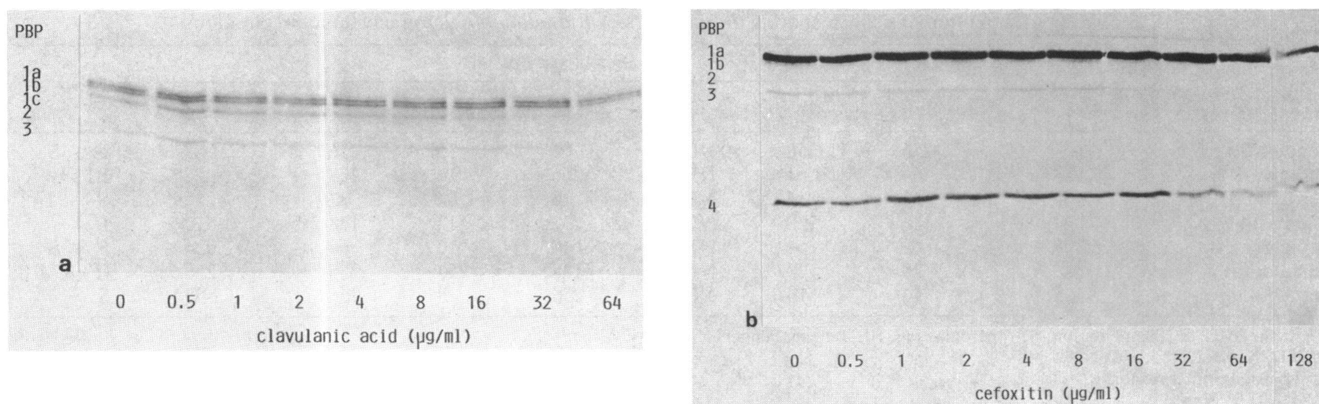


FIG. 3. PBP competition assay: (a) *B. fragilis* with clavulanic acid; (b) *B. thetaiotaomicron* with cefoxitin.

weight proteins 1′ and 1″ in intact cell assays was perplexing, as these proteins did not show affinity for any of the β-lactam compounds examined in competition assays. The extended time of incubation necessary for the PBPs in growing cells to bind to ^{14}C-penicillin G (as opposed to PBPs in envelope preparations or whole cell suspensions) may be indicative of the unavailability of the PBPs to β-lactam antibiotics in vivo.

Growth-stage-dependent effects have also been shown for PBPs from envelope preparations of *E. coli*, *Bacillus subtilis*, and *Streptococcus faecium* (5, 9, 22). In this study, no change in the PBP profile was seen by the stage at which the cells were harvested, and this may be because the PBPs from *B. fragilis* are not regulated during growth by the amount of PBP produced but by the topographical distribution in the cell. It seems unlikely that the enzymic activity of the PBPs is switched on and off, as this would alter the affinity of the PBPs for [^{14}C]benzylpenicillin. The PBPs from *Streptococcus pneumoniae* were shown to be unaltered with the growth phase (26), so it may be that there are species-specific PBP regulatory systems.

The morphological responses to most of the β-lactam compounds studied compared well with the data published for the same compounds for aerobic gram-negative bacilli (3, 4, 11, 12, 14, 23). The primary morphological response seen with most compounds was filamentation, and after prolonged incubation or at higher concentrations there was spheroplasting and lysis. For all compounds with these responses, PBP 2 was shown to bind at concentrations correlating to the initial morphological response of filamentation; some compounds (e.g., cephalexin with *B. vulgatus*) bound to PBP 2 specifically, and only filamentation was observed. The response of spheroplasting and lysis correlated with binding to PBP 1 and was confirmed by some compounds having affinity for this PBP only (ceftazidime with *B. vulgatus*). Most compounds bound to PBP 2 and the components of PBP 1 at concentrations correlating to the MIC, and it therefore seems that the killing target for most β-lactam antibiotics in species of the genus *Bacteroides* is PBP 2 and PBP 1.

The correlation of the components of PBP 1 with the response of spheroplasting and lysis agrees well with the information available for PBP 1 in aerobic gram-negative bacilli (13, 16, 23–25). It can also be seen (Fig. 3a) that upon sodium dodecyl sulfate-polyacrylamide electrophoresis PBP 1 from *Bacteroides* species separated into a doublet or a triplet of bands, as does PBP 1b from *E. coli* and other gram-negative bacilli (3), and has a similar molecular weight. The acylation and deacylation of ^{14}C-penicillin G by PBP 1 in *Bacteroides* species was also similar to that of PBP 1 from *E. coli* (23), in that acylation was rapid (<10 min) and deacylation was slow (>70 min). A component with a molecular weight similar to that of PBP 1a seen in aerobic gram-negative bacilli was not apparent in PBP profiles from species of the genus *Bacteroides*, although very rarely a protein with a molecular weight of 84,000, which does participate in binding to unlabeled β-lactam antibiotics, was resolved. It is thought that an equivalent PBP 1a (equivalent to that seen in *E. coli* and *P. aeruginosa*) was not present and that PBP 1a, 1b, and 1c are the proteins analogous to the triplet of PBP 1b; the sporadically seen protein with a molecular weight of 84,000 may have been due to incomplete reduction of one of the proteins forming PBP 1a, b, and c. From the evidence, it may therefore be deduced that the components of PBP 1 in species of the genus *Bacteroides* are enzymes involved in cell elongation.

The binding of PBP 2 has been correlated with the morphological response of filamentation. This response (with the same compounds) has been seen in aerobic gram-negative bacilli and is correlated with PBP 3. It seems that the PBP in *Bacteroides* spp. involved in septation (PBP 2) has a higher molecular weight than its counterpart in aerobic organisms (PBP 3), but to all intents and purposes it fulfills the same enzymic role. Unlike PBP 3 in *E. coli*, PBP 2 in *Bacteroides* spp. forms less stable complexes with radiolabeled penicillin, and deacylation is complete after 50 min.

β-Lactams that are known to bind preferentially to PBP 2 in aerobic gram-negative bacilli (e.g., mecillinam, clavulanic acid, and imipenem) had the unexpected morphological response of filamentation at low concentrations, and only at the MIC (or at higher concentrations) was the expected response of round forms seen; for compounds such as imipenem, the transition from filaments to round forms was never seen, and bulging and distorted filaments were seen before lysis. The response seen most frequently in *Bacteroides* species for compounds such as mecillinam or clavulanic acid at the MIC was bulging filaments. The morphological response of filamentation to mecillinam has been observed with a strain of *Fusobacterium nucleatum* (20), which is a member of the family *Bacteroidaceae*, and it was observed by the same workers that the morphological response was indistinguishable from that produced by piperacillin. The morphological responses seen with the species examined in this study at concentrations approximating the MIC were similar to those seen with imipenem and *P. aeruginosa* (7) and with 6-aminopenicillanic acid in *E.*

coli (personal observations), so it may be that these responses are not unique to anaerobic bacteria. The PBP affinity data for *Bacteroides* species for mecillinam, clavulanic acid, imipenem, and Sch 34343 show that PBP 3 is initially bound and at higher concentrations PBP 2 and PBP 1 are bound. At concentrations 10 times below the MIC, filamentation and inhibition of growth with these compounds was observed, although at this concentration none of the PBPs reached 50% saturation of binding. This indicates that less than 50% inhibition of a PBP can influence growth and cell morphology. At a high concentration of clavulanic acid (40 μg/ml), which is 10 times the MIC, only classical round forms were seen, even though both PBP 2 and 3 were bound by more than 50%, indicating that at high concentrations this compound affected PBP 3 preferentially to PBP 2. This may be because there are fewer molecules of PBP 3 (approximately 520 molecules of PBP 3 to approximately 960 molecules of PBP 2 as calculated by the method of Spratt [23]); in the cell at high concentrations, all the PBP 3 molecules are bound, whereas some PBP 2 molecules are still unbound. At low concentrations, there is competition for the two PBPs, and unbound PBPs of both types are available. Filamentation at low concentrations of compounds such as imipenem is not a lethal event, although some bacteriostasis is observed (i.e., cell division is inhibited). As classical round forms were obtained with the same compounds that cause this response in *E. coli* and binding to PBP 3 was seen initially in competition assays, it is thought that PBP 3 is involved in the maintenance of cell shape and is therefore analogous to PBP 2 in aerobic bacilli. The binding of compounds such as mecillinam and clavulanic acid to PBPs other than PBP 3 (equivalent to PBP 2 in *E. coli*) may be due to some molecular instability of these compounds in anaerobic environments (while retaining microbiological activity), as the response of sub-MIC filamentation has not been widely reported. It may also be that the active site of PBP 2 is more flexible in *Bacteroides* species than is that of its counterpart PBP 3 in *E. coli*.

The affinity of β-lactam compounds with the low-molecular-weight PBPs from *Bacteroides* species (when detected) was not seen to correlate with any morphological response, and it seems likely that these PBPs correlate to the low-molecular-weight carboxypeptidases seen in aerobic organisms (PBP 4, 5, and 6 in *E. coli*). The presence (or absence) of detectable low-molecular-weight PBPs in *B. fragilis* may indicate that these enzymes are often absent in this species. Curtis et al. (3) did not elucidate PBP 4 for *Klebsiella aerogenes* and hypothesized that this enzyme was dispensable. This may be the situation for *B. fragilis*. However, it may be that a low-molecular-weight PBP with extremely slow kinetics of acylation (>70 min) is present (as in *Streptococcus faecium* [9]). The rate of kill of *B. fragilis* by the β-lactams studied was correlated to the major killing target (data not shown). Compounds that bound to PBP 1 at the MIC, such as cefoxitin and cephaloridine, killed more rapidly than compounds that bound to PBP 2 and 3. The kill rate of compounds at the MIC binding to PBP 2, e.g., piperacillin and cephalexin, was slow, frequently the compound was inactivated (due either to breakdown by β-lactamase hydrolysis or inherent chemical instability), and regrowth was therefore seen after 24 h of incubation. Hence, it may be assumed that compounds that are β-lactamase stable and kill quickly (by binding to PBP 1) are the most efficient agents against *Bacteroides* spp.; this may be why compounds such as cefoxitin, imipenem, and Sch 34343 are so effective in in vitro studies.

The interpretation of PBP I_{50} data and their correlation to MICs and hence the role of the PBPs in the susceptibility of the organism to β-lactam compounds has been used in several studies in recent years. However, the PBP affinity data obtained are dependent on a variety of experimental conditions and can be taken only as an indicator of the in vivo situation. The use of the PBP competition assay to assess the role of a permeability barrier to β-lactam antibiotics was first used by Curtis et al. (4), who examined the affinity of PBPs from a non-β-lactamase-producing *E. coli* for several compounds and estimated a crypticity factor, which was the ratio of the I_{50} (i.e., concentration at which the PBP is inhibited by 50% when compared with the antibiotic-free control) of a compound for its primary target and the MIC; the lower the crypticity value, the greater the permeability barrier. Unfortunately, a valid crypticity figure can never be ascertained from PBP data for *Bacteroides* species, as most strains produce β-lactamase. However, the crypticity value obtained can be used to indicate a penetration problem (whether by the outer membrane or hydrolysis of the incoming β-lactam by β-lactamase or a combination of these phenomena). The affinity data for PBPs from species of the genus *Bacteroides* and the β-lactam compounds studied indicate that for most compounds a penetration problem exists, as the I_{50} of the primary target is frequently fourfold lower than the MIC, e.g., *B. fragilis* and cefuroxime (PBP 2), *B. vulgatus* and mezlocillin (PBP 1 and 2), *B. thetaiotaomicron* and cephalexin (PBP 2), and *B. ovatus* and methicillin (PBP 2 and 3). Measurements of a permeability barrier have also been made by using a ratio of the rate of hydrolysis of a β-lactam by intact cell preparation to the rate of hydrolysis of a β-lactam by sonicated cell suspensions (crude β-lactamase) (28). Theoretically, this procedure is applicable to *Bacteroides* species. However, the assay depends on the β-lactamase being secure in the periplasm and on no leakage into the external environment in the whole cell preparation. It has been estimated that 97% of the β-lactamase in *Bacteroides* species is located in the periplasm (19). However, during the assay of the test compound using intact cells, significant amounts of β-lactamase were found in the suspending buffer, which interfered substantially with the calculation of a crypticity factor (J. Maskell, personal communication). Using the method of Zimmerman and Rosselet (28), Olsson et al. (17) demonstrated a permeability barrier to cephaloridine for five highly resistant strains of *B. fragilis*, but as yet there is no published data for a permeability barrier to any other β-lactam agents. For compounds such as the broad-spectrum cephalosporins (or penems and carbapenems), which are the β-lactamase-stable compounds, the crypticity value from PBP data may relate to the penetration barrier being due to a component of the outer membrane rather than β-lactamase hydrolysis. Further evidence for this is that the MICs of these compounds are not lowered by the β-lactamase inhibitor clavulanic acid.

From the PBP data, it is clear that the decreased susceptibility of *Bacteroides* spp. to some β-lactam antibiotics may be due to low affinity between the PBP and the compound. This is illustrated by compounds such as temocillin (for which in all four species the MIC is equivalent to the PBP I_{50} and is equal to 16 μg/ml or higher). However, the low affinity of *E. coli* PBPs to temocillin has been shown to be artifactual, and when the PBP assay conditions were altered, affinity was demonstrated. Several other compounds also demonstrated low affinity for *Bacteroides* spp. PBPs. Some compounds, although hydrolyzed by β-lactamase, also

showed low affinity for PBPs, e.g., cefamandole with *B. thetaiotaomicron*. It can be seen that one of the mechanisms of decreased susceptibility in *Bacteroides* species is low affinity of the target PBPs for the β-lactam, and when compared with *E. coli* (3), the PBPs from species of the genus *Bacteroides* exhibited lower affinity for most of the agents examined.

In summary, the PBPs of four species of the genus *Bacteroides* were examined and found to have PBP profiles similar to those observed for aerobic gram-negative bacilli, and on the basis of morphological studies and PBP affinities of 35 β-lactam compounds, functions similar to those of the major PBPs in *E. coli* were implicated.

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