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THE PERMEABILITY PROPERTY OF THE OUTER MEMBRANE OF *Bacteroides fragilis*, A STRICTLY ANAEROBIC OPPORTUNISTIC PATHOGEN

YORIKO KOBAYASHI and TAIJI NAKAE

Institute of Medical Sciences and School of Medicine,

Tokai University, Isehara 259-11, Japan

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SUMMARY It was concluded that the exclusion limit of the outer membrane of *Bacteroides fragilis* is close to the size of uncharged saccharides of M_r, 340-400 and of anionic solutes of M_r, 500 on the basis of the following lines of evidence. (i) Penetration of methylhexoses and salicin into the NaCl-expanded periplasm appeared to be 85 and 50%, respectively, of that of pentoses, whereas penetration of the saccharides of M_r larger than 340-400 was negligibly small. (ii) The cells treated with 500 mosM di- through pentasaccharides were about 36% lighter than the isotonic NaCl-treated cells, whereas the 500 mosM pentoses- or hexoses-treated cells appeared to be 16 or 21%, respectively, lighter. The result indicates that the saccharides of M_r more than 340 exerted osmolarity at the outer membrane and hence these saccharides are assumed merely permeable. (iii)The phosphorylated solutes and β-lactam antibiotics of M_r a little larger than the above threshold exclusion limit are partially diffusible.

The outer membrane of gram-negative bacteria constitutes a molecular sieve with a defined exclusion limit, e.g. M_r 600 to 700 in *S. typhimurium* and *E. coli* (see ref. 1 for review). Recent studies on the outer membrane permeability of an opportunistic pathogen, *Pseudomonas aeruginosa* revealed the presence of even smaller diffusion pore (2,3), although this conclusion contradicts from the earlier publication (4). Though a good deal of information is available in the permeability of the outer membrane of aerobic bacteria, little is known about that of the anaerobic gram-negative pathogen. An awaiting information among them is that from geneus *Bacteroides*, as this species of bacteria are often identified as opportunistic pathogens and show high intrinsic resistance to a number of the structurally unrelated antibiotics.

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This study shows that the diffusion pore in the outer membrane of this organism is smaller than that of E. coli and S. typhimurium, and close to that of P. aeruginosa.

MATERIALS and METHODS

Bacterial strains and growth conditions. Bacteroides fragilis ATCC25285 was grown to a late log-phase in GAM broth at 35°C under a mixed gas (N_2:H_2:CO_2 = 80:10:10). Cells were washed once with 0.85% of NaCl in Buffer A (25 mM phosphate buffer, pH7.0 - 5 mM $MgCl_2$), or in Buffer B (10 mM Hepes buffer, pH7.6 - 5 mM $MgCl_2$), and resuspended in the same medium.

Penetration assay. Three different assay methods were used. (i) Determination of the solute penetration into the expanded periplasm was reported earlier (2). (ii) Determination of pellet weight after treating cells with the hypertonic saccharide was reported (2). (iii) Determination of the diffusion rate in intact cells is based on "crypticity" of the periplasmic enzymes as reported earlier (5,6).

Quantitative methods. Activity of glucose-6-phosphate dehydrogenase was determined spectrophotometrically at 340 nm as before (2). Activity of β-galactosidase was determined spectrophotometrically at 400 nm in the reaction mixture containing 5 mM o-nitrophenyl-β-D-galactopyranoside and 50 mM Tris-HCl, pH7.0. Saccahride was quantified by the phenol sulfuric acid reaction (7). Cefoxitin (CFX), cefmetazol (CMZ) and latamoxef (LMOX) were quantified by the bioassay measuring the growth inhibition of the indicator strains.

Materials. Chemicals and enzymes used were of highest quality commercially available. None of the solute used was metabolized by the bacteria.

RESULTS

Penetration of saccharides into the periplasm of the plasmolyzed cells. When the cell was plasmolyzed with hypertonic NaCl in the presence of test solute, the outer membrane-permeable solute penetrates into the periplasm. Therefore, it is possible to measure the extent of solute penetration by quantifying the solute recovered from the centrifuged supernatant of the deplasmolyzed cell. Fig. 1 shows that ribose (M_r, 150) and α-methylmannoside (Me-man, M_r, 194) penetrated into the periplasm efficiently equilibrating within about 4 min and salicin (M_r, 286) penetrated a little less than these saccharides. Turanose (M_r, 342) and melezitose (M_r, 504) were poorly permeable.

Penetration of the saccharides of various M_r into the periplasm was measured. The result shows that pentoses diffused through the outer membrane very efficiently (Fig. 2). Methylhexoses and salicin penetrated about 85 and 50%, respectively, compared with that of pentoses. Extent of

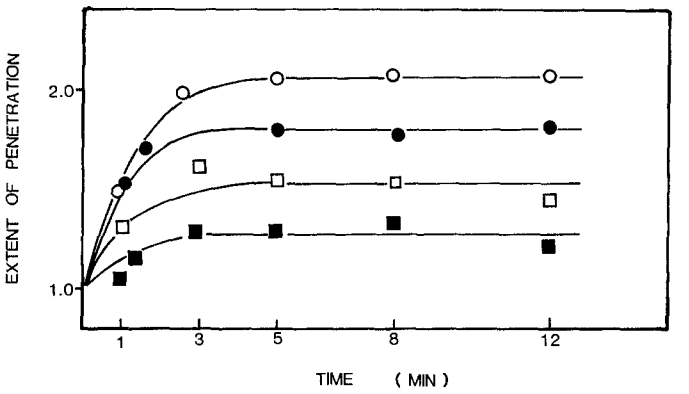

Fig. 1. Time course of the saccharide penetration. A hundred μl of the cell suspension was placed in a microcentrifuge tube (4.4 mg protein), mixed with 30 μl of 0.8 M NaCl in Buffer A and the mixture was kept at 23°C for 3 min. To the mixture were added 30 μl of a solution containing 150 mM test solute and NaCl (total 600 mosM) in Buffer A and 0.5 units of glucose-6-phosphate dehydrogenase. After incubating for appropriate time at 23°C, the mixture was centrifuged with an air-driven microcentrifuge (Beckman) for 40 sec at 58,000 x g and a portion of the supernatant was saved (supI). The remaining liquid was removed carefully and the pellet was resuspended in 100 μl of Buffer A. After 4 min, the suspension was centrifuged for 1 min as above and the supernatant was saved (supII). Extent of solute penetration was calculated as below. (test solute/glucose-6-phosphate dehydrogenase)$_{supII}$/(test solute/glucose-6-phosphate dehydrogenase)$_{supI}$. Symbols: ○,ribose; ●,Me-man; □,salicin; ■,melezitose.

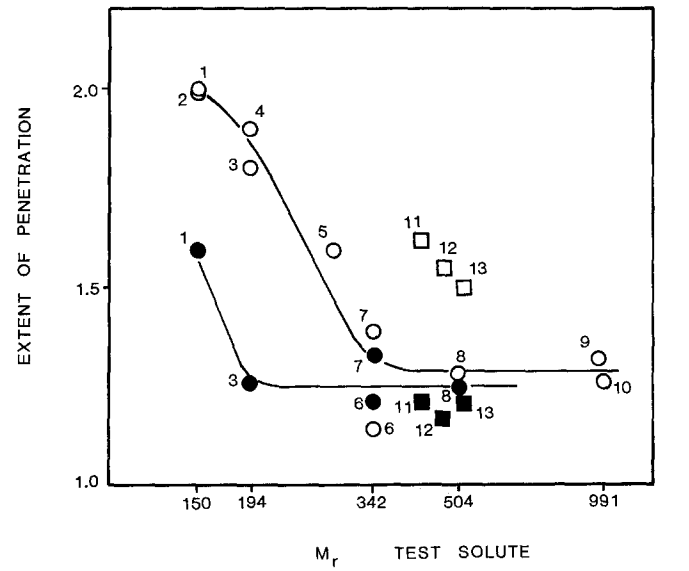

Fig. 2. Penetration of the uncharged saccharides and the cephem antibiotics into the periplasm of the plasmolyzed cells. The penetration assay was carried out by the procedure described in the legend to Fig. 1 except that the plasmolyzed cells were incubated with the test solute for 4 min. Saccharides used were 1, ribose; 2, arabinose; 3, Me-man; 4, α-methylgalactoside; 5, salicin; 6, turanose; 7, trehalose; 8, melezitose; 9, α-cyclodextrin; and 10, maltohexaose. Cephem antibiotics used were; 11, CFX; 12, CMZ; and 13, LMX; at 2 mM. Symbols: ○, □,cells treated with 600 mosM NaCl; ●, ■,cells treated with 220 mosM NaCl for the unplasmolyzed control.

penetration of di- through hexasaccharides appeared to be all same, that is less than 20% that of pentoses.

Determination of pellet weight after treating the cells with hypertonic saccharide. The outer membrane-impermeable hypertonic solute exerts osmotic pressure at the cell surface resulting in dehydration, shrinking and weight loss of the treated cell (2). Whereas the cell treated with the outer membrane-permeable hypertonic solute shows no significant change in its weight (2). As shown in Fig. 3, the cells treated with 500 mM di- through pentasaccharides appeared to be about 36% lighter than the isotonic NaCl-treated cells, suggesting that the

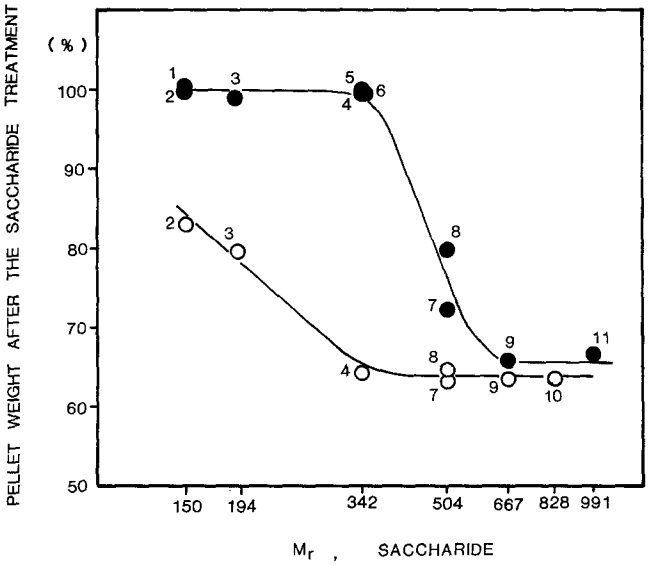

Fig. 3. Relative weight of the cells treated with the hypertonic saccharide. A hundred µl of cell suspension (4.4 mg protein) was centrifuged for 40 sec at 58,000 x g. After removing the supernatant, the tube was weighed. Cells were mixed with 100 µl of 500 mM saccharide of various sizes and 0.5 units of β-galactosidase, and incubated at 23°C for 4 min. After centrifugation under appropriate conditions, the supernatant was saved (1st sup) and the tube was weighed again. The pellet was resuspended in 100 µl of 500 mM sucrose in Buffer A and incubated at 23°C for 4 min. After centrifugation at 58,000 x g for 2 min, the supernatant was saved (2nd sup). Weight of saccharide-treated cells was expressed as (net weight of saccharide-treated cells)/(net weight of the isotonic NaCl-treated cells), x 100. Net weight was calculated as (gross weight) - (tube weight) - (weight of the intercellular water). Weight of the intercellular water in the centrifuged pellet was computed from a volume of water occupied by β-galactosidase, multiplied with the solute density. A volume of intercellular space was derived from the β-galactosidase activity of the 1st and 2nd sup as 2nd sup/1st sup, x 100. Saccharides used were 1, ribose; 2, arabinose; 3, Me-man; 4, trehalose; 5, sucrose; 6, cellobiose; 7, melezitose; 8, raffinose; 9, stachyose; 10, maltopentaose; and 11, maltohexaose. Symbols: ○,*B. fragilis*; ●,*S. typhimurium*.

penetration of these saccharides through the outer membrane was very poor. This is consistent with the results of the penetration experiment (Fig. 2). The cells treated with 500 mM arabinose and Me-man were 16 and 21%, respectively, lighter than the cells treated with isotonic NaCl, suggesting that even these small saccharides do not pass through the outer membrane freely. Thus, it became evident that the exclusion limit of the outer membrane of B. fragilis is close to the size of uncharged disaccharide. Reliability of this assay method was examined by using a strain of the established outer membrane permeability (8). The result confirmed that the outer membrane of S. typhimurium allows the penetration of mono-, di-, and trisaccharides, but excludes the saccahrides larger than tetrasaccharide (Fig. 3).

Penetration of the negatively charged solutes and antibiotics. B. fragilis is susceptible to the negatively charged β-lactam antibiotics, e.g. LMOX, CMZ and CFX, of which M_r is larger than the above exclusion limit. It is conceivable therefore that the outer membrane pore of B. fragilis selects anion. Determination of the extent of such antibiotic penetration shows that the diffusion of LMOX, CMZ and CFX is comparable with that of salicin despite of the fact that M_r of these cephems are relatively large (Fig. 2).

The hydrolysis rate of the phosphorylated small compouds such as 4-methylumbelliferyl phosphate (MeUmbP, M_r, 256) and p-nitrophenyl phosphate ($_p$NPP, M_r, 263) in intact cell appeared to be 21 and 13%, respectively, compared with that of the cell free system (Fig. 4). The hydrolysis rate of phenolphthalein diphosphate (PPDP, M_r, 491) appeared to be 7% (Fig. 4). The hydrolysis rate of cephaloridine (CER, M_r, 415), cephalothin (CET, M_r, 418), cefsulodin (CFS, M_r, 531), cefamandole (CMD, M_r, 461), and cefoperazone (CPZ, M_r, 644) appeared to be 6.8, 5.9, 12.9, 6.4, and less than 2%, respectively, compared with that of the cell free system (Fig. 4). These results indicate that the carboxylated cephem antibiotics diffused through the outer membrane pore, though their M_r is above the exclusion limit (see

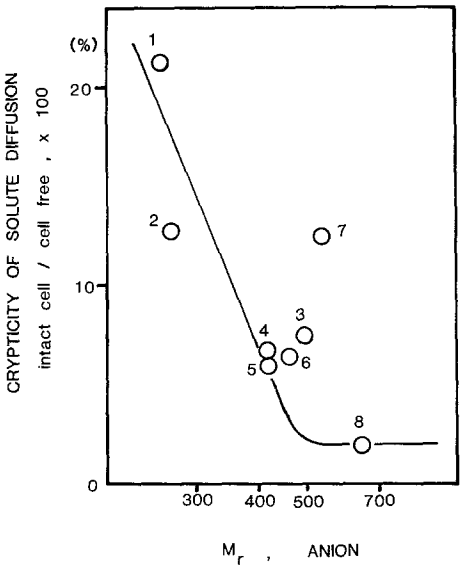

Fig. 4. Diffusion rate of the negatively charged solutes and the cephem antibiotics. Diffusion rate of NPP, MeUmbP and PPDP was measured by determining the rate of hydrolysis of these solutes by the periplasmic and cell free alkaline phosphatase as reported earlier (6). K_m of the alkaline phosphatase to NPP was determined to be 9×10^{-6}M. Cell was resuspended in 0.85 % NaCl in Buffer B (1.8 mg protein/ml). A half aliquot of the cell suspension was passed through a French pressure cell at 500 Kg/cm^2 (a cross section area 1.8 cm^2), centrifuged at 103,000 x g for 3 min with an air-driven microcentrifuge and the supernatant was isolated. A typical reaction mixture contained 2 mM solute in a solution of 0.85 % NaCl in Buffer B and the cells (18 µg protin). Hydrolysis of NPP and MeUmbP was recorded spectrophotometrically at 405 nm and fluorophotometrically at 445 nm (exciting at 360nm), respectively, at 25°C. Hydrolysis of PPDP was carried out in the mixture described above at 25°C and the mixture was boiled for 2 min in the presence of SDS. Absorption at 550 nm at pH11 was recorded. Diffusion rate of cephems was assayed as crypticity of the periplasmic β-lactamase (K_ms of β-lactamase for CER, CET, CMD, CPZ and CFS were determined to be 10, 5.5, 2, 6.6 and 20 $\times 10^{-5}$M, respectively). The reaction mixture containing 100µl of the cell suspension (4.5 mg protein) and 60 µl of 5.3 mM cephem was incubated at 23°C. The mixture was centrifuged at 58,000 x g for 40 sec and the supernatant was separated. Unhydrolyzed CER, CET, CMD, CPZ and CFS in the supernatant were quantified spectrophotometrically at 260, 255, 275, 270 and 263 nm, respectively. Solutes used were 1, NPP; 2, MeUmbP; 3, PPDP; 4, CER; 5,CET; 6, CMD; 7, CFS; and 8, CPZ.

above). Relatively high diffusion rate of CFS may be due to the presence of two negative charges.

DISCUSSION

Data presented in this paper clearly demonstrated that the outer membrane of B. fragilis acts as a penetration barrier against the uncharged saccharides of M_r higher than 340-400 and the negatively charged solutes of M_r about 500. This result is close to the permeability of the outer membrane of P. aeruginosa (2,3). Thus, it is evident that the diffusion

pores in the outer membrane of these opportunistic pathogens are substantially smaller than that of E. coli and S. typhimurium (see ref. 1 for review), that seems to be ultimately related to the intrinsic drug resistance of these organisms.

One may wonder how B. fragilis utilizes the oligo- and polysaccharides of M_r far above the exclusion limit of the outer membrane. It is conceivable, however, that B. fragilis induces the specific diffusion pore for the diffusion of such oligo-(or poly-)saccharides, when the inducer is present, analogous to the LamB protein for the maltodextrin diffusion in E. coli (9). In fact, the outer membrane of B. fragilis allows the accelerated diffusion of monosaccahrides, maltose- and some of the non-maltose oligosaccharides, when the cells were grown in the presence of maltose (data not shown). The outer membrane of such cells exhibited the altered protein composition as examined the stained protein band after SDS-gel electrophoresis. This is consistent with the observation that B. thetaiotaomicron induces the new outer membrane protein as was grown in the polysaccharide containing medium (10).

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