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IN VITRO DEMONSTRATION BY THE RATE ASSAY OF THE PRESENCE OF SMALL PORE IN THE OUTER MEMBRANE OF *Pseudomonas aeruginosa*

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SUMMARY Determination of the rates of saccharide diffusions by the proteoliposomes showed that the outer membrane of *Pseudomonas aeruginosa* only possesses small diffusion pores and that protein F might have not been involved in the pore formation. Proteoliposomes containing stachyose or Dextan T-10 showed the same relative diffusion rates as measured by the liposome swelling method. Slopes of the lines, diffusion rate vs saccharide M_r, in the liposomes made of the *P. aeruginosa* and *E. coli* B outer membranes appeared to be -7.4 and -3.5, respectively. Intercepts of the lines with x-axis in the liposomes containing the *P. aeruginosa* and *E. coli* B outer membrane appeared to be about M_r, 220 and 320, respectively. Relative diffusion rates of saccahrides through the liposome membranes reconstituted from the protein F-deficient outer membrane were superimposable with that of the protein F-sufficient outer membrane.

P. aeruginosa known as a problem pathogen for immunocompromised patients is highly resistant to a number of structurally unrelated antibiotics (1,2). One of the important factors contributing to this intrinsic drug resistance has been thought to be the poor antibiotic permeability across the outer membrane (2,3). A puzzling results reported earlier were that protein F of *P. aeruginosa* formed a large diffusion pore through which polysaccharides of M_r, 3,000 to 7,000 could penetrate (4,5). On the other hand, Caulcott et al. (6) and Yoneyama et al. (7,8) presented lines of evidence that the outer membrane of *P. aeruginosa* only allows free diffusion of saccharides less than about di- to trisaccharides by measuring the equilibrium of test solutes in the intact outer membrane.

Present study was conducted to verify the presence of small diffusion pores in the outer membrane and explain the intrinsic drug resistance of

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P. aeruginosa. To do so, the diffusion rates of uncharged saccharides were determined by using the proteoliposomes reconstituted from the purified outer membrane of the protein F-sufficient or -deficient strains. The results fully supported the notion that the P. aeruginosa outer membrane contains small diffusion pores and did not support the notion that protein F is the porin and forms a large pore.

MATERIALS AND METHODS

Bacterial strains and growth conditions P. aeruginosa PAO1 is a wild type laboratory strain and KG1077 (9) is a protein F-deficient mutant derived from a substrain of PAO1. E. coli B was also used. Cells were grown in 1 l of L-broth containing 5 mmol of $MgCl_2$, pH7.4 at 37° C for 4 h with vigorous aeration (200 rpm) after dilution of a fully grown 100 ml of preculture. The outer membranes were purified from P. aeruginosa and E. coli B according to the procedures described by Mizuno and Kageyama (10) or Smit et al. (11) and Mizushima and Yamada (12), respectively.

Reconstitution of the proteoliposomes and the liposome swelling assay Proteoliposomes were reconstituted by the procedure described earlier (13) by using the mixture of 97 parts of phosphatidylcholine (PC) and 3 parts of dicetylphosphate (DCP) (14) or using crude phospholipids extracted from Salmonella typhimurium LT2M1 (13,15), and the outer membrane.

The diffusion rate was calculated from the swelling rate of the proteoliposome as described earlier (13,14). Proteoliposomes (0.26 μmol lipids/ 35 μl) were diluted into 665 μl of the isotonic test solute and a optical density at 450 nm was recorded at range 0.2 with a chart speed 120 mm/min for 60 sec.

Materials Reagents used were the best grades commercially available.

RESULTS AND DISCUSSION

Experiments with the proteoliposomes containing stachyose or Dextran T-10. In order to clarify the discrepancy of the reported pore sizes, the proteoliposomes were prepared entrapping either Dextran T-10 (average M_r, 10,000) or stachyose (M_r, 666), and the diffusion rates of pentoses, hexoses, disaccharides and trisaccharide were determined by the liposome swelling method. If the outer membrane possesses small pores only allowing the diffusion of trisaccharides or less, the diffusion rates of the above listed saccharides should be measurable with either proteoliposomes regardless of the intraliposomal solutes. On the other hand, if the pores are large enough to allow free diffusion of large oligosaccharides, entrapped stachyose should readily leak out of the liposomes resulting in little change

in optical density. While, the diffusion of the above listed solutes should be measurable using the proteoliposomes containing Dextran T-10. The results of such experiments showed that the relative diffusion rates of hexoses in the stachyose or the Dextran T-10 entrapped proteoliposomes appeared to be 40 to 60% or 70 to 75%, respectively, of the rate of ribose (Fig. 1). Relative diffusion rates of methylhexoses and N-acetylglucosamine in both liposomes appeared to be about 10 to 35% of the rate of ribose. Diffusion rates of di- and trisaccharides were undetectably low under the conditions. Slopes of the lines appeared to be -7.4 and -7.3 for the stachyose and Dextran T-10 containing proteoliposomes, respectively and intercepts with x-axis of both lines appeared to be about M_r, 210 to 220, respectively. These results clearly indicated that the pore(s) in the outer membrane of P. aeruginosa is smaller than that of E. coli as was shown in intact cells (6,7,8). Both bacterial crude phospholipids and PC/DCP showed essentially the same results (data not shown). Rate of optical density change of the stachyose or Dextran T-10 entrapped proteoliposomes for glucose appeared to be 56 and 36 arbitrary units, respectively. Methods of outer membrane preparation (refs. 10 or 11) did not affect on the result.

Comparison of the size of the outer membrane pore of P. aeruginosa with that of E. coli B In order to compare the size of the P. aeruginosa pore with that of E. coli B, diffusion rates of saccharides were determined using the liposomes reconstituted from the outer membrane of E. coli B. A slope of the line of diffusion rates vs log M_r appeared to be -3.52 and an intercept of the line with x-axis was close to saccharide M_r, about 320 (Fig. 2). These data essentially confirmed the earlier results reported from this and other laboratories (15,16). Since a slope of the line for the permeability of P. arugninosa was shown to be -7.4 (Fig. 1), comparison of the slope with that of E. coli B clearly indicated that the size of the P. aeruginosa outer membrane pore is smaller than that of the outer membrane pore of E. coli B. The intercepts for P. aeruginosa and E.

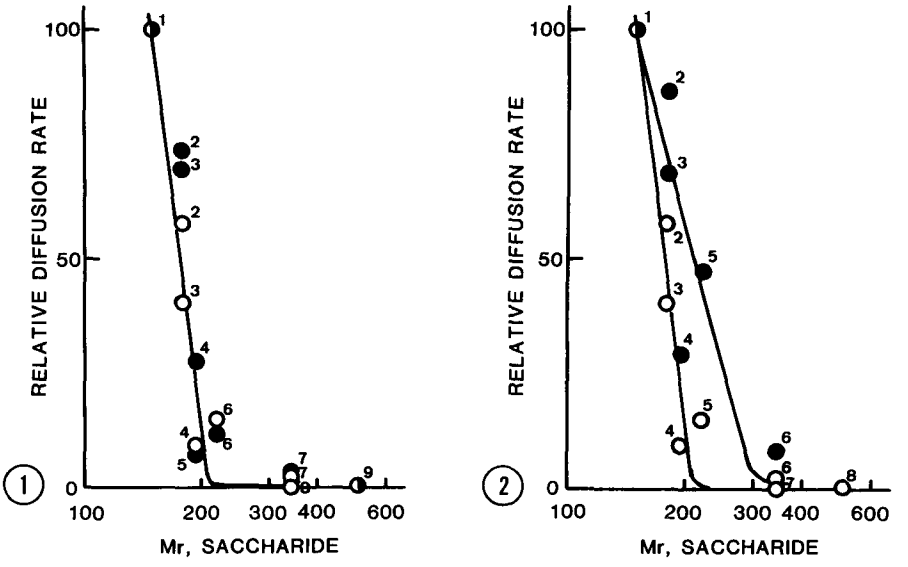

Fig. 1. Diffusion rates of saccharides through the liposome membranes containing stachyose or Dextran T-10. Proteoliposomes were reconstituted from 1 μmol of phospholipid from S. typhimurium LT2M1 and 50 μg of proteins of the P. aeruginosa PAO1 outer membrane in the presence of 40 mosM stachyose or 6% of Dextran T-10 in 1 mM Mops, pH 7.2. The liposome swelling assay was performed as described in Materials and Methods. Diffusion rates were normalized to that of ribose of the respective proteoliposomes. Saccharides used were; (1), ribose; (2), glucose; (3), galactose; (4), α-methylmannoside; (5) α-methylgalactoside; (6), N-acetylglucosamine; (7), lactose; (8), sucrose; and (9), raffinose. Symbols; ○ , liposomes containing stachyose; ● , liposomes containing Dextran T-10.

Fig. 2. Diffusion rates of saccharides through the liposome membranes reconstituted from the outer membranes of P. aeruginosa PAO1 or E. coli B. Proteoliposomes were reconstituted from 1 μ mol of phospholipid from S. typhimurium and 5 and 50 μg of outer membrane proteins of E. coli B and P. aeruginosa PAO1, respectively, in the presence of 40 mosM stachyose in 1 mM Mops, pH 7.2. Diffusion rates were determined by the liposome swelling assay as described in Materials and Methods. Data for P. aeruginosa PAO1 are taken from Fig. 1. Saccharides used were; (1), ribose; (2), glucose; (3), galactose; (4), α-methylmannoside; (5), N-acetylglucosamine; (6), lactose; (7), sucrose; and (8), raffinose. Symbols; ○ , P. aeruginosa PAO1; ● , E. coli B.

coli obtained from Fig. 1 and 2 appeared to be about M_r, 210 and 320, respectively. These results suggested again that the outer membarane pore(s) of P. aeruginosa is substancially smaller than that of E. coli B, supporting the previously reported conclusion (6,7,8). The same result to Fig. 1 was obtained with the octylglucoside soluble materials obtained from the outer membrane of P. aeruginosa as reconstituted into the liposomes after removing the surfactant (data not shown).

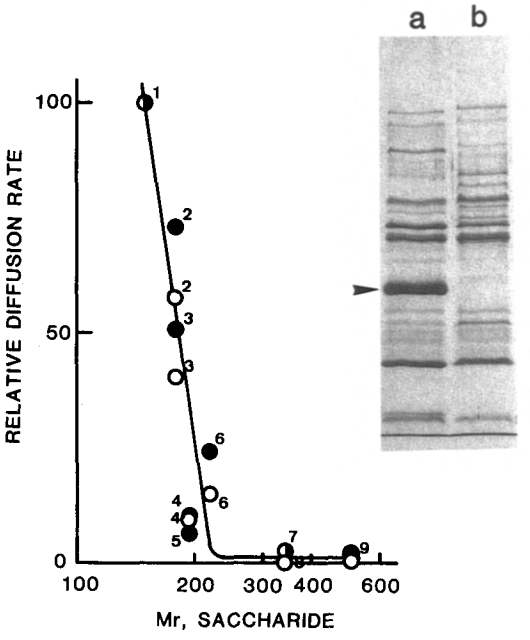

Fig. 3. Diffusion rates of saccharides through the liposomes containing the protein F-deficient and -sufficient outer membranes of P. aeruginosa. Proteoliposomes were reconstituted from 50 μg of proteins of the outer membranes purified from P. aeruginosa PAO1 or KG1077 and 1 μ mol of phospholipid purified from S. typhimurium in the presence of 40 mosM stachyose in 1 mM Mops, pH 7.2. Diffusion rates were determined as described in Materials and Methods and that was normalized to the diffusion rate of ribose. Saccharides used were; (1), ribose; (2), glucose; (3), galactose; (4), α-methylmannoside; (5), α-methylgalactoside; (6), N-acetylglucosamine; (7) lactose; (8), sucrose; and (9), raffinose. Data in PAO1 are taken from Fig. 1. Symbols; ○, PAO1; ●, KG1077.

Inset, Acrylamide gel electrophoregram of the outer membrane proteins. The purified outer membrane was solubilized in the sample buffer, heated at 95° C for 5 min and applied 40 μ g of proteins per lane. The buffer system employed was the Laemmli's formula (23). a, PAO1 (wild type); b, KG1077 (a protein F-deficient mutant). A arrow head indicated the position for protein F. Five other mutants showd the same protein profiles to that of KG1077.

Permeability of the liposome membranes reconstituted from the protein F-free outer membrane A question to be asked now is whether the previously reported pore-forming protein, F forms the diffusion pore of small size. We had isolated 6 protein F-deficient mutants of P. aeruginosa (9). Polyacrylamide gel electrophoregrams of the outer membrane proteins showed no visible band of protein F (Fig. 3, inset). Permeability assay using the proteoliposomes reconstituted from such outer membrane indicated that the membranes allowed the diffusion of pentoses, and hexoses well but did not allow the diffusion of saccahrides larger than di- or trisaccharide

(Fig. 3). The outer membranes from five other protein F-deficient mutants showed the same results (data not shown). The slope of the plots and the intercept with x-axis are indistinguishable from that of the experiment using the protein F-sufficient outer membrane (Fig. 1). These data did not favor the previously published results (4,14,17).

The reason why the previous data suggested the presence of large pore is not clear. However, the following interpretations of the previous data may be possible. (i) Sucrose had been used as a smallest test solute in most experiments (4,5,18) except ref. 14 that was shown to be poorly permeable across the P. aeruginosa outer membrane (6-8). Therefore, earlier data might not be reliable. (ii) Permeability of the intact outer membrane of P. aeruginosa was shown to be roughly close to that of enteric bacteria as mentioned by the investigators themselves (18), yet the in vitro permeability assays in the same laboratory showed the presence of large pores (4,5,14). The investigators provided no clear interpretation for this discrepancy. (iii) Dialysis of the purified proteins against 5% of ethanol solution for 22 days (4) might have caused denaturation of genuine porin(s) and possibly created the artifact by protein F. We experienced that the purified P. aeruginosa porins are relatively sensitive to prolonged storage at 4° C (unpublished result). Following lines of evidence support our conclusion. The imipenem resistant strains from the imipenem-treated patients or by the in vitro selection were missing protein D but fully retained protein F (19,20,21). The deduced amino acid sequence from the DNA sequencing of the gene coded for protein F showed no apparent homology with the known amino acid sequences of E. coli porins, Omp F, Omp C. Pho E or Lam B, but that showed the closest homology to the Omp A proteins of different enteric bacteria (22). All these results suggested that protein F of P. aeruginosa might have not been the porin.

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