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Cloning of the protein D2 gene of *Pseudomonas aeruginosa* and its functional expression in the imipenem-resistant host

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Protein D2 forms the water-filled pore across the outer membrane of *Pseudomonas aeruginosa* and allows the penetration of imipenem. We cloned the protein D2 gene by the antibody screening technique. When the imipenem-resistant mutant lacking protein D2 harbored the plasmid with the cloned D2 gene, the mutant overproduced protein D2 in the outer membrane. These transformants exhibited fully-restored imipenem susceptibility. The results prove unequivocally that protein D2 forms the imipenem-permeable pore in the *P. aeruginosa* outer membrane.

Pseudomonas aeruginosa; Protein D2; Imipenem; Permeability; Porin; Outer membrane

1. INTRODUCTION

Pseudomonas aeruginosa is a major agent of nosocomial infections. The organism is highly resistant to many structurally unrelated antibiotics that is most likely due to the production of antibiotic-modifying enzyme(s) and the presence of a tight diffusion barrier [1,2]. Caulcott et al. and ourselves have reported that the tight diffusion barrier is attributable to the presence of only small outer membrane pores [3,4]. Alternatively, the presence of the inefficient large pore was reported [5,6]. Imipenem is a low M_r carbapenem having potent antipseudomonal activity. The efficient permeability of imipenem through the D2 pore has been demonstrated [7,8]. Therefore most imipenem-resistant clinical isolates lack D2 [9]. To ascertain the role of D2 in imipenem diffusion, we cloned the gene coding for D2 and expressed it in an imipenem-resistant D2-defective host.

2. MATERIALS AND METHODS

2.1. *Bacterial strains and culture conditions*

P. aeruginosa PAO1, PAO2003 (*arg*H32, *rec*A2, FP$^-$), and *E. coli* XL1-BLUE (Stratagene) were used. pKT240 is a broad-spectrum vector with ABPC^r and KM^r markers. L-broth containing 10 g of tryptone, 5 g of yeast extract and 5 g of NaCl per liter was generally used.

2.2. *DNA techniques*

Most recombinant DNA techniques used were described in [10]. *P. aeruginosa* was transfected with the fusion plasmid as in [11].

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Abbreviations: D2, protein D2; CBPC, carbenicillin; ABPC, ampicillin; KM, kanamycin; MIC, minimum inhibitory concentration

2.3. *Other techniques*

The spontaneous D2-defective derivatives of strain PAO2003 were selected for imipenem resistance at 3.13 μg/ml. The outer membrane protein was analyzed by SDS-polyacrylamide gel electrophoresis according to the method in [12]. MIC of the antibiotic was determined by the 2-fold agar dilution method using Mueller Hinton Medium (Difco). Protein was quantified by the method of Lowry et al. [13]. Outer membrane was purified according to the procedure described in [14]. The diffusion rates of ribose and imipenem were determined by the liposome swelling method [15]. Gold staining was carried out following the manufacturer's manual (Janssen).

3. RESULTS

3.1. *Specificity of anti-D2 IgG*

Rabbit anti-D2 was purified by a D2-coupled Sepharose 4B column. When whole cell lysate of strain PAO1 was analyzed by the Western blotting technique and visualized with the anti-D2 IgG, only D2 band was seen (Fig. 1). Whole cell lysate of *E. coli* XL1-BLUE showed no detectable D2 band (Fig. 1).

3.2. *Immunological screening and construction of the fusion plasmid*

DNA was isolated from strain PAO1 as in [16] and partially digested with *Sau*3AI. DNA fragments of 3–10 kb were ligated to pBluescript II SK(+) (Stratagene) digested with *Bam*HI. *E. coli* XL1-BLUE was transfected with pBluescript II SK(+) and transformants were grown on L-agar containing 50 μg/ml of CBPC. We screened about 5000 clones with the rabbit anti-D2 IgG [10,17] and obtained seven D2-positive clones. A recombinant plasmid with a 4.5 kb insert (pTN001) was digested with *Bam*HI and ligated to a plasmid pKT240 (ABPC^r, KM^r) treated with *Bam*HI and alkaline phosphatase. *E. coli* harboring the fusion plasmid was screened on L-plates containing 12.5 μg/ml

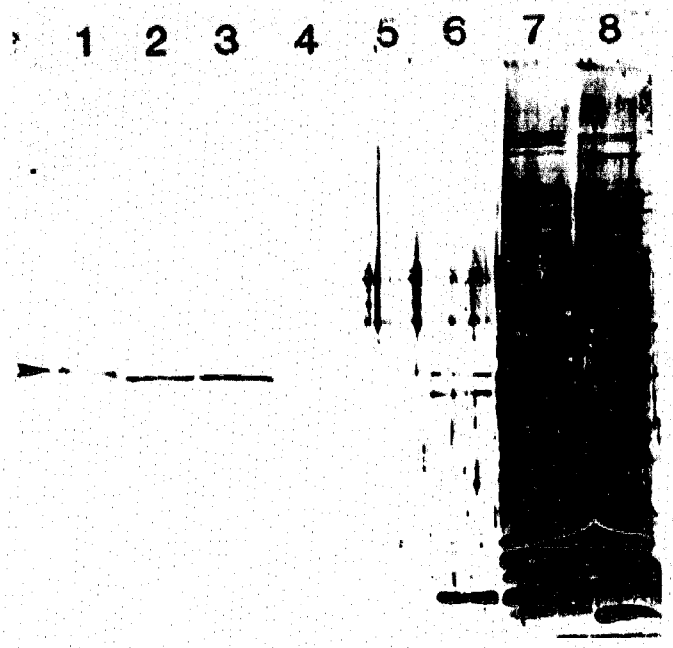

Fig. 1. Western blotting analysis of D2. Samples were subjected to SDS-polyacrylamide gel (10%) electrophoresis. Protein was blotted to polyvinylidene difluoride membrane (Millipore) at 300 mA for 30 min. Lanes 1 through 4 were stained with the anti-D2 IgG. Lanes 5 through 8 were visualized with gold stain. Lane 1 and 5, purified D2 (70 ng); lane 2 and 6, strain PAO1 outer membrane (1 μg protein); lane 3 and 7, whole cell lysate of strain PAO1 (10 μg protein); lane 4 and 8, whole cell lysate of *E. coli* XL1-BLUE (10 μg protein). An arrowhead represents protein D2.

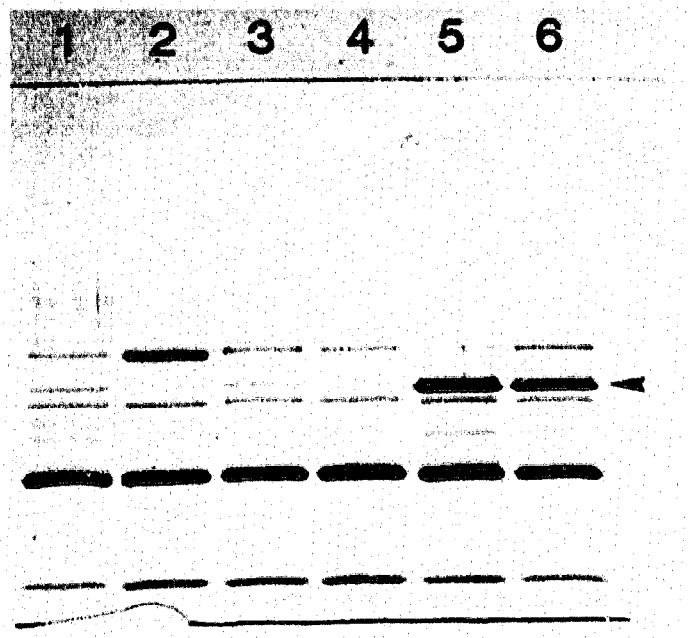

Fig. 2. Electrophoretic analysis of the outer membrane proteins. Purified outer membrane (15 μg protein) was subjected to SDS-polyacrylamide gel (10%) electrophoresis. Lane 1, strain PAO2003; lane 2, TNP031; lane 3, TNP033; lane 4, TNP035; lane 5 TNP038; lane 6, TNP040. An arrowhead represents protein D2.

KM. The *E. coli* transformants harboring the recombinant plasmid (pTN003) expressed a full-sized D2 as judged by the Western blotting analysis of whole cell lysate (data not shown), indicating that there was no *Bam*HI site in the D2 gene.

3.3. *Antibiotic susceptibility*

To determine the physiological role of the plasmid-encoded D2, the D2-defective *P. aeruginosa* TNP031 was transfected with pTN003 and MICs of the transformants to antibiotics were determined. Strain PAO2003 and strain TNP033 (harboring uncloned fusion vector pTN002) showed low MICs to imipenem, 0.78 μg/ml (Table I). The D2-defective strains TNP031 and TNP035 (the strain harboring the uncloned plasmid, pTN002) showed high MICs to imipenem, 12.5 μg/ml (Table I). TNP037 through TNP041 were the derivatives of TNP031 harboring the plasmid carrying the cloned D2 gene (pTN003). All these strains exhibited restored susceptibility to imipenem to the level of PAO2003 (Table I). These results unequivocally demonstrate that D2 forms the diffusion pore through

Table I

MICs of antibiotics against the strains used

Strains	Relevant property		MICs of antibiotics (μg/ml)							
	D2	D2-gene	IPM	CER	CEZ	CAZ	GM	OFLX	CP	TC
PAO2003	+	chromosome	0.78	>800	>800	1.56	1.56	0.2	50	12.5
TNP031	−		12.5	>800	>800	1.56	3.13	0.39	50	12.5
TNP033	+	chromosome	0.78	>800	>800	1.56	3.13	0.39	25	12.5
TNP035	−		12.5	>800	>800	1.56	1.56	0.39	50	12.5
TNP037	+	pTN003	0.78	>800	>800	1.56	3.13	0.39	25	12.5
TNP038	+	pTN003	0.78	>800	>800	1.56	1.56	0.39	25	12.5
TNP039	+	pTN003	0.78	>800	>800	1.56	1.56	0.39	25	12.5
TNP040	+	pTN003	0.78	>800	>800	1.56	1.56	0.39	25	12.5
TNP041	+	pTN003	0.78	>800	>800	1.56	3.13	0.39	25	12.5

About 5×10^3 colony forming units of cells per 5 μl were inoculated into Mueller Hinton medium containing antibiotic and growth of the cells was scored after 18–20 h of incubation at 37°C. The medium used for the preculture of strains TNP033 through TNP041 contained 200 μg/ml of CBPC, since these strains harbored plasmid with ABPC^r marker. Abbreviations: IPM, imipenem; CER, cephaloridine; CEZ, cefazolin; CAZ, ceftazidime; GM, gentamicin; OFLX, ofloxacin; CP, chloramphenicol; TC, tetracycline.

Table II

Permeability of the outer membrane to ribose and imipenem

Strains	Relevant property		Relative permeability	
	D2	D2-gene	Ribose	Imipenem
PAO2003	+	chromosome	100	100
TNP031	−		103.3 ± 7.52	75.2 ± 8.71
TNP033	+	chromosome	98.2 ± 12.33	97.3 ± 21.03
TNP035	−		106.2 ± 10.72	65.2 ± 24.16
TNP038	+	pTN003	256.0 ± 57.11	198.6 ± 51.96
TNP040	+	pTN003	240.9 ± 46.20	226.8 ± 42.10

Proteoliposomes were reconstituted from 1 μmol phospholipid (phosphatidylcholine/dicetylphosphate, 97/3 molar ratio) and 50 μg of outer membrane protein in the presence of 20 mM stachyose and 5 mM MOPS, pH 7.2. The diffusion rate was determined as described in Section 2 and was expressed as the relative value to the rate in strain PAO2003 outer membrane. The values are mean ±SD of 5–6 independent assays.

which imipenem is permeable. MICs of other antibiotics showed no detectable difference among the strains tested.

3.4. *Outer membrane permeability*

To ascertain that the cloned gene encodes intact D2, we analyzed the outer membrane proteins of the strain harboring plasmid pTN003. An SDS-polyacrylamide gel electrophoretogram of the outer membranes of TNP038 and TNP040 showed overproduced protein corresponding to D2 (Fig. 2). The electrophoretic mobility of the protein was indistinguishable from that of authentic D2 in PAO2003. To assess the functional aspect of the plasmid-encoded D2, we determined the diffusion rates of ribose and imipenem through liposome membranes reconstituted from the outer membranes of TNP038 and TNP040. The diffusion rates of ribose and imipenem in the outer membrane of these strains appeared to be about 2.5 and 2 times, respectively, higher than those of the PAO2003 outer membrane (Table II). The diffusion rates of imipenem in TNP038 and TNP040 were 2.75 and 3.0 times higher, respectively, than those in TNP031. These results demonstrate that the cloned gene encodes functionally active D2.

4. DISCUSSION

P. aeruginosa is highly resistant to most antibiotics that are effective against *E. coli* and other Gram-negatives. One of the factors contributing to this natural drug resistance is the outer membrane barrier that is attributable to the presence of only small diffusion pores [3,4] or the inefficient large pore [5,6]. Potent antipseudomonal drugs developed recently are carbapenems, such as imipenem. Imipenem having an M_r of 299 fulfills the conditions for passing through the porin-pores consisting of proteins C, D2, or E [15]. The D2 pore is the most efficient among these for the diffusion of imipenem [7,8] and hence imipenem-resistant *P. aeruginosa* often lacks D2 [7,9].

We cloned the gene encoding D2 and expressed it in the D2-deficient host (Fig. 2). The D2 protein expressed in the D2-deficient host is identical to chromosomally encoded D2 in several criteria (Fig. 2 and Tables I, II). Most importantly, the plasmid-encoded D2 was functionally active in the diffusion of saccharide and imipenem (Table II). The imipenem susceptibility of the D2-defective mutant was fully restored by transfection of the plasmid carrying the D2 gene (Table I). This is the first case to our knowledge where the D2 gene was cloned and functionally expressed in a D2-defective mutant. The only data we had difficulty in interpreting were the diffusion rates of ribose in the outer membrane from the imipenem-resistant strains, TNP031 and TNP035, were comparable with that of PAO2003. One possible interpretation is that the contribution of D2 to the diffusion of ribose is relatively small compared with that of other porins as suggested earlier [15].

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