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Nucleotide Sequence of the Protein D2 Gene of *Pseudomonas aeruginosa*

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Protein D2 of the outer membrane of *Pseudomonas aeruginosa* was shown to form the imipenem-permeable pore. We cloned and sequenced the protein D2 gene. The protein D2 gene encodes a polypeptide with 443 amino acids consisting of 23 and 420 amino acid residues for the signal peptide and mature polypeptide (M_r, 46,010), respectively. Protein D2 contains the highest molar ratio of glycine and no cysteine. The polar amino acids are scattered throughout the sequence.

Imipenem resistance in *Pseudomonas aeruginosa* is often associated with the loss of the outer membrane protein D2 (OprD) (1, 7, 11). OprD is one of the pore-forming proteins in the outer membrane as reported previously (17), and the gene coding for OprD was mapped between 71 and 75 min of the *P. aeruginosa* chromosome (6). This protein was shown to have affinity towards basic amino acids and imipenem (12–14). In fact, the MIC of imipenem became higher in the presence of basic amino acids (2). We have cloned the gene coding for protein D2 (*oprD*), and it was functionally expressed in an OprD-defective host (16). This paper reports the nucleotide sequence of the *oprD* gene and its deduced amino acid sequence.

Bacterial strain, plasmids, and medium. The bacterial strain used was *Escherichia coli* XL1-BLUE (Stratagene). The subcloning vectors used were pBluescript II SK(+) and pBluescript II SK(−) (Stratagene). L broth contains 10 g of tryptone, 5 g of yeast extracts, and 5 g of NaCl per liter, pH 7.2.

DNA techniques and sequencing. Most recombinant DNA techniques have been described previously (16). Nested sets of deletion plasmids were prepared by treating DNA with exonuclease III and mung bean nuclease as described elsewhere (9). Both strands of DNA were sequenced by using the dideoxy chain termination method (10).

Western blot (immunoblot) analysis. An overnight culture of *E. coli* XL1-BLUE harboring recombinant plasmid was harvested, washed once, mixed with the sample buffer described by Laemmli (3), and heated at 95°C for 5 min. Whole-cell lysate was subjected to sodium dodecyl sulfate-polyacrylamide gel (10%) electrophoresis as described previously (3). Protein bands were blotted onto a polyvinylidene difluoride membrane (Millipore) at 300 mA for 30 min and visualized by using anti-OprD rabbit immunoglobulin G.

Determination of protein and the N-terminal amino acid sequence of purified OprD. Protein was quantified by the method of Lowry et al. (4). OprD purified according to the method of Yoshihara and Nakae (17) was blotted onto a polyvinylidene difluoride membrane. The membrane filter with the OprD band was excised, and the protein was subjected to Edman degradation in a gas-phase sequencer (Model 477A; Applied Biosystems).

Restriction map and localization of *oprD*. Details for the cloning of *oprD* were reported earlier (16). To determine the

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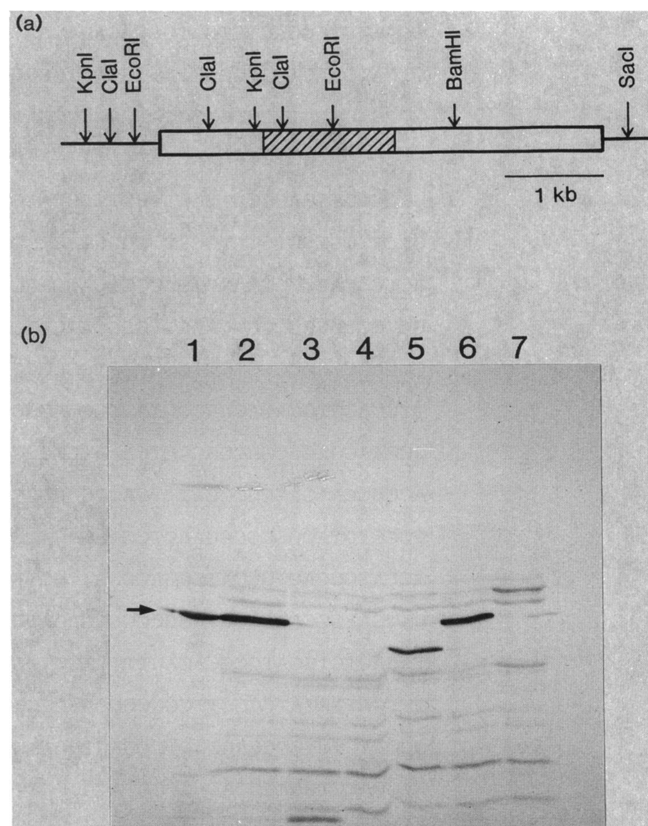

FIG. 1. Restriction map and Western blot analysis of the product of cloned *oprD*. (a) Restriction map of the insert of plasmid pTN001. The box represents the chromosomal DNA fragment of *P. aeruginosa* PAO1 (4.5 kb), and the lines represent the vector DNA. Arrows show the relevant cloning sites. The hatched box shows the location of the OprD structural gene. The physical distances between the restriction sites are arbitrary. (b) Western blot analysis of whole-cell lysate from *E. coli* XL1-BLUE harboring a subcloned insert. Lanes: 1, whole-cell lysate of *P. aeruginosa* PAO1; 2 to 6, *E. coli* XL1-BLUE harboring the following: pTN001 (lane 2), a large *Eco*RI fragment (lane 3), a small *Eco*RI fragment (lane 4), a large *Cla*I fragment (lane 5), a large *Kpn*I fragment (lane 6); 7, *E. coli* XL1-BLUE. The amounts of protein applied were 10 μg for lane 1 and 20 μg for lanes 2 through 7.

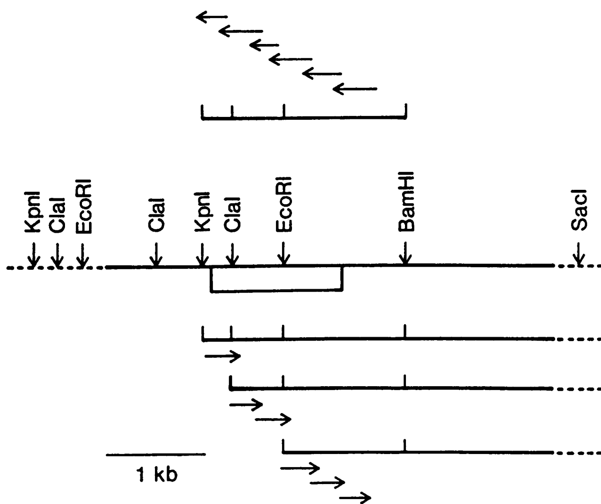

FIG. 2. Sequencing strategy of *oprD*. Solid lines represent the choromosomal DNA. Dotted lines represent the vector DNA marked with a few relevant cloning sites. OprD coding region is indicated by an open box. Recombinant plasmids containing *Kpn*I through *Bam*HI, large *Kpn*I, large *Cla*I, and large *Eco*RI fragments were constructed as described in the text. Recombinant plasmids, except that containing large *Kpn*I-*Bam*HI, were treated with exonuclease III and mung bean nuclease.

location of the OprD structural gene in the 4.5-kb chromosomal fragment in the plasmid (pTN001), we treated the plasmid DNA with various restriction enzymes and mapped the restriction sites in the insert (Fig. 1a). Neither *E. coli* XL1-BLUE cells harboring the recombinant plasmid with the small *Eco*RI fragment (1.7 kb) nor the large *Eco*RI fragment (2.8 kb) on pTN001 produced anti-OprD immuno-

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ATGCGACATGCGTCATGCAATTTTGCGACAGCACGGTAAAGAATCCGTCGCTTCGGAACC  -115
TCAACTATCGCCAAGAAACACTGCGTGCTATAAGTTAGCGCCGACAAGAAGAACTAGCCG   -55
TCACTGCGGCACTGTGATGGCAGAGATAATTTCAAAACCAAAGGAGCAATCACAATGAAA     6
                                                         M  K
GTGATGAAGTGGAGCGCCATTGCACTGGCGGTTTCCGCAGGTAGCACTCAGTTCGCCGTG    66
 V  M  K  W  S  A  I  A  L  A  V  S  A  G  S  T  Q  F  A  V
GCCGACGCATTCGTCAGCGATCAGGCCGAAGCGAAGGGGTTCATCGAAGACAGCAGCCTC   126
 A  D  A  F  V  S  D  Q  A  E  A  K  G  F  I  E  D  S  S  L
GACCTGCTGCTCCGCAACTACTATTTCAACCGTGACGGCAAGAGCGGCAGCGGGGACCGC   186
 D  L  L  L  R  N  Y  Y  F  N  R  D  G  K  S  G  S  G  D  R
GTCGACTGGACCCAAGGCTTCCTCACCACCTATGAATCCGGCTTCACCCAAGGCACTGTG   246
 V  D  W  T  Q  G  F  L  T  T  Y  E  S  G  F  T  Q  G  T  V
GGCTTCGGCGTCGATGCCTTCGGCTACCTGGGCCTGAAGCTCGACGGCACCTCCGACAAG   306
 G  F  G  V  D  A  F  G  Y  L  G  L  K  L  D  G  T  S  D  K
ACCGGCACCGGCAACCTGCCGGTGATGAACGACGGCAAGCCGCGCGATGACTACAGCCGC   366
 T  G  T  G  N  L  P  V  M  N  D  G  K  P  R  D  D  Y  S  R
GCCGGCGGCGCCGTGAAGGTGCGCATCTCCAAGACCATGCTGAAGTGGGGCGAGATGCAA   426
 A  G  G  A  V  K  V  R  I  S  K  T  M  L  K  W  G  E  M  Q
CCGACCGCCCCGGTCTTCGCCGCTGGCGGCAGCCGCCTGTTCCCGCAGACCGCGACCGGC   486
 P  T  A  P  V  F  A  A  G  G  S  R  L  F  P  Q  T  A  T  G
TTCCAGCTGCAGAGCAGCGAATTCGAAGGGCTCGACCTCGAGGCAGGCCACTTCACCGAG   546
 F  Q  L  Q  S  S  E  F  E  G  L  D  L  E  A  G  H  F  T  E
GGCAAGGAGCCGACCACCGTCAAATCGCGTGGCGAACTCTATGCCACCTACGCAGGCGAG   606
 G  K  E  P  T  T  V  K  S  R  G  E  L  Y  A  T  Y  A  G  E
ACCGCCAAGAGCGCCGATTTCATTGGGGGCCGCTACGCAATCACCGATAACCTCAGCGCC   666
 T  A  K  S  A  D  F  I  G  G  R  Y  A  I  T  D  N  L  S  A
TCCCTGTACGGCGCCGAACTCGAAGACATCTATCGCCAGTATTACCTGAACAGCAACTAC   726
 S  L  Y  G  A  E  L  E  D  I  Y  R  Q  Y  Y  L  N  S  N  Y
ACCATCCCACTGGCATCCGACCAATCGCTGGGCTTCGATTTCAACATCTACCGCACAAAC   786
 T  I  P  L  A  S  D  Q  S  L  G  F  D  F  N  I  Y  R  T  N
GATGAAGGCAAGGCCAAGGCCGGCGACATCAGCAACACCACTTGGTCCCTGGCGGCAGCC   846
 D  E  G  K  A  K  A  G  D  I  S  N  T  T  W  S  L  A  A  A
TACACTCTGGATGCGCACACTTTCACCTTGGCCTACCAGAAGGTCCATGGCGATCAGCCG   906
 Y  T  L  D  A  H  T  F  T  L  A  Y  Q  K  V  H  G  D  Q  P
TTTGATTATATCGGCTTCGGCCGCAACGGCTCTGGCGCAGGTGGCGACTCGATTTTCCTC   966
 F  D  Y  I  G  F  G  R  N  G  S  G  A  G  G  D  S  I  F  L
GCCAACTCTGTCCAGTACTCCGACTTCAACGGCCCTGGCGAGAAATCCTGGCAGGCTCGC  1026
 A  N  S  V  Q  Y  S  D  F  N  G  P  G  E  K  S  W  Q  A  R
TACGACCTGAACCTAGCCTCCTATGGCGTTCCCGGCCTGACTTTCATGGTCCGCTATATC  1086
 Y  D  L  N  L  A  S  Y  G  V  P  G  L  T  F  M  V  R  Y  I
AATGGCAAGGACATCGATGGCACCAAGATGTCTGACAACAACGTCGGCTATAAGAACTAC  1146
 N  G  K  D  I  D  G  T  K  M  S  D  N  N  V  G  Y  K  N  Y
GGCTACGGCGAGGATGGCAAGCACCACGAAACCAACCTCGAAGCCAAGTACGTGGTCCAG  1206
 G  Y  G  E  D  G  K  H  H  E  T  N  L  E  A  K  Y  V  V  Q
TCCGGTCCGGCCAAGGACCTGTCGTTCCGCATCCGCCAGGCCTGGCACCGTGCCAACGCC  1266
 S  G  P  A  K  D  L  S  F  R  I  R  Q  A  W  H  R  A  N  A
GACCAGGGCGAAGGCGACCAGAACGAGTTCCGCCTGATCGTCGACTATCCGCTGTCGATC  1326
 D  Q  G  E  G  D  Q  N  E  F  R  L  I  V  D  Y  P  L  S  I
CTGTAATCGACCGACAGGCAACGAAAAAACCCGGCATCGCCGGGTTTTTCTTCTTGGCG  1386
 L ***
GCAACGCGCCTATAAAGGAAGGGCGTAGGTACC                             1419
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FIG. 3. Nucleotide sequence of *oprD* and neighboring DNA and the deduced amino acid sequence of OprD. The numbers on the right represent the numbers of nucleotides from the initiation codon ATG. The underlined sequences represent the putative −35 and −10 sequences, the Shine-Dalgarno sequence, and the symmetry dyad that acted as the transcription termination signal. The stop codon is indicated by asterisks. The signal peptide is indicated in italics. The N-terminal amino acid sequence of purified OprD as determined by the Edman degradation is indicated in bold letters.

globulin G-reactive protein (Fig. 1b, lanes 3 and 4). The *E. coli* XL1-BLUE transformant harboring plasmid with deletion of *Kpn*I fragment (3.6 kb) produced a single anti-OprD immunoglobulin G-positive protein with the same electrophoretic mobility as intact OprD (Fig. 1b, lanes 1, 2, and 6). On the other hand, a transformant containing a *Cla*I large fragment (3.4 kb) produced a fast-moving anti-OprD immunoglobulin G-reactive polypeptide, indicating that the structural gene of OprD should place between the *Bam*HI site and the *Kpn*I site.

We used the *Bam*HI-*Kpn*I fragment and the DNA fragments with a deletion between the *Kpn*I site of the plasmid DNA and the *Kpn*I site of the insert for the sequence analysis of coding and anticoding strands, respectively.

Nucleotide sequence of *oprD*. A strategy for the nucleotide sequence analysis of *oprD* is shown in Fig. 2. For the analysis of the coding strand, the *Bam*HI-*Kpn*I restriction fragment from plasmid pTN001 was subcloned into the phagemid pBluescript II SK(−). For the analysis of the anticoding strand, the fragments inclusive of the *Bam*HI site to *Kpn*I, *Cla*I, or *Eco*RI (large fragments) were ligated at the respective restriction sites (far left in Fig. 2).

Figure 3 shows the nucleotide sequence of the OprD coding region and its 5′ and 3′ flanking regions. The sequence shows an open reading frame consisting of 1,332 bp. A putative Shine-Dalgarno sequence (GGAG) was located at the ninth nucleotide upstream (nucleotides −12 to −9) from the initiation codon. At the 79th and 102nd nucleotides upstream from the initiation codon, putative −10 and −35 sequences (TAAGTT and TCGCCA, respectively) were found. In the 3′-nontranslated region, there was an AT-rich sequence, possibly forming a stable stem-and-loop structure, a ρ-factor independent transcription termination site. The G+C content of the coding region was 61%, which is in good agreement with the mean G+C content (65.2%) of the 15 *P. aeruginosa* chromosomal genes (15).

Deduced amino acid sequence of OprD. Figure 3 shows the amino acid sequence of OprD as deduced from the primary nucleotide sequence. The potential cleavage sites were at the 23rd and 25th amino acid residues containing the Ala-X-Ala sequence (5). To ascertain the N-terminal amino acid of mature OprD, the amino acid sequence of the N-terminal region of purified OprD protein was determined. The sequence appeared to be Asp-Ala-Phe-Val-Ser-Asp-Gln-Ala-Glu-Ala-Lys. Thus, the N-terminal amino acid was determined to be Asp at the 24th amino acid residue. Consequently, the N-terminal signal sequence was determined to be the first 23 amino acid residues. Mature OprD (amino acid residues 24 to 443) has 420 amino acid residues having an M_r of 46,010, as shown previously (17). OprD lacked cysteine. The highest amino acid content appeared to be glycine. These features seem to be general for porins of gram-negative bacteria (8). Of 420 amino acids in the mature OprD, 55 and 39 were acidic and basic ones, respectively. The charged amino acids were scattered throughout the sequence. The information obtained in this study may be useful in studies of imipenem selectivity and the channel-forming activity of OprD.

Nucleotide sequence accession number. The sequence of *oprD* has been assigned GenBank/EMBL/DDBJ accession no. X63152.

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