FEMS Microbiology Letters 97 (1992) 267–274

© 1992 Federation of European Microbiological Societies 0378-1097/92/$05.00
Published by Elsevier

FEMSLE 05098

Analysis of two gene regions involved in the expression of the imipenem-specific, outer membrane porin protein OprD of *Pseudomonas aeruginosa*

Hongjin Huang, Richard J. Siehnel, Francis Bellido, Eileen Rawling and Robert E.W. Hancock

Department of Microbiology, University of British Columbia, Vancouver, B.C., Canada

Received 14 July 1992
Accepted 20 July 1992

Key words: Outer membrane protein; OprD; *Pseudomonas aeruginosa*; Gene expression

1. SUMMARY

A Tn*501* mutant of *Pseudomonas aeruginosa* resistant to imipenem and lacking the imipenem-specific outer membrane porin protein OprD was isolated. The mutation could be complemented to imipenem susceptibility and OprD-sufficiency by a cloned 6-kb *Eco*RI–*Pst*I fragment of DNA from the region of chromosome of the wild-type strain surrounding the site of Tn*501* insertion. However, this fragment did not contain the *oprD* structural gene as judged by its inability to hybridize with an oligonucleotide corresponding to the N-terminal amino acid sequence of OprD. DNA sequencing of 3.9 kb of the region surrounding the Tn*501* insertion site revealed three large open reading frames, one of which would be interrupted by the Tn*501* insertion in the mutant. This latter open reading frame, named *opdE* (for putative regulator of *oprD* expression), predicted a hydrophobic protein of M_r 41 592. Using the above-mentioned oligonucleotide, the *oprD* structural gene was cloned and expressed in *Escherichia coli* on a 2.1-kb *Bam*HI–*Kpn*I fragment. DNA sequencing predicted a 420 amino acid mature OprD protein with a 23 amino acid signal sequence.

2. INTRODUCTION

Imipenem is a new broad spectrum carbapenem β-lactam antibiotic that is highly active against *Pseudomonas aeruginosa* [1]. However, during clinical therapy of *P. aeruginosa*, imipenem-resistant isolates arise at a significant rate [2]. The major cause of resistance is loss of a specific outer membrane protein OprD (formerly called D2) [2,3]. Interestingly, *oprD* mutants are not cross-resistant to other classes of β-lactam antibiotics. Instead resistance is limited to the zwitterionic carbapenem antibiotics [2,4]. This was explained by Trias, Nikaido and colleagues [4–6] who demonstrated that OprD was an imipenem- and basic amino acid-specific, channel-forming

Correspondence to: R.E.W. Hancock, Department of Microbiology, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z3.

protein, by virtue of its possession of a specific binding site in its channel.

In this communication, we have cloned and sequenced two sets of sequences which influence the expression of the *oprD* gene. One of these has been shown to be the *oprD* gene. Some of the data described here were originally presented in outline at the American Society of Microbiology Annual Meeting, 1990 (Abstract No. D-40).

3. MATERIALS AND METHODS

3.1. Bacterial strains and media

P. aeruginosa PA01 strain H103 [7] was used as the wild-type strain and source of wild-type DNA. Strain H673 was isolated by transposon mutagenesis with Tn*501*, as previously described [7], and selection for resistance to 50 μg ml^{-1} mercuric chloride and 8 μg ml^{-1} imipenem. *Escherichia coli* DH5α (BRL, Bethesda, MD) was used as the host strain for cloning experiments. OprD was expressed in *E. coli* strain CE1248 [8]; a strain with mutations preventing the production of porins OmpF, OmpC (due to an *ompB* mutation), and PhoE. Luria broth (1% Bacto-tryptone, 0.5% yeast extract), containing 1% NaCl for *E. coli*, was used as the liquid medium, and, when solidified with 2% Bacto-agar, was used as the solid medium.

3.2. Cloning of the opdE gene

In general, DNA procedures followed the protocols outlined by Sambrook et al. [9] with modifications described previously [10]. Chromosomal DNA was isolated from *P. aeruginosa* strain H673 and the Tn*501* and flanking DNA was cloned as a 11.5-kb *Pst*I fragment into pTZ18R (USB, Cleveland, OH) to create plasmid pER1, using the method described previously [7]. A 2.7-kb *Pst*I–*Eco*RI fragment from pER1, comprising *P. aeruginosa* DNA flanking Tn*501*, was used as a hybridization probe to clone a 4.8-kb *Eco*RI fragment from the wild-type *P. aeruginosa* strain H103 into plasmid pTZ18R to create plasmid pD2-29. This fragment, when subcloned into the mobilizable vector pRK767 [11] to create plasmid pD2-18E, failed to complement the mutation in H673.

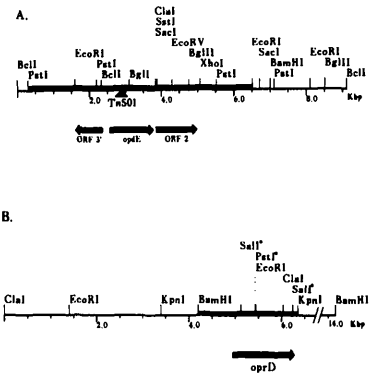

Fig. 1. Restriction endonuclease maps of *P. aeruginosa* PA01 strain H103 chromosomal DNA derived by Southern hybridizations as described in MATERIALS AND METHODS. (**A**) Map of the region surrounding the insertion site of Tn*501* (marked by the solid triangle) in strain H673. (**B**) Map of the region surrounding the *oprD* gene. Asterisks indicate restriction sites discovered by DNA sequencing only. Thick bars indicate DNA cloned in pD2-45 (**A**) and pBK-19R (**B**).

Therefore a 1.8-kb *Pst*I fragment overlapping the left-hand *Eco*RI site (Fig. 1A) was cloned from *P. aeruginosa* into plasmid pD2-18E, to create plasmid pD2-45.

3.3. Cloning of the oprD gene

OprD was partially purified from *P. aeruginosa* PA01 strain H103 grown in BM2 minimal medium containing succinate as a carbon source, using the protocol previously described for protein D1 [12]. The protein was separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE [12]), transferred to Immobilon paper and amino terminal sequenced by Sandy Kielland, University of Victoria, Canada [7] to yield the sequence D A F V S D Q A E A K G F I E D S. Taking into account codon bias in *Pseudomonas aeruginosa*, a corresponding 29-mer oligonucleotide pool was deduced from amino acids 6–15. This was then radiolabelled with ^{32}P and used in a Southern hybridization analysis of *P. aeruginosa* PA01

chromosomal DNA that had been singly or pairwise digested with several restriction endonucleases, following previously described protocols [7,9]. A 2.1-kb *Kpn*I–*Bam*HI fragment was cloned, in both orientations relative to the *lac* promoter, into pTZ18R and pTZ19R to create plasmids pBK18R (inverse orientation) and pBK19R (correct orientation). A 4-kb *Eco*RI fragment was cloned in both orientations into pTZ18R to create plasmids pE37 (correct orientation) and pE65 (inverse orientation).

3.4. Sequencing and other assays

DNA sequencing utilized an Applied Biosystems Incorporated (ABI) (Foster City, CA) automated fluorescence sequenator and dye terminator chemistry as detailed in protocols from ABI. Both strands were sequenced using either timed exonuclease III digestions (Erase-a-base, Promega, Madison, WI) to create ordered deletions (for the *oprD* gene) or a combination of subcloning and building of oligonucleotide primers (for the *opdR* gene region) as detailed previously [9,13]. Outer membranes [8,12] and cell envelope proteins [13,14] were isolated as previously described. SDS-PAGE [12] was performed as described previously. Antibiotic minimal inhibitory concentrations (MIC) were determined by the agar dilution method [2].

4. RESULTS

In an attempt to mutagenize the *oprD* gene, strain H673 *opdE*::Tn*501* was isolated as detailed in MATERIALS AND METHODS. The MIC of imipenem for H673 was 12 μg ml^{-1}, whereas the MIC for the parent strain H103 was 1.5 μg ml^{-1}, and SDS-PAGE of outer membranes revealed that H673 was OprD-deficient (Fig. 2, lane 2). MICs of ceftazidime (0.3 μg ml^{-1}) and norfloxacin (1.5 μg ml^{-1}) were unaltered by the mutation in strain H673. Therefore the region of the chromosome equivalent to that surrounding the transposon insertion site was cloned from the parent strain H103 into the vector pRK767, to create plasmid pD2-18E containing a 4.8-kb *Eco*RI fragment, and plasmid pD2-45 containing

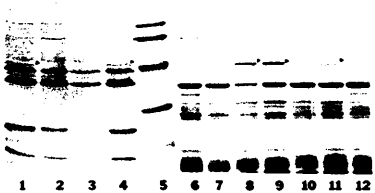

Fig. 2. SDS polyacrylamide gel electrophoretograms demonstrating the mutagenesis and cloning of DNA segments influencing the expressing of OprD. The banding position of OprD is shown by arrowheads. Lanes 1–4, *P. aeruginosa* outer membranes. The predominant bands under OprD are in descending order OprE, OprF, OprG and OprL. Lanes 6–12, *E. coli* outer membranes. Lane 1, strain H103; lane 2, strain H673 *opdR*::Tn*501*; lane 3, H673/pRK767; lane 4, H673/pD2-45; lane 5, standards with M_r values of 94000, 67000, 43000, 30000 and 20000. Lane 6, CE1248; lane 7, CE1248/pTZ18R; lanes 8, 9, CE1248/pBK19R; lane 10, CE1248/pTZ19R; lane 11, CE1248/pBK18R; lane 12, CE1248/pE37. IPTG was added to all *E. coli* cultures to induce expression from the *lac* promoter.

an overlapping 6-kb *Eco*RI–*Pst*I fragment. When mobilized back into strain H673 by triparental mating, pD2-45 was able to complement this strain to imipenem susceptibility (MIC = 1.5 μg ml^{-1}) and OprD sufficiency (Fig. 2, lane 4) whereas H673 (pD2-18E) and the vector plasmid containing strain H673 (pRK767) remained imipenem-resistant (MIC = 12 μg ml^{-1}) and OprD-deficient (Fig. 2, lane 3). In no case was ceftazidime or norfloxacin susceptibility affected.

It was at first assumed that the *oprD* gene had been cloned. However, sequencing of 3931 bp of DNA surrounding the site of transposon insertion in strain H673 failed to reveal a sequence corresponding to the N-terminal amino acid sequence of OprD, and no open reading frame equivalent to an outer membrane protein (i.e. containing a signal sequence) was predicted. In addition, no new protein bands were observed in *E. coli*, containing plasmid pD2-45. Furthermore an oligonucleotide specific for the N-terminal sequence failed to hybridize to plasmid pD2-45 and indeed hybridized with sequences in the *P. aeruginosa* chromosome with an entirely different restriction pattern (cf. Fig. 1, maps A and B).

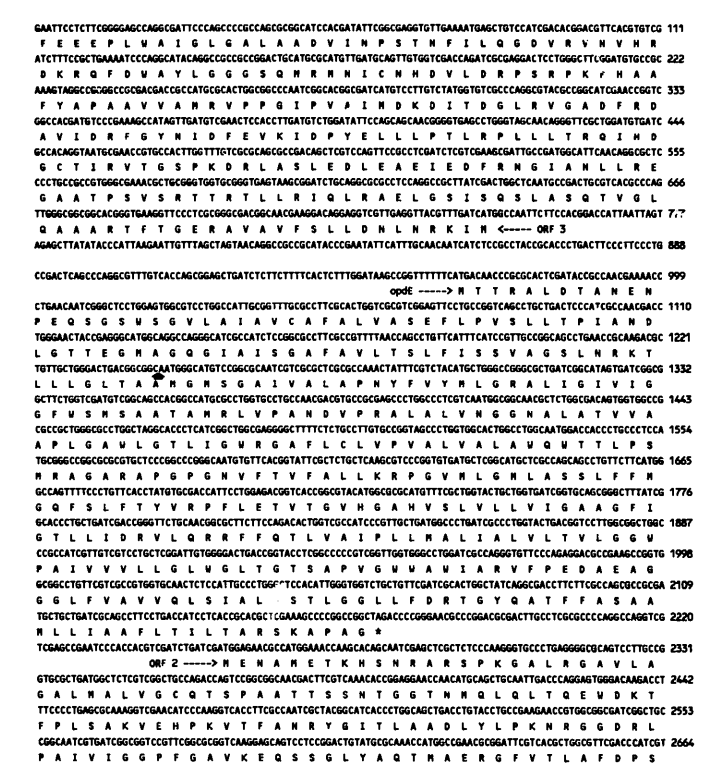

Fig. 3. Nucleotide sequence and predicted open reading frames of the sequence surrounding the transposon insertion site from H673 (indicated by an arrowhead after nucleotide 1243). The sequence is oriented in the same direction as the map given in Fig. 1A, and goes from the leftmost *Eco*R1 site to the third *Pst*1 site (at 5.5 kb in Fig. 1A). ORF 3′ was on the complimentary strand and began at base 747, read to the left and proceeded beyond the beginning of this sequence (no stop codon was encountered). These sequence data will appear in the EMBL data library under accession number Z14064.

```
ATCAGCCGCCCTACAACGAACTGAAGGGTGGTGAGGGACAGTTCCTCGTCGACTACCACGACTACTACATGACACCCCGTGGCTACCACCCGCGGGCAGTCAACTCCGGTA 3108
Y Q P P Y N E L K G G E G Q F L V D Y H D Y Y M T P R G Y H P R A V N S G
ACGCCTGGACGATGACCACGCCGCTGTCGTTCATGAACATGCCGATCCTCACCTACATCAAGGAGATCTCGCCACGCCCGATCCTGTTAATCCACGCGCAAAGGGCCCATT 3219
N A W T M T T P L S F M N M P I L T Y I K E I S P R P I L L I H G E R A H
CACGCTACTTCAGCGAGACCGCCTACGCCGCTGCCGCAGAGCCAAAGGAGCTGCTGATCGTTCCGGGAGCCAGTCATGTCGACCTGTACGACCGGCTGGACAGGATTCCTT 3330
S R Y F S E T A Y A A A A E P K E L L I V P G A S H V D L Y D R L D R I P
TCGATCGGATTGCCGGATTCTTCGACGAGCATCTGTAGCGTCGTGCACGCCAGGGCAACAGCGCCGGGAGATTGATTCGGCCCGCTCCCCCGCGTCCTGTCGCGCACCTCT 3441
F D R I A G F F D E H L *
CCGGCTTTTTCCGCGCCAGCGAGGTCCCGCTCCGCTCGAGACCTCGCCCCTCCCTGGCACCCCTTTCAAGCAACCGCCGCCCGCGTCACGATCCCGTCCACCAACCGCGCA 3552

ATCCCCAATGGGTTGCCATCCTTCAGCGCTTCCGGCAGCAACGCGTCCGGGTAGTTCTGGTAGCACACCGGGCGCAGGAAACGGTCGATGACCAGGGTGCCCACCGAGGTA 3663

CCGCGGGCGTCCGAAGTGACCGGGTACGGCCCACCGTGGACCATCGCGTCGCAGACTTCCACACCGGTCGGGTAGCCGTTGAGCAGCAGGCGTCCTGCCTTCTGTTCCAGG 3774

AGCGGTACCAGGTCGCCGAAGGACGCCAGGTCTTCCGCTTCGGCGATCAGGGTCGCGGTGACCTGCCCGTGCAGCCCATGCAGCGCGCGCTTCAGTTCGGCGTGGTCGGCG 3885

ACCTCGACGACCACGCTGGCCGGGCCGTTGACTTCTTCCTGCAG                                                                    3929
```

Fig. 3 (continued).

The DNA sequenced (Fig. 3) predicted four large open reading frames, three of which had a codon usage typical of *P. aeruginosa* genes (> 80% G + C in position 3 of codons). One of these open reading frames overlapped the region of transposon insertion in *P. aeruginosa* strain H673 and was thus named *opdE* (for putative regulator of OprD expression). This open reading frame was 402 amino acids long with a predicted M_r value of 41592. The sequence was quite hydrophobic with 61.3% non-polar amino acids (A, I, L, M, F, P, W, V), 29.4% uncharged amino acids (N, C, Q, G, S, T, Y) and only 34 charged residues. All secondary structure prediction methods used suggested that this protein was an integral membrane protein containing as many as 12 membrane spanning α-helices. Only 85 nucleotides after the end of the *opdE* gene, another large open reading frame (1110 bp, predicted to encode a 370 amino acid protein) was predicted, whereas a third open reading frame of greater than 747 bp (predicted by single-stranded sequencing past the *Eco*RI site to be 978 bp in length) was predicted to be encoded by the complementary strand. These sequences, called *orf2* and *orf3* (Fig. 1), might also be involved in OprD expression since no obvious terminator appears between *opdE* and *orf2*, suggesting a potential operon structure, whereas the divergently transcribed *orf3* contained DNA required to complement the transposon mutation in H673, based on the above complementation studies. A screen of the EMBL Swiss pro database failed to reveal genes homologous to any of these three reading frames.

The *oprD* gene was cloned as a sequence homologous to an N-terminal-specific oligonucleotide probe. A 2.1-kb *Kpn*I–*Bam*HI fragment cloned in plasmid pBK19R in the same orientation as the *lac* promoter revealed expression in *E. coli* of a cell envelope protein migrating with the same mobility as OprD (Fig. 2, lanes 8, 9). When cloned in the inverse orientation to the *lac* promoter in pBK18R (Fig. 2, lane 11), only weak expression was observed. Strong expression was almost certainly driven by the *lac* promoter since it was isopropylthiogalactoside-inducible (data not shown). The vector controls (Fig. 2, lanes 7 and 10), plasmidless control (Fig. 2, lane 6) or plasmids pE37 (Fig. 2, lane 12) or pE65 containing a 4-kb *Eco*RI insert (that proved not to contain the entire gene) did not result in production of a band of equivalent M_r to OprD.

The 2.1-kb *Kpn*I–*Bam*HI fragment containing *oprD* was sequenced (Fig. 4). Within this fragment, a 443 amino acid protein was predicted. Amino acids 24–40 were identical in sequence to the N-terminal sequence obtained from the purified protein, whereas the first 23 amino acids had features typical of a bacterial signal sequence. The 420-amino acid sequence of the mature protein predicted certain typical features observed for other outer membrane proteins including overall negative charge and a typical [13] hy-

drophobicity plot with alternating hydrophobic and hydrophilic stretches. However, less typical was the large number of stretches of uncharged amino acids [14], with 10 stretches of between 9 and 15 amino acids in length. An attempt was made to match the putative OprD sequence to other outer membranc protein sequences obtained from *P. aeruginosa* [9,13,15] and to the OmpF and TolC porins from *E. coli* [14], using the method of Neddleman and Wunsch [16] as

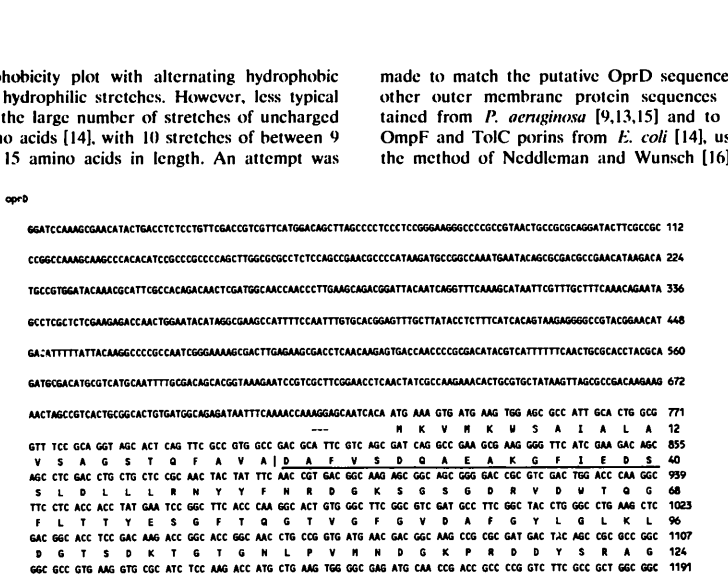

Fig. 4. Nucleotide sequence and predicted protein sequence of the DNA region including the *oprD* gene. The sequence is oriented in the same direction as the map in Fig. 1B and goes from the *Bam*HI site to the rightmost *Kpn*I site. The sequence corresponding to the N-terminal sequence determined from the mature protein is underlined. The end of the leader peptide is denoted by a vertical line. A typical Shine-Dalgarno sequence appears between nucleotides 723–726 while a predicted terminator stem–loop appears between nucleotides 2084 and 2112 (underlined with a dashed broken line). These sequence data will appear in the EMBL data library under accession number Z14065.

implemented by Dayhoff [17] with a bias parameter of 0 and a gap penalty of 4 with 10 random runs. The alignment scores obtained were 1.4, −0.5, 1.4, 2.6 and 1.1 for OprF, OprH, OprP and *E. coli* OmpF and TolC, respectively. None of these scores were considered significant above three standard deviations.

5. DISCUSSION

In this communication, we have described two sequences that influence the expression of OprD in *Pseudomonas aeruginosa*. One of these is the *oprD* gene itself. Although the cloning of this gene and its ability to complement mutants in *P. aeruginosa* has been reported [18], no restriction map or nucleic acid sequence has appeared in the literature as yet. Interestingly, the mature OprD sequence has insignificant homology with the known *P. aeruginosa* outer membrane protein sequences including the general porin OprF [9], the phosphate-specific porin OprP [13] and the Mg^{2+}-regulated protein OprH [15] or with two other porins from *E. coli*.

Our data also indicate a second gene region involved in OprD expression in that inactivation of this region by transposon mutagenesis eliminated *OprD* expression (Fig. 1). Analysis of the nucleic acid sequence revealed a long open reading frame, named here the *opdE* gene, and two adjacent open reading frames. We hypothesize that the *opdE* gene, and possibly one or two of the other open reading frames, encodes a protein that influences the expression of OprD. We can not, at this stage, state how expression is influenced but possibilities include transcriptional, translational or post-translational regulation. In keeping with this hypothesis, the *oprD* gene was expressed poorly from its own promoter in *E. coli*, in contrast to the non-regulated *oprF* gene, but similar to the regulated *oprP* [13] and *oprH* genes [14]. In addition, it is known that the level of OprD observed in the outer membrane is influenced strongly by the growth medium and carbon source [12]. However, the exact function of the coding regions indicated in Fig. 3 will only be determined by further studies.

ACKNOWLEDGEMENTS

This work was financially supported by the Medical Research Council of Canada and its early stages by the B.C. Health Care Research Foundation. E.R. and F.B. were the recipients of a Canadian Cystic Fibrosis Summer Studentship and Postdoctoral Fellowship, respectively. Howard Meadows isolated the original mutant H673.

REFERENCES

[1] Wise, R. (1986) Antimicrob. Agents Chemother. 30, 343–349.
[2] Qunn, J.P., Dudeck, E.J., Divincenzo, C.A., Lucks, D.A. and Lerner, S.A. (1986) J. Infect. Dis. 154, 289–294.
[3] Lynch, M.J., Drusano, G.L. and Mobley, H.L. (1987) Antimicrob. Agents Chemother 31, 1892–1896.
[4] Trias, J., Dufresne, J., Levesque, R.C. and Nikaido, H. (1989) Antimicrob. Agents Chemother. 33, 1201–1206.
[5] Trias, J. and Nikaido, H. (1990) Antimicrob. Agents Chemother. 34, 52–57.
[6] Trias, J. and Nikaido, H. (1990) J. Biol. Chem. 265, 15680–15684.
[7] Worobec, E.A., Siehnel, R.J., Gladman, P. and Hancock, R.E.W. (1988) FEMS Microbiol. Lett. 52, 235–238.
[8] Hancock, R.E.W., Egli, C., Benz, R. and Siehnel, R.J. (1992) J. Bacteriol. 174, 471–476.
[9] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[10] Ullstrom, C.A., Siehnel, R.J., Woodruff, W.A., Steinbach, S. and Hancock, R.E.W. (1991) J. Bacteriol. 173, 768–775.
[11] Ditta, G., Schmidhauser, T., Yakobson, E., Lu, P., Liang, X.W., Finlay, D.R., Gruney, D. and Helinski, D.R. (1985) Plasmid 13, 149–153.
[12] Hancock, R.E.W. and Carey, A.M. (1980) FEMS Microbiol. Lett. 8, 105–109.
[13] Siehnel, R.J., Martin, N.L. and Hancock, R.E.W. (1990) Mol. Microbiol. 4, 831–838.
[14] Mizuno, T., Chou, M.Y. and Inouye, M.A. (1983) J. Biol. Chem. 25, 6932–6940.
[15] Bell, A. and Hancock, R.E.W. (1989) J. Bacteriol. 171, 3211–3217.
[16] Needleman, S.B. and Wunsch, C.D. (1970) J. Mol. Biol. 48, 443–453.
[17] Dayhoff, O. (1978) Atlas of Protein Sequence and Structure, Vol. 5, Suppl. 3., National Biomedical Research Foundation, Washington, D.C.
[18] Yoneyama, H. and Nakae, T. (1991) FEBS Lett. 283, 177–179.