Protein C (OprC) of the outer membrane of *Pseudomonas aeruginosa* is a copper-regulated channel protein

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Protein C (OprC) of the outer membrane of *Pseudomonas aeruginosa* forms small channels, as assayed by the liposome swelling method. We report here that OprC functions as a channel-forming and copper-binding protein. OprC purified to homogeneity formed a channel in planar lipid bilayers with an ion conductance of about 200 pS in 1 M NaCl. Cloning and sequencing of the gene encoding OprC revealed that it specified a polypeptide comprising 723 and 668 amino acid residues for the precursor and mature polypeptides (M_r 73372), respectively. The amino acid sequence of OprC showed the highest degree of similarity with that of NosA of *Pseudomonas stutzeri* (65% sequence identity) which conveys Cu^{2+} to intracellular acceptor(s). OprC showed high copper-binding activity ($K_d = 2.6 \mu\text{M}$) in aqueous solution containing surfactant. The expression of OprC appeared to be repressed by exogenous Cu^{2+} and derepressed by anaerobiosis in the presence of nitrate. These results suggest that OprC might be involved in copper utilization.

Keywords: *Pseudomonas aeruginosa*, porin, protein C (OprC), copper-binding protein, outer membrane

INTRODUCTION

The outer membrane of *Pseudomonas aeruginosa* forms a tight barrier against the penetration of large hydrophilic solutes. This barrier function is due largely to the predominance of small diffusion pores (Yoneyama *et al.*, 1986; Yoneyama & Nakae, 1986). Using the liposome swelling assay, we identified outer-membrane proteins (OMPs) OprC, OprD2 and OprE1 to be the proteins having a porin function that allowed the diffusion of small hydrophilic solutes, such as saccharides, with M_r below 350–400 (Yoshihara & Nakae, 1989). Additionally, studies using planar lipid bilayers have shown that OprD2 and OprE1 form smaller ion conductive channels than those of the *Escherichia coli* porins (Ishii & Nakae, 1993; Obara & Nakae, 1992).

Biochemical analysis shows that OprD2 is equipped with a gate- and a channel-forming domain, suggesting that it somehow regulates channel activity by this means (Yoshihara & Nakae, 1992). Flickering of the OprD2-

channel was also demonstrated using planar lipid bilayers in the presence of lipopolysaccharide (Ishii & Nakae, 1993), consistent with the finding of the gate domain. Earlier studies showed that OprD2 serves as a selective diffusion pore for a small β -lactam antibiotic, imipenem (Büscher *et al.*, 1987; Quinn *et al.*, 1986; Satake *et al.*, 1990; Trias & Nikaido, 1990; Yoneyama & Nakae, 1991). The primary structures of OprD2 and OprE1 have been deduced from the nucleotide sequences of *oprD* and *oprE*, respectively, and showed that the proteins have a high degree of similarity to each other (Yoneyama *et al.*, 1992; Yamano *et al.*, 1993).

In contrast, the physiological function of OprC remains to be elucidated. For a better understanding of the physiological function of OprC, we investigated the channel activity of OprC in planar lipid bilayers and cloned the *oprC* gene. Here, we report that OprC is the channel-forming and copper-binding protein. We also discuss the bifunctionality of OprC in terms of copper utilization.

METHODS

Bacterial strain, plasmid and growth conditions. *P. aeruginosa* PAO1 was grown aerobically in L-broth containing 10 g Tryptone (Difco), 5 g Yeast Extract (Difco), and 5 g NaCl l^{-1} (pH 7.2) at 37 °C overnight or anaerobically in L-broth con-

Abbreviations: C_{12}E_8 , octaethylene glycol dodecylether; diPhy-PC, L- α -diphytanoylphosphatidyl choline; OMP, outer-membrane protein.

The DDBJ accession number for the nucleotide sequence reported in this paper is D28119.

taining 40 mM KNO₃. DNA was cloned in *E. coli* XL1-Blue (Stratagene) and JM109 (Toyobo). A *P. aeruginosa* chromosomal DNA library was constructed and the isolated DNA fragment was cloned using the phagemid vector pBluescript II SK(+) (Stratagene).

Formation of planar lipid bilayers and conductivity measurements. We formed planar lipid bilayers by the procedure described previously (Ishii & Nakae, 1993; Obara & Nakae, 1992; Benz *et al.*, 1978). A Teflon chamber, separating two aqueous phases by a septum having an orifice of 0.5–1 mm in diameter, was used. The orifice edge was precoated with about 0.3 µl of a 2 mg ml⁻¹ solution of L- α -diphytanoylphosphatidylcholine (diPhy-PC) dissolved in hexane. Two microlitres of a 10 mg ml⁻¹ solution of diPhy-PC, dissolved in *n*-decane on a brass wire sleeved with Teflon tubing, was applied to the orifice of the septum immersed in a salt solution containing 1 M NaCl and 2.5 mM HEPES (pH 7.0) in the chamber. Electrical resistance of the membrane appeared to be consistently higher than 400 GΩ cm⁻². The protein to be tested was added to the *cis* compartment in which a 10 GΩ probe was immersed. The conductivity was recorded through Ag/AgCl₂ electrodes connected to a patch-clamp amplifier CEZ-2200 (Nihon-Koden) in voltage-clamping mode. A membrane potential was applied through a stimulator (SEN-3301, Nihon-Koden). Signals were recorded by a digital recording oscilloscope 2232 (Tektronix) and a pen recorder LR4201 (Yokogawa).

Cloning of *oprC*. We cloned and sequenced *oprC* according to standard procedures (Sambrook *et al.*, 1989). The DNA library of *P. aeruginosa* PAO1 was constructed by ligating *Sau*3AI-digested DNA fragments (4–10 kb) with *Bam*HI-digested pBluescript II SK(+) using *E. coli* XL1-Blue as the host strain (Yoneyama & Nakae, 1991). Transformants were screened using the rabbit anti-OprC antibody (Yoneyama & Nakae, 1991; Yoshihara *et al.*, 1991).

Immunological screening and Western blots. Rabbit anti-OprC antiserum was prepared as described previously (Yoshihara *et al.*, 1991). The antibody was further purified by means of a Sepharose 4B column with immobilized OprC. Immunological screening and Western blotting were carried out as described previously (Yoneyama & Nakae, 1991; Yoshihara *et al.*, 1991).

DNA sequencing and analysis. Nested sets of deletion plasmids were prepared by digesting plasmid DNA with exonuclease III and mung bean nuclease (Yoneyama *et al.*, 1992; Sambrook *et al.*, 1989). Both coding and non-coding strands were sequenced by the dideoxy chain-termination method (Sanger *et al.*, 1977) using BcaBEST (Takara) and Sequenase Version 2.0 (USB). The derived sequence was analysed using TBLASTN software (Altschul *et al.*, 1990), from the NCBI BLAST server, and also TFasta software (Lipman & Pearson, 1985; Pearson & Lipman, 1988) implemented on a FLAT DB e-mail network server.

Restriction map of cloned gene. Restriction maps of DNA from the anti-OprC antibody-positive clones were constructed by digesting recombinant plasmids with one or a combination of several restriction enzymes (Yoneyama *et al.*, 1992).

Purification of OprC and determination of the N-terminal amino acid sequence. Mature OprC was purified as described previously (Yoshihara & Nakae, 1989) with minor modifications. The fraction containing OprC eluted from a DEAE-HPLC column (TSKgel DEAE-5PW, 7.5 × 0.75 cm i.d.) was mixed with a solution containing 2 M (NH₄)₂SO₄ and 0.01 % octaethylene glycol dodecylether (C₁₂E₈). The mixture was applied to a hydrophobic HPLC column (TSKgel Ether-5PW, 7.5 × 0.75 cm i.d.) equilibrated with 100 mM Tris/HCl (pH

7.5), 2 M (NH₄)₂SO₄, 0.01 % C₁₂E₈ and 20 % (v/v) glycerol, and the column was eluted with a linear gradient of (NH₄)₂SO₄ (2–0 M) in 100 mM Tris/HCl (pH 7.5) containing 0.04 % C₁₂E₈ and 20 % glycerol. After dialysis against a large excess of 10 mM Tris/HCl (pH 8) containing 1 mM C₁₂E₈, the OprC preparation was resolved by the SDS-PAGE (10 %, w/v, polyacrylamide) and the protein band was blotted on a PVDF membrane at 1 mA cm⁻² for 45 min (Hirano & Watanabe, 1990). The OprC band was excised and subjected to Edman degradation in a gas-phase sequencer (Model 477A, Applied Biosystems).

Determination of the copper-binding activity of OprC. A Teflon cell comprising two chambers separated by a dialysis membrane (Spectrum Medical Industries) was used for equilibrium dialysis. One chamber was filled with purified OprC (2.9 µM) in a solution containing 5 mM C₁₂E₈, 10 mM NaCl and 10 mM Tris/HCl (pH 7.5). Another chamber contained an appropriate concentration of CuSO₄ dissolved in the same solution. After dialysis by rotation at 16 r.p.m. using a 5-Cell Equilibrium Dialyzer (Spectrum) at 20 °C for 2 h, the Cu²⁺ concentration in both compartments was determined as described below.

Other techniques. Cu²⁺ was quantified according to the method of Hirayama (1976) with some modifications. A sample (50 µl) was placed in a microtube containing 20 µl 1 M ammonium buffer (pH 9.3) and 350 µl water. After the addition of 82.2 µl 0.05 % *o*-aminophenol (Wako) containing 0.005 M sulphuric acid, the mixture was incubated at 23 °C for 30 min, then chilled on ice for 2 min. The oxidized phenoxazine derivative was extracted with 500 µl chloroform and the chloroform phase was dried with about 100 mg Na₂SO₄. Developed colour was determined spectrophotometrically (Shimadzu, Model UV-2200) at 414 nm (path length 1 cm). Protein was quantified by the method of Lowry. SDS-PAGE was carried out according to the method of Laemmli (1970).

RESULTS

Channel activity of OprC

We have previously shown, by the liposome swelling method, that OprC functions as a porin (Yoshihara & Nakae, 1989). To test channel activity using different methodology, we measured the ion conductivity of the protein reconstituted in planar lipid bilayers. When about 1.6 × 10⁻¹⁵ mol of highly purified OprC was added into the *cis* compartment of the chamber filled with 1 M NaCl/2.5 mM HEPES buffer (pH 7.0), stepwise conductance increments were observed (Fig. 1a). A histogram of the conductance showed two discrete peaks at about 200 and 450 pS (Fig. 1b). The channels showed occasional closing with a conductance of about 200 pS (Fig. 1c). These results suggest that the mean single channel conductance of OprC is about 200 pS in 1 M NaCl and that the large conductance values of about 400 pS might be the result of simultaneous insertion of two channel units or because the protein exists in two different conformational states.

Cloning, mapping and nucleotide sequencing of the *oprC* gene

We isolated three anti-OprC antibody-reactive clones by screening a chromosomal DNA library of *P. aeruginosa* PAO1 constructed in *E. coli* XL1-Blue. Restriction maps

Fig. 1. Channel conductivity of *OprC* in a diPhy-PC membrane. (a) Typical conductivity increments in the presence of *OprC*. Purified *OprC* (about 1.6×10^{-15} μ mol) in 5 mM $C_{12}E_8$, 1 mM EDTA, 10 mM Tris/HCl (pH 8) was added to the *cis*-compartment. The final $C_{12}E_8$ concentration was less than 2 μ M and this concentration of $C_{12}E_8$ showed no detectable influence on the conductivity. The applied potential was +50 mV. (b) Distribution of channel conductance. The histogram represents 717 independent channel events. (c) An example of flickering of *OprC* channel.

of the three clones indicated chromosomal DNA inserts of about 5.7, 6.5 and 8 kb, respectively. However, restriction sites over a 4.5 kb region of each of the DNA fragments were common to all three (data not shown). Additionally, a protein band corresponding to *OprC* on an electrophoretogram of total cellular extracts from each transformant was reactive with anti-*OprC* antibody (data not shown). To localize the region containing the *oprC*

Fig. 2. Restriction fragments and Western blot analysis of the products from the genes in the fragments. (a) Restriction fragments derived from pTN100 were subcloned. The hatched box indicates the location of the structural gene for *OprC*. A, *Apal*; H, *HindIII*; K, *KpnI*; P, *PstI*; S, *Sall*; Sc, *SacI*. (b) Western blots of whole cell lysates from *E. coli* XL1-Blue harbouring DNA inserts subcloned into pBluescript II SK(+). Lanes: 1, purified outer membrane of *P. aeruginosa* PAO1; 2–7, *E. coli* XL1-Blue harbouring pTN100 (2), the 2.4 kb *Apal* fragment (3), the 1.1 kb *HindIII* fragment (4), the 4.6 kb *HindIII* fragment (5), the 3.5 kb *PstI* fragment (6) and the 2.2 kb *PstI* fragment (7); 8, *E. coli* XL1-Blue. The amounts of protein applied were 2 μ g for lane 1 and 30 μ g for lanes 2–8. The arrowhead indicates the location of mature *OprC*.

gene, we subcloned *Apal*, *HindIII* and *PstI* fragments into the phagemid vector pBluescript II SK(+) (Fig. 2a). A transformant harbouring a plasmid with an insert of 5.7 kb (pTN100) or a plasmid containing a 4.6 kb *HindIII* fragment produced a single band corresponding to authentic *OprC* as determined by immunoblotting analysis (Fig. 2b, lanes 2 and 5). Transformants harbouring the plasmid containing other fragments showed undetectable or truncated proteins. Since the M_r of *OprC* is about 70000 (Yoshihara & Nakae, 1989), the size of *oprC* was expected to be around 2.1 kb. Therefore, we concluded that the location of the 5' end of *oprC* would be close to

OprC	-----HSQHQ	DHA--VELAP	SVVTGVAQSS	PLTIVTNPKE	PRQPVPAASDG	43
NosA	AESVDESEHA	HHASSAELAP	MVITGVAQOS	PLTVATDPKI	PRQPVPAASDA	50
OprC	ADYLKTIPGF	AVIRNGGSG	DPVLRGMFGS	RLNIETNGGM	MLGACPNRMD	93
NosA	GDYLQTIPGF	SAVRGGGSGS	DPVLRGMFGS	RLKLLANGAE	MLGACPSRMD	100
OprC	APTSIISPET	YDKLTVIKGP	QTVLWGPAS	AGTILFEREP	ERFGELGSRV	143
NosA	SPSSYITPEN	YDALTVIKGP	QTVLWGPNS	AATILLERDP	EDFSELGGRI	150
OprC	NASLLAGSNG	RFDKVLDAAA	GNRLGYLRFT	GNHAQSDDEE	DGAGNTVPSR	193
NosA	DASFLVGS DG	RFDNRIDAAA	GGEQGYIRLL	ANRSDDEEYQ	DGNGDDVHSR	200
OprC	WKKWNGDVAV	GWTPEDETLI	ELTAGKGDGE	ARYAGRGMDG	SQFKRESLGL	243
NosA	WKKWSTDLVL	GWTPEDETLI	ELTVGRGDGE	ARYAGRMMDG	SQFERESVAL	250
OprC	RFVKSNSVDV	LEKVEAQVYY	NYADHIMDNF	RLRTPDPSSM	MPMPASQVD	293
NosA	RFEKTNLGEN	LKKIARVYY	NYADHVMDNY	SLRTP-P----	M-MOMATNVD	295
OprC	RRTLGGRILAA	TWRWDDFKLV	TGVDAMRNEH	RARGSKYDMM	TDIYTDADQF	343
NosA	RRTLGGRMAA	TNQLDEYELV	TGVDAQTNEH	RREGG-----	VDYKSK----	336
OprC	PHSKDAVFHN	YGAFGELTWF	AAERDLIGG	LRLDRASVKD	YRQTLKSGHM	393
NosA	PWEKDAVFHN	YGLFGELTWT	LNDDSRVIGG	ARLDHATAKD	YRSTGPGS---	383
OprC	GHAMANPTAN	DTRADTLPSG	FVRYEHDLD	SPTTLTAGLG	HAERFPDYWE	443
NosA	-----AG	DSRSDNLPSG	FLRYEHDLS	LPATAYVGLG	ETORFPDYWE	425
OprC	LFSPKRGPNQ	SVNAPDKIKP	EKTQLDFGL	QYNGDKLOAN	ASGYVGIVQD	493
NosA	LFS-----G	GADAFKLDP	EKTQLDFGL	QYSKGPLDAN	VSAIVGQVRD	469
OprC	FILFSYREGM	MGSSTQATNV	DARIMGGELG	ASYQLTGNNK	TDASLAYANG	543
NosA	YILFSY----	-SPSKYSENI	DARIMGGELG	ATYRLTSNNK	TDASLAYANG	514
OprC	KNSDDRALP	QIPPLEARFG	LTYEEDWSA	GSLNRVVAPO	NRIARDQGNV	593
NosA	KNSSDGEALP	QMPPLEGRIG	LTYEQDWSA	AGLNRVVAQA	NRVAEGKGNV	564
OprC	VGKDFDKSAG	PGVPSLNGAY	RVTRNVKLSA	GVDNLFDRDY	TEHLNKAGDA	643
NosA	TSKDFDESSG	PGVPSLNGAY	RVNQNFKLST	GIDNLFDRAY	SEHLNQAGNA	614
OprC	GFGFSANSTV	PEPGRIFWTK	VDFSF			668
NosA	GIGLSADERI	NEPGRIWMAR	VDMSF			639

Fig. 3. Alignment of the amino acid sequence of the mature polypeptides of OprC and NosA. The alignment was carried out using the software package GENEWORKS (IntelliGenetics). Gaps were introduced to optimize the alignment. Identical amino acid residues are shaded.

the *Apa*I site located between the *Hind*III and *Pst*I sites, and that the 3' end would be near another *Apa*I site (Fig. 2a).

The coding strand of the 4.6 kb *Hind*III fragment and the anticoding strand of the 2.2 kb and 3.5 kb *Pst*I fragments of pTN100 were sequenced by the dideoxy chain-

termination method. The sequence contains an ORF having 2169 bp encoding 723 amino acid residues. There were three putative RNA polymerase binding sites: a σ^{70} recognition site (Deretic *et al.*, 1989) at nucleotides -311 to -287, an *anr* site (for anaerobic regulation of arginine deiminase and nitrate reduction; Galimand *et al.*, 1991) at -124 to -111, and σ^{54} sites (Deretic *et al.*, 1989) at -90 to -77, -48 to -35 and -44 to -31. A ribosome binding site (GGAaGT) (Toschka *et al.*, 1988) was located at -16 to -11 from the adenine of the initiation codon. A self-complementary sequence without a run of Ts appeared at the end of the sequence, suggesting that this site may act as a ρ -dependent transcription terminator (Lundrigan & Kadner, 1986; Küpper *et al.*, 1978; Rosenberg & Court, 1979).

Amino acid sequence of OprC

The ORF encoded a protein of 723 amino acid residues with a calculated M_r of 79215. To confirm whether the isolated gene encoded authentic OprC, we compared the N-terminal amino acid sequence of purified OprC with that deduced from the nucleotide sequence of *oprC*. The sequence of the OprC protein appeared to be H-S-Q-H-Q-D-X-A-V-E, which was identical to a stretch of amino acids encoded by the ORF (amino acid residues 56–65). Thus, we concluded that the cloned gene encodes OprC. The second in-frame initiation codon with an appropriate 16S rRNA binding site (AtGGA) was found at the fourth amino acid residue. It is not clear at present whether OprC translation starts from either one of these initiation codons or from both sites. However, an extraordinarily long leader sequence is known to exist for the OMP of *P. putida*, the ferric-pseudobactin receptor PupA (Bitter *et al.*, 1991). Mature OprC consists of 668 amino acid residues and the M_r appears to be 73372. The content of hydrophobic and polar amino acids of OprC was similar to that of other OMPs. Moreover, the C-terminal residue of OprC was Phe. These are all common features of channel-forming OMPs in bacteria (Yoneyama *et al.*, 1992; Yamano *et al.*, 1993; Lundrigan & Kadner, 1986; Dean & Poole, 1993; Heller & Kadner, 1985; Lee *et al.*, 1991; Nau & Konisky, 1989; Sauer *et al.*, 1990).

Sequence similarity search

A computer-aided similarity search of the amino acid sequence of mature OprC was performed. The amino acid sequence of OprC showed the highest degree of similarity with NosA of *P. stutzeri*, with 65% sequence identity (Fig. 3). To assess the statistical similarity/dissimilarity between OprC and NosA, we used the shuffling program, RDF2, of Lipman & Pearson (1985) with 100 random shuffles. The degree of similarity between OprC and NosA appeared to be 113.5 of the optimized Z value (in terms of SD above the mean). Other proteins found to be similar to OprC were PfeA, FepA, Cir, FhuE and BtuB with optimized Z values 13.3, 7.4, 6.2, 10.2 and 6.3, respectively. A Z value greater than 10 indicates significant relatedness and that of 6–10 indicates a probable relationship. Therefore, the similarity of OprC with these

(a)		
OprC	DKLTVIKGPQTVLWGPGA	122
NosA	DALTVIKGPQTVLWGPGN	129
PfeA	ERIEVIRGPAAARYGNGA	139
FepA	ERIEVLRGPARARYGNGA	137
Cir	ERIEVVVRGPMSSLYGSDA	123
FhuE	ERVEVVRGATGLMTGTGN	137
BtuB	QRVEYIRGPRSAVYGSDA	122
	* *	
(b)		
OprC	LSAGVDNLFDK	631
NosA	LSTGIDNLFDK	602
PfeA	LGAGVDNLFDK	684
FepA	LTGGVDNLFDK	680
Cir	LRAGVLNLGDK	614
FhuE	LQGNVNNLFDK	667
BtuB	VRGKIANLFDK	571
	** **	

Fig. 4. Similarity of sequences of OprC and the TonB-dependent OMPs. The number of amino acid residues at the end of the similar region of the mature protein is indicated on the right. (a) A well-conserved sequence in the central region; (b) a conserved sequence in the C-terminal region. Multiple alignment was carried out using the molecular evolutionary analysis system *ODEN*, programmed by Y. Ina (National Institute of Genetics, Japan), loaded on a DDBJ computer.

proteins appeared to be statistically significant. The proteins found to be similar to OprC are the TonB-dependent OMPs (Fig. 4). Multiple alignment showed that two glycine residues are conserved in the central region of OprC (Fig. 4a) and 4 amino acid residues, N-L-X-D-K (where X is F or G), are conserved in the C-terminal region (Fig. 4b). The latter 4 amino acid residues were assumed to be the site involved in substrate binding (Lee *et al.*, 1991).

Regulation of OprC expression

Since the amino acid sequence of OprC deduced from the nucleotide sequence showed a high degree of similarity with NosA of *P. stutzeri*, expression of which is regulated by Cu^{2+} , we tested the effect of Cu^{2+} on OprC expression. When the Cu^{2+} concentration in the medium was increased stepwise from 0.1 to 100 μM , synthesis of OprC became clear at 0 and 0.1 μM , but was barely detectable above 1 μM (Fig. 5a). This result contrasts with the expression of NosA in *P. stutzeri* where 11 μM Cu^{2+} completely repressed its synthesis (Lee *et al.*, 1989). To confirm that the faint band that appeared was OprC and not another protein of similar size, we probed the protein band by Western blotting (Fig. 5b). The result clearly showed the presence of OprC. The effect of other divalent cations such as Pb^{2+} , Mn^{2+} , Ni^{2+} , Cd^{2+} , Co^{2+} , Fe^{2+} and Zn^{2+} at 38–90 μM on OprC expression was undetectable (data not shown). Thus the effect of Cu^{2+} on the regulation of OprC synthesis seemed specific. Since *oprC* has a

Fig. 5. Effect of CuSO_4 and KNO_3 on the OMP profile of *P. aeruginosa* PAO1. Cells were grown in the presence or absence of Cu^{2+} and the outer membranes were purified as described in Methods. (a) Purified outer membrane (20 μg protein) was resolved by SDS-PAGE (10% gel). Lanes: 1, without Cu^{2+} ; 2, 0.1 μM CuSO_4 ; 3, 1 μM CuSO_4 ; 4, 10 μM CuSO_4 ; 5, 100 μM CuSO_4 ; 6, 40 mM KNO_3 . The molecular mass markers were myosin (200 kDa), β -galactosidase (116 kDa), albumin (66.3 kDa), aldolase (42.4 kDa) and carbonic anhydrase (30 kDa). (b) Western blot of the purified outer membranes. Lanes: 1, purified OprC; 2, without added Cu^{2+} ; 3, 0.1 μM CuSO_4 ; 4, 1 μM CuSO_4 ; 5, 10 μM CuSO_4 ; 6, 100 μM CuSO_4 ; 7, 40 mM KNO_3 . Amounts of protein applied were about 0.1 μg in lanes 2–6, and 1 μg in lane 7. The arrowhead indicates the location of authentic OprC.

putative *anr* recognition site and because NosA is induced under anaerobic conditions in *P. stutzeri*, we tested the effect of anaerobiosis on the expression of OprC. Fig. 5

Fig. 6. Binding of Cu^{2+} to OprC. Data were from three independent experiments. An experiment in the presence of 100 mM of NaCl showed almost the same result.

shows that OprC was overexpressed under anaerobic culture conditions, confirming a report by Yamano *et al.* (1993).

Binding of Cu^{2+} by OprC

Since OprC expression was regulated by Cu^{2+} (Fig. 5) and OprC has a high degree of similarity with NosA, the copper-binding protein, it was conceivable that OprC binds with Cu^{2+} . We therefore determined the binding affinity of Cu^{2+} to highly purified OprC by the equilibrium dialysis method. The results depicted in Fig. 6 clearly show that OprC bound Cu^{2+} . Scatchard plots showed a K_d value of 2.6 μM and two to three ligand binding sites per molecule. Since the production of OprC was derepressed under anaerobiosis in the presence of nitrate and its amino acid sequence showed a high degree of similarity with NosA, it is highly likely that OprC is also involved in copper utilization by *P. aeruginosa*.

DISCUSSION

OprC was first identified as one of three porins (OprC, OprD2 and OprE1) in the outer membrane of *P. aeruginosa* that allow the diffusion of small hydrophilic solutes with M_r below 350–400 (Yoshihara & Nakae, 1989). To confirm this function, we determined the ion conductivity through the OprC channel by incorporating it into planar lipid bilayers. OprC in 1 M NaCl showed mean conductance of 200 pS and 400–450 pS (Fig. 1). Small and large conductance values were close to those of OprE1 (Obara & Nakae, 1992) and OprD2 (Ishii & Nakae, 1993), respectively, of *P. aeruginosa*. Flickering of the channel was often observed at a conductance 200 pS in 1 M NaCl (Fig. 1c). Therefore, the unit conductance of OprC seemed to be 200 pS. Alternatively, it is possible that OprC can take two conformational states that create two different sizes of channel. Two states of channel size

have been observed in the OMPs LamB of *E. coli* (Benz *et al.*, 1986) and OprD2 of *P. aeruginosa* (Ishii & Nakae, 1993).

We cloned the gene encoding OprC using an anti-OprC antibody. The nucleotide sequence of *oprC* showed an ORF of 2169 bp encoding a polypeptide with 723 amino acid residues. A search of the DNA data libraries found similarity between OprC and several bacterial OMPs. The highest degree of similarity found was with the NosA protein of *P. stutzeri* that is indispensable for the supply of Cu^{2+} to the periplasmic N_2O reductase (Mokhele *et al.*, 1987). NosA-deficient mutants of *P. stutzeri* are unable to grow anaerobically using N_2O as an electron acceptor. A low concentration of Cu^{2+} in the culture medium repressed OprC to a minimum level (Fig. 5). OprC expression was specifically repressed in the presence of Cu^{2+} (data not shown).

It is likely, therefore, that OprC binds Cu^{2+} and conveys the ions to an acceptor protein. We tested the copper-binding affinity of OprC by equilibrium dialysis. We found high affinity binding of OprC with Cu^{2+} *in vitro* with a calculated K_d value of 2.6 μM . These results suggest the involvement of OprC in copper utilization. A question arose as to whether Cu^{2+} affects channel activity. Our preliminary measurement of single channel activity in the presence of Cu^{2+} showed no significant effect.

A question also arises about how the channel-forming activity and the copper-binding property of OprC are coordinated in copper utilization. We assume that the size of the OprC, D2 and E1 channels are large enough to accommodate the free passage of Cu^{2+} across the outer membrane and that Cu^{2+} readily equilibrates in the periplasm. If so, what would be the role of the specific binding of Cu^{2+} to OprC? It is possible that N_2O reductase cannot utilize free Cu^{2+} in the periplasm directly. Instead, N_2O reductase may require an auxiliary protein which binds Cu^{2+} and conveys it to the reductase. We speculate that OprC serves as this auxiliary protein. The presence of such auxiliary binding proteins and their roles are well documented in bacterial chemotaxis and the ATP-dependent active transport system (Adler *et al.*, 1973; Doige & Ames, 1993). This does not mean that OprC may function as a Cu^{2+} -specific channel. In fact, the mutant carrying a defective *oprC* gene, constructed by gene replacement, grew anaerobically in the presence of 20 mM NaNO_2 (data not shown). Under these conditions, the copper-requiring redox protein, azurin, binds with free Cu^{2+} in the periplasm and transfers electrons to nitrite reductase (Zannoni, 1989). This indicates that Cu^{2+} diffuses across the outer membrane even when *oprC* is defective. This result supports the notion that Cu^{2+} equilibrates across the outer membrane readily and is utilized by azurin, indicating that OprC is not essential for copper entry into the periplasmic space. We speculate that a physiological role for OprC under anaerobiosis is to convey periplasmic Cu^{2+} efficiently to N_2O reductase, otherwise it functions as a channel protein.

The sequence of OprC exhibits statistically significant similarity with some OMPs involved in iron uptake in a

ligand-specific manner. FepA functions through protein-protein interaction with the inner membrane energy transducer protein, TonB (Skare *et al.*, 1993). It is intriguing to speculate that OprC conveys Cu^{2+} by interacting with a putative TonB-like protein in *P. aeruginosa*.

There are well characterized copper resistance operon products in *Enterococcus hirae* (Solioz & Odermatt, 1995) and *Pseudomonas syringae* (Cha & Cooksey, 1991). A protein of *P. syringae*, CopB, is an OMP that is induced in the presence of Cu^{2+} . We could not find any similarity between OprC and these copper-resistant proteins in the similarity search analysis. Furthermore, an OprC-deficient mutant showed no difference in copper susceptibility (data not shown). Therefore, the physiological function of OprC is not related to copper susceptibility. This is accordance with the repression of OprC expression in the presence of Cu^{2+} .

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REFERENCES

- Adler, J., Hazelbauer, G. L. & Dahl, M. M. (1973). Chemotaxis toward sugars in *Escherichia coli*. *J Bacteriol* **115**, 824–847.
- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403–410.
- Benz, R., Janko, K., Boos, W. & Läuger, P. (1978). Formation of large, ion-permeable membrane channels by the matrix protein (porin) of *Escherichia coli*. *Biochim Biophys Acta* **511**, 305–319.
- Benz, R., Schmid, A., Nakae, T. & Vos-Scheperkeuter, G. H. (1986). Pore formation by LamB of *Escherichia coli* in lipid bilayer membranes. *J Bacteriol* **165**, 978–986.
- Bitter, W., Marugg, J. D., de Weger, L. A., Tommassen, J. & Weisbeek, P. J. (1991). The ferric-pseudobactin receptor PupA of *Pseudomonas putida* WCS358: homology to TonB-dependent *Escherichia coli* receptors and specificity of the protein. *Mol Microbiol* **5**, 647–655.
- Büscher, K. H., Cullmann, W., Dick, W., Wendt, S. & Opferkuch, W. (1987). Imipenem resistance in *Pseudomonas aeruginosa* is due to diminished expression of outer membrane proteins. *J Infect Dis* **156**, 681–684.
- Cha, J. & Cooksey, D. A. (1991). Copper resistance in *Pseudomonas syringae* mediated by periplasmic and outer membrane proteins. *Proc Natl Acad Sci USA* **88**, 8915–8919.
- Dean, C. R. & Poole, K. (1993). Cloning and characterization of the ferric enterobactin receptor gene (PfeA) of *Pseudomonas aeruginosa*. *J Bacteriol* **175**, 317–324.

- Deretic, V., Konyecsni, W. M., Mohr, C. D., Martin, D. W. & Hibler, N. S. (1989). Common denominators of promoter control in *Pseudomonas* and other bacteria. *Bio/Technology* **7**, 1249–1254.
- Doige, C. & Ames, G. F. L. (1993). ATP-dependent transport systems in bacteria and humans: relevance to cystic fibrosis and multidrug resistance. *Annu Rev Microbiol* **47**, 291–319.
- Galimand, M., Gamper, M., Zimmermann, A. & Haas, D. (1991). Positive FNR-like control of anaerobic arginine degradation and nitrate respiration in *Pseudomonas aeruginosa*. *J Bacteriol* **173**, 1598–1606.
- Heller, K. & Kadner, R. J. (1985). Nucleotide sequence of the gene for the vitamin B12 receptor protein in the outer membrane of *Escherichia coli*. *J Bacteriol* **161**, 904–908.
- Hirano, H. & Watanabe, T. (1990). Microsequencing of proteins electrotransferred onto immobilizing matrices from polyacrylamide gel electrophoresis: application to an insoluble protein. *Electrophoresis* **11**, 573–580.
- Hirayama, H. (1976). Spectrophotometric determination of trace amounts of copper based on its catalytic oxidation of *o*-aminophenol. *J Chem Soc Jpn* **5**, 768–772.
- Ishii, J. & Nakae, T. (1993). Lipopolysaccharide promoted opening of the porin channel. *FEBS Lett* **320**, 251–255.
- Küpper, H., Sekiya, T., Rosenberg, M., Egan, J. & Landy, A. (1978). A rho-dependent termination site in the gene coding for tyrosine tRNA su_3 of *Escherichia coli*. *Nature* **272**, 423–428.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Lee, H. S., Hancock, R. E. W. & Ingraham, J. L. (1989). Properties of a *Pseudomonas stutzeri* outer membrane channel-forming protein (NosA) required for production of copper-containing N_2O reductase. *J Bacteriol* **171**, 2096–2100.
- Lee, H. S., Abdelal, A. H. T., Clark, M. A. & Ingraham, J. L. (1991). Molecular characterization of *nosA*, a *Pseudomonas stutzeri* gene encoding an outer membrane protein required to make copper-containing N_2O reductase. *J Bacteriol* **173**, 5406–5413.
- Lipman, D. J. & Pearson, W. R. (1985). Rapid and sensitive protein similarity searches. *Science* **227**, 1435–1441.
- Lundrigan, M. D. & Kadner, R. J. (1986). Nucleotide sequence of the gene for the ferrienterochelin receptor FepA in *Escherichia coli*: homology among outer membrane receptors that interact with TonB. *J Biol Chem* **261**, 10797–10801.
- Mokhele, K., Tang, Y. J., Clark, M. A. & Ingraham, J. L. (1987). A *Pseudomonas stutzeri* outer membrane protein inserts copper into N_2O reductase. *J Bacteriol* **169**, 5721–5726.
- Nau, C. D. & Konisky, J. (1989). Evolutionary relationship between the TonB-dependent outer membrane transport proteins: nucleotide and amino acid sequences of the *Escherichia coli* Colicin I receptor gene. *J Bacteriol* **171**, 1041–1047.
- Obara, M. & Nakae, T. (1992). Porin of *Pseudomonas aeruginosa* forms low conductance ion channel in planar lipid bilayers. *Biochem Biophys Res Commun* **186**, 645–651.
- Pearson, W. R. & Lipman, D. J. (1988). Improved tools for biological sequence comparison. *Proc Natl Acad Sci USA* **85**, 2444–2448.
- Quinn, J. P., Dudek, E. J., DiVincenzo, C. A., Lucks, D. A. & Lerner, S. A. (1986). Emergence of resistance to imipenem during therapy for *Pseudomonas aeruginosa* infections. *J Infect Dis* **154**, 289–294.
- Rosenberg, M. & Court, D. (1979). Regulatory sequences involved in the promotion and termination of RNA transcription. *Annu Rev Genet* **13**, 319–335.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74**, 5463–5467.
- Satake, S., Yoshihara, E. & Nakae, T. (1990). Diffusion of β -lactam antibiotics through liposome membrane reconstituted from purified porins of the outer membrane of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* **34**, 685–690.
- Sauer, M., Hantke, K. & Braun, V. (1990). Sequence of the *fbuE* outer-membrane receptor gene of *Escherichia coli* K12 and properties of mutants. *Mol Microbiol* **4**, 427–437.
- Skare, J. T., Ahmer, B. M. M., Seachord, C. L., Darveau, R. P. & Postle, K. (1993). Energy transduction between membranes. TonB, a cytoplasmic membrane protein, can be chemically cross-linked *in vivo* to the outer membrane receptor FepA. *J Biol Chem* **268**, 16302–16308.
- Soliz, M. & Odermatt, A. (1995). Copper and silver transport by CopB-ATPase in membrane vesicles of *Enterococcus hirae*. *J Biol Chem* **270**, 9217–9221.
- Toschka, H. Y., Höpfel, P., Ludwig, W., Schleifer, K. H., Ulbrich, N. & Erdmann, V. A. (1988). Complete nucleotide sequence of a 16S ribosomal RNA gene from *Pseudomonas aeruginosa*. *Nucleic Acids Res* **16**, 2348.
- Trias, J. & Nikaido, H. (1990). Outer membrane protein D2 catalyzes facilitated diffusion of carbapenems and penems through the outer membrane of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* **34**, 52–57.
- Yamano, Y., Nishikawa, T. & Komatsu, Y. (1993). Cloning and nucleotide sequence of anaerobically induced porin protein E1 (OprE) of *Pseudomonas aeruginosa* PAO1. *Mol Microbiol* **8**, 993–1004.
- Yoneyama, H. & Nakae, T. (1986). A small diffusion pore in the outer membrane of *Pseudomonas aeruginosa*. *Eur J Biochem* **157**, 33–38.
- Yoneyama, H. & Nakae, T. (1991). Cloning of the protein D2 gene of *Pseudomonas aeruginosa* and its functional expression in the imipenem-resistant host. *FEBS Lett* **283**, 177–179.
- Yoneyama, H., Akatsuka, A. & Nakae, T. (1986). The outer membrane of *Pseudomonas aeruginosa* is a barrier against the penetration of disaccharides. *Biochem Biophys Res Commun* **134**, 106–112.
- Yoneyama, H., Yoshihara, E. & Nakae, T. (1992). Nucleotide sequence of the protein D2 gene of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* **36**, 1791–1793.
- Yoshihara, E. & Nakae, T. (1989). Identification of porins in the outer membrane of *Pseudomonas aeruginosa* that form small diffusion pores. *J Biol Chem* **264**, 6297–6301.
- Yoshihara, E. & Nakae, T. (1992). Separation of gate- and channel-forming domains in the pore-forming protein of the outer membrane of *Pseudomonas aeruginosa*. *FEBS Lett* **306**, 5–8.
- Yoshihara, E., Yoneyama, H. & Nakae, T. (1991). *In vitro* assembly of the functional porin trimer from dissociated monomers in *Pseudomonas aeruginosa*. *J Biol Chem* **266**, 952–957.
- Zannoni, D. (1989). The respiratory chains of pathogenic pseudomonads. *Biochim Biophys Acta* **975**, 299–316.

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