Sequence and relatedness in other bacteria of the *Pseudomonas aeruginosa* oprP gene coding for the phosphate-specific porin P

R. Siehnel,* N. L. Martin and R. E. W. Hancock
Department of Microbiology, University of British Columbia, 300-6174 University Boulevard, Vancouver, B.C., Canada V6T 1W5.

Summary
The oprP gene encoding the *Pseudomonas aeruginosa* phosphate-specific outer membrane porin protein OprP was sequenced. Comparison of the derived amino acid sequence with the known sequences of other bacterial porins demonstrated that OprP could be no better aligned to these porin sequences than it could to the periplasmic phosphate-binding protein PhoS of *Escherichia coli*. Southern hybridization and restriction mapping of the oprP gene in 37 clinical isolates and the 17 serotype strains of *P. aeruginosa* revealed that restriction sites in the vicinity of the oprP gene were highly conserved. Several species from the *Pseudomonas fluorescens* rRNA homology group contained DNA that hybridized to an oprP gene probe.

Introduction
The outer membranes of Gram-negative bacteria constitute a barrier to hydrophilic molecules above a certain size (the exclusion limit; Hancock, 1987). Transmembrane diffusion of molecules smaller than this exclusion limit is mediated by the water-filled channels of proteins termed porins. These porins fit into two functional classes. The majority of the known porins form general diffusion pores that are chemically non-specific, although they may be weakly selective (Hancock, 1987). In contrast, the specific porins contain saturable binding sites for specific solutes. Only three porins are known to fit into this latter class, namely the maltodextrin-specific LamB (Lucky and Nikaido, 1980) and nucleoside-specific Txs proteins of *Escherichia coli* (Maier et al., 1988), and the phosphate-porin, protein P (called here OprP after the gene designation) of *Pseudomonas aeruginosa* (Hancock and Benz, 1986). The gene and derived amino acid sequences of many of the general diffusion porins from several bacteria are now known (Mizuno et al., 1983; Gotschlich et al., 1987; Barlow et al., 1989; Munson and Tolan, 1989). However, the sequence is known for only one of the specific porins, namely LamB (Sauvin et al., 1989). We report here the sequence of the oprP gene for the phosphate-specific porin, OprP.

OprP has been demonstrated to be involved in the phosphate-starvation-inducible high-affinity phosphate-uptake system of *P. aeruginosa*. This phosphate-specific transport system also involves a periplasmic phosphate-binding protein PhoS of *E. coli* (Siehnel et al., 1988b). The equivalent phosphate-regulated porin in *E. coli* is the PhoE porin. Genes for protein components of the PhoE uptake systems form a regulon, whose regulatory mechanism is well conserved between *E. coli* and *P. aeruginosa*. A sequence similar to the well-characterized, cis-acting 'pho-box' consensus sequence has been identified preceding the oprP gene of *P. aeruginosa* and shown to function in *E. coli* (Siehnel et al., 1988b). In addition, genes cloned from *P. aeruginosa* have been shown to complement the phoB and phoR regulatory genes of *E. coli* (Filloux et al., 1988). However, although the regulatory system is well conserved between *E. coli* and *P. aeruginosa*, the phosphate-starvation-inducible porins are functionally different. PhoE forms a general diffusion pore with large (1.1 nm diameter), weakly anion-selective channels (Benz et al., 1984). In contrast, OprP forms constricted (0.6 nm diameter), anion-specific channels that contain a saturable binding site (Hancock and Benz, 1986). Although this binding site can bind a variety of anions (e.g. $K_d$ for Cl$^-$ binding = 40 mM), its affinity for phosphate is at least 100-fold greater ($K_d$ at pH 7 for phosphate = 0.3 mM), demonstrating its substrate-specific character. The OprP phosphate-binding site has been characterized, in part, by chemical modification experiments, and probably involves three lysine residues (one from each monomer of the OprP trimer; Hancock and Benz, 1986). The existence of such a binding site confers a 16-fold increase in the maximal rate of phosphate passage through OprP relative to PhoE at a physiologically significant phosphate concentration (0.15 mM). Because of these substantial differences in function of OprP and PhoE, despite their well-conserved biochemical properties (Worobec et al., 1988) and regulatory systems (Siehnel et al., 1988b), we were very interested in the
Fig. 1. DNA sequence of the oprP gene and predicted amino acid sequence of the OprP protein. The mature amino terminus, preceded by a 29-amino-acid signal sequence, was identified by comparison with the known amino-terminal OprP protein sequence (Worobec et al., 1988). The pho-box, Pribnow (-10) site and Shine-Dalgarno (S.D.) sequence discussed previously (Siehnel et al., 1988b) are indicated upstream of the first codon of the gene. The predicted terminator was identified by the computer program SEQNGE (Delaney Software, Vancouver, B.C., Canada). Restriction sites for common 6-base-cutter enzymes are indicated. These sequence data will appear in the EMBL/GenBank/DDBJ Nucleotide Sequence Data Libraries.
We show here that these two proteins are not very similar, although the oprP gene appears well conserved in \textit{P. aeruginosa} strains and related species.

**Results**

**Sequence of the oprP gene**

The oprP gene was sequenced from both strands, from the HindIII site preceding the previously localized (Siehnel \textit{et al.}, 1988b) pho-box to 153 nucleotides past the stop codon (Fig. 1). The amino terminus of the mature protein started at residue 30 of the derived amino acid sequence, since the next 36 amino acids matched the known N-terminal amino acid sequence of OprP, as described previously (Siehnel \textit{et al.}, 1988b; Worobec \textit{et al.}, 1988). Prior to the start of the mature protein there was a predicted 29-amino-acid signal sequence with features typical of the signal sequences of prokaryotic exported proteins. The mature protein was predicted to be 411 amino acids long and the derived amino acid composition was similar to the known amino acid composition (Table 1). The G+C composition of the oprP gene was 64% overall and 90% in the third position of codons, like that of other \textit{P. aeruginosa} genes, as summarized by West and Iglewski (1988). There was a strong preference (62.6%) for cytosine at the third position and the codon preference for given amino acids was similar to that for other \textit{P. aeruginosa} genes (West and Iglewski, 1988). As demonstrated previously (Siehnel \textit{et al.}, 1988b), the coding sequence was preceded by a typical pho-box and associated consensus Pribnow –10 box. A further regulatory element might be present at the putative 3' start of the mRNA. Between nucleotides 9 and 53, downstream from the end of the –10 site, a 15bp stem-loop structure could be predicted in the putative mRNA. Nine base pairs after the termination codon of the gene, an inverted repeat was observed which predicted a GC-rich stem-loop mRNA structure with a 12bp stem followed by three uracil residues, features typical of a rho-independent terminator.

**Structural features of OprP**

Previous biochemical studies have demonstrated that OprP has many features similar to those of other bacterial porins, notably a high content of $\beta$-sheet (68%), a native trimer structure, resistance to denaturation by heating in sodium dodecyl sulphate, resistance to proteolysis by several enzymes, and an inability of antibodies to the OprP trimer to interact with the native monomer (suggesting the almost exclusive presence of conformational epitopes) (Poole and Hancock, 1986; Worobec \textit{et al.}, 1988). Nevertheless, OprP is only the second specific porin sequenced to date and it has been suggested that it has a structure fundamentally different from that of other porins. Functional studies have indicated a single channel per OprP trimer, whereas other porins have one channel per monomer (Hancock, 1987). Therefore we examined the deduced sequence of OprP for features similar to other porins. One common feature of all porins examined to date is the paucity of long uncharged stretches of amino acids with more than 12 amino acids. \textit{E. coli} porins LamB and PhoE and \textit{Haemophilus influenzae} porin P2 have, respectively, 1, 0 and 1 stretch of 13 uncharged amino acids and no longer stretches. In contrast, between residues 145 and 168 of the mature OprP sequence there was a stretch of 24 consecutive uncharged residues, and three other stretches of 13 or more amino acids are present in the OprP sequence. Three of these four stretches were evident as peaks in the hydropathy plot (Fig. 2). Interestingly, one other porin, protein I of \textit{Neisseria gonorrhoeae}, has been recently shown to have a stretch of 19 consecutive uncharged residues between residues 163 and 181 (Barlow \textit{et al.}, 1989). This stretch could be matched to the long uncharged stretch of OprP (six direct matches, four conservative substitutions). We feel that such a sequence could be important for OprP structural stability (Worobec \textit{et al.}, 1988), forming part of a $\beta$-barrel
structure lining the channel. The strong hydrophobic character of this long, uncharged sequence would permit interaction both with other β-strands and with the membrane interior.

A common feature of porin proteins is the regular alternating peak and trough appearance of their hydropathy plots (Barlow et al., 1989). This feature was evident in the carboxy-terminal half of OprP, but was less evident in the amino-terminal half (Fig. 2).

Extent of conservation of the OprP protein sequence

We compared the predicted amino acid sequences of the OprP protein with the known sequences of other porins (data not shown). This was done using a combination of the computer program SEQUENCE, which employs a FAST-P algorithm, and visual alignment with the assistance of the program, Eyeball Sequence Editor (ESEE) (Table 2). Since the pair-wise alignment of even strongly related porins (e.g. PhoE versus OmpC; Mizuno et al., 1983 or N. gonorrhoeae versus Neisseria meningitidis protein I; Barlow et al., 1989) involved the introduction of nine gaps (Table 2), we allowed up to double this number of gaps. However, while the strongly related porins could be shown to align to a very high degree (i.e. with more than 60% identity and 88% alignment), OprP could be no better aligned to the four other porins tested than it could be to the E. coli periplasmic phosphate-binding protein PhoS.

### Table 2. Degree of alignment of the sequences of P. aeruginosa porin OprP with E. coli porins, PhoE, and LamB, H. influenzae porin P2, N. gonorrhoeae porin IB and E. coli periplasmic phosphate-binding protein PhoS. For comparison the two distinct classes of E. coli porins, the weakly anion-selective PhoE porin and the maltodextrin-specific LamB porin were aligned, as were two closely related E. coli porins PhoE and OmpC and the two closely related Neisseria porins, IB (N. gonorrhoeae) and I (N. meningitidis).

<table>
<thead>
<tr>
<th>Porins compared</th>
<th>Gaps introduced</th>
<th>Direct matches</th>
<th>Conservative substitutions</th>
<th>% alignment</th>
<th>Alignment score</th>
</tr>
</thead>
<tbody>
<tr>
<td>OprP versus PhoS</td>
<td>15</td>
<td>61</td>
<td>125</td>
<td>58</td>
<td>-1.316</td>
</tr>
<tr>
<td>OprP versus PhoE</td>
<td>13</td>
<td>71</td>
<td>94</td>
<td>50</td>
<td>0.764</td>
</tr>
<tr>
<td>OprP versus LamB</td>
<td>17</td>
<td>73</td>
<td>143</td>
<td>53</td>
<td>-1.165</td>
</tr>
<tr>
<td>OprP versus P2</td>
<td>10</td>
<td>57</td>
<td>146</td>
<td>60</td>
<td>0.567</td>
</tr>
<tr>
<td>OprP versus IB</td>
<td>15</td>
<td>70</td>
<td>116</td>
<td>56</td>
<td>1.032</td>
</tr>
<tr>
<td>IB versus OmpC</td>
<td>15</td>
<td>59</td>
<td>142</td>
<td>63</td>
<td>4.114</td>
</tr>
<tr>
<td>PhoE versus LamB</td>
<td>17</td>
<td>69</td>
<td>120</td>
<td>57</td>
<td>-0.720</td>
</tr>
<tr>
<td>PhoE versus OmpC</td>
<td>17</td>
<td>206</td>
<td>86</td>
<td>88</td>
<td>51.779</td>
</tr>
<tr>
<td>IB versus I</td>
<td>9</td>
<td>166</td>
<td>134</td>
<td>91</td>
<td>30.021</td>
</tr>
</tbody>
</table>

a. Sequences were aligned in a linear fashion (i.e. proceeding from the N-terminus to the C-terminus) using the computer program SEQUENCE (Delaney Software, Vancouver, B.C., Canada) and visual alignment with the assistance of the program ESEE (Eric Cabot, Simon Fraser University, Vancouver, Canada) and analysed using ROGENE (Intelligenticns, Inc., Mountain View, CA). Sequences were from the following references: E. coli PhoE, OmpC (Mizuno et al., 1983) and LamB (Sauvin et al., 1989); H. influenzae P2 (Munson and Tolan, 1989); N. gonorrhoeae IB (Gotschlich et al., 1987); N. meningitidis I (Barlow et al., 1989); E. coli PhoS (Surin et al., 1984).
b. Conservative substitutions were assessed by the Dayhoff (1978) minimum mutation matrix with a cut-off score of 0.8.
c. Assessed as direct matches + conservative substitutions divided by the total number of amino acids in the smaller of the two proteins and multiplied by 100.
d. Assessed by the method of Needleman and Wunsch (1970), as implemented by Dayhoff (1978) and Feng et al., 1985 using the genetic code matrix with a gap penalty of 4 and a bias parameter of 0. A value >3.0 suggests significant similarity.
Similarly, PhoE was no more strongly related to OprP than it was to E. coli maltoporin LamB. Alignment scores computed by comparing a matrix alignment of two sequences with 100 trials in which the second sequence was randomly arranged also emphasized the lack of similarity between OprP and any of the other porins tested (Table 2).

To compare genes for which a greater similarity was expected, we examined nucleotide sequence relatedness by Southern hybridization of an oprP gene-specific DNA probe (represented by the sequence between sites PstI and PstI in Fig. 3) to restriction digests of chromosomal DNA from other P. aeruginosa strains. Similar studies with a probe from the upstream region of the P. aeruginosa exotoxin A structural gene showed enormous diversity in the sizes of hybridizing fragments of individual P. aeruginosa strains (Ogle et al., 1987), and a pilin-specific probe also revealed substantial diversity (Pasloske et al., 1988).

In contrast, the restriction patterns of DNA hybridizing to the oprP gene probe were strongly conserved amongst 37 clinical isolates and the 17 type strains of the International Antigen Typing Scheme (IATS). Of the eight sites shown in Fig. 3, three (KpnI, PstI, SalI) were conserved in all tested strains, three (KpnI, PstI, and SalI) were present in all except one of the tested strains, one (PstI) was missing in only two of the 54 tested strains and the most distant (KpnI) was present at the same location in 34 of the 54 strains. Thus, we conclude that the oprP gene is highly conserved. The presence of a second 1.7 kb PstI fragment (PstI to PstI) which hybridized to our oprP probe (PstI to PstI) in 51 of the 54 strains suggested that the P.R. (see below) region, hypothesized to have arisen from the oprP gene by a gene duplication event (Siehnel et al., 1988a), was also present in these strains. (NB: two of the other strains lacked PstI and could not be examined for the P.R. region and the third contained a second PstI fragment with altered size.)

Several other related Pseudomonas species have previously been shown to produce a phosphate-regulated outer membrane protein that cross-reacts immunologically with OprP (using trimer-specific antiserum). Four of these have been purified and shown to be functionally similar to P. aeruginosa OprP (Poole et al., 1987). Therefore, a range of Pseudomonas sp. strains were examined for a gene which hybridized to the oprP gene probe. However, since the oprP gene has been duplicated in P. aeruginosa (Siehnel et al., 1988a; R. Siehnel, unpublished data), and the duplicated gene is not phosphate-starvation-inducible, we could not rule out the possibility that it was this second gene (called the P.R. region) that was present in these species.

Discussion

Previous data has shown that OprP forms a trans-outer membrane channel that is anion-specific and which strongly selects phosphate over other anions. Despite many biochemical similarities to other porins (Worobec et al., 1988), it is functionally different from the equivalent phosphate-starvation-inducible outer membrane porin PhoE of E. coli, which is not selective for phosphate (Benz et al., 1984), or apparently polyphosphate (Rao and Torriani, 1988), and which is only weakly anion-selective (Benz et al., 1984). The basis for this substantial difference in channel properties has been demonstrated to be the presence of a phosphate-binding site in OprP (Kd for
phosphate, pH 7 = 0.3 mM) (Hancock and Benz, 1986). Chemical modification experiments have demonstrated that the phosphate-binding site contains charged lysine side-chains, and experiments with anions of different sizes, as well as symmetry arguments, have been utilized to suggest that OprP contains a single phosphate-binding site per trimer (Hancock and Benz, 1986). In contrast, a recent examination of PhoE using two-dimensional electron crystallographic techniques has suggested three independent channels per trimer (Jap, 1989).

Despite these substantial differences in function, the basic design principles of porins seem to be preserved for OprP. It has a molecular weight in the normal range for porins, comprises more than 60% β-sheet structure and forms SDS-resistant trimers that are both lipopolysaccharide- and peptidoglycan-associated (Worebec et al., 1988). Thus, it was of interest to see the extent of similarity between OprP and other sequenced porins. We attempted to align the OprP sequence with those of other porins (Table 2). However, we could demonstrate no better alignment between OprP and general porins from three different species or the maltodextrin-specific porin LamB from E. coli than we could with the control protein PhoS. In each case we could observe approximately 17–22% identity (after introduction of gaps), a total of 53–60% sequence alignment and consistently low alignment scores (Table 2). The lack of clear homology with PhoE was surprising since the regulatory systems (Filloux et al., 1988) and the DNA sequences on which they act, the pho-box (Siehnel et al., 1988b), are strongly conserved. Furthermore, PhoE and OprP show immunological cross-reaction at the trimer level (but not at the denatured monomer level), suggesting one or more conserved conformational epitopes (Poole and Hancock, 1986). Nevertheless, there was no stretch of identical amino acids longer than four residues which could be aligned between these two proteins.

It should be stated that porins have the unusual property of comprising largely of β-structure, and recent data for OmpF porin suggest that the most predominant secondary structure feature that can be observed is β-strands running almost perpendicular to the plane of the membrane (Kleffel et al., 1985). Thus, tertiary associations between adjacent β-strands in the β-sheet structure will be extremely important in determining structure. Since most structure prediction and alignment formulae consider primary and secondary structure only, it could be argued that such analyses are of little relevance in considering overall porin structure conservation. Nevertheless, the availability of the OprP sequence will be useful in identifying the key lysine residues of the phosphate-binding site and in the eventual solving of the structure of OprP.

In contrast to its lack of conservation in less related species, sequences homologous to the oprP gene were found in several pseudomonads belonging to the P. fluorescens rRNA homology group (to which P. aeruginosa belongs). Furthermore, in 37 clinical isolates and 17 serotype strains of P. aeruginosa there were very few restriction fragment length polymorphisms identified within the gene or in the adjacent DNA. This, in addition to the strong functional conservation of OprP in four of the studied species (Poole et al., 1987), suggests that the oprP gene has been somewhat conserved during the recent evolutionary past.

**Experimental procedures**

**Bacterial strains and media**

*P. aeruginosa* PA01 strain H103 (Hancock and Carey, 1979) was the source of the oprP gene. DNA cloning was performed using *E. coli* strain DH5α F− (BRL, Bethesda, MD). Strains were maintained on Luria broth (Maniatis et al., 1982). Ampicillin was added to 50 µg ml−1 when necessary. Other bacterial strains were received from the American Type Culture Collection (ATCC; Rockville, MD; Table 3).

**DNA sequencing**

Restriction fragments from plasmid pRS-XP, which contains the oprP gene (Siehnel et al., 1988a) were subcloned into the multicloning site of the Gene Scribe Z™ vectors pTZ18R, pTZ18U, pTZ19R or pTZ19U (USB, Cleveland, OH). Single-stranded DNA was prepared by a modification of the procedure of Dente et al. (1983) using the NaCl/polyethylene glycol (PEG) precipitation step of Zinder and Boeke (1982). Both strands of DNA were sequenced using the chain-termination DNA sequencing method (Sanger et al., 1977) with Sequenase™ (USB) enzyme, as described by Tabor and Richardson (1987). When areas of the sequencing gel showed compressions, the template was sequenced again using Taq DNA polymerase (BRL) and the TaqTrack™ Sequencing System (Promega Corp., Madison, WI) with 7-deaza dGTP instead of dGTP at 70°C. All restriction sites used for subcloning were verified as single sites by sequencing across them on larger restriction fragments. The BamHI to SalI fragment was sequenced with the aid of synthetic 23-mer oligonucleotide primers in order to clearly read the sequence past these restriction sites.

**Southern hybridization**

Bacterial chromosomal DNA was isolated by the CTAB (hexa-decytrimethyl ammonium bromide) method described by Wilson (1987). Restriction enzyme-digested DNA was separated on 0.8% agarose gels and transferred to Zeta-Probe nylon membranes (Bio-Rad Laboratories, Richmond, CA) by the method of Reed and Mann (1985). Hybridizations were done by the standard protocol recommended by the supplier at 50°C with washes at 42°C.
Table 3. Interaction of Pseudomonas species DNA with OprP.

<table>
<thead>
<tr>
<th>Source of Partially Digested Chromosomal DNA</th>
<th>rRNA Homology Group</th>
<th>Size of Hybridizing Fragments (kb)</th>
<th>Molecular Weight of Phosphate-Regulated Outer Membrane Protein</th>
<th>Antigenic Cross-reactivity</th>
<th>$K_d$ of Channel for Chloride (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. aeruginosa</em> (PAO1)</td>
<td>I</td>
<td>1.1; 1.7*</td>
<td>48000</td>
<td>++</td>
<td>153*</td>
</tr>
<tr>
<td><em>P. fluorescens</em> (ATCC no. 13525)</td>
<td>I</td>
<td>3.7*</td>
<td>50000</td>
<td>+</td>
<td>220*</td>
</tr>
<tr>
<td><em>P. chlorophosphorica</em> (ATCC no. 9446)</td>
<td>I</td>
<td>2.6*</td>
<td>48000</td>
<td>+</td>
<td>204*</td>
</tr>
<tr>
<td><em>P. putida</em> (ATCC no. 12633)</td>
<td>I</td>
<td>5.5*</td>
<td>192*</td>
<td>+</td>
<td>ND*</td>
</tr>
<tr>
<td><em>P. putida</em> (ATCC no. 12633)</td>
<td>II</td>
<td>2.6*</td>
<td>39000</td>
<td>+</td>
<td>297*</td>
</tr>
<tr>
<td><em>P. aerofaciens</em> (ATCC no. 13985)</td>
<td>II</td>
<td>3.7*</td>
<td>48000</td>
<td>+</td>
<td>ND*</td>
</tr>
<tr>
<td><em>P. chioraphis</em> (ATCC no. 13525)</td>
<td>III</td>
<td>2.6*</td>
<td>45000</td>
<td>+</td>
<td>192*</td>
</tr>
<tr>
<td><em>P. maltophilia</em> (ATCC no. 13637)</td>
<td>V</td>
<td></td>
<td>35000</td>
<td>+</td>
<td>&gt;3000*</td>
</tr>
<tr>
<td><em>E. coli</em> phoE* (DH5aF*)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a. Data from Poole and Hancock (1987).
b. + +, strong cross-reactivity on Western blots; +, visible cross-reactivity; -, no observed cross-reacting protein (Poole and Hancock, 1986).c. Weaker interaction with probe.d. Signifies either no hybridizing fragment (column 3) or no protein present (columns 4, 5 and 6).e. These chloride-binding sites within the channel had at least a 100-fold higher affinity for phosphate (Poole et al., 1987).f. Not determined.g. No chloride- or phosphate-binding site present.

Acknowledgements

This work was performed with the assistance of a grant from the Natural Sciences and Engineering Research Council of Canada. We thank Wendy Woodruff and Suzanne Steinbach for preparing some of the DNA digests and Southern blots.

References


This document is a scanned copy of a printed document. No warranty is given about the accuracy of the copy. Users should refer to the original published version of the material.