Molecular Microbiology (1992) 6(16), 2319–2326

Polyphosphate-selective porin OprO of *Pseudomonas aeruginosa:* expression, purification and sequence

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Summary

The *oprO* gene of *Pseudomonas aeruginosa* codes for a polyphosphate-specific porin and terminates 458 bp upstream of the start codon for the phosphate-specific porin OprP. OprO was found to be expressed only under phosphate-starvation conditions in both wild-type and *oprP*::Tn*501* mutant *P. aeruginosa* strains. However, unlike the rest of the genes of the Pho regulon, including *oprP*, expression of *oprO* required cells to be in the stationary growth phase in addition to phosphate starvation. Wild-type *P. aeruginosa* cells were grown in fermentor culture under these conditions and fractionated by selective solubilization in octylpolyoxyethylene detergent solution. Solubilized OprO was separated from OprP by application to a Mono Q FPLC column and elution with a salt gradient and shown to be functionally identical to cloned OprO produced in *Escherichia coli*. DNA sequencing of *oprO* showed the gene product to be highly homologous to OprP, with 76% identity and 16% conserved substitutions. Most genes of the Pho regulon possess a modified –35 region called the Pho box. Two such elements, separated by 4 bp were found in *oprO*. DNA sequencing also revealed a second Pho box in the *oprP* gene with the same spacing.

Introduction

The outer membranes of Gram-negative bacteria can be considered molecular sieves, the 'holes' of which are due to a class of channel-forming proteins called porins (Hancock, 1987). The porins fall into two classes: non-specific porins which form channels that permit general diffusion of hydrophilic molecules below a certain size, and thus are responsible for the exclusion limit of the outer membrane, and substrate-specific porins that act as facilitated diffusion channels for specific substrates by virtue of having a specific substrate-binding site in their channel (Hancock, 1987). Many non-specific porins are known (Hancock, 1987), but to date only six substrate-specific porins have been defined, namely the maltodextrin-specific porin LamB (Luckey and Nikaido, 1980) and nucleoside-specific porin Tsx (Maier *et al.*, 1988) of *Escherichia coli*, and imipenem/basic amino acid-specific porin OprD (Trias and Nikaido, 1990), phosphate-specific porin OprP (Hancock *et al.*, 1982; Hancock and Benz, 1986), glucose-specific porin OprB (Trias *et al.*, 1988) and recently described polyphosphate-specific porin OprO (Hancock *et al.*, 1992) of *Pseudomonas aeruginosa*.

In the process of cloning the *oprP* gene, we obtained a DNA fragment containing both *oprP* and a neighbouring homologous region (Siehnel *et al.*, 1988a). Insertion of transposon Tn*501* (Poole and Hancock, 1986) into the *oprP* gene (Siehnel *et al.*, 1988a) led to prevention of production of OprP during logarithmic growth under phosphate limitation. Results with the cloned gene in *E. coli* and with *P. aeruginosa* mutants affecting regulation of phosphate-starvation-inducible proteins, indicated that the *oprP* gene was regulated by the PhoB/PhoR two component regulatory system in both bacteria (Siehnel *et al.*, 1988b). Consistent with this observation, *oprP* was observed to have a typical Pho box upstream of the gene (Siehnel *et al.*, 1988b) and the cloned *phoB* and *phoR* genes of *P. aeruginosa* could complement defects in these genes in *E. coli* (Filloux *et al.*, 1988).

The region of DNA adjacent to and upstream of the *oprP* gene (previously called PR but now renamed *oprO*), cross hybridized with *oprP* DNA, had a similar restriction pattern at its amino-terminus encoding end, and when gene-fused to the amino terminus of the *lacZ* gene, produced a protein product that cross-reacted immunologically with OprP (Siehnel *et al.*, 1988a). However given the lack of presence of an OprP cross-reactive band in an *oprP*::Tn*501* insertion mutant growing logarithmically under phosphate-deficient conditions, and the deficiency of this mutant in phosphate transport (Poole and Hancock, 1986a), we reasoned that the putative OprO protein was unlikely to have an important role in phosphate uptake in *P. aeruginosa*. Therefore, we overexpressed OprO in *E. coli* by subcloning the cloned *oprO* gene behind the *lac* promoter, purified it, and studied it in model

Received 3 March, 1992; revised 27 April, 1992; accepted 14 May, 1992.
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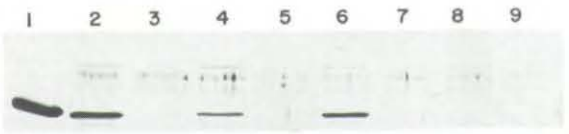

Fig. 1. Interaction of OprP monomer-specific polyclonal antisera with a Western blot of whole cell lysates separated by an 11% SDS–PAGE gel and transferred to nitrocellulose. Only that part of the Western immunoblot corresponding to proteins of 40 kDa or greater molecular mass are shown. Lane 1, a purified OprP; lane 2, phosphate-deficient-medium grown (low Pi), stationary-phase (stat), wild-type strain (H103) cells; lane 3, phosphate-sufficient-medium grown (high Pi), stat, H103; lane 4, low Pi, stat, *oprP*::Tn*501* strain (H576) cells; lane 5, high Pi, stat, H576; lane 6, low Pi, logarithmic-phase-grown (log), H103; lane 7, high Pi, log, H103; lane 8, low Pi, log, H576; lane 9, high Pi, log, H576.

membrane systems (Hancock *et al.*, 1992). To our surprize, the OprO protein had channel properties that were quite analogous but distinct from those of OprP. In particular the OprO channel had a lower affinity and higher capacity for both phosphate and chloride than the OprP channel. However, while its affinity for monophosphate was 10-fold lower, OprO had a 10-fold higher affinity for pyrophosphate than OprP and maintained a high affinity even for tripolyphosphate.

In this paper we have taken this functional information and used it in an attempt to see whether *oprO* is a silent gene or whether, and under what conditions, it is actually produced in *P. aeruginosa*. Furthermore, we have sequenced the gene to permit comparison of sequences with the *oprP* gene.

Results

Expression of OprO in P. aeruginosa

Our earlier finding that OprO formed polyphosphate-selective channels when purified from *E. coli* cells containing the *oprO* gene expressed behind the lac promoter, (Hancock *et al.*, 1992), led us to look for expression of OprO in *P. aeruginosa* under phosphate-deficient growth conditions. Figure 1 shows an immunoblot of such an experiment. The immunological crossreactivity of OprP and OprO (Hancock *et al.*, 1992) permitted use of an OprP-specific antibody in these experiments. Wild-type *P. aeruginosa* strain H103 cells produced a band cross-reactive with OprP in both mid-logarithmic growth phase (OD_{600} = 0.5) and overnight stationary phase (OD_{600} = 1.1) cells grown on phosphate-deficient medium (Fig. 1, lanes 2 and 6). In contrast no such band was observed in strain H103 grown on phosphate-sufficient medium (Fig. 1, lanes 3 and 7). Since OprP and OprO have similar molecular weights (Hancock *et al.*, 1992), an isogenic *oprP*::*Tn501* mutant strain H576 was utilized to permit specific examination of OprO induction. OprO was only observed in the stationary phase of growth (Fig. 1, lane 4) on phosphate deficient medium, but not in the mid-logarithmic phase (Fig. 1, lane 8) or at any phase of growth on phosphate-sufficient medium (Fig. 1, lanes 5 and 9). Thus both phosphate deficiency and stationary phase were required for OprO induction.

To observe if other conditions could give rise to OprO induction, we examined a wide range of growth conditions for strain H576. These included growth with choline chloride under conditions that induce *P. aeruginosa* phospholipase C (Shortridge *et al.*, 1992), substitution of limiting pyrophosphate or tripolyphosphate for orthophosphate, use of gluconate or glycerol as alternative carbon sources, carbon source limitation, higher (41°C) or lower (25°C) growth temperatures, anaerobic growth conditions in the presence of 1% nitrate or O_2-saturating conditions in the fermentor, omission of trace elements from the medium, reduction in medium Fe^{3+} or Mg^{2+} concentrations, N_2 limitation, and inclusion of phosphorylated substrates such as glucose-6-phosphate, glucosamine-6-phosphate or ATP in the medium. Each of these studies was performed under both phosphate sufficient (1 mM phosphate) and deficient (0.1 mM phosphate) conditions. Although small differences in the levels of OprO were observed, the only general conclusion obtained was that OprO was not induced unless cells were grown to stationary phase under phosphate-deficient conditions.

Purification of OprO

To prove that OprO was produced in wild-type *P. aeruginosa* strain H103 under the same conditions as above, cells grown in phosphate-deficient medium to stationary phase, were fractionated by differential solubilization in octyl-POE detergent and the octyl-POE/Tris/EDTA soluble fractions were applied to a Mono Q FPLC column and eluted with a salt gradient. Fractions containing OprP and OprO were pooled and reapplied to the Mono Q column. The resultant chromatogram, after elution with a salt gradient showed four peaks (Fig. 2). Peak A (Fraction 25) contained pure OprO (Fig. 3, lane 2). Peak D (fractions 28 and 29) contained pure OprP (Fig. 3, lanes 8 and 10). The minor peaks B and C contained OprO and OprP in approximately 2:1 and 1:2 ratios, respectively (Fig. 3, lanes 4 and 6). All peaks when run on SDS–polyacrylamide gel electrophoresis after solubilization at room temperature, showed oligomer bands (Fig. 3, lanes 1, 3, 5, 7 and 9), which have been shown, in the case of OprP (Angus *et al.*, 1983), to be trimers. This, together with the ratios of OprO:OprP observed in peaks B and C suggested that peaks B and C, might contain mixed trimers, as observed for other porins; however, this possibility was not confirmed directly.

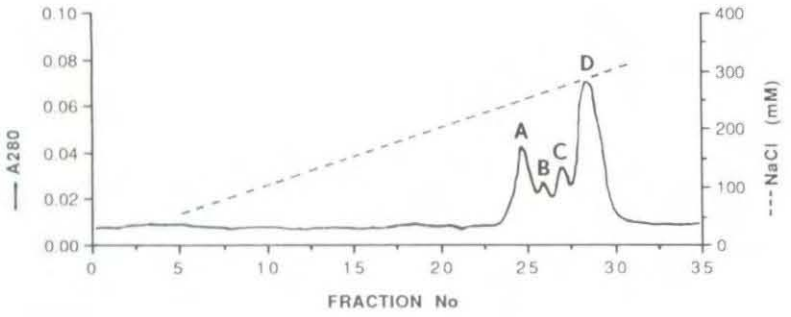

Fig. 2. FPLC chromatography of OprP and OprO. OprP and OprO, selectively solubilized with octyl-POE/Tris/EDTA buffer from outer membrane of H103 were separated on an FPLC Mono Q column as described in the *Experimental procedures*. OprO (Peak A) and OprP (Peak D) were eluted from the column with 250 and 300 mM NaCl in column buffer, respectively.

The identity of the proteins in peaks A and D were confirmed directly by model membrane studies of the purified proteins. Thus OprO, when reconstituted into planar bilayers bathed by 1 M KCl, had a single channel conductance of 510 pS whereas OprP had a single-channel conductance of 260 pS (Fig. 4). Furthermore experiments measuring inhibition of macroscopic Cl^- conductance through OprO channel demonstrated I_{50} values of 2 mM for phosphate, 0.3 mM for pyrophosphate and 1.5 mM for tripolyphosphate, whereas an I_{50} value of 0.5 mM for phosphate was confirmed for OprP. These results were consistent with previous measurements (Hancock and Benz, 1986; Hancock *et al.*, 1992).

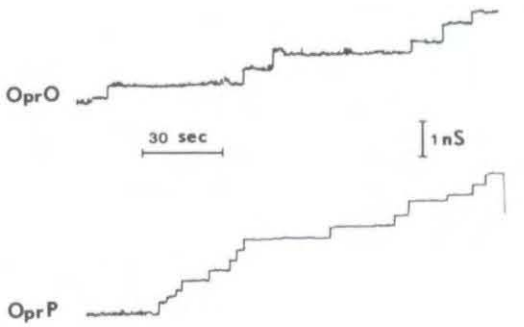

Fig. 4. Analysis of single-channel conductance events due to incorporation of OprO and OprP porins into black lipid membranes. The addition of 40 ng ml^{-1} OprO or OprP resulted in stepwise increases of the membrane current. Lipid membranes were formed from a 1% solution of oxidized cholesterol in *n*-decane, and a voltage of 10 mV was applied. The average single-channel conductance of OprO and OprP was 510 and 260 pS, respectively.

DNA sequence studies

The sequence of OprO and surrounding regions was determined (Fig. 5). The reading frame was determined by previous gene fusion experiments (Siehnel *et al.*, 1988a), in which the amino terminus of *lacZ* was fused to a *Pst* site at nucleotide 550 (Fig. 5) (i.e. amino acid 35 of mature OprO), whereas the boundaries of OprO were implied from previous subcloning experiments (Hancock *et al.*, 1992). The DNA sequence predicted a protein of 438 amino acids which had an amino-terminal 24-amino-acid stretch with features typical of a prokaryotic signal sequence. Thus the predicted sequence of mature OprO was 414 amino acids or just three amino acids longer than OprP (Fig. 6). The predicted sequence of OprO was highly similar to that of OprP (Fig. 6). The mature protein sequences could be aligned with only three single amino acid gaps in OprP such that 74% of the amino acids were identical and a further 16% were similar. This reflected the DNA sequences which demonstrated 79% identity. Of the 275 different nucleotides, 126 were in position three of codons. Despite this, the *oprO* and *oprP* genes had similar G + C contents (62.2% and 64.2%, respectively). The largest difference between the *oprO* and *oprP* coding sequences were in the regions encoding the putative signal sequences. Thus *oprO* had an apparent 15 base pair (three-amino-acid) deletion compared with *oprP*, although the general features of signal sequences were conserved in both. Examination of the non-coding regions of *oprO* revealed a G:C-rich inverted repeat capable of forming a strong stem-loop followed by six thiamine residues, just 29 nucleotides after the end of the OprO coding region. This region thus contained features typical of a rho-independent transcriptional terminator. The OprO and OprP coding regions were separated by a sequence of 458 base pairs.

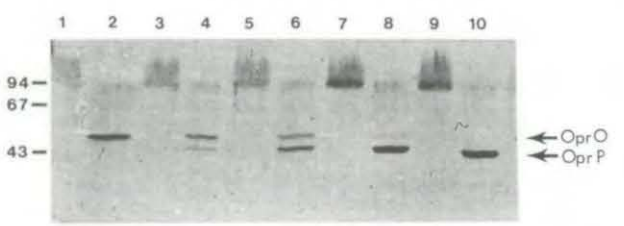

Fig. 3. SDS-PAGE analysis of the FPLC peaks shown in Fig. 2. OprO (Peak A, lanes 1 and 2) and OprP (Peak D, lanes 7, 8, 9 and 10) were separated on a 9% SDS–polyacrylamide gel. Peak B (lanes 3, 4) and Peak C (lanes 5, 6) represent possible heterotrimers of Opr and OprP. Lanes 1, 3, 5, 7 and 9 represent trimeric forms (solubilized at room temperature). Fractions in lanes 2, 4, 6, 8 and 10 were boiled for 10 min prior to electrophoresis and represent the monomeric form of the porins OprO and OprP. Molecular mass markers are given in kilodaltons.

As expected, based on regulation of OprO production

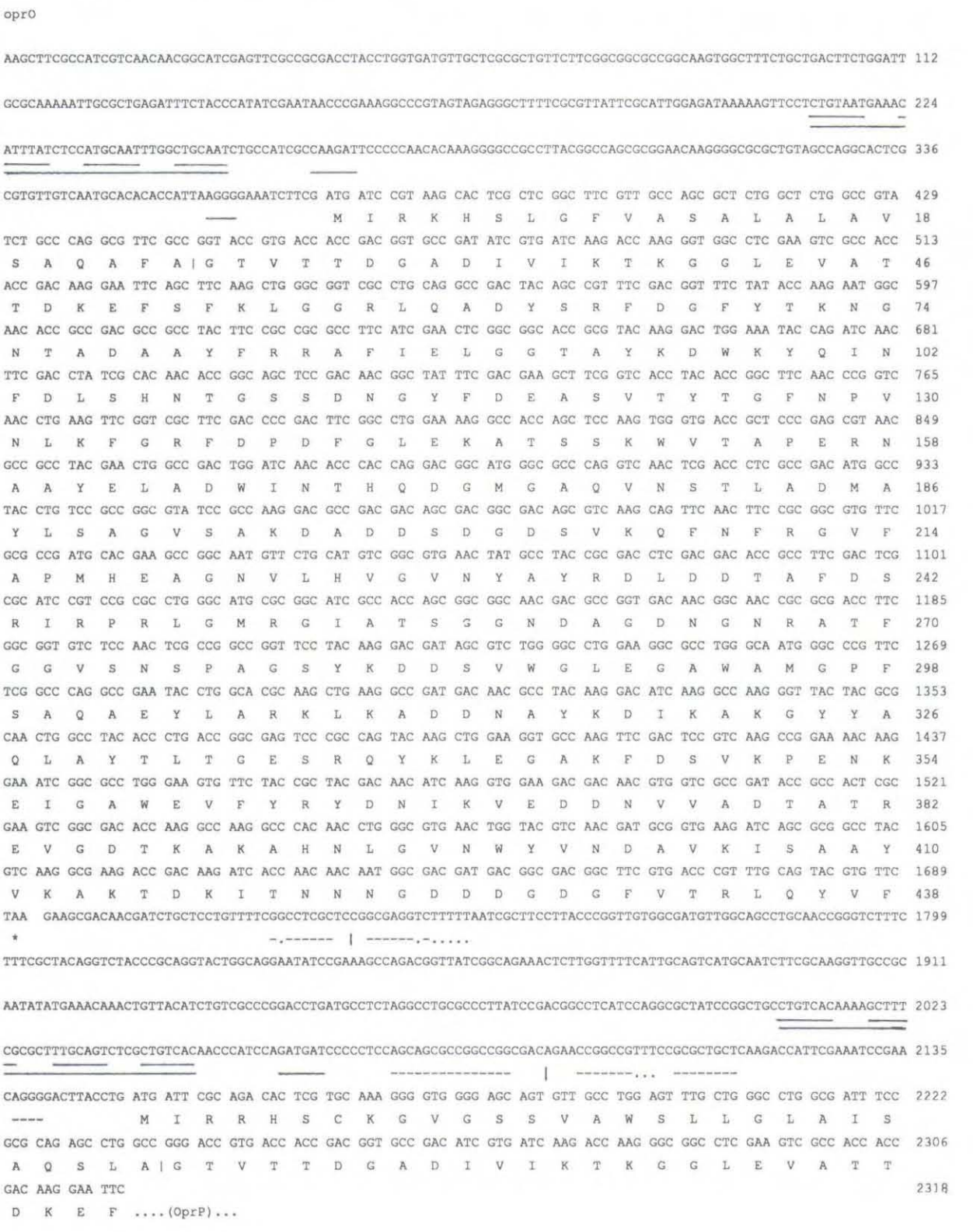

Fig. 5. DNA sequence of the *oprO* gene and predicted amino acid sequence of the OprO protein. The mature *N*-terminus was preceded by a 24-amino-acid signal sequence as delineated by a vertical line. The Pho box, Pribnow (–10) site and Shine–Dalgarno sequences are underlined. The repeated regions in the Pho box are double underlined. Sequences predicted to form stem-loop structure in mRNA are indicated by dashed lines, dots indicate mismatches or poly U tracts. The sequence terminates within the *oprP* gene at an *Eco*RI site. See Siehnel *et al* (1990) for the complete *oprP* sequence. These sequence data (*oprO* and *oprP*) appear in the GenBank data library under accession number M86648.

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OprO  MIRKHSLGFVAS     ALALAVSAQAFAGTVTTDGADIVIKTKGGLEVATTDKEFSFKL 55
      |||.||   |.|      |.||.|||..|||||||||||||||||||||||||||||
OprP  MIRRHSCKGVGSSVAWSLLGLAISAQSLAGTVTTDGADIVIKTKGGLEVATTDKEFSFKL 60

OprO  GGRLQADYSRFDGFYTKNGNTADAAYFRRAFIELGGTAYKDWKYQINFDLSHNTGSSDNG 115
      ||||||||.||||.||.|||||||||||||..|.|||||.|||||||.|||.| |    |
OprP  GGRLQADYGRFDGYYTNNGNTADAAYFRRAYLEFGGTAYRDWKYQINYDLSRNVGNDSAG 120

OprO  YFDEASVTYTGFNPVNLKFGRFDPDFGLEKATSSKWVTAPERNAAYELADWINTHQDGMG 175
      |||||||||||||||||||||  |||||||||||||| ||| .|..|||.| .. | |
OprP  YFDEASVTYTGFNPVNLKFGRFYTDFGLEKATSSKWVTALERNLTYDIADWVN DNVGTG 179

OprO  AQVNSTLADMAYLSAGVSAKDADDSDGDSVKQFNFRGVFAPMHEAGNVLHVGVNYAYRDL 235
        | .| ...||.||..| . . .|.|||.||..|.||||||.||.|||.|.|..|||||
OprP  IQASSVVGGMAFLSGSVFSENNNDTDGDTVKRYNLRGVFAPLHEPGNVVHLGLQYAYRDL 239

OprO  DDTAFDSRIRPRLGMRGIATSGGNDAGDNGNRATFGGVSNSPAGSYKDDSVWGLEGAWAM 295
      .|.| |.|||||.||||..|.|||||| |||| ||| |   | .|||||||||||||.
OprP  EDSAVDTRIRPRMGMRGVSTNGGNDAGSNGNRGLFGGSSAVE GLWKDDSVWGLEGAWAL 298

OprO  GPFSAQAEYLARKLKADDNAYKDIKAKGYYAQLAYTLTGESRQYKLEGAKFDSVKPENKE 355
      |.|||||||| | .||. .   |.|| ||||||||||||.| |||.|||||..||||||
OprP  GAFSAQAEYLRRTVKAERD REDLKASGYYAQLAYTLTGEPRLYKLDGAKFDTIKPENKE 357

OprO  IGAWEVFYRYDNIKVEDDNVVADTATREVGDTKAKAHNLGVNWYVNDAVKISAAYVKAKT 415
      |||||.|||||.|||||||.| |.|||||||.|.|.. |||||| |.|||.|| ||||||
OprP  IGAWELFYRYDSIKVEDDNIVVDSATREVGDAKGKTRALGVNWYANEAVKVSANYVKAKT 417

OprO  DKITNNNGDDDGDGFVTRLQYVF                                      438
      |||.| |||| |||.| ||||||
OprP  DKISNANGDDSGDGLVMRLQYVF                                      440
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Fig. 6. Comparison of the deduced amino acid sequences of OprO and OprP. Vertical lines indicate identity. Dots indicate conservative substitutions determined by the Dayhoff minimum-mutation matrix using a matching score of 0.9 as a cutoff (Dayhoff, 1978). The OprP sequence is from Siehnel *et al* (1990).

by phosphate limitation, the *oprO* gene contained a conserved Pho box region that matched the *E. coli* consensus Pho box and the previously identified *P. aeruginosa* Pho box from *oprP* (Fig. 7). Moreover careful examination revealed that the upstream sequences of *oprO* contained two tandem Pho box regions separated by 4 base pairs whereas the definition of additional *oprP* upstream sequences in this study permitted identification of similar pair of tandem Pho boxes. Exactly 10 nucleotides downstream from the last Pho box upstream of the *oprP* and *oprO* genes was a typical –10 site (Fig. 7). The upstream regions of *oprP* and *oprO* had very high (60–70% based on 50 bp intervals) A + T contents.

Discussion

Previous data have indicated that OprO and OprP have certain functional similarities, in that they are anion specific and contain an anion-binding site within their channels with a much higher affinity for phosphate than chloride ions (Hancock and Benz, 1986; Hancock *et al.*, 1992). Nevertheless, they are also functionally distinct since OprO has a distinctly higher affinity for pyrophosphate than orthophosphate (Hancock *et al.*, 1992); in OprP this is reversed (Hancock and Benz, 1986). Thus OprO, which also binds tripolyphosphate with high affinity (Hancock *et al.*, 1992) forms a polyphosphate-selective channel. Other data showed that OprO, when produced in *E. coli* under the control of the *lac* promoter, was of a similar molecular size and was immunologically cross-reactive with OprP. Previous studies have shown that a *oprP*::Tn*501* insertion mutant of *P. aeruginosa* did not produce an immunologically cross-reactive band (Poole and Hancock, 1986a). This was confirmed here for cells

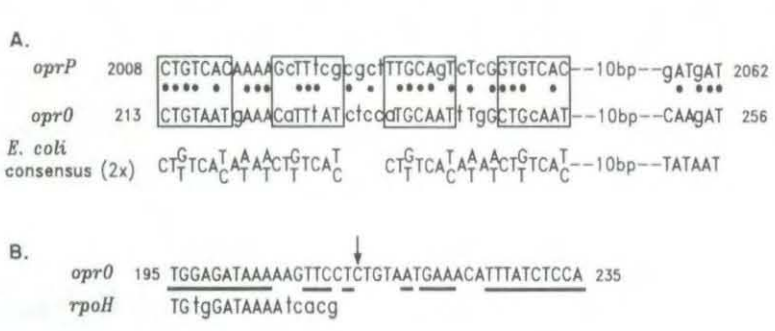

Fig. 7. Putative regulatory elements of the *oprP* and *oprO* genes. Numbers refer to the nucleotide number in Fig. 5. A. Comparison of Pho boxes from *oprO*, *oprP* and the *E. coli* consensus Pho box. Dots indicate matches between the *oprO* and *oprP* Pho boxes. Upper case letters indicate matches with known Pho boxes. B. Inverted repeat in the *oprO* promoter. The arrow indicates the start of the *oprO* Pho box. The lower sequence is the CRP-binding site of the *E. coli* rpoH gene (Nagai *et al.* 1990). Upper case letters in the rpoH sequence indicate homology with the *oprO* inverted repeat.

growing in the logarithmic phase of growth and demonstrated that only in phosphate-deficient media during the stationary phase was OprO observable in the outer membranes. Consistent with this, upstream of the *oprO* coding sequence, a pair of tandem Pho boxes could be observed (Fig. 7). The Pho box is a sequence found in the promoter regions of phosphate-starvation-inducible genes in *E. coli* (Makino *et al.*, 1986) and *P. aeruginosa* (Siehnel *et al.*, 1988a) and is the DNA-binding site for the PhoB activator protein (Makino *et al.*, 1988). The Pho boxes of OprO matched the consensus Pho box of *E. coli* in 14 out of 18 positions and 10 out of 18 positions for the first and second Pho boxes, respectively. If known substitutions in *E. coli* Pho boxes were taken into account (Siehnel *et al.*, 1988b), the matching was 15 and 14 out of 18, respectively (Fig. 7). Furthermore, as demonstrated for all other phosphate-starvation-inducible promoters, there was a conserved –10 site exactly 10 nucleotides after the most downstream Pho box. Interestingly, the *phoE* gene, encoding the phosphate-starvation-inducible porin PhoE also contains two Pho boxes in its promoter region and deletion of one of these reduces gene expression (Thomassen *et al.*, 1987). However, these Pho boxes are inverted relative to one another.

Looking in more detail at the Pho boxes of OprP and OprO there was a consistent pattern of a heptanucleotide motif with a consensus CTGTAAT (cf. *E. coli* CTGTCAT) repeated four times with a spacing of exactly four nucleotides. Interestingly the sequences in the *oprO* Pho box region matched this consensus better than do the equivalent sequences in the *oprP* Pho box region. In contrast OprP expression is more readily induced under phosphate-deficient conditions, whereas OprO induction requires growth into the stationary phase. Presumably some other factor regulates the expression of OprO. An extensive search for the specific factor resulting in OprO induction in cells grown into stationary phase was unsuccessful. However, we noted that there is an inverted repeat region overlapping three of the four heptanucleotide motifs in the Pho box region (Fig. 7). Although we have no direct proof that this region is involved in stationary-phase regulation, the largest portion, a decamer involved in the *oprO* inverted repeat, matches a sequence from the *rpoH* P5 promoter CRP-binding sequence (Nagai *et al.*, 1990). This promoter, which controls transcription of the heat-shock sigma factor, is extremely poorly transcribed in the absence of cAMP/CRP.

Although we cannot be certain about the exact mode of regulation of *oprO*, the induction of OprO under phosphate starvation in the stationary phase seems consistent with its function as a polyphosphate channel (Hancock *et al.*, 1992). *P. aeruginosa* can be found in high abundance in the soil. In many soils phosphate availability is limited. One reason for this is that most microorganisms sequester and store phosphate as polyphosphate (Harold, 1966). Thus it seem reasonable, that along with its arsenal of microbiocidal compounds (Leisinger and Magraff, 1979), *P. aeruginosa* should have a mechanism to sequester polyphosphates released by the action of these compounds. It would be of interest to see if *Pseudomonas* produced a periplasmic polyphosphatase under the same conditions that induce OprO.

The sequences of OprP and OprO were found to be 90% conserved. This is equivalent to the extent of conservation between the porins OmpF, OmpC and PhoE of *E. coli*. However, to obtain this extent of conservation for these *E. coli* proteins, nine or more gaps of varying size must be introduced into these sequences (Mizuno *et al.*, 1983). In contrast only three single amino acid gaps must be introduced into OprP to permit alignment with OprO. Thus the genes for these two seem to have diverged less than other porin-encoding genes, an observation that may reflect the fact that they are not produced under many growth conditions, rather than the time in evolution at which they diverged. In agreement with this proposal, we could find no sequences consistent with an IS-element-induced gene duplication event in the regions surrounding the *oprO* and *oprP* genes.

OprP and OprO form quite similar proteins with similar molecular weights (45232 and 45357, respectively), theoretical isoelectric points (4.79 and 4.68, respectively) and oligomeric structures. Furthermore, although OprO has six more lysine residues than OprP — lysine being the amino acid involved in the phosphate-binding site within the OprP channel (Hancock and Benz, 1986) — every single lysine in OprP is found in an equivalent position in OprO. Thus we assume that the differences in channel properties of these two proteins derives from subtle structural rearrangements. We feel that these two proteins represent an interesting example of molecular evolution.

Experimental procedures

Strains and media

All experiments with *P. aeruginosa* utilized strain H103 and its *oprP*::Tn*501* mutant strain H576 (Poole and Hancock, 1986a). OprO was sequenced using the subclones of plasmid pRS-XP described by Siehnel *et al.* (1988a), cloned into *E. coli* strain DH5α (BRL). For routine induction of OprP and OprO, *P. aeruginosa* was grown in a HEPES-buffered minimal medium containing 0.15 mM potassium phosphate pH 7.2, as described by Hancock *et al.*, (1982).

OprO purification

P. aeruginosa strain H103 cells were grown overnight in a fer-

mentor as described by Worobec *et al.* (1988). Outer membranes were isolated as described previously (Hancock and Carey, 1979) and resuspended at a concentration of 10 mg ml^{-1} in 10 mM Tris-HCl pH 8.0, 0.5% octyl-polyoxyethylene (octyl-POE, Bachem) by sonication. After centrifugation at 150 000 × *g* for 1 h, the pellet was resuspended in 10 mM Tris-HCl, pH 8.0, 3% octyl-POE by sonication. After centrifugation at 150 000 × *g* for 1 h this latter solubilization step followed by centrifugation was repeated. The resulting pellet was then resuspended in 10 mM Tris-HCl, pH 8, 50 mM EDTA, 3% octyl-POE by sonication and centrifuged, and these steps were repeated for the pellet after centrifugation. The supernatants from the octyl-POE/EDTA/Tris-HCl solubilizations were pooled, diluted 1:2 with the 10 mM Tris-HCl, pH 8.0 buffer and loaded onto a Mono Q (HR 5/5) column on a Pharmacia fast protein liquid chromatograph. The protein was eluted by applying a gradient of 0 to 0.4 M NaCl in 10 mM Tris-HCl pH 8.0, 10 mM EDTA, 1% octyl-POE. Fractions containing OprP and OprO were rechromatographed under the same conditions.

DNA sequencing

DNA sequencing was performed using an ABI (Foster City, CA) automated sequencer. Sequencing involved double-stranded DNA templates and either fluorescent-labelled primers or fluorescent-labelled dideoxynucleotides according to ABI protocols. All sequencing was done on both DNA strands and utilized overlapping clones or chromosome walking strategies.

Other techniques

SDS–polyacrylamide gel electrophoresis utilized 11% acrylamide gels as described by Hancock and Carey (1979). Western immunoblotting utilized the technique of Mutharia and Hancock (1985), and anti-OprP antibody was prepared according to the protocol of Poole and Hancock (1986b). Black lipid bilayer methods were done as described previously by Hancock and Benz (1986).

Acknowledgements

This research was supported by funding from the Medical Research Council of Canada. C.E. received postdoctoral support from the Swiss National Science Foundation.

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