Cloning and expression of the phosphotriesterase gene hocA from Pseudomonas monteilii C11

Irene Horne, Tara D. Sutherland, John G. Oakeshott and Robyn J. Russell

The cloning of a gene encoding the novel phosphotriesterase from Pseudomonas monteilii C11, which enabled it to use the organophosphate (OP) coroxon as its sole phosphorus source, is described. The gene, called hocA (hydrolysis of coroxon) consists of 501 bp and encodes a protein of 19 kDa. This protein had no sequence similarity to any proteins in the SWISS-PROT/GenBank databases. When a spectinomycin-resistance cassette was placed in this gene, phosphotriesterase activity was abolished and P. monteilii C11 could no longer grow with coroxon as the sole phosphorus source. Overexpression and purification of HocA as a maltose-binding protein fusion produced a protein having a broad substrate specificity across oxon and thion OPs. Michaelis–Menten kinetics were observed with the oxon OPs, but not with the thion OPs. End-product inhibition was observed for coroxon-hydrolytic activity. Increased expression of hocA was observed from an integrative hocA–lacZ fusion when cultures were grown in the absence of phosphate, suggesting that it might be part of the Pho regulon, but the phosphate-regulated promoter was not cloned in this study. This is believed to be the first study in which a gene required for an organism to grow with OP pesticides as a phosphorus source has been isolated.

Keywords: organophosphate hydrolase, coroxon

INTRODUCTION

Phosphatases and other phosphoester hydrolases are produced by bacteria to cope with phosphate-limiting conditions in the environment. These enzymes collectively hydrolyse a wide range of substrates to obtain phosphate bound covalently in an organic form. They are generally not required under phosphate-rich conditions, and the expression of many is repressed by phosphate in the medium (Wanner, 1983). Furthermore, phosphate can also inhibit enzymic activity (von Tigerstrom & Stelmaschuk, 1986).

Synthetic organophosphates (OPs) are widely used as insecticides. OPs contain three phosphoester linkages, and hydrolysis of any one of the phosphoester bonds dramatically reduces the toxicity of the pesticides by virtue of their inability to inactivate acetylcholinesterase. The OPs can be divided into two groups: those containing a P=O bond (oxon OPs) and those containing a P=S bond (thion OPs). Several enzymes capable of detoxifying OPs have been isolated from micro-organisms capable of using OPs as carbon sources (Dumas et al., 1989; Cheng et al., 1996) and are being evaluated for application in various pesticide bioremediation processes.

The most widely characterized phosphotriesterase enzyme identified is the OPH protein from Flavobacterium sp. ATCC 27551 (Mulbry & Karns, 1989). This bacterial strain is capable of using the OP diazinon (O,O-diethyl O-[6-methyl-2-(1-methylethyl)-4-pyrimidinyl] phosphorothioate) as a carbon source (Sethunathan & Yoshida, 1973). A similar gene was also identified in a Pseudomonas strain capable of using methyl parathion (O,O-dimethyl O-p-nitrophenyl phosphorothioate) as a carbon source (Chaudhry et al., 1988). OPH is a 31 kDa protein that is metal-requiring, with Zn(II) identified in the native enzyme and a reaction mechanism that does not involve a phosphorylated intermediate (Lewis et al.,...
The OPAA (organophosphate acid anhydrolase) proteins from Alteromonas spp. are much larger, at approximately 60 kDa. These proteins are also metalloenzymes [with Mn(II)] and have sequence similarity with prolidases (Cheng et al., 1996). However, OPs have not been reported to serve as a nutrient source for these organisms. Two further OP-hydrolytic enzymes have been identified in Nocardia-related species isolated for their ability to use coumaphos (3-chloro-4-methyl-7-coumarinyl diethyl phosphorothioate) as a carbon source (Shelton & Sornich, 1988). Mulbry (1992) identified a hydrolytic enzyme (AdpB) from a Nocardia strain which possessed very different physical properties to OPH, including the lack of a requirement for metal ions. Mulbry (2000) subsequently identified a phosphotriesterase in the related Nocardioides simplex NRRL B-24074. This enzyme was suggested to be a metalloenzyme, and had a molecular mass of 45 kDa.

Many studies have identified bacterial strains capable of using OPs as phosphorus sources (Cook et al., 1978; Rosenberg & Alexander, 1979), but as yet no genes involved in OP metabolism have been characterized from these organisms. A recent study by Horne et al. (2002b) described the isolation of a Pseudomonas monteilii strain (C11) capable of using the oxon OP coroxon (3-chloro-4-methyl-7-coumarinyl diethyl phosphate) as a phosphorus source (with glucose as a carbon source). P. monteilii C11 appeared to possess a single enzyme capable of hydrolysing coumaphos and coroxon, and this enzyme was associated with the soluble fraction of the cell. OP-hydrolytic activity appeared to be due to a novel phosphotriesterase enzyme that was not a metalloenzyme, and this activity was regulated by the presence of phosphate in the medium. Here, we describe the isolation of this novel phosphotriesterase gene/enzyme system and present a preliminary characterization of its properties.

METHODS

Strains and growth conditions. All strains and plasmids used in this study are listed in Table 1. All cultures were routinely grown with modified LB medium (Harcourt et al., 2002). When grown on minimal media, Pseudomonas strains were grown on the medium of Sutherland et al. (2000) with the modification of Horne et al. (2002b). For the examination of various phosphorus sources (which were added to a final concentration of 100 µM) in the medium, phosphate was omitted from this minimal medium, which was then buffered with Tris/HCl (Horne et al., 2002b). Both Escherichia coli and Pseudomonas strains were grown at 37 °C. When used in the medium for E. coli, ampicillin, kanamycin, tetracycline and trimethoprim were used at concentrations of 100, 25, 10 and 25 µg ml⁻¹, respectively. When included in the medium for Pseudomonas, rifampicin, tetracycline, streptomycin and kanamycin were used at 100, 1, 25 and 25 µg ml⁻¹, respectively.

DNA manipulations. All standard DNA manipulations were carried out according to Sambrook et al. (1989). For PCR detection of insertional mutants, chromosomal DNA was extracted according to the method of Rainey et al. (1992). Isolation of chromosomal DNA from P. monteilii C11, for library construction, was performed according to the method of Gardiner et al. (1996): a size-fractionated genomic library of P. monteilii C11 DNA was constructed. DNA fragments (from a partial EcoRI digestion) in the range 12–20 kb were cloned into the EcoRI site of plasmid pK18 and transformed into E. coli DH10β. Tri-parental matings of the library clones were performed with E. coli JM109 pR751::Tn813 as previously described (Horne et al., 2002a) but with P. aeruginosa BENNY, a Pseudomonas strain that did not have OP-hydrolytic activity, as the recipient and matings were performed on a reduced-phosphate medium (Horne et al., 2002b). Conjugal transfer of plasmids from E. coli S17-1 into Pseudomonas strains was carried out by the method of Bonnett et al. (1995). All PCR reactions were performed using buffer supplied by the manufacturer (Gibco-BRL) and 2 mM MgCl₂. The DNA was denatured at 94 °C for 3 min, after which 0·5 µTag polymerase (Gibco-BRL) was added. Thirty cycles of 94 °C for 35 s, 48 °C for 70 s and 72 °C for 2 min were routinely used, followed by a final extension period of 72 °C for 5 min.

Assays and biochemical techniques. Protein concentrations in crude extracts and pure proteins were determined by previously described methods (Bradford, 1976; Gill & von Hippel, 1989). SDS-PAGE (10%; acrylamide/bisacrylamide 30:1) was performed according to the method of Laemmli (1970). Hydrolysis of coroxon, coumaphos, O₂,O-dimethylumbelliferyl phosphate, 4-methylumbelliferyl phosphate, 4-methylumbelliferyl acetate and 4-methylumbelliferyl β-d-galactopyranoside was measured by examining the formation of fluorescent hydrolysis products (Roth, 1969; Harcourt et al., 2002). Parathion (O,O-diethyl p-nitrophenyl phosphorothioate), paraoxon (O,O-diethyl p-nitrophenyl phosphate), methyl parathion and bis-p-nitrophenyl phosphate hydrolyses were determined colorimetrically by measuring the formation of p-nitrophenol (Dumas et al., 1989). All assays were performed in 50 mM Tris/HCl (pH 7·5) at 25 °C. The effect of EDTA as a metal-chelator was tested by dialysis of pure protein against three changes of EDTA (5 mM) in 50 mM Tris/HCl (pH 7·5). When included in assays, MnSO₄, MgSO₄, ZnCl₂ and CoCl₂ were added to a final concentration of 1 mM. All assays were performed at least twice on two separate samples. Standard errors are displayed throughout the text. Cell density measurements were performed as previously described (Sutherland et al., 2000).

Construction of a plasmid for HocA overexpression and purification. To construct a maltose-binding protein (MBP)–HocA overexpression plasmid, the hocA gene was amplified using PCR. The upstream and downstream oligonucleotide primers, hoc5 (5′-GTCTAAGGATCCATGAAAGAAC-TAAAAACC-3′) and hoc3 (5′-GTCTAAGGATCCAGTCTAA-TCTACC-AATTAGTTTAG-3′) contained, respectively, a BamHI restriction site at the hocA start codon and a HindIII restriction site at the stop codon (underlined bases). The PCR fragment was subsequently cloned into the BamHI–HindIII restriction sites of pMAL-c2X (New England Biolabs) to generate the recombinant plasmid pMALhoc2. The correct sequence of the insert was confirmed. Optimal production of MBP–HocA was obtained when mid-exponential-phase cells (OD₅₈₀ 0·6) were induced with 0·1 mM IPTG for 5 h at 37 °C. Harvested cells were disrupted by sonication and the soluble fraction loaded onto an amylase resin (New England Biolabs) equilibrated with 50 mM Tris/HCl (pH 7·5). MBP–HocA was eluted with 10% maltose in 50 mM Tris/HCl (pH 7·5). The purity of fractions was assessed by SDS-PAGE, and fractions that appeared to be homogeneous were pooled. Approximately 720 µg pure protein was obtained from 500 ml culture.

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**Construction of hocA–lacZ transcriptional fusions.** A 30 kb ClaI–XbaI lacZ-containing fragment from pEM32m (Machowski et al., 2000) was ligated with similarly digested pBluescript DNA to create plasmid pBSlac. The 5′ region of hocA (containing approximately 162 bp upstream of the hocA start codon) and the upstream region were amplified by PCR from pBSRK7(1), using the vector primer T3 (5′-GATGATGACAGAATCC-3′) and the RK7T3r primer (5′-CTGCTAGCAATAGTGGATGATGACAGAATCC-3′), containing an XbaI restriction site at the 5′ end (underlined). This generated a 500 bp PCR fragment that contained an XbaI site at either end, the second generated from vector sequence. This PCR fragment was digested with XbaI and ligated into similarly digested pBSlac, and the correct orientation confirmed by digestion with PstI to produce a 3·2 kb fragment and a 2·9 kb fragment. This plasmid was called pC11lac. A 6·9 kb HindIII fragment containing the mobilization genes (mob) and tetracycline-resistance cassette from pR459II was ligated into pC11lac to create the integrative lacZ fusion plasmid, pC11lacmob. A replicative transcriptional hocA–lacZ fusion was also constructed. A 3·5 kb PstI fragment from pC11lac, containing the 5′ region of hocA with lacZYA, was cloned into the broad-host-range plasmid pDSK519, to create pDSKCIlac. Both integrative and replicative lacZ fusion vectors were transferred into P. monteilii C11 using E. coli S17-1.

**Construction of a defective HocA mutant.** The 5′ PSI fragment generated by amplification (see above) using the T3

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**Table 1. Strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics*</th>
<th>Source or reference</th>
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<td><strong>E. coli strains</strong></td>
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<td>Strain for α-complementation cloning</td>
<td>Gibco-BRL</td>
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<td>Mobilizing strain; carries chromosomally integrated derivative of RP4</td>
<td>Simon et al. (1983)</td>
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<td>S17-1 Δpir</td>
<td>S17-1 lysogenized with Δpir</td>
<td>Penfold &amp; Pemberton (1992)</td>
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<td>JM109 λ</td>
<td>Host for α-complementation of cloning vectors (lysogenized with phage λ carrying the pir gene; allows for replication of R6K-based suicide vectors)</td>
<td>Penfold &amp; Pemberton (1992)</td>
</tr>
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<td><strong>Pseudomonas strains</strong></td>
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<td>Horne et al. (2002b)</td>
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<td>P. monteilii C11lac</td>
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<td>This study</td>
</tr>
<tr>
<td>P. aeruginosa BENNY</td>
<td>Wild-type strain</td>
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<td><strong>Plasmids</strong></td>
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<td>Cloning vector, KnR</td>
<td>W. Klipp</td>
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<td>pK18 + 12 kb P. monteilii C11 DNA in opposite orientation to pK16</td>
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<td>pBSlac + 500 bp of hocA with upstream region</td>
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<td>pBSK+ with tandemly repeated double polylinker with ΩSm/Sp</td>
<td>Eraso &amp; Kaplan (1994)</td>
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<td>Conjugative, co-integrative broad-host-range plasmid</td>
<td>Bowen &amp; Pemberton (1985)</td>
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<td>Broad-host-range plasmid, KnR</td>
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<td>Promega</td>
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<td>800 bp PCR fragment in pGEMT Easy</td>
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<td>pGhocA3 + 5</td>
<td>800 bp SpeI fragment from pGhocA3(3) in pGhocA5</td>
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</table>

* Abbreviations: Ap, ampicillin; Cor, coroxon-hydrolytic activity; Kn, kanamycin; mob, mobilization genes; ΩSpR, spectinomycin-resistant cassette bordered by transcription terminators; R, resistant; Tc, tetracycline.
primer and RK7T3f (containing an XhoI restriction site at the 5’ end), and using pBSRK7(1) as a template was ligated into pGEM-T Easy (Promega) to generate the plasmid pGhocA5. A 3’ region of hocA was also amplified by PCR with pBSRK7(1) as a template, using the primers RK7T3f (5’-GAACCTGAAC-AATCAGCCTTA-3’) and the T7 vector primer (5’-TAATA-GCAGTCTACTATAGGGAGA-3’). This generated an 800 bp fragment that was cloned into pGEM-T Easy to create pGhoc3(3). Digestion of pGhoc3(3) with SpeI released the 800 bp PCR fragment, which was then ligated with SpeI-digested pGhocA5. Transformants were screened for correct orientation by a positive PCR amplification using the hoc3 and hoc5 primers. One was chosen and designated pGhocA5+3. A 1.2 kb BamHI fragment from pGhocA5+3 that contained both the 5’ and 3’ fragments and only one XhoI site (between the 5’ and 3’ fragments) was ligated with similarly digested pJP5603, to create pJPhoc. The streptomycin-resistance cassette from pUI1188Sp was cloned into the unique XhoI site in pJPhoc to create pJPhocSp, which was transferred into P. monteilii C11 using E. coli S17-1 [pir]; kanamycin-sensitive, streptomycin-resistant exconjugants were selected. One exconjugant was chosen and shown by PCR to lack an intact hocA gene. Rather than the 0.5 kb PCR product produced for the wild-type strain (C11), a PCR fragment of 2 kb was obtained, demonstrating the insertion of the 2 kb streptomycin-resistance cassette in hocA. The PCR was performed using the hoc3 and hoc5 primers.

Chemicals. Methyl parathion and parathion were obtained from Riedel-de Haan. Coumaphos (3-chloro-4-methyl-7-coumarinyl diethyl phosphorothioate) was a gift from Bayer. Coroxon was purchased from Alltech. Paraoxon, bis-p-nitrophenyl phosphate, 4-methylumbelliferyl phosphate, 4-methylumbelliferyl β-D-galactopyranoside were obtained from Sigma. O,O-Dimethyl 4-methylumbelliferyl phosphate was synthesized by Professor Alan Devonshire (A. L. Devonshire and others, unpublished).

RESULTS

Construction of a genomic library and subcloning of the OP-hydrolytic gene

It was not known whether the phosphotriesterase gene would be expressed adequately in E. coli to detect enzymic activity in library clones. In a previous study, an OP-degrading gene was successfully isolated from an Agrobacterium strain by mobilizing a genomic library into a related strain lacking activity (Horne et al., 2002a). A similar approach was taken here, using Pseudomonas aeruginosa BENNY, a strain previously isolated in our laboratory and unable to hydrolyse OPs. The library of P. monteilii C11 genomic DNA, constructed in pK18 in E. coli DH10β, was transferred to P. aeruginosa BENNY in a tri-parental mating procedure. Approximately 500 transformants were transferred. Matings were assayed for coumaphos-hydrolytic activity, yielding two resultant positive clones (pK16 and pK265), which were shown by restriction enzyme analysis (using EcoRI, BamHI, HindIII and KpnI) to be identical. Since neither of these clones conferred detectable coumaphos-hydrolytic activity on E. coli DH10β, all subcloning was performed in the broad-host-range plasmid pRK415 and transferred via E. coli S17-1 into P. aeruginosa BENNY. A subclone containing a 1.2 kb BamHI fragment (Fig. 1) was obtained that conferred coumaphos-hydrolytic activity on P. aeruginosa BENNY [820 ± 7 pmol min⁻¹ (mg protein)⁻¹] and 13-fold less activity on E. coli DH10β when expressed from the lacZ promoter [62 ± 2 pmol min⁻¹ (mg protein)⁻¹].

The sequence of the 1.2 kb EcoRI–BamHI fragment contains one ORF

Restriction mapping demonstrated that the 1.2 kb BamHI fragment was contained at one end of the 12 kb EcoRI fragment. Sequence from the opposite end, and of other subclones of the EcoRI fragment, had similarity to a segment of the P. aeruginosa PAO1 genome (83:5 %; Stover et al., 2000). The sequence of the 1.2 kb BamHI genome and contained a single ORF of 504 bp, revealed using the GenScan program in Bionavigator (Burge & Karlin, 1997). This ORF was preceded by a ribosome-binding site (AGGAG; 7 bp upstream of the putative ATG start codon) and, in constructs that produced coumaphos-hydrolytic activity in E. coli DH10β, this gene was in the same orientation as the lacZ promoter. This ORF was designated hocA (hydrolysis of coroxon). The GenBank accession number for the hocA gene is AF469117. HocA is predicted to be 19 kDa and composed of 167 amino acids. The protein has a predicted pl of 6.50, and a hydrophathy plot of this protein by the method of Kyte & Doolittle (1982) revealed no obvious membrane-spanning domains, indicating that it is likely to be a soluble protein, probably contained in the cytoplasm. This is consistent with the previous finding of Horne et al. (2002b), i.e. that OP-hydrolytic activity in P. monteilii C11 is located in the soluble fraction of the cell.
The HocA protein demonstrated no significant sequence similarity to any of the previously identified OP-hydrolytic enzymes, OPH/OpdA (GenBank accession numbers M20392 and AY043245, respectively), OPAA-2 (GenBank accession number U29240) or AdpB (GenBank accession number M91040). Some sequence similarity was observed with a putative cytoplasmic protein from Salmonella typhimurium LT2 that was identified in the genome sequencing project (GenBank accession number AE008708). This putative protein showed 41% sequence identity at the amino acid level to HocA over the entire protein, and 59% sequence similarity. The function of this putative Salmonella protein is not known. No other strong sequence similarity to any other proteins in the GenBank, PDB, EMBL, SWISS-PROT and Prosite databases were observed using the BLASTP program. However, some similarity (43%) was seen with isocitrate dehydrogenase from Methanococcus jannaschii (Bult et al., 1996) over the entire protein (23% identity), while the central third showed 60% similarity (36% identity) with phospholipase C from Arabidopsis thaliana (Sato et al., 2000). Both of these proteins have some involvement with phosphate. Phosphatidyl-inositol-specific phospholipase C catalyses the cleavage of the membrane lipid, phosphatidylinositol or its phosphorylated derivatives, to produce diacylglycerol (Griffith & Ryan, 1999). Isocitrate dehydrogenase is an enzyme that is regulated by phosphorylation of a serine residue in its active site (Hurley et al., 1990). Key residues in isocitrate dehydrogenase and phospholipase C that bind phosphate are not conserved in HocA, nor are key catalytic residues. It is likely that these sequence similarities have no functional significance. No conserved domains or motifs were identified in this protein using either EMBL or Prosite, and so this appears to be a novel protein with no obvious homologues.

P. monteilii HOC, which contains an insertional mutation in hocA, showed limited phosphotriesterase activity with coroxon as a substrate (16 ± 0.3% activity of the wild-type). Furthermore, P. monteilii HOC was unable to grow with coroxon as a phosphorus source. This confirms that HocA is a protein required for P. monteilii C11 to grow with coroxon as a phosphorus source.

**Purification and substrate specificity of HocA**

To confirm that hocA did indeed encode a phosphotriesterase, the enzyme activity of an MBP fusion was examined. The purified protein possessed phosphotriesterase activity against the substrates coumaphos, coroxon, O,O-dimethylumbelliferyl phosphate, parathion, and methyl parathion. The kinetics of hydrolysis against the oxon OPs coroxon, parathion and methyl parathion were shown in Fig. 2a. Reactions with the thion OPs did not appear to follow Michaelis–Menten kinetics (Fig. 3). This may be due to the $K_m$ of the enzyme being quite low, and the substrate concentrations tested being greater than the $K_m$. Specific activities for these substrates are approximately 30–50% of the activity with oxon OPs at the same substrate concentration (Fig. 2b). While it is possible that fusion with MBP interfered with these activities, the activity of untagged HocA expressed from the lacZ promoter in E. coli DH10β (pBSRK7(1)) demonstrated the same $K_m$ for coroxon as for the fusion protein, so we find this unlikely. In a previous study, the
Diethyl phosphate, the hydrolysis product of coroxon, specifically inhibited coroxon-hydrolytic activity (Fig. 4). While the $K_m$ values showed little difference, the $V_{max}$ values decreased with increased concentrations of diethyl phosphate. This demonstrated that diethyl phosphate produced a non-competitive inhibition with respect to coroxon, and the $K_i$ was determined to be 67 $\mu$M. Interestingly, this is approximately the same as the $K_m$ for the protein for coroxon, suggesting that HocA has as much affinity for the hydrolysis product as it does for coroxon. No inhibition was observed with inorganic phosphate.

The effect of metal-chelators on the coroxon-hydrolytic activity of HocA was examined because most of the previously described phosphotriesterase enzymes appear to be metalloenzymes. Extensive dialysis of MBP–HocA against EDTA had no effect on coroxon-hydrolytic activity. Nor did the metal ions Co(II), Zn(II), Mg(II) or Mn(II) affect coroxon-hydrolytic activity when added directly to the assay mixture.

### Phosphate levels regulate hocA expression

Previously, coumaphos-hydrolytic activity in *P. monteilii* C11 appeared to be regulated by phosphate levels (Horne et al., 2002b). In many organisms, including the pseudomonads, genes of the Pho regulon are either induced or repressed by the PhoP-R sensor–regulator pair upon phosphate starvation. The PhoR protein binds to a distinctive segment of DNA (5’-TTGCAGT-CTCGCTGTCACAA-3’). The region contained in the 1.2 kb EcoRI–BamHI fragment did not possess any obvious promoter region or regulatory DNA sequence such as the Pho DNA-binding sequence. To examine the regulation of hocA expression, both an integrative and a replicative lacZ fusion were constructed. With the integrated hocA–lacZ fusion, transcription from upstream regions not contained in the 1.2 kb EcoRI–BamHI fragment can contribute to hocA expression (and therefore $\beta$-galactosidase expression). The $\beta$-galactosidase activity from this fusion was examined in media containing either excess phosphate or 4-methylumbelliferyl phosphate (limited phosphate). The $\beta$-galactosidase activity in cells grown without phosphate was twice that of cells grown with phosphate ($109.4 \pm 1.3$ nmol min$^{-1}$ mg$^{-1}$ compared with $51.7 \pm 0.2$ nmol min$^{-1}$ mg$^{-1}$). This suggests that expression of the hocA gene is regulated by phosphate levels in the medium.

A replicative lacZ fusion (pDSKC11lac) was used to determine whether the 1.2 kb EcoRI–BamHI fragment contained immediate upstream sequences involved in phosphate regulation. Greater $\beta$-galactosidase activity was observed from this construct in *P. monteilii* C11 compared with the integrative hocA–lacZ fusion, presumably because of a copy-number effect. Therefore, no overall comparison can be made between the integrative and replicative lacZ fusions with respect to the extent to which upstream promoters contribute to overall hocA expression. However, it is clear that the replicative plasmid conveyed identical $\beta$-galactosidase activity in cells grown with either phosphate (141.9 $\pm$

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**Fig. 3.** Reaction rates of HocA against coumaphos, a thion OP, with increasing substrate concentration, demonstrating that Michaelis–Menten kinetics are not observed.

**Fig. 4.** Lineweaver–Burk plots for coroxon-hydrolytic activity in the presence of increasing amounts of diethyl phosphate (DEP).
7.7 nmol min⁻¹ mg⁻¹) or 4-methylumbelliferyl phosphate (142.2 ± 3.8 nmol min⁻¹ mg⁻¹). This suggests that regions involved in phosphate regulation are contained on DNA sequences much further upstream of hocA than those cloned in this study. The hocA gene may be contained in a much larger operon that is regulated by phosphate. Alternatively, the regulatory region is much further upstream. For example, the ‘Pho box’ is almost 600 bp upstream of the phosphate-regulated phoA gene of P. aeruginosa (Stover et al., 2000). It was not possible to identify such upstream regulatory regions in this study, as the cloned fragment containing the hocA gene contained only 162 bp of sequence upstream of the start codon.

**DISCUSSION**

A gene (hocA) was isolated from *P. monteilii* C11; its product is required for this strain to grow with coroxon as a phosphorus source. The hocA gene appears to be contained on the chromosome of *P. monteilii* C11, as sequences of subclones of the 12 kb EcoRI fragment containing hocA had sequence similarity to sequences on the *P. aeruginosa* genome. A hocA homologue was not identified on the *P. aeruginosa* genome, and the *P. aeruginosa* strain BENNY did not demonstrate phosphotriesterase activity. This gene is either unique to *P. monteilii*, and possibly some other *Pseudomonas* strains, or was acquired via lateral gene transfer. The 1.2 kb BamHI fragment containing hocA has a 46.9 mol% G + C content, in contrast to the 66.6 mol% G + C content of the *P. aeruginosa* genome, which is typical of pseudomonads (Stover et al., 2000). Differences in G + C contents have been suggested to imply lateral gene transfers (Folkesson et al., 1999). *Pseudomonas* strains have been frequently reported to have acquired genes via lateral gene transfer (Sinclair et al., 1986; Williams & Murray, 1974) among soil microbial communities. This may be the case here.

The expression of hocA in *P. monteilii* C11 was regulated by phosphate levels. While this suggests a role for HocA in phosphate metabolism, it is highly unlikely that the native role is to hydrolyse phosphotriesters, because OPs have only been in existence for the last 50 years. HocA may have evolved from a pre-existing phosphatase or phosphodiesterase as it has been shown that a phosphotriesterase (OPH) could be altered by only one amino acid to possess phosphodiesterase activity (Shim et al., 1998), demonstrating how a minor change can allow a protein to acquire a new activity. Likewise, HocA may have been a pre-existing phosphodiesterase that has acquired phosphotriesterase activity.

It is common for phosphatases and phosphodiesterases to be inhibited by their phosphorus-containing hydrolysis product (von Tigerstrom & Stelmachuk, 1986). End-product inhibition of HocA activity was observed by the phosphorus-containing moiety. The severe end-product inhibition of HocA by diethyl phosphate probably contributed to the lack of parathion/paraoxon hydrolysis by *P. monteilii* C11 observed in our previous study (Horne et al., 2002b). Furthermore, the activity is quite low for these substrates and may not be seen until the protein is in sufficient concentrations, as seen here with purified enzyme. The unusual kinetics observed for HocA with the thion OPs may be a result of end-product inhibition by the phosphorothioates, diethyl thiophosphate and dimethyl thiophosphate, the hydrolysis products of coumaphos and methyl parathion, respectively. Alternatively, the enzyme has a low *Kₘ* for the thion OPs, and substrates were tested at concentrations far exceeding the *Kₘ* of the enzyme. We find this unlikely, as this would be the first report of a phosphotriesterase having an extremely low *Kₘ* for thion OPs relative to the oxon versions. In general, the reverse is true and the enzymes have a higher affinity and catalytic activity for the naturally occurring P = O-containing OPs rather than the bigger, unnatural P = S-containing OPs.

*P. monteilii* C11 was isolated by virtue of its ability to utilize OPs as a source of phosphorus. Phosphotriesterase activity is the first step in obtaining phosphate from coroxon. The doubling time of *P. monteilii* C11 was not increased when inorganic phosphate, rather than coroxon, was provided as the sole source of phosphorus (data not shown), suggesting that phosphotriesterase activity is not a rate-limiting step in providing inorganic phosphate for *P. monteilii* C11. This implies that there is no requirement for a kinetically better phosphotriesterase in this organism. HocA (the only enzyme in this organism responsible for phosphotriesterase activity) is less efficient at hydrolysing OPs than are other phosphotriesterases isolated from organisms capable of using OPs as a carbon source. In general, phosphotriesterase activity would be the first step in the use of OPs as a carbon source. The OPH enzyme of *Flavobacterium* sp. ATCC 27551 can hydrolyse paraoxon at rates close to the limits of diffusion. We find it unlikely that the kinetics of HocA would be able to support growth of an organism with OPs as a carbon source. Bacteria require much higher levels of carbon than phosphorus for growth. Stanier et al. (1986) suggested that a *Pseudomonas putida* strain required 7 mM carbon, whereas 100-fold less phosphorus is needed for growth; therefore an enzyme that is providing the only source of phosphorus can be 100-fold less active than an enzyme providing the only source of carbon. In accordance with this, the *kₗ* values of OPH for both coroxon and paraoxon appear to be 100-fold higher than those of HocA (based on the results of Horne et al., 2002a). We propose that the isolation of enzymes from organisms using OPs as carbon sources rather than phosphorus sources potentially yields more efficient enzymes.

The kinetics of the OPH/OpdA proteins suggest that they are far better suited to bioremediation than HocA. However, HocA does not require a metal ion. It might, therefore, be better suited than OpdA for bioremediation in certain environments such as heavily polluted waters containing inhibitory metals or metal-chelating agents. It might be possible to improve HocA by *in vitro*
evolution (MacBeath et al., 1998) for better kinetics and reduced end-product inhibition.

ACKNOWLEDGEMENTS

We would like to thank Michelle Williams for technical assistance in this study. This research was supported by Orica Australia Ltd and Horticulture Australia Ltd (HG97034).

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Received 28 January 2002; revised 3 May 2002; accepted 21 May 2002.