Expression of the phosphonoalanine-degradative gene cluster from *Variovorax* sp. Pal2 is induced by growth on phosphonoalanine and phosphonopyruvate

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Abstract
The phosphonopyruvate hydrolase (PalA) found in *Variovorax* sp., Pal2, is a novel carbon–phosphorus bond cleavage enzyme, which is expressed even in the presence of high levels of phosphate, thus permitting phosphonopyruvate to be used as the sole carbon and energy source. Analysis of the regions adjacent to the palA gene revealed the presence of the five structural genes that constitute the 2-amino-3-phosphonopropionic acid (phosphonoalanine)-degradative operon. Reverse transcriptase-PCR (RT-PCR) experiments demonstrated that all five genes in the operon are transcribed as a single mRNA and that their transcription is induced by phosphonoalanine or phosphonopyruvate. Transcriptional fusions of the regulatory region of the phosphonoalanine degradative operon with the gfp gene were constructed. Expression analysis indicated that the presence of a LysR-type regulator (encoded by the palR gene) is essential for the transcription of the structural genes of the operon. Similar gene clusters were found in the sequenced genomes of six bacterial species from the Alpha-, Beta- and Gammaproteobacteria, and analysis of metagenomic libraries revealed that sequences related to palA are widely spread in the marine environment.

Introduction
The natural occurrence of 2-amino-3-phosphonopropionic acid (phosphonoalanine), the phosphonate analogue of aspartic acid, was first reported by Kittredge & Hughes (1964) in the sea anemone *Zoanthus sociatus* and the protozoon *Tetrahymena pyriformis*. It has since been established to be one of the most widely distributed of the biogenic C–P compounds, particularly among the lower marine invertebrates (Quin & Quin, 2001). As in the case of many natural phosphonates, however, details of the enzymology and genetics of phosphonoalanine metabolism are poorly understood. The need for an improved understanding is clear, however, given the increasing awareness of the natural abundance of C–P compounds (e.g. in the marine environment; Benitez-Nelson *et al.*., 2004) and their likely involvement in global P cycling and ecosystem productivity.

Furthermore, the essential structural roles thought to be played in many protozoa and other pathogenic microorganisms by phosphonates in peptide, glycan or lipid conjugates make disruption of the pathways of C–P bond metabolism a possible therapeutic option.

We have isolated soil strains of *Burkholderia cepacia* (Ternan *et al.*, 2000) and *Variovorax* sp. (Kulakova *et al.*, 2003) that utilize the L-enantiomer of phosphonoalanine as a sole source of carbon, nitrogen and phosphorus; near-quantitative levels of Pi are excreted. Both isolates were found to contain a novel C–P hydrolase (EC 3.11.1.3) specific to 3-phosphonopyruvate, the transamination product of the parent compound; the products are pyruvate and Pi. The purified enzyme from *Variovorax* sp. Pal 2 shows highest levels of sequence identity to phosphoenolpyruvate phosphomutase – an enzyme that catalyses the intramolecular rearrangement and interconversion of phosphonopyruvate
and phosphoenolpyruvate (Chen et al., 2006). Phosphoenolpyruvate production by this route is a reaction common to the biosynthetic pathways of all natural products that contain the C–P bond.

Microbial cleavage of the C–P bond has generally been thought to be mediated by the C–P lyase multienzyme complex, which is expressed only under conditions of Pi starvation. The phosphonopyruvate hydrolase from Variovorax sp. Pal2 therefore belongs to an emerging group of novel microbial P metabolism enzymes whose expression is independent of Pi starvation (Quinn et al., 2007). We now report the structural and functional analysis of the clustered genes involved in phosphonoalanine degradation in Variovorax sp. Pal2, and demonstrate that their expression is co-ordinately induced by phosphonopyruvate (or phosphonoalanine) and requires the presence of a LysR-type transcriptional regulator.

Materials and methods

Bacterial strains and plasmids

Variovorax sp. Pal2 has been described previously (Kulakova et al., 2003). Escherichia coli DH5α-TIR and Pseudomonas putida KT2440 (Bagdasarian et al., 1981) were used for cloning and expression of the phosphonoalanine degradation genes. The broad host range promoter probe vector pPROBE-NT (Miller et al., 2000) was a gift from Professor Steven Lindow (University of California, Berkeley).

DNA techniques and sequencing

Standard methods of DNA manipulation were used (Sambrook et al., 1989). Recovery and purification of DNA fragments from agarose was achieved with the GFX™ PCR DNA and Gel Band Purification Kit (GE Inc.). Fragments containing sequences adjacent to the palA gene (Kulakova et al., 2003) were obtained using a GenomeWalker Kit (Clontech) and inverse PCR. Primer synthesis was performed by Sigma-Genosys (UK). DNA sequencing of the fragments produced was carried out at the University of Dundee Sequencing Service (http://www.dnaseq.co.uk/); the nucleotide sequences of both strands were determined. Alignment of sequences was performed using CLUSTALW (Thompson et al., 1994) with parameters set at default values. Searches for nucleotide and amino acid sequence similarities were carried out using the BLAST program (Pearson & Lipman, 1988) in the EMBL, GenBank and CAMERA (http://camera.calit2.net/) databases. The nucleotide sequence of the phosphonoalanine degradation gene cluster has been deposited in GenBank (Accession Number AY179862).

RNA isolation and Reverse transcriptase-PCR (RT-PCR)

Cultures of Variovorax sp. Pal2 were grown in minimal medium (McGrath et al., 1997) to a cell density of $1 \times 10^9$ CFU mL$^{-1}$ with phosphonoalanine (1 mM), phosphonopyruvate (0.5 mM) or Pi (1 mM) as sole sources of P. Total RNA preparations were obtained using the Fast RNA ProBlue kit (Qiagen, Irvine, CA) according to the manufacturer’s instructions and subsequently treated with 14 U of RNase-free DNase (Qiagen) for 30 min at room temperature to remove genomic DNA. The RNA was finally purified with an RNeasy MinElute Cleanup kit (Qiagen). RT-PCR was carried out in a two-stage process; for this cDNA synthesis was performed using Avian Myeloblastosis Virus Reverse Transcriptase (Invitrogen) in 25 μL volumes using 0.5 μg of total RNA as recommended by the manufacturer. Aliquots (2 μL) of the reaction mixtures were used for PCR amplification in 25 μL reactions as follows: initial heating was at 94°C for 2 min followed by 30 cycles of 20 s at 94°C, 30 s at 58°C and 90 s at 72°C. Negative control reactions to ensure that no amplification of the genomic DNA had occurred were carried out using the same samples but without the reverse transcription step.

Construction and analysis of clones with green fluorescent protein (GFP) fusions

pPROBE-NT contains a multiple cloning site (MCS) that allows the construction of transcriptional fusions between a cloned sequence and a promoterless gfp reporter gene. Two T1 E. coli transcriptional terminators situated downstream of gfp prevent read through transcription from cloned promoters (Miller et al., 2000). The pPROBE-NT plasmid confers resistance to kanamycin. Two primer pairs were designed as follows to amplify a putative promoter/transcriptional regulatory region (palR–palE; Fig. 1) of the phosphonoalanine degradation genes from Variovorax sp. Pal2: (1) forward primer AF193, 5′-GAG GAA GCT TCT GAG GGG CTG GTG CGG CTG CTG TC-3′; reverse primer AR1884, 5′-CCA TGA GCT CGG TCA CGT GGA CGT TGG TG-3′; (2) forward primer AF1265, 5′-GGG CGG ATG ATC CGG TGG TG-3′; reverse primer AR1884, 5′-CCA TGA GCT CGG TCA CGT GGA CGT TGG TG-3′.

The numbering of these primers corresponds to their 5′-ends on the nucleotide sequence of the region. HindIII restriction sites (underlined) were introduced into all forward and Sacl sites into all reverse primers. The Fail Safe PCR System (Epicentre Technologies) was used to amplify DNA with PCR conditions as follows: 95°C for 1 min followed by 25 cycles consisting of 15 s at 95°C and 135 s at 68°C, with a final extension step at 68°C for 4 min. The resulting products were purified, digested with restriction enzymes and ligated into plasmid pPROBE-NT using T4 DNA ligase. Plasmids were transformed into E. coli DH5α, and the transformants were selected on LB plates containing both kanamycin and ampicillin. Kanamycin-resistant colonies were then tested for the expression of gfp.
enzymes HindIII and SacI and ligated into the MCS of pPROBE-NT. This resulted in transcriptional fusions in which the gfp gene is located downstream of the promoter region of the pal operon. Ligated DNAs were transformed into E. coli DH5α-TIR chemically treated competent cells, and into P. putida KT2440 and Variovorax sp. Pal2 electro-competent cells. Transformants were selected on Luria–Bertani plates with kanamycin (50 μg mL⁻¹) and phosphonoalanine (0.5 mM). The cloning of the fragment amplified by primer pair (1) resulted in the construct designated pNTPALR1-69 (with an insert of 1.69 kb containing palR and the predicted promoter region of palE–palA); cloning of the fragment amplified by pair (2) resulted in pNT-ΔR (which has an insert of 0.6 kb that contains the palE–palA promoter region only).

**Enzyme assays**

Fluorescence produced by the pPROBE-NT constructs was measured according to Miller et al. (2000) using a Tecan microplate reader (Maennedorf, Switzerland). Briefly, fluorescence measurements were taken by setting the excitation
wavelength to 485 nm and measuring emission at 535 nm. A relative fluorescence unit (RFU) was defined as the culture fluorescence relative to culture biomass at OD600 nm. Specific fluorescence was determined as the RFU of induced cells divided by the RFU of uninduced, Pi-grown, cells (Stiner & Halverson, 2002).

Results and discussion

Characterization of the phosphonoalanine-degradative gene region

To study expression of the genes involved in the degradation of phosphonoalanine, a sequencing analysis was carried out as described in Materials and methods. The results of this analysis are presented in Table 1 and a map of the region is shown in Fig. 1. In addition to the palA gene that has already been shown to encode phosphonopyruvate hydrolase (Kulakova et al., 2003) five further ORFs that encode putative proteins with various degrees of similarity to known genes were found (Table 1). BLAST searches and analysis of the corresponding alignments indicate that the palE, palC and palD genes encode putative components of an ABC-type Fe3+ transport system and that PalB shows high similarity to the aspartate aminotransferase protein from Rhizobium meliloti (Table 1; Capela et al., 2001). The ORF found immediately upstream of palE is encoded on a complementary strand and was designated as palR. The predicted product of this gene showed significant similarity to several proteins belonging to the LysR family of transcriptional regulators, with greatest homology to the glycine cleavage system transcriptional activator GcvA from E. coli (Wilson & Stauffer, 1994; Perna et al., 2001). Comparative analysis of PalR (not shown) suggests that this protein is most closely related to the RbcR subfamily (Schell, 1993) of LysR regulators.

Identification of transcripts from the palA region

To analyse transcription of the phosphonoalanine degradation genes, total RNA preparations were isolated from Variovorax sp. Pal2 cells grown with Pi, phosphonoalanine or phosphonopyruvate, as sole P sources. RNA samples were analysed by RT-PCR. The oligonucleotide primers were designed so as to cover all of the genes identified as well as the intergenic regions (Fig. 1 and Table 2). This analysis identified two separate transcriptional units; the first included the palA, palB, palC, palD and palE genes, and the second, the independently transcribed palR gene (Fig. 1). Transcription of the palA–palE genes was demonstrated in cells grown on phosphonoalanine (Fig. 2), and confirmed in phosphonopyruvate-grown cells. Transcription was similarly observed in cells grown on either phosphonoalanine or phosphonopyruvate in the presence of 1 mM Pi (results not shown). However no transcription was detected in cells grown on Pi as the sole P source (Table 2). The palR gene was transcribed in both induced (with phosphonoalanine or phosphonopyruvate) and uninduced cells, although in the latter, a significantly lower amount of RT-PCR product was detected (Fig. 2; lanes 1 and 2).

Table 2. RT-PCR analysis of the phosphonoalanine-degradative genes in Variovorax sp. Pal2

<table>
<thead>
<tr>
<th>Primer pairs (position, nt)</th>
<th>Primer sequence (5’–3’)</th>
<th>Region</th>
<th>Amplification of cDNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>F607 (607–629)</td>
<td>GACGTAAGCGCAGGATCGCAGC</td>
<td>palR</td>
<td>+</td>
</tr>
<tr>
<td>R1359 (1339–1359)</td>
<td>GTGCGCCGCCAGTGGCAACTTC</td>
<td>palR</td>
<td>+</td>
</tr>
<tr>
<td>F607 (607–629)</td>
<td>GACGTAAGCGCAGGATCGCAGC</td>
<td>palD</td>
<td>–</td>
</tr>
<tr>
<td>Ar61 (2757–2736)</td>
<td>GATGACCGCGCACTGGGTCAC</td>
<td>palD</td>
<td>–</td>
</tr>
<tr>
<td>PPHF4 (1806–1826)</td>
<td>CAGTTTGAGCCACCGAATCC</td>
<td>palE</td>
<td>+</td>
</tr>
<tr>
<td>Ar61 (2757–2736)</td>
<td>GATGACCGCGCACTGGGTCAC</td>
<td>palD</td>
<td>–</td>
</tr>
<tr>
<td>A390f (3590–3608)</td>
<td>GTGCAAGGCCGATGATGTC</td>
<td>palC</td>
<td>+</td>
</tr>
<tr>
<td>A101f (4716–4734)</td>
<td>CGTGTCGATTGATGTCAG</td>
<td>palC</td>
<td>–</td>
</tr>
<tr>
<td>PPHF6 (3820–3841)</td>
<td>GTCCAGCGGCACTCGATGTC</td>
<td>palC</td>
<td>+</td>
</tr>
<tr>
<td>PPHR11 (5245–5266)</td>
<td>GATGCACCGGCACGGTGTCAC</td>
<td>palD</td>
<td>+</td>
</tr>
<tr>
<td>PPHF7 (6148–6165)</td>
<td>CTCGACCGGGCGATGAGGAC</td>
<td>palD</td>
<td>–</td>
</tr>
<tr>
<td>PPHR14 (7388–7368)</td>
<td>CATGCCGTGTCGCGCTCAG</td>
<td>palA</td>
<td>–</td>
</tr>
<tr>
<td>JQ127 (6805–6826)</td>
<td>CAACGGCGTAGCTCAGTAC</td>
<td>palC</td>
<td>+</td>
</tr>
<tr>
<td>JQ153 (7528–7545)</td>
<td>AGGCCCGCTTCTTCTCTCT</td>
<td>Outside</td>
<td>–</td>
</tr>
</tbody>
</table>

RT-PCR experiments were conducted as described in Materials and methods. Total RNA was isolated from Variovorax sp. Pal2 cells grown on phosphonoalanine or Pi. Experiments were carried out in triplicate. +, RT-PCR product of the correct size was detected; –, no RT-PCR product detected.
Analysis of expression of the pal gene region

To analyse regulation of the expression of the phosphonoalanine degradative genes, the putative palA–palE promoter region together with palR was cloned upstream of the gfp gene in the promoter probe vector pPROBE-NT, to produce plasmid pNTPALR1-69. The same region, but with a partially deleted palR, was also cloned (plasmid pNT-ΔR). Expression of gfp by these constructs was analysed in E. coli DH5α-TIR, P. putida KT2440 and Variovorax sp. Pal2 (Table 3). Only low levels of expression were detected in E. coli cells, possibly due to poor functioning of the Variovorax transcriptional regulator (PalR) in this bacterial host. In Variovorax sp. Pal2 gfp was expressed in the presence of either phosphonoalanine or phosphonopyruvate. Analysis of gfp expression in P. putida KT2440 (whose genome does not contain a phosphonoalanine-degradative gene cluster) unexpectedly indicated that this took place only following addition of phosphonopyruvate. Such regulation of biodegradative pathways by a catabolic intermediate has been shown previously; the regulation of the naphthalene-degradative genes by salicylate is a particularly well-studied example (Jones et al., 2003). The results also indicate that a functional palR is needed for expression of the phosphonoalanine-degradative genes (Table 3). This result is in accordance with our suggestion that palR encodes a LysR-type transcriptional regulator of the phosphonoalanine degradation operon.

The distribution of phosphonoalanine-degradative genes in nature

Database searches have revealed the presence of similar operon systems in six sequenced bacterial strains. In two other members of the Betaproteobacteria – Acidovorax avenue AAC00-1 (Genome Accession Number NC_008752) and Bordetella bronchiseptica RB50 (Parkhill et al., 2003; NC_002927) – the gene order was identical to that described above and the amino acid sequences of the putative proteins showed from 69% to 86% identity to their respective Variovorax sp. Pal2 homologues. In the Alphaproteobacteria Rhodopseudomonas palustris BisB18 (NC_007925) and Mesorhizobium loti MAFF303099 (NC_002678), and the Gammaproteobacteria Pseudomonas fluorescens Pf-5 (NC_004129) and Pseudomonas entomophila L48 (NC_008027), levels of amino acid identities were lower (33–39% with PalA); nevertheless putative aminotransferase gene and transporter genes were always present. In P. fluorescens Pf-5, a putative LysR-encoding gene was also found.

The most significant difference between the active sites of phosphoenolpyruvate (PEP) mutase (which catalyses intramolecular phosphoryl group transfer within phosphonopyruvate) and phosphonopyruvate hydrase consists in the substitution of Asn122 for Thr118 (Chen et al., 2006). It has been suggested that Thr 118 in phosphonopyruvate hydrase may bind and polarize a water molecule to facilitate hydrolysis (Chen et al., 2006). It is important to note that the Thr residue that corresponds to Thr118 of Pal2 is conserved in the Acidovorax and Bordetella strains, whereas the four other strains have Asn (similar to PEP mutase) in the relevant positions. Analysis of available marine metagenomic libraries (from the CAMERA database, Seshadri et al., 2007) revealed that genes similar to palA (with translated amino acid identities of 30% and more) are present at all 45 sampling points from across the world’s oceans; in most instances these are likely to encode PEP mutases (on the basis of the amino acid substitution described above). Importantly, however, a putative palA (encoding Thr 118) was identified at the Rangiorora Atoll

Table 3. Expression in various hosts of plasmid-borne GFP fusions involving the regulatory region of the phosphonoalanine-degradative genes from Variovorax sp. Pal2

<table>
<thead>
<tr>
<th>Plasmid (P source for growth of host cell)</th>
<th>Specific fluorescence*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E. coli DH5α-ΔR/Pal2</td>
</tr>
<tr>
<td>pNTPALR1-69 (p’alanine)</td>
<td>1.1</td>
</tr>
<tr>
<td>pNTPALR1-69 (p’pyruvate)</td>
<td>1.9</td>
</tr>
<tr>
<td>pNT-AR (p’alanine)</td>
<td>1.1</td>
</tr>
<tr>
<td>pNT-AR (p’pyruvate)</td>
<td>1.0</td>
</tr>
</tbody>
</table>

*Fluorescence was measured as described in Materials and methods. The values presented are the means of three replicate samples. RFU was defined as the culture fluorescence relative to culture biomass at OD600 nm. Specific fluorescence was determined as the RFU of induced cells divided by the RFU of uninduced, Pi-grown, cells.
near French Polynesia: site GS051, Accession Number JCVI_PEP_1105117198929 (source DNA: JCVI_READ_1093010128719). This result corroborates previous investigations of the distribution of genes encoding phosphate-degrading enzymes in the marine environment (Quinn et al., 2007).

**Conclusion**

Our study indicates a probable mechanism by which expression of the phosphonoalanine-degradative pathway occurs in *Variovorax* sp. Pal2. Only in the case of phosphonoacetate metabolism has any P-containing compound been previously shown to induce the expression of the genes required for its own catabolism (Kulakova et al., 2001). In the light of other studies in this laboratory (McGrath et al., 1997), which showed that phosphonopyruvic acid can also be degraded by environmental microorganisms in a Pi-insensitive manner, it seems likely that further C–P cleavage enzymes may prove to be similarly regulated. The analysis presented also indicates that phosphonoalanine-degradative genes are present in a wide diversity of bacteria and in the marine environment.

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**References**


