4-Methylphthalate catabolism in *Burkholderia (Pseudomonas) cepacia* Pc701: a gene encoding a phthalate-specific permease forms part of a novel gene cluster

Christopher P. Saint† and Pauline Romas

Author for correspondence: Christopher P. Saint. Tel: +61 8 259 0378. Fax: +61 8 259 0228.
e-mail: chris.saint@sawater.sa.gov.au

Department of
Microbiology, Monash
University, Clayton, Victoria
3168, Australia

We have determined the entire nucleotide sequence of a 4.4 kbp fragment of pMOP, a plasmid involved in 4-methylphthalate catabolism in *Burkholderia cepacia* (formerly *Pseudomonas cepacia*) Pc701. Two complete ORFs were identified and termed *mopA* and *mopB*. *mopB* encodes a 4-methylphthalate permease which is a member of a superfamily of symport proteins found in both prokaryotes and eukaryotes. Functionality was assigned to MopB by detailed analysis of the predicted amino acid sequence, resulting in the identification of 12 hydrophobic membrane-spanning domains and motifs associated with this class of protein. An assay was developed to demonstrate MopB function in substrate uptake. Of 4-methylphthalate, 4-hydroxyisophthalate, benzoate, *p*-toluate and phthalate, only uptake of 4-methylphthalate and phthalate was demonstrated, suggesting that two carboxyl groups in the *ortho* position are essential for substrate recognition. The predicted protein MopA showed significant levels of homology to reductase proteins implicated in aromatic and aliphatic catabolism, and contained motifs recognized as binding the ADP and flavin moieties of FAD/NAD. Northern hybridization experiments determined that *mopA* and *mopB* are cotranscribed, but expression was only seen in cells grown on 4-methylphthalate and not in cells grown on closely related structural analogues, including phthalate. *mopA* and *mopB* may be situated at the 3' terminus of a cistron about 10 kbp in size. The isolation and characterization of a 4-methylphthalate permease gene may lead to the identification of other permeases involved in bacterial biodegradation processes and possibly the construction of strains with enhanced degradative abilities.

Keywords: *Burkholderia (Pseudomonas)*, phthalates, gene organization, phthalate-specific permease

INTRODUCTION

Phthalate wastes are discharged into the environment by the plastics, paper and paint industries. Phthalates can accumulate at air–water interfaces and reach toxic levels in the tissues of animals that feed at such interfaces, and so

their presence in the environment is of some concern (Autian, 1973; Peakall, 1975; Keith & Telliard, 1979). Studies on the genetics and biochemistry of phthalate degradation by micro-organisms may result in improved biodegradation of industrial wastes prior to environmental release and the conversion of such wastes to useful intermediates for the organic synthetic chemicals industry.

Aerobic phthalate degradation appears to proceed via phthalate dioxygenase, consisting of phthalate oxygenase and phthalate oxygenase reductase, and a dehydrogenase, yielding 4,5-dihydroxyphthalate in *Pseudomonas fluorescens* (Pujar & Ribbons, 1985), *Pseudomonas testosteroni* (Naka-

† **Present address:** The Cooperative Research Centre for Water Quality and Treatment, Australian Water Quality Centre, Bolivar, SA5108, Australia.

Abbreviations: DIG, digoxigenin; MCS, multiple cloning site.

The GenBank accession number for the sequence reported in this paper is U29532.

zawa & Hayashi, 1977) and *Comamonas acidovorans* (Dutton *et al.*, 1995), or 3,4-dihydroxyphthalate in *Micrococcus* sp. (Eaton & Ribbons, 1982). The products are subsequently decarboxylated to yield protocatechuate, which is further converted to amphibolic intermediates via an *ortho*-cleavage pathway. Anderson (1980) isolated a soil pseudomonad capable of the degradation of 4-methylphthalate, 4-hydroxyisophthalate and phthalate. Through enzyme assays and analysis of mutants which accumulated pathway intermediates, a tentative pathway for 4-methylphthalate degradation was proposed (Anderson, 1980; Saint, 1986). A 4-methylphthalate 2,3-dioxygenase followed by a dehydrogenase convert the substrate to 2,3-dihydroxy-*p*-toluate, which is subsequently subjected to *meta*-cleavage at the 3,4 position. The ring cleavage product is then decarboxylated and converted to tri-carboxylic acid cycle intermediates. The ability to utilize 4-methylphthalate (Mop) was unstable and Mop⁻ derivatives were also found to have lost the ability to utilize 4-hydroxyisophthalate (Hip) but retained the ability to grow on phthalate.

Subsequently the ability to utilize 4-methylphthalate and 4-hydroxyisophthalate was demonstrated to be plasmid-encoded (Saint & Ribbons, 1990). The plasmid was sized at between 226 and 232 kbp and Mop⁺ and Hip⁺ phenotypes could be reintroduced to an isogenic cured (pMOP⁻) derivative of the wild-type isolate *Burkholderia cepacia* Pc701. Transposon mutagenesis with Tn1 produced a Mop⁻ Hip⁺ derivative of *B. cepacia* Pc701, termed *B. cepacia* Pc704. It was confirmed that Tn1 had inserted into a 2.1 kbp *Hind*III-derived fragment of pMOP in this isolate. A partial *Hind*III digestion of pMOP was performed and the resultant fragments were cloned into pKT230. One recombinant, pCS1 (pMOP1000), was able to restore a Mop⁺ phenotype to *B. cepacia* Pc704 by complementation, and was found to contain two contiguous *Hind*III fragments of 2.3 and 2.1 kbp derived from pMOP. This was the first report of genes involved in the catabolism of phthalates being plasmid-encoded, although subsequently Nomura *et al.* (1990) and Dutton *et al.* (1995) have described large plasmids involved in phthalate catabolism in *Pseudomonas putida* and *C. acidovorans*, respectively.

In this paper, we report the entire nucleotide sequence of a pMOP-derived 4.4 kbp insert in pMOP1000. We present evidence that the region involved in complementation encodes a 4-methylphthalate/phthalate-specific permease and identify an ORF upstream which may encode a 4-methylphthalate reductase. To our knowledge, this is the first molecular and functional demonstration of a permease involved in aromatic uptake as a prelude to catabolism.

METHODS

Bacterial strains and plasmids. The *Burkholderia cepacia* and *Escherichia coli* strains and the plasmids used and constructed during the course of this study are detailed in Table 1.

Chemicals and enzymes. All chemicals were of analytical grade and purchased from BDH, Sigma or Boehringer Mannheim

unless otherwise indicated. Restriction endonucleases and other DNA- and RNA-modifying enzymes and kits were purchased from Boehringer Mannheim unless otherwise indicated.

Media and culture conditions. L agar and L broth were prepared according to Sambrook *et al.* (1989). For growth of *B. cepacia* strains, solid and liquid minimal media were prepared according to Eaton & Ribbons (1982) and carbon sources were added to a final concentration of 5 mM from a 500 mM sterile stock solution. *E. coli* hosts containing plasmid clones were maintained on L agar containing ampicillin (100 µg ml⁻¹) for derivatives of pKK233-3 and pUC18, and L agar containing streptomycin (25 µg ml⁻¹) or kanamycin (20 µg ml⁻¹) for pKT230 derivatives. Where pRK311 was used for cloning, *E. coli* recombinants were maintained on L agar containing tetracycline (10 µg ml⁻¹).

Complementation studies. Bacterial conjugations were carried out as previously described (Saint *et al.*, 1990). *E. coli* recombinants containing either pMOP1210 or pMOP1211 were transformed with pMOP1100 or pMOP1101 using standard techniques (Sambrook *et al.*, 1989). Transformants were selected on LB agar incorporating 25 µg streptomycin ml⁻¹. Colonies were subsequently tested for retention of either pMOP1210 or pMOP1211 by growth on LB agar containing 10 µg tetracycline ml⁻¹. Transformants containing one of these two plasmids and pMOP1100 or pMOP1101 were used in triparental filter matings with *E. coli*(pRK2013) and *B. cepacia* Pc704. pRK2013 provides mobilization functions which permit transfer of the recombinant plasmids into *B. cepacia* Pc704. Selection for transconjugants was made on minimal medium containing 4-methylphthalate. Other complementation studies involving pMOP1300, pMOP1400 and pMOP1730 were carried out using similar methods; however, *E. coli* DH5α(pNJ5000) was used in place of DH5α(pRK2013) to provide mobilization functions. Transfer of pMOP1000 from *E. coli* DH5α to *B. cepacia* Pc704 was employed as a positive control in all of these experiments.

Plasmid DNA extraction and manipulation. Plasmid DNA for restriction enzyme analysis and subcloning was extracted from *E. coli* hosts by the alkaline lysis procedure described by Sambrook *et al.* (1989), with the addition of the phenol/chloroform extraction step. In addition, where DNA was to be used for cloning procedures, a final RNase treatment was employed. RNase stock (10 mg ml⁻¹) (in 10 mM Tris/HCl, 1 mM EDTA, pH 8) was added to a final concentration of 1 µg ml⁻¹ and the sample was incubated at 37 °C for 20 min. Restriction endonuclease digestion, treatment with calf intestinal phosphatase and ligation with T4 ligase were all performed in the buffer provided under the conditions specified by the manufacturer. On occasion, DNA fragments were gel-purified and extracted using GeneClean (Biolab 101) following the manufacturer's protocol.

pMOP1430 was constructed using the following protocol. pKK233-3 contains two *Bam*HI sites; one is upstream of P_{lac} and the other is within the multiple cloning site (MCS). pKK233-3 was partially digested with *Bam*HI, and singly cut vector gel purified and recovered using GeneClean. The recovered DNA was then digested to completion with *Eco*RI, purified and ligated to a 3.5 kbp *Bam*HI-*Eco*RI fragment of pMOP1420, which included *mopB*. Ligation into the MCS downstream of P_{lac} was confirmed by digestion with *Sal*I. pMOP1430 yields a 1 kbp fragment when digested with *Sal*I.

DNA-RNA hybridization. *B. cepacia* Pc701 was grown on minimal medium containing 0.05 % (w/v) Casamino acids and the appropriate carbon source at 5 mM concentration. A 5 ml starter culture was inoculated and grown overnight at 37 °C with shaking. This culture (2 ml) was used to inoculate 200 ml

Table 1. Bacterial strains and plasmids

Strain or plasmid	Description	Source/reference
Strains		
<i>Escherichia coli</i>		
JM105	<i>tbi rpsL endA sbcB15 asdR4 supE Δlac-proAB F' (traD36 proAB⁺ lacI^q lacZΔM15)</i>	Yanisch-Perron <i>et al.</i> (1985)
DH5α	F ⁻ <i>endA1 hsdR17 (r_K⁻ m_K⁺) tbi-1 recA1 gyrA96 relA1 Q80dlacZΔM15</i>	Bethesda Research Laboratories
<i>Burkholderia cepacia</i>		
Pc701	Mop ⁺ Hip ⁺ , pMOP	Saint & Ribbons (1990)
Pc704	Mop ⁻ Hip ⁺ , pMOP::Tn1 (Ap ^R)	Saint & Ribbons (1990)
Plasmids		
pRK2013	RK2 derivative, Km ^R Mob Tra	Figurski & Helinski (1979)
pNJ5000	RP4 derivative, Tc ^R Mob Tra	Grinter (1983)
pKT230	Km ^R Sm ^R	Bagdasarian <i>et al.</i> (1981)
pRK311	Tc ^R <i>lacZ cos</i>	Ditta <i>et al.</i> (1985)
pWSK29	Ap ^R <i>lacZ</i>	Wang & Kushner (1991)
pKK233-3	Ap ^R P _{tac}	Brosius & Holy (1984)
pUC18	Ap ^R <i>lacZ</i>	Yanisch-Perron <i>et al.</i> (1985)
pMOP1000	2.3 and 2.1 kbp <i>Hind</i> III pMOP fragments in pKT230, Sm ^R Km ^S , formerly pCS1	Saint & Ribbons (1990)
pMOP1100	2.3 kbp <i>Hind</i> III fragment from pMOP1000 in pKT230, Sm ^R Km ^S , formerly pCS4	This study
pMOP1101	pMOP1100 insert in reverse orientation in pKT230, Sm ^R Km ^S , formerly pCS5	
pMOP1210	2.1 kbp <i>Hind</i> III fragment from pMOP1000 in pRK311, Tc ^R	
pMOP1211	pMOP1210 insert in reverse orientation in pRK311, Tc ^R	
pMOP1300	3.3 kbp <i>Xho</i> I fragment from pMOP1000 in pKT230, Km ^S Sm ^R	
pMOP1400	4.85 kbp <i>Eco</i> RI fragment from pMOP1000 in pKT230, Sm ^S Km ^R	
pMOP1800	1.2 kbp <i>Eco</i> RI- <i>Xho</i> I fragment of pMOP1000 in pWSK29, Ap ^R	
pMOP1730	1.2 kbp <i>Xho</i> I- <i>Sma</i> I fragment from pMOP1800 in pKT230, Km ^S Sm ^R	
pMOP1120	2.3 kbp <i>Hind</i> III fragment of pMOP1000 in pUC18, Ap ^R	
pMOP1220	2.1 kbp <i>Hind</i> III fragment of pMOP1000 in pUC18, Ap ^R	
pMOP1250	1.2 kbp <i>Xho</i> I- <i>Eco</i> RI fragment of pMOP1000 cloned into the <i>Eco</i> RI- <i>Sal</i> I site of pUC18, Ap ^R	
pMOP1420	4.85 kbp <i>Eco</i> RI fragment of pMOP1000 in pUC18, Ap ^R	This study
pMOP1430	3.5 kbp <i>Bam</i> HI- <i>Eco</i> RI fragment of pMOP1420 in pKK233-3, Ap ^R	

medium in a 500 ml conical flask, which was incubated with shaking at 37 °C. In initial experiments, samples were taken at early- (OD₆₀₀ = 0.04), mid- (OD₆₀₀ = 0.15) and late-exponential phase (OD₆₀₀ = 0.4), rapidly cooled on ice and centrifuged at 3000 *g* for 10 min at 4 °C. Cell pellets were resuspended in 200 ml 100 mM phosphate buffer (pH 6.8) at 4 °C and recentrifuged. Pellets were frozen at -70 °C until used. For slot blot experiments, cells were routinely harvested at an OD₆₀₀ of 0.3.

Total RNA was extracted from cell pellets using the RNeasy protocol incorporating spin column purification (Qiagen). Pellets were adjusted so that approximately 10⁹ cells per extraction were used. The final elution volume was 40 µl diethyl pyrocarbonate (DEPC)-treated water. DNase treatment was performed by adding 1 µl (1 U) DNase (Promega), 1 µl RNasin (Promega), 4.7 µl 10 × DNase buffer (100 mM MgCl₂, 200 mM Tris/HCl, pH 8.0) and incubating for 45 min at 37 °C. If at this stage RNA required concentration, this was achieved by ethanol precipitation and the vacuum-dried pellet was resuspended in DEPC treated water.

For Northern transfer following gel electrophoresis of samples, RNA was electrophoresed through formaldehyde agarose gels in appropriate buffer (Sambrook *et al.*, 1989). Hybond N⁺ nylon membrane (Amersham) was used for all experiments. A Minifold II system (Schleicher and Schell) attached to a vacuum pump was used for slot blot analysis and the procedure of sample preparation and transfer was essentially as described by Sambrook *et al.* (1989). Prehybridization and hybridization buffer consisted of 7% (w/v) SDS, 50% (v/v) formamide, 5 × SSC (75 mM sodium citrate, 750 mM sodium chloride), 2% (w/v) blocking reagent, 0.1% *N*-lauroylsarcosine and 0.1% (v/v) sodium phosphate, pH 7.0.

DNA probes were prepared by PCR incorporating digoxigenin (DIG)-dUTP. A standard reaction consisted of: 10 ng template DNA, 18 µl 1 mM dNTP solution [prepared from stocks of dCTP, dATP, dGTP and dTTP (100 mmol l⁻¹)], 2 µl DIG-DNA labelling mixture [dATP, dCTP, dGTP (0.65 mmol l⁻¹); 0.35 mmol DIG-dUTP l⁻¹], 10 µl 10 × PCR reaction buffer, oligonucleotide 1 (100 µM), oligonucleotide 2 (100 µM), 1 U *Taq* DNA polymerase and double glass-distilled water to 100 µl.

For the internal probe to *mopA*, the template was pMOP1120 and oligonucleotides were *mopAF* (GGCGAGATTATGATGCTCGG) position 898–917, and *mopAR* (TCCGGAAGGATGCTCGAACC), position 1652–1671. For the probe to *mopB*, the template was pMOP1420 and oligonucleotides were *mopBF* (AGCTTTTGAAACCTCGCAGG), position 2197–2216, and *mopBR* (CGAAGGCGAGGCAGAGATAG), position 3463–3482. PCR was performed under the following conditions: 94 °C 1 min, 55 °C 1 min, 72 °C 1 min, 30 cycles; 94 °C 1 min, 55 °C 1 min, 72 °C 5 min, 1 cycle. Reactions yielded fragments of expected size and control reactions lacking DIG-dUTP yielded identical fragments which, when analysed by restriction digestion, confirmed the correct regions had been amplified. DIG-dUTP-labelled products were electrophoresed through low-melting-point agarose and the relevant band was excised and boiled for 15 min before being added directly to the hybridization solution. Approximately 1 µg of labelled probe was used per hybridization.

Following hybridization at 65 °C for 16 h, membranes were subjected to two washes in $2 \times$ SSC, 0.1% (w/v) SDS for 5 min at room temperature, followed by two washes in $0.1 \times$ SSC, 0.1% (w/v) SDS for 15 min at 65 °C. Detection of hybridization was performed using a DIG chemiluminescent detection kit according to the manufacturer's protocol, using disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl) phenyl phosphate as the substrate.

Primer extension analysis. RNA extracted from cells used in the Northern blotting experiments was also used for primer extension studies. Two oligonucleotides were designed to bind just downstream of the putative translation start codons of *mopA* (GATTGGTTGAGTGGTACG), position 797–814, and *mopB* (CTGCGAGGTTTCAAAGC), position 2198–2215. End-labelling of oligonucleotides and primer extension reactions were performed using a kit according to the manufacturer's instructions (Promega). Fifty micrograms of total RNA from *B. cepacia* Pc701 was used in each reaction.

Aromatic uptake assays. *E. coli* JM105 containing pKK233-3 or pMOP1430 was grown in M9 minimal medium (Sambrook *et al.*, 1989) containing 0.05% (v/v) Casamino acids, 10 µg thiamin ml⁻¹ and 5 mM sodium succinate. Initially 5 ml starter cultures were grown overnight with shaking at 37 °C. Two millilitres of culture was added to 200 ml of fresh medium in a 500 ml conical flask and the culture was incubated at 37 °C with shaking until an OD₆₀₀ of 0.6 (late-exponential phase) was reached. Cells were collected by centrifugation at 3000 g for 10 min, and the pellet was resuspended in 40 ml M9 minimal medium containing 2.5 mM sodium succinate and the relevant aromatic substrate at a concentration of 50 µM.

The culture was shaken at 37 °C for 1 h. IPTG was then added to a final concentration of 100 µM. Samples (1.5 ml) were removed at 10 min intervals, rapidly cooled on ice, centrifuged at 16000 g for 2 min at 4 °C, and the supernatant was scanned directly between 190 and 300 nm using a Variant DMS 1005 model UV/visible spectrophotometer. Disappearance of aromatic substrate from the medium was revealed by a reduction in absorbance at approximately 210 nm.

DNA sequencing. Sequencing was performed using an Applied Biosystems 373A DNA sequencer and raw data were collected using the manufacturer's software installed on a Macintosh system. DNA extraction and sequencing reactions were performed using a PRISM ready reaction DyeDeoxy terminator cycle sequencing kit, according to the manufacturer's protocol. Plasmids pMOP1120, pMOP1220 and pMOP1250 were used to acquire sequence data. pUC18 forward (5'-TGTAACG-

ACGGCCAGT-3') and reverse (5'-CAGGAAACAGCTATGAC-3') primers were used, along with a series of 18-mers designed to 'walk' along the cloned DNA in both the forward (F) and reverse (R) directions to complete the sequence of both strands. The coordinates of these oligonucleotides were as follows: 207–224F, 207–224R, 532–549F, 575–592R, 559–576F, 924–941F, 885–902R, 902–919R, 1198–1215F, 1242–1259R, 1548–1565R, 1815–1832F, 1850–1867R, 1833–1850F, 2112–2129F, 2139–2156F, 2262–2279R, 2550–2566F, 2897–2914F, 2901–2918R, 3192–3209R, 3198–3215R, 3206–3223F, 3613–3629F, 3614–3631R, 3980–3997F, 3980–3997R.

Computer analysis. DNA sequence contigs were constructed using GeneJockey II (Biosoft) and screening of the sequence for secondary structure and possible promoter sequences was carried out using The DNA Inspector IIe program (Textco). Primers for PCR were designed using the Amplify I program (University of Wisconsin, USA). Examination of proteins by BLAST, Clustal V, Scrutineer (Sibbald & Argos, 1990), the algorithm of Kyte & Doolittle (1982), and the Alom program of Kaneshisa (1982) was performed via the Australian National Genomic Information Service (ANGIS) facility at the University of Sydney. BLAST analysis was performed at high stringency using a single-letter match score of 5, single-letter mismatch score of -4 and word size (ktup) of 5 (Altschul *et al.*, 1990).

RESULTS AND DISCUSSION

Subcloning and complementation analysis

A series of subclones containing regions within the 4.4 kbp insert of pMOP1000 was constructed in pKT230 and pRK311 and introduced into *B. cepacia* Pc704 by conjugation. Fig. 1 shows these plasmids and their varying ability to restore a Mop⁺ phenotype. We previously reported that plasmids pCS2–5, containing either the 2.1 or 2.3 kbp *Hind*III fragment in either orientation in pKT230, could not complement *B. cepacia* Pc704 (Saint & Ribbons, 1990). We cloned the 2.1 kbp fragment in both orientations into pRK311 to give pMOP1210 and pMOP1211. pRK311 is compatible with pKT230; therefore pMOP1210 and pMOP1211 were mobilized to *B. cepacia* Pc704 along with either pMOP1100 or pMOP1101. None of the four possible combinations resulted in complementation. This confirmed that a region spanning the central *Hind*III site of pMOP1000 was essential for complementation.

A 3.3 kbp *Xho*I fragment of pMOP1000, containing the 2.3 kbp *Hind*III fragment and 0.5 kbp of the 2.1 kbp *Hind*III fragment, was cloned into pKT230 to give pMOP1300 (Fig. 1). By digestion of pMOP1000 with *Eco*RI and ligation of the resulting 4.8 kbp fragment into pKT230, all the 2.1 kbp *Hind*III fragment and 700 bp of the 3' region of the 2.3 kbp *Hind*III fragment were cloned to give pMOP1400. A region of 1.2 kbp bounded by *Eco*RI and *Xho*I sites and encompassing the central *Hind*III site was cloned into pWSK29 to give pMOP1800. The MCS of pWSK29 allowed this region to be conveniently excised on a *Sma*I–*Xho*I fragment and subcloned into pKT230 to give pMOP1730. Neither pMOP1300 or pMOP1730 restored a Mop⁺ phenotype to *B. cepacia* Pc704, whereas pMOP1400 did. This defined the start of the complementing region, with respect to Fig. 1,

Fig. 1. pMOP1000 and its subclones. Insert DNA is represented by a line, vector pKT230 by an open box and vector pRK311 by a hatched box. Arrows denote the direction of transcription from the vector promoter. The ability of each plasmid to restore a Mop⁺ phenotype to *B. cepacia* Pc704 is indicated in the right-hand column. The subsequently mapped positions of *mopA* and *mopB* and their direction of transcription are indicated by arrows above pMOP1000. H, *Hind*III; E, *Eco*RI; X, *Xho*I.

as downstream of the *Eco*RI site and running into the 2.1 kbp *Hind*III fragment beyond the *Xho*I site. Both the 2.3 and 2.1 kbp *Hind*III fragments and the central 1.2 kbp *Eco*RI-*Xho*I region were cloned into pUC18 to give pMOP1120, pMOP1220 and pMOP1250, respectively. Initial sequence analysis was performed on pMOP1250 and pMOP1220, which encompass the complementing region, followed by pMOP1120, to complete the entire sequence of the original pMOP1000 insert.

Identification of *mopA* and *mopB*

Fig. 2 shows the complete nucleotide sequence of the insert in pMOP1000 derived from pMOP. There are three predicted ORFs, which appear to be transcribed from left to right with respect to pMOP1000 (Fig. 1). The first ORF is incomplete and terminates at position 779. The putative translated product showed significant homology to PdxA, a pyridoxal phosphate biosynthetic protein of *E. coli* (Roa *et al.*, 1989). There was 54% similarity and 32% identity over the complete translated region of ORF1. *pdxA* encompasses approximately 1 kbp DNA, producing a polypeptide of 35.1 kDa in size (Roa *et al.*, 1989). If ORF1 is of comparable size, then we estimate that between 200 and 300 bp of the 5' end are missing from pMOP1000.

ORF2 begins 13 bp downstream of ORF1 and there is a putative Shine-Dalgarno sequence, AGGAG, 8 bp upstream of the putative ATG start codon (Shine & Dalgarno, 1975; Fig. 2). The predicted polypeptide is 431 amino acids in length and has a predicted molecular mass of 45.6 kDa. Predicting functionality for this protein was done by alignment with known proteins from the sequence database and the identification of motifs of known function. A BLAST analysis of the putative translated product of ORF2 revealed significant homologies to reductase proteins of both aromatic and aliphatic degradative pathways: 33% identity and 47% similarity to TodA, a reductase component of *P. putida* toluene 1,2-dioxygenase (Zylstra & Gibson, 1989); 31% identity and 47% similarity to BphA, a reductase component of biphenyl dioxygenase from *Pseudomonas pseudoalcaligenes* (Erickson & Mondello, 1992); 31% identity and 45%

similarity to BedA, the reductase component of benzene dioxygenase from *P. putida* ML 2 (Tan *et al.*, 1993); 31% identity and 43% similarity to TerA, a terpredoxin reductase from *Pseudomonas* sp. (Peterson *et al.*, 1992); 30% identity and 41% similarity to CamA, an NADH-putidaredoxin reductase involved in camphor metabolism in *P. putida* (Koga *et al.*, 1989); and 26% identity and 38% similarity to AlkT, the rubredoxin reductase component of alkane hydroxylase from *Pseudomonas oleovorans* (Eggink *et al.*, 1990). We have called ORF2 *mopA* and refer to its putative protein product as MopA.

There are two conserved motifs which are associated with reductase proteins. The first is GXGX₂GX₃AX₆G (where X is any amino acid), which forms a $\beta\alpha\beta$ fold responsible for binding the ADP moiety of FAD or NAD (Wierenga *et al.*, 1985). The second consensus is TX₆AXGD, which is responsible for binding the flavin moiety of FAD (Eggink *et al.*, 1990). When a Clustal V alignment of MopA was performed with the six reductases showing high homologies, three conserved regions were identified in MopA. The first was at the N-terminal end between amino acids 17 and 33, followed by a second region between amino acids 159 and 175. Both these regions match the FAD and NAD consensus motif, respectively, except that an alanine replaces the third glycine in the first motif. A third motif situated between amino acids 274 and 281 matches the second consensus, known to bind the flavin moiety of FAD.

We investigated further the presence of an alanine in place of glycine in the first motif. The third conserved glycine is considered important to allow for a close interaction between the β strands and the α helix (Wierenga *et al.*, 1985). Wierenga *et al.* (1986) went on to propose that these three glycine residues are strictly required for NAD- or FAD-binding $\beta\alpha\beta$ units. Eggink *et al.* (1990) found that the second fingerprint, which binds the ribityl moiety of FAD/NAD, was an excellent predictor for FAD-binding oxidoreductases. We examined the Swiss-Prot and PIR databases for proteins which invariably retained the second fingerprint but which contained either glycine or alanine at the third position of the first motif. The analysis was carried out using the Scrutineer program (Sibbald & Argos, 1990). The sequence search tag initially was

AAGCTTTCGGGTCGGGCGCATCGTGGTCTGGATCCGCTCGATCTTGCGCCGACAGATATGTACGGGCGAGCCTTCCGCCGCGCGGACGAGCGCTCGTCCGCTGGATGGCGCTCG 120
 A F A S G R I V V L D P L D L A P H E Y V T G Q P S A A A G R A V V R W M A L A 40
 CGGACCGGATCGCGCATGACGGAATCGGCGGATGGATCGGGGCGATCGATTTCAGCGTCCGCTCAAGCTCGCGGGGAAGTGGCAAGCATCGACGAATCCAGCCCGAGCATTCCT 240
 D R I A H D G L I G G W I M G P I D S T S L K L A G E V A S I D E L Q P E H S F 80
 TCATGTTTCGATGAGCGGAGACTGCGCGTGGTCCGTTGACGGAGCACATCCCGCTGCGCGACGTGCCGCCACCGTGACTTTCGATGCGGTGCTCAACCTGGTCACGCTGGTCAATG 360
 M F R M S G R L R V V P L T E H I P L R D V P A T V T F D A V L N L V T L V N E 120
 AGCGTTTGAAGACTGGGGATCGAGGCGCCGCGCATCGCGTTCGCGGGCTCAACCCGACGCCATGTTTCGAGGAGGAAGAGCGAATCATCGGTCCTCGGTCGAACAAGCCAGAGCGC 480
 R L K D W G I E A P R I G V A G L N P H A M F E E E E R I I G P A V E Q A R A Q 160
 AAGGATCGACGTGCGGGGCTGTTTCGCCCGACTCGGTATTCGTCATGAGCTGACGGCGCTATGACCGGTGCTCAGCATGTATCAGGACAGGGGAGATCGCACTAAAGTCG 600
 G I D V R G P V S P D S V F R H G L D G R Y D A V V S M Y H D Q G Q I A L K S A 200
 CTGCGTTCGAAGGGGCTGACGCTCTATCTTGGGGTCCCTACGTGCGCGGACCGTTCCTCAGGGGACTGCAATGGGTATTGCGGAGCGGAACCTGGCGAGCATCACAGCATGCTG 720
 A F E G A C T L Y L G V P Y V R A T V P H G T A M G I A G R N L A Q H H S M V A 240
 CCGCGTTGAGGACGCGCGCGCGCTTGCACGAAGCGCGGCTTCTGAACGAACAATAAGGATCGCGCGCATGGCTACCACTCAACCAATCGTTACTTTCACGCGTGGTGTGCTG 840
 A L R T A A A L A T K R G F L N E Q * M A Y H S T N P L L S R V I V G 17
 GAGCGGGGAGGCGCGCGGACCTTGGCGGAGGACCTTGGCGGAGGACCTTGGCGGAGGATATGATGCTCGGCGCTGAACCGGTGGGTCCGTATGAGCGGCGCGCTTGTTCGAAG 960
 A G Q A A A A A V A A K T L R A E G H R G E I M M L G A E R V G P Y E R P P L S K A 57
 CGTGGCTGAGCGCGGAAACGTGCCGAAATAGCGCTCTGCTCGAAAAGTCTGAAGCGGGGAGTCCGATGTCGACTTGGCGGACCGGGTGAACGTACCGGATCGACCGCGCTCAT 1080
 W L S A E N V P E I A A L L E K S E A G Q S D V D L R T G V N V T R I D R P S C 97
 GCACCGTGACGTCGACGATGGATCGGAGATTGCTTCGATCGTCTCGTATCGCAACGGGCGGACGCGCGCGCGGCTGGCGGTGCTGGCGACGCGAGCGACCATCGCTATCTG 1200
 T V H V D F D R L V D S E I C F D R L V I A T G G R A R R L A V P G D A S D G I A Y L R 137
 GTACGATCGACGAGCTTCGATATCCGCGCGGATGAAAGCAGGCAAGCGGCTGTGCTCATCGGGGCGGATGGATCGGATTGGAACCGCGTGCAGCGCGCGGAGCTAGGCGTGC 1320
 T I D D A L H I R R G L K A G K R L L L I G G W I G L E T A C S A R K L G V D 177
 ACGTGGTGTGTCGAAGCGGCGAGCGGTTATCGAAGCGGACCTCCCGCGATCGTGGTGAGCGTCTGCTCGGATTTCAGCGTTCGCTTGGCGTCGAGTGCCTTGGCGCGGCA 1440
 V V L V E A A Q R L C E R T V P A I V G E R L L G I Q R S L G V D V R L G A G I 217
 TCAGCAACTGTCAAGCTGCCGGCGGAGCTTATGCCGCTTCGCTATCGCGGCTTCGCGAAGAAATTCGACCTGGTGGTTGCGGGGTGGGATGGTGGCGAAGCAGAACTCGCGGTG 1560
 E Q L S K P L S G R Y A A S L S G V R E E F D L V A G V G M V A D E L A A E 257
 AAGCAGGTCTCCCTTGTGCGGCGCGGTGTTATGCGAGCTTGAAGGCGCGACGCTCGATCCCGCGTTCGCGTCCGCGGATGTCGCGTTCGAGCATCCTTCGCGACCATCGGGA 1680
 A G L P C A G G V L C D V E G R T V D P H V F A C G D V A S F E H P S G P I G M 297
 TCGCGCGCTGGAGTCTGGGACAACGCGCAGCAGCGGTCGCGGTGTGCCCGCGCATCCTCGGCAAGCGGCGCGCGCGCATCGTTGCTTGGTTCGTCGATCAGGCGGAGC 1800
 R R L E S W D N A Q Q Q G A A C A R A I L G K R A A A H P L P W F W S D Q G D V 337
 TGAATATCCAGATCCTCGGCTTCCCGAATGCGACGCGCACACCGGTGTCGCGGAAGCGCAGCGCAAGGCGAGCTGGTTCGCGTGGAGGAGCAGCAGCGGAGCGCGCGCAATCG 1920
 N I Q I L G F P N A T P V R E E G D G A C T L V W L E E H A A D E P A C I V 377
 TCGCGCGGTATGCATCAACGCGCGCGGACATGCGGATGCTGCGCGCATGTGCGAGAAGGCGTTCGCGCTCGACCGAAGACGCTCGCGTGCAGGAGGTGAGTCTCAAGAGCCTTC 2040
 A A V C I N A P A D M P I L R R M W Q K G V R V D R K T L A V Q E V S L K S L L 417
 TGCAGCGTTCGCGGCGCGCGGAGGATGAGGTCGGAACACTTAGAAGCGCATCAGCGATCGGACTTTTGCATTCACATCGACAGAGCACATCAGAGCGGGCGGTACCACTCGTGGC 2160
 Q R S A G A A R N G G A N T * 431
 GCTGACCGTCCACGCCAAAGGAGACAGCGGCTGAAGCTTTTGAACCTCGCAGGAGTGGCAGCGCACAGTCACGAGCAGGAATCCGCTTCGCGCGCAACACAACGGAATCGGGATCA 2280
 M K A F E T S Q E W H G T V T S T E S A S G A N T T E S G S 30
 CGACGACTGTTCTTTCGCGAGCTTTCGCGCACAATGGTCGAGTGGTACGACTTTTTCCTTACGCTTTATCGCGCGCTCGTTTTCGAAGAGTTGTTCTTCCCGAAGCTGAACGCACTT 2400
 R R L F F A S F L G T M V E W Y D F F L Y G F I A P L V F E E L F F P K L N A L 70
 AGCGGACCATCGCGGTATGCGACCTTCGCGGTGGCTATGCGAGCGGCGCGCTTCGCGGAGTCTGTTTCGCTCATTTTCGTCGACAAAATCGGCGCGCAATCAATGATGCTCGTCACA 2520
 S G T I A V Y A T F A V G Y A S R P L G L G L V F G H F G D K I G R K S M M L V T 110
 CTGCTGATGATGGGTATCGTATGCGGCAATGCGGCTCTTTCGCGAGCGTCGCGCAATGAGCGGACCTGCTGCTGCTGCGGCTGCGCTTCGTCAGGCTTCGCGCTAGG 2640
 L L M M G I G L L P S V A I G M S A T C L L V A L R F V G F A L G 150
 GGTGAGTCGCGTGTGCCAGCTGATGGTCTCGAGAGCGCGCAACACGTCGCGGGGCGCTGCTTGGGCGAGCGCTTCAGGCGGAGGACCGCTGGGCGTGTGCTGCGCTCGCTGGCG 2760
 G E S A A A S L M V L E S A Q Q R R R G L L G A A L Q A A G P L G V L L A S L A 190
 GTATTCGTGTCGCTGTCGCGCAGCAGCGCTTGTTCGCTGGGCTGGCGGTGCGCTTCCTTGTAGCGCGCTGCTGGTGGTATCGGTCGTATATCCGTTTCGAGTCGAGGAA 2880
 V F V V S R L P H D A L L A W G R V P F L V A V L V I G L Y L R L R V E E 230
 ACGGTCGCTTCGCGAAGCAGGTAAGCAGAACTAACCCGATCCCGCGTGGGAAACGTTGCGCAGCTACAAGTGGCTGTCTTCGTGCTGCTGGTAGTCAGCATTTAGGAGAGC 3000
 T V A F R K Q Q S K H E L T R I P A W E T L R S Y K W P V F V V L V V S I M E S 270
 ACGTTCTACTATCTGACCGCGCTTATTCATCTCTACGTACCAAGACGCTTCATATCGCGGGTTCGTCGCGATCGCGCGGATCGCTGTGCTAACGCTTTTGCCTCGTTCGGTA 3120
 T F Y Y L T G V Y S I S Y V T K T L H M P G S V A I G A I A C A N A F A L V S V 320
 CCGCTCTATGGCGCGCTTCCGACCGCTCGGCGCAAGGGCGTGTATTGCTCGGCATCGTGGCGTTCGCGGATTACCTGAACCTTCTTCTTACGATGCTCGACGCGCTTCGCGGATG 3240
 P L Y G A L S D R L G R K G V Y L L G I V A S I Y L N F F F T M L D F F R S A M 360
 GTCGTGGTGTGCGCGTGTGGCAGCGGTGCGGCTGATTCATGACCGATGTACGCCATGTTTCGCGAGCTTCTATGGCGAAGCTTTTCCACGCGAGTTCGCTTACGGGCTTCTGATG 3360
 V V V L A V V A A V G V I H A P M Y A M F G S F Y G E L F P T R V R F T G F S M 400
 GGCAAGCGGTCGCGCAGGTGCTCGCGCGCGCATCGCGCGATGATCGCGCGCTCGCTCGCGCGCGCAATCACGGAGACCCCTCGCTATCGGTCCTACTATCTTCGCTCGCTTC 3480
 G K A F G T V L G G G I A P M I A A S L A A R N H G D P S A I G L Y Y L C L A F 440
 GTCGCGTGTGCGTGGTGTGCGCGTGGCGAAGCGGAGCAGCAGCATACCGCTTAGCAACCTCGTTTTCACGTAGCCATTGGACGAGCGTCTGTGCTCTCGTGGAGGCGCACC 3600
 V A L C V L R V R E T R N D I T V * 459
 CGGCGCGGCTTTTGTTCGCTCTCACTCTCTAATAACATCATATGAAGAAGAACAGGCTTCTCTCGGCGTTCGCGCTTCGCGCGCGCGCGGACGCGGACGCGTGAACGCT 3720
 CTATGGCTCATCGTACTGGCCCCGATACGTCAACAATGTAAAGGGCAGTCATTGTTTCGAATTCGCGAAGCGGCTGATTCAGGGTAATCGTTTCGTTTGAAGGGGCGGAGGATCT 3840
 CGCGGAGGCGATGTCGCGCATCTTCACTCTGGAGATGGCTTCGACGGCAACCGGTCGCTGGGGCAAGGCGGCGCATGTTTCGAGCGGCGGCGATTCGTTGGGCTTTCGCGCGGAGC 3960
 ATACGCGAAGCTGACCTTGGGTCGTCAGTACGATACCAATACGATTTCATGGCGGATACGCGCGCGGCAAACTGTTTCGCGGATGATCCGATAACGACCACTGTTTCGCGCGGCTG 4080
 GCTTCGCAAGCTCGTCAAGTACACGAGCGCGAGCTGGGCGGCTTCAGCTTCGTCGATGATGATGAGCAACGCTCGCGGCGACTTCGCGAATAATCGCGCTACAGCGCGAGCG 4220
 CGGCATATCGCAATGGTAGCTGAAGCTT

Fig. 2. For legend see facing page.

Table 2. Glycine substitution in the ADP-binding moiety of reductase proteins

Organism	Protein	Sequence	Accession no./reference
<i>Escherichia coli</i>	Glutathione reductase	174 GAGYI A VELAGVINGLG 190	GSHR_ECOLI Greer & Perham (1986)
Pea		253 GGGYI A LEFAGIFNGLK 269	GSHR_PEA
<i>Pseudomonas aeruginosa</i>		173 GGGYL A VEFASIFNGLG 189	S15236
<i>Streptococcus thermophilus</i>		174 GAGYI A VEVAGVLNALG 190	S41386
Human		195 GAGYI A VEVAGVLSALG 211	S08979 Tutic <i>et al.</i> (1990)
Mouse		182 GAGYI A VEIAGVLSALG 198	
<i>Shigella flexneri</i>	Mercuric(II) reductase	104 GSGGA A MAAALKAVEQG 120	RDEBHA Misra <i>et al.</i> (1985)
<i>Thiobacillus ferrooxidans</i>		91 GTGGA A MALALKAVERG 107	JQ0153 Inoue <i>et al.</i> (1989)
<i>Pseudomonas aeruginosa</i>		105 GSGGA A MAAALKAVEQG 126	MERA_PSEAE Fox & Walsh (1983)
<i>Streptomyces lividans</i>		14 GSGAG A FAAAIAARNKG 30	MERA_STRLI Sedlmeier & Altenbuchner (1992)
<i>Staphylococcus aureus</i>		92 GSGGA A FSAAIKANENG 108	MERA_STAAU Laddaga <i>et al.</i> (1987)
<i>Escherichia coli</i>	Thioredoxin reductase	152 GGGNT A VEEALYLSNTA 168	TRXL_ECOLI Russel & Model (1988)
<i>Arabidopsis thaliana</i>		135 GGGDS A MEEANFLTKEYG 151	S44026
<i>Burkholderia cepacia</i> Pc701	MopA	17 GAGQ A AAVAKTLRAEG 33	
	Consensus	G-G--G---A-----G	

GXGX₂[GA]X₃A, and all proteins extracted were then searched with the second consensus sequence, TX₆AXGD. The results are presented in Table 2. There are clearly a variety of both prokaryotic and eukaryotic reductases which contain alanine in place of the third glycine in the ADP-binding motif. We found that the second consensus sequence by itself only extracted proteins identified or putatively identified as ferredoxins, reductases or dehydrogenases. Our analysis suggests that the second consensus is the more definitive search tool for the putative identification of reductase proteins.

Downstream of *mopA* there is another ORF which we have termed *mopB*. The ORF is preceded by a putative Shine–Dalgarno sequence and begins with the rare codon GTG (Fig. 2). *mopB* is 1347 nucleotides and encodes a predicted protein of 449 amino acids in length and 48.3 kDa in size. Further evidence to suggest that GTG is the start codon of *mopB* is provided by the position of the *Hind*III site just downstream. Previous subcloning demonstrated that the *Hind*III site bisects a gene functional in complementation. The predicted amino acid sequence for the protein encoded by *mopB* shows a proportion of non-polar amino acid residues typical of integral membrane proteins (69.5% Ala, Cys, Phe, Gly,

Ile, Leu, Met, Pro, Val, Trp and Tyr) (Culham *et al.*, 1993). Using the BLAST protein alignment program, the predicted protein showed high overall identity, between 48 and 57%, with several members of a superfamily of symport-type transporter proteins, including *E. coli* ProP, a proline–betaine transporter (Culham *et al.*, 1993), *E. coli* KgtP, a 2-oxoglutarate (α -ketoglutarate) transporter (Seol & Shatkin, 1991), and several citrate–proton symport proteins, namely Cit1 (Sasatsu *et al.*, 1985) and Cit2 (Ishiguro & Sato, 1985) of *E. coli*; CitA of *Salmonella typhimurium* (Shimamoto *et al.*, 1991) and CitH of *Klebsiella pneumoniae* (van der Rest *et al.*, 1990). Fig. 3 shows a Clustal V alignment of representatives of this group with MopB. Additionally, all the proteins shown were analysed using the algorithms of Kyte & Doolittle (1982) and Kaneshisa (1982) to predict possible hydrophobic membrane-spanning domains. The analysis revealed 12 predicted highly hydrophobic membrane-spanning regions, shown in Fig. 3. The regions were in good agreement with those previously predicted for these proteins. CitA has been found to be a member of a membrane transporter gene superfamily that includes a number of eukaryotic transporter proteins (Maiden *et al.*, 1987). Seol & Shatkin (1991) aligned KgtP with CitA, *E. coli* AraE and the human hepatoma glucose carrier (Glu). They

Fig. 2. Nucleotide sequence and predicted translation products of the pMOP1000 insert. Some restriction sites are shown for ease of orientation with regard to the data presented in Fig. 1. Putative Shine–Dalgarno sequences are shown in bold type whilst the positions of primers used for primer extension analysis are underlined. Arrows indicate the positions of inverted repeat sequences which may form a transcription termination signal.

Fig. 3. Alignment of MopB with members of a family of bacterial symport proteins. Asterisks indicate identities across all proteins and dots indicate conservative substitutions. Boxed amino acids are those predicted to form hydrophobic transmembrane domains. Motifs which are typical of this class of proteins are indicated. 1, 2 and 3 are motifs described by Culham *et al.* (1993). The duplicated motif described by Jessen-Marshall *et al.* (1995) is shown underlined within motifs 1 and 3. Kp CitH, *K. pneumoniae* citrate permease; St CitA, *S. typhimurium* citrate permease; Ec KgtP, *E. coli* 2-oxoglutarate (α -ketoglutarate) permease; Ec ProP, *E. coli* proline permease; Bc MopB, *B. cepacia* 4-methylphthalate permease.

identified a duplicated motif (R/K)XGR(R/K) common to these proteins which is also present in MopB (Fig. 3). Culham *et al.* (1993), in their analysis of ProP, defined three conserved sequence motifs. Two of these include the duplicated motif whilst another bridges the sixth hydrophobic domain (Fig. 3). MopB shows 79% identity and 97% similarity of amino acid residues over these conserved regions, with only a single amino acid difference in region 1. Recently, a precise study of LacY of *E. coli* further defined the duplicated motif as

GX₃(D/E)(R/K)XG(R/K)(R/K), which is common to a wide variety of both eukaryotic and prokaryotic permeases, including MopB (Jessen-Marshall *et al.*, 1995) (Fig. 3).

Jung *et al.* (1995) recently presented some studies on LacY which provided insight into the structure of this class of permeases. They replaced glycine residues at various positions in LacY and monitored the effects on activity. There were three glycine residues which, when replaced by cysteine, completely inactivated the protein, at positions 64, 115 and 147, respectively. However, when Gly was replaced by Ala at these positions, activity was restored to almost wild-type levels. LacY shows a low overall level of homology to MopB, but contains both copies of the short (R/K)XGR(R/K) consensus proposed by Scol & Shatkin (1991), between hydrophobic domains 2 and 3 and 8 and 9, and a single copy of the GX₃(D/E)(R/K)XG(R/K)(R/K) motif proposed by Jessen-Marshall *et al.* (1995) between hydrophobic domains 2 and 3. Alignment of LacY with MopB (data not presented) revealed that Gly₆₄ of LacY corresponds to Gly₉₅ of MopB, which is the first Gly of the GX₃(D/E)(R/K)XG(R/K)(R/K) consensus between hydrophobic domains 2 and 3 (Fig. 3). Gly₁₁₅ corresponds to Gly₁₄₆ of MopB found in the fourth hydrophobic domain, which is also present in the four other proteins aligned in Fig. 3. However, Gly₁₄₇ corresponds to Ala₁₇₉ in MopB, and is represented by Thr and Ser, as well as Gly, in the four other permeases in Fig. 3. In a detailed study of the GX₃(D/E)(R/K)XG(R/K) motif, Jessen-Marshall *et al.* (1995) produced a variety of site-directed mutations at the first, fifth, sixth, eighth, ninth and tenth positions in LacY. They found that an amino acid with a bulky side chain at position 1, equivalent to Gly₆₄ in the Jung *et al.* (1995) study, caused inactivation. The acidic amino acid at position five was critical and could not be replaced; this is retained as aspartate in all the proteins analysed in Fig. 3. They suggested that since this motif is conserved among proteins that transport a wide range of solutes, it is unlikely to be concerned with solute binding. The identification of MopB and its retention of the consensus would tend to support this theory.

mopA and *mopB* are cotranscribed

We conducted a detailed search of the DNA sequence presented here for possible -12/-24- and -10/-35-like promoter sequences which might be present, but there were none apparent. However, we could not rule out the possibility that there might be a sequence upstream of *mopA* or *mopB* capable of being recognized as a promoter in *B. cepacia*. Two primers were designed and primer extension experiments were performed to distinguish this possibility. The two primers are indicated in Fig. 2, and are positioned just downstream of the putative start codons of *mopA* and *mopB*. No primer extension product was seen with either oligonucleotide (data not presented). Total RNA was extracted from mid-exponential-phase cells of *B. cepacia* Pc701 grown on succinate or 4-methylphthalate, electrophoresed and Northern blot

Fig. 4. Northern hybridization analysis of total RNA extracted from *B. cepacia* Pc701 employing *mopA* and *mopB* probes. DNA probes were hybridized to total RNA extracted from cells grown on a range of substrates. Total amounts of RNA used (μg) are indicated. (a) *mopB* probe; (b) *mopA* probe. Lanes 1–5 in each experiment contained the same RNAs, prepared from cells grown on the following substrates: 1, succinate; 2, 4-methylphthalate; 3, phthalate; 4, 4-hydroxyisophthalate; 5, benzoate. Positive controls were employed for each probe: single slots contained 0.1 μg pMOP1220 in lane 6 of (a) and 0.1 μg pMOP1120 in lane 6 of (b).

analysis was performed using DIG-labelled *mopA* and *mopB* internal probes. We saw hybridization with both probes, but only to RNA extracted from 4-methylphthalate-grown cells. However, partial degradation of RNA was always seen for repeat isolations and also using an alternative extraction procedure. The resulting smeared band had a maximum size of about 10 kbp, suggesting that a single long transcript was produced which was difficult to extract in its entirety. Sequence analysis revealed a possible rho-independent transcription termination signal downstream of *mopB* (Fig. 2). The structure had a calculated ΔG of $-18 \text{ kcal mol}^{-1}$ (-75 kJ mol^{-1}) (Tinoco *et al.*, 1973).

Induction of the transcript encoding MopA and MopB with a range of substrates was investigated. *B. cepacia* Pc701 readily grows in medium containing phthalate or benzoate. Total RNA was extracted from cells grown to mid-exponential phase on these substrates or succinate, 4-methylphthalate or 4-hydroxyisophthalate. Slot blot analysis was performed using between 5 and 0.1 μg of total RNA and identical probes to *mopA* and *mopB* used in the large-scale Northern blot experiments. Fig. 4 shows the resultant autoradiograph. Only hybridization with RNA extracted from 4-methylphthalate-grown cells was seen, and was visible down to the 0.1 μg level. Interestingly, although phthalate is a substrate for MopB, it does not bring about induction of the MOP transcript, suggesting regulation is tightly controlled.

B. cepacia Pc701 degrades 4-methylphthalate via a diol intermediate which is dehydrogenated to give 2,3-dihydroxy-*p*-toluate; this is subsequently cleaved by an extradiol dioxygenase reaction (Anderson, 1980; Saint, 1986). Subsequent products are substrates for the tri-carboxylic acid pathway. The initial conversion of 4-methylphthalate to its respective diol is a reaction type

usually associated with multicomponent dioxygenases incorporating a reductase component (Harayama *et al.*, 1992). Sequence analysis combined with transcriptional studies suggest that MopA is a reductase directly associated with 4-methylphthalate degradation. Previously, multicomponent dioxygenases have been found to consist of discrete genetic units with tandemly arranged components (van der Meer *et al.*, 1992). Whilst *mopA* may form part of the same transcriptional unit as the other essential dioxygenase components, there is clearly a locational separation.

MopB is a phthalate-specific permease

To facilitate functional studies on MopB, a 3.5 kbp *Bam*HI–*Eco*RI fragment of pMOP1420 was cloned into pKK233-3 downstream of the P_{tac} promoter. With respect to pMOP1000 (Fig. 1), the fragment contained all pMOP-derived DNA to the right of the *Eco*RI site and a small section (0.7 kbp) of pKT230 up to the vector-encoded *Bam*HI site. This includes the complete *mopB* ORF, but only the 3' region of *mopA*. Directional cloning into pKK233-3 resulted in transcription proceeding from P_{tac} towards *mopB*. Sequence and primer extension analysis confirmed that there was no functional promoter upstream of *mopB*; therefore we expected pMOP1430 to contain *mopB* under the regulatory control of P_{tac} . Uptake assays were performed in *E. coli* transformed with pMOP1430. The host was grown on a supplemented minimal medium with succinate as carbon and energy source. Late-exponential-phase cells were harvested and resuspended in minimal medium containing succinate and either 4-methylphthalate, 4-hydroxyisophthalate, phthalate, benzoate or *p*-toluate. After incubation for 1 h, the non-metabolizable inducer of P_{tac} , IPTG, was added and supernatant samples were removed every 10 min and

Fig. 5. Aromatic uptake by *E. coli* JM105 (pMOP1430). Full details of the assay technique are given in Methods. A summary of the aromatics tested along with their uptake profiles are presented: (a) 4-methylphthalate; (b) 4-hydroxyisophthalate; (c) phthalate; (d) benzoate; (e) *p*-toluate. For clarity, only data for phthalate and 4-methylphthalate are presented graphically, as the patterns for all other substrates were identical to the negative control, *E. coli* JM105(pKK233-3). ■, JM105(pMOP1430)+4-methylphthalate; □, JM105(pKK233-3)+4-methylphthalate; △, JM105(pMOP1430)+phthalate; ▲, JM105(pKK233-3)+phthalate.

scanned spectrophotometrically for aromatic substrate removal. Only 4-methylphthalate and phthalate were seen to be removed from the medium. Fig. 5 shows the pattern of removal up to and following the addition of IPTG. There was no removal of any substrate from medium containing *E. coli*(pKK233-3), and a similar pattern was seen for media containing 4-hydroxyisophthalate, benzoate and *p*-toluate when incubated with induced *E. coli*(pMOP1430). For simplicity, these data have been omitted from Fig. 5. There was both a faster and greater removal of 4-methylphthalate than phthalate, but both substrates did not continue to be removed beyond 100 min. These studies suggested that the presence of two *ortho*-placed carboxyl groups is critical for permease recognition.

The methodology developed for functional studies on MopB should be applicable to other bacterial uptake systems. The technique is particularly useful where the expense or lack of availability of radiolabelled substrates makes comprehensive uptake studies impossible. The combination of a high level of induction coupled with high cell numbers in the assay was sufficient to permit detection of substrate removal from the supernatant. We never detected the appearance of new absorption peaks when scanning culture media, further suggesting that MopB actively removes substrate and does not merely convert phthalate or 4-methylphthalate to their respective dihydrodiols, which might be expected to accumulate in the culture medium. Removal stopped abruptly with both phthalate and 4-methylphthalate. Possibly destabilization of the cell membrane due to permease overproduction, the toxicity of substrate build-up in the cells, or the dissolution of the energy gradient required for uptake could explain this. We never saw substrate levels subsequently rise in the supernatant or monitored a decrease in optical density of the cell suspension, suggesting that cells remained intact.

The identification of MopB as a permease involved in aromatic uptake is of significance. Previous genetic and biochemical analysis of aromatic degradative pathways in bacteria have not revealed the presence of specific transport mechanisms. Cloning and sequencing of *mopB* should allow the design of probes to search for other aromatic permeases in closely related genera.

ACKNOWLEDGEMENTS

The authors wish to express their gratitude to Dr N. C. McClure for his critical appraisal of the manuscript.

REFERENCES

- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403–410.
- Anderson, B. N. (1980). *4-Methylphthalic acid utilization by soil pseudomonads: biochemistry and genetics of plasmid encoded functions*. PhD thesis, University of Miami, USA.
- Autian, J. (1973). Toxicity and health threats of phthalate esters: review of the literature. *Environ Health Perspect* **4**, 3–26.
- Bagdasarian, M., Lurz, R., Rukert, B., Franklin, F. C. H., Bagdasarian, M. M., Frey, J. & Timmis, K. N. (1981). Specific purpose plasmid cloning vectors. II. Broad host range, high copy number, RSF1010 derived vectors, and a host vector system for gene cloning in *Pseudomonas*. *Gene* **16**, 237–247.
- Brosius, J. & Holy, A. (1984). Regulation of ribosomal RNA promoters with a synthetic *lac* operator. *Proc Natl Acad Sci USA* **81**, 6929–6933.
- Culham, D. E., Lasby, B., Marangoni, A. G., Milner, J. L., Steers, B. A., van Nues, R. W. & Wood, J. M. (1993). Isolation and sequencing of *Escherichia coli* gene *proP* reveals unusual structural features of the osmoregulatory proline/betaine transporter, ProP. *J Mol Biol* **229**, 268–276.
- Ditta, G., Schmidhauser, T., Yakobson, E., Lu, P., Liang, X. P., Finlay, D. R., Guiney, D. & Helinski, D. R. (1985). Plasmids related to the broad host range vector, pRK290, useful for gene cloning and for monitoring gene expression. *Plasmid* **13**, 149–153.

- Dutton, J. R., Venables, W. A. & Saint, C. P. (1995). *Corynebacterium acidovorans* UCC61 catabolizes *o*-phthalate via a 4,5-oxygenation pathway that is encoded on a 70 kbp section of plasmid pOPH1 bounded by directly repeated sequences. *Microbiology* **141**, 1673–1682.
- Eaton, R. W. & Ribbons, D. W. (1982). Metabolism of dibutylphthalate and phthalate by *Micrococcus* sp. strain 12B. *J Bacteriol* **151**, 48–57.
- Eggink, G., Engel, H., Vriend, G., Terpstra, P. & Witholt, B. (1990). Rubredoxin reductase of *Pseudomonas oleovorans*: structural relationship to other flavoprotein oxidoreductases based on one NAD and two FAD fingerprints. *J Mol Biol* **212**, 135–142.
- Erickson, B. D. & Mondello, F. J. (1992). Nucleotide sequencing and transcriptional mapping of the genes encoding biphenyl dioxygenase, a multicomponent polychlorinated-biphenyl-degrading enzyme in *Pseudomonas* strain LB400. *J Bacteriol* **174**, 2903–2912.
- Figurski, D. H. & Helinski, D. R. (1979). Replication of an origin containing derivative of RK2 dependent on a plasmid function in *trans*. *Proc Natl Acad Sci USA* **76**, 1648–1652.
- Fox, B. S. & Walsh, C. T. (1983). Mercuric reductase: homology to glutathione reductase and lipoamide dehydrogenase. Iodoacetamide alkylation and sequence of the active site peptide. *Biochemistry* **22**, 4082–4088.
- Greer, S. & Perham, R. N. (1986). Glutathione reductase from *Escherichia coli*: cloning and sequence analysis of the gene and relationship to other flavoprotein oxidoreductases. *Biochemistry* **25**, 2736–2742.
- Grinter, N. J. (1983). A broad host range cloning vector transposable to various replicons. *Gene* **21**, 133–143.
- Harayama, S., Kok, M. & Neidle, E. L. (1992). Functional and evolutionary relationships among diverse oxygenases. *Annu Rev Microbiol* **46**, 565–601.
- Inoue, C., Sugawara, K., Shiratori, T., Kusano, T. & Kitagawa, Y. (1989). Nucleotide sequence of the *Thiobacillus ferrooxidans* chromosomal gene encoding mercuric reductase. *Gene* **84**, 47–54.
- Ishiguro, N. & Sato, G. (1985). Nucleotide sequence of the gene determining plasmid-mediated citrate utilization. *J Bacteriol* **164**, 977–982.
- Jessen-Marshall, A. E., Paul, N. J. & Brooker, R. J. (1995). The conserved motif, GXXX(D/E)(R/K)XG[X](R/K)(R/K), in hydrophilic loop 2/3 of the lactose permease. *J Biol Chem* **270**, 16251–16257.
- Jung, K., Jung, H., Colacurcio, P. & Kaback, H. R. (1995). Role of glycine residues in the structure and function of lactose permease, an *Escherichia coli* membrane transport protein. *Biochemistry* **34**, 1030–1039.
- Kaneshisa, M. (1982). Los Alamos sequence analysis package for nucleic acids and proteins. *Nucleic Acids Res* **10**, 183–196.
- Keith, L. H. & Tellard, W. A. (1979). Priority pollutants; I-A perspective view. *Environ Sci Technol* **13**, 416–423.
- Koga, H., Yamaguchi, E., Matsunaga, K., Aramaki, H. & Horiuchi, T. (1989). Cloning and nucleotide sequences of NADH-putidaredoxin reductase gene (*camA*) and putidaredoxin gene (*camB*) involved in cytochrome P-450cam hydroxylase of *Pseudomonas putida*. *J Biochem* **106**, 831–836.
- Kyte, J. & Doolittle, R. F. (1982). A simple method for displaying the hydropathic nature of a protein. *J Mol Biol* **157**, 105–132.
- Laddaga, R. A., Chu, L., Misra, T. K. & Silver, S. (1987). Nucleotide sequence and expression of the mercurial-resistance operon from *Staphylococcus aureus* plasmid p1258. *Proc Natl Acad Sci USA* **84**, 5106–5110.
- Maiden, M. C. J., Davis, E. O., Baldwin, S. A., Moore, D. C. M. & Henderson, F. J. F. (1987). Mammalian and bacterial sugar transport proteins are homologous. *Nature* **325**, 641–643.
- van der Meer, J. R., de Vos, W. M., Harayama, S. & Zehnder, A. J. B. (1992). Molecular mechanisms of genetic adaptation to xenobiotic compounds. *Microbiol Rev* **56**, 677–694.
- Misra, T. K., Brown, N. L., Haberstroh, L., Schmidt, A., Goddette, D. & Silver, S. (1985). Mercuric reductase structural genes from plasmid R100 and transposon Tn501: functional domains of the enzyme. *Gene* **34**, 253–262.
- Nakazawa, T. & Hayashi, E. (1977). Phthalate metabolism in *Pseudomonas testosteroni*: accumulation of 4,5-dihydroxyphthalate by a mutant strain. *J Bacteriol* **131**, 42–48.
- Nomura, Y., Takada, N. & Oshima, Y. (1990). Isolation and identification of phthalate-utilizing bacteria. *J Ferment Bioeng* **67**, 297–299.
- Peakall, D. B. (1975). Phthalate esters; occurrence and biological effects. *Residue Rev* **54**, 1–41.
- Peterson, J. A., Lu, J.-Y., Geisselsoder, J., Graham-Lorence, S., Carmona, C., Witney, F. & Lorence, M. C. (1992). Cytochrome P-450_{terp}: isolation and purification of the protein and cloning and sequencing of its operon. *J Biol Chem* **267**, 14193–14203.
- Pujar, B. G. & Ribbons, D. W. (1985). Phthalate metabolism in *Pseudomonas fluorescens* PHK: purification and properties of 4,5-dihydroxyphthalate carboxylase. *Appl Environ Microbiol* **49**, 374–376.
- van der Rest, M. E., Schwarz, E., Oesterheld, D. & Konings, W. N. (1990). DNA sequence of a citrate carrier of *Klebsiella pneumoniae*. *Eur J Biochem* **189**, 401–407.
- Roa, B. B., Connolly, D. M. & Winkler, M. E. (1989). Overlap between *pdxA* and *ksgA* in the complex *pdxA-ksgA-apaG-apaH* operon of *Escherichia coli* K-12. *J Bacteriol* **171**, 4767–4777.
- Russel, M. & Model, P. (1988). Sequence of thioredoxin reductase from *Escherichia coli*. *J Biol Chem* **263**, 9015–9019.
- Saint, C. P. (1986). *Genetics and biochemistry of catabolic plasmids in Pseudomonas*. PhD thesis, University of Wales, UK.
- Saint, C. P. & Ribbons, D. W. (1990). A catabolic plasmid involved in 4-methyl-*o*-phthalate and 4-hydroxy-*iso*-phthalate degradation in *Pseudomonas cepacia*. *FEMS Microbiol Lett* **69**, 323–328.
- Saint, C. P., McClure, N. C. & Venables, W. A. (1990). Physical map of the aromatic amine and *m*-toluate catabolic plasmid pTDN1 in *Pseudomonas putida*: location of a unique *meta*-cleavage pathway. *J Gen Microbiol* **136**, 615–625.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sasatsu, M., Misra, T. K., Chu, L., Laddaga, R. & Silver, S. (1985). Cloning and DNA sequence of a plasmid determined citrate utilization system in *Escherichia coli*. *J Bacteriol* **164**, 983–993.
- Sedlmeier, R. & Altenbuchner, J. (1992). Cloning and DNA sequence analysis of the mercury resistance genes of *Streptomyces lividans*. *Mol Gen Genet* **236**, 76–85.
- Seol, W. & Shatkin, A. J. (1991). *Escherichia coli* *kgtP* encodes an α -ketoglutarate transporter. *Proc Natl Acad Sci USA* **88**, 3802–3806.
- Shimamoto, T., Izawa, H., Daimon, H., Ishiguro, N., Shinagawa, M., Sakano, Y., Tsuda, M. & Tsuchiya, T. (1991). Cloning and nucleotide sequence of the gene (*citA*) encoding a citrate carrier from *Salmonella typhimurium*. *J Biochem* **110**, 22–28.
- Shine, J. & Dalgarno, L. (1975). Determinant of cistron specificity in bacterial ribosomes. *Nature* **254**, 34–38.

- Sibbald, P. R. & Argos, P. (1990).** Scrutineer: a computer program which flexibly seeks and describes motifs and profiles in protein sequence databases. *Comput Appl Biosci* **6**, 279–288.
- Tan, H.-M., Tang, H.-Y., Joannou, C. L., Abdel-Wahab, N. H. & Mason, J. R. (1993).** The *Pseudomonas putida* ML2 plasmid-encoded genes for benzene dioxygenase are unusual in codon usage and low in G + C content. *Gene* **130**, 33–39.
- Tinoco, L., Borer, P. N., Dengler, B., Levine, M. D., Uhlenbeck, O. C., Crothers, D. M. & Gralla, J. (1973).** Improved estimation of secondary structure in ribonucleic acids. *Nature* **246**, 40–41.
- Tutic, M., Lu, X., Schirmer, R. H. & Werner, D. (1990).** Cloning and sequencing of mammalian glutathione reductase cDNA. *Eur J Biochem* **188**, 523–528.
- Wang, R. F. & Kushner, S. R. (1991).** Construction of versatile low copy number vectors for cloning, sequencing and gene expression in *E. coli*. *Gene* **100**, 195–199.
- Wierenga, R. K., De Maeyer, C. H. & Hol, W. G. J. (1985).** Interaction of pyrophosphate moieties with α -helices in dinucleotide binding proteins. *Biochemistry* **24**, 1346–1357.
- Wierenga, R. K., Terpstra, P. & Hol, W. G. J. (1986).** Prediction of the occurrence of the ADP-binding $\beta\alpha\beta$ -fold in proteins, using an amino acid sequence fingerprint. *J Mol Biol* **187**, 101–107.
- Yanisch-Perron, C., Vieira, J. & Messing, J. (1985).** Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**, 103–119.
- Zylstra, G. J. & Gibson, D. T. (1989).** Toluene degradation by *Pseudomonas putida* F1 nucleotide sequence of the *todC1C2BADE* genes and their expression in *Escherichia coli*. *J Biol Chem* **264**, 14940–14946.
-

Received 22 February 1996; revised 15 April 1996; accepted 22 April 1996.