***Comamonas 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**

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Twenty-three bacteria capable of growing with *o*-phthalate as sole carbon source were isolated from sewage sludge. One of these, named UCC61, was identified as a strain of *Comamonas acidovorans* and was selected for further study. UCC61 was found to carry a single plasmid (named pOPH1) of about 270 kbp in size. The phthalate-utilizing phenotype of UCC61 was unstable and could be lost either by complete curing of pOPH1, or by the deletion of a specific 70 kbp segment from pOPH1. This segment, termed the Pht element, was extensively restriction mapped (using four restriction endonucleases), and was found to be flanked by directly repeated sequences greater than 1.9 kbp in length. Data from comparative restriction analysis of pOPH1 and its deleted derivative were consistent with a deletion mechanism that involves homologous recombination between the direct repeats. UCC61 was found to catabolize *o*-phthalate via 4,5-oxygenation, dehydrogenation and decarboxylation to protocatechuate. These three activities were encoded within the Pht element, with the genes clustered near the right-hand terminus. Protocatechuate metabolism was chromosomally encoded, as also was the ability to catabolize the *m*- and *p*-isomers of phthalate.

Keywords: *Comamonas*, phthalate, degradation, plasmid-encoded

INTRODUCTION

In recent decades phthalic acid esters (PAEs) have been manufactured in large amounts by chemical and textile industries, most commonly for use as plasticizers in a variety of polymers. A consequence of this widespread use is the ubiquitous presence of PAEs in the biosphere resulting both from leaching from plastic products and from direct entry. Considerable interest has been directed at the toxicity of PAEs in the environment, and also at the potential of phthalate-degrading micro-organisms to carry out commercially and environmentally important biotransformations of phthalate-related molecules.

Microbial biodegradation of isomers of phthalic acid and their esters by both Gram-negative and Gram-positive

bacteria has been documented by numerous studies under both aerobic and anaerobic conditions. Although ultimate mineralization of the entire ester molecule was not universally demonstrated, it has been shown that various micro-organisms are able to metabolize PAEs via *o*-phthalic acid (Keyser *et al.*, 1976; Kurane *et al.*, 1977a, b; Engelhardt & Wallnofer, 1978; Eaton & Ribbons, 1982a, b, c, d, e). These investigations have illustrated two major pathways employed in the initial steps of *o*-phthalate degradation. Most Gram-negative phthalate degraders, including all the pseudomonads tested, have been found to metabolize *o*-phthalate via 4,5-dihydroxyphthalate and protocatechuate (Nakazawa & Hayashi, 1977, 1978; Nomura *et al.*, 1989a), whilst *Micrococcus* spp. have been shown to utilize 3,4-dihydroxyphthalate as an intermediate (Eaton & Ribbons, 1982a, b; Nomura *et al.*, 1989a) (Fig. 1).

The ability of many bacteria to degrade xenobiotics is conferred by degradative plasmids (Sayler *et al.*, 1990). Eaton & Ribbons (1982b) suggested that part of the

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Fig. 1. Established pathways for phthalate catabolism (adapted from Ribbons *et al.*, 1984). (a) 3,4-Dihydroxyphthalic acid; (b) 4,5-dihydroxyphthalic acid; (c) *o*-cleavage reaction; (d) *m*-cleavage reaction.

pathway enabling *Micrococcus* strain 12B to degrade phthalate and phthalate esters was encoded by a plasmid possessed by this organism, however only recently has conclusive evidence for plasmid involvement in phthalate degradation been presented. Saint & Ribbons (1990) determined that a 230 kbp plasmid termed MOP carried the genes enabling *Pseudomonas cepacia* Pc701 to grow at the expense of 4-methyl-*o*-phthalate and 4-hydroxy-*m*-phthalate. During the course of the study reported here Nomura *et al.* (1989b) showed that a number of phthalate degraders isolated in their study harboured large plasmids. One of these strains, *Pseudomonas putida* NMH102-2, isolated from an activated sludge unit at a plastics factory, was able to utilize 3% (w/v) *o*-phthalate as a carbon source via 4,5-dihydroxyphthalate and protocatechuate. This ability was found to be encoded by a 200 kbp conjugative plasmid termed PHT (Nomura *et al.*, 1990a).

The purpose of the study reported here was to investigate the molecular biology of phthalate degradation, and to identify phthalate-degrading genes that would be amenable to genetic manipulation. To this end a number of phthalate-degrading bacteria were isolated and studied for the presence of plasmids involved in phthalate degradation. One such plasmid is described, and evidence is presented that the phthalate-degrading genes are clustered on an unstable region of this plasmid.

METHODS

Bacterial strains. The bacterial strains and plasmids used and constructed in this study are described in Table 1.

Culture media and conditions. Solid and liquid minimal media used in the isolation and maintenance of phthalate-utilizing organisms were prepared as described by Eaton & Ribbons (1982b) with carbon sources provided at a final concentration of 5 mM unless otherwise stated. Nutrient agar and nutrient broth were prepared as described by the manufacturer (Difco). Luria broth and SB broth were prepared as described by Sambrook *et al.* (1989). *Escherichia coli* strains were cultured at 37 °C; all

other organisms were grown at 30 °C. Antibiotics were employed at the following concentrations: 25 µg kanamycin ml⁻¹, 100 µg ampicillin ml⁻¹, 1 mg streptomycin ml⁻¹, 500 µg chloramphenicol ml⁻¹, 30 µg tetracycline ml⁻¹ (10 µg ml⁻¹ for *E. coli*).

Isolation and identification of *o*-phthalate degraders. Activated sludge (1 g) from Pontypridd sewage works (South Wales) was used to inoculate 200 ml minimal medium containing 5 mM dibutylphthalate as sole carbon source. After incubation for 10 d at 30 °C, 5 ml of this culture was used to inoculate 200 ml *o*-phthalate minimal medium. The turbidity of this culture increased over a 72 h period of incubation at 30 °C after which aliquots were plated onto solid *o*-phthalate media. Phthalate-degrading isolates were maintained in pure culture and identified using the API 20NE system according to the manufacturer's instructions (API catalogue no. 2005).

Plasmid DNA isolations and manipulations. Large plasmids were extracted from the phthalate-degrading isolates by the method of Wheatcroft & Williams (1981). Miniprep plasmid DNA was routinely isolated from *E. coli* by the method of Holmes & Quigley (1981). Cosmid DNA was prepared by an alkaline lysis method described by Birnboim & Doly (1979), and caesium-chloride-purified DNA from plasmids of less than 30 kbp was prepared as described by Guerry *et al.* (1973). Routine DNA manipulations were carried out as described by Sambrook *et al.* (1989). Non-radioactive detection of DNA-DNA hybridization was carried out with biotinylated DNA probes labelled using the Bio-Rad Random Priming Biotinylation kit. Hybrid detection was achieved by a chemiluminescent protocol, Southern Light, supplied by Tropix.

Preparation of a cosmid library of plasmid DNA from UCC61.

A library of partially *Sau*3A-digested plasmid DNA from strain UCC61 was cloned in the cosmid vector pLAFR3 (Staskawicz *et al.*, 1987). Packaging and infection protocols were taken from the Gigapack Plus *in vitro* packaging kit (Stratagene).

Plasmid curing. The isolation of strains cured of plasmids was achieved by daily L-broth subculture at 30 °C over 5 d with or without mitomycin C at 10 mg ml⁻¹. Percentage curing was estimated by plating suitable dilutions onto succinate minimal media and testing colonies for loss of the ability to grow on *o*-phthalate.

Matings. Donor, recipient and mobilizing strains (where necessary) were harvested, resuspended in 0.95% saline and mixed on a cellulose acetate filter placed on a nutrient agar plate. After overnight incubation at 30 °C the bacteria on the filter were resuspended in 1 ml 0.95% saline, and aliquots plated on selective media.

Conversion of quinolinic acid by organisms growing on *o*-phthalate. Demonstration of activity of enzymes involved in the initial reactions in the degradation of *o*-phthalate was achieved as described by Nomura *et al.* (1989a). Essentially, organisms grown on minimal media supplemented with 5 mM quinolinic acid were challenged with the addition of a diazotized *p*-nitroaniline reagent (50 vols of a 0.3%, w/v, solution of *p*-nitroaniline in 0.8 M HCl and 3 vols 5%, w/v, NaNO₂ solution mixed immediately before use.) The production of an immediate red colour (maximum *A*₅₁₂) indicates the generation of 2,3-pyridinediol from quinolinic acid, a reaction that has been shown to be catalysed specifically by phthalate 4,5-dioxygenase.

Preparation of cell-free extracts. Cell cultures grown in the presence of aromatic substrates were resuspended in assay buffer and sonicated on ice using an MSE Soniprep 150 at an amplitude

Table 1. Bacterial strains and plasmids

Strain/plasmid	Genotype/phenotype	Reference
Bacterial strain		
<i>C. acidovorans</i> UCC61	Wild-type, Pht ⁺ carrying pOPH1	This study
<i>C. acidovorans</i> UCC60	Pht ⁻ mitomycin-C-cured derivative of UCC61	This study
<i>C. acidovorans</i> UCC62	Pht ⁻ derivative of UCC61 carrying pOPH2, a deleted derivative of pOPH1	This study
<i>P. putida</i> KT2440	<i>P. putida</i> mt2 derivative	Franklin <i>et al.</i> (1981)
<i>P. putida</i> PaW340	Trp ⁻ Sm ^r	Jeenes & Williams (1982)
Plasmid		
pRK2013	Km ^r Tra ⁺ mobilizing plasmid	Ditta <i>et al.</i> (1980)
pKT231	Km ^r Sm ^r cloning vector	Bagdasarian <i>et al.</i> (1981)
pLAFR3	Tc ^r cosmid vector	Staskawicz <i>et al.</i> (1987)
pNJ5000	Tc ^r unstable derivative of RP4; mobilizing plasmid	Grinter (1983)
pEP1	Partial <i>Eco</i> R1 digest fragment of HA from pOPH1 cloned in pKT231	This study

of 10–14 μ for 6 $\times$ 30 s cycles employing a 20 s cooling period between cycles. The extract was centrifuged in a microfuge for 1 min to remove cell debris, and the resulting supernatant (termed the cell-free extract) was used immediately. In the assay of protocatechuate-4,5-dioxygenase activity, ethanol at a 10 % (v/v) final concentration was added to the cell-free extract.

Assay of enzymes involved in o-phthalate degradation.

Demonstration of protocatechuate *meta*-cleavage was achieved by monitoring the production of γ -carboxy- α -hydroxymuconic semialdehyde as described by Stanier *et al.* (1966). The activity of protocatechuate 4,5-dioxygenase was assayed as described by Ono *et al.* (1970). The activity of 4,5-dihydroxyphthalate decarboxylase was measured as described by Nakazawa & Hayashi (1977) recording the change in A_{230} due to the conversion of 4,5-dihydroxyphthalate to protocatechuate. Activity of 4,5-dihydro-4,5-dihydroxyphthalate dehydrogenase was estimated by following the reduction of NAD⁺ to NADH, recording the change in A_{340} . Protein content in cells and cell-free extracts was estimated by the Lowry method. Purified preparations of the substrates 4,5-dihydro-4,5-dihydroxyphthalate and 4,5-dihydroxyphthalate were generously provided by Professor D. W. Ribbons (Institute of Organic Chemistry, University of Technology, Graz, Austria).

Oxygen uptake assays were performed with suspensions of freshly harvested, washed cells in a Clarke-type oxygen electrode as described by Evans & Venables (1990).

RESULTS

Isolation and characterization of o-phthalate-degrading bacteria

A total of 23 phthalate-utilizing strains were isolated from activated sludge by selective culture at 30 °C in minimal medium containing phthalate as sole source of carbon and energy. Each of these strains was investigated, using the method of Wheatcroft & Williams (1981), for the presence

Table 2. Identification of phthalate-utilizing bacteria

Strain designation	Tentative identity
A	<i>Pseudomonas</i> sp.
F	<i>Pseudomonas paucimobilis</i>
H	<i>C. acidovorans</i>
K	<i>C. acidovorans</i>
Q	<i>Agrobacterium radiobacter</i>
S	?
U	<i>A. radiobacter</i>

of plasmids. Seven strains were found to harbour single large plasmids which were approximately sized in the range 100–250 kbp by restriction endonuclease digestion and gel-electrophoresis. As subsequent genetic investigation of phthalate genes would be facilitated if they were plasmid-borne, these seven strains were selected for further characterization.

All seven plasmid-bearing strains were found to be Gram-negative, oxidase-positive rods that failed to produce fluorescent pigments on King's B-medium (King *et al.*, 1964). These and other metabolic tests resulted in the tentative identification shown in Table 2. Organism 'S' was not attributable to any known genus on the basis of the tests used. All strains showed strong growth on *o*-, *m*- and *p*-phthalate, *p*-hydroxybenzoate, protocatechuate and *m*-hydroxybenzoate after incubation at 30 °C on solid medium for 72 h, except for F, Q and U, which failed to grow on *m*-hydroxybenzoate. Additionally, F grew weakly on 4-methyl-*o*-phthalate.

Table 3. Growth rate of *o*-phthalate utilizers, and oxygenation of quinolinate

The carbon source for growth rate measurement is *o*-phthalate. Quinolinate oxygenation is measured as production of 2,3-pyridinediol [A_{512} units (mg protein) $^{-1}$ after incubation for 24 h].

Strain	Mean generation time (h)	Quinolinate oxygenation
A	1.4	37
F	1.9	8.3
H	2.0	35
K	2.2	34
Q	4.0	40
S	1.6	43
U	3.2	15

Table 3 shows the mean generation times of the seven strains when growing on *o*-phthalate minimal medium, and also shows the amounts of 2,3-pyridinediol produced in the quinolinate-conversion test of Nomura *et al.* (1989a). This test measures the activity of phthalate 4,5-dioxygenase (see Methods). Data in Table 3 indicate that all seven strains produced 2,3-pyridinediol in the quinolinate test, and therefore must possess phthalate 4,5-dioxygenase. This indicates that all isolates use the catabolic route involving 4,5-dihydroxyphthalate which has been found to be used by most other Gram-negative phthalate utilizers (Nomura *et al.*, 1989b).

As strains A and H were metabolically identical and showed identical plasmid restriction profiles, they were considered to be different isolates of the same organism. Strain H was designated *Comamonas acidovorans* UCC61, and all further work was carried out on this strain.

Plasmid involvement in *o*-phthalate catabolism by *C. acidovorans* UCC61

The phthalate-degrading ability of UCC61 was found to be unstable. After approximately 100 generations in nutrient broth, 2% of cells showed spontaneous loss of ability to utilize *o*-phthalate (Pht⁻ phenotype), and the appearance of such Pht⁻ variants was accelerated by treatment with the plasmid-curing agent mitomycin C. Neither spontaneous nor mitomycin-C-induced Pht⁻ variants showed any detectable reversion to Pht⁺. Spontaneous Pht⁻ strains were shown to retain a large plasmid, but with the loss of specific restriction fragments totalling about 70 kbp. In mitomycin-C-induced Pht⁻ strains no plasmid could be detected. An example of each type of Pht⁻ strain was retained for further study. These were designated UCC62 (containing the plasmid that has undergone deletion) and UCC60 (no longer containing a plasmid).

In the presence of the RP4-derived mobilizing plasmid pNJ5000, the plasmid of UCC61 was conjugally trans-

Fig. 2. Restriction digests of pOPH1 and pOPH2. Lanes: 1, lambda/HindIII size standard; 2, pOPH1/HindIII; 3, pOPH2/HindIII.

ferred to a Cm^r Sm^r derivative of UCC60 at a frequency of 2.5×10^{-5} per donor, and with concomitant restoration of the Pht⁺ phenotype. This indicated that the plasmid of UCC61 was involved in encoding *o*-phthalate catabolism and it was, accordingly, designated pOPH1. The deleted form of pOPH1 that is present in UCC62, and which no longer encodes the Pht⁺ phenotype, was designated pOPH2. Transfer of plasmid pOPH1 to UCC60 in the absence of a mobilizing plasmid was not detected, so pOPH1 is presumably a non-conjugative plasmid. Mobilized transfer of pOPH1 to *P. putida* PaW40 could not be detected using *o*-phthalate utilization as the selection, but this may reflect lack of Pht expression in *P. putida* rather than lack of transfer.

Physical characterization of plasmid pOPH1

Digestion of pOPH1 with restriction endonuclease HindIII yielded 32 fragments, of size range 25–0.75 kbp, and totalling 270 kbp; digestion with EcoRI yielded 48 fragments, of size range 16.5–0.7 kbp, and totalling 274 kbp; XhoI digestion generated 19 fragments in the size range 0.45–12.2 kbp (total 63 kbp) plus a fragment too large to size accurately. The size of pOPH1 is therefore about 270 kbp. A gel showing the larger fragments from HindIII digests is presented in Fig. 2.

The deleted plasmid, pOPH2, differed from pOPH1 in the loss of restriction fragments totalling about 70 kbp in

Fig. 3. Restriction endonuclease map of the Pht element of plasmid pOPH1. The arrows labelled LHS and RHS indicate the known extent of the directly repeated sequences that flank the Pht element. Lines with labels prefixed CC or HC represent the extents of cosmid clones used in the map construction. The map was constructed from restriction digest analysis and hybridization probing using the following fragments as probes: BK, CC6, ET, EZ37/38, H25.4, HJ, HL, HZ7/8 and probes 1–4 (extents of the latter are shown as dashed lines). EP1 is a fragment derived from partial digestion with *EcoRI*, and carries the genes that encode the Pht phenotype.

size; several of the lost *HindIII* fragments can be identified in Fig. 2. pOPH2 lacked all *XhoI* fragments except the largest (fragment XA). Independently generated examples of pOPH2 had always lost the same fragments, and formation of pOPH2 did not produce any new fragments not seen in pOPH1 digests. These observations are consistent with the formation of pOPH2 by deletion of a defined element of DNA from pOPH1 by recombination between directly repeated flanking sequences as seen in the catabolic plasmid pTDN1 (Saint *et al.*, 1990). This element must carry sequences involved in coding the Pht⁺ phenotype, and is referred to subsequently as the Pht element.

Construction of a restriction map of the Pht element of pOPH1

To investigate the Pht element, a library of pOPH1 DNA was prepared in *E. coli* by cloning *Sau3A* partial digest fragments into the cosmid vector pLAFR3. Clones containing DNA from the Pht element were selected initially by a hybridization screen using fragment HJ (excised from a gel and cloned into pHG327) as a hybridization probe for Pht DNA. HJ is a 10.5 kbp *HindIII* fragment (Fig. 2) which is present in pOPH1 but not pOPH2 and is therefore part of the Pht element. Of 480 cosmids probed with fragment HJ, about 25% gave a positive hybridization signal. These were screened for size of cloned fragment, and one of the largest,

designated HC25.4 and containing an insert of about 32 kbp, was selected for further study. Cosmid HC25.4 was mapped by restriction digest and DNA–DNA hybridizations, and its physical extent is shown in Fig. 3. As all of the fragments assigned to cosmid HC25.4 were missing from the deleted plasmid pOPH2, it was concluded that it represented an internal segment of the Pht element.

Cosmids containing Pht DNA were also selected by mobilizing clones into the Pht[−] strain UCC60 and selecting for growth on o-phthalate. Of 960 cosmids tested in this way about 30% complemented the Pht[−] phenotype of UCC60 and were designated ‘complementing cosmids’ (prefixed by ‘CC’). Several complementing cosmid clones were mapped by restriction and hybridization. The sections of pOPH1 that are represented by these cosmids are shown in Fig. 3. CC6 contained most of *HindIII* fragment HO, and therefore overlapped with hybridizing clone HC25.4. In addition, CC6 contained a small fragment (HZ8) which was retained in the deleted plasmid pOPH2, thus indicating that this cosmid covered the right-hand terminus of the Pht element. CC5 and CC19 also contained HZ8 together with numerous other fragments that are retained by pOPH2 (e.g. EZ24 and EZ31), indicating that the inserts in these cosmids extended well beyond the right-hand terminus of the Pht element. The hybridizing clone HC25.4 and the complementing clone CC6 together accounted for all the

Fig. 4. Hybridization of restriction fragment BK with restriction digests of plasmids pOPH1 and pOPH2. Hybridized Southern blots are: lanes 1 and 2, pOPH1/*Hind*III and pOPH2/*Hind*III; lanes 3 and 4, pOPH1/*Eco*RI and pOPH2/*Eco*RI. Fragments referred to in the text are labelled using names shown in Fig. 3.

restriction fragments of the Pht element except for XC (partially present in HC25.4), HP, EJ, BE, and BM, which had, therefore, to be located at the left-hand end.

The discovery, described below, that the Pht element is flanked by repeated sequences suggested the use of a cloned HZ8 fragment to probe for hybridizing cosmid clones that might represent either terminus of the element. This approach produced cosmid HC17 which represents the left-hand terminus and was used to complete the Pht map shown in Fig. 3.

The Pht element is flanked by repeated sequences

Fig. 3 shows a sequence of five restriction sites enclosing fragments HZ7 and EZ30 which is directly repeated at the other end of the map around HZ8 and EZ31. Evidence for the existence of repeated sequences at the ends of the Pht element was first indicated by hybridization studies with the 3.2 kbp *Bam*HI fragment BK. Fig. 4 shows that fragment BK hybridized with HZ2, HZ8 and EZ31 which are retained in the deleted plasmid pOPH2,

and with HA, ET and EZ16 which are lost in pOPH2, indicating that this fragment spans the right-hand terminus of the Pht element. However, BK also hybridized with fragments clustered around the left-hand terminus, including HP, HF, EZ19 (Fig. 4) and BC (not shown), indicating sequence repetition at the two termini. Other hybridizations using fragments HZ8 and EZ31 as probes supported this conclusion.

The most likely mechanism for deletion of the Pht element from pOPH1 is by recombination between its terminal repeats, as shown for the plasmid pTDN1 (Saint *et al.*, 1990). If this mechanism is in fact operative, any restriction fragments that are contained entirely within each repeated sequence should be retained by the parent plasmid, but only as single copies. Fig. 4 shows that the strength of the hybridization signals for HZ8 and EZ31 was considerably weaker in preparations of pOPH2 than pOPH1, even though total amounts of DNA loaded on the gel were approximately equal for the two plasmids. The difference in signal strength must therefore arise from a lower copy number of these fragments in pOPH2, and supports the view that HZ8 and EZ31 are internal fragments of directly repeated sequences.

Hybridization of fragment BK with the right-hand terminus fragments HA, HZ8, ET, EZ16 and EZ31 can be explained by direct overlap of the probe and its target sequences. However, the hybridization observed with fragments HZ2 and EZ24 suggests that in these cases the probe is interacting with a duplication within the repeated sequence (as in the repeated ends of an IS element). Similarly at the left-hand terminus, BK hybridizes with fragments on both sides of the terminal sequence, again suggesting repetition within the sequence. BK also showed weak hybridization with a few fragments that were located outside of the Pht element but otherwise unmapped.

In summary, Fig. 3 shows the Pht element to be approximately 70 kbp in length, and to be flanked by two direct repeats that may be similar in structure to IS elements. Each repeat includes restriction fragments HZ8 and EZ31, and as these overlap only marginally, their combined size of about 1.9 kbp represents the minimum size of the repeat. As neither EZ16 nor EZ24 are contained entirely within the repeat, the 5.2 kbp between their distal termini must represent a little over its maximum size.

The location of *pht* genes in the Pht element

As cosmid clones CC19 and CC20 both complement the Pht⁻ strain UCC60, the genes for *o*-phthalate utilization must lie between their termini. This was confirmed by cloning the right-hand end of fragment HA from CC19, and subcloning from this the fragments ET and EW, and (from a partial *Eco*RI digest) the composite fragment EW-EZ32-ET into vector pKT231. When these clones were introduced into UCC60 by pRK2013-mobilized conjugation, only clone pEP1 (containing the composite fragment) restored growth on *o*-phthalate, indicating that neither of the single *Eco*RI fragments carries all the *pht* genes.

Table 4. Activities of enzymes involved in phthalate catabolism

Phthalate oxygenase is measured by conversion of quinolinate as detailed in Methods. DHase (4,5-dihydro-4,5-dihydroxyphthalate dehydrogenase) [nmol NAD⁺ reduced min⁻¹ (mg protein⁻¹)] and DCase (4,5-dihydroxyphthalate decarboxylase) [nmol substrate utilized min⁻¹ (mg protein⁻¹)] were measured in cell-free extracts. All data are means of at least three independent determinations.

Strain	Growth substrate	Phthalate oxygenase	DHase	DCase
UCC61	Succinate	2.0	< 1	< 1
UCC61	Succinate + phthalate	59	210	210
UCC62	Succinate + phthalate	1.6	< 1	< 1
UCC60	Succinate + phthalate	1.6	< 1	< 1
UCC62(pEP1)	Succinate + phthalate	27	86	90
UCC60(pEP1)	Succinate + phthalate	16	350	250

Table 5. Oxidation of o-phthalate and protocatechuate by wild-type and deleted strains

Data shown are rates of oxygen uptake [nmol min⁻¹ (mg protein⁻¹)] by suspensions of whole cells at 30 °C as described in Methods. UCC61 contains the plasmid pOPH1; UCC62 contains pOPH1 from which the Pht element has been deleted; PCA, protocatechuate. High levels of succinate oxidation were recorded in all cell suspensions. Data are means of at least three independent determinations.

Growth substrate	Strain	Assay substrates	
		PCA	o-Phthalate
Succinate	UCC61	< 1	< 1
	UCC62	< 1	< 1
PCA	UCC61	88	< 1
Phthalate	UCC61	82	147
Succinate + PCA	UCC61	86	5
	UCC62	106	< 1
Succinate + phthalate	UCC61	43	75
	UCC62	< 1	< 1

The biochemistry of phthalate catabolism by *C. acidovorans* UCC61

As indicated earlier, the quinolinate conversion test had indicated that UCC61 catabolized o-phthalate by 4,5-dioxygenation, a step that would be likely to result in protocatechuate production via 4,5-dihydroxyphthalate as shown in Fig. 1. Direct evidence for the operation of this pathway was obtained by measuring the activities of the three relevant enzymes. Table 4 shows the activities of o-phthalate 4,5-dioxygenase, 4,5-dihydro-4,5-dihydroxyphthalate dehydrogenase and 4,5-dihydroxyphthalate decarboxylase in a variety of strains grown on either succinate minimal medium, or succinate minimal medium supplemented with phthalate. All three activities were significantly induced in UCC61 (carries pOPH1) by inclusion of o-phthalate in the growth medium. In contrast to this, no significant activity of any of the enzymes was

Table 6. Induction of protocatechuate-4,5-dioxygenase activity during growth with different carbon sources

Specific activities [nmol min⁻¹ (mg protein⁻¹)] were measured in cell-free extracts. Data are means of at least three independent determinations. PCA, Protocatechuate.

Strain	Carbon source in growth medium			
	Succinate	PCA	Phthalate	Succinate + phthalate
UCC61	0.01	1.6	1.4	0.36
UCC60	0.01	1.7	—	0.01
UCC62	0.01	1.4	—	0.01

induced in the Pht⁻ derivatives UCC60 (carries no plasmid) and UCC62 (carries deleted plasmid). All three activities were restored to the Pht⁻ derivatives by the clone pEP1 which carries the composite EW-EZ32-ET fragment of plasmid pOPH1. These data provide strong evidence for the catabolism of o-phthalate by the pathway shown in Fig. 1(b), and indicate that the first three enzymes are plasmid encoded. Additional evidence for the proposed pathway was provided by respiration studies with the predicted intermediate 4,5-dihydro-4,5-dihydroxyphthalate. In UCC61, respiration of this compound was induced over 150-fold by inclusion of o-phthalate in the growth medium, whereas in UCC62, no detectable activity was induced by o-phthalate.

The pathway constituted by the above three enzymes would generate protocatechuate. The observation that UCC61, 60 and 62 all utilized protocatechuate as a carbon source indicated the existence of a chromosomally encoded pathway for the degradation of this compound. In oxygen uptake studies using whole cells the above three strains all showed inducible oxidation of protocatechuate (Table 5), and cell-free extracts of all three showed inducible protocatechuate-4,5-dioxygenase activity (Table 6), confirming that this compound is degraded by a chromosomally encoded pathway, and

Table 7. Mean generation times during growth on phthalate isomers

Bacteria were grown on minimal medium supplemented with the appropriate phthalate isomer.

Strain	Phthalate isomer		
	<i>ortho</i>	<i>meta</i>	<i>para</i>
UCC61	1.98	1.40	1.55
UCC60	0	1.30	1.51
UCC62	0	1.44	1.55

identifying a 4,5-cleavage as the initial step. Protocatechuate was an effective inducer of its own metabolism in all three strains, but phthalate was an inducer only in strain UCC61, which carries pOPH1. This indicates that during catabolism of phthalate by UCC61, protocatechuate oxidation is not induced directly by phthalate but by one or more of its catabolic products (including protocatechuate).

UCC61 utilized both *m*-phthalate and *p*-phthalate as carbon sources, and these isomers supported more rapid growth than *o*-phthalate (Table 7). Pht⁻ strains UCC60 and 62 (which have lost the ability to grow on *o*-phthalate) retained full capacity to grow on *m*- and *p*-phthalate (Table 7), indicating that these isomers are catabolized by a different, and chromosomally encoded, pathway.

Growth of UCC61 on *o*-phthalate did not induce oxidation of either *m*- or *p*-phthalate showing that the different pathways are under different regulatory systems.

DISCUSSION

C. acidovorans UCC61 was found to metabolize the *o*-, *m*- and *p*-isomers of phthalic acid. *o*-Phthalate degradation was shown to be encoded by the 270 kbp plasmid pOPH1, but degradation of the *m*- and *p*-isomers appeared to be chromosomally encoded. UCC61 utilizes the 4,5-dihydroxylation pathway for *o*-phthalate degradation, with protocatechuate as the ring-cleavage substrate, a route described previously for a closely related species *Pseudomonas testosteroni* (Nakazawa & Hayashi, 1977, 1978). No evidence was found for the existence of a 3,4-dioxygenase cleavage pathway in UCC61, though in some bacteria the presence of this oxygenase can be hidden by the sensitivity of the 4,5-enzyme (D. W. Ribbons, personal communication). If such a hidden pathway is present in UCC61, it would have to be encoded on the Pht element along with the 4,5-pathway, as it would otherwise be exposed by the deletion of the Pht element in UCC62. Metabolism of all three phthalate isomers by a single strain has previously been reported only once, again in a *P. testosteroni* strain (strain EN5A, Keyser *et al.*, 1976), highlighting the metabolic diversity of this subset of the pseudomonads.

The genes for *o*-phthalate degradation on plasmid pOPH1 were located within a 70 kbp section (termed the Pht element) that is flanked by direct repeats, and is frequently excised as a result of recombination between the repeats. Loss of the Pht element caused complete loss of the Pht⁺ phenotype. Reintroduction of a 10.7 kbp fragment of the deleted region (cloned as pEPI) restored the activities of phthalate-degrading enzymes to both cured and deleted-plasmid strains of UCC61. The fact that parts of pEPI (maximum size 5.2 kbp) cloned and reintroduced separately into UCC62 were unable to restore any enzyme activity associated with the Pht⁺ phenotype suggests that the *pht* genes may be present as an operon spanning the central region pEPI insert. This interpretation fits the predicted size of such an operon based on the known sizes of the enzyme proteins. Two of the enzymes have been purified and characterized from pseudomonads: the phthalate oxygenase complex of *Pseudomonas fluorescens* comprised phthalate oxygenase and phthalate oxygenase reductase components of combined molecular mass 82 kDa (Batie *et al.*, 1987), while the 4,5-dihydroxy phthalate decarboxylase of *P. cepacia* was sized at 66 kDa (Pujar & Ribbons, 1985). Adding in a gene of at least 1 kbp for the diol dehydrogenase, the minimum size for the *pht* operon would thus be around 6 kbp, easily accommodated on a 10.7 kbp fragment but certainly disrupted by *Eco*R1 digestion to give sub-fragments of maximum size 5.2 kbp. In the only other reported *o*-phthalate-degrading plasmid, PHT, a 7 kbp *Eco*R1 fragment was able to restore the Pht⁺ phenotype to mutant derivatives of *P. putida* NHM101-2, and this fragment was also able to confer the Pht⁺ phenotype to other naturally Pht⁻ strains of *P. putida* (Nomura *et al.*, 1990a).

The flanking of a catabolic region by direct repeats appears as a common feature in the structure of metabolic plasmids, and the loss of such regions following recombination between the repeats has been demonstrated in TOL plasmids (Meuleien *et al.*, 1981), the isopropylbenzene-degrading plasmid pRE4 (Eaton & Timmis, 1986), the chlorobenzoate-degrading plasmid pBR60 (Wyndham *et al.*, 1988) and the toluidine-degrading plasmid pTDN1 (McClure & Venables, 1987; Saint *et al.*, 1990; Saint & Venables, 1990). In the latter plasmid the direct repeats that flank the catabolic region have an apparently identical restriction map to the equivalent repeats in pOPH1, indicating that there is likely to be a high degree of homology between them. Although pOPH1 and pTDN1 were characterized in the same laboratory, geographic proximity is not considered a significant reason for the similarity of their direct repeats as plasmid pBR60 isolated in Canada also has directly repeated regions with the same restriction map as those of pOPH1 (Wyndham & Straus, 1988; Wyndham *et al.*, 1988). The presence of these related repeats in analogous situations on diverse plasmids suggests that they may direct the evolution of these plasmids. Roles for insertion sequences (IS) have been described in plasmid rearrangement (Gaffney & Lessie, 1987) and transposition (Grindley & Reed, 1985), and the failure of an IS-mediated

cointegrate to resolve provides a scenario for the formation of a region flanked by repeated copies of an element, as apparent in these plasmids. However, although catabolic regions have been shown to act as transposons, for example Tn4652 from the TOL plasmid pWW0 (Tsuda & Iino, 1988), there is no evidence that either the repeats or catabolic regions of pOPH1 or pTDN1 possess any functional transposition activity. Elucidation of the precise relationships between the related repeats of pOPH1, pTDN1 and pBR60 awaits a comparison of full nucleotide sequences for these elements.

A survey by Nomura *et al.* (1989b) has reported the occurrence of the phthalate 4,5-dihydroxylation pathway in a wider range of Gram-negative genera than found in a survey some 20 years previous. They conclude that this expansion of the phthalate phenotype may be evidence of the spread of *pht* genes via a common mechanism, possibly involving phthalate plasmids such as PHT. Although this seems a tempting explanation on a local basis, it must be noted that our Pht⁺ isolates from the UK (Table 2) covered a similar range of genera to those found in the Nomura survey (carried out exclusively in Japan), and achievement of such global distribution in two decades by gene transfer seems unlikely. It is also apparent that many of the 4,5-dihydroxylation genes in other phthalate degraders are apparently chromosomally encoded, and sequence homology between these and the plasmid-encoded genes, and between the various plasmids themselves, should be established to verify any possibility of horizontal gene transfer.

Finally, it is the 4,5-dihydrodiol and 4,5-dihydroxy derivatives of *o*-phthalic acid, shown as intermediates in the pathway utilized by *C. acidovorans* UCC61, that are of most interest in biotechnology research (Nomura *et al.*, 1990b). These reactive compounds are raw materials for a variety of polymer manufacturing processes but are recalcitrant to artificial chemical synthesis. Thus patents have been sought for their isolation through biotransformation of *o*-phthalic acid using pseudomonad strains with lesions introduced in the phthalate 4,5-dihydroxylation pathway. The plasmid pOPH1 provides a source of phthalate degradation genes for further exploitation of this pathway through directed study of individual enzymes.

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