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Conserved and Hybrid *meta*-Cleavage Operons from PAH-degrading *Burkholderia* RP007

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We have compared the sequence and gene order of *meta*-cleavage pathway operons from α- and γ-subgroups of the *Proteobacteria* with operons from *Burkholderia* sp. strain RP007 which belongs to the β-subgroup of the *Proteobacteria*. *Burkholderia* RP007 was isolated for its ability to degrade phenanthrene and contains two *meta*-cleavage operons. One exhibits a comparable gene order to previously characterised γ-subgroup *Proteobacterial* (*Pseudomonas*) *meta* operons, whilst the other has distinctive features present in both α- and γ-subgroup *Proteobacterial* (*Sphingomonas* and *Pseudomonas*) *meta* operons. Gene sequence conservation, highlighted by examining the phylogeny of *Proteobacterial* catechol 2,3-dioxygenase sequences, reveals that sequences generally cluster in a manner which correlates with the taxonomic grouping of the *Proteobacterial* subgroup from which they originated.

During the microbial degradation of aromatic compounds initial substrates are converted by distinct enzyme systems to dihydroxylated intermediates that are substrates for a few key ring-cleavage enzymes (1). An example are the extradiol dioxygenases which are central to *meta*-cleavage pathways (2). *Meta*-cleavage pathways catalyse the conversion of catechols (or substituted catechols), via 2-hydroxymuconic semialdehyde, to pyruvate, acetaldehyde and acetate. The *meta*-cleavage operon gene which encodes the enzyme catechol 2,3-dioxygenase (C23O) is often used in environmental biotechnology for monitoring the biodegradation of aromatic hydrocarbons in contaminated soils (3).

Sequence conservation amongst *Proteobacterial meta*-cleavage operon genes suggests they are all closely related and derived from a common ancestor (4). Evolution has subsequently conspired to produce different gene arrangements amongst *Proteobacterial meta*-cleavage operons, and this is particularly evident for *Pseudomonas* and *Sphingomonas* spp. The widely studied *Pseudomonas* (γ-subclass of the *Proteobacteria*) *meta*-cleavage operons (5–9) have a characteristic gene order, whilst *Sphingomonas* (α-subclass of the *Proteobacteria*) *meta*-cleavage operons (10–13) have a different gene order. In this study we were interested in comparing β-subclass *Proteobacterial meta*-cleavage operons with those from other *Proteobacterial* subgroups. *Burkholderia* RP007, originally isolated for its ability to degrade phenanthrene, is a member of the β-subclass of the *Proteobacteria* and contains a distinctive set of PAH catabolic genes (14, 15). We will describe the isolation, characterisation, and subsequent comparison of two distinctive *meta*-cleavage gene clusters from *Burkholderia* RP007 with previously described sequences.

MATERIALS AND METHODS

Bacterial strains. *Burkholderia* sp. RP007 (14) has been deposited in the ICMP culture collection (Landcare Research, Private Bag 92170, Auckland, New Zealand) as strain number ICMP 13529. The organism was routinely maintained on Plate Count Agar (Difco Laboratories), or on solid mineral salts medium using a phenanthrene overlay. Minimal medium (MM) contained Na_2HPO_4 (4 g/L), KH_2PO_4 (2 g/L), $(NH_4)_2SO_4$ (1 g/L), and 2 ml/L mineral salts (16). Incubation was at 28°C. *Escherichia coli* DH5α was used as the host strain for pUC18 plasmids and derivatives. The strains and plasmids used in this work are described in Table 1.

DNA manipulation and sequencing. Genetic manipulations were performed as described by Sambrook *et al.* (17). The library of RP007 plasmid DNA was constructed using pUC18 and recombinant plasmids were isolated from *E. coli* using QIAprep spin columns (Qiagen, Germany). Derivatives of subclones for nucleotide sequencing were obtained using a double stranded Nested Deletion kit (Pharmacia, Sweden). Nucleotide sequencing was performed by the Waikato DNA Sequencing facility using a PRISM Ready Reaction DNA Terminator Cycle Sequencing kit (Perkin-Elmer), and the reactions were resolved using an Applied Biosystems Incorporated (ABI) model 377 sequencer. Completed sequence in both directions was assembled using the ABI Fractura and Assembler software, and analysed using ClustalW (18), and Omiga (Version 1) (Oxford Molecular Group,

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TABLE 1

Bacterial Strains and Constructs

Bacteria	Genotype/Phenotype	Reference/Source
Burkholderia sp. strain RP007	Degrades phenanthrene and naphthalene	(14)
Escherichia coli XL1 Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F' *proAB lacl*^{q}*ZΔM15* Tn*10* (Tet^r)]	Stratagene

Plasmids	Construction	Reference/Source
pUC18	General cloning vector	Pharmacia Biotech
pH1	10.5 kb *Hind*III fragment from RP007 inserted into the *Hind*III site of pUC18	This study
pH5	2.433 kb *Eco*RI derivative of pH1 in pUC18	This study
pH2	11.2 kb *Hind*III fragment from RP007 inserted into the *Hind*III site of pUC18	This study
pH2–E3	3.5 kb *Eco*RI/*Eco*RV fragment from pH2 in pUC18	This study
pH2–P2	3.8 kb *Pst*I fragment from pH2 in pUC18	This study
pH2–E2	6 kb *Eco*RI/*Sma*I fragment from pH2 in pUC18	This study

Oxford, England). Phylogenetic trees were constructed using the SEQBOOT, PROTDIST, NEIGHBOR, and CONSENSE programmes of the PHYLIP software package (version 3.57c) (19), and the trees were redrawn using the ClustalW homology data to determine branch lengths.

The nucleotide sequence of the pH1 and pH2 *meta*-cleavage genes are available in GenBank under accession numbers AF112136 and AF112137.

Enzyme analysis. Catechol 2,3-dioxygenase activity was assayed according to Nozaki (20). Absorbance maxima and extinction coefficients for the *meta* cleavage products of substrates assayed are: catechol, 375 nm and 36000 $M^{-1}cm^{-1}$; 3-methylcatechol, 388 nm and 15000 $M^{-1}cm^{-1}$; 4-methylcatechol, 382 nm and 31500 $M^{-1}cm^{-1}$; 2,3-dihydroxybiphenyl, 434 nm and 22000 $M^{-1}cm^{-1}$. Protein concentration in the cell-free extracts was estimated using the Bio-Rad Protein Assay kit calibrated with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

meta-Cleavage Pathway Genes from RP007

The *meta*-cleavage genes from *Burkholderia* RP007 are located on *Hind*III fragments of 10.5 and 11.2 kb, and were isolated from a pUC18 library of RP007 DNA by screening for catechol *meta*-cleavage activity. Clones pH1 and pH2 hybridised to plasmid DNA isolated from RP007 (data not shown). The activity of the C23Os encoded by pH1 and pH2 were distinctive and point toward different roles for these two enzymes in RP007 (Table 2). The pH1 C23O (*phnE1*) shows maximum activity toward catechol and significantly less activity towards substituted catechols, which corresponds to the activity seen in crude cell extracts of RP007 grown on phenanthrene. pH1 therefore contains part of a lower *meta*-pathway operon in RP007 that degrades catechol derived from salicylic acid which is formed following the degradation of naphthalene or phenanthrene. The pH2 C23O (*phnE2*) has a high activity towards both catechol and 3-methylcatechol which corresponds to the activity in RP007 crude cell extract following growth on benzoate. From this we can infer that induction of the pH2 *meta* pathway occurs in response to the presence of benzoate as a growth substrate. The low level of *meta*-cleavage activity seen in acetate-grown RP007 may result from low level constitutive expression of either one or both these C23Os, or even an additional extradiol dioxygenase. The PAH upper pathway extradiol dioxygenase PhnC also shows some activity towards catechol, however it is only

TABLE 2

Wild-Type and Recombinant *meta* Cleavage Activities from *Burkholderia* sp. RP007

	Burkholderia sp. RP007[a]			*E. coli* C23O clones[b]	
Enzyme substrate	Acetate	Benzoate	Phenanthrene	pH1	pH2
Catechol	49	251	251	1735 ± 294 (100%)	781 ± 78 (89%)
3-Methylcatechol	34	175	62	257 ± 25 (15%)	873 ± 102 (100%)
4-Methylcatechol	12	64	76	529 ± 105 (30%)	416 ± 47 (48%)
2,3-Dihydroxybiphenyl	—	—	—	0	64 ± 16 (7%)

[a] Specific activity (μmol/min/mg protein) against catechol and methylcatechols of crude cell extracts of *Burkholderia* sp. RP007 grown at the expense of different substrates.

[b] Specific activity (μmol/min/mg protein) and relative activity (in parentheses) of catechol 2,3-dioxygenases expressed by the pH1 and pH2 clones. Each value is the mean from three different cultures, showing standard deviation.

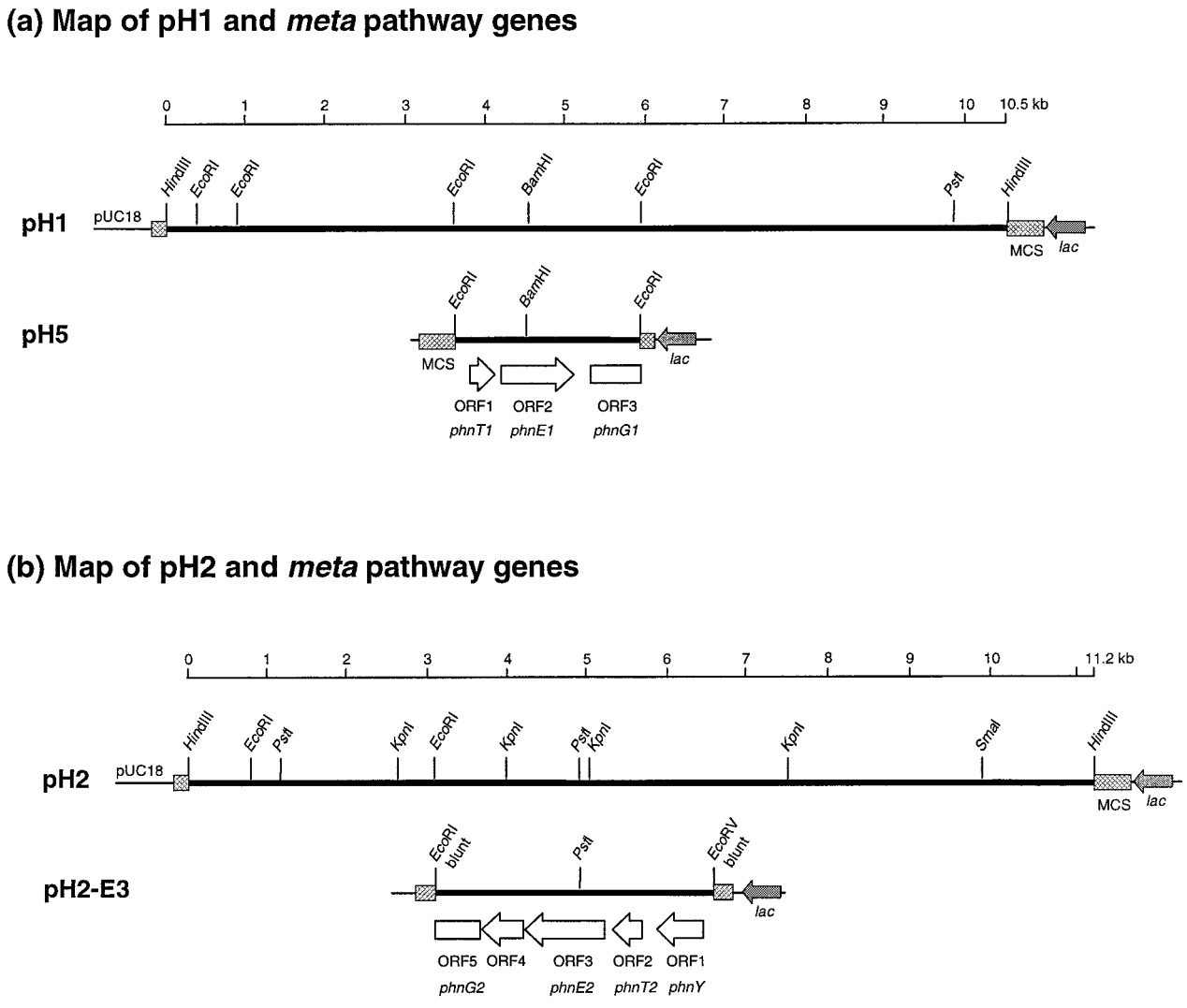

FIG. 1. (a) Physical map of the 10.5 kb *Hind*III insert of pH1 showing cleavage sites of common restriction enzymes, the orientation of the *lac* promoter, and the subcloned fragment pH5, showing the location of the open reading frames identified by nucleotide sequencing. (b) Physical map of the 11.2 kb *Hind*III insert of pH2 showing cleavage sites of common restriction enzymes, the orientation of the *lac* promoter, and the subcloned fragment pH2-E3, showing the location of the open reading frames identified by nucleotide sequencing.

expressed during growth at the expense of naphthalene or phenanthrene (14).

The nucleotide sequence of the *meta*-cleavage and associated genes present on clones pH1 and pH2 was determined. The 2433 bp nucleotide sequence of the pH5 insert (Fig. 1a) carries the C23O gene of pH1, and also contains two complete open reading frames (ORFs) and one partial ORF which extends beyond the 3' end of this fragment. The properties of these ORFs are summarised in Table 3. Upstream of the C23O gene (*phnE1*) lies a chloroplast-like ferredoxin (*phnT1*) (21), and downstream of *phnE1* the partially sequenced ORF is a putative 2-hydroxymuconic semialdehyde dehydrogenase (HMSD) that is initiated by a GTG start codon. The pH2 C23O gene was present on a 3.5 kb pH2-E3 subclone of pH2. Nucleotide sequencing of this region (3511 bp) revealed the presence of four complete ORFs and one partial ORF (Fig. 1b). The properties of the ORFs identified in the sequenced region are also summarised in Table 2. In common with the pH1 C23O gene *phnE1*, the pH2 C23O gene (*phnE2*) is preceded by a chloroplast-like ferredoxin gene (*phnT2*) which is initiated by a GTG start codon. Upstream of *phnT2* is a gene which shows low homology to several α subunits of aromatic 1,2-dioxygenases, and is possibly that for benzoate dioxygenase. Downstream of *phnE2* is ORF 4 a gene designated U and of no known function. The partially sequenced ORF downstream of ORF 4 is a putative HMSD gene.

The deduced amino acid sequence of ORF U shows significant homology to ORFs with no assigned function that are associated with other catabolic gene clus-

TABLE 3

Summary of Predicted Polypeptides Identified on 2433 bp of the pH1 Fragment, and 3511 bp of the pH2 Fragment from *Burkholderia* sp. RP007

Clone	ORF	Gene	Nucleotide position	No. of aa	Protein feature	% Similarity to analogous enzymes
pH1	1	*phnT1*	214–542	109	Chloroplast-like ferredoxin	32% *xylT*, 32% *nahT*
	2	*phnE1*	626–1576	316	C23O	54% *tdnC*, 56% C23OII (mt-15), 39% *nahH*
	3	*phnG1*	1799–	211[a]	HMSD	68% *dmpC*, 58% *xylG*
pH2	1	*phnY*	114–707	197	α subunit of aromatic 1,2 dioxygenase	18% *xylY*, 18% *benB*
	2	*phnT2*	951–1292	113	Chloroplast-like ferredoxin	39% *phnT1*, 30% *xylT*, 28% *nahT*
	3	*phnE2*	1330–2274	314	C23O	81% *tdnC*, 69% C23OII (mt-15), 57% *phnE1*
	4	U	2296–2745	149	Unknown function	41% *cmpX*, 41% *phnF*, 41% orf1126
	5	*phnG1*	2812–	233[a]	HMSD	73% *dmpC*, 62% *xylG*, 65% *cmpC*, 67% *phnG1*

[a] Number of amino acids encoded by the sequenced region of this gene; the amino acid similarity to analogous enzymes was calculated based on this N-terminal truncated amino acid sequence.

ters. These catabolic gene clusters include *meta*-cleavage genes in *Sphingomonas* spp. and *Bacillus*, and glycerol fermetation operons from *Citrobacter*, *Klebsiella*, and *Clostridium* spp. ORF U shares 41% amino acid sequence homology with a group of *Sphingomonas meta* pathway ORFs (that share >97% homology), which include cmpX of *Sphingomonas* sp. HV3 (10), phnF from *Pseudomonas* (*Sphingomonas* (22)) sp. DJ77 (23), and the translation of orf1126 from the *Sphingomonas aromaticivorans* plasmid pNL1 (13). Interestingly ORF U also shares 52% homology over 52 amino acids corresponding to the last third of a partially sequenced ORF preceding the C23O gene (*pheB*) of *Bacillus thermoleovorans* (24). Perhaps more suprising is the homology between the predicted amino acid sequences of ORF U and three ORFs of unknown function associated with the glycerol fermentation operons of *Citrobacter freundii* (25) (33%), *Clostridium pasteurianum* (26) (30%), and *Klebsiella pneumoniae* (Genbank accession U30903) (25%).

Phylogeny of meta-Cleavage Genes

The phylogeny of the RP007 C23O enzymes relative to other representative C23Os is shown in Fig. 2. PhnE1 and PhnE2 fall into a subfamily of the extradiol dioxygenases defined by Harayama & Rekik (27), and further refined by Eltis & Bolin (28) into five subfamilies I.2.A–I.2.E. The RP007 C23Os belong to the I.2.C subfamily of the C23Os which led us to consider the characteristics of this group. A feature of the I.2.C subclass, which is conserved in the RP007 C23Os, is the presence of an additional 24 bp (nt 474–498) in these C23O genes which is absent from all other classes. The contribution made by these additional 8 codons to enzyme function is not known. Another characteristic of the I.2.C phylogenetic group was proposed by Kukor & Olsen (29) who suggested that these C23Os may have arisen in response to low concentrations of substrates.

We view this phylogeny-based classification system for C23Os (28) from a different perspective. One in which the I.2 subfamilies bear the hallmarks of taxonomic confinement and therefore appear to mirror the taxonomic grouping of the host bacterium. Evolutionary pressures within the different *Proteobacterial* subclasses may subsequently contribute to distinctive properties for C23Os from different I.2 subfamilies (29). Thus the I.2.A subfamily contains C23O enzymes from the γ-subclass of the *Proteobacteria* (e.g. *Pseudomonas* spp.), C23Os from the α-subclass of the *Proteobacteria* (e.g. *Sphingomonas* spp.) belong to the I.2.B subfamily, whilst β-subclass C23Os belong to the I.2.C subclass. PheB which was isolated from *Bacillus thermoleovorans* would appear to belong to either the I.2.E subfamily (28) or to its own I.2.F subfamily (24) which may also mirror the taxonomic grouping of the host bacterium.

We concede that, although C23O sequences do bear the hallmarks of taxonomic confinement, horizontal transfer (30) makes taxonomic inferences based on catabolic gene sequences somewhat risky. Examples of a lack of phylogenetic congruence between aromatic catabolic genes present in β-subclass *Proteobacteria* and isolate taxonomy (16S rDNA gene phylogeny) include polycyclic aromatic hydrocarbon degrading bacteria (31), and 2,4-dichlorophenoxyacetic acid degrading bacteria (32, 33). Nevertheless within the I.2 subclasses we see 100% correlation for the I.2.A subfamily which contains C23O enzymes from only the γ-subclass of the *Proteobacteria*. The only apparent anomaly in the I.2.B group is the C23O gene from *Pseudomonas* DJ77 (23), however recently acquired taxonomic data indicates that strain DJ77 belongs to the *Sphingomonas* genus (22). Sequences such as *tdnC* and C23OII, which phylogenetically map to the I.2.C class, serve to highlight that horizontal transfer events do occur between different subgroups of the *Proteobacteria*. Both *tdnC* and C230II are atypical *Pseudomonas* C23Os,

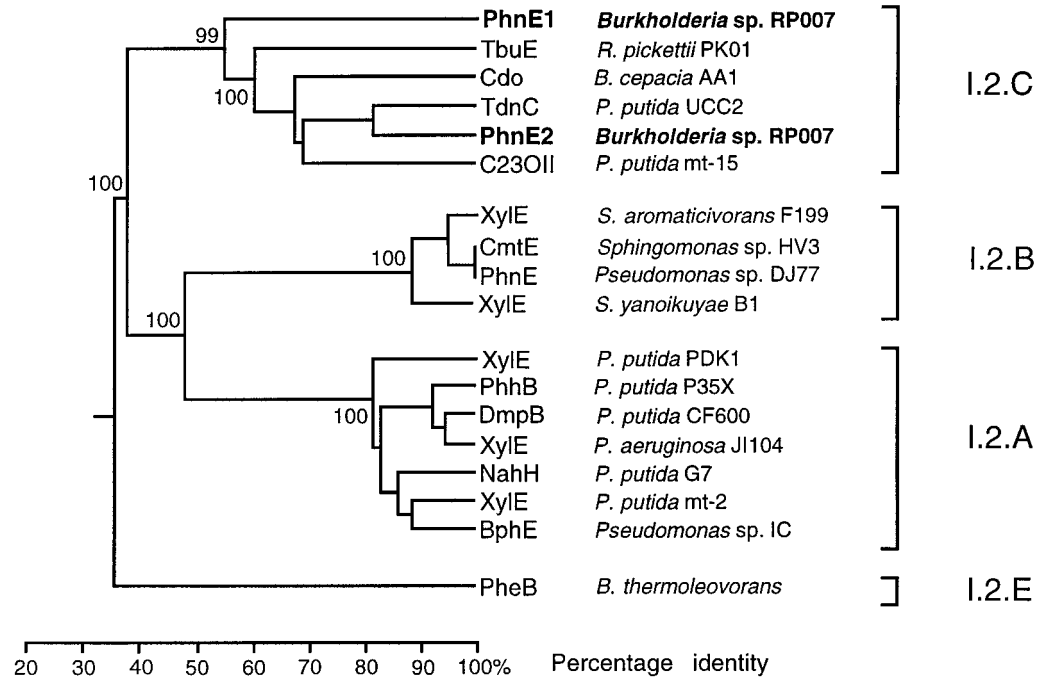

FIG. 2. Dendrogram based on a comparison of the complete deduced amino acid sequence of genes encoding catechol 2,3-dioxygenase enzymes (307 and 316 amino acid residues). The unrooted tree is rerooted using PheB (*B. thermoleovorans*) as an outgroup. Bootstrap values of the major branches are shown. The tree was constructed using the PHYLIP software package (version 3.57c) (19) using the SEQBOOT, PRODIST, NEIGHBOR and CONSENSE programmes. Dendrograms were redrawn to scale using the ClustalW (18) homology data. C23O subgroups according to Eltis & Bolin (1996) (28) are highlighted. Genbank accession numbers are as follows: PhnE1 (AF112136); TbuE (U20258); Cdo (U47111); TdnC (X59790); PhnE2 (AF112137); C23OII (U01826); XylE (F199) (AF079317); CmtE (L10655); PhnE (U83882); XylE (B1) (U23375); XylE (PDK1) (M65205); PhhB (X77856); DmpB (M33263); XylE (JI104) (X60740); NahH (M17159); XylE (mt-2) (J01845); BphE (U01825); PheB (X67860).

and it is possible that these genes may have been acquired during laboratory cultivation and isolation, *tdnC* having been acquired by *Pseudomonas putida* mt-2 during selective adaptation for growth on aromatic amines (34), whilst C23OII is also plasmid borne (35) and also appears to have been acquired as a consequence of a relatively recent horizontal transfer event (29). This lack of congruence between catabolic genes, such as I.2.C subclass C23Os, and isolate taxonomy presumably reflects the natural competence of *γ-Proteobacteria* which are able to both take up and express genetic material acquired from other *Proteobacterial* sources.

The phylogeny of the other *meta* cleavage enzymes (Fn and HMSD) appears to follow the same trend as the C23O sequences. Though since the database of sequences for these genes is more limited the evidence is less robust and is not shown.

Gene Order of RP007 meta Pathway Operons

The conservation of gene order amongst characterised operons emphasises the stability of gene arrangements during the evolution of *Proteobacterial meta*-cleavage pathways. The two commonly described *meta* operons we refer to as P-type (Fig. 3a) (characteristic of *Pseudomonas* spp.) and S-type *meta* operons (Fig. 3b) (characteristic of *Sphingomonas* spp.) are a case in point. *Burkholderia* sp. strain RP007 has two different *meta* operons (Fig. 3c), the pH1 *meta* operon has a P-type gene order previously only described in members of the γ subclass of the *Proteobacteria,* whilst the pH2 *meta* operon is also unusual with a P-type gene order interrupted by the presence of the unidentified gene (U) between C23O and HMSD. This ORF is similar to an ORF of unknown function also found in *Sphingomonas* (α subclass), and *Bacillus meta*-pathway gene clusters where it is also located upstream of the C23O gene. In *Sphingomonas meta*-pathway gene clusters it is located between C23O and HMSD genes (Fig. 3b). The absence of U, which is a common feature in *Sphingomonas meta* operons, from archetypal P-type operons suggests this gene is either not required by, or may not function in, *Pseudomonas* strains. We propose that since U has been maintained in the pH2 operon of RP007, this gene must have some advantageous function in *Burkholderia,* however despite many sequenced examples of this gene its function remains to be established. This interesting feature of the pH2 *meta* operon may have arisen by homologous recombination producing a hybrid with compo-

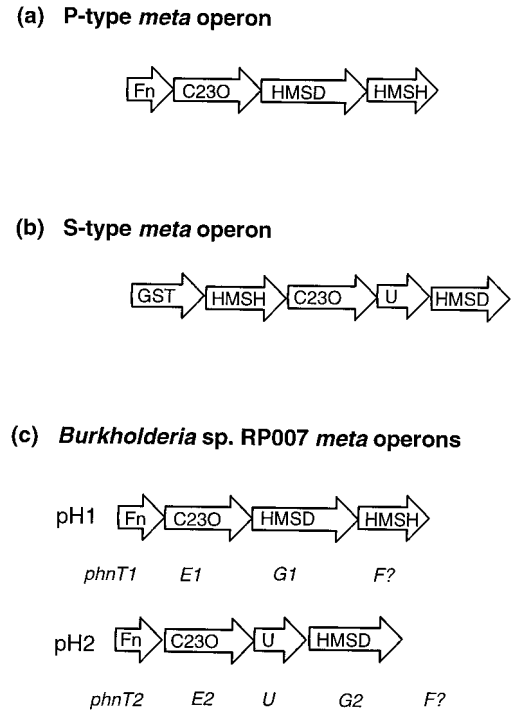

FIG. 3. Gene organization of C23O and neighboring genes of; (a) P-type (*Pseudomonas*) *meta* pathway operons, (b) S-type (*Sphingomonas*) *meta* pathway operons, and (c) Gene organization of the pH1 *meta* pathway of *Burkholderia* sp. RP007 (P-type) and the pH2 *meta* pathway of RP007 (P-type/S-type). Gene abbreviations are: Fn, chloroplast-like ferredoxin; C23O, catechol 2,3-dioxygenase; HMSD, 2-hydroxymuconic semialdehyde dehydrogenase; HMSH, 2-hydroxymuconic semialdehyde hydrolase; U, gene of unknown function.

nents of both P-type and S-type operons. Closer analysis of the nucleotide sequence of pH2 did not reveal any other obvious features of an S-type operon such as contiguous GST and HMSH genes. Similarly there was no evidence of hybrid genes, or differences in codon usage within these genes, which could identify a recombination site which suggests that the unidentified gene (U) was acquired during an ancient event in the evolution of the pH2 *meta* operon that was subsequently masked by evolutionary drift.

Assuming that evolution of *meta* pathways occurred independently from a common precursor in different subgroups of the *Proteobacteria,* the principal contributing factor for iterations of *meta* operons we see today is the isolation of these genes in different taxonomic groups. It is possible that the *Proteobacterial meta*-cleavage pathways have co-evolved with the different *Proteobacterial* subgroups that are all derived from a common ancestor (36). Certainly this segregation occurred sufficiently long ago to allow the evolutionary drift of nucleotide sequences to reflect, for example, the 60% difference in amino acid sequence evident between modern γ, α, and β *Proteobacterial* C23O sequences. This is not the only factor contributing to the evolution of these operons since there is plenty of evidence for the exchange of catabolic genes between *Proteobacterial* subgroups (31–35).

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