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Characterisation of bacterial cultures enriched on the chlorophenoxyalkanoic acid herbicides 4-(2,4-dichlorophenoxy) butyric acid and 4-(4-chloro-2-methylphenoxy) butyric acid

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Abstract The aim of this study was to enrich and characterise bacterial consortia from soils around a herbicide production plant through their capability to degrade the herbicides 4-(2,4-dichlorophenoxy) butyric acid (2,4-DB) and 4-(4-chloro-2-methylphenoxy) butyric acid (MCPB). Partial 16S rRNA gene sequencing revealed members of the genera *Stenotrophomonas*, *Brevundimonas*, *Pseudomonas*, and *Ochrobactrum* in the 2,4-DB- and MCPB-degrading communities. The degradation of 2,4-DB and MCPB was facilitated by the combined activities of the community members. Some of the members were able to utilise other herbicides from the family of chlorophenoxyalkanoic acids. During degradation of 2,4-DB and MCPB, phenol intermediates were detected, indicating ether cleavage of the side chain as the initial step responsible for the breakdown. This was also verified using an indicator medium. Repeated attempts to amplify putatively conserved *tfd* genes by PCR indicated the absence of *tfd* genes among the consortia members. First step cleavage of the chlorophenoxybutyric acid herbicides is by ether cleavage in bacteria and is encoded by divergent or different *tfd* gene types. The isolation of mixed cultures capable of degrading 2,4-DB and MCPB will aid future investigations to determine both the metabolic route for dissimilation and the fate of these herbicides in natural environments.

Keywords 2,4-DB · MCPB · Biodegradation · Chlorophenoxyalkanoic acids · Herbicides

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Introduction

Chlorinated phenoxyalkanoic acids are broadly distributed in the environment due to their widespread use as herbicides. Many bacteria have been isolated that are able to degrade halogenated aromatic compounds [8, 16, 20]. In particular, biodegradation of the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) has been well researched and the degradation pathway is well characterised in a number of bacteria isolated from diverse geographical locations [2, 17, 18, 29]. The catabolism of 2,4-D involves a number of steps including side-chain removal, hydroxylation of the resulting 2,4-dichlorophenol (2,4-DCP), opening of the 3,5-dichlorocatechol ring and then conversion of 2,4-dichloro-cis,cis-muconate to succinate for cell metabolism. In 2,4-D-degrading bacteria, these activities are encoded by the 2,4-D degradation genes (*tfdA-F*) [3, 4, 11, 29, 30]. This serves as a paradigm for the study of related phenoxyalkanoate herbicides.

Until recently, few studies have focused on the microbial degradation of the structurally related herbicide mecoprop [2-(2-methyl-4-chlorophenoxy)propionic acid]. However, there are now several reports describing pure culture degradation with mecoprop as the sole carbon and energy source [15, 21, 25, 31] and the degradation pathway has been elucidated [26]. The mecoprop degradation pathway has been shown to be convergent with the 2,4-D pathway [26]. The related chlorophenoxybutyric acid herbicides 4-(2,4-dichlorophenoxy) butyric acid (2,4-DB) and 4-(4-chloro-2-methylphenoxy) butyric acid (MCPB) have also been applied in weed control since the 1940s but to date only a few studies have described the microbial metabolism of these compounds [14]. They are structurally related to 2,4-D and mecoprop but possess a butyric acid side chain rather than an acetic or propionic acid side chain. As 2,4-DB and MCPB are complex compounds it is likely that, in natural environments, the complete degradation of 2,4-DB and MCPB may occur only through

the combined activities of a community of microorganisms.

There are many studies describing the role of conserved *tfd* genes in 2,4-D-degrading bacterial species isolated from many geographic locations worldwide [28, 30]. These genes have also been detected in bacteria isolated from pristine soils with no history of 2,4-D application [30]. Our recent work has shown that *tfd*-like genes are also present in bacteria that can degrade the structurally related herbicide mecoprop [21, 22].

As there are few reported 2,4-DB- and MCPB-degrading bacteria, we needed to enrich and characterise bacteria with this capability. The constituent members of each community were characterised and their ability to utilise other related phenoxyalkanoate herbicides was investigated. This enabled us to investigate whether *tfd*-like genes were present in the degrading communities.

Materials and methods

Enrichment of 2,4-DB- and MCPB-degrading communities

Soil samples were obtained from the vicinity of a herbicide manufacturing plant (Marks, Bradford, UK). Samples were transferred to sealed glass jars and stored at 4°C under aerobic conditions. Initial culture enrichments by using 10% (w/v) soil in 100 ml (in 250 ml conical flasks) minimal salts medium with the following compounds dissolved in 1 l distilled water: $MgSO_4$, 0.2 g; $(NH_4)_2SO_4$, 0.5 g; KH_2PO_4, 0.5 g; K_2HPO_4, 1.5 g; Na_2 EDTA, 0.12 g; NaOH, 0.02 g; $ZnSO_4$, 0.004 g; $CuSO_4$, 0.001 g; Na_2SO_4, 0.0001 g; Na_2MoO_4, 0.001 g; $CoCl_2$, 0.0001 g; $MnSO_4$, 0.0004 g; and 0.5 ml concentrated H_2SO_4 (pH 7.0) containing 0.5 g carbon l^{-1} 2,4-DB or MCPB (Marks, Bradford, UK). 2,4-DB and MCPB solutions were prepared by dissolving the powder in sterile water containing NaOH, followed by filter sterilisation. The enrichments were incubated in an orbital incubator at 25°C for 3 days.

Isolation of community members

Dilutions of the 2,4-DB and MCPB consortia were spread onto nutrient agar (Oxoid, UK) and minimal selective agar containing (in g l^{-1}): $MgSO_4$, 0.2; $(NH_4)_2SO_4$, 0.5; KH_2PO_4, 0.5; K_2HPO_4, 1.5; Na_2EDTA, 0.12; NaOH, 0.02; $ZnSO_4$, 0.004; $CuSO_4$, 0.001; Na_2SO_4, 0.0001; Na_2MoO_4, 0.001; $CoCl_2$, 0.0001; $MnSO_4$, 0.0004; and 0.5 ml concentrated H_2SO_4 (pH 7.0) containing 2,4-DB or MCPB at concentrations of 1.0 g carbon l^{-1}. The plates were incubated at 30°C for 48 h and 96 h, respectively. Single colonies from both consortia were isolated by repeated streaking on both nutrient agar and minimal selective agar containing the herbicides. Isolates were stored frozen in 15% glycerol at −70°C.

16S rRNA gene amplification, sequencing and phylogenetic analysis

The genomic DNA of isolates was extracted [19]. PCR amplification of the 16S rRNA gene of each isolate was performed using universal 16S rRNA gene prokaryotic primers [5] with a Hybaid (Omn-E) thermocycler according to the conditions presented by Lane [12]. A 10 μl aliquot of the PCR products was analysed on horizontal 1.5% agarose gels (BDH) stained with ethidium bromide in TBE buffer [19]. Electrophoresis was carried out for 1 h at 60 V. Sequencing reactions were performed by MWG Biotech (Ebersberg, Germany). FASTA and BLAST subroutines [1] were used to search for related sequences in the GenBank database. The CLUSTAL W program [27] was used for multiple sequence alignments. Phylogenetic analysis was performed using the heuristic search algorithm with branch-swapping and total-branch recombination in the program PAUP*4.0s [24].

Nucleotide sequence accession numbers

The 16S rRNA gene sequences for the 2,4-DB- and MCPB-degrading isolates have been deposited in GenBank under accession numbers AF309078–AF309081 and AY008333–AY008336, respectively.

Growth of the communities enriched on 2,4-DB and MCPB

Growth was measured by determining the culture absorbance at 420 nm using a Cecil 3000 series spectrophotometer (Cecil Instrument, Milton, Cambridge, UK). The growth medium was the same as the enrichment medium, with 2,4-DB and MCPB added to concentrations of 0.5 g carbon l^{-1}. The culture was aerated by shaking in an orbital shaker at 120 rpm at 25°C.

Degradation of 2,4-DB and MCPB

Degradation of 2,4-DB and MCPB was measured directly by monitoring the change in maximum absorbance peak (A_{279}) in a Cecil 3000 UV spectrophotometer. A decrease in A_{279} indicated breakdown of the herbicides.

Determination of phenolic compounds released during degradation of 2,4-DB and MCPB using a colorimetric method

A 1 ml sample of culture was taken from the growth vessel and centrifuged at 10,000 *g* for 5 min to remove bacterial cells. Subsequently, the supernatant was analysed for the release of 2,4-DCP and 4-chloro-2-methylphenol (MCP) formed during degradation of 2,4-DB and MCPB. This was quantitatively determined in the supernatant by the formation of a red antipyrine dye [9] measured at its specific maximum of 510 nm on a Cecil 3000 series spectrophotometer. Quantities of released phenol (μM) were calibrated as described by King et al. [9] using phenol as standard. There are specific differences in the wavelength for the two released phenols (MCP and 2,4-DCP) and therefore it is important to maintain a degree of caution when using the data to specifically identify the intermediate phenols.

Growth of the constituent members on alternative substituted and unsubstituted compounds using modified Loos medium

All isolated strains from the 2,4-DB- and MCPB-consortia were grown to an optical density at 420 nm of 0.6–1.0 (OD_{420}; measured using a Cecil 3000 series spectrophotometer) before plating on modified Loos agar plates [23]. In short, this medium indicates microbial dehalogenation of the herbicide compounds, a process that usually follows cleavage of the aromatic ring. The cultures were serially diluted in one-quarter strength Ringers solution (Oxoid, UK) and a 10^{-5} dilution of each strain was then plated on modified Loos agar plates with the following composition (in g l^{-1} distilled water): $(NH_4)_2SO_4$, 0.1; eosin B (Sigma), 0.04; yeast extract, 0.25; Na_2EDTA, 0.12; NaOH, 0.02; $ZnSO_4$, 0.004; $CuSO_4$, 0.001; Na_2SO_4, 0.0001; Na_2MoO_4, 0.001; $CoCl_2$, 0.0001; $MnSO_4$, 0.0004 and 0.5 ml concentrated H_2SO_4. Alkaline methylene blue was added until the medium became dark violet (approximately 1–2 ml of a

1% w/v solution). Separate carbon sources, 2,4-DB, MCPB, (*R*/*S*)-mecoprop, (*R*)-mecoprop, (*R*/*S*)-2-(2,4-dichlorophenoxy)propionic acid (2,4-DP), 2,4-D, 2-methyl 4-chlorophenoxyacetic acid (MCPA), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), phenoxyacetate and 4-nitrophenoxyacetic acid (4NPA) were added at concentrations of 1 g carbon l^{-1} (3 mmol l^{-1}). Plates were set up in triplicate for each strain on each substrate type and incubated aerobically at 25°C for up to 4 days.

tfd gene screening of the constituent members

Total genomic DNA was extracted from each of the isolates as described by Sambrook et al. [19]. PCR primers targeted to putatively conserved areas of the *tfdA*, *tfdB* [28] and *tfdC* genes [30] were used to amplify *tfdA*, *tfdB* and *tfdC* fragments, respectively, using a Hybaid (omn-E) thermocycler. PCR conditions described by Vallaeys et al. [28, 30] were used for amplification of the *tfdA*, *tfdB* and *tfdC* genes. As a positive control, DNA from the 2,4-D-degrading *Ralstonia eutropha* JMP134 was extracted and amplified using the *tfd* gene primer sets.

Results

Growth and degradation of 2,4-DB and MCPB by enrichment cultures

Although initial degradation of 2,4-DB and MCPB was slow, complete loss was observed within 2 months. Continual enrichment via successive subculturing resulted in the loss of 2,4-DB at an overall degradation rate of 0.0036 g h^{-1} after 176 h and MCPB after 200 h at a degradation rate of 0.0041 g h^{-1} (Fig. 1). Although the rate of degradation of 2,4-DB was slower than that of MCPB, it had a shorter lag phase of approximately 48 h in comparison to 96 h. At the end of growth, 94% and 89% maximum utilisation of 2,4-DB and MCPB was calculated, respectively. This activity was maintained at the same steady rate for subsequent enrichments (data not shown).

Isolation of 2,4-DB and MCPB culture members

The 2,4-DB- and MCPB-degrading enrichments were subcultured and constituent members were isolated. All three members of the MCPB culture grew on minimal selective agar containing MCPB as the sole carbon source (Table 1). Two isolates of five from the 2,4-DB community were able to grow on 2,4-DB minimal selective agar (Table 1).

Determination of phenolic compounds released during degradation of 2,4-DB and MCPB

During degradation, the supernatants from the 2,4-DB and MCPB cultures were analysed for the production of 2,4-DCP and MCP. This was detected just prior to an increase in culture growth (Fig. 2) and during the start of herbicide degradation. The 2,4-DB and MCPB cultures accumulated 198 μM and 216 μM phenol, respectively, during growth (Fig. 2).

Growth of the constituent members on alternative substituted and unsubstituted compounds using modified Loos medium

Growth of the 2,4-DB and MCPB community members on alternative substituted and unsubstituted phenoxyalkanoate compounds, MCPB, 2,4-DB, (*R*/*S*)-mecoprop, (*R*)-mecoprop, (*R*/*S*)-2,4-DP, 2,4-D, MCPA, 2,4,5-T, phenoxyacetate, and 4-NPA was investigated. All members from both 2,4-DB- and MCPB-enriched cultures were able to use 2,4-D as a growth substrate as indicated by use of modified Loos medium. All members of the MCPB culture could utilise (*R*/*S*)-2,4-DP (Table 1). One member of the MCPB culture showed greatest overall activity by utilising all the substrates tested apart from MCPA and 4-NPA (Table 1). Interestingly, none of the MCPB isolates could grow on the phenoxyacetic herbicide MCPA (Table 1).

Only one member of the 2,4-DB culture could utilise all of the substrates tested (with the exception of 2,4,5-T). Three of the 2,4-DB culture isolates were unable to grow on 2,4-DB without the other members (Table 1).

Phylogenetic analysis

Phylogenetic analysis was performed based on the partial 16S rRNA gene sequences (approximately 500 nucleotides) of all isolates. Species closely related to the isolates were found by searching the GenBank database, and similarities were calculated using FASTA and BLAST subroutines [1]. Sequences were then aligned using the CLUSTAL W program [27]. Two of the 2,4-DB community members belong to the genus *Stenotrophomonas* in the γ-subdivision and were most closely related to *Stenotrophomonas* sp. BO (AF156709). These were designated as *Stenotrophomonas* sp. DB1 and DB4 (Fig. 3A). The other members belong to the genus *Brevundimonas* and were most closely related to *Brevundimonas diminuta* (AJ227779) (Fig. 3B). They were designated *Brevundimonas* sp. DB2, DB3 and DB5. Although they are phylogenetically very close, substrate specificity data demonstrates distinct substrate utilisation profiles (Table 1).

Two members of the MCPB community belong to the genus *Pseudomonas* and were designated *Pseudomonas* sp. MCPB1 and MCPB3. They were most closely related to *Pseudomonas putida* (L37365) and *Pseudomonas* strain IPA (X96787), respectively (Fig. 3C). The third MCPB member was found to belong to the genus *Ochrobactrum* in the α-subunit of the proteobacterium. It was designated as *Ochrobactrum* sp. MCPB2 and was closely related to *Ochrobactrum* sp. 3CB5 (AF229884) (Fig. 3D).

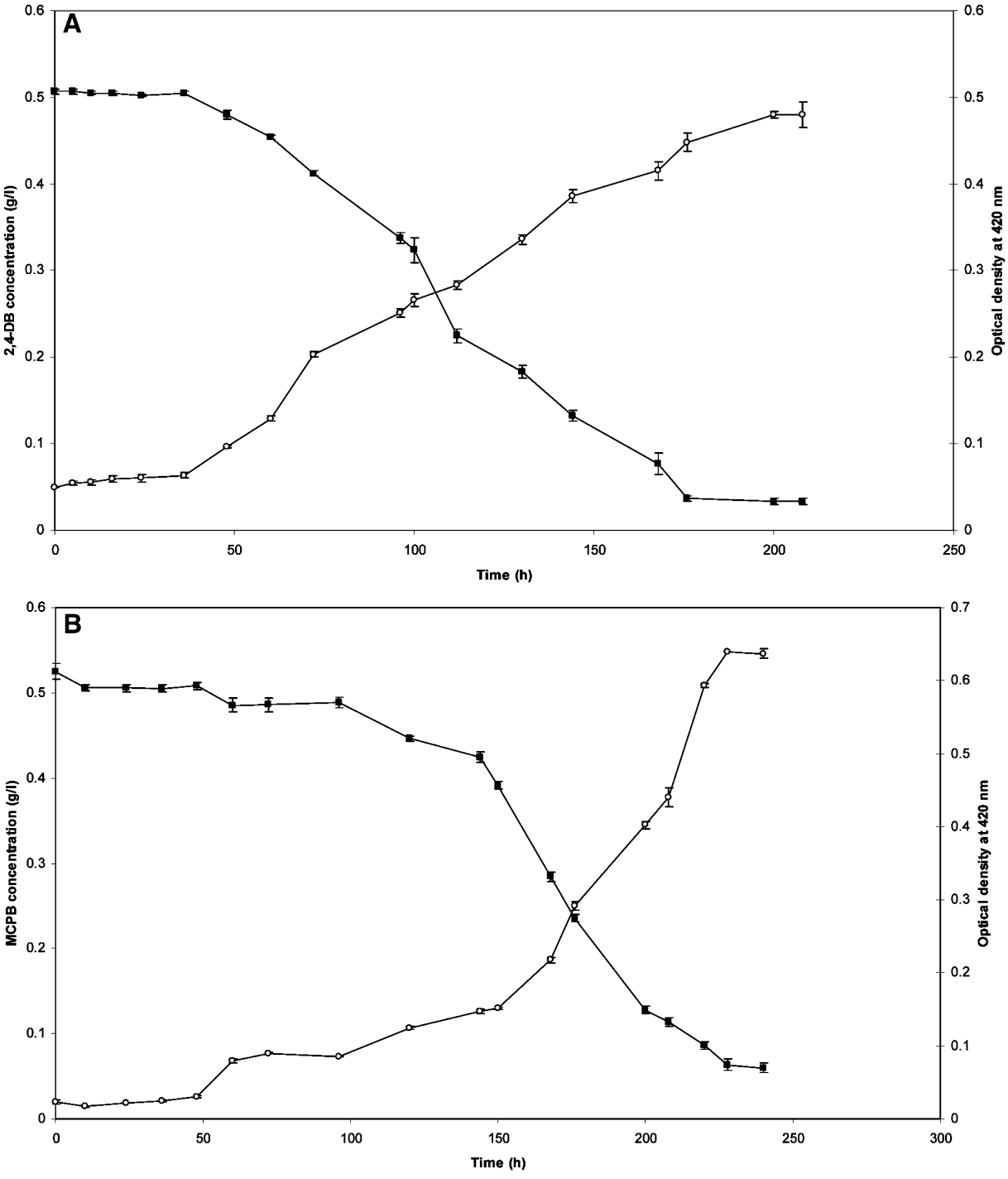

Fig. 1 Degradation (■) and growth (O) of **A** 4-(2,4-dichlorophenoxy) butyric acid (*2,4-DB*) and **B** 4-(4-chloro-2-methylphenoxy) butyric acid (*MCPB*) by mixed communities enriched on each of these herbicides at an initial concentration of 0.5 g carbon l^{-1}

tfd gene screening of the constituent community members

Repeated attempts to PCR amplify *tfd* genes using the *tfdA*, *tfdB* and *tfdC* gene-specific primer sets showed no positive amplification for any isolate from the 2,4-DB and MCPB consortia.

Discussion

2,4-DB- and MCPB-degrading communities were enriched from soils taken from the vicinity of a herbicide production plant through their capacity to use these compounds as sole sources of carbon and energy. Progressive enrichments resulted in the isolation of five members in the 2,4-DB-degrading culture and three members in the MCPB culture and these were assessed for their catabolic activities.

Table 1 Growth of the 4-(4-chloro-2-methylphenoxy) butyric acid (MCPB) and 4-(2,4-dichlorophenoxy) butyric acid (2,4-DB) consortia members on modified Loos agar plates containing a range of substituted and unsubstituted phenoxy compounds. All plates were incubated at 25°C for 4 days. *2,4-D* 2,4-Dichlorophenoxyacetic acid, *MCPA* 2-methyl 4-chlorophenoxyacetic acid, *2,4-DP* 2-(2,4-dichlorophenoxy)propionic acid, *2,4,5-T* 2,4,5-trichlorophenoxyacetic acid, *4NPA* 4-nitrophenoxyacetic acid

Strain	Original substrate	MCPB	2,4-DB	(*R*/*S*)-mecoprop	(*R*)-mecoprop	(*R*/*S*)-2,4-DP	2,4-D	MCPA	2,4,5-T	Phenoxy-acetate	4-NPA
Pseudomonas sp. MB1	MCPB	+[a]	+	+	+	+	+	–	+	+	–
Ochrobactrum sp. MB2	MCPB	+	–	+	–	+	+	–	–	–	–
Pseudomonas sp. MB3	MCPB	+	+	–	–	+	+	–	+	+	+
Stenotrophomonas sp. DB1	2,4-DB	–	–	+	–	+	+	–	–	–	–
Brevundimonas sp. DB2	2,4-DB	–	–	–	–	–	+	–	–	–	–
Brevundimonas sp. DB3	2,4-DB	+	+	+	–	+	+	+	–	–	–
Stenotrophomonas sp. DB4	2,4-DB	–	–	+	–	–	+	–	–	–	–
Brevundimonas sp. DB5	2,4-DB	+	+	+	+	+	+	+	–	+	+

[a] + Growth observed,– no growth observed

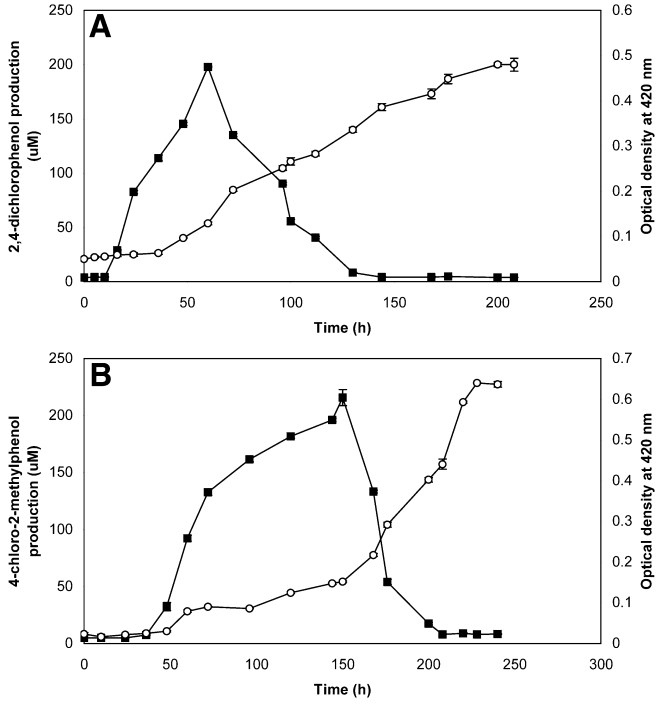

Fig. 2 Production of 2,4-dichlorophenol (DCP) and 4-chloro-2-methylphenol (MCP) during growth of the **A** 2,4-DB- and **B** MCPB-degrading communities. ■ DCP/MCP production, O growth

2,4-D is a soil metabolite of 2,4-DB due to a rapid β-oxidative process [7]. However, it is not known whether this process is microbially mediated. Conversion of MCPB to MCPA in soils by a similar mechanism has not previously been recorded, although some studies have unsuccessfully tried to establish the presence of MCPA in MCPB-treated soils [10]. Our results show the production of 2,4-DCP and MCP during degradation by cultures containing 2,4-DB and MCPB, respectively, as carbon sources. The phenols were detected just prior to an increase in culture growth, indicating the possibility of ether cleavage of the alkanoic acid side chain to form the intermediate phenols. This mechanism, if correct, is in agreement with the first step of 2,4-D [8] and mecoprop [26] breakdown. The initial ether cleavage step in 2,4-D and mecoprop degradation is facilitated by an α-ketoglutarate-dependent dioxygenase enzyme (TfdA) [6, 22], which is encoded by the *tfdA* gene [4, 21]. Based on this, as with 2,4-D and mecoprop, it is possible that the first step in the breakdown of 2,4-DB and MCPB by bacteria is by ether cleavage and not, as previously thought, by β-oxidation. However, further investigations are needed to fully elucidate this mechanism. In addition, all of the consortia members were able to utilise 2,4-D (Table 1), indicating that similar enzymatic mechanisms may be involved. Attempts at amplifying *tfd*-like genes by PCR from the 2,4-DB and MCPB community members were unsuccessful. It is therefore likely that a divergent set of enzymes is responsible for catabolic activity in these strains as is the case with other chlorophenoxyalkanoic acid-degrading bacteria that exhibit similar enzymatic activities although phylogenetic analysis of the *tfd* gene family shows divergent results [28, 30]. For example, *Sphingomonas* sp. AW5, isolated on 2,4,5-T, has been reported not to express the *tfdA* gene using *tfdA* primer sets [28]. However, weak fdA-like activity in this strain has been detected with 2,4-D as a growth substrate [22].

The results of growth on modified Loos medium containing different substituted and unsubstituted aromatic compounds showed that the constituent members of each community have distinct substrate utilisation profiles regardless of genus in accordance with a previous study where this medium was applied [23]. In the study by Mertingk et al. [14] *Rhodococcus* and *Aureobacterium* species isolated from a demolished herbicide production plant were not able to utilise phenoxyacetic and phenoxypropionic acid derivatives, whereas in this study many of the isolated strains demonstrated the ability to degrade a wide range of this family of herbicides.

The variability in substrate utilisation by the culture members implies that there may be specific uptake mechanisms governing substrate specificity. A recent study by Leveau et al. [13] demonstrated that a gene designated *tfdK* is involved in the uptake of 2,4-D by *R. eutropha* JMP134. This encodes a protein belonging

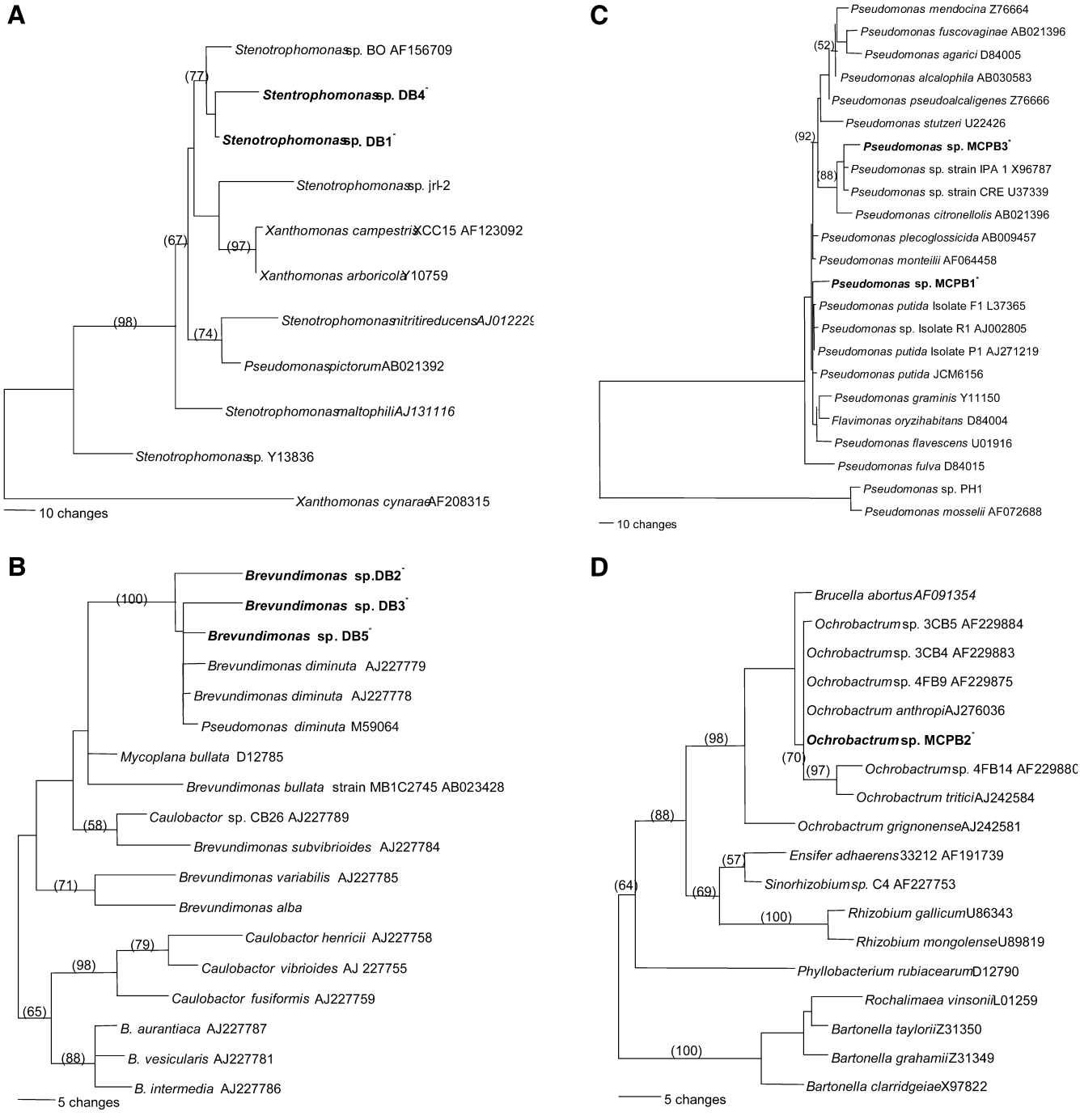

Fig. 3A–D Phylogenetic analysis was performed based on the partial 16S rRNA gene sequences of all members of the 2,4-DB- and MCPB-degrading consortia (strains indicated with an *asterisk*). Phylograms **A**–**D** were constructed using the heuristic search algorithm with branch-swapping and total-branch recombination in the program PAUP*4.0 s [24] with 100 replicates. Bootstrap confidence limits (percentages) are indicated above each branch

to the major facilitator superfamily group of transporter proteins and is related to proteins responsible for the transport of carboxylated aromatic substances. The fdK enzyme in *R. eutropha* JMP134 is specific only to the uptake of 2,4-D and MCPA. This could also be true for the constituent community members, which may have transport proteins exhibiting specificity to particular compounds.

The isolation of mixed cultures capable of degrading 2,4-DB and MCPB should help in future investigations to determine both the metabolic route for dissimilation and the fate in natural environments of these widely applied herbicides.

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