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Comparison of 16S rRNA gene phylogeny and functional *tfdA* gene distribution in thirty-one different 2,4-dichlorophenoxyacetic acid and 4-chloro-2-methylphenoxyacetic acid degraders

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ABSTRACT

31 different bacterial strains isolated using the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) as the sole source of carbon, were investigated for their ability to mineralize 2,4-D and the related herbicide 4-chloro-2-methylphenoxyacetic acid (MCPA). Most of the strains mineralize 2,4-D considerably faster than MCPA. Three novel primer sets were developed enabling amplification of full-length coding sequences (CDS) of the three known *tfdA* gene classes known to be involved in phenoxy acid degradation. 16S rRNA genes were also sequenced; and in order to investigate possible linkage between *tfdA* gene classes and bacterial species, *tfdA* and 16S rRNA gene phylogeny was compared. Three distinctly different classes of *tfdA* genes were observed, with class I *tfdA* sequences further partitioned into the two sub-classes I-a and I-b based on more subtle differences. Comparison of phylogenies derived from 16S rRNA gene sequences and *tfdA* gene sequences revealed that most class II *tfdA* genes were encoded by *Burkholderia* sp., while class I-a, I-b and III genes were found in a more diverse array of bacteria.

Introduction

Bacterial degradation of the phenoxyacetic acid herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) has been intensively studied, and pure cultures of 2,4-D degrading bacteria have been isolated from numerous locations worldwide [1–3]. The similar compound 4-chloro-2-methylphenoxyacetic acid (MCPA) has been less studied, and to our knowledge no bacterial degraders has been isolated based on this compound. Despite structural similarity between MCPA and 2,4-D, recent studies on expression of the functional gene *tfdA* (involved in the first step of degradation of both compounds) during degradation of the compounds *in situ* in soil, have revealed that marked differences in functional diversity exist between the degrading populations [4,5].

Most of the organisms that are able to degrade 2,4-D have been isolated from agricultural fields or other locations previously treated with 2,4-D [3], but also few 2,4-D degrading organisms have been isolated from more pristine areas as well [1,6]. Typically, isolates from 2,4-D-naïve locations belong to the group of α-proteobacteria, while organisms isolated from sites with historical exposure typically belong to β- and γ-proteobacteria [6]. Even though the first step in the degradation of MCPA and 2,4-D is the same, only few of the strains isolated on 2,4-D have been investigated for their ability to degrade MCPA [23]. Based on uniqueness investigated by repetitive extragenic palindromic PCR (REP–PCR) (data not shown), we chose 31 strains from a collection of four novel and 46 previously described 2,4-D degraders and investigated their abilities towards mineralization of 2,4-D and MCPA (for details on isolation strategy and origin of the novel degraders see S1). This experiment was performed by addition of similar cell numbers of the individual strains to mineral salt medium containing 2.3 mM of the respective phenoxy acid. Mineralization was quantified by radiorespirometry (for further details see S1). Most of the strains mineralized ≥40% of the applied 2,4-D relatively rapidly, typically within 5 days, while ≥40% of the applied MCPA was mineralized much slower (Table 1). Only strains TFD44, TFD47, TFD48, and TFD49 required more than 5 days to mineralize 2,4-D, while only strains TFD34, TFD36, TFD38, and *C. necator* JMP134 mineralized MCPA in less than 35 days. Thus, all of the strains except strain TFD38 (which mineralized either compound in 5 days) exhibited a strong preference towards 2,4-D as substrate.

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; MCPA, 4-chloro-2-methylphenoxyacetic acid; CDS, coding sequence; α-KG, α-ketoglutarate; bp, base pair

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Table 1
2,4-D degrading strains, GenBank accession numbers of sequences, and ability of strains to degrade 2,4-D and MCPA.

Strain	Genus/species[a]	16S GenBank acc. #	*tfdA* GenBank acc. #	*tfdA* class[b]	2,4-D degradation[c]	MCPA degradation[c]	Ref.
TFD2	*Burkholderia* sp.	EU827471	EU827440	II	5	35	3
TFD4	*Burkholderia* sp.	EU827473	EU827442	II	5	35	3
TFD6	*Burkholderia* sp.	EU827470	EU827439	II	5	35	3
TFD9	*Achromobacter xylosoxidans*	EU827474	EU827458	I-b	5	35	3
TFD17	*Burkholderia* sp.	EU827476	EU827443	II	5	35	3
TFD19	*Burkholderia* sp.	EU827477	EU827462	I-a	5	–	3
TFD20	*Burkholderia* sp.	EU827478	EU827444	II	5	35	3
TFD21	*Burkholderia* sp.	EU827479	EU827445	II	2	–	3
TFD23	*Burkholderia* sp.	EU827480	EU827446	II	5	–	3
TFD28	*Burkholderia* sp.	EU827481	EU827447	II	2	35	3
TFD29	*Burkholderia* sp.	EU827482	EU827461	I-b	2	35	3
TFD31	*Burkholderia* sp.	EU827483	EU827448	II	2	35	3
TFD32	*Burkholderia* sp.	EU827484	EU827449	II	5	–	3
TFD33	*Cupriavidus* sp.	EU827485	EU827463	I-a	5	35	3
TFD34	*Burkholderia* sp.	EU827486	EU827450	II	5	12	3
TFD35	*Variovorax koreensis*	EU827487	EU827452	III	5	35	3
TFD36	*Burkholderia* sp.	EU827468	EU827438	II	2	12	3
TFD38	*Cupriavidus* sp.	EU827488	EU827464	I-a	5	5	3
TFD39	*Pseudomonas* sp.	EU827489	EU827465	I-a	5	–	3
TFD40	*Cupriavidus* sp.	EU827490	EU827454	III	5	35	3
TFD42	*Cupriavidus* sp.	EU827491	EU827460	I-b	5	–	3
TFD44	*Sphingomonas* sp.	EU827494	ND	ND	12	35	3
TFD45	*Cupriavidus* sp.	EU827492	EU827453	III	5	35	3
TFD47	*Achromobacter xylosoxidans*	EU827475	EU827459	I-b	12	35	This study
TFD48	*Cupriavidus respiraculi*	EU827493	EU827455	III	12	35	3
TFD49	*Pseudomonas putida*	EU827496	EU827456	III	12	35	This study
TFD50	*Pseudomonas* sp.	EU827497	EU827457	III	5	–	This study
TFD51	*Cupriavidus* sp.	EU827498	EU827466	I-a	5	–	This study
TFD52	*Burkholderia* sp.	EU827469	EU827451	II	5	–	3
JMP134	*Cupriavidus necator*	EU827495	EU827467	I-a	2	5	11
RASC	*Burkholderia tropicana*	EU827472	EU827441	II	2	–	2

[a] The genus and/or species designation is based on the classification of the most closely related rRNA gene sequence in GenBank.
[b] ND, a *tfdA* gene was not detected.
[c] 2,4-D and MCPA degradation is noted as the day when $\geq$40% cumulative mineralization of that compound was detected as $^{14}CO_2$ captured in the NaOH trap.

Recently, we suggested that degradation of MCPA in soil is linked to class III *tfdA*-harboring bacteria [4,5], and therefore the lack of correlation between the different classes of *tfdA* genes and MCPA substrate specificity might reflect the difference between the natural soils studied and the selective nature of analyzing bacterial isolates. Also, a possible explanation is that the strains in our collection were biased by their substrate of isolation being 2,4-D. Furthermore, it is unclear whether the relatively fast mineralization of 2,4-D generally observed compared to that of MCPA is due to the fact that all of the strains were isolated using 2,4-D as the only source of carbon, or whether MCPA is more difficult, or in some way less energetically favorable, to metabolize. In the current study, we initially intended to isolate pure cultures based on their ability to use MCPA as sole source of carbon (using the same strategy of isolation as Ka et al. [7], but replacing 2,4-D with MCPA), but we were not successful. Indeed, to our knowledge, no bacterial strains have ever been isolated using MCPA as sole carbon source, so the existence of strains with preference for this compound over 2,4-D has yet to be demonstrated.

A generally accepted paradigm in biodegradation microbiology is that, the more halogen moieties a compound contains, the more difficult it is for microorganisms to degrade (for reviews see [24,25]). However, in extant experiments with phenoxyacetic acid application to soil samples, there is a clear trend that MCPA is more difficult to degrade than 2,4-D in the soil environment [5]. This is somewhat surprising given that 2,4-D contains two halogens (Cl^-) while MCPA only contains one, but is consistent with our culture-based results and the dearth of known MCPA degraders in the literature.

The pathways of 2,4-D biodegradation and the genes involved have been described for several of the existing isolates [8–10]. Numerous phylogenetically diverse α-ketoglutarate (α-KG)-dependent dioxygenases have been shown to be able to initiate the first step in the degradation. Streber et al. [11] isolated, sequenced and characterized the first gene, the canonical *tfdA*, encoding an α-KG dioxygenase involved in 2,4-D degradation from the bacterium *Cupriavidus necator* JMP134 (formerly *Alcaligenes eutrophus* JMP134). Subsequently, a number of additional α-KG dioxygenase genes related to the canonical *tfdA* gene have been isolated and characterized [12]. Grouping of these *tfdA* genes into three classes (I, II and III) showing 78–95% identity was suggested by Mcgowan et al. [13]. Also, organisms harboring the genes *tfdAα* and *cadA* have been isolated and identified as 2,4-D degraders as well. The majority of extant studies on *tfdA* gene sequence diversity and phylogeny have been performed based on partial (typically ~300 bp) coding sequences (CDS) [13,14]. The entire *tfdA* CDS is 861, 891, and 861 bp for classes I, II and, III, respectively, [11,12,15] and a significant amount of phylogenetic information is thereby lost when only partial sequences are analyzed.

In the current study, full length CDS of the *tfdA* genes were amplified and bi-directionally sequenced using primers designed for this purpose (Table 2). Depending on how strictly the different phylogenetic classes are defined, the *tfdA* sequences studied herein fall into 3–4 different phylogenetic branches (Fig. S2). The sequences in the branch previously classified as class I *tfdA* genes [13] can be subdivided into two new classes based on differences in the nucleic acid sequence (Fig. S4) as well as in their amino acid sequences (Fig. S5). Therefore, we propose the two new sub-

Table 2
Primers used for PCR and sequencing.

Primer name	Target gene	Primer (5′–3′)	Fragment size (bp)[a]	Annealing temp. (°C)	Ref.
tfdA (CI)	*tfdA*-class I	F: GTGAGCGTCGTCGCAAAT R: GCATCGTCCAGGGTGGTC	856	58	This study
tfdA (CII)	*tfdA*-class II	F: TGAGCATCAATTCCGAATACC R: AAGACTGACCCCGTGGACT	882	55	This study
tfdA (CIII)	*tfdA*-class III	F: TGAGCATCACTTCCGAATACC R: ACAGCGTCGTCCAACGTC	856	58	This study
16S-27f/1492r	General 16S rRNA gene	F: GAGTTTGATCMTGGCTCAG[b] R: GGYTACCTTGTTACGACTT[b]	1329–1405	55	[26]

[a] Due to the sequencing technique, the entire amplicon could not be obtained.
[b] M, A/C; Y, T/C.

classes, I-a and I-b, within which the sequences in each individual sub-class are 100% identical, while 99% identity was observed between the two sub-classes.

Among the class II *tfdA* genes, only the *Burkholderia* sp. RASC *tfdA* gene differs from the others in that class and only by one bp (#264–see Fig. S4). Among the class III genes, only TFD35 was 100% identical to the reference class III *tfdA* sequence *B. cepacia* 2a, while the other sequences differed at bp #252 (Fig. S4). Neither the difference in the class II *tfdA* genes nor the difference in the class III *tfdA* genes gave rise to differences in the amino acid sequence (i.e. they were synonymous) and therefore no sub-class partitioning of these groups is suggested. Based on the 856 bp present in all of the sequences analyzed herein, the DNA sequence identity between classes I and II is 76%, between classes I and III it is 78%, and between classes II and III it is 92%.

Collectively, these data demonstrate that diversity indeed exists among *tfdA* sequences, but that this diversity is distributed into four distinct clades (three classes with class I comprised of two sub-classes) within which the *tfdA* genes are highly identical. We suggest that the partitioning of class I *tfdA* sequences in two separate sub-classes is justified by the fact that between these two sub-classes the amino acid sequences differ by three residues (Fig. S5) and thereby they are not the same enzyme. Actually, Vallaeys et al. [16] have previously suggested the existence of a fourth class of *tfdA* genes based on PCR-restriction length fragment polymorphism (RLFP) analysis. While that work potentially reflected the two sub-classes of class I suggested here, it is not possible to relate their RFLP data to our study, as no sequence information was given. The sequence data and phylogenetic tree provided herein demonstrate that diversity *within* the classes and sub-classes of *tfdA* genes is very low. In accordance with a previous report [13], TFD44 did not produce an amplification product using any of the *tfdA* primer sets suggesting that its etherase activity for 2,4-D and MCPA may be encoded by an entirely different enzyme and thus it remains unclassified within the *tfdA* scheme.

Also, we sequenced the 16S rRNA genes bi-directionally in the region between 27 and 1492 (according to *E.coli*) (Table 2). 16 of the 31 strains could be classified as *Burkholderia* sp. strains, while many of the remaining strains also belonged to the β-subdivision of the proteobacteria, e.g. *Achromobacter* sp., *Ralstonia* sp., *Variovorax* sp., and *Cupriavidus* sp. Three strains were *Pseudomonas* sp. belonging to the γ-proteobacteria, while one strain only was a *Sphingomonas* sp. which belongs to the α-proteobacteria (Table 1). Others have isolated collections of 2,4-D degrading bacteria primarily belonging to α- and γ- proteobacteria subdivisions using other isolation techniques [1,6]. It is well known that the isolation technique may have a strong influence on the types of bacteria isolated [17,18]. Therefore, the fact that 26 of 31 strains in the current study belong to the β-subdivision, and that 16 strains of these are *Burkholderia* spp. suggests that the relatively small phylogenetic variation reported here may reflect the consistency of the isolation technique employed across the suite of starting samples.

When comparing the phylogenetic tree of the 16S rRNA gene sequences of these strains with the phylogenetic tree of *tfdA* gene sequences (Fig. S3), an interesting pattern was revealed. All of the class II genes were found in the *Burkholderia* branch only, while class I and III *tfdA* genes were obtained from bacteria belonging to many different families. This suggests that class I and III *tfdA* genes are more mobile, potentially being distributed readily by horizontal gene transfer. Strong evidence exist that these genes are most often located on self-transmissible plasmids with a broad spectrum of hosts [13,19–21]. The class II *tfdA* gene, in contrast, does not have the tendency to transfer horizontally, but the reason for this is unknown. It has been suggested that the class II *tfdA* gene in *Burkholderia* sp. RASC is located on the chromosome [12,19,22], which would, of course, limit its distribution via horizontal gene transfer, but it is not yet clear whether all class II *tfdA* genes are chromosomally encoded.

In summary, this study corroborates prior work suggesting that three classes of *tfdA* gene exist. Further, our work goes on to suggest that *tfdA* Class I be divided into two sub-classes based on three distinct amino acid differences between the two sub-classes, but are highly conserved at the amino acid and DNA level (100% identity) within sub-classes. These differences were revealed by the more extensive sequence analysis and comparison in the current study. We also assessed the distribution of the various *tfdA* classes across phylogenetic taxa based on essentially full-length 16S rRNA gene sequences and show that classes I and III exhibit distribution across multiple taxa, which is consistent with gene mobility based on horizontal gene transfer. By contrast, class II was found only in *Burkholderia* spp., which is consistent with prior evidence indicating that it is chromosomally encoded. The inclusion of a larger suite (31) of 2,4-D and MCPA degraders, the recovery and analysis of bidirectional 16S rRNA gene sequences, and the utilization of current software tools and databases in the present study lend support to these findings.

Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.syapm.2010.01.001.

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