Diversity of *tfd*C genes: distribution and polymorphism among 2,4-dichlorophenoxyacetic acid degrading soil bacteria

Lucia Cavalca [a],*, Alain Hartmann [b], Nadine Rouard [b], Guy Soulas [b]

[a] *DISTAM, Sez. MAAE, Università degli Studi di Milano, via Celoria 2, 20133 Milan, Italy*
[b] *INRA, CMSE, Laboratoire de Microbiologie des Sols, 17 rue Sully, BV 1540, 21034 Dijon Cedex, France*

Abstract

The aim of the present work was to study the occurrence, distribution and diversity of 1,2-dichlorocatechol dioxygenase genes among 2,4-dichlorophenoxyacetic acid degrading bacteria. Phylogenetic relationships between the 31 strains or isolates were evaluated by amplified ribosomal DNA restriction analysis of the 16S rDNA gene. All the strains could be assigned to the β or γ subdivisions of the Proteobacteria. *tfd*C genes were detected by PCR amplification using degenerated primers. Two specific probes were produced from *Ralstonia eutropha* strain JMP134 and from a soil isolate strain PLAE6 which was grouped with *Variovorax paradoxus*. Sequence analysis of the probes revealed that they were homologous to the *tfd*C genes of JMP134 located on plasmid pJP4 and to the *tfd*C gene of *Pseudomonas putida* strain PaW85 located on plasmid pEST4011. The localization and the copy number of *tfd*C genes were determined by hybridization of plasmid profiles and genomic DNA restriction fragment length polymorphism profiles with the two probes. Most of the strains were found to bear *tfd*C genes on plasmids ranging from 78 to 532 kb; two strains without any plasmids were also found to hybridize with the probes, revealing a chromosomal localization of catabolic genes. Sequence analysis of the PCR products from different strains confirmed that four different classes of chlorocatechol 1,2-dioxygenase genes were present in the strains and isolates studied. © 1999 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

1. Introduction

Halogenated aromatics are used world-wide in agriculture and industrial activities. They are released into the environment, particularly soils and water, and concerns have been raised about possible harmful effects of these compounds on non-target organisms. A wide range of soil microorganisms have developed novel metabolic capacities enabling them to degrade halogenated aromatics. These novel metabolic activities or altered enzymatic specificities may have evolved from existing enzymatic systems effective towards naturally occurring aromatic compounds [1]. Genetic diversity may have resulted from these adaptive processes which enlarge the substrate utilization pattern of the soil microflora and help maintain its metabolic activity under environmental stress. Among environmentally significant catabolic activities, aromatic ring cleavage is the limiting step

* Corresponding author. Tel.: +39 (2) 23955825;
Fax: +39 (2) 70630829; E-mail: vinandre@imiucca.csi.unimi.it

of the degradation of a number of natural and xeno-biotic compounds which are potential pollutants. Aromatic ring cleavage capabilities are of major interest for depollution and bioremediation of sites contaminated by chlorinated herbicides such as 2,4-dichlorophenoxyacetic acid (2,4-D). Knowledge about the diversity of the genes encoding (chloro)catechol 1,2-dioxygenases might be useful to understand how extended catabolic capacities have evolved from a few metabolic pathways involved in the aerobic degradation of aromatic compounds by bacteria.

Chlorocatechol dioxygenases of the modified *ortho* pathway (or type II dioxygenases) are a distinct subgroup of ring cleaving enzymes [1]. Many of these are encoded by plasmid-located genes found in Gram-negative strains, *clc*A on plasmid pAC27 from *Pseudomonas putida* strain AC867 [2], *tcb*C on plasmid pP51 from *Pseudomonas* sp. strain P51 [3]. *tfd*C genes were found on plasmid pJP4 from *Ralstonia eutropha*, formerly *Alcaligenes eutrophus* JMP134 [4], on plasmid pEML159 from *Alcaligenes* sp. [5], on plasmid pRC10 from *Flavobacterium* sp. [6], on plasmid pEST4011 from *Pseudomonas putida* PaW85 [7] and on plasmid pMAB1 from *Burkholderia cepacia*, formerly *Pseudomonas cepacia* CSV90 [8]. On the other hand, the characterization of plasmid-cured derivatives of *Burkholderia* sp. strain RASC suggests that 2,4-D catabolic genes are located on the chromosome of this bacterium [9]. A *tfd*C-like gene has also been detected in a Gram-positive bacterium: *Rhodococcus erythropolis* strain 1CP [10,11].

With the exception of the chlorocatechol 1,2-dioxygenase of *R. erythropolis* which exhibits only 15–22% amino acid sequence homology with other catechol 1,2-dioxygenases and chlorocatechol 1,2-dioxygenase, many of the type II pyrocatechases, although having different substrate specificity, show a significant degree of homology in their amino acid sequences [11]. This relatedness has been interpreted as the result of sequence divergence among genes deriving from a common ancestor. Acquisition of new phenotypes from pre-existing genes (able to degrade structurally similar compounds) is likely to be based on gene duplication and subsequent mutation of the duplicated copy which broadens the range of substrates which could be degraded [12]. Duplication has been reported for different genes of the 2,4-D

degradative pathway on plasmid pJP4 harbored by *R. eutropha*: *tfd*A [13], *tfd*D [14] and *tfd*C [15]. Moreover, a high degree of interspecies diversity has been described for the 2,4-D dioxygenase gene *tfd*A and the 2,4-dichlorophenol hydroxylase gene *tfd*B among different 2,4-D degrading soil isolates [16]. These observations are in agreement with the assumption that gene duplication and sequence divergence are important for enlarging the range of substrates degraded by soil microorganisms. Type II pyrocatechases from Gram-negative bacteria show substrate specificity to some extent: for example TcbC, TfdC and ClcA preferentially degrade 3,4-dichlorocatechol, 3,5-dichlorocatechol and 3-chlorocatechol respectively [3]. By comparison, the *R. erythropolis* pyrocatechase has a distinct preference for the 4-substituted catechols [11].

Chlorocatechol 1,2-dioxygenase genes are often found as part of a relatively well conserved gene cluster known as the chlorocatechol oxidative pathway [1]. Three to four structural genes form these clusters, *tcb*CDEF in *Pseudomonas* sp. strain 51, *clc*ABD in *P. putida* and *tfd*CDEF in *R. eutropha*, which are regulated respectively by the *tcb*R, *clc*R and *tfd*R (*tfd*S) genes. Regulatory genes encode proteins which are all members of the LysR family of transcriptional activators. A *tfd*C gene was also found associated with a *tfd*B gene to form an operon in the degradative plasmid pEST4011 [17]. A second copy of the *tfd*C and *tfd*D genes has been detected on plasmid pJP4 from *R. eutropha* [12] [15] [14]. These findings suggest that different levels of organization can be found among isofunctional genes indicating different degrees of evolution. Some of these genes are integrated in regulated polycistronic operons which are likely to have evolved under a natural selective process. This evolution may have started before contamination of soils with chlorinated xenobiotic compounds. Other genes occur in more simply regulated structures or even as proximal pairs with one structural gene being under the direct control of a regulatory gene.

The objective of this study was to analyze the diversity of chlorocatechol 1,2-dioxygenase genes from 2,4-D ring degrading bacteria isolated from soils of a wide geographical origin and to describe their organization, localization and copy number. Molecular tools (probes and primers) were developed and tested

for the detection of these genes in various strains of soil bacteria. The detection of *tfd*C genes is of great interest since chlorocatechol 1,2-dioxygenase is the key enzyme of degradation of chloroaromatics which allows a complete mineralization of these potential pollutants.

2. Materials and methods

2.1. Bacterial strains, culture media and phenotypic characterization

Bacterial strains used in this study are listed in Table 1. 2,4-D degrading isolates from the MSDJ (Microbiologie des Sols Dijon) collection were isolated in 1995 from a grassland soil (Dijon soil)

with no previous known application of 2,4-D, and from an agricultural gleyic luvisol of Citeaux in the Dijon area, with no previous history of 2,4-D treatments. An enrichment step of the soil samples in 2,4-D minimal liquid medium (2,4-D MM) [16] was carried out as previously described [18]. Strains were then isolated on 2,4-D MM plates, solidified by 15 g l^{-1} agar and supplemented with 200 mg l^{-1} of 2,4-D. All strains were maintained on 2,4-D minimal medium agar slopes supplemented with 50 mg l^{-1} of 2,4-D. For long-term storage strains were kept frozen at $-80°C$ in 12.5% glycerol.

Strains were screened for their capacity to utilize both side chain labeled [^{14}C]2,4-D and uniformly ring labeled [U-^{14}C]2,4-D. Two concentrations were used: 20 and 200 mg l^{-1} of 2,4-D. Ring cleavage capacity was measured for each strain on liquid

Table 1
Bacterial strains used in this study

Strain	Species	ARDRA type	Origin	Reference
JMP134	*Ralstonia eutropha*	E	Australia	[32]
134I17A1	*Ralstonia eutropha*	E	derivative of JMP134	This work
134I17A2	*Ralstonia eutropha*	E	derivative of JMP134	This work
134AcA2	*Ralstonia eutropha*	E	derivative of JMP134	This work
JD1	undetermined	E	USA (Michigan)	Dunbar (unpublished)
JD2	undetermined	E	USA (Michigan)	Dunbar (unpublished)
JD16	undetermined	E	USA (Michigan)	Dunbar (unpublished)
JD17	undetermined	E	USA (Michigan)	Dunbar (unpublished)
S1(TV1)	*Variovorax paradoxus*	C	France (Dijon soil)	[16]
PLAE3	undetermined	C	France (Citeaux soil)	[47]
PLAE4	undetermined	C	France (Citeaux soil)	[47]
PLAE6	undetermined	C	France (Citeaux soil)	[47]
D8	undetermined	C	France (Dijon soil)	[47]
F7-2	undetermined	C	France (Dijon soil)	[47]
B197	undetermined	C	France (Dijon soil)	[47]
1D9	undetermined	A	France (Citeaux soil treated with DNOC 50 mg kg soil^{-1})	[47]
JMP131	*Alcaligenes eutrophus*	D	Australia	[32]
JMP131B	*Alcaligenes eutrophus*	D	derivative of JMP131	This work
PLAE2	undetermined	D	France (Citeaux soil)	[47]
D2(1)Ba	undetermined	D	France (Dijon soil)	[47]
C11CL	undetermined	D	France (Dijon soil)	[47]
C1CL	undetermined	D	France (Dijon soil)	[47]
B6-9	*Rhodoferax fermentans*	F	Canada (Ontario, lake water microcosm with 2,4-D)	[27]
JMP133	*Alcaligenes paradoxus*	H	Australia	[32]
TFD6	*Burkholderia mallei*	I	USA (Michigan, soil with a history of 2,4-D application)	[46]
Pcep	*Burkholderia cepacia*	G	Indonesia	[45]
PCD4	*Burkholderia cepacia*	G	Indonesia	[45]
RASC	*Burkholderia cepacia*	G	USA (Oregon, sewage sludge)	[9]
TCP	*Ralstonia eutropha*	E	Italy (activated sludge)	[25]
D10	undetermined	E	France (Dijon soil)	[47]
82	undetermined	B	France (Citeaux soil)	This work

2,4-D MM supplemented with 200 mg l^{-1} of 2,4-D and with 1.1 MBq l^{-1} of ring labeled [U-^{14}C]2,4-D (specific activity 37 MBq mmol^{-1}) or side chain labeled [^{14}C]2,4-D (specific activity 463 MBq mmol^{-1}). The degradation assays were done in 96-well microtiter plates containing 100 µl of medium per well. Each well was inoculated with 4 µl of a preculture of each strain on TY liquid medium [19]. ^{14}C-CO produced was trapped on a filter paper soaked in a saturated solution of barium hydroxide. Over each well, a spot of precipitated barium ^{14}C-carbonate was formed and analyzed with a Phosphorimager Storm (Molecular Dynamics). The radioactivity present on each spot was expressed as the percentage of the radioactivity initially added to the corresponding well.

2.2. Amplification of tfdC by PCR and restriction analysis

PCR primers were chosen from the published *tfd*C sequences of *Pseudomonas putida* strain PaW85, GenBank accession number U32188 [17], of *Ralstonia eutropha* strain JMP134, GenBank accession numbers M36280, M35097 and U16782 [12,13,20], and of *Burkholderia cepacia* strain CSV90, GenBank accession number D16356 [8]. Sequences of *clcA* gene of *P. putida* strain AC867, GenBank accession number M36279 [12] and of the *tcbC* (chlorocatechol 1,2-dioxygenase) gene of *Pseudomonas* sp. strain P51 GenBank accession number M57629 [3] were also used in the alignments. Sequences were aligned using the Clustal W software (version 1.7) and primers were designed within the conserved regions. Primers TFDCF (20-mers, position 259–279 of the sequence M35097) 5′ GGC CGG CTS AAG ACH TAC GA 3′, and TFDCR (19-mers, position 721–740 of the sequence M35097) 5′ GCG GGY TCG ATV ACG AAG T 3′, were chosen using OLIGO 5 0 (Primer analysis software, ©1989–95 by Wojciech Rychlik, National Bioscience) and synthesized by Appligene (France).

PCR reactions were done in a total reaction volume of 50 µl, containing: 200 µM of each dNTP, 0.25 µM of each primer, 1.5 mM MgCl$_2$, 5 µl of the buffer (10×) supplied with Taq polymerase, 1.25 U of Taq polymerase (Gibco BRL, France). 5 ng of template DNA was added per reaction. PCR was

performed with an Omnigene DNA thermal cycler (Hybaid, UK). Cycles used were as follows: 1 cycle at 94°C for 4 min; 40 cycles at 94°C for 1 min, at 57°C for 1 min, at 72°C for 2 min; 1 cycle at 72°C for 15 min. The *R. eutropha* strain JMP134 was used as a positive control.

The *tfd*C PCR products were then digested with the two restriction endonucleases *Hae*III and *Hha*I. Restriction fragments were separated on 3.5% Metaphor agarose gels (FMC, Tebu, France) in TBE buffer. Gels were stained with ethidium bromide (0.1 µg ml^{-1}), and pictures were recorded with the imaging system Bioprint (Vilber Lourmat, France). Sizes of the fragments were estimated using the molecular mass marker VIII (Boehringer Mannheim, France) with the Bioprofil Bio1D++ software (Vilber Lourmat, France).

2.3. Amplification of the 16S rDNA gene and restriction analysis (ARDRA)

16S rDNA was amplified for each strain using the forward primer 27f (5′ AGA GTT TGA TC(A/C) TGG CTC AG 3′, positions 8–27 of the *Escherichia coli* 16S rDNA and the reverse primer 1492r (5′ TAC GG(A/T/C) TAC CTT GTT ACG ACT T 3′, positions 1492–1513 of the *E. coli* 16S rDNA sequence). PCR reactions were done in the same conditions as for *tfd*C amplification, except that the annealing temperature was set at 55°C.

PCR products were then digested with the three restriction endonucleases *Hae*III, *Hha*I and *Alu*I. Restriction fragments were analyzed as described for *tfd*C PCR products.

Similarity among strains was estimated from the proportion of shared restriction fragments generated by the three restriction endonucleases, using the simple matching coefficient to compute the distance matrix (NTSYSpc 2.0, Exeter Software, New York) [22]. The unweighted pair group method with arithmetic mean (UPGMA) [21] was used to build the similarity tree from the distance matrix using the NTSYSpc 2.0 analysis software.

2.4. DNA sequencing and sequence alignments

PCR products corresponding to the internal fragment of *tfd*C genes were purified from 2% agarose

gel using the QIAEX II kit (Qiagen) and sequenced by Genome Express (France). The sequencing reaction was performed by PCR amplification in a final volume of 20 µl using 100 ng of PCR products, 5 pmol of primer and 9.5 µl of DyeTerminators pre-mix according to Applied Biosystems protocol. After heating to 94°C for 2 min, the reaction was cycled as following: 25 cycles of 30 s at 94°C, 30 s at 55°C, and 4 min at 60°C (9600 thermal cycler, Perkin El-mer). Removal of excess of DyeTerminators was performed using Quick Spin columns (Boehringer Mannheim). The samples were dried in a vacuum centrifuge and dissolved in 4 µl of deionized forma-mide EDTA pH 8.0 (5/1). The samples were then loaded onto an Applied Biosystems 373A sequencer and run for 12 h on a 4.5% denaturing acrylamide gel. New partial sequences were aligned together with published sequences from GenBank using Clus-tal W. Phylip 3 software was used to built an un-rooted phylogenetic tree, based on the Kimura dis-tance and the neighbor joining method.

2.5. Total DNA extraction, probe labeling and Southern hybridization

Strains were grown on TY liquid medium at 28°C at 150 rpm shaking for 24 h. Cells were pelleted (2 ml of culture adjusted to an OD_{620nm} of 0.5) by cen-trifugation at $6000 \times g$. From the cell pellets total DNA was extracted and purified using the Non-or-ganic DNA extraction kit (Oncor Appligene, France) according to the manufacturer's instructions.

DNA was digested independently with *Eco*RI, *Hin*dIII and *Xho*I (Boehringer Mannheim) electro-phoresed on 0.9% agarose gel in TAE buffer. The Dig labeled DNA molecular mass marker II (Boeh-ringer Mannheim) was also loaded on each gel. DNA fragments were then transferred to a Nylon membrane (Nytran NY13, Schleicher and Schuell) using a Vacu Gene apparatus (Pharmacia). The membranes were then washed in $5 \times$ sodium saline citrate (SSC) and air-dried. DNA was cross-linked to the membrane by UV irradiation for 3 min. Hy-bridization with *tfd*C probes was carried out under highly stringent conditions, i.e. posthybridization stringency washes were performed twice in $2 \times$SSC and 0.1% SDS solution for 5 min at room temper-ature and twice in $0.1 \times$SSC and 0.1% SDS solution

for 15 min at 68°C. The detection of the hybridized probe was realized using the Dig Luminescent De-tection Kit (Boehringer Mannheim) and CDP Star as chemiluminescent substrate.

Two probes for *tfd*C were produced by PCR using the primers TFDCF and TFDCR and diluted total DNA from JMP134 (probe A) and PLAE6 (probe P) as template DNA. The reaction conditions were as described above, except that the dNTP concentra-tions were as follows: dATP, dCTP and dGTP 200 mmol l^{-1} each; dTTP 26 mmol l^{-1}; Dig 11-dUTP 13 mmol l^{-1} (Boehringer Mannheim). For each prob-ing, 2 µl of the amplified product was heat-denatured and incorporated in 10 ml of hybridization solution. The two PCR products used as probes were se-quenced and their sequences were aligned with known *tfd*C sequences.

2.6. Plasmid profiling

Plasmids were visualized by the method of Eck-hardt [23] as modified by Wheatcroft et al. [24]. The method was adapted by using 15% SDS instead of 10% SDS for the in gel lysis of the cells. *Rhi-zobium meliloti* strain 41, carrying plasmids of 500 kb, 225 kb and 60 kb, and *Agrobacterium tumefa-ciens* strain C58 harboring plasmids pAT and pTi of 414 kb and 195 kb respectively were used as molec-ular mass standards according to Wheatcroft et al. [24]. Pictures of the gels were recorded as described previously after ethidium bromide staining.

2.7. Nucleotide sequence accession numbers

The new *tfd*C sequences were deposited in Gen-Bank under the following accession numbers: AF035159 (isolate PLAE6), AF043451 (*B. cepacia* strain RASC), AF041364 (*Alcaligenes paradoxus* strain JMP133) and AF047032 (isolate C1CL).

3. Results

3.1. 2,4-D degradation capacities of the strains

Thirty-one strains or isolates from geographically different sites were considered in this study. They were tested for their capacity to grow on media con-

Table 2
Phenotypic and genotypic characterization of strains

Strain	Utilization of side chain [14C]2,4-D	% of ring [14C]2,4-D mineralized	ARDRA type	*Eco*RI RFLP (kb)[a] Probe A	Probe P	PCR *tfd*C	PCR-*tfd*C restriction types	Plasmid profile (kb)[b]	Hybridization of plasmid profile
JMP134	+	65	E	**19.2**+8.7	19.2+**8.7**	+	A	410, **86**	(A, P)
134I17A1	+	67	E	**19.2**+8.7	19.2+**8.7**	+	A	395, **88**	(A, P)
134I17A2	+	67	E	**19.2**+8.7	19.2+**8.7**	+	A	416, **78**	(A, P)
134AcA2	+	70	E	**19.2**+8.7	19.2+**8.7**	+	A	406, **92**	(A, P)
JD1	+	64	E	20.0	21.0+**5.9**	+	A	404, **194**	(A, P)
JD2	+	52	E	**20.0**+5.9	21.0+**5.9**	+	A	377, **194**	(A, P)
JD16	+	70	E	**21.0**+5.9	21.0+**5.9**	+	A	547, **209**	(A, P)
JD17	+	51	E	**21.0**+5.9	21.0+**5.9**	+	A	**166**	(A, P)
S1 (TV1)	+	69	C	13.7	**13.7**	+	P	**442**, 350	(P)
PLAE3	+	74	C	13.7	**13.7**	+	P	**321**	(P)
PLAE4	+	69	C	13.7	**13.7**	+	P	**331**	(A, P)
PLAE6	+	67	C	13.7	**13.7**	+	P	376, **305**	(P)
D8	+	69	C	13.7	**13.7**	+	P	**321**	(P)
F7-2	+	71	C	13.7	**13.7**	+	P	**350**	(P)
B197	+	69	C	13.7	**13.7**	+	P	**308**	(P)
1D9	+	41	A	13.7	**13.7**	+	P	**225**	(P)
JMP131	+	67	D	21.8	**21.8**	+	E	268, **107**	weak (A, P)
JMP131B	+	68	D	21.8	**21.8**	aspecific	nd	248, **99**	weak (A, P)
PLAE2	+	57	D	21.8	**21.8**	aspecific	nd	229, **96**	weak (A, P)
D2(1)Ba	+	60	D	21.8	**21.8**	aspecific	nd	236, **95**	weak (A, P)
C11CL	+	68	D	21.8	**21.8**	+	A	229, **96**	weak (A, P)
C1CL	+	65	D	22.0+17.5	22.0+17.5	+	C	234, **97**	weak (A, P)
B6-9	+	73	F	–	**22.5**	+	P	no plasmid	well (A, P)
JMP133	+	73	H	–	23.0	+	C	208, **87**	(P)
TFD6	+	0	I	9.1	9.1+2.6	+	A	**265**	weak (P)
Pcep	+	84	G	**20.0**	20.0	+	A	435, 326, 117, 85, 29	well (A)
PCD4	+	1	G	9.7	9.7+2.4	+	A	**409**, 287, 232, 90	(P), well (A)
RASC	+	1[c]	G	–	7.2+4.7	+	D	**532**, 331	(P)
TCP	−	1	E	–	–	−	nd	601, 466	–
D10	+	0	E	17.0	**17.0**	−	nd	153, **72**	(A, P)
82	+	1	B	–	16.8	−	nd	no plasmid	well (P)

[a]Fragments indicated in bold numbers hybridize strongly to the probe, other fragments hybridize weakly.
[b]Plasmids indicated in bold numbers hybridize with the probe as shown in the next column, others do not hybridize with the probes used.
[c]RASC was able to grow on ring labeled [U-14C]2,4-D in the presence of 20 mg l^{-1} of 2,4-D.

taining 2,4-D as the only carbon and energy source. All the strains were able to utilize the side chain carbon except strain TCP, while only 26 strains were shown to mineralize carbon from the ring (Table 2).

3.2. Phylogenetic grouping of the strains

The 31 strains or isolates were characterized by ARDRA analysis. They were grouped into nine different 16S rDNA types, on the basis of the polymorphism of restrictions profiles with three endonucleases: *Alu*I, *Hae*III, *Hha*I (Fig. 1). Most of the Dijon and Citeaux soil isolates could be assigned to the three genera *Variovorax*, *Alcaligenes* and *Ralstonia*, on the basis of their ARDRA profiles. A first cluster of isolates from Dijon soil (B197, D8 and F7-2) and from Citeaux soil (PLAE3, PLAE4, PLAE6) could not be distinguished from the *V. paradoxus* strain S1, and are therefore closely related to this
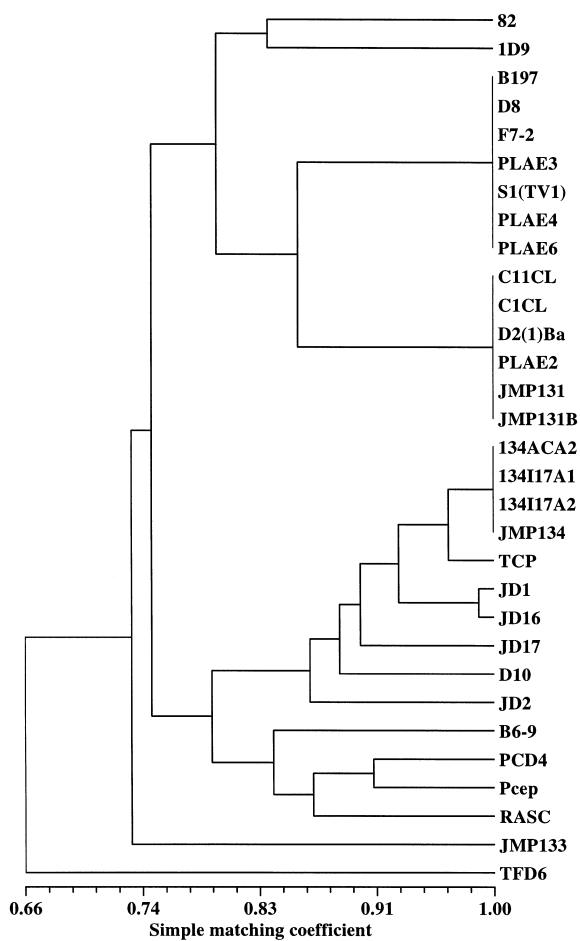
Fig. 1. ARDRA (SSU rDNA RFLP) clustering of 2,4-D degrading strains and soil isolates, based on restriction profiles obtained with the three restriction endonucleases *Hha*I, *Hae*III and *Alu*I. The simple matching coefficient was used to compute the distance matrix and the UPGMA method was used to build the similarity tree from the distance matrix.

3.3. PCR detection and polymorphism of tfdC-like genes

Detection of *tfd*C-like genes was performed on all strains by PCR using degenerated primers. PCR products were hybridized with both *R. eutropha* JMP134 and PLAE6 *tfd*C probes, 'A' and 'P' respectively (data not shown) in order to verify their homology with *tfd*C. Twenty-five strains out of 31 tested showed a PCR product of 461 bp, homologous to one or both of the two probes used. Three strains gave non-specific amplification products, and
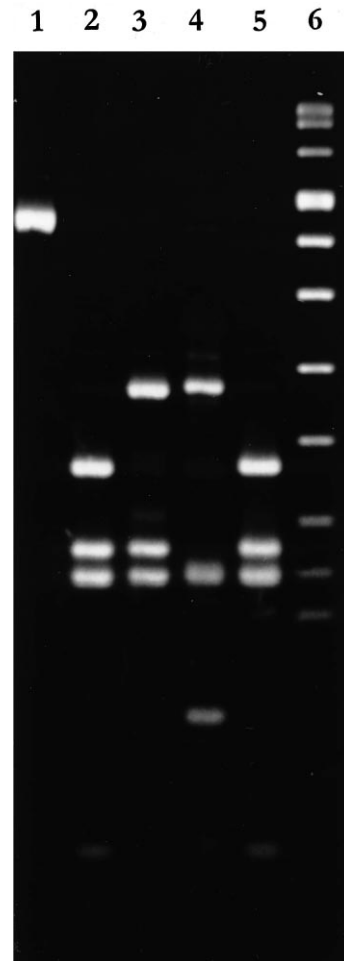

Fig. 2. Restriction profiles of the *tfd*C PCR products with *Hae*III. Lane 1: *Ralstonia eutropha* strain JMP134. Lane 2: strain PLAE6. Lane 3: *Burkholderia cepacia* strain RASC. Lane 4: *Alcaligenes paradoxus* strain JMP133. Lane 5: strain 1D9. Lane 6: molecular mass marker VIII (Boehringer Mannheim).

species (Fig. 1). A second set of isolates from Dijon (C11CL, C1CL, D2(1)Ba) and Citeaux (PLAE2) soils are clustered with *Alcaligenes eutrophus* strain JMP131. Dijon soil isolate D10 is closely related to the genus *Ralstonia*. None of the Dijon or Citeaux isolates is clustered with *Burkholderia* strains PCD4 and RASC, or with *Rhodoferax fermentans* strain B6-9. Strains JMP134 and TCP, both identified as *R. eutropha*, are clustered at a similarity level of 97%. Other strains and isolates show a wide diversity in their ARDRA profiles.

three strains gave no amplification products (Table 2).

Polymorphism of the *tfd*C PCR products was estimated by restriction analysis with two endonucleases (*Hha*I and *Hae*III) for the 25 strains giving positive amplification. Examples of *Hae*III restriction profiles are shown in Fig. 2, and results are reported in Table 2. Five distinct restriction profiles named A, P, C, D, and E were found among the 25 strains. The profiles A and P corresponded to the restriction patterns deduced from the sequences of the *tfd*C gene of *R. eutropha* JMP134 on plasmid pJP4 and *P. putida* PaW85 on plasmid pEST4410, respectively. These two profiles are dominant among the 25 strains tested. The restriction profiles of strains JMP133 and C1CL (type C) are identical. Profiles D and E are unique to *B. cepacia* strain RASC and *A. eutrophus* strain JMP131, respectively.

3.4. Sequence analysis

A 450-bp internal fragment of *tfd*C putative genes was sequenced for strains presenting new *tfd*C restriction patterns. The *tfd*C PCR products from strains JMP134, RASC, JMP133 and from local soil isolates PLAE6, C1CL, 1D9 were sequenced (both strands). Sequences were aligned and the phylogenetic tree shown in Fig. 3 was built. The product from JMP134 showed 100% homology with the published canonical *tfd*C sequence from JMP134 (GenBank accession number M35097), thus demonstrating the specificity of the primers used. The *tfd*C sequence from PLAE6 was 100% homologous to that from *P. putida* PaW85 (GenBank accession number U32188). The PLAE6 *tfd*C sequence was deposited (GenBank accession number AF035159) and was chosen as an alternative probe for Southern hybridization, because it shared only 63% homology with *tfd*C from *R. eutropha* JMP134. The *tfd*C sequences from *P. putida* PaW85 and PLAE6 are more closely related to the isofunctional gene *tfd*CII of *R. eutropha* JHP134 (86% homology) than to the canonical *tfd*C of *R. eutropha* JMP134 (Fig. 3).

The *tfd*C gene from *Burkholderia* sp. strain RASC (GenBank accession number AF043451) showed 93% of homology to that of *Pseudomonas putida* PaW85. The *tfd*C gene of JMP133 and C1CL (GenBank accession numbers AF041364, AF047032) showed 94% of homology to the *tfd*CII gene of *Ralstonia eutropha* (GenBank accession number U16782) and 83% of homology to *clc*A gene located on plasmid pAC27 of *Pseudomonas putida* strain AC867 (GenBank accession number M36279). The *tfd*C gene from isolate 1D9 is 100% homologous to
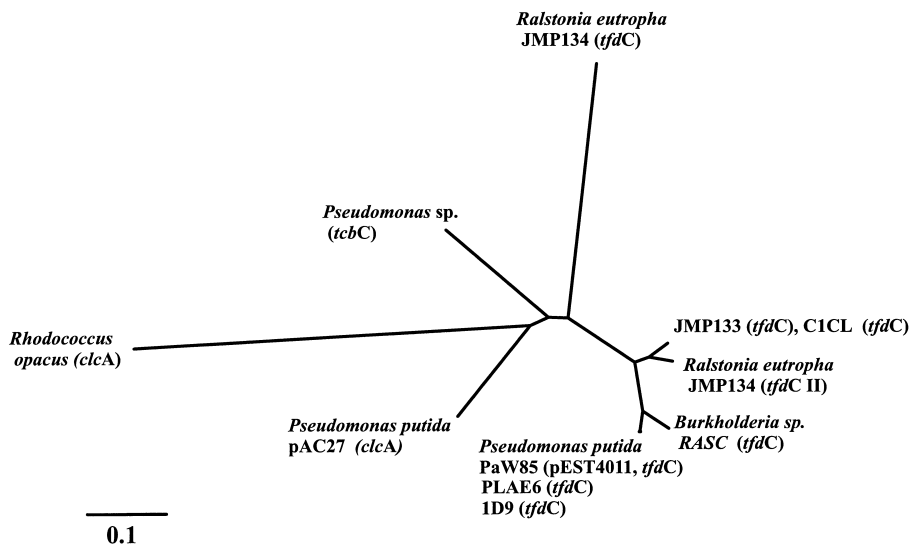

Fig. 3. Phylogenetic tree of the *tfd*C genes and related pyrocatechase genes. Sequences were aligned together with published sequences from GenBank using Clustal W. Phylip 3 software was used to build an unrooted phylogenetic tree, based on the Kimura distance and the neighbor joining method. Bar = 0.1% sequence divergence.
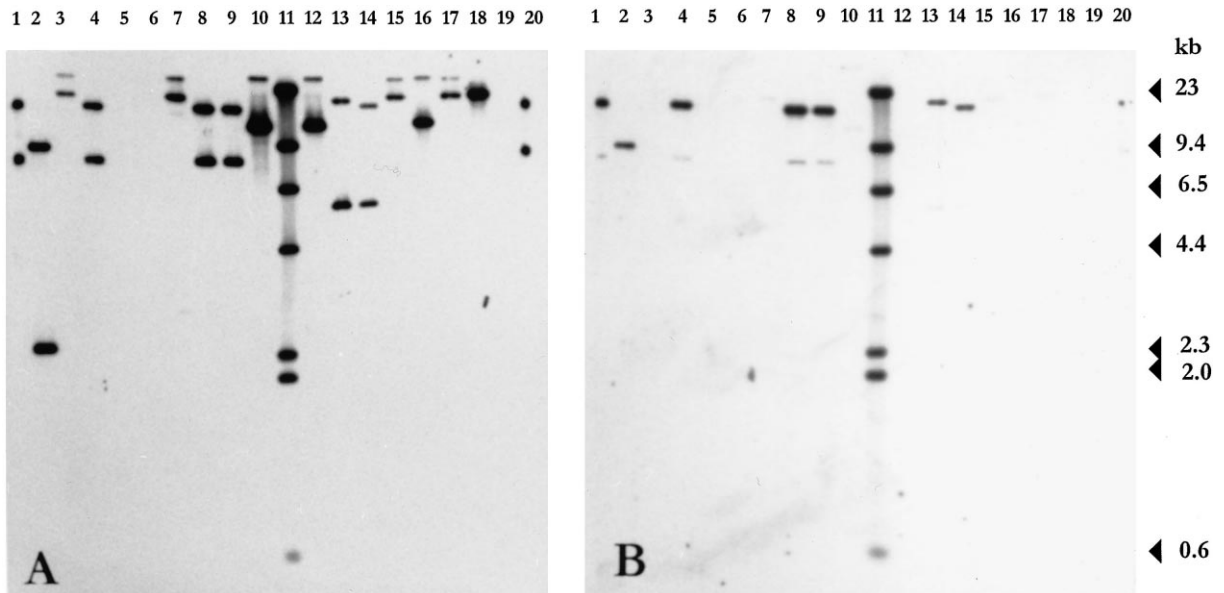
Fig. 4. Hybridization patterns of total DNA restricted by *Eco*RI using *tfd*C probe 'P' from isolate PLAE6 (A) and *tfd*C probe 'A' from *R. eutropha* (B). Lanes 1: JMP134, 2: PCD4, 3: PLAE2, 4: JMP134, 5: TCP, 6: non-degrading strain, 7: JMP131, 8: 134I17A1, 9: 134I17A2, 10: S1, 11: molecular mass marker II Dig-labelled (Boehringer Mannheim), 12: PLAE6, 13: JD17, 14: JD2, 15: D2(1)Ba, 16: PLAE3, 17: C11CL, 18: B6-9, 19: non-degrading strain, 20: JMP134.

the *tfd*C gene of *Pseudomonas putida* PaW85 on plasmid pEST4011. None of the soil isolates carried a *tfd*C gene highly homologous to *tfd*C of *R. eutropha* JMP134.

3.5. RFLP hybridization experiments

Total DNA restrictions were performed by three endonucleases: *Hind*III, *Eco*RI, *Xho*I. They were chosen for their inability to cut *tfd*C genes, as deduced from the published GenBank sequences; this allowed us to detect the polymorphism of gene organization and the gene copy number in the different strains studied. Restriction profiles were independently hybridized with the two probes produced from *R. eutropha* JMP134 and from isolate PLAE6 *tfd*C genes (probes 'A' and 'P', respectively). Hybridization experiments were all carried out at high stringency. They showed a great diversity in gene organization among the strains (Fig. 4). Sizes of hybridizing bands for *Eco*RI digests are given in Table 2. The hybridization patterns obtained with the two other restriction enzymes were in agreement with

data obtained from *Eco*RI digests, thus data for other enzymes are not shown. Seven strains, including JMP134, showed two restriction fragments hybridizing with the probes whatever the restriction enzyme used, thus indicating the occurrence of two copies of *tfd*C genes. In strains JMP134 and derivatives, and in strains JD16, JD17 and JD2 one restriction fragment (with a size of 19–21 kb) hybridizes strongly with probe 'A' and weakly with probe 'P', the second fragment (with a size of 8.7 or 5.9 kb) showing a strong hybridization with probe 'P' and only weak homology with probe 'A'. This result indicates that these strains carry two different copies of the *tfd*C gene, one homologous to *R. eutropha* JMP134 *tfd*C and the other homologous to *P. putida* PaW85 *tfd*C. Strain JD1 has two hybridizing fragments but showed strong hybridization only with probe 'P'. Strains PCD4 and TFD6 showed only weak hybridization with the two probes.

A second group of strains and soil isolates (AR-DRA types C, A and D) showed only one fragment (with a size of 13.7 and 21.8 kb, respectively) hybridizing strongly with probe 'P' from PLAE6 and

weakly with probe 'A' from JMP134. C1CL, although it belongs to ARDRA group D, had two *tfd*C copies located on 22-kb and 17.5-kb *Eco*RI fragments. Strains B6-9, JMP133, RASC and 82 hybridized only with probe 'P'. Other strains showed unique patterns.

Finally, the *R. eutropha* strain TCP, able to use 2,4,6-trichlorophenol as sole carbon and energy source [25]. did not give any positive hybridization signal (Fig. 4), reflecting the specificity of the two probes for 3,5-chlorocatechol dioxygenase genes.

3.6. Plasmid profiles and localization of tfdC genes

The plasmid profile of each strain was determined. The in gel lysis technique allowed us to visualize very large plasmids and to estimate their size. The localization of *tfd*C-like genes was estimated by hybridization of Southern blots of plasmids profile with probes 'A' and 'P'. Examples of plasmid profiles and corresponding hybridization with probe 'A' are given in Fig. 5. The number of plasmids per strain varied from 0 to 6 among the strains studied, with sizes varying from 36 kb to 601 kb. Plasmid profiles and hybridization data are given in Table 2. In 27 out of 31 strains tested, the *tfd*C-like genes were shown to be localized on one single plasmid with a size varying from 72 kb to 532 kb. Sixteen of these

plasmids hybridized with the two probes used and 11 hybridized only with probe 'P' from PLAE6. For strains B6-9 and 82 it was not possible to visualize any plasmid, only the well of the gel showed hybridization. *B. cepacia* strain Pcep carried five plasmids, but none of these was found to carry *tfd*C genes, and a hybridization signal was also observed in the well. Plasmid-borne *tfd*C genes were also found in *Burkholderia* sp. strain TFD6 and in *B. cepacia* strain RASC.

4. Discussion

4.1. Detection and polymorphism of tfdC genes, using degenerated primers

By combining PCR amplification with degenerated primers and hybridization with two DNA probes, we were able to detect catabolic *tfd*C genes from different strains and from local soil isolates. There is a good agreement, for 28 strains out of 31 tested, between the capacity of the strains to cleave (or not) the 2,4-D ring and the presence (or absence) of a PCR product using the primers designed in this work (Table 2). However, three strains degrading 2,4-D ring (JMP131B, PLAE2, D2(1)Ba), which grouped in the same ARDRA type D and had sim-
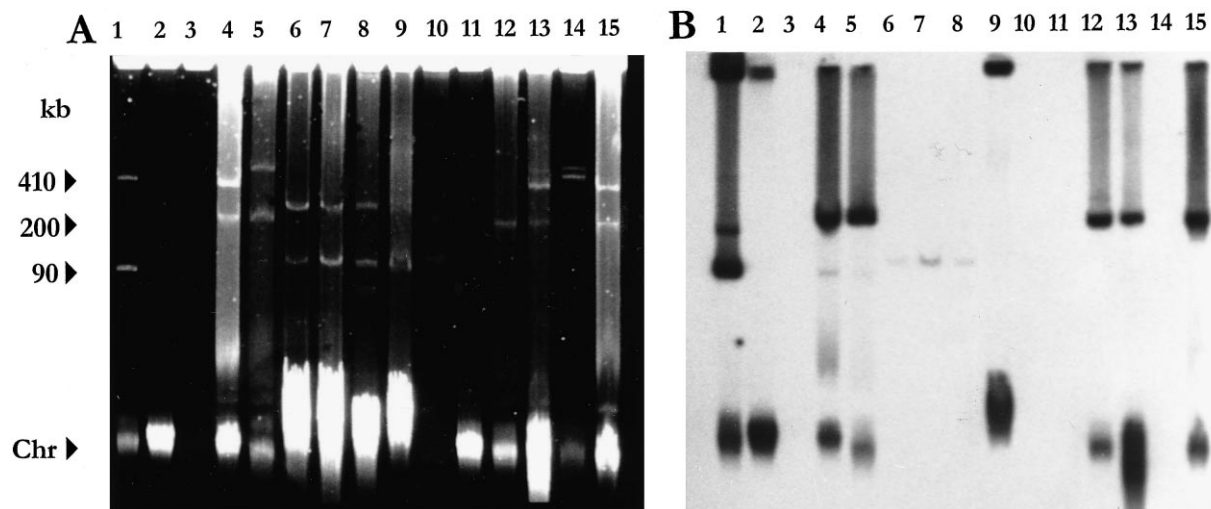

Fig. 5. Plasmid profiles of some strains degrading 2,4-D (A) and corresponding hybridization with *tfd*C probe 'A' from *R. eutropha* (B). Lanes 1: JMP134, 2: Pcep, 3: D10DEH4, 4: JD2, 5: JD16, 6: PLAE2, 7: C11CL, 8: C1CL, 9: PCD4, 10: JMP131A, 11: RASC, 12: JD17, 13: JD1, 14: TCP, 15: JD6.

ilar plasmids profiles, failed to give specific amplification, even when positive signals were detected by hybridization of total DNA and of plasmid profiles. This discrepancy could be explained by sequence divergence in the *tfd*C regions chosen as PCR priming sites. Three strains (RASC, PDC4, TFD6) failed to utilize the carbon of the ring, although they have been reported to degrade the 2,4-D ring [9,16,26]. These strains gave 460-bp amplification products which did hybridize with at least one of the two probes. This discrepancy could be due to the preculture step on TY medium which might have caused the loss of the genes encoding the initial steps of 2,4-D degradation, but not the loss of the *tfd*C gene, thus impairing the functioning of *tfd*C. For the three strains TCP, D10 and 82, which were unable to cleave the 2,4-D ring, no PCR product was observed.

PCR amplification of the chlorocatechol 1,2-dioxygenase gene was demonstrated to be specific for microorganisms able to cleave the 2,4-D aromatic ring. Restriction profiling of *tfd*C amplicons was a convenient tool to evaluate polymorphism of *tfd*C-type catabolic genes and was consistent with hybridization results and sequencing analysis. It allows a rapid screening of numerous isolates.

4.2. Sequence divergence leads to specialization of genes

Together, PCR/RFLP and sequence data obtained from some strains underlined a great diversity of *tfd*C genes. We found that the dominant *tfd*C gene type in local soil isolates was homologous to that of *P. putida* PaW85 isolated in Estonia. *tfd*C genes homologous to the isofunctional copy *tfd*CII of *R. eutropha* JMP134 were found in *A. paradoxus* strain JMP133 isolated in Australia and in the French soil isolate C1CL. The *tfd*CII gene of *R. eutropha* was found to be more closely related to the *tfd*C gene of *P. putida* PaW85 than to the canonical *tfd*C gene of *R. eutropha* JMP134. This data indicated that the *P. putida* PaW85 *tfd*C gene type could be more widely distributed than the canonical *tfd*C from *R. eutropha* JMP134 and it could explain why recent studies, using pJP4-based probes [16,18,27], failed to detect *tfd*C genes in 2,4-D degrading strains isolated from soil. The *tfd*C gene of strain RASC, isolated in the USA, is closely related to those of *P.*

putida PaW85, JMP133, C1Cl, and *tfd*CII of *R. eutropha* (Fig. 3). The *tfd*CII gene of *R. eutropha* JMP134 has been found to share 63% sequence similarity with the *clc*A gene of *P. putida* AC867 [12], although TfdC and ClcA pyrocatechases showed different substrate specificity.

All strains or isolates, originated from different locations, but grouped within the β and γ subdivisions of Proteobacteria, carry chlorocatechol 1,2-dioxygenase sequences more closely related to each other than to the canonical *tfd*C gene of *R. eutropha* JMP134. The second copy of the *tfd*C gene in strain JMP134 (Fig. 5) is more closely related to the *tfd*C gene of PaW85 than to the first copy located on pJP4. The *tfd*C genes homologous to that of PaW85 might be fossil footprints along the evolution of 2,4-D catabolic pathways.

Our data support the hypothesis that the *tfd*C gene of *P. putida* PaW85 and the other related *tfd*C genes might be ancestor genes for chlorocatechol 1,2-dioxygenases involved primarily in the degradation pathway of natural compounds and later recruited for 2,4-D degradation. Further evolution has taken place and led to more specialized enzymes like those encoded by the *tfd*C gene of *R. eutropha* JMP134 located on the pJP4 plasmid.

4.3. Genomic localization of tfdC genes and copy number

The *tfd*C genes were found to be located on plasmids with a size ranging from 80 to 500 kb, in 27 out of 31 strains and isolates tested. Plasmids carrying *tfd*C genes are highly variable in size, suggesting that they are diverse. The PLAE6 probe 'P' homologous to *P. putida* PaW85 hybridized to many plasmids that the pJP4 probe 'A' from JMP134 failed to detect. Three out of the 31 strains showed hybridization only with the well of the gel and with the chromosomal band area: strain B6-9, previously identified as *Rhodoferax fermentans*, had no visible plasmid band. Strain Pcep, assigned to *B. cepacia* by ARDRA, carried five plasmids but none of them hybridized; strain 82, distant from the previous two ARDRA groups, did not show any hybridizing plasmid. Thus chlorocatechol 1,2-dioxygenase genes might be chromosomally encoded in the strains B6-9, Pcep and 82.

Previous studies have reported that the *tfd*A genes of strains TFD6 and RASC were chromosomally located [9,26]. In the present study, we have demonstrated that their *tfd*C genes are located on large plasmids with a size of 265 and 532 kb for strains TFD6 and RASC, respectively. For strain RASC, plasmid localization of the *tfd*C gene is in agreement with the recent work reported by McGowan et al. [28]. These authors found that *B. cepacia* strain RASC was able to transfer degradative abilities to a non-degrading recipient strain.

Ka et al. [29] have reported that *tfd* genes are located on plasmids in 75% of the isolates from their soils. We have demonstrated in the present study that this proportion is higher (87%), at least for *tfd*C genes. This might be due to the method that we used for plasmid analysis which allows us to visualize very large plasmids.

4.4. Evidence of tfdC gene transfer

Transfer and recruitment of catabolic pathways between different soil bacteria has already been demonstrated for many catabolic pathways due to the presence of transposons, insertion sequence elements, or to the localization on conjugative plasmids [30–37].

In the present study, we have demonstrated that strongly homologous *tfd*C genes can be found in various chromosomal backgrounds (ARDRA groups), suggesting the occurrence of *tfd*C gene transfer. No correlation was found between ARDRA grouping of the strains and either the sequence or the organization of the *tfd*C genes. This observation is consistent with recent data from McGowan et al. [28] on *tfd*A genes. These authors hypothesized that gene transfer could occur between species due to the lack of correlation between ARDRA grouping of the strains and *tfd*A diversity. Thus, phylogenetically diverse strains may carry strongly homologous or identical *tfd*C genes. As these genes are located on plasmids in most of the strains studied, the transfer mechanism might be conjugative plasmid transfer.

4.5. Duplication of tfdC and gene copy number

Strains and isolates which carry a *tfd*C gene homologous to that of *P. putida* PaW85 had one copy of *tfd*C and showed similar RFLP patterns. On the other hand, strains carrying a *tfd*C gene homologous to the *R. eutropha* JMP134 *tfd*C gene showed more variable RFLP patterns, and carried two copies of *tfd*C, one homologous to the canonical *tfd*C of *R. eutropha* JMP134 and the other to *tfd*C of *P. putida* PaW85. This second signal probably corresponds to the *tfd*CII gene, since hybridization is stronger with the *P. putida* PaW85 probe as a consequence of their sequence relatedness (Fig. 3). Duplication of genes in catabolic pathways could occur for xenobiotics recently introduced into the environment [30,38]. Duplicated sequences have been previously described within the *tfd* operon for *tfd*A [13], *tfd*C [12] and *tfd*D [14] genes. The presence of partially homologous copies of isofunctional genes has also been reported for the *nif* genes found among nitrogen fixing soil bacteria [39–41] and for catabolic pathways of various xenobiotic compounds [12,13,42–44].

Our results demonstrate the usefulness of a polyphasic approach to study gene diversity and distribution. The combination of PCR with specific primers, RFLP of PCR products, hybridization with two divergent probes and plasmid profile analysis, allowed us to get a good insight into the mechanisms that have led to the construction of the 2,4-D catabolic pathway.

References

[1] van der Meer, J.R. (1994) Genetic adaptation of bacteria to chlorinated aromatic compounds. FEMS Microbiol. Rev. 15, 239–249.

[2] Ghosal, D., You, I.S., Chatterjee, D.K. and Chakrabarty, A.M. (1985) Genes specifying degradation of 3-chlorobenzoic acid in plasmids pAC27 and pJP4. Proc. Natl. Acad. Sci. USA 82, 1638–1642.

[3] van der Meer, J.R., Eggen, R.I., Zehnder, A.J.B. and de Vos, W.M. (1991) Sequence analysis of the *Pseudomonas* sp. P51 *tcb* gene cluster, which encodes metabolism of chlorinated catechols: evidence for specialization of catechol 1,2-dioxygenases for chlorinated substrates. J. Bacteriol. 173, 2425–2434.

[4] Don, R.H., Weightman, A.J., Knackmuss, H.J. and Timmis, K.N. (1985) Transposon mutagenesis and cloning analysis of the pathway for degradation of 2,4-dichlorophenoxyacetic acid and 3-chlorobenzoate in *Alcaligenes eutrophus* JMP134(pJP4). J. Bacteriol. 161, 85–90.

[5] Amy, P.S., Schulke, J.W., Frazier, L.M. and Seidler, R.J. (1985) Characterization of aquatic bacteria and cloning of

genes specifying partial degradation of 2,4-dichlorophenoxy-acetic acid. Appl. Environ. Microbiol. 49, 1237–1245.

[6] Chaudhry, G.R. and Huang, G.H. (1988) Isolation and characterisation of a new plasmid from *Flavobacterium* sp. which carries the genes for degradation of 2,4-dichlorophenoxyacetic acid. J. Bacteriol. 170, 3897–3902.

[7] Mäe, A.A., Marits, R.O., Ausmess, N.R., Koiv, V.M. and Heinaru, A.L. (1993) Characterization of a new 2,4-dichlorophenoxyacetic acid degrading plasmid pEST4011: physical map and localisation of catabolic genes. J. Gen. Microbiol. 139, 3165–3170.

[8] Bhat, M.A., Tsuda, M., Horiike, K., Nozaki, M., Vaidyanathan, C.S. and Nakazawa, T. (1994) Identification and characterization of a new plasmid carrying genes for degradation of 2,4-dichlorophenoxyacetate from *Pseudomonas cepacia* CSV90. Appl. Environ. Microbiol. 60, 307–312.

[9] Suwa, Y., Wright, A.D., Fukimori, F., Nummy, K.A., Hausinger, R.P., Holben, W.E. and Forney, L.J. (1996) Characterization of a chromosomally encoded 2,4-dichlorophenoxyacetic acid/alpha-ketoglutarate dioxygenase from *Burkholderia* sp. strain RASC. Appl. Environ. Microbiol. 62, 2464–2469.

[10] Maltseva, O.V., Solyanikova, I.P. and Golovleva, L.A. (1991) Catechol 1,2-dioxygenases of a chlorophenol-degrading strain of *Rhodococcus erythropolis*: purification and properties. Biochemistry 56, 1548–1555.

[11] Maltseva, O.V., Solyanikova, I.P. and Golovleva, L.A. (1994) Chlorocatechol 1,2-dioxygenase from *Rhodococcus erythropolis* 1CP. Kinetic and immunochemical comparison with analogous enzymes from Gram negative strains. Eur. J. Biochem. 226, 1053–1061.

[12] Ghosal, D. and You, I.S. (1988) Nucleotide homology and organization of chlorocatechol oxidation genes of plasmids pJP4 and pAC27. Mol. Gen. Genet. 211, 113–120.

[13] Perkins, E.J. and Lurquin, P.F. (1988) Duplication of a 2,4-dichlorophenoxyacetic acid monooxygenase gene in *Alcaligenes eutrophus* JMP134 (pJP4). J. Bacteriol. 170, 5669–5672.

[14] Matrubutham, U. and Harker, A.R. (1994) Analysis of duplicated gene sequences associated with *tfd*R and *tfd*S in *Alcaligenes eutrophus* JMP134. J. Bacteriol. 176, 2348–2353.

[15] Kaphammer, B., Kukor, J.J. and Olsen, R.H. (1990) Regulation of *tfd*CDEF by *tfd*R of the 2,4-dichlorophenoxyacetic acid degradation plasmid pJP4. J. Bacteriol. 172, 2280–2286.

[16] Vallaeys, T., Fulthorpe, R.R., Wright, A.M. and Soulas, G. (1996) The metabolic pathway of 2,4-dichlorophenoxyacetic acid degradation involves different families of *tfd*A and *tfd*B genes according to PCR-RFLP analysis. FEMS Microbiol. Ecol. 20, 163–172.

[17] Koiv, V., Marits, R. and Heinaru, A. (1996) Sequence analysis of the 2,4-dichlorophenol hydroxylase gene *tfd*B and 3,5-dichlorocatechol 1,2-dioxygenase gene *tfd*C of 2,4-dichlorophenoxyacetic acid degrading plasmid pEST4011. Gene 174, 293–297.

[18] Vallaeys, T., Persello-Cartieaux, F., Rouard, N., Lors, C., Laguerre, G. and Soulas, G. (1997) PCR-RFLP analysis of 16S rRNA, *tfd*A and *tfd*B genes reveals a diversity of 2,4-D degraders in soil aggregates. FEMS Microbiol. Ecol. 24, 269–278.

[19] Beringer, J.E. (1974) R Factor transfer in *Rhizobium leguminosarum*. J. Gen. Microbiol. 84, 188–198.

[20] Leveau, J.H. and van der Meer, J.R. (1996) The tfdR gene product can successfully take over the role of the insertion element-inactivated TfdT protein as a transcriptional activator of the *tfd*CDEF gene cluster, which encodes chlorocatechol degradation in *Ralstonia eutropha* JMP134(pJP4). J. Bacteriol. 178, 6824–6832.

[21] Sneath, P.H. and Sokal, R.R. (1973) Numerical Taxonomy. W.H. Freeman and Co., San Francisco, CA.

[22] Rohlf, F.J. (1987) NTSYS-pc: Numerical Taxonomy and Multivariate Analysis. System for the IBM PC Microcomputer (and Compatibles), Version 1.3. Applied Biostatistics, New York.

[23] Eckhardt, T. (1978) A rapid method for the identification of plasmid desoxyribonucleic acid in bacteria. Plasmid 1, 584–588.

[24] Wheatcroft, R., McRae, D.G. and Miller, R.W. (1990) Changes in the *Rhizobium meliloti* genome and the ability to detect supercoiled plasmids during bacteroid development. Mol. Plant-Microbe Interact. 3, 9–17.

[25] Andreoni, V., Baggi, G., Colombo, M., Cavalca, L., Zangrossi, M. and Bernasconi S. (1998) Degradation of 2,4,6-trichlorophenol by a specialized organism and by indigenous soil microflora: bioaugmentation and self-remediability for soil restoration. Lett. Appl. Microbiol. 27, 86–92.

[26] Matheson, V.G., Forney, L.J., Suwa, Y., Nakatsu, C. H., Sexstone, A.J. and Holben, W.E. (1996) Evidence for acquisition in nature of a chromosomal 2,4D-dichlorophenoxyacetic acid/alpha-ketoglutarate dioxygenase gene by different *Burkholderia* spp. Appl. Environ. Microbiol. 62, 2457–2463.

[27] Fulthorpe, R.R., McGowan, C., Maltseva, O.V., Holben, W.E. and Tiedje, J.M. (1995) 2,4-Dichlorophenoxyacetic acid-degrading bacteria contain mosaics of catabolic genes. Appl. Environ. Microbiol. 61, 3274–3281.

[28] McGowan, C., Fulthorpe, R., Wright, A. and Tiedje, J.M. (1998) Evidence for interspecies gene transfer in the evolution of 2,4-dichlorophenoxyacetic acid degraders. Appl. Environ. Microbiol. 64, 4089–4092

[29] Ka, J.O., Holben, W.E. and Tiedje, J.M. (1994) Genetic and phenotypic diversity of 2,4-dichlorophenoxyacetic acid (2,4-D)-degrading bacteria isolated from 2,4-D treated field soils. Appl. Environ. Microbiol. 60, 1106–1115.

[30] Feng, X., Ou, L.T. and Ogram, A. (1997) Plasmid-mediated mineralization of carbofuran by *Sphingomonas* sp. Strain CF06. Appl. Environ. Microbiol. 63, 1332–1337.

[31] Wyndham, R.C., Cashore, A.E., Nakatsu, C.H. and Peel, M.C. (1994) Catabolic transposons. Biodegradation 5, 323–342.

[32] Don, R.H. and Pemberton, J.M. (1981) Properties of six pesticide degradation plasmids isolated from *Alcaligenes paradoxus* and *Alcaligenes eutrophus*. J. Bacteriol. 145, 681–686.

[33] DiGiovanni, G.D., Neilson, J.W., Pepper, I.L. and Sinclair, N.A. (1996) Gene transfer of *Alcaligenes eutrophus* JMP134 plasmid pJP4 to indigenous soil recipients. Appl. Environ. Microbiol. 62, 2521–2526.

[34] Kamagata, Y., Fulthorpe, R.R., Tamura, K., Takami, H.,

Forney, L.J. and Tiedje, J.M. (1997) Pristine environments harbor a new group of oligotrophic 2,4-dichlorophenoxyacetic acid-degrading bacteria. Appl. Environ. Microbiol. 63, 2266–2272.

[35] Herrick, J.B., Stuart-Keil, K.G., Ghiorse, W.C. and Madsen, E.L. (1997) Natural horizontal transfer of a naphthalene dioxygenase gene between bacteria native to a coal tar-contaminated field site. Appl. Environ. Microbiol. 63, 2330–2337.

[36] Zhou, J.Z. and Tiedje, J.M. (1995) Gene transfer from a bacterium injected into an aquifer to an indigenous bacterium. Mol. Ecol. 4, 613–618.

[37] Madsen, E.L., Thomas, C.T., Wilson, M.S., Sandoli, R.L. and Bilotta, S.E. (1996) In situ dynamics of aromatics hydrocarbons (AHs) and bacteria capable of AH metabolism in a cool tar waste-contaminated field site. Environ. Sci. Technol. 30, 2412–2416.

[38] Daubaras, D. and Chakrabarty, A.M. (1992) The environmental microbes and bioremediation: microbial activities modulated by the environment. Biodegradation 3, 125–135.

[39] Quinto, C., De La Vega, H., Flores, M., Fernandez, L., Ballado, T., Soberon, G. and Palacios, R. (1982) Reiteration of nitrogen fixation gene sequences in *Rhizobium phaseoli*. Nature 299, 724–726.

[40] Premkumar, R., Lenos, E.M. and Bishop, P.E. (1984) Evidence for two dinitrogenase reductase regulatory control by molybdenum in *Azotobacter vinelandii*. Biochim. Biophys. Acta 797, 64–70.

[41] Bishop, P.E., Rizzo, T.M. and Bott, K.F. (1985) Molecular cloning of *nif* DNA from *Azotobacter vinelandii*. J. Bacteriol. 162, 21–28.

[42] Neidle, E.L., Hartnett, C., Bonitz, S. and Ornston, L.N. (1988) DNA sequence of the *Acinetobacter calcoaceticus* catechol 1,2-dioxygenase I structural gene *cat*A: evidence for evolutionary divergence of intradiol dioxygenases by acquisition of DNA sequence repetitions. J. Bacteriol. 170, 4874–4880.

[43] Keil, H., Lebens, M.R. and Williams, P.A. (1985) TOL plasmid pWW15 contains two non-homologous, independently regulated catechol-2,3-oxygenase genes. J. Bacteriol. 163, 248–255.

[44] Dorn, E. and Knackmuss, H.J. (1978) Chemical structure and biodegradability of halogenated aromatic compounds. Two catechol 1,2-dioxygenases from a 3-chlorobenzoate-grown pseudomonad. Biochem. J. 174, 73–84.

[45] Gunalan (1991) Etude du Comportement dans le Sol d'un Inoculum Bactérien Dégradant l'Herbicide 2,4-D. PhD. Thesis, Université Claude Bernard, Lyon.

[46] Tonso, N.L., Matheson, V.G. and Holben, W.E. (1995) Polyphasic characterisation of a suite of bacterial isolates capable of degrading 2,4-D. Microb. Ecol. 30, 3–24.

[47] Lors, C. (1997) Impact des Produits Phytosanitaires sur la Diversité Spécifique et Fonctionnelle de la Microflore du Sol. PhD Thesis, Université de Metz.