A transposon encoding the complete 2,4-dichlorophenoxyacetic acid degradation pathway in the alkalitolerant strain *Delftia acidovorans* P4a

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**INTRODUCTION**

Pollution of the environment with compounds derived from fossil sources and the anthropogenic production of xenobiotics is a global problem. Organochlorines are one group of compounds having a most serious impact on the environment; these compounds were applied extensively as herbicides, pesticides and solvents in the past. Some of them prove recalcitrant, as shown, for example, for poly-chlorinated biphenyl or dioxin, whereas others have been shown to be fairly well degradable by micro-organisms. Nevertheless, these compounds are known to accumulate in the environment. Therefore, a challenge in solving pollution problems is to better exploit and stabilize degradative machineries of the general cell metabolism. The genes of the ortho cleavage pathway are homologous and clustered in operons of similar structure. In many cases, the modified ortho cleavage pathway is encoded on catabolic plasmids (Don & Pemberton, 1981; Chatterjee et al., 1981; van der Meer et al., 1991c; Mäe et al., 1993; Bhat et al., 1994; Ogawa & Miyashita, 1995; Poh et al., 2002), which hints to the distribution of these genes by horizontal gene transfer. High similarity has also been observed for the genes and their products which are required to catalyse steps of certain upper pathways. This is the case for TfdA [2,4-dichlorophenoxyacetic acid (2,4-D)/2-oxoglutarate dioxygenase] and TfdB (2,4-dichlorophenol hydroxylase), respectively, which are responsible for the degradation of the commonly used herbicide 2,4-D to 3,5-dichlorocatechol via 2,4-dichlorophenol. Moreover, the genes and operons of the various steps/sequences are organized in a mosaic-like manner (Fulthorpe et al., 1995; Vallaeyss et al., 1999).

2,4-D can be used as a carbon and energy source by various soil bacteria and therefore has become a model compound to study the distribution and evolution of catabolic genes for chloroaromatic compounds (Ka et al., 1994; Fulthorpe et al., 1995, 1996; Top et al., 1995; Vallaeyss et al., 1996, 1999; Hogan et al., 1997; Kamagata et al., 1997; McGowan et al., 1998; Itoh et al., 2002). Although micro-organisms capable of mineralizing 2,4-D have been investigated intensively, knowledge on the microbial degradation of chloroaromatics in extreme environments such as highly alkaline habitats is still very limited (Maltseva et al., 1996). However, in addition to contaminated soils and (ground)water,
pollution problems in the chemical industry often extend to the facilities themselves. One special problem in this context is toxic residues in pesticide factories and the microbial decontamination of building rubble obtained after their demolition, since aqueous eluates from this material are very alkaline. Microbes occupying such an ecological niche have to face both extreme pH values and toxic substrates.

In the case described here, the building rubble of a former herbicide production plant was heavily contaminated with organochlorines, especially chlorinated and methylated phenoxyalkanoates and phenols, and generated pH values of up to 12 in an aqueous environment (Müller et al., 1999a). A microbial consortium was enriched from this material which was able to mineralize the cocktail of contaminants under alkaline conditions. One of the strains isolated from this consortium was Delftia [formerly Comamonas (Wen et al., 1999)] acidovorans P4a, which can utilize 2,4-D as sole sources of carbon and energy at pH values of up to 10, and has also exhibited degradative activity on concrete material in situ at overall pH values of up to 11.5 (Hoffmann et al., 1996; Müller et al., 1996).

Biodegradation of 2,4-D is catalysed sequentially by six degradative enzymes, encoded by tfdA, tfdB, tfdC, tfdD, tfdE and tfdF in the case of the well-characterized strain Ralstonia eutropha JMP134 (Don & Pemberton, 1985; Pieper et al., 1988, 1993). Enzymes with regulatory or transport functions encoded by tfdR, tfdS and tfdK, respectively, are also known (Kaphammer et al., 1990; Kaphammer & Olsen, 1990; Leveau & van der Meer, 1996; Leveau et al., 1998). The presence of tfd genes in D. acidovorans P4a has been revealed by analysis of PCR products generated using tfd-specific primers and genomic DNA of D. acidovorans P4a as a template (Hoffmann et al., 2001). In the present study, the organization of the genes of the entire 2,4-D degradative pathway in strain P4a was elucidated, and the relationships of the individual genes to those of other strains were probed. In addition, the localization of the respective genes was looked for. Both these aspects were considered essential to understand the function and stability of 2,4-D biodegradation. These aspects are of special interest if one intends to use strains such as D. acidovorans P4a to initiate or improve bioremediation efficiency in such problematic biotopes as building rubble.

METHODS

Genomic library. Genomic DNA of D. acidovorans P4a was isolated according to the protocol supplied with the Supercos 1 Cosmid Vector Kit (Stratagene), which was used to construct a genomic DNA library of D. acidovorans P4a. The library was amplified in Escherichia coli XL-1 Blue MR according to the Supercos 1 Cosmid Library (Stratagene) instruction manual.

Screening of recombinant library clones. To prepare DNA of the recombinant E. coli clones, the biomass of each freshly grown single colony was suspended in 50 μl sterile distilled water, boiled for 10 min, left on ice for 10 min and sedimented by centrifugation at 23 000 g and 4 °C for 10 min. From each supernatant, 4 μl was dripped onto a nylon membrane (Roche Diagnostics) and the DNA was fixed by UV irradiation. Clones were screened for genes encoding the 2,4-D degradation pathway with probes specific for the tfdA, tfdB, tfdC, tfdD and tfdF genes, obtained by PCR using genomic DNA from D. acidovorans P4a as a template and primers derived from conserved amino acid sequence motifs of corresponding homologous enzymes (Hoffmann et al., 2001). Oligonucleotides were synthesized by MWG BIOTECH. PCR and purification of the PCR products were performed as described by Hoffmann et al. (2001). The PCR products were digoxigenin (DIG)-labelled using the DIG DNA Labelling Kit (Roche). For hybridization with the DIG-labelled probes and immunodetection, the DIG Easy Hyb Wash and Block Buffer Set and DIG Nucleic Acid Detection Kit (Roche) were used. Hybridizations with tfdA, tfdB and tfdC probes were performed at 50 °C. tfdD and tfdF probes were hybridized at 60 °C. Two library clones, 59 and 1183, which showed hybridization signals with various tfd probes, were selected for further investigation.

Characterization of positive library clones and subcloning. Cosmid DNA of the tfd-positive E. coli clones was isolated with the NucleoBond PC Kit and NucleoBond AX columns as recommended by the manufacturer (Macherey-Nagel). Restriction analyses were initiated by digesting the cosmid DNA with various restriction endonucleases (Roche; New England Biolabs). DNA fragments were then separated electrophoretically in 0.8% agarose gels, blotted onto nylon membranes by standard procedures according to Sambrook et al. (1989) and fixed as described above. Southern blotting was performed with an Appligene vacuum blower. Hybridizations with the tfdA, tfdB, tfdC, tfdD and tfdF probes were carried out as described above. The positive DNA fragments of the two library clones 59 (subclones A34, B108 and C93) and 1183 (subclone N11) were subcloned using pUC18 and pGEM3-Zf (+) as vectors and E. coli DH5α as a host strain. Recombinant subclones were screened by blue/white selection and plasmid DNA of selected clones was isolated as described above. The presence of the expected insert was verified by restriction analysis.

DNA sequencing and sequence analysis. The nucleotide sequences of the tfd-positive and subcloned restriction fragments of D. acidovorans P4a (inserts of A34, B108, C93 as well as N11 subclones) were determined according to the method of Sanger et al. (1977) using an ABI PRISM 310 Genetic Analyser (PE Applied Biosystems). To complete the sequence data, sequencing was extended to neighbouring regions in the cosmid DNA of the library clones 59 and 1183 applying the primer-walking technique (Alphay, 1998; Strauss et al., 1986). Special primers were derived from respective insert sequences. Each sequence was determined twice. Sequence data were analysed by the SEQUENCE NAVIGATOR software (PE Applied Biosystems) and contigs were assembled by the AUTOASSEMBLER software (PE Applied Biosystems).

The comparison of sequences with DNA and protein sequences in sequence databases was performed with BLAST (Altschul et al., 1997; http://www.ncbi.nlm.nih.gov/blast). The sequence determined will appear in the GenBank/EMBL/DDJB sequence databases under accession number AY078159.

Location of the tfdCDF and tfdCαβEαβBKA operons. Chromosomal DNA of D. acidovorans P4a was isolated by using the NucleoSpin C&T Kit (Macherey-Nagel), while plasmid DNA was isolated by using the NucleoBond PC Kit (Macherey-Nagel) following the standard protocol of the supplier. DNA was digested with EcoRI and BglII (double digestion). DNA fragments were separated electrophoretically, blotted and fixed as described above, and subjected to Southern blot hybridization with the labelled PCR fragments tfdCDF (3-4 kb) and tfdAKB (2-5 kb) as probes at 60 °C. The tfdCDF probe was obtained by PCR carried out with genomic DNA of strain P4a...
using the specific tfdD forward and tfdF reverse primers described by Hoffmann et al. (2001). To prepare the tfdAKB probe, PCR was carried out with genomic DNA of strain P4a as template using 5′-CAT (A/G)TC (A/G)CA GAA CTC CGT-3′ as the tfdA primer and 5′-GA(A/G) ATG AA(C/T) CAG CGC TAT-3′ as the tfdB primer (complementary reverse sequences to the tfdA2 and tfdB1 primers described by Vallaey et al., 1996).

RESULTS

Characterization of the tfd-positive subclones

The respective tfd genes of library clone 59 were found on three different DNA fragments subcloned in A34 (2 kb), B108 (1.1 kb) and C93 (4.6 kb). Sequence analysis of these inserts revealed tfdA (partial) and a further open reading frame (ORF), orfL, on the A34 insert, tfdK and tfdA (partial) on the B108 insert as well as tfdB, tfdE, tfdCII and a partial tfdK gene on the C93 insert. The tfd-positive DNA fragment of library clone 1183 subcloned in N11 (8.2 kb) revealed the presence of genes encoding putative enzymes of the chlorocatechol pathway: tfdR, tfdC, tfdD, tfdE as well as tfdF (partial). Two further ORFs, orfI and orfII, were detected in this fragment in addition to a partial IS1380 sequence.

The 2,4-D degradation pathway of strain P4a

The genome fragments of library clones 59 and 1183 were found to carry different tfd genes and several ORFs; nevertheless, in both library clones orfL, tfdA, tfdB and tfdK occurred. Based on these data, the sequences were assembled, resulting in a 28.4 kb segment of the genome of strain P4a, suggesting the organization of the degradative genes of the entire 2,4-D degradation pathway in P4a (Fig. 1). Accordingly, the degradation pathway apparently consists of the two different gene clusters tfdCDEF and tfdCG1E1BKA, arranged in opposite transcriptional directions. The tfdR genes evidently associated with each of the two clusters are transcribed in the opposite direction to the respective clusters. Furthermore, the tfdE and tfdC genes occur twice, once in each cluster.

The tfdCDEF cluster. Some of the genes in the tfdCDEF cluster overlap in their coding regions, as this applies to the tfdC and tfdD as well as to the tfdE and tfdF genes in their start and stop codons, respectively. Other genes are separated from each other by intergenic regions: 1 bp between tfdD and orfI, 21 bp between the stop and start codons of orfI and tfdE, and 149 bp between tfdR and tfdC. The intergenic region of tfdR and tfdC has a typical characteristic of a divergent promoter region subjected to regulation by a LysR-type transcriptional regulator, as the nucleotide sequence differs in only one nucleotide compared with the homologous region in Pseudomonas sp. P51 (van der Meer et al., 1991b). Downstream of tfdF, the additional ORFs orfIII (1029 bp) and orfL (720 bp) were located, whereof orfL is transcribed divergently. Upstream of tfdC, another ORF, orfII, was detected which is transcribed in the same direction as the tfdCDEF operon.

![Fig. 1](http://mic.sgmjournals.org)
The \textit{tfdCDEII} cluster. The second gene cluster comprises five ORFs. An additional \textit{tfdC} gene was detected which is identical in nucleotide sequence to that within the \textit{tfdCDEF} cluster except for a short sequence of 19 bp at the 3' terminus. By contrast, a further \textit{tfdE} gene was found, the sequence of which differs substantially compared to \textit{tfdE} in the \textit{tfdCDEF} cluster, corresponding to gene products with only 28% identity. For differentiation, the \textit{tfdC} and \textit{tfdE} genes in this cluster were indexed. The gene cassette is arranged in the order \textit{tfdCII}EII\textit{BKA} and transcribed in the opposite direction to the \textit{tfdCDEF} cluster. The \textit{tfdR} gene, which presumably encodes the positive regulator of the \textit{tfdCDEII}BKA cluster, is located upstream of \textit{tfdCII}. Downstream of \textit{tfdR}, another ORF is located which is identical to \textit{orfII} in the vicinity of the \textit{tfdCDEF} cluster.

\textbf{IS sequences.} Sequence data from the 28.4 kb contig show that the genes of the 2,4-D degradation pathway are flanked by insertion elements of the IS1071 and IS1380 families (Mahillon & Chandler, 1998). This indicates that the \textit{tfd} genes of strain P4a are located within a transposon-like structure.

The insertion elements belonging to the IS1380 families were completely sequenced and found to be 1744 bp long. The transposase gene (1.4 kb) is flanked on either side by inverted and direct repeats. The inverted repeats consist of 17 bp and their nucleotide sequences are nearly identical: 5'\text{-}CCCGGATGTTTCATAAA-3' and 5'\text{-}CCCGATGTTTCATAAA-3'. The nucleotide sequence of the direct repeat is CTAG; it is located in front of the inverted repeat.

The insertion element belonging to the IS1071 family was completely sequenced in the right part of the segment (Fig. 1); the one on the left was only partially sequenced. A fragment of a further transposase gene, \textit{tnpA}, is located in the intergenic region of the insertion elements IS1380 and IS1071 and showed high similarity to the transposase gene of Tn21.

\textbf{Comparison of the sequence with sequence databases.} The chlorocatechol degradative operon of strain P4a exhibits high similarity in both its nucleotide sequence and in the arrangement of the genes to the homologous operons in other strains such as \textit{R. eutropha} NH9(pEN9H1) [99% identity (Ogawa & Miyashita, 1995, 1999)], \textit{Pseudomonas chlororaphis} RW71 [99% identity (Potrawfke et al., 1998, 2001)] and \textit{Pseudomonas} sp. P51(pP51) [97% identity (van der Meer et al., 1991a); Fig. 2]. The lengths of the homologous ORFs, their overlaps and the lengths of the intervening sequences are identical in these gene clusters. Comparison of the nucleotide sequences of the complete operons indicated that the \textit{D. acidovorans} P4a sequence differs in only five nucleotides in relation to \textit{R. eutropha} NH9.

The \textit{tfdR} and \textit{tfdCII} genes of the \textit{tfdCDEII}BKA cluster (except for a short nucleotide sequence at the 3' end of...
Rearrangements within the transposon-like structure

Sequence analysis of the transposon-like structure revealed that a nucleotide sequence of 7-8 kb upstream of tfdD, including IS1071 and IS1380 insertion elements as well as the orfII, tfdR and tfdC genes (Fig. 1; 0–7.8 kb, DNA segment in the box), is identical to another 7-8 kb nucleotide sequence located immediately upstream of the tfdEII-BKA cluster (Fig. 1; 19-2–27 kb). The sequences are oriented in opposite directions to each other. The tfdC gene and the sequence upstream of it are highly similar to homologous sequences of R. eutropha NH9(pENH91), Pseudomonas sp. P51(pP51) and P. chlororaphis RW71. This is also true for the nucleotide sequence of tfdCII. However, a short sequence upstream of the stop codon of tfdCII (19 nt) is identical to the corresponding sequences of A. xylosoxidans subsp. denitrificans EST4002(pEST4011) and V. paradoxus TV1(pTV1) (Fig. 3). The latter also applies to a further 5 kb sequence (98–99% identity) in the downstream direction. These facts are considered indications of rearrangements that took place in the formation of this transposon-like structure, probably by recombination of DNA fragments (Fig. 1). If this hypothesis is correct, the 7-8 kb DNA fragment (Fig. 1, in the box, on the left side of the transposon-like structure) was presumably transferred into the intragenic region of an ancient tfdC gene by recombination. Hence, the nucleotide sequence of tfdCII represents a combination of two genes rearranged without any frame shifts.

Chromosomal localization of the tfd genes in D. acidovorans P4a

In the hybridization experiment with a tfdAKB probe (2-5 kb), in which both chromosomal and plasmidal DNA from D. acidovorans P4a were tested (Fig. 4), a strong signal was generated from a 6 kb DNA fragment derived from the chromosomal moiety (lane 2). No signals were obtained, by contrast, with the plasmidial DNA (lane 1). On the basis of the hybridization results, a chromosomal localization of the tfd genes clustered in tfdCII-EII-BKA in D. acidovorans P4a is highly probable. This was confirmed by the results of another hybridization experiment using the tfdDEF (3-4 kb) probe (not shown).

2,4-D-negative mutants

After prolonged cultivation under non-selective conditions, mutants occurred repeatedly which were unable to degrade 2,4-D. PCR was performed by using the genomic DNA as a template and by applying the appropriate primers specific for the tfd genes. The results of the PCR experiments were negative; no PCR products were detected with any of the tfd primers. Moreover, no signals were obtained by hybridizing the genomic DNA of these 2,4-D-negative mutants using the labelled PCR products of the tfd genes as probes, indicating that all the tfd genes are completely lost in these mutants.

DISCUSSION

Micro-organisms are able to adapt to changing environmental conditions and to protect themselves from potentially damaging agents such as toxicants (Loffhagen et al., 1997, 2001). They may even exploit such agents by acquiring novel genetic capacity to open up new metabolic niches. This is frequently found with the utilization of xenobiotic compounds, a well-established example being the degradation of chloroaromatics via the modified ortho pathway. The genes of these pathways are arranged in mosaic-like structures (Fulthorpe et al., 1995; van der Meer, 1997). For instance, the ability to utilize chlorocatechols by means of the tfdCDEF genes can be extended to the degradation of chlorophenols and chlorophenoxalkanoates by acquiring the genes tfdB and tfdA (Top et al., 1995; McGowan et al., 1998; Vallaeys et al., 1996, 1999). In many cases, the metabolic versatility of micro-organisms can be quickly and conveniently extended using appropriate plasmids (Don & Pemberton, 1981, 1985; Chaudhry & Huang, 1988; van der Meer et al., 1991c; Mää et al., 1993; Bhat et al., 1994; Ka & Tiedje, 1994; Vallaeys et al., 1998; Xia et al., 1998; Vedler et al., 2000) and other mobile elements such as transposons and integrons (van der Meer et al., 1991d, 2001; Nakatsu et al., 1991, 1995, 1997; Springael et al., 1993; Nakatsu & Wyndham, 1993; Wyndham et al., 1994a, b; Merlin et al., 1997; Xia et al., 1998; Ogawa & Miyashita, 1999; Müller et al., 2003).

Assuming that the tfd genes detected in the present study were adequately expressed, D. acidovorans P4a provides an excellent example of this kind of adaptation. Indeed, implication of the tfd genes in the metabolism of 2,4-D is highly reliable as this strain exhibits growth on this substrate being accompanied, moreover, by expressing respective enzyme activities as measured for TfdA, TfdB, TfdC and TfdD (D. Hoffmann, unpublished data). The genes of the chlorocatechol pathway (i.e. the tfdCDEF genes encoding the enzymes of the modified ortho pathway and the regulatory unit tfdR, controlling their transcriptional activity as shown for R. eutropha JMP134 by Leveau &
### Table 1. Putative gene products of strain P4a compared to gene products in other bacterial strains

Comparisons were performed with BLAST (Altschul et al., 1997). The percentage identity represents the ratio of identical amino acids to the total number of amino acids in common. The full lengths of the putative gene products of strain P4a are shown in parentheses. Hyp. prot., hypothetical protein; Put. reg. prot., putative regulatory protein; Tnp., transposase.

<table>
<thead>
<tr>
<th>D. acidovorans P4a putative gene product</th>
<th>Gene product (product, percentage identity, no. identical amino acids/total no. amino acids in common) from</th>
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<tr>
<td></td>
<td><strong>Ralstonia eutropha NH9(pENH91)</strong> (Ogawa &amp; Miyashita, 1999)</td>
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<td></td>
<td><strong>Pseudomonas chlororaphis RW71</strong> (Potrawfke et al., 2001)</td>
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<td></td>
<td><strong>Pseudomonas sp. P51(pP51)</strong> (van der Meer et al., 1991a)</td>
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<tr>
<td>TfdR (294 aa)</td>
<td>ChnR, 99 %, 293/294</td>
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<tr>
<td>TfdC (251 aa)</td>
<td>ChnA, 99 %, 250/251</td>
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<tr>
<td>TfdD (370 aa)</td>
<td>ChnB, 100 %, 370/370</td>
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<tr>
<td>OrfI (336 aa)</td>
<td>Orf, 99 %, 335/336</td>
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<tr>
<td>TfdE (238 aa)</td>
<td>ChnC, 100 %, 238/238</td>
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<tr>
<td>TfdF (352 aa)</td>
<td>ChnD, 100 %, 352/352</td>
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<tr>
<td>OrfI (240 aa)</td>
<td>Orf, 100 %, 240/240</td>
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<tr>
<td>TfdGII (249 aa)</td>
<td>ChnA, 99 %, 246/248</td>
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<td><strong>Variovorax paradoxus TV1(pTV1)</strong> (Vallaecs et al., 1999)</td>
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<td><strong>Achromobacter xylosoxidans subsp. denitrificans EST4002(pEST4011)</strong> (Vedler et al., 2000)</td>
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<td>TfdEII (235 aa)</td>
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<tr>
<td>TfdB (586 aa)</td>
<td>TfdB, 99 %, 582/586</td>
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<td>TfdK (463 aa)</td>
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<tr>
<td>TfdA (287 aa)</td>
<td>TfdA, 98 %, 104/106</td>
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<td></td>
<td><strong>Burkholderia cepacia 2a(pIJB1)</strong> (Poh et al., 2002)</td>
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<td>OrfII (336 aa)</td>
<td>BenR, 26 %, 69/262</td>
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<td></td>
<td><strong>Pseudomonas putida</strong> (Cowles et al., 2000)</td>
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<td></td>
<td><strong>Pseudomonas sp. IC(pWW110)</strong> (Odgen, 1998)</td>
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<td></td>
<td><strong>Pseudomonas putida 01G3</strong> (Chablain et al., 2001)</td>
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<td></td>
<td>BphS, 26 %, 70/263</td>
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<td></td>
<td><strong>Pseudomonas resinovorans pCAR1</strong> (Maeda et al., 2003)</td>
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<td>Put. reg. prot., 34 %, 117/341</td>
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<td><strong>Sphingomonas paucimobilis UT26</strong> (Miyauchi &amp; Nagata, BAA36333)</td>
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<td><strong>Put. Tnp. IS1380</strong></td>
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<td><strong>Put. Tnp. IS1380</strong></td>
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<td>Tnp. IS107 (956 aa)</td>
<td>Tnp. IS107, 99 %, 932/936</td>
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<td><strong>Comamonas testosterone</strong> (Nakatsu et al., 1991)</td>
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<tr>
<td></td>
<td><strong>Pseudomonas putida UCC22</strong> (Fukumori, BAA12804)</td>
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<td></td>
<td>TnpA, 100 %, 531/531</td>
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van der Meer, 1996) are clustered (Fig. 2), as they are in a range of other strains (van der Meer et al., 1991a, b, c, d; Leveau et al., 1994; Potrawfke et al., 1998, 2001; Ogawa & Miyashita, 1999). Sequence similarity even extends to the overlapping regions of some genes as well as to their intergenic regions. Clearly this kind of arrangement has emerged during the course of evolution, enabling the effective use of chlorocatechols, and is distributed as a module for the recruitment of degradative pathways.

A second cluster found in some organisms is distinguished by the presence of *tfdA*, encoding a 2,4-D/2-oxoglutarate dioxygenase (Streber et al., 1987; Fukumori & Hausinger, 1993), in addition to *tfdK*, which was shown to encode a transport protein (Leveau et al., 1998). The combination of these genes seems logical if active transport is required for exploiting phenoxyalkanoates as carbon and energy sources. Active transport of these compounds has been found to play a significant role in *Sphingomonas herbicidovorans* MH, in which inducible and substrate-specific uptake systems have been observed (Zipper et al., 1998). By contrast, the lack of *tfdK* appears to have only minor negative effects in a mutant strain of *R. eutropha* JMP134 (Leveau et al., 1998). Accordingly, uptake may also proceed by simple diffusion, which is a mechanism known for (uncharged) organic acids. In these two studies, the strains were grown under neutral pH conditions. With strain P4a, which is adapted to alkaline conditions, the uptake characteristics remain to be proven. One should take into account, however, that the dissociation equilibrium is shifted strongly towards the anion of the target compounds during growth under alkaline conditions, which means pH values of up to 10 with this strain (Hoffmann et al., 1996). Hence, uptake by simple diffusion ought to be limited by the concentration of the free acid of 2,4-D, making it likely or even essential to support utilization of this compound by a specific uptake system.

The second *tfd* cluster, *tfdC*$_{II}$BKA, also includes *tfdB*, which is known to encode a chlorophenol hydroxylase (Perkins et al., 1990). This apparently completes the 2,4-D degradative pathway in strain P4a. The structure of this gene

Fig. 3. Alignment of the 3’ termini of the *tfdC* and *tfdC*$_{II}$ genes of *D. acidovorans* P4a as well as the *tfdC* genes of *V. paradoxus* TV1 and *A. xylosoxidans* subsp. *denitrificans* EST4002 (49 and 43 nt upstream of the stop codon, respectively). The *tfdC*$_{II}$ gene of strain P4a is composed of nucleotide sequences descending from two different *tfdC* genes. The 5’ terminus of *tfdC*$_{II}$ is identical to the *tfdC* gene in the chlorocatechol operon of strain P4a. However, the 19 nt at the end 3’ terminus are identical to homologous regions in *V. paradoxus* TV1 and *A. xylosoxidans* subsp. *denitrificans* EST4002. Black underlined, identical regions; black framed, putative target region of recombination events; white with black background, nucleotide sequence of *tfdC* in the *tfdCDEF* operon of *D. acidovorans* P4a; black with grey background, nucleotide sequence of *tfdC* in *V. paradoxus* TV1 and *A. xylosoxidans* subsp. *denitrificans* EST4002.

Fig. 4. Southern blot hybridization of chromosomal and plasmidal DNA of *D. acidovorans* P4a. Chromosomal and plasmidal DNA were digested with *EcoRI* and *BglII*. The DNA fragments were separated electrophoretically in an 0-8% agarose gel (a), blotted onto a nylon membrane and fixed by UV irradiation. Southern blot hybridization was performed with a *tfdAKB*-specific probe (2-5 kb) at 60 °C (b). Raoul size marker was hybridized with labelled *pBR322* fragments. Lanes: 1, plasmid DNA 1 (containing all plasmids) of strain P4a, digested with *EcoRI* and *BglII*; 2, genomic DNA of strain P4a, digested with *EcoRI* and *BglII*; 3, plasmid DNA 2 (containing the 21 kb plasmid only) of strain P4a, digested with *EcoRI* and *BglII*; 4, Raoul marker (Appligene).
cassette appears to be identical to that found in other strains (Fig. 2; Vallaeyts et al., 1996; Vedler et al., 2000; Poh et al., 2002) and, again, transcription of the genes is presumably governed by tfdR. Two sets of tfdC and tfdE genes are found in strain P4a. It remains to be elucidated whether the expression of both these genes is essential for the proper function of the 2,4-D pathway in this strain or whether one of each is merely rudimentary. In R. eutropha JMP134, two complete sets of the chlorocatehol genes were also found, the expression of which was shown to speed up growth of this organism on 2,4-D (Leveau et al., 1999; Laemmli et al., 2000; Pérez-Pantoja et al., 2000). According to the differences in their nucleotide sequences, both tfd modules must have been acquired from different evolutionary origins in R. eutropha JMP134. This can also be concluded for strain P4a when the differences between tfdE and tfdEII are considered: their gene products share only 28% identity with each other but are highly similar to TfdE homologues in different bacterial strains (Table 1).

This high degree of coincidence between the tfd genes and their arrangement suggests horizontal gene transfer as the likely explanation for the acquisition of the 2,4-D cassettes in strain P4a rather than being a feature evolved in this strain itself. The two different cassettes (tfdCDEF and tfdCIIIEIIIBKA) hint at two origins, although it cannot be excluded that a complete arrangement was captured by D. acidovorans from any strain. With special reference to building rubble, from which strain P4a was isolated, some questions regarding the generation of the ability to degrade 2,4-D remain obscure. These refer to the fact that the highly alkaline environmental conditions, which exceed pH 11.5 in aqueous surroundings of this material, preclude growth of D. acidovorans on this material. Thus, any conjugative processes are rather unlikely to occur in this very habitat. One can speculate, however, that micro-niches with moderate pH conditions, for example, mixing zones between building rubble and soil, are appropriate breeding areas for this characteristic. Starting from any primitive strain carrying and expressing the genetic information for 2,4-D degradation, strain P4a might have evolved in one of these micro-niches by gene transfer, genetic rearrangements and selection of the fittest. This also applies to the strains D. acidovorans MC1 and Rhodoferax sp. P230, which were isolated from the same habitat and are highly similar to strain P4a with reference to some of their tfd genes (Müller et al., 2001).

The nucleotide sequences found outside the 2,4-D degradation genes, i.e. downstream of the tfdR genes, are indicative of possible maturation processes and complete the picture of the ‘recent’ strain D. acidovorans P4a. The tfd genes are flanked by transposons of the IS1380 and IS1071 families, each comprising a transposase gene in this DNA segment. Remarkably, the two segments are identical in structure. Moreover, the identity is extended to an ORF with unknown function, named orfII, and to both tfdR and tfdC. A further observation that may help elucidate the evolution of the 2,4-D degradation pathway in strain P4a is that a short sequence of tfdCII (19 bp, located downstream) deviates from that of tfdC in P4a and is identical to homologous sequences of other tfdC genes found in the tfdCEBKA cassettes of some other strains (Fig. 2). This implies that recombination events took place at this position with a gene descending from a different ancestor.

Another finding that should be emphasized is that the complete set of tfd genes is located on the chromosome in strain P4a, while in other strains known to carry chromosomally located tfd genes only parts of the 2,4-D degradation pathway are encoded on the chromosome (Ka et al., 1994; Matheson et al., 1996; Suwa et al., 1996). When we started investigating strain P4a we noted plasmids of various sizes ranging from 5 to about 60 kb, and found indications that tfd genes were located on them (Hoffmann et al., 1996). However, we have had serious problems assigning the tfd genes to the respective plasmids. Recent investigations have shown that strain P4a now carries only one plasmid of about 21 kb, which is not associated with 2,4-D degradation. Therefore, under the selective pressure applied during laboratory cultivation, events occurred in the genetic structure that stabilized the degradative activities, leading to the emergence of the strain we describe as ‘recent’. The transposon structure is likely to have promoted these events in a kind of evolutionary optimization process, similar to phenomena observed in various other strains, raising questions about the pros and cons of carrying plasmids (Deutz et al., 1991; Stouthamer & Kooijman, 1993).

The chromosomal location of the genes of a pathway should certainly have consequences. Genes localized in the chromosomal matrix of the genome can be considered established, in contrast to plasmid-derived properties which can be classed as temporary or transitional. Taking into account that transposons are elements appropriate to quickly spread genetic information to suitable carriers, and plasmids are carriers capable of distributing genetic information into respective host strains, strains with plasmids harbouring respective information on transposons are fragile for this characteristic as they include 2 d.f. of losing this information. When it becomes localized on the chromosome, by contrast, even as a transposon, the d.f. is accordingly reduced. This prompts the question of whether P4a, as a strain of a species that prefers organic acids as carbon and energy sources (Tamaoka et al., 1987; Wen et al., 1999), is in the process of adding a further acid, i.e. 2,4-D, to its spectrum of preferred substrates. Only a few mutation events which prevent this genetic element from transposing, for example, by changing target sites of transposase attack, avoiding expression of transposases or leading to inactive transposases, would be required to stabilize this property, making the ability to use the compound an inherent property of the strain. Stability of the degradative performance is by no means under selective pressure but becomes decisive if any strain is intended...
to be used for bioaugmentation as effective production of biomass will rely more on conventional carbon and energy sources. Even in recent times we have observed the occasional loss of the 2,4-D degradative capability in some clones, accompanied by the lack of all tfd genes.

The metabolic constellation of this alkali tolerant strain (P4a) enables the productive degradation of 2,4-D at pH values up to 10 in laboratory cultures (Hoffmann et al., 1996). Moreover, we have detected activity at up to pH 11.5 when strain P4a has been applied as an inoculum in a bioremediation process with building rubble (Müller et al., 1996, 2000). The capacity of the species to metabolize phenoxyalkanoates was also demonstrated with another strain, D. acidovorans MC1, which was isolated from the same alkaline environment and was able to utilize an even wider spectrum of compounds (Müller et al., 1999b, 2001). In contrast to these alkali tolerant strains, true alkaliophiles are unlikely to be able to degrade 2,4-D productively. This conclusion is consistent with results obtained with a soda lake isolate, Halomonas sp. EF43, supplied with plasmid pJP4 by conjugative transfer (Kleinsteuber et al., 2001). Despite the expression of the tfd genes, this strain was unable to use 2,4-D as a sole source of carbon and energy, even though it was able to degrade the compound, at least in the presence of an additional carbon source (Kleinsteuber et al., 2001). This may well also apply to another alkaliophilic strain belonging to the Halomonadaceae described by Maltseva et al. (1996), since the published data do not indicate that this isolate can make productive use of 2,4-D.

The property of catching and getting rid of genetic information is considered a mechanism to effectively settle niches and contributes to the metabolic resilience of biotopes. The present results suggest that the uptake of any genetic information for completing a degradative pathway, even as preformed and ‘optimized’ cassettes, is only a precondition to enable the degradation of a given compound but may not be sufficient to do this in an effective, ultimately competitive, way. This refers to growth rate first but may include stability too, both of which are an expression of adaptation. The latter also applies for D. acidovorans MC1, a further strain isolated in this laboratory in 1999. In this case, we observed loss of the degradative trait under non-selective conditions in almost all overnight cultures. Today, derivatives of this strain exist which harbour this activity for at least 80 generations during growth on complex medium. Thus, stability is gained at the expense of flexibility.

REFERENCES


Ogawa, N. & Miyashita, K. (1999). The chlorocatechol-catabolic transposon Tn5707 of Alcaligenes eutrophus NH9, carrying a gene cluster highly homologous to that in the 1,2,4-trichlorobenzene-degrading bacterium Pseudomonas sp. strain P51, confers the ability to grow on 3-chlorobenzoate. Appl Environ Microbiol 65, 724–731.


Poh, R. P.-C., Smith, A. R. W. & Bruce, I. J. (2002). Complete characterization of Tn5530 from Burkholderia cepacia strain 2a(pIPB1) and studies of 2,4-dichlorophenoxyacetate uptake by the organism. Plasmid 48, 1–12.


