2,4-Dichlorophenoxyacetate Degraders in the Real World, Do We Know Who They Are?

Robert A. Fairlough, John Dunbar, Bill Hoben, Larry Forney, Olga Maltseva, Grace Matheson, Catharine McGowan, Patrick Oriel, Albert Rhodes, Hideko Taira, James Tiedje, Nancy Tono, Tatiana Vallaeyer, Alice Wright
Michigan State University, East Lansing, MI 48824, USA

Our current understanding of the degradation of 2,4-dichlorophenoxyacetate (2,4-D) is based on detailed studies of pJP4, a catabolic plasmid isolated from an Alcaligenes eutrophus strain isolated in Australia that encodes mercury resistance and the 2,4-D catabolic genes (tfdACDEFB). The tfd genes appear to be globally distributed. They have been isolated from disparate places of the world, either on pJP4 or various other plasmid backbones. For example, Amy et al. (1) described pJP4 in a different host (EML159) isolated in Oregon and plasmid borne tfd genes that highly homologous to those of pJP4, albeit rearranged, have been found in Pseudomonas putida (pEST4011; 10), P. cepacia (pMAB1; 2) and Flavobacterium (pRC10; 3).

We were interested in discerning biogeographic patterns in the distribution of 2,4-D catabolic genes. In other words, do the 2,4-D catabolic genes show any dependence on ecosystem type, on location of origin, or are they ubiquitous? Moreover, the canonical tfd genes are not the only ones found in environmental isolates. Work by Ka et al. (9), Matheson (11) and Amy et al. (1) has shown that populations carrying genes nonhomologous to the tfd genes are present in soils and other ecosystems. To address this question, we have undertaken a systematic effort to evaluate the global distribution of chloroaromatic catabolic genes. To assess the global distribution of bacteria able to degrade 2,4-D, in pristine soils (with no known exposure to 2,4-D), catabolic activity was measured in enrichment cultures using 672 soil samples from 28 sites on five continents and Hawaii. Soils were enriched in media that contained either radioactively labeled 3-chlorobenzoate or 2,4-D. The
mineralization of these compounds was followed using carbon dioxide traps, and soils that showed mineralization of the target compound were transferred to secondary enrichment cultures prior to isolation of axenic cultures able to degrade the target compound.

Soils from all the regions sampled mineralized both 2,4-D and 3-chlorobenzoate, but 3-chlorobenzoate was mineralized without a lag period while 2,4-D was generally not mineralized until the second week (Table 1). 3-chlorobenzoate was mineralized more rapidly than 2,4-D, in spite of the structural similarities of the two chemicals and the fact that the catabolic genes for both compounds are often found on the same plasmids. The degradative capabilities of many of the soils were retained after transfer to fresh liquid media (secondary enrichments). Microorganisms able to metabolize 3-chlorobenzoate were readily isolated and 375 isolates that could release carbon dioxide from ring-labelled 3-chlorobenzoate were obtained. Although 63% of all the enrichment cultures mineralized $^{14}$C-2,4-D, axenic cultures able to degrade 2,4-D were only obtained from ~1% of these enrichment cultures (five isolates). There are at least two explanations for this outcome: one is that 2,4-D strains were unculturable with the methods used and the second is that 2,4-D was mineralized by microbial consortia. Since the same methodology has routinely been used to obtain axenic cultures of 2,4-D strains from soils with a history of exposure to 2,4-D, we postulate that 2,4-D metabolism by microbial consortia is more common than previously realized. These data also suggest that the organisms responsible for degrading 3-chlorobenzoate in these soils are not the same as those degrading 2,4-D, since the former were readily isolated. Thus, it appears that culturable strains able to completely degrade 2,4-D are not ubiquitous in nature and are rather rare in pristine soils from different ecosystems.

To gain insight on the characteristics of microorganisms able to metabolize 2,4-D, we have undertaken studies of microorganisms previously isolated from agricultural
soils, freshwater microcosms, sewage sludge or other waste treatment systems. The
genomic fingerprints of fifty strains were determined using PEP-PCR (18). Seventeen
genotypes could be distinguished among these strains and the majority carried one of
four plasmid types (Table 2). Studies on the distribution of genes homologous to tfdA, 
tfdB and tfdC among these 2,4-D* strains were done by hybridization analyses of
genomic DNAs using probes based on the tfdA, tfdB and tfdC genes of pJP4. These
strains occur in two broad groups: group I consists of Pseudomonas, Burkholderia,
Alcaligenes and related species that carry one or more of the tfd genes or their
homologues, while group II consists of Sphingomonas strains that lack significant
homology to tfdA, tfdB and tfdC. Among group I were diverse pseudomonads that
carry genes that were highly homologous to tfdABC of pJP4. This suggests that a
common pathway may have spread among these strains. Others had weak homology
to tfdA but high homology to tfdB. These strains were isolated from geographically
separated sites but had nearly identical genomic DNA fingerprints suggesting these
strains were closely related and widely distributed. Another subgroup comprised
entirely of α-proteobacteria showed no homology to tfdA or tfdC and only weak
homology to tfdB. These data suggest that some bacteria carry genes that are either
isofunctional but nonhomologous to those of pJP4 or metabolize 2,4-D by novel
means. Subsequent work has shown that the tfd genes of these strains are not
necessarily plasmid borne, but nonetheless the plasmid harbored by each strain is
generally indicative of the type of catabolic genes it carries.

Type I plasmids are highly homologous to pJP4 and are found in a variety of
different genotypes. Hybridization analysis of two of these species showed that they
carry tfdA, B, and C homologues or DNA fragments the same size as those found in
pJP4. However, not all of the type I plasmids proved to be pJP4 itself. We have four
non-pJP4 type I plasmids; only one of these (TFD41) carries tfdABC, while the other
three carry genes highly homologous to only one or two of the tfd genes. It seems
therefore, that the tfd(pJP4) genes do not travel as a well ordered unit, but are subjected to recombination with iso-functional homologues from other sources. This recombination may be facilitated by the presence of these genes on conjugative, type I plasmids.

Strains carrying type II or type IV plasmids seem to carry the tfd catabolic genes on the chromosome and share a tfdA gene that differs from tfdA(pJP4). Genomic catabolic genes were first noted in strain RASC, where the 12 kb plasmid was proven to be unrelated to 2,4-D degradation activity (15). Interstrain transfer of at least 20 kb of genomic DNA harboring these catabolic genes via an unknown mechanism has been demonstrated (13). The tfdA(RASC) gene has been sequenced and is 73% similar to tfdA(pJP4) (15). The identical gene was also cloned from TFD6 (12). TFD6 and TFD4 carried DNA fragments of the same size that hybridized to tfdA, suggesting they share the same gene.

Regions of DNA sequence conserved between tfdA(pJP4) and tfdA(RASC) were used to design DNA primers for the PCR amplification of DNA internal to tfdA-like genes. We were able to amplify ~300 bp DNA fragments from all the strains in the collection that hybridize with tfdA(pJP4). The partial sequences obtained confirm that RASC, TFD2 and TFD6 harbor the same tfdA gene. The catabolic genes of TFD2 have not been localized to the chromosome, however this strain carries the same small plasmid (type IV) that was shown to be unrelated to 2,4-D metabolism in RASC, therefore a chromosomal location is likely. Together, strains with a gene similar or identical to tfdA(RASC) constitute 16 of the 50 strains in our collection.

Sphingomonas strains carry type III plasmids and are phylogenetically more distant from the rest of the strains in our collection. TFD26, TFD44, B6-5, and B6-10, and K1443 were all members of this genus. We found that these strains may hybridize weakly to tfdB but not at all to tfdA or tfdC. These yellow-pigmented bacteria are clearly of ecological importance since they were easily isolated from lake water,
sewage sludge, and soil systems alike. While they do not show homology to ttdC nor to ctaA, they do exhibit chlorocatechol dioxygenase activity. In this laboratory researchers have been unable to find ttdA-like activity in these strains using published methods (4, Takami, unpublished data).

The structure of the pJP4 plasmid has lead to the hypothesis that ttdA seems to have been recruited via horizontal gene transfer from a different source. The data we have obtained supports this hypothesis since ttdA genes are not always associated with the same ttdB or ttdC fragments in different strains. In addition, the ttdA(RASC) gene is distributed among phylogenetically distinct strains, suggesting that horizontal gene transfer is responsible for the current host-gene associations (Figure 1). Similarly, a third partial ttdA sequence was amplified from phylogenetically distinct strains that had been isolated from widely separated areas. This partial sequence, which is 94% similar to that of ttdA(RASC) and 76% similar to ttdA(pJP4), was amplified from Rhodocerax fermentans TF031 from Saskatchewan, Rhodocerax fermentans B6-9 from Ontario, Alcaligines sp. st. TV-1 isolated in France, and in Halomonas sp. st. 118, isolated from highly saline and alkaline 2,4-D contaminated ponds in Oregon (Matseva and Oriel, unpublished data). This distribution of this sequence also suggests horizontal gene transfer, probably by conjugation since we have no evidence that these genes are chromosomal (Figure 1).

Our data also indicate there is substantial variation in the ttdC-like fragments present in our collection. Many strains do not hybridize with the ttdC(pJP4) gene probe at high stringencies. Others do not hybridize with the ttdC(pJP4) gene probe at all, but still exhibit chlorocatechol dioxygenase (CCD) activity (5). In most cases, it is likely that these strains harbor ttdC homologues that are less than 80% similar to our gene probes and therefore cannot be detected by our hybridization techniques. It is not uncommon for isofunctional genes to be less than 60% homologous, depending on how much of the protein structure needs to be conserved in order to maintain the
enzymatic function. We have shown, therefore, that $tdfC$ gene probes are insufficient
to detect these CCD genes in environmental samples or strains, because any further
reduction in stringency conditions can lead to problematic non-specific probe binding.
The detection of CCD is of considerable importance because of the central role that
these enzymes play in the degradation of many simple chlorinated aromatic
compounds (7). A search for these less similar CCD's will help in finding more
universal detection tools for these important enzymes.

Does our collection represent strains that are active in the environment? In the
cases where 2,4-D populations have been examined using gene probes, strains
similar to those in our collection are present. A high diversity of degraders seems to
be maintained, both temporally and spatially. Ka et al. (9) have demonstrated that
dominant populations in one site can vary from year to year. He found pJP4 like
genes dominant in KBS in one year, but *Sphingomonas* like strains dominant in
another, although the soils were from the same location. Matheson (11) found that
experimentally enriched KBS soils harbored populations non-homologous to pJP4 but
soils from Saskatchewan treated in the same way selected for populations that have
high homology to $tdfA$ and $tdfB$ but, interestingly, not $tdfC$ (8). The latter population
may have been dominated by TFD9-like organisms. TFD9 was isolated in Indian
Head, Saskatchewan, but it carries a plasmid that was also found in K712, a different
genotype isolate from the Kellog Biological Station in Michigan. The plasmid has
genes highly homologous to $tdfA$ and $tdfB$, but shows low homology to $tdfC$.

We have shown that the diversity of 2,4-D degraders can be quite high within a
given soil at any one time. Plasmid capture experiments using a Michigan soil
showed the presence of nine distinctly different catabolic plasmids in the soil
population at any one time (17). Rifampin resistant, 2,4-D- mutants *Alcaligenes
eutrophus* strains were mated with a suspension of organisms from 0.5-1 gram of soil.
Transconjugants receiving and expressing catabolic plasmids from the soil microflora
were isolated on rifampicin, 1000 ppm 2,4-D media. Plasmids closely related to that found in TFD9, were found in the majority of plasmid recipients. Another strain harbored a pJP4-like plasmid. Even in the small amount of soil examined, a high diversity of plasmid types is revealed by this technique.

The diversity uncovered by the plasmid capture method underestimates the diversity of 2,4-D degrading organisms present in soils. When a sensitive isolation procedure and soils from the same source were used, a total of 54 different genotypes that could degrade 2,4-D were isolated from unexposed soils (Figure 2). After only 7 days of exposure to 10 ppm or 100 ppm, the diversity of strains was dramatically reduced and only a few populations dominated. In total 15 genotypes were detected following selection; the bulk of which belonged to three highly variable populations of the same species (Figure 3). This work shows that the populations that can be detected after enrichment is still poorly representative of the diversity that can be detected prior to that enrichment.

In spite of the multiple combinations of tol gene homologues and analogues that we find in various strains in contaminated systems, these recombinations do not seem to have occurred in pristine soils. The absence of culturable 2,4-D degraders in these suggests either a) the degraders are presently uncultured and/or b) the various recombination events required to assemble the genes into a single host have not occurred. Two more things have been learned about the 2,4-D degraders present in these “ancestral” systems. Plasmid capture experiments on enriched pristine soils do not produce transconjugants. Even plasmid capture experiments carried out on 2,4-D degrading mixed cultures derived from these soils do not yield transconjugants. (Tu and Fulthorpe, unpublished data). This is fairly good evidence that conjugative plasmids are not present in the mixed 2,4-D catabolic cultures from these soils.

Nonetheless, the same mixed cultures do harbor a population of tolA carrying strains. When total DNA from these mixed cultures are used as templates for the amplification
of _tfdA_ gene fragments, _tfdA_ sequences identical to those from TFD31, B6-9, TV-1, and I-18 have been obtained from pristine soil enrichment cultures. However, when isolates obtained from these enrichments were tested using the same PCR amplification protocol, no sequences homologous to _tfdA_ were obtained. We hypothesize that the _tfd_-like catabolic genes are present in the these cultures, but they are not present on conjugative plasmids and they may not be present in association with other catabolic genes necessary for 2,4-D metabolism. One or all the bacteria carrying these genes appears to be unculturable at present. We are now doing experiments to determine if with continued selection pressure that recombination and gene transfer events will occur and result in single strains with the ability to degrade 2,4-D. It remains to be seen if the evolutionary solutions to continued application of 2,4-D will be the same in soils from completely different sources.

In summary, pJP4-like plasmids do not appear to be widespread in nature, nor are they frequently isolated. The available evidence suggests that in pristine systems the _tfd_ genes have not recombined into specific organisms, but rather a mosaic pattern of homologous confer the 2,4-D⁺ phenotype. We need to continue work on the distribution an occurrence of _tfd_ like genes in pristine systems, and to focus our work on these more widespread species and their transfer and rearrangement mechanisms.
REFERENCES


<table>
<thead>
<tr>
<th>Source of inocula(^a)</th>
<th>CAL</th>
<th>CHILE</th>
<th>SA</th>
<th>AUS</th>
<th>SASK</th>
<th>RUSSIA</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of enrichments able to mineralize 3-CBA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary enrichments</td>
<td>99%</td>
<td>86%</td>
<td>98%</td>
<td>90%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Secondary enrichments</td>
<td>92%</td>
<td>69%</td>
<td>98%</td>
<td>50%</td>
<td>91%</td>
<td>84%</td>
</tr>
<tr>
<td>Number of axenic cultures able to degrade 3-CBA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total number</td>
<td>58</td>
<td>46</td>
<td>72</td>
<td>69</td>
<td>83</td>
<td>47</td>
</tr>
<tr>
<td>Number that were stably 3-CBA(^*)</td>
<td>15</td>
<td>13</td>
<td>56</td>
<td>7</td>
<td>30</td>
<td>23</td>
</tr>
<tr>
<td>% of enrichments able to mineralize 2,4-D</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary enrichments</td>
<td>50%</td>
<td>54%</td>
<td>63%</td>
<td>53%</td>
<td>100%</td>
<td>57%</td>
</tr>
<tr>
<td>Secondary enrichments</td>
<td>41%</td>
<td>35%</td>
<td>18%</td>
<td>3%</td>
<td>100%</td>
<td>26%</td>
</tr>
<tr>
<td>Number of axenic cultures able to degrade 2,4-D</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total number</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

\(^a\)Soils from California, USA (CAL); Chile (CHILE); South Africa (SA); Australia (AUS); Saskatchewan (SASK); Russia (RUSSIA).
Figure 2. 2,4-D degrading populations in control microcosms.
Figure 3. Response of 2,4-D degrading populations in microcosms exposed to 100 ppm 2,4-D