Evidence for Interspecies Gene Transfer in the Evolution of 2,4-Dichlorophenoxyacetic Acid Degraders
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Small-subunit ribosomal DNA (SSU rDNA) from 20 phenotypically distinct strains of 2,4-dichlorophenoxyacetic acid (2,4-D)-degrading bacteria was partially sequenced, yielding 18 unique strains belonging to members of the alpha, beta, and gamma subgroups of the class Proteobacteria. To understand the origin of 2,4-D degradation in this diverse collection, the first gene in the 2,4-D pathway, tfdA, was sequenced. The sequences fell into three unique classes found in various members of the beta and gamma subgroups of Proteobacteria. None of the α-Proteobacteria yielded tfdA PCR products. A comparison of the dendrogram of the tfdA genes with that of the SSU rDNA genes demonstrated incongruency in phylogenies, and hence 2,4-D degradation must have originated from gene transfer between species. Only those strains with tfdA sequences highly similar to the tfdA sequence of strain JMP134 (tfdA class I) transferred all the 2,4-D genes and conferred the 2,4-D degradation phenotype to a Burkholderia cepacia recipient.

Bacteria capable of mineralizing 2,4-dichlorophenoxyacetic acid (2,4-D), a commonly used herbicide, are found in many different phylogenetic groups (2, 3, 7, 11, 22, 23). Evidence suggests that numerous variants of 2,4-D catabolic genes exist and that catabolic operons consist of a near-random mixing of these variants (7). Interspecies gene transfer is a well-documented phenomenon (13), and horizontal gene transfer of the 2,4-D-degrading plasmid pJP4 has been shown (3, 5). However, not all 2,4-D catabolic operons are found on plasmids (10, 11, 16, 20). The extent to which other 2,4-D genes have been exchanged in nature is unknown. The aim of this research was to assess the role of horizontal gene transfer in the evolution of 2,4-D-degrading strains. This article summarizes the results of two aspects of this work—the study of the 2,4-D-catabolic operons and one group of the 2,4-D degradation pathway, tfdA, was sequenced. The sequences fell into three unique classes found in various members of the beta and gamma subgroups of Proteobacteria. None of the α-Proteobacteria yielded tfdA PCR products. A comparison of the dendrogram of the tfdA genes with that of the SSU rDNA genes demonstrated incongruency in phylogenies, and hence 2,4-D degradation must have originated from gene transfer between species. Only those strains with tfdA sequences highly similar to the tfdA sequence of strain JMP134 (tfdA class I) transferred all the 2,4-D genes and conferred the 2,4-D degradation phenotype to a Burkholderia cepacia recipient.

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automatic sequencer (Perkin-Elmer Cetus) by using fluorescently labeled dye termination at the Michigan State University Sequencing Facility. The sequencing primer used for SSU rDNA fragments was 519R (5'-GTA TTA CCG CGG CTG CTG G-3'). For tfdA fragments, the sequencing primers were the same as the amplification primers. GenBank accession numbers for these sequences are given in Table 1.

The SSU rDNA sequences were compared to sequences in GenBank by using the Basic Local Alignment Search Tool (BLAST) (1), and those strains with the highest maximal segment similarity of SSU rDNA sequences.

### Table 1. 2,4-D-degrading strains, geographic origins, and GenBank accession numbers

| Strain | GenBank accession no. (SSU rDNA) | Origin | Most similar to genus and/or species
d | Transfer
d | tfdA type
| Reference or source |
|---|---|---|---|---|---|---|---|
| JMP134 | AF049542 | Australia | Ralstonia eutropha | + | I | M16730 | 3 |
| EML1549 | AF049546 | Oregon | Burkholderia sp. | + | I | U43197 | 23 |
| TFD39 | AF049539 | Saskatchewan | Burkholderia sp. | + | I | U43197 | 23 |
| K712 | AF049543 | Michigan | Alcaligenes xylosoxidans | + | I | U43276 | 11 |
| TFD9 | AF049537 | Saskatchewan | Ralstonia eutropha | + | I | U43276 | 23 |
| TFD41 | AF049541 | Michigan | Ralstonia eutropha | + | I | U43276 | 23 |
| TFD38 | AF049540 | Michigan | Ralstonia eutropha | + | ND | U43276 | 23 |
| TFD23 | AF049536 | Michigan | Rhodobacter fermentans | + | I | U43276 | 23 |
| RASC | AF049544 | Oregon | Burkholderia sp. | (+) | II | U25717 | 2 |
| TFD6 | AF049546 | Michigan | Burkholderia sp. | – | II | U43276 | 23 |
| TFD2 | AF049545 | Michigan | Burkholderia sp. | – | II | U43276 | 23 |
| TFD31 | AF049536 | Saskatchewan | Rhodobacter fermentans | + | II | U43276 | 23 |
| B6-9 | AF049538 | Ontario | Rhodobacter fermentans | ND | III | U43196 | 9 |
| I-18 | U22836 | Oregon | Halomonas sp. | ND | III | U22499 | 15 |
| K1443 | AF049531 | Michigan | Sphingomonas sp. | + | ND | R. Sanford | 11 |
| 2,4-D1 | AF049535 | Montana | Sphingomonas sp. | + | ND | R. Sanford | 11 |
| B6-5 | AF049533 | Ontario | Sphingomonas sp. | – | ND | R. Sanford | 9 |
| B6-10 | AF049534 | Ontario | Sphingomonas sp. | – | ND | R. Sanford | 9 |
| EML146 | AF049532 | Oregon | Sphingomonas sp. | – | ND | R. Sanford | 9 |
| M1 | AF049530 | French Polynesia | Rhodospseudomonas sp. | – | ND | R. Fulthorpe | 9 |

* The genus and/or species most similar to the strain is given based on similarities of SSU rDNA sequences.

* Symbols: +, able to transfer 2,4-D degradation to *B. cepacia* D5; (+), able to transfer at very low frequency; –, no transfer detected.

* ND, not determined.

* d, no amplificate was obtained.

FIG. 1. Neighbor-joining dendrogram (Jukes-Cantor distances) of SSU rDNA from 2,4-D-degrading bacteria (indicated in boldface type) and reference strains (indicated in italic type). Class I (●), class II (▲), and class III (▲) types of tfdA genes are indicated. Bootstrap confidence limits (percentages) are indicated above each branch. Scale bar represents a Jukes-Cantor distance of 0.01.
pair scores were retrieved from GenBank and included in the phylogenetic analysis. Sequences were aligned manually with the software SeqEd (Applied Biosystems) and with MacClade (14). Sites where nucleotides were not resolved for all sequences were deleted from the alignment, as were those nucleotides corresponding to the small loop in this region that is absent in the alpha subgroup of the class Proteobacteria. These deletions left 283 unambiguous sites for the construction of the SSU rDNA phylogenies. Phylogenetic trees were constructed by using the neighbor-joining analysis of pairwise Jukes-Cantor distances (4), and the topology was confirmed by using the maximum parsimony method PAUP (21). Desulfofomicine tiedjei of the 6-Proteobacteria was used as an outgroup. Bootstrap analysis based on 100 replicates was used to place confidence estimates on the tree. Only bootstrap values of greater than 50 were used.

2,4-D degrader diversity. The 2,4-D degraders in this study were distributed throughout the alpha, beta, and gamma subgroups of the Proteobacteria (Fig. 1). The lack of representation of gram-positive bacteria is likely a reflection of isolation methods, not of the lack of gram-positive 2,4-D degraders. The majority of these strains were members of the beta subgroup of Proteobacteria, five of which were most closely related to the genus Burkholderia, having at least 92% sequence similarity with each other. Three were closely related to Rhodoferax fermentans (close to the class Comamonadaceae), three were related to Ralstonia eutropha, and one was related to Allocigenes xylosoxidans. TFD39 falls outside any clear cluster. One member of the $\gamma$-Proteobacteria, strain I-18, a haloalkaliphile, was found to be closely related to the salt-loving genus Halomonas (15). The remaining six strains all clustered in the alpha branch of Proteobacteria (Fig. 1). Of this subgroup, five were most closely related to the genus Sphingomonas. One member of the $\alpha$-Proteobacteria, strain M1, which is the most oligotrophic and slow growing of all the strains used in this study, is 97% similar to Rhodopseudomonas palustris. The character of strain M1 correlates well with its phylogenetic placement near the slow-growing genus Bradyrhizobiun.

tfdA gene fragments. tfdA gene fragments were successfully amplified and sequenced from 10 strains of $\beta$-Proteobacteria and 1 strain of $\gamma$-Proteobacteria. None of the strains from the $\alpha$-Proteobacteria gave any amplificates with these primers. These 313 contiguous nucleotides were aligned with additional tfdA sequences from JMP134 and from strain RASC (Fig. 2). Three distinct classes of tfdA gene sequences with slight variations in each class were found. Class I included fragments from JMP134, TFD39, TFD23, K712, and TFD9 that differed from each other by 2 bp at the most. Class II tfdA genes are probably plasmid encoded. All strains with a class I tfdA gene examined so far contained broad-host-range, self-transmissible plasmids containing 2,4-D genes (2, 3, 11, 17). All of the strains with a class I tfdA gene were able to transfer the 2,4-D phenotype in the mating studies reported above. The class II tfdA sequences included identical fragments amplified from RASC, TFD6, and TFD2 which were 76% similar to those in class I. Class III included identical fragments from strains TFD31, B6-9, and I-18 which were 77% similar to class I genes and 80% similar to class II genes. Both class II and III tfdA genes differed from each other and from class I genes in the same nine sites corresponding to the third base pair of the codons. The tfdA phylogenetic tree is a simple one, with three distinct branches that are incongruent with the SSU rDNA-derived phylogeny (Fig. 3). Class I tfdA sequences were found in Burkholderia-like strains, in strains related to the Comamonas-Rhodoferax group, and in the Ralstonia-Acaligenes group, all in the $\beta$-Proteobacteria. Class II sequences are less widely distributed, found only in Burkholderia-like branches. However, even in this subgroup, this tfdA variant is found in strains that differ by 7% at the SSU rDNA level (RASC and TFD2). However, the class III sequences were most interesting, being found both in the Comamonas-Rhodoferax group and in a strain of the $\gamma$-Proteobacteria, I-18, strains that differ by 24% at the SSU rDNA level. Class III genes have since been found in a collection of randomly isolated non-2,4-D degraders, including gram-positive bacilli, as well as in various gram-negative bacteria, even though the gene is not expressed (10).

An interesting result was the detection of two different tfdA gene variants in sibling strains. TFD23 and TFD31 are identical at the ribosomal gene level, but one harbors a class I gene and the other harbors a class III gene. Similarly, TFD6 and...
tfdA

SSU rDNA

FIG. 3. Phylogenetic incongruency of tfdA genes and SSU rDNA from di- verse 2,4-D-degrading bacteria. Dendrograms for tfdA and SSU rDNA are indicated. Shading indicates the type of tfdA sequence, either class I, II, or III. Note that branch lengths are not drawn to scale.

EML159 are rRNA siblings that carry a class II and class I gene, respectively.

None of the α-Proteobacteria yielded a PCR product when amplified with the conserved tfdA primers. This finding complements our observation that none of these bacteria hybridized to the tfdA gene, even under conditions of low stringency, indicating that any tfdA-like genes in the α-Proteobacteria are likely to be more divergent from the ones sequenced here (7, 11). In addition, none of the Sphingomonas strains in the study hybridized with a whole pJP4 probe, and similarly, no Sphingomonas strains scored positive for transfer of 2,4-D-degrading ability to recipient B. cepacia D5. Together these results suggest a reduced gene flow between members of the α- and β- or γ-Proteobacteria or poor gene expression of β- or γ-derived genes by α-Proteobacteria. Although plasmid pJP4 is a broad-host-range plasmid and has been known to transfer to α-Proteobacteria such as Rhizobium and Agrobacterium species and to α-Proteobacteria such as Pseudomonas putida, Pseudomonas fluorescens, and Pseudomonas aeruginosa, the 2,4-D pathway is not expressed in these strains of the α- or γ-Proteobacteria (3). Phylogenetically limited expression of plasmid-borne 3-chlorobenzoate-degradative genes has also been noted for the pseudomonads (8). Subsequent studies have found divergent but related sequences for the tfdB and tfdC genes in 2,4-D-degrading Sphingomonas strains (7, 12, 24).

With the exceptions of the minor differences within the class I pJP4-like tfdA sequences, there were no intermediate tfdA sequences. The most likely explanation of this is that the rate of horizontal transfer of the tfd genes is high relative to the rate at which mutations can accumulate. Examination of sequences of tfdA genes from a greater variety of organisms may turn up more intermediate variation.

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