Carbofuran degradation mediated by three related plasmid systems

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Abstract

Two carbofuran-metabolizing Sphingomonas strains, TA and CD, were isolated from soils with differing histories of exposure to carbofuran. These strains were compared with a previously described strain, Sphingomonas sp. CFO6, with regard to growth rate, formation of metabolites, and plasmid content and structure. Extensive regions of similarity were observed between the three different plasmid systems as evidenced by cross hybridization. In addition, all three systems harbor IS1412, an insertion sequence (IS) element involved in heat-induced loss of carbofuran phenotype in CFO6, and heat-induced carbofuran deficient mutants of all three strains correlated with loss of IS1412. A carbofuran deficient mutant of TA generated by induction of IS elements was complemented by reintroduction of the wild-type plasmid, confirming the presence of genes required for carbofuran metabolism on this plasmid. Carbofuran metabolism in these three strains is clearly linked via plasmids of different numbers and sizes that share extensive common regions, and carbofuran-degrading genes may be associated with active IS elements. © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Genetic systems encoding metabolism of pesticides provide an attractive framework for studying development of catabolic pathways and relationships between plasmids in soil bacteria. Most pesticides currently in use have been present in the biosphere less than 40 years, yet many of these compounds are rapidly biologically degraded in soil, suggesting that soil bacteria possess efficient mechanisms for recruitment and assembly of novel biochemical pathways. As with biodegradation of many complex-aromatic compounds, development of many pesticide-degrading pathways likely involves recruitment of catabolic genes from various sources by horizontal gene exchange mediated by plasmids, and assembly of novel pathways catalyzed by mobile elements such as insertion sequence (IS elements) elements and transposons [1,2]. An understanding of the development of pathways involved in metabolism of pesticides will provide a greater understanding of evolutionary processes in soil bacteria.

We are interested in bacterial metabolism of the insecticide carbofuran (furadan; 2,3-dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate) (Fig. 1). Carbofuran was introduced in 1967 by FMC Corporation (Princeton, NJ, USA) and is used extensively in the United States, Europe and Asia [3]. It is of environmental importance due to its high mammalian toxicity (LD50 = 2 mg kg⁻¹ in mice) [4] and potential for contamination of ground waters. Carbofuran is metabolized by a variety of bacteria, indicating that genetic systems controlling its metabolism have either rapidly evolved de novo, or existing systems involved in the metabolism of other naturally occurring or xenobiotic compounds were recruited by the degrading organisms. Many strains capable of completely metabolizing carbofuran to CO₂ harbor multiple plasmids, although few have been extensively characterized [5,6].

We are interested in studying the development of car-
bofuran-degrading pathways in soil bacteria, and in defining relationships between carbofuran-degrading plasmids harbored by different strains of soil bacteria. We recently described the initial characterization of a carbofuran-degrading bacterium, \textit{Sphingomonas} sp. CFO6 [6]. The genetic topology of carbofuran metabolism in CFO6 appears to be complex; maintenance of five plasmids appears to be required for metabolism of the insecticide by this strain and extensive regions of similarity exist between four of the five plasmids. As might be expected from a system poised for rapid recruitment of foreign genes, CFO6 plasmids are rich in IS elements, with at least one (IS1412) implicated in instability of the carbofuran-degrading phenotype. Growth of CFO6 on carbofuran is rather slow, with stationary phase growth typically reached in 4 days. This slow growth might be expected of a strain carrying large amounts of plasmid DNA (five separate plasmids) and a system that may not be efficiently regulated with respect to catabolic functions. One might expect that more efficient systems (including fewer plasmids required for carbofuran metabolism, and consolidation of genes into well-regulated operons) might occur with time.

Two other carbofuran-metabolizing \textit{Sphingomonas} strains, TA and CD, were recently isolated from soil. Our objectives for the work presented here include analysis of: (1) phylogenetic relationships between TA, CD and CFO6; (2) possible involvement of plasmids in carbofuran metabolism by TA; (3) similarities and differences between plasmids in TA, CD and CFO6; and (4) possible presence of common IS elements between the three strains. Comparison of plasmids encoding similar functions, particularly those in newly evolving systems, will shed light on evolutionary processes among soil bacteria, and the development of novel metabolic pathways.

2. Materials and methods

2.1. Chemicals

Technical grade carbofuran (99% purity), analytical grade carbofuran phenol (99.3% purity), uniformly ring-labeled (URL) \(^{14}\text{C}\)-carbofuran and carbonyl-labeled (CAL) \(^{14}\text{C}\)-carbofuran were gifts from FMC Corporation (Princeton, NJ, USA). Prior to use, the two radiolabeled compounds were purified to >98% radiopurity by thin-layer chromatography (TLC) using preparative silica gel G TLC plates [7,8].

2.2. Soil and sampling

Soil samples (0–15 cm depth) were collected from an experimental field site near Hastings, FL, USA, after four consecutive annual applications of carbofuran. After two annual applications, this site exhibited enhanced degradation toward carbofuran (Ou, unpublished observation). Soil samples were also collected from a nearby control site, which had never been treated with carbofuran. Soil samples were stored in the dark at 4°C and used within 1 month of collection.

2.3. Isolation and screening of carbofuran degraders

A batch culture enrichment technique [9] was used to isolate carbofuran-degrading bacteria from different soil samples. Strains TA and CD were selected due to their rapid growth on carbofuran as a sole source of carbon.

2.4. Growth and mineralization of carbofuran by TA

Five ml of 1-day-old bacterial culture was inoculated into a 250-ml biometric flask (Bellco, Vineland, NJ, USA) containing 50 ml of minimal medium (BMM) [6], 50 \(\mu\)g ml\(^{-1}\) of technical grade carbofuran, and 30 Bq ml\(^{-1}\) of URL or CAL \(^{14}\text{C}\)-carbofuran. The side arm of the flask contained 5 ml of 0.5 M KOH for trapping evolved \(^{14}\text{CO}_2\). At predetermined time intervals, KOH was removed from the side arm and replaced with fresh KOH. At the end of incubation (72 h), 10 ml of culture fluid was withdrawn and used for vacuum filtration through a 0.2-\(\mu\)m Nylon filter (Micron Separation, Westboro, MA, USA). The filter was washed three times under vacuum with 5 ml of BMM. \(^{14}\text{C}\) activity in the KOH (\(^{14}\text{CO}_2\)), washed filter and filtered solution was quantified by liquid scintillation counting (LSC). In conjunction with the sampling of the KOH traps, 100 \(\mu\)l of culture fluid was removed and diluted with an equal volume of 0.1 M phosphate buffer, pH 7.2. After mixing, two drops of the diluted fluid were deposited on the counting chamber of a Petro£-Hasuer bacteria counter, and cells were counted under a phase contrast microscope [10].

2.5. Degradation and metabolite formation

Ten ml of 1-day-old bacterial culture was inoculated into a 1000-ml Erlenmeyer flask containing 500 ml BMM, technical grade carbofuran (50 \(\mu\)g ml\(^{-1}\)) and URL \(^{14}\text{C}\)-carbofuran (80 Bq ml\(^{-1}\)). After inoculation, mineralization was monitored by the method described by Ou [11].

Metabolites present in the culture medium were determined by extraction from cell-free filtrates. Filtrates (10 ml) were acidified with concentrated HCl to pH<2, and extracted twice with 25 ml of ethyl acetate. After removal of moisture by anhydrous sodium sulfate, the ethyl acetate extracts were evaporated to dryness by a roto-evaporator. The residues were dissolved in anhydrous methanol and concentrated under a gentle stream of \(\text{N}_2\) gas to 0.3 ml. Carbofuran and its metabolites in the concentrated extracts were separated and quantified by TLC autoradiographic analysis and LSC as described previously by Trabue et al. [12].
2.6. 16S rDNA sequencing and phylogenetic analysis

Genomic DNA was isolated from TA by a standard cetyltrimethylammonium bromide and isopropanol precipitation technique [13]. The 16S rDNA gene was amplified by PCR by standard procedures using primers 27f and 1492r according to Lane [14]. PCR products were ligated into a TA cloning vector (Invitrogen, San Diego, CA, USA) according to the vendor’s instructions and transformed into *Escherichia coli* cells. Recombinant plasmids were purified and used as templates for direct DNA sequencing with standard primers. In the case of CD strain, the genomic DNA was obtained by boiling the cell suspension in sterilized water for 5 min, and appropriate dilutions were used as templates in the PCR reaction using 27f and 1492r primers. The PCR product was cloned into pGEM-T vector system (Promega, Madison, WI, USA). The resulting sequences were assembled to produce contigs of ca. 1400 bases, which were aligned using the Pileup function of GCG (Wisconsin Package Version 10.0, GCG, Madison, WI, USA). Phylogenetic trees were constructed using maximum parsimony (Pau*4.0b2a, Sinauer, Sunderland, MA, USA) and neighbor-joining using a Jukes and Cantor method [15] with bootstrap analysis (100 replicates) in both cases.

2.7. Plasmid isolation and characterization

Plasmids from strains TA, CD, CFO6 and transconjugants were isolated by a modification of the method of Feng et al. [6], utilizing Qiagen mini prep columns (Valencia, CA, USA). Plasmids were further purified by CsCl-ethidium bromide density gradient ultracentrifugation [16].

2.8. Mutagenesis and complementation

Single colonies of TA and CD were inoculated into LB broth, and grown at 40°C or 41°C for 24-48 h. Colonies from the heat-treated bacteria were screened for carbofuran catabolism deficient mutants as described by Feng et al. [6]. Clones from heat-treated bacteria were examined for differences from the wild-type strain by examination of restriction patterns resulting from digestion of the resident plasmid with *Bam*HI. Mutant TA50 (carbofuran deficient and spontaneous kanamycin and ampicillin resistant) was used as recipient in triparental matings and electroporation with the wild-type plasmid [6]. Plasmid pCT001 in transconjugants was confirmed by plasmid isolation and Southern blot hybridization as described by Feng et al. [6,16].

2.9. Restriction enzyme digestions, blotting and hybridization

Plasmid DNAs were digested with *Bam*HI and separated by 0.7% agarose gel electrophoresis, and blotted onto Nylon+ membrane using alkali methods according to the manufacturer’s suggestion (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The DNAs were hybridized with probes generated from CFO6 or TA plasmids or IS1412 and labeled with [32P]dCTP by random priming according to the vendor’s recommendations (Gibco BRL, Gaithersburg, MD, USA).

3. Results

3.1. Isolation and identification

Strain TA was isolated from soil after four successive annual field treatments of carbofuran, and CD was isolated simultaneously from soil taken from an adjacent site with no history of direct applications of carbofuran. Both strains were Gram-negative motile short rods with a single polar flagellum. When grown on LB agar, TA and CD were pigmented yellow. Both strains utilized carbofuran as a sole source of C or N for growth, and also utilized carbofuran phenol and methyamine (hydrolysis products of carbofuran) as sole sources of C for growth. In addition, both strains utilized two other carbamate pesticides, carbaryl and baygon, as sole sources of C or N for growth, and also utilized carbofuran phenol and methyamine (hydrolysis products of carbofuran) as sole sources of C for growth. When grown in BMM-carbofuran, a water-soluble red metabolite was formed by both strains. This red metabolite was also produced during metabolism of carbofuran by other bacteria [17], including *Sphingomonas* sp. CFO6 [6].

Phylogenetic analysis by 16S rDNA analysis placed TA and CD firmly within the *Sphingomonas* group of the α-proteobacteria (Fig. 2). 16S rDNA phylogeny indicates that TA and CD are phylogenetically very similar. *Sphingomonas* sp. CFO6 belongs to a separate subgroup of *Sphingomonas*.

3.2. Growth and mineralization

Both TA and CD grew well on carbofuran and rapidly mineralized URL and CAL [14]C-carbofuran to [14]CO2 (Fig. 3). Patterns of the mineralization of CAL [14]C-carbofuran by the two isolates were similar to the growth patterns. After 22 h of incubation, 85% and over 90% of the applied CAL [14]C-carbofuran were mineralized by TA and CD, respectively. It should be pointed out that when CAL [14]C-carbofuran is hydrolyzed, the carbonyl [14]C is instantly converted to [14]CO2, methyamine and carbofuran phenol (Fig. 1).

At the end of 72 h of incubation, approximately 47% of the [14]C URL label versus approximately 85% CAL was mineralized by TA and CD, respectively (Table 1). The initial step in carbofuran degradation is typically hydrolysis of the carbamate linkage resulting in liberation of [14]CO2 for CAL.
Fig. 1. Proposed conversion of carbofuran to carbofuran phenol and methylamine [3].

Fig. 2. Phylogenetic tree identifying placements of Sphingomonas spp. TA, CD and CFO6.
14C-carbofuran (Fig. 1), and hence no 14C should be associated with biomass. Small amounts of 14C were found to be associated with biomass, however. It is likely that the 14C associated with biomass was due to incomplete washing or from impurities that might have been converted to biomass by the isolates. Both TA and CD utilized the aromatic ring for growth, as indicated by the incorporation of URL 14C into biomass (Table 1). Approximately 24.8% and 16.3% of applied 14C remained in cell-free spent growth media for TA and CD; the residual label was likely associated with non-metabolized carbofuran and metabolites.

**Table 1**

Distribution of 14C activity after 72 h growth in liquid media with 50 W gm l⁻³ carbofuran. Numbers represent percentages of applied 14C. Numbers in parentheses are S.D.

<table>
<thead>
<tr>
<th>Strain</th>
<th>14C-CO₂</th>
<th>14C in medium</th>
<th>14C in biomass</th>
<th>14C recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>[¹⁴C-CAL]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TA</td>
<td>85.4 (1.1)</td>
<td>5.2 (0.9)</td>
<td>2.6 (0.0)</td>
<td>93.3 (1.9)</td>
</tr>
<tr>
<td>CD</td>
<td>87.8 (2.3)</td>
<td>6.5 (0.5)</td>
<td>1.1 (0.2)</td>
<td>97.0 (3.1)</td>
</tr>
<tr>
<td>[¹⁴C-URL]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TA</td>
<td>47.4 (1.5)</td>
<td>24.8 (0.9)</td>
<td>27.6 (1.0)</td>
<td>99.8 (1.9)</td>
</tr>
<tr>
<td>CD</td>
<td>46.5 (0.1)</td>
<td>16.3 (0.8)</td>
<td>32.6 (1.0)</td>
<td>98.4 (0.7)</td>
</tr>
</tbody>
</table>

Fig. 3. Growth and mineralization of URL and CAL ¹⁴C-carbofuran by *Sphingomonas* spp. TA and CD on carbofuran as a sole source of carbon. A: *Sphingomonas* sp. TA. B: *Sphingomonas* sp. CD.

Both TA and CD grew much faster in pure culture than did CFO6 under similar conditions (data not shown). CFO6 requires several days growth to reach late exponential growth phase, whereas only 2 days are required for TA and CD to reach this stage, suggesting that carbofuran metabolism may be more efficient in these strains than in CFO6.

Fig. 4. Plasmid profiles and hybridization of IS1412 to *Sphingomonas* spp. CD, TA and CFO6. A: CHEF gel electrophoresis. Lanes: 1, CD; 2, TA; 3, CFO6. B: Southern blot of gel in A; hybridization with IS1412. Lanes: 1, CD; 2, TA; 3, CFO6. Unlabeled arrows on the left of the figures indicate location of pCD2 and pCTOO1.

Fig. 5. Southern blots of restriction enzyme digestions of plasmids from wild-type and mutant strains. A: Hybridized with pCTOO1 DNA. Lanes: 1, λ x *HodIII*; 2, CFO6; 3, CD; 4, TA; 5, TA50; 6, TA-TC. B: Hybridized with all CFO6 plasmids. Lanes: same as A.
Table 2
Mineralization of $[^{14}\text{C-CAL}]$carbofuran and $[^{14}\text{C}]$carbofuran by wild-type, mutant and transconjugant strains. Numbers in parentheses are S.D.

<table>
<thead>
<tr>
<th>Strain</th>
<th>$[^{14}\text{C-carbonyl}]$carbofuran (S.D.)</th>
<th>$[^{14}\text{C}]$carbofuran (S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TA</td>
<td>65.8 (0.7)</td>
<td>46.0 (7.4)</td>
</tr>
<tr>
<td>TA50</td>
<td>64.5 (1.2)</td>
<td>2.1 (2.8)</td>
</tr>
<tr>
<td>TA–TC</td>
<td>66.7 (1.1)</td>
<td>47.5 (3.9)</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Average and S.D. based on three replicates.

3.3. Plasmid content and comparisons

Strain TA harbors a single plasmid (pCTOO1) of approximately 100 kb as determined by restriction digestion, and CD harbors four plasmids (Fig. 4A). Note that pCD2 and pCTOO1 are approximately the same size. These plasmids are compared with those of Sphingomonas sp. CFO6, which harbors five plasmids ranging in size from 5.5 kb to over 200 kb [6].

These three systems share significant amounts of sequence similarity, including at least one common IS element (IS1412) (Fig. 4B). IS1412 hybridizes with the similarly sized plasmids pCTOO1 and pCD2, and extensive regions of similarity between restriction digests of total plasmid DNA hybridized with pCTOO1 (Fig. 5A) and CFO6 plasmid DNA (Fig. 5B). Extensive similarity between the systems is evident from the similarly sized hybridized electrophoretic bands common between CFO6 and CD, and shared sequences between pCTOO1 and the other two systems are evident from the differently sized bands hybridizing in pCTOO1.

3.4. Carbofuran metabolism is mediated by pCTOO1 in Sphingomonas sp. TA

In order to investigate the potential role of pCTOO1 in carbofuran metabolism, various strategies to cure TA of its plasmid were attempted. Repeated efforts to cure TA of its plasmid by growth at high temperatures (38–42°C) and by repeated passage on non-selective growth media failed. Repeated attempts to introduce pCTOO1 into a neutral background (Pseudomonas fluorescens M480R) with selection on carbofuran as a sole source of either carbon or nitrogen also failed, possibly suggesting that some chromosomally encoded functions may be required for metabolism of carbofuran in this strain.

Mutants lacking the ability to grow on carbofuran were generated by growth at 41°C, yielding an approximately 50-kb deletion in pCTOO1 (TA50; Fig. 5, lane 5). Also deleted in this mutant was IS1412 (data not shown); mobilization of IS1412 was previously shown to be associated with loss of carbofuran metabolism in CFO6 [2]. These mutants failed to grow on carbofuran as a sole source of carbon and did not mineralize the aromatic ring of the pesticide, although functions encoding mineralization of the chain (carbofuran hydrolase) remained (Table 2). The loss of numerous CFO6-like restriction fragments in TA50 suggests common sequences required for carbofuran metabolism (Fig. 5A, lanes 4 and 5).

The carbofuran phenotype was recovered by mating a spontaneous kanamycin/ampicillin resistant derivative of TA50 with wild-type TA, which resulted in displacement of the mutant pCTOO1 in the transconjugant TA–TC (Fig. 5, lane 6; Table 2). Displacement of the resident pCTOO1 in the deletion mutant was also confirmed by electrotransformation of TA50 with pCTOO1 (data not shown). This confirms that at least some functions required for carbofuran metabolism are encoded by pCTOO1.

4. Discussion

Carbofuran metabolism in Sphingomonas spp. TA, CD and CFO6 is linked to a common ancestor via their resident plasmids, and it is likely that IS elements such as IS1412 were responsible for recruitment and rearrangements linking carbofuran metabolism in these strains. Arrangements of genes in CD and CFO6 are more similar to each other than either is to TA, as indicated by the numbers of plasmids and commonly hybridizing restriction fragments. Growth rates of CD and TA are more similar to each other than either is to CFO6, suggesting that the CD plasmids encode relatively small, but significant, differences in carbofuran metabolism than does CFO6. The additional plasmid DNA in CD relative to TA does not appear to adversely affect its growth on carbofuran relative to TA, suggesting that the genetics of carbofuran metabolism in CD may ultimately be more similar to that in TA than to CFO6. Carbofuran metabolism in these three strains is therefore related via their resident plasmids, but significant differences in carbofuran metabolism exist between the three strains. Future studies will focus on defining the nature of the related and different sequences, which should help elucidate the development of carbofuran metabolism in soil bacteria.

It is not possible to retrace the precise steps involved in evolution of carbofuran metabolism in these three strains, but speculations regarding the relationships are possible at this time. It is clear that genetic exchange, mediated by plasmids, allowed the passage of carbofuran genes between an unknown number of intermediate strains, resulting in common genes on different plasmids in CD, TA and CFO6. These plasmids have linked carbofuran metabolism in phylogenetically similar, yet geographically diverse strains. It is also likely that IS elements such as IS1412 were responsible for initial recruitment of the carbofuran genes from disparate sources, and for the differences in structure of plasmids between the three strains. Loss of IS1412 corresponded with a loss of carbofuran metabo-
ism in all mutants studied, suggesting that IS1412 is linked with these genes, and may have been responsible for their recruitment from various host DNAs.

It is tempting to speculate that the core fragments shared between CD and CFO6 (Fig. 5) were subject to IS-mediated rearrangement to form TA, resulting in a single plasmid (pCT001). This might be expected in a soil that was subject to repeated carbofuran applications, as TA was isolated from; smaller amounts of plasmid DNA are likely to yield more efficient growth in soils. Knowledge of the replication functions of the plasmids in these strains will provide us with greater understanding of the relatedness of the plasmids, as will more precise mapping of the carbofuran-degrading genes and associated IS elements. These studies are currently underway.

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References


