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Plasmid-Mediated Mineralization of Carbofuran by *Sphingomonas* sp. Strain CF06

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A bacterial strain (CF06) that mineralized both the carbonyl group and the aromatic ring of the insecticide carbofuran and that is capable of using carbofuran as a sole source of carbon and nitrogen was isolated from a soil in Washington state. Phospholipid fatty acid and 16S rRNA sequencing analysis indicate that CF06 is a *Sphingomonas* sp. CF06 contains five plasmids, at least some of which are required for metabolism of carbofuran. Loss of the plasmids induced by growth at 42°C resulted in the inability of the cured strain to grow on carbofuran as a sole source of carbon. Introduction of the plasmids confers on *Pseudomonas fluorescens* M480R the ability to use carbofuran as a sole source of carbon for growth and energy. Of the five plasmids, four are rich in insertion sequence elements and contain large regions of overlap. Rearrangements, deletions, and loss of individual plasmids that resulted in the loss of the carbofuran-degrading phenotype were observed following introduction of Tn*5*.

Carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranoyl *N*-methylcarbamate) was first introduced in 1967 as a pesticide (1) and is extensively used as a soil-incorporated *N*-methylcarbamate insecticide to control a variety of insect pests that infest crops such as corn, potatoes, and strawberries (1). Carbofuran is of concern due to its relatively high mammalian toxicity (8) and its relatively high mobility in soil (29). The environmental fate of carbofuran is of great concern in many areas such as Washington State, where it has been detected in groundwater (7).

The biochemistry and genetics of carbofuran metabolism by bacteria have not been well characterized, although a number of bacteria capable of partially mineralizing the insecticide have been isolated and partially described (1, 13, 17, 32). The first step in metabolism is generally thought to be hydrolysis of the labile methylcarbamate linkage, yielding carbofuran-7-phenol (2,3-dihydro-2,2-dimethyl-7-benzofuranol) and methylamine (Fig. 1) (1). Three different enzymes that catalyze carbamate hydrolysis have been isolated from three different species (2, 16, 20), and two of these are capable of attacking carbofuran. Only one of the genes encoding these enzymes has been cloned (16).

The carbofuran hydrolase produced by *Achromobacter* sp. strain WM111 is encoded by a plasmid-borne gene (*mcd*) (30) that apparently shows little sequence similarity with many carbofuran-degrading microorganisms, as evidenced by a lack of hybridization with *mcd* (24). This lack of similarity indicates that other genetic sequences coding for the *N*-methylcarbamate hydrolase function must exist in many carbofuran-degrading soil bacteria, and it may be that a variety of different pathways are carried by different strains.

Bacterial plasmids may play a significant role in the evolution and dissemination of many genes required for the metabolism of pesticides such as carbofuran. Most of the work on the genetics of carbofuran degradation has focused on strains that are capable of mineralizing only the methylcarbamate moeity and not the aromatic ring, with only one preliminary study indicating that plasmids are present in a strain capable of completely mineralizing the insecticide (13). No detailed studies characterizing possible plasmids involved in modifying the benzofuran ring of carbofuran have been presented to date, nor have any studies describing enzymes or genes involved in the complete mineralization of carbofuran been reported.

It is not surprising that plasmids may be involved in the complete mineralization of the insecticide, due to the great number of previous reports of plasmid-encoded degradation of aromatic compounds (5, 26). Many catabolic plasmids may be transferred between species and genera, increasing both their distribution among microorganisms as well as the potential for recombination with other pieces of DNA. Recombination in these plasmids may be affected by mobile DNA elements such as transposons and insertion sequence (IS) elements. These rearrangements may facilitate evolution of novel pathways for metabolism of recently introduced compounds such as carbofuran (34).

In this study, we report the isolation and characterization of a *Sphingomonas* sp. capable of mineralizing the aromatic ring of carbofuran and present the most detailed characterization to date of plasmids encoding the complete mineralization of the pesticide. In addition, we have investigated the possible presence of IS elements harbored by the resident plasmids. These studies are necessary preliminaries to understanding the evolution of catabolic pathways for recently introduced compounds such as carbofuran.

MATERIALS AND METHODS

Insecticides and their metabolites. Analytical grade carbofuran (99.4% purity), uniformly labeled [U-^{14}C]carbofuran (specific activity, 39.4 mCi/mmol; 98% purity), carbonyl-1-[^{14}C]carbofuran (specific activity, 13.3 mCi/mmol; 98% purity), 3-hydroxycarbofuran, and 3-ketocarbofuran were gifts of FMC Corp. (Middleport, N.Y. or Princeton, N.J.). Analytical grade methylamine was purchased from Sigma Chemical Co. (St. Louis, Mo.). Purity of the labeled material was assessed by thin-layer chromatography and scintillation counting (23).

Bacterial strains and growth conditions. The bacterial strains and plasmids described in this study are listed in Table 1. *Sphingomonas* and *Pseudomonas* strains were grown in either Luria-Bertani (LB) medium (25) or minimal medium (MM) that contained the following ingredients (amounts per liter are

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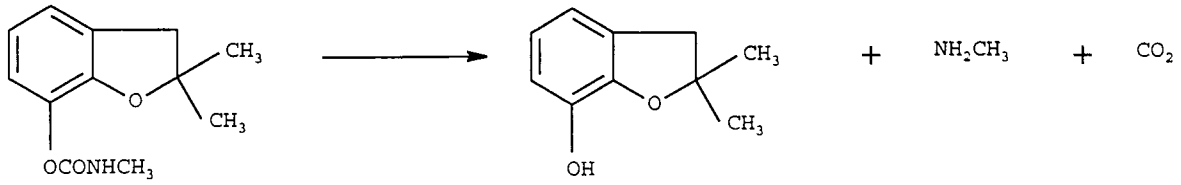

FIG. 1. Proposed conversion of carbofuran to carbofuran-7-phenol and methylamine (1).

given): K_2HPO_4, 2.4 g; KH_2PO_4, 1.2 g; NH_4NO_3, 1 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2 \cdot 2H_2O$, 25 mg; $Fe_2(SO_4)_3$, 8 mg; and carbofuran, 300 mg, for the sole carbon source. Nitrogen-free MM (NFM) lacked NH_4NO_3, and carbofuran served as a sole source of carbon and nitrogen. Glucose-supplemented NFM contained 0.1% glucose and carbofuran as a sole nitrogen source. Antibiotics were incorporated into media at the following final concentrations (in micrograms per milliliter): ampicillin, 100; kanamycin, 50; rifampin, 100; tetracycline, 30; and gentamicin, 50. All antibiotics were purchased from Sigma Chemical Co.

Enrichment and isolation of carbofuran-degrading bacteria. Carbofuran-degrading bacteria were isolated from a soil in western Washington that had been exposed to carbofuran for at least 3 years but had not been exposed to the chemical in recent years. Standard enrichment procedures (24) were used for isolating carbofuran-degrading bacteria that were capable of growth on MM, NFM, or glucose-supplemented NFM plus carbofuran.

16S ribosomal DNA (rDNA) sequencing. Genomic DNA was isolated from CF06 by a standard chloroform-isoamyl alcohol extraction procedure. Twenty nanograms of DNA was used as a template for PCR amplification of a 1,451-base segment of the 16S rRNA gene by standard procedures (19). The PCR primers were 27f (AGAGTTTGATCMTGGCTCAG) and 1492r (TACGGYTACCTTG TTACGACTT) as indicated by Lane (19). The PCR product was ligated into a TA cloning vector (Invitrogen, San Diego, Calif.) according to the vendor's instructions and transformed into *Escherichia coli* DH5α cells. Plasmid DNA was purified from positive recombinant transformants and used as a template for direct DNA sequencing of both strands with standard primers (19).

Analysis of 16S rDNA sequence data. The resulting sequences were assembled to produce 1,451-base contiguous ribosomal DNA (rDNA) sequences corresponding to *E. coli* positions 27 to 1492. The basic local alignment search tool (Fasta) network service was used to search for homologous DNA sequences in the database, and Pileup was used to align 16S rDNA sequences of different species closely related to CF06 in the alpha group of *Proteobacteria* (10). The sequence alignment was analyzed by the distance matrix method with the PHYLIP package of computer programs. Distances were calculated by the method of Kimura, and the statistical significance of the phylogenetic tree was tested by bootstrap analysis, after which phylogenies were estimated with the FITCH option (14).

Phospholipid fatty acid analyses. After nutrient broth (LB) cultures (500 ml) of CF06 were incubated at room temperature for 2 days, the cells were harvested by centrifugation, washed with phosphate-buffered saline (25), and lyophilized dry. Analysis of phospholipid fatty acids was conducted by the Center for Environmental Biotechnology at the University of Tennessee (21).

Plasmid isolation and analysis. Plasmids from strain CF06 and transconjugants were isolated by a modification of the method of O'Sullivan and Klaenhammer (22). Plasmids were further purified by CsCl-ethidium bromide density gradient ultracentrifugation, followed by butanol extraction and ethanol precipitation (25).

Plasmids were digested with restriction enzymes according to the vendor's specifications (Life Technologies). Restriction enzyme digestion fragments were separated by clamped homogeneous electric field (CHEF) gel electrophoresis at 14°C for 11 h and blotted onto nylon membranes (Hybond-N) (Amersham, Arlington Heights, Ill.) by standard procedures (25, 33). Hybridization was performed overnight at 42°C (12).

Probes. A 3-kb *Sca*I-*Cla*I fragment containing *mcd* from plasmid pJK340 (30), a 2.2-kb *Xho*I fragment containing *xylE* (encoding catechol 2,3-diooxygenase) from plasmid pJDC230 (*Xho*I fragment from pWW0 cloned into pBS+; gift of J. Dutton, University of Idaho), and a 1.4-kb *Sal*I fragment containing *catA* (encoding catechol 1,2-diooxygenase) from plasmid pPX30 (15) were used as probes to screen for the presence of these genes. Individual plasmids from CF06 were excised from gels after CHEF gel electrophoresis and used as probes to identify similarities and differences between the plasmids. An internal fragment of an IS element (IS*1412*) isolated from plasmids of CF06 (9) was also used as a probe to screen for the distribution of IS elements on CF06 plasmids. Radiolabeled probes ([α-^{32}P]dCTP) (Amersham) were generated by random priming (Promega) to a specific activity of approximately 10^8 dpm/μg of DNA.

Plasmid curing. Single colonies of CF06 were inoculated into liquid LB medium and grown at 42°C with shaking for 2 days. A series of dilutions were made from this 2-day culture by using sterile 0.85% NaCl buffer as the diluent, and 100 μl of an appropriate dilution was spread onto LB plates and incubated at 28°C for 2 days. Single colonies from LB plates were screened for carbofuran catabolism-deficient mutants by using microtiter plates with MM supplemented with

TABLE 1. Bacterial strains and plasmids used in this study[a]

Organism and strain	Plasmids and comments	Relevant phenotype	Reference or source
Sphingomonas			
CF06	Wild-type carbofuran degrader; pCF01–pCF05	CFD$^+$ Rif^s Gm^s	This study
CF06-1	Cured derivative of CF06; no plasmids	CFD$^-$ Rif^s Gm^s	This study
CF06-26	Cured derivative of CF06; deletion within pCF04	CFD$^-$ Rif^s Gm^s	This study
TF01-20	Transposon mutants of CF06	CFD$^-$ Rif^s Gm^r	This study
Pseudomonas			
M480R	Recipient strain for conjugation	CFD$^-$ Rif^r Gm^s	L. Thomashow
CMTR	Transconjugant strain obtained by mating CF06 and M480R	CFD$^+$ Rif^r Gm^s	This study
E. coli			
HB101	pRK2013	Helper; tra$^+$ mob$^+$ Km^r	L. Thomashow
S17-1	pSUP102	Derivative of Tn*5* (B22), Gm^r Tc^r	27
DH5α	Host strain of derivatives of pUCD800 with IS elements trapped into *sacB* gene	Suc^r Km^r	This study
DH5α	pRK415	Tc^r	18
DH5α	pJK340	Clone of *mcd*	30
DH5α	pJDC230	*xylE*	J. Dutton
DH5α	pPX30	*catA*	15

[a] Abbreviations: CFD$^+$, ability to grow on carbofuran; CFD$^-$, inability to grow on carbofuran; Rif^r, rifampin resistant; Rif^s, rifampin sensitive; Gm^r, gentamicin resistant; Gm^s, gentamicin sensitive; Km^r, kanamycin resistant; Tc^r, tetracycline resistant; Suc^r, sucrose resistant.

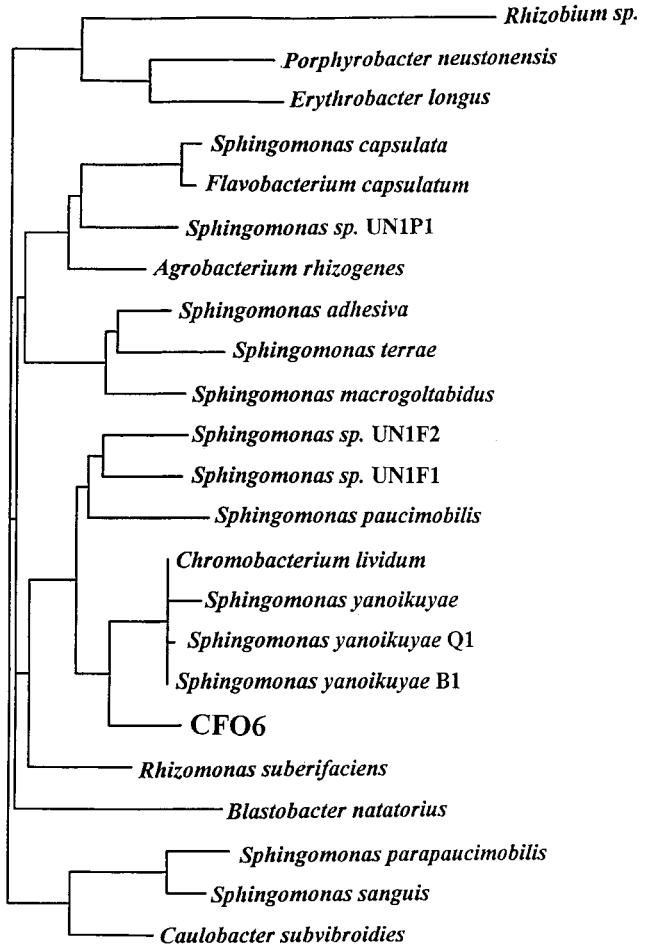

FIG. 2. Consensus phylogenetic tree for carbofuran-degrading bacterium CF06 and 22 selected strains of eubacteria.

carbofuran as the sole carbon source. Those mutants unable to utilize carbofuran as a growth substrate were screened for the presence of plasmids.

Plasmid transfer to *Pseudomonas fluorescens* M480R. Plasmids were transferred by conjugation to the rifampin-resistant *P. fluorescens* M480R. Triparental matings were conducted with *Sphingomonas* sp. strain CF06 as the donor, plasmid pRK2013 in *E. coli* HB101 as the helper, and strain M480R as the recipient (27). Rifampin-resistant colonies were screened for the presence of plasmid DNA.

Plasmids from CF06 were also introduced into *P. fluorescens* M480R by electroporation with a Gene Pulser apparatus (Bio-Rad Laboratories, Richmond, Calif.) (6), and transformants were selected for their ability to grow on MM with carbofuran as the sole carbon or nitrogen source. pRK415 was used as a positive electroporation control in these studies (18).

Mineralization assays. A 1-ml portion of MM containing either [^{14}C-carbonyl]carbofuran or [U-^{14}C]carbofuran was added to 15-ml vials and inoculated with 10 μl of cultures. The vials were sealed with screw caps and incubated at room temperature with shaking (150 rpm) for 5 days. Negative controls consisted of recipient *P. fluorescens* M480R and autoclaved CF06.

The assay was stopped by addition of 0.2 ml of concentrated HCl, which also resulted in the volatilization of $^{14}CO_2$. Ten milliliters of scintillation solution was added, and samples were counted with a Packard model C1900TR liquid scintillation analyzer (Packard, Downers Grove, Ill.).

Transposon mutagenesis. Suicide donor plasmid vector pSUP102 containing Tn*5*:B22 (27) was introduced into CF06 by electroporation as described above. The mutants were selected on LB plates amended with gentamicin and were screened for the absence of degradative functions by using microtiter plates with MM containing carbofuran as the sole carbon and energy source and amended with gentamicin. Colonies that were unable to grow on MM with carbofuran as the sole carbon and energy source but that could grow on MM with glucose as the sole carbon and energy source were assumed to be carbofuran-deficient mutants. These mutants were chosen for further characterization of plasmid profiles by CHEF gel electrophoresis.

Cloning of IS elements. A positive selection vector for trapping IS elements (pUCD800) (11, 28) was introduced into CF06 by electroporation as described above, and transformants were selected for their kanamycin-resistant phenotype. Sucrose-resistant mutants were selected by plating cultures at 28°C on LB plates supplemented with kanamycin with 5% sucrose. The plasmids isolated from sucrose-resistant mutants were transferred into *E. coli* DH5α by a standard transformation procedure (25).

Nucleotide sequence accession number. The GenBank accession number for the assembled 16S rDNA sequence of CF06 is U52146.

RESULTS AND DISCUSSION

Isolation of carbofuran-degrading bacteria. Fifteen bacterial strains that could use carbofuran as a sole source of either carbon, nitrogen, or carbon and nitrogen were isolated from the study soil. All isolates were capable of mineralizing the side chain and of using carbofuran as either C, N, or C and N for growth, but only two of these also mineralized the aromatic ring of carbofuran. One of these carbofuran-degrading bacteria, designated strain CF06, was selected for further studies due to its relatively high mineralization rate.

Characterization of CF06. CF06 could utilize carbofuran, carbofuran-7-phenol, carbaryl, gentisate, protocatechuic acid, and methylamine as the sole carbon and energy sources but

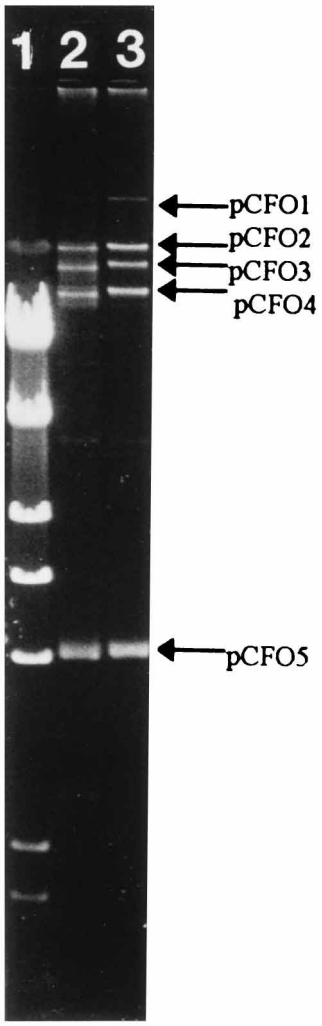

FIG. 3. Plasmid profiles of strain CF06. Lanes: 1, λ DNA plus λ DNA digested with *Hin*dIII; 2, plasmids from CF06; 3, plasmids from *Pseudomonas* sp. strain CMTR.

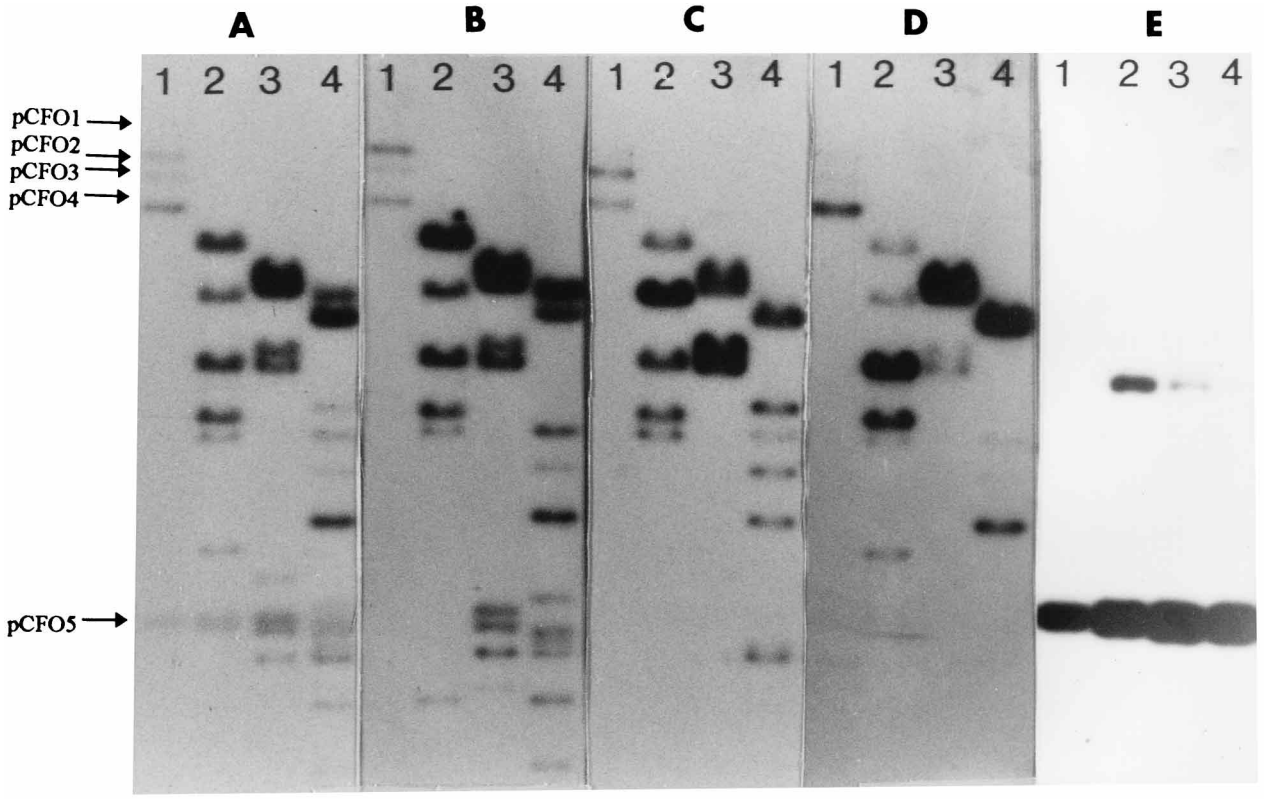

FIG. 4. Southern blot analysis of plasmids digested with different restriction enzymes. (A) plasmid pCF01 as probe; (B) plasmid pCF02 as probe; (C) plasmid pCF03 as probe; (D) plasmid pCF04 as probe; (E) plasmid pCF05 as probe. Lanes: 1, plasmids from CF06; 2, plasmids digested with *Bam*HI; 3, plasmids digested with *Eco*RI; 4, plasmids digested with *Hin*dIII.

could not utilize toluene, naphthalene, benzofuran, dibenzofuran, or 2,4-dichlorophenoxyacetate as sole carbon and energy sources.

CF06 grew well in MM with carbofuran as the sole carbon source, and an unidentified red metabolite that correlated with the disappearance of carbofuran was observed after 48 h. A yellowish color appeared after prolonged incubation up to 30 days. The red color was used as a screening tool in subsequent studies.

Analysis of the 16S rDNA of CF06 indicated that this strain belongs to the genus *Sphingomonas*, a member of the alpha group of *Proteobacteria* (Fig. 2).

The phospholipid fatty acid of CF06 contains 16:0, 16:1w7c, and 18:0w7c, constituting 52% of the total (data not shown). This indicated the presence of anaerobic desaturase fatty acid biosynthesis, a pathway utilized by *Sphingomonas* spp. Sphinganine, a defining characteristic of the genus *Sphingomonas* (10), was also detected in CF06.

Plasmid profiles. Five bands were consistently observed when the plasmids were separated by CHEF gel electrophoresis (Fig. 3). Hybridization of individual bands with fragments of total plasmid DNA digested with different restriction enzymes indicated that there are five different plasmids, designated pCF01, pCF02, pCF03, pCF04, and pCF05 (Fig. 4). It should be noted that a very weak band may be seen between pCF04 and pCF05 in Fig. 3, immediately below the second band of the λ (undigested) + λ × *Hin*dIII size marker in lane 1 (see the legend to Fig. 3). This band was commonly observed in plasmid preparations from CF06 and is believed to have resulted from breakage of one or more of the larger plasmids during preparation. Isolation of pCF01, pCF02, pCF03, and pCF04 from an agarose gel followed by purification of the bands by GeneClean (Bio 101, La Jolla, Calif.) resulted in the detection of this band when the purified plasmids were electrophoresed (data not shown).

Hybridization with the individual bands indicated that pCF01 and pCF02 have large regions of sequence similarity, as do pCF03 and pCF04 (Fig. 4A to D). It is not known at this time what functions, if any, are encoded by these common regions. Explanations for duplications of genes in catabolic systems have included compensation for the lack of a positive regulatory system (4), such as might be expected in a system that has not achieved a high degree of organization and efficiency. pCF05 (5.5 kb) (Fig. 4E) does not hybridize with any of the other plasmids.

The carbofuran hydrolase (*mcd*) gene from *Achromobacter* sp. strain WM111 (30), the catechol 2,3-dioxygenase (*xylE*), and the catechol 1,2-dioxygenase (*catA*) (15) genes did not hybridize with any of these plasmids, nor did they hybridize with total genomic DNA from CF06 (data not shown).

Plasmid-associated catabolic functions. In order to determine whether these plasmids are required by CF06 for growth on carbofuran, CF06 was cured of the plasmids by growth at 42°C. Most of the mutants characterized had lost all five plasmids, although some contained deletions in plasmids or losses of single plasmids. Loss of all five plasmids corresponded with the loss of ability to mineralize both the carbonyl group and the aromatic ring of carbofuran (Table 2).

The ability to mineralize the carbonyl and aromatic ring groups of carbofuran could be transferred from CF06 to *P. fluorescens* M480R by conjugation, as evidenced by the observation that all five plasmids were present in all transconjugants

TABLE 2. Mineralization of [^{14}C-carbonyl]carbofuran and [U-^{14}C]carbofuran by donor, recipient, transconjugant, and cured strains

Strain	Plasmids present and not visibly modified	% Mineralization (SD)[a] of:	
		[^{14}C-carbonyl] carbofuran	[U-^{14}C] carbofuran
Donor (CF06)	+	77 (0.7)	39 (1.4)
Transconjugant (CMTR)	+	78 (0.71)	43 (15.6)
Recipient (M480R)	−	0	0
Cured strain (CF06-1)	−	0	0

[a] Based on two replicates.

selected (Table 2). The curing and mating experiments demonstrated that these plasmids mediate mineralization of the carbonyl group and the aromatic ring of carbofuran.

In an effort to determine which of the five plasmids are required for growth on carbofuran, a variety of combinations of plasmids were introduced into *P. fluorescens* M480R, including pCF05 alone; a combined mixture of pCF01, pCF02, pCF03, and pCF04; and a mixture of all five plasmids. Selection of transformants on carbofuran as a sole source of carbon or nitrogen following transformation of these mixtures would likely result in significantly higher recoveries of transformants containing only those plasmids required for growth under the specific selection. Utilization of carbofuran as a nitrogen source may require only one enzyme (2, 16, 20) that is likely to be encoded on only one or two plasmids. Transformants were isolated only from transformation of all five plasmids, and no transformants were isolated from mixtures of the four largest plasmids or from pCF05 alone (data not shown). The positive control for these studies, electroporation of pRK415, indicated that DNA was successfully taken up by these cells under the experimental conditions. It is unlikely that all five plasmids are required for utilization of the pesticide as a sole source of nitrogen, but it is not known at this time why these strategies to separate the plasmids have failed.

Tn*5*:B22 mutagenesis and instability of CF06 plasmids. In a further attempt to identify the location of genes required by CF06 for growth on carbofuran, a derivative of Tn*5* was introduced into CF06. After transposon mutagenesis of CF06, 1.2% of the Gm^r mutants (29 of 2,375) were auxotrophs and 0.8% of the Gm^r mutants were deficient in carbofuran catabolism (20 of 2,375). Plasmid profiles of all transposon mutants and cured strains were different from those of CF06, with many rearrangements, deletions, insertions, and losses of plasmid DNA being observed, frequently affecting multiple plasmids (data not shown). Since these rearrangements, insertions, deletions, and losses of plasmids occurred simultaneously, it was not possible to determine the location of the catabolic genes or to determine which plasmids were responsible for the catabolic functions. Multiple rearrangements following Tn*5* mutagenesis have been reported previously (3), including one report describing rearrangements in a carbofuran-degrading bacterium (13). These rearrangements may be a result of induction of IS elements.

IS elements. Six different putative IS elements were cloned from CF06, and three of these were chosen for further study. The three putative IS elements (designated IS*1412*, IS*1487*, and IS*1488*) hybridized with three of the five different plasmids: pCF01 hybridized strongly with IS*1488* (Fig. 5A, lane 2) and weakly with IS*1487* and IS*1412* (Fig. 5A, lanes 3 and 4). pCF02 hybridized with all three putative IS elements (Fig. 5B, lanes 2, 3, and 4), pCF03 hybridized with IS*1488* (Fig. 5C, lane 2), and pCF04 and pCF05 did not hybridize with any of these three IS elements (Fig. 5D). No hybridization of any of these IS elements was detected with the chromosome of CF06, strongly suggesting that they are localized to the plasmids (data not shown). These results support the conclusion that these five plasmids are different, as was also indicated by the results of hybridization presented in Fig. 3. The characterization of IS*1412* and evidence of its mobility between the CF06 plasmids have been reported in a separate manuscript (9).

IS elements have been implicated in the evolution and recruitment of genes controlling the degradation of other pesticides (31), and it is possible that the IS elements present in the CF06 plasmids were instrumental in recruiting genes for metabolism of this relatively new compound from other microorganisms.

The genetics of carbofuran metabolism by CF06 appears to be significantly more complex and probably less well organized than most single-plasmid catabolic systems. The apparent involvement of five plasmids with extensive regions of duplication between plasmids and the presence of several active IS

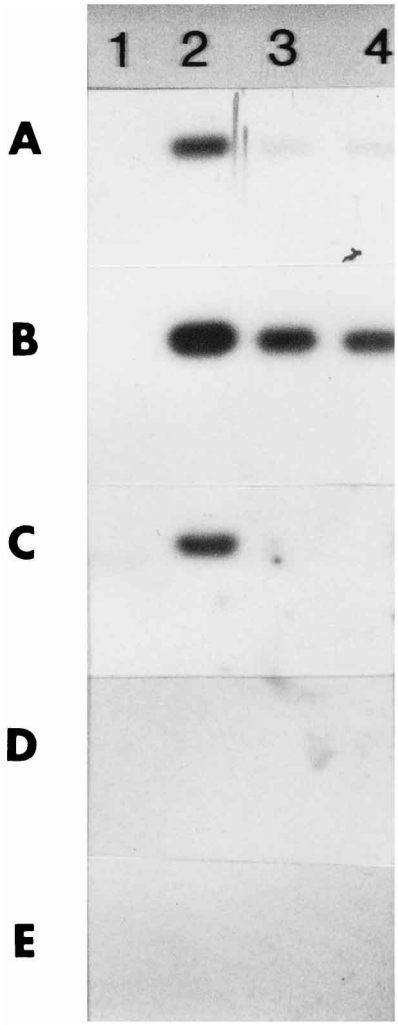

FIG. 5. Southern blot analysis of different IS elements isolated from CF06 hybridized with each individual plasmid. (A) plasmid pCF01 as probe; (B) plasmid pCF02 as probe; (C) plasmid pCF03 as probe; (D) plasmid pCF04 as probe; (E) plasmid pCF05 as probe. Lanes: 1, pUCD800 digested with *Eco*RI; 2 to 4, IS derivatives of pUCD800 (IS*1488*, IS*1487*, and IS*1412*, respectively) digested with *Eco*RI.

elements may be representative of a system at an early stage of its evolution. Such a system might be expected for the metabolism of a xenobiotic such as carbofuran that had been introduced within the past 30 years. One would expect that, with time and selective pressure, the system may evolve toward a more efficient and well-organized system. Future studies will focus on the organization of this system and on elucidation of the possible role of IS elements in the organization of the plasmids.

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