Characterization of a new 2,4-dichlorophenoxyacetic acid degrading plasmid pEST4011: physical map and localization of catabolic genes

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Plasmid pEST4011 enables *Pseudomonas putida* PaW85 to degrade 2,4-dichlorophenoxyacetic acid (2,4-D) and 3-chlorobenzoate (3-CBA). This new 2,4-D degradative plasmid has considerable homology with the regions of pJP4 containing the 2,4-D degradative genes (*tfd*). Restriction fragment *Bam*HI-B of plasmid pEST4011, which has homology with this region, was cloned into the broad-host-range vector pKT240 and studied in *P. putida* PaW85. Restriction mapping, hybridization analysis and enzyme assays established the location of the genes for 2,4-D monooxygenase (*tfdA*), 2,4-dichlorophenol hydroxylase (*tfdB*), chlorocatechol 1,2-dioxygenase (*tfdC*) and the *tfdR* and *tfdS* regulatory genes on this fragment. Plasmid pEST4012 is a derivative of pEST4011 derived through the spontaneous deletion of a 42 kbp DNA fragment, which results in the loss of the 2,4-D⁺ and 3-CBA⁺ phenotype. We present here the physical maps of pEST4011 and pEST4012. In spite of the similarities in functions, the size (70 kbp), order of catabolic genes and restriction pattern of pEST4011 are clearly different from those of pJP4.

Introduction

Plasmids involved in the degradation of 2,4-dichlorophenoxyacetic acid (2,4-D) have been under considerable investigation (Pemberton & Fisher, 1977; Pierce *et al.*, 1981; Amy *et al.*, 1985; Chaudhry & Huang, 1988). The 2,4-D degradative plasmid pJP4 from *Alcaligenes eutrophus* JMP134 (isolated in Australia) has become a model for the investigation of 2,4-D degradation (Don & Pemberton, 1985; Don *et al.*, 1985; Ghosal & You, 1988, 1989; Harker *et al.*, 1989; Kaphammer & Olsen, 1990; Kaphammer *et al.*, 1990; Perkins *et al.*, 1990). A second plasmid, pRC10, present in a 2,4-D-degrading *Flavobacterium* sp. isolated in the USA, has also been characterized in detail (Chaudhry & Huang, 1988). Little information is, however, available on the nature and physical structure of other 2,4-D degradative plasmids.

Despite differences in the size and restriction pattern of 2,4-D plasmids there is considerable homology between the 2,4-D degradative genes (Don & Pemberton, 1981; Amy *et al.*, 1985; Chaudhry & Huang, 1988; Ditzelmüller *et al.*, 1989). Comparison of other plasmids which determine the degradation of chlorinated compounds with pJP4 would be of great value in understanding the evolutionary process by which enzymes with novel specificities are evolved by bacteria and how they have spread amongst bacteria.

Recently, we isolated from 2,4-D-pretreated soils in Estonia several plasmid-containing strains with the ability to degrade 2,4-D. Restriction analysis revealed that they all harboured an identical 78 kbp plasmid, designated pEST4002 (Ausmees & Heinaru, 1990).

In this paper, we present the physical maps of the 70 kbp plasmid pEST4011, a derivative of pEST4002 which encodes enzymes for the catabolism of 2,4-D, and pEST4012, a spontaneous 2,4-D⁻ derivative of pEST4011, which has 42 kbp deleted. DNA–DNA hybridization showed extensive homology between the catabolic regions of pJP4 and pEST4011. The structural genes 2,4-D monooxygenase (*tfdA*), chlorocatechol 1,2-dioxygenase (*tfdC*), 2,4-dichlorophenol hydroxylase (*tfdB*) and the regulatory genes *tfdR* and *tfdS* have been cloned and localized on pEST4011.

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Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; 3-CBA, 3-chlorobenzoate.

Methods

Bacterial strains and plasmids. Bacterial strains and plasmids used or constructed in this study are described in Table 1.

Media and culture conditions. The complete medium was Luria broth (LB) and the minimal medium (MS) was M9 salts solution (Maniatis *et al.*, 1982) supplemented with trace elements (Bauchop & Elsdén, 1960). Concentrated stock solution (20 mg ml⁻¹) of 2,4-D was prepared as the sodium salt and added to MS medium at a final concentration of from 4.0 to 20.0 mM. Antibiotics were incorporated into LB medium at the following concentrations: 30 µg ampicillin (Ap) ml⁻¹, 1000 µg carbenicillin (Cb) ml⁻¹, 1000 µg streptomycin (Sm) ml⁻¹ and 25 µg kanamycin

(Km) ml⁻¹. *Pseudomonas* strains were incubated at 30 °C and *Escherichia coli* strains at 37 °C, respectively. Cultures (30 ml) for enzyme assays were grown in MS medium supplemented with Casamino acids (0.3%, w/v). 2,4-D and 2,4-dichlorophenol (2,4-DCP) were added to final concentrations of 0.025% (w/v) and 0.007% (w/v), respectively. After 4 h growth, a 30 ml culture was used to inoculate 70 ml of the same medium and allowed to grow to mid-exponential phase.

DNA transformation. For transformation of *P. putida* cells with plasmid DNA, we followed the method described by Bagdasarian & Timmis (1982). Transformation of *E. coli* cells was as described by Hanahan (1983).

DNA isolation, analysis and manipulation. Plasmid DNA was isolated by the procedures of Hansen & Olsen (1978) and Connors & Barnsley (1982) and purified by ethidium bromide–CsCl density gradient centrifugation. DNA cleavage by restriction endonucleases, agarose gel electrophoresis and isolation of restriction fragments from low-melting agarose gels were performed as described by Maniatis *et al.* (1982). The DNA fragments were cloned in *E. coli* into pUC18 (Yanisch-Perron *et al.*, 1985) and in *P. putida* into pKT240 (Bagdasarian *et al.*, 1983). The sizes of DNA fragments in agarose gels were calculated by comparing their mobilities with fragments of plasmid pWW0 of known size (Downing & Broda, 1979).

Southern blotting and hybridization. DNA fragments were separated on 1.0% (w/v) agarose gels and transferred to nitrocellulose filters according to Southern (1975). DNA probes were labelled to a specific activity of 1–3 × 10⁷ c.p.m. (µg DNA)⁻¹ by nick-translation (Maniatis *et al.*, 1982).

Oxygen uptake assay. Activities of 2,4-D monooxygenase were measured by monitoring oxygen uptake in the presence of 2,4-D using a Clark oxygen electrode (Yellow Springs Instruments Co.) and the YSI model 5300 biological oxygen monitor. Reaction mixtures contained approximately 10⁸ washed cells ml⁻¹ and 2.5 mM-2,4-D in 100 mM-potassium phosphate buffer (pH 7.6). The endogenous oxygen uptake was measured for 4 min in the absence of substrate and the reaction was started by injecting 20 µl of 2,4-D into the chamber. The oxygen uptake was monitored for 5 min.

Extract preparation and enzyme assays. Cell extracts for 2,4-dichlorophenol hydroxylase (*tdB*) and chlorocatechol 1,2-dioxygenase (*tdC*) assay were prepared by harvesting mid-exponential phase cultures by centrifugation at 8000 g for 10 min. The cell pellets were washed with 10 ml 100 mM-potassium phosphate buffer (pH 7.6), centrifuged again and suspended in 5 ml 100 mM-potassium phosphate buffer (pH 7.6) containing 1 mM-2-mercaptoethanol, 0.1 mM-EDTA and 2 µM-flavin adenine nucleotide. Cell-free extracts were prepared and 2,4-DCP hydroxylase activity was determined by the procedure of Kaphammer & Olsen (1990).

Chlorocatechol 1,2-dioxygenase assays were performed as described by Hageman (1966) using 3-methylcatechol as the substrate instead of chlorocatechols. *P. putida* PaW85 did not express catechol 1,2-dioxygenase activity at detectable levels when grown in MS medium containing 0.3% Casamino acids with or without 2,4-D or 2,4-DCP.

Protein concentrations were determined by the method of Bradford (1976).

Results

Characterization of *Pseudomonas putida* EST4021(pEST4011)

We have previously reported the isolation of a new 2,4-D degradative plasmid pEST4002 in *Pseudomonas*

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant markers*	Source or reference
<i>E. coli</i>		
HB101	<i>hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44</i>	Maniatis <i>et al.</i> (1982)
TG1		Gibson (1984)
<i>P. putida</i>		
PaW85	Prototroph; Tn4652 in chromosome	Bayley <i>et al.</i> (1977)
PaW340	Sm ^r Trp ⁻ ; Tn4652 in chromosome	Bayley <i>et al.</i> (1977)
EST4021	Prototroph; 2,4-D ⁺	This study
EST4022	Prototroph; 2,4-D ⁻	This study
<i>A. eutrophus</i>		
JMP134	Prototroph; 2,4-D ⁺	Dr H.-J. Knackmuss†
<i>Pseudomonas</i> sp.		
EST4002	Prototroph; 2,4-D ⁺	Ausmees & Heinaru (1990)
Plasmids		
pUC18	Cloning vector (Ap ^r)	Yanisch-Perron <i>et al.</i> (1985)
pKT240	Cloning vector (Cb ^r Km ^r)	Bagdasarian <i>et al.</i> (1983)
pJP4	Plasmid from JMP134 (2,4-D ⁺ Hg ^r 3-CBA ⁺)	Don & Pemberton (1981)
pEST4002	Plasmid from EST4002 (2,4-D ⁺ ; wild-type 2,4-D plasmid)	Ausmees & Heinaru (1990)
pEST4011	Del. mutant of pEST4002 (2,4-D ⁺)	This study
pEST4012	Del. mutant of pEST4002 (2,4-D ⁻)	This study
pEST4013	pKT240 carrying 10.5 kbp <i>Bam</i> HI fragment of pEST4011	This study
pEST4014	pKT240 carrying 7.8 kbp <i>Bam</i> HI– <i>Eco</i> RI fragment of pEST4013	This study
pEST4015	pKT240 carrying 4.5 kbp <i>Bam</i> HI– <i>Kpn</i> I fragment of pEST4013	This study
pEST4016	pKT240 carrying 2.5 kbp <i>Xho</i> I fragment of pEST4013	This study

* Abbreviations: Ap, ampicillin; Cb, carbenicillin; Km, kanamycin; Sm, streptomycin; Hg, mercury; Trp⁻, requirement for tryptophan; 2,4-D⁺, ability to grow on 2,4-dichlorophenoxyacetic acid; 3-CBA⁺, ability to grow on 3-chlorobenzoate.

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Fig. 1. Restriction digest map of plasmids pJP4 (Kaphammer *et al.*, 1990; Perkins *et al.*, 1990), pEST4011 and pEST4012. The black box in plasmid pEST4012 shows the deleted region.

sp. EST4002 (Ausmees & Heinaru, 1990), which could also utilize 3-CBA in addition to 2,4-D as a sole source of carbon. The growth characteristics are similar to those of *A. eutrophus* (Don & Pemberton, 1981) which harbours plasmid pJP4 (Don & Pemberton, 1985; Don *et al.*, 1985). The 2,4-D⁺ phenotype was very unstable in EST4002 and similar isolates (results not presented). In EST4002, 80% of the cells had lost their ability to grow on 2,4-D after growth in a non-selective LB medium for 4 h. In most cases the 2,4-D⁻ clones were either plasmid-free or contained a smaller plasmid, derived from pEST4002 by a deletion of 20–30 kbp (Ausmees & Heinaru, 1990). Due to the instability of the 2,4-D⁺ phenotype and the difficulties with DNA extraction from the wild-type strains the exact location and size of the deletion was not determined.

To overcome the above difficulties pEST4002 was transferred by conjugation from EST4002 into the plasmid-free recipient strain *P. putida* PaW340. Restriction analysis showed that 2,4-D⁺ transconjugants of PaW340 contained a 70 kbp plasmid pEST4011, which had lost a 7.8 kbp *EcoRI* fragment present in pEST4002 (78 kbp).

P. putida strain PaW85 was transformed with pEST4011 and subcultured to obtain stable 2,4-D⁺ 3-CBA⁺ transformants. A single plasmid, pEST4011 in strain EST4021 was detected. In batch cultures the growth rates of EST4021(pEST4011) were the same at 2,4-D concentrations between 4.0–20.0 mM; 4 mM-2,4-D (1 mg ml⁻¹) was degraded within 14 h after a lag period of 1–3 h.

In spite of its similar functions, the size (70 kbp) and restriction pattern of plasmid pEST4011 (Fig. 1) are considerably different from those of pJP4 (80 kbp).

In contrast to pEST4002 in EST4002, pEST4011 was relatively stable in EST4021. However, growth of EST4021 in LB medium for 30 generations resulted in a putative cured strain which had simultaneously lost its ability to grow on 2,4-D and 3-CBA. One 2,4-D⁻ strain. EST4022 did not revert to the 2,4-D⁺ phenotype at detectable levels (< 1 in 10¹⁰) and contained a 28 kbp plasmid pEST4012. Five such 2,4-D⁻ 3-CBA⁻ clones, isolated in separate experiments after prolonged growth of EST4021 in LB medium, contained plasmids with the same restriction patterns as pEST4012.

2,4-D monooxygenase, 2,4-DCP hydroxylase and chlorocatechol 1,2-dioxygenase activities were detected in EST4021(pEST4011), but not in EST4022(pEST4012). This suggests that the catabolic genes are located on the excised DNA segment.

Physical maps of the plasmids pEST4011 and pEST4012

By using restriction analysis, gene cloning and Southern hybridization a physical map of pEST4011 and pEST4012 was determined with restriction endonucleases *Bam*HI, *Dra*I, *Hind*III and *Kpn*I (Fig. 1). Comparison of pEST4012 with pEST4011 indicates the excision of a 42 kbp fragment and the restriction mapping reveals that the excised region is a continuous segment of the 2,4-D plasmid pEST4011.

Homology between pJP4 and pEST4011

The homology between pEST4011 and the 2,4-D⁺ plasmid pJP4 was investigated by Southern hybridization. pEST4011 hybridized to all of the restriction fragments of pJP4 except fragments *Bam*HI-D, *Hind*III-A and E to H. This indicated that pEST4011 had homology with the regions of pJP4 encoding 2,4-D degradative enzymes (Fig. 2*a, b*). Subsequently, we cloned *Bam*HI-C, *Bam*HI-F and *Hind*III-B fragments from pJP4 into pUC18. The preliminary location of the degradative genes on plasmid pEST4011 was determined by preparing three probes using isolated pJP4 fragments *Bam*HI-*Cla*I (from *Bam*HI-C), *Bam*HI-*Xho*I (from *Bam*HI-E) and *Bam*HI-*Eco*RI (from *Hind*III-B). Those fragments contain *tfdC*-*tfdB* (chlorocatechol 1,2-dioxygenase, 2,4-DCP hydroxylase) genes, *tfdR* (regulator), *tfdC* (chlorocatechol 1,2-dioxygenase) genes and *tfdA* (2,4-D monooxygenase), *tfdS* (regulator) genes respectively (Perkins *et al.*, 1990). All of these fragments of pJP4 hybridized with the *Bam*HI-B restriction fragment of pEST4011, thus indicating that catabolic genes for 2,4-D degradation are located on pEST4011 in the 10.5 kbp *Bam*HI-B fragment.

Localization of *tfd* genes in pEST4011

Plasmid pEST4013 was constructed by cloning the *Bam*HI-B fragment of pEST4011 into the *Bam*HI site of the vector plasmid pKT240.

Strain PaW85, containing pEST4013 showed inducible expression of the *tfdA* (2,4-D monooxygenase), *tfdB* (2,4-DCP hydroxylase) and *tfdC* (chlorocatechol 1,2-dioxygenase) genes when 2,4-D and 2,4-dichlorophenol were used as inducers (Fig. 2*b*).

Further hybridization experiments are summarized in Fig. 2*a, b*. The hybridization of the 2.1 kbp *Sal*I-*Bam*HI (*tfdB*, 2,4-DCP hydroxylase) and the 1.6 kbp *Hind*III fragment of pJP4, which contains the *tfdC* (chlorocatechol 1,2-dioxygenase) and a part of the *tfdD* (chloromuconate cycloisomerase) gene only with the same 2.5 kbp *Xho*I fragment of pEST4013 shows that the *tfdB* and *tfdC* genes in pEST4011 are more closely located as compared with the corresponding genes of pJP4. The 1.0 kbp *Bam*HI-*Xho*I fragment of pJP4, which contains the *tfdR* (regulator) gene hybridized only to the 1.0 kbp *Bam*HI-*Xho*I fragment of pEST4013. Hybridization was also detected between the cloned 1.5 kbp *Eco*RI-*Bam*HI fragment of pJP4, which contains the *tfdA* (2,4-D monooxygenase) and part of the *tfdS* (regulator) gene and the 2.7 kbp *Hind*III fragment of pEST4013.

The 7.8 kbp *Eco*RI-*Bam*HI fragment from *Bam*HI-B of pEST4011 was subcloned into the vector plasmid pKT240 to give pEST4014. The *tfdA* locus is either

absent or defective in this plasmid as no 2,4-D monooxygenase (*tfdA*) activity could be detected in PaW85 (pEST4014). However, 2,4-DCP hydroxylase (*tfdB*) and chlorocatechol 1,2-dioxygenase (*tfdC*) activities were inducible in this strain (Fig. 2*b*). This revealed that the regulatory genes *tfdR* and *tfdS* are present on the 7.8 kbp *Eco*RI-*Bam*HI fragment.

The 4.5 kbp *Kpn*I-*Bam*HI fragment from pEST4013 was subcloned into pKT240 to give pEST4015. Chlorocatechol 1,2-dioxygenase (*tfdC*) was inducible in PaW85 (pEST4015) when 2,4-DCP was used as an inducer, whereas no 2,4-DCP hydroxylase (*tfdB*) activities was detected (Fig. 2*b*). The regulatory gene, *tfdS*, must therefore be located somewhere left from *Kpn*I-*Bam*HI (4.5 kbp) in the *Eco*RI-*Bam*HI (7.8 kbp) fragment.

The 2.5 kbp *Xho*I fragment from pEST4013 was subcloned into pKT240 to give pEST4016. Strain PaW85(pEST4016) exhibited uninducible expression of chlorocatechol 1,2-dioxygenase (*tfdC*) activity and no 2,4-DCP hydroxylase (*tfdB*) activity (Fig. 2*b*). This suggests that the regulatory gene, *tfdR*, is located at the right-end of the 10.5 kbp *Bam*HI fragment in pEST4013.

Discussion

In this report we have characterized a new 2,4-D plasmid pEST4002 determining catabolism of 2,4-D and 3-CBA. This plasmid originated from *Pseudomonas* sp. EST4002 isolated from soil in Estonia (Ausmees & Heinaru, 1990). The size (70 kbp) and restriction pattern of the derived plasmid pEST4011 (Fig. 1) differed considerably from those of pJP4 previously reported in *Alcaligenes eutrophus* JMP134 (Don & Pemberton, 1985; Harker *et al.*, 1989).

Loss of the 2,4-D⁺ and 3-CBA⁺ phenotype in EST4011 occurred at a high frequency and spontaneously by the deletion of approximately 42 kbp of DNA (Fig. 1). One possible mechanism for specific excision is reciprocal recombination between two DNA sequences present as direct repeats at the end of the excised region. If there are more than two repeats present on the plasmid molecules, the deleted sequence will be determined by the recombination switch points between specific repeats. pJP4 contains three such direct repeats (Ghosal *et al.*, 1985) and undergoes deletions of a 15 to 18 kbp segment when selected for growth on 3-CBA alone (Ghosal *et al.*, 1985), and about 40 kbp are deleted with the loss of growth on 2,4-D⁺ and 3-CBA⁺ (Don & Pemberton, 1985). A similar situation may be the case for pEST4011, in which the loss of the 2,4-D⁺ and 3-CBA⁺ phenotypes occurs by the excision of a 42 kbp sequence.

It is interesting to note that pEST4011 showed homology only with the regions coding for the degradative genes (*tfdA*-*F*) and regulatory genes *tfdS* and *tfdR*

Fig. 2. (a) Map of pJP4 based on data from Kaphammer *et al.* (1990) and Perkins *et al.* (1990). The approximate locations of *tfdA*, *tfdS*, *tfdR*, *tfdCDE* and *tfdB* are shown. (b) Cloning of DNA fragments of pEST4011 in *P. putida* PaW85 using the broad-host-range vector pKT240. Relevant restriction sites of pEST4011 DNA fragments used for cloning are shown for the 10.5 kbp *Bam*HI fragment (B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; M, *Mlu*I; S, *Sal*I; X, *Xho*I). Each plasmid listed on the left was subcloned from pEST4013 (pKT240 carrying the 10.5 kbp *Bam*HI-B fragment of pEST4011). The fragments of pEST4013 hybridizing with the pJP4 fragments containing *tfdA* and *tfdS* (□), *tfdR* (■), and *tfdC,D,B* (▨) are shown. Expression of *tfd* genes in *P. putida* PaW85 is shown on the right: MO, 2,4-dichlorophenoxyacetic acid monooxygenase activity; HY, 2,4-dichlorophenol hydroxylase activity; CO, chlorocatechol 1,2-dioxygenase activity. ++, strong inducible activity; +, inducible activity; ±, uninducible activity; -, no activity. By the terms 'inducible' and 'uninducible' we mean the following: in an inducible system the enzyme activities differ at least fivefold when an inducer compound is used or not; in the uninducible system the enzymes exhibit a low constitutive level of expression independent of the presence or absence of the inducer.

of pJP4. This supports other evidence that 2,4-D degradative plasmids demonstrate appreciable homology between the genes encoding enzymes that catalyse the same steps in breakdown of 2,4-D and related compounds (Chaudhry & Huang, 1988).

By using DNA-DNA hybridization, DNA cloning techniques and enzyme assays we established the location of at least three catabolic genes encoding the first three steps in the 2,4-D degradation pathway: 2,4-D monooxygenase (*tfdA*), 2,4-D dichlorophenol hydroxylase (*tfdB*) and chlorocatechol 1,2-dioxygenase (*tfdC*) on the 10.5 kbp *Bam*HI-B fragment of pEST4011. On the basis of hybridization with the *tfdR* probe from pJP4 and the results of gene expression from pEST4015 and pEST4016, we have assigned the *tfdR* gene to the right-

hand of the *Bam*HI-B fragment of plasmid pEST4011 (Fig. 2b). Testing enzyme activities in pEST4014 and pEST4015 verified the location of the second regulatory gene, *tfdS* needed for the inducible expression of 2,4-DCP hydroxylase, the product of gene *tfdB*. It is possible that this regulator could be an analogue for *tfdS* present in pJP4.

In spite of the strong homology between catabolic and regulatory genes of 2,4-D pathways in pJP4 and pEST4011, the order of these genes on the two plasmids is completely different (Fig. 2a, b). A further comparative study of the nucleotide sequence of catabolic genes in pEST4011, pJP4 and related plasmids would widen our understanding of the unique differences and evolutionary relationship between different 2,4-D degradative plas-

mids in strains isolated from different geographical regions of the world.

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