The Formation of an R-prime Carrying the Fraction I Dehalogenase Gene from *Pseudomonas putida* PP3 using the IncP Plasmid R64.44

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Plasmid R68.44 was transferred to *Pseudomonas putida* PP3 at a frequency of approximately 10^{-6} . In the new strain, *P. putida* PPW1, the plasmid, designated pUU1, lost a 2.0 kb fragment corresponding to IS21 which had previously been implicated in enhanced chromosome mobilization, but gained between 3.4 and 5.7 kb of DNA. Plasmid pUU1 was used to mobilize the fraction I dehalogenase gene at a low frequency of approximately 10^{-10} into another strain of *P. putida*. The R-prime plasmid, designated pUU2, contained an additional 9.2 to 11.0 kb fragment which carried the fraction I dehalogenase.

INTRODUCTION

Pseudomonas putida strain PP3, isolated by Senior *et al.* (1976) as a result of its acquired capacity to grow on 2,2-dichloropropionic acid (22DCPA; the herbicide Dalapon) and 2-monochloropropionic acid (2MCPA), has been shown to contain two dehalogenases (Weightman *et al.*, 1979; Weightman & Slater, 1980). Dehalogenases, or alternatively halohydrolases, catalyse the hydrolytic removal of halides from halogenated alkanolic acids, particularly chlorinated acetic and propionic acids (Goldman *et al.*, 1968; Little & Williams, 1971; Slater *et al.*, 1979). So far, a limited range of microbial genera have been grown on halogenated alkanolic acids (Slater *et al.*, 1979; Berry *et al.*, 1979), although some evidence suggests that a greater number of different bacterial species with dehalogenating capabilities can be isolated from environments which have been challenged by the presence of chlorinated alkanolic acids (Burge, 1969; Hardman & Slater, 1981) compared to those from unchallenged environments.

We are interested in the mechanisms of transfer of genes coding for catabolic functions in natural populations of loosely or closely associated communities (Slater 1978; Slater & Godwin, 1980; Slater & Bull, 1982). It would be of particular interest to know how the dehalogenase gene (or genes) of *P. putida* PP3 may be transferred initially to other pseudomonads and ultimately to other genera by mechanisms involving plasmid transfer. We have argued (Hardman & Slater, 1981) that the plasmid-mediated transfer of dehalogenase genes might explain the increased occurrence of different bacteria possessing dehalogenases after challenging the mixed microbial flora with halogenated alkanolic acids. Furthermore, plasmid-mediated mobilization of dehalogenase genes provides a possible mechanism for the evolution of strains carrying multiple dehalogenase genes (Hardman & Slater, 1981), and recent evidence suggests that the dehalogenase genes in a number of soil bacteria are plasmid-borne (Kawasaki *et al.*, 1981, 1982; D. J. Hardman, P. C. Gowland and J. H. Slater, unpublished observations).

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Abbreviations: MCA, monochloroacetic acid; DCA, dichloroacetic acid; 2MCPA, 2-monochloropropionic acid; 22DCPA, 2,2-dichloropropionic acid.

Plasmid R68.44 is a derivative of R68 which exhibits enhanced chromosome-mobilizing ability (Haas & Holloway, 1976) and has been shown to be physically identical to the more stable R68.45 (Leemans *et al.*, 1980). R68.44 differs from R68 by a 2.12 kb duplicated region which is correlated with the possession of enhanced chromosome-mobilizing ability (Riess *et al.*, 1980; Leemans *et al.*, 1980; Willetts *et al.*, 1981).

In this paper, we report the introduction of plasmid R68.44 (Haas & Holloway, 1976) into *P. putida* PP3, its structural modification in this strain and the mobilization of chromosomal DNA carrying a dehalogenase gene into another strain of *P. putida*.

METHODS

Bacterial strains and maintenance. *Pseudomonas putida* PP3 (2Mcpa⁺ 22Dcpa⁺ Ap^S Km^S Sm^S Tc^S) was obtained as previously described (Senior *et al.*, 1976). *Pseudomonas putida* PaW340 (2Mcpa⁻ 22Dcpa⁻ Trp⁻ Ap^S Km^S Sm^S Tc^S) was kindly provided by Dr P. A. Williams (University College of North Wales, Bangor). *Pseudomonas aeruginosa* PAC174 (2Mcpa⁻ 22Dcpa⁻ Lys⁻) containing plasmid R68.44 (Ap^R Km^R Tc^R) was kindly provided by Professor P. H. Clarke (University College, London). *Pseudomonas aeruginosa* PAO1162 (*leu-38 rmo-11*) (Dunn & Holloway, 1971) was kindly provided by Professor K. N. Timmis (University of Geneva, Switzerland). A spontaneous rifampicin-resistant derivative of PAO1162, designated *P. aeruginosa* PAU2, was selected on nutrient agar containing rifampicin at 250 µg ml⁻¹. The parent and derivative strains capable of growing on 2MCPA were grown on defined medium containing 2MCPA (Slater *et al.*, 1979) supplemented, where appropriate, with amino acids (25 µg ml⁻¹), ampicillin (50 µg ml⁻¹), kanamycin (50 µg ml⁻¹), tetracycline (150 µg ml⁻¹), rifampicin (250 µg ml⁻¹) and streptomycin (750 µg ml⁻¹). The other strains were maintained on nutrient agar (Oxoid) supplemented as necessary with drugs at the above concentrations. King's A and B media were made as described by King & Phillips (1978).

Measurement of dehalogenase activity. The dehalogenase activity was determined in organisms grown in medium containing 2MCPA at 0.5 g Cl⁻¹. The cultures were incubated overnight at 30 °C on an orbital shaker rotating at 200 r.p.m. Late-exponential phase cultures were harvested by centrifugation at 5000 g for 15 min, and washed and resuspended in 0.02 M-Tris/sulphate buffer, pH 7.9. Crude cell-free extracts were prepared by two passages through a French pressure cell (Aminco International) at 83 MPa. The remaining whole organisms and cell debris were removed by centrifugation at 30000 g for 45 min.

Dehalogenase activity in the cell-free extract against MCA, DCA, 2MCPA and 22DCPA was determined as previously described (Weightman & Slater, 1980). The assay depended on measuring free chloride ion release in a Marius Chlor-O-Counter (F. T. Scientific, Tewkesbury) as previously described (Slater *et al.*, 1979). Protein concentration of the extracts was estimated by the Biuret method using BSA as the reference protein. Enzyme specific activities were expressed as µmol substrate converted min⁻¹ (mg protein)⁻¹.

PAGE. The two dehalogenases of *P. putida* PP3 were separated and identified in derivative strains by an analytical discontinuous PAGE technique as described by Weightman & Slater (1980) and Hardman & Slater (1981).

Plasmid transfer and chromosome mobilization. A modified membrane mating technique due to Hedges & Jacob (1977) was used. The parent strains were grown in either LB broth (Miller, 1972) or defined medium containing 2MCPA and supplemented as appropriate. Samples of mid-exponential phase donor culture (5 ml) or maximum population phase recipient culture (5 ml) were centrifuged and each resuspended in 5 ml of 0.1 M-phosphate buffer pH 7.0. The suspensions were mixed and vacuum-filtered through a sterile 0.45 µm pore size membrane filter (Millipore). The filter was transferred to the surface of an LB agar Petri dish and incubated at 30 °C for 6 h. The membrane was transferred to a sterile test tube and the organisms resuspended in 2.5 ml 0.1 M-phosphate buffer pH 7.0. The suspension was serially diluted in 0.1 M-phosphate buffer and samples (0.1 ml) of appropriate dilutions spread on suitable selective media to screen for transconjugants.

Screening for the presence of plasmid DNA. The method developed by Wheatcroft & Williams (1981) was used.

Plasmid DNA isolation for restriction endonuclease analysis. The method of Birnboim & Doly (1979) was used.

Plasmid DNA digestion by restriction endonuclease. The restriction endonucleases *EcoRI*, *HindIII*, *PstI* and *SmaI* were used in accordance with the manufacturer's instructions (Boehringer, Mannheim). After 2 h the digestion was terminated by heat inactivation at 65 °C for 10 min. The resulting DNA fragments were separated by electrophoresis in horizontal 0.7% (w/v) agarose gels in TB buffer containing 89 mM-Tris, 2.5 mM-EDTA and 89 mM-boric acid. The gels were run at 30 to 60 mA for 16 h, stained with ethidium bromide and photographed. The plasmid DNA fragments were sized by comparison with the DNA fragments produced by a double *EcoRI* and *HindIII* restriction endonuclease digest of λ DNA (Boehringer Mannheim). The standard sizes for the λ fragments were taken from Southern (1979).

Transformation. This was performed as described by Bagdasarian & Timmis (1981).

Materials. All materials for the growth and enzyme assays were of the highest commercially available purity as described previously (Slater *et al.*, 1979; Weightman & Slater, 1980), as were the materials used for the plasmid analyses.

RESULTS

The introduction of plasmid R68.44 into P. putida PP3

Plasmid R68.44 was transferred by conjugation to *P. putida* PP3 in a membrane mating with *P. aeruginosa* PAC174, selecting for transconjugants on defined medium containing 2MCPA, ampicillin, kanamycin and tetracycline. Plasmid transfer occurred at low frequencies of about 10^{-6} . The putative *P. putida* strains carrying R68.44 were checked for characteristic growth on King's A and B media. The transconjugants produced cream/white colonies with no pigment on King's A medium, characteristic of *P. putida* strains, whereas *P. aeruginosa* produced a green diffusible pigment. On King's B medium the transconjugants produced lime-green, yellow-fluorescing colonies, again characteristic of *P. putida* and in contrast to *P. aeruginosa* which produced darker green, white/blue-fluorescing colonies. A number of transconjugants were screened for the presence of plasmid DNA, including the strain *P. putida* PPW1 selected for the mobilization studies, and were shown to contain a plasmid of the same size as that in the donor strain.

A comparison of the digests using restriction endonucleases *Pst*I and *Sma*I showed unexpected, significant differences between the plasmids in *P. aeruginosa* PAC174 and *P. putida* PPW1 (Fig. 1). This was a consistent modification since in two other independent matings using *P. putida* PP3 as recipient, the same changes in the digests were obtained. In view of the differences, the plasmid found in *P. putida* PPW1 was designated pUU1. The second largest fragment (21.0 kb) in the *Pst*I digest of plasmid R68.44 was missing in the equivalent digest of plasmid pUU1 (Fig. 1*a*). It was replaced by two additional fragments of sizes 15.0 and 11.7 kb in pUU1. Thus the combined size of the additional DNA was 5.7 kb greater than the replaced 21.0 kb fragment of R68.44. However, the complete size of pUU1 calculated from the *Pst*I fragments was 61.7 kb compared with the size of R68.44 which was calculated to be 58.0 kb [this size for R68.44 includes the second copy of the 0.74 kb fragment identified by Riess *et al.* (1980)]. The difference of 3.7 kb was significantly smaller than the insert of DNA into the fragment of size 21.0 kb and was due to the concomitant loss of two *Pst*I fragments at some time during the formation of pUU1 from R68.44. The loss of these two fragments (1.3 kb and 0.74 kb) with a total of 2.0 kb and the addition of the 5.7 kb insert accounted for the net increase in size of pUU1 compared with plasmid R68.44.

With restriction endonuclease *Sma*I, the second largest fragment (16.6 kb) was also lost in plasmid pUU1 and replaced by a single, larger fragment of 20.0 kb, giving an increase in size of 3.4 kb (Fig. 1*b*). This is comparable with the extra DNA (5.7 kb) determined from the *Pst*I digestion. Furthermore, in agreement with the *Pst*I digestion data, one *Sma*I fragment of 2.0 kb was lost, resulting in a net increase in the size of plasmid pUU1 of 1.4 kb.

The mobilization of the fraction I dehalogenase gene into P. putida PaW340

Pseudomonas putida PPW1 was mated on a membrane with *P. putida* PaW340 with selection for transconjugants on medium containing 2MCPA, tryptophan, ampicillin, kanamycin, streptomycin and tetracycline. Transconjugants were selected at a frequency of 1.3×10^{-10} per donor cell. This compared with a frequency of 0.1×10^{-6} to 1.3×10^{-6} per donor cell for the transfer of plasmid pUU1 into *P. putida* PaW340 in the absence of selection for growth on 2MCPA. The transconjugants proved to be tryptophan auxotrophs and contained a plasmid; one was designated *P. putida* PPW2.

Dehalogenase activity was demonstrated in *P. putida* PPW2 but the activity towards DCA was substantially lower than the activities in *P. putida* strains PP3 and PPW1 (Table 1). Indeed, the substrate activity ratios were indicative of an organism synthesizing only the fraction I dehalogenase, such as *P. putida* PP411-006, and compared favourably with the activity ratio for partially purified fraction I dehalogenase (Table 1). This was confirmed by discontinuous PAGE of *P. putida* strains PP3, PPW1 and PPW2 (Fig. 2). These results suggest the formation of a plasmid which carries *P. putida* chromosomal DNA encoding the fraction I dehalogenase gene.

Further evidence for the formation of such a plasmid was obtained by mating *P. putida* PPW2 with *P. aeruginosa* PAU2 as recipient and selecting for transfer of drug resistance markers.

Fig. 1. Restriction endonuclease digest fragment patterns for (a) enzyme *Pst*I and (b) enzyme *Sma*I for plasmids R68.44, pUU1 and pUU2 in *P. putida* PPW2. The fragment sizes are given in kb by calculation from a double digest of λ DNA with restriction endonucleases *Eco*RI and *Hind*III and using standard DNA fragment sizes given by Southern (1979). The asterisk indicates the position of two fragments of the same size.

Fig. 2. Distribution of dehalogenases from *P. putida* strains PP3, PPW1 and PPW2 after separation by PAGE.

Table 1. *Dehalogenase specific activities and substrate activity ratios for various strains of P. putida and P. aeruginosa*

Strain	Dehalogenase specific activities [$\mu\text{mol substrate min}^{-1} (\text{mg protein})^{-1}$]*			
	MCA	DCA	2MCPA	22DCPA
<i>P. putida</i> PP3	0.45 (1.00)	0.63 (1.40)	0.15 (0.33)	0.08 (0.18)
<i>P. putida</i> PPW1	0.32 (1.00)	0.40 (1.28)	0.14 (0.44)	0.08 (0.26)
<i>P. putida</i> PPW2	0.66 (1.00)	0.08 (0.12)	0.28 (0.42)	0.14 (0.21)
<i>P. putida</i> PP411-006†	ND (1.00)	ND (0.17)	ND (0.49)	ND
Partially purified fraction I dehalogenase‡	0.62 (1.00)	0.07 (0.11)	0.40 (0.65)	0.19 (0.31)

ND, Not determined.

* Numbers in parentheses are dehalogenase substrate activity ratios with respect to MCA.

† Data from Weightman *et al.* (1982).

‡ Data from Weightman *et al.* (1979).

Transconjugants were selected at a frequency of 3.8×10^{-4} per donor cell. Of these transconjugants 95% co-inherited the ability to grow on 2MCPA as the sole carbon and energy source. As additional evidence, plasmid pUU2 was obtained from *P. putida* PPW2 and transformed into *P. aeruginosa* PAO1162 and transformants selected on the basis of drug resistance. Fifty of the transformants were tested and all grew on defined medium containing 2MCPA. No colonies grew on control plates on which recipient cells alone had been spread.

Restriction endonuclease digestion of plasmid pUU2 from P. putida PPW2

Digestion of plasmid pUU2 with restriction endonucleases *Pst*I and *Sma*I revealed that fragments were generated in the same positions as the equivalent digestions for the parent plasmid, with the exception of one fragment in both cases (Fig. 1). For the *Pst*I digestion the 11.7 kb fragment was missing whilst in the *Sma*I digestion the 20.0 kb fragment was not present in pUU2 (Fig. 1).

There were, however, a number of new DNA fragments in plasmid pUU2 which were not present in plasmid pUU1. Using the restriction endonuclease *Pst*I, six extra bands were formed with sizes of 12.0, 3.16, 3.15, 1.1, 0.86 and 0.58 kb; and for the *Sma*I digestion four new fragments were detected with sizes of 16.0, 8.7, 3.7 and 2.4 kb (Fig. 1). Thus, with these additional DNA fragments, plasmid pUU2 had a size of 70.8 to 76.3 kb, depending on which restriction endonuclease was used for the size determination. This represented an increase of 9.2 to 11.0 kb as a result of the formation of the R-prime. The inserted DNA carrying the fraction I dehalogenase gene therefore had an additional four *Pst*I sites and two *Sma*I sites.

DISCUSSION

The restriction endonuclease digest data for enzymes *Pst*I and *Sma*I were consistent with the previously published information (Riess *et al.*, 1980; Leemans *et al.*, 1980; Nayudu & Holloway, 1981; Willetts *et al.*, 1981) enabling the construction of a plasmid map showing the positions of the various restriction cleavage sites (Fig. 3). Plasmid R68.44 contained region I (Fig. 3) with two *Pst*I and one *Sma*I sites whereas plasmid pUU1 had lost this region; this was demonstrated by loss of two *Pst*I fragments totalling 2.0 kb and the equivalent loss of a single *Sma*I fragment of 2.0 kb size. The region lost has previously been associated with the enhanced chromosome-mobilizing capability of plasmid R68.44 and is probably the only difference with plasmid R68 which has no enhanced chromosome-mobilizing ability (Jacob *et al.*, 1977; Riess *et al.*, 1980;

The additional 9.2 to 11.0 kb of DNA inserted into plasmid pUU1 during the formation of the dehalogenase R-prime, plasmid pUU2, was inserted within the common *Pst*I and *Sma*I DNA contained between sites *d* (*Pst*I site) and *t* (*Sma*I site) (Fig. 3). This was determined on the basis of the loss of a single *Sma*I band (20.0 kb) which itself was a composite of the original R68.44 16.6 kb fragment (between sites *t* and *u*) and the added 3.4 kb to produce pUU1 which did not

have any further *Sma*I sites (Fig. 3). This was confirmed by the *Pst*I digest showing that the extra DNA of pUU2 was added into the fragment of 11.7 kb of pUU1 which had in turn been generated by the addition of segment II into the original *Pst*I fragment between sites *c* and *d* (Fig. 3). The exact location of the dehalogenase-encoding DNA cannot be determined with precision from the present information; however, the results do demonstrate that this must be between *Pst*I site *d* and *Sma*I site *t* (Fig. 3).

These results demonstrate the formation of an R-prime carrying the gene for a catabolic enzyme. Furthermore, it is clear that the mobilization of the fraction I dehalogenase gene was not linked topographically with the IS21 region implicated in the mobilization of other pseudomonad genes (Willetts *et al.*, 1981). Although we cannot pinpoint the time at which the 2.0 kb (IS21) segment was eliminated, its absence in pUU1 suggests that the R-prime formation proceeded without the involvement of the duplicate IS21 region. It is also clear that the site of integration of the dehalogenase gene is near the smaller segment of foreign DNA originally inserted during the formation of pUU1 (segment II, Fig. 3). It is possible that this region acts as a site for recombination with chromosomal DNA and hence a focal point for mobilizing chromosomal DNA. An analogous situation has been described for R68 and R68.45-like plasmids in *Escherichia coli* where the derivative plasmids contained 1.2 kb of *E. coli* K12 chromosomal DNA, possibly transposable elements, and were shown to have enhanced chromosome-mobilizing ability (Holloway, 1979). However, if this 3.4 to 5.7 kb region is associated with chromosome-mobilization in *P. putida* PPW1, it does not appear to be very efficient since the dehalogenase gene was transferred at very low frequencies (approximately 10^{-10}). In part this is due to the low rate of transfer of plasmids R68.44 and pUU1 between the various strains of *P. putida* and *P. aeruginosa* used in this study (approximately 10^{-6} to 10^{-7}). This contrasts with considerably higher rates of plasmid transfer demonstrated elsewhere. For example, Johnston *et al.* (1978) demonstrated frequencies of 10^{-2} for the transfer of R68.45 from *Rhizobium leguminosarum* to *Rhizobium meliloti* which was similar to the rates observed between different strains of *R. leguminosarum* (Beringer *et al.*, 1978). Thus, making the adjustment for poor plasmid transfer, the frequency of dehalogenase gene mobilization is about 10^{-6} which is comparable with the poorer mobilization frequencies recorded (Holloway, 1979).

Finally, we cannot exclude mechanisms of R-prime formation which have nothing to do with enhanced chromosome-mobilizing capabilities. It is possible, for example, that the plasmids act as suitable vectors for transposable elements which leave the chromosome independently of any plasmid-determined mechanism. Indeed, preliminary evidence suggests that the dehalogenase genes of *P. putida* PP3 are borne on transposable elements (J. H. Slater, A. J. Weightman and B. G. Hall, unpublished observations).

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