Chromosomal insertion of TOL transposons in *Pseudomonas aeruginosa* PAO

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Insertions of the TOL plasmid transposons Tn4651 and Tn4653 into the *Pseudomonas aeruginosa* PAO chromosome were isolated by a temperature selection technique. The locations and orientations of 16 insertions were determined by pulsed field gel electrophoresis and Southern hybridization with genomic and TOL DNA probes. All insertions occurred within a 334 kb region of the chromosome (representing less than 6% of the genome) with nine of the inserts clustered within a 10 kb area. Each transposon was able to insert in either orientation. An internal duplication of the 39 kb excisable region of pWW0 was seen in two independent insertions.

Introduction

The 115 kb TOL plasmid pWW0 from *Pseudomonas putida* mt-2 was the first *Pseudomonas* catabolic plasmid to be subjected to detailed physical analysis (Downing *et al.*, 1979; Downing & Broda, 1979) and remains one of the best characterized of all such plasmids, although the complex genetic and physical organization of the pWW0 molecule is not yet fully understood. Irreversible loss of the TOL phenotype from *P. putida* has been shown to occur by two mechanisms, either by loss of the entire plasmid or by deletion of a specific segment from the plasmid genome (Bayley *et al.*, 1977). Subsequent physical analysis has revealed that loss of this 39 kb segment occurs by homologous recombination between two 1.4 kb repeat sequences located at the boundaries of the region (Meulien *et al.*, 1981), and that all of the structural and regulatory genes of the toluene catabolic pathway are carried on the excised segment (Franklin *et al.*, 1981; Inouye *et al.*, 1981, 1983; Harayama *et al.*, 1984).

Transposability of part of the TOL plasmid was suggested by numerous independent reports of cointegrate formation between pWW0 and various R plasmids (including White & Dunn, 1977; Nakazawa *et al.*, 1978; Jacoby *et al.*, 1978; Chakrabarty *et al.*, 1978; Franklin & Williams, 1980), and the finding that TOL markers could persist in strains which carried only the deleted form of the plasmid or no detectable plasmid DNA (Cane & Williams, 1982; Jeenes & Williams, 1982; Jeenes *et al.*,

1982). Physical characterization of several cointegrate plasmids revealed that different amounts of TOL plasmid DNA were present in the cointegrate molecules (Lehrbach *et al.*, 1982), and that in all cases the region of TOL DNA incorporated extended beyond the boundaries of the 39 kb excisable segment. Whereas these findings appeared to contradict the hypothesis that a discrete transposable region of DNA was responsible for the observed movement of the TOL markers between different replicons, subsequent studies (Tsuda & Iino 1987, 1988) have shown the existence of at least two independent transposable elements on the pWW0 plasmid. A 56 kb transposon (Tn4651) is entirely contained within a larger 70 kb transposable element (Tn4653), these elements each having a unique, non-interchangeable transposase but sharing cointegrate resolution functions. The 39 kb excisable segment is encompassed by both transposons but appears not to encode any functions required for the transposition process, as derivatives lacking this internal region show unchanged transposition activity.

Southern hybridization and DNA sequence comparisons have shown a substantial degree of DNA sequence homology between the *xytXYZL* genes of the pWW0 plasmid and the isofunctional chromosomal *ben* genes of *P. aeruginosa* PAO and *P. putida* PPN (Holloway *et al.*, 1990) and *Acinetobacter calcoaceticus* (Harayama & Rekik, 1990), thus suggesting the likelihood of a common origin for these gene clusters. The chromosomal insertion of segments of the pWW0 plasmid in some *P. putida* strains has been inferred from physical evidence (Jeenes

Abbreviation: PFGE, pulsed field gel electrophoresis.

& Williams, 1982; Meulien & Broda, 1982) and in one case confirmed by genetic mapping studies (Sinclair *et al.*, 1986).

The plasmid pWW0 is temperature-sensitive for replication in *P. aeruginosa* strain PAO (Nakazawa, 1978). In this paper we report a simple temperature selection protocol which allows the isolation of insertions of pWW0 plasmid DNA into the chromosome of the genetically well-characterized *P. aeruginosa* strain PAO. These insertions were physically mapped by pulsed field gel electrophoresis. A marked regional specificity of insertion of the TOL plasmid DNA into the *P. aeruginosa* PAO chromosome was observed.

A preliminary account of this work was presented at the 6th International Symposium on the Genetics of Industrial Micro-organisms, Strasbourg, August 1990.

Methods

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are described in Table 1.

Media and cultural conditions. Nutrient yeast broth (NYB) and nutrient agar (NA) have been described elsewhere (Stanisich &

Holloway 1972). KNO₃ (0.4%, w/v) was added to NYB to provide an alternative electron acceptor in the absence of aeration. Minimal medium (MM) was as described by Ornston *et al.* (1969), *m*-xylene was supplied in vapour form by taping a cotton plugged vial containing the hydrocarbon to the lid of a Petri dish and *m*-toluate was used at a concentration of 5 mM.

Isolation of putative TOL insert strains. The TOL plasmid pWW0 was introduced into PAO110, PAO313, PAO688 and PAO899 by conjugal transfer of the plasmid from *P. putida* PaW1, with selection for growth on *m*-toluate as sole carbon source. The donor strain was contraselected with rifampicin or streptomycin, and the recipient strains were pregrown at 43 °C to induce a restrictionless phenotype (Holloway, 1965). The PAO(pWW0) transconjugant strains were purified twice on the same selective medium prior to use in subsequent experiments. All transconjugant strains showed the expected phenotype with respect to growth on TOL plasmid substrates and parental strain growth factor requirements. Four single colonies of each PAO(pWW0) strain were inoculated into NYB and grown overnight at 37 °C with shaking. These cultures were diluted 1 in 2000 into pre-warmed NYB supplemented with 0.4% KNO₃ and incubated overnight at 43 °C without shaking. The 43 °C subculture procedure was repeated once, then serial 10-fold dilutions of the final cultures were plated on MM with *m*-toluate or *m*-xylene as the sole carbon source and incubated at 37 °C for 48 h. Viable counts were plated on NA at 37 °C and incubated for 24 h.

Colonies from the *m*-toluate and *m*-xylene selections were patched onto selective master plates of the same medium and incubated

Table 1. *Bacterial strains and plasmids*

Abbreviations used as in Holloway & Zhang (1990), except for *cml* (chloramphenicol resistance) and *znn* (transposon insertion).

Strain	Genotype/characteristics	Reference/comments
<i>P. putida</i> mt-2 PaW1	Prototroph, pWW0	Murray <i>et al.</i> (1972)
<i>P. aeruginosa</i> PAO110	<i>leu-38 str-60</i>	Carrigan <i>et al.</i> (1978)
PAO313	<i>argB18 str-20 cml-2</i>	B. W. Holloway collection
PAO688	<i>pur-67 rif-93</i>	Moore <i>et al.</i> (1983)
PAO899	<i>met-28 str-1</i>	Moore <i>et al.</i> (1983)
PAO1293	Prototroph	Ratnaningsih <i>et al.</i> (1990)
PAO1416	<i>met-28 str-1 znn-54::Tn4651</i>	This study, TOL insertion in <i>SpeI</i> fragment 4
PAO1417	<i>met-28 str-1 znn-55::Tn4651</i>	This study, TOL insertion in <i>SpeI</i> fragment 24
PAO1418	<i>met-28 str-1 znn-56::Tn4651</i>	This study, TOL insertion in <i>SpeI</i> fragment 4
PAO1439	<i>met-28 str-1 znn-57::Tn4651</i>	This study, TOL insertion in <i>SpeI</i> fragment 10
PAO1444	<i>met-28 str-1 znn-58::Tn4651</i>	This study, TOL insertion in <i>SpeI</i> fragment 4
PAO1536	<i>leu-38 str-60 znn-59::Tn4651</i>	This study, TOL insertion in <i>SpeI</i> fragment 24
PAO1537	<i>leu-38 str-60 znn-60::Tn4651</i>	This study, TOL insertion in <i>SpeI</i> fragment 24
PAO1538	<i>leu-38 str-60 znn-61::Tn4653</i>	This study, TOL insertion in <i>SpeI</i> fragment 4
PAO1539	<i>argB18 str-20 cml-2 znn-62::Tn4651</i>	This study, TOL insertion in <i>SpeI</i> fragment 4
PAO1540	<i>argB18 str-20 cml-2 znn-63::Tn4651</i>	This study, TOL insertion in <i>SpeI</i> fragment 24
PAO1541	<i>argB18 str-20 cml-2 znn-64::Tn4651</i>	This study, TOL insertion in <i>SpeI</i> fragment 24
PAO1542	<i>argB18 str-20 cml-2 znn-65::Tn4653</i>	This study, TOL insertion in <i>SpeI</i> fragment 4
PAO1543	<i>pur-67 rif-93 znn-66::Tn4651</i>	This study, TOL insertion in <i>SpeI</i> fragment 24
PAO1544	<i>pur-67 rif-93 znn-67::Tn4651</i>	This study, TOL insertion in <i>SpeI</i> fragment 4
PAO1545	<i>pur-67 rif-93 znn-68::Tn4651</i>	This study, TOL insertion in <i>SpeI</i> fragment 24
PAO1546	<i>pur-67 rif-93 znn-69::Tn4651</i>	This study, TOL insertion in <i>SpeI</i> fragment 4
Plasmid		
pMO2048	Clone carrying the <i>SpeI</i> 4–24 junction	B. W. Holloway collection
pMO2057	Clone carrying the <i>SpeI</i> 6–21 junction plus a repeat sequence which hybridizes to <i>SpeI</i> fragment 4 near the 4–12 junction.	
pMO1977	Cosmid carrying the <i>SpeI</i> 10–17 junction	
pMO010921	Cosmid carrying the <i>SpeI</i> 10–24 junction	

overnight at 37 °C. After incubation, the master plates were replica plated onto MM + *m*-toluate plates pre-spread with strain PAO1293 as a test for conjugal transfer of the TOL markers. Parental PAO(pWW0) strains were included as positive controls. Putative TOL inserts were identified by their inability to transfer the TOL markers to PAO1293. These strains were purified to single colonies on MM + *m*-xylene then retested for transfer ability and thermostability of the TOL markers during passage at 43 °C on NA. One putative TOL insertion strain was retained from each single colony broth culture.

DNA isolation, Southern blotting and hybridization. Large scale plasmid DNA isolations were done as described by Nayudu & Holloway (1981), and alkaline miniprep plasmid DNA extractions by the method of Maniatis *et al.* (1982). Genomic DNA isolations were performed as described by Willetts *et al.* (1981). Restriction enzyme digestions were performed according to the manufacturers' instructions. Southern blotting (Smith & Summers, 1980), hybridization to nylon membranes (Amersham Hybond-N) and autoradiography were done using standard procedures.

Pulsed field gel electrophoresis (PFGE). The techniques used were based on those of Smith *et al.* (1986) with minor modifications as described by Ratnaningsih *et al.* (1990). Electrophoresis was carried out using an LKB/Pharmacia Pulsaphor (OFAGE) apparatus with hexagonal electrode array. The gel pictured in Fig. 3 was run in 0.2 × TBE buffer, 1.2% (w/v) agarose at 170 V with a pulse regime of 60 s for 12 h, 40 s for 12 h, 20 s for 24 h and 10 s for 17 h.

Results

Initial characterization of TOL insert strains

The viable counts of PAO(pWW0) cultures subjected to 43 °C passage were in the range of $1-5 \times 10^8$ c.f.u. ml⁻¹, and colonies were obtained on *m*-toluate and *m*-xylene MM plates at frequencies of 2×10^{-5} – 1×10^{-3} per viable cell. It was found that 96% of colonies selected on either *m*-toluate or *m*-xylene MM were unable to transfer the TOL markers to the PAO1293 recipient, and that the TOL markers in such colonies had become thermostable. In contrast, the remaining 4% of surviving TOL⁺ colonies and the parental plasmid-containing strains (not subjected to 43 °C passage) were transfer proficient for the TOL markers and rapidly lost these markers at 43 °C. Plasmid DNA could not be detected by miniprep extractions in the putative TOL insert strains but could be readily demonstrated in the parental PAO(pWW0) strains.

Fifteen strains carrying putative TOL insertions were isolated by this method, three from PAO110(pWW0) and four from each of PAO313(pWW0), PAO688(pWW0) and PAO899(pWW0). In addition, one spontaneous insert strain (PAO1439) was fortuitously isolated from a frozen stock culture of PAO899(pWW0) which had not been subjected to any deliberate selection procedure (see Table 1).

Genomic DNA samples from the putative TOL insert strains were digested with *Hind*III, separated by horizontal agarose gel electrophoresis then transferred to nylon

Fig. 1. Physical map of plasmid pWW0 showing cleavage sites for *Hind*III and *Xho*I and approximate locations of catabolic genes (from Franklin *et al.*, 1981). The location of the *Spe*I site is indicated by an arrowhead and the extents of the 39 kb excisable segment (dotted line), and the transposons Tn4651 (56 kb, solid line) and Tn4653 (70 kb, dashed line) are indicated by the arcs outside the map (Tsuda & Iino, 1987, 1988).

membranes and probed with nick-translated pWW0 DNA. The hybridization patterns (data not shown) revealed that fourteen of the sixteen strains carried *Hind*III fragments consistent with the presence of the 56 kb transposon Tn4651 (*Hind*III fragments A, D, F, H, L, M, N, O, P and Q present, fragments B, C, E, G, I, J and K absent, see Fig. 1). The remaining two strains, PAO1538 and PAO1542, appeared to carry the 70 kb transposon Tn4653 (*Hind*III fragments A, D, E, F, H, I, K, L, M, N, O, P and Q present, fragments B, C, G and J absent). The smaller *Hind*III fragments (R and S) were not detectable on these gels.

PFGE analysis

A physical and genetic map of the *P. aeruginosa* PAO chromosome has been constructed using the enzyme *Spe*I (Römling *et al.* 1989; Ratnaningsih *et al.*, 1990). Restriction enzyme digestion of the plasmid pWW0 revealed only one *Spe*I site. This site was located to the centre of the *Hind*III D fragment by *Hind*III/*Spe*I double digestion, but no alteration to *Xho*I fragments could be detected in *Xho*I/*Spe*I double digests. Therefore, the *Spe*I site on pWW0 must be located very close to the *Xho*I site which separates the *Xho*I F and *Xho*I G fragments (see Fig. 1). This *Spe*I site is located within the 39 kb

Fig. 2. Diagram of the *SpeI* restriction digest patterns of the reference strain PAO1293 and the 16 TOL insertion strains. Numbers immediately to the left of the PAO1293 column designate the *SpeI* fragments in descending size order; numbers at the extreme left indicate the fragment sizes in kb; fragments below number 34 are not shown. Fragment sizes below 60 kb have been amended from the values reported by Ratnaningsih *et al.* (1990) following improvements in PFGE techniques which allowed better resolution and sizing of small fragments. New fragments generated by TOL insertion are indicated by an arrowhead to the left of the fragment. Strains have been grouped according to the affected *SpeI* fragment: PAO1416, PAO1418, PAO1444, PAO1538, PAO1539, PAO1542, PAO1546 and PAO1544 have inserts in *SpeI* fragment 4; PAO1439 has an insert in *SpeI* fragment 10; PAO1417, PAO1536, PAO1537, PAO1540, PAO1541, PAO1543 and PAO1545 have inserts in *SpeI* fragment 24. The smallest TOL-generated fragments in tracks 2, 3, 6 and 16 were not visible on gels but were detected by hybridization.

excisable segment of pWW0 and thus will be present in strains carrying chromosomal insertions of either the 56 kb (Tn4651) or the 70 kb (Tn4653) regions of the TOL plasmid.

Agarose blocks containing genomic DNA from each of the putative TOL insert strains, the parental PAO strains and the reference strain PAO1293, were digested with *SpeI* and run on pulsed field gels with varying pulse time regimes so that 34 of the 37 *SpeI* fragments of strain PAO could be visualized. As expected, the parental strains PAO110, PAO313, PAO688 and PAO899 showed a *SpeI* fragment pattern identical to that of PAO1293 (data not shown). All of the putative TOL insert strains showed alterations in their *SpeI* fragment pattern relative to PAO1293 and these data are displayed in Fig. 2. In seven strains it could be seen that the doublet band of *SpeI* fragments 4 and 5 had been replaced by a single band and that two or three additional bands were present (PAO1416, PAO1418, PAO1444, PAO1538, PAO1539, PAO1542 and PAO1546). In one strain the doublet band of *SpeI* fragments 10 and 11 had been

replaced by a single band and two new fragments were also present (PAO1439). Seven strains were lacking *SpeI* fragment 24 and had acquired two new bands (PAO1417, PAO1536, PAO1537, PAO1540, PAO1541, PAO1543 and PAO1545). In the remaining strain, one new *SpeI* fragment could be seen but none of the existing *SpeI* fragments appeared to have been lost (PAO1544).

Southern hybridization analysis of SpeI fragments

The DNA fragments from pulsed field gels were transferred to nylon membranes then hybridized with various radioactively labelled probes to confirm the identity of the chromosomal bands affected by the TOL DNA insertion. Probes included cosmids from a PAO genomic bank known to complement specific genetic markers and cloned genomic fragments carrying *SpeI* recognition sites (junction clones). The hybridization patterns of these probes to PAO1293 *SpeI* fragments had been previously determined (Ratnaningsih *et al.*, 1990).

Fig. 3. Southern hybridization of *Spe*I fragments with pWW0 fragment probes. (a) Pulsed field gel showing large *Spe*I fragments of PAO1293 (track 1), PAO1416 (track 2), PAO1418 (track 3), PAO1444 (track 4), PAO1538 (track 5), PAO1539 (track 6), PAO1542 (track 7), PAO1544 (track 8), PAO1546 (track 9) and PAO1439 (track 10). Tracks 0 and 11 contain bacteriophage λ DNA concatamers as size standards (partially degraded). *Spe*I fragment numbers are shown to the left of panel (a). Fragments hybridizing to the *Xho*I C fragment of pWW0 (see b) are marked with a white cross to the left of the track. Fragments hybridizing to the *Xho*I H fragment of pWW0 (see c) are marked with a white circle to the left of the track. (b) Southern hybridization of a filter prepared from the gel in (a), and probed with the *Xho*I C fragment of pWW0. Note there is no hybridization to tracks 4, 7 and 9 as *Spe*I fragments smaller than number 21 are not present on this filter. (c) As for (b) except the probe was the *Xho*I H fragment of pWW0. Note there is no hybridization in tracks 2, 3 and 6 as *Spe*I fragments smaller than number 21 are not present on this filter.

Table 2. Southern hybridization analysis of *SpeI* fragments generated by TOL transposon insertion

+, Hybridization detected; -, no hybridization detected; ND, not determined. This table summarizes hybridization results only for those fragments affected by Tn4651 or Tn4653 insertion and does not include other unaffected fragments which also hybridized, e.g. for the pMO2048 probe (4-24 junction) strains with an insertion in fragment 4 showed an unaltered fragment 24 on the autoradiograph, and, conversely, strains with an insertion in fragment 24 showed an unchanged fragment 4.

Strain	Affected <i>SpeI</i> fragment	Sizes of new <i>SpeI</i> fragments (kb)	Size gain (kb)	Hybridization with probes:					
				pMO 2048	pMO 2057	pMO 010921	pMO 1977	pWW0 <i>XhoI</i> C	pWW0 <i>XhoI</i> H
PAO1416	4 (360 kb)	415	101	-	+	ND	ND	+	-
		34		-	-			+	-
		12		+	-			-	+
PAO1418	4	415	101	-	+	ND	ND	+	-
		34		-	-			+	-
		12		+	-			-	+
PAO1444	4	355	58	-	+	ND	ND	-	+
		63		+	-			+	-
PAO1538	4	270	85	-	+	ND	ND	+	-
		175		+	-			-	+
PAO1539	4	415	67	-	+	ND	ND	+	-
		12		+	-			-	+
PAO1542	4	380	77	-	+	ND	ND	-	+
		57		+	-			+	-
PAO1544	4	360	51	-	+	ND	ND	-	+
		51		+	-			+	-
PAO1546	4	300	60	-	+	ND	ND	-	+
		120		+	-			+	-
PAO1417	24 (60 kb)	65	53	-	ND	+	ND	-	+
		48		+		-		+	-
PAO1536	24	62	52	-	ND	+	ND	-	+
		50		+		-		+	-
PAO1537	24	62	52	-	ND	+	ND	-	+
		50		+		-		+	-
PAO1540	24	69	54	+	ND	-	ND	+	-
		45		-		+		-	+
PAO1541	24	65	54	-	ND	+	ND	-	+
		45		+		-		+	-
PAO1543	24	100	53	+	ND	-	ND	+	-
		13		-		+		-	+
PAO1545	24	69	54	+	ND	-	ND	+	-
		45		-		+		-	+
PAO1439	10 (245 kb)	165	45	ND	ND	+	-	+	-
		125				-	+	-	+

The nylon membranes were also probed with pWW0 plasmid DNA and individual pWW0 restriction fragments to identify those bands containing TOL DNA and to determine the orientation of the TOL insertions in the different strains. The result of one such hybridization experiment is presented in Fig. 3 and a summary of all relevant data is shown in Table 2. It can be seen that the estimated size gain in strains with a Tn4651 insertion ranges from 45 kb in PAO1439 to 67 kb in PAO1539. PAO1416 and PAO1418 have an additional TOL duplication, giving a larger insert than the expected increase of 56 kb. Strains carrying Tn4653 show size increases of 77 and 85 kb in contrast to the expected 70 kb increase. We believe that these discrepancies are likely to reflect

the inaccuracy of fragment size measurements on pulsed field gels, although at this stage we cannot rule out the possibility of deletion or duplication of genomic DNA due to transposon insertion.

Analysis of the data from hybridizations with genomic probes showed that one strain had a TOL DNA insertion in *SpeI* fragment 10, seven strains had an insertion in *SpeI* fragment 24 and eight strains had an insertion in *SpeI* fragment 4. In PAO1544 one of the new fragments produced by Tn4651 insertion was similar in size to the original *SpeI* fragment 4; thus, the location of the insertion was not apparent by examination of the *SpeI* fragment pattern, but was evident from the hybridization results. *SpeI* fragments 4 (360 kb), 10 (245 kb) and 24

Fig. 4. Physical map of the PAO chromosome in the region of the TOL transposon insertions. Tn4651 inserts are represented by filled triangles. Tn4653 inserts by open triangles. Inserts in the 'left hand' orientation (arbitrarily defined as having the *XhoI* H fragment of pWW0 at the left end of the insertion as drawn in the figure) are positioned above the line, inserts in the 'right hand' orientation below the line. *SpeI* fragment 24 has been expanded in the upper part of the figure for clarity. Locations of the genetic markers *ben* (benzoate utilization) and *gcu* (glycine utilization) have been determined by physical mapping; the *cys-54* locus is known to be located on *SpeI* fragment 12 but has not been physically mapped.

(60 kb) are contiguous in the 50 minute region of the PAO chromosome with 24 being flanked by 4 and 10. As expected, the pWW0 plasmid probe hybridized to all of the new *SpeI* fragments generated by TOL insertion but not to any other fragments (data not shown). The pWW0 fragment probes each hybridized only to one of the two new bands in each insert strain (except for PAOI416 and PAOI418; see below), and these data in combination with the results of the genomic probes allowed the orientation of each insertion to be determined (see Fig. 4). The estimated distance from each insertion to the nearest chromosomal *SpeI* site was calculated by subtracting the amount of TOL DNA present on the fragment from the total fragment size. Using restriction mapping data (M. Tsuda, personal communication) and assuming the *SpeI* site on the TOL DNA to be located at the boundary of the *XhoI* F and G fragments, Tn4651 should produce segments of 8 and 48 kb upon *SpeI* digestion, and Tn4653 will yield segments of 18 and 52 kb. In both cases the smaller end of the transposon is the 'left hand' end as defined in Fig. 4. All seven insertions in *SpeI* fragment 24 had the same orientation thus allowing an unequivocal order to be determined. The insert closest to the 4–24 junction (PAOI541) has been placed at 0 kb from this *SpeI* site (the distance calculated from Table 2 was –3 kb due to the error factor in size measurements) and other insertions have been placed relative to this. Two pairs of strains appeared to have identically located insertions (PAOI536 and PAOI537 at 5 kb, PAOI540 and PAOI545 at 24 kb from the 4–24 junction) while the remaining two strains had different insertion sites

(PAOI417 at 3 kb from the 4–24 junction, PAOI543 at 5 kb from the 10–24 junction). For *SpeI* fragment 4, the presence of insertions in both orientations close to the 4–24 junction makes the determination of their relative order difficult. The two insertions in 'right hand' orientation (PAOI544 at 3 kb and PAOI542 at 5 kb) can be placed relative to each other but their position with respect to the three 'left hand' inserts (PAOI416, PAOI418, and PAOI539, all at 4 kb) is unconfirmed. The three other inserts in fragment 4 were widely separated and could be ordered without ambiguity (PAOI444 at 15 kb, PAOI546 at 72 kb and PAOI538 at 157 kb from the 4–24 junction). The insert in *SpeI* fragment 10 (PAOI439) was placed near the centre of that fragment at 117 kb from the 10–24 junction.

Thus all 16 TOL insertions were confined to an approximately 334 kb region of the PAO chromosome and a further localized clustering of 9 insertions was observed within an estimated 10 kb area at the junction of *SpeI* fragments 4 and 24. Since the size gain estimates vary from the expected value for each transposon these map positions are subject to some degree of error; nevertheless, this small region is likely to be no greater than 20 kb in size.

Analysis of the 'extra' *SpeI* fragment in PAOI416 and PAOI418

Two strains with TOL inserts in *SpeI* fragment 4, PAOI416 and PAOI418, carried three new *SpeI* fragments (estimated at 415, 34 and 12 kb from PFGE

analysis) instead of the expected two fragments. The largest and smallest of these fragments hybridized with probes specific for the 'right hand' end of fragment 4 and the 4–24 junction respectively, thus indicating that they were derived from the genomic *SpeI* fragment 4. The 34 kb fragment hybridized with the pWW0 *XhoI* C probe but not with *XhoI* H or either of the genomic probes. In order to determine the origin of these 'extra' bands, the 34 kb fragments were isolated from a pulsed field gel, radioactively labelled then probed to *HindIII* and *XhoI* digests of pWW0. In both strains the 'extra' *SpeI* fragment hybridized only to pWW0 fragments from the 39 kb excisable region of the plasmid and did not hybridize to other regions of pWW0 (data not shown). Therefore, it appears likely that this 'extra' fragment in PAO1416 and PAO1418 has arisen by tandem duplication of the 39 kb segment of pWW0 within a 56 kb (Tn4651) insertion. The additional internal *SpeI* fragment generated in this manner would be 39 kb in size, which is in good agreement with the 34 kb size estimate obtained by PFGE. From fragment size estimates on PFGE gels these two strains and PAO1539 appear to have identically located insertions although the latter strain has not undergone the internal duplication.

Discussion

The development of PFGE and related methods for the separation of large DNA molecules has made possible the physical mapping of prokaryote and smaller eukaryote genomes (reviewed by Smith & Condemine, 1990). In this study the PFGE technique has been used to map Tn4651 and Tn4653 insertions in the *P. aeruginosa* PAO chromosome and more accurate locations were obtained than could have been estimated by conventional genetic mapping techniques. The mapping analysis was greatly facilitated by the fortuitous presence of a *SpeI* site within these transposable elements and the asymmetric location of the site allowed a reasonably accurate estimation of the physical distance from each insertion to neighbouring *SpeI* sites in the chromosome. Tn4651 and Tn4653 insertions were isolated in both orientations, with the former element being the majority class. Among the 16 inserts studied 12 different insertion sites could be distinguished, and all insertions fell within an estimated 334 kb region of the PAO chromosome, representing less than 6% of the 5868 kb genome of this organism. A further clustering of nine insertions within a small sub-region (estimated at 20 kb or less) at the junction of *SpeI* fragments 4 and 24 was evident from the mapping experiments. This region is within one of the regions of the PAO chromosome that is rich in catabolic markers, but is about 370 kb distant from the *ben* genes

which share DNA homology with the *xyI*/*XYZL* genes of the TOL plasmid.

The observation of a possible internal duplication of the 39 kb excisable region of the pWW0 plasmid in two strains was unexpected and such duplications have not been previously reported in the literature. Such a structure may have arisen by homologous recombination between an intact copy of Tn4651 and a 39 kb circular molecule generated by excision of the central region from another copy of the transposon. This event may have occurred before or after chromosomal insertion of the transposon. A similar mechanism has been proposed to explain the tandem amplification of segments of R factor DNA in *Streptococcus* and *Proteus* species (Clewett *et al.*, 1978). Further physical analysis of the 'extra' *SpeI* fragment in these strains would be necessary to confirm whether this model for their origin is correct.

P. A. Williams and co-workers have proposed that the present structures of pWW0 and other toluene degradative plasmids may have evolved via the separate acquisition of three different 'modules' of DNA comprising the upper and lower pathway genes, and the two regulatory genes *xyI*/*R* and *xyI*/*S* (Shaw & Williams, 1988). Harayama & Rekik (1990) have suggested the likelihood that the lower pathway operon itself has arisen by fusion of two formerly separate entities comprising the *xyI*/*XYZL* genes which encode enzymes for the conversion of toluates to catechols, and the genes for the remainder of the pathway which encode the further catabolism of catechols. The observed DNA sequence homology between the *xyI*/*XYZL* gene cluster of pWW0 and the chromosomal *ben* genes in *Acinetobacter calcoaceticus* (Harayama & Rekik, 1990), *P. aeruginosa* and *P. putida* (Holloway *et al.*, 1990) is evidence that the *xyI*/*XYZL* DNA module has spread horizontally among bacterial genera.

Our observations on the non-random nature of TOL insertions into the PAO chromosome indicate some degree of specificity in the interactions of the plasmid and bacterial genomes. It has been proposed that contemporary *P. aeruginosa* and *P. putida* chromosomes have evolved by sequential non-random acquisition of genes by a smaller ancestral chromosome which originally encoded only essential 'housekeeping' functions (Holloway & Morgan, 1986), and that the present day *ben* genes of *Pseudomonas* species may be a remnant of a catabolic plasmid insertion. Further analysis of the chromosomal 'hot region' for TOL transposon insertion may reveal characteristics of the genomic DNA which cause this region to be the preferred target of these transposable elements and aid in our understanding of plasmid–chromosome interactions and their possible role in chromosomal evolution.

This study has supported the conclusions of Smith &

Kolodner (1988) on the utility of PFGE techniques in combination with Southern hybridization for the rapid and precise mapping of chromosomal insertions, provided that a suitable range of genomic probes is available and a fairly extensive physical/genetic map has already been constructed for the organism in question.

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