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Genetic organization of a plasmid from an industrial wastewater bioreactor

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Abstract *Pseudomonas* strain CT14 was isolated from activated sludge. Strain CT14 contained a 55, 216 bp plasmid that was characterized by sequence analysis. The plasmid had a modular structure with 51 open reading frames (ORFs) that were distributed between two clearly demarcated domains. Domain I primarily contained genes for plasmid-related functions and a novel origin of replication. Domain II bore evidence of extensive transposition and recombination. Domain II contained several genes from a *meta*-cleavage pathway for aromatic rings. These genes appeared to have been recruited from different hosts. This observation suggests that sequencing pCT14 may have revealed an intermediate stage in the evolution of a new assemblage of *meta*-cleavage pathway genes.

Introduction

The degradation genes for many xenobiotic organic compounds have been found on catabolic plasmids and other mobile genetic elements (Nojiri et al. 2004). Several large catabolic plasmids have now been sequenced (e.g., Chattoraj 2000; Greated et al. 2002; Igloi and Brandsch 2003; Li et al. 2004; Maeda et al. 2003; Romine et al. 1999; Vedler et al. 2004). Although these plasmids are diverse and encode a variety of different degradation pathways, all of these plasmids have a modular organization (Thomas 2000). Each module is a cluster of genes encoding related functions, e.g., genes for replication and stable segregation form a distinct cluster, and genes for conjugation form a different distinct cluster. The modular organization of the Tol pathway *xyl* genes on plasmid pWW0 is typical for plasmid-associated degradation genes (Greated et al. 2002). The *xyl* genes are organized into two operons. The upper pathway operon encodes enzymes for oxidation of toluene to benzoate. The lower pathway operon encodes enzymes that cleave the aromatic ring and produce intermediates of the TCA cycle. It is most likely that clustering of functionally related genes has evolved to minimize the probability that recombination will disrupt a set of genes that are useful only in the presence of each other (Thomas 2000).

The catabolic plasmids that have been sequenced are generally from bacteria that were originally isolated from soil. However, because the selective conditions in wastewater bioreactors are different than in soil and other natural environments, the microbial diversity in wastewater bioreactors provides a framework in which new biochemical pathways can evolve (Bramucci and Nagarajan 2000). One factor influencing natural selection in industrial wastewater bioreactors, particularly those that recycle biomass as part of the activated sludge process, is higher concentrations of metabolically active bacteria than are found in many natural environments (Bitton 1994). Accordingly, the unique growth conditions in wastewater bioreactors should facilitate formation of novel biochemical pathways through horizontal gene transfer and recruitment of different genes from diverse hosts into a single host. Indeed, bacteria with novel genes and biochemical pathways have been isolated from industrial wastewater bioreactors (Bramucci et al. 2002a,b; Cheng et al. 2000; Kostichka et al. 2001).

In this report, we describe the sequence of a plasmid from a *Pseudomonas* strain isolated from activated sludge. The plasmid had a modular structure and a novel origin of replication. One region of the plasmid bore evidence of extensive transposition and recombination. This same region contained an incomplete *meta*-cleavage operon for aromatic rings. Inasmuch as the *meta*-cleavage operon had genes that were apparently derived from different sources, it is possible that the plasmid represents an intermediate stage in the formation of a new assemblage of *meta*-cleavage pathway genes.

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Materials and methods

Isolation of strain CT14

Liquid S12 medium, S12 agar, Luria–Bertani (LB) medium, and LB agar were used for culturing bacteria (Bramucci et al. 2002a; Sambrook et al. 1989). S12 liquid medium was supplemented with toluene by adding the toluene directly to the culture medium. S12 agar plates were supplemented with toluene by placing 20 μl of toluene on the interior of each petri dish lid. The petri dishes were sealed with parafilm and incubated upside down at 25°C.

Activated sludge from an industrial wastewater bioreactor was inoculated into 10 ml of S12 medium with 100 mg/l toluene in a 125-ml screw-cap Erlenmeyer flask. The enrichment culture was incubated at 25°C with reciprocal shaking. The enrichment culture was maintained by adding 100 mg/l of toluene every 2–3 days. The culture was diluted 1:10 every 10 days. Bacteria that utilize toluene as a sole source of carbon and energy were isolated at 25°C by spreading samples of the enrichment culture onto S12 agar supplemented with toluene. Representative bacterial colonies were tested for the ability to use toluene as a sole source of carbon and energy on S12 agar.

The 16S rRNA gene sequence of strain CT14 was amplified by the polymerase chain reaction with primers HK12 and HK13 and sequenced essentially as previously described (Bramucci et al. 2002a). The 16S rRNA gene sequence was used as the query sequence for a BLAST search of GenBank for similar sequences (Altschul et al. 1997). Isolate CT14 was deposited with the American Type Culture Collection (Manassas, VA, USA) and assigned accession number PTA-4995.

Isolation of plasmid DNA

Strain CT14 was inoculated into 100 ml of LB medium and incubated for 17 h at 37°C with reciprocal shaking. The cells were collected by centrifugation (10 min, 2,300×*g*, 4°C), resuspended in 8 ml of Solution 1 (50 mM Tris–HCl, pH 7.5, 10 mM EDTA, 100 ug/ml RNase A), and lysed by adding 8 ml of Solution 2 (0.2 M NaOH, 1% SDS). The cleared lysate was neutralized by adding 8 ml of Solution 3 (1.32 M potassium acetate, pH 4.8) and gently inverting until a white precipitate formed. The supernatant was collected by centrifugation (30 min, 13,000×*g*, 4°C) and transferred to new tubes. The DNA was precipitated by adding 4M NaCl (final concentration 0.4 M) and 2.5 vol of 100% ethanol and placing the tubes at −20°C for 17 h. The precipitated DNA was recovered by centrifugation (45 min, 13,000×*g*, 4°C). The white pellet was washed with cold 70% ethanol, dried by rotary evaporation, dissolved in 250 μl of deionized water, and stored at −20°C.

Plasmid DNA was purified by field inversion gel electrophoresis (FIGE) using a Hoeffer Scientific Instruments PS500XT DC power supply and PC500 SwitchBack Pulse Controller. Total genomic DNA was electrophoresed through 1.0% low melting temperature agarose (SeaPlaque) in 0.5× TBE buffer at 6 to 9 V/cm with a 10-min run-in, 50–80 s pulse time, and 3:1 For/Rev switch time for 24 to 28 h (Sambrook et al. 1989). The eletrophoretic gels were stained in 0.5× TBE buffer containing ethidium bromide (1 ug/ml). The DNA bands that corresponded to pCT14 were excised and placed into microfuge tubes. The gel slices were melted at 70°C for 5 min and extracted with an equal volume of tris-saturated phenol. The samples were centrifuged in a microfuge at the highest speed for 10 min The aqueous layer was transferred to a new microfuge tube and extracted twice with 1:1 chloroform/phenol (EM Science) and 24:1 chloroform/isoamyl alcohol (EM Science). After the final extraction, the aqueous layer was adjusted to 0.4M NaCl final concentration. The DNA was precipitated with 2 vol of ethanol at −70°C overnight. The precipitated DNA was centrifuged in a microfuge at the highest speed for 15 min, and the pellet was washed with 70% ethanol. The pellet was dried and resuspended in 50 μl of sterile deionized water.

Sequencing of pCT14

Plasmid DNA was diluted to a final volume of 1.5 ml and placed in a modified Aeromist nebulizer. The modifications consisted of disassembling the nebulizer and removing the outer lip of the inner cone and then replacing the cone upside-down within the nebulizer. The nebulizer was then reassembled. The plasmid DNA was transferred to the nebulizer. Using the included tubing, the nebulizer was attached to a filtered compressed air line with a pressure regulator set for 18 psi. Air was blown through the nebulizer for 30 s while tapping the nebulizer on the tabletop to dislodge any droplets attached to the sides of the nebulizer. Five hundred microliters of the DNA sample was removed, and compressed air was reapplied for an additional 15 s. Another 500 μl of the sample was removed, and compressed air was reapplied for another 15 s. The three samples were then recombined and run on a 1% 1X TEA low melting temperature gel. A section of the gel corresponding to 2 to 4 kb based was excised from the gel and purified using a commercial kit (Qiagen catalog #). The fragments were polished with T4 DNA polymerase ligated into SmaI digested pUC18 (Ready-to-Go, Pharmacia-Biotech) at 22°C for 16 h. The ligated DNA was transformed into DH5-α Maximum Efficiency Competent cells (Gibco-BRL). Cells were plated on Q-plates containing LB+ Ampicillin (50 μg/ml)+X-gal. Plates were placed at 37°C overnight. White colonies containing inserts were picked using a Q-bot colony picker and inoculated into 96 or 384 well microtiter plates for high-throughput sequencing.

Plasmid sequence assembly

Sequence data obtained from the high-throughput sequencing facility were assembled using Sequencher (GeneCodes) and Phred/Phrap. Assembled data were manually edited and annotated. All potential open reading frames (ORFs)

were compared against the GenBank database using BLASTX and BLASTP (Altschul et al. 1997). The complete pCT14 nucleotide sequence was deposited in GenBank (Accession number DQ126685).

Results

Isolation and characterization of strain CT14

Strain CT14 was isolated from a toluene enrichment culture. This isolate utilized toluene, *m*-xylene, and the corresponding intermediates of the Tol pathway as sole sources of carbon and energy (data not shown). Microscopic examination revealed that strain CT14 was a Gram-negative rod. The DNA encoding 16S rRNA in strain CT14 was amplified by PCR, sequenced, and compared to 16S rRNA gene sequences contained in GenBank. The CT14 16S rRNA gene sequence had 100% similarity to *Pseudomonas veronii* (GenBank Accession number AB056120).

An agarose gel electrophoresis procedure that involved in situ lysis of bacteria was used to initially test strain CT14 for plasmid DNA (Eckhardt 1978). A single plasmid was detected (data not shown). The plasmid was sequenced, and the BlastX algorithm was used to assign putative functions to ORFs by comparison to sequences in GenBank.

pCT14

Plasmid pCT14 was 55,216 bp in size and had at least 51 ORFs that were divided between two clearly demarcated domains (Fig. 1). Most of the ORFs in Domain I (1–26,829) were predicted to be genes for plasmid replication and stability (Table 1). Domain II (26,830–55,216) contained a set of genes encoding part of a *meta*-cleavage pathway for chlorinated aromatic rings.

Most of the putative translation products derived from the ORFs in Domain I were similar to polypeptides encoded by various broad host range plasmids. However, pCT14 did not have extensive nucleotide base sequence homology to any characterized plasmid except for regions associated with transposable elements. The interaction of catabolic transposons and mobilizable plasmids is a key factor in the distribution of degradation genes among bacteria exposed to xenobiotic compounds (Nojiri et al. 2004; Top and Springael 2003). Comparison of pCT14 with several different catabolic plasmids indicated that pCT14 had more transposase genes relative to its size than other plasmids (Table 2). This observation suggests that pCT14 has passed through one or more environments that were dynamic for transposition.

pCT14 replication and partitioning functions

The putative replication region was contained within Domain I and was similar to the replication regions of

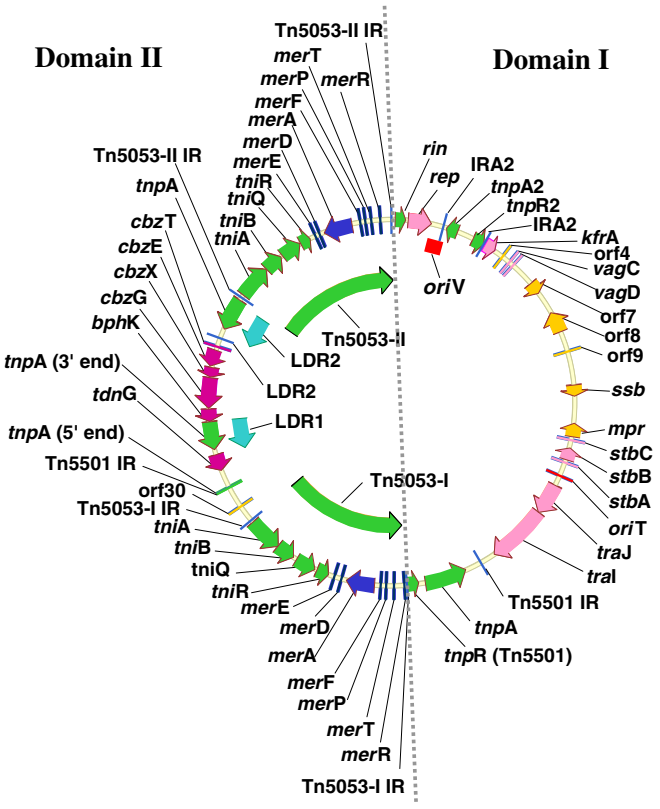

Fig. 1 Map of pCT14 genetic features identified by sequence analysis. The *broken line* separates Domain I from Domain II. Predicted functions of proteins as indicated by color: *pink*, plasmid replication, stability, and conjugation; *green*, transposition and site-specific recombination; *blue*, mercury resistance; *magenta*, *meta*-cleavage pathway enzymes; *yellow*, conserved hypothetical proteins and other functions. Other features: *light blue*, long direct repeats (*LDR*), inverted repeats (*IR*); *red*, *ori*V and *ori*T

iteron-containing plasmids. The replication regions of iteron-containing plasmids typically have several 20-bp direct repeats (iterons), inverted repeats and DnaA boxes that form the *ori* and are located immediately upstream from a *rep* gene (Chattoraj 2000). The iterons bind the cognate Rep protein so that DnaA can initiate melting within *ori*. In addition to being necessary for initiation of replication, the inverted repeats and iterons act as operators for the *rep* gene. Although the distinguishing sequence features of most iteron-containing plasmids are located upstream of the *rep* gene, IncHI plasmids and IncQ differ. The IncHI2 plasmid R478 has two different iteron-regulated replicons (Gardner et al. 2001). Iterons are located upstream and downstream of the *rep*A genes for both replicons. The iterons of the IncQ-like plasmid pTC-F14 are downstream from a *rep*ABC operon (Page et al. 2001).

The pCT14 *rep* gene was adjacent to a series of four direct 20-bp repeats, two 20-bp inverted repeats and two putative DnaA boxes (Fig. 2). One of the DnaA boxes overlapped one of the inverted repeats. The pCT14 *ori* region differed from typical iteron-containing plasmids in that the direct repeats, inverted repeats, and DnaA boxes comprising the *ori* region were downstream of the *rep*

Table 1 pCT14 open reading frames

ORF	Position	GenBank homolog (source plasmid or organism)	Identity (%)
res	109–726	Putative DNA invertase (*Acetobacter pasteurianus*)	62
rep	713–1849	Plasmid replication initiator (*Rhodobacter sphaeroides*)	41
*tnp*A2	2603–3151	Transposase-like protein (pKLC102)	100
*tnp*R2	3973–4539	Invertase/recombinase (pKLC102)	100
*kfr*A	4607–5257	KfrA protein (uncultured bacterium)	39
4	5620–6045	Conserved hypothetical protein (pIPO2T)	37
*vag*C	6055–6291	Virulence-associated protein (*Salmonella enterica* plasmid)	60
*vag*D	6288–6725	Virulence-associated protein (pLVPK)	67
7	7502–8095	Hypothetical exonuclease (Phage CP81)	45
8	9204–10238	Conserved hypothetical protein (pDC3000A)	84
9	11246–11593	Putative transcriptional repressor (pWW0)	44
ssb	12878–13429	Putative SSB protein (pWW0)	79
mpr	14501–15280	Metalloproteinase (plasmid R46)	56
*stb*C	15548–15931	Stable plasmid inheritance (plasmid R46)	27
*stb*B	15951–16655	Stable plasmid inheritance (plasmid R46)	47
*stb*A	16659–17072	Stable plasmid inheritance (plasmid R46)	28
*tra*J	17845–19383	Mobilization protein (pCU1)	46
*tra*I	19384–22339	Conjugal nickase and helicase (plasmid R46)	53
*tnp*A	23125–26154	Transposase (Tn5501)	99
*tnp*R	26138–26773	Resolvase (Tn5501)	100
*mer*R	26949–27383	Regulatory protein (Tn5053)	100
	54663–55097		
*mer*T	27455–27805	Mercury ion transport protein (Tn5053)	100
	54241–54591		
*mer*P	27821–28096	Periplasmic mercuric ion binding protein (Tn5053)	100
	53950–54225		
*mer*F	28099–28344	Mercuric ion transport protein (TN5053)	100
	53702–53947		
*mer*A	28341–29987	Mercuric reductase (Tn5053)	99
	52059–53705		
*mer*D	30004–30369	Coregulator protein (Tn5053)	100
	51677–52042		
*mer*E	30366–30602	Mercury resistance protein (Tn5053)	100
	51444–51680		
*tni*R	30655–31269	Resolvase (Tn5053)	99
	50777–51391		
*tni*Q	31330–32547	Needed for transposition of Tn5053	99
	49502–50716		
*tni*B	32544–33452	Putative ATP-binding protein needed for transposition of Tn5053	99
	48597–49505		
*tni*A	33455–35134	Transposase (Tn5053)	99
	46915–48594		
30	36092–36424	Putative TnpA repressor protein (pND6-1)	100
*tnp*A	37183–37344	5′ end of transposase (pADP-1)	100
*tdn*G	38060–38854	2-oxopent-4-dienoate hydratase (pTDN1)	37
*tnp*A	39107–40483	3′ end of transposase (pADP-1)	73
*bph*K	40485–41096	Glutathione S-transferase	70
*cbz*G	41133–42593	2-hydroxymuconic semialdehyde dehydrogenase	77
*cbz*X	42631–43095	Unknown function	100
*cbz*E	43176–44120	Chlorocatechol 2,3-dioxygenase	99
*cbz*T	44132–44491	Ferredoxin	100
*tnp*A	45043–46623	Putative transposase	75

Table 2 The number of annotated transposase genes carried by various catabolic plasmids

Plasmid name	Plasmid size (kb)	Number of transposase genes	Number of transposase genes/kb
pCT14	55.2	5	0.091
pWW0	116.6	5	0.043
pCAR1	199.0	11	0.055
pNL1	184.5	4	0.022
pDTG1	83.0	3	0.036
pND6-1	101.9	6	0.059
pEST4011	77.0	3	0.039

gene. No interon-like sequences or long inverted repeats were located upstream of the pCT14 *rep* gene as is the case for both replicons associated with R478 (Gardner et al. 2001). In addition, the genes encoding putative replication and partitioning functions for pCT14 were not in a single operon as on pTC-F14 and other *rep*ABC replicons (Fig. 1; Venkova-Canova et al. 2004). Hence, control of replication for pCT14 is most likely not the same as the IncHI and IncQ plasmids.

The *rep* gene was separated from putative stability genes by a presumptive transposon. The presumptive transposon contained putative genes for a transposase (*tnp*A2) and a recombinase (*tnp*R) that were located between inverted 26-bp repeats. The presumptive transposon was flanked by two 6-bp direct repeats (...GATACT...), suggesting the possibility that a target site had been duplicated during transposition.

Plasmid pCT14 carried two sets of ORFs that were similar to genes associated with plasmid stability. Neither set of genes has been extensively studied. The *vag*C and *vag*D genes were originally recognized on the basis of plasmid mutations that decreased virulence of *Salmonella dublin* (Pullinger and Lax 1992). Genes that are closely related to *vag*C and *vag*D have been implicated in plasmid maintenance (Radnedge et al. 1997). Mutations in *stb* genes have been demonstrated to cause increased rates of plasmid loss (Paterson et al. 1999). Although homologs of both systems have been observed to be located on a single plasmid such as *Salmonella typhimurium* plasmid R64 (GenBank accession number NC_005014), this occurrence seems to be rare.

Plasmid mobilization functions

Domain I contained two putative conjugation genes. However, pCT14 lacked most of the genes that would be necessary for self-mobilization. The sequence upstream from *tra*J contained a possible origin for DNA transfer (*ori*T). The putative *ori*T was located in an A/T rich region and consisted of a 29-bp sequence that included the *nic* site of plasmid pMUR274 (Paterson and Iyer 1997).

Genes for *meta*-cleavage of aromatic rings

A portion of Domain II was essentially the same as a fragment of DNA that was previously cloned from *Pseudomonas putida* GJ31 (Mars et al. 1999). The cloned GJ31 DNA includes four genes that encode part of a *meta*-cleavage pathway: *cbz*T (ferredoxin), *cbz*E (chlorocatechol 2,3-dioxygenase), *cbz*X (unknown function), and the 5′ end of *cbz*G (2-hydroxymuconic semialdehyde dehydrogenase). These genes have been found in several bacterial isolates that degrade chlorocatechol via a *meta*-cleavage pathway (Goebel et al. 2004). The pCT14 sequence included the entire *cbz*G gene. In addition, two ORFs were located downstream of *cbz*G. The first ORF (*bph*K) encoded a putative glutathione S-transferase that may be involved in dehalogenation of intermediates from degradation of

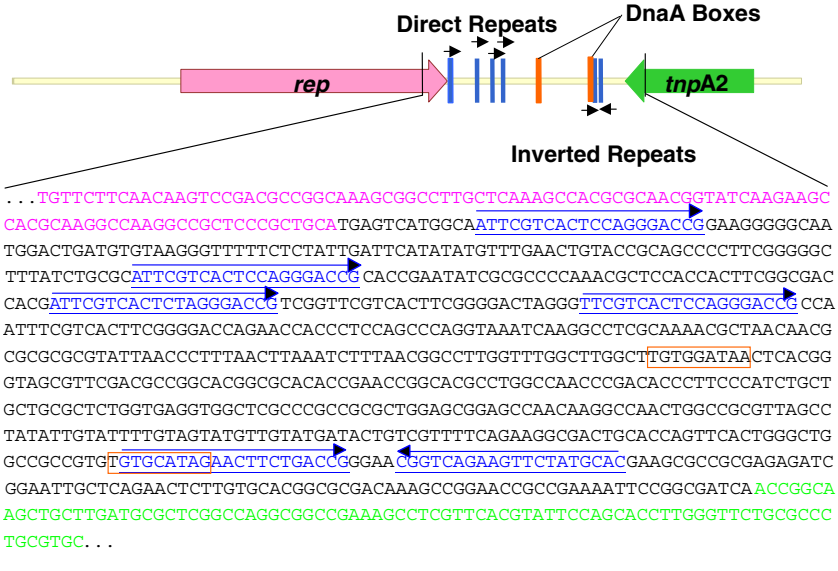

Fig. 2 Map of the pCT14 *ori*V region. Sequence colors: *pink*, *rep*; *green*, *tnp*A2; *light blue*, direct repeats and inverted repeats; *orange*, DnaA boxes

polychlorinated biphenyls (Hofer et al. 1994). The second ORF (*tdn*G) encoded another *meta*-cleavage pathway enzyme, 2-oxopent-4-dienoate hydratase.

If the pCT14 *meta*-cleavage pathway genes enable strain CT14 to degrade chlorinated aromatics, then strain CT14 should grow with at least one of the isomers of chlorobenzoic acid as a source of carbon and energy. Although strain CT14 readily utilized benzoic acid as a sole source of carbon and energy, strain CT14 failed to do so with the isomers of chlorobenzoic acid (data not shown).

All of the *meta*-cleavage pathway genes except *tdn*G were flanked by two long direct repeats (LDR1 and LDR2) that were each 1693 bp in length (Fig. 1). Both repeats had a single ORF that corresponded to the 3′ portion of a transposase gene. The LDR2 ORF extended in the 5′ direction beyond the upstream border of LDR2, resulting in a complete ORF (*tnp*A1) whose deduced translation product was similar to an IS801-like transposase encoded by pADP-1 (Martinez et al. 2001). The *tdn*G gene was located between *tnp*A1′ (the 3′ portion of *tnp*A1 that was located on LDR1) and *tnp*A5, which was the 5′ portion of another transposase gene. The deduced *tnp*A5 transcription product had 100% identity to the corresponding portion of an IS1071-like transposase (GenBank accession number AAK50306), indicating that *tnp*A1′ and *tnp*A5 were unlikely to be parts of the same gene. Differences in base composition as reflected by differences in % G+C are commonly accepted as indications of different origins for genes (Lawrence and Roth 1996; Ochman and Lawrence 1996). Such differences are frequently used to argue for exogenous origin of a gene or set of genes (Dogra et al. 2004). The difference in % G+C for *tnp*A1′ (66.2% G+C) and *tnp*A5 (56.5% G+C) supported the conclusion that these sequences were derived from different genes.

The position of *tdn*G provided a strong indication that *tdn*G had been recruited onto pCT14 separately from the other *meta*-cleavage pathway genes. The *tdn*G sequence was located between two apparently unrelated transposase sequences (i.e., *tnp*A1′ and *tnp*A5). Furthermore, *tdn*G was separated by LDR1 from the other *meta*-cleavage pathway genes that were clustered between LDR1 and LDR2 (Fig. 1). The G+C content for *tdn*G was 71.4%, whereas the other *meta*-cleavage pathway genes on pCT14 ranged from 55.8 to 62.3% (Table 3). Hence, the differences in % G+C values for *tdn*G and the other *meta*-cleavage pathway genes supported the conclusion that *tdn*G had been recruited onto pCT14 separately from the other *meta*-cleavage pathway genes.

Table 3 % G+C values for the pCT14 *meta*-cleavage pathway genes

Gene	% G+C
*tdn*G	71.4
*bph*K	55.8
*cbz*G	59.6
*cbz*X	62.3
*cbz*E	55.8
*cbz*T	59.2

Tn5053

The two inverted copies of mercury resistance transposon Tn5053 that flanked the ends of Domain II formed an interesting feature of pCT14 (Kholodii et al. 1993, 1995). Tn5053 displays a marked preference for inserting into the *res* regions associated with site-specific recombinases of the resolvase/DNA invertase family (Leschziner et al. 1995; Minakhina et al. 1999). Independent Tn5053 insertions usually have the same orientation, i.e., the *mer*R end of the transposon is adjacent to the site-specific recombinase of the target element. Because insertion of Tn5053 involves duplication of the target site, the transposon is flanked by 5-bp direct repeats. If Domain II functioned as a Tn5053-based transposable element, then Domain II should be flanked by 5-bp direct repeats that represent duplication of a single insertion site. Otherwise, the two copies of Tn5053 should have different sets of 5-bp direct repeats that represent duplication of independent target sites.

Tn5053-I was oriented on pCT14 so that *mer*R was adjacent to an ORF whose putative translation product was identical to the Tn5501 resolvase (Lauf et al. 1998). The region containing Tn5053-I was flanked by 38-bp inverted repeats that were identical to the terminal inverted repeats of cryptic Tn3-family transposon Tn5501 (Lauf et al. 1998). Deleting the Tn5053-I sequence from the pCT14 sequence resulted in the remaining nucleotide sequence between the two inverted repeats being 97% identical to Tn5501. Hence, Tn5053-I had inserted into a transposon that was closely related to Tn5501. The Tn5053-I insertion site on Tn5501 was marked 4-bp direct repeats (...TACC...).

Tn5053-II was oriented so that *mer*R was adjacent to an ORF whose translation product was similar to putative resolvases and invertases (*res*; 109–726). The insertion site of Tn5053-II was between *res* and an ORF whose translation product was similar to the IS801-like transposase encoded by pADP-1 (Martinez et al. 2001). Although Tn5053-II was associated with an external transposase and resolvase that presumably recruited Tn5053-II into pCT14, inverted repeats that defined the ends of a corresponding transposable element could not be identified. The Tn5053-II insertion site was marked by 4-bp direct repeats (...CAGG...) that differed from Tn5053-I. These observations indicated that Tn5053-II most likely inserted into pCT14 independently of Tn5053-I.

Discussion

Inasmuch as the *xyl* genes for degradation of toluene are present on catabolic plasmids in 85–100% of the bacterial strains that express the Tol pathway, it was expected that pCT14 would encode toluene degradation in strain CT14 (Sentchilo et al. 2000; Williams and Worsey 1976).

Although no *xyl* genes were present on pCT14, the plasmid contained several *meta*-cleavage pathway genes that have been implicated in degradation of chloroaromatics. The role of the pCT14 *meta*-cleavage pathway in strain CT14 is unclear because strain CT14 did not grow on chlorobenzoates. Nevertheless, these genes are interesting because they are located on a portion of the plasmid that has undergone several transpositions and possibly other recombination events.

It is tempting to speculate that Domain II transposed into pCT14 as a single complex element because the two copies of Tn5053 and the corresponding target elements flanked the *meta*-cleavage pathway genes. This scenario is unlikely because the Tn5053 transpositions apparently occurred independently of each other. The presence of Tn5501, the tnpA1/res element, and portions of two additional transposases indicated that other transposition events have occurred during the formation of Domain II. At least four transpositions have shaped the complex structure of Domain II, i.e., two insertions of Tn5053 and insertions for the two elements that recruited Tn5053. It was evident that insertion of Tn5501 proceeded insertion of Tn5053-I. Similarly, insertion of the tnpA1/res element proceeded insertion of Tn5053-II. However, the exact order in which these different sets of insertions occurred could not be determined.

The *meta*-cleavage pathway genes apparently have been assembled from different sources. The *tdn*G gene was separated from the other *meta*-cleavage pathway genes by a partial transposase gene and one of the two repeated sequences. Furthermore, the % G+C content of *tdn*G was significantly higher than the other *meta*-cleavage pathway genes. Novel pathways seem to form by an assembly process that involves horizontal gene transfer and recruitment of different parts of the novel pathway from different hosts into one single host (Top and Springael 2003). Hence, sequencing pCT14 may have revealed an intermediate stage in the evolution of a new assemblage of *meta*-cleavage pathway genes.

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