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**Plasmid pBRCe0 DNA Integration into
the *Comomonas* sp. Genome**

by

Alisa Evelyn Cashore

A thesis submitted to
The Ottawa-Carleton Institute of Biology
Carleton University
in partial fulfilment of
the requirements for the degree of
Master of Science

1 March, 1993
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Plasmid pBRC60 DNA Integration into
the *Comomonas* sp. Genome

submitted by Alisa Evelyn Cashore
B.J. (Hons.), B.Sc. (Hons)
in partial fulfillment of the requirements for
the degree of Master of Science

Thesis Supervisor

Chair, Department of Biology

Carleton University

Abstract

The plasmid pBRC60 carries a transposon, Tn5271, which specifies 3-chlorobenzoate degradation and is deficient in its cointegrate resolution system. pBRC60 has previously been shown to exhibit chromosomal recombination in its host strain, *Alcaligenes* sp. BR60, mediated by homology between chromosomal and plasmid copies of the insertion element IS1071, which flanks Tn5271. pBRC60 was mated into *Comomonas acidovorans* and *C. testosteroni*, neither of which has a chromosomal copy of IS1071. An incompatible plasmid, pGS65, was subsequently mated in with selection for the determinants on both plasmids. The transconjugants carried pGS65 in the cytoplasm and a chromosomal insertion of the entire plasmid, pBRC60. Upon removal from 3-chlorobenzoate selection, the integrated plasmids underwent several rearrangements. Analysis of these derivatives by DNA hybridization showed that a 40 kb segment of the integrated plasmid had been deleted, as well as the 3Cba genes. It is suggested that pBRC60 carries a second transposon encompassing Tn5271, which was responsible for the chromosomal integration and subsequent rearrangements.

Acknowledgements

This thesis research could not have been completed without the support and assistance I received from many people. I would like to thank my supervisor, Dr. Cam Wyndham, for accepting me into his lab and for his encouragement through all my occasionally cryptic results. The past two years have been enjoyable thanks to my colleagues in the lab: Cindy Nakatsu, Varsha Kamal, Michelle Peel, Maysoon Salih and Barb Holland. Finally, I would like to thank my husband Harvey for his constant support and encouragement.

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1. Introduction

1.1 Evolution of xenobiotic degradation pathways

One of the consequences of industrialization has been the release of a large number of xenobiotic compounds into the environment. These synthetic compounds include herbicides, pesticides, solvents, and other industrially useful compounds. However, many of these compounds are extremely persistent in the environment, and most of them are toxic to humans and animals (Frantz and Chakrabarty, 1986; Ghosal et al., 1985b; Sayler et al., 1990).

The release of unusual organic carbon sources into the environment provides a unique niche in which to study evolution (Ghosal et al., 1985b). The xenobiotic compounds provide strong selection pressure for bacterial populations, whose genetic flexibility allows them to develop new degradative abilities quickly in response to the new substrates (Frantz and Chakrabarty, 1986; Rothmel et al., 1989).

There are two ways in which the evolution of new catabolic pathways is thought to occur. Existing genes can be recruited from degradative pathways for similar compounds, or novel genes can arise by fusion or recombination of genes for related enzymes (Ghosal et al., 1985b; Rothmel et al., 1989). To this end, genetic rearrangements like deletions and amplifications are thought to be significant in the evolution of new catabolic pathways

(Ghosal et al., 1985b).

1.2 Role of plasmids in xenobiotic degradation

Newly evolved degradative genes for xenobiotic compounds are often located on plasmids (Ghosal et al., 1985b; Rothmel et al., 1989; Sayler et al., 1990). The first description of a plasmid-encoded degradative pathway, in 1971, was for camphor and octane degradation in *Pseudomonas* (Chakrabarty and Gunsalus, 1971). Since then, a number of catabolic plasmids have been described; they have been isolated from many bacterial strains, although primarily from *Pseudomonas* spp., and encode degradation of a diverse group of natural and synthetic substrates (Frantz and Chakrabarty, 1986; Sayler et al., 1990). The first plasmid-encoded degradative pathway for a synthetic compound was described in 1977, for 2,4-dichlorophenoxyacetic acid (2,4-D) degradation by the plasmid pJP4 in *Alcaligenes eutrophus* (Pemberton and Fisher, 1977).

The frequency of catabolic plasmids tends to increase in environments with high amounts of pollution (Sayler et al., 1990). The presence of the pollutants imposes strong selection pressure on the bacterial populations in that environment. Because plasmids can transfer both within and between bacterial species, the bacterial strains carrying the plasmids are provided with a greater degree of genetic flexibility and can adapt faster to their environment

(Fulthorpe and Wyndham, 1991; Sayler et al., 1990).

Consequently, the newly evolved plasmid-encoded phenotypes can be more readily maintained in the bacterial population (Sayler et al., 1990).

Genetic rearrangement of the genes encoding catabolic pathways on plasmids is a common event. The genes undergo deletions at high frequencies, in which either the entire operon is lost, or a smaller fragment is lost, inactivating the operon. Such rearrangements may indicate the involvement of either repeated DNA sequences or transposable elements in the evolution of catabolic pathways. The presence of these elements confers an even greater degree of adaptability in the arrangement and expression of the newly evolved genes, allowing for genetic rearrangements within the plasmid, and also between the plasmids and the chromosome. Integration of the plasmid into the chromosome can occur by transposition or homologous recombination or both (Sayler et al., 1990; Wyndham et al., 1988).

Similarities among degradative genes from bacteria isolated from diverse hosts carry significant evolutionary implications in terms of the origins of the genes. For example, plasmids pAC27 and pWR1 were isolated from different strains of *P. putida*, and the plasmid pJP4 was isolated from *Alcaligenes eutrophus* (Sayler et al., 1990). All three plasmids carry genes which specify degradation of 3-chlorobenzoate (3Cba). There is considerable homology

among the genes, suggesting they evolved via related mechanisms or from related ancestral pathways (Ghosal et al., 1985a).

The Tol plasmids provide another example. Plasmids specifying degradation of toluenes and benzenes have been isolated from locations around the world, from Japan to Minnesota to Wales (Shaw and Williams, 1988). These plasmids vary in size, but the encoded pathways are very similar. The archetypal Tol plasmid pWWO undergoes a variety of genetic rearrangements, mediated by homologous recombination between repeated sequences and transposition (Tsuda et al., 1989). These rearrangements are not confined to the plasmid; a segment of Tol-derived DNA has been found integrated into the chromosome of the host strain (Sinclair et al., 1986). Homology among the Tol plasmids suggests an evolutionary relationship among them, and the variety of the observed genetic rearrangements demonstrates the capacity for change conferred by transposable elements.

1.3 *Alcaligenes* sp. BR60

1.3.1 Isolation of pBRC60

The highly industrialized Niagara region was a natural environment for the evolution of a catabolic pathway for 3-chlorobenzoate (3Cba). Bloody Run Creek is a tributary of the Niagara River, which collects surface runoff waters from the Hyde Park industrial land fill in New York state. The

area is contaminated with a variety of xenobiotic compounds, the major contaminants being phenols and chlorobenzoates (Wyndham and Straus, 1988).

In 1988, *Alcaligenes* sp. BR60 was isolated from a mixed bacterial community from Bloody Run Creek that had been enriched with 3Cba (Wyndham and Strauss, 1988). BR60 exhibited several characteristics of having a newly evolved catabolic pathway. The strain carries a large (88 kb) unstable conjugative plasmid which was found to specify 3Cba degradation (Wyndham and Straus, 1988; Wyndham et al., 1988). When BR60 was cured of the plasmid, designated pBRC60, the 3Cba degradative functions were lost (Wyndham et al., 1988).

The plasmid exhibited a spontaneous 14 kb deletion, and the resulting deletion mutant strain BR40 (pBRC40) was not capable of 3Cba degradation (Wyndham et al., 1988). The deletion from pBRC60 to pBRC40 occurred at a frequency of 1.6×10^{-3} per cell per generation when 3Cba selection was removed (Nakatsu et al., 1991). When the resistance plasmid R68.45 was conjugated into BR60, the resulting transconjugants no longer carried pBRC60, but maintained R68.45. However, 30% of these transconjugants still retained the ability to degrade 3Cba. DNA hybridization using probes derived from the region of pBRC60 involved in the original deletion event revealed the presence of this region in the chromosome of the transconjugants, as well as

a repeated sequence flanking this region (Wyndham et al., 1988).

1.3.2 Structure and characteristics of Tn5271

The deleted region was identified as a 17 kb transposon, designated Tn5271 (Figure 1.1). Copies of the transposon are carried both on the plasmid pBRC60 and in the chromosome of *Alcaligenes* sp. BR60. The transposon is flanked by directly repeated segments of 3.2 kb each, designated IS1071. Each of these is in turn flanked by 110 bp inverted repeats (Nakatsu et al., 1991). The insertion element IS1071, which is found in seven copies in the BR60 genome, is thought to mediate the recombination of pBRC60 into the chromosome (Ng and Wyndham, 1993).

IS1071 contains one open reading frame which encodes a transposase related to the TnpA transposases of the class II transposable elements. DNA homology has shown that the 110 bp inverted repeats are related to class II inverted repeats. The outer 38 base pairs are DNA-binding and cutting sites for class II transposases. However, no corresponding resolvase gene has been found encoded within IS1071 or Tn5271, nor has a *res* site been located. The absence of a resolvase gene suggests that these elements are capable of the first step of class II transposition only. The fact that transposition is blocked at the cointegrate stage was confirmed by the formation of stable

Figure 1.1 Restriction map of Tn5271. Tn5271 is flanked by two directly repeated copies of the insertion element IS1071. These in turn are flanked by terminal inverted repeats (IRs) of 110 bp. Both the IRs and the transposase encoded by the single ORF within IS1071 are similar to those of the class II transposable elements.

Tn5271

cointegrates, mediated by IS1071 transposition, in a recombination deficient strain of *E. coli* (Nakatsu et al., 1991).

1.4 Genetic rearrangements mediated by Tn5271

In addition to the inherent instability of pBRC60 shown by the spontaneous deletions, pBRC60 has been shown to exhibit other genetic rearrangements in response to various genetic manipulations. Conjugation of incompatible plasmids pGS65 or R68 into *Alcaligenes* sp. BR60 induced the recombination of Tn5271 into the chromosome. The new chromosomal copy of Tn5271 was detected by DNA hybridization. The complete transposon was shown to have integrated by mobilization experiments, in which pBRC40 or R68 could mobilize the newly created chromosomal copy of Tn5271 from BR60 into a 3Cba⁻ derivative of *Alcaligenes* sp. BR60 (Ng and Wyndham, 1993).

The plasmid pBRC60 was shown to exhibit reversible recombination into the chromosome. Conjugation of pGS65 into *Alcaligenes* sp. BR6024 (pBRC60) with no 3Cba selection generated transconjugants with pBRC60 integrated into the chromosome. When selection for pGS65 was removed, that plasmid was lost and a cytoplasmic copy of pBRC60 was recovered. It was proposed that a recombinational equilibrium existed between cytoplasmic and chromosomal copies of pBRC60, mediated by IS1071, in that pBRC60 or

pBRC40 recombined in and out of the chromosome by homologous recombination between plasmid and chromosomal copies of IS1071 (Ng and Wyndham, 1993).

Genetic rearrangements have also been shown to occur in Tn5 mutagenized *Alcaligenes* sp. BR60 cells. As with the incompatible plasmids, Tn5 mutagenesis was sufficient to induce recombination of Tn5271 into the chromosome of its host strain, BR60. Integration of Tn5271 into the chromosome of BR60 seemed not to depend on the location of Tn5 integration (Ng and Wyndham, 1993).

There is also evidence of intramolecular inversion mediated by IS1071 on the plasmid pBRC60. One such plasmid (pBRC54) was similar in size to pBRC60, but restriction digest profiles with *Eco*RI and *Hin*DIII showed a novel 1.72 kb *Eco* fragment and 7.9 kb *Hin*d fragment. Further restriction mapping and hybridization of pBRC54 revealed the insertion of a new copy of IS1071 adjacent to the transposon in an inverted orientation. Restriction mapping revealed that the intervening DNA had undergone an inversion. Since inversions require only transposase, not resolvase or *res* sites, IS1071 would be capable of mediating an inversion event (Ng, 1991; Ng and Wyndham, 1993).

1.5 Objectives

The objective of this project was to characterize the mechanism of pBRC60 integration into the chromosomes of

heterologous host bacteria lacking chromosomal copies of IS1071. pBRC60 had already shown itself capable of integration into the chromosome of its host strain *Alcaligenes* sp. BR60, following the mating in of an incompatible plasmid (Ng, 1991). It was reasoned that this chromosomal integration could be generated in other hosts using a similar system. It was hypothesized that the chromosomal integrations would be mediated by transposition by Tn5271 or by IS1071.

2. Literature Review

2.1 Evolutionary significance of transposable elements in bacteria

Transposable elements are discrete segments of DNA which are able to move within and between chromosomes and plasmids (Galas and Chandler, 1981; Schmitt and Rogowsky, 1985). They are normal constituents of most bacterial genomes, and have been implicated in chromosomal and plasmid rearrangements (Kleckner, 1981). These elements require no homology for recombination, and most appear to insert randomly into the genome (Schmitt and Rogowsky, 1985). Each element carries its own regulatory information, and controls the potentially lethal over-expression of its mutational activity (Syvanen, 1984).

Simple insertion elements were first described in *E. coli* in the late 1960s (Jordan et al., 1968; Shapiro, 1969). More complex elements encoding antibiotic resistance were described a few years later; the transposon Tn3, which encodes ampicillin resistance, was the first (Hedges and Jacob, 1974).

The simplest rearrangement mediated by transposable elements is the excision of the element (Syvanen, 1984), which may or may not be accompanied by the deletion of adjacent host DNA (Saedler, 1985). The adjacent DNA may also be inverted (Syvanen, 1984). Other rearrangements include insertions, inversions of intervening DNA,

duplication of intervening sequences, or translocation of DNA sequences (Schmitt and Rogowsky, 1985). Replicative transposition can result in amplification and dispersal of DNA sequences (Sherratt et al., 1983). The rearrangements can be caused by inter- or intramolecular events, and are all independent of the host-encoded *recA* function (Kleckner, 1981).

Transposable elements modify the organization and expression of genes; the most common effects involve changes in the regulation of existing enzymes, by mutations in the regulatory proteins or in promoter activation (Syvanen, 1984). These changes occur at higher frequencies than spontaneous mutation rates (Schmitt and Rogowsky, 1985). Transposition occurs at frequencies of 10^{-4} to 10^{-7} per cell per generation (Kleckner, 1981).

Genetic rearrangements like those mediated by transposable elements are essential in the evolution of bacterial genomes (Saedler, 1985; Schmitt and Rogowsky, 1985). Two theories have been proposed to explain the biological function of genetic rearrangements. The selectionist theory proposes that genetic rearrangements provide the genetic diversity required to drive natural selection, so that transposable elements may have evolved as tools for creating a diversity of selectable phenotypes, thereby causing increased survival of individual organisms or population of organisms (Kleckner, 1981; Syvanen, 1984).

Alternatively, the selfish DNA theory suggests that since transposition is fundamentally a replicative process, transposable elements are simply selfish DNA, functioning like genetic parasites and ensuring their own survival (Kleckner, 1981; Syvanen, 1984).

The majority of evidence seems to support the first theory, that genetic rearrangements provide a potential selective advantage to the bacteria (Syvanen, 1984). A comparison of species evolving slowly and rapidly showed a much higher frequency of changes in the organization of the genome in the rapidly evolving species. Furthermore, isolation of bacterial strains which have acquired new metabolic traits has revealed that the most common mutations involve changes in the regulation of existing enzymes, implicating genetic rearrangements, rather than the creation of new enzymes (Syvanen, 1984).

The selective advantage conferred by the ability for genetic rearrangements has been shown in competition experiments with *E. coli*. Strains carrying the transposon Tn10, which is flanked by two copies of the insertion element IS10, were shown to have an evolutionary advantage in a chemostat, provided that the strain had a high enough starting ratio to generate the necessary beneficial mutations (Chao et al., 1983; Syvanen, 1984). The Tn10-carrying population increased about 200-fold over the competing strain without the transposable element; the

increase in the population following the first 50 to 150 generations was due to appearance of a new, faster growing strain. The same result was obtained in four separate experiments (Chao et al., 1983; Syvanen, 1984).

Furthermore, the faster-growing strains all contained a new copy of IS10 in the same site in the chromosome, suggesting the same mutation event was being selected (Chao et al., 1983).

The role played by transposable elements in the evolution of new genes has been shown in the development of *P. cepacia* strain AC1100, which acquired the ability to grow on 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) following growth in a chemostat in a mixed culture derived from various sources (Tomasek et al., 1989). A 1477 base pair (bp) sequence, designated RS1100, was found repeated many times on both the chromosome and the plasmid, pAC1100. The structure of the sequence was similar to that of a transposable element; it had 38 and 39 bp terminal inverted repeats, flanked by 8 bp direct repeats. The open reading frame (ORF) within the segment was similar to the transposase encoded by the Mu bacteriophage. Neither RS1100 nor the 2,4,5-T genes hybridized to any *Pseudomonas* strains, indicating they must have had a foreign origin in other genera present in the chemostat (Tomasek et al., 1989).

There are three classes of transposable elements. Class I elements are simple insertion elements, class II

elements belong to the Tn3 family of transposable elements, and class III elements are transposing bacteriophages, like Mu (Grindley and Reed, 1985; Kleckner, 1981). Class I insertion sequences (ISs) occur either individually or as a composite transposon in which two copies of an IS element flank a central segment which usually carries a selectable marker. Each IS element encodes its own transposition function, and show some insertion site specificity. The composite transposon Tn10, for example, has been shown to prefer hotspots; variations in the target sequence change the insertion frequency by 1000 times (Bender and Kleckner, 1992). IS elements are diverse in terms of both their structure and their sequence (Kleckner, 1981). Class II elements are more complex in both their structure and function, and will be examined in more detail. Class III elements will not be discussed.

2.2 Mechanisms of class II transposition

2.2.1 Intermolecular transposition

Intermolecular transposition mediated by class II transposable elements (also known as the Tn3 family) is a two-step process (Galas and Chandler, 1981). The first step is the formation of a cointegrate structure between the donor and recipient molecules, in which there is a copy of the transposing element at both junctions. The second step is a site-specific recombination in which the two original

molecules are regenerated; the donor maintains its copy of the element, and the recipient carries a new copy of the element (Figure 2.1) (Bennett et al., 1983; Sherratt, 1989). Transposition occurs at a frequency of 10^{-4} to 10^{-7} per cell per generation (Sherratt, 1989). The insertion of the transposon into a staggered cut on the target molecule causes a five bp segment of the target DNA to be duplicated, resulting in flanking direct repeats (Grindley and Reed, 1985; Kleckner, 1981; Schmitt and Rogowsky, 1985).

All class II transposons have short flanking inverted repeats of about 40 bp; the internal region encodes the transposition functions and occasionally additional determinants (Schmitt and Rogowsky, 1985). Cointegrate formation requires the product of the *tnpA* gene, transposase (Sherratt et al., 1983). Cointegrate resolution involves site-specific recombination mediated by the *tnpR* product, resolvase, acting on the *res* sites located within the two copies of the transposing element (Arthur et al., 1984; Sherratt et al., 1983).

This replicative mechanism of transposition is characteristic of class II elements (Schmitt and Rogowsky, 1985). Recognition of the cointegrate intermediate followed from several observed features of transposition. Transposition was known to be replicative because the donor molecules retained their copy of the element, and no excision products were found due to deletion of the element

Figure 2.1 Mechanism of class II transposition Class II transposable elements transpose through a two step process. A cointegrate structure is formed in which the donor and target molecule are joined with directly repeated copies of the element at the two junctions. Cointegrate formation generates a 5 bp duplication of target DNA adjacent to the transposon, indicated by the small arrows. Cointegrate resolution occurs via site-specific recombination between the two elements, to give the original two molecules with a copy of the transposon located on the target molecule, flanked by the short direct repeats.

from the donor (Kleckner, 1981). Class II elements encode their own site-specific resolution system, which functions independently of the host-encoded *recA* system (Bennett et al., 1983; Kleckner, 1981). Furthermore, cointegrate structures can be resolved if the resolvase is provided in *trans* (Sherratt, 1989).

2.2.2 Cointegrate formation

The *tnpA* genes of class II elements encode transposase proteins that mediate cointegrate formation. Transposases are large proteins; Tn3 transposase, to which others are compared, contains 1015 amino acids (Kleckner, 1981). Transposition activity is limited by the amount of transposase available, so that increased levels of transposase lead to increased transposition activity (Sherratt et al., 1983; Sherratt, 1989). However, overproduction of transposases is bad for the host cell, presumably because of deleterious mutations caused by excessive transposition (Kleckner, 1981).

The ends of the transposons are also essential for transposition (Sherratt et al., 1983). Class II elements are all flanked by inverted repeat (IR) sequences of 38 bp; the homology between the two is perfect or nearly perfect (Grindley and Reed, 1985). The IRs provide the recognition sites for the transposase protein, which can be supplied in *trans* (Grindley and Reed, 1985). In order for normal

transposition to occur, both IRs must be present in *cis*, and in an inverted orientation (Grindley and Reed, 1985; Huang et al., 1986).

Studies with derivatives having two left or two right IRs of Tn3 have shown that there is no preference between the two (Arthur et al., 1984). Such derivatives were able to transpose normally, and the transposition frequency was the same regardless of combination of ends as long as they were inverted (Arthur et al., 1984). Reducing the distance between the two IRs was shown not to affect the transposition frequency (Huang et al., 1986). It was also shown that the transposase is sometimes specific for the IRs, so that despite similarities in the sequences of IRs between closely related transposons like Tn3 and $\gamma\delta$, the transposase functions were not interchangeable (Sherratt, 1989).

Studies involving *in vitro* binding of the Tn3 transposase to its IRs have shown that the Tn3 IR sequence has two domains. Domain B, which occupies positions 13 to 38, is involved in transposase binding; in footprinting experiments, the Tn3 transposase protected the inner 25 bp of the IR. Domain A, which occupies positions 1 to 10, is not involved in binding the transposase. Mutations in domain A do not inhibit transposase binding, while mutations in domain B do. However, although domain A does not bind transposase, it is essential for cointegrate formation

(Ichikawa et al., 1990).

Class II elements show some insertion site specificity, preferring AT rich sequences, or sequences with some homology to the IRs (Sherratt et al., 1983; Sherratt, 1989). Some transposons show regional specificity, inserting at different sites within a short region (Kleckner, 1981). When both Tn3 and Tn501 are present in the same cell, they tend to insert close to each other (Sherratt, 1989).

A low percentage of transposition events do not proceed through a cointegrate intermediate (Sherratt, 1989). These have been identified in systems in which the resolution function has been disabled (Bennett et al., 1983). If cointegrates were an absolutely obligatory intermediate, they would be the only structures found using a TnpR deficient element in a *recA* host, other than the donor and target molecules. Experiments with a Tn3 *tnpR* mutant, in which the donor and target molecules had different selectable markers, showed that between one and five percent of transposition events do not go through the cointegrate intermediate, in which the donor and target molecules each carried a copy of the element. The transposons on the products of this direct transposition were shown still to be functional (Bennett et al., 1983).

Models explaining the mechanism of transposition must account for the generation of the direct repeats flanking the transposon on the target molecule. The current models

suggest that the transposase creates a single-stranded nick on the donor molecule at the junction with the transposon, and a double-stranded staggered nick in the target DNA (Sherratt, 1989). The models can be divided into asymmetric and symmetric mechanisms (Grindley and Reed, 1985).

The asymmetric models suggest replication of the transposon is initiated at one end of the transposon. The replication fork would travel through the transposon to the second IR, where replication would be terminated. The second strand of the target DNA would be cut, and depending on which strand of donor DNA was cut, the result would be either cointegrate formation or direct transposition. Both of these would be replicative mechanisms, in which the donor molecule would be preserved (Galas and Chandler, 1981; Grindley and Reed, 1985).

The symmetric models also propose the single-stranded nick in the donor DNA, but at both ends of the transposing element, so that replication would be initiated at both ends of the transposon. This would result in two replication forks travelling towards each other through the transposon. If replication proceeded through the entire transposon, the result would be cointegrate formation. If the single strands that attached the transposon to the donor molecule were broken, the result would be direct transposition by a non-replicative mechanism in which the donor molecule would be destroyed (Grindley and Reed, 1985).

2.2.3 Cointegrate resolution

The *tnpR* gene in class II transposons encodes a resolvase protein containing 185 amino acids (Kleckner, 1981; Sherratt, 1989). The TnpR protein mediates site-specific recombination between the *res* sites in the cointegrate, and like the TnpA protein, it is trans acting (Grindley and Reed, 1985). Although cointegrates are usually very transient structures in class II transposition, in the absence of a functional *tnpR* gene, cointegrates will remain stable in *recA*- hosts (Kleckner, 1981).

Both the *tnpR* product and the *res* sites are required for cointegrate resolution (Grindley and Reed, 1985). The resolvase binds to the *res* sites, and the site-specific recombination occurs at a unique site within the *res* sequences. The direction and specificity of cointegrate resolution is such that the *res* sites must be directly repeated on the same molecule; resolution frequencies are much lower with inverted *res* sites or sites on different molecules (Sherratt, 1989).

Resolvase proteins have two domains. The smaller carboxy (C) terminal domain binds to the *res* sites, while the amino (N) terminal domain is responsible for strand exchange during recombination and for interactions between proteins. Resolvases show extensive homology in their amino acid sequences, particularly at the N-terminal end. Unlike the transposase proteins, the resolvase proteins are

functionally interchangeable between related elements (Sherratt, 1989).

In addition to mediating cointegrate resolution, the resolvase proteins act as transcriptional repressors of both the *tnpA* and *tnpR* genes (Grindley et al., 1982; Grindley and Reed, 1985). The promoters for both genes lie within the *res* site, so in binding to the *res* site, the resolvase exercises negative control over the expression of both products (Kleckner, 1981; Sherratt et al., 1983; Sherratt, 1989). It has been shown that transposition of Tn3 increases 100-fold if the regulatory function of TnpR is blocked (Kleckner, 1981).

The *res* sites are 120 bp in length, and contain three resolvase binding sites which are highly conserved (Grindley and Reed, 1985; Sherratt, 1989). Site I overlaps the crossover point for recombination and both transcriptional promoters. Sites II and III cover most of the DNA between site I and the beginning of the *tnpR* coding sequence. Site I is not sufficient to promote site-specific recombination; all three sites are required (Grindley et al., 1982).

The recombination point in site I occurs within a 19 bp sequence, which is identical in Tn3 and $\gamma\delta$. The resolvase protein cleaves this sequence at a specific location thought to be the site of DNA breakage and reunion. Binding at sites II and III may serve a regulatory function. It has been shown that mutations in site II reduce the regulatory

effect of resolvase binding on the expression of the *tnpR* gene (Grindley et al., 1982).

Structurally, the three sites are very similar. On transposons within the Tn3 subfamily, each site consists of a pair of nine bp inverted repeats which flank a central spacer. The size of spacer varies among the three sites; it is 10 bp in site I, 16 bp in site II and seven bp in site III. The distances between the sites also varies. There are 53 bp separating the centres of sites I and II and 34.5 bp separating centres of sites II and III (Sherratt, 1989).

The ability to express their own site-specific resolution system is unique to the class II transposons. The molecular mechanism of the interaction between the resolvase and the *res* sites is addressed by the two-step synapsis model, which suggests that a resolvase dimer binds to each site within the *res* region. The *res* regions are brought together so the three sites are brought into contact and site-specific recombination occurs at site I (Sherratt, 1989).

2.2.4 Intramolecular transposition

Intramolecular transposition by class II elements occurs at similar frequencies to intermolecular transposition (Bishop and Sherratt, 1984; Sherratt, 1989). It is not a two-step process like intermolecular transposition, so the *tnpR* product and *res* sites are not

necessary. All that is required for intramolecular transposition is the *tnpA* product (Arthur et al., 1983; Bishop and Sherratt, 1984).

Replicative intramolecular mechanisms result either in adjacent deletions or inversion-insertions, depending on the orientation of linkage between the target site and the transposon ends (IRs). Direct orientation generates adjacent deletions, in which the DNA adjacent to one end of the transposon is deleted as a circular DNA molecule containing a single copy of the transposon (Figure 2.2). Inverse orientation generates inversion insertions, in which the adjacent DNA sequence is inverted and a new copy of the transposon is inserted. There is a third possible mechanism, in which one end of the transposon is joined to an adjacent target site. This is a non-replicative mechanism, so a new copy of the transposon would not be generated, and the deleted segment would simply be lost (Grindley and Reed, 1985).

2.2.5 Transposition immunity

The phenomenon of transposition immunity is unique to class II elements (Grindley and Reed, 1985). It serves to block transposition of one element into a replicon which already contains a copy of that element (Sherratt et al., 1983). This immunity is a *cis* acting process, as other replicons in the same cell can still act as targets.

Figure 2.2 Intramolecular transposition. Intramolecular transposition will generate different products depending on the orientation of the target site relative to the element. If the target is oriented in the same direction, transposition results in an adjacent deletion in which the deleted DNA is circular and carries a copy of the transposon (A). If the target is inversely oriented, transposition results in an inversion-insertion in which a second copy of the element is generated and the intervening DNA is inverted (B). Both these mechanisms are replicative. An alternate mechanism involves one end of the transposon moving to an adjacent target site (C), in which case the deleted DNA is not excised as a circular product as in the first two mechanisms. This is a non-replicative mechanisms, so no new copy of the transposon is generated.

However, replicons already containing the element are used as targets at a frequency of less than 5% of the normal frequency (Grindley and Reed, 1985).

A single 38 bp IR carried on a replicon is sufficient to cause immunity (Heritage and Bennett, 1984; Sherratt, 1989). The specificity of immunity is similar to that of transposase. The transposase from $\gamma\delta$ does not cause transposition with IRs from Tn3, and the $\gamma\delta$ IR does not cause immunity to Tn3 transposition (Arthur et al., 1984; Sherratt, 1989). The degree of immunity depends on the level of transposase, so that increased expression from *tnpA* can partially overcome the effects of immunity (Heritage and Bennett, 1984). Mutations in the IRs have also been shown to diminish the effects of immunity (Sherratt, 1989).

Transposition immunity appears not to affect intramolecular transposition on replicons carrying only one copy of a transposable element. The nonimmunity of intramolecular transposition is consistent with the theory that both IRs are complexed with the transposase protein before searching for a target site (Grindley and Reed, 1985). In keeping with this theory, the presence of a third IR on a replicon has been shown to reduce the frequency of intramolecular transposition. The mechanism of this restriction is unknown, although it is presumed to be similar to immunity (Bishop and Sherratt, 1984).

2.2.6 Single-ended transposition

All class II transposons have two IRs, both of which are involved in transposition. However, it has been shown that only a single IR is necessary for transposition, as long as transposase is provided in *cis* or in *trans* (Heritage and Bennett, 1985; Schmitt and Rogowsky, 1985). Single ended transposition occurs at frequencies of 10 to 1000 times lower than for normal transposition (Sherratt, 1989). The product of single-ended transposition is analogous to a cointegrate; it consist of the fusion of the two replicons (Grindley and Reed, 1985). These so-called plasmid fusions occur independently of the host *recA* system (Heritage and Bennett, 1985).

Experiments using single-ended derivatives of Tn1 and Tn3 have shown that either end is capable of mediating this transposition (Heritage and Bennett, 1984). Derivatives with either the left or right IR produced cointegrates with essentially no duplication of transposon DNA. Sequences with partial homology to the inverted repeat were presumed to be acting as an alternate end (Arthur et al., 1984).

More complete characterization of single-ended transposition has been carried out with derivatives of Tn21 and Tn1721 (Grindley and Reed, 1985). The plasmid fusions from these derivatives had a complete copy of the donor replicon, and most also showed some duplication of donor DNA (Avila et al., 1984). Analysis of the junction sequences

showed that one junction had a single copy of the IR, while the sequence was variable at the other end. Both junctions were flanked by direct repeats from the target DNA, as would be expected from normal transposition. It was thought that since there was no second IR, replication would proceed around the donor molecule; termination would not occur precisely at the IR because it would not be in the correct orientation (Mötsch et al., 1985).

2.3 Organization of class II elements

2.3.1 Class II element subfamilies

Class II elements are similar in both structure and function, which suggests a common evolutionary origin (Kleckner, 1981). Differences in the degree of similarity among the class II elements have prompted their division into the Tn3 and Tn501 subfamilies (Sherratt, 1989). The Tn501 family has been further divided into the Tn21 and Tn1721-Tn501 subfamilies (Tsuda et al., 1989).

The division of the class II elements into the Tn3 and Tn501 subfamilies is based on several factors. Members of the subfamilies are structurally different. In the Tn3 subfamily, the *tnpA* and *tnpR* genes are transcribed divergently from an intervening region which includes the *res* site. In the Tn501 subfamily, the two genes are in the same orientation, and the *res* site is located outside, rather than between the two genes (Grindley and Reed, 1985;

Schmitt and Rogowsky, 1985; Sherratt, 1989). There is no apparent reason for the differences in organization between the two subfamilies (Sherratt, 1989).

Strong similarities in both the transposition and resolution functions encoded by the elements within each subfamily may suggest divergent evolution of the two subfamilies (Sherratt, 1989). Within the Tn3 subfamily, the sequences of the IRs are similar, with several conserved motifs and organization. Very closely related elements can complement each other's transposition function (Sherratt, 1989). The *res* sites of closely related elements also show strong homology, and their resolution functions can be complemented (Kleckner, 1981).

Organizational similarity, sequence homology and complementation tests therefore provide the basis for the two subfamilies. For example, Tn21 encodes resistance to mercury, sulphonamide and streptomycin, Tn501 encodes mercury resistance and Tn1721 encodes tetracycline resistance (Diver et al., 1983). Their resolution systems are similar, so that a *tnpR* mutant of Tn21 could be complemented by Tn21, Tn1721 and Tn501. However, the *res* sites show no clear homology to those of Tn3 subfamily elements, and Tn3 cannot complement the *tnpR*- mutant of Tn21 (Diver et al., 1983). The similarities also extend to the transposition functions. Tn21, Tn1721 and Tn501 have similar inverted repeats, and their *tnpA* products can

complement each other, whereas the *tnpA* product of Tn3 cannot (Diver et al., 1983; Kleckner, 1981).

2.3.2 Nested transposons

The occurrence of several nested transposons carries some interesting implications with respect to the evolution of new transposons. Tn3, which encodes ampicillin resistance, is nested within Tn4, which in addition to the ampicillin resistance also carries streptomycin and sulfonamide resistance (Kopecko et al., 1976). Similarities between the Tn4 sequences and those of Tn21 suggest that Tn4 was formed by the insertion of Tn3 into the mercury resistance gene of Tn21 (Michiels and Cornelis, 1984).

A similar relationship exists for the transposons Tn2501 and Tn951. Tn951, which encodes lactose fermentation, is a defective transposon, in that it does not encode the first step of transposition. However, it will transpose when the *tnpA* product of Tn3 is provided in trans (Cornelis et al., 1981). Tn2501 is a cryptic transposon contained within Tn951. Its structural organization is similar to that of Tn3, but its transposition functions show more homology with Tn21 and Tn501, so that it appears to represent a new subfamily of the class II elements, sharing characteristics of both the Tn3 and Tn501 groups. Sequence homology between Tn951 and Tn3 suggests that Tn951 was created by the insertion of Tn2501 into Tn3 (Michiels et

al., 1987).

This arrangement is also demonstrated by the Tol transposons Tn4651 and Tn4653. Tn4651 carries the *xyl* genes for toluene degradation, and is contained within Tn4653 (Tsuda and Iino, 1988). There is extensive homology in the IRs of Tn4653 and Tn1721, and in the ends of the *tnpR* genes. There is further homology in sites II and III of their *res* regions. This suggests that the insertion of Tn4651 into a Tn1721-like element led to the creation of Tn4653 (Tsuda and Iino, 1989).

There is a slightly different relationship between Tn1721 and Tn1722. Tn1721 encodes both of its transposition functions, as well as tetracycline resistance, with the tetracycline (*tet*) region located adjacent to the transposition genes (Schmitt and Rogowsky, 1985). The entire element is flanked by two IRs. However, a third IR, located between the *tet* region and the transposition genes enables the smaller segment, designated Tn1722, to transpose without the *tet* region (Schöffl et al., 1981). The difference in organization from the earlier example lies in that transposition of both elements is encoded by the same genes. However, the arrangement of Tn1721 still suggests that it was generated by the insertion of Tn1722 into a related element, with considerable deletion of adjacent sequences.

2.4 IncP plasmids

2.4.1 Incompatibility

Genetic rearrangements are not the only means by which bacteria acquire new phenotypes. Genetic exchange can occur via conjugation, in which plasmids are transferred through contact between two bacteria. The ability for conjugation is usually encoded on the plasmids. Transfer can occur within and between species; many conjugative plasmids have a broad host range, so their replication and transfer are therefore not limited to a single species. Interspecies transfer is important in bacterial evolution, and in the dissemination of plasmids (Guiney and Lanka, 1989).

The IncP group of plasmids have a very broad host range, covering most Gram negative bacteria (Holloway, 1986; Smith and Thomas, 1989). The incompatibility group defines plasmids which are unable to coexist autonomously in the same cell; they share replication functions and thereby compete for stable inheritance (Bayley et al., 1979; Thomas and Smith, 1987). This incompatibility reflects an evolutionary relationship, in that the incompatible plasmids share a great deal of homology (Bayley et al., 1979).

IncP plasmids can transfer to and be maintained in almost all Gram negative species of bacteria; the only exceptions are *Myxococcus* and *Bacterioides*. The plasmids are capable of transfer but not maintenance within these two species (Thomas and Helinski, 1989).

The IncP backbone consists of a block of conjugal transfer genes and a block of replicative and maintenance functions (Thomas and Helinski, 1989). The order of the genetic loci on all the plasmids is essentially the same, indicating that the backbone region is well conserved (Smith and Thomas, 1989).

There are three subgroups of IncP plasmids: the α subgroup, the β subgroup, and those that do not fit into the other two (Thomas and Helinski, 1989). IncP α and IncP β are defined by similarities in the replication and transfer loci. In addition, almost all the IncP α plasmids encode resistance to tetracycline and kanamycin. There are similarities between the two subgroups in the arrangement of the regions for maintenance and transfer, but not in the restriction patterns (Smith and Thomas, 1989).

Two different IncP plasmids should not be able to coexist in the same cell; however, if one plasmid is integrated into the chromosome, it can be compatible with another (Grinter, 1984). The phenomenon of integrative compatibility has been shown using cells carrying two copies of the IncP plasmid RP4, one in the chromosome and one replicating autonomously. When a different IncP plasmid was transferred into the cells, the autonomous copy of RP4 was lost but the integrated copy remained. When selection for the second plasmid was removed, the integrated copy of RP4 was restored to its autonomous state. It showed no mutation

in either the incompatibility or replication functions (Watson and Scaife, 1980).

The ability of an IncP plasmid to integrate into the chromosome can be enhanced by the presence of transposons on the plasmid, or by regions of homology between the plasmid and the chromosome (Guiney and Lanka, 1989). While an integrated IncP plasmid will not block the replication of a second IncP plasmid, it can affect the copy number of the second plasmid. This suggests that replication control may involve diffusible regulatory molecules (Grinter, 1984).

Incompatibility occurs due to the replication control mechanism of the plasmids. A second mechanism, plasmid entry exclusion, also prevents coexistence of similar plasmids in the same cell. Conjugative plasmids have been shown to encode an exclusion property which discourages transmission of similar plasmids into the same cell (Heinemann and Ankenbauer, 1993).

2.4.2 Transfer functions

IncP plasmids are self-transmissible at high frequencies (Thomas and Smith, 1987). Transfer requires the *oriT* site in *cis*, and many *trans* acting functions which are encoded in the *Tral*, 2 and 3 regions (Guiney and Lanka, 1989).

The *oriT* region, or origin of transfer, is the site from which transfer is initiated by a single-stranded nick

in the plasmid (Guiney and Lanka, 1989). The nick occurs at one end of the 38 bp inverted repeats within the *oriT* region (Thomas and Smith, 1987). *oriT* and the nick site are located near the end of the *TraI* region (Guiney and Lanka, 1989).

The 2.5 kb segment of DNA which encodes *oriT* also encodes several proteins. The complex of the proteins and plasmid DNA at the *oriT* site is known as the relaxosome, and its formation is essential for conjugation (Thomas and Smith, 1987).

TraI is transcribed divergently from two promoters located near the *oriT* region, although the number of proteins encoded is unclear (Guiney and Lanka, 1989; Thomas and Smith, 1987). *Tra2* and 3 may form a single unit on the plasmid, although insertions between the two regions do not block transfer (Thomas and Smith, 1987).

Although it is not essential, the *pri* gene is also involved in conjugation; inactivation of *pri* lowers the conjugation frequency (Thomas and Smith, 1987). *pri* is located in the *TraI* region. The gene encodes a DNA primase which converts single stranded circular DNA to its duplex form (Guiney and Lanka, 1989).

The transfer functions are partially interchangeable between *IncPa* and *IncP β* plasmids. The *oriT/TraI* region of plasmids from either subgroup can be complemented by the *Tra2/Tra3* regions of the other (Smith and Thomas, 1989).

There are limits to this interchangeability. IncP β plasmids can mobilize plasmids with the sequence encoding *oriT* and the relaxosome proteins from IncP α plasmids, but only other IncP α plasmids can mobilize plasmids with the minimal *oriT* sequence (Thomas and Smith, 1987).

2.4.3 Replication functions

Replication of IncP plasmids requires two essential loci, *oriV* and *trfA*. *oriV* is the origin of vegetative replication, and *trfA* encodes a trans-acting replication function which activates *oriV*. *oriV* is required in *cis*, and *trfA* can be supplied in *trans* (Thomas and Smith, 1987; Thomas and Helinski, 1989).

The minimum length of *oriV* required for replication is 393 bp, but the regulatory functions are included in a longer segment of about 1000 bp. *oriV* contains five 17 bp tandem repeats, each separated by 22 or 23 bp. Three additional directly repeated but unevenly spaced copies are located upstream; these seem to be involved in plasmid incompatibility and copy number control. Two other, inexact copies are located downstream, one being directly repeated and the other inversely repeated. Their role is not certain (Thomas and Helinski, 1989; Thomas and Smith, 1987).

trfA is the second gene in the *trfA* operon, and is expressed from a broad host range promoter. The gene encodes two polypeptides of different sizes due to two

translation start sites in the same ORF (Thomas and Smith, 1987). Footprinting experiments have shown that these proteins bind to the direct repeats in the *oriV* region (Thomas and Helinski, 1989).

The *oriV* regions from IncP α and IncP β plasmids show 65% homology to each other, and the *trfA* products from either subgroup can complement the *oriV* regions from the other (Smith and Thomas, 1989).

2.4.4 Regulatory functions

The *trfA* operon consists of more than the *trfA* gene; it also encodes functions which are lethal to certain hosts, or which inhibit plasmid maintenance. Other IncP plasmid genes encode similar potentially lethal functions. Without regulatory functions to repress transcription of these genes, the plasmids could not be maintained (Thomas and Smith, 1987).

Many of the regulatory functions are encoded in the *trfB* operon. Proteins encoded within this operon operate individually or with others to repress potentially lethal phenotypes or to control plasmid copy number (Figure 2.3). Many of the regulatory features are interchangeable between the IncP α and IncP β subgroups (Thomas and Helinski, 1989).

Therefore, the replication functions are not sufficient for stable maintenance of IncP plasmids. The minimal *oriV* segment and the *trfA* gene have been shown to be sufficient

Figure 2.3 Regulation of IncP plasmids. The known regulatory functions of proteins encoded within the IncP backbone of the plasmid RK2 are shown. The dark arrows indicate negative regulation, and the open arrows show positive regulation. *kil*, host lethal functions; *trf*, trans-acting replication functions; *oriV*, origin of vegetative replication; *kor*, *kil* override; *kcr* and *kfr*, regulated by KorC and KorF respectively (from Thomas and Helinski, 1989).

to express incompatibility. However, the association of the lethal *KilD* phenotype with the *trfA* operon creates the necessity for functions to suppress expression of *kilD*. Within the IncP backbone there are three other *kil* genes; although their role is not understood, their expression is lethal to the host. The regulation of these genes is essential (Thomas and Smith, 1987).

While the complexities of the regulatory networks are not completely understood, it is clear that IncP plasmids depend upon more than the minimal replication functions. Products encoded by the *trfB* operon are strongly involved in regulating a series of operons. Many of these would otherwise be lethal to the host, indicating the importance of the *trfB* operon to the stable inheritance of IncP plasmids (Thomas and Helinski, 1989).

2.5 Plasmids carrying catabolic pathways

2.5.1 Tol plasmids

The Tol plasmids are a diverse group of plasmids which encode degradation of *m*- or *p*-methylbenzene, 1,2,4-trimethylbenzene, 3-ethyltoluene, toluene and their alcohol and acid derivatives (Frantz and Chakrabarty, 1986). They have been found mostly in *Pseudomonas* spp. isolated all over the world. For example, pWVO was found in *P. putida* mt-2 which was isolated in Japan in the 1950s, pDK1 was found in *P. putida* HS1 isolated in Minnesota, and pWW53 was found in

P. putida MT53 isolated in North Wales (Shaw and Williams, 1988). The various Tol plasmids range in size, transmissibility and incompatibility groups, but all carry toluene degradation genes (Frantz and Chakrabarty, 1986).

The most characterized Tol plasmid is the 117 kb, IncP plasmid pWWO (Burlage et al., 1989). *P. putida* mt-2 was thought to have toluene and benzene degradation genes carried on a plasmid because of the unusually high loss of the Tol phenotype. Spontaneously derived Tol⁻ derivatives were shown to have lost a unique plasmid found in the parent strain, or to have had a large fragment of the plasmid deleted (Bayley et al., 1977; Williams and Murray, 1974).

The catabolic pathways encoded on pWWO are organized into three units. These encode genes for the oxidation of the methyl group of toluene to give benzoate, genes for benzoate metabolism through the meta ring-fission pathway, and two regulatory genes (Burlage et al., 1989). Comparison of the organization of pDK1 to that of pWWO showed many similarities. The pathway was organized into the same three units, and the gene order was conserved within each unit, shown by conservation of the restriction sites. However, the conservation existed only in the coding regions. There were differences in the relative positions of the three units, indicating that the acquisition of the genes was not due to insertion of a single continuous segment of DNA (Shaw and Williams, 1988).

Plasmid pWWO was shown to undergo spontaneous excision of a 39 kb fragment, resulting in the loss of the Tol phenotype. This excision was thought to occur by reciprocal recombination between directly repeated sequences of 1.4 kb (Meulien et al., 1981), and was RecA dependent (Frantz and Chakrabarty, 1986). Independently isolated hybrids of R-factor plasmids and pWWO showed the amount of pWWO plasmid DNA which had incorporated varied from 56 to 104 kb. This showed that if transposition had occurred, it did not involve a unique segment of pWWO DNA (Lehrbach et al., 1982). It also suggested that the 39 kb segment flanked by the direct repeats was not a transposon, since all the cointegrates formed carried more than 39 kb of pWWO DNA (Meulien et al., 1981).

Further genetic rearrangements of Tol DNA were shown in *P. putida* strain MW1000, which had the same growth characteristics as strains carrying a pWWO plasmid. The degradative genes were shown by DNA hybridization to be on the chromosome of the new strain. A 56 kb segment of the chromosome carrying the Tol genes transposed onto the IncP plasmid R18-18, and the fragment was almost identical to the same 56 kb region of pWWO (Sinclair et al., 1986).

The 56 kb fragment was identified as a transposon, designated Tn4651. Its internal deletant, in which the 39 kb fragment carrying the *xyl* genes was lost, was designated Tn4652. Tn4651 was able to transpose in *recA* strains of *E.*

coli. It was identified as a class II transposon, in that it transposed through a cointegrate intermediate. A transposase gene was identified at one end of the element. The resolution activity of Tn4651 was weaker than that of other class II elements, so unstable cointegrates were often isolated. Two resolution genes were found (*tnpS* and *tnpT*), but they were not located near the *tnpA* gene, and were not similar to *tnpR* genes. However, the resolvase of the transposon $\gamma\delta$ has two functional domains, one for recognition of the *res* sites, and the other for mediating site-specific recombination. It is possible that these two domains are represented by the *tnpS* and *tnpT* products from Tn4651 (Tsuda and Iino, 1987).

A second transposon, Tn4653, was also identified. The 79 kb element included Tn4651. Like Tn4651, it forms an internal deletant in which the *xyl* genes are lost, designated Tn4654. Tn4653 is also a class II transposon, going through a cointegrate intermediate. A transposase gene was found encoded at one end of Tn4653 (Tsuda and Iino, 1988). Tn4653 carries a *tnpR* gene which complements resolvases from other class II elements. It also carries a *res* region adjacent to the *tnpR* gene, but its resolution system does not function properly, due to the incomplete structure of the *res* region (Tsuda et al., 1989).

Further characterization of Tn4651 and Tn4653 placed them in the appropriate subfamilies of the class II

elements. The two elements have 46 and 38 bp inverted terminal repeats respectively, and both generate 5 bp duplications upon transposition. Complementation tests with Tn3, Tn21 and Tn1721 showed that the transposase from Tn4651 was not interchangeable with any other element, the Tn4653 resolvase complemented Tn21 and Tn1721, and the Tn4653 transposase was interchangeable with that of Tn1721. Based on these results, Tn4653 was placed in the Tn501-Tn1721 subgroup and Tn4651 was placed in an entirely new subgroup (Tsuda et al., 1989).

2.5.2 Chloroaromatic degradative plasmids

Perhaps one of the most recalcitrant groups of environmental pollutants is the chlorinated aromatic compounds. Such compounds include chlorobenzoates and chlorobenzenes. These compounds are degraded slowly or not at all by microorganisms (Feineke and Knackmuss, 1988). However, many bacteria capable of metabolizing such compounds have been isolated, with the degradative genes carried either on the chromosome or on plasmids (Sayler et al., 1990).

Plasmids encoding 3Cba degradation have been isolated from a variety of sources. Plasmid pAC25 was found in the 3Cba degrading strain of *P. putida* AC858 isolated from sewage samples (Chatterjee et al., 1981). The plasmid pWR1 was found in *Pseudomonas* sp. strain B13, which could grow on

3Cba, 4-fluorobenzoate and 4-chlorophenol (Weisshaar et al., 1987). This plasmid was later shown to have extensive homology to pAC27, a derivative of pAC25 (Ghosal et al., 1985b). Plasmid pJP4 was found in *Alcaligenes eutrophus*. This 80 kb IncP β plasmid encodes resistance to mercuric chloride and phenyl mercury acetate and degradation of 2,4-dichlorophenoxyacetic acid (2,4-D), 2-methyl-4-chlorophenoxyacetic acid and 3Cba (Don and Pemberton, 1985). Another 3Cba degradative plasmid, pBRC60, was found in *Alcaligenes* sp. BR60, isolated from surface runoff water from an industrial landfill site (Wyndham and Straus, 1988).

The genes specifying degradation of 3Cba on pAC25 are clustered on a 4.2 kb region. On pJP4, the 3Cba degradative genes are carried on a 10 kb segment of DNA. Homology between these two DNA fragments, shown by hybridization as well as the similarity of the biochemical pathways they encode, suggests a common evolutionary origin. However, there is a significant difference in the regulation of the genes; pAC25 genes are readily expressed, but the genes from pJP4 behave like a cryptic operon, with occasional activation by complex rearrangements (Ghosal et al., 1985a).

The role played by plasmids in conferring new phenotypes was demonstrated when *P. putida* carrying pAC25 was grown in the presence of cells carrying the Tol plasmid pWWO. Selection on 4Cba resulted in the isolation of cells which could degrade both 3- and 4Cba. These were found to

carry a derivative of pAC25, designated pAC27, and to have a 41.5 kb segment of pWWO DNA inserted into the chromosome (Chatterjee and Chakrabarty, 1982). pAC27 was seven kb smaller than pAC25 (Chatterjee and Chakrabarty, 1984).

Further enrichment with 3,5-dichlorobenzoate (3,5dCba) still in the presence of pWWO resulted in the isolation of cells with a 3,5dCba degradative phenotype. Plasmid analysis showed the appearance of a third plasmid, pAC29, which was derived mainly via duplication of a fragment of pAC27 and a fragment of pWWO DNA (Chatterjee and Chakrabarty, 1982). A fourth plasmid, pAC31, also encoded degradation of 3,5dCba (Chatterjee and Chakrabarty, 1984).

Plasmid pAC27 encodes three enzymes which degrade chlorobenzoate (Cba) to chlorocatechol (Clc). Similarities have been shown in the organization and regulation of these genes and the analogous genes for degradation of benzoate to catechol. The *clc* gene cluster is organized as the operon *clcABD* under control of a regulatory protein encoded by *clcR*. The chromosomally encoded catechol (*cat*) genes are organized in a similar operon, *catBC*, with a positive regulatory protein encoded by *catR*. The promoters for the two operons show considerable homology. Within the two operons there is high sequence homology at both the DNA and amino acid level. This suggests that the organisms expressing *clcABD* and *clcR* evolved from a benzoate degrading organism expressing ancestral *cat* genes (Rothmel et al.,

1989).

Recruitment of chromosomal genes in the evolution of a new degradative pathway is also shown by the recombinant *P. putida* strain CB1-9. The parent strains *P. putida* R5-3 and *P. alcaligenes* C-0 were able to metabolize a range of aromatic hydrocarbons, and 3Cba respectively. The recombinant strain was able to use 3Cba, chlorobenzene and 1,4-dichlorobenzene. It carried a 33 kb plasmid, pKFL3, which showed no homology to a 15 kb plasmid pKFL1 in its *P. alcaligenes* parent, but showed some homology to the 55 kb plasmid pKFL2 in its *P. putida* parent. The plasmid also showed homology to chromosomal DNA from its *P. alcaligenes* parent. The development of the novel phenotype appeared to be associated with the mobilization of chromosomal DNA (Carney et al., 1989).

The 3Cba degradative plasmids are not the only known chloroaromatic degradative plasmids. The plasmid pP51, found in *Pseudomonas* sp. strain P51, encodes genes for the degradation of 1,2-dichloro-, 1,4-dichloro- and 1,2,4-trichlorobenzene (van der Meer et al., 1991a; 1991b). Chlorobenzene metabolism is encoded in two gene clusters on pP51. Conversion of chlorobenzenes to chlorocatechols is encoded by the operon *tcbAB*, which is similar to the *xylXYZ* operon on pWWO. Chlorocatechol oxidation by a modified ortho cleavage pathway is encoded by the operon *tcbCDEF*, which is similar to the *clcABD* operon in *P. putida* (van der

Meer et al., 1991a).

The *tcbAB* operon is located on a composite class I transposon, designated Tn5280. The transposon is flanked by the inversely oriented insertion elements IS1066 and IS1067, which differ by a single base pair. The identification of the transposable element suggests that the chlorobenzene degradative pathway evolved by the recruitment of the *tcbAB* operon to combine with the *tcbCDEF* operon, thereby creating a complete chlorobenzene degradative pathway (van der Meer et al., 1991b).

There is considerable homology among the chloroaromatic degradative plasmids. Plasmid pAC25 may have evolved by recruiting genes from other plasmids. There is some homology between pAC25 and pJP4, even though they have different incompatibilities and host ranges (Frantz and Chakrabarty, 1986). The non-halogenated aromatic degradative plasmids, like pWWO, the NAH plasmids which encode naphthalene degradation and the SAL plasmids which encode salicylate degradation also show similarities to one another (Harayama et al., 1987). These similarities suggest evolutionary relationships among degradative plasmids (Frantz and Chakrabarty, 1986).

3. Methods and Materials

3.1 Bacterial strains and growth conditions

All the strains used are listed in Table 3.1.

Alcaligenes sp. BR60 has been previously described (Wyndham and Straus, 1988; Wyndham et al., 1988). The strain was grown on 4 mM 3-chlorobenzoate (Aldrich) in minimal medium A (Wyndham, 1986) and 1.6% agar (Difco) (3Cba). *Alcaligenes* sp. BR6024 is a tryptophan requiring auxotroph, derived from BR60 by selection for spontaneous chloramphenicol resistant mutants, followed by mutagenesis with N-methyl-N'-nitro-N-nitrosoguanidine (Fulthorpe and Wyndham, 1991). BR6024 was grown on 3Cba supplemented with 0.1 mM tryptophan (Sigma) and 1.6% agar (3Cba + Trp_{0.1}).

Both *Comomonas acidovorans* and *C. testosteroni* were maintained on 1% tryptone (Difco), 1% yeast extract (Difco), 0.5% NaCl (TYE) with 1.6% agar. The *E. coli* strains were grown on TYE supplemented with the appropriate antibiotic: 50 µg/mL ampicillin (Sigma), 50 µg/mL kanamycin (Sigma) or 20 µg/mL tetracycline (Sigma).

3.2 Generation of experimental isolates

3.2.1 pBRC60 matings

The first set of matings were performed to conjugate the plasmid pBRC60 from *Alcaligenes* sp. BR6024 into *C. acidovorans* or *C. testosteroni*. Standard filter matings were performed using 25 mm millipore filters, 0.22 µm pore

Table 3.1 Strains and plasmids

<u>Strain</u>	<u>Plasmid (phenotype)</u>	<u>Source</u>
<i>Alcaligenes</i> sp.		
BR60	pBRC60 (3Cba ⁺)	R.C. Wyndham
BR6024	pBRC60 (3Cba ⁺ Trp ⁻ Cm ^r)	R.C. Wyndham
<i>Comomonas acidovorans</i>		ATCC # 15668
<i>Comomonas testosteroni</i>		ATCC # 11996
<i>E. coli</i>		
BW97	pGS65 (Km ^r Tc ^r IncP mob)	V.N. Iyer
MM294	pRK2013 (Km ^r tra ⁺)	D.R. Helinski
JM109	pBRH2	R.C. Wyndham
JM109	pBRH4	R.C. Wyndham
JM109	pBRN4029	R.C. Wyndham
JM101	pBRE7/8	R.C. Wyndham
JM101	pBRE9	R.C. Wyndham
JM101	pBRE17	R.C. Wyndham

size. The required strains were grown overnight in 5 mL of TYE broth, then 50 μ L of the donor and recipient strains were placed on filters on individual TYE plates. A third filter on a TYE plate received 50 μ L of each strain. After the filters had dried, all three plates were incubated overnight at 32 °C.

The growth on the filter was removed by vortexing the filter in a test tube of 4.5 mL medium A. This was considered the 10^0 dilution. A serial dilution was taken down to a concentration of 10^{-6} , and 50 μ L of the required concentrations were plated onto the appropriate selective plates. The donor and recipient strains were plated onto 3Cba + Trp_{0.1} and TYE respectively, from the 10^{-4} to 10^{-6} concentrations. Transconjugants from every dilution of the mating mixture were selected on 3Cba.

3.2.2 Triparental IncP matings

Triparental filter matings were performed to mobilize the IncP plasmid pGS65 into transconjugants selected from the first matings. Three control filters were placed on separate TYE plates, each receiving 50 μ L of *E. coli* MM294(pRK2013), *E. coli* BW97(pGS65) or the recipient. A fourth mating filter on a fourth TYE plate carried 50 μ L of each of the three strains. The four plates were incubated overnight at 32 °C.

Growth from the filters was removed as before, and the

same dilution series carried out in medium A. The recipients were selected on 3Cba. *E. coli* BW97 and MM294 were selected on TYE + Km₅₀Tc₂₀ and TYE + Km₅₀ respectively. The secondary transconjugants from the mating filter were selected on 3Cba + Km₅₀Tc₂₀. Frequencies of conjugation were expressed as numbers of transconjugants per potential recipient.

3.2.3 Replica plating for deletion derivatives

Secondary transconjugants were taken from a 3Cba + Km₅₀Tc₂₀ plate and grown overnight in 5 mL of TYE + Km₅₀Tc₂₀. A dilution series in medium A was taken to 10⁻⁵, and 50 µL were plated onto TYE + Km₅₀Tc₂₀. The plates were incubated overnight at 32 °C. When the colonies had developed, usually after two days, 500 colonies were patched onto 3Cba + Km₅₀Tc₂₀ and TYE + Km₅₀Tc₂₀. Replicates which did not grow on 3Cba were screened by colony hybridization for the presence of IS1071.

3.3 DNA extractions

3.3.1 Large-scale plasmid extractions

Many of the required probes had previously been cloned into a pUC18 vector and grown in *E. coli* (Wyndham et al., 1988). These plasmids were extracted using a large-scale alkaline lysis method (Sambrook et al., 1989). The clones were grown overnight in 5 mL of TYE + Ap₅₀, then 2.5 mL were

transferred to 25 mL of TYE + Ap₅₀. When that culture had grown to mid-log phase, 12.5 mL were transferred to 250 mL of TYE, and again grown to mid-log phase. Chloramphenicol (Sigma) was added to a final concentration of 50 µg/mL, and the cultures were again incubated overnight at 37 °C.

The cells were harvested by centrifugation (Sorvall) at 7K rpm, 4 °C for 15 minutes, then washed in 50 mL of 100 mM NaCl, 10 mM Tris pH 7.6 and 1 mM EDTA (STE buffer). The pellets were resuspended in 2.5 mL 25 mM Tris pH 8, 10 mM EDTA, 50 mM glucose (TEG buffer); 2 mL of lysozyme (12 mg/mL in 25 mM Tris pH 8) were added and the tubes were incubated at room temperature for 5 minutes. Fresh 0.2 M NaOH/2% SDS was mixed and 9 mL were added to each tube, then the tubes were placed on ice for 10 minutes. Potassium acetate solution containing 9 mL of 5M potassium acetate, 1.73 mL of glacial acetic acid, and 4.28 mL of distilled water was mixed; 6.5 mL were added to each of the tubes, which were again left on ice for 10 minutes. The precipitate was removed by centrifugation (IEC) at 12K rpm for 20 minutes at 4°C, and the supernatants were transferred to clean tubes.

The supernatant was extracted once with 1:1 phenol (BDH):chloroform (BDH), then once with chloroform alone, with the aqueous layer being transferred to a clean tube. The DNA was precipitated with two volumes of cold absolute ethanol, overnight at -20°C.

DNA was pelleted by centrifuging for 20 minutes (IEC)

10K rpm, 4°C. The DNA pellets were rinsed with cold 70% ethanol, then dessicated dry, dissolved in a total of 200 µL of buffer (10 mM tris, 1 mM EDTA, pH 8) (TE) and stored at -20°C.

3.3.2 Small-scale plasmid extractions

Plasmids from the experimental isolates were screened using a small-scale alkaline lysis method (Sambrook et al., 1989). Isolates were grown overnight in 5 mL of TYE broth, with selection if necessary; 1.5 mL of these cultures were transferred to microfuge tubes, and the cells harvested by centrifuging for 30 seconds at 12K rpm, 4°C. As much of the supernatant as possible was removed.

The pellets were resuspended in 100 µL of cold solution 1 (50 mM glucose, 25 mM Tris, 10 mM EDTA, pH 8) by vortexing. Fresh 0.2M NaOH/1% SDS was mixed, then 200 µL were added to each tubes. The tubes were mixed by inverting and placed on ice. Following the addition of 150 µL of solution 3 (60 mL potassium acetate, 11.5 mL glacial acetic acid, 28.5 mL distilled H₂O) the tubes were gently vortexed and incubated on ice for 5 minutes.

The precipitate was removed by centrifugation for 10 minutes at 12K rpm and 4°C, and the supernatants were transferred to fresh tubes. They were extracted once with phenol:chloroform and the aqueous lay . transferred. The DNA was precipitated overnight at -20°C with 2 volumes of

absolute ethanol, then pelleted, washed with cold 70% ethanol and dried. Each pellet was resuspended in 50 μ L of TE buffer, and stored at -20°C .

3.3.3 Genomic DNA extraction

Genomic DNA was extracted using minipreps (Ausubel et al., 1989). Isolates were grown overnight in 5 mL of TYE, with selection if necessary. The cells were pelleted by centrifuging 5 minutes, 10K rpm, 4°C , and as much as possible of the supernatant was removed. The pellets were resuspended in 1.9 mL of TE buffer. 50 μ L of 20% SDS and 10 μ L of proteinase K (20 mg/mL) (Sigma) were added, and the tubes were gently vortexed and incubated for one hour at 37°C .

Following the incubation, 333 μ L of 5M NaCl were added and the tubes were mixed. A solution of 10% hexadecyltrimethyl ammonium bromide (Sigma) in 0.7 M NaCl (CTAB/NaCl) was pre-warmed, and 267 μ L were added and the contents of the tubes were mixed, then incubated for 10 minutes at 65°C . Each tube was extracted with 5 mL of chloroform, vortexed for 30 seconds and centrifuged for 10 minutes at 10 K rpm, 4°C . The aqueous layer was transferred to a clean tube and extracted with 5 mL phenol:chloroform, and the aqueous layer was transferred. The DNA was precipitated overnight with 6 mL of cold isopropanol. The DNA was pelleted, washed with cold 70%

ethanol, dried and dissolved in 250 μ L of TE buffer, and stored at -20 °C.

3.4 Restriction digests

Standard restriction digests with *Bgl*II, *Eco*RI, *Nhe*I, *Nru*I, *Sal*I, *Sma*I (New England Biolabs) and *Sac*I (Boehringer Mannheim) were performed (Sambrook et al., 1989). Molecular biology grade agarose (Bethesda Research Laboratories) was heated in Tris-acetate buffer (0.04 M Tris-acetate, 0.001 M EDTA) by microwaving on high power for 1 to 3 minutes, depending on the concentration and the size of the gel. Gels were poured at 1.0%, except those for plasmids (0.7%) and the gels used for mapping (0.6%). The restricted DNA was loaded into the gels in horizontal gel electrophoresis chambers (BIO-RAD). Following electrophoresis, the gels were stained in 0.5 μ g/mL ethidium bromide (Sigma) and photographed on Polaroid Type 57 high-speed film, using a Polaroid MP-4 Land camera with the f-stop at 4.5 and an exposure time of 1 second.

3.5 Southern blots

A standard capillary transfer procedure was carried out to bind the DNA to nylon membranes (Sambrook et al., 1989). The gels were trimmed and exposed to UV light for 5 minutes. They were soaked for 45 minutes in denaturing solution (1.5 M NaCl, 0.5 M NaOH) at room temperature, rinsed with

distilled water and soaked for another 45 minutes in neutralizing solution (1 M Tris pH 7.4, 1.5 M NaCl) at room temperature.

Approximately 400 mL of transfer buffer (3 M NaCl, 0.3 M sodium citrate, pH 7.0) (20XSSC) was used for each blot. Two pieces of 3MM Whatman chromatography paper cut to the size of the gel were placed on the support. The gel was inverted and placed on the Whatman papers. A nylon filter (Magnagraph nylon transfer membrane), cut just larger than the gel, was placed on the inverted gel, followed by two more pieces of cut Whatman paper. The sides of the gel were blocked with strips of parafilm. A stack of cut paper towels was placed on top, followed by a glass plate and a weight. The transfer was allowed to proceed overnight.

When the transfer was dismantled, the positions of the wells were marked on the filter with a pencil before the gel was removed. The filter was soaked for 5 minutes in 6XSSC to remove any agarose, then air dried. The gel was checked on the UV transilluminator to ensure all the DNA had transferred, and the DNA side of the filter was exposed to UV radiation for 5 minutes to fix the DNA.

3.6 DNA Hybridizations

3.6.1 Probe preparation

Some probes had previously been cloned into pUC18 (Wyndham et al., 1988). The rest were cut directly from the

appropriate restriction digest of pBRC40 plasmid DNA. The required fragments were cut out of 1% SeaPlaque (FMC Mandel) agarose gels and purified using the Geneclean system (Bio/Can Scientific) according to the manufacturer's instructions. Between 10 ng and 3 μ g of DNA in 15 μ L of sterile distilled water was placed in a microfuge tube and denatured by boiling for 10 minutes; following this the tubes were placed immediately on ice. To label the probes for non-radioactive detection, 2 μ L of hexanucleotide random primer (Boehringer Mannheim) and 2 μ L of dNTP labelling solution containing the digoxigenin-11-dUTP (Boehringer Mannheim) were added, and the contents of the tubes were mixed and then briefly centrifuged. Finally, 1 μ L of Klenow polymerase was added, the contents of the tube were mixed again, and the labelling mixture was incubated overnight at 37 °C. Between 3 and 5 μ L of probe was used for each hybridization.

3.6.2 Filter hybridizations

All hybridizations were carried out in a Tyler hybridization incubator model HI-12000. The fixed filters were wetted in 2XSSC/0.1% SDS, then prehybridized at 68 °C for at least two hours in 10 mL of hybridization solution (5XSSC, 0.1% N-lauroylsarcosine, 0.02% SDS, 2% Carnation skim milk powder), which had been prepared in advance and heated to 70 °C. For the hybridization, 3 to 5 μ L of the

appropriate probe was suspended in 0.5 mL of hybridization solution and boiled for 10 minutes. The prehybridization solution on the filters was replaced with fresh hybridization solution, the probe was added and the filters were replaced into the incubator to hybridize overnight.

The filters were washed twice for 5 minutes at room temperature with 2XSSC/0.1%SDS, then twice for 15 minutes at 68 °C with 0.1XSSC/0.1%SDS. All subsequent incubations were at room temperature. The filters were washed with buffer 1 (100 mM Tris, 150 mM NaCl, pH 7.5), then for 30 minutes with 1% Carnation skim milk powder in buffer 1, which had been prepared in advance at 70 °C. The filters were again washed in buffer 1, then incubated for 1 hour with 2 µL of alkaline phosphatase labelled anti-digoxigenin antibody (Boehringer Mannheim) in 10 mL of buffer 1. Finally, the filters were washed twice for 15 minutes in buffer 1, then briefly in buffer 3 (100 mM Tris, 100 mM NaCl, 50 mM MgCl₂, pH 9.5). For the final colour reaction, each filter was sealed in a bag with 10 mL of colour solution (45 µL 4-nitro blue tetrazolium chloride (Boehringer Mannheim), 35 µL 5-brom-4-chlor-3-indolyl-phosphat (Boehringer Mannheim), 10 mL buffer 3), and stored in the dark. Most colour reactions took less than an hour to develop.

Filters were photographed on Polaroid Type 55 positive/negative film, with the F-stop at 4.5 and an exposure time of 1/30 seconds. The negatives were developed

for one minute into 10% sodium sulfite (Kodak), rinsed in distilled water and dried. The sizes of the DNA bands were determined using Bandsizer, an IBM PC program developed in Toronto (Wyndham et al., 1988).

3.6.3 Stripping filters

For filters that were to be reprobed, the colour was removed as completely as possible by soaking the filter in dimethylformamide (BDH) at room temperature, for up to two hours, with frequent changes of DMF. The probe was stripped by soaking the filter in 0.2 M NaOH, 0.1% SDS for 30 minutes at 37 °C, then washing 3 times in 2XSSC for 15 minutes each. These filters were never allowed to dry, and were kept in the dark as much as possible.

3.6.4 Colony hybridizations

Large numbers of isolates were screened for the presence of the 3Cba genes or of the insertion element IS1071 by rapid colony screening on nylon filters (Buluwela et al., 1989). Colonies were patched onto a nylon filter (Magnagraph nylon transfer membranes) on a TYE plate, with antibiotic selection if required, and incubated overnight at 30°C. Strains known to probe positive and negative were included as controls.

Filters were placed on Whatman paper soaked with 2XSSC/5%SDS for 15 minutes at room temperature to lyse the

cells, then microwaved on high power for 40 seconds to fix the DNA to the filters. Subsequent hybridization was performed at the same stringency and with the same procedure as the filter hybridizations.

3.7 Ligations

Chromosomal fragments to be amplified were cut out of a gel of the appropriate restriction digest. Since the exact fragment could not be identified on the gel, several thin bands were excised and the DNA purified using the Geneclean (Bio/Can Scientific) method. A small amount from each sample was run down a small gel by electrophoresis, then transferred and probed for the presence of IS1071. The sample which hybridized was used for the ligation.

Because the DNA to be amplified was outside of the insertion element on the fragment, the fragment was first circularized by ligating the ends together (Triglia *et al.*, 1988). For the ligation reaction, concentrated (2,000,000 U/mL) T4 DNA ligase was used (New England Biolabs). A standard ligation reaction was performed (Sambrook *et al.*, 1989) except that half the required concentration of supplied buffer was used, to satisfy conditions for self-ligation. The ligation reactions were incubated for 4 hours at room temperature, and the ligation product was used directly in the PCR reactions.

3.8 Polymerase Chain Reaction

The primers used were ISOUT3070 and ISOUT124c (Oligonucleotide Synthesis Laboratory, Queen's University), which face outward from the insertion element (see Appendix 1 for details). For each 100 μ L PCR reaction, the reagent concentrations were 300 μ M of each dNTP (BRL), 0.5 μ M of each primer, 10 μ L of the ligation product for template, 1 U of Vent DNA polymerase (New England Biolabs) and 1X Vent buffer. For amplifying the larger control fragments, the MgSO_4 concentration was increased from 2 mM to 3-5 mM.

The DNA was amplified in a programmable Thermal Controller, model PTC-100, from Hypercell Biological. After an initial denaturation at 95 $^{\circ}\text{C}$ for 1 minute, the reactions were taken through 30 cycles of 1 minute denaturation at 94 $^{\circ}\text{C}$, 1 minute annealing at 52 $^{\circ}\text{C}$ and 2 minutes extension at 72 $^{\circ}\text{C}$. This was followed by 10 minutes at 72 $^{\circ}\text{C}$. The PCR products were detected by gel electrophoresis.

4. Results and Discussion

4.1 Generation of chromosomal recombinants

4.1.1 Experimental rationale

pBRC60 is a broad host range plasmid; in freshwater ecosystems it has been shown to transfer to a variety of Gram negative recipients. Lab matings showed pBRC40 which had been marked with Tn5 (for kanamycin resistance) could be conjugated into species in the β - and γ -subgroups of the proteobacteria (Fulthorpe and Wyndham, 1991).

Because pBRC60 belongs to the IncP incompatibility group (Burlage et al., 1990), it cannot coexist autonomously with a second IncP plasmid, due to competition of their replication control functions (Thomas and Smith, 1987). However, pBRC60 should be able to coexist with another IncP plasmid if one of them were integrated into the chromosome, due to the phenomenon of integrative compatibility (Watson and Scaife, 1980). Therefore, selection for another IncP plasmid in the same cell should force pBRC60 either to be lost from the host strain, or to recombine into the chromosome. The presence of the IncP plasmid pGS65 has been shown to force chromosomal recombination of pBRC60 in its host strain, *Alcaligenes* sp. BR60 (Ng and Wyndham, 1993). The recombination was thought to occur via homology between plasmid and chromosomally located copies of the insertion element IS1071 (Ng and Wyndham, 1993; Ng, 1991).

It was reasoned that similar pressure could force

pBRC60 to integrate into the chromosome of other host strains, even in the absence of chromosomal copies of IS1071. The presence of transposable elements IS1071 and Tn5271 on pBRC60 would augment its integration ability (Guiney and Lanka, 1989). A series of two matings would be required for each host (Figure 4.1). In the first, pBRC60 would be transferred into a new host. A second mating to transfer pGS65 into the same host would force pBRC60 to recombine into the chromosome, or to be diluted out of the population. Selection for determinants on both plasmids would ensure isolation of cells in which the former had occurred.

4.1.2 Conjugation of pBRC60 into *C. acidovorans* and *C. testosteroni*

The donor strain for the first set of matings was *Alcaligenes* sp. BR6024 carrying pBRC60. Because this strain is a tryptophan auxotroph, it could be selected against using a minimal medium (Fulthorpe and Wyndham, 1991). The recipient strains were obtained from the American Type Culture Collection. Neither had any resident plasmids, and neither was able to degrade 3Cba. Thus, transconjugants could be selected simply by growth on 3Cba in minimal medium A.

The recipients chosen for the matings were from the family Comamonadaceae, *C. acidovorans* and *C. testosteroni*.

Figure 4.1 Generation of chromosomal recombinants.

Chromosomal recombinants are generated by two successive matings to transfer incompatible plasmids into the recipients. In the first mating pBRC60 is transferred in, conferring a 3Cba⁺ phenotype on the recipient. In the second mating, pGS65 is mated in by a triparental mating, conferring resistance to kanamycin and tetracycline. Continuation of selection for 3Cba degradation in addition to kanamycin and tetracycline resistance selects for secondary transconjugants in which pBRC60 is integrated into the chromosome and pGS65 is replicated autonomously in the cytoplasm.

Preliminary experiments showed these to be the most successful of an assortment of potential recipients representing both the β and γ proteobacteria. Table 4.1 shows the conjugation frequencies for each mating. Both had high frequencies, although the frequency for *C. acidovorans* was ten times higher, at about 10^{-1} . Colony hybridizations of the isolates from the matings using the probe H4, which is specific for the insertion element (Figure 1.1) were positive for every isolate. The presence of IS1071 and the newly acquired ability to catabolize 3Cba indicated that pBRC60 had successfully transferred.

Plasmid analyses of several transconjugants from the two matings confirmed the presence of pBRC60 (Figure 4.2). The double plasmid band shows the presence of both pBRC60 and pBRC40, which results from the spontaneous deletion of the transposon region carrying the 3Cba genes. The deletion likely occurred when the transconjugants were taken off 3Cba selection overnight for growth for the plasmid extraction.

4.1.3 Conjugation of pGS65

pGS65 was derived from the IncP plasmid RK2. It contains the RK2 origin of transfer (*oriT*) and can be mobilized by transfer proficient IncP helper plasmids, like pRK2013 (Selvaraj and Iyer, 1985). Using triparental matings, pGS65 was conjugated into the transconjugants from the first set of matings which had been confirmed to carry

Table 4.1 Frequency of conjugation of pBRC60 into *C. acidovorans* and *C. testosteroni*

<u>Mating</u>	<u>CFU/mL</u>	<u>Frequency</u>
BR6024 X <i>C. acidovorans</i>		
transconjugants	2.8 x 10 ⁷	1.7 x 10 ⁻¹
recipients	1.7 x 10 ⁸	
BR6024 X <i>C. testosteroni</i>		
transconjugants	8.7 x 10 ⁶	1.4 x 10 ⁻²
recipients	6.1 x 10 ⁸	

Figure 4.2 Plasmids from the primary transconjugants. pBRC60 isolated from the transconjugants CT5 and CT7 from the mating of *C. testosteroni* with pBR6024, using alkaline lysis. The double band indicates the presence of both pBRC60 and pBRC40. pBRC60 isolated from *Alcaligenes* strain BR6024 is included as a control. Lane 1) λ /HindIII markers: 23.1 kb, 9.4 kb, 6.7 kb; 2) CT5; 3) CT7; 4) BR6024

1

2

3

4

pBRC60. pGS65 specifies resistance to both kanamycin and tetracycline, while pRK2013 specifies only kanamycin resistance. In addition, pRK2013 is not able to replicate in *Comomonas* species. Thus, transconjugants selected on 3Cba supplemented with kanamycin and tetracycline were presumed to have acquired pGS65, but still to carry pBRC60. Since the two plasmids belong to the same incompatibility group, one of them, presumably pBRC60, was reasoned to have integrated into the chromosome.

The conjugation frequencies for the second set of matings are shown in Table 4.2. Again, the results for both *C. acidovorans* and *C. testosteroni* are similar, showing frequencies in the order of 10^{-7} . These frequencies are much lower than for the first mating. However, all the secondary transconjugants were capable of 3Cba degradation and were resistant to both kanamycin and tetracycline. Colony hybridizations of selected isolates using the H2 probe which is specific for both the insertion element and for the degradative genes (Figure 1.1) were all positive.

Plasmid analyses of selected isolates were as expected in that pBRC60 could no longer be isolated from the secondary transconjugants. Interestingly, pGS65 was also seldom isolated from the transconjugants. This plasmid was also difficult to isolate from its host strain in parallel control experiments.

Table 4.2 Frequency of conjugation of pGS65 into recipients already carrying pBRC60

<u>Mating</u>	<u>CFU/mL</u>	<u>Frequency</u>
CA47 X BW97 X MM294		
2° transconjugants	2.7×10^2	6.9×10^{-7}
recipients	3.9×10^8	
CT3 X BW97 X MM294		
2° transconjugants	7.6×10^2	1.2×10^{-7}
recipients	6.5×10^9	
CT5 X BW97 X MM294		
2° transconjugants	1.0×10^2	1.7×10^{-7}
recipients	5.9×10^9	
CT7 X BW97 X MM294		
2° transconjugants	4.0×10^2	1.1×10^{-7}
recipients	3.8×10^9	

4.1.4 Discussion

The two recipients, *C. acidovorans* and *C. testosteroni*, are closely related members of the rRNA superfamily III, the β group of the proteobacteria (De Ley, 1992). Previous work on the transfer of pBRC60 in natural freshwater environments has shown that most recipients of the plasmid which successfully express the 3Cba catabolic genes fall within this group. *Alcaligenes* sp. BR60 is also related to the rRNA superfamily III (Fulthorpe and Wyndham, 1991).

The reason for this observed host range relates to the pathway of 3Cba metabolism. The great majority of recipients able to grow on 3Cba express the meta ring-fission pathway for *p*-hydroxy-benzoate and protocatechuate catabolism. Recent analysis of the degradative pathway encoded by pBRC60 suggests that Tn5271 encodes the dioxygenation of 3Cba to yield protocatechuate and 5-chloro-protocatechuate (Nakatsu et al., 1993; Nakatsu and Wyrdham, 1993). Therefore, recipients able to express 3Cba genes successfully are constrained by their repertoire of ring-fission pathways.

The suitability of *C. acidovorans* and *C. testosteroni* as recipients is reflected in the high conjugation frequencies. pBRC60 transferred into these host species at frequencies greater than 10^{-2} . This figure corresponds to previously reported conjugation frequencies for *C. acidovorans*, in which the Tn5271 deletion derivative pBRC40

was marked by Tn5 insertion. The plasmid conjugated into *C. acidovorans* at a frequency that was 10 to 1000 times higher than for other recipient species (Fulthorpe and Wyndham, 1991).

The frequencies of pGS65 conjugations into both pBRC60-containing hosts were much lower, at about 10^{-7} . However, frequencies for conjugation of pGS65 into strains already harbouring pBRC60 were previously shown to be relatively low. Conjugation of pGS65 into *Alcaligenes* sp. BR6024 (pBRC60) had a frequency of 7.8×10^{-5} , three orders of magnitude lower than the conjugation frequency into plasmid-free BR6024 (Ng and Wyndham, 1993).

A conjugation of 10^{-7} was not unexpected as it reflects not only the frequency of pGS65 conjugation, but also the frequency of pBRC60 integration into the chromosome, presumably by transposition. The product of these two frequencies would naturally be lower than either individual frequency. The conjugation frequency of pGS65 would be reduced because of the presence of pBRC60 in the recipients. Incompatibility of the two plasmids would lower the expected conjugation frequency, as would the phenomenon of entry exclusion (Heinemann and Ankenbauer, 1993).

Previous work on the conjugation of pGS65 in the host *Alcaligenes* sp. BR60 used slightly different selection. Following the mating in of pGS65, transconjugants were selected only for pGS65, but not for pBRC60 by 3Cba

degradation. This selection was sufficient to maintain cytoplasmic copies of pGS65. No pBRC60 could be detected in these transconjugants. When 3Cba selection was subsequently reapplied, pBRC60 had been eliminated from 15 percent of the transconjugants so that they were unable to express *cba* genes. In the rest, pBRC60 was maintained in its entirety in the chromosome. When antibiotic selection for pGS65 was removed, pBRC60 reappeared in the cytoplasm. It was thought to recombine in and out of the chromosome by homology between copies of IS1071 (Ng, 1991; Ng and Wyndham, 1993). The higher frequency of homologous recombination, compared to transposition, would account for the observed difference in frequency of Tc^r, Km^r, 3Cba⁺ transconjugants (10^{-5} into *Alcaligenes* sp. BR60 and 10^{-7} into *Comomonas* spp.) following pGS65 conjugation.

Since neither *C. acidovorans* nor *C. testosteroni* had copies of IS1071 in their chromosomes, the mechanism of integration must have been different than in the *Alcaligenes* strain. In the secondary transconjugants from *C. acidovorans* and *C. testosteroni*, there is in fact no evidence for how much of pBRC60 integrated into the chromosome. The 3Cba degradative phenotype and the positive colony hybridizations only indicate the presence of the transposon, Tn5271. However, if either IS1071 or Tn5271 transposition was responsible for the integration, the rest of pBRC60 would be present in the chromosome at least

transiently, since Tn5271 is deficient in its cointegrate resolution system. Also in that situation, either one copy of IS1071 or all of Tn5271 would be duplicated (Figure 2.1). Therefore, the rest of pBRC60 could be deleted via homologous recombination.

4.2 Analysis of the involvement of Tn5271 in chromosomal integration

4.2.1 Experimental rationale

The structure of Tn5271 places it between the composite class I transposons and the class II elements. The flanking insertion elements, IS1071, encode a transposase which shows similarity to the TnpA proteins of the class II elements, and are flanked by inverted terminal repeats (IRs) which also show similarity to the IRs of the class II elements. The insertion element IS1071 has been shown to be capable of the first step of class II transposition. Alternatively, the entire Tn5271 element is presumed to act as a single unit in carrying out the first step of transposition. Regardless of the transposing element, this step would generate short flanking direct repeats in keeping with transposition mechanisms reported for all other class II elements (Figure 2.1).

No resolvase sequence or *res* sites have been found within Tn5271. Therefore, whether transposition is mediated by IS1071 or by Tn5271, the process could not be completed

by the usual site-specific recombination mechanism. If pBRC60 integrated into the chromosome via either IS1071- or Tn5271-mediated transposition, there should be a cointegrate formed between pBRC60 and the chromosome of the host.

Homologous recombination in the RecA positive hosts between the directly repeated copies of IS1071 or Tn5271 should result in the deletion of the intervening plasmid sequence in the cointegrate structure. Further deletion of the 3Cba catabolic genes would occur in the absence of 3Cba selection (Figure 4.3). Regardless of whether the transposition was mediated by IS1071 or by Tn5271, the result of these two deletions should be a single copy of IS1071 in the chromosome, flanked by the direct repeats generated upon transposition. The flanking chromosomal DNA can be analyzed by isolating a DNA fragment carrying the single insertion element, recircularizing it and amplifying it using PCR primers which face outward from the ends of the element (Figure 4.4).

4.2.2 Generation of deletion derivatives

The secondary transconjugants from the two sets of matings were removed from 3Cba selection, although selection with kanamycin and tetracycline was maintained in order to ensure the presence of pGS65, thereby maintaining the chromosomal integration. Replica plating of 500 colonies back onto 3Cba and onto TYE, both supplemented with the

Figure 4.3 Hypothesis for the generation of deletion derivatives. Transposition by either IS1071 or Tn5271 would result in a cointegrate structure formed between pBRC60 and the recipient chromosome, with direct repeats flanking the outer copies of the transposing element (black arrows). Removal from 3Cba selection would result in a series of deletions, through two possible intermediate products. The final result of the deletions would be a single copy of IS1071 on the chromosome, flanked by the direct repeats.

Figure 4.4 Inverse PCR protocol. In order to amplify the chromosomal segments flanking IS1071, the linear fragment must be circularized. PCR primers facing outward from the insertion element can then be used to amplify the flanking sequences; the result will be a linear product with the direct repeats at either end.

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antibiotics, resulted in a varying number of isolates which had lost the 3Cba phenotype (Table 4.3). The frequency with which the cells lost the degradative genes varied widely among the four isolates, ranging from less than 10% to more than 90%.

With each set of *C. testosteroni* deletion derivatives, randomly chosen isolates were checked for the presence of the insertion element by colony hybridization with the H4 probe (Figure 1.1). Positive and negative controls on the filters hybridized as expected, but a very small percentage of the isolates still probed positive for the element (Table 4.3). Although the 3Cba- H4+ isolates comprised only a small fraction of the isolates, these were used for further analysis as they potentially represented the hypothesized integration events.

The *C. acidovorans* isolate CA47P12 grew well on the selective medium, but would not grow on nylon filters for the colony hybridizations. The deletion derivatives could therefore not be screened as extensively as those from *C. testosteroni*. Following replica plating, ten *C. acidovorans* isolates which appeared not to be growing on 3Cba were selected and their genomic DNA was isolated. Following restriction digests and electrophoresis, the genomic DNA was probed separately with E13/14 for the presence of the 3Cba genes, and Nh6 for the presence of IS1071 (Figure 1.1). Of the ten isolates tested, only two were negative with E13/14

Table 4.3 Frequency of loss of 3Cba degradative phenotype upon removal from 3Cba selection and of retention of IS1071 in the genome as shown by H4 hybridization

Parent	% 3Cba-	% H4+ ^a
CA47P12	20.0 ^b	-- ^c
CT3P15	96.2	1.0
CT5P3	8.0	20.0
CT7P6	69.2	1.0

^a expressed as a percentage of the 3Cba- isolates

^b represents only 10 isolates for this secondary transconjugant

^c H4 colony hybridizations not done with these isolates

and positive with Nh6. None were negative with both probes.

Since the secondary transconjugants used to generate the deletion derivatives did not carry a cytoplasmic copy of pBRC60, the plasmid would not be expected to be present in the deletion derivatives either. Even though they had been removed from 3Cba selection, the antibiotic pressure was still present, keeping pGS65 in the cells and presumably preventing pBRC60 from replicating as an autonomous plasmid in the cytoplasm. Plasmid analyses were performed with the deletion derivatives to confirm the absence of the plasmid. Plasmid pBRC60 was not detected in these deletion derivatives, although it was readily detected in the control culture (BR6024). As with the previous set of plasmid extractions, pGS65 was not isolated either. This plasmid was rarely detected using the alkaline extraction procedure, even when applied to the control, donor *E. coli* BW97 (pGS65).

From the pools of 3Cba- H4+ deletion derivatives, a few were selected for each parent. Table 4.4 lists all the isolates with which further analysis was performed.

4.2.3 Restriction analysis of deletion derivatives

A. *Bgl*III restrictions

The restriction enzyme *Bgl*III is particularly useful in analysing pBRC60 as it cuts the plasmid into only five fragments of varying lengths, but does not cut within the

Table 4.4 Derivation of secondary transconjugants and deletion derivatives used in this study

<u>Parent</u>	<u>1° Transconjugant (X BR6024)</u>	<u>2° Transconjugant (X BW97 X MM294)</u>	<u>Deletion Derivative</u>
<i>C. acidovorans</i>	CA47	CA47P12	CA47P12-A3
<i>C. testosteroni</i>	CT3	CT3P15	CT3P15-A32 CT3P15-F37
	CT5	CT5P3	CT5P3-H5 CT5P3-I26
	CT7	CT7P6	CT7P6-D26 CT7P6-F1

insertion elements, keeping them each on different fragments (Figure 4.5; Table 4.5). Each secondary transconjugant and its corresponding deletion derivative was digested with *Bgl*III and hybridized with E7/8, E13/14 and Nh6 to compare the presence of the rest of the plasmid, the 3Cba genes and the number of insertion sequences.

The *Bgl*III digests showed variations of the same pattern. The digest of CT3P15 and its two deletion derivatives, -A32 and -F37, are typical (Figure 4.6). The *Bgl*III restriction patterns of all the secondary transconjugants are summarized in Table 4.6.

All of the secondary transconjugants derived from *C. testosteroni* hybridized as predicted by chromosomal integration of pBRC60. As expected, the E13/14 probe for the degradative genes hybridized to a fragment the same size as the B4 fragment (Figure 4.5; Table 4.5) on which the genes are located. They all probed positive with E7/8, which hybridizes to a region of the plasmid outside of the transposon. Furthermore, the size of fragment was similar to that of the B2 fragment. The hybridizations with Nh6, which is specific for the insertion element, showed variations. Fragments matching B2 (IS1071), B3 (IS1071) and B5 in size hybridized in all of them. B5 hybridizes with the insertion element in all strains carrying pBRC60, as previously shown (Fulthorpe and Wyndham, 1992). There appears to be significant similarity between the *tnpA*

Figure 4.5 Restriction digest map of pBRC60. The map shows the relative restriction sites determined by digestion of plasmid DNA with the restriction enzymes in combination with probing with digoxigenin-labelled plasmid fragments. The sizes of the restriction fragments are given in Table 4.5. Outlined fragments Nh6 (N6), E13/14 and E7/8 indicate the locations of the probes used to screen deletion derivatives (from Nakatsu et al., 1993).

Table 4.5 Restriction fragment sizes in kilobases of plasmid pBRC60 and the corresponding identification numbers used on the restriction map (Figure 4.5)

<u>EcoRI</u>	<u>SalI</u>	<u>SmaI</u>	<u>NruI</u>	<u>PvuII</u>	<u>BglII</u>
E1, 16.0	S1, >30	Sm1, >30	Nr1, >30	P1, >30	B1, >30
E2, 12.5	S2, 19.3	Sm2, 15.1	Nr2, 23.8	P2, 28.0	B2, 24.5*
E3, 10.1	S3, 7.2	Sm3, 12.2*	Nr3, 8.3	P3, 11.5	B3, 11.6*
E4, 7.9	S4, 4.4	Sm4, 10.0*	Nr4, 8.2*	P4, 7.0	B4, 8.4
E5, 7.1	S5, 4.3	Sm5, 9.3	Nr5, 6.5	P5, 4.5	B5, 6.1
E6, 6.4	S6, 4.0	Sm6, 8.5	Nr7, 3.8	P6, 4.1	
E7, 4.9	S7, 3.4	Sm7, 7.2	Nr8, 3.5	P7, 1.7	
E8, 4.7	S8, 3.4	Sm8, 3.0	Nr9, 3.3*	P8, 1.6	
E9, 3.6	S9, 3.0	Sm0, 0.9	Nr10, 1.6	P9, 0.6	
E10, 3.5	S10, 2.5	Sm10, 0.6			
E11, 2.9	S11, 2.3	Sm11, 0.3			
E12, 1.8	S12, 1.9				
E13, 1.6	S13, 1.7				
E14, 1.5	S14, 0.5				
E15, 1.1					
E16, 0.8					
E17, 0.6					
E18, 0.6					

<u>NheI</u>	<u>SacI</u>	<u>BamHI</u>	<u>HindIII</u>
N1, >30	Sac1, >30*	Bam1, >30	H1, >30
N2, 27.4	Sac2, 20.0*	Bam2, 8.9	H2, 8.4
N3, 10.8	Sac3, 4.5	Bam3, 3.3	H3, 4.4
N4, 7.1	Sac4, 2.7	Bam4, 0.8	H4, 1.1
N5, 4.9	Sac5, 1.4	Bam5, 0.7	
N6, 3.1*	Sac6, 0.3		

* these fragments carry IS1071 copies from pBRC60

Figure 4.6 *Bgl*III hybridization patterns of CT3P15 and its deletion derivatives. Genomic DNA from CT3P15 and its deletion derivatives CT3P15-A32 and CT3P15-F37 was digested with *Bgl*III and hybridized with E7/8, E13/14 and Nh6 which are specific for plasmid DNA outside Tn5271, the 3Cba genes and IS1071 respectively. Genomic DNA from *Alcaligenes* strain BR6024 is included as a control. Lane 1) CT3P15; 2) CT3P15-A32; 3) CT3P15-F37; 4) BR6024. λ /*Hind*III size markers are shown on the left: 23.1, 9.4, 6.7, 4.4 kb.

1 2 3 4

1 2 3 4

1 2 3 4

Table 4.6 Fragment sizes in kilobases of secondary transconjugants digested with *Bgl*III and hybridized with E7/8, E13/14 and Nh6

<u>2° Transconjugant</u>	<u>E7/8</u>	<u>E13/14</u>	<u>Nh6</u>
CA47P12	-- ^a	9 (B4)	11 (B3) 6 6 (B5)
CT3P15	>20 (B2)	9 (B4)	>20 (B2) 18 14 (B3) 7 (B5)
CT5P3	>20 (B2)	9 (B4)	>20 (B2) 13 (B3) 6 (B5)
CT7P6	>20 (B2)	9 (B4)	>20 (B2) 12 (B3) 6 (B5)

^a E7/8 did not probe positive for CA47P12

sequence and part of the B5 fragment. The reason for this is not known; however, it may relate to the presence of Tn501-like mercury resistance genes in this region of pBRC60 (see Final Discussion). The CT3P15 hybridization also showed a novel band of about 17.5 kb; CT5P3 and CT7P6 showed no novel bands.

The secondary transconjugant from *C. acidovorans*, CA47P12, showed a different pattern. It hybridized as expected with E13/14, but did not hybridize at all with E7/8, indicating the loss of at least part of the B2 fragment. The Nh6 hybridization also revealed that some loss had occurred. The B3 and B5 fragments were present, but the B2 fragment was absent. However, a novel fragment of about 6.2 kb was present. This data indicated substantial loss of pBRC60 sequences.

The deletion derivatives from all the secondary transconjugants hybridized mostly as expected. None of them hybridized with either E7/8 or with E13/14, indicating that the degradative genes and at least a segment of the rest of the plasmid had been lost in the deletion events. In the Nh6 hybridizations, all the deletion derivatives showed only one novel band which did not correspond to any bands in the parent. This would be expected if all that remained of the plasmid was one copy of the insertion element (Figure 4.3). In that case, the length of the BglIII fragment carrying IS1071 would depend on the location of chromosomal

restriction sites for *Bgl*III.

However, the sizes of these bands varied among deletion derivatives from the same secondary transconjugant, which was a somewhat surprising result. Each secondary transconjugant would be presumed to have the plasmid integrated in one location in the chromosome, and therefore the deletion derivatives from the same transconjugant would be expected to yield the same size *Bgl*III fragment carrying the insertion element. Variation in the *Bgl*III fragment sizes was seen in all the secondary transconjugants from *C. testosteroni*, with the fragment sizes in deletion derivatives from the same secondary transconjugant differing by more than 7 kb in some cases. The variation in sizes was not seen for the *C. acidovorans* derivative CA47P12, although this does not mean it did not occur. Only two deletion derivatives were isolated from CA47P12, so it was not possible to look for the size variation.

The stability of the Nh6 hybridization patterns of *Bgl*III restriction digests of the *C. testosteroni* deletion derivatives was tested by repeatedly restreaking each deletion derivative on TYE + Km₅₀Tc₂₀ over a five-week period. Genomic DNA was again isolated, digested with *Bgl*III and hybridized with Nh6. Figure 4.7 shows the IS1071-carrying *Bgl*III fragments from all the deletion derivatives before and after the restreaking. There is no change in any of them, indicating that the deletions were stable.

Figure 4.7 Stability of *Bgl*III fragment sizes from the *C. testosteroni* deletion derivatives. The stability of the fragment sizes in the *C. testosteroni* deletion derivatives was confirmed by continuously restreaking the deletion derivatives, and reisolating the genomic DNA after 5 weeks. The DNA was digested with *Bgl*III and hybridized with Nh6 which is specific for IS1071. DNA from both extractions was included for comparison of the fragment sizes. Lanes 1 and 2) CT3P15-A32; 3 and 4) CT3P15-F37; 5 and 6) CT5P3-H5; 7 and 8) CT5P3-I26; 9 and 10) CT7P6-D26; 11 and 12) CT7P6-F1. λ /*Hind*III size markers are shown on the left: 23.1, 9.4, 6.7, 4.4 kb.

1 2 3 4 5 6 7 8 9 10 11 12

The two parent species, *C. acidovorans* and *C. testosteroni*, were hybridized with E7/8, E13/14 and Nh6. None of the three probes gave a positive result, thereby confirming that the positive results for the secondary transconjugants and their deletion derivatives were due to the presence of pBRC60-derived DNA.

B. Multiple restrictions

A second set of restriction digests was performed with each deletion derivative and its immediate parent. They were digested with a series of enzymes known not to cut within the insertion element, and hybridized with Nh6. This served two purposes; it would confirm that only a single copy of IS1071 was present in the chromosome, and establish which restriction enzyme would yield a fragment that was a suitable size for subsequent polymerase chain reaction work. Again, the multiple digest analysis of CT3P15 and its deletion derivative CT3P15-F37 is representative of the results (Figure 4.8).

The restriction patterns for all the secondary transconjugants, from both *C. testosteroni* or *C. acidovorans*, confirm the presence of Tn5271 (Table 4.7). The BglIII patterns have already been summarized (Table 4.6). Hybridization of the NheI-digested DNA showed the presence of the Nh6 fragment. NheI cuts just within the terminal inverted repeats of IS1071, so if there have been no

Figure 4.8 Nh6 hybridization of multiple digests of CT3P15 and CT3P15-F37. CT3P15 and its deletion derivative CT3P15-F37 were digested with 5 restriction enzymes known not to cut within IS1071. The DNA was hybridized with Nh6 which is specific for IS1071. DNA from CT3P15 and CT3P15-F37 was run in adjacent lanes for comparison. A *Bgl*II digest of genomic DNA from *Alcaligenes* strain BR6024 was included as a control. Lanes 1 and 2) *Bgl*II digest; 3 and 4) *Nhe*I digest; 5 and 6) *Nru*I digest; 7 and 8) *Sac*I digest; 9 and 10) *Sma*I digest; lane 11) BR6024 *Bgl*II digest. λ /*Hin*DIII size markers are shown on the left: 23.1, 9.4, 6.7, 4.4, 2.3 kb.

Table 4.7 Fragment sizes in kilobases of secondary transconjugants digested with enzymes known not to cut within IS1071 and hybridized with Nh6

<u>Isolate</u>	<u>NheI</u>	<u>NruI</u>	<u>SacI</u>	<u>SmaI</u>
CA47P12	3 (Nh6)	8 (Nr3)	>20 (S2)	>20 (Sm1)
		8 (Nr4)	11	16 (Sm3)
		3 (Nr9)	5 (S3)	10 (Sm4)
CT3P15	3 (Nh6)	9 (Nr3)	>30 (S1)	>20 (Sm1)
		8 (Nr4)	>20 (S2)	16 (Sm3)
		4	5 (S3)	10 (Sm4)
		3 (Nr9)		
CT5P3	3 (Nh6)	8 (Nr3)	>30 (S1)	>20 (Sm1)
		7 (Nr4)	>20 (S2)	13 (Sm3)
		4	5 (S3)	9 (Sm4)
		3 (Nr9)		
CT7P6	3 (Nh6)	8 (Nr3)	>30 (S1)	>30 (Sm1)
		8 (Nr4)	>20 (S2)	15 (Sm3)
		5	5 (S3)	10 (Sm4)
		3 (Nr9)		

rearrangements within IS1071, all the bands should be about three kb (Figure 4.5, Table 4.5).

In the *Nru*I digests, all of the secondary transconjugants showed the presence of the Nr4 and Nr9 fragments, which contain the two copies of IS1071 in pBRC60 (Figure 4.5). The Nr3 fragment also hybridized, which was unexpected as it is outside the area of the plasmid for which the Nh6 probe is specific. A four or five kb *Nru*I fragment hybridized faintly in the *C. testosteroni* secondary transconjugants. This was the same size as the *Nru*I fragment in the deletion derivatives (Table 4.8).

In the *Sac*I digested DNA from the *C. testosteroni* secondary transconjugants, the Sac1 and Sac2 fragments hybridized. These large bands contain the two copies of IS1071 on pBRC60. The Sac3 band also hybridized, which was surprising because, like the Nr3 band, it does not overlap IS1071 and should therefore not hybridize with Nh6. The *Sac*I hybridization patterns for the *C. acidovorans* secondary transconjugant are slightly different than those for the *C. testosteroni* transconjugants. The Sac2 and 3 fragments hybridized, but the Sac1 fragment was replaced by a novel 11 kb fragment. This size does not correspond to any *Sac*I fragments from pBRC60 or pBRC40, and therefore may indicate that one end of the fragment is located in chromosomal DNA.

The *Sma*I digested DNA from all the secondary transconjugants showed the Sm3 and Sm4 fragments, which

Table 4.8 Fragment sizes in kilobases of deletion derivatives digested with enzymes known not to cut within IS1071 and hybridized with Nh6

<u>Derivative</u>	<u>BglII</u>	<u>NheI</u>	<u>NruI</u>	<u>SacI</u>	<u>SmaI</u>
CA47P12-A3	13	3	4	>20	>20
CT3P15-A32	>20	3	5	>20	15
CT3P15-F37	>20	3	4	>20	13
CT5P3-H5	19	3	4	>20	8
CT5P3-I26	15	3	4	>20	>20
CT7P6-D26	13	3	4	>20	>20
CT7P6-F1	>20	3	4	>20	18

contain IS1071 on pBRC60. A third *Sma*I fragment also hybridized. Its large size, >20 kb, suggests it may be the *Sma*I fragment, although again this does not overlap IS1071 and should therefore not hybridize with the Nh6 probe.

The restriction sizes for the deletion derivatives derived from these secondary transconjugants are summarized in Table 4.8. The *Bgl*II fragments vary in size, from 13 kb to >20 kb, suggesting that at least one end of the fragment is located in the chromosome. Interestingly, the sizes of the *Bgl*II fragments vary even between deletion derivatives from the same secondary transconjugant. If the same mechanism was responsible for all the deletions, these fragments would be expected to be the same size.

In addition to the *Bgl*II fragments, only the *Sma*I bands showed a great amount of variation, with sizes ranging from less than 8 kb to more than 20 kb. This variation likely indicates that for the *Sma*I fragment containing IS1071, at least one of the *Sma*I sites is located in the chromosomal DNA flanking IS1071.

The *Nhe*I fragments in all the deletion derivatives are the same size, which was also seen in the secondary transconjugants. The restriction patterns from the next two enzymes were more revealing. The deletion derivatives come from four different secondary transconjugants, each representing a separate chromosomal integration event. Thus, there should have been a great variety of fragment

sizes if the remaining insertion element was flanked by chromosomal DNA. However, the patterns for all the deletion derivatives were strikingly similar. The *Nru*I fragment was invariably 4 kb, and the *Sac*I fragment was always larger than 20 kb.

Even more intriguing was the fact that the patterns shown were remarkably similar to what would be produced if pBRC40 was cut with the same set of enzymes (Figure 4.9; Table 4.9). In fact, the size of the *Nru*I fragments containing IS1071 was identical to that from pBRC40. Although the E7/8 hybridizations of the *Bgl*II digests showed that part of the plasmid had been lost (Table 4.6), the results from these multiple digests would seem to indicate that the part of the plasmid flanking the insertion element IS1071 remained following the deletions.

4.2.4 PCR analysis

The IS1071-containing *Nru*I fragment from each derivative was used for PCR since it was the smallest size which included the entire element. The *Nhe*I bands were smaller, but because *Nhe*I cuts within the ends of the element, these bands would not contain flanking sequences. The *Nru*I fragments were all 4 kb. Because outward facing primers were used, the internal part of IS1071 would not be amplified. The PCR products were therefore expected to be about 1 kb.

Figure 4.9 Restriction digest map of pBRC40. The map shows the relative restriction sites determined by digestion of plasmid DNA with the restriction enzymes in combination with probing with digoxigenin-labelled plasmid fragments. The sizes of the restriction fragments are given in Table 4.9.

Table 4.9 Restriction fragment sizes in kilobases of plasmid pBRC40 and the corresponding identification numbers used on the restriction map (Figure 4.9)

<u>EcoRI</u>	<u>SalI</u>	<u>SmaI</u>	<u>NruI</u>	<u>PvuII</u>	<u>BglII</u>
E1, 16.1	S1, >30	Sm1, >30	Nr1, >30	P1, >30	B1, >30
E2, 12.9	S2, 19.3	Sm2, 15.1	Nr2, 23.8	P2, 28.0	B2, 29.4
E3, 10.1	S3, 7.2	Sm5, 9.3	Nr3, 8.3	P3, 11.5	B5, 6.1
E4, 7.9	S5, 4.3	Sm6, 8.5	Nr6, 4.3	P5, 4.5	
E6, 6.4	S6, 4.0	Sm7, 7.2	Nr7, 3.8	P9, 0.6	
E7, 4.9	S7, 3.4	Sm8, 3.0	Nr8, 3.5		
E8, 4.7	S9, 3.0	Sm9, 0.9	Nr10, 1.6		
E9, 3.6	S11, 2.3				
E10, 3.5	S14, 0.5				
E12, 1.8					
E15, 1.1					
E16, 0.8					
E17, 0.6					
<u>NheI</u>	<u>HindIII</u>				
N1, >30	H1, >30				
N2, 27.4	H4, 1.1				
N4, 7.1					
N5, 4.9					
N6, 3.1					

Fragments of about 4 kb were excised from gels of *NruI*-digested total genomic DNA, and the DNA was isolated. The control used for the ligation and PCR was pBRN4029, in which the IS1071-containing 4.3 kb *NruI* fragment from pBRC40 was cloned into pDPL13 (Figure 4.10) (Nakatsu et al, 1991). With each of the deletion derivatives, pBRN4029 was used as a control. The DNA was cut with *NruI*, and the ligations and PCR reactions were performed concurrently.

The PCR products from all the deletion derivatives were the same size, equivalent to the size of the control product (Figure 4.11a). Negative control preparations yielded no product. This was taken as evidence that the deletion derivatives carried the Nr6 fragment of pBRC40 in their chromosomes. The double bands in the PCR product were likely caused by incorrect primer annealing.

Further confirmation that the deletion derivatives carried the Nr6 fragment from pBRC40 was obtained from hybridization of the PCR products. A probe was made from pBRN4029, covering the flanking pBRC40-derived DNA not included within the insertion element (Figure 4.10). This probe hybridized very strongly, not only to the control PCR product, but to the products from all the deletion derivatives, including the double bands (Figure 4.11b). This evidence showed conclusively that part of pBRC40 flanking the insertion element remained in the chromosomes of the deletion derivatives.

Figure 4.10 Plasmid map of pBRN4029. pBRN4029 consists of the 4.3 kb Nr6 fragment from pBRC40, which contains IS1071, cloned into pDPL13. The location of the *Nru*I restriction sites are indicated, as are the *Nhe*I restriction sites located just within the inverted repeats of IS1071. The outward-facing PCR primers (hatched arrows) are located interior to the *Nhe*I sites. Therefore the 1.1 kb probe cut from between the *Nhe*I site in IR-R and the nearest *Nru*I site does not hybridize to the primers when used to probe the PCR products.

Figure 4.11 PCR products from the *C. testosteroni* deletion derivatives. *Nru*I chromosomal fragments from the *C. testosteroni* deletion derivatives were self-ligated and amplified using outward facing primers. The *Nru*I fragment from pBRN4029 was included as a control. Lane 1) CT3P15-A32; 2) CT3P15-F37; 3) CT5P3-E23; 4) CT5P3-H5; 5) CT5P3-I26; 6) CT7P6-D26; 7) CT7P6-F1; 8) pBRN4029. Panel A: gel electrophoresis of the PCR products. Panel B: hybridization of the PCR products with the 1.1 kb probe cut from pBRN4029 which is specific for pBRC40-derived DNA. λ /HindIII size markers are shown on the left: 6.7, 4.4, 2.3, 2.0 kb.

B

4.2.5 Discussion

The percentages of deletion derivatives for each of the secondary transconjugants were not at all similar, ranging from 8% to 96%. Factors which may have contributed to the range of frequencies include the fact that the derivatives were grown for varying lengths of time on TYE + Km₅₀Tc₂₀ before being replica plated. Because the isolates grew at different rates, there was no consistency in the length of time they were grown under non-selective conditions. As well, the growth on 3Cba + Km₅₀Tc₂₀ was difficult to identify following the replica plating, as they were all growing very slowly. Previous work has shown much more consistent figures; in the absence of 3Cba selection, the frequency of Tn5271 deletion is about 10⁻³ per cell per generation (Wyndham et al., 1988; Ng and Wyndham, 1993).

Cointegrate structures formed by IS1071 transposition have been shown to be resolved, in spite of the absence of a transposon-encoded resolution system, by the homologous recombination system in the host (Nakatsu et al., 1991). Thus it seems reasonable to expect that deletion in the secondary transconjugants would occur by the same system, mediated by homology between the directly repeated copies of IS1071. However, regardless of the insertion mechanism, this deletion would leave one copy of the insertion element in the chromosome (Figure 4.3).

The variation in sizes of the IS1071-containing BglII

fragments in the deletion derivatives is difficult to explain. It is surprising that derivatives from the same integration would generate different sized fragments. However, the variations seen appear not to be completely random; instead, there are two or three sizes which are generated most often. *Bgl*II digests comparing DNA extracted before and after five weeks of transfers showed that the deletions were very stable.

If transposition was the mechanism of the chromosomal integration, the secondary transconjugants would have a cointegrate formed between pBRC60 and the chromosome, with a copy of the transposing element at each junction. It would then be possible for intramolecular transposition to be responsible for the deletion, with one of the elements transposing across the intervening cointegrate to a site on the chromosome. Variations in the location of this site would result in varying distances to the next chromosomal *Bgl*II site; this would in turn generate IS1071-carrying *Bgl*II fragments of different sizes.

The fact that so many of the deletion derivatives did not probe positive for the insertion element is equally difficult to explain. Their failure to probe positive also suggests that IS1071 may not have been involved in the majority of deletions. The existence of a plasmid-chromosome cointegrate structure could explain the loss of IS1071. Precise excision of the flanking transposing

elements and the intervening plasmid sequence would result in the loss of the insertion element in addition to the loss of the degradative phenotype.

It was hypothesized that either IS1071- or Tn5271-mediated transposition was responsible for the integration of pBRC60 into the chromosomes of *C. acidovorans* and *C. testosteroni*. The results from the restriction analyses and the PCR were examined for evidence either supporting or refuting this hypothesis. Because the results would be the same whether transposition was mediated by IS1071 or by Tn5271 (Figure 4.3), for the ease of discussion both mechanisms will be referred to as IS1071-mediated transposition.

The results from the *Bgl*II restriction analyses are ambiguous. The novel bands in the *Bgl*II digests of CA47P12 and CT3P15 would suggest that transposition had occurred. Class II transposition is a replicative process; if IS1071 or Tn5271 was responsible for this integration, at least part of the sequence would be duplicated, thereby generating a novel *Bgl*II band that would hybridize with a probe specific for the insertion element. However, there appear to be no novel bands carrying the insertion element in either CT5P3 or in CT7P6. The absence of a novel band suggests a non-replicative method of integration.

The presence of the E7/8 band in the secondary transconjugants and its subsequent loss in the deletion

derivatives suggests that transposition had occurred. IS1071-mediated transposition would result in the intervening segment of pBRC60 being flanked by directly repeated homologous regions. Recombination between these would delete the intervening plasmid sequence and account for the loss of E7/8 probing (Figure 4.3). Transposition is the only integration mechanism that would result in flanking copies of IS1071, because again, at least one copy of IS1071 would have to have been duplicated.

The failure of E7/8 to hybridize to DNA from the secondary transconjugant CA47P12 can also be explained by transposition. Because of the low frequency of isolation of deletion derivatives from this isolate, it was maintained on 3Cba + Km₅₀Tc₂₀ for a long time. Although the 3Cba selection would ensure the maintenance of the degradative genes, there was no pressure to prevent the deletion of the intervening plasmid via homologous recombination between copies of IS1071, provided that the intervening region was flanked by two copies of IS1071. Therefore, E7/8 would not probe positive. However, this does not account for the presence of the B5 fragment, which would be lost along with the E7/8 fragment in this model.

Some of the results from the multiple restriction digests support the hypothesis that the integration mechanism was IS1071-mediated transposition. One novel band was seen in the SacI digest of CA47P12 (Table 4.7). The 11

kb fragment does not correspond to any *SacI* fragments from pBRC60, so at least one end of this fragment may be located in the adjacent chromosomal DNA. The variation in the *BglII* and *SmaI* fragment sizes in the deletion derivatives (Table 4.8) suggests that at least one end of these fragments are chromosomally located. These results implicate IS1071-mediated transposition as the mechanism for the chromosomal integration.

However, many of results from the multiple restriction digests discount the possibility of IS1071-mediated transposition as the integration mechanism. With the exception of the 11 kb *SacI* fragment from CA47P12, the restriction fragments from all the secondary transconjugants appear to be plasmid bands. Because the restriction enzymes used do not cut within IS1071, IS1071-mediated transposition should have generated novel bands since both ends of the restriction fragments would be chromosomally located.

Several unexpected plasmid bands hybridized: Nr3, S3 and Sm1, and the four or five kb fragment from the *NruI* digest. These do not correspond to IS1071-containing plasmid fragments. However, these bands were the same size for all the independently-derived secondary transconjugants, and their sizes corresponded to known plasmid bands. Nr3, S3 and Sm1 are all adjacent to bands which contain IS1071, and which cut close to the end of IS1071 (Figure 4.5), so there may be some overlap that would account for their

hybridization. The four kb *Nru*I fragment corresponds to the Nr6 fragment in pBRC40 (Figure 4.9). Its presence can be accounted for by spontaneous deletion of the 3Cba genes when the isolates were removed from 3Cba selection prior to the DNA extraction.

IS1071-mediated transposition would have resulted in copies of IS1071 flanking all of the pBRC60 sequences in the chromosome; deletions resulting in a single remaining copy of IS1071 would entail the loss of all of pBRC60-derived DNA, so that the remaining copy of IS1071 would be flanked by chromosomal DNA (Figure 4.3), therefore generating a great variety of sizes among deletion derivatives. The similarities in the multiple digests of all the deletion derivatives are likely not coincidental. Their similarity to a pBRC40 digest is also unlikely to have arisen by chance. These results strongly suggest the presence of more plasmid DNA than IS1071-mediated transposition would predict.

The most conclusive evidence against IS1071-mediated transposition came from the PCR results. The control DNA was the cloned Nr6 fragment from pBRC40, the Tn5271 deletion derivative of pBRC60. The PCR products from the chromosomal deletion derivatives were from *Nru*I fragments. All were identical in size to the control. Hybridization of the inverse PCR products with a fragment of plasmid DNA adjacent to the insertion element verified the presence of plasmid-

derived DNA flanking the insertion element in the chromosomes.

Overall, the restriction analysis showed that the secondary transconjugants, including CA47P12, did contain more of pBRC60 than just the transposon in their chromosomes. However, although there was some evidence that transposition had occurred, the presence of pBRC40 DNA in the deletion derivatives suggested that neither IS1071- nor Tn5271-mediated transposition could have been the mechanism for chromosomal integration.

4.3 Mapping the chromosomal recombinants

4.3.1 Experimental rationale

A large part of pBRC60 was known to be present in the secondary transconjugants because of the *Bgl*III restriction patterns. Although much of this was deleted upon removal from 3Cba selection, there was still a portion of pBRC40 in the chromosomes of the deletion derivatives, shown by the PCR results. Because IS1071-mediated transposition appeared not to have been involved in the original integration, further hybridizations were undertaken to ascertain the extent of the deletions, and to try to figure out the mechanism responsible for the integration.

Restriction enzymes known to cut pBRC60 frequently were used to digest four of the deletion derivatives and their corresponding secondary transconjugants. A series of probes

covering almost all of pBRC60 were used to show how much of pBRC60 was present in the secondary transconjugants and how much of pBRC40 was left following the deletions. A mechanism is proposed which explains the rearrangements.

4.3.2 Chromosomal rearrangements of pBRC60

The mapping showed similar patterns in all the deletion derivatives, and in all the secondary transconjugants except CA47P12 (see Appendix 2 for details). There were two details which stood out.

First, in all secondary transconjugants from *C. testosteroni*, not only was all of pBRC60 present, but a segment including Tn5271 was duplicated (Figure 4.12). This was apparent in hybridizations with the probes E4, E6, E17, Nr8, S3 and Sm2. All the bands predicted by the presence of pBRC60 hybridized, as did novel bands.

Second, all the deletion derivatives from *C. testosteroni*-derived secondary transconjugants showed two deletion events (Figure 4.12). As was shown by the earlier *Bgl*III digests, the internal section of Tn5271 which contains the 3Cba genes was lost, presumably by homologous recombination between the two directly repeated copies of IS1071. In addition, a larger segment located opposite Tn5271 on the plasmid was also lost. The size and location of the deletion was similar in all the *C. testosteroni* deletion derivatives. The ends of this deleted area are

Figure 4.12 Mapping results from the secondary transconjugants and their deletion derivatives. The secondary transconjugants from *C. testosteroni* contained all of pBRC60 as well as a duplicated segment, indicated by the hatched area. This duplication was shown with probes E4, E6, E17, Nr8, S3 and Sm2. The secondary transconjugant from *C. acidovorans* only carried one copy of the hatched area, and the rest of the plasmid was absent. The *C. testosteroni* deletion derivatives showed two deletions, indicated by the shaded areas. The smaller deletion in which the 3Cba genes were lost was shown in the *C. acidovorans* deletion derivative.

located within the E6 and E4 fragments, so that the size of the deletion would have to be between 38.2 and 52.5 kb, depending on how much of the E4 and E6 fragments remain following the deletion. The stability exhibited by the deletion derivatives (Figure 4.7) indicates the products of the combined smaller 3Cba deletion and the larger deletion were stable.

The deletion derivative CA47P12-A3 showed hybridization patterns similar to those of the *C. testosteroni* deletion derivatives. That is, IS1071 and flanking DNA identical to pBRC40 DNA was present, but a large part of the pBRC40 plasmid opposite IS1071 was lost (Figure 4.12). The corresponding secondary transconjugant, CA47P12, had the same hybridization patterns as CA47P12-A3, except with probes from within Tn5271, which showed the degradative genes still to be present. Thus it appears that by the time the secondary transconjugant was isolated, the large deletion opposite Tn5271 on the plasmid had already taken place. Since the deleted region does not encode 3Cba degradation, there was no pressure preventing this deletion in the secondary transconjugant.

4.3.3 A mechanism explaining the mapping results

Any mechanism proposed to explain the chromosomal integration in these isolates has to satisfy a number of criteria. First, it must account for the duplication of a

segment of pBRC60 seen in the secondary transconjugants. The occurrence of the duplication suggests a replicative mode of integration, pointing to a possible transposition.

Second, it would have to account for both deletions. The deletion of the 3Cba genes must have occurred by recombination between the flanking copies of IS1071. The larger deletion is not so easily explained. This occurred between the E6 and the end of the E4 fragments, across the intervening plasmid DNA in all the derivatives (Figure 4.12). It is not possible for this deletion to have occurred by homologous recombination involving IS1071, or any part of Tn5271.

Of several possible mechanisms of chromosomal integration, there is one which seems to account for the patterns shown in the mapping. It would seem that pBRC60 carries a second, larger transposon of about 45 kb, in which Tn5271 is included. Transposition mediated by this encompassing transposon would account for both the duplicated section of pBRC60 in the secondary transconjugants, and the large deletion (Figure 4.13).

The duplication indicates a replicative means of integration must have occurred; transposition satisfies this requirement. The duplication of the pBRC60 segment from E6 to E4, covering Tn5271, would indicate a possible transposing element encompassing that section. This is the structure of the element as it was found in the C.

Figure 4.13 Chromosomal integration of pBRC60 mediated by the encompassing transposon. A potential second transposon on pBRC60, within which Tn5271 is contained, would explain the hybridization patterns in the secondary transconjugants and their deletion derivatives. Transposition would generate a cointegrate formed between pBRC60 and the chromosome, with directly repeated copies of the second element at the junctions. A first deletion, not dependent on removal from 3Cba selection, would occur via homologous recombination between the two copies of the encompassing element. The result would be a single copy of the element on the chromosome. Removal from 3Cba selection would result in a second deletion in which the 3Cba genes were lost by homologous recombination between the two remaining copies of IS1071. The final product of these deletions would be a pBRC40-like segment of plasmid DNA in the chromosome; most of the encompassing element would remain, with only one copy of IS1071.

recipient
chromosome

transposition

deletion of
intervening cBR 060
segment

deletion of
to 5271

testosteroni secondary transconjugants.

The existence of a second transposing element would also explain the deletion of the rest of the plasmid. Homologous recombination between the directly repeated copies of the element in the cointegrate structure would result in the large deletion, leaving a single copy of the large element in the chromosome. This is the structure of the element as it was found in the *C. acidovorans* secondary transconjugant.

The different hybridization patterns and mapping results for CA47P12 and for the *C. testosteroni* secondary transconjugants are not inconsistent. As previously stated, 3Cba selection would not provide pressure to maintain the intervening plasmid sequences in the chromosome. Homologous recombination between the two copies of the encompassing element would result in the large deletion in CA47P12, with Tn5271 still maintained by the 3Cba selection. This would cause the observed hybridization patterns.

Homologous recombination between the two copies of IS1071 contained within the larger element would result in the deletion of the degradative genes, and a single remaining copy of IS1071. This is the structure of the element as it was found in all deletion derivatives from both *Comomonas* species. The remaining encompassing element would account for the results shown by the early restriction analyses and the PCR, in which a large part of pBRC40

flanking a single copy of IS1071 was still present.

4.3.4 Discussion

The isolates used for the mapping represented both *C. acidovorans* and *C. testosteroni* recipients. The three *C. testosteroni* derivatives were from three separate IncP matings, thereby resulting from three independent chromosomal integrations. Thus, the four isolates were all independently derived, and the similarities in their hybridization patterns indicate that the same mechanism of chromosomal integration occurred four separate times.

The evidence suggesting the existence of a second transposon on pBRC60 is incomplete; at this time it is still speculative. Certainly this model explains the chromosomal integrations in both *C. acidovorans* and *C. testosteroni*. Perhaps the most appealing aspect of this model is the way it explains the large deletion, which was too precise and too stable to be a random occurrence.

The mapping results from CA47P12 show that the large deletion is not necessarily dependent on removal from 3Cba selection. It is possible that in the three *C. testosteroni* derived isolates, this deletion had simply not yet occurred in the secondary transconjugants. If they were maintained for a time on 3Cba + Km₅₀Tc₂₀, it is likely that isolates would show the 40 kb deletion and still carry Tn5271, as in the case of CA47P12.

This would not be the first occurrence of a transposon within a transposon. The archetypal class II element, Tn3, is itself contained within the much larger element Tn4 (Kopecko et al., 1976). Nested transposons have been shown to exist in the well-characterized Tol system carried on plasmid pWWO; the 56 kb element Tn4651 is contained within the 70 kb element Tn4653 (Tsuda and Iino, 1988). A cryptic transposon, Tn2501, has been found within the lactose transposon Tn951 (Michiels and Cornelis, 1984).

If chromosomal integration had occurred by homologous recombination or some other simple insertion mechanism, the plasmid would have been broken open at some site, presumably in the B1 fragment since all the other *Bgl*III fragments were found intact in the secondary transconjugants. This would linearize the plasmid from within the large deleted region, and thus the loss of the 40 kb segment would have to occur via two deletions covering the junctions of the plasmid and the chromosome to two fairly precise points in the plasmid in the E4 and E6 fragments. The proposed mechanism of deletion by homologous recombination between two copies of a larger transposing element encompassing Tn5271 is more efficacious.

5. Final Discussion

The work presented here shows that neither IS1071 nor Tn5271 mediated-transposition caused the chromosomal integration of pBRC60 in derivatives from *C. acidovorans* and *C. testosteroni*. However, mapping showed duplication of part of the plasmid, indicating a replicative means of integration, and a large deletion which could not be explained by recombination involving either of the known elements. The hybridization patterns suggest the existence of a second transposon on pBRC60, within which Tn5271 is contained.

The proposed structure of this new element can explain the hybridization patterns seen in all the secondary transconjugants and their respective deletion derivatives. The secondary transconjugants from *C. testosteroni* show the presence of a cointegrate structure formed between pBRC60 and the chromosome with a copy of the new element at each junction. The secondary transconjugant from *C. acidovorans* does not carry the intervening plasmid segment, and has only one copy of the new element, suggesting either homologous recombination between the two elements to delete the intervening DNA, or cointegrate resolution which would also delete the intervening DNA. The persistence of the cointegrate in the *C. testosteroni* derivatives suggests that like Tn5271, the resolution system of the encompassing element is deficient, or that the encompassing element is

not a typical class II element.

The absence of the cointegrate structure in the *C. acidovorans* derivative shows that the large deletion is not dependent on removal from 3Cba selection. This is a property that would be predicted by the location of the encompassing element; since the 3Cba genes would be contained within the element, homologous recombination between the two copies of the element would not delete the genes (Figure 4.13). This large deletion would therefore be independent of 3Cba selection.

All the deletion derivatives from both recipients have one copy of IS1071 flanked by a portion of pBRC40-derived DNA. This has been shown by restriction analysis and by PCR. Again, this would be predicted by the structure of the encompassing transposon. The remaining pBRC40-derived DNA covers the area thought to be included in the encompassing element. The loss of the 3Cba phenotype would be accompanied by the loss of the degradative genes, presumably by recombination between the two copies of IS1071 (Figure 4.13).

Interestingly, the mapping hybridizations did not show the presence of the B5 fragment in CA47P12, which should have hybridized with the E2 and E9 probes (Figure 4.5). The presence of this fragment in earlier hybridizations for IS1071 is enigmatic to begin with. The absence of this fragment in CA47P12 in hybridizations with the E2 and E9

probes, to which it should be homologous, raises questions about its identification in CA47P12. It would seem from the E2 and E9 hybridizations that this fragment is not B5, although there seems to be no explanation for a band this size to hybridize with the Nh6 probe.

The presence of a second transposon in which Tn5271 is contained may account for the failure of such a high percentage of deletion derivatives to probe positive for the insertion element. All that is required for this element to transpose are terminal inverted repeat sequences, which can be as short as 38 base pairs. This is the minimal recognition sequence for the transposases of class II transposable elements. However, if this element were to have a composite transposon structure similar to that of Tn5271 or class I composite transposons, there would be much greater homology at the ends, since the element would be flanked by two copies of an insertion element. If these were directly oriented to each other, homologous recombination between the extreme outside copies would result in the loss of the intervening sequence, including all copies of IS1071.

Alternatively, the failure of a large percent of deletion derivatives to probe positive for IS1071 could be explained by precise excision of the element. All transposons are capable of precise excision from DNA via deletion between the direct repeats formed by the

transposition. This excision is independent of transposon functions, and the excised DNA is usually lost (Kleckner, 1981; Grindley and Reed, 1985). The excision could occur either before or after the large deletion between the two copies of the encompassing element. That is, either the cointegrate would be excised or the deletion would occur via homologous recombination and the single remaining copy of the element would be precisely excised.

The variation in the sizes of the IS1071-carrying *Bgl*III fragments from the *C. testosteroni* deletion derivatives from the same secondary transconjugant can also be explained (Figure 4.7). Starting from the cointegrate structure, the deletion could occur by intramolecular transposition by one of the repeated copies of the encompassing element. Intramolecular transposition across the intervening plasmid segment to a target site on the chromosome would result in the deletion not only of most of the cointegrate but also of some flanking chromosomal DNA. Transposition immunity does not block intramolecular transposition, although the presence of a third IR on the molecule has been shown to reduce the frequency of transposition (Bishop and Sherratt, 1984). Alternatively, following the deletion event one of the ends of the transposon could delete adjacent chromosomal DNA (Figure 2.2). Variation in the locations of the target site would alter the distance to the next chromosomal *Bgl*III site, thereby resulting in *Bgl*III fragments of different

lengths in the deletion derivatives.

The results from the multiple restriction digests of the secondary transconjugants and their deletion derivatives are also supported by this model. The absence of novel bands in the secondary transconjugants CT5P3 and CT7P6 (Table 4.7) indicates that IS1071-mediated transposition could not have been the mechanism of chromosomal integration. However, transposition mediated by the encompassing element would not be expected to generate novel IS1071-carrying bands in digests with enzymes which cut close to IS1071, like *Nru*I (Figure 4.5). Novel *Sac*I bands would be expected since the ends of IS1071 and the encompassing element are located in the same *Sac*I fragment (Figure 4.5). However, the *Sac*I fragments that did hybridize were so large (Table 4.7) that the presence of novel bands may have been masked; the larger bands would not necessarily separate well enough to be distinguishable.

The variation in the sizes of the Nh6-hybridizing *Sma*I bands among the deletion derivatives (Table 4.8) is interesting. Since the end of the encompassing transposon is not in the same *Sma*I fragment as IS1071 (Figure 4.5), the *Sma* bands would be predicted to show no variation. There is a *Sma*I site very close to the end of IS1071, so it is possible that adjacent deletions by IS1071 resulted in the loss of that restriction site. Since the end of the encompassing transposon is in the adjacent *Sma*I fragment

(Figure 4.5) the loss of the intervening *Sma*I restriction site would result in the variation in sizes, as the next *Sma*I site would be located in the adjacent chromosomal DNA.

pBRC60 was shown to belong to the IncP incompatibility group by homology to the replication, maintenance, incompatibility and conjugal transfer regions of other IncP plasmids (Burlage et al., 1990). The arrangement of the IncP backbone of pBRC60 is remarkably similar to that of R751, the prototype of the IncP β subgroup (Figure 5.1). The differences lie in the insertion to the backbone. Where R751 has Tn402, encoding resistance to trimethoprim, inserted between the *pri* gene and the Tra2 region, pBRC60 has shown homology to the mercury resistance gene probes derived from Tn501 (Burlage et al., 1990). It is not known if the *mer* genes are within a transposable element on pBRC60.

Perhaps more interesting in terms of the work presented here is the location of Tn4321 on R751. Tn4321 is a putative, cryptic transposon which has not been shown to transpose (Smith and Thomas, 1989). The locations of the ends of Tn4321 on R751 are similar to those for the encompassing transposon on pBRC60. One end is in the same *Eco*RI fragment (E4) as the *trfA* operon, while the other end overlaps the *oriV* probe positive *Eco*RI (E10) fragment. It would seem that these two plasmids arose from an earlier progenitor plasmid by insertions in approximately the same

Figure 5.1 Comparison of the IncP plasmids R751 and pBRC60.

The arrangement of IncP backbones of the two plasmids are very similar; the order is the same for both and two interruptions occur at similar locations. The locations of the various IncP functions on R751 are very precise; on pBRC60 they have only been localized to restriction fragments. The locations of the ends of the encompassing transposon on pBRC0 are tentative. *oriV*, origin of vegetative replication; *kil*, host lethal functions; *kor*, *kil* override; *oriT*, origin of conjugal transfer; *Tra*, other transfer functions; *pri*, primase; *trf*, trans-acting replication functions; *mer*, mercury resistance.

new transposon

locations. In pBRC60 there is a third insertion of Tn5271 nested within the larger transposon. It would be worth comparing Tn4321 with sequences of the encompassing transposon on pBRC60, to look for further similarities.

There is other evidence that class II transposons seem to evolve frequently via insertion of another element. The transposon Tn4 has been shown to consist of Tn3 inserted into the mercury resistance gene of Tn21. As well, the mercury resistance transposon Tn501 is very closely related to Tn1721, and it has a Tn21 inverted repeat close to one of its own inverted repeats. This suggests that Tn501 was generated by transposition of a Tn21-like element, encoding mercury resistance, into a Tn1721-like element (Michiels and Cornelis, 1984).

Another example is the transposon Tn2501, which is located within the lactose transposon Tn951 (Michiels et al., 1987). Tn951 is a defective transposon, in that it does not encode a transposase protein. It has been found that the Tn2501 sequence starts exactly where the sequence of Tn951 diverges from that of Tn3, suggesting that Tn951 arose from the insertion of Tn2501 into Tn3, and that the *tnpA* genes were inactivated by the Tn2501 insertion. There are no inverted repeats flanking Tn2501 within Tn951, suggesting that deletions have occurred adjacent to Tn2501 (Michiels and Cornelis, 1984).

A similar arrangement is demonstrated by Tn4653 which

is nested within Tn4651 on the Tol plasmid pWWO. The IRs, *tnpR* gene and *res* sites of Tn4651 show extensive homology to those of Tn1721, suggesting that Tn4651 was created by the insertion of Tn4653 into Tn1721 (Tsuda and Iino, 1989).

Thus it is plausible that there is a second transposon on pBRC60 into which Tn5271 inserted. However, although there is strong evidence for its existence, much of the analysis remains speculative. Further work needs to be done to confirm the presence of the encompassing transposon. The locations of the ends of the elements are uncertain; more detailed mapping and sequencing are required in order to pinpoint their locations and define them. Finally transposition of the encompassing element must be confirmed by characterizing cointegrate structures in a *recA* host, and by sequencing the integration point in search of direct repeats.

Appendix 1: Nucleotide sequence of IS1071

The complete nucleotide sequence of IS1071 is shown in Figure A1.1. The positions of the PCR primers ISOUT3070 and ISOUT124c are shown, as are the *Nhe*I sites located just within the IRs of IS1071. Because these *Nhe*I sites are outside the locations of the primers, they would be inside the PCR products generated by these primers (Figure 4.10). Therefore, the 1.1 kb probe cut from the PCR control plasmid pBRN4029 at one of these *Nhe*I sites would not overlap the primers, and would hybridize to the products only if they contained pBRC40-derived DNA (Figure 4.10).

Figure A1.1 Nucleotide sequence of IS1071. The numbering is relative to the first G in the IR sequence (underlined). Short sequences on either side of the insertion element are shown. The translation product of the open reading frame beginning at position 146 is shown. The position of the PCR primers ISOUT3070 and ISOUT124c are indicated (double underlined) as are the positions of the *Nhe*I restriction sites at the outer ends of the IRs (boxed) (from Nakatsu et al., 1991).

[illegible]

Appendix 2: Mapping results from four secondary transconjugants and their deletion derivatives

Hybridization mapping results from four independently derived secondary transconjugants and their deletion derivatives are given in Tables A2.1 to A2.4. The digested DNA was hybridized with digoxigenin-labelled fragments from pBRC60. *EcoRI*-digested pBRC40 was used as a control in all the hybridizations. All the sizes are given in kilobases.

CA47P12 and CA47P12-A3 were not mapped as extensively as the *C.testosteroni* derivatives. Only *EcoRI*-digested total genomic DNA was used. Data from all the probes is presented; the control DNA hybridized with all the probes, even those which did not hybridize with CA47P12 or CA47P12-A3.

The three *C. testosteroni* secondary transconjugants and their deletion derivatives were mapped much more extensively, using total genomic DNA digested with *BglII*, *EcoRI*, *NruI*, *SalI* or *SmaI*. Only data from probes which hybridized to the deletion derivatives is presented. Probes E2, E3, E9, E12, Nr7 and S9 hybridized to the appropriate sized DNA fragments from the secondary transconjugants, but did not hybridize at all with the deletion derivatives. For each secondary transconjugant and its deletion derivative, the same filter was used repeatedly (except for the Sm2 hybridization of CT5P3 and CT5P3-H5) so the fragment sizes are consistent within each set of data.

Table A2.1 Mapping results for CA47P12 and CA47P12-A3

<u>probe</u>	<u>CA47P12</u>	<u>CA47P12-A3</u>
E2	-- ^a	-- ^a
E3	-- ^a	-- ^a
E6	18	18
E9	-- ^a	-- ^a
E12	-- ^a	-- ^a
Nr3	16 (E1)	16 (E1)
Nr8	16 (E1)	16 (E1)
S3	16 (E1)	16 (E1)
Sm2	>20	>20

^a no detectable signal in this lane

Table A2.2 Mapping results for CT3P15 and CT3P15-F37

probe	digest	CT3P15	CT3P15-F37
E4	<i>Bgl</i> II	>30(B1), 19	15
	<i>Eco</i> RI	8(E4)	7
	<i>Nru</i> I	25(Nr2), 11	4
	<i>Sal</i> I	20(S2), 14, 7(S3)	14, 7(S3)
	<i>Sma</i> I	15(Sm2)	3
E6	<i>Bgl</i> II	30(B2), 25	23
	<i>Eco</i> RI	6(E6)	7
	<i>Nru</i> I	28(Nr1), 8	8
	<i>Sal</i> I	30(S1), 4	4
	<i>Sma</i> I	29(Sm1)	10
E17	<i>Bgl</i> II	>30(B1), 19	15
	<i>Eco</i> RI	1(E17)	1(E17)
	<i>Nru</i> I	26(Nr2), 11	4
	<i>Sal</i> I	7(S3)	7(S3)
	<i>Sma</i> I	7(Sm7)	7(Sm7)
Nr3	<i>Bgl</i> II	12(B3)	23
	<i>Eco</i> RI	17(E1)	17(E1)
	<i>Nru</i> I	8(Nr3)	8(Nr3)
	<i>Sal</i> I	4(S5), 4(S4)	4(S5), 4(S4)
	<i>Sma</i> I	9(Sm6), 3(Sm8)	10(Sm1), 3(Sm8)
Nr8	<i>Bgl</i> II	>30(B1), 19	14
	<i>Eco</i> RI	17(E1)	17(E1)
	<i>Nru</i> I	3(Nr8)	3(Nr8)
	<i>Sal</i> I	7(S3)	7(S3)
	<i>Sma</i> I	7(Sm7)	7(Sm7)
S3	<i>Bgl</i> II	>30(B1), 19	15
	<i>Eco</i> RI	13(E1)	13(E1)
	<i>Nru</i> I	25(Nr2)	4, 3(Nr8)
	<i>Sal</i> I	7(S3)	7(S3)
	<i>Sma</i> I	7(Sm7)	7(Sm2)
Sm2	<i>Bgl</i> II	>30	15
	<i>Eco</i> RI	8(E4)	7
	<i>Nru</i> I	24(Nr2), 11	4
	<i>Sal</i> I	19(S2)	14
	<i>Sma</i> I	15(Sm2)	3
Sm8	<i>Bgl</i> II	11(B3)	22, 15
	<i>Eco</i> RI	17(E1)	17(E1)
	<i>Nru</i> I	8(Nr3)	8(Nr3)
	<i>Sal</i> I	7(S3), 4(S6)	7(S3), 4(S6)
	<i>Sma</i> I	3(Sm8)	3(Sm8)

Table A2.3 Mapping results for CT5P3 and CT5P3-H5

probe	digest	CT5P3	CT5P3-H5
E4	<i>Bgl</i> II	27	17
	<i>Eco</i> RI	8 (E4)	8 (E4)
	<i>Nru</i> I	23 (Nr2), 11	11
	<i>Sal</i> I	20 (S2)	14
	<i>Sma</i> I	15 (Sm2)	12
E6	<i>Bgl</i> II	26 (B2), 22	17
	<i>Eco</i> RI	6 (E6)	8
	<i>Nru</i> I	25 (Nr1)	-- ^a
	<i>Sal</i> I	26 (S1)	14
	<i>Sma</i> I	26 (Sm1)	13
E17	<i>Bgl</i> II	26	17
	<i>Eco</i> RI	1 (E17)	1 (E17)
	<i>Nru</i> I	23 (Nr2)	11
	<i>Sal</i> I	7 (S3)	7 (S3)
	<i>Sma</i> I	7 (Sm7)	7 (Sm7)
Nr3	<i>Bgl</i> II	11 (B3)	17
	<i>Eco</i> RI	16 (E1)	16 (E1)
	<i>Nru</i> I	8 (Nr3)	8 (Nr3)
	<i>Sal</i> I	4 (S5), 4 (S4)	4 (S5), 4 (S4)
	<i>Sma</i> I	8 (Sm6)	8 (Sm6)
Nr8	<i>Bgl</i> II	26	17
	<i>Eco</i> RI	16 (E1)	16 (E1)
	<i>Nru</i> I	3 (Nr8)	3 (Nr8)
	<i>Sal</i> I	7 (S3)	7 (S3)
	<i>Sma</i> I	7 (Sm7)	7 (Sm7)
S3	<i>Bgl</i> II	-- ^a	17
	<i>Eco</i> RI	-- ^a	16 (E1)
	<i>Nru</i> I	-- ^a	11
	<i>Sal</i> I	-- ^a	7 (S3)
	<i>Sma</i> I	-- ^a	7 (Sm2)
Sm2^b	<i>Bgl</i> II	>30 (B1), 26	19
	<i>Eco</i> RI	8 (E4), 5	15, 8 (E4)
	<i>Nru</i> I	26 (Nr2)	9
	<i>Sal</i> I	20 (S2), 7 (S3)	13, 7 (S3)
	<i>Sma</i> I	13	9
Sm8	<i>Bgl</i> II	11 (B3)	17
	<i>Eco</i> RI	16 (E1)	16 (E1)
	<i>Nru</i> I	8 (Nr3)	8 (Nr3)
	<i>Sal</i> I	-- ^a	4 (S6)
	<i>Sma</i> I	3 (Sm8)	3 (Sm8)

^a no detectable signal in this lane^b results from a different filter

Table A2.4 Mapping results for CT7P6 and CT7P6-D26

probe	digest	CT7P6	CT7P6-D26
E4	<i>Bgl</i> II	>30(B1), 23	26
	<i>Eco</i> RI	14, 8(E4)	16
	<i>Nru</i> I	25(Nr2), 4	5
	<i>Sal</i> I	20(S2), 7(S3), 4	7(S3), 5
	<i>Sma</i> I	15(Sm2), 3	4
E6	<i>Bgl</i> II	22	13
	<i>Eco</i> RI	7(E6)	19
	<i>Nru</i> I	29(Nr1), 16	-- ^a
	<i>Sal</i> I	29(S1), 22	-- ^a
	<i>Sma</i> I	29(Sm1), 23	-- ^a
E17	<i>Bgl</i> II	>30(B1), 23	25
	<i>Eco</i> RI	1(E17)	1(E17)
	<i>Nru</i> I	25(Nr2), 4	5
	<i>Sal</i> I	7(S3)	7(S3)
	<i>Sma</i> I	7(Sm7)	7(Sm7)
Nr3	<i>Bgl</i> II	12(B3)	25
	<i>Eco</i> RI	17(E1)	18(E1)
	<i>Nru</i> I	8(Nr3)	8(Nr3)
	<i>Sal</i> I	4(S5), 4(S4)	4(S5), 4(S4)
	<i>Sma</i> I	9(Sm6), 3(Sm8)	3(Sm8)
Nr8	<i>Bgl</i> II	>30(B1), 24	25
	<i>Eco</i> RI	18(E1)	18(E1)
	<i>Nru</i> I	3(Nr8)	3(Nr8)
	<i>Sal</i> I	7(S3)	7(S3)
	<i>Sma</i> I	7(Sm7)	7(Sm7)
S3	<i>Bgl</i> II	23	25
	<i>Eco</i> RI	18(E1)	18(E1)
	<i>Nru</i> I	3(Nr8)	3(Nr8)
	<i>Sal</i> I	7(S3)	7(S3)
	<i>Sma</i> I	7(Sm7)	7(Sm7)
Sm2	<i>Bgl</i> II	>30(B1), 23	25
	<i>Eco</i> RI	8(E4)	16
	<i>Nru</i> I	25(Nr2), 4	5
	<i>Sal</i> I	29, 20(S2)	5
	<i>Sma</i> I	30, 16(Sm2)	4
Sm8	<i>Bgl</i> II	12(B3)	25
	<i>Eco</i> RI	18(E1)	18(E1)
	<i>Nru</i> I	8(Nr3)	8(Nr3)
	<i>Sal</i> I	4(S6)	7(S3), 4(S6)
	<i>Sma</i> I	3(Sm8)	3(Sm8)

^a no detectable signal in this lane

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