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**SURVIVAL, ACTIVITY AND TRANSFER OF A
BACTERIAL CATABOLIC TRANSPOSON IN AN AQUATIC ECOSYSTEM**

by

Roberta R. Fulthorpe

**A thesis submitted to
the Faculty of Graduate Studies and Research
in partial fulfilment of
the requirements for the degree of**

Doctor of Philosophy

Department of Biology

Carleton University

Ottawa, Ontario

November 30th, 1990

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**SURVIVAL, ACTIVITY AND TRANSFER OF A
BACTERIAL CATABOLIC TRANSPOSON IN AN AQUATIC ECOSYSTEM**

submitted by

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in partial fulfilment of the requirements
for the degree of Doctor of Philosophy.

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DEDICATION

**This thesis is dedicated to the memory of my father,
Robert Alexander Wood Fulthorpe,
who couldn't wait for it to be finished.**

ABSTRACT

Alcaligenes sp. BR60, carrying a 3-chlorobenzoate (3Cba) catabolic transposon (Tn5271) on a conjugative plasmid (pBRC60), was introduced to twelve 50L flow-through lake water and sediment mesocosms exposed to 3-chlorobenzoate concentrations from 0 to 25 μ M. The frequency of the 3Cba catabolic genes on Tn5271 was monitored using specific DNA probes and the most probable number DNA hybridization technique. This method provided catabolic population estimates highly correlated to the [U- 14 C]-3Cba uptake rates in the mesocosms - demonstrating showing the correlation between the size and activity of a specific catabolic population in a natural mixed community for the first time.

Transfer of pBRC60 was evident since 3Cba degrading, pBRC60-carrying bacteria were isolated from the mesocosms which were distinct from BR60. The plasmid recipients expressed the catabolic genes in different ways. The majority of pBRC60 recipients were motile yellow pigmented gram negative rods related to the Group III Pseudomonads and to BR60 according to their substrate utilization patterns. They were capable of complete 3Cba degradation at both mM and μ M concentrations. *Pseudomonas fluorescens* strain PR24b(pBRC60) and *Pseudomonas* sp. strain PR120(pBRC60) were more distantly related to BR60 and both produced chlorocatechol when exposed to 3Cba at mM concentrations in the presence of yeast extract. These species showed poor growth in liquid 3Cba minimal media but could degrade 3Cba in continuous culture dosed with μ M levels of the chemical. Laboratory matings confirm that pBRC60 can transfer from BR60 to species in both

the beta and gamma sub-groups of the proteobacteria, and that 3Cba gene expression is variable between species.

BR60 inoculated mesocosms were exposed to 50 nM, 1 μ M, and 50 μ M 4-chloroaniline (PCA), 3-chlorobiphenyl (CBP), or 2,4-dichlorophenoxyacetate (2,4-D). Tn5271 carrying organisms distinct from BR60 reached significantly higher than control populations in the control 25 μ M 3Cba, the 50 μ M PCA and 50 μ M CBP dosed mesocosms after 60 days exposure. A dose response relationship between probe positive populations and PCA dose was apparent.

3Cba degrading bacteria taxonomically distinct from BR60 were isolated from CBP dosed mesocosms that carried pBRC60. An isolated chlorobiphenyl oxidizing strain carries a large plasmid unrelated to pBRC60, and is metabolically similar to other large-plasmid-bearing strains isolated on chlorobenzoate and chlorophenol agar from experimental mesocosms. *P. delafieldii* T6-11 was repeatedly isolated from PCA dosed mesocosms - carrying one copy of IS1071 (the insertion sequence that brackets Tn5271) on a large plasmid unrelated to pBRC60.

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LIST OF ABBREVIATIONS

AODC	acridine orange direct counts of bacteria
BglII	restriction endonuclease
BR40	<i>Alcaligenes</i> sp. BR40, carrying plasmid pBRC40
BR60	<i>Alcaligenes</i> sp. BR60, carrying plasmid pBRC60
BR6025	A chloramphenicol resistant, tryptophan requiring mutant of BR60, lacking pBRC60
BR611	<i>Alcaligenes</i> sp. BR611, carrying plasmid pBRC60 and capable of 3-chlorobenzoate and 4-chlorobenzoate utilization.
3Cba	3-chlorobenzoate
3Cba+	capable of using 3-chlorobenzoate
4Cba	4-chlorobenzoate
CBP	3-chlorobiphenyl
CFU	colony forming units
Cm ^r	chloramphenicol resistant
CPS	casitone-peptone-starch medium
2,4-D	2,4-dichlorophenoxyacetic acid
EcoRI	restriction endonuclease
E-11	DNA probe made from restriction fragment EcoRI-11 of pBRC60
FL	artificial lake water medium
HindIII	restriction endonuclease
IS1071	DNA insertion sequence that brackets either end of transposon Tn5271

K _m	the concentration at which uptake is one half its maximum value
PCA	parachloroaniline = 4-chloroaniline
pBRC40	plasmid pBRC40, derivative of the larger pBRC60, lacking Tn5271 and the 3-chlorobenzoate utilization genes
pBRC60	plasmid pBRC60, carrying the transposon Tn5271 harbouring the 3-chlorobenzoate utilization genes
probe+	probe positive bacteria, having DNA homologous to a Tn5271 DNA probe
pUC18	small plasmid vector used to clone fragments of DNA
SDS	sodium dodecyl sulphate
SSC	sodium chloride, sodium citrate solution
stdev	standard deviation
TAE	Tris-acetate-EDTA, electrophoresis buffer
TBE	Tris-borate-EDTA, electrophoresis buffer
TE	buffer composed of 10 mM Tris, 1 mM EDTA, pH = 8.
Tn5	a transposon carrying kanamycin resistance
Tn5271	transposon carried by pBRC60 encoding 3Cba utilization
Trp ⁻	denotes the strain requires tryptophan for growth
TYE	tryptone-yeast extract medium
U- ¹⁴ -C	aromatic compound labelled with carbon-14 in an unspecified position on an aromatic ring
V _{max}	maximum uptake rate of compound

Codes used for various mesocosm treatments are defined in the chapters where these treatments are discussed, see Table 4-2 and Table 6-1.

CHAPTER 1:BACKGROUND AND RATIONALE FOR STUDIES UNDERTAKEN

CHLORINATED AROMATIC COMPOUNDS IN THE ENVIRONMENT

Industrial organic chemicals and their byproducts are often xenobiotic, that is, foreign to the biochemical make up of all living organisms. Many of these chemicals persist after release to the environment. They are resistant to chemical change because of their stable chemical structures and resistant to bacterial attack because of their dissimilarities to normal organic substrates. Some accumulate to toxic levels in the tissues of living organisms. The benzene ring is the basis of many of the problematic toxic and recalcitrant industrial chemicals, yet natural forms of it are ubiquitous in nature and micro-organisms readily exploit this abundant carbon source. Biochemical pathways for the degradation of aromatic compounds are similar for many bacterial species and genera. Under aerobic conditions, degradation proceeds via the dihydroxylation of the aromatic structure followed by cleavage of the ring and production of simple metabolites that feed into the Krebs cycle. The pre-cleavage product is normally based on one of six structures, the most common of which is catechol (Figure 1-1). Catechol and substituted catechols are enzymatically cleaved in two ways, between the two hydroxyl groups (intradiol or ortho cleavage) or immediately adjacent to one of them (extradiol or meta cleavage) (Dagley 1986). Enzymes catalyzing this fission are variously referred to as pyro-catechases, catechol oxygenases, ring fission enzymes, or catechases.

Figure 1-1: Some simple aromatic compounds degraded via catechol. The meta cleavage pathway of catechol is depicted on the left, the ortho cleavage pathway on the right. The fate of chlorocatechol in these two pathways is illustrated. Adapted from Stanier and Ornston (1976), Dagley (1987), and Reineke and Knackmus (1988).

Chlorinated aromatic compounds are synthesized for industrial use specifically because the highly electro-negative chloride stabilizes the aromatic bonds of benzene and yields a molecule resistant to abiotic chemical change. The large size of chlorine atoms hinders enzymatic attack, such that benzene rings with 2 or more chlorines or a chlorine adjacent to another functional group are particularly resistant to microbial transformation. The monochlorinated rings are easily cleaved but chlorine continues to cause problems. Meta cleavage of chloro-catechols generates acyl chlorides (termed "suicide metabolites" because of their inhibitory effect on enzyme activities) while ortho cleavage is followed by loss of the chloride and production of utilizable muconate and succinate. However ortho cleavage of natural intermediates such as methyl catechol produces a methyl substituted muconolactone that cannot be processed by subsequent enzymes. (Bartels *et al.* 1984). The most commonly encountered pathway for utilization of aromatic substrates, such as xylenes, toluenes and their alcohol and acid derivatives is the TOL pathway that employs meta fission catechol oxygenases (Frantz and Chakrabarty 1986). More unusual bacteria capable of growth on simple chlorinated aromatics found to date carry ortho cleavage catechol oxygenases with broadened substrate specificities (Spain and Gibson 1988, Pierce *et al.* 1983, Reineke and Knackmuss, 1988)

In laboratory cultures and natural bacterial communities that cannot metabolize chlorinated aromatics, chlorinated catechols are produced by virtue of attack by ring oxidation enzymes. Chlorocatechols and their polymerization products accumulate and persist (Spain and Gibson 1988; Schmidt 1987; DiGeronimo 1979; Spokes and Walker 1974; Horvath and Alexander 1970; Remberger *et al.* 1986).

Studies show that the introduction of an ortho cleavage catechase to individual strains or to mixed cultures broadens the degradative abilities of those strains or systems (Krockel and Focht 1987; Reineke and Knackmuss 1980; Hartman *et al.* 1979; Schmidt 1987; Schmidt *et al.* 1983). Currently, research teams are focussing on the construction of new catabolic strains by artificially recombining catabolic genes and regulatory sequences in vitro (Timmis *et al.*, 1988) or by mixing together strains with useful genes and subjecting the mixture to severely restrictive selective conditions (Krockel and Focht 1987, Kellogg *et al.* 1981).

CHLORINATED AROMATIC DEGRADERS IN SITU

While this "bacterial husbandry" is producing versatile strains and useful insights into microbial genetics and catabolic pathways, questions abound concerning the performance of laboratory selected strains in natural systems. Current environmental technology standards have created a demand for catabolic bacterial strains for waste treatment and for release to contaminated sites, but our understanding of the potential for inoculant or catabolic gene survival in natural systems is poor.

Isolated bacterial populations form the basis of our knowledge of the metabolism, genetics and ecology of chlorinated aromatic degraders but the population dynamics and natural activity of these organisms within complex microbial communities remains poorly understood, primarily because of technical difficulties of making measurements in situ. In environments such as the water and benthos of rivers or lakes, chlorinated organic contaminants may occur at micromolar levels or

less. Although biodegradation of synthetic organics does occur at low concentrations (Lewis *et al.* 1988), studies suggest that the organisms responsible differ from those operating at higher concentrations (Rubin *et al.* 1982). The usefulness of laboratory selected strains as inocula in natural situations has been demonstrated in only a few cases (Dwyer *et al.* 1988, Edgehill *et al.* 1983, Karns *et al.* 1984, Focht and Shelton 1987, Brunner *et al.* 1985) and there are reports of inocula that failed to enhance biodegradation of their substrate *in situ* (Ramadan *et al.* 1990, Goldstein *et al.* 1985, McClure *et al.* 1990). or that survived poorly (Kilbane *et al.* 1983). Natural conditions present a number of problems for laboratory selected contaminant degraders re-introduced into the environment. Low substrate concentrations may not support the growth of these specialized strains, or the introduced organisms may abandon metabolism of the contaminant in favor of other substrates (Goldstein *et al.*, 1985). Naturally occurring toxins, predators, and interspecific competition for nutrients may all reduce the survival of the introduced strain (Liang *et al.* 1982, Scheuerman *et al.* 1988). On the other hand, alternate carbon sources available in natural ecosystems may enhance the survival of introduced strains and hence the degradation of low level contaminants (Schmidt and Alexander, 1985).

Assuming an inoculum survives, the degree to which catabolic genes are disseminated in natural systems is unknown. Abundant evidence exists that bacterial genetic exchange processes occur in nature, including bacterial conjugation, phage transduction and natural transformation. (Coughter and Stewart 1989; Trevors *et al.* 1987; Stotsky and Babich 1986, Morrison *et al.* 1978, Stewart and Sinigalliano, 1990; Carlson *et al.* 1985). However the rate and the importance of these processes to the

degradation of xenobiotic chemicals is unknown.

MODEL CHLORINATED AROMATIC DEGRADER - *Alcaligenes* sp. BR60

Using a chlorobenzoate degrading bacterium as a model organism, I set out to explore basic questions about the ability of this strain to survive in a natural system and the potential for transfer of the catabolic genes in systems treated with chlorobenzoate and with similar simple chlorinated aromatics. I used a chlorobenzoate degrader that was isolated by Dr. R.C. Wyndham from chlorobenzoate and chlorophenol contaminated waters in the Niagara region (Wyndham and Straus, 1988). This bacterium had three desirable characteristics - a) it degrades a simple chlorinated compound via an ortho cleavage pathway; b) the catabolic genes are encoded on a mobile DNA element; and c) it was a natural isolate from a freshwater aquatic system. The latter characteristic meant it had a reasonable chance of surviving in a new system, and that release experiments would not be restricted by regulations applicable to genetically engineered strains. *Alcaligenes* sp. BR60 is capable of growth on 3-chlorobenzoate (3Cba) because of genes found within a transposable DNA element, Tn5271, carried by conjugative 85 kb plasmid, pBRC60 (Wyndham *et al.* 1988). Tn5271 is presumed to be mobile by virtue of the transposase encoding insertion sequence, IS1071, that brackets it. Homologous recombination between IS1071 elements deletes the internal catabolic region at high frequencies from the carrier plasmid, leaving behind a single copy of IS1071 in a derivative plasmid called pBRC40. IS1071's transposition function also facilitates the insertion of Tn5271 into the chromosome of BR60. Ongoing

Figure 1-2: Restriction enzyme map of Tn5271, the catabolic element carried on pBRC60. Tn5271 is deleted at high frequencies, resulting in a smaller plasmid, pBRC40 with only one copy of the bracketing insertion sequence IS1071.

sequencing work on Tn5271 has revealed the presence of 7 genes in the catabolic region (Nakatsu *et al.* 1991, Figure 1-2). Complete loss of Tn5271 from the organism is always accompanied by loss of 3Cba catabolic activity. *Alcaligenes* sp. BR60 cultures growing on 3Cba accumulate chloromuconic acid, indicating that an ortho cleavage chlorocatechol oxygenase is active in this organism (Wyndham and Strauss 1988). The cloning of fragments of Tn5271 allowed me to use specific DNA probes to determine the frequency of homologous DNA in the natural systems.

HYPOTHESES TESTED

Using *Alcaligenes* sp. BR60, I explored four basic questions, each one leading from the previous:

- 1) Could BR60 survive in a new aquatic environment after release?
- 2) Would it utilize 3-chlorobenzoate *in situ* and if so, would it require 3Cba to survive?
- 3) Would pBRC60 transfer to other bacteria in the community?
- 4) Would the chlorobenzoate catabolic genes on Tn5271 be used by community bacteria in the degradation of other chlorinated aromatic compounds that share part of the degradative pathway of 3-chlorobenzoate?

This thesis addresses these questions in turn. General methods are described in the chapter that follows. Preliminary information presented in Chapter 3 describes the origin of BR60 and the selection of a study site for release experiments. Chapter 4 describes those release experiments and the relationship between the BR60 inoculum and chlorobenzoate utilization *in situ*. Chapter 5

presents evidence for the horizontal transfer of pBRC60. Chapter 6 describes the response of BR60 and Tn5271 to three other chloroaromatic chemicals over a 100 day period. Chapter 7 describes those organisms that responded to those chemicals and their genetic makeup, and presents a number of suggestions for future studies. The final chapter summarizes the main findings of this work.

CHAPTER 2: BASIC METHODS AND MATERIALS

BACTERIAL STRAINS

Alcaligenes sp. BR60 is described in the previous chapter. *Alcaligenes* sp. BR611 is a mutant of BR60 that can utilize 4Cba and 3Cba and also carries pBRC60 (Wyndham *et al.* 1988). Both BR60 and BR611 are naturally streptomycin resistant. Freeze dried samples of the following were purchased from the American Type Culture Collection: *Pseudomonas acidovorans* (ATCC 15668), *P. alcaligenes* (ATCC 14909), *P. cepacia* (ATCC 25416), *P. fluorescens* (ATCC 13525), *P. pseudoflava* (ATCC 33668), *P. palleronii* (ATCC 17724), *P. putida* Biotype A (ATCC 12633), *Chromobacterium violaceum* (ATCC 12472) *Alcaligenes* sp. H850 was provided by General Electric Company in Schenectedy, New York. *Acinetobacter calcoaceticus* DON2, an aniline degrading isolate from the Don River was described by Wyndham (1986). *Moraxella* sp. G. was provided by Dr. Josef Zeyer of Geneva, Switzerland (Zeyer *et al.* 1985). *P. putida* PRS2015 was provided by Dr. Chakrabarty (Chatterjee and Chakrabarty 1983).

GROWTH MEDIA

General media used for strain maintenance and/or purification included 0.1% TYE agar (0.1% tryptone, 0.1% yeast extract, 1.8% agar), CPS agar (0.5g casitone, 0.5g peptone, 0.5g soluble starch, 1g glycerol, 0.04 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2g K_2HPO_4 , 1g agar in 1L water) or nutrient agar. Nutrient agar was only used if strains had previously demonstrated growth on this medium. Lake water or sediment microflora

were enumerated or purified on 0.1%TYE or CPS because a greater diversity of heterotrophic strains could be cultured on these media versus nutrient agar. Selective media for isolation or maintenance of catabolic strains were based on minimal medium A (Wyndham, 1986), and contained 1.8% agar and 50 mg/L bromothymol blue as a pH indicator. 2,4-D, 3Cba and 4Cba plates contained 3mM of substrate. PCA/ye plates contained 2 mM PCA supplemented with 0.005% yeast extract. CBP-agar contained no carbon source, 50 μ L CBP was placed on the lid of the petri dishes after plating. The pH was adjusted to 8.0 for all plates. Batch cultures were grown in identical media, lacking agar. Populations of BR60 and BR611 were enumerated by triplicate plate counts on 3Cba or 4Cba agar, respectively, containing 25 μ g/mL streptomycin. Fluorescent pigments were determined by streaking on King's B agar (King *et al.* 1984).

MESOCOSM DESIGN

Queen's University Biological Station is situated on Lake Opinicon near Elgin, Ontario. The toxicology facility at this station was used to set up 12 mesocosms representative of an epilimnetic sediment/water interface. The mesocosms were large aquaria (72cm x 40cm x 22cm high) containing surficial sediments, complete with small macrophytes, detritus and snails, taken directly from a shallow bay of the lake. A 5 cm layer of epilimnetic sediment was allowed to settle and was not subsequently disturbed. The 50 L of overlying water was aerated gently with porous stones. Epilimnetic lake water was continuously pumped from an intake pipe in the middle of an adjacent 2m deep bay into an overflowing header

tank before delivery to the various mesocosms at a controlled dilution rate. Sterile aqueous solutions of the chloroaromatic treatment chemical were also delivered at fixed rates to selected tanks. Overflow water from experimental tanks was passed through a carbon filter before entering a seepage bed. In 1989 a plastic wading pool (120 cm in diameter by 30 cm deep) retained the overflow water before release to the carbon filter and chemical concentrations in this treatment pool were monitored. No nutrients were added and all experiments took place at ambient temperatures.

Lake water and chemical delivery systems.

Initial Design, 1987: Lake water was siphoned from the header tank to the mesocosms using aquarium air tubing constricted with plastic clamps such that the dilution rate approximated 0.05 hr^{-1} . 3Cba was delivered from sterile 4 mM reservoirs using commercially available intravenous apparatus.

Technical improvements 1988-1989: Water and chlorobenzoate delivery systems were altered in 1988 to allow finer control on the feed concentration of 3Cba. A 50 X 32 X 16 cm plexiglass header tank containing an 11 cm baffle was constructed from 1/4 inch plexiglass. Water flowed from the header tank through 1 mL plastic syringes capped with upturned fine gauge stainless steel needles. Turning the needles tip up eliminated most problems associated with funnelling of lake water particulates and subsequent blockage of the needles, but as an extra precaution incoming lake water was also filtered through a 170 μM mesh screen prior to the header tank. Dilution rates were set at 0.01 hr^{-1} by using a 20G needle 3 cm below the water surface in the header tank. Chemicals were delivered from sterile

Figure 2-1: Schematic of mesocosm system set up at Queen's University Biological Station.

reservoirs to the water delivery lines via a 10 rpm 10-channel peristaltic pump (Watson-Marlow 501U) at the rate of 0.008 mL/min. Aquaria were sealed with plexiglass lids containing input and output tubes sealed in place. These lids were designed to eliminate the transfer of aerosols or water spray between mesocosms.

DNA HYBRIDIZATION TECHNIQUES

DNA probe preparation and hybridization conditions: Cloned fragments of Tn5271 or purified preparations of pBRC60 were used to make DNA probes. A pUC18 library of EcoRI and HindIII pBRC60 fragments in *E. coli* JM101 was available. EcoRI fragments 11 or 5 or HindIII fragment 2 were generally used as DNA probes for Tn5271. pUC18 plasmids were isolated from *E. coli* JM101 using alkaline lysis and were purified by chromatography on Sepharose 4B columns (Pharmacia) as described previously (Wyndham and Straus, 1988). The vectors were cut using either EcoRI or HindIII and loaded onto a low-temperature melting agarose gel (Seaplaque Agarose, FMC Corp) for electrophoresis. The pBRC60 derived fragment was cut from the agarose and diluted 1:3 with sterile distilled water, and stored frozen until needed. ³²P labelled probes were prepared by denaturing the fragment at 100°C for 5-10 minutes, and labelling with ³²P deoxycytosine triphosphate (Amersham) using the random primer - Klenow polymerase method (Feinberg and Vogelstein, 1984). The labelled probe was separated from unincorporated ³²P dCTP on a Sephadex G-50 column equilibrated with 10 mM Tris buffer before use. The probe was used at approximately 10⁷ cpm per 20 mL of hybridization solution. The hybridization solution used with these

probes was composed of 5X Denhardt's solution, 5x SSPE, 0.1% SDS, and 50% formamide, and hybridizations were always carried out at 42°C. 3 washes were carried out using 0.1% SDS and 0.1X SSC at room temperature, plus one at 55°C. After drying samples were exposed to KODAK X OMAT AR X-ray film.

Digoxigenin labelled probes were also made by the random primer method. For 50 μ L of probe, 38 μ l of denatured probe in dilute (1:3) Seaplaque agarose was mixed with 5 μ L of random primers (Boehringer Mannheim) and 5 μ L of deoxyribose nucleotides/digoxigenin labelled dUTP solution (10 mM each dATP, dCTP, dGTP, 6.5 mM dTTP, 3.5 mM digoxigenin-11-dUTP), and 2 μ L Klenow polymerase enzyme. This was left to label overnight (up to 20 hours) at 37°C. The probe was not purified further, but was added directly to hybridization solution after denaturing at a concentration of 1 μ L probe per mL of hybridization solution. Although Boehringer Mannheim recommends purification from low-melt agarose, in my hands the unpurified preparation had high specificity and sensitivity, and DNA losses during precipitation were avoided. The hybridization solution used was 0.1% SDS, 0.1% sarcosyl, 0.5% blocking agent and 3X SSC for highly stringent conditions or 5X SSC for less stringent conditions, as recommended by Boehringer Mannheim. Hybridizations were carried out overnight at 66-68°C. The remainder of the procedure was exactly as described in the Boehringer Mannheim manual for non-radioactive probing (1989).

***In situ* colony hybridization:** Bacterial growth on nitrocellulose filters was fixed by placing them on Whatman 3M paper soaked in 10% SDS for 3 minutes, denaturing solution (0.5 M NaOH, 1.5M NaCl) for 5 minutes, neutralizing solution

(0.5 M Tris, 1.5 M NaCl) for 5 minutes, and finally SSPE for 5 minutes. Filters were dried and baked at 80°C and stored at -20°C prior to probing. *In situ* fixed growth was probed with ³²P labelled probes only. DNA probes made with non-radioactive labels such as biotin or digoxigenin exhibited too much non-specific binding to colonies unless filter bound colonies were treated with protease as outlined by Taub and Thompson (1982).

MPN:DNA hybridizations: A method adapted from Frederickson *et al.* (1988) was used to quantify population levels of bacteria carrying DNA homologous to our probes in mesocosm water and sediments or laboratory cultures. Undiluted mesocosm water or 10-1 diluted sediment was inoculated with 5 replicates for each sample and diluted a further 3-4 orders of magnitude in microtitre plates containing a growth medium. After 4-5 days growth, contents of the plates were filtered onto nitrocellulose or nylon (Amersham Hybond) membranes using a vacuum manifold designed for the filtration of microtitre plate wells (Shleicher and Schuell). Nitrocellulose or nylon membranes were processed in the same way as colony growth. DNA was fixed onto nylon membranes by exposure to the short wave radiation of a transilluminator for 5 minutes for cross linking. Again, only ³²P labelled probes were used with these preparations. Those wells showing hybridization of a density comparable to BR60 control wells were counted and an estimate of "probe positive" bacteria was derived from MPN tables (de Man, 1975).

Hybridization of Southern Blots: Gels were blotted onto nylon membranes using the capillary transfer technique (Sambrook *et al.* 1989). After drying and cross

Figure 2-2: Typical result of MPN:DNA hybridizations. Lake water (w) and 10^1 diluted sediment (s) samples were inoculated into 5 wells and diluted three orders of magnitude in the rows below the inoculum. On the right lake water and sediment from mesocosms inoculated with BR60 are shown, on the left are lake water and sediment from mesocosms not inoculated with BR60. Hybridizations were more intense using a general growth media because of the denser bacterial growth.

Uninoculated**Inoculated** **General
Medium** **Selective
Medium**

-linking, hybridization was carried out using ^{32}P labelled probes, or in later work, using digoxigenin labelled probes.

HETEROTROPHIC POTENTIALS

The degradation rates of the treatment chemicals in bacterial cultures, mesocosm waters and sediments were determined using ^{14}C labelled substrates obtained from Sigma radiochemicals. Their specific activities (mCi/mmole) were: 3Cba - 12.3, 2,4-D - 16.6, PCA - 15.6, 3CBP - 10.06. Each compound was prepared as a 300 μM aqueous solution and frozen before use. Heterotrophic potentials of mesocosm waters were determined from either the rate of uptake of the compound (1987 and 1988 studies) or from the rate of uptake and the rate of production of $^{14}\text{CO}_2$ (1989). Background levels of treatment chemicals were used to correct uptake and CO_2 production data where applicable.

Waters: Water was pipetted into rubber stoppered glass test tubes in 3 mL triplicate samples for each of 5 concentrations of added ^{14}C labelled 3-Cba ranging from 1 to 100 μM . Tubes were incubated at room temperature for a fixed period (1-3 hours) before the contents were filtered through 0.22 μm nucleopore membranes using a vacuum manifold. The filters were rinsed with 6 mL of distilled water, dissolved in 0.5 mL of ethanolamine and counted in a LKB scintillation counter using a 15% methanol/POP-Bis toluene scintillation cocktail (Wyndham 1986). Uptake controls were carried out using formaldehyde treated water.

The evolution of $^{14}\text{CO}_2$ was measured by adding labelled substrate at 5 concentrations between 0.1 and 10 μM to triplicate 3 mL samples and incubating for 2 hours in rubber capped serum tubes. Samples were acidified and recapped

with ethanolamine soaked filter paper strips suspended from the tops of the tubes, boiled briefly, cooled and left overnight. Samples tested with PCA or CBP were not boiled but were left in a 30°C incubator overnight, and for the latter small pieces of activated charcoal were added after acidification to bind any free CBP and to minimize its volatilization overnight. Filter paper strips were removed and immersed in scintillation cocktail (85% toluene containing 4 g/L POPOP:15% methanol, or PCS from Amersham) and counted in a Beckman scintillation counter. Triplicate controls inoculated with 25 μL ^{14}C NaHCO_3 (Sigma Radiochemicals, specific activity = 10 mCi/mmol) were used to determine the efficiency of capture of CO_2 . Values of maximum uptake rates (V_{max}) and half saturation constants (K_m) were determined from the Lineweaver-Burk linearization of the Michaelis-Menten equation, i.e.;

$$1/v = K_m/V_{\text{max}} * 1/s + 1/V_{\text{max}}$$

where s = substrate concentration and v = the sum of substrate taken up and respired. This commonly employed method was chosen in spite of its statistical faults because the uptakes rates were of interest not for their absolute values but for correlation of their relative magnitudes to bacterial population parameters. The preferred option of fitting data directly to the Michaelis-Menten non-linear model was impractical because of the paucity of good non-linear regression procedures available at the time of writing. The reader is referred to Robinson (1985) for a good discussion of the application of nonlinear regression to kinetic analysis.

Sediments: Heterotrophic potentials of sediments were determined using CO_2 production alone. Sediment skimmed from the surface was allowed to settle in sterile tubes before 0.25 mL of settled sediment was subsampled and added to 2.75

mL of mesocosm water. The procedure was then the same as for water samples. After analysis, values of $V_{\max}$ obtained for mesocosm waters was subtracted from the $V_{\max}$ of the water and sediment combined. The remaining activity was attributed to the sediment microflora.

CHEMICAL CONCENTRATION DETERMINATION

Mesocosm water samples (10 mL) were preserved with 100 μ L 0.25M HgCl_2 and stored frozen in glass scintillation vials before analysis. The concentration of chemicals in mesocosm waters and culture samples was determined using high pressure liquid chromatography with a Waters 501 solvent pump, Nova-Pak C-18 reverse phase column and Lambda-Max Model 481 LC spectrophotometer set at 237 nm to detect 3Cba, 2,4-D, and at 254 nm to detect CBP and PCA. The solvent used was a 1:1 mixture of methanol and 1% acetic acid. Standards were made using column solvent. Using these conditions retention times equalled 5.7, 6.5 and 7.2 minutes for 3Cba, 2,4-D and PCA respectively. Chlorobiphenyl standards formed a broad peak at 8.5 minutes.

ISOLATE CHARACTERIZATION

Phenotype Fingerprints/Preliminary Identification: BIOLOG GN ID kits from BIOLOG Inc. Hayward, California were used to characterize gram negative isolates of interest. Colonies were streaked onto CPS agar and grown for 18 hours or less at 30°C. Growth was rolled onto a sterile cotton swab and suspended in 1 mL of medium A and centrifuged at 3,000 g for 3 minutes in an Eppendorf centrifuge to give a loose pellet. 100 μ L of this pellet was resuspended in 20 mL of

sterile saline (0.85%) or distilled water, vortexed and pipetted into BIOLOG GN ID plates and incubated for 24 hours. The binary results from the 95 substrate utilization tests in the BIOLOG GNID plates were used to generate a matrix of Jaccard similarity coefficients using a BASIC program (Appendix I). This matrix was used to cluster the isolates using the single linkage CLUSTER procedure of SYSTAT (SYSTAT Inc., Evanston, Illinois).

General Characterization: Tests for gram stain, oxidase reaction, catalase activity, poly- β -hydroxybutyric acid accumulation, and fluorescent pigment production were carried out as described by Smibert and Kreig (1981). Liquid broth cultures of various compositions and ages were examined to determine cell shape and motility. Cultures containing motile cells were stained using Gray's Mordanting Solution or Liefson's Stain to determine flagellar type. Gray's mordanting solution (5 parts saturated potassium aluminum sulphate, 2 parts 20% tannic acid, 2 parts saturated mercuric chloride, 0.8 parts saturated alcoholic basic fuchsin) was applied to the edge of a wet mount, flagella became visible under phase contrast after a few minutes, but were still extremely thin and difficult to see. Liefson's stain proved more reliable. The stain was made by mixing equal parts of 1.2% alcoholic basic fuchsin, 3% tannic acid and 1.5% sodium chloride, and allowing the mixture to settle for 24 hours. Small batches were stored at -20°C and warmed to room temperature before use. A 1 mL sample of a motile culture was centrifuged at 2,000 g for 3 minutes, washed in sterile TE and spun again. Pellets were resuspended in a small volume of 1% formalin and applied to a clean (never used) glass slide. After drying, a rectangle was drawn around the preparation with a grease pencil. Liefson's

stain was applied for up to 20 minutes. When the surface of the stain appeared covered with precipitate, the preparation was rinsed in distilled water, dried and examined under oil immersion. Properly stained cells exhibited thickened, highly visible flagella.

Rapid Plasmid Screens: Rapid plasmid screens were performed on fresh plate or broth cultures growing either on selective media or on general media inoculated with a colony from selective media if possible.

i) **In-well lysis.** Cell suspensions obtained from suspending fresh (1-2 day old) plate growth in TE or from logarithmic phase 5 mL cultures were centrifuged at 6,000 g for 5 minutes, and the supernatant replaced with 4 volumes (50 to 200 μ L) of lysis buffer (50 mM Tris, 10 mM EDTA, 2 mg/mL lysozyme, 25% sucrose, 100 ug/ml RNAase, pH = 8) and the cells resuspended and left at room temperature for 2 hours. Cell lysis buffer suspensions were diluted with an equal volume of xylene cyanol/30% glycerol loading buffer and pipetted into the wells of a 0.7% agarose gel which had been pre-loaded with 3 μ L of 10% ultrapure SDS. Cell lysis occurred immediately in most cases. The gel was then transferred to an electrophoresis tray and run in TAE at 20 V overnight or at 40V for 4 hours.

ii) **Sheared Lysate Electrophoresis:** Fresh plate growth was rolled onto a sterile cotton swab, resuspended in 1 mL TE(10mM Tris, 1 mM EDTA, pH=8), spun at 3,000 g for 3 minutes in an Eppendorf centrifuge. This cell pellet was resuspended in 50 to 200 μ L of lysis buffer (50 mM Tris, 10 mM EDTA, 2 mg/mL lysozyme, 25% sucrose, 100 ug/ml RNAase, pH = 8) and left on ice for 2 hours. 1% SDS was added to lyse the cells. The crude lysate was vortexed for 2 minutes

to shear the chromosomal DNA and decrease lysate viscosity. An equal volume of loading buffer was added and the lysate loaded into the wells of a 0.6% agarose/TBE gel (Sambrook *et al.* 1989) and run in 1/2X TBE at 20 V overnight or at 40V for 4 hours. This procedure always gave a large chromosomal band but plasmid bands were also more intense than with the in well lysis procedure, presumably because of the greater release of plasmid DNA from association with large masses of chromosomal DNA.

Plasmid Extractions: Plasmid extractions were carried out according to the general method of Crosa and Falkow (1981) in which lysis is carried out at pH 12.3-12.6. Lysis at this pH is thought to minimize losses of large plasmid DNA during denaturation, although minimal disturbance of the alkaline lysate prior to neutralization may be a more important factor.

i) **Large Scale:** 3Cba degrading strains were cultured in 300 mL of 3Cba/ye broth, non-3Cba degrading strains were cultured in 0.1% TYE or CPS broth. Cells were harvested from a log phase culture by centrifuging at 6,000 g for 10 minutes in a Sorval RCB-2. Pellets were resuspended in 1 mL TE (10 mM Tris, 1 mM EDTA, pH=8) containing 2 mg/mL lysozyme and left on ice for 10 minutes. The cell suspension was pipetted slowly into 15 mL lysis buffer (50 mM Tris, 10 mM EDTA, pH=12.5, 3% SDS) in glass corex tubes. Cells were lysed with a minimum of disturbance for 10 minutes. 7 mL of 3 M sodium acetate (pH=4.5) was added, the tubes were slowly inverted to mix, and placed on ice for 2 hours with intermittent inversions. The chromosomal DNA was removed by centrifuging at 15,000 g and the supernatant extracted twice with 5 mL pH 8 Tris buffered-phenol

and 5 mL chloroform. The extracted prep was made 0.3 M NaCl and 0.6 volumes of isopropanol added. Plasmid precipitation took place overnight at -20°C. Plasmids were centrifuged at 20,000 g and washed in 70% ethanol before dissolution in 100-400 μ L TE. The plasmid preparation was treated with RNAase A prior to restriction digestions and electrophoresis.

ii) Small Scale: Small scale plasmid extraction volumes were taken from Gealt *et al.* (1985). 50 mL cultures of cells grown in a selective medium or in 0.1% TYE were harvested in an IEC Centra 4R centrifuge at 10,000 g at 4°C. The pellet was suspended in 1-4 mL of TE (depending on cell yield), divided between 1.5 mL Eppendorf microcentrifuge tubes, recentrifuged and resuspended in 40 μ L TE per tube. 600 μ L of lysis buffer (4% SDS in 50 mM Tris, 10 mM EDTA, pH=12.4, prepared fresh) was added to the suspension which was gently inverted and incubated at 37°C for 20 min. 30 μ L of 2 M Tris-hydrochloride, pH=7, was added for neutralization followed by 240 μ L 5M sodium chloride to precipitate the chromosomal DNA. Precipitation took place on ice for 2-4 hours, followed by centrifugation at 20,000 g for 15 min at 4°C. The supernatant was transferred to a fresh tube prior to precipitation of plasmid DNA with 600 μ L isopropanol, incubation at -20°C and centrifugation at 20,000 g at 4°C. Pellets were dissolved in 50 μ L of TE.

Total Genomic DNA Extractions: Total genomic DNA was extracted from 5 mL nutrient broth or CPS stationary phase cultures after incubation with shaking at room temperature. Cells were harvested at 6,000 g in a Sorval RCB2 centrifuge for 10 minutes. The cell pellet was resuspended in 0.3 mL of TE, 5 μ L of 10

mg/mL RNAase, and 100 μ L of 1 mg/mL Proteinase K. After incubation on ice for 10 minutes and lysis with 100 μ l of 10% sodium sarcosyl, the lysate was incubated at 37°C overnight. 3 mL sterile TE was added prior to extraction with one volume of 200mM Tris, pH 8, 50mM NaCl buffered phenol, followed by two to three extractions with chloroform-/isoamylalcohol (24:1) until no white precipitate formed. DNA was precipitated with 0.6 volumes isopropanol, spooled out using a glass rod, dissolved in 400 μ l of TE and reprecipitated using 2 volumes of cold ethanol. Total genomic preparations were digested with EcoRI or BglII restriction endonucleases.

BACTERIAL CONJUGATION EXPERIMENTS

To test the host range of the pBRC60 replicon, I used a Tn5 (kanamycin resistant - Km^r) labelled plasmid constructed by M. Swallow from pBRC40, the deletion derivative of pBRC60 that lacks the inherently unstable catabolic transposon Tn5271. A suicide plasmid donor of Tn5, pGS9 (Selvarag and Iyer 1983) was conjugated from *E. coli* HB101 into *Alcaligenes* sp. BR40 (pBRC40), and pBRC40::Tn5 transposition derivatives were selected by mating the pool of Km^r transposon recipients with a plasmid-free chloramphenicol resistant (Cm^r), tryptophan auxotroph (Trp⁻) derivative of *Alcaligenes* sp. BR60 (BR6025). (BR6025 was isolated by selection for spontaneous resistance to 80 μ g/mL Cm, followed by mutagenesis with 200 μ g mL⁻¹ N-methyl-N-nitrosoguanidine (MNNG, Sigma Chem. Co.) for 35 minutes at 30°C. Following mutagenesis, Trp⁻auxotrophs were isolated using the method of Carhart and Hegeman (1975). BR6025 recipients of pBRC40::Tn5 were selected on medium A, 12 mM succinate, 50 μ g Cm, 0.1 mM Trp, 50 μ g/mL Km.

Overnight nutrient broth cultures of BR6025(Cm^r, Trp^r, pBRC40::Tn5) and recipients filter mated for 12 h on NA and transconjugants were selected on medium A agar supplemented with 1g sodium succinate, 1g sodium acetate, 1g glycerol and 50 mg Km per L.

DIRECT COUNTS OF BACTERIA

Total numbers of bacteria in samples were from acridine orange direct counts (AODC). Nucleopore 0.22 μ m membrane filters were stained with sudan black by soaking them in an sudan black saturated 70% ethanol for at least 24 hours. 2 to 10 mL of sample to be counted was filtered through a stained membrane, and stained with acridine orange (0.01% in 15.4 mM Na₂HPO₄·2H₂O, 7.3 mM citric acid, pH 4) for 5 minutes. The preparation was rinsed with filter-sterilized distilled water, air dried and placed on a clean glass slide between two drops of immersion oil and covered with a cover slip. 20 fields were counted using a Zeiss Photomicroscope 3 with epifluorescence illumination. Average numbers per field were converted to cells per mL by multiplying by [filtered area diameter/(field diameter*sample volume)]. Stocks of stained filters and filter sterilized waters were regularly examined for bacterial growth and replaced if significant numbers were found.

CHAPTER 3: DISTRIBUTION OF ORGANISMS HYBRIDIZING WITH DNA HOMOLOGOUS TO pBRC60 - NIAGARA REGION AND LAKE OPINICON

INTRODUCTION

Alcaligenes sp. BR60 was isolated from a continuous culture of microflora from Bloody Run Creek amended with 200 μ M 3Cba (Wyndham and Straus, 1988). Located just south of the Power Dam in Lewiston/Niagara Falls, New York, Bloody Run Creek collects surface and near-surface runoff from land adjacent to the Hyde Park industrial landfill before spilling its contents down the sides of the Niagara Gorge into the Niagara River. The Hyde Park landfill, 6.1 hectares in size, contains over 60 million kg of chemical waste from industrial operations of 1953 to 1975. Moving underground from the landfill are aqueous and non-aqueous plumes of chemical contaminants including various heavy metals and chlorinated and non-chlorinated hydrocarbons. Mixed chloro-benzoic acids are found at high concentrations (189 ppm) (Wagner and Collin 1984). After remedial attention in 1987, Bloody Run Creek was isolated from the landfill plumes.

Circumstantial evidence suggested that pBRC60 could be an important genotype in a chemically contaminated community. pBRC60 was dominant in the laboratory continuous culture of Bloody Run Creek water. Homologous DNA was found in 8 of 24 isolates and close examination of two distinct 3Cba degraders revealed the presence of the complete plasmid (Fulthorpe *et al.* 1989). However, the frequency of pBRC60 or homologous DNA elements in the *in situ* community of Bloody Run Creek or in similar aquatic systems was unknown. I sought information

on the frequency of this genotype in Bloody Run Creek to confirm suspicions that it was an important element in this historically contaminated system. I sought background frequencies in similar aquatic environments to see if it was a common genotype independent of chemical contamination and to assess the feasibility of tracking BR60 in a natural aquatic community using gene probes.

To this end the microflora of Bloody Run Creek were sampled and probed with a pBRC60 probe. For comparison the sediments of Theatre Creek and Lake Opinicon were also sampled and probed. Theatre Creek is a small stream which flows free from chemical contamination from a woodlot at the base of the Niagara escarpment at Lewiston, New York, to the Niagara River just north of Bloody Run Creek. Lake Opinicon, approximately 500 km away, was chosen as a study site for three reasons. Its water chemistry was similar to that of the dolomite-shale based surface waters of the Niagara region. Situated on a marble/granite base on the Frontenac Axis of Eastern Ontario, Lake Opinicon waters are dominated by carbonates and the pH is frequently over 8.0.

The Lake Opinicon watershed is also relatively pristine. To meet my objective of studying the survival of a chloroaromatic degrader and the fate of its degradative plasmid under known levels of contamination, I needed a site that was clean and a bacterial community that had not been previously subjected to selection by chloroaromatic contamination. In addition to these two factors, Lake Opinicon is adjacent to the laboratory facilities of Queen's University Biological Station and close to those of Carleton University. Figure 3-1 illustrates the sampling sites in the Niagara Region and near the Queens University Biological Station.

METHODS AND MATERIALS

In February, 1987, 10 mL water and sediment samples from four sites in Bloody Run Creek, water from the Niagara River at the base of the Creek and water and sediment from two sites in Theatre Creek were collected in sterile tubes and stored on ice overnight. Samples were diluted in medium A and plated on 82.5 mm circular nitrocellulose filters (Biorad Laboratories) overlying 2 media - 2 mM 3Cba/0.05% ye and 2 mM 3Cba/0.005% ye. After 18 days filter growth was replicated to fresh media using velveteen pads. Filters were peeled from the plates and colony DNA was fixed *in situ* and hybridized to a general pBRC60 DNA probe made from ³²P labelling of EcoRI digested fragments. Replicas of colonies showing hybridization to pBRC60 were later tested for growth on 3Cba solid and liquid media and grown on fresh nitrocellulose disks overlying 3Cba/0.005% yeast extract. Filters were fixed and probed using ³²P labelled pBRC60 EcoRI fragment 11 (E-11).

In early April of 1987, sediments were collected from the shallows of Lake Opinicon around the peninsula that encompasses Queen's University Biological Station. Ten sites from Lake Opinicon were sampled, and one from a creek several kilometres away just off Highway 15 (Figure 3-1). Sediments were stored on ice for 2 days prior to dilution and plating at 10⁻¹ and 10⁻² on nitrocellulose disks on 2mM 3Cba/0.005% yeast extract solid media.

After 2 weeks growth, 10⁻¹ dilution growth filters were fixed and split into two groups, one of which was hybridized to a total pBRC60 probe, the other to an E-11 probe. 10⁻² dilution plates were examined for colonies exhibiting good growth - four such colonies of apparently different types were picked from each site, patched

Figure 3-1: Sites sampled for *in situ* colony hybridization.

Figure 3-1: Sites sampled for in situ colony hybridization with pBRC60 probe

onto fresh media and tested for growth on 3Cba media. After a week, 2 nitrocellulose lifts of these colonies were made and the DNA fixed prior to hybridization. From each site, one colony lift was hybridized to a total pBRC60 probe, the other to an E-11 probe.

RESULTS AND DISCUSSION

Colonies grew well on both selection media containing 2mM 3-chlorobenzoate and yeast extract. Colony formation in the presence of these concentrations of yeast extract is not indicative of the use 3Cba in the media as a source of energy or carbon. The majority of bacteria on these plates co-metabolized 3-chlorobenzoate to chlorocatechol and all plates quickly took on the purple-black colour indicative of the accumulation of oxidation products of chlorocatechol.

Results of the hybridization of pBRC60 to colony growth are shown in Table 3-1. When small colonies (< 2mm in diameter) are included in the total count of colony-forming-units per mL, all sites appear to have some population level homologous to pBRC60 DNA. In the case of Niagara River water, positive hybridization signals suggest a higher number of cfu's per mL than were visibly counted. This higher sensitivity is reasonable given that poorly growing micro-colonies that were invisible against the white background of the nitrocellulose filters may have yielded a probe-hybridization signal. If we accept this explanation, a large proportion of bacteria in the Niagara River harbour DNA homologous to some portion of pBRC60 but are not selected for by our 3Cba/ye media. This is reasonable given that functions encoded by pBRC60 other than 3Cba metabolism

are unknown, and plasmid specific genes such as the origin of transfer and mobilization genes might be expected to be common in local populations.

The lowest population of total hybridizing cfu's were from Lake Opinicon sediments. When only strong positive hybridization signals larger than 2 mm were counted, a clear pattern emerged. These colonies were only found in Bloody Run Creek water and sediment and in the Niagara River samples. No large colonies gave strong positive hybridization signals in the Theatre Creek or Lake Opinicon samples. Lake Opinicon signals were weak in comparison to those from Bloody Run Creek (Figure 3-2). The five Lake Opinicon filters that were hybridized to the Tn5271 specific E-11 probe yielded a few extremely weak signals that did not correspond to the position of visible colonies (Figure 3-3). There were no large colonies that hybridized to the E-11 probe.

Of 10 replica pBRC60 positive colonies picked from Bloody Run Creek plates, 2 exhibited 3Cba utilization on further testing and also hybridized to E-11. Of 44 colonies sampled from Lake Opinicon and Morton Creek plates showing apparent good growth on 3Cba/ye, none exhibited 3Cba utilization. Of these, only 14 hybridized weakly to pBRC60 and none showed homology to probe E-11. Of the 14 weakly pBRC60+ colonies, 13 cometabolized 3Cba to chlorocatechol.

Thus pBRC60-homologous organisms from Lake Opinicon were weakly homologous, less frequent, and not associated with 3Cba degradation or with homology to E-11. Current sequence analysis confirms that E-11 overlaps the 3Cba catabolic region and the repeated sequence IS1071 - so it will hybridize to organisms carrying all of Tn5271 or IS1071 alone i.e. it is an effective probe for the 3Cba

Table 3-1: Number of colony forming units greater than 2 mm and less than 2 mm in size showing homology to pBRC60 probe.

SITE	%YE in media*	Colonies Hybridizing to Probe x 10 ³ cells per mL		
		<2mm	>2mm	%total**
WATER				
Niagara River	0.005	3.0	0.2	> 100
	0.05	1.0	0	> 100
Bloody Run	0.005	0.1	0	36
	0.05	0.2	0.2	13
Theatre	0.005	0	0	0
	0.05	0.4	0	17
SEDIMENT				
Bloody Run	0.005	17.0	5.0	71
	0.05	47.0	11.0	12
Theatre	0.005	4.0	0	15
	0.05	12.0	0	0.3
Opinicon	0.05	2.5	0	

* percentage of yeast extract added to 2 mM 3Cba agar media to supplement growth.

** (total number of hybridizing cfu/total culturable cfu)x100%

Figure 3-2: Audioradiograms of the results of *in situ* colony hybridization of filter blots of sediment colonies from Bloody Run Creek, Theatre Creek and Lake Opinicon to a pBRC60 probe. For Bloody Run Creek and Theatre Creek, filter blots of the growth from 10^0 and 10^{-2} dilutions of samples are shown. For Lake Opinicon, a filter blot of growth from a 10^{-2} dilution is shown. The bottom right autoradiogram is of a filter blot of the 3Cba/ye growth of BR60 (bottom half) and 4 isolates from Lake Opinicon samples. Note the intense hybridization to colonies from Bloody Run Creek and Theatre Creek, and the fainter more non-specific hybridization to growth from Lake Opinicon samples.

Bloody Run
Creek

Theatre Creek

Lake Opinicon

10^2

BR60

Figure 3-3: Audioradiograms of results of *in situ* hybridization of five 10^{-1} dilutions of Lake Opinicon sediment samples to an EcoRI-11 DNA probe. Also shown, bottom left, is the autoradiogram of filter blots of 3Cba/ye growth of 10 isolates from Bloody Run Creek. The two positively hybridizing isolates exhibited utilization of 3Cba.

Lake Opinicon Sediments 10¹

Probe EcoRI 11

Niagara
Isolates

catabolic region but is not entirely specific for it. All 44 isolates from Lake Opinicon and the 8 isolates from Bloody Run Creek showing no homology to E-11 lacked both the 3Cba catabolic genes and the insertion sequence IS1071 - so this insertion sequence is not commonly found independent of Tn5271.

The data presented here do not constitute absolute proof that Tn5271 did not exist in the vicinity of Lake Opinicon, because enrichment was restricted to plating on selective media. Enrichment steps in which populations were selectively increased with low 3Cba concentrations might have revealed a different story. However, the fact that Tn5271 homologous colonies were not detected, the distance of this site from the Niagara region, and the presumed lack of 3Cba exposure at Opinicon all support the idea that the lake sediments in the area around the biological station supported a microbial community that did not carry Tn5271 in the collective gene pool. Tn5271 may represent a locally evolved catabolic element in the highly contaminated Niagara region or in Bloody Run Creek alone, however I have no evidence that the catabolic element is not found elsewhere. At any rate, Lake Opinicon emerged as a suitable area in which to release and track BR60 using Tn5271 probes.

At the time that this study was carried out, the complete sequence of Tn5271 was not known, and the exact location of the chlorobenzoate degradative genes on this element was yet to be determined. In the release studies that followed, described in Chapters 4 and 6, DNA probes were constructed from the fragments within the region deleted from pBRC60 during loss of Tn5271 without complete knowledge of the structure of the element. Cloned fragment E-11 was used as a

probe for the 3Cba catabolic genes because, in this preliminary study and previous studies, Niagara region microorganisms showing homology to E-11 were all 3Cba utilizers (Fulthorpe *et al.* 1989). The following year, sequencing work showed the presence of repeated sequences at either end of Tn5271 and initial work suggested that E-11 was located in these repeated sequences rather than with sequences internal to the element, so larger probes encompassing regions known to be internal were chosen as 3Cba catabolic probes - EcoRI fragment 5 and HindIII fragment 2. All three probes overlap as least a portion of the 3Cba catabolic region and all proved to be equally effective in monitoring 3Cba utilizing populations, so in the final year of release studies (1989) E-11 was again used as the Tn5271 probe.

CHAPTER 4: SURVIVAL AND ACTIVITY OF BR60 AND pBRC60 IN LAKE OPINICON MESOCOSMS

INTRODUCTION

I released BR60 into flow-through freshwater and sediment mesocosms designed to closely simulate the conditions in the shallow bay of a lake. The mesocosms were dosed with different amounts of 3Cba. The frequency of the catabolic genotype in each mesocosm was monitored using DNA probes specific for Tn5271. Because of the inherent mobility of this genetic material, the data describe the survival and activity of a specific genotype, not a specific population of bacteria, in a natural system. What follows is evidence that the frequency of the catabolic genotype differs from that of the originally introduced bacteria, and that the genotype frequency is of greater importance to 3Cba metabolism in the system than the survival of the original inoculum.

MATERIALS AND METHODS

Initial Release Experiments: In July and August of 1987 mesocosms were set up and dosed with 3Cba at four different approximate concentrations. Four mesocosms received 3Cba at 0.04 mL/min and four at 0.4 mL/min. For exposure to maximal 3Cba concentrations, two mesocosms were dosed by floating gauze bags of powdered 3Cba in the water rather than by increasing the chemical delivery rates. 3Cba reservoirs limited to 4 mM concentrations, and practical considerations precluded using high chemical delivery rates. Two mesocosms received no 3Cba.

These treatments exposed mesocosms to mean 3Cba concentrations ranging from 0 to 36 μM with a high level of variation due to imprecise water flow rates.

Alcaligenes sp. BR60 was introduced to one half of the tanks (representative of the full range of 3Cba concentrations) on June 29th, 1987. Cultures in 100 mL of Medium A and 3 mM 3Cba plus approximately 20 g of white sterilized sand were grown to stationary stage and then used to inoculate the mesocosms. All mesocosms were monitored for 8 weeks. Viable counts were made using 2mM 3Cba/0.005% yeast extract and 2mM 3Cba/0.05% yeast extract agar plates. Water or sediment samples were diluted in Medium A. The population of bacteria carrying the catabolic genotype in each mesocosm was determined using the MPN:DNA hybridization technique every 2-3 weeks. Two determinations were made each time: one using a general medium (0.2% yeast extract) in the initial culture step, the other using a more selective medium (2 mM 3Cba plus 0.005% yeast extract). 3Cba uptake rates in mesocosm waters were determined in mid-August and again at the close of the experiment on September 2nd. Total phosphorus, nitrogen and dissolved organic carbon levels in the intake water were determined biweekly by the Ontario Ministry of the Environment.

Dose Response Quantification Experiments: During the 1988 field season, reservoir concentrations and peristaltic pump rates were adjusted to give 3Cba feed concentrations ranging from 10 nM to 25 μM with an overall lake water dilution rate of 0.01/hr. A naturally occurring streptomycin resistant strain of *Alcaligenes* sp. BR60 was used for mesocosm inoculation. The strain was grown to stationary phase on medium A supplemented with 2 mM 3-chlorobenzoate and 25 $\mu\text{g.mL}^{-1}$ of

streptomycin and introduced at $100,000 \text{ cells.mL}^{-1}$ to all mesocosms on June 25th, 1988. Inocula cell densities were determined by plating on 3Cba/Str and 3Cba agar. Water and sediment viable counts were made weekly on agar containing 3mM 3Cba as a sole carbon source, and on similar agar containing $25 \mu\text{g.mL}^{-1}$ streptomycin. MPN:DNA hybridizations were carried out weekly using only a general growth medium (0.1% yeast extract/0.1% tryptone). EcoRI fragment 5 or HindIII fragment 2 were used as DNA probes for Tn5271. 3Cba uptake rates in the mesocosm waters were determined at the close of the experiment 4 weeks after inoculation.

Effect of nutrients on BR60 survival: At the end of July, 1988, all mesocosms were flushed with lake water at 0.05/hr for three days. The dilution rate was adjusted back to 0.01/hr and a second inoculation of BR60 was made on August 6th to three of these mesocosms - one was not treated, a second was supplemented with $15 \mu\text{M}$ phosphate, and a third was supplemented with non-specific dissolved organic matter (DOM). A concentrated solution of the latter was prepared by boiling approximately 2 kg (wet weight) of crushed *Valisneria* and *Meriophyllum* plants and associated periphyton and trapped fauna with 4 l of distilled water. The resulting dark brown extract was strained and autoclaved was pumped to the DOM treatment mesocosm at 0.08 mL/min (1/100 dilution of extract). The survival of BR60 and probe positive organisms was compared between each treatment.

Effect of Dilution Rate on BR60/pBRC60 response to 3Cba: Three flushed mesocosms were re-inoculated on August 6th with BR60 and dilution rates adjusted to 0.01/hr, 0.03/hr and 0.1/hr using 20G, 18G and 16G needles in the header tank, respectively. Stock solutions of 3Cba feed to these mesocosms contained 1.0 mM, 3.0

mM and 10 mM 3Cba, respectively, to give final 3Cba concentrations of 10 μ M in each. BR60 and probe positive populations were monitored over the next four weeks and compared between these three mesocosms.

RESULTS

Mesocosm Parameters. In June, 1987, lake water and sediment was diluted in general medium MPN microtitre plates and subsequent growth probed with EcoRI fragment 11. Again, only faint hybridization, indicative of non-specific binding, was detected.

Lake water total phosphorus inputs for July and August of 1987 varied from 2 to 62 μ g per L and averaged $30 \pm$ a standard deviation of 20 μ g/L. Dissolved organic carbon and total Kjeldahl nitrogen levels were more constant at 5.2 ± 0.7 mg/L and 0.52 ± 0.09 mg/L, respectively. Changes in lake water nutrients had no discernable effect on BR60 populations. Ambient temperatures varied between 20°C and 32°C over both the 1987 and 1988 experimental periods.

Chlorobenzoate Dosages. Results are presented and discussed with reference to the chlorobenzoate dosage received by the various mesocosms. This is always reported as the concentration of chlorobenzoate in the incoming lake water - termed here the 3Cba feed rate. In 1987 the incoming water and 3Cba did not mix before delivery to the tanks, in 1988 they did, but in both cases the 3Cba feed rate was calculated as the 3Cba reservoir concentration x 3Cba input rate/water input rate for those mesocosms receiving 3Cba from the reservoirs of aqueous solutions.

Initial Release Experiments - 1987. For the tanks with 3Cba delivered as a solid suspension the 3Cba concentration was measured in the mesocosms after equilibration with the substrate and before introduction of BR60. There was a significant amount of variation over a 24 hour period in the water input rate and it was adjusted daily to compensate for this problem. In addition there was some variation over a much longer period of time in the 3Cba input rates. Both these sources of variation made the estimated 3Cba feed rate fairly approximate and no attempt was made to quantify results with respect to this parameter.

(i) Growth of BR60 on Agar Plates. On June 29th, 1987, BR60 was introduced to the mesocosms at approximately 10^6 CFU.mL⁻¹. On July 3rd, BR60 colonies were discernable on the 3Cba/0.005% yeast extract plates from the inoculated mesocosms. Eight days after introduction, similar colonies were discernable in samples from uninoculated control mesocosms and thereafter MPN probe positive counts in the control mesocosms rose to levels not significantly different from those in the inoculated mesocosms. Inter-mesocosm transfer of bacteria via aerosols was suspected as all aquaria were uncovered. Thereafter the uninoculated tanks were treated as replicates. The size of the original inoculum of BR60 did not seem to be an important factor in establishing BR60 in the mesocosms.

(ii) Detection using MPN:DNA Hybridization. The average population levels of organisms carrying DNA homologous to our probe (termed here the "probe positives" for brevity) in the mesocosms is depicted in Figure 1. Each bar shows the average of the log probe positive population from four estimates taken over the two month period. Estimates depended on the media used in the initial enrichment step

in the MPN-microtitre plates - those levels estimated on general media were higher than those estimated on selective media. Estimates derived from general media tended to remain the same (for [3Cba] < 20 μM) or to increase (for [3Cba] > 23 μM) over the 10 month sampling period. In contrast estimates derived from selective media increased for 3Cba exposures of 36, 31 and 17 μM , but remained the same or decreased in all other mesocosms.

Using this technique, the theoretical lowest detectable level of probe positive organisms was 8 cells.mL⁻¹ in the waters or 80 cells.mL⁻¹ in the sediments. Probe positive organisms persisted in water and sediments of all of the mesocosms, except those lacking 3Cba inputs for the full 56 days of the experiment. In both the inoculated and uninoculated zero 3Cba mesocosms, probe positive counts in the sediments dropped to undetectable levels after 41 days. In the overlying waters, probe positives persisted at less than 100 cells.mL⁻¹ after 56 days. Their persistence in the waters may have been due to continuing aerosol contamination from adjacent high 3Cba dosed tanks.

After release of *Alcaligenes* sp. BR60, average probe positive estimates over the 8 week period were clearly related to the concentration of chlorobenzoate delivered to the mesocosms (Figure 1). The variance in probe positive counts for each mesocosm was proportional to the mean, so a log transformation was performed before any analyses of these data. The mean log probe positive estimates were highly correlated to the log of the estimated 3Cba feed concentration, and those obtained from the use of general media were more highly correlated than those from selective media. (General media: $r^2(\text{water}) = 0.83$; $r^2(\text{sediments}) = 0.92$. Selective

media: $r^2(\text{water}) = 0.49$, $r^2(\text{sediments}) = 0.45$; $n=48$, all are $p<0.01$). Regression parameters were not calculated for these relationships because of the large amount of error associated with estimating the 3Cba concentration. In addition, in the higher dosage mesocosms the MPN probe positive counts were minimum estimates because dilutions were not sufficient to yield a definite estimate from the MPN tables. Therefore the coefficient of variation are indicative of an association between these variables, but should not be used to estimate the variance explained in the probe positive estimates by the 3Cba feed rate. This was estimated in the 1988 experiment.

(iii) **Chlorobenzoate Uptake Rates.** The heterotrophic potential of the water in the mesocosms was determined between August 10th and September 2nd, 1987. Two measurements were performed on each tank at temperatures between 17 and 20°C. The estimate of $V_{\max}$ for 3Cba uptake reflected the estimated average 3Cba feed concentration ($r^2=0.85$, $p<0.01$). In addition $\log(V_{\max})$ was highly correlated to the $\log(\text{probe positives})$ estimate, again more so to the general media estimate than to the selective media estimate ($r^2=0.79$, $p<0.01$, vs $r^2=.48$, $p<0.05$). The data are shown in Table 4-1.

Figure 4-1: Average log(probe positives) for 1987 initial release experiment versus average 3-chlorobenzoate feed concentrations in mesocosms. Error bars are standard errors, $n = 4$.

Table 4-1: 1987 mesocosm average 3Cba feed concentration, 3Cba uptake rates and probe positive populations.

Code	3Cba μM	$V_{\max}$ nM.hr^{-1}	$\text{Log}(V_{\max})$	Probe Positives $\text{Log}(\text{cells.ml}^{-1})$	
				G*	S*
4	0	0.8	-0.10	1.05	0.67
3	0	1.2	0.08	1.74	1.15
11	0	1.2	0.08	3.05	0.53
9	5	16.4	1.21	2.60	1.08
12	6	5.3	0.72	3.01	1.45
10	7	19.5	1.29	3.82	2.78
5	17	71.0	1.85	3.97	2.68
7	19	46.8	1.67	3.80	0.56
6	21	41.0	1.61	4.77	4.25
8	23	60.0	1.78	4.23	1.99
1	31	55.0	1.74	3.92	2.98
2	36	79.0	1.90	4.52	4.08

* G- denotes determined from general media

* S -denotes determined from selective media

Dose Response Quantification - 1988: Technical improvements made to the mesocosm fluid input systems stabilized the flow rates dramatically and allowed much more precise control of the 3Cba feed concentrations. Input water flow rates varied little over the four week period, remaining at $7.25 \pm$ a standard deviation of 0.67 mL/min. 3Cba reservoir concentrations were measured after 4 weeks by HPLC showed no change from their initial concentrations.

(i) **Viable plate counts of BR60.** After inoculation into the mesocosms, BR60 was easily detected in the waters and sediments four days later. It formed distinct colonies on 3Cba or 3Cba/Str agar, having translucent cream coloured colonies with slightly irregular margins and exhibiting the smooth/irregular colony dimorphism

seen in the laboratory. On 3Cba/Str agar BR60- like colonies were virtually the only growth seen. On 3Cba agar, many colonies formed but BR60-like colonies were evident from their much larger size and rapidity of formation. BR60-like colonies recovered from some mesocosms exhibited a variance in chlorobenzoate metabolism evident from the accumulation of dark brown chlorocatechol oxidation products in the centre of the colony. Subsamplings of these colonies and culturing in 3Cba liquid culture resulted in "normal" non-chlorocatechol accumulating colonies. BR60 grew more quickly after sampling from the mesocosm than after repeated culturing in the laboratory in Medium A plus 3Cba. According to viable counts made on CBA/Str agar, 3 days after inoculation BR60 populations averaged 1.0×10^4 CFU.mL⁻¹ in mesocosm waters, and 1.1×10^5 CFU.mL⁻¹ in the surficial sediments. The highest populations were found in those mesocosms receiving 10 nM, 40 nM, 700 nM, 10 and 20 μ M 3Cba, exhibiting no correlation with chlorobenzoate concentration. After 14 days counts across all tanks averaged 68 CFU.mL⁻¹ in the waters; 4.0×10^3 CFU.mL⁻¹ in the sediments. No colony forming BR60 were detected in mesocosm waters after 32 days and sediment populations had dropped to 1.7×10^3 CFU.mL⁻¹. There was a similar decrease in viable counts made on 3Cba agar, indicating that a loss of streptomycin resistance was not the cause of this result.

(ii) **Detection using MPN:DNA hybridizations.** Probe positive estimates were determined using a general medium. The counts obtained gave considerably higher estimates of Tn5271 containing bacteria than the viable counts of BR60 in some mesocosms. The MPN technique can yield different estimates of a culturable population than plate counts. In our experience with BR60 growing in pure culture,

MPN population estimates never differ from plate count estimates more than 3-fold higher. In contrast, the MPN probe positive estimates from the mesocosms were often orders of magnitude higher than the viable counts of BR60 (Table 4-2). The actual discrepancy in values is depicted in Figure 4-2. The overestimation was most obvious in the waters where viable BR60 were undetectable but MPN probe positive counts were significant. This phenomena was not a function of the sample volume used in the two methods, since a triplicate viable count took 150 μ L of sample, and an MPN probe positive estimate, 100 μ L.

Table 4-2: Average Ratio of Probe Positive Counts:BR60 counts

Mesocosm [3Cba] Code	μ M	Ratio in Water*	Ratio in Sediment
B3	0	162 +	1.2
B5	0.011	45	7.9
B1	0.043	12	1.9
B2	0.070	69	2.5
T1	0.12	46 +	13.5
T2	0.43	564	52.0
T3	0.73	580	7.0
T4	1.26	101	8.4
T5	5.80	498	16.1
B6	10.9	2476 +	52.8
T6	25.4	606 +	66.2

* + denotes ratio = infinity in some cases, BR60 counts = 0.

Figure 4-2: MPN-DNA probe positives counts minus viable BR60 counts plotted against 3Cba feed concentration for 1988 mesocosms. Sediments = filled circles, water = open circles.

The mean log estimates of the probe positive populations were significantly related to the log of the 3Cba feed concentration (Figure 4-4). Untransformed estimates were regressed on 3Cba feed concentration in order to determine the average yield in terms of probe positive cells.nmole⁻¹ of 3Cba. They averaged 2.35×10^3 cells.nmole⁻¹ in the mesocosm waters and 1.07×10^4 cells.nmole⁻¹ in the sediments. In continuous culture of BR60 in sterile creek water containing $200 \mu\text{M}$ 3Cba the yield averages 4.7×10^4 cells.nmole⁻¹ (Wyndham and Straus, 1988).

(iii) **Chlorobenzoate Uptake Rates.** 3Cba uptake rates in the mesocosm waters were measured on July 24th, at the end of the four week experiment. $V_{\max}$ does not increase above background levels until the 3Cba concentration is greater than $1 \mu\text{M}$ (Figure 4-3). $V_{\max}$ was significantly correlated to the 3Cba feed concentration as expected (Figure 4-4, $r^2 = 0.92$, $V_{\max} = 1.88[3\text{Cba}] + 3.99$). Log $V_{\max}$ was again significantly correlated to the log of the probe positive counts (Figure 4-5). The relationship between probe positives and $V_{\max}$ is similar for the 1987 initial release experiments and for the 1988 experiment, but the slopes of the regressions are significantly different ($F=12.4$, $0.0001 < p < 0.0005$). In 1988, $V_{\max}$ was lower for a given population of probe positives than in 1987. The 3Cba uptake rates could not be predicted from the plate counts of BR60 in the waters. Correlations between $V_{\max}$ and mesocosm average plate counts were weak ($r^2=0.47$ and 0.25 for counts on 3Cba and 3Cba/Str agar respectively). During the week that $V_{\max}$ was actually measured, viable counts had dropped to zero in most of the mesocosms, including the one receiving $10 \mu\text{M}$ 3Cba. The four mesocosms retaining viable BR60 bore no relationship to either 3Cba exposure or the $V_{\max}$ of the system.

Figure 4-3: Relationship between average log (probe positives) and log of 3Cba feed concentration in mesocosms, 1988

water — ● — , $r^2 = 0.56$, $y = 0.477x + 3.65$, $p < 0.01$

sediments — ▲ — , $r^2 = 0.73$, $y = 0.555x + 4.40$, $P < 0.01$

Figure 4-4: 3Cba uptake rate ($V_{\max}$) in mesocosm waters versus log (3Cba feed concentration). X-axis is shown as log scale for clarity. Linear relationship is $r^2 = 0.92$; $V_{\max} = 1.88[3\text{Cba}] + 3.99$.

Figure 4-5: Relationship between $\log(V_{\max})$ and $\log(\text{probe positives})$ in mesocosm waters.

$$1987, r^2 = 0.80; y = 0.697x - 1.20, p < 0.01$$

$$1988, r^2 = 0.69, y = 0.496x - 0.9, p < 0.01$$

Effect of nutrients on BR60 survival: Table 4-3 summarizes the average BR60 and probe positive populations in phosphate and dissolved organic matter supplemented mesocosms. Population estimates are averaged over the four weeks of the experiment. Standard deviations are high because BR60 estimates and probe positive estimates both exhibit die off over this period. Neither the addition of phosphate nor of DOM retards this die off. In fact, BR60 colonies were not detected in the waters of either treatment mesocosm or in the sediments of the phosphate treated mesocosm although they were evident in the control. A Mann-Whitney-Wilcoxon comparison of the medians shows that water probe positive populations are marginally lower than the control in both the phosphate treatment ($p=0.078$) and the DOM treatment mesocosms ($p=0.057$). Sediment populations are also lower but not significantly so.

Table 4-3: Effect of nutrient additions on BR60 and probe+ populations in uncontaminated mesocosms. Values in parenthesis are standard deviations, $n = 4$.

Treatment	Water log (cells.mL ⁻¹)		Sediment log (cells.mL ⁻¹)	
	BR60	probe+	BR60	probe+
Control	0.65(0.66)	2.49(0.90)	1.44(1.49)	2.41(1.46)
15 μ M PO ₄	0.00(0.00)	1.07(0.52)	0.00(0.00)	1.08(1.10)
DOM	0.00(0.00)	0.52(0.89)	1.90(1.14)	1.10(1.12)

Effect of dilution rate on BR60/probe positive response to 10 μ M 3Cba.

As shown in Table 4-4, even a ten-fold increase in the flow-through rate of lake water had no discernable effect on the BR60 and probe positive population estimates in water or sediment.

Table 4-4: Effect of dilution rate on BR60 and probe positive populations in mesocosms dosed with 10 μ M 3Cba. Values in parenthesis are standard deviations, n = 4.

Dilution hr ⁻¹	Water log (cells.mL ⁻¹)		Sediments log (cells.mL ⁻¹)	
	BR60	probe +	BR60	probe +
0.01	2.01(0.45)	3.79(0.67)	3.77(0.31)	4.67(0.69)
0.03	1.26(0.79)	3.91(0.63)	3.60(0.18)	4.02(0.51)
0.10	1.51(0.88)	4.04(0.33)	3.72(0.63)	4.53(0.79)

DISCUSSION

The results presented here indicate the catabolic element Tn5271, carrying 3Cba degradation information, is a viable genotype in a natural aquatic community. The substrate, 3Cba, acts as a strong selective force and at micromolar levels promotes the rapid establishment of Tn5271 harbouring organisms from inocula as small as that apparently provided by aerosol contamination. The genotype is selected for in a natural system in proportion to the chlorobenzoate added to the system, and

expresses metabolic activity even though the original inoculum loses culturability on selective media. The addition of scarce nutrients in the form of inorganic phosphate or as a macrophyte-periphyton extract does not enhance the survival of BR60. This suggests that BR60 is unable to effectively compete with other bacteria for more commonplace carbon sources and for scarce phosphorus - if it could its populations would reflect the growth potential afforded by these nutrient additions. The response of BR60 to phosphate additions in these mesocosms echoes its competitive behaviour in continuous culture with *Pseudomonas fluorescens* st. BR52 - where the addition of phosphate stimulated the growth of BR52 at the expense of BR60 (Wyndham *et al.*, 1988).

Selection for the Tn5271 genotype becomes noticeable only after chlorobenzoate concentrations reach 1 μM or more. Below that concentration, both the numbers of probe positive organisms and the V_{max} for the community are not significantly different from those found in mesocosms lacking chlorobenzoate inputs. Schmidt and Alexander (1985) have theorized that below a certain threshold concentration, a particular substrate cannot support the growth of cells catabolizing it. They place this threshold at around 2 $\mu\text{g/L}$ or approximately 10 nM of a typical simple aromatic compound. Janke (1987) has argued that growth requirements may be the primary consideration for compounds degraded by constitutive enzymes, but the concentration required for induction of enzyme synthesis is more important for other compounds. He found a phenol-oxidation induction threshold at 2 μM of the inducing compound. Similarly, induction of chlorobenzoate catabolism in an *Acinetobacter calcoaceticus* strain occurred at comparable levels (Reber 1982). Our

data suggest that chlorobenzoate does not select for Tn5271 below 1 μM . This supports the idea that the induction threshold is more relevant to the survival of catabolic bacteria than a growth threshold.

Chlorobenzoate is not the only determinant of the survival of Tn5271 in the mesocosms. Probe positive organisms presumably carried by aerosols spread to and reproduced in mesocosms receiving no 3Cba inputs in the 1987 initial release experiments. After introduction to mesocosms with a full range of 3Cba concentrations in 1988, Tn5271 carrying organisms flourished in some of the mesocosms independent of their 3Cba inputs. After 3 weeks, positively hybridizing organisms existed in two of the mesocosms, that receiving 11 nM 3Cba and that receiving 10.9 μM 3Cba, at levels much higher than would be predicted from their 3Cba concentration alone (Figure 4-4). Being adjacent to one another, these two mesocosms likely received similar "batches" of sediment from a heterogeneous sediment source at the start of the experiment. An unidentified alternate substrate or nutrient may have enhanced the survival of probe positives in these two mesocosms. Other genes carried on the pBRC60 plasmid may have determined the fitness of these organisms in these particular mesocosms.

3Cba uptake rates in the mesocosms were best predicted by the contaminant exposure levels. This result is not new (Rubin *et al.* 1982). What is new is the strong association between the heterotrophic potential and the population of bacteria carrying a specific genotype in this system. The relationship between V_{max} and probe positive organisms in the two experiments (Figure 4-5) demonstrates that the MPN:DNA hybridization technique gives a meaningful estimate of the

3-chlorobenzoate degrading population. Each year exhibits a different predictive relationship: the mesocosm populations sampled in 1987 were more active per probe positive organism than those sampled in 1988. The slope of the 1987 relationship is probably artificially high because some of the higher probe positive estimates are minimum estimates only. The overall nutrient levels in the 1988 mesocosms would have been reduced by the 170 μm pre-filter in the water intake, but since nutrient additions hamper rather enhance the survival of probe positive organisms, I do not expect that this contributed to lower activity levels. The dilution rate in the 1987 mesocosms averaged 0.05 hr^{-1} , but was reduced to 0.01 hr^{-1} in the 1988 experiment. While the dilution rate doesn't affect overall probe positive populations, the lower rate might have selected for a population with a lower growth rate in the 1988 experiment.

Not all of the variance in 3Cba uptake rates was explained by our MPN:DNA probe positive estimates. The possibility remains that Tn5271 was transferred to species that are not culturable even on general liquid media and therefore not detectible by the MPN:DNA hybridization technique. Some of the variation in 3Cba uptake rates not explained by our probe positive count might be accounted for by populations of truly non-culturable Tn5271 harbouring bacteria. Nevertheless, the dependence of chlorobenzoate uptake rates on the frequency of Tn5271 carrying populations, as determined by the MPN:DNA hybridization method, lends credence to the expectation that catabolic gene frequencies are a meaningful measure of biodegradative potential in a natural system. The predictive value of this measurement would be most useful in systems where the contaminant exposure

history, to date still the best predictor of biodegradative potential, is unknown.

The exact nature of the probe positive organisms remains unclear. There were discrepancies between probe positive estimates derived from general and selective media in 1987. Colonies failed to form on 3Cba plates in 1988 when positively hybridizing organisms were abundant (Figure 4-2). These two observations indicate that some Tn5271 genes detected were carried by organisms culturable in general liquid medium, but not culturable on 3 mM 3Cba plates or in 3Cba liquid media. Bacteria fitting this description might include BR60 cells with an altered 3Cba metabolism, or bacteria other than BR60. There is evidence for both of these possibilities. First, phenotypic variation in 3Cba metabolism was evident in BR60 colonies on 3Cba plates; many colonies accumulated the brown products of chlorocatechol oxidation, a toxic metabolic intermediate not accumulated in high 3Cba adapted populations. Chlorocatechol accumulation, a manifestation of a genotypic or phenotypic adaptation to low 3Cba levels encountered in the mesocosms, might have caused a loss of culturability on 3Cba agar plates. Incipient colonies may have failed to grow because of high levels of production of this intermediate. Both phenotypic and genotypic changes have been documented in strains adapting to new substrate regimes.(Munro *et al.* 1987, Rangnekar 1988).

Second, pBRC60 is a conjugative plasmid that transfers at a high frequency (10^4 per donor) under laboratory conditions with conspecific strains. DNA probing of samples from Bloody Run Creek in the Niagara region (BR60's site of origin) demonstrated a high frequency of probe positive organisms, among them a *Pseudomonas acidovorans* carrying pBRC60. (Fulthorpe *et al.* 1989, Wyndham and

Straus 1988). A few strains isolated from mesocosms during these experiments have been found that differ from BR60, but carry pBRC60 and exhibit 3Cba catabolism (Chapter 5).

Probe positive estimates account for most of the variation in the 3Cba uptake rates while the viable BR60 counts can not. These "extra" probe positives play an active role in 3Cba degradation, but the data presented in this chapter do not clarify if they are predominately comprised of "adapted" BR60 or new hosts of Tn5271.

Jain *et al.* (1987) examined the persistence of plasmid-bearing bacteria in ground aquifer material and concluded that catabolic plasmids can be maintained in a microbial community in the absence of any known selection pressure. I have examined the persistence of an unstable catabolic element, rather than an entire plasmid, in a highly realistic setting. In experiments described here, probe positive organisms were undetectable in the sediments of the zero 3Cba control mesocosms in the 1987 release experiment by the end of 8 weeks. In the 1988 experiment, sediment probe positive populations had declined to 10^2 cells.mL⁻¹ in three of the four mesocosms receiving less than 70 nM 3Cba for three weeks. In the absence of known selection, the likelihood of Tn5271 being lost from the system is high, but not a foregone conclusion. The higher population of probe positives in mesocosm B5 (11 nM feed, Figure 4) is evidence that other selective factors might be important. Other functions encoded by pBRC60 have not been examined, they might have played a role in maintaining the plasmid in these systems. A separate mesocosm set up in 1988 in a different building approximately 500 m away from the toxicology facility was dosed with high levels of 3Cba and developed a healthy population of

streptomycin sensitive BR60 in 1988. The most likely source of this population was the sediments collected from the lake and used in the mesocosm. This would imply that the streptomycin sensitive strain used in release experiments in 1987 survived the winter in the sediments but was not detected by the MPN:DNA probe hybridizations the next spring, when 3Cba exposure began again. For their plasmid persistence studies Jain *et al.* (1987) used sealed glass vials containing aquifer material as microcosms. These were stored at a constant temperature and closed to nutrient inputs. Experimental mesocosms at Lake Opinicon provided a more rigorous test of the fitness of a very specific DNA element in a natural system. Organisms carrying Tn5271 were under constant pressure to compete with incoming lake microflora, to adapt to changing nutrient conditions, diurnal temperature changes, and the predators and toxins associated with lake ecosystems. The dilution rate used challenged the organisms to grow, obtain refuge in the sediments or be washed out of the mesocosm waters. In the absence of selection, the response of the probe positive organisms was to drop to extremely low levels, but not low enough to be considered eliminated from the system.

Finally, this study showed that viable counts are clearly an unreliable method of determining the survival of this particular catabolic genotype in a natural system. The phenotypic plasticity of bacterial strains and their capacity for genetic transfer must be considered. This is of particular importance when the unit of interest is not a bacterial strain but a gene sequence that it carries.

CHAPTER 5:IN SITU TRANSFER AND EXPRESSION OF THE CATABOLIC PLASMID pBRC60 IN MESOCOSM RECIPIENTS.

INTRODUCTION

Bacterial genetic elements encoding the degradation of aromatic and chloroaromatic hydrocarbons are frequently carried on conjugative plasmids. Thus they are potentially mobile in natural bacterial communities. Based on our knowledge of the spread of plasmid borne antibiotic and metal resistance elements (Rochelle *et al.*, 1989, Bale *et. al* 1987, Genther *et al.* 1988, Stotsky and Babich 1986, review in Trevors *et al.* 1987, Pickup 1989) catabolic plasmids may be expected to show broad distributions. Interspecific transfer of degradative elements is suggested by the broad distribution of plasmids encoding toluene and xylene degradation (the TOL plasmids) that show strong homology (Chatfield and Williams, 1986). Similarly the chlorobenzoate catabolic plasmids pAC27, pWR1 and pJP4, isolated on three different continents, show extensive homology (Ghosal 1985, Chatterjee 1983).

Direct studies on the survival, expression and host range of a degradative element in natural systems are rare. The wide spread of antibiotic and other resistance elements in bacterial communities can be explained by the extreme selection pressure acting against those bacteria that lack the resistance element. However, the degree of selective pressure exerted by an additional carbon substrate on a degradative element is unknown. Pertsova *et al.* (1984) studied the fate of chlorobenzoate degradative plasmids in small soil samples and found that chlorobenzoate degrading isolates differed from the species originally inoculated,

indicating a transfer of the genetic information. Unfortunately this study involved the use of artificially high levels of nutrients (16 mM phosphate) and is of limited value in predicting the behaviour of degradative elements in the nutrient limited natural environment. Similarly most studies on plasmid behaviour come from work performed with sterilized systems or systems where introduced donors and recipients are isolated from the natural microflora via dialysis bags or chambers (i.e. Pickup *et al.* 1990)

Lake Opinicon mesocosms allowed the study of the behaviour of a degradative element in a natural system experiencing normal levels of nutrients, predators, competitors and environmentally relevant concentrations of a chemical contaminant. *Alcaligenes* sp. BR60 was introduced to mesocosms where they could interact with natural microfauna continually refreshed from an adjacent lake. Those organisms isolated from mesocosm waters and sediments that carry pBRC60 and that I believe were responsible for 3Cba degradation in the mesocosms are described in this chapter. They include species from both inside and outside the known host range of the plasmid pBRC60.

METHODS AND MATERIALS

Throughout the the 1988 field season, isolations of bacteria were made from colonies appearing on 3Cba agar from samples plated from 3Cba exposed mesocosms. Colonies were transferred to 3Cba liquid media. Those cultures showing turbidity after approximately 1 week were maintained and purified until the organism responsible for 3Cba metabolism was isolated.

Isolate Characterization: Bacteria isolated from the mesocosms were subjected to rapid plasmid screens and preliminary identification using BIOLOG GN ID kits.

Rapid plasmid screens were carried out by the in-well lysis method. Blots of these screens were probed with a P-32 labelled DNA probe prepared from EcoRI restriction enzyme fragments E1, E5 and E14.

Continuous Culture: In a 1.2L continuous culture vessel, cells were diluted at 0.01 to 0.04 hr⁻¹ with sterile artificial lake medium (FL) amended with 10 μ M 3Cba. FL medium composition was based on the nitrogen and dissolved inorganic carbon levels found in Lake Opinicon, and on inorganic ion concentrations typical for hardwater lakes (Wetzel, 1973). It contained 1.25 mM CaCO₃, 650 μ M MgCl₂, 300 μ M MgSO₄, 450 μ M NaHCO₃, 10 μ M NaNO₃, 5 μ M (NH₄)₂SO₄, 1 μ M FeSO₄, 1 μ M MnSO₄, 0.2 μ M CuSO₄, 0.2 μ M ZnSO₄, 0.01 μ M CoCl₂, 0.01 μ M Na₂MoO₄. The final pH was 8.2. When supplemented with 10 μ M 3Cba, this medium was capable of supporting approximately 10⁶ cells.mL⁻¹ of BR60.

Populations of total culturable cells in the continuous cultures were enumerated using CPS agar. Culturable cells using 3Cba were enumerated using 3Cba agar dyed with bromothymol blue (50 mg/L) to indicate the release of HCl following dehalogenation of 3Cba metabolites. AODC total cell counts were also determined in some cases.

Kinetic Parameters: Measurements were carried out on batch cultures of FL medium amended with 50 μ M 3Cba or on samples taken from 10 μ M 3Cba, FL medium continuous cultures grown at a dilution rate of 0.01 hr⁻¹. All continuous

cultures were inoculated with a batch culture of the appropriate species grown in 3Cba or 3Cba/ye and left to equilibrate for at least three volume changes (12 days) before uptake and carbon dioxide production measurements were made. Populations were enumerated on CPS, 3Cba and 3Cba/ye agar. Rates of losses of the 3Cba+ phenotype were calculated from

$$\text{loss rate} = 1 - (N+/N)^{1/g}$$

where g = number of generations

= hours cultured/generation time

and $N+$ = number of cells 3Cba+

and N = total number of cells.

RESULTS

Cluster Analysis of Isolates: All isolates showing some degree of growth in liquid 3Cba medium were metabolically fingerprinted using the BIOLOG GNID plates. Type species of typical freshwater bacteria were also fingerprinted. Of the 27 3Cba degrading isolates that were probe positive, 16 had BIOLOG fingerprints indistinguishable from that of BR60. Only one of these isolates was included in the analysis. The cluster analysis grouped the isolates and reference strains into 5 groups: 1) BR60 like species; 2) the PR117 group of yellow pigmented rods; 3) a fluorescent pseudomonad group; 4) Moraxella/Acinetobacter group and 5) remaining species distantly related to the other 4 major groups and to each other

(Figure 5-1). Two fingerprints for PR117 are included in the analysis, and PR12 is a BR60 colony re-isolated from the experimental mesocosms. These replicate fingerprints indicate that a cluster distance less than about 2.7 (similarity coefficient = 0.27) represents the practical limit of resolutions of strains. Only isolates found to carry pBRC60 (indicated by asterisks) retained the 3Cba⁺ phenotype on subculturing from the initial isolation. One isolate carrying a plasmid homologous to pBRC60 (strain PR63) showed no 3Cba metabolism. The remaining isolates lost the 3Cba⁺ phenotype during isolation.

Group 1, BR60 Donor and Related Recipients: The BIOLOG metabolic fingerprint and other diagnostic tests (Table 5-1) place the original inoculum, *Alcaligenes* sp. BR60 in a group with *P. acidovorans* (an rRNA group III pseudomonad) and *Alcaligenes* sp H850. It is identified according to the BIOLOG software as being *Comomonas testosteroni* (also an rRNA group III pseudomonad). *Alcaligenes* and *Comomonas testosteroni* are members of the non-saccharolytic group in Pickett's schema for identifying gram-negative nonfermentative bacilli (Pickett and Pederson, 1970), and the only key difference seems to be that of flagellar type. When motile, BR60 exhibits variable forms of flagellation but multitrichous polar flagellation, characteristic of *C. testosteroni*, is common. In many cases BR60 is non-motile and forms chains, as do *Alcaligenes* species, but unlike the latter, it cannot grow at 37°C or higher temperatures. Since both *Comomonas* and *Alcaligenes* are found in the beta rRNA homology group of the proteobacteria (Stackebrandt, 1988), we can only be sure that BR60 belongs in this group and is most closely allied with these two species.

Group 2, PR117 recipients: Eight of the 3Cba+, probe positive isolates, PR122, PR81B, PR68B, BL10, PR117, PR25B, PR118, PR23A1 (not shown in Figure 5-1) were morphologically similar and isolated from microcosms receiving 20 μ M, 10 μ M, 10 μ M, 10 μ M, 20 μ M, 0.73 μ M, 20 μ M, 0.12 μ M of 3Cba respectively. All these isolates were motile, aerobic, slightly curved gram negative, narrow rods bearing multiple polar or peritrichous flagella (preparations gave variable results). Colonies developed a non-fluorescent yellow pigment on CPS and KING's B agar, and to a lesser degree, on nutrient agar. Cells accumulated poly- β -hydroxybutyric acid as determined by the formation of sudan black crystals. BIOLOG GN plates showed that organic acid and amino acid utilization dominated, with limited use of carbohydrates (Table 5-1). All the strains give the same profile with some variation in the use of m-inositol, maltose and mannose. PR117 was selected as the type strain for this group in further studies. The BIOLOG system could not identify these isolates, giving PR117 a "poor" identification of *Alcaligenes faecalis* with a similarity of 0.49. All these isolates are tentatively identified as members of the Group III Pseudomonads, but they are not any of the three known yellow pigmented Group III Pseudomonads (*P. pseudoflava* (ATCC 33668) and *P. palleroni* (ATCC 11724) in Figure 5-1, nor *P. flava* which is known to utilize arabinose, cellobiose, fructose, galactose, maltose, mannitol, mannose, rhamnose and sucrose.

PR117 isolates are similar to the host strain BR60 in their BIOLOG fingerprint, and they are streptomycin and ampicillin resistant as is BR60. However PR117 grows slower on general and selective (3Cba) liquid or solid media (Figure 5-2).

Figure 5-1: Dendrogram of strains isolated from mesocosms using 3Cba, and key type strains. Distance was calculated from Jaccard similarity coefficients based on reactions in BIOLOG GN ID plates. Asterisks indicate those strains carrying pBRC60. Arrows indicate laboratory strains that receive pBRC60 via conjugation during filter mating with BR60 overnight.

2

MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS
STANDARD REFERENCE MATERIAL 1010a
(ANSI and ISO TEST CHART No. 2)

0 1 2 3 4 5 6 7 8 9 10

Group 3, Fluorescent Pseudomonads: Most strains in this group lost the 3Cba⁺ phenotype upon isolation and oxidized 3Cba to chlorocatechol as indicated by the accumulation of purple oxidation products of this metabolite in the media. Many of the chlorocatechol accumulating strains harboured large plasmids, bearing no homology to pBRC60. Only strain PR24B, isolated from a mesocosm receiving only 0.12 μ M 3Cba, retained full 3Cba metabolism and it was found to carry pBRC60. It is a motile, aerobic gram-negative oval shaped rod with polar monotrichous flagella. On KING'S B, colonies accumulate a fluorescent yellow green pigment. The BIOLOG GN ID plate identifies it as *Pseudomonas fluorescens* BIOVAR IV. PR24B grows rapidly on general media, but slowly on 3Cba minimal media. In minimal liquid media containing 3 mM 3Cba, PR24B exhibits either no growth at all or the accumulation of a small amount of chlorocatechol. In liquid culture amended with 0.005% yeast extract, PR24B first metabolizes 3Cba to chlorocatechol which accumulates in the media, and then metabolizes chlorocatechol to yield a high biomass (Figure 5-2).

Group 4, Moraxella/Acinetobacter: These strains were capable of acquiring and maintaining the pBRC60 replicon (pBRC40::Tn5) in laboratory matings. However, no natural recipients were found in this group.

Group 5, Distantly Related Recipients: PR42A gives a unique substrate utilization pattern (Table 5-1), but is indistinguishable from PR117 in its colony morphology and growth on general media and on 3Cba media. The BIOLOG system gives PR42A a "poor" identification of *Comomonas acidovorans* with a similarity of 0.45. I assume that it is a Group III pseudomonad and that it is closely

Table 5-1: Substrate utilization and other characteristics of pBRC60 carrying strains.

	BR60	PR117	PR42A	PR24B	PR120	PR63
Pigment	-	+	+	-	-	-
Oxidase	+	+	+	+	+	+
Catalase	+	+	+	+	+	+
Flagellar type*	(pl)	per	per	pm	pm	pm
3-chlorobenzoate	+	+	+	+	+	-
chlorocatechol accum.	-	-	-	+	+	-
distance***	0.45	10.4	11.4	40.3	16.9	21.4
dextrin	-	-	-	+	-	-
tween 40	+	+	+	+	+	+
tween 80	+	+	+	+	+	+
n-acetyl-D-glucosamine	-	-	-	+	-	-
L-arabinose	-	-	-	+	-	+
D-arabitol	-	-	+	+	-	+
D-fructose	-	-	+	+	-	+
D-galactose	-	-	-	+	-	+
alpha-D-glucose	-	-	-	+	-	+
m-inositol	-	D**	-	+	+	-
maltose	-	D	-	+	-	-
D-mannitol	-	-	+	+	-	+
D-mannose	-	D	-	+	-	+
D-melibiose	-	-	-	+	-	-
psicose	-	-	-	+	-	-
D-sorbitol	-	-	-	+	-	+
methyl pyruvate	+	+	+	+	+	+
methyl succinate	+	-	-	-	-	+
acetate	-	+	+	+	-	-
cis-aconitate	-	-	+	+	+	-
citrate	-	-	-	+	-	-
formate	+	+	-	+	-	-
D-galactonic lactone	-	-	-	+	-	-
D-gluconate	-	+	+	+	+	+
D-glucosamine	-	-	-	+	-	-
alpha-hydroxy butyrate	+	-	-	-	+	-
beta-hydroxy butyrate	+	+	+	+	+	+
p-hydroxy phenylacetate	-	-	+	-	+	+

alpha-keto butyrate	+	-	-	-	+	-
alpha-keto- glutarate	-	-	+	+	+	+
alph keto valerate	+	-	-	-	-	-
lactate	+	+	+	+	+	+
malate	-	-	+	+	-	-
propionate	+	+	+	+	+	+
quinat	-	+	+	+	+	+
D-saccharate	-	-	+	+	+	-
sebacate	+	+	+	+	+	+
succinate	+	+	+	+	-	+
bromo-succinate	+	-	+	+	-	+
succinamate	+	-	+	+	+	+
alaninamide	-	-	-	+	-	-
D-alanine	-	+	-	+	-	+
L-alanine	-	+	-	+	-	+
L-alanyl-glycine	-	-	-	+	-	+
L-asparagine	+	+	+	+	+	+
L-aspartate	+	+	+	+	+	+
L-glutamate	+	+	+	+	+	+
glycyl-L-glutamate	-	-	-	+	-	-
L-histidine	-	-	+	+	-	+
hydroxy L-proline	-	-	-	+	-	-
L-leucine	+	+	+	+	-	+
L-ornithine	-	+	-	+	-	-
L-phenyl alanine	-	+	+	+	+	-
L-proline	+	+	+	+	-	+
Lpyroglutamate-	+	-	+	+	-	+
D-serine	-	-	-	+	-	+
L-serine	-	+	-	+	-	-
L-threonine	-	+	-	+	-	-
D,L-camitine	-	-	-	+	-	-
gamma-amino butyrate	-	-	-	+	-	+
urocanate	+	+	+	+	-	-
inosine	-	+	-	+	-	-
glycerol	-	+	-	+	-	+
alpha glycerol phosphate	-	-	-	+	-	-

* (pl) = polar multitrichous, variable; pm = polar monotrichous, per = peritrichous.

** D = variable between strains in the PR117 group.

*** = distance between utilization pattern of strain and that of Comomonas testosteroni in number of substrate tests different from type strain. From Biolog GN ID software.

Figure 5-2: Growth of pBRC60 bearing strains in 3Cba. Open circles = [3Cba] in mM. Closed Circles = Biomass of Cells in Absorbance or Cells.mL⁻¹. Triangles = concentration of chlorocatechol in UV absorbance units from HPLC analysis.

related to PR117 in spite of the substrate utilization differences that place it in a separate group in the cluster analysis. The group III pseudomonads as a whole are not clustered together by their BIOLOG fingerprints (note positions of *P. pseudoflava* and *P. palleronii*).

Strain PR120 was isolated from a mesocosm receiving 20 μ M of 3Cba. It is a motile, aerobic gram negative oval shaped rod with polar monotrichous flagella. According to BIOLOG GN ID profile it is similar to *Psychrobacter immobilis* (similarity = 0.458). However the cell shape and motility of PR120 indicate that it is not a *Psychrobacter* species. PR120 grows very poorly on general media, forming small, translucent colourless colonies after 2 to 3 days. By contrast it grows well on 3Cba agar or 3Cba agar amended with 0.005% yeast extract. In 3Cba liquid cultures PR120 accumulates chlorocatechol and then metabolizes this intermediate in a manner similar to *P. fluorescens* PR24B (Figure 5-2).

PR63 was isolated from a mesocosm receiving 10 μ M 3Cba. It is also a motile aerobic, gram-negative rod with polar monotrichous flagella. On first isolation, it exhibited scant but positive growth on 3Cba agar. This property was difficult to reestablish, nevertheless the organism hybridized to a pBRC60 probe (Fragments E1+E5+E14). The BIOLOG GN ID profile suggests that it is *Pseudomonas delafieldii*; it has a similarity value of only 0.368 to this species, but does share the characteristics of the group III Pseudomonads.

Plasmids in Natural Recipients: Figure 5-3 shows the plasmids isolated from four strains representative of natural recipients in Groups 2, 3, and 5. PR117, PR24B and PR120 all carry pBRC60, while PR63 is carrying a smaller derivative of

Figure 5-3: EcoRI digestion of plasmids extracted from

- A) BR60
- B) PR24B
- C) PR117
- D) PR120
- E) PR63
- F) BR40

Size markers indicated at: (top to bottom) 9.4, 6.6, 4.4, 2.3 and 2.0 kb.

A B C D E F

pBRC60. The stability of the 3Cba⁺ phenotype was determined for each of PR117, PR24B and PR120 by several transfers in general media (TYE or NB). For each strain the loss rate was approximately 7% per generation under these conditions.

Competition experiments in cultures of BR60 and a natural plasmid recipient:

During 100 days of continuous culture of BR60 with 10 μ M 3Cba, the total free-living population fell from 1×10^7 cells.mL⁻¹ to 1×10^6 cells.mL⁻¹ as phosphorous was removed from the medium and wall growth occurred.

During this time BR60 maintained the ability to grow on 3Cba, showing only a small loss in the proportion of 3Cba⁺ cells (Figure 5-4). When it was clear that BR60 was stable in this system after 100 days, an introduction of 3Cba utilizing PR117 was made at 1×10^4 cells.mL⁻¹. PR117 was not detectible after 3 days, therefore a second introduction was made at 1×10^5 cells.mL⁻¹. Thereafter BR60 decreased from 10^6 cells.mL⁻¹ to 10^5 cells.mL⁻¹. Over the subsequent weeks, numbers of 3Cba⁺ BR60 dropped to 10% of the total BR60 population. PR117 stabilized at 1×10^5 cells.mL⁻¹ (equal to the total population of BR60). Estimates of total PR117 populations from CPS plates gave slightly lower counts than those made on 3Cba plates, since BR60 tended to inhibit PR117 on general media. BR60 rapidly outgrew PR117 on NA or KING's B, but inhibition was minimized on CPS plates. The two species were distinguished by their pigmentation on general media (PR117 is yellow) and by the slower rate of growth and smaller colony size of PR117 on chlorobenzoate plates. Plating on general media also revealed that the culture maintained a small population of contaminating yeast (10^4 cells.mL⁻¹) after the establishment of PR117.

Figure S-4: Continuous co-culture of BR60 in FL media supplemented with 10 μ M 3Cba. PR117 was introduced at day 100, see text.

Kinetics of 3Cba Utilization in BR60 and Natural pBRC60 Recipients:

Preliminary experiments on the uptake of 3Cba by BR60 and pBRC60 recipients were performed on overnight batch cultures of the strains in FL media amended with 50 μM 3Cba. The V_{max} of PR81b, BL10 and PR42A (3.4×10^{-16} , 1.5×10^{-16} , and 1.2×10^{-16} mole.cell⁻¹.hr⁻¹, respectively) compared favourably with the V_{max} values for BR60 and for *Pseudomonas putida* PRS2015(pAC27), a 3Cba degrader carrying different chlorobenzoate genes: 6.2×10^{-17} and 3.6×10^{-16} mole.cell⁻¹.hr⁻¹ respectively. To determine the kinetic parameters for uptake in a more realistic situation, BR60, PR117, PR120, PR24B and PR42A were each continuously cultured with artificial lake water amended with 10 μM 3Cba. All of the strains except PR42A were capable of degrading 3Cba at the greatest V_{max} measured in the original mesocosms (50 nM/hr). The results (Table 5-2) show that in these monocultures, BR60 outperforms the three other pBRC60 hosts with respect to overall V_{max} , stability of the chlorobenzoate genes, and affinity for 3Cba (A K_m value for PR24B could not be obtained since the uptake rate did not vary with substrate concentration for this species). However the specific maximum uptake rates indicate that PR117 and PR24B may have higher activities per cell. PR117 and PR24B 3Cba+ cells were present at low frequencies and the resulting cell specific uptake rates appear to be higher than for BR60 and PR120. In addition the culture of PR117 became contaminated with yeast at approximately 100 cells.mL⁻¹, as did the continuous co-culture of BR60/PR117. A pure culture of PR42A lost viability after three volume changes. These results suggest that these isolates survive poorly in pure culture. PR117, and the related strain PR42A, may require alternative nutrients for growth

in continuous cultures which may be provided by the contaminating yeast.

Table 5-2: Kinetic Parameters of Chlorobenzoate Using Strains in Continuous Culture with 10 μ M 3Cba

STRAIN	$V_{\max}$ nM/hr	TOTAL cells/L	3CBA+ cells/L	$V_{\max}$ moles/ cell.hr	K_m μ M	LOSS* %
BR60	771	6.5E9	6.5E9	1.2E-16	0.77	0
PR117	267	6.4E9	8.0E6	3.3E-14	2.37	79
PR120	100	1.3E9	5.3E8	1.9E-16	1.30	6
PR24B	66	1.0E9	1.0E5	6.6E-13	<0.5	23

* Loss rate of 3Cba+ phenotype, in percent per generation.

Laboratory Host Range of pBRC60: Using BR6025(Cm^r, Trp⁻)(pBRC40::Tn5) as a donor, only a few species could be tested for the host range of the pBRC60 replicon. Counterselection against the BR6025 donor necessitated the use of minimal media supplemented with simple carbon sources, so fastidious strains could not be tested. The growth of BR6023 trp⁻ was quite different depending on the carbon sources added to the minimal media. For instance on succinate/acetate plates, growth is negligible; while on plates containing succinate, acetate, citrate, glucose, glycerol (designed to select for a wide range of recipient strains) - growth is slow but considerable, making counterselection quite difficult. In addition a number of potential recipients were naturally resistant to kanamycin. The results of the filter matings are shown in Table 5-3.

Table 5-3: Results of filter mating between BR6025(pBRC40::Tn5) and various potential recipients.

Species	ATCC#	Ratio*	Rate**
<i>Acinetobacter</i> sp. DON2		9	6.2×10^{-5}
<i>Moraxella</i> sp. G.		19	4.5×10^{-4}
<i>P. acidovorans</i>	15668	0.2	5.4×10^{-2}
<i>P. cepacia</i>	25416	82	2.2×10^{-3}
<i>P. putida</i> Biotype A	12633	44	5.5×10^{-4}
<i>Chromobacterium violaceum</i>	12472	200	nd
<i>P. fluorescens</i>	13525	100	nd
<i>P. palleronii</i>	17724	2000	nd
<i>P. pseudoflava</i>	33668	6.5	nd

* Ratio of donor cells to recipients

** Transconjugant cells per donor

nd = transconjugants not detected

Transfers into *P. putida* and *Moraxella* were confirmed by electrophoresis of crude cell lysates; that into *Acinetobacter* was confirmed by hybridization of a southern blot with a pBRC60 specific probe. Transfer to *P. alcaligenes* (ATCC 14909) could not be detected because of the poor growth of this strain on minimal media. Transfers of pBRC40::Tn5 from BR6025 to *Alcaligenes* H850 and to *E. coli* JM109 were observed in concurrent studies by P. Stewart and Jim Ng of this laboratory.

DISCUSSION

3Cba degrading bacteria differing from *Alcaligenes* sp BR60 in their taxonomic characteristics have been isolated from mesocosm waters and sediments inoculated with BR60. They all carry the catabolic plasmid pBRC60. There are two alternate explanations for the presence of this particular plasmid in these strains. The first is that the plasmid is indigenous to the microflora of Lake Opinicon, and the selective pressure of 3Cba in the mesocosms increased the plasmid bearing population. However, data presented in Chapter 3 suggest that pBRC60 bearing bacteria are localized in the Niagara region where there has been strong selection for 3Cba degradation. The second explanation is that pBRC60 transferred from BR60 into PR117, PR24B and PR120 via conjugation, and this is the more parsimonious for a number of reasons.

Representatives of three of the four probe-positive groups isolated carried pBRC60 with no changes in restriction pattern (Figure 5-3). pBRC60 is a conjugative plasmid and laboratory matings indicate that a broad range of bacteria act as recipients of a Tn5 marked pBRC60 replicon. Under laboratory conditions this plasmid transfers into species in both the beta and gamma subgroups of the proteobacteria (Stackebrandt *et al.* 1988), although not consistently into either one. Recent studies in this lab also confirm that transfer to *E. coli* HB101 is detectible when strong counter-selection is applied (J. Ng, pers comm.). It transfers at very high frequencies into some strains (10^1 per donor into conspecifics and 10^2 per donor into *P. acidovorans*).

Plasmid transfer has been demonstrated in natural aquatic environments. Bale *et al.* (1987) demonstrated transfer of a natural large plasmid between *Pseudomonas aeruginosa* strains on filters applied to the surface of non-sterile stones in a river bed. Rates of transfer equalled 5×10^6 transconjugants/donor at 11°C. O'Morchoe *et al.* (1988) documented transfer of R68.45 and PFP5 between *P. aeruginosa* strains in test chambers suspended in 20-28°C lake water at rates ranging from 10^6 to 10^4 transconjugants/donor even though donor and recipient densities were low (10^3 to 10^4 cells.mL⁻¹). In our study, assuming conservatively that each pBRC60-bearing isolate was from a unique colony on its original isolation plate, 500 transconjugants.mL⁻¹ of sediments or 50 transconjugants.mL⁻¹ of water must have been present in the mesocosms. Given that 10^5 cells.mL⁻¹ of BR60 were originally introduced to the mesocosm waters, a comparable conjugation rate of 5×10^{-4} transconjugants per donor is calculated if we assume that the transconjugants did not increase in number. If they did increase, the conjugation rate would be less than 5×10^{-4} per donor, and still within the range of that observed in the natural environment. The mesocosm environment was conducive to plasmid transfer - temperatures were above 20°C and the sediment layer and natural lake water provided an abundance of solid surfaces known to enhance conjugative transfer rates (Genther *et al.* 1988). BR60 died off in these mesocosms, but Wellington *et al.* (1990), O'Morchoe *et al.* (1988), and Gealt *et al.* (1985) have all documented plasmid transfer from donors that did not actively grow in the test media. Genther *et al.* (1988) have demonstrated that a large proportion of freshwater gram negative bacteria are "recipient-active" for a broad-host-range plasmid.

The previous chapter showed that the frequency of pBRC60-probe positive bacteria in lake mesocosms dosed with high levels of 3Cba was greater than the introduced population of BR60 in the mesocosms. At that time I hypothesized that this might be due to a) a loss of culturability of BR60 on 3Cba plates or b) the existence of other pBRC60 bearing bacteria in the aquatic system. While the latter has been demonstrated, the former cannot be completely discounted. Changes in culturability have been observed in a number of organisms after exposure to the nutrient limitations of natural aquatic systems (Roszak and Colwell 1987). In this study BR60 was cultured for 100 days in a low nutrient artificial lake water in order to mimic conditions in the original mesocosms. It failed to obtain a state of non-culturability on chlorobenzoate or on CPS agar. BR60 survived well in FL media until challenged by the presence of PR117 and contaminating yeast at which time its population dropped to 10% of its former value and 3Cba metabolism was abandoned by the majority of the population. After PR117 was introduced to the BR60 cultures, culturable counts of the two did not sum to the AODC counts. Based on the observation that PR117 exhibited a loss of culturability following prolonged continuous culture in FL medium in the absence of BR60, I believe that the discrepancy between total plate counts and AODC in the co-culture was largely due to non-culturable PR117 cells.

Previous work indicated that 3Cba degradation continued in experimental mesocosms after BR60 died off. Maximum 3Cba uptake rates could be predicted from the total population of probe positive organisms. I suggest the bacteria isolated in this study were responsible for 3Cba degradation *in situ*. The continuous culture

work shows that most of the organisms carrying Tn5271 were capable of 3Cba metabolism at substrate concentrations of 10 μ M, even those that exhibited poor 3Cba degradation at mM levels. While they exhibited comparable or higher levels of specific maximum activity than did BR60, all the wild recipients showed a lower affinity for 3Cba and/or greater losses of the 3Cba⁺ phenotype (and therefore presumably of Tn5271) than did BR60 under these conditions. These results were surprising since a priori I expected isolated pBRC60 recipients to be better than BR60 at sequestering small amounts of 3Cba within the natural environment. In hindsight I speculate that ecological factors, not mimicked in our continuous cultures, selected for the recipients isolated in this study. I suggest that pBRC60 was found predominantly in these species not because the 3Cba genes were most efficiently expressed in them, but because the acquired 3Cba⁺ phenotype enhanced pre-existing levels of fitness superior to that of BR60. Scheuerman et. al. (1988) demonstrated the importance of protozoan predation and competition for available carbon, nitrogen and phosphorus in the survival and growth of bacteria in lake water. Klein and Alexander (1986) documented the seasonal occurrence of chemical inhibitors in lake water. The alternate pBRC60 hosts might be superior to BR60 in their ability to utilize other carbon substrates typical of Lake Opinicon waters, their abilities to sequester nitrogen and phosphorus in a competitive environment, or their resistance to predation and chemical inhibition. The relatively low affinity of PR117, PR24B and PR120 for 3Cba leads to more interesting questions about the factors that actually determined their fitness in the mesocosms, or about what is wrong with the way these kinetic determinations are made. Henis and Alexander (1990) have

proposed that the apparent K_s , μ_{max} , length of lag phase and resistance to stress of bacteria in lake water receiving low levels of organic nutrients are important parameters in predicting the behaviour of those bacteria. Results indicate that the 3Cba kinetic parameters of the pBRC60 carriers and their resistance to the low nutrient stress of the continuous mono-cultures were of limited value in predicting the behaviour of these species *in situ*. An interesting study by de Taxis du Poet *et al* (1987) shows that characteristics such as plasmid stability and plasmid borne gene activities are affected by cell immobilization - possibly the attachment of pBRC60 hosts to the surfaces in the aquatic environment affects the stability and expression of the 3Cba genes. Study of the recipient species in biofilm systems such as those described by Bauda *et al.* (1990) is recommended.

The alternate hypothesis that the particular pBRC60 bearing isolates characterized in this work maintained the plasmid but did not express the 3Cba genes *in situ*, and that our techniques failed to isolate those bacteria actually degrading 3Cba, must also be acknowledged. Recent studies have reported the stability of plasmids after introduction to soil or aquatic systems (Caldwell *et al.* 1989, Cruz-Cruz 1988, Devenas *et al.* 1986) or in continuous cultures (Caulcott *et al.* 1989, Filinov *et al.* 1985, Whan Lee and Edlin 1985). Host bacteria experience long term growth disadvantages only if plasmids carry constitutive genes or large pieces of heterologous DNA (Boronin *et al.* 1985, Whan Lee 1985, Warnes and Stephenson 1985). Thus the persistence of the parent plasmid independent of 3Cba selective pressure is feasible. However, in this study I have documented the persistence of a transposable element that under laboratory conditions is deleted at a rate of 0.07

per generation via a recombinational event. Slow growth rates in the natural environment may mean that losses of Tn5271 are infrequent relative to losses in rich media batch cultures.

The majority of pBRC60 recipients isolated from mesocosms were in the PR117 group. The identification of this group remains unclear. From their BIOLOG fingerprint they appear to be extremely close to BR60, but they are not closely related to the other yellow pigmented polar flagellated Group III pseudomonads. It is tempting to conclude that pBRC60 transferred most frequently from BR60 to closely related organisms, and less frequently to more distantly related species such as *P. fluorescens* or *Pseudomonas* sp. PR120. However culture techniques optimized isolation of strains with properties similar to those of BR60; that is reasonable growth on 3Cba agar plates and in 3Cba liquid culture. These properties were shared by the PR117 group but not by the other two organisms. PR120 and PR24B, more distantly related to BR60, exhibited dysfunctional expression of the 3Cba genes at high nutrient concentrations. The fact that PR24B and PR120 were isolated under less than optimal conditions for their growth, suggests that other recipients of pBRC60, non-culturable under the conditions employed, were present in the mesocosms.

A number of factors would contribute to a failure to detect the full range of bacteria harbouring pBRC60. A poor or a complete lack of expression of 3Cba genes in alternate hosts exposed to high 3Cba concentrations is an important problem. As an example *P. putida* (ATCC 12633) will express the Tn5 kanamycin resistance gene when carried by pBRC40::Tn5, however this species does not appear

to express the 3Cba genes on pBRC60. Repeated attempts to transfer pBRC60 into *P. putida* yielded no transconjugants when chlorobenzoate was the selective agent, in spite of the fact that *P. putida* is the normal host of another 3Cba catabolic plasmid (pAC25, Chatterjee and Chakrabarty 1983). More common bacteria may also fail to express these genes under laboratory conditions. Other potential hosts may not have been detected because of their requirements for growth factors in the isolation media. No supplement such as yeast extract was used in the isolation of 3Cba degraders because this encouraged abundant growth of oligotrophs which were difficult to separate from true 3Cba degraders. Some recipients of pBRC60 may simply have been nonculturable. Finally, Tn5271 is lost from pBRC60 at a rate of 7% per generation. This instability makes it difficult to maintain the 3Cba+ phenotype through successive culture purification steps.

Overall, this study shows that a catabolic plasmid, introduced to a freshwater system in one bacteria, can transfer via conjugation to other proteobacteria and can be actively expressed in the presence of micromolar substrate concentrations. The proliferation of the plasmid in chlorobenzoate dosed mesocosms was mediated by gene transfer to alternate hosts that were better adapted *a priori* to conditions in the mesocosms than the original host. The ability of catabolic plasmids to transfer to and be expressed in indigenous bacteria is clearly beneficial from the point of view of remediating contaminated waters.

At the same time the data presented here emphasize gaps in our knowledge about plasmid behaviour after introduction. Prediction of host range is complicated by our limited catalogue of non-pathogenic bacteria and problems of culturability

and counterselection inherent in the methods used to study conjugation. Under laboratory conditions, none of the pBRC60 carrying recipients isolated in this study were efficient 3Cba degraders relative to the originally introduced strain. We need better methods to assess the fitness of particular plasmid-host associations in the natural environment.

**CHAPTER 6: INVOLVEMENT OF TN52⁺ IN COMMUNITY
ADAPTATION TO CHLOROBIPHENYL, CHLOROANILINE, AND
2,4-DICHLOROPHENOXYACETATE.**

INTRODUCTION

Simple chlorinated aromatic compounds are often degraded via oxidation of the chlorinated ring to a chlorocatechol intermediate. Subsequent cleavage of this chlorocatechol product often forms the bottleneck in bacterial chloroaromatic degradation since catechol oxygenases are highly substrate specific and are inhibited by chlorinated substituents (Dorn and Knackmuss, 1978). However chlorocatechol oxygenases do occur in nature, and have been characterized from several chlorobenzoate degrading bacteria (Reineke and Knackmuss 1988). The chlorocatechol oxygenase associated with the chlorobenzoate degrader *Pseudomonas* sp. B13 is well studied (Weisshaar *et al.* 1987). The genes for chlorocatechol cleavage from *Pseudomonas* B13 have been used to broaden the degradative capacity of mixed cultures (Schmidt *et al.* 1983) or of individual strains either through natural genetic exchange or through artificial recombination (Chatterjee and Chakrabarty 1982; Jeenes *et al.* 1982; Latorre *et al.* 1984; Lehrbach *et al.* 1984; McClure *et al.* 1989; Oltmanns *et al.* 1988; Reineke *et al.* 1982; Schwien and Schmidt 1982). Other donors of the genetic requirements for 3Cba degradation have also been applied to isolate novel catabolic strains (Krockel and Focht 1987). In those studies not involving artificial genetic recombination, nutrient rich media, high chloroaromatic substrate concentrations, and dense parent populations were used to

enhance the frequency of rare genetic exchange events, so the relevance of this artificial selection to the natural situation is questionable.

Since it encodes the breakdown of 3- and 4- chlorobenzoate through chlorocatechol, I was interested in the fate of Tn5271 in natural freshwater ecosystems dosed with low levels of chloroaromatic contaminants that are known to be degraded through similar pathways. Studies outlined in the previous two chapters indicate that conjugal spread of pBRC60 exposes it to different genomic backgrounds. It was logical to hypothesize that, through transfer, the genes found on Tn5271 might complement catabolic pathways found in other bacteria to allow chloroaromatic utilization.

To this end, three chloroaromatic compounds were chosen for mesocosm dosing, 4-chloroaniline (PCA), 3-chlorobiphenyl (CBP) and 2,4-D. The choice of compounds was influenced primarily by their known degradative pathways but also by the availability of radio-labelled substrate. CBP was chosen because it is commonly metabolized to chlorobenzoate but no further. PCA was chosen because it is a common industrial/agricultural pollutant, forming part of three herbicides (monolinuron, monuron, and buturon). Fairly resistant to microbial attack, PCA undergoes numerous abiotic transformations in soil and aquatic environments, including acylation, oxidation and condensations of the amine group, and binding to natural macromolecules (Freitag *et al.* 1984). Only two PCA degraders have been found and both metabolize the compound through chlorocatechol (Zeyer *et al.* 1985; Latorre *et al.* 1984). 2,4-D was chosen because studies on 2,4-D degrading *Arthrobacter* (Tiedje *et al.* 1969), *Alcaligenes eutrophus* JMP134 (pJP4) (Don *et al.*

1985), and *Xanthobacter* sp CP (Ditzelmuller *et al.* 1989) show the breakdown is mediated through ortho cleavage of dichlorocatechol. In JMP134, the plasmid pJP4 encodes genes for the metabolism of chlorocatechols that permit the breakdown of both 2,4-D and 3Cba (Don *et al.* 1985).

Each of these chemicals was fed to mesocosm waters at three treatment concentrations over a 100 day period, and the frequency of Tn5271 was monitored and related to the degradative potential in the systems. The following describes the work that shows that Tn5271 played no role in 2,4-D degradation, some role in community adaptation to PCA, and a useful role in CBP degradation.

METHODS AND MATERIALS

Mesocosm Treatments: Mesocosms were continuously fed with non-sterile water from adjacent Lake Opinicon at 7.5 mL/min (dilution rate 0.01/hr) over a period of three months from May 20th to September 5th, 1989. Each mesocosm received a specific treatment chemical in the dilution water, summarized in Table 6-1. 2,4-D, 3Cba and PCA stock solutions were sterile aqueous solutions of the anions or cation respectively. Acetone, chosen because of its high volatility, was used to solubilize CBP in its stock solution. Percentages of acetone in the stock solution were as follows: high dose - 50%, medium dose - 10%, low dose - 2.5%. CBP solutions were prepared by sterilizing the appropriate amount of water in a 4L glass container and adding the acetone and chlorobiphenyl. Stock solutions were prepared in 4L nalgene containers. 3Cba, 2,4-D and PCA chemical treatments began on June 1st, 1989 (day 0). The same day, 10^6 cells.mL⁻¹ of BR60 were introduced to the 3Cba

and 2,4-D treatment mesocosms and to the inoculated control mesocosm from a 2 day old CPS culture, and 10^5 cells.mL⁻¹ of BR611 (capable of degrading 4Cba) were introduced to the PCA treatment mesocosms from a 2 day old 4Cba culture. 3-chlorobiphenyl stock solutions were connected to the mesocosms on June 9th. BR60 was introduced at the same time from a CPS broth culture.

Quantification of Catabolic Genotypes: Approximately every 10 days throughout the experimental period, undiluted water or 10^{-1} diluted sediment was inoculated with 5 replicates and diluted to 10^{-3} to 10^{-4} in microtitre plates containing CPS broth. After incubation for 2 days at 30°C growth was filtered onto Hybond nylon membranes (Amersham), lysed, fixed and probed with fragment EcoRI-11 (E-11). Populations of BR60 and BR611 were enumerated by triplicate plate counts on 3Cba or 4Cba agar, respectively, containing 25 µg.mL⁻¹ streptomycin.

From day 37, mesocosm waters and sediments were plated on specific substrate plates. 3Cba and CBP dosed mesocosm samples were plated on 3Cba agar. CBP dosed samples were also plated on CBP agar. PCA dosed samples were plated on PCA/ye and 4Cba agar. 2,4-D dosed samples were plated on 2,4-D and 3Cba agar. Any colonies forming on these specific substrate plates that showed signs of chloroaromatic degradation (that is a decrease in the pH indicated by a yellowing of the colonies or agar) were restreaked to a fresh substrate plate and to CPS plates for purification and future study.

Heterotrophic Potentials: Heterotrophic potentials of mesocosm waters were determined from both the rate of uptake of the compound and the rate of production of ¹⁴CO₂. Heterotrophic potentials of sediments were determined using

Table 6-1: Summary of Mesocosm Treatments

Mesocosm	Date	Treatment	Date	Treatment
C1*	1/6-14/8	0 μ M 3Cba	15/8-5/9	50 μ M 3Cba
C2	1/6-14/8	0 μ M 3Cba	15/8-5/9	50 μ M 3Cba
C3	1/6-5/9	25 μ M 3Cba		
TFDI	1/6-29/7	50 nM 2,4D	30/7-5/9	50 μ M 3Cba
TFDm	1/6-29/7	1 μ M 2,4D	30/7-5/9	50 μ M 3Cba
TFDh	1/6-29/7	50 μ M 2,4D		50 μ M 2,4D
CBPI	1/6-5/9	50 nM CBP		
CBPm	1/6-5/9	1 μ M CBP		
CBPh	1/6-5/9	50 μ M CBP		
PCAI	1/6-5/9	50 nM PCA		
PCAm	1/6-5/9	1 μ M PCA		
PCAh	1/6-5/9	50 μ M PCA		

* Not inoculated with BR60 or BR611

CO₂ production alone. Background levels of treatment chemicals were used to correct uptake and CO₂ production data from high dose treatments. Because substrate concentrations used in uptake measurements were higher than the feed concentration of the low and medium dose treatments, background concentrations were assumed to be negligible for these mesocosms.

Statistical analyses: Treatments encompassed a wide range of chemical concentrations and three different chemicals to maximize the likelihood of producing conditions selective for Tn5271. However this allowed the use of only one mesocosm per treatment level and population estimates collected over the exposure period were averaged to determine mean populations for each treatment. These data were sequential and therefore not strictly independent of each other, even though sampling intervals exceeded the mesocosm turnover time. Statistical comparisons require random (independently sampled) data, so the collection of sequential data, strictly speaking, violated this assumption. Since small sample sizes were involved and different population dynamics took place in each mesocosm, time series analyses could not be used to compare population parameters. However, as stated on page 364 of Gibbons (1985), if sequential data points act as if they are random according to the "runs up and down" test (pp 371-377 of Gibbons 1985), comparisons between data sets can be carried out with some degree of confidence. Accordingly, data were log transformed to make variances homogeneous, and the "runs up and down" test was performed to determine if the data departed from randomness for the period of time of interest. If behaving randomly, non-parametric methods were used to compare populations on the assumption that the data were not necessarily normally

distributed. Intra-mesocosm comparisons were carried out using the sign test (pp 94-99, Gibbons 1985) and inter-mesocosm comparisons carried out using a Mann-Whitney-Wilcoxon test (pp 159-165, Gibbons 1985).

RESULTS

The first five sections of results that follow describe the response of BR60 or BR611 and probe positive (probe+) populations to the chemical treatments. Section 1 details the response of the bacterial populations to no treatment (mesocosms C1 and C2). Section 2 describes the responses to 3Cba exposure (mesocosm C3). Sections 3, 4 and 5 describe responses to PCA, CBP and 2,4-D exposures respectively. Statistical comparisons are performed on log transformed data and only if the data exhibited random behaviour according to a runs up and down test. Following these details on population changes is a summary of the average probe positive populations for each treatment (Section 6). Finally the in situ degradation rates of the treatment chemicals are compared to the probe positive populations existing at the time of measurement (Section 7).

1. Survival of BR60/probe+ in uncontaminated mesocosms. Control mesocosms, C1 (uninoculated) and C2 (inoculated), were both left uncontaminated for 77 days, after which 50 μ M 3Cba was delivered to both mesocosms to test for recovery of BR60 or Tn5271-carrying bacteria. Fig. 6-1 a-d illustrate the population dynamics of BR60, the total probe+ population, and the population of 3Cba degraders other than BR60 over this period. In C1, BR60 populations were negligible and probe+ populations remained undetectible up to day 77 as expected

(Figure 6-1 a,b). In C2, although inoculated with 10^6 cells.mL⁻¹ BR60, populations dropped to $10^{2.45}$ cells.mL⁻¹) in the water and $10^{3.11}$ in the sediments five days later. Thereafter, up to day 77, BR60 was undetectable in mesocosm waters and decreased in the sediments (Spearman's $r = -0.64$, $p = 0.048$, Figure 6-1c). Probe+ populations were higher than counts of BR60 in the first 20 days, but mirrored the BR60 counts thereafter - exhibiting a significant decrease with time (Spearman's $r = -0.69$, $p = 0.035$, Figure 6-1-d).

After delivery of 50 μ M 3Cba probe+ populations increased from levels below detection to $10^{6.0}$ cells.mL⁻¹ in sediments and $10^{2.0}$ to $10^{3.0}$ cells.mL⁻¹ in waters in both mesocosms (Figure 6-1b and d). In C1 this probe+ bloom was completely independent of BR60, in C2 it was entirely due to the recovery of BR60.

2. Survival of BR60/probe+ in 3-Chlorobenzoate Dosed Control Mesocosm (Mesocosm C3). BR60 populations initially persisted longer in 3Cba-dosed-mesocosm C3 than in the uncontaminated control C2. They decreased to $10^{2.38}$ cells.mL⁻¹ in the waters and to $10^{4.18}$ cells.mL⁻¹ in the sediments five days after introduction, and persisted in waters at low levels until day 58. They persisted in sediments at average levels higher than the control until day 58 ($10^{3.79}$ versus $10^{2.66}$ cells.mL⁻¹, Mann-Whitney-Wilcoxon $p = 0.12$, ns). However, BR60 was not detected in either water or sediments on or after day 69, when high populations of other 3Cba degraders became dominant (Figure 6-1e).

Probe+ populations were found at populations equal to or higher than the total 3Cba degraders, persisting well after BR60's disappearance. Five days after introduction probe+ populations were high in both waters and sediments ($10^{5.23}$ and

Figure 6-1: Summary of seasonal changes in BR60 and probe+ populations in mesocosm

C1 - uninoculated, uncontaminated (a and b)

C2 - inoculated with BR60, uncontaminated (c and d)

C3 - inoculated with BR60 and contaminated with 25 μM 3-chlorobenzoate (e and f).

After day 77, 50 μM was fed to control mesocosms C1 and C2.

$10^{5.15}$ cells.mL⁻¹, respectively). Although variable, sediment probe+ populations did not decrease over time (Spearman's $r = -0.19$, $p = 0.302$, ns, Figure 6-1f). Water probe+ populations were consistently 2 orders of magnitude higher than BR60 populations, gradually decreasing with time (Spearman's $r = -0.648$, $p = 0.025$). Up to day 60, sediment probe+ populations were higher but not significantly so from BR60 populations ($10^{4.73}$ vs $10^{3.79}$ cells.mL⁻¹, sign test, $p = 0.19$), and after this plate counts of 3Cba degraders other than BR60 account for most of the probe+ population. Water 3Cba degrader populations are of comparable magnitude to probe+ populations (Probe+ = $10^{2.97}$, 3Cba+ = $10^{2.31}$ cells.mL⁻¹). In sediments, probe+ populations are higher but not significantly different than 3Cba degrader populations (Probe+ = $10^{4.5}$, 3Cba+ = $10^{3.1}$ cells.mL⁻¹, sign test, $p = 0.23$).

Chlorobenzoate was incompletely degraded until day 37 when concentrations dropped to zero following a peak of probe+ in the sediments of 7.63 (Figure 6-1f).

3. Survival of BR611/probe+ in Chloroaniline Dosed Mesocosms. In mesocosms PCAI, PCAm and PCAh, BR611 became undetectable within 4 weeks and remained so in both the waters and sediments for the duration of the summer (Figure 6-2b,d). Probe+ levels initially reflected this decrease, but in PCAh, probe+ populations recovered to $10^{3.04}$ in the water by day 48. Probe+ populations in PCAh subsequently reached a peak of about $10^{4.9}$ between days 59 and 77. This recovery in the water was followed by a larger probe+ bloom in the sediments where populations reached $10^{5.85}$ before decreasing again. Smaller probe+ population blooms were also seen in PCAI and PCAm after 60 days (Figure 6-2b,d). There was a dose response relationship between mesocosm PCA dose and probe+ population

Figure 6-2: Summary of seasonal changes in BR60 and probe+ populations in mesocosms

PCAl - 50 nM PCA (a and b)

PCAm - 1 μ M PCA (c and d)

PCAh - 50 μ M PCA (e and f)

averaged over this bloom period (day 46 to 98, $r = 0.95$ for water; $r = 0.97$ for sediments, ns at $n=3$). A relationship between dose and lag phase of probe+ recovery is suggested - PCAh developed probe+ populations in the waters about 20 days before lower dosed mesocosms. The appearance of probe+ organisms in PCAh waters was concurrent with a large decrease in PCA concentrations (Figure 6-2f).

Growth on PCA/ye plates was not indicative of chloroaniline degraders - colonies were never large nor was any pH change evident during colony formation. Counts do not correspond well with probe+ populations during the same period. In spite of slightly elevated average counts in the high PCA mesocosm, there was no significant difference in PCA/ye plate counts between mesocosms.

4. Survival of BR60/probe+ in Chlorobiphenyl Dosed Mesocosms (Figure 6-3). On day 12, 2 L of a 5.7 mM solution of chlorobiphenyl spilled onto the plexiglass lid of CBPI and was at least partially absorbed into the plexiglass before the spill was discovered. Given that water spray from the aerator condensed on mesocosm lids and dripped back into the water, it is not inconceivable that some additional chlorobiphenyl exposure occurred in CBPI due to the exchange of the chemical between the plexiglass and organic particles in the water droplets forming on the lid. In all CBP treatment mesocosms, BR60 population decreases were similar to those seen in C2 but probe+ populations were quite variable. The lower dosed tanks showed die offs followed by short-lived sediment probe+ population blooms after day 63 (Figure 6-3b,d). These peaks coincided with small recoveries in the BR60 populations but were much higher than the BR60 levels.

Figure 6-3: Summary of seasonal changes in BR60 and probe+ populations in chlorobiphenyl dosed mesocosms

CBPl - 50 nM CBP

CBPm - 1 μ M CBP

CBPh - 50 μ M CBP

Probe+ populations were persistent in the water and sediments of CBPh although levels are variable (Figure 6-3f). Chlorobenzoate degraders were also enumerated in CBPm and CBPh (Figure 6-3c,e) and in the latter account for the probe+ populations. The 3Cba degrader population averaged $10^{1.2}$ and $10^{3.0}$ cells.mL⁻¹ in waters and sediments respectively, the corresponding probe+ populations averaged $10^{1.9}$ and $10^{2.9}$ cells.mL⁻¹.

Growth on chlorobiphenyl plates was abundant so only large colonies associated with a decrease in pH were enumerated. Counts of putative chlorobiphenyl degraders made in this way were not significantly different between tanks.

4. Survival of BR60/probe+ in 2,4-D dosed mesocosms. In TFDl, BR60 populations exhibited die offs similar to those of the control tank C2 (Figure 6-4a). BR60 populations in TFDm persist at levels higher than in control C2 but this phenomenon is not related to 2,4D dose, BR60 does not persist in TFDh. Probe+ populations decline in all three mesocosms and show an anomalous underestimation of BR60 populations in TFDm (Figure 6-4b,d,f).

Culturable 2,4-D degraders were readily detected in TFDh from day 37 to the end of the experimental period, indicating that genetic elements coding for 2,4-D degradation were common in the bacterial community and not homologous to Tn5271 (Figure 6-4e). Therefore 50 μ M dosing of TFDh was continued to confirm that Tn5271 would remain uninvolved with 2,4-D degradation, and the lower dosed mesocosms were used to contrast probe+ population responses in a mixture of 2,4 D and 3Cba versus 3Cba alone.

On day 60, TFDI was fed with 50 μM 3Cba while TFDm was fed with 50 μM 3Cba and 50 μM 2,4-D. Increases in the probe+ populations in response to these treatments spanned several orders of magnitude (Figure 6-4b,d) and was probably due to the growth of 3Cba degraders. 3Cba and 2,4-D degraders and probe+ populations in all three mesocosms over this period are tabulated in Table 6-2. There were three interesting differences between the 3Cba (TFDI) and the 3Cba/2,4-D mixed (TFDm) mesocosms. First, water probe+ populations were significantly higher in TFDI than in TFDm (Mann-Whitney-Wilcoxon, $p=0.048$). Second, the chemical concentrations in the mesocosm waters suggested that TFDI harboured a more efficient 3Cba degrading population than did TFDm. However, TFDI and TFDm waters and sediments exhibited similar rates of 3Cba uptake and respiration when measured on day 97 (Table 6-2). Third, 3Cba degraders enumerated from TFDI corresponded to probe+ populations, but counts of neither BR60, other 3Cba degraders, or 2,4-D degraders could account for the probe+ levels in TFDm, indicating that the probe+ population was mostly not culturable on 3Cba and 2,4-D. 3Cba degraders that arose immediately after the introduction of the chemical mixture to TFDm did not persist. The only culturable 3Cba catabolizers detectable on day 98 were sediment BR60 populations, and yet considerable 3Cba degradation was evident from the uptake and respiration measurements. Similarly TFDm exhibited high rates of 2,4-D degradation in spite of a low population of culturable 2,4-D degraders in the waters.

Figure 6-4: Summary of seasonal changes in BR60 and probe+ populations in 2,4-D dosed mesocosms

TFDI - 50 nM 2,4-D; 50 μ M 3Cba

TFDm - 1 μ M 2,4-D; 50 μ M 3Cba + 50 μ M 2,4-D

TFDh - 50 μ M 2,4-D

Table 6-2: Mesocosms receiving 3Cba, 2,4-D and a Mixture of 3Cba and 2,4-D.

Site	Day	Vmax of 3Cba 2,4D nM.hr ⁻¹		BR60	Populations of 3Cba+ 2,4D+ log(cells.mL ⁻¹)		Probe +	Concentrations [Cba] [2,4D] μM	
TFDI water	68			2.06	4.47	.	4.03	2.2	0.0
	75			1.32	4.75	.	5.03	0.7	0.0
	87			0.00	3.20	.	5.09	2.4	0.0
	97	136	120	0.00	4.97	0.00	4.85	2.41	0.0
	mean			0.84	4.35	.	4.75		
	stdev			1.02	0.79	.	0.49		
TFDI sed	68			3.30	6.35	.	4.60		
	75			2.82	5.78	.	6.03		
	87			3.08	4.86	.	6.09		.
	97	1994	17	0.00	6.00	.	6.09		
	mean			2.30	5.75	.	5.70		
	stdev			1.55	0.64	.	0.74		
TFDI _m water	68			1.32	2.85	.	4.03	2.2	7.0
	75			1.45	2.26	2.94	3.49	5.8	8.2
	87			0.00	0.00	0.00	3.35	14.1	19.8
	97	129	84	0.00	0.00	2.33	4.35	9.6	15.3
	mean			0.69	1.28	1.76	3.81		
	stdev			0.80	1.5	1.55	0.47		
TFDI _m sed	68			3.91	4.51	.	5.03		
	75			4.17	5.34	5.28	4.49		
	87			3.53	3.90	4.00	5.85		
	97	9229	3510	3.56	0.00	5.83	5.85		
	mean			3.79	3.44	5.04	5.31		
	stdev			0.31	2.40	0.94	0.67		
TFDI _h water	68			0.00	1.3	.	0.00		3.5
	75			0.00	0.00	4.44	1.00		2.0
	87			0.00	0.00	3.30	0.00		0.0
	97	0	60	0.00	0.00	3.56	0.00		0.0
	mean			0.0	0.32	4.41	0.25		
	stdev			0.0	0.65	0.81	0.50		
TFDI _h sed	68			3.91	3.78	.	0.00		
	75			2.82	3.60	5.28	2.69		
	87			0.00	0.00	5.00	0.00		
	97	0	1174	0.00	0.00	4.88	0.00		
	mean			1.68	1.84	5.05	0.67		
	stdev			1.99	2.13	0.21	1.34		

6. Overall Summary of Chemical Treatments. Since the primary aim of this study was to test the hypothesis that mesocosms stressed with simple chloroaromatics would harbour higher than control levels of probe+ organisms, treatment mesocosm probe+ populations were each compared to the inoculated control population. Data collected within the five weeks immediately after BR60 introduction are excluded from this analysis so that probe+ populations do not reflect the artificially high variation associated with BR60's initial die off. Only data collected after day 37 are used. The period after five weeks is defined as representing the "acclimated" community even though significant changes in community composition may have taken place after this time. This objective definition of the acclimatized period increases the variability in the data, but makes the statistical analysis more conservative.

Acclimated average probe+ populations are plotted in Figure 6-5. Data on the 3Cba and 3Cba/2,4-D mixed dosing of TFDl and TFDm are excluded. When compared to the inoculated control mesocosms, water probe+ populations of the high dose chlorobenzoate, chlorobiphenyl and chloraniline mesocosms are significantly higher (Table 6-3), as are the probe+ populations of the sediments of the high dose chlorobenzoate mesocosm. Sediment probe+ populations of the medium and high dose chloroaniline mesocosms and the high dose chlorobiphenyl mesocosm are marginally higher (Table 6-3).

Figure 6-5: Average probe + populations in water (open circles) and sediments (dark circles) after acclimation (see text) for each treatment. Data for control mesocosm C2 is plotted at chemical dose 10^{-2} in all four graphs for comparison. Bars are standard errors, $n = 6$.

Table 6-3: Comparison of average acclimated probe positive populations between treated mesocosms and inoculated control mesocosm.

Phase	Mesocosm	Dose	probability*
Water	C3	25 μ M 3Cba	p = 0.019
	PCA _h	50 μ M PCA	p = 0.010
	CBPh	50 μ M CBP	p = 0.019
Sediment	C3	25 μ M 3Cba	p = 0.010
	CBPh	50 μ M CBP	p = 0.071
	PCA _h	50 μ M PCA	p = 0.13
	PCAm	1 μ M PCA	p = 0.083

* Mann-Whitney-Wilcoxon test

7. Correlation between mesocosm degradation rates and probe positive populations:

In the correlations that follow, log transformed probe positive populations are compared to log transformed uptake data.

Measurements of the 3Cba degradative potentials of mesocosms C1, C2, C3, TFD_l, TFD_m and TFD_h mesocosms are plotted against corresponding probe+ populations in Figure 6-6a. There is a significant relationship between V_{max} and the probe+ population in both the waters and sediments. The regression of the water V_{max} on the probe+ population is significantly different from that found for mesocosms in the two previous years, the slope is less in the current regression. 3Cba degradative potentials do not correlate significantly to total plate counts of 3Cba degraders (BR60 + others) in waters ($r^2 = 0.28$), but are correlated to total plate counts in sediments ($r^2 = 0.66$, $p < 0.01$).

For all PCA dosed mesocosms, as shown in Figure 6-6b, the water V_{max} rates are weakly but significantly correlated to the probe+ populations with $r^2 = 0.55$ ($p < .05$). Sediment V_{max} rates and probe+ populations are correlated with $r^2 =$

0.68 ($p < .05$). None of the measured sediment CO_2 production rates were very high but no measurements were taken during the peak of the probe + population blooms.

In CBP dosed mesocosms, no chlorobiphenyl respiration could be detected using the techniques described until the incubation period was increased from 2 to 5 hours for the last set of measurements (Table 6-4). High rates of degradation were detected only in CBPh.

Table 6-4: Summary of Degradation Rates of 3Cba and CBP in Chlorobiphenyl Dosed Mesocosms.

Code day	Uptake of		Probe Positives			
	3Cba water	3Cba sed.	CBP water	CBP sed.	water	sed.
CBPI						
46	0.0	0.0	.	.	0.0	0.0
69	.	.	2.0	0.0	0.0	3.25
95	.	36.5	.	18.4		5.80
CBPm						
46	0.0	0.0	.	.	0.0	0.0
69	.	.	1.2	15.6	0.0	2.25
95	.	8.9	0	0		0.0
CBPh						
46	0.0	0.0	.	.	2.55	4.01
69	.	.	0.34	1.1	2.79	2.60
95	.	13.7	1.7	180.0	3.09	2.95

Rates are maximum uptakes in $\text{nM}\cdot\text{hr}^{-1}$, probe + populations in $\log(\text{cells}/\text{hr})$

on day 95 and a low but discernable rate CO_2 production was evident in CBPI on day 95.

Figure 6-6: Relationship between maximum uptake rates of 3Cba, PCA and 2,4-D and probe+ populations in 3Cba, PCA and 2,4-D dosed mesocosms respectively. Dark symbols are for sediments, open symbols for water. Numbers in lower left corner denote number of overlapping data points.

a) Water: $y = 0.228 x + 0.98$, $r^2 = 0.74$, $n = 8$, $p < 0.01$

Sediment: $y = 0.584 x + 0.03$, $r^2 = 0.84$, $n = 9$, $p < 0.01$

b) Water: $y = 0.177 x + 1.83$, $r^2 = 0.55$, $n = 10$, $p < 0.05$

Sediment: $y = 0.526 x + 0.51$, $r^2 = 0.71$, $n = 7$, $p < 0.05$

c) Water: $r^2 = 0.25$, $n = 10$, ns

Sediment: $r^2 = 0.10$, $n = 10$, ns

Chlorobenzoate uptake and sediment respiration was measured in these three mesocosms on day 46. No significant uptake or respiration could be detected, even in CBPh where probe+ populations were at 4.0. On day 95 respiration of 3Cba was measured again, all three showed detectable levels of CO₂ production from 3Cba that mirrored the probe+ populations present at that time.

2,4-D heterotrophic potentials measured in TFDm and TFDh on day 46 and day 60 show significant uptake and respiration of 2,4-D was taking place in both mesocosms when probe+ populations were generally low. The relationship between probe+ populations and 2,4 D degradation is not significant (Figure 6-6c).

DISCUSSION

The probe positive populations measured in this study using MPN dilutions and DNA-hybridization are our best estimation of the number of bacterial cells that carry the degradative transposon, Tn5271, *in situ*. Other studies have shown the MPN:DNA hybridization technique to be an adequate method of enumerating genetically marked cells (Bentjen *et al.* 1989, Steffan *et al.* 1989, Fredrickson *et al.* 1988). In this study the technique was valuable because of its relative independence from the specific phenotypes of the host bacteria.

The specificity of the DNA probe for Tn5271 carrying cells is confirmed in this longer term study. The dependence of probe+ populations on 3Cba inputs and the correlation between 3Cba uptake and probe+ populations in 3Cba dosed mesocosms remains significant. Probe positive populations responded dramatically to the input of 3Cba. 25 μ M 3Cba maintained significantly higher levels of total

probe+ organisms in both the waters and sediments of mesocosm C3. Late summer addition of 3Cba at 50 μM to inoculated and uninoculated control mesocosms and to mesocosms TFDl and TFDm stimulated rapid growth of probe positive organisms from very low population levels. The probe+ population measurement is significantly correlated to the 3Cba degradative potential in 3Cba dosed mesocosms while plate counts of BR60 and other 3Cba degraders are less so. Even so, the regression is weaker than that determined for 1988 mesocosms exposed to 3Cba for only 4 weeks. The relationship is weakened by late summer samples from the 25 μM dosed mesocosm that had high probe+ populations but generally low uptake/degradation rates. In this mesocosm ambient 3Cba concentrations had declined to zero or less than 1 μM before degradation measurements were made. Probe positive organisms sampled at this time might not have been actively expressing the 3Cba genes. They may have been a persistent population fed by the occasional input of 3Cba, generally declining as an active biofilm population grew near the input tubing.

The strongest demonstration of the dependability of the MPN:DNA probe+ population estimate was found in TFDm, the 3Cba/2,4-D mixed mesocosm, where at the end of the summer the waters showed significant 3Cba uptake and degradation, a healthy probe+ population but no culturable 3Cba degraders. The selective detection of active pBRC60::Tn5271 bearing organisms in a mixed chemical environment emphasizes the utility of the technique.

The dominance of BR60 over other 3Cba degraders varies both over time and between mesocosms. The ecological factors that control the dominance of one

pBRC60 host over another are poorly understood - but this study reveals that factors other than temperature, the inorganic chemistry, and the biotic composition of the incoming waters were important. Temperature and lake water inputs were identical between treatments. The nature of the predatory and competitive biotic community was most probably different in each isolated mesocosm at the outset of the experiment and diverged as time passed.

There is clearly no involvement of Tn5271 in the degradation of 2,4-D in these systems. Significant 2,4-D catabolism in TFDm and TFDh occurred in the absence of probe+ organisms. Plate counts of 2,4-D degraders indicate populations much higher than concurrent probe+ populations in TFDh throughout the summer (Figure 2e and 2f). This result is not surprising, in hindsight, given the opportunity for historical contamination of Lake Opinicon waters by 2,4-D via spraying activities of the local hydro companies along hydro rights of way and of local cottage and small farm residents within the Lake Opinicon watershed.

There is limited evidence for interference between 2,4-D and 3Cba metabolism in these mesocosms. The 3Cba/2,4-D mixed mesocosm exhibited lower water probe+ populations, poorer degradation of incoming contaminants and the degrader populations were non-culturable on minimal media plates, differing from those found in the 3Cba only or 2,4-D only dosed mesocosms. The two chemicals have similar enough structures that initial oxidation enzymes may have led to the accumulation of toxic intermediates. Interference between 2,4-D and 2,4,5-trichlorohenoxyacetic acid has been demonstrated in two-membered cultures able to degrade these substrates individually (Haugland *et al.* 1990). Uptake and respiration

measurements taken on day 98 do not support the suspicion that inefficient 3Cba degraders existed in TFDm. By this time, however, probe positive populations had increased again, and most culturable degraders had dropped out of the system, indicating the development of a different type of degrader population, perhaps one better able to deal with the chemical mixture. Had degradation measurements been taken over the full course of the four weeks, some interference might have been detected.

Genetic elements homologous to Tn5271 are involved with mesocosm adaptation to 4-chloroaniline. All three chloroaniline dosed mesocosms experienced population blooms of probe positive organisms that were not seen in either control tanks, the 2,4-D or chlorobiphenyl dosed tanks. A dose-response correlation is evident. The water probe+ population bloom in the highest dose mesocosm was statistically significantly higher than control mesocosm probe positive populations. The production of CO₂ from labelled chloroaniline in sediments and the uptake rates of chloroaniline in waters correlate to the probe positive populations even though actual degradation rates at the time of measurement were quite low.

Chloroaniline degraders capable of forming colonies on PCA/ye agar could not be cultured from these mesocosms. This result does not mean that chloroaniline degradation was not taking place. PCA/ye plates, containing 2 mM chloroaniline, were probably toxic to organisms that were capable of degrading 50 μ M or less. Also, there is no reason to assume that only a complete chloroaniline degrader would be selected for in the mesocosms. A bacterial population might arise that carries out only part of the degradation sequence. Probe positive organisms might

therefore be responsible only for chloroaniline oxygenation, or for chlorocatechol cleavage.

Overall the chlorobiphenyl mesocosms gave the most variable probe positive population estimates. Chlorobiphenyl is an extremely hydrophobic molecule and it could be expected to sorb onto particles quite quickly after reaching mesocosm waters. The concentration of chlorobiphenyl within the sediments of these mesocosms would therefore be patchy, and correspondingly the bacterial populations responding to its presence would be irregularly distributed. This would lead to highly variable probe positive population estimates. The fact that CBPh developed probe positive populations that were significantly higher than control levels in spite of high variation is strong evidence that Tn5271 is selected for by this chemical. The complete degradation of chlorinated biphenyls requires two types of degraders - one that oxygenates and cleaves the compound to yield chlorobenzoates, and a second that metabolizes chlorobenzoate (Sylvestre and Fauteux 1982, Masse *et al.* 1984, Furukawa *et al.* 1978, Sylvestre *et al.* 1985). Bailey *et al.* (1983) recovered 50% of radioactively labelled CBP as carbon dioxide when the substrate was introduced to river water, but about 80% of that introduced as biphenyl. The CBP was degraded through a metabolite that was probably chlorobenzoate and the 50% radioactivity remaining in the river water was most likely end products of chlorobenzoate oxygenation. Reports of single organisms that attack both chlorobiphenyls and chlorobenzoates are rare. Parsons *et al.* (1988) describe a *Pseudomonas* strain JB1 that is able to degrade chlorobiphenyls to corresponding chlorobenzoates, and to further degrade some of the chlorobenzoates via cometabolism after induction by

benzoate. Smith and Rutledge (1989) describe a *Nocardia* species that can transform non-chlorinated biphenyl to catechol and further to cis,cis-muconate (indicative of ortho cleavage). The minimal response of probe positive populations to chlorobiphenyl relative to 3Cba (200 cells per nmole chlorobiphenyl versus 10,000 or more cells per nmole chlorobenzoate in sediments) suggests that they are selected for by the slowly released metabolites of chlorobiphenyl transformers. The organisms responsible for this increase in probe+ populations were most probably all 3Cba degraders.

CHAPTER 7: CHARACTERIZATION OF MESOCOSM PROBE

POSITIVE POPULATIONS

INTRODUCTION

In the previous chapter the frequency of organisms carrying DNA homologous to Tn5271 in mesocosms contaminated with different chemicals is described. As a follow up to that study, representative organisms of those probe positive populations were sought for three reasons: a) to confirm the specificity of the DNA probes for organisms carrying elements of Tn5271, b) to glean more information about the host range of pBRC60 and Tn5271 in these systems; and c) to determine the probable *in situ* phenotype of probe positive organisms in the chlorobiphenyl and chloroaniline dosed mesocosms.

METHODS AND MATERIALS

Mesocosms dosed with 3Cba, PCA, 2,4-D and CBP as described in the previous chapter were sampled approximately every 10 days. Sediment and water samples were plated on appropriate single substrate agar plates. After incubation at 30°C for 1-2 weeks, colonies of putative catabolizers were restreaked to general media (CPS) and to fresh substrate plates for purification prior to preservation. In addition, because probe positive organisms were seen to increase in the PCA dosed mesocosms and because growth on PCA/ye agar remained poor, samples from these mesocosms were also plated on CPS. Dominant colony types from this plating were also purified and preserved.

Each strain was tested for growth on appropriate substrates by patching them to single substrate plates supplemented with 0.05% yeast extract. Colonies exhibiting degradative activity were streaked from the substrate plate to fresh CPS plates. After incubation at 30°C for 18 hours, growth was used for BIOLOG fingerprints and for a rapid plasmid screen using the sheared lysate method. Isolates not exhibiting degradative activity but still deemed to be of interest were streaked from a single colony forming on a plate grown from freezer stock to CPS plates. Plasmid screen gels were blotted on Hybond nylon and probed with dioxygenin labelled EcoRI-fragment 5. Total genomic extractions were performed on probe positive isolates and strains not tested by the rapid plasmid screen method. In this case the test colony was inoculated to 5 mL CPS and incubated with shaking prior to DNA extraction. Total genomic DNA was digested using BglII, electrophoresed, blotted and hybridized to a dioxygenin labelled EcoRI-11.

BIOLOG results were combined with those of other mesocosm species and a new similarity matrix was generated. All strains were clustered together using the single linkage option of the CLUSTER procedure of SYSTAT.

Plasmid extractions were performed on selected strains. Plasmids were cut with EcoRI and probed with other fragments of Tn5271 to confirm the presence or absence of this element.

RESULTS

A summary of all probe positive isolates and their associated phenotypes is given in Table 7-1. The result of the cluster analysis on these strains and key strains from previous experiments is shown in Figure 7-1.

1. Chlorobenzoate Degraders: Five mesocosms were dosed with 3Cba during the course of the 1989 experiments. Only BR60 was isolated on 3Cba plates from mesocosms C2 and TFDm. Chlorobenzoate degraders distinct from BR60 were isolated from three chlorobenzoate dosed mesocosms - C1, C3, and TFDI, and also from the chlorobiphenyl dosed mesocosm - CBPh. They were all of one or two distinct types, both slow growing. One strain was a motile yellow pigmented, gram negative rod bearing a single polar flagella. The other was a motile, non-pigmented gram negative rod with peritrichous flagella. When clustered with strains described in Chapter 5, they fell clearly into familiar groups. All the yellow pigmented strains are phenotypically indistinguishable from the PR117 group (Figure 7-1, Group 3). The non-pigmented strains are indistinguishable from PR120 (Figure 7-1, Group 1). No 3Cba degraders in the fluorescent group could be maintained. Duplicate BIOLOG fingerprints performed on three of these strains (B2-1, T3-3, and PR117) and fingerprints performed on BR60 using a new shipment of GN ID plates emphasize the high degree of intra-strain fingerprint variability.

Total DNA extracted from representative strains from Groups 1 and 3 was cut with BglII and hybridized to a dioxygenin DNA probe of EcoRI-11.

Table 7-1: List of Probe Positive Isolates from 1989 mesocosms.

Mesocosm	Strain	Probe	Phenotype	Cluster
C1	B2-1	+	3Cba+	1
	B2-2	+	3Cba+	3
C2	BR60	+	3Cba+	4
C3	T3-2	+	3Cba+	3
	T3-3	+	3Cba+	3
	T3-5	+	3Cba+	1
	T3-6	+	3Cba+	1
	T3-7	+	3Cba+	3
	T3-11	+	3Cba+	3
	T3-12	+	3Cba+	1
	T3-13	+	3Cba+	3
CBPh	B1-2	+	3Cba+	3
	B1-8	-	3Cba ^o	-
CBPm	T1-9W	+	3Cba+	3
	T1-9Y	-	CBP+	7
PCAh	T6-11	+	?	5
	T6-13	+	?	5
	T6-14	+	?	5
	T6-15	+	?	5
TFDI	B4-3	+	3Cba+	1
TFDh	B6-3	-	2,4D+	-
	B6-4	-	2,4D+	-
	B6-5	-	2,4D+	-
	B6-6	-	2,4D+	-
	B6-7	-	2,4D+	-
	B6-8	-	2,4D+/ 3Cba+	6
	B6-9	-	2,4D+	-
	B6-11	-	2,4D+	-

Figure 7-1: Cluster analysis of isolates from 1989 chlorobiphenyl, chloroaniline, 2,4-D and 3-chlorobenzoate dosed mesocosms combined with key type strains and mesocosm strains. Cluster analysis is based on Jaccard similarity coefficients. A code ending in "R" indicates this is a repeat determination of the BIOLOG reactions.

- 1 - Non pigmented 3Cba degraders
- 2 - Strains similar to BR60
- 3 - Yellow pigmented 3Cba degraders
- 4 - Fluorescent pseudomonads and similar strains
- 5 - *Pseudomonas delafieldii* like strains
- 6 - Distantly related strains
- 7 - large plasmid bearing coccoids, bright yellow

Codes

- * - pBRC60 recipient from mesocosm
- - pBRC60 recipient in laboratory
- ** - host of rearranged pBRC60 plasmid
- *** - carries IS1071
- # - host of large plasmid not homologous to pBRC60, isolated on chloroaromatic agar plates, see text.

A BglII cut of pBRC60 has five hybridizing fragments, BglII-1, BglII-2 and BglII-4 and to a lesser extent BglII-5, that show homology with the EcoRI E11. BglII-3 and BglII-4 contain all of Tn5271. Hybridization to BglII-3 gives the strongest signal because E11 and BglII-3 overlap completely, the other fragments to E11 because they all contain part of the repeated sequences of Tn5271. All of the chlorobenzoate degraders contain fragments BglII-2,3,4 of pBRC60 (Figure 7-2).

2. Organisms isolated from chlorobiphenyl dosed mesocosms: Chlorobiphenyl utilizers proved difficult to isolate and maintain. Initial growth on chlorobiphenyl agar plates was dominated by large, bright yellow colonies. Isolates from four of these colonies exhibited initial growth on CBP plates, and one (B1-8) formed large colonies on 3Cba without pH change, i.e. there was no yellowing of the agar indicative of hydrogen chloride release. The only culture capable of maintaining complete CBP degradation, i.e. growth accompanied by a pH change in agar, proved to be a mixture of two strains, T1-9y and T1-9w. Both types formed colonies on CBP agar, but T1-9y had better growth. The other three isolates similar to T1-9 produced dark metabolites on CBP agar. T1-9y is a non-motile, bright yellow gram negative cocci. There is no homology between Tn5271 and T1-9y total DNA, in spite of the fact that this strain and similar strains isolated from colonies on CBP agar carried high molecular weight plasmids. The BIOLOG fingerprint of this species clusters it with three other bright yellow strains isolated from mesocosms dosed in 1988 (Group 7). Both PR44a and PR13 were isolated from 3Cba dosed mesocosms on 3Cba agar as putative 3Cba degraders. In the laboratory their 3Cba metabolism was restricted to the production of chlorocatechol. PR113 was isolated on chlorophenol agar from

Figure 7-2: Hybridization of EcoRI-11 to Bgl-II digest of total genomic DNA and plasmid enrichments of chlorobenzoate degrading strains and isolates from chlorobiphenyl and chloroaniline dosed mesocosms. pBRC60 fragments Bgl-II-2, 3 and 4 are marked. Lanes as follows:

- 1) pBRC40
- 2) pBRC60
- 3) B2-1, 3Cba+, total genomic
- 4) T3-7, 3Cba+, total genomic
- 5) B6-5, 2,4-D+, total genomic
- 6) B6-7, 2,4-D+, total genomic
- 7) pBRC40
- 8) pBRC60
- 9) plasmid enrichment of T6-11
- 10) T6-11, from PCAh mesocosm, total genomic
- 11) T1-9w, 3Cba+, total genomic
- 12) pBRC60
- 13) pBRC40
- 14) same as lane 9.

1 2 3 4 5 6 7 8 9 10 11 12 13 14

a mesocosm dosed with chlorophenol and 3Cba. Rapid plasmid screens performed using the in well lysis method revealed the presence of large plasmids in PR13 and PR44a, but not in PR113. Large unidentified plasmids were found in a number of isolates, all of which involved bright yellow colony formers, but only PR13 and 44a were fingerprinted. None of the three strains showed homology to Tn5271. Plasmids were extracted from PR13 and PR44a and digested with EcoRI (Figure 7-3), both organisms harbour a plasmid with at least 8 bands. An extraction and restriction digest of pT1-9y shows that it carries a plasmid with only 2 EcoRI restriction sites yielding two large fragments.

T1-9w, from the chlorobiphenyl degrading mixed culture, and B1-2, isolated from the high dose chlorobiphenyl mesocosm, proved to be probe positive, capable of growth on 3Cba, and members of Group 3 cluster. T1-9w contains BglII fragments 2, 3 and 4 along with some bands not present in pBRC60 (Figure 7-2). However, a plasmid extraction confirms the presence of unaltered pBRC60 in T1-9w (Figure 7-4).

3. 2,4-D Degradars: A number of 2,4-D degraders were isolated but none showed homology to Tn5271. A BIOLOG fingerprint was performed on only one of these, B6-8. It resembled Group 3 strains in its colony characteristics, and exhibited the ability to degrade both 2,4-D and 3Cba. However no plasmids were evident in rapid plasmid screens of this species nor any of the other 2,4-D degraders analyzed. It also proved to be metabolically distinct from the Group 3 strains in spite of superficial similarities. It is also distinct from PR42a, another organism visibly indistinguishable from Group 3 strains.

Figure 7-3: EcoRI digest of plasmids extracted from PR13 and pPR44a of Group 7, strains isolated on chlorobenzoate agar but failing to utilize 3Cba on subculturing. Strains have metabolic fingerprint similar to that of the chlorobiphenyl oxidizer, T1-9y. Eight bands are discernable, corresponding roughly to 40, 28, 23, 18, 14, 12, 10 and 6.5 kb.

- A) pPR13**
- B) pBRC40 for reference**
- C) pPR44a**
- D) pPR63 for reference**

Size markers at (top to bottom) 23.1, 9.4, 6.6, 4.3, 2.3, and 2.0 kb.

4. Colonies from 4-Chloroaniline Dosed Mesocosms: Several isolates picked from 4-chloroaniline plates initially showed homology in rapid plasmid screens, but on later investigation appeared to lose this homology. Only those isolates originally taken from CPS plates from the probe positive bloom period maintained homology to pBRC60 probes. All four of these isolates, T6-11, T6-13, T6-14 and T6-15 proved to be of the same strain, a large, motile rod with single polar flagella, a coral colour to the cell pellet and a foul odour in liquid media. T6-11 and sister isolate T6-13 cluster in a small group (Group 5) with PR63, distinct from the bulk of the strains. PR63 is a putative 3Cba degrader isolated from 1988 mesocosms on the basis of 3Cba utilization that subsequently lost this phenotype. It carries a derivative of pBRC60 (see Chapter 5). It is similar to T6-11 in its shape, size, motility, cell pellet colour, ease of cell lysis, as well as in its BIOLOG fingerprint. Both strains are identified by the BIOLOG GNID system as *Pseudomonas delafieldii*. *P. delafieldii* T6-11 can form colonies on PCA/ye agar, but cannot grow in PCA/ye broth. This strain carries a large plasmid that is lost after prolonged exposure to PCA. When cut with BglII and probed with E11, plasmid enriched and total genomic preparations give banding patterns different from that of pBRC60, but a homologous fragment equal in size to BglII-4 seems to be present (Figure 7-2).

A plasmid enriched preparation of DNA of this strain was cut with EcoRI and probed with HindIII-2. HindIII-2 is a Tn5271 fragment that encompasses all the catabolic genes and most of IS1071. Strong homology to a band equal in size to EcoRI-16 and to another band approximately 4.5 kb in length is seen. The

presence of EcoRI-16 is indicative of the presence of IS1071, but none of the other Tn5271 bands are present, and the strong 4.5 kb band is probably indicative of IS1071 inserted in a plasmid non-homologous to pBRC60. 6 other bands from the chromosomal background reveal homology also (Figure 7-5). Hybridization of the same blot to EcoRI-13, a fragment of Tn5271 not overlapping IS1071, showed no high stringency homology to T6-11. Similarly, a plasmid enriched and total genomic preparation were digested with EcoRI and probed with HindIII-2 minus the IS1071 ends (made by cutting the HindIII-2/Puc18 clone with BglII and excising the 5.7 kb fragment from a low melt-agarose gel). This hybridization was carried out at slightly more relaxed conditions, at 66°C with 5X SSC in the hybridization fluid and still no homology was seen to this probe.

Figure 7-4: EcoRI digest of plasmids extracted from

- A) BR60
- B) T6-11, from chloroaniline mesocosm, 2 lanes
- C) T6-13, from chloroaniline mesocosm, 2 lanes
- F) T1-9y, chlorobiphenyl oxidizer
- G) T1-9w, 3Cba degrader

Size markers on Tn5271 fragments: at (top to bottom) 7.0, 2.9, 1.7, 1.6, 0.9 kb.

Figure 7-5: Hybridization of Tn5271 fragment HindIII-2 probe to Southern blot of EcoRI digestion of plasmids extracted from

- A) BR60
- B) T6-11, from chloroaniline mesocosm, 2 lanes
- C) T6-13, from chloroaniline mesocosm, 2 lanes
- F) T1-9y, chlorobiphenyl oxidizer
- G) T1-9w, 3Cba degrader

Note that hybridization to plasmids from BR60 and T1-9w results in hybridization to 6 bands, corresponding to EcoRI fragments 5 (7.0kb), 10, 11 (2.9kb), 13 (1.7 kb), 14 (1.6 kb and 16 (0.9kb), all of which overlap part of Tn5271. Probing of plasmids from T6-11 and T6-13 show intense hybridization to a fragment the same size as EcoRI fragment 16, and to a larger fragment approximately 4.5 kb in size.

DISCUSSION

The repeated isolation of 3Cba degraders that carry pBRC60::Tn5271 from mesocosms harbouring high levels of probe positive organisms supports the assumption that the probes used in the MPN:DNA hybridization technique employed in the mesocosm studies were specific for elements of the introduced genotype.

The clustering of the 3Cba degrading strains places them all into two distinct groups that include organisms isolated from the previous season. I assume that both Group 1 and Group 3 each represent only one distinct species or strain because of the high level of intra-strain variability seen with BIOLOG fingerprints. However Group 2, containing BR60, *Alcaligenes*, *Pseudomonas acidovorans*, *Moraxella*, and *Acinetobacter*, exhibits no more variability than does a single strain. This fact belies the power of the GN ID system to distinguish between phenotypically related species without incorporation of additional taxonomic information. That being said, observations on the morphology, pigmentation, growth and motility characteristics of the two dominant groups of mesocosm 3Cba degraders suggest that only two distinct species are involved.

The re-isolation of members from these two species underscores the viability of these two particular host-plasmid associations in Lake Opinicon waters. This makes them ideal candidates for further competition studies between them and BR60. Each form distinguishable colonies (group 1 are white with distinct margins, BR60 is off-white with irregular margins, and group 3 all produce a yellow pigment) so their individual responses to ecologically relevant parameters such as the availability of micronutrients, surfaces, the presence of protozoa, changes in pH and

temperature could all be examined.

The isolation of probe positive chlorobenzoate degraders only from the chlorobiphenyl dosed mesocosms is consistent with the population data from the dosing experiments and with the characteristics of known chlorobiphenyl degraders. Probe positive populations were generally low in chlorobiphenyl dosed mesocosms. Known chlorobiphenyl degraders produce chlorobenzoates as ultimate metabolites.

This confirms the utility of a chlorobenzote degrading genotype in a chlorobiphenyl contaminated system. Without chlorobenzoate degraders, persistent metabolites might be formed in chlorobiphenyl dosed systems. Bailey *et al.* (1983) have shown that monochlorobiphenyls are incompletely degraded in river water. Sylvestre *et al.* (1985) have shown a co-culture of a chlorobenzoate degrader and a chlorobiphenyl metabolizer transforms chlorobiphenyl more efficiently than does the chlorobiphenyl metabolizer alone, presumably because the chlorobenzote degrader reduces the build up of toxic metabolites in the culture.

It is curious that the two halves of the degradation pathway are only known to occur naturally in two separate organisms, particularly in two-membered cultures or where conjugative plasmids are involved. There are two theoretical reasons why an organism that could both cleave the unchlorinated ring and fully metabolize the chlorinated ring might not be selected for. First of all, if a given organism can derive x amount of energy and carbon from the metabolism of the unchlorinated ring of monochlorinated biphenyls, it could only derive a maximum of $2x$ that energy and carbon from the complete metabolism of both rings. This small growth

advantage might be outweighed if the chlorocatechol produced by chlorobenzoate oxygenation were to interfere with the efficiency of the meta-cleavage enzyme acting on the non-chlorinated ring. Or, if efficient use of both rings would require two types of cleavage enzymes, these might be generally incompatible.

In this study the chlorobiphenyl oxidizing strain, T1-9y, was metabolically and morphologically similar to other strains isolated during selection for chlorobenzoate and chlorophenol degradation, i.e. those in Group 7. Originally put aside with the assumption that their large plasmids were cryptic and not involved in chloroaromatic degradation, the isolation of a related strain capable of transforming chlorobiphenyl raises new questions about the members of Group 7. Large plasmids found in these strains might share common elements since they share related hosts. The recurring appearance of these strains under quite different isolation conditions suggests that their involvement with chlorobenzoate or chlorophenol degradation was real rather than coincidental.

The repeated isolation of T6-11 and sister strains suggests that it was at least partially responsible for the probe positive organisms enumerated during the population blooms. This organism harbours IS1071, the insertion sequence that brackets pBRC60's catabolic genes. Two explanations exist for its presence in this organism. The first is that it is a common insertion sequence in gram negative bacteria and its presence in *P. delfieldii* is independent of pBRC60. The second is that conjugation of pBRC60 or pBRC40 into this strain led to the formation of a cointegrate with the resident plasmid via the transposase gene on IS1071. This would generate direct repeats of IS1071 (or Tn5271) and could be followed by

homologous recombination to eliminate pBRC40, the catabolic sequences, or all of pBRC60. There is no proof that any portion of Tn5271 or PBRC60 other than IS1071 formed part of the genome of this organism, but we do know that deletion events are frequent for this transposon, that maintenance of Tn5271 was poor in a related organism (PR63), and that the isolation of this strain did not involve continuous selection for chloroaniline degradation. It was isolated on general media and exposed to 2 mM PCA prior to genetic analysis, conditions that might well have been counter-selective rather than selective.

The presence of copies of IS1071 independent of the catabolic genes suggests the hypothesis that IS1071 itself mediated adaptational changes in T6-11 that were selected for in the presence of chloroaniline. Insertion sequences can mobilize portions of DNA, or provide a mechanism for gene amplification. Insertion sequences can carry complete or partial promoter sequences that can switch on or enhance the transcription of downstream sequences (Scordilis *et al.* 1987), or produce major phenotypic changes such as affecting the host range of a pathogen by inactivating gene products (Kearney, 1988). The nature of T6-11's adaptational phenotype is not immediately evident. Although T6-11 can form colonies on chloroaniline/yeast extract (a property not shared by the majority of culturable bacteria from these mesocosms), chloroaniline degradation has not been established. More sophisticated culture techniques have to be employed. Conditions mimicking those in the chloroaniline dosed mesocosms, for example lake water media supplemented with 50 μ M PCA or less, might reveal an adaptive phenotype in this organism that is related to the presence of IS1071. As an alternate approach,

chloroaniline or similar dosing experiments could be repeated using separate probes specific to IS1071 and to Tn5271's catabolic region to test the hypothesis that it was really the former and not the latter that was selected for under these conditions.

Either way, the isolates characterized from the mesocosm studies confirm that pBRC60 played a role in the complete degradation of chlorobiphenyl *in situ*, and that elements of the catabolic transposon, Tn5271, were associated with community adaptation to 4-chloroaniline. In both cases, organisms other than *Alcaligenes* sp. BR60 mediated these roles, emphasizing the key importance of horizontal genetic transfer to adaptational changes.

CHAPTER 8: GENERAL DISCUSSION - ANSWERS TO QUESTIONS POSED AND NEW HORIZONS

In these final pages I summarize the main findings of this work with reference to the four questions posed in the introduction.

1) Can BR60 survive after release to a new environment?

In enumerating *Alcaligenes* sp. BR60 using selective plate counts I have enumerated only those cells that maintain the pBRC60 replicon. It is clear from my studies that BR60(pBRC60) is able to persist in Lake Opinicon flow-through mesocosms. While populations are highest when 3Cba is available, BR60 exhibits persistence in the absence of this chemical, being detectable at 200 cells/mL in the sediments after 60 days in the absence of selection. Even after populations drop to undetectable levels, the addition of 3Cba induces rapid growth of populations, indicating the presence of a small reservoir population. Additions of phosphorus and nonspecific organic matter do not improve the survival of BR60(pBRC60), indicating that growth of this host/plasmid association is limited primarily by the availability of the unique substrate for which it can effectively compete. In the absence of the substrate the population can be very small, and yet still of potential importance to the community. This confirms the current trend to assess the ability of specific genotypes to be maintained in a bacterial community over the long term using techniques with very low detection limits such as community DNA extraction and amplification, or amplification through selective culturing.

2) Will BR60 break down 3Cba in situ and if so does it require it for its survival?

The use of two techniques to enumerate BR60(pBRC60) led to the discovery that the frequency of the plasmid in a 3Cba dosed community differs from the frequency of the host BR60, and that it is the former and not the latter that determines 3Cba degradation rates in the mesocosms. Thus BR60 contributed to 3Cba degradation in the mesocosm systems in competition with other pBRC60 hosts - it was a dominant 3Cba degrader in some cases and a minor member of the degradative community in others. The ephemeral dominance of alternate pBRC60 hosts over BR60 cannot be explained by the growth characteristics of these alternate hosts under laboratory conditions. The discovery of two successful 3Cba degrading strains, carriers of pBRC60 and morphologically distinguishable from BR60, opens the door to fruitful experiments on ecological factors that determine the dominance of one strain over another. Competition experiments could be devised that measured strain performance at different temperatures and pH's, in the presence of competing bacteria, of protozoa, of alternate carbon sources or in biofilms, for example.

As for the key issue of whether or not 3Cba is required for the maintenance of the valuable 3Cba catabolic genes in the system, my conclusions are only tentative, since longer term experiments are necessary to answer that question. I have no evidence that the genes were ever eliminated from the mesocosm systems. The tendency for Tn5271 carrying cells to fall to low levels under non-selective

conditions and to recover rapidly in the presence of selection indicates that Tn5271 survives by virtue of the periodic benefits it allows the bacterial hosts. Presumably losses during non-selective periods are balanced by these periods of rapid growth and plasmid transfer - but the following question remains - how long a non-selective period can Tn5271 survive? Will low rates of transposition and plasmid conjugation maintain this element indefinitely in a bacterial community? Is it maintained in dormant bacteria where minimal growth rates minimize losses due to recombination and plasmid segregation?

It is interesting that Charlesworth (1987) in considering the ability of transposons to be maintained in eucaryotic genomes in the absence of selection, concludes rates of transposon replication are adequate to account for the survival of "selfish" transposons. Condit *et al.* (1988) conclude the opposite about procaryotic transposons. They maintain that known rates of transposition and plasmid transfer are too low to account for the maintenance of non-functional or "parasitic" transposons in bacterial populations. However the authors base their conclusions on a model derived for bacterial growth in chemostats, where differences in bacterial growth rates are critical in the face of a constant wash out rate. This model in no way characterizes the bulk of natural bacterial populations which tend to be found in biofilms on surfaces (Costerton *et al.* 1987), where there is a good possibility that plasmid stabilities are higher than in the free living population (de Taxis du Poet *et al.* 1986, 1987). In my work, the mesocosm sediments always harboured larger populations of BR60 under non-selective conditions than did the waters, clearly acting as genetic reservoirs. Successful pBRC60 hosts isolated from the mesocosms

exhibited poor growth and poor maintenance of the 3Cba+ phenotype under continuous culturing. Plasmid and transposon stabilities may well be substantially higher in the biofilms of aquatic sediments than in the continuously renewed and stirred waters of a chemostat. If so, I expect Tn5271 to persist at a low level in exposed Lake Opinicon sediments even in the absence of selection.

3) Will pBRC60 genes transfer to other bacteria in the community?

The isolation of three groups of bacteria carrying pBRC60 from waters and sediments that did not previously harbour bacteria homologous to Tn5271 is strong evidence that pBRC60 transferred from BR60 to these strains. Members of another group, tentatively identified as *Pseudomonas delafieldii*, were found with a pBRC40 derived plasmid and, from the chloroaniline-dosed mesocosms, another a nonhomologous large plasmid carrying IS1071. Both of these plasmids are assumed to be derived from pBRC60. Laboratory matings confirmed that pBRC60 is conjugative and that it transfers into members of the alpha and beta proteobacteria.

While allowing some ambiguity over the initial origin of pBRC60 in the non-BR60 hosts, the introduction of BR60 to the undefined biotic community of the mesocosms allowed it to interact with a full range of aquatic bacteria, including the numerous species not yet described. These studies demonstrated that the range of culturable pBRC60 hosts was restricted to three or four distinct types, the majority of which were metabolically similar to BR60. This may reflect a true tendency for pBRC60 to fare well in a few metabolic types because of efficiency of expression, or it may reflect the fact that only some of pBRC60 recipients were robust enough to survive subculturing on 3Cba or 3Cba/ye agar. The 3Cba dosing experiments

showed that probe+ populations could exceed the total number of bacteria enumerated on 3Cba agar media, and of the isolates representing those enumerated, not all survived multiple transfers required to obtain pure cultures. More recipients must have been active in the mesocosms. To identify a wider range of pBRC60 recipients, purification of isolates could be done on a weak general media such as lake water agar, followed by subculturing of isolates in filtered lake water supplemented with micronutrients and micromolar levels of 3Cba. A large number of isolates could be subcultured in microtitre plates, filtered and probed to determine the presence or absence of Tn5271.

Expression of the 3Cba genes can vary between recipients and within a given strain. Two of the natural recipients, PR120 and PR24B consistently accumulate chlorocatechol when cultured on 3mM 3Cba. Even BR60, when freshly re-isolated from low dose (μ M) 3Cba mesocosms, accumulates chlorocatechol on 3Cba agar, but loses this characteristic after further culturing on 3mM 3Cba. Efficient uptake and oxidation of 3Cba to chlorocatechol is probably beneficial when 3Cba is rarely encountered, but at high concentrations the rate of 3Cba uptake and oxidation probably exceeds the rate of chlorocatechol cleavage. While BR60 exhibits plasticity in its 3Cba use, other pBRC60 mesocosm recipients do not.

The tendency for known 3Cba degraders to exhibit poor 3Cba degradation or chlorocatechol accumulation after isolation from low dose 3Cba mesocosms makes us question the role of other strains isolated from 3Cba agar that on subculturing exhibited poor growth and chlorocatechol accumulation. Some of these strains were probably involved with 3Cba degradation, particularly the large plasmid-bearing

strains related to T1-9y, a chlorobiphenyl oxidizer. Their large plasmids might be TOL-like plasmids involved in chloroaromatic oxidation only (Reineke and Knackmuss 1988), or they might have carried genes for further degradation that only express well at low concentrations or were lost due to genetic instability. There is no reason to believe that only one 3Cba catabolic genotype could function in the mesocosms, since two or three different pBRC60 bearing strains were active at any one time in 3Cba dosed mesocosms - why not also a different genetic determinant in a different group of hosts?

The phenotypic variability between and within hosts of pBRC60 necessitates the use of the MPN-DNA hybridization procedure to enumerate the catabolic community. Dependent only on the ability of the bacteria to grow in general liquid media, the procedure is less time consuming and less expensive than in situ colony hybridization. The procedure suffers from variability, but this could be overcome with the use of more replicates per sample. It also suffers from dependence on the media, so a range of different media could be used to maximize the detection of probe positive organisms. Techniques not dependent on initial culturing of the sample such as the Taq Polymerase Chain Reaction (Steffan and Atlas 1988) are being developed for the detection of specific pieces of DNA in environmental samples. PCR could be applied to MPN samples to enumerate populations as well, but problems of enzyme inhibition and primer specificity arising from the highly heterogeneous nature of the samples need to be addressed. The cost of the enzyme and the primers for multiple samples such as those required for an MPN determination is also currently quite high.

4) Will the chlorobenzoate catabolic genes on Tn5271 be used by community bacteria in the degradation of other chlorinated aromatic compounds?

As for this final question, the answer is a qualified yes - depending on the chemical in question. pBRC60 clearly had no role to play in the breakdown of 2,4-D because the substrate was sequestered by an existing population of 2,4-D degraders. However pBRC60 hosts grew in mesocosms dosed with 3-chlorobiphenyl, allowing the complete degradation of a chemical that often is degraded only to chlorocatechol.

A proliferation of Tn5271 homologous genotypes was seen in response to 4-chloroaniline. The repeated isolation of bacteria carrying IS1071 on an unrelated plasmid is a clue to the involvement of Tn5271 in chloroaniline adaptation. It remains to be seen if IS1071 was involved with the mobilization of the 3Cba catabolic genes on Tn5271, or with the mobilization of a different genetic determinant, catabolic or otherwise, that encoded a phenotypic adaptation to the presence of chloroaniline.

A third possibility is that the proliferation of IS1071 was not associated with any phenotypic adaptation. Terzaghi and Ohara (1989) in a thought provoking article raise the possibility that "some classes of rearrangement are under the global control of a yet to be identified environmental signal". In theory there would be some adaptational value in releasing inhibition on elements controlling DNA rearrangements when physiological stress is encountered, but hints that environmental signals may play a role are few. Palmer *et al.* (1984) describe a recoverable loss of "extractability" of four plasmids in *Escherichia coli* after exposure

to sterile Chesapeake Bay water; O'Morchoe *et al.* (1988) report an unusually high number of plasmid rearrangements in recipients suspended in lake water. Did IS1071 increase in frequency in PCA dosed waters because of a stress related trigger that increased the frequency of transposition events and/or the rate of plasmid transfer? Could this precede adaptational changes? Exposure of mixed communities to chloroaniline is an experiment that bears repeating, contrasting the fate of Tn5271 with that of IS1071 alone using more specific gene probes.

The introduction of BR60 to a natural aquatic system has amply demonstrated the potential for introduced catabolic genetic determinants to play a role in environmental remediation. I have shown the inoculum to be viable at three different levels. BR60 remains viable in the bacterial community, the catabolic plasmid pBRC60 can be active in different bacterial hosts, and part or all of the catabolic transposon participated in bacterial adaptation to chlorobiphenyl and chloroaniline contamination.

APPENDIX I **BASIC PROGRAM USED TO GENERATE JACCARD SIMILARITY** **COEFFICIENTS FROM BIOLOG DATA.**

The following basic program receives input from data files arranged by strain id code followed on the next line(s) by the binary results of Biolog substrate tests. The program reads 96 results, test results can be entered in any format into the data source file provided they are separated by at least one space,

E.G. SAMPLE DATA FILE FORMAT

PR24B

0 1 1 1 1 1 0 1 0 1 1 0	0 1 0 1 1 1 1 0 0 0 1 1
0 0 1 0 0 1 1 1 0 0 1 0	1 1 1 1 1 1 1 1 1 1 1 1
1 0 1 1 1 1 1 1 1 1 1 1	1 1 1 1 1 1 1 1 1 1 0 1
1 1 1 1 1 1 1 1 1 1 1 1	1 1 1 0 0 1 1 0 1 0 0 0

AH60

0 0 0 0 1 1 0 0 0 0 0 0	0 0 0 0 0 0 0 0 0 0 0 0
0 0 0 0 0 0 0 0 0 0 1 1	1 0 0 0 0 0 0 0 0 0 1 0
0 0 1 1 0 1 0 1 1 0 1 1	1 1 0 0 1 1 0 0 0 1 0 0
1 0 0 0 0 1 1 1 0 0 1 1	0 0 0 0 0 0 0 0 1 0 0 0

ATCC306

0 0 0 0 1 1 0 0 0 0 0 0	0 0 0 0 0 1 0 0 0 0 0 1
0 0 0 0 0 0 0 0 0 0 1 0	1 1 1 1 0 0 1 0 0 1 1 0
0 0 1 1 0 1 1 1 1 1 1 1	1 1 1 0 1 1 0 1 1 1 0 0
0 0 0 0 0 1 0 0 0 0 0 0	0 0 0 0 0 0 0 0 0 0 0 0

To run the program you need to input the name of the ASCII file containing the data, (as above), and the number of strains in the file. After the data has been read, the program is instructed to "beep" to let you know it is proceeding. After the

similarity coefficients for one strain have been completed, the ID of that strain is printed on the printer so that you have a record of the order of strains, because strain IDs are not written to the output file. The output file is called "jaccall" but this can be altered by editing line 130. "Jaccall" will contain a complete matrix of similarity coefficients of $N \times N$ dimensions (where N = the number of strains) with 10 coefficients per line in the output file. The output file may require further editing (using a word processor that can output text-only files) if your clustering procedure will not accept a complete matrix, i.e. to use the cluster procedure of SYSTAT only one half of the matrix can be read. The complete matrix can be inputted to SPSS.

**PROGRAM TO GENERATE JACCARD SIMILARITY
COEFFICIENTS FOR BASIC COMPILERS**

```

10  INPUT "NAME OF BIOLOG FILE"; FILE$
20  INPUT "NUMBER OF STRAINS IN THIS FILE";N
30  DIM ID$(N), TEST(N,96), JACCSIM(N,N)
40  OPEN FILE$ FOR INPUT AS #1
50  FOR J=1 TO N
60      INPUT #1, ID$(J)
70          FOR I = 1 TO N
80              INPUT #1, TEST(J,I)
90          NEXT I
100 NEXT J
110 CLOSE
120 BEEP
130 OPEN "JACCALL" FOR OUTPUT AS #2
140 FOR J = 1 TO N
150     FOR K = 1 TO N
160         PP = 0
170         MP = 0
180         PM = 0
190         FOR I = 1 TO 96
200             IF TEST(J,I)=1 AND TEST(K,I)=1 THEN PP=PP+1
210             IF TEST(J,I)=0 AND TEST(K,I)=1 THEN MP=MP+1
220             IF TEST(J,I)=1 AND TEST(K,I)=0 THEN PM=PM+1
230         NEXT I
240         JACCSIM(J,K) = PP/(PP+MP+PM)
250         IF K = 10 THEN GOTO 340
260         IF K = 20 THEN GOTO 340
270         IF K = 30 THEN GOTO 340
280         IF K = 40 THEN GOTO 340
290         IF K = 50 THEN GOTO 340
300         IF K = 60 THEN GOTO 340
310         IF K = N THEN GOTO 340
320         PRINT #2, USING "#.### "; JACCSIM(J,K),
330         GOTO
340         PRINT #2, USING "#.### "; JACCSIM(J,K)
350     NEXT K
360 LPRINT ID$(J)
370 NEXT J
380 CLOSE
390 PRINT "PROGRAM IS FINISHED"
400 END

```

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