To the Graduate Council:

I am submitting herewith a dissertation written by Janet Packard entitled "Distribution and Molecular Relatedness of PSS50-homologous Plasmids in Bacterial Environmental Isolates." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the Doctor of Philosophy, with a major in Ecology.

Gary S. Sayler, Major Professor

We have read this dissertation
and recommend its acceptance:

Accepted for the Council:

Vice Provost
and Dean of The Graduate School

DISTRIBUTION AND MOLECULAR RELATEDNESS OF PSS50-HOMOLOGOUS PLASMIDS IN BACTERIAL ENVIRONMENTAL ISOLATES

A Dissertation

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Degree

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ABSTRACT

The 51 kilobase plasmid pSS50, a broad host range IncP1 plasmid from *Alcaligenes eutrophus* A5, a 4-chlorobiphenyl (4-CBP)-degrading organism isolated from lake sediment, has been used as a probe in colony hybridization of lakewater microcosms and soil lysimeters to monitor population levels of organisms with pSS50-homologous sequences. In this study, organisms with pSS50-homologous plasmids from the colony hybridizations were isolated, checked for the ability to degrade 4-CBP and screened for similarity to A5 or among themselves by chromosomal restriction enzyme analysis combined with pulsed field gel electrophoresis. Also, their pSS50-homologous plasmids were screened for variations by restriction enzyme analysis.

Results from characterization of isolates from Lake Loudon lakewater microcosms demonstrated that isolates with chromosomal restriction digest patterns identical to A5 and harboring the pSS50 plasmid were present in Lake Loudon lakewater several years after the isolation of A5 from Lake Loudon sediment. Results of this study also suggest conjugal transfer of pSS50 from A5 to a 4-CBP-degrading lakewater isolate tentatively identified as *Pseudomonas facilis* in a 4-CBP-enriched, A5-inoculated lakewater microcosm. No large unstable pSS50-homologous plasmids were detected from the lakewater microcosms or soil lysimeters, possibly because they had undergone deletions prior to plasmid analysis. However, a 61 kb pSS50-homologous plasmid, pSS60, which did not undergo deletions, was found in two chromosomal restriction digest groups from 4-CBP-enriched lakewater

microcosms. LBS1C1 is a unique isolate harboring pSS60 which degrades 4-CBP. Several isolates which harbor pSS60 but do not degrade 4-CBP and have identical chromosomal restriction digest patterns were also found. Results from characterization of soil lysimeter isolates demonstrate that isolates harboring pSS50 with chromosomal restriction digest patterns identical to that of A5 were present a year after inoculation of A5 into the soil lysimeters, indicating maintenance of the inoculated A5 in the lysimeters. It is possible that the pSS50 plasmids found in five different *Pseudomonas* strains which did not degrade 4-CBP in A5-inoculated soil lysimeters were transferred to them by A5, although there is no available evidence to determine the source of the pSS50 plasmid in these strains.

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I. INTRODUCTION

Polychlorinated biphenyls (PCBs) introduced into the environment are hazardous to organisms which are exposed to them either directly or through the food web; they accumulate in fatty tissues causing damage due to chronic PCB exposure. Hooper et al. (1990) have written a review on the fate and effects of PCBs in the environment; PCBs in the environment comprise many different chlorobiphenyl molecules containing primarily from one to six chlorine atoms on the rings of the biphenyl molecule (mono- to hexachlorobiphenyls).

Microorganisms exist in soil and sediment which participate in the biodegradation of these PCB congeners. Aerobic organisms which mineralize (convert to CO_2) lower-chlorinated PCBs have been reported (Shields et al. 1985, Barton and Crawford 1988, Parsons et al. 1988, Pettigrew et al. 1990). Many aerobic organisms have been isolated which degrade lower-chlorinated PCB congeners to their respective chlorobenzoates; recent reports of these organisms comprise those by Bedard and coworkers (1986), Furukawa and coworkers (1989) and Pettigrew and coworkers (1990). In addition, two other aerobic organisms (*Alcaligenes eutrophus* H850 and *Pseudomonas sp.* LB400) have been isolated which degrade higher-chlorinated PCBs to their respective chlorobenzoates (Bedard et al. 1987a). There are also aerobic organisms which mineralize chlorobenzoates (Chatterjee and Chakrabarty 1982 and 1984, Wyndham et al. 1988, Pettigrew et al. 1990). Anaerobic organisms involved in PCB degradation comprise organisms which dechlorinate higher-chlorinated PCB congeners to lower-chlorinated PCB congeners (Quensen et

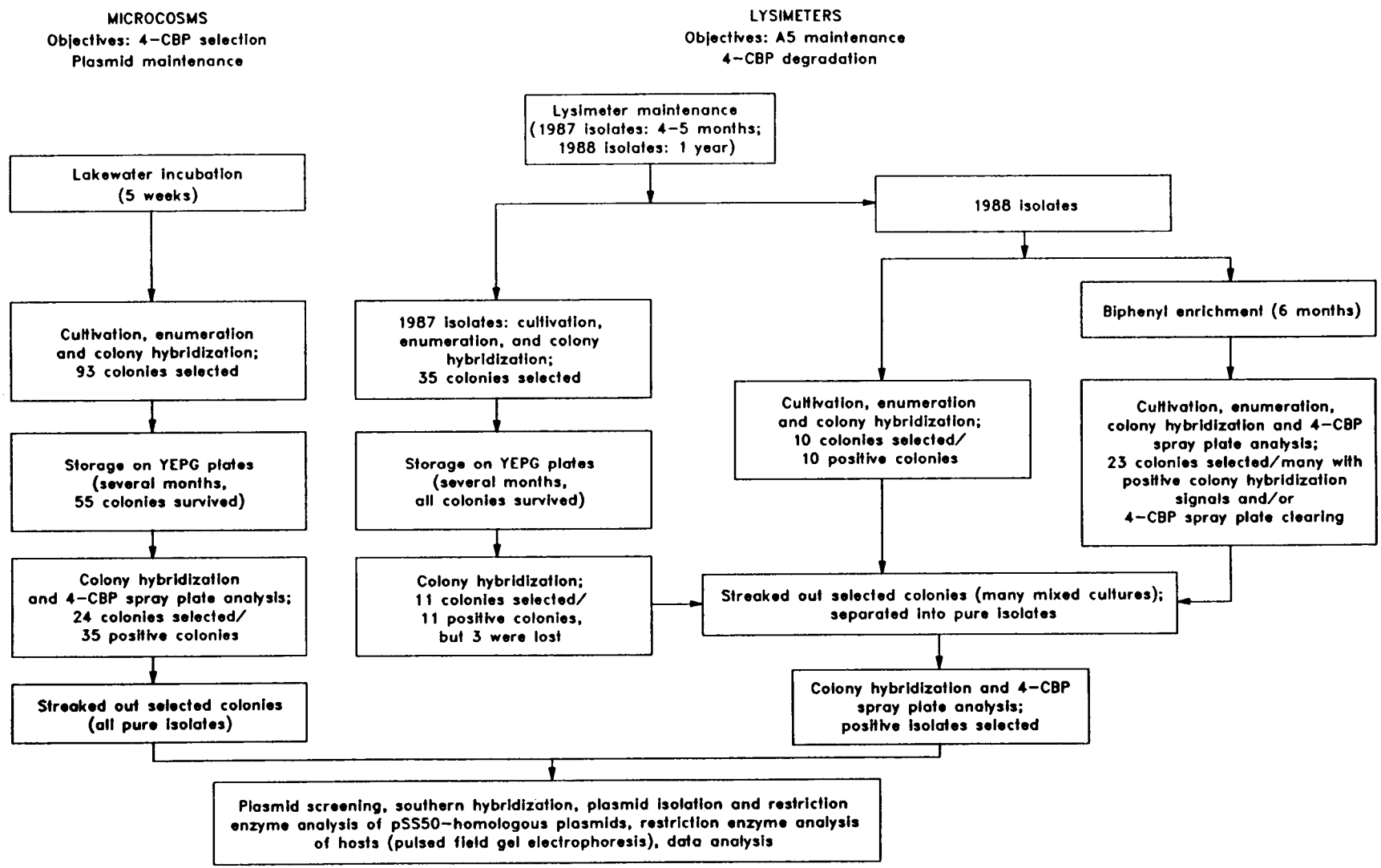
al. 1988) and organisms which mineralize chlorobenzoates (Genther et al. 1989a and b). Consortia of native microorganisms and genetically engineered microorganisms are being studied for possible use in bioremediation of PCB-contaminated sites (Hooper et al. 1990).

Future bioremediation practices will employ molecular technology to monitor native and/or introduced catabolic strains in contaminated sites in order to analyze the diversity of microbial populations involved in bioremediation, their molecular stability and gene transfer among organisms. Colony hybridization is a molecular technique which uses gene probes to detect organisms which have been cultured on agar plates which have specific plasmids, catabolic operons, taxonomic status or markers; these organisms can subsequently be isolated for investigation (Grunstein and Hogness 1975, Sayler et al. 1985). DNA restriction enzyme analysis (REA) uses restriction enzymes to cut DNA into fragments which are size-separated by electrophoresis. REA includes plasmid REA (Thompson et al. 1974) and chromosomal REA combined with pulsed field gel electrophoresis (Smith et al. 1988). These molecular techniques can be used to identify isolated plasmids and organisms, respectively, and to detect genetic rearrangements which have occurred in them. Southern hybridization (Southern 1975) of the restriction digest fragments can be employed to detect the existence and/or location of plasmids, specific operons or markers in the isolated organisms homologous to the probe.

Positive isolates with pSS50-homologous plasmids which were detected by colony hybridizations with a pSS50 plasmid probe of samples from lakewater

microcosms and soil lysimeters were isolated and identified by restriction enzyme analysis in this study; the pSS50 plasmid which was used as the probe in the colony hybridizations was from the 4-chlorobiphenyl (4-CBP)-degrading organism *Alcaligenes eutrophus* A5. Both the lakewater microcosm and lysimeter experiments contained treatments which had been inoculated with A5. The objectives of this study were to increase knowledge of the variability of pSS50-homologous plasmids in environmental isolates and of the variability of their hosts and to compare this variability to the relationship of these organisms to convert 4-CBP to 4-CBA. The steps involved in achieving these objectives are outlined in a processing flow diagram (Figure 1). The isolates obtained were further investigated by other investigators for plasmid characteristics and PCB catabolic operons. The final goal is to understand the biology and ecology of organisms with pSS50-homologous plasmids in the environment and their role in the biodegradation of PCBs.

Figure 1. Processing flow diagram.


MICROCOSMS
Objectives: 4-CBP selection
Plasmid maintenance
LYSIMETERS
Objectives: A5 maintenance
4-CBP degradation
Lysimeter maintenance
(1987 isolates: 4-5 months;
1988 isolates: 1 year)
Lakewater incubation
(5 weeks)
1988 isolates
Cultivation, enumeration
and colony hybridization;
93 colonies selected
1987 isolates: cultivation,
enumeration, and colony
hybridization;
35 colonies selected
Biphenyl enrichment (6 months)
Cultivation, enumeration
and colony hybridization;
10 colonies selected/
10 positive colonies
Cultivation, enumeration,
colony hybridization and 4-CBP
spray plate analysis;
23 colonies selected/many with
positive colony hybridization
signals and/or
4-CBP spray plate clearing
Storage on YEPG plates
(several months,
55 colonies survived)
Storage on YEPG plates
(several months,
all colonies survived)
Colony hybridization
and 4-CBP spray plate analysis;
24 colonies selected/
35 positive colonies
Colony hybridization;
11 colonies selected/
11 positive colonies,
but 3 were lost
Streaked out selected colonies (many mixed cultures);
separated into pure isolates
Streaked out selected colonies
(all pure isolates)
Colony hybridization and 4-CBP
spray plate analysis;
positive isolates selected
Plasmid screening, southern hybridization, plasmid isolation and restriction
enzyme analysis of pSS50-homologous plasmids, restriction enzyme analysis
of hosts (pulsed field gel electrophoresis), data analysis

II. LITERATURE REVIEW

PCB-degrading organisms

Bacterial biodegradation of PCBs occurs in both anaerobic and aerobic environments. Environmental data and laboratory results demonstrate reductive dechlorination of PCB congeners by microorganisms under anaerobic conditions (Quensen et al. 1988). The reductive dechlorination of PCB congeners results in lower-chlorinated congeners which are more readily degraded by aerobic bacteria (Quensen et al. 1988). Anaerobic microbial biodegradation of chlorobenzoates, end products of PCB biodegradation by many aerobic organisms, has also been demonstrated (Genther et al. 1989a and 1989b).

Aerobic bacterial mineralization of PCBs generally requires two pathways (Furukawa and Chakrabarty 1982). The first pathway converts PCBs to chlorobenzoates by oxidizing one of the rings of the biphenyl molecule (Ahmed and Focht 1973) and the second pathway mineralizes the remaining chlorobenzoates to CO_2. The organisms which have been reported to mineralize PCBs comprise the *Alcaligenes* and *Acinetobacter* spp. from Lake Loudon sediment containing the pSS50 plasmid (Sayler et al. 1984, Shields et al. 1985); LBS1C1, a Lake Loudon lakewater isolate which harbors a 61 kb pSS50-homologous plasmid (Pettigrew et al. 1990); a *Pseudomonas* sp. MB86 (Barton and Crawford 1988) and another *Pseudomonas* sp. (Parsons et al. 1988). The genes responsible for 4-CBP mineralization in these species have not been identified.

The majority of the PCB-degrading organisms which have been identified

produce chlorobenzoates as end products of PCB degradation. These PCB-degrading organisms have been found among many gram negative species world-wide and one gram positive species which has been positively identified, *Corynebacterium* sp. MB1 (Bedard et al. 1984). In addition, aerobic organisms exist which are capable of mineralizing chlorobenzoates (Chatterjee and Chakrabarty 1982 and 1984, Wyndham et al. 1988, Pettigrew et al. 1990). Bacterial cocultures containing a strain capable of transforming PCBs to chlorobenzoates and a strain capable of mineralizing chlorobenzoates to CO_2 are capable of mineralizing PCBs. This has been demonstrated for 4-chlorobiphenyl mineralization (Furukawa and Chakrabarty 1982, Sylvestre et al. 1985, Pettigrew et al. 1990) and 4,4′-dichlorobiphenyl mineralization (Adriaens et al. 1989).

Although the ability of PCB-degrading organisms to use biphenyl as a sole carbon source is variable among organisms (Barton and Crawford 1988, Pettigrew et al. 1990), it is generally accepted that biphenyl-oxidizing enzymes with a wide substrate range in organisms which can use biphenyl as a carbon source are responsible for metabolizing the various PCB congeners to chlorobenzoates. The biphenyl-oxidizing enzymes which oxidize biphenyl to benzoate and PCBs to chlorobenzoates make up the 2,3-dioxygenase pathway (Furukawa et al. 1979) which is encoded by four genes, *bphA*, *-B*, *-C*, and *-D*. The 2,3-dioxygenase pathway is named for the first enzyme in the pathway, biphenyl 2,3-dioxygenase, which is encoded by *bphA*. Biphenyl 2,3-dioxygenase inserts oxygen atoms at unsubstituted 2 and 3 (or 5 and 6) positions on the nonchlorinated or less-chlorinated ring to

produce a dihydrodiol. The second enzyme in the pathway, dihydrodiol dehydrogenase, which is encoded by *bphB*, dehydrogenates the dihydrodiol. The third enzyme in the pathway, which is encoded by *bphC*, is 2,3-dihydroxybiphenyl dioxygenase which cleaves the 2,3-dihydroxybiphenyl between carbons 1 and 2. This produces a *meta*-cleavage product which has a yellow color. The production of this enzyme and the concomitant yellow product is commonly used to indicate the presence of the 2,3-dioxygenase pathway. The fourth enzyme in this pathway, encoded by *bphD*, is the *meta*-cleavage product hydrase, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid hydrase, which hydrolyzes the *meta*-cleavage product to the corresponding benzoate or chlorobenzoate.

Biphenyl/PCB-degrading genes have been isolated from chromosomes of PCB-degrading organisms and have been cloned into other organisms for expression of the respective enzymes to confirm that they encode the PCB-degrading activity. *Bph* genes have been cloned from *Pseudomonas pseudoalcaligenes* KF707 (Furakawa and Miyazaki 1986), *Pseudomonas* sp. strain LB400 (Mondello 1989), *Pseudomonas putida* OU83 (Khan and Walia 1989) and *Pseudomonas testosteroni* strain B-356 (Ahmad et al. 1990). The gene order of the *bphABC* genes in KF707 is *bphA*, *bphB*, *bphC*. The gene order of the *bphABCD* genes in LB400 is *bphA*, *bphB* or *bphC*, *bphD*. It is likely that the *bphABCD* genes are organized into a common regulatory unit forming an operon since the *bph* genes are clustered and inducible (Furakawa et al. 1986).

Individual bacterial isolates exhibit broad differences in the number and type of PCB congeners that they oxidize (Furakawa 1982, Bedard et al. 1986). Furukawa

and coworkers established several correlations between PCB congener structure and biodegradability based on data obtained from two strains of bacteria that oxidize PCBs via the 2,3-dioxygenase pathway (Furukawa et al. 1978, Furukawa 1982). (i) Biodegradability decreases as chlorine substitution increases. (ii) Congeners with two *ortho* chlorines are extremely resistant to degradation. (iii) PCBs containing an unsubstituted ring are generally degraded more rapidly than isomers that are chlorinated on both rings. (iv) Tetra- and pentachlorobiphenyls containing a 2,3-chlorophenyl ring are more susceptible to biodegradation than other tetra- and pentachlorobiphenyls. (v) Ring fission generally occurs on the unchlorinated or less-chlorinated ring of the congeners. These correlations have been found to apply to the majority of the bacteria which have been isolated which degrade PCBs.

Two bacteria, *Alcaligenes eutrophus* H850 and *Pseudomonas* sp. strain LB400, have been discovered that degrade an unusually broad variety of PCBs including hexachlorobiphenyls (Bedard et al. 1987a). The congener selectivity patterns of H850 and LB400 differ substantially from those of other bacteria which follow the correlations established by Furukawa. The novel patterns of H850 and LB400 are consistent with the interpretation that H850 and LB400 use a 3,4-dioxygenase, which inserts oxygen atoms at carbons 3 and 4 as the first step in the pathway, in addition to the 2,3-dioxygenase pathway commonly found in PCB-degrading bacteria. It is not known whether 2,3- and 3,4-dioxygenase activities represent separate enzymes or a single enzyme with broad substrate specificity (Bedard et al. 1987b). The genes encoding the metabolism by the 2,3-dioxygenase pathway, *bphABCD*, and the 3,4-

dioxygenase activity were found to be encoded within a 12.4 kb region of LB400 (Mondello 1989).

PCB/biphenyl-degrading genes among many bacterial isolates have been investigated by DNA-DNA hybridization and, in one case, sequencing to determine the extent of homology among the genes in different organisms in order to determine molecular relatedness among them. The *bph* genes of *Alcaligenes eutrophus* H850 and *Pseudomonas* sp. LB400, the organisms which degrade higher-chlorinated PCBs, were found to be homologous by Southern hybridization, hybridization of the DNA restriction digests of the two organisms with the cloned PCB operon from LB400 (Yates and Mondello 1989). Because it is not probable for such similar genes to have been developed by two different genera, Yates and Mondello state that the genes must have been acquired through some form of DNA transfer, although the *bph* genes did not appear to be on plasmids, since no plasmids could be detected in these species. No homology was found between the PCB-degrading genes of LB400 and H850 and PCB-degrading genes of bacteria which do not degrade higher-chlorinated PCBs.

Furukawa and coworkers (1989) used the *bphABC* genes from *Pseudomonas pseudoalcaligenes* sp. KF707 as a probe in hybridization of restriction digests of many PCB-degrading soil bacteria. Five *Pseudomonas* strains of different species and one *Alcaligenes* strain had homologous *bph* genes which had similar if not identical restriction enzyme digestion patterns to the KF707 pattern. Although all of the strains they examined harbored one to three plasmids whose molecular sizes were up

to about 150 kb, the *bphABC* probe did not hybridize with any plasmid DNAs. Therefore, all of the PCB/biphenyl-degrading operons that they examined which had homology to their probe were on the chromosomes. Because of the existence of nearly identical chromosomal genes among different strains, the investigators suggest that the chromosomal segments containing the *bphABCD* genes may have a mechanism for transferring the genes from one strain to another.

Three strains investigated by Furukawa and coworkers had *bph* genes which were homologous to the *bph* genes from KF707 but had different restriction profiles. Five additional biphenyl-degrading strains showed weak or no significant homology to the *bph* genes from KF707. The sequences of the *bphC* genes from KF707 and *Pseudomonas paucimobilis* Q1, which had *bph* genes which did not hybridize with the KF707 *bph* genes, were compared. Although the *bphC* gene products, 2,3-dihydroxybiphenyl dioxygenases, from strains KF707 and Q1 were enzymatically very similiar, the overall sequence homology of the enzymes at the amino acid level was as low as 38% between the two enzymes. However, the enzymes possessed some homologous regions in which some amino acid sequences were significantly conserved. Furukawa and coworkers concluded that *bph* operons are widely distributed and that some of them are highly conserved. They surmised that since there are biphenyl-related compounds in plant lignin, which is composed of many aromatic skeletons, biphenyl/PCB-catabolic genes might originally have been involved in the degradation of certain components of decomposed lignin. If so, they observed that these genes could have evolved over a long period and that the

divergence of *bph* genes in biphenyl-utilizing strains might support this hypothesis.

Ahmad and coworkers (1990) probed blots of genomic DNA from various PCB-degrading organisms using the PCB/biphenyl-degrading genes from *Pseudomonas testosteroni* strain B-356 as the probe. Of the 12 strains probed, six showed distinct homology, one showed some homology and five showed no homology to the *Pseudomonas testosteroni bphABCD* genes. DNA from *Alcaligenes eutrophus* A5 and the pSS50 plasmid of A5 was among the DNA that they probed; the A5 genome had 19% homology to the *bph* genes of B-356, while the pSS50 plasmid had no homology to the B-356 *bph* genes. Because of the homology among the *bph* genes from several different strains, Ahmad and coworkers concluded that many PCB-degrading pathways have a common phylogenetic origin and that the differences may be due to adaptation of the pathway in the course of evolution. They hypothesize that the PCB degradation pathway originally evolved to degrade many naturally occurring biphenyl analogs which would explain its broad substrate specificity.

The role of the pSS50 plasmid in the environment

In a study of halogenated biphenyl biodegradation, a 4-chlorobiphenyl (4-CBP) enrichment culture of PCB-contaminated sediment from Lake Loudon, near Knoxville, Tennessee, was found to mineralize (completely degrade to CO_2) 4-CBP (Kong and Sayler 1983). 4-CBP is a monochlorinated PCB congener which contains a chlorine atom at the *para* position on one of the rings of the biphenyl molecule. Several *Alcaligenes* isolates and an *Acinetobacter* isolate were isolated from the 4-CBP enrichment culture. Mineralization assays demonstrated that the *Alcaligenes* and

Acinetobacter isolates mineralized 4-CBP (Sayler et al. 1984, Shields et al. 1985); they were the first bacteria to be isolated which mineralize PCBs.

Plasmids, extrachromosomal DNA molecules, in the *Alcaligenes* and *Acinetobacter* isolates were found by gel electrophoresis of isolate DNA. In one of the original gels, the plasmids were around 75 kilobases (kb) in most of the *Alcaligenes* isolates and the *Acinetobacter* isolate, and 56 kb in A5, one of the *Alcaligenes* isolates (Sayler et al. 1984). In another gel, both 75 and 56 kb plasmids were found in the lane of a culture of A5; in the same gel the plasmids in the other *Alcaligenes* isolates apparently had undergone deletions to produce a single plasmid which was about 56 kb. The 56 kb plasmid was named pSS50 (Shields et al. 1985). Later, A5 also harbored only the pSS50 plasmid (Sayler et al. 1984, Shields et al. 1985). The pSS50 plasmids in the isolates were determined to be identical by restriction digests (Shields et al. 1985). The exact size of the pSS50 plasmid was later determined by sizing of restriction fragments to be 51 kb (Burlage et al. in press). The mineralization of 4-CBP by these organisms was not affected by the deletions which created the pSS50 plasmid (Sayler et al. 1984, Shields et al. 1985). Later, during subculture, most of the *Alcaligenes* isolates and the *Acinetobacter* isolate containing the pSS50 plasmid lost the ability to mineralize 4-CBP, retaining the ability to convert 4-CBP to 4-chlorobenzoate (4-CBA, Sayler et al. 1984, Shields et al. 1985). A5 harboring the pSS50 plasmid retained the ability to mineralize 4-CBP (Sayler et al. 1984, Shields et al. 1985); A5 was identified as *Alcaligenes eutrophus* by mol % G+C (Hooper et al. 1989).

The genes for the breakdown of many toxic compounds occur on plasmids; environmental catabolic plasmids are reviewed by Sayler and coauthors (1990). Some of the well-known catabolic plasmids include the TOL plasmids (toluene catabolism, Keil et al. 1985), the NAH plasmids (napthalene catabolism, Yen and Serdar 1988), the OCT plasmids (n-alkane catabolism, Owen et al. 1987) and plasmids which contain the genes for the catabolism of chlorobenzoates (Chatterjee and Chakrabarty 1984, Wyndham et al. 1988). The catabolic genes in these plasmids are clustered into operons which are positively regulated.

The pSS50 plasmid was investigated by Sayler and coworkers by a curing experiment and a conjugation (plasmid transfer) experiment to determine if it demonstrated that it has the genes which encode 4-CBP mineralization. Results of the curing and conjugation experiments indicated that the pSS50 plasmid contains the genes necessary for 4-CBP mineralization (Sayler et al. 1984, Shields et al. 1985). Later, A5 harboring pSS50 lost the ability to mineralize 4-CBP while retaining the ability to convert 4-CBP to 4-CBA (Pettigrew and Sayler 1990). After mineralization ability of A5 was lost, it was assumed that the pSS50 plasmid was still responsible for the conversion of 4-CBP to 4-CBA.

Recently, however, in conjugation experiments between A5 harboring a marked pSS50 plasmid, pSS50:Tn4431, and recipient *Alcaligenes eutrophus*, *Pseudomonas putida* and *E. coli* strains, Springael and Mergeay (1990) have shown that the pSS50 plasmid in A5 is not responsible for conversion of biphenyl to benzoate. Although transfer of the plasmid into these strains was 100%, expression

of the biphenyl degrading genes was only encountered in the *Alcaligenes eutrophus* strains with a frequency of 10^{-5} to 10^{-6}. Springael and Mergeay do not extrapolate their data to the conversion of 4-CBP to 4-CBA, but it is generally accepted that the pathway is the same for the conversion of biphenyl to chlorobenzoate and the conversion of 4-CBP to 4-CBA. Their data corroborates genetic evidence that the pSS50 plasmid does not contain the genes which encode 4-CBP to 4-CBA. Burlage et al. (in press) have shown that the pSS50 plasmid is a member of the IncP1 incompatibility group (this group is called the IncP group in *Eschericia coli*) and that pSS50 is comprised almost entirely of the IncPβ-sub-group plasmid "backbone" encoding replication, maintenance, incompatibility, conjugation and mercury resistance determinants; the 4 kb region which is unaccounted for is not large enough to encode conversion of 4-CBP to 4-CBA. Burlage et al. (in press) propose that the original 75 kb 4-CBP catabolic plasmid may have lost the 4-CBP degradation pathways by homologous recombination between flanking repeats to create the pSS50 plasmid.

Bacterial plasmids that share either replication control or partitioning functions compete for stable inheritance, are termed incompatibile and are placed in the same incompatibility group. Members of the IncP1 incompatiblity group are broad host range plasmids; they are capable of conjugal transfer among and stable maintenance in almost all gram-negative bacterial species (Thomas and Smith 1987). IncP1 plasmids are also capable of mobilization and retromobilization of poorly self-transferrable or nontransferrable plasmids (Top et al. 1990). Retromobilization is

the capture of nonmobilizable plasmids from recipient strains and is specific to IncP1 plasmids. Because the host of the IncP1 plasmid acts as both donor and recipient of plasmids or genes, IncP1 plasmids are called "hermaphroditic" plasmids (Top et al. 1990).

The IncP plasmids have been divided into two sub-groups, α and β, based on the divergent genetic relatedness of the entire plasmids within the IncP group (Yakobson and Guiney 1983, Smith and Thomas 1987); IncPα sub-group members are all antibiotic resistance plasmids while IncPβ sub-group members are phenotypically distinct comprising the antibiotic resistance plasmid R751, the degradative plasmid pJP4 which encodes the degradation of 2,4-dichlorphenoxyactetate (2,4-D) and 3-chlorobenzoate (3-CBA), the degradative plasmid pBR60 which encodes degradation of 3-CBA, and the pSS50-homologous plasmids (Smith and Thomas 1987, Burlage et al. in press). The antibiotic resistance genes of the IncPα plasmids and the phenotypic genes of the IncPβ are found in respectively similar places in the IncP1α and IncP1β plasmids (Smith and Thomas 1987).

In a conjugation experiment between A5 harboring pSS50 and recipient *Alcaligenes eutrophus* strains which did not degrade biphenyl, Springael and Mergeay discovered transconjugant *Alcaligenes eutrophus* strains capable of degrading biphenyl. These transconjugant strains contained 100 kb plasmids called pSSD-plasmids, which did not undergo deletions, in which the biphenyl catabolism pathway from A5 had been inserted into the pSS50 plasmid. Restriction digest patterns of these plasmids

showed slight modifications indicating that the biphenyl degrading genes were inserted at different locations in pSS50. The pSSD-plasmids were transferred to other *Alcaligenes eutrophus* strains; they were shown to be responsible for biphenyl catabolism in the *Alcaligenes eutrophus* strains. However, *Pseudomonas putida* and *E. coli* transconjugant strains containing the pSSD-plasmids did not express biphenyl catabolism. Springael and Mergeay also found the creation of a plasmid which degraded biphenyl when they introduced an IncPα plasmid, the antibiotic resistance plasmid RP4, into *Alcaligenes eutrophus* var. *metallotolerans* AE868 containing the biphenyl catabolic pathway on the chromosome. The resulting RP4::Tn*bph* plasmid which was resonsible for biphenyl degradation was created at a frequency of 10^{-5}.

Springael and Mergeay propose that the biphenyl catabolism genes are found on transposons (Tn*bph*) of chromosomal origin in A5 and AE868. Transposons (Tn) are discrete regions of DNA which comprise genes which encode proteins which are flanked by insertion sequences. Insertion sequences are discrete segments of DNA capable of translocation within a genome independent of the host general recombination system. Transposons may be involved in nonhomologous rearrangements of DNA including insertions, deletions, inversions and other more complex structural changes (Kleckner 1981). Deletion and/or insertion derivatives of plasmids involved in the metabolism of toluene (Lehrbach et al. 1983), chlorobenzoates (Chatterjee and Chakrabarty 1982, Wyndham et al. 1988) and napthalene (Yen and Sendar 1988) have been described. At least in the case of the toluene catabolic plasmid pWWO, toluene catabolic genes have been found to be on

a transposon which is capable of recombination with antibiotic resistance plasmids and has also been found inserted into the *Pseudomonas putida* chromosome (Sinclair et al. 1986).

In a conjugation experiment in which the transconjugant *Alcaligenes eutrophus* strains containing the pSSD-plasmids were cured of the pSSD-plasmids by the introduction of RP4, which is incompatible to pSS50, Springael and Mergeay (1990) found that most of the strains lost their ability to degrade biphenyl. However, one cured strain retained the ability to degrade biphenyl. This suggests that the 100 kb pSSD-plasmid acted as a vehicle to transfer its biphenyl transposon into the chromosome of the transconjugant *Alcaligenes eutrophus* strain before it was cured from the strain. Springael and Mergeay (1990) suggest that mobilization of biphenyl degrading genes to soil bacteria by means of the broad host range plasmid pSS50 implies the role of broad host range plasmids in the acquisition of new degradative pathways by gathering and rearranging genes from various hosts and in the potential to spread DNA (for example, genetically engineered DNA sequences).

Colony hybridizations with the pSS50 plasmid probe

The pSS50 plasmid from A5 was used in colony hybridization of Lake Loudon sediment from the PCB-contaminated site from which the *Alcaligenes* and *Acinetobacter* strains had been isolated to determine if organisms could be detected which had pSS50-homologous sequences and the ability to convert 4-chlorobiphenyl to 4-chlorobenzoate. All of the colonies which were detected by colony hybridization with pSS50 had the ability to convert 4-CBP to 4-CBA (Pettigrew and Sayler 1986).

All of these colonies contained one or two isolates which converted 4-CBP to 4-CBA. Three isolates comprised the group of isolates with the ability to convert 4-CBP to 4-CBA; LPS3B was identified as *Alcaligenes* sp. and it contained the pSS50 plasmid (Pettigrew et al. 1990); the other two isolates with the ability to degrade 4-CBP were tentatively identified as a gram positive species and a *Flavobacterium* species (Pettigrew and Sayler 1986).

The pSS50 plasmid probe was also used in colony hybridization to monitor population dynamics of organisms with pSS50-homologous sequences in Lake Loudon lakewater microcosms which had been enriched with 4-CBP (+4-CBP) and/or inoculated with *Alcaligenes eutrophus* A5 containing the pSS50 plasmid (+A5, Breen et al. 1987, Steffan et al. 1989a). Results from the -A5, +4-CBP treatment showed that populations of native lakewater isolates containing pSS50-homologous sequences were increased above the colony hybridization detection limit by 4-CBP enrichment (Breen et al. 1987, Steffan et al. 1989a). Organisms containing pSS50-homologous sequences were not detected in the -A5, -4-CBP treatment. However, colony hybridization has a high detection limit when used on nonselective medium when population levels are high because of the dilutions which are made before plating; if target organisms are a small percentage of the total population, they may be lost by dilution (Steffan et al. 1989a, Steffan et al. 1989b). Therefore, organisms containing pSS50-homolgous sequences may have been present in the -A5, -4-CBP treatment, but not detected. A positive population in the +A5, -4-CBP treatment indicated that A5 was present in the treatment because there was no positive

population in the -A5, -4-CBP treatment. Also, because of the high positive population (around 10% of the total population on the YEPG plates) in the +A5, +4-CBP treatment, A5 appeared to have been maintained along with the native pSS50-homologous population at higher levels than in the +A5, -4-CBP treatment because of the 4-CBP enrichment (Breen et al. 1987, Steffan et al. 1989a).

The pSS50 plasmid was also used to monitor population levels of organisms with pSS50-homologous sequences in soil lysimeters at a PCB-contaminated site near Oak Ridge, Tennessee (Donaldson et al. 1988, Hill et al. 1989). Half of the lysimeters had been inoculated with A5; these lysimeters had been maintained with water, stirring, minerals and nutrients. Results from colony hybridization of the soil lysimeters indicated that A5 was maintained in the soil lysimeters six months after it had been inoculated into the lysimeters (Donaldson et al 1988, Hill et al. 1989) and also one year after the inoculation (Packard et al. 1990). In addition to positive colony hybridization signals from the A5-inoculated lysimeters, there were positive signals detected in two of the uninoculated lysimeters (Donaldson et al. 1988, Hill et al. 1989). Organisms with pSS50-homologous sequences were always less than 4% of the total population found on the YEPG plates (Donaldson et al. 1988, Hill et al. 1989, Packard et al. 1990).

III. MATERIALS AND METHODS

Sources of bacterial isolates

The lakewater microcosms contained 20 l of lakewater from Lake Loudon near Knoxville, TN which had been supplemented with 100 mg/l glucose plus other nutrients and stirred for aeration. A5 had been added at 10^5 cells/ml to the A5-inoculated treatments and 4-CBP had been added at 25 mg/l to the 4-CBP-enriched treatments (Breen et al. 1987, Steffan et al. 1989a). Colony hybridization was conducted on samples from the microcosms each week during the eight week incubation. Colonies were selected for investigation from the colony hybridizations conducted after five weeks incubation. Colonies including those which corresponded to positive colony hybridization signals were selected from the +A5, +4-CBP and -A5, +4-CBP treatments; no attempt was made to obtain all of the colonies which had positive signals. Colonies also were selected from the -A5, -4-CBP treatment which did not have any positive signals.

Six soil lysimeters were set up at a PCB-contaminated site near Oak Ridge, Tennessee in June 1987 (Donaldson et al. 1988, Hill et al. 1989). The lysimeters were halves of 55 gal steel drums which were placed into the ground and filled with soil contaminated with 5-10 mg/kg of PCBs from the site. Lysimeters 4, 5 and 6 had been inoculated with A5 at the start of the lysimeter experiment at a concentration of approximately 10^7 cells/gram of soil. Treatments to the lysimeters had been carried out biweekly except during the winter for one year: lysimeter 1 received water only; lysimeter 2 received water and stirring for aeration; lysimeters 3 through

6 received various combinations of minerals, yeast extract and biphenyl in addition to water and stirring (Donaldson et al. 1988, Hill et al. 1989). Colony hybridization was conducted on samples from two sampling dates in the fall of 1987, one sampling date in July 1988, and samples from a biphenyl enrichment most probable number (MPN) enumeration dilution series from the July 1988 sampling date.

Colonies including those which corresponded to positive signals from lysimeters 3 and 4 from the 1987 colony hybridizations (Donaldson et al. 1988, Hill et al. 1989) were obtained from Dr. Anthony V. Palumbo of Oak Ridge National Laboratory (Oak Ridge, Tennessee). Dilution series of samples (Donaldson et al. 1988, Hill et al. 1989) from all of the lysimeters from the July 1988 sampling date were obtained from Dr. Tommy J. Phelps of the Institute of Applied Microbiology (University of Tennessee, Knoxville) from which plates for colony hybridizations were made. The colony hybridization of samples from the July 1988 sampling date had ten signals varying in intensity from strong to very faint; all of the colonies corresponding to the positive signals were selected for investigation, regardless of their signal intensity. A biphenyl enrichment MPN enumeration dilution series from the July 1988 sampling date was also obtained from Dr. Phelps. The medium which had been used for the MPN enumeration series was the following in grams per liter: $NaNO_3$, 4.0; KH_2PO_4, 1.5; $MgSO_4$-$7H_2O$, 0.2; $CaCl_2$-$2H_2O$, 0.01; $FeCl_3$-$6H_2O$, 0.5; biphenyl, 0.1; yeast extract, 0.01; plus minerals and vitamins (Lynd et al. 1982). Colony hybridization of samples from these enrichment cultures was done in order to obtain a greater selection of isolates from the 1988 sampling date. Also, colony

hybridization with the pSS50 plasmid probe of biphenyl enrichment cultures is more sensitive than hybridization of the unenriched samples for isolates with pSS50-homologous plasmids which degrade biphenyl and 4-chlorobiphenyl because of selection for biphenyl-degrading organisms. Two microliter aliquots from each of the biphenyl enrichment MPN tubes were placed on replicate YEPG plates; the plates were incubated and the resulting colonies were hybridized with pSS50 or sprayed with 4-CBP. Positive colonies were selected for identification.

Bacterial isolate maintenance and purification

The selected lakewater microcosm colonies were named using the plate number followed by the coordinates of their location and streaked out on fresh YEPG medium (yeast extract, polypeptone, glucose medium; Sayler et al. 1979). Since the colonies had been on YEPG medium for several months, no attempt to change them to 4-CBP selective medium was made. Fifteen out of 20 isolates from the -A5, -4-CBP treatment, 26 out of 49 isolates from the -A5, +4CB treatment and 14 out of 24 isolates from the +A5, +4-CBP treatment were recovered. All of the colonies were pure cultures as determined by identical morphology on agar plates. They were transferred to gridded YEPG plates.

The colonies provided from the soil lysimeters from the 1987 sampling dates were on YEPG medium and were transferred to fresh YEPG medium. Colony hybridization was conducted on these colonies; the colonies which had positive signals were selected for further analysis. On successive hybridizations of these positive colonies, some did not produce positive signals. Later, it was discovered that most

of these colonies were mixed cultures which contained from two to four isolates. For several colonies, when the mixed cultures were transferred to fresh plates, the positive isolates were not transferred along with the colonies, resulting in colonies without positive signals. Some of the original colonies with pSS50-homologous plasmids were not recovered. Subsequently, colonies were streaked out on YEPG medium and mixed cultures were separated into pure cultures before they were transferred to gridded plates. This proved to be the key to isolating organisms with pSS50-homologous plasmids. Later, when variable results from colony hybridizations or 4-CBP spray plates from one colony occurred, it was always found that the colony was still a mixed culture. Once the colony was separated into pure cultures, results from the isolates were consistent.

The colonies selected from the colony hybridization of the July 1988 samples were transferred to YEPG plates and hybridized again. All of the colonies had positive signals. Most of the colonies were mixed cultures as demonstrated by different morphological characteristics on agar plates and were separated into what was thought to be pure cultures, although it was later discovered that some of these were still mixed cultures and they had to separated into pure cultures again; one colony could not be separated into pure cultures and was eliminated.

Colonies from the biphenyl enrichment cultures which cleared 4-CBP spray plates or had positive colony hybridization signals were selected for further investigation. Many of the colonies were contaminated by adjacent colonies when the colonies were selected, so that not all colonies could be recovered. Most of the

23 colonies obtained were mixed cultures; they were separated into pure cultures and transferred to replicate gridded YEPG plates. Colony hybridization and 4-CBP spray plating were conducted to obtain the isolates with positive colony hybridization signals and/or positive spray plate results. Only one isolate from each mixed culture had a positive colony hybridization signal and/or cleared 4-CBP spray plates.

Colonies and isolates were maintained on YEPG medium plates throughout the investigation (about one year). They were restreaked on fresh YEPG medium every several months. There was no evidence that any of the positive isolates were cured of their plasmids, had plasmid rearrangements or lost their ability to degrade 4-CBP in successive replicate tests over the year. However, there is no way to know what changes might have occurred in the plasmids or PCB/biphenyl operons before the first plasmid screening and spray plating. The isolates which were found to have pSS50-homologous plasmids were put into frozen stock.

Colony hybridization and 4-CBP spray plating

Colony hybridization was carried out by the protocol supplied with Biotrans nylon hybridization membranes (ICN Biomedicals, Cleveland OH). Colonies on gridded plates including control colonies were transferred to the nylon membranes by placing the membranes on the plates. The colonies on the membranes were labelled for reference to the colonies on the plates, lysed to expose their DNA, and the DNA was denatured to make it a single-stranded target for homologous probe DNA. The membranes were then baked to fix the denatured DNA in place on the membrane. The positive control was *Alcaligenes eutrophus* A5 harboring the pSS50

plasmid. Randomly selected negative controls comprised *Pseudomonas putida* G7 containing the NAH7 plasmid responsible for napthalene degradation (obtained from I. C. Gunsalus, The University of Illinois, Urbana); *Pseudomonas putida* mt-2 (ATCC 23973) containing the TOL plasmid responsible for degradation of toluene; LOS1, a *Pseudomonas fluorescens* strain isolated from Lake Loudon, near Knoxville, Tennessee; ABS10, a *Pseudomonas geomorphus* groundwater isolate; and ACS8, the *Acinetobacter* sp. strain from Lake Loudon sediment which had been cured of the pSS50 plasmid.

The pSS50 plasmid was labeled with ^{32}P-dCTP by nick translation with kits purchased from Bethesda Research Laboratories (Gaithersberg MD). The membranes were hybridized with the hybridization solution recommmended by the suppliers of the Biotrans membranes (5X Denhardt's solution, 0.5X SSC, 0.1% SDS and 10 mg/ml denatured salmon sperm DNA). The hybridization solution contained the denatured ^{32}P-labelled pSS50 plasmid probe at a concentration of 10^6 cpm/ml to allow binding of the probe to homologous target DNA. After hybridization at 65 ° F, the membranes were washed at high stringency in 10 mM NaCl, 20 mM Tris, 1 mM Na_2EDTA and 0.5% SDS at 65 ° F. The high stringency wash was necessary to wash weakly bound probe off of the membranes to eliminate detection of the negative controls. Less than a 12 h exposure of the membranes to x-ray film (X-mat AR, Kodak, Rochester NY) with one intensifier screen at -80 ° C was necessary to produce strong signals from the positive controls. The signals on the film were compared to the membranes to identify positive isolates.

The positive control, A5 harboring the pSS50 plasmid, had a positive signal on all films after autoradiography. None of the negative controls had signals, demonstrating that false positives of at least equal homology to the negative control with the most homology to pSS50 were eliminated in all colony hybridizations. The sensitivity of the detection of positive colonies from plates is 100%. Growth of the colonies on the medium amplifies the number of cells and therefore amplifies the number of copies of the target DNA in the colony for binding to the ^{32}P-labelled probe. Therefore, the number of ^{32}P molecules in the probe bound to each target colony is well above the number needed to exceed the detection limit for β emissions from the ^{32}P to produce a signal on the x-ray film.

The ability of the isolates to clear 4-CBP spray plates was used to test for 4-CBP degradation (Sylvestre 1980). 4-CBP spray plating was conducted by spraying colonies on agar plates with a 2% solution of 4-CBP in diethyl ether. Positive isolates produce a zone of clearing around themselves as they degrade 4-CBP. The 4-CBP spray plates were monitored for one week.

Plasmid identification

Plasmid DNA from lakewater, lysimeter and sediment isolates was isolated for plasmid screening by the method for isolating large plasmid DNA developed by Anderson and McKay (1983). Plasmid DNA for identification of the plasmids by restriction enzyme analysis (REA, Thompson et al. 1974) was isolated by a procedure developed by Promega (Madison WI) and purified by cesium chloride density gradient separation (Maniatis et al. 1982). Both the Anderson and McKay and

Promega procedures were successfully carried out on all isolates. Restriction digests of plasmid DNA with *Eco*RI, *Hin*dIII and *Sal*I (Promega, Madison WI) were conducted according to the supplier's directions.

Separation of plasmid DNA from chromosomal DNA for plasmid screening and separation of plasmid restriction digest fragments were conducted by gel electrophoresis. Lanes of 0.75% agarose gels were loaded with isolate DNA and electrophoresed on a vertical gel apparatus. After electrophoresis, the gels were stained with ethidium bromide, which intercolates into the DNA and fluoresces under ultraviolet light. The isolate plasmid restriction digests were compared to plasmid restriction digests of pSS50 from A5 and among themselves.

Southern hybridization (Southern 1975) is a technique for hybridizing DNA separated by electrophoresis on gels to locate molecules or fragments with homology to the probe. Southern hybridization was conducted with the pSS50 plasmid as a probe on plasmid screening gels to confirm that the pSS50 homology was on the pSS50-sized plasmids. Southern hybridization was also conducted on gels of restriction digests of the pSS50-homologous plasmids when other plasmids were present and it was necessary to discriminate which fragments were homologous to pSS50. DNA from *Pseudomonas putida* G7, harboring the NAH7 plasmid, was used as a negative control on the gels. The DNA from the gels was transferred to Biotrans membranes by vacuum blotting with an LKB Vacu Gene Vacuum Blotting System (LKB Pharmacia, Bromma, Sweden). The membranes were hybridized and autoradiographed as described for colony hybridization.

Isolate identification

Chromosomal restriction enzyme analysis (REA) was used to differentiate among the isolates. The chromosomal restriction digest patterns were produced by pulsed field gel electrophoresis (PFGE) of DNA restriction fragments; PFGE is designed to separate large DNA molecules or fragments. The restriction fragments were produced with a low frequency restriction enzyme which produces relatively few large DNA fragments. This method has been found to be an improvement in resolution over traditional restriction digest and gel electrophoresis which creates many small fragments which can be resolved only up to 50 kb. PFGE combined with the use of low frequency restriction enzymes allows clear distinction between strains found in different epidemiological situations (Allerdet-Servent et al. 1989) and has been applied to investigation and mapping of genomes of numerous species.

Pulsed field gel electrophoresis and preparation of chromosomal DNA in agarose gel inserts were developed by D. Schwartz and C. Cantor for separation and sizing of yeast chromosomes (Schwartz et al. 1982, Schwartz and Cantor 1984). Detailed protocols for preparing DNA from diverse organisms and restricting DNA in gel inserts have been developed by C. Smith (Smith et al. 1988). The bacterial cells are embedded in agarose for PFGE; the DNA preparation and restriction digestion are accomplished by diffusion of reagents into the agarose inserts to eliminate the pipetting of DNA which shears large DNA fragments which is required in traditional DNA preparation.

During electrophoresis, DNA fragments move in gels towards the positive pole

because they are negatively charged; smaller fragments move more quickly through the gel matrix than larger fragments resulting in separation of the fragments with an upper limit of about 50 kb for traditional 0.75% agarose gels. The electric pulses of a pulsed field gel electrophoresis system alternate from a north-south direction to an east-west direction. Although small fragments move rapidly through the gel, larger fragments get caught as they turn the corners created by the alternation of the direction of the electric field for lengths of time proportional to their length. The larger fragments, therefore, take longer to migrate down the gel and they can be separated by PFGE, increasing restriction fragment size resolution by more than an order of magnitude over fragment size resolution by traditional gel electrophoresis. The pulse time is generally varied to produce the desired range of fragments to be separated (Birren et al. 1988, Lai et al. 1989).

Bacterial DNA of the lakewater microcosm and lysimeter isolates was prepared and restricted with the low frequency restriction enzyme *Xba*I as described by the suppliers of the InCert agarose (FMC Bioproducts, Rockland ME). *Xba*I was chosen as the low frequency restriction enzyme because it has a restriction enzyme recognition sequence (TCTAGA) which results in the production of relatively few fragments in organisms with 50-70% mol % G+C such as *Alcaligenes* and *Pseudomonas* species (McClelland et al. 1987). The DNA preparation and restriction digests were successful for all isolates.

The gel inserts containing the bacterial DNA digested by *Xba*I were loaded into a 1% SeaKem GTG agarose gel (FMC Bioproducts, Rockland ME). The gel

was electrophoresed by a Pulsaphor unit (LKB Pharmacia Biotechnology, Bromma, Sweden) using a point electrode array; the point electrode array creates a double inhomogeneous field which creates curved lanes on the gels. Standard run conditions for optimum separation of bacterial low frequency enzyme restriction fragments (330V, 25 second pulse time and a 40 h run time) were used. These conditions produce separation of fragments from around 50 to around 400 kb. Since pSS50 plasmid fragments are less than 50 kb they were not separated by PFGE; therefore, chromosomal fragments created the digestion pattern. Lambda DNA (c18757) oligomers starting at the monomer length of 48.5 kb and *Saccharomyces cereviseae* (YPH80) chromosomes were used as high molecular weight standards (Pharmacia LKB Biotechnology, Piscataway NJ). The gels were stained with ethidium bromide for visualization using an ultraviolet light transilluminator. The patterns produced were compared to the A5 pattern and among themselves.

Whole cell fatty acid profile analysis of A5 and selected isolates from the soil lysimeters with the pSS50 plasmid which cleared 4-CBP spray plates was conducted by Dr. Tommy Phelps of the Institute of Applied Microbiology (University of Tennessee, Knoxville) to determine if the selected isolates were identical to the inoculated A5. Whole cell fatty acid analysis of selected lakewater microcosm isolates was conducted by Micro Check Bacterial Analysis Lab (Northfield VT). The identification by fatty acid profiles of the lakewater microcosm isolates was conducted to provide preliminary taxonomic identification for the isolates. Identification by fatty acid profiles is based on the facts that a large number of fatty acids are

produced by bacteria and that there are unique fatty acid patterns for given taxa.

The selected isolates for fatty acid analysis were streaked out on YEPG medium and were submitted to the laboratories where they were subcultured on trypticase soy broth agar (TSBA) and incubated aerobically at 28°C. After 48 h incubation, the cells were harvested for extraction of whole cell fatty acids. The fatty acid extracts were injected into a chromatograph with a flame ionization detector by an autosampler. An integrator processed the chromatographic data and sent it to a computer which named the fatty acids and compared the fatty acid profile of the unknown to the profile of the 7000 strains in the data base; the TSBA+ data base was used. Each unknown was assigned a match or matches along with similarity indices for each match. The similarity index is a numerical value which expresses how closely the fatty acid composition of the unknown corresponds to the fatty acid composition of the match. A similarity index of 0.5 to 0.99 is an excellent match, as well as a single match identification of 0.3 or higher. For similarity indices between 0.1 and 0.3, the species of the unknown may not have been in the TSBA+ version of the data base. For similarity indices below 0.1, the genus of the unknown is questionable.

IV. RESULTS AND DISCUSSION

Lakewater microcosm isolates

Thirty-five out of the 55 lakewater microcosm isolates examined had either positive colony hybridization signals or cleared 4-CBP spray plates. From among the 26 isolates from all three treatments which had positive colony hybridization signals, five cleared 4-CBP spray plates; they were distributed among the three treatments (Table 1). The other 21 isolates which had positive colony hybridization signals did not clear 4-CBP spray plates. Results from plasmid screening of selected lakewater microcosm isolates which had positive colony hybridization signals showed that all 20 of the isolates which had positive colony hybridization signals had single plasmids which appeared similar in size to pSS50 (Table 2); Southern hybridization showed that the homology found in these isolates was on the plasmids rather than the chromosomes. Six selected isolates which had negative colony hybridization signals, but cleared 4-CBP spray plates, did not have plasmids (Table 2).

Plasmids from 12 out of the 20 lakewater microcosm isolates containing pSS50-homologous plasmids were compared to the pSS50 plasmid from A5 using restriction digest patterns. The restriction digest patterns showed that the pSS50-homologous plasmids in the lakewater microcosm isolates were either pSS50 or pSS60 (Figure 2). Four of the plasmids were pSS50 and eight were pSS60 (Table 3). PSS60 is a 61 kb plasmid comprised of the pSS50 plasmid and an additional 10 kb fragment (Pettigrew et al. 1990, Burlage et al. in press, Figure 3). The additional 10 kb fragment is found within two 2.2 kb inverted repeats (defined by restriction

Table 1. Colony hybridization and 4-CBP spray plate results from the colonies provided from the lakewater microcosms.

Name	Treatment	Colony hybridization signal	4-CBP spray plate result
3A1	-A5, -4-CBP	+	+
3A4	"	-	-
3A5	"	-	-
3B1	"	-	-
3B2	"	-	-
3B3	"	-	-
3B4	"	-	-
3B5	"	-	-
3C3	"	-	-
3C4	"	-	-
3C5	"	-	-
3D1	"	-	-
3D3	"	-	-
3D4	"	-	-
3D5	"	-	-
1A3	-A5, +4-CBP	-	+
1A4	"	+	-
1A5	"	-	-
1A6	"	+	-
1B2	"	-	-
1B3	"	-	-
1B4	"	-	-
1B5	"	-	-

Table 1 (continued)

Name	Treatment	Colony hybridization signal	4-CBP spray plate result
1C1	-A5, +4-CBP	+	+
1C3	"	-	+
1D4	"	-	-
1D5	"	-	-
2A1	"	+	+
2A2	"	+	-
2A4	"	+	-
2A5	"	+	-
2A6	"	+	-
2B2	"	+	-
2B4	"	-	-
2B6	"	-	-
2C2	"	+	-
2C3	"	+	-
2C4	"	-	+
2C5	"	+	-
2C6	"	+	-
2D2	"	+	+
4A1	+A5, +4-CBP	+	+
4A2	"	-	-
4A3	"	-	-
4A4	"	+	+
4A5	"	-	+
4A6	"	+	-

Table 1 (continued)

Name	Treatment	Colony hybridization signal	4-CBP spray plate result
4A3	+A5, +4-CBP	-	+
4A4	"	+	+
4A5	"	-	+
4A6	"	+	-
4B1	"	+	-
4B2	"	+	-
4B3	"	+	-
4B4	"	+	-
4B5	"	+	-
4B6	"	+	-
4C1	"	+	-
4C2	"	-	+
4C3	"	+	-

Table 2. The presence of pSS50-homologous plasmids in lakewater microcosm isolates which were examined which have positive colony hybridization signals and/or clear 4-CBP spray plates.

Name	Treatment	Colony hybridization signal	4-CBP spray plate result	PSS50-homologous plasmid
3A1	-A5, -4-CBP	+	+	+
1A3	-A5, +4-CBP		+	-
1C1	"	+	+	+
1C3	"	-	+	-
2A1	"	+	+	+
2A4	"	+	-	+
2A6	"	+	-	+
2C2	"	+	-	+
2C3	"	+	-	+
2C4	"	-	+	-
2C5	"	+	-	+
2C6	"	+	-	+
2D2	"	+	-	+
4A1	+A5, +4-CBP	+	+	+
4A3	"	-	+	-
4A4	"	+	+	+
4A5	"	-	+	-
4A6	"	+	-	+
4B1	"	+	-	+
4B2	"	+	-	+
4B3	"	+	-	+
4B4	"	+	-	+

Table 2 continued

Name	Treatment	Colony hybridization signal	4-CBP spray plate result	PSS50-homologous plasmid
4B5	+A5, +4-CBP	+	-	+
4B6	"	+	-	+
4C1	"	+	-	+
4C2	"	-	+	-

Figure 2. Restriction digest patterns of the pSS50 and pSS60 plasmids.

A. *Eco*RI plasmid restriction digestions. Lane 1 is the pSS50 plasmid from A5. Lanes 2 through 8 are plasmids with the pSS60 pattern from seven 2A isolates (2A4, 2A6, 2C6, 4A6, 4B1, 4B2 and 4B4). It appears that there is a mutation in one of the *Eco*RI fragments in the pSS60 plasmid in Lane 6 which created a new *Eco*RI recognition sequence and therefore the fragment is divided into two fragments.

B. *Hin*dIII plasmid restriction digestions. Lane 1 is the pSS50 plasmid from A5. Lanes 3 through 8 are plasmids with the pSS60 pattern from seven 2A isolates (2A4, 2A6, 2C6, 4A6, 4B1, 4B2 and 4B4). Lane 9 is the plasmid with the pSS50 pattern from a lysimeter biphenyl enrichment isolate (MPN 20). Lanes 10 and 11 are pSS50 plasmids plus extra fragments from extra small plasmids; these plasmids are from two biphenyl enrichment isolates (MPN21 and MPN23).

A.



B.

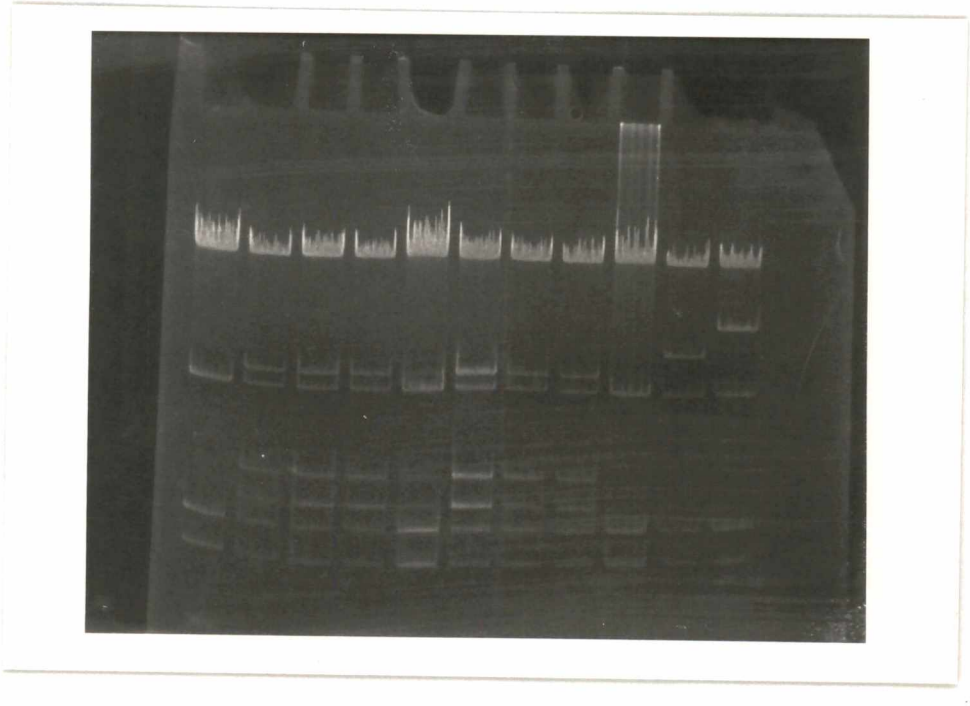
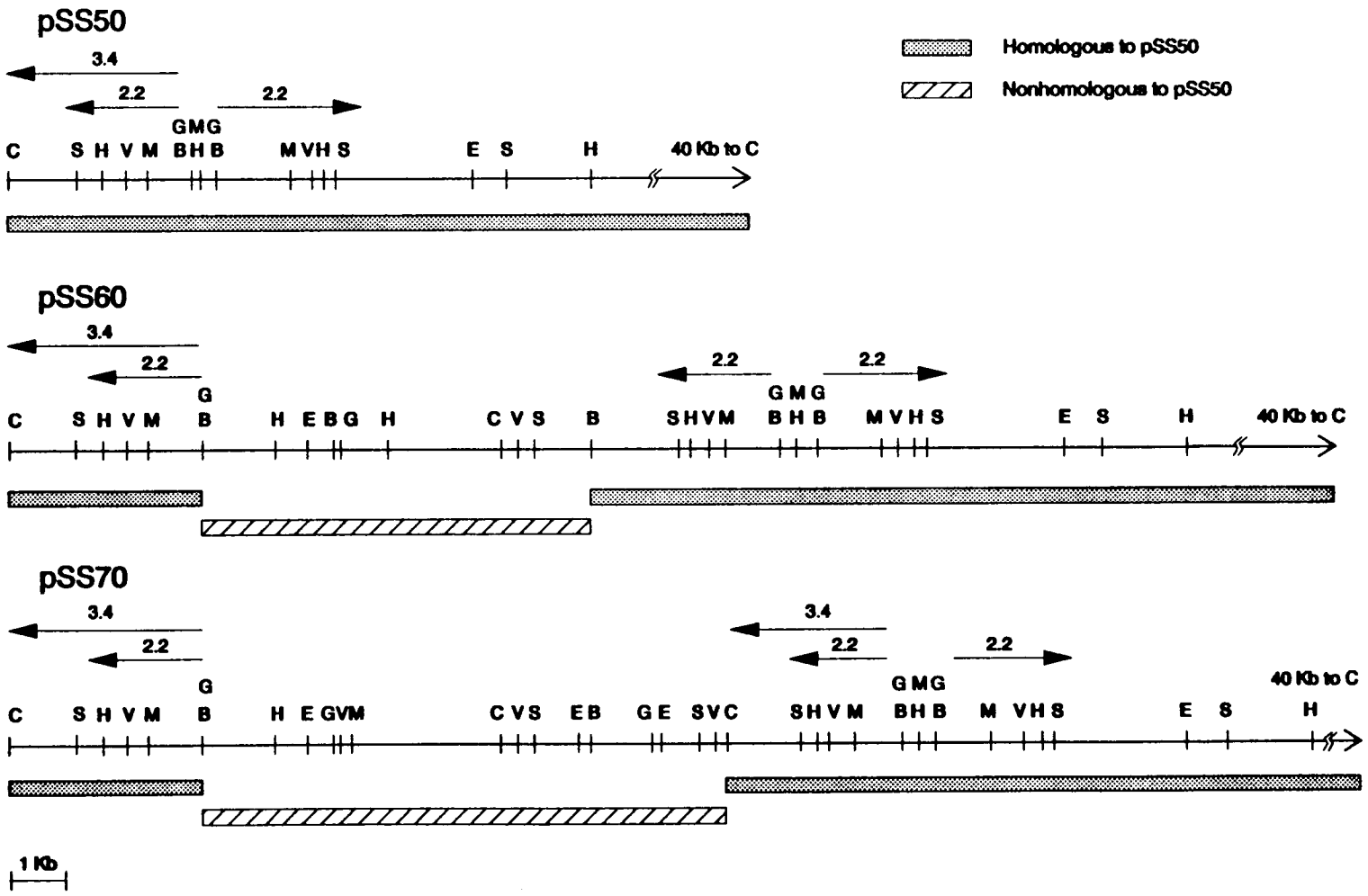


Figure 2

Table 3. Identification of lakewater microcosm isolates harboring pSS50-homologous plasmids by chromosomal restriction enzyme analysis (REA) and pulsed field gel electrophoresis and identification of their plasmids by plasmid REA.

Name	Treatment	4-CBP spray plate result	Plasmid	Chromosomal identification
3A1	-A5, -4-CBP	+	pSS50	A5
1C1	-A5, +4-CBP	+	pSS60	1C1
2A1	"	+	pSS50	A5
2A4	"	-	pSS60	2A
2A6	"	-	pSS60	2A
2C3	"	-	not done	2A
2C6	"	-	pSS60	2A
2D2	"	-	not done	2A
4A1	+A5, +4-CBP	+	pSS50	A5
4A4	"	+	pSS50	6A
4A6	"	-	pSS60	2A
4B1	"	-	pSS60	2A
4B2	"	-	pSS60	2A
4B3	"	-	not done	2A
4B4	"	-	pSS60	2A
4B5	"	-	not done	2A
4B6	"	-	not done	2A
4C1	"	-	not done	2A

Figure 3. Restriction digest maps of the inverted repeat area of the pSS50 plasmid and the insertions between the inverted repeats in the pSS60 and pSS70 plasmids. E, *Eco*RI; S, *Sal*I; H, *Hin*dIII; M, *Sma*I; C, *Cla*I; G, *Bgl*II; B, *Bam*HI; V, *Pvu*II. This figure was provided by Dr. A. Layton.


pSS50
3.4
2.2
2.2
GMG
C S H V M BHB M VH S E S H 40 Kb to C
Homologous to pSS50
Nonhomologous to pSS50
pSS60
3.4
2.2
2.2
2.2
G
GMG
C S H V M B H E B G H C V S B S H V M B H B M V H S E S H 40 Kb to C
pSS70
3.4
2.2
3.4
2.2
2.2
G
GMG
40 Kb to C
C S H V M B H E GVM C V S E B G E S V C S H V M BHB M VHS E S H
1 Kb

enzyme patterns) in the pSS50 plasmid. About 7 kb of the extra 10 kb fragment is unique; the 10 kb fragment found in pSS60 also contains a copy of the 2.2 kb repeat, which creates adjacent inverted 2.2 kb repeats on one end of the unique fragment and direct 2.2 kb repeats flanking the unique segment (Burlage et al. in press, Figure 3).

Four different chromosomal restriction digest patterns were found among the lakewater microcom isolates: A5, 1C1, 6A and 2A (Table 3, Figure 4). The two isolates analyzed from the 2A group had nearly identical fatty acid analysis matches. However, the similarity indices were so low that the isolates from the 2A group could not be assigned to a genus (Table 4). The three isolates from the 6A group, one containing the pSS50 plasmid (4A4), all had *Pseudomonas facilis* as the only match with similarity indices which were greater than 0.7 (Table 4). A5 was identified as *Pseudomonas pickettii* with a similarity index of 0.574; the identification of A5 as *Pseudomonas pickettii* by fatty acid profile analysis contrasts the identification of A5 as *Alcaligenes eutrophus* (Hooper et al. 1989). However, since physical and biochemical data demonstrated that A5 was an *Alcaligenes* species (Shields et al. 1985) and the similarity index of the fatty acid identification was only 0.574, the fatty acid identification was determined to erroneous. LBS1C1 had four matches with very low similarity indices: *Chromobacterium violaceum* (0.016), *Azotobacter chroococcum* (0.011), *Vibrio anguillarum* (0.010) and *Vibrio fluvialis* (0.007).

Three isolates with the A5 chromosomal restriction digest pattern (Figure 4) were found among the lakewater microcosm colonies which were examined (Table

Figure 4. Chromosomal restriction digest patterns created by pulsed field gel electrophoresis of lakewater microcosm, soil lysimeter and sediment isolate DNA. Restriction was with the low frequency restriction enzyme *Xba*I. Run conditions were 330 V, a 25 sec pulse time and a 40 h run time.

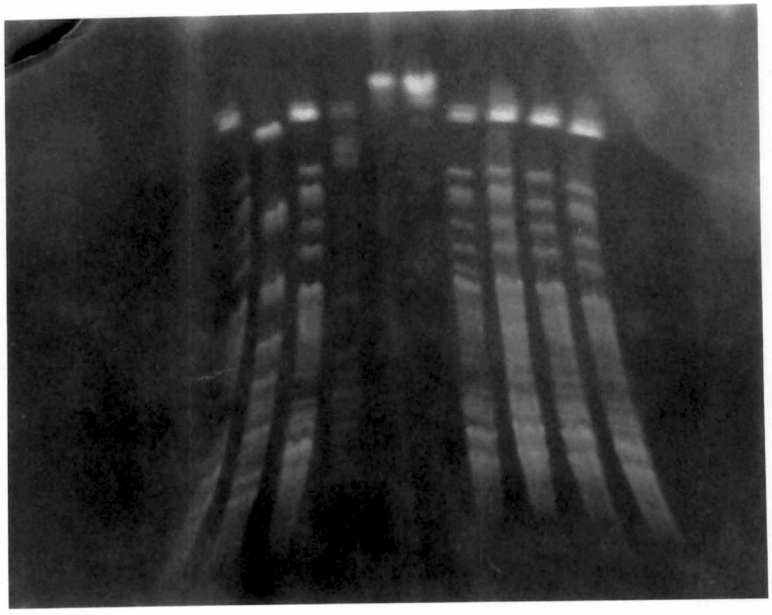
A. Lane 1 is A5 with the A5 pattern. Lane 2 is ACS8, an *Acinetobacter* isolate from Lake Loudon sediment which was cured of the pSS50 plasmid with the ACS8 pattern. Lane 3 is LPS3B, a Lake Loudon sediment isolate, with the A5 pattern. Lane 4 is LBS1C1, a lakewater microcosm isolate, with the 1C1 pattern. Lane 7 is a lakewater microcosm isolate (3A1) with the A5 pattern. Lanes 8 through 10 are lysimeter isolates (7A, 7C and 7F) with the A5 pattern.

B. Lane 1 is a lysimeter isolate (14) with the A5 pattern. Lanes 2 through 4 and 6 are lakewater microcosm isolates (2C3, 2D2, 4A6 and 4B3) with the 2A pattern. Lane 7 is the lambda oligomer ladder starting at 48.5 kb. Lane 8 is yeast chromosomes (220, 280 and 360 kb). Lanes 9 through 11 and 13 are lysimeter biphenyl enrichment isolates and a lysimeter isolate (MPN7, MPN12, 19 and MPN21) with the A5 pattern. The faint extra bands in Lanes 10 and 13 are the result of incomplete enzymatic digestion.

C. Lanes 1 through 4 are lysimeter isolates from the biphenyl enrichments (MPN5, MPN13, MPN14 and MPN19) which have the A5 pattern. Lanes 7 through 9 and 11 through 13 are lakewater microcosm isolates (1C3, 2C4, 4A3, 4A5, 4C2 and 4A4) with the 6A pattern. 4A4 is the 6A isolate which harbors the pSS50 plasmid. Lanes 15 and 17 through 19 are lysimeter isolates (28A, 7A, 7C and 7F) with the A5 pattern. Lane 16 is a lakewater microcosm (3A1) with the A5 pattern.

D. Lanes 2 through 6 and 9 through 11 are lysimeter biphenyl enrichment isolates (MPN4, MPN6, MPN8, MPN11, MPN15, MPN16, MPN17 and MPN18) with the A5 pattern. Lane 14 is a lysimeter isolate (9D) with the 9D pattern. Lane 15 is a lysimeter isolate (34A) with the 34A pattern.

A.



B.

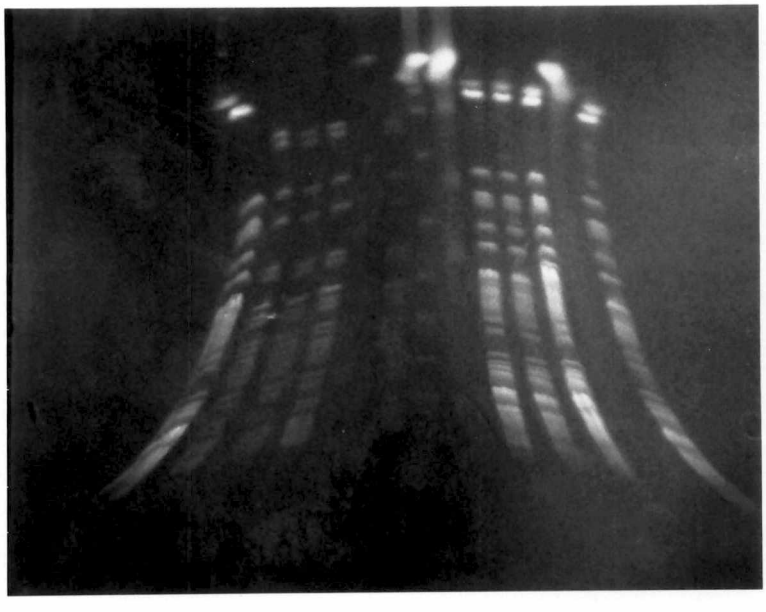
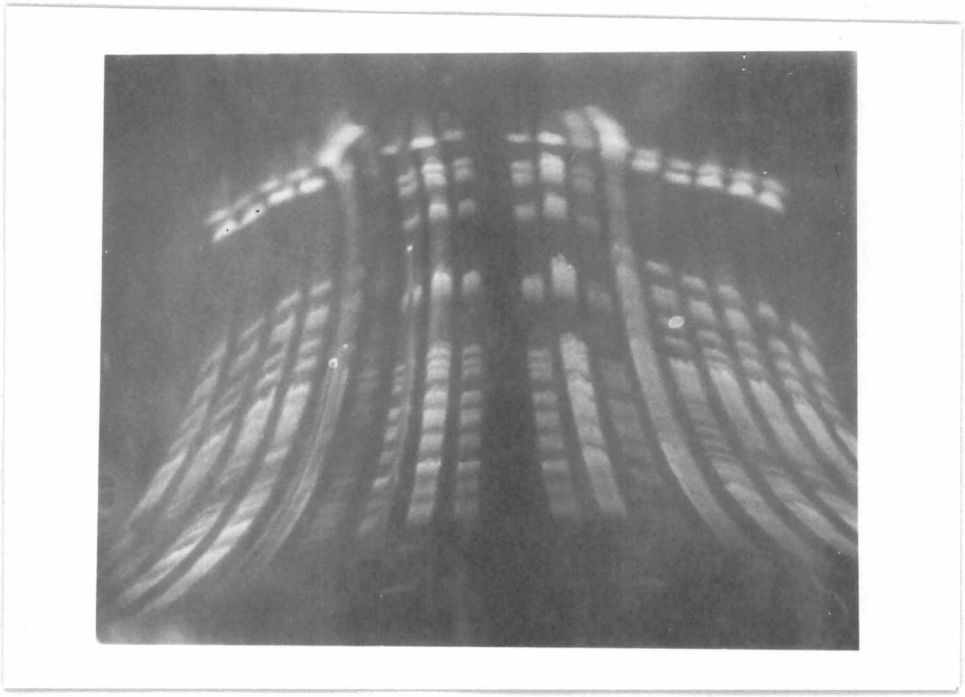


Figure 4

C.



D.



Figure 4 (continued)

Table 4. Identification by fatty acid profile analysis of the isolates from the lakewater microcosms labeled 2A and 6A by chromosomal restriction digest patterns.

Name	Identification	Similarity index
	2A	
2A4	Listonella anguillarum	0.119
	Pseudomonas facilis	0.119
	Azotobacter chroococcum	0.094
	Neisseria meningitidis	0.072
	Vibrio anguillarum	0.061
	Vibrio fluvialis	0.034
4B4	Azotobacter chroococcum	0.077
	Neisseria meningitidis	0.067
	Vibrio anguillarum	0.056
	6A	
4A4	Pseudomonas facilis	0.722
1A3	Pseudomonas facilis	0.757
4A3	Pseudomonas facilis	0.780

3). One A5 isolate was found in the A5-inoculated treatment and two other A5 isolates were found in the uninoculated treatment. All three of the A5 isolates harbored pSS50 and cleared 4-CBP spray plates. Seven isolates with the 6A chromosomal restriction digest pattern (Figure 4C) were also among the lakewater microcosm isolates examined. All of the 6A isolates cleared 4-CBP spray plates. Six of the 6A isolates did not have positive colony hybridization signals or pSS50-homologous plasmids (Table 5). However, one 6A isolate (4A4) did harbor a pSS50 plasmid (Table 3). Since A5 harboring the pSS50 plasmid and 6A isolates which did not harbor the pSS50 plasmid were found in the same treatment as 4A4, there is a possibility that the origin of pSS50 in 4A4 was conjugal transfer from A5 during the microcosm incubation.

One isolate with the 1C1 pattern, LBS1C1 (Figure 4A, Table 3), and thirteen isolates with the 2A pattern (Figure 4B, Table 3) were also among the isolates examined from the lakewater microcosms. The plasmids in these isolates (with the exception of the pSS50-homologous plasmids in six 2A isolates which were not analyzed) were pSS60 plasmids. The pSS60 plasmid was stably maintained in these isolates and did not undergo deletions to create the pSS50 plasmid. LBS1C1 cleared 4-CBP spray plates, but the 2A isolates did not (Table 3). The 2A isolates were readily distinguished from other isolates by smaller size on YEPG plates and slower growth rate in YEPG broth than other isolates. LBS1C1 harboring pSS60 and isolates with the 2A pattern harboring pSS60 were found in the same treatment, so there was the possibility of conjugal transfer of pSS60 between them.

Table 5. Identification by chromosomal REA and PFGE of lakewater microcosm isolates which were examined which clear 4-CBP spray plates and do not have pSS50-homologous plasmids.

Name	Treatment	Chromosomal identification
1A3	-A5, +4-CBP	6A
1C3	"	6A
2C4	"	6A
4A3	+A5, +4-CBP	6A
4A5	"	6A
4C2	"	6A

The existence of the isolates with the 2A pattern in the 4-CBP enriched microcosms and their absence from the microcosms which were not enriched with 4-CBP suggests the possibility of involvement of the isolates with the 2A pattern in 4-CBP degradation, although they do not clear 4-CBP spray plates. There are preliminary data available from experiments with LBS1C1 which suggests that 7 kb of the extra 10 kb fragment present in the pSS60 plasmid may contain the genes responsible for degradation of 4-chlorobenzoate (4-CBA), the endproduct of the first pathway of 4-chlorobiphenyl degradation (A. Layton, personal communication).

In summary, isolates from the lakewater microcosms can be divided into three groups determined by the colony hybridization and 4-CBP spray plate clearing results. (i) The five lakewater microcosm isolates which had positive colony hybridization signals and cleared 4-CBP spray plates were comprised of three isolates identical to A5 harboring the pSS50 plasmid, an isolate with the 6A pattern harboring the pSS50 plasmid, and the LBS1C1 isolate harboring the pSS60 plasmid. These isolates were distributed among the three treatments (Table 6). (ii) Thirteen isolates with the 2A pattern comprised the group of isolates which had positive colony hybridization signals but did not clear 4-CBP spray plates. The recovery of the 2A isolates with the pSS50 probe demonstrates that the pSS50 probe detects organisms which do not degrade 4-CBP. The 2A isolates comprised greater than half of the isolates examined from both 4-CBP-enriched treatments (Table 6). The pSS50-homologous plasmids in seven of the 2A isolates were pSS60 plasmids; the plasmids from the other six 2A isolates were not examined. The determination by chromosomal

Table 6. Summary of results from colonies examined from lakewater microcosms which have positive colony hybridization signals and/or clear 4-CBP spray plates.

Treatment	Number of strains examined	+ Hybridization + clearing (%)	- Hybridization + clearing (%)	+ Hybridization -clearing (%)
-A5, -4-CBP	1	100	0	0
-A5, +4-CBP	12	7	25	58
+A5, +4-CBP	13	15	23	62

restriction digest analysis that the 2A isolates were identical was corroborated by fatty acid analysis. (iii) The isolates which did not hybridize with the plasmid, but which cleared 4-CBP spray plates were comprised of six isolates with the 6A pattern, identified as *Pseudomonas facilis* by fatty acid analysis; these isolates did not have plasmids. They comprised about 25% of the isolates examined from both 4-CBP-enriched treatments (Table 6). The determination by chromosomal restriction digest analysis that the 6A isolates were identical was also corroborated by fatty acid analysis.

Lysimeter isolates

Eight isolates with pSS50-homologous plasmids from the 1987 sampling dates were recovered. Six of these isolates cleared 4-CBP spray plates (Table 7); two of the isolates (8C and 9D) did not clear 4-CBP spray plates (Table 7). From the July 1988 sampling date, single isolates from each of six of the nine colonies with positive signals were found to have pSS50-homologous plasmids. Four of these isolates cleared 4-CBP spray plates; two isolates (13A and 34A) did not clear 4-CBP spray plates (Table 7). The other three colonies with positive signals from July 1988 (12, 33 and 35B) were found to contain isolates which had positive signals but which did not have pSS50-homologous plasmids; these isolates did not clear 4-CBP spray plates. In a colony hybridization of pure cultures which included three isolates with pSS50 plasmids and the three isolates without the plasmids, the isolates with the plasmids had stronger signals than the isolates which did not have pSS50-homologous plasmids. However, colonies which had pSS50-homologous plasmids could not be identified on

Table 7. 4-CBP spray plate results and identification by chromosomal REA and PFGE of soil lysimeter isolates which were examined which harbor pSS50 plasmids.

Name	4-CBP spray plate result	Chromosomal identification
	Lysimeter 3 1987	
7A	+	A5
7C	+	A5
7F	+	A5
8C	-	8C
9D	-	9D
	Lysimeter 4 1987	
10F	+	A5
28A	+	A5
29	+	A5
	Lysimeter 3 1988	
13A	-	13A
14	+	A5
	Lysimeter 4 1988	
19	+	A5
	Lysimeter 6 1988	
34A	-	34A
34B	+	A5
35A	+	A5

Table 7 (continued)

Name	4-CBP spray plate result	Chromosomal identification
	Lysimeter 2 biphenyl enrichment 1988	
MPN3	+	A5
	Lysimeter 3 biphenyl enrichment 1988	
MPN4	+	A5
MPN5	+	A5
MPN6	+	A5
MPN7	+	A5
MPN8	+	A5
	Lysimeter 4 biphenyl enrichment 1988	
MPN11	+	A5
MPN12	+	A5
	Lysimeter 5 biphenyl enrichment 1988	
MPN13	+	A5
MPN14	+	A5
MPN15	+	A5
	Lysimeter 6 biphenyl enrichment 1988	
MPN16	+	A5
MPN17	+	A5
MPN18	+	A5
MPN19	+	A5
MPN20	+	A5
MPN21	+	A5
MPN23	-	23

the original colony hybridization by signal strength.

Twenty two of the pure cultures from the biphenyl enrichment MPN dilution series had positive colony hybridization signals; eighteen had strong signals and four had weak signals. Plasmid screening of these isolates showed that the 18 with strong signals had single pSS50-sized plasmids; all but one of these cleared 4-CBP spray plates (Table 7). The four with weak signals, MPN2 from lysimeter 1, MPN9 and MPN10 from lysimeter 3 and MPN22 from lysimeter 6, did not have plasmids or clear 4-CBP spray plates. There was no correlation between signal strength and the presence of the pSS50 plasmid on the original colony hybridization.

All of the pSS50-homologous plasmids from the lysimeter isolates were identical to pSS50; no pSS60 plasmids or other variations of pSS50 plasmids were found in the lysimeter isolates. Single pSS50 plasmids were found in all of the lysimeter isolates with several exceptions. 9D from the 1987 colony hybridization had an extra plasmid in addition to and nonhomologous to pSS50 which was similar in size to pSS50. 9D was identified as a *Pseudomonas* sp. soil isolate by fatty acid analysis; it could have received the pSS50 plasmid by conjugal transfer from A5. 19 from the 1988 colony hybridization had a small extra plasmid in one preparation, but not in others; 19 had the A5 pattern (Table 7). MPN16 from the biphenyl enrichment cultures had an extra plasmid which was larger than pSS50 but not homologous to pSS50. In one of the large scale preps, MPN16 was cured of the pSS50 plasmid but retained the other plasmid which was nonhomologous to pSS50. Southern hybridization was done on this gel, and there was no signal on the

chromosome, demonstrating that the plasmid had not integrated into the chromosome. In all other preparations of MPN16, both plasmids were seen. MPN16 had the A5 pattern (Table 7); therefore, MPN16 appears to have acquired the large plasmid while in the soil or biphenyl enrichment cultures.

Six of the eighteen isolates with pSS50-homologous plasmids from the biphenyl enrichments (MPN4, MPN5, MPN6, MPN8, MPN21 and MPN23) had single small plasmids in addition to pSS50. Southern hybridization conducted on the gels of the plasmid DNA of MPN4, MPN5 and MPN6 showed that the extra plasmids and fragments were not homologous to pSS50. All of the isolates with extra small plasmids had chromosomal restriction digest patterns identical to A5 with the exception of MPN23; the isolates with the A5 pattern appear to have acquired the extra small plasmids in the soil or during the biphenyl enrichment.

Twenty seven out of the total number of the lysimeter isolates (including those from the biphenyl enrichments) which contained pSS50 also cleared 4-CBP spray plates; five isolates with pSS50 did not clear 4-CBP spray plates. Fatty acid analysis was conducted on three of the 1988 isolates with pSS50 plasmids which cleared 4-CBP spray plates to determine if they were the same as the A5 which had been used to inoculate the lysimeters at the start of the experiment. All three of these isolates (14 from lysimeter 3 and 19 and 35A from lysimeter 4) had nearly identical matches to A5. This was the first evidence that isolates identical to A5 were present in the lysimeters one year after the A5 inoculation. Also, the existence of an isolate identical to A5 in lysimeter 3 suggested that lysimeter 3 had been contaminated with

A5. Chromosomal restriction digests with the low frequency restriction enzyme *Xba*I were conducted on all of the lysimeter isolates which had pSS50 plasmids. After PFGE, the restriction digest patterns were compared to the A5 pattern. All 27 of the isolates containing pSS50 which cleared 4-CBP spray plates had identical chromosomal restriction digest patterns to A5 (Table 7), corroborating the fatty acid profile data that isolates identical to A5 were present in the lysimeters one year after inoculation of A5.

The possibility of A5 occurring in the soil before inoculation cannot be eliminated because no isolates were characterized from samples taken before A5 had been inoculated or from soil samples physically removed from the lysimeters. However, the distribution of A5 isolates suggests that they were from the original A5 inoculum. Many isolates similar to A5 were recovered from lysimeters 3, 4, 5 and 6; one A5 isolate was recovered from lysimeter 2 from the biphenyl enrichment and no A5 isolates were recovered from lysimeter 1 (Table 7). This uneven distribution of A5 isolates suggests that the A5 isolates which were recovered were from the original A5 inoculum in lysimeters 4, 5, and 6 which spread to lysimeter 3 and to a lesser extent to lysimeter 2. This would be logical due to the adjacent numerical order-line up of the lysimeters. However, the possibility that the A5 distribution was due to stimulation by nutrients in lysimeters 3, 4, 5 and 6 and the absence of nutrients in lysimeters 1 and 2 makes the contamination hypothesis equivocal. Therefore, due to the same restriction patterns between the original A5 inoculum and the A5 isolates recovered from the soil, it is likely but not confirmed that the

isolates detected from the lysimeters by colony hybridization were from the original A5 inoculum and had been maintained in the soil lysimeters.

The five isolates containing pSS50 plasmids which did not clear 4-CBP spray plates (8C, 9D, 13A, 34A and MPN23) had restriction digest patterns different from the A5 pattern, different from each other and different from the lakewater microcosm isolates (Table 7). All of the soil isolates harboring pSS50 plasmids were identified as *Pseudomonas* species by fatty acid analysis (Table 8). 8C and 9D were identified as different species from each other and from A5, but mutiple matches were given for both so that no species identification could be made for them. 13A and 34A were identified as the same species, *Chryseomonas luteola* (the new name for *Pseudomonas* VEI) with the similarity indices differing by 0.1. MPN23 was given the same match as A5, *Pseudomonas pickettii*; the similarity indices differed between the two by 0.2. The soil isolates harboring pSS50 plasmids could harbor pSS50 in uninoculated soil or they could have acquired the pSS50 plasmid by conjugal transfer from A5.

All seventeen of the isolates from the biphenyl enrichment cultures which had the pSS50 plasmid and cleared 4-CBP spray plates were A5 isolates (Table 7). Only one isolate, MPN23, from the biphenyl enrichment cultures had the pSS50 plasmid and did not clear 4-CBP spray plates. The chromosomal restriction digest pattern of MPN23 showed that it is a unique soil isolate (Table 7). The pSS50 plasmid may have been transfered from A5 to MPN23. Colony hybridization with the pSS50 plasmid of biphenyl enrichment cultures is more sensitive than direct colony

Table 8. Identification by fatty acid profile analysis of soil lysimeter isolates harboring pSS50 plasmids which do not clear 4-CBP spray plates and have chromosomal restriction digest patterns different from A5 and from each other.

Name	Identification	Similarity index
8C	Pseudomonas aureofaciens	0.527
	Pseudomonas putida biotype D	0.192
9D	Pseudomonas putida biovar B	0.820
	Pseudomonas fluorescens B	0.713
	Pseudomonas fluorescens G	0.483
	Pseudomonas chlororaphis	0.069
13A	Chryseomonas luteola (Pseudomonas VEI)	0.610
34A	Chryseomonas luteola (Pseudomonas VEI)	0.707
MPN23	Pseudomonas pickettii	0.774

hybridization of samples for organisms which degrade biphenyl and 4-chlorobiphenyl and have pSS50-homologous plasmids. The absence of any isolates from the biphenyl enrichment cultures, other than A5, which contained pSS50 and cleared 4-CBP spray plates is evidence that organisms with pSS50-homologous plasmids which grow well on biphenyl and degrade 4-CBP did not occur in these lysimeters. In summary, colony hybridization data from the lysimeters indicates that organisms with pSS50-homologous plasmids which degrade 4-CBP, other than A5, did not occur in the lysimeters.

One of the isolates from the biphenyl enrichments, MPN1 from lysimeter 1, cleared 4-CBP spray plates but did not have a positive colony hybridization signal or a pSS50-homologous plasmid. It was identified by fatty acid analysis as *Comomonas testosteroni* with a similarity index of 0.911 and put into frozen stock; *Comomonas testosteroni* is the new name for *Pseudomonas testosteroni* (Tamaoka 1987). This is the only soil isolate that was recovered that degraded 4-CBP; its recovery was due to the fact that 4-CBP spray plating was conducted on the original colonies from the biphenyl enrichments in order to detect 4-CBP-degrading organisms. LPS10A, a Lake Loudon sediment isolate which converts 4-CBP to 4-CBA and does not have a pSS50-homologous plasmid (Pettigrew et al. 1990), and strain B-356, a 4-CBP degrading strain isolated from activated sludge (Ahmad et al. 1990), have also been identified as *Comomonas testosteroni.*

In summary, all of the 27 lysimeter isolates with pSS50-homologous plasmids (isolated by colony hybridization of the lysimeter samples and colony hybridization

of the biphenyl enrichments from the lysimeter samples) had pSS50 plasmids; no variations of the pSS50 plasmids were found in the soil lysimeters. All of the lysimeter isolates with the pSS50 plasmid which cleared 4-CBP spray plates were identical to A5 as determined by chromosomal restriction digest patterns and corroborated by fatty acid analysis. The presence of A5 isolates in the lysimeters indicates maintenance of the inoculated A5 for one year in the lysimeters which were maintained with biweekly stirring, water, minerals and nutrients. Five other isolates with the pSS50 plasmid did not clear 4-CBP spray plates. All of these five isolates were determined to be different from A5 by chromosomal REA. The recovery of these five *Pseudomonas* isolates with the pSS50 probe demonstrates that the pSS50 probe detects organisms which do not degrade 4-CBP.

Sediment isolates

A sediment isolate, ALP-1-83, which has a larger pSS50-homologous plasmid in addition to pSS50 on gels was recovered from frozen stocks of isolates from the 4-CBP enrichment culture of Lake Loudon sediment conducted in 1983. ALP-1-83 was recovered by A. Layton by colony hybridization of frozen stock cultures with the extra fragment found in pSS60. Chromosomal restriction digestion with the low frequency restriction enzyme *Xba*I and pulsed field gel electrophoresis were conducted on a culture from the ALP-1-83 frozen stock which had both plasmids; ALP-1-83 had a chromosomal restriction digest pattern identical to A5. The two ALP-1-83 plasmids seen on a plasmid screening gel of an ALP-1-83 culture are pSS50 and a larger form of pSS50, the pSS70 plasmid (A. Layton, personal communication).

Individual ALP-1-83 cells may contain either the pSS70 plasmid or the deletion derivative, the pSS50 plasmid (A. Layton, personal communication).

Comparison of the restriction map of the pSS70 plasmid to the restriction maps of pSS50 and pSS60 shows that PSS70 is a 63 kb plasmid comprised of pSS50 plus an extra 12 kb fragment which is often deleted and that 7 kb of this extra fragment is homologous but not identical to 7 kb in the extra fragment in pSS60 (A. Layton, personal communication; Figure 3). Research is presently being conducted to determine if the homologous 7 kb fragments found in pSS60 and pSS70 are responsible for degradation of 4-chlorobenzoate. One end of the extra 12 kb fragment contains a 3.4 kb repeat which is a direct repeat in relation to a 3.4 kb repeat (including the original 2.2 kb repeat) in pSS50 at the other end of the unique 12 kb fragment. 2.2 kb inverted repeats are found on one end of the unique fragment in pSS70 as they are in pSS60 (A. Layton, personal communication; Figure 3). The 12 kb fragment which is deleted from pSS70 is not inserted into the A5 chromosome (A. Layton, personal communication).

LPS3B, an isolate which degrades 4-CBP to 4-CBA (Pettigrew and Sayler 1986) and harbors the pSS50 plasmid (Pettigrew et al. 1990), was recovered by colony hybridization of the PCB-contaminated sediment from which A5 had been recovered two years earlier (Pettigrew and Sayler 1986). LPS3B had tentatively been identified as *Alcaligenes* sp. by biochemical and physicial data (Pettigrew et al. 1990); it has a restriction digest pattern and fatty acid identification identical to A5 (Figure 4A). This indicates the persistence of isolates resembling A5 in Lake Loudon sediment,

at least for two years after the initial isolation of A5.

In summary, the fact that ALP-1-83 harbors the unstable pSS70 plasmid which undergoes deletions to create the pSS50 plasmid provided evidence that pSS50 plasmids in other isolates, such as those from this study, could be a deletion derivatives of larger plasmids, as indicated by the gels of the original *Alcaligenes* isolates (Sayler et al. 1984, Shields et al. 1985). Burlage and coworkers (in press) propose that the original 75 kb 4-CBP catabolic plasmid may have lost the 2,3-dioxygenase pathway (which converts 4-CBP to 4-CBA) by homologous recombination between the repeats to create a plasmid that resembles pSS60. They also proposed that a subsequent recombination event of the direct repeats flanking the unique sequence in pSS60 (which is under investigation for genes which encode 4-chlorobenzoate degradation) would produce the present form of pSS50.

V. CONCLUSIONS

Isolates identical to A5 (as determined by chromosomal restriction digest patterns and fatty acid analysis), harboring the 51 kb pSS50 plasmid and demonstrating the ability to degrade 4-CBP were isolated from Lake Loudon lakewater several years after the isolation of A5 from Lake Loudon sediment. The pSS50 plasmid probe also detected two groups of organisms from the lakewater microcosms which harbored the pSS60 plasmid. PSS60 is a 61 kb plasmid, which did not undergo deletions, which is comprised of the pSS50 plasmid plus an extra 10 kb fragment (Pettigrew et al. 1990, Burlage et al. in press). LBS1C1 harboring pSS60 has the ability to degrade 4-CBP and is the only isolate representative of the 1C1 chromosomal restriction digest pattern. Several isolates harboring the pSS60 plasmid which do not have the ability to degrade 4-CBP and have similar restriction digest patterns, the 2A pattern, were also found. The recovery of the 2A isolates with the pSS50 plasmid probe demonstrates that the pSS50 probe detects organisms which do not degrade 4-CBP.

The pSS50 and pSS60 plasmids are IncP1 plasmids which can be transferred and maintained in almost all gram negative organisms (Burlage et al. in press). There is circumstantial evidence for conjugal transfer in the lakewater microcosms of the pSS50 plasmid from A5 to a 4-CBP-degrading lakewater isolate 4A4 (which was tentatively identified as *Pseudomonas facilis* by fatty acid analysis). Several isolates with identical chromosomal restriction digest patterns to 4A4 which do not harbor the pSS50 plasmid and the A5 isolate harboring the pSS50 plasmid were

found in the same treatment as the 4A4 isolate.

Isolates identical to the A5 inoculum (as determined by chromosomal restriction digest patterns and corroborated by fatty acid analysis), harboring the pSS50 plasmid and demonstrating the ablity to degrade 4-CBP were found in the soil lysimeters one year after inoculation with A5. This indicates maintenance of the A5 inoculum in the lysimeters which were maintained with water, stirring and nutrients. In addition, the pSS50 plasmid was found in five different *Pseudomonas* strains which did not degrade 4-CBP in the soil lysimeters. Since these lysimeters also contained A5 isolates, presumably from the A5 inoculum at the start of the experiment, it is not known if these isolates contained the pSS50 plasmids before inoculation or received them by conjugal transfer from A5. The recovery of the five *Pseudomonas* isolates with the pSS50 plasmid probe further demonstrates that the pSS50 probe detects organisms which do not degrade 4-CBP.

The discovery by Dr. A. Layton of the 63 kb pSS70 plasmid which undergoes deletions of 12 kb to produce the pSS50 plasmid in ALP-1-83 (which has an identical chromosomal restriction digest pattern to A5) demonstrates that large unstable pSS50-homologous plasmids, possibly containing the 2,3-dioxygenase pathway for conversion of 4-CBP to 4-CBA and/or the pathway for degradation of 4-chlorobenzoate, may not be detected in environmental isolates because of similar deletions. No isolates with unstable pSS50 homologous plasmids were detected in this study; however, deletions resulting in the pSS50 plasmid may have occurred before plasmid analysis. Also, the discovery that the PCB/biphenyl operon is most

likely on a chromosomal transposon in A5 which can be transposed into pSS50 plasmid during conjugation creating stably maintained pSSD-plasmids argues for the possible existence of pSSD-plasmids in the environment with the potential for transferring the *bph* operon to other strains (Springael and Mergeay 1990), although pSSD-plasmids were not detected in this study.

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LITERATURE CITED

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VITA

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