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Characteristics and Restriction Analysis of the 4-Chlorobiphenyl Catabolic Plasmid, pSS50

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The plasmid pSS50 is a 53-kilobase self-transmissible plasmid of broad host range that has been isolated from several *Alcaligenes* and *Acinetobacter* species. This plasmid has previously been shown to mediate the mineralization of 4-chlorobiphenyl to carbon dioxide and water. Physical characterization of this plasmid by restriction analysis indicates that most hexanucleotide cleavage sites are clustered in a 5-kilobase region, leaving large regions without restriction sites. The paucity of restriction sites is not due to DNA methylation.

The 53-kilobase (kb) plasmid pSS50, found in *Alcaligenes* and *Acinetobacter* species, was the first plasmid reported which mediated the complete mineralization of 4-chlorobiphenyl (4-CB) (a model compound for polychlorinated biphenyl [PCB] degradation) to carbon dioxide and water (16, 18). Other organisms have been described that are capable of PCB catabolism (4, 7), but no mineralization capability has been reported. Plasmids which mediate only chlorobiphenyl-to-chlorobenzoate conversion (pKF1 [6]), or only chlorobenzoate degradation (pAC25 [4, 5, 8] and pBR60 [19]), are known. Recently, a *Pseudomonas* sp. that can utilize both 4-chlorobenzoate and 4-CB has been described (2). Spent culture medium was found to consist mainly of 4-chloroacetophenone and a small amount of 4-chlorobenzoate, making it probable that this organism is capable of complete mineralization of 4-CB.

Strains bearing the plasmid pSS50 were isolated from a sediment microbial consortium, obtained from a PCB-contaminated reservoir, capable of mineralizing 4-CB. Upon dissection of this consortium, individual isolates were discovered which independently exhibited the same catabolic phenotype. Strains that were cured of their plasmid (pSS50) lost the mineralization phenotype (18). The role of pSS50 in the catabolism and complete mineralization of 4-CB was demonstrated by plasmid curing of the *Acinetobacter* strain A2 and by conjugative transfer of pSS50 into the cured *Acinetobacter* strain, which restored mineralization (18).

PCBs are worldwide environmental pollutants of anthropogenic origin (10). Owing to possible toxic, mutagenic, and carcinogenic effects and proven bioaccumulation properties, they are considered to be a health hazard for humans (9, 15, 17). The stability of the benzene rings and carbon-halogen bonds makes PCBs extremely resistant to chemical degradation (10). Because microbial degradation may be a feasible method for eliminating these types of pollutants and because of the evolutionary significance of this degradative plasmid, studies were undertaken to better characterize pSS50. During these studies, *Alcaligenes eutrophus* A5 and *Acinetobacter* strain A2 were cultured as previously described (18).

In an attempt to characterize the extent of the plasmid-mediated degradation activity, a wide range of substrates were tested for their differential ability to support growth of plasmid-bearing strains over that of cured strains. Organisms were grown in basal minimal salts buffer that consisted of the following components (per liter): 4.0 g of $NaNO_3$, 1.5 g of KH_2PO_4, 0.5 g of Na_2HPO_4, 0.005 g of $FeCl_2$ or 0.0011 g of $FeSO_4$, 0.2 g of $MgSO_4 \cdot 7H_2O$, and 0.01 g of $CaCl_2 \cdot 2H_2O$, final pH of 7. Carbon substrate was added to a concentration of 100 mg/liter. *Acinetobacter* and *Alcaligenes* strains grew on benzoic acid, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, biphenyl, and protocatechuic acid regardless of whether pSS50 was present.

Since no antibiotic resistance markers for pSS50 are known (M.S. Shields, Ph.D. dissertation, The University of Tennessee, Knoxville, 1985), experiments were done to determine whether pSS50 encoded resistance to any heavy metals. pSS50 does not encode resistance to cadmium, cobalt, or zinc. At the concentration found to be diagnostic for resistance to mercury in *A. eutrophus* (14), pSS50 does not encode resistance to mercury. For nickel and lead, all strains were resistant at the diagnostic concentrations determined for *A. eutrophus* (14).

A restriction endonuclease cleavage map of pSS50 was constructed through the analysis of single, double, and triple digests of pSS50 DNA. Plasmid DNA was obtained through a modified procedure of Anderson and McKay (1), in which cells were frozen after pelleting and then thawed in the warmed buffer containing lysozyme. This modification was found to increase plasmid yield substantially. The plasmid DNA was then purified on CsCl/ethidium bromide density gradients. Restriction digestion was performed as specified by the vendor or by the modified method of Maniatis (12). Deductive reasoning and the results of multiple digests were used to determine the order of the fragments. A map of the plasmid is shown in Fig. 1; Table 1 summarizes the restriction fragment sizes.

Two possible explanations for the relative paucity of hexanucleotide-recognizing restriction endonuclease cleavage sites are methylated DNA and repetitive DNA. pSS50 was digested with tetranucleotide-recognizing restriction endonucleases which recognize the same basic sequences but are affected differently by methylation. *Dpn*I and *Mbo*I recognize and cleave the same GATC sequence, but *Dpn*I requires a methylated adenine residue in order to cleave. Methylation of the adenine residue blocks *Mbo*I cleavage. Similarly, *Hpa*II and *Msp*I recognize the sequence CCGG, but *Hpa*II is blocked by methylation at either cytosine residue, while *Msp*I is blocked only if the 5′ cytosine residue

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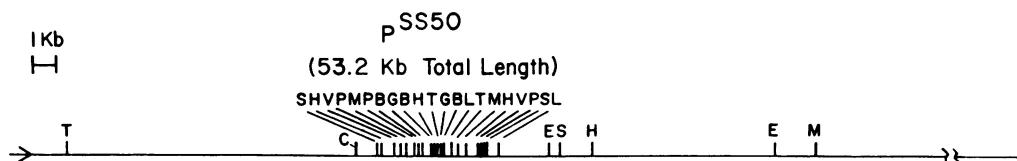

FIG. 1. Restriction endonuclease cleavage map of pSS50 (as isolated from *A. eutrophus* A5). Abbreviations for the restriction enzymes are given in Table 1.

TABLE 1. Profile of restriction fragments for pSS50

Enzyme (abbreviation)[a]	No. of fragments	Length (kb)
*Bam*HI (B)	3	0.2, 0.4, ~52
*Bcl*I (L)	2	2.2, ~51
*Bgl*II (G)	2	0.8, ~52
*Bst*EII (T)	3	1.0, 16.8, 35.4
*Cla*I (C)	1	Linearized
*Eco*RI (E)	2	10.3, ~43
*Hin*dIII (H)	4	2.0, 2.4, 5.2, ~41
*Kpn*I	0	
*Pst*I (P)	3	0.4, 3.1, ~50
*Pvu*II (V)	2	3.9, ~49
*Sal*I (S)	3	3.4, 4.9, ~45
*Sma*I (M)	3	2.6, 15.2, ~35
*Xho*I	0	

[a] The one-letter abbreviations are used in Fig. 1.

is methylated (11). The enzymes *Mbo*I, *Hpa*II, and *Msp*I cleave pSS50 into very small fragments such that most are less than 600 base pairs in length. Because *Mbo*I cuts pSS50 and *Dpn*I does not cut pSS50, the adenine residues within all GATC sequences were not methylated. The banding patterns of the *Hpa*II and *Msp*I digests were identical; thus, the internal cytosine of the sequence CCGG is not methylated and the 5′ cytosine of the CCGG sequence cannot be completely methylated. To check for extensive repetitive sequences, the relative intensities of the bands were compared visually. Since no particular band was substantially brighter than any of the others, extensive repetitive sequences are not prevalent in pSS50.

A clustering of most of the hexanucleotide-recognizing endonuclease cleavage sites on pSS50 became apparent once the restriction profiles were integrated into a map. Furthermore, there is an apparent symmetry of these cleavage sites about a central axis. In order to investigate whether this symmetry is due to homologous stretches of DNA, this region was cleaved such that the right and left halves of the symmetry were on fragments of different sizes. This was accomplished through *Bgl*II/*Cla*I/*Eco*RI triple digestion of whole pSS50 DNA. As a result, the left-hand portion of this region (with respect to the map in Fig. 1) is on a 3.2-kb fragment and the right-hand portion is on a 4.3-kb fragment. The smaller, 3.2-kb, fragment was collected and radiolabeled with ^{32}P along with bacteriophage lambda DNA (as a size marker). This probe hybridized with both the 3.2-kb left-hand fragment and the 4.3-kb right-hand fragment of the *Bgl*II/*Cla*I/*Eco*RI triple digest but with neither the smaller nor larger fragments of pSS50. The results from this experiment indicate homology on each side of the restriction-site-rich region.

pSS50 has an unusual paucity and distribution of hexanucleotide-recognizing restriction endonuclease cleavage sites. This phenomenon is not due to either extensive methylation or extensive repetitive DNA. Most of these sites are clustered within a 5-kb region of the plasmid. Other broad-host-range plasmids, such as RSF1010, tend to have a clustering of cleavage sites, but not to the extent seen in pSS50. Recently, new restriction endonucleases (*Sph*I, *Not*I, and *Sfi*I) have been discovered that digest pSS50 at sites throughout its length. These results, along with the acquisition of a transposon that is stably maintained, will facilitate a more in-depth genetic and biochemical analysis of this plasmid.

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