REPORT DOCUMENTATION PAGE

AD-A284 492

0768

Public reporting burden for this collection of information is estimated to average ... hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY (Leave blank)

2. REPORT DATE
Aug 94

4. TITLE AND SUBTITLE
Distribution and Catabolic Diversity of 3-Chlorobenzoic Acid Degrading Bacteria Isolated from Geographically Separated Pristine Soils

5. FUNDING NUMBERS

6. AUTHOR(S)
Albert Nute Rhodes III

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
AFIT Students Attending:
Michigan State University

8. PERFORMING ORGANIZATION REPORT NUMBER
AFIT/CI/CIA
94-036D

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
DEPRTMENT OF THE AIR FORCE
AFIT/CI
2950 P STREET
WRIGHT-PATTERSON AFB OH 45433-7765

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for Public Release IAW 190-1
Distribution Unlimited
MICHAEL M. BRICKER, SMSgt, USAF
Chief Administration

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 words)

14. SUBJECT TERMS

15. NUMBER OF PAGES
113

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT

18. SECURITY CLASSIFICATION OF THIS PAGE

19. SECURITY CLASSIFICATION OF ABSTRACT

20. LIMITATION OF ABSTRACT

Standard Form 298 (Rev. 2-89)

DISTRIBUTION AND CATABOLIC DIVERSITY OF 3-CHLOROBENZOIC ACID DEGRADING BACTERIA ISOLATED FROM GEOGRAPHICALLY-SEPARATED PRISTINE SOILS

By

Albert Nute Rhodes III

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Crop and Soil Science

1994

ABSTRACT

DISTRIBUTION AND CATABOLIC DIVERSITY OF 3-CHLOROBENZOIC ACID DEGRADING BACTERIA ISOLATED FROM GEOGRAPHICALLY-SEPARATED PRISTINE SOILS

By

Albert Nute Rhodes III

Chlorinated aromatic compounds have been used extensively in industry and agriculture for over the past 50 years. Even though chloroaromatic compounds are generally viewed as xenobiotic, numerous bacteria have been isolated that are able to mineralize these compounds. However, it is not known whether these isolates have recently evolved new catabolic traits or if they are genetically preadapted for xenobiotic degradation. In this study, 3-chlorobenzoic acid (3-CBA) was chosen as a model substrate to test the hypothesis that natural bacterial populations are preadapted for chloroaromatic degradation. Pristine soil ecosystems, defined as those with no documented history of exposure to chlorinated aromatic compounds, were sampled to determine if isolates could be cultivated without long-term enrichment. Two ecosystems were selected: 1) Mediterranean, sclerophyllous woodlands in California, Chile, South Africa, and Australia; and 2) boreal forests in Canada and Russia. A two-step enrichment protocol using 3-CBA-UL-ring-^{14}C was used to detect mineralization in mixed communities. Primary soil enrichments were treated with 50 μg 3-CBA g•dry•soil^{-1} and secondary enrichments were performed with 50 μg 3-CBA ml^{-1} defined medium. Isolates were obtained by

plating on R2A agar. In all, 610 3-CBA degrading isolates were cultured from these sites indicating that the distribution of the 3-CBA catabolic activity is widespread in pristine soil ecosystems. Catabolic activity of individual strains was assessed by HPLC analysis. The amount of 1.0 mM 3-CBA degraded in one week ranged from approximately 1 to 100%. Population structures of 3-CBA degraders were compared by rank ordering the percent transformation in these incubations. Cluster analysis revealed the profiles formed two major population structures; one with a uniform distribution in 3-CBA transforming capacity and the other with a high proportion of low capacity degraders. The variation in the ability of different strains to transform 3-CBA is attributed to natural variation in the specificity of catabolic enzymes. To determine if variability was present in other enzymes, a subset of fifty-eight genotypically-distinct strains able to degrade >80% 1.0 mM 3-CBA in one week were examined for their catabolic versatility using other substituted aromatic compounds. Forty-seven different compounds representing mono and dichlorinated benzoate, phenol, aniline, toluene, nitrobenzene, chlorobenzene, and 2,4-D were used with a novel ^{35}S incorporation assay. The data reveal appreciable diversity in the range of other chloroaromatic compounds metabolized by the 3-CBA isolates. Cluster analysis of the substrate utilization profiles revealed five metabolic groups which formed around the type and number of substrates used.

DEDICATION

Mrs. Sharon Williams, Murray Elementary School, Dublin, California

and

Mr. Dennis Boyle, Anderson Union High School, Anderson California

ACKNOWLEDGEMENTS

The following individuals are recognized for providing invaluable assistance during this research project:

Prof. James Tiedje, my major professor, for his guidance and direction during this project and for encouraging me to finish my degree under my rigid time constraints.

Profs. Michael Klug, Stephen Boyd, and Craig Criddle for serving as my graduate committee.

Dr. Roberta Fulthorpe, my coinvestigator, for collecting soil samples, providing guidance and encouragement. I look forward to continued collaborations in the future.

Robert Sroufe, my laboratory assistant, for assisting in all phases of the enrichment and isolation of strains; it could not have been done without him.

Dr. Larry Forney, for technical guidance in the use of the ^{35}S assimilation technique used in substrate use assays.

Dr. Cindy Nakatsu, for critical discussions and review of this manuscript.

The Department of Biology, United States Air Force Academy, for giving me the opportunity to pursue this degree.

This work was support by grants from the United States Air Force Armstrong Laboratory and the NSF Center for Microbial Ecology.

TABLE OF CONTENTS

LIST OF TABLES

Table Page

Chapter One

Chapter Two

Chapter Three

LIST OF FIGURES

Chapter Three

Chapter One

Literature Review

Introduction

Early investigations demonstrated chlorinated aromatic compounds are susceptible to biodegradation in natural communities. However, these early studies generally involved measuring changes in the concentration of the original compounds and did not attempt to determine metabolites or if transformations were biologically mediated. As a result, it was not clear whether disappearance of a compound resulted from leaching, sorption, volatilization, or microbial activity. Audus (2) provided indirect evidence for the biological transformation of 2,4-dichlorophenoxyacetic acid (2,4-D) using garden soil perfusion columns. Although direct evidence was not provided by this study, future experiments bear testimony that Audus correctly deduced 2,4-D removal was biological as disappearance rates of 2,4-D in perfusion fluids resembled the kinetics of microbial growth in the soil. Dewey *et al.* (10) demonstrated biological activity accounted for the removal of the herbicide trichlorobenzoic acid in biologically-active treated soil. Using radioisotopic methods, MacRae and Alexander (30) found chlorobenzoic acid biodegradability in soil microcosms was inversely related to the number of chlorine substitutions. Additionally, they were able to show chlorobenzoate metabolism was not carried out by benzoate-degrading populations in soil implying a specific subpopulation was responsible for chloroaromatic catabolism. But it was several more years before a bacterium able to use 3-chlorobenzoic acid (3-CBA) as a sole-source of carbon and energy was obtained in pure culture (26). Since that time, our understanding of the biochemical and genetic components required for chlorinated aromatic catabolism has steadily increased. Since these compounds are used extensively as herbicides and pesticides, many chloroaromatic compounds can become significant environmental pollutants if

improperly used. From a practical standpoint, scientific understanding of how natural microbial communities respond to chlorinated aromatic contaminants is essential to accurately predicting their environmental fate. Although much progress has been made, the scientific community still does not fully understand either the distribution or ecology of 3-CBA degrading bacteria or the origin of the genes required for degradation.

Believing chlorinated aromatic compounds are inherently xenobiotic, many have assumed biochemical pathways for chloroaromatic catabolism have arisen during recent evolution. This assumption has often lead investigators to search for isolates in previously exposed communities; most logically contaminated soils, sediments, aquifers, or wastewater treatment facilities. After collection, samples are often enriched in mineral salts media using increasing concentrations of the target compound as a sole source of carbon. In some cases, yeast extract or casamino acids are added to meet growth factor requirements. Natural selective pressures are relied upon to bring out isolates able to use the substrate. Once isolated, organisms are cultivated and characterized on defined media under controlled laboratory conditions. The resulting body of knowledge, although important in understanding the basic biochemistry and genetics of catabolism, can not be used to directly address the ecology of chlorinated aromatic degrading organisms in pristine environments.

Development of Biological Activity Against Chlorinated Aromatics in Natural Bacterial Communities

The fundamental question that must be addressed, regarding the distribution of bacteria with the capacity to degrade chlorinated aromatics, is what is the origin of these catabolic pathways. In a recent review by van der Meer *et al.* (40), three mechanisms were used to explain the adaptive response of natural bacterial communities to xenobiotic aromatic compounds. These mechanisms are: 1) induction of specific catabolic enzymes, 2) outgrowth of specific populations present at low densities, and 3) selection of mutants with novel metabolic activities. In the first two mechanisms organisms are genetically preadapted for biodegradation, but due to difficulties in induction or low population densities, initial degradation is slow. In other words, specific enzymes are present in the genomes within the community to allow catabolism of xenobiotic aromatic compounds. The third mechanism requires the development of catabolic activity absent from the original community. In this case, natural selection favors mutations or gene rearrangements which confer enzymatic activity on a clonal subpopulation that was absent from the community at the outset of exposure. Therefore, it is reasonable to expect at least two mechanisms explain catabolic activity observed in natural communities: 1) expression of traits from within existing preadapted populations, or 2) natural selection of novel clonal subpopulations. In pristine environments, either may explain observed catabolic activity, however, they differ markedly in the way they affect community structure following exposure.

Genetic preadaptation is based upon the the premise that enzymes within the community have substrate ranges sufficiently broad to accept xenobiotic compounds. It is well known that enzyme specificity is determined by

the amino acid sequence in the region of the active site. Since enzymes with broad specificity could explain the observed degradation of xenobiotic compounds in previously unexposed communities, structural relatedness to natural analogs would be an important factor determining susceptibility to biodegradation. As a result, xenobiotic compounds may be degraded in nature through fortuitous interactions with existing catabolic enzymes and not through evolution of new pathways.

Selection of novel clonal subpopulations, on the other hand, requires the development of catabolic activity only after exposure to a substrate. In this case, the introduction of a compound into natural systems is a prerequisite to the development of the phenotypic trait. Genetic information for the degradation of xenobiotic substrates is either not present or genes are not in suitable arrangement for proper expression in unexposed communities. As a result, no phenotypic traits should be observed in a pristine natural community without first experiencing previous exposure and sufficient time for natural selection to choose the best adapted strains. During the adaptation period, clonal subpopulations are selected for their increased fitness and competitiveness arising from their ability to degrade a novel substrate. It is not known how frequently mutational changes or genetic rearrangements lead to new phenotypic traits in nature, but bacteria have been shown to readily evolve new metabolic traits under laboratory conditions (5, 28, 29, 33, 35).

The rate of adaptation in this case is controlled by both frequency of mutation and genetic rearrangement and by clonal reproduction rates. In order for a new phenotype to be observed, clonal growth rates must be large enough to produce population densities high enough to produce detectable levels of substrate transformation. Dissemination of genetic information through horizontal transmission may increase both the rate and host range of a trait

within the community, but documented evidence of this phenomenon in soil is inconclusive (38).

Natural Occurrence of Chlorinated Compounds

Although chlorinated organic compounds are generally associated with environmental pollution and xenobiotic recalcitrance, halogenated compounds are produced naturally and are biologically important. Gribble (19) referenced more than 1500 halogenated compounds in a recent survey of organochlorine compounds in the environment (Table 1.1). All were produced naturally by biosynthesis or through combustion of organic matter in the presence of a halogen. At least 130 different chlorinated compounds are produced by higher plants and ferns alone (15). Most are either plant hormones, toxins, or antifeedants. Of primary importance to this study are the chlorinated aromatic compounds. Gribble (19) points out the extreme chemical conditions required to achieve halogenation of a benzene molecule under laboratory conditions. As a result most naturally occurring chlorination reactions occur using activated benzene rings. Examples of activated benzene rings include benzoates, phenols, and phenolic ethers. The presence of hydroxl- and carboxyl-functional groups on the ring reduce the stability of the benzene nucleus and make chlorination reactions more favorable. Natural production of dichlorobenzoates has been reported in Mt. St. Helens volcanic ash (19), but the distribution of these compounds should be limited. Chloroperoxidases and lactoperoxidases have been found in fungi, algae, and mammalian milk and can catalyze the chlorination of phenols and phenolic ethers.

Table 1.1. Classes of naturally-occurring haloorganic compounds.

Alkaloids	Benzofuran
Alkanes	Indoles
Amino Acids and Peptides	Indolcarbazoles
Aromatics	Lipids
Phenols	Steroids
Phenolic Ethers	Fatty Acids
Benzoates	Prostaglandins
Anthraquinones	Nucleic Acids
Carbazoles	Pyrroles
Carbolines	Quinolines
Dibenzodioxins	Terpenes
Dibenzofurans	Thiophenes
Furans	

Examples of biogenic chlorophenols include: 2,4-dichlorophenol from the soil fungus *Penicillium* (1); 2,5-dichlorophenol, an ant repellent produced by the grasshopper *Romalea microptera* (14); and 2,6-dichlorophenol, a sex pheromone of several hard ticks (3). Eisner *et al.* (14), however, hypothesized the 2,5-dichlorophenol was from breakdown of ingested herbicide and was not synthesized by the grasshoppers. Several basidiomycetes, common wood- and litter-degrading fungi, produce significant levels of chlorinated anysil metabolites (8). In particular, *Bjerkandera* sp BOS55 produced 3-chloro- and 3,5-dichloroanisyl *de novo* from glucose (9). These investigators believe chloroanisyls are important in the extracellular production of H_2O_2 required for lignin degradation by lignin peroxidases. The environmental fate of biogenic chloroanisyls has been postulated to be either mineralization, detoxification to chlorohumus, or biotoxification to dioxins (Figure 1.1). No reports of the biogenic formation of chlorobenzoate have been reported, however, it is well known

chlorobenzoates are produced from the degradation of PCBs in natural systems.

It is evident xenobiotic compounds alone are not the only selective pressures for the development of chloroaromatic degradative enzymes, although this is a long-held belief in the field of biodegradation. This could be a reflection of current interest in studying bacterial evolution, therefore, rapid development of new pathways is an attractive explanation. However, it is not valid to assume that previous exposure to a particular substrate is required for the development of chloroaromatic phenotypes in soil bacteria. Xenobiotics may closely resemble natural analogs occurring in communities.

Bacterial Degradation and Assimilation of 3-Chlorobenzoic Acid as a Sole-Source of Carbon

Several bacteria have been reported to degrade 3-CBA (Table 1.2). The first pure culture isolate able to use 3-CBA as a sole source of carbon for energy and biosynthesis was reported by Johnston *et al.* (26). These authors noted that previous reports of 3-CBA metabolism resulted in incomplete degradation indicated by the accumulation of chlorocatechols in the culture fluid. This strain, identified as a *Pseudomonas* species, was cultured from soil enrichments in minimal medium and could completely degrade 3-CBA without the production of detectable levels of chlorocatechol intermediates. The production of chlorocatechol intermediates is observed in bacteria lacking a chlorocatechol pathway, and therefore, do not mineralize 3-CBA. This isolate required yeast extract for growth and no other mono- or dichlorobenzoic acids were metabolized. 3-Hydroxybenzoic acid transiently accumulated during growth

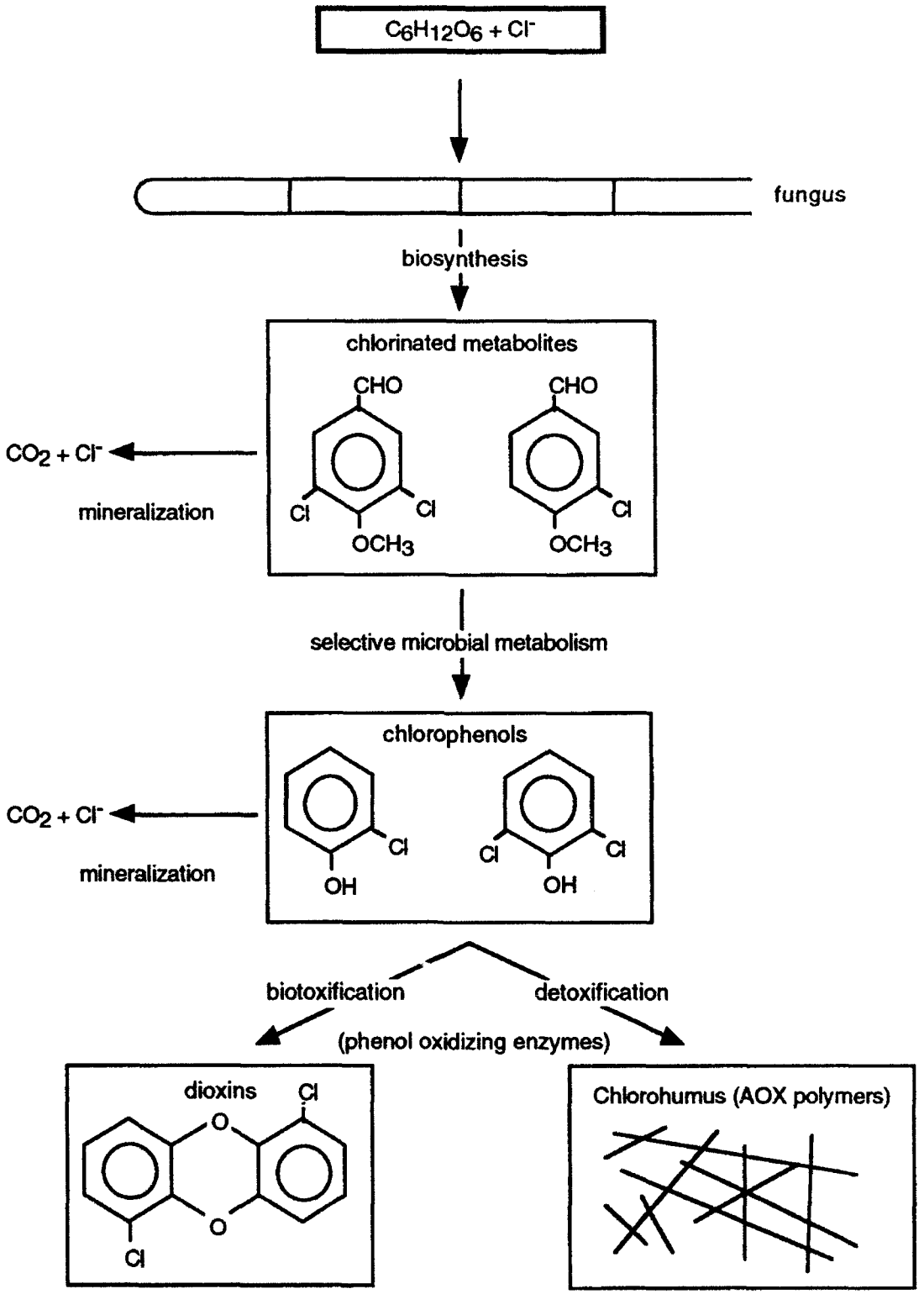

Figure 1.1. Environmental fate of fungal produced choloraromatic compounds (from reference 8).

Table 1.2. Bacteria reported to use 3-Chlorobenzoic Acid as a growth substrate.

Strain	Cleavage[a]	Origin	Reference
Pseudomonas sp.		soil,	26
Pseudomonas sp. B13	*ortho*	sewage, Göttingen, Germany	12
Pseudomonas sp. WR912		soil, Göttingen, Germany	22
Pseudomonas sp. H1 & H2		soil and sewage	21
Pseudomonas putida AC858 (pAC25)	*ortho*	sewage, Niskayuna, N.Y.	6
Alcaligenes eutrophus JMP134 (pJP4)	*ortho*	2,4-D treated soil, Australia	11
Pseudomans putida 87		pesticide-treated soil, Russia	20
Acinetobacter calcoaceticus INMI-KZ-3	*ortho*	cultivated soil Moscow region, Russia	45
Pseudomonas alcaligenes C-0		activated sludge, Ontario, Calif.	16
Pseudomonas sp. KTB4	*ortho*	soil and mud, Wuppertal area, Germany	39
Alcaligenes sp. BR60	*meta*	Bloody Run Creek, Hyde Park, N.Y.	44
Pseudomonas aeruginosa JB2		biphenyl-contaminated soil Fontana, Calif.	24
Pseudomonas putida P111		industrial sewage effluent Panama City, Panama	23

[a]ring cleavage as reported.

leading these authors to propose the first step in this 3-CBA pathway involved hydrolytic dehalogenation.

Using long-term chemostat enrichments, Dorn *et al.* (12) isolated *Pseudomonas* sp. B13 from sewage sludge. Initially the chemostats were filled with mineral salts medium amended with yeast extract and succinate and seeded with sludge. Gradually over a period of two weeks, succinate was replaced as the sole carbon source by benzoate, and ultimately, 3-CBA. After five weeks, constant turbidity and chloride production were measured in the chemostat. Phenotypic characteristics revealed this strain to be related to *Pseudomonas fluorescens.* Oxygen uptake studies demonstrated 3-CBA induced cells could simultaneously metabolize 3-CBA and benzoate using the ß-ketoadipate pathway through 3- and 4-chlorocatechol. Cells grown on benzoate alone could not immediately degrade 3-CBA and instead produced oxidized chlorinated catechols. These observations were in agreement with previous studies showing benzoate oxidase could transform 3-CBA to chlorinated metabolites, but without a functional 3-CBA pathway, these intermediates accumulate in culture fluids (25, 41). They concluded 3-CBA metabolism required a unique pyrocatechase (catechol-1,2-dioxygenase) possessing greater affinity for chlorinated catechols than the normal pyrocatecase induced during benzoic acid metabolism. This implied B13 contained two functional dioxygenase genes: one specific for catechol dioxygenase, the other a broad-specificity chlorocatechol dioxygenase. In contrast to the strain described by Johnston *et al.* (26) above, *Pseudomonas* B13 cleaved the aromatic ring prior to dechlorination. The genes for chlorocatechol degradation are encoded by the plasmid pWR1, (pB13) (34, 42).

Haller and Finn (21) substantiated the reports of Dorn *et al.* (12) by isolating two strains (H1 and H2) capable of degrading 3-CBA as a sole source

of carbon. Haller and Finn reported the isolates were probably *Pseudomonas*, and it was not clear if the isolates were similar to *Pseudomonas* B13 since a detailed description was not provided. Unlike previous studies, Haller and Finn were able to culture their isolates directly from soil and sewage without using cosubstrates or extensive enrichments. Strain H1 behaved metabolically much like *Pseudomonas* B13 and tended to produce colored metabolites from the accumulation of chlorocatechols. Strain H2, on the other hand, did not accumulate colored intermediate metabolites even following growth on benzoate.

Previous to the reports of Chatterjee *et al.* (6), the genetics of 3-CBA enzymes were unknown. Following isolation of a 3-CBA degrading *Pseudomonas putida* AC858 from sewage, they were able to tentatively localize the genes for 3-CBA degradation to a 68 MD transmissible plasmid named pAC25. The plasmid nature of the 3-CBA genes was inferred from curability and transferability experiments. In difference to *Pseudomonas* B13, this isolate metabolized chlorocatechols by *ortho* cleavage through maleylacetate in place of ß-ketoadipate. Further comparisons revealed pB13 from *Pseudomonas* B13 and pAC25 from *Pseudomonas putida* were homologous in DNA-DNA hybridization studies, and with the exception of a 6 kb deletion in pB13, physical maps of the two plasmids were nearly identical (7). Similar studies involving pAC25 and pJP4 from *Alcaligenes eutrophus* JMP134 (11), a 2,4-D degrading strain with the 3-CBA genes, found that although the structural genes had high sequence homology, gene clustering patterns of regulation differed significantly between the two strains (18). While the degradative genes were clustered on both pAC25 and pJP4, efficient expression of the pJP4 genes occurs only after genetic rearrangement.

In an exhaustive study of 760 3-CBA degrading bacteria isolated directly from agricultural soils with a long history of pesticide treatment, Grishchenkov *et al.* (20) selected 14 isolates for in-depth examination. From this collection, two stable strains, *Pseudomonas putida* 87 and *Pseudomonas putida* 203, were capable of assimilating 3-CBA after repeated cultivation on nonselective media. Only *Pseudomonas putida* 87 was studied in depth after it was found to harbor a 40 MD plasmid. In studies with 3-CBA mutants, their data suggested the catechol dioxygenase genes induced in benzoic acid grown cells were chromosomal while chlorocatechol dioxygenase genes were carried by the 40 MD plasmid. On the basis of intermediate analysis, they proposed 3-CBA metabolism proceeded through 3-chlorophenol followed by chlorocatechol and subsequent *ortho*-cleavage to chloromuconic acid.

Acinetobacter calcoaceticus INMI-KZ-3 isolated by Zaitsev and Baskunov (45) represented another non-pseudomonad obtained from cultivated soil in the vicinity of Moscow, Russia. The major difference between this strain and other previously reported degraders was in the specificity of the benzoate oxygenase. While some isolates catalyze a mixture of 3- and 4-chlorocatechol from 3-CBA, this strain could metabolized only 4-chlorocatechol to 3-chloro-*cis,cis*-muconic acid and ultimately to complete degradation and assimilation. This strain was reported to grow well on 3-CBA, but metabolites accumulate during growth and only 50-60% of the available chlorine is released as chloride. The metabolites and the balance of the chloride is presumably from 3-chlorocatechol being metabolized to dead-end products. The identity of the dead-end product was 2-chloro-*cis,cis*-muconic acid based on its UV absorption spectrum.

For most 3-CBA degrading isolates, chlorocatechols produced from 3-CBA are metabolized by *ortho*-cleaving chlorocatechol- 1,2-dioxygenases. Naturally occurring aromatic compounds, on the other hand, such as phenol,

aniline, benzene, salicylate, and substituted toluenes, are generally metabolized by *meta*-cleaving catechol-2,3-dioxygenases. Biochemically, the *meta*-cleaving enzymes tend to produce toxic dead-end products thereby inhibiting growth. Taeger *et al.* (39) were able to enrich and isolate from soil a gram negative, non-motile, short rod named strain KTB4 that expressed *ortho* and *meta* activity against 3-CBA and 3-methylbenzoate, respectively. However, these enzymes were not specific and chlorinated *meta*-cleavage products did accumulate. *Meta*-cleavage products of 3-chlorocatechol produced suicide inactivation while 4-chlorocatechol products could not be assimilated.

An alternative to the *ortho* pathway was found in *Alcaligenes* BR60 isolated from a tributary receiving surface runoff from a phenol and chlorophenol contaminated industrial landfill in Hyde Park, New York (43). Using genetic and biochemical evidence, Nakatsu (32) and Nakatsu and Wyndham (31) were able tc conclude that the 3-CBA aromatic ring is cleaved by a unique *meta*-cleavage pathway. In the case of *Alcaligenes* BR60, *meta*-cleavage did not produce toxic dead-end products. This strain carries the 3-CBA pathway on a transposable element (44) and is transferable to other hosts (17). Other investigators have found similar transposable elements in 2-chlorocatechol degrading isolates (4).

In most cases, 3-CBA degrading isolates are believed to possess relatively narrow specificity chlorobenzoate-dioxygenases. This often effectively limits the range of other degradable mono-, di-, or trichlorobenzoic acids to a single compound. Two unique isolates, *Pseudomonas aeruginosa* JB2 (24) from polychlorinated biphenyl contaminated soil and *Pseudomonas putida* P111 (23) from industrial sewage effluent, have been reported to utilize a number of chlorinated benzoates. *Pseudomonas aeruginosa* JB2 degrades 2-,

3-, 2,3-di-, 2,5-di-, and 2,3,5-trichlorobenzoate while *Pseudomonas putida* P111 degrades 2-, 3-, 4-, 2,3-di-, 2,4-di-, and 2,3,5-trichlorobenzoic acid. These authors state these two isolates provide evidence for the development of specialized chlorobenzoate pathways and regulatory strategies. It is important, however, to note P111 was obtained following 13 months of enrichment on 2,5-dichlorobenzoate and may not be representative of native degraders.

An examination of the origin of isolates in Table 1.2 indicates principle investigators were not specifically interested in obtaining materials from pristine sites. Many isolates were cultivated from samples collected from cultivated soils, contaminated sites, and wastewater treatment facilities. Potential bacterial exposure to xenobiotic substances in these habitats is drastically different from natural and undisturbed sites. Often cultivated soils and contaminated sites were purposefully selected because they experienced long-term exposures to chlorinated aromatic herbicides and pesticides. When soil sample origins were not specifically reported, it was assumed soils were collected from cultivated soil as this would be the most logical place to begin looking for isolates with novel catabolic traits. In sewage sludges, bacteria are exposed to a vast variety of organic substrates at concentrations not normally seen in natural settings. Given the heterogeneity and unpredictability of wastewater streams, it is unlikely one could rule out previous exposure to chlorinated aromatic compounds given their ubiquity in both industrial and municipal applications. Collecting samples from all of these types of communities would be in keeping with the generally held convention that 3-CBA is a xenobiotic substrate and organisms require previous exposure before degradation can be observed. Since samples were not collected in accordance with a rigorous experimental design, inferences about the distribution of 3-CBA degrading bacteria in pristine environments is not possible. Thus, it is not

known how widespread this trait is in pristine environments. These isolates do provide extensive information on the biochemical mechanisms employed by known bacteria to degrade 3-CBA.

Biochemical Pathways for 3-Chlorobenzoate Degradation

The detailed pathways for 3-CBA degradation are found in Figure 1.2. With the exception of hydrolytic dehalogenation reported by Johnston *et al.* (26), 3-CBA pathways cleave the aromatic ring prior to dechlorination. The major difference between the pathways occurs during ring cleavage. In one case, the ring is cleaved by an dioxygenase (Figure 1.2, A) while the other uses an extradiol dioxygenase (Figure 1.2, B). Early studies using the enzymes for the initial oxidation of benzoic and anthranilic acid revealed the initial dioxygenase enzymes could also use a number of substituted compounds as substrates although complete mineralization was not observed.

Using cell-free extracts, Ichihara *et al.* (25) found *3-CBA* was degraded more readily than either 2- or 4-chloro- substituents. In comparison to benzoate oxidase activity using benzoic acid, activity on 3-CBA was 29.6, 11.3, and 43.1% in *Pseudomonas aeruginosa, Micrococcus urece,* and *Pseudomonas fluorescens*, respectively. Oxygen consumption and carbon dioxide production by *Pseudomonas aeruginosa* using benzoate and 3-CBA as substrates were 10.2 and 4.2 and 4.2 and 3.9 μmoles per 5 μmoles substrate, respectively. After allowing the compounds to react, the benzoate assays were colorless while 3-CBA assays turned violet. Paper chromatography demonstrated the colored end product was 3-chlorocatechol. No catechol-1,2-dioxygenase activity was observed with 3-chlorocatechol as a substrate although 4-chlorocatechol was transformed at slow rates. These observations indicated benzoate oxidase was

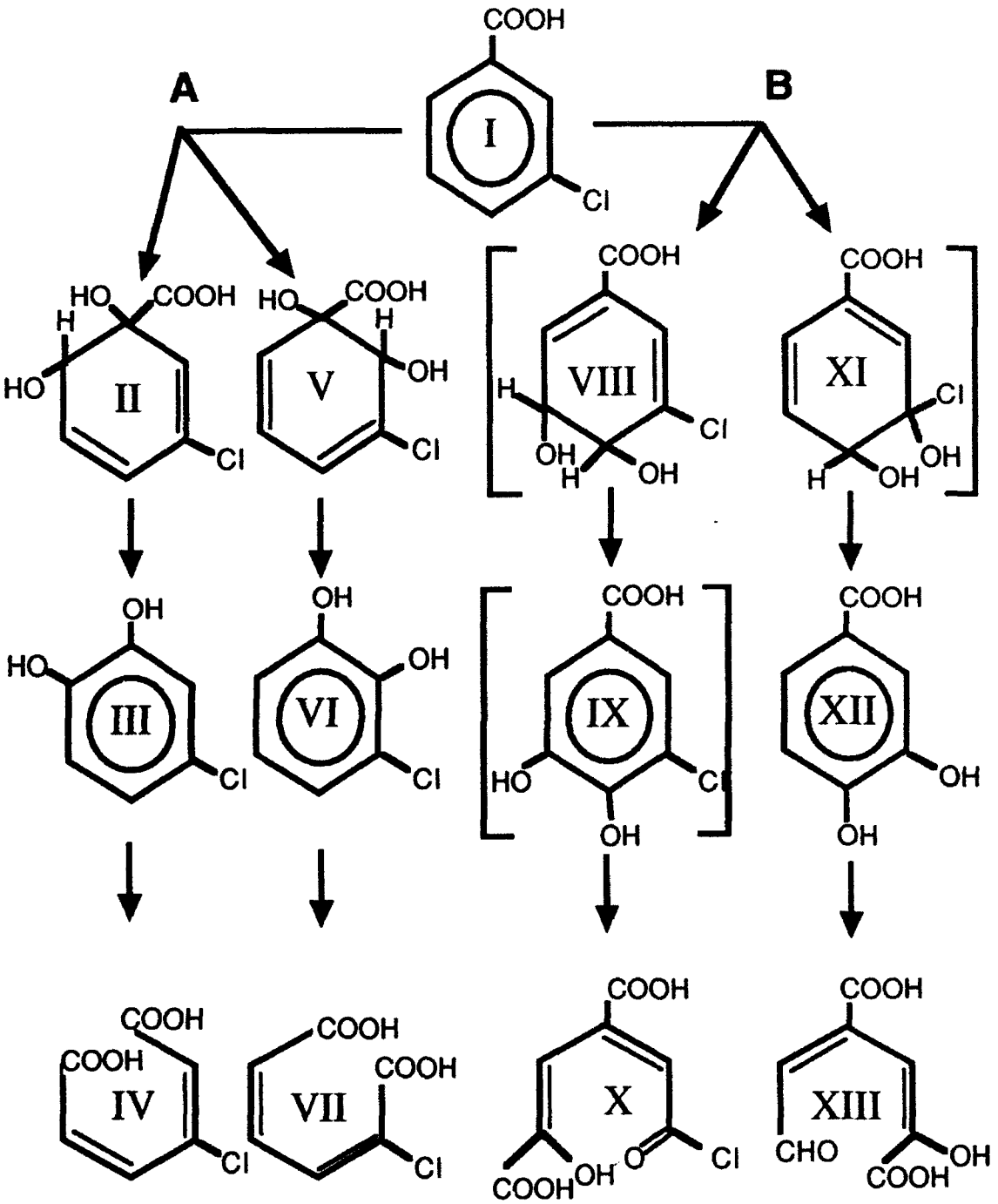

Figure 1.2. Biochemical pathways for the degradation of 3-chlorobenzoic acid. (A) The modified *ortho* pathway in *Pseudomonas* sp B13. (B) The proposed *meta* cleavage pathway of *Alcaligenes* sp. BR60. Compound I, 3-chlorobenzoic acid; II, 1-carboxy-5-chloro-1,2-dihydroxycyclohexa-3,5-diene; III, 4-chlorocatechol; IV, 3-chloro-*cis,cis*-muconic acid; V, 1-carboxy-3-chloro-1,2-dihydroxycyclohexa-3,5-diene; VI, 3-chlorocatechol; VII, 2-chloro-*cis,cis*-muconic acid; VIII, 1-carboxy-3-chloro-4,5-dihydroxycyclohexa-2,5-diene; IX, 5-chloroprotocatechuic acid; X, 2-hydroxy-4-carboxy-6-chloro-muconic semialdehyde; XI, 1-carboxy-3-chloro-3,4-dihydroxycyclohexa-1,5-diene; XII, protocatechuic acid; XIII, 2-hydroxy-4-carboxy-muconic semialdehyde (from reference 32).

able to oxidize 3-CBA but further transformation of the chlorocatechols was not possible in these strains. They correctly concluded the first stable intermediate in benzoate degradation was catechol, or chlorocatechol in the case of 3-CBA. This was early evidence for the possibility of a unique pathway for 3-CBA utilization.

In depth biochemical analysis of *Pseudomonas* sp. B13 provided evidence for the elucidation of the first entire 3-CBA pathway (Figure 1.2, A). While Ichihara *et al.* (25) found benzoate oxidase could oxidize 3-CBA (I) thus initiating degradation. Unstable dihydroxy intermediates (II and V) occur transiently and are spontaneously decarboxylated and dehydrogenated to allow rearomatization of the ring and form chlorocatechols (III and VI). Dorn and Knackmuss (13) demonstrated *Pseudomonas* sp. B13 had two catechol-1,2-dioxygenases, named pyrocatechase I and pyrocatechase II, for the subsequent degradation of catechol and chlorocatechols, respectively. Not only did the two enzymes differ in substrate affinity, but they also had different molecular weights, elution characteristics, chromatography and electrophoretic mobilities and chemical stabilities. They hypothesized *Pseudomonas* sp. B13 oxidized 3-CBA primarily through 4-chlorocatechol as 3-chlorocatechol was more resistant to ring cleavage. Subsequent studies by Schmidt *et al.* (36) and Schmidt and Knackmuss (37) found 3- and 4-chlorocatechol were transformed by 3-CBA specific enzymes to 2- and 3-chloro-*cis,cis*-muconic acid (IV and VII) and maleylacetic acid, respectively. Chloride was removed spontaneously following cycloisomerization of the muconic acids (37). The benzoate and 3-CBA pathways converge at maleylacetic acid and both form of 3-oxoadipate (27).

An alternative pathway for 3-CBA degradation (Figure 1.2, B) was found in *Alcaligenes* sp. strain BR60 (31, 32). As mentioned above, this strain carries the genes for 3-CBA degradation on a transmissible element, Tn5271. In this

strain, the aromatic ring of 3-CBA is oxidized to form either a 3,4- or 4,5-dihydroxy intermediate (VIII and XI). Dehydrogenation results in the either the loss of water and rearomatization to 5-chloroprotocatechuate (IX) or loss of chloride and rearomatization to protocatechuate (XII). Chlorocatechuate can be metabolized further but it is not known what degradation products are formed. Protocatechuate is oxidized by a chromosomally-encoded protocatechuate-4,5-dioxygenase into *meta* cleavage products. This dioxygenase is also believed to express activity on chlorinated analogs, but this has not yet been proven (31).

Summary

Chlorinated aromatic compounds are widely used in the production of herbicides and pesticides. In modern agriculture, these compounds have been used extensively to maintain high and sustained crop yields. Although generally associated with xenobiotic environmental pollutants, numerous chlorinated compounds are synthesized through natural biological and chemical processes. Therefore it is possible for bacteria to develop the 3-CBA trait from metabolism of naturally occurring chlorinated compounds produced in their environments.

Several isolates have been reported to degrade 3-CBA as a sole-source of carbon. In a few, the 3-CBA pathway was extensively studied. From these studies several conclusions can be drawn about the biochemistry and genetics of 3-CBA degradation. In general, most isolates degrade 3-CBA use an intradiol ring cleavage commonly known as the *ortho* pathway. The first degradative step involves the dioxygenation of 3-CBA to chlorocatechol by a chromosomally encoded benzoate dioxygenase. Ring cleavage is facilitated by a plasmid encoded chlorocatechol-1,2-dioxygenase to chloromuconic acid.

The lactonization and isomerization of chloromuconate releases chloride spontaneously with the formation of maleylacetate. Maleylacetate is degraded through ß-ketoadipate by enzymes of the general benzoate pathway.

In two cases, the degradation of 3-CBA was reported to differ from the pathway elucidated in B13. A unique *meta* pathway was recently described in *Alcaligenes* sp. strain BR60 (31, 32). Hydrolytic dehalogenation of 3-CBA to chloride and phenol also occurred prior to ring cleavage in a soil pseudomonad, however, this pathway was never described in detail (26).

Inferences from available data imply that although ability to degrade 3-CBA might be rare among bacterial populations in pristine ecosystems, isolates are widely distributed in nature. The distribution of 3-CBA degrading bacteria in soil with a no history of exposure to chlorinated aromatic compounds has never been the subject of rigorous investigation. Most isolates have been collected from cultivated soils with a long history of herbicide treatment, soil and water from contaminated sites, or sewage sludges. No unambiguous evidence exists for the distribution of this trait in pristine environments. Thus it is not possible to make predictions about the evolutionary history of the 3-CBA genes without the complications of previous exposure and artificially imposed natural selection.

Literature Cited

1. **Ando, K., A. Kato, and S. Suzuki.** 1970. Biochem. Biophys. Res. Commun. 39:1104.

2. **Audus, L. J.** 1951. The biological detoxification of hormone herbicide in soil. Plant Soil 2:170-192

3. **Berger, R. S.** 1972. 2,6-Dichlorophenol, sex pheromone of the Lone Star tick. Science 177:704-705.

4. **Brenner, V., B. S. Hernandez, and D. D. Focht.** 1993. Variation in chlorobenzoate catabolism by *Pseudomonas putida* P111 as a consequence of genetic alterations. Appl. Environ. Microbiol. 59:2790-2794.

5. **Bruhn, C., R. C. Bayly, and H.-J. Knackmuss.** 1988. The in vivo construction of 4-chloro-2-nitrophenol assimilatory bacteria. Arch. Microbiol. 150:171-177.

6. **Chatterjee, D. K., S. T. Kellogg, E. Hamada, and A. M. Chakrabarty.** 1981. Plasmid specifying total degradation of 3-chlorobenzoate by a modified *ortho* pathway. J. Bacteriol. 146:639-646.

7. **Chatterjee, D. K. and A. M. Chakrabarty.** 1983. Genetic homology between independently isolated chlorobenzoate-degradative plasmids. J. Bacteriol. 153:532-534.

8. **deJong, E., J. A. Field, H.-E. Spinnler, J. B. P. A. Wijenberg, and J. A. M. deBont.** 1994. Significant biogenesis of chlorinated aromatics by fungi in natural environments. Appl. Environ. Microbiol. 60:264-270.

9. **deJong, E., A. E. Cazemier, J. A. Field, and J. A. M. deBont.** 1994. Physiological role of chlorinated aryl alcohols biosynthesized de novo by the white rot fungus *Bjerkandera* sp. strain BOS55. Appl. Environ. Microbiol. 60:271-277.

10. **Dewey, O. R., R. V. Lyndsay, and G. S. Hartley.** 1962. Biological destruction of 2,3,6-trichlorobenzoic acid in soil. Nature 195:1232.

11. **Don, R. H. and J. M. Pemberton.** 1981. Properties of six pesticide degradation plasmids isolated from *Alcaligenes paradoxus* and *Alcaligenes eutrophus*. J. Bacteriol. 145:681-686.

12. **Dorn, E., M. Hellwig, W. Reineke, and H.-J. Knackmuss.** 1974. Isolation and characterization of a 3-chlorobenzoate degrading pseudomonad. Arch. Microbiol. 99:61-70.

13. **Dorn, E. and H.-J. Knackmuss.** 1978. Chemical structure and biodegradability of aromatic compounds: two catechol 1,2-dioxygenases from a 3-chlorobenzoate-grown pseudomonad. Biochem J. 174:73-84.

14. **Eisner, T., L. B. Hendry, D. B. Peakall, and J. Meinwald.** 1971. 2,5-Dichlorophenol (from ingested herbicide?) in defensive secretion of grasshopper. Science 172:277-278.

15. **Engvild. K. C.** 1986. Chlorine-containing natural compounds in higher plants. Phytochem 25:781-791.

16. **Focht, D. D. and D. Shelton.** 1987. Growth kinetics of *Pseudomonas alcaligenes* C-0 relative to inoculation and 3-chlorobenzoate metabolism in soil. Appl. Environ. Microbiol. 53:1846-1849.

17. **Fulthorpe, R. R. and R. C. Wyndham.** 1991. Transfer and expression of the catabolic plasmid pBRC60 in wild bacterial recipients in a freshwater ecosystem. Appl. Environ. Microbiol. 57:1546-1553.

18. **Ghosal, D., I.-S. You, D. K. Chatterjee, and A. M. Chakrabarty.** 1985. Genes specifying degradation of 3-Chlorobenzoic acid in plasmids pAC27 and pJP4. Proc. Natl. Acad. Sci. 82:1638-1642.

19. **Gribble, G. W.** 1992. Naturally occurring organohalogen compounds - a survey. J. Nat. Prod. 55:1353-1395.

20. **Grishchenkov, V. G., I. E. Fedevichkina, B. P. Baskunov, L. A. Anisimova, A. M. Boronin, and L. A. Gololeva.** 1984. Degradation of 3-chlorobenzoic acid by a *Pseudomonas putida* strain. Mikrobiol. 52:771-776.

21. **Haller, H. D. and R. K. Finn.** 1979. Biodegradation of 3-chlorobenzoate and formation of black color in the presence and absence of benzoate. European J. Appl. Microbiol. 8:191-205.

22. **Hartmann, J., W. Reineke, H.-J. Knackmuss.** 1979. Metabolism of 3-chloro-, 4-chloro-, and 3,5-dichlorobenzoate by a Pseudomonad. Appl. Environ. Microbiol. 37:421-428.

23. **Hernandez, B.S., F. K. Higson, R. Knodrat, and D. D. Focht.** 1991. Metabolism of and inhibition by chlorobenzoates in *Pseudomonas putida* P111. Appl. Environ. Microbiol. 57:3361-3366.

24. **Hickey, W. J. and D. D. Focht.** 1990. Degradation of mono-, di-, and trihalogenated benzoic acids by *Pseudomonas aeruginosa* JB2. Appl. Environ. Microbiol. 56:3842-3850.

25. **Ichihara, A., K. Adachi, K. Hosokawa, and Y. Takeda.** 1962. the enzymatic hydroxylation of aromatic carboxylic acids; substrate specificities of anthranilate and benzoate oxidases. J. Biol. Chem. 237:2296-2302.

26. **Johnston, H. W., G. G. Briggs, and M. Alexander.** 1972. Metabolism of 3-chlorobenzoic acid by a pseudomonad. Soil Biol. Biochem. 4:187-190.

27. **Kaschabek, S. R. and W. Reineke.** 1992. Maleylacetate reductase of *Pseudomonas* sp. strain B13: dechlorination of chloromalelylacetates, metabolites in the degradation of chloroaromatic compounds. Arch. Microbiol. 158:412-417.

28. **Krockel, L., and D. D. Focht.** 1987. Construction of chlorobenzene-utilizing recombinants by progenitive manifestation of a rare event. Appl. Environ. Microbiol. 53:2470-2475.

29. **Latorre, J., W. Reineke, and H.-J. Knackmuss.** 1984. Microbial metabolism of chloroanilines: enhanced evolution by natural genetic exchange. Arch. Microbiol. 140:159-165.

30. **MacRae, I. C. and M. Alexander.** 1965. Microbial degradation of selected herbicides in soil. J. Agr. Food Chem. 13:72-76.

31. **Nakatsu, C. H. and R. C. Wyndham.** 1993. Cloning and expression of the transposable chlorobenzoate-3,4-dioxygenase genes of *Alcaligenes* sp. strain BR60. Appl. Environ. Microbiol. 59:3625-3633.

32. **Nakatsu, C. H.** 1992. Ph.D. thesis. Carleton University.

33. **Ramos, J. L., and K. N. Timmis.** 1987. Experimental evolution of catabolic pathways of bacteria. Microbiol. Sci. 4:228-237.

34. **Reineke, W., S. W. Wessels, M. A. Rubio, J. Latorre, U. Schwien, E. Schmidt, M. Schlomann, and H. J. Knackmuss.** 1982. Degradation of monochlorinated aromatics following transfer of genes encoding chlorocatechol catabolism. FEMS Microbiol. Lett. 14:291-294.

35. **Rojo, F., D. H. Pieper, K.-H. Engesser, H.-J. Knackmuss, and K. N. Timmis.** 1987. Assemblage of ortho cleavage route for simultaneous degradation of chloro- and methylaromatics. Science 238:1395-1398.

36. **Schmidt, E., G. Remberg, and H.-J. Knackmuss.** 1980. Chemical structure and biodegradability of halogenated aromatic compounds: halogenated muconic acids as intermediates. Biochem. J. 192:331-337.

37. **Schmidt, E. and H.-J. Knackmuss.** 1980. Chemical structure and biodegradability of halogenated aromatic compounds: conversion of chlorinated muconic acids into maleylacetic acid. Biochem. J. 192:339-347.

38. **Stotzky, G.** 1989. Gene transfer among bacteria in soil. In, S. B. Levy and R. V. Miller (ed.), Gene transfer in the environment, McGraw-Hill, Inc., New York.

39. **Taeger, K., H. J. Knackmuss, and E. Schmidt.** 1988. Biodegradability of mixtures of chloro- and methylsubstituted aromatics: simultaneous degradation of 3-chlorobenzoate and 3-methylbenzoate. Appl. Microbiol. Biotechnol. 28:603-608.

40. **van der Meer, J. R., W. M. de Vos, S. Harayama, and A. J. B. Zehnder.** 1992. Molecular mechanisms of genetic adaptation to xenobiotic compounds. Microbiol. Rev. 56:677-694.

41. **Walker, N. and D. Harris.** 1970. Metabolism of 3-chlorobenzoic acid by *Azotobacter* species. Soil Biol. Biochem. 2:27-32.

42. **Weisshaar, M.-P., F. C. H. Franklin, and W. Reineke.** 1987. Molecular cloning and expression of the 3-chlorobenzoate-degrading genes from *Pseudomonas* sp. strain B13. J. Bacteriol. 169:394-402.

43. **Wyndham, R. C. and N. A. Straus.** 1988. Chlorobenzoate catabolism and interactions between *Alcaligenes* and *Pseudomonas* species from Bloody Run Creek. Arch. Microbiol. 150:230-236.

44. **Wyndham, R. C., R. K. Singh, and N. A. Straus.** 1988. Catabolic instability, plasmid gene deletion and recombination in *Alcaligenes* sp. BR60. Arch. Microbiol. 150:237-243.

45. **Zaitsev, G. M. and B. P. Baskunov.** 1985. Utilization of 3-chlorobenzoic acid by *Acinetobacter*. Mikrobiol. 54:203-208.

Chapter Two

Distribution and Transformation Capacity of 3-Chlorobenzoic Acid Degrading Bacteria in Geographically-Separated Pristine Soils

Introduction

In comparison to naturally-occurring organic compounds, man-made chlorinated aromatics are relatively recent additions to the extensive list of compounds bacteria experience and metabolize in natural environments. As a result, these compounds are markedly more recalcitrant than structurally-similar nonhalogenated compounds. Although man-made halogenated compounds have only been synthesized in large amounts for approximately 50 years, bacteria have evolved degradative systems to deal with xenobiotics (14). Catabolic pathways have either evolved in nature due to widespread use of chlorinated aromatic compounds or are a preadapted variant of a pathway that fortuitously degrades xenobiotic compounds (5). The evolution of catabolic activity against xenobiotic chlorinated compounds assumes bacteria experience appropriate selective pressures after exposure to a compound and evolutionary processes occur rapidly in natural communities. Thus, only organisms in habitats experiencing chloroaromatic compounds, such as sewage sludge (7), agricultural soils (16), or contaminated sites (1, 11, 27), would be expected to have sufficient selective pressure for the evolution of novel catabolic traits for the complete degradation of xenobiotic compounds. Alternatively, genetic and biochemical preadaptation allows bacteria to degrade naturally-occurring and structurally-similar xenobiotic analogs without the requirement for previous exposure. A plausible explanation for this phenomenon is the natural variation of substrate specificity among catabolic enzymes found in aromatic degrading populations. This mechanism can explain the occurrence of catabolic activity against man-made halogenated compounds in pristine environments; those with no documented exposure to man-made chloroaromatic compounds. In order to determine if this

mechanisms is observable in nature, this study examined the presence of chloroaromatic catabolic traits in communities with no previous exposure to these compounds using 3-chlorobenzoic acid (3-CBA) as a model substrate.

Chlorobenzoates are known to occur naturally, but they are reported to be present only in recent volcanic ash (12). Although numerous investigators have isolated and characterized 3-chlorobenzoic acid (3-CBA) mineralizing bacteria from soil (6, 13, 15, 16, 17, 19, 25, 29), there is no clear evidence 3-CBA degrading bacteria are widely distributed in pristine environments. Since many of these isolates were cultured from sites with histories of exposure to chloroaromatics, questions of genetic preadaptation can not be addressed. In addition, several reports indicate 3-CBA is potentially recalcitrant in soil and aquatic habitats (4, 8, 10, 11, 18, 21) implying the 3-CBA trait is potentially rare in natural communities. Early investigators shared the view that chlorinated benzoates were not normal physiological compounds (15) and were surprised by the number of organisms which have evolved this xenobiotic degrading trait (16). Since the distribution of 3-CBA degrading bacteria in undisturbed habitats is not known, and 3-CBA is not expected to be present in soil without human intervention, understanding the distribution of the 3-CBA phenotype in pristine environments may provide insight into the genetic origin and dissemination of other chloroaromatic catabolic pathways.

In this study, soil samples were systematically collected from six geographically-separated pristine soil communities to assess the biogeography of 3-CBA degrading bacteria. In this chapter, I describe the distribution of catabolic activity in these soils measured by $^{14}CO_2$ evolution from 3-CBA-UL-ring-^{14}C, the site specific variation in degradation rates, and the transformation capacities of pure culture isolates in defined medium. I conclude that the 3-CBA

phenotype is widely distributed in the pristine soil communities sampled. Also, the 3-CBA degrading populations is metabolically diverse based on $^{14}CO_2$ evolution and HPLC transformation studies. These findings suggest the 3-CBA phenotype is derived from degradation of naturally-occurring aromatic compounds which predispose bacteria for the degradation of structurally related chlorinated compounds and not from previous exposure to anthropogenic chlorinated aromatic compounds.

Materials and Methods

Soil sampling. Soils were collected aseptically from five different sites within six geographically-separated regions in two ecosystem types: Mediterranean sclerophyllous woodlands and boreal forests (Table 2.1, Figures 2.1 to 2.6). If possible, sites were within nature preserves and park lands to insure pristine conditions. When these criteria were not met, sites were chosen from areas with no documented exposure to chloroaromatic pesticides. In all cases, the soils were undisturbed. Mediterranean sites were sampled in the spring while boreal forest sites were sampled in the summer. Approximately 30 individual soil samples were collected at evenly spaced locations along a 200 m transect. At the sampling site, the surface organic layer and 1 to 2 cm of the mineral soil was removed. Samples were collected as 25 cm deep cores using a 2.5 cm diameter core sleeve. Soil cores were placed into individual plastic bags and stored on ice until they could be shipped to the laboratory. Between samples, excess soil was removed from the coring device by brushing with a wire brush followed by dipping the entire corer in 70% ethanol. The corer was then flame sterilized to prevent cross contamination between samples.

Table 2.1. Locations and characteristics of global soil sampling sites.

Site[a]	Climate	FAO[b] Soil Class	Vegetation	Coordinates[c]	pH	Moisture Content (%)
Australia[1]						
Bridgetown (BN)	Mediterranean	Chromic luvisol	sclerophyllous, *Eucalyptus*, Acacia	34°S - 116°15'E	6.58	0.18
Geraldton (GE)				N.R.	7.21	0.03
Jarrahdale (JD)				32°23' - 116° - 07'	6.66	0.15
Kellerberrin (KE)				31° 25' - 117° 46'	6.04	0.06
Merredin (ME)				31°23' - 118° 41'	9.09	0.09
California[2]						
Chabot (CH)	Mediterranean	Chromic luvisol	sclerophyllous, oak meadow, *Eucalyptus* chapparal	37°45'N - 122°10'W	6.16	0.14
Cloverdale (CL)				40°29' - 122°29'	5.80	0.14
Hillgate (HG)				40°10' - 122°30'	6.39	0.17
Murrieta (MU)				33°25' - 117°13'	6.04	0.14
Venice Hills (VH)				36°20' - 119°41'	6.42	0.11
Chile[3]						
Fray Jorge (FJ)	Mediterranean	Chromic luvisol	sclerophyllous oak meadow, *Eucalyptus* chapparal	30° 38'S - 71°35'W	6.80	0.26
Los Campanas (LC)				32°57' - 71°05'	6.30	0.11
Lagos Peñeulas (LP)				N.R.	6.10	0.10
Rio Clarillo (RC)				33°51' - 70°29'	6.35	0.08
Eucalyptus Grove (EG)				N.R.	n.d.	0.04

Table 2.1 (cont'd).

Site	Climate	FAO Soil Class	Vegetation	Coordinates	pH	Moisture Content (%)
South Africa[4]						
Helshoogte (HH)	Mediterranean	Chromic luvisol	sclerophyllous, renosterveld, fynbos, *Eucalyptus*	33°55'S - 18°55'E	5.45	0.09
Mooresburg (MB)				33°04' - 18°40'	6.23	0.05
Mamreweg (MR)				33°28' - 18°28'	5.76	0.08
Paarl Mountain (PM)				33°44' - 18°56'	6.09	0.14
Welgevallen (WG)				33°57' - 18°52'	6.04	0.12
Russia[5]						
R1	Temperate	Albic luvisol	boreal forest, spruce, poplar, birch	60-61°N - 38-39°E[d]	4.87	0.38
R2					4.83	0.29
R3					4.59	0.33
R4					4.81	0.27
Saskatchewan[4]						
Bittern (BT)	Temperate	Albic luvisol	boreal forest, jack pine, spruce, poplar, birch	53°55'N - 105°27'W	5.68	0.14
Napatak (NP)				54°52' - 105°21'	5.82	0.22
Porcupine (PC)				52°39' - 102°23'	6.35	0.29
Waskesiu (WK)				54°24' - 106°35'	6.05	0.13
Waitville (WV)				53°41' - 105°22'	3.10	0.28

[a]Samples collected by: [1]J. Tiedje and I. Dadour; [2]R. Fulthorpe and A. Rhodes; [3]P. Ravest; [4]R. Fulthorpe; [5]Russian Academy of Sciences.

[b] Food and Agriculture Organization of the United Nations

[c] Approximate coordinants. N.R., Not Reported

[d] All sites sampled within these coordinates

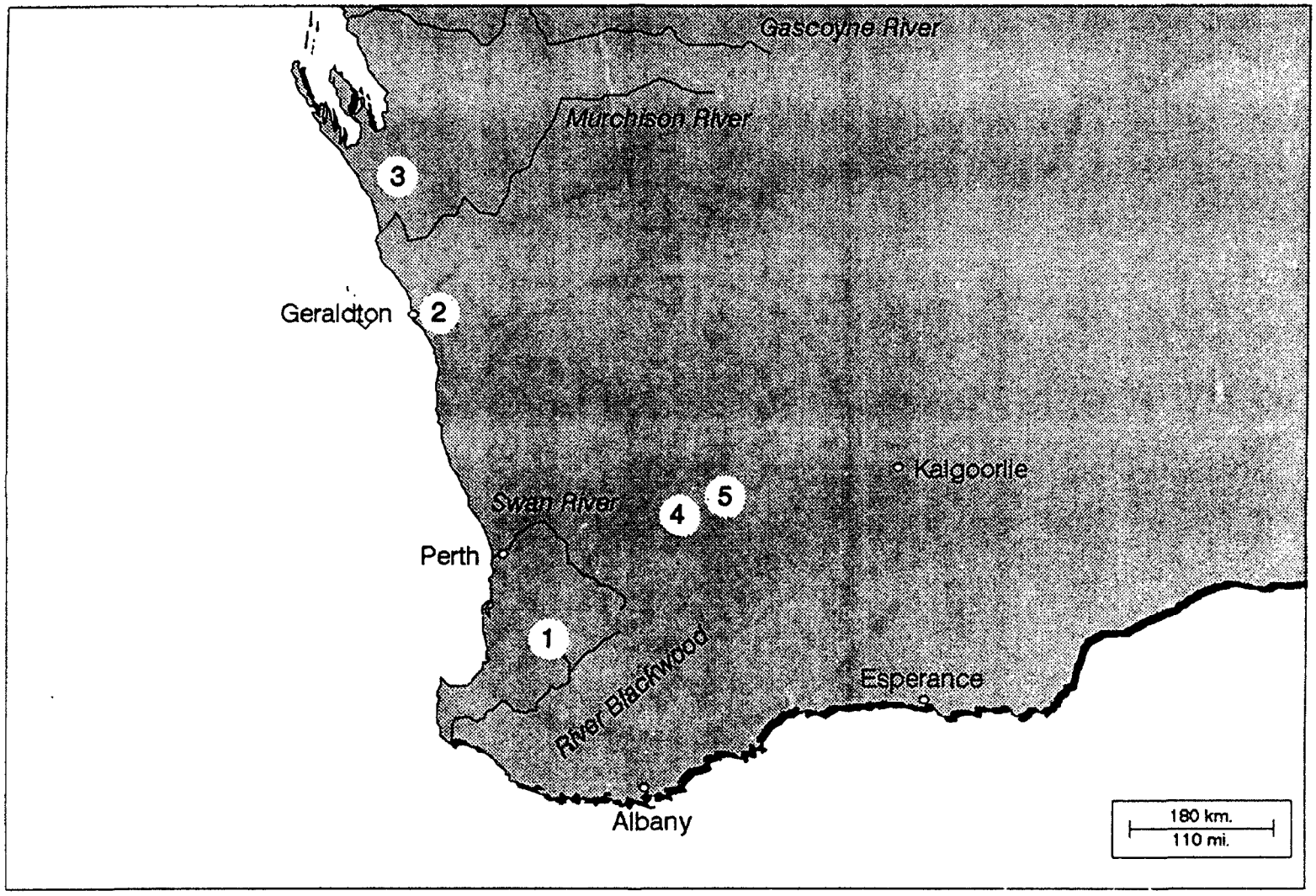

Figure 2.1. Location of sampling sites within Australia. Site names are: 1, Bridgetown; 2, Geralton; 3, Jarrahdale; 4, Kellerberrin; and 5, Merredin.

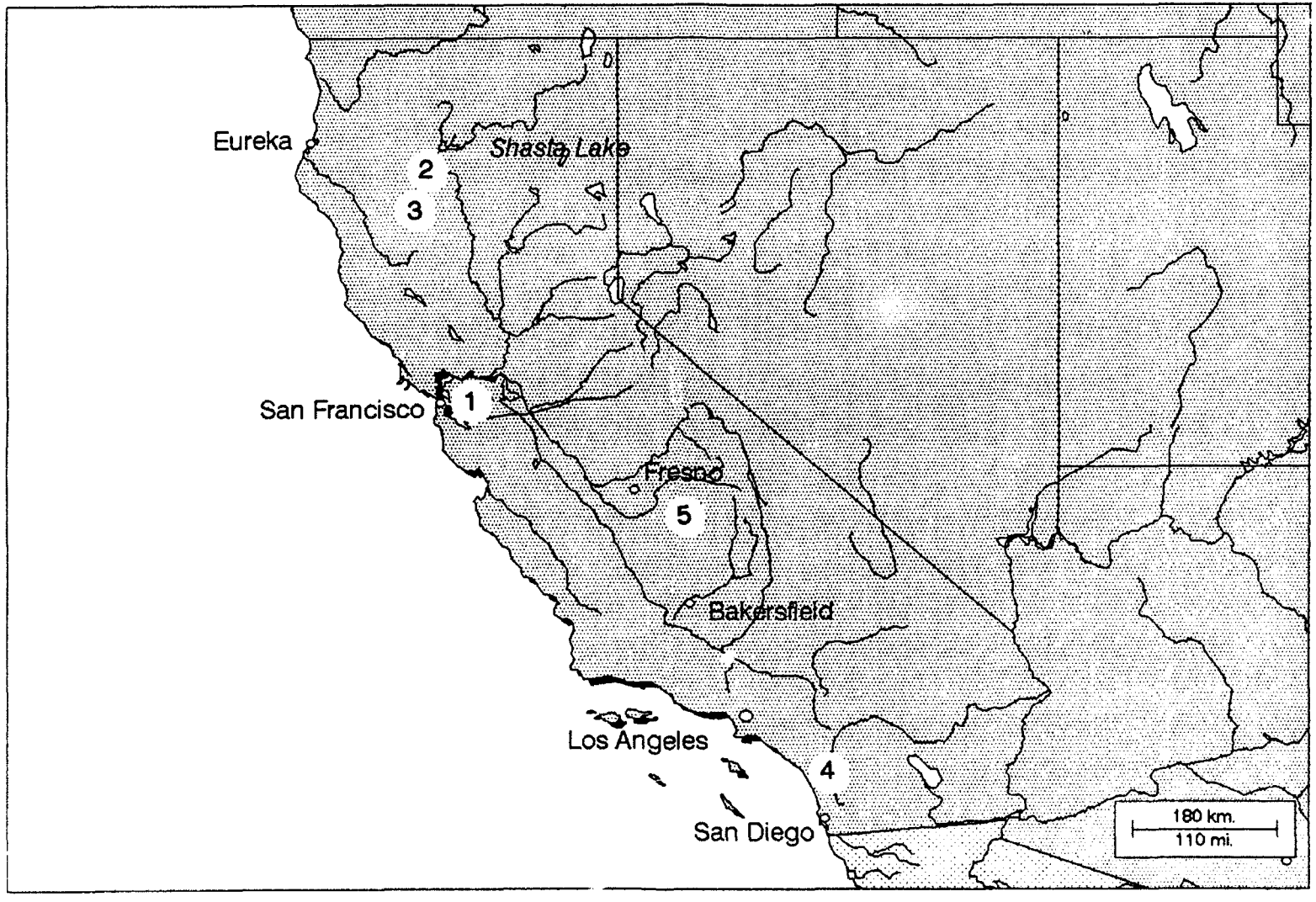

Figure 2.2. Location of sampling sites within California. Site names are: 1, Chabot; 2, Cloverdale; 3, Hillgate; 4, Murrieta; and 5, Venice Hills.

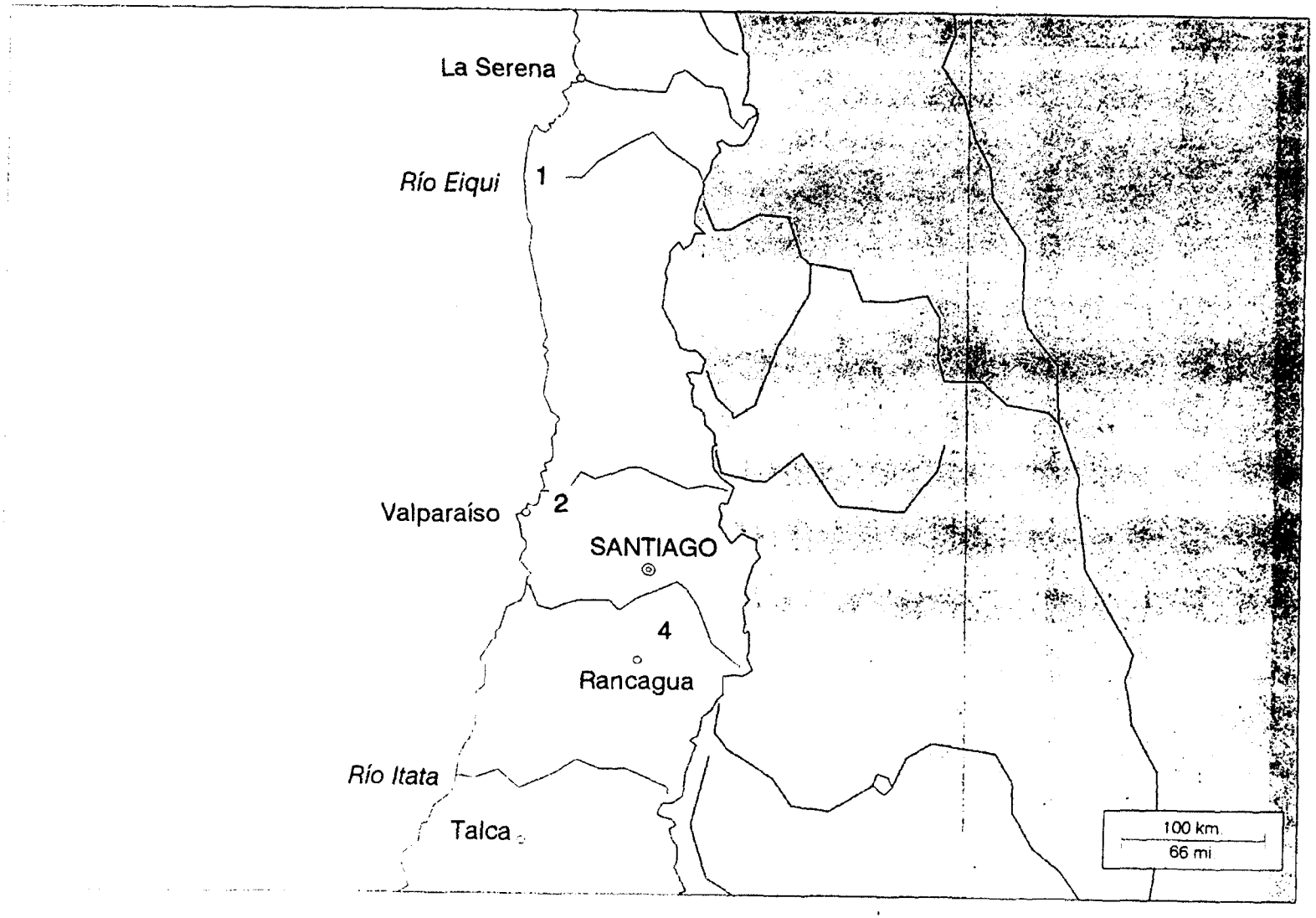

Figure 2.3. Location of sampling sites within Chile. Site names are: 1, Fray Jorge; 2, Los Campanas; 3, Los Peñeulas; and 4, Rio Clarillo.

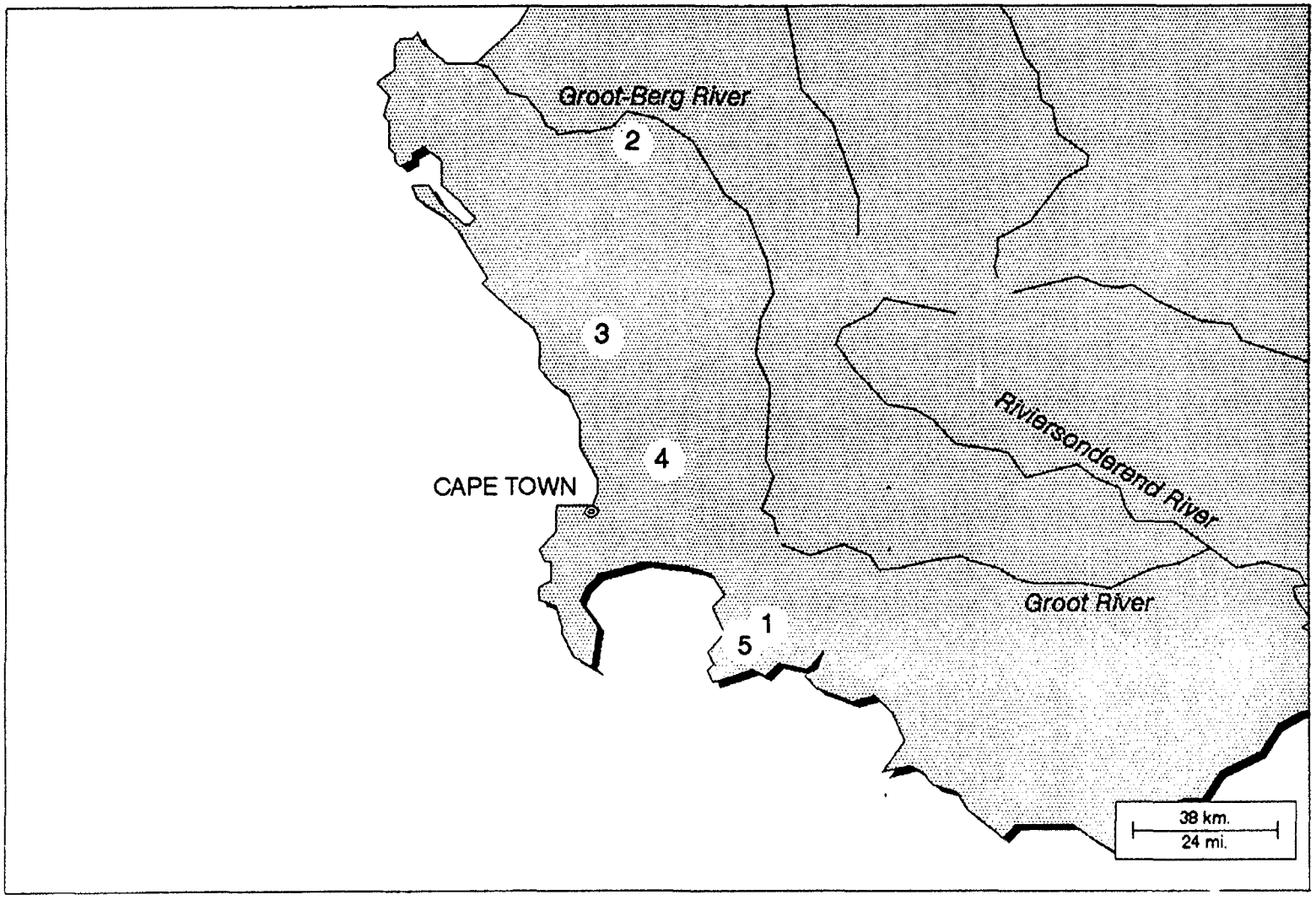

Figure 2.4. Location of sampling sites within South Africa. Site names are: 1, Helshoogte; 2, Mooresburg; 3, Mamreweg; 4, Paarl Mountain; and 5, Welgevallen.

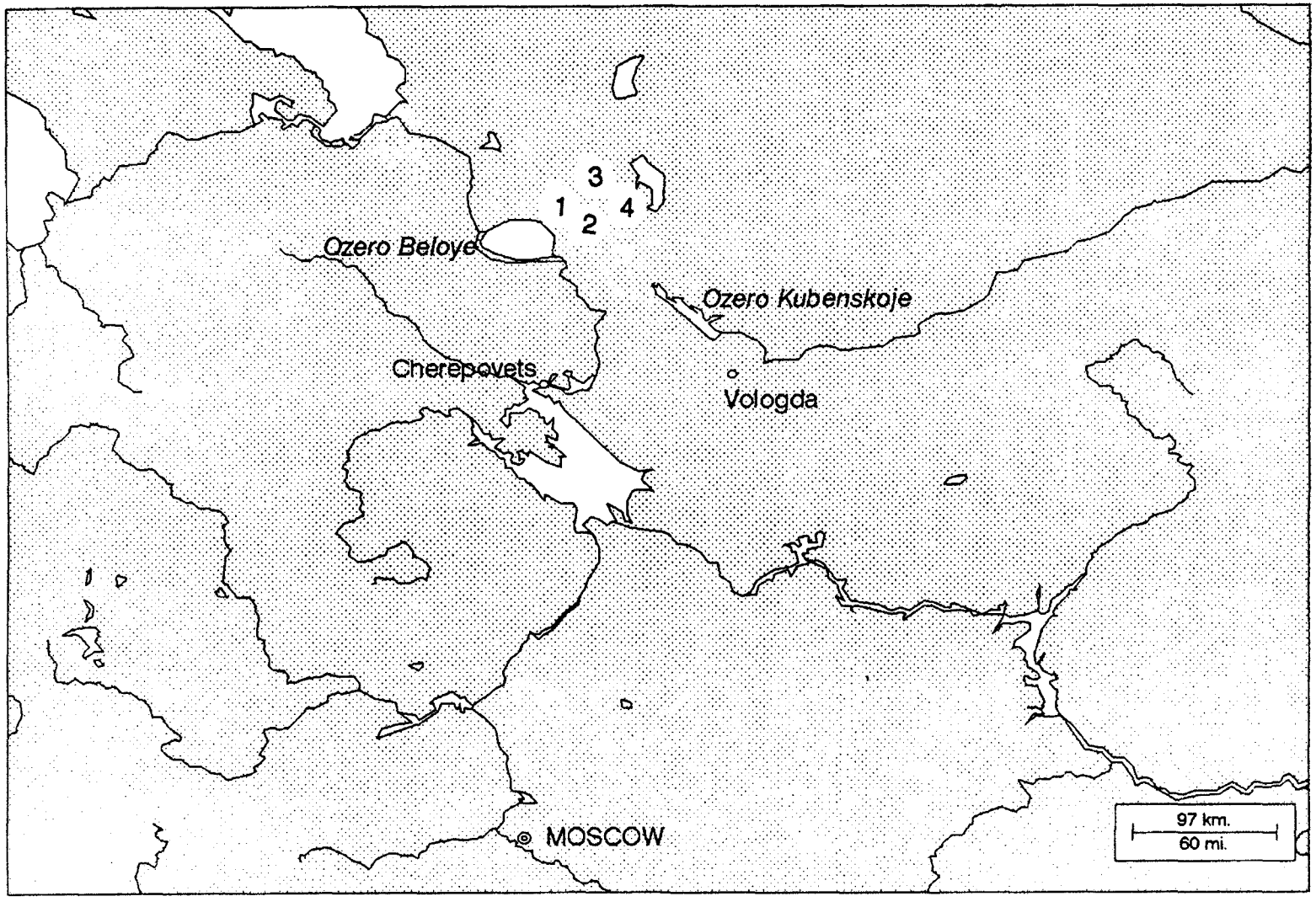

Figure 2.5. Location of sampling sites within Russia. Site names are: 1, R1; 2, R2; 3, R3; and 4, R4.

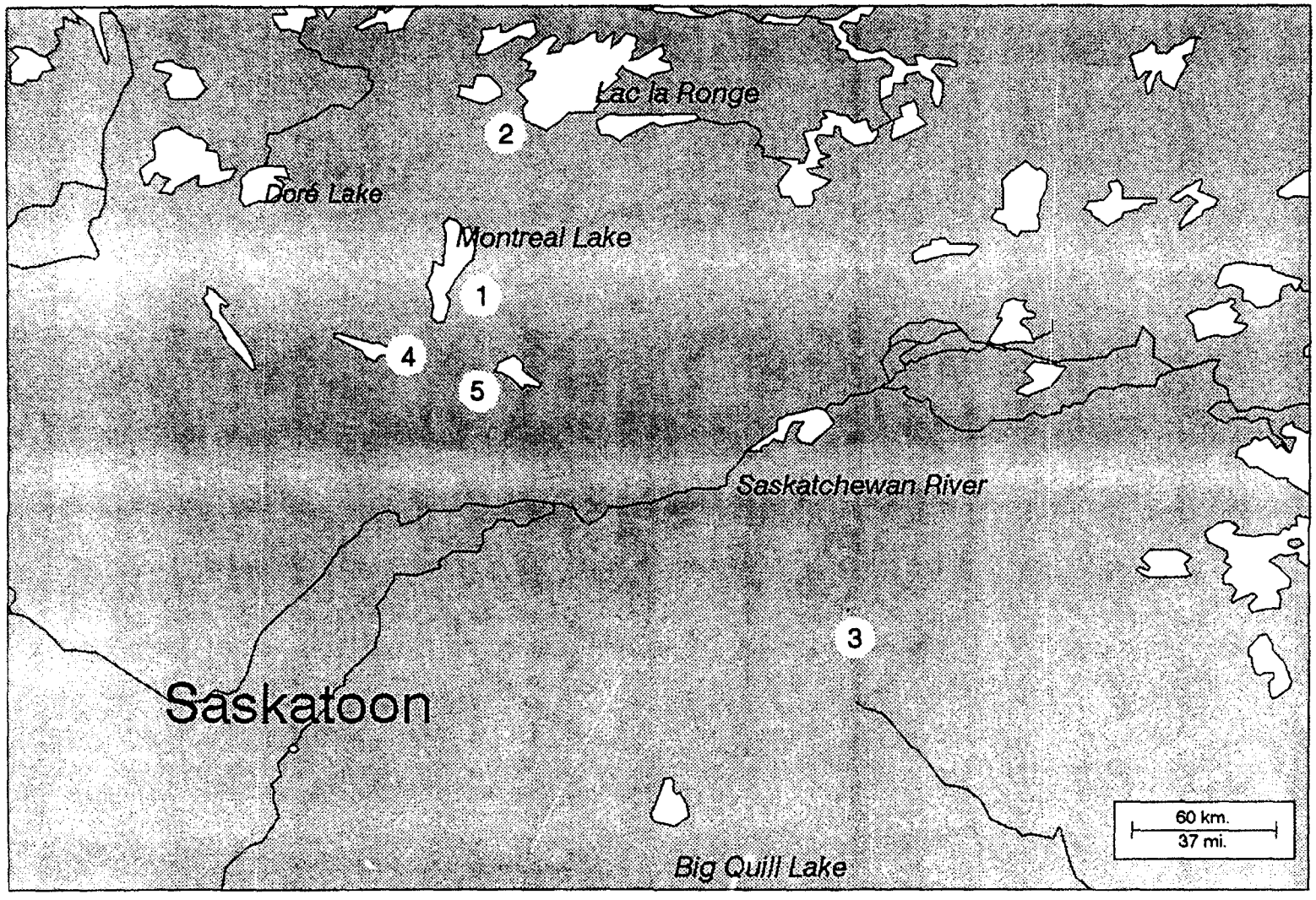

Figure 2.6. Location of sampling sites within Saskatchewan. Site names are: 1, Bittern; 2, Napatak; 3, Porcupine; 4, Waskesiu; and 5, Waitville.

Once in the laboratory, samples were immediately stored at 4°C and soil moisture content was determined by drying overnight at 100° C. Soil pH was determined on a randomly selected subset of the samples by suspending 1.0 g soil in 1 ml distilled water. Soil pH was measured by test paper and electrode.

Media and reagents. Defined aerobic basal medium (DAB, Table 2.2) is a phosphate buffered, mineral salts medium amended with amino acids, vitamins, and elements common required by fastidious aerobic bacteria (9). DAB salts medium (DABS) was used for dilution fluid and is of the same composition as DAB but without vitamin and amino acid amendment. Chloride-free A+N medium was prepared following the formulation of Wyndham (28). 3-Chlorobenzoic acid, 99+%, was purchased from Aldrich Chemical Comp., Inc. (Milwaukee, Wisc.) and 3-CBA-UL-ring-^{14}C, (98%, 11.4 mCi mol^{-1}) was obtained from California Bionuclear Corp. (Los Angeles, Calif.)

Analytical methods. Disappearance of 3-CBA in broth culture was determined using a Hewlett-Packard (Palo Alto, Calif.) Series 1050 high performance liquid chromatograph (HPLC) equipped with a LiChrosorb RP-18 (10 µm) reverse phase column (E.Merck, Darmstadt, Germany) and a multiple wavelength detector simultaneously scanning 218, 230, and 280 nm. Mobile phase was 70% methanol:30% 0.1% aqueous H_3PO_4. Chromatograms were recorded and integrated using Hewlett-Packard ChemStation® software running on a Gateway 2000 personal computer (North Sioux City, So. Dak.). Chloride release from 3-CBA was quantified after Bergmann and Sanik (2) using Spectroquant® reagents (E. Merck, Darmstadt, Germany) and measured spectrophotometrically at 450 nm with a Bio-Tek EL312e automated plate reader (Bio-Tek Instruments, Inc., Winooski, Ver.). Quantification of $Ba^{14}CO_3$ in alkaline blotting paper traps was achieved using an AMBIS Radioanalytic

Table 2.2. Composition of defined aerobic basal medium (DAB). All quantities are $mg \cdot l^{-1}$ distilled water.

Part A			
$Na_2HPO \cdot 7H_2O$	2680.0	Pantothenic acid	0.025
KH_2PO_4 (anhyd.)	1740.0	L-glutamate	0.94
NH_4Cl	1060.0	L-leucine	0.38
$Na_2EDTA \cdot 2H_2O$	5.0	L-proline	0.32
$FeSO_4 \cdot 7H_2O$	2.0	L-lysine	0.30
$MnCl_2 \cdot 2H_2O$	1.86	L-serine	0.30
$Na_2MoO_4 \cdot 2H_2O$	1.53	L-isoleucine	0.26
H_3BO_3	0.3	L-tyrosine	0.26
$CoCl_2 \cdot 6H_2O$	0.321	L-valine	0.26
$ZnSO_4 \cdot 7H_2O$	0.161	L-aspartate	0.25
$NiCl_2 \cdot 6H_2O$	0.02	L-alanine	0.22
$CuCl_2 \cdot 2H_4O$	0.01	L-pheylalanine	0.20
Pyridoxine•HCl	0.05	L-arginine	0.18
Thiamine•HCl	0.025	L-threonine	0.16
Nicotinic Acid	0.025	L-methionine	0.14
p-Aminobenzoic Acid	0.025	L-asparagine	0.10
Biotin	0.01	L-histidine	0.08
Folic Acid	0.01	L-tryptophan	0.04
Pyridoxal Phosphate	0.0005	L-glycine	0.02
Riboflavin	0.025	L-hydroxyproline	0.02
Thioctic Acid	0.025		

Part B	
$Na2SO_4$	140.0
$MgCl_2 \cdot 6H_2O$	102.0
$CaCl_2$ (anhyd.)	88.0

Imaging System (AMBIS, Inc., San Diego, Calif.) equipped with a 3.2 mm x 3.2 mm resolution screen and scanned for 60 min. Scans were calibrated against known amounts of $^{14}CO_2$ (Figure 2.7).

3-CBA catabolic activity in soil amendments. Enrichment protocols were designed to use autoradiographic determination of $^{14}CO_2$ evolution from soil amended with radioisotopically-labelled 3-CBA in microtiter plate incubations (24). A schematic representation of the protocols used in this study are illustrated in Figure 2.8. In primary enrichments twenty-four samples were selected from the 30 samples representing each site. A 1.0 g•dry•weight equivalent of each soil sample was placed separately into the wells of a 24-well tissue culture plate (Falcon #3047, Beckton Dickinson and Comp., Lincoln Park NJ). Concentrated aqueous stock solutions of unlabeled 3-CBA and 3-CBA-UL-ring-^{14}C were added to each sample to obtain 50 μg 3-CBA g•dry•soil^{-1} and 0.05 μCi g•dry•soil^{-1}, respectively. Sufficient sterile distilled water was added to the substrate stock solutions to adjust the final soil moisture content to 25%. This aqueous solution was evenly distributed over the surface of the soil. Approximately 5 ml sterile distilled water was placed in the voids between adjacent wells to reduce desiccation. Plates were covered with sterile blotting paper (GB002, Schleicher and Schuell, Keene, New Hamp.) premoistened with a saturated solution of BaOH to serve as a $^{14}CO_2$ trap. Plates were incubated in sealed plastic containers at room temperature until all samples evolved CO_2 or for up to 2 months, whichever was longer. Blotting paper traps were removed weekly for the first month and exposed to x-ray film (Kodak X-Omat AR, Rochester, New York) for autoradiography. After the first month, CO_2 traps were replaced biweekly. 3-CBA mineralization by indigenous microorganisms

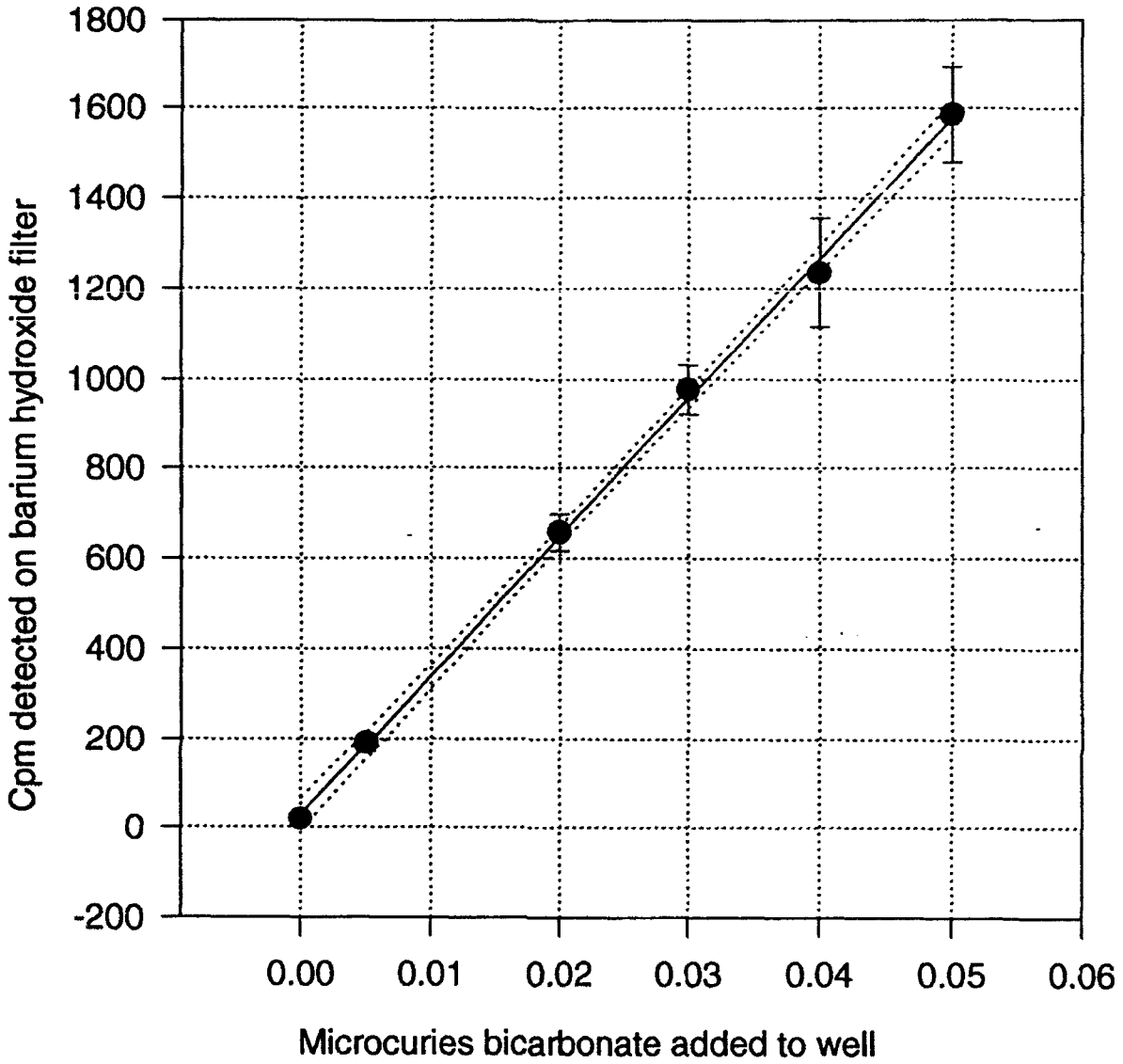

Figure 2.8. Correlation between counts per minute of $^{14}CO_2$ detected in BaOH filters with µCi $Na^{14}CO_3$ added to each well of a 24-well tissue culture plate. Analyses were performed in triplicate. Both sides of the filter were quantified using an AMBIS Radioanalytic detector. Errors bars represent 1 standard deviation from the mean while dashed lines represent 95% confidence interval for regression line (r^2=0.996).

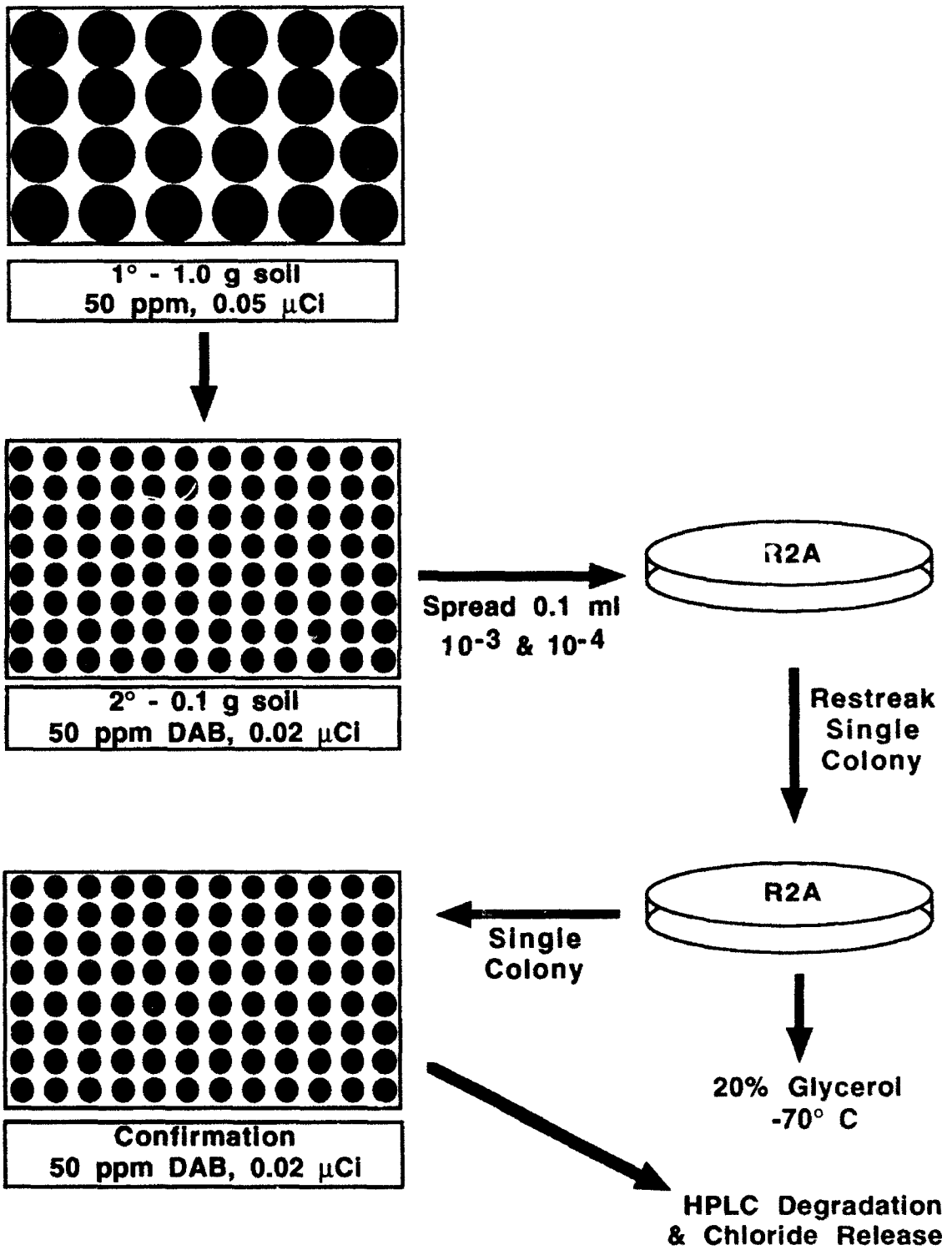

Figure 2.8. Schematic representation of enrichment and isolation protocols used to obtain 3-chlorobenzoic acid degrading bacteria from geographically-separated soils.

was determined based on the presence or absence of exposed regions of the autoradiograph corresponding to individual wells. In total, 687 soil samples were examined for their ability to mineralize 3-CBA.

Soil samples exhibiting activity were transferred to secondary enrichments in DAB. Approximately 0.1 g of well blended soil from each of the positive primary enrichment wells was transferred into 1.0 ml sterile distilled water and mixed by vortexing for 30 seconds. A 40 µl aliquot of soil suspension was transferred into individual wells along in the top row of wells of 96-well microtiter plates (Falcon 3077, Beckton Dickinson and Comp., Lincoln Park, NJ) filled with 160 µl DAB amended with 50 µg 3-CBA ml^{-1} and 0.1 µCi ml^{-1} (0.02 µCi $well^{-1}$). Suspensions in the top row were serially diluted five-fold into the remaining wells with a final dilution of 2.56×10^{-6}. The plates were covered with a BaOH saturated blotting paper trap, and incubated up to 4 weeks at room temperature. Blotting paper traps were removed weekly and autoradiographs exposed as above. When activity was first observed on autoradiographs, the most dilute well mineralizing the substrate was used for isolation of bacteria responsible for degradation.

Isolation of 3-CBA degrading bacteria. From the highest-dilution secondary-enrichment well releasing $^{14}CO_2$, 20 µl were transferred into 180 µl of DABS in 96-well microtiter plates and serial dilutions were performed through 10^{-4} using a multichannel pipettor. R2A agar plates were inoculated with 100 µl from each of the 10^{-3} and 10^{-4} dilutions, spread with a sterile glass rod, and incubated from 2-5 days at room temperature until colonies sizes were large enough for transfer. A single colony representative of each morphology present on the isolation plate was streaked onto R2A and incubated at room

temperature for 2-5 days to insure purity. Single, well-isolated, colonies were transferred, in parallel, from purification plates to 3-CBA confirmation medium and phosphate-buffered 20% glycerol for storage at -70° C.

Confirmation of 3-CBA degradation. Medium for confirmation of 3-CBA degradation was the same as used for secondary enrichment. Isolates were transferred from R2A plates using sterile wooden applicators into individual wells of a 96-well microtiter plate containing 200 µl of 50 µg 3-CBA ml^{-1} DAB. Plates were covered with traps as above. Autoradiography was performed on traps every 2 weeks Glycerol stocks of isolates releasing $^{14}CO_2$ in confirmation tests were inoculated into 3.0 ml of 50 µg 3-CBA ml^{-1} A+N broth. Percent 3-CBA disappearance was determined by HPLC and the accumulation of colored metabolites, if present, recorded at 7 days and 21 days Chloride release was monitored spectrophotometrically at 21 days as described above. As a test for phenotypic stability, 24 h. cultures were transferred five times in R2A broth prior to 3-CBA HPLC tests (3).

Cluster analysis. Dendrograms of 3-CBA transformation capacities were generated using NTSYS software (Applied Biostatistics, Inc., Setauket, N.Y.). An Euclidean dissimilarity matrix was computed from data standardized using the Z statistic. Distances between strains were then calculated using unpaired group mean averages. Goodness of fit between the Euclidean matrix and resulting tree was compared using the cophenetic correlation coefficient (23). To reduce the complexity of the data set, ten data points were selected along each transformation capacity profile every 0.1 frequency units between 0.1 and 1.0.

Results

Mineralization of 3-CBA in mixed-culture enrichments. Microbial mineralization of radioisotopically-labelled 3-CBA in pristine soils was found to be widely distributed between the geographic regions sampled during this study (Table 2.3). The percentage of individual soil samples within a site evolving $^{14}CO_2$ ranged from 53% in a Chilean soil collected under a *Eucalyptus* grove (EG) to 100% in 20 different sites representing all six geographic regions. The sites with 3-CBA mineralization in all samples collected along the transect represent 69% of all the sites sampled. On a regional basis, 3-CBA mineralization was observed in 100% of the primary enrichments from the boreal forest sites, while mineralization occurred in 93% of the primary enrichments from the Mediterranean sclerophyllous woodland sites. Not surprising, field gravimetric soil moisture content did appear to influence the activity of soil microbial communities (Figure 2.9). Samples with high field moisture content also had the highest percent of active samples in primary soil enrichments. Although soil moisture content and mineralization are not linearly related, this data indicates moisture contents below 10% in field soils results in lower overall 3-CBA catabolic activity. Percent activity was greatest in soils with an average pH below 6.5 (Figure 2.10). Higher pH tended to reduce activity but one sample, Australia ME, had 100% activity at a pH of 9.09. Habitat, vegetation, and soil type does not appear to affect the distribution and activity of 3-CBA metabolizing communities.

The quantity and rate of 3-CBA mineralization was highly variable between the sampling sites indicating the composition and density of 3-CBA populations were highly influenced by site specific factors (Figure 2.11 and 2.12). In comparison to the percent activity data above, no correlation could be

Table 2.3. Frequency of 3-chlorobenzoic acid degrading activity. Frequency calculated from number of samples transferred at each point.

	Frequency			Total 3-CBA	No.
	1°	2°	Isolates	strains	>80%[a]
Australia					
BN	0.96	1.00	0.56	44	5
GE	0.84	0.38	0.75	8	1
JD	0.87	0.86	0.94	28	0
KE	0.83	0.30	0.17	1	0
ME	1.00	0.92	0.68	20	1
Average	**0.90**	**0.69**	**0.62**	**20**	**1.4**
California					
CH	1.00	1.00	0.54	22	13
CL	1.00	0.96	0.56	26	2
HG	1.00	0.88	0.36	11	0
MJ	1.00	1.00	0.58	19	1
VH	0.96	0.83	0.50	14	0
Average	**0.99**	**0.93**	**0.51**	**18**	**3.2**
Chile					
FJ	0.67	0.67	0.27	4	3
LC	1.00	1.00	0.50	29	1
LP	1.00	0.83	0.95	13	4
RC	1.00	0.88	0.57	23	5
EG	0.53	0.13	0.00	0	0
Average	**0.84**	**0.70**	**0.46**	**14**	**2.6**
South Africa					
HH	0.96	1.00	0.30	17	8
MB	0.96	0.96	0.55	18	11
MR	1.00	1.00	0.71	24	15
PM	1.00	1.00	0.75	46	14
WG	1.00	1.00	0.75	43	7
Average	**0.98**	**0.99**	**0.61**	**30**	**11**
Russia					
R1	1.00	1.00	0.29	8	6
R2	1.00	0.92	0.68	31	11
R3	1.00	0.54	0.77	13	2
R4	1.00	0.92	0.68	28	7
Average	**1.00**	**0.85**	**0.61**	**20**	**6.5**
Saskatchewan					
BT	1.00	1.00	0.83	39	9
NP	1.00	0.54	0.76	14	3
PC	1.00	1.00	0.66	22	2
WK	1.00	1.00	0.71	27	10
WV	1.00	1.00	0.50	18	6
Average	**1.00**	**0.91**	**0.69**	**24**	**6**

[a]Isolates degrading >80% 3-CBA in 7 d. and releasing chloride.

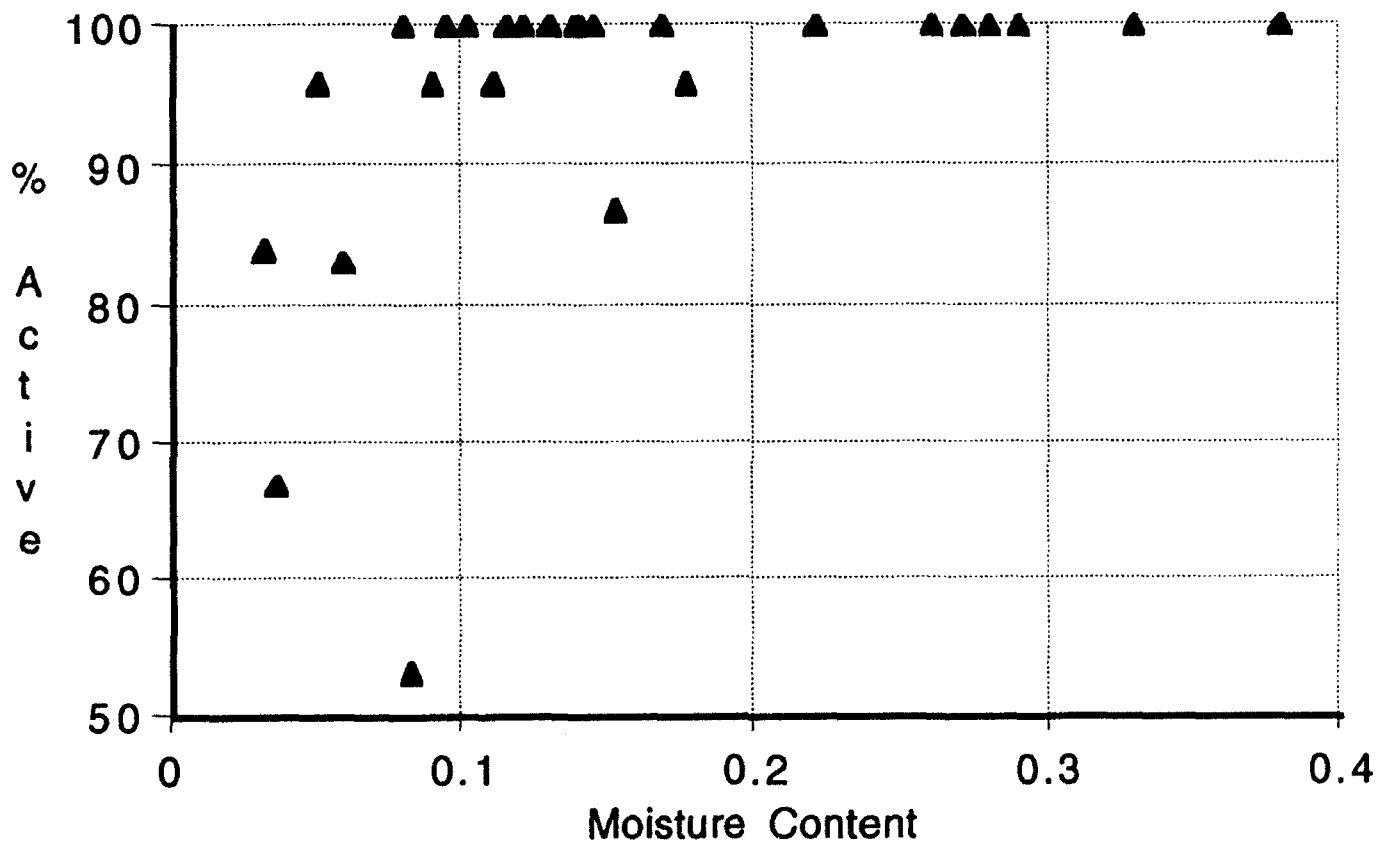

Figure 2.9. Relationship between gravimetric soil moisture content and 3-CBA mineralization in primary soil enrichments. Activities represent the percentage of samples from each where $^{14}CO_2$ was detected in BaOH filters.

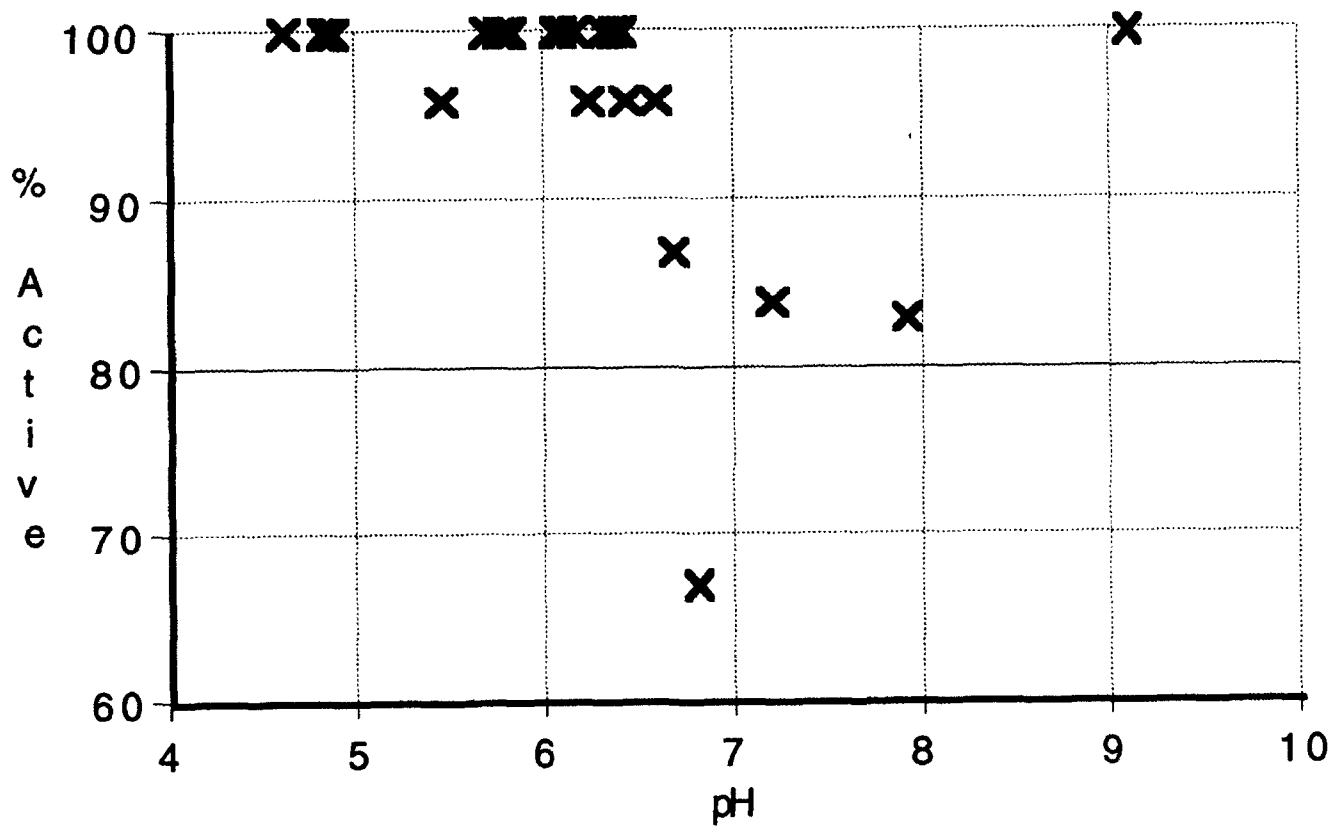

Figure 2.10. Relationship between average soil pH and percent activity at each sampling site. pH computed from five randomly selected samples from each site.

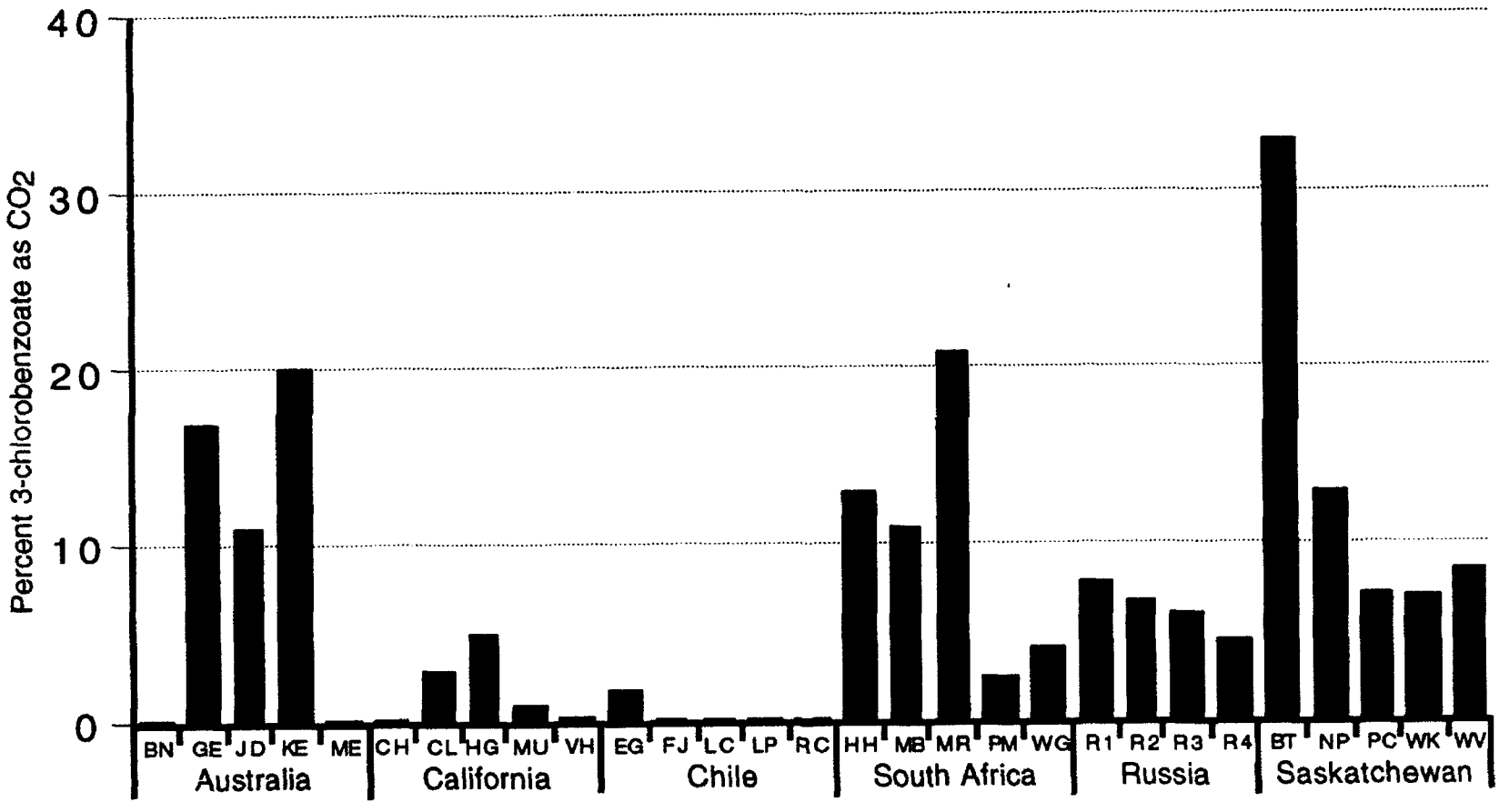

Figure 2.11. Initial mineralization of 3-chlorobenzoate in primary soil enrichments after 7 d. Values represent percent labled substrate release as carbon dioxide.

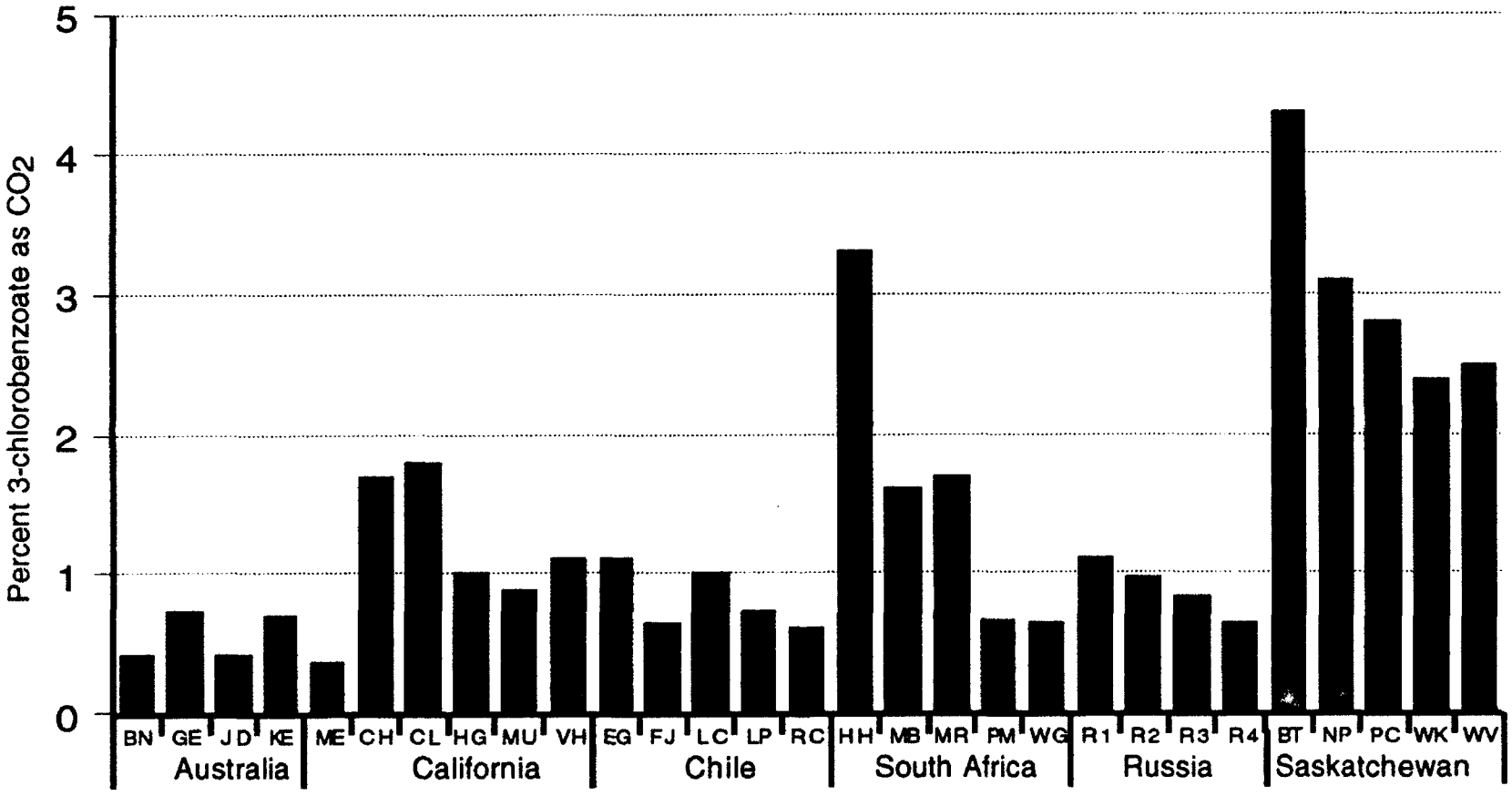

Figure 2.12. Daily average of 3-chlorobenzoate degradation rate in primary soil enrichments. Values represent percent labled substrate release as carbon dioxide.

found between quantity and rate of 3-CBA mineralization and soil moisture content, percent activity, or region. These data do show the initial catabolic activity of 3-CBA degraders in soil. Samples from Saskatchewan, South Africa, Australia, and Russia had higher activities after 7 days than samples from California and Chile. This trend was not evident in overall mineralization rates. While Saskatchewan soils continued to have high rates, the other samples with high initial activity decreased. California and Chile samples increased in activity with time and surpassed those of Australia and Russia.

Activity was usually transferable from 50 μg 3-CBA g•dry•soil^{-1} primary enrichments to secondary enrichments in 50 μg 3-CBA ml^{-1} DAB but, was lost in 16% of the transfers. Most notable were losses in activity for KE, Australia and EG, Chile where only 30 and 13%, respectively, of the samples active in primary enrichments continued to mineralize 3-CBA after transfer to defined medium.

Transformation capacities of 3-CBA degrading bacteria. The relative change in 3-CBA concentration between inoculated and uninoculated controls measured after 7 days was considered the transformation capacity of an isolate. Of the 610 isolates chosen because they released $^{14}CO_2$ in confirmation test, 533 were subjected to HPLC and chloride release analysis to determine their ability to transform 3-CBA. Isolates varied markedly in their ability to degrade 3-CBA revealing that while all isolates could partially mineralize 3-CBA, a majority of the strains could not completely degrade 3-CBA. The rank ordered transformation capacity profiles for each geographic region illustrated in Figure 2.13 show that the 3-CBA populations are catabolically diverse. While most regional profiles are fairly similar in appearance, the profiles from South Africa and Chile deserve notice. South Africa isolates were distinctive in both their proportion of isolates with high

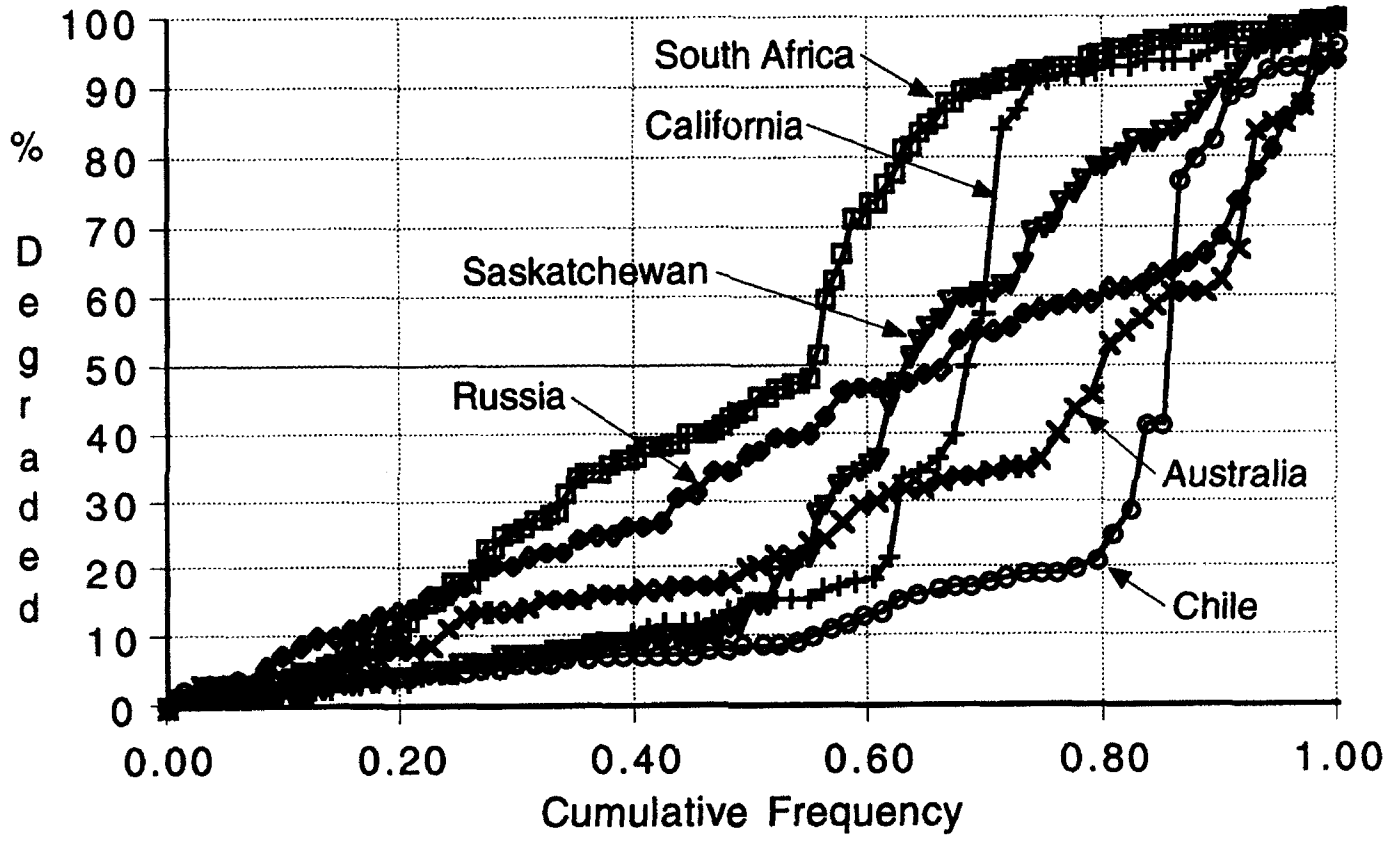

Figure 2.13. Transformation capacity profiles of 3-CBA degrading isolates from six geographically separated regions. Transformation capacitites were measured after 7 d.in 1.0 mM3-CBA A+N by HPLC. Release of chloride was measured after 21 d. by spectrophotometry.

transformation capacities (37% with transformation capacities >80%) and the overall number of isolates obtained (148 isolates, 24% of total). Chile, on the other hand, had a total of 69 isolates with only 13 (19%) having transformation capacities >80%. Overall Chile represented 11% of the total isolates cultured. This low proportion of the total does not explain the relatively low transformation capacities of the isolates. Russia, with 80 isolates (13% of total), had 33% of its isolates with transformation capacities >80%.

While HPLC analysis demonstrated 22% of the isolates could degrade >80% of 1.0 mM 3-CBA A+N in 7 days, this percentage increased only slightly to 29% after 21 days Chloride release measured at 21 days showed 37% of the isolates could dechlorinate 3-CBA even though they could not facilitate complete degradation. Discoloration of the medium, presumably due to the accumulation of polyphenolic intermediates, was observed in 22% of the strains, of which, 34% (7% of the total) also released chloride. These isolates transformed approximately 60% of the 3-CBA in the medium. Thus, the majority of the strains exhibited only incomplete transformation of 3-CBA as evidenced in Table 2.4 by the low mean and median transformation capacity values. Overall mean and median transformation capacities computed from all sites were 36% and 19%, respectfully.

Transformation capacity profiles were also used to examine similarities in the structure of catabolic populations between sites within a region (Figures 2.14 to 2.19). In general, individual site profiles from within a region appear to be repetitive indicating similarities in catabolic population structures between sites. Most striking are the catabolic populations from Russia (Figure 2.18) where similar profiles are observed at all four sites. This was also observed in South Africa (Figure 2.17) where sites MB and MR and sites HH and PM form two different similarity groups. As a group, these four sites maintain the general

Table 2.4. Transformation capacity statistics of 3-CBA degrading isolates from each sampling site.

	No.	Mean	Median	Range
Australia				
BN	33	29%	17%	3-100%
GE	6	52	57	14-99
JD	16	36	33	24-67
KE	0	0	0	0
ME	17	16	9	4-59
Total	**72**	**29%**	**18%**	**3-100%**
California				
CH	12	74%	94%	4-98%
CL	26	45	22	1-100
HG	10	7	6	1-17
MU	14	21	6	1-94
VH	11	25	6	2-92
Total	**73**	**37%**	**10%**	**1-100%**
Chile				
EG	0	0%	0%	0%
FJ	4	67	80	4-93
LC	29	9	7	4-25
LP	13	32	9	4-92
RC	22	26	18	2-96
Total	**68**	**23%**	**10%**	**2-96%**
South Africa				
HH	17	58%	61%	1-99%
MB	15	62	85	1-99
MR	24	66	92	2-99
PM	36	52	40	2-96
WG	43	42	36	1-100
Total	**135**	**53%**	**40%**	**1-100%**
Russia				
R1	5	49%	46%	12-93%
R2	27	32	23	1-86
R3	11	31	25	3-69
R4	27	46	49	1-94
Total	**70**	**39%**	**26%**	**1-94%**
Saskatchewan				
BT	39	29%	8%	1-99%
NP	13	51	55	1-96
PC	19	11	8	1-99
WK	26	52	60	1-98
WV	18	44	36	1-99
Total	**115**	**36%**	**10%**	**1-99%**

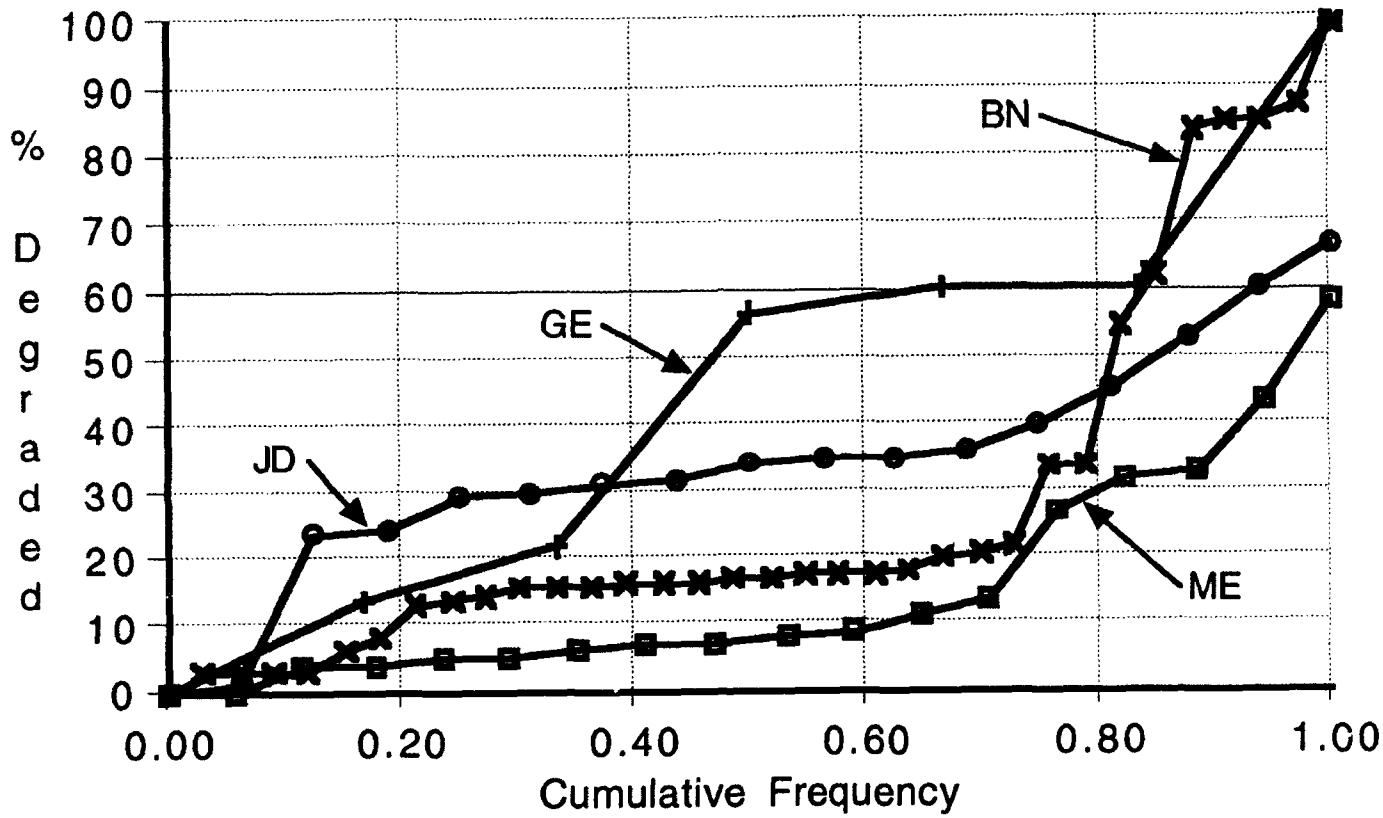

Figure 2.14. Transformation capacity profiles of 3-CBA isolates from Australia. Transformation capacitites were measured after 7 d.in 1.0 mM 3-CBA A+N by HPLC. Release of chloride was measured after 21 d. by spectrophotometry.

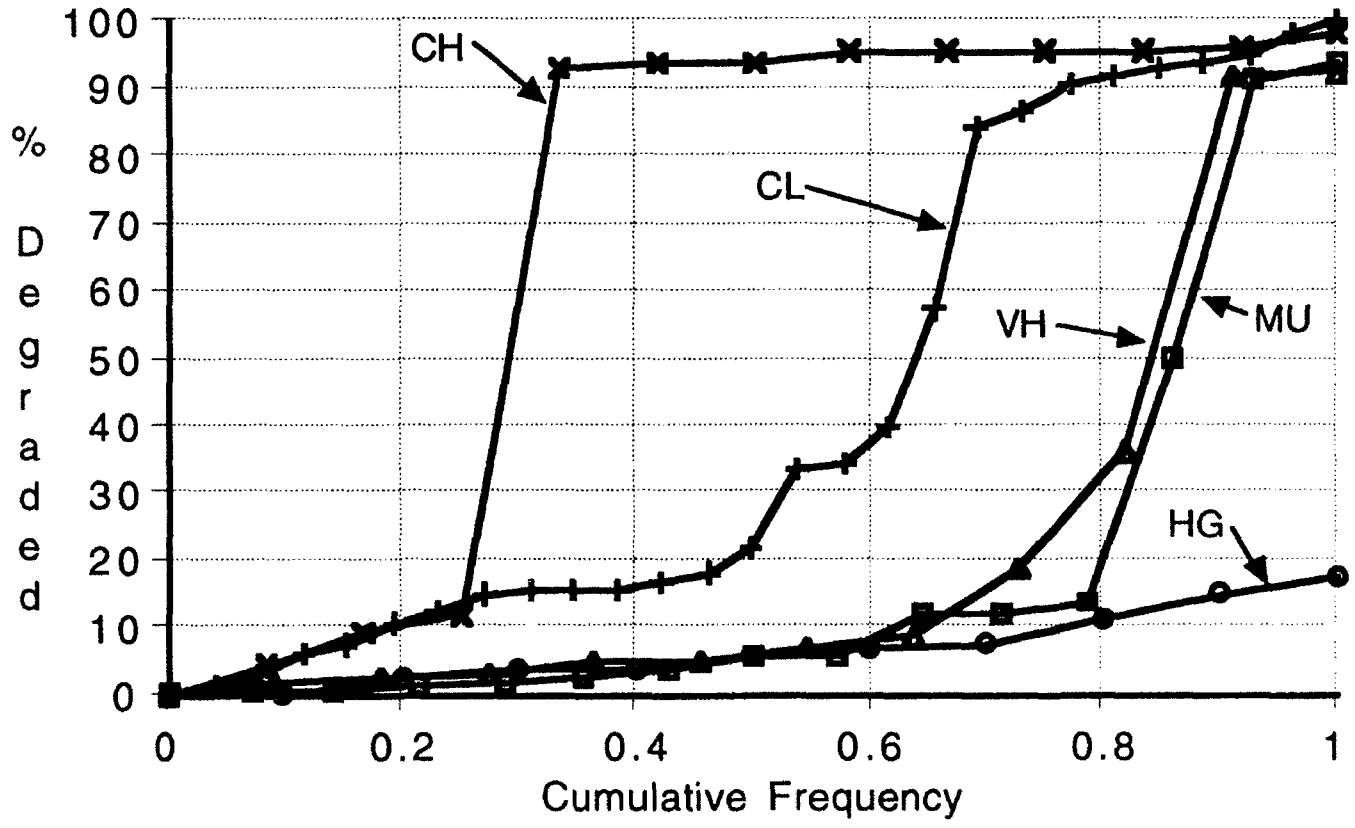

Figure 2.15. Transformation capacity profiles of 3-CBA isolates from California. Transformation capacitites were measured after 7 d.in 1.0 mM 3-CBA A+N by HPLC. Release of chloride was measured after 21 d. by spectrophotometry.

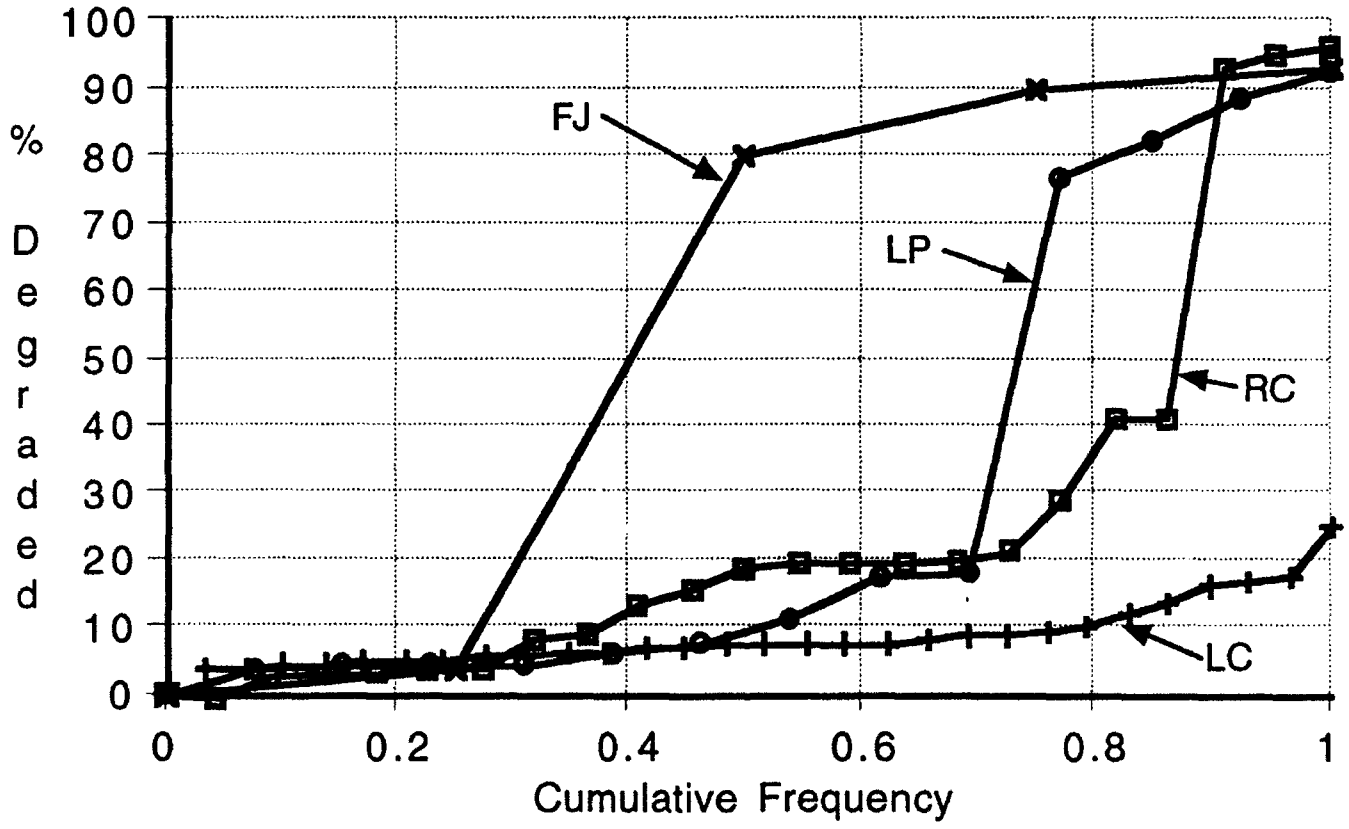

Figure 2.16. Transformation capacity profiles of 3-CBA isolates from Chile. Transformation capacitites were measured after 7 d.in 1.0 mM 3-CBA A+N by HPLC. Release of chloride was measured after 21 d. by spectrophotometry.

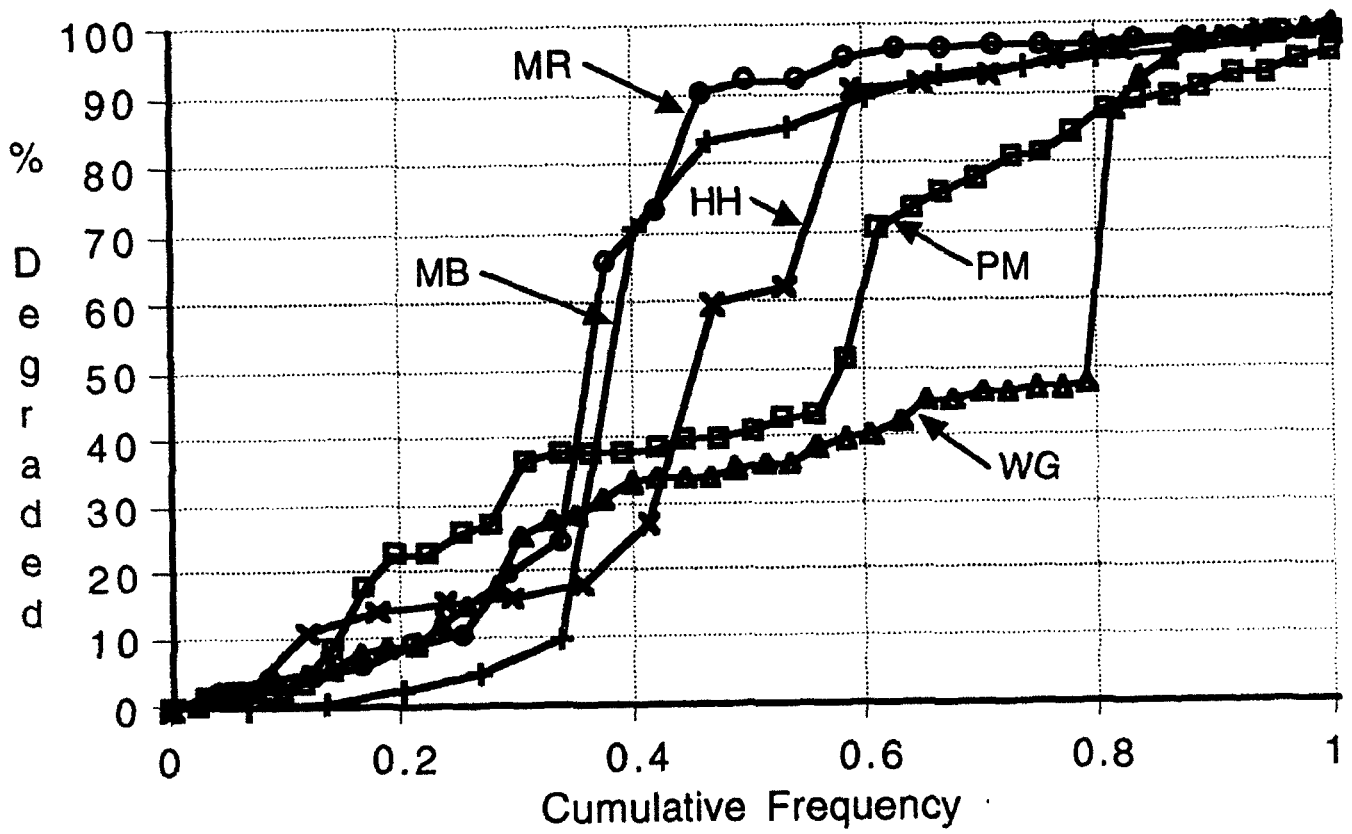

Figure 2.17. Transformation capacity profiles of 3-CBA isolates from South Africa. Transformation capacitites were measured after 7 d.in 1.0 mM 3-CBA A+N by HPLC. Release of chloride was measured after 21 d. by spectrophotometry.

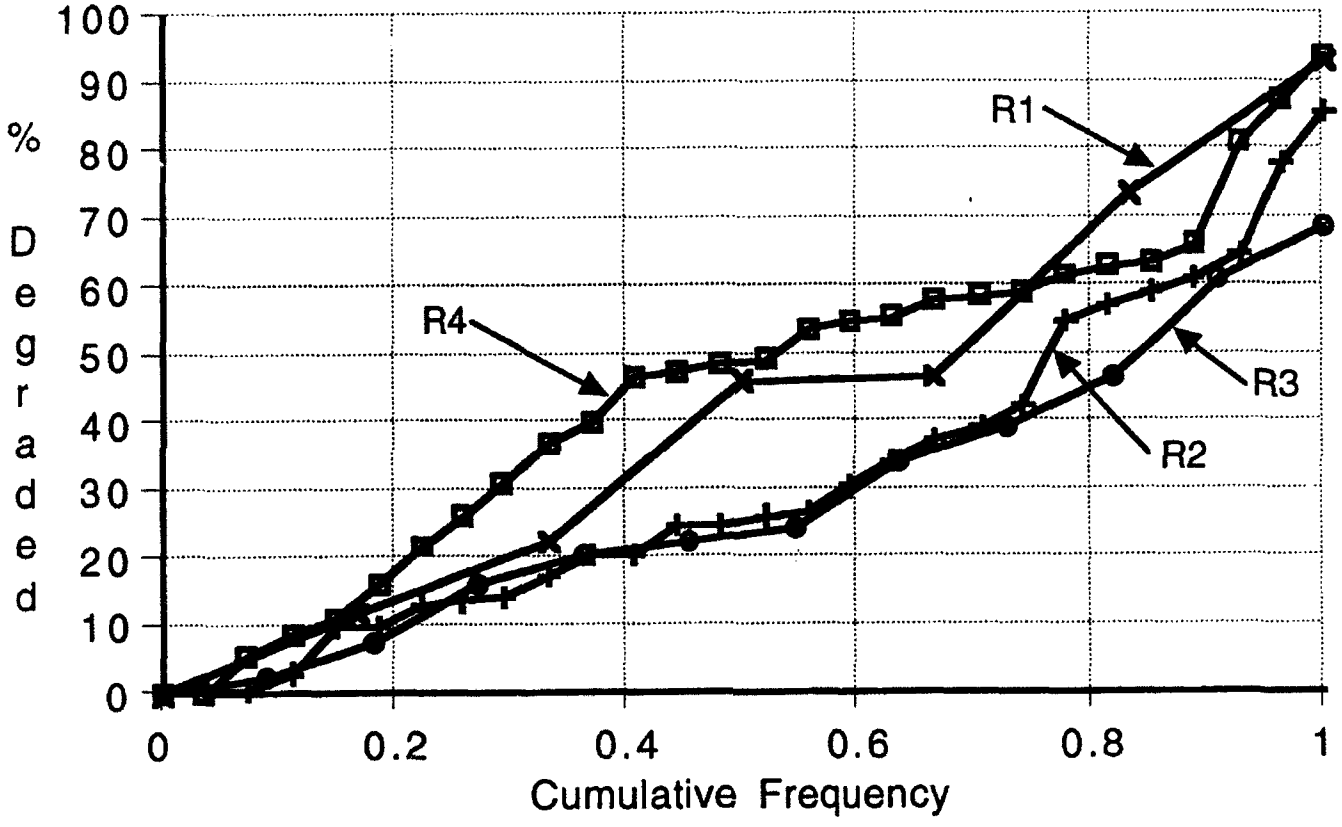

Figure 2.18. Transformation capacity profiles of 3-CBA isolates from Russia. Transformation capacitites were measured after 7 d.in 1.0 mM 3-CBA A+N by HPLC. Release of chloride was measured after 21 d. by spectrophotometry.

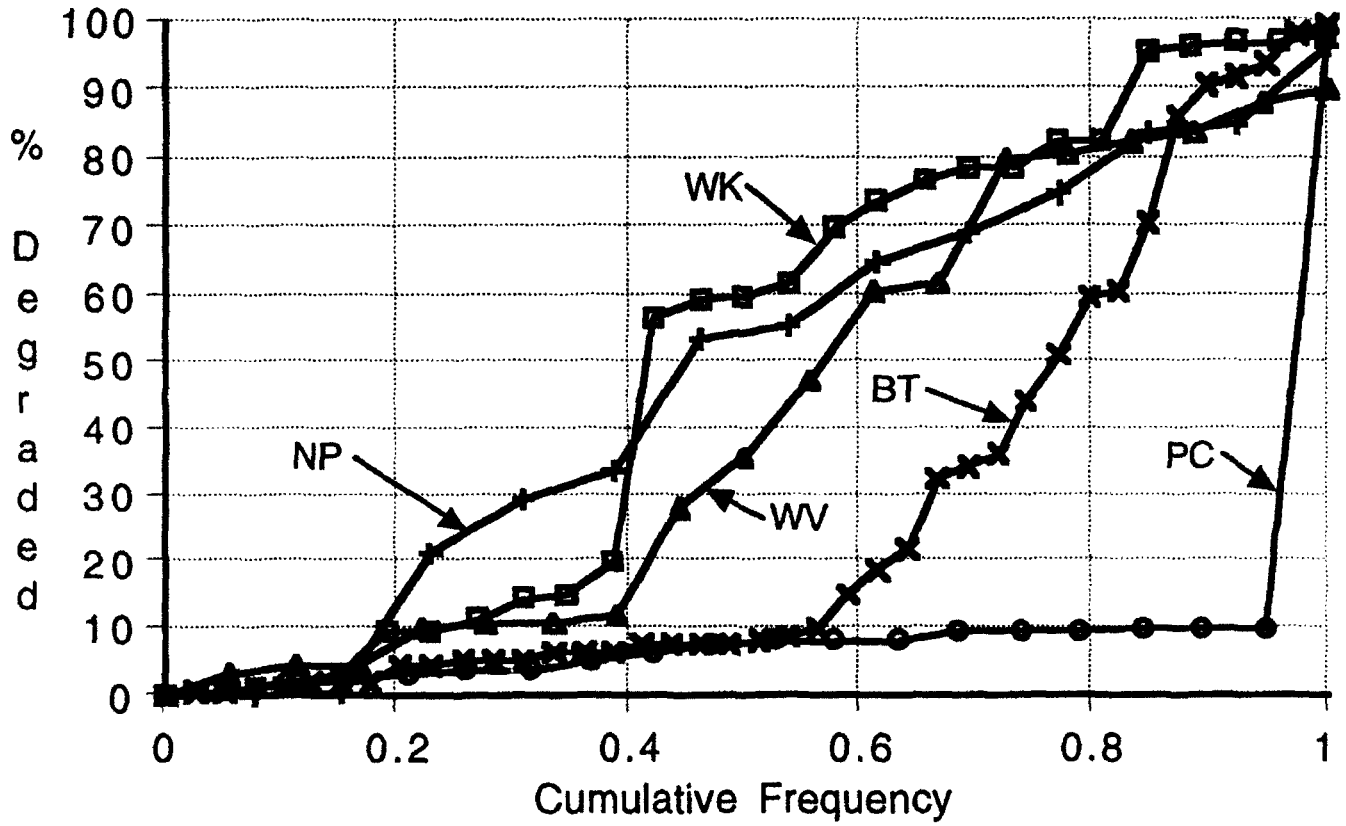

Figure 2.19. *Transformation capacity profiles of 3-CBA isolates from Saskatchewan.* Transformation capacitites were measured after 7 d.in 1.0 mM 3-CBA A+N by HPLC. Release of chloride was measured after 21 d. by spectrophotometry.

regional pattern. The relative shape of the WG site is similarity to the other South African patterns but is different in magnitude because of a greater proportion on intermediate degraders. With the exception of the strains comprising the 0.6 to 0.8 classes, WG also show regional similarity with slight variation. In the other regions, catabolic profiles were more diverse. Saskatchewan profiles (Figure 2.19) were similar in 4 of the 5 sites but the PC site is obviously different in catabolic structure. Although sampled in the same proximity as the other sites, PC was a successional old field and was dominated by young poplar and not the coniferous forest present at the other sites. Australia profiles (Figure 2.14) have more diverse profiles for the four sites with isolates. No isolates were collected from the fifth site (EG) indicating that while a population profile is representative of the region as a whole, it does not mean catabolic isolates can be expected at every site.

Catabolic profiles from sites in California (Figure 2.15) and Chile (Figure 2.116) were the most diverse, but together have very similar regional patterns. In both regions, one site had a high proportion of degraders with capacities >80% (California CH and Chile FJ) while at another site, no degraders were found with capacities > 20% (California HG and Chile LC). The CH and FJ profiles appear to be similar to the South African profiles, but the lack of any intermediate capacity degraders at these two sites indicates they are catabolically distinct from the South African groups. The most similar group is composed of California CL, VH and MU and Chile LP and RC with CL being slightly more different than the others. This group also bears similarity to the Saskatchewan BT profile with the CL profile between BT and the Saskatchewan NP, WK, and WV group. As was the case in Australia, one site in Chile (EG) did not produce any 3-CBA isolates.

Cluster analysis of regional profiles provided more detailed analysis of each transformation profile. When regional profiles were examined, three distinctive groups were found (Figure 2.20). California and Saskatchewan, representing both of the North American regions have the greatest similarity. Another cluster of intermediate similarity is comprised of Australia and Chile; regions from Mediterranean sclerophyllous woodlands in the southern hemisphere. The last cluster of South Africa and Russia are only slightly similar and no clear relationship can be found between these two sites since they represent both Mediterranean woodland and boreal forest habitats, respectively. Analysis of all individual sampling sites revealed two diverse catabolic groups at a Euclidean dissimilarity value of 4.67. These groups are identified as I and II in Figure 2.21. Each of these clusters were divided into two additional subgroups, designated a and b, with dissimilarities of 3.47 and 3.70 for groups I and II, respectively. Generalized transformation capacity profiles for groups I and II were prepared from the individual sites in each cluster (Figure 2.22). The resulting profiles show group I sites have a greater percentage of degraders with relatively high 3-CBA transformation capacities while group II sites are dominated by low capacity isolates.

Overall, the cluster analysis did not reveal any regional fidelity and the clusters with greatest similarity (Euclidean dissimilarity < 1.38) were interregional and equally divided between sites in like climates (e.g., boreal/boreal or Mediterranean/Mediterranean) and those from different climates (e.g., Mediterranean/boreal). Only two cases were found where two sites from the same region formed closely related clusters. Sites R2 and R3 from Russia and sites NP and WK from Saskatchewan had Euclidean

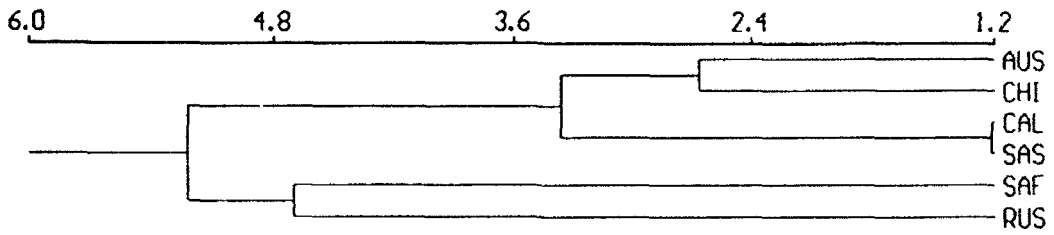

Figure 2.20. Dendrogram of regions generated by Euclidean dissimilarities of transformation profiles. ($r_{x,y}$=0.89)

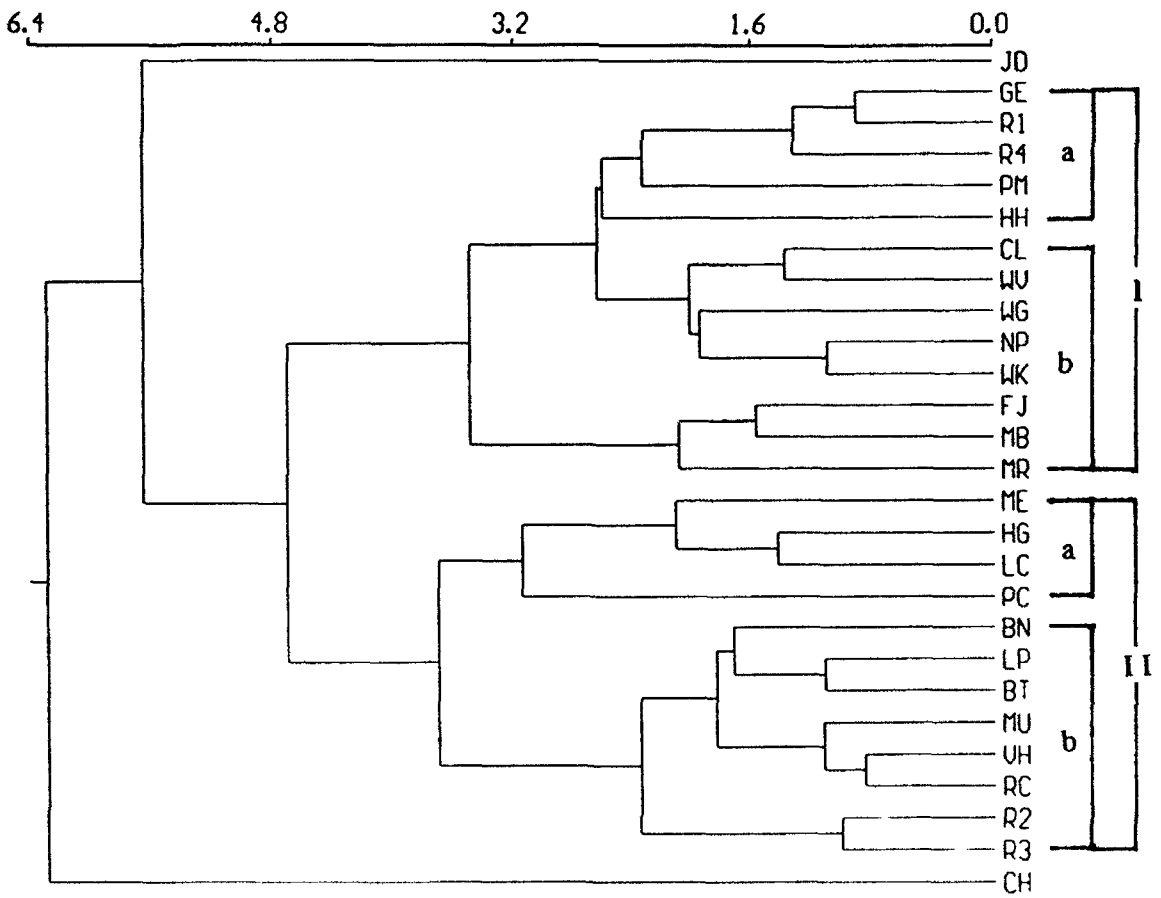

Figure 2.21. Dendrogram of site profiles generated by Euclidean dissimilarities of transformation profiles. ($r_{x,y}$=0.73)

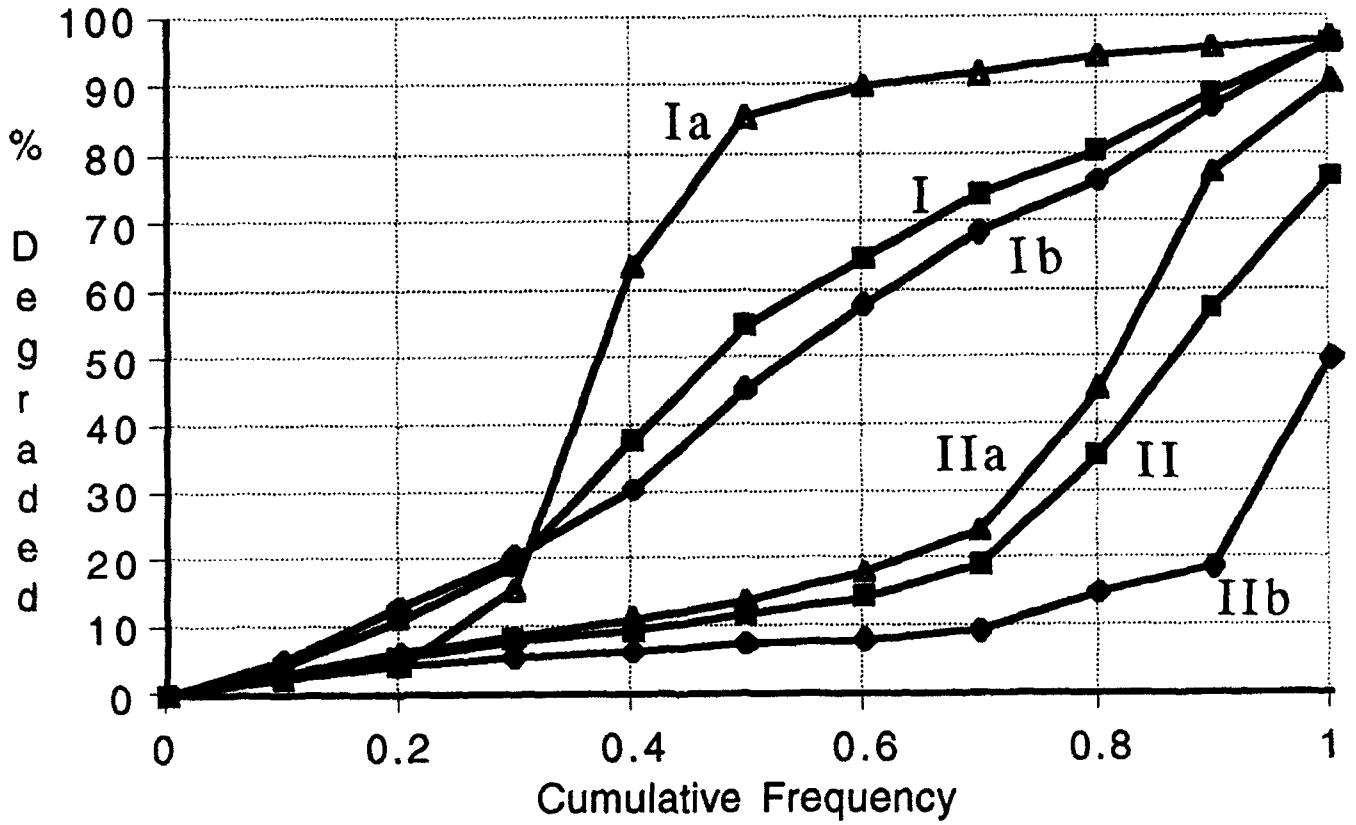

Figure 2.22. Combined transformation capacity profiles derived from cluster analysis. Groups I and II represent the major clusters found in Figure 2.21. Subgroups are designated by a and b.

dissimilarity values of 1.02 and 1.11, respectively. Clusters along climatic or vegetational parameters were not evident in any of the analyses.

Catabolic stability of 3-CBA isolates. Stability tests measuring the change in 3-CBA transformation capacity following serial transfers of 3.3% inoculum in R2A broth, a nonspecific growth medium, show less than 20% of the isolates tested had reduced transformation capacities (Figure 2.23). Another 20% showed stability by transforming the same amount of 3-CBA before and after growth on R2A. The remaining 60%, however, increased their transformation capacities after growth in R2A.

Discussion

Degradation of 3-CBA was observed in soils sampled from geographically separated, pristine sites representing boreal forest and Mediterranean sclerophyllous woodland ecosystems. 3-CBA mineralization was observed in all 29 sites and axenic cultures were obtained from 93% of these sites indicating the 3-CBA phenotype is not particularly rare in natural communities. The widespread distribution of 3-CBA degrading strains from sites with no documented history of exposure to anthropogenic chloroaromatic compounds provides support for the genetic and biochemical preadaptation model of xenobiotic degradation. Although mineralization of 3-CBA is observed at a high frequency in both primary and secondary enrichments, isolates were not easily obtained from all samples indicating a possible community-level role in 3-CBA metabolism. Microbial populations in California and Chile soils required a period of acclimation before maximum activity was observed. Other sites having high initial rates of activity may be preadapted to a structural analog present in soil organic matter.

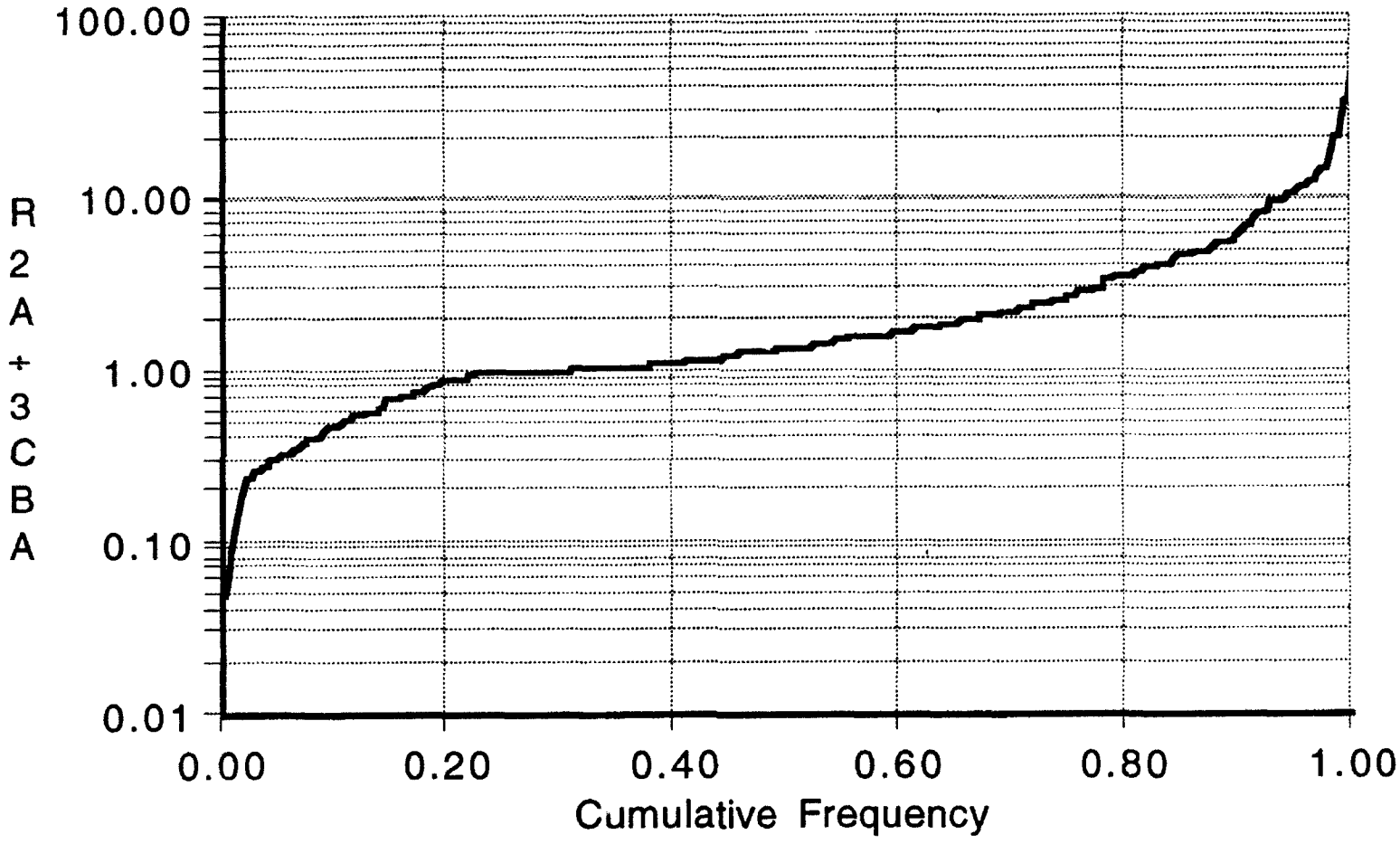

Figure 2.23. Stability of the 3-CBA phenotype following sequential transfers in R2A medium. Profile indicates the relative change in transformation capacity between R2A and 3-CBA grown isolates. Values <1 show catabolic instability, while values >1 show catabolic stimulation.

In previous studies, pollutant degrading isolates were selected due to their near complete removal of the parent substrate. This study shows only 22% of the bacteria isolated satisfy this criteria. Since the remaining 78% cannot completely degrade 3-CBA, but were shown to mineralize 3-CBA in radioisotope studies, it is apparent the majority of potential 3-CBA degraders in soil can only partially transform 3-CBA. The contribution of this later group of organisms may have significant ecological importance. By examining only the best degraders, an incomplete picture of 3-CBA transformations in natural environments is produced. In future ecological studies it may be important to examine the entire spectrum of organisms to gain greater understanding of the role the entire community plays in the transformation of xenobiotic compounds in natural environments.

The reduced frequency in 3-CBA mineralization following the transfer of samples from primary soil enrichments to secondary enrichments in chemically defined medium is attributed to a combination of nonculturable organisms, cometabolism, or the loss of required growth factors contained in the soil. Although these are often used to rationalize the inability of the investigator to obtain isolates, they are important biological phenomena in bacterial populations and underscores the extreme heterogeneity of both the biological and abiotic components of soils. From the data shown in this study, these organisms may have greater importance ecologically than those with high transformation capacities. A further reduction in potential degrader diversity occurred when secondary enrichments were plated on R2A agar. R2A was chosen because it consistently allowed the cultivation of the greatest diversity of bacterial isolates from Michigan agroecosystem long-term ecological research (LTER) soils (20). Even with this medium, only 59% of positive secondary enrichments yielded 3-CBA degrading strains in pure culture. Almost half the

degradation observed in individual soil samples may result from community level metabolism or by nonculturable organisms. For the purposes of this study, this was not a problem since sufficient numbers of organisms were cultured to allow analysis of populations from different geographic regions.

Transformation capacities varied widely between strains and ranged from approximately 1 to 100%. The diversity in the transformation capacity profiles indicates a single population was not favored at any particular site. The trait also appears to be quite stable since over 80% of the strains continued to degrade 3-CBA at or above their previous levels following sequential transfers in R2A broth. Stimulation of 3-CBA catabolic activity, observed by an increase in transformation capacity following R2A growth, occurred with a majority of the isolates. A likely explanation is the isolates were cultured under metabolic stress in 3-CBA A+N medium alone. Previous growth in R2A alleviated this stress and allowed the organisms to regain full metabolic potential prior to reinoculation into 3-CBA broth. Under these conditions, the isolates could express higher transformation capacities. After the initial return to 3-CBA, further transfers into fresh 3-CBA medium were not conducted, therefore, it is not known if the higher transformation capacities could be maintained by these isolates.

Cluster analysis of transformation profiles did not reveal any strict relationships between any of the geographic regions (Figure 2.20). Since the sites were selected to represent two different ecosystem types, the lack of relationship between regions and transformation profiles seems to indicate soil type and vegetation do not play a significant role in the distribution of the 3-CBA trait. However, these sites were not extensively characterized and future analyses may reveal relationships between isolates and the overlying plant community. Alternatively, the occurrence of the trait may be the result of

secondary processes arising during the decomposition of plant matter. Cluster analysis of transformation profiles from each site revealed two major metabolic groups. Averaged profiles from each of these groups revealed two distinct transformation profiles (Figure 2.22). These profiles suggest the possibility of at least two distinct functional structures among 3-CBA degrading populations.

Even though widely distributed geographically, variation in activity among soils should be anticipated when collecting samples in the field. In this study, spatial variability was reduced by analyzing a larger sample set which was facilitated by the use of a novel microtiter plate/autoradiography method. This method was developed to measure the mineralization of 3-CBA-UL-ring-^{14}C in soil and proved to be both reliable and efficient. In this study, 687 individual soil samples from six geographic regions were analyzed. This effort would have been unmanageable using traditional respirometry methods. Samples could be rapidly screened for activity using autoradiography, and with the AMBIS system, the amount of $^{14}CO_2$ evolved quantified. The ability to handle a large number of samples makes this method ideal in situations where a high number of replicates is desired to reduce experimental error and between treatment variation. As this method requires far less handling than conventional alkali traps, it is safer to use in the laboratory. This method was very precise when using the AMBIS system to measure the radioactivity of BaOH saturated filter paper. Regression analysis of cpm $^{14}CO_2$ trapped in the filters against known activity of $Na^{14}CO_2$ in the wells produced a correlation coefficient of 0.996. This level of correlation was found only when both surfaces of the trap were quantified. Apparently the surface of the filter closest to the culture became saturated with time and $^{14}CO_2$ diffused farther into the filter before forming $BaCO_3$. This high level of correlation indicates the precision of

the method. Because of the small size of each individual sample, there are a few potential drawbacks to this method. The opportunity for extensive analysis of each sample either during incubation or after experimentation is reduced by the the available material in each well. In this study, 1.0 g·dry·soil was added to each well. Although this is sufficient for bacteriological analysis, larger quantities of soil may be desired for chemical analysis. Using the 24-well plates, there is sufficient volume to hold approximately. 3-4 $g \cdot well^{-1}$ at similar bulk densities. Larger welled plates are available, but the trade-off is a reduction in the number of samples which can treated per plate. Thus, the design of sampling protocols should judiciously use the limited amount of material in each well. Drying of soil samples could be a problem if care is not taken during incubation. The addition of sterile water to the intrawell void space and keeping the plates in sealable plastic containers greatly reduced soil water loss.

This study has shown that a trait for the degradation of a model xenobiotic compound is common in pristine ecosystems. This provides strong support for the hypothesis that microbial populations are preadapted for the degradation of certain chloroaromatic substrates. Preadaptation was concluded to arise from the natural variation in substrate specificity among aromatic degrading populations. As a result, the functional structure of chloroaromatic populations is complex. Our current understanding of the biochemical and genetic basis of 3-CBA degradation is based on a few isolates. Comparison of the isolates from this study with those previously described will show how well the current biochemical and genetic models for 3-CBA degradation represents isolates from pristine ecosystems.

Acknowledgements

I thank Frank Krist for assistance in the preparation of site maps.

Literature Cited

1. **Ahmed, M. and D. Focht.** 1973. Degradation of polychlorinated biphenyls by two species of *Achromobacter*. Can. J. Microbiol. 19:47-52.

2. **Bergmann, J. G. and J. Sanik.** 1957. Determination of trace amounts of chlorine in naptha. Anal. Chem. 29:241-243.

3. **Brenner, V., B. S. Hernandez, and D. D. Focht.** 1993. Variation in chlorobenzoate catabolism by *Pseudomonas putida* P111 as a consequence of genetic alterations. Appl. Environ. Microbiol. 59:2790-2794.

4. **Brunsbach, F. R. and Reineke, W.** 1993. Degradation of chlorobenzoates in soil slurry by special organisms. Appl. Microbiol. Biotech. 39:117-122.

5. **Chatterjee, D. K., S. T. Kellogg, E. Hamada, and A. M. Chakrabarty.** 1981. Plasmid specifying total degradation of 3-chlorobenzoate by a modified *ortho* pathway. J. Bacteriol. 146:639-646.

6. **Don, R. H. and J. M. Pemberton.** 1981. Properties of six pesticide degradation plasmids isolated from *Alcaligenes paradoxus* and *Alcaligenes eutrophus*. J. Bacteriol. 145:681-686.

7. **Dorn, E., M. Hellwig, W. Reineke, and H.-J. Knackmuss.** 1974. Isolation and characterization of a 3-chlorobenzoate degrading pseudomonad. Arch. Microbiol. 99:61-70.

8. **Focht, D. D. and D. Shelton.** 1987. Growth kinetics of *Pseudomonas alcaligenes* C-0 relative to inoculation and 3-chlorobenzoate metabolism in soil. Appl. Environ. Microbiol. 53:1846-1849.

9. **Forney, L. (Michigan State University).** 1992. Personal communication.

10. **Fulthorpe, R. R. and R. C. Wyndham.** 1989. Survival and activity of a 3-chlorobenzoate-catabolic genotype in a natural system. Appl. Environ. Microbiol. 55:1584-1590.

11. **Furukawa, K., N. Tomizuka, and A. Kamibayashi.** 1979. Effect of chlorine substitution on the bacterial metabolism of various polychlorinated biphenyls. Appl. Environ. Microbiol. 38:301-310.

12. **Gribble, G. W.** 1992. Naturally occurring organohalogen compounds - a survey. J. Nat. Prod. 55:1353-1395.

13. **Grishchenkov, V. G., I. E. Fedevichkina, B. P. Baskunov, L. A. Anisimova, A. M. Boronin, and L. A. Gololeva.** 1984. Degradation of 3-chlorobenzoic acid by a *Pseudomonas putida* strain. Mikrobiol. 52:771-776.

14. **Häggblom, M. M.** 1992. Microbial breakdown of halogenated aromatic pesticides and related compounds. FEMS Microbiol. Rev. 103:29-72.

15. **Haller, H. D. and R. K. Finn.** 1979. Biodegradation of 3-chlorobenzoate and formation of black color in the presence and absence of benzoate. European J. Appl. Microbiol. 8:191-205.

16. **Hartmann, J., W. Reineke, H.-J. Knackmuss.** 1979. Metabolism of 3-chloro-, 4-chloro-, and 3,5-dichlorobenzoate by a Pseudomonad. Appl. Environ. Microbiol. 37:421-428.

17. **Hickey, W. J. and D. D. Focht.** 1990. Degradation of mono-, di-, and trihalogenated benzoic acids by *Pseudomonas aeruginosa* JB2. Appl. Environ. Microbiol. 56:3842-3850.

18. **Hickey, W. J., D. B. Searles, and D. D. Focht.** 1993. Enhanced mineralization of polychlorinated biphenyls in soil inoculated with chlorobenzoate-degrading bacteria. Appl. Environ. Microbiol. 59:1194-1200.

19. **Johnston, H. W., G. G. Briggs, and M. Alexander.** 1972. Metabolism of 3-chlorobenzoic acid by a pseudomonad. Soil Biol. Biochem. 4:187-190.

20. **Klug, M. J. (Michigan State University).** 1992. Personal communication.

21. **Krumme, M. L.** 1993. Development of aquifer microcosms and in situ methods to test the fate and function of pollutant-degrading microorganisms. Ph.D. dissertation. Technischen Universität Carolo-Wilhelmina, Braunschweig, Germany.

22. **Pertsova, R. N., F. Kunc, and L. A. Golovleva.** 1984. Degradation of 3-chlorobenzoate in soil by pseudomonads carrying biodegradative plasmids. Folia Microbiol. 29:242-247.

23. **Romesburg, H. C.** 1984. Cluster analysis for researchers. Lifetime Learning Publications, Belmont, Calif.

24. **Tabor, H. C., C. W. Tabor, and E. W. Hafner.** 1975. Convenient method for detection of $^{14}CO_2$ in multiple samples: application to rapid screening for mutants. J. Bacteriol. 128:485-486.

25. **Taeger, K., H. J. Knackmuss, and E. Schmidt.** 1988. Biodegradability of mixtures of chloro- and methylsubstituted aromatics: simultaneous degradation of 3-chlorobenzoate and 3-methylbenzoate. Appl. Microbiol. Biotechnol. 28:603-608.

26. **Weisshaar, M.-P., F. C. H. Franklin, and W. Reineke.** 1987. Molecular cloning and expression of the 3-chlorobenzoate-degrading genes from *Pseudomonas* sp. strain B13. J. Bacteriol. 169:394-402.

27. **Wyndham, R. C. and N. A. Straus.** 1988. Chlorobenzoate catabolism and interactions between *Alcaligenes* and *Pseudomonas* species from Bloody Run Creek. Arch. Microbiol. 150:230-236.

28. **Wyndham, R. C.** 1986. Evolved aniline catabolism in *Acinetobacter calcoaceticus* during continuous culture of river water. Appl. Environ. Microbiol. 51:781-789.

29. **Zaitsev, G. M. and B. P. Baskunov.** 1985. Utilization of 3-chlorobenzoic acid by *Acinetobacter*. Mikrobiol. 54:203-208.

Chapter Three

Catabolic Diversity of Genotypically-Distinct 3-Chlorobenzoic Acid Degrading Bacteria Determined by Assimilation of [^{35}S]Sulfate

Introduction

Although man-made chlorinated compounds do not occur naturally in the environment, the ability to use these compounds as growth substrates has been reported for several genera of bacteria (1, 2, 9, 14, 16). Most studies of chloroaromatic degrading bacteria have generally focused on the biochemistry and genetics of chloroaromatic degrading enzymes. These studies often concentrate on the degradation of a single chlorinated compound in chemically defined media. In reality, however, organisms often face a mixture of several chlorinated compounds when pollutants are inadvertently released into the environment. If a wider range of compounds is examined, it is generally limited to those of the same class, such as mono- and dichlorinated benzoates using 3-chlorobenzoate degrading bacteria (10, 11). Less frequent are studies where a wide range of chlorinated compounds are examined to see if relaxed substrate specificity is evident in other aromatic catabolic pathways in indigenous soil bacteria (19).

Soil bacteria naturally experience a wide variety of aromatic compounds, therefore, one can reason that the ability to use aromatic substrates for growth and energy is a common trait among soil bacteria. Many of these compounds may be derived from humic materials formed during the decomposition of organic residues in soils. Humic materials are chemically complex and can be best described as amorphous, hydrophillic, and partially aromatic compounds. Estimates of the humic fraction of soil organic matter range from 65 to 75% (15). As a result, soil bacteria have a wide variety of enzymes for catabolism of natural aromatic substrates. Metabolic versatility of soil bacteria may be attributable to either relatively few broad specificity enzymes or multiple highly specific enzymes. The former strategy would allow organisms to express a

wide range of catabolic activity using relatively few enzyme systems, thus avoiding the additional metabolic and genetic burden of maintaining many different enzyme systems. Thus, several natural enzymes may have sufficiently reduced substrate specificities to allow the catalysis of xenobiotic compounds.

Chapter Two reported the wide-spread distribution of 3-chlorobenzoic acid (3-CBA) degrading bacteria in geographically-separated, pristine soils. 3-CBA degradation, however, varies widely among the isolates from pristine habitats. In all, 610 bacteria were isolated; of these, 19% had a 3-CBA transformation capacity in excess of 80% after 7 days of incubation in chemically defined medium. Although these strains are similar in their ability to degrade 3-CBA, their genetic similarity and the extent of their catabolic capabilities for the degradation of other mono- and disubstituted chloroaromatic compounds was not known. Fulthorpe (8) determined which strains were genotypically-distinct by genomic fingerprinting.

In this chapter, I describe the substrate use patterns of representatives of each genotypically-distinct 3-CBA degrading strain. I used a novel radioisotopic procedure, the assimilation of $^{35}SO_4^{2-}$ during growth, to evaluate if other chloroaromatic compounds would support growth. My results reveal a broad continuum of chloroaromatic catabolism ranging from strains capable of transforming only 3-CBA and benzoate to isolates which can transform over 30 different chloroaromatic compounds. This suggests soil bacteria isolated from pristine habitats also have the capability of degrading other chloroaromatic substrates, but these phenotypes are diverse and are not related to 3-CBA catabolism.

Materials and Methods

Media and reagents. The composition of defined aerobic basal medium (DAB) was described in Chapter Two (Table 2.2). Routine culture of strains was on R2A agar (Difco Laboratories, Detroit, Mich.). Bacto agar (Difco, Detroit, Mich.), Agar Noble (Difco), agarose (Bio-Rad Laboratories, Richmond, Calif.), and Gelrite™ (Scott Labs., Fiskeville, R.I.) were used as solidifying agents. Chemicals used in substrate range and their purities, if known, are listed in Table 3.1. Sodium [^{35}S]sulfate was obtained from New England Nuclear (Boston, Mass.). 2,4-D-UL-ring-^{14}C was purchased from Sigma Chemical Corp. (St. Louis Mo.).

Strains. For preliminary studies, forty-four 2,4-dichlorophenoxyacetic acid (2,4-D) degrading bacteria were chosen from the Center for Microbial Ecology, Research on Microbial Evolution stock cultures (TFD strains). The TFD isolates were collected from a variety of sources and previously characterized by plasmid type, substrate use profiles, and fatty acid methyl ester content (Table 3.2) (18). Genotypically-distinct 3-CBA degrading strains were chosen from the isolates cultured from geographically-separated pristine soils described in Chapter 2 (Table 3.3). One hundred and sixteen strains have the ability to transform greater than 80% of 1.0 mM 3-CBA in 7 days of incubation in chemically defined medium. Nucleotide fragment patterns generated by repetitive extragenic palindromic-polymerase chain reaction (REP-PCR) and amplified ribosomal DNA restriction analysis (ARDRA) revealed these organisms contained 61 genotypically-distinct strains (8).

FAME analysis. 3-CBA degrading strains were grown for 2 to 5 days at room temperature on R2A agar and harvested by scrapping the agar surface

Table 3.1. Compounds selected for use in substrate range assays.

Compound	Abbreviation	Purity (%)	Source[a]
benzoic acid, Na salt	BA	-	Sigma
phenol	PH	99	Mallinckrodt
aniline	AN	99	Mallinckrodt
toluene	TN	100	Baker
nitrobenzene	NB	99	Aldrich
chlorobenzene	CB	99.6	Aldrich
2,4-D	24D	-	Sigma
2-Hydroxybenzoic acid	2HB	-	Sigma
3-Hydroxybenzoic acid	3HB	99	Aldrich
4-Hydroxybenzoic acid, Na salt	4HB	-	Sigma
2-chlorobenzoic acid	2BA	98	Aldrich
3-chlorobenzoic acid	3-CBA	99+	Aldrich
4-chlorobenzoic acid	4BA	99	Aldrich
2,3-dichlorobenzoic acid	23BA	97	Aldrich
2,4-dichlorobenzoic acid	24BA	98	Aldrich
2,5-dichlorobenzoic acid	25BA	97	Aldrich
2,6-dichlorobenzoic acid	26BA	98	Aldrich
3,4-dichlorobenzoic acid	34BA	99	Aldrich
3,5-dichlorobenzoic acid	35BA	99	Aldrich
2-chlorophenol	2PH	98	Aldrich
3-chlorophenol	3PH	-	Eastman
3-chlorophenol	4PH	-	Aldrich
2,3-dichlorophenol	23PH	-	AccuStand.
2,4-dichlorophenol	24PH	-	AccuStand.
2,5-dichlorophenol	25PH	-	AccuStand.
2 6-dichlorophenol	26PH	-	AccuStand.
3,4-dichlorophenol	34PH	-	AccuStand.
3,5-dichlorophenol	35PH	-	AccuStand.
2-nitrophenol	2NPH	98%	Aldrich
3-nitrophenol	3NPH	99%	Aldrich
4-nitrophenol	4NPH	99+%	Aldrich
2,4-dinitrophenol	24NPH	-	AccuStand.
2-chloroaniline	2AN	98+%	Aldrich
3-chloroaniline	3AN	99%	Aldrich
4-chloroaniline	2AN	98%	Aldrich
2- chlorotoluene	2TN	99%	Aldrich
3- chlorotoluene	3TN	98%	Aldrich
4- chlorotoluene	4TN	98%	Aldrich
2-nitrotoluene	2NTN	99+%	Aldrich
3-nitrotoluene	3NTN	99%	Aldrich
4-nitrotoluene	4NTN	99%	Aldrich
2,4-dinitrotoluene	24NTN	-	AccuStand.
1-chloro-2-nitrobenzene	2NB	99+%	Aldrich
1-chloro-3-nitrobenzene	3NB	95%	Aldrich
1-chloro-4-nitrobenzene	4NB	99%	Aldrich

[a] AccuStandard
Baker Chem. Comp., Phillipsburg, NJ
Mallinckrodt Chem. Comp., St. Louis, Mo.
Aldrich Chem. Comp., Milwaukee, Wisc.
Eastman Chem. Comp., Rochester, NY
Sigma Chem. Comp., St. Louis, Mo.

Table 3.2. 2,4-D degrading isolates used in preliminary investigations (from reference 18).

TFD No.	Strain	Origin	Classes REP	FAME	Biolog	Plasmid
1	4/6/M	Sask.	D	5	5	α
2	1c-LWM	KBS	B	4	4	δ
3	RASC	Oreg.	U	4	4	δ
4	4e-LWM	KBS	B	4	4	δ
5	1e-WRL	KBS	B	4	4	δ
6	1/4/D	KBS	A	4	4	β
7	1d-WRL	KBS	B	4	4	δ
9	2/5/G	Sask.	U	n.d.	2	α
11	2/5/A	Sask.	D	5	5	n.d.
13	4/4/J	KBS	A	4	4	γ
14	3/4/C	KBS	A	4	4	β
15	1/4/H	KBS	A	4	4	β
16	1/4/L	KBS	A	4	4	n.d.
17	EML155	Oreg.	A	4	4	β
18	4/6/K	Sask.	A	4	4	β
19	EML159	Oreg.	U	4	4	α
21	2/4/K	KBS	A	4	4	β
22	4/4/H	KBS	A	4	4	β
23	4/4/C	KBS	n.d.	1	1	α
24	1/6/M	Sask.	e	1	1	α
26	EML146	Oreg.	U	n.d.	4	ε
27	1/4/F	KBS	A	4	4	β
28	2/6/A	Sask.	U	1	4	α
29	5R1-2b	Sask.	g	1	1	α
30	1/6/D	Sask	e	1	1	ε
31	5/5/E	Sask	U	1	1	α
32	EML148	Oreg.	U	n.d.	3	γ
33	2e-WRL	KBS	C	1	1	α
34	5/4/C	KBS	A	4	4	β
35	2/4/M	KBS	U	1	1	ε

Table 3.2 (cont'd).

TFD No.	Strain	Origin	Classes REP	FAME	Biolog	Plasmid
36	1a-WRL	KBS	B	4	4	ε
37	3/6/B	Sask.	f	1	1	α
38	3c-WRL	KBS	C	1	1	α
39	1/6/N	Sask.	f	1	4	ε
40	EML157	Oreg	U	4	4	α
41	3c-LYR	KBS	C	1	1	α
42	5R3-4I	Sask.	g	1	4	α
43	JMP134	Aus.	U	1	1	α
44	2,4-D1	Wash	U	3	3	γ

Table 3.3. Genotypically distinct strains isolated from geographically-separated pristine soils.

Region Strain	FAME ID	Matching Index	Site
Australia			
BN-I-302	n.d.[a]	-	Bridgetown
BN-I-304	n.d.	-	Bridgetown
BN-L-304	n.d.	-	Bridgetown
GE-D-301	*Pseudomonas gladioli*	0.294	Geraldton
California			
CH-J-303	*Pseudomonas gladioli*	0.237	Chabot
CH-U-302	*Pseudomonas putida*	0.291	Chabot
CL-AB-303	*Morganella moganii*	0.111	Cloverdale
CL-C-303	*Pseudomonas gladioli*	0.218	Cloverdale
MU-B-305	*Acinetobacter baumannii*	0.160	Murrieta
VH-Q-302	No match	-	Venice Hills
Chile			
FJ-A-302	n.d.	-	Fray Jorge
LC-G-302	n.d.	-	Los Campanas
RC-01-304	*Arthrobacter oxydans*	0.443	Rio Clarillo
RC-13-306	*Aureobascterium liquefaciens*	0.081	Rio Clarillo
RC-E-302	*Pseudomonas putida*	0.431	Rio Clarillo
RC-J-305	*Paracoccus denitrificans*	0.373	Rio Clarillo
South Africa			
HH-04-304	n.d.	-	Helshoogte
HH-08-302	*Pseudomonas gladioli*	0.169	Helshoogte
HH-08-303	No match	-	Helshoogte
HH-D-301	n.d.	-	Helshoogte
HH-D-302	*Pseudomonas gladioli*	0.243	Helshoogte
HH-D-303	*Pseudomonas gladioli*	0.067	Helshoogte
MB-16-303	*Pseudomonas gladioli*	0.036	Mooresberg
MB-B-304	n.d.	-	Mooresberg
MB-G-302	*Pseudomonas chlororaphis*	0.206	Mooresberg
MB-G-303	*Aureobacterium saperdae*	0.255	Mooresberg
MB-M-302	No match	-	Mooresberg
MR-10-301	*Pseudomonas cepacia*	0.176	Mamreweg
PM-01-303	No match	-	Paarl Mountain

[a]n.d. Not Determined

Table 3.3 (cont'd).

Region Strain	FAME ID	Matching Index	Site
South Africa			
PM-N-301	*Pseudomonas cepacia*	0.060	Paarl Mountain
PM-P-301	*Pseudomonas gladioli*	0.111	Paarl Mountain
WG-15-304	*Pseudomonas cepacia*	0.138	Welgelvallen
WG-H-301	No match	-	Welgelvallen
WG-M-301	n.d.	-	Welgelvallen
Russia			
R2-06-301	*Pseudomonas gladioli*	0.121	
R2-19-301	*Xenorhabdus nematophilus*	0.136	
R2-38-302	*Rhodococcus equi*	0.021	
R2-39-302	*R. equi*	0.013	
R3-32-301	No match	-	
R4-12-301	*Arthrobacter oxydans*	0.199	
R4-16-301	*Pseudomonas gladioli*	0.129	
R4-29-302	*Pseudomonas gladioli*	0.299	
R4-32-301	*Escherichia coli*	0.030	
Saskatchwan			
BT-01-301a	*Pseudomonas gladioli*	0.039	Bittern
BT-I-301	*Pseudomonas gladioli*	0.067	Bittern
BT-K-302	n.d.	-	Bittern
BT-L-302	*Pseudomonas cepacia*	0.104	Bittern
BT-M-302	*Pseudomonas cepacia*	0.064	Bittern
NP-13-301	No match	-	Napatak
NP-C-302	*Pseudomonas gladioli*	0.032	Napatak
PC-C-302	*Pseudomonas cepacia*	0.121	Porcupine
WK-03-303	*Pseudomonas gladioli*	0.028	Waskesiu
WK-11-302	*Pseudomonas gladioli*	0.124	Waskesiu
WK-A-302	*Pseudomonas cepacia*	0.043	Waskesiu
WK-F-302	*Brevibacterium linens*	0.635	Waskesiu
WK-K-302	No match	-	Waskesiu
WV-07-301	*Pseudomonas gladioli*	0.219	Waitville
WV-15-301	*Pseudomonas putida*	0.107	Waitville
WV-I-301	*Pseudomonas cepacia*	0.018	Waitville
WV-N-302	*Pseudomonas putida*	0.254	Waitville

with a Pasteur pipette bent to form a loop. The cell pellet was placed into a screw-capped test tube and stored at -20°C until prepared for analysis. Cellular membrane phospholipids were saponified, methylated, and extracted using the MIDI protocols and gas chromatograms compared to a fatty acid identification library (Microbial Identification Systems, Inc., Neward, Del.).

[^{35}S]Sulfate substrate range assay. DAB-agarose medium was routinely prepared using one of two methods. For the preparation of low numbers of plates (<20), 10 ml of molten double-strength DAB part A containing 1.5% agarose were dispensed into French square bottles and autoclaved. Prior to use, 2X DAB agar was melted in boiling water, and 1.0 ml of sterile part B was added after cooling to 50°C. Sterile concentrated aqueous substrate solutions and sterile distilled water were combined and added to achieve a final concentration of 50 $\mu g\, ml^{-1}$ DAB. Toxicity was anticipated with the dichlorobenzoates and the chlorinated phenols, these compounds were used at a final substrate concentration of 10 and 25 $\mu g\, ml^{-1}$ DAB. Individual plates were poured and immediately amended with 1.0 μCi $Na_2{}^{35}SO_4{}^{2-}\, ml^{-1}$, gently swirled to disperse the isotope, and allowed to harden. For larger experiments (>20), stock solutions, DAB part B, and 1.0 μCi $Na_2{}^{35}SO_4{}^{2-}\, ml^{-1}$ were dispensed individually into petri dishes. DAB part A with 1.5% agarose was prepared in bulk and aseptically dispensed into petri dishes using a sterile self-filling syringe. In both cases, control plates were prepared following the procedures above but only sterile distilled water was added. When solid, a sterile surfactant-free 0.45 μm x 85 mm HATF nitrocellulose filter (Millipore Corp., Bedford, Mass.) was placed on each plate. Plates were incubate 2-3 days at

room temperature to dry filters thus preventing excessive spreading of inoculum and to avoid any gradients in the concentration of the isotope.

Stock cultures were revived from -70°C glycerol stocks by plating onto R2A agar and incubating for 2 days at room temperature. Following incubation, well isolated colonies were suspended in DAB salts base (DAB without vitamin supplement) and incubated 48 hours at room temperature to deplete cell energy reserves. After starvation, 100 µl of cell suspension was transferred into assigned wells of a 96-well microtiter plate. Filter-covered plates with and without substrates were inoculated with the starved cell suspension using a 48-prong inoculator (Sigma Chemical Comp., St. Louis, Mo.). After incubating for 2 weeks at room temperature, the filters were removed from the substrate plates and unincorporated $^{35}SO_4^{2-}$ was removed from the filters by washing on phosphate-buffered 0.2 M Na_2SO_4 agar for no less than 1 hour. Six washed filters were taped onto a 8 in. x 10 in. backing paper, covered with plastic wrap and exposed to Kodak XAR x-ray film (Eastman Kodak Comp., Rochester, New York) for 2 days at room temperature and developed using an X-Omat automated film processor (Eastman Kodak Comp., Rochester, New York). Organisms capable of degrading the test substrates were determined by comparing exposure densities of autoradiographic films between substrate versus no substrate control plates.

Confirmation of 2,4-D degradation. Mineralization of 2,4-D was determined by $^{14}CO_2$ evolution using the 96-well microtiter plate assay of Tabor *et al.* (17) using 50 µg 2,4-D ml^{-1} and 0.1 µCi 2,4-D-UL-ring-^{14}C ml^{-1} in 200 µl DAB. Degradation of 2,4-D in DAB broth culture was measured by high-performance liquid chromatography (HPLC) with a Hewlett-Packard (Palo Alto,

Calif.) series 1050 HPLC equipped with a LiChrosorb RP-18 (10 μm) column (E. Merck, Darmstadt, Germany) using 70% methanol:30% 1% H_3PO_4 as the mobile phase. The detector was set to record absorbance at 230 nm. ^{14}C incorporation (6) and $^{35}SO_4^{2-}$ assimilation assays were conducted using DAB solidified with agarose amended with 0.02 μCi 2,4-D-UL-ring-^{14}C ml^{-1} and 1.0 μCi $Na_2{}^{35}SO_4^{2-}$ ml^{-1}, respectively.

Method calibration. The amount of inoculum transferred to filters were determined gravimetrically. The inoculator was placed into a 96-well plate containing 150 μl distilled water $well^{-1}$, transferred to an HATF filter placed on a dry agar plate, and held in place for 5 seconds with gentle pressure. Autoradiographic detection levels were determined by titrating $^{35}SO_4^{2-}$ in 2-fold dilutions in distilled water, transferring to HATF filters with the 48-prong inoculator, and exposing to x-ray film for 48 hours Several solidifying agents were examined with the ^{35}S method above. Plates were prepared without the addition of a carbon source, inoculated with a 24 h starved cell suspension, and incubated for 5 days at room temperature. Autoradiographs were compared to determine if the solidifying agents affected nonspecific ^{35}S background.

Cluster analysis. Relationships between strains and their substrate use profiles were analyzed using NTSYS software (Applied Biostatistics, Inc., Setauket, N.Y.). A simple matching similarity matrix was computed from the results of the substrate range test. Dendrograms were generated using unpaired group mean averages.

Results and Discussion

Method calibration. The volume of inoculum transferred per inoculator prong was determined gravimetrically from the mass of water retained by HATF filters on dry agar plates and averaged 1.70 µl (SD=0.23). The diameter for a single prong wetted zone was 4 mm. The minimum detection level for ^{35}S on HATF filters following 48 hours exposure at room temperature was calculated to be 2.13×10^{-5} µCi and the level of saturation (activity above which no additional resolution is observed on autoradiographs) was 0.01 µCi. A conservative estimate of the amount of degradation required to produce a minimum detectable signal is outlined below. Since DAB contains 0.998 mM SO_4^{2-} and 1.25 µCi ml^{-1}, the specific activity of ^{35}S plates is 1253 µCi mol^{-1}. Using a C:S of 50 (13), the equivalent amount of carbon required to produce this signal was determined to be 8.5×10^{-7} mMol. Using 3-CBA as a model substrate, this is equivalent to 19.1 ng 3-CBA. With an effective medium volume of 44 µl (the volume of medium immediately beneath the wetted zone from which an organism can obtain substrate) and 50 µg 3-CBA ml^{-1}, the total available mass of 3-CBA is 2.2 µg. Assuming 40% assimilation, the detectable level of 3-CBA degradation is 2.18%.

The practical detection limit, defined to be the level of ^{35}S assimilation required to resolve growth on a substrate from background assimilation was higher due to the presence of carbon presumably from nutrients and organic contaminants in solidifying agents. Comparisons of autoradiographs from substrate-free control plates with those from the detection level calculations above revealed a practical detection level between 0.33×10^{-3} to 1.33×10^{-3}

μCi. Using the same rationale as above, the practical limit of degradation required at 50 μg 3-CBA ml^{-1} is between 13 and 54%. However, this is a very conservative estimate since the effective volume is much larger due to diffusion.

The assimilation of $^{35}SO_4^{2-}$ has been used successfully in aquatic and marine microbial ecology to indirectly measure the assimilation of organic compounds (3, 12). Since the method can be generally applied with most growth substrates, it overcomes the need for numerous radiolabeled compounds that may be required if examination of an extensive range of substrates is desired. However, as the method is indirect, care must be taken to reduce background incorporation of ^{35}S resulting from the metabolism of organic matter in the medium or solidifying agents. DAB was formulated to provide most growth factors required by fastidious aerobic microorganisms at nutrient concentrations low enough to produce acceptable levels of background (7). Previous experience in this laboratory has shown sufficient levels of organic contaminants are present in bacteriological grade agar to support the growth of oligotrophic environmental isolates. Since one of the objectives of this study was to achieve high sensitivity, several solidifying agents were evaluated to find one with optimal characteristics of low nonspecific $^{35}SO_4^{2-}$ assimilation, ease of use, and cost effectiveness. In parallel studies using Bacto agar, Agar Noble, agarose, and Gelrite™, agarose and Gelrite™ produced the lowest background. Of the two, agarose was chosen for its ease of use and cost effectiveness. However, background levels for agarose and Gelrite™ were not drastically lower than Bacto agar or Agar Nobel. It should be noted that in certain circumstances the additional cost of using agarose may not be

warranted as satisfactory results may be obtained with less costly solidifying agents.

2,4-D degradation studies. Using the TFD strains, a comparison was made between $^{35}SO_4^{2-}$ assimilation, $^{14}CO_2$ evolution, ^{14}C incorporation, and substrate disappearance using HPLC (Table 3.4). Thirty-five strains exhibited 2,4-D metabolism using the $^{35}SO_4^{2-}$ assimilation method and were supported by similar results in the other three methods. One isolate (TFD 28) did not assimilated $^{35}SO_4$ but was shown to degrade 2,4-D using the other methods. Five isolates (TFD 1, 11, 12, 16 and 39) assimilated $^{35}SO_4$ in the presence of 2,4-D but did not release $^{14}CO_2$, assimilate ^{14}C, or decrease the concentration of 2,4-D in HPLC analyses. Five isolates (TFD 24, 26, 31, 40 and 42) assimilated $^{35}SO_4^{2-}$ at one 2,4-D concentration but not at the other. All demonstrated 2,4-D metabolism in the other methods and were, therefore, considered to be in agreement.

The $^{35}SO_4^{2-}$ assimilation assay duplicated the results of HPLC, $^{14}CO_2$ evolution, and ^{14}C incorporation in 86% of the strains. This demonstrated the method could be used as an initial assay for other catabolic activities. Based upon the above evaluation, an error rate of 14% could be anticipated. False negative results would lead to the conclusion that an organism lacks a specific phenotype when it would otherwise be observed in other assays. Unfortunately, these organisms would probably not be examined in subsequent analyses. False positive results, on the other hand, would cause an overestimation of the frequency of a trait in a collection of organisms. Positive $^{35}SO_4^{2-}$ results can be verified by additional analyses and corrected. With this confirmation, the overall error observed with the TFD collection would be

Table 3.4. Comparison of 2,4-D degradation using HPLC, $^{14}CO_2$ evolution, ^{14}C incorporation, and ^{35}S assimilation.

TFD No.	mM 2,4-D in DAB							
	HPLC		$^{14}CO_2$ Evol.		^{14}C Incorp.		^{35}S Assim.	
	1.25	0.25	1.25	0.25	1.25	0.125	1.25	0.125
1	-	-	-	-	+	-	-	+
2	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+
5	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+
7	+	+	+	+	+	+	+	+
8	+	+	+	+	+	+	+	+
11	-	-	-	-	-	-	-	+
12	-	+	-	-	-	+	-	+
13	+	+	-	+	+	+	+	+
14	+	+	+	+	+	+	+	+
15	+	+	+	+	+	+	+	+
16	-	-	-	+	+	-	+	-
17	+	+	+	+	+	+	+	+
18	+	+	+	+	+	+	+	+
19	+	+	+	-	+	+	+	+
20	+	+	+	+	+	+	+	+
21	+	+	+	+	+	+	+	+
22	+	+	+	+	+	+	+	+
23	+	+	+	+	+	+	+	+
24	+	+	+	+	+	+	-	+
25	+	+	+	+	+	+	+	+
26	+	+	+	+	+	+	+	-
27	+	+	+	+	+	+	+	+
28	+	+	+	+	+	+	-	-
29	+	+	+	+	+	+	+	+
30	+	+	+	+	+	+	+	+
31	+	+	+	+	+	+	-	+
32	+	+	+	+	+	+	+	+
33	+	+	-	-	+	+	+	+
34	+	+	+	+	+	+	+	+
35	+	+	+	+	+	+	+	+
36	+	+	+	+	+	+	+	+
37	+	+	+	+	+	+	+	+
38	+	-	+	+	+	+	+	+
39	-	+	-	-	-	-	-	+
40	+	+	+	-	+	+	+	-
41	+	+	+	+	+	-	+	+
42	+	+	+	+	+	+	+	-
43	+	+	+	+	+	+	+	+
44	+	+	+	+	+	+	+	+

reduced to 2% resulting in an equivalent 98% level of accuracy. At this level of accuracy, the method offers a great deal of promise in the initial screening of large numbers of organisms for novel catabolic traits.

When other aromatic substrates were tested, the $^{35}SO_4^{2-}$ assimilation method demonstrated TFD strains increased protein synthesis when incubated on medium containing a wide range of other substituted aromatic compounds (Table 3.5). There was no correlation between the 2,4-D phenotype and the ability of organisms to degrade other compounds. Thus, no predictions could be made about the range of metabolizable substrate based on activity against a a single chlorinated compound. However, it was evident the method could be used to analyze the substrate ranges of large populations.

Catabolic diversity of 3-CBA degrading strains. Fatty acid methyl ester identification of the isolates generated very low matching indices (Table 3.1). This has been observed in this laboratory previously and is probably due to limitations inherent in the database as it was developed primarily for the identification of clinical isolates (data not shown). However, the genera represented by the genotypically-distinct 3-CBA strains reveal 61% are *Pseudomonas*. The predominance of *Pseudomonas* within this group is not surprising as they are widely known to be metabolically versatile.

The substrate range of a 3-CBA isolate was defined to be the number of other chloroaromatic compounds which would cause the assimilation of $^{35}SO_4^{2-}$. Fifty-eight genotypically-distinct 3-CBA degrading bacteria isolated from geographically-separate pristine soil were examined using 47 different aromatic compounds. Duplicate $^{35}SO_4^{2-}$ assimilation analysis revealed the method was reproducible in 81% of the cases and had disparate results (positive in one trial, negative in the other) in 19% of the cases. Since this

Table 3.5. Substrate range of 2,4-D degrading bacteria determined by ^{35}S assimilation. Abbreviations: Ba, benzoic acid; An, aniline; CBn, chloro-benzene; NBn, nitrobenzene; Ph, phenol; 2NT, 2-nitrotoluene; 2NP, 2-nitrophenol; Pa, phenoxyacetic acid; 2Pa, 2-chlorophenoxyacetic acid; 4Pa, 4-chlorophenoxyacetic acid; 34D, 3,4-dichlorophenoxyacetic acid; 24Pa, 2-(2,4-dichlorophenoxy)-propionic acid.

Table 3.5 (cont'd.)

TFD No.	Ba	An	CBn	NBn	Ph	2NT	2NP	Pa	2Pa	4Pa	34D	24Pa
1												
2	•											
3												
4	•	•	•									
5		•										
6		•										
7		•										
9												
10												
11		•	•	•						•	•	
12			•	•								
13												
14			•	•		•	•	•				
15												
16												
17				•			•					
18				•			•					
19				•					•			
21							•					
22							•			•		
23			•			•						
24			•			•	•	•				
26	•		•			•	•	•				
27			•									
28	•					•						
29	•		•	•		•		•				
30			•			•						
31						•						
32	•		•			•						
33	•	•	•	•		•		•				
34	•											
35	•		•									
36	•		•	•						•		
37			•			•						
38	•	•		•		•		•			•	
39	•			•								
40	•			•								
41	•	•	•	•		•		•			•	
42				•					•			•
43	•		•	•					•	•		
44		•		•					•			

method had an 80% level of reproducibility, it is adequate for the screening of environmental isolates.

Results of the substrate range assays indicate a wide continuum of activity within the 3-CBA strains tested (Table 3.6). These environmental isolates are metabolically versatile and implies they synthesize a number of different enzymes to deal with natural, as well as man-made, aromatic compounds. The range of activity extended from three strains, WK-F-302, GE-D-301, and BN-I-304, which used only two substrates to strain R4-29-302 which assimilated $^{35}SO_4^{2-}$ with 37 compounds. The average number of substrates used was 11 (SD=8) with a median of 9 (Figure 3.1). Benzoate was used most frequently by 93% of strains. As would be expected, 3-CBA was the most often used chloroaromatic compound and stimulated $^{35}SO_4^{2-}$ assimilation in 86% of the strains (Table 3.7). Eight strains (14%) were not able to use 3-CBA in $^{35}SO_4^{2-}$ tests. This is consistent with the 20% instability frequency observed in Chapter Two. No strains were able to grow using 2-chlorobenzoate, and 2- and 3-nitrophenol at 50 μg substrate ml^{-1}.

There appears to be a decreasing preference in the use of hydroxy- and chlorobenzoate substitutions in the *meta* and *para* positions, to the *ortho* position (Table 3.7). The frequency of use for dichlorobenzoic acids was from 5 to 67% of the isolates. 2,6- and 3,4-Dichlorobenzoate were the most frequently used at 67 and 64%, respectively, however dichlorobenzoates as a whole were not used by a majority of the isolates. The use of 3,4-dichlorobenzoate is consistent with the use preferences of *meta* and *para* monosubstituted benzoate. The ability to use 2,6-dichlorobenzoate does not fit this model and implies that another mechanism may be responsible for the degradation of this compound. The trend in position preference was not observed in chlorophenol

Table 3.6. Substrate ranges of genotypically-distinct 3-CBA degrading bacteria determined by ^{35}S incorporation. Abbreviations for substrates are listed in Table 3.3. Strains are arranged in accordance to their positions in Figure 3.2

Strain	Metabolic Group	No. Used	2CB	2NPH	3NPH	4NB	4NPH	3AN	4AN	35BA	4TN	2AN	TN	AN	23PH	24PH	25PH	2TN
BN-I-302	I	6					●											
MB-G-303	I	4																
R2-19-301	I	4																
BN-I-304	I	2																
CH-J-303	I	3																
BN-L-304	I	4																
HH-D-301	I	3																
WK-F-302	I	2																
FJ-A-302	I	4																
R2-06-301	I	4																
RC-01-304	I	5																
PM-01-303	I	4																
GE-D-301	I	2																
CL-C-303	I	4																
MR-10-301	I	5																
BT-01-301a	II	8																
MB-16-303	II	9																
BT-K-302	II	8																
BT-M-302	II	7																
HH-D-302	II	7																
HH-D-303	II	7																
WG-M-301	II	8																

Table 3.6. (cont'd.)

Strain	Metabolic Group	3TN	3NTN	2NB	3NB	35PH	NB	24D	3PH	26PH	34PH	2NTN	4NTN	CB	4PH	2AS	24NTN	4BA
BN-I-302	I																	
MB-G-303	I																	
R2-19-301	I																	
BN-I-304	I																	
CH-J-303	I																	
BN-L-304	I																	
HH-D-301	I																	
WK-F-302	I																	
FJ-A-302	I																	
R2-06-301	I																	
RC-01-304	I								●									
PM-01-303	I																	
GE-D-301	I																	
CL-C-303	I															●		
MR-10-301	I															●		
BT-01-301a	II															●		
MB-16-303	II																	
BT-K-302	II																	
BT-M-302	II																	
HH-D-302	II																	
HH-D-303	II																	
WG-M-301	II																	

Table 3.6. (cont'd.)

Strain	Metabolic Group	2PH	25BA	23BA	24BA	3AA	pAA	2HB	34BA	PH	26BA	3HB	4HB	3CB	BA
BN-I-302	I							●				●	●	●	●
MB-G-303	I											●	●	●	●
R2-19-301	I											●	●	●	●
BN-I-304	I													●	●
CH-J-303	I							●						●	●
BN-L-304	I							●				●		●	●
HH-D-301	I											●		●	●
WK-F-302	I											●		●	
FJ-A-302	I									●		●	●	●	
R2-06-301	I									●		●		●	●
RC-01-304	I									●		●		●	●
PM-01-303	I										●	●	●		●
GE-D-301	I						●					●			
CL-C-303	I						●							●	●
MR-10-301	I							●					●	●	●
BT-01-301a	II						●	●		●		●	●	●	●
MB-16-303	II				●		●	●		●	●	●	●	●	●
BT-K-302	II					●			●	●	●	●	●	●	●
BT-M-302	II								●	●	●	●	●	●	●
HH-D-302	II								●	●	●	●	●	●	●
HH-D-303	II							●	●		●	●	●	●	●
WG-M-301	II						●	●	●		●	●	●	●	●

Table 3.6. (cont'd.)

Strain	Metabolic Group	No. Used	2CB	2NPH	3NPH	4NB	4NPH	3AN	4AN	35BA	4TN	2AN	TN	AN	23PH	24PH	25PH	2TN
NP-C-302	II	8																
PM-P-301	II	9																
WK-11-302	II	9																
CL-AB-303	II	11																
HH-08-303	II	11																
WK-K-302	II	10																
WK-A-302	II	9																
NP-13-301	II	6																
MB-G-302	II	9																
LC-G-302	II	6																
PC-C-302	II	9																
BT-I-301	II	12																●
R4-29-302	II	10																●
BT-L-302	II	8																
WG-15-304	II	6																●
MB-B-304		6																
MB-M-302		6																
HH-04-304	III	13																
R3-32-301	III	15																
RC-J-305	III	12																
HH-08-302	IV	18											●					
PM-N-301	IV	14																

Table 3.6. (cont'd.)

Strain	Metabolic Group	3TN	3NTN	2NB	3NB	35PH	NB	24D	3PH	26PH	34PH	2NTN	4NTN	CB	4PH	2AS	24NTN	4BA
NP-C-302	II																	
PM-P-301	II																	
WK-11-302	II																	
CL-AB-303	II																	
HH-08-303	II																	
WK-K-302	II																	
WK-A-302	II																	
NP-13-301	II																	
MB-G-302	II																	●
LC-G-302	II														●			
PC-C-302	II														●			
BT-I-301	II	●								●								
R4-29-302	II	●																
BT-L-302	II																	
WG-15-304	II																	
MB-B-304			●									●						
MB-M-302																		
HH-04-304	III								●						●			
R3-32-301	III								●						●			●
RC-J-305	III										●				●			
HH-08-302	IV		●									●		●			●	●
PM-N-301	IV																	●

Table 3.6. (cont'd.)

Strain	Metabolic Group	2PH	25BA	23BA	24BA	3AA	pAA	2HB	34BA	PH	26BA	3HB	4HB	3CB	BA
NP-C-302	II						●	●	●	●	●	●	●		●
PM-P-301	II						●	●	●	●	●	●	●	●	●
WK-11-302	II						●	●	●	●	●	●	●	●	●
CL-AB-303	II	●		●		●	●		●	●	●	●	●	●	●
HH-08-303	II			●		●	●	●	●	●	●	●	●	●	●
WK-K-302	II					●	●	●	●	●	●	●	●	●	●
WK-A-302	II					●	●		●	●	●	●	●	●	●
NP-13-301	II							●	●	●	●		●		●
MB-G-302	II			●			●	●		●	●	●	●		●
LC-G-302	II					●				●			●	●	●
PC-C-302	II					●		●	●	●	●		●	●	●
BT-I-301	II				●			●	●	●	●	●	●	●	●
R4-29-302	II				●			●	●		●	●	●	●	●
BT-L-302	II		●		●			●	●		●		●	●	●
WG-15-304	II								●		●		●	●	●
MB-B-304								●	●				●		●
MB-M-302		●		●					●	●	●		●		
HH-04-304	III	●		●		●		●	●	●	●	●	●	●	●
R3-32-301	III	●	●		●	●		●	●	●	●	●	●	●	●
RC-J-305	III	●			●	●			●	●	●	●	●	●	●
HH-08-302	IV		●	●	●		●	●	●	●	●	●	●	●	●
PM-N-301	IV	●	●	●	●		●	●	●	●	●	●	●	●	●

Table 3.6. (cont'd.)

Strain	Metabolic Group	No. Used	2CB	2NPH	3NPH	4NB	4NPH	3AN	4AN	35BA	4TN	2AN	TN	AN	23PH	24PH	25PH	2TN
R2-38-302	IV	12																
R2-39-302	IV	12																
WV-N-302	IV	14								●				●				
RC-13-306	IV	13												●	●			
WG-H-301		16																
WV-15-301		18														●		●
CH-U-302		16				●												
WV-I-301		17						●	●		●	●	●					●
MU-B-305	V	29					●								●	●	●	
VH-Q-302	V	22														●	●	
R4-12-301	V	30								●				●	●	●	●	
R4-16-301	V	28										●			●	●	●	
RC-E-302	V	36						●			●	●	●	●	●	●	●	
R4-32-301	V	37							●	●	●	●	●	●	●		●	●

Table 3.6. (cont'd.)

Strain	Metabolic Group	3TN	3NTN	2NB	3NB	35PH	NB	24D	3PH	26PH	34PH	2NTN	4NTN	CB	4PH	2AS	24NTN	4BA
R2-38-302	IV													●				●
R2-39-302	IV													●				●
WV-N-302	IV																	●
RC-13-306	IV			●									●					●
WG-H-301						●			●	●	●				●	●	●	
WV-15-301		●					●			●						●		
CH-U-302			●	●	●		●	●				●	●	●			●	
WV-I-301		●	●				●					●	●				●	
MU-B-305	V		●		●	●	●	●	●	●	●	●	●	●			●	●
VH-Q-302	V					●		●		●	●		●				●	●
R4-12-301	V			●	●	●	●	●	●		●		●	●	●	●	●	●
R4-16-301	V			●	●	●		●	●	●	●	●			●	●	●	●
RC-E-302	V	●		●	●	●	●	●	●	●	●	●	●	●	●	●	●	
R4-32-301	V	●	●	●	●	●	●	●		●	●	●	●	●		●	●	●

Table 3.6. (cont'd.)

Strain	Metabolic Group	2PH	25BA	23BA	24BA	3AA	pAA	2HB	34BA	PH	26BA	3HB	4HB	3CB	BA
R2-38-302	IV		●	●	●				●	●	●	●	●	●	●
R2-39-302	IV		●	●	●	●			●	●	●	●	●		●
WV-N-302	IV		●	●	●	●		●	●		●	●	●	●	●
RC-13-306	IV		●		●				●		●	●	●	●	●
WG-H-301		●				●	●		●	●	●		●	●	●
WV-15-301		●			●	●	●	●	●	●	●	●	●	●	●
CH-U-302						●	●	●					●	●	●
WV-I-301										●		●	●	●	●
MU-B-305	V	●	●		●	●		●	●	●	●	●	●	●	●
VH-Q-302	V	●	●	●	●	●		●	●	●	●	●	●	●	●
R4-12-301	V	●	●	●	●			●	●	●	●	●	●	●	●
R4-16-301	V	●	●	●		●	●	●	●	●	●		●	●	●
RC-E-302	V	●	●	●	●	●	●	●	●	●	●		●	●	●
R4-32-301	V		●	●	●	●	●	●	●	●	●	●	●	●	●

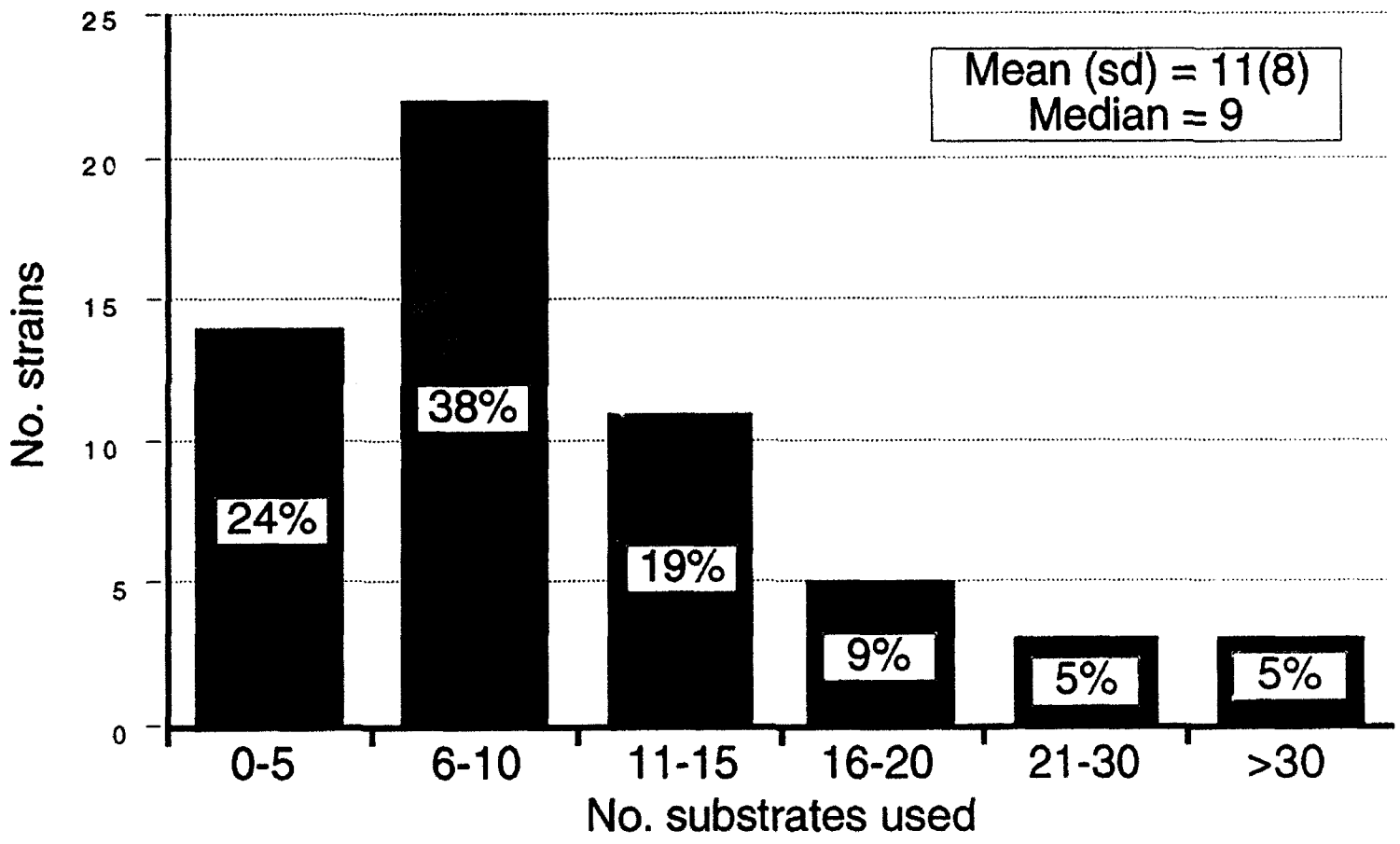

Figure 3.1. Range of additional chloroaromatic compounds used by genotypically-distinct 3-CBA degrading isolates. Percentage represents proportion of strains represented in each class.

Table 3.7. Percentage of 3-CBA isolates degrading other substituted aromatic compounds. Metabolic groups are derived from cluster analysis in Figure 3.2.

Substrate[a]	Metabolic Group (% Strains)					All Strains (%)
	I	II	III	IV	V	
3AA	0	32	100	33	83	34
pAA	13	50	0	33	50	36
AN	0	0	0	33	50	9
2AN	0	0	0	0	50	7
3AN	0	0	0	0	17	3
4AN	0	0	0	0	17	3
2AS	13	5	0	0	67	16
BA	80	100	100	100	100	93
2HB	27	68	67	50	100	57
3HB	73	77	100	100	67	74
4HB	40	100	100	100	100	84
2BA	0	0	0	0	0	0
3BA	87	86	100	83	100	86
4BA	0	5	33	100	83	22
23BA	0	14	33	83	83	26
24BA	0	18	67	100	83	31
25BA	0	5	33	100	100	24
26BA	7	91	100	100	100	67
34BA	0	82	100	100	100	64
35BA	0	0	0	17	33	5
CB	0	0	0	50	67	14
24D	0	0	0	0	100	12
NB	0	0	0	0	67	12
2NB	0	0	0	17	67	10
3NB	0	0	0	0	83	10
4NB	0	0	0	0	0	2

[a] Compound abbreviations listed in Table 3.1.

Table 3.7 (cont'd).

Substrate	Metabolic Group (% Strains)					All Strains (%)
	I	II	III	IV	V	
PH	20	77	100	67	100	64
2PH	0	5	100	17	83	22
3PH	7	0	67	0	67	14
4PH	0	9	100	0	50	16
23PH	0	0	0	17	83	10
24PH	0	0	0	0	83	10
25PH	0	0	0	0	100	10
26PH	0	5	0	0	83	14
34PH	0	0	33	0	100	14
35PH	0	0	0	0	100	12
2NPH	0	0	0	0	0	0
3NPH	0	0	0	0	0	0
4NPH	7	0	0	0	17	3
TN	0	0	0	17	33	7
2TN	0	14	0	0	17	10
3TN	0	9	0	0	33	10
4TN	0	0	0	0	33	5
2NTN	0	0	0	17	67	14
3NTN	0	0	0	17	33	10
4NTN	0	0	0	17	83	14
24NTN	0	0	0	17	100	17
Avg. no. used	4	9	13	13	30	11
No. strains	15	22	3	5	6	58
% Total	26	38	5	9	10	100

metabolism. Phenol was used by 61% of the isolates but 2-, 3-, and 4-chlorophenol were used by only 22, 14, and 16% of the strains, respectively. Chlorinated aniline, nitrobenzene, and toluene were used by approximately 10% of the strains. This frequency is similar to that for the nonsubstituted substrates.

The nonsubstituted molecule was used by an isolate in most cases where chloro-substituted benzoate (3), phenol (2), aniline (2), toluene (3) and nitrobenzene (1) were metabolized (numbers in parentheses indicate number of strains which did not use the nonsubstituted molecule while degrading a substituted one). This suggests that the enzymes used for the degradation of the substituted molecules are not unique to chloroaromatic metabolism and possibly share pathways with the nonsubstituted molecule. The ability of 35% of the isolates to degrade 3-chloroanisaldehyde is not surprising as this compound was recently reported to be naturally produced by lignin degrading fungi (4, 5). In contrast to chloro- substituted aromatics, seven strains using nitrotoluene did not use toluene. Phenol, on the other hand, was used by all strains using nitrophenol.

Cluster analysis was performed on the substrate use patterns to find evidence of metabolic relatedness among the strains (Figure 3.2). Placement of metabolic divisions was decided by inspecting the dendrogram for the presence of coherent groups. When done, five groups were evident. The average number of compounds used by each group shows the clusters closely approximate the number of substrates used (Table 3.7) and range from 4 to 30 compounds for Groups I to V, respectively. Group I is the least metabolically versatile with an average of only four substrates used. These compounds are predominantly benzoate, 3-hydroxy- and 3-chlorobenzoate. Group II is the predominate group and contains 38% of the strains. The average number of

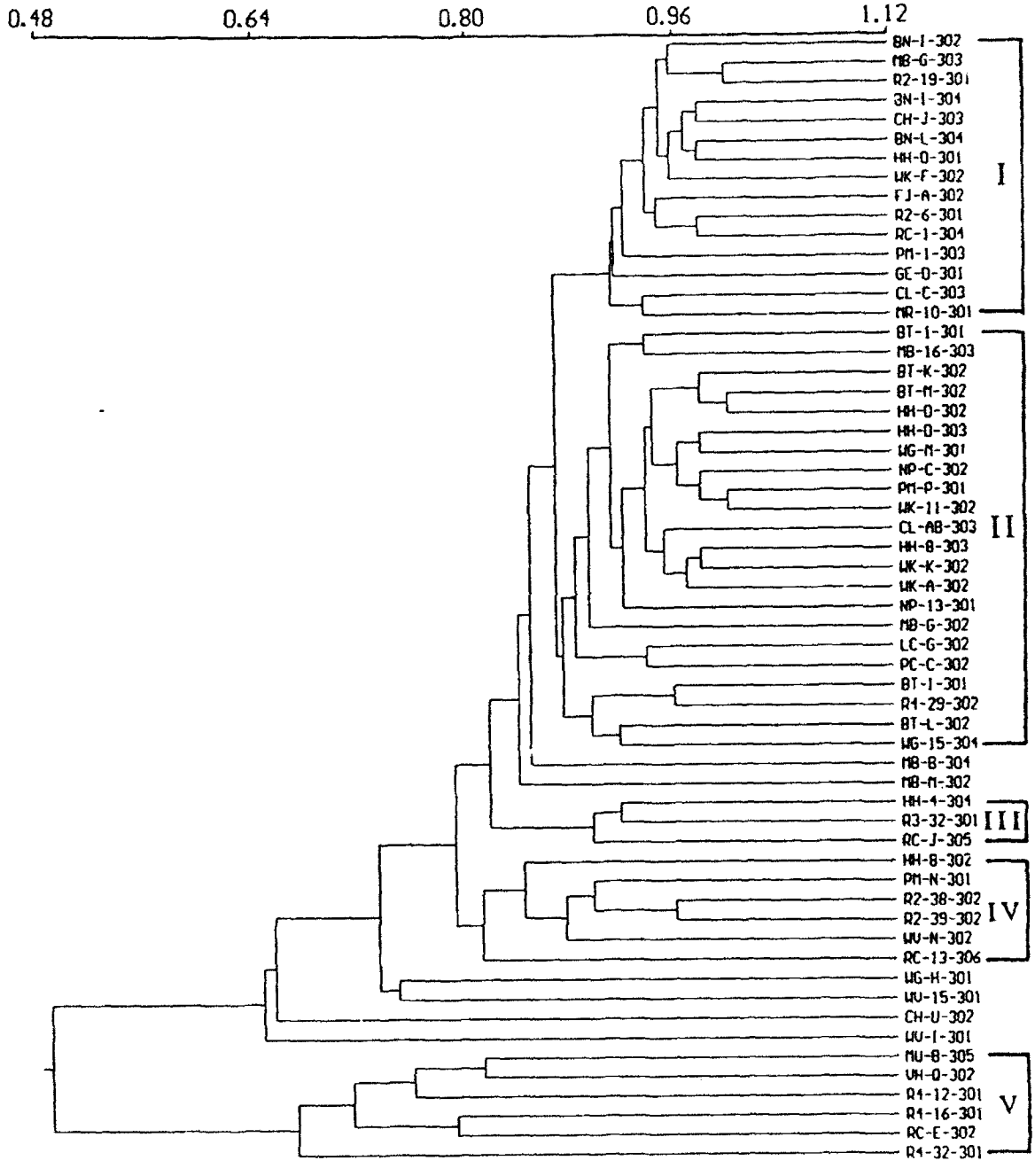

Figure 3.2. Dendrogram of substrate use patterns of genotypically-distinct 3-CBA degrading bacteria. ($r_{x,y}$=0.926)

substrates used by this group is nine with most using phenol and substituted benzoates. Together Groups I and II account for 64% of the strains. Since these isolates tend to use only the benzoates, this implies most metabolic versatility is contained within a single enzyme class. Substrate range in this group appears to be determined by variation in benzoate degrading enzymes. Group V, on other hand, is very versatile and is represented by strains which can use every substrate tested with the exception of 2-chlorobenzoate, 1-chloro-4-nitrobenzene, 2- and 3-nitrophenol; the four substrates not used by any isolate. Metabolic versatility in this group cannot be explained by enzyme variation since the compounds used cross several metabolic classes.

While these strains formed five metabolic clusters, there does not appear to be any regional, climatic, soil, or vegetational fidelity within any of the clusters. However, 33 and 50% of the Group V strains are from California and Russia, respectively. Interestingly, the three isolates from Russia come from the same site and both California strains come from the two southern sites. The significance of this site preference is not clear. Among the three pairs of isolates with identical substrate ranges, all are combinations of boreal forest/sclerophyllous woodland associations. While edaphic, climatic, and vegetational factors may play a role in structuring microbial communities in general, they do not have a major influence on the structure of 3-CBA degrading populations. This suggests that if substrate exposure is important, secondary metabolites produced during the degradation of organic residues may be a reasonable explanation for the development of substrate specificity.

Due to the variety of organic compounds produced in natural environments, many bacteria isolated from pristine soil environments can metabolize a broad range of aromatic substrates. It is surprising, however, to

find organisms which can readily use chlorinated aromatic compounds; compounds thought to be xenobiotic and possibly requiring evolutionary changes prior to the observed expression of the phenotype. This explanation accounts for the separate and specific pathways observed in previously characterized 3-CBA isolates. Activity can also occur if an organism is preadapted to xenobiotic degradation by possessing enzymes, while not specifically evolved for xenobiotic degradation, do degrade them presumably due to their of structural similarity to natural compounds. This is most likely the case of the 3-CBA degrading strains examined during this study as all were isolated from soils without any documented exposure to man-made chloroaromatic pesticides. These results indicate wide spread distribution of catabolic activity against compounds thought to be xenobiotic and support the hypothesis that soil bacteria are preadapted to degrade chlorine substituted aromatic compounds.

Acknowledgements

I thank Helen Garchow, Kellogg Biological Station, for conducting FAME identification of the isolates.

Literature Cited

1. **Chaudhry, G. R., and S. Chapalamadugu.** 1991. Biodegradation of halogenated organic compounds. Microbiol. Rev. 55:59-79.

2. **Commandeur, L. C. M., and J. R. Parsons.** 1990. Degradation of halogenated aromatic compounds. Biodegradation 1:207-220.

3. **Cuhel, R. L., C. D. Taylor, and H. W. Jannasch.** 1982. Assimilatory sulfur metabolism in marine microorganisms: considerations for the application of sulfur incorporation into protein as a measurement of natural population protein synthesis. Appl. Environ. Microbiol. 43:160-168.

4. **deJong, E., J. A. Field, H.-E. Spinnler, J. B. P. A. Wijenberg, and J. A. M. deBont.** 1994. Significant biogenesis of chlorinated aromatics by fungi in natural environments. Appl. Environ. Microbiol. 60:264-270.

5. **deJong, E., A. E. Cazemier, J. A. Field, and J. A. M. deBont.** 1994. Physiological role of chlorinated aryl alcohols biosynthesized de novo by the white rot fungus *Bjerkandera* sp. strain BOS55. Appl. Environ. Microbiol. 60:271-277.

6. **Dunbar, J., A. Rhodes, D. Wong, M. Yarou, and L. Forney.** Unpublished data.

7. **Forney, L. J. (Michigan State University).** 1992. Personal communication.

8. **Fulthorpe, R. F. (Michigan State University).** 1994. Personal communication.

9. **Häggblom, M. M.** 1992. Microbial breakdown of halogenated aromatic pesticides and related compounds. FEMS Microbiol. Rev. 103:29-72.

10. **Hartmann, J., W. Reineke, H.-J. Knackmuss.** 1979. Metabolism of 3-chloro-, 4-chloro-, and 3,5-dichlorobenzoate by a Pseudomonad. Appl. Environ. Microbiol. 37:421-428.

11. **Hickey, W. J. and D. D. Focht.** 1990. Degradation of mono-, di-, and trihalogenated benzoic acids by *Pseudomonas aeruginosa* JB2. Appl. Environ. Microbiol. 56:3842-3850.

12. **Jordan, M. J., and B. J. Peterson.** 1978. Sulfate uptake as a measure of bacterial production. Limnol. Oceanogr. 23:146-150.

13. **Luria, S. E.** 1960. The bacterial protoplasm: composition and organization, p. 15. *In* I. C. Gunslaus and R. Y. Stanier (ed.), The bacteria: a treatise on structure and function, Academic Press, New York.

14. **Reineke, W., and H.-J. Knackmuss.** 1988. Microbial degradation of haloaromatics. Ann. Rev. Microbiol. 42:263-287.

15. **Schnitzer, M.** 1982. Organic matter characterization, p. 581-594. *In* A. L. Page, R. H. Miller, and D. R. Keeney (ed.), Methods of soil analysis, part 2. American Society of Agronomy, Inc., and Soil Science Society of America, Inc., Madison.

16. **Schraa, G. and A. J. B. Zehnder.** 1985. Biodegradation of chlorinated compounds, p. 278-291. *In*, A. Gorseth (ed.), Organic micropollutants in the aquatic environment. 4th Euro. Sym. Vienna.

17. **Tabor, H. C., C. W. Tabor, and E. W. Hafner.** 1975. Convenient method for detection of $^{14}CO_2$ in multiple samples: application to rapid screening for mutants. J. Bacteriol. 128:485-486.

18. **Tonso, N. L., V. G. M. Calabrese, and W. E. Holben.** Submitted for publication.

19. **Vandenbergh, P. A., R. H. Olsen, and J. P. Colaruoltolo.** 1981. Isolation and genetic characterization of bacteria that degrade chloroaromatic compounds. Appl. Environ. Microbiol. 42:737-739.

ABSTRACT

DISTRIBUTION AND CATABOLIC DIVERSITY OF 3-CHLOROBENZOIC ACID DEGRADING BACTERIA ISOLATED FROM GEOGRAPHICALLY-SEPARATED PRISTINE SOILS

By

Albert Nute Rhodes III

Chlorinated aromatic compounds have been used extensively in industry and agriculture for over the past 50 years. Even though chloroaromatic compounds are generally viewed as xenobiotic, numerous bacteria have been isolated that are able to mineralize these compounds. However, it is not known whether these isolates have recently evolved new catabolic traits or if they are genetically preadapted for xenobiotic degradation. In this study, 3-chlorobenzoic acid (3-CBA) was chosen as a model substrate to test the hypothesis that natural bacterial populations are preadapted for chloroaromatic degradation. Pristine soil ecosystems, defined as those with no documented history of exposure to chlorinated aromatic compounds, were sampled to determine if isolates could be cultivated without long-term enrichment. Two ecosystems were selected: 1) Mediterranean, sclerophyllous woodlands in California, Chile, South Africa, and Australia; and 2) boreal forests in Canada and Russia. A two-step enrichment protocol using 3-CBA-UL-ring-^{14}C was used to detect mineralization in mixed communities. Primary soil enrichments were treated with 50 μg 3-CBA g•dry•soil^{-1} and secondary enrichments were performed with 50 μg 3-CBA ml^{-1} defined medium. Isolates were obtained by

plating on R2A agar. In all, 610 3-CBA degrading isolates were cultured from these sites indicating that the distribution of the 3-CBA catabolic activity is widespread in pristine soil ecosystems. Catabolic activity of individual strains was assessed by HPLC analysis. The amount of 1.0 mM 3-CBA degraded in one week ranged from approximately 1 to 100%. Population structures of 3-CBA degraders were compared by rank ordering the percent transformation in these incubations. Cluster analysis revealed the profiles formed two major population structures; one with a uniform distribution in 3-CBA transforming capacity and the other with a high proportion of low capacity degraders. The variation in the ability of different strains to transform 3-CBA is attributed to natural variation in the specificity of catabolic enzymes. To determine if variability was present in other enzymes, a subset of fifty-eight genotypically-distinct strains able to degrade >80% 1.0 mM 3-CBA in one week were examined for their catabolic versatility using other substituted aromatic compounds. Forty-seven different compounds representing mono and dichlorinated benzoate, phenol, aniline, toluene, nitrobenzene, chlorobenzene, and 2,4-D were used with a novel ^{35}S incorporation assay. The data reveal appreciable diversity in the range of other chloroaromatic compounds metabolized by the 3-CBA isolates. Cluster analysis of the substrate utilization profiles revealed five metabolic groups which formed around the type and number of substrates used.