Molecular Mechanisms of Adaptation of Soil Bacteria to Chlorinated Benzenes

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Molecular mechanisms of adaptation of soil bacteria to chlorinated benzenes

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Welke platgetreden paden kan ik betreden om iedereen te bedanken, zonder wiens hulp tenslotte geen enkele promovendus het tot een goed einde brengt? Ik weet het niet. Een schouderklopje dan, of een aaitje voor diegenen die mij bijzonder genegen waren? Een rijtje namen wellicht, waar men in kan zoeken of de eigen naam er in staat, of juist niet? Ik doe dat liever niet en wil daarom het liefst in het algemeen iedereen bedanken aan de vakgroep en alle bekenden daarbuiten die mij geholpen hebben met het directe onderzoek, de begeleiding, de sfeer erom heen of anderszins. Ik heb het altijd zeer goed naar mijn zin gehad op de vakgroep Microbiologie, en hoop dat ik iedereen tijdens mijn verblijf daar altijd al voldoende dankbaarheid en plezier heb getoond.

Dit promotieonderzoek werd uitgevoerd binnen het Speerpuntprogramma Bodemonderzoek en begeleid door de Programmacommissie Basiskennis Bodemonderzoek .
De afronding van de promotie werd tevens mogelijk gemaakt door bijdragen van de Landbouwuniversiteit en de vakgroep Microbiologie.

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Stellingen behorende bij het proefschrift
Molecular mechanisms of adaptation of soil bacteria to chlorinated benzenes
door **Jan Roelof van der Meer**

1. Het heeft gunstige veiligheids-, milieu- en economische effecten om autowegen voortaan ondergronds aan te leggen: i) auto's kunnen dan altijd met het licht aan rijden, ii) automobilisten hebben geen last meer van weersinvloeden, iii) uitlaatgassen kunnen centraal gereinigd worden, en iv) de asfalteer- en bouwdriften van Bouwkundig Nederland zijn weer voor lange tijd gestild.

2. Volksdansers in Nederland realiseren zich onvoldoende dat het bewegen op muziek in discotheken eigenlijk de hedendaagse volksdans is; omgekeerd beseffen ook discogangers niet dat zij eigenlijk aan volksdansen doen en dus minder denigrerend over die term hoeven te denken.

3. De Landbouwuniversiteit kan haar naam als milieu-universiteit beter eer aan doen door meteen over te schakelen op kringloop- of niet chloorgebleekt papier als haar officiële briefpapier.

4. Een samenleving die het zichzelf toestaat om omwille van een "heilige koe" -de auto- per jaar duizenden slachtoffers te laten maken, is in moreel opzicht nog niet verder gevorderd dan de zogenaamde primitieve samenlevingen waar zij zich graag boven verheven ziet.

5. De beste aanwijzing voor het bestaan van buitenaardse beschavingen is wellicht het feit dat ze nog nooit kontakt met ons hebben gezocht.

6. In de ogen van bacteriën bestaat er geen milieuverontreiniging.

7. De prachtig vormgegeven "handgreep" aan de buik van de nieuwe melkfles gaat helaas voorbij aan het feit dat je een fles niet bij z'n buik uit de schappen van de supermarkt kunt pakken, maar alleen bij de hals.

8. Het ongebreidelde optimisme over het nut van gentechnologische "verbeteringen" aan planten en dieren doet vermoeden dat hier alleen belangen van de betrokken bedrijven en wetenschappers bij gediend zijn.
   Bio/Technology, september 1991

9. De term "viable-but-nonculturable" duidt eerder op de onmacht van de microbioloog om de betreffende bacterie tot groeien te bewegen, dan op een aparte fysiologische status van het organisme; deze status kan daarom beter weergegeven worden door de term "dormant".
   Roszak, D. B., R. R. Colwell. Microbiol. Rev. 51, 1987,365-379.
   Nilsson, L., J. D. Oliver, S. Kjelleberg. J. Bacteriol. 173, 1991, 5054-5059.

10. Primaire resultaten die verkregen zijn door het gebruik van digitale beeldverwerkingsapparatuur en -programma's zouden niet als bewijsmateriaal voor publikatie geaccepteerd moeten worden, vanwege het reële risico van "kosmetische veranderingen".

11. Herinneringen bestaan dankzij taal.

12. Er is een sterke bias in de wetenschappelijke wereld om alleen positieve resultaten te publiceren.

13. Het gebruik van de metafoor "kennisklоof" leidt niet tot een goede aanpak om het gebrek aan begrip en interesse van het publiek in wetenschap op te lossen; beter is het om kennis te zien als een "berglandschap", waarbij het de taak is voor de wetenschapper om de juiste paden aan te geven (het begrip) om de kennisbergen te bestijgen, en om goede uitzichtspunten te vinden van waaruit een goed overzicht over het "berggebied" verkregen kan worden.

Wetenschapsbijlage "De Kloof", de Volkskrant, 21 december 1991.

14. De laatste stelling is meestal een teleurstelling.

Table of contents

Preface

This thesis describes the results of a research project entitled "Molecular mechanisms of adaptation in soil bacteria", which started in 1986 and was financed for 4 years by The Netherlands Integrated Soil Research Programme. The aim of this project was to study genetic events that occur in soil bacteria during adaption to xenobiotic pollutants (e. g. chlorinated benzenes) as novel carbon and energy sources. The project arose from previous studies which were set up in collaboration with the Amsterdam Waterworks in Leiduin to evaluate the fate and biodegradation of halogenated aromatic and aliphatic compounds during infiltration and passage through soil. These compounds occur as micropollutants in water of the river Rhine and are undesirable when this water is used as a source for drinking-water. Soil column experiments which were carried out in the Laboratory of the Amsterdam Waterworks and at the Department of Microbiology showed the possibilities for biodegradation of a number of selected halogenated compounds under different natural conditions (such as oxic and anoxic). However, most of the tested compounds, and especially chlorinated benzenes, were not readily degraded in the soil columns, but long adaptation (or lag-) times were observed before removal of the compounds was measurable. This led to the idea that those microorganisms which were finally capable of growing on chlorinated benzenes, did not possess these properties initially, but either were single or multiple mutants or had acquired genes or gene fragments from different other microorganisms. We decided that chlorinated benzenes were good model compounds to test this idea of adaptation, since no organisms had been described which could use chlorobenzenes as single carbon and energy source. Thus our approach was to isolate bacterial strains which could completely metabolize chlorobenzenes and then characterize these in detail on the genetic level to determine the unique characteristics of chlorobenzene metabolism. This would give us molecular tools to further study the genetic mechanisms which lead to adaptation of bacteria in the environment to xenobiotic chemicals.

The thesis has the following outline. Chapter 1 presents a review of the most relevant literature on the molecular mechanisms of adaptation. It describes the different aspects of adaptation phenomena in biodegradation studies, the existing knowledge on catabolic genes from different aromatic pathways, and the numerous possible evolutionary relationships among those genes and gene clusters. Furthermore, it deals with the genetic events and mechanisms which may cause adaptation, and finally with the possibilities to study genetic interactions in microbial ecosystems. Chapter 2 describes the results of experiments to enhance

biodegradation of chlorobenzenes in soil percolation columns by adding a previously enriched bacterial strain, *Pseudomonas* sp. strain P51, our pet organism in this research. This chapter also presents our observations of adaptation of the natural population of soil microorganisms to chlorinated benzenes. The detailed genetic characterization of the novel metabolic pathway for chlorobenzene degradation in *Pseudomonas* sp. strain P51 is covered in chapters 3 to 6. Important aspects include: i) the presence of a catabolic plasmid in strain P51 with two separate gene clusters which contain the genetic information for chlorobenzene degradation (Chapter 3), ii) the specialization of the catechol 1,2-dioxygenase enzyme from the chlorocatechol pathway of strain P51 for dichlorinated substrates, but yet a high overall homology of the chlorocatechol oxidative genes of strain P51 to those from two other species (Chapter 4), iii) a LysR-type specialized regulator controlling expression from the chlorocatechol pathway genes, which showed strong homology to other known bacterial regulator genes (Chapter 5), and iv) the discovery of a novel transposable element which contained the chlorobenzene dioxygenase genes, which presented strong evidence that the complete catabolic pathway in strain P51 had actually been put together from different gene modules (Chapter 6). The last chapter (Chapter 7) deals with the experiments and techniques that we set up to investigate the genetic interactions and events that determine adaptation under natural conditions. Therefore, we studied the distribution and variation of catabolic genes in soil by using DNA-DNA hybridization with gene probes derived from chlorobenzene genes, and by in vitro amplification of DNA sequences specific for chlorocatechol dioxygenase genes.

Chapter 1

Molecular mechanisms of genetic adaptation to xenobiotic compounds

Molecular Mechanisms of Genetic Adaptation to Xenobiotic Compounds

GENERAL INTRODUCTION

The pollution of the environment with organic compounds of man-made origin has become so evident, that it needs no further introduction. Microorganisms play a major role in the final breakdown of these pollutants (5). Although microbes have been shown to degrade a considerable range of pollutants, the kinetics of this process may be much slower than desired. Numerous field tests and laboratory experiments have revealed a wide range of harmful organic compounds which are slowly biodegradable. These include halogenated aromatics (such as benzenes, biphenyls, and anilines), halogenated aliphatics, and several pesticides (6, 86, 116, 123, 198, 215, 231). This slow biodegradation is partly caused by environmental factors that may determine the rate of disappearance under natural conditions. These factors include physico-chemical parameters (temperature, pH, redox potential, salinity, oxygen concentration), availability of nutrients, accessibility of the substrates, predation, etc. (6, 63, 103, 166, 203). The majority of microorganisms present in the natural environment, however, may not be able to deal effectively with those pollutants that have uncommon structures or properties. Such compounds, which are alien to existing enzyme systems, are called xenobiotics. Xenobiotic compounds are not recognized by those microorganisms as potential substrates, and -in the best case- partially metabolized when resembling a non-xenobiotic analog.

Despite the slow biodegradation of xenobiotic compounds under environmental conditions, microorganisms have been isolated - sometimes after a long period of incubation - that metabolize them completely and at considerable rates. It is therefore of great interest to investigate what determines the apparent adaptation of these microorganisms to use xenobiotics as novel growth substrates. Here we shall focus on the possible genetic events that are underlying this adaptation process. The nature and sequence of those events will be illustrated using examples of the degradation of aromatic compounds. The last part then briefly describes the new genetic approaches and techniques that can be used to study the complex genetic interactions in natural communities.

The study of genetic adaptation of microorganisms to xenobiotic compounds suits a twofold purpose: i) it provides essential information on the versatility and capacity of indigenous microorganisms to clean our environment, and ii) it is an example of rapid bacterial evolution, the study of which will contribute to a better understanding of the nature and frequency of genetic events that determine evolutionary changes.

ADAPTATION OF MICROORGANISMS TO XENOBIOTIC COMPOUNDS

Laboratory experiments carried out under environmental conditions with microbial communities to determine mineralization of individual organic compounds or resistance to heavy metals have shown the adaptive responses from microorganisms to pollutant stress (2, 11, 86, 123, 192, 198, 203, 215). In the adaptive response no initial biodegradation of the tested compounds is measurable. Only after a certain time period, ranging from hours to months, mineralization starts and the compounds are removed rapidly. Adaptation was thus defined operationally as a change in the microbial community that causes an increase of mineralization rates as a result of prior exposure to a certain compound (198). Different mechanisms may lead to an adaptive response. Depending on the nature and concentration of the compound tested, these mechanisms may be induction of specific enzymes or growth of a specific subpopulation in a microbial community (192, 198). A good example of adaptation caused by growth of a subpopulation was observed in experiments which tested the mineralization of *p*-nitrophenol. Adaptation times varied in different ecosystems between a couple of days and a month, however, the increase of biodegradation rates was accompanied by an increase in numbers of microrganisms growing on *p*-nitrophenol (2). A similar direct growth-related adaptation was found for the occurrence of Hg^{2+}-resistance in aquatic bacteria (9, 10, 11).

A different kind of adaptation is the result of selection of a subpopulation in the community, which members have altered enzymatic activities or novel metabolic pathways, owing to infrequent genetic events (11, 198). We can distinguish this genetic adaptation from the other two processes (i. e. induction and growth) since it may take a considerably longer time to become evident and does not necessarily need to be reproducible as it is based on stochastic processes. Genetic adaptation could very well be responsible for the adaptive processes observed in mineralization of recalcitrant xenobiotics, such as halogenated aromatics (2, 64, 86, 174, 186, 197, 198, 215). The nature of genetic adaptation is not fully understood, and several genetic mechanisms may underly this phenomenon both in whole microbial communities or in individual strains.

COMPARISON OF GENES FOR AROMATIC DEGRADATION PATHWAYS

The metabolism of aromatic compounds by bacteria has been extensively studied in all its details and varieties (reviewed in references 27 - 29, 32, 61, 162, 164). Although aerobic mineralization processes are certainly not the only important biodegradation processes in the environment, genetic studies have mainly focused on aerobic pathways. In the following we will limit a discussion to these pathways. A comparison of the major aromatic pathways discovered in bacteria has revealed that essentially all compounds are degraded through a number of variable enzymatic steps to a limited number of central intermediates, the (substituted) catechols (27, 28, 29, 32, 44, 61, 162, 164). This has sometimes been called the "catabolic funnel" (158). In the catabolic funnel, enzymes

of the peripheral routes catalyze the different primary reactions which convert aromatic compounds to catecholic intermediates, and these are then channeled into two possible pathways (73), i) a *meta* cleavage pathway, or ii) an *ortho* cleavage pathway (Fig. 1). Both pathways finally lead to intermediates which are converted by the central metabolic routes, such as the tricarboxylic acid cycle. The concept implies that microorganisms have extended their substrate range by variations in peripheral enzymes, and still kept the central catechol cleavage pathways. The current genetic information on aromatic pathways supports this idea, but in addition suggests that these variations arose through divergence or recombination of a limited number of genes. For example, numerous evolutionary relationships have been found between genes encoding the enzymes of different peripheral branches (Fig. 2). On the other hand, the idea of two non-changing central catechol cleavage pathways has to be adjusted, since important differences in substrate specificity have been discovered in the catechol pathway enzymes (16, 51, 109, 138, 154, 182, 184, 185, 213, 221). The following paragraphes will discuss a number of important gene and enzyme families from aromatic pathways, which have been revealed by genetic studies.

Extradiol dioxygenases

The extradiol dioxygenases form a superfamily of enzymes that catalyze *meta*-fission of aromatic substrates (Fig. 1). One group of this family is formed by the catechol 2,3-dioxygenases. Probably the best characterized representative in bacteria is encoded by the TOL plasmid pWW0 and related TOL plasmids (19, 41, 47, 73, 74, 76, 145, 173, 230). These self-transmissable plasmids contain two large operons which encode the complete degradation of methylbenzenes, such as toluene, xylene, 1,2,4-trimethylbenzene, and methyl-substituted benzoates (45, 87, 131, 229). One operon (*xylCMABN*) codes for the upper pathway enzymes, which oxidize methylbenzenes to (methylated) benzoates (71, 77). The second comprises 13 genes that are all coordinately transcribed and encodes the *meta* cleavage pathway (45, 46, 70, 74). The *meta* pathway enzymes transform (methylated) benzoate via catechol to pyruvate, acetaldehyde and acetate. The catechol 2,3-dioxygenase which catalyzes *meta* cleavage of catechol is encoded by the *xylE* gene (Fig. 1). A second member of this group is the catechol 2,3-dioxygenase encoded by *nahH* (76, 236), which is located on catabolic plasmid NAH7. This plasmid encodes the metabolism of naphtalene, salicylate and benzoate via an upper and a *meta* cleavage pathway (236). Two operons, the *nah* and *sal* operon, contain the required genetic information and encompass more than 20 kb of DNA on this plasmid (Fig. 2) (178, 236). XylE and NahH share 80% identical amino acid residues (76), which suggests a close similarity in function and indicate that they share a common ancestor. All the central genes for the *meta* cleavage route of both TOL and NAH plasmids (e. g. *xylEGFJ* and *nahHINL*) are well conserved between the two plasmids. This has been shown by hybridization studies (8, 117), genetic mappings and in more detail by DNA sequencing data (73, 76).

A separate branch of the superfamily of extradiol dioxygenases was recently proposed (73) and consists of members distantly related to the catechol 2,3-dioxygenases XylE and NahH. To this group belongs the 1,2-dihydroxynaphtalene dioxygenase, encoded by the *nahC* gene, which is present in the *nah*-operon of plasmid NAH7 (Fig. 2). NahC showed only 16% sequence identity to XylE and NahH (73), but 35% and 70% homology with BphC1 (54) and BphC2 (204), the 2,3-dihydroxybiphenyl 1,2-dioxygenases from *P. pseudoalcaligenes* KF707 and *P. paucimobilis* Q1, respectively. TodE, the 3-methylcatechol 2,3-dioxygenase from *P. putida* belongs also to this group sharing 54% homology to BphC1 and only 23% to XylE (239). Further possible candidates of this separate branch of extradiol enzymes are CbpC, a 2,3-dihydroxybiphenyl 1,2-dioxygenase from *P. putida* (93) and a 1,2-dihydroxynaphtalene dioxygenase, which was isolated from a bacterium degrading naphtalenesulfonic acid and shown to be strongly related with BphC2 and NahC in its N-terminal region

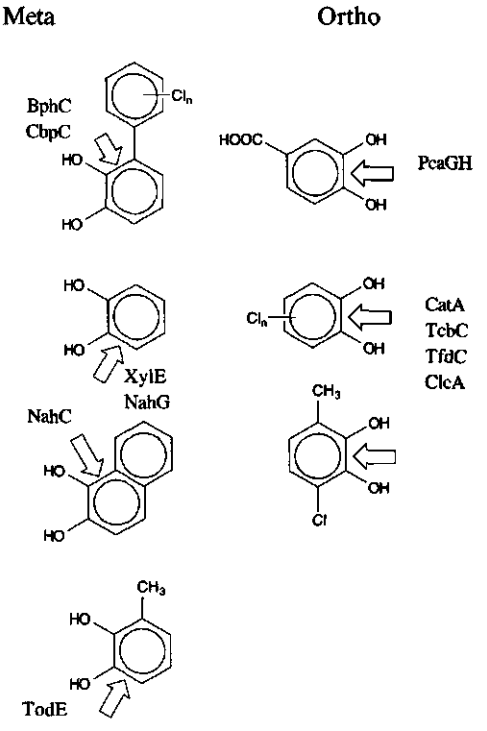

FIG. 1. Extradiol and intradiol dioxygenase enzymes. The archaetype catechol 2,3-dioxygenases XylE and NahG (73) catalyze *meta* cleavage of catechol as indicated by the arrow. The superfamily of extradiol enzymes further includes TodE (239, 240), NahC (73), BphC (54, 204), and CbpC (93). The preferential substrates and the sites of cleavage are indicated. *Ortho* cleavage is catalyzed by intradiol dioxygenases. The superfamily of intradiol dioxygenases includes the protocatechuate 3,4-dioxygenases (PcaGH) (40, 78, 241, 242), the catechol 1,2-dioxygenase CatA (134), and the chlorocatechol 1,2-dioxygenases TcbC (213), TfdC (59), and ClcA (48). A catechol 1,2-dioxygenase activity which could convert 3-methyl-6-chlorocatechol was detected in a mutant of *Pseudomonas* sp. strain JS6 (65).

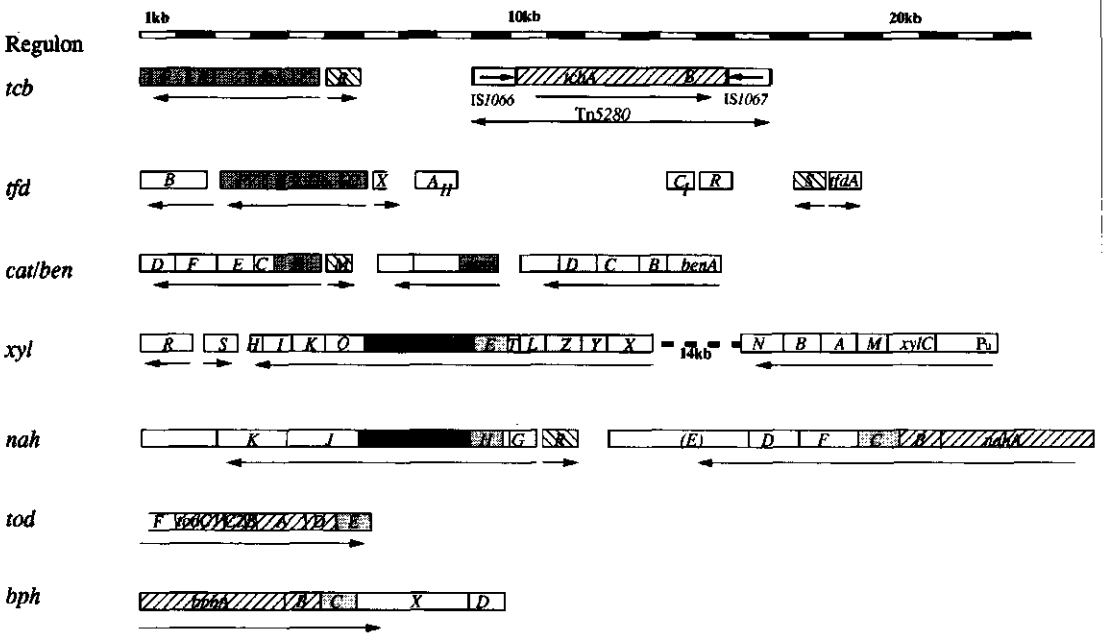

FIG. 2. Comparison of catabolic gene clusters encoding aromatic pathways from different microorganisms. The localization and sizes of the different genes are presented by open horizontal bars and are drawn to scale. In addition, the direction of transcription from the genes is indicated by arrows. Similar patterns of hatching and shading between genes from different organisms indicate significant amino acid similarity or DNA sequence homology. The *tcb* genes were characterized of *Pseudomonas* sp. strain P51, and encode chlorobenzene degradation (213, 214, 216, 217). The *tcbAB* gene cluster is flanked by two iso-insertion elements, IS*1066* and IS*1067*, which form transposable element Tn*5280* (217). The *tfd* genes encode metabolism of 2,4-dichlorophenoxyacetic acid in *A. eutrophus* JMP134(pJP4) (37, 59, 60, 96, 149, 150, 202). The *tfdX* gene is the assignment to an open reading frame which showed homology to *tfdS* and *tcbR*, but which was only partially sequenced. The *cat* and *ben* genes encode metabolism of benzoate in *A. calcoaceticus* (134, 135, 136, 137). Blocks in the horizontal bars without gene assignment represent other open reading frames that were revealed by DNA sequencing. The *xyl* genes of the TOL plasmid encompass four different transcriptional units (19, 74, 76, 77). The distance between the upper pathway genes *xylCMABN* and the *meta* pathway genes *xylXYZLTEGFJQKIH* is around 14 kb, as indicated in the figure. The *nah* genes of the NAH7 plasmid encode naphtalene and salicylate degradation, and are organized in two large gene clusters and a regulatory gene *nahR* (for reviews, see references 178, 236). The last two gene clusters shown, represent the chromosomally located *tod* genes for toluene metabolism of *P. putida* F1 (239, 240), and the *bph* genes for conversion of (chloro-)biphenyls of *P. pseudoalcaligenes* KF707 (54, 56). Homology families and their patterns are: ▩, modified *ortho* cleavage pathway genes and related *ortho* cleavage genes; ▩, extradiol dioxygenase genes; ▨, gene clusters encoding the three component dioxygenase and the dihydrodiol dehydrogenase; ▩, gene clusters encoding the two component dioxygenase and dehydrogenase; ▩, regulatory genes belonging to the LysR family of transcriptional activators.

(110). The genes encoding the extradiol dioxygenases of this subgroup are not directly coupled to a *meta*-pathway operon, but are linked to a aromatic dioxygenase multienzyme complex and a dehydrogenase (Fig. 2, see below). It was suggested that a third categorie of extradiol dioxygenases would exist, namely catechol 2,3-dioxygenases with a high relative activity for 3-methylcatechol compared to the activity for catechol (187, 221). However, no complete DNA or amino acid sequence information exists at present to support this hypothesis for an additional group.

Intradiol dioxygenases

The family of intradiol dioxygenases includes three different groups of enzymes: catechol 1,2-dioxygenase, protocatechuate 3,4-dioxygenase, and chlorocatechol 1,2-dioxygenase. The first two enzymes have been found in the *ortho* cleavage route (or β-ketoadipate pathway) of *P. putida*, *P. aeruginosa*, *P. cepacia*, *Acinetobacter calcoaceticus*, and several other organisms (4, 40, 78, 100, 111, 134, 137, 241, 242) (Fig. 3, 4). These enzymes cleave catecholic intermediates which are formed during the metabolism of benzoate, phenol and p-hydroxybenzoate between the two hydroxyl groups. The three-dimensional structure of the protocatechuate 3,4-dioxygenase of *P. putida* has been determined and showed that two His and Tyr residues are involved in binding of the ferric iron and positioning of the catecholic substrate (140, 209). The His and Tyr residues appear to be strongly conserved among all members of the intradiol oxygenases (78, 100, 134, 149, 213). The overall amino acid sequence homologies between members of the catechol 1,2-dioxygenases and protocatechuate 3,4-dioxygenase α- and β-subunits were relatively low (18 - 22%), which suggests an early evolutionary divergence (78, 134).

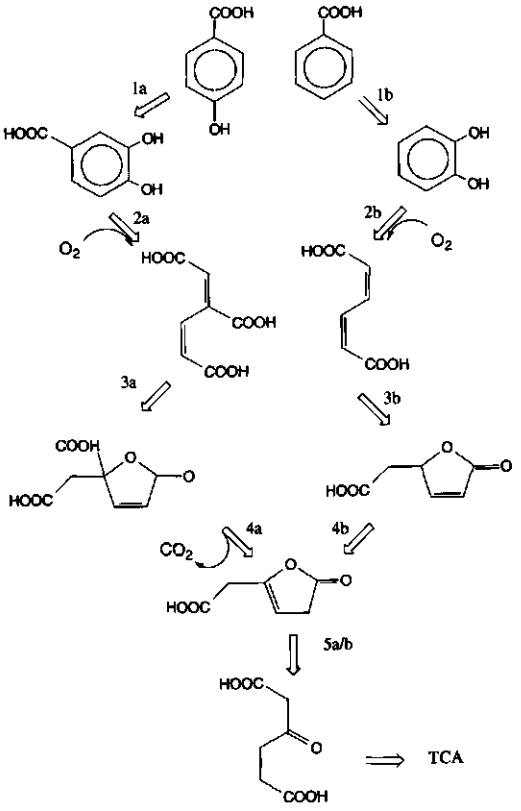

FIG. 3. The *ortho* cleavage pathways (or β-ketoadipate pathways) for benzoate and *p*-hydroxybenzoate degradation (adapted from reference 143). Conversion steps catalyzed in a single reaction are represented by a closed arrow; others by open arrows. Sequential enzymatic steps are: *p*-hydroxybenzoate hydroxylase (1a) or benzoate dioxygenase and dehydrogenase (1b); protocatechuate 3,4-dioxygenase (2a) or catechol 1,2-dioxygenase (2b); muconate cycloisomerase or lactonizing enzyme (3a, 3b); decarboxylase (4a) or muconolactone isomerase (4b); enol-lactone hydrolase (5a, 5b); transferase and thiolase.

The genes encoding the *ortho* pathway were found to be located on the chromosome (4, 40, 85, 137, 223, 226). Two separate but equivalent pathways occur for the metabolism of benzoate (encoded by the *cat* operons) and *p*-hydroxybenzoate (encoded by the *pca* genes) (Fig. 3). The genetic organization of the *cat* and *pca* operons of different species was overall very similar but showed interesting variations in gene order which suggested that gene rearrangements had taken place (40, 85, 137, 143). In some microorganisms such as *A. calcoaceticus* both *cat* and *pca* operons are present, but in *P. putida* the *catEFD* genes are missing and their role is taken over by equivalent enzymes encoded by the *pcaDEF* genes (reviewed in reference 107). Interestingly, the genes encoding the catechol 1,2-dioxygenase (*catA*), and the two different subunits of protocatechuate 3,4-dioxygenase (*pcaH* and *pcaG*), are in most cases not associated with the other genes of the pathway (Fig. 2) (4, 78, 137, 143, 241, 242).

The isolation and genetic characterization of a number of bacterial strains capable of degrading xenobiotic compounds, such as chlorinated benzenes (213, 216), chlorinated benzoates (24, 25, 26, 48, 59, 60, 223), and 2,4-dichlorophenoxyacetic acid (36, 37, 149), presented strong evidence for another branch of the intradiol dioxygenase family with important differences in substrate specificity. Most of these novel strains converted chlorinated aromatic compounds via a chloro-substituted catechol, which was cleaved in *ortho* fission by specific chlorocatechol 1,2-dioxygenases. The conversion of chlorocatechols in these strains was mediated by a modified *ortho* cleavage pathway, by enzymes that had different substrate potential than their β-ketoadipate counterparts (Fig. 4). The individual enzymatic steps of the modified *ortho* cleavage pathway or chlorocatechol oxidative pathway have been characterized in full detail in *Pseudomonas* sp. strain B13 (38, 39, 162, 164, 184, 185, 189), in *Alcaligenes eutrophus* JMP134 (37, 109, 149, 154, 182, 183, 202), and in other bacteria which metabolize chlorinated benzenes (64, 142, 152, 174, 186, 197, 216). The enzymes of the modified *ortho* pathways convert of a wide range of substrates, which indicates evolution of these enzymes towards novel (xenobiotic) substrates (16, 109, 138, 154, 189, 213). Purification and characterization of several enzymes of the modified *ortho* pathway, which is in progress in several laboratories, will give important information on the evolutionary changes in the catalytic domains (16, 109, 147). Contrary to the β-ketoadipate pathway genes, the genes for the modified *ortho* cleavage route have in many cases been located on catabolic plasmids (24, 25, 36, 37, 48, 216, 223, 234), and their organization differs substantially from that of the chromosomally *cat* and *pca* gene clusters (Fig. 2). Three cases have been described in detail, i) the *clcABD* operon of *P. putida* (pAC25) (24, 25, 26, 48, 59, 60), ii) the

tfdCDEF operon from *A. eutrophus* JMP134(pJP4) (37, 59, 96, 149), and iii) the *tcbCDEF* operon of *Pseudomonas* sp. strain P51(pP51) (213, 216). In these three organisms, the chlorocatechol 1,2-dioxygenase genes appeared to be linked to the rest of the pathway genes in a single operon. Besides the chlorocatechol 1,2-dioxygenase gene, only the chlorocycloisomerase gene of these operons is significantly homologous to a counterpart in the β-ketoadipate pathway (47). No other genes of the modified *ortho*-pathway have been reported to be significantly related to genes from the *cat* or *pca* gene clusters, which renders speculation about their ancestry currently impossible.

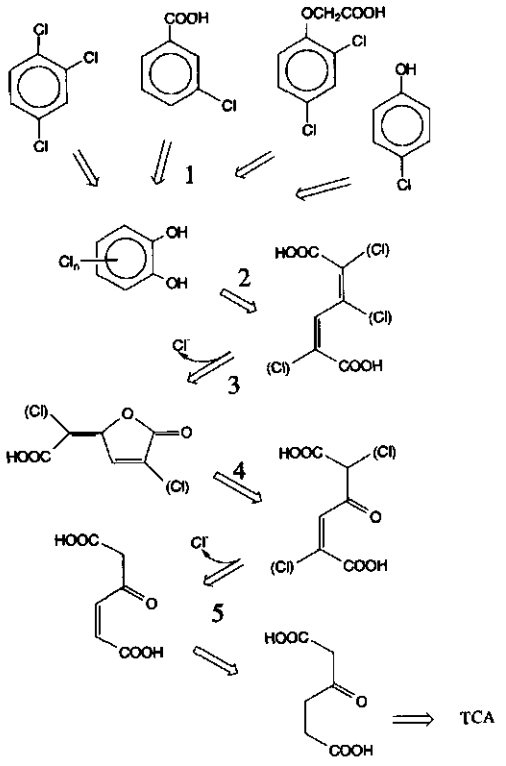

FIG. 4. The modified *ortho* cleavage or chlorocatechol oxidative pathway. This pathway is used as central metabolic route for the degradation of chlorinated catechols, which arise in conversion of chlorinated benzenes, chlorinated benzoates, 2,4-dichlorophenoxyacetic acid, or chlorophenol (reviewed in references 27, 32, 164). A variety of peripheral enzymes lead to the formation of chlorocatechols (1). Cleavage of chlorocatechols proceeds via *ortho* cleavage by a chlorocatechol 1,2-dioxygenase (2). A chloromuconate cycloisomerase catalyzes the formation of chlorocarboxymethylenebut-2-en-4-olide after spontaneous release of one chlorine atom (3). This intermediate is further converted by a dienelactone hydrolase (4). Maleylacetate reductase presumably catalyzes the last steps which also lead to a final dechlorination (5), and to the formation of 3-oxoadipate.

Peripheral routes

The enzymes of the peripheral routes of aromatic degradation exhibit a greater variation than the catechol cleavage pathways. Nevertheless, related genes and their encoded enzymes have been found for the first conversion steps of many aromatic pathways. A first important group includes the dioxygenase multienzyme complexes, which occur in many of the described aromatic pathways (Fig. 2 and 5). These enzyme complexes catalyze a NADH-dependent oxidation of aromatic compounds, such as benzene, toluene, naphtalene, (chloro-)biphenyl, chlorobenzenes, benzoates, and chlorobenzoates, to the corresponding *cis*-dihydrodiol (43, 56, 62, 75, 90, 129, 136, 174, 201, 216, 219). The enzyme complex is formed of two or three different components, a terminal oxygenase (also called Iron-Sulphur-Protein or hydroxylase protein) which consists of two subunits, further a ferredoxin, and a NADH-ferredoxin reductase (62). The genetic organization of the three component systems is as follows: first the two genes for the subunits of the terminal oxygenase, followed by the ferredoxin and reductase genes. This was revealed for the *todC1C2BA*-encoded toluene dioxygenase of *P. putida* F1 (62, 239, 240), and the benzene dioxygenase of *P. putida* (90). The gene order of terminal oxygenase and ferredoxin appeared to be reversed in the *ndoABC* gene cluster, encoding the naphtalene dioxygenase of *P. putida* NCIB9816. In this case the gene for the reductase protein was not yet described (113). Other examples of three component systems, which have not yet been characterized on DNA sequence level, include the naphtalene dioxygenase from the NAH7 plasmid (encoded by the *nahA* locus) (236), the biphenyl dioxygenases from *P. pseudoalcaligenes* KF707 (encoded by the *bphA* locus) (56, 80), *Pseudomonas* sp. LB400 (129), and *P. paucimobilis* Q1 (204). Recently, the gene cluster for a putative three component dioxygenase system was cloned from *Pseudomonas* sp. strain P51, which catalyzes the oxidation of chlorinated benzenes (216).

Two component dioxygenases have been characterized from toluate and benzoate metabolism in *P. putida* (74, 75) and *A. calcoaceticus* (136). In these two dioxygenases, encoded by *benABC* (136) and *xylXYZ* (74, 75) respectively, there is no separate ferredoxin protein. Instead the electron transport function is fulfilled by a single protein with a ferredoxin-like N-terminal part and an oxidoreductase-like C-terminal region (136). In the two component dioxygenases of *benABC* and *xylXYZ*, the genes encoding large and small subunit of the terminal oxygenase (*benAB* and *xylXY*) are followed by the gene encoding the ferredoxin oxidoreductase (*benC* and *xylZ*) (74, 75, 136). The subunits of the terminal oxygenases (or hydroxylases) of both two and three component systems showed significant deduced sequence identity, which was between 20 and 24% for BenA, TodC1, NdoB, and BnzA (P1) (136). The deduced sequence identity was higher among members of the same group (e. g. BenA and XylX share 62% identical residues; NdoB and BnzA(P1) share 34% identity) (113, 136). It has been suggested that the naphtalene dioxygenase system encoded by the *ndo* genes of *P. putida* NCIB 9816 differs from the other three and two component dioxygenases by having both ferredoxin (NdoA) and ferredoxin-oxi-

doreductase activities (136). In all the multicomponent dioxygenase gene clusters, the genes for the dioxygenase are followed by a gene encoding the dihydrodiol dehydrogenase. A homology of 54% has been reported between TodD and BphB dehydrogenases of *P. putida* F1 and *P. pseudoalcaligenes* KF707 (239), but no information to date is available about other dihydrodiol dehydrogenases.

Several variations exist on the theme of multienzyme dioxygenases, which suggests evolutionary relations of each of the individual components with other systems. A ferredoxin was recently characterized from the toluene-4-monooxygenase multicomponent complex (235). This ferredoxin, which is encoded by the *tmoC* gene, showed 31.6% deduced amino acid sequence identity with the TodA ferredoxin and 28.1% with the $NahA_b$ ferredoxin from the naphtalene dioxygenase (235). The other components of this monooxygenase, which is encoded by five genes, *tmoABCDE*, were not significantly related to proteins of the dioxygenase multienzyme systems. The gene products of *tmoA* and *tmoE*, which are thought to constitute the oxygenase component, were found to be 26% and 22% identical to two polypeptides (DmpN and DmpL, respectively) of the phenol hydroxylase multicomponent system (139, 235). This hydroxylase complex is encoded by the *dmpKLMNOP* operon (139). The *dmpP* gene product was shown to contain a N-terminal ferredoxin-like domain, but otherwise was not significantly related to other components of monooxygenases or dioxygenases (139).

Other enzymes of peripheral routes include single-component hydroxylases and monooxygenases, which are found in various pathways, such as TfdA (202), TfdB (149), NahG (236), and PheA (101), but their evolutionary relationships have not yet been determined. The xylene oxygenase component XylM (75) was recently shown to be similar to the alkane hydroxylase component AlkB (233) (27% sequence identity). Furthermore the 4-toluene sulfonate methyl monooxygenase, which was purified and biochemically characterized, also resembled XylMA, although this enzyme was apparently not membrane associated (121).

Regulatory genes

Catabolic pathways are controlled by specific regulatory proteins, which respond to the presence of inducer molecules and interact with promoter-operator regions of the catabolic operons by binding the DNA. A number of regulatory genes of catabolic pathways and their products have been well characterized. In the TOL-encoded pathways, XylR and XylS coordinately regulate the full transcription of the *xyl* operons (88, 89, 125, 132, 199). XylR responds to xylenes and benzylalcohols and activates transcription of the upper pathway genes (*xylCMABN*) and of *xylS* in the presence of RpoN and IHF (34, 83, 106). XylS on its turn, activates transcription of the *meta* cleavage genes by hyper-expression due to activation by XylR, but also independently by benzoate effector molecules (89, 125). To date, the regulatory genes of the TOL-encoded pathways, *xylR* and *xylS*, have no counterparts in other catabolic operons. The XylS protein, however, was found

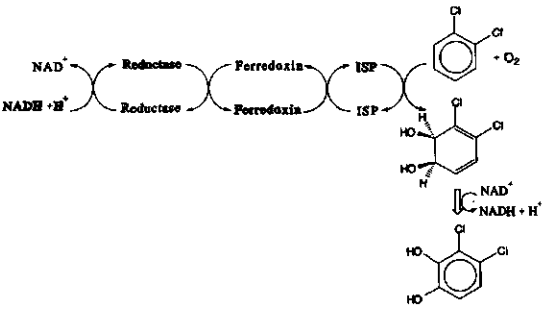

FIG. 5. Schematic representation of the reactions catalyzed by the three component dioxygenase and dihydrodiol dehydrogenase (adapted from reference 62). Molecular oxygen is incorporated into the aromatic ring by a multienzyme complex as electrons are transferred from NADH to the terminal oxidase. The reduced state of the proteins is indicated by a shadowed style. The terminal oxidase (or hydroxylase) is abbreviated by ISP, the iron-sulfur protein, which contains two subunits. In the two component dioxygenases, the ferredoxin and reductase activities are coupled in an electron transfer oxidoreductase.

to belong to the AraC family of bacterial transcriptional activator proteins (156). Contrary to the regulation of the *meta* cleavage pathway of the TOL plasmid by XylS, the *meta*-pathway of NAH7 is regulated by NahR (177, 178). NahR is a member of a different class of bacterial activator proteins, the LysR family (81, 178, 181). The NahR protein binds to the promoter regions of both the *sal*-operon and the *nah*-operon (180). In response to its inducer salicylate NahR changes the contacted nucleotides in its bound region and is able to activate transcription from the *nah* and *sal* promoters (84, 179). Several other regulatory genes and putative regulatory genes which belong to the LysR family have been identified from catabolic pathways, such as *catR* (172), *catM* (135), *tfdS* (81), *tcbR* (214), and *clcR* (31). All of these genes have about the same size of 1 kb and are located in opposite direction from the first gene of the operon at a small spacing of about 200 base-pairs. Interestingly, most of them were reported to encode transcriptional activator proteins, except *catM* which was shown to code for a repressor (135). CatR and CatM were induced by *cis,cis*-muconate (3, 135, 172), but for the others the effector molecules have not been characterized. The widespread occurrence of members of the LysR family of transcriptional regulators among bacteria raises the question about their evolutionary lineage. Although N-terminal parts of the encoded proteins contain similar helix-turn-helix motifs (82), presumed to be involved in DNA-binding, C-terminal parts of the proteins are often very dissimilar (81). This suggests that perhaps not all members of this family arose from a single ancestor regulatory gene, but instead converged from different regulatory circuits to one type (178).

MECHANISMS OF GENETIC ADAPTATION

The numerous relationships between the different aromatic pathways and gene clusters here discussed, reveal

the evolutionary changes that accompanied the development of metabolic routes. The next paragraphes will deal with the different genetic mechanisms which may be the driving forces behind the evolution of aromatic pathways in general and especially behind the adaptation of microorganisms to xenobiotic substrates, which can be seen as a relatively fast evolutionary process. The different mechanisms will be dealt with in three groups, i) mutational selection, ii) genetic recombination and transposition, and iii) gene transfer. Some of these genetic mechanisms are difficult to prove experimentally, since we can only observe their final results (e. g. the existence of homologous genes between different organisms). The importance of the different evolutionary mechanisms will be further clarified, however, by a number of studies in which researchers have tried to establish evolution of catabolic pathways experimentally.

Mutational selection

It is generally accepted that mutational selection or mutational drift is the driving force behind the evolution of genes (20). Mutational selection is caused by base pair changes in the DNA, which can have a direct phenotypic effect, or be without a direct phenotypic effect. In that case base pair changes may accumulate, which results in the slow divergence of genes (mutational drift).

Several examples illustrate the importance of single site mutations on the adaptive process, either by altering the substrate specificity of key catabolic enzymes or by altering the inducer recognition of regulatory proteins. In very detailed studies Clarke et al. (reviewed in reference 28, 29) were able to isolate mutants with altered substrate specificities of the AmiE amidase of *P. aeruginosa*, which could be attributed to single base pair changes. Sequential mutations in the cryptic *ebg* genes of *E. coli* were shown to result in active enzymes for lactose-utilization and other sugars (recently reviewed in reference 67). The TOL-plasmid encoded enzymes and XylS regulatory protein have served as a rewarding system for testing experimental evolution and selection for novel substrate specificities. By creating mutations in *xylE*, Ramos et al. (159) was able to expand the substrate range of the catechol 2,3-dioxygenase enzyme to 4-ethylcatechol, a compound normally not used. A similar strategy was followed later to create a mutant xylene oxidase, encoded by *xylAM*, able to transform *p*-ethyltoluene (1). The same authors showed further the importance of mutations in regulatory genes for expansion of existing pathways. By creating mutations in *xylS* it was possible to isolate mutants of XylS with an altered recognition of effector molecules, i. e. these could be activated by novel compounds such as 4-ethylbenzoate, salicylate, 3,5-dichlorobenzoate, 2,5-dichlorobenzoate and 2,6-dichlorobenzoate (157). Other mutations were obtained in which the *meta* pathway genes were constitutively activated by XylS (238).

Under natural conditions single site mutations are believed to arise continously and at random, due to errors in DNA replication or induced by mutagenic substances. However, some authors have suggested that directed mutations in response to a selective pressure are possible (15, 20, 66). Specific *E. coli* mutants were shown to form revertants in lactose metabolism at frequencies which suggested an effect of the presence of lactose (20, 66). One hypothesis for these observations was that a cell at a certain stage synthesizes a pool of variable mRNAs from one gene rather than a single mRNA, resulting in the production of many different proteins (20). The best performing proteins are selected if the cell can grow, and the corresponding mRNA would then be reverse-transcribed to DNA by a bacterial reverse transcriptase in one of the cell's descendants and incorporated into the DNA. A variation to this hypothesis was recently suggested, proposing the occurence of mismatches in one of the strands of the DNA, which results in the formation of an altered mRNA. When selectively successful, these mismatches should become incorporated in both strands (15). It seems that bacteria have the possibility to increase the mutation rate in certain regions of their genome by altering the activity of *mut* mismatch repair genes. Mutations in several *mut* genes, *mutS*, *mutH*, *mutL*, and *mutD5*, have been shown to increase the mutation rates in *E. coli* (30, 33, 151, 161, 208). Most authors suggest that it is not unlikely that bacterial cells have mechanisms to generate genetic variations as a response to environmental changes. Similar mechanisms could also function when cells adapt to xenobiotic substrates.

Despite the important direct effects of a single base pair mutation on the adaptive process, most base pair substitutions probably have no direct effect and lead to the slow divergence of genes. It is likely that the accumulation of single base pair changes is not sufficient to explain the divergence of related genes and gene clusters in catabolic operons for natural and xenobiotic aromatic substrates (Fig. 2). Assuming rates of spontaneous mutation of 10^{-8} to 10^{-10} per replicative round, it was calculated for example that *xylE* and *nahH* diverged from a common ancestor more than 50,000 years ago (76). Such a slow divergence is certainly not sufficient to account for the arisal of very specialized enzymes of the modified *ortho* cleavage pathway if one assumes that these evolved due to a selection for chlorinated xenobiotic compounds.

Recently, a model was proposed which allows a much faster divergence of DNA sequences by a process called slipped-strand mispairing (78, 120, 134, 143). In this model melted DNA single strands are assumed to have a possibility to shift during replication with respect to their original position, and be stabilized in a heterologous duplex due to the presence of short repetitive motifs which can form perfect matching base-pairs (68, 144, 205). Base pairs which mismatch in this heterologous duplex can then be repaired from either one of the strands as template (120, 127). Model experiments showed that this slipped-strand mispairing occurred during DNA replication (94, 112, 114, 124). The remnants of such a process have been proposed recently by Ornston et al. to be present in DNA sequences of members of the intradiol dioxygenase family, CatA, PcaG, and ClcA (78, 134, 143, 144). Interestingly, the process is thought to act both as a conservational step and as a possible way for fast divergence (144). Repetitive motifs around the very conserved sites that encode the iron-binding histidine residues in intradiol dioxygenases are thus assumed to protect this region from adverse mutations by creating numerous possibilities for repair by slipped-strand mispairing. In regions which have less selective constraint, a faster divergence is possible due to sequence repetitions. The model of slipped-strand mispairing can explain readily

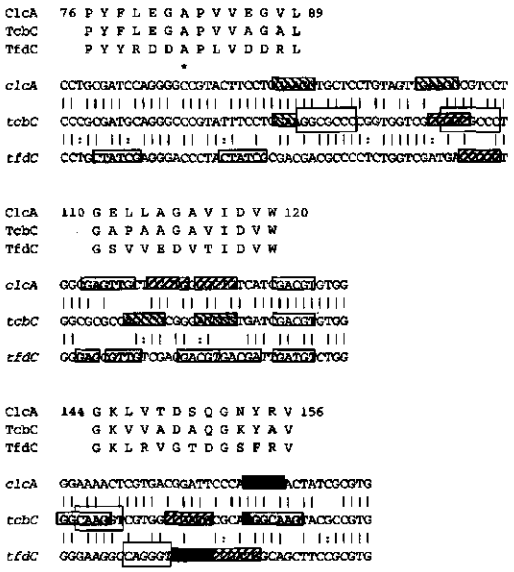

FIG. 6. Short sequence repetitions in chlorocatechol 1,2-dioxygenase genes. Three sites of regional amino acid divergence in the overall highly conserved chlorocatechol 1,2-dioxygenases TcbC, ClcA, and TfdC, are shown with the corresponding DNA sequence. Divergence may have been promoted by the process of slipped-strand mispairing. Short sequence repetitions which account for most of the changes are indicated by similar boxed regions in the DNA sequence. These repetitions may serve as sites for stabilization of a mispaired heteroduplex, or conversely, be remnants of previous mismatched regions, which were repaired from the false template. The asterix above the DNA sequence of the first region indicates the reading frame of the Pro residue. Amino acid residues are represented by the one-letter code and numbers correspond with the sequential numbering of amino acid residues of the complete primary sequences.

the most important sites of variation between three highly conserved chlorocatechol 1,2-dioxygenase genes (213), that form an evolutionary important subgroup of the intradiol dioxygenases (Fig. 6).

Recombination and transposition events

A second possibility by which genetic events can effect adaptation is through recombinational processes and transposition. These can be of diverse nature and have very different effects. First we will consider the possibilities of gene rearrangements in creating novel combinations of existing catabolic gene clusters, then we will review shortly the role of insertion elements in these processes.

The comparison of catabolic operons and gene clusters presented above suggests that gene rearrangements have taken place in the evolution of aromatic pathways. Probably the most extensively studied examples of gene rearrangements occur in the β-ketoadipate pathways in *A. calcoaceticus* and *P. putida*. Several genes have exchanged place in the operons encoding the β-ketoadipate pathways (reviewed in reference 143). In comparison to the β-ketoadipate operons the catechol 1,2-dioxygenase genes of the modified *ortho* pathways (*tcbC*, *tfdC*, and *clcA*) have been transcriptionally fused with the chlorocycloisomerase genes (48, 149, 213). Further evidence for gene rearrangements comes from a comparison of the different chlorocatechol oxidative gene clusters. In the *clcABD* and *tcbCDEF* operons an extra open reading frame was found, which was missing from the *tfdCDEF* operon although no function has been attributed to this open reading frame (48, 213). A comparison of TOL and NAH-encoded pathways showed that a certain part of the *meta* cleavage operons (*xylTEGFJ* and *nahTHJNL*) are identical in gene order and highly homologous (74, 76) (see Fig. 2). However, downstream genes in both operons have changed order and upstream of the catechol 2,3-dioxygenase genes, different genes are found (e. g. *xylXYZL* for benzoate dioxygenase, and the *nahG* salicylate hydroxylase gene). The close relationship of the *xylXYZL* genes with *benABCD* (74, 136) suggests that these genes form a modulelike gene cluster which was inserted in different places in catabolic operons. A similar module could be formed by the genes for the three component dioxygenases (e. g. *todC1C2BAD*) (239, 240), which is found as such in other operons as well (e. g. *bphAB*, *tcbAB*, *nahAB*) (56, 216, 236). The individual genes of this putative module may have undergone gene rearrangements themselves (e. g. *ndoABD* vs. *todC1C2BA*) (113, 240). Apparently, organisms have mechanisms to recombine genes and modulelike gene clusters to form novel combinations. Clear indications of what may direct these rearrangements, such as longer direct or inverted repeats which would allow general recombination, or the activity of insertion elements, are presently missing.

Duplications of specific genes or gene clusters from catabolic operons have been found frequently. Studies with various TOL-type plasmids have shown that upper and lower pathway operons have sometimes switched positions, occur inverted, or have more copies of one catabolic operon or *xylS* regulatory gene present on a single plasmid (23, 145, 191, 228). A pair of 1.4 kb directly repeated sequences, which are present on the TOL plasmid, has been presumed to direct several recombinational processes and the insertion of the *xyl*-operons on the chromosome (126, 193). In various other bacterial strains, isoenzymes of catechol 2,3-dioxygenase or catechol 1,2-dioxygenase occur, which in some cases are encoded by genes on duplicated regions on catabolic plasmids (97, 130, 187). The plasmid pJP4 which contains the genes for 2,4-dichlorophenoxyacetic acid degradation appears to have undergone several duplications (37, 149). Two copies have been found of the chlorocatechol 1,2-dioxygenase gene *tfdC* (58, 59, 96), the 2,4-D monooxygenase gene *tfdA* (150), and possibly of the putative regulatory genes *tfdS* and *tfdX* (81, 82, 214). However, the exact DNA sequence of all these putative gene copies has not been reported, preventing comparison on sequence level. It was further shown that pJP4 contains three sites of directly repeated sequences which were thought to play a role in the formation of a large duplication occurring under specific growth conditions (58). The duplicated region includes all catabolic genes for 2,4-dichlorophenoxyacetic acid degradation (58). A tandem amplification of the catabolic region for 3-chlorobenzoate metabolism was also shown for *Pseudomonas* sp. B13 to occur as a response to changes in growth conditions (155).

Gene duplications have been considered an important mechanism for catabolic evolution in microorganisms (13). Once duplicated, the extra gene copy could essentially be free of selective constraints and thus diverge much faster by the accumulation of mutations. These mutations could eventually lead to a full inactivation, rendering this gene copy silent. Reactivation could occur through the action of insertion elements (see below). Evidence for a silent duplicated gene copy has been reported in *Flavobacterium* sp. strain K172 (141). This strain harbours two isoenzymes of the 6-aminohexanoate dimer hydrolase, one of the enzymes involved in the degradation of nylon oligomer. The two isoenzymes differ 100-fold in their activity toward 6-amino-hexanoate dimer, despite their amino acid sequence identity of 88% (133). Both enzymes were encoded by genes on plasmid pOAD2, located in regions that were proposed to be duplicated.

Insertion elements have been shown to play an important role in rearrangement of DNA fragments, in gene transfer, and in activation or inactivation of silent genes. For catabolic pathways several examples of insertion elements are known. The *xyl*-operons of the TOL plasmid are part of a large transposable element, Tn*4651*, that belongs to the family of Tn*3*-type transposons (210, 211, 212). This transposon was later found to be part of an even larger mobile element, Tn*4653* (211). The bacterial strain *P. cepacia* 249 was shown to carry at least nine different insertion elements, which were present in 1 to 13 copies in the genome of this strain. These IS-elements are thought to be responsible for the extraordinary adaptability and catabolic potential of this strain (119). One particular element, IS*931*, was detected in multiple copies around the *chq* gene locus for 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) degradation (79, 207). IS*931* is presumed to have played a role in the development of the 2,4,5-T pathway, since it was shown to mobilize adjacent DNA fragments (31, 79). Interestingly, this IS-element apparently did not originate from *P. cepacia*. It was not found in other strains of this species, which suggested that IS*931* and perhaps (part of) the 2,4,5-T genes were acquired from a complete different organism (31, 175). IS-elements carrying catabolic genes for aromatic degradation were also discovered or postulated in other bacterial strains. The dehalogenase genes of *P. putida* PP3 are located on a transposable element (194), and the genes for 4-chlorobenzoate degradation of *Alicaligenes* sp. BR60 were shown to be instable and proposed to be localized on a mobile element on plasmid pBR60 (53, 194, 234). Recently, it was shown that the chlorobenzene dioxygenase genes of *Pseudomonas* sp. strain P51 were flanked by two copies of iso-insertion elements, IS*1066* and IS*1067*, and that this complete element, Tn*5280*, was a functional transposon capable of inserting at random into the genome (217). The origin of these two insertion elements remained unclear, although they were shown to be related to insertion elements from *Bradyrhizobium japonicum* (95) and *Agrobacterium rhizogenes* (195).

An other important role of insertion elements is the activation or inactivation of (silent) genes (7, 146, 237). The ends of insertion elements often contain promoterlike sequences, which upon insertion nearby the start of a gene can function as recognition sites for RNA polymerase and as such be able to activate expression of the gene (57). In *P. cepacia*, this was shown for activation of *lacZ* by insertion of IS*406* and IS*407* (190, 232). The elements IS*931* and IS*932* were also able to activate expression of adjacent genes (79). The mechanisms of activation of genes by insertion elements can be considerably complicated. *E. coli* requires for the utilization of β-glucosides the activation of the cryptic *bgl* operon (165), which can occur upon insertion of IS*1* and IS*5* (122). Additional studies showed that the *bgl* operon itself does contain a suitable promoter sequence, which, however, is not available under normal circumstances. It was suggested that cAMP binding protein binds at sites near the promoter and further upstream, which leads to the formation of a strong loop in the DNA and thus would hinder transcription initiation (122). Insertion of an IS in this region prevents the formation of this loop and in this manner cause activation of *bgl* expression.

Gene transfer

A third type of genetic mechanisms that can lead to adaptation to xenobiotic substrates is gene transfer. In this process DNA fragments pass from one microorganisms to another and provide the new host with gene functions that it did not previously contain. Gene transfer takes place through the action of mobile elements, such as plasmids, transposons, and bacteriophages. Since many examples are found of self-transmissable plasmids that carry genes for degradation of aromatic and xenobiotic compounds [for reviews see (22, 27, 47, 69)], their role in spreading these genes to other microorganisms is evident. Early studies on TOL, NAH, and SAL plasmids revealed strong homologies between these plasmids (117), and many examples of naturally occurring related TOL plasmids are known (41, 47). Recently, Burlage et al. (18) have shown that many of the catabolic plasmids for chloroaromatic degradation, such as pJP4, pAC25, pSS50 and pBRC60, have a strong homologous plasmid backbone which contains the genes for replication and transfer. The chlorobenzene plasmid pP51 was also shown to be homologous to pJP4 outside the regions of the catabolic genes. This suggests that a common plasmid ancestor may have acquired different catabolic modules, which subsequently spread throughout the microbial ecosystem.

The importance of gene transfer for adaptation can be illustrated on many studies on experimental evolution of novel metabolic pathways (17, 69, 91, 128, 142, 158, 163). The rationale behind those studies was to identify biochemical blockades in existing pathways which prohibited the degradation of novel substrates, and to suggest the transfer of the proper genes from another organism to remove this blockade. Pathways could be expanded by replacing narrow specificity proteins for broader specificity isoproteins (horizontal expansion), or by providing extra peripheral enzymes to extend pathways on the basis of a central degradation sequence (vertical expansion) (158). The catabolic pathways of *Pseudomonas* sp. strain B13 were horizontally expanded from 3-chlorobenzoate to 4-chlorobenzoate and 3,5-dichlorobenzoate by transfer of the TOL plasmid pWW0 from *P. putida* (91, 163). This transfer provided strain B13 with the XylXYZ toluate dioxygenase of the TOL plasmid, an enzyme with a broader substrate range than the chlorobenzoate dioxygenase of strain B13 itself. Various other derivatives of strain B13 have

been constructed which could degrade chlorosalicylates (118), chlorobiphenyls (128), chloroanilines (115) and chloronitrophenols (17). It should be noted that transfer of catabolic operons on plasmids can lead to regulatory and metabolic problems for the cell. Often additional mutations or genetic rearrangements in the primary transconjugants had to take place before the final strains could be isolated (91, 163). In strains which carried the *meta* and *ortho* cleavage pathways simultaneously, the catechol 2,3-dioxygenase had to be inactivated for proper growth on chlorinated aromatics (163, 171). Since the catechol 2,3-dioxygenase has a higher specific activity than the catechol 1,2-dioxygenase, most chlorinated catechols arising as intermediates in degradation of chlorianted aromatics, were subject to *meta* cleavage. However, *meta* cleavage of chlorocatechols leads to dead-end metabolites or toxic intermediates (12, 102, 171). Recently, bacterial strains have been obtained that induce *meta* and *ortho* pathways enzymes simultaneously without apparent problems, such as *Pseudomonas* sp. strain JS6 (65, 152).

Another interesting case where gene transfer techniques were applied to construct a novel metabolic pathway was that of chlorinated benzenes. Until recently, no bacterial strains were isolated which could metabolize chlorinated benzenes completely. A complete pathway for chlorobenzene degradation would require a specific combination of a broad substrate benzene dioxygenase and dehydrogenase (to oxidize chlorobenzenes to chlorocatechols) and a chlorocatechol oxidative pathway. By mating *P. putida* F1, a strain that carried the *tod*-encoded toluene degradation pathway (Fig. 2), with *Pseudomonas* sp. strain B13, carrying the modified *ortho* cleavage pathway (Fig. 4), Oltmanns and Reineke (142) were able to obtain transconjugants which could metabolize 1,4-dichlorobenzene completely. Similar transconjugant strains were isolated by using chemostatic growth and ceramic bead columns to mate *P. putida* R5-3 (harboring a TOL-like plasmid) and *P. pseudoalcaligenes* C-0, a strain capable of mineralizing benzoate and 3-chlorobenzoate (21, 108). Interestingly, the transconjugants which showed chlorobenzene mineralization were derivatives of strain R5-3 that carried a chromosomal DNA fragment of strain C-0 on the TOL-like plasmid (21). Evidence that similar gene transfer processes in the evolution of the chlorobenzene pathway may take place in nature, came from studies with *Pseudomonas* sp. strain P51, which was isolated by selective enrichment from Rhine sediment (215). A catabolic plasmid in this strain harbors two operons that encode degradation of chlorinated benzenes. One operon was shown to encode a chlorocatechol oxidative pathway (Fig. 4) (213, 216), and the other encoded a chlorobenzene dioxygenase homologous to other aromatic dioxygenases (Fig. 2) (216, 217). This chlorobenzene dioxygenase gene cluster was part of a transposable element, suggesting that it was acquired by a catabolic plasmid which previously contained only a chlorocatechol oxidative operon (217).

THE USE OF MOLECULAR TOOLS TO STUDY GENETIC ADAPTATION IN THE ENVIRONMENT

The study of invidual microorganisms that have been enriched from the environment or constructed by *in vivo* cloning techniques is important for our understanding of genetic mechanisms that underly adaptational processes, but has its limitations for a proper assessment of the importance of genetic adaptation under natural conditions. A thorough understanding of adaptational processes in nature and of the potential of indigenous microorganisms to adapt to novel (xenobiotic) substrates, requires detailed study of the genetic interactions among microorganisms in microbial communities, of the influence of environmental parameters on the occurrence and rate of dissemination of catabolic genes, and of the distribution of microorganisms in the environment that harbor important catabolic genes for xenobiotics degradation.

Genetic interactions in microbial communities are effected by the mechanisms that determine the mobility of DNA sequences between microbial cells, such as conjugation via plasmid replicons and transposable elements, transduction via phages, and transformation of naked DNA molecules [reviewed in: (160, 227)]. Virtually all mechanisms that are known to take place in the laboratory, have been found under environmental conditions as well. Many studies have focused on the presence of plasmids in microorganisms from indigenous sources (49, 50, 92, 105, 167, 188, 225, 230). The occurrence of plasmids in bacterial strains in the environment is certainly a general phenomenon, and the fact that several known self-transmissable plasmids spread to indigenous strains show that an important pool of genetic information may flow among microorganisms (52, 53, 104, 196, 218). The transfer of catabolic plasmid pBRC60 encoding 3-chlorobenzoate degradation of *Alcaligenes* sp. strain BR60 and its expression in indigenous recipient bacteria was investigated in several mesocosm experiments (52, 53). Monitoring the gene flow in natural systems poses considerable problems, and direct extrapolations from in vitro mating experiments to environmental conditions do not take into account the different factors that influence transfer in *situ*. The transfer frequencies of conjugation of an epilithic plasmid encoding mercury resistance (pQM1) dropped a 100-fold in microcosm studies relative to those in filter mating experiments (168, 169).

Since many catabolic genes from different bacteria have been characterized in detail, DNA-DNA hybridization experiments may be carried out with specific gene probes and DNA from indigenous soil microorganisms, in order to obtain an idea of the distribution of these genes in the environment. Results from several comparative studies with soil bacteria isolated by selective enrichment, indicated that homologous catabolic gene clusters are present in microorganisms from different environmental sources. This was shown for genes for the degradation of biphenyls (55), chlorinated catechols (18, 24), naphtalene and xylenes (8, 23, 41, 117). Selective enrichment is likely to bias the true distribution of these genes in the microbial ecosystem, since a large proportion of the bacteria from soil and aquatic environments are not easily culturable, or may carry catabolic genes but not express them. The DNA-DNA hybridization technique is a relatively novel experimental approach in microbial ecology to solve this problem (35, 53, 153, 176, 220). Colonies which are not previously selectively enriched can be hybridized with specific gene probes to detect the presence of DNA sequences in the strains. This techniques allowed a highly specific detection,

and bacterial strains have been detected that carried *mer* resistance genes (170), genes for resistance to cadmium (35), toluene and naphtalene degradation genes (176), chlorobiphenyl genes (153, 220), or chlorobenzoate genes (53). Alternatively, specific indicator gene fragments may be amplified by using the polymerase chain reaction (PCR) with DNA isolated from environmental sources, and subsequently detected by hybridization (148, 200, 222). PCR amplification allowed the detection of specific bacterial strains at very low numbers in samples of various origins (14, 200). These studies, however, could not show the existing variation of genotypes in the environment. Such an indication would require non-specific probes or mixtures of probes in DNA-DNA hybridization, and non-specific or universal primers in PCR-amplification. Experiments which have attempted such an approach are relatively scarce. Using genetic markers from chlorobenzene and chlorocatechol pathways to study the variation of such genes among microrganisms in soil, we encountered a number of problems, such as low specificity and false positive reactions in DNA-DNA colony hybridization (this thesis). To cover a broad range of variations in catabolic genes which are likely to be found among indigenous microorganisms, we developed mixed gene probes derived from a class of highly related genes, the chlorocatechol 1,2-dioxygenases *tcbC*, *clcA*, and *tfdC*. Existing variations in catabolic genes could also be studied by sequence analysis of DNA fragments amplified by PCR from soil DNA, primed with degenerate oligonucleotides for conserved regions in related genes, such as the chlorocatechol 1,2-dioxygenase genes (this thesis). This approach was first demonstrated for the characterization of variations in 16S rRNA genes from microorganisms in microbial communities (222, 224). Other groups were able to detect and isolate homologous gene fragments of the ribulose biphosphate decarboxylase gene (*rbcL*) and the nitrogenase gene (*nifH*), by PCR amplification of DNA from environmental samples with primers for highly conserved regions in these genes (99, 148).

CONCLUSIONS

The available information suggests that microorganisms may eventually be able to deal with any kind of organic compound. We have seen that microorganisms possess a wide range of genetic mechanisms which determine evolutionary changes in existing metabolic pathways. Very specialized enzyme systems and metabolic pathways exist already which deal with a variety of xenobiotic compounds. The question remains if these specialized enzymes evolved from more common isoenzymes by selection after the large scale introduction of xenobiotic chemicals into the environment, which would require a relatively fast evolutionary divergence. There is virtually no information on the time scale of evolutionary divergence for the catabolic genes under natural conditions. Nor is it known if microorganisms can speed up this process in response to the presence of novel (xenobiotic) substrates by increasing mutation rates or favoring replicative errors. On the other hand, the processes of recombination, transposition and gene transfer can be considerably faster and lead to expansion of existing pathways by interchanging catabolic gene modules. To speed up the evolutionary process a little bit, many people have tried to engineer specific microbial strains and equip those with extra catabolic genes (72, 98, 158, 159, 171, 206). However, on many polluted sites the environmental conditions may be the rate-determining factors in the degradation of xenobiotic compounds. We believe that suitable microorganisms will evolve by themselves, and that by creating optimal conditions for microbial activity the degradation of harmful organic chemicals in the environment may be enhanced. The application of engineered strains in the near future will be useful (42) but necessarily limited to closed systems, to treat industrial waste-water streams or heavily contaminated groundwater and soil.

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Chapter 2

Degradation of low concentrations of dichlorobenzenes and 1,2,4-trichlorobenzene by Pseudomonas sp. strain P51 in nonsterile soil columns

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Degradation of low concentrations of dichlorobenzenes and 1,2,4-trichlorobenzene by *Pseudomonas* sp. strain P51 in nonsterile soil columns

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1. SUMMARY

A newly isolated *Pseudomonas* species, strain P51, growing aerobically on all dichlorobenzene isomers and on 1,2,4-trichlorobenzene as sole carbon and energy sources was tested for its ability to mineralize these components also in a nonsterile soil environment. Untreated sand from the river Rhine in which none of the dichloro- and trichlorobenzenes were degraded was placed in a percolation column and inoculated with *Pseudomonas* sp. strain P51. The column was fed continuously with synthetic river water containing the chlorinated compounds at concentrations between 10 μg/l and 1 mg/l. The inoculated microorganisms were able to degrade the chlorinated benzenes and survived for at least 60 days in the column. For each compound a specific threshold concentration was observed below which no further degradation took place, and which was independent of the initial concentration. These thresholds were 6 ± 4 μg/l for 1,2-dichlorobenzene, 20 ± 5 μg/l for 1,2,4-trichlorobenzene and more than 20 μg/l for the 1,3- and 1,4-isomers. Repeated inoculation of the column with strain P51 did not affect this minimal concentration. In noninoculated soil columns the native microbial population adapted to degrade 1,2-dichlorobenzene after a long lag phase, and reduced it from 25 μg/l to a threshold concentration of 0.1 μg/l.

2. INTRODUCTION

Microbial degradation plays an important role in the elimination of organic micropollutants in the environment [1], and may be enhanced by inoculation with genetically engineered bacteria or bacteria isolated by specific selection procedures, capable of degrading certain pollutants [2,3]. The use of these kinds of specific bacteria might be necessary for the breakdown of a number of organic compounds present in high concentrations at heavily polluted sites or in waste-water streams.

Examples of bacteria which have been isolated via enrichment techniques are *Pseudomonas* sp.

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strain B13 which can grow on 3-chlorobenzoate [4] and *Pseudomonas cepacia* AC1100 which is able to use 2,4,5-trichlorophenoxy acetic acid (2,4,5-T) [5]. Recently, bacteria have been isolated in our laboratory by specific selection procedures which can degrade various chlorinated compounds, e.g., *Alcaligenes* sp. strain A175 is able to use 1,3- and 1,4-dichlorobenzene as sole carbon and energy source [6]. New degradation characteristics in bacteria were also developed by genetic recombination techniques [7], e.g., the construction of a pathway for catabolism of chlorosalicylate in *Pseudomonas* sp. B13 [8].

Although much is known about the degradation capacities of these bacteria under laboratory conditions, little information exists on the behavior of such isolated or engineered bacteria when released into the environment or applied to in situ treatment of polluted soils [9]. Chatterjee et al. [10] examined the degradation of 2,4,5-T in soil by using cells of *P. cepacia* AC1100. Strain AC 1100 was able to use 2,4,5-T in the soil, but cell numbers of the strain decreased as the amount of 2,4,5-T in the soil diminished. Pertsova et al. [11] observed degradation of 3-chlorobenzoate in soil by inoculated strains of *Pseudomonas* spp. able to grow on 3-chlorobenzoate. In an article by Goldstein et al. [12] suggestions are given for the possible success or failure when inoculating laboratory-cultivated strains into environmental samples in order to metabolize specific chemicals. One of the factors affecting the possible outcome of inoculation is the concentration of the xenobiotic compound in nature. In many cases these concentrations are very low (μg/l to ng/l range) and under certain circumstances they may be too low to support growth of bacteria. Another important factor might be the presence or absence of other metabolizable organic substrates. Goldstein et al. [12] and Klein and Alexander [13] found, in addition, several other factors which may cause the failure of introduced strains to degrade certain chemicals. These factors are: the presence of toxic inhibitory substances, the pH of the environment and the rate of predation by protozoa.

In this study we report the use of *Pseudomonas* sp. strain P51 for inoculation experiments in soil percolation columns. This strain P51 is able to grow on 1,2,4-trichlorobenzene (TCB), 1,2-, 1,3- and 1,4-dichlorobenzene (DCB) as sole carbon and energy sources. The survival of this strain and its activity in mineralizing dichlorobenzenes and trichlorobenzenes under simulated bank infiltration conditions was tested in a concentration range between 10 and 1000 μg/l for each compound present. Parallel, noninoculated soil columns were used to investigate the degradation of these compounds by the native soil population.

3. MATERIALS AND METHODS

3.1. Isolation and identification

Strain P51 was isolated with the method as described for the isolation of *Alcaligenes* sp. strain A175 [6]. The inoculum consisted of a mixture of sediment from the river Rhine and sand from the dunes (recharge area of the Amsterdam Water Works) near Haarlem, The Netherlands. 1,2,4-TCB served as the sole carbon and energy source and was added in excess to the mineral medium which contained (per liter of demineralized water): 2.9 g $Na_2HPO_4 \cdot 2H_2O$, 1.5 g KH_2PO_4, 0.1 g $MgSO_4 \cdot 7H_2O$, 1.0 g NH_4NO_3, 0.05 g $Ca(NO_3)_2 \cdot 4H_2O$, 1 ml of a trace-element solution [14] and 1 ml of a vitamin solution [6]. It took about 6 months before degradation of 1,2,4-TCB was observed and another 3 months before a bacterium was isolated which was capable of growing on 1,2,4-TCB as sole carbon and energy source. The isolate, strain P51, was a strictly aerobic, Gram-negative, motile, rod-shaped organism with a single polar flagellum. It was catalase and oxidase positive and showed a positive reaction in the Hugh-Leifson test [15]. On the basis of these characteristics, the organism was tentatively classified as a *Pseudomonas* species [16].

3.2. Growth conditions

Cells were grown in 100-ml serum bottles with 40 ml of the above-described mineral medium (without vitamin solution), and with 1,2,4-TCB or 1,2-DCB as the sole carbon and energy source. The pH of the medium was 7.2. 1,2,4-TCB was added to the bottles after sterilization in amounts of 10–20 μl per 40 ml medium, by injecting the

pure substance with a microliter syringe. This corresponded to a final concentration of 2–4 mM. In cultures growing on 1,2-DCB, about 20 μl of the substrate were added after sterilization into a small tube inside the serum bottle. From this tube 1,2-DCB evaporated into the head-space and equilibrated with the medium. Direct contact of growing cells with droplets of 1,2-DCB turned out to be inhibitory for growth. To prevent loss by volatilization of the chlorobenzenes from the medium, the bottles were closed with aluminum caps containing Viton septa (Iso-versinic, Rubber B.V., Hilversum, The Netherlands). The head-space in the bottles (gas/liquid ratio 2 : 1) served as the source of oxygen. The bottles were incubated at 30 °C on a rotary shaker, and cells, needed for inoculation experiments, were harvested by centrifugation after 4–5 days.

3.3. Experimental set-up

Inoculation experiments and native biodegradation experiments were performed in soil percolation columns made of hard PVC with a length of 25 cm and a diameter of 3.4 or 5.5 cm. Stainless steel capillaries (2.0 mm in diameter) reaching into the center of the soil column served as sampling ports at various heights (0, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0, 20.0 and 25.0 cm above the influent port). A coarse filter inside the capillaries prevented blockage by soil particles. The columns were filled with sandy sediments from the river Rhine near Wageningen, The Netherlands. The sand was sieved to remove stones and particles larger than 2 mm. A detailed description of the column system is given in Ref. 17. The columns were percolated continuously in an upflow mode under saturated conditions with a medium that closely resembled the salts composition of Rhine water [18]. It contained, per liter: 27 mg NH_4Cl, 102 mg $MgCl_2 \cdot 6H_2O$, 12 mg K_2HPO_4, 222 mg $CaCl_2$, 215 mg $NaHCO_3$, 7 mg Na_2SO_4 and 0.15 ml of the trace-element solution mentioned above. The medium was made up with highly purified water (Milli-Q Systems, Millipore Company, U.S.A.) to minimize traces of dissolved organic carbon present in the demineralized water. The synthetic Rhine water was continuously aerated and stirred with an excess of granulated marble which served as carbonate buffer in combination with CO_2 in the air. The column system was operated aseptically up to the influent port of the soil columns, where a bacterial filter (Cellulose-nitrate, 0.2 μm, Sartorius GmbH, F.R.G.) prevented back growth of microorganisms from the soil into the feeding lines. The synthetic Rhine water was pumped into the columns by a peristaltic pump (Watson Marlow cassette pump, 502 S/502 AA-19, Watson Marlow Ltd., U.K.) at a flow rate of 10 ml/h. Mixtures of di- and trichlorobenzenes at various concentrations were added continuously with a syringe pump (Perfusor VI, B. Braun Medical B.V., F.R.G.) at a flow rate of 0.6 ml/h. These mixtures were prepared from stock solutions which were made by saturating water with each individual compound. The appropriate dilutions were autoclaved before use. Mixing of the di- and trichlorobenzenes and the synthetic Rhine water took place in a mixing chamber just before entering the soil column. Three sets of concentration ranges of chlorinated benzenes were tested in the inoculation experiments: 5–20 μg/l, 30–200 μg/l and 300–1000 μg/l. In the experiments in which the degradation potential of the native microbial soil population was tested, concentration ranges of 7.5–25 μg/l and 75–400 μg/l (Table 1) were used. All column studies were done at 20 °C. In the inoculation experiments cells from a batch culture of *Pseudomonas* sp. strain P51, pregrown on 1,2,4-TCB, were inoculated in the soil column through the sample ports at either 0.5 cm or 10 cm. About 10^9 cells in 1 ml culture

Table 1

Influent concentrations in the soil percolation experiments (all values in μg/l)

Compound	Inoculation experiment			Experiments with native soil population	
	1	2	3	4	5
1,2-DCB	1000	200	20	25	400
1,3-DCB	1000	150	15	15	400
1,4-DCB	600	80	10	12	150
1,2,4-TCB	350	80	10	10	150
1,2,3-TCB	300	30	5	7.5	150
1,3,5-TCB	–	40	5	10	75

medium were injected with a syringe. In addition, 2 ml of culture medium were also injected to rinse the capillary tube. Inoculation of a column took place upon complete breakthrough of all tested chlorobenzenes in the soil column, thus assuring complete adsorption equilibrium of the chlorinated benzenes with the soil at the concentrations used.

3.4. Analytical methods

Growth of strain P51 in batch cultures was determined by cell counting, and by measuring Cl^--release during growth. Chloride ion measurements were made potentiometrically with a Marius Micro-chlor-o-counter (Marius, Utrecht, The Netherlands) using a NaCl solution as standard. Total cell counts were determined by direct microscopic counting using a Bürker Türk counting chamber. Water samples from the soil columns were taken with a glass syringe connected to a sample port. The syringe was allowed to be filled by the flow pressure of the pumps in the system (10.6 ml/h). Analysis of chlorobenzenes in the water samples was performed by hexane extraction followed by on-column injection into a GC 436 gas chromatograph (United Technologies, Delft, The Netherlands) equipped with a ^{63}Ni-high-temperature electron capture detector and a 25 m capillary column (Sil 5CB, 1.22 μm, Chrompack, The Netherlands). Soil samples were taken with a syringe through the sample ports where a new capillary was installed without the coarse soil filter. A fluorescent antibody technique was used to estimate the cell-number of strain P51 in both water and soil samples from the columns. To remove the bacteria from the soil particles, the soil samples were repeatedly sonicated during 30 s and centrifuged as described by Zvyagintsev [19]. Cells in the resulting water samples were counted under ultraviolet light after incubation with fluorescent-labeled antibodies [20,21].

3.5. Chemicals

1,2-, 1,3- and 1,4-dichlorobenzene and 1,2,4-trichlorobenzene were purchased from E. Merck, Darmstadt, F.R.G. 1,2,3-Trichlorobenzene and 1,3,5-trichlorobenzene were kind gifts from the Organic Chemistry Department, Agricultural University, Wageningen, The Netherlands. All chemicals were of analytical grade and were used without further purification.

4. RESULTS

Our experiments with the native soil microbial population from the Rhine sediments show very poor degradation of the dichlorobenzenes and trichlorobenzenes under simulated bank infiltration conditions. The soil columns were operated for 200 days continuously and fed with a mixture of all di- and trichlorobenzene isomers. After 100 days, degradation of 1,2-DCB was observed in the column with an influent concentration of 25 μg/l (Fig. 1). The other compounds were not affected at either of the concentrations tested (Table 1) within the time period of the experiments. The occurrence of such a long adaptation period for the degradation of 1,2-DCB was confirmed in a second soil column experiment. Once initiated, degradation of 1,2-DCB was stable. The effluent concentration of 1,2-DCB was always in the order of 0.1 μg/l.

Upon inoculation of cells of strain P51 into a soil column with influent concentrations ranging from 300–1000 μg/l (experiment 1), we observed

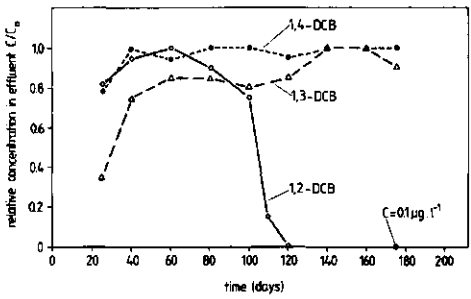

Fig. 1. Breakthrough of the dichlorobenzene isomers in a soil percolation column and the adaptation of the natural microbial population to the degradation of 1,2-DCB (experiment 4, Table 1). Except for 1,2-DCB, none of the other chlorobenzenes was affected by microbial degradation. Influent concentrations: 1,2-DCB 25 μg/l, 1,3-DCB 15 μg/l and 1,4-DCB 12 μg/l. C/C_0 is effluent concentration divided by influent concentration.

a rapid degradation of all dichlorobenzene isomers and of 1,2,4-trichlorobenzene. 1,2,3-TCB was not affected, while 1,3,5-TCB was not added in this experiment. Analysis of chlorobenzene concentrations along the column length showed an immediate degradation of 1,2-DCB at and beyond the point of inoculation (10 cm), while 1,3-DCB, 1,4-DCB and 1,2,4-TCB were degraded at a much lower rate (not shown). After approximately 40 days, rapid degradation of all DCB-isomers and 1,2,4-TCB was observed in the first 10 cm of the column (Fig. 2), although the cells were initially inoculated at a height of 10 cm above the inlet. The overall removal of 1,2-DCB in the column at this concentration was more than 99%. 1,2-DCB was degraded at a maximum rate of about 17 $nmol \cdot d^{-1} \cdot cm^{-3}$ column material in the first 10 cm of the column (pore volume 0.38). The rate of removal of 1,2,4-TCB in the first 10 cm of the column was lower, namely 2.2 $nmol \cdot d^{-1} \cdot cm^{-3}$. Although degradation occurred throughout the column, the effluent still contained low concentrations of the chlorinated benzenes (Table 2, Figs. 3 and 4).

After 77 days of continuous operation during which the three dichlorobenzene isomers and 1,2,4-TCB were readily degraded in the column, the addition of these compounds was stopped. The column received only synthetic Rhine water for 28 days. Hereafter the input of the chlorinated benzenes was resumed at the original concentration. However, none of these compounds was converted anymore, even after another 50 days of operation.

Table 2

Threshold concentrations observed in aerobic degradation of di- and trichlorobenzenes

All values are given in μg/l. n.d., no degradation observed. –, no data available.

Compound	Batch cultures of P51	Inoculated soil columns	Native soil columns	Field studies
1,2-DCB	0.1–0.5	6 ± 4	0.1	0.1–1.0 [a]
1,3-DCB	10 ± 2	50 ± 10 [b]	n.d.	–
1,4-DCB	10 ± 2	30 ± 5 [b]	n.d.	< 0.01 [c]
1,2,3-TCB	n.d.	n.d.	n.d.	–
1,2,4-TCB	20 ± 5	20 ± 5	n.d.	–
1,3,5-TCB	n.d.	n.d.	n.d.	–

[a] Total DCB- and TCB-isomers [15].
[b] Only degradation observed in experiment 1.
[c] From Ref. 14.

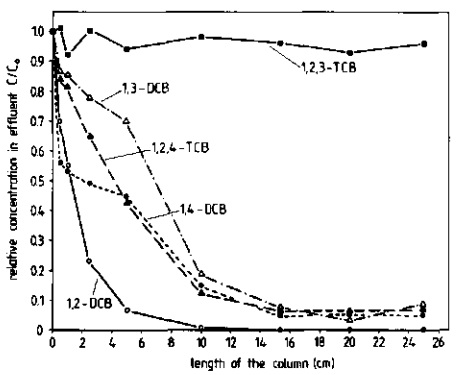

Fig. 2. Concentration profiles of DCB- and TCB-isomers in the column during experiment 1, 65 days after inoculation of *Pseudomonas* sp. strain P51. The strain was originally added at a height of 10 cm. Influent concentrations: 1,2-DCB 1000 μg/l, 1,3-DCB 1000 μg/l, 1,4-DCB 600 μg/l, 1,2,4-TCB 350 μg/l, and 1,2,3-TCB 300 μg/l.

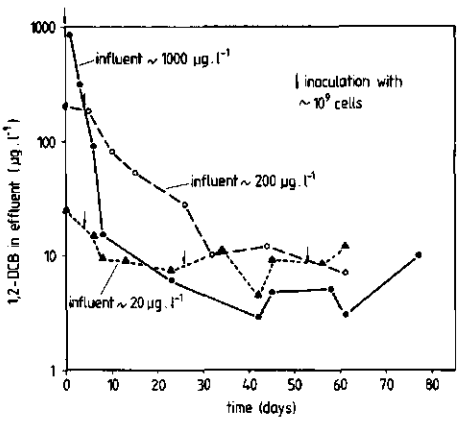

Fig. 3. A comparison of the degradation of 1,2-DCB in inoculated columns at different influent concentrations. Here, the effluent concentration is drawn as a measure for degradation. The figure shows the threshold concentration observed in all three inoculation experiments for 1,2-DCB of 6 ± 4 μg/l. Inoculations with strain P51 are indicated by arrows.

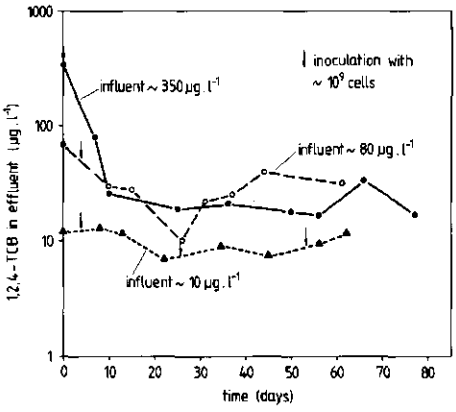

Fig. 4. Threshold concentrations observed for the degradation of 1,2,4-TCB during the inoculation experiments at different influent concentrations. Measurements of 1,2,4-TCB have been made concomitantly with the other isomers (Fig. 3). Inoculations are indicated by arrows.

In the second inoculation experiment with a new soil column, rapid degradation of 1,2-DCB at an influent concentration of 200 µg/l at and beyond the point of inoculation (10 cm) was again observed, immediately after introduction of the cells into the column. Although 1,2-DCB and 1,2,4-TCB were again degraded, 1,4-DCB and 1,3-DCB remained recalcitrant in the column. The overall removal of 1,2-DCB was greater than 90%. However, a threshold of 5–10 µg/l remained in the effluent (Fig. 3). 1,2,4-TCB behaved in a similar way. The effluent concentration in this case was also never lower than about 20 µg/l (Fig. 4). This column was followed for 60 days when the degradative activity disappeared. No cells of strain P51 were found in this experiment 40 days after inoculation at a point 5 cm below the point of inoculation. However, 10^5–10^6 cells per gram of soil (dry weight) were counted at the point of inoculation and 5 cm above. Less than 10^5 cells per gram of soil were counted at day 70 at the point of inoculation (when the degradation had already stopped), which is about the lower limit of the counting technique using fluorescent antibodies. This suggests a possible decline and loss of the active population.

In a third experiment the same column as in experiment 2 but a lower substrate concentration was used. Upon inoculation, 1,2-DCB was reduced from 20 µg/l to 10 µg/l, while no degradation was observed for 1,2,4-TCB. This was not surprising, since the influent concentration (10 µg/l) was below the previously found threshold concentration of around 20 µg/l. Repeated inoculations with cells of P51 had no effect on this threshold concentration (Fig. 3). Between the inoculations, the effluent was tested regularly for the presence of cells of P51 but no cells were detected. As in experiment 2, 1,4-DCB and 1,3-DCB were not degraded at these low concentrations. In all three experiments with inoculated bacteria, the 1,2,3-TCB and 1,3,5-TCB isomers remained recalcitrant.

Between experiments 2 and 3, we collected the effluent from the soil column system and, after autoclaving, used it as a medium for fresh cells of strain P51 in batch cultures. A 1 ml aliquot of a culture of strain P51 pregrown on 1,2,4-TCB (about $2.0 \cdot 10^8$ cells per ml) was added to 30 ml of the effluent in 35-ml serum bottles. The batch cultures were incubated at 20 °C and after 0, 1 and 3 days concentrations of the di- and trichlorobenzenes were determined. At an initial concentration of 40 µg/l for 1,2-, 1,3-, 1,4-DCB and 1,2,4-TCB, 30 µg/l for 1,2,3-TCB and 15 µg/l for 1,3,5-TCB, we found significant degradation of all DCB-isomers and 1,2,4-TCB. The concentration of 1,2-DCB was lowered to 0.1 µg/l after 3 days (Table 2).

5. DISCUSSION

In this study we examined the ability of strain P51 to degrade di- and trichlorobenzenes at concentrations found in the environment (5–1000 µg/l). For this purpose, we inoculated the strain into a nonsterile soil column system simulating a typical river bank infiltration site [17,22]. The results obtained from both inoculated and native soil columns show a strong enhancement of biodegradation of dichlorobenzenes and 1,2,4-TCB by the introduction of a laboratory-cultivated strain. Strain P51 was able to survive for at least 60 days

in a nonsterile environment and to retain its activity there. However, too low substrate levels or complete absence of the specific substrate resulted in the loss of the degradation ability, as was shown in experiment 1, when feeding with DCBs and TCBs was interrupted for 4 weeks. These results suggest that *Pseudomonas* sp. strain P51 did not survive during this nonselective period. In biodegradation experiments which were performed by researchers from the Amsterdam Water Works, the degradation of 1,2- and 1,4-DCB by the native microbial population in similar soil percolation columns was studied. Their results show that an interruption for 16 days of the substrate supply did not result, except for a short lag period of 5 days, in the loss of the capability to degrade 1,2- and 1,4-DCB (Smeenk, J.G.M., personal communication). The microorganisms in the column did retain their degradation capacity. Chatterjee et al. [10] found a decrease in cell numbers of *Pseudomonas cepacia* AC 1100 inoculated in soil when their specific growth substrate, 2,4,5-trichlorophenoxyacetic acid, was absent. Unfortunately, at the time of experiment 1, we were not yet able to use the fluorescent antibody technique to detect cells of strain P51 in the soil. Therefore, it cannot be excluded that strain P51 did survive for longer periods, but was not able to degrade chlorinated benzenes anymore. Such a disappearance of a specific activity could be explained by the loss of a plasmid, containing part of the genetic information for the degradation of these xenobiotic compounds [8], in an environment where the selection pressure (presence of di- and trichlorobenzenes) was removed [9]. As a matter of fact, we have recently found a large plasmid in strain P51 (Van der Meer, J.R., unpublished results).

Various other factors may be involved in the survival of an introduced strain and, consequently, the success of inoculation. Experiments by the group of Alexander [12,13,23] indicated that growth-inhibiting compounds, susceptibility to predators, or inaccessibility of the substrate may cause a possible failure of an inoculation. We did not obtain evidence for inhibiting effects of these factors. In addition, when strain P51 was introduced halfway in the column, we observed a phenomenon that might best the described by chemotaxis (experiment 1). After 42 days the main degradation activity towards DCBs and 1,2,4-TCB had moved to the first 5 cm of the column. Such a chemotactic response is facilitated by the water-saturated conditions in the column and the mobility of strain P51. Mathematical modelling of 1,4-DCB degradation (Bosma, T.N.P. et al., unpublished data) by native microorganisms in experiments similar to those described here [17], strongly indicates the importance of chemotaxis in water-saturated soil systems.

All inoculation experiments showed the presence of a threshold concentration below which no degradation of the xenobiotic compounds occurred (Table 2). This concentration level was independent of the influent concentration used in the soil columns, and it could not be lowered further by repeated inoculations with freshly grown cells (experiment 3). A washout of these cells, which might have affected this concentration, did not occur. We have never detected cells of *Pseudomonas* sp. P51 in the effluent. Interestingly, strain P51 could reduce the threshold concentration when incubated in batch experiments with the effluent of the soil column (Table 2). This observation would make it less plausible to assume that strain P51 – isolated in the presence of relatively high substrate concentrations (mg/l range) – has intrinsic physiological properties of eutrophic growth and its therefore not able to grow on very low substrate levels as is stated for other organisms by Poindexter [24] and Alexander [25]. It would suggest, rather, that a complex number of interactions between soil adsorption sites, metabolic characteristics of the bacteria, and the substrates present, is responsible for the observed threshold values. One of the factors affecting the threshold could be the presence of other metabolizable substrates in the soil columns. It is known that secondary substrates can have both beneficial and maleficial effects on primary substrate removal [23,26]. The sandy material used in our columns, contained about 0.5% (w/w) of organic carbon, which could partially be used by the microorganisms as additional substrate. The availability of this organic carbon for microorganisms is supported by the fact that the sand in the column already contained

an autochthonous population of about 10^8 bacteria per gram of soil (dry weight) (Van der Meer, J.R., unpublished data). In order to support such a population, some of the soil organic matter must serve as carbon and energy source. Therefore, it cannot be excluded that it could also serve as a secondary substrate for strain P51, inhibiting uptake and metabolism of xenobiotic compounds at very low substrate concentrations. In the column studies performed with the native soil population only, we observed degradation of 1,2-DCB to a concentration of 0.1 μg/l. This means that the native soil population is able – when adapted – to reduce low levels (25 μg/l 1,2-DCB) of xenobiotic compounds even in the presence of other metabolizable substrates. This agrees with observations in various field sites (Table 2) where very low concentrations of these xenobiotic compounds were measured as a result of biodegradation [17]. Experiments will be performed using inoculated soil columns and reactors where cells are immobilized in calcium alginate, to study the effect of easily degradable compounds, such as acetate, on the biodegradation of xenobiotic compounds like the di- and trichlorobenzenes.

From our experiments we conclude that prolonged survival of laboratory-cultivated strains with interesting catabolic properties in their original environment is possible. They do not lose their activity for at least as long as selective pressure in the form of specific substrates is present. For as yet unknown reasons, *Pseudomonas* sp. strain P51 is not able to degrade DCB-isomers and 1,2,4-TCB below a certain threshold concentration in soil columns. In addition, this threshold is different for various substrates. Such a relatively high threshold could be a major obstacle in the use of these kinds of organisms in cleaning sites with low concentrations of pollutants. Waiting for the native soil population to adapt to degrade polluting chemicals, however, might take a long time in some cases. Therefore, careful and rational use of laboratory organisms can be very helpful in the immediate breakdown of various xenobiotic compounds in the environment, or in process-controlled industrial reactors.

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Chapter 3

Cloning and characterization of plasmid-encoded genes for the degradation of 1,2-dichloro-, 1,4-dichloro-, and 1,2,4-trichlorobenzene of Pseudomonas sp. strain P51

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Cloning and Characterization of Plasmid-Encoded Genes for the Degradation of 1,2-Dichloro-, 1,4-Dichloro-, and 1,2,4-Trichlorobenzene of *Pseudomonas* sp. Strain P51

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***Pseudomonas* sp. strain P51 is able to use 1,2-dichlorobenzene, 1,4-dichlorobenzene, and 1,2,4-trichlorobenzene as sole carbon and energy sources. Two gene clusters involved in the degradation of these compounds were identified on a catabolic plasmid, pP51, with a size of 110 kb by using hybridization. They were further characterized by cloning in *Escherichia coli*, *Pseudomonas putida* KT2442, and *Alcaligenes eutrophus* JMP222. Expression studies in these organisms showed that the upper-pathway genes (*tcbA* and *tcbB*) code for the conversion of 1,2-dichlorobenzene and 1,2,4-trichlorobenzene to 3,4-dichlorocatechol and 3,4,6-trichlorocatechol, respectively, by means of a dioxygenase system and a dehydrogenase. The lower-pathway genes have the order *tcbC-tcbD-tcbE* and encode a catechol 1,2-dioxygenase II, a cycloisomerase II, and a hydrolase II, respectively. The combined action of these enzymes degrades 3,4-dichlorocatechol and 3,4,6-trichlorocatechol to a chloromaleylacetic acid. The release of one chlorine atom from 3,4-dichlorocatechol takes place during lactonization of 2,3-dichloromuconic acid.**

In recent years, several bacteria have been isolated that were able to degrade chlorinated aromatic compounds, such as chlorinated benzoic acids, chlorinated phenols (27, 39), and even chlorinated benzenes and chlorinated biphenyls (12, 24). Bacteria able to use chlorinated benzenes as sole carbon and energy substrates (15, 25, 30, 35) were found to oxidize the chlorinated benzene to a chlorocatechol by the action of a dioxygenase enzyme and a dehydrogenase. The chlorocatechol could then be degraded via a pathway similar to the one described for 3-chlorobenzoate metabolism in *Pseudomonas* sp. strain B13, i.e., ring cleavage by a catechol 1,2-dioxygenase II enzyme, lactonization by a cycloisomerase II, and hydrolysis by a hydrolase II, yielding chloromaleylacetic acid (7, 8, 27–29). These enzymes were shown to have higher affinity toward chlorinated substrates than did their counterparts in benzoate metabolism. Chloromaleylacetic acid would then be converted further by the enzyme maleylacetate reductase to yield β-ketoadipate. One chlorine atom is thought to be released from the aromatic ring during lactonization (28).

The genetic information encoding metabolic pathways for xenobiotic compounds in bacteria often resides on catabolic plasmids (3, 19, 41). In the case of chlorinated aromatic compounds, gene clusters have been described that code for the conversion of chlorinated catechols, such as *tfdCDEF*, present on plasmid pJP4 (6, 13, 14, 26), and *clcABD*, present on plasmid pAC27 (4, 10). Furthermore, genes have been identified and characterized that encode the conversion of chlorinated benzoic acids (38, 39) and chlorinated biphenyls (12, 24).

In this report, we describe the cloning and genetic characterization of two gene clusters encoding the degradation of 1,2,4-trichlorobenzene (1,2,4-TCB), 1,2-dichlorobenzene (1,2-DCB), and 1,4-dichlorobenzene (1,4-DCB) of *Pseudomonas* sp. strain P51, which was isolated previously (37). To our knowledge, strain P51 is the first organism described to degrade 1,2,4-TCB as well as 1,2- and 1,4-DCB (37). The genetic and biochemical characterization of this catabolic pathway offers a unique possibility to study the evolution of new metabolic routes and the adaptation of microorganisms to xenobiotic substrates.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used are listed in Table 1. *Pseudomonas* sp. strain P51 was grown in Z3 minimal medium at 30°C as previously described (37). 1,2,4-TCB was added as liquid at 400 μl (3.2 mmol) per liter of culture medium. The solubility of 1,2,4-TCB in water was estimated by gas chromatography (GC) analysis to be 83 μM. When cells were grown on 1,2-DCB or 1,4-DCB, 3.5 mmol of these compounds was dissolved in 20 ml of heptamethylnonane (Sigma Chemical Co., St. Louis, Mo.), which was then added as organic phase to 1 liter of culture volume. Cultures used for enzyme activity measurements were grown to late exponential phase on 1,2,4-TCB or, when uninduced, on 10 mM succinate. *Alcaligenes eutrophus* JMP222 and *Pseudomonas putida* KT2442 were grown routinely at 30°C on LB (22). When tested for enzyme activity, *A. eutrophus* JMP222 harboring plasmid pTCB1 was grown on 10 mM succinate and induced with 1 mM 3-chlorobenzoate as described by Don et al. (6). *P. putida* KT2442 strains harboring plasmid pTCB1, pTCB39, or pTCB13 were cultivated to late exponential phase in 50 ml of M9 medium containing either 10 mM succinate or 10 mM 3-chlorobenzoate as the carbon source supplemented with the appropriate antibiotics. *Escherichia coli* strains were cultivated on LB at 37°C. When tested for enzyme activity, strains were grown in 50 ml of LB plus ampicillin to an A_{620} of 1.0. For detection of chlorobenzene dioxygenase activity in *E. coli*, media were supple-

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Source or reference
Bacterial strains		
Pseudomonas sp. strain P51	Tcb$^+$ Dcb$^+$	
Escherichia coli		
DH5α	F$^-$ *lacZΔM15 recA1 hsdR17 supE44* Δ (*lacZYA-argF*)	Bethesda Research Laboratories
HB101	*recA13 leuB6 proA2 thi-1 hsdR hsdM* Sm^r	S. Harayama
Pseudomonas putida KT2442	*P. putida* mt-2, *hsdR1 hsdM*$^+$ Rif^r	11
Alcaligenes eutrophus JMP222	2,4-D$^-$ 3CB$^-$ Sm^r	6
Plasmids		
pP51	110-kb native plasmid of *Pseudomonas* sp. strain P51	This work
pUC18	Ap^r	40
pUC19	Ap^r	40
pKT230	Km^r Sm^r	1
pRK2013	ColE1::pRK2 Tra$^+$ Km^r	5
pCBA3	pKT230 carrying the 1.6-kb *Xho*I fragment of pJP4 containing *tfdC*; Sm^r	J. R. van der Meer
p261.39	pBR322 carrying a putative benzene dioxygenase gene from *Moraxella* sp. strain RST 67-1; Ap^r	H. G. Rast
pTCB1	pKT230 carrying the 13-kb *Sst*I fragment of pP51; Km^r	This work
pTCB13	pKT230 carrying the 2.3-kb *Xho*I fragment of pP51; Sm^r	This work
pTCB39	*Cla*I deletion derivative of pTCB1; Km^r	This work
pTCB42	pUC18 carrying the 4.5-kb *Sst*I-*Cla*I fragment of pTCB45; Ap^r	This work
pTCB43	pUC19 carrying the 3.1-kb *Pst*I fragment of pTCB42; Ap^r	This work
pTCB44	Orientation opposite that of pTCB43;Ap^r	This work
pTCB45	pUC19 carrying the 13-kb *Sst*I fragment of pTCB1; Ap^r	This work
pTCB48	pUC18 carrying the 1.7-kb *Pst*I-*Hin*dIII fragment of pTCB43; Ap^r	This work
pTCB51	pUC18 carrying the 1.3-kb *Pst*I-*Xho*I fragment of pTCB48; Ap^r	This work
pTCB51Δ	Derivative of pTCB51 in which the *Bam*HI site was removed; Ap^r	This work
pTCB60	pUC18 carrying the 12-kb *Hin*dIII fragment of pP51; Ap^r	This work
pTCB62	pUC18 carrying the 5.2-kb *Eco*RI-*Kpn*I fragment of pTCB45; Ap^r	This work
pTCB63	pUC18 carrying the 2.5-kb *Eco*RI-*Sal*I fragment of pTCB43; Ap^r	This work
pTCB64	pUC18 carrying the 3.8-kb *Eco*RI-*Pst*I fragment of pTCB45; Ap^r	This work
pTCB64Δ4	Derivative of pTCB64 in which the 4th *Sal*I site was removed; Ap^r	This work
pTCB69	pUC18 carrying a 6.3-kb *Sau*3AI partial fragment of pTCB60; Ap^r	This work
pTCB70	pUC18 carrying a 6.8-kb *Sau*3AI partial fragment of pTCB60; Ap^r	This work
pTCB71	pUC19 carrying the 4.9-kb *Xho*I-*Eco*RI fragment of pTCB60; Ap^r	This work
pTCB72	pUC19 carrying the 5.8-kb *Xho*I-*Bam*HI fragment of pTCB60; Ap^r	This work
pTCB73	pUC18 carrying the 5.8-kb *Xho*I-*Bam*HI fragment of pTCB60; Ap^r	This work

[a] Abbreviations: Tcb$^+$, growth on 1,2,4-TCB; Dcb$^+$, growth on 1,2-DCB; Sm, streptomycin; Rif, rifampin; r, resistance; 2,4-D$^-$, no growth on 2,4-dichlorophenoxyacetic acid; 3CB$^-$, no growth on 3-chlorobenzoate; Ap, ampicillin; Km, kanamycin.

mented with 1 mM indole as described by Stephens et al. (36). Antibiotics were incorporated at the following concentrations: ampicillin, 50 μg/ml; kanamycin, 50 μg/ml; rifampin, 50 μg/ml; and streptomycin, 25 μg/ml (for *E. coli*) or 250 μg/ml (for *A. eutrophus* or *P. putida*). 5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) was added to a final concentration of 0.004%.

DNA manipulations and hybridizations. Plasmid DNA was isolated from *Pseudomonas* sp. strain P51 cultivated on 1,2,4-TCB, using the procedure of Hansen and Olsen (18). Crude plasmid fractions were further purified by CsCl-ethidium bromide density gradient centrifugation and dialysis according to established procedures (22).

Miniscale and large-scale preparations of recombinant plasmids in *E. coli*, *A. eutrophus*, and *P. putida* strains were done by alkaline lysis as described by Maniatis et al. (22). *E. coli* strains were transformed according to Hanahan (17).

Restriction enzymes, Klenow large-fragment polymerase, and T4 DNA ligase were obtained from GIBCO/BRL Life Technologies Inc. (Gaithersburg, Md.) or Pharmacia LKB Biotechnology (Uppsala, Sweden) and used according to the specifications of the suppliers. DNA fragments were recovered from agarose gels by using Geneclean (Bio 101, Inc., La Jolla, Calif.).

For DNA hybridizations, DNA from agarose gels was transferred to a GeneScreen Plus membrane (Dupont, NEN Research Products, Boston, Mass.) by the method of Southern (34). DNA was fixed to the membrane by illumination with UV light for 3 min. Hybridization and washing conditions were applied as specified by the supplier. DNA fragments used in the hybridization were labeled with [α-^{32}P]dATP (3,000 Ci/mmol; Amersham International plc, Amersham, United Kingdom), using a nick translation kit (GIBCO/BRL). The DNA probes were a 1.6-kb *Xho*I fragment of plasmid pCBA3, containing the chlorocatechol 1,2-dioxygenase gene *tfdC* present on pJP4 (6, 13, 14, 26), and a 2-kb *Pst*I fragment of plasmid p261.39, containing part of a benzene dioxygenase gene cluster obtained from *Moraxella* sp. strain RST 67-1 (26a). This fragment was a gift from H. G. Rast, Bayer AG, Leverkusen, Federal Republic of Germany.

Conjugation of recombinant plasmids into *A. eutrophus* or *P. putida*. DNA fragments that were cloned into the broad-host-range vector pKT230 were mobilized from *E. coli* to

either *A. eutrophus* JMP222 or *P. putida* KT2442, using a triparental mating with *E. coli* HB101(pRK2013) as the helper strain (5).

Resting-cell and enzyme assays. Conversion of 1,2-DCB and 1,2,4-TCB by resting cells of *E. coli* was determined as follows. *E. coli* DH5α(pTCB69) was grown in 500 ml of LB plus ampicillin to an A_{620} of 1.5 and harvested by centrifugation for 10 min at 6,000 rpm and 4°C in a Sorvall GSA rotor. Cells were washed twice with 25 mM sodium-potassium phosphate buffer (pH 7.5) and resuspended to make a final volume of 50 ml. Glass vials of 37 ml were filled with 1 ml of cell suspension to which either 8 ml of 25 mM sodium-potassium phosphate buffer (pH 7.5) and 1 ml of a saturated solution of 1,2-DCB in the same buffer or 9 ml of a saturated solution of 1,2,4-TCB in 25 mM phosphate buffer was added. The concentration of 1,2-DCB in this saturated solution was estimated to be 75 mg/liter (0.5 mM), and that of 1,2,4-TCB was estimated to be 30 mg/liter (166 μM). Vials were closed with Viton stoppers (Iso-versinic; Rubber B.V., Hilversum, The Netherlands) to prevent loss of the chlorinated benzenes during the course of the experiment. Cells were incubated at 37°C in a shaking water bath. For each time point, three vials were analyzed for chlorobenzene concentration, and in three other vials the concentration of chlorocatechol was determined. Samples for chlorocatechol production were centrifuged and the supernatants were stored at −20°C until analyzed by high-performance liquid chromatography (HPLC).

Cultures used for enzyme assays were harvested by centrifugation, washed twice in 50 mM Tris hydrochloride (pH 8.0), or in 25 mM sodium-potassium phosphate buffer (pH 7.5) when tested for chloride release, resuspended in a final volume of 3 ml, and subsequently disrupted in a miniature French pressure cell (Aminco, Inc., Silver Spring, Md.) in the case of *E. coli* and *Pseudomonas* sp. strain P51. *P. putida* and *A. eutrophus* strains were broken by sonication six times for 15 s each time at 50 W and 0°C (Vibracell; Sonic Materials Inc., Danbury, Conn.). The crude extract was cleared by centrifugation at 19,000 rpm for 10 min at 4°C in a Sorvall SS34 rotor. The supernatant after centrifugation is referred to as the cell extract. Protein content of the cell extract was determined according to Bradford (2).

Enzymatic activities of catechol 1,2-dioxygenase II and hydrolase II were determined as described by Dorn and Knackmuss (7, 8) and Schmidt et al. (28, 29).

Cycloisomerase II activity was measured by a modified procedure, monitoring the disappearance of 2-chloromuconic acid upon addition of cell extract by HPLC analysis. The enzyme assay consisted of 0.66 ml of 40 mM Tris hydrochloride (pH 8.0), 0.30 ml of 0.3 mM 2-chloromuconic acid (dissolved in 25 mM Tris hydrochloride [pH 8.0]), 10 μl of 100 mM $MnCl_2$, and 30 μl of cell extract (approximately 100 μg of protein) (31).

Enzymes of the chlorocatechol pathway were further analyzed in cell extracts of *E. coli* strains by monitoring the conversion of 3,4-dichlorocatechol (3,4-DCC) and appearance of transformation products. These assays were performed by incubating 0.96 ml of 40 mM Tris hydrochloride (pH 8.0), 30 μl of cell extract, and 10 μl of 10 mM 3,4-DCC at 30°C. Reactions were monitored by spectral analysis on a double-beam spectrophotometer (model 25; Beckman Instruments Inc., Fullerton, Calif.). Chloride release was measured in a similar reaction mixture except that chloride-free 25 mM sodium-potassium phosphate buffer (pH 7.5) was used instead of Tris hydrochloride. Reactions were monitored spectrophotometrically. When no more changes occurred, 0.8 ml of the reaction mixture was analyzed for Cl^-.

Chemical analysis. Chlorinated catechols and their transformation products were analyzed on an LKB model 2150 HPLC (LKB, Bromma, Sweden) equipped with a UV detector (LKB 2158 Uvicord SD) and a rapid spectral detector (LKB model 2140). Transformation products of chlorocatechols were analyzed by using a Hibar 10-μm LiChroSorb RP-8 column (E. Merck, Darmstadt, Federal Republic of Germany) with 30% acetonitrile and 70% 20 mM phosphoric acid as the mobile phase at a flow rate of 0.6 ml/min.

Formation of 3,4-DCC from 1,2-DCB and of 3,4,6-trichlorocatechol (3,4,6-TCC) from 1,2,4-TCB was analyzed by using a Chromospher C-8 column (Chrompack, Middelburg, The Netherlands) operated at a flow rate of 0.5 ml/min with a mobile phase of 50% acetonitrile and 50% 20 mM phosphoric acid.

Chlorobenzene concentrations were measured by GC as described by Van der Meer et al. (37). Chloride concentrations in media and enzyme reactions were analyzed as described by Schraa et al. (30).

Chemicals. 3-Chlorocatechol, 3,4-DCC, 2-chloro-*cis*, *cis*-muconate, and *cis*-4-carboxymethylenebut-2-en-4-olide were kind gifts from W. Reineke, Wuppertal, Federal Republic of Germany. 3,4,6-TCC was synthesized from 2,4,5-trichlorophenol and was a gift from Gineke van Bergen of the Department of Organic Chemistry, Wageningen Agricultural University, Wageningen, The Netherlands.

RESULTS

Degradation of 1,2,4-TCB by strain P51. The complete conversion by *Pseudomonas* sp. strain P51 of 1,2,4-TCB, 1,2-DCB, and 1,4-DCB simultaneously is shown in Fig. 1. Growth resulted in the concomitant release of 3 mol of chloride per mol of 1,2,4-TCB and 2 mol of chloride per mol of 1,2- and 1,4-DCB into the medium. Strain P51 showed a slight preference for 1,2-DCB rather than 1,4-DCB and 1,2,4-TCB. 1,3-DCB was also shown to be converted by strain P51 in soil columns (37) but could not be used as the sole carbon and energy source in liquid culture. The only additional aromatic substrates that were found to be used as carbon and energy sources were toluene, monochlorobenzene, and 4-chlorophenol.

Cultures grown on 1,2,4-TCB appeared to contain elevated levels of a catechol 1,2-dioxygenase activity specific for chlorinated substrates such as 3-chlorocatechol (Table 2).

Degradation of 1,2,4-TCB is plasmid encoded in strain P51. After several transfers in nonselective media, it was observed that the ability of strain P51 to grow on 1,2,4-TCB as the sole carbon and energy source was lost irreversibly (results not shown). From cultures of strain P51 that were cultivated on 1,2,4-TCB, a catabolic plasmid of 110 kb could be isolated by CsCl-ethidium bromide gradient centrifugation. This plasmid was found to be absent in strains that could no longer grow on 1,2,4-TCB. In addition, total DNA of those cured strains did not hybridize with pP51 DNA probes (results not shown). A physical map of plasmid pP51 was constructed by using the enzymes *Kpn*I, *Hpa*I, *Hin*dIII, *Xho*I, and *Bam*HI (Fig. 2).

Transfer of plasmid pP51 could not be shown in direct plate mating experiments with recipient strains such as *P. putida* KT2442, *A. eutrophus* JMP222, or plasmid-free strains of P51 (results not shown), possibly because of poor growth on agar plates containing 1,2,4-TCB as the sole

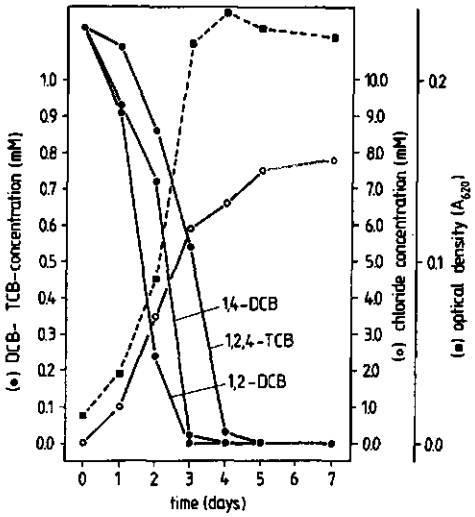

FIG. 1. Growth of *Pseudomonas* sp. strain P51 on 1,2,4-TCB, 1,2-DCB, and 1,4-DCB simultaneously present in the culture medium. Symbols: ●, concentration of 1,2-DCB, 1,4-DCB, or 1,2,4-TCB as determined by GC analysis; ○, chloride concentration; ■, A_{620}.

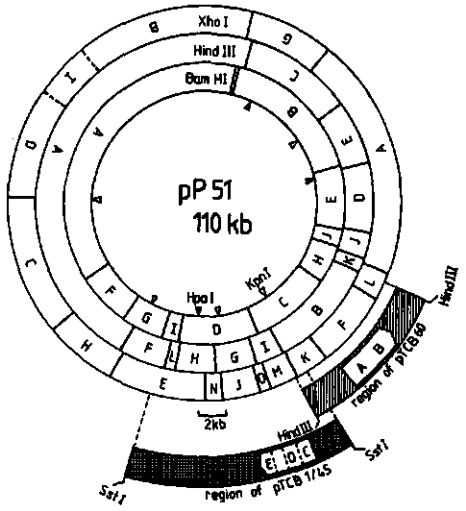

FIG. 2. Physical map of the catabolic plasmid pP51 from *Pseudomonas* sp. strain P51. Capital letters indicate the order of the generated restriction enzyme fragments according to size. The size of the total plasmid was estimated to be 110 kb. Shown are the two gene clusters that were cloned and characterized in this study: (i) the upper-pathway genes *tcbA* and *tcbB*, which are located on the *Hind* B fragment of plasmid pP51, and (ii) the genes *tcbC*, *tcbD*, and *tcbE* of the lower or catechol pathway, which are located on the 13-kb *Sst*I fragment of pP51. Arrows in the gene clusters show the deduced orientations of the genes. Dotted lines (e.g., at *Xho*I-I) indicate possible ambiguity in locating the fragment.

carbon source (supplied as vapor) and consequently a weak counterselection of the recipients.

Cloning of the lower-pathway genes encoding chlorocatechol metabolism. To locate the genes involved in degradation of 1,2,4-TCB, we hybridized pP51 DNA to a 1.6-kb *Xho*I fragment of plasmid pJP4, containing the catechol 1,2-dioxygenase II gene *tfdC* from *A. eutrophus* JMP134 (6, 13, 26). The results (Fig. 3A) showed that a 13-kb *Sst*I fragment of pP51 hybridized under stringent washing conditions. This fragment was subsequently cloned in *E. coli* by using the broad-host-range vector pKT230 (1). The resulting plasmid, pTCB1, was mobilized into the plasmid-free recipient strains *A. eutrophus* JMP222 (6) and *P. putida* KT2442. The KT2442 transconjugant harboring pTCB1 was found to be able to grow on 3-chlorobenzoate as the sole carbon and energy source, whereas the parent strain KT2442 was unable to do so. High catechol 1,2-dioxygenase II activities were detected in *P. putida* KT2442(pTCB1) when this strain was cultivated on 3-chlorobenzoate (Table 2). This strain was found not to degrade 1,2,4-TCB or 1,2-DCB. Both orientations of the 13-kb *Sst*I fragment in pKT230 appeared to be functional in *A. eutrophus* as well as *P. putida* (results not shown), which suggests that expression signals from pP51 can be recognized in both recipient strains.

When a *Cla*I deletion derivative of pTCB1 was constructed, the ability to degrade 3-chlorobenzoate appeared to be lost (pTCB39; Fig. 4A). A smaller construct, pTCB13, did not confer growth on 3-chlorobenzoate in strain KT2442. These results indicate that pP51 encodes a catechol 1,2-dioxygenase II activity and a functional pathway for degradation of chlorinated catechols.

TABLE 2. Catechol 1,2-dioxygenase activities in *Pseudomonas* sp. strain P51 and in *A. eutrophus* and *P. putida* strains

Strain	Plasmid	Growth substrate	Catechol 1,2-dioxygenase activity[a]	
			Catechol	3-Chlorocatechol
Pseudomonas sp. strain P51	pP51	1,2,4-TCB	79.5	139.1
	pP51	Succinate	40.0	4.4
A. eutrophus JMP222		Succinate + 3-CBa[b]	265.0	0.0
	pTCB1	Succinate + 3-CBa	430.2	17.6
P. putida KT2442	pKT230	Succinate + 3-CBa	0.0	0.0
	pTCB1	Succinate	3.3	10.0
	pTCB1	3-CBa	185.2	170.1

[a] Activity in cell extracts, expressed as nanomoles of muconic acid per milligram of protein per minute. 3-Chlorocatechol was used as a specific substrate for catechol 1,2-dioxygenase II.
[b] 3-CBa, 3-Chlorobenzoate.

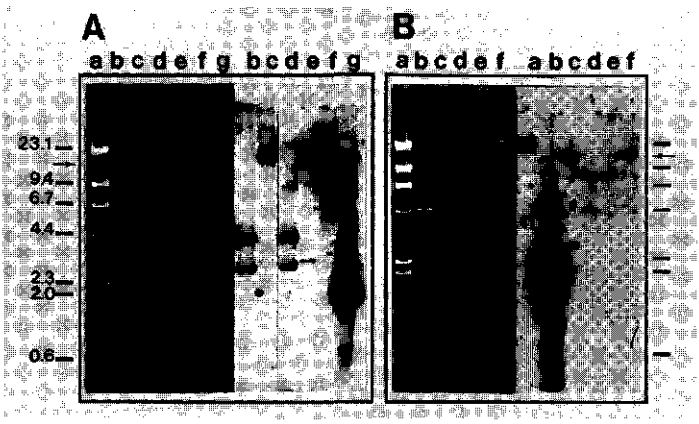

FIG. 3. (A) Hybridization of pP51 DNA with the 1.6-kb *Xho*I fragment of pJP4, containing the catechol 1,2-dioxygenase II gene *tfdC*. Lanes: a, λ DNA, *Hin*dIII; b, pP51, *Xho*I; c, pP51, *Sst*I; d, pTCB45, *Xho*I; e, pTCB45, *Sst*I; f, pJP4, *Sst*I; g, pCBA3, *Xho*I. The arrow indicates the 13-kb *Sst*I fragment of pP51. Sizes on the left are indicated in kilobases. (B) Hybridization of pP51 DNA with the 2-kb *Pst*I fragment of plasmid p261.39, containing part of the benzene dioxygenase gene of Moraxella sp. strain RST 67-1 (26a). Lanes: a, λ DNA, *Hin*dIII; b, plasmid p261.39, *Pst*I; c, pTCB60, *Hin*dIII; d, pTCB60, *Xho*I; e, pP51, *Xho*I; f, pP51, *Hin*dIII. A weak cross-hybridization was also detected between the pUC vector bands and the pBR322-based plasmid p261.39. The arrow shows the 12-kb *Hin*d B fragment of plasmid pP51.

Identification and location of the lower-pathway genes *tcbC*, *tcbD*, and *tcbE*. To characterize this pathway further, we cloned various pP51 fragments of pTCB1 in *E. coli*, using pUC18 and pUC19. The constructs used for analyzing the lower-pathway genes are shown in Fig. 4B. When the 13-kb *Sst*I fragment from pTCB1 was cloned into pUC18 in both directions, only one orientation (pTCB45) was found to give very low catechol 1,2-dioxygenase II activity (Table 3). A similar level of low activity was detected in subclone pTCB42. Further hybridization studies with the *tfdC* gene from pJP4 showed hybridization with two small *Sal*I fragments (Fig. 4B). Deletion derivatives of pTCB45 that started at either the *Eco*RI or *Pst*I site upstream of the *Sal*I fragments were found to give higher catechol 1,2-dioxygenase activities in cell extracts than did pTCB45 or pTCB42. Clones constructed in the other orientation, such as pTCB44, exhibited no enzyme activity. This finding indicated that in *E. coli*, the catechol 1,2-dioxygenase gene was under the control of a vector-located promoter and suggested the orientation of the gene as indicated in Fig. 4B. The catechol 1,2-dioxygenase II activity was completely absent in *E. coli* harboring pTCB51Δ. In this plasmid, the unique *Bam*HI site of pTCB51 was removed by digestion with *Bam*HI, filling in of the 3′ recessed ends with Klenow polymerase, and religation of the plasmid, thereby causing a frameshift mutation. This result showed that we had localized the catechol 1,2-dioxygenase II gene from pP51. This gene was designated *tcbC*.

The chlorocatechol pathway was studied in more detail by measuring activities of the other known pathway enzymes (6, 21, 27–29) in *E. coli* (Fig. 4 and Table 3). Cycloisomerase II activity was found in cell extracts of *E. coli* containing pTCB63, pTCB43, pTCB64, or pTCB62 at low levels ranging from 0.3 to 4.3 nmol/mg of protein per min. Since no activity of this enzyme could be detected in *E. coli* strains harboring pTCB51 and pTCB48, it is very likely that in those plasmids the cycloisomerase gene of pP51 was interrupted. These experiments located the gene encoding a cycloisomerase II, which was named *tcbD* (Fig. 4B).

Hydrolase II activity measured in *E. coli* cell extracts was higher than cycloisomerase II or catechol 1,2-dioxygenase II activity, in the range of 25 to 30 nmol/mg of protein per min. Activity was detected only in constructs pTCB64 and pTCB62. This gene was designated *tcbE* (Fig. 4B).

To locate *tcbE*, we constructed a frameshift mutation in the *Sal*I site present between the deduced locations of *tcbD* and *tcbE*. Therefore, pTCB64 was partially digested with *Sal*I, filled with Klenow polymerase, and religated (Fig. 4B). *E. coli*(pTCB64Δ4) retained all of the enzyme activities that were also found to be present in *E. coli* harboring pTCB64. Furthermore, we did not observe other conversion steps after incubation with 3,4-DCC (see below). This finding indicated, however, that the hydrolase gene *tcbE* is not located adjacent to the cycloisomerase gene *tcbD* but downstream from the *Sal*I site. The overall deduced gene order was found to be *tcbC-tcbD-tcbE* (Fig. 4B).

To test whether the cloned fragments also coded for a maleylacetate reductase, we measured the activity of this enzyme as described by Oltmanns et al. (25), using the product of *cis*-4-carboxymethylenebut-2-en-4-olide after hydrolysis at pH 12 as a substrate (26b). However, none of the *E. coli* strains tested showed maleylacetate reductase activity (results not shown).

Conversion reactions of 3,4-DCC. Four types of products were formed during incubation of 3,4-DCC with cell extracts of the various *E. coli* strains (Fig. 5). Clones pTCB51 and pTCB48 contained only catechol 1,2-dioxygenase II activity (Table 3) and accumulated one product. This product I was stable at pH 8.0 during the course of the incubation experiments (2 h), as observed in the spectrophotometric assay. At this pH, the spectrum of product I showed the features of a muconic acid (15, 28, 31), which in this case would be 2,3-dichloro-*cis*,*cis*-muconic acid, with an absorption maximum at 268 nm (Fig. 5A). When injected into an HPLC, the

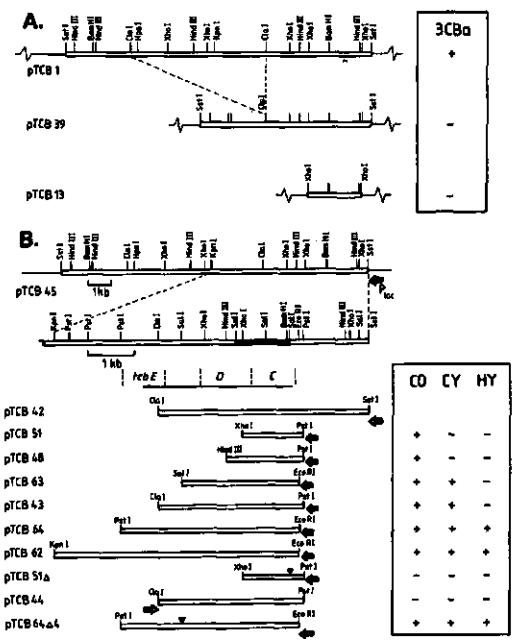

FIG. 4. (A) Cloning of DNA fragments of pP51 in *P. putida* and *A. eutrophus*, using the broad-host-range vector pKT230. □, pP51 fragments that when ligated into pKT230 form the plasmids indicated on the left. Restriction sites of relevant enzymes are shown on the pP51 fragments. pTCB1 contains the 13-kb *Sst*I fragment of pP51 cloned into the unique *Sst*I site present in the Sm^r gene of pKT230. pTCB39 was derived from pTCB1 by deleting a 5.8-kb *Cla*I fragment (– – –). pTCB13 contains the *Xho*I M fragment of pP51. At the right are shown the phenotypes of *P. putida* KT2442 harboring these plasmids. +, Growth on 3-chlorobenzoate (3CBa); –, no growth. (B) Cloning of pP51 DNA fragments in *E. coli*, using pUC18 and pUC19. Relevant restriction sites of the pP51 DNA fragments used for cloning are shown for the 13-kb *Sst*I fragment and in more detail in the enlarged fragment (– – –). Each plasmid listed on the left was subcloned from pTCB45 and contains the fragment indicated by the horizontal bar. The location and direction of the *lac* promoter present on pUC18 or pUC19 are indicated by an arrow. The two small *Sal*I fragments of pP51 hybridizing with the pJP4 fragment containing *tfdC* (results not shown) are shown (■) in the detailed physical map of pTCB45. pTCB51Δ and pTCB64Δ4 were derived from pTCB51 and pTCB64, respectively. The order and locations of the genes for chlorocatechol degradation as derived from the cloning and expression studies are shown: *tcbC*, catechol 1,2-dioxygenase II; *tcbD*, cycloisomerase II; *tcbE*, hydrolase II. Expression of the genes in *E. coli* is shown on the right. CO, Catechol 1,2-dioxygenase II activity; CY, cycloisomerase II activity; HY, hydrolase II activity. +, Activity; –, no activity.

compound was unstable and two peaks with different retention times (7.2 and 8.3 min) were found.

E. coli harboring pTCB63 and pTCB43 converted 3,4-DCC first to product I and subsequently to product II (8.7 min) as a result of the activity of the cycloisomerase II (Fig. 5B). This compound was not stable at pH 8.0, but its adsorption maximum at 295 nm slowly changed to 252 nm (retention time of product III on HPLC, 3.8 min). The product after cycloisomerization would be 5-chloro-4-carboxymethylenebut-2-en-4-olide (15). Product IV was observed with use of clones pTCB64, pTCB64Δ4, and pTCB62 (Fig. 5C). Upon incubation of 3,4-DCC, product I was formed first, followed by product II and then product IV, which was found to be stable in the reaction mixture. The absorption maximum at pH 8.0 was 242 nm, but with HPLC (retention time, 7.1 min) we detected a different spectrum with the highest absorption at 190 nm, suggesting that this compound was a maleylacetate (in this case 5-chloromaleylacetate), which decarboxylated under acidic conditions to yield chloroacetyl acrylic acid (15). During the conversion of product I to product IV, we could detect only product II, which suggests that product III is a nonenzymatically formed compound.

TABLE 3. Enzyme activities of chlorocatechol pathway enzymes in *E. coli* strains

Plasmid	Enzyme activity[a]			Chloride release (μmol)[b]
	Catechol 1,2-dioxygenase II	Cycloisomerase II	Hydrolase II	
pTCB51	6.2	0.0	0.0	ND[c]
pTCB51Δ	0.0	ND	ND	ND
pTCB48	5.5	0.0	0.0	0.00
pTCB63	5.8	2.8	0.0	0.09
pTCB43	7.7	4.3	0.0	0.10
pTCB64	9.5	1.2	25.5	0.06
pTCB62	11.0	0.3	27.8	0.08
pTCB45	0.4	ND	ND	ND
pTCB42	0.2	ND	ND	ND
pTCB44	0.0	ND	ND	ND
pTCB64Δ4	6.3	11.7	4.5	0.11

[a] Expressed as follows: for catechol 1,2-dioxygenase II, nanomoles of muconic acid per milligram of protein per minute, using 3-chlorocatechol as the substrate; for cycloisomerase II, nanomoles of 2-chloromuconate converted per milligram of protein per minute; for hydrolase II, nanomoles of *cis*-4-carboxymethylene-but-2-en-4-olide converted per milligram of protein per minute.

[b] Expressed as the total amount of chloride formed per milliliter of assay medium after the reaction was complete.

[c] ND, Not determined.

Chloride release was analyzed in cell extracts incubated with 3,4-DCC (Table 3). Cell extracts of *E. coli* harboring pTCB51 or pTCB48 did not show chloride release with 3,4-DCC, whereas those of *E. coli* harboring pTCB63, pTCB43, pTCB64, pTCB64Δ4, or pTCB62 showed the release of a maximum of 0.1 μmol of chloride after incubation with 0.1 μmol of 3,4-DCC.

Cloning and location of the upper-pathway genes *tcbA* and *tcbB*. To detect the genes encoding the conversion of 1,2,4-TCB to the corresponding chlorinated catechol, we probed for the presence of a benzene dioxygenase gene. The benzene dioxygenase gene was obtained from *Moraxella* sp. strain RST 67-1 and had been cloned previously (26a). We could detect a weak hybridization signal in pP51 plasmid DNA with the *Moraxella* probe under stringent washing conditions (Fig. 3B). Since the hybridization was limited to the *Hind* B fragment of pP51 (Fig. 2), this fragment was cloned into pUC18, resulting in pTCB60 (Fig. 6). From pTCB60 we constructed a plasmid bank, using partial digestion with *Sau*3AI. Fragments ranging from 5 to 6 kb were recovered from an agarose gel after digestion, ligated with pUC18 digested with *Bam*HI, and transformed into *E. coli*. To test the expression of the benzene dioxygenase gene, transformants were selected on LB containing 1 mM indole (9, 36). Among 2,000 transformants, we detected two colo-

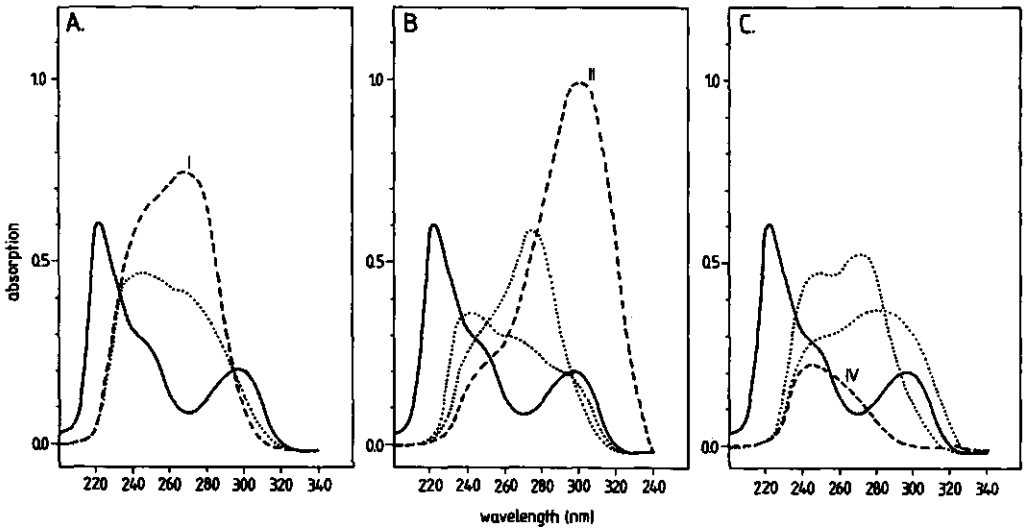

FIG. 5. Conversion reactions of 3,4-DCC. Shown are UV spectra of the products accumulating in the spectrophotometric assay when *E. coli* cell extracts harboring plasmid pTCB48, pTCB63, pTCB43, pTCB64, pTCB64Δ4, or pTCB62 were incubated with 3,4-DCC. Symbols: —, UV spectrum of 3,4-DCC: – – –, product I accumulating upon incubation with *E. coli* cell extract harboring pTCB48 (A), product II after incubation with *E. coli* cell extract harboring plasmid pTCB63 or pTCB43 (B), and product IV after incubation with *E. coli* cell extract harboring plasmid pTCB64, pTCB64Δ4, or pTCB62 (C); · · ·, intermediate spectra during transformation of 3,4-DCC to the final product, as recorded every 10 min. The UV spectrum of product III, arising after the spontaneous conversion of product II, is not shown.

nies that turned blue. Plasmid DNA was isolated from these clones (pTCB69 and pTCB70), and the inserts were aligned with that of pTCB60 by restriction mapping (Fig. 6). *E. coli* strains harboring deletion derivatives constructed from pTCB60, such as pTCB71 and pTCB72, that contained inserts similar to those of pTCB69 and pTCB70, were also found to express the benzene dioxygenase but to a smaller extent, as suggested from coloration on indole media. *E. coli* carrying plasmid pTCB71 showed only a very slight coloration of the medium. When the same fragment as in plasmid pTCB72 was cloned in the opposite direction (pTCB73), no expression of the benzene dioxygenase gene was found.

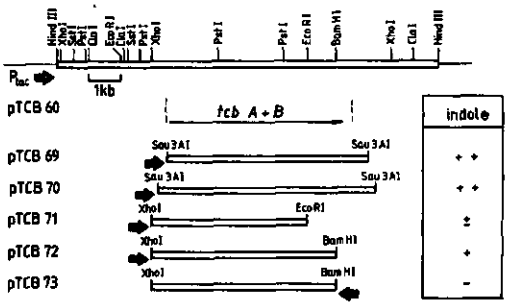

FIG. 6. Cloning of pP51 DNA fragments containing the upper-pathway genes *tcbA* and *tcbB* in *E. coli*, using pUC18 and pUC19. pP51 fragments (▭) form the plasmids indicated on the left when ligated with pUC18 or pUC19. The relevant restriction sites of pP51 used for cloning are indicated. The location and direction of the vector-located *lac* promoter are shown by an arrow. pTCB60 contains the 12-kb *Hind* B fragment of pP51. pTCB69 and pTCB70 were obtained by partial digestion of pTCB60 with *Sau*3AI and selection on indole media as indicated in the text. The extent of indigo formation by *E. coli* strains harboring these plasmids as judged from coloration on indole-containing agar plates is shown on the right. ++, Strong blue color, +, brownish color; −, no coloration visible.

The construct with the highest expression (pTCB69) was used in a resting-cell assay to examine the conversion of 1,2-DCB and 1,2,4-TCB by *E. coli* to the corresponding catechols 3,4-DCC and 3,4,6-TCC, respectively. Upon addition of 1,2-DCB or 1,2,4-TCB, the corresponding chlorinated catechols were produced and excreted into the medium (Fig. 7). Although the production of chlorinated catechols was not very high within the time course of the experiment (2 h), the accumulation of catechols was found to be significant and equimolar to the decrease in DCB and TCB concentrations. The results indicated that we had cloned and successfully expressed the upper-pathway genes from plasmid pP51 in *E. coli*. These genes were tentatively named *tcbA* and *tcbB*, consistent with the nomenclature for dioxygenase and dehydrogenase genes in similar metabolic routes, such as *todAB* (42) and *bphAB* (24).

DISCUSSION

Pathway for 1,2,4-TCB degradation. *Pseudomonas* sp. strain P51 was found to be able to completely mineralize 1,2,4-TCB and 1,2- and 1,4-DCB, a property not previously reported to occur within one microorganism. The metabolic pathway of 1,2,4-TCB degradation in *Pseudomonas* sp. strain P51 was studied in detail by cloning two gene clusters that are involved in this pathway and analyzing their func-

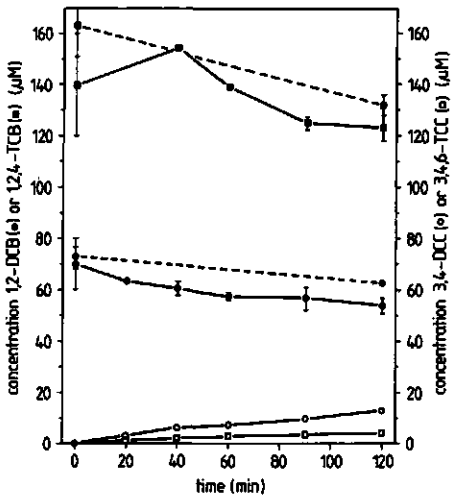

FIG. 7. Conversion of 1,2-DCB and 1,2,4-TCB to 3,4-DCC and 3,4,6-TCC, respectively, by resting-cell suspensions of *E. coli* harboring pTCB69. Shown are concentrations of 1,2-DCB (●) and 1,2,4-TCB (■) measured in cell suspensions (——) and in controls without bacterial cells added (– – –) by using GC analysis during the time course of the incubation (2 h) and concentration of 3,4-DCC (○) and 3,4,6-TCC (□) measured in cell suspensions by using HPLC analysis. Each error bar indicates the standard deviation of a mean value of three independent samples. No formation of chlorinated catechols was detected in controls with chlorinated benzenes but without cells added or in controls without chlorinated benzenes but with cells added. No formation of chlorinated catechols was detected when *E. coli* without recombinant plasmid was incubated with 1,2-DCB or 1,2,4-TCB (results not shown).

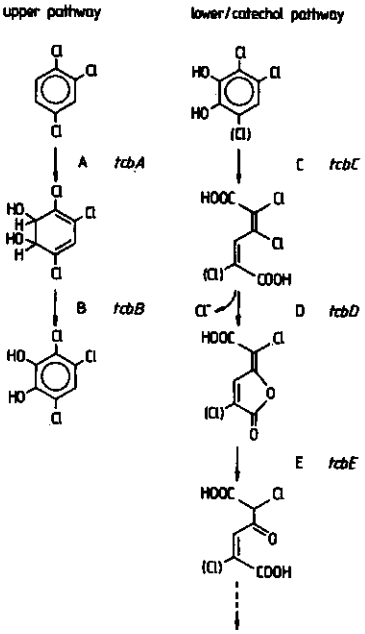

FIG. 8. Proposed pathways for the degradation of 1,2,4-TCB, 1,2-DCB, and 1,4-DCB by *Pseudomonas* sp. strain P51. The structural genes located on plasmid pP51 encoding the intermediate conversion steps are indicated (see text). A, *tcbA* (dioxygenase); B, *tcbB* (dehydrogenase); C, *tcbC* (catechol 1,2-dioxygenase II); D, *tcbD* (cycloisomerase II); E, *tcbE* (hydrolase II). Further metabolism of chloromaleylacetic acid is probably chromosomally encoded (21, 27) and is indicated by the dashed arrow. (Cl), Proposed position of the residual chlorine atom of 1,2,4-TCB. Absolute stereochemical assignments are not intended.

tions primarily in *E. coli*, as well as in *A. eutrophus* and *P. putida*. As a working model, we used a pathway for degradation of 1,2,4-TCB similar to that described for 1,2-DCB by Haigler et al. (15) and for 1,4-DCB by Schraa and co-workers (30) and Spain and Nishino (35). A schematic representation of the pathway is shown in Fig. 8. We propose two steps in which 1,2,4-TCB (or 1,2-DCB and 1,4-DCB) is transformed to the corresponding chlorinated catechol. The first reaction is thought to be an oxidation performed by a dioxygenase enzyme complex. This dioxygenase complex involved in DCB and TCB degradation will also convert indole to indigo, as was reported for the naphthalene dioxygenase by Ensley et al. (9). Similar dioxygenases were also described for oxidation of toluene in *P. putida* F1 (42) and in *P. putida* NCIB 11767 (36), oxidation of naphthalene (41) and xylenes (20), and oxidation of chlorinated biphenyls (24). The second step in the conversion would then necessarily be a dehydrogenase reaction, yielding the chlorinated catechol. We have obtained no evidence for release of chloride during the first two transformation steps, as was proposed by Markus et al. (23) and Schweizer et al. (32) in the case of 4-chlorophenylacetate and recently for the oxidation of 1,2,3,4-tetrachlorobenzene (38a). At present, it is unclear whether the first chlorobenzene dioxygenase complex is also involved in conversion of toluene in *Pseudomonas* sp. strain P51. Degradation of 1,4-DCB and toluene simultaneously was observed for *Pseudomonas* sp. strain JS6 (16). Furthermore, the toluene dioxygenase from *P. putida* F1 was reported to oxidize 1,2-DCB to 3,4-DCC (41a). It is conceivable that a toluene dioxygenase was recruited in the DCB/TCB pathway of strain P51 to function as the first conversion step.

The chlorinated catechols formed from dichlorobenzenes and 1,2,4-TCB were shown to be subsequently degraded through a pathway similar to the archetype described for *Pseudomonas* sp. strain B13 by Knackmuss et al. (7, 8, 27–29). Only one chlorine atom is released from 3,4-DCC after cycloisomerization, probably finally yielding 5-chloromaleylacetate. This view is in agreement with the pathways for 3,4-DCC degradation proposed by Haigler et al. (15), for 3-chlorocatechol proposed by Schmidt et al. (29), and for 3,5-dichlorocatechol (31).

Organization of the *tcb* clusters. The degradation pathway for 1,2,4-TCB consists of two gene clusters that were shown to be present on a catabolic plasmid of 110 kb. The upper-pathway genes, tentatively designated *tcbA* and *tcbB*, were studied only as a cluster. However, subcloning experiments showed that for proper functioning the region cannot be shortened to less than approximately 5 kb (as in plasmid pTCB71), which suggests that the gene cluster contains more than two genes. This would be in agreement with the findings for other dioxygenases, which are multicomponent enzyme

systems. In the case of the *nah*, *xyl*, and *tod* gene clusters, the dioxygenase genes were found to consist of four genes, followed by the gene encoding the dehydrogenase, and were approximately 4 kb in size (20, 41, 42).

Separated from this gene cluster by approximately 4 kb are the genes *tcbC*, *tcbD*, and *tcbE*, encoding the catechol or lower pathway. Analysis of subclones of this gene cluster showed that the orientation of *tcbCD* and *tcbE* is opposite that of the upper-pathway genes *tcbA* and *tcbB*. The lower pathway is organized similarly to the other chlorocatechol-oxidizing pathways *clcABD* (10) and *tfdCDEF* (6, 13, 14, 26). These pathways differ in the presence of an extra open reading frame, which is located between the *clcB* and *clcD* genes of plasmid pAC27 (10) but absent from the *tfd* operon (14, 26). Furthermore, a functional counterpart of the gene *tfdF* was not found in the *clc* cluster, although a region with close DNA homology to *tfdF* was detected directly downstream of *clcD* (26). Gene *tfdF* is proposed to encode a *trans*-dienelactone isomerase, converting *trans*-chlorobutenolide to *cis*-chlorobutenolide, which was shown to be the substrate for the hydrolase II (31). Consequently, this proposed enzyme activity would be a necessary step in the degradation of 3,5-dichlorocatechol (6, 26, 31). However, this isomerization reaction apparently does not take place during conversion of 3-chlorocatechol. This was reported by Knackmuss and co-workers (29), who showed that the intermediate product of the cycloisomerization of chloromuconate is spontaneously transformed and that both *trans*- and *cis*-butenolide are substrates for the hydrolase II. We have found that the *tcbE* gene is not adjacent to *tcbD* (Fig. 4), which allows an additional gene to be located in between *tcbD* and *tcbE*. Introduction of a mutation in this intergenic region did not affect the overall enzyme activities. A *tfdF*-like gene may be located downstream of *tcbE*, but we found no difference in enzyme activities in clones extending in this region (such as pTCB62) compared with the activity pTCB64. This observation suggests that no additional function is required for the transformation of 3,4-DCC to chloromaleylacetate.

In conclusion, the abilities to degrade dichlorobenzenes and 1,2,4-TCB are unique in *Pseudomonas* sp. strain P51. The finding that the genes for degradation of these compounds are clustered in two separate regions on a catabolic plasmid and their similarity to other known catabolic operons could imply that they originated from different sources and were acquired successfully by strain P51. The genetic and biochemical results for DCB and TCB degradation in strain P51 presented here will allow a detailed comparison of the TCB pathway with other known catabolic pathways in order to provide more information on the process of genetic adaptation to xenobiotic substrates in microorganisms.

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Chapter 4

Sequence analysis of the Pseudomonas sp. strain P51 tcb gene cluster which encodes metabolism of chlorinated catechols: evidence for specialization of catechol 1,2-dioxygenases for chlorinated substrates

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Sequence Analysis of the *Pseudomonas* sp. Strain P51 *tcb* Gene Cluster, Which Encodes Metabolism of Chlorinated Catechols: Evidence for Specialization of Catechol 1,2-Dioxygenases for Chlorinated Substrates

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***Pseudomonas* sp. strain P51 contains two gene clusters located on catabolic plasmid pP51 that encode the degradation of chlorinated benzenes. The nucleotide sequence of a 5,499-bp region containing the chlorocatechol-oxidative gene cluster *tcbCDEF* was determined. The sequence contained five large open reading frames, which were all colinear. The functionality of these open reading frames was studied with various *Escherichia coli* expression systems and by analysis of enzyme activities. The first gene, *tcbC*, encodes a 27.5-kDa protein with chlorocatechol 1,2-dioxygenase activity. The *tcbC* gene is followed by *tcbD*, which encodes cycloisomerase II (39.5 kDa); a large open reading frame (ORF3) with an unknown function; *tcbE*, which encodes hydrolase II (25.8 kDa); and *tcbF*, which encodes a putative *trans*-dienelactone isomerase (37.5 kDa). The *tcbCDEF* gene cluster showed strong DNA homology (between 57.6 and 72.1% identity) and an organization similar to that of other known plasmid-encoded operons for chlorocatechol metabolism, e.g., *clcABD* of *Pseudomonas putida* and *tfdCDEF* of *Alcaligenes eutrophus* JMP134. The identity between amino acid sequences of functionally related enzymes of the three operons varied between 50.6 and 75.7%, with the *tcbCDEF* and *tfdCDEF* pair being the least similar of the three. Measurements of the specific activities of chlorocatechol 1,2-dioxygenases encoded by *tcbC*, *clcA*, and *tfdC* suggested that a specialization among type II enzymes has taken place. TcbC preferentially converts 3,4-dichlorocatechol relative to other chlorinated catechols, whereas TfdC has a higher activity toward 3,5-dichlorocatechol. ClcA takes an intermediate position, with the highest activity level for 3-chlorocatechol and the second-highest level for 3,5-dichlorocatechol.**

Bacterial degradation of xenobiotic organic pollutants involves in many cases the use of altered metabolic functions (23) and can be regarded as a system of genetic adaptation to new substrates. As such, it presents a good model for studying evolution of enzymes and catabolic pathways. Of the vast array of synthetic compounds that have been introduced into the environment, chlorinated aromatic compounds are particularly refractory to bacterial degradation (11). In the past decade, various reports have described the degradation of chlorinated aromatic compounds (reviewed in reference 24). In many of these pathways chlorinated catechols are the central metabolites, whereas the only productive pathway for conversion of the chlorinated catechols was found to be the *ortho*-cleavage route (24). The *ortho*-cleavage pathway of chlorinated catechols was first described for *Pseudomonas* sp. strain B13 (7, 40), *Pseudomonas putida* (pAC27) (3), and *Alcaligenes eutrophus* JMP134(pJP4) (5). These bacterial strains were able to grow on 3-chlorobenzoate and, in the case of *A. eutrophus*, also on 2,4-dichlorophenoxyacetic acid as the sole carbon and energy source.

Recently, degradation of chlorinated benzenes was reported, and it was shown that the enzymes of the *ortho*-cleavage route were also present in this catabolic pathway (12, 20, 31, 33, 37). In *Pseudomonas* sp. strain P51, the genes encoding the metabolism of 1,2,4-trichlorobenzene (1,2,4-TCB) and 1,2-dichlorobenzene (1,2-DCB) are located on a catabolic plasmid of 110 kb called pP51 (38). These genes were cloned, characterized, and found to encompass two gene clusters. The upper pathway cluster, *tcbAB*, encodes the conversion of chlorinated benzenes to chlorinated catechols by means of a benzene dioxygenase system and a dehydrogenase. The lower pathway gene cluster encodes the enzymes of the chlorocatechol pathway, which were found to include a catechol 1,2-dioxygenase II (chlorocatechol 1,2-dioxygenase; EC 1.13.11.1) encoded by *tcbC*, a chloromuconate cycloisomerase (cycloisomerase II; EC 5.5.1.1) encoded by *tcbD*, and a dienelactone hydrolase (hydrolase II; EC 3.1.1.45) encoded by *tcbE*. In *P. putida*(pAC27) and *A. eutrophus* JMP134(pJP4), this pathway is encoded by the *clcABD* and *tfdCDEF* gene clusters, respectively (5, 8–10, 21). DNA sequence analysis of the *clc* and *tfd* gene clusters showed that the encoded enzymes are strongly homologous (9, 10, 21). Furthermore, the genes for those pathways were found to be distantly related to the *cat* operons encoding the metabolism of catechol (1, 13, 17).

This paper describes the sequence analysis of the *tcb* chlorocatechol-degradative gene cluster. In addition, we have measured the activities of the type II catechol 1,2-dioxygenases encoded by the *tcbC*, *clcA*, and *tfdC* genes with various chlorinated catechols. The results show that these dioxygenases have different substrate specificities which reflect their specialized function in the metabolism of chlorinated aromatics.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are listed in Table 1.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant properties[a]	Source or reference
Strains		
Pseudomonas sp. strain P51	DCB^+ TCB^+	37
Pseudomonas sp. strain B13	$3CBa^+$	6
A. eutrophus JMP134	2,4-D^+ $3CBa^+$	5
E. coli BL21 (DE3)	F^- *hsdS gal* r_B^- m_B^- (*lacUV5*-T7 gene 1)	34
E. coli DH5α	F^- *lacZ*ΔM15 *recA1 hsdR17 supE44* Δ(*lacZYA-argF*)	Bethesda Research Laboratories
E. coli TG1	F'[*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15] *supE hsd*Δ*5* Δ(*lac-proAB*)	25
Plasmids		
pUC18	Ap^r	41
pT7-5	Ap^r	35
pTCB58	1.8-kb *Pst*I-*Hin*dIII fragment of pTCB42 in pT7-5, Ap^r	38; this work
pTCB58Δ	Same as pTCB58 but contains a frameshift mutation obtained by digestion with *Bam*HI, filling with Klenow enzyme, and religation; Ap^r	This work
pTCB62	5.5-kb *Eco*RI-*Kpn*I fragment of pP51 in pUC18, Ap^r	38
pTCB68	1.6-kb *Mae*I-*Hin*dIII fragment of pTCB62 in pUC18, Ap^r	This work
pTCB82	1.3-kb *Mae*I-*Xho*I fragment of pTCB62 in pT7-5, Ap^r	This work
pTCB83	2.5-kb *Mae*I-*Sal*I fragment of pTCB62 in pT7-5, Ap^r	This work
pTCB84	1.2-kb *Xho*I-*Cla*I fragment of pTCB62 in pT7-6, Ap^r	This work
pTCB85	1.1-kb *Sma*I-*Pst*I fragment of pTCB62 in pT7-5, Ap^r	This work
pTCB86	1.8-kb *Sst*II-*Kpn*I fragment of pTCB62 in pT7-5, Ap^r	This work
pCBA4	1.6-kb *Hin*dIII fragment of pJP4 in pUC18, contains *tfdC*, Ap^r	This work
pDC100	4.2-kb *Bgl*II fragment of pAC27 in pMMB22, Ap^r	8

[a] Abbreviations: DCB, growth on 1,2- and 1,4-DCB; TCB, growth on 1,2,4-TCB; 3CBa, growth on 3-chlorobenzoate; 2,4-D, growth on 2,4-dichlorophenoxyacetic acid; Ap^r, resistance to ampicillin.

Pseudomonas sp. strain P51 was cultivated in Z3 mineral medium supplied with 3.2 mM 1,2,4-TCB as the sole carbon and energy source as described elsewhere (37). *A. eutrophus* JMP134 (5) and *Pseudomonas* sp. strain B13 (6) were grown at 30°C in Z3 mineral medium with 10 mM 3-chlorobenzoate as the sole carbon and energy source. *Escherichia coli* strains were cultivated at 37°C in Luria broth (25). When appropriate, 50 μg of ampicillin per ml, 0.004% 5-bromo-4-chloro-3-indolyl-β-D-galactoside, or 1.0 mM isopropyl-β-D-thiogalactopyranoside was added to the media.

DNA manipulations and sequence analysis. Plasmid DNA isolations and transformations and other DNA manipulations were carried out as described earlier (38) or according to established procedures (25). Restriction enzymes, T4 DNA ligase, T7 RNA polymerase and Klenow DNA polymerase were obtained from GIBCO/BRL Life Technologies Inc. (Gaithersburg, Md.) or Pharmacia LKB Biotechnology (Uppsala, Sweden) and used according to the specifications of the manufacturer.

DNA fragments to be sequenced were cloned into M13mp18 and M13mp19 and propagated in *E. coli* TG1. The sequencing strategy is shown in Fig. 1. Oligonucleotides used as primers in sequencing reactions were synthesized in a Cyclone DNA synthesizer (Biosearch). DNA sequencing was performed by the dideoxy-chain termination method of Sanger et al. (26) using a Sequenase kit (version 2.0; United States Biochemical Corp., Cleveland, Ohio) with [α-^{32}P]dATP (3,000 Ci/mmol; Amersham International plc, Amersham, United Kingdom). To overcome sequence compressions, we routinely incorporated dITP in the reactions instead of dGTP. Computer analysis of DNA and amino acid sequences was performed with the programs PC/GENE (Genofit, Geneva, Switzerland) and GCG (J. Devereux, University of Wisconsin).

Detection of gene products of the *tcb* gene cluster. To detect the gene products of the different open reading frames (ORFs) predicted from the DNA sequence, we subcloned pP51 DNA fragments containing one or more ORFs from plasmid pTCB62 into the T7 expression vector pT7-5 or pT7-6 (35). The resulting plasmids are shown in Fig. 1. Expression of the ORFs from the T7 promoter was analyzed with *E. coli* BL21 (34), and proteins were radioactively labeled with L-[^{35}S]methionine (Amersham) upon induction of the T7 RNA polymerase in the presence of rifampin (35). Further studies were performed by in vitro transcription-translation. For this purpose, mRNA was synthesized from pP51 DNA cloned under control of the T7 promoter by using T7 RNA polymerase (39) and subsequently translated in vitro with an *E. coli* S30 extract as described by the manufacturer (Amersham). Protein samples were denatured by boiling for 5 min and separated on a sodium dodecyl sulfate-polyacrylamide gel (10% polyacrylamide) according to the method of Laemmli (15). After electrophoresis, the gels were electroblotted on nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) with an LKB 2117-605/250 Multiphor II NOVA-blot (LKB, Bromma, Sweden). Dried membranes were exposed to X-ray film at room temperature.

Analysis of catechol 1,2-dioxygenase II activities. To test for catechol 1,2-dioxygenase II activity, *Pseudomonas* sp. strain P51 was cultivated on 1,2,4-TCB, while *A. eutrophus* JMP134 and *Pseudomonas* sp. strain B13 were grown on 3-chlorobenzoate. The activities of the chlorocatechol 1,2-dioxygenases were also studied in *E. coli* after cloning the respective genes. A 1.6-kb *Mae*I-*Hin*dIII fragment containing the *tcbC* gene was isolated from plasmid pP51 (38) and inserted into pUC18 digested with *Sma*I and *Hin*dIII to yield plasmid pTCB68. The *tfdC* gene from *A. eutrophus* JMP134 was isolated on a 1.6-kb *Hin*dIII fragment of plasmid pJP4 (5) and inserted into *Hin*dIII-linearized pUC18 to obtain pCBA4. The *clcA* gene from *P. putida*(pAC27) had been cloned previously in pMMB22, resulting in pDC100 (8). This

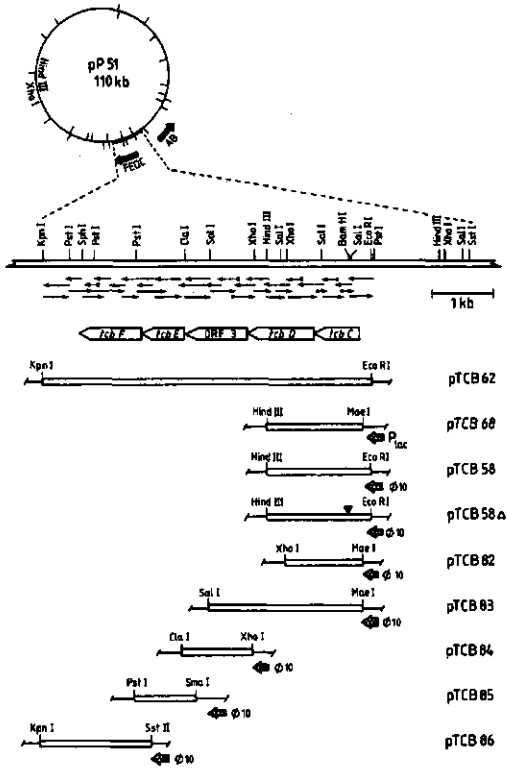

FIG. 1. Sequencing strategy and constructs used in this study. The upper part shows the enlarged region of catabolic plasmid pP51 containing the *tcbCDEF* gene cluster. The direction and size of the determined sequences are indicated. Arrows starting with a solid square indicate that a specific oligonucleotide primer was used. Open arrows represent the locations of the ORFs and genes. The lower part gives the physical maps of the plasmids that were used in this study. In pTCB58Δ, the unique *Bam*HI site was filled in with Klenow polymerase (▼). The direction of transcription from the vector-located promoters (P_{lac} and ϕ10) is depicted by an arrow.

plasmid was kindly provided by W. M. Coco, University of Chicago.

All strains were harvested in their logarithmic phase, and cell extracts were prepared with a French pressure cell or by sonication as described previously (38). Samples containing between 100 and 200 μg of protein were assayed for catechol 1,2-dioxygenase II activity by measuring the formation of chloro-substituted muconic acids at 260 nm after addition of chloro-substituted catechols (30). Enzyme activity was expressed as the amount of muconic acid produced per milligram of protein per minute by using the extinction coefficients given by Schmidt et al. (30). The extinction coefficient of 2,3,5-trichloromuconic acid was estimated from spectroscopic data to be 10,000 liters · mol^{-1} · cm^{-1}. Chlorinated catechols used as substrates in the activity measurements were a generous gift from Walter Reineke, University of Wuppertal, Wuppertal, Germany. 3,4,6-Trichlorocatechol (3,4,6-TCC) was a gift from Gineke van Bergen, Organic Chemistry Department, Wageningen Agricultural University, Wageningen, The Netherlands.

Nucleotide sequence accession number. The nucleotide sequence present in this article has been deposited in the GenBank data base under accession number M57629.

RESULTS

Nucleotide sequence analysis and expression of the *tcbCDEF* genes. The *tcbCDE* genes encoding the chlorocatechol degradation pathway of *Pseudomonas* sp. strain P51 had previously been found to be located on a 5-kb region of catabolic plasmid pP51 (38). Plasmid pTCB62 (Fig. 1) contains a 5.5-kb *Eco*RI-*Kpn*I fragment of plasmid pP51 harboring *tcbC*, *tcbD*, and *tcbE* and was used to generate DNA fragments suitable for cloning into M13mp18 and M13mp19. The complete nucleotide sequence of the 5,499 bp of the pP51 DNA fragment of pTCB62 and the derived amino acid sequences are shown in Fig. 2. The sequence showed the presence of five complete large ORFs, which were all colinear. Putative promoter sequences were localized upstream of the first ORF in a region containing two boxes (TTGGAC and TAGCAT at positions 130 to 154) which were spaced by 18 bp and showed good homology with the *nahA* and *catB* promoter regions recognized by the σ^{70}-activated RNA polymerase in *Pseudomonas* spp. (2, 4, 27). The first ORF (positions 211 to 966) coded for a protein of 251 amino acids with a calculated mass of 27,554 Da. The start codon at position 211 is preceded by a putative ribosome binding site, AGGAG, starting at position 200. The position of this ORF corresponded with the previously determined location of the *tcbC* gene (38). Expression of the *tcbC* gene under control of the T7 promoter was analyzed with plasmids pTCB58 and pTCB82, resulting in the selective labeling of a 27-kDa protein (Fig. 3A). This size agreed with that predicted for the *tcbC* gene product. This protein band was absent when the in vitro system was primed with RNA transcribed from pTCB58Δ, a pTCB58 derivative containing a frameshift mutation at the unique *Bam*HI site. Similar results were obtained when the in vivo expression of the *tcbC* gene was studied with *E. coli* BL21 harboring pTCB58 or pTCB58Δ (results not shown). No catechol 1,2-dioxygenase II activity could be measured in cell extracts of *E. coli* BL21(pTCB58Δ) upon induction, whereas the activity in *E. coli* BL21 (pTCB58) was 10 nmol · mg of $protein^{-1}$ · min^{-1}. On the basis of these results, we conclude that this ORF is the *tcbC* gene.

The second ORF showed an ATG codon at nucleotide 962 and a stop codon at position 2073, which indicated that this ORF could code for a protein of 370 amino acid residues with a predicted mass of 39,487 Da. In addition to the labeled *tcbC* gene product, we observed a protein band with an approximate size of 39 kDa by using an in vitro expression system primed with RNA transcribed from pTCB83 (Fig. 3A). On the basis of the gene order that was determined previously (38) and the homology observed with isofunctional enzymes encoded by *clcB* and *tfdD* (see below), we conclude that this second ORF is the *tcbD* gene.

Between bases 2077 and 3085, a third large ORF, designated ORF3, which could encode a protein of 35,887 Da is found. No consensus ribosome binding site for this ORF was observed preceding the ATG at position 2077 or at downstream initiation codons. No specifically labeled protein could be detected when the expression of pTCB84 containing ORF3 under control of the T7 promoter in *E. coli* BL21 was analyzed in vivo or in vitro (results not shown).

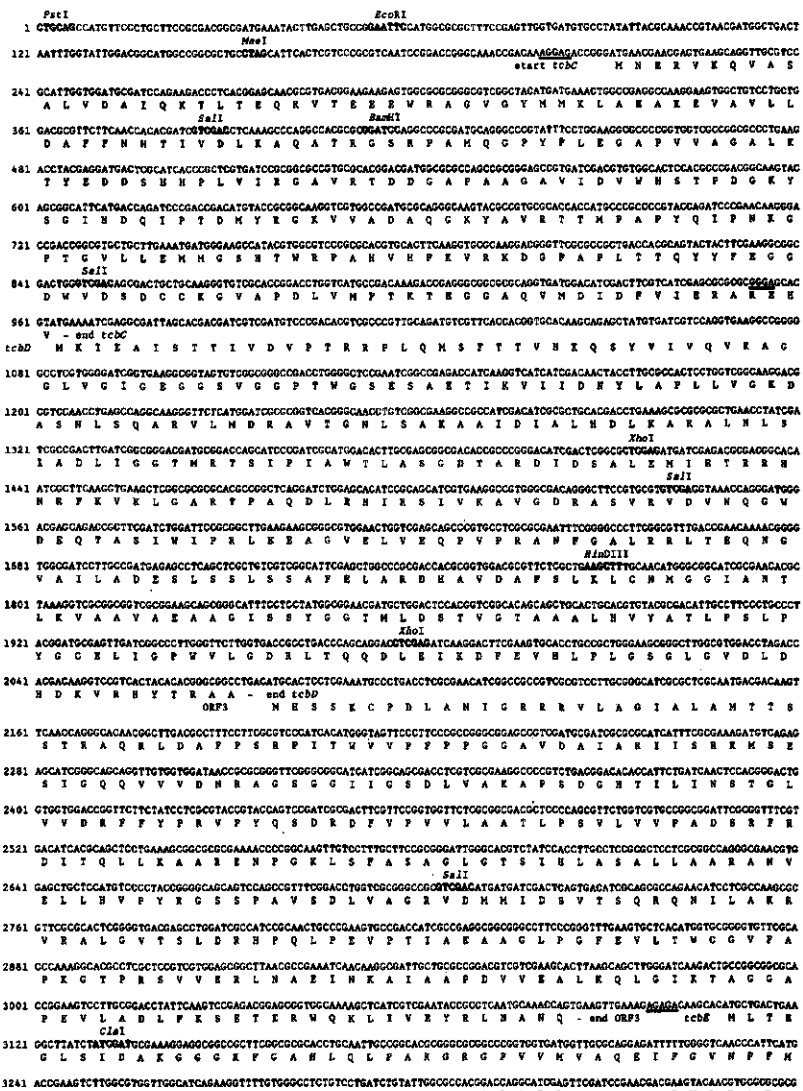

FIG. 2. Nucleotide sequence and deduced amino acid sequence of the 5,499-kb *PstI-KpnI* fragment of pP51 containing the *tcbCDEF* gene cluster. Bases are numbered relative to the initial *Pst*I site. Relevant restriction sites are shown in boldface type and indicated above the nucleotide sequence. Potential ribosome binding sites are underlined.

At position 3109, the putative start of a fourth ORF of 713 nucleotides was found and was preceded by a consensus ribosome binding site around position 3096. This ORF was located at the position previously assigned to the *tcbE* gene (38), which encodes dienelactone hydrolase II. The size of the predicted gene product is 25,828 Da, which agrees well with the observed size of the induced protein band detected in *E. coli* BL21 harboring pTCB85 (Fig. 3B). Therefore, we conclude that this ORF is the *tcbE* gene.

Another ORF started at position 3822. This ORF had a

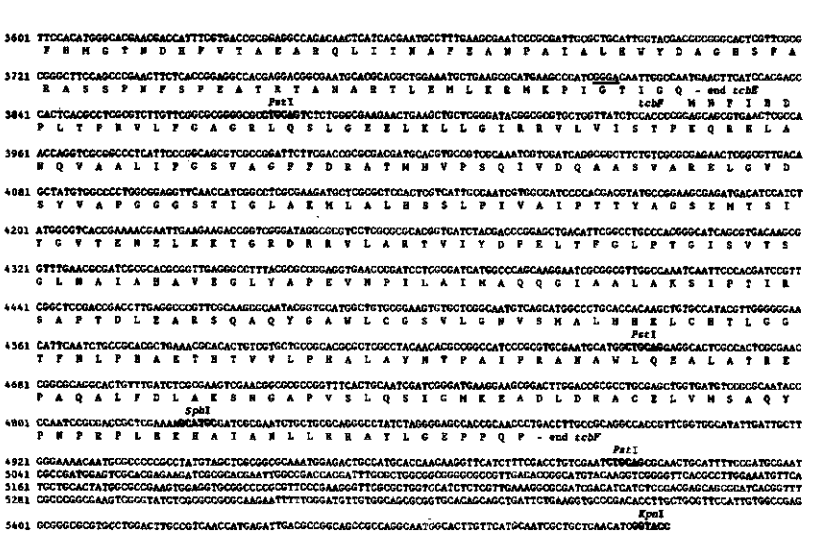

FIG. 2—*Continued.*

size of 1,058 bp, and its predicted gene product had a calculated size of 37,499 Da, which is consistent with the observed size of the protein produced by pTCB86 in *E. coli* BL21 (Fig. 3B). The sequence of this ORF showed high homology with that of the *tfdF* gene (see below), which has been proposed to encode a *trans*-dienelactone isomerase (5, 32). On the basis of the homology with the *tfdF* gene, we have tentatively designated this ORF *tcbF*.

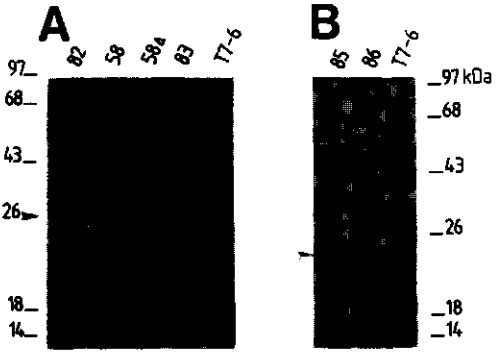

FIG. 3. Expression products of the *tcbCDEF* gene cluster. (A) Autoradiogram of results obtained by in vitro translation using mRNA transcribed from plasmids pTCB82, pTCB58 (containing *tcbC*), pTCB58Δ, and pTCB83 (containing *tcbC* and *tcbD*). The putative expression products of the *tcbC* gene (27 kDa) and *tcbD* gene (39 kDa) are indicated by arrowheads. The other strong protein bands in the pTCB83 extract are nonspecific labeled proteins which occasionally appeared during the reaction. (B) Autoradiogram obtained from [^{35}S]methionine-labeled proteins by using T7-directed expression in *E. coli* BL21 harboring plasmid pTCB85 (containing *tcbE*) or pTCB86 (containing *tcbF*). The arrowheads indicate the putative gene products of *tcbE* (25.8 kDa) and *tcbF* (37.5 kDa).

Homologies among chlorocatechol-degrading enzymes. The predicted amino acid sequences of the gene products of the *tcb* pathway and the corresponding DNA sequences were compared with those of other known catechol-degrading enzymes encoded by the *cat* (1, 17), *clcABD* (8), and *tfdCDEF* (9, 10, 21) gene clusters. Figure 4A shows the alignment obtained with the different catechol 1,2-dioxygenases (CatA, TcbC, ClcA, and TfdC). The degree of identity of CatA with the type II enzymes was approximately 22%, whereas the identity between the members of the latter group was between 53.5 and 63.1% (Fig. 5). All amino acid sequences contained the residues that are involved in the binding of the ferric ion in the *P. putida* protocatechuate 3,4-dioxygenase β subunit, e.g., Tyr-118, Tyr-147, His-160, and His-162 (19) (in the type II sequences, Tyr-130, Tyr-164, His-188, and His-190, respectively). Other strongly conserved residues among both catechol 1,2-dioxygenases and protocatechuate 3,4-dioxygenases are indicated in Fig. 4A. The sequences of the type II enzymes all showed the same gaps compared with the CatA sequence.

The alignment of the cycloisomerases TcbD, ClcB, TfdD, and CatB is shown in Fig. 4B. The amino acid sequences were slightly more conserved than the catechol 1,2-dioxygenase sequences (Fig. 5). All cycloisomerases from the chlorocatechol operons had a size of 370 amino acid residues. TcbD was found to be 43.9% identical to CatB in a 369-amino-acid overlap. The ORF3 of the *tcb* gene cluster could be aligned with the ORF3 found in *clcABD* (Fig. 4C) (8). The hydrolase II enzymes of the three pathways, TcbE, ClcD, and TfdE, were found to be approximately 52% identical (Fig. 4D). The fifth ORF, designated *tcbF*, was found to be homologous to *tfdF*. Their deduced amino acid sequences showed 54.9% identity (Fig. 4E). The DNA sequence similarity of the *tcb* and *tfd* gene clusters was extended in the region downstream of *tcbF*, which showed 40.7% identity with the region preceding *tfdB*.

Activity of chlorocatechol 1,2-dioxygenases with chlorinated catechols. In a first attempt to correlate the structures of the

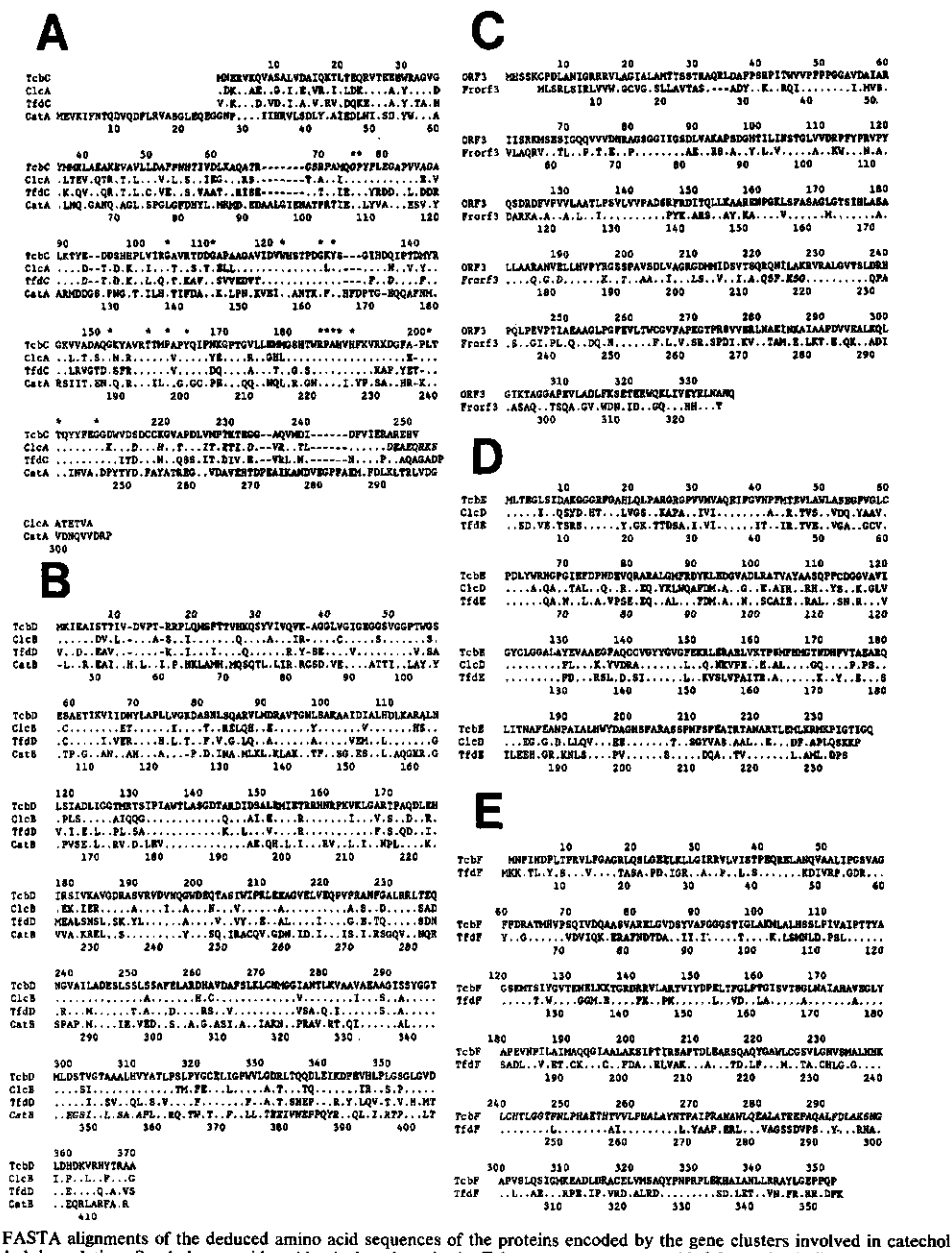

FIG. 4. FASTA alignments of the deduced amino acid sequences of the proteins encoded by the gene clusters involved in catechol or chlorocatechol degradation. Symbols: ., residues identical to those in the Tcb sequences; –, gaps added for optimal alignment; *, strongly conserved residues in catechol 1,2-dioxygenases as well as in protocatechuate 3,4-dioxygenases (19). Numbers above the sequences represent the amino acid positions in the type II enzymes. Numbers under the sequences refer to the positions of amino acids in CatA and CatB. (A) Alignment of the catechol 1,2-dioxygenases TcbC, ClcA, TfdC, and CatA. The FASTA alignment was corrected on the basis of the three-dimensional structure of the protocatechuate 3,4-dioxygenase (19). (B) Alignment of the cycloisomerases encoded by *tcbD*, *clcB*, *tfdD*, and *catB*. (C) Alignment of the gene products of ORF3 from the *tcb* (ORF3) and *clc* (Frorf3) gene clusters. (D) Alignment of hydrolase II enzymes encoded by *tcbE*, *clcD*, and *tfdE*. (E) Alignment of TcbF and TfdF.

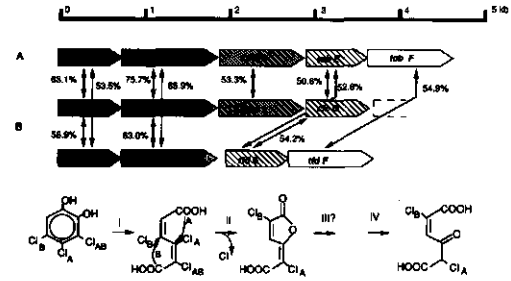

FIG. 5. Organization of the gene clusters *tcbCDEF*, *clcABD* (8), and *tfdCDEF* (21), which encode chlorocatechol metabolism. The different ORFs of the gene clusters are drawn to scale. Similar patterns indicate DNA and amino acid sequence homology between equivalent genes of the three gene clusters, and percentages of identity between deduced amino acid sequences are given. DNA sequence identities were found to lie in the same range (56.7 to 72.1%). Symbols: ▥, catechol 1,2-dioxygenase II (encoded by *tcbC*, *clcA*, and *tfdC*); ▤, cycloisomerase (encoded by *tcbD*, *clcB*, and *tfdD*); ▨, ORF 3; ▧, hydrolase (encoded by *tcbE*, *clcD*, and *tfdE*); □, *tcbF* and *tfdF*. The ORF *tfdF* encodes a putative *trans-cis* isomerase (5). A region of sequence homology with *tfdF* is found downstream from *clcD* (21). The lower part of the figure shows the conversion steps mediated by the gene products of the chlorocatechol clusters (3,4-DCC for the *tcb* cluster [A] and 3,5-DCC for *tfd* and *clc* [B]). Step I, Activity of the chlorocatechol 1,2-dioxygenase, leading to the formation of 2,3- or 2,4-dichloromuconic acid. Step II, Cycloisomerizaton (TcbD, ClcB, or TfdD) yielding a dienelactone (*cis* or *trans*). Cl_A indicates the residual chlorine atom in the *tcb*-mediated pathway; Cl_B indicates the one in the *tfd* or B13 pathway (22, 32). Step III, Putative *cis-trans* isomerization reaction mediated by TfdF or TcbF. Step IV, Activity of the hydrolase II enzyme (TcbE, ClcD, or TfdE) yielding 5-chloromaleylacetate (Cl_A) or 2-chloromaleylacetate (Cl_B).

different type II enzymes with their ability to convert chlorinated catechols with different degrees and positions of the chlorine atoms, we measured catechol 1,2-dioxygenase II activities in cell extracts of the respective wild-type strains and *E. coli* strains containing type II enzymes encoded by the *tcbC*, *clcA*, and *tfdC* genes (Fig. 5). Table 2 shows the relative activities of the different catechol 1,2-dioxygenase II enzymes in cell extracts with various chlorinated catechols compared with the activity obtained with 3-chlorocatechol. The overall patterns of relative activities were similar in cell extracts of the wild-type strains and *E. coli* strains harboring the respective type II genes. The results indicate that the catechol 1,2-dioxygenase TcbC from *Pseudomonas* sp. strain P51 has a high relative activity on 3,4-dichlorocatechol (3,4-DCC) compared with the other tested chlorocatechols, whereas type II enzymes of *A. eutrophus* JMP134 and *Pseudomonas* sp. strain B13 (TfdC and ClcA, respectively) have a high relative activity on 3,5-DCC. Table 2 shows that the type II enzymes of all the strains tested were able to convert 3,6-DCC and 3,4,6-TCC, although at a lower rate than that obtained with their optimal substrates, whereas none was able to convert 3,4,5-TCC or 3,4,5,6-tetrachlorocatechol (results not shown).

DISCUSSION

Molecular organization of the *tcbCDEF* gene cluster. The lower pathway gene cluster of plasmid pP51 encodes the enzymes for the conversion of chlorocatechols to chloromaleylacetate in *Pseudomonas* sp. strain P51 (38). Five ORFs were revealed by sequence analysis of a 5.5-kb region comprising the *tcbCDEF* cluster; these ORFs were preceded by a consensus promoter sequence (Fig. 2). The positions of the *tcbC*, *tcbD*, and *tcbE* genes correlated well with their previously determined locations (38). The overall gene order found by sequence analysis was *tcbC-tcbD-tcbE-tcbF*, with an extra ORF3 located between *tcbD* and *tcbE*. The *tcbC* and *tcbD* genes as well as the *tcbE* and *tcbF* genes showed overlapping stop and start codons (ATGA), indicating a possible translational coupling of the genes. This organization of the *tcbCDEF* gene cluster strongly suggests an operon structure.

The organization of the *tcbCDEF* gene cluster showed high similarity to the *clcABD* cluster of plasmid pAC27 (8) and the *tfdCDEF* gene cluster of plasmid pJP4 (9, 10, 21) (Fig. 5). However, both the *tcb* and *clc* clusters contained an additional ORF3, which appears to be missing in the *tfd* cluster (21). The absence of ORF3 in the *tfdCDEF* operon of *A. eutrophus* may be the result of a deletion. However, the only indication of this seems to be the conservation of short nucleotide motifs (maximum of 7 bp) in the DNA sequence between *tfdD* and *tfdE* and the end of the ORF3 sequences of the *tcbCDEF* and *clcABD* gene clusters (results not shown). The function of ORF3 in these chlorocatechol pathways remains unclear. No expression product from this region of the *clc* operon were detected in experiments with maxicells (8). Also, in our experiments, we were unable to find expression products from ORF3 by using T7 RNA polymerase-directed expression. Furthermore, construction of a frameshift mutation in the ORF3 of the *tcb* operon did not affect the conversion of 3,4-DCC in *E. coli* cell extracts (38). However, the experimental results achieved so far made use of *E. coli*, which may be an unsuitable host for this type of experiment.

In contrast to the *clc* operon (8, 21), it appears that the *tcb*

TABLE 2. Enzyme activities of chlorocatechol 1,2-dioxygenases in cell extracts with chlorinated catechols

Strain	Sp act (relative activity) with substrate[a]:					
	3-CC	3,4-DCC	3,5-DCC	3,6-DCC	3,4,5-TCC	3,4,6-TCC
Pseudomonas sp. strain P51	89 (100)	66 (74)	11 (12)	12 (14)	0 (0)	12 (14)
A. eutrophus JMP134	584 (100)	31 (5)	402 (69)	126 (20)	0 (0)	75 (13)
Pseudomonas sp. strain B13	360 (100)	7 (2)	175 (49)	91 (25)	0 (0)	30 (13)
E. coli(pTCB68)	30 (100)	38 (126)	3 (11)	7 (25)	0 (0)	6 (21)
E. coli(pCBA4)	146 (100)	9 (6)	160 (110)	45 (31)	ND	32 (22)
E. coli(pDC100)	49 (100)	3 (5)	33 (68)	16 (32)	0 (0)	3 (6)

[a] Specific activity is expressed in nanomoles of chloromuconate per milligram of protein per minute. Relative activity is expressed as the percentage of the specific activity with 3-chlorocatechol (3-CC; set as 100%). ND, Not determined.

gene cluster contains a gene, *tcbF*, which is homologous to the *tfdF* gene. Although the *tcbF* gene is expressed in *E. coli* (Fig. 3B), we could not detect any *trans*-chlorodienelactone isomerase activity, which is ascribed to the *tfdF* gene product (5, 21, 32, 38). These suggestions, however, are not supported by biochemical studies of the *tfd*-encoded enzymes, which indicated that *cis*-2-chloro-4-carboxymethylenebut-2-en-4-olide is formed directly from 2,4-dichloro-*cis*,*cis*-muconate by the activity of chloromuconate cycloisomerase (14, 22, 28). Instead, it has been suggested that *tfdF* could encode a protein with 5-chloro-3-oxoadipate-dehalogenating activity (28).

The high identity between enzymes encoded by the three gene clusters and between their DNA sequences (Fig. 4 and 5) suggests that *tcbCDEF* and *clcABD* are more closely related to each other than to *tfdCDEF*. This can also be concluded from the conservation of a complete ORF3 in both *tcbCDEF* and *clcABD*. The cycloisomerase gene appeared to have the highest degree of sequence conservation among the different genes of the clusters, followed by the catechol 1,2-dioxygenase gene. The overall organization of the chlorocatechol gene clusters is different from the organization of the *cat* genes in *P. putida* (1) and *Acinetobacter calcoaceticus* (17). In these cases, the *catA* gene encoding the catechol 1,2-dioxygenase is separated from *catB*, which encodes muconate-lactonizing enzyme.

Activity of catechol 1,2-dioxygenase II enzymes. Catechol 1,2-dioxygenase enzymes can be divided into two types, I and II, according to Dorn and Knackmuss (7). Both type I and type II enzymes can occur in the same cell (7, 22), and even more isozymes of catechol 1,2-dioxygenase can be found in one strain (16), although significant differences in substrate range are not always observed (16). Type I enzymes are relatively specific enzymes that use primarily catechol as a substrate. Chlorinated catechols are not used as substrates, with the exception of 4-chlorocatechol (7). Type II enzymes are induced upon growth with a chlorinated carbon source, such as 3-chlorobenzoate, and are relatively nonspecific enzymes which have a wider substrate range. They convert chlorinated catechols more rapidly than catechol (7). The wider substrate range of the type II enzymes, however, may have resulted in a substantially lower specific activity (18). The specialization of chlorocatechol pathway enzymes was further extended by characterizing the chloromuconate cycloisomerases and hydrolase II enzymes from *Pseudomonas* sp. strain B13 and *A. eutrophus* JMP134 (14, 28–30). We were interested in determining whether differences among catechol 1,2-dioxygenase II enzymes occur with respect to their substrate range, which could reflect their function in metabolism of chlorinated compounds. In those experiments, we also examined expression of the cloned genes in *E. coli* in order to prevent interference with endogenous catechol 1,2-dioxygenases present in the wild-type strains.

The observed relative activities of the type II enzymes with chlorinated catechols show that there are clear differences in conversion rates. Our results with the TcbC enzyme indicate that, of the tested chlorocatechols, 3,4-DCC is the optimal substrate for this enzyme. In contrast, the TfdC enzyme shows a higher rate of conversion with 3,5-DCC as a substrate, which is in agreement with the findings of Pieper et al. (22). In *A. eutrophus* JMP134, 3,5-DCC occurs as an intermediate in the conversion of 2,4-dichlorophenoxyacetic acid by enzymes of the *tfd* pathway (5), indicating that this pathway is specialized for conversion of 3,5-DCC (22). This phenomenon was also observed by Kuhm et al. (14), who analyzed the kinetic properties of the chloromuconate isomerase of *A. eutrophus* and found a high specific activity of this enzyme toward 2,4-dichloromuconate, the product of intradiol cleavage of 3,5-DCC. However, 3,5-DCC is not an intermediate occurring in the metabolism of chlorinated compounds in *Pseudomonas* sp. strain B13, since the wild-type strain can grow on 3-chlorobenzoate only (6). Our results with the ClcA enzyme confirm a previous finding that, for *Pseudomonas* sp. strain B13 (7, 32), the activity of the type II enzyme is not the limiting factor in metabolizing 3,5-DCC. The higher rate of conversion of 3,4-DCC by TcbC is consistent with the fact that 3,4-DCC occurs as an intermediate in the metabolism of 1,2-DCB by *Pseudomonas* sp. strain P51 (38). A similar preference for 3,4-DCC observed in the relative activity of the catechol 1,2-dioxygenase was also reported for a *Pseudomonas* sp. (12) which can grow on 1,2-DCB as the sole carbon and energy source. Strain P51 grows more slowly on 1,4-DCB or 1,2,4-TCB than on 1,2-DCB (38). This could be explained by the lower level of activity of the catechol 1,2-dioxygenase II toward the chlorinated catechols which are formed as intermediates in the metabolism of these compounds, e.g., 3,6-DCC and 3,4,6-TCC. 3,5-DCC is an intermediate which is formed during conversion of 1,3-DCB. Although 3,5-DCC can be converted by the type II enzyme of strain P51, 1,3-DCB cannot serve as the sole carbon and energy source (37, 38). Characterization of the catechol 1,2-dioxygenase II from microorganisms capable of growing solely on 1,4-DCB, such as *Alcaligenes* sp. strain A175 (31) or *Pseudomonas* sp. strain JS6 (33), could yield more insight into the diversity of type II enzymes.

Homology among catechol 1,2-dioxygenase genes. Recently, a number of articles reporting the homology among catechol 1,2-dioxygenases and protocatechuate 3,4-dioxygenases have been published (10, 13, 17). The data presented here reinforce the hypothesis that the intradiol dioxygenases have a common ancestor. With the inclusion of the *tcbC* sequence, it has become more obvious that the catechol 1,2-dioxygenase II enzymes form a separate group. The amino acid residues which are conserved in all sequences include the ones shown to be involved in binding the ferric ion (19) and other residues such as glycine, proline, and leucine, which, on the basis of the high homology with the protocatechuate 3,4-dioxygenase, are supposed to be of structural importance (Fig. 4A). The DNA sequences of structural genes *tcbC*, *clcA*, and *tfdC* provide examples of three relatively recently evolved genes sharing close homology but otherwise differing subtly and as such may reflect mechanisms that are acting on sequence conservation and divergence (13, 17).

Although the deduced amino acid sequences of the type II enzymes are highly homologous (53.5 to 63.1%), we observed a specialization in the specific activities for different chlorinated substrates. Until now, the basis for the extended substrate range of the catechol 1,2-dioxygenase II enzymes, compared with that of the type I enzymes, had been unknown. Recent evidence about substrate binding in the protocatechuate 3,4-dioxygenase β subunit (36) has indicated that the catecholic substrates directly interact with the ferric ion via their hydroxyl groups. This suggests that the observed differences in substrate specificities are due not to substrate binding itself but to a total conformational change of the enzyme to fit the substrate. In addition, further conformational changes are probably needed to account for the substrate specificities of TfdC and ClcA on the one hand and TcbC on the other. The analysis of other type II

enzymes could reveal whether this specialization is a more general phenomenon and provide insight into the amino acid residues which are involved in these functional differences.

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Chapter 5

Characterization of the Pseudomonas sp. strain P51 gene tcbR, a LysR-type transcriptional activator of the tcbCDEF chlorocatechol oxidative operon, and analysis of the regulatory region

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Characterization of the *Pseudomonas* sp. Strain P51 Gene *tcbR*, a LysR-Type Transcriptional Activator of the *tcbCDEF* Chlorocatechol Oxidative Operon, and Analysis of the Regulatory Region

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Plasmid pP51 of *Pseudomonas* sp. strain P51 contains two gene clusters encoding the degradation of chlorinated benzenes, *tcbAB* and *tcbCDEF*. A regulatory gene, *tcbR*, was located upstream and divergently transcribed from the chlorocatechol oxidative gene cluster *tcbCDEF*. The *tcbR* gene was characterized by DNA sequencing and expression studies with *Escherichia coli* and pET8c and appeared to encode a 32-kDa protein. The activity of the *tcbR* gene product was analyzed in *Pseudomonas putida* KT2442, in which it appeared to function as a positive regulator of *tcbC* expression. Protein extracts of both *E. coli* overproducing TcbR and *Pseudomonas* sp. strain P51 showed specific DNA binding to the 150-bp region that is located between the *tcbR* and *tcbC* genes. Primer extension mapping demonstrated that the transcription start sites of *tcbR* and *tcbC* are located in this region and that the divergent promoter sequences of both genes overlap. Amino acid sequence comparisons indicated that TcbR is a member of the LysR family of transcriptional activator proteins and shares a high degree of homology with other activator proteins involved in regulating the metabolism of aromatic compounds.

Pseudomonas sp. strain P51 is a recently isolated bacterium able to use chlorobenzenes as sole carbon and energy sources (49, 50). With the current interest in environmental pollution, increasing numbers of bacterial strains that degrade organic chemicals are being described (35). These strains offer the unique possibility of studying the evolution of bacterial metabolism in response to new substrates, such as xenobiotic compounds.

Bacteria that degrade chlorinated catechols via the chlorocatechol oxidative pathway (35), such as *Pseudomonas* sp. strain P51 (49, 50) and strain B13 (11, 12), *Pseudomonas putida*(pAC27) (7, 8), and *Alcaligenes eutrophus* JMP134 (pJP4) (10, 43), express specialized enzymes capable of converting chlorinated substrates. Sequence analysis showed a strong homology among the *tcbCDEF* (48), *clcABD* (16), and *tfdCDEF* (18, 19, 32, 33) gene clusters. The high similarity in the functions and deduced sequences of the key enzymes in this metabolic pathway, such as catechol 1,2-dioxygenases (12, 18, 21, 28, 33, 48), cycloisomerases (19, 26, 33, 43, 48), and hydrolases (33, 43, 44, 48), suggests that the chlorocatechol oxidative pathway originated from common metabolic pathways, such as that of catechol and protocatechuate degradation in *Acinetobacter calcoaceticus* (21, 28, 30) or pseudomonads (3, 30). However, new pathways, such as the chlorocatechol oxidative pathway, need, in addition to altered enzymatic activities (12, 26, 35, 43, 44, 48), fine-tuning of regulatory functions, such as inducer recognition. Mutation studies with TOL plasmid-encoded regulatory gene *xylS* showed that new metabolic substrates for the TOL pathway could be selected on the basis of their ability to function as inducers for the altered XylS protein (1, 34).

Preliminary studies on the regulation of the *tfdCDEF* cluster and of the *tfdA* and *tfdB* genes (20, 24, 25) and (partial) sequence analysis of flanking regions of the *tfdCDEF* (32, 33) and *clcABD* (16, 32) clusters indicated that the expression of those gene clusters was regulated by proteins that showed homology to the LysR family of transcriptional activator proteins (22, 23). This group also includes the well-studied NahR (41, 42, 52), CatR (37), and CatM (29) proteins, which are all involved in regulating the metabolism of aromatic compounds. In previous studies, preliminary evidence for the presence of a regulatory gene of the *tcbCDEF* gene cluster which would be located upstream of *tcbC* was obtained (48, 50). This paper describes the cloning and characterization of this regulatory gene, *tcbR*, as well as an analysis of the promoter regions of both the *tcbC* and *tcbR* genes, on which TcbR exerts its activity. The results show that TcbR is a member of the LysR family of transcriptional activator proteins.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Pseudomonas* sp. strain P51 (49, 50) contains plasmid pP51 and is able to use dichlorobenzenes (Dcb^+) and 1,2,4-trichlorobenzene (Tcb^+) as sole carbon and energy sources. *P. putida* KT2442 (15) is a rifampin-resistant (Rif^r), plasmid-free derivative of strain mt-2 and was used as a recipient strain for pKT230-derived plasmids containing pP51 DNA fragments. *Escherichia coli* DH5α and TG1 (38) were used for routine cloning experiments with plasmids and M13 phages, respectively. *E. coli* BL21(DE3) carrying the T7 RNA polymerase gene under the control of the *lacUV5* promoter and harboring plasmid plysS, which expresses the T4 lysozyme gene (47), was used for the T7-directed expression of pET8c-derived plasmids (36). *E. coli* HB101(pRK2013) (13) was the helper strain used for mobilizing pKT230-derived plasmids in triparental mat-

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ings with *P. putida* KT2442. Plasmids pUC18 and pUC19 (51) were used as general cloning vehicles. Plasmid pKT230 (5) is a mobilizable broad-host-range vector. pET8c (36), an ATG vector derived from pBR322, contains the ϕ10 promoter, ribosome binding site, and terminator and is optimized for T7-directed expression. For sequencing, we used M13mp18 and M13mp19 (51). pTCB1 and pTCB45 (50) contain the *tcbCDEF* chlorocatechol oxidative gene cluster and the *tcbR* gene of plasmid pP51 and were used as the sources for the cloning and expression experiments described here. Plasmids pTCB66 and pTCB77 contain an intact *tcbR* gene (see Fig. 1A). A frameshift mutation was introduced into the *tcbR* gene of plasmid pTCB77, resulting in plasmid pTCB77Δ. This was done by removing the 3′-protruding ends of the *Sst*II-linearized plasmid by using the exonuclease activity of Klenow polymerase, recircularizing the plasmid, and transforming *E. coli*. Plasmids pTCB75 and pTCB76 carry the *tcbCDEF* gene cluster and an intact *tcbR* gene on a 10.0-kb *Hpa*I-*Sst*I fragment isolated from pTCB45. This fragment was inserted into pKT230 which was digested with *Hpa*I and *Sst*I (pTCB75) or into pKT230 which was first digested with *Eco*RI, then subjected to Klenow polymerase treatment, and finally digested with *Sst*I (pTCB76). The *tcbR* gene was inactivated in plasmid pTCB74 (see Fig. 1B). The mutation was introduced in *tcbR* by first cloning the 1.5-kb *Eco*RI-*Sst*I fragment containing *tcbR* separately in pUC19 (pTCB56) and subsequently digesting the resulting plasmid with *Sst*II and treating it with Klenow polymerase (pTCB56Δ). The 1.5-kb *Eco*RI-*Sst*I fragment of pTCB56Δ was then isolated and ligated with the 8.5-kb *Hpa*I-*Eco*RI fragment of pTCB45 containing *tcbC-DEF* and with pKT230 which was digested with *Hpa*I and *Sst*I. After transformation in *E. coli*, plasmid pTCB74 resulted.

Media and culture conditions. *Pseudomonas* sp. strain P51 was grown on minimal medium containing 3.2 mM 1,2,4-trichlorobenzene (1,2,4-TCB) or 10 mM succinate at 30°C (50). *P. putida* was grown at 30°C on LB (38) or on M9 minimal medium (38) containing one of the following carbon sources: 10 mM succinate, 10 mM 3-chlorobenzoate (3-CB), or 10 mM benzoate. *E. coli* was cultivated at 37°C on LB. Antibiotics were added in the following amounts: ampicillin, 50 μg/ml; kanamycin, 50 μg/ml; and rifampin, 50 μg/ml. When necessary, media were supplemented with 0.004% 5-bromo-4-chloro-3-indolyl-β-D-galactoside or 1.0 mM isopropyl-β-D-thiogalactopyranoside (IPTG).

DNA manipulations and sequence analysis. Plasmid DNA isolations, transformations, conjugative crosses, and other DNA manipulations were carried out as described earlier (50) or by established procedures (38). DNA sequencing was performed by the dideoxy chain termination method of Sanger et al. (39) as described elsewhere (48). Computer analysis and processing of sequence information were done with the program PC/GENE (Genofit, Geneva, Switzerland) and the GCG package (J. Devereux, University of Wisconsin). Restriction enzymes and other DNA-modifying enzymes were obtained from Life Technologies Inc. (Gaithersburg, Md.) or Pharmacia LKB Biotechnology (Uppsala, Sweden).

RNA isolation and primer extension studies. RNA was isolated from 500-ml cultures of *Pseudomonas* sp. strain P51 cultivated on 1,2,4-TCB or succinate and harvested in the logarithmic phase by the acid phenol extraction procedure of Aiba et al. (2). For primer extension experiments, 0.2 μg of a synthetic oligonucleotide was annealed to 10 or 30 μg of RNA. Oligo 11 (5′ GAGGGTCTTCTGGATCG 3′) was complementary to a region between 42 and 60 nucleotides downstream from the ATG codon of *tcbC*; oligo 12 (5′ TGCAGCCATGTTCCCTG 3′) was complementary to a region between 43 and 60 nucleotides downstream from the putative start of *tcbR* (see Fig. 2). Oligonucleotides were synthesized on a Cyclone DNA synthesizer (Biosearch) and end labeled with [γ-^{32}P]ATP (3,000 Ci/mmol; Amersham International plc., Amersham, United Kingdom) by using T4 kinase. The primer-RNA hybrid was extended with 200 U of Moloney murine leukemia virus reverse transcriptase for 1 h at 37°C (38). Extension products were separated on a 6% denaturing polyacrylamide gel and compared with the products derived from DNA sequencing reactions primed with the same oligonucleotides.

Catechol 1,2-dioxygenase activity measurements. For studying the induction of *tcbC* in *P. putida*, were grown 100-ml cultures to an A_{620} of 1.0, harvested by centrifugation, washed once in 50 ml of 50 mM Tris hydrochloride (pH 7.5), resuspended in 1.0 ml of the same buffer, and subsequently disrupted by sonication (50). Catechol 1,2-dioxygenase *tcbC* was induced with either 3-CB or benzoate as the sole carbon source in the growth medium. Catechol 1,2-dioxygenase activity was assayed with 3-chlorocatechol (3-CC) as a specific substrate for *tcbC*-mediated activity (12, 50) and catechol for both endogenous and *tcbC*-derived catechol 1,2-dioxygenase activities.

DNA binding experiments. Cell extracts of *Pseudomonas* sp. strain P51 or *E. coli* harboring cloned pP51 DNA fragments with the *tcbR* gene were tested for DNA binding activity by an electrophoretic mobility shift assay. Crude cell extracts were prepared from exponentially growing cultures of *Pseudomonas* sp. strain P51 on 1,2,4-TCB as described previously (50). *E. coli* BL21(DE3) was grown to an A_{620} of 0.6, after which IPTG was added and incubation was continued for another 2 h. Subsequently, cells were harvested, washed, and disrupted as described above. Crude cell extracts were then cleared by centrifugation at 30,000 rpm (80,000 × *g*) for 30 min at 4°C and kept on ice until further use. The DNA binding assay was performed with a total volume of 15 μl of 10 mM HEPES buffer (pH 7.9) containing 10% glycerol, 100 mM KCl, 4 mM spermidine, 0.1 mM EDTA, 0.25 mM dithiothreitol, 2 mM $MgCl_2$, 1.5 μg of bovine serum albumin, and 1 μg of poly(dI-dC) (Boehringer GmbH, Mannheim, Germany). Typically between 1 and 10 μg of protein was used in the assay. DNA fragments tested for binding were labeled with [α-^{32}P]dATP (3,000 Ci/mmol; Amersham) by filling in of 3′-recessive ends with Klenow DNA polymerase (38). In each assay, approximately 10,000 cpm of a labeled fragment was used. Binding reactions were carried out for 15 min at 20°C, after which the samples were electrophoresed through a 5% native polyacrylamide gel. Subsequently, the gels were dried and exposed to X-ray film.

Protein determinations. Concentrations of proteins in cell extracts were determined as described by Bradford (6).

Nucleotide sequence accession number. The nucleotide sequence presented in this article has been deposited at GenBank under accession number M57629.

RESULTS

DNA sequence and expression analysis of *tcbR*. In a previous study (48), we showed that the expression of the *tcbCDEF* gene cluster in *E. coli* was affected by an upstream region. Therefore, the region of plasmid pP51 immediately preceding the *tcbCDEF* chlorocatechol oxidative gene cluster (48, 50) was analyzed for the presence of a putative

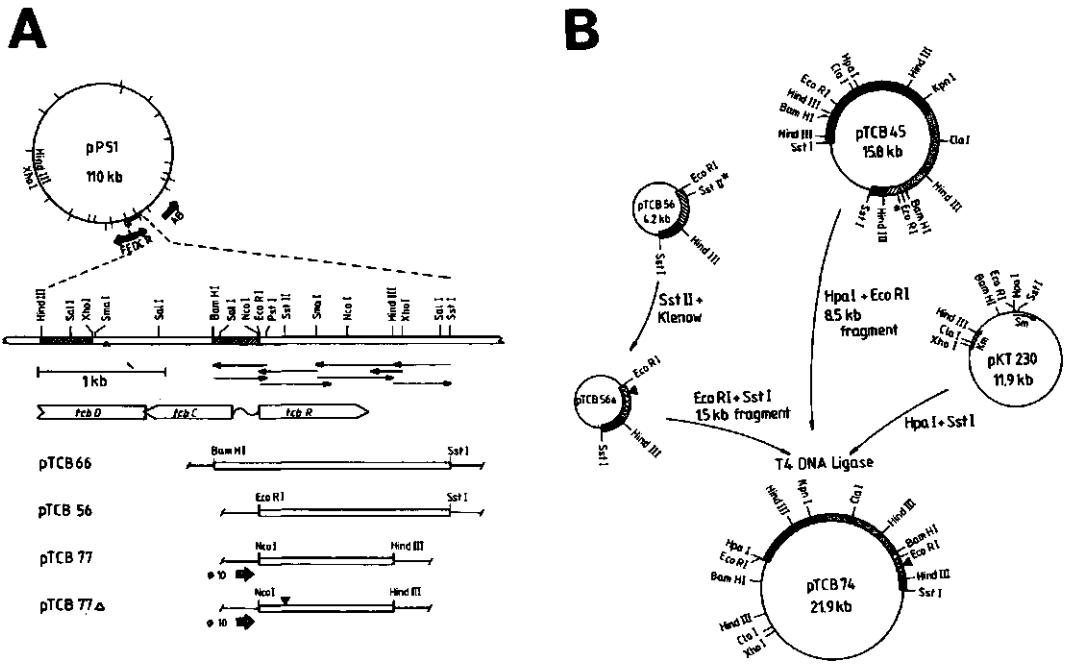

FIG. 1. (A) Enlarged region of the part of catabolic plasmid pP51 containing the *tcbR* gene and the start of the *tcbCDEF* gene cluster (48, 50). The relevant restriction sites are shown. The directions and sizes of DNA fragments used for sequencing are indicated by arrows. Open bars represent the locations and directions of the determined genes. The names of the plasmids refer to the constructs used in this study. The replicons of pTCB77 and pTCB77Δ are from pET8c (36), and the direction of the vector-localized ϕ10 promoter is indicated. Symbols: ▨, fragment containing the promoter regions of *tcbC* and *tcbR* used in the electrophoretic mobility shift assay; ▩, negative control fragment used in the binding assay; ▼, position of the *Sst*II site which was removed in pTCB77Δ. (B) Cloning strategy of plasmid pTCB74 carrying a frameshift mutation in *tcbR*. The strategy is explained in the text. Symbols: *, position of the *Sst*II site to be mutated in *tcbR*; ▼, *Sst*II site removed in pTCB56Δ and pTCB74; ▩, region of *tcbCDEF* on plasmid pTCB45 and pTCB74 (50); ▨, region of *tcbR*; ▩, mutated *tcbR* expressing a truncated protein; ■, remaining parts of pP51 insert; ▭, promoter region of *tcbC* and *tcbR*. Relevant restriction sites and sizes are indicated.

regulatory gene (Fig. 1A). The nucleotide sequence of the 1,877-bp *Bam*HI-*Sst*I fragment of pP51 DNA preceding the *tcbCDEF* gene cluster was determined. The results (Fig. 2) revealed one large open reading frame from positions 357 to 1241 (designated *tcbR*; see below), starting with an ATG start codon and preceded by a potential ribosome binding site (45). The open reading frame was found to be oriented in the opposite direction to the *tcbCDEF* gene cluster. The *tcbR* gene could encode a protein of 294 amino acid residues with a calculated molecular mass of 32,025 daltons. A second possible start codon was found at position 408 and was also preceded by a potential ribosome binding site.

Expression of the *tcbR* gene was analyzed in *E. coli* BL21(DE3) by cloning of the 1.1-kb *Nco*I-*Hin*dIII fragment of pTCB66 in vector pET8c, which had been digested with *Nco*I and *Hin*dIII, yielding plasmid pTCB77. In this construction, the *tcbR* coding region is fused with the ATG start codon present on the T7 expression vector pET8c (36). The expression of a polypeptide of approximately 30 kDa could be seen upon induction of *E. coli* BL21(DE3) harboring plasmid pTCB77 (Fig. 3). This size was in agreement with that predicted from the sequence of the *tcbR* gene. When a frameshift mutation was introduced into the *tcbR* gene, as in plasmid pTCB77Δ, the 30-kDa protein was no longer produced. Instead, a smaller, truncated protein of 12 kDa was synthesized (Fig. 3); this size corresponded to that predicted from the out-of-frame gene fusion from the first ATG at position 357. Both protein bands were absent in *E. coli* BL21(DE3) harboring the vector pET8c. Furthermore, a second protein band of 19 kDa was observed upon induction of *E. coli* BL21(DE3) harboring plasmid pTCB77. Because the introduction of the frameshift mutation abolished the synthesis of this protein (Fig. 3, lane 4), we assume that it was a degradation product of TcbR.

Homology of TcbR with LysR-type transcriptional regulators. Comparison of the deduced amino acid sequence of TcbR with those of members of the LysR family by a FASTA alignment (31) revealed clear similarities (Fig. 4). Assuming that the *tcbR* gene starts at position 357 (Fig. 2), the homology was highest in the N-terminal helix-turn-helix motif which is presumed to be the DNA binding region of these proteins (22, 23). Good homology was observed with amino acid sequences deduced from regulatory genes involved in aromatic metabolism, such as those for CatR of *P. putida* (31.4% identity in 283 amino acids [37]), CatM of *A. calcoaceticus* (31.2% in 247 amino acids [29]), or NahR of *P.*

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     BamHI                                SalI
  1  GGATCCGCGCGTGGCCTGGGCTTTGAGGTCGACGATCGTGTGGTTGAAGAACGCGTCCAG
     CCTAGGCGCGCACCGGACCCGAAACTCCAGCTGCTAGCACACCAACTTCTTGCGCAGGTC

 61  CAGGACAGCCACTTCCTTGGCCTCGGCCAGTTTCATCATGTAGCCGACGCCCGCGCCCA
     GTCCTGTCGGTGAAGGAACCGGAGCCGGTCAAAGTAGTACATCGGCTGCGGGCGCCGGGT

121  CTCTTCTTCCGTCACCCGTTGCTCCGTGAGGGTCTTCTGGATCCGATCCACCAATGCGGA
     GAGAAGAAGGCAGTGCGCAACGAGGCACTCCCAGAAGACCTAGCGTAGGTGGTTACGCCT

181  CGCAACCTGCTTCACTCGTTCGTTCATCCCGGTCTCCTTTGTCGGTTTGCCCCGTCCGGA
     GCGTTGCACGAAGTGACCAAGCAAGTA  start tcbC

241  TTGACGCGGGACGAGTGAATGCTAGGCAGCGCCGGCCATGCCGTCCAATACCAAATTAGT
                                                          NcoI
301  CAGCCATCGTTACCGTTTGCGTAATATAGCGCACATCACCAACTCCGAAAGCGCCCCATGC
   1                                              start tcbR     M E
     EcoRI                                                  PstI
361  AATTCCGGCAGCTCAAGTATTTCATCGCCGTCGCCGAAGCAGCGAACATGGCTGCAGCAG
   3   F R Q L K Y F I A V A E A C N M A A A A

421  CCAAGCGCCTGCACGTCTCACAGCCCCCCATCACGCGGCAGATGCAAGCCCTGGAAGCGC
  23   K R L H V S Q P P I T R Q M Q A L E A D
                                  SstII
481  ACTTGGGCGTCGTGCTTCTCGAGCGAAGCCACCGCGGGGATCGAACTCACCGCCGCAGGTC
  43   L G V V L L E R S H R G I E L T A A C H

541  ACGCTTTCCTGCAGCATGCGCGCCCCATCCTGCAGTTCGCAGGCCCTTCGGGTGATCGCT
  63   A F L E D A R R I L E L A G R S G D R S

601  CCGCGCCGCCGCCCCGGGCGACCTGGGCGAGTTCACCCTGGCGTATTTCGCAACGCGCA
  83   R A A A R G D V G E L S V A Y F G T P I

661  TCTACCGCAGGCTGCCCCTTTGCTGCGCGCTTTCGTTACGTCCACGCCTACGGGCGACGG
 103   Y R S L P L L L R A F L T S T P T A T V

721  TATCCCTCACGCACATGACCAAGCACGAGCAGCTCGAGCGCCTGCTCGCGCGGCACCATCC
 123   S L T H M T K D E Q V E G L L A G T I H
                                  SmaI
781  ACGTGGGCTTCAGCCGCTTCTTTCCCCGGCATCCCGGGGATCGAGATCGTCAACATCGCGC
 143   V G F S R F F P R H P G I E I V N I A Q

841  AAGAGGATCTCTACCTCGCGCTGCACCGCTCCGAGTCGCGGAAGTTCGCGCAAGACCTGCA
 163   E D L Y L A V H R S Q S G K F G K T C K

901  AGCTCGCGGACCTGCGCGCGGCTGGAACTCACACTGTTTCCGCCGCGGCGCCGGCCGAGCT
 183   L A D L R A V E L T L F P R G G R P S F

961  TCGCCGATGAGGTGATCGGCCTGTTCAAGCACGCGGGTATCGAGCCCCGCATCGCCCGAG
 203   A D E V I G L F K H A G I E P R I A R V
                                  NcoI
1021  TCGTGGAGGACGCCACGGCCGCGCTCGCCTGACCATGGCCGGTGCGGCGTCGAGCATCG
 223   V E D A T A A L A L T M A G A A S S I V

1081  TCCCTGCGTCTGTCCAGCGATCCGTTGCCCGATATCGCGTTCGCCCGGATCGTCGGGA
 243   P A S V A A I R W P D I A F A R I V G T

1141  CACGGGTCAAGGTGCCCATCAGCTGCACCTTCCGCAAGCAGAAACAGCCGCCCATCCTGG
 263   R V K V P I S C T F R K E K Q P P I L A

1201  CAAGGTTCGTGGAACACGTGCGGCGATCCGCGAAGGACTGAGGCAAATGGTTATTCGCTC
 283   R F V E H V R R S A K D - end tcbR      294

1261  AACCACGTTTCAGTTCGCAAGGTCCGATCGTCACGCTCGACACGCGATCGCGTTCCGGTCG
1321  CAAGCTACGAGTTCGTAGCCCAGCCTTCATCGCCCAGCCCGCCGTCACAGATGCAAAAGA
                                                        HindIII
1381  GTCAGAAAGGTTGCAGTTCTGATGGCAAGCATTCGTAGCTACCGTCAAGCAAGCTTATCG
1441  GATCACTTGAGGTGGGCTCGATTGACTCGCTCGATGGTCTGCGAGGGTAGTTCCCCAAAC
      XhoI
1501  TCGAGTCGGTACTCTTGAGAGAAACGCCCCCAGGTGATCGAGTGCGCATTCTCCAGCCACC
1561  TCCGTCACGCAAGCCCCACGTTCGCGTCGATCGAGCATCTTTCTAGCCTGCGTCAATCGGT
1621  GGAGGCGCAGGTACGCCATAGGACTCGTATTTCGAAATCGCCTGAAATTTATACAAAGGC
1681  AGCGCTTAGTTACTCCCGCCGCTGACGCAACCTCCGCCAGCGTAATCGCTTCCCGAAAGT
1741  TCTCGCGCATGAAGGCTTCCGCTTTTTGCACATGGAACGGCACGGCGCCCAAGCAACGTC
      SalI
1801  GACCTGATTCGGCCGAAGATAACGAATCATCAGGTCAACGATCAGATCGGCCATCCGCT
                SstI
1861  CGGACGTGCGCCGAGCTC  1877
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FIG. 2. Nucleotide sequence of the 1,877-bp *Bam*HI-*Sst*I fragment of pP51 containing *tcbR*. Also shown is the derived amino acid sequence for the largest open reading frame, designated *tcbR*. Putative consensus ribosome binding sites are indicated by double underlining. Nucleotides printed in italic type represent those of the divergently transcribed *tcbC* gene. Relevant restriction sites are indicated above the nucleotide sequence (nucleotides are printed in boldface type). Bases are numbered relative to the initial *Bam*HI site; amino acid residues are numbered relative to the putative start site of *tcbR*.

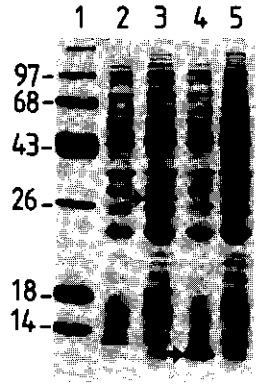

FIG. 3. Expression of the *tcbR* gene in *E. coli* BL21(DE3) cell extracts visualized on a Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gel. Lanes: 1, molecular weight markers (in kilodaltons); 2, *E. coli* BL21(DE3)(pET8c) plus IPTG; 3, *E. coli* BL21(DE3)(pTCB77) plus IPTG; 4, *E. coli* BL21(DE3)(pTCB77Δ) plus IPTG; 5, same as lane 3 but without IPTG. The arrows in lanes 3 and 4 indicate the 30-kDa *tcbR* gene product and its 12-kDa truncated form, respectively.

putida (18.2% in 176 amino acids [41, 52]). Very good homologies were found when the TcbR deduced primary sequence was compared with the amino acid sequences translated from parts of open reading frames from upstream regions of the *clcABD* gene cluster of *P. putida* (16) or the *tfdA* gene (46) and *tfdCDEF* gene cluster (18, 19, 32, 33) of *A. eutrophus*, which are also presumed to encode regulatory proteins (20, 24, 25, 32, 37) (76.3% [in 76 residues], 62.6% [in 155 residues], and 68.0% [in 47 residues], respectively).

Analysis of the activity of the *tcbR* gene product in *P. putida*. *P. putida* KT2442 harboring plasmid pTCB1, which contained the *tcbCDEF* gene cluster, had previously been found to be able to degrade 3-CB (50). We decided to use this strain to analyze the function of the *tcbR* gene product. Therefore, plasmids that carried either *tcbCDEF* and an intact *tcbR* gene (pTCB75 and pTCB76) or *tcbCDEF* and an inactivated *tcbR* gene (pTCB74) were constructed (Fig. 1B).

Table 1 shows the expression of catechol 1,2-dioxygenase activity obtained when *P. putida* strains harboring different constructs were grown on different media. To distinguish between endogenous catechol 1,2-dioxygenase activity and the activity of the *tcbC* gene product, we used both catechol and 3-CC as substrates in the enzyme assay (12, 50). Growth on succinate did not induce catechol 1,2-dioxygenase activity in *P. putida*(pKT230). However, *P. putida* harboring *tcbC* showed low catechol 1,2-dioxygenase activity when grown on succinate, which was highest when 3-CC was used as a substrate in the enzyme assay. Since 3-CC is not a substrate for the endogenous catechol 1,2-dioxygenase of *P. putida*, as observed with *P. putida*(pKT230), this result indicated that the activity was due to the expression of *tcbC*. Upon growth on aromatic substrates, such as benzoate or 3-CB, both catechol 1,2-dioxygenase activities were induced (Table 1). The expression of *tcbC* in *P. putida* containing wild-type genes (pTCB1, pTCB75, and pTCB76) was induced at least 3-fold by growth on benzoate and up to 17-fold by growth on 3-CB. The introduction of a mutation in *tcbR* in plasmid pTCB74 affected expression in *P. putida* as

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                    .          .     **  ** ***   **       .
TcbR   1 MEFRQLKY-----FIAVAEAGNMAAAAKRLHVSQPPITRQMQALEADLGV 45
TfdS   1 ......R.-----.V.A..E..VG...R...I....V...I....QH... 45
TfdX   1 ..I........-----.V......GFGT..Q.M.I....L...I....R.I.A 45
ClcR   1 ......R.-----.....E..IG...R...I.........I....Q.H.. 45
CatR   1 ..L.H.R.-----.KVL..TL.FTR..EL..IA...LS..ISQ..DQ..T 45
CatM   1 ..L.H.R.-----.VT.V.EQSISK..EK.CIA...LS..I.K..EE..I 45
NahR   1 ..L.D.DLNLLVV.NQLLVDRRVSIT.EN.GLT..AVSNALKR.RTS.QD 50
LysR   3 VNL.HIEI-----.H..MT..SLTE..HL..T...TVS.ELARF.KVI.L 48

                .         .         .         .         .
TcbR  46 VLLERSHRGIE-----------LTAAGHAFLEDARRILELAGRSGDRSRA  84
TfdS  46 L.F...A..VQ-----------..P..A........M...GRT.V.....  84
TfdX  46 K.
ClcR  46 ..F..T...V.---------------.....TT.......L.HVT
CatR  46 L.VV.-E.PLR------------..E..RF.Y.QSCTV.Q.Q-NIS.NT.R  82
CatM  46 Q.F..GF.PAK-----------V.E..MF.YQH.VQ..THTAQASSMAKR  84
NahR  51 P.FV.T.Q.M.PTPYAAHLAEPV.S.M..LRNALQHHESFDPLTSE.TFT 100
LysR  49 K.F..VRGRLH-----------P.VQ.LRLF.EVQ.SWYGLD.IVSAAES  87

                .         .         .         .         .
TcbR  85 AARGDVGELSVAYFGTPIYRSLPLLLRAFLTSTPTATVSLTHMTKDEQVE 134
TfdS  85 .S..EI.Q.DIG.L..A..QTV.A..H..TQAV.G..L..AL.P.VR.I. 134
CatR  83 IGQ.QRQW.GIGFAPSTL.KV..E.I.E-.RQDSELELG.NE..TLQ... 131
CatM  85 I.TVS-QT.RIG.VSSLL.GL..EIIYL.RQQN.EIHIE.IECGTKD.IN 133
NahR 101 L.MT.I..I---.--------M.R.MDVLAHQA.NCVI.TVRDSSMSLMQ 140
LysR  88 LREFRQ....I.CLPVFSQSF..Q..QP..ARY.DVSLNIVPQESPLLE. 137

                .         .         .         .         .
TcbR 135 GLLAGTIHVGFSRFFPRHPGIEIVNIAQEDLYLAVHRSQSG------KFG 178
TfdS 135 A.R.....L.VG..Y.QE..
CatR 132 A.KS.R.DIA.G.IRIDD.A.HQQVLCEDP.VAVLPKDHPL------A-S 174
CatM 134 A.KQ.K.DL..G.-LKTD.A.RRIVLHK.Q.K..I.KHHHL-----NQ.A 177
NahR 141 A.QN..VDLAVG.LL.NLQTGFFQRRLLQNH.VCLC.KDHPVTREPLTLE 189
LysR 138 W.S.QRHDL.LTETLHTPA.T.RTELLSL.EVCVLPPGHP------LAVK 181

                .         .         .         .         .
TcbR 179 -KTCKLADLR---------AVELTLFPRGGRPSFADEVIGLFKHAGIEPR 218
CatR 175 -SPLT..Q.A---------GEAFI.Y.ANP...Y..H.LA..A.H.MSIH 214
CatM 178 A.GVH.SQII---------DEPML.Y.VSQK.N..TFIQS..TEL.LV.S 218
NahR 190 -RF.SYGHV.VIAAGTGHGE.DTYMTRV.I.RDIRL..PHF---.AVGHI 235
LysR 182 -.VLTPD.FQ---------GENYISLS.TD--.YRQLLDQ..TEHQVKR. 219

                .         .         .         .         .
TcbR 219 IARVVEDATAALALTMAGAASSIVPASVAAIRWPDIAFARIVGTRVKVPI 268
CatR 215 VSQWANELQT.IG.VAV.VGVTL.....QQQHRT..EYVSLLDSGAVS.. 264
CatM 219 KLTEIAEIQL..G.VA..EGVC.....AWILG  end             249
NahR 236 LQ.TDLL..VPIR.ADCCVEPFGLS.LPHPVVL.E..INMFWHAKYHKDL 285
LysR 220 MIVETHS.ASVC.MVR..VGI.V.-NPLT.LDYAASGLV-VRRFSIA..F 267

                .         .
TcbR 269 SCTFRKEKQPPILARFVEHVRRSAKD end 294
CatR 265 ILSR..GDVS..VQ.CLTLIAQQAE end  289
NahR 288 ANIWLRQLMPDLFTD  end           300
LysR 268 TVSLIRPLHR.SSALVQAFSGHLQAGLPKL
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FIG. 4. FASTA alignment of the deduced amino acid sequences of proteins encoded by regulatory genes of aromatic catabolism within the LysR family of transcriptional regulator proteins relative to TcbR. The ClcR, TfdS, and TfdX sequences are partial sequences that were translated from nucleotide sequences of upstream regions of *clcABD* (16), *tfdA* (46), and *tfdCDEF* (32, 33), respectively, and their homology to LysR was observed previously (23). Symbols: ., identical amino acid residues; -, gaps needed for optimal alignment; *, strongly conserved residues involved in the helix-turn-helix N-terminal parts of the proteins. Numbers indicate the positions of the amino acid residues in the total sequence as well as the total lengths of the deduced proteins.

follows: growth on benzoate still resulted in the induction of *tcbC*-encoded catechol 1,2-dioxygenase activity, whereas growth on 3-CB no longer resulted in the elevated expression of *tcbC*, which was measured with 3-CC as a substrate. Surprisingly, *P. putida*(pTCB74) was still able to grow on 3-CB as the sole carbon and energy source, albeit at a much lower rate than *P. putida* harboring pTCB1, pTCB75, or pTCB76. This growth was probably the result of the residual expression of *tcbC*, also observed under noninducing conditions, such as growth on succinate. We conclude from these results that an active *tcbR* gene product is required for the induction of the expression of *tcbC* upon growth on a chlorinated compound such as 3-CB.

Determination of transcription start sites of *tcbC* and *tcbR*. The transcription start sites of *tcbC* and *tcbR* in *Pseudomonas* sp. strain P51 were determined by primer extension mapping of RNA isolated from strain P51 cultivated on either 1,2,4-TCB or succinate (Fig. 5). A single transcription start site was detected for *tcbC*. The extension product of *tcbC* was only observed with RNA isolated from cultures that had been grown on 1,2,4-TCB and not with RNA isolated from succinate-grown cultures. This result indicated that the transcription of *tcbC* is induced by growth on 1,2,4-TCB but not by growth on succinate. In contrast, when RNA was isolated from cultures grown on 1,2,4-TCB or succinate, the synthesis of an identical extension product specific for *tcbR* resulted. This result indicated that *tcbR* transcription is at least partially constitutive and initiated at the same start site. The start sites of the divergent transcripts were located in close vicinity on the opposite strands.

TABLE 1. Catechol 1,2-dioxygenase activities measured in cell extracts of *P. putida* KT2442 harboring different constructs containing *tcbCDEF* and *tcbR*

Plasmid	Growth substrate[a]	Catechol 1,2-dioxygenase activity[b] with the following substrate:	
		Catechol	3-CC
pKT230	SU	—	—
	BE	644	—
	3-CB	—	—
pTCB1	SU	3	10
	BE	382	48
	3-CB	185	170
pTCB75	SU	3	10
	BE	372	31
	3-CB	105	30
pTCB76	SU	8	20
	BE	500	65
	3-CB	142	69
pTCB74	SU	7	19
	BE	390	39
	3-CB	165	12

[a] SU, succinate; BE, benzoate.

[b] Nanomoles of muconate per milligram of protein per minute. —, not detectable.

DNA binding activity of the *tcbR* gene product. To determine whether the activation of *tcbC* gene expression was due to a direct interaction of the *tcbR* gene product with the mapped *tcbC* promoter region, we performed a series of DNA binding experiments. Cell extracts of *E. coli* BL21 (DE3) harboring pTCB77 and induced by IPTG and of *Pseudomonas* sp. strain P51 were tested for the ability to bind in an electrophoretic mobility shift assay to a 0.38-kb *Eco*RI-*Bam*HI fragment that contained the promoter regions of *tcbC* and *tcbR* (Fig. 1A). As a negative control, a 0.40-kb *Xho*I-*Hin*dIII fragment originating from the *tcbD* gene was used (Fig. 1A). Figure 6A shows a clear shift of the labeled *Eco*RI-*Bam*HI fragment incubated with a cell extract of *E. coli*(pTCB77), in which *tcbR* was expressed. This shift was not observed with the labeled *Xho*I-*Hin*dIII control fragment. Similarly, no retardation was observed with induced *E. coli* cell extracts harboring pET8c alone or pTCB77Δ, which expresses a truncated TcbR protein. The retardation of the *Eco*RI-*Bam*HI fragment by TcbR could be abolished

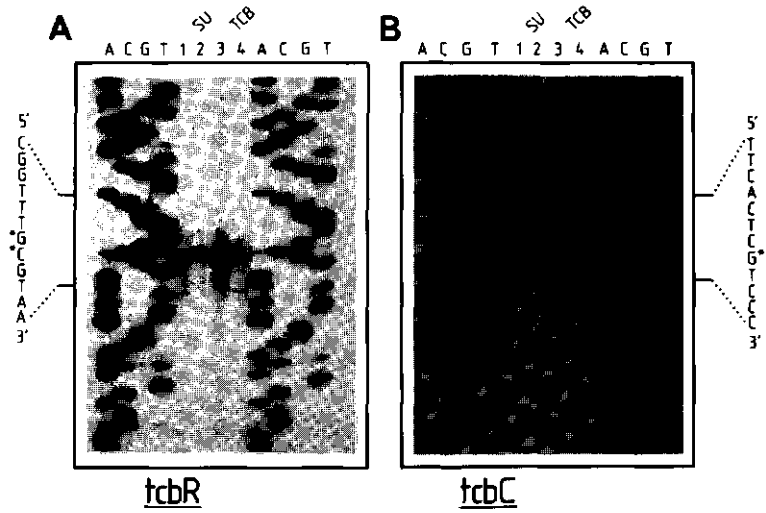

FIG. 5. Primer extension products of RNA transcribed from *tcbR* and *tcbC*. (A) Transcription start sites determined for *tcbR*. (B) Transcription start sites determined for *tcbC*. The relevant DNA sequences are indicated (5' to 3' direction), and the determined transcription start sites are marked by asterisks. Lanes: A, C, G, T, sequencing reactions carried out as described in Materials and Methods; SU, cells pregrown on succinate; TCB, cells cultivated on 1,2,4-TCB; 1, 30 μg of RNA; 2, 10 μg of RNA; 3, 30 μg of RNA; 4, 10 μg of RNA.

by adding 0.5 μg of unlabeled plasmid pTCB48 (50), which contains the cloned promoter region and the *tcbC* gene, but not by adding 0.5 μg of unlabeled plasmid pUC18. Preliminary purification of the TcbR DNA binding activity from cell extracts of *E. coli* BL21(DE3) harboring pTCB77 showed that the activity could be attributed to the 30-kDa protein (27). A similar specific retardation of the promoter fragment was observed with cell extracts of *Pseudomonas* sp. strain P51 (Fig. 6B), indicating that this strain contains DNA binding activity most probably caused by the *tcbR* gene product. From these results, we conclude that the TcbR protein binds specifically to a fragment containing the divergently located promoter regions of the *tcbC* and *tcbR* genes.

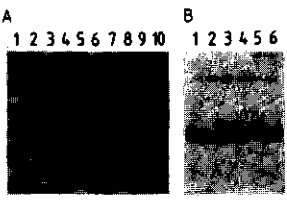

FIG. 6. Electrophoretic mobility shift assays of *tcbC* promoter fragments with cell extracts containing TcbR. (A) Assays performed with IPTG-induced cell extracts of *E. coli* BL21(DE3) containing various expression plasmids and the pTCB48 *Eco*RI-*Bam*HI promoter fragment (48EB) or with the pTCB48 *Xho*I-*Hin*dIII control fragment (48XH). Lanes: 1, 48EB, no extract added; 2, 48EB plus 0.5 μg of cell extract of *E. coli*(pTCB77); 3, 48EB plus 1.0 μg of cell extract of *E. coli*(pTCB77); 4, 48EB plus 2.0 μg of cell extract of *E. coli*(pTCB77); 5, 48EB plus 2.0 μg of cell extract of *E. coli* (pTCB77Δ); 6, 48EB plus 2.0 μg of cell extract of *E. coli*(pET8c); 7, 48XH, no extract added; 8, 48XH plus 2.0 μg of *E. coli*(pTCB77); 9, 48EB plus 1.0 μg of cell extract of *E. coli*(pTCB77) and 0.5 μg of pTCB48; 10, 48EB plus 1.0 μg of cell extract of *E. coli*(pTCB77) and 0.5 μg of pUC18. (B) Assays performed with *Pseudomonas* sp. strain P51 cell extracts and 48EB or with 48XH. Lanes: 1, 48EB, no extract added; 2, 48EB plus 15 μg of cell extract; 3, 48EB plus 30 μg of cell extract; 4, 48EB plus 45 μg of cell extract; 5, 48XH, no extract added; 6, 48XH plus 45 μg of cell extract.

DISCUSSION

In this report, we describe the cloning and characterization of the *Pseudomonas* sp. strain P51 *tcbR* gene, which encodes a regulatory protein involved in expression of the *tcbCDEF* chlorocatechol oxidative gene cluster. The functionality of the *tcbR* gene was confirmed by analysis of the inducible expression of catechol 1,2-dioxygenase encoded by *tcbC* in *P. putida*. Furthermore, the nucleotide sequence of the *tcbR* gene was determined and the deduced 294-amino-acid product of this gene was identified in overproducing *E. coli* as a protein with an apparent mass of 30 kDa.

The TcbR regulatory protein showed all the features of members of the LysR family of regulatory proteins (22, 23): considerable homology with LysR (21.5% identity in 279 amino acids [22]), a helix-turn-helix motif in the N-terminal part, divergent transcription from the gene cluster which is regulated, and specific binding to a DNA fragment containing the promoter region of this cluster. Although the complete sequences of the regulatory genes from chlorocatechol oxidative gene clusters have not been reported, the high homology (62.6 to 76.3%) of the deduced amino acid sequences of the N-terminal parts of ClcR (translated from part of the nucleotide sequence upstream of the *clcABD* operon [16]), TfdS (translated from part of the sequence upstream of *tfdA* [46]), and TfdX (translated from part of the sequence upstream of the *tfdCDEF* gene cluster [32, 33]) strongly suggests that these proteins constitute a subgroup within the

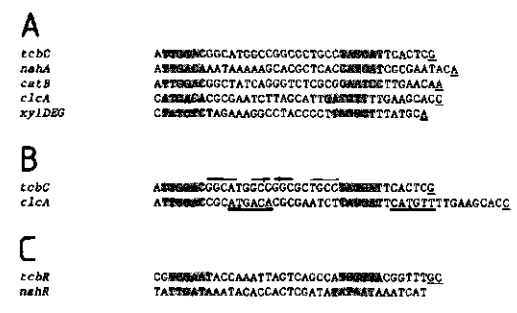

FIG. 7. (A) Alignment of promoter sequences of *Pseudomonas* catabolic genes transcribed by the σ^{70}-activated RNA polymerase (9). (B) Homology between the derived promoter sequence of *tcbC* and a region 9 nucleotides upstream of the deduced promoter sequence of *clcA* (16). The −35 and −10 regions of the *clcA* promoter (16) are double underlined. Arrows indicate a nonperfect inverted repeat observed within the −35 and −10 regions of the *tcbC* promoter. (C) Comparison of the *tcbR* promoter sequences with *Pseudomonas* constitutive promoter sequences (9). Derived transcription start sites are singly underlined, and −35 and −10 regions are shaded.

LysR family. Except for TfdS, which was reported to regulate *tfdB* expression (25), no clear role or function was ascribed to the other two putative proteins. Like TcbR, they could be involved in the regulation of the divergently transcribed gene clusters which start in their immediate vicinity, e.g., *clcABD* and *tfdCDEF*. This possibility, however, is not in agreement with the suggestion that the *tfdCDEF* gene cluster is regulated by the *tfdR* gene, which has been localized elsewhere on plasmid pJP4 (20, 24). Preliminary studies have shown that cell extracts of *E. coli* overproducing TcbR also retard DNA fragments that contain regions immediately upstream of *tfdCDEF* (27), suggesting similar mechanisms of regulation of the chlorocatechol oxidative operons. This possibility would reinforce the close similarity that was also observed for the genetic organization and DNA sequence of these clusters (18, 19, 33, 48).

Among the regulatory proteins of the LysR family, both negative and positive regulators are found. CatM was shown to be a negative regulator (29), as was TfdS (25), whereas NahR (41, 42, 52), CatR (37), and LysR (22) belong to the class of positive regulators. Our results suggest that TcbR is a positive regulator, since in its presence *tcbC*-encoded catechol 1,2-dioxygenase activity was much higher in *P. putida* KT2442 (Table 1). However, low constitutive expression of *tcbC* was observed in the absence of induction in both *P. putida* and *Pseudomonas* sp. strain P51 (50). Analysis of the *tcbR-tcbCDEF* regulatory system is hindered by several factors. In *E. coli*, transcription from *Pseudomonas* promoters by *E. coli* RNA polymerases was found to be inefficient (48, 50). In *P. putida* KT2442 grown on benzoate, the role of the *tcbR* gene product can probably be taken over by the product of the *catR* gene, which regulates the expression of the chromosomal *catA* gene. Although our results (Table 1) showed that in *P. putida* containing the *tcbCDEF* genes *catA* expression can be induced by CatR even upon growth on 3-CB, they also showed that CatR probably is not able to activate the transcription of *tcbC* upon growth on 3-CB. It is very likely that 3-CB itself is not the inducing compound for the *tcbCDEF*-encoded pathway, since 3-CB is not a substrate of the wild-type strain P51 (50). The inducer may be 2-chloromuconic acid, similar to the situation in the *catA*- and *catBC*-encoded pathways of *P. putida* and *A. calcoaceticus*, in which muconate is the inducer (28, 29, 37).

Mapping of the transcription start sites of *tcbC* and *tcbR* in *Pseudomonas* sp. strain P51 allowed us to identify their promoter sequences (Fig. 7 and 8). Both promoter sequences were located on opposite strands and partially overlapped (Fig. 8). The operon organization of the *tcbCDEF* gene cluster, as determined previously (48), suggests that these genes are transcribed from the same, *tcbC*, promoter. The sequence of the promoter region of *tcbC* could be aligned with several promoter region sequences derived for catabolic genes in *Pseudomonas* spp. (Fig. 7) (9). It showed good homology with the *nahA* (40) and *catB* (4) promoter region sequences with respect to the −35 box TTGGAC recognized by the σ^{70}-activated RNA polymerase in *Pseudomonas* spp. (9). The −10 box was more variable among the promoter region sequences, but the sequence TAGCAT of the *tcbC* promoter resembled that of the promoters of both *nahA* and *catB*. A striking difference was found when the *tcbC* promoter region was compared with the reported *clcA* promoter sequence (16). However, we found that the nucleotide sequences of both regions were highly conserved (Fig. 7B) and that the *clcA* region contained sequences identical to the −35 and −10 boxes of *tcbC*. The different start site that was reported for *clcA* (16) could have been due to the different and indirect method that was used in its determination. The promoter region of *tcbR* partially overlapped that of *tcbC*, which was located on the opposite strand. The −35 box TCCAAT and the −10 box TCGTTA of the *tcbR* promoter showed, however, less homology with the consensus promoter sequences described for constitutive *Pseudomonas* promoters (Fig. 7C) (9).

Analogous to other systems, the ability of the TcbR protein to activate the transcription of *tcbC* would depend on

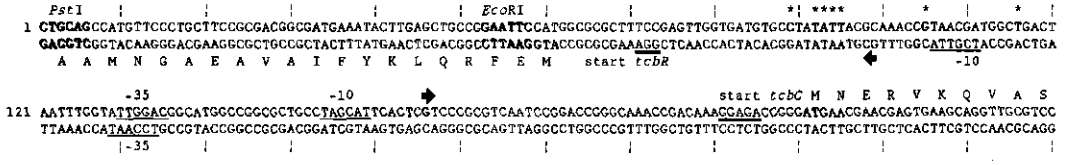

FIG. 8. Overview of the promoter-operator regions of *tcbR* and the *tcbCDEF* gene cluster. The relevant nucleotide sequences of the regions are shown, as are the proposed start sites of the *tcbR*- and *tcbC*-encoded proteins. Potential ribosome binding sites are doubly underlined, and the −35 and −10 regions of the derived promoter sequences are singly underlined. Arrows indicate transcription start sites and directions of transcription; *, nucleotides homologous to regions protected by NahR and NodD from DNase I digestion (14, 41). Relevant restriction sites are indicated as explained in the legend to Fig. 2.

the binding of this protein to a specific target site upstream from the promoter of *tcbC* and after interaction with a suitable inducer, to form an open transcription complex (17). In fact, the TcbR protein binds specifically with a region containing the promoters of *tcbC* and *tcbR*. Comparison of this region with the binding sites of the NahR and NodD proteins (14, 41) revealed a number of consensus nucleotides (Fig. 8) which are known to be involved in the binding of those regulatory proteins.

Further analysis of the specific specialized features of TcbR and the *tcbR-tcbCDEF* regulatory circuit, which arose in response to environmental stress with chlorinated benzenes, may provide more insight into the importance of the fine-tuning of regulatory systems as a means of adaptation to novel substrates.

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Chapter 6

Identification of a novel composite transposable element, Tn5280, carrying chlorobenzene dioxygenase genes of Pseudomonas sp. strain P51

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Identification of a Novel Composite Transposable Element, Tn*5280*, Carrying Chlorobenzene Dioxygenase Genes of *Pseudomonas* sp. Strain P51

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Analysis of one of the regions of catabolic plasmid pP51 which encode chlorobenzene metabolism of *Pseudomonas* sp. strain P51 revealed that the *tcbA* and *tcbB* genes for chlorobenzene dioxygenase and dehydrogenase are located on a transposable element, Tn*5280*. Tn*5280* showed the features of a composite bacterial transposon with iso-insertion elements (IS*1066* and IS*1067*) at each end of the transposon oriented in an inverted position. When a 12-kb *Hin*dIII fragment of pP51 containing Tn*5280* was cloned in the suicide donor plasmid pSUP202, marked with a kanamycin resistance gene, and introduced into *Pseudomonas putida* KT2442, Tn*5280* was found to transpose into the genome at random and in single copy. The insertion elements IS*1066* and IS*1067* differed in a single base pair located in the inner inverted repeat and were found to be highly homologous to a class of repetitive elements of *Bradyrhizobium japonicum* and distantly related to IS*630* of *Shigella sonnei*. The presence of the catabolic genes *tcbA* and *tcbB* on Tn*5280* suggests a mechanism by which gene clusters can be mobilized as gene cassettes and joined with others to form novel catabolic pathways.

Chlorinated aromatic compounds have until now been considered one of the most problematic categories of environmental pollutants which are nondegradable or slowly degradable by microorganisms (36). Nevertheless, bacteria that partially transform chlorinated aromatic compounds, such as biphenyls or other polyaromatic compounds, and even completely mineralize chlorinated benzoates and benzenes (36, 38, 44) have been isolated. Metabolism of this last class of compounds is particularly interesting, because it requires a specific combination of existing pathways, which each have their limitations with respect to chlorobenzene degradation (31). The initial attack of the aromatic ring is carried out in most cases by dioxygenase multienzyme complexes and (substituted) catechols are formed as central pathway intermediates (36). Chlorinated catechols which arise as intermediates in chlorobenzene degradation can then be successfully metabolized only via a modified *ortho* cleavage or chlorocatechol oxidative pathway (36). The *meta* cleavage route will in this case lead to dead-end products (2, 25). However, in microorganisms such as *Pseudomonas putida* F1 (15, 16, 56) that were shown to be able to oxidize chlorobenzenes, *meta* cleavage follows the initial conversion by the dioxygenase complex. The modified *ortho* cleavage pathway is the central degradation sequence used by microorganisms that utilize 2,4-dichlorophenoxyacetic acid and 3-chlorobenzoate (8, 9). However, these organisms fail to perform the initial oxidation of chlorobenzenes (31, 36).

Pseudomonas sp. strain P51 was isolated from river sediments and is able to use 1,2-dichloro-, 1,4-dichloro-, and 1,2,4-trichlorobenzene, as the sole carbon and energy source (51). Strain P51 was found to harbor catabolic plasmid pP51 of 110 kb (52). With the use of expression studies and deletion analysis, two regions of pP51 that contain the genetic information for the metabolism of chlorobenzenes have been identified and characterized (49, 52) (Fig. 1). One region was found to contain a gene cluster, *tcbCDEF*, which encoded a modified *ortho* cleavage pathway (49, 52). The organization of the *tcbCDEF* gene cluster was found to be very similar to that of the *tfdCDEF* operon of *Alcaligenes eutrophus* JMP134 (8, 14, 34) and the *clcABD* operon of *P. putida* (11). The other region of pP51 contained the *tcbAB* gene cluster which encoded a chlorobenzene dioxygenase complex and a dehydrogenase, catalyzing the conversion of chlorobenzenes to chlorocatechols (52). This gene cluster exhibited size, organization, and function characteristics similar to those of other known bacterial dioxygenases, such as *todC1C2BA* from *P. putida* F1 (16, 56), *bphAB* (12, 23, 30), or *xylXYZ* (20). We were interested in analyzing the mechanisms that could give rise to bacterial strains capable of metabolizing chlorobenzenes, such as *Pseudomonas* sp. strain P51, in order to allow a better understanding of the evolution of new pathways in bacteria. This article describes the transposable nature of the *tcbAB* gene cluster of *Pseudomonas* sp. strain P51, and our results support a mechanism for evolving novel, functional metabolic routes by cassettelike combination of existing pathways.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Escherichia coli* DH5α [*supE44* Δ*lacU169* (ϕ80 *lacZΔM15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*] and TG1 [*supE hsdΔ5 thi* Δ(*lac-proAB*) F′ (*traD36 proAB*$^+$ *lacI*^{q} *lacZΔM15*)] (37) were routinely used for cloning experiments with plasmids and M13 phages, respectively. *E. coli* S17-1 (41) was used as the donor strain for mobilization of pSUP202-derived plasmids. *Pseudomonas putida* KT2442 (*hsdR1 hsdM*$^+$) (10) was the recipient strain in mating experiments with *E. coli* S17-1 and was used to study transposition events. The strain is a rifampin-resistant (Rif^r) plasmid-free derivative of strain *P. putida* mt-2. Plasmids pUC18 and pUC19 (55) were used as general

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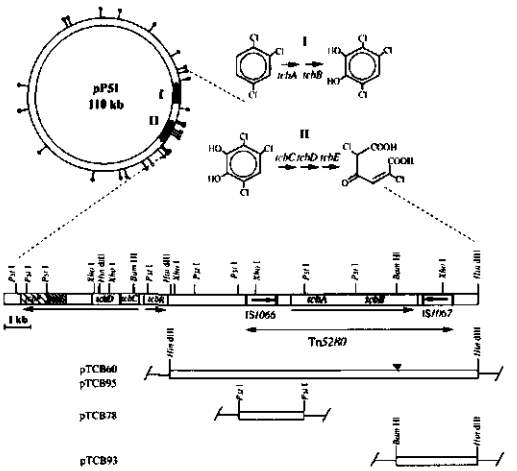

FIG. 1. Physical maps of the two gene clusters and Tn*5280* on plasmid pP51 encoding chlorobenzene metabolism and of relevant clones used in this study. The map of pP51 is shown for restriction enzymes *Xho*I (♀) and *Hin*dIII (♀). The sizes of the relevant gene clusters and their directions of transcription are shown in the physical map of pP51 as solid arrows and below the detailed physical map. The upper pathway (pathway I) genes *tcbA* and *tcbB* mediate the conversion of 1,2,4-trichlorobenzene to 3,4,6-trichlorocatechol; the lower pathway (pathway II) genes encode metabolism of 3,4,6-trichlorocatechol to 2,5-dichloromaleylacetic acid (52). Detailed analysis of the genes and gene products of this region showed that it was composed of at least three structural genes: *tcbC*, encoding chlorocatechol 1,2-dioxygenase; *tcbD*, encoding chloromuconate cycloisomerase, and *tcbE*, encoding dienelactone hydrolase. The function of *tcbF* has not yet been revealed (49, 52). The *tcbR* gene was found to be involved in regulation of the chlorocatechol oxidative pathway (50). The solid triangle indicates the position at which the Km^r gene cassette has been inserted in pTCB95R3.

cloning vectors. For DNA sequencing, we applied M13mp18 and M13mp19 (55). Plasmid pSUP202 (41) is a pBR325-derived vector carrying the *mob* genes of plasmid RP4. It can be mobilized to other gram-negative bacteria by the transfer system of *E. coli* S17-1, but it does not replicate in *P. putida*. The plasmid was used to deliver Tn*5280* transposon constructs in *P. putida* KT2442. pTCB60 contains Tn*5280* and the *tcbAB* gene cluster on a 12-kb *Hin*dIII fragment of catabolic plasmid pP51 of *Pseudomonas* sp. strain P51 in pUC18 (52) (Fig. 1). pTCB78 contains a 2.8-kb *Pst*I fragment harboring IS*1066*, and pTCB93 contains a 3.2-kb *Bam*HI-*Hin*dIII fragment with IS*1067*. Plasmid pTCB95 was constructed by cloning the 12-kb *Hin*dIII pP51 DNA fragment of pTCB60 in pSUP202. This plasmid was then partially digested with *Bam*HI and ligated with a DNA fragment containing the kanamycin resistance (Km^r) gene of Tn*903* (17), derived from plasmid pRME1 (19), with *Bam*HI ends. Transformants of *E. coli* were subsequently analyzed for their plasmid content, and a plasmid carrying the Km^r gene in the *Bam*HI site on the *tcbB* locus was used (pTCB95R3) (Fig. 1). Plasmid pSUP202::Km was created by cloning the Km^r gene into the unique *Bam*HI site present on pSUP202.

Media and culture conditions. *E. coli* strains were cultivated at 37°C on LB (37), whereas *P. putida* strains were grown at 30°C in the same medium. For mating experiments, *E. coli* S17-1 and *P. putida* KT2442 strains were diluted 20-fold from cultures grown overnight into fresh medium, to which the relevant antibiotics were added, and grown to an A_{620} of approximately 1.0. Strains were then mixed in a 1:1 ratio, filtered over a 0.2-μm-pore-size nitrocellulose filter, washed with 5 ml of sterile LB medium, and incubated on the surface of an LB agar plate for 12 h at 30°C. After this incubation time, the bacteria were washed from the filter, plated onto selective medium containing rifampin and kanamycin, and incubated for at least 18 h at 30°C after which transconjugants were screened. Antibiotics were added as follows: ampicillin, 50 μg/ml; kanamycin, 50 μg/ml; and rifampin, 50 μg/ml. When necessary, the medium was supplemented with 0.004% 5-bromo-4-chloro-3-indolyl-β-D-galactoside and 1.0 mM isopropyl-β-D-thiogalactopyranoside.

DNA techniques and sequence analysis. Plasmid DNA isolations, transformations, and other DNA techniques were carried out as described elsewhere (49, 52) or by established procedures (37). Total DNA was isolated from 1.5-ml cultures of *P. putida* transconjugants by the procedure of Marmur (28). DNA sequencing was performed by cloning appropriate DNA fragments into M13 phages and carrying out sequencing reactions by a modified version (49) of the dideoxy-chain termination method of Sanger et al. (39). Computer analysis and processing of sequence information were done using the program PC/GENE (Genofit, Geneva, Switzerland) and the GCG package (7). Restriction enzymes and other DNA-modifying enzymes were obtained from Life Technologies Inc. (Gaithersburg, Md.) or Pharmacia LKB Biotechnology (Uppsala, Sweden).

Nucleotide accession number. The nucleotide sequence presented in this article was deposited at GenBank under accession number M61114.

RESULTS

Identification and characterization of IS*1066* and IS*1067*. Analysis of the start of the *tcbA* chlorobenzene dioxygenase gene cluster of plasmid pP51 by DNA sequencing revealed not only the beginning of the gene encoding the large iron sulfur protein of the dioxygenase complex (48) but also a large upstream open reading frame (ORF) (Fig. 2). This ORF and its flanking regions were compared with sequences in the GenBank data base and found to show considerable homology with presumed repetitive or insertion elements (see below). The 1,068-bp ORF was found to be flanked by short inverted repeats of 13 bp (Fig. 2), giving the entire element within these borders a size of 1,142 bp, which was tentatively designated IS*1066*. When translated, the ORF could encode a polypeptide of 355 amino acids, with a calculated molecular mass of 39.7 kDa. We were interested to see whether more copies of IS*1066* occurred on plasmid pP51, so we hybridized restriction fragments of pP51 generated with *Hin*dIII and *Xho*I with a ^{32}P-labelled 0.7-kb *Xho*I-*Bgl*II fragment of IS*1066*, derived from plasmid pTCB78. This revealed a single hybridizing band with the 12-kb *Hin*dIII and 7.5-kb *Xho*I fragments of pP51, containing the *tcbAB* cluster (results not shown). However, fine mapping within the 12-kb *Hin*dIII fragment using hybridization with the same DNA probe showed that another copy of IS*1066* was present on the right side of the presumed *tcbB* locus (Fig. 3) (52). The DNA region that hybridized with the IS*1066* probe was located on the 3.2-kb *Bam*HI-*Hin*dIII fragment of pTCB60. This region was further analyzed by DNA sequencing and found to contain an iso-IS*1066* copy, designated IS*1067*. The sequence of this copy was identical to IS*1066*

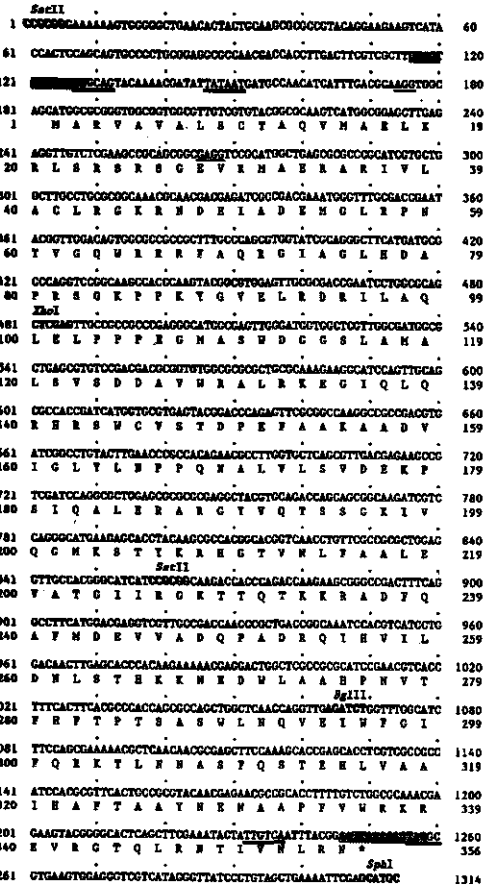

FIG. 2. Nucleotide sequence of a 1,315-bp *Sst*II-*Sph*I fragment of pTCB78 containing IS*1066*. The figure also shows the predicted amino acid sequence of the major ORF from IS*1066* of 356 residues. The single-base-pair difference (C → A) of IS*1067* with IS*1066* occurred at position 1255 in the nucleotide sequence of IS*1066*. Putative ribosome binding sites are underlined in the DNA sequence. The inverted repeats which are proposed to be the border sequences of IS*1066* are shaded. Sequences that show homology to consensus −35 and −10 promoter regions in *Pseudomonas* species (6), are underlined with a thick line. Relevant restriction sites are presented above the nucleotide sequence. Bases are numbered relative to the initial *Sst*II site; amino acid residues are numbered relative to the putative start of the major ORF of IS*1066*.

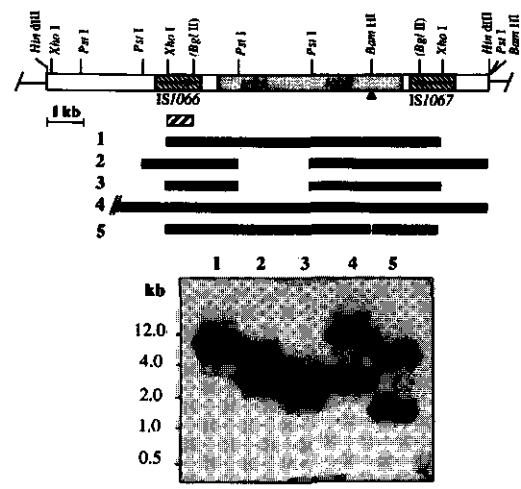

FIG. 3. Detection of IS copies flanking the *tcbAB* gene cluster of pP51. The 12-kb *Hin*dIII fragment of pTCB60 is shown here with the locations of relevant restriction sites. The locations and sizes of IS*1066* and IS*1067*, as determined by DNA sequencing, are indicated in the physical map of pTCB60. The directions and sizes of the large ORFs of IS*1066* and IS*1067* are given by the arrow inside the bar. The lower part of the figure shows the autoradiogram of hybridization of plasmid pTCB60 with a ^{32}P-labeled 0.7-kb *Xho*I-*Bgl*II fragment derived from IS*1066* (hatched box below the physical map). Plasmid pTCB60 was digested with *Xho*I (lane 1), *Pst*I (lane 2), *Xho*I and *Pst*I (lane 3), *Bam*HI (lane 4), or *Xho*I and *Bam*HI (lane 5). Hybridizing fragments of pTCB60 are shown in the middle part of the figure as solid bars under the physical map of the 12-kb *Hin*dIII fragment. The solid triangle in the physical map indicates the position at which the Km^r gene has been inserted into pTCB95R3.

except for a 1-bp difference in the inner repeat (Fig. 2), and it showed that the orientation of IS*1067* was opposite to IS*1066*. The architecture of the *tcbAB* gene cluster and the flanking insertion elements (ISs) resembled thus the organization of a bacterial composite transposon (18).

Functionality of transposon Tn*5280*. In subsequent experiments, we established the functionality of the transposon-like structure, designated Tn*5280*. For this purpose, the 12-kb *Hin*dIII fragment of pP51 DNA containing Tn*5280* was cloned into the suicide donor vector pSUP202 (41) and marked with a Km^r gene (resulting in plasmid pTCB95R3) (Fig. 4A). This plasmid was mobilized to *P. putida* KT2442, by first transforming it into *E. coli* S17-1 (41) and then mating this strain with *P. putida* KT2442. After 12-h mating time, the cell mixtures were plated onto agar medium containing rifampin (which counterselected for the *E. coli* donor strain) and kanamycin (which selected for possible *P. putida* transconjugants that had acquired the resistance in their genome, since the plasmid itself cannot replicate in *Pseudomonas* species [41]). Using this approach, we detected kanamycin-resistant *P. putida* transconjugants, whereas no significant resistance was observed in control experiments (Table 1).

From a number of these *P. putida* transconjugants, total DNA was isolated and analyzed by DNA hybridization to determine the location of Tn*5280*. About half of the transconjugants with acquired kanamycin resistance showed actual integration of Tn*5280* (Fig. 4), whereas the others presumably carried a fully integrated plasmid pTCB95R3 (results not shown). Digestion of total DNA of the transconjugants with integrated Tn*5280* with *Xho*I showed similar-sized DNA fragments that hybridized with the 0.7-kb *Xho*I-*Bgl*II fragment of IS*1066* (Fig. 4B). This was to be expected, since *Xho*I cleaves at two sites within Tn*5280* and one site within the inserted Km^r gene and hybridization was thus to two internal fragments. In contrast, *Hin*dIII cleaves at only one site in the transposon on pTCB95R3 within the inserted Km^r gene. Hybridization of *Hin*dIII-digested DNA with the

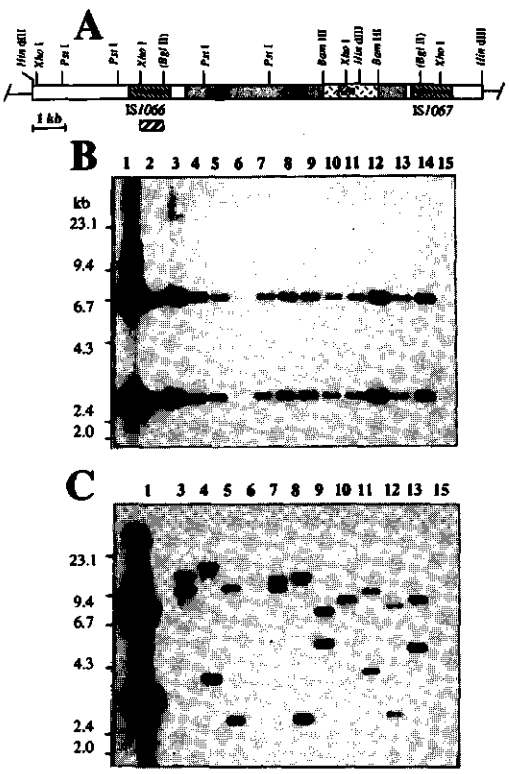

FIG. 4. Analysis of *P. putida* KT2442 transconjugants containing Tn*5280*. (A) Physical map of plasmid pTCB95R3 which contains the Km^r gene inserted into Tn*5280*. (B and C) Autoradiograms of total DNA and plasmid control DNA, digested with *Xho*I (B) or *Hin*dIII (C), and hybridized with the ^{32}P-labelled 0.7-kb *Xho*I-*Bgl*II fragment from IS*1066* (hatched bar below the physical map in Fig. 4A). Plasmid pTCB95R3 DNA (lanes 1) and total DNAs from different *P. putida* KT2442 transconjugants with Tn*5280* insertions (lanes 2 to 14) and from *P. putida* KT2442, the parent strain (lanes 15) are shown.

same IS*1066* probe yielded two fragments which varied in size in different strains, indicating insertion of Tn*5280* in different sites of the genome (Fig. 4C). This analysis further showed that only a single copy of Tn*5280* had been integrated into the genome in each case. Hybridization with DNA probes containing fragments of the *tcbAB* region or with DNA fragments containing the Km^r gene gave the same results as those obtained with the IS*1066* probe (not shown). No hybridization was detected with the vector pSUP202 (not shown). These results led us to the conclusion that Tn*5280* introduced on a suicide delivery system is able to transpose in *P. putida* KT2442 in single copy and apparently inserts randomly.

TABLE 1. Frequencies of kanamycin and rifampin resistance obtained by mating *E. coli* S17-1 harboring different suicide constructs and *P. putida* KT2442

Strain	Frequency of resistance[a]
P. putida KT2442 (control)	$<10^{-9}$
E. coli S17-1(pTCB95R3) (control)	$<10^{-8}$
E. coli S17-1(pSUP202::Km)	$<10^{-8}$
P. putida KT2442 + *E. coli* S17-1(pTCB95R3)	10^{-6}–10^{-7}
P. putida KT2442 + *E. coli* S17-1(pSUP202::Km)	$<10^{-9}$

[a] Frequencies are given per (recipient) cell, and the values were similar in three independent experiments.

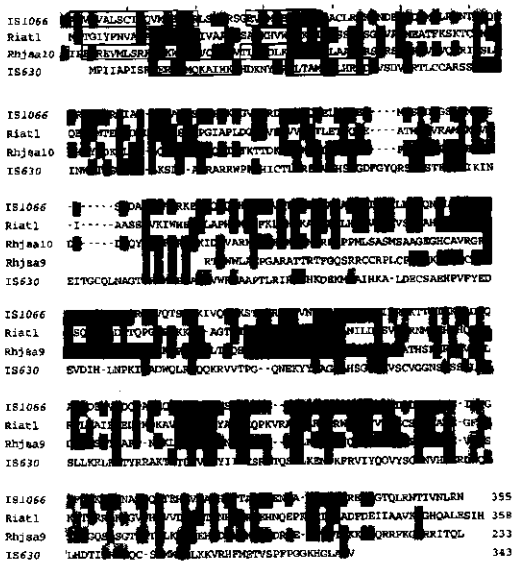

FIG. 5. FASTA alignment (33) of the deduced amino acid sequences of putative proteins encoded by the major ORF of the ISs and repetitive elements IS*1066*, Riatl (43), Rhjsα9 and Rhjsα10 (24), and IS*630* (29). Identical residues in two or more proteins are shaded. The N-terminal region of the polypeptides that was predicted to contain a helix-turn-helix motif by using the algorithms of the GCG program (7) is boxed in the sequence. Rhjsα10 presents the part of the translated first ORF of these repetitive elements that was homologous to the ORF of IS*1066*, and Rhjsα9 contains part of the second ORF. Gaps introduced in the sequence for optimal alignment are indicated by hyphens.

Homology of IS*1066* with other insertion elements. A TFASTA search (33) of the putative gene product of the large ORF of IS*1066* with sequences present in the GenBank catalogue revealed homology with one other IS (IS*630* from *Shigella sonnei*) (29), with a class of repetitive elements from *Bradyrhizobium japonicum* (24), and with an uncharacterized ORF on the integrated Ri plasmid of *Agrobacterium rhizogenes* (43) (Fig. 5). The homology of IS*1066* was highest with the Rhjsα elements of *B. japonicum* (61.5% DNA sequence identity in 1,010 bp). These elements, however, do not encode one large ORF but two smaller overlapping ORFs (24). Percentages of identity of IS*1066* with these ORFs on the amino acid level were 44.7% with ORF1 (in 141 amino acids overlap) and 55.4 to 57.7% with ORF2 (in 182 residues overlap). The ORF from *A. rhizogenes* could encode a protein of 355 residues, and its identity to IS*1066* was calculated to be 36.4% in 338 amino acids overlap (53.1% DNA sequence identity in 727 bp). All these three types

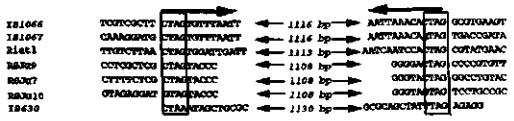

FIG. 6. Comparison of sizes and border sequences of a class of repetitive elements and ISs homologous to IS*1066* and IS*1067*. The sizes of the inverted repeats differ from 9 to 14 bp. The boxed sequence indicates the duplicated CTAG found in some of the sequences. The single-base-pair difference between IS*1066* and IS*1067* is underlined in the inverted repeat of IS*1067*. Riatl, *A. rhizogenes* (43); RSJα9, RSJα7 and RSJα10, *B. japonicum* (24); IS*630*, *S. sonnei* (29).

were slightly related to IS*630* (identity of IS*1066* with IS*630* was 21.8% in 312 amino acids overlap). The putative proteins of all these ORFs were calculated to have an isoelectric point of between 10.6 and 11.6, which is a characteristic of IS-encoded transposases (13). Further, secondary structure predictions suggested a helix-turn-helix motif for all putative proteins between residues 30 and 60 (Fig. 5), which could be involved in DNA binding and recognition. The sizes of the elements and their border sequences were similar (Fig. 6). The border sequences of the elements consisted of inverted repeats of between 9 and 14 bp in size. A possible target site duplication of 4 bp due to transposition with the sequence CTAG could be observed in some of the inverted repeats which, however, could also be part of the inverted repeat itself.

DISCUSSION

This report describes the initial characterization of a novel bacterial transposable element, Tn*5280*, which occurs on catabolic plasmid pP51 of *Pseudomonas* sp. strain P51. Our results show that Tn*5280* is a composite transposon, formed by two isocopies of an IS (IS*1066*) which are oriented in opposite directions. These elements, IS*1066* and its twin with a single-base-pair difference (IS*1067*), flank the complete *tcbAB* gene cluster on pP51 which encodes chlorobenzene dioxygenase and dehydrogenase (52). We showed by analyzing transposition events in *P. putida* KT2442 that Tn*5280* is a functional transposon, transposing in single copy and inserting randomly into the genome.

The similarity of the ISs of Tn*5280* with other repetitive elements or ISs such as those from *B. japonicum* (24), *A. rhizogenes* (43), and *S. sonnei* (29) suggests that this class of elements can be found in different bacterial species. The large ORF present in IS*1066* and related elements could encode a putative transposase, with features similar to other IS-encoded tranposases such as isoelectric point and helix-turn-helix motif (13). The DNA sequence of IS*1066* upstream of the putative start codon at position 184 further showed a possible ribosome binding site and promoter sequences (Fig. 2) (6). The end of IS*1066* also contained a sequence homologous to the −35 promoter box TTGACA, facing outward (Fig. 2), which is a characteristic of other bacterial ISs as well (13).

Transposable elements and ISs are commonly found in bacteria and are known to contribute to the spread of genes and to trigger other genetic events by their insertion, such as activation or inactivation of gene expression (1, 18). In particular, composite transposons, such as Tn*5* (3) and Tn*10* (26), are examples of mobile genetic elements that promote the spread of DNA (in this case, of antibiotic resistance genes) that is captured between the ISs. In catabolic pathways, transposable elements are presumed to play a role in generating rearrangements of existing routes (4, 5, 54). Probably the best-characterized transposable elements and catabolic pathways are the TOL transposons, Tn*4361* and Tn*4362* (46, 47), which belong to the Tn*3* family and contain the *xyl* operons which encode xylene and/or toluene metabolism. Studies of different TOL plasmids have shown that various combinations of the individual *xyl* operons are found, as well as duplications, which may be caused by the activity of these transposable elements or by general recombination events (32). In addition, the dehalogenase genes of *P. putida* PP3 were discovered to be located on transposable elements (42). Finally, ISs were presumed to play a role in the development of the catabolic pathway for 2,4,5-trichlorophenoxyacetic acid (45), and two functional ISs, *IS*931 and *IS*932, that were able to activate gene expression of adjacent genes upon insertion and to mobilize adjacent DNA sequences were characterized (21, 22).

The characterization of the transposable nature of the *tcbAB* gene cluster with its ISs, IS*1066* and IS*1067*, is to our knowledge the first documented example of the functionality of a composite transposon carrying catabolic genes which code for degradation of aromatic compounds. The discovery of the mobile nature of the *tcbA* and *tcbB* genes may help to propose a mechanism by which the pathway for chlorobenzene degradation has evolved in *Pseudomonas* sp. strain P51. Most existing routes of aromatic metabolism in bacteria will not be able to successfully catalyze the complete conversion of chlorobenzenes but will either direct metabolites, such as chlorinated catechols into dead-end pathways (2, 25), or fail to perform the initial oxidation of the chlorinated substrate (35). This has led research groups to efforts to combine two pathways operating in different strains into a single progenitor, by using mating techniques to transfer one pathway, encoded on a catabolic plasmid, from one strain to a strain with the other pathway. In this manner, new strains that completely mineralized 1,4-dichlorobenzene and monochlorobenzene have been constructed, starting with strains that carry a toluene degradation pathway, such as *P. putida* F1 (15, 16, 56), or *P. putida* R5-3 (27) and strains that use 3-chlorobenzoate or benzoate as the sole carbon and energy source, such as *Pseudomonas* sp. strain B13 (9, 31) or *P. alcaligenes* C-0 (27). The transconjugants in these experiments were all found to use the modified *ortho* cleavage route to metabolize chlorinated catechols, although they still contained detectable catechol 2,3-dioxygenase activity (27, 31). Our findings with *Pseudomonas* sp. strain P51 suggest that in this strain two separate pathways were combined as well. The chlorocatechol oxidative pathway is present in this strain, encoded by the *tcbCDEF* gene cluster that is located on plasmid pP51 (49, 52). The initial conversion of chlorobenzenes to chlorocatechols is performed by the enzymes encoded by the *tcbAB* gene cluster (52) which are very much akin to other dioxygenases, such as those encoded by the *todC1C2BA* (16, 56), *xylXYZ* (20), or *bphAB* genes (12, 23, 30). The *tcbAB* gene cluster of pP51 forms part of a composite transposon, which suggests that it was recruited on a catabolic plasmid that already contained the genes for the chlorocatechol oxidative pathway. The combination of the two gene clusters on a single plasmid may have been necessary for a proper regulation of the two pathways. However, preliminary DNA binding experiments with the regulator of the chlorocatechol oxidative pathway, TcbR (50), and upstream regions of *tcbA* did not indicate any interaction (48), which suggests that both operons are not

regulated by a single regulatory gene. Furthermore, cultures of *Pseudomonas* sp. strain P51 or other chlorobenzene-degrading strains such as *Alcaligenes* sp. strain A175 (40) growing on chlorinated benzenes, showed in certain cases for unknown reasons excretion of chlorinated intermediates resulting in cell death (53), which suggests misrouting and improper regulation of the two different pathways. This misrouting could be caused by the presence of a functional gene for a catechol 2,3-dioxygenase which could be a remnant of the toluene dioxygenase pathway (15, 56), although its activity has so far not been detected in *Pseudomonas* sp. strain P51 (53).

The recruitment of the *tcbAB* gene cluster in Tn*5280* raises the interesting question of how the ISs became inserted near a dioxygenase gene cluster, e.g., present on the chromosome as in the case of the *todC1C2BA* genes (16, 56), thereby creating a transposable element. The transposon could subsequently have been acquired by a passing mobilizable catabolic plasmid that already contained a chlorocatechol oxidative gene cluster. If so, it seems as though nature's patchwork assembly has worked in a similar manner to the genetic engineering strategies we have developed at our desks and laboratory benches.

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Chapter 7

Use of genetic markers from chloroaromatic metabolism in studying the distribution and variation of catabolic genes among soil microorganisms

Use of Genetic Markers from Chloroaromatic Metabolism in Studying the Distribution and Variation of Catabolic Genes among Soil Microorganisms

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Different methods were applied, which made use of genetic markers from chloroaromatic metabolism, to study the distribution and variation of catabolic genes among soil microorganisms. In the first method bacterial strains that were enriched for growth on (chloro-) aromatic substrates were analyzed for their plasmid content, and hybridized with gene probes derived from catabolic plasmid pP51, which encodes chlorobenzene metabolism. This revealed a class of plasmids which were indistinguishable from, or homologous to pP51. In addition, microorganisms were extracted from soil and screened for the presence of catabolic genes by DNA-DNA colony hybridization with a set of probes for three chlorocatechol 1,2-dioxygenase genes. Positively reacting colonies were obtained under selective conditions with a frequency of 1 to 5 per 2000. The total DNA from these strains was analyzed by DNA hybridization. Isolated soil bacteria were further tested for growth on chlorinated aromatic substrates. In the third method the variation of chlorocatechol 1,2-dioxygenase genes from soil microorganisms was analyzed by isolating total DNA from soil samples and using it as target in the polymerase chain reaction. Discrete fragments hybridizing with a *tcbC* gene probe were obtained after amplification primed with degenerate oligonucleotides which were derived from conserved regions in three chlorocatechol 1,2-dioxygenase genes. Cloning of these DNA fragments and sequence analysis showed six different types with the expected size, of which only one was related to the chlorocatechol 1,2-dioxygenase gene (identical to the *tcbC*-type). This illustrated the possibility to detect and isolate chlorocatechol 1,2-dioxygenase sequences from soil DNA although the selectivity of the amplification reaction was relatively low.

Many bacterial strains have been isolated which degrade a variety of xenobiotic compounds such as chlorinated aromatics (for reviews, see (6, 7, 27)). However, little information is available on the distribution of these strains in the environment and the variations which occur in catabolic pathways of indigenous microorganisms. A technique which used DNA-DNA colony hybridization with the TOL and NAH plasmid genes was applied to detect bacteria able to degrade toluene and naphtalene (31). This technique has further been used to isolate bacterial strains that degrade 4-chlorobiphenyl from freshwater sediment samples (26), to detect biphenyl metabolizing bacterial colonies from waste disposal sites with chlorobiphenyl gene probes (39), and to study the distribution of heavy metal resistance genes among bacteria in various environments (9, 28). The validity and sensitivity of DNA-DNA colony hybridizations in these studies was assessed by comparing the numbers of microorganisms hybridizing with a specific gene probe with those growing on the substrate for which the gene probe was selective (9, 26, 28, 31, 39). Similarly, the presence of specific genes in bacteria of a certain environment has been studied by hybridization of DNA or RNA directly purified from environmental samples, by using known gene probes for naphtalene and toluene (30, 31), nitrogen fixation (18), or for mercury resistance (1). The detection of specific bacterial strains, such as genetically engineered or pathogenic strains, has been made more sensitive by first amplifying specific DNA target sequences in the Polymerase Chain Reaction (PCR) (2, 33, 34). The PCR-reaction has also been used to study specific variable regions in genes that are overall very conserved, such as 16S rRNA genes (40), or to study classes of very related genes among different microorganisms, such as the gene for ribulose biphosphate carboxylase *rbcL* (24).

Our previous studies have focused on the analysis of genes encoding chlorobenzene degradation in *Pseudomonas* sp. strain P51, which we believe have evolved by natural selection due to the presence of chlorinated benzenes in the environment. The structural genes for chlorobenzene metabolism in this strain are located on a 110-kilobase catabolic plasmid pP51 and comprised two distinct regions (35, 36, 37, 38). In this chapter, we describe the use of various gene probes to study the distribution of genes for degradation of chloroaromatic compounds among soil microorganisms and in environmental samples. Marker sequences that were used for this purpose were derived from genes encoding the chloroaromatic pathways and especially from those for the chlorocatechol 1,2-dioxygenases of *Pseudomonas* sp. strain P51, of *Alcaligenes eutrophus* JMP134 (25), and of *Pseudomonas putida* pAC27 (13). We have analyzed the usefulness of different hybridization methods to screen soil microorganisms and isolate strains with genetic material homologous to chloroaromatic markers, and have further used PCR-amplification to detect and isolate chlorocatechol 1,2-dioxygenase genes from purified soil-DNA.

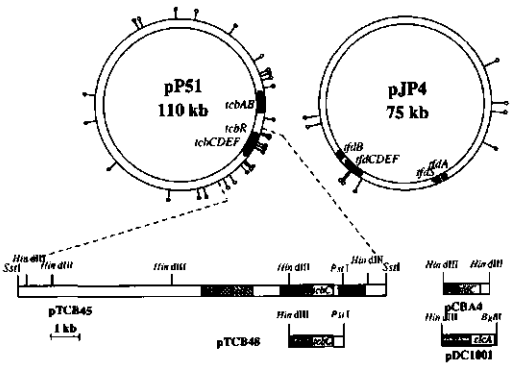


FIG. 1. Plasmids and constructs used in this study. The figure shows the catabolic genes of plasmids pP51 and pJP4 and their direction of transcription (derived from (10, 15, 37)). The physical map of pP51 is drawn for *Xho*I (•) and *Hin*dIII (o), the map of pJP4 for *Hin*dIII (o) only. The enlarged regions show the fragments which were used in the different hybridization experiments. Relevant restricition sites are indicated in the physical maps. Plasmid pTCB45 contains the chlorocatechol pathway genes from pP51; pTCB48 contains the chlorocatechol 1,2-dioxygenase gene *tcbC* (35, 37), pCBA4 contains *tfdC* (10, 16, 25), and pDC1001 *clcA* (13). Symbols: ■ , regulatory gene; ▨ , chlorocatechol 1,2-dioxygenase gene; ■ , cycloisomerase gene; ▧ , dienelactone hydrolase gene; □ , ORF3; ■ , *tcbF*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids that were used in this study are listed in Table 1. *Pseudomonas* and *Alcaligenes* strains were grown in Z3 minimal medium at 30°C to stationary phase as described earlier (37). The following carbon and energy sources were added for the cultivation of the strains (per liter of Z3 medium): *Pseudomonas* sp. strain P51, 1,2,4-trichlorobenzene (3.2 mmol); *Alcaligenes* sp. strain A175, 1,4-dichlorobenzene (1,4-DCB) (3.5 mmol dissolved in 20 ml heptamethylnonane) (HMN) (Sigma Chemical Co., St. Louis, Mo.) ; unidentified strains K3-A, K3-B, K3-2, and *Pseudomonas* sp. strain GJ60, 1,2-DCB (3.5 mmol in 20 ml HMN); *Pseudomonas* sp. strain P59, γ-hexachlorocyclohexane (10 mmol); *P. putida* mt-2, 3-methylbenzoate (10 mmol); *A. eutrophus* JMP134, 3-chlorobenzoate (10 mmol) ; and *Pseudomonas* sp. strain 2A, 1,3,5-trimethylbenzene (5 mmol).

For cultivation of soil microorganisms a low nutrient medium was used (5% PTYG), which contained per liter: 0.25 g peptone, 0.25 g tryptone, 0.5 g yeast extract, 0.5 g glucose, 0.6 g $MgSO_4 \cdot 6H_2O$, and 0.1 g $CaCl_2 \cdot 2H_2O$. Incubations with soil microbial strains were carried out at 20°C.

DNA techniques. Plasmid DNA was isolated from the above-mentioned bacterial strains cultivated on the aromatic substrate, by using the procedure of Hansen and Olsen (17). Further purification was done by CsCl-ethidium bromide density gradient centrifugation and dialysis according to established procedures (29). Total DNA from soil microorganisms was isolated by the method of Marmur (21). Other DNA techniques such as restriction digestions, Southern blottings, and DNA sequencing, were carried out according to standard procedures (29) or as described earlier (35, 37). Computer analysis of DNA sequences was done with the program GCG (8).

Isolation and growth of soil microorganisms. Microorganisms were extracted from soil samples taken from the sandy sediment of the river Rhine (nearby Wageningen, The Netherlands). Samples were withdrawn from the first 25 cm of the sediment by pushing a 30 cm long and 5 cm wide perspex cylinder, which had been decontaminated by ethanol washings, into the under water sediment. Sediment cores were kept in the sample cylinder, closed with sterilized stoppers and then immediately transported to the laboratory and transferred into sterile erlenmeyer flasks. From these flasks sterile 50 ml polypropyleen tubes were filled with about 7.5 ml water-saturated soil slurry to which then 25 ml of a 0.1% sodiumpyrophosphate solution (pH 7.0) was added. The tubes were shaken on a rotary shaker at 100 rpm, 30°C for 30 min, then incubated for 5 min in a Bransonic 32 ultrasonic water bath at 150 W (Branson Ultrasonics, Connecticut), centrifuged for 10 min at room temperature at 100 x *g* to settle the soil particles, and decanted to collect the supernatant containing the bacterial fraction. This procedure was repeated three times and supernatants from separate rounds were pooled. The pooled supernatant fractions were subsequently diluted 10- and 100-fold in sterilized tap water and 0.1 ml fractions were plated on 5% PTYG agar plates, which were incubated at 20°C for one to two weeks. In this way, between 10^6 and 10^7 CFU per gram of soil could be obtained.

Hybridization analysis of soil microorganisms. Plates containing 100 to 200 colonies were selected for colony blotting. Hereto filter membranes (Colonyscreen, NEN Dupont) were placed on the agar plates, and allowed to absorb for about 5 min. Filters were then peeled of the plates, and treated according to the manufacturer's descriptions, except that the filters were first placed in a 0.75 ml pool of 1% sodium dodecylsulphate (SDS) for 3 min. Filters were air-dried and subsequently illuminated with UV at 315 nm for 3 min to cross-link the DNA. Excess of colony material was removed by washing the filters at 60°C in 3X SSC (29), 0.1% SDS, for 3 h. Filters were subsequently prehybridized in a solution containing 7% SDS, 1% Bovine serum albumin (Fraction V), and 0.5 M sodium phosphate (pH 7.2) for 1 h at 62°C, and allowed to react with the denatured ^{32}P labeled DNA probes in the same solution for 16 h at 62°C. After this period the filters were removed from the hybridization solution, washed at different temperatures or salt concentrations (2X SSC or 0.2X SSC), and exposed to X-ray film. Plates were kept at 20°C after blotting to allow regrowth of the colonies.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Source or reference
Bacterial strains		
Pseudomonas sp. strain P51	Tcb$^+$, Dcb$^+$	
Pseudomonas sp. strain GJ60	1,2-Dcb$^+$	D.J. Janssen
Pseudomonas sp. strain P59	Hch$^+$	J. Huntjens
Pseudomonas sp. strain 2A	Tmb$^+$	A. van Neerven
Pseudomonas putida mt-2	Tol$^+$, Xyl$^+$	
Alcaligenes sp. strain A175	1,4-Dcb$^+$, 1,3-Dcb$^+$	(32)
Alcaligenes eutrophus JMP134	2,4-D$^+$, 3-CBa$^+$	(10)
soil isolate, sp. strain K3-A	Tcb$^+$, 1,2-Dcb$^+$	T. Bosma
soil isolate, sp. strain K3-B	Tcb$^+$, 1,2-Dcb$^+$	T. Bosma
soil isolate, sp. strain K3-2	1,2-Dcb$^+$	T. Bosma
Escherichia coli TG1	F'[*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15] *supE hsd*Δ5 Δ(*lac-proAB*)	(29)
Plasmids		
pP51	110-kb native plasmid of *Pseudomonas* sp. strain P51, encodes Tcb- and Dcb-metabolism	(37)
pJP4	75-kb native plasmid of *A. eutrophus* JMP134, encodes metabolism of 2,4-D and 3-Cba	(10)
pTCB45	13-kb *Sst*I fragment of pP51 in pUC19, contains *tcbR* and *tcbCDEF* gene cluster	(35, 37)
pTCB60	12-kb *Hin*dIII fragment of pP51 in pUC18, contains *tcbAB* gene cluster	(37)
pTCB48	1.7-kb *Pst*I-*Hin*dIII fragment of pP51 in pUC18, containing *tcbC*	(37)
pCBA4	1.6-kb *Hin*dIII fragment of pJP4 in pUC18, contains *tfdC*	(35)
pDC1001	2.0-kb *Bgl*II-*Hin*dIII fragment of pDC100 in pUC18, containing *clcA*	(35, 13)

[a]Abbreviations: Tcb$^+$, growth on 1,2,4-TCB; Dcb$^+$, growth on all DCB-isomers; 1,2-Dcb$^+$, growth on 1,2-DCB; 1,4-Dcb$^+$, growth on 1,4-DCB; 1,3-Dcb$^+$, growth on 1,3-DCB; Hch$^+$, growth on γ-hexachlorocyclohexane; Tmb$^+$, growth on 1,3,5-trimethylbenzene; Tol$^+$, growth on toluene; Xyl$^+$, growth on *meta*- and *para*-xylene; 2,4-D$^+$, growth on 2,4-dichlorophenoxyacetic acid; 3-Cba$^+$, growth on 3-chlorobenzoate.

Hybridizing colonies were identified and transferred with a tooth-pick from the original plates into fresh 5% PTYG-medium or streaked on a fresh agar plate, regrown at 20°C and rescreened by colony blotting (see above). Further characterization was done by purifying total DNA from 5 ml cultures and analyzing the DNA by Southern blotting and hybridization with chlorocatechol 1,2-dioxygenase probes.

DNA probes that were used in the screening experiments were the plasmids pP51 and pJP4 (Fig. 1, Table 1), or cloned DNA fragments containing parts of the operons encoding chlorobenzene metabolism: i) the 13-kb *Sst*I fragment of pTCB45 containing the *tcbCDEF* and *tcbR* genes, ii) the 6.2-kb *Eco*RI fragment of pTCB60 containing the *tcbAB* genes, iii) the 1.7-kb *Pst*I-*Hin*dIII fragment of pTCB48 containing the *tcbC* gene (35, 37), iv) the 1.6-kb *Hin*dIII fragment of pCBA4 containing the *tfdC* gene (10, 25), and v) the 2.0-kb *Bgl*II-*Hin*dIII fragment of pDC1001 containing the *clcA* gene (13) (Fig. 1).

Colonies showing positive hybridization were further inoculated and regrown in microtiter plates containing 5% PTYG-medium at 20°C. 10 µl of these cultures was then dot-blotted on nylon membrane (Genescreen Plus, Dupont) for hybridization analysis and another 10 µl was further transferred to new microtiter plates containing the following carbon and energy sources in Z3-minimal medium: 1,2-DCB (3.5 mmol in HMN as described above), toluene (5 mmol per liter Z3 in HMN), 1 mM benzoate, 1mM 3-chlorobenzoate, or 1 mM 4-chlorobenzoate. Growth was determined by comparing the bacterial pellets formed after two weeks incubation at 20°C with those formed after incubation in Z3-medium without carbon

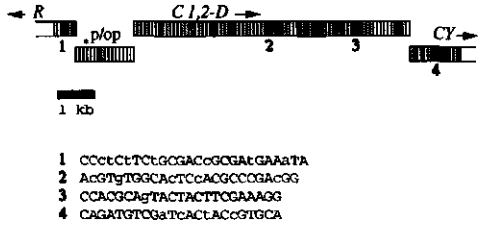


FIG. 2. Schematic representation of the alignment of chlorocatechol 1,2-dioxygenase genes and flanking regions, and conserved sequences selected for development of PCR primers. Boxes give the number of nucleotides identical in the three sequences per group of 10 nucleotides. Capital letters in the DNA sequences shown below indicate fully conserved nucleotides, small caps present nucleotides of divergence. The sequence shown is the sequence of the *tcbC* gene. Abbreviations: R, regulatory gene; p/op, promoter/operator region; C 1,2-D, catechol 1,2-dioxygenase gene; CY, cycloisomerase gene. Symbols: □, less than three nucleotides identical; ▨, 4 nucleotides identical; ▨, 5 identical; ▨, 6 identical; ▨, 7 identical; ■, 8 identical; ■, 9 identical; ■, 10 identical.

source added. Cultures of *Pseudomonas* sp. strain P51, *A. eutrophus* JMP134, and *P. putida* mt-2 were inoculated as controls in duplo in the microtiter plates.

Amplification and analysis of chlorocatechol 1,2-dioxygenase genes from soil DNA using polymerase chain reaction. DNA was extracted from soil samples from the river Rhine essentially as described by Holben et al. (18). The DNA was isolated from the pooled bacterial fraction that was extracted from the sediment samples as described above, although now scaled up for the complete soil core (approximately 350 g). The crude DNA fraction was purified by CsCl-ethidium bromide density gradient centrifugation and dialysis (29). The amount of DNA extracted from the soil sediment in this way was approximately 0.2 mg DNA per g soil. Purified DNA samples were analyzed for the presence of chlorocatechol 1,2-dioxygenase genes by using PCR to amplify possible target fragments. The PCR was primed with oligonucleotides that were derived for strongly conserved regions in chlorocatechol 1,2-dioxygenase genes and regions directly flanking these genes (Fig. 2, Table 2). The reactions were performed in a total volume of 50 μl containing 50 mM KCl, 2.5 mM $MgCl_2$, 20 mM Tris hydrochloride (pH 8.4), 0.1 mg/ml BSA, 0.2 mM of each deoxynucleotide (dATP, dCTP, dGTP, dTTP), 1 ng of each primer-DNA, 40 to 400 ng (target-) soil DNA, and 1 U Taq DNA polymerase (BRL Life Technologies). An amplification program of 35 cycli was run on a Techne PHC-1 (New Brunswick Scientific, Nijmegen, The Netherlands).

The following temperature settings were used: i) annealing at 35°C for 1.5 min, extension for 2 min at 72°C and denaturation at 94°C for 1.5 min (primers 1 and 4), ii) as i, except that the annealing step was performed at 50°C (primers 2 and 3). Amplification reactions with soil DNA and primers 2 and 3 were subsequently digested with

TABLE 2. Primers for chlorocatechol 1,2-dioxygenase genes.

Region	Sequence 5'-3'	Size
1	CCIGCITCIGCGACIGCGAIG	21-mer
2	GTITGGCACTCGAGGCCCGAIG	22-mer
3	GCAAGCTTCGAAGTAGTAITGCGTG	25-mer
4	GCACIGTIGTIAICGACATCTG	22-mer

Region numbers correspond to regions in Fig. 2. Shown in bold case are the introduced restriction sites for *Xho*I and *Hin*dIII. Inosine nucleotides are abbreviated with I in the sequences.

*Xho*I and *Hin*dIII after adjusting the salt concentration and pH, and fragments were purified by gel electrophoresis through 1.5% agarose gels. Fragments of approximately 200 bp in size were recovered from the gel and purified by using Geneclean (Bio 101, Inc. La Jolla, Calif.). Purified fragments were then ligated with vector M13mp18 (41), which was digested with *Sal*I and *Hin*dIII, and transformed into *E. coli* TG1. White plaques were picked and replated, screened by hybridization with chlorocatechol 1,2-dioxygenase probes and analyzed by DNA sequencing.

RESULTS

Hybridization studies with different plasmids from catabolic strains and *tcb* gene probes. To determine if any homology existed between plasmid pP51 and plasmids from various catabolic strains, hybridization experiments were performed in which we used DNA fragments of pP51 as gene probes. The hybridizations showed a strong homology between plasmids isolated from the unidentified soil isolates K3-A, K3-B, and K3-2 and plasmid pP51 (Fig. 3), whereas the strains themselves were clearly different in colony morphology (3). Except for a minor extra band which appeared in some isolations of pP51 (Fig. 3C), the plasmids from K3-A, K3-B, and pP51 showed identical restriction digests and hybridization patterns. The plasmid from K3-2 carried some restriction fragments which were similar to those of pP51 (Fig. 3A, B, C and D), although others were clearly different (Fig. 3D, E, and F). A region homologous to *tcbCDEF* was still present on pK3-2, which was confirmed later by subcloning the 13-kb *Sst*I fragment of pK3-2. However, the restriction map of this fragment was slightly different from the *tcbCDEF*-region (results not shown). In contrast, the *tcbAB* gene cluster could not be detected on this plasmid. Although hybridization was obtained with the 6.2-kb *Eco*RI fragment of pTCB60 (Fig. 3F), no hybridization was found with a gene probe containing the insertion element IS*1066* (38), which suggests that Tn*5280* was missing from this plasmid (not shown). DNA probes from pP51 also hybridized with plasmid pJP4 and a plasmid isolated from *Pseudomonas* sp. strain GJ60. The strong hybridization of pJP4 with the 13-kb *Sst*I fragment of pP51 (Fig. 3C and D) was not found with a gene probe consisting of *tcbC* alone (Fig. 3E). This suggests that pJP4 and pP51 are more homologous in other regions than the chlorocatechol oxidative gene cluster per se. The strong hybridization obtained between pP51 gene probes and

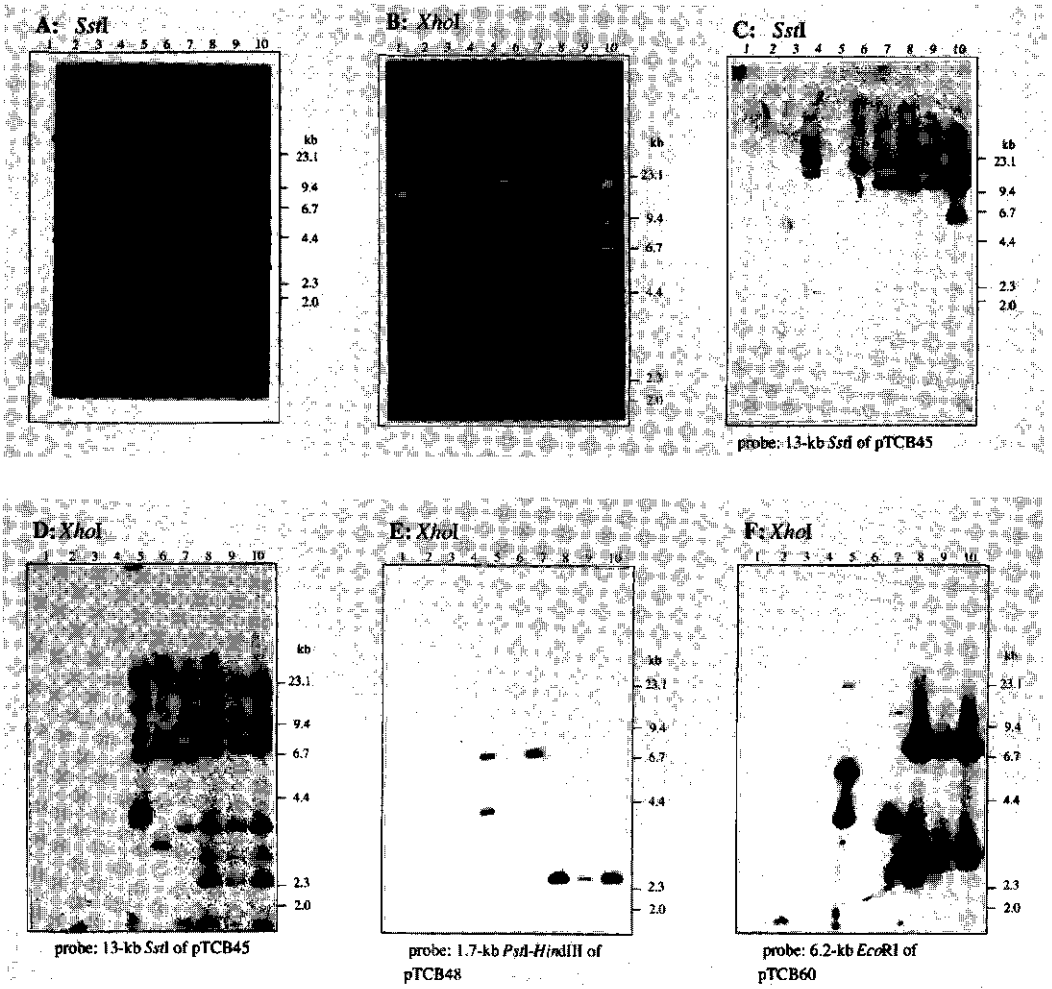


FIG. 3. Analysis of catabolic plasmids and plasmids from catabolic strains using hybridizations with gene probes derived from *tcb* genes. Lanes: 1, plasmid isolated from *Pseudomonas* sp. strain P59; 2, plasmid from *Pseudomonas* sp. strain 2A; 3, TOL plasmid; 4, plasmid from *Pseudomonas* sp. strain GJ60; 5, plasmid from *Alcaligenes* sp. strain A175; 6, plasmid pJP4; 7, plasmid from strain K3-2; 8, plasmid from strain K3-B; 9, plasmid from strain K3-A; 10, plasmid pP51. The last lane in A and B shows λ DNA, digested with *Hin*dIII. In panels B, D, E, and F lanes 4 and 5 are exchanged. (A) Restriction digests of all plasmids by *Sst*I, visualized on agarose gel by Ethidium bromide staining. (B) Ibidem, digested with *Xho*I. (C) Autoradiogram of A, probing with the 13-kb *Sst*I fragment of pTCB45. (D) Autoradiogram of B, probing with the 13-kb *Sst*I fragment of pTCB45. (E) Autoradiogram of B, using 1.8-kb *Pst*I-*Hin*dIII fragment of pTCB48 as probe. (F) Autoradiogram of B, using 6.2-kb *Eco*RI fragment of pTCB60 as probe .

plasmid pGJ60 (Fig. 3C, D, E, and F), indicates that plasmid pGJ60 may contain both *tcbCDEF*-like and *tcbAB*-like gene clusters. Hybridization patterns observed with plasmid pP51 itself suggested that certain regions on pP51 are duplicated (Fig. 3C). A region which was hybridizing to the 13-kb *Sst*I fragment of pTCB45 is located around *Xho*I fragment A (Fig. 1), but it did not hybridize with the *tcbC* gene probe (Fig. 3E), which suggests that the *tcbCDEF* catabolic genes themselves are not duplicated. No hybridization signals were obtained between pP51 gene probes and plasmids isolated from *Alcaligenes* sp. strain A175 and *Pseudomonas* sp. strain P59, nor with the TOL-plasmid or the plasmid-DNA isolated from *Pseudomonas* sp. strain 2A.

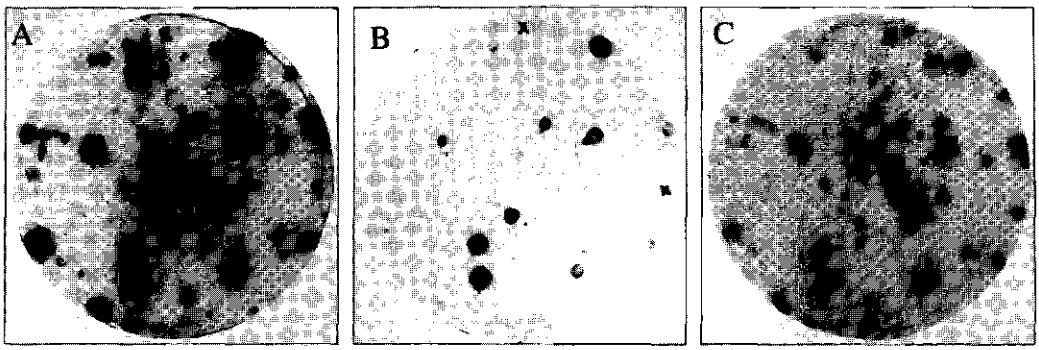


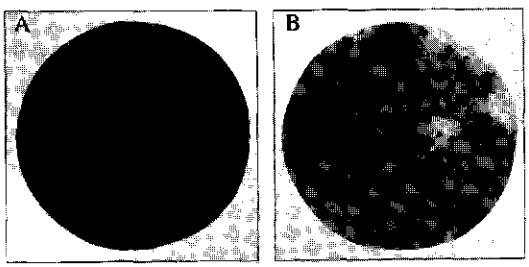
FIG. 5. Effect of different stringencies and probes on hybridization signals. Colony hybridizations were performed with plates which contained approximately 10^1 cells of strain P51 (estimated by separate selective plate counts) per plate in a background of soil bacteria. (A) Hybridization obtained under non-stringent washings conditions with pP51 as probe. (B) Same as in A, stringent washing conditions. (C) Hybridization of the same blot, now with plasmid pJP4 as a probe under stringent washing conditions. Crosses mark orientation spots of the filter.

Screening soil microorganisms for the presence of DNA sequences homologous to chlorocatechol oxidative pathway genes. To obtain more information on the dissemination of chlorocatechol genes in the soil microbial ecosystem, we developed screening methods using genetic markers from the *tcbCDEF* and related operons. Two procedures were tested for their feasibility, and the effect of various factors on the methods were assessed. Procedure A consisted of a first screening of soil microorganisms by colony hybridizations, followed by isolation of positive colonies, regrowth on fresh media, rescreening, and finally analysis of purified total DNA of the selected strains by Southern blotting and hybridization. In procedure B a similar screening by colony hybridization as in (A) was followed by inoculation of positive colonies into micro-titer plates which were then tested by dot-blotting and hybridization, and for growth on different media. We tried to determine the effects of the following factors in the procedures on the obtained hybridization signals: i) stringency of hybridization and washing, ii) application of multiple probes, and iii) lysis of individual colonies and aspecific hybridization to cell wall components.

Microorganisms extracted from the soil showed an enormous heterogeneity in colony size and morphology on 5% PTYG-medium after growth at 20°C for two weeks. A "reprint" of all colonies on the plates could be obtained by using the colony blotting procedure and hybridization with a relatively non-specific probe (the complete plasmid pP51) under non-stringent conditions (Fig 4A). This suggested that all bacterial colony types lysed sufficiently to release their DNA (see below). The blotting, however, resulted very often in the complete loss of individual colonies, which could then not be regrown upon prolonged incubation of the plates, or which were fully overgrown by other faster growing species. This heterogeneity made replica plating very difficult.

To test the effects of different probes and stringencies on the signal strength obtained in the colony hybridization, we mixed cells of strain P51 with the extracted soil bacteria prior to plating (Fig. 5). Colony blotting of those plates and hybridization with a fully homologous DNA probe (in this case plasmid pP51) resulted in spots due to the

FIG. 4. Example of the heterogeneity in colony size and morphology observed with bacteria extracted and grown from soil samples on non-selective media. (A) Non-stringent conditions using whole pP51 plasmid as probe. (B) Same as A, now under stringent washing conditions, same exposure time as A.



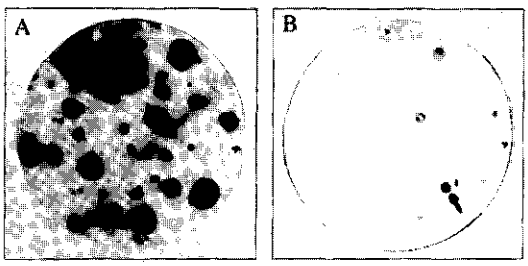


FIG. 6. Hybridizations obtained under different conditions with colony blots from plates containing bacteria extracted from Rhine sediment. (A) Non-stringent conditions using the multiple chlorocatechol 1,2-dioxygenase probes (isolated fragments of pTCB48, pCBA4, and pDC1001). (B) Same blot as in A, now under stringent conditions. Arrow marks a putative positive colony which was selected (isolate 1G6, see Fig. 8A, lane 9). Exposure time was chosen such that a control hybridization with colonies of strain P51 showed clear spots.

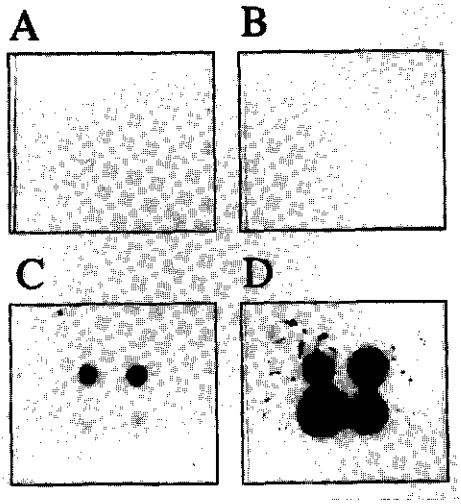


FIG. 7. Tests of the different individual steps in the procedure of colony blotting. Hybridizations were performed under non-stringent conditions using the chlorocatechol 1,2-dioxygenase fragment of plasmid pCBA4 as probe. Four different colonies of soil bacteria that reacted positively with this probe were inoculated and 10 µl samples were dot-blotted. (A) Blotted cultures, no further treatment. (B) Cultures lysed with SDS, but not treated with NaOH. (C) Cultures that underwent the full procedure of lysis, denaturation and renaturation. (D) Samples of purified denatured DNA, isolated from these cultures.

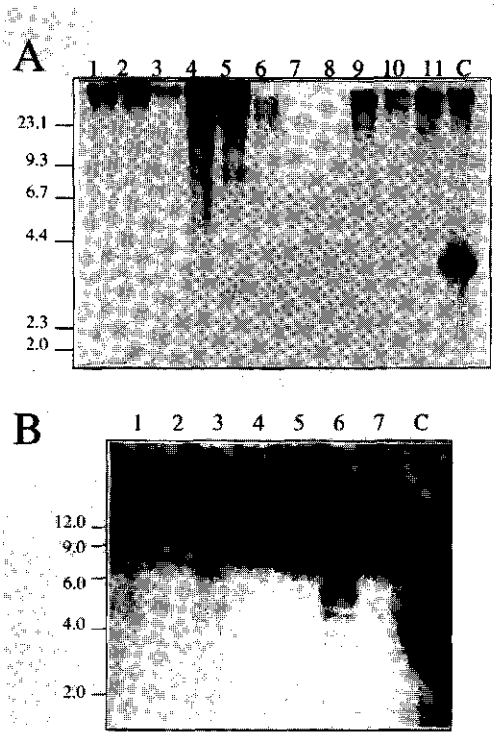


FIG. 8. Southern hybridizations of different soil isolates selected from the colony hybridizations. Probes used in these hybridizations were the multiple chlorocatechol 1,2-dioxygenase gene fragments of pTCB48, pCBA4, and pDC1001. On the left side of the figures a size marker in kb is given. (A) DNA samples digested with *Hin*dIII. Lanes: 1, isolate 1G7; 2, isolate R123; 3, isolate DG2; 4, isolate RH21; 5, isolate R2M21; 6, isolate DD1B; 7, isolate RK22; 8, isolate R2I21; 9, isolate 1G6; 10, isolate R91012; 11, isolate R4811; C, *Pseudomonas* sp. strain P51. (B) DNA samples digested with *Bam*HI. Lanes: 1, isolate 7C3; 2, isolate 5H1; 3, isolate 7C2; 4, isolate 8B1; 5, isolate D8; 6, isolate 6J1; 7, isolate 5A1; 8, *Pseudomonas* sp. strain P51.

presence of colonies of strain P51 that became clearly distinguishable from the background when stringent conditions were used (washing temperature 64°C, salt concentration 0.2xSSC) (Fig. 5A and B). However, by applying a heterologous probe such as plasmid pJP4 the signal strength was reduced and the colonies of strain P51 could no longer be discriminated among background colonies (Fig. 5C). This indicated that only bacterial strains carrying DNA fully homologous to the used probes could be discriminated in a heterogenous population of soil bacteria.

We tried to increase the sensitivity of hybridization by applying multiple probes for chlorocatechol 1,2-dioxygenase genes, in order to be able to detect strains in a heterogenous population that carried DNA not completely homologous to one of the individual probes. Hybridizations with the multiple probe set containing the DNA fragments of *tcbC* (35), *clcA* (13) and *tfdC* (10, 25), were applied under non-stringent (Fig. 6A) and stringent conditions (Fig. 6B). In this way, between 1 and 5 positive signals per 1000 to 2000 colonies screened were consistently obtained, which suggests that bacterial strains were present in the soil microbial population that carried DNA homologous to the chlorocatechol 1,2-dioxygenase genes.

To test whether other factors could affect signal strength such as aspecific adherence to cell wall components or insufficient lysis, we performed hybridizations with material after successive steps of the procedure and made use of an oligonucleotide which was complementary to a universally conserved domain of the bacterial 16S rRNA (primer 1115) (11). On colony blots hybridized with the labeled 16S rRNA probe essentially all colonies showed a signal, which suggests that lysis of the colonies in the colony blotting procedure was sufficient (results not shown). However, we sometimes observed insufficient lysis with dot-blotted samples. This was probably due to irregularities in the blotting membrane which were caused by the suction of the vacuum pump used for the dot-blot apparatus. Hybridizations with bacterial material after successive steps in the procedure made clear that no aspecific reactions of the labeled probe took place with other components of the cell than the DNA itself (Fig. 7).

Colonies that were identified as putative positives on the basis of their hybridization signal, were transferred from the original plate to fresh plates, regrown and screened by the same procedure of colony blotting and hybridization.

This showed that in many cases the positive signal in the first round could not be reproduced, indicating that either not the right colony had been transferred from the original plate (possibly due to the heterogeneity of the colonies on the original plate) or that the sequences that initially hybridized were unstable in these strains and lost by segregation.

Identification and characterization of soil isolates. Total DNA was purified from colonies which hybridized positively with the multiple chlorocatechol 1,2-dioxygenase gene probe set (see above) and subjected to restriction digestion, Southern blotting and hybridization. This was done to further verify the selectivity of the colony blotting. In two cases of eighteen selected cultures positive hybridization was obtained with the multiple probe set containing the chlorocatechol 1,2-dioxygenase DNA fragments (Fig. 8A, lane 5; Fig. 8B, lane 6). Most other samples showed only aspecific hybridizations of the probes with the bulk of the isolated DNA, which may have accounted for the positive hybridization reaction in the colony blotting experiments (such as culture 1G6, Fig. 8A lane 9 and Fig. 6B). The two hybridizing isolates R2M21 and 6J1 (Fig. 8) were able to grow on benzoate, but not on other tested aromatic substrates (3-chloro-, 4-chlorobenzoate or 1,2-dichlorobenzene).

Relation of genotypic hybridization to phenotypic characters. To relate hybridization signals obtained with DNA probes from chloroaromatic catabolism with growth on (chloro-)aromatic substrates, colonies of soil bacteria were transferred from the original plates to microtiter plates containing different media. A total of 48 colonies identified as putative positive in the first round of screening were inoculated in the first four rows (Fig. 9, lanes a-d), whereas another 42 were picked at random (lanes e, f, g, and h1-6). Dot blot hybridizations with pP51 derived probes gave very few positive reactions in which the hybridization signal compared to the control strains P51 (h7, h8) and JMP134 (h9, h10) did not decrease after the two washings under different stringencies. In two separate experiments 35% and 54% of colonies initially identified as positives, were judged positively a second time in the dot blots, whereas of randomly picked colonies this was 26% and 40%, respectively. When the results from the hybridizations were compared with those from growth experiments of the cultures on various aromatic carbon sources (Fig. 9C), we did not find combinations of positive hybridization and growth on the tested substrates.

Detection and isolation of chlorocatechol 1,2-dioxygenase genes from a soil environment by PCR amplification. The DNA sequences of three chlorocatechol 1,2-dioxygenase genes were aligned. This revealed regions of very high homology, not only in the structural parts of the genes, but also in regions directly upstream and downstream (Fig. 2) (35). Using oligonucleotides which carried inosine residues at sites of degeneration, we tried to amplify fragments of chlorocatechol 1,2-dioxygenase genes from soil DNA. We anticipated to increase the specificity of the amplification which was low as a consequence of the use of degenerate primers and heterogenous soil DNA, by using two subsequent amplification rounds with two different sets of primers (Fig. 2). For unknown reasons primers 1 and 4

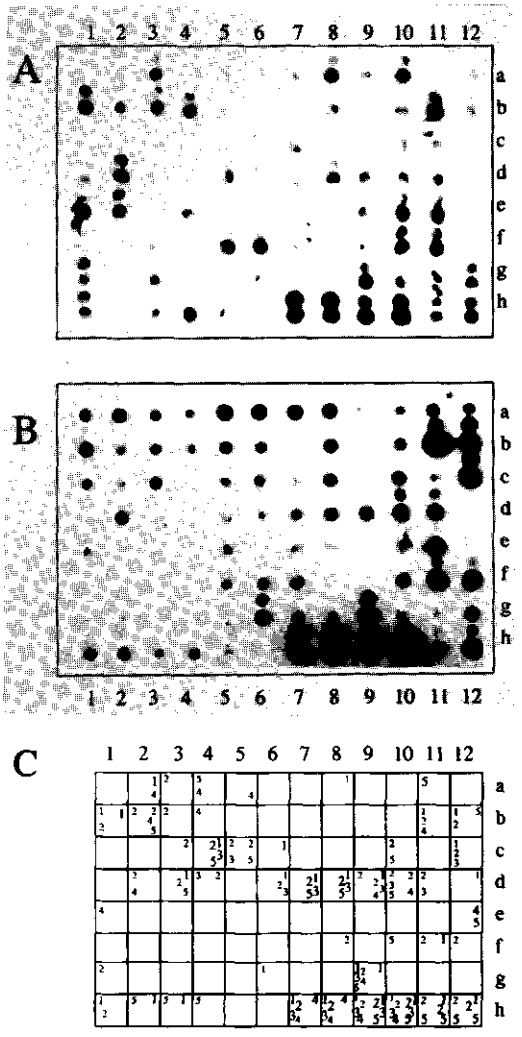


FIG. 9. Dot-blot hybridizations of soil isolates selected from colony hybridizations. Two autoradiograms obtained from different stringencies (high stringency on top) were superimposed. Exposure times were chosen such that the signal for the control strains P51 remained the same (positions h7, h8). (A) Hybridization obtained with the pTCB48 fragment as probe. (B) Hybridization of the same cultures as A with plasmid pP51 as probe. (C) Summary of hybridization results with different probes, and growth experiments with different substrates. Large cap size on the left side of the titer plate squares indicates a similar reaction with the probe under stringent conditions as under non-stringent conditions; small cap size indicates weak reaction with the used probe. Similarly for the right side numbers: large size indicates strong growth (as pellet formation), small size indicates weak growth. Symbols: (probes; left side) 1, pTCB48 fragment; 2, pP51 plasmid; 3, pCBA4 fragment; 4, pJP4 plasmid; 5, TOL plasmid; (substrates; right side) 1, growth on benzoate; 2, 4-chlorobenzoate; 3, 3-chlorobenzoate; 4, 1,2-dichlorobenzene; 5, toluene.

gave a detectable product only at very low annealing temperatures of 30°C, even in control reactions with pTCB48 plasmid DNA. With soil DNA as template and primers 1 and 4 amplification resulted in a smear ranging in size of 0.2 kb to more than 4 kb (results not shown). When DNA fragments with a size around 1.0 kb were excised from this smear, purified and submitted to a second round of amplification, we always observed false positive reactions with our blancs (i.e. amplification reactions without DNA added). This was not found in a single amplification round with primers 2 and 3, which lead to a discrete band of approximately 0.2 kb (Fig. 10A). Subsequent hybridization showed that these bands hybridized with the *tcbC* probe (Fig. 10B). The amplified fragments were then cloned into M13 phages by making use of the restriction sites that were devised in the oligonucleotides. About 50 recombinant plaques were obtained of which 5 hybridized clearly to the *tcbC* probe (not shown). Next, 48 plaques were analyzed by T-track sequencing, and we found 12 different types of inserts. About half of these consisted of very short inserts of only 10 to 50 base-pairs, whereas the others carried inserts ranging between 132 and 250 base-pairs. One type, which occurred in 5 of the 48 tested plaques (which also previously had shown positive hybridization with the *tcbC* gene probe), was fully identical to the *tcbC* sequence (35). The other sequences, however, did not have any significant homology with one another or with chlorocatechol 1,2-dioxygenase genes (percentages DNA similarity ranged from 33.1 to 46.4 estimated with the program GAP, whereas percentages amino acid similarity were less than 30%). None of the determined sequences, except for the *tcbC*-type, predicted the conservative H(V/I)H-box that forms part of the active centre of the intradiol dioxygenases (22).

DISCUSSION

In this chapter we have evaluated various methods for obtaining information on the distribution and variation of genes for chloroaromatic catabolism among soil microorganisms. The focus was mainly on the chlorocatechol 1,2-dioxygenase genes since they encode a central step in metabolism of many chloroaromatic compounds (27). In analyzing strains that were enriched by growth on chlorobenzenes or other (chloro-) aromatics, we found that a class of plasmids from a number of those strains carried large regions of extensive homology to plasmid pP51, which were not limited to the catabolic genes, but also extended to other regions. Our results showed that plasmids identical or nearly identical to pP51 could be isolated from three other soil microorganisms than the original wild-type strain *Pseudomonas* sp. strain P51, which suggests that these plasmids are transmissable. Not all plasmids isolated from strains capable of metabolizing chlorobenzenes, i. e. *Alcaligenes* sp. strain A175 (32) and *Pseudomonas* sp. strain P59 (19), showed detectable homology with plasmid pP51. Catabolic plasmids have been observed to belong to different homology classes, such as TOL, NAH and SAL (12, 20), or pJP4, pAC27, and pSS50 (4, 5, 16). Our hybridizations indicate that pP51 may very well belong to this last group (Fig. 3). We also detected duplication of a region on plasmid pP51, which was also found for different TOL-plasmids and pJP4 (15, 23).

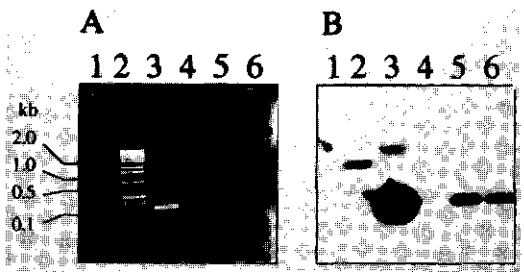


FIG. 10. Amplification of target fragments from soil DNA with chlorocatechol 1,2-dioxygenase primers 2 and 3, and hybridization with *tcbC* gene probe. (A) Amplified fragments visualized on agarose gel. (B) Autoradiogram of hybridization of Southern blot of A using pTCB48 fragment as probe, under stringent conditions. The hybridization of the size marker is caused by vector parts that were not fully removed during purification of the pTCB48 fragment. Lanes: 1, no DNA; 2, 1 kb size marker; 3, amplification product of 1 ng pTCB48 plasmid DNA, linearized with *Hin*dIII; 4, control PCR without DNA added; 5, and 6, amplification products of 400 ng soil DNA.

In order to study the distribution of catabolic genotypes in the soil microbial ecosystem we screened a population of microorganisms by DNA-DNA colony hybridization which were not previously enriched or selected by growth on aromatic substrates. A major consideration of this approach is the selectivity of the technique, which is high when using specific probes (1, 9, 14, 26, 28, 31, 39). Most of these studies suggested a positive relation between the numbers of bacteria hybridizing with DNA probes and those found on the basis of growth experiments, and incidentally this was confirmed by isolation of strains (26). However, it is likely that both the DNA-DNA colony hybridization technique and the method for determining numbers by growth experiments bias the true number of microorganisms capable of metabolizing the xenobiotic substrate, since organisms may be present that carry a gene but not express it, or that show the expected phenotype, but express uncharacterized genes for this (28). We have tried to cover as broad as possible a class of chlorocatechol 1,2-dioxygenase genes by using three gene probes which had an overall 60% DNA sequence identity (35). We detected rather strong hybridizing colonies even under the most stringent conditions in colony hybridizations (Fig. 6), which suggests that organisms were present which carried genes homologous to the chlorocatechol pathway genes. However, we found no organisms that reacted positively with the chlorocatechol 1,2-dioxygenase gene probes and could degrade a chloroaromatic compound. This could be caused by the low numbers of organisms present in the soil samples that would actually grow on the tested chloroaromatic compounds. The validity of the DNA-DNA colony hybridization could not be clearly confirmed by analysis of purified DNA samples (Fig. 8), although two cultures were found which showed weak hybridizing bands on Southern blots. Testing the different steps of the procedures also made clear that colonies were lost easily from the original plates because of heterogeneity of the bacterial colonies and overgrowth by the fastest growing species, which may have resulted in transferring other microorganisms than the

selected ones (Fig. 8 and 9). This problem was also observed in experiments when screening aquatic microorganisms for mercury resistance genes (28).

The third strategy to study the occurrence and variation of chlorocatechol 1,2-dioxygenase genes was to amplify gene fragments from purified soil DNA by using PCR and analyze these. This approach had been shown to be valid for the isolation and characterization of novel 16S rRNA sequences extracted from environmental samples (40). 16S rRNA sequences are present more abundantly than single copy genes, and occur in every organism. They are therefore easier to detect. Furthermore, conserved regions from single copy genes were amplified from environmental samples and detected by hybridization (24). Our results showed that amplification of discrete gene fragments of chlorocatechol 1,2-dioxygenase genes from heterogenous soil DNA was possible by using degenerate primers for conserved regions in the chlorocatechol 1,2-dioxygenase gene. We can not completely rule out the possibility that the cases of the detected *tcbC*-fragments were the result of a contamination of cells or DNA of *Pseudomonas* sp. strain P51 during the sampling procedure of the soil or the extraction of the soil bacterial DNA, since this strain was being handled in our laboratory as well. No separate blanc experiment was run for the sampling and extraction procedure to account for this effect. Negative controls in the PCR showed no evidence for contamination of DNA from strain P51 during this stage of the experiment, however.

Conclusions. By using a class of conserved genetic markers derived from chloroaromatic metabolism it was possible to obtain direct and indirect evidence that genes for these pathways are present among soil bacteria from the river Rhine sediment. The assessment of the different applied techniques provided a good basis for judging their validity and usefulness, and for future efforts to further study the diversity of these genes among soil organisms. New efforts are needed to develop more diverse multiple probe sets such as for the chlorocatechol 1,2-dioxygenase genes. This would improve the accurate estimation of the presence of microorganisms with related catabolic genes in the environment, and allow isolation and characterization of new microorganisms which have not yet been described, or which carry incomplete pathways that give further insight into the mechanisms of evolution of catabolic routes.

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Summary and concluding remarks

The pollution of our environment with a large number of different organic compounds poses a serious threat to existing life, since many of these chemicals are toxic or are released in such quantities that exceed the potential of biological detoxification and degradation systems. Bacteria and other microorganisms play an essential role in the breakdown of xenobiotic compounds. Microbes use these compounds as carbon and energy source and metabolize them to harmless end-products. However, not all compounds are easily metabolized and some structures resist the action of existing enzyme systems in bacteria. Nevertheless, bacterial species have been isolated which have overcome these metabolic barriers and completely metabolize chemicals that were previously considered to be persistent.

The project of this thesis was initiated to study the genetic mechanisms in bacteria that cause adaptation to use xenobiotic compounds as novel growth substrates (see Chapter 1 for a review). The work presented here mainly focused on one class of compounds, i. e. lower chlorinated benzenes such as dichlorobenzenes (DCB) and 1,2,4-trichlorobenzene (1,2,4-TCB). These compounds were known to be very resistant to biodegradation by bacteria. A number of bacterial species was isolated by enrichment techniques which were able to use DCB's and/or 1,2,4-TCB as sole carbon and energy source for growth. One of these bacteria, *Pseudomonas* sp. strain P51, was investigated further in this study. We have obtained strong evidence that the pathway for chlorobenzene metabolism arose as a consequence of the unique combination of two gene clusters, each specifying part of the complete pathway. These individual gene clusters are not uncommon and probably exist separately in different bacteria. Our results suggest that one of the gene clusters is contained in a novel transposable element that may have been acquired by strain P51 and integrated into a catabolic plasmid that already contained the other gene cluster. A further fine-tuning of the new pathway may have occurred through specialization of individual enzymes towards novel metabolic intermediates and by changes in the regulatory system in response to novel inducer molecules.

The degradation of DCB's and 1,2,4-TCB was studied at concentrations between 10 µg/l and 1 mg/l in soil percolation columns filled with sediment of the Rhine river, which in some cases were inoculated with *Pseudomonas* sp. strain P51 (Chapter 2). In the inoculated columns, DCB's and 1,2,4-TCB were instantly degraded. Strain P51 remained viable in the column as long as the chlorinated benzenes were fed in the influent. Interestingly, minimal concentrations of the chlorinated benzenes were measured in the effluent of the columns, independently of the influent concentrations used (6 ± 4 µg/l for 1,2-DCB; 20 ± 5 µg/l for 1,2,4-TCB;

more than 20 µg/l for 1,3-DCB and 1,4-DCB), which could not be lowered by additional inoculations with strain P51. The native microbial population in the non-inoculated columns adapted to degrade 1,2-DCB after a lag phase of about 60 days, and was then able to remove a concentration of 25 µg/l of 1,2-DCB in the influent to less than 0.1 µg/l.

Detailed genetic studies were carried out with *Pseudomonas* sp. strain P51 to characterize the genetic determinants for chlorobenzene metabolism. A large plasmid of 110 kilobase-pairs (kb) (pP51) could be isolated from cells that were cultivated on 1,2,4-TCB (Chapter 3). This plasmid could be cured from the strain by successive inoculations on non-selective media, rendering the strain incapable of metabolizing chlorinated benzenes. Subsequent cloning and deletion experiments in *Escherichia coli*, *Pseudomonas putida*, and *Alcaligenes eutrophus* showed that two regions on plasmid pP51 were responsible for chlorobenzene metabolism. Expression studies in *E. coli* revealed that a 5-kb region encoded the activity to convert 1,2,4-TCB and 1,2-DCB to 3,4,6-trichlorocatechol and 3,4-dichlorocatechol, respectively. This activity was determined using whole cell incubations, and in analogy with other described catabolic pathways it was proposed that the activity was caused by a chlorobenzene dioxygenase multienzyme complex and a dehydrogenase (encoded by *tcbA* and *tcbB*, respectively). Separated from the chlorobenzene dioxygenase gene cluster by approximately 6 kb a region was located which contained the genes for the conversion of chlorocatechols. Different DNA fragments of this region of pP51 were cloned in expression vectors in *E. coli*, *P. putida* and *A. eutrophus*. Both *P. putida* KT2442 and *A. eutrophus* JMP222 could be complemented for growth on 3-chlorobenzoate by a 13-kb fragment of pP51, which indicated that a functional pathway for degradation of chlorocatechols was encoded on this fragment. Enzyme activity measurements and transformation reactions with 3,4-dichlorocatechol in cell extracts of *E. coli* harboring cloned pP51 DNA fragments showed the activity of three enzymes, chlorocatechol 1,2-dioxygenase (catechol 1,2-dioxygenase II), chloromuconate cycloisomerase (cycloisomerase II), and dienelactone hydrolase II. The genes encoding these activities were designated *tcbC*, *tcbD*, and *tcbE*, respectively, and their deduced gene order was found to be *tcbC-tcbD-tcbE*. It was thus proposed that 3,4-dichlorocatechol was converted via a chlorocatechol oxidative pathway (or modified *ortho* cleavage pathway), similar to that described in *Pseudomonas* sp. strain B13 (5) and *A. eutrophus* JMP134 (4), leading finally to the formation of 5-chloromaleylacetate. The release of one chlorine atom from 3,4-dichlorocatechol was shown to take place spontaneously during lactonization in the cycloisomerization reaction.

The genes of the chlorocatechol oxidative pathway of strain P51 are organized in a single operon, comprising a region of 5.5 kb, which was fully sequenced and contained five large open reading frames (Chapter 4). The gene products of the different open reading frames were analyzed by subcloning appropriate pP51 DNA fragments in *E. coli* expression vectors. Expression studies confirmed the previously determined gene order and could attribute three open reading frames to the gene loci *tcbC*, *tcbD*, and *tcbE*, respectively. In between *tcbD* and *tcbE* an 1,022 bp open reading frame was present (ORF3), but we could not detect any protein encoded by this ORF. Immediately downstream of *tcbE* another ORF was found, designated *tcbF*, which encoded a 38 kDa protein. Until now, no clear function has been attributed for the *tcbF* gene product. The *tcbCDEF* genes and their encoded gene products showed high (50.6% - 75.7%) homology to two other chlorocatechol oxidative gene clusters, *clcABD* of *P. putida* (pAC27) and *tfdCDEF* of *A. eutrophus* JMP134(pJP4). Furthermore, a homology of 22% and 43.9% was found of TcbC and TcbD to CatA and CatB, respectively, the catechol 1,2-dioxygenase and cycloisomerase of the β-ketoadipate pathway of *Acinetobacter calcoaceticus*. This suggests that the chlorocatechol oxidative pathway originated from other, more common, metabolic pathways. Despite the strong DNA and amino acid sequence homology of the genes and enzymes of the chlorocatechol oxidative pathways, the substrate range of the pathway enzymes from the three organisms differed subtly. This was demonstrated for the chlorocatechol 1,2-dioxygenases TcbC, ClcA, and TfdC. In contrast to ClcA and TfdC, which showed a high relative activity for 3,5-dichlorocatechol, TcbC exhibited a strong preference for 3,4-dichlorocatechol and a weak affinity for the 3,5-isomer. This suggested that the *tcb*-encoded pathway enzymes had become specialized for intermediates (i.e. 3,4-dichlorocatechol) which arise in the metabolism of the novel compound 1,2-dichlorobenzene. Different genetic mechanisms may cause the divergence of genes and allow a specialization of encoded proteins (see also Chapter 1). Recently it has been proposed that slippage of short sequence repetitive motifs and subsequent mismatch repair would be the major driving force for rapid evolutionary divergence, rather than single base-pair substitutions (3). Detailed DNA sequence comparisons between the chlorocatechol 1,2-dioxygenase genes *tcbC*, *clcA*, and *tfdC* gives evidence for slippage of short sequence repetitions in regions of strong divergence in amino acid sequence.

The transcription of the *tcbCDEF* operon was found to be regulated by the gene product of *tcbR*, a gene located upstream of and divergently transcribed from the *tcbC* gene. The *tcbR* gene was characterized by DNA sequencing and expression studies in *E. coli* and appeared to encode a 32 kDa protein (Chapter 5). The

activity of the *tcbR* gene was analyzed in *P. putida* KT2442 harboring the cloned *tcbR* and *tcbCDEF* genes by determining the activity of the chlorocatechol 1,2-dioxygenase TcbC during growth on 3-chlorobenzoate. Strains of *P. putida* KT2442, which carried a frame shift mutation in the *tcbR* gene, could no longer induce *tcbC* expression during growth on 3-chlorobenzoate, suggesting that TcbR functions as a positive regulator of *tcbC* expression. A region of 150-bp is separating *tcbR* and *tcbC*, the first gene of the *tcbCDEF* cluster, and contains the expression signals needed for the transcriptional activation of *tcbCDEF* by the *tcbR* gene product. The transcriptional start sites of *tcbR* and *tcbC* were determined by primer extension analysis and this showed that the two divergent promoter sequences of the genes overlap. Protein extracts of both *E. coli* overproducing TcbR and of *Pseudomonas* sp. strain P51 showed specific DNA binding to this 150-bp region. TcbR probably regulates *tcbCDEF* expression and autoregulates its own expression, by binding the DNA region containing the promoters of *tcbC* and *tcbR*. It is likely that an inducer molecule interacts with TcbR, which may cause alterations or partially unwinding of the bound region and stimulation of RNA polymerase to start transcription of the *tcbCDEF* operon (1). Amino acid sequence comparisons indicated that TcbR is a member of the LysR family of transcriptional activator proteins and shares a high degree of homology with other activator proteins involved in regulating the catabolism of aromatic compounds, such as CatM, CatR and NahR. Detailed studies have recently been carried out to determine the precise interaction of TcbR with its operator region by DNaseI footprinting (2). It would be interesting to see if in analogy with the specialization of TcbC, TcbR has diverged from a more common regulator protein such as CatM or CatR, and became specialized in recognizing chorinated inducer molecules.

DNA sequence analysis of the start of the chlorobenzene dioxygenase cluster revealed the presence of an insertion element, IS*1066* (Chapter 6). An almost exact copy of this element, IS*1067*, was discovered on the other side of this gene cluster, although oriented in an inverted position. Thus, the complete genetic element formed by IS*1066*, the *tcbAB* gene cluster, and IS*1067*, resembled a composite bacterial transposon. The functionality of this transposon, which was designated Tn*5280*, was established by inserting a 12-kb *Hin*dIII fragment of pP51 containing Tn*5280*, marked with a kanamycin resistance gene in between the IS-elements, into the suicide donor plasmid pSUP202 followed by conjugal transfer to *P. putida* KT2442. Analysis by DNA hybridization of transconjugants with acquired kanamycin resistance showed that Tn*5280* had transposed into the genome of this strain at random and in single copy. The insertion elements IS*1066* and IS*1067* were found to be highly homologous to a class of repetitive elements of

Bradyrhizobium japonicum and *Agrobacterium rhizogenes*, and were distantly related to IS*630* of *Shigella sonnei*. The presence of the *tcbAB* genes on Tn*5280* suggested a mechanism by which a chlorobenzene dioxygenase gene cluster was mobilized as a gene module by the mediation of IS-elements. This gene module was then joined with the chlorocatechol gene cluster to form the novel chlorobenzene pathway.

To obtain more information on the distribution of chlorobenzene degradation genes in the environment, different methods were applied which were based on DNA-DNA hybridization with gene probes derived from chloroaromatic metabolism (Chapter 7). A number of bacterial strains which were isolated by selective enrichment from soil samples for growth on chloroaromatic compounds was screened for the presence of catabolic plasmids. Hybridization of these plasmid-DNA's with DNA fragments of the *tcbAB* or *tcbCDEF* genes revealed a class of plasmids which were identical or homologous to plasmid pP51 of strain P51. In other experiments soil microorganisms were directly extracted from soil samples, plated on nonselective media and screened by DNA-DNA colony hybridization for the presence of catabolic genes with a set of probes for three chlorocatechol 1,2-dioxygenase genes (*tcbC*, *clcA*, and *tfdC*). Positively reacting colonies were obtained under selective conditions with a frequency of 1 to 5 per 2000, which indicated that in the soil samples microorganisms were present which contained DNA sequences homologous to the used probes. However, from additional screening and hybridization experiments of these positively reacting colonies it became clear that some of these were false positives. Furthermore, positive strains were lost easily during transfer from the original agar plates due to the heterogeneity in colony types of the different soil microorganisms. In a third method the variation of chlorocatechol 1,2-dioxygenase genes among soil microorganisms was analyzed by amplifying total DNA from soil samples in the polymerase chain reaction, which was primed with degenerate oligonucleotides derived for conserved regions in *tcbC*, *clcA*, and *tfdC*. Discrete amplified fragments were obtained in this manner, which were cloned and analyzed by hybridization and DNA sequencing. We found six different types of fragments which had the expected size, only one of which was related significantly to the chlorocatechol 1,2-dioxygenase (and in fact was identical to the *tcbC*-type). This indicated that it was possible to detect and isolate chlorocatechol 1,2-dioxygenase sequences from soil DNA although the selectivity of the amplification reaction was relatively low.

In this study, we have entered a field of microbial research which will have continuing evolutionary and environmental interest. A detailed genetic characteriza-

tion of bacteria which adapted to use xenobiotic compounds as novel growth and energy subsrates, suggested different mechanisms by which novel metabolic pathways evolve in bacteria. Our results presented evidence for i) a specialization of enzyme systems and ii) an exchange and combination of pre-existing gene modules. Still we do not know what the capacity of microorganisms present in the natural environment is to adapt rapidly to metabolize xenobiotic substrates, nor do we know how and which environmental factors influence genetic adaptation. Astonished by the diversity of genetic mechanisms displayed in bacteria which govern evolutionary change, we shouldn't be surprised to find mechanisms which direct and regulate genetic adaptation in response to changing environmental conditions.

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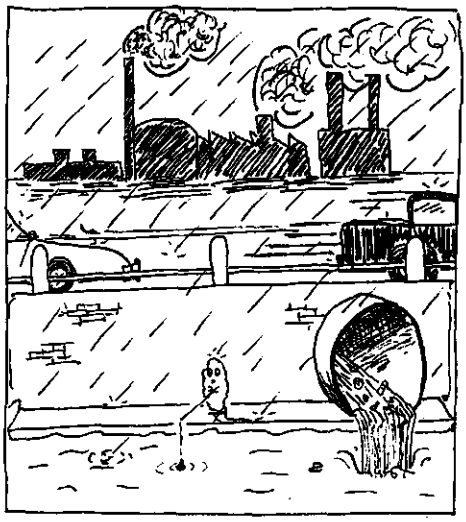
Samenvatting
Voor wie weten wil waar dit proefschrift over gaat

Bacteriën ..

Dit proefschrift gaat over bacteriën en over milieuverontreiniging. Wat hebben bacteriën en milieuverontreiniging met elkaar te maken? Om hierop een antwoord te kunnen geven, moeten we eerst iets weten over bacteriën. Bacteriën zijn er in alle soorten en maten (figuurlijk gesproken, want de meesten zijn niet groter dan 0,002 mm). Sommige soorten zijn minder aardig voor mens en dier (ziekteverwekkende bacteriën), maar andere zijn heel gewoon en doen nuttig werk. Waarom is het werk dat bacteriën doen nuttig? Net als alle andere levende wezens, hebben bacteriën voedsel nodig om te kunnen leven en te groeien. Maar dit voedsel is van zeer eenvoudige aard. Soms is het niet meer dan koolzuurgas (CO_2) en waterstof, andere keren is het glucose, azijnzuur en zuurstof. Sommige bacteriën kunnen wel honderd verschillende moleculen gebruiken als voedselbron, en andere maar enkele. De organische verbindingen (het voedsel) worden door de bacterie opgenomen en geheel of gedeeltelijk omgezet in andere verbindingen. Hoe doet een bacterie dit? Een hele machinerie van eiwitten (die men enzymen noemt) in de bacterie is voortdurend bezig om stapje voor stapje de organische verbindingen af te breken tot kleinere brokstukken (zie figuur 2). Hieruit kan een bacterie energie krijgen en tevens bouwmateriaal voor de instandhouding van zijn cel en voor de aanmaak van nieuwe cellen.



Figuur 1. Ten einde raad gingen de bacteriën op zoek naar nieuwe voedselbronnen.

..en milieuverontreiniging

Wat is milieuverontreiniging? Milieuverontreiniging is een omvattende term voor allerlei schadelijke aantastingen van ons leefmilieu. Ook milieuverontreiniging is er in soorten en maten. We moeten ons hier een beetje beperken, dus we zullen

het alleen hebben over milieuvervuiling die veroorzaakt wordt doordat schadelijke chemische verbindingen in het milieu terecht komen. Wie hiervoor verantwoordelijk is, is een heel andere kwestie, niet minder belangrijk, maar niet aan de orde. Hoe komen we er van af, dat is een vraag die we ons hier wel stellen. Veel van wat wij schadelijke verbindingen noemen, zijn chemisch gezien organische moleculen waar allerlei rare atomen en groepen aan vast zitten, zoals chlooratomen of nitrogroepen. Deze verbindingen kunnen hun schadelijke werking uitoefenen omdat ze niet of verkeerd omgezet worden door bestaande enzymen in levende wezens (zelfs niet in bacteriën). Hoewel? Onderzoekers hebben vastgesteld dat bacteriën zich relatief snel kunnen aanpassen aan bepaalde schadelijke verbindingen. Als je een potje met vervuilde grond van een vuilstortplaats een tijdje laat staan met verschillende schadelijke organische moleculen erin, tien tegen één dat je dan na verloop van tijd ziet dat deze verdwenen zijn en dat je een bacterie uit de grond kunt isoleren die deze stoffen als voedselbron kan gebruiken! Dit proefschrift gaat over de vraag hoe het kan dat bacteriën zich zo snel wennen aan nieuwe voedselbronnen. En dan niet zo maar aan "lekkere hapjes", zoals bijvoorbeeld suikers, maar aan vreemde organische moleculen die ons milieu vervuilen, zoals chloorbenzenen.

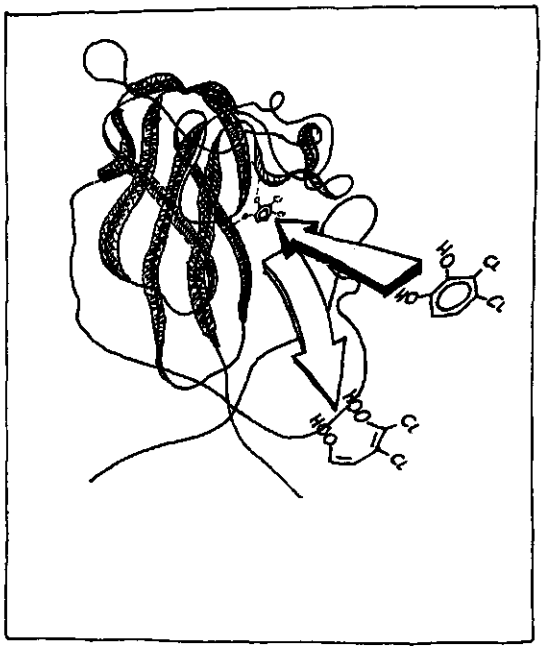
Bacteriën die chloorbenzenen als voedsel gebruiken

We weten nu al heel wat; bacteriën kunnen erg nuttig zijn omdat ze schadelijke verontreinigingen kunnen afbreken tot onschadelijke eindprodukten. Ook weten we dat wanneer je maar lang genoeg wacht, er bacteriën zullen gaan groeien, bijvoorbeeld in een potje vervuilde grond, die stoffen kunnen afbreken die nog niet eerder als voedselbron gebruikt konden worden. Iets dergelijks hebben we in dit onderzoek als eerste experiment gedaan. Zo verkregen we na lange tijd (een aantal maanden) een tweetal bacteriën die chloorbenzenen als voedsel konden gebruiken. Niet iedereen zal chloorbenzenen van dichtbij kennen, maar bijvoorbeeld de verbinding 1,4-dichloorbenzeen werd vaak toegepast in wc-blokjes, zogenaamd omdat het fris rook. De andere chloorbenzenen worden veel gebruikt als oplosmiddel in de industrie om van alles en nog wat te ontvetten. Eén van deze twee bacteriën, behorend tot de soort *Pseudomonas*, kon het grootste aantal verschillende chloorbenzenen afbreken; daarom besloten we om deze bacterie in ons onderzoek verder te gebruiken. We hebben ons toen vooral gericht op de vraag wat er nu zo bijzonder aan deze bacterie was; waarom net deze *Pseudomonas* chloorbenzenen als voedsel kon gebruiken en waarom andere bacteriën niet. Verder hebben we geprobeerd er achter te komen hoe de eigenschappen van deze bacterie

waren ontstaan, en of er nog andere bacteriën met vergelijkbare eigenschappen in de natuur voorkwamen. Dit alles zou ons misschien kunnen helpen te begrijpen hoe bacteriën in de natuur zich kunnen aanpassen aan schadelijke verbindingen.

Aanpassing van bacteriën

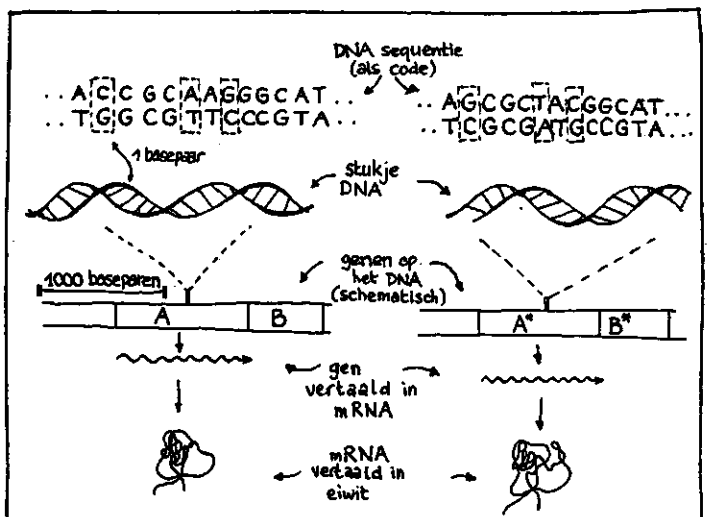
Misschien is het goed om nog een stapje dieper in te gaan op dat "aanpassen" van bacteriën aan nieuwe voedselbronnen. Wát is het in de bacterie dat zich aanpast? Verschillende onderdelen van de "spijsvertering" van de bacterie kunnen hierbij een rol spelen. Op de eerste plaats moet een bacterie een molecuul naar binnen krijgen (bacteriën hebben geen "mond"), en moet het dan kunnen herkennen als potentieel voedsel. Hiervoor zijn in een bacterie gespecialiseerde eiwitten aanwezig. Maar het kan best zijn dat deze het nieuwe voedsel niet herkennen, omdat ze gericht zijn op een iets andere verbinding. Op de tweede plaats moet een bacterie de nieuwe verbinding ook daadwerkelijk kunnen verteren. Voor de vertering bezit een bacterie zoals we gezien hebben enzymen, die stapje voor stapje een verbinding af kunnen breken tot brokstukken die voor de bacterie nuttig zijn. Als de bacterie normaal gesproken heel andere voedingsbronnen gebruikt, kan het best zijn dat de juiste enzymen niet door de bacterie aangemaakt kunnen worden, waardoor nieuwe verbindingen niet omgezet worden. In het beste geval lijken schadelijke verbindingen erg op natuurlijk voorkomende moleculen die wel als voedsel worden gebruikt. Dan kunnen ze soms een klein beetje worden omgezet door de bestaande enzymen van een bacterie. Maar



Figuur 2. Schematische voorstelling van een eiwitmolecuul. Het eiwit is hier weergegeven als een lange, opgewikkelde slinger. De slinger wordt gevormd door de afzonderlijke aminozuren waaruit de eiwitketen is opgebouwd. Dit is maar één voorstelling die een beetje de vorm en struktuur van het eiwit weergeeft. In werkelijkheid is er eigenlijk geen loze ruimte in het eiwit. Het eiwit wat hier is uitgebeeld is een enzym. Moleculen kunnen naar "binnen" (zie pijl), waar de omzetting plaats vindt, en gaan dan weer naar buiten. De plaats in het reactieve centrum van het enzym is dan weer vrij voor een volgend molecuul. Het enzym hier afgebeeld kan deze omzetting onder optimale omstandigheden ongeveer 10.000 keer per seconde uitvoeren.

sommige enzymen zijn erg selectief. Alleen bepaalde moleculen kunnen worden omgezet en als daar bijvoorbeeld één extra chlooratoom aan vast zit, dan gebeurt er niets meer.

Om dit nog eens te herhalen; er zijn dus drie belangrijke redenen waarom de meeste bacteriën niet zomaar in staat zijn om een vreemde verbinding af te breken: i) de verbinding wordt niet als voedsel herkend, ii) de bacterie kan niet de juiste enzymen maken om de verbinding te verteren, en iii) er zijn wel enzymen, maar die doen het niet of ze doen het onvolledig. Wat voor mogelijkheden zijn er nu voor bacteriën om zich aan te passen? Op de eerste plaats is het mogelijk dat enzymen en voedselherkenningseiwitten een beetje veranderen. Dit is het gevolg van veranderingen die in het erfelijk materiaal van de bacterie kunnen optreden (zie figuur 3). Op de tweede plaats is het mogelijk dat een bacterie de beschikking krijgt over nieuw erfelijk materiaal, en zo eiwitten kan aanmaken die hij voorheen niet kon maken. Dit is mogelijk doordat genetische informatie van de ene naar de andere bacterie kan worden overgedragen (zie figuur 4). Ik wil deze twee punten verder proberen uit te leggen aan de hand van het onderzoek dat we gedaan hebben aan onze voorbeeldbacterie, *Pseudomonas* (we hebben hem P51 genoemd, naar zijn nummer in de catalogus van de vakgroep), die chloorbenzenen kan als voedsel kan



Figuur 3. Enkele genetische begrippen. Het erfelijk materiaal (het DNA) heeft de vorm van een langgerekt molecuul. Twee strengen spiraleren om elkaar heen, en zijn met elkaar verbonden door waterstofbruggen tussen de basen van het DNA. Er zijn vier verschillende basen: adenine (afkorting A), guanine (G), cytosine (C) en thymine (T). Slechts twee baseparen worden normaal gevormd: A - T, en C - G. De volgorde van de basen (de DNA-sequentie) wordt meestal als code geschreven, bijvoorbeeld ACCGCAAGGGCAT (zie de figuur). Schematisch wordt het DNA vaak weergegeven als twee parallelle lijnen met als lengtemaat het aantal baseparen. Tussen de lijnen kun je de grootte, plaats en richting van een gen weergeven (A, B). De basenvolgorde van een gen wordt in de cel vertaald in RNA, en dit dient als matrijs voor de aanmaak van het eiwit. Veranderingen in de basenvolgorde van een gen (bijvoorbeeld A en A) geven aanleiding tot een andere vertaling in mRNA en een ander eiwit. De genen A en A* die hier staan getekend, zijn erg verwant (de basenvolgorde in het DNA is bijna hetzelfde). Door evolutionaire processen kunnen genen in hun basenvolgorde steeds meer van elkaar gaan verschillen. Dit vormt de basis voor nieuwe eigenschappen.*

gebruiken.

Het bijzondere aan de bacterie *Pseudomonas* P51

Het is nu nodig dat we onze bacterie P51 eens gaan open maken en kijken welke bijzondere eigenschappen hij bezit. In ons onderzoek hebben we vooral gekeken naar erfelijke eigenschappen van P51. In bacteriën zijn de erfelijke eigenschappen gelegen op één groot langgerekt molecuul, het chromosoom. Het chromosoom is gemaakt van DNA (dit is de veelgebruikte afkorting voor de chemische naam van het erfelijke materiaal). Daarnaast bezitten bacteriën vaak nog kleinere DNA moleculen die men plasmiden noemt. Het is voor onderzoekers gebruikelijk om de grootte van DNA moleculen weer te geven in het aantal baseparen (zie figuur 3). Plasmiden zijn er in groottes van 2000 baseparen tot zo'n 750.000 baseparen. Een bacteriechromosoom is al gauw tussen 2 en 7 miljoen baseparen. Van stam P51 hebben we niet alle miljoenen baseparen in kaart gebracht. We hebben een plasmide onderzocht in deze bacterie, dat een grootte had van ongeveer 110.000 baseparen. Dit plasmide bleek een bijzondere functie voor de bacterie te hebben, want zonder het plasmide kon de bacterie niet langer chloorbenzenen afbreken. Op het plasmide DNA waren de erfelijke eigenschappen aanwezig voor enzymen en herkenningseiwitten, waarmee de bacterie chloorbenzenen als voedsel kon herkennen en kon verteren. Twee kleine gebiedjes op dit plasmide DNA, ieder van ongeveer 7000 baseparen, bleken bij nader onderzoek de genen voor deze eigenschappen te bevatten. Van de afzonderlijke genen (we zullen ze verder de "chloorbenzeen-genen" noemen) werd ook precies de volgorde van de DNA baseparen bepaald (zie figuur 3).

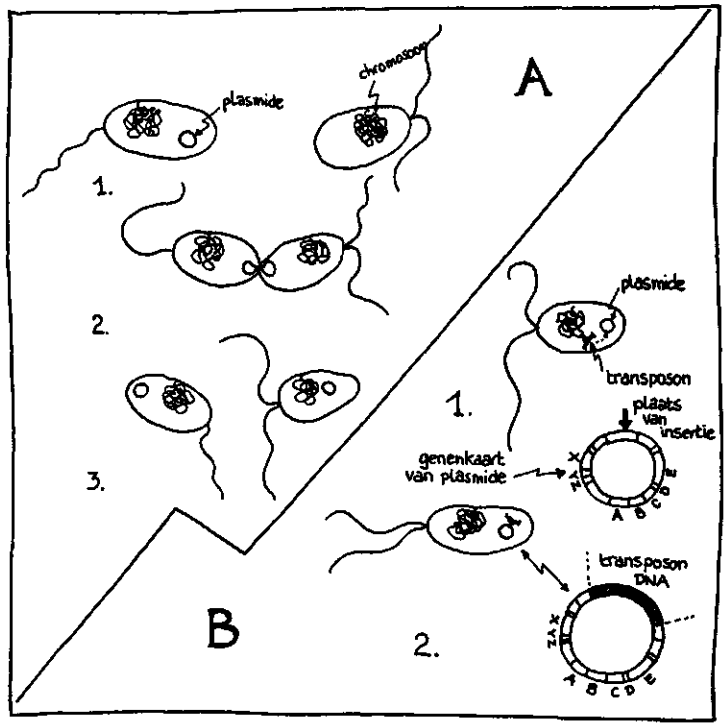
Verwante genen en veranderingen in het DNA

Het bijzondere van bacterie P51 bleek pas, toen we de chloorbenzeen-genen konden gaan vergelijken met genen van andere bacteriën. Met behulp van de basenvolgorde konden we de verwantschap tussen de genen van bacterie P51 en van verschillende andere bacteriën onderzoeken. Toen we dit deden, bleek dat soortgelijke genen als die we vonden in bacterie P51, ook voorkwamen in andere bacteriën. Ook verschillende van de enzymen waarmee P51 chloorbenzenen kon verteren, bleken bij andere bacteriën voor te komen. Alleen, de basenvolgordes van deze verwante genen uit verschillende bacteriën waren niet *precies* hetzelfde. En voor de soortgelijke enzymen bleek, dat ze niet precies hetzelfde *konden*. Voor het ene of het andere enzym van dezelfde klasse maakte het wel degelijk uit of er een extra chlooratoom aan een molecuul zat. (De enzymen uit bacterie P51 konden hier duidelijk beter mee overweg.)

Onderzoekers menen dat verwantschap van genen iets zegt over de evolutie ervan. Doordat er allerlei veranderingen in de basenvolgorde van het DNA kunnen optreden (bijvoorbeeld als gevolg van mutaties), kan een gen in een verre voorouder zich geëvolueerd hebben tot vele afgeleide, verwante genen. Op deze manier kunnen toevallige veranderingen in de basenvolgorde van een gen, aanleiding geven tot kleine veranderingen in een enzym, dat immers vertaald wordt vanuit deze basenvolgorde (zie figuur 3). Hier ligt een belangrijk mechanisme voor aanpassing van bacteriën. Een enzym kan een iets andere werking krijgen door een verandering in de basenvolgorde van een gen, van waaruit het enzym vertaald wordt. Dit iets veranderde enzym is nu misschien in staat om wel het nieuwe, vreemde voedsel om te zetten. Voor zover we weten, is het ontstaan van veranderingen in het DNA een toevallig proces; als een verandering aanleiding geeft tot een beter enzym, dan kan de bacterie die deze verandering in zijn DNA heeft, beter overleven dan een ander. Zijn nakomelingen zullen dan allemaal deze genetische verandering hebben. Dit is het proces dat men natuurlijke selectie noemt. Bacterie P51 heeft blijkbaar een aantal veranderingen in zijn DNA ondergaan, waardoor hij "betere" enzymen kon aanmaken om chloorbenzenen af te breken. Wat we nog niet goed begrijpen is de snelheid waarmee dit proces van selectie is verlopen bij bacteriën als P51. Het lijkt er op dat deze selectie veel sneller gaat dan we zouden verwachten op grond van het optreden van toevallige mutaties in het erfelijk materiaal.

Overdracht van genen

Dit was nog niet het eind van het verhaal. Bacterie P51 bleek niet alleen een aantal "betere" enzymen te kunnen maken voor omzetting van chloorbenzenen, maar gebruikte ook een nieuwe combinatie van verschillende enzymen (het lijkt wel een wasmiddel). Toen we alle chloorbenzeen-genen en enzymen uit P51 met verwante genen en enzymen van andere bacteriën vergeleken, zagen we dat deze nooit allemaal tegelijk in één andere bacterie voorkwamen. Stel, bacterie P51 gebruikte zes enzymen voor de omzetting van chloorbenzenen tot eenvoudige brokstukken, enzym A, B, C, D, E, en F. Enzymen A en B konden we vinden bij sommige andere bacteriën, net als enzymen C, D, E, en F. Maar de combinatie nooit. Bacterie P51 had dus de beschikking gekregen over de erfelijke informatie voor zowel enzymen A en B, als C, D, E, en F. Zoals we gezien hadden, lagen deze erfelijke eigenschappen op een plasmide in twee gebieden. Bij nader onderzoek bleek dat de erfelijke eigenschappen voor enzymen A en B onderdeel waren van een niet stabiel stuk DNA. Dit stuk DNA kon zichzelf verplaatsen naar



Figuur 4. Overdracht van erfelijke informatie. Genen liggen wel min of meer "vast", maar DNA is toch in beweging. In A staat weergegeven hoe een plasmide van de ene bacterie naar de andere gaat. Het plasmide DNA komt zo in een andere bacterie terecht, die nu de beschikking krijgt over nieuwe eigenschappen. Niet alleen plasmide DNA is mobiel; kleine stukjes DNA op een plasmide of op het chromosoom kunnen ook bewegen, bijvoorbeeld transposons. In B staat getekend hoe een transposon vanaf het chromosoom in een plasmide inserteert ("springt"). Op dit plasmide komt zo nieuw DNA terecht met de erfelijke eigenschappen van het transposon. Deze eigenschappen kunnen zich daarna verder verspreiden door bijvoorbeeld overdracht van het plasmide naar een andere bacterie.

een ander gebied van het DNA. Het was al veel langer bekend dat bacteriën dit soort mobiele stukjes DNA hebben. Men noemt ze "transposons" (zie figuur 4). Het bijzondere van dit transposon was dat er een aantal chloorbenzeen-genen op voorkwamen. We denken nu dat dit transposon ervoor heeft gezorgd dat een aantal genen vanuit een *andere* bacterie in P51 terecht zijn gekomen. In die andere bacterie werden deze genen niet gebruikt voor chloorbenzeen vertering. Maar in P51 vormden ze een nieuwe combinatie met andere (al aanwezige) erfelijke eigenschappen. Al deze genetische informatie samen in één bacterie was voldoende voor de aanmaak van enzymen die de volledige omzetting van chloorbenzenen konden bewerkstelligen.

Natuurlijke selectie, snelle evolutie van eigenschappen, overdracht van genen... Hoe meer je er over nadenkt, hoe vreemder het wordt; maar het is eigenlijk een wilde wereld daar in dat DNA.

Curriculum vitae

Als typisch kind van de zestiger jaren werd Jan Roelof (geboren in1961 te Leeuwarden) al ras aangetrokken tot het grote leed van de wereld, de milieuverontreiniging. Tijdens zijn gymnasiale opleiding in Leeuwarden (1973-1979) dwaalde hij eerst nog wel eens af door zijn blikken alleen omhoog te werpen - van de Aarde af - in de richting van sterren en planeten, maar eenmaal gesteld voor de belangrijkste keuze na het eindexamen, viel het pleit voor de studie Milieuhygiëne in plaats van sterrenkunde. Hij begon de studie in Wageningen met zeer veel inzet (1979), maar kwam toen toch in een periode van diepe twijfel over de keuze van zijn studie terecht (1980), want deze kon (helaas) niet aan zijn verwachtingen voldoen. Veel discussiegroepjes later, pas toen het mogelijk was om zélf onderzoek te gaan doen - bij de sectie Bodemverontreiniging en Bodemhygiëne - keerde het enthousiasme bij hem terug. Onverwacht - voor hem zelf - werd hij aangetrokken tot het milieuonderzoek aan de vakgroep Microbiologie en de wondere wereld van de microkosmos (dus toch!). Wellicht droeg zijn enthousiasme er toe bij dat hij na zijn afstuderen (in 1985) kon blijven werken aan de vakgroep en zich kon omscholen tot een halve moleculair bioloog in een promotieonderzoek (1986-1990). Gelukkig hoeft een mens nooit te oud te zijn om iets nieuws te leren, als hem deze mogelijkheid maar geboden wordt. Het had uiteindelijk heel wat voeten in de aarde, maar na drie jaar kwam het onderzoek op gang en kon uiteindelijk een proefschrift geschreven worden. Momenteel verricht Jan Roelof zijn vervangende diensttijd aan het NIZO in Ede, waar hij tijdelijk het milieuonderzoek heeft verruild voor wetenschappelijk zuivelonderzoek. Hierna zal hij de grote sprong in het duister van een langdurig buitenlands verblijf gaan wagen.