細菌のクロロカテコール及び2,4-ジクロロフェノキシ酢酸分解遺伝子群の構造と転写調節
The Structure and Transcriptional Regulation of the Bacterial Degradative Genes for Chlorocatechols and 2,4-Dichlorophenoxyacetate

Naoto Ogawa*

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The structures of bacterial genes for the degradation of chlorocatechols and 2,4-dichlorophenoxyacetate were studied and were deduced to have been formed by some genetic recombination events including transposition. In vivo and in vitro analysis of chlorocatechol degradative operon cbnR-ABCD of a 3-chlorobenzoate degradative bacterium *Ralstonia eutropha* NH9 showed the transcriptional activation of the cbnA promoter by the LysR-type regulator CbnR upon recognition of the inducer molecules, (chloro)muconates produced from (chloro)catechols. Further in vivo analysis of LysR-type transcriptional regulators of (chloro)aromatic degradative operons indicated evolutionary divergence of these regulators in terms of the inducer recognition specificity and suggested specialization of some regulators of chlorocatechol operons for recognition of (chloro)muconates.

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* Department of Environmental Chemistry
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Chapter I Introduction

Contamination of environment and its adverse effects are some of the major problems that we are facing today. The environment is the shared gift to all organisms and thus responsibilities of all people, no matter where they live. Global contamination of environment is so severe, and accelerating so rapidly, that we will soon be forced to accept protection of the environment as our first priority as a matter of sheer survival (Drucker, 1994). Bioremediation is the process using microorganisms to transform hazardous chemical compounds in an environment to nonhazardous end products. It is an effective method of decontaminating pollutants in the environment, with less environmental impact than other methods (Bandyopadhyay et al., 1994; Fletcher, 1994). Most of other methods are less cost effective and are not suitable for the in situ treatment. Some of them are not effective in treating a complex array of different pollutants. Biological treatment appears to offer solutions to these limitations. The basic advantages of bioremediation can be outlined as follows (Bandyopadhyay et al., 1994): (1) Bioremediation is a natural process in which naturally occurring microorganisms are used for treatment of the wastes. (2) The residues or the by-products of biological processes (CO₂, H₂O) are usually geochemically cycled in the environment as harmless products. (3) Microorganisms have a wide range of abilities to metabolize different chemicals. (4) It is possible to utilize and improve native microorganisms that have
been demonstrated to degrade the pollutants on the site. In some cases, microorganisms from another site, which are known to metabolize the pollutants, can be introduced to improve biodegradation. (5) For treatment of soils, sludge, and ground water, bioremediation is less expensive and less disruptive than options frequently used for treatment, such as excavation followed by incineration or landfilling.

Since bioremediation harnesses the activities of the degradative enzymes that microorganisms produce, it is critical to study the biochemical and genetic basis of microbial degradation to find ways to improve the degradative ability of microorganisms. Furthermore, functional expression of the genes is essential for the production of appropriate degradative enzymes. Various environmental factors are known to activate or repress gene expression and thereby modulate microbial activities (Bandyopadhyay et al., 1994; Daubaras and Chakrabarty, 1992). To develop methods of bioremediation, it is imperative that we learn how the environment controls the expression of microbial genes and the formation of degradative pathways in microorganisms (Daubaras and Chakrabarty, 1992). To obtain insights for future application to bioremediation and for evolution of degradative genes, structure of bacterial genes for biodegradation and their expression are examined and discussed in this study.

Among all types of pollutants, chlorinated aromatic compounds pose one of the most serious contemporary environmental problems worldwide, because they have been produced and used in large quantities. Agrochemicals such as herbicides and pesticides have been used and some of them have accumulated in the environment. Chlorinated aromatics including solvents and polychlorinated biphenyls (PCBs) have been used in industry and released into the environment accidentally. By-products of the above chemicals, such as chlorinated dioxins, have been unintentionally produced and released into the environment. Although some of these compounds persist in the environment for long periods, others are degraded by microorganisms in soil and water (Alexander, 1981). In the oxidative bacterial degradation of chlorinated aromatics, the modified ortho-cleavage pathway plays a pivotal role in the degradation of chlorocatechols produced through converging pathways from various chlorinated aromatics (Fig. 1) (reviewed in Daubaras and Chakrabarty, 1992; Harwood and Parales, 1996; Reinke, 1998; Schlimann, 1994; van der Meer et al., 1992, van der Meer, 1997). Chlorocatechols, however, are toxic to bacterial cells (Fritz et al., 1992) and cannot be used as substrates for growth of bacteria in most cases. Undisturbed growth of organisms with some chlorinated aromatics requires well-balanced production of the enzymes involved in the degradation to avoid accumulation of toxic metabolites such as chlorocatechols (Fritz et al., 1992). On the other hand, chlorobenzoates, which can be growth substrates of bacteria, have been used frequently to study the degradation of chlorinated aromatics because they are intermediates of cometabolism of PCBs and are soluble in water (Chatterjee et al., 1981). Among the isomers of chlorobenzoates, 3-chlorobenzoate (3-CB) has been a representative model substrate for degradation and is degraded through 3- or 4-chlorocatechol via the modified ortho-cleavage pathway in the most common cases of aerobic soil bacteria (Fig. 1). The herbicide 2,4-dichlorophenoxyacetate (2,4-D) has been another representative model of biodegradation because it has been a major agrochemical and is soluble in water. 2,4-D is also degraded via the modified ortho-cleavage pathway through 3,5-dichlorocatechol (Fig. 1). Thus, bacterial genes for degradation of 3-CB or 2,4-D are characterized in this report, concentrating on those encoding the enzymes of the modified ortho-cleavage pathway because of their indispensable role.

Three evolutionarily related sets of genes of gram-negative bacteria that encode enzymes in the modified ortho-cleavage pathway have been well described. These are clcABDE, tfdCDEF, and tcbCDEF. The clcABDE genes are responsible for degradation of 3-chlorocatechol (3-CC) and were cloned from plasmid pAC27 of Pseudomonas putida A C866, which is a 3-CB-degrading bacterium (Chatterjee and Chakrabarty, 1982; Chatterjee et al., 1981; Frantz and Chakrabarty, 1987; Kasberg et al., 1997). The tfdCDEF genes are present on plasmid pJP4 and are responsible for the
degradation of 3,5-dichlorocatechol, which is produced from 2,4-D, by the products of the tfdAB genes in Ralstonia eutropha (Alcaligenes eutrophus) JMP134 (Don and Pemberton, 1981; Don et al., 1985; Perkins et al., 1990). The tcbCDEF genes are located on plasmid pP51 and are responsible for the degradation of 3,4,6-trichlorocatechol, generated from 1,2,4-trichlorobenzene by the products of the tcbAB genes in Pseudomonas sp. P51 (van der Meer et al., 1991a, c). These three gene clusters of gram-negative bacteria are thought to have evolved from common ancestral chlorocatechol genes (van der Meer et al., 1991a). Recently, the study of both of the catechol and the chlorocatechol ortho-cleavage pathway of Rhodococcus opacus 1CP has shown that the chlorocatechol ortho-cleavage genes of this strain have evolved independently of those of gram-negative bacteria from common origin of the catechol ortho-cleavage genes in all bacteria (Eulberg et al., 1997; Eulberg et al., 1998).

The process of dissemination of the genes for biodegradation is interesting from the viewpoint of evolution of bacterial strains capable of biodegradation. The worldwide distribution of genes for chlorocatechol degradation has been speculated by several isolates from different places (Amy et al., 1985; Bhat et al., 1994; Chatterjee and Chakrabarty, 1983; Chatterjee et al., 1981; Chaudhry and Huang, 1988; Don and Pemberton, 1981; Ka et al., 1994; Mie et al., 1993; Matheson et al., 1996; Suwa et al., 1996; Top et al., 1995; van der Meer et al., 1991c) and has recently been demonstrated more systematically by the studies by Fulthorpe et al. (Fulthorpe et al., 1995; Fulthorpe et al., 1998; Leander et al., 1998). However, as to the means of the dissemination of the gene clusters, there have been only a few examples of identical or highly homologous plasmids carrying the modified ortho pathway genes to indicate gene distribution by plasmid transfer (Amy et al., 1985; Chatterjee and Chakrabarty, 1983; Don and Pemberton, 1981). Although the similar operon-like structures of the genes for the modified ortho-cleavage pathway suggest they might have spread as parts of transposable elements, there have been only a few documented examples of transposable elements that carry the modified ortho pathway genes. This sparsity makes clear contrast to abundant reports on other catabolic transposons, such as the toluene transposons on TOL plasmids and the catabolic genes mobilized by IS1071 (Nakatsu et al., 1991) (for reviews, see Di Gioia et al., 1998; Tsuda, 1996; Tsuda et al., 1999; Wyndham et al., 1994). The examples of transposable elements carrying modified ortho pathway genes include the ISJ4P composite transposon (Leveau and van der Meer, 1997) and the clc element (Ravatn et al., 1998b). The ISJ4P composite transposon carries the genes tfdSRD, C, E, F, B, K on plasmid pJP4 in R. eutropha JMP134. The 105-kb clc element has recently been found to carry

Fig. 1. Pathways for degradation of chlorinated aromatic compounds via modified ortho-cleavage pathway.
the clcRABDE genes in Pseudomonas sp. strain B13 (Ravatn et al., 1998a). This element also takes the form of the plasmid pB13 especially at stationary phase of the growth of the cell and integrates into chromosomes of various bacterial strains at glycine tRNA structural genes using a site-specific recombinase, Int-B13, which belongs to the bacteriophage P4 integrase family (Ravatn et al., 1998b). The clc element has been proposed to be considered as a “degradation island” (Ravatn et al., 1998b).

In contrast to the above two transposable elements, this report presents an example of the class I composite transposon containing the modified ortho pathway genes which are captured between the two identical insertion sequences (ISs).

A few reports of transposons that carry the genes for degradation of chlorinated aromatics other than 3-CB have appeared in the literature (reviewed in Tsuda, 1996; Tsuda et al., 1999; Wyndham et al., 1994). The tcbAB gene cluster of plasmid pP51 encoding enzymes that convert 1,2,4-trichlorobenzene to 3,4,6-trichlorocatechol, is located on a composite transposon (Tn5280) with two slightly different insertion sequences (IS1066 and IS1067), in inverted orientation, at its ends (van der Meer et al., 1991d). A 17-kb segment of plasmid pBRC60, specifying enzymes for degradation of 3-CB in R. eutropha BR60, was found to be a composite transposon (Tn5271) flanked by directly repeated 3.2-kb segments of class II insertion sequences (IS1071) (Nakatsu et al., 1991). Strain BR60 is unique in that it degrades 3-CB through protocatechuate meta-ring-fission. The catabolic genes of strain BR60 have derived from origins which are different from that of the modified ortho-cleavage pathway genes (Nakatsu and Wyndham, 1993; Wyndham et al. 1988). The catabolic transposon Tn4371 (59 kb) found in R. eutropha A5 carries the genes to convert biphenyl and 4-chlorobiphenyl to benzoate (Ben) and 4-chlorobenzoate, respectively (Springael et al., 1993). This transposon has an integrase system and conjugative transfer genes, which are characteristics of the conjugal transposons. Although the conjugative transfer of Tn4371 has not been demonstrated, its transposition between replicons has been observed and the second mobile element, Tn-bph, which shares the right half of Tn4371 was shown to transfer by conjugation (Merlin et al., 1999).

In general, the processes of the dissemination of the degradative genes are not clear in terms of their evolutionary time scale. In this study, however, the strong homology between the cbnR-ABCD genes and the tcbR-CDEF genes illustrates the recent horizontal transfer of these genes for the modified ortho cleavage pathway.

Genetic rearrangements of the degradative genes by general recombination have been another focus of interest. There are a few reports of genetic recombination related to the degradation of 3-CB. Amplification and deamplification of the 4.3-kb fragment that contains the structural genes for degradation of 3-CC was demonstrated in Pseudomonas sp. strain B13 (Rangnekar, 1988). Amplification of cloned fragments containing the clc structural genes from plasmid pAC27 and from pJP4 was reported (Ghosal et al., 1985). Inverted duplication of catabolic genes on pJP4 and pre-existing tandem duplication of catabolic genes on a related plasmid, pJP2, were also described (Ghosal and You, 1988; Ghosal et al., 1985). Although each of these events seemed to have been mediated by homologous recombination, the nucleotide sequences responsible for the recombination have not been determined in any of these cases. The amplification and deamplification of the clc element in several bacterial strains have been described (Ravatn et al., 1998a). While the deamplification of the element was attributed to homologous recombination, the process of amplification has not been elucidated.

This study underscores the significance of the recombination events, including general recombination, in the assembly and reconstruction of the gene clusters for the degradation of chlorinated aromatics with two examples, 3-CC degradative genes on plasmid pENH91 in R. eutropha NH9 and 2,4-D degradative genes on plasmid pMAB1 in Alcaligenes sp. CSV90.
Diversities are exhibited not only in the overall structures of the gene clusters and the sequences of the respective degradative enzymes but also in the regulation of the expression of the genes. Sequence homology and overall structural similarity among the modified ortho-cleavage pathway operons and the catechol ortho-cleavage pathway operon catBCA (Aldrich et al., 1987; Houghton et al., 1995) indicate an evolutionary relationship among them (Schlömann, 1994; van der Meer et al., 1992). Each operon has a lysR-type regulatory gene (Schell, 1993), which is located upstream of and is divergently transcribed from the degradative gene cluster (Coco et al., 1993; Rothmel et al., 1990; van der Meer et al., 1991b). For the tfdCDEF operon of plasmid pJP4, however, tfdT, the lysR-type regulatory gene originally located upstream of the operon, is inactivated by an insertion-sequence element, and its function has been taken over by distantly located tfdR (Leveau and van der Meer, 1996; You and Ghosal, 1995).

With regard to the regulatory mechanism of the expression of the degradative genes, the operons catR-BCA and clcR-ABD have been studied both in vivo and in vitro (Chugani et al., 1998, 1997; Coco et al., 1994, 1993; Houghton et al., 1995; McFall et al., 1997a, b, c; Parsek et al., 1995, 1994b,c, 1992; Rothmel et al., 1990; reviewed in McFall et al., 1998). The regulators CatR and ClcR bind specifically to the catB and clcA promoter regions, respectively, and activate the expression of the degradative genes upon recognition of inducer. The inducers of the catBCA and clcABD operons have been identified as intermediates of their respective pathways, cis,cis-muconate (CCM) (Parsek et al., 1992) and 2-chloro-cis,cis-muconate (2CM) (McFall et al., 1997c). In the 2,4-D degradation process, 2,4-dichloro-cis,cis-muconate (2,4-DCM), the intermediate produced by TfdC, has been identified as an inducer of the tfdCDEF operon in vivo (Filer and Harker, 1997). The binding of the regulator TcbR to the tcbC promoter has been described (Leveau et al., 1994), and the role of tcbR has been described based on in vivo experiments (Leveau and van der Meer, 1996; van der Meer et al., 1991b). P. putida KT2442 harboring a plasmid containing the tcbR-CDEF genes was found to grow on 3-CB, while KT2442 containing the tcbCDEF genes and the inactivated tcbR gene grew on 3-CB at a much lower rate (van der Meer et al., 1991b). P. putida and R. eutropha strains harboring plasmids containing the tcbR-CDEF genes showed elevated (chloro)catechol 1,2-dioxygenase activity towards 3-CC after cultivation on 3-CB. This activity was not observed with cells of R. eutropha containing the tcbCDEF genes with tcbR inactivated (Leveau and van der Meer, 1996; van der Meer et al., 1991b). These results indicated that tcbR was required for the efficient expression of tcbC which encodes chlorocatechol dioxygenase.

The cbnR-ABCD operon on plasmid pENH91, found in a 3-CB degradative bacterium R. eutropha NH9, is highly homologous to the tcbR-CDEF operon (95.6%~100% identity at the amino acid sequence level) including 150-bp divergent promoter region identical to that of the tcb operon. It is responsible for the degradation of 3-CC. This report describes the results on transcriptional regulation of the cbnR-ABCD operon, which includes the identification of inducers and a change in the bending angle of the promoter region upon recognition of inducer. These observations illustrate a conserved transcriptional mechanism of regulation plus the independent evolution of inducer-recognition and DNA-binding properties among the regulators of the ortho-cleavage pathway. Together with the results from in vivo analysis of the transcriptional activation of the tfdC and the tfdA promoters by tfdS of Alcaligenes sp. CSV90, the divergence of the LysR-type regulators are discussed in terms of their inducer-recognition specificity.

Chapter II
Materials and Methods

Bacteria, plasmids, media, chemicals and enzyme assays. The strains, phages, and plasmids used in this study are listed in Table 1. All strains of Escherichia coli were grown in Luria-Bertani medium (Sambrook et al., 1989)
Table 1. Bacterial strains, phages, and plasmids used in this study

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<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
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<td>E. coli</td>
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<td>B378</td>
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<tr>
<td>B387</td>
<td>DH1(pPSA842) Ap' Sm' Tc' mob' tra Δcos</td>
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<td>XLI-Blue</td>
<td>supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac' F' [proA B lacI lacZ Δ M15 Tn10(εTet)]</td>
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<td>T G1</td>
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<td>DH5</td>
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<td>pET 16b</td>
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Strains of *Ralstonia eutropha* and *Pseudomonas putida* were grown at 30 °C. Strain NH9 was routinely maintained on basal salts medium [per liter: (NH₄)₂SO₄, 1.1 g; K₂HPO₄, 2.29 g; KH₂PO₄, 0.9 g; MgSO₄·7H₂O, 0.1 g; MnSO₄·4~6H₂O, 0.025 g; FeSO₄·7H₂O, 0.005 g; L-ascorbic acid, 0.005 g] that contained either 0.1% (ca. 6.39 mM) or 5 mM 3-chlorobenzoate (3-CB) (final pH 7.0). Luria-Bertani medium, nutrient broth medium (per liter: beef extract, 5 g; peptone, 5 g; NaCl, 2.5 g; [pH unadjusted]), glucose-yeast extract (GY) medium [per liter: glucose, 1 g; yeast extract, 1 g; K₂HPO₄, 0.5 g; MgSO₄·7H₂O, 0.2 g; Fe₂(SO₄)₃·7H₂O, trace; (pH 6.8)] were also used for strains of *R. eutropha*.

As a selective medium for the transconjugants of *R. eutropha* NH9D and *P. putida* KT2440, either Davis-minimal medium (K₂HPO₄, 7 g; KH₂PO₄, 2 g; MgSO₄·7H₂O, 0.1 g; (NH₄)₂SO₄, 1 g) or basal salts medium for NH9 (above) was used supplemented with 0.2% sodium citrate. M9 minimal medium (Sambrook et al., 1989) with 0.2% mannitol, 1 mM MgSO₄·0.1 mM CaCl₂, and 1 mM thiamine hydrochloride was used as the minimal medium for the selection of recipient strains in conjugations with transfers of plasmids from *R. eutropha* to *E. coli*. To obtain transformants of *P. putida* PRS4020, Pseudomonas Isolation Agar (Difco) was used.

Antibiotics were incorporated into media at the following final concentrations for the strains of *E. coli* and *R. eutropha*: ampicillin (Ap), 100 µg/ml; kanamycin (Km), 50 µg/ml; streptomycin (Sm), 25 µg/ml; tetracycline (Tc), 15 µg/ml. For *P. putida* PRS4020: carbenicillin (Cbpc), 1000 µg/ml; gentamicin (Gm) 10 µg/ml; Tc 200 µg/ml.

The ability of strain NH9 to grow on dichlorobenzene and on 1,2,4-trichlorobenzene was tested basically as described by van der Meer et al. (1991c). A preculture of NH9 was inoculated into liquid basal salts medium (above) supplemented either with 3.5 mmol of 1,2- or 1,4-dichlorobenzene/liter and was incubated at 30 °C.

2-Chloromuconate was produced by the enzymatic conversion of 3-chlorocatechol with chlorocatechol 1,2-dioxygenase (ClcA) by the method of McFall et al. (1997b). Quantitative determination of β-galactosidase activity in the reporter assay was performed by the method of Miller (Miller, 1972; McFall et al., 1997c). Each experiment was performed in triplicate.
DNA manipulations. Recombinant plasmids from E.coli were prepared either by the alkaline lysis method of Kieser (1984) or by the “small-scale preparations” method described by Sambrook et al. (1989). Total DNA from R. eutropha was prepared as described by Schmidt et al. (1986). Other recombinant DNA techniques including digestion with restriction endonucleases, DNA ligations and transformation of E. coli with plasmid DNA were performed basically as described by Sambrook et al. (1989). Plasmid pUC18, pUC19, pBluescript KS(-) and SK(-) were used routinely for subcloning of DNA fragments. Transformation of E. coli JM109, XLI-Blue, or DH5 was carried out either by the method of Hanahan (1983) or by electroporation with a Gene Pulser apparatus (Bio-Rad Laboratories, Richmond, Calif.) (Smith et al., 1990). Transformation of the plasmid pQF50 and its derivatives into P. putida PRS4020 were performed as described previously (McFall et al., 1997c). Mobilization of the plasmid pCP13 and its derivatives into P. putida PRS4020 and R. eutropha NH9D were conducted based on the method of Franklin (1985).

Cloning of genes for the catabolism of 3-CB. Large plasmids from R. eutropha were prepared by the method of Casse et al. (1979), followed by cesium chloride/ethidium bromide centrifugation. Plasmid pENH91 was digested partially with Sau3A to generate fragments of predominantly 30 to 50 kb and subjected to centrifugation in a 10% to 40% sucrose density gradient. Fractions containing DNA fragments of 30 to 50 kb were pooled. This DNA was inserted into BamHI-digested broad-host-range cosmid pPSA842, packaged with a packaging extract (Gigapack Plus; Stratagene) by the procedure described by the manufacturer, and transduced into E. coli B378. The individual cosmid clones were mobilized into strain NH9D, a 3-CB- derivative of NH9 that had been cured of plasmid pENH91, as described by Franklin (1985). Transconjugants were then screened for growth on plates that contained 0.1% 3-CB and streptomycin (25 µg/ml). Plasmid pKT230 was used for subcloning of the genes for catabolic enzymes to test complementation of growth. P. putida KT2440 was used as the host strain to test complementation of growth by DNA fragments subcloned in pKT230.

Conjugation and incompatibility test. Conjugation was performed as described by Franklin (1985). The conclusion of incompatibility tests was based on the observation of the appearance of the transconjugants of R. eutropha that retained the ability to use 3-CB and exhibited the phenotype of antibiotic resistance of introduced plasmid.

Southern hybridization. Southern blotting to nylon membranes (Hybond-N; Amersham International plc, A mersham, UK) was performed by the standard procedure (Sambrook et al., 1989) or with a vacuum blotting apparatus (VacuGene; Pharmacia LKB Biotechnology Inc., Uppsala, Sweden). DNA restriction fragments were labeled by nick-translation or random-priming (Sambrook et al., 1989) with [α-32P]dCTP (Amersham) except for cloning of the 3.7-kb SalI fragment from strain P51. Hybridizations were performed overnight at 42 °C as described by Sambrook et al. (1989). The hybridization solution consisted of (per 10 ml) 5.08 ml formamide, 0.50 ml Hapes (0.5 M, pH 7.5), 2.25 ml of a solution of nonhomologous DNA in water (1 mg/ml), 0.10 ml 100xDenhardt’s solution, 0.56 ml distilled water and 1.50 ml 20 x SSC. Washing was performed under high-stringency conditions: twice for 15 min with 2 × SSC at 65 °C, once for 30 min with 2 x SSC, 0.1% SDS at 65 °C, and then once for 10 min with 0.1 x SSC at 65 °C. Southern hybridization experiments for cloning of the 3.7-kb SalI fragment from strain P51 were performed with a DIGoxigenin Labeling Kit (Boehringer Mannheim, Mannheim, Germany) according to the protocol from the manufacturer.

Sequencing. For sequencing of the 3.3-kb SalI-SphI fragment containing DR2 and the relevant fragments containing the degradative genes of pENH91, they were cloned into M13mp18 and M13mp19, and nested deletions were introduced in both directions and clones with inserts of appropriate lengths were selected. Restriction fragments subcloned in pUC vectors and pBluescript vectors were also used for sequencing. Sequences were determined by the dideoxy chain-termination method (Sanger et al., 1977) with automated sequencers (373A [Perkin-Elmer-Applied Biosystems Inc.,
Foster City, Calif., A LFred [Pharmacia, Uppsala, Sweden], DSQ-1000L [Shimazu, Kyoto, Japan], and LIC-4200L2 [Li-Cor, USA], with the dye-primer or dye-terminator kits recommended by the respective manufactures.

**Construction of plasmids for the study of transcriptional activation by cbnR.** Plasmids to test growth complementation by cbnR were constructed as follows: a plasmid, pBLcbn2, containing the cbnA-BCD genes with cbnA promoter region in pBluescript KS(-) was constructed using a 5.6-kb insert from pEKC1 which was cut out by a unique EcoRI site in the cbnR-ABCD operon (This EcoRI site which is 4 bp within the N-terminal of cbnR will be referred to as EcoRI* and a XhoI site located downstream of the cbnD gene (Table 1). A 1.1-kb EcoRI fragment containing cbnR truncated at the EcoRI* site was cut out from pBLcbn1 and inserted into the EcoRI site of pBLcbn2 to restore the original structure of the cbnR-ABCD operon, yielding pBLcbn3. From pBLcbn2 and pBLcbn3, the relevant fragments were excised and inserted into the cloning site of pCP13, yielding pCbn13ABCD and pCbn13RABCD, respectively. The broad-host-range vector pQF50 was used to produce the transcriptional fusion constructs (Table 1, Fig. 12, 24, and 25).

To make constructs for expression of cbnR in E. coli, DNA fragments containing cbnR with NdeI and BamHI restriction sites were synthesized by PCR using plasmid pBLcbn1 as a template. A PCR-generated fragment using primers CBNR1 (5'-TTT TCA TAT GGA ATT CCG GCA GCT-3') and CBNR2 (5'-TTT TGG ATC CCT GTC CAG CGT GA-3') was digested and inserted into the cloning sites of pT7-7, yielding plasmid pT7cbnR. To make a construct of cbnR with 6 His codons on its carboxyl-terminus (cbnRHis), a PCR-generated fragment using primers CBNR1 and CBNRHis1 (5'-TTT TAA GCT TCA ATG ATG ATG ATG ATG ATG ATG CTG CCT CGT CAT GCA GAA CC-3') was digested with NdeI and HindIII and was inserted into pT7-7, yielding pT7cbnRHis. Nucleotide sequences of the PCR-derived inserts of the two plasmids were verified by sequencing analysis. In these two constructs, the initiation codons of cbnR or cbnRHis were ligated with the NdeI site of the vector and thus were located 8-bp downstream of a vector derived ribosomal binding site, and transcription was initiated from the T7 promoter of the vector. The insert cbnR was excised from pT7cbnR by digestion with NdeI and BamHI and cloned into pET16b, yielding pEHiscbnR. In this construct, cbnR was fused downstream of 10 His codons and a Factor Xa site.

To demonstrate that CbnRHis has the same function as wild type CbnR, plasmids were constructed as follows: A 0.95-kb EcoRI*-XbaI fragment containing cbnRHis truncated near the amino-terminal was excised from a pUC18-based construct which was made using the insert from pT7cbnRHis, and was inserted into XbaI-EcoRI* site of pBLcbn2. The resulting plasmid, pBLcbn3H, contained a reconstructed cbnR-ABCD operon with 6 His codons attached to C-terminal of cbnR. Relevant fragments from pBLcbn3H were used (Table 1) to construct pCbn13RHABCD for growth complementation test and pNO50RHAB for reporter assay in which cbnB' gene was connected to a promoterless lacZ.

A supercoiled template for in vitro transcription assay, designated pMPcbn1, was constructed using the vector pMP7. The inserted fragment spanned positions -163 to +254 with respect to the cbnA transcriptional start site.

**Purification of protein.** Induction and extraction of protein from E. coli BL21(DE3)pLysS containing a construct of pT 7-7 or pET 16b and further purification of the protein with the histidine tag were conducted according to the His·Bind resin manual (Novagen, Madison, Wis.). In order to partially purify CbnR and also to eliminate a contaminating protein of 27 kd from crude lysate of cells with pT7cbnRHis, a heparin-agarose column was used as described previously (Coco et al., 1994). The fractions that showed specific binding to the cbnA promoter fragment by gel retardation assay were pooled and concentrated by Centricon 10 (A micon, Mass.). To prepare the vector (pT 7-7) control for pT7cbnR, the fractions corresponding to the ones containing activity with pT7cbnR were used. CbnRHis was purified further by His·Bind resin.

**Gel retardation assay.** DNA binding reactions were performed in 20 µl volumes consisting of 10 mM HEPES (pH7.9), 10% glycerol, 100 mM KCl, 4 mM spermidine, 0.1 mM EDTA, 0.25 mM dithiothreitol, 1.5 µg of bovine serum
albumin, 5 μg of heparin (Sigma, H-3125), approximately 0.1 ng of a DNA fragment and the tested protein. The DNA fragments were synthesized by PCR and labeled internally with [α-32P]dCTP, followed by a purification procedure (Parsek et al., 1994a). The binding reactions were initiated by the addition of CbnR and incubated for 20 min at room temperature. The binding reactions were then electrophoresed through 5% native polyacrylamide gel in 0.5 X TBE buffer for 2 hours at 130 V with circulation of cooling water in Bio-Rad Protean apparatus. The DNA probe containing the 252-bp cbnR-A promoter region was made by PCR with primers, CBNFT1 (5′-TGT GCT GCA GCC ATG TTC CC-3′) and CBNFT2 (5′-AAT GCG GAC GCA ACC TGC TTC ACT CG-3′), and pBLcbn1 as a template. A 336-bp fragment containing a part of hydroxyquinol 1,2-dioxygenase gene from Burkholderia cepacia AC1100 was used as a nonspecific DNA binding probe. The fragment was synthesized by PCR using primers, ORF4P1 (5′-GCC TGC AGC GGC CCC TTC CAT GTG-3′) and ORF4P2 (5′-GCC TGC AGC CT C AAG CAT TTT G ACC-3′), and pKS100 as a template (Daubaras et al., 1995).

**S1 nuclease analysis.** Bacterial RNA was isolated via the RNeasy total RNA isolation kit (Qiagen, Chatsworth, Calif.) from a 9-ml culture of *P. putida* PRS4020 containing the plasmid pNO50RAB’ cultivated in Luria broth supplemented with 5 mM 3-CB. To prepare the DNA probe, primer CBNFT2 was end-labeled with T4 kinase (Gibco BRL, Gaithersburg, Md.) and [α-32P]ATP followed by PCR synthesis of a 252-bp fragment containing the promoter region with 2nd primer, CBNFT1 and pBLcbn1 as a template. The PCR product was purified by QIA quick PCR purification kit (Qiagen). This double stranded fragment was denatured by the addition of 1/10th volume of 2N NaOH, 2 mM EDTA, pH 8.0 and incubated at room temperature for 5 minutes. The denatured DNA was ethanol precipitated, washed with 70% ethanol and resuspended in water. The labeled DNA fragment was hybridized with the RNA at 45°C overnight and incubated with S1 nuclease using S1-Assay kit (Ambion, Austin, Tex.) according to the manufacturer’s instructions. Sequencing reactions to juxtapose the protected DNA fragment resulting from the S1 reaction were performed using SequiTherm cycle sequencing kit (Epicentre Technologies, Madison, Wis.) with CBNFT2 as the primer.

**DNase I protection assay.** DNase I footprinting experiments were conducted based on the method described previously (Parsek et al., 1994a), except that the binding reaction was performed in 20 μl solution consisting of 100 mM Tris-HCl (pH7.9), 1 mM EDTA, 4% glycerol, 100 mM KCl, 1 μg of bovine serum albumin, 1 μg of poly(dI-dC), approximately 10 ng of a DNA fragment plus the purified protein indicated. PCR was used to generate a 252-bp fragment spanning the cbnA promoter region using pBLcbn1 as a template. In each reaction, one of the primers, CBNFT1 or CBNFT2, was end-labeled with T4 kinase (Gibco BRL) and [α-32P]ATP as described previously (Parsek et al., 1994a). To locate the footprint, sequencing reactions were performed as described above with either of the end-labeled primers, CBNFT1 or CBNFT2, and pBLcbn1 as the template.

**In vitro transcription assays.** In vitro transcription assays were performed as described previously (Hershberger et al., 1995; McFall et al., 1997c). The supercoiled template, pMPCbn1 and *E. coli* holo RNA polymerase (Epicentre Technologies) were used. For each reaction, 100 ng of purified CbnRHis and 1 mM each of the chemicals tested as effector molecule were used.

**DNA bending by circular permutation gel shift assay.** Circular permutation gel shift assays were conducted by the same method as the gel retardation assay with the following modifications: the binding reactions contained 0.2 μg of purified CbnRHis, approximately 0.3 ng labeled DNA fragments and 2 μg heparin. In the inducer containing samples, CCM was added at 1 mM in the binding reactions and at 0.5 mM in the running buffer. Electrophoresis was performed at 130 V for 5 hours. Five DNA fragments of 257 bp containing the CbnR-binding sites at different positions from the ends to the middle were generated and labelled internally by PCR with [α-32P]dCTP using the following
primer pairs: MCBD1, 5'-GTT CGA TCC CGC GGT GGC TTC GCT CCA GAA GC-3' and MCBD2, 5'-GCC CCG GCC ATG CCG TCC AAT ACC-3'; MCBD3, 5'-TCC AGG GCT TGC ATC TGC CCG GTG ATG G-3' and MCBD4, 5'-TTG TCG GGT TGC CGG GTCC-3'; MCBD5, 5'-GAA ATA CTT GAG CTG CCGG-3' and MCBD6, 5'-TTT CCG GTT TGC CCG GTCC-3'.

The DNA fragments were purified with QIA quick PCR purification kit (Qiagen). The bending angles were calculated as described previously (McFall et al., 1997b).

Accession number of the nucleotide sequence. The nucleotide sequence of the 6959-bp SacI-KpnI region containing the cbnR-ABCD genes and the deduced amino acid sequences are deposited in the DDBJ, GenBank, and EMBL databases under accession number AB019032. The nucleotide sequence of IS1600 (DR2) and orfL (from the SphI site to outside of the KpnI site of pENH91 in Fig. 4) and the deduced amino acid sequences are deposited under accession number D64144. The nucleotide sequence containing the 1300-bp region from strain P51, homologous to part of IS1600, is deposited under accession number AB019033.

Chapter III
Results

1. The structure of the 3-chlorocatechol degradative transposon Tn5707

R. eutropha NH9, isolated in Japan, grew on 3-chlorobenzoate (3-CB) abundantly. The genes responsible for 3-CB degradation cloned from a large plasmid of NH9 were suggested to be those for the modified ortho-cleavage pathway by a hybridization experiment and thus further characterized. A deletion event of the degradative genes from the plasmid led to the elucidation of the composite transposon structure capturing the degradative genes on the plasmid.

(1) R. eutropha NH9 and its plasmids

R. eutropha NH9 was isolated from a soil sample at our institute under conditions where 3-CB was the sole source of carbon and energy, after repeated subculturing on 3-CB-containing mineral salts agar plates. Taxonomic identification was carried out by K. Katoh (Personal communication), and was confirmed later by analysis of the sequence of 16s ribosomal DNA.

Mutants of NH9 unable to utilize 3-CB (3-CB-) arose spontaneously at high frequency (>10%) after repeated subculture (5 times) on glucose-yeast extract (GY) liquid medium. It appeared, therefore, that some of the functions necessary for degradation of 3-CB were plasmid encoded.

With a slight modification to the method of Casse et al. (1979), it was shown that NH9 harbored two large plasmids (Fig. 2, Lane 1). The yield of the larger plasmid (designated pENH92) was much lower than that of the smaller one (pENH91). Many of the 3-CB- mutants obtained as independent colonies after subculture on GY medium had lost pENH91 (Fig. 2, Lanes 2-5, 7, 8). Although pENH91 appeared to be retained in one of the 3-CB- mutants (Lane 6; designated NH9d5), the plasmid seemed to be smaller than the original one. These results suggested that some of the genes essential for the degradation of 3-CB were plasmid encoded.

pENH91 was transferred from wild-type NH9 cells to spontaneous 3-CB-negative Smr cells by conjugation. Transfer into the recipient strain, which generated 3-CB-positive transconjugants, occurred at a frequency of 10^4 per donor cell.
pENH91 was tested for its incompatibility with pKT230, pPSA842 (IncQ) and RP4 (IncP). Plasmids pKT230 and pPSA842 were mobilized from E. coli S17-1 to NH9, and RP4 was transferred from E. coli B378 to NH9. The test was based on the observation of the appearance of transconjugants of R. eutropha, which can grow on 3-CB agar plates supplemented with either Km (for pKT230 and RP4) or Ap (for pPSA842). Transconjugants resistant to Km and Ap were obtained after introduction of pKT230 and pPSA842, respectively. The presence of the two plasmids (pENH91 plus either pKT230 or pPSA842) was verified after reintroduction of the plasmids into E. coli. The plasmids were identified by restriction analysis. Thus, pENH91 was found to be compatible with pKT230 and pPSA842. But it was found that pENH91 could not be maintained with RP4 in a stable manner. Thus, pENH91 was considered to be incompatible with RP4 and therefore is likely an IncP group plasmid. A physical map of pENH91 is shown in Fig. 3.

Fig. 2. Agarose gel electrophoresis of the plasmids from strain NH9 and 3-CB− segregants (0.7% gel and TBE buffer). Lanes: M, lambda DNA digested with HindIII; 1, R. eutropha NH9; 2 to 8, 3-CB− segregants of R. eutropha NH9.

Fig. 3. Restriction sites are abbreviated as follows: Bl, BamHI; Bg, BglII; E1, EcoRI; Ev, EcoRV; K, KpnI; N, NheI; S, Sad.
(2) Cloning of the genes for catabolic enzymes

A library of genes in pENH91 was constructed in E. coli B378 by use of the broad-host-range cosmid pPSA842. The individual cosmid clones were mobilized from E. coli into R. eutropha NH9D, a 3-CB− derivative that had been cured of pENH91, and transconjugants were selected on minimal agar plates that contained 3-CB and streptomycin. Among about 200 clones examined, eight had the 3-CB+ phenotype. A comparison of the restriction maps of the inserts of the positive clones showed that all included a common 13-kb region (Fig. 4). A physical map of pENH91 was then constructed by further restriction analysis (Fig. 3). For subcloning, a 9.2-kb SacI fragment (Fig. 4) from this 13-kb region was inserted into the broad-host-range vector pKT230 to yield pEKC1, which was mobilized into R. eutropha NH9D (3-CB−) and P. putida KT2440. Cells of both strains harboring pEKC1 grew on 3-CB-supplemented mineral salt agar plates. These results showed that the genes for catabolism of 3-CB were located within the 9.2-kb SacI fragment.
Hybridization with chlorocatechol degradative genes from *P. putida*

To identify the determinants responsible for catabolism of 3-CB on pENH91, hybridization experiments were conducted with the chlorocatechol catabolic genes of pAC27 (Chatterjee and Chakrabarty, 1982), which had been originally isolated from *P. putida* AC866, as a probe. The 4.3-kb fragment containing the clcABD genes of pAC27, isolated from an EcoRI digest of pDC100, hybridized to a 5.8-kb BamHI-BglII fragment from within the 9.2-kb SacI fragment (data not shown). Therefore, the catabolic genes on pENH91 appeared to encode enzymes of the modified ortho-pathway. However, the 5.8-kb BamHI-BglII fragment inserted into pKT230 did not confer the 3-CB+ phenotype on strain NH9D or strain KT2440. Thus, the 5.8-kb fragment did not contain all the genetic determinants necessary to confer the degradative ability of 3-CB.

Deletion of the catabolic genes from pENH91 by homologous recombination

A 12.5-kb region containing the 9.2-kb SacI fragment (Fig. 4) of pENH91 was found to have been deleted in the plasmid harbored by one of the 3-CB-negative segregants (Fig. 2, Lane 6 [designated pENH91d5]). (A result of hybridization experiments is shown in lanes 3 of the Fig. 8a and b). Two regions of pENH91, which encompassed either the left or right boundary region of the deleted portion, showed strong homology to each other in hybridization experiments (Fig. 5; fragments B and D in Fig. 4). A 2.5-kb EcoRI-SacI fragment (fragment A in Fig. 4) hybridized with a 2.3-kb KpnI-SacI fragment (fragment C) (data not shown). These results suggested that the two regions contained a
set of directly-oriented elements and that the deletion event had been due to homologous recombination between the two elements. On the restriction map (Fig. 4), the left element was designated as DR1, and the right element was designated as DR2. When the 2.0-kb SacI-KpnI fragment (fragment B in Fig. 4) isolated from pENH91 was used as a probe, only one hybridized band appeared for pENH91d5 instead of two bands for pENH91 (data not shown). Further restriction analysis with pENH91d5 showed that this plasmid retained one copy of the elements, and thus confirmed the deletion of catabolic genes was due to homologous recombination between the two elements.

(5) Identification of the direct repeats, DR1 and DR2, as identical ISs

After the boundary regions of DR1 and DR2 were determined by hybridization experiments, a 3.3-kb SalI-SphI fragment containing DR2 was subcloned into M13 vectors and pUC18 (yielding pEUDR2) and was sequenced. DR2 was 2,520 bp long including the inverted repeats (Fig. 6). Two long open reading frames (ORFs) were found on one DNA strand of DR2 (Fig. 6 and Fig. 7a). The amino acid sequences deduced from the two ORFs showed the highest homology to IstA and IstB of IS1326 (Brown et al., 1996) of the IS21 family (Fig. 7a). Since DR2 was found to have the perfect structure of an IS of the IS21 group, it was designated IS1600 and the two genes were designated istA and istB (Fig. 6 and 7a). IS1600 contained a set of 16-bp inverted repeats at its ends, with two mismatches (Fig. 7b).

Interestingly, although the terminal inverted repeats of IS1326 (26 bp) were considerably longer than those of IS1600,
some nucleotides of IS1600 proximal to the inverted repeats matched the nucleotides within the inverted repeats of IS1326 (shown by asterisks in Fig. 7b).

A 4.7-kb EcoRI fragment containing DR1 was cloned from plasmid pENH91 (Fig. 4) to yield plasmid pELDR1. Sequencing analysis revealed that DR1 was identical to DR2. There were no target-site duplications flanking the extreme ends of the 15-kb region containing the two elements or the termini of each of DR1 or DR2.

There were two possible initiation codons for istA of IS1600. This gene had the capacity to encode proteins of 518 or 515 amino acids, depending on the codon of translation initiation was the GTG codon at position 106 or that at position 115, respectively (Fig. 6). Each of these codons was preceded by a potential ribosome-binding site (SD1, SD2). istB encoded a putative protein of 264 amino acids, if the ATG codon of position 1,652 (Fig. 6) was used as an initiation codon. This codon was preceded by a possible ribosome-binding site (SD3).

The G+C contents were 63.6% for IS1600, 52.1% for IS1326, and 52.3% for IS21. The G+C content of IS1600

Fig. 7 (b) Terminal regions of the elements shown in panel a and IS21. The solid arrows indicate the inverted repeats and their orientations (Brown et al., 1996; Reimann et al., 1989). The dotted arrow below the nucleotide sequence of the SalI fragment from P51 shows a putative left inverted repeat that was delineated on the basis of similarity to the sequence of IS1600. Note that here the left and right designations refer to the directions of the orfs found in IS1600, as discussed in the text, and thus, they are the reverse of the directions shown in Fig. 4. The asterisks indicate identical nucleotides in the juxtaposed sequences that are not included in the inverted repeats of IS1600 and the SalI fragment from P51.
was similar to but slightly lower than that of the total genomic DNA of *R. eutropha* (66.3 to 66.8% [Davis et al., 1969]) and was closer to the chromosomal G+C content of species of *Pseudomonas* (e.g., *P. putida*, 59.6% to 63.4% [Mandel, 1966]). The G+C contents of the third positions in the codons used for IstA and IstB of IS1600 were 83.4% and 80.4%, respectively. Those for IstA and IstB of IS21 were 62.6 and 60.9%, respectively. By contrast to the genes of IS21, the codon usage in IstA and IstB of IS1600 was highly biased in favor of G or C in the third position. This phenomenon has also been observed in other genes from *R. eutropha* (Andersen and Caton, 1987; Hein and Steinbüchel, 1994; Priefert et al., 1991).

(6) Duplication of the catabolic genes

After successive transfers of NH9 to fresh 3-CB-containing liquid medium at approximately two-week intervals for a year, the cells in the culture were compared with NH9 by hybridization experiments with both plasmid DNAs and total DNAs to examine whether any recombination events had occurred. The cells and the catabolic plasmid from the one-year-old subculture were designated strain NH9A and pENH91A, respectively.

The 5.8-kb BamHI-BglII fragment of the cloned 9.2-kb SacI fragment was labeled and used as a probe to examine EcoRI digests of plasmid DNAs and total DNAs from the two strains (Fig. 8). In the EcoRI digest of pENH91, a 40-kb EcoRI fragment containing the probe region was found to hybridize to the probe (Lane 1). In the EcoRI digest of pENH91A, an additional 10-kb fragment appeared, and this novel fragment also hybridized strongly with the probe (Lane 2). Hybridization with EcoRI digests of total cellular DNA from the two strains gave the same patterns as those of the respective plasmid DNAs (Lanes 4 and 5). These results suggested that the novel 10-kb EcoRI fragment on pENH91A also contained the catabolic genes.

![Fig. 8](image)

*Fig. 8.* Southern hybridization of restriction digests of plasmid DNA and total DNA from NH9 and derivative strains with the 5.8-kb BamHI-BglII fragment as the labeled probe. (a) Agarose gel electrophoresis (0.7% gel and TBE buffer) of EcoRI digests of the samples of DNA. Lanes: 1, pENH91; 2, pENH91A; 3, pENH91d5; 4, total DNA from NH9; 5, total DNA from NH9A; M, fragments of lambda DNA digested with HindIII as size standards. (b) Autoradiogram of the corresponding Southern blot after hybridization with the 5.8-kb BamHI-BglII fragment as the labeled probe. The lanes correspond to those in panel a.
Several restriction profiles of pENH91 and pENH91A were compared. One NheI site, one BamHI site, two EcoRV sites, and two EcoRI sites were present within the intervening region between DR1 and DR2 on pENH91, but there were no sites for any of these four restriction endonucleases in the direct repeat sequences (Fig. 4). On the contrary, BgII sites were found in the direct repeats of pENH91 as well as within the intervening region. All the fragments that were present in the digests of pENH91 were observed in the corresponding restriction profile of pENH91A. In addition, restriction profiles of pENH91A with NheI, BamHI, EcoRV, or EcoRI gave an additional fragment (12.5 kb, 12.5 kb, 11 kb, and 10 kb, respectively). The profiles of BgII digests of the two plasmids were identical (data not shown).

A 1.0-kb HindIII-BgII fragment of IS1600 (DR2), when used as a probe, hybridized with two fragments that contained either DR2 or DR1 in each digest and with the additional fragments of pENH91A listed above (data not shown). The intensities of the three hybridization bands in each of digests of pENH91A with NheI, BamHI, EcoRV, or EcoRI were nearly equal. This result suggested that the copy numbers of the three fragments containing DR1, DR2, and the novel fragment in pENH91A were equal.

When the 5.8-kb BamHI-BgII fragment of the cloned 9.2-kb SacI fragment was used as a probe, it hybridized with the novel fragments that appeared in the digests of pENH91A with NheI, BamHI, EcoRV, or EcoRI as well as with the fragments that initially contained the 5.8-kb region. Hybridization patterns with the BgII digests of pENH91 and pENH91A were the same; this probe hybridized only with the 10.4-kb fragment that included the 5.8-kb region.

These results indicated that the novel fragment contained both the catabolic gene region and the direct repeat and that there were doublet band(s) in the electrophoretic profile of the BgII digest of pENH91A. If the novel fragment had been located at a distant site on the plasmid, for example, by transposition, it would have altered the sizes of the original fragments of pENH91. Because all the restriction fragments of pENH91 were retained in pENH91A, it was clear that the novel fragment was located close to the original region in a way that kept the pattern of the BgII digest the same.

The recombination event related to IS1600 must have occurred with one of the following two patterns: i) -DR-CB-DR-CB-DR-, by homologous recombination between the direct repeats; or ii) -DR-CB-DR-CB-DR-, by replicative transposition (DR and CB indicate IS1600 and the intervening region of 3-CB-catabolic genes, respectively).

The length (12.5 kb) of the novel fragments generated by digestion with either NheI or BamHI was equal to the sum of the length of the intervening region (10 kb) and one IS element (2.5 kb). This result is in accord with the pattern (i). In addition, the identical profiles obtained with the BgII digests of the two plasmids allow us to exclude the pattern (ii). If the pattern (ii) were to describe what had occurred, the BgII digest of pENH91A should have given an additional fragment of (at least) 2.5 kb, derived from the junctional region (-DR-DR-).

The lengths of the novel fragments generated by the four enzymes other than BgII corresponded to the lengths of fragments that would be produced by tandem duplication and not by inverted duplication.

These considerations lead to the conclusion that tandem duplication of the 12.5-kb region that contained the catabolic genes took place via reciprocal recombination between the direct repeats, as illustrated in Fig. 9.

(7) Determination of sequences of genes for degradative enzymes

Sequencing analysis of the 9.2-kb SacI fragment revealed seven long open reading frames (orfs; Fig. 4). Six of the orfs formed a cluster and exhibited strong homology to orfs in the following clusters of chlorocatechol-degradative genes (in the order of relatedness): i) the tcbR-CDXEF genes on plasmid pP51 in Pseudomonas sp. P51 (van der Meer et al., 1991a,b); ii) the clcR-ABXDE genes on plasmids pAC27 in P. putida AC866 and pB13(pWR1) in Pseudomonas sp. B13 (Coco et al., 1993; Frantz and Chakrabarty, et al., 1987; Kasberg et al., 1997); and iii) the tfdR and tfdCDEF genes on
plasmid pJP4 in R. eutropha JMP134 (Matrubutham and Harker, 1994; Perkins et al., 1990) (X denotes the third orf in the clusters of tcbCDEF and clcABDE degradative genes; the functions of these genes are unknown). In particular, the extent of homology between six orfs of NH9 and orfs of the tcbR-CDXEF genes of Pseudomonas sp. strain PS1 was very great (Table 2).

From the high homology to known chlorocatechol-degradative genes and by analogy to the pathways formed by the products of these gene clusters (van der Meer et al., 1991a), it was apparent that the sequenced degradative genes of strain NH9 encoded enzymes of the modified ortho-cleavage pathway (Fig. 10). Since strain NH9D (a derivative of strain NH9 that had been cured spontaneously of plasmid pENH91) grew on benzoate, strain NH9 was assumed to harbor genes for benzoate 1,2-dioxygenase and 1,2-dihydro-1,2-dihydroxybenzoate dehydrogenase, which might convert (chloro)benzoate into (chloro)catechol, either on its chromosome (Johnson and Stanier, 1971) or on the additional plasmid pENH92 (Fig. 2).

![Restriction map of the duplicated region of pENH91A. Hatched areas indicate the fragments that were used as probes in hybridization experiments and the corresponding hybridized regions. The numbers on the map for each restriction enzyme are the lengths of fragments in kilobases. The fragments with numbers in bold type are the fragments that were not seen in the restriction profiles of pENH91. The fragments with numbers in parentheses are those whose entire lengths are not included in this figure. The abbreviations for the restriction sites are the same as those in the legend to Fig. 3 and 4.](image)

**Table 2. Homology between cbn genes and tcb genes**

<table>
<thead>
<tr>
<th>Genes</th>
<th>cbnR</th>
<th>cbnA</th>
<th>cbnB</th>
<th>orf3cbn</th>
<th>cbnC</th>
<th>cbnD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corresponding and G+C content</td>
<td>(66.1%)</td>
<td>(65.0%)</td>
<td>(63.3%)</td>
<td>(64.3%)</td>
<td>(61.0%)</td>
<td>(62.9%)</td>
</tr>
<tr>
<td>tcbR</td>
<td>(66.2%)</td>
<td>(65.2%)</td>
<td>(63.7%)</td>
<td>(63.3%)</td>
<td>(61.0%)</td>
<td>(62.9%)</td>
</tr>
<tr>
<td>TcB</td>
<td>885 bp</td>
<td>756 bp</td>
<td>1113 bp</td>
<td>1011 bp</td>
<td>717 bp</td>
<td>1059 bp</td>
</tr>
<tr>
<td>Amino acids</td>
<td>294 aa</td>
<td>251 aa</td>
<td>370 aa</td>
<td>336 aa</td>
<td>238 aa</td>
<td>352 aa</td>
</tr>
<tr>
<td>Nucleotide level (%)</td>
<td>99.9</td>
<td>98.0</td>
<td>88.9</td>
<td>97.9</td>
<td>99.9</td>
<td>100</td>
</tr>
<tr>
<td>Amino acid level (%)</td>
<td>99.7</td>
<td>95.6</td>
<td>97.0</td>
<td>97.0</td>
<td>99.6</td>
<td>100</td>
</tr>
</tbody>
</table>
Since the enzymes encoded by the chlorocatechol-degradative genes of NH9 were responsible for degradation of 3-CB, the genes were designated cbnR-ABCD, with cbnA, B and C encoding chlorocatechol 1,2-dioxygenase, chloromuconate cycloisomerase, and dienelactone hydrolase, respectively (van der Meer et al., 1991a, c). Recent studies suggested that cbnD, corresponding to tcbF, might encode maleylacetate reductase (Kasberg et al., 1995; Kasberg et al., 1997; Seibert et al., 1993). cbnR was presumed to be a regulatory gene that belonged to the lysR family (Schell, 1993; van der Meer et al., 1991b). orfX, located between cbnB and cbnC, corresponded to the third orf in the tcbCDXEF and clcABXDE gene clusters, whose products have unknown functions. The strongly conserved amino acid sequences encoded by orfX in cbnABXCD and in tcbCDXEF suggest that the products of these orfs might play a role that is indispensable for the function of the gene cluster in some as yet unknown fashion.

The extent of the homology of each gene in the cbnR-ABXCD cluster to the corresponding gene in the clcR-ABXDE cluster (Coco et al., 1993; Frantz and Chakrabarty, et al., 1987; Kasberg et al., 1997) ranged from 59% to 72% at the nucleotide level and from 51% to 76% at the amino acid level. Homology to the tfdR and tfdCDCEF genes of strain JMP134 (Matrubutham and Harker, 1994; Perkins et al., 1990) was 58% to 66% at the nucleotide level and 52% to 67% at the amino acid level. The degradative genes cbnA, cbnB and cbnR are considered to be evolutionarily related to the functionally similar genes in the catechol ortho-cleavage pathway, namely, catA, catB, and catC (Schlömann, 1994). The homology between the cbnR-AB genes and the corresponding cat genes of P. putida strain PRS2000 (Houghton, 1995) and P. putida strain RB1 (Aldrich et al., 1987; Rothmel et al., 1990) was 51% to 57% at the nucleotide level and 31% to 45% at the amino acid level.

(8) Comparison between the cbn and tcb gene clusters

The regions containing the chlorocatechol-degradative genes of the two plasmids, namely, the 6959-bp SacI–KpnI regions of pENH91 and pP51, were compared with one another (Fig. 4, 11 and Table 2). All of the corresponding orfs were of the same respective lengths (Table 2). Hence, all the differences between the coding regions of the cbnR-ABXCD

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**Ralstonia eutropha NH9 (pENH91)**

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**Pseudomonas sp. P51 (pP51)**

Fig. 10. Pathways for degradation of 3-chlorobenzoic acid by Ralstonia eutropha NH9 and for degradation of 1,2,4-trichlorobenzene by Pseudomonas sp. P51. The pathway for degradation of 1,2,4-trichlorobenzene by Pseudomonas sp. P51 was first described by van der Meer et al. (1991c).
and tcbR-CDXEF genes were substitutions. The cbnA and cbnB genes overlapped by 4 bp. One nucleotide was present between cbnB and orfX in the cbn degradative operon. The intergenic region between orfX and cbnC consisted of 21 bp. cbnC overlapped with cbnD by 4 bp. All of these structural features of the cbn genes were the same as those of the corresponding regions of the tcb genes. The nucleotide sequence of the promoter region between cbnR and cbnA (150 bp) was identical to that between tcbR and tcbC.

Nucleotide substitutions between cbnA and tcbC resulted in a slight decrease in the percentage homology between the deduced amino acid sequences (Table 2). Eleven of 13 nucleotide substitutions caused non-synonymous substitutions at the amino acid level. However, the four amino acids that are supposed to coordinate the ferric ion at the active site were conserved in CbnA (Tyr-130, Tyr-164, His-188, and His-198), as were other 28 amino acids that are conserved among catechol 1,2-dioxygenases (Nakai et al., 1995). Although homology at the nucleotide level was the lowest between the cycloisomerase genes cbnB and tcbD among the genes listed in Table 2, the majority of nucleotide substitutions between cbnB and tcbD were synonymous substitutions (in 102 out of 113 codons), reflecting the high frequency of nucleotide substitutions at the third base in the codon (107 out of 124 nucleotides; Fig. 11). Consequently, the homology at the amino acid level between CbnB and TcbD remained high, in contrast to the case of CbnA and TcbC (Table 2). There was one nucleotide substitution both between cbnR and tcbR and between cbnC and tcbE, resulting in one amino acid substitution in each pair. The nucleotide sequence of cbnD was identical to that of tcbF. All of the putative ribosome-binding sites for the cbn genes were located at the corresponding spacing from the initiation codons and all had the same sequences as those for the tcb genes (van der Meer et al., 1991a, b).

![Fig.11. Nucleotide substitutions and other changes in the region of the cbn gene cluster in comparison to the region of the tcb gene cluster.](image)

The numbers per 200 nucleotides are shown, counted from the SacI site in the 6959-bp region that contains the cbn gene cluster. The corresponding regions of nucleotides in the two clusters are as follows: cbn 1 to 287 and tcb 1 to 287, cbn 289 to 6578 and tcb 288 to 6577, and cbn 6579 to 6959 and tcb 6579 to 6959.
The nucleotide sequences of the flanking regions of the two gene clusters within the SacI-KpnI fragments were nearly identical: the 637-bp nucleotide sequence of the downstream flanking region of cbnR was identical to that of tcbR except that one nucleotide was missing from the latter. The 618-bp nucleotide sequence of the downstream flanking region of cbnD was identical to that of tcbF except for insertion of one nucleotide in the latter case. These results suggested that the highly homologous regions of the two plasmids extended beyond both the SacI site and the KpnI site (Fig. 4).

(9) An additional orf in the region between cbnD and DR2

In a 2-kb region between cbnD and DR2, an orf of considerable length was found, which exhibited some similarity to known proteins at the amino acid level (Fig. 4; designated orfL). The deduced amino acid sequence of orfL (240 a.a.) was homologous to many bacterial polypeptides that are known to be components of membrane-bound transport systems for amino acids. In particular, LivF of E. coli (Adams et al., 1990) and BraG of Pseudomonas aeruginosa PAO (Hoshino and Kose, 1990) were 42% and 39% homologous to orfL at the amino acid level, respectively. orfL seemed to have complete length in comparison with LivF (237 a.a.) and BraG (233 a.a.). Both LivF and BraG are located at the downstream end (in the direction of transcription) in their respective gene clusters. Because of the location and apparent direction of transcription of orfL in the region bracketed by the two ISs, it appeared as if orfL might have been cut out from the rest of the genes in the cluster by an excision event involving IS1600 (DR2).

(10) Cloning and sequencing of a DNA fragment from strain P51 with homology to IS1600

It was examined whether the tcbR-CDEF gene cluster was also associated with an IS1600 (or IS1600-like) sequence on plasmid pP51. Total DNA was extracted from cells of strain P51 that had been grown on Luria-Bertani (LB) medium (Sambrook et al., 1989). In a Southern hybridization experiment, the presence of plasmid pP51 DNA in the restriction digests of total DNA was confirmed with a 3.5-kb BamHI-PstI fragment containing a part of the cbn gene cluster as the probe. Then the cbn gene probe was removed from this membrane, which was next used in a hybridization experiment with a 2.3-kb HindIII-SphI fragment containing a part of IS1600 (DR2) as the probe. A hybridizing band was observed and this fragment was cloned from the total DNA of strain P51 into pUC19 as a 3.7-kb SalI fragment. In a subsequent hybridization experiment, using the cloned 3.7-kb SalI fragment as the probe, the SalI-digested total DNA from strain P51 (which retained the tcb genes) and from derivative strains of P51, that had been cured spontaneously of the plasmid P51 after successive transfer in LB liquid medium, gave the same pattern of signals. Therefore, it appeared that the cloned 3.7-kb SalI fragment resided on the chromosome of strain P51.

Sequence of a ca. 2-kb region of the cloned 3.7-kb SalI fragment revealed that a 1,300 bp region in the fragment was homologous to IS1600 with nucleotide homology of 81%. The 1,300-bp region was flanked by nonrelated marginal regions of ca. 0.2 kb and 0.5 kb on each side. Thus, the homologous region had not been truncated by the SalI sites. The homologous 1,300-bp region started with a 15-bp sequence that resembled the left inverted repeat of IS1600 (Fig. 7b). It was followed by a 115-bp intervening region and then by an open reading frame (designated orfSA; 1,170 bp) which showed the highest homology with a part of IstA of IS1600 (83% at amino acid level; Fig. 7a and 26) in the database. In addition to the fact that orfSA seemed to lack the 3′ portion of IstA, it was obviously not followed by IstB and an inverted repeat at the other end. Thus, this fragment seemed to be a remnant of an IS.

Summary of results section 1: A ca. 5.7-kb six gene cluster responsible for chlorocatechol degradation was cloned from the transmissible plasmid pENH91 of 3-CB degradative bacterium R. eutropha NH9: the cbnABCD operon (including
orfX of unknown function) encoding the degradative enzymes and the divergently transcribed cbnR encoding the LysR-type transcriptional regulator of the cbn operon. The cbnR-ABCD genes showed the highest homology to the tcbR-CDEF genes on plasmid pP51 of the 1,2,4-trichlorobenzene-degrading bacterium Pseudomonas sp. P51, which was isolated in the Netherlands (89% to 100% identity at the nucleotide level). The cbnR-ABCD genes were found to be located between two directly-oriented identical insertion sequences (ISs) of 2,520 bp, designated IS1600, thus forming a composite transposon designated Tn5707 (ca. 15 kb). Both deletion and duplication events of the cbnR-ABCD gene cluster on the plasmid pENH91 were mediated by homologous recombination between the two copies of IS1600.

2. Transcriptional activation of the 3-chlorocatechol degradative genes cbnR-ABCD

The structure of the cbnR-ABCD genes suggested that CbnR was a LysR-type transcriptional activator for the expression of the cbnA promoter by analogy with the structures of the homologous tcbR-CDEF and clcR-ABD genes. The function of CbnR in the expression of the cbnA promoter was examined in vivo and in vitro.

(1) Complementation by CbnR of growth on 3-CB of bacteria harboring cbnABCD genes

The cbnABCD genes encode enzymes of the modified ortho-cleavage pathway, which degrade 3-chlorocatechol converted from 3-CB (Fig. 10). To examine if cbnR was required for growth of NH9 on 3-CB, a complementation test was conducted. Either pCbn13RABCD or pCbn13ABCD, which retained cbnA promoter region, was mobilized into R. eutropha NH9D, a derivative of NH9 cured of the cbnR-ABCD genes, or P. putida PRS4020, a catR knockout strain. The growth of the resultant four strains on 3-CB were compared together with strains containing the vector control, pCP13. Strains NH9D and PRS4020 with pCbn13RABCD showed growth on the 3-CB plate, while the strains with pCbn13ABCD or pCP13 did not. These results indicated that cbnR was necessary for the growth of these bacteria on 3-CB.

(2) Activation of the cbnA promoter by CbnR during growth in the presence of benzoate or 3-chlorobenzoate

It was presumed that CbnR may activate the cbnA promoter in analogy to the regulatory systems of other ortho cleavage operons described in chapter I (Leveau et al., 1994; McFall et al., 1998; van der Meer et al., 1991b). To analyze the function of cbnR for the expression of the degradative genes in vivo, P. putida PRS4020 (catR knockout strain) was used as a host for reporter plasmids depicted in Fig. 12. The cells were grown in basal synthetic medium (BSM) (Aldrich et al., 1987) supplemented with 10 mM glucose alone, 10 mM glucose and 5 mM benzoate (Ben), or 10 mM glucose and 5 mM 3-CB for 18 hours at 30 °C. When the cells containing pNO50RAB' were grown on glucose with either Ben or 3-CB, the transcription from cbnA promoter was activated 17 fold and 6.8 fold, respectively, compared to the cells grown on glucose alone (Table 3). The activation in the presence of Ben or 3-CB was not seen for the cells with pNO50AB' which did not contain cbnR. These results indicated that cbnR was a positive regulator of the transcription from cbnA promoter.

Cells containing pNO50A' did not induce substantially in the presence of 3-CB. When the same cells were grown in the presence of Ben, the transcription was activated 12-fold. The intact cbnA gene was necessary for the activation upon addition of 3-CB and also for the higher activation upon addition of Ben observed with pNO50RA'B'. 3-CB and Ben are converted to 3-chlorocatechol and catechol, respectively, by enzymes encoded on the host chromosome, and CbnA (chlorocatechol dioxygenase) can further convert these (chloro)catechols to 2-chloro-cis,cis-muconate (2-CM) and
cis,cis-muconate (CCM), respectively. The above results suggested that 2-CM and CCM were the inducers of the cbnA promoter.

(3) Purification of CbnRHis protein
Specific binding of CbnR to the cbnA promoter was demonstrated by gel retardation assay using the crude protein from E. coli BL21(DE3)pLysS containing pT 7cbnR. In order to simplify protein purification, a plasmid expressing cbnR with 6 His codons on its C-terminal, pT 7cbnRHis, was constructed. The crude soluble protein from E. coli BL21(DE3)pLysS containing pT 7cbnRHis showed binding activity to cbnA promoter, although a considerable portion of the protein was secluded in inclusion bodies. When the crude protein was directly purified with the nickel affinity column, two major bands appeared in the SDS-PAGE profile. One of the bands apparently corresponded to the calculated molecular weight of CbnRHis (32.9 kD), and the other was a polypeptide of about 27 kD. This 27-kD protein was also produced from the cells of the vector control. To remove this contaminating protein, a heparin-agarose column was employed followed by the nickel affinity column, and the 32.9-kD protein was recovered at more than 90% purity (Fig. 13, lane 3). The amino acid sequence of the N-terminal of this protein was found to be identical to that of the deduced sequence of CbnRHis. The function of CbnRHis was evaluated with respect to partially-purified wild-type CbnR in vivo and in vitro in the following sections.

(4) Confirmation of the function of CbnRHis
Partially purified CbnR and purified CbnRHis were subjected to gel retardation assays with a 252-bp fragment

![Diagram of the inserts of the constructs used for the cbnA promoter activity assay](image)

**Fig.12. Diagram of the inserts of the constructs used for the cbnA promoter activity assay**

<table>
<thead>
<tr>
<th>P. putida PRS4020 /plasmid construct</th>
<th>Glucose 10mM</th>
<th>Glucose 10mM + Benzoeate 5mM</th>
<th>Glucose 10mM + 3-CB 5mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>vector control (pQF50)</td>
<td>3.5( ±0.2 )</td>
<td>1.8( ±0.2 )</td>
<td>2.0( ±0.2 )</td>
</tr>
<tr>
<td>pNO50RA B'</td>
<td>61.8( ±2.3 )</td>
<td>1038.4( ±46.8 )</td>
<td>424.9( ±22.4 )</td>
</tr>
<tr>
<td>pNO50A B'</td>
<td>116.6( ±3.2 )</td>
<td>56.5( ±3.9 )</td>
<td>71.1( ±5.5 )</td>
</tr>
<tr>
<td>pNO50RA '</td>
<td>21.6( ±0.7 )</td>
<td>261.9( ±27.0 )</td>
<td>56.4( ±8.7 )</td>
</tr>
</tbody>
</table>

□-galactosidase units: nmol/min/mg extract( □ ): standard deviation
containing the cbnA promoter region. The two proteins showed the same retardation mobility of the cbnA promoter fragment (Fig. 14, lanes 3-5 and lanes 6-8). To test if CbnRHis had the same function as CbnR in vivo, growth complementation tests and reporter analysis were conducted. In growth complementation, strains NH9D and PRS4020 harboring pCbn13RHA BCD grew on 3-CB as did strains with pCbn13RA BCD. In reporter analysis, PRS4020 containing pNO50RHB' exhibited activation of transcription in the presence of either Ben or 3-CB to the same levels as PRS4020 containing pNO50RA'B' did (data not shown). These results indicated that CbnRHis had the same function as CbnR in these in vitro and in vivo experiments.

(5) Features of the cbnA promoter region bound by CbnR

The transcriptional start site of cbnA promoter was determined by S1 nuclease analysis; it was the A on the template strand marked by an arrow in Fig. 15 and was located 47-bp upstream from the translational start codon of

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**Fig. 13.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purification of CbnRHis. Lanes: 1, total protein from induced cells of BL21(DE3)pLysS containing pT7cbnRHis (100 μg); 2, protein purified by heparin-agarose column (50 μg); 3, CbnRHis purified by heparin-agarose column and nickel column (5 μg); M, protein molecular mass markers. Sizes are shown in kilodaltons.

**Fig. 14.** Gel retardation assay demonstrating specific binding of CbnR and CbnRHis to the cbnA promoter region. A 252-bp fragment containing the cbnA promoter region was used as probe in lanes 1 to 8. A 336-bp fragment containing part of the hydroxyquinol 1,2-dioxygenase gene of Burkholderia cepacia A C1100 was used in lanes 9 to 12. Lanes: 1 and 9, no protein; 2 and 10, partially purified protein from BL21(DE3)pLysS/pT 7-7 at 0.5 μg; 3, 4, 5, and 11, partially purified CbnR at 0.05, 0.1, 0.5, and 0.5 μg, respectively; 6, 7, 8, and 12, CbnRHis at 0.05, 0.1, 0.5, and 0.5 μg, respectively.
cbnA gene. The site of CbnR binding on the cbnA promoter was determined by DNase I protection assay (Fig. 16 and 17). In the absence of inducer, approximately 60-bp from -76 to -19 (relative to the +1 of transcription) were protected from DNase I cleavage by CbnR. Both partially purified CbnR and CbnRHis exhibited the same protected patterns on the sense and anti-sense strands. These footprints were very similar to those of TcbR bound to the tcbC promoter (Leveau et al., 1994). The addition of CCM or 2CM up to 1 mM in the binding reaction did not change the footprinting pattern of CbnR to cbnA promoter. Besides the inverted repeat containing T-N11-A located in the RBS (Schell, 1993), there was a second inverted repeat (Fig. 17) whose location is similar to those described recently by the study of BlaA, a LysR type regulator for ß-lactamase genes of Streptomyces cacaoi (Magdalena et al., 1997). The role or functionality of the second inverted repeats is not known.

(6) Identification of the inducers by in vitro transcription assay

The results of the LacZ assays suggested that CCM and 2-CM act as the inducers of the cbnA promoter. Considerable activity, however, was also shown when the cells containing a truncated CbnA gene (pNO50RA') were grown in glucose with Ben (Table 3). The possibility that either Ben or catechol may also serve as inducers could not
To examine the effect of these compounds on the transcriptional activation by CbnR, in vitro transcription assays were performed with purified CbnRHis and potential inducer compounds. Figure 18 shows that cbnA transcripts were produced only when either CCM or 2-CM was added to the reaction (lanes 12 and 14). No transcript was produced with other compounds including Ben (lane 4) or catechol (lane 8). Therefore Ben or catechol does not serve as an inducer for the cbnA promoter. The transcriptional start sites of the products with CCM or 2-CM were determined by S1 nuclease assay and were confirmed to be the same as that derived by in vivo assay (data not shown).

Fig. 16. DNase I footprint of CbnRHIs and CbnR on the cbnA promoter region. (a and b) Top strand (a) and bottom strand (b) of the cbnA promoter region. Lanes: 1, no protein; 2, CbnRHis (0.5 μg); 3, partially purified CbnR (1 μg); 4, partially purified protein from BL21(DE3)pLysS/pT7-7 (1 μg).

Fig. 17. Schematic diagram of the cbnA promoter region protected from DNase I digestion by CbnR(His). The protected nucleotides are shown by the brackets. The vertical arrows indicate sites of hypersensitivity to DNase I digestion. The thick horizontal arrows show the inverted repeats containing T-N11-A, regarded as a motif of LysR regulatory systems (Schell, 1993). The horizontal dotted arrows above the bottom strand indicate the second imperfect inverted repeat similar to those described recently (Magdalena et al., 1997). The horizontal solid lines under the top strand and above the bottom strand indicate the -35 and -10 regions of the cbnA promoter and the divergently transcribed cbnR promoter. The regions from nucleotides -76 to -49 and from -44 to -19 are suggested to be the recognition binding site (RBS) and the activation binding site (ABS), respectively (McFall et al., 1998). The numbering is relative to the transcriptional start site of cbnA.
(7) Bending of the \textit{cbnA} promoter region

The presence of hypersensitive sites in the center of the footprint region of the \textit{cbnA} promoter suggested that changes in DNA conformation might occur upon binding of CbnRHis. To examine this potential change with or without inducer, circular permutation gel shift assays were performed. Lanes 1 to 5 of Fig. 19 indicate that binding of...
CbnRHis caused bending of the promoter fragment; the bending angle was estimated to be 78°. When CbnRHis was bound to the promoter in the presence of CCM, relaxation of the bending to 54° was observed (lanes 6 to 10). The bending angles of three independent experiments varied by no more than 7.7%. Partially purified CbnR gave the same bending angles as CbnRHis (data not shown.)

Summary of results section 2: CbnR was demonstrated to regulate the expression of the cbnA promoter positively by in vivo and in vitro experiments. The inducers for the expression of the cbnA promoter by CbnR were found to be CCM and 2-CM. Specific binding of CbnR protein to the cbnA promoter region was demonstrated by gel shift and DNaseI footprinting analysis. In the absence of inducer, a region of ca. 60 bp from -20 to -80 positions of the cbnA transcriptional start point was protected from DNaseI cleavage by CbnR, with a region of hypersensitivity to DNaseI cleavage clustered at around -50. Circular permutation gel shift assays demonstrated that CbnR bent the cbnA promoter region to an angle of 78° and that this bending was relaxed to 54° upon the addition of inducer, CCM.

3. The structure and transcriptional activation of the 2,4-dichlorophenoxyacetate degradative genes of Alcaligenes sp. CSV90

The tfdC gene encoding the dichlorocatechol dioxygenase cloned from the large plasmid pMAB1 of the 2,4-dichlorophenoxyacetate (2,4-D) degradative bacterium Alcaligenes sp. CSV90 has been found to be identical with that of the well-characterized plasmid pJP4 of the 2,4-D degradative bacterium R. eutropha JMP134. The sequence of the upstream region of the tfdC gene on pMAB1, however, has turned out to be different from that on pJP4 (Bhat et al., 1994; Leveau and van der Meer, 1996). Therefore, the structure of the 2,4-D degradative genes on pMAB1 was examined in comparison with that of pJP4. Also, the expression of the degradative genes was analyzed in vivo using the same system as that used to test the expression of the cbnA promoter to compare the function of the regulators.

(1) The structure of the 2,4-D degradative genes from Alcaligenes sp. CSV90

2,4-D is one of the major herbicides and has been used as a model compound for the study of the evolution of the degradative genes for chlorinated aromatic compounds. In the well studied catabolic pathway of 2,4-D by aerobic bacteria, the side chain is firstly removed by the 2,4-D/Đ-ketoglutarate dioxygenase encoded by the tfdA gene (Fukumori and Hausinger, 1993a, b) and the resulting 2,4-dichlorophenol is converted to 3,5-dichlorocatechol (3,5-DCC) by the 2,4-dichlorophenol hydroxylase encoded by the tfdB gene (Perkins et al., 1990). 3,5-DC is then degraded to a common intermediate, Đ-keto-dicarboxylate, by the enzymes of the chlorocatechol (modified) ortho-cleavage pathway encoded by the tfdCDEF genes (Fig. 20) (Don et al., 1985).

2,4-D degradative bacterium Alcaligenes sp. CSV90 was found to harbor a 90-kb plasmid pMAB1 and the tfdC gene encoding 3,5-dichlorocatechol dioxygenase was cloned from the plasmid (Bhat et al., 1993, 1994). The nucleotide sequence of the tfdC gene and a part of the tfdD gene contained in the cloned 1.6-kb HindIII fragment from pMAB1 was identical to the corresponding region of the tfdCD genes of pJP4 (Bhat et al., 1994; this study). Sequencing analysis of the downward flanking region of the tfdC gene of pMAB1 revealed the presence of the complete tfdD gene and the tfdDEF-B genes, which were nearly identical to those of pJP4. In the upstream region of the tfdC gene of pMAB1, a gene apparently encoding a LysR-type regulator was found in the opposite orientation, which was highly homologous to the tfdT gene located in the corresponding part of pJP4. The 633-bp nucleotide sequence of the 5'-end of the gene on pMAB1, designated tfdT, was identical to the corresponding part of the tfdT gene of pJP4 (Fig. 21). The tfdT gene (687
Fig. 20. Pathway for degradation of 2,4-dichlorophenoxycacetate

Fig. 21. Alignment of the nucleotide sequences of the tfd genes from pMAB1 and pJP4. The underline indicates 33 nucleotides of the tfd gene from pMAB1 that are not highly similar to either corresponding portions of tfd gene from pJP4.

1st Nucleotide Sequence: tfdT(pMAB1), 888 bp
2nd Nucleotide Sequence: tfdT(pJP4), 687 bp

Unit Size to Compare = 2
Pick up Location = 1

[100.0% / 633 bp] INT/OPT.Score : < 2532/ 2532 >

1' ATGGAAAATA GACAGTTGAA ATACTGGTGC GCGTGCCCGG AGGCAGGAAG TTTCGGAACG GCGGCCAGAG CAGAGCACT

81' ATGCGGAGCCA CGGCGTACTG CCTGAGCGCA GCGTGGAGGA GGGCAAGGG TTTGACGAGG CAGCCACGG GAGAGCA

161' GGCTGAGACTC ACACGCCGCC GAAAGGATGT TTTGTCGAAGC GCGCTGCGCG TTGTTGACGG GTCTGTGGCG

241' CGATTGGCAAG CTGCCAGCTAG AGGCACACCT GCTCGACCTA GCTGTGGCA GAGCGTCC

321' GGGCGTCGCTC AGAGACGGTC TCGCGGATGA AAGCGGGCCG ACCGGCGGCG TGTCGCGCG GACACAGGAG GTCCGCAGG

401' AGTCGTTTCT TCGAGGCCAT GTGAGCTGGA GTTCTGCGG ATTCATCCAG TGGTGGCAGA CTGCTCGGAG TTGAACATT

561' CGATGACGCC GTACATTCTT ATTCCAGCGG GAGCGGACCA AGGTGTCAGG TTGATGAAAG AGAGAGAG

641' GAGTAGGCGG CCCAGGCGTC GTGCAGTGGT TGGCAGTGA GAGCGGACCA
bp) of pJP4 was nonfunctional because it was disrupted by the ISJP4 composite transposon (Leveau and van der Meer, 1996). In contrast, the tfdT gene on pMAB1 had a length of 888 bp thus seemed to encode a full-length LysR-type regulator. Curiously, the 222-bp nucleotide sequence of the 3'-end of the tfdT gene of pMAB1 was nearly identical to the corresponding part of the tfdR (or tfdS) gene of pJP4 (Fig. 22). Nucleotide sequence of 33 bp between these two sequences in the tfdT gene (underlined in both Fig. 21 and 22) did not exhibit high similarity to known sequences of pJP4 or examined sequence of pMAB1 shown in Fig. 23. Further sequencing analysis of the downward flanking region of the tfdT gene revealed another lysR-type regulatory gene, tfdS, and the tfdA gene, both of which were nearly identical to those of pJP4 (Fig. 23).

Fig.22. Alignment of the nucleotide sequences of the tfdT gene from pMAB1 and the tfdR gene from pJP4. The underline indicates 33 nucleotides of the tfdT gene from pMAB1 that are not highly similar to either corresponding portions of tfdR (or tfdS) gene of pJP4 (Fig. 22).
(2) Activation of the tfdC promoter by TfdS

To differentiate the possible role of the tfdT and tfdS genes in transcriptional regulation of the tfdC and tfdA promoters, several constructs for each promoter were made including those in which either of the two lysR-type genes was disrupted, and used for reporter analysis (Fig. 24 and 25). The same method was used as that used for the analysis of activation of the cbnA promoter. It was presumed 2-CM could be an inducer for the tfdC promoter because this strain can degrade 3-CB by using the enzymes encoded by the tfdCDEF genes which are homologous to the clcABDE genes and the cbnABCD genes both regulated by the corresponding LysR-type activators recognizing 2-CM as an inducer (McFall et al., 1997c; this study).

When the cells containing pCS50STCD’, which contained intact tfdSTC genes, were grown on glucose in the presence of 3-CB, the transcription from the tfdC promoter was increased 2-fold compared to that in the cells grown on glucose alone (Table 4). This elevation in the presence of 3-CB was also seen for the cells with pCS50ST ‘CD’ (tfdT disrupted) but was not seen for the cells with pCS50S ‘TCD’ (tfdS truncated) or pCS50T CD’ containing only tfdT. These results indicated that tfdS activated the tfdC promoter while tfdT did not.

Cells containing pCS50T C’ or pCS50STC’, whose tfdS genes are incomplete, exhibited very low basal level activity when grown on glucose alone, compared to those containing the constructs with the full length tfdC gene, and did not show any increased transcriptional activity in the presence of either 3-CB or Ben. These results indicated neither of Ben or 3-CB could induce the transcriptional activation of the tfdC promoter by tfdS or tfdT. The results, together with the above results of activation of the tfdC promoter shown with pCS50STCD’ and pCS50ST ‘CD’, indicate that 2-CM, which was produced only in the presence of 3-CB and the complete tfdC gene in the constructs, was the inducer for the activation of the tfdC promoter. Catechol and 3-CC, which are produced in the cells from Ben and 3-CB before conversion into CCM and 2-CM, respectively, could not be inducers. The high basal level observed with the cells containing the constructs with truncated tfdD gene could be ascribed to the absence of a cognate regulator of the tfdC promoter and to some cis effect of the region of the tfdC gene (see Discussion).

In order to examine the effect of the distance and orientation of the tfdS gene and the tfdC promoter on the transcriptional activity, pCS50SCD’1 and pCS50SrCD’1, which lacked tfdT and were different in distance and orientation, were used for reporter analysis and the results are shown in Table 4. The reporter activity of pCS50SCD’1 was increased 2-fold in the presence of 3-CB, whereas the reporter activity of pCS50SrCD’1 was not increased. These results indicated that the transcriptional activity of the tfdC promoter was enhanced by the presence of tfdS in the constructs but not by the presence of tfdT. The results, together with the above results of activation of the tfdC promoter shown with pCS50STCD’ and pCS50ST ‘CD’, indicate that 2-CM, which was produced only in the presence of 3-CB and the complete tfdC gene in the constructs, was the inducer for the activation of the tfdC promoter. Catechol and 3-CC, which are produced in the cells from Ben and 3-CB before conversion into CCM and 2-CM, respectively, could not be inducers. The high basal level observed with the cells containing the constructs with truncated tfdD gene could be ascribed to the absence of a cognate regulator of the tfdC promoter and to some cis effect of the region of the tfdC gene (see Discussion).

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Fig. 23. Schematic representation of regions containing 2,4-D degradative genes on pJP4 and pMAB1

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orientation of tfdS and the tfdC promoter, were constructed and used for reporter analysis. While the cells with pCS50SCD'1 did not show substantial difference in the activation by 3-CB from those with pCS50ST CD' and pCS50ST 'CD', the cells with pCS50SrCD'1 induced the highest activity among all the constructs for the analysis of tfdC promoter activity in the presence of 3-CB (5-fold activation compared to the cells grown with glucose alone) (Table 4). These results suggested the effect of local DNA structure in the upstream of the tfdC promoter (see Discussion).

(3) Activation of the tfdA promoter by TfdS

Transcriptional activation of the tfdA promoter was tested using PRS4020 cells with each of the four constructs.

![Diagram of the inserts of the constructs used for activity assay of the tfdC promoter of pMAB1.](image)

Table 4: The effect of Ben and 3-CB on transcriptional activation at the tfdC promoter of Alcaligenes sp. CSV90 (pMAB1)

<table>
<thead>
<tr>
<th>P. putida PRS4020 /plasmid construct</th>
<th>Glucose 10mM</th>
<th>Glucose 10mM + Benzoate 5mM</th>
<th>Glucose 10mM + 3-CB 5mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCS50TC'</td>
<td>7.8 (0.6)</td>
<td>5.7 (1.8)</td>
<td>5.5 (0.2)</td>
</tr>
<tr>
<td>pCS50ST C'</td>
<td>8.6 (1.7)</td>
<td>7.9 (1.4)</td>
<td>7.7 (0.2)</td>
</tr>
<tr>
<td>pCS50CD'</td>
<td>136.9 (10.3)</td>
<td>93.0 (1.3)</td>
<td>72.7 (5.4)</td>
</tr>
<tr>
<td>pCS50ST CD'</td>
<td>109.0 (4.2)</td>
<td>84.9 (18.6)</td>
<td>91.4 (16.6)</td>
</tr>
<tr>
<td>pCS50ST 'CD'</td>
<td>191.3 (20.6)</td>
<td>141.2 (5.0)</td>
<td>109.0 (11.9)</td>
</tr>
<tr>
<td>pCS50ST CD'</td>
<td>115.8 (10.9)</td>
<td>139.0 (10.9)</td>
<td>234.7 (12.7)</td>
</tr>
<tr>
<td>pCS50ST 'CD'</td>
<td>104.4 (8.5)</td>
<td>136.7 (12.3)</td>
<td>225.6 (21.7)</td>
</tr>
<tr>
<td>pCS50SCD'1</td>
<td>160.7 (3.8)</td>
<td>152.7 (7.6)</td>
<td>284.9 (15.5)</td>
</tr>
<tr>
<td>pCS50SrCD'1</td>
<td>126.4 (4.1)</td>
<td>118.5 (11.0)</td>
<td>630.3 (36.6)</td>
</tr>
</tbody>
</table>

<k box>-galactosidase units: nmol/min/mg extract ( □ ): standard deviation
When the cells containing pCS50CTSA', which included intact tfdTS, or pCS50CT'SA' (tfdT disrupted) were grown on glucose with 3-CB, the transcription from the tfdA promoter was increased 37- and 38-fold, respectively, compared to those in the respective cells grown on glucose alone (Table 5). In contrast, the cells with pCS50CT'SA' (tfdS disrupted) did not exhibit any activation upon the addition of 3-CB. These results indicated that tfdS was the positive regulator for the transcription at the tfdA promoter. No activation observed for the cells with pCS50CT'SA' (tfdC truncated) in the presence of 3-CB further indicated that 2-CM was the inducer for the activation of the tfdA promoter by tfdS.

The basal level obtained with the cells grown on glucose alone was quite low throughout the four experimental sections. The cells containing either of the three constructs with the intact tfdS gene showed a constant level of activation in the presence of Ben (11- to 15-fold), whether or not the construct contained the full length of the tfdC gene. This activation in the presence of Ben was not induced for the cells with pCS50CT'SA' (tfdS disrupted). These results suggest that either Ben or its metabolite, catechol, might serve as an inducer for the transcriptional activation at the tfdA promoter by tfdS.

Summary of results section 3: The tfdA-S-T-CDEFB genes on plasmid pMAB1 (90 kb) from Alcaligenes sp. CSV90 have

Table 5. The effect of Ben and 3-CB on transcriptional activation at the tfdA promoter of Alcaligenes sp. CSV90 (pMAB1)

<table>
<thead>
<tr>
<th>P. putida PRS4020</th>
<th>Glucose 10mM</th>
<th>Glucose 10mM</th>
<th>Glucose 10mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>/plasmid construct</td>
<td>+ Benzoate 5mM</td>
<td>+ 3-CB 5mM</td>
<td>+ 3-CB 5mM</td>
</tr>
<tr>
<td>pCS50CTSA'</td>
<td>2.0±0.3</td>
<td>29.2±0.2</td>
<td>4.0±1.1</td>
</tr>
<tr>
<td>pCS50CT'SA'</td>
<td>2.6±0.3</td>
<td>29.3±0.1</td>
<td>97.5±7.7</td>
</tr>
<tr>
<td>pCS50CT'SA'</td>
<td>2.1±0.2</td>
<td>32.7±0.7</td>
<td>80.2±10.5</td>
</tr>
<tr>
<td>pCS50CT'SA'</td>
<td>2.3±0.1</td>
<td>2.2±0.0</td>
<td>1.8±0.2</td>
</tr>
</tbody>
</table>

* galactosidase units: nmol/min/mg extract; ( ) standard deviation

Fig 25. Diagram of the inserts of the constructs used for activity assay of the tfdA promoter of pMAB1.
turned out to be highly homologous (>98% at the nucleotide level) with the corresponding genes of the 2,4-D degradative gene cluster tfdA-S-R-D\_IICIIEIIIFB\_IICIIEIIIFB\_K-T\_CDEFB on plasmid pJP4 (80 kb) of R. eutropha JMP134 which contained the additional genes (tfdR-D\_IICIIEIIIFB\_IICIIEIIIFB\_K). The genes tfdS and tfdT seemed to encode LysR-type transcriptional regulators. These results indicated evolutionarily close relationship between the two degradative gene regions, suggesting recent genetic recombination event(s). Transcriptional fusion assays showed that the tfdC promoter was not regulated by tfdT but that it was activated by the distantly-located tfdS in the presence of 2-CM which is an analogous compound of 2,4-dichloromuconate, the metabolite from 3,5-dichlorocatechol by TfdC. TfdS also activated the tfdA promoter in the presence of 2-CM.

Chapter IV
Discussion

1. The structure of the degradative genes for 3-chlorocatechol and 2,4-dichlorophenoxyacetate

(1) A novel 3-chlorocatechol catabolic plasmid pENH91

Plasmid pENH91 from R. eutropha strain NH9 that carries genes for catabolism of chlorocatechols was characterized in this study. The restriction pattern of pENH91 (Fig. 3) was different from those of other plasmids with chlorocatechol degradative genes (Chatterjee and Chakrabarty, 1984; Chaudhry and Huang, 1988; van der Meer et al., 1991c). NH9 could not grow on 2,4-D, and the restriction profile of pENH91 was different from those of 2,4-D degrading plasmids (Bhat et al., 1994; Don and Pemberton, 1985; Mæ et al., 1993). Therefore, it was apparent that pENH91 was a novel chlorocatechol degradative plasmid.

(2) The structure of IS\_1600 and the chlorocatechol catabolic transposon Tn\_5707

The chlorocatechol degradative genes on plasmid pENH91 were found to be located between two copies of IS\_1600 thus the ca. 15-kb region formed the composite class I-type transposon Tn\_5707 (Fig. 3 and 4).

The homologies and the overall structural similarity among IS\_1600, IS\_1326, and IS\_21 indicated an evolutionary relationship (Fig. 7 and 26).

The genes istA and istB of IS\_1600 could encode transposase/cointegrase and a helper protein, respectively, by analogy with those of IS\_21 (Schmid et al., 1998, 1999). The istA gene of IS\_21 encodes both transposase and cointegrase. While the transposase of IS\_21 is the full-length product (46 kDa) of the istA gene, its cointegrase (45 kDa) is translated from an internal translational start of istA and devoids of eight amino acids of the amino (N-) terminus of the transposase. The functional specialization of the transposase was attributed to the eight amino acid residues (MLSREDYF) and it was suggested that the highly polar terminus might help the protein to bring the ends of IS\_21 together in a putative mechanism for simple insertion (transposition of one copy of IS\_21 element) (Schmid et al., 1998). The N-terminus of IstA of IS\_21 contains six amino acids (M, S, R, E, D, and probably Y) that are polar or charged. The N-terminus of the deduced amino acid sequence of IstA of IS\_1600 is MIDVATLS (Fig. 6). Four amino acids (M, D, T, and S) out of the eight are polar or charged. Alternatively, if the translation starts at the second GTG codon, the N-terminus is MATLSVIR and four amino acids (M, T, S, and R) are polar or charged. The function of the protein(s) encoded by the istA gene of IS\_1600 remains to be elucidated.

Although the terminal inverted repeats of IS\_1600, IS\_1326, and IS\_21 differed in length (Fig. 7b), there was some similarity between the nucleotide sequences. Besides the high similarity between the inverted repeats of IS\_1600 and
IS1326, the inverted repeats of the three ISs conserved a dinucleotide CA-3 at their extreme ends. This dinucleotide is known to be significantly preferred for cleavage and joining by retroviral integrases containing the catalytic domain of DDE amino acid residues during integration process of retroviruses to host DNA (Goff, 1992; Katz and Skalka, 1994; Hindmarsh and Leis, 1999). The DDE triad, which is proposed to coordinate a divalent metal ion essential for catalytic activity, are conserved among the IstAs of the ISs of IS21 family as well as among transposases of many bacterial ISs and retroviral integrases (Haren et al., 1999). The dinucleotide has also been suggested to be critical for the cointegrate formation of plasmids containing two copies of IS21 (Haas et al., 1996) and thus could be important for the function of IstA of IS1600.

Because pENH91 was found not to coexist stably with RP4, RP4 was introduced into NH9 by conjugation, expecting to obtain isolates in which the catabolic genes were transposed onto either RP4 or the chromosome of NH9. Although a few colonies appeared on plates prepared with 3-CB and Km, none of these isolates was stable and no evidence of transposition has yet been obtained. Thus, the transposability of the 15-kb region remains to be demonstrated.

The structure of the 15-kb region might indicate that this region had been transposed into a plasmid that was an ancestral plasmid of pENH91. But this region was not flanked by duplicated sequences of the target site, which was supposed to be generated by transposition. Some explanations are possible for this structure. (i) Integration of an ancestral plasmid of pENH91 with IS1600 element(s) into a target site flanking chromosomal chlorocatechol genes, followed by insertion of another IS1600 element into a site beyond the chlorocatehol genes and deletion between the

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**Fig. 26.** Phylogenetic tree of members of the IS21 family based on the alignment of IstA or IstA-like proteins. The alignment was performed with ClustalW software and adjusted manually to incorporate the results reported by Haas et al. (1996). The tree was constructed with the Genetyx software program (Software Development Co., Tokyo, Japan) by the unweighted-pair group method with mathematical averages. A accession numbers (and the references) except those for IS1600 and P5 orfSA are as follows (from top to bottom): X67861 (Xu et al., 1993), Z32853 (Podladchikova et al., 1994), X14793 (Reimmann et al., 1989), L49438 (Xu et al., 1997), U38187 (Brown et al., 1996), A F002247 (Nagy et al., 1997), M 38870 (M enou et al., 1990), X79443 (Solinas et al., 1995), U67315 (Yeo and Poh, 1997), L09108 (Byrne and Lessie, 1994), and U05888 (Rogers et al., 1994).
distal copies of IS1600 (Fig. 27). The scheme for this mechanism has been modified from that proposed for mobilization of catabolic genes into plasmids by IS1071 (Wyndham et al., 1994; Di Gioia et al., 1998) and accounts for the lack of target-site duplications. (ii) Insertion of an IS1600 element proximal to chlorocatechol genes of an antecedent of pENH91 that had originally contained the chlorocatechol genes, followed by insertion of another IS1600 element. This case has been observed for the degradative genes for 2,4,5-trichlorophenoxyacetate and associated insertion sequences (Haugland et al., 1990). This scheme can account for the observed 15-kb structure of pENH91 if the transposition of the element did not generate target-site duplication, as was found for certain kinds of IS elements (Diaz-Aroca et al., 1987; Joset and Guespin-Michel, 1993).

(3) Duplication and deletion of the catabolic genes on the plasmid pENH91

Duplication and deletion of the degradative genes were demonstrated to be consequences of recombination.

Fig. 27. A model for the acquisition of the cbnRABCD genes by an ancestral plasmid of pENH91. The model is drawn based on the model of chromosome mobilization by IS1071 proposed by Wyndham et al. (Wyndham et al., 1994; Di Gioia et al., 1998). While class II transposable elements to which IS1071 belongs transfer by a replicative mechanism, IS1600 could transpose via a cut-and-paste (nonreplicative) mechanism in analogy with IS21. 1. An ancestral plasmid of pENH91 containing a single copy of IS1600. 2. Tandem formation of IS1600 on the plasmid, which facilitates the formation of cointegrate. 3. Bacterial chromosome with a cbnRABCD gene cluster. The target sites (1 and 2) are written arbitrarily. No consensus target sequence is apparent for IS21. 4. Cointegrate formation by the plasmid with the chromosome. Two to three nucleotides in the junction of IS1600 tandem are lost during the reaction. Further insertion of another copy of IS1600 beyond the catabolic region and subsequent deletion by homologous recombination between the two distal copies of IS1600 results in the formation of a plasmid with a composite transposon structure in 5.
between the two IS1600 elements.

There have been a few reports of the amplification of genes for 3-CB (or 3-CC) degrading enzymes. The nucleotide sequences involved in these recombination events have not been reported. Amplification of the 4.3-kb region in *Pseudomonas* sp. strain B13 seemed to be necessary to avoid the accumulation of intermediate products of the degradation of 3-CB (Rangnekar, 1988). Similar amplification of a cloned 4.2-kb fragment from pAC27 and a 15-kb fragment from pJP4 were also ascribed to the necessity for high gene-dosage, for example, in order to compensate for the absence of a positive regulatory element (Ghosal et al., 1985). In the case of the inverted duplication on pJP4 and the pre-existing tandem duplication on pJP2, the advantage was attributed to mutation of the duplicated copy with resultant acquisition of new catabolic phenotypes, rather than to a gene-dosage effect (Ghosal and You, 1988). Recently, tandem amplification of the 105-kb clc element containing the clcR-ABCD genes on the chromosome of *P. putida* F1 has been described (Ravatn et al., 1998a). The amplification was prerequisite for the growth of the strain on chlorobenzene. It seemed that the strain could obtain enough enzymatic activity for the efficient conversion of chlorocatechol, which is a critical step in the chlorobenzene degradation pathway, only by expressing the clc genes from multiple copies (Ravatn et al., 1998a). While the genetics and function of the recombinase of the clc element as the bacteriophage P4 type integrase have been described (Ravatn et al., 1998b), the process of the amplification to more than two copies at one locus has not been fully characterized as to whether it is due to the replicative transposition of the clc element or due to homologous recombination between directly oriented clc elements.

Amplification of chromosomal genes due to general recombination mediated by homologous IS elements have been reported for *Salmonella typhimurium* under laboratory selection condition (Haack and Roth, 1995) and for *E. coli* strain W3110 (Hesslinger and Sawers, 1998). The finding of duplication of the genes in NH9(A) is rather unique in that it was found in Ralstonia and on a plasmid. Interestingly, the transcription of the IS200-encoded orf increased the frequency of homologous recombination between directly repeated chromosomal IS200 elements (Haack and Roth, 1995). It remains to be examined if IstA of IS1600 could play a role in the duplication event of the cbn genes on pENH91, as well as the recombinase of the clc element for its tandem amplification.

No major differences between NH9 and NH9A have yet been observed when their growth in 3-CB-containing liquid medium or their tolerance to 3-CB are compared. In Fig. 8b (lanes 1 and 4) (plasmid DNA and total DNA of NH9, respectively), very weakly hybridized bands can be seen at the same size of the extra fragment of the duplicated plasmid of NH9A. This suggested that culture of NH9 would contain NH9A at a low ratio. However, the fact that NH9A became dominant after 1 year of successive subculturing indicates that duplicated catabolic genes provide cells with an advantage under our laboratory conditions. The functional significance of the gene duplication on pENH91 could be slightly higher growth rate by virtue of efficient conversion of chlorocatechol resulting from increased production of the enzymes or mutation of the genes.

(4) **The similarity between the cbnR-ABXCD genes and the tcbR-CDXEF genes and their different roles in their respective genetic backgrounds**

The chlorocatechol-degradative genes of NH9 (cbnR-ABCD) were found to be highly homologous to those of *Pseudomonas* sp. PS1 (tcbR-CDEF) (van der Meer et al., 1991a, b). The lengths of the corresponding orfs, the overlaps of orfs, and the intervening sequences between orfs were the same between the two gene clusters. Highly conserved nucleotide sequences and the identical overall structures of the two gene clusters suggested that the horizontal transfer and the divergence at the nucleotide level of the two clusters had occurred relatively recently in the evolutionary history of the clusters of genes in the modified ortho pathway (Schlümann, 1994).
The nucleotide divergence between the regions that contained cbnB and tcbD was significantly higher than that between the other corresponding regions of the two clusters (Table 2 and Fig. 11). The genes for chloromuconate cycloisomerase are generally more conserved than the other three genes in the cluster of modified ortho pathway genes (van der Meer et al., 1991a) and, in fact, the sequences of cbnB and tcbD are highly conserved at the amino acid sequence level. The high homology kept at amino acid level might reflect constraint for the function of chloromuconate cycloisomerases. Thus, the reason of the divergence of cbnB and tcbD could be discussed focusing mainly on the pressure to cause nucleotide substitutions, which may not be related to function of the enzyme. The G+C contents of cbnB (63.3%) and tcbD (63.7%) are similar and in the middle range of those of all the genes in the respective clusters. Therefore, the nucleotide divergence between cbnB and tcbD cannot be attributed to "GC pressure" from the host.

Alternatively, one possible explanation for the rapid divergence of cbnB and tcbD is that some pattern of biased codon usage forced the bacteria in which these genes were located to replace nucleotides in an effort to adapt to their genetic background, for example, the pool of transfer RNAs. However, no significant differences in codon-usage patterns between cbnB and tcbD or between cbnB (tcbD) and the other genes in the clusters have been found. At present, it is unknown whether any selective pressure forced the rapid nucleotide divergence of cbnB and tcbD.

The fact that numerous nucleotide substitutions were not limited to the cbnB (tcbD) gene but spanned a region of ca. 1.3 kb that contained cbnB (tcbD) (Fig. 11) suggests that this divergence might have been caused by physical conditions in this region of DNA and not by the genetic nature of cbnB (tcbD). The rate of substitutions is high throughout cbnB (tcbD). Substitutions in the flanking genes tended to be localized in regions proximal to cbnB (tcbD): most of the nucleotide substitutions (18 out of 21) between orfXs (next to cbnB or tcbD) were located within 138 bp of the 5' portion of orfXs (1011 bp), and 5 out of 14 nucleotide substitutions between cbnA and tcbC were located within 24 bp of the 3' region of the genes (756 bp). In the other corresponding parts of the two 6959-bp SacI-KpnI fragments, the rate of mutations, including substitutions, insertions and deletions, was one in several hundred base pairs, which might reflect the basal rate of mutation. Some unidentified local structure of the DNA in the region containing cbnB (tcbD) might have made this region more vulnerable to substitutions during replication.

There was a difference between the chloroaromatic compounds that the two strains, NH9 and P51, utilized as substrates for growth. Strain P51 grew on 1,2- and 1,4-dichlorobenzenes and it also grew on 1,2,4-trichlorobenzene (van der Meer et al., 1991c). Strain NH9 was tested for growth on these compounds, but it failed to grow in liquid medium in the presence of these chlorobenzenes. On the other hand, strain P51 did not grow on 3-CB (van der Meer et al., 1991c). This discrepancy in growth substrates is explained by the difference in available "upper-pathway" enzymes between the two strains. Strain NH9 probably synthesizes enzymes that convert 3-CB to chlorocatechol but not the enzymes that convert chlorobenzenes to any further-metabolizable compounds. Strain P51 has been reported to synthesize the enzymes that convert chlorobenzenes to the corresponding chlorocatechols (van der Meer et al., 1991c; Werlen et al., 1996), but it does not seem to synthesize enzymes that convert chlorobenzoates to chlorocatechols. The recruitment of the homologous chlorocatechol-degradative gene clusters by the two strains, which utilize different chloroaromatics as growth substrates, may illustrate a simple way to adapt to xenobiotic compounds.

(5) The diverse origins of the catabolic transposons carrying chlorocatechol-degradative genes

There have been only a few documented examples of transposable elements that carries genes for the modified ortho pathway, including ISJP4 composite transposon (Leveau and van der Meer, 1997) and the 105-kb clc element (Ravatn et al., 1998b). These two and Tn5707 are diverse in terms of the origin of the transposable elements.

The insertion sequence ISJP4 copy A and its incomplete copy C captured the genes tfdS-R-D1,C,E,F,B,K to form a...
composite transposon on plasmid pJP4 in R. eutropha JMP134 (Leveau and van der Meer, 1997; Leveau et al., 1998). Although the transposition of this whole composite transposon has not been observed, transposition of an artificial composite transposon comprising two copies of ISJP4 has been detected (Leveau and van der Meer, 1997). With regard to their characteristics as transposable elements, there are some differences between Tn5707 and the composite transposon formed by ISJP4. IS1600 of Tn5707 belongs to the IS21 family while ISJP4 belongs to the IS5 group of the IS4 family. The ISJP4 transposon was suggested to have transposed to pJP4 as a composite transposon by the presence of target-site duplication at both ends (Leveau and van der Meer, 1997). By contrast, the absence of such duplication at both ends of Tn5707 on pENH91 suggests mobilization of the chromosome that resulted from plasmid integration and subsequent excision mediated by IS1600 (Wyndham et al., 1994). The functionality of Tn5707 as a composite transposon remains to be examined. The catabolic genes carried by the ISJP4 transposon are different from those carried by Tn5707 as follows. Although the tfdDIICIIEIIFII genes are the most homologous to the tcbCDEF genes, the homology between the corresponding genes ranged from 58% to 70% at the nucleotide level and from 27% to 65% at the amino acid level. The ISJP4 transposon contains duplicated regulatory genes, tfdR and tfdS (Matrubutham and Harker, 1994; You and Ghosal, 1995), as well as additional genes, namely, tfdBII that might encode chlorophenol monooxygenase and tfdK that encodes an active transporter of 2,4-D (Leveau et al., 1998; Perkins et al., 1990).

The differences in inherent characteristics between Tn5707 and the other two chlorocatechol transposons and the strong homology between the two clusters, cbnR-A BCD and tcbR-CDEF, may illustrate the role of the IS elements in the recent dissemination of genes in the modified ortho pathway.

On the phylogenetic tree of IstAs of the IS21 family, IstA of IS1600 formed a distinct cluster together with orfSA from strain P51, IstA of IS1326, and NmoT (Fig. 26). The branching point of the IstA of IS1326 and the other three elements suggests that these four elements diverged relatively recently in the evolution of the members of IS21 family. IS1326 was found in integrons in antibiotic-resistant clinical isolates (Brown et al., 1996). NmoT is a putative transposase that corresponds to IstAs and was found proximal to nitrilotriacetate-degradative genes in Chelatobacter heintzii (Xu et al., 1997). The diverse origins of the four elements indicate the recent wide distribution of the related IS(-like) elements among bacteria, which in turn raises the possibility that these IS(-like) elements might have been involved in recent genetic rearrangements of various kinds. Recent findings of the ISs of IS21 family including the highly reiterated IS1631 on the chromosome of Bradyrhizobium japonicum (Isawa et al., 1999) and several members distributed on the chromosome of Mycobacterium tuberculosis H37Rv (Gordon et al., 1999) may illustrate the diversity of the host bacterial species for the ISs of the family.

### (6) The structure of the 2,4-D degradative gene cluster on plasmid pMAB1

In the 2,4-D degradative bacterium Alcaligenes sp. CSV90, the degradative genes are located within a ca. 12-kb region on its plasmid pMAB1 (90kb) and are organized as two clusters, tfdA-S genes and tfdT-CDEFB genes. The genes tfdS and tfdT seemed to encode LysR-type transcriptional regulators and are located in the opposite orientation from the degradative gene(s) in their respective units. The tfdA-S-T-CDEFB genes from CSV90 have turned out to be highly homologous (>98% at the nucleotide level) with the corresponding genes of the 2,4-D degradative gene cluster (tfdA-S-R-D,C,E,F,B,K-T' -CDEFB) from R. eutropha JMP134 (pJP4 [80kb]) which contained the additional genes (tfdR-Dn,Cn,E,F,Bn,K).

In contrast to the apparent difference between the backbones of the plasmids pMAB1 and pJP4, the above results indicate evolutionarily close relationship between the two degradative gene regions, suggesting recent genetic recombination event(s) (Fig. 23). The structure of the gene clusters on pMAB1 and the high similarities of 5' portion
and 3' end of the tfdT gene of pMAB1 to 5' portion of the tfdT gene of pJP4 and 3' end of the tfdR gene of pJP4, respectively, may suggest that reciprocal recombination between the tfdR and tfdT genes on pJP4 resulted in the structure of the genes on pMAB1. However, the presence of the 33-bp nucleotide sequence of unknown origin between the two parts in the tfdT gene of pMAB1 may suggest an additional process. The process of formation of the gene clusters on pMAB1 remains to be elucidated and so is the question whether tfdT of pMAB1 is a pseudogene or not.

One of the possible advantages of the regulons with LysR-type regulators which share divergent promoter regions with the regulated promoters could be their self-containment (Beck and Warren, 1988). The function of the regulons with LysR-type regulators could be less vulnerable to change of their location during genetic recombination events because of this autonomy; for example, they could be resistant to read-through effect by transcription from neighboring genes. The structures of gene modules for 2,4-D degradation on pMAB1 and pJP4 illustrate this advantage; each of the modules tfdA-S and tfdR-DIICIIEIIFIIBIIK could be independently functional.

As to the tfd(T)-CDEF-B module either on pMAB1 and pJP4, although the respective regulatory gene tfdT seems to be non-functional for both, the activation of the tfdC promoter has been functionally fulfilled by the TfdR(S) on pJP4 (Leveau et al., 1996) and by TfdS on pMAB1 (this study). This was achieved by the recruitment of closely related regulators, which allow cross-activation upon recognition of the pathway intermediate (Leveau et al., 1996; Filer and Harker, 1997; this study).

The ca. 22-kb region of pMAB1 containing the 2,4-D degradative genes was delimited by fragments homologous to parts of Tn5501 (Lauf et al., 1998) (Fig. 23). It remains unknown if these elements were involved in the recruitment of the degradative genes to form plasmid pMAB1.

2. Transcriptional activation of the degradative genes for 3-chlorocatechol and 2,4-dichlorophenoxyacetate

(1) Transcriptional activation of the cbnA promoter by CbnR

Transcriptional activation of the cbnA promoter by cbnR has been characterized both in vivo and in vitro. In growth complementation studies, cbnR was demonstrated to be necessary for growth on 3-CB of strains R. eutropha NH9D (3-CB- derivative of NH9) and P. putida PRS4020 (catR knockout strain), when they harbor the cbnABCD degradative genes. CbnR has also been demonstrated to be a positive regulator by transcriptional reporter and in vitro transcription assays. Both CCM and 2-CM have been identified as inducer molecules. In reporter analysis, the PRS4020 cells containing a plasmid with truncated cbnA gene, pNO50RA'·, exhibited some elevated transcription in the presence of Ben. Therefore, it remained possible that the activity of the cbnA promoter could be induced by Ben or catechol. However, neither Ben nor catechol induced transcriptional activation by purified CbnRHIs in vitro. The reason for the in vivo activation of the pNO50RA'· construct is unclear. It is possible that the activity observed may have been induced by CCM supplied by the host-encoded catechol dioxygenase (CatA). Although catR, the activator of the host catA gene (Houghton et al., 1995), is disrupted in PRS4020, there may be some low level constitutive expression of catA which results in the conversion of Ben to CCM allowing the activation of the cbnA promoter. It is also possible that there are additional CbnR binding sites in the cbnA gene which add to the complexity of the cbnA promoter regulation. Both repressor and activator internal binding sites have been observed with other LysR family members including CatR (Chugani et al., 1998; Cowan et al., 1993).

It is puzzling that CbnR recognizes CCM as well as 2-CM as inducing molecules. Some enzymes of the cbn operon
could catabolize catechol to a certain extent. Chlorocatechol 1,2-dioxygenase (CbnA) could utilize catechol as a substrate (Dorn and Knackmuss, 1978; Reineke, 1998), and chloromuconate cycloisomerase (CbnB) could have some activity against nonchlorinated CCM (Reineke, 1998; Schmidt and Knackmuss, 1980). However, 3-oxoadipate enol-lactone, the product from CCM by CbnB, was presumed not to be catabolized further by the enzymes encoded by cbnCD (Schlömann, 1994). CatR and ClcR activated their regulated promoters significantly only in the presence of the intermediates of their respective pathway, CCM for CatR (Parsek et al., 1992) and 2-CM for ClcR (McFall et al., 1997c). The clcABDE operon was expressed when cells are grown in benzoate because CatR binded to and activates the clcA promoter (McFall et al., 1997b, c; Parsek et al., 1994b). However, CatR cannot bind to or activate the cbnABCD promoter (unpublished observations). It is possible that in addition to utilizing chlorocatechols as carbon substrates, the chlorocatechol dioxygenase enzymes act as important detoxifying agents against catechol. Catechol could be toxic to pseudomonads as chlorocatechols are (Fritz et al., 1992). Because CatR cannot activate the expression of CbnA to detoxify catechol as it would ClcA, CbnA expression by CbnR upon recognition of CCM could be beneficial for the cell to decompose catechol rapidly.

It has been demonstrated that 2,4-dichloromuconate acts as an inducer of tfdCDEF expression (Filer and Harker, 1997). However, the range of inducing molecules for the modified ortho-cleavage pathways, especially as to highly substituted catechols or corresponding muconates, has not been explored. Since the products from the tcbCDEF operon degrade dichlorocatechols and 3,4,6-trichlorocatechol, TcbR probably recognizes corresponding chloromuconates as inducer. It is interesting that the homologous CbnR has the ability to recognize CCM as an inducer, which is characteristic of CatR. The recognition specificity of CbnR remains to be further elucidated.

The specific binding of CbnRHis and CbnR to the cbnA promoter has been shown by gel retardation and DNase I protection assays. CbnRHis had the same function as CbnR in growth complementation, reporter assay, and bending assay of cbnA promoter with and without inducer. Therefore, functional equality of CbnRH with CbnR has been verified in vivo and in vitro. Similar observation about a His tag on C-terminal has been reported for NhaR, a LysR type regulator of Na+/H+ antiporter gene of Escherichia coli (Carmel et al., 1997), suggesting the unaltered function of a C-terminally His-tagged LysR-type protein. On the other hand, the crude soluble protein from E. coli BL21(DE3)pLysS containing pEHiscbnR did not show binding activity to cbnA promoter (data not shown). Although the N-terminal part of LysR family proteins functions as the DNA binding domain, adding additional amino acids on the N-terminal does not necessarily abolish binding activity. The addition of a polypeptide to the N-terminal of SpvR, a LysR-type regulator of virulence genes of Salmonella dublin, showed little effect on the specific binding to the regulated promoters and activation of the promoters (Grob and Guiney, 1996). It is not known whether the protein produced from the construct pEHiscbnR does not have the binding activity to cbnA promoter or is exclusively retained in the inclusion bodies.

Using established conditions for gel retardation assays (Parsek et al., 1994a; van der Meer et al., 1991b), crude soluble protein from BL21(DE3)pLysS containing either pT7cbnR or pT7cbnRHs always formed aggregates with the cbnA promoter fragment in the well of the electrophoresis gel. The addition of heparin in the binding reaction dissolved the aggregate and the protein/DNA complex was able to migrate in the gel. It was presumed that CbnR formed these aggregates because of its highly basic nature [calculated pl=10.3 by Genetyx Software (Software Development Co., Tokyo, Japan)]. Heparin has been used successfully in the study of the formation of splicing complex on mRNA's where it was presumed to quench the nonspecific binding of components in the nuclear extract with the highly negatively charged RNA (Konarska and Sharp, 1986). In our study, it is possible that the negatively charged heparin was able to cancel the electrostatic force among the complexes of CbnR(His)/DNA but not the specific binding between CbnR(His) and DNA fragment.
The footprinting pattern of CbnR to cbnA promoter, with hypersensitive sites localized in the center, resembles those of CatR to the catB promoter (in the presence of the inducer CCM) (Parsek et al., 1992) and ClcR to the clcA promoter (Coco et al., 1994; McFall et al., 1997c). Thus, based on the results of ClcR, it is suggested that the region from nucleotides -76 to -49 is the recognition binding site (RBS) and the region from -44 to -19 is the activation binding site (ABS) (McFall et al., 1998) as shown in Fig. 17. RBS contains the conserved T-N11-A motif critical for binding of the LysR-type regulators (McFall et al., 1998; Schell, 1993). Further binding of the regulator to ABS helps RNA polymerase bind to the promoter region, thus to activate transcription (McFall et al., 1998). The extended footprinting pattern of CbnR in the absence of the inducer, encompassing both RBS and ABS of the cbnA promoter, resembles that of ClcR to the clcA promoter (Coco et al., 1994; McFall et al., 1997c). This is in contrast to the restricted footprinting pattern of CatR to RBS of the catB promoter in the absence of CCM (Parsek et al., 1992).

The addition of inducer, CCM or 2CM, to the binding reaction did not cause a change in the footprinting pattern of CbnR to the cbnA promoter, while addition of 2CM caused a shift in the footprinting pattern of ClcR to the ABS of the clcA promoter region (McFall et al., 1997c). The relaxation of the bending angles of cbnA promoter region bound by CbnR(His) upon the addition of inducer was similar to those shown by CatR (Parsek et al., 1995) and ClcR (McFall et al., 1997b). The footprinting patterns and changes in DNA bending upon addition of inducers of the cat, clc and cbn promoters suggest that the outline of the transcriptional activation mechanism of these regulatory systems is conserved.

(2) Transcriptional activation of the tfdC promoter and the tfdA promoter by TfdS in vivo

Transcriptional fusion assays showed that the tfdC promoter was not regulated by tfdT but that it was activated by the distantly-located tfdS in the presence of 2-CM which is an analogous compound of 2,4-DCM, the metabolite from 3,5-dichlorocatechol (3,5-DCC) by TfdC. TfdS also activated the tfdA promoter in the presence of 2-CM (Fig. 25 and Table 5). These results indicated the degradative abilities of the two separate units, tfdA and tfdCDEFB, are interconnected functionally by one regulator, TfdS, to constitute the 2,4-D degradative pathway in CSV90.

In the assay of the activity of the tfdC promoter, high basal level transcription was observed for the cells containing the constructs with the full length tfdC gene in contrast to the cells containing the constructs with only 5'-terminus of the tfdC gene. High basal level might be the result of the following two factors. (i) The absence of intact cognate regulatory gene which should regulate the promoter strictly thus repress the transcription when substrate is not available might allow leaky transcription. (ii) Some structure resulting from the sequence of the tfdC (or truncated tfdT) gene might make the promoter region more vulnerable to contact by RNA polymerase. This tendency was parallel with the activation of the cbnA promoter by CbnR (See the result for pNO50AB+ Table 3). In any case, the alternative regulator, tfdS, could regulate the tfdC promoter only in a relaxed manner.

In the presence of 3-CB, the PRS4020 cells containing pCS50SrCD+1 exhibited much higher activity than those containing pCS50SCD+1. In fact, it was the highest activity observed among the constructs for the assay of the tfdC promoter activity. In the construct pCS50SrCD+1, the promoter regions of the two genes, tfdS and tfdC, are located closely but not overlapped. The primary reason for this high activity could be the contribution of the read-through transcription from the tfd(S-)A promoter, as well as the transcription from the tfdC promoter, for the expression of the 8-galactosidase gene in the construct pCS50SrCD+1. In the constructs, pCS50SCD+1 and pCS50SrCD+1, the -10 portion of the putative promoter sequence for tfdA (thus, for tfdC in pCS50srCD+1) was altered from T A G A C T to T A G A G C by the cloning procedure using the overlapping XbaI site (T CT A GA), while -35 portion of the tfdA promoter and the whole putative promoter for tfdS was retained.
Some of the other reasons why divergent promoter region(s) could contribute to the transcriptional activation, even though the promoters are not shared, are as follows (Beck and Warren, 1988); (a) Regulatory proteins can act more efficiently in cis, which allows effective control at low concentrations of the regulatory molecules. (b) Multiple promoters in divergent promoter regions might attract RNA polymerase independently, resulting in a higher local concentration of the protein. (c) Binding of RNA polymerase to a promoter deforms DNA thus affect DNA topology. The different activities exerted by 3-CB from pCS50SCD'1 and pCS50SrCD'1 could not likely be attributed to the point (a) but possibly to (b) and (c).

In contrast to the leaky control of the tfdC promoter by tfdS, the regulation of the tfdA promoter by tfdS is rather strict. The structure of the gene clusters on pJP4 seems to indicate tfdR belongs to tfd chlorocatechol gene module II. It is possible that a duplication event, which gave rise to the tfdS gene from the tfdR gene, resulted in simultaneous duplication of the (tfdR-)tfdD_u promoter region to supply a promoter for the tfdA gene on pJP4. This structure of the tfdS-A region might have been kept on pMAB1, provided that the structure of the gene cluster on pMAB1 is the descendant resulted from recombination event(s) happened to the gene clusters of pJP4. Thus, while tfdS and the divergent promoter region might not have been the original regulatory system for the expressin of the tfdA gene of pMAB1, the tfdA gene might have been accidentally connected under the divergent promoter to be strictly controlled by TfdS.

2,4-D itself does not induce the expression of the tfdA gene in strain JMP134 (Filer and Harker, 1997). The apparent discrepancy between the substrate of TfdA (i.e. 2,4-D) (Fukumori and Hausinger, 1993b) and the inducers recognized by TfdSR (2-CM [this study] and, for the transcriptional activation of the tfdC promoter in JMP134, 2,4-DCM [Filer and Harker, 1997]) seems to agree with the above possibility that tfdS might not have co-evolved with the tfdA gene. Recent study with the 2,4-D pathway encoded by plasmid pEST4011 also showed that the regulator of the pathway, TfdR, which shared 90% amino acid identity with TfdR of pJP4, activated the cognate tfdC promoter with 2,4-DCM as an effector molecule (Vedler et al., 2000).

The transcription at the tfdA promoter of pMAB1 was weakly activated by TfdS in the presence of Ben (Table 5). Since the cells with pCS50CTSA' exhibited comparable activity with those by pCS50CTSA' and pCS50CT'SA', the effector molecule could be either Ben, catechol, or CCM. However, a recent study of the strain JMP134 in vivo strongly suggested CCM could induce the expression of the tfdA gene (Radnoti de Lipthay et al., 1999). Thus, it is highly likely that CCM is the inducer also for the tfdA gene of pMAB1. The activity shown with pCS50CTSA' might have been exerted by CCM produced by the constitutive activity of host encoded CatA.

Since both of the tfdA-S genes and the tfd(T)-CDEF-B (module I) genes are nearly identical between pMAB1 and pJP4, further study with the genes of pMAB1 and comparative study with strains CSV90 and JMP134 could cast light upon the overall regulation of the expression of the 2,4-D degradative genes.

(3) Divergence of the LysR-type transcriptional regulators of the (chloro)benzoate/(chloro)catechol degradative pathway

A phylogenetic tree was constructed with the amino acid sequences of LysR-type transcriptional regulators which activate the promoters of the degradative genes for (chloro)catechols or (chloro)benzoates of Gram negative bacteria and for which information of the inducers is available (Fig. 28). NahR was used as the out-group.

The regulators are apparently divided into two large groups. One group (group I; upper portion of the tree) comprises CbeR(NK8), CatR(RB1), BenM(A DP1) and CatM(A DP1). These regulators activate the expression of catechol ortho-cleavage genes or benzoate dioxygenase genes. The other group (group II; lower portion except NahR) consisted
of the regulators for the expression of the chlorocatechol ortho-cleavage genes. The chlorocatechol ortho-cleavage genes are considered to have diverged from catechol ortho-cleavage genes after the common ancestor had recruited a LysR-type regulator. The amino acid sequences of the regulators have accordingly diverged, which is reflected as the two large clusters as a whole. However, there seem to be distinctive characteristics for each group which may not be explicable only by co-evolution with the cognate degradative genes.

While the regulators, ClcR, TfdS(R), and CbnR, of group II seem to have specialized to recognize chloromuconates or CCM, the other regulators seem to acquire the ability to recognize Ben or 3-CB fortuitously. Considering that CbnR recognize CCM and 2-CM equally well as inducer, ClcR and TfdS might have been more specialized for 2-CM. When the responses of these regulons to dichloromuconates produced in vivo from dichlorocatechols were compared by reporter

![Phylogenetic tree of LysR-type regulators of (chloro)benzoate/ (chloro) catechol degradative pathway based on the deduced amino acid sequences.](image)

Fig. 28. Phylogenetic tree of LysR-type regulators of (chloro)benzoate/ (chloro) catechol degradative pathway based on the deduced amino acid sequences.

**Inducers known to activate the regulated promoter**

<table>
<thead>
<tr>
<th>Inducers</th>
<th>Benzoylate</th>
<th>3-Chlorobenzoate</th>
<th>Catechol</th>
<th>3-Chlorocatechol</th>
<th>CCM</th>
<th>2-CM</th>
<th>Others</th>
</tr>
</thead>
<tbody>
<tr>
<td>CbeR(NK8)</td>
<td>+</td>
<td>+</td>
<td>(±?)</td>
<td>(±?)</td>
<td>-</td>
<td>4-CB</td>
<td>+</td>
</tr>
<tr>
<td>CatR(RB1)</td>
<td>-</td>
<td>-</td>
<td>(±?)</td>
<td>+</td>
<td>-</td>
<td>4-CB</td>
<td>+</td>
</tr>
<tr>
<td>BenM(ADB1)</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>DCM +</td>
<td>2,3-CB</td>
</tr>
<tr>
<td>CatM(ADB1)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>DCM +</td>
<td>2,3-CB</td>
</tr>
<tr>
<td>ClcR(pAC27)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>DCM +</td>
<td>2,3-CB</td>
</tr>
<tr>
<td>TfdS(R) (pMAB1&amp;PJ4)</td>
<td>(±?) -</td>
<td>(±?) -</td>
<td>±? ±</td>
<td>+ ±</td>
<td>+</td>
<td>3,4-DCC ±</td>
<td>2,4-DCC ±</td>
</tr>
<tr>
<td>CbnR (pEH91)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>DCM +</td>
<td>2,3-CB</td>
</tr>
<tr>
<td>TfdT(NK8)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+?</td>
<td>+</td>
</tr>
<tr>
<td>TfdT(pMAB1)</td>
<td>(defective as an activator?)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>NahR(NAH7)</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

CbeR (NK8); catechol/(chloro)benzoate operon of Burkholderia sp. NK8
(CFrancisco, Ogawa, Suzuki, and Miyashita, A B024746)

CatR (RB1); catechol operon of Pseudomonas putida RB1 (Rothmel et al., 1990; M 33817)

BenM (ADB1); benzoate operon of Acinetobacter sp. A DP1 (Collier et al., 1998; A F009224)

CatM (ADB1); catechol operon of Acinetobacter sp. A DP1 (Neidle et al., 1989; A F009224)

ClcR (pAC27); chlorocatechol operon from pAC27 of Pseudomonas putida A C866 (Coco et al., 1993; L06464)

TfdS (pMAB1); 2,4-D/chlorocatechol operon from pMAB1 of Alcaligenes sp. CSV90 (this study). Nearly identical with TfdS and TfdR of 2,4-D/chlorocatechol operon from pJP4 of Ralstonia eutropha JMP134. (Matrubutham and Harker, 1994; M 98445, You and Ghosal, 1995; S80112)

CbnR (NH9); chlorocatechol operon from pEN91of Ralstonia eutropha NH9 (this study; A B019032). Nearly identical with TcbR of chlorocatechol operon from pPS1 of Pseudomonas sp. P51 (van der Meer et al., 1991b)

TfcR (NK8); chlorocatechol operon from a large plasmid of Burkholderia sp. NK8
(Liu, Ogawa, and Miyashita, A B050198)

TfdT (pMAB1); 2,4-D/chlorocatechol operon from pMAB1 of Alcaligenes sp. CSV90 (Bhat et al., 1994; D16356, this study)

NahR (NA H7); naphthalene operon from plasmid NA H7 of Pseudomonas putida (Schell and Sukordhaman, 1989; J04233)
analysis, ClcR responded to 2,3-DCM more preferentially than to 2,4-DCM while TfdS(R) and CbnR responded to 2,4-DCM more strongly. TfdC is known to have the highest specificity constant against 3,5-DCC among catechol derivatives, which has been considered as adaptation of the enzyme for 2,4-D degradation pathway (Bhat et al., 1993). CbnA has also preference for 3,5-DCC compared to 3,4-DCC. These results suggest the possibility that TfdS(R) and CbnR have adapted for the recognition of the intermediate produced from the favored substrate 3,5-DCC by the cognate chlorocatechol dioxygenases. These three regulators of group II seem to have highly specialized for (chloro)muconates or seem to have lost the flexibility of the structure to resume the recognition of (chloro)benzoate. Since the inducer-recognizing specificity could be altered by subtle change in the amino acid sequence of the regulator (Cebolla et al., 1997), the specialization of these three regulators is rather conspicuous.

CbeR of Burkholderia sp. NK8 recognize Ben and 3-CB to induce the expression of the catechol ortho-cleavage genes and the chlorobenzoate dioxygenase genes. The recognition of 3-CB by CbeR was necessary for the cell to degrade this compound because the chlorobenzoate 1,2-dioxygenase genes responsible for the conversion of 3-CB in NK8 are located at downstream of and cotranscribed with the catechol 1,2-dioxygenase gene. It should be noted that the regulators, ClcR, TfdS and CbnR, may not have had a chance to be in such a situation that necessitate them to recognize Ben or 3-CB. TfdT of strain NK8 is a regulator of chlorocatechol degradative genes, which are similar to tfd genes (module I) of R. eutropha JMP134. Transcriptional fusion study suggested TfdT recognize 3-CB, 3-CC and 3,4-DCC as well as 2,4-DCM to activate the cognate promoter thus implying the diversity of the inducer recognition among the group II. The present study demonstrated the diversity of inducer recognition by the LysR-type regulators involved in the degradation of (chloro)benzoates/ (chloro)catechols and the specialization of ClcR, TfdS, and CbnR to recognize (chloro)muconates.

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This report is based on Ph.D thesis of Naoto Ogawa and modified from it.

Appendix A
List of abbreviations

Ben: benzoate
3-CB: 3-chlorobenzoate
4-(or 2-) CB: 4-(or 2-) chlorobenzoate
3-CC: 3-chlorocatechol
CCM: cis,cis-muconate
2-CM: 2-chloro-cis,cis-muconate
2,4-D: 2,4-dichlorophenoxyacetate
DCC: dichlorocatechol
DCM: dichloro-cis,cis-muconate
PCBs: polychlorinated biphenyls

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Summary

Ralstonia eutropha NH9, isolated in Japan, degrades 3-chlorobenzoate (3-CB) via the modified ortho-cleavage pathway. A ca. 5.7-kb six gene cluster responsible for chlorocatechol degradation was cloned from the transmissible plasmid pEN91 of NH9: the cbnABCD operon encoding the degradative enzymes (including orfX of unknown function) and the divergently transcribed cbnR encoding the LysR-type transcriptional regulator of the cbn operon.

The cbnR-ABCD genes were found to be located between two directly-oriented identical insertion sequences (ISs) of 2,520 bp, designated IS1600, thus forming a composite transposon designated Tn5707 (ca. 15 kb).

The cbnR-ABCD genes showed the highest homology to the tcbR-CDEF genes on plasmid pPS1 of the 1,2,4-trichlorobenzene-degrading bacterium Pseudomonas sp. PS1, which was isolated in the Netherlands (89% to 100% identity at the nucleotide level). The structure of the operon, including the lengths of open reading frames and intervening sequences, was completely conserved between the cbn and tcb genes. Most nucleotide substitutions were localized within and proximal to the cbnB (tcbD) gene. The difference in utilizable chloroaromatics as growth substrates between the two strains seemed to be due to differences in enzymes that convert substrates to chlorocatechols.

Although the tcbR-CDEF genes were not associated with similar ISs, a DNA fragment homologous to IS1600 was cloned from the chromosome of strain PS1. The sequence of the fragment suggested that it might be a remnant of an IS. The two sequences, together with IS1326 and nmoT, formed a distinct cluster on a phylogenetic tree of the IS21 family. The diversity of the sources of these IS or IS-like elements suggests the prevalence of ISs of this type among related bacteria.

In one of the 3-CB segregants, the plasmid had undergone deletion of a segment of about 12.5 kb that covered the catabolic genes. The deletion event seemed to be the result of reciprocal recombination between two elements of IS1600. During repeated subculturing of NH9 on liquid media with 3-CB, the culture was taken over by a derivative strain (designated NH9A), in which the degradative plasmid carried a duplicate copy of the 12.5-kb region that contained the cbnR-ABCD genes. The duplication of these genes seemed again to have been mediated by recombination between the direct repeat sequences.

Transcriptional fusion studies demonstrated that cbnR regulates the expression of cbnABCD positively in the presence of either 3-CB or benzoate which are catabolized via 3-chlorocatechol and catechol, respectively. In vitro transcription assays confirmed that 2-chloro-cis,cis-muconate (2-CM) and cis,cis-muconate (CCM), intermediate products from 3-chlorocatechol and catechol, were inducers of this operon. This inducer-recognizing specificity is different from those of the homologous catechol (catBCA) and chlorocatechol (clcABD) operons of Pseudomonas putida, in which only the intermediates of the regulated pathway, CCM for catBCA and 2-CM for clcABD, act as significant inducers.

Specific binding of CbnR protein to the cbnA promoter region was demonstrated by gel shift and DNaseI footprinting analysis. In the absence of inducer, a region of ca. 60 bp from -20 to -80 upstream of the cbnA transcriptional start point was protected from DNaseI cleavage by CbnR, with a region of hypersensitivity to DNaseI cleavage clustered at -50. Circular permutation gel shift assays demonstrated that CbnR bent the cbnA promoter region to an angle of 78º and that this angle was relaxed to 54º upon the addition of inducer. While a similar relaxation of bending angles upon the addition of inducer molecules observed with the catBCA and the clcABD promoters may indicate a conserved transcriptional activation mechanism of ortho-cleavage pathway genes, CbnR is unique in having a different specificity of inducer recognition and the extended footprint as opposed to the restricted footprint of CatR without CCM.
In the 2,4-dichlorophenoxyacetate (2,4-D) degradative bacterium Alcaligenes sp. CSV90, the degradative genes were located within a ca. 12-kb region on its plasmid pMAB1 (90kb) and were organized as two gene clusters, tfdA-S and tfdT-CDEFB. The genes tfdS and tfdT apparently encoded LysR-type transcriptional regulators and were located in the opposite orientation from the degradative gene(s) in their respective units. The tfdA-S-T-CDEFB genes from CSV90 have turned out to be highly homologous (>98% at the nucleotide level) with the corresponding genes of the 2,4-D degradative gene cluster (tfdA-S-R-D,E,F,B,K-T-CDEFB) from R. eutropha JMP134 (pJP4[80kb]) which contained the additional genes (tfdR-D,E,F,B,K). These results indicated evolutionarily close relationship between the two degradative gene regions, suggesting recent genetic recombination event(s).

Transcriptional fusion assays showed that the tfdC promoter was not regulated by tfdT but that it was activated by the distantly-located tfdS in the presence of 2-CM which is an analogous compound of 2,4-dichloromuconate, the metabolite from 3,5-dichlorocatechol by TfdC. TfdS also activated the tfdA promoter in the presence of (chloro)muconate. It was suggested that the three regulators, CbnR, TfdS, and ClcR, have specialized to recognize (chloro)muconates as inducer molecules while some of other LysR-type regulators of (chloro)benzoate/(chloro)catechol pathway seemed to recognize (chloro)benzoates or chlorocatechols as inducer molecule(s).
細菌のクロロカテコール及び2,4-ジクロロフェノキシ酢酸分解遺伝子群の構造と転写調節

小川 直人

概要

PCBや農薬、有機溶剤のような芳香族塩素化合物は分解しにくく、環境中に長く残存して汚染問題を引き起こすが、環境中の微生物には、進化、適応の結果、このような化合物を分解する能力を獲得したものがいる。この微生物の分解能力を研究することは、バイオレメディエーション技術の開発のため、また、微生物の進化、適応機構を解明するためにも重要である。このような目的のもと、本研究では、芳香族塩素化合物の一種であるPCBの分解中間産物である3-クロロ安息香酸（3-CB）の分解菌Ralstonia eutropha NH9株の3-クロロカテコール分解遺伝子群、及び農薬として使われた2,4-ジクロロフェノキシ酢酸（2,4-D）の分解菌Alcaligenes sp. CSV90株の分解遺伝子群の構造と転写調節機構の解析を行った。

細菌による3-CBや2,4-Dなどの芳香族塩素化合物の代表的な好気的分解経路は、これらの化合物がクロロカテコールにまで変換される前半過程（上流経路）と、クロロカテコールが完全分解される後半過程（修飾オルソ開裂経路）から成る（図1）。修飾オルソ開裂経路は、芳香族塩素化合物を完全分解するために重要な経路である。日本で分離された3-CB分解菌R. eutropha NH9株の修飾オルソ開裂経路の遺伝子群は、オペロン様構造をなす分解酵素遺伝子群cbnABCDと、その上流に双方向プロモーター（divergent promoter）領域を介して逆向きに存在する、LysR型の調節因子の遺伝子cbnRから成ることが判明した（図2）。さらにcbnR-ABCD遺伝子群（5.7kb）は、NH9株の分解プラスマドpENH91（78kb）上に、両側をそれぞれ約2.5kbの挿入配列IS600に挟まれ、全体として約15kbのclassI型複合トランスポゾンTn5707として存在することができるになった。

cbnR-ABCD遺伝子群は、オランダで分離された1,2,4-トリクロロベンゼン分解菌Pseudomonas sp. P51株の分解プラスマドpP51（110kb）上の3,4,6-トリクロロカテコール分解遺伝子群tcbR-CDEFとほぼ同一の配列を持つことから、修飾オルソ開裂経路遺伝子群の分化の過程でも、最近水平伝達されたことが示唆された。両菌株が分解する化合物が異なるのは、保持している分解の上流経路が異なるためであった。ほぼ同じ修飾オルソ開裂経路の遺伝子群を使用しながら、生育する芳香族塩素化合物が異なることは、既存の分解遺伝子群モジュールを組み合わせることが、細菌の新たな分解経路形成の有効な戦略であることを示している。また、NH9株ではTn5707の両端のIS600を介した相同組換えにより、プラスマド上で分解遺伝子群の欠失や増幅が起こることが判明し、挿入配列が転移以外でも細菌の遺伝子群の再編成に寄与していることが示された。

(pP4)  (組換えによる欠失 (？))  つdA-S-T-CDEF-B遺伝子群(CSV90株(pMAB1)), という過程で再編成を経てきたことが推測された。

これらの分解遺伝子群の発現調節機構を解明するために、まず、NH9株(pENH91)のcbnA BCD分解酵素遺伝子群について、cbnA プロモーター領域や前後の遺伝子から構築したレポーター-アッセイ

図１．3・クロロ安息香酸と2,4・ジクロロフェノキシ酸の分解経路

図２．複合型トランスポゾンTn5707の模式図

図3．プラスマドpJP4とpMAB1上の2,4-D分解遺伝子群の模式図
系による実験を行った。その結果、cbnA プロモーター活性の誘導には cbnR が必須であり、正の調節を行うことが判明した。また、安息香酸（Ben）や 3-CB の分解経路における CbnA (chlorocatechol dioxygenase) による反応産物 (cis,cis-ジスチクロン酸 (CCM) 及び 2-クロロ-cis,cis-ジスチクロン酸 (2CM)) が CbnA プロモーターの真の発現誘導物質であると推定された。以上の点を、精製した CbnR、cbnA プロモーター、大腸菌 RNA polymerase、誘導物質候補の各種化学物質を用いた in vitro transcription 実験により確認した。これまでに発現調節機構が詳細に解析されている類縁のカテコールオルソ開裂経路の分解遺伝子群 catR-BCA (Pseudomonas putida RB1 株)、及びクロロカテコール分解遺伝子群 clcR-ABDE (Pseudomonas putida AC866 (pAC27) 株、Pseudomonas sp. B13 株) では、それぞれの分解経路における中間代謝産物（前者は CCM、後者は 2CM）1 種類のものが主要な誘導物質であるのに対し、cbnR-A BCD 部分遺伝子群ではこの両化合物が誘導物質であることが示された。一方、in vitro での結合の解析により CbnR は cbnA の転写開始点の上流約 20 割-80bp の領域に LysR family の調節系に特徴的なパターンで結合すること、及び cbnA promoter 領域は CbnR の結合により 78 度曲がり、誘導物質 CCM の添加によりこの Bending の角度が 54 度まで緩和することが判明した。catR-BCA (RB1) 分解遺伝子群及び clcR-ABDE (pAC27) 分解遺伝子群も同様のプロモーター領域の折れ曲がり角度の変化を示すことなどから、これら 3 つの分解遺伝子群の転写活性化の基本的な機構は共通であるとして推察された。以上、CbnR のよる cbnA プロモーターの転写活性化について、in vivo、in vitro で解析を行った結果、catR-BCA、clcR-ABDE 各分解遺伝子群の発現調節機構との相違点、類似点が明らかになった。

次に CSV90 株 (pMAB1) の tfdCDEF(-B) 遺伝子群の発現調節機構を解析するためにレポーター実験を行った。その結果、TfdT には tfdC プロモーターを活性化する機能はないことが示唆され、一方、TfdS が 2CM の存在下で tfdC プロモーターの発現を正に調節することが判明した。また TfdS が 2CM の存在下で tfdA プロモーターの活性化も行うことが示唆し、CSV90 株では tfdS が 2 つの制御単位に分かれた分解遺伝子群をそれぞれ正に制御していると考えられた。TfdS によるこれらのプロモーターの活性化は、CCM の存在下ではごく弱かった。tfdT (pMAB1) がレポーター実験で tfdC プロモーターの活性化を行わなかったことからは、この遺伝子が、pMAB1 上の 2,4-D 分解遺伝子群を形成した組み換えの結果生じた偽遺伝子である可能性が示唆された。そして、tfdS が近傍の tfdA の発現のみならず、離れたところに位置する tfdCDEFB の発現も活性化することは、本来の調節因子が機能しない場合にも、同族の調節因子によるクロストークが分解経路遺伝子の発現に寄与することが示された。

以上の LysR-type 調節因子 (CbnR, TfdS, ClcR, CatR) はいずれも、分解経路の中間産物（あるいはそのアナログ）である 2CM や CCM を認識して分解遺伝子群の発現を活性化する。これに対し、2,3,4-CB 分解菌 Burkholderia sp. NK8 株は、Ben 及び 2-CB の分解中間産物として生じるカテコールをオルソ開裂経路により分解するが、その調節因子 CbcR は分解中間産物ではなく Ben, 3-CB, 4-CB そのものを認識して分解遺伝子群プロモーターの発現を活性化することがレポーター実験により判明した。また NK8 株の 3 及び 4-クロロカテコール分解遺伝子群の発現調節因子 TfdT は、レポーター実験により 3-クロロカテコールを認識して分解遺伝子群の発現を活性化することが示唆された。このように芳香族塩素化合物分解遺伝子群の発現調節に関与する LysR-type の調節因子は、その誘導物質の認識スペクトラルが多様であることが明らかになった。

本研究はクロロカテコールと 2,4-D の分解遺伝子群を対象としてその構造、発現調節機構を解明することで、細菌の芳香族塩素化合物分解能力獲得における遺伝子群の再編成の意義と、LysR-type 調節因子による発現調節機構の役割を明らかにした。