MICROBIOLOGICAL REVIEWS, Dec. 1992, p. 677–694
0146-0749/92/040677-18$02.00/0
Copyright © 1992, American Society for Microbiology
Vol. 56, No. 4

Molecular Mechanisms of Genetic Adaptation to Xenobiotic Compounds

JAN ROELOF VAN DER MEER,[1]†* WILLEM M. DE VOS,[1] SHIGEAKI HARAYAMA,[2] AND ALEXANDER J. B. ZEHNDER[1]†

Department of Microbiology, Wageningen Agricultural University, 6703 CT Wageningen, The Netherlands,[1] and Department of Medical Biochemistry, Faculty of Medicine, University Medical Centre, 1211 Geneva 4, Switzerland[2]

GENERAL INTRODUCTION

The pollution of the environment with manmade organic compounds has become such an evident issue that it needs no further introduction. Microorganisms play a major role in the breakdown and mineralization of these pollutants (5); however, the kinetics of the biodegradation process may be much slower than desired from public health or environmental considerations. Numerous field tests and laboratory experiments have identified many harmful organic compounds which are slowly biodegradable. These include halogenated aromatics (such as benzenes, biphenyls, and anilines), halogenated aliphatics, and several pesticides (6, 94, 125, 135, 221, 249, 265). The slow biodegradation of these compounds in the natural environment may be caused by unfavorable physicochemical conditions (such as temperature, pH, redox potential, salinity, oxygen concentration) or may be affected by the availability of other nutrients, the accessibility of the substrates (solubility, dissociation from adsorbed materials, etc.), or predation (6, 70, 113, 183, 231). On the other hand, the low biodegradability may be due to the incapacity of microorganisms present in the natural environment to effectively metabolize pollutants with uncommon chemical structures or properties. Such compounds, which are alien to existing enzyme systems, are called xenobiotics. Nevertheless, microbial communities exposed to xenobiotic compounds could often adapt to these chemicals, and microorganisms that metabolize them completely and at considerable rates have been isolated. Little is known about the molecular events that lead to adaptation of microbial communities. Increased knowledge about these events may provide a better insight into the metabolic capacities of microorganisms to use xenobiotics as novel growth substrates and may reveal the underlying principles of metabolic diversification in bacteria in general.

The term "adaptation" used in biodegradation literature is confusing since it refers to phenomena which can be encountered both in mixed microbial communities and in individual microbial lineages. In a typical adaptive response to organic compounds, virtually no biodegradation occurs immediately after the first exposure of the microbial community to the compound. However, after a period ranging from hours to months, mineralization starts. Adaptive acquisition of degradative abilities for some organic compounds or of resistance to heavy metals has been demonstrated for bacteria in laboratory ecosystems (2, 12, 94, 135, 215, 221, 231, 249). Different molecular and biochemical processes may cause such an adaptive response. (i) The first is induction of specific enzymes in members of the community, resulting in an increase of the observed degradative capacity of the total

* Corresponding author.
† Present address: EAWAG/ETH, CH-8600 Dübendorf, Switzerland.

community (215, 221). (ii) Another process is growth of a specific subpopulation of a microbial community able to take up and metabolize the substrate. This was supposed to be the likely explanation for the adaptation of aquatic bacteria to *p*-nitrophenol (2) or to Hg^{2+} resistance (10–12). (iii) Adaptation can also involve the selection of mutants which acquired altered enzymatic specificities or novel metabolic activities and which were not present at the onset of exposure of the community to the introduced compounds (12, 221). Such a selective process may require longer adaptation times than the other two processes (i.e., induction and growth). It may well be responsible for the adaptation observed in mineralization of recalcitrant xenobiotics, such as halogenated aromatics (2, 71, 94, 191, 208, 220, 221, 249).

In this review, the possible genetic mechanisms which may contribute to the adaptive response of bacteria to xenobiotic compounds are discussed. Unfortunately, it is almost impossible to study these mechanisms directly, since current knowledge does not allow us to predict adaptive events in time and space. Thus, we cannot search for them in a single microorganism at a given time. However, mechanisms for the evolution of metabolic pathways can be deduced to some extent from the comparison of related catabolic enzymes and homologies between catabolic genes. In the following discussion, the organization of different genes encoding aromatic degradation pathways in bacteria is first reviewed. The aromatic degradation pathways have been studied most extensively and form a good model for the genetics of xenobiotics degradation. The second part deals with different genetic mechanisms operating in microorganisms (i.e., gene transfer, mutation, recombination, and transposition), which contribute to metabolic adaptation. The last part of this review briefly discusses the available information on genetic processes occurring under natural conditions in the environment and the molecular tools which are nowadays available to study these processes.

ORGANIZATION OF AROMATIC DEGRADATION PATHWAY GENES

An important class of xenobiotics comprises aromatic compounds carrying different chemical substituents (e.g., halogen atoms, nitro groups). These substituents often decrease the biodegradability of the compounds. The close structural resemblance of these compounds to nonxenobiotic analogs makes them very suitable for use in studies of metabolic adaptation. Although aerobic mineralization processes are certainly not the only important biodegradation processes in the natural environment, genetic studies have focused mainly on aerobic pathways, and many details of these metabolic routes have been described in excellent reviews (30–32, 35, 68, 179, 181). A general comparison of the major pathways for catabolism of aromatic compounds in bacteria has revealed that the initial conversion steps are carried out by different enzymes but that the compounds are transformed to a limited number of central intermediates, such as protocatechuate and (substituted) catechols (30–32, 35, 49, 68, 179, 181). These dihydroxylated intermediates are channeled into one of two possible pathways, either a *meta* cleavage-type pathway or an *ortho* cleavage-type pathway (Fig. 1) (80). Both types of pathways lead to intermediates of central metabolic routes, such as the tricarboxylic acid cycle. This generalized scheme of catabolic pathways for aromatic compounds suggests that microorganisms have extended their substrate range by developing peripheral

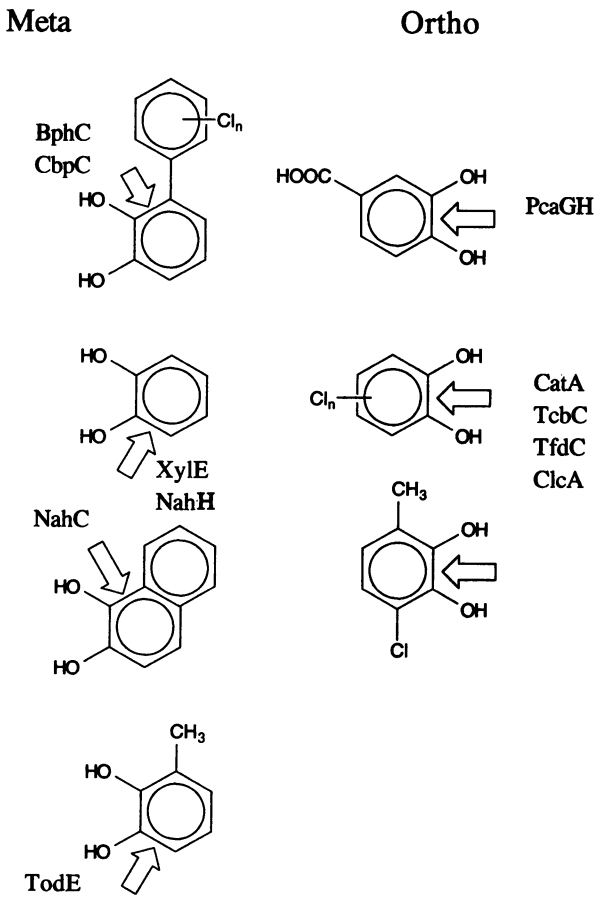

FIG. 1. Extradiol and intradiol dioxygenase enzymes. Catechol 2,3-dioxygenases XylE, NahH (80), and DmpB (14) catalyze *meta* cleavage of catechol as indicated by the arrow. The superfamily of extradiol enzymes also includes TodE (274), NahC (80), BphC (60, 232), and CbpC (107). The preferential substrates and the sites of cleavage are indicated. *ortho* cleavage is catalyzed by intradiol dioxygenases. The superfamily of intradiol dioxygenases includes protocatechuate 3,4-dioxygenases (PcaGH) (42, 86, 157, 276, 277), catechol 1,2-dioxygenase (CatA) (150), and chlorocatechol 1,2-dioxygenases (TcbC, TfdC, and ClcA) (54, 66, 247). The catechol 1,2-dioxygenase activity which converts 3-methyl-6-chlorocatechol was detected in a mutant of *Pseudomonas* sp. strain JS6 (72).

enzymes, which are able to transform initial substrates into one of the central intermediates.

meta Cleavage Pathway Genes

TOL plasmids are self-transmissible and contain two or more operons which encode enzymes required for the degradation of methylbenzenes, such as toluene, xylenes, and 1,2,4-trimethylbenzene, via methylbenzoates (9, 22, 43, 51, 53, 80, 81, 84, 96, 146, 162, 190, 263, 264). On the TOL plasmid pWW0, one operon (*xylCMABN*) codes for the "upper-pathway" enzymes, which oxidize methylbenzenes to methylbenzoates (78, 85) while the "lower-pathway" or *meta* operon is composed of 13 genes that encode enzymes for the conversion of methylbenzoates to pyruvate, acetaldehyde, and acetate via (methyl)catechols (51, 52, 77, 81). The *meta* cleavage pathway enzymes in stricter sense are encoded by the genes *xylE*, *xylG*, *xylF*, *xylJ*, *xylQ*, *xylK*, *xylI*, and *xylH*, in that order, which are part of the *meta*

operon (Fig. 2). The catabolic genes of plasmid NAH7, which encode the enzymes required for the degradation of naphthalene via salicylate (269), are clustered in two operons, the *nah* and *sal* operons (199, 269) (Fig. 2). The *sal* operon contains similar *meta* cleavage pathway genes to those present on pWW0, and DNA sequences of these parts of both operons (e.g., *xylEGFJ* and *nahHINL*) are homologous. This has been shown by Southern hybridizations (8, 126), by genetic mapping experiments, and in more detail by DNA sequence comparisons (80, 84). Genes related to *xylF* and *xylJ* were also found in the toluene degradation pathway encoded by the *tod* operon, although these genes, *todF* and *todJ*, are separated in this operon by the genes encoding an aromatic ring dioxygenase and 3-methyl 2,3-dioxygenase (92). Another *meta* cleavage pathway was characterized in *Pseudomonas* sp. strain CF600, which uses phenol, cresols, and 3,4-dimethylphenol as the sole carbon and energy source (156, 216). This *meta* cleavage pathway is encoded by the *dmpQBCDEFGHI* genes present on plasmid pVI150 and was shown by biochemical analysis, polypeptide analysis, and nucleotide sequence determination to be very closely related to the *xyl*-encoded *meta* cleavage pathway (216). The *meta* cleavage pathway genes of plasmid pVI150 form one operon with the *dmpKLMNOP* genes, encoding phenol hydroxylase (Fig. 2).

Extradiol dioxygenases. Catechol 2,3-dioxygenases encoded by TOL and NAH plasmids not only are similar to each other but also show sequence similarities to other extradiol ring cleavage enzymes, such as 1,2-dihydroxynaphthalene dioxygenase encoded by the *nahC* gene in the *nah* operon of plasmid NAH7 (80) (Fig. 2), 2,3-dihydroxybiphenyl 1,2-dioxygenases encoded by the *bphC* genes from *Pseudomonas pseudoalcaligenes* KF707 (60) and from *P. paucimobilis* Q1 (232), and 3-methylcatechol 2,3-dioxygenase encoded by *todE* from *P. putida* F1 (274). 2,3-Dihydroxybiphenyl 1,2-dioxygenase (*cbpC*) from *P. putida* (107) and 1,2-dihydroxynaphthalene dioxygenase from a bacterium that degrades naphthalenesulfonic acid (119) may also be members of the catechol 2,3-dioxygenase family. Other extradiol dioxygenases which are not related to the above-mentioned catechol 2,3-dioxygenase family have also been found, including catechol 2,3-dioxygenase from *Alcaligenes eutrophus* (102) and protocatechuate 4,5-dioxygenase from *P. paucimobilis* (154).

ortho Cleavage Pathway Genes

ortho cleavage pathways are involved in the degradation of catechol and protocatechuate. These compounds are transformed to a common intermediate, 3-oxoadipate enol-lactone, which is further converted to succinate and acetyl coenzyme A (Fig. 3). The genes encoding *ortho* cleavage pathway enzymes are located on the chromosome (4, 42, 93, 153, 276, 277). The organizations of the *cat* genes encoding catechol degradation and the *pca* genes for protocatechuate degradation of different species show interesting variations in gene order and operonic clustering (42, 93, 153, 160). For example, in some microorganisms, such as *Acinetobacter calcoaceticus*, two sets of equivalent enzymes exist for the degradation of 3-oxoadipate enol-lactone (encoded by *catEFD* and *pcaDEF*), while in *P. putida*, only one set of enzymes is encoded by apparently unlinked genes (160).

Genes of the modified *ortho* cleavage pathways. The isolation and characterization of a number of bacterial strains capable of degrading chlorinated aromatics such as chlorinated benzenes (71, 208, 220, 247, 249, 250), chlorinated benzoates (27–29, 54, 66, 67, 259), and 2,4-dichlorophenoxyacetic acid (38, 39, 165) demonstrated the presence of another set of *ortho* cleavage pathway enzymes. Most of these strains convert chlorinated aromatic compounds via a chloro-substituted catechol, which is always *ortho* cleaved by a chlorocatechol 1,2-dioxygenase (Fig. 4). The enzymes involved in the mineralization of chlorocatechols have wider substrate specificities than the ordinary *ortho* cleavage pathway enzymes do. Therefore, the chlorocatechol degradative pathway is also called the modified *ortho* cleavage pathway. In *Pseudomonas* sp. strain B13 (40, 41, 179, 181, 204, 205, 212), in *Alcaligenes eutrophus* JMP134 (39, 118, 165, 171, 172, 201, 203, 229), and in other bacteria which metabolize chlorinated benzenes (71, 159, 168, 191, 208, 220, 250), these pathways have well been characterized. In contrast to the ordinary *ortho* cleavage pathway genes, the genes for the modified *ortho* cleavage pathways are generally located on catabolic plasmids (27, 28, 38, 39, 54, 250, 259, 267), and their organization into operon structures differs substantially from that of the chromosomally encoded *cat* and *pca* genes (Fig. 2). Modified *ortho* cleavage pathway genes from three bacteria have been extensively characterized: (i) the *clcABD* operon of *P. putida*(pAC27) (27–29, 54, 66, 67), (ii) the *tfdCDEF* operon of *Alcaligenes eutrophus* JMP134(pJP4) (39, 66, 104, 165), and (iii) the *tcbCDEF* operon of *Pseudomonas* sp. strain P51(pP51) (247, 250). In these three organisms, the chlorocatechol 1,2-dioxygenase genes appeared to be linked to genes for the rest of the pathway in a single operon. The chlorocatechol 1,2-dioxygenase genes and the chlorocycloisomerase genes of these operons are significantly homologous to the counterparts of the ordinary *ortho* cleavage pathway genes (Fig. 2) (53, 165, 247). Although no clear function has been attributed to the TfdF and TcbF proteins of the modified *ortho* cleavage pathway, the amino acid sequences predicted from the nucleotide sequence show 30% identity with those of iron-containing alcohol dehydrogenases (246). This suggests that the two enzymes require NADH for their functioning, which would make them a likely candidate for the NADH-consuming activity which cocatalyzed the conversion of chloromaleylacetate in cell extracts of *Alcaligenes eutrophus* JMP134 (202).

Intradiol dioxygenases. The family of intradiol dioxygenases includes three subgroups of enzymes: catechol 1,2-dioxygenase, protocatechuate 3,4-dioxygenase, and chlorocatechol 1,2-dioxygenase. These enzymes cleave the aromatic ring between two adjacent hydroxyl groups of catechol or protocatechuate. The genes encoding catechol 1,2-dioxygenase (*catA*) and two different subunits of protocatechuate 3,4-dioxygenase (*pcaH* and *pcaG*) are transcribed separately from the other genes of the *ortho* cleavage pathway (4, 86, 153, 160, 276, 277). These two enzymes have been found in many bacterial species including *P. putida*, *P. aeruginosa*, *P. cepacia*, and *Acinetobacter calcoaceticus* (4, 42, 86, 110, 120, 150, 153, 276, 277) (Fig. 1 and 3). The three-dimensional structure of protocatechuate 3,4-dioxygenase from *P. putida* has been determined (157), and two His and two Tyr residues were shown to be involved in the binding of the catalytic ferric iron (157, 241). These His and Tyr residues appear to be strongly conserved among all members of the intradiol dioxygenases (86, 110, 150, 165, 247). The overall amino acid sequence homologies between the members of catechol 1,2-dioxygenases and the α- and β-subunits of protocatechuate 3,4-dioxygenase were relatively low (18 to 22%) (86, 150). Chlorocatechol 1,2-dioxygenases encoded by *clcA*, *tfdC*, and *tcbC* cleave chlorinated catechols at a higher rate than the rate of oxidation of the

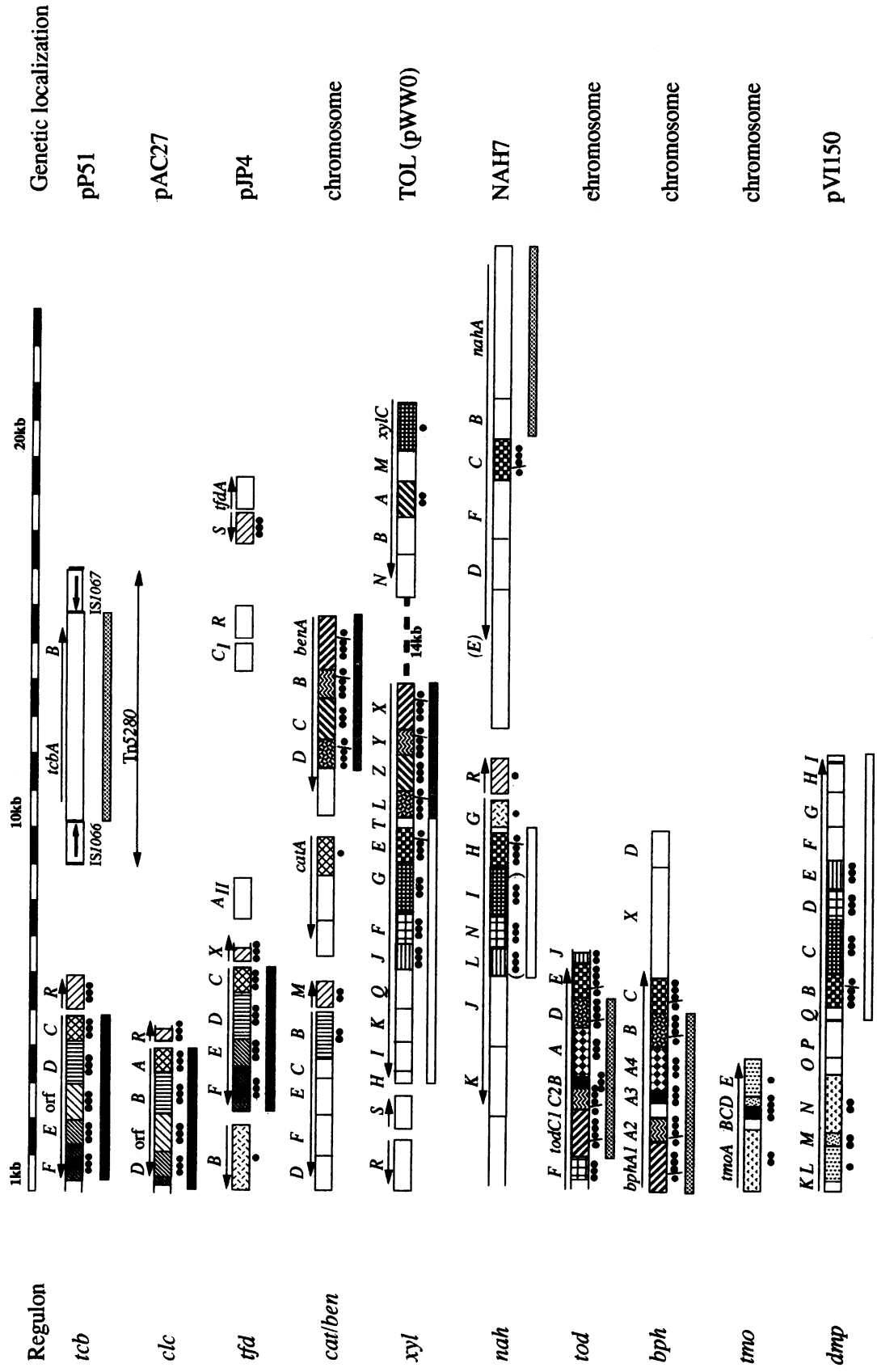
Regulon
Genetic localization
1kb
10kb
20kb
tcb
pP51
clc
pAC27
tfd
pJP4
cat/ben
chromosome
xyl
TOL (pWW0)
nah
NAH7
tod
chromosome
bph
chromosome
tmo
chromosome
dmp
pVI150
tcbA
IS1066
IS1067
Tn5280
14kb

FIG. 2. Comparison of catabolic regulons encoding aromatic pathways from different microorganisms. The localization and sizes of the different genes are indicated by open horizontal bars and are drawn to scale. In addition, the direction of transcription from the genes is indicated by arrows. Similar patterns of hatching and shading between genes from different organisms indicate significant amino acid similarity of the translated gene products or DNA sequence homology. The number of black dots below the genes indicates the category of overall amino acid sequence identity between the majority of the members of that group of genes: •, 18 to 25%; ••, 25 to 50%; •••, more than 50%. When two subfamilies exist within a group of related genes (e.g., within the extradiol dioxygenases), two categories are given below the genes of that group: ••/•, more than 50% identity within the first subfamily but 18 to 25% identity with members of the second subfamily; •/••, vice versa. Numbers within parentheses indicate DNA sequence homology as derived by DNA-DNA hybridization. Blocks in the horizontal bars without gene assignment represent other open reading frames that were revealed by DNA sequencing. Thin horizontal bars below the gene clusters indicate putative gene modules that occur in different catabolic pathways. ■, modified *ortho* cleavage pathway genes; ▩, two-component aromatic ring dioxygenase and dehydrodiol dehydrogenase; ▨, three-component aromatic ring dioxygenase and dehydrodiol dehydrogenase; □, *meta* cleavage pathway genes. The *tcb* genes were characterized from *Pseudomonas* sp. strain P51 and encode chlorobenzene degradation (247, 248, 250, 251). The *tcbAB* gene cluster is flanked by two iso-insertion elements, IS*1066* and IS*1067*, which form transposable element Tn*5280* (251). The *clc* operon encodes 3-chlorobenzoate metabolism in *P. putida* (pAC27) (28, 29, 54). The *tfd* genes encode metabolism of 2,4-dichlorophenoxyacetic acid in *A. eutrophus* JMP134(pJP4) (39, 66, 67, 104, 105, 165, 166, 229). The *tfdX* gene is the assignment to an open reading frame which showed homology to *tfdS* and *tcbR* but which was only partially sequenced; $tfdA_{II}$ and $tfdC_I$ depict the locations of the presumed duplicated gene copies of *tfdA* and *tfdC*, respectively. The *cat* and *ben* genes encode metabolism of benzoate in *Acinetobacter calcoaceticus* (150-153). The *xyl* genes of the TOL plasmid encompass four different transcriptional units (22, 81, 85). The *nah* genes of the NAH7 plasmid encode naphthalene and salicylate degradation and are organized in two large gene clusters and a regulatory gene, *nahR* (199). The chromosomally located *tod* genes for toluene metabolism of *P. putida* F1 (274, 275) and the *bph* genes for conversion of (chloro)biphenyls of *P. pseudoalcaligenes* KF707 are shown below (61, 233). The last two regulons show the genes for the multicomponent monooxygenases toluene-4-monooxygenase (*tmo*) (268) and phenol hydroxylase (*dmpKLMNOP*) and the *meta* cleavage pathway genes (*dmpQBCDEFGHI*) (14, 155, 156, 216). Recognized gene families include the following: ▩, intradiol dioxygenases; ▥, (chloro-) muconate cycloisomerases; ▩, dienelactone hydrolases; ▨, modified *ortho* cleavage pathway genes with homology to genes encoding iron-containing alcohol dehydrogenases; ■, extradiol dioxygenases; ■, *meta* cleavage pathway genes of the aldehyde dehydrogenase superfamily (92); ▦, 2-hydroxymuconic semialdehyde hydrolases; ■, 2-hydroxy-pent-2,4-deionate hydratases; ▧, large (α) hydroxylase subunit of the multicomponent aromatic ring dioxygenases; ▩, small (β) subunit of the hydroxylases; ▨, electron transfer component (oxidoreductases) of the two-component aromatic ring dioxygenases (152); ▥, ferredoxin component of *tod*, *bph* and *tmo*; ▩, *cis*-dehydrodiol dehydrogenases belonging to the short-chain alcohol dehydrogenase superfamily (149); ▨, single-component hydroxylases; ▥, ▩, and ▨, homologous genes in multicomponent monooxygenases; ▨, regulatory components belonging to the LysR family of transcriptional activators (89, 90).

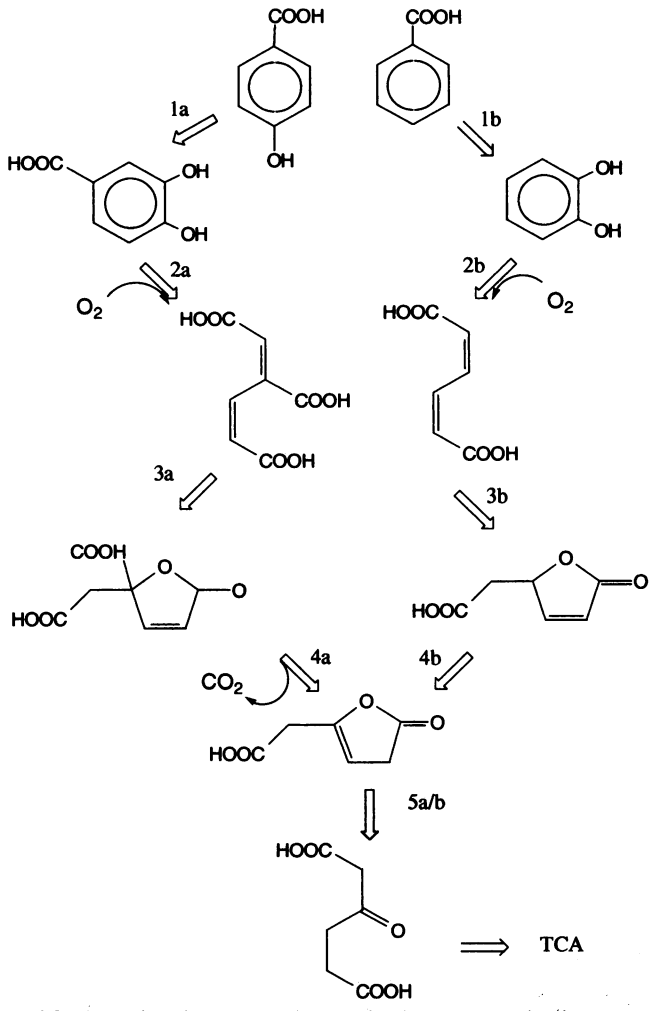

FIG. 3. *ortho* cleavage pathways for benzoate and *p*-hydroxybenzoate degradation (160). Conversion steps catalyzed in a single reaction are represented by closed arrows; others are represented by open arrows. Sequential enzymatic steps: *p*-hydroxybenzoate hydroxylase (1a) or benzoate dioxygenase and diol dehydrogenase (step 1b); protocatechuate 3,4-dioxygenase (step 2a) or catechol 1,2-dioxygenase (step 2b); muconate cycloisomerase or lactonizing enzyme (steps 3a and 3b); decarboxylase (step 4a) or muconolactone isomerase (step 4b); enol-lactone hydrolase (steps 5a and 5b); and transferase and thiolase. TCA, tricarboxylic acid cycle.

nonsubstituted catechol (19, 247). These three enzymes are highly homologous and exhibit between 53.5 and 63.1% amino acid sequence identity. However, they differ subtly in substrate range, and this makes them important to the study of adaptive changes of enzymes to xenobiotic compounds (19, 165, 247).

Genes for Peripheral Enzymes

Multicomponent aromatic ring dioxygenases. The substrate specificities of enzymes involved in the initial transformation of aromatic substrates into central intermediates exhibit a greater variety than those of the aromatic ring cleavage pathways described above. Nevertheless, genes for some of these enzymes are evolutionary related to each other. One illustrative example is a group of multicomponent dioxygenases involved in the NADH-dependent incorporation of

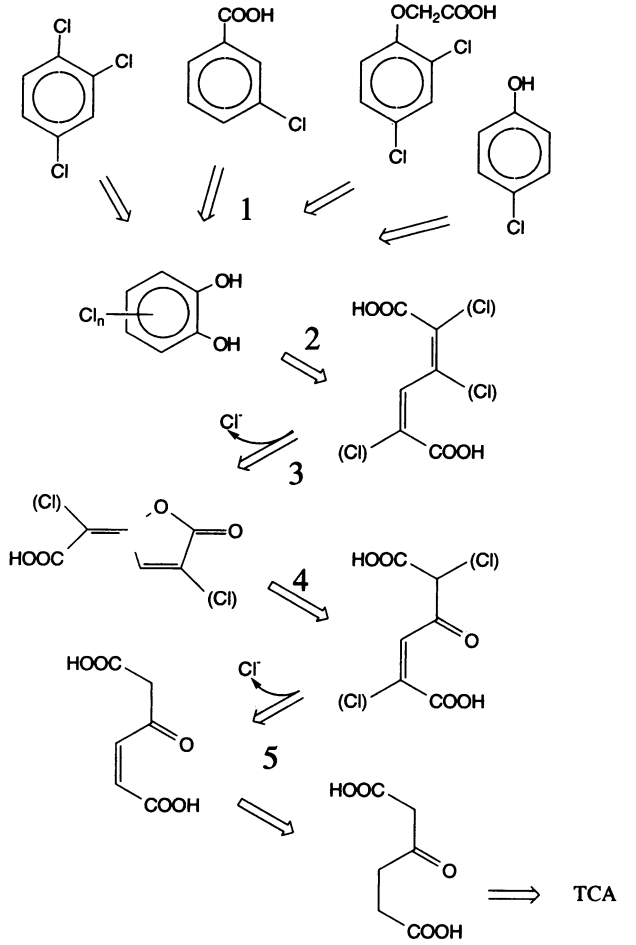

FIG. 4. Modified *ortho* cleavage pathway. This pathway is used as central metabolic route for the degradation of chlorinated catechols, which arise in the conversion of chlorinated benzenes, chlorinated benzoates, 2,4-dichlorophenoxyacetic acid, or chlorophenol (30, 35, 181). A variety of peripheral enzymes lead to the formation of chlorocatechols (step 1). Cleavage of chlorocatechols proceeds via *ortho* cleavage by a chlorocatechol 1,2-dioxygenase (step 2). A chloromuconate cycloisomerase catalyzes the formation of chlorocarboxymethylenebut-2-en-4-olide after spontaneous release of one chlorine atom (step 3). This intermediate is further converted by a dienelactone hydrolase (step 4). Maleylacetate reductase and an uncharacterized NADH-consuming enzyme presumably catalyze the last steps, which also lead to a final dechlorination (step 5) and to the formation of 3-oxoadipate (171, 202). TCA, tricarboxylic acid cycle.

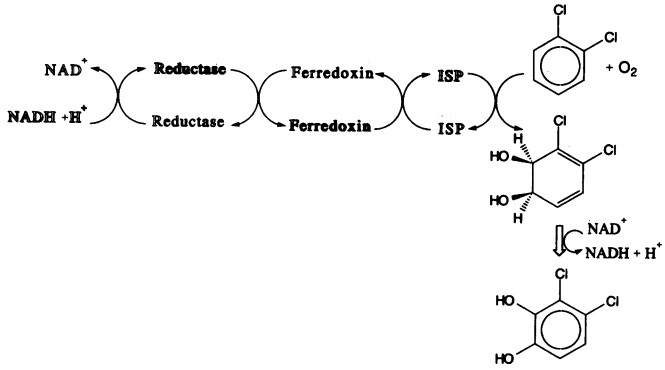

FIG. 5. Schematic representation of the reactions catalyzed by the three-component dioxygenase and dihydrodiol dehydrogenase (69). Molecular oxygen is incorporated into the aromatic ring by a multienzyme complex as electrons are transferred from NADH to the terminal oxidase. The reduced state of the proteins is indicated by shadowed type. The terminal oxidase (or hydroxylase), which contains two subunits, is abbreviated by ISP (iron-sulfur protein). In the two-component dioxygenases, the ferredoxin and reductase activities are coupled in one protein (152).

two adjacent hydroxyl groups on the aromatic ring of (chloro)benzene, toluene, naphthalene, (chloro)biphenyl, and (chloro)benzoates (Fig. 5) (46, 63, 69, 83, 99, 143, 152, 191, 227, 250, 254). The enzyme complex is generally formed from three different components, a terminal oxygenase (also called iron-sulfur-protein or hydroxylase protein) which consists of two different subunits (α and β), a ferredoxin, and an NADH-ferredoxin reductase (69). The genes for the components of toluene dioxygenase from *P. putida* F1 (69, 274, 275), of benzene dioxygenase from *P. putida* (99), of biphenyl dioxygenase from *P. pseudoalcaligenes* (63, 88, 233) and from *Pseudomonas* sp. strain LB400 (47, 143), and of naphthalene dioxygenase from *P. putida* NCIB9816 (122) are all clustered, but the orders of equivalent genes can be different. Other examples of three-component systems, which have not yet been characterized on the DNA sequence level, include naphthalene dioxygenase from plasmid NAH7 (encoded by the *nahA* locus) (269), biphenyl dioxygenase from *P. paucimobilis* Q1 (232), and chlorobenzene dioxygenase which was cloned from *Pseudomonas* sp. strain P51 (250).

Toluate dioxygenase from *P. putida* and benzoate dioxygenase from *Acinetobacter calcoaceticus* have two components instead of three. In these two dioxygenases, encoded by *xylXYZ* (81, 83) and *benABC* (152), respectively, the electron transport function is fulfilled by a single protein which possesses a ferredoxin-like structure in its N-terminal part and an NADH-ferredoxin reductase-like structure in its C-terminal region (152). A novel two-component dioxygenase, 2-halobenzoate 1,2-dioxygenase, which may be structurally related to toluate dioxygenase was recently isolated from *P. cepacia* 2CBS (48). Similarly to 4-chlorophenylacetate 3,4-dioxygenase from *Pseudomonas* sp. strain CBS3 (136, 211), this enzyme shows dehalogenation activity. The α- and β-subunits of the different terminal oxygenases show significant deduced amino acid sequence similarity to other α- and β-subunits (Fig. 2) (122, 152). In all the multicomponent dioxygenase gene clusters, the genes for the dioxygenases are followed by a gene encoding dihydrodiol dehydrogenase. These enzymes are members of the short-chain alcohol dehydrogenase family (149).

Monooxygenases. Electron transfer components of some of the multicomponent dioxygenases have structures similar to those of some monooxygenases involved in different metabolic functions. Toluene-4-monooxygenase multicomponent enzyme complex from *P. mendocina* KR1 catalyzes the formation of *p*-cresol from toluene and is encoded by five genes, *tmoABCDE* (Fig. 2) (268). The ferredoxin component of toluene-4-monooxygenase, encoded by the *tmoC* gene, shows 32 and 28% amino acid sequence identity with the ferredoxins of toluene and naphthalene dioxygenases, respectively (268). Other components of toluene-4-monooxygenase are not significantly related to proteins of the multicomponent dioxygenases. However, three components of toluene-4-monooxygenase are related to the components of

phenol hydroxylase from *Pseudomonas* sp. strain CF600 (Fig. 2) (155, 268). Phenol hydroxylase is a multicomponent enzyme complex which transforms phenol into catechol and is encoded by the *dmpKLMNOP* genes (Fig. 2) (155). Both multicomponent monooxygenases contain polypeptides which are structurally related to components of methane monooxygenase (223).

Single-component hydroxylases and monooxygenases, which are found in various pathways, also share conserved domains. Salicylate hydroxylase NahG, encoded on the NAH7 plasmid (269, 270), was shown to be 25% homologous in amino acid sequence to *p*-hydroxybenzoate hydroxylase from *P. fluorescens* (257). Higher local sequence similarities occur in specific domains, such as the presumed FAD-binding region. A lower global sequence similarity of salicylate hydroxylase is found with other hydroxylases, such as with dichlorophenol hydroxylase encoded by *tfdB* from *Alcaligenes eutrophus* (165) and phenol hydroxylase encoded by *pheA* (111). These single-component monooxygenases, however, are not structurally related to 2,4-dichlorophenoxyacetate monooxygenase, encoded by the *tfdA* gene (229).

Xylene monooxygenase has two components, XylM and XylA (83). The XylM component was shown to be related to the alkane hydroxylase component AlkB (230). The XylA component was proposed to have an N-terminal domain similar to that in chloroplast-type ferredoxins and a C-terminal sequence similar to that in ferredoxin-$NADP^+$ reductases (230). Although 4-toluene sulfonate methyl monooxygenase catalyzes a reaction similar to that catalyzed by xylene monooxygenase, the biochemical properties of these two enzymes are very different (131).

Dehalogenases. Three polypeptides with sizes of 57, 30, and 16 kDa make up the 4-chlorobenzoate dehalogenase activity in *Pseudomonas* sp. strain CBS3 (207). This activity was proposed to be the sum of the individual activities of a 4-chlorobenzoate:coenzyme A ligase, a 4-chlorobenzoate: coenzyme A dehalogenase existing as a heterodimer of the 57- and 30-kDa components, and a 4-hydroxybenzoate:coenzyme A thioesterase (207). A stretch of homology was found between the 57-kDa polypeptide and several magnesium-dependent ATPases (207). A dehydrochlorinase activity which functions in the dehalogenation of γ-hexachlorocyclohexane to 1,2,4-trichlorobenzene via γ-pentachlorocyclohexene was isolated from *P. paucimobilis* UT26 (95). The activity could be attributed to the gene product of the *linA* gene, encoding a 17-kDa polypeptide which has no significant similarity with other protein sequences (95). Hydrolytic dehalogenases are also key enzymes in transformation reactions of halogenated aliphatic compounds. Two categories are generally distinguished: haloalkane dehalogenases and 2-haloacid dehalogenases. The 2-haloacid dehalogenase from *Xanthobacter autotrophicus* GJ10M50, the gene product of the *dhlB* gene, was recently shown to be 61% similar to the primary sequences of two haloacid dehalogenases encoded by *dehCI* and *dehCII* from *Pseudomonas* sp. strain CBS3 (206, 252). The primary sequences of the 2-haloacid dehalogenases are not significantly similar to those of the product of *dhlA*, which encodes the haloalkane dehalogenase in *X. autotrophicus* (252). Interestingly, the tertiary structure of haloalkane dehalogenase resembles that of dienelactone hydrolase, although no significant homology between the primary sequences of those two proteins is observed (50).

Regulatory Genes

Catabolic pathways are controlled by specific regulatory proteins, which recognize inducer molecules and interact with promoter-operator regions of the catabolic operons. A number of regulatory genes of catabolic pathways and their products have been well characterized. In the TOL-encoded pathways, XylR and XylS coordinately regulate the full transcription of the *xyl* operons (97, 98, 137, 147, 222). XylR responds to xylenes and (methyl)benzylalcohols and activates transcription of the upper-pathway genes (*xylCMABN*) and of the *xylS* gene (91). The transcription is mediated by an alternative RNA polymerase containing the RpoN sigma factor (116). In addition, an integration host factor (IHF)-like protein is required for the expression of the upper operon (36). XylS recognizes benzoate and its analogs and activates transcription of the *meta* cleavage genes. The hyperexpression of XylS after the induction of the *xylS* gene by XylR also activates the *meta* cleavage pathway genes, even in the absence of the benzoate effector (98, 137). The XylS protein belongs to the AraC family of bacterial transcriptional activator proteins (174), whereas the XylR protein is similar to the regulatory components of two-component systems. The *nah* and *sal* catabolic operons on plasmid NAH7 are regulated by a single regulator protein, NahR (198, 199). NahR is a member of the LysR family (89, 199, 200), which comprises several other regulatory proteins for catabolic genes, such as those encoded by *catR* (189), *catM* (151), *tfdS* (89), *tcbR* (248), and *clcR* (34). All of these regulatory genes have sizes of approximately 1 kb, are transcribed in the opposite direction from the target operons, and are separated from these by approximately 200 bp. Interestingly, most of the regulatory proteins encode transcriptional activator proteins, except *catM*, which was shown to code for a repressor (151). The effector of NahR is salicylate (198, 199) and that of CatR and CatM is *cis,cis*-muconate (3, 151, 189), but those of TfdS, TcbR, and ClcR have not yet been identified. Although the N-terminal parts of the LysR family regulators contain similar helix-turn-helix motifs (90), presumed to be involved in DNA binding, C-terminal parts of the proteins are often very dissimilar (89). This suggests that perhaps each protein in this family arose by fusion of at least two different subunits, one of which is shared by all the members of the LysR family.

MECHANISMS OF GENETIC ADAPTATION

Amino acid similarities of the different aromatic pathway enzymes and the diverged organization of catabolic gene clusters discussed above (Fig. 2) suggest that several different gene clusters (e.g., modified *ortho* cleavage pathway genes, *meta* cleavage pathway genes, and three- and two-component aromatic ring dioxygenase and dihydrodiol dehydrogenase genes) may be combined as modules, to which other peripheral genes may be added. Furthermore, it shows that many DNA rearrangements have occurred during the evolution of different pathways. The next paragraphs will deal with the different genetic mechanisms which may be involved in the evolution of aromatic pathways in general and in the process of adaptation of microorganisms to xenobiotic substrates in particular. The different mechanisms will be dealt with in three groups: (i) gene transfer, (ii) mutational drift, and (iii) genetic recombination and transposition. Some of these mechanisms are difficult to prove experimentally, since we can observe only their final results (e.g., the existence of homologous genes between different

organisms). The importance of the different evolutionary mechanisms will be further clarified by discussing a number of studies which have tried to establish evolution of catabolic pathways experimentally.

Gene Transfer

The many examples illustrated above strongly suggest that common ancestral genes have spread among different microorganisms. The divergence of descendants in different organisms, however, does not necessarily correspond to the evolutionary distances determined for those organisms by rRNA lineage. This observation suggests the occurrence of extensive horizontal gene transfer during evolutionary processes. Genetic interactions in microbial communities are effected by several mechanisms, such as conjugation via plasmid replicons, transduction (193, 272), and transformation (133, 134). The occurrence of plasmids in bacteria in the natural environment is certainly a general phenomenon (55, 56, 101, 115, 184, 210, 261, 264), and an important pool of genetic information residing on plasmid vehicles may flow among indigenous microorganisms (57, 58, 114, 219, 253). Since many examples of self-transmissible plasmids that carry genes for degradation of aromatic or of other organic compounds are known (25, 30, 53, 76, 195), their role in spreading these genes to other microorganisms is undebatable. Early studies of TOL, NAH, and SAL plasmids revealed strong DNA homologies among these plasmids in regions both inside and outside the catabolic gene clusters (9, 43, 53, 126). Recently, Burlage et al. (21) have shown that pJP4, pAC25, pSS50, and pBRC60, catabolic plasmids carrying genes for chloroaromatic degradation, have a strongly homologous plasmid backbone that determines replication and transfer functions. The chlorobenzene plasmid pP51 was also shown to be homologous to pJP4 outside the regions of the catabolic genes (246). These observations suggest that a few common self-transmissible ancestor replicons may have been involved in the acquisition and spread of different catabolic modules.

The importance of gene transfer for adaptation of host cells to new compounds has been illustrated in many studies on experimental evolution of novel metabolic activities (20, 76, 100, 142, 159, 176, 180). Such studies could identify biochemical blockades in natural pathways which prevented the degradation of novel substrates and could overcome these barriers by transferring appropriate genes. Pathways could be expanded by replacing narrow-specificity enzymes by broader ones (horizontal expansion) or by providing peripheral enzymes which could direct substrates into existing degradative pathways (vertical expansion) (176). For example, the range of biodegradative capacity of *Pseudomonas* sp. strain B13 was horizontally expanded from 3-chlorobenzoate to 4-chlorobenzoate and 3,5-dichlorobenzoate by transfer of the TOL plasmid pWW0 from *P. putida* mt-2 (100, 180). This transfer provided strain B13 with the XylXYZ toluate dioxygenase of the TOL plasmid, an enzyme with a broader substrate range than chlorobenzoate dioxygenase of strain B13. Various other derivatives of strain B13 which could degrade chlorosalicylates (127), chlorobiphenyls (142), chloroanilines (124), and chloronitrophenols (20) have been constructed. It should be noted that transfer of catabolic plasmids can lead to regulatory and/or metabolic problems for the cell, and therefore additional mutations in the primary transconjugants are often required to construct strains with the desired metabolic activities (100, 180). In strains which carried the *meta* and *ortho* cleavage pathways simultaneously, catechol 2,3-dioxygenase had to be inactivated for proper growth on chlorinated aromatics (13, 112, 180, 188). However, bacterial strains, such as *Pseudomonas* sp. strain JS6, that induce *meta* and *ortho* pathways enzymes simultaneously without apparent problems for the cell have been obtained recently (72, 168).

Gene transfer techniques were further applied to construct a novel metabolic pathway for degradation of chlorinated benzenes. Until recently, no bacterial strains which could metabolize chlorinated benzenes completely had been isolated. A complete pathway for chlorobenzene degradation would require a broad-substrate-specificity benzene dioxygenase and a broad-substrate-specificity benzene glycol dehydrogenase, together with a modified *ortho* cleavage pathway. By mating *P. putida* F1, a strain that contains the *tod* genes (Fig. 2) encoding a broad-substrate-specificity benzene dioxygenase and benzene glycol dehydrogenase, with *Pseudomonas* sp. strain B13, which carries a modified *ortho* cleavage pathway on a plasmid, Oltmanns et al. (159) were able to obtain transconjugants which could metabolize 1,4-dichlorobenzene completely. Similar transconjugant strains were isolated by using cultures from a chemostat and ceramic bead columns to mate *P. putida* R5-3, harboring a TOL-like plasmid, with *P. pseudoalcaligenes* C-0, a strain capable of mineralizing benzoate and 3-chlorobenzoate (24, 117). Interestingly, the transconjugants which showed chlorobenzene mineralization were derivatives of strain R5-3 and carried a chromosomal DNA fragment of strain C-0 on the TOL-like plasmid (24). Evidence that similar gene transfer processes in the evolution of the chlorobenzene pathway may take place in nature came from studies with *Pseudomonas* sp. strain P51, which was isolated by selective enrichment from Rhine sediment (249). This strain harbors a catabolic plasmid containing two catabolic operons, one encoding a modified *ortho* cleavage pathway (247, 250) and the other encoding chlorobenzene dioxygenase and chlorobenzene glycol dehydrogenase (Fig. 2) (250, 251). The complete chlorobenzene dioxygenase gene cluster is located on a transposable element, suggesting that the pP51 plasmid was formed by the transposition of the chlorobenzene dioxygenase transposon onto an ancestral plasmid of pP51 that contained only the modified *ortho* cleavage pathway genes (251).

Point Mutations

Several examples have illustrated that single-site mutations can alter substrate specificities of enzymes or effector specificities. Clarke, for example, isolated mutants with altered substrate specificities of the AmiE amidase of *P. aeruginosa*, which were provoked by single-base-pair changes (31, 32). Sequential mutations in the cryptic *ebg* genes of *Escherichia coli* were shown to result in active enzymes capable of metabolizing lactose and other sugars (74). The substrate range of catechol 2,3-dioxygenase encoded by TOL plasmid pWW0 has been extended to 4-ethylcatechol by single substitutions of amino acids (177). Similarly, the substrate specificity of xylene monooxygenase, encoded by *xylMA*, was extended to *p*-ethyltoluene, which is not a natural substrate (1). The specificity of the XylS regulatory protein was modified by mutagenesis, resulting in recognition of other compounds, such as 4-ethylbenzoate, salicylate, and 3,5-, 2,5-, and 2,6-dichlorobenzoate, as effector molecules (175). Furthermore, XylS mutants capable of activating the *meta* cleavage pathway genes in the absence of its effector were isolated (273).

Single-site mutations are believed to arise continuously and at random as a result of errors in DNA replication or repair. However, some authors have suggested that directed mutations in response to a selective pressure are possible: the frequencies of Lac^+ revertants in *E. coli* increased in the presence of lactose (18, 23, 73). Several mechanisms that account for this observation have been proposed, but whether some environmental factors can control the direction of mutations is still debatable (139, 140). However, it is firmly established that induction of the SOS response increases mutation frequencies via the highly regulated UmuCD replication pathway (33, 45, 167). It is possible that a diversity of stress factors including chemical pollutants (17) stimulate error-prone DNA replication and hence accelerate DNA evolution.

Despite the important effects of a single-base-pair mutation on the adaptive process, as has been demonstrated experimentally, the accumulation of single-base-pair changes may not be the sole mechanism for the divergence of properties in contemporary catabolic enzymes. Other mechanisms which would allow faster divergence of DNA sequences include gene conversion or slipped-strand mispairing (86, 129, 150, 160). In the model of slipped-strand mispairing, partially melted DNA duplexes are assumed to have the possibility of shifting during replication with respect to their original position and of being stabilized in a heterologous duplex by the presence of repetitive motifs which can form perfect matching base pairs (75, 161, 234). Base pairs which mismatch in this heterologous duplex can then be repaired from either one of the strands as template (45, 129, 141). Model experiments showed that slipped-strand mispairing occurs during DNA replication in vitro (121, 123). The remnants of such a process have been proposed to be visible in DNA sequences of members of the intradiol dioxygenase family, encoded by *catA*, *pcaG*, and *clcA* (86, 150, 160, 161), and in the diverged sequences of *xylZ* and *benC* (82).

Recombination and Transposition

DNA rearrangements. The orders of the genes encoding the *ortho* cleavage pathways of *Acinetobacter calcoaceticus* and *P. putida* differ from one another (160) and from those of other organisms, suggesting that various DNA rearrangements have occurred (54, 165, 247). Gene rearrangements are also evident even between the different operons for the modified *ortho* pathway enzymes. The *clcABD* and *tcbC DEF* operons contain an extra open reading frame with 53.3% deduced amino acid sequence identity, which has no apparent function. This open reading frame is missing from the *tfdCDEF* operon (Fig. 2), which has only a small region with nucleotide sequence homology to the boundaries of this open reading frame, perhaps the remnants of the excision event (54, 247). Further examples are found in a comparison of TOL- and NAH-encoded catabolic genes. The gene orders of part of the *meta* cleavage operons (*xylTEGFJ* and *nahTHJNL*) are identical (Fig. 2) (81, 84) but are different in downstream genes. Furthermore, different genes are located upstream of *xylTEGFJ* and *nahTHJNL* (i.e., the *xylXYZL* toluate dioxygenase genes and the *nahG* salicylate hydroxylase gene). The close relationship of the *xylXYZL* genes with *benABCD* (81, 82, 152) suggests that these genes form a DNA module which was inserted in different catabolic operons. The genes for the three-component dioxygenases (e.g., *todC1C2BAD*) (274, 275) may have been inserted in an ancestor of a *meta* cleavage pathway operon between *todF* and *todJ* to form a novel toluene degradation pathway (92). Furthermore, such a DNA module for the three-component dioxygenases may have inserted into other catabolic operons as well (e.g., *bphABCXD*, *tcbAB*, *nahABCFD*) (63, 250, 269). Genes for other multicomponent dioxygenases are also clustered, but their gene orders can be different (e.g., *ndoABD* versus *todC1C2BA*) (122, 275). The biphenyl dioxygenase gene clusters *bphA1A2A3A4* and *bphAEFG* are very similar to *todC1C2BA* in gene order and nucleotide sequence but have an extra small open reading frame between the genes *bphA2* (*bphE*) and *bphA3* (*bphF*) compared with *todC1* and *todC2* (47, 233). There are as yet no clear indications of what mechanisms may direct these rearrangements, such as longer direct or inverted repeats which would allow general recombination, or the activity of insertion elements.

Gene duplications. Gene duplications have been considered an important mechanism for the evolution of microorganisms (15). Once duplicated, the extra gene copy could essentially be free of selective constraints and thus diverge much faster by accumulating mutations. These mutations could eventually lead to full inactivation, rendering this gene copy silent. Reactivation of the silent gene copy could then occur through the action of insertion elements (see below). Duplications of specific genes or gene clusters have been frequently found. *Flavobacterium* sp. strain K172 harbors two isoenzymes of 6-aminohexanoate dimer hydrolase, one of the enzymes involved in the degradation of nylon oligomers (158). Both enzymes were encoded by plasmid pOAD2 in regions that were proposed to be duplicated. The two isoenzymes differ 100-fold in their activity toward 6-aminohexanoate dimer, despite their amino acid sequence identity of 88% (148). Studies with various TOL-type plasmids have shown that upper- and lower-pathway operons, as well as the *xylS* and *xylR* regulatory genes, have sometimes switched positions, inverted, or increased their copy number (9, 26, 162, 214, 262). A pair of 1.4-kb directly repeated sequences present on the TOL plasmid has been presumed to direct several recombinational processes and the insertion of the TOL catabolic operons in the chromosome (138, 217). In various other bacterial strains, genes for catechol 2,3-dioxygenase or catechol 1,2-dioxygenase are duplicated (106, 144, 209). Plasmid pJP4, which contains the genes for the degradation of 2,4-dichlorophenoxyacetic acid, appears to have undergone several gene duplications (39, 165). By using DNA-DNA hybridization, two copies of the chlorocatechol 1,2-dioxygenase gene *tfdC* (65, 67, 104), of the 2,4-dichlorophenoxyacetic acid monooxygenase gene *tfdA* (166), and of the putative regulatory genes *tfdS* and *tfdX* (89, 90, 248) have been found. The exact DNA sequence of these duplicated copies is unfortunately not yet available. It was further shown that pJP4 contains three directly repeated sequences which were thought to play a role in the formation of a large duplication accumulating in cells grown under specific growth conditions (65). The duplicated region appeared to include the catabolic genes for 2,4-dichlorophenoxyacetic acid degradation (65). A tandem amplification of the catabolic region for 3-chlorobenzoate metabolism was also found in *Pseudomonas* sp. strain B13 when cultivated under nonselective conditions (178).

Transposition. Insertion elements have been shown to play an important role in rearrangement of DNA fragments, in gene transfer, and in activation or inactivation of silent genes. For catabolic pathways several examples of insertion elements are known. The TOL catabolic operons are part of a large transposable element, Tn*4651*, that belongs to the family of Tn*3*-type transposons (242, 243, 245). This trans-

poson was later found to be part of an even larger mobile element, Tn*4653* (243). A similar Tn*3*-type element, Tn*4655*, contains the catabolic genes on plasmid NAH7, although it is defective in its transposase function (244). *P. cepacia* 249 was shown to carry at least nine different insertion elements, which were present in 1 to 13 copies in its genome. These IS elements are thought to be responsible for the extraordinary adaptability and catabolic potential of this strain (128). One particular element, IS*931*, was detected in multiple copies around the *chq* gene locus for 2,4,5-trichlorophenoxyacetic acid degradation (87, 238). IS*931* is presumed to have played a role in the development of the 2,4,5-trichlorophenoxyacetic acid pathway, as it could mobilize adjacent DNA fragments (34, 87). Interestingly, this IS element apparently did not originate from *P. cepacia*. It was not found in other strains of this species, which suggested that IS*931* and perhaps (part of) the 2,4,5-trichlorophenoxyacetic acid genes were acquired from a completely different organism (34, 192). Other mobile and IS elements carrying catabolic genes include the *DEH* element, which carries the dehalogenase genes of *P. putida* PP3 (235, 236), and Tn*5271*, which is present on plasmid pBRC60 and specifies the genes for 3-chlorobenzoate degradation by *Alcaligenes* sp. strain BR60 (58, 145, 267). This transposable element is formed by two copies of a 3.2-kb repeated sequence, IS*1071*, which itself is related to the Tn*3* family of transposable elements (145). The chlorobenzene dioxygenase genes of *Pseudomonas* sp. strain P51 were shown to be flanked by two copies of iso-insertion elements, IS*1066* and IS*1067*. The composite element, called Tn*5280*, consists of IS*1066*, the chlorobenzene dioxygenase genes, and IS*1067* and was a functional transposon capable of inserting at random into the genome (251). The origin of Tn*5280* remained unclear, although IS*1066* and IS*1067* resemble IS elements from *Bradyrhizobium japonicum* (103) and *Agrobacterium rhizogenes* (218).

Insertional activation. Another important role of IS elements is the activation or inactivation of (silent) genes (7, 163, 271). One end of an IS element often contains promoterlike sequences, which can activate the expression of genes outside the IS element (64). In *P. cepacia*, insertion of IS*406* and IS*407* could lead to activation of *lacZ* (213, 266). The IS elements IS*931* and IS*932* were also able to activate the expression of adjacent genes (87). The mechanisms of activation of genes by insertion elements can be complicated, as was shown for the insertional activation of the cryptic *bgl* operon in *E. coli* by IS*1* and IS*5* (132, 182). The *bgl* operon itself is preceded by a promoter sequence, which, however, is not active under normal circumstances. It was suggested that the cyclic AMP-binding protein binds at sites near the promoter and further upstream, which leads to the formation of a loop in the DNA structure that prevents transcription initiation (132). Insertion of an IS element in this region prevents the formation of this loop and hence activates *bgl* expression.

MOLECULAR TOOLS TO STUDY GENETIC ADAPTATION IN THE NATURAL ENVIRONMENT

Although characterization of individual microorganisms that have been isolated from the natural environment or constructed in laboratories is important for our understanding of genetic mechanisms that govern adaptational processes, it has its limitations for a proper assessment of the importance of different genetic events in the adaptative response of bacteria in the natural environment. For the understanding of adaptational processes in nature, in situ genetic interactions among microorganisms and the influence of environmental parameters on the selection and dissemination of specific catabolic genes should be studied. Many recent studies address the possibilities and frequencies of gene transfer under various environmental conditions (59, 114, 130, 133, 193, 228, 239, 240). In mesocosm experiments, catabolic plasmid pBRC60 encoding 3-chlorobenzoate degradation of *Alcaligenes* sp. strain BR60 was transferred to and expressed in indigenous recipient bacteria (57, 58). The addition of low concentrations of 3-chlorobenzoate, 4-chloroaniline, or 3-chlorobiphenyl to microcosms inoculated with *Alcaligenes* sp. strain BR60 significantly affected the population size of microorganisms that finally obtained Tn*5271* (59). To assess the importance of gene flow in nature, its rate has to be measured under environmental conditions. For example, the transfer frequencies of conjugation of an epilithic plasmid encoding mercury resistance (pQM1) were about 100-fold lower in microcosm studies than in filter-mating experiments (185, 186).

How widespread are bacterial species capable of degrading xenobiotic compounds in the natural environment? Many bacterial species that can degrade biphenyls (62), chlorinated catechols (21, 27), or naphthalene and xylenes (8, 26, 43, 126) have been isolated from different environments, suggesting that such species are ubiquitous in natural environments. However, the distribution of bacteria exhibiting specific genetic traits is difficult to measure since a large proportion of the bacteria from soil and aquatic environments are not easily culturable, and some genes may not be expressed. The DNA-DNA hybridization technique is a relatively novel experimental approach in microbial ecology to overcome the problems of culturability or gene expression (37, 58, 169, 170, 194, 196, 197, 226, 256). With this technique, the distribution of specific genes in the natural environment can be determined by hybridization of DNA from indigenous soil microorganisms with specific gene probes. The technique allowed a highly specific detection of bacterial strains which carried Hg^{2+} resistance genes (187), genes for resistance to cadmium (37), toluene and naphthalene degradation genes (197), chlorobiphenyl genes (169, 256), or chlorobenzoate genes (58). Series of related genes could in principle be detected when mixed probes, such as for the different chlorocatechol 1,2-dioxygenases (see above), are applied. Specific DNA sequences could also be detected in environmental sources by hybridization with probes after amplification of those sequences by using the polymerase chain reaction (164, 224, 225, 258). Polymerase chain reaction amplification allowed the detection of specific bacterial strains at very low levels in samples of various origins (16, 224). Theoretically, the use of conserved DNA sequences in a gene family as universal primers in polymerase chain reaction amplification and subsequent cloning of the amplified fragments would allow the detection and isolation of a wider variation of genotypes from the environment. Such an approach was used for the characterization of variations in 16S rRNA genes from microorganisms in natural communities (258, 260). Also, gene fragments of the ribulose biphosphate decarboxylase gene (*rbcL*) and the nitrogenase gene (*nifH*) could be isolated after polymerase chain reaction amplification of DNA from environmental samples by using primers for highly conserved regions in these genes (109, 164).

CONCLUDING REMARKS

The presently available information suggests that microorganisms may eventually be able to deal with any kind of organic compound, provided that the compounds are intrinsically degradable. This review has shown that microorganisms possess a range of genetic mechanisms allowing evolutionary changes in existing metabolic pathways. Specialized enzyme systems and metabolic pathways for the degradation of manmade compounds such as chlorobiphenyls or chlorobenzenes have been found in bacteria from geographically separated areas of the world. The question whether enzymes specialized for the degradation of xenobiotics in these bacteria evolved from more common isozymes only after the large-scale introduction of xenobiotic chemicals into the environment cannot be answered yet. There is virtually no information concerning the mutation rates in bacteria living in nature and the response of such rates to the presence of potential (xenobiotic) substrates or combinations of substrates. Enough evidence is available to conclude that recombination, transposition, and gene transfer can accelerate the evolution of catabolic pathways by recruiting and combining new catabolic activities. Now we need quantitative data for these processes in the natural environment and for the effect of environmental parameters on the rate of evolution.

Many microbial strains have been engineered and equipped with extra catabolic genes (79, 108, 176, 177, 188, 237). These studies have given much insight into the molecular events and regulatory mechanisms responsible for evolution. It has been suggested that engineered strains be used for bioremedial purposes. Experiments in soil and wastewater systems have shown that such organisms can be successful under the selective pressure of a specific substrate or a toxic chemical (44, 173, 255). However, these organisms will in general not succeed in a multisubstrate, multiorganism environment without specific selection pressure. The application of genetically modified organisms for bioremediation without physical containment and the related biosafety aspects are much-debated issues. Because of biosafety aspects and the limited competitiveness, it is questionable whether laboratory-derived genetically modified microorganisms should be used in open systems. The current knowledge summarized in this review clearly evidences the potential of microorganisms to evolve new and desired pathways themselves. In fact, naturally evolved microorganisms tend to be more competitive since they have already survived natural selection.

Under natural conditions or during treatment processes, the degradation of pollutants is controlled most often by a variety of physical and chemical parameters, such as temperature, pH, and availability of the substrate, and not by the presence or absence of the appropriate population of microorganisms. Optimal physical and chemical conditions will allow eventual evolution and growth of the best-adapted microbial population. In case adaptation is too slow under optimal conditions, the addition of a variety of microbial strains carrying some desired genes and/or parts of necessary pathways may speed up evolution. Such a "directed evolution" has proven to be successful in many laboratory experiments and may have considerable potential to increase the "infallibility" of microorganisms in the natural environment.

REFERENCES

1. **Abril, M.-A., C. Michan, K. N. Timmis, and J. L. Ramos.** 1989. Regulator and enzyme specificities of the TOL plasmid-encoded upper pathway for degradation of aromatic hydrocarbons and expansion of the substrate range of the pathway. J. Bacteriol. **171**:6782–6790.
2. **Aelion, C. M., C. M. Swindoll, and F. K. Pfaender.** 1987. Adaptation to and biodegradation of xenobiotic compounds by microbial communities from a pristine aquifer. Appl. Environ. Microbiol. **53**:2212–2217.
3. **Aldrich, T. L., and A. M. Chakrabarty.** 1988. Transcriptional regulation, nucleotide sequence, and localization of the promoter of the *catBC* operon in *Pseudomonas putida*. J. Bacteriol. **170**:1297–1304.
4. **Aldrich, T. L., B. Frantz, J. F. Gill, J. J. Kilbane, and A. M. Chakrabarty.** 1987. Cloning and complete nucleotide sequence determination of the *catB* gene encoding *cis,cis*-muconate lactonizing enzyme. Gene **52**:185–195.
5. **Alexander, M.** 1981. Biodegradation of chemicals of environmental concern. Science **211**:132–138.
6. **Alexander, M.** 1985. Biodegradation of organic chemicals. Environ. Sci. Technol. **18**:106–111.
7. **Aronson, B. D., M. Levinthal, and R. L. Somerville.** 1989. Activation of a cryptic pathway for threonine metabolism via specific IS*3*-mediated alteration of promoter structure in *Escherichia coli*. J. Bacteriol. **171**:5503–5511.
8. **Assinder, S. J., and P. A. Williams.** 1988. Comparison of the *meta* pathway operons on NAH plasmid pWW60-22 and TOL plasmid pWW53-4 and its evolutionary significance. J. Gen. Microbiol. **134**:2769–2778.
9. **Assinder, S. J., and P. A. Williams.** 1990. The TOL plasmids: determinants of the catabolism of toluene and the xylenes. Adv. Microb. Physiol. **31**:1–69.
10. **Barkay, T.** 1987. Adaptation of aquatic microbial communities to Hg^{2+} stress. Appl. Environ. Microbiol. **53**:2725–2732.
11. **Barkay, T., C. Liebert, and M. Gillman.** 1989. Hybridization of DNA probes with whole-community genome for detection of genes that encode microbial responses to pollutants: *mer* genes and Hg^{2+} resistance. Appl. Environ. Microbiol. **55**:1574–1577.
12. **Barkay, T., and H. Pritchard.** 1988. Adaptation of aquatic microbial communities to pollutant stress. Microbiol. Sci. **5**:165–169.
13. **Bartels, I., H.-J. Knackmuss, and W. Reineke.** 1984. Suicide inactivation of catechol 2,3-dioxygenase from *Pseudomonas putida* mt-2 by 3-halocatechols. Appl. Environ. Microbiol. **47**:500–505.
14. **Bartilson, M., I. Nordlund, and V. Shingler.** 1989. Nucleotide sequence and expression of the catechol 2,3-dioxygenase-encoding gene of phenol catabolizing *Pseudomonas* CF600. Gene **85**:233–238.
15. **Beacham, I. R.** 1987. Silent genes in prokaryotes. FEMS Microbiol. Rev. **46**:409–417.
16. **Bej, A. K., R. J. Steffan, J. DiCesare, L. Haff, and R. M. Atlas.** 1990. Detection of coliform bacteria in water by polymerase chain reaction and gene probes. Appl. Environ. Microbiol. **56**:307–314.
17. **Blom, A., W. Harder, and A. Matin.** 1992. Unique and overlapping pollutant stress proteins of *Escherichia coli*. Appl. Environ. Microbiol. **58**:331–334.
18. **Boe, L.** 1990. Mechanism for induction of adaptive mutations in *Escherichia coli*. Mol. Microbiol. **4**:597–601.
19. **Broderick, J. B., and T. V. O'Halloran.** 1991. Overproduction, purification, and characterization of chlorocatechol dioxygenase, a non-heme iron dioxygenase with broad substrate tolerance. Biochemistry **30**:7349–7358.
20. **Bruhn, C., R. C. Bayly, and H.-J. Knackmuss.** 1988. The in vivo construction of 4-chloro-2-nitrophenol assimilatory bacteria. Arch. Microbiol. **150**:171–177.
21. **Burlage, R. S., L. A. Bemis, A. C. Layton, G. S. Sayler, and F. Larimer.** 1990. Comparative genetic organization of incompatibility group P degradative plasmids. J. Bacteriol. **172**:6818–6825.
22. **Burlage, R. S., S. W. Hooper, and G. S. Sayler.** 1989. The TOL (pWW0) catabolic plasmid. Appl. Environ. Microbiol. **55**: 1323–1328.
23. **Cairns, J., J. Overbaugh, and S. Miller.** 1988. The origin of

mutants. Nature (London) **335**:142–145.
24. **Carney, B. F., L. Kröckel, J. V. Leary, and D. D. Focht.** 1989. Identification of *Pseudomonas alcaligenes* chromosomal DNA in the plasmid DNA of the chlorobenzene-degrading recombinant *Pseudomonas putida* strain CB1-9. Appl. Environ. Microbiol. **55**:1037–1039.
25. **Chakrabarty, A. M., D. A. Friello, and L. H. Bopp.** 1978. Transposition of plasmid DNA segments specifying hydrocarbon degradation and their expression in various microorganisms. Proc. Natl. Acad. Sci. USA **75**:3109–3112.
26. **Chatfield, L. K., and P. A. Williams.** 1986. Naturally occurring TOL plasmids in *Pseudomonas* strains carry either two homologous or two nonhomologous catechol 2,3-oxygenase genes. J. Bacteriol. **168**:878–885.
27. **Chatterjee, D. K., and A. M. Chakrabarty.** 1983. Genetic homology between independently isolated chlorobenzoate-degradative plasmids. J. Bacteriol. **153**:532–534.
28. **Chatterjee, D. K., and A. M. Chakrabarty.** 1984. Restriction mapping of a chlorobenzoate degradative plasmid and molecular cloning of the degradative genes. Gene **27**:173–181.
29. **Chatterjee, D. K., S. T. Kellogg, S. Hamada, and A. M. Chakrabarty.** 1981. Plasmid specifying total degradation of 3-chlorobenzoate by a modified *ortho* pathway. J. Bacteriol. **146**:639–646.
30. **Chaudhry, G. R., and S. Chapalamadugu.** 1991. Biodegradation of halogenated organic compounds. Microbiol. Rev. **55**: 59–79.
31. **Clarke, P. H.** 1982. The metabolic versatility of pseudomonads. Antonie Leeuwenhoek **48**:105–130.
32. **Clarke, P. H.** 1984. The evolution of degradative pathways, p. 11–27. *In* D. T. Gibson (ed.), Microbial degradation of organic compounds. Marcel Dekker, Inc., New York.
33. **Claverys, J.-P., and S. A. Lacks.** 1986. Heteroduplex deoxyribonucleic acid base mismatch repair in bacteria. Microbiol. Rev. **50**:133–165.
34. **Coco, W. M., U. M. X. Sangodkar, and A. M. Chakrabarty.** 1989. Recruitment of *tft* and *clc* biodegradative pathway genes: modes of evolution. Adv. Appl. Biotechnol. **4**:44–59.
35. **Commandeur, L. C. M., and J. R. Parsons.** 1990. Degradation of halogenated aromatic compounds. Biodegradation **1**:207–220.
36. **de Lorenzo, V., M. Herrero, M. Metzke, and K. N. Timmis.** 1991. An upstream XylR- and IHF-induced nucleoprotein complex regulates the σ^{54}-dependent Pu promoter of TOL plasmid. EMBO J. **10**:1159–1169.
37. **Diels, L., and M. Mergeay.** 1990. DNA probe-mediated detection of resistant bacteria from soils highly polluted by heavy metals. Appl. Environ. Microbiol. **56**:1485–1491.
38. **Don, R. H., and J. M. Pemberton.** 1981. Properties of six pesticide degradation plasmids isolated from *Alcaligenes paradoxus* and *Alcaligenes eutrophus*. J. Bacteriol. **145**:681–686.
39. **Don, R. H., A. J. Weightman, H.-J. Knackmuss, and K. N. Timmis.** 1985. Transposon mutagenesis and cloning analysis of the pathways for degradation of 2,4-dichlorophenoxyacetic acid and 3-chlorobenzoate in *Alcaligenes eutrophus* JMP134(pJP4). J. Bacteriol. **161**:85–90.
40. **Dorn, E., M. Hellwig, W. Reineke, and H.-J. Knackmuss.** 1974. Isolation and characterization of a 3-chlorobenzoate degrading pseudomonad. Arch. Microbiol. **99**:61–70.
41. **Dorn, E., and H.-J. Knackmuss.** 1978. Chemical structure and biodegradability of halogenated aromatic compounds. Two catechol 1,2-dioxygenases from a 3-chlorobenzoate-grown pseudomonad. Biochem. J. **174**:73–84.
42. **Doten, R. C., K.-L. Ngai, D. J. Mitchell, and L. N. Ornston.** 1987. Cloning and genetic organization of the *pca* gene cluster from *Acinetobacter calcoaceticus*. J. Bacteriol. **169**:3168–3174.
43. **Duggleby, C. L., S. A. Bayley, M. J. Worsey, P. A. Williams, and P. Broda.** 1977. Molecular sizes and relationships of TOL plasmids in *Pseudomonas*. J. Bacteriol. **130**:1274–1280.
44. **Dwyer, D. F., F. Rojo, and K. N. Timmis.** 1988. Fate and behaviour in an activated sludge microcosm of a genetically-engineered micro-organism designed to degrade substituted aromatic compounds, p. 77–88. *In* M. Sussmann, C. H. Collins, F. A. Skinner, and D. E. Stewart-Tull (ed.), The release of genetically-engineered micro-organisms. Academic Press, Ltd., London.
45. **Echols, H., and M. F. Goodman.** 1991. Fidelity mechanisms in DNA replication. Annu. Rev. Biochem. **60**:477–511.
46. **Ensley, B. D., B. J. Ratzkin, T. D. Osslund, M. J. Simon, L. P. Wackett, and D. T. Gibson.** 1983. Expression of naphthalene oxidation genes in *Escherichia coli* results in the biosynthesis of indigo. Science **222**:167–169.
47. **Erickson, B. D., and F. J. Mondello.** 1992. Nucleotide sequencing and transcriptional mapping of the genes encoding biphenyl dioxygenase, a multicomponent polychlorinated-biphenyl-degrading enzyme in *Pseudomonas* strain LB400. J. Bacteriol. **174**:2903–2912.
48. **Fetzner, S., R. Müller, and F. Lingens.** 1992. Purification and some properties of 2-halobenzoate 1,2-dioxygenase, a two-component enzyme system from *Pseudomonas cepacia* 2CBS. J. Bacteriol. **174**:279–290.
49. **Fewson, C. A.** 1988. Microbial metabolism of mandelate: a microcosm of diversity. FEMS Microbiol. Rev. **54**:85–110.
50. **Franken, S. M., H. J. Rozeboom, K. H. Kalk, and B. W. Dijkstra.** 1991. Crystal structure of haloalkane dehalogenase: an enzyme to detoxify halogenated alkanes. EMBO J. **10**:1297–1302.
51. **Franklin, F. C. H., M. Bagdasarian, M. M. Bagdasarian, and K. N. Timmis.** 1981. Molecular and functional analysis of the TOL plasmid pWW0 from *Pseudomonas putida* and cloning of genes for the entire regulated aromatic ring-cleavage pathway. Proc. Natl. Acad. Sci. USA **78**:7458–7462.
52. **Franklin, F. C. H., P. R. Lehrbach, R. Lurz, B. Rueckert, M. Bagdasarian, and K. N. Timmis.** 1983. Localization and functional analysis of transposon mutations in regulatory genes of the TOL catabolic pathway. J. Bacteriol. **154**:676–685.
53. **Frantz, B., and A. M. Chakrabarty.** 1986. Degradative plasmids in *Pseudomonas*, p. 295–323. *In* J. R. Sokatch (ed.), The biology of *Pseudomonas*. Academic Press, Inc., New York.
54. **Frantz, B., and A. M. Chakrabarty.** 1987. Organization and nucleotide sequence determination of a gene cluster involved in 3-chlorocatechol degradation. Proc. Natl. Acad. Sci. USA **84**:4460–4464.
55. **Fredrickson, J. K., F. J. Brockman, D. J. Workman, S. W. Li, and T. O. Stevens.** 1991. Isolation and characterization of a subsurface bacterium capable of growth on toluene, naphthalene, and other aromatic compounds. Appl. Environ. Microbiol. **57**:796–803.
56. **Fredrickson, J. K., R. J. Hicks, S. W. Li, and F. J. Brockman.** 1988. Plasmid incidence in bacteria from deep subsurface sediments. Appl. Environ. Microbiol. **52**:2916–2923.
57. **Fulthorpe, R. R., and R. C. Wyndham.** 1989. Survival and activity of a 3-chlorobenzoate-catabolic genotype in a natural system. Appl. Environ. Microbiol. **55**:1584–1590.
58. **Fulthorpe, R. R., and R. C. Wyndham.** 1991. Transfer and expression of the catabolic plasmid pBRC60 in wild bacterial recipients in a freshwater ecosystem. Appl. Environ. Microbiol. **57**:1546–1553.
59. **Fulthorpe, R. R., and R. C. Wyndham.** 1992. Involvement of a chlorobenzoate-catabolic transposon, Tn*5271*, in community adaptation to chlorobiphenyl, chloroaniline, and 2,4-dichlorophenoxyacetic acid in a freshwater ecosystem. Appl. Environ. Microbiol. **58**:314–325.
60. **Furukawa, K., N. Arimura, and T. Miyazaki.** 1987. Nucleotide sequence of the 2,3-dihydroxybiphenyl dioxygenase gene of *Pseudomonas pseudoalcaligenes*. J. Bacteriol. **169**:427–429.
61. **Furukawa, K., N. Hayase, and K. Taira.** 1990. Biphenyl/polychlorinated biphenyl catabolic gene (*bph* operon): organization, function, and molecular relationship in various pseudomonads, p. 111–121. *In* S. Silver, A. M. Chakrabarty, B. Iglewski, and S. Kaplan (ed.), *Pseudomonas*: biotransformations, pathogenesis, and evolving biotechnology. American Society for Microbiology, Washington, D.C.
62. **Furukawa, K., N. Hayase, K. Taira, and N. Tomizuka.** 1989. Molecular relationship of chromosomal genes encoding biphe-

nyl/polychlorinated biphenyl catabolism: some soil bacteria possess a highly conserved *bph* operon. J. Bacteriol. **171**:5467–5472.

63. **Furukawa, K., and T. Miyazaki.** 1986. Cloning of a gene cluster encoding biphenyl and chlorobiphenyl degradation in *Pseudomonas pseudoalcaligenes*. J. Bacteriol. **166**:392–398.
64. **Galas, D. J., and M. Chandler.** 1989. Bacterial insertion sequences, p. 109–163. *In* D. E. Berg and M. M. Howe (ed.), Mobile DNA. American Society for Microbiology, Washington, D.C.
65. **Ghosal, D., and I.-S. You.** 1988. Gene duplication in haloaromatic degradative plasmids pJP4 and pJP2. Can. J. Microbiol. **34**:709–715.
66. **Ghosal, D., and I.-S. You.** 1988. Nucleotide homology and organization of chlorocatechol oxidation genes of plasmids pJP4 and pAC27. Mol. Gen. Genet. **211**:113–120.
67. **Ghosal, D., and I.-S. You.** 1989. Operon structure and nucleotide homology of the chlorocatechol oxidation genes of plasmids pAC27 and pJP4. Gene **83**:225–232.
68. **Gibson, D. T., and V. Subramanian.** 1984. Microbial degradation of aromatic hydrocarbons, p. 181–252. *In* D. T. Gibson (ed.), Microbial degradation of organic compounds. Marcel Dekker, Inc., New York.
69. **Gibson, D. T., G. J. Zylstra, and S. Chauhan.** 1990. Biotransformations catalyzed by toluene dioxygenase from *Pseudomonas putida* F1, p. 121–133. *In* S. Silver, A. M. Chakrabarty, B. Iglewski, and S. Kaplan (ed.), *Pseudomonas*: biotransformations, pathogenesis, and evolving biotechnology. American Society for Microbiology, Washington, D.C.
70. **Goldstein, R. M., L. M. Mallory, and M. Alexander.** 1985. Reasons for possible failure of inoculation to enhance biodegradation. Appl. Environ. Microbiol. **50**:977–983.
71. **Haigler, B. E., S. F. Nishino, and J. C. Spain.** 1988. Degradation of 1,2-dichlorobenzene by a *Pseudomonas* sp. Appl. Environ. Microbiol. **54**:294–301.
72. **Haigler, B. E., and J. C. Spain.** 1989. Degradation of *p*-chlorotoluene by a mutant of *Pseudomonas* sp. strain JS6. Appl. Environ. Microbiol. **55**:372–379.
73. **Hall, B. G.** 1988. Widespread distributions of deletions of the *bgl* operon in natural isolates of *Escherichia coli*. Mol. Biol. Evol. **5**:456–467.
74. **Hall, B. G.** 1990. Directed evolution of a bacterial operon. Bioessays **12**:551–558.
75. **Hancock, J. M., and G. A. Dover.** 1990. 'Compensatory slippage' in the evolution of ribosomal RNA genes. Nucleic Acids Res. **18**:5949–5954.
76. **Harayama, S., and R. H. Don.** 1985. Catabolic plasmids: their analysis and utilization in the manipulation of bacterial metabolic activities, p. 283–307. *In* J. K. Setlow and A. Hollaender (ed.), Genetic engineering: principles and methods. Plenum Publishing Co., New York.
77. **Harayama, S., P. R. Lehrbach, and K. N. Timmis.** 1984. Transposon mutagenesis analysis of *meta*-cleavage pathway operon genes of the TOL plasmid of *Pseudomonas putida* mt-2. J. Bacteriol. **160**:251–255.
78. **Harayama, S., R. A. Leppik, M. Rekik, N. Mermod, P. R. Lehrbach, W. Reineke, and K. N. Timmis.** 1986. Gene order of the TOL catabolic plasmid upper pathway operon and oxidation of both toluene and benzyl alcohol by the *xylA* product. J. Bacteriol. **167**:455–461.
79. **Harayama, S., J. L. Ramos, and K. N. Timmis.** 1986. Experimental evolution of plasmid-specified functions. Banbury Rep. **24**:389–402.
80. **Harayama, S., and M. Rekik.** 1989. Bacterial aromatic ring-cleavage enzymes are classified into two different gene families. J. Biol. Chem. **264**:15328–15333.
81. **Harayama, S., and M. Rekik.** 1990. The *meta* cleavage operon of TOL degradative plasmid pWW0 comprises 13 genes. Mol. Gen. Genet. **221**:113–120.
82. **Harayama, S., M. Rekik, A. Bairoch, E. L. Neidle, and L. N. Ornston.** 1991. Potential DNA slippage structures acquired during evolutionary divergence of *Acinetobacter calcoaceticus* chromosomal *benABC* and *Pseudomonas putida* TOL pWW0 plasmid *xylXYZ*, genes encoding benzoate dioxygenases. J. Bacteriol. **173**:7540–7548.
83. **Harayama, S., M. Rekik, and K. N. Timmis.** 1986. Genetic analysis of a relaxed substrate specificity aromatic ring dioxygenase, toluate 1,2-dioxygenase, encoded by TOL plasmid pWW0 of *Pseudomonas putida*. Mol. Gen. Genet. **202**:226–234.
84. **Harayama, S., M. Rekik, A. Wasserfallen, and A. Bairoch.** 1987. Evolutionary relationships between catabolic pathways for aromatics: conservation of gene order and nucleotide sequences of catechol oxidation genes of pWW0 and NAH7 plasmids. Mol. Gen. Genet. **210**:241–247.
85. **Harayama, S., M. Rekik, M. Wubbolts, K. Rose, R. A. Leppik, and K. N. Timmis.** 1989. Characterization of five genes in the upper-pathway operon of TOL plasmid pWW0 from *Pseudomonas putida* and identification of the gene products. J. Bacteriol. **171**:5048–5055.
86. **Hartnett, C., E. L. Neidle, K.-L. Ngai, and L. N. Ornston.** 1990. DNA sequences of genes encoding *Acinetobacter calcoaceticus* protocatechuate 3,4-dioxygenase: evidence indicating shuffling of genes and of DNA sequences within genes during their evolutionary divergence. J. Bacteriol. **172**:956–966.
87. **Haugland, R. A., U. M. X. Sangodkar, and A. M. Chakrabarty.** 1990. Repeated sequences including RS1100 from *Pseudomonas cepacia* AC1100 function as IS elements. Mol. Gen. Genet. **220**:222–228.
88. **Hayase, N., K. Taira, and K. Furukawa.** 1990. *Pseudomonas putida* KF715 *bphABCD* operon encoding biphenyl and polychlorinated biphenyl degradation: cloning, analysis, and expression in soil bacteria. J. Bacteriol. **172**:1160–1164.
89. **Henikoff, S., G. W. Haughn, J. M. Calvo, and J. C. Wallace.** 1988. A large family of bacterial activator proteins. Proc. Natl. Acad. Sci. USA **85**:6602–6606.
90. **Henikoff, S., J. C. Wallace, and J. P. Brown.** 1990. Finding protein similarities with nucleotide sequence databases. Methods Enzymol. **183**:111–133.
91. **Holtel, A., M.-A. Abril, S. Marques, K. N. Timmis, and J. L. Ramos.** 1990. Promoter-upstream activator sequences are required for expression of the *xylS* gene and upper-pathway operon on the *Pseudomonas* TOL plasmid. Mol. Microbiol. **4**:1551–1556.
92. **Horn, J. M., S. Harayama, and K. N. Timmis.** 1991. DNA sequence determination of the TOL plasmid (pWW0) *xylGFJ* genes of *Pseudomonas putida*: implications for the evolution of aromatic catabolism. Mol. Microbiol. **5**:2459–2474.
93. **Hughes, E. J., M. K. Shapiro, J. E. Houghton, and L. N. Ornston.** 1988. Cloning and expression of *pca* genes from *Pseudomonas putida* in *Escherichia coli*. J. Gen. Microbiol. **134**:2877–2887.
94. **Hutchins, S. R., M. B. Tomson, J. T. Wilson, and C. H. Ward.** 1984. Microbial removal of wastewater organic compounds as a function of input concentration in soil columns. Appl. Environ. Microbiol. **48**:1039–1045.
95. **Imai, R., Y. Nagata, M. Fukuda, M. Takagi, and K. Yang.** 1991. Molecular cloning of a *Pseudomonas paucimobilis* gene encoding a 17-kilodalton polypeptide that eliminates HCl molecules from γ-hexachlorocyclohexane. J. Bacteriol. **173**:6811–6819.
96. **Inouye, S., A. Nakazawa, and T. Nakazawa.** 1981. Molecular cloning of TOL genes *xylB* and *xylE* in *Escherichia coli*. J. Bacteriol. **145**:1137–1143.
97. **Inouye, S., A. Nakazawa, and T. Nakazawa.** 1983. Molecular cloning of regulatory gene *xylR* and operator-promoter regions of the *xylABC* and *xylDEGF* operons of the TOL plasmid. J. Bacteriol. **155**:1192–1199.
98. **Inouye, S., A. Nakazawa, and T. Nakazawa.** 1987. Expression of the regulatory gene *xylS* on the TOL plasmid is positively controlled by the *xylR* gene product. Proc. Natl. Acad. Sci. USA **84**:5182–5186.
99. **Irie, S., S. Doi, T. Yorifuji, M. Takagi, and K. Yano.** 1987. Nucleotide sequencing and characterization of the genes encoding benzene oxidation enzymes of *Pseudomonas putida*. J. Bacteriol. **169**:5174–5179.

100. **Jeenes, D. J., W. Reineke, H.-J. Knackmuss, and P. A. Williams.** 1982. TOL plasmid pWW0 in constructed halobenzoate-degrading *Pseudomonas* strains: enzyme regulation and DNA structure. J. Bacteriol. **150:**180–187.
101. **Jobling, M. G., S. E. Peters, and D. A. Ritchie.** 1988. Plasmid-borne mercury resistance in aquatic bacteria. FEMS Microbiol. Lett. **49:**31–37.
102. **Kabisch, M., and P. Fortnagel.** 1990. Nucleotide sequence of metapyrocatechase I (catechol 2,3-oxygenase I) gene *mpcI* from *Alcaligenes eutrophus* JMP222. Nucleic Acids Res. **18:** 3405–3406.
103. **Kaluza, K., M. Hahn, and H. Hennecke.** 1985. Repeated sequences similar to insertion elements clustered around the *nif* region of the *Rhizobium japonicum* genome. J. Bacteriol. **162:**535–542.
104. **Kaphammer, B., J. J. Kukor, and R. H. Olsen.** 1990. Regulation of *tfdCDEF* by *tfdR* of the 2,4-dichlorophenoxyacetic acid degradation plasmid pJP4. J. Bacteriol. **172:**2280–2286.
105. **Kaphammer, B., and R. H. Olsen.** 1990. Cloning and characterization of *tfdS*, the repressor-activator gene of *tfdB*, from the 2,4-dichlorophenoxyacetic acid catabolic plasmid pJP4. J. Bacteriol. **172:**5856–5862.
106. **Keil, H., M. R. Lebens, and P. A. Williams.** 1985. TOL plasmid pWW15 contains two nonhomologous, independently regulated catechol 2,3-oxygenase genes. J. Bacteriol. **163:**248–255.
107. **Khan, A. A., and S. K. Walia.** 1991. Expression, localization, and functional analysis of polychlorinated biphenyl degradation genes *cbpABCD* of *Pseudomonas putida*. Appl. Environ. Microbiol. **57:**1325–1332.
108. **Kilbane, J. J.** 1986. Genetic aspects of toxic chemical degradation. Microb. Ecol. **12:**135–145.
109. **Kirsthein, J. D., H. W. Paerl, and J. Zehr.** 1991. Amplification, cloning, and sequencing of a *nifH* segment from aquatic microorganisms and natural communities. Appl. Environ. Microbiol. **57:**2645–2650.
110. **Kivisaar, M., L. Kasak, and A. Nurk.** 1991. Sequence of the plasmid-encoded catechol 1,2-dioxygenase-expressing gene, *pheB*, of phenol-degrading *Pseudomonas* sp. strain EST1001. Gene **98:**15–20.
111. **Kivisaar, M. A., J. K. Habicht, and A. L. Heinaru.** 1989. Degradation of phenol and *m*-toluate in *Pseudomonas* sp. strain EST1001 and its *Pseudomonas putida* transconjugants is determined by a multiplasmid system. J. Bacteriol. **171:**5111–5116.
112. **Klecka, G. M., and D. T. Gibson.** 1981. Inhibition of catechol 2,3-dioxygenase from *Pseudomonas putida* by 3-chlorocatechol. Appl. Environ. Microbiol. **41:**1159–1165.
113. **Klein, T. M., and M. Alexander.** 1986. Bacterial inhibitors in lake water. Appl. Environ. Microbiol. **52:**114–118.
114. **Klingmüller, W.** 1991. Plasmid transfer in natural soil: a case by case study with nitrogen-fixing *Enterobacter*. FEMS Microbiol. Ecol. **85:**107–116.
115. **Kobori, H., C. W. Sullivan, and H. Shizuya.** 1984. Bacterial plasmids in antarctic natural microbial assemblages. Appl. Environ. Microbiol. **48:**515–518.
116. **Köhler, T., S. Harayama, J.-L. Ramos, and K. N. Timmis.** 1989. Involvement of *Pseudomonas putida* RpoN σ factor in regulation of various metabolic functions. J. Bacteriol. **171:** 4326–4333.
117. **Kröckel, L., and D. D. Focht.** 1987. Construction of chlorobenzene-utilizing recombinants by progenitive manifestation of a rare event. Appl. Environ. Microbiol. **53:**2470–2475.
118. **Kuhm, A. E., M. Schlömann, H.-J. Knackmuss, and D. H. Pieper.** 1990. Purification and characterization of dichloromuconate cycloisomerase from *Alcaligenes eutrophus* JMP134. Biochem. J. **266:**877–883.
119. **Kuhm, A. E., A. Stolz, K.-L. Ngai, and H.-J. Knackmuss.** 1991. Purification and characterization of a 1,2-dihydroxynaphthalene dioxygenase from a bacterium that degrades naphthalenesulfonic acids. J. Bacteriol. **173:**3795–3802.
120. **Kukor, J. J., R. H. Olsen, and D. P. Ballou.** 1988. Cloning and expression of the *catA* and *catBC* gene clusters from *Pseudomonas aeruginosa* PAO. J. Bacteriol. **170:**4458–4465.
121. **Kunkel, T. A., and A. Soni.** 1988. Mutagenesis by transient misalignment. J. Biol. Chem. **263:**14784–14789.
122. **Kurkela, S., H. Lehväslaiho, E. T. Palva, and T. H. Teeri.** 1988. Cloning, nucleotide sequence and characterization of genes encoding naphthalene dioxygenase of *Pseudomonas putida* strain NCIB9816. Gene **73:**355–362.
123. **Lai, M.-D., and K. L. Beattie.** 1988. Influence of DNA sequence on the nature of mispairing during DNA synthesis. Biochemistry **27:**1722–1728.
124. **Latorre, J., W. Reineke, and H.-J. Knackmuss.** 1984. Microbial metabolism of chloroanilines: enhanced evolution by natural genetic exchange. Arch. Microbiol. **140:**159–165.
125. **Leahy, J. G., and R. R. Colwell.** 1990. Microbial degradation of hydrocarbons in the environment. Microbiol. Rev. **54:**305–315.
126. **Lehrbach, P. R., I. McGregor, J. M. Ward, and P. Broda.** 1983. Molecular relationships between *Pseudomonas* Inc P-9 degradative plasmids TOL, NAH and SAL. Plasmid **10:**164–174.
127. **Lehrbach, P. R., J. Zeyer, W. Reineke, H.-J. Knackmuss, and K. N. Timmis.** 1984. Enzyme recruitment in vitro: use of cloned genes to extend the range of haloaromatics degraded by *Pseudomonas* sp. strain B13. J. Bacteriol. **158:**1025–1032.
128. **Lessie, T. G., M. S. Wood, A. Byrne, and A. Ferrante.** 1990. Transposable gene-activating elements in *Pseudomonas cepacia*, p. 279–292. *In* S. Silver, A. M. Chakrabarty, B. Iglewski, and S. Kaplan (ed.), *Pseudomonas*: biotransformations, pathogenesis, and evolving biotechnology. American Society for Microbiology, Washington, D.C.
129. **Levinson, G., and G. A. Gutman.** 1987. Slipped-strand mispairing: a major mechanism for DNA sequence evolution. Mol. Biol. Evol. **4:**203–221.
130. **Levy, S. B., and B. M. Marshall.** 1988. Genetic transfer in the natural environment, p. 61–75. *In* M. Sussmann, C. H. Collins, F. A. Skinner, and D. E. Stewart-Tull (ed.), The release of genetically-engineered micro-organisms. Academic Press, Ltd., London.
131. **Locher, H. H., T. Leisinger, and A. M. Cook.** 1991. 4-Toluene sulfonate methyl-monooxygenase from *Comamonas testosteroni* T-2: purification and some properties of the oxygenase component. J. Bacteriol. **173:**3741–3748.
132. **Lopilato, J., and A. Wright.** 1990. Mechanisms of activation of the cryptic *bgl* operon of *Escherichia coli* K-12, p. 435–445. *In* K. Drlica and M. Riley (ed.), The bacterial chromosome. American Society for Microbiology, Washington, D.C.
133. **Lorenz, M. G., B. W. Aardema, and W. Wackernagel.** 1988. Highly efficient genetic transformation of *Bacillus subtilis* attached to sand grains. J. Gen. Microbiol. **134:**107–112.
134. **Lorenz, M. G., D. Gerjets, and W. Wackernagel.** 1991. Release of transforming plasmid and chromosomal DNA from two cultured soil bacteria. Arch. Microbiol. **156:**319–327.
135. **Madsen, E. L., J. L. Sinclair, and W. C. Ghiorse.** 1991. In situ biodegradation: microbiological patterns in a contaminated aquifer. Science **252:**830–833.
136. **Markus, A., D. Krekel, and F. Lingens.** 1986. Purification and some properties of component A of the 4-chlorophenylacetate 3,4-dioxygenase from *Pseudomonas* species strain CBS. J. Biol. Chem. **261:**12883–12888.
137. **Mermod, N., J. L. Ramos, A. Bairoch, and K. N. Timmis.** 1987. The *xylS* gene positive regulator of TOL plasmid pWW0: identification, sequence analysis and overproduction leading to constitutive expression of *meta* cleavage operon. Mol. Gen. Genet. **207:**349–354.
138. **Meulien, P., R. G. Downing, and P. Broda.** 1981. Excision of the 40kb segment of the TOL plasmid from *Pseudomonas putida* mt-2 involves direct repeats. Mol. Gen. Genet. **184:**97–101.
139. **Mittler, J. E., and R. E. Lenski.** 1990. New data on excisions of Mu from *E. coli* MCS2 cast doubt on directed mutation hypothesis. Nature (London) **344:**173–175.
140. **Mittler, J. E., and R. E. Lenski.** 1992. Experimental evidence for an alternative to directed mutation in the *bgl* operon. Nature (London) **356:**446–448.
141. **Modrich, P.** 1987. DNA mismatch correction. Annu. Rev. Biochem. **56:**435–466.

142. **Mokross, H., E. Schmidt, and W. Reineke.** 1990. Degradation of 3-chlorobiphenyl by in vivo constructed hybrid pseudomonads. FEMS Microbiol. Lett. **71:**179–186.
143. **Mondello, F. J.** 1989. Cloning and expression in *Escherichia coli* of *Pseudomonas* strain LB400 genes encoding polychlorinated biphenyl degradation. J. Bacteriol. **171:**1725–1732.
144. **Nakai, C., K. Horiike, S. Kuramitsu, H. Kagamiyama, and M. Nozaki.** 1990. Three isozymes of catechol 1,2-dioxygenase (pyrocatechase), αα, αβ, and ββ, from *Pseudomonas arvilla* C-1. J. Biol. Chem. **265:**660–665.
145. **Nakatsu, C., J. Ng, R. Singh, N. Straus, and C. Wyndham.** 1991. Chlorobenzoate catabolic transposon Tn*5271* is a composite class I element with flanking class II insertion sequences. Proc. Natl. Acad. Sci. USA **88:**8312–8316.
146. **Nakazawa, T., S. Inouye, and A. Nakazawa.** 1980. Physical and functional mapping of RP4-TOL plasmid recombinants: analysis of insertion and deletion mutants. J. Bacteriol. **144:**222–231.
147. **Nakazawa, T., S. Inouye, and A. Nakazawa.** 1990. Regulatory systems for expression of *xyl* genes on the TOL plasmid, p. 133–141. *In* S. Silver, A. M. Chakrabarty, B. Iglewski, and S. Kaplan (ed.), *Pseudomonas*: biotransformations, pathogenesis, and evolving biotechnology. American Society for Microbiology, Washington, D.C.
148. **Negoro, S., S. Nakamura, and H. Okada.** 1984. DNA-DNA hybridization analysis of nylon oligomer-degradative plasmid pOAD2: identification of the DNA region analogous to the nylon oligomer degradation gene. J. Bacteriol. **158:**419–424.
149. **Neidle, E., C. Hartnett, L. N. Ornston, A. Bairoch, M. Rekik, and S. Harayama.** 1992. *Cis*-diol dehydrogenases encoded by the TOL pWW0 plasmid *xylL* gene and the *Acinetobacter calcoaceticus* chromosomal *benD* gene are members of the short-chain alcohol dehydrogenase superfamily. Eur. J. Biochem. **204:**113–120.
150. **Neidle, E. L., C. Hartnett, S. Bonitz, and L. N. Ornston.** 1988. DNA sequence of the *Acinetobacter calcoaceticus* catechol 1,2-dioxygenase I structural gene *catA*: evidence for evolutionary divergence of intradiol dioxygenases by acquisition of DNA sequence repetitions. J. Bacteriol. **170:**4874–4880.
151. **Neidle, E. L., C. Hartnett, and L. N. Ornston.** 1989. Characterization of *Acinetobacter calcoaceticus catM*, a repressor gene homologous in sequence to transcriptional activator genes. J. Bacteriol. **171:**5410–5421.
152. **Neidle, E. L., C. Hartnett, L. N. Ornston, A. Bairoch, M. Rekik, and S. Harayama.** 1991. Nucleotide sequences of the *Acinetobacter calcoaceticus benABC* genes for benzoate 1,2-dioxygenase reveal evolutionary relationships among multicomponent oxygenases. J. Bacteriol. **173:**5385–5395.
153. **Neidle, E. L., and L. N. Ornston.** 1986. Cloning and expression of *Acinetobacter calcoaceticus* catechol 1,2-dioxygenase structural gene *catA* in *Escherichia coli*. J. Bacteriol. **168:**815–820.
154. **Noda, Y., S. Nishikawa, K.-I. Shiozuka, H. Kadokura, H. Nakajima, K. Yoda, Y. Katayama, N. Morohoshi, T. Haraguchi, and M. Yamasaki.** 1990. Molecular cloning of the protocatechuate 4,5-dioxygenase genes of *Pseudomonas paucimobilis*. J. Bacteriol. **172:**2704–2709.
155. **Nordlund, I., J. Powlowski, and V. Shingler.** 1990. Complete nucleotide sequence and polypeptide analysis of multicomponent phenol hydroxylase from *Pseudomonas* sp. strain CF600. J. Bacteriol. **172:**6826–6833.
156. **Nordlund, I., and V. Shingler.** 1990. Nucleotide sequences of the *meta*-cleavage pathway enzymes 2-hydroxymuconic semialdehyde dehydrogenase and 2-hydroxymuconic semialdehyde hydrolase from *Pseudomonas* CF600. Biochim. Biophys. Acta **1049:**227–230.
157. **Ohlendorf, D. H., J. D. Lipscomb, and P. C. Weber.** 1988. Structure and assembly of protocatechuate 3,4-dioxygenase. Nature (London) **336:**403–405.
158. **Okada, H., S. Negoro, H. Kimura, and S. Nakamura.** 1983. Evolutionary adaptation of plasmid-encoded enzymes for degrading nylon oligomers. Nature (London) **306:**203–206.
159. **Oltmanns, R. H., H. G. Rast, and W. Reineke.** 1988. Degradation of 1,4-dichlorobenzene by constructed and enriched strains. Appl. Microbiol. Biotechnol. **28:**609–616.
160. **Ornston, L. N., J. Houghton, E. L. Neidle, and L. A. Gregg.** 1990. Subtle selection and novel mutation during evolutionary divergence of the β-ketoadipate pathway, p. 207–225. *In* S. Silver, A. M. Chakrabarty, B. Iglewski, and S. Kaplan (ed.), *Pseudomonas*: biotransformations, pathogenesis, and evolving biotechnology. American Society for Microbiology, Washington, D.C.
161. **Ornston, L. N., E. L. Neidle, and J. E. Houghton.** 1990. Gene rearrangements, a force for evolutionary change: DNA sequence arrangements, a source of genetic constancy, p. 325–334. *In* M. Riley and K. Drlica (ed.), The bacterial chromosome. American Society for Microbiology, Washington, D.C.
162. **Osborne, D. J., R. W. Pickup, and P. A. Williams.** 1988. The presence of two complete homologous *meta* pathway operons on TOL plasmid pWW53. J. Gen. Microbiol. **134:**2965–2975.
163. **Parker, L. L., P. W. Betts, and B. G. Hall.** 1988. Activation of a cryptic gene by excision of a DNA fragment. J. Bacteriol. **170:**218–222.
164. **Paul, J. H., L. Cazares, and J. Thurmond.** 1990. Amplification of the *rbcL* gene from dissolved and particulate DNA from aquatic environments. Appl. Environ. Microbiol. **56:**1963–1966.
165. **Perkins, E. J., M. P. Gordon, O. Caceres, and P. F. Lurquin.** 1990. Organization and sequence analysis of the 2,4-dichlorophenol hydroxylase and dichlorocatechol oxidative operons of plasmid pJP4. J. Bacteriol. **172:**2351–2359.
166. **Perkins, E. J., and P. F. Lurquin.** 1988. Duplication of a 2,4-dichlorophenoxyacetic acid monooxygenase gene in *Alcaligenes eutrophus* JMP134(pJP4). J. Bacteriol. **170:**5669–5672.
167. **Peterson, K. R., N. Ossanna, A. T. Thliveris, D. G. Ennis, and D. W. Mount.** 1988. Derepression of specific genes promotes DNA repair and mutagenesis in *Escherichia coli*. J. Bacteriol. **170:**1–4.
168. **Pettigrew, C. A., B. E. Haigler, and J. C. Spain.** 1991. Simultaneous biodegradation of chlorobenzene and toluene by a *Pseudomonas* strain. Appl. Environ. Microbiol. **57:**157–162.
169. **Pettigrew, C. A., and G. S. Sayler.** 1986. The use of DNA:DNA colony hybridization in the rapid isolation of 4-chlorobiphenyl degradative bacterial phenotypes. J. Microbiol. Methods **5:**205–213.
170. **Pickup, R. W.** 1991. Development of molecular methods for the detection of specific bacteria in the environment. J. Gen. Microbiol. **137:**1009–1019.
171. **Pieper, D. H., A. E. Kuhm, K. Stadler-Fritzsche, P. Fischer, and H.-J. Knackmuss.** 1991. Metabolization of 3,5-dichlorocatechol by *Alcaligenes eutrophus* JMP 134. Arch. Microbiol. **156:**218–222.
172. **Pieper, D. H., W. Reineke, K.-H. Engesser, and H.-J. Knackmuss.** 1988. Metabolism of 2,4-dichlorophenoxyacetic acid, 4-chloro-2-methylphenoxyacetic acid and 2-methylphenoxyacetic acid by *Alcaligenes eutrophus* JMP134. Arch. Microbiol. **150:**95–102.
173. **Pipke, R., I. Wagner-Döbler, K. N. Timmis, and D. F. Dwyer.** 1992. Survival and function of a genetically engineered pseudomonad in aquatic sediment microcosms. Appl. Environ. Microbiol. **58:**1259–1265.
174. **Ramos, J. L., F. Rojo, L. Zhou, and K. N. Timmis.** 1990. A family of positive regulators related to the *Pseudomonas putida* TOL plasmid XylS and the *Escherichia coli* AraC activators. Nucleic Acids Res. **18:**2149–2152.
175. **Ramos, J. L., A. Stolz, W. Reineke, and K. N. Timmis.** 1986. Altered effector specificities in regulators of gene expression: TOL plasmid *xylS* mutants and their use to engineer expansion of the range of aromatics degraded by bacteria. Proc. Natl. Acad. Sci. USA **83:**8467–8471.
176. **Ramos, J. L., and K. N. Timmis.** 1987. Experimental evolution of catabolic pathways of bacteria. Microbiol. Sci. **4:**228–237.
177. **Ramos, J. L., A. Wasserfallen, K. Rose, and K. N. Timmis.** 1987. Redesigning metabolic routes: manipulation of TOL plasmid pathway for catabolism of alkylbenzoates. Science **235:**593–596.

178. **Rangnekar, V. M.** 1988. Variation in the ability of *Pseudomonas* sp. strain B13 cultures to utilize *meta*-chlorobenzoate is associated with tandem amplification and deamplification of DNA. J. Bacteriol. **170:**1907–1912.
179. **Reineke, W.** 1984. Microbial degradation of halogenated aromatic compounds, p. 319–360. *In* D. T. Gibson (ed.), Microbial degradation of organic compounds. Marcel Dekker, Inc., New York.
180. **Reineke, W., D. J. Jeenes, P. A. Williams, and H.-J. Knackmuss.** 1982. TOL plasmid pWW0 in constructed halobenzoate-degrading *Pseudomonas* strains: prevention of *meta* pathway. J. Bacteriol. **150:**195–201.
181. **Reineke, W., and H.-J. Knackmuss.** 1988. Microbial degradation of haloaromatics. Annu. Rev. Microbiol. **42:**263–287.
182. **Reynolds, A. E., J. Felton, and A. Wright.** 1981. Insertion of DNA activates the cryptic *bgl* operon in *E. coli* K12. Nature (London) **293:**625–629.
183. **Rijnaarts, H. H. M., A. Bachmann, J. C. Jumelet, and A. J. B. Zehnder.** 1990. Effect of the desorption and intraparticle mass transfer on the aerobic biomineralization of α-hexachlorocyclohexane in a contaminated calcareous soil. Environ. Sci. Technol. **24:**1349–1354.
184. **Rochelle, P. A., M. J. Day, and J. C. Fry.** 1988. Occurrence, transfer and mobilization in epilithic strains of *Acinetobacter* of mercury-resistance plasmids capable of transformation. J. Gen. Microbiol. **134:**2933–2941.
185. **Rochelle, P. A., J. C. Fry, and M. J. Day.** 1989. Factors affecting conjugal transfer of plasmids encoding mercury resistance from pure cultures and mixed natural suspension of epilithic bacteria. J. Gen. Microbiol. **135:**409–424.
186. **Rochelle, P. A., J. C. Fry, and M. J. Day.** 1989. Plasmid transfer between *Pseudomonas* spp. within epilithic films in a rotating disc microcosm. FEMS Microbiol. Ecol. **62:**127–136.
187. **Rochelle, P. A., M. K. Wetherbee, and B. H. Olson.** 1991. Distribution of DNA sequences encoding narrow- and broad-spectrum mercury resistance. Appl. Environ. Microbiol. **57:** 1581–1589.
188. **Rojo, F., D. H. Pieper, K.-H. Engesser, H.-J. Knackmuss, and K. N. Timmis.** 1987. Assemblage of ortho cleavage route for simultaneous degradation of chloro- and methylaromatics. Science **238:**1395–1398.
189. **Rothmel, R. K., T. L. Aldrich, J. E. Houghton, W. M. Coco, L. N. Ornston, and A. M. Chakrabarty.** 1990. Nucleotide sequencing and characterization of *Pseudomonas putida catR*: a positive regulator of the *catBC* operon is a member of the LysR family. J. Bacteriol. **172:**922–931.
190. **Saint, C. P., N. C. McClure, and W. A. Venables.** 1990. Physical map of the aromatic amine and *m*-toluate catabolic plasmid pTDN1 in *Pseudomonas putida*: location of a unique *meta*-cleavage pathway. J. Gen. Microbiol. **136:**615–625.
191. **Sander, P., R.-M. Wittich, P. Fortnagel, H. Wilkes, and W. Francke.** 1991. Degradation of 1,2,4-trichloro- and 1,2,4,5-tetrachlorobenzene by *Pseudomonas* strains. Appl. Environ. Microbiol. **57:**1430–1440.
192. **Sangodkar, U. M. X., P. J. Chapman, and A. M. Chakrabarty.** 1988. Cloning, physical mapping and expression of chromosomal genes specifying degradation of the herbicide 2,4,5-T by *Pseudomonas cepacia* AC1100. Gene **71:**267–277.
193. **Saye, D. J., O. A. Ogunseitan, G. S. Sayler, and R. V. Miller.** 1990. Transduction of linked chromosomal genes between *Pseudomonas aeruginosa* strains during incubation in situ in a freshwater habitat. Appl. Environ. Microbiol. **56:**140–145.
194. **Sayler, G. S., J. Fleming, B. Applegate, C. Werner, and K. Nikbakht.** 1989. Microbial community analysis using environmental nucleic acid extracts, p. 658–662. *In* T. Hattori, Y. Ishida, Y. Maruyama, R. Y. Morita, and A. Uchida (ed.), Recent advantages in microbial ecology. Japan Scientific Societies Press, Kyoto, Japan.
195. **Sayler, G. S., S. W. Hooper, A. C. Layton, and J. M. H. King.** 1990. Catabolic plasmids of environmental and ecological significance. Microb. Ecol. **19:**1–20.
196. **Sayler, G. S., and A. C. Layton.** 1990. Environmental application of nucleic acid hybridization. Annu. Rev. Microbiol. **44:**625–648.
197. **Sayler, G. S., M. S. Shields, E. T. Tedford, A. Breen, S. W. Hooper, K. M. Sirotkin, and J. W. Davis.** 1985. Application of DNA-DNA colony hybridization to the detection of catabolic genotypes in environmental samples. Appl. Environ. Microbiol. **49:**1295–1303.
198. **Schell, M. A.** 1985. Transcriptional control of the *nah* and *sal* hydrocarbon-degradation operons by the *nahR* gene product. Gene **36:**301–309.
199. **Schell, M. A.** 1990. Regulation of naphthalene degradation genes of plasmid NAH7: example of a generalized positive control system in *Pseudomonas* and related bacteria, p. 165–177. *In* S. Silver, A. M. Chakrabarty, B. Iglewski, and S. Kaplan (ed.), *Pseudomonas*: biotransformations, pathogenesis, and evolving biotechnology. American Society for Microbiology, Washington, D.C.
200. **Schell, M. A., and M. Sukordhaman.** 1989. Evidence that the transcription activator encoded by the *Pseudomonas putida nahR* gene is evolutionarily related to the transcription activators encoded by the *Rhizobium nodD* genes. J. Bacteriol. **171:**1952–1959.
201. **Schlömann, M., P. Fischer, E. Schmidt, and H.-J. Knackmuss.** 1990. Enzymatic formation, stability, and spontaneous reactions of 4-fluoromuconolactone, a metabolite of the bacterial degradation of 4-fluorobenzoate. J. Bacteriol. **172:**5119–5129.
202. **Schlömann, M., D. H. Pieper, and H.-J. Knackmuss.** 1990. Enzymes of haloaromatics degradation: variations of *Alcaligenes* on a theme by *Pseudomonas*, p. 185–197. *In* S. Silver, A. M. Chakrabarty, B. Iglewski, and S. Kaplan (ed.), *Pseudomonas*: biotransformations, pathogenesis, and evolving biotechnology. American Society for Microbiology, Washington, D.C.
203. **Schlömann, M., E. Schmidt, and H.-J. Knackmuss.** 1990. Different types of dienelactone hydrolase in 4-fluorobenzoate-utilizing bacteria. J. Bacteriol. **172:**5112–5118.
204. **Schmidt, E., and H.-J. Knackmuss.** 1980. Chemical structure and biodegradability of halogenated aromatic compounds. Conversion of chlorinated muconic acids into maleoylacetic acid. Biochem. J. **192:**339–347.
205. **Schmidt, E., G. Remberg, and H.-J. Knackmuss.** 1980. Chemical structure and biodegradability of halogenated aromatic compounds. Halogenated muconic acids as intermediates. Biochem. J. **192:**331–337.
206. **Schneider, B., R. Müller, R. Frank, and F. Lingens.** 1991. Complete nucleotide sequences and comparison of the structural genes of two 2-haloalkanoic acid dehalogenases from *Pseudomonas* sp. strain CBS3. J. Bacteriol. **173:**1530–1535.
207. **Scholten, J. D., K.-H. Chang, P. C. Babbitt, H. Charest, M. Sylvestre, and D. Dunaway-Mariano.** 1991. Novel enzymic hydrolytic dehalogenation of a chlorinated aromatic. Science **253:**182–185.
208. **Schraa, G., M. L. Boone, M. S. M. Jetten, A. R. W. van Neerven, P. J. Colberg, and A. J. B. Zehnder.** 1986. Degradation of 1,4-dichlorobenzene by *Alcaligenes* sp. strain A175. Appl. Environ. Microbiol. **52:**1374–1381.
209. **Schreiner, A., K. Fuchs, F. Lottspeich, H. Poth, and F. Lingens.** 1991. Degradation of 2-methylaniline in *Rhodococcus rhodochrous*: cloning and expression of two clustered catechol 2,3-dioxygenase genes from strain CTM. J. Gen. Microbiol. **137:**2041–2048.
210. **Schütt, C.** 1989. Plasmids in the bacterial assemblage of a dystrophic lake: evidence for plasmid-encoded nickel resistance. Microb. Ecol. **17:**49–62.
211. **Schweizer, D., A. Markus, M. Seez, H. H. Ruf, and F. Lingens.** 1987. Purification and some properties of component B of the 4-chlorophenylacetate 3,4-dioxygenase from *Pseudomonas* species strain CBS3. J. Biol. Chem. **262:**9340–9346.
212. **Schwien, U., E. Schmidt, H.-J. Knackmuss, and W. Reineke.** 1988. Degradation of chlorosubstituted aromatic compounds by *Pseudomonas* sp. strain B13: fate of 3,5-dichlorocatechol. Arch. Microbiol. **150:**78–84.
213. **Scordilis, G. E., H. Ree, and T. G. Lessie.** 1987. Identification of transposable elements which activate gene expression in *Pseudomonas cepacia*. J. Bacteriol. **169:**8–13.

214. **Shaw, L. E., and P. A. Williams.** 1988. Physical and functional mapping of two cointegrate plasmids derived from RP4 and TOL plasmid pDK1. J. Gen. Microbiol. **134**:2463–2474.
215. **Shimp, R. J., and F. K. Pfaender.** 1987. Effect of adaptation to phenol on biodegradation of monosubstituted phenols by aquatic microbial communities. Appl. Environ. Microbiol. **53**:1496–1499.
216. **Shingler, V., J. Powlowski, and U. Marklund.** 1992. Nucleotide sequence and functional analysis of the complete phenol/3,4-dimethylphenol catabolic pathway of *Pseudomonas* sp. strain CF600. J. Bacteriol. **174**:711–724.
217. **Sinclair, M. I., P. C. Maxwell, B. R. Lyon, and B. W. Holloway.** 1986. Chromosomal location of TOL plasmid DNA in *Pseudomonas putida*. J. Bacteriol. **168**:1302–1308.
218. **Slightom, J. L., M. Durand-Tardif, L. Jouanin, and D. Tepfer.** 1986. Nucleotide sequence analysis of TL-DNA of *Agrobacterium rhizogenes* agropine type plasmid. J. Biol. Chem. **261**:108–121.
219. **Smit, E., and J. D. van Elsas.** 1990. Determination of plasmid transfer frequency in soil. Consequences of bacterial mating on selective agar media. Curr. Microbiol. **21**:151–157.
220. **Spain, J. C., and S. F. Nishino.** 1987. Degradation of 1,4-dichlorobenzene by a *Pseudomonas* sp. Appl. Environ. Microbiol. **53**:1010–1019.
221. **Spain, J. C., and P. A. van Veld.** 1983. Adaptation of natural microbial communities to degradation of xenobiotic compounds: effects of concentration, exposure, time, inoculum, and chemical structure. Appl. Environ. Microbiol. **45**:428–435.
222. **Spooner, R. A., K. Lindsay, and F. C. H. Franklin.** 1986. Genetic, functional and sequence analysis of the *xylR* and *xylS* regulatory genes of the TOL plasmid pWW0. J. Gen. Microbiol. **132**:1347–1358.
223. **Stainthorpe, A. C., V. Lees, G. P. C. Salmond, H. Dalton, and J. C. Murrell.** 1990. The methane monooxygenase gene cluster of *Methylococcus capsulatus* (Bath). Gene **91**:27–34.
224. **Steffan, R. J., and R. M. Atlas.** 1988. DNA amplification to enhance detection of genetically engineered bacteria in environmental samples. Appl. Environ. Microbiol. **54**:2185–2191.
225. **Steffan, R. J., and R. M. Atlas.** 1991. Polymerase chain reaction: applications in environmental microbiology. Annu. Rev. Microbiol. **45**:137–161.
226. **Steffan, R. J., A. Breen, R. M. Atlas, and G. S. Sayler.** 1989. Application of gene probe methods for monitoring specific microbial populations in freshwater ecosystems. Can. J. Microbiol. **35**:681–685.
227. **Stephens, G. M., J. M. Sidebotham, N. H. Mann, and H. Dalton.** 1989. Cloning and expression in *Escherichia coli* of the toluene dioxygenase gene from *Pseudomonas putida* NCIB11767. FEMS Microbiol. Lett. **57**:295–300.
228. **Stotzky, G., and H. Babich.** 1986. Survival of, and genetic transfer by, genetically engineered bacteria in natural environments. Adv. Appl. Microbiol. **31**:93–135.
229. **Streber, W. R., K. N. Timmis, and M. H. Zenk.** 1987. Analysis, cloning, and high-level expression of 2,4-dichlorophenoxyacetate monooxygenase gene *tfdA* of *Alcaligenes eutrophus*. J. Bacteriol. **169**:2950–2955.
230. **Suzuki, M., T. Hayakawa, J. P. Shaw, M. Rekik, and S. Harayama.** 1991. Primary structures of xylene monooxygenase: similarities to and differences from the alkane hydroxylation system. J. Bacteriol. **173**:1690–1695.
231. **Swindoll, C. M., C. M. Aelion, and F. K. Pfaender.** 1988. Influence of inorganic and organic nutrients on aerobic biodegradation and on the adaptation response of subsurface microbial communities. Appl. Environ. Microbiol. **54**:212–217.
232. **Taira, K., N. Hayase, N. Arimura, S. Yamashita, T. Miyazaki, and K. Furukawa.** 1988. Cloning and nucleotide sequence of the 2,3-dihydroxybiphenyl dioxygenase gene from the PCB-degrading strain of *Pseudomonas paucimobilis* Q1. Biochemistry **27**:3990–3996.
233. **Taira, K., J. Hirose, S. Hayashida, and K. Furukawa.** 1992. Analysis of *bph* operon from the polychlorinated biphenyl-degrading strain of *Pseudomonas pseudoalcaligenes* KF707. J. Biol. Chem. **267**:4844–4853.
234. **Tautz, D., M. Trick, and G. A. Dover.** 1986. Cryptic simplicity in DNA is a major source of genetic variation. Nature (London) **322**:652–656.
235. **Thomas, A. W., J. H. Slater, and A. J. Weightman.** 1992. The dehalogenase genes *dehI* from *Pseudomonas putida* PP3 is carried on an unusual mobile genetic element designated *DEH*. J. Bacteriol. **174**:1932–1940.
236. **Thomas, A. W., A. W. Topping, J. H. Slater, and A. J. Weightman.** 1992. Localization and functional analysis of structural and regulatory dehalogenase genes carried on *DEH* from *Pseudomonas putida* PP3. J. Bacteriol. **174**:1941–1947.
237. **Timmis, K. N., F. Rojo, and J. L. Ramos.** 1990. Design of new pathways for the catabolism of environmental pollutants. Adv. Appl. Biotechnol. **4**:61–82.
238. **Tomasek, P. H., B. Frantz, U. M. X. Sangodkar, R. A. Haugland, and A. M. Chakrabarty.** 1989. Characterization and nucleotide sequence determination of a repeat element isolated from a 2,4,5-T degrading strain of *Pseudomonas cepacia*. Gene **76**:227–238.
239. **Trevors, J. T., T. Barkay, and A. W. Bourquin.** 1987. Gene transfer among bacteria in soil and aquatic environments: a review. Can. J. Microbiol. **33**:191–198.
240. **Trevors, J. T., and K. M. Oddie.** 1986. R-plasmid transfer in soil and water. Can. J. Microbiol. **32**:610–614.
241. **True, A. E., A. M. Orville, L. L. Pearce, J. D. Lipscomb, and L. Que.** 1990. An EXAFS study of the interaction of substrate with the ferric active site of protocatechuate 3,4-dioxygenase. Biochemistry **29**:10847–10854.
242. **Tsuda, M., and T. Iino.** 1987. Genetic analysis of a transposon carrying toluene degrading genes on a TOL plasmid pWW0. Mol. Gen. Genet. **210**:270–276.
243. **Tsuda, M., and T. Iino.** 1988. Identification and characterization of Tn*4653*, a transposon covering the toluene transposon Tn*4651* on TOL plasmid pWW0. Mol. Gen. Genet. **213**:72–77.
244. **Tsuda, M., and T. Iino.** 1990. Naphthalene degrading genes on plasmid NAH7 are on a defective transposon. Mol. Gen. Genet. **223**:33–39.
245. **Tsuda, M., K.-I. Minegishi, and T. Iino.** 1989. Toluene transposons Tn*4651* and Tn*4653* are class II transposons. J. Bacteriol. **171**:1386–1393.
246. **van der Meer, J. R.** Unpublished results.
247. **van der Meer, J. R., R. I. L. Eggen, A. J. B. Zehnder, and W. M. de Vos.** 1991. Sequence analysis of the *Pseudomonas* sp. strain P51 *tcb* gene cluster, which encodes metabolism of chlorinated catechols: evidence for specialization of catechol 1,2-dioxygenases for chlorinated substrates. J. Bacteriol. **173**: 2425–2434.
248. **van der Meer, J. R., A. C. J. Frijters, J. H. J. Leveau, R. I. L. Eggen, A. J. B. Zehnder, and W. M. de Vos.** 1991. Characterization of the *Pseudomonas* sp. strain P51 gene *tcbR*, a LysR-type transcriptional activator of the *tcbCDEF* chlorocatechol oxidative operon, and analysis of the regulatory region. J. Bacteriol. **173**:3700–3708.
249. **van der Meer, J. R., W. Roelofsen, G. Schraa, and A. J. B. Zehnder.** 1987. Degradation of low concentrations of dichlorobenzenes and 1,2,4-trichlorobenzene by *Pseudomonas* sp. strain P51 in nonsterile soil columns. FEMS Microbiol. Ecol. **45**:333–341.
250. **van der Meer, J. R., A. R. W. van Neerven, E. J. de Vries, W. M. de Vos, and A. J. B. Zehnder.** 1991. Cloning and characterization of plasmid-encoded genes for the degradation of 1,2-dichloro-, 1,4-dichloro-, and 1,2,4-trichlorobenzene of *Pseudomonas* sp. strain P51. J. Bacteriol. **173**:6–15.
251. **van der Meer, J. R., A. J. B. Zehnder, and W. M. de Vos.** 1991. Identification of a novel composite transposable element, Tn*5280*, carrying chlorobenzene dioxygenase genes of *Pseudomonas* sp. strain P51. J. Bacteriol. **173**:7077–7083.
252. **van der Ploeg, J., G. van Hall, and D. B. Janssen.** 1991. Characterization of the haloacid dehalogenase from *Xanthobacter autotrophicus* GJ10 and sequencing of the *dhlB* gene. J. Bacteriol. **173**:7925–7933.
253. **van Elsas, J. D., J. T. Trevors, and M. E. Starodub.** 1988. Bacterial conjugation between pseudomonads in the rhizo-

sphere of wheat. FEMS Microbiol. Ecol. **53**:299–306.
254. **Wackett, L. P., and D. T. Gibson.** 1988. Degradation of trichloroethylene by toluene dioxygenase in whole-cell studies with *Pseudomonas putida* F1. Appl. Environ. Microbiol. **54**: 1703–1708.
255. **Wagner-Döbler, I., R. Pipke, K. N. Timmis, and W. F. Dwyer.** 1992. Evaluation of aquatic sediment microcosms and their use in assessing possible effects of introduced microorganisms on ecosystem parameters. Appl. Environ. Microbiol. **58**:1249–1258.
256. **Walia, S., A. Khan, and N. Rosenthal.** 1990. Construction and applications of DNA probes for detection of polychlorinated biphenyl-degrading genotypes in toxic organic-contaminated soil environments. Appl. Environ. Microbiol. **56**:254–259.
257. **Weijer, W. J., J. Hofsteenge, J. M. Vereijken, P. A. Jekel, and J. J. Beintema.** 1982. Primary structure of *p*-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens*. Biochim. Biophys. Acta **704**:385–388.
258. **Weisburg, W. G., S. M. Barns, D. A. Pelletier, and D. J. Lane.** 1991. 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. **173**:697–703.
259. **Weisshaar, M.-P., F. C. H. Franklin, and W. Reineke.** 1987. Molecular cloning and expression of the 3-chlorobenzoate-degrading genes from *Pseudomonas* sp. strain B13. J. Bacteriol. **169**:394–402.
260. **Weller, R., J. W. Weller, and D. M. Ward.** 1991. 16S rRNA sequences of uncultivated hot spring cyanobacterial mat inhabitants retrieved as randomly primed cDNA. Appl. Environ. Microbiol. **57**:1146–1151.
261. **Wickham, G. S., and R. M. Atlas.** 1988. Plasmid frequence fluctuations in bacterial populations from chemically stressed soil communities. Appl. Environ. Microbiol. **54**:2192–2196.
262. **Williams, P. A., S. J. Assinder, and L. E. Shaw.** 1990. Construction of hybrid *xylE* genes between the two duplicate homologous genes from TOL plasmid pWW53: comparison of the kinetic properties of the gene products. J. Gen. Microbiol. **136**:1583–1589.
263. **Williams, P. A., and K. Murray.** 1974. Metabolism of benzoate and the methylbenzoates by *Pseudomonas putida* (*arvilla*) mt-2: evidence for the existence of a TOL plasmid. J. Bacteriol. **120**:416–423.
264. **Williams, P. A., and M. J. Worsey.** 1976. Ubiquity of plasmids in coding for toluene and xylene metabolism in soil bacteria: evidence for the existence of new TOL plasmids. J. Bacteriol. **125**:818–828.
265. **Wilson, J. T., C. G. Enfield, W. J. Dunlap, R. L. Cosby, D. A. Foster, and L. B. Baskin.** 1981. Transport and fate of selected organic pollutants in a sandy soil. J. Environ. Qual. **10**:501–506.
266. **Wood, M. S., C. Lory, and T. G. Lessie.** 1990. Activation of the *lac* genes of Tn*951* by insertion sequences from *Pseudomonas cepacia*. J. Bacteriol. **172**:1719–1724.
267. **Wyndham, R. C., R. K. Singh, and N. A. Straus.** 1988. Catabolic instability, plasmid gene deletion and recombination in *Alcaligenes* sp. BR60. Arch. Microbiol. **150**:237–243.
268. **Yen, K.-M., M. R. Karl, L. M. Blatt, M. J. Simon, R. B. Winter, P. R. Fausset, H. S. Lu, A. A. Harcourt, and K. K. Chen.** 1991. Cloning and characterization of a *Pseudomonas mendocina* KR1 gene cluster encoding toluene-4-monooxygenase. J. Bacteriol. **173**:5315–5327.
269. **Yen, K.-M., and C. M. Serdar.** 1988. Genetics of naphthalene catabolism in pseudomonads. Crit. Rev. Microbiol. **15**:247–268.
270. **You, I.-S., D. Ghosal, and I. C. Gunsalus.** 1991. Nucleotide sequence analysis of the *Pseudomonas putida* PpG7 salicylate hydroxylase gene (*nahG*) and its 3′-flanking region. Biochemistry **30**:1635–1641.
271. **Zafarullah, M., D. Charlier, and N. Glansdorff.** 1981. Insertion of IS*3* can "turn-on" a silent gene in *Escherichia coli*. J. Bacteriol. **146**:415–417.
272. **Zeph, L. R., M. A. Onaga, and G. Stotzky.** 1988. Transduction of *Escherichia coli* by bacteriophage P1 in soil. Appl. Environ. Microbiol. **54**:1733–1737.
273. **Zhou, L., K. N. Timmis, and J. L. Ramos.** 1990. Mutations leading to constitutive expression from the TOL plasmid *meta*-cleavage pathway operon are located at the C-terminal end of the positive regulator protein XylS. J. Bacteriol. **172**: 3707–3710.
274. **Zylstra, G. J., and D. T. Gibson.** 1989. Toluene degradation by *Pseudomonas putida* F1. J. Biol. Chem. **264**:14940–14946.
275. **Zylstra, G. J., W. R. McCombie, D. T. Gibson, and B. A. Finette.** 1988. Toluene degradation by *Pseudomonas putida* F1: genetic organization of the *tod* operon. Appl. Environ. Microbiol. **54**:1498–1503.
276. **Zylstra, G. J., R. H. Olsen, and D. P. Ballou.** 1989. Cloning, expression, and regulation of the *Pseudomonas cepacia* protocatechuate 3,4-dioxygenase genes. J. Bacteriol. **171**:5907–5914.
277. **Zylstra, G. J., R. H. Olsen, and D. P. Ballou.** 1989. Genetic organization and sequence of the *Pseudomonas cepacia* genes for the alpha and beta subunits of protocatechuate 3,4-dioxygenase. J. Bacteriol. **171**:5915–5921.