Instituto Juan March
de Estudios e Investigaciones

39 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Molecular Basis for Biodegradation of Pollutants

Organized by

K. N. Timmis and J. L. Ramos

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INTRODUCTION

Kenneth N. Timmis and Juan L. Ramos

The workshop titled "Molecular Basis for Biodegradation of Pollutants" was organized to discuss the mechanisms microbes use to mineralize organic pollutants, and to define the state of the present knowledge as a starting point to design future research. This was addressed through five independent sessions, namely, 1) The Ecology of Biodegradative Microbes provided insights on the habitats and behavior of microbes in different niches and under different environmental conditions. The participants explained how and why a microbe colonizes a given ecosystem. 2) The Biochemistry of Catabolic Pathways examined the mechanisms microbes use to remove substituents on aromatic rings, and how the aromatic ring is activated and then cleaved. This session was essential to understand key metabolic steps that prevent the metabolism of certain compounds. 3) The session Genetics of Catabolic Pathways discussed how genes are organized and which genes encode different enzymatic activities. Plasmid- and chromosomal-borne pathways are critical to our understanding of the spread of catabolic pathways in nature. 4) The session on Regulation of Catabolic Genes was aimed at elucidating which signals are critical for the synthesis of the enzymes of a given catabolic pathway; the participants discussed the circuits and functions microbes use to avoid cross-noise. 5) The session on Molecular Evolution and Design of Enzymes and Pathways looked at how specific proteins can be manipulated to expand their catabolic potential, and how different genes from different pathways can be assembled to create new pathways for the elimination of recalcitrant compounds. The evolution (at the molecular level) of genes for the degradation of aromatic carboxylic acids was also addressed.

In all, the sessions allowed the participants to review the state of the art and use this workshop as a springboard to new science in this exciting area of research, which has important social connotations.

Juan L. Ramos

Kenneth N. Timmis

FIRST SESSION: MICROBIAL ECOLOGY IN BIODEGRADATION

Chairperson: Peter A. Williams

MICROBES VORACIOUS APPETITE FOR BTX

James M. Tiedje, Joanne Chee-Sanford, Marcos Fries and Jizhong Zhou. Michigan State University, East Lansing, MI USA.

BTX (benzene, toluene and xylene) is one of the two most common classes of hazardous groundwater pollutants. Microorganisms that aerobically degrade these chemicals are common and a few strains with this capability have been well studied. What is not known is the extent of physiologic and kinetic diversity that exists for metabolism of this class of chemicals. If such diversity exists, it should help insure that BTX is more effectively degraded in the diverse niches in nature. We have characterized a new class of organism, *Azoarcus tolulyticus*, which is a widely distributed, N_2-fixing, denitrifying, anaerobic, toluene-degrader. This anaerobic toluene-degrader is not tolerant of toluene concentrations above 50 ppm and will not grow well on complex media, which may explain why it has been overlooked before. The type strain of this species, Tol-4, appears to metabolize toluene by activating the ring by an acetyl CoA attack on the methyl group forming hydrocinnamoyl CoA, cinnamoyl CoA and benzoate in a mineralization pathway. *Azoarcus* strains examined are also capable of aerobic toluene metabolism and some hybridize to probes from the known aerobic toluene pathway genes. We have also examined the prevalence of the gene sequences for the 5 aerobic toluene pathways in a wide variety of aerobic toluene degraders. Nearly 1/2 of the strains hybridized to the ortho-monohydroxylase probe while only a few percent hybridized to the other 4 pathway probes. This is particularly interesting because this probe is from the best known TCE co-oxidizing organism. We have also examined the kinetic diversity of aerobic benzene degraders since effective degradation of benzene at ppb levels is essential for meeting regulatory targets which in some cases is as low as 1 ppb. We have found a wide range of Km and Vmax values for benzene for organisms from nature. Particularly interesting are two strains which have a high Vmax/Km ratio and rapidly reduce benzene to concentrations below 1 ppb. This kinetic diversity suggests that the microbial community is well equipped to metabolize benzene at a wide variety of concentrations.

Biological Treatment Systems For Remediation And Pollution Control

Ronald Unterman

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The release of organic compounds into our environment poses one of the most significant problems confronting our society today. These problems include both historical contamination that requires remediation, as well as the current production and release of industrial chemicals. These compounds include solvents, pesticides, wood preservatives, fire retardent fluids, pulp and paper wastes, and industrial byproducts. This seminar will present the status of current studies into the development of new and more effective biocatalysts with emphasis on their application in pilot and commercial biotreatment systems for soil, water, and air emissions. Specific examples will include the bioremediation of PCB and TNT contaminated soils and sediments, and TCE, nitrobenzene and aniline contaminated ground water, as well as biotreatment systems for chlorinated solvents in industrial waste water and VOCs in industrial air effluents. To date, field system development has focused on the use of superior, naturally occurring strains under optimized process conditions, however, future applications will include the use of genetically engineered microorganisms in bioreactors and eventually in ground water and field applications.

SULFATE STARVATION-INDUCED REGULATION OF GENE EXPRESSION IN GRAM-NEGATIVE BACTERIA.

Thomas Leisinger, Michael Kertesz, Alasdair Cook, Stefan Beil, Jürgen Ragaller, Martina Weiss, and Jan van der Ploeg, Mikrobiologisches Institut, Eidgenössische Technische Hochschule, ETH-Zentrum, CH-8092 Zürich, Switzerland.

Bacteria growing in soil environments obtain their sulfur for growth almost entirely in the form of organically bound sulfur, and not as inorganic sulfate. We have recently observed that under sulfate starvation conditions bacteria produce a set of proteins whose synthesis is repressed by sulfate or cysteine (1). The identity of these sulfate-repressed proteins is under investigation in *Pseudomonas aeruginosa* PAO and in *E. coli*. The methods used include determination of the N-termini of proteins purified by 2D-polyacrylamide electrophoresis, enzyme activity measurements, and - in *E. coli* - screening of translational *lacZ*-fusions in the chromosome for sulfate starvation-regulated expression of β-galactosidase fusion proteins.

In *P. aeruginosa* 12 proteins are upregulated under conditions of sulfate-starvation, but none of these could be correlated to a number of enzyme acitivities involved in the assimilation of non-sulfate sulfur sources. Arylsulfatase, however, proved to be coregulated with the sulfate starvation-induced proteins and thus was used as a marker enzyme. We have purified and characterized this enzyme from strain PAO, and have cloned and sequenced its structural gene *atsA*. The AtsA protein was 38% identical to the arylsulfatase from *Klebsiella aerogenes*. In contrast to other authors, we have found no evidence for multiple forms of the enzyme or the *atsA* gene in *P. aeruginosa*. Transposon insertion mutants of *P. aeruginosa* were generated which synthesize arylsulfatase at high levels in the

presence of sulfate, and these are being used to explore sulfate regulation of *atsA* expression.

Seven sulfate starvation-induced proteins from *E. coli* were purified by preparative 2D-polyacrylamide gel electrophoresis, and their N-terminal sequences were determined. Five of the sequences obtained were novel, and two were identified by comparison with computer databases as sulfate-binding protein and cysteine synthase. The latter was also present after growth with sulfate, but was slightly upregulated under sulfate-limitation. Three sulfate-regulated translational *lacZ*-fusions in the *E. coli* chromosome were also analyzed. Sequencing of the DNA-regions upstream of the insertion sites revealed two sequences whose function is unknown as well as dmsA, the gene encoding the catalytic subunit of dimethyl sulphoxide reductase. Together with the largely novel N-terminal sequences found for the sulfate starvation-regulated proteins excised from 2D-gels, it appears that we have discovered a group of genes which is not expressed under routine laboratory conditions, and whose members are therefore as yet quite uncharacterized.

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Anaerobic Mineralization of Mineral Oil in a Contaminated Aquifer, in Aquifer Columns and in Microbial Cultures

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In situ bioremediation of aquifers contaminated with mineral oil is considered to be a promising concept. The technique is based on the stimulation of the microbial transformation of pollutants and ideally leads to complete mineralization of the contaminants. However, particularly in anaerobic zones of the aquifer, the efficiency of this concept is poorly documented. The first objective of our study was to define a network of microbial, geochemical and physical processes that allows to quantify the mineral oil degradation in a polluted aquifer and in laboratory aquifer columns. The second goal was to study the enzymatic steps involved in the denitrifying microbial metabolism of selected components of mineral oil.

The study site consisted of a mineral oil contaminated aquifer which was bioremediated by recirculating ground water supplemented with O_2, NO_3^-, NH_4^+ and PO_4^{3-} to stimulate the activity of the indigenous microbial populations. Aquifer samples were examined by using the MPN-method and $2.3 \cdot 10^6$, $150 \cdot 10^6$, and $2.3 \cdot 10^6$ culturable cells g^{-1} were found under aerobic, denitrifying- and sulfate-reducing conditions, respectively. In the contaminated zone, O_2 and NO_3^- were consumed and elevated concentrations of total inorganic carbon, NO_2^-, N_2O, Mn^{2+}, Fe^{2+} and CH_4 were observed. The isotopic ratio ($\delta^{13}C$) of the inorganic carbon in the groundwater decreased from -12‰ in the uncontaminated zone to -17‰ in the contaminated zone. This indicates that the inorganic carbon originates mainly from the mineralization of mineral oil ($\delta^{13}C$ = -28‰) and only partially from the dissolution of carbonate ($\delta^{13}C$ = +1‰). The presence of sulfate reducing bacteria and the evolution of methane suggest that the aquifer is inhomogeneous and includes reduced niches with very low redox potentials. The mineralization processes in the aquifer were simulated in aerobic and denitrifying laboratory aquifer columns which were filled with aquifer material (<4.5 mm) from the contaminated zone. In these columns, production rates of total inorganic carbon and consumption rates of O_2 and NO_3^-,

respectively, were within the same order of magnitude as the rates observed in the field. However, a production of CH_4 was not observed which can be explained by the homogeneity of the column matrix and the absence of niches with low redox potentials. The balance of microbial, geochemical and physical processes involved in the mineralization of mineral oil was examined by calculating electron and alkalinity balances and it was clearly demonstrated that mineral oil is efficiently mineralized in the polluted subsurface.

The denitrifying microbial metabolism of some selected components of mineral oil was studied in enrichment cultures and under pure culture conditions. The mineralization of toluene, *m*-xylene, *p*-xylene, ethylbenzene, naphthalene and hexadecane was confirmed by electron and carbon mass balances. Detailed biochemical studies with a pure strain (*Pseudomonas* sp. strain T) revealed that the anaerobic degradation of toluene was initiated by direct oxidation of the methyl group. Benzaldehyde and benzoate accumulated sequentially after addition of toluene when cell suspensions were incubated at 5°C. In addition the methyl group oxidizing enzyme system of strain T also catalyzed the oxidation of each isomer of the chlorotoluenes and fluorotoluenes to the corresponding halogenated benzoate derivatives. During anaerobic growth with toluene strain T produced two transformation products: benzylsuccinate and benzylfumarate. About 0.5% of the toluene carbon was converted to these products. In addition, pure cultures isolated from the contaminated zone of the aquifer are phylogenetically characterized on the basis of 16S rRNA sequence comparison. Oligonucleotide probes will subsequently be used to determine the significance of these strains in the field.

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SECOND SESSION: BIOCHEMISTRY OF CATABOLIC PATHWAYS

Chairperson: L. Nicholas Ornston

Biodegradation of crude oil

Shigeaki Harayama*, Haruhisa Toki, Shoko Komukai, Kasthuri Venkateswaran, Yukie Yamauchi, Keiji Sugiura, Masayoshi Asaumi, Toshitsugu Shimauchi and Masami Ishihara, Marine Biotechnology Institute, Kamaishi Laboratories, 3-75-1 Heita, Kamaishi City, 026 Iwate, Japan

Contamination by oil spills occurs in marine, coastal and inland areas. Crude oil-degrading microorganisms were isolated in order to evaluate the biodegradability of crude oil and to use them into bioremediation of spilled oil. Many enriched cultures of mixed population removed 50% of the saturate fraction and 20% of the aromatic fraction of Arabian light crude oil. Marine bacteria which can grow on either *n*-tetradecane, pristane or *n*-propylbenzene were isolated from the enriched cultures. Taxonomic studies identified an *n*-tetradecane-degrading bacterium (strain T4) as *Acinetobacter* sp., a pristane-degrading bacterium (strain PR4) as *Rhodococcus* sp. and a *n*-propylbenzene-degrading bacterium (strain PB4) as *Pseudomonas putida*. *Acinetobacter* sp. T4 degraded *n*-alkanes of C_7 to C_{20}. Pristane was also decomposed by this strain. *Rhodococcus* sp. PR4 exhibited a broad specificity of hydrocarbon degradation similar to *Acinetobacter* sp. T4. The alkane-degrading enzymes were constitutively expressed in srain PR4. This strain produced a biosurfactant. *P. putida* PB4 degraded benzene and C_1-, C_3- and C_4-alkylbenzenes. *Rhodococcus* sp. PR4 and *Acinetobacter* sp. T4 exhibited a growth on crude oil while *P. putida* PB4 does not. A culture constituted from these three strains degraded and mineralized crude oil as efficiently as oil-degrading cultures enriched from natural population.

The degradation of four different crude oils, namely Arabian light, Dubai, Maya and Shengli crude oils, by *Acinetobacter* sp. T4 or by a mixed population of oil-degrading microorganisms was examined. The biodegradation of the crude oils by both strain T4 and

the mixed population was found to be higher in the order of Arabian light > Dubai > Shengli > Maya. The mixed population exhibited better degradation than strain T4 of all four crude oils. The biodegradability of the crude oils was correlated with the sum of their contents of saturates and aromatics, but inversely related to their viscosity. When the saturate- and aromatic fractions of biodegraded oils were analyzed by fractionating with a column chromatography, it was found that the saturate fraction of all the four crude oils were more susceptible to biodegradation than their aromatic fraction. Individual and groups of compounds in these fractions were further analyzed by a gas chromatography, a gas chromatography-mass spectrometry, a field desorption mass spectrometry and a nuclear magnetic resonance spectrometry.

The resin fraction of crude oil is hardly biodegraded in the natural environment; however, *P. putida* PB4 and a newly isolated *Pseudomonas* sp. UN3-3 degraded the resin fraction. These bacteria also transformed tetralin to 3,4-dihydro-1(2H)naphthalenone and 1,2,3,4-tetrahydro-1-naphthol. The possibility that tetralin could be served as a model compound of resins is currently investigated.

BACTERIAL DEGRADATION OF NITROAROMATIC COMPOUNDS UNDER AEROBIC CONDITIONS

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Reductive transformation of nitroaromatic compounds by bacteria has been widely reported. In contrast, only a few strains able to mineralize nitro compounds have been reported. The most common strategy used by bacteria seems to involve an initial oxidative attack with subsequent release of the nitro group as nitrite. Two mechanisms have been demonstrated for the oxidative removal of the nitro group. Monooxygenase enzymes catalyze the insertion of one atom of molecular oxygen with elimination of nitrite from a variety of nitrophenols. Recent evidence strongly indicates that one mole of NADH is required for the reaction and that the product of the reaction is a benzoquinone which is subsequently reduced to form a diphenol. The second mechanism involves the dioxygenase catalyzed insertion of two atoms of molecular oxygen on the ring of less polar compounds such as 2-nitrotoluene, 1,3-dinitrobenzene, and 2,4-dinitrotoluene. The proposed product of the reaction is a dihydroxy nitro cyclohexadiene which is thought to spontaneously rearomatize with elimination of nitrite. Recent evidence indicates that the dioxygenases involved in the removal of the nitro group may be closely related to naphthalene dioxygenase. The diphenols resulting from either monooxygenase or dioxygenase catalyzed reactions are subsequently degraded by well established pathways of aromatic metabolism.

Nitrobenzene can be degraded by either oxidative or reductive pathways. A gram positive isolate uses an initial dioxygenation as described above to produce catechol and remove the nitro group as nitrite. In contrast, a strain of Pseudomonas pseudoalcaligenes reduces the nitro group to form hydroxylaminobenzene, which is converted to 2-aminophenol by an unusual Bamberger type of internal rearrangement. The ring of 2-aminophenol is opened by a dioxygenase mechanism analogous to that catalyzed by catechol 2,3-dioxygenase. Subsequent reactions release ammonia and allow the ring fission product to serve as a source of carbon. This unusual pathway provides significant advantages over the pathways initiated by oxygenase enzymes. The oxygen and NADH requirements are considerably lower and the nitrogen is released as ammonia rather than nitrite.

Results presented here and other recent research has demonstrated that a variety of nitroaromatic compounds are amenable to biodegradation by aerobic bacteria.

DEGRADATION OF DIOXIN-LIKE COMPOUNDS

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Unsubstituted dibenzo-p-dioxin (DBD) and dibenzofuran (DBF) are good carbon sources for some bacteria. The complete mineralization occurs not only in liquid cultures but also in soil samples if the conditions like water content, pH, temperature, and cell concentration are optimized. Like diphenylether, dibenzo-p-dioxin and dibenzofuran are attacked by a dioxygenase at a carbon atom forming the ether bridge and an unsubstituted neighbouring carbon. This angular dioxygenation gives rise to a hemiacetal structure which is unstable and spontaneously decomposes. All subsequent degradation steps of the originating hydroxylated derivatives follow established pathways.

The initial dioxygenase will also accept DBD and DBF substituted with halogen atoms or other substitutents. Here the numbers, positions, and the nature of the substituent are decisive. For example in the case of 2,3-dichloro-dibenzofuran degradation halts at the stage of dichloro-salicylate as dead end product. However, other bacteria exist which can utilize this dead end product as a carbon source and thus can be used for its complete mineralisation.

Some reports state that bacteria degrade even highly chlorinated dioxins. In all cases, however, these conclusions are based on finding that no dioxins were measurable in extracts of treated samples. In no case was the stoichometric conversion of the original molecules into metabolites or CO_2 demonstrated. The presumptive bacterial

activities have not thus far been rigorously proven. We have chosen a different approach to eliminate highly chlorinated derivatives. Initially dehalogenation is achieved by eletroreduction of pure substances, aritifcial mixtures, or extracts of real-life samples. Subsequently, the unsubstituted or monohalogenated products are treated with our bacteria with degradative capacity. This procedure results in complete destruction of highly chlorinated DBDs and DBFs. It is still hampered by the fact that the medium for the electrolysis is methanol which must be eliminated in an additional step before the products can be transferred into the bacterial cultures.

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Current Aspects on Polycyclic Aromatic Hydrocarbon Biodegradation Processes. Dr. Carl E. Cerniglia, National Center for Toxicological Research, Jefferson, AR 72079

Given the myriad of natural sources of polycyclic aromatic hydrocarbons (PAHs) in the environment, it follows that over eons many microorganisms would adapt toward the use and degradation of these naturally occurring and anthropogenic compounds. Accordingly, many bacterial, fungal and algal strains have been shown to degrade a wide variety of PAHs containing from two to five aromatic rings. The microbial degradation of PAHs has been studied and the biochemical pathways and mechanisms of enzymatic oxidation have been elucidated in many laboratories. Current aspects of these biodegradative pathways will be presented.

In general, high molecular weight PAHs are slowly degraded by indigenous microorganisms and may persist in soils and sediments. The recalcitrance of these priority pollutants is due in part to the strong adsorption of higher molecular PAHs to soil organic matter and in part to low solubility, which decreases their bioavailability for microbial degradation.

There are fundamental differences in the mechanisms of PAH metabolism used by microorganisms. Bacteria and some green algae initiate the oxidation of PAHs by incorporating both atoms of molecular oxygen, catalyzed by a dioxygenase, into the aromatic ring to produce a *cis*-dihydrodiol, which is then dehydrogenated to give a catechol. Since PAHs. such as phenanthrene, pyrene, benzo[a]pyrene and benz[a]anthracene, are complex fused ring structures, bacteria metabolize PAHs at multiple sites to form isomeric *cis*-dihydrodiols.

Further metabolism of *cis*-dihydrodiols by bacteria involved NAD^+ - dependent dehydrogenation reactions that produce catechols. This is an important step in the catabolism of PAHs since ring-fission dioxygenases cleave the aromatic ring to give aliphatic intermediates. These intermediates funnel into central pathways of metabolism, which can then be oxidized to provide cellular energy or for biosynthesis of cell constituents.

In contrast to bacteria, non-ligninolytic fungi and prokaryotic algae (cyanobacteria) metabolize PAHs in pathways that are generally similar to those used by mammalian enzyme systems. Many fungi oxidize PAHs via a cytochrome P-450 monooxygenase by incorporating one atom of the oxygen molecule into the PAH to form an arene oxide and the other into water. The monooxygenases, like the bacterial aromatic ring dioxygenases, are complex, multi-component systems, usually membrane-bound with broad substrate specificities. Most arene oxides are unstable and can undergo either enzymatic hydration via epoxide hydrolase to form *trans*-dihydrodiols or non-enzymatic rearrangement to form phenols, which can be conjugated with glucose, sulfate, xylose or glucuronic acid. A diverse group of non-ligninolytic fungi have the ability to oxidize PAHs to *trans*-dihydrodiols, phenols, tetralones, quinones, dihydrodiol epoxides and various conjugates of the hydroxylated intermediates, but only a few have the ability to degrade PAHs to CO_2. The best studied non-ligninolytic fungus is the zygomycete, *Cunninghamella elegans*, which metabolizes PAHs from 2 to 5 aromatic rings. Like most fungi, *C. elegans* doesn't utilize PAHs as the sole source of carbon and energy but biotransforms or cometabolizes these recalcitrant compounds to detoxified products. Although *C. elegans* metabolizes PAHs in a manner similar to mammalian systems, there are differences in the regio-

and stereospecificities of the fungal and mammalian enzymes. The bacterial metabolic pathways have shown a greater tendency toward detoxification than the bioactivation pathways found more commonly in mammals.

The ligninolytic fungi which cause white-rots on wood have been screened for their PAH degrading ability when grown under ligninolytic and non-ligninolytic culture conditions. The best studied strain *Phanerochaete chrysosporium* has been used as a model organism to study the potential of white-rot fungi to degrade PAHs. The initial attack on PAHs by *P. chrysosporium* under culture conditions which favor ligninolysis is catalyzed by extracellular enzymes known as lignin peroxidase and manganese peroxidase as well as by other H_2O_2-producing peroxidases. Ligninolytic enzymes have been shown *in vitro* to oxidize PAHs with ionization potentials less than 7.35 to 7.55 ev. Lignin peroxidases in the presence of H_2O_2 can abstract one electron from a PAH molecule, such as anthracene, pyrene, and benzo[a]pyrene, thus creating an aryl cation radical, which undergoes further oxidation to form quinones. Other white-rot fungi, such as *Trametes versicolor* and *Bjerkandera* sp. have also been evaluated and show promise for their ability to degrade PAHs to CO_2 more rapidly than *P. chrysosporium*.

Cyanobacteria (blue-green algae) and eukaryotic algae oxidize PAHs under photoautotrophic conditions to form hydroxylated intermediates. Cyanobacteria oxidize naphthalene and phenanthrene to metabolites that are similar to those formed by mammals and fungi. In contrast, the green alga *Selenastrum capricornutum* oxidizes benzo[a]pyrene to isomeric *cis*-dihydrodiols similar to bacterial metabolites. Although the level of metabolism is extremely low, both prokaryotic and eukaryotic algae could be important in the overall degradation of PAHs, since they occur in high abundance and are widely distributed in aquatic ecosystems.

All of these studies indicate that the microbial world has developed a wide variety of mechanisms to biodegrade PAHs. However, the specific, fundamental differences in catabolic machinery between microorganisms can be factors to consider in the bioremediation of PAH contaminated soils and waste water systems when the objective is detoxification in addition to removal of the parent compound. It is clear that further studies are necessary to characterize the enzymes and mechanisms involved in the mineralization of high molecular weight PAHs.

APPLICATIONS OF BIODEGRADATIVE ENZYMES IN ORGANIC SYNTHESIS

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Alkane hydroxylase and xylene monooxygenase are non-heme iron monooxygenases, which enable Pseudomonads to grow on alkanes and aromatics. These enzymes oxidize terminal methyl groups and terminal double bonds of alkanes, ring carbons in cyclic alkanes, and alkyl substituents of substituted aromatic and heterocyclic rings. In addition to highly *regio*- and *substituent* specific oxidations, oxidation of terminal double bonds occurs *stereo* specifically to produce chiral epoxides which are potentially useful synthons. Both biocatalysts have wide substrate ranges, so that several dozen substrates can be oxidized stereo- and regioselectively, something that is quite difficult to do with conventional chemistry. Hence the biotechnological potential of these enzyme systems.

The enzymology of the two enzyme systems is partially established and bioconversions can be carried out *in vivo* and *in vitro*. For now, cells growing continuously or in fed-batch processes in two-liquid phase media are best suited for such bioconversions. We have maximized cell growth in such media, reaching cell densities of 40 g/l (dry mass) for *E. coli* recombinants and 100 g/l (dry mass) for *Pseudomonas* strains. In continuous cultures, cell densities between 3 - 12 g/l (dry mass) can be maintained.

The above processes will be illustrated by our work on the production of chiral styrene epoxides, chiral epoxyalkanes, terminal alkanols and alkanoic acids. Down stream processing of such products from the separated phases is now being developed.

For some of these bioconversions, productivities equivalent to 3-5 tons/m^3•yr have been attained. These productivities are beginning to be attractive from an industrial point of view, and several industries have in fact begun exploring biological oxidations for the production of chemicals.

Maleylacetate reductase functioning in the degradation of chloroaromatics

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Introduction

Widespread use of chlorinated organic compounds gives rise to persistent problems in the environment, particularly because many of these substances are resistant to biological degradation. Aerobic bacteria use some chloroaromatics as sources of carbon and energy, whereby chlorinated catechols are the central metabolites. The chlorocatechol degradation pathway starts with *ortho* ring cleavage caused by catechol 1,2-dioxygenase generating chloro-*cis*,*cis*-muconates. The cycloisomerization of the ring cleavage products by chloromuconate cycloisomerase is coupled with chloride elimination leading to (chloro)dienelactones. (Chloro)maleylacetates, the compounds resulting from the hydrolysis of the dienelactones by dienelactone hydrolase, are further converted to (chloro)3-oxoadipates by maleylacetate reductase.

Maleylacetate reductases from the 3-chlorobenzoate-degrading *Pseudomonas* sp. strain B13, from the 2,4-dichlorophenoxyacetate-degrading *Alcaligenes eutrophus* JMP134, and from the 1,4-dichlorobenzene-degrading *Pseudomonas aeruginosa* RHO1 will be compared with respect to structure and catalytic properties. In addition, information on the genetic basis for the maleylacetate reductase of *Pseudomonas* sp. strain B13 will be presented.

Biochemistry

Maleylacetate reductase of *Pseudomonas* sp. strain B13

The maleylacetate reductase was purified to homogeneity by chromatography on DEAE-cellulose, Butyl-Sepharose, Blue-Sepharose, and Sephacryl S100 from 3-chlorobenzoate-grown cells of *Pseudomonas* sp. strain B13. The final preparation gave a single band by polyacrylamide gel electrophoresis under denaturing conditions and a single symmetrical peak by gel filtration under non-denaturing conditions. The subunit M_r value was 37,000 (determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis). Estimation of the native M_r value by gel filtration gave a value of 74,000 with a Superose 6 column, indicating that the enzyme is dimeric. The pH and temperature optima were 5.4 and 50°C, respectively. The pI of the enzyme was estimated to be 7.0. The apparent K_m value for NADH was 30 µM. NADPH was also used as a cofactor instead of NADH with nearly the same V_{max} value, but its K_m value was estimated to be 77 µM.

Reductase activity was inhibited by a range of thiol-blocking reagents. The absorption spectrum indicated that there was no bound cofactor or prosthetic group in the enzyme.

Maleylacetate reductase from *Pseudomonas aeruginosa* RHO1 and *Alcaligenes eutrophus* JMP134

Maleylacetate reductase from *Pseudomonas aeruginosa* RHO1, grown with 1,4-dichlorobenzene, was purified to homogeneity by various steps of chromatography. Its properties were compared with the properties of the corresponding enzyme from *Pseudomonas* sp. B13 and *Alcaligenes eutrophus* JMP134. The molecular weight of the enzyme from strain RHO1 was determined by gel filtration to be approximately 74,000. Investigation of the M_r value of the subunits by SDS-PAGE resulted in 37,000 indicating that the enzyme is dimeric. These data as well as pH and temperature optima, isoelectric point and the effects of different inhibitors on the enzyme activity were similar to those found for the maleylacetate reductase from *Pseudomonas* sp. B13. The enzyme from strain JMP134 is dimeric with a size of 35,000 of the identical subunits.

Catalytic activity

Maleylacetate reductase of *Pseudomonas* sp. strain B13

Several substituted maleylacetates were prepared *in situ* by alkaline or enzymatic hydrolysis of dienelactones as the precursor. The conversion of these methyl-, chloro-, fluoro-, and bromo-substituted maleylacetates by maleylacetate reductase was studied. Two moles of NADH per mole of substrate were consumed for the conversion of maleylacetates which contain a halogen substituent in the 2 position. In contrast, only 1 mol of NADH was necessary to convert 1 mol of substrates without a halogen substituent in this position. The conversion of 2-fluoro-, 2-chloro-, 2,3-dichloro-, 2,5-dichloro-, 2,3,5-trichloro-, 2-bromo-, 2,3-dibromo-, 2,5-dibromo-, 2-bromo-5-chloro-, 2-chloro-3-methyl-, and 2-chloro-5-methylmaleylacetate was accompanied by the elimination of halide from the 2 position and the temporary occurrence of the corresponding dehalogenated maleylacetate. The reduction of this intermediate consumed another mole equivalent of NADH. Halogen substituents influenced the affinity to the enzyme in the following manner: K_m values increased with increasing van der Waals radius and simultaneously decreasing electronegativity of the halogen substituent (i.e., low steric hindrance and high electronegativity positively influenced the binding). The K_m values obtained with 2-methyl-, 3-methyl-, and 5-methylmaleylacetate showed that a methyl substituent negatively affected the affinity in the following order: 2 position > 3 position >> 5 position. A reaction mechanism explaining the exclusive elimination of halogen substituents from the 2 position is proposed.

Maleylacetate reductases from *Pseudomonas aeruginosa* RHO1 and *Alcaligenes eutrophus* JMP134

The maleylacetate reductases from *Pseudomonas aeruginosa* RHO1 and *Alcaligenes eutrophus* JMP134 showed nearly the same affinities to the substrates. Both enzymes reduced 2-halogenated maleylacetates with the

consumption of two moles of NADH per mole of substrate accompanied by the elimination of one mole equivalent of the corresponding halide. In contrast, halogen substituents in positions 3 and 5 resisted. In these cases only 1 mol of NADH was consumed. This behaviour has also been found to be typical for the maleylacetate reductase of *Pseudomonas* sp. B13. The comparison of the k_{cat}/K_m values revealed that the reductase of strain JMP134 was slightly better adapted for the conversion of substituted maleylacetates than the enzymes of the strains B13 and RHO1.

Genetic basis of the maleylacetate reductase

The maleylacetate reductase from *Pseudomonas* sp. strain B13 was digested with trypsin. The polypeptides separated by high performance liquid chromatography were sequenced. Alignments were obtained with the polypeptides predicted from the *tfdF* and *tcbF* genes located on the plasmids pJP4 of the 2,4-dichlorophenoxyacetate-degrading *Alcaligenes eutrophus* strain JMP134 and pP51 of the 1,2,4-trichlorobenzene-degrading *Pseudomonas* sp. strain P51 as well as from the *tftE* gene located on the chromosome of the 2,4,5-trichlorophenoxyacetate-degrading *Pseudomonas cepacia* strain AC1100. In addition, the deduced amino acid sequence encoded by the nucleotide sequence downstream of *clcD* on plasmid pAC27 of the 3-chlorobenzoate-degrading *Pseudomonas putida* strain AC866 was tested towards homology. The high identity with the polypeptides encoded by the *tfdF*, *tcbF* and *tftE* genes (up to 80 %) and the nucleotide sequence downstream of *clcD* gene (100 %) indicates that these genes encode maleylacetate reductases. A NAD-binding motif in an βαβ-element was detected.

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THIRD SESSION: REGULATION OF CATABOLIC GENES

Chairperson: Teruko Nakazawa

TRANSCRIPTIONAL CONTROL OF THE PSEUDOMONAS PUTIDA TOL PLASMID CATABOLIC PATHWAYS.

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TOL plasmid pWW0 of Pseudomonas putida contains two operons that specify a pathway for the degradation of aromatic hydrocarbons. The upper pathway operon encodes the enzymes for the oxidation of toluene/xylenes to benzoate/toluates, and the meta-cleavage pathway operon encodes the enzymes for the further oxidation of these compounds to Krebs cycle intermediates. Their expression is controlled by the gene products of two divergently transcribed regulatory genes, xylR and xylS. The XylR protein, which belongs to the NtrC family of regulators, is expressed from two tandem promoters and autoregulates its synthesis. XylR stimulates transcription from the xylS gene promoter (Ps) and the upper pathway operon promoter (Pu) in the presence of pathway substrates. Both promoters are σ^{54} dependent, and Pu also requires the presence of integration host factor (IHF) for activation of transcription. Binding sites for XylR and IHF in the Pu promoter and for XylR in the Ps promoters have been defined. The XylS protein, which belongs to the AraC family of regulators, stimulates transcription from the meta-cleavage pathway operon promoter (Pm) in the presence of benzoates. The

effector binding pocket and DNA-binding region of XylS have been defined through the isolation of mutants that exhibit altered effector specificity and modified transcriptional patterns, respectively. Expression of the meta-cleavage pathway operon is also induced by xylene-activated XylR protein via a cascade regulatory system in which this protein, in combination with σ^{54}, stimulates the expression from the xylS promoter. The increased concentration of XylS in turn leads to high level expression of the meta-cleavage pathway operon independently of meta-cleavage pathway substrates.

Regulatory noise in catabolic promoters of *Pseudomonas* : How bacteria learn to respond to novel aromatic substrates

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Analysis of DNA and protein sequences of the enzymes which form catabolic pathways in *Pseudomonas* has revealed that, as a rule, isoenzymes which catalyze similar or identical steps within catabolic pathways tend to be highly homologous even in cases where the substrates of the pathway are very different. These homologies suggest a common evolutive origin for the enzymes and even for different portions of the pathways. The mechanism of transcriptional control under which these operons occur, is however, extremely different regardless of the structural similarity between pathway inducers. The *meta*-NAH pathway, activated by salicylate, uses a transcriptional control system based on the NahR protein, which belongs to the LysR family of regulators. On the contrary, the *meta*-TOL pathway is regulated by an activator, XylS, of the AraC family. This is particularly striking, since XylS mutants can be easily found which respond to salicylate. This suggest that transcriptional control evolves quite independently of the structural genes of the catabolic operons. To gain some insight in the way control elements get assembled within biodegradative pathways we have examined in detail two cases in which a single promoter region located in front of a polycistronic operon can respond to at least two regulators which respond themselves to different aromatic effectors and even to totally alien signals.

The first case is that of the *Pu* promoter of the archtypical TOL (toluene degradation) plasmid pWW0 of *Pseudomonas putida*. This is a σ^{54}-dependent promoter which requires the binding of a cognate activator, XylR, to upstream activating sequences (UAS) in order to initiate transcription when cells are exposed to toluene. A distinct IHF sequence placed between the UAS and the σ^{54}-RNApol binding site ensures not only a maximum activity under induced conditions, but also enhances the fidelity and the strictness of the response to the right inducer/activator. This effect is due to the DNA bending caused by IHF and can be mimicked by substituting the IHF site within the promoter by a statically curved DNA sequence. In spite of such barriers, *Pu* can also be "illegitimately" activated by the regulator of a totally different catabolic pathway, namely DmpR, of the phenol degrading pathway of plasmid pVI150 of *Pseudomonas* CF600. Both in the absence and the presence of XylR, DmpR makes *Pu* to respond to phenol by binding the very same UAS which are the target sites for XylR. Furthermore, in the absence of IHF, the *Pu* promoter responds significantly to heterologous regulators of the σ^{54}-related group, such as NifA, in a fashion independent of the binding of the alien activator to DNA. This suggests that the rigid conformation which DNA may adopt upon IHF binding, increases activation specificity by preventing access to σ^{54}-RNApol of activators other than that properly bound and positioned in the UAS. It seems, therefore, that the role of IHF in this system is not only as a co-activator but also as an active supressor of cross-regulation.

The second example is the XylS-dependent and independent activation of the *Pm* promotor of the TOL plasmid : The *Pm* promoter of the *meta*-cleavage operon carried by the plasmid becomes activated by the plasmid-encoded XylS regulator in the presence of a variety of benzoate effectors. XylS belongs to the AraC family of transcriptional regulators and acts upon *Pm* by binding a distinct directly repeated sequence within the promoter region. In the absence of XylS, *Pm* is still responsive to unsubstituted benzoate but with an induction kinetics and within a range of activity which differs substantially of the XylS-mediated activation. XylS-independent induction by benzoate is dependent on σ^{54} and regulated by growth-phase. However, XylS-dependent

and independent transcription is initiated at the very same nucleotide. Furthermore, a series of deletions and mutations at the *Pm* promoter sequence showed the same overall pattern of responsiveness to benzoate with or without XylS, thus providing genetic evidence that the same promoter structure is recognized and activated by at least two different regulators. One of them is XylS, while the other, provided by the host bacterium, could be related to the chromosomally-encoded benzoate-degrading pathway.

All these data indicate that activation of catabolic promoters is not a very specific process. Unrelated regulators present in the same cell or acquired occasionally from other members of the community can make promoter sequences to shift totally its specificity and transcribe catabolic genes or operons in response to signals for which they do not seem to be initially programmed. This "regulatory noise" may further enhance the capacity of bacterial communities to quickly adapt to changing environmental conditions by circulating "regulatory cassettes" which can be recruited for the assembly of transcriptional control elements in new degradative pathways.

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MULTIPLE ALLELES OF XylS-LIKE REGULATOR PROTEINS

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The regulation of the two xyl operons on the archetypal TOL plasmid pWW0 has been elucidated in great detail by the combined work of Nakazawa, Timmis, Ramos, De Lorenzo and their coworkers. Other TOL plasmids which confer the same phenotype as pWW0 but are found in independently isolated Pseudomonas strains have been described and their regulation has been assumed to be analogous to that of pWW0 because of the presence of genes homologous to xylR and xylS. In two such plasmids, pWW53 and pDK1, there is evidence for the presence of two or more genes homologous to xylS which mirrors the situation on pWW53 at least where there is also a duplication of the meta pathway operon xylXYZLTEGFQKJIH. Apart from their role in regulating the operon, these genes may have played a part in the past history of these plasmids by serving as regions of homology within which recombination has occurred. Recently we have found a plasmid-encoded meta pathway operon in a biphenyl-degrading strain, Pseudomonas strain IC, which appears highly homologous to the xyl operon and which also appears to carry a homologue of xylS.

CATABOLITE REPRESSION CONTROL IN *PSEUDOMONAS*: ANALYSES OF THE *crc* GENE AND ITS PROTEIN PRODUCT

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Pseudomonas aeruginosa utilizes tricarboxylic acid cycle intermediates and acetate preferentially to glucose, other carbohydrates, and many other carbon and energy sources. Metabolism of these organic acids, but not pyruvate or lactate, elicits strong catabolite repression control (CRC) over the induction of enzymes and transport systems required for catabolism of the alternative carbon sources. Unlike CRC elicited by glucose in *Escherichia coli*, CRC appears to be cyclic-AMP independent in *P. aeruginosa* and *P. putida*. Mutations in the *P. aeruginosa vfr* gene, which encodes a homologue of the *E. coli* cyclic-AMP receptor protein (CRP) (courtesy of S. West and colleagues), have no apparent effects on the CRC phenotype. Mutants of *P. aeruginosa* PAO1 that are defective in CRC of multiple, independently regulated catabolic systems have been isolated and characterized. These *crc* mutations map between the *pyrE* and *plcA* loci at about 11 min on the PAO1 chromosome. The wild-type *crc* allele, which complements the *crc* mutations *in trans*, was cloned on a 922 bp fragment of the PAO1 chromosome and the complete nucleotide sequence was determined in both directions. Sequence analysis identified a single open reading frame (ORF) which would encode a 259 residue polypeptide with a molecular weight of 28.5 kDa. DNA fragments containing this ORF, cloned behind either T7 or *lacZ* promoters, were used to overexpress and visualize a protein of about 30 kDa in both *P. aeruginosa* and *E. coli*. Native Crc protein and a Crc fusion protein (with a hexahistidyl amino terminus tail) were isolated, purified, and shown by amino acid sequence analysis to correspond to the protein predicted by the *crc* nucleotide sequence. Polyclonal antibodies to purified Crc fusion protein reacted with proteins of similar size in Western blot analyses of crude extracts of *P. putida* and *P. fluorescens*, suggesting a common mode of CRC in these three species. Antibody specific cross reacting proteins were not detected in Western blots with extracts from either *E. coli* or a *crc* knockout mutant of *P. aeruginosa* ($\Delta crc::Tc^r$).

The deduced amino acid sequence of Crc was used to perform homology searches of known proteins in the Genebank and Swiss Protein Bank data bases. The only similar proteins identified belong to a family of eukaryotic and prokaryotic DNA repair enzymes (AP endonucleases) with sequence identity ranging from 25% to 32% and similarity from 51% to 57%. Neither purified Crc nor the fusion protein exhibited detectable AP endonuclease activity. Moreover, neither purified protein exhibited exonuclease activity which was shared by all of the prokaryotic members of the family of similar proteins. Extensive effort to demonstrate Crc-DNA binding activity in gel mobility shift assays also were uniformly negative. These assays were performed with *P. aeruginosa* DNA fragments containing upstream sequences of the *crc* gene and of selected genes known to be sensitive to

catabolite repression control. Thus, no specific catalytic or DNA binding activities of Crc protein have been identified and the molecular mechanism of CRC remains unknown.

Though the *crc* gene clearly is required for catabolite repression control of a wide range of catabolic regulatory units, it appears not to be required for CRC of all inducible catabolic systems. For example, histidase and urocanase, of the *hut* operon, remain sensitive to strong repression by succinate in *crc* mutants of *P. aeruginosa*. On the other hand, the *crc* gene may affect the expression of genes other than those of inducible catabolic systems. As examples: *crc* mutants of *P. aeruginosa* are observed to produce more blue pigment (pyocyanin) than wild-type strains; and there is preliminary evidence that expression of reporter genes fused to both *las*R and *alg*D promoters is reduced 2- to 3-fold in strains containing *crc* mutations.

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The composition of the toluene-degrading bacterial population in soil: the occurrence of different pathways, genera and their susceptibility to catabolite repression.

On many separate occasions in the past individual toluene-degrading bacterial strains have been isolated. A number of different genera and species were found to be represented and in biochemical studies five different degradation pathways were found.

In order to get an indication of the relative importance of the different species and pathways, we studied the toluene-degrading bacterial population in oil-contaminated and pristine soil samples by a direct-plating procedure. Gram-positive bacteria were found to predominate in most pristine soil samples while the majority of the oil-contaminated soil samples were dominated by Pseudomonads. It was found that a vast majority of the toluene-degrading strains degraded toluene through pathways involving direct oxygenation of the aromatic ring rather than oxidation of the methyl group.

A number of isolated strains were compared with respect to their competition behaviour under toluene-limiting conditions in chemostat culture. First results at moderate dilution rate (0.05 h^{-1}) indicated a competitive advantage of Pseudomonads in comparison to Gram-positive strains harbouring a similar pathway for the degradation of toluene

The susceptibility to catabolite repression of the toluene-degradation pathways in a number of laboratory strains and isolated strains was compared. *P. putida* mt-2 was found to be subject to catabolite repression by succinate, both under conditions of maximal growth rate and under ammonium limiting conditions. Other strains are currently under investigation.

FOURTH SESSION: GENETICS OF CATABOLIC PATHWAYS

Chairperson: Dick B. Janssen

Aromatic Hydrocarbon Degradation by *Comamonas testosteroni*

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The simple compounds naphthalene and phenanthrene have long been model compounds for analysis of polycyclic aromatic hydrocarbon degradation. The genetics and biochemistry of naphthalene degradation by the analogous strains *P. putida* NCIB 9816-4 and *P. putida* G7 (NAH plasmid) have been well documented. Several investigators over the last decade have attempted to identify new catabolic genes for the degradation of polycyclic aromatic hydrocarbons but all of these attempts have resulted in the isolation of genes that are nearly identical to the classical *nah* genes. Indeed, there are five published *nah*-like gene sequences in the literature and many undocumented, unpublished *nah*-like sequences circulated among the scientific community. All of these sequences are greater than 90% homologous to one another. Since the classical *nah* genes are the ones that are available, and seem to be widespread in the environment, they are the ones utilized as paradigms for genetic ecology and biodegradation studies of polycyclic aromatic hydrocarbons. However, the presence of a single, highly homologous group of genes for polycyclic aromatic hydrocarbon degradation does not reflect the true metabolic diversity and evolutionary potential of microorganisms. The present studies were undertaken in order to investigate the genetic diversity of polycyclic aromatic hydrocarbon degradation and to identify novel organisms and genes for polycyclic aromatic hydrocarbon degradation.

We have determined that the chronically polluted Passaic River in New Jersey is a rich source of diverse microorganisms capable of aromatic hydrocarbon degradation. Several strains were isolated from Passaic River sediment for the ability to degrade the polycyclic aromatic hydrocarbon phenanthrene. Six of these bacterial strains were initially chosen for the present investigation. Three of the strains were classified as *P. putida* (GZ41, GZ44, and GZ45) and three were classified as *C. testosteroni* (GZ38A, GZ39, and GZ42). All of the isolated strains have the ability to grow on naphthalene as well as phenanthrene except for *C. testosteroni* GZ38A which grows only on phenanthrene. Southern blots using the cloned genes for the conversion of naphthalene to salicylate from *P. putida* NCIB 9816-4 as a probe were performed using total genomic DNA prepared from the six isolated strains. *Eco*RI restricted genomic DNA from the three newly isolated *P. putida* strains showed a hybridization band similar in size and intensity to the positive control *P. putida* NCIB 9816-4. However, genomic DNA prepared from the three *C. testosteroni* strains did not show any hybridizing bands even under low stringency conditions and long exposures to x-ray film. In order to determine if the lack of hybridization

indicates the presence of novel genes for naphthalene/phenanthrene degradation the genes for this catabolic activity were cloned from *C. testosteroni* GZ39. A genomic cosmid library was prepared and screened for clones capable of transforming indole to indigo, indicative of aromatic dioxygenase activity. Two cosmid clones each approximately 40 kilobase pairs in size were obtained and were subsequently shown to overlap by about 18 kilobase pairs. Biotransformation experiments with the two cosmid clones showed that the clones possessed the ability to transform both naphthalene and phenanthrene to oxidized products including the corresponding *cis*-dihydrodiol and dihydroxylated compounds. Southern blot experiments using the newly cloned genes for naphthalene/phenanthrene degradation (designated as the *phd* genes) as a probe showed that no detectable hybridization could be seen using genomic DNA prepared from *P. putida* NCIB 9816-4. This confirms the previous hypothesis that *C. testosteroni* GZ39 possesses a new novel set of genes for polycyclic aromatic hydrocarbon degradation that are distinctly different from those found in *P. putida* NCIB 9816-4. In addition, Southern blots with the *phd* genes as a probe indicate that the genes for phenanthrene degradation in *C. testosteroni* GZ38A are similar but not identical to those cloned from *C. testosteroni* GZ39. However, the Southern blots also indicate that the *phd* genes from *C. testosteroni* GZ39 do not have any detectable homology to the analogous genes in *C. testosteroni* GZ42. The three *C. testosteroni* strains thus represent at least two new classes of genes involved in polycyclic aromatic hydrocarbon degradation.

The *phd* genes coding for the initial enzymes involved in naphthalene and phenanthrene degradation by *C. testosteroni* GZ39 were further localized to a 5.5 kilobase pair *Nco*I-*Pst*I fragment by subcloning and expression analysis in *E. coli*. The nucleotide sequence of this region was determined. Functions of the open reading frames were assigned based on subclone characteristics and homology to other proteins of known function. Five genes were located and implicated in the first two catabolic steps of phenanthrene degradation. The gene order is *phdAb*, coding for the ferredoxin component of the aromatic dioxygenase; *phdAa*, coding for the reductase component of the aromatic dioxygenase; *phdB*, coding for an aromatic *cis*-dihydrodiol dehydrogenase; *phdAd*, coding for the large subunit of the terminal oxygenase component of an aromatic dioxygenase; and *phdAd* coding for the small subunit of the terminal oxygenase component of an aromatic dioxygenase. The deduced amino acid sequences for each protein show similarities to the amino acid sequences of analogous proteins from other oxygenase enzyme mediated catabolic pathways. However, in every case sequence analysis indicates that the *phd* gene-encoded proteins represent a class distinct from proteins involved in toluene, biphenyl, or naphthalene degradation.

Gene organization and regulation of catabolic pathways in *Pseudomonas* strains degrading alkylsubstituted aromatic hydrocarbons.

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The existence of different genetic mechanisms which contribute to metabolic adaptation together with a relaxed substrate specificity of enzymes involved in the catabolic pathways of aromatic hydrocarbons allows the rapid evolution of new metabolic pathways in response to environmental changes. The complexity of the catabolic routes implies sophisticated systems of regulation to control the expression and achieve the coordination of catabolic abilities.

Although degradative activities of pseudomonads are very different, degradative routes are convergent and lead to a rather limited number of common intermediates such as catechols, which are channeled into one of two possible pathways either a *meta* or an *ortho* cleavage-type pathway.

This generalized scheme for aromatic hydrocarbons degradation suggests that pseudomonads as well other microorganisms have extended their substrate range by developing peripheral enzymes which are responsible of the initial substrate transformation into common central intermediates.

Several *Pseudomonas* strains have been isolated and described for their ability to degrade different alkylsubstituted aromatic hydrocarbons. The introduction of substituent group(s) onto the benzene ring opens up the possibility of alternative modes of biodegradation: either alkyl group attack or ring attack.

The simplest of such molecules is toluene, for which so far five different pathways have been identified among members of the genus *Pseudomonas*. One of them, the TOL plasmid encoded pathway, can also catabolize dimethylated compounds such as *m*- and *p*-xylene or trimethylated compounds such as 1,2,4-trimethylbenzene. *P putida* TMB, isolated in the presence of 1,2,4 - trimethylbenzene is functionally and genetically homologous to *P. putida* PaW1 carriyng the TOL plasmid pWW0. The catabolic genes of strain TMB, located on the chromosome, share homology with pWW0, but differ significantly in gene organization

and regulation. In addition at least part of the *meta*-pathway operon is duplicated.

None of the pathways for toluene, *p* and m-xylene has been reported to allow the growth on *o*-xylene.

A novel pathway chromosomally encoded, for the degradation of both toluene and *o*-xylene has been investigated in the strain *P.stutzeri* OX1, whose activity results in the formation of a mixture of cresols from toluene, and of 2,3-and 3,4- dimethylphenol from *o*-xylene. It has been assumed that a same multicomponent enzyme could be involved in the initial attack of toluene and *o*-xylene, as well of other hydrocarbons such as benzene, ethylbenzene, styrene.

A mutant M1 of OX1 has lost the ability to grow on *o*-xylene but has acquired the ability to grow on *p* and *m*-xylene by recruitment of enzymes for the successive oxidation of xylenes to the corresponding alcohols, aldehydes and carboxylic derivatives.

A revertant strain R1 was successively isolated, capable to grow on the three isomers *p*-, *m*- and *o*-xylene. This mutant seems to have rescued all the metabolic activities of the wild strain OX1, still retaining the ability, expressed in mutant M1 but undetectable in OX1, to oxidize a methyl group of *m*- and *p*-xylene to alcoholic group.

Chromosomal rearrangements due to insertion-deletion of a 3 kb sequence in the region containing upper pathway genes, have been evidenced in OX 1, M1 and R1, leading to activation of catabolic pathways pre-existing but not functioning or to extinction of other pathways that may be potentially suicide in some conditions.

MICROORGANISMS FOR BIOREMEDIATION: LESSONS FROM THE STUDIES OF *Pseudomonas* PLASMIDS

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The study of D plasmids controlling the degradation of e-caprolactam or naphthalene reveals great diversity of their properties [1, 2]. Natural bacterial populations of *Pseudomonas* consist of plasmids carrying genetic systems for degradation of the same compounds, though these systems differ in some features [2]. The transfer of the same NAH plasmid into several *P. putida* strains results in obtaining strains significantly differing from each other in growth characteristics with naphthalene or salicylate as the sole carbon source. A similar effect is produced by the use of the same *P. putida* strain as a recipient of different NAH plasmids [3].

Pseudomonas strains containing the well-known NAH7 plasmid, as well as most other NAH plasmids, perform naphthalene degradation with the use of the meta-pathway of catechol cleavage. However, other strains with the NAH plasmids exhibit enzymic activity of the ortho-pathway cleavage of the above compound [4]. There is reason to beleive that in part this is due to the fact that genetic systems which control degradation of naphthalene and salicylate also allow some catabolism of their numerous chloro- or methyl derivatives. Therefore, the bacteria modify these genetic systems to adjust them to the environmental conditions directing in particular, the catechol catabolysm vire either the ortho- or the meta-pathway of its cleavage [5]. This changes the rate of microbial growth with utilization of these carbon sources, at different concentrations and under different growth conditions, which in turn, alters the relative competitiveness of microorganisms.

When constructing microorganisms for bioremediation, it would be useful to consider the already exisiting results of genetic engineering experiments performed by microorganisms themselves in nature.

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A MOLECULAR APPROACH FOR ELUCIDATING THE CATABOLIC PATHWAY OF 4-HYDROXYPHENYLACETIC ACID IN ESCHERICHIA COLI

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The ability to use aromatic compounds as sources of carbon and energy for growth is a property widely distributed among bacteria (1). This property has been mainly studied on Pseudomonads and other soil bacteria since they are attractive candidates for detoxification of a wide range of environmental pollutants (2). However, several enteric bacteria including *Escherichia coli* have been also shown to possess aromatic degradative pathways (3). *E. coli* B, C, and W are able to degrade 4-hydroxyphenylacetic acid (4-HPA) and homoprotocatechuate (HPC; 3,4-dihydroxyphenylacetate) (two substrates that cannot be metabolized by K-12) via an inducible chromosomally encoded meta-cleavage pathway (4). We have recently cloned a DNA fragment of *E. coli* W ATCC11105 encoding an aromatic hydroxylase having a broad substrate range that was tentatively classified as a 4-HPA 3-hydroxylase (5). The nucleotide sequences of the *hpa*B and *hpa*C genes encoding the 4-hydroxyphenylacetate 3-hydroxylase have been determined (6). These genes appear to be part of an operon and encode two proteins of 58,781 and 18,679 Da, respectively, that are required for hydroxylase activity. This aromatic hydroxylase is NADH-dependent and uses FAD as the redox chromophore. The largest component (HpaB) has been purified by affinity chromatography in Cibacron Blue. *E. coli* cells that express exclusively *hpa*B showed only a very low hydroxylase activity that was enhanced in the presence of extracts containing the smallest protein HpaC. This behavior resembles that of the coupling protein of the 4-hydroxyphenylacetate-3-

hydroxylase from *Pseudomonas putida* and it might prevent the wasteful oxidation of NADH in the absence of substrate (7). Using a promoter-probe plasmid we have demonstrated that the *hpa*BC operon is expressed by a promoter inducible by 4-HPA. A gene, named *hpa*A, encoding a protein homologous to the *XylS/AraC* family of regulators was identified upstream of the hydroxylase operon. The role played by HpaA in the regulation of the hpaBC operon has been partially elucidated. Since HpaB is not homologous to other aromatic hydroxylases, we suggest that the *E. coli* 4-hydroxyphenylacetate 3-hydroxylase is the first member of a new family of two component aromatic hydroxylases sequenced so far. More recently the complete 4-HPA degradative pathway of *E. coli* W has been cloned and sequenced. An *E. coli* K12 strain containing the 4-HPA cluster in a plasmid or a transposon was able to grow using 4-HPA as the sole carbon source. This pathway is composed of a complex system of operons and regulatory genes which is currently under study.

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Metabolism of substituted Catechols via Intradiol Cleavage

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In the recent years, a variety of microorganisms have been isolated which mineralize single ring chlorinated aromatic compounds. Even though reactions have been demonstrated by which chloro-substituents were removed prior to ring cleavage, including oxygenolytic, hydrolytic and reductive eliminations by usually very specific enzymes, in most cases chloroaromatics have been shown to be metabolized via chlorocatechols, with the elimination of chloride occuring after ring cleavage. Those microorganisms able to mineralize chloroaromatics via chlorocatechols have generally been described to contain enzmyes of the so-called chlorocatechol pathway (*ortho* II pathway), which was assumed to occur in parallel to the well described 3-oxoadipate pathway (*ortho* I pathway), both pathways catalysing intradiol cleavage and cycloisomerization of the formed muconate. Chlorocatechol 1,2-dioxygenase and chloromuconate cycloisomerase of the *ortho* II pathway were assumed to differ from their counterparts in the *ortho* I pathway (catechol 1,2-dioxygenase and muconate cycloisomerase) only by their broader substrate specificity. Dehalogenation in both pathways was postulated to be a spontaneous secondary reaction by which dienelactones were formed from unstable chloromuconolactones.

A great number of microorganisms isolated on the basis of their ability to degrade unchlorinated aromatic compounds have been shown to harbour enzymes which could transform a variety of chloro-aromatics into chlorocatechols. Therefore, combination of the *ortho* II pathway genes with genes coding for unspecific peripheric enzymes could be used to construct microorganisms able to mineralize a variety of chlorinated pollutants. In the absence of *ortho* II pathway genes, catechols will be subject to either extradiol (*meta*) or intradiol cleavage by type I enzymes.

Substrate misrouting and even formation of toxic or highly reactive metabolites has initially been observed when microorganisms were confronted with mixtures of chloro- and methylaromatics. For mineralization of chlorocatechols, extradiol- (*meta*) cleaving activities, which were believed to be essential for mineralization of methylcatechols, have to be prevented. After discovering a new, modified *ortho*-cleavage pathway for methylcatechol metabolism (*ortho* III) with a 4-methylmuconolactone methylisomerase as key enzyme, an organism able to simultaneously degrade methyl- and chloroaromatics was successfully constructed. Limitations and possible extensions of this strategy will be discussed.

It has now been shown that muconate cycloisomerases and chloromuconate cycloisomerases catalyze different reactions when confronted with chloromuconates. Based on the observation of unexpectedly stable chlorinated intermediates in chloromethylaromatic metabolism, the dogma of spontaneous dehalogenation in the metabolism of halocatechols was doubted. Evidence will be given that dehalogenation of 2-chloromuconate, when channeled into the *ortho* I pathway, is due to the concerted action of muconate cycloisomerase and muconolactone isomerase, while chloro-muconate cycloisomerases directly catalyzes dehalogenation to form *trans*-dienelactone.

Various muconate cycloisomerases are now shown to produce protoanemonin instead of *cis*-dienelactone as a product. Protoanemonin is a known antibiotic compound and will always be formed if chloroaromatics are transformed into 4-chlorocatechol (which is then, in turn, metabolized by enzymes of the *ortho* I pathway with reasonably high activity). Formation of proto-anemonin led to a drastic drop in viability of bacterial cells. We presume that protoanemonin will be formed, for example, during metabolism of 4-chlorobiphenyl with the resulting poisoning of the microbial community including the PCB degraders. Strategies to prevent protoanemonin formation or accumulation will be discussed and indications for ecotoxicological effects be given.

As discussed above, effective degradation of chloroaromatics is achieved by the *ortho* II pathway only, and misrouting into the *meta*- as well as into the *ortho* I pathway disturb metabolism.

FIFTH SESSION: MOLECULAR EVOLUTION AND LABORATORY DESIGN OF ENZYMES AND PATHWAY

Chairperson: James Tiedje and Víctor de Lorenzo

The design of performant bacteria for the elimination of environmental pollutants

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Microbial catabolic pathways offer great promise for the accelerated, economic and safe removal of chemical pollutants from the environment. Natural catabolic activities are not, however, optimized for biotechnological applications and can be improved in a variety of ways. Moreover, natural catabolic activities have not yet been identified for a number of the more toxic and recalcitrant pollutants with which we are currently faced. Effective exploitation of biological solutions to environmental pollution will therefore require both improvement of known catabolic activities and the design of new ones.

We have developed a number of genetic tools (1-6) and three general strategies for the design of improved or novel pathways, namely (a) the restructuring of existing pathways to expand their substrate range (7-9), (b) the assembly of novel pathways through the judicious combination of different pathway segments from different organisms, in order to channel substrates through unusual routes (10), and (c) the combination of different pathways to accomplish the metabolism of distinct toxic chemical moieties present in some pollutants (11).

The development and characteristics of a novel bacterium designed by the patchwork assembly strategy (b) to metabolize problematic mixtures of chloro- and methylaromatic pollutants will be described, and evidence which refutes the dogma that laboratory organisms are not able to survive and function in natural environments (12) will be presented. The biochemical basis of the poor survival of some inoculants used for *in situ* bioremediation of chloroaromatic-contaminated sites has been elucidated and shown to involve the unproductive conversion of 4-chlorocatechol to protoanemonin, a normally plant-derived antibiotic, by the indigenous microflora. This antibiotic kills both inoculants and members of the indigenous microflora. A strategy to design microorganisms able to degrade chloroaromatics without permitting the formation of protoanemonin will be discussed.

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Modification of haloalkane dehalogenase activity and specificity by protein engineering

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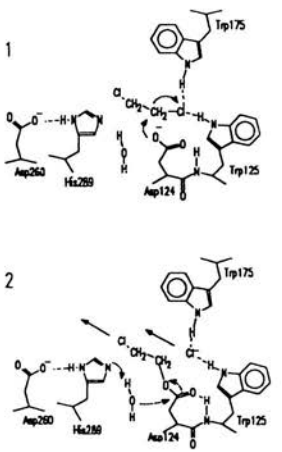
Haloalkane dehalogenase catalyzes the hydrolytic cleavage of carbon-halogen bonds in a broad range of 1-halo-n-alkanes. The structure and catalytic mechanism of the enzyme were solved by X-ray crystallography (Verschueren et al., 1993a, 1993b) and biochemical studies (Pries et al., 1994a). The enzyme is composed of two domains, designated main domain and cap domain. The main domain has an α/β hydrolase fold structure that is found in a variety of hydrolytic proteins, including other dehalogenases, epoxide hydrolases, lipases and diene lactone hydrolases (Ollis et al., 1992). The cap domain is not conserved and plays an important role in the determination of substrate specificity (Pries et al., 1994b). The dehalogenase reaction proceeds by nucleophilic attack of the carboxylate oxygen of Asp124 on the Cα of the substrate, which results in displacement of the halogen. This is facilitated by Trp125 and Trp175, which stabilize the leaving group. The covalent alkyl-enzyme intermediate is subsequently hydrolyzed by a water molecule that is probably activated by His289.



The effect of various active-site mutations on the activity and specificity of haloalkane dehalogenase was investigated. Mutants in which the halide-binding residues Trp125 and Trp175 were replaced by glutamines, phenylalanines, or arginines all showed a decreased activity and reduced affinity for halide ions. In most of the mutants, the rate-limiting step had changed from cleavage of the carbon-halogen bond and product release to formation of the covalent intermediate.

Mutants in which the nucleophilic aspartate was replaced were inactive, except an Asp124→Asn mutant, which slowly reactivated by hydrolysis of the side-chain amide bond of Asn124. This indicated a high sensitivity to nucleophilic attack of the side chain carbonyl carbon of residue 124.

Mutation of His289, which was proposed to activate the water that cleaves the covalent intermediate, led to a mutant enzyme in which the alkyl-enzyme accumulated and was not further converted. In addition, the rate of (substrate-independent) exchange of

carboxylate oxygens with oxygen from solvent water, which in the wild type enzymes is significantly higher than expected from the usual chemical reactivity of a carboxylate, was strongly reduced in the His289→Gln mutant.

Two different strategies were employed to obtain mutants with an altered substrate specificity. First, several spontaneous mutants were selected on basis of their activity with 1-chlorohexane (Pries et al., 1994b). This led to either substitutions, repeats, or a large deletion in the cap domain of the enzyme. The mutations caused an increase in activity towards long-chain chloroalkanes, which probably was caused by a higher flexibility or a different position of residues of the N-terminal part of the cap domain. Some of these amino acids line the active-site cavity. Loss of a surface-located salt bridge between Asp170 and Lys261 also could influence enzyme specificity. The activity of the mutants with short-chain chloroalkanes was lower than in the wild type.

Other mutants were constructed by site-directed mutagenesis. Mutating Phe172 to tryptophan or tyrosine increased the catalytic rate of the dehalogenase with 1,2-dibromoethane, probably by destabilizing the covalent intermediate. This may have led to an increase in the rate of the second step, i.e. cleavage of the intermediate and halide plus proton export. An even stronger increase of k_{cat} with 1,2-dibromoethane was found with mutants of Val226, another residue lining the active site cavity.

The prospects of obtaining further mutant enzymes with improved applicability for specific chlorinated pollutants will be discussed.

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Sequence Analysis of Catechol 1,2-Dioxygenases of *Pseudomonas putida(arvilla)* mt-2 and *Pseudomonas arvilla* C-1

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Many aromatic hydrocarbons are degraded by pseudomonads and other soil bacteria via an intermediate catechol. Catechol ring cleavage reactions are catalyzed by two types of dioxygenases, intradiol(ortho-cleavage) and extradiol(meta-cleavage). Catechol 2,3-dioxygenase(C23O) is an extradiol type of enzyme and encoded by the *xylE* gene on the TOL plasmid harbored by *Pseudomonas putida* mt-2. In contrast to C23O, catechol 1,2-dioxygenase (C12O) of *P. putida* mt-2 is encoded by a chromosomal gene, *catA*. We have shown that C12O of the mt-2 strain consists of two identical subunits of the molecular weight of 32 kDa (1). We now cloned the *catA* gene from the mt-2 strain by using a PCR product of amino acid sequence-based primers as a probe. The amino acid sequence deduced from the 930-nucleotide sequence was in perfect agreement with the chemically determined sequence of the enzyme protein. Crude extract of *Escherichia coli* cells carrying the *catA* plasmid had the C12O activity.

Pseudomonas arvilla C-1 contains three isozymes of C12O, which are formed by combinations of two nonidentical subunits as dimers, $\alpha\alpha$, $\alpha\beta$, and $\beta\beta$ (2). The amount of $\beta\beta$ was twice as much as $\alpha\alpha$ when the cells were grown in the benzoate-yeast extract medium, and the enzymatic properties such as substrate specificity and kinetic parameters of the three isozymes were essentially the same. By using the same probe for the mt-2 enzyme, we successfully cloned and sequenced the $catA_\beta$ gene for the $\beta\beta$ isozyme from *P. arvilla* C-1. There was a very high homology between C12Os of the mt-2 strain and of the $\beta\beta$ isozyme of the C-1 enzyme in both the amino acid (98%) and DNA sequences (97%). A preference for the use of codons terminating in C and G was found in the coding region of both enzymes that contributed to the high G + C content of the genes (66%).

Search for the $catA_\alpha$ gene encoding the $\alpha\alpha$ subunit of the C-1 enzyme by using the same DNA probe or sequencing the flanking region of the $catA_\beta$ gene was unsuccessful, suggesting a distinct structure and distant location of the $catA_\alpha$ gene from the $catA_\beta$ gene. Thus we determined the whole amino acid sequence of the α subunit by direct sequencing, and the sequence revealed 78% homology with that of the β subunit.

The activity of intradiol dioxygenases depends on ferric iron which is ligated by two histidyl and two tyrosyl side chains. Determination of the crystal structure of a *Pseudomonas* protocatechuate 3,4-dioxygenase established the position of the iron ligands within the primary sequence of the β subunit. All the available sequences of intradiol dioxygenases exhibit a high level of amino acid sequence conservation in the presumed iron-binding regions.

Multiple alignment of nine C12Os revealed distinct divergence of the two typing groups that have been proposed, except C12O of *Arthrobacter* sp. mA3. The type I group includes five enzymes, i.e. C12Os of strain mt-2, $\alpha\alpha$ and $\beta\beta$ isozymes of strain C-1, *Acinetobacter calcoaceticus*, and *Pseudomonas* sp. EST1001, whereas the type II group includes three enzymes encoded by *clcA*, *tcbC* and *tfdC*.

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CONTRIBUTIONS OF RECOMBINATION TO METABOLIC EVOLUTION

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Chromosomal genes for catabolic pathways have undergone numerous rearrangements during their divergence in different bacterial species. In the absence of selective pressure for their clustering, such rearrangements might be expected to have scattered independently transcribed genes to separate chromosomal regions. In defiance of such predictions, genes for related catabolic functions are closely clustered in *Acinetobacter calcoaceticus*. The 11 *ben-cat* structural genes required for metabolism of benzoate *via* catechol are contained within a 16 kbp chromosomal segment, and a separate 18 kbp chromosomal segment contains the 12 *pca-qui-pob* structural genes required for dissimilation of *p*-hydroxybenzoate, quinate and shikimate *via* protocatechuate. These findings draw attention to selective forces that may favor clustering of physiologically interdependent genes.

Gene rearrangement placed *catA* directly downstream from *catC* in *Pseudomonas putida*. These genes can be traced to separate ancestry. Nevertheless, a 13 bp nucleotide sequence within *P. putida catC* is directly repeated at the beginning of *P. putida catA*. This and additional patterns of sequence repetition suggest that mismatch repair between slipped DNA strands may have contributed to the evolution of the neighboring genes. This possibility is strengthened by identification of a repetitive genetic element, 85 bp in length, at different locations in the *cat* gene cluster of fluorescent pseudomonads. An interpretation of these findings is that exchange of nucleotide sequence between the slipped DNA strands of neighboring genes may contribute to their acquisition of the codon usage pattern characteristic of the host DNA. Such sequence exchange also may contribute to the maintenance of genes through mismatch repair.

In *A. calcoaceticus*, *catIJF* and *pcaIJF* are nearly identical 2.5 kbp DNA segments encoding isofunctional enzymes associated with different metabolic pathways. Through gene conversion, the wild type nucleotide sequence in one DNA segment can serve as template for repair of mutations in the other DNA sequence. This allows organisms containing copies of both wild type sequences to be maintained in bacterial populations.

Isofunctional enzymes also are encoded by *catD* and *pcaD* in *A. calcoaceticus*. These genes have diverged substantially as demonstrated by a difference of 6% in the G+C content of their DNA. A possible mechanism for divergence is suggested by the presence of potential DNA slippage structures within *pcaD*. Acquisition of such structures could have fostered rapid divergence of *catD* and *pcaD*, and this would have been favored if the genes had diverged past a threshold that would make recombination between them deleterious.

Introduction of a deletion into *A. calcoaceticus pcaBDK* provides a genetic background in which null mutations inactivating *pobA*, *pobR* and *pcaH,G* can be selected. The genes are required for expression of *p*-hydroxybenzoate hydroxylase or protocatechuate 3,4-dioxygenase. Analysis of spontaneous mutations in *pcaH,G* suggests DNA slippage structures that may leave particular nucleotides susceptible to modification. It has been possible to construct an *A. calcoaceticus* strain in which recombinants between *pcaIJF* and *catIJF* can be selected by demanding null *pcaH,G* mutations. Such recombination is relatively slight in growing cells and is the predominant form of mutation after cells cease to grow. Thus the physiological state of cells appears to influence the destiny of their DNA.

POSTERS

THE BIPHENYL/POLYCHLORINATED BIPHENYLS-DEGRADATION CLUSTER OF *Rhodococcus globerulus* P6

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Polychlorinated biphenyls (PCB) are a family of man-made compounds that have been of public and scientific concern for several decades because of their persistence in the environment, ability to bioaccumulate, and potential carcinogenicity. There is considerable interest in developing biodegradative processes to destroy PCB safely. However three major hurdles to such applications have emerged, namely the poor competitivity and robustness of the best degraders, the poor stability of the PCB degradation phenotype in the absence of selection, and the inability of such strains to degrade the second aromatic ring of PCB, which results in accumulation of chlorobenzoates. The cloning and characterization of the genes responsible for PCB degradation will facilitate the development of bacterial strains possessing a superior ability to mineralize different PCB congeners.

The Gram$^+$ *Rhodococcus globerulus* P6 that catabolizes a wide range of PCB congeners was first designated as *Acinetobacter* sp. P6 and *Corynebacterium* sp. MB1, and has been now reclassified based on polyphasic analyses including phenotypic characterization, chemotaxonomic determination and characterization of cell wall amino acids and sugars, plasmid content, genomic DNA fingerprinting and 16S ribosomal DNA sequence determination [Asturias *et al.* (1994) *Syst. Appl. Microbiol.* **17**, 226]. The PCB degradation cluster and two additional *bphC* genes, cloned for the first time from a Gram$^+$ bacterium, were studied [Asturias and Timmis (1993) *J. Bacteriol.* **175**, 4631].

Sequence comparisons of the six clustered cistrons *bphA1A2A3A4BC1*, encoding the four subunits of biphenyl dioxygenase, 2,3-dihydroxy-4-phenylhexa-4,6-diene dehydrogenase, and 2,3-dihydroxybiphenyl 1,2-dioxygenase, respectively, show homologies to equivalent enzymes from Gram$^-$ bacteria with the highest homology to those of the toluene degradation pathway of *Pseudomonas putida* F1 [Asturias *et al.*, *Gene*, in press]. This fact suggests that PCB degradation genes of *R. globerulus* P6 may have originated in Gram$^-$ microorganisms, probably *Pseudomonas*, and subsequently transferred to this Gram$^+$ bacterium.

The other two *bphC* genes, not belonging to the *bph* operon, shown no homology with any other sequence in the database. At least two of the three *meta* cleavage enzymes of *R. globerulus* P6 are inducible by biphenyl [Asturias *et al.* (1994) *J. Biol. Chem.* **269**, 7807]. The extradiol dioxygenase encoded by the *bphC2* gene, was purified in anaerobic conditions and used in crystallization studies that are now in progress. Fundamental differences between this dioxygenase and other known extradiol dioxygenases will be dicussed.

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From Xenobiotic to Antibiotic: Ecotoxicity of some Chloroaromatic Pollutants due to Microbial Formation of Protoanemonin

Chloroaromatics, a major class of industrial pollutants, may be oxidatively metabolised to chlorocatechols by soil and water microorganisms which have evolved catabolic activities towards these xenobiotics. We show here that 4-chlorocatechol can be further transformed by enzymes of the ubiquitous 3-oxoadipate pathway in a novel reaction to protoanemonin, an ordinarily plant-derived antibiotic which is toxic to microorganisms. This finding provides one explanation for the ecotoxicity of chloroaromatics.

Cloning and analysis of Rhizobial dehalogenase genes

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The widespread use of halogenated organic compounds as herbicides, pesticides, solvents and intermediates in the chemical industry makes them one of the largest groups of environmental pollutants. Consequently, there is much interest in reactions which lead to detoxification of such compounds.

We have shown previously that a *Rhizobium* sp., isolated through its ability to grow at the expense of the herbicide 2,2-dichloropropionic acid, contains three distinct, inducible, dehalogenase enzymes with individual substrate specificities.

To investigate the genes encoding these enzymes a plasmid-based Rhizobial genomic library was constructed in *Escherichia coli* NM522 and screened for the ability to grow with D,L-2-chloropropionic acid. Two clones were obtained, pSC1 and pSC530, that contained, respectively, a 6.5 kb and 3.0 kb *Eco*RI insert fragment. Restriction mapping and Southern blotting analysis has shown that the two clones are unrelated to each other but both hybridised to Rhizobial genomic DNA.

A 2.2 kb subclone of pSC1 strongly expressed dehalogenases I and III and these two enzymes were purified for determination of their N-terminal amino acid sequences. The nucleotide sequence of the 2.2 kb fragment has been determined and the *hadL* and *hadD* genes encoding, respectively, dehalogenase I and III located. Consideration of the deduced amino acid sequences of these two dehalogenases has shown them to be not obviously related to each other or to any other dehalogenase presently sequenced.

Isolation of Mutants and Clones Involved in the Anaerobic Toluene Utilization (tut) Pathway of the Denitrifying Strain T1. P.W. COSCHIGANO* and L.Y. YOUNG. Rutgers, The State University of New Jersey, New Brunswick, N.J.

Strain T1 can use toluene as the sole source of carbon and energy under denitrifying conditions. A pathway has been proposed in which toluene utilization proceeds through phenylpropionyl-CoA and benzoyl-CoA. A side reaction results in the generation of the dead-end products benzylsuccinate and benzylfumarate from toluene. Mutants of strain T1 which are unable to utilize toluene as a source of carbon have been isolated by N-methyl-N'-nitro-N-nitrosoguanidine mutagenesis. The mutants have been divided into several classes based on their range of growth substrates and other characteristics.

The tutB class of mutants is blocked early in the toluene utilization pathway. These mutants are able to grow on such substrates as benzoate and phenylpropionate but not toluene. We have focused on the tutB-16 mutant, one that appears to be blocked in a key step of toluene metabolism.

A cosmid library (constructed in pLAFR3) has been generated from total DNA isolated from strain T1. Triparental matings were used to identify a clone that restored the ability of the tutB-16 mutant to grow with toluene serving as the carbon source. This cosmid clone carries DNA which also has the ability to complement tut alleles of other classes. Subcloning experiments have better defined the region essential for complementation. Further characterization to identify the gene(s) that lie in this region is underway.

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SINGLE MUTATIONS IN THE SIGNAL RECEPTOR DOMAIN OF XYLR RESULTED IN MUTANTS THAT STIMULATE TRANSCRIPTION IN THE ABSENCE OF EFFECTORS

The XylR protein positively controls expression from the *Pseudomonas putida* TOL plasmid σ_{54}-dependent "upper" pathway operon promoter (Pu) and the *xylS* gene promoter (Ps), in response to the presence of aromatic effectors. Two mutant XylR regulators able to stimulate transcription from Pu and Ps in the absence of effectors were isolated. These mutants exhibited single point mutations, namely Asp135-Asn and Pro85-Ser. Both mutations are located in the amino-termini domain of XylR, which is thought to be responsible for interactions with effectors. The effector profile of XylRP85S was similar to that of wild-type XylR protein; however, XylRD135N exhibited an altered pattern of effector recognition: with *m*-nitrotoluene it stimulated transcription from the Pu promoter above the high basal level, whereas this nitroarene inhibited the wild-type regulator. Previous work showed that residue 172 was involved in effector interactions, as mutant XylRE172K also recognize *m*-nitrotoluene. However, a double mutant XylRD135N/E172K did not stimulate transcription in the absence of effector, but retained the ability to stimulate transcription with *m*-nitrotoluene. Transcription mediated by XylRD135N and XylRP85S from Pu::lacZ was analyzed in detalil. Like the wild-type regulator, XylRD135N and XylRP85S required σ_{54} for full transcription activation, but in contrast with the wild-type regulator, XylRD135N -but not XylRP85S- stimulated transcription from Pu in the absence of the integration host factor protein. XylRD135N, also in contrast with XylR and XylRP85S, mediated transcription from a mutant Pu promoter that lacked one of the upstream regulator binding sites, but not when both UASs were deleted. The level of autoregulation of XylRD135N was at least 2-fold higher than that found with the wild-type XylR regulator and the mutant XylRP85S.

ANALYSIS OF THE ACTIVE SITE RESIDUES IN THE 2-HYDROXYMUCONIC SEMIALDEHYDE HYDROLASE OF THE *PSEUDOMONAS PUTIDA* TOL PATHWAY.

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Biotechnological treatment of waste materials and bioremediation of polluted environments is based on the capacity of microorganisms to transform pollutants to non-toxic products. The attention in the enzymes catalyzing conversion of synthetic compounds has strongly increassed when it became clear that several xenobiotics are ubiquitously present in the environment due to their slow rates of biodegradation. Whereas there is an increasing understanding of *meta*-cleavage enzymes much less is known about the hydrolytic cleavage of carbon-carbon bonds in the degradation of aromatic compounds, which are of the most environmentally relevant xenobiotics

The 2-hydroxymuconic semialdehyde hydrolase (XylF) of the *Pseudomonas putida* TOL pathway catalyses one of the rarest of all enzyme reaction types (EC 3.7.1.9), the hydrolysis of a carbon-carbon bond in the ring-fission product of preferably 3 alkyl-substituted catechols. Equivalent hydrolases cleaving carbon-carbon bonds in pathways responsible for the biodegradation of phenol, toluene and polychlorinated biphenyls, have been cloned and their primary structure determined. In some cases, these hydrolases have been shown to be determinants of substrate specificity of the catabolic pathways. In this study, amino acid sequence comparisons between XylF and other hydrolases, and analysis of the similarity between the predicted secondary structure of XylF and the known secondary structure of the haloalkane dehalogenase from *Xanthobacter autotrophicus* strain GJ10, led us to identify several conserved residues likely to have a functional role in the catalytic center of XylF. Three amino acids were found to be arranged in a sequential order similar to the one present in α/β hydrolase-fold enzymes. To explore the participation of these residues in the catalytic triad of XylF, we performed amino acid modification studies and *in vitro* site-directed mutagenesis experiments. Our results provide direct demonstration of the involvement of the above three residues as the potential active amino acids, and confirm previous hypothesis that XylF is a serine hydrolase of the α/β hydrolase-fold family of enzymes. These studies also provide information on other conserved residues in XylF-related enzymes.

Styrene Degradation by *Pseudomonas putida* CA-3; induction and repression of the degradative pathway.

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Styrene is used in the chemical industry both as a starting material for synthetic polymers and as a solvent in the polymer processing industry. Consequently it is present in many industrial effluents, including airborne gaseous emissions where it is particularly problematic due to the malodorous and potential carcinogenic properties of the compound.

Styrene metabolism in *Pseudomonas putida* CA-3 has been shown to proceed via styrene oxide, phenylacetaldehyde and phenylacetic acid. Induction studies suggest that the styrene degradative pathway is divided into an "upper" and "lower" regulon, with styrene, styrene oxide and phenylacetaldehyde being upper pathway intermediates affecting degradation of themselves and phenylacetic acid. Whereas phenylacetic acid is a lower pathway intermediate affecting the degradation of itself and phenylacetaldehyde.

Respiration studies performed on whole washed cells grown in the presence of styrene and a variety of non-aromatic carbon sources revealed that styrene degradation is repressed in the presence of some of these carbon sources. Non aromatic carbon sources such as citrate and α ketoglutarate and amino acids such as aspartate, glutamate and proline repress styrene degradation; while non aromatic carbon sources such as oxaloacetate, malate, succinate and glucose together with the amino acids alanine, tryptophan and valine do not. Cells grown under the same repressing conditions in the presence of phenylacetic acid also failed to oxidise phenylacetic acid, indicating that both the "upper" and "lower" regulons are repressed in the same way. This repression does not appear to be cAMP mediated given that exogenous addition of cAMP to the growth medium does not aleviate the observed diauxic growth patterns.

The genes encoding the first two enzymes in the styrene degradative pathway in *P. putida* CA-3, namely styrene monooxygenase and styrene oxide isomerase, have been cloned in an attempt to study the mechanisms of induction and repression control being exerted on the operon.

Supression of mutations at the TOL meta-cleavage pathway operon promoter Pm by XylS mutant regulators.

M. Trinidad Gallegos

In vivo stimulation of transcription from Pm, the TOL *meta*-cleavage pathway operon promoter, requires the XylS regulator activated by a benzoate effector. The C-terminal end of the XylS protein is highly homologous to the C-terminal end of at least 27 different proteins involved in transcription stimulation in carbon metabolism, pathogenesis and response to alkylating agents in bacteria. A helix-turn-helix motif is located within this conserved region, found between residues 230-250 of XylS, and is probably involved in DNA binding. This set of regulators constitutes the XylS/AraC family.

In Pm, about 85 bps upstream of the main transcription initiation point are required to stimulate transcription. An almost perfect direct repeat motif rich in As between positions -44 and -72, may constitute part of the sequence recognized by the XylS regulator. Mutations at -68 C→G (Pm4) and -47 C →G, -44 A→G (Pm5) within this motif, gave promoters which in vivo exhibit only about 10% transcription level with respect to that of wild-type promoter.

We had previously isolated mutant regulators (XylSPro37Leu and XylSGly44Ser) that constitutively stimulated transcription from the wild type promoter, increasing 8 to 14-fold the level mediated by the wild-type protein in the absence of effector. These two mutants are also able to further increase transcription from Pm when 3-methylbenzoate is added to the media. We have observed that these mutant regulators are able to stimulate transcription from both mutant promoters at high levels, if and only if the effector was present in the medium.

To rule out that the mutant Pm::*lacZ* fusions used to measure ß-galactosidase activity contained artificial promoters, the transcription initiation point for the wild-type and mutant Pm promoters with wild type and mutant XylS, were determined by primer extension in cultures growing with and without 3-methylbenzoate. In all cases the main transcription initiation point was at the same position. We therefore suggest that the mutant XylS regulators do suppressed Pm4 and Pm5 mutations.

These results suggest that the substitutions Pro37Leu and Gly44Ser partially mimic the active form of XylS regulator when interacting with the Pm promoter, but subtle alterations produced by effector binding are still needed to suppress the mutations in Pm4 and Pm5. These data also support the theory that binding of effector to XylS produces conformational changes in the protein that facilitate transcription activation from Pm.

Expansion of degradative abilities towards environmental pollutants in *A. eutrophus* JMP134 (pJP4). González, B., *Clément, P., Matus, V., Ehrenfeld, N., Maturana, A., Valenzuela, J. and #Céspedes, R. Laboratorio de Microbiología, Departamento de Biología Molecular y Celular. Facultad de Ciencias Biológicas, P. Universidad Católica de Chile. Santiago, CHILE.

Because their use as biocides and their presence in treated chlorine pulp bleaching effluents, chlorinated guaiacols and phenols reach soils and water. Although the degradation of chlorophenols has been studied, catabolism of chloroguaiacols and some di and trichlorophenols remains poorly understood. The biological removal of chlorophenols in polluted environments is also inadequately known.

In this work, the degradation of chlorophenols by *Alcaligenes eutrophus* JMP134 (pJP4) was studied. This strain grow on 2,4-dichlorophenoxyacetic (2,4-D) and 3-chlorobenzoic acids and phenol; and it is resistant to Hg^{2+}. Except degradation of phenol, all these properties are encoded in pJP4. It is hypothesized that i) strains with increased degradative abilities or tolerance to higher levels of pollutants may be obtained through adaptation/selection procedures, and ii) such strains will express their improved abilities in complex systems. Then, the use of specialized chlorophenol-degrading bacteria in bioremediation of polluted environments would be explored.

Although this bacterium was unable to grow on several chlorophenols or chloroguaiacols as sole carbon and energy source, it proliferated in 4-chlorophenol, 2,4-dichlorophenol and 2,4,6-trichlorophenol (2,4,6-TCP). During growth, complete degradation of each compound was detected by chloride release, UV spectroscopy and gas chromatography (for 2,4,6-TCP). Degradation of 2,4,6-TCP (up to 0.4 mM) did not depend on the presence of pJP4, since the cured strain JMP222 also expressed this property. In chemostats fed with 2,4,6-TCP (0.4 mM) or phenol (1mM) plus 0.05 mM 2,4,5-TCP, the strain was adapted to mineralize (CO_2 evolution) 2,4,5-TCP.

The ability of strain JMP134 to survive and degrade chlorophenols in pulp bleaching effluent treatment microcosms was assessed in aerated batch cultures. If this biodegradable organic matter- and chlorophenol-containing effluent was previously sterilized by filtration, it allowed the proliferation of the strain JMP134. When 2,4-D, 2,4,6-TCP or phenol were added, complete degradation of these compounds was detected. With non sterile effluents, removal of these compounds took place but strain JMP134 represented about 1% of total bacterial population, after 15 days of incubation.

Strain JMP134 resists higher levels of $HgCl_2$ (0.4 mM) or merbromin (2 mM) in rich medium, but the resistance to mercury dropped significatively in the presence of chloroorganics as carbon sources. We are now attempting the adaptation of strain JMP134 to tolerates higher levels of mercury while grow on chloroorganics. This resistance will be used as a marker during conjugal transfer from *A.eutrophus* JMP134 to other catabolic strains. The near term goal is to transfer pJP4 encoded functions to *Acinetobacter junii* 5ga, a guaiacol-degrading strain able to metabolize mono and dichloroguaiacols but not able to degrade mono, di- or trichlorophenols.

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Engineering of mobile *catabolic segments* in *Pseudomonas* for enhanced biodegradation of chloro-aromatic compounds

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Adaptation of bacteria to novel carbon sources frequently accompanied by the acquisition or loss of single or multiple DNA segments which encode the functions required for survival. One archtypical case of adaptation is the sequence of molecular events which lead to the assembly of metabolic pathways for the degradation of xenobiotic compounds, in which analysis of the DNA sequences of the corresponding catabolic genes and operons suggests that they have resulted from joining DNA segments recruited from preexisting pathways. Modularity of the genetic complement of bacteria implies the existence of *natural genetic engineering systems* to assemble the various sequence components of the different elements. Catabolic operons are frequently included within transposons, which use to have the capacity to exchange DNA segments through site-specific recombination systems. A series of transposon vectors derived from Tn5 have been constructed in the past, which permit the insertion and stable inheritance of heterologous DNA elements into the chromosome of target bacteria, a process which resembles to some extent the existing mechanisms of acquisition or loss of adaptation-related phenotypes. In a further step to develop tools for metabolic engineering inspired by natural processes of DNA insertion/excision, the ability of the multimer resolution system (*mrs*) of broad host range plasmid RP4 has been exploited to add heterologous DNA segments, devoid of any phenotypic marker, to the genome of target gram-negative bacteria. Utilization of this new type of *mrs*-vectors leaves little room for the somewhat arbitrary distinction between "recombinant" and "natural" bacteria, since they mimic faithfully the natural processes of DNA shuffling between distant locations of the bacterial genome.

Various DNA segments encoding portions of the TOL (toluene/xylenes), TOD (Toluene), NAH (naphtalene/salicylate), *bph* (biphenyl/PCBs) and *tcb* (trichloro bencene) pathways of *Pseudomonas* have been constructed and introduced in transposon vectors with excisable or environmentally harmless selection markers such as resistance to potassium tellurite. Insertion of one or more of these segments into the chromosome of bacteria with other natural biodegradative properties results in the generation of novel strains with enhanced catabolic phenotypes. In some archtypical cases, such as aerobic degradation of PCBs, the efficiency of the pathways is limited by the poor inducibility of the catabolic genes by the substrates of interest, and the problem of feedback inhibition by intermediates at early stages of the biodegradation process. To enhance expression of catabolic genes in response to the desired substrates, the regulatory elements of each of the native pathways have been replaced by a single heterologous transcriptional control based on a transcriptional cascade responsive to the presence of benzoates in the medium. The construction of modified pathways for improved metabolism of various PCB congeners and chloro-toluenes using this approach will be discussed.

Expression of the Xylene Monooxygenase Encoding Genes

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The TOL plasmid pWW0 of *Pseudomonas putida* determines the oxidative catabolism of toluene/xylenes down to Krebs cycle intermediates via initial conversion into benzoate/toluates (upper pathway) followed by *meta*-ring cleavage of a derived catechol (*meta* pathway). The first enzyme of this pathway is the xylene monooxygenase, which oxidizes toluene and xylenes to (methyl)benzyl alcohols. The enzyme is composed of two different polypeptides. XylA, the NADH:acceptor reductase component of xylene monooxygenase consists of two domains, an amino-terminal ferredoxin [2Fe-2S] domain and a carboxy-terminal ferredoxin reductase domain. The proposed electron flow is from NADH > FAD(XylA) > [2Fe-2S] (XylA) to XylM, the hydroxylase component. XylM is a transmembrane polypeptide and its primary structure resembles that of AlkB, a component of the alkane monooxygenase system of *Pseudomonas oleovorans*. The two components of the xylene monooxygenase are encoded by the genes *xylM* and *xylA*. Two different expression systems were used to generate translational fusions and express these genes in *Escherichia coli*. Application of the P*alk* /AlkS expression system resulted in an over-production of the membrane protein XylM. In addtion, the T7-RNA polymerase expression system was used for specifical labeling of the two components.

Genetic Organization of the Genes for Polycyclic Aromatic Hydrocarbon Degradation from *Beijerinckia* sp. strain B1

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Beijerinckia sp. strain B1 is known to be versatile in its ability to metabolize a number of monocyclic and polycyclic aromatic hydrocarbons (PAHs) as carbon and energy sources, including *m*-xylene, biphenyl, naphthalene, and phenanthrene. In addition, *Beijerinckia* B1 can cometabolize a number of PAHs, such as acenaphthene, benz[a]anthracene, dibenzothiophene, and acenaphthylene, following induction by either biphenyl or *m*-xylene. The wide range of substrates oxidized by *Beijerinckia* B1 may be attributed to the relaxed specificity of the initial enzymes involved in biphenyl degradation, which proceeds through dioxygenase attack on the aromatic ring. Mutagenesis and reversion experiments demonstrated that *Beijerinckia* sp. strain B1 could lose and recover the ability to grow on both biphenyl and *m*-xylene simultaneously, suggesting that genes for the two catabolic pathways may be in the same operon or may be under control of the same regulatory gene(s). In order to study the genetic basis of these pathways in more detail, the genes responsible for the degradative ability were cloned. A genomic library was constructed in *E. coli* S17-1 using the cosmid pHC79. Colony blot experiments were performed against the cosmid clones using a previously cloned 3.0 kilobase pair *Cla*I DNA fragment from *Beijerinckia* B1 containing the catechol dioxygenase gene (*xylE*) as a probe. One cosmid clone out of 700 clones tested showed homology to the probe and was subsequently shown to express catechol dioxygenase activity. Restriction mapping, subcloning, and site-directed mutagenesis data suggests involvement of three adjacent operons in the metabolism of biphenyl and other aromatic hydrocarbons. Nucleotide sequence homology studies show that *bphA1* and *xylMA* are present in the same operon. A second, adjacent operon has significant homology with the *meta* operon of the TOL plasmid, and contains the genes for the degradation of *m*-toluate. This second operon is transcribed in the opposite direction from the first operon. Interestingly, the genes for cis-biphenyl dihydrodiol dehydrogenase (*bphB*) is located at the end of the *meta* cleavage operon. A third operon contains the genes for 2,3-dihydroxybiphenyl 1,2-dioxygenase (*bphC*) and 2-hydroxy-6-oxo-6-phenyl-hexa-2,4-dienoic acid hydrolase (*bphD*). Therefore, it is speculated that the gene clusters may have been recruited through a recombinational event(s). A 22 kilobase pair *Hin*dIII DNA fragment, containing the *xyl* gene clusters, was cloned into the broad host range vector pRK415 and subsequently conjugated into *Pseudomonas putida* PPO200. The recombinant strain showed growth on *m*-toluate, an intermediate of the metabolic pathway. In addition, the cloned genes of two catechol dioxygenases have been expressed in *E. coli* at higher levels than in the parent strain, and found to be able to cleave a wide range of 1,2-dihydroxylated compounds, which reflects the wide substrate range of the parental strain. Full understanding of the genetic structure of the genes for aromatic hydrocarbon degradation by *Beijerinckia* B1 supplies needed information for applications in the environmental field.

Naphthalene dioxygenase of *Pseudomonas stutzeri* AN10: Isolation and characterization of dihydrodiols as novel metabolic products.

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Pseudomonas stutzeri AN10 is a naphthalene degrading strain isolated from marine sediments. The strain is able to grow with naphthalene and 2-methylnaphthalene as the only carbon and energy source. Recently, the upper pathway genes of this strain have been cloned, and it's organization, and expression both in the wild strain and in *E. coli*, have been elucidated. The metabolic pathway seems to be rather non-specific, and the strain is able to co-metabolise 1-methyl, 2-bromo, 1-chloro, and 2-chloronapthalene to the corresponding salicylates.

Mutagenesis with transposon Tn1000 of the cloned genes in pUC118 rendered a set of catabolic mutants, each of them lacking expression of a gene, or a group of genes. One of them, described as γδ-14, expressed only the first gene of the operon, *nahA*, encoding naphthalene dioxygenase, the enzyme responsible for the conversion of naphthalene to 1,2-dihydroxy-1,2-dihydronaphthalene. Restriction mapping showed that Tn1000 was inserted, in that mutant, downstream of *nahA*, presumably affecting the expression of the gene *nahB*, coding for the 1,2-dihydroxy-1,2-dihydronaphthalene dehydrogenase. In the presence of naphthalene, γδ–14 was unable to metabolise the dihydrodiol formed, and as a consequence, it was accumulated and excreted into the medium.

A study of the metabolites accumulated from different substrates —alkylic and halogenated naphthalenes, phenanthrene and anthracene— has been carried out. In all cases, the product detected and isolated has been the corresponding dihydrodiol. HPLC, H^1-NMR and ROESY bi-dimensional NMR experiments were the techniques used for the isolation and structural determination.

In all cases, no hydroxylation or oxidation in the alkylic substituent was detected. The dihydoxylation affected the aromatic ring in the 7,8 position in the case of naphthalene derivatives, except when 1-bromo, 1-chloro, and 1,3-dimethylnaphthalene were tested. In that case, two metabolites were detected, one showing the 7,8 substitution, and a different one in which the dihydroxylation was found in the 5,6 position. This was the main metabolite in the case of 1,3-dimethylnaphthalene, in contrast to the halosubstitution in position 1, in which 7,8 substitution was the more prominent. On the other hand, dihydroxylation of phenantrene was produced in the 3,4 position. No metabolite was detected in the case of anthracene.

In *E. coli*, the level of enzymatic activity —NahA— depends on the substrate compound; aromatics with a large substituent in the 1- position and the polysubstituted ones, are metabolised at a rather low rate.

INHIBITION OF AROMATICS DEGRADATION BY ALTERNATIVE SUBSTRATES IN Pseudomonas putida.

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Pseudomonas putida bearing the TOL plasmid pWW0 can mineralize toluene, xylenes and related hydrocarbons to Krebs cycle intermediates. This degradation occurs via the sequential upper and meta-cleavage pathways, encoded by the TOL plasmid-borne xyl genes, clustered in the upper and meta operons, respectively. The expression of these operons is under the positive control of two regulatory proteins, XylR and XylS, that are activated in the presence of effectors of the pathway.

Usually, this process has been analyzed under conditions in which the target compound is present as the sole growth-supporting substrate. Several observations have been made in different conditions indicating that the presence of alternative substrates has negative effects on the degradative capability of P. putida. In batch cultures, the degradation of benzyl alcohol by P. putida (pWW0) is inhibited in the presence of glucose. In continuous cultures, the levels of the TOL enzymes involved in degradation was determined in the presence and in the absence of succinate, a primary carbon and energy source for pseudomonads. We observed that, when growing on limiting concentrations of succinate, the enzymes were expressed to high levels in response to the addition of an upper pathway inducer. However, when succinate was supplied at nonlimiting concentrations, no expression of the TOL enzymes was observed after addition of the inducer. The analysis of mRNA synthesis from the four pWW0 plasmid promoters involved in the degradation of aromatics, both in batch and in continuous cultures, showed that this inhibition was exherted at the level of transcription from both the upper pathway operon promoter Pu and the xylS promoter Ps. However, the expression from the meta-cleavage pathway promoter Pm was not affected by the presence of additional carbon sources.

ENZYMES INVOLVED IN THE DEGRADATION OF AROMATIC COMPOUNDS BY LIGNINOLYTIC FUNGI FROM THE GENUS *Pleurotus*

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The white-rot basidiomycetes are the only organisms carrying out extensive degradation of lignin, the most recalcitrant biopolymer in nature. In order to depolymerize and mineralize this aromatic heteropolymer they developed an unspecific degradation mechanism which has been defined as "enzymatic combustion" (4). Most investigations on lignin degradation by these fungi focus on the development of alternative - environmentally friendly - biological procedures for the removal of lignin during paper manufacture. However, several studies have demonstrated that the same fungi can degrade different xenobiotic compounds involved in soil and water contamination. One of the ways to introduce ligninolytic fungi in contamined soils, is the use of a lignocellulosic substrate - e.g. cereal straw - which, after fungal development under solid-state fermentation conditions, is mixed with the soil. The fungi from the genus *Pleurotus* are very well adapted to grow on cereal straw and, consequently, they are adequate organisms for soil bioremediation. Moreover, the study of their degradative abilities has been considered of interest due to the capacity to delignify agricultural wastes (5). At the present, four groups of degradative enzymes are being investigated in these fungi: extracellular aryl-alcohol oxidase (AAO), Mn-dependent peroxidase (MnP), and phenoloxidase (laccase), and intracellular reductases of aromatic compounds. The three extracellular enzymes have been already purified and partially characterized with respect to protein characteristics and catalytic properties. *Pleurotus* laccase oxidizes different substituted phenols but, in the presence of adequate intermediates, it can also oxidize non-phenolic compounds (6). *Pleurotus* MnP differs in several aspects (e.g. its low inactivation by hydrogen peroxide) from similar enzymes described in *Phanerochaete chrysosporium*, the most studied ligninolytic organism. However, all of them produce Mn(III), and strong oxidant which, in the form of chelates, can play an important role in biodegradation. AAO (2), in cooperation with intracellular dehydrogenases reducing aromatic acids and aldehydes, is responsible for the relatively high levels of peroxide detected in cultures of *Pleurotus*. Peroxide generation by the redox cycling of aromatic compounds, synthesized by the fungus (3) has been for the first time described in *Pleurotus* (1) but it can also operate in other white-rot fungi. This compound is necessary for the activity of MnP, but it can be also involved in the generation of reduced oxygen species, namely hydroxyl radicals in the presence of Fe(II). In the reduction of Fe(III), the most frequent state of iron, to Fe(II) a second group of intracellular enzymes could be involved. Effectively, several experiments demonstrate that different quinones are quickly reduced by myceliar reductases to the corresponding hydroquinones and that hydroquinone oxidation generates superoxide radicals, which can reduce Fe(III) to Fe(II). The hydroxyl radicals, formed from hydrogen peroxide in the presence of Fe(II), are highly reactive and can be involved in the degradation of aromatic compounds.

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CHARACTERIZATION OF A REGULATOR OF THE 4-HYDROXYPHENYLACETATE 3-HYDROXYLASE OPERON OF *Escherichia coli*. A MEMBER OF XylS/AraC FAMILY.

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In recent years, there has been a considerable interest to explore the ability of microorganisms to degrade and detoxify the increasing amounts of phenol and structurally related compounds which enter the environment as by-products of many industrial processes. Most of the present knowledge on bacterial aromatic catabolism stems from investigations with the genus *Pseudomonas*. Enteric organisms have also been studied occasionally, but the ability of *Escherichia coli* to degrade certain aromatic compounds such as 3- or 4-hydroxyphenylacetic acids (3-HPA, 4-HPA) was not explored until recently. These catabolic pathways have been found to be biochemically identical to those occurring in various soil bacteria.

We have cloned and expressed in *E.coli* K12 a DNA fragment of *E. coli* W (ATCC 11105) that contains the genes encoding the two-protein components of the 4-hydroxyphenylacetate 3-hydroxylase. These genes belong to the *hpa*BC operon. Recently, we have been able to clone another DNA fragment of this strain which contains the catabolic *meta* pathway of the 4-HPA. Molecular analysis of this fragment revealed that it is located adjacent to the 4-HPA hydroxylase operon. The *hpa*BC operon is expressed by a promoter inducible by 4-HPA. A gene, named *hpa*A, has been identified 274 pb upstream the *hpa*BC operon. The aminoacid sequence of HpaA shows a significant similarity with MelR, a member of the XylS/AraC family of regulators. The C-terminal region of HpaA shows the conserved motif and a putative DNA binding domain organized as a helix-turn-helix motif found in the C-terminal domain of this family of regulators. Our data also provide experimental evidence suggesting that the *hpa*A gene is a regulator of the *hpa*BC operon.

ANALYSIS OF THE UPPER PATHWAY OF PCB-DEGRADATION ENCODED BY THE *bph* LOCUS OF *Pseudomonas* sp. LB400. M. Seeger [1,2], C.A. Jerez[1], K.N. Timmis[2] and B. Hofer[2] [1]Departamento de Bioquímica, Facultad de Medicina, Universidad de Chile, Santiago, Chile, [2]Bereich Mikrobiologie, Gesellschaft für Biotechnologische Forschung, Braunschweig, Germany.

Polychlorinated biphenyls (PCBs) consist of 209 congeners that differ in number and/or position of the chlorines. Properties of these xenobiotics such as persistence, bioaccumulation, toxicity and carcinogenicity make them a serious environmental and health problem. For decontamination of large areas, bioremediation seems to be a promising approach. *Pseudomonas* sp. LB400 is capable of attacking an unusually broad spectrum of PCBs. In order to determine the substrate spectra of the individual PCB catabolic enzymes of LB400, and threreby their contribution to the global specificity of the pathway PCB degradation products and the extent of PCB degradation by *E. coli* K-12 derivatives carrying hybrid plasmids containing appropiate *bph* genes from LB400 was investigated. PCB metabolites were analysed by analytical techniques such as GC-MS, HPLC, UV and visible spectroscopy. All of the mono- and almost all of the dichlorinated biphenyls were degraded to chlorobenzoates. Most of the tri- and the tetrachlorinated biphenyls analysed were incompletely metabolized yielding different dead-end products. Interestingly, biphenyl 2,3-dioxygenase attack on some *ortho*-chlorinated biphenyls was found to directly lead to dihydroxy biphenyls through elimination of hydrochloric acid.

ALKANE OXIDATION BY PSEUDOMONAS STRAINS

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Many Pseudomonads are known to metabolize linear alkanes. In several cases, the enzymology of alkane oxidation by these strains has been investigated. However, only *Pseudomonas oleovorans* has been analyzed in detail with respect to both the enzymology and the genetics of alkane metabolism. In *P. oleovorans*, a three component alkane hydroxylase system, an aldehyde and an alcohol dehydrogenase catalyze the consecutive oxidation of alkanes to 1-alkanols, 1-alkanals, and the corresponding carboxylic acids. The genes which encode these functions are organized in two operons, localized on the OCT-plasmid.

We have collected bacteria that, like *P. oleovorans*, are able to grow on linear alkanes, and have started to characterize the genes and enzyme systems that allow these bacteria to metabolize alkanes. Previous reports indicate that while some of the strains may possess completely unrelated enzyme systems, other strains contain enzyme systems that are closely related to the *P. oleovorans* system.

In Southern blots we found that several *P. aeruginosa* strains contain genes that are virtually identical to the *P. oleovorans alk*-genes (most restriction sites conserved). An example is the paraffin hydroxylase of *P. aeruginosa* KSLA 473. In a Western blot analysis we found that antibodies directed against the three components of the alkane hydroxylase system can be used to detect the three components of the *P. aeruginosa* paraffin hydroxylase. In addition, we found that the substrate range of the two enzyme systems is strikingly similar. Other strains contain more divergent genes (some or no sites conserved in Southern blots, or no signal at all).

Workshop on Molecular Basis for Biodegradation of Pollutants
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POSTER ABSTRACT

Detection of dichloromethane dehalogenase genes by PCR

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Over the last 15 years, 14 strains have been isolated and characterized by our laboratory which possess the ability to grow on dichloromethane as the sole carbon and energy source (1). In the case of *Methylobacterium sp.* DM4 (2) and of *Methylophilus sp.* DM11 (3), the gene encoding the dehalogenase enzyme has been cloned and sequenced. The enzymes are moderately related at the gene and protein level (56% and 75% identity, respectively). From a functional point of view as well as basing on their sequences, these enzymes belong to the theta-subclass of eukaryotic glutathione-S-transferase enzymes. Interestingly, only very few other glutathione-S-transferase sequences are available so far for bacteria, and they are only distantly related to other members of this enzyme family. Dichloromethane being a man-made compound previously unknown in nature, we are interested in a better understanding of how such dehalogenase genes have arisen, and in the detection and characterization of related dehalogenase and glutathione-S-transferase genes.

Different sets of degenerate primers were designed which correspond to nucleotide sequences conserved between DM4 and DM11 dehalogenase genes. Such primers have permitted the specific amplification of dehalogenase genes in all DM strains characterized in this laboratory and thus complement and extend the data obtained by Gälli and Leisinger using DNA hybridization techniques (4, and R. Gälli, Ph. D. Thesis). No amplification was observed with other methylotrophic bacteria such as *Methylobacterium extorquens* AM1 and *Methylobacterium organophilum* XX, which cannot grow on dichloromethane. In addition, PCR amplification using primers corresponding to the N-termini of DM4 and DM11 dehalogenases have confirmed that the dehalogenase genes of all other DM strains are of the DM4-type, confirming previously observed DNA hybridization patterns (5).

The availability of these primers now allows an easy and rapid screening of the bacterial world for the presence of dichloromethane dehalogenase genes using the PCR technique. It will hopefully contribute to a better knowledge of the occurrence of dehalogenase genes in bacteria and allow the detection and characterization of glutathione-S-transferase related genes in sites which have been contaminated by dichloromethane.

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