Biodegradation of Aromatic Compounds by Nocardioform Actinomycetes

CHANG-JUN CHA and CARL E. CERNIGLIA

Division of Microbiology, National Center for Toxicological Research,

U.S. FDA, Jefferson, AR 72079, U.S.A.

Abstract

Mycolic acid-containing gram-positive bacteria, so called nocardioform actinomycetes, have become a great interest to environmental microbiologists due to their metabolic versatility, multidegradative capacity and potential for bioremediation of priority pollutants. For example, *Rhodococcus rhodochrous* N75 was able to metabolize 4-methylcatechol via a modified β-ketoadipate pathway whereby 4-methylmuconolactone methyl isomerase catalyzes the conversion of 4-methylmuconolactone to 3-methylmuconolactone in order to circumvent the accumulation of the 'dead-end' metabolite, 4-methylmuconolactone. *R. rhodochrous* N75 has also shown the ability to transform a range of alkyl-substituted catechols to the corresponding muconolactones. A novel 3-methylmuconolactone-CoA synthetase was found to be involved in the degradation of 3-methylmuconolactone, which is not mediated in a manner analogous to the classical β-ketoadipate pathway but activated by the addition of CoA prior to hydrolysis of lactone ring, suggesting that the degradative pathway for methylaromatic compounds by gram-positive bacteria diverges from that of proteobacteria. *Mycobacterium* sp. Strain PYR-1 isolated from oil-contaminated soil was capable of mineralizing various polyaromatic hydrocarbons (PAHs), such as naphthalene, phenanthrene, pyrene, fluoranthrene, 1-nitropyrene, and 6-nitrochrysene. The pathways for degradation of PAHs by this organism have been elucidated through the isolation and characterization of chemical intermediates. 2-D gel electrophoresis of PAH-induced proteins enabled the cloning of the dioxygenase system containing a dehydrogenase, the dioxygenase small (β)-subunit, and the dioxygenase large (α)-subunit Phylogenetic analysis showed that the large α subunit did not cluster with most of the known sequences except for three newly described α subunits of dioxygenases from *Rhodococcus* spp. and *Nocardioides* spp. 2-D gel analysis also showed that catalase-peroxidase, which was induced with pyrene, plays a role in the PAH metabolism. The survival and performance of these bacteria raised the possibility that they can be excellent candidates for bioremediation purposes.

1. Introduction

An ecosystem consists of a complex ecological community and environment that forms a functioning whole in nature. Microorganisms occupy niches within all of the earth's ecosystems and, due to their metabolic and growth capabilities, are important determinants of the chemical nature of the ecosystems in which they reside. It is notable that microorganisms present in some natural ecosystems that have been contaminated with xenobiotic chemicals have been isolated and shown to be capable of modifying and degrading the contaminants. A large proportion of organic carbon in

nature is contributed by aromatic compounds containing benzene nuclei and are subsequently locked up in ecosystem. Lignin, a major component of plant tissue, is known as the most abundant natural source of the aromatic ring. From a geological point of view, such a huge amount of organic carbon must be recycled within the ecosystem. Increasing numbers and amount of man-made organic pollutants including pesticides, herbicides, detergents, pharmaceuticals, dye-stuffs and industrial effluents, are also challenging the environment and are of growing concern. Persistence of these xenobiotics in the environment causes a serious human health hazard as many of these chemicals are known to be highly toxic, mutagenic, or carcinogenic. Microbial degradation is the main force to remove the majority of such chemicals in the natural environment.

Nocardioform actinomycetes are a group of mycolic acid-containing gram-positive bacteria which display fragmentation of vegetative hyphae, encompassing the genera *Corynebacterium, Gordona, Mycobacterium, Nocardia, Rhodococcus, and Tsukamurella*. Their catabolic capabilities encompass not only a wide range of natural organic compounds, but also a variety of xenobiotic compounds including methylaromatics, chloroaromatics, nitroaromatics, halogenated alkanes, alicyclic compounds, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyl, and steroids. Therefore, the range of biotransformations mediated by these bacteria suggests that they could be seen to complement the pseudomonads in metabolic versatility. Tolerance to starvation, frequent lack of catabolite repression, and environmental persistence make them excellent candidates for bioremediation treatments (1). In this paper, two cases of biodegradation studies by nocardioform actinomycetes will be described, alkylaromatic degradation by a *Rhodococcus*, and PAH degradation by a *Mycobacterium* species.

2. Degradation of alkylaromatics by *Rhodococcus rhodochrous* N75

Alkyl-substituted aromatics are commonly found in petroleum, coal tar and in the liquors of coking oven waste, as well as many synthetic compounds. Microbial degradation of alkylcatechols generally proceeds via *meta*-cleavage pathway. *Ortho*-cleavage of alkyl-substituted diphenols had been considered for a long time to be excluded from microbial metabolism. *Rhodococcus rhodochrous* N75 and other nocardioform actinomycetes are able to utilize *p*-toluate as a sole source of carbon. 4-Methylcatechol is metabolized via a modified β-ketoadipate pathway whereby 4-methylmuconolactone methyl isomerase catalyzes the conversion of 4-methylmuconolactone to 3-methylmuconolactone. Thus, these distinct bacterial groups have circumvented the accumulation of the 'dead-end' metabolite (4-methylmuconolactone) resulting from the misrouted lactonization by evolving a new isomerase enzyme (2,3) *R. rhodochrous* N75 has also shown the ability to transform a range of alkyl-substituted catechols to the corresponding muconolactones since the two enzymes initiating the pathway, catechol 1,2-dioxygenase and *cic,cis*-muconate cycloisomerase, possess broad substrate specificities (4).

A novel 3-methylmuconolactone-CoA synthetase was found to be involved in the degradation of 3-methylmuconolactone, which is not mediated in a manner analogous to the classical β-ketoadipate pathway but activated by the addition of CoA prior to hydrolysis of lactone ring (5). It is specifically induced by growth of *R. rhodochrous* N75 on *p*-toluate as a sole source of carbon. The enzyme is highly specific for its substrate and little or no activity with other monoene and diene

lactone analogues. The high specificity of the CoA synthetase may imply a physiological role of the methyl isomerization from the 4-isomer to the 3-isomer. Thus, a new modified *ortho*-cleavage pathway is postulated (Figure 1), suggesting that the degradative pathway for methylaromatic compounds by gram-positive bacteria diverges from that of proteobacteria; *Ralstonia eutropha* JMP134 employs a pathway analogous to the classical β-ketoadipate pathway by evolving an isomeric muconolactone isomerase for the metabolsim of 4-methylmuconolactone (6). The new modified pathway also implies a metabolic divergence between prokaryotes and eukaryotes. In nocardioforms, CoA-activation of β-ketoadipate occurs before the lactone ring opening, whereas it takes place after the delactonization in the yeast *Trichosporon cutaneum* (7).
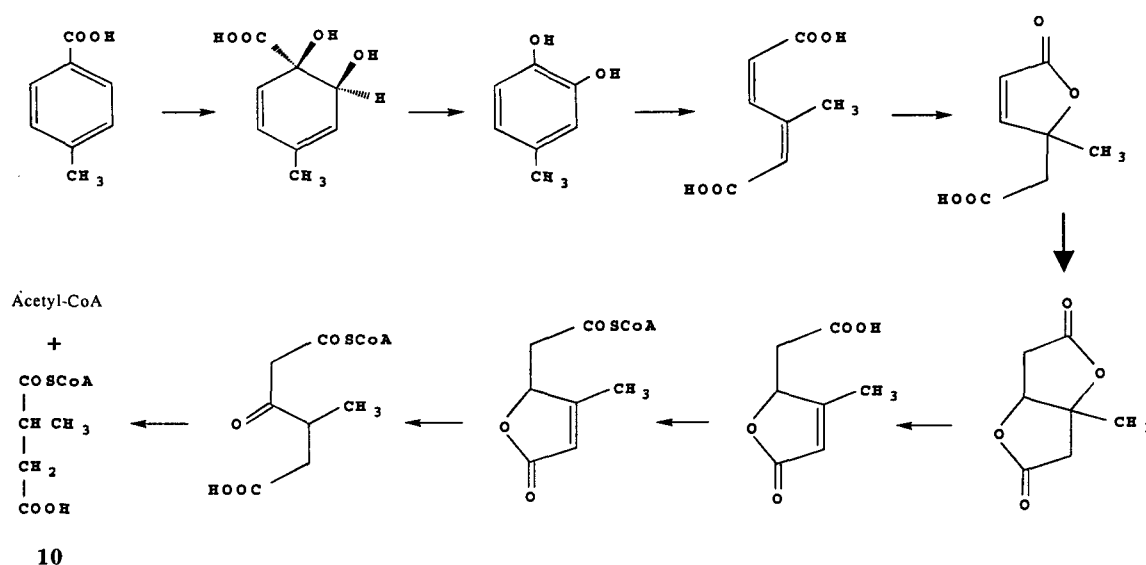

FIG. 1. Proposed pathway for the dissimilation of *p*-toluate in *R. rhocochrous* N75

3. PAH degradation by *Mycobacterium* sp. PYR-1

PAHs are an important class of toxic contaminates that have been shown to be metabolized by microorganisms (8). PAHs are compounds having 2 or more fused rings. They are environmentally persistent compounds that are ubiquitous in aquatic and terrestrial ecosystems. Some molecular structures of PAHs are present naturally in fossil fuels, whereas others are generated by combustion of fuels, as byproducts of industrial processes and in the cooking of foods. Exposure to PAHs constitutes a significant health risk for people living in industrialized areas of the world. Several of these compounds, notably anthracene, phenanthrene, chrysene, fluoranthrene, pyrene and benzo[*a*]pyrene are toxic to mammals. Some PAHs, including several mentioned above, are also carcinogenic and genotoxic. Because of their genotoxicity, the U.S. Environmental Protection Agency has listed 16 PAHs as priority pollutants to be monitored in industrial effluents (9). Of additional concern, is the increasing presence of nitropolycyclic aromatic hydrocarbons, which are present in cigarette smoke

and emissions from gasoline and diesel engines, wood-burning stoves and coal-burning power plants. These compounds are mutagenic in bacteria and carcinogenic in experimental animals.

PAHs are hydrophobic compounds and their persistence within ecosystems is due chiefly to their low water solubility. These compounds are taken up readily by suspended particles in aquatic ecosystems and are deposited into sediments upon settling of the particles. The concentration of PAHs varies widely depending on the level of area development and contamination with petroleum products. For example, PAH contamination ranged from a low of 5 ng/g of soil in an undeveloped area to 1.79 x 10^6 ng/g at an oil refinery (8). Additionally, the concentration of PAHs in marine sediments can exceed 100,000 ng/g in urban estuaries. The PAHs in soils and aquatic ecosystems can constitute a threat to public health by entering ground water supplies and by entering the food chain through exterior contamination of food substances and through the feeding habits of lower organisms.

A wide variety of bacteria, fungi, and algae have the ability to metabolize PAHs (8). The number of bacterial genera documented to metabolize PAHs in soil and marine environments ranges from 22 to 25 with the most often isolated genera being *Achromobacter*, *Acinetobacter*, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Flavobacteria*, *Nocardia*, *Sphingomonas*, *Mycobacterium*, and *Pseudomonas*. As many as 31 genera of fungi have been documented to metabolize PAHs. Bacteria, like fungi and algae, begin the metabolism of PAHs through enzymatic activity that involves the incorporation of molecular oxygen. However, there is a difference in the initial incorporation of oxygen because bacteria utilize dioxygenase enzymes to metabolize the PAHs to *cis*-dihydrodiols, whereas fungi and other eukaryotic organisms utilize monooxygenases to yield *trans*-dihydrodiols (Figure 2) (8). The bacterial oxidation of PAHs may proceed by additional attack by dioxygenase enzymes yielding dihydroxylated intermediates ultimately resulting in ring fission and assimilation of the PAH carbon into
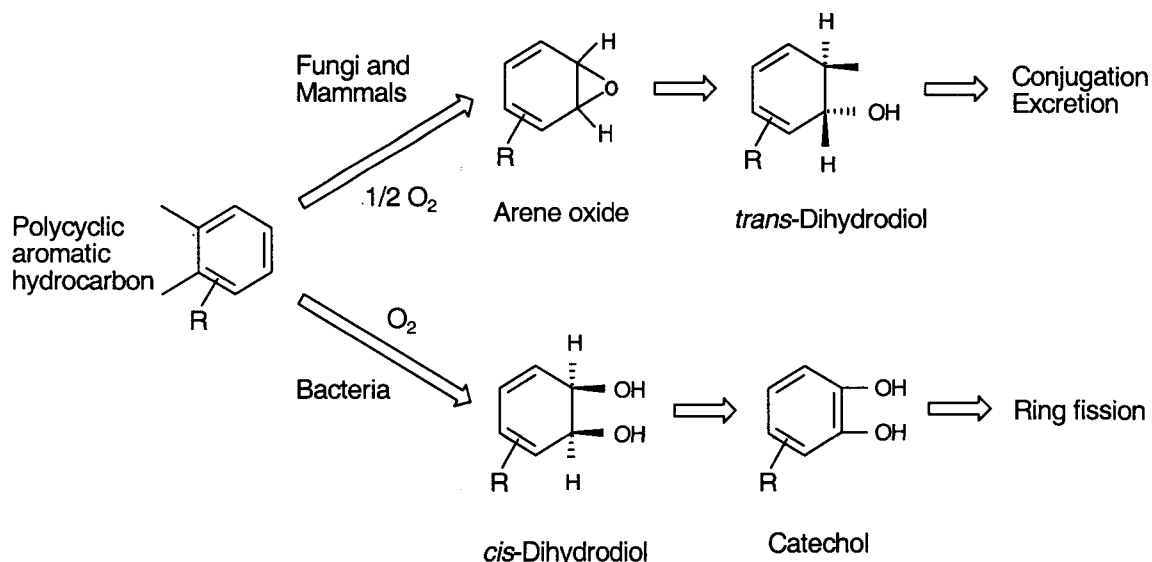

FIG. 2. Initial reactions in the oxidation of polycyclic aromatic hydrocarbons

cellular material. In general, many bacterial genera can rapidly metabolize the lower weight PAHs such as naphthalene, whereas few bacteria have been isolated that can degrade higher molecular weight molecules such as benzo[a]anthracene, fluoranthrene, pyrene, or benzo[a]pyrene. The metabolism of these molecules is generally much slower than that for naphthalene (8).

Using enrichment culture techniques, Heitkamp and Cerniglia isolated a mycobacterium from oil-contaminated soil and showed that it could completely mineralize naphthalene, pyrene, fluoranthrene, 1-nitropyrene, 6-nitrochrysene, and 3-methylcholanthrene (10-17). The results of tests for classifying this organism suggest that it is a new (undescribed) species of the genus Mycobacterium (18). The pathways for degradation of various PAHs by this organism have been elucidated through the isolation and characterization of intermediates. This organism has been shown to be unique among prokaryotes in two ways; it could metabolize the high molecular weight PAHs phenanthrene, pyrene, and fluoranthrene more rapidly than naphthalene in microcosms (11,12), and a trans-dihydrodiol characteristic of monooxygenase-mediated attack carried out by eukaryotic organisms was isolated from a pure culture. Production of cis-dihydrodiols by dioxygenase-mediated attack was the preferred method of metabolism (15). Initial attempts to isolate plasmid DNA were unsuccessful suggesting that the ability to metabolize PAHs is contained within genes on the chromosome. It is notable that other strains of Mycobacterium have been isolated that possess plasmids that are thought to contain genes for PAH degradation. Because PAHs vary in structural complexity and solubility, these results raise the possibility that the organism contains more than one dioxygenase enzyme capable of mediating the initial attack on these molecules. The findings also raise the possibility that the organism has unique structure or functional attributes that contribute to its ability to metabolize the higher molecular weight compounds. It is hypothesized that the most likely cellular attribute is the cell envelope that is hydrophobic with a waxy-like consistency from the presence of mycolic acids.

4. A new approach to biodegradation research

An emerging field for the analysis of biological system is the study of the complete protein complement of the genome, the 'proteome'. Two-dimensional (2-D) gel electrophoresis has been widely used for proteomic analysis (19). Using this technique, a 81-kDa protein from Mycobacterium sp. PYR-1 was recovered after it was expressed in response to exposure of the strain to the polycyclic aromatic hydrocarbon pyrene (20). Following the N-terminal amino acid determination, the gene encoding the 81kDa protein was isolated and identified as katG for catalase-peroxidase. In addition, 2-D gel electrophoresis of PAH-induced proteins from the cultures of Mycobacterium sp. PYR-1 was used to detect proteins that increased after PAHs exposure. Comparison of proteins from induced and uninduced cultures on 2D gels indicated that at least six major proteins were expressed (105, 81, 52, 50, 43, and 13 kDa) (Figure 3). The N-terminal sequence of the 50-kDa protein was similar to those of dioxygenases, which introduce both atoms of molecular oxygen to PAH rings. The genes encoding the dioxygenase system were a dehydrogense, the dioxygenase small (β)-subunit, and the dioxygenase large (α)-subunit genes, arranged in a sequence different from those of genes encoding other bacterial dioxygenase systems (21). Phylogenetic analysis showed that the large α subunit did not cluster with

most of the known α-subunit sequences but rather with three newly described alpha subunits of dioxygenases from *Rhodococcus* spp. and *Nocardioides* spp.

5. Conclusion

Nocardioform actinomycetes were shown to employ different metabolic pathways for the degradation of methylaromatics and polycyclic aromatic hydrocarbons. The ability of these bacteria to metabolize methylaromatics via a modiefied *ortho*-cleavage pathway raises the possibility of their use to construct engineered microorganisms for the simultaneous treatment of chloroaromatics and methylaromatics (22). It has also been suggested that enzymes mediating the aromatic degradative pathway might have arisen independently by functionally convergent evolution among gram-positive and proteobacteria. Further, a characteristic cell wall structure of mycobacteria appears to contribute to their exceptional ability to degrade high molecular weight PAHs. The survival and performance of these
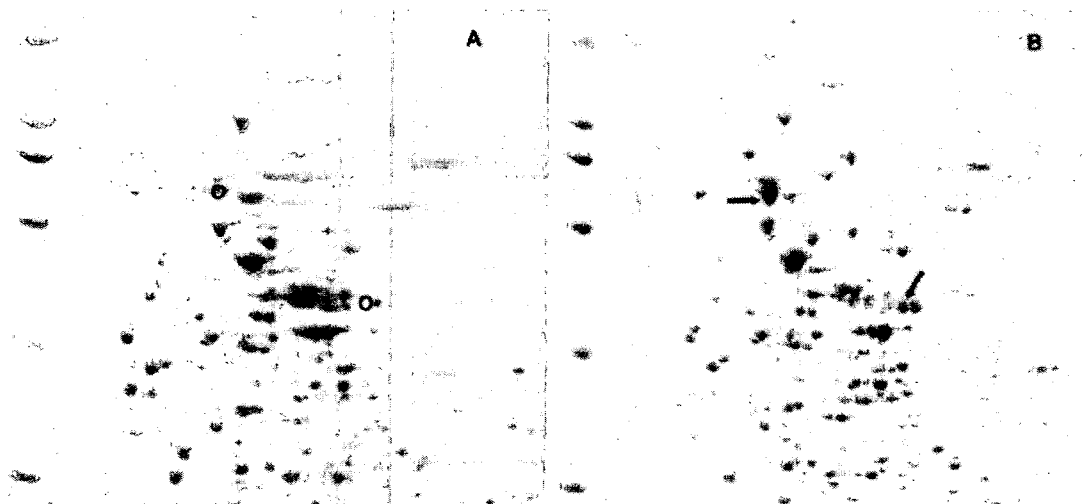

FIG. 3. 2-D gel identification of pyrene-induced proteins of *Mycobacterium* sp. PYR-1. (A) Uninduced proteins of strain PYR-1. (B) Pyrene-induced proteins of strain PYR-1. Arrows, pyrene-induced proteins; circle, same positions in the uninduced sample.

bacteria in microcosms simulating terrestrial and aquatic ecosystems suggest that these bacteria have a high potential for the bioremediation purposes in the natural ecosystem.

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