Catabolism of Aromatic Compounds and Steroids by *Rhodococcus*

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Abstract Aromatic compounds and steroids are among the remarkable variety of organic compounds utilized by rhodococci as growth substrates. This degradation helps maintain the global carbon cycle and has increasing applications ranging from the biodegradation of pollutants to the biocatalytic production of drugs and hormones. The catabolism of aromatic compounds and steroids converge as steroid degradation proceeds via aromatic intermediates. Consistent with the aerobic

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lifestyle of rhodococci, these pathways are rich in oxygenases. Analysis of five rhodococcal genomes confirms the modular nature of the aromatic compound catabolic pathways: peripheral pathways degrade compounds such as biphenyl and phthalate to common intermediates, while central pathways transform these intermediates, such as catechol and phenylacetate, to central metabolites. Studies of *Rhodococcus jostii* RHA1 in particular have revealed a similar modular structure of steroid degradation pathways, which is also conserved in related actinobacteria, such as *Mycobacterium tuberculosis*. Indeed, steroid degradation appears to be a very common, potentially ubiquitous characteristic of rhodococci. Nevertheless, the steroid catabolic pathways appear to be more redundant than the aromatic compound catabolic pathways. Finally, studies in rhodococci have helped elucidate the role of key steroid-degrading proteins including the Mce4 steroid uptake system which define a new class of ABC transporters. The significance of some of these recent discoveries for industrial processes and pathogenesis is discussed.

1 Introduction

Aromatic compounds are widely distributed in the biosphere, being produced by a variety of biological and chemical processes. They range in size from low-molecular-mass compounds such as benzene to the large, insoluble biopolymer lignin. The defining characteristic of aromatic compounds is a planar, fully conjugated, ring-shaped moiety possessing $(4n+2)$ π electrons, where n is a non-negative integer (Hückel's rule) (Fig. 1a) (McMurry 1992). The exceptional stability of these compounds arises from the delocalization of their π orbitals, also called resonance energy. It is this stability that has contributed to the widespread production and use of natural and xenobiotic aromatic compounds for a variety of industrial applications. For example, polychlorinated biphenyls (PCBs) have been used as dielectric fluids and coolants (Field and Sierra-Alvarez 2008), while polybrominated diphenyl ethers (PBDEs) are used as flame retardants (Sjodin et al. 2003). Such compounds are among the most stable and persistent organic pollutants. Finally, polycyclic aromatic hydrocarbons (PAHs) constitute a family of compounds possessing fused aromatic rings. These compounds occur in hydrocarbon deposits and are also produced as byproducts of incomplete combustion of fossil fuels or biomass (Harvey 1991).

Steroids are a class of terpenoid lipids characterized by a carbon skeleton of four fused rings, labeled A to D, and side chains consisting of up to ten carbons. Hundreds of steroids have been identified in plants, animals, and fungi, varying in functional groups attached to the four fused rings. Bacteria contain the structurally related five-ringed hopanoids (Fernandes et al. 2003). The most important physiological roles of steroids are as hormones and in modulating membrane fluidity. In addition, these bioactive compounds have a range of therapeutic applications including as anti-inflammatory agents (Ko et al. 2000), antifungals (Chung et al. 1998), and contraceptives (Tuba et al. 2000). The discovery of the 11α-hydroxylation activity of the

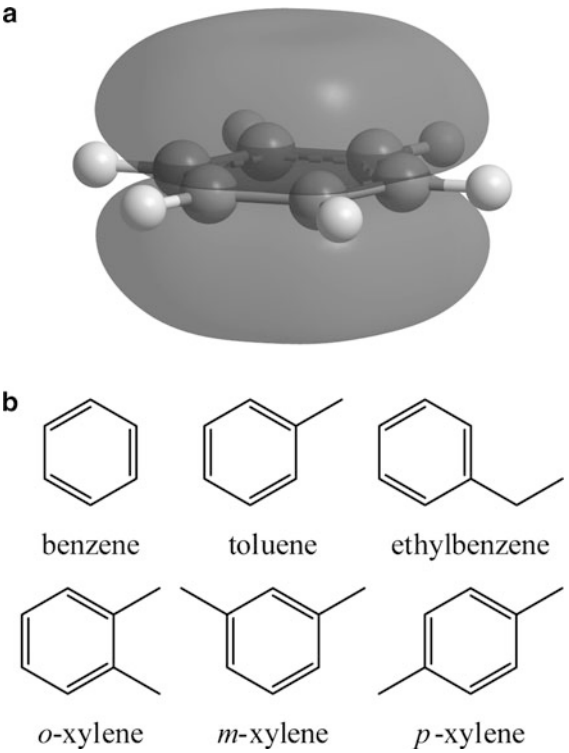


Fig. 1 Aromatic compounds. (**a**) A representation of π-orbital delocalization in a benzene ring. The *orbitals* shown represent the lowest occupied molecular orbital configuration. (**b**) BTEX hydrocarbons

fungus *Rhizopus* in 1949 enabled the transformation of simple sterols to corticosteroids and sparked interest in the synthesis and production of active steroid molecules (Hogg 1992). Cholesterol, obtained from animal fats and oils, and phytosterols, such as stigmasterol, β-sitosterol, and campesterol, are major starting materials for the production of steroid drugs and hormones owing to their low cost and ease of transformation.

In light of the exceptional ability of rhodococci to utilize a wide range of organic compounds as growth substrates, particularly hydrophobic ones, it is hardly surprising that these organisms figure prominently among known degraders of aromatic compounds and steroids (van der Geize and Dijkhuizen 2004). Indeed, *Rhodococcus jostii* RHA1, isolated from lindane-contaminated soil (Seto et al. 1995a), is one of the most potent PCB degraders characterized to date, contains up to four steroid-degrading pathways, and has recently been reported to degrade lignin. The catabolic activities of *Rhodococcus* likely help sustain the biosphere, as these organisms are found in a broad range of environments including various soils, sea water, and eukaryotic cells. Indeed, in at least one study of *o*-xylene-contaminated soils, rhodococci were the most prominent

species (Taki et al. 2007). The exceptional ability of rhodococci to degrade such compounds may be due in part to their mycolic-acid-containing outer membrane (see chapter "The Rhodococcal Cell Envelope: Composition, Organisation and Biosynthesis" by Sutcliffe et al.) as well as their production of surfactants (Iwabuchi et al. 2002; Vogt Singer et al. 1990). Recent genomic, molecular genetic, microbiological, and biochemical studies have increased our understanding of this degradation in rhodococci as well as in related mycolic-acid-producing actinomycetes such as *Corynebacterium*, *Nocardia*, and *Mycobacterium*.

This chapter focuses on the catabolic pathways utilized by rhodococci to degrade aromatic compounds and steroids. We first discuss the overall strategies used by these bacteria to degrade naturally occurring mononuclear aromatic compounds. The underlying principles are illustrated using several pathways. We then discuss the catabolism of more complex compounds, including lignin, PAHs, some halogenated pollutants, and steroids. Differences and similarities of rhodococcal catabolism with that of other bacteria are highlighted by genomic analyses of five rhodococci: *R. opacus* B4, *R. erythropolis* PR4, *R. jostii* RHA1,[1] *R. erythropolis* SK121, and *R. equi* 103S. Particular emphasis is placed on recent discoveries that provide new insights into how this degradation occurs. These advances have important implications for industrial processes, ranging for bioremediation to biocatalysis, as well as for the pathogenesis of *Mycobacterium tuberculosis*, the leading cause of mortality from bacterial infection, and *R. equi*, a horse pathogen that can infect immunocompromised humans (Prescott 1991).

2 Mononuclear Aromatic Compounds

Mononuclear aromatic compounds possess a single aromatic ring within their structure. While these compounds are chemically simpler than the others considered in this chapter, their catabolism illustrates a number of features that are central to the catabolism of all aromatic compounds and steroids in rhodococci, if not aerobic bacteria in general. Mononuclear aromatic compounds are the most prevalent aromatic compounds in the biosphere, being produced by a variety of biological and geochemical processes. Due to their stability, compounds such as benzene and its derivatives are used extensively in the chemical, agriculture, and petroleum industries. For example, gasoline contains a mixture of benzene, toluene, ethylbenzene, and xylene isomers, collectively known as BTEX hydrocarbons (Fig. 1b). BTEX compounds are frequently found as groundwater contaminants as a result of leaking fuel tanks (Cozzarelli et al. 1990).

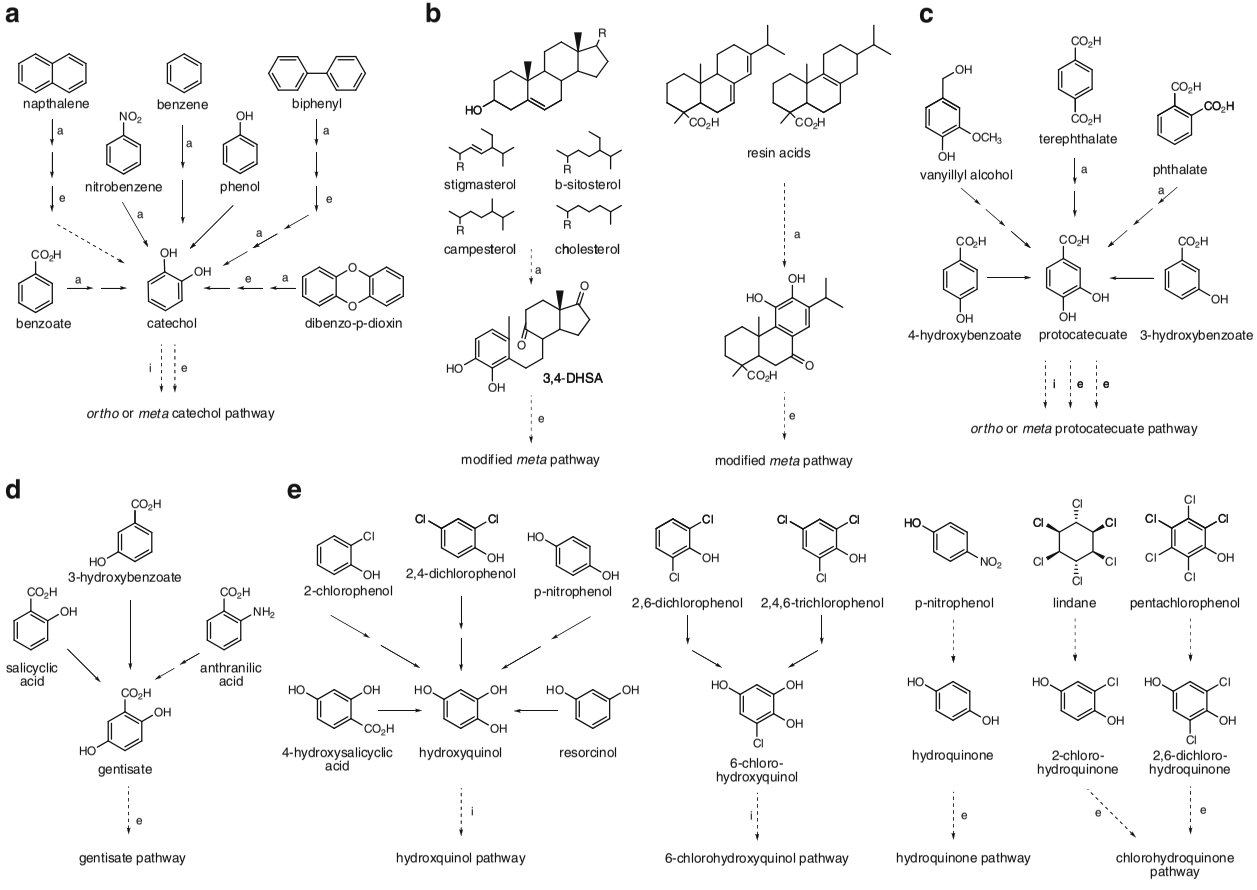
[1]Herein, *R. opacus* B4, *R. erythropolis* PR4, and *R. jostii* RHA1 are referred to by their strain names: B4, PR4 and RHA1, respectively.

2.1 *Underlying Strategies of Aromatic Compound Catabolism in Rhodococci*

The bacterial catabolism of aromatic compounds involves two key steps: the activation of the thermodynamically stable benzene ring, and its subsequent cleavage. While bacteria have evolved diverse anaerobic and aerobic strategies to effect these two steps, rhodococci utilize predominantly the latter strategies, consistent with their aerobic lifestyle. More particularly, rhodococci make extensive use of Rieske non-heme iron oxygenases and other oxygenases to activate the benzene ring by catalyzing the incorporation of hydroxyl groups (Mason and Cammack 1992). Such reactions eventually yield central aromatic metabolites such as catechol, protocatechuate (dihydroxylated at positions 1,2), gentisate (dihydroxylated at positions 1,4), and hydroquinone (dihydroxylated in a *para* position). The critical step of ring fission is then catalyzed by ring-cleaving oxygenases (Vaillancourt et al. 2006). This cleavage can occur either between the hydroxyl groups (intradiol, *ortho*-cleavage) or adjacent to the hydroxyl groups (extradiol, *meta*-cleavage). Each of these four central aromatic metabolites occurs in various catabolic pathways, as summarized in Fig. 2. Steps that are catalyzed by Rieske non-heme iron oxygenases are indicated with "a," whereas extradiol and intradiol dioxygenases are indicated with "e" or "i," respectively.

The pathways summarized in Fig. 2 illustrate an important principle that has been recognized in rhodococci and other bacterial species including the well-studied pseudomonads (Luengo et al. 2001); a wide variety of aromatic compounds are transformed to central metabolites via a relatively limited number of dihydroxylated metabolites. Indeed, the efficiency of this catabolic strategy is such that it has been adapted to degrade polyalicyclic compounds such as steroids (van der Geize et al. 2007) (Fig. 2b). While this figure summarizes our knowledge of the aerobic catabolism of aromatic compounds in all bacteria, all of the intermediates and most of these pathways are known to occur in rhodococci.

A second aerobic catabolic strategy involves the derivatization of aromatic acids by co-enzyme A (CoA) and nonoxygenolytic ring fission (Denef et al. 2006; Navarro-Llorens et al. 2005; Olivera et al. 1998), reminiscent of CoA-dependent reductive pathways responsible for the anaerobic cleavage of aromatic nuclei. However, in the aerobic CoA-dependent pathways, an oxygenase transforms the aromatic acyl-CoA ester prior to ring fission. While, these types of pathways have been called hybrid pathways (Ferrandez et al. 1998), the evolutionary relationship of the aerobic and anaerobic CoA-dependent pathways is unclear. Despite being strict aerobes, rhodococci contain at least one hybrid pathway, the phenylacetate (Paa) pathway described below (Navarro-Llorens et al. 2005). However, they do not appear to contain the Box pathway, which transforms benzoate to β-ketoadipyl-CoA in *Burkholderia*, perhaps under O_2-limiting conditions (Denef et al. 2006).


a
napthalene
benzene
biphenyl
nitrobenzene
phenol
benzoate
catechol
dibenzo-p-dioxin
ortho or meta catechol pathway
b
stigmasterol
b-sitosterol
campesterol
cholesterol
3,4-DHSA
modified meta pathway
resin acids
modified meta pathway
c
vanyillyl alcohol
terephthalate
phthalate
4-hydroxybenzoate
protocatecuate
3-hydroxybenzoate
ortho or meta protocatecuate pathway
d
3-hydroxybenzoate
salicyclic acid
anthranilic acid
gentisate
gentisate pathway
e
2-chlorophenol
2,4-dichlorophenol
p-nitrophenol
4-hydroxysalicyclic acid
hydroxyquinol
resorcinol
hydroxquinol pathway
2,6-dichlorophenol
2,4,6-trichlorophenol
6-chloro-hydroxyquinol
6-chlorohydroxyquinol pathway
p-nitrophenol
hydroquinone
hydroquinone pathway
lindane
pentachlorophenol
2-chloro-hydroquinone
2,6-dichloro-hydroquinone
chlorohydroquinone pathway

3 Peripheral Versus Central Aromatic Pathways

Analyses of bacterial genomic sequences has revealed that the catabolism of aromatic compounds is organized such that a large number of "peripheral" aromatic pathways funnel a range of growth substrates into a restricted number of "central" aromatic pathways. The latter complete the transformation of these compounds to tricarboxylic acid (TCA) cycle intermediates. Thus, the organizational logic of the pathways follows the logic of the chemistry outlined in Fig. 2. The term "catabolon" has been used to define each set of peripheral pathways and corresponding central pathway in a given organism (Luengo et al. 2001). Thus, each catabolon is a complex functional unit of integrated catabolic pathways which transform related compounds via common metabolites. This organization was first described in pseudomonads, where analyses of the genomic sequences of four strains together with functional studies have identified at least 38 peripheral pathways, some of which are strain-specific, and five conserved central pathways (Luengo et al. 2001). Subsequent analyses have confirmed that aromatic catabolic pathways are similarly organized in other bacteria, including rhodococci (McLeod et al. 2006).

3.1 Central Pathways

Up to eight central aromatic pathways have been identified in rhodococci to date. The β-ketoadipate pathway is encoded by the *pca* and *cat* genes, and transforms catechol and protocatechuate to acetyl-CoA and succinyl-CoA via the intradiol cleavage of the catecholic intermediate (Harwood and Parales 1996). The Paa pathway is encoded by the *paa* genes and involves the derivatization of aromatic acids by CoA, ring hydroxylation by an oxygenase, and nonoxygenolytic ring fission (Navarro-Llorens et al. 2005). The 2-hydroxypentadienoate (Hpd) pathway, encoded by the *bphIJK* in *Burkholderia xenovorans* LB400 (Erickson and Mondello 1992), the *bphEFG* in RHA1 (Masai et al. 1997) and the similar *hsaEFG* genes in RHA1 (van der Geize et al. 2007), transforms 2-hydroxypentadienoates to acetyl-CoA and pyruvate through the successive actions of a hydratase, an aldolase, and a dehydrogenase. The gentisate pathway, characterized in *R. erythropolis* strain S1 (Suemori et al. 1995), transforms gentisate to pyruvate and fumarate. The homogenisate pathway (Hmg), characterized in *P. putida* (Arias-Barrau et al. 2004) and

Fig. 2 Overall strategy for the aerobic bacterial catabolism of aromatic compounds. Pathways degrade growth substrates to one of four central intermediates: (**a**) catechol; (**b**) substituted catechol in steroid and cryptic aromatic catabolism; (**c**) protocatechuic acid; (**d**) gentisic acid; and (**e**) (chloro) hydroquinol and (chloro) hydroquinone. *Solid arrows* indicate a single enzymatic reaction, while *dotted arrows* represent multiple steps. *Letter labels* designate catalysis via the following enzymes: "a" = Rieske non-heme iron oxygenase; "i" = intradiol dioxygenase; and "e" = extradiol dioxygenase. Adapted from Vaillancourt et al. (2006)

predicted to occur in several rhodococci, involves the extradiol-type cleavage of homogentisate followed by C–C bond hydrolysis to yield fumarate and acetoacetate. The hydroxyquinol pathway, encoded by the *dxn* genes in *Sphingomonas wittichii* RW1 (Armengaud et al. 1999), involves the intradiol cleavage of hydroxyquinol to acetyl-CoA and succinyl-CoA. The homoprotocatechuate (3,4-dihydroxyphenylacetate) pathway, encoded by the *hpc* genes, involves the extradiol-type cleavage of homoprotocatechuate. The resulting product (5-carboxymethyl-2-hydroxymuconic semialdehyde) is transformed to TCA cycle intermediates via a dehydrogenative route. Finally, an eighth pathway comprising a hydroxylase, an extradiol dioxygenase, and hydrolase has been assigned as a central pathway in RHA1 (McLeod et al. 2006) although its substrate has not yet been identified.

Genomic analyses indicate that B4 contains nearly all the same central pathways compared to RHA1. By contrast, the genes encoding the gentisate, homoprotocatechuate, and the above-mentioned unidentified pathways are absent in the two *R. erythropolis* strains, PR4 and SK121. Moreover, these latter two strains have fewer copies of the Hpd pathway. Despite these differences, all of the central aromatic pathways are chromosomally encoded in these rhodococcal strains when present. This is consistent with the notion that these pathways are core to the bacterium's catabolic capabilities. As noted above, the genes, enzymes and regulatory mechanisms of the central pathways are found in a broad range of bacterial species. However, aspects of their organization are unique to *Rhodococcus* as discussed in more detail below for the β-ketoadipate and Paa pathways.

3.1.1 β-Ketoadipate Pathway

The β-ketoadipate pathway, also known as the *ortho*-cleavage pathway, was first identified in *Pseudomonas putida* (Ornston 1966a, b) and was one of first central aromatic pathways to be analyzed in different genera (Harwood and Parales 1996). The pathway has separate branches that catabolize catechol and protocatechuate, which differ by a carboxylate group, to TCA cycle intermediates (Fig. 3, upper panel). Accordingly, the intermediates of the pathway, and therefore the enzymes, are distinct until decarboxylation of the protocatechuate metabolite, catalyzed by PcaC, the third enzyme in this branch of the pathway. Other than this, the steps of the two branches are equivalent, and are catalyzed by homologous enzymes. Briefly, catechol and protocatechuate are cleaved by intradiol dioxygenases (CatA and PcaGH, respectively) to yield muconates which are cyclized by CatB and PcaB to muconolactones. The muconolactone of the catechol branch is isomerized by CatC, whereas that of the protocatechuate branch is decarboxylated by PcaC. These two reactions yield β-ketoadipate enol-lactone, which is hydrolyzed by PcaD to β-ketoadipate and transformed to TCA cycle intermediates in two CoA-dependent steps (PcaIJ and PcaF).

Among rhodococci, the β-ketoadipate pathway has been functionally characterized in each of *R. erythropolis* AN-13 (Aoki et al. 1983), *R. opacus* 1CP (Eulberg et al. 1998a), and RHA1 (Patrauchan et al. 2005). Moreover, genomic analyses

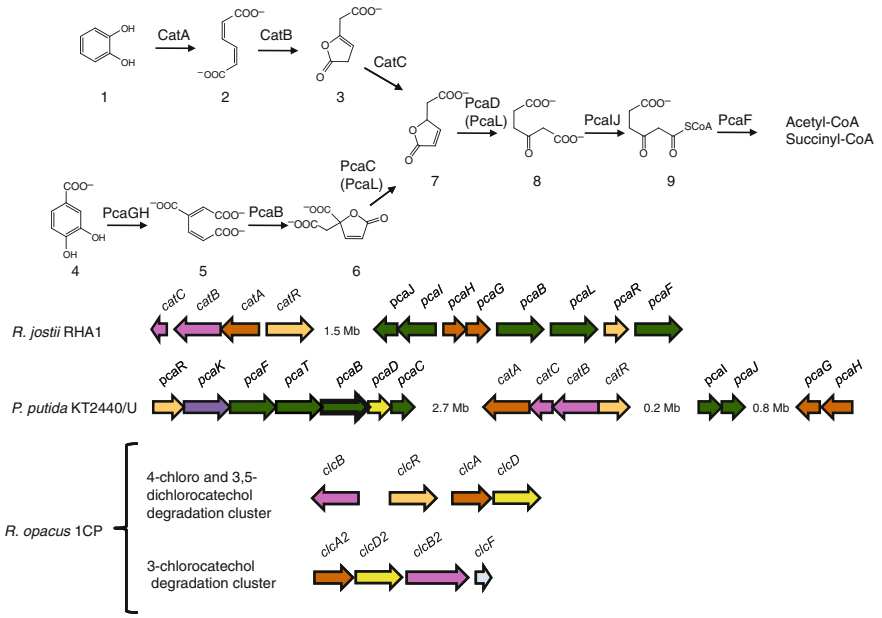


Fig. 3 The β-ketoadipate pathway. The enzymatic steps and the metabolites for the catechol (*upper*) and protocatechuate (*lower*) branches of the pathway are depicted in the *upper panel*. Catabolites are *numbered* as follows: 1, catechol; 2, *cis,cis*-muconate; 3, mucolactone; 4, protocatechuate; 5, γ-carboxymuconate; 6, γ-carboxymucolactone; 7, β-ketoadipate enol-lactone; 8, β-ketoadipate; 9, β-ketoadipyl CoA. The enzymes are: CatA, catechol 1,2 dioyxgenase; CatB, muconate cycloisomerase; CatC, mucolactone isomerase; PcaGH, protocatechuate dioxygenase; PcaB, carboxy-*cis,cis*-mucolactone isomerase; PcaC, 4-carboxymuconolactone decarboxylase; PcaD, β-ketoadipate enol-lactone hydrolase; PcaIJ, β-ketoadipate:succinyl-CoA transferase; and PcaF, β-ketoadipate:succinyl-CoA thiolase. The *lower panel* summarizes the organization of the β-ketoadipate pathway genes in *R. jostii* RHA1 and *P. putida* KT2440 (same as in *P. putida* U) together with the modified β-ketoadipate gene clusters for degrading chlorocatechols in *R. opacus* 1CP. Genes are *colored* by function as follows: *brown*, intradiol dioxygenases; *pink*, catabolism of (chloro) catechol; *dark green*, catabolism of protocatechuate; *yellow*, hydrolase; *pale blue*, dehalogenase; *indigo*, transporter; and *peach*, gene regulators. *Numbers* between genes correspond to the intergenic distance. Adapted from McLeod et al. (2006)

indicate that it occurs in B4 and PR4. The organization of the *pca* gene clusters is identical in three sequenced rhodococcal genomes in which it occurs. Comparison of the gene clusters with those in *Pseudomonas* and *Burkholderia* highlights several features of the rhodococcal pathway. First, rhodococci and other actinomycetes contain a bifunctional enzyme, PcaL, which catalyzes the decarboxylation and hydrolysis of the enol-lactone, the first shared intermediate of the pathway branches. By contrast, these activities are catalyzed by PcaC and PcaD, respectively, in the two *P. putida* strains KT2440 and U (Fig. 3, lower panel). In addition to rhodococci, PcaL has been identified in *Streptomyces* sp. 2065 (Iwagami et al. 2000) and is predicted to occur *S. coelicolor* A3(2) (Bentley et al. 2002) as well as in *Nocardia* and *Corynebacterium* genomes.

An apparent *Rhodococcus*-specific feature of the β-ketoadipate pathway is that the *pca* genes are clustered together in two divergently transcribed operons. More specifically, an analysis of the completed RHA1, B4, and PR4 genomes support our previous analysis that the chromosomal organization of the *pca* and *cat* genes in RHA1 appears to be unique to rhodococci and most similar to that of the closely related corynebacteria (Patrauchan et al. 2005). The *pca* genes are organized in a single cluster in all actinomycetes in which they have been found, as well as in *K. rhizophila* DC2201 (Takarada et al. 2008), *C. crescentus* (Nierman et al. 2001), and *A. baylyi* ADP1 (Brzostowicz et al. 2003), which is a γ-proteobacterium. Non-rhodococcal actinomycetes containing the *pca* genes include *C. glutamicum* ATCC 13032 (Kalinowski et al. 2003), *Streptomyces* sp. 2065 (Iwagami et al. 2000), *S. coelicolor* A3(2) (Bentley et al. 2002), and *S. avermitilis* MA-4680 (Omura et al. 2001). In contrast, the *pca* genes can be arranged in up to three clusters in pseudomonads (Jiménez et al. 2002). Multiple *pca* clusters also occur in β-proteobacteria such as *Burkholderia pseudomallei* and *R. metallidurans* (Jiménez et al. 2002). Nevertheless, the organization of the *pca* genes in a single cluster of two divergently transcribed operons with the gene order of RHA1 appears to be unique to rhodococci; in *C. glutamicum*, the gene order is different, and in streptomycetes, the genes appear to be arranged in a single operon. In all bacteria, the *cat* genes are usually organized in a single cluster (reviewed in Jiménez et al. 2002). In Gram-negative bacteria and *Arthrobacter*, *catR* encodes a LysR-type transcriptional regulator (Murakami et al. 2004), which activates transcription of the adjacent catabolic genes through induction by *cis*,*cis*-muconate. In contrast, the rhodococcal *catR* encodes an IclR-type regulator (Eulberg and Schlömann 1998), which has been shown to function as a repressor in *R. erythropolis* CCM 2595 (Vesely et al. 2007). IclR-type regulators in most cases control the protocatechuate catabolic operons (Eulberg et al. 1998a; Gerischer et al. 1998). However, permutations occur with respect to gene order (e.g., *catRBAC* in the streptomycetes sequenced to date) and the presence of additional genes in the transcriptional unit (e.g., the main *cat* operon in *A. baylyi* ADP1 contains six genes). The order of the genes in RHA1, *catRABC*, is seen in the five sequenced rhodococcal genomes as well as *R. erythropolis* CCM 2595 (Vesely et al. 2007), but not in *Rhodococcus* sp. AN-22 (Matsumura et al. 2006). This aniline-degrading strain was found to constitutively express the *catABC* operon because of a disrupted regulatory *catR* gene. In two *C. glutamicum* isolates (ATCC 13032 and R), *catR* is not adjacent to the other genes.

3.1.2 Modified β-Ketoadipate Pathways

A number of rhodococci possess modified β-ketoadipate pathways to metabolize substituted catechols, thereby expanding the range of aromatic compounds that can be used as a growth substrate. Generally, the modified pathway is incomplete, feeding into the chromosomally encoded classical pathway. Moreover, the modified pathway genes appear to occur on plasmids. The best characterized modified β-ketoadipate pathways are those that degrade chlorocatechols in *R. opacus* 1CP

(formerly *R. erythropolis* 1CP), isolated for its ability to utilize 2,4-dichlorophenol and 4-chlorophenol, which are degraded via 3,5-dichloro and 4-chlorocatechol, respectively. *R. rhodochrous* N75 utilizes a modified pathway to catabolize 4-methylcatechol. This pathway includes a 3-methyl-muconolactone-CoA synthetase (Cha et al. 1998) and a 4-methylmuconolactone isomerase (Bruce et al. 1989).

R. opacus 1CP contains two modified β-ketoadipate pathways, both of which include a chlorocatechol dioxygenase (ClcA), a chloromuconate cycloisomerase (ClcB), and a dienelactone hydrolase (ClcD) (Eulberg et al. 1998b), which correspond to CatA, CatB, and CatD, respectively, of the catechol branch of the β-ketoadipate pathway (Fig. 3, lower panel). In the pathway responsible for the degradation of 3,5-dichloro and 4-chlorocatechols, the *trans*-dienelactone resulting from dehalogenation and cyclization is cleaved by the hydrolase to give maleylacetate, which is then reduced to produce β-ketoadipate. The second pathway, encoded by the *clcA2B2D2F* genes, is specific for the degradation of 2-chlorophenol, 3-chlorophenol, and 3-chlorobenzoate (Moiseeva et al. 2002). The encoded enzymes are only distantly related to the previously known chlorocatechol enzymes and include a dechlorinating enzyme related to mucolactone isomerase (ClcF).

The modified β-ketoadipate pathways for degrading chlorocatechols are distinct from the classical pathway in four respects. Firstly, the enzymes in the modified pathway are highly specific for their chlorinated substrates. Secondly, the modified pathways possess enzymes capable of dehalogenation including cycloisomerases, (ClcB and ClcB2), a maleylacetate reductase (ClcE), and a dehalogenase (ClcF). Thirdly, since the modified pathway lacks the protocatechuate branch of the standard β-ketoadipate pathway, the *clc* and *clc2* clusters encode for dienelactone hydrolases (ClcD and ClcD2) alone, replacing the bifunctional PcaL. Finally, the *clc* and *clc2* operons occur on a 740-kbp plasmid, p1CP (Konig et al. 2004). Among the *Actinobacteria*, chlorocatechol metabolism has been investigated in some detail only for *R. opacus* ICP, and the presence of these chlorocatechol gene clusters has not been reported for other rhodococcal strains that degrade chloroaromatic compounds.

3.1.3 Phenylacetate (Paa) Pathway

Phenylacetate (Paa) arises in the catabolism of a variety of compounds including phenylalkanoates, tropate, and homophthalate. In addition, phenylacetate is the first common intermediate in the degradation of phenyldecane by *R. opacus* PD630 (Alvarez et al. 2002). The chromosomally encoded central pathway of the phenylacetyl-CoA catabolon has been described in both Gram-negative bacteria such as *P. putida* (Olivera et al. 1998) and *E. coli* (Ferrandez et al. 1998; Luengo et al. 2001), as well as Gram-positive bacteria such as RHA1 (Navarro-Llorens et al. 2005). However, the Paa pathway has yet to be functionally characterized. In the representative pathway shown in Fig. 4 (upper panel), phenylacetate is first transformed to phenylacetyl-CoA through the addition of CoA in an ATP-dependent fashion by PaaF, a phenylacetate-CoA ligase, first characterized in *P. putida* (Martinez-Blanco et al. 1990). This suggests that intermediates are processed as CoA-thioesters, an unconventional strategy for aerobic aromatic metabolism. Next, a multicomponent di-iron

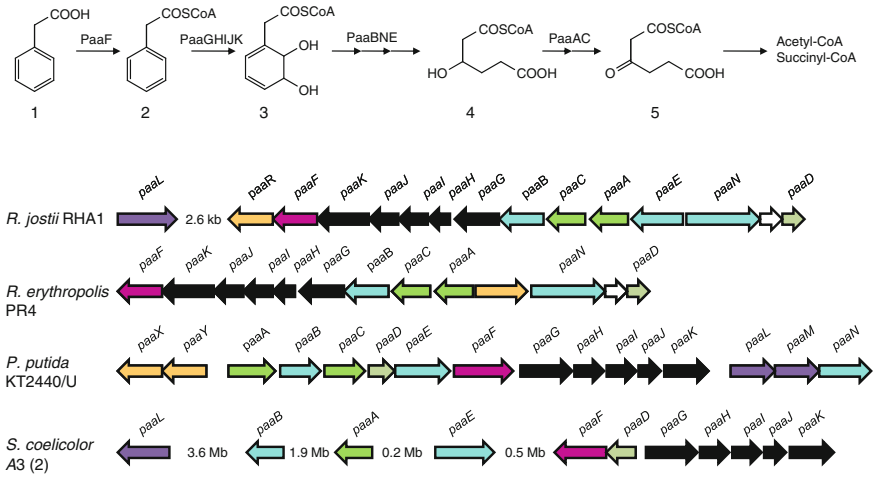


Fig. 4 The phenylacetic acid pathway. The enzymatic steps and the proposed metabolites of the pathway are depicted in the *upper panel*. Presumed catabolites are *numbered* as follows: 1, phenylacetate; 2, phenylacetate-CoA; 3, 2,3-dihydroxy-2,3-dihydrophenylacetate CoA thioester; 4, β-hydroxyadipyl-CoA; and 5, β-ketoadipyl CoA. Other catabolites may occur in this pathway. The enzymes are PaaF, phenylacetate-CoA ligase; PaaGHIJK, a multicomponent oxygenase; PaaN, a non-oxygenolytic ring-cleavage enzyme; PaaB, 2-cyclohexenylcarbonyl CoA isomerase; PaaE, a β-ketoadipyl CoA thiolase.; PaaA, enoyl-CoA hydratase; PaaC, 3-hydroxyl-butyryl-CoA dehydrogenase. The *lower panel* summarizes the organization of the pathway genes in *R. jostii* RHA1, *R. erythropolis* PR4, *P. putida* KT2440 (same as in *P. putida* U), and *S. coelicolor* A3(2). Genes are *colored* by function as follows: *purple*, phenyl-CoA ligase; *black*, ring-hydroxylating oxygenase components; *aqua*, ring-cleavage and associated functions; *lime green*, *paaAC*; *gray*, *paaD*; *indigo*, transporters; and *peach*, gene regulators. *Numbers* between genes correspond to the intergenic distance. Adapted from McLeod et al. (2006)

oxygenase encoded by paaGHIJK is then postulated to catalyze the 2,3-dihydroxylation of the aromatic ring, yielding a cis-dihydrodiol. The ring of this intermediate is non-oxygenolytically cleaved in a reaction that is thought to be catalyzed by PaaN, an aldehyde dehydrogenase. Consistent with its predicted role, a *paaN* knockout strain of RHA1 completely abolished growth on substrates known to be degraded via the Paa pathway and produced tropone, 2-coumaranone, and the methyl ester of 2-methoxyphenylacetate. The latter two metabolites were presumed derivatives of the expected substrate for PaaN (Navarro-Llorens et al. 2005). Together, PaaN, an enol-CoA hydratase (PaaB), and a ketothioesterase (PaaE) are thought to transform the dihydrodiol-CoA thioester to acetyl-CoA and β-hydroxyadipyl-CoA in poorly characterized reactions that utilize water and CoA. Finally, β-hydroxyadipyl-CoA is transformed by an enol-CoA hydratase (PaaA) and a 3-OH-acyl-CoA dehydrogenase (PaaC) to β-ketoadipyl-CoA, which is transformed to succinyl-CoA and a second equivalent of acetyl-CoA. Despite the ubiquitous nature of the Paa pathway, the identity of the metabolites and the function of each gene product are still unknown.

Genomic analyses of the *paa* clusters in three rhodoccocal species (RHA1, B4, and PR4), two pseudomonads (*P. putida* KT2440 and *P. putida* U), and two

non-rhodococcal actinomycetes (*S. coelicolor* A3(2) and *K. rhizophila* DC2201 (Takarada et al. 2008)) reveal several genus-specific features of the pathway (Fig. 4, lower panel). First, genes encoding two core functional units of the pathway are consistently clustered: *paaGHIJK*, encoding a ring-hydroxylating system, and *paaABC*, encoding a β-oxidation system. Other genes commonly occurring in *paa* gene clusters include *paaN* and *paaF*. Some *paa* gene clusters also contain genes encoding a transport system (*paaLM*) and a regulatory system (*paaXY*). In many Gram-positive organisms including *Rhodococcus*, there is no homolog of the *paaM*–encoded porin, consistent with such a protein being unnecessary in organisms lacking an outer cell membrane (Navarro-Llorens et al. 2005). In RHA1, PaaR may function to regulate the *paa* genes, replacing PaaXY found in some Gram-negative bacteria such as *P. putida* (Olivera et al. 1998). Interestingly, the *paa* cluster of PR4 lacks the *paaE*, *paaR*, and *paaL*, which encode a β-ketoadipyl-CoA thiolase, an AraC-type transcriptional regulator, and a transporter, respectively. It is possible that these functions are encoded by different genes. For example, genes encoding an Rrf2 DNA-binding protein or a TetR-type transcriptional regulator are positioned 1.9 and 5.4 kbp, respectively, from *paaF* in PR4. Similarly, a gene encoding a divalent anion-sodium symporter (DASS) is located 9.6 kbp upstream of the cluster in PR4. The PR4 cluster does not include an obvious candidate gene encoding a β-ketoadipyl-CoA thiolase.

The most notable distinguishing feature of the *paa* genes in rhodococci is their organization. In these bacteria, the principal cluster appears to be organized in two divergently transcribed operons despite their different gene contents. The two clusters minimally comprise *paaACBGHIJKF* and *paaN–orfX–paaD*, where *orfX* is a gene of unknown function. By contrast, the *paa* genes in some Gram-positive bacteria, most notably in *Arthrobacter* and *Streptomyces*, are dispersed in the chromosome. For example, in each of three *Arthrobacter* species (*A. oxydans* CECT386, *A. strain* FB24, and *A. aurescens*), soil-dwelling actinomyces, the *paa* genes, are organized in two distinct clusters: *paaDF–tetR–paaN* and *paaGHIJK* (Navarro-Llorens et al. 2008). Moreover, the *paaA, paaC,* and *paaE* genes have not been reported to date in these species. Similarly, the *paa* genes in *S. coelicolor* A3 (2) (Bentley et al. 2002) and *S. avermitilis* (Omura et al. 2001) are distributed throughout the genome with only the *paaGHIJK* genes clustered (Fig. 4, lower panel). Finally, the *paa* genes are also clustered in different ways in Gram-negative organisms, occurring as a single chromosomal cluster in *E. coli* (Ismail et al. 2003) and *P. putida* (Olivera et al. 1998) or multiple clusters separated by over 200 kbp, as in *B. xenovorans* LB400 (Chain et al. 2006).

3.2 Peripheral Pathways

In contrast to the central pathways, the peripheral pathways can be found on both plasmid and chromosome, consistent with the expansion of catabolic capabilities through the exchange of genes on mobile elements (van der Geize and Dijkhuizen 2004). The redundancy of peripheral pathway genes in rhodococci further

contributes to the catabolic diversity of these microorganisms. RHA1 is predicted to contain 26 peripheral pathways. However, only a few have been functionally confirmed. Among the best characterized are the catabolic pathways responsible for the degradation of biphenyl, ethylbenzene (Iwasaki et al. 2006; Sakai et al. 2002, 2003; Seto et al. 1995a), phthalate, and terephthlalate degradation (Hara et al. 2007). These pathways are typical of peripheral aromatic pathways in that oxygenases catalyze the hydroxylation of the aromatic ring, activating it for subsequent cleavage. Accordingly, these pathways are discussed in more detail below as illustrative examples. The degradation of other compounds such as naphthalene, salicylate, and 3-hydroxybenzoate, which are ultimately degraded via the central gentisate pathway (Suemori et al. 1995), will not be discussed in detail.

3.2.1 Biphenyl and Alkylbenzene Pathways

The biphenyl (Bph) pathway has been characterized in a number of rhodococci, including RHA1 (Masai et al. 1995), *R. globerulous* P6 (Asturias et al. 1995), *R. erythropolis* TA421 (Kosono et al. 1997), *Rhodococcus* strain M5 (Labbe et al. 1997), and three *R. rhodochrous* strains: K37, HA99, and TA431 (Taguchi et al. 2007). Much of the interest in this pathway has been driven by its ability to at least partially transform PCBs, discussed below. The Bph pathway in *Rhodococcus* is very similar to that in other bacteria (Furukawa 2000), comprising four enzymes that transform biphenyl into Hpd and benzoate (Fig. 5, upper panel). Degradation is initiated by biphenyl dioxygenase (BPDO), a three-component Rieske-type oxygenase (RO) comprising a reductase, a ferrodoxin, and a catalytic oxygenase. BphB, a member of the short chain dehydrogenases reductases (SDR) superfamily, catalyzes the NAD^+-dependent dehydrogenation of the resulting *cis*-diol to 2,3-dihydroxybiphenyl, a catechol. The latter is cleaved by the BphC extradiol dioxygenase to yield a *meta*-cleavage product (MCP). In the final step, an MCP hydrolase, BphD, adds water across a C–C bond to afford Hpd and benzoate. Hpd is further degraded via a central aromatic pathway, while benzoate is transformed to a catechol by the benzoate (Ben) peripheral pathway before being degraded by the β-ketoadipate pathway (Patrauchan et al. 2008). In RHA1, the expression of the *bph* genes is regulated by a two-component regulatory system: BphS, the sensor kinase, and BphT, the response regulator (Takeda et al. 2004).

The rhodococcal Bph pathway illustrates how rhodococci have developed catabolic versatility and efficiency through genetic redundancy. It also provides a sobering lesson on the challenges of gene annotation. RHA1 carries two copies of an ethylbenzene (Etb) catabolic pathway that is highly similar to the Bph pathway (Fig. 5). This includes two copies of an ethylbenzene dioxygenase (EBDO), two copies of an EtbC extradiol dioxygenase, and two copies of the EtbD MCP hydrolase. Indeed, sequence analyses revealed 54 potential *bph* genes in RHA1 including a total of 13 *bphC* homologs (Goncalves et al. 2006). A variety of studies have revealed that the Etb and Bph pathways are involved in a range of alkylbenzenes in RHA1, including biphenyl, ethylbenzene, styrene, and benzene (Patrauchan et al.

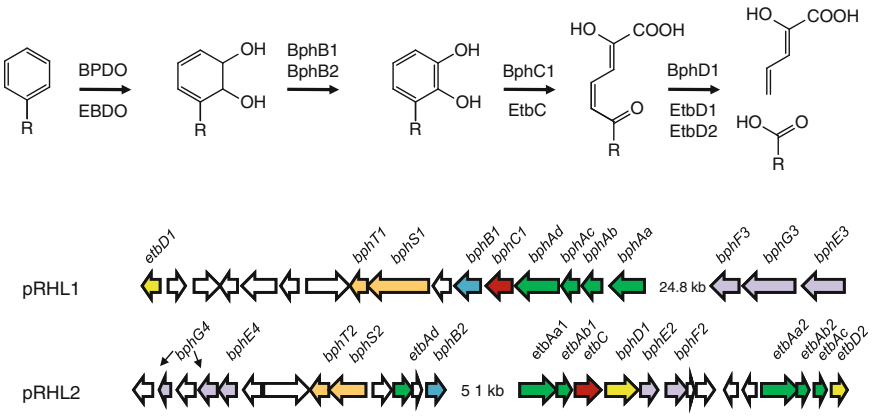


Fig. 5 The proposed catabolic pathways for biphenyl, ethylbenzene, benzene, and styrene. R = phenyl for biphenyl, CH_2CH_3 for ethylbenzene, $CH{=}CH_2$ for styrene, and H for benzene. (In benzene transformation, the resulting catechol is directly degraded by the Cat-PCA branches of the β-ketoadipate pathway.) The enzymatic steps and metabolites of the Bph pathway are depicted in the *upper panel*. The enzymes are: BPDO and EBDO, ring-hydroxylating dioxygenase; BphB1 and BphB2, *cis*-dihydrodiol dehydrogenase; BphC1 and EtbC, extradiol dioyxgenase; BphD1, EtbD1, and EtbD2, *meta*-cleavage product hydrolase. The resulting Hpd is transformed by the Hpd pathway to central metabolites. The *lower panel* summarizes the organization of the pathway genes in clusters located on RHA1 plasmids pRHL1 and pRHL2. Genes are *colored* by function as follows: *green*, Rieske oxygenase components; *blue*, dehydrogenases; *red*, extradiol dioxygenases; *yellow*, MCP hydrolases; *lavender*, Hpd pathway enzymes; and *peach*, gene regulators. *Numbers* between genes correspond to the intergenic distance. Adapted from Patrauchan et al. (2008)

2008; Seto et al. 1995b). Moreover, BPDO of RHA1 shares 98% amino acid sequence identity with isopropylbenzene dioxygenase of *R. erythropolis* BD2 (Stecker et al. 2003), while EBDO of RHA1 shares 100% amino acid sequence identity with *o*-xylene dioxygenase (oXYDO) of *Rhodococcus* sp. DK17 (Kim et al. 2004). oXYDO/EBDO transforms a range of alkylbenzenes (Kim et al. 2007a) and appears to transform larger substrates than BPDO (Iwasaki et al. 2006). Finally, four of the *bphC* homologs of RHA1 are involved in steroid catabolism, as discussed below. In the absence of functional data, it is difficult to know which of the many Bph enzymes that have been identified in rhodococci function primarily in biphenyl catabolism. *R. globerulus* P6, RHA1, and *R. erythropolis* TA421 all clearly contain homologous Bph pathways encoded by similarly organized gene clusters. However, the order and sequences of the *bph* genes in *R. rhodochrous* K37, HA99, and TA431 are clearly different from those in other rhodococci species (Taguchi et al. 2007).

3.2.2 Phthalate and Terephthalate Pathways

Phthalates are widely used as plasticizers to impart flexibility and durability to polyvinyl chloride (PVC) products used in building materials, food packaging,

lubricants, and cosmetics. They are ubiquitous contaminants in food, indoor air, soils, and sediments (Stales et al. 1997). Although toxicity profiles vary according to the phthalate ester, this class of xenobiotic has been implicated in cancer, malformations, and reproductive toxicity in laboratory animals (Gray et al. 1999; Kluwe et al. 1982). The aerobic degradation of phthalate isomers was first reported in pseudomonads in the late 1950s (Ribbons and Evans 1960). Since then, various strains of microorganisms have been found to utilize them as growth substrates (Vamsee-Krishna and Phale 2008). The rhodococcal strains that have been reported to degrade them include *Rhodococcus* sp. DK17 (Choi et al. 2005), *R. erythropolis* S-1 (formerly *Nocardia erythropolis*) (Kurane et al. 1980), and *Rhodococcus* sp. L4 (Lu et al. 2009). *R. rhodochrous* is an interesting case, as it apparently requires hexadecane to degrade various phthalate isomers (Nalli et al. 2002).

RHA1 utilizes both phthalate and terephthalate as growth substrates, degrading them via the Pad and Tpa pathways, respectively (Hara et al. 2007). RHA1 carries two identical sets of *pad* and *tpa* genes on linear plasmids (Fig. 6, lower panel), as does *Rhodococcus* sp. DK17 (Choi et al. 2005). This duplication is required for maximal rates of growth of the latter on phthalate (Choi et al. 2007). The Pad and Tpa pathways are very similar: degradation is initiated by cognate multicomponent RO dioxygenases encoded by *padAaAbAcAd* and *tpaAaAbB*. While both systems comprise large and small oxygenase subunits and a reductase, the phthalate RO system has an additional ferrodoxin component. PadB and TpaC are the respective SDR dihydrodiol dehydrogenases of the pathways (Fig. 6, upper panel). Finally, a decarboxylase, PadC, is required to yield protocatechuate in the Pad pathway. The protocatechuate generated by each of the Pad and Tpa is degraded via the β-ketoadipate pathway. Interestingly, gene knockout and transcriptomic studies indicate that terephthalate can also be transformed to catechol via a bifurcated pathway and can thus feed into the Cat branch of the β-ketoadipate pathway (Hara et al. 2007).

4 Polymeric and Halogenated Aromatic Compounds

The catabolism of lignin, PAHs, and many xenobiotic aromatic compounds is more complex than that described above. Nevertheless, the aromatic nuclei of these compounds are degraded according to the same underlying principles, with some of the resulting metabolites being funneled into peripheral and central aromatic pathways.

4.1 Lignin Degradation

Lignin is the second most abundant polymer in nature after cellulose, comprising 30% of the nonfossil organic carbon (Boerjan et al. 2003) and is arguably the most important aromatic compound in the biosphere (Fig. 7). This polymer is

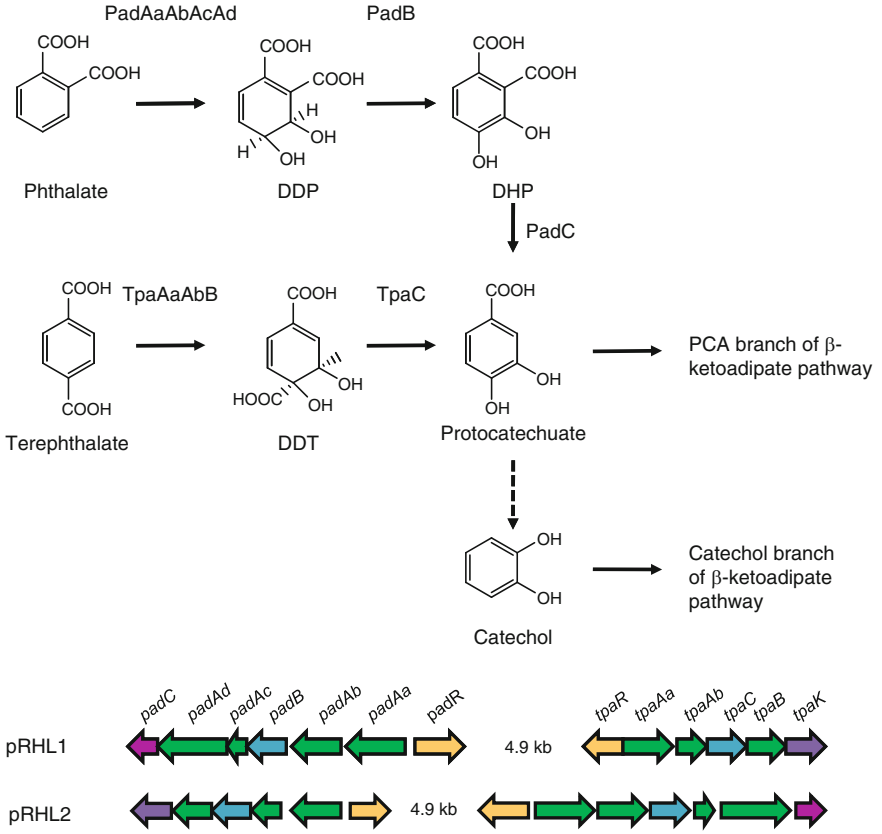


Fig. 6 The degradation pathways for phthalate and terephthalate. The *upper panel* illustrates the degradation of phthalate via the PCA branch of the β-ketoadipate pathway, whereas terephthalate is degraded via a bifurcated pathway that utilizes both PCA and catechol branches. The metabolites are: DDP, *cis*-3,4-dihydroxy-3,4-dihydrophthalate; DHP, 3,4-dihydroxyphthalate; and DTT, *cis*-1,2-dihydroxy-1,2-dihydroterephthalate. The enzymes are: PadAaAbAcAd, a multicomponent ring-hydroxylating 3,4-dioxygenase system; PadB, dihydrodiol dehydrogenase; PadC, decarboxylase; TpaAaAbB, a ring-hydroxylating 1,2-dioxygenase system; and TpaC, dihydrodiol dehydrogenase. The *lower panel* summarizes the organization of the pathway genes in clusters located on RHA1 plasmids pRHL1 and pRHL2. Genes are *colored* by function as follows: *green*, Rieske oxygenase components; *blue*, dehydrogenase; *purple*, decarboxylase; *indigo*, transporters; and *peach*, gene regulators. *Numbers* between genes correspond to the intergenic distance. Adapted from Hara et al. (2007)

synthesized by plants and algae in a radical process from the cinnamyl precursors derived from *p*-hydroxyphenyl, guaiacyl, and syringyl alcohols. The best characterized lignin-degrading organisms are white rot fungi, such as *Phanerochaete chrysosporium* (Gold and Alic 1993), which first break down the polymer into smaller aromatic units using extracellular peroxidases and laccases (Singh and Chen 2008). Lignocellulose is currently of great interest as a feedstock for

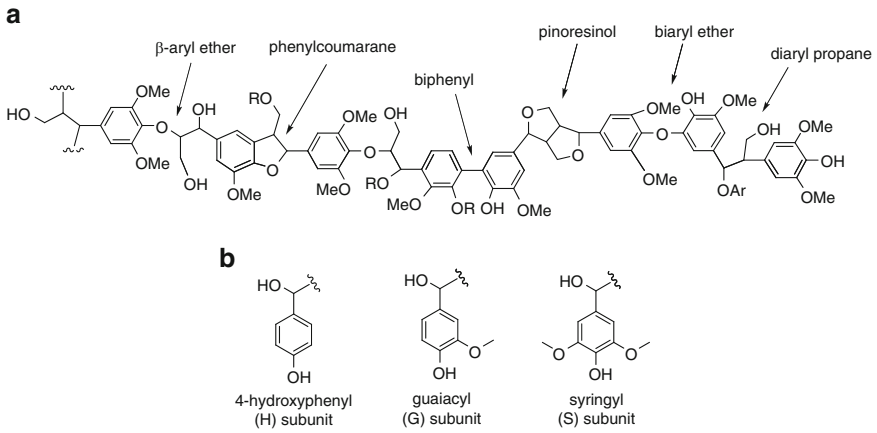


Fig. 7 Structure of lignin complex. (**a**) Schematic of different types of linkages that occur in lignin. (**b**) Three different types of lignin monomers. Adapted from Ahmad et al. (2010)

second-generation biofuel production due to its high energy content, abundance, and renewable status, and represents the most scalable alternative fuel source (Hill et al. 2006). In nature, lignin forms an insoluble, unreactive layer around the energy-rich cellulose, where it plays a role in the vascular system of the plant and in protection from pathogens. Access to the energy stored in the plant material requires breakdown of the lignocellulosic biomass, separation of the cellulose component, and conversion of the fermentable sugars to ethanol (Rubin 2008). Currently, industrial processes for lignin removal are dependent on high heat, pressure, and acid treatments, which tend to be expensive, slow, and relatively inefficient (Ward and Singh 2002). Biological pretreatment for lignocelluloses decomposition is currently being explored.

Bacteria, including rhodococci, have long been recognized to contribute to the mineralization of the lignin break-down products initially generated by fungi (Vicuna et al. 1988; Zimmermann 1990). However, degradation of the lignin polymer by Gram-positive actinomycetes such as *Nocardia*, *Rhodococcus*, and *Streptomyces* has only been observed at a low level, likely due to the heterogeneity of polymeric lignin. Thus, much of the present knowledge of the mechanism of lignin degradation by bacteria has been obtained using lignin model compounds (LMC) as substrates. *R. rhodochrous* (Andreoni et al. 1991) was versatile in utilizing a number of aromatic lignin-related monomers as a sole carbon source. *R. equi* DSM 43349 (Rast and Engelhardt 1980) was able to degrade veratryl-glycerol-β-phenyl ether, a lignin-like synthetic compound. More recently, RHA1 was observed to degrade the lignin polymer (Ahmad et al. 2010). Interestingly, this activity was not dependent on extracellular peroxidases, unlike in other bacterial lignin degraders such as *Streptomyces viridosporus* and *Nocardia autotrophica*.

4.2 Polyaromatic Hydrocarbons

PAHs contain two or more fused benzene rings in linear or cluster arrangements (Fig. 8). The molecular size of PAHs correlates with their lipophilicity, environmental persistence, and toxicity (Jacob et al. 1986). Soil bacterial communities, especially the Nocardioform actinomycetes (e.g., *Rhodococcus, Nocardia,* and *Mycobacterium*), play a crucial role in the mineralization of PAH in contaminated soil (Kästner et al. 1994). However, microbial degradation of PAHs is strongly influenced by a multitude of biotic and abiotic factors, most notably the physical–chemical properties of the PAHs. Lower molecular weight PAHs, such as naphthalene and phenanthrene, are degraded relatively rapidly, whereas higher molecular weight PAHs, such as benz[α]anthracene, chrysene, and benzo[α]pyrene, are more resistant to microbial attack (Cerniglia 1992). While several rhodococcal species can completely mineralize naphthalene, such as B4 and *R. opacus* R7, the metabolic pathway utilized by these Gram-positive organisms differs when compared to the Gram-negatives. In *P. putida* NAH7, naphthalene is degraded to salicylic acid, which is then transformed to central metabolites via catechol. However, in rhodococci, salicylic acid, the common intermediate in naphthalene metabolism, is metabolized to gentisate (Di Gennaro et al. 2001; Grund et al. 1992).

Rhodococcal species are able to metabolize aromatics with up to four rings. For example, *Rhodococcus* sp. UW1 utilizes pyrene, phenanthrene, fluoranthrene, and chrysene as growth substrates and could cometabolize naphthalene, dibenzofuran, fluorine, and dibenzothiophene (Walter et al. 1991). Although complete degradation pathways for four-ringed PAHs have not been described in a *Rhodococcus*, the pyrene and fluorine degradation pathways are likely to resemble the pathways recently described in *Mycobacterium vanbaalenii* PYR-1 (Kim et al. 2007b; Kweon et al. 2007), in which these compounds are eventually transformed to phthalate. Finally, while a small number of bacteria have been reported to degrade PAHs containing more than four rings (Kanaly and Harayama 2000), these do not include rhodococci.

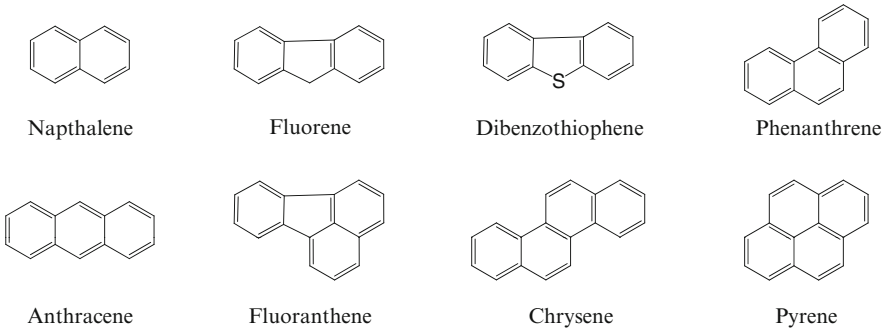


Fig. 8 Structure of various polyaromatic hydrocarbons degraded by rhodococci

4.3 Halogenated Aromatic Compounds

Halogenated aromatic compounds are frequently used in the manufacture of solvents, pesticides, and fire retardants. The substitution of fluorine, chlorine, or bromine on the aromatic ring increases their resistance to microbial degradation. Rhodococci can degrade a wide range of halogenated aromatic compounds, and a number of strains utilize various chlorinated aromatic compounds as sole carbon and energy sources such as *R. percolatus* MBS1 (Briglia et al. 1996), *R. opacus* GM-14 (Zaitsev et al. 1995), *Rhodococcus* strain MS11 (Rapp and Gabriel-Jurgens 2003), and *R. phenolicus* (Rehfuss and Urban 2005). Function studies have established that rhodococci can dechlorinate compounds by either hydroxylation or reduction (Bondar et al. 1999; Haggblom et al. 1988).

R. chlorophenolicus PCP-1 was found to efficiently degrade polychlorinated phenols including penta-, tetra-, and trichloro phenols (Apajalahti and Salkinoja-Salonen 1986, 1987a, b). This strain catalyzes a novel hydroxylation at position 4, regardless of whether a chlorine substituent occupies this position. Dechlorination of penta- and tetrachlorophenols is catalyzed by reductive dehalogenation prior to ring cleavage. Although this strain has been reclassified as a *Mycobacterium* based on mycolic acid analyses (Haggblom et al. 1994), this pathway likely could exist in rhodococci.

4.3.1 PCBs and PBDEs

PCBs and PBDEs are toxic and persistent aromatic compounds that continue to pose an environmental problem. Both compounds usually exist as mixtures of 209 congeners differing in number and position of the halogen substituents. PCBs were once frequently used in the production of plastics and adhesives, but now remain among the most pervasive and recalcitrant of pollutants. They have been linked to cancer, childhood neurological deficits, and endocrine disruption (Cogliano 1998; Walkowiak et al. 2001; Winneke et al. 2002). PBDEs are a class of flame retardants that have been used in a wide variety of manufactured materials (de Wit 2002). PBDEs have varying degrees of chemical and toxicological properties.

Rhodococcal strains capable of degrading PCBs include *R. globerulus* P6 (Asturias et al. 1995), *R. erythropolis* TA421 (Maeda et al. 1995), RHA1 (Masai et al. 1995), *Rhodococcus* sp. M5 (Lau et al. 1996), *Rhodococcus* sp. R04 (Yang et al. 2007b), and three *R. rhodochrous* strains: K37, HA99, and TA431 (Taguchi et al. 2007). Moreover, in a survey of soil microbial populations associated with mature trees growing in a contaminated site, the majority of culturable PCB-degraders were identified as rhodococci (Leigh et al. 2006). As in other aerobic bacteria, PCB degradation often involves their cometabolism by the above-described Bph pathway. Accordingly, the less substituted congeners are subject to dihydroxylation, usually in the 2,3-positions. The extent of further degradation is congener-specific and largely depends on the specificity of the extradiol dioxygenase (Fortin et al. 2005)

and MCP hydrolase (Seah et al. 2001) as has been established in studies of the *R. globerulus* P6 isozymes. The elimination of chlorine or bromine is thought to be a fortuitous event which occurs in later metabolic steps, although the initial dioxygenase can catalyze some dechlorination (Haddock et al. 1995). The potent PCB-degrading properties of RHA1 have been attributed in part to the multiplicity of Bph and Etb isozymes in this strain (Goncalves et al. 2006). Interestingly, EBDO transformed more highly chlorinated, and thus larger, PCB congeners than BPDO (Iwasaki et al. 2006).

The degradation of PBDE has not been as well studied as that of PCBs. However, a recent study established that RHA1 efficiently transforms PBDE congeners containing up to five bromines (Robrock et al. 2009). Analogously to what was observed for the PCB congeners, EBDO transformed more highly brominated congeners than did BPDO.

5 Steroids

Steroids consist of a four-ringed nucleus and a branched alkyl side chain varying in complexity (Fig. 9). Rhodococci have long been known to degrade a range of naturally occurring steroids including cholesterol and phytosterols (van der Geize and Dijkhuizen 2004), although the ubiquity of this ability in this genus is only now becoming clear. In one study, 16 rhodococcal isolates were found to utilize cholesterol as a growth substrate, including strains of *R. equi*, *R. erythropolis*, *R. rhodochrous*, *R. fascians*, and *R. rhodnii* (Watanabe et al. 1986). Natural estrogens, including 17β-estradiol, estrone, and estriol, were also found to be degraded by strains of *R. equi* and *R. zopfii* (Yoshimoto et al. 2004). Recent genomic studies have demonstrated that RHA1 contains four clusters of genes potentially encoding distinct catabolic pathways (McLeod et al. 2006). Of these, the cholesterol catabolic pathway (Fig. 10a) is the best characterized (van der Geize et al. 2007). *R. opacus* B4, *R. erythropolis* PR4. erythropolis SK121, and *R. equi* 103S are all predicted to encode this pathway. Indeed, B4 and PR4 carry two additional gene

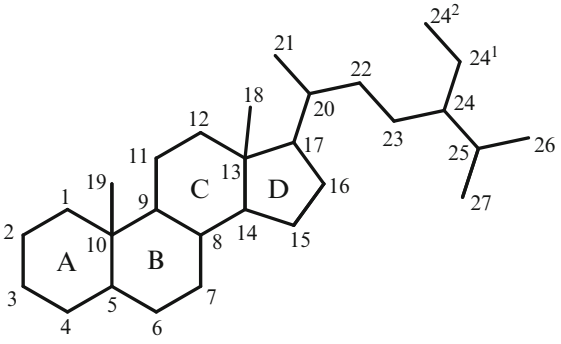


Fig. 9 The steroid skeleton. Rings are labeled A to D. Carbons are numbered according to established nomenclature of steroids

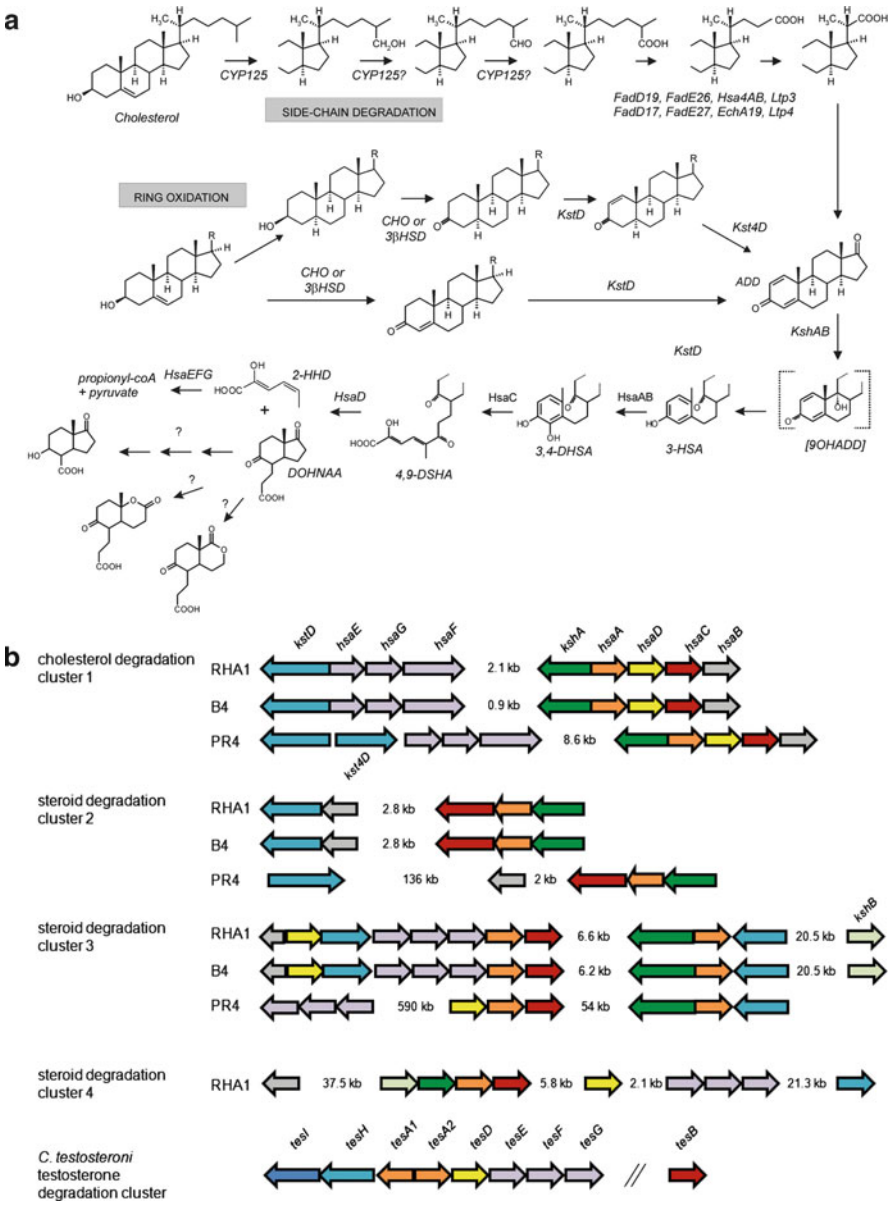


Fig. 10 (**a**) The proposed degradation of the cholesterol side chain and rings A and B. *Brackets* indicate nonenzymatic hydrolysis. CYP125, a steroid 26-monooxygenase, is thought to perform the C26 hydroxylation and oxidations to yield the acid intermediate. The metabolites are: AD, 4-androstene-3-17-dione; ADD, 1,4-androstadiene-3,17-dione; 9-OHADD, 9α-hydroxy-1,4-androstadiene-3-17-dione; 3-HSA, 3-hydroxy-9,10-seconandrost-1,3,5(10)-triene-9,17-dione; 3,4-DHSA, 3,4-dihydroxy-9,10-seconandrost-1,3,5(10)-triene-9,17-dione; 4,9-DSHA, 4,5-9,10-diseco-3-hydroxy-5-9-17-trioxoandrosta-1(10),2-diene-4-oic acid; 2-HHD, 2-hydroxy-hexadieno-ate; DOHNAA, 9,17-dioxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid. The enzymes are: CHO,

clusters initially identified in RHA1 (Fig. 10b). Overall, it appears that steroid degradation is a common, perhaps ubiquitous, characteristic of rhodococci.

A common intermediate in bacterial steroid degradation is 4-androstene-3,17-dione (AD) (Fig. 10a). Nevertheless, it is unclear whether side-chain degradation precedes all ring degradation steps or whether these two processes occur concurrently. Recent evidence indicates that the oxidation of the alkyl side chain occurs before transformation of 3-hydroxysterols to 3-oxo sterols (Rosloniec et al. 2009), but it is unknown whether full cleavage of the side chain is necessary before ring degradation can begin. Moreover, cultures of *R. rhodochrous* IFO3338 are capable of selective side-chain cleavage of sterols in the presence of Fe^{2+}-chelating agents, chemically inactivating enzymatic ring degradation resulting in the accumulation of pharmaceutically interesting intermediates such as C-22-oic acid steroid catabolites and 1,4-androstadiene-3,17-dione (ADD) (Arima et al. 1978). In the following text, we summarize first the sterol uptake and then, side-chain degradation and ring degradation.

5.1 Uptake of Sterols

The transport of cholesterol and some other sterols across the cell membrane, periplasm, and thick mycolic-acid-containing cell wall of rhodococci is performed by a multicomponent ATP-dependent uptake system encoded by the *mce4* locus (Mohn et al. 2008). This locus comprises an 11-gene operon consisting of *supAB, mce4ABCDEF, mceHI*, and *ro04706*. SupAB, Mce4ABCDEF, and Mce4HI, all are essential components of the system that transports steroids that have a long, hydrophobic side chain such as cholesterol, 5α-cholestanol, 5α-cholestanone, and β-sitosterol. The SupAB proteins constitute the permease subunits. The roles of Mce4ABCDEF and MceHI in steroid transport remain unknown. However, each of the six Mce4ABCDEF proteins has a signal sequence, suggesting that they are located outside of the cell. Moreover, the homologous Mce1ABECDEF proteins of

Fig. 10 (Continued) cholesterol oxidase; 3βHSD, 3β-hydroxysteroid dehydrogenase; KstD, 3-ketosteroid-Δ1-dehydrogenase; KshAB, 3-ketosteroid-9α-hydroxylase (oxygenase and reductase); HsaAB, 3-HSA hydroxylase (oxygenase and reductase); HsaC, 3,4-DHSA dioyxgenase; HsaD, 4,9-DSHA hydrolase; and HsaEFG, 2-HHD hydratase, aldolase, and dehydrogenase, respectively. Further transformation of DOHNAA is unknown. 2-HHD is degraded by the lower Bph pathway to central metabolites. Adapted from van der Geize et al. (2007). (**b**) The gene organization of the steroid catabolic pathways in *R. jostii* RHA1, *R. opacus* B4, *R. erythropolis* PR4 and *C. testosteroni* TA441. Genes are *colored* by function as follows: *blue*, dehydrogenase; *green*, Rieske oxygenase; *orange*, hydroxylase; *red*, extradiol dioxygenase; *yellow*, MCP hydrolase; *lavender*, lower Bph pathway enzymes; *gray*, reductase; and *pale green*, reductase component of KshA. *Numbers* between genes correspond to the intergenic distance. Identity of the other steroids degraded by the other rhodococcal gene clusters is unknown

M. tuberculosis have been localized to the cell envelope (Shimono et al. 2003). Finally, these six Mce proteins have a shared sequence predicted to fold into five β-strands and eight α-helices, suggestive of a common function (Casali and Riley 2007). The ATPase driving the RHA1 Mce4 transporter may be either Ro01974 or Ro02744, which are orthologs of the ATPase MceG of *M. tuberculosis* H37Rv. The *mce4* locus is conserved in *R. equi* 103S (Meijer and Prescott 2004; van der Geize et al. 2008). Inactivation of the *supAB* genes impaired growth of *R. equi* on cholesterol, but did not affect the intracellular survival of the pathogen (van der Geize et al. 2008). Similarly, cholesterol import by *M. tuberculosis* H37Rv was found not to be required for establishing infection in mice or for growth in resting macrophages, but does appear to be important for persistence of the pathogen (Pandey and Sassetti 2008).

Although Mce4 takes up cholesterol, it appears that not all steroids are taken up by an Mce system and not all Mce systems take up steroids. Thus, while the RHA1 genome encodes two complete Mce systems and four steroid degradation pathways, described further below, the *mce4* cluster is the only one that is proximal to a steroid degradation gene cluster. A similar result is obtained by analyzing the other rhodococcal genome sequences, which revealed the presence of five complete *mceABCDEF* gene clusters in PR4 and six in B4. Overall, it appears that sterols that do not have a long hydrophobic side chain are taken up by other transport systems.

5.2 Side-Chain Degradation

Microbial side-chain degradation was first observed in a strain of *Nocardia*, where cholesterol was poorly transformed to C22-oic acid pathway intermediates, AD and ADD (Whitmarsh 1964). Most notably, the complete pathway by which the cholesterol side chain is removed, resulting in the 17-keto substituent, was elucidated in *Nocardia* (Sih et al. 1968). Subsequently, *Mycobacterium* sp. mutants were isolated that selectively degraded the cholesterol side chain to produce AD and ADD without transforming the steroid rings (Marsheck et al. 1972). The accumulation of AD and ADD suggests that the side-chain degradation occurs prior to sterol ring degradation. In general, microorganisms appear to shorten the side chain (C-21 to C-28) of sterols such as stigmasterol, β-sitosterol, campesterol, and cholesterol (Fig. 10a) by a mechanism similar to that of β-oxidation of fatty acids (Szentirmai 1990).

Recent molecular genetic and spectroscopic data indicate that a cytochrome P450, CYP125, initiates sterol side-chain degradation by catalyzing the oxidation of C-26 (Rosloniec et al. 2009). CYP125 was found to bind tightly to cholesterol and 5α-cholestane-3β-ol, and a *cyp125* knockout in RHA1 was impaired in growth on cholesterol and other 3-hydroxysterols with long aliphatic side chains (Rosloniec et al. 2009). It is unclear whether CYP125 oxidizes C-26 to the carboxylic acid or whether dehydrogenases are involved after an alcohol is formed. Once formed, this acid is activated by an acyl-CoA ligase that is CoA-, ATP-, and magnesium-dependent (Chen 1985). Following CoA activation, dehydrogenation of C-24 and

C-25 occurs, mediated by an acyl-CoA dehydrogenase, followed by hydration of the double bond by an enoyl-CoA hydratase. Subsequent dehydrogenation of the C24-hydroxy moiety, catalyzed by a β-hydroxyacyl-CoA dehydrogenase, and thiolytic cleavage result in shortening of the cholesterol side chain with the release of propionyl-CoA and acetyl-CoA in the first and second cycles of β-oxidation, respectively. The remaining three-carbon side chain of the C-22-oic acid is thought to be released via aldolytic fission. Degradation of the C24-branched side chains of sterols such as β-sitosterol requires cleavage of the C-24 substituent. β-Sitosterol side-chain degradation is initiated by C-28 carboxylation and subsequent CoA-activation (Fujimoto et al. 1982). After dehydrogenation and hydration, the C24-branched chain is released as propionyl-CoA by a reverse-aldol reaction. Further side-chain degradation occurs as in cholesterol. RHA1 has multiple sets of genes that encode the types of enzymes necessary to perform β-oxidation, and many are highly upregulated during growth on cholesterol (van der Geize et al. 2007). Two sets of β-oxidation enzymes are predicted to perform the cycles of β-oxidation, resulting in the formation of the C-24 and C-22-oic acid intermediates (Fig. 10a) (van der Geize et al. 2007).

5.3 Nucleus Degradation

While aspects of steroid degradation have been well documented in several microorganisms, including those of the genera *Nocardia* (Sih et al. 1967), *Pseudomonas* (Owen et al. 1983), and *Mycobacterium* (Marsheck et al. 1972), genes for steroid degradation have only recently been identified. Steroid catabolic genes were first identified in *Comamonas testosteroni* TA441 (Horinouchi et al. 2004). Shortly thereafter, genomic studies of RHA1 led to the identification of four clusters of genes potentially encoding distinct steroid-degrading pathways, one of which is specific for cholesterol (McLeod et al. 2006; van der Geize et al. 2007). These pathways are all predicted to involve aromatization of ring A and are rich in oxygenases (Fig. 10a). Rhodococcal steroid catabolism appears to be analogous to the process in other actinobacteria, and our knowledge of the process is based on studies of several genera.

Degradation of the steroid nucleus is initiated by either an NAD^+-dependent 3β-hydroxysteroid dehydrogenase (3β-HSD) (Yang et al. 2007a) or an O_2-dependent cholesterol oxidase (CHO) (MacLachlan et al. 2000). In either case, the 3β-hydroxy-Δ^5 sterol is oxidized to a 3-keto-Δ^5 intermediate, which then spontaneously isomerizes to the 3-keto-Δ^4 configuration. In the case of cholesterol, 4-cholestene-3-one is produced. Rhodococci displaying moderate cholesterol-degradation activity were found to possess both extracellular and intracellular oxidase activity (Aihara et al. 1986). Further metabolism of AD to 9α–hydroxy-1,4-androstadiene-3,17-dione (9-OHADD) involves two enzymes: 3-ketosteroid-Δ^1-dehydrogenase (KstD), a flavoprotein, catalyzes C-1(2)-dehydrogenation of ring A; and 3-ketosteroid-9α-monooxygenase (KshAB), a two-component RO, catalyzes the 9α-

hydroxylation. The order of these two steps does not appear to be obligate (van der Geize et al. 2000, 2002a, 2002b). The ratio of the respective intermediate metabolites 1,4-androstadiene-3,17-dione (ADD) and 9α-hydroxy-4-androstene-3-17-dione (9-OHAD) produced by KstD or KshAB, respectively, from AD is unknown.

Four phylogenetically distinct types of KstD enzymes have been identified in actinobacterial genomes, three of which were characterized from *R. erythropolis* SQ1 (Knol et al. 2008). Two of these, KstD1 and KstD2, were shown to display broad substrate specificities towards diverse 3-ketosteroids. By contrast, the KstD3-type enzymes are highly specific 3-keto-5α-steroid Δ^1-dehydrogenases, displaying highest activity towards 5α-androstane-3,17-dione and 17β-hydroxy-5α-androstane-3-one (Knol et al. 2008). Interestingly, the cholesterol catabolic gene cluster of RHA1 contains a KstD3, but neither a KstD1 nor a KstD2, suggesting that 5α-H steroids having saturated A-rings may be intermediates in the degradation of sterols (Fig. 10a) (Knol et al. 2008). While it is unclear why such an intermediate would occur during sterol degradation, it is possible that it is formed by a steroid Δ5-reductase to prevent steroid ring degradation from occurring prior to complete side-chain degradation. Located next to *kstD3* on the *R. erythropolis* SQ1 chromosome is a gene encoding a probable 3-ketosteroid-Δ^4-(5α)-dehydrogenase, Kst4D, homologous to TesI of *C. testosteroni* TA441 (Horinouchi et al. 2003a). TesI catalyzes the desaturation of the C4–C5 bond in sterol A ring, a step that is required for the degradation of 5α-H steroids. Thus, Kst4D appears to be involved in cholesterol degradation via a 5α-H steroid intermediate (Fig. 10a) (Knol et al. 2008). As with the 3-ketosteroid Δ^1-dehydrogenases, several phylogenetically distinct groups of 3-ketosteroid Δ^4-dehydrogenases can be distinguished in actinobacterial genomes. TesI and Kst4D belong to one phylogenetic type, whereas the RHA1 genome appears to encode a single Δ^4-dehydrogenase (Ro05698) of a different type (Knol et al. 2008).

KshAB transforms both AD and ADD, as demonstrated by molecular genetic studies in *R. erythropolis* SQ1 (van der Geize et al. 2002b). Purified KshAB of *R. rhodochrous* DSM43269 catalyses the NADH-dependent 9α-hydroxylation of a range of steroids (Capyk et al. 2009; Petrusma et al. 2009), although the *M. tuberculosis* enzyme has higher specificity for ADD than AD, producing 9-OHADD (Capyk et al. 2009). The latter undergoes aromatization and cleavage of ring B via a nonenzymatic reverse-aldol reaction to produce 3-hydroxy-9,10-secondandrost-1,3,5(10)-triene-9,17-dione (3-HSA). Ring A of 3-HSA is hydroxylated by a two-component oxygenase and reductase (HsaAB), requiring molecular oxygen and NADH, to yield a catecholic 3,4-dihydroxy-9,10-seconandrost-1,3,5(10)-triene-9,17-dione (3,4-DHSA) and subsequently cleaved by an extradiol dioxygenase (HsaC). 3,4-DHSA was deduced as a metabolite in the catabolism of AD by *N. restrictus* ATCC 14887 nearly 50 years ago, based on the limited knowledge of bacterial degradation of aromatic compounds (Sih et al. 1965). An MCP hydrolase then cleaves the C-5:C-6 bond of 4,5-9,10-diseco-3-hydroxy-5-9-17-trioxoandrosta-1(10),2-diene-4-oic acid (4,9-DSHA) through addition of water, resulting in 2-hydroxyhexadienoate (2-HHD) and 9,17-dioxo-1,2,3,4,10,19-

hexanorandrostan-5-oic acid (DOHNAA) as products. 2-HHD is transformed to central metabolites via a homolog of the Hpd pathway involving the successive actions of a hydratase (HsaE), an aldolase (HsaF), and an acetaldehyde dehydrogenase (HsaG). Degradation of the propionate moiety of DOHNAA occurs via a cycle of β-oxidation, first proposed by Lee and Sih (1967) and supported by subsequent studies (Miclo and Germain 1990, 1992). The first step in DOHNAA degradation in *R. equi* is suggested to involve ATP-dependent CoA activation, followed by reduction of the 5′-keto moiety by a DOHNAA-CoA reductase. The CoA activation is required for reduction (Miclo and Germain 1990). The rhodococcal enzymes involved in degradation of the propionate moiety of DOHNAA have yet to be identified. It is predicted that ring D of DOHNAA is degraded by a Baeyer–Villiger monooxygenase and a lactone hydrolase (Ro06698 and Ro06693 in RHA1, respectively) (McLeod et al. 2006). While these genes are upregulated during growth on cholesterol, they are not clustered with the other cholesterol catabolic genes.

Each of the four clusters of steroid degradation genes in RHA1 codes for homologs of each of the four enzymes which together cleave rings B and then A: KstD, KshAB, HsaAB, and HsaC (Fig. 10b), although it is unknown which steroids are degraded by the enzymes encoded in each cluster. The KshA homologs encoded in each gene cluster share at least 52% amino acid sequence identity with KshA of *R. erythropolis* SQ1 (van der Geize et al. 2002b). Indeed, many of these proteins share significant sequence similarity with the Tes proteins that specify growth of *C. testosteroni* TA441 on testosterone (Fig. 10b). For example, each of the four HsaC homologs of RHA1 shares greater sequence identity (at least 37%) with the TesB dioxygenase of *C. testosteroni* TA441 (Horinouchi et al. 2003b) than with any other extradiol dioxygenase.

A notable feature of the rhodococcal steroid degradation pathways is their apparent redundancy. This is perhaps most striking for *hsaEFG*, which encodes a pathway homologous to the Hpd central aromatic pathway involved in the degradation of biphenyl. Three of the four RHA1 clusters encode homologs of HsaEFG to transform 2-HHD to central metabolites. This redundancy is in contrast to the organization of many aromatic pathways. For example, a single β-ketoadipate pathway transforms catechol and protocatechuate generated from a range of aromatic compounds.

6 Conclusion and Prospects

It is clear from the number and range of publications relating to *Rhodococcus* in recent years that the genus is of considerable interest in a wide variety of fields (Fernandes et al. 2003; Martinkova et al. 2009). The metabolic activities of this genus underline the latter's tremendous potential for bioremediation and as biocatalysts in the production of bioactive molecules. The currently sequenced genomes provide important insights into the aerobic degradation of aromatic compounds and pollutants. However, our understanding of how these pathways are regulated and

how we might exploit them for industrial applications is still nascent. The large genome of RHA1 consists of many copies of catabolic genes which are organized in complex pathways (McLeod et al. 2006). Gene redundancy, multiple gene activation, and diversity of metabolic pathways have allowed members of the rhodococcal genus to use mixtures of aromatic compounds as growth substrates. However, this very property has hampered efforts to engineer strains for bioremediation that also survive well in a changing environment (Cases and de Lorenzo 2005). While several transcriptional regulators (Eulberg and Schlömann 1998; Iida et al. 2009; Nga et al. 2004) are associated with rhodococcal catabolic pathways, the mechanisms of gene expression are not fully understood. Regardless, undesirable pathways and genes can be tightly controlled in a number of ways as a result of advances in the tools for rhodococcal genetic engineering. For example, the construction of plasmid vectors for gene transfer in *R. erythropolis* CCM 2595 enabled the study of the P-*catA* and P-*catR* promoters of the β-ketoadipate pathway (Vesely et al. 2003, 2007). Similarly, the development of unmarked gene deletion techniques for constructing multiple gene deletion mutants in *Rhodococcus* should facilitate the further characterization of the catabolic pathways of aromatic compounds and steroids (van der Geize et al. 2000, 2008). Ultimately, greater knowledge of rhodococcal physiology, genetics, and enzymology will contribute to engineering improved transformation of a vast array of aromatic compounds for industrial and environmental purposes.

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