

THE β -KETOADIPATE PATHWAY AND THE BIOLOGY OF SELF-IDENTITY

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ABSTRACT

The β -ketoadipate pathway is a chromosomally encoded convergent pathway for aromatic compound degradation that is widely distributed in soil bacteria and fungi. One branch converts protocatechuate, derived from phenolic compounds including *p*-cresol, 4-hydroxybenzoate and numerous lignin monomers, to β -ketoadipate. The other branch converts catechol, generated from various aromatic hydrocarbons, amino aromatics, and lignin monomers, also to β -ketoadipate. Two additional steps accomplish the conversion of β -ketoadipate to tricarboxylic acid cycle intermediates. Enzyme studies and amino acid sequence data indicate that the pathway is highly conserved in diverse bacteria, including *Pseudomonas putida*, *Acinetobacter calcoaceticus*, *Agrobacterium tumefaciens*, *Rhodococcus erythropolis*, and many others. The catechol branch of the β -ketoadipate pathway appears to be the evolutionary precursor for portions of the plasmid-borne *ortho*-pathways for chlorocatechol degradation. However, accumulating evidence points to an independent and convergent evolutionary origin for the eukaryotic β -ketoadipate pathway. In the face of enzyme conservation, the β -ketoadipate pathway exhibits many permutations in different bacterial groups with respect to enzyme distribution (isozymes, points of branch convergence), regulation (inducing metabolites, regulatory proteins), and gene organization. Diversity is also evident in the behavioral responses of different bacteria to β -ketoadipate pathway-associated aromatic compounds. The presence and versatility of transport systems encoded by β -ketoadipate pathway regulons is just beginning to be explored in various microbial groups. It appears that in the course of evolution, natural selection has caused the β -ketoadipate pathway to assume a characteristic set of features or identity in different bacteria. Presumably such identities have been shaped to optimally serve the diverse lifestyles of bacteria.

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INTRODUCTION

The microbial degradation of aromatic compounds has tremendous practical significance. In addition to the well publicized problem of environmental contamination by toxic aromatic hydrocarbons, huge amounts of aromatic material are contributed to the biosphere from natural sources. The complex aromatic polymer lignin comprises about 25% of the land-based biomass on earth, and the recycling of this and other plant-derived aromatic material is a vital component of the earth's carbon cycle. The resonance energy that stabilizes the carbon-carbon bonds of aromatic rings presents microorganisms with a significant biochemical challenge. Both aerobic and anaerobic microorganisms have been isolated that degrade aromatic compounds, but much more is known about aerobic pathways. In general, degradation proceeds in two phases. First, an aromatic compound is prepared for ring cleavage by a variety of ring modification reactions. Of the many diverse pathways that have been identified, all have in common a mono- or dioxygenation step that results in the formation of a dihydroxylated benzene ring. The second phase of degradation includes ring fission and subsequent reactions leading to the generation of tricarboxylic acid cycle intermediates. Ring fission is catalyzed by dioxygenases and is termed *ortho*-cleavage when it occurs between the hydroxyl groups (intradiol cleavage) and *meta*-cleavage when it occurs adjacent to one of the hydroxyls (extradiol cleavage). A third aerobic ring cleavage pathway, the gentisate

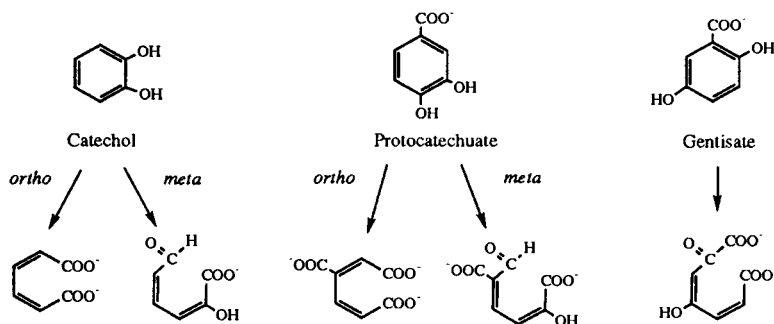


Figure 1 Major aerobic routes of aromatic ring cleavage: *ortho*-, *meta*-, and gentisate cleavage.

pathway, is followed when the two hydroxyl groups on the aromatic ring are *para* to each other, and cleavage occurs between the carboxyl-substituted carbon and the adjacent hydroxylated carbon (23) (Figure 1). Of the three pathways, the best studied are the *meta*-cleavage pathway and the *ortho*-cleavage or, as it is commonly known, the β -ketoacid pathway. The latter name derives from the fact that β -ketoacid is a key intermediate of the *ortho*-cleavage pathway.

The *meta*-cleavage and the β -ketoacid pathways each catalyze the dissimilation of the archetypal ring cleavage substrates, catechol and protocatechuate. *Meta*-fission pathway enzymes differ from those of the *ortho*-pathway in their ability to also catalyze the degradation of methylated catecholic substrates, and thus they have been well studied in connection with the degradation of methylated aromatic hydrocarbons such as toluene and xylene. Modified *ortho*-cleavage pathways include enzymes that are closely related to those of the β -ketoacid pathway but that have evolved to handle chlorinated substrates. These pathways appear to be used primarily for the dissimilation of chlorinated catechols generated from the metabolism of chlorobenzoates, chlorobenzenes, and chlorophenoxyacetate. The modified *ortho*-cleavage pathways are encoded on catabolic plasmids. *Meta*-cleavage pathways specifying the degradation of phenol, toluene, and naphthalene have been described that are plasmid encoded. Because they contribute to the degradation of environmental pollutants, the *meta*-cleavage pathway and modified *ortho*-pathways have been the subject of a number of recent reviews (8, 131, 140, 148).

The β -ketoacid pathway is widely distributed among taxonomically diverse eubacteria and fungi (Figure 2). It is almost always chromosomally encoded and plays a central role in the processing and degradation of naturally-occurring aromatic compounds derived from lignin and other plant components,

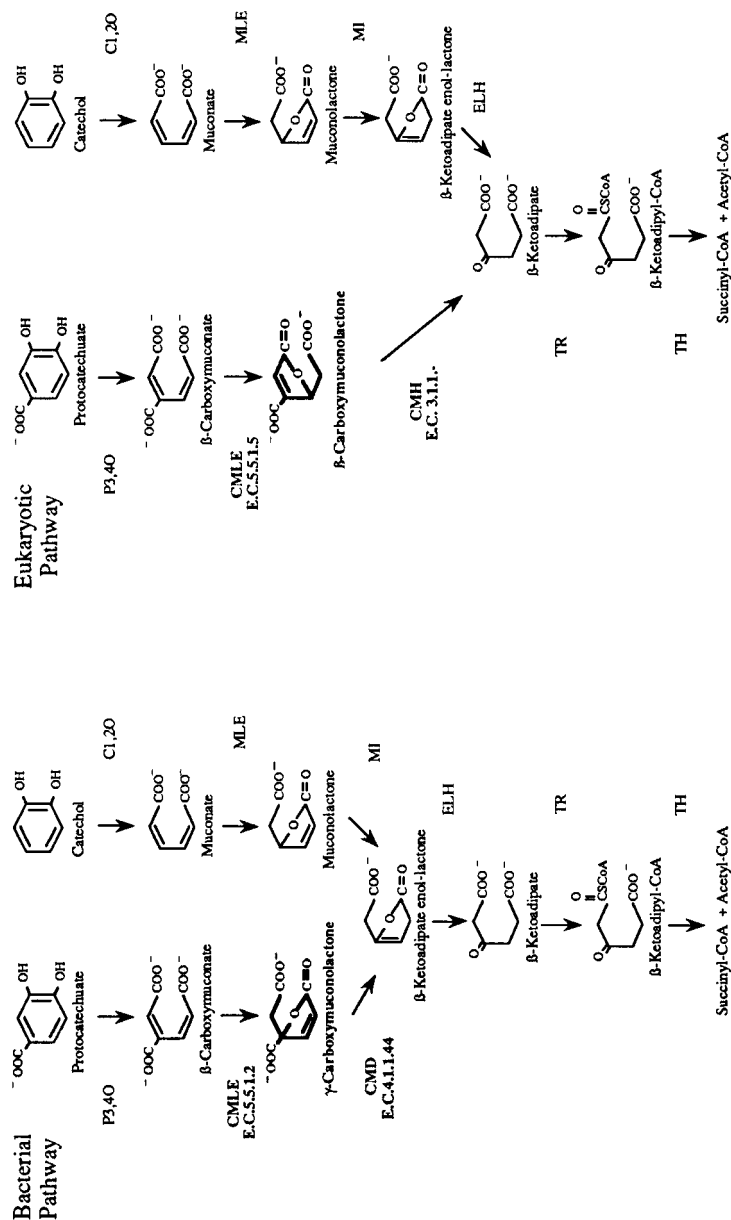


Figure 2 Bacterial and eukaryotic β -ketoadipate pathways. Enzymatic steps known to differ are indicated by EC numbers. Enzyme abbreviations: P3,4O, protocatechuate 3,4-dioxygenase; CML, β -carboxy-*cis*-muconate lactonizing enzyme; CMD, γ -carboxymuconolactone decarboxylase; C1,2O, catechol 1,2-dioxygenase; MLE, *cis*,*cis*-muconate lactonizing enzyme; MI, muconolactone isomerase; ELH, enol-lactone hydrolase; TR, β -ketoadipate:succinyl-CoA transferase; TH, β -ketoadipyl-CoA thiolase; CMH, β -carboxymuconolactone hydrolase.

as well as in the degradation of some environmental pollutants. This pathway often coexists in bacteria with plasmid-encoded *meta*-pathways (8), and it is always present in bacterial strains that degrade chlorinated aromatic compounds by means of plasmid-encoded modified *ortho*-cleavage pathways. This is because the final steps in the complete degradation of chlorinated catechols are catalyzed by the final two enzymes of the β -ketoadipate pathway (140). These characteristics underscore the importance of the β -ketoadipate pathway as a major utility pathway for aromatic compound degradation.

The rough outlines of the β -ketoadipate pathway were established prior to 1966, but the publication of four landmark papers by L. Nicholas Ornston firmly established the chemical and enzymatic steps of the reactions in the two branches. Important features of the regulation of the pathway as it exists in *Pseudomonas putida* were also described (97–99, 106). It seems appropriate that, in the 30th anniversary year of the publication of Dr. Ornston's papers, the β -ketoadipate pathway be reviewed. Here we focus on progress on molecular and biochemical aspects of the β -ketoadipate pathway made in the last decade and since this topic was last comprehensively summarized (105, 107, 145). We also review work on accessory features, including substrate transport and chemotaxis, which properly should be considered part of this degradation sequence. Emphasis is placed on the β -ketoadipate pathway as it operates in diverse groups of soil bacteria. As should be evident from the discussion below, the β -ketoadipate pathway has assumed a characteristic gene organization and set of regulatory features, or identity, that is specific to the bacterial group in which it resides. Because of this, the β -ketoadipate pathway provides an ideal model system for examining how a widely distributed, yet highly conserved series of enzymes, can diversify without compromising its core catalytic activities. Such information may aid in the identification of environmental selective forces that result in the tailoring of a particular pathway to best serve the lifestyle of a particular organism.

BIOLOGICAL DISTRIBUTION

Prokaryotes

Bacterial representation includes species of the gram-positive organisms of *Bacillus* (22), *Rhodococcus* (6, 150), *Arthrobacter* (10), and *Nocardia* (133), as well as gram-negative bacteria representing the alpha, beta, and gamma subdivisions of the proteobacteria. As discussed below, the β -ketoadipate pathway has been examined in most detail in the following gram-negative genera: *Acinetobacter* (15), *Alcaligenes* (62), *Burkholderia* (162, 163), *Comamonas* (108), *Enterobacter* (28), *Pseudomonas*, and *Azotobacter* (46), as well as in

the nitrogen-fixing plant symbiotic species *Rhizobium* and *Bradyrhizobium*, and the plant pathogen *Agrobacterium tumefaciens* (118, 121). *Ortho*-cleavage dioxygenase activities indicative of the β -ketoadipate pathway have been reported in free-living nitrogen fixers belonging to *Azomonas*, *Azospirillum*, and *Beijerinckia* species (20). The β -ketoadipate pathway has not yet been reported in members of the Archaea.

Eukaryotes

A eukaryotic version of the β -ketoadipate pathway has been identified in members of the ascomycetous and basidiomycetous yeasts and fungi (13, 82). The fungal pathway has been studied in *Rhodotorula* spp. (21, 30), *Trichosporon cutaneum* (40, 79, 132), *Aspergillus* spp. (64, 72), and *Neurospora crassa* (78). The catechol branch of the eukaryotic pathway is present in some but not all eukaryotes and, when present, appears to match that of the prokaryotic pathway. The protocatechuate branch differs from its prokaryotic counterpart in that β -carboxy-*cis*,*cis*-muconate, the product of protocatechuate ring cleavage, is cyclized to give β -carboxymuconolactone rather than γ -carboxymuconolactone (13, 14) (Figure 2). The two branches of the fungal β -ketoadipate pathway converge at β -ketoadipate rather than at β -ketoadipate enol-lactone, as in the bacterial pathway (Figure 2).

Funneling Pathways

Despite its widespread taxonomic distribution, the β -ketoadipate pathway has been identified almost exclusively in soil microorganisms, with strong representation from bacterial groups that are found associated with plants. This makes sense in view of the large number of phenolic compounds synthesized by plants during growth.

A great variety of soluble phenolic compounds are released in plant rhizospheres where they serve as chemical signals that can mediate a diverse array of plant-microbe interactions, both symbiotic and virulent (129). Lignin, a major component of wood, is a polymer of phenolic aromatic compounds. Lignin-related monomers, as by-products of decaying plant material, have been detected in soil in micromolar quantities (151). Coniferyl alcohol, ferulate, vanillate, and 4-coumarate are converted to protocatechuate prior to degradation via the β -ketoadipate pathway (24, 121). Unsubstituted lignin-related compounds such as cinnamate are metabolized via the catechol branch of the pathway (4). The naturally occurring aromatics mandelate, anthranilate, and tryptophan are also converted to catechol and further catabolized by *ortho*-cleavage (100, 130). Quinate and shikimate, two hydroaromatic compounds released in abundant quantities from decaying plant material (55), are converted to protocatechuate before entry into the β -ketoadipate pathway (11, 117) (Figure 3). An

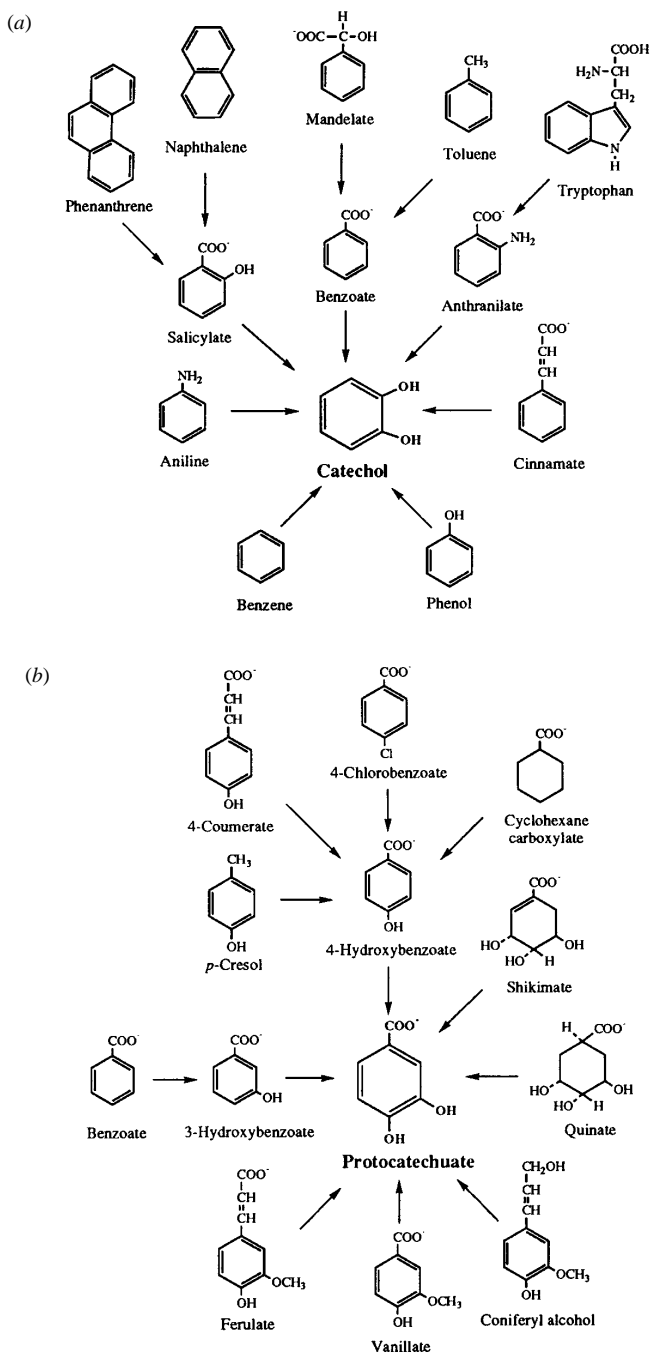


Figure 3 Compounds funneled to (a) catechol and (b) protocatechuate.

aromatization pathway leading to the formation of 4-hydroxybenzoate with subsequent metabolism by the β -ketoadipate pathway is followed in the catabolism of the alicyclic compound cyclohexanecarboxylate by *Arthrobacter* sp. (10).

Benzoate and 4-hydroxybenzoate almost always serve as growth substrates for microbes possessing the β -ketoadipate pathway, and the pathway has been studied most extensively in connection with the degradation of these two aromatic acids, which are only one (4-hydroxybenzoate) or two (benzoate) enzymatic steps removed from protocatechuate and catechol.

Aromatic hydrocarbons, aminoaromatics, and chlorinated aromatic compounds representative of industrially produced toxic substances are sometimes degraded by the β -ketoadipate pathway (Figure 3). Routes for aniline, benzene, naphthalene, *p*-cresol, and 4-chlorobenzoate degradation have been described that proceed through *ortho*-cleavage (6, 9, 29, 57, 154). A 4-chlorobenzoate pathway that has been studied in *Pseudomonas*, *Acinetobacter*, and *Arthrobacter* species is of particular interest because it involves an unusual conversion of 4-chlorobenzoate to 4-hydroxybenzoate via coenzyme A-esterified intermediates (29). The three enzymes that catalyze this conversion are a coenzyme A ligase, a dehalogenase, and a thioesterase. The 4-hydroxybenzoate that is generated is degraded via the β -ketoadipate pathway (29).

There is one well-documented example of a plasmid-encoded β -ketoadipate pathway. A 200-kb plasmid has been described from *Acinetobacter calcoaceticus* that encodes benzene degradation via the catechol branch of the β -ketoadipate pathway (154).

CONSERVATION OF ENZYME STRUCTURE AND FUNCTION

Prokaryotes

The chemistry and enzymology of the β -ketoadipate pathway have been studied primarily as they occur in *P. putida*. All nine enzymes catalyzing the conversion of protocatechuate and catechol to tricarboxylic acid cycle intermediates have been purified and characterized, and in several cases, crystal structures are available (Table 1). The best-studied enzyme is protocatechuate 3,4-dioxygenase (P3,4O), which catalyzes the intradiol cleavage of protocatechuate by incorporating two atoms from molecular oxygen to form β -carboxy-*cis,cis*-muconate. Enzyme activity requires the participation of a ferric iron ion that is located at the interface between the α and β polypeptide chains and is ligated by histidyl and tyrosyl residues within the β subunit (93). A refined crystal structure of the *Pseudomonas aeruginosa* (now reclassified as *P. putida*) enzyme has been reported (94). All the P3,4Os studied to date have a $\alpha\beta\text{Fe}^{3+}$ protomeric

structure, with the α and β subunits sharing substantial amino acid sequence identity (30%). However there is considerable diversity in the number of promoters that constitute active enzymes (Table 1).

Several of the parallel steps in the two branches of the β -ketoadipate pathway appear to be catalyzed by analogous reactions, raising the question of whether or not catalysis is mediated by homologous enzymes. This seems to be the case for the ring fission step; catechol 1,2-dioxygenase (C1,2O) catalyzes the incorporation of oxygen to form *cis,cis*-muconate and resembles P3,4O because it is a dimeric, ferric iron-containing enzyme. The two dioxygenases share noticeable amino acid sequence identity (about 25% identity between the *P. putida catA* and *pcaG* gene products), and the C1,2O sequences that have been reported have conserved tyrosyl and histidyl residues at positions corresponding to those that have been proposed to ligate Fe^{3+} at the active site of P3,4O (85). Most C1,2Os studied to date are homodimers of identical subunits. An exception is the enzyme from *Pseudomonas arvilla* strain C-1, which forms isozymes from combinations of two different subunits to give $\alpha\alpha$, $\alpha\beta$, and $\beta\beta$ dimeric forms of the enzyme (83). The C1,2Os associated with the β -ketoadipate pathway (encoded by *catA*) are often referred to as type I enzymes to distinguish them from type II catechol 1,2-dioxygenases, which catalyze the cleavage of chlorinated catechols and thus initiate degradation via modified *ortho*-cleavage pathways. Type I and type II C1,2Os share 25–35% overall amino acid sequence identity and show a high degree of sequence conservation in the proposed active site region (85).

The next parallel set of enzymes in the sequence, β -carboxy-*cis,cis*-muconate lactonizing enzyme (CMLE) and *cis,cis*-muconate lactonizing enzyme (MLE), also appear to catalyze analogous reactions (Figure 2). However, a determination of the stereochemistries of the lactonizations has shown that the reaction mechanisms of the two enzymes are actually quite different. CMLE mediates an *anti*-cycloisomerization whereas MLE catalyzes a *syn* addition to the double bond of *cis,cis*-muconate (17). The amino acid sequences of these two enzymes (deduced from the *A. calcoaceticus* and *P. putida pcaB* and *catB* genes) are not similar. Based on sequence analysis, CMLE belongs to the class II fumarase family of proteins (153), whereas MLE is a member of a different family that includes mandelate racemase (MR). MLE and MR have remarkably similar three-dimensional shapes, a finding that has heightened interest in the natural evolution of enzymes because the two enzymes catalyze chemically distinct reactions (86, 130). A probable common ancestry for MLE and MR is made more intriguing by the fact that both enzymes are part of the same metabolic pathway (mandelate feeds into the catechol branch of the β -ketoadipate pathway as shown in Figure 4; 38).

Table 1 Properties of bacterial β -ketoadipate pathway enzymes

Enzyme	E.C. No.	Gene designation	Subunit, mol. mass ^a	Oligomeric structure	Purified from	Crystal structure	Gene sequence	Amino acid identity Pp/Ac ^b
P3,4O Protocatechuate 3,4-dioxygenase	1.13.11.3	<i>pcaGH</i> (formerly <i>pcaA</i>)	PcaG (α), 23 kDa; PcaH (β), 27 kDa	$\alpha\beta$ protomer; <i>P. putida</i> , A. <i>calcoacetatus</i> ($\alpha\beta\text{Fe}^{3+}$) ₁₂ , <i>Burkholderia</i> <i>cepacia</i> ($\alpha\beta\text{Fe}^{3+}$) ₄ , <i>Brevibacterium</i> <i>fuscum</i> ($\alpha\beta\text{Fe}^{3+}$) ₆ <i>Azotobacter</i> <i>vinelandii</i> ($\alpha\beta\text{Fe}^{3+}$) ₁₀ ,	<i>P. putida</i> (12), A. <i>calcoacetatus</i> (149), <i>Rhizobium trifolii</i> (18), <i>Nocardia erythropolis</i> (71), <i>B. cepacia</i> (75), <i>B. fuscum</i> (152), <i>A. vinelandii</i> (30a)	<i>P. putida</i> (94), A. <i>calco-</i> <i>ceticus</i> (149), <i>B.</i> <i>cepacia</i> (75), <i>B.</i> <i>fuscum</i> (32)	<i>P. putida</i> (39), <i>B. cepacia</i> (163), A. <i>calcoacetatus</i> (47)	PcaG 53% PcaH 54%
CMLE β -Carboxy- <i>cis,cis</i> -muconate lactonizing enzyme (cycloisomerase)	5.5.1.2	<i>pcaB</i>	42 kDa	Not known	<i>P. putida</i> (127,153)	—	<i>P. putida</i> (153), A. <i>calcoacetatus</i> (69)	PcaB 45%
CMD γ -Carboxy- muconolactone decarboxylase	4.1.1.44	<i>pcaC</i>	15 kDa	Hexamer	<i>P. putida</i> (115) <i>A. vinelandii</i> , <i>A. calcoacetatus</i> (156, 158)	—	<i>P. putida</i> , ^c A. <i>calcoacetatus</i> (47), A. <i>tumefaciens</i> ^d	PcaC 53%
ELH β -Ketoadipate enol-lactone hydrolase	3.1.1.24	<i>pcaD</i> , <i>catD</i>	PcaD (ELH I), 29 kDa; CatD (ELH II), 29 kDa	Monomer	<i>P. putida</i> (80,160), A. <i>calcoacetatus</i> (157)	—	<i>P. putida</i> , ^c A. <i>calcoacetatus</i> (47, 142) A. <i>tumefaciens</i> ^d	PcaD 45%
TR β -Ketoadipate succinyl-CoA transferase	2.8.3.6	<i>pcaIJ</i> , <i>catIJ</i> (formerly <i>pcaE</i> and <i>catE</i>)	PcaI, CatI (α), 24 kDa; PcaJ, CatJ (β), 22 kDa	$\alpha_2\beta_2$	<i>P. putida</i> , A. <i>calcoacetatus</i> (159)	—	<i>P. putida</i> (109), A. <i>calcoacetatus</i> (69, 142)	PcaI 68% PcaJ 66%

TH	2.3.1.-	<i>pcaF, catF</i>	PeaF, 43 kDa	Tetramer ^e	<i>P. putida</i> ^e	—	<i>P. putida</i> (50), <i>A. calcoaceticus</i> (69,142)	PeaF 62%
β -Ketoadipyl-CoA thiolase								
Cl ₁ ,20	1.13.11.1	<i>catA</i>	32–34 kDa	Dimer	<i>P. putida</i> (84), <i>P. arvilla</i> (31) (83), <i>A. calcoaceticus</i> (126), <i>Frateuria</i> sp. (5)		<i>P. putida</i> PRS2000 (60), mt-2 (85), <i>Pseudomonas</i> sp. EST1001 (68), <i>P. arvilla</i> C-1 (85), <i>A. calcoaceticus</i> ADP1 (87), NCIB8250 (34), <i>Arthrobacter</i> (33)	CatA 55%
Catechol 1,2-dioxygenase								
MLE	5.5.1.1	<i>catB</i>	40 kDa	Octamer	<i>P. putida</i> (81)	<i>P. putida</i> (42, 86)	<i>P. putida</i> (3,60), <i>A. calcoaceticus</i> (142), <i>Rhodococcus erythropolis</i> ^f	CatB 53%
<i>cis, cis</i> -Muconate lactonizing enzyme (cycloisomerase)								
MI	5.3.3.4	<i>catC</i>	11 kDa	Decamer	<i>P. putida</i> (66, 81)	<i>P. putida</i> (66)	<i>P. putida</i> (3, 60), <i>A. calcoaceticus</i> (142), <i>R. erythropolis</i> ^f	CatC 57%
Muconolactone isomerase								

^aCalculated from deduced amino acid sequences.

^bAmino acid identities of deduced amino acid sequences from *P. putida* and *A. calcoaceticus*.

^cJE Houghton, personal communication.

^dD Parke, personal communication.

^eQ Li, K-L Ngai & LN Ormston, personal communication.

^fD Eulberg, LA Golovleva & M Schlömann, personal communication.

Mechanistic distinctions between the two branches of the β -ketoadipate pathway extend to γ -carboxymuconolactone decarboxylase (CMD) and muconolactone isomerase (MI). The genes (*pcaC* and *catC*) encoding these proteins share little sequence similarity, and a stereochemical analysis of the reactions catalyzed has indicated that the enzymes have opposite stereofacial specificities for their substrates (16). The crystal structure of MI has revealed that it is a decamer consisting of two pentamers stacked on top of each other. Five active sites, arranged around a central hydrophilic core, are contributed by five dimers (66).

Whereas most of the enzymes of the β -ketoadipate pathway are specialized and are not closely related to other proteins of wide biological distribution, the final two steps of the pathway are catalyzed by enzymes representative of tightly conserved groups that are generally involved in coenzyme A transfer and thiolytic cleavage. The two β -ketoadipate:succinyl-CoA transferase subunits from *P. putida* share about 40% amino acid sequence identity with the two subunits of acetoacetyl-CoA:acetate/butyrate transferase from *Clostridium acetobutylicum* and pig heart succinyl-CoA:3-ketoacid transferase (109). Sequence alignments suggest that the homodimeric structure of the mammalian enzyme probably derived from a gene fusion of the bacterial α and β subunits during

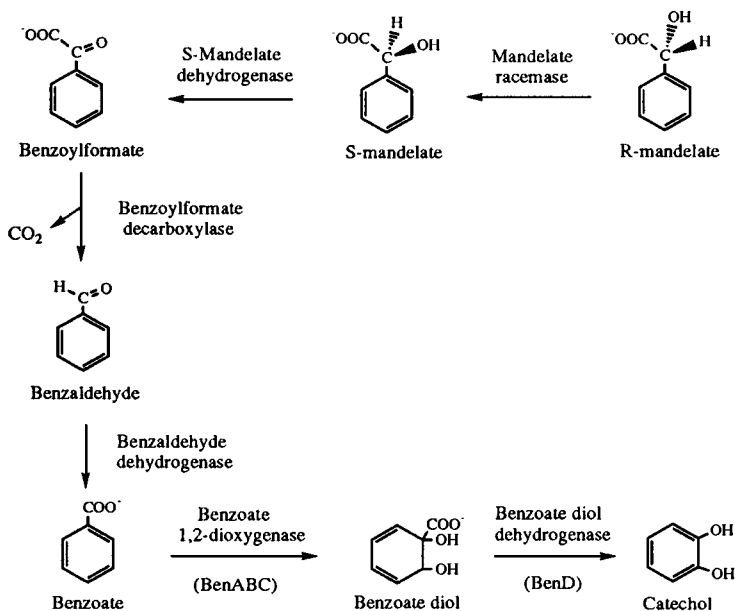


Figure 4 Conversion of mandelate and benzoate to catechol (38, 91).

evolution. Amino acid residues shown to be critical for catalysis are conserved in all three proteins (109, 134). The *P. putida* β -ketoadipyl-CoA thiolase amino acid sequence is about 45% identical to the deduced amino acid sequences of thiolases involved in poly- β -hydroxybutyrate biosynthesis by *Alcaligenes eutrophus* (128), fatty acid degradation by human mitochondria (1), and butyrate production by *C. acetobutylicum* (147). Most thiolases resemble the β -ketoadipyl-CoA thiolase purified from *P. putida* in that they are homotetramers (128). Even though a great deal of amino acid sequence conservation is seen with other transferases and thiolases, the β -ketoadipate:succinyl-CoA transferases and β -ketoadipyl-CoA thiolases synthesized by *P. putida* and *A. calcoaceticus* are specific to the β -ketoadipate pathway. Mutations in the genes encoding these enzymes block growth on benzoate and/or 4-hydroxybenzoate (27, 50, 109).

All of the genes of the β -ketoadipate pathway from *P. putida* and *A. calcoaceticus* have been cloned and sequenced. These two organisms, although both members of the gamma subdivision of the proteobacteria, have diverged sufficiently at the DNA level to have G+C contents that differ by 20%. Despite this, pairwise comparisons of isofunctional enzymes reveal amino acid sequence identities ranging from 45 to 68% (Table 1), an obvious indication that the β -ketoadipate pathways from *P. putida* and *A. calcoaceticus* are closely derived from a common ancestor. Comparisons of selected enzyme sequences from other organisms, along with enzymological data from other species, extend this conclusion to eubacteria in general. For instance, CatB and CatC from the gram-positive organism *R. erythropolis* share 39% and 49% amino acid identity with their counterparts from *P. putida* (D Eulberg, LA Golovleva, & M Schlömann, personal communication).

Eukaryotes

Two of the reactions in the protocatechuate branch of the fungal pathway are unique to eukaryotes and are not found in bacteria (14, 44). These are the cyclization of β -carboxy-*cis,cis*-muconate to give β -carboxymuconolactone, rather than γ -carboxymuconolactone as seen in prokaryotes, and the apparently direct conversion of β -carboxymuconolactone to β -ketoadipate (Figure 2). Recently, β -carboxy-*cis,cis*-muconate lactonizing enzyme (CMLE) has been purified from *N. crassa*, and the corresponding cDNA clone and gene sequence obtained (78). The sequence of the gene encoding *cis,cis*-muconate lactonizing enzyme, (MLE) from the fungus *T. cutaneum* has also been obtained (79). Inspection of the deduced amino acid sequences and mechanistic studies with purified enzymes strongly indicate that the fungal enzymes have evolved independently from the bacterial CMLEs and MLEs. Furthermore, the cycloisomerases from *T. cutaneum* and *N. crassa* appear to represent a novel eukaryotic motif in the cycloisomerase enzyme family. The finding that MLE and CMLE

from *T. cutaneum* and *N. crassa*, respectively, catalyze a *syn* lactonization that has an identical absolute stereochemistry to that of the *P. putida* MLE (67, 79) initially raised the possibility that the eukaryotic CMLE was related to the bacterial MLE (78). However, there are differences in the regiochemical course of the reaction with halogenated muconates between the *T. cutaneum* MLE and the *P. putida* chloromuconate cycloisomerase (79). Also, the *P. putida* MLE requires a divalent metal ion for activity, whereas the eukaryotic enzymes do not (78, 79). Finally, the *T. cutaneum* MLE has no discernable sequence similarity to bacterial MLE. Likewise, the *N. crassa* CMLE is not at all similar at the amino acid sequence level to bacterial cycloisomerases, either CMLE or MLE (78). However, the *T. cutaneum* MLE and *N. crassa* CMLE share 21% amino acid identity (79). The inference from these studies is that the fungal and bacterial β -ketoadipate pathways have evolved independently and convergently. Many of the other fungal β -ketoadipate enzymes catalyze reactions that are seemingly identical to their bacterial counterparts. It will be interesting to see whether the sequences of additional fungal genes, as they become available, reveal common or disparate evolutionary ancestries with prokaryotic β -ketoadipate pathway genes.

Relationship to the Modified Ortho-Pathways

DNA sequence analysis and enzyme studies indicate that the catechol branch of the β -ketoadipate pathway is the evolutionary precursor of at least portions of the modified *ortho*-pathways for chlorocatechol degradation. This is almost certainly the case for the initial two steps of the pathways, catalyzed by catechol 1,2-dioxygenase, and a cycloisomerase (Figure 5). The chloromuconate cycloisomerases catalyze dechlorination and formation of dienelactones. In the case of the β -ketoadipate pathway, the corresponding enol-lactone is generated by two enzymatic steps catalyzed by muconate cycloisomerase (muconate lactonizing enzyme, MLE) and muconolactone isomerase. The enzymes catalyzing the lactone hydrolyzing step (e.g. PcaD/CatD and ClcD) show significant differences, and the final step of the modified *ortho*-pathways, catalyzed by maleylacetate reductase, lacks any counterpart at all in the β -ketoadipate pathway.

In addition to the close structural relationship between type I (acting primarily on unmodified catechol) and type II (acting on chlorocatechols) catechol 1,2-dioxygenases, mentioned above, muconate and chloromuconate cycloisomerases are closely related, sharing on the order of 40% amino acid identity. Despite their close structural similarity, the cycloisomerases differ with respect to substrate specificity as well as dehalogenation ability (140). Although the dienelactone hydrolases of the modified *ortho*-pathways and the enol-lactone hydrolases of the β -ketoadipate pathway were initially assumed to be closely related, sequence data have shown that this is not the case; the two

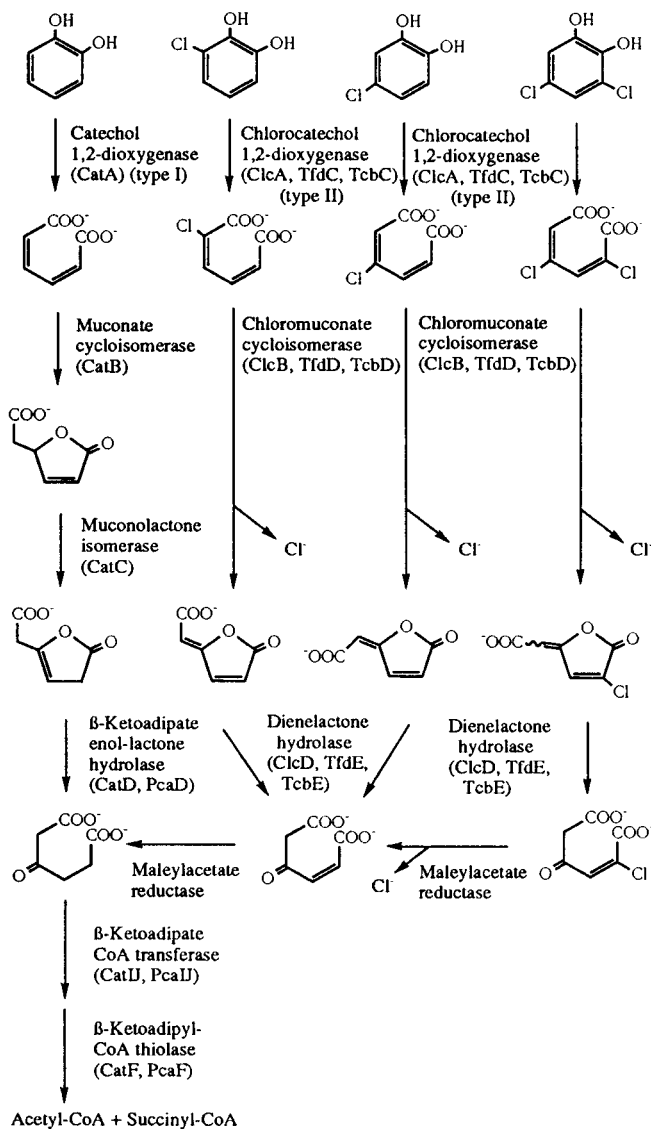


Figure 5 Comparison of the *ortho*-(β -ketoadipate) and modified *ortho*-pathways (140).

groups of enzymes share no obvious amino acid sequence identity. However, close inspection of the sequences does suggest that enol-lactone hydrolases have features indicative of α/β hydrolase fold enzymes, a group to which the dienelactone hydrolases belong (95, 140). Dienelactone hydrolases catalyze the formation of maleylacetates, which in turn are converted to β -ketoadipate by maleylacetate reductases (Figure 5) (65, 139). When taken together, available comparative sequence and enzyme data suggest that the β -ketoadipate and the modified *ortho*-pathways are related. However, the evolutionary path connecting the two appears to be more tortuous than initially supposed.

It is also important to point out that the use of chlorocatechols as carbon and energy sources by bacterial cells requires the synthesis of the last two enzymes of the β -ketoadipate pathway, β -ketoadipate:succinyl-CoA transferase and β -ketoadipyl-CoA thiolase. Thus modified *ortho*-pathways, all of which are plasmid encoded, depend on the presence of the β -ketoadipate pathway in the host strain in order to operate.

ACCESSORY FEATURES: CHEMOTAXIS AND TRANSPORT

Chemotaxis

An aspect of aromatic biology that is undoubtedly important for biodegradation is the detection and acquisition of aromatic compounds from the environment, processes that are governed by chemotaxis and transport. Aromatic acids and hydroaromatic compounds are good chemoattractants for motile gram-negative soil bacteria, including *P. putida*, *Rhizobium leguminosarum* biovars trifolii and phaseoli, *Bradyrhizobium japonicum*, and *Azospirillum* species (2, 7, 49, 54, 74, 119, 120). The ability of bacteria to sense and swim towards micromolar concentrations of phenolic compounds could be a factor guiding the association of bacteria with plant roots, sites expected to be rich in aromatic material. Ferulate, vanillate, 4-coumarate, 4-hydroxybenzoate, protocatechuate, shikimate, and quinate all derive from plant material; all are also growth substrates and chemoattractants for selected soil bacteria.

In some cases, bacteria are chemotactically attracted to compounds that they cannot immediately degrade. The strain of *P. putida* (PRS2000) that has been most studied with respect to chemotaxis is attracted to chlorobenzoates and toluates, compounds that it cannot ordinarily metabolize because it does not harbor catabolic plasmids encoding the requisite biodegradation genes (51, 53). The chemotactic response may still be important in natural situations, however, because sources of attractant—say a chemical waste spill containing chlorobenzoates as a component—would also be sites enriched in strains

carrying degradative plasmids (58). Higher densities of plasmid-bearing strains at the point source of attractant coupled with chemotaxis of strains to the source would increase the chances for plasmid transfer by conjugation with the chemotactic strain. In such a scenario, chemotaxis could be an important mechanism of plasmid distribution and strain recruitment to sites of toxic waste bioremediation. Interestingly, the TOL plasmid (for toluene/toluene degradation) and plasmid pAC27 (for chlorobenzoate degradation) do not themselves appear to encode chemotaxis functions (51, 53).

The β -ketoadipate pathway is present in most of the bacteria for which chemotaxis to aromatic compounds has been reported. *B. japonicum* expressed a constitutive chemotactic response to β -ketoadipate, while protocatechuate-grown cells were very chemotactic to protocatechuate (120) findings that suggest that chemotaxis to at least some compounds by this microbe is a β -ketoadipate pathway-associated trait. This is definitely the case for *P. putida* strain PRS2000. Chemotactic responses to the growth substrates benzoate and 4-hydroxybenzoate and to the nonmetabolizable compounds 3- and 4-toluate and 3- and 4-chlorobenzoate were coordinately induced in cells grown on β -ketoadipate (53, 54). Work with blocked mutants verified that β -ketoadipate, generated intracellularly as a metabolite of benzoate or 4-hydroxybenzoate degradation, is the direct inducer of the chemotactic response to aromatic acids (53, 54).

PcaK: A Dual Function 4-Hydroxybenzoate Chemotaxis/Transport Protein

Analysis of a *P. putida* mutant that was specifically nonchemotactic to 4-hydroxybenzoate led to the identification of a new gene associated with the β -ketoadipate pathway termed *pcaK* (50). The phenotype of *pcaK* mutants suggests that they are defective in the synthesis of a chemoreceptor for 4-hydroxybenzoate and related aromatic acids. These mutants failed to form chemotactic rings on soft agar swarm plates containing 4-hydroxybenzoate as a sole carbon source, and the mutants did not respond to aromatic acid chemoattractants in quantitative computer-assisted assays. The chemotactic responses of *pcaK* mutants to succinate and other nonaromatic compounds, including Casamino acids, were normal (50). An important additional feature of *pcaK* mutants is that they grew at wild-type rates on 4-hydroxybenzoate at neutral pH. That 4-hydroxybenzoate chemotaxis can be uncoupled from metabolism suggests that this response is receptor-mediated.

Although the *pcaK* phenotype suggests that the gene encodes a chemoreceptor protein, the deduced amino acid sequence of PcaK indicates that it is unrelated to known chemoreceptors. Rather, PcaK strongly resembles a

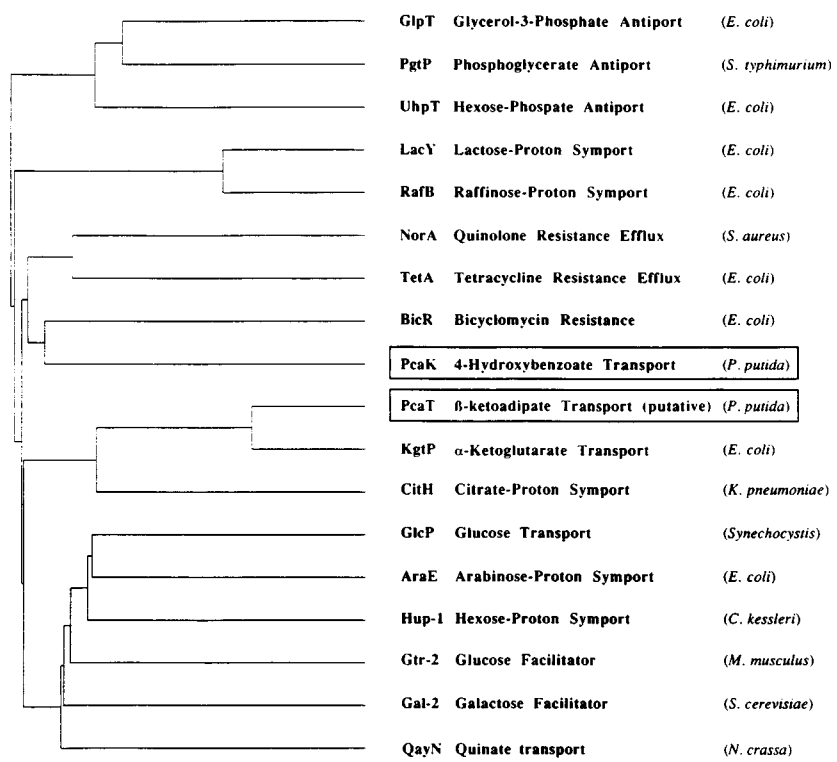


Figure 6 Relationship between PcaK and PcaT and representative members of the five clusters of the major superfamily of transmembrane facilitators (50, 76; JE Houghton, personal communication). The dendrogram was constructed by using the PILEUP program (Wisconsin Sequence Analysis Package, Genetics Computer Group, Madison, Wisconsin). The length of the line connecting any two sequences is a relative measure of relatedness.

transport protein (50). The PcaK sequence predicts a 47.2-kDa membrane protein that belongs to a large group of transport proteins termed the major facilitator super family of proteins, or USA proteins (for uniporter, symporter, and antiporter) (43, 76). PcaK is most closely related to a subgroup of proteins that function in drug efflux (Figure 6). When expressed in *Escherichia coli*, PcaK catalyzed the intracellular accumulation of 4-hydroxybenzoate against a concentration gradient at rates that were at least as great as those measured in wild-type *P. putida*, confirming its suspected role as an active transport system for 4-hydroxybenzoate. This protein is quite specific for 4-hydroxybenzoate and does not catalyze the transport of benzoate (50).

An intact 4-hydroxybenzoate transport system is critical for optimal growth of *P. putida* at high pHs, where only a small percentage of the available 4-hydroxybenzoate is present in the membrane permeable (4-hydroxybenzoic acid) form, as well as for optimal growth at the very low external 4-hydroxybenzoate concentrations that are known to occur in natural environments. Wild-type and *pcaK* mutant cells grew at similar rates on 4-hydroxybenzoate at pHs 6.3 and 6.8, but *pcaK* mutants grew at only about 40% the rate of wild-type cells at pH 8.2 (50). In addition, *pcaK* mutant cells were significantly impaired, relative to wild-type cells, in their ability to accumulate [^{14}C] 4-hydroxybenzoate when it was supplied at a concentration (70 μM) that is sufficient to saturate the transport system as expressed in *E. coli* (50). This concentration is on the order of the concentrations of 4-hydroxybenzoate that are seen in soils (151).

The most straightforward interpretation of the available data is that PcaK has dual functions as a chemoreceptor and a 4-hydroxybenzoate transporter in *P. putida*. Because PcaK is not homologous to methyl-accepting chemotaxis proteins or to periplasmic binding proteins, the two classes of characterized chemoreceptors, it would appear to represent a previously unrecognized type of chemoreceptor. This is an exciting possibility because it raises the suggestion that a member of the major facilitator superfamily, a large and relatively conserved group of proteins, may be able to initiate sensory signaling by a mechanism distinct from the well-defined *E. coli* system (37, 56). At this point much more work is required, however, to determine whether PcaK mediates direct transmission of sensory input to the central chemotaxis machinery of *P. putida* by some novel means, or whether it functions indirectly to modulate aromatic acid-stimulated transduction of sensory information.

Chemotaxis is certainly not an obligatory function of PcaK, because a homologous gene that is 57% identical at the amino acid level to the *P. putida* PcaK has been identified in *A. calcoaceticus*, a nonmotile organism (69). Although the *A. calcoaceticus* PcaK is presumed to mediate 4-hydroxybenzoate transport, its function in this bacterium has not yet been explored in detail. The *pcaK* genes from both species are located within clusters of *pca* structural genes for protocatechuate degradation (see below). Very recently, a second gene has been identified in *A. calcoaceticus* whose deduced amino acid sequence is about 30% identical to that of *P. putida* PcaK. This gene lies upstream of the benzoate dioxygenase genes and has tentatively been designated *benK* (EL Neidle & LS Collier, personal communication). It will be interesting to see whether *benK* encodes a benzoate-specific transporter.

β -Keto adipate Transport

P. putida synthesizes a transport system for β -keto adipate. This transporter was first identified based on the ability of 4-hydroxybenzoate-grown cells to

accumulate ^{14}C -labelled adipate, a nonmetabolizable analogue of β -ketoadipate (104). β -Ketoadipate appears to be the natural substrate for the transport system because adipate uptake is inducible by β -ketoadipate, and it is also competitively inhibited by β -ketoadipate with a K_i of 40 μM , which is lower than the K_m of 230 μM observed for adipate (96, 104). Furthermore, regulatory mutants have been isolated that constitutively synthesize the transport system as well as three of the enzymes of the protocatechuate branch of the pathway (CMLE, CMD, and ELH) (116). This latter result is consistent with the idea that the transporter and the *pcaB*, *C*, and *D* genes belong to a common transcriptional unit. In fact, a gene predicted to encode a protein with 67% amino acid sequence identity to α -ketoglutarate permease from *E. coli* (Figure 6) (141) has been identified immediately upstream and adjacent to the *pcaBDC* genes in *P. putida* (JE Houghton, personal communication). This gene, designated *pcaT*, is a strong candidate to encode the β -ketoadipate transporter.

Discovery of a transport system for β -ketoadipate was surprising because previous experiments had shown that growth of *P. putida* was limited by restricted permeability to this compound (144). Indeed β -ketoadipate supports a doubling time of only 140 min, as compared to a doubling time of 75 min for growth on a comparable concentration of 4-hydroxybenzoate (96). Together with the observation that the transport system is induced only severalfold by a concentration of β -ketoadipate that causes a 50-fold induction of the enzymes of the β -ketoadipate pathway (116), this suggests that overexpression of the β -ketoadipate transport system may be disadvantageous to cells under some circumstances. One such circumstance might be the energy-dependent transport of a nonmetabolizable compound such as adipate. Transport of adipate might harm cells by depleting precious energy reserves during carbon starvation. This idea was tested by comparing the starvation-survival rates of wild-type *P. putida* cells with those of a hyperconstitutive mutant that expresses 20-fold higher levels of the transport system. When the two strains were co-starved in minimal medium containing adipate but lacking a metabolizable carbon source, 99.99% of the high-level transport constitutive mutant cells lost viability in 50 h, whereas the viability of wild-type cells was unaffected. The mutant cells remained viable when starved in the absence of adipate (52). These results emphasize the importance of tight control over nutrient acquisition systems, especially under conditions of nutritional stress. Adipate is a naturally occurring dicarboxylic acid that *P. putida* would be likely to encounter in its environment. *P. aeruginosa*, a fluorescent pseudomonad that is closely related to *P. putida* and that occupies a similar ecological niche, can utilize adipate as a growth substrate (146).

The question of how widely distributed β -ketoadipate transport systems are in bacteria has not been extensively addressed. The transport system is expressed

in several biotypes of *P. putida* and *Pseudomonas fluorescens* (96). The presence of a β -ketoadipate transport system in *P. aeruginosa* or other bacteria does not appear to have been examined.

PATHWAY ORGANIZATION AND REGULATION IN DIVERSE BACTERIA

Branch Convergence and Isozymes

Although the enzymes of the β -ketoadipate pathway are conserved among bacteria, the pathway shows great diversification in the forms of regulation and pathway organization that have evolved. Some organisms carry only portions of the pathway, and the two branches of the pathway converge at different points in different bacteria. For example, in *P. putida*, the pathway converges at β -ketoadipate enol-lactone, and one set of enzymes is present to complete the conversion to tricarboxylic acid cycle intermediates (50, 99, 109). *A. eutrophus* has two isofunctional hydrolases that convert β -ketoadipate enol-lactone to β -ketoadipate, the branch convergence point (63). In *A. calcoaceticus*, the two branches never converge: two independently regulated sets of genes encode isofunctional enzymes for the last three steps of the pathway (Figure 7) (15, 69, 142). *Comamonas* species (formerly known as the acidovorans group of pseudomonads) metabolize protocatechuate through the *meta*-pathway. However they are capable of growth with the β -ketoadipate pathway intermediate β -carboxy-*cis,cis*-muconate using a truncated version of the protocatechuate branch of the β -ketoadipate pathway (108). A complete catechol branch of the β -ketoadipate pathway is present in *Comamonas acidovorans* (12a). Some organisms, such as *Azotobacter* spp., synthesize only the protocatechuate branch of the β -ketoadipate pathway and metabolize catechol and related substrates exclusively through a *meta*-cleavage route (46). The catechol branch of the pathway appears to be present only in some members of the rhizobial/agrobacterial phylogenetic groups (19, 117).

Inducing Metabolites

The genes of the β -ketoadipate pathway are inducible by pathway intermediates. Among different bacteria, however, many variations in inducing metabolites are seen. For example, protocatechuate induces all of the genes of the protocatechuate branch in *A. calcoaceticus* (15), and none of the *pca* genes in *Nocardia* (133) or *Agrobacterium* (111) species (Figure 8). β -Ketoadipate, on the other hand, is an important inducing metabolite in every organism except *A. calcoaceticus*. β -Carboxy-*cis,cis*-muconate is an inducer of protocatechuate-branch degradation genes in species of *Comamonas* (108), *Agrobacterium* (111), and *Rhizobium*

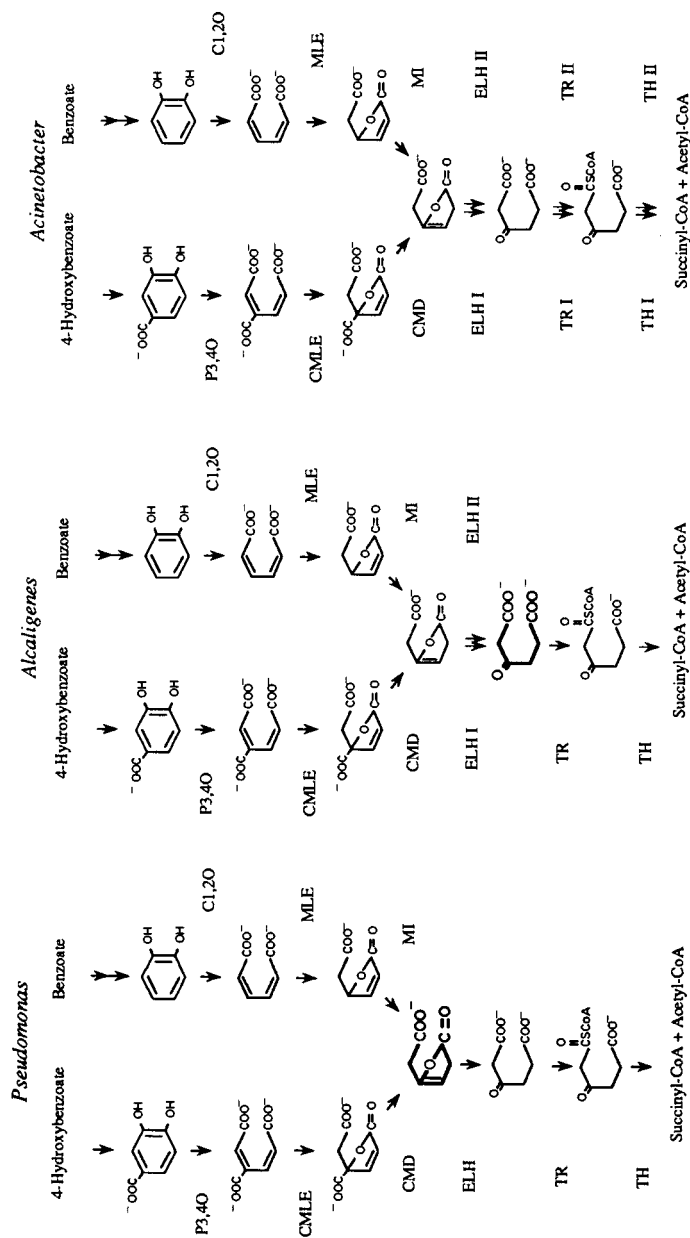
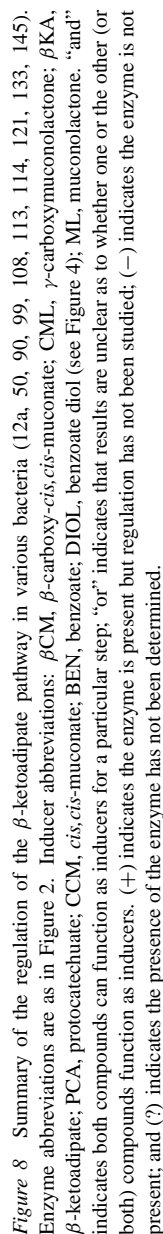


Figure 7 Convergence points of the *pca* and *cat* branches of the β -ketoadipate pathways in different bacteria (15, 63, 99). Enzyme abbreviations are as in Figure 2.



(121), but not, apparently, in other bacteria. In some instances two different compounds can act to induce the synthesis of a single enzyme. In *Burkholderia* (*Pseudomonas*) *cepacia*, for example, CMLE synthesis is induced by β -ketoadipate and 4-hydroxybenzoate (162). More than one inducer can also effect the synthesis of some of the enzymes in *Agrobacterium*, *Alcaligenes*, and *Acinetobacter* spp. (Figure 8). Examples of both substrate induction and product induction are seen, and in diverse *Bradyrhizobium* species, most of the enzymes of protocatechuate catabolism are produced constitutively (118). The results of physiological regulation studies of β -ketoadipate pathway enzymes in diverse bacterial species are shown in Figure 8.

Gene Organization

Molecular studies of the β -ketoadipate pathway carried out with *P. putida*, *A. calcoaceticus*, and *A. tumefaciens* have confirmed that molecular diversity as revealed by disparate induction patterns extends to diversity in gene organization and transcriptional regulation. Current information about the organization of the genes involved in catechol and protocatechuate degradation in *P. putida* and *A. calcoaceticus* is summarized in Figure 9. A complete set of information (with the exception of *pcaF*) is also available for *A. tumefaciens* A348, a strain that has only the protocatechuate branch of the β -ketoadipate pathway. Recently three *cat* genes have been identified and sequenced from the gram-positive species *R. erythropolis* 1CP (143) (D Eulberg, LA Golovleva, & M Schlömann, personal communication). Features of the gene clusters that are obvious from a superficial inspection are that *cat* genes and *pca* genes are generally clustered, but within these clusters, no particular gene order is maintained from species to species. Exceptions are *pcaIJ* and *pcaGH*, which encode two-subunit enzymes and thus would be expected to evolve as a unit.

Supraoperonic Clustering

Supraoperonic clustering of genes has been observed; operons encoding enzymes for related metabolic functions are contiguous on the chromosome. The close physical association of *cat* genes with *ben* genes and *pca* genes with *pob* genes, the former for conversion of benzoate to catechol and the latter for conversion of 4-hydroxybenzoate to protocatechuate, is striking. Not only are the *ben* genes located directly upstream from the *cat* genes in *A. calcoaceticus*, but BenM, a regulator that positively activates *ben* gene expression, can also function to activate *cat* genes (135; EL Neidle & LS Collier, personal communication). That this is a relatively recent evolutionary event is suggested by the observation that the *A. calcoaceticus* *ben* genes are homologous to the *xylXYZL* genes from the *P. putida* *meta*-pathway—encoding TOL plasmid (45). This indicates that the evolutionary ancestries of the *cat* and *ben* genes are distinct

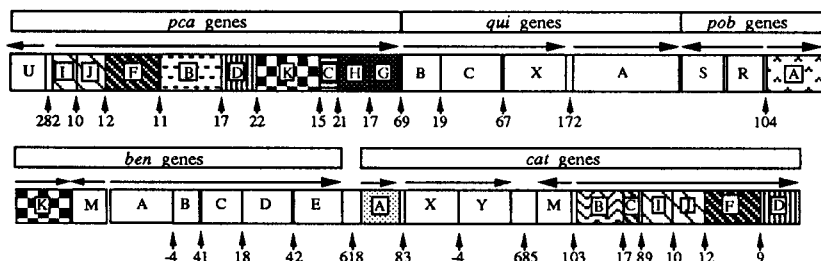
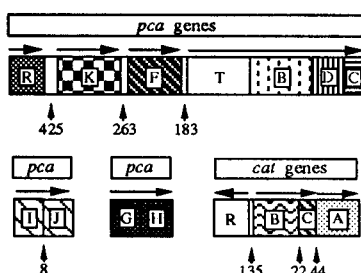
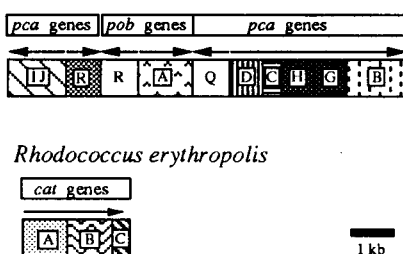
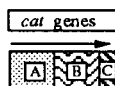
Acinetobacter calcoaceticus*Pseudomonas putida**Agrobacterium tumefaciens**Rhodococcus erythropolis*

Figure 9 The *pca* and *cat* gene organization in various bacteria (35, 36, 39, 47, 48, 50, 60, 69, 87–89, 109, 113, 142, 153). Transcriptional units, designated by horizontal arrows, are in some cases putative. Where known, spacing between genes (in base pairs) is indicated below the genetic maps. Gene designations are as given in Table 1. Functions for *benE*, *catX*, and *catY* are unknown. The genes *qui* and *benABCD* are for quinate and benzoate degradation, respectively (see Figures 4 and 10). Sequence comparisons indicate that *quiX* may encode a porin (36). No direct involvement of this gene in quinate degradation has been shown. *pobA* encodes 4-hydroxybenzoate hydroxylase. *catB*, *catM*, *pcaR*, *pcaU*, *pobR*, and *benM* are regulatory genes described in the text. *pobS* encodes a regulator that lowers expression of *pobA* by an unidentified mechanism (AA DiMarco & LN Ornston, personal communication). *pcaK* and *benK* are proposed aromatic acid transport genes as described in the text. *pcaT* is proposed to encode a β -ketoacid transporter.

and that selection pressures may have brought patches of independently derived genes together. *P. putida* cosmid clones have been obtained that include both *ben* and *cat* genes, and mapping studies indicate that these two clusters are contiguous on both the *P. aeruginosa* and *P. putida* chromosomes (161). These observations substantiate the perceived importance by some investigators of benzoate as a favored growth substrate that is funneled into the catechol branch of the β -ketoacid pathway and of 4-hydroxybenzoate as a favored substrate to enter the protocatechuate branch. Genes for conversion of quinate to protocatechuate are located between *pca* and *pob* genes in *A. calcoaceticus* (Figure 9). Quinate is also a good growth substrate for this soil-dwelling species

(Figure 10). This is perhaps not surprising in view of the fact that quinate has been estimated to account for 10% by weight of leaf litter (55).

Genes for the two branches of the β -keto adipate pathway do not necessarily map close to each other on the chromosome. In *A. calcoaceticus*, for example, the *cat* and *pca* genes are separated by a distance of about 290 kb (EL Neidle & B Gralton, personal communication). The relative positions of *cat* and *pca* genes have not been determined in *P. putida*, but in the closely related organism *P. aeruginosa*, they are located on practically opposite sides of the chromosome, 2000 kb apart (59). The relative positions of *pcaIJ*, *pcaGH*, and *pcaRKFTBDC* on the *P. putida* chromosome have not been determined, and the possibility that these gene clusters are closely linked, or even contiguous, has not been excluded.

DNA sequence data point to a common ancestry for genes encoding iso-functional enzymes from *P. putida* and *A. calcoaceticus* (Table 1) and have also revealed some remarkable features of the genes (*catIJF*, *catD*, *pcaIJF*, and *pcaD*) that encode the three isozymes of the last three steps of the *A. calcoaceticus* β -keto adipate pathway. The nucleotide sequences of *catIJF* and *pcaIJF* are virtually (99%) identical (69) and can freely exchange genetic information to repair mutations in the homologous set (27). Why are they so stable? Why isn't one of the sets lost through recombination? In contrast, the *pcaD* and *catD* genes appear to have evolved for avoidance of genetic exchange. These genes share just 52% nucleotide sequence identity, and the deduced amino acid sequences of PcaD and CatD from *A. calcoaceticus* are no more similar to each

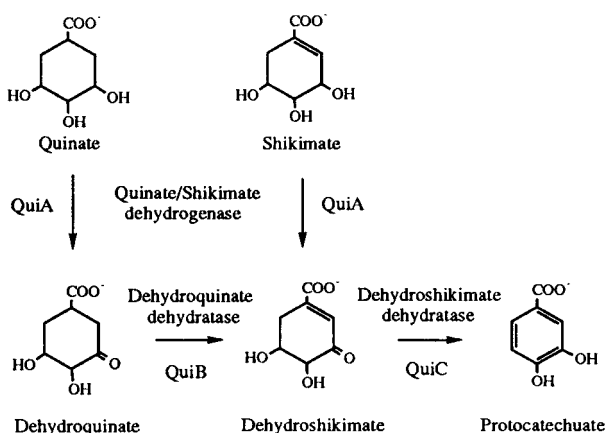


Figure 10 The quinate/shikimate degradation pathway in *Acinetobacter calcoaceticus* (36).

other than they are to the PcaD protein from *P. putida* (48; JE Houghton, personal communication). Why are these genes so different? Hartnett & Ornston (48) have proposed that in the case of *catIJF/pcaIJF*, genetic recombination and accompanying repair of mutations may help cells to maintain an accurate protein product. However, in the case of the *catD/pcaD* gene sequences, divergence somehow occurred beyond a threshold to a point where genetic exchange between genes became detrimental, and defective proteins were generated. This favored the selection of rapidly diverged genes.

Regulators and Operons

Structural genes are often flanked by regulatory genes, many of which are divergently transcribed. The best studied regulatory protein in the β -ketoacid pathway is CatR, which positively regulates expression of *catBC* and *catA* genes in *P. putida* in response to the inducer *cis,cis*-muconate (60, 137). Although *catBC* and *catA* are contiguous on the *P. putida* PRS2000 chromosome, *catA* can be independently transcribed from *catBC* (60). In *P. aeruginosa*, *catA* is located about 3 kb away from *catBC* (70).

CatR is a member of the LysR family of regulators (137). A closely related subgroup of the family includes proteins [ClcR (123), TfdR (77), and TcbR (73)] that are regulators for modified *ortho*-pathways involved in the degradation of monochlorocatechol, dichlorocatechol, and trichlorocatechol, respectively. The CatR protein has been purified, and its DNA binding properties and mechanism of *catBC* operon activation investigated (124, 125, 138). An inducer-independent binding site, also referred to as a repression binding site (RBS), has been localized to a 27-bp region between bases – 79 and – 53 relative to the transcription start site of *catB*. Two CatR dimers have been proposed to bind at this site, one bound to the DNA and the second stacked on the first dimer (124). Addition of the inducer (100 μ M *cis,cis*-muconate) to the system increases the affinity of CatR for *catB* promoter DNA by about twofold, and it also stimulates the binding of a CatR dimer to a site called the activation binding site (ABS) at nucleotides – 47 to – 34 (124, 125). Parsek et al have proposed that in the presence of inducer there is one dimer bound to the RBS and one bound to the ABS (124, 125). Experiments with DNA bending vectors show that binding of CatR to the RBS in the absence of inducer results in bending of DNA. In the presence of inducer, the binding of a CatR dimer to the ABS causes a relaxation of the bend angle. These observations have led to the proposal that a relaxation in the bending of DNA in the *catB* promoter region facilitates activation of transcription, possibly by allowing direct interaction of CatR with the α subunit of RNA polymerase in the – 35 region. There is evidence that this general type of transcription activation mechanism may also be used by other LysR proteins (122).

All of the genes for catechol degradation in *A. calcoaceticus* are activated by CatM, a homologue of CatR (41% amino acid identity) that, like CatR, acts in concert with the inducer *cis,cis*-muconate to positively regulate *cat* gene expression (135). In vivo footprinting has been used to identify a CatM recognition and binding site that is nearly identical to the proposed binding site for CatR and related LysR proteins (135). Analysis of *catM* mutants has been complicated by the fact that *A. calcoaceticus* synthesizes a second LysR-type regulator, BenM, which can activate *cat* gene transcription in response to *cis,cis*-muconate. BenM also induces *catA* gene expression in response to benzoate (90; EL Neidle, personal communication). The deduced amino acid sequences of CatM and BenM are 57% identical (EL Neidle & LS Collier, personal communication). It will be interesting to see if, and how, these two regulators interact at the *catA* promoter to bind and activate transcription.

A fourth LysR family member, PcaQ, operates in *A. tumefaciens* to induce expression of *pca* genes required for conversion of protocatechuate to β -ketoadipate (111, 114). PcaQ recognizes both β -carboxy-*cis,cis*-muconate and the unstable pathway intermediate γ -carboxymuconolactone as inducers. Although a LysR family member, PcaQ is not closely related to CatR subgroup members. CatR and PcaQ share just 15% amino acid sequence identity.

P. putida regulates most of the genes of the protocatechuate branch of the β -ketoadipate pathway with PcaR, a protein that belongs to a newly described family of regulatory proteins called the PobR family (25). PcaR functions to activate the expression of at least four separate transcriptional units in *P. putida* in response to β -ketoadipate. A functional *pcaR* gene is required for inducible expression of genes (*pcaBDC*, *pcaF*, *pcaIJ*) required for conversion of β -carboxy-*cis,cis*-muconate to TCA cycle intermediates, as well as for expression of the *pcaK* gene encoding the 4-hydroxybenzoate transport protein (61, 92, 110, 136) (Figure 8). The *pcaGH* genes, encoding protocatechuate 3,4-dioxygenase are induced by protocatechuate in *P. putida*, but the regulatory protein involved in *pcaGH* expression has not yet been identified. The PcaR protein, overexpressed in *E. coli*, was found to bind specifically to the *pcaIJ* promoter fragment in DNA mobility shift assays. Addition of β -ketoadipate did not alter migration of the complex (136). In complementary experiments with *lacZ* transcriptional fusions, a deletion analysis of the *pcaI* promoter region indicated that no specific sequences upstream of the -35 region are required for full induction by PcaR (110). This indicates that PcaR binds unusually closely to the transcription start site, and raises the possibility that PcaR has a novel mechanism of transcriptional activation. Comparisons between the regions upstream of *pcaR* and *pcaIJ* have revealed a 15-bp conserved nucleotide

sequence, centered at -10 relative to each transcription start site, that has been proposed to serve as a PcaR binding site (136).

PobR regulates 4-hydroxybenzoate-inducible expression of *pobA*, the gene encoding 4-hydroxybenzoate hydroxylase in *A. calcoaceticus* (25, 26). PobR, like PcaR, has an N-terminal helix-turn-helix motif, is about 30 kDa in size, and exhibits inducer-independent binding to the promoter region of the gene it positively regulates. Despite superficial similarities, PobR and PcaR do not cluster with LysR proteins or with other defined families of transcriptional regulators. Instead, sequence comparisons indicate that PobR forms the basis for a new cluster of evolutionarily related proteins that includes, in addition to PcaR, IclR, the acetate operon repressor in *E. coli*, and GylR, a regulator of glycerol catabolism in *Streptomyces* spp. (25). PobR from *A. calcoaceticus* and PcaR from *P. putida* share 32% amino acid sequence identity. *pcaU* is divergently transcribed from the *pcaJFBDKCHG* gene cluster. Its product functions as an activator of the protocatechuate degradation genes and presumably acts in concert with protocatechuate, the inducer of *pca* gene expression in *A. calcoaceticus* (41; LN Ornston, personal communication). PobR and PcaU from *A. calcoaceticus* share 51% amino acid sequence identity, whereas PcaR from *P. putida* is 34% identical to PcaU.

Genes have recently been identified in *A. tumefaciens* and *R. leguminosarum* that have been named *pobR* based on their proposed function as regulators of *pobA* expression (112, 155). It is not yet known if these genes are homologous to the *A. calcoaceticus* *pobR* gene. The *pcaR* gene from *A. tumefaciens* is homologous to *pcaR* from *P. putida* (D Parke, personal communication).

Communication Between Branches: A New Layer of Regulation

A study of *P. putida* *pcaF* and *pcaK* regulation showed that, as expected, these two independently transcribed genes depend on PcaR and β -ketoadipate for transcriptional activation. However, the additional surprising finding was made that expression of the *pcaK* gene for 4-hydroxybenzoate transport is repressed by growth on benzoate (92). This previously unrecognized layer of regulatory control in the β -ketoadipate pathway appears to extend to the first two steps of 4-hydroxybenzoate degradation, as illustrated by repression of levels of 4-hydroxybenzoate hydroxylase and protocatechuate 3,4-dioxygenase activities by benzoate when cells were grown on a mixture of benzoate and 4-hydroxybenzoate. As would be predicted from these results, *P. putida* cells presented with both substrates degraded benzoate in preference to 4-hydroxybenzoate (92). Although the biological basis for this preference is not entirely obvious, an argument can be made that the initial steps of benzoate degradation are slightly less energetically demanding than the initial steps of 4-hydroxybenzoate degradation (92). Whatever the explanation, it is clear that

this level of regulation is not unique to *P. putida*. Studies using nuclear magnetic resonance to follow the metabolism of aromatic acids by *A. calcoaceticus* cells have shown that this organism uses multiple aromatic carbon sources according to a preferred hierarchy, including the metabolism of benzoate in preference to 4-hydroxybenzoate (GL Gaines III & EL Neidle, personal communication). A regulatory mechanism to ensure a preferred sequence of substrate use may have evolved in response to a need to use mixtures of available substrates in the most efficient manner possible.

WHAT ARE THE SELECTION FORCES THAT SHAPE SELF-IDENTITY?

The β -ketoadipate pathway is a wonderful example of diversity in the context of constancy. Isofunctional enzymes of the pathway are highly conserved in bacteria, but bacterial groups display great diversity in inducing metabolites, regulators, and gene organization. Diversity is also seen in the range of behavioral responses of various bacterial species to β -ketoadipate-associated aromatic compounds and in the repertoire of transport systems for aromatic compounds and pathway intermediates. We hope this review serves to reinforce the concept that metabolic pathways can be fully understood only when examined in the biological context of the particular organism in which they reside. It would be a mistake to view chromosomally encoded metabolic pathways in particular only in modular terms, as entities that can be facily transferred from one organism to another with any degree of confidence that they will operate efficiently. Analyses of the β -ketoadipate pathway in diverse bacteria verify the premise that selection pressures on a conserved biochemical sequence are exerted at the level of the whole organism and are subject to the requirements of a whole organism to be metabolically and ecologically successful. Among the mechanisms proposed to generate genetic diversity are DNA sequence slippage and exchange, gene transfer and transposition, and mutational drift. Many of these mechanisms can be experimentally addressed and are the subject of much attention with respect to the β -ketoadipate pathway (101–103) and other degradation pathways (148). By contrast, our ability to identify specific environmental selection pressures that have shaped the β -ketoadipate pathway in different organisms is much less well developed.

From a human's perspective, these selective forces as they are exerted in nature are often subtle. Some clues may come from considering that bacteria perceive agents of environmental selection differently according to their different phenotypic attributes. These attributes might include the overall catabolic versatility of an organism, whether or not a particular organism is normally

found in association with plants or with lignin-degrading fungi, and whether an organism has a sedentary (nonmotile) or pioneering (motile) lifestyle. Above all, it is clear that bacteria know who they are; they have an identity. If we can better understand how these identities are shaped, then we will have moved closer to describing fundamental principles of metabolic capabilities, including the β -ketoadipate pathway.

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