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MINI-REVIEW

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Biological conversion of cyclic alkanes and cyclic alcohols into dicarboxylic acids: biochemical and molecular basis

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Abstract Biological oxidation of cyclic alkanes and cyclic alcohols normally results in formation of the corresponding dicarboxylic acids, which are further metabolized in the cell. The biochemical pathways for oxidative conversion of cyclic compounds are similar in various phylogenetically diverse bacteria. Significant progress has been made in the past 2 years in the isolation and characterization of genes involved in cyclic alkane oxidation pathways in several bacterial species. In this article, we review recent advancements in the field of cyclic alcohol oxidation with focus on the biochemical and genetic characterization of the gene functions. Phylogenetic relationships of the analogous enzymes in the pathways are analyzed. Potential biocatalysis applications of these enzymes are also discussed.

Introduction

Alicyclic compounds are a major component of petroleum and are also found as secondary metabolites in plants and microbes. They are used in herbicides and insecticides, flavors and fragrances, and serve as solvents and intermediates in the chemical industry. Microbes can degrade them either aerobically or anaerobically (Dangel et al. 1988; Dangel and Fuchs 1989; Harder and Probian 1995) through different mechanisms. Microbial aerobic degradation of short chain cyclic alkanes and cyclic alcohols has been well studied (Griffin and Trudgill 1972; Donoghue and Trudgill 1975; Hasegawa et al. 1983; Trower et al. 1985; Brzostowicz et al. 2000; Cheng et al. 2000) and the degradation pathways involve a Baeyer-Villiger monooxygenase that requires molecular oxygen (Griffin and Trudgill 1976; Trower et al. 1989; Trudgill 1990; Brzostowicz et al. 2000; Cheng et al. 2000). Recently, degradation of large cyclic alkanes was described and the degradation pathway is analogous to that of the small cyclic alkanes (Schumacher and Fakoussa 1999).

The first gene encoding a cyclohexanone monooxygenase involved in cyclic alkane degradation was cloned from *Acinetobacter* sp. NCIB 9871 in 1988 (Chen et al. 1988). In the past 2 years, more genes involved in cyclic compound degradation have been cloned from different bacterial species. In some cases the gene cluster for the entire pathway has been identified. This review will summarize the recent research findings on the genetic and biochemical characterization of oxidative degradation of cyclic alkanes and cyclic alcohols. The genetic organization of the genes from different bacteria will be compared, and the function and the phylogenetic relationships of analogous enzymes from different bacteria will be analyzed.

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Biochemical and molecular analysis of cyclic alkane oxidation in Eubacteria

Oxidation of small cyclic alkanes and cyclic alcohols

Oxidation of small cyclic alkanes and cyclic alcohols has been extensively studied biochemically and genetically with cyclohexanol. Although a *Xanthobacter* sp. was reported to degrade cyclohexane (Trower et al. 1985), and the cyclohexane hydroxylase has been partially purified (Trickett et al. 1991), the gene encoding cyclohexane hydroxylase has not yet been cloned. This discussion will focus on cyclohexanol degradation by *Acinetobacter*, *Brevibacterium*, and *Arthrobacter*. The biochemical conversion of cyclohexanol to adipic acid in different bacteria proceeds through the same pathway: cyclohexanol→cyclohexanone→epsilon-caprolactone→6-hydroxyhexanoic acid→6-oxohexanoic acid→adipic acid (Fig. 1). The enzymes responsible for each step of the conversion are: cyclohexanol dehydrogenase, NADPH-linked cyclohexanone monooxygenase, epsilon-caprolac-

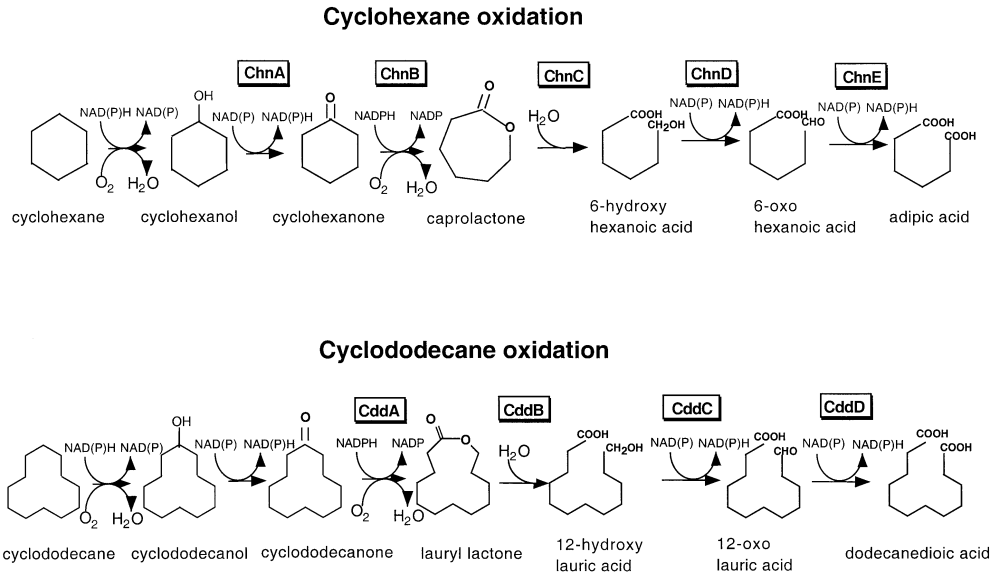

Fig. 1 Pathway for cyclic alkane oxidation. Enzymes encoded by the identified genes are *boxed* and listed above the reaction steps they catalyze

tone hydrolase, NAD (NADP)-linked 6-hydroxyhexanoic acid dehydrogenase and 6-oxohexanoic acid dehydrogenase.

Cyclohexanol oxidation in Acinetobacter *sp. NCIB 9871 and* Acinetobacter *sp. SE19*

Acinetobacter sp. NCIB 9871 has been the model strain used to study cyclohexanol oxidation since its isolation almost 3 decades ago. A majority of the early studies focused on the cyclohexanone monooxygenase of NCIB 9871. The function of this enzyme was first characterized in 1976 (Donoghue et al. 1976) as oxidizing cyclohexanone to caprolactone in an apparent Baeyer-Villiger reaction. The potential utility of this enzyme as an enantioselective and/or chemoselective oxidant for synthesis of organic molecules was also explored (Branchaud and Walsh 1985). Cloning of the gene encoding the enzyme (Chen et al. 1988) made it possible to produce this enzyme in large quantities in a recombinant host. This enzyme has thus far been a model with which to study the mechanism, structure and applications of the family of Baeyer-Villiger monooxygenases (Willetts 1997).

More genes involved in cyclohexanol oxidation were recently identified from *Acinetobacter* NCIB 9871 (Iwaki et al. 1999). In addition to *chn*B, encoding the cyclohexanone monooxygenase that had been previously cloned, two new genes (*chn*E and *chn*R) were found downstream of *chn*B. The sequence of *chn*E showed homology to NAD(P)$^+$-dependent aldehyde dehydrogenases. Expression of *chn*E in *Escherichia coli* showed 6-oxohexanoic acid dehydrogenase activity when the crude protein extract was incubated with adipic semialdehyde methyl ester as the substrate. The sequence of *chn*R showed homology to the AraC-XylS family of transcriptional regulators. The activity of the transcriptional activator of *chn*R was suggested by induction of a *lacZ* transcriptional fusion to the monooxygenase gene *chn*B and was shown to be required for the expression of the cyclohexanone monooxygenase; *chn*R is the first regulatory gene identified for bacterial degradation of monocyclic cycloparaffin compounds.

Another *Acinetobacter* sp., strain SE19, was isolated in 2000 and shown to use cyclohexanol as a sole carbon and energy source. All of the SE19 genes required for cyclohexanol oxidation to adipic acid were identified, and their functions were thoroughly characterized (Cheng et al. 2000). A 14-kb gene cluster contains nine genes with six of them required for cyclohexanol oxidation (Fig. 2), including a transcriptional regulator and the five enzymes for each of the five steps of the biochemical conversion. The genetic organization of the three genes *chnBER* in SE19 is identical to the organization of the corresponding genes in NCIB 9871. In fact, these three genes were 99–100% identical at the nucleotide level in SE19 and NCIB 9871. The SE19 cluster contains three more genes, *chn*ADC, for cyclohexanol oxidation that are transcribed in opposite orientation to *chn*BER. Recombinant *E. coli* carrying this gene cluster was shown to be able to convert cyclohexanol to adipic acid. The function of each gene was assigned by transposon mutagenesis coupled with analysis of accumulated oxidation intermediates. The *chn*B, *chn*E and *chn*R of SE19 were confirmed by this approach to have the same functions as the corresponding genes of NCIB 9871. Sequence analysis suggested that *chn*A and *chn*D both encode putative alcohol dehydrogenases, and *chn*C encodes a protein with homology to known hydrolases. The in vivo function of *chn*A and *chn*D was unambiguously determined by the intermediates that accumulated in *chn*A and *chn*D mutants. The *chn*A gene encodes cyclohexanol dehydrogenase, since cyclohexanol accumulated in the *chn*A mutant. The *chn*D gene encodes 6-hydroxyhexanoic acid dehydrogenase, since 6-hydroxyhexanoic acid accumulated in the *chn*D mutant. Accumulation of caprolactone in the *chn*C mu-

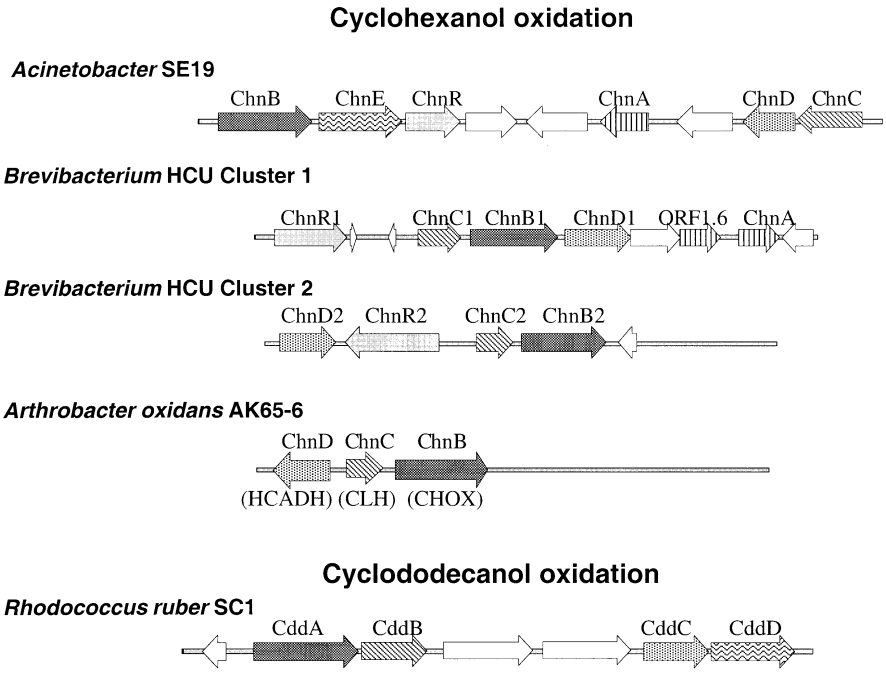

Fig. 2 Genetic organization of the gene clusters involved in cyclic alcohol oxidation. The *arrows* represent the ORFs identified in the clusters. Direction of arrows indicates direction of transcription of the genes. Enzymes involved in cyclic alcohol oxidation are labeled using the designation as shown in Fig. 1. The original designation of the enzymes used for *Arthrobacter* (Cho et al. 1991) is shown in parenthesis. ORF1.6 is a short chain alcohol dehydrogenase that does not have ChnA activity (Brzostowicz et al. 2002). *Dark gray arrows* Baeyer-Villiger monooxygenases, *vertically striped arrows* short-chain alcohol dehydrogenases, *diagonally striped arrows* hydrolases, *dotted arrows* long-chain alcohol dehydrogenases, *wavy line-filled arrows* aldehyde dehydrogenases, *light gray arrows* transcriptional regulators (ChnR)

tant suggested that *chn*C encodes the caprolactone hydrolase that is responsible for the conversion of caprolactone to 6-hydroxyhexanoic acid. Furthermore, this genetic analysis also showed that the transcriptional regulator ChnR in SE19 positively regulates the cyclohexanol dehydrogenase, ChnA, needed for the first step of cyclohexanol oxidation. This powerful genetic approach for assigning gene function in vivo gave insights into function, specificity and regulation of genes, bypassing the need for expression, purification and assay development for each individual protein.

Cyclohexanol oxidation in Brevibacterium epidermidis *HCU*

Brevibacterium epidermidis HCU was isolated for its ability to grow on cyclohexanol and cyclohexanone under halophilic conditions (Brzostowicz et al. 2000). The genes encoding two cyclohexanone monooxygenases were identified from the HCU strain by the mRNA-based technique of differential display on the basis of their induction by cyclohexanone. Both enzymes are homologs of *Acinetobacter* ChnB. Both genes were cloned and expressed in *E. coli*, and the activity of the enzymes they encode was characterized.

The sequence of the DNA surrounding each monooxygenase was determined (Brzostowicz et al. 2002). The genes surrounding *chn*B1 are organized in an operon as shown in Fig. 2. The first gene in this group, *chnR1*, belongs to the *ntrC* family of transcription regulators. It is followed by a 1.5 kb segment of DNA that lacks any significant similarity to known genes except for two short regions related to a transposase. Downstream are two genes encoding the caprolactone hydrolase ChnC1 and the cyclohexanone monooxygenase ChnB1. Both proteins are homologous to the ChnC and ChnB of the *Acinetobacter* strains. A gene homologous to *chn*D encoding 6-hydroxyhexanoic acid dehydrogenase is not found in this gene cluster. Rather, four dehydrogenase genes follow the monooxygenase gene. *chn*D1 encodes an alcohol dehydrogenase belonging to the family of Fe-dependent alcohol dehydrogenases. It shows some 6-hydroxyhexanoic acid oxidation activity when expressed in *E. coli*. Downstream lies the gene homolog of the 3-hydroxyacyl-CoA dehydrogenase typical of a fatty acid β-oxidation pathway, followed by two members of the short-chain alcohol dehydrogenase family. The second short-chain dehydrogenase, designated ChnA, is most closely related to the cyclohexanol dehydrogenase of *Acinetobacter* and was shown to oxidize cyclohexanol. This putative operon lacks a *chn*E gene homolog for 6-oxohexanoic acid dehydrogenase. It is not known if this activity is not necessary, as postulated earlier (Cho et al. 1991; Cheng et al. 2000), or if the corresponding gene is located somewhere else on the chromosome. Despite the differences observed with the *Acinetobacter* gene cluster, heterologous expression of this operon in *E. coli* suggests that it encodes all the enzymatic functions needed for the conversion of cyclohexanol into 6-oxohexanoic acid.

The gene for the second cyclohexanone-inducible monooxygenase is found in a cluster of four genes. A transcription regulator gene, *chnR2*, which belongs to the *ntrC* family of regulators, is divergently transcribed from two genes encoding functional enzymes: hydrolase and monooxygenase. The hydrolase ChnC2 belongs to a different family of hydrolases than ChnC1. Heterologous expression in *E. coli* indicates that ChnC2 can also hydrolyze caprolactone. The cyclohexanone-inducible cyclohexanone monooxygenase ChnB2 has a range of sub-

strates that is similar but not identical to that of ChnB1. In particular, ChnB2 is capable of oxidizing cyclopentanone, a growth substrate for this *Brevibacterium*, while ChnB1 has little activity with this substrate. The fourth gene of this cluster (*chn*D2) lies in another operon immediately upstream of the *chnR2-chnC2-chnB2* genes. When expressed in *E. coli*, ChnD2 oxidizes 6-hydroxyhexanoic acid, and in a native gel-based activity stain, co-migrates with a cyclohexanone-induced 6-hydroxyhexanoic acid dehydrogenase from *Brevibacterium* HCU extract. This strongly suggests that *chn*D2 is also involved in cyclohexanone oxidation.

Cyclohexanol oxidation in Arthrobacter oxidans *AK65–6*

Arthrobacter oxidans AK65–6 can use cyclohexanol as a sole carbon source (Cho et al. 1991). Four enzymes, cyclohexanol dehydrogenase ChnA (CHDH), cyclohexanone monooxygenase ChnB (CHOX), caprolactone hydrolase ChnC (CLH) and 6-hydroxyhexanoic acid dehydrogenase ChnD (HCADH), have been purified and extensively characterized biochemically. The optimal pH, optimal temperature, pH stability, temperature stability, cofactor requirements, molecular weight, isoelectric point, and N-terminal amino acid sequence were determined for each enzyme. A degenerate 32-nucleotide oligomer based on the N-terminal amino acid sequence of ChnD was used as a probe to clone a DNA fragment containing the three genes *chn*BCD (Fig. 2). The caprolactone hydrolase gene, *chn*C, and the cyclohexanone monooxygenase gene, *chn*B, in this cluster are linked the same way as the two genes in the *Brevibacterium* clusters (Brzostowicz et al. 2002). *E. coli* expressing *chn*D was sufficient to convert 6-hydroxyhexanoic acid to adipic acid. The 6-oxohexanoic acid dehydrogenase was not identified in *Arthrobacter*, like in *Brevibacterium*, and is apparently not required for adipic acid production. Analysis of the *Acinetobacter* SE19 *chn*E mutant also indicated that the *chn*E gene, encoding the 6-oxohexanoic acid dehydrogenase, is not essential for cyclohexanol oxidation (Cheng et al. 2000). Spontaneous oxidation of the 6-oxohexanoic acid to adipic acid and/or an *E. coli* nonspecific aldehyde dehydrogenase might account for adipic acid production without ChnE.

Oxidation of large cyclic alkanes and cyclic alcohols

Cyclododecane oxidation in Rhodococcus ruber *CD4*

The oxidation of large cyclic alkanes by *R. ruber* CD4 was recently reported (Schumacher and Fakoussa 1999). *R. ruber* CD4 used cyclododecane as a sole carbon source, and metabolites of cyclododecane were analyzed in experiments including growth of CD4 on cyclododecane, biotransformation of cyclododecane with resting cells of CD4, and in vitro conversion using cell-free extract of CD4. The postulated degradation pathway of cyclododecane (Fig. 1) was analogous to that of small cyclic alkane degradation. The Baeyer-Villiger monooxygenase was purified from CD4 and required oxygen and NADPH. The purified enzyme exhibited strong substrate specificity towards large cyclic ketones (C12 and C15). The activity for C6 or C8 cyclic ketones was insignificant or not detectable. This is different from the specificity of cyclohexanone monooxygenases favoring short chain cyclic ketones (Trower et al. 1989; Brzostowicz et al. 2000). The gene encoding the monooxygenase or other enzymes involved in the cyclododecane oxidation has not yet been identified from CD4.

Cyclododecanol oxidation in R. ruber *SC1*

Another *Rhodococcus* strain, *R. ruber* SC1, that can use cyclododecanol as a sole carbon source has been isolated (Kostichka et al. 2001). One obvious difference between SC1 and CD4 may be the lack of cyclododecane hydroxylase in SC1 that renders it unable to use cyclododecane. Cyclododecanol oxidation in SC1 has been extensively studied and a gene cluster responsible for oxidation of cyclododecanone to 1,12-dodecanedioic acid (DDDA) was identified (Fig. 2). Although SC1 could use cyclododecanol as a carbon source, the gene encoding the cyclododecanol dehydrogenase was not found on the cluster. The order of the four *cdd* genes on the cluster matched well with the sequence of the biochemical reactions for cyclododecanone oxidation. The gene functions were determined in *E. coli* based on the acquired ability of recombinant *E. coli* to convert cyclododecanone. *E. coli* containing only the monooxygenase gene (*cdd*A) produced lauryl lactone. *E. coli* containing both the monooxygenase gene (*cdd*A) and the lactone hydrolase gene (*cdd*B) produced 12-hydroxy lauric acid. *E. coli* containing two additional dehydrogenase genes, 12-hydroxylauric acid dehydrogenase gene (*cdd*C) and 12-oxolauric acid dehydrogenase gene (*cdd*D) produced 12-hydroxylauric acid and DDDA. Bioconversion of different cyclic ketones indicated that the Baeyer-Villiger type of cyclododecanone monooxygenase of SC1 also exhibited substrate specificity towards long chain cyclic ketones (C11-C15), as shown by the in vitro enzyme assay for the cyclododecanone monooxygenase in CD4. The substrate range of the bioconversions correlated well with the range of the growth substrates of the SC1 strain. This is a first, and thorough, analysis of the molecular basis of biological oxidation of large cyclic compounds.

Phylogenetic analysis of enzymes for cyclic alcohol oxidation pathways

Although cyclic alkanes are degraded by similar biochemical pathways in different bacteria, genetic organization of the genes involved is diverse. Comparison of the amino acid sequences of the analogous enzymes in the cyclic alcohol oxidation pathways sheds light on the evolutionary relationships of these enzyme families (Fig. 3).

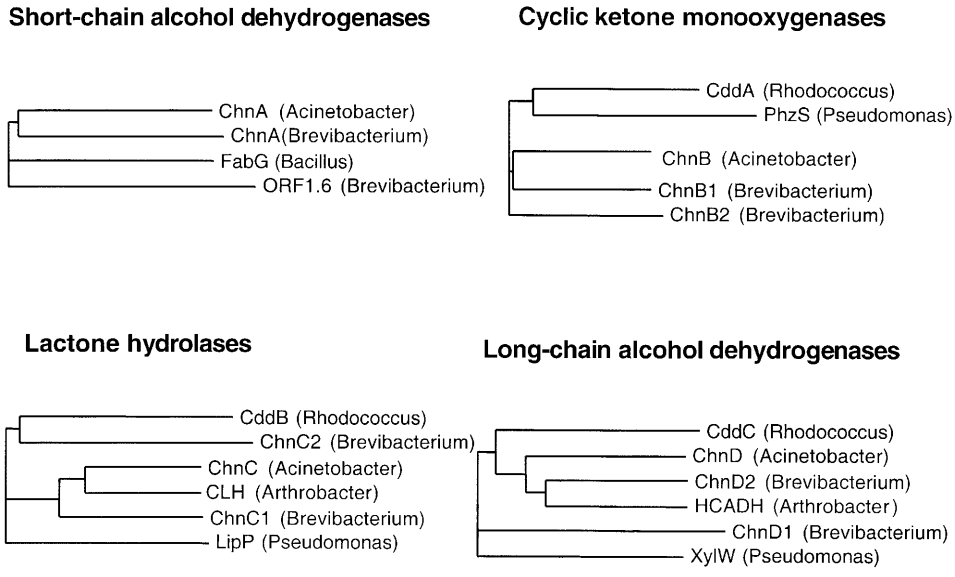

Fig. 3 Phylogenetic relationships of the families of enzymes involved in cyclic alcohol oxidation. Phylogenetic trees are generated using the AlignX analysis tools in the Vector NTI Suite (InfoMax, Bethesda, Md.). A non-related enzyme was added to each family of enzymes as an outgroup member. FabG is a 3-oxoacyl-acyl carrier protein reductase (accession no. AAC44307) involved in lipid biosynthesis (Morbidoni et al. 1996), which belongs to the family of short-chain alcohol dehydrogenases. PhzS is a flavin-containing monooxygenase (PA4217 of *P. aeruginosa* PAO1 genome) with hydroxylative decarboxylation activity for pyocyanin biosynthesis (Mavrodi et al. 2001). LipP is a cold-adapted lipase (accession no. AAC38151) from a psychrotrophic Pseudomonas (Choo et al. 1998). XylW is a benzyl alcohol dehydrogenase (accession no. AAB70823) belonging to the family of long-chain zinc-dependent alcohol dehydrogenases. The rest of the enzymes are from the clusters shown in Fig. 2

The cyclohexanol dehydrogenases belong to the very large family of short chain alcohol dehydrogenases (Krozowski 1994). Three short chain alcohol dehydrogenases have been identified in the cyclic alcohol degradation pathways, two from the *Brevibacterium* gene cluster 1 and one from *Acinetobacter* SE19. Only one of the *Brevibacterium* short chain alcohol dehydrogenases (ChnA) showed activity in cyclohexanol bioconversion, and the phylogenetic tree revealed its closer relationship to cyclohexanol monooxygenase ChnA from *Acinetobacter* SE19. The complete sequence of the cyclohexanol dehydrogenase of *Arthrobacter* is not known. It is expected to be closely related to the *Brevibacterium* ChnA, since 13 of its 17 N-terminal amino acids are identical to those of the *Brevibacterium* ChnA. The second *Brevibacterium* short chain alcohol dehydrogenase (ORF1.6), which lacks cyclohexanol conversion activity, is more distantly related to ChnA.

The cyclic ketone monooxygenases belong to the family of flavin monooxygenases. They all share two dinucleotide binding motifs: a FAD binding motif (GXGXXG) at the N-terminal part of the protein and an NADP-binding motif [GXGXX(G/A)] in the middle of the protein. The phylogenetic tree reflects the difference in their substrate specificities. The *Acinetobacter* cyclohexanone monooxygenase is more closely related to the *Brevibacterium* ChnB1 with specificity for cyclohexanone than the *Brevibacterium* ChnB2, which has specificity for cyclopentanone. The *Rhodococcus* cyclododecanone monooxygenase CddA, with its specificity for large cyclic ketones, lies on a branch distant from that of the small cyclic ketone monooxygenases.

The five identified lactone hydrolases all showed activity towards caprolactone or lauryl lactone. However, the two lactone hydrolases from *Brevibacterium* do not share significant homology to each other. The ChnC1 showed the greatest homology to the hydrolase ChnC from *Acinetobacter* SE19 and is grouped closely with many esterases/lactone hydrolases from other bacteria. The ChnC2 belongs to a poorly characterized protein family which includes a fungal lactone hydrolase shown to catalyze the hydrolysis of various aldonate lactones and aromatic lactones.

The hydroxy acid dehydrogenases belong to the long chain alcohol dehydrogenases (Reid and Fewson 1994). They are zinc-dependent enzymes with the exception of *Brevibacterium* ChnD1. ChnD1 is Fe-dependent and, like *Brevibacterium* ChnD2, shows activity towards 6-hydroxyhexanoic acid when expressed in *E. coli*.

The phylogenetic tree for the aldehyde dehydrogenases was not generated since only two of the enzymes were identified: one from the cyclohexanol degradation pathway from *Acinetobacter* SE19 and one from the cyclododecanone degradation pathway from *R. ruber* SC1. They shared 31% amino acid identity and 64% amino acid similarity.

Three transcriptional regulators were identified from the cyclohexanol oxidation pathways. The ChnR of *Acinetobacter* belongs to the AraC-LysR family of transcriptional regulators and its function for positive regulation has been demonstrated through a biotransformation experiment in SE19 (Cheng et al. 2000) and a *lac*Z-fu-

sion experiment in NCIB9871 (Iwaki et al. 1999). Two putative transcriptional regulators identified in *Brevibacterium*, ChnR1 and ChnR2, both belong to the NtrC family of transcriptional activators; however their function has not been demonstrated.

The similarities between enzymes in each family shown in Fig. 3 are mostly very low (30–40% identities) compared to enzymes in other degradative pathways (Sentchilo et al. 2000). In the family of cyclic ketone monooxygenases, the distant member is the *Rhodococcus* CddA as expected from its different substrate specificity. Surprisingly, the rest of the enzyme families all have a distant member from *Brevibacterium*. It is interesting to note that enzymes from the *Brevibacterium* clusters performing analogous functions are quite different, and the distant member is not always from the same cluster. It seems unlikely that the genes for the degradation of cyclic alcohols and cyclic ketones co-evolved as a unit as have the degradative genes for some other compounds like toluene and xylene (Sentchilo et al. 2000). Many degradative enzymes identified in the organisms mentioned in this review have broad substrate specificities. This substrate flexibility allows a mix-and-match type of evolution (Copley 2000), which might explain the diverse gene sequences and diverse gene organizations used to perform similar biochemical functions.

Biocatalysis applications

Baeyer-Villiger monooxygenases

Baeyer-Villiger monooxygenases catalyze a wide range of reactions and accept as substrates cyclic ketones, terpenoids and steroids. The biochemical diversity of the Baeyer-Villiger monooxygenase enzymes has been reviewed (Willetts 1997; Stewart 1998), and this versatility can be exploited for applications in agro-chemical, food/flavor, and pharmaceutical synthesis. For example, chiral lactones made using a Baeyer-Villiger monooxygenase are important synthons for the production of prostaglandins (Banerjee 2000). To date, the majority of bioconversions describing enzymatic Baeyer-Villiger oxidations have used the enzyme from *Acinetobacter sp* NCIB 9871 because of its availability in a recombinant form. The additional Baeyer-Villiger monooxygenases described above will expand possible applications by providing different substrate ranges (ring sizes or ring substitutions) and different enantioselectivity. As an example, products with large ring size, which could not be made previously with the *Acinetobacter* enzyme, can be made with the cyclododecanone monooxygenase from *Rhodococcus*. Combining enzyme evolution with the increasing number of characterized Baeyer-Villiger monooxygenase genes available, the properties of this family of enzymes could also be enhanced to function under non-natural conditions at higher space-time productivities.

Hydrolases

Esterase/lipase enzymes are one of the most extensively used classes of enzymes for biocatalysis, organic transformations, and industrial applications. Broad use of esterases stems from the combination of properties that these enzymes exhibit: robust activity in organic solvents, no cofactor requirement, high enantioselectivity, and broad substrate range. Esterases are commonly used for hydrolysis, direct and trans-esterification reactions, and for the kinetic resolution of racemic mixtures. Another class of reactions involves the formation of intra- and intermolecular esters via condensation reactions to make either lactones or polyesters depending on the reaction conditions (Gross et al. 2001). Description of the extensive chemistry carried out by esterases has been previously reviewed (Haraldsson and Gudmundur 1992; Jaeger and Reetz 1998; Berglund and Hult 2000) and is beyond the scope of this section. The lactone hydrolases described above provide alternative biocatalysts for these applications. High-throughput biological techniques (sequencing, mutagenesis, and screening) will speed up the search for highly selective esterases for preparative synthesis and large-scale chemical production.

Dehydrogenases

The dehydrogenases (oxidoreductases) found in cyclic alkane oxidation pathways could be used for the synthesis of structurally complex molecules that may not be amenable to modification via chemical dehydrogenation. For example, the cyclohexanol short chain alcohol dehydrogenases could be used to catalyze the conversion of a structurally complex cyclic alcohol to the corresponding ketone in the presence of a cofactor regeneration reaction. Additionally, by coupling the short chain dehydrogenase and the Baeyer-Villiger monooxygenase in conjunction with a transhydrogenase, a range of lactones could be produced; these compounds have broad applications in the food and flavor markets. Combinatorial synthesis using the genes described above will generate novel sets of enzymes which can be rapidly evaluated using high throughput analytical screens such as LC/MS-MS, GC-MS and magnetic resonance imaging. Several dehydrogenases are available commercially and the substrate ranges of these enzymes were summarized in a recent review (Kula and Kragl 2000). Although the dehydrogenase enzymes described above may find uses in lab-scale preparations, an efficient and cost effective NAD(P) recycle/regeneration mechanism is needed for economical large-scale production systems using oxidoreductases. New methods of cofactor regeneration and the demand for chiral compounds will drive the development and use of dehydrogenase reactions for commercial applications.

Enzyme combinations

Characterization of the pathways of cyclic alcohol oxidation revealed that many of the genes involved in the pathways are clustered. This feature provides the advantage of using combinations of enzymes for designing multi-step bioconversion processes. Cyclic alcohols or cyclic ketones, or a mixture of both, could be used as substrates in biocatalysis to produce lactones, hydroxy acids or dicarboxylic acids. The lactones could be used industrially, for example as solvents or fragrances. The hydroxy acids and dicarboxylic acids could be used for a number of industrial applications including as precursors for synthesis of fibers (polyesters or nylons). One example is the long-chain dicarboxylic acids of interest to the chemical industry, which could be biologically produced from aliphatic alkanes using metabolically engineered yeast (Picataggio et al. 1992). Characterization of the large cyclic alkane/cyclic alcohol oxidation pathway from bacteria provides an alternative method to synthesize long chain dicarboxylic acids from cyclic alkanes/cyclic alcohols. Whole cell bioconversion might be a preferred choice over enzyme bioconversion for this process since multiple enzymes and cofactors are required for the latter. Cloning of the genes for large cyclic alcohol oxidation makes it feasible to coordinate the expression of genes for each step of the pathway in order to optimize the bioconversion yield and to minimize the buildup of undesirable intermediates or byproducts.

Conclusions

Knowledge of cyclic alkane oxidation has greatly increased in the past 2 years with the genetic elucidation of several pathways from different bacteria. The variety of enzymes uncovered will undoubtedly expand their applications by providing different substrate specificity, different stereoselectivity or different kinetic properties, although detailed biochemical characterization of these enzymes is rather limited at this point. Gene identification will also greatly facilitate recombinant protein expression and enzyme engineering for application optimization and mechanistic studies. One challenge with the well-characterized cyclohexanone monooxygenase from *Acinetobacter* NCIB9871 has been to obtain a crystal structure to better understand the Baeyer-Villiger monooxygenases. *B. epidermidis* and *R. ruber* provide alternative sources of the enzymes with different substrate specificities for comparative crystal structure study of Baeyer-Villiger monooxygenases.

Some questions still remain: the gene for the cyclic alkane hydroxylase has not been cloned, although a cyclohexane degrader (*Xanthobacter* sp.) and a cyclododecane degrader (*R. ruber* CD4) have been isolated. A partial ORF homologous to an alkane hydroxylase was identified adjacent to an esterase and a Baeyer-Villiger monooxygenase in *Pseudomonas fluorescens* (Khalameyzer et al. 1999). However, the function of these enzymes in alkane degradation is not clear since the bacterium could not use cyclic ketones as sole carbon sources. Enzymes of similar function as those in the cyclic alkane pathway, such as Baeyer-Villiger monooxygenase and short chain alcohol dehydrogenase (van der Werf et al. 1999, 2000), have been identified as being involved in monoterpene degradation. The physiological roles of these enzymes remain unknown. It is postulated that secondary metabolites might be the physiological substrates of these enzymes, which in some cases are co-opted by bacteria to degrade non-natural xenobiotics.

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