Molecular characterization of benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II of *Acinetobacter calcoaceticus*

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The nucleotide sequences of *xylB* and *xylC* from *Acinetobacter calcoaceticus*, the genes encoding benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II, were determined. The complete nucleotide sequence indicates that these two genes form part of an operon and this was supported by heterologous expression and physiological studies. Benzaldehyde dehydrogenase II is a 51654 Da protein with 484 amino acids per subunit and it is typical of other prokaryotic and eukaryotic aldehyde dehydrogenases. Benzyl alcohol dehydrogenase has a subunit M_r of 38923 consisting of 370 amino acids, it stereospecifically transfers the pro*R* hydride of NADH, and it is a member of the family of zinc-dependent long-chain alcohol dehydrogenases. The enzyme appears to be more similar to animal and higher-plant alcohol dehydrogenases than it is to most other microbial alcohol dehydrogenases. Residue His-51 of zinc-dependent alcohol dehydrogenases is thought to be necessary as a general base for catalysis in this category of alcohol dehydrogenases. However, this residue was found to be replaced in benzyl alcohol dehydrogenase from *A. calcoaceticus* by an isoleucine, and the introduction of a histidine residue in this position did not alter the kinetic coefficients, pH optimum or substrate specificity of the enzyme. Other workers have shown that His-51 is also absent from the TOL-plasmid-encoded benzyl alcohol dehydrogenase of *Pseudomonas putida* and so these two closely related enzymes presumably have a catalytic mechanism that differs from that of the archetypal zinc-dependent alcohol dehydrogenases.

INTRODUCTION

The Gram-negative bacterium *Acinetobacter calcoaceticus* NCIB 8250 can use a wide range of aromatic compounds as sole sources of carbon and energy for growth. Benzyl alcohol is oxidized to benzoate by the chromosomally encoded benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II; benzaldehyde dehydrogenase I is a quite different enzyme that is involved in the metabolism of mandelate. Benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II have been purified and characterized and shown to be soluble NAD-dependent enzymes [1,2]. Similar enzymes in *Pseudomonas putida* are encoded by TOL plasmids [3]. The genes for benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase from the TOL plasmid pWW0, *xylB* and *xylC* respectively, have been cloned and sequenced [4]. The present paper describes the cloning and sequencing of the genes for benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II from *A. calcoaceticus*. Benzyl alcohol dehydrogenase was found to be a member of the family of zinc-dependent long-chain alcohol dehydrogenases and this paper describes experiments which support the work of Harayama and colleagues [5] in indicating that the bacterial benzyl alcohol dehydrogenases have a catalytic mechanism that is different from that believed to operate in horse-liver alcohol dehydrogenase, the archetypal enzyme of this family.

EXPERIMENTAL

Materials

Chemicals were of the best quality available commercially and most were from BDH Chemicals (Poole, Dorset, U.K.). Coenzymes were from Boehringer (Lewes, Sussex, U.K.). Horse-liver and yeast alcohol dehydrogenases were from Sigma (Poole, Dorset, U.K.).

Construction of a λ genomic-DNA library of *A. calcoaceticus* NCIB 8250 and generation of probes for *xylC* and *xylB*

A. calcoaceticus genomic DNA was generally prepared as described previously for *Ps. putida* [6]. A genomic-DNA library was made by partially digesting *A. calcoaceticus* NCIB 8250 genomic DNA with *Sau*IIIA1 and size fractionating on an NaCl gradient in a Beckman Quick-Seal™ centrifuge tube (Beckman, Palo Alto, CA, U.S.A.) to enrich the amount of DNA in the 14–20 kb range. Fragments of this size were ligated into the vector λGEM-11 (Promega) digested with *Bam*HI, and used to infect the host *Escherichia coli* KW252. This library was screened with probes for *xylC* and *xylB* that had been generated by PCR from genomic DNA using primers designed against the N-terminal protein sequences of benzaldehyde dehydrogenase II and benzyl alcohol dehydrogenase determined previously [7]. The sequences of these primers were CGGGATCCAARGARYTNTGGGA (oligo BZDHII-I) and CGGAATTCGCRTANCCDATYTC (oligo BZDHII-II) for *xylC* and CGGAATTCCACATNCCNGTNGC (oligo 1108) and CGGGATCCTGYAARGGNGCNGA (oligo 1109) for *xylB*. These primers contained *Bam*HI or *Eco*RI sites (underlined) to facilitate cloning. *A. calcoaceticus* genomic DNA was used as template for the PCR reactions and products were cloned into pUC18 for sequencing. Clones corresponding to the N-terminal sequences of *xylC* or *xylB* were 5′-end labelled with ^{32}P using T4 polynucleotide kinase (Promega) and used to screen plaque lifts on Hybond-N membranes (Amersham) as described by Sambrook et al. [8]. Positive clones were digested, mapped and sub-cloned by standard procedures [8]. All clones were sequenced on both strands using the chain-termination method [9]. Synthetic oligonucleo-

Abbreviations used: IPTG, isopropyl β-D-thiogalactoside; ACBADH-His51, benzyl alcohol dehydrogenase mutant with histidine at residue 51.

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tides designed about 250 bp apart on the template DNA were used to prime sequencing reactions. The nucleotide sequences of *xylC* and *xylB* have been deposited in the GenBank data base with the accession number U61983.

Site-directed mutagenesis

Site-directed mutagenesis of the *xylB* gene encoding benzyl alcohol dehydrogenase was carried out using the PCR method described by Higuchi et al. [10]. Vent DNA polymerase (New England Biolabs) was used for the PCR reactions since this enzyme has $3' \rightarrow 5'$ exonuclease activity. To create benzyl alcohol dehydrogenase-His51, the codon for this residue was changed from ATT to CAT using the oligo TGCCACACCGATTTGCATGTACGTGATCAATA and its reverse and complement (TATTGATCACGTACATGCAAATCGGTGTGGCA). Mutagenized *xylB* was cloned into the *Nde*I and *Bgl*II sites of the T7 expression vector pTB361 [11] and clones were sequenced on both strands using the chain-termination method [9].

Measurement of enzyme activity

The activities of purified benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II were measured, and kinetic parameters determined, as described by MacKintosh and Fewson [1,2]. Horse-liver and yeast alcohol dehydrogenases were assayed as described by Plapp [12].

Purification of benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II

Cultures of *E. coli* JM109 or JM109(DE3) transformed with plasmids expressing recombinant benzyl alcohol dehydrogenase and/or benzaldehyde dehydrogenase II were grown at 30 °C until attenuance at 600 nm of approximately 0.4 was reached, at which point expression was induced with 0.5 mM IPTG (isopropyl β-D-thiogalactoside). Recombinant benzyl alcohol dehydrogenase (and mutants) and benzaldehyde dehydrogenase II were purified as described by MacKintosh and Fewson [1] for the enzymes from *A. calcoaceticus* NCIB 8250.

Electrospray MS

Electrospray MS of purified recombinant benzaldehyde dehydrogenase II and benzyl alcohol dehydrogenase was carried out as described by Krell et al. [13].

Determination of stereospecificity of hydride transfer of benzyl alcohol dehydrogenase

Stereospecificity of hydride transfer was determined for purified benzyl alcohol dehydrogenase as described by Baker et al. [14].

RESULTS AND DISCUSSION

Nucleotide sequences of *xylC* and *xylB* and the derived amino acid sequences of benzaldehyde dehydrogenase II and benzyl alcohol dehydrogenase

Clones harbouring *xylC* and *xylB* were isolated from a λ chromosomal library of *A. calcoaceticus* NCIB 8250 by screening with probes generated by PCR using primers designed against the N-terminal amino acid sequences of benzaldehyde dehydrogenase II and benzyl alcohol dehydrogenase purified from the wild-type organism [7]. The complete nucleotide sequence of a total of 3131 base pairs of *A. calcoaceticus* genomic DNA was determined (Figure 1). Two open reading frames were identified within this region corresponding to *xylC* and *xylB*, encoding benzaldehyde dehydrogenase II and benzyl alcohol dehydrogenase respectively. No further open reading frames could be found upstream of *xylC* or downstream of *xylB*. The nucleotide sequence, which indicates there to be only 33 bp between the translational stop codon of *xylC* and the translational start codon of *xylB*, plus the facts that both benzaldehyde dehydrogenase II and benzyl alcohol dehydrogenase can be co-ordinately expressed from the *lac* promoter of the vector pBluescript SKII after induction with IPTG (data not shown) and that the two enzymes are co-ordinately induced and repressed in *A. calcoaceticus* [15] and are also co-ordinately repressed during the metabolism of mandelate [16], suggest that the two genes form an operon transcribed in the direction *xylC* → *xylB*. Putative Shine–Dalgarno sequences were identified upstream of the translational start codons of the two genes and a possible rho-independent transcription termination sequence was identified downstream of *xylB* using the method of Brendel and Trifonov [17] (Figure 1).

Subclones were developed such that both benzaldehyde dehydrogenase II and benzyl alcohol dehydrogenase could be expressed independently of each other, and the two proteins were purified as described for the enzymes from the wild-type organism [1].

The *xylC* structural gene is 1452 bp in length and encodes a 51654 Da protein consisting of 484 amino acids per subunit. The subunit molecular mass was confirmed by electrospray MS of purified recombinant benzaldehyde dehydrogenase II (data not shown). The *xylB* structural gene is 1110 bp in length and encodes a 370 amino acid protein with a deduced relative molecular mass of 38923 Da. This value was confirmed by electrospray MS of purified recombinant benzyl alcohol dehydrogenase (data not shown). The two structural genes had codon-usage patterns typical for *Acinetobacter* structural genes (data not shown) [18] and had G+C contents of 44% and 42% for *xylC* and *xylB*, respectively. These values are typical for acinetobacters [19].

Genetic organization of the benzyl alcohol pathway in *A. calcoaceticus*

The organization of the genes encoding the enzymes of the benzyl alcohol pathway in *A. calcoaceticus* is different from that of the TOL-plasmid pWW0 since the latter has an operon with additional genes [3,4]. In the TOL-system, genes are transcribed in the order *xylUWCMABN* where *xylU* encodes a small protein consisting of 131 amino acid residues, but has no significant identity with any known protein sequences, and *xylW* encodes a 341 amino acid protein that is homologous with members of the long-chain zinc-dependent alcohol dehydrogenases. As yet, these two proteins have no known function. Gene *xylC* encodes benzaldehyde dehydrogenase, *xylM* and *xylA* encode the two subunits of xylene monoxygenase, *xylB* encodes benzyl alcohol dehydrogenase and *xylN* encodes another polypeptide of unknown function [4]. The fact that *xylM* and *xylA* are absent from *A. calcoaceticus* is not surprising since this organism is not able to degrade toluene and toluates [20]. The start and finish of the putative *A. calcoaceticus xyl* operon needs to be confirmed to conclude unequivocally that this operon does not contain genes equivalent to *xylU*, *xylW* and *xylN*, although it appears that the *A. calcoaceticus* operon does not contain these genes.

Benzaldehyde dehydrogenase II is similar to other prokaryotic and eukaryotic aldehyde dehydrogenases

Benzaldehyde dehydrogenase II of *A. calcoaceticus* shares a high degree of amino acid sequence identity with benzaldehyde

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   1 aattctaaacaagagaatcgccttcggttatgccgcaaattatgggggtgaacaacagct  60
  61 ggatggaatcaaactggatcagaacatggaaaagcaacaagcgcgattggagtttcagca 120
 121 aatgctgaatgcaaaaactcaattaagtgcacagttgctttcagacacacgaacagaaag 180
 181 cggatttaagaaagattacggtctaaatttttcgtgtgttttacatttttttaatttaaagc 240
 241 aggtgtaacacattgattgcgtcatttttacaaggatgcagttggaacagtaaagcaatt 300

 301 gtctaaataaatttttgaa**gagg**tcatcatgtctatattcacaaaagagctttgggataaa 360
                           M  S  I  F  T  K  E  L  W  D  K    11

 361 aaattatttaacggttcatggcagtcagcccaagacacctacagtgtgattgaggttgca 420
     K  L  F  N  G  S  W  Q  S  A  Q  D  T  Y  S  V  I  E  V  A   31

 421 acaggtcaagtgcttggtgaaatcggttatgccactgccgctgatgtggtgagtgctgca 480
     T  G  Q  V  L  G  E  I  G  Y  A  T  A  A  D  V  V  S  A  A   51

 481 caacaagccaaagccgctcaacaacagtggtgggctttaaattatcaagagcgtcaggca 540
     Q  Q  A  K  A  A  Q  Q  Q  W  W  A  L  N  Y  Q  E  R  Q  A   71

 541 gtgtttgagcgagctgctgctttactgacagaaaatcaagcggaagtgattgagtggctg 600
     V  F  E  R  A  A  A  L  L  T  E  N  Q  A  E  V  I  E  W  L   91

 601 gttaaggaaagtggctcattacaactgaaagcaggctttgaagtgagtattgcgattcaa 660
     V  K  E  S  G  S  L  Q  L  K  A  G  F  E  V  S  I  A  I  Q  111

 661 gtgcttaaacattgtattgcttcacctacaaatgaacagggcacattattacctacgcaa 720
     V  L  K  H  C  I  A  S  P  T  N  E  Q  G  T  L  L  P  T  Q  131

 721 aatggcaaactaagtatagcgaaacgcttgcccttaggtgttgttggggtcatttctcca 780
     N  G  K  L  S  I  A  K  R  L  P  L  G  V  V  G  V  I  S  P  151

 781 tttaactttccactgtacttagctttacgtgctgtggcacctgctttggcctttggtaat 840
     F  N  F  P  L  Y  L  A  L  R  A  V  A  P  A  L  A  F  G  N  171

 841 gcggtggtgttaaaacctgatgagcgtacagcagtgtgcagtggctatgtgattgcacgg 900
     A  V  V  L  K  P  D  E  R  T  A  V  C  S  G  Y  V  I  A  R  191

 901 atttttgaacttgcaggattaccaaaggggctgctgcatgtattgccaggtggggtagaa 960
     I  F  E  L  A  G  L  P  K  G  L  L  H  V  L  P  G  G  V  E  211

 961 gttggtgaagcactgactttggatcaaaatattgccagtatccaatttacgggatcaacc 1020
     V  G  E  A  L  T  L  D  Q  N  I  A  S  I  Q  F  T  G  S  T  231

1021 caagttggacggattgttggggcgaatgcggcaaagaccttaaagaaagtttctctagaa 1080
     Q  V  G  R  I  V  G  A  N  A  A  K  T  L  K  K  V  S  L  E  251

1081 ctgggtgggaaaaactcactgattattttggatgatgcggatattgagttagcagcagaa 1140
     L  G  G  G  K  N  S  L  I  I  L  D  D  A  D  I  E  L  A  A  E  271

1141 aatattgcatggggtgcatttttacattctgggcagatttgtatgacttctggcaagatt 1200
     N  I  A  W  G  A  F  L  H  S  G  Q  I  C  M  T  S  G  K  I  291

1201 ttgattcaccaaaaaatttatcaacaggtcaaacagcgtgtaattgaaaaagtgcagaat 1260
     L  I  H  Q  K  I  Y  Q  Q  V  K  Q  R  V  I  E  K  V  Q  N  311

1261 tttgtggtagggaatccttgtgacaagaatgtcaccattgggccattgatcaatgctaaa 1320
     F  V  V  G  N  P  C  D  K  N  V  T  I  G  P  L  I  N  A  K  331

1321 caagcgcaacgtgtagaacaattggtcagtgctgcggttaatgaaggcgcaacattggaa 1380
     Q  A  Q  R  V  E  Q  L  V  S  A  A  V  N  E  G  A  T  L  E  351

1381 atcggcgggcatgccgatggggtatttttttcaaccgactgtactgacagatgtgactgca 1440
     I  G  G  H  A  D  G  V  F  F  Q  P  T  V  L  T  D  V  T  A  371

1441 aacaattcgatttttagtgaagagatttttggtcctgttgcggtattgatcccgtttagc 1500
     N  N  S  I  F  S  E  E  I  F  G  P  V  A  V  L  I  P  F  S  391

1501 tcagatgaacaggccattgaattagccaatgacggcgattatgggctttcagcaggcatt 1560
     S  D  E  Q  A  I  E  L  A  N  D  G  D  Y  G  L  S  A  G  I  411

1561 attacctccaatgtgggacgaggtatgcaactcggtgcgcagctcaaagtgggcttgcta 1620
     I  T  S  N  V  G  R  G  M  Q  L  G  A  Q  L  K  V  G  L  L  431

1621 catatcaacgatcagaccgtaaatgatgaaacggtcaatccatttggtggttttggttca 1680
     H  I  N  D  Q  T  V  N  D  E  T  V  N  P  F  G  G  F  G  S  451

1681 tcaggtaatggtacgcgtattggcggccctgccaatgccgatgaatttacccagtggcaa 1740
     S  G  N  G  T  R  I  G  G  P  A  N  A  D  E  F  T  Q  W  Q  471

1741 tggattacagtacaggctcaagcgccacattatccttttaaacaattaaataaatcatg 1800
     W  I  T  V  Q  A  Q  A  P  H  Y  P  F  *                   484

1801 ac**aggac**gttataccatgagtgaattaaaagatattattgccgcagtgaccccatgtaaa 1860
                        S  E  L  K  D  I  I  A  A  V  T  P  C  K   14

1861 ggtgctgactttgagctgcaagctttaaaaatacgtcagccgcaaggtgatgaagtattg 1920
     G  A  D  F  E  L  Q  A  L  K  I  R  Q  P  Q  G  D  E  V  L   34

1921 gtaaaagtggttgctacaggaatgtgccacaccgatttgattgtacgtgatcaatattat 1980
     V  K  V  V  A  T  G  M  C  H  T  D  L  I  V  R  D  Q  Y  Y   54

1981 ccggtaccgctgcctgccgtccttggacatgaagggtcaggaattattgaggcgattggc 2040
     P  V  P  L  P  A  V  L  G  H  E  G  S  G  I  I  E  A  I  G   74

2041 cctaacgtaaccgagcttcaagtcggcgatcatgtggttttaagctatggttattgtggg 2100
     P  N  V  T  E  L  Q  V  G  D  H  V  V  L  S  Y  G  Y  C  G   94

2101 aaatgtacccaatgtaatactggtaatcctgcctattgttcagagtttttggacgaaat 2160
     K  C  T  Q  C  N  T  G  N  P  A  Y  C  S  E  F  F  G  R  N  114

2161 tttagtggggcagattcagaagggaatcatgcgctttgtacccatgatcaaggcgtggta 2220
     F  S  G  A  D  S  E  G  N  H  A  L  C  T  H  D  Q  G  V  V  134

2221 aatgatcatttttttgcccaatcctcctttgcaacctatgctttaagtcgtgaaaataac 2280
     N  D  H  F  F  A  Q  S  S  F  A  T  Y  A  L  S  R  E  N  N  154

2281 acggttaaagtgaccaaagacgtaccaattgaactattaggaccattaggttgtggtatt 2340
     T  V  K  V  T  K  D  V  P  I  E  L  L  G  P  L  G  C  G  I  174

2341 caaacaggtgctggcgcatgtataaatgctttaaaagtgacacctgccagtagtttggtg 2400
     Q  T  G  A  G  A  C  I  N  A  L  K  V  T  P  A  S  S  L  V  194

2401 acttggggtgccggcgctgttggtctgagtgctttacttgcggcaaaagtgtgtggcgct 2460
     T  W  G  A  G  A  V  G  L  S  A  L  L  A  A  K  V  C  G  A  214

2461 tcgatcattatcgctgttgatattgttgaatcacgtttggaattggcaaaacaattgggt 2520
     S  I  I  I  A  V  D  I  V  E  S  R  L  E  L  A  K  Q  L  G  234

2521 gcaactcatgtgatcaatagtaaaactcaagatcctgtggctgcgattaaagagattact 2580
     A  T  H  V  I  N  S  K  T  Q  D  P  V  A  A  I  K  E  I  T  254

2581 gatggtggagtcaactttgcattggaatctactgggcgacctgagatattaaaacaaggt 2640
     D  G  G  V  N  F  A  L  E  S  T  G  R  P  E  I  L  K  Q  G  274

2641 gttgatgcgctgggtattttaggtaaaatcgccgtggtaggtgcacctcaattaggcaca 2700
     V  D  A  L  G  I  L  G  K  I  A  V  V  G  A  P  Q  L  G  T  294

2701 actgcacaatttgatgtaaatgatctgttattgggtgggaaaacgattttaggtgtcgtt 2760
     T  A  Q  F  D  V  N  D  L  L  L  G  G  K  T  I  L  G  V  V  314

2761 gaggggagtggttcgcccaagaaatttatccctgagttagtccgcttatatcaacaaggg 2820
     E  G  S  G  S  P  K  K  F  I  P  E  L  V  R  L  Y  Q  Q  G  334

2821 aaatttccctttgaccaacttgtaaaattctatgcatttgatgaaatcaatcaagctgcg 2880
     K  F  P  F  D  Q  L  V  K  F  Y  A  F  D  E  I  N  Q  A  A  354

2881 atagatagtcacaagggtattacactcaaaccgattattaaaattgcataatcttgcatg 2940
     I  D  S  H  K  G  I  T  L  K  P  I  I  K  I  A  *           370

2941 gcatgctcattgaggagcatgccatttttgattataagttttttaagtttaaattttgggt 3000
3001 ttagtcttagattggaggtttagtgttttaagctagttggatgactttagagctttcttc 3060
3061 gcttttccagtaattttccaaataataaacccagctcaaacaataaccacattgggatcg 3120

3121 caagcataatc 3131
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Figure 1 Nucleotide and deduced amino acid sequence of the genes encoding benzaldehyde dehydrogenase II (*xylC* open reading frame) and benzyl alcohol dehydrogenase (*xylB* open reading frame)

Putative ribosome-binding sequences are shown in bold type. Translation stop codons are marked by *. A putative transcription terminator sequence is underlined.

dehydrogenase encoded by the TOL-plasmid pWW0 of *Ps. putida* (56%), *Streptococcus mutans* non-phosphorylating NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (48%) and the thermostable aldehyde dehydrogenase from *Bacillus stearothermophilus* (47%). Residues Cys-302 and Glu-268 of human aldehyde dehydrogenase have been shown to be essential for catalytic activity [21] and these residues are both conserved in benzaldehyde dehydrogenase II, suggesting that this enzyme is typical of the large family of NAD^+-linked aldehyde dehydrogenases [22]. Members of this family contain 23 invariant amino acid residues, of which 22 are conserved in benzaldehyde dehydrogenase II, with Thr-456 replacing a Gly residue. In addition, members of this family also contain 27 nearly invariant residues, of which 24 are conserved in benzaldehyde dehydrogenase II, and 41 invariant similarities, of which 36 are present in benzaldehyde dehydrogenase II. Benzaldehyde dehydrogenase II also contains the consensus sequence FTGSTxVG proposed to constitute the NAD^+-binding domain of other aldehyde dehydrogenases (residues 227-FTGSTQVG-234) [22].

Benzyl alcohol dehydrogenase is a member of the family of NAD(P)-dependent long-chain zinc-dependent alcohol dehydrogenases

The amino acid sequence of benzyl alcohol dehydrogenase from *A. calcoaceticus* was found to share identity with members of the family of long-chain zinc-dependent alcohol dehydrogenases. Crystallographic evidence has shown that horse-liver alcohol dehydrogenase, the archetypal enzyme of this family, binds two zinc atoms per enzyme subunit, with a catalytic zinc atom bound at the active site to the ligands Cys-46, His-67 and Cys-174, and a structural zinc atom bound to the cysteine residues 97, 100, 103 and 111. These zinc ligands are all found to be conserved in the *A. calcoaceticus* enzyme (Figure 1). It is therefore likely that benzyl alcohol dehydrogenase binds two zinc atoms per enzyme subunit.

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                30          40          50          60
                |           |           |           |
  popADH  YTYSLRDTGP EDVFIKVISC G.VCHTDIHQ IK..NDLGMS .HYPMVPGHE
   BSADH  KEVEKPKISY GEVLVRIKAC G.VCHTDLHA AH..GDWPVK PKLPLIPGHE
   ZMADH  KDTKLRPLKY GEALLEMEYC G.VCHTDLHV KN..GDFGDE T..GRITGHE
  ANADHI  KQIPVPKPGP DQILVKIRYS G.VCHTDLHA MM..GHWPIP VKMPLVGGHE
   SADHI  KDIPVPKPKA NELLINVKYS G.VCHTDLHA WH..GDWPLP TKLPLVGGHE
   KADHI  KDIPVPKPKA NELLINVKYS G.VCHTDLHA WK..GDWPLP TKLPLVGGHE
   SPADH  EEVPVAEPGQ DEVLVNIKYT G.VCHTDLHA LQ..GDWPLP AKMPLIGGHE
   CBADH  IEKERPVAGS YDAIVRPLAV S.PCTSDIHT VFEGALGDRK ...NMILGHE
   TBADH  IEKEKPAPGP FDAIVRPLAV A.PCTSDIHT VFEGAIGERH ...NMILGHE
    EADH  IEKKIPECGP LDALVRPLAL A.PCTSDTHT VWAGAIGDRH ...DMILGHE
   AEADH  ADKPIPDIGP NDALVRITTT T.ICGTDVH. ILKGEYPVAK ...GLTVGHE
    ECTH  TDVPVPELGH N.DLLIKIRK TAICGTDVHI YNWDEWSQKT IPVPMVVGHE
    xylW  DDVAAPKEAH STDVLVKVEA AGICGTDLLI YKWGEFA.KR MKLPTILGHE
   HLADH  EEVEVAPPKA HEVRIKMVAT G.ICRSDDHV V...SGTLVT P.LPVIAGHE
  humADH  EDVEVAPPKA YEVRIKMVAV G.ICRTDDHV V...SGNLVT P.LPVILGHE
  BarADH  EEVEVAPPQA MEVRVKILFT S.LCHTDVYF W...EAKGQI PMFPRIFGHE
    MADH  EEVEVAPPQA MEVRVKILFT S.LCHTDVYF W...EAKGQT PVFPRIFGHE
  ACBADH  QALKIRQPQG DEVLVKVVAT G.MCHTDLIV R...DQYYPV P.LPAVLGHE
TOL-BADH  EHVALNEPAE DQVLVRLVAT G.LCHTDLVC R...DQHYPV P.LPMVFGHE
                              *  *                    ***
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Figure 2 Comparison of a segment of the primary sequence of benzyl alcohol dehydrogenase from *A. calcoaceticus* with equivalent segments of microbial, horse-liver, human and various plant NAD(P)-dependent long-chain zinc-dependent alcohol dehydrogenases

Sequences were aligned using the pileup program on the UNIX GCG system. Gap weight = 3.0. Gap weight length = 0.1. * Corresponds to conserved residues. The sequences are numbered according to HLADH (horse-liver alcohol dehydrogenase). The comparison is based on sequences obtained from the SwissProt and GenEMBL databases except that ACBADH (*A. calcoaceticus* benzyl alcohol dehydrogenase) was obtained from this work and ECTH (*E. coli* L-threonine dehydrogenase) was obtained from Aronson et al. [40]. Abbreviations used: popADH, *Populus deltoides* (poplar tree) cinnamyl-alcohol dehydrogenase; BSADH, *B. stearothermophilus* alcohol dehydrogenase; ZMADH, *Zymomonas mobilis* type I alcohol dehydrogenase; ANADHI, *Aspergillus nidulans*, type I alcohol dehydrogenase; SADHI, *Saccharomyces cerevisiae* isoenzyme I; KADHI, *Kluyveromyces lactis* isoenzyme I; SPADH, *Schizosaccharomyces pombe* fermentative alcohol dehydrogenase; CBADH, *Clostridium beijerinckii* alcohol dehydrogenase; TBADH, *Thermoanaerobium brockii* secondary alcohol dehydrogenase; EADH, *Entamoeba histolytica* alcohol dehydrogenase; AEADH, *Alcaligenes eutrophus* fermentative alcohol dehydrogenase; xylW, xylW gene product; humADH, human class I β-subunit alcohol dehydrogenase; BarADH, *Hordeum vulgare* (barley) alcohol dehydrogenase I; MADH, *Zea mays* (maize) alcohol dehydrogenase I; TOL-BADH, *Pseudomonas putida* (pWW0) benzyl alcohol dehydrogenase.

Besides the strong identity (54%) that benzyl alcohol dehydrogenase from *A. calcoaceticus* shares with the pWW0 enzyme, the *A. calcoaceticus* enzyme also shares 35% sequence identity with the human class I $\beta\beta$ alcohol dehydrogenase, 31% with maize alcohol dehydrogenase and 32% with horse-liver alcohol dehydrogenase. Interestingly, benzyl alcohol dehydrogenase from *A. calcoaceticus* shows a higher degree of sequence identity with many mammalian and plant alcohol dehydrogenases than it does with fungal and most other bacterial alcohol dehydrogenases. This observation raises questions concerning the evolutionary origins of benzyl alcohol dehydrogenase. Is it possible that the benzyl alcohol dehydrogenases and the plant and mammalian alcohol dehydrogenases all originated from a relatively recent common evolutionary ancestor? Presumably it is possible that *xylB* was recruited from a plant or animal source by horizontal gene transfer.

The alignment of the primary sequences of various long-chain zinc-dependent alcohol dehydrogenases, part of which is shown in Figure 2, shows that there are 12 residues conserved across all of the sequences. These are Cys-46, Asp-49, His-67, Glu-68, Val-80 and glycine residues 66, 71, 77, 86, 201, 204 and 235. There have not been any proposed catalytic roles for Val-80 and the conserved glycine residues, but they all have structural roles, some of which involve coenzyme binding [23–26]. Val-80, along with three of the conserved glycine residues (66, 71 and 77), His-67 and Glu-68, constitutes the characteristic zinc-dependent alcohol dehydrogenase motif GHEXXGXXXXGXXV [27]. Cys-46 and His-67 have been shown by protein crystallography techniques to be two of the four ligands involved in binding the catalytic zinc atom in the active site of horse-liver alcohol dehydrogenase [23–25,28].

Table 1 Relative activities of benzyl alcohol dehydrogenase and benzyl alcohol dehydrogenase-His51 with various substrates

Substrate concentrations were fixed at 200 μM and activities were measured as described in the Experimental section. The values shown are averages of the duplicate separate experiments shown in parentheses. ACBADH, benzyl alcohol dehydrogenase; ACBADH-His51, benzyl alcohol dehydrogenase mutant with histidine residue in position 51.

	% Activity relative to benzyl alcohol	
Alcohol substrate	ACBADH	ACBADH-His51
Benzyl alcohol	100	100
2-Methylbenzyl alcohol	34 (36, 32)	39 (40, 37)
3-Methylbenzyl alcohol	82 (93, 71)	74 (73, 74)
4-Methylbenzyl alcohol	97 (100, 93)	99 (93, 105)
Perillyl alcohol	55 (64, 46)	50 (47, 53)
Cinnamyl alcohol	48 (49, 46)	47 (45, 48)
Coniferyl alcohol	8 (9, 6)	12 (9, 15)

Cleft structure, active-site structure and substrate specificity of benzyl alcohol dehydrogenase

Benzyl alcohol dehydrogenase from *A. calcoaceticus* and benzyl alcohol dehydrogenase encoded by the TOL-plasmid pWW0 of *Ps. putida* are enzymes that display activity against a wide range of alcohol substrates [2,29–31]. Both enzymes can use substrates with substituents at the *ortho*, *meta*, and *para* positions of the benzene ring. The *A. calcoaceticus* enzyme has a preference for *meta* and *para* substitutions. 2-Methylbenzyl alcohol is a relatively poor substrate for the enzyme, producing rates of oxidation only about one third of those found with benzyl alcohol (Table 1). An apparent difference between the benzyl alcohol dehydrogenase from *A. calcoaceticus* and that encoded by the TOL-plasmid pWW0 is that the TOL-encoded enzyme has a very strong preference for 3-methyl- and 3-methoxybenzyl alcohols as substrates compared with benzyl alcohol and 4-methyl- and 4-methoxybenzyl alcohols. Also, the TOL-enzyme can oxidize 2-methylbenzyl alcohol more rapidly than it can oxidize unsubstituted benzyl alcohol [2,30,31]. Benzyl alcohol dehydrogenase purified from the TOL-plasmid pWW53 also shows a strong preference for 3-methylbenzyl alcohol, although this enzyme does not use this substrate as efficiently as the pWW0 enzyme, whereas 2-methylbenzyl alcohol is a relatively poor substrate for the pWW53 enzyme [30]. These results suggest that there may be differences in the structures of the active sites of these enzymes which bring about these slight differences in substrate specificities. Despite their affinities for a wide range of (substituted) aromatic alcohols, neither benzyl alcohol dehydrogenase from *A. calcoaceticus* nor the TOL-enzyme is able to oxidize aliphatic alcohols [2,29], although the *A. calcoaceticus* enzyme can oxidize the cyclohex-1-ene compound perillyl alcohol [2]. This is in marked contrast to horse-liver alcohol dehydrogenase which can oxidize straight and branched chain primary alcohols, secondary alcohols, cyclohexanol and benzyl alcohol, and is also in contrast to yeast alcohol dehydrogenase which can oxidize short chain primary alcohols but little else [32,33].

The inner part of the substrate-binding cleft in the active sites of the benzyl alcohol dehydrogenases from *A. calcoaceticus* and *Ps. putida* (pWW0) may be smaller than that in horse-liver

alcohol dehydrogenase. In the horse-liver enzyme 13 amino acid residues line what is apparently a deep and hydrophobic cleft [24]. This substrate-binding cleft is found within individual subunits of the enzyme [24]. The inner part of the substrate-binding cleft is lined with the catalytic zinc-liganding residues (Cys and His) and residues Ser-48, Phe-93, Phe-140 and Leu-141. In the benzyl alcohol dehydrogenases, Ser-48 and Leu-141 are replaced by larger residues, Thr and Phe respectively. The *A. calcoaceticus* enzyme also replaces Phe-93 with Tyr. It therefore appears that in this inner region the benzyl alcohol dehydrogenases may have four aromatic residues clustered together, forming an internal hydrophobic core. This hydrophobic core may be necessary to facilitate interaction with the hydrophobic aromatic ring. Phe-110 of horse-liver alcohol dehydrogenase is replaced by tyrosine in benzyl alcohol dehydrogenase from *A. calcoaceticus* and by serine in the TOL-encoded enzyme. Position 110 appears to be the only position within the putative substrate-binding cleft in which there are what could be seen as significant differences between the two benzyl alcohol dehydrogenases. The replacement of Phe-110 with Ser in TOL-encoded benzyl alcohol dehydrogenase has been proposed to make the mouth of the active-site pocket larger [5], and since this position may be the only significant difference between the putative substrate-binding clefts of the two benzyl alcohol dehydrogenases, with the *A. calcoaceticus* enzyme having tyrosine at the mouth of the pocket, it is likely that this difference is responsible for the slight differences in substrate specificities of the *A. calcoaceticus* and *Ps. putida* (pWW0) enzymes. The TOL-encoded enzyme has a preference for substituted benzyl alcohols. It is possible that tyrosine at the mouth of the *A. calcoaceticus* enzyme pocket restricts the entry of substituted benzyl alcohols, notably 2-substituted benzyl alcohols. By sequencing the gene encoding benzyl alcohol dehydrogenase from the TOL-plasmid pWW53 it would be interesting to see which residue this enzyme has at position 110 since it has substrate specificities more similar to the *A. calcoaceticus* enzyme than the pWW0 enzyme [30].

Amino acid changes at positions 48 and 93 have been shown to influence substrate specificity. All of the long-chain zinc-dependent alcohol dehydrogenases have a serine or threonine residue in position 48 (Figure 2) and this residue has been proposed to be involved in the proton transfer mechanism of these enzymes [25]. It is interesting, considering that a Thr-48 → Ser yeast alcohol dehydrogenase mutant had gained some activity against benzyl alcohol [34] and that the human γ subunit (Ser-48) has greater activity against benzyl alcohol than the β subunit (Thr-48), that the presence of serine at position 48, rather than threonine, seems to facilitate activity against benzyl alcohol, since both the benzyl alcohol dehydrogenases from *A. calcoaceticus* and *Ps. putida* (pWW0) have a threonine in position 48 (Figure 2). The benzyl alcohol dehydrogenases also have an additional aromatic residue in the inner part of their putative substrate-binding clefts (Phe-141) that will narrow the active site further. The substrate specificity of benzyl alcohol dehydrogenase from *A. calcoaceticus*, in which substituent groups on the benzene ring are preferred if situated away from the reactive carbinol group, i.e. at the *para* rather than the *ortho* position on the aromatic ring (Table 1), suggests that the active site of the enzyme may have a cleft structure. Despite the overall similarity between the active-site structures of benzyl alcohol dehydrogenase from *A. calcoaceticus* and of horse-liver, human and yeast alcohol dehydrogenases, the enzymes have different substrate specificities due to amino acid replacements that slightly alter the size and shape of the active-site-substrate-binding cleft. For example, yeast and horse-liver alcohol dehydrogenases have active sites with very similar structures, despite the enzymes sharing only 25% sequence identity [35], yet the yeast enzyme has an active site which contains a number of bulky residues that reduce the volume of the substrate-binding cleft. It has been described how this decrease in space in the active site has been shown to restrict the substrate specificity of the enzyme to short-chain primary alcohols [34,36]. The amino acid replacements thus far described in the putative substrate-binding site of benzyl alcohol dehydrogenase from *A. calcoaceticus* compared with that of the human, yeast, and in particular, horse-liver alcohol dehydrogenases suggest that the *A. calcoaceticus* enzyme has a narrower substrate-binding cleft than the horse-liver enzyme. The benzyl alcohol dehydrogenases from *A. calcoaceticus* and *Ps. putida* (pWW0) show the highest sequence conservation within their putative substrate-binding clefts with the human class II (π subunit) alcohol dehydrogenase. As would be expected, the human class II enzyme has similar substrate specificities to the benzyl alcohol dehydrogenases [37]. In particular, the human class II enzyme exhibits strong activity with benzyl alcohol and 4-hydroxy-3-methoxybenzyl alcohol (vanillyl alcohol), the enzyme has very high K_m values for ethanol and cyclohexanol (120 mM and 210 mM respectively) and will not oxidize methanol.

Considering the substrate specificities of the human and yeast alcohol dehydrogenases, it seems strange that neither of the benzyl alcohol dehydrogenases shows detectable activity with ethanol or longer-chain aliphatic alcohols [2,29]. The human class II enzyme has the same residues lining the inner substrate-binding cleft as benzyl alcohol dehydrogenase from *A. calcoaceticus* but displays weak activity against ethanol and strong activity against pentanol and octanol [37]. This suggests that residues outside the active site are influencing substrate specificity.

Stereospecificity of hydride transfer by benzyl alcohol dehydrogenase

The stereospecificity of hydride transfer was determined by measuring the ratio of label (^{3}H) in NAD^+ and benzyl alcohol after benzyl alcohol dehydrogenase had been incubated in the presence of pro*S*-[^{3}H]NADH or pro*R*-[^{3}H]NADH and benzaldehyde. When the enzyme was incubated with benzaldehyde and pro*S*-[^{3}H]NADH the ratio of counts incorporated into the resultant NAD^+ and benzyl alcohol was 98.2%:1.8%, respectively, i.e. the enzyme was stereospecific for the pro*R* hydride of NADH. This was confirmed by incubation of the enzyme with benzaldehyde and pro*R*-[^{3}H]NADH, for which the ratio of counts incorporated into the resultant NAD^+ and benzyl alcohol was 4.8%:95.2%, respectively. This means that benzyl alcohol dehydrogenase is a class A enzyme with respect to hydride transfer, as is horse-liver alcohol dehydrogenase [38], and so the nicotinamide ring is bound in the *anti* position. The side chains of residues Thr-178 and Val-203 of horse-liver alcohol dehydrogenase, residues that are conserved in benzyl alcohol dehydrogenase, have been predicted to obstruct binding of the *syn* conformer of the co-enzyme [26]. It seems likely that benzyl alcohol dehydrogenase binds NAD^+ in a manner analogous to binding by horse-liver alcohol dehydrogenase [24,26].

His-51 seems not to be required by alcohol dehydrogenases in general

His-51 of horse-liver alcohol dehydrogenase has been proposed to contribute to the proton-relay system of the enzyme [25]. The hydroxyl group of the alcohol substrate is ligated to the catalytic zinc atom and linked to the imidazole group of His-51 through a hydrogen-bonded system containing the hydroxyl groups of

Table 2 Comparison of kinetic coefficients of benzyl alcohol dehydrogenase and benzyl alcohol dehydrogenase-His51

Enzyme activity was measured as described in the Experimental section. The NAD^+ concentration was fixed at 2 mM. The k_{cat} values were calculated as (apparent maximum velocity)/(concentration of active sites) assuming a subunit molecular mass value of 38923 Da (this study) and one active site per subunit. NA, no detectable activity, ACBADH, benzyl alcohol dehydrogenase; ACBADH-His51, benzyl alcohol dehydrogenase mutant with histidine residue in position 51; HLADH, horse-liver alcohol dehydrogenase; YADH, yeast alcohol dehydrogenase.

Enzyme	Substrate	Apparent K_m (μM)	Apparent V_{max} (U/mg protein)	k_{cat} (s^{-1})	Specificity constant k_{cat}/K'_m (s^{-1} mM^{-1})
ACBADH	Benzyl alcohol	19	81	53	2.79×10^3
ACBADH-His51	Benzyl alcohol	44	211	137	3.11×10^3
HLADH	Ethanol	1300	2	1.34	1.03
	Benzyl alcohol	15	0.12	0.08	5.53
YADH	Ethanol	4700	124	75	16
	Benzyl alcohol	NA	NA	NA	NA

Ser-48 and the nicotinamide ribose [24,25,28]. The His residue is proposed to act as a general base during catalysis by abstracting a proton from the alcohol substrate, with proton transfer from the alcohol substrate to His-51, and then to the surface of the enzyme, mediated by this hydrogen-bonding system (proton-relay system) [25]. His-51 is conserved in the majority of long-chain zinc-dependent alcohol dehydrogenases (Figure 2). Some plant (e.g. barley and maize; Figure 2) zinc-dependent alcohol dehydrogenases have a tyrosine in this position, as is the case with the human χ subunit [39]. A tyrosine residue could fulfil the role of His-51 by hydrogen bonding to the 2′-hydroxyl group of the nicotinamide ribose [39]. However, the benzyl alcohol dehydrogenases from *A. calcoaceticus* and *Ps. putida* (pWW0) have isoleucine and valine residues, respectively, in position 51 (Figure 2). Also, the product of the *xylW* gene of *Ps. putida*, a protein of unknown function that shows strong similarity to the zinc-dependent alcohol dehydrogenases, has leucine in this position (Figure 2) and the human class II (π subunit) has serine or threonine at position 51 (the gene is polymorphic) [39]. These residues are either incapable of hydrogen-bonding in the manner of His-51 (or Tyr-51) or, as in the case of Ser, the distance that would be involved to form the hydrogen bond is too great. Since the remainder of the proposed proton-relay system is conserved in these enzymes (Figure 2) the question, as first raised by Harayama and colleagues [5], needs to be asked as to how proton transfer occurs in the enzymes. For some reason, during the evolution of benzyl alcohol dehydrogenase, a catalytically important residue has been changed. Reasons for the change were investigated by introducing His-51 into benzyl alcohol dehydrogenase from *A. calcoaceticus*.

Benzyl alcohol dehydrogenase-His-51 (ACBADH-His51) was expressed, purified and characterized along with wild-type benzyl alcohol dehydrogenase. The K'_m, V'_{max} and k_{cat} values determined for ACBADH-His51 are all about 2.5 times greater than the values determined for the wild-type enzyme, but consequently the k_{cat}/K'_m values are similar (Table 2). These results suggest that the insertion of a histidine residue into benzyl alcohol dehydrogenase at position 51, so potentially reconstituting the proton-relay system of the long-chain zinc-dependent alcohol dehydrogenases, has little effect on the overall effectiveness of the enzyme at oxidizing benzyl alcohol. The relative activities against a range of substrates of the wild-type enzyme and of the His-51 mutant enzyme were virtually identical (Table 1).

Benzyl alcohol dehydrogenase from *A. calcoaceticus* is a much more effective enzyme than horse-liver alcohol dehydrogenase, which also has benzyl alcohol dehydrogenase activity (Table 2). The bacterial enzyme has an apparent V_{max} that is 645-fold higher for benzyl alcohol oxidation than the mammalian enzyme, and has a 'specificity constant' about 500-fold higher. Yeast alcohol dehydrogenase was found to have no detectable activity against benzyl alcohol. The specificity constants of both the horse-liver and yeast alcohol dehydrogenases with ethanol as substrate are about three and two orders of magnitude lower than the specificity constant of the bacterial enzyme for benzyl alcohol, respectively.

The effect of pH on the activity of wild-type benzyl alcohol dehydrogenase and ACBADH-His51 was found to be identical (data not shown). Since the two enzymes behaved in a similar manner, it seems likely that the insertion of His-51 has not significantly altered the pK_a of any proton-release group involved in benzyl alcohol oxidation.

CONCLUSIONS

The results in this paper, together with previous work, show that the enzymes of the benzyl alcohol degradation pathway in *A. calcoaceticus* NCIB 8250 are chromosomally encoded on an operon and are very similar to the TOL-plasmid-encoded equivalent enzymes of *Ps. putida*. Benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II seem to differ with respect to their evolutionary origins. Benzyl alcohol dehydrogenase appears to be more similar to mammalian and higher plant alcohol dehydrogenases than to most other microbial alcohol dehydrogenases, whereas benzaldehyde dehydrogenase II is typical of other microbial aldehyde dehydrogenases. There is no significant sequence identity between *xylB* and *xylC*, the genes encoding benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II respectively, thus ruling out the idea of retrograde evolution having occurred during the evolution of the benzyl alcohol pathway. Benzyl alcohol dehydrogenase is a member of the family of long-chain zinc-dependent alcohol dehydrogenases and is apparently a closer relative of other mammalian alcohol dehydrogenases than it is of other bacterial alcohol dehydrogenases; however, it is apparent that benzyl alcohol dehydrogenase has a catalytic mechanism that is different from the 'classical' mechanism that has been proposed for horse-liver alcohol dehydrogenase, the archetypal enzyme in this family.

Parts of this work were supported by grant GR/F74158 from the Biotechnology and Biological Sciences Research Council and D.J.G. is grateful for the award of one of their postgraduate studentships. Dr. T. Krell participated in the work on electrospray MS. Dr. M. Wales and Dr. D. P. Baker determined the stereospecificity of hydride transfer.

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Received 4 July 1997/17 November 1997; accepted 16 December 1997