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ORIGINAL PAPER

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Acquisition of a potential marker for insect transformation: isolation of a novel alcohol dehydrogenase gene from *Bactrocera oleae* by functional complementation in yeast

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Abstract The alcohol dehydrogenase genes make up one of the best studied gene families in Drosophila, both in terms of expression and evolution. Moreover, alcohol dehydrogenase genes constitute potential versatile markers in insect transformation experiments. However, due to their rapid evolution, these genes cannot be cloned from other insect genera by DNA hybridization or PCR-based strategies. We have therefore explored an alternative strategy: cloning by functional complementation of appropriate yeast mutants. Here we report that two alcohol dehydrogenase genes from the medfly *Ceratitis capitata* can functionally replace the yeast enzymes, even though the medfly and yeast genes have evolved independently, acquiring their enzymatic function convergently. Using this method, we have cloned an alcohol dehydrogenase gene from the olive pest *Bactrocera oleae*. We conclude that functional complementation in yeast can be used to clone alcohol dehydrogenase genes that are unrelated in sequence to those of yeast, thus providing a powerful tool for isolation of dominant insect transformation marker genes.

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Introduction

The enzyme alcohol dehydrogenase (ADH; alcohol: NAD^+ oxidoreductase; EC 1.1.1.1.) catalyses the reversible conversion of a variety of alcohols to their corresponding aldehydes and ketones. The reduction of acetaldehyde to ethanol is the final step in alcoholic fermentation in yeast and the reverse reaction is the initial step in the catabolism and detoxification of ethanol in a variety of organisms. Insect ADHs belong to the superfamily of short-chain dehydrogenases/reductases (SDR) (Jörnvall et al. 1995); members of which have been found in organisms as diverse as bacteria and humans. Interestingly, all four yeast *Adh* genes, as well as those from other organisms, belong to the medium-chain (formerly "long-chain") dehydrogenase family. In light of the major differences in structure and catalytic mechanism between the two dehydrogenase families, it is believed that they have evolved independently and acquired the same function by convergence (Jörnvall et al. 1981).

Until recently, insect *Adh* genes had been cloned exclusively from species of the genus Drosophila, where this gene-enzyme system has been the subject of intensive studies on gene evolution and expression (for a recent review, see Ashburner 1998), as well as its response to natural selection (Kreitman 1983; Bodmer and Ashburner 1984; Kreitman and Aguade 1986; Kreitman and Hudson 1991; McDonald and Kreitman 1991). Because of their low levels of sequence similarity to the Drosophila genes, cloning of the two *Adh* genes from the medfly *Ceratitis capitata* was only accomplished by first purifying the proteins to homogeneity (Gasperi et al. 1994) and using peptide sequences and RT-PCR to amplify the corresponding cDNA sequences (Brogna et al., in preparation). The deduced medfly ADH peptide sequences are 83% identical to each other, but show only about 35% identity with the Drosophila proteins.

Using a similar approach, Horio et al. (1996) cloned one *Adh* gene from *Sarcophaga peregrina* (a member of the family Calyptratae), which is also 38% identical to Drosophila proteins. The low degree of sequence similarity between Drosophila and non-Drosophila *adh* genes suggests that cloning them from non-Drosophilid insects by conventional techniques will not be straightforward. Thus there is a need for the development of an alternative cloning technique.

Functional complementation of yeast mutants has been used successfully in the past for cloning conserved genes from various species, such as the gene encoding the Drosophila *ade3*, and human and Drosophila cyclins (Henikoff et al. 1981; Léopold and O'Farrell 1991; Lew et al. 1991). *Saccharomyces cerevisiae* has four alcohol dehydrogenase genes (Williamson and Paquin 1987), all of which belong to the medium-chain dehydrogenase family (Young and Pilgrim 1985). A previous attempt to detect complementation of ADH-deficiency in yeast cells following transformation with the Drosophila *Adh* gene failed; although the insect ADH protein was detected by immunoblotting (Atrian et al. 1990). In the present study, we show that two short-chain alcohol dehydrogenases from medfly *Ceratitis capitata* can complement ADH deficiency in yeast, thus allowing mutant cells to grow when respiration is inhibited. We have used this finding to develop a method that allows cloning of short-chain alcohol dehydrogenase genes. Using this method we have successfully cloned an *Adh* gene from another major agricultural pest, the olive fruitfly *Bactrocera oleae* (Dacus).

Materials and methods

Strains

C. capitata flies are derived from the stock 'Benakeio', and were raised at 22–25° C as described previously (Rina and Savakis 1991). *Bactrocera oleae* flies were provided by Dr. Chaniotakis (Entomology Laboratory, Research Center "Demokritos", Greek Atomic Energy Commission). The *Adh*-deficient yeast strain MC892-1C (*MAT*α; *adh1*, *adh2, adh3*, *ura3-52*, *trp1*, *leu2*, *his3*) and MC71-27A (*MAT***a**, *adh1*, ADH2, *adh3*, *ura3-52*, *trp1-289*) were used to select for insect *Adh* genes, and the wild type RH1168 (*MAT*α, *mal*, *gal2*, *ura3-52*, *leu2-2*) was used as a positive control. Initial propagation of the *B. oleae* cDNA library was accomplished in *E. coli* strain XL1-Blue MRF′ (Stratagene). In all other cases, the *E. coli* strain DH5α [F′ *endA1 hsdR17 supE44 thi-1 recA1 gyr A relA1* Δ(*lacZYA-argF*)] was used as a plasmid host.

Transformation in *E. coli* and yeast

Transformation of *E. coli* cells was generally done as described by Hanahan (1983). The *B. oleae* cDNA library was introduced into the host cells by electroporation (0.1 cm electroporation cuvette; pulse at 25 μF, 200 Ω, 1.65 kV). All yeast cells were transformed as described by Ito et al. (1983).

Plasmid constructs

The two medfly genes were cloned in the yeast expression vector pDB20 (Fikes et al. 1990). Total RNA was isolated from adult flies, as described in Holmes and Bonner (1973), and single-stranded, oligo(dT)-primed cDNA was synthesized by standard methods (Sambrook et al. 1989). *Adh1* and *Adh2* cDNAs were amplified by PCR, using Vent polymerase (New England Biolabs) to increase fidelity. The following set of primer pairs was used to amplify the two cDNAs. 5′-CTAAGCTTCATAATGAGTTTGGCCGGTAAAAAT-3′ and 5′-CTAAGCTTCTAGTAAGTGGGTTCCCAGTA-3′ for *Adh-1*; and 5′-CTAAGCTTCATAATGAGTTTGGCCGGTAAAAAT-3′ and 5′-CTAAGCTTCTAGTGGTAGGTGGGCTGCCA-3′ for *Adh-2*. The PCR was first run for five cycles at the annealing temperature of 52° C and then for another 35 cycles at 62° C. Southern analysis using an *Adh-2* cDNA clone as a probe under stringent conditions (60° C), verified that the amplified fragments were indeed derived from the two medfly genes. The constructs pDBCcA1 and pDBCcA2 resulted from cloning the two PCR products into vector pDB20. Construct pDBScA1, containing the *S. cerevisiae ADH1* gene, was used as a positive control for the complementation experiments. The *Sph*I fragment of the vector pDB20 (extending from the *ADC1* promoter to the termination signal) was replaced by the yeast genomic fragment that extends from 414 bp upstream of ATG to 528 bp downstream of the termination codon of the *ADH1* gene.

Screening for ADH activity

Transformed yeast cells were plated on MIN plates (2% glucose, 0.7% yeast nitrogen base without amino acids, and 2% agar), with the addition of the appropriate amino acids, and allowed to grow for three days. They were then replica plated onto plates of the same medium supplemented with 0.5 ppm antimycin A, which inhibits respiration. To clone the *B. oleae Adh* gene, DNA was recovered from yeast that were capable of growing in the presence of antimycin, and was used to re-transform yeast cells, which were then re-screened for ADH activity. This method revealed that three out of the 18 clones initially recovered were false positives.

Construction of the *B. oleae* cDNA library

Total RNA was prepared from adult *B. oleae* as described by Holmes and Bonner (1973). mRNA was subsequently isolated on an oligo-d(T) column. cDNA was synthesized using the cDNA synthesis reagents provided by Stratagene, according to supplier's instructions. The resulting cDNAs contained *Eco*RI and *Xho*I overhangs at the 5′- and 3′-end, respectively. This allowed directional cloning into a modified version of the vector pDB20.

Sequencing

The *B. oleae Adh* cDNA clone was sequenced using the transposon-based method described by Strathmann et al. (1991). Whenever single-pass sequencing was used, this was done as described by Sambrook et al. (1989).

Results and discussion

Complementation of ADH-deficient yeast strains by medfly genes

Since all yeast *Adh* genes belong to the medium-chain dehydrogenase family and are thus substantially different from the insect enzymes, we tested whether a short-chain ADH could adequately complement the yeast deficiency, before attempting to use complementation to clone new *Adh* genes from other insect species. We first cloned the two medfly *Adh* cDNAs (EMBL Accession

Nos. Z30194 and Z30195) separately in the shuttle vector pDB20 (Fikes et al. 1990). pDB20 is a multicopy vector that can be propagated and selected in both *E. coli* and yeast, using ampicillin resistance and uracil auxotrophy, respectively. Expression in yeast cells is controlled by the promoter of the *Adh1* gene (*ADC1*), which is one of the strongest constitutively active yeast promoters (Nasmyth and Hall 1980). In order to maximize the efficiency of translation, both constructs with medfly cDNAs contained the CATA sequence immediately upstream of the initiator ATG, which matches the consensus at this position in yeast genes that are highly expressed (Cigan and Donahue 1987). Furthermore, we confirmed, by single-pass sequencing, the absence of any upstream ATGs that might cause a decrease in expression levels. The vector without insert was used as a negative control and the vector containing the *S. cerevisiae ADH1* gene (construct pDBScA1) as a positive control.

These four constructs were used to transform the yeast strains MC892-1C and MC71-27A. Strain MC892-1C is deficient for the three major alcohol dehydrogenase genes; whereas MC71-27A is deficient for *ADH1* and *ADH3*, but retains the *ADH2* gene intact. Three independent colonies of each of these transformants were screened for ADH activity, using antimycin A plates. Antimycin A, at a concentration of 0.5 ppm, blocks cell respiration, leaving the glycolytic pathway as the only source of NAD^+. Thus, only cells that are able to metabolize acetyl Coenzyme A to ethanol can grow. The results are shown in Fig. 1. Each of the two medfly *Adh* genes, as well as the yeast *ADH1* gene, complemented both mutant yeast strains, allowing them to grow under anaerobic conditions i.e. in the presence of antimycin A (Fig. 1). When transformed with the empty vector, the triple mutant (strain MC892-1C) failed to grow; whereas the double mutant (strain MC71-27A) did exhibit some limited growth. This residual growth must be due to its intact *ADH2* gene (which would be repressed, by the glucose in the medium, during early phases of growth). Both mutant strains transformed with medfly genes grew about 20% more slowly than the wild type strain and that transformed with the yeast *ADH1* gene.

To verify expression of the insect proteins in vivo, crude extracts from transformants of strain MC892-1C, carrying the plasmids pDBCcA1, pDBCcA2, pDBScA1 and pDB20 were fractionated by SDS-polyacrylamide gel electrophoresis. The proteins on the gel were electrotransferred to a membrane and immunostained with a rabbit polyclonal antibody against medfly ADH1, which cross-reacts with ADH2 (Gasperi et al. 1994). We confirmed the expression of the medfly ADHs (Fig. 2). No other protein cross-reacted with this antibody.

These results support the view that the short-chain alcohol dehydrogenases can functionally replace the yeast medium-chain enzymes. This is a clear case where molecules that are completely unrelated, in both their primary structure and the mode of formation of the

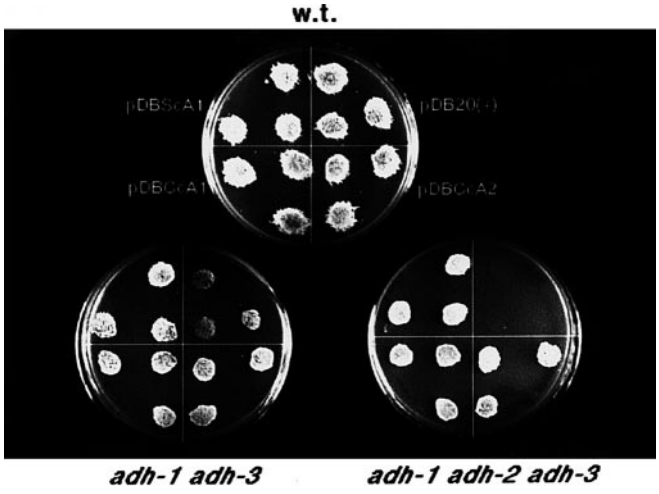

Fig. 1 Complementation of alcohol dehydrogenase-deficient yeast by *C. capitata Adh* genes. The *ADH*-deficient yeast strains MC71-27A (*MAT***a**, *adh1*, *adh3*, *ura3-52*, *trp1-289*), which expresses *ADH2* (*bottom left*) and MC892-1C (*MATα*, *adh1*, *adh2*, *adh3*, *ura3-52*, *trp1*, *leu2*, *his3*), which completely lacks *ADH* activity (*bottom right*), were transformed with the plasmids pDBCcA1 (*lower left sector of the plates*) and pDBCcA2 (*lower right sector of the plates*). Both these plasmids are derived from the yeast expression vector pDB20 and carry the *C. capitata* genes *Adh-1* and *Adh-2*, respectively. We used the vector containing the *S. cerevisiae ADH1* gene (plasmid pDBScA1) as a positive control (*top left sector of the plates*); and the empty vector (pDB20) as a negative control (*top right sector of the plates*). Three individual transformants from each plate were replica-plated onto agar plates containing antimycin A (Sigma), at a concentration of 0.5 ppm, which blocks cell respiration. Cells transformed with pDBCcA1, pDBCcA2 or pDBScA1 can grow, whereas cells carrying the empty vector did not grow, under these conditions

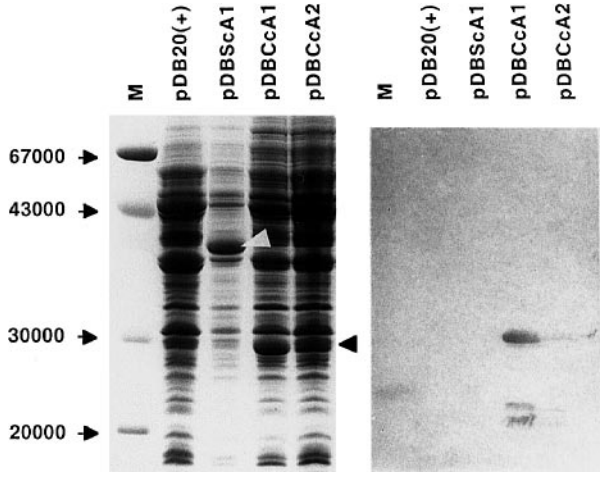

Fig. 2 Electrophoretic and immunoblot analysis of the *C. capitata* proteins expressed in yeast. Crude extracts from cells of the yeast strain MC892-1C, transformed with the plasmids indicated, were fractionated on a 12% SDS-polyacrylamide gel. *Left panel* Gel stained with Coomassie Brilliant Blue. The positions of the ADH proteins are indicated by the *arrowheads*. *Right panel* An identical gel was electroblotted onto a nitrocellulose filter, which was probed with a polyclonal antibody (diluted 1:1000) against *C. capitata Adh1* (Gasperi et al. 1994), and stained with an anti-rabbit antibody (Boehringer Mannheim), conjugated to horseradish peroxidase. Low molecular weight bands in the pDBCcA1 lane are probably degradation products

active enzyme complex, are found to replace each other functionally in vivo.

Cloning of a novel gene from *B. oleae* by complementation of a yeast mutant

A small cDNA library from *B. oleae* was constructed in the shuttle vector pDB20. The library consisted of about 12,500 clones, of which more than 80% contained inserts greater than 200 bp long. The sizes of the inserts varied from 100 bp to 2.5 kb, with the majority being around 1000 bp long. Plasmid DNA from this library was used to transform yeast cells of strain MC892-1C; these were subsequently screened for ADH activity in the presence of antimycin A. Library plasmids were recovered from a total of 18 candidate positive colonies, all of which could grow in the presence of antimycin A. Of these, 15 had inserts of about 1000 bp in length; the other three were about 900, 500 and 450 bp long. Single-pass sequencing of the 5′ ends of the 15 larger clones proved that they were identical. DNA from each of the four classes of clones was used to re-transform yeast, which were plated on minimal medium containing antimycin A. The clone containing the 1-kb insert successfully complemented yeast ADH deficiency upon re-transformation (Fig. 3); whereas the three shorter clones failed, indicating that they were false positives.

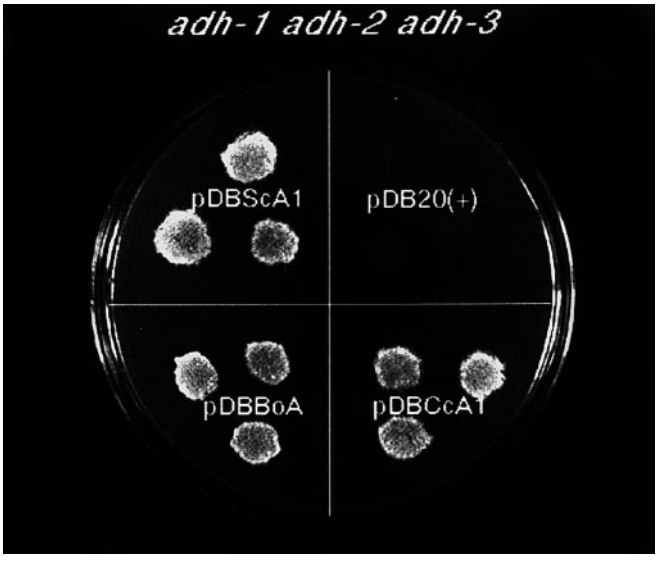

Fig. 3 Cloning of an alcohol dehydrogenase gene from *B. oleae*. A cDNA library was constructed in the yeast expression vector pDB20, using poly(A+) RNA from adult flies. Plasmid DNA from this library was used to transform MC892-1C cells (*MATα*, *adh1*, *adh2*, *adh3*, *ura3-52*, *trp1*, *leu2*, *his3*), which were subsequently plated on minimal medium plates. Approximately 10,000 yeast colonies were then replica-plated onto the same medium containing 0.5 ppm antimycin A (Sigma) and 18 putative clones were recovered. Three of these failed to complement the *ADH* deficiency phenotype upon retransformation; the other 15 proved to be identical. Three of the 15 clones were plated onto a minimal medium plate containing antimycin A (*lower left quadrant*), together with three yeast colonies transformed with one of the following plasmids pDBScA1 (*top left*), pDB20 (*top right*) and pDBCcA1 (*bottom right*)

The complete sequence of this cDNA clone was determined (EMBL Accession No. AJ2500007). It is 967 bp long and contains an ORF of 777 bp (starting at the first ATG), with a 50-bp 5′ UTR and a 3′ UTR of 140 bp [excluding the poly(A) tail]. The corresponding protein is predicted to be 258 amino acids long. It shows 78% and 84% overall sequence identity with medfly ADH-1 and ADH-2 respectively; and is 33% identical to *D. melanogaster* ADH (Fig. 4). The sequence contains also the "short-chain dehydrogenase" motif (PROSITE Accession number PS00061; ADH_SHORT). There appears to be one single amino acid insertion and two double amino acid deletions in the *D. melanogaster* ADH relative to those from Tephritids.

A number of studies have attempted to identify the amino acid residues that are important for the structure and function of the *D. melanogaster* enzyme. Site-directed mutagenesis has shown that glycine-14, glycine-129, glycine-132, tyrosine-152, lysine-156 and glycine-183 are amongst the most important; mutations in each of these positions resulted in a non-functional protein (Abalat et al. 1992; Chen et al. 1990, 1993; Cols et al. 1993). In agreement with these studies, the *B. oleae* ADH (as well as that from *S. peregrina* and both *C. capitata* proteins) retain all these amino acids in the corresponding positions (Fig. 4), despite the relatively low overall sequence similarity. Another gene (*Adhr* or *Adh-related*), located at the 3′ to *Adh*, has been characterised in many Drosophilids. *Adh* and *Adhr* have the same intron-exon structure (Schaeffer and Aquadro 1987) and it has been shown that in *D. melanogaster* these two genes are initially transcribed as a single dicistronic message from the same promoter (Brogna and Ashburner 1997). It is generally believed that they originated from a common ancestor by gene duplication (Schaeffer and Aquadro 1987; Kreitman and Hudson 1991; Jeffs et al. 1994). *Adhr* is a highly conserved gene in Drosophilids; even more conserved than *Adh*. *D. melanogaster* ADH and ADHR share only 37.6% overall sequence identity (calculated with the program GAP in the GCG software suite). Based on the high degree of divergence between ADH and ADHR and on the fact that, in the latter, glycine-14 is replaced by aspartic acid, it has been suggested that *Adhr* might have no alcohol dehydrogenase activity (Jeffs et al. 1994; Brogna and Ashburner 1997). This notion is in agreement with our observations. When yeast ADH-deficient strains were transformed with *D. melanogaster Adhr* cDNA, they failed to grow in the presence of antimycin (data not shown).

These data support the hypothesis that the gene from *B. oleae*, which was cloned by functional complementation in yeast, indeed represents an alcohol dehydrogenase gene. By cloning a novel *Adh* gene from another member of the Tephritid family, we have demonstrated that yeast complementation can, in principle, be used as a tool for cloning short-chain alcohol dehydrogenase genes from insect species that are remote from Drosophila.

Fig. 4 Multiple alignment of insect *Adh* genes. Although the overall degree of sequence conservation between the Sarcophaga, Tephritid and *D. melanogaster* ADHs is rather low (approximately 30%), all residues that have been characterized as critical for the enzymatic activity (*bold*) are conserved. The alignment was done with the ClustalW program (Thompson et al. 1994)

Adh gene duplication in Tephritids?

It is known that the *Adh* locus has undergone multiple duplication events in insects. In Drosophilids the remnants of these duplications include (depending on the species or group): a second copy of the gene, a pseudogene and the *Adh-related* gene. Russo et al. (1995) have proposed a scenario according to which as many as four independent duplications occurred in the last 180 Myr, resulting in the present status of the *Adh* region in the Drosophilid species. Apart from the four proposed duplications in Drosophilids, there is at least one duplication in Tephritids. Medfly *C. capitata* has two copies of the *Adh* gene, tandemly arranged on the chromosome. The two genes are expressed in different tissues of the fly. ADH2 is the fat body/midgut enzyme (like the one in *D. melanogaster*) and ADH1 is expressed in muscle (Brogna et al., submitted).

The *B. oleae* ADH appears to be more similar to the *C. capitata* ADH-2 (*B. oleae* ADH is 84% identical to *C. capitata* ADH-2, compared to 77% to *C. capitata* ADH-1), which is consistent with the expression profile of the ADH-2 protein. These data also show that the duplication of the *Adh* gene in Tephritids is more likely to have occurred prior to the divergence of the genera *Bactrocera* and *Ceratitis*. Further studies will be required to reveal whether *B. oleae* has a second copy of the *Adh* gene and/or an *Adhr* gene.

Conclusion

We have shown that insect short-chain alcohol dehydrogenases can functionally substitute for the yeast enzymes in vivo. Using this observation, we have developed a cloning method for insect *Adh* genes, based on functional complementation of yeast mutants. This lifts the barrier to cloning *Adh* genes from genera other than Drosophila, and allows the further evolutionary study of this locus and its products. With this method, we have cloned a novel *Adh* gene from another Tephritid species, *B. oleae*, which we used to make a preliminary estimation of the amino acid substitution rate within Tephritids. Our results suggest that this rate is higher than that within Drosophilids. Further studies are needed to address this problem more definitively.

By conferring resistance to environmental ethanol, *Adh* genes constitute dominant (selectable) markers. Until recently, transformation was possible only in members of the genus Drosophila. Advances in transformation of non-Drosophila insects, including the agricultural pest *C. capitata* (Loukeris et al. 1995; Handler et al. 1998) and the yellow fever mosquito *Aedes aegypti* (Coates et al. 1998), make the development of pest control methods based on gene-transfer technology potentially feasible. Thus, the method we have reported for cloning insect *Adh* genes, apart its usefulness as a tool for evolutionary studies of the locus, will also provide a tool for insect transformation.

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References

Abalat R, Gonzalez-Duarte R, Atrian S (1992) Protein engineering of *Drosophila* alcohol dehydrogenase. The hydroxyl group of Tyr152 is involved in the active site of the enzyme. FEBS Lett 308:235–239

Ashburner M (1998) Speculations on the subject of alcohol dehydrogenase and its properties in *Drosophila* and other flies. Bioessays 20:949–954

Atrian S, Gonzàlez-Duarte R, Fothergill-Gilmore LA (1990) Synthesis of *Drosophila melanogaster* alcohol dehydrogenase in yeast. Gene 93:205–212

Bodmer M, Ashburner M (1984) Conservation and change in the DNA sequences coding for alcohol dehydrogenase in sibling species of *Drosophila*. Nature 309:425–430

Brogna S, Ashburner M (1997) The *Adh-related* gene of *Drosophila melanogaster* is expressed as a functional dicistronic messenger RNA: multigenic transcription in higher organisms. EMBO J 16:2023–2031

Chen Z, Lu L, Shirley M, Lee WR, Chang SH (1990) Site-directed mutagenesis of glycine-14 and two "critical" cysteinyl residues in *Drosophila* alcohol dehydrogenase. Biochemistry 29:1112–1118

Chen Z, Jiang JC, Lin ZG, Lee WR, Baker ME, Chang SH (1993) Site-specific mutagenesis of *Drosophila* alcohol dehydrogenase: evidence for involvement of tyrosine-152 and lysine-156 in catalysis. Biochemistry 32:3342–3346

Cigan AM, Donahue TF (1987) Sequence and structural features associated with translational regions in yeast – a review. Gene 59:1–18

Coates CJ, Jasinskiene N, Miyashiro L, James AA (1998) *Mariner* transposition and transformation of the yellow fever mosquito, *Aedes aegypti*. Proc Natl Acad Sci USA 95:3748–3751

Cols N, Marfany G, Atrian S, Gonzalez-Duarte R (1993) Effect of site-directed mutagenesis of conserved positions of *Drosophila* alcohol dehydrogenase. FEBS Lett 319:90–94

Fikes JD, Becker DM, Winston F, Guarente L (1990) Striking conservation of TFIID in *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*. Nature 346:291–294

Gasperi G, Kafetzopoulos D, Christodoulidou A, Bouriotis V, Savakis C (1994) Isolation and partial characterization of two alcohol dehydrogenase isozymes from the medfly *Ceratitis capitata*. Insect Biochem Mol Biol 24:87–94

Hanahan D (1983) Studies on transformation of *Escherichia coli* with plasmids. J Mol Biol 166:557–580

Handler AM, McCombs SD, Fraser MJ, Saul SH (1998) The lepidopteran transposon vector, piggyBac, mediates germ-line transformation in the Mediterranean fruit fly. Proc Natl Acad Sci USA 95:7520–7525

Henikoff S, Tatchell K, Hall B, Nasmyth K (1981) Isolation of a gene from *Drosophila* by complementation in yeast. Nature 289:33–37

Holmes DS, Bonner J (1973) Preparation, molecular weight, base composition, and secondary structure of giant nuclear ribonucleic acid. Biochemistry 12:2330–2338

Horio T, Kubo T, Natori S (1996) Purification and cDNA cloning of the alcohol dehydrogenase of the flesh fly *Sarcophaga peregrina*. A structural relationship between alcohol dehydrogenase and a 25-kDa protein. Eur J Biochem 237:698–703

Ito H, Fukuda Y, Murata YK, Kimura A (1983) Transformation of intact yeast cells treated with alkali cations. J Bacteriol 153:163–168

Jeffs PS, Holmes EC, Ashburner M (1994) The molecular evolution of the alcohol dehydrogenase and alcohol dehydrogenase-related genes in the *Drosophila melanogaster* species subgroup. Mol Biol Evol 11:287–304

Jörnvall H, Persson M, Jeffery J (1981) Alcohol and polyol dehydrogenases are both divided into two protein types, and structural properties cross-relate the different enzyme activities within each type. Proc Natl Acad Sci USA 78:4226–4230

Jörnvall H, Persson B, Krook M, Atrian S, Gonzalez-Duarte R (1995) Short-chain dehydrogenases/reductases (SDR). Biochemistry 34:6003–6013

Kreitman M (1983) Nucleotide polymorphism at the alcohol dehydrogenase locus of *Drosophila melanogaster*. Nature 304:412–417

Kreitman M, Aguade M (1986) Excess polymorphism at the *Adh* locus in *Drosophila melanogaster*. Genetics 114:93–110

Kreitman M, Hudson RR (1991) Inferring the evolutionary histories of the *Adh* and *Adh-dup* loci in *Drosophila melanogaster* from patterns of polymorphism and divergence. Genetics 127:565–582

Léopold P, O'Farrell P (1991) An evolutionarily conserved cyclin homolog from *Drosophila* rescues yeast deficient in G1 cyclins. Cell 66:1207–1216

Lew D, Duclic V, Reed S (1991) Isolation of three novel human cyclins by rescue of G1 cyclin (Cln) function in yeast. Cell 66:1197–1206

Loukeris TG, Livadaras I, Arcà B, Zabalou S, Savakis C (1995) Gene transfer into the medfly *Ceratitis capitata*, with a *Drosophila hydei* transposable element. Science 270:2002–2005

McDonald J, Kreitman M (1991) Adaptive protein evolution at the *Adh* locus in *Drosophila*. Nature 351:652–654

Nasmyth K, Hall B (1980) Isolation of the structural gene for alcohol dehydrogenase by genetic complementation in yeast. Nature 283:214–216

Rina M, Savakis C (1991) A cluster of vitellogenin genes in the Mediterranean fruit fly *Ceratitis capitata*: sequence and structural conservation in dipteran yolk proteins and their genes. Genetics 127:769–780

Russo CAM, Takezaki N, Nei M (1995) Molecular phylogeny and divergence times of Drosophilid species. Mol Biol Evol 12:391–404

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual (2nd edn). Cold Spring Harbor Laboratory, Cold Spring Harbor, New York pp. Cold Spring Harbor Laboratory Press

Schaeffer SW, Aquadro CF (1987) Nucleotide sequence of the *Adh* gene region of *Drosophila pseudoobscura*: evolutionary change and evidence for an ancient gene duplication. Genetics 117:61–73

Strathmann M, Hamilton BA, Mayeda CA, Simon MI, Meyerowitz EM, Palazzolo MJ (1991) Transposon-facilitated DNA sequencing. Proc Natl Acad Sci USA 88:1247–1250

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680

Williamson VM, Paquin CE (1987) Homology of *Saccharomyces cerevisiae* ADH4 to an iron-activated alcohol dehydrogenase from *Zymomonas mobilis*. Mol Gen Genet 209:374–381

Young ET, Pilgrim D (1985) Isolation and DNA sequence of ADH3, a nuclear gene encoding the mitochondrial isozyme of alcohol dehydrogenase in *Saccharomyces cerevisiae*. Mol Cell Biol 5:3024–3034