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A tomato enzyme catalyzing the phosphorylation of 3,4-dihydroxy-2-butanone

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Abstract

A riboflavin biosynthesis *ribB* mutant of *Escherichia coli* deficient of 3,4-dihydroxy-2-butanone 4-phosphate synthase was complemented with a cDNA library from *Lycopersicon esculentum*. The complementing gene was isolated and expressed in *E. coli*. The resulting protein was shown to specify a 62 kDa protein which phosphorylates dihydroxyacetone, both enantiomers of 3,4-dihydroxy-2-butanone, and several other aldoses and ketoses. Sequence analysis revealed homology to dihydroacetone kinases (*dak*) genes from plants, animals, fungi and some eubacteria. Genes with similarity to the 5′ part of the *dak* gene from tomato were found in many other eubacteria. The physiological role of the *dak* gene is still incompletely known.

Keywords: 3,4-Dihydroxy-2-butanone 4-phosphate; Riboflavin; 3,4-Dihydroxy-2-butanone; Dihydroxyacetone; *Lycopersicon esculentum*; Solanaceae; Tomato; Kinase; Phosphorylation

1. Introduction

The riboflavin precursor, L-3,4-dihydroxy-2-butanone 4-phosphate (**2**), is biosynthesized from ribulose 5-phosphate (**1**) by a rearrangement reaction under release of carbon atom 4 as formate (Fig. 1) (Volk and Bacher, 1988, 1990; Bacher, 1991; Bacher et al., 1997a, b). In *Escherichia coli*, this complex reaction is catalyzed by the homodimeric 3,4-dihydroxy-2-butanone 4-phosphate synthase specified by the *ribB* gene (Richter et al., 1992; Bacher et al., 1996). The bacterial enzyme has been studied in some detail (Richter et al., 1992, 1999; Kelly et al., 1999, 2001; Liao et al., 2001). A bifunctional enzyme with 3,4-dihydroxy-2-butanone 4-phosphate synthase and GTP cyclohydrolase II activity has been cloned more recently from *Arabidopsis thaliana* (Herz et al., 2000). This enzyme is similar to bifunctional orthologs from various bacteria and catalyzes both initial reactions of the convergent riboflavin pathway.

Prior to the cloning of the bifunctional plant enzyme (Herz et al., 2000), we performed complementation experiments using a *ribB* mutant of *E. coli* (Katzenmeier, 1991) and a cDNA library from tomato (*Lycopersicon esculentum*). The *ribB* mutant is deficient of 3,4-dihydroxy-2-butanone 4-phosphate synthase and requires riboflavin in very high concentration for growth. A plant gene was cloned that could complement the 3,4-dihydroxy-2-butanone 4-phosphate synthase deficiency of the *E. coli ribB* mutant. Strangely, however, complementation required the presence of yeast extract in the culture medium.

The cloned plant gene was subsequently found to specify a kinase which is not involved in the riboflavin biosynthetic pathway. Putative orthologs of that enzyme are present in plants, animals and certain microorganisms.

2. Results

In an attempt to clone 3,4-dihydroxy-2-butanone 4-phosphate synthase genes from plants, we performed complementation experiments using a *ribB* mutant of *E. coli* deficient of 3,4-dihydroxy-2-butanone 4-phosphate synthase (Katzenmeier, 1991). A cDNA library from tomato (Schmid et al., 1992) in the plasmid pBlueskript

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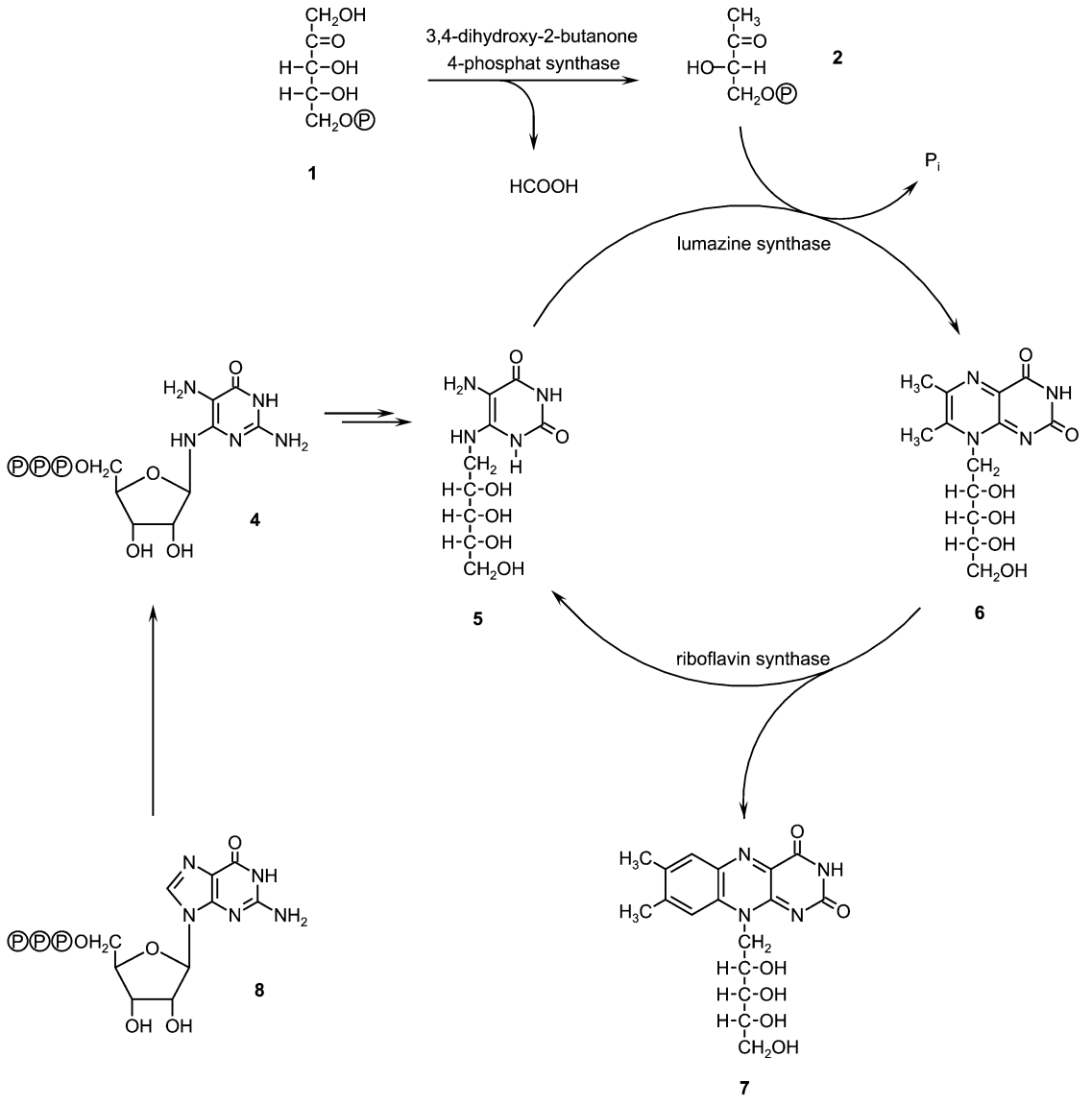

Fig. 1. Biosynthesis of riboflavin.

SK II$^-$ was transformed into *E. coli ribB* cells which were plated on Luria Bertani (LB) medium. The riboflavin content of this medium is well below the concentration level required for growth of riboflavin deficient *E. coli* mutants (Shavlovskii et al., 1982; Katzenmeier, 1991). Colonies growing on this medium were found to harbor a plasmid designated p10–20 containing an insert of 2.2 kbp. Retransformation of plasmid p10–20 into two different *ribB* mutants (rib5 and rib10) of *E. coli* reproducibly afforded clones growing on LB medium without added riboflavin.

The growth rate of the recombinant *E. coli* strains on LB medium was relatively low and could be increased by the addition of riboflavin in high concentration (400 mg l^{-1}). We concluded tentatively that the plasmid p10–20 contained a tomato gene which is capable of restoring the function of the defective *ribB* gene, albeit with limited efficiency.

The DNA insert of plasmid p10–20 was sequenced (EMBL Acc. No. Y12090) and was shown to contain a putative open reading frame of 1782 bp specifying a predicted peptide of 594 amino acid residues. Moreover, the insert contained 138 untranslated nucleotides at the 5′ end and 300 untranslated bp including a polyA sequence of 18 residues at the 3′ end. This gene is subsequently designated *dak*.

Sequence comparison of the predicted tomato protein showed similarity to putative ATP-dependent dihydroxyacetone kinases from eukaryotes and several eubacteria (Figs. 2 and 3). Dihydroxyacetone kinases are required for glycerol and methanol metabolism in eubacteria and yeast (Kato et al., 1988; Daniel and

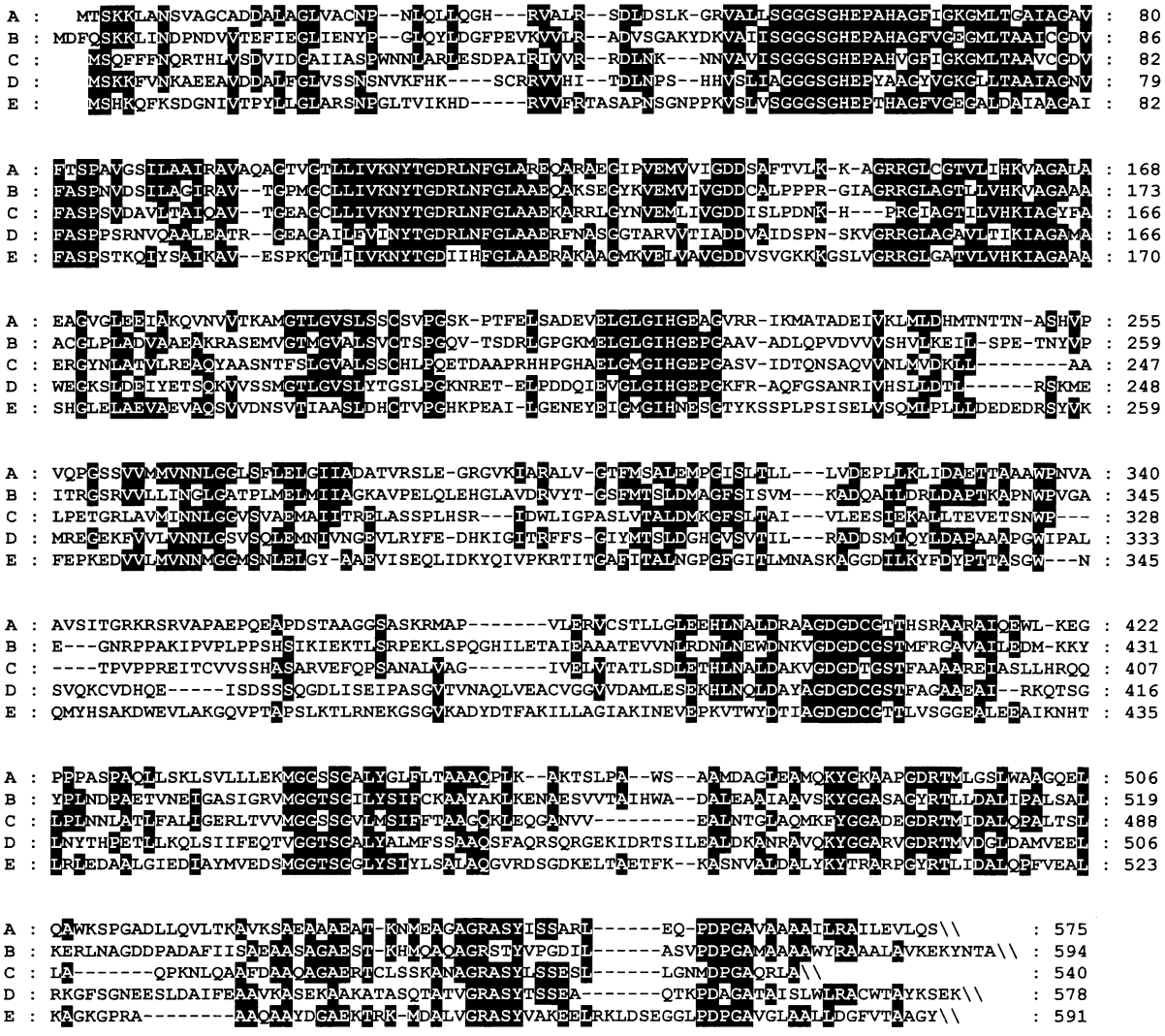

Fig. 2. Comparison of protein sequences specified by putative *dak* orthologs. A, *Homo sapiens*; B, *Lycopersicon esculentum*; C, *Citrobacter freundi*; D, *Caenorhabditis elegans*; E, *Saccharomyces cerevisiae* (DAK2). The sequences were aligned with the PILEUP program using the default parameters (GCG, Madison, WI, USA). Identical residues conserved in at least 60% of the sequences were shown in inverse contrast.

Gottschalk, 1992; Kimura et al., 1998). In order to rule out the possibility that the isolated gene could originate from a microbial DNA contamination in the tomato cDNA library, PCR experiments were performed with chromosomal tomato DNA. Several segments of the *dak* gene could be amplified from the chromosomal tomato DNA. These experiments leave no doubt that the *dak* gene is an authentic tomato gene.

The open reading frame of the tomato *dak* gene was cloned into the plasmid vector pNCO113 under control of a T5 promoter and *lac* operator affording plasmid pDAK. A recombinant *E. coli* strain carrying this plasmid was shown to express a 62 kDa peptide at an estimated yield of 5% based on total cell protein. The recombinant protein was purified to about 95% homogeneity as judged by SDS-PAGE (Fig. 4) by two chromatographic steps as described under Experimental (Table 1).

The recombinant tomato protein was shown to phosphorylate dihydroxyacetone at a rate of 2.6 μmol min^{-1} mg^{-1} (Table 2). Moreover, both enantiomers of 3,4-dihydroxy-2-butanone can serve as substrate for the tomato kinase, but the D enantiomer is phosphorylated at a considerably higher rate. Several other hydroxyketones and hydroxyaldehydes were phosphorylated at low rates. Polyalcohols are unable to serve as substrates. Mg^{2+} is required for catalytic activity.

These findings suggested that the complementation experiment conducive to the isolation of the tomato clone p10–20 had involved the formation of the riboflavin intermediate, 3,4-dihydroxy-2-butanone

4-phosphate (**2**), by phosphorylation of the corresponding alcohol, 3,4-dihydroxy-2-butanone (**3**), which might have been present in the LB medium used for the selection of colonies. In line with this hypothesis, we found that *ribB* mutants of *E. coli* carrying the plasmid p10–20 could not grow on mineral salt medium, whereas growth could be restored by the addition of 3,4-dihydroxy-2-butanone to the minimal medium (data not shown). *RibB* mutants without the plasmid were unable to grow on LB medium but could use diacetyl

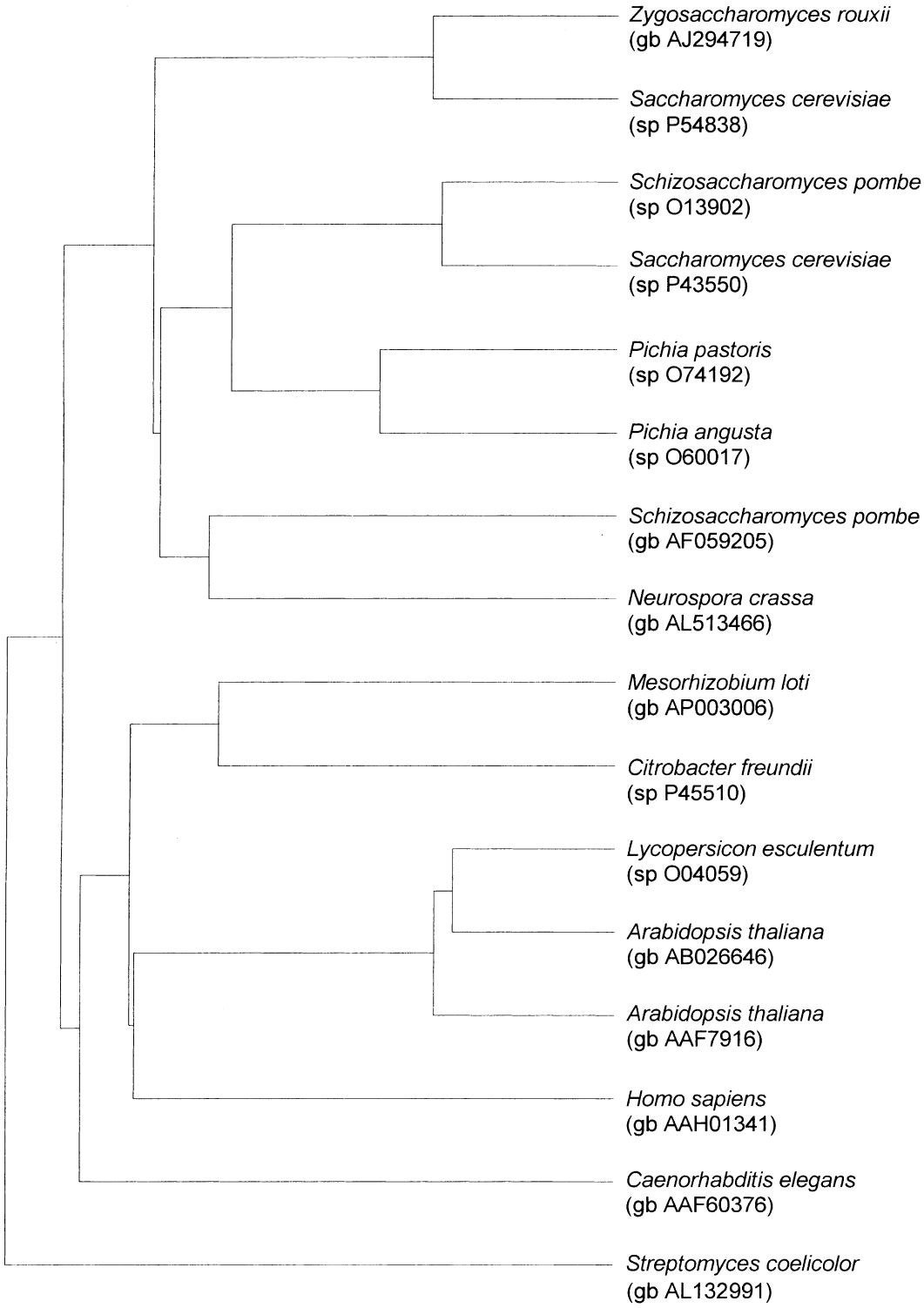

Fig. 3. Sequence comparison of proteins or predicted proteins with similarity to 3,4-dihydroxyacetone kinases from tomato. The dendrogram was constructed with the PILEUP program using the default parameters. The GenBank and Swissprot accession numbers, respectively are indicated in parentheses.

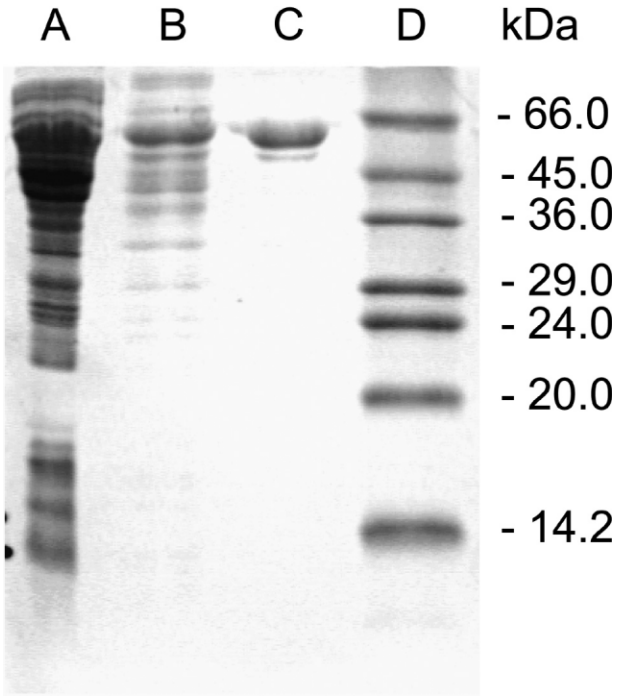

Fig. 4. Purification of the DAK protein from tomato. Lane A, crude cell extract of XL1-pDAK; lane B, DAK protein after anion exchange chromatography with Sepharose Q; lane C, DAK protein after anion exchange chromatography with Mono Q; lane D, molecular weight markers.

Table 1
Purification of recombinant dihydroxyacetone kinase

Procedure	Protein (mg)	Specific activity[a] (μmol min^{-1} mg^{-1})
Cell extract	73	0.06
QA52 cellulose	17	0.28
Mono Q	2.0	0.78

[a] The activity was determined with D-3,4-dihydroxy-2-butanone as substrate.

Table 2
Phosphorylation of different substrates by dihydroxyacetone kinase from tomato at 37 °C

Substrate	Specific activity (μmol min^{-1} mg^{-1})
Dihydroxyacetone	2.6
D-3,4-Dihydroxy-2-butanone	0.78
L-3,4-Dihydroxy-2-butanone	0.04
D,L-Glyceraldehyde	0.13
Hydroxyacetone	0.06
D-Erythrose	0.12
L-Erythrulose	0.07
D-Ribulose	0.03
D-Ribose	0.31
D-Xylulose	0.24
Glycerol	<0.01
Erythritol	<0.01
D-Threitol	<0.01
L-Threitol	<0.01

Enzyme activity was determined with the photometric assay described under Experimental.

instead of riboflavin for growth since diacetyl **9** condenses spontaneously with **5** under formation of **6** (Fig. 5) (Plaut and Harvey, 1971; Bacher and Mailänder, 1978). Quantitative growth experiments suggested that the yeast extract used for the preparation of LB medium used in this study contained approximately 1 mg of 3,4-dihydroxy-2-butanone per g of dry powder.

3. Discussion

The *dak* gene of tomato specifies a kinase accepting a variety of simple carbohydrates as substrate. The presence of a carbonyl group and a chain length of 3–5 appears to be required for substrate activity. The recombinant gene could be expressed efficiently in a bacterial host strain.

Two putative orthologs of this were found on chromosomes 1 and 3 of *Arabidopsis thaliana*, and a putative ortholog fragment was reported from *Glycine maxima*. Putative orthologs were also found in the human genome and in *Caenorhabiditis elegans* but not in *Drosophila melanogaster*. The yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* each contain two putative orthologs. Genes with similarity to the tomato *dak* gene over its full length were also found in the eubacteria *Citrobacter freundii*, *Mesorhizobium loti* and *Streptomyces coelicolor* (Fig. 3). The two DAK proteins of the eubacteria *Citrobacter freundii* and *Mesorhizobium loti* are surprisingly similar to the orthologous proteins of the plants *L. esculentum* and *A. thaliana* (about 43% identity). This may reflect a horizontal transfer of plant genes to plant-associated microorganisms.

The DAK protein of tomato shows a significant degree of internal sequence similarity between the *N*-terminal and *C*-terminal parts (34 identical amino acids equivalent to about 17% identity) (Fig. 6). This suggests tentatively that the peptide folds into two domains with similar topology. This hypothesis is well in line with the finding that substantially shorter genes with similarity to the 5′ part of the plant *dak* genes are found in numerous eubacteria where they have been annotated as putative dihydroxy acetone kinases. The second putative domain of the eukaryotic *dak* kinases is a putative phosphatase (see Conserved Domain Database at http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml).

The expression of dihydroxyacetone kinase from *S. cerevisiae* specified by *dak1* increases fourfold under

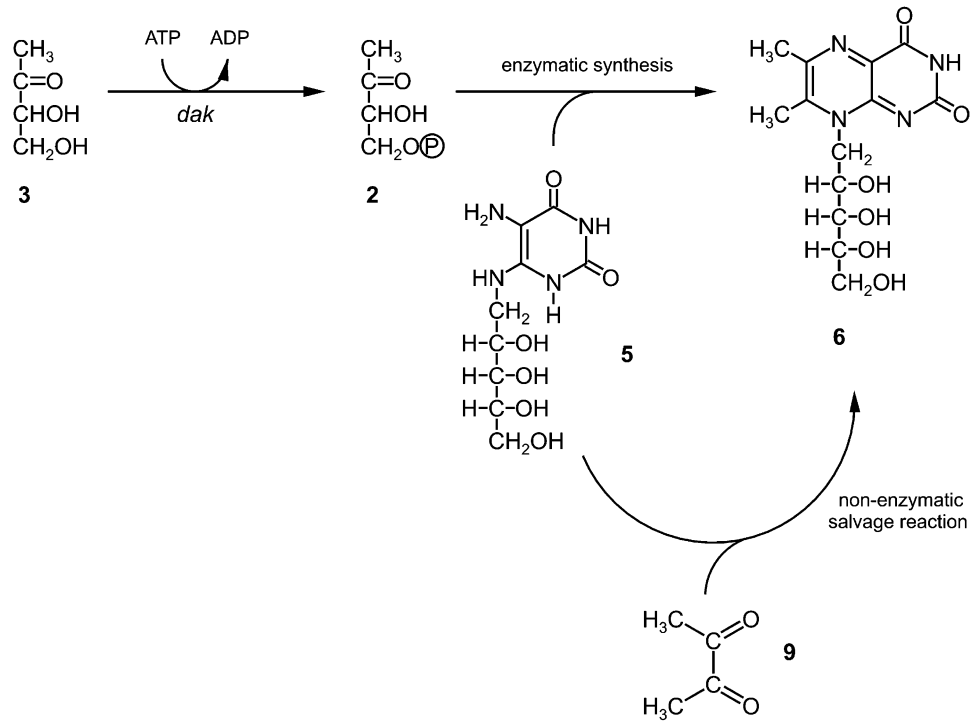

Fig. 5. Formation of 3,4-dihydroxy-2-butanone 4-phosphate (**2**) from 3,4-dihydroxy-2-butanone (**3**) under catalysis of dihydroxyacetone kinase and detection of **2** as lumazine **6**.

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  1 MDFQSKKLINDPNDVVTEFIEGLIENYPGLQYLDGFPEVKVVLRADVSGAKYDKVAIISG  60
395           VNLRDNLNEWDNKVGDGDCGSTMFRG--AVAILEDMKKYYPLNDPAETVNE 443

 61 GGSGHEPAHAGFVGEGMLTAAICGDVFA--SPNVDSILAGIRAVTGPMGCLLIVKNYTGD 118
444 IGASIGRVMGG--TSGILYSIFCKAAYAKLKENAESVVTAIHWADALEAAIAAVSKYGGA 502

119 RLNFGLAAEQAKSEGYKVEM-VIVGDDCALPPPRGIAGRRGLAGTLLVHKVAGAAAACGL 177
503 ------------SAGYRTLLDALIPALSALKER--LNAGDDPADAFII--SAEAASAGAE 545

178 PLADVAAEAKRASEMVGTMGVALSVCTSPGQVTSDRLGPGKMELGLGIHGEPGAAVADLQ 237
546 STKHMQAQAGRSTYVPG--DILASVPDPGAMAAAAWYRAAALAVKEKYNTA\\         594

238 PVDVVVSHVLKEILSPETNYVPITRGSRVVLLINGLGATPLMELMIIAGKAVPELQLEHG 297
298 LAVDRVYTGSFMTSLDMAGFSISVMKADQAILDRLDAPTKAPNWPVGAEGNRPPAKIPVP 358
359 LPPSHSIKIEKTLSRPEKLSPQGHILETAIEAAATEV                         394
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Fig. 6. Internal sequence homology of the kinase (*N*-terminus) and putative phosphatase (*C*-terminus) domain of dihydroxyacetone kinase from tomato. The sequences were aligned with the PILEUP program using the default parameters. Identical residues were shown in inverse contrast, similar residues were shaded in grey.

osmotic stress (Norbeck and Blomberg, 1997). It was also reported that the accumulation of glycerol is the main response to osmotic stress in *S. cerevisiae* (Ölz et al., 1993). Therefore, it has been proposed that the enzyme is required for glycerol catabolism and thus regulates the glycerol concentration in yeast cells (Norbeck and Blomberg, 1997). On the hypothesis, that the mechanism for osmotolerance is conserved in eukaryotes it can be suggested, that the plant homolgs have similar functions (Lee et al., 1999).

The outcome of the complementation experiment reported in this paper is best explained by the hypothesis that the yeast extract used for the preparation of the culture medium contained an appreciable amount of 3,4-dihydroxy-2-butanone as estimated by bioassay with the recombinant *E. coli* strain XL1-pDAK. 3,4-Dihydroxy-2-butanone did not originate during heat sterilization of the medium since similar results were obtained with autoclaved and sterile-filtered medium, respectively.

Table 3
Bacterial strains and plasmids used

Strain/plasmid	Relevant characteristics	Reference
Strains		
E. coli XL1-Blue	recA1,endA1,gyrA96,thi-1,hsdR17,supE44,relA1, lac [F′,proAB,lacI^{q}ZM15,Tn10(tet^r]]	Stratagene, La Jolla, USA
Rib 5, Rib 10	*F*$^-$, *thi*$^-$, *leu*$^-$ *pro*$^-$, *lac*$^-$, *gal*$^-$, *ara*$^-$, *mtl*$^-$, *xyl*$^-$, *supE44*, *endA*$^-$, *hsd*(*r*$^-$,*m*$^-$), *pheS*, *recA*$^-$, *ribB*	Richter et al., 1993; Katzenmeier, 1991
Plasmids		
pBluescript II SK$^-$	High-copy-number phagemid vector	Stratagene, La Jolla, USA
p10–20	pBluescriptIISK- with 2.2 kb fragment of tomato DNA	This study
pDAK	pNCO113 with *dak*-gene (tomato)	This study
pNCO113	Expression vector	Richter et al., 1997

Table 4
Oligonucleotides used in this study

Designation	Sequence
MU	5′-CGCAGCTGCTCTGTACCAC-3′
MR	5′-CTGACTGCAGCTATCTGTGGG-3′
MU2	5′-CATGGTCTTCAAGTATAGCCAC-3′
MR2	5′-CACGCTTCTTGTTCACAAGGTTG-3′
MU3	5′-CATTAATCAGGAGCACGACTCTAC-3′
MR3	5′-GGCTTTTCAATTTCGGTAATGAAGG-3′
MU4	5′-GCAGTCAAACCAAAATTCAGTCG-3′
MR4	5′-GCGGTATCTTATACAGTATATTCTG-3′
DHK1	5′-GAGGAGAAATTAACTATGGATTTTCAGAGCAAAAAAC-3′
DHK2	5′-TATTATGGATCCTTATGCTGTAATTATACTTCTCC-3′
BSECORI	5′-CAATTTGAATTCATTAAAGAGGAGAAATTAACTATG-3′

A literature search for the occurrence of 3,4-dihydroxy-2-butanone in natural materials retrieved no results except for cigarette smoke condensate (Schumacher et al., 1977), and the origin of 3,4-dihydroxy-2-butanone which appears to be present in yeast extract remains unknown. An anaerobic fermentation process similar to those affording butanediol, 3-hydroxy-2-butanone and propanediol can be considered. The recombinant *E. coli ribB* mutant carrying the pDAK plasmid could be used to screen for the formation of 3,4-dihydroxy-2-butanone by a simple bioassay.

4. Experimental

4.1. Materials

5-Amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione (**5**) was freshly prepared by catalytic hydrogenation of 5-nitro-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione (Cresswell and Wood, 1960). DL-, D- and L-3,4-dihydroxy-2-butanone (**3**) and 6,7-dimethyl-8-ribityllumazine (**6**) were prepared as described (Fischer et al., 1937; Kis et al., 1995; Plaut and Harvey, 1971). Recombinant 6,7-dimethyl-8-ribityllumazine synthase of *Bacillus subtilis* was prepared by published procedures (Bacher et al., 1997b). Oligonucleotides were custom-synthesized by MWG Biotech, Ebersberg, Germany. Polyols and carbohydrates were purchased from Fluka (Buchs, Switzerland) and Sigma (Deisenhofen, Germany).

4.2. Bacterial strains, media and plasmids

Bacterial strains and plasmids used in this study are summarized in Table 3. *E. coli* strains were cultured in Luria Bertani (LB) medium or minimal medium (Sambrook et al., 1989) containing 10 mg l^{-1} thiamine, 50 mg l^{-1} leucine and 50 mg l^{-1} proline at 37 °C. Ampicillin was added at a concentration of 150 mg l^{-1} and riboflavin at a concentration of 400 mg l^{-1} (dissolved at 65 °C) as required. A cDNA library of tomato flowers (Schmid et al., 1992) was a gift of J. Schmid.

4.3. Isolation of tomato DNA

Lycopersicon esculentum pulp (5 g) was powdered in liquid nitrogen. The frozen powder was suspended in

30 ml of 100 mM Tris–hydrochloride, pH 8.0, containing 50 mM EDTA, 500 mM NaCl and 10 mM 2-mercaptoethanol. SDS-solution (20%, 2 ml) was added, and the mixture was incubated for 10 min at 65 °C. After the addition of 10 ml of 5 M potassium acetate, the mixture was incubated for 20 min at 0 °C. The suspension was centrifuged and passed through a 0.2 μm filter. DNA was precipitated with 20 ml *iso*-propanol and redissolved in 1.4 ml of 50 mM Tris–hydrochloride containing 10 mM EDTA. The solution was centrifuged, and DNA was precipitated by the addition of 0.15 ml of 3 M sodium acetate and 1 ml of *iso*-propanol to the supernatant. The pellet was washed twice with 70% ethanol and redissolved in 0.2 ml redistilled H_2O.

4.4. Plasmid isolation

Plasmid DNA was isolated from 5 ml of fresh overnight culture using the mini plasmid isolation kit from Quiagen (Hilden, Germany).

4.5. DNA sequence determination

Sequencing was performed by the automated dideoxynucleotide method (Sanger et al., 1977) using an ABI Prism 377 DNA sequencer from Perkin Elmer (Foster City, USA) with the ABI Prism Sequencing Analysis Software.

4.6. Construction of an expression clone

A segment of plasmid p10–20 was amplified by PCR using the primers DHK1 and DKH2 (Table 4). The amplificate served as template in a second PCR amplification using the oligonucleotides BSECORI and DHK2 as primers. The amplificate was treated with *BamH*1 and *EcoR*I. The DNA fragment was purified using the PCR purification kit from Qiagen and was then ligated into the expression vector pNCO113 which had been pretreated with the same restriction enzymes. The resulting plasmid pDAK was transformed into *E. coli* XL1-Blue yielding strain XL1-pDAK.

4.7. Protein purification

The recombinant *E. coli* strain XL1-Blue harboring plasmid pDAK was grown at 37 °C in 2 l shaking flasks containing 0.5 l of LB medium containing 75 mg of ampicillin. At an optical density of 0.8 (600 nm), isopropylthiogalactopyranoside was added to a final concentration of 1 mM. The suspension was incubated with shaking at 37 °C for 5 h. The cells were harvested by centrifugation and were washed with 0.9% NaCl. Wet cell mass (2 g) was suspended in 10 ml of 20 mM Tris–hydrochloride, pH 8.5, containing 6 mM phenylmethanesulfonyl fluoride and 1 mg of lysozyme. The suspension was incubated for 1 h at 37 °C, cooled on ice, disrupted by sonication, and centrifuged. The supernatant was applied to an anion exchange column Sepharose Q (Amersham Pharmacia Biotech; volume, 30 ml) which had been equilibrated with 20 mM Tris–hydrochloride, pH 8.5. The column was developed with a gradient of 0 to 1 M NaCl containing 20 mM Tris–hydrochloride, pH 8.5. Fractions were collected, desalted with a Highprep 26/10-column (Amersham Pharmacia Biotech) and applied to a Mono Q column (Amersham Pharmacia Biotech; volume, 35 ml) which had been equilibrated with 20 mM Tris–hydrochloride, pH 8.5. The column was developed with a gradient of 0 to 1 M KCl containing 20 mM Tris–hydrochloride, pH 8.5. Fractions of the protein which appeared at a gradient concentration of about 250 mM were collected and concentrated by ultrafiltration using 30 kDa membranes (Pall, Ann Arbor, MI, USA).

4.8. Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed as described by Lämmli (1970).

4.9. Fluorimetric assay of 3,4-dihydroxy-2-butanone kinase activity of dihydroxyacetone kinase

Assay mixtures contained 250 mM K_2HPO_4, pH 7.5, 10 mM $MgCl_2$, 16 mM ATP, 20 mM DTT, 0.9 mM **5**, 6.7 mM 3,4-dihydroxy-2-butanone, and 90 μg of recombinant lumazine synthase from *B. subtilis* in a total volume of 75 μl. The assay was started by the addition of protein solution (25 μl), and the mixture was incubated at 37 °C. Aliquots (10 μl) were retrieved at intervals, and 15% trichloroacetic acid was added to a final volume of 100 μl. The samples were analyzed by reversed-phase HPLC using a column of Nucleosil RP18 (4×300 mm). The eluent contained 30 mM formic acid and 10% methanol. The effluent was monitored fluorimetrically (excitation, 408 nm; emission, 487 nm); synthetic **6** was used as standard.

4.10. Photometric assay of polyol kinase activity of dihydroxyacetone kinase

Assay mixtures contained 50 mM Tris–hydrochloride, pH 8.0, 50 mM KCl, 1 mM EDTA, 4 mM $MgCl_2$, 1 mM phosphoenol pyruvate, 0.5 mM ATP, 0.12 mM NADH, 5 U (μmol min^{-1}) lactate dehydrogenase, 5 U pyruvate kinase and 2 mM substrate in a total volume of 1 ml at 37 °C. The assay was started by the addition of 10 μl of protein solution (18 μg) and the absorbance at 340 nm was recorded ($\varepsilon_{NADH} = 6{,}220\ M^{-1}\ cm^{-1}$). Control experiments were performed without addition of substrate.

Acknowledgements

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