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RESEARCH ARTICLE

Genetic analysis of coenzyme A biosynthesis in the yeast *Saccharomyces cerevisiae*: identification of a conditional mutation in the pantothenate kinase gene *CAB1*

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Abstract Coenzyme A (CoA) is a ubiquitous cofactor required for numerous enzymatic carbon group transfer reactions. CoA biosynthesis requires contributions from various amino acids with pantothenate as an important intermediate which can be imported from the medium or synthesized de novo. Investigating function and expression of structural genes involved in CoA biosynthesis of the yeast *Saccharomyces cerevisiae,* we show that deletion of *ECM31* and *PAN6* results in mutants requiring pantothenate while loss of *PAN5* (related to *panE* from *E. coli*) still allows prototrophic growth. A temperature-sensitive mutant defective for fatty acid synthase activity could be functionally complemented by a gene significantly similar to eukaryotic pantothenate kinases (*YDR531W*). Enzymatic studies and heterologous complementation of this mutation by bacterial and mammalian genes showed that *YDR531W* encodes a genuine pantothenate kinase (new gene designation: *CAB1*, "coenzyme A biosynthesis"). A G351S missense mutation within *CAB1* was identified to cause the conditional phenotype of the mutant initially studied. Similar to *CAB1*, genes *YIL083C*, *YKL088W*, *YGR277C* and *YDR106C* responsible for late CoA biosynthesis turned out as essential. Null mutants could be complemented by their bacterial counterparts *coaBC*, *coaD* and *coaE*, respectively. Comparative expression analyses showed that some CoA biosynthetic genes are weakly de-repressed with ethanol as a carbon source compared with glucose.

Keywords Coenzyme A · Pantothenate · Pantothenate kinase · *Saccharomyces cerevisiae*

Introduction

Coenzyme A (CoA) is a ubiquitous co-substrate for a large number of enzymes involved in the transfer of acyl groups. Acetyl-CoA as the most important thioester derivative at the sulfhydryl group of CoA plays a central role for the oxidative degradation of sugars and fatty acids via the tricarboxylic acid cycle but is also indispensable for anabolic pathways such as fatty acid biosynthesis, sterol biosynthesis, formation of ketone bodies in mammals and gluconeogenesis from C_2 substrates in many microorganisms. For biosynthesis of CoA, pantothenate (vitamin B_5) is a general intermediate that may be formed de novo from several amino acids or can be taken up from outside the cell, using a specific permease. Once inside the cell, five reactions are required to convert pantothenate into CoA (reviewed by Leonardi et al. 2005).

The yeast *Saccharomyces cerevisiae* is able to synthesize pantothenate de novo (White et al. 2001) although growth of some laboratory strains requires pantothenate supplementation (Stolz and Sauer 1999). Although biosynthesis of pantoate in *S. cerevisiae* presumably follows the pathway established in bacteria (structural genes *panB* and *panE*), a homolog of aspartate decarboxylase (*panD* in *E. coli*) providing β-alanine is absent from yeast. Instead, β-alanine is obtained by oxidative conversion of polyamines (White et al. 2001, 2003; summarized in Fig. 1). The use of external

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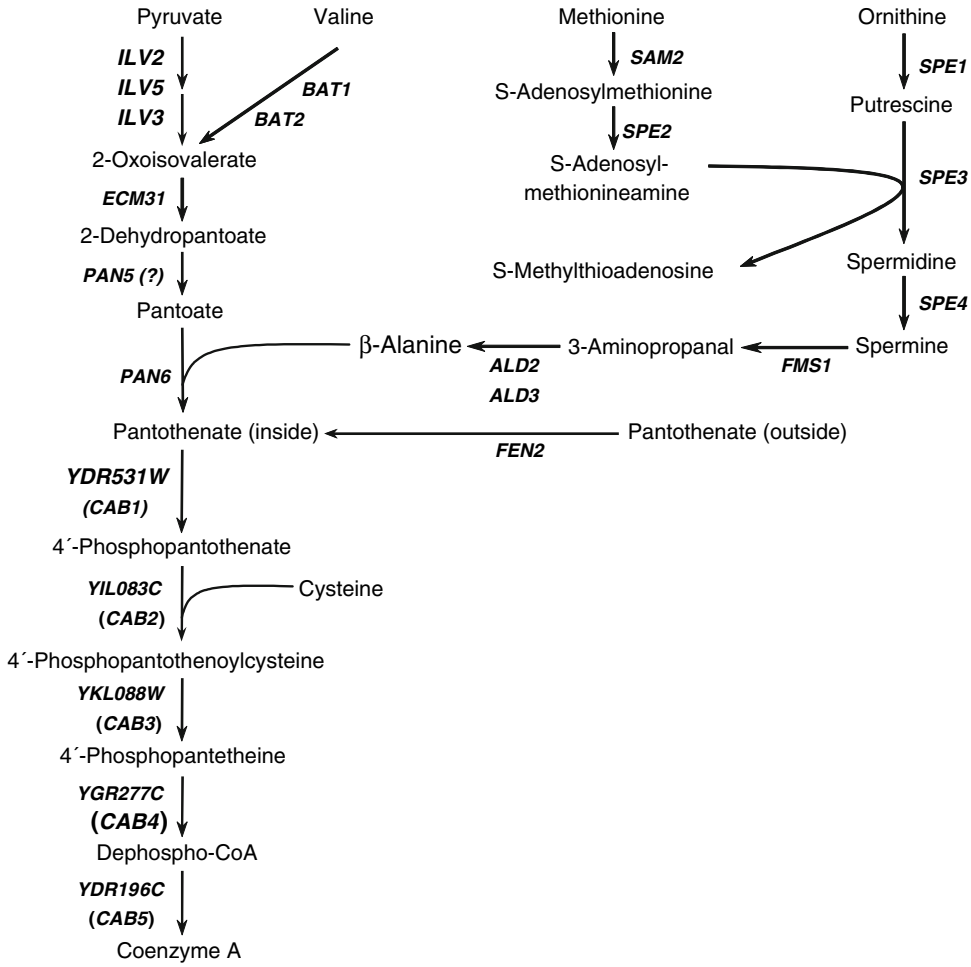

Fig. 1 Outline of coenzyme A biosynthesis in the yeast *S. cerevisiae*

pantothenate requires the high-affinity transporter of the plasma membrane, Fen2 (Stolz and Sauer 1999).

To finally synthesize CoA, pantothenate must react with cysteine and ATP. As an initial step, pantothenate is phosphorylated by the ATP-dependent pantothenate kinase (panK; encoded by *coaA* in *E. coli*) which is considered as the rate-limiting enzyme for the entire pathway. Indeed, CoA could competitively inhibit panK activity in *E. coli* presumably by its interference with ATP binding (Vallari et al. 1987) while acylated CoA thioesters were less efficient. In contrast to bacteria, mammalian panK enzymes are strongly inhibited by acetyl-CoA (and malonyl-CoA, although less effective) while CoA, surprisingly, is a mild activator (Rock et al. 2000). In the following reaction, 4′-phosphopantothenate forms an amide bond with cysteine which is subsequently decarboxylated to give 4′-phosphopantetheine. The nucleotide moiety of CoA is then provided by ATP and the resulting dephospho-CoA finally needs to be phosphorylated. Interestingly, an alternative pathway catalyzed by the CoA-synthesizing protein complex CoA-SPC of 400 kDa has been postulated for yeast (Bucovaz et al. 1980, 1997), proposing an early transfer of the ADP moiety directly to pantothenate. While this pathway would bypass the need of 4′-phosphopantothenate, genomic data from yeasts and fungi support the existence of genuine panK enzymes (Calder et al. 1999).

Completion of the mammalian pathway of CoA biosynthesis by comparitive genomics (Daugherty et al. 2002) allowed the identification of genes encoding 4′-phosphopantothenoylcysteine synthetase (PPCS), 4′-phosphopantothenoylcysteine decarboxylase (PPCDC), 4′-phosphopantetheine adenylyltransferase (PPAT) and dephospho-CoA kinase (DPCK) for which putative homolog exist in *S. cerevisiae*. These findings suggest that biosynthesis of CoA follows a uniform pathway in all living systems. Not only CoA as a mobile cofactor is essential for metabolism but also its phosphopantethein component which becomes covalently linked to eukaryotic fatty acid synthases (Fichtlscherer et al. 2000).

In this work, we analyzed biosynthesis of CoA in the yeast *S. cerevisiae* by genetical and biochemical methods. Focusing on yeast panK, we characterized a temperature-sensitive mutant which has been previously isolated in a screen for fatty acid-requiring mutants. We could identify a missense mutation at a completely conserved residue within panK causing this defect.

Materials and methods

Strains of *S. cerevisiae* and *E. coli*, media and growth conditions

All strains of *S. cerevisiae* used in this work (compiled in Table 1) are isogenic to strain JS91.15-23. Synthetic complete (SC) media used for selective growth of transformants have been described (Schüller et al. 1992). To obtain selective medium lacking pantothenate (SCD-Pan), a mixture of pure substances composed identical to yeast nitrogen base (Invitrogen) was used. Strains were incubated at 30 or 37°C as indicated.

For bacterial expression of the *GST-CAB1* fusion gene, strain BL21-CodonPlus (Stratagene/Agilent) grown in YT-G and supplemented with ampicillin and chloramphenicol was used.

Plasmid constructions and site-directed mutagenesis

Plasmids constructed and used for this work are listed in Table 2. The murine panK3 cDNA clone was purchased from OriGene via AMS Biotechnologie (Wiesbaden, Germany). To overexpress and epitope-tag CoA biosynthetic genes, *MET25*-containing vector p426-MET25HA (2 µm *URA3* $MET25_{prom}$-HA_3; Mumberg et al. 1994) was used. Bacterial genes *coaA*, *coaBC*, *coaD* and *coaE* were amplified using gene-specific primers and subsequently cloned into p426-MET25HA. To achieve a regular gene dosage, plasmid YCplac33 (Gietz and Sugino 1988) was used. Reporter gene fusions for genes of pantothenate and CoA biosynthesis were constructed using YEp356 and related vectors (Myers et al. 1986). For molecular characterization of the $cab1^{ts}$ mutant allele, primers CAB1-Bam and CAB1-Hind were used to amplify the reading frame of *YDR531W/CAB1*. The PCR product obtained was cloned into pUC19 to give pJO19. Plasmids from two independent amplifications were subsequently used for DNA sequencing. The *ydr531w*(G351S) missense mutation was introduced into the coding region of wild-type *YDR531W* using the QuikChange site-directed mutagenesis kit (Stratagene/Agilent). The desired mutant allele in the resulting plasmid pJO62 was confirmed by DNA sequencing.

For bacterial expression of GST fusion genes by IPTG induction, derivatives of pGEX-2TK (GE Healthcare) were used.

Enzyme assays

Assay of pantothenate kinase followed the procedure previously described by Vallari et al. (1987). In brief, D-[1-^{14}C] pantothenate (^{14}C-pan; supplied by Biotrend, Cologne, Germany) was converted into phosphopantothenate which was subsequently bound to ion-exchange filter and analyzed by liquid scintillation counting. 5500 Bq of ^{14}C-pan (2.75 nmol) were incubated in buffer (100 mM Tris/HCl, 2.5 mM $MgCl_2$, 2.5 mM ATP, pH 7.4) with 75 µg of total protein in a volume of 40 µl. After incubation at 30 or 37°C for 10 min, the mixture was transferred to a DE-81 ion-exchange filter disk and washed three times with 1% acetic acid in technical ethanol. Dried filter disks were transferred into scintillation solution (Beckman Ready-Solv MP) and

Table 1 Strains of *S. cerevisiae*

Strain	Genotype
JOY1	*MATa/MATα ura3/ura3 his3/his3 trp1/TRP1 leu2/LEU2 Δyil083c::HIS3/ YIL083C*
JOY2	*MATa/MATα ura3/URA3 his3/his3 leu2/LEU2 YDR531W/cab1*^{ts}
JOY2D	*MATa/MATα ura3/URA3 his3/his3 leu2/LEU2 Δydr531w::HIS3/cab1*^{ts}
JS91.14-24	*MATa ura3 his3 cab1*^{ts}
JS91.15-23	*MATα ura3 leu2 trp1 his3*
JS42	*MATα ura3*
JS01.3	MAT*a/MATα ura3/ura3 his3/his3 leu2/LEU2 trp1/TRP1*
JS07.1-6	*MATa ura3 leu2 his3 Δfen2::LEU2*
JWH2	*MATα ura3 leu2 trp1 his3 Δpan6::HIS3*
JWH3	*MATα ura3 leu2 trp1 his3 Δecm31::HIS3*
KLY4-17	*MATα ura3 leu2 trp1 his3 Δpan5::HIS3*
KLY5 + pKL7	*MATα ura3 leu2 trp1 his3 Δydr531w::HIS3 + ARS CEN URA3 GAL1-CAB1*
KLY6	*MATα ura3 leu2 trp1 his3 Δpan5::HIS3 Δydl144c::kanMX*
KLY16	MAT*a/MATα ura3/ura3 his3/his3 leu2/LEU2 trp1/TRP1 Δykl088w::HIS3/ YKL088W*
SBY1	*MATa/MATα ura3/ura3 his3/his3 leu2/LEU2 trp1/TRP1 Δygr277c::HIS3/ YGR277C*
SBY2	*MATa/MATα ura3/ura3 his3/his3 leu2/LEU2 trp1/TRP1 Δydr196c::HIS3/ YDR196C*
SSH1	*MATa/MATα ura3/ura3 his3/his3 leu2/LEU2 trp1/TRP1 Δydr531w::HIS3/ YDR531W*

Table 2 Plasmids used for this work

Plasmid	Genotype
(a) Gene disruption constructs	
pWJ2	Δ*ecm31::HIS3*
pWJ11	Δ*pan5::HIS3*
pKL9	Δ*ydl144c::kanMX*
pCW118	Δ*pan6::HIS3*
pKH3	Δ*fen2::LEU2*
pSBS7	Δ*ydr531w::HIS3* (Δ*cab1*)
pJO9	Δ*yil083c::HIS3*
pKL24	Δ*ykl088w::HIS3*
pSB2	Δ*ygr277c::HIS3*
pSB5	Δ*ydr196c::HIS3*
(b) Expression plasmids	
pJO57	*ARS CEN URA3 CAB1*
pJO62	*ARS CEN URA3 cab1*(G351S)
pKL7	*ARS CEN URA3 GAL1-HA$_3$-CAB1*
pSBS5	2 μm *URA3 MET25-HA$_3$-CAB1*
pFK1	2 μm *URA3 MET25*-HA$_3$-*coaA*
pJO73	2 μm *URA3 MET25*-HA$_3$-*panK3*
pJO74	2 μm *URA3 MET25*-HA$_3$-*coaBC*
pJO75	2 μm *URA3 MET25*-HA$_3$-*coaD*
pJO76	2 μm *URA3 MET25*-HA$_3$-*coaE*
pJO72	2 μm *URA3 MET25*-HA$_3$-*YGR205W*
pJO1	2 μm *URA3 MET25*-HA$_3$-*YIL083C*
pJO3	2 μm *URA3 MET25*-HA$_3$-*YKL088W*
pJO2	2 μm *URA3 MET25*-HA$_3$-*SIS2*
pJO26	2 μm *URA3 MET25*-HA$_3$-*VHS3*
pSB3	2 μm *URA3 MET25*-HA$_3$-*YGR277C*
pSB6	2 μm *URA3 MET25*-HA$_3$-*YDR196C*
pJO59	tac$_{prom}$-*GST-CAB1*
pJO68	tac$_{prom}$-*GST-coaA*
(c) Reporter gene fusions	
pWJ6	2 μm *URA3 ECM31-lacZ*
pWJ8	2 μm *URA3 PAN6-lacZ*
pJO60	2 μm *URA3 CAB1-lacZ*
pJO29	2 μm *URA3 YIL083C-lacZ*
pJO48	2 μm *URA3 YKL088W-lacZ*
pJO58	2 μm *URA3 YGR277C-lacZ*
pJO51	2 μm *URA3 YDR196C-lacZ*

analyzed with a Perkin Elmer Packard Tri-Carb 2900TR scintillation counter. Assay of β-galactosidase activities has been previously described (Schwank et al. 1995).

Miscellaneous procedures

Transformation of *S. cerevisiae* and PCR amplification have been previously described (Schwank et al. 1995). DNA sequencing was performed by Agowa (Berlin, Germany).

Results

Genes of *S. cerevisiae* involved in pantothenate biosynthesis

De novo biosynthesis of pantothenate utilizes derivatives of amino acid metabolism, 2-oxoisovalerate and β-alanine (cf. Fig. 1). Three enzymes are specifically required for the conversion of these molecules into pantothenate, 2-oxoisovalerate hydroxymethyltransferase, dehydropantoate reductase and pantoate-β-alanine ligase which are encoded by genes *panB*, *panE* and *panC* in *E. coli*, respectively. Since *ECM31* (=*YBR176W*) of *S. cerevisiae* shows significant similarity to *E. coli panB* (36.4% identity, 55% similarity), we constructed a deletion mutant (strain JWH3, Δ*ecm31::HIS3*) which indeed failed to grow on a selective medium lacking pantothenate (Fig. 2; also shown by White et al. 2001). The same result was obtained with a null mutant defective for *PAN6* (=*YIL145C*; strain JWH2, Δ*pan6::HIS3*) which is similar to *panC* from *E. coli* (40% identity, 52% similarity).

In contrast, a null mutant lacking *PAN5* (=*YHR063C*; strain KLY4-17, Δ*pan5::HIS3*) which is similar to *E. coli* dehydropantoate reductase gene *panE* (22.8% identity, 40% similarity) was still able to grow in the absence of pantothenate, although slightly less efficient than the wild-type (cf. Fig. 2). We thus reasoned that a second gene encoding an isoenzyme may exist in *S. cerevisiae*. Indeed, the gene product of *YDL144C* is also similar to *E. coli panE* over its entire length (21.4% identity, 33.3% similarity). However, even a strain with a double deletion (KLY6, Δ*pan5::HIS3* Δ*ydl144c::kanMX*) was not auxotrophic for pantothenate (Fig. 2). Identical results were obtained with double deletion mutations introduced into other strain backgrounds

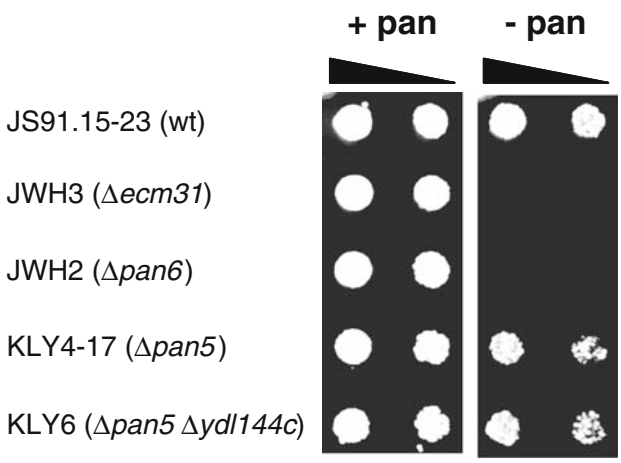

Fig. 2 Growth phenotype of mutants involved in biosynthesis of pantothenate. Serial dilutions of mutant strains were transferred to synthetic complete media with or without pantothenate (+pan, −pan)

(not shown). We conclude that the remaining dehydropantoate reductase is encoded by a *S. cerevisiae* gene unrelated in sequence to bacterial enzymes.

Mutations *pan6* and *fen2* show synthetical lethality

Owing to the existence of the high-affinity pantothenate transporter Fen2 (Stolz and Sauer 1999), biosynthetic mutants of *S. cerevisiae* can utilize external pantothenate. We thus wished to investigate whether a *pan6 fen2* double mutant is still viable. After mating of haploid single mutants JWH2 (Δ*pan6::HIS3*) and JS07.1-6 (Δ*fen2::LEU2*) and subsequent sporulation of the resulting diploid strain no viable progeny with a His^+ Leu^+ phenotype could be observed (18 tetrads). Media for sporulation, spore germination, and phenotypic characterization of ascospores were supplemented with an excess of pantothenate (50 μM). We, thus, conclude that *pan6* and *fen2* are synthetically lethal. Our data also argue against the existence of a low-affinity pantothenate transporter and suggest that passive diffusion of pantothenate across the membrane does not occur.

Characterization of a temperature-sensitive mutant defective for pantothenate kinase activity

A previous screen for mutants defective for fatty acid synthase (FAS) activity also led to the identification of strains containing full-length FAS subunits α and β but were devoid of pantetheine (Schweizer et al. 1973). Strains carrying the *cab1*^{ts} mutation (for explanation of the gene designation see below) show regular growth at 30°C but fail to proliferate at 37°C. The temperature-sensitive mutant JS91.14-24 was used to isolate the wild-type gene which could restore growth at 37°C. Plasmids obtained by functional complementation all contained the *APA2-YDR531W* gene pair. Since *YDR531W* encodes a protein with significant similarity to the pantothenate kinase of *A. nidulans* (43.8% identity, 55% similarity; not shown), failure to synthesize CoA may cause the mutant phenotype. Indeed, plasmid pSBS5 containing the coding region of *YDR531W* under control of the *MET25* promoter could also complement the temperature-sensitive phenotype of strain JS91.14-24 (Fig. 3a). However, this finding could be also the result of dosage-dependent suppression of a mutation in a distinct gene.

We thus constructed a null mutant allele (Δ*ydr531w::HIS3*; in plasmid pSBS7) which was subsequently introduced into the diploid strain JS01.3 (*his3/his3 YDR531W/YDR531W*). Following sporulation of the resulting transformant SSH1 (containing a single mutant allele Δ*ydr531w::HIS3*), no viable ascospores with a His^+ phenotype were obtained. Since the same result was obtained

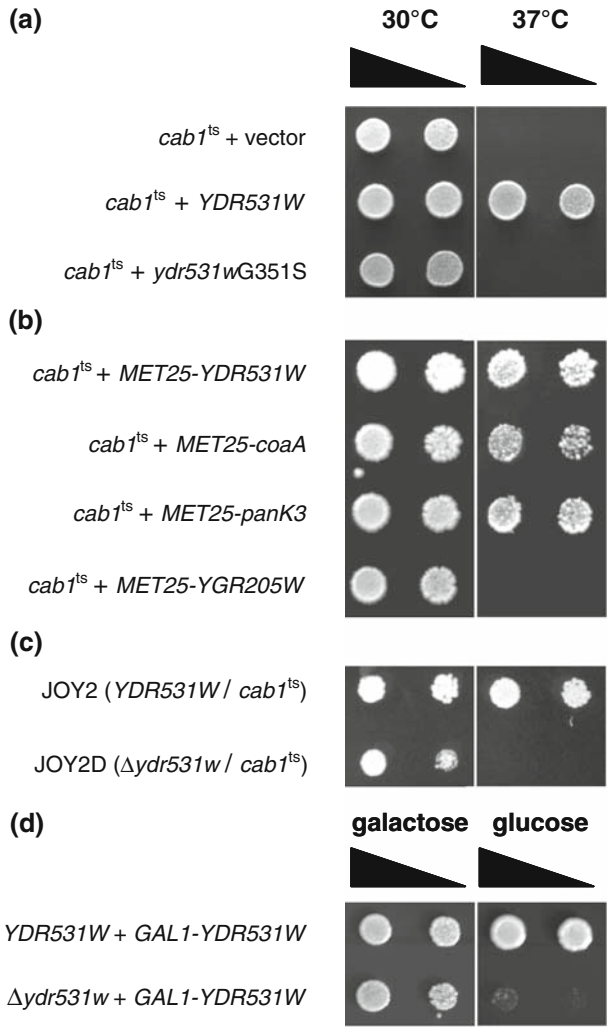

Fig. 3 Complementation of conditional phenotypes of pantothenate kinase mutants. **a** Strain JS91.14-24 (*cab1*^{ts}) was transformed with plasmids pJO57 and pJO62 encoding wild-type *YDR531W* (*CAB1*) and a G351S variant activated by the natural control region (YCp vector). **b** Strain JS91.14-24 (*cab1*^{ts}) was transformed with plasmids pSBS5, pFK1, pJO73 and pJO72 encoding homologous and heterologous panK genes (*coaA*: *E. coli*, *panK3*: *M. musculus*) and the *coaA*-related gene *YGR205W* (*S. cerevisiae*). Genes were activated by the *MET25* promoter (2 μm vector). **c** Test for allelism of *ydr531w* and *cab1*^{ts}. Serial dilutions of strains JOY2 and JOY2D were grown on rich medium (YEPD) at 30 and 37°C, respectively. **d** Carbon source-dependent complementation of *ydr531w* null mutant (strain KLY5) by *GAL1*-activated *YDR531W* (plasmid pKL7)

with a medium containing fatty acids, *YDR531W* is an essential gene, at least under conditions tested. This finding agrees with the data of the yeast systematic gene deletion project (Winzeler et al. 1999; Giaever et al. 2002) and is further supported by the finding that a haploid Δ*ydr531w* mutant transformed with a *GAL1-YDR531W* fusion can grow in galactose-containing medium but not in the presence of glucose (Fig. 3d). We thus repeated the disruption experiment of *YDR531W* with a diploid obtained by mating of JS91.14-24 with a wild-type strain (JOY2; *his3/his3*

YDR531W/*cab1*^{ts}). Considering *cab1*^{ts} as a mutant allele of *YDR531W*, introduction of the *ydr531w* null allele into JOY2 should result in transformants with a temperature-sensitive phenotype. Indeed, the predicted phenotype was observed for strain JOY2D (Δ*ydr531w::HIS3*/*cab1*^{ts}; Fig. 3c), confirming that *cab1*^{ts} is a mutant allele of *YDR531W*.

For a precise mapping of the mutation responsible for the temperature-sensitive phenotype, we amplified the coding region of *YDR531W* using DNA from strain JS91.14-24 as a template. Comparative DNA sequencing revealed the existence of five missense mutations (D208E, N255H, S261P, A327T and G351S). Phylogenetic comparison of pantothenate kinase (panK) sequences from fungal and higher eukaryotic species showed that among these variants G351 is the only residue which is entirely conserved (cf. Fig. 4). We, thus, introduced the G351S mutation into a functional *YDR531W* gene by site-directed mutagenesis. In contrast to the wild-type sequence, the expression of *ydr531w*(G351S) driven by its natural promoter failed to complement the temperature-sensitivity of strain JS91.14-24 (Fig. 3a). We conclude that alteration of the highly conserved residue G351 is indeed responsible for the conditional phenotype caused by the mutant allele studied initially.

Although the *coaA* gene encoding panK in *E. coli* fails to display any significant similarity to the gene product of *YDR531W*, we expressed the bacterial gene in strain JS91.14-24. Interestingly, introduction of a *MET25-coaA* fusion (plasmid pFK1) could substantially restore growth at 37°C (Fig. 3b), supporting *YDR531W* as the genuine panK gene of *S. cerevisiae*. Yeast expression of the murine *panK3* gene (its product is 31.6% identical and 47.7% similar to *YDR531W*) with plasmid pJO73 could functionally complement temperature-sensitivity of strain JS91.14-24 as well. Interestingly, *S. cerevisiae* gene *YGR205W* encodes a protein which is distantly related to bacterial *coaA* (15.2% identity, 29.1% similarity) but resembles prokaryotic pantothenate kinases at the structural level (de La Sierra-Gallay et al. 2004). In contrast to *coaA* of *E. coli*, overexpression of *YGR205W* using multi-copy plasmid pJO72 did not confer growth of strain JS91.14-24 at the non-permissive temperature (Fig. 3b).

As a final proof of function, we assayed panK activity in crude extracts from strain JS91.14-24 and multicopy transformants overexpressing *YDR531W*. As is shown in Table 3, enzyme activity in strain JS91.14-24 was reduced to about 50% of the wild-type level at 30°C and was undetectable at 37°C. The use of the heterologous *MET25* promoter on a multicopy plasmid increased enzyme activity by

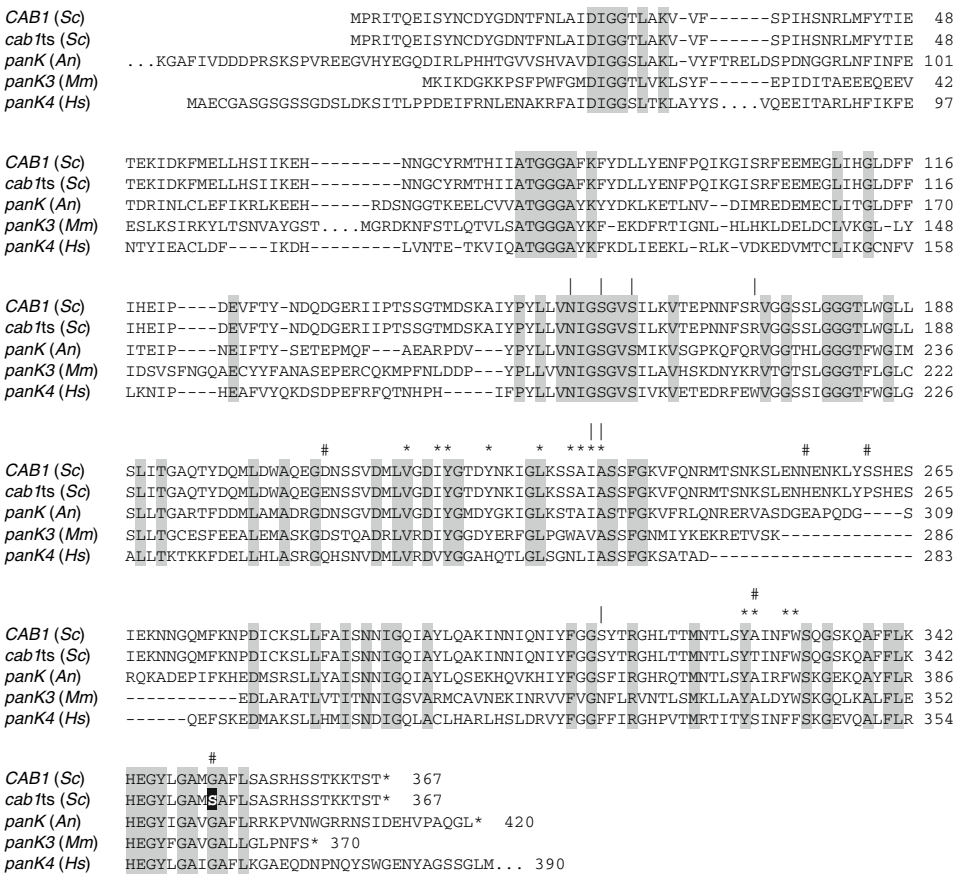

Fig. 4 Sequence comparison of eukaryotic pantothenate kinases. Entirely conserved residues are emphasized by *gray* shading. Missense mutations identified in the *cab1*^{ts} allele are indicated by #. The G351S mutation (depicted inverse) is sufficient to confer the temperature-sensitive phenotype. Residues involved in binding of the competitive inhibitor acetyl-CoA are indicated by *vertical line* (hydrogen bond) or * (hydrophobic interaction). *An Aspergillus nidulans*, *Hs Homo sapiens*; *Mm Mus musculus*, *Sc Saccharomyces cerevisiae*

Table 3 Pantothenate kinase (panK) activity in mutant strain and multicopy transformants of *S. cerevisiae* and *E. coli*

Strain (genotype)	Plasmid (genotype)	Spec. panK activity (cpm/μg)	
		30°C (SD)	37°C (SD)
S. cerevisiae			
JS91.15-23 (wild-type)	p426MET25 (2μm *URA3*)	31 (3)	29 (3)
JS91.14-24 (*cab1*^{ts})	p426MET25 (2μm *URA3*)	15 (2)	b. d. (–)
JS91.15-23 (wild-type)	pSBS5 (2μm *URA3 MET25-YDR531W*)	1,710 (240)	1,520 (220)
JS91.15-23 (wild-type)	pFK1 (2μm *URA3 MET25-coaA*)	120 (20)	95 (20)
E. coli			
BL21	pGEX-2TK (*GST*)	110 (15)	n. t.
BL21	pJO68 (*GST-coaA*)	3,230 (500)	n. t.
BL21	pJO59 (*GST-YDR531W*)	2,870 (450)	n. t.

Strains were grown at 30°C under selective conditions (SCD-Ura or YTG + ampicillin + chloramphenicol)

For each assay, 75 μg of total protein was used

Enzyme assays were performed at the temperature indicated

Enzyme activities are given in cpm 1-^{14}C-phosphopantothenate formed per μg protein within 10 min

b. d. below detection, *n. t.* not tested, *SD* standard deviation

a factor of more than 50. A substantial panK activity was also assayed in yeast transformants expressing bacterial *coaA*, explaining the result of the heterologous complementation experiment described above. Conversely, the expression of *YDR531W* as a GST fusion in *E. coli* similarly increased enzyme activity in protein extracts. From these results, we conclude that *YDR531W* indeed encodes the initial enzyme of coenzyme A biosynthesis from pantothenate and suggest *CAB1* (coenzyme A biosynthesis) as its new adequate gene designation.

Genetic analysis of genes presumably involved in late CoA biosynthesis

Because of sequence similarity, the four remaining steps to finally synthesize coenzyme A from phosphopantothenate may be catalyzed by the products of *S. cerevisiae* genes *YIL083C* (phosphopantothenoylcysteine synthetase, PPCS), *YKL088W* (phosphopantothenoylcysteine decarboxylase, PPCDC), *YGR277C* (phosphopantetheine adenylyltransferase, PPAT) and *YDR196C* (dephospho-CoA kinase, DPCK). Similar to what has been described for *YDR531W*/*CAB1*, null mutant alleles for these four genes were individually introduced into a diploid strain and the heterozygous situation at all four loci could be shown. Following sporulation, no haploid progeny carrying the selection marker used for disruption of the respective locus could be identified, again confirming the results of the systematic gene deletion project (Winzeler et al. 1999; Giaever et al. 2002). Haploid strains with a chromosomal deletion of one the four genes were obtained only in the presence of a plasmid containing the respective wild-type gene.

It should be mentioned that two genes (*VHS3* and *SIS2*/*HAL3*) exhibit significant sequence similarity to *YKL088W*. Vhs3 and Sis2 are highly related to each other over their entire length; both proteins are able to bind to and inhibit the Ser/Thr-specific phosphatase Ppz1 involved in regulation of halotolerance and ion homeostasis. Null mutation *vhs3* and *sis2* are synthetically lethal (Ruiz et al. 2004). Similarity of Ykl088w with Vhs3 and Sis2 is most apparent at the C-terminus of the respective proteins (Ykl088w and Vhs3: 22.2% identity, 37.6% similarity; Ykl088w and Sis2: 20.8% identity, 34.5% similarity over entire length). To assay for dosage-dependent suppression, we introduced multi-copy plasmids containing *MET25* fusions of *VHS3* and *SIS2*, respectively, into a diploid with a heterozygous Δ*ykl088w* null mutation. Following sporulation, we were unable to obtain haploid progeny with a chromosomal Δ*ykl088w* mutation (only 2 viable spores per tetrad). In contrast, introduction of a *MET25-YKL088W* fusion plasmid allowed growth of haploid Δ*ykl088w* spores. We conclude that even at an elevated level of gene expression, *VHS3* and *SIS2* are unable to replace *YKL088W* and may not be considered as genuine genes of coenzyme A biosynthesis.

To obtain further evidence for a function of *YIL083C*, *YKL088W*, *YGR277C* and *YDR196C* in coenzyme A biosynthesis, we assayed for heterologous complementation of mutants by the corresponding bacterial genes. *E. coli* genes *coaBC* (encoding bifunctional PPCS and PPCDC), *coaD* (encoding PPAT) and *coaE* (encoding DPCK) were fused with the *MET25* promoter and subsequently introduced into heterozygous diploids carrying deletion mutations Δ*yil083c*, Δ*ykl088w* (*MET25-coaBC*), Δ*ygr277c* (*MET25-coaD*) and

Table 4 Influence of nutrient variation on the expression of coenzyme A biosynthetic genes

Fusion gene	Specific β-galactosidase activity (U/mg) after growth in			
	SCD-Ura	SCE-Ura	SM	SCD-Ura-Pan
ECM31-lacZ	50 (15)	125 (20)	55 (10)	60 (15)
PAN6-lacZ	90 (20)	230 (40)	110 (25)	95 (15)
CAB1-lacZ	135 (25)	205 (40)	110 (25)	n. t.
YIL083C-lacZ	115 (20)	210 (40)	200 (45)	n. t.
YKL088W-lacZ	40 (5)	30 (5)	60 (10)	n. t.
YGR277C-lacZ	15 (4)	20 (4)	20 (5)	n. t.
YDR196C-lacZ	12 (3)	25 (4)	20 (4)	n. t.

Plasmids containing translational fusions with *lacZ* of the genes indicated were transformed into strain JS42 (*ura3*, regulatory wild-type)

Transformants were grown selectively under conditions of carbon source variation (SCD-Ura, glucose; SCE-Ura, ethanol), absence of amino acids (SM) and absence of pantothenate (SCD-Ura-Pan)

Addition of pantothenate in excess did not alter expression of *ECM31* and *PAN6* (not shown)

Specific β-galactosidase activities are given in nmol oNPG hydrolyzed per min per mg of protein (U/mg)

Standard deviations are shown in parentheses

n. t. not tested

Δ*ydr196c* (*MET25-coaE*), respectively. Similar to authentic *S. cerevisiae* genes, plasmid-dependent expression of *coaBC* allowed growth of haploid null mutants Δ*yil083c* and Δ*ykl088w* (not shown). The same result could be obtained for *coaD* in the Δ*ygr277c* mutant and *coaE* in the Δ*ydr196c* mutant. We conclude that bacterial genes of CoA biosynthesis are able to fulfill their function even in *S. cerevisiae*, at least when adequately overexpressed. This finding supports the hypothesis that yeast null mutants used for heterologous complementation are indeed defective for CoA biosynthesis.

Expression analysis of coenzyme A biosynthetic genes

Since coenzyme A and its derivatives influence several metabolic pathways, we wished to investigate whether the expression of the biosynthetic genes is affected by the carbon source and by availability of amino acids and pantothenate, respectively. We thus constructed *lacZ* fusion genes for *ECM31*, *PAN6*, *CAB1*, *YIL083C*, *YKL088W*, *YGR277C* and *YDR196C* and transformed the resulted plasmids into regulatory wild-type strain JS42. Transformants were grown under selective conditions in SCD (2% glucose, supplementation with amino acids), SCE (3% ethanol as the sole carbon source), SM (no amino acids added) and SCD-Pan (2% glucose, no pantothenate), respectively. As shown in Table 4, for some fusions a modest increase of reporter gene expression was found when transformants were cultivated in the presence of the non-fermentable carbon source ethanol, compared with glucose as a substrate. However, a de-repression factor of 2.5 (for *ECM31*) or below does not justify to use the designation "glucose repression". Similarly, the complete absence of amino acids (in SM) did not significantly alter gene expression when compared with the supplementation in SCD medium. Surprisingly (at least for pantothenate biosynthetic genes *ECM31* and *PAN6*), variation of pantothenate supply failed to influence reporter gene expression, as well. We thus consider the seven genes for which expression was assayed as typical "house-keeping" genes which are transcribed at a constitutive low level.

Discussion

The yeast *S. cerevisiae* is able to synthesize pantothenate de novo from intermediates of amino acid metabolism. Comparative in silico analyses indicated that yeast genes *ECM31*, *PAN5* and *PAN6* (similar to *E. coli panB*, *panE* and *panC*, respectively) may be involved in pantothenate biosynthesis. In agreement with a previous study (White et al. 2001), null mutants *ecm31* and *pan6* were indeed auxotrophic for pantothenate, in contrast to the *pan5* mutant phenotype. Yeast gene *YDL144C* encodes a second protein related to *panE* but even a double mutant *pan5 ydl144c* could still grow in the absence of pantothenate. This indicates that activity of dehydropantoate reductase may be provided by a distinct protein which is not similar to the bacterial enzyme.

It has been reported that *S. cerevisiae* strains auxotrophic for pantothenate show a defect of biosynthesis of β-alanine (Stolz and Sauer 1999) which in yeast is derived from polyamines via a complex oxidative pathway (White et al. 2001, 2003) instead of using decarboxylation of aspartate (*panD* gene product in *E. coli*; Cronan et al. 1982). In the absense of de novo biosynthesis of pantothenate, growth is rescued by external supplementation requiring a functional pantothenate

transporter (Fen2; Stolz and Sauer 1999). From our failure to obtain a *pan6 fen2* double mutant, we conclude that viability of yeast requires either a functional pantothenate transporter (Fen2) or intact biosynthetic genes (*ECM31*, *PAN5* and *PAN6*). In contrast to what was found for the fission yeast *Schizosaccharomyces pombe* (viability of a *pan6 liz1* [=*fen2*] double null mutant at 10 μM pantothenate; Stolz et al. 2004), pantothenate uptake via passive diffusion may not be effective in *S. cerevisiae*. Synthetic lethality of mutations *pan6* and *fen2* also argues against the existence of a low-affinity pantothenate transporter. This finding parallels the situation in *E. coli* where *panC panF* double mutants could not be constructed (*panF* encoding pantothenate permease; Vallari and Rock 1985).

While previous work on the enzymology of yeast CoA biosynthesis from pantothenate proposed the existence of an alternative pathway (Bucovaz et al. 1980, 1997) comparative genomic analyses taking advantage of the characterization of the human enzymes (Daugherty et al. 2002) supported a uniform order of events. Owing to the absence of a CoA uptake system, biosynthetic genes must be considered as indispensable for cellular viability. Even with fatty acid supplementation, we were unable to obtain viable haploid mutants for the five candidate genes (*YDR531W*, *YIL083C*, *YKL088W*, *YGR277C* and *YDR196C*), confirming the results of genome-wide deletion studies (Winzeler et al. 1999; Giaever et al. 2002). In this work, we focused on the essential *YDR531W* gene which encodes the yeast pantothenate kinase (panK) according to the following criteria: (1) similarity of its gene product to the human enzyme; (2) characterization of a temperature-sensitive mutant (*cab1*^{ts}) lacking panK activity under non-permissive conditions; (3) functional complementation of this mutant by *YDR531W* as well as by the bacterial panK gene *coaA* and mammalian *panK3*; (4) lack of complementation of the *ydr531w* deletion mutation and *cab1*^{ts} mutation; (5) identification of missense mutations within the *ydr531w* allele of the ts mutant strain; (6) strongly increased panK activity after overexpression of *YDR531W* in a yeast wild-type strain. Taken together, we consider *YDR531W* as the genuine yeast panK gene and consequently suggest *CAB1* (coenzyme A biosynthesis) as its adequate designation. Our finding of heterologous complementation of *cab1*^{ts} by mammalian *panK3* offers the opportunity of functional in vivo studies with gene variants from other eukaryotes.

Among the five missense mutations found within the *cab1*^{ts} mutant, A327T and G351S appeared as most promising to cause the temperature-sensitive phenotype. G351 is the only residue which is entirely conserved among eukaryotic panK enzymes. Superimposition of the Cab1 sequence to the recently solved crystal structure of human panK (panK3; Hong et al. 2007) suggests that G351 should be located in the interior of the enzyme and may not be involved in ATP binding or dimerization. Introduction of the G351S mutation into a functional *CAB1* gene led to a variant which could no longer complement *cab1*^{ts}. However, the remaining four missense mutations were not assayed individually for their influence on enzyme function. The strong conservation of residues responsible for acetyl-CoA binding among panK3 and the *CAB1* gene product (7 residues involved in hydrogen bonds, 13 residues mediating hydrophobic interactions; cf. Fig. 4; Hong et al. 2007) indicates that the yeast panK may also show competitive inhibition by this nucleotide.

Similar to what was found for *E. coli coaA* and yeast *cab1*^{ts}, heterologous complementation studies showed that bacterial genes could also replace yeast genes of late CoA biosynthesis (complementation of Δ*yil083c* and Δ*ykl088w* by bifunctional *coaBC*, Δ*ygr277c* by *coaD* and Δ*ydr196c* by *coaE*). This result provides evidence that the corresponding yeast wild-type genes are indeed involved in coenzyme A biosynthesis. Interestingly, the *coaBC* gene product shows significant similarity to the carboxy-terminal half of Ykl088w. In contrast, no similarity of protein CoaBC and Yil083c could be detected. Sequences of CoaD and Ygr277c are also unrelated while CoaE and Ydr196c show similarities almost over their entire length (28.2% identity, 40.2% similarity). To introduce a systematic gene nomenclature providing functional information, we thus suggest to rename *YIL083C* (new designation: *CAB2*), *YKL088W* (*CAB3*), *YGR277C* (*CAB4*) and *YDR196C* (*CAB5*).

Beyond its central metabolic function, CoA biosynthesis may exhibit a (direct or indirect) function for ribosomal biogenesis. Using a genome-wide strategy ("diploid shuffle"), temperature-sensitive alleles for all essential genes of *S. cerevisiae* have been constructed recently (Ben-Aroya et al. 2008). Interestingly, the functional characterization of the mutant alleles obtained indicated that *YGR277C* and *YDR196C* influence processing of rRNA precursors. Based on the incomplete cleavage of the 35S rRNA precursor the authors conclude that a CoA derivative may be important for processing of the primary rRNA transcript.

Expression analysis of pantothenate and CoA biosynthetic genes showed that these genes are transcribed at a low level which is hardly affected by external stimuli. Although the existence of a specific transporter for external pantothenate may suggest a regulatory feedback on its de novo biosynthesis, transformants containing *lacZ* fusions of *ECM31* and *PAN6* did not provide evidence for pantothenate repression of these biosynthetic genes. This is in contrast to several other anabolic pathways which are transcriptionally controlled by key metabolites such as inositol (Chen et al. 2007) and thiamin (Nosaka et al. 2005). Similarly, variation of amino acid availability (minimal

medium vs. synthetic complete medium) did not significantly alter expression of seven *lacZ* fusions assayed. Variation of the carbon source (glucose vs. ethanol) resulted in a 2-2.5-fold increase of reporter gene expression for four of the seven tested fusion genes. These findings essentially agree with the previous microarray data analyzing gene expressions patterns in the course of the diauxic shift (DeRisi et al. 1997; Gasch et al. 2000). De-repression of CoA biosynthetic genes makes some sense, considering the requirement of acetyl-CoA for gluconeogenesis in the presence of a C_2 substrate. The moderate increase of gene expression for *ECM31*, *PAN6*, *CAB1,* and *YIL083C* with ethanol instead of glucose as the sole carbon source was no longer observed in mutants *snf1* and *cat8 sip4* (not shown) lacking important regulators of the gluconeogenic pathway (Schüller 2003). Since neither promoter contains a sequence reminiscent of a Cat8 and Sip4 binding sites (Roth et al. 2004), the increase of gene expression observed may be indirectly influenced by these transcription factors. Instead, in silico inspection of the promoter sequences of CoA biosynthetic genes gave evidence for binding sites of pleiotropic transcription factors such as Abf1 (ATCTTAC ATGACG; −180/−168 of *CAB1*), Reb1 (TGACCCG; −196/−190 of *YIL083C*) and Rap1 (GTCCATACGC; −185/−176 of *YKL088W*) possibly mediating their almost constitutive expression at a low level.

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