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Characterization of Recombinant YakC of *Schizosaccharomyces pombe* Showing YakC Defines A New Family of Aldo-keto Reductases

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The *yakC* gene in *Schizosaccharomyces pombe*, which encodes yakC protein (YakC), a potential member of an aldo-keto reductase (AKR) family, was cloned and expressed in *Escherichia coli* cells. The recombinant YakC purified to homogeneity catalyzed the reduction of 2-nitrobenzaldehyde (k_{cat}, 44.1 s^{-1}, K_m, 0.185 ± 0.018 mM), 2-phthalaldehyde (19.8, 0.333 ± 0.032), and pyridine-2-aldehyde (7.64, 0.302 ± 0.028). Neither pyridoxal nor other compounds examined acted as substrates. NADPH, but not NADH, was a hydrogen donor. The enzyme is a monomer with a molecular weight of 38,900 ± 6,600 (SDS-PAGE). The amino acid sequence deduced from *yakC* showed the highest (34%) identity with that of pyridoxal reductase (AKR8A1) among the identified AKRs. Twenty-one function-unknown proteins showed 40% or higher identity to the deduced amino acid sequence: DR2261 protein of *Deionococcus radiodurans* showed the highest (50%) identity. The predicted secondary structure of YakC is similar to that of human aldose reductase, a representative AKR. The results establish YakC as the first member of a new AKR family, AKR13. The yeast cells contained enzyme(s) other than YakC and pyridoxal reductase with the ability to reduce 2-nitrobenzaldehyde: total (100%) activity in the crude extract consisted of about 23% YakC, about 44% pyridoxal reductase, and about 33% other enzyme(s).

Key words: aldo-keto reductase, pyridoxal reductase, *Schizosaccharomyces pombe*, YakC, *yakC* gene.

The aldo-keto reductases (AKRs) are one of three enzyme superfamilies encompassing a range of oxidoreductases. Members of the AKR superfamily are monomeric (alpha/beta)$_8$-barrel proteins, about 320 amino acids in length, that bind NAD(P)(H) to metabolize an array of substrates (*1*). AKRs found throughout the biological kingdoms have been divided into 12 families on the basis of sequence identity: AKRs that share greater than 40% identity are placed together as members of a family (*2*). Recently, we have found that pyridoxal reductase [EC 1.1.1.65] from a fission yeast, *Schizosaccharomyces pombe*, as a founding member of family 8 (*3*). The enzyme catalyzes the reduction of pyridoxal to pyridoxine and may be involved in the salvage synthesis of pyridoxine in yeast cells (*4*). The enzyme also shows high reactivity toward 2-nitrobenzaldehyde. The amino acid sequence of the enzyme shows identities to those of function-unknown proteins, such as YakC in *S. pombe* (34% identity) and F8A5.20 in *Arabidopsis thaliana* (37%).

YakC (Accession No. Q09923, Swiss-Prot), encoded by the SPAC1F7.12 gene in yeast, has been proposed to be a putative AKR. In the NiceProt View of Swiss-Prot, the protein is described as belonging to the AKR 2 family based on its deduced protein sequence. However, it is classified as a potential AKR member and is not assigned a consistent nomenclature by the systematic nomenclature system (*2*). Although YakC has been suggested to be an isozyme of pyridoxal reductase based on the similarity of their protein sequences (*3*), its function has not been assigned.

Here we cloned and overproduced recombinant YakC (rYakC) in *Escherichia coli*. The purified rYakC catalyzed the reduction of 2-nitrobenzaldehyde but not pyridoxal. Its primary structure showed that the enzyme is the founding member of a 13th AKR family.[2] Several function-unknown proteins were found to belong to this family.

MATERIALS AND METHODS

Microorganisms and Culture Conditions—*S. pombe* IFO 0346 was used for the partial purification of YakC. *S. pombe* TP4-5A (*h⁻ leu 1 ura4 ade6-M 210*) was obtained from Dr. Kaoru Takegawa (Kagawa University). A pyridoxal reductase–coding gene-disrupted strain (*h⁻ leu 1 ura4 ade6-M 210 Δplr1::ura4*) was prepared from *S. pombe* TP4-5A (the details of the preparation will be presented elsewhere). The yeast cells were grown at 30°C with shaking in Edinburgh minimal medium (EMM) containing supplements as required (*5*). The cells were harvested by centrifugation at 7,000 ×*g* for 10 min, washed twice with 0.9% NaCl, and then stored at –20°C.

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[2] Dr. T.M. Penning, a founding member of the nomenclature system for the AKR superfamily, personal communication.

Abbreviations: AKR, aldo-keto reductase; KPB, potassium phosphate buffer; PMSF, phenylmethanesulfonyl fluoride; EMM, Edinburgh minimal medium; LB, Luria-Bertani; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Enzyme and Protein Assays—Catalytic activity of the enzyme was measured by following the initial rate of the decrease in the A_{366} (ε: 3090 M^{-1} cm^{-1}) of NADPH at 37°C in 1.0 ml of reaction mixture. Each reaction mixture contained 2.0 mM 2-nitrobenzaldehyde, 0.2 mM NADPH, 0.1 M MOPS-KOH buffer (pH 7.5), and the enzyme. The reaction was started by the addition of 2-nitrobenzaldehyde. One unit of activity was defined as the amount of enzyme required to reduce 1 μmol of 2-nitrobenzaldehyde per min.

The protein concentration was determined by the dye-binding method of Bradford (*6*) with bovine serum albumin as the standard.

Construction of Plasmids Carrying the yakC Gene—The *yakC* gene on the *S. pombe* genome was amplified by PCR using the two oligonucleotides 5′-GTCGACCCGGGTGAACTAAACCCATTACCACTCGC-3′ (primer N, sense) and 5′-AGATCTGCAGCATAAATGAAGGTAACTATAAACAT-3′ (primer C, antisense) to introduce *Sma*I (underlined in primer N) and *Pst*I (underlined in primer C) sites. The reaction mixture (50 μl) of PCR consisted of Takara LA-PCR buffer II (Mg^{2+} plus), 20 nmol of each dNTP, 2.5 units of Takara LA *Taq* polymerase, 0.5 μg of *S. pombe* genomic DNA (as a template), and 100 pmol of each primer. The mixture was heated at 98°C for 20 s and then incubated at 68°C for 20 min. The programmed temperature shift was repeated 16 times, and then, without delay, an "autosegment extension" program was done as follows: heating at 95°C for 20 s and incubation at 68°C for 20 min + *t* s, where *t* denotes the segment extension time that increases by 15 s at each cycle; the temperature shift was repeated 14 times. Finally, the mixture was held at 72°C for 10 min. The amplified fragments were digested by *Sma*I and *Pst*I, and then ligated into the *Sma*I–*Pst*I site of pUC19. The constructed plasmid was designated as pUYA. The 1,150 bp fragment carrying the *yakC* gene was cut out from plasmid pUYA with *Sma*I and *Pst*I, and then ligated into the *Sma*I-*Pst*I site of pTrc99A (Amersham Pharmacia), which is a bacterial overexpression vector. The recombinant plasmid was designated as pTYA. A plasmid pTYASD, in which a typical bacterial ribosome-binding sequence was bound to the 5′ end of the *yakC* coding sequence, was constructed in the same way except that an oligonucleotide 5′- GCGGAATTC**AGGAGGAGGA**TCAGAAATGTCTATCCCTACTCG TAAAATT-3′ was used as a sense primer to introduce ribosome-binding (boldface types) and *Eco*RI (underlined) sites. The plasmids pUYA, pTYA, or pTYASD were introduced into Takara *E. coli* JM109 competent cells. The clone cells were used for the analysis of enzyme production in *E. coli*.

Enzyme Purification—YakC protein from *S. pombe* cells was partially purified by the purification steps described for pyridoxal reductase with 2-nitrobenzaldehyde as a substrate (*3*). Recombinant YakC protein was purified from *E. coli* JM109 cells harboring pTYASD. The cells were grown in 1 liter of LB medium (*7*) containing ampicillin (50 μg/ml) and isopropyl-β-D-thiogalactopyranoside (1 mM) at 37°C for 16 h. The cells (wet weight, 18.9 g) were suspended in 37.8 ml of 20 mM potassium phosphate buffer (KPB), pH 6.0, containing stabilizing reagents (1 mM PMSF, 0.01% 2-mercaptoethanol, 1mM EDTA). The suspension was sonicated and centrifuged, and the supernatant was used as a crude extract. NaCl (final 4M) was added to the crude extract, and the solution was applied to a butyl-Toyopearl column (1.8 × 18 cm) equilibrated with 10 mM Tris-acetate buffer, pH 6.0, containing 2 M NaCl, 0.01% 2-mercaptoethanol, and 1 mM EDTA. The column was washed with 10 mM Tris-acetate buffer, pH 6.0, containing 2 M NaCl, 0.01% 2-mercaptoethanol, 1 mM EDTA, and 0.005% Tween 40. The enzyme activity was eluted as a single peak with the washing buffer.

The active fractions obtained by butyl-Toyopearl column chromatography (27 ml) were dialyzed at 4°C overnight against 1 liter of 20 mM KPB, pH 7.0, containing 1 mM EDTA, 0.01% 2-mercaptoethanol, 0.005% Tween 40, and 0.1 M NaCl. The dialyzed solution was applied to an Orange A column (0.9 × 3.2 cm) equilibrated with 20 mM KPB, pH 7.0, containing 1 mM EDTA, 0.01% 2-mercaptoethanol, 0.005% Tween 40, and 0.1 M NaCl. The column was then washed with 150 ml of the equilibration buffer, and the enzyme was eluted with the same buffer containing 1 mM NADP. The active fraction (15 ml) was the purified recombinant enzyme preparation.

Molecular Weight Determination of rYakC—The purity of rYakC and the subunit molecular weight were estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli (*8*). The molecular weight of the enzyme was estimated by gel filtration at 4°C, using a Superose 12 HR column at a flow rate of 0.3 ml/min. A calibration curve was made from the elution pattern of bovine liver catalase (M_r = 240,000), pig heart mitochondrial aspartate aminotransferase (90,000) and malate dehydrogenase (67,000), and horse heart cytochrome *c* (12,400).

Substrate Specificity of rYakC—Substrates were screened at concentrations of 0.2–1.0 mM. K_m and V_{max} values were determined by measuring the initial reaction velocities with concentrations of substrate between 0.1 × K_m and 10 × K_m, with the NADPH concentration held at 0.2 mM. Kinetic parameters were determined by employing curve-fitting software (KaleidaGraph) to fit the Michaelis-Menten equation using the Levenberg Marquardt algorithm.

Amino Acid Sequencing—The rYakC (250 μg) was digested at 25°C for 2 h with 10 μg of V-8 protease in a reaction mixture (270 μl) consisting of 100 mM Tris-HCl, pH 7.8, and 2 M urea. The digestion mixture was applied to an ODS-AM reverse-phase column (YMC, 4.6 × 100 mm). The peptides were eluted at flow rate of 1 ml/min by gradients between solvent A (0.1% trifluoroacetic acid) and solvent B (0.07% trifluoroacetic acid dissolved in acetonitrile): a linear gradient to 5% solvent B for 5 min, 5 to 50% for 60 min, and 50 to 80% for 10 min, and isocratic elution with 80% solvent B for 10 min. Sequencing of the separated peptides was done by automated Edman degradation with an Applied Biosystems 492 protein sequencer.

Homology Search of rYakC with Other Proteins—The DNA and protein databases in the DDBJ/EMBL/GenBank were searched for proteins homologous with the deduced amino acid sequence of *yakC* by use of the BLAST algorithm (*9*). An unrooted tree of aldo-keto reductase proteins was drawn by the neighbor-joining method (*10*) with CLUSTALW.

Immunoprecipitation of YakC in Crude Extracts—Rabbit anti-rYakC antiserum, prepared by immunizing a rabbit three times with 0.1 mg of purified rYakC mixed with complete Freund adjuvant, or control preimmune serum (0, 1, 2, or 3 μl), 50 μl of crude extract of *S. pombe* TP4-5A cells or pyridoxal reductase gene-disrupted cells, prepared by

disrupting the cells with glass beads as described previously (11), were mixed with an appropriate volume of 0.9% NaCl to make a final volume of 65 μl. The mixtures were incubated for 60 min at 37°C, after which 5 μl of Zysorbin (40 mg/ml) was added. The immune complexes (Zysorbin-antibody-YakC) were removed by centrifugation, and the 2-nitrobenzaldehyde reductase activity remaining in supernatant was assayed.

RESULTS

Peptide Sequences of YakC and rYakC—YakC was co-purified with pyridoxal reductase from *S. pombe*. When pyridoxal reductase was purified, Orange A affinity column chromatography was used as the third step in the purification procedure. The proteins in the active fraction obtained by affinity chromatography consisted of about 85% pyridoxal reductase and about 15% YakC; the ratio changed with each purification. Thus, analyses of lysylendopeptides among the proteins in the Orange A fraction showed internal sequences of YakC as shown in Fig. 1. The sequences of the four peptides coincided with the amino acid sequences deduced from the *yakC* gene. The results show that the *yakC* gene is expressed in *S. pombe* cells.

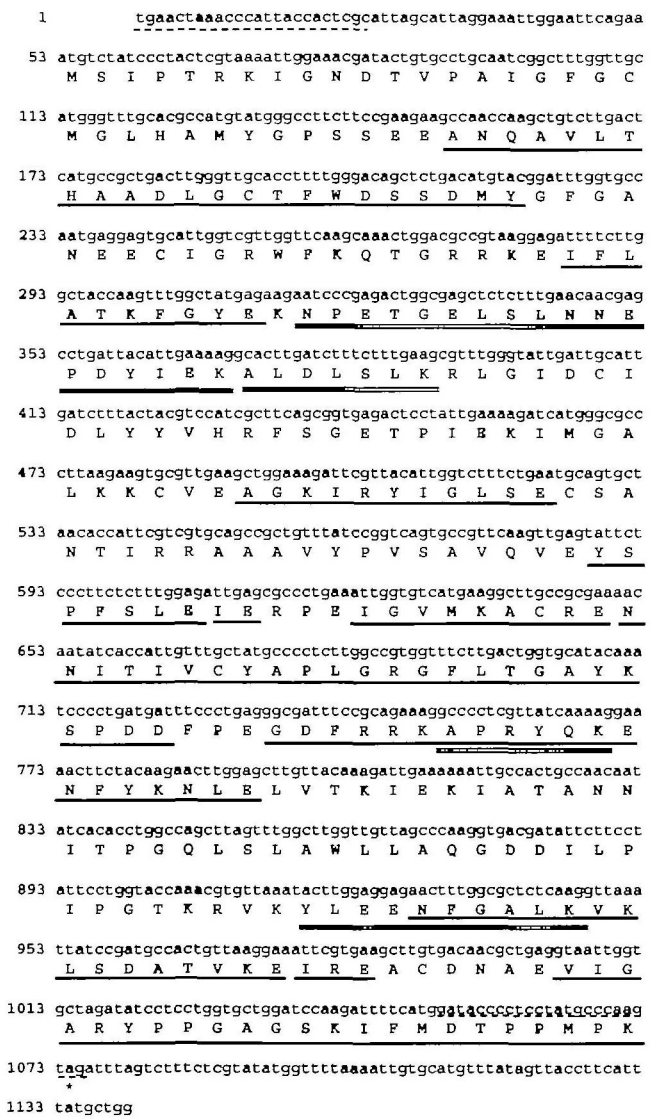

Fig. 1. **Peptide sequences of YakC and rYakC.** The lysyl endopeptides of YakC sequenced by the method described previously (3) are shown as double-lines along with the nucleotide and deduced amino acid sequences of the yakC gene of *S. pombe*. The single lines show amino acid sequences determined by sequencing V-8 peptides from rYakC. The broken lines show the nucleotide sequences used for the design of PCR primers.

Cloning and Expression of the yakC Gene—Because the deduced amino acid sequence of *yakC* shares 34% identity with pyridoxal reductase, it was expected that rYakC would show catalytic activity toward pyridoxal or other substrates of pyridoxal reductase. So, 2-nitrobenzaldehyde, the best synthetic substrate for pyridoxal reductase, was used to measure the expression of rYakC. Indeed, the crude extract of *E. coli* JM 109/pUYA showed low but measurable 2-nitrobenzaldehyde reduction activity. Higher activity was observed when the vector was changed to pTrc99A from pUC19. The addition of the ribosome binding nucleotide sequence to the 5′-terminal end increased the activity further. Thus, the specific activities of the crude extracts of *E. coli* JM109/pTYA and /pTYASD were 0.618 ± 0.099 and 5.88 ± 0.169, respectively. The latter crude extract showed a protein band in SDS-PAGE gels with a molecular weight of about 38,000 that coincides with the predicted molecular weight (37,700) of YakC.

Purification and Molecular Weight of rYakC—Recombinant YakC was purified to homogeneity in two steps of column chromatography (Table I). The purified protein showed a single protein band with a molecular weight of 38,900 ± 6,600 (average and S.D. of three experiments) in an SDS-PAGE gel (Fig. 2). The molecular weight of native rYakC was estimated to be 40,000 ± 4,500 by gel filtration column

TABLE I. **Purification of rYakC from transformant *E. coli* JM 109/pTYASD cells.**

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Crude extract	152.3	1,078.2	7.1	100.0
Butyl-Toyopearl	50.8	1,214.4	23.9	112.6
Orange A	1.8	211.0	117.2	19.6

(wet cells 2.9 g)

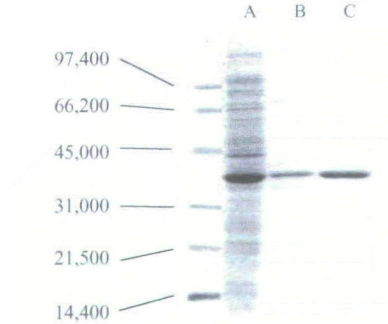

Fig. 2. **SDS-PAGE of crude and purified preparations of rYakC.** Lane A, crude extract (10 μg of protein); lane B, purified fraction from the butyl-Toyopearl column (5 μg); and lane C, the fraction from the Orange A column (5 μg).

chromatograpy. Thus, rYakC is a monomeric protein.

Optimum pH and Substrate Specificity—The enzymatic properties of rYakC were examined. The optimal pH for 2-nitrobenzaldehyde reduction was 7.5 when the activity was measured with MOPS-KOH and sodium phosphate buffers. NADPH (K_m = 0.012 ± 0.001 mM at pH 7.5) was required for the reduction of 2-nitrobenzaldehyde; NADH was inactive as a coenzyme.

Steady-state kinetic parameters for rYakC are given in Table II. All substrates showed Michaelis-Menten kinetics in the concentration range studied. 2-Nitrobenzaldehyde was an excellent substrate. 2-Phthalaldehyde and pyridine-2-aldehyde were moderate substrates. The compounds to which rYakC showed no measurable activity were pyridoxal, pyridoxal 5′-phosphate, DL-glyceraldehyde, D-glucose, D-galactose, D-fructose, D-mannose, D-glucronate, D-xylose, L-arabinose, D-erythrose, indolaldehyde, 2-aminobenzaldehyde, 2-carboxybenzaldehyde, isovaleraldehyde, acetaldehyde, formaldehyde, succinic semialdehyde, 3-nitrobenzaldehyde, 4-nitrobenzaldehyde, 5-nitrosalicylaldehyde, benzaldehyde, 3-phthalaldehyde, 4-phthalaldehyde, pyridine-3-aldehyde, pyridine-4-aldehyde, 9,10-phenanthrenequinone, and 2-nitroacetophenone. Thus, rYakC is not an isozyme of pyridoxal reductase, and its natural substrate is unknown.

Primary Sequence of rYakC—The partial amino acid sequence of rYakC was determined to confirm the identity of the expressed protein with YakC. The amino-terminal sequence could not be determined for an unknown reason. Ten V-8 peptides were sequenced and 149 residues, including the carboxyl-terminal region, were determined (Fig. 1). The sequences coincide with the predicted sequences.

Homologous Proteins—Because YakC was expected to be an AKR as described above, its deduced amino acid sequence was compared with other protein sequences in the database to find homologous proteins. No proteins belonging to the AKR superfamily showed greater than 40% identity. Pyridoxal reductase showed the highest (34%) identity. However, several function-unknown proteins were found to have identities greater than 40% as shown in Table III. The DR 2261 protein from *Deinococcus radiodurans* showed the highest identity. These proteins may comprise a new 13th ARK family as described below.

Sequence and Secondary Structure Comparisons—Because the amino acid sequence deduced from the *yakC*

TABLE II. **Substrate specificities of rYakC and pyridoxal reductase from *S. pombe*.**

Substrate	rYakC		Pyridoxal reductase	
	K_m (mM)	K_{cat}/K_m (sec/mM)	K_m (mM)	K_{cat}/K_m (sec/mM)
Pyridoxal	NMA		0.909 ± 0.080	61.7
2-Nitrobenzaldehyde	0.185 ± 0.018	238.0	0.096 ± 0.009	350.0
3-Nitrobenzaldehyde	NMA		NMA	
4-Nitrobenzaldehyde	NMA		0.012 ± 0.001	16.3
2-Phthalaldehyde	0.333 ± 0.032	59.3	0.118 ± 0.010	10.8
3-Phthalaldehyde	NMA		NMA	
4-Phthalaldehyde	NMA		NMA	
Pyridine-2-aldehyde	0.302 ± 0.028	25.3	1.31 ± 0.090	11.5
Pyridine-3-aldehyde	NMA		0.349 ± 0.029	0.6
Pyridine-4-aldehyde	NMA		1.13 ± 0.102	1.6

NMA: no measurable activity. Values for pyridoxal reductase were reexamined in this study.

TABLE III. **Hypothetical proteins similar to the amino acid sequence deduced from the *yakC*.** In the column headings, Accession indicates the accession numbers. Length is the number of amino acid residues of the hypothetical protein of DDBJ/EMBL/GenBank or Swiss-Prot. Identity and similarity score of the hypothetical protein with the amino acid sequence deduced from the *yakC* were obtained by BLAST search in the DDBJ Homology Search System.

Source	Abbreviation	Accession	Length	Identity	Similarity
				%	%
Deinococcus radioduruns	DR2261	AE002058-9	327	50	68
Pseudomonas aeruginosa	PA2535	AE004681-5	331	48	67
Zea mays	IP2	P49249	306	46	61
Mesorhizobium loti	mlr1801	AP002998-108	334	45	62
Nicotiana tabacum	NTAUCX115	X56267	307	45	61
Arabidopsis thaliana	F8A5.20	AC002292	340	45	59
A. thaliana	F8A5.23	AC002292-23	287	45	59
Sinorhizobium meliloti	SMc00368	AL591783-59	252	44	61
Agrobacterium tumefaciens	AGRC447	007965-8	369	43	57
A. thaliana	atb2	AF057715-1	345	44	63
A. thaliana	F8A5.21	AC002292-21	345	43	59
Thermotoga maritima	TM1006	H72307	333	42	60
Caulobacter crescentus	CC3002	AE005963	333	42	60
A. thaliana	F8A5.24	AF361576-1	345	42	58
M. loti	mlr5051	AP003005-210	332	41	60
Helianthus annuus	HaAC1	AF030301-1	338	41	59
Streptomyces clavuligerus	Cvm1	AF124928-3	344	41	56
S. meliloti	SMa0563	AE07222-2	329	40	58
Yersinia pestis	YPO2806	AJ414153-202	329	40	58
Streptomyces coelicolor	2SCK31.03	AL451182-3	330	40	58
A. thaliana	F8A5.26	AC002292-26	374	40	55
Schizosaccharomyces pombe	plr	D89205-1	333	34	50

showed only low sequence identity with the representative aldo-keto reductase, human aldose-reductase, the conservation of essential residues and secondary structure elements was examined (Fig. 3). The DR2261 protein of *D. radiodurans*, which showed the highest identity with YakC, and pyridoxal reductase, which showed some relatedness to YakC based on unrooted phylogenic tree analysis as discussed below, were also aligned. The distributions of essential amino acid residues involved in substrate binding or catalysis and elements of the secondary structure are well conserved among the proteins. However, a region corresponding to loop A in human aldose reductase is not found in the other proteins.

Immunoprecipitation—In order to confirm that YakC is actually expressed in *S. pombe* cells, a crude extract of *S. pombe* TP4-5A cells was titrated with anti-rYakC antiserum (Fig. 4A). The specific activity of 2-nitrobenzaldehyde reduction in the crude extract was 0.73 and decreased by about 23% (0.17 × 100/0.73) after titration with the anti-rYakC antiserum, showing that YakC is expressed in the

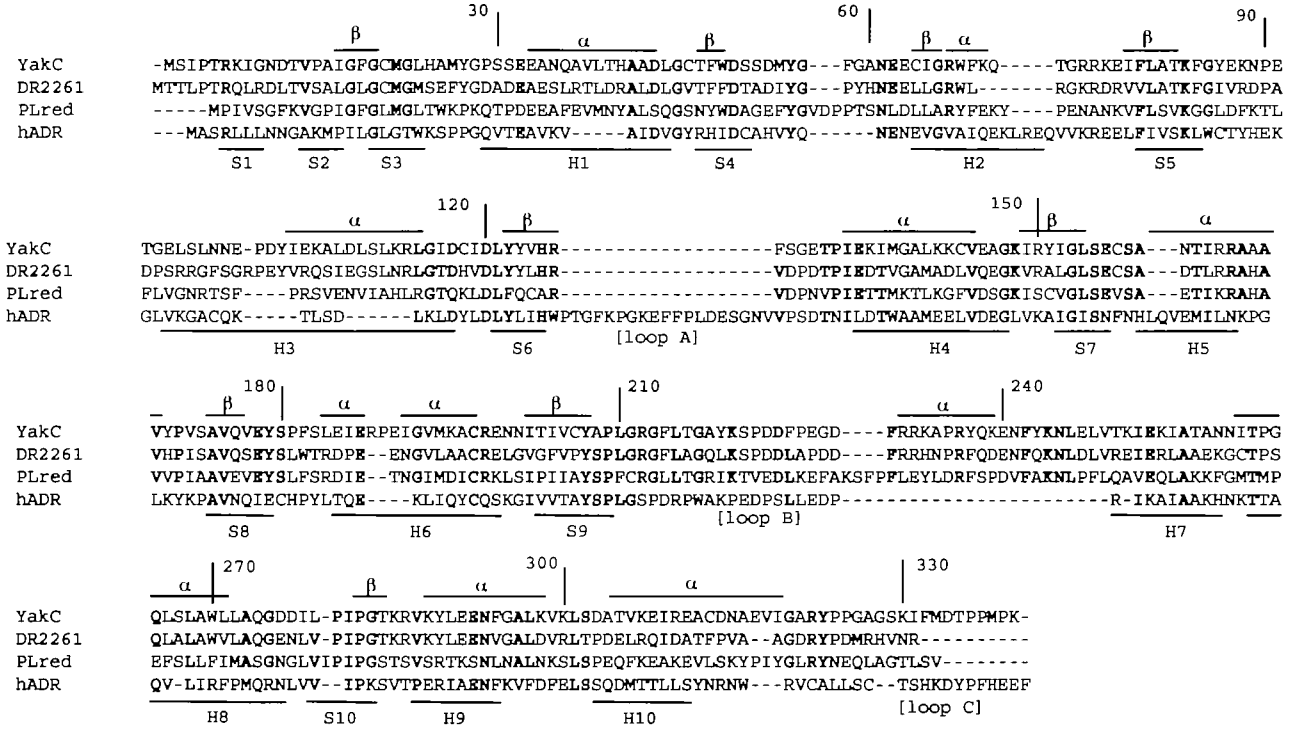

Fig. 3. **Sequence and secondary structure comparisons.** The amino acid sequence deduced from the *yakC*, and the sequences of DR 2261 protein, pyridoxal reductase (PLred), and human aldose reductase (hADR) were aligned. Amino acid residues comprising predicted α-helices and β-sheets in YakC are shown by a solid lines labeled α and β, respectively. The secondary structures, α-helix and β-sheet structures, determined by X-ray crystallography are shown for human aldose reductase as H1–H10 and S1–S10, respectively. Amino acid residues common in 3 or 4 sequences are shown in boldface type.

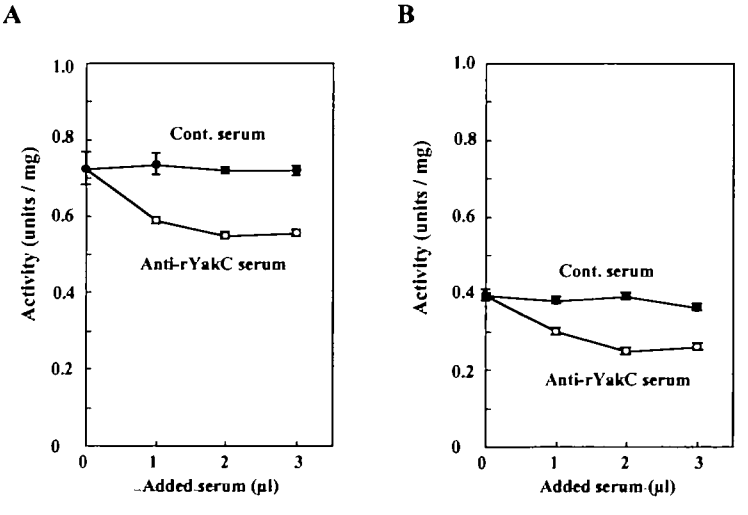

Fig. 4. **Immunotitration of YakC.** The 2-nitrobenzaldehyde reduction activities in crude extracts of *S. pombe* wild type TP4-5A (A) and pyridoxal-reductase-coding-gene–disrupted (B) cells were assayed after immunoprecipitation of YakC with various amounts of antiserum (○) or preimmunized control serum (●).

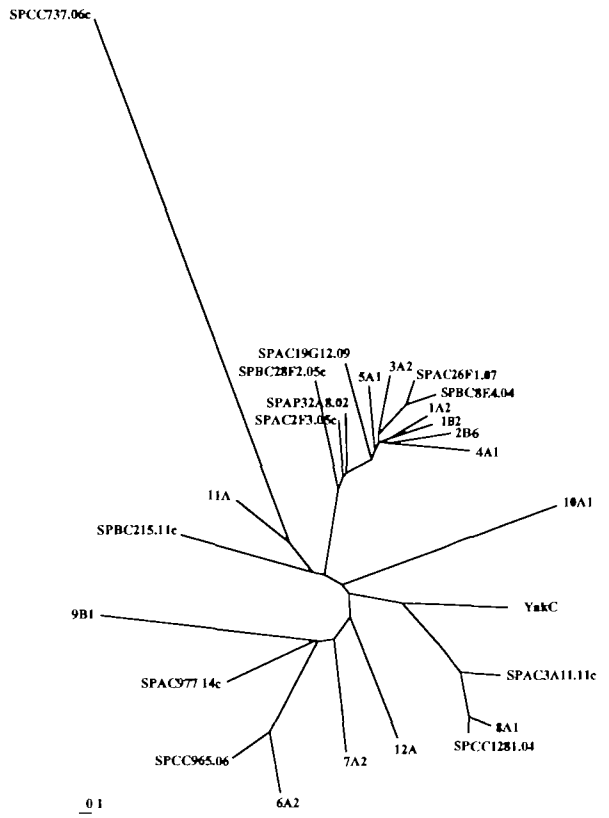

Fig. 5. **Unrooted phylogenic tree of aldo-keto reductases.** A representative AKR was selected from each family. AKR1A2 is aldehyde reductase, 1B2 is aldose reductase, 2B6 is Gre 3p, 3A2 is Ypr1p, 4A1 is chalcone polyketide reductase, 5A1 is reductase, 6A2 is shaker channel β-subunit, 7A2 is aflatoxin inducible aldehyde reductase, 8A1 is pyridoxal reductase, 9B1 is aryl-alcohl dehydrogenase, 10A1 is bluensomycin aldo-keto reductase, 11A is vegetative protein 147, and 12A is NDP-hexose-2,3-enoyl reductase. Defined or putative AKRs found in *S. pombe* are also shown. SPCC737.16 is a putative glutamate-cysteine ligase regulatory subunit, SPAC-2F3.05c is an aldo/keto reductase family oxidoreductase, SPAC-32A8.02 is a putative aldo/keto reductase, SPBC28F2.05c is a putative oxidoreductase, SPAC19G12.09 is a putative aldose reductase, SPAC26F1.07 is a putative oxidoreductase, SPBC8E4.04 is a putative oxidoreductase, SPBC215.11c is a putative oxidoreductase;aldo-keto family, SPAC977.14c is a putative oxidoreductase, SPCC965.05 is a putative potassium channel subunit, SPCC1281.04 is a pyridoxal reductase homolog, and SPAC3A11.11c is a pyridoxal reductase homolog.

cells. To estimate the contribution of pyridoxal reductase, which also has high reactivity toward 2-nitrobenzaldehyde, to the activity, a crude extract of pyridoxal-reductase–coding gene-disrupted cells was also titrated (Fig. 4B). The average specific activity of the crude extract from the gene-disrupted cells was 0.41. Thus, pyridoxal reductase accounts for about 44% [(0.73–0.41) × 100/0.73] of the total activity in the crude extract of wild-type cells. Anti-rYakC antiserum decreased the specific activity of the crude extract of gene-disrupted cells by 0.16, showing that the activity levels of YakC are almost the same in the wild type and gene-disrupted cells. The residual activity (about 33% of total activity) is due to other enzyme(s).

DISCUSSION

This study demonstrates that rYakC is indeed a reductase with high reactivity towards 2-nitrobenzaldehyde, but not pyridoxal. Recombinant YakC shows a somewhat higher hydrogen acceptor specificity than pyridoxal reductase as shown in Table II. Recombinant YakC shows no reactivity toward pyridoxal, 4-nitrobenzaldehyde, pyridine-3-aldehyde or pyridine-4-aldehyde, which are substrates for pyridoxal reductase. Furthermore, rYakC shows higher reactivity toward 2-phthalaldehyde than pyridoxal reductase, and is not inactivated during the catalysis of 2-phthalaldehyde, which is a suicide substrate for pyridoxal reductase (*3*). The results suggest that the structures of the active center of the enzymes differ somewhat from each other to allow them to exert their specific reactivities although the enzymes share high reactivity toward 2-nitrobenzaldehyde.

Figure 5 shows an unrooted phylogenic tree of AKRs in which one representative protein is selected from each family. The amino acid sequence deduced from *yakC*, pyridoxal reductase (8A1), a vegetative protein 147 from *B. subtilis* (11A), and NDP-hexose 2,3-enoyl reductase (12A) are relatively similar to each other. However, the identity of the amino acid sequence deduced from *yakC* with the other proteins is less than 40%. The delineation of families among the AKR superfamily occurs at approximately 40% sequence identity (*2*). Thus, YakC defines a new 13th family of AKRs[2].

It can be pointed out that AKRs can be separated roughly into three groups, those with reactivity towards 4-nitrobenzaldehyde, *e.g.*, AKR2B6, AKR3A2, and AKR9B1 (*12*), those with reactivity towards 2-nitrobenzaldehyde, *e.g.* AKR8A1 (*3*) and YakC (rYakC), and those with reactivity towards both 4- and 2-nitrobenzaldehyde, *e.g.* AKR7A2 (*13*).

Although elements of the predicted secondary structure of YakC are shared with those of human aldose reductase, with the exception that loop A structure that is found only in human aldose reductase, the identity of the primary structures is low. However, amino acid residues that may be involved in the catalytic function are well conserved. Asp-51, Tyr-56, and Lys-83 may be active site residues, and have a spatially conserved arrangement as seen in human aldose reductase (*14, 15*). Ser-156, Tyr-207, and Asn-293 may likewise be involved in the binding of NADPH (*15*).

AKRs that contain loop A structure are rather limited (*16*). The proteins AKR1A2, AKR1B2, AKR2B6, AKR3A2, AKR5A1, and AKR4A1, which occupy branches located on an upper right of the phylogenic tree (Fig. 5), contain loop A. No other AKRs contain loop A. In contrast, all AKRs contain loop B, suggesting that this is necessary to the function of AKRs. Analyses of the tertiary structures of AKRs without loop A are necessary to understand the role of the loop in the function of the enzymes.

The crude extract of *S. pombe* cells contains other enzyme(s) with 2-nitrobenzaldehyde reductase activity, accounting for about 33% of the total activity, besides YakC and pyridoxal reductase. The results of the *S. pombe* genome sequencing project show that the organism contains 14 genes coding defined or putative AKR proteins. These proteins are also shown in the phylogenic tree (Fig. 5). Homologues of pyridoxal reductase (AKR8A1), SPAC-3A11.11c and SPCC1281.04, are the first candidates for

other enzymes with the activity. A putative oxidoreductase, SPAC977.14c and SPBC215.11c, could also contribute. But, a putative potassium channel subunit, SPCC965.06, is less likely to be involved. Gene disruption experiments will demonstrate which enzyme(s) reduce the synthetic substrate, 2-nitrobenzaldehyde. The natural substrates of the enzymes, including YakC, should be elucidated.

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