The yeast gene *YJR025c* encodes a 3-hydroxyanthranilic acid dioxygenase and is involved in nicotinic acid biosynthesis

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Abstract We have deleted the yeast gene *YJR025c* and shown that this leads to an auxotrophy for nicotinic acid. The deduced protein sequence of the gene product is homologous to the human 3-hydroxyanthranilic acid dioxygenase (EC 1.13.11.6) which is part of the kynurenine pathway for the degradation of tryptophan and the biosynthesis of nicotinic acid. In cell-free extracts the 3-hydroxyanthranilic acid dioxygenase activity is proportional to the copy number of the *YJR025c* gene. As *YJR025c* encodes the yeast 3-hydroxyanthranilic acid dioxygenase, we have named this gene *BNA1* for biosynthesis of nicotinic acid.

Key words: 3-Hydroxyanthranilic acid dioxygenase; Kynurenine pathway; Nicotinic acid biosynthesis; Tryptophan degradation; *Saccharomyces cerevisiae*

1. Introduction

During the systematic sequencing of the genome of *Saccharomyces cerevisiae* we identified the gene *YJR025c* on chromosome X [1]. Analysis of the sequence revealed strong similarities to the human 3-hydroxyanthranilic acid dioxygenase (EC 1.13.11.6; 3-HOA). This enzyme was initially characterized and purified from bovine liver and kidney [2–4], and is involved in the catabolism of tryptophan via the kynurenine pathway. Precursors for the biosynthesis of the nicotinamide moiety of NAD may also be provided by this pathway. Recently, kynurenine metabolites have been implicated in several neuropathological conditions (see [5] for a review). This has led to the cloning and characterization of the human 3-HOA [6]. Here we present genetic and biochemical evidence that *YJR025c* encodes the yeast 3-HOA.

2. Materials and methods

2.1. Strains, media and genetic methods

The *Escherichia coli* strain used was DH5αF′ (*supE44*, Δ*lacU169* {Φ*80 lacZ*ΔM15} *hsdR17*, *recA1*, *endA1*, *gyrA96*, *thi-1*, *relA1*, *F′*), the media used for the propagation of *E. coli* were as described in [7]. The *S. cerevisiae* strains used were all derived from the homozygous diploid strain W303 (*MAT*α/*MAT*α, *ade2-1*, *his3-11*, *15*, *leu2-3*, *12*, *trp1-1*, *ura3-1*, *can1-100*) [8] and are described in the text. The media used for the cultivation of yeast were as described in [9]. Nicotinic acid was added to the G0 synthetic minimal medium at a concentration of 0.5 μg/ml. Solid G0 medium was made by adding 1.5% agarose. Standard genetic manipulations of yeast were performed as described in [10].

2.2. Preparation of cell-free extracts and 3-hydroxyanthranilic acid dioxygenase assays

Cells were grown to early stationary phase in G0 synthetic minimal medium containing nicotinic acid, harvested, washed and resuspended in 33 mM Tris-HCl. The cells were disrupted in a Brown homogenizer for 2 min, centrifuged for 5 min at 4°C in a microfuge and supernatant was passed over a G25 NAP column (Pharmacia) equilibrated with 33 mM Tris-HCl. This desalted extract was used for 3-HOA assays as described [2]. The product, 2-amino-3-carboxymuconic acid semialdehyde, was measured at 360 nm and a molar extinction coefficient of 47 500 M^{-1} cm^{-1} was used for the calculation of the activity. Proteins were assayed using the method of Lowry [11].

2.3. Nucleic acid manipulation and transformation

Restriction enzymes, Taq DNA polymerase and T4 DNA ligase were obtained from standard sources and were used in accordance

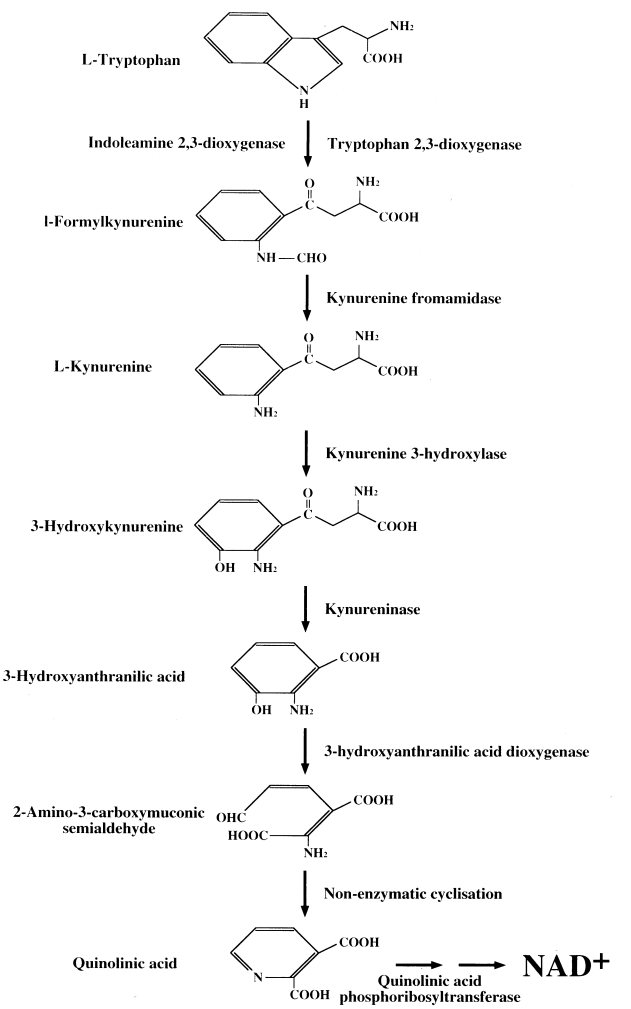

Fig. 1. Simplified schematic view of the intermediates and enzymes involved in the kynurenine pathway.

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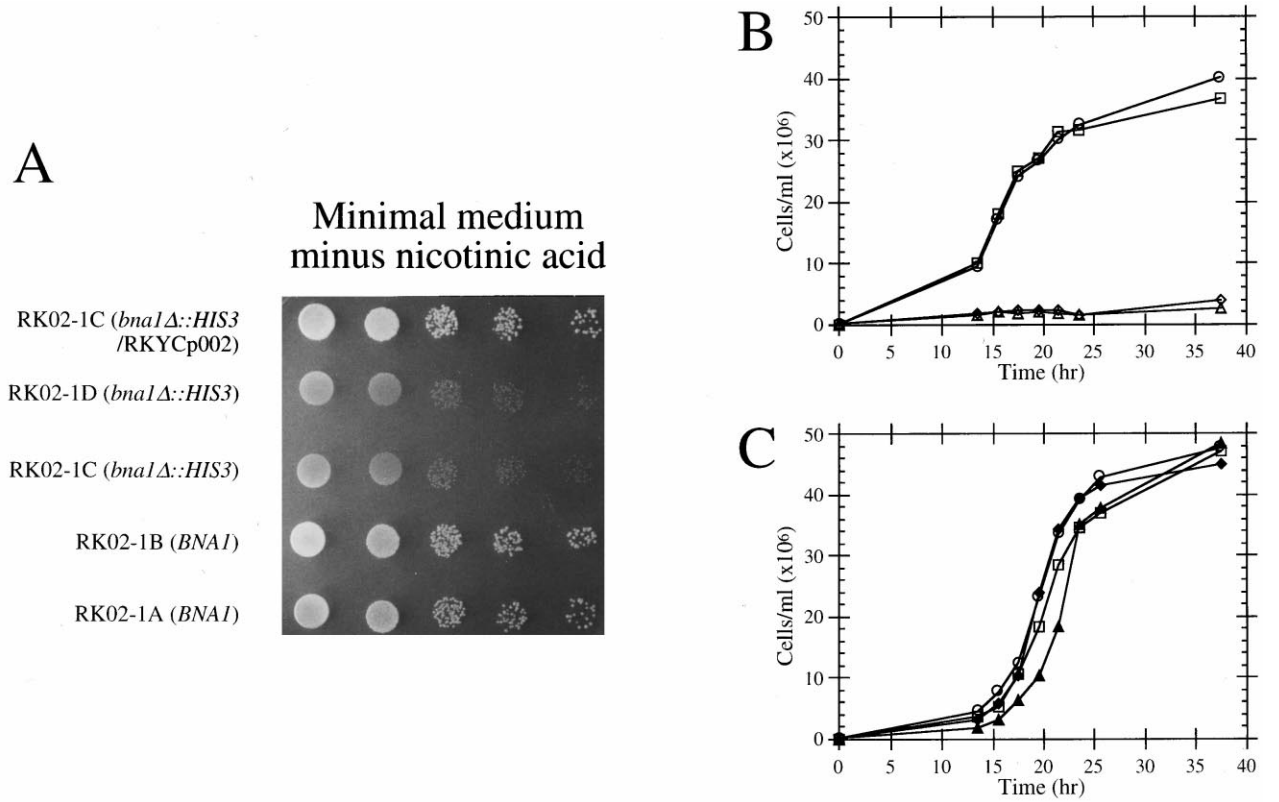

Fig. 2. A: Growth of wild type, *BNA1* deleted and *BNA1* deleted/complemented strains on solid G0 minimal medium without nicotinic acid. The plate was incubated at 28°C for three days. B: Growth of wild type (RK02-2A ○ and RK02-2B □) and *BNA1* deleted strains (RK02-2C ◇ and RK02-2D △) on liquid G0 minimal medium without nicotinic acid. C: Growth of wild type (RK02-2A ○ and RK02-2B □) and *BNA1* deleted/complemented strains (RK02-2C/RKYCp002 ♦ and RK02-2D/RKYCp002 ▲) on liquid G0 minimal medium without nicotinic acid.

A Specific activity of 3-HAO in cell extracts

Strain	RK02-1B (*BNA1*)	RK02-1C (*bna1Δ::HIS3*)	RK02-1C /RKYCp002 (*bna1Δ::HIS3*)	RK02-1C /RKYEp003 (*bna1Δ::HIS3*)
Specific activity (mmol/min/mg protein)	0.21 (2)	0.0 (2)	0.62 (3)	4.93 (3)

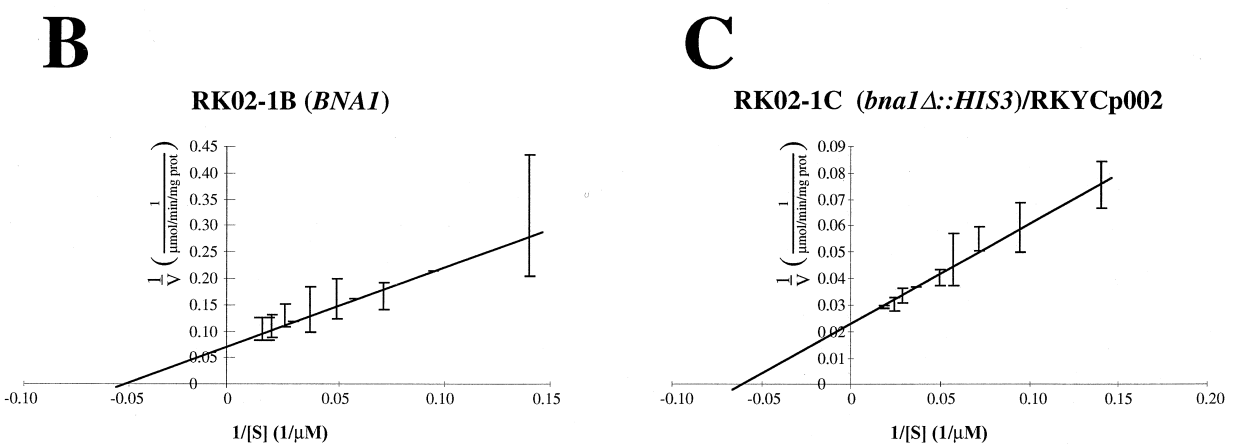

Fig. 3. A: Specific activity of 3-HOA in cell extracts. The specific activity is expressed as mmol of 2-amino-3-carboxymuconic acid semialdehyde produced per min per mg of protein. The figure in parentheses indicates the number of independent determinations. B, C: Determination of the K_m for 3-hydroxyanthranilic acid in cell extracts RK02-1B (*BNA1*) 19.2 µM and RK02-2C (*bna1Δ::HIS3*)/RKYCp002 15.9 µM.

with the manufacturers' instructions. Southern blot analysis was performed as described [12] using radiolabelled probes made with the Amersham Megaprime DNA kit. *E. coli* was transformed using the $CaCl_2/RbCl_2$ method or by electroporation [12]. Yeast was transformed using the LiCl procedure [13].

2.4. Plasmid construction and deletion of the gene YJR025c

The wild type *YJR025c* gene was cloned as a 1709 bp *Hin*dIII-*Xho*I fragment, derived from the cosmid pEJ103 [1]. This fragment was cloned into the *Hin*dIII-*Xho*I sites of the centromeric plasmid pRS416 to give RKYCp002 and the *Hin*dIII-*Sal*I sites of the multicopy plasmid YEp352 to give RKYEp003. All plasmids carry a *URA3* marker for selection in yeast. To delete the chromosomal copy of the *YJR025c* gene the method of Reiger et al. [14] was used. The *HIS3* gene was amplified by PCR using hybrid primers that contained long tails (~50 bp) identical to the extremities of the *YJR025c* gene: oligo-pro AGGCCTTTTGAAACCACCGGTGAATAATTATTGCTTACATAAAGGGGGATTCGTTCAGAATGACACG; oligo-term TTAATTAGATTGAGGGCGTGCGTAGTTTAACGTCTTGCAATGGAAACATGCTCTTGGCCTCCTCTAG.

In the resulting fragment a 384 bp region of the coding sequence of the *YJR025c* gene has been replaced by 1108 bp encoding the *HIS3* gene. This PCR product was used to transform the yeast strain W303, histidine prototrophs were selected and screened by PCR to determine if the integration had occurred at the *YJR025c* locus.

3. Results and discussion

As a first step in our analysis of *YJR025c* we decided to delete the chromosomal copy of the gene in the diploid strain W303. This was done using a PCR based strategy as described in Section 2. A tetrad analysis of the resulting heterozygous diploid, RK02, showed that the gene was not essential. We had previously noted a strong similarity between the predicted product of *YJR025c* and human 3-HOA [1], an enzyme of the kynurenine pathway. This suggested that *YJR025c* may be involved in the degradation of tryptophan and the biosynthesis of nicotinic acid (see Fig. 1). To test this hypothesis the wild type strain and strains with a deleted *YJR025c* gene were grown on G0 minimal medium in the presence and absence of 0.5 μg/ml nicotinic acid. The results in Fig. 2 show that in liquid medium the strains with a deleted *YJR025c* gene were unable to grow in the absence of added nicotinic acid, on solid medium some residual growth was seen. In both cases a wild type level of growth was restored when the deleted strains were transformed by the plasmid RKYCp002, a centromeric plasmid which carries the wild type *YJR025c* gene.

In order to determine if the nicotinic acid auxotrophy of the *YJR025c* deleted strains was due to a deficiency in 3-HOA, the activity of the enzyme was determined in cell-free extracts made from a wild type strain, a strain with a deleted *YJR025c* gene and *YJR025c* deleted strains carrying the wild type gene on a centromeric plasmid (RKYCp002) or a multicopy plasmid (RKYEp003). The results in Fig. 3A show that in the deleted non-complemented strain 3-HOA activity cannot be detected and that in the other strains 3-HOA activity is proportional to the copy number of the *YJR025c* gene.

In a final series of experiments the K_m for 3-hydroxyanthranilic acid was determined in cell-free extracts of a wild type strain and a *YJR025c* deleted strain carrying the wild type gene on a centromeric plasmid (RKYCp002). The results are presented in Fig. 3B,C. The K_m values observed, 19.2 μM (wild type strain) and 15.9 μM (deleted complemented strain), are similar to those obtained previously: 26 μM for bovine liver enzyme [2], 5.6 μM for rat liver enzyme and 2.3 μM for the cloned human enzyme [6].

Taken together these results clearly demonstrate that *YJR025c* encodes 3-HOA, an enzyme of the kynurenine pathway of tryptophan degradation, and that in yeast this enzyme is involved in the biosynthesis of nicotinic acid. We propose that the gene be called *BNA1* (biosynthesis of nicotinic acid).

Previously Ahmad and Moat [15] demonstrated that under aerobic conditions nicotinic acid is derived from tryptophan via quinolinic acid and were able to detect two enzymes of the kynurenine pathway in cell extracts. With the completion of the sequence of the yeast genome sequence it is now possible to tentatively identify the genes encoding some other enzymes in this pathway: *YBL098w* (kynurenine 3-hydroxylase, EC 1.14.13.9), *YLR231c* (kynureninase, EC 3.7.1.3) and *YFR047c* (quinolinic acid phosphoribosyltransferase, EC 2.4.2.19) (see Fig. 1). Our sequence analysis suggests that yeast does not contain a tryptophan 2,3-dioxygenase and to our knowledge no sequence data are available for indoleamine 2,3-dioxygenase or kynurenine formamidase to allow comparisons to be made, but kynurenine formamidase activity has been detected in yeast [15]. Thus there is some evidence for the existence of most of the enzymes of the kynurenine pathway in yeast.

The reason for the leaky growth of the *BNA1* deleted strains see in Fig. 2 remains unclear. This growth may be due to residual nicotinic acid in the medium, but this is unlikely as a completely defined medium solidified with agarose was used. Ahmad and Moat [15] provided evidence for an alternative pathway of nicotinic acid biosynthesis from aspartic acid or glutamic acid which operates under anaerobic conditions and it is possible that this pathway may be responsible for the residual growth of the *BNA1* deleted strains.

We have presented the first genetic evidence for the existence of the kynurenine pathway of tryptophan degradation and nicotinic acid biosynthesis in yeast and partially characterized the 3-hydroxyanthranilic acid dioxygenase. This study also shows how the availability of the complete sequence of the yeast genome can facilitate metabolic studies and should lead to a rapid increase in our understanding of intermediary metabolism in yeast.

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