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Aerobic and anaerobic NAD$^+$ metabolism in *Saccharomyces cerevisiae*

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Abstract In *Saccharomyces cerevisiae* the nicotinic acid moiety of NAD$^+$ can be synthesized from tryptophan using the kynurenine pathway or incorporated directly using nicotinate phosphoribosyl transferase (*NPT1*). We have identified the genes that encode the enzymes of the kynurenine pathway and for *BNA5* (YLR231c) and *BNA6* (YFR047c) confirmed that they encode kynureninase and quinolinate phosphoribosyl transferase respectively. We show that deletion of genes encoding kynurenine pathway enzymes are co-lethal with the Δ*npt1*, demonstrating that no other pathway for the synthesis of nicotinic acid exists in *S. cerevisiae*. Also, we show that under anaerobic conditions *S. cerevisiae* is a nicotinic acid auxotroph.

Key words: Aerobic; Anaerobic; NAD$^+$ metabolism; Tryptophan degradation; *NPT1*; *Saccharomyces cerevisiae*

1. Introduction

The systematic sequencing of complete genomes has provided a wealth of information that can be exploited in many different ways to further our understanding of diverse biological systems. One of the most common uses of the data provided by the sequencing projects is to perform sequence comparisons between proteins of known function in one organism and hypothetical proteins encoded by uncharacterized open reading frames from other organisms, in an attempt to associate them with a function. This approach can be of great help to the biologist, but independent biological corroboration is necessary to confirm these functional inferences.

We have previously used this approach to show that in *Saccharomyces cerevisiae BNA1* encodes the 3-hydroxyanthranilic acid dioxygenase, which is an enzyme of the kynurenine pathway, involved in NAD$^+$ biosynthesis [1]. Here we present an extension of this study. We have identified the genes encoding other enzymes in the kynurenine pathway, showed that the deletion of these genes is co-lethal with the deletion of *NPT1*, which encodes the nicotinate phosphoribosyl transferase (NAPRTase) and showed that wild-type *S. cerevisiae* is a nicotinic acid auxotroph under conditions of anaerobic growth.

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2. Materials and methods

2.1. Strains, media and genetic methods

The *Escherichia coli* strain used was DH5αF′ (*supE44*, Δ*lacU169* {*Φ80 lacZ*ΔM15} *hsdR17*, *recA1*, *endA1*, *gyrA96*, *thi-1*, *relA1*, *F′*), the media used for the propagation of *E. coli* were as described in [2]. The *S. cerevisiae* strains used were all derived from the homozygous diploid strain W303 [3] and are described in Table 1. The standard media used for the cultivation of yeast were as described in [4], nicotinic acid was added to G0 synthetic minimal medium at 0.5 μg/ml and complete synthetic medium (CSM) was from Bio 101. Where appropriate, strains for growth tests were starved overnight in liquid G0 medium without nicotinic acid, cell densities were adjusted prior to plating. For anaerobic growth, media were supplemented with 0.2% Tween 80 and 30 μg/ml ergosterol (TE) and the anaerobic environment was generated using the Anaerocult P system from Merck. Standard genetic manipulations of yeast were performed as described in [5].

2.2. Nucleic acid manipulation, plasmid construction and gene deletion

Taq DNA polymerase was obtained from standard sources and was used in accordance with the manufacturer's instructions. Southern blot analysis was performed as described in [6] using radiolabeled probes made with the Amersham Megaprime DNA kit. Yeast cells were transformed by the lithium acetate procedure of [7]. Plasmids were constructed by 'gap repair' after PCR amplification of the gene to be cloned. All the genes cloned in this way were able to complement the phenotype of the corresponding deletion. Gene deletions were constructed using the PCR based strategy with either the G418 resistance marker or a heterologous *HIS3* marker [8,9]. All gene deletions were performed on diploid strains, showed a correct segregation of the inserted marker and were confirmed by Southern blot.

2.3. Preparation of cell-free extracts and the assays of enzymatic activities of quinolinic acid phosphoribosyltransferase (QPRTase) and kynureninase

Cells were grown to the early stationary phase in G0 minimal medium containing nicotinic acid (200 ml or 400 ml for strains carrying multi-copy plasmids), washed in deionized water and resuspended in the buffer used for the enzymes assays. The cells were disrupted by shaking for 6 min with glass beads (0.45 mm diameter) and centrifuged for 5 min at 4°C in a microfuge. The enzymatic activities were assayed in the supernatants. The QPRTase activity was measured essentially as described in [10] with [^{3}H]quinolinic acid as substrate. Radioactive nicotinic acid mononucleotide retained on Dowex 1×8 was eluted with formic acid and was quantitated by liquid scintillation spectrometry. Kynureninase was measured fluorimetrically as described in [11] with L-kynurenine and DL-3-OH-kynurenine as substrates. The formation of the products of the kynureninase reaction, L-3-hydroxyanthranilate and anthranilate, was measured using a Cary 3 spectrophotometer (Varian Inc.) with a total fluorescence accessory set at excitation wavelength 315 nm and the BG12 glass filter (band pass 325–500 nm with the maximum at 400 nm). The increase in the fluorescence was followed for 10 min at room temperature. The amount of L-3-hydroxyanthranilate or anthranilate was calculated using anthranilic acid solution as fluorescence standard. Protein concentration was assayed using the Bio-Rad Protein Assay Reagent in accordance with the manufacturer's instructions.

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3. Results

3.1. NPT1 is essential for anaerobic growth in S. cerevisiae

We have used insertion mutagenesis to identify genes that are essential for anaerobic growth in *S. cerevisiae*. To do this, the haploid strain CN026-1A was transformed by a *LacZ* yeast genomic DNA fusion bank [12]. In this way the majority of the fusions obtained are null alleles of the host gene. Individual transformants were grown up in 384-well microtiter plates, replicated onto two CSM+TE-leu plates and incubated under aerobic or anaerobic conditions. Transformants unable to grow under anaerobic conditions were retested and in the case of positive transformants the host gene was identified using a PCR strategy. In a pilot experiment, 3840 transformants were tested (~25 000 transformants are needed to give an average of one insertion every 500 bp). Only one gene clearly essential for anaerobic growth was identified. This figure was much lower than expected. Control experiments with the *HAP1* gene which is know to be essential for strict anaerobic growth [13] indicated that the system used to generate the anaerobic environment produced a severe oxygen limitation rather than strict anaerobic conditions and the screen was not continued.

The gene identified in the pilot experiment was *NPT1*, which encodes the NAPRTase, an enzyme involved in NAD^+ biosynthesis [14], which has also been implicated in silencing [15] and aging [16]. To confirm that *NPT1* is essential for anaerobic growth we have deleted the gene in the strain IW303 as described in Section 2 to give the strain CN028. The results in Fig. 1 show that after sporulation and micro-dissection of the heterozygote CN028 (*NPT1*/*Δnpt1::G418*) the anaerobic growth deficiency co-segregated with the deletion marker, demonstrating that *NPT1* is essential for growth under anaerobic conditions. In yeast, the nicotinate moiety of NAD can be generated in two ways: either by the kynurenine pathway from tryptophan [1] or by recycling from degraded NAD^+ [17] (see Fig. 2). An examination of the kynurenine pathway shows that oxygen is required at three steps, thus under anaerobic conditions the nicotinate required for NAD^+ biosynthesis must be incorporated via the *NPT1*-encoded NAPRTase, explaining why *NPT1* is an essential gene under anaerobic conditions.

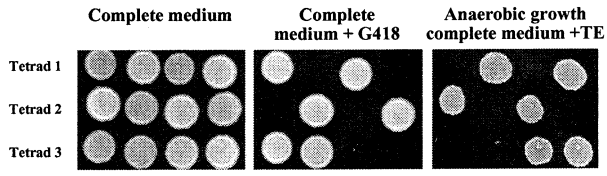

Fig. 1. NPT1 is essential for anaerobic growth. The heterozygote *NPT1*/*Δnpt1::G418* (CN028) was sporulated, micro-dissected and growth of the haploid strains tested on complete medium, complete medium+G418 (to select for the *Δnpt1::G418* allele) and under anaerobic conditions. The plates were incubated for 2 days at 28°C, the *Δnpt1::G418* allele co-segregates with the inability to grow under anaerobic conditions.

3.2. S. cerevisiae is a nicotinic acid auxotroph under anaerobic conditions

An examination of the pathways for NAD^+ biosynthesis in Fig. 2 leads to two hypotheses: first, wild-type *S. cerevisiae* strains should be nicotinic acid auxotrophs under anaerobic conditions, and second, mutations in the kynurenine pathway should be co-lethal with the deletion of *NPT1*. In order to test the first hypothesis the diploid strain IW303 was plated on solid G0+TE medium ± nicotinic acid and incubated under aerobic and anaerobic conditions. The results presented in Fig. 3A show that in the absence of nicotinic acid the cells are unable to grow without oxygen. Thus wild-type yeast cells are nicotinic acid auxotrophs under anaerobic conditions.

Table 1
S. cerevisiae strains used in this study

Strain	Genotype	Plasmid
IW303	*MATa*/*MATα*, *ade2-1*, *his3-11*, *15*, *leu2-3*, *12*, *TRP1*, *ura3-1*, *can1-100*	none
CN026-1A	*MATα*, *ade2-1*, *his3-11*, *15*, *leu2-3*, *12*, *trp1-1*, *ura3-1*, *can1-100*	none
CN028	*MATa*/*MATα*, *ade2-1*, *his3-11*, *15*, *leu2-3*, *12*, *trp1-1*, *ura3-1*, *can1-100*, *NPT1*/*Δnpt1::G418*	none
CN028-1B	*MATα*, *ade2-1*, *his3-11*, *15*, *leu2-3*, *12*, *trp1-1*, *ura3-1*, *can1-100*, *Δnpt1::G418*	none
CN028-1D	*MATa*, *ade2-1*, *his3-11*, *15*, *leu2-3*, *12*, *trp1-1*, *ura3-1*, *can1-100*, *Δnpt1::G418*	*NPT1*, *URA3* (CPYCp001)
RKO2-1C	*MATa*, *ade2-1*, *his3-11*, *15*, *leu2-3*, *12*, *trp1-1*, *ura3-1*, *can1-100*, *Δbna1::HIS3*	none
CN009	*MATa*/*MATα*, prototroph	none
CN008-1A	*MATa*, *leu2-3*, *12*	none
CP002-3C	*MATa*, *ade2-1*, *his3-11*, *15*, *leu2-3*, *12*, *ura3-1*, *can1-100*, *Δbna3::HIS3*	none
CP003-5B	*MATa*, *ade2-1*, *his3-11*, *15*, *leu2-3*, *12*, *ura3-1*, *can1-100*, *Δbna2::HIS3*	none
CP004-1A	*MATa*, *ade2-1*, *his3-11*, *15*, *leu2-3*, *12*, *ura3-1*, *can1-100*, *Δbna4::HIS3*	none
CP005-2D	*MATa*, *ade2-1*, *his3-11*, *15*, *leu2-3*, *12*, *ura3-1*, *can1-100*, *Δbna5::HIS3*	none
CP006-4B	*MATa*, *ade2-1*, *his3-11*, *15*, *leu2-3*, *12*, *ura3-1*, *can1-100*, *Δbna6::HIS3*	none
MNY03-1C	*MATa*, *leu2*, *Δbna5::G418*	none
MNY01-3B	*MATa*, *leu2*, *Δbna6::G418*	none
CP001-2C	*MATa*, *ade2-1*, *his3-11*, *15*, *leu2-3*, *12*, *trp1-1*, *ura3-1*, *can1-100*, *Δnpt1::G418*	*NPT1*, *URA3* (CPYCp001)
CP001-5B	*MATa*, *ade2-1*, *his3-11*, *15*, *leu2-3*, *12*, *trp1-1*, *ura3-1*, *can1-100*, *Δnpt1::G418*, *Δbna1::HIS3*	*NPT1*, *URA3* (CPYCp001)
CP001-15D	*MATα*, *ade2-1*, *his3-11*, *15*, *leu2-3*, *12*, *trp1-1*, *ura3-1*, *can1-100*, *Δnpt1::G418*, *Δbna1::HIS3*	*BNA1*, *URA3* (RKYCp002)
CP001-17A	*MATα*, *ade2-1*, *his3-11*, *15*, *leu2-3*, *12*, *trp1-1*, *ura3-1*, *can1-100*, *Δbna1::HIS3*	*BNA1*, *URA3* (RKYCp002)
CP007-3A	*MATα*, *ade2-1*, *his3-11*, *15*, *leu2-3*, *12*, *ura3-1*, *can1-100*, *Δnpt1::G418*, *Δbna3::HIS3*	*NPT1*, *URA3* (CPYCp001)
CP008-1D	*MATα*, *ade2-1*, *his3-11*, *15*, *leu2-3*, *12*, *ura3-1*, *can1-100*, *Δnpt1::G418*, *Δbna2::HIS3*	*NPT1*, *URA3* (CPYCp001)
CP009-7B	*MATα*, *ade2-1*, *his3-11*, *15*, *leu2-3*, *12*, *ura3-1*, *can1-100*, *Δnpt1::G418*, *Δbna5::HIS3*	*NPT1*, *URA3* (CPYCp001)
CP010-3A	*MATα*, *ade2-1*, *his3-11*, *15*, *leu2-3*, *12*, *ura3-1*, *can1-100*, *Δnpt1::G418*, *Δbna6::HIS3*	*NPT1*, *URA3* (CPYCp001)
CP011-1A	*MATα*, *ade2-1*, *his3-11*, *15*, *leu2-3*, *12*, *ura3-1*, *can1-100*, *Δnpt1::G418*, *Δbna4::HIS3*	*NPT1*, *URA3* (CPYCp001)

All the strains used are derived from the homozygous diploid strain W303, *MATa*/*MATα*, *ade2-1*, *his3-11*, *15*, *leu2-3*, *12*, *trp1-1*, *ura3-1*, *can1-100* [3].

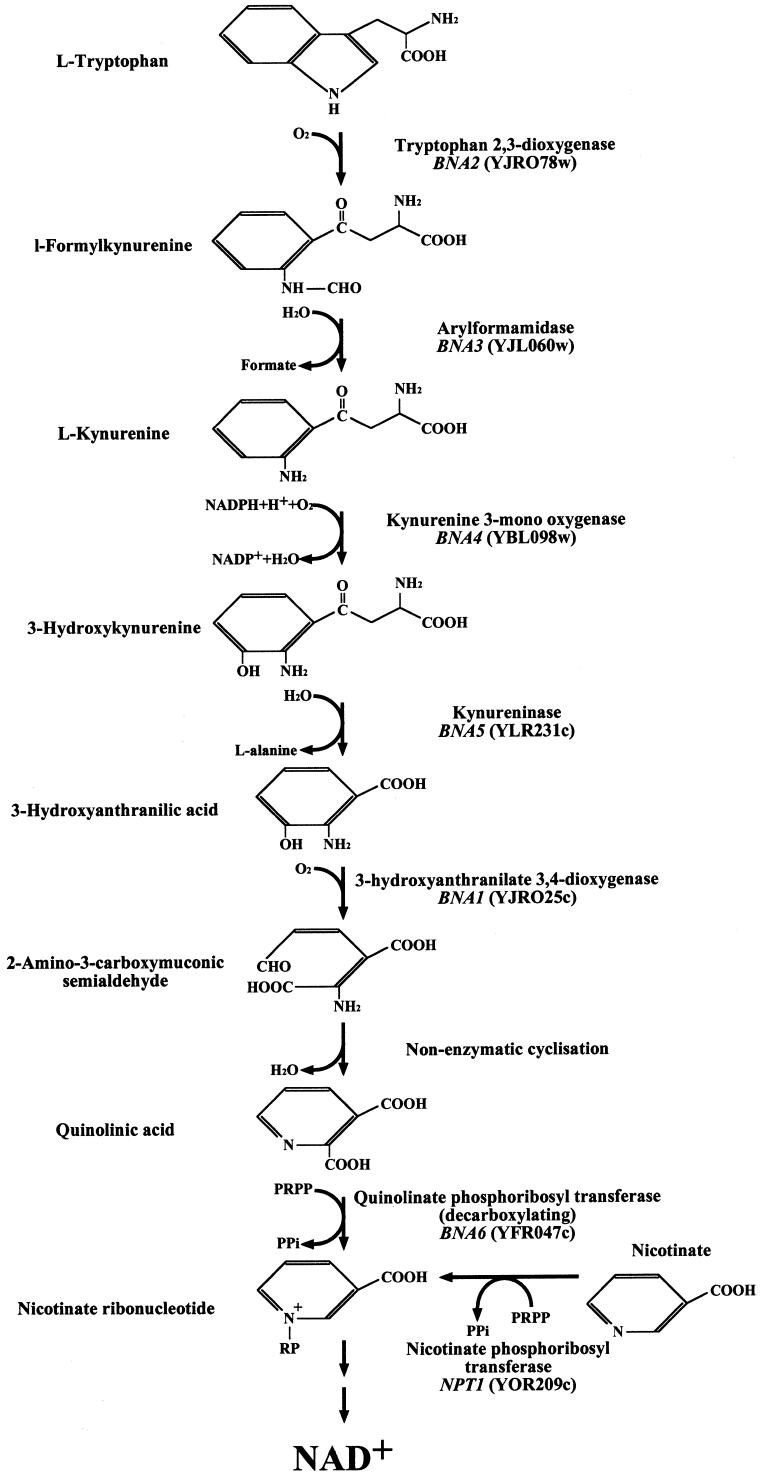

Fig. 2. Biosynthesis of NAD^+. Schematic view of the intermediates, enzymes and genes involved in NAD^+ biosynthesis via the kynurenine pathway or by direct incorporation of nicotinic acid.

3.3. Δbna1 and Δnpt1 are co-lethal

We have previously shown that *BNA1* encodes 3-hydroxyanthranilate 3,4-dioxygenase, which is an enzyme of the kynurenine pathway [1]. To determine if Δ*bna1* and Δ*npt1* are co-lethal, RK02-1C (Δ*bna1::HIS3*) was crossed to CN028-1B (Δ*npt1::G418*) to give the diploid CP001. Upon sporulation and micro-dissection no spores carrying both the *BNA1* and *NPT1* deletions were obtained suggesting that this combination is co-lethal. To confirm this, CP001 was transformed by the plasmids RKYCp002 (*BNA1*, *URA3*) [1] or CPYCp001 (*NPT1*, *URA3*), the resulting diploids were micro-dissected. In the presence of either plasmid the double mutant (Δ*bna1*, Δ*npt1*) could be obtained. To test the ability of these strains and the control single deletions to loose the plasmids, they

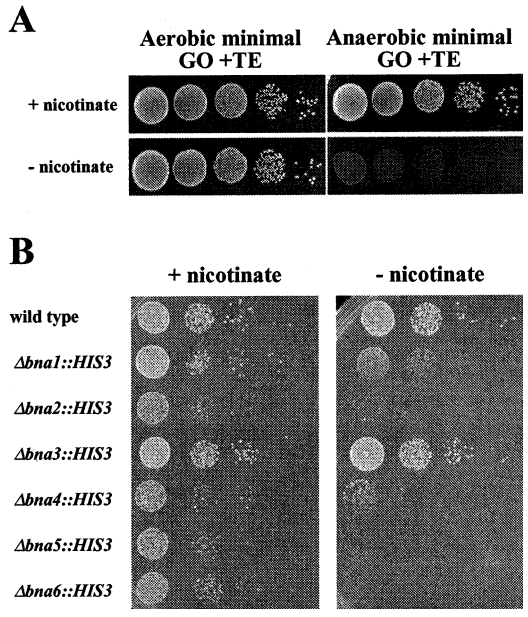

Fig. 3. The nicotinic acid auxotroph phenotype of wild-type *S. cerevisiae* and the Δ*bna* mutants. A: Wild-type *S. cerevisiae* (IW303) was plated on minimal G0+TE medium ± nicotinic acid and incubated for 3 days at 28°C in aerobic and anaerobic conditions. In the absence of nicotinic acid cells were only able to grow under aerobic conditions. B: The wild-type and Δ*bna* mutants (wild-type (CN026-1A), Δ*bna1::HIS3* (RK02-1C), Δ*bna2::HIS3* (CP003-5B), Δ*bna3::HIS3* (CP002-3C), Δ*bna4::HIS3* (CP004-1A), Δ*bna5::HIS3* (CP005-2D) and Δ*bna1::HIS3* (CP006-4B)) were plated on G0 minimal medium ± nicotinic acid. The phenotype is shown after 3 days growth at 28°C.

were grown overnight in non-selective liquid medium and then plated on minimal medium ± 5-fluoro orotic acid (5FOA). In the presence of 5FOA only *ura3* strains, i.e. strains that have lost the plasmid, will be able to grow. Fig. 4A shows that the single deletions are able to loose the *URA3* plasmid, whereas the double deletions cannot. This demonstrates that Δ*bna1* and Δ*npt1* are co-lethal.

3.4. The kynurenine pathway in S. cerevisiae

The observation that a mutation in the kynurenine pathway (Δ*bna1*) and Δ*npt1* are co-lethal provides a way of identifying other members of the kynurenine pathway in *S. cerevisiae*. A comparison of the sequence of the different enzymes in the kynurenine pathway in other organisms with the *S. cerevisiae* genome sequence allowed us to predict the *S. cerevisiae* genes that encode these enzymes. In each case a single gene was identified (data not shown), we have named these genes *BNA2–BNA6* for biosynthesis of nicotinic acid. The correspondence between the genes and the enzymes is shown in Fig. 2.

To determine if these genes are involved in nicotinic acid biosynthesis they were deleted in the diploid strain IW303 using a heterologous *HIS3* marker as described in Section 2 to give the strains CP002, CP003, CP004, CP005 and CP006, which are heterozygous for the deletion of *BNA3*, *BNA2*, *BNA4*, *BNA5* and *BNA6* respectively. These diploids were all sporulated and micro-dissected and haploid segregants carrying the deleted *BNA* genes were tested for their ability to grow in the absence of nicotinic acid. The results are presented in Fig. 3B and show that with the exception of the Δ*bna3* strain the deletion mutants show a more or less pronounced reduction of growth in the absence of nicotinic acid. This is clearest with the Δ*bna6* strain and least evident with the Δ*bna1* strain; we have already noted that the growth reduction of the Δ*bna1* strain in the absence of nicotinate is much clearer in liquid medium than on solid medium [1].

If the new *BNA* genes do encode enzymes that are part of the kynurenine pathway we would expect them to be co-lethal with the deletion of *NPT1*. To confirm this haploid strains carrying deletions of the individual *BNA* genes were crossed to CN028-1D (*MATa*, Δ*npt1::G418* centromeric plasmid *NPT1*, *URA3*) and haploid segregants carrying the double deletion Δ*bna*, Δ*npt1::G418* and the centromeric plasmid *NPT1*, *URA3* were isolated after sporulation and micro-dissection. These strains were grown in non-selective liquid medium and then plated onto minimal medium ± 5FOA. The results in Fig. 4B show that the only double mutant able to grow on 5FOA medium, and hence able to loose the *NPT1* plasmid is Δ*bna3::HIS3*, Δ*npt1::G418*. Therefore, we can conclude that the deletion of *BNA2*, *BNA4*, *BNA5* or *BNA6* is co-lethal with Δ*npt1*.

3.5. BNA5 encodes kynureninase and BNA6 encodes quinolate phosphoribosyl transferase

To confirm that the new *BNA* genes do encode enzymes of the kynurenine pathway we have determined the levels of kynureninase and quinolinate phosphoribosyl transferase activity in wild-type and strains deleted and deleted/complemented for *BNA5* and *BNA6*. To do this *BNA5* and *BNA6* were deleted and replaced by the G418 resistance marker in the strain CN009 (diploid prototroph). After sporulation and micro-dissection spores carrying the deleted genes were crossed to CN008-1A (*leu2*) and further segregated to give MNY03-1C (*MATa*, *leu2*, Δ*bna5::G418*) and MNY01-3B (*MATa*, *leu2*, Δ*bna6::G418*). The wild-type *BNA5* (pMN231) and *BNA6* (pMN047) genes were cloned after PCR amplification and transformed into the corresponding deleted strain. Cell-free extracts were prepared and the kynureninase and phosphoribosyl transferase activities were deter-

Table 2
Kynureninase and quinolinate phosphoribosyl transferase activities in wild-type, *BNA5* and *BNA6* deleted and deleted complemented strains

Strain	Kynureninase (IU × 10^{-4}/mg protein (S.D.))		QPRTase (pmol/min/mg protein (S.D.))
	kynurenine	3-(OH)-kynurenine	[^{3}H]quinolinic acid
Wild-type	9.2 (2.4)	17.4 (4.4)	0.37 (0.11)
Δ*bna5::G418*	0	0	nd
Δ*bna5::G418*+p*BNA5*	10.9 (4.7)	21.3 (5.1)	nd
Δ*bna6::G418*	nd	nd	0
Δ*bna6::G418*+p*BNA6*	nd	nd	0.51 (0.25)

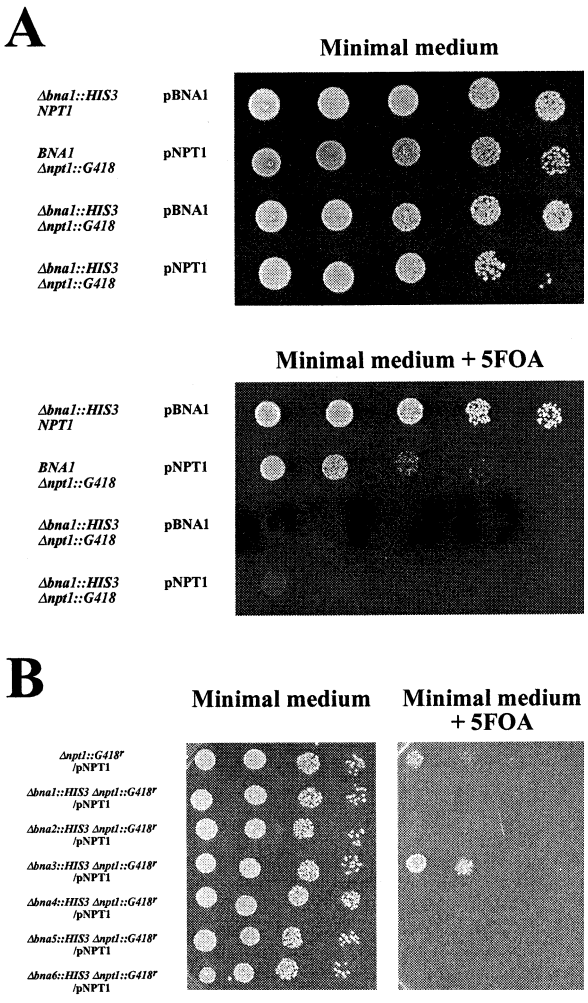

Fig. 4. The co-lethality of Δ*npt1* with the Δ*bna* mutations. A: Strains carrying different combinations of deletions of *BNA1* and *NPT1* and complementing plasmids (Δ*bna1::HIS3*, *NPT1*, pBNA1 (CP001-17A), *BNA1*, Δ*npt1::G418*, pNPT1 (CP001-2C), Δ*bna1::HIS3*, Δ*npt1::G418*, pBNA1 (CP001-15D) and Δ*bna1::HIS3*, Δ*npt1::G418*, pNPT1 (CP001-5B)) were grown in non-selective medium and plated onto minimal medium and minimal medium+5-FOA. The strains carrying the double deletion were not able to loose the complementing plasmid and grown on 5FOA medium, shown is that Δ*bna1* and Δ*npt1* are co-lethal. B: A control strain (Δ*npt1::G418* (CN028-1B)) and Δ*npt1*, Δ*bna* double mutants (Δ*bna1::HIS3*, Δ*npt1::G418* (CP001-5B), Δ*bna2::HIS3*, Δ*npt1::G418* (CP008-1D), Δ*bna3::HIS3*, Δ*npt1::G418* (CP007-3A), Δ*bna4::HIS3*, Δ*npt1::G418* (CP011-1A), Δ*bna5::HIS3*, Δ*npt1::G418* (CP009-7B) and Δ*bna1::HIS3*, Δ*npt1::G418* (CP010-3A)) all containing the *URA3*, *NPT1* plasmid, CPYCp001, were grown on non-selective medium and plated onto minimal medium and minimal medium+5-FOA. After 3 days growth at 28°C only the control and Δ*bna3::HIS3*, Δ*npt1::G418* (CP007-3A) strains were able to grow on 5FOA medium.

mined as described in Section 2. The results in Table 2 show that when *BNA5* is deleted, kynureninase activity is undetectable and it is restored to wild-type levels when the wild-type gene is introduced on a centromeric plasmid. Similarly, phosphoribosyl transferase activity is undetectable when *BNA6* is deleted, but is restored to wild-type levels in the presence of the cloned gene. These results confirm that *BNA5* and *BNA6* are part of the kynurenine pathway and encode kynureninase and phosphoribosyl transferase respectively.

4. Discussion

We have identified the genes encoding the different enzymes of the kynurenine pathway in *S. cerevisiae* and for three of them: *BNA1*, *BNA5* and *BNA6*, confirmed the enzyme attribution by direct assays ([1] and Table 2). A previous study [16] suggested that *YFR047c* (*BNA6*) and *NPT1* function in two redundant NAD^+ biosynthesis pathways in yeast and we have shown that mutations in these two pathways are co-lethal (Fig. 4). This co-lethality suggests that no other pathway for NAD^+ biosynthesis exists in *S. cerevisiae*.

Three steps of the kynurenine pathway require oxygen, therefore in the absence of oxygen NAD^+ must be synthesized either by the salvage pathway from NAD^+ or by the direct incorporation of nicotinic acid via *NPT1* (Fig. 2). Therefore, *S. cerevisiae* is a nicotinic acid auxotroph under anaerobic conditions (Fig. 3A). This is consistent with the observations of [18] who noted that under aerobic conditions labeled tryptophan was preferentially incorporated into NAD^+, whereas under anaerobic conditions it was labeled aspartate and glutamate that were preferentially incorporated into NAD^+. We can now explain these results as follows: under aerobic conditions, labeled tryptophan will be transformed into the nicotinic acid moiety of NAD^+ using the kynurenine pathway, and under anaerobic conditions, labeled aspartate and glutamate will be converted into oxaloacetate and α-ketoglutarate and enter cellular metabolism via the Krebs cycle, thus labeling the adenine dinucleotide moiety of NAD^+.

There are two phenomena that remain to be clarified: the poor nicotinic acid auxotroph phenotype of the Δ*bna* mutants and the absence of phenotype for the Δ*bna3* mutation. It is now clear that cells can survive with very reduced levels of NAD^+, the Δ*npt1* strain has a wild-type growth phenotype but contains only 35% of the wild-type level of NAD^+ [17]. Thus it is possible that in drop tests recycling NAD^+ may give rise to significant residual growth. This is consistent with our observations that the nicotinic acid auxotroph phenotype is clearer in liquid medium or in drop tests where the strains have been starved overnight in nicotinic acid minus medium ([1] and Fig. 3B). Two hypotheses can be advanced to account for the lack of phenotype associated with the Δ*bna3* mutation: either *BNA3* does not encode the arylformamidase or another enzyme can perform the same reaction. The computer analysis clearly indicated *BNA3* as the gene encoding arylformamidase. However, *S. cerevisiae* contains at least three other formyltransferases: *ADE8*, which encodes phosphoribosylglycinamide formyltransferase [19], and *ADE16* and *ADE17*, which encode 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase isozymes [20]. So it is possible that one, or a combination of these enzymes may be able to replace the putative arylformamidase encoded by *BNA3*. We have created a Δ*bna3*, *ade8* double mutant, but no nicotinic acid auxotroph phenotype could be detected (data not shown). Therefore further experiments will be necessary to resolve this question and to determine if a combination of mutations in these different formyltransferases can lead to a nicotinic acid auxotroph phenotype.

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