Identification of oxygen-independent pathways for pyridine-nucleotide and Coenzyme-A synthesis in anaerobic fungi by expression of candidate genes in yeast

Thomas Perli†, Aurin M. Vos†, Jonna Bouwknegt, Wijb J. C. Dekker, Sanne J. Wiersma, Christiaan Mooiman, Raúl A. Ortiz-Merino, Jean-Marc Daran, Jack T. Pronk*

Department of Biotechnology, Delft University of Technology, Van der Maasweg 2629 Delft, The Netherlands.

*Corresponding author: Department of Biotechnology, Delft University of Technology, Van der Maasweg 9, 2629 Delft, The Netherlands. Tel: +31-15-27-83214; E-mail: J.T.Pronk@tudelft.nl

†These authors contributed equally.

Thomas Perli	T.Perli@tudelft.nl	0000-0002-4910-3292
Aurin M. Vos	A.M.Vos@tudelft.nl	
Jonna Bouwknegt	J.Bouwknegt@tudelft.nl	0000-0316-1238-7873
Wijb J. C. Dekker	W.J.C.Dekker@tudelft.nl	
Sanne J. Wiersma	S.J.Wiersma@tudelft.nl	
Christiaan Mooiman	C.Mooiman@tudelft.nl	
Raúl A. Ortiz-Merino	Raul.Ortiz@tudelft.nl	0000-0003-4186-8941
Jean-Marc Daran	J.G.Daran@tudelft.nl	0000-0003-3136-8193
Jack T. Pronk	J.T.Pronk@tudelft.nl	0000-0002-5617-4611

Abstract

Neocallimastigomycetes are rare examples of strictly anaerobic eukaryotes. This study investigates how these anaerobic fungi bypass reactions involved in synthesis of pyridine nucleotide cofactors and coenzyme A that, in canonical fungal pathways, require molecular oxygen. Analysis of Neocallimastigomycete proteomes identified a candidate L-aspartate-decarboxylase (AdcA), and L-aspartate oxidase (NadB) and quinolinate synthase (NadA), constituting putative oxygen-independent bypasses for coenzyme A synthesis and pyridine nucleotide cofactor synthesis, respectively. The corresponding gene sequences indicated acquisition by ancient horizontal gene transfer event involving bacterial donors. To test whether these enzymes suffice to bypass corresponding oxygen-requiring reactions, they were introduced into *fms1Δ* and *bna2Δ Sacharomyces cerevisiae* strains. Expression of *nadA* and *nadB,* and *adcA* from the Neocallimastigomycetes *Piromyces finnis* and *Neocallimastix californiae*, respectively, conferred cofactor prototrophy under aerobic and anaerobic conditions. This study simulates how horizontal gene transfer can drive eukaryotic adaptation to anaerobiosis, and provides a basis for elimination of auxotrophic requirements in anaerobic industrial applications of yeasts and fungi.

Introduction

Neocallimastigomycetes are obligately anaerobic fungi with specialised metabolic adaptations that allow them to play a key role in the degradation of recalcitrant plant biomass in herbivore guts [1]. Despite complicated cultivation techniques and lack of genetic-modification tools [2], several evolutionary adaptations of these eukaryotes to an anaerobic lifestyle have been inferred from biochemical studies [3-5]. Sequence analysis implicated extensive horizontal gene transfer (HGT) events as a key mechanism in these adaptations [6-8]. For example, instead of sterols, which occur in membranes of virtually all other eukaryotes [9] and whose biosynthesis involve multiple oxygen-dependent reactions [10], Neocallimastigomycetes contain tetrahymanol [3, 6]. This sterol surrogate [11] can be formed from squalene by a squalene:tetrahymanol cyclase (STC), whose structural gene in Neocallimastigomycetes showed evidence of acquisition by HGT from prokaryotes [6, 12]. Expression of an STC gene was recently shown to enable sterol-independent anaerobic growth of the model eukaryote *S. cerevisiae* [13].

Further exploration of oxygen-independent bypasses in Neocallimastigomycetes for intracellular reactions that in other eukaryotes require oxygen is relevant for a fundamental understanding of the requirements for anaerobic growth of eukaryotes. In addition, it may contribute to the elimination of nutritional requirements in industrial anaerobic applications of yeasts and fungi.

Most fungi are capable of *de novo* synthesis of pyridine-nucleotide cofactors (NAD^+ and $NADP^+$) and Coenzyme A (CoA) when grown aerobically. As exemplified by the facultatively anaerobic yeast *S. cerevisiae* [14], canonical fungal pathways for synthesis of these cofactors are oxygen dependent. In *S. cerevisiae,* biosynthesis of CoA involves formation of β-alanine by the oxygen-requiring polyamine oxidase Fms1 [15]. This intermediate is then condensed with pantoate to yield the CoA precursor pantothenate

[16, 17] (Fig. 1A). Similarly, the yeast kynurenine pathway for *de novo* synthesis of NAD^+ involves three oxygen-dependent reactions, catalyzed by indoleamine 2,3-dioxygenase (Bna2; EC 1.13.11.52), kynurenine 3-monooxygenase (Bna4; EC 1.14.13.9), and 3-hydroxyanthranilic-acid dioxygenase (Bna1; EC 1.13.11.6) [14] (Fig. 1B). The Neocallimastigomycete *Neocallimastix patricianum* has been shown to grow in synthetic media lacking precursors for pyridine-nucleotide and CoA synthesis [18]. This observation indicates that at least some anaerobic fungi harbour oxygen-independent pathways for synthesizing these essential cofactors. Genomes of Neocallimastigomycetes lack clear homologs of genes encoding the oxygen-requiring enzymes of the kynurenine pathway. Instead, their genomes were reported to harbour genes encoding an L-aspartate oxidase (NadB) and quinolinate synthase (NadA), two enzymes active in the bacterial pathway for NAD^+ synthesis [6] (Fig. 1A). Since bacterial and plant aspartate oxidases can, in addition to oxygen, also use fumarate as electron acceptor [19, 20], it is conceivable that NadA and NadB may allow for oxygen-independent NAD^+ synthesis in anaerobic fungi. No hypothesis has yet been forwarded on how these fungi may bypass the oxygen requirement for the canonical fungal CoA biosynthesis route.

The goals of this study were to identify the pathway responsible for oxygen-independent synthesis of CoA in Neocallimastigomycetes and to investigate a possible role of NadA and NadB in oxygen-independent synthesis of pyridine-nucleotide cofactors. A candidate L-aspartate decarboxylase (Adc) encoding gene was identified by genome analysis of Neocallimastigomycetes and its phylogeny investigated. Candidate Neocallimastigomycete genes for L-aspartate oxidase and quinolinate synthase, previously reported to have been acquired by HGT [6], as well as the candidate Adc gene, were then functionally analysed by expression in *S. cerevisiae* strains devoid of essential steps in the native cofactor synthesis pathways. As controls, previously characterized

genes involved in oxygen-independent NAD^+ biosynthesis by *Arabidopsis thaliana* [21], and a previously characterized Adc encoding gene from the red flour beetle *Tribolium castaneum* (*TcPAND*) [22] were also expressed in the same *S. cerevisiae* strains. The results demonstrate how heterologous expression studies in yeast can provide insight into evolutionary adaptations to anaerobic growth and selective advantages conferred by proposed HGT events in Neocallimastigomycetes. In addition, they identify metabolic engineering strategies for eliminating oxygen requirements for cofactor biosynthesis in anaerobic industrial applications of *S. cerevisiae*.

Material and Methods

Strains, media and maintenance

S. cerevisiae strains used and constructed in this study (Table 1) were derived from the CEN.PK lineage [23]. Yeast cultures were routinely propagated in YP (10 g L^{-1} Bacto yeast extract [Becton, Dickinson and Co., Sparks, MD], 20 g L^{-1} Bacto peptone [Becton, Dickinson and Co]) or synthetic medium (SM) [24]. YP and SM were autoclaved at 121 °C for 20 min. SM was then supplemented with 1 mL L^{-1} of filter-sterilized vitamin solution (0.05 g L^{-1} D-(+)-biotin, 1.0 g L^{-1} D-calcium pantothenate, 1.0 g L^{-1} nicotinic acid, 25 g L^{-1} myo-inositol, 1.0 g L^{-1} thiamine hydrochloride, 1.0 g L^{-1} pyridoxol hydrochloride, 0.20 g L^{-1} 4-aminobenzoic acid). Where indicated, nicotinic acid or pantothenic acid were omitted from the vitamin solution, yielding SM without nicotinic acid (SMΔnic) and SM without pantothenic acid (SMΔpan), respectively. A concentrated glucose solution was autoclaved separately for 15 min at 110 °C and added to SM and YP to a concentration of 20 g L^{-1} or 50 g L^{-1}, yielding SMD and YPD, respectively. SMD with urea or acetamide instead of ammonium sulfate (SMD-urea and SMD-Ac, respectively) were prepared as described previously [25, 26]. For anaerobic growth experiments, sterile media were supplemented

with Tween 80 (polyethylene glycol sorbate monooleate, Merck, Darmstadt, Germany) and ergosterol (≥95 % pure, Sigma-Aldrich, St. Louis, MO) as described previously [27]. Yeast strains were grown in 500-mL shake flasks containing 100 mL medium or in 100-mL shake flasks containing 20 mL medium. Shake-flask cultures were incubated at 30 °C and shaken at 200 rpm in an Innova Incubator (Brunswick Scientific, Edison, NJ). Solid media were prepared by adding 15 g L^{-1} Bacto Agar (Becton, Dickinson and Co) and, when indicated, 200 mg L^{-1} G418 (Thermo Scientific, Waltham, MA). After genotyping, engineered strains were restreaked twice to select single clones. Removal of the gRNA carrying plasmid was done as previously described [28]. Stock cultures were prepared by adding glycerol to a final concentration of 33 % (v/v), frozen and stored at -80°C.

Molecular biology techniques

DNA was PCR amplified with Phusion Hot Start II High Fidelity Polymerase (Thermo Scientific) and desalted or PAGE-purified oligonucleotide primers (Sigma Aldrich) by following manufacturers' instructions. DreamTaq polymerase (Thermo Scientific) was used for diagnostic PCR. Oligonucleotide primers used in this study are listed in Supplementary Table S1. PCR products were separated by gel electrophoresis using 1 % (w/v) agarose gel (Thermo Scientific) in TAE buffer (Thermo Scientific) at 100 V for 25 min and purified with either GenElute PCR Clean-Up Kit (Sigma Aldrich) or with Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA). Plasmids were purified from *E. coli* using a Sigma GenElute Plasmid Kit (Sigma Aldrich). Yeast genomic DNA was isolated with the SDS/LiAc protocol [29]. Yeast strains were transformed with the lithium acetate method [30]. Four to eight single colonies were re-streaked three consecutive times on selective media and diagnostic PCR were performed to verify their genotype. *Escherichia coli* XL1-blue was used for chemical transformation [31]. Plasmids were then

isolated and verified by either restriction analysis or by diagnostic PCR. Lysogeny Broth (LB; 10 g L^{-1} Bacto Tryptone, 5 g L^{-1}, Bacto Yeast Extract with 5 g L^{-1} NaCl) was used to propagate *E. coli* XL1-Blue. LB medium was supplemented with 100 mg L^{-1} ampicillin for selection of transformants. The overnight grown bacterial cultures were stocked by adding sterile glycerol at a final concentration of 33 % (v/v) after which samples were frozen and stored at -80 °C.

Plasmid construction

Plasmids used and cloned in this study are shown in Table 2. Plasmids carrying two copies of the same gRNA were cloned by Gibson assembly [28, 32]. In brief, an oligo carrying the gene-specific 20 bp target sequence and a homology flank to the plasmid backbone was used to amplify the fragment carrying the 2µm origin of replication sequence by using pROS13 as template. The backbone linear fragment was amplified using primer 6005 and pROS11 as template [33]. The two fragments were then gel purified and assembled *in vitro* using the NEBuilder HiFi DNA Assembly Master Mix (New England BioLabs, Ipswich, MA) following manufacturer's instructions. Transformants were selected on LB plates supplemented with 100 mg L^{-1} ampicillin or 50 mg L^{-1} kanamycin. Primer 11861 was used to amplify the 2µm fragment containing two identical gRNA sequences for targeting *BNA2*. The PCR product was then cloned in a pROS11 backbone yielding plasmid pUDR315.

The coding sequences for *AtNADA*, *AtNADB*, *PfnadA*, *PfnadB*, and *NcadcA* were codon-optimized for expression in *S. cerevisiae* and ordered as synthetic DNA through GeneArt (Thermo Fisher Scientific). The plasmids carrying the expression cassettes for *TcPAND, AtNADA*, *AtNADB*, *PfnadA* and *PfnadB* were cloned by Golden Gate assembly using the Yeast Toolkit (YTK) DNA parts [34]. These plasmids were cloned using the pYTK096 integrative backbone that carries long homology arms to the *URA3 locus* and a *URA3*

163 expression cassette allowing for selection on SM lacking uracil. The *TcPAND* coding
164 sequence was amplified using the primer pair 11877/11878 and pCfB-361 as template.
165 Then, the linear *TcPAND* gene and plasmids pUD1096, pUD1097, pUD652, and pUD653
166 carrying the coding sequence for *AtNADA*, *AtNADB*, *PfnadA*, and *PfnadB*, respectively, were
167 combined together with YTK-compatible part plasmids in BsaI (New England BioLabs)
168 golden gate reactions to yield plasmid pUDI168, pUDI245, pUDE931, pUDI243, and
169 pUDI244, respectively. A detailed list of the YTK-compatible parts used for constructing
170 each plasmid can be found in Supplementary Table S2.
171 The plasmid carrying the expression cassette for *NcadcA* was cloned by Gibson assembly.
172 The *pTDH3* promoter, the *NcadcA* coding sequence, the *tENO2* terminator and the
173 pYTK0096 backbone were amplified by PCR using primer pairs 16721/16722,
174 16723/16724, 16725/16726, and 16727/16728 respectively, using pYTK009, pUD1095,
175 pYTK055, and pYTK096 as template, respectively. Each PCR product was then gel purified
176 and combined in equimolar amounts in a Gibson reaction that yielded pUDI242.

177 **Strain construction**

178 *S. cerevisiae* strains were transformed using the LiAc/SS-DNA/PEG and CRISPR/Cas9
179 method [28, 30, 35]. For deletion of the *BNA2* gene, IMX585 (*can1Δ::Spycas9-natNT2*) was
180 transformed with 500 ng of the *BNA2* targeting gRNA plasmid pUDR315 together with
181 500 ng of the annealed primer pair 11862/11863 as repair dsDNA oligo, yielding strain
182 IMK877. The resulting strain was then used for the integration of the two heterologous
183 *NADB-A* pathways. Expression cassettes for *AtNADA*, *AtNADB*, *PfnadA*, *PfnadB*, were
184 amplified from plasmids pUDI245, pUDE931, pUDI243, pUDI244, respectively, using
185 primer pairs 13123/13124, 13125/10710, 13123/13124, 13125/10710, respectively.
186 Then, 500 ng of each pair of gel purified repair cassettes were co-transformed in IMK877

together with 500 ng the *SGA1* targeting gRNA plasmid, yielding IMX2302 (*sga1::AtNADA AtNADB*) and IMX2301 (*sga1::PfnadA PfnadB*).

For deletion of the *FMS1* gene, IMX581 (*can1Δ::Spycas9-natNT2 ura3-52*) was transformed with 500 ng of the *FMS1* targeting gRNA plasmid pUDR652 together with 500 ng of the annealed primer pair 13527/13528 as repair dsDNA oligo, resulting in IMX2293. Then, 500 ng of plasmids pUDI168 and pUDI242 carrying the expression cassettes for *TcPAND* and *NcadcA,* respectively, were NotI (Thermo Fisher) digested and separately transformed in IMX2293, yielding IMX2305, and IMX2300, respectively. Selection of IMX2305 and IMX2300 was done on SMD agar plate since the integration of each Adc encoding cassette also restored the *URA3* phenotype. In contrast, selection of IMK877 was done on SMD-Ac agar plates while selection of IMX2302, IMX2301, and IMX2293 was done YPD-G418 agar plates. Strains IMK877, IMX2300, IMX2302, and IMX2301 were stocked in SMD, while IMX2305 and IMX2293 were stocked in SMDΔpan and YPD, respectively.

Aerobic growth studies in shake flasks

For the determination of the specific growth rate of the engineered strains under aerobic conditions, a frozen aliquot was thawed and used to inoculate a 20 mL wake-up culture that was then used to inoculate a pre-culture in a 100 mL flask. The exponentially growing pre-culture was then used to inoculate a third flask to an initial OD_{660} of 0.2. The flasks were then incubated, and growth was monitored using a 7200 Jenway Spectrometer (Jenway, Stone, United Kingdom). Specific growth rates were calculated from at least five time-points in the exponential growth phase of each culture. Wake-up and pre-cultures of IMX2301 and IMX2302 were grown in SMDΔnic. Wake-up and pre-cultures of IMX2300

and IMX2305 were grown in SMDΔpan while wake-up and pre-cultures of IMK877 and IMX2292 were grown in SMD.

Anaerobic growth studies in shake flasks

Anaerobic shake-flask based experiments were performed in Lab Bactron 300 anaerobic workstation (Sheldon Manufacturing Inc., Cornelius, OR) containing an atmosphere of 85 % N_2, 10 % CO_2, and 5 % H_2. Flat-bottom shake flasks of 50-mL were filled with 40 mL SMD-urea media containing 50 g L^{-1} glucose as carbon source, to ensure depletion of the vitamin/growth factor of interest, and 20 g L^{-1} glucose for the first transfer. Media weer supplemented with vitamins, with and without pantothenic acid or nicotinic acid as indicated, and in all cases supplemented with Tween 80 and ergosterol. Sterile medium was placed inside the anaerobic chamber 24 h prior to inoculation for removal of oxygen. Traces of oxygen were continuously removed with a regularly regenerated Pd catalyst for H_2-dependent oxygen removal placed inside the anaerobic chamber. Aerobic overnight shake-flask cultures on SMD-urea were used to inoculate the anaerobic shake flask without pantothenic acid or without nicotinic acid at an initial OD_{600} of 0.2. Cultures were cultivated at 30 °C with continuous stirring at 240 rpm on IKA KS 260 Basic orbital shaker platform (Dijkstra Verenigde BV, Lelystad, the Netherlands). Periodic optical density measurements at a wavelength of 600 nm using an Ultrospec 10 cell density meter (Biochrom, Cambridge, United Kingdom) inside the anaerobic environment were used to follow the growth over time. After growth had ceased and the OD_{600} no longer increased the cultures were transferred to SMD-urea with 20 g L^{-1} glucose at an OD_{600} of 0.2 [27].

Anaerobic bioreactor cultivation

Anaerobic bioreactor batch cultivation was performed in 2-L laboratory bioreactors (Applikon, Schiedam, the Netherlands) with a working volume of 1.2 L. Bioreactors were

tested for gas leakage by applying 0.3 bar overpressure while completely submerging them in water before autoclaving. Anaerobic conditions were maintained by continuous sparging of the bioreactor cultures with 500 mL N_2 min^{-1} (≤0.5 ppm O_2, HiQ Nitrogen 6.0, Linde Gas Benelux, Schiedam, the Netherlands). Oxygen diffusion was minimized by using Fluran tubing (14 Barrer O2, F-5500-A, Saint-Gobain, Courbevoie, France) and Viton O-rings (Eriks, Alkmaar, the Netherlands). Bioreactor cultures were grown on either SMDΔpan or SMDΔnic with ammonium sulfate as nitrogen source. pH was controlled at 5 using 2 M KOH. The autoclaved mineral salts solution was supplemented with 0.2 g L^{-1} sterile antifoam emulsion C (Sigma-Aldrich). Bioreactors were continuously stirred at 800 rpm and temperature was controlled at 30 °C. Evaporation of water and volatile metabolites was minimized by cooling the outlet gas of bioreactors to 4 °C in a condenser. The outlet gas was then dried with a PermaPure PD-50T-12MPP dryer (Permapure, Lakewood, NJ) prior to analysis. CO_2 concentrations in the outlet gas were measured with an NGA 2000 Rosemount gas analyser (Emerson, St. Louis, MO). The gas analyser was calibrated with reference gas containing 3.03 % CO_2 and N6-grade N_2 (Linde Gas Benelux, Schiedam, The Netherlands).

Frozen glycerol stock cultures were used to inoculate aerobic 100 mL shake flask cultures on either SMDΔpan or SMDΔnic. Once the cultures reached OD_{660} > 5, a second 100 mL aerobic shake-flask pre-culture on the same medium was inoculated. When this second pre-culture reached the exponential growth phase, biomass was harvested by centrifugation at 3000 g for 5 min and washed with sterile demineralized water. The resulting cell suspension was used to inoculate anaerobic bioreactors at an OD_{660} of 0.2.

Analytical methods

Biomass dry weight measurements of the bioreactor batch experiments were performed using pre-weighed nitrocellulose filters (0.45 µm, Gelman Laboratory, Ann Arbor, MI). 10 mL culture samples were filtrated and then the filters were washed with demineralized water prior to drying in a microwave oven (20 min at 360 W) and weight measurement. Metabolite concentrations in culture supernatants were analysed by high-performance liquid chromatography (HPLC). In brief, culture supernatants were loaded on an Agilent 1260 HPLC (Agilent Technologies, Santa Clara, CA) fitted with a Bio-Rad HPX 87 H column (Bio-Rad, Hercules, CA). The flow rate was set at 0.6 mL min^{-1} and 0.5 g L^{-1} H_2SO_4 was used as eluent. An Agilent refractive-index detector and an Agilent 1260 VWD detector were used to detect culture metabolites [36]. An evaporation constant of 0.008 divided by the volume in liters, was used to correct HPLC measurements of ethanol in the culture supernatants, taking into account changes in volume caused by sampling [37]. Statistical analysis on product yields was performed by means of an unpaired two-tailed Welch's t-test.

Homology and phylogenetic analyses

A set of 51 aminoacid sequences previously used to discriminate between glutamate decarboxylases and L-aspartate decarboxylases [38] was re-used to identify candidate Neocallimastigomycete Adc sequences. These sequences were used as queries against a database containing all 58109 Neocallimastigomycete proteins deposited in Uniprot trembl (Release 2019_02), which represented 5 species (*Neocallimastix californiae*, *Anaeromyces robustus*, *Piromyces sp* E2, *Piromyces finnis*, and *Pecoramyces ruminatum*), and extracted according to the NCBI taxid 451455. Sequence homology was analysed using BLASTP 2.6.0+ [39] with 10^{-6} as e-value cut-off resulting in 13

Neocallimastigomycete sequences as shared hits from all 51 queries (Supplementary Table S3). Four of these sequences showing homology to experimentally characterised proteins with L-aspartate-decarboxylase (Adc) activity originated from *N. californiae*, and were checked for RNAseq read coverage and splicing junction support revealing A0A1Y1ZL74 as best candidate (Supplementary Fig. S1).

A0A1Y1ZL74, also referred to as *NcadcA,* was used for a second round of homology search using HMMER 3.2 [40] against a database with a balanced representation of taxa across the 3 domains of life. This database was built from Uniprot Release 2019_02 to include all refseq sequences from Bacteria (taxid 2), Eukarya (taxid 2759), and Archaea (taxid 2157; TrEMBL and Swiss-Prot categories were also included in this case). Selection for hits with more than 60% alignment length and evalue < 10^{-6} resulted in a total of 325 sequences (103 from Bacteria, 101 from Eukaryotes, and 121 from Archaea).

The set of 325 A0A1Y1ZL74 homologous sequences, together with those from Tomita *et. al.* (2015) [38] were aligned with Clustal Omega 1.2.4 [41] and then used to build a maximum likelihood phylogenetic tree with RAxML-NG 0.8.1 [42] using default parameters with the exception of the use of the PROTGTR+FO model and 100 bootstrap replicates. The resulting phylogenetic tree drawn with iTOL [43] is shown in Fig. 2, corresponding alignments and trees are provided in Supplementary Files 1 and 2.

Multiple sequence alignment was also performed with Clustal omega 1.2.4 [41] to compare selected aminoacid sequences showing candidate and experimentally characterised Adcs, against bacterial PanDs. These sequences and alignments are shown in Supplementary File 3.

Whole-genome sequencing and analysis.

Genomic DNA of strains IMX2300 and IMX2300-1 was isolated with a Blood & Cell Culture DNA Kit with 100/G Genomics-tips (QIAGEN, Hilden, Germany) according to the manufacturers' instructions. The Miseq Reagent Kit v3 (Illumina, San Diego, CA), was used to obtain 300 bp reads for paired-end sequencing. Genomic DNA was sheared to an average of 550 bp fragments using an M220 ultrasonicator (Covaris, Wolburn, MA). Libraries were prepared by using a TruSeq DNA PCR-Free Library Preparation kit (Illumina) following manufacturer's instructions. The samples were quantified by qPCR on a Rotor-Gene Q PCR cycler (QIAGEN) using the Collibri Library quantification kit (Invitrogen Carlsbad, CA). Finally, the library was sequenced using an Illumina MiSeq sequencer (Illumina, San Diego, CA) resulting in a minimum 50-fold read coverage. Sequenced reads were mapped using BWA 0.7.15-r1142-dirty [44] against the CEN.PK113-7D genome [45] containing an extra contig with the relevant integration cassette. Alignments were processed using SAMtools 1.3.1 [46], and sequence variants were called using Pilon 1.18 [47], processed with ReduceVCF 12 (https://github.com/AbeelLab/genometools/blob/master/scala/abeel/genometools/reducevcf/ReduceVCF.scala), and annotated using VCFannotator (http://vcfannotator.sourceforge.net/) against GenBank accession GCA_002571405.2 [48].

Data availability

DNA sequencing data of the *Saccharomyces cerevisiae* strains IMX2300 and IMX2300-1 were deposited at NCBI (https://www.ncbi.nlm.nih.gov/) under BioProject accession number PRJNA634013. All measurement data and calculations used to prepare Fig. 3-4 and Tables 3-4 of the manuscript are available at the 4TU.Centre for research data

repository (https://researchdata.4tu.nl/) under doi: 10.4121/uuid:c3d2326d-9ddb-469a-b889-d05a09be7d97.

Results

Identification of a candidate oxygen-independent L-aspartate decarboxylase involved in CoA synthesis in anaerobic fungi

Decarboxylation of L-aspartate to β-alanine by L-aspartate decarboxylase (Adc), an enzyme that occurs in all domains of life [38], enables an oxygen-independent alternative for the canonical fungal pathway for CoA synthesis (Fig. 1A). A set of 51 amino acid sequences of Adcs homologs listed by Tomita *et al*. (2015)[38] were used as queries against all Neocallimastigomycete proteins deposited in the TrEMBL section of the UNIPROT database. This search yielded 13 Neocallimastigomycete hits (e-value < 10^{-6}, Supplementary Table S3), 4 of which originated from *N. californiae*. Only one of these hits, A0A1Y1ZL74, did not reveal annotation errors upon RNAseq read mapping and showed the highest read coverage (Supplementary Fig. S1) and was selected as best candidate Adc encoding gene.

The sequence A0A1Y1ZL74 (hereafter refered to as *Nc*AdcA) was used for a second round of homology search against a broader set of Adc sequences, with a similar sequence representation of taxa across the 3 domains of life (103 sequences from Bacteria, 101 from Eukarya, and 121 from Archaea). The resulting set of *Nc*AdcA homologs, together with the set definded by Tomita *et al*. (2015) [38], were subjected to multiple sequence alignment. A subsequent phylogenetic tree (Fig. 2) showed that *Nc*Adc sequences are closely related to those of chytrid fungi (A0A0L0HIP1 from *Spizellomyces punctatus*, A0A1S8W5A4 from *Batrachochytrium salamandrivorans* and F4NWP2 from *Batrachochytrium dendrobatidis*) and from the rumen-associated anaerobic bacterium

Clostridium cellulolyticum (B8I983). Neocallimastigomycete, chytrid and *C. cellulolyticum* Adc homologs were more closely related to each other than to characterised eukaryotic Adc and bacterial PanD sequence*s.* These results indicate that an ancestor of *C. cellulolyticum* donated an Adc-encoding sequence to a common ancestor of chytrids and Neocallimastigomycetes.

Comparison of bacterial PanDs (Q0TLK2 from *E. coli* and P9WIL2 from *Mycobacterium tuberculosis*) against Adcs from other bacteria (B8I983 from *C. cellulolyticum*), and eukaryotes (including A7U8C7 from *Tribolium castaneum*) showed only little sequence homology between *Nc*Adc*s,* known bacterial PanDs, and eukaryotic Adcs (Supplementary File 3). The only conserved region encompassed the full length of PanDs (126-139 amino acids), which represents less than 60 % of the full length of other Adc sequences (*e.g. Nc*Adc*A* is 625 amino acids long).

Neocallimastigomycete *PfnadB, PfnadA* and *NcadcA* genes support aerobic pyridine-nucleotide and CoA synthesis in yeast.

Neocallimastigomycetes were previously reported to have acquired an L-aspartate oxidase (*nadB*) and a quinolinate synthase gene (*nadA*) by HGT [6]. Hence, UNIPROT entries A0A1Y1V2P1 and A0A1Y1VAT1 from *Piromyces finnis* were functionally reassigned as NadA and NadB candidates and the corresponding genes were tentatively named *PfnadB* and *PfnadA.* These sequences, together with *NcadcA,* were codon-optimised and tested to bypass the corresponding oxygen-requiring reactions in *S. cerevisiae*.

The *BNA2* and *FMS1* genes of *S. cerevisiae* were deleted by Cas9-mediated genome editing. The inability of strain IMK877 (*bna2Δ*) to synthesize quinolinic acid and of strain IMX2292 (*fms1Δ*) to synthesize β-alanine was evident from their inability to grow on

glucose synthetic medium (SMD) lacking nicotinic acid or pantothenate, respectively (Table 3). Strain IMK877 was used for heterologous complementation studies with codon-optimized expression cassettes for *PfnadB* and *PfnadA,* while an expression cassette for *N. californiae NcadcA* (A0A1Y1ZL74) was introduced into strain IMX2292. Congenic strains expressing previously characterized *NADB* and *NADA* genes from *Arabidopsis thaliana* (*At*NadB and *At*NadA, Q94AY1 and Q9FGS4)[21], and a previously characterized gene from *Tribolium castaneum* encoding an aspartate decarboxylase (*Tc*PanD, A7U8C7)[22] were tested in parallel.

Aerobic growth of the engineered *S. cerevisiae* strains was characterized in shake-flask cultures on SMD or on either SMDΔnic or SMDΔpan (Table 1). In contrast to the reference strain IMK877 (*bna2Δ*), *S. cerevisiae* IMX2301 (*bna2Δ PfnadB PfnadA*) grew in SMDΔnic, indicating complementation of the *bna2Δ*-induced nicotinate auxotrophy by *PfnadB* and *PfnadA*. However, the specific growth rate of the engineered strain in these aerobic cultures was approximately 3-fold lower than that of the reference strain IMX585 (*BNA2*, Table 1). Strain IMX2302 (*bna2Δ AtNADB AtNADA*) did not grow in SMDΔnic, suggesting that the plant NadB and/or NadA proteins were either not functionally expressed or not able to complement the nicotinate auxotrophy in these aerobic yeast cultures.

Strain IMX2300 (*fms1Δ NcadcA*) grew in SMDΔpan, indicating complementation of the panthotenate auxotrophy. However, this strain reproducibly showed a lag phase of approximately 48 h upon its first transfer from SMD to SMDΔpan, and grew exponentially thereafter at a rate of 0.34 ± 0.01h^{-1}. To explore whether the lag phase of strain IMX2300 reflected selection of a spontaneous mutant, it was subjected to three sequential transfers in SMDΔpan. A single-colony isolate, IMX2300-1 from the adapted population showed a specific growth rate of 0.34 ±0.01 h^{-1} in both SMD and SMDΔpan (Table 1). Whole-genome sequencing of IMX2300-1 did not reveal any mutations in coding DNA sequences that

were considered physiologically relevant in this context when compared to the non-adapted strain IMX2300 (Bioproject accession number: PRJNA634013). When both strains were compared to the reference CEN.PK113-7D sequence [45], a total of 16 mutations were found including eight non-synonymous and eight synonymous, with most mutations occurring in either Y' helicases or Ty elements (Supplementary File 4). These elements resided in highly repetitive chromosomal regions and were therefore prone to biased variant calling when using short-read sequencing technologies. The only non-synonymous mutation found in both IMX2300 and IMX2300-1 involved a leucine to methionine change in amino acid 315 of an Mtm1 homolog predicted to be a high affinity pyridoxal 5'-phosphate (PLP) transporter, involved in delivery of the PLP cofactor to mitochondrial enzymes. Overall, these observations indicate that the lag phase of strain IMX2300 most likely reflected a physiological adaptation or culture heterogeneity rather than a mutational event [49].

The specific growth rate of *S. cerevisiae* IMX2305 (*fms1Δ TcPAND*) on SMDΔpan did not significantly differ from that of the reference strain IMX585 on SMD, and it was almost four-fold higher than the specific growth rate of the reference strain on SMDΔpan. These results are consistent with a previous study on functional expression of *TcPAND* in *S. cerevisiae* [50].

Expression of Neocallimastigomycete *PfnadB, PfnadA,* and *NcadcA* suffice to enable anaerobic pyridine-nucleotide and CoA synthesis in yeast

To investigate whether expression of heterologous *PfnadB, PfnadA,* and *NcadcA* was sufficient to enable anaerobic growth in the absence of nicotinate and pantothenate, respectively, growth of the engineered *S. cerevisiae* strains on SMD, SMDΔnic and/or SMDΔpan was monitored in an anaerobic chamber (Fig. 3).

Growth experiments on SMDΔnic or SMDΔpan were preceded by a cultivation cycle on the same medium, supplemented with 50 g L^{-1} instead of 20 g L^{-1} of glucose to ensure complete depletion of any surplus cellular contents of pyridine nucleotides, CoA, or relevant intermediates. Indeed, upon a subsequent transfer to SMDΔnic or SMDΔpan, the reference strain IMX585 (*BNA2 FMS1*), expressing the native oxygen-dependent pathways for nicotinate and β-alanine synthesis, showed no growth (Fig. 3 panels A, B and C).

Both engineered strains IMX2301 (*bna2Δ PfnadB PfnadA*) and IMX2302 (*bna2Δ AtNADB AtNADA*) grew anaerobically on SMDΔnic. This provided a marked contrast with the aerobic growth studies on this medium, in which strain IMX2302 did not grow. Strains IMX2305 (*fms1Δ TcPAND*) and the aerobically pre-adapted IMX2300-1 (*fms1Δ NcadcA*) both grew on SMDΔpan under anaerobic conditions (Fig. 3 panels D, E and F).

Characterization of engineered yeast strains in anaerobic batch bioreactors

The anaerobic chamber experiments did not allow quantitative analysis of growth and product formation. Therefore, growth of the *S. cerevisiae* strains expressing the Neocallimastigomycetes genes, IMX2301 (*bna2Δ PfnadB PfnadA*) and IMX2300-1 (*fms1Δ NcadcA*) was studied in anaerobic bioreactor batch cultures on SMDΔnic or SMDΔpan and compared with growth of *S. cerevisiae* IMX585 (*BNA2 FMS1*) on the same media.

The reference strain IMX585, which tipically grows fast and exponentially in anaerobic bioreactors when using complete SMD [51], exhibited extremely slow, linear growth on SMDΔnic and SMDΔpan (Fig. 4). Similar growth kinetics in 'anaerobic' bioreactor cultures of *S. cerevisiae* on synthetic medium lacking the anaerobic growth factors Tween 80 and ergosterol were previously attributed to slow leakage of oxygen into laboratory bioreactors [27, 52, 53].

In contrast to the reference strain IMX585, the engineered strains IMX2301 and IMX2300-1 exhibited exponential anaerobic growth on SMDΔnic and SMDΔpan, respectively (Fig. 4; Table 2). The specific growth rate of strain IMX2301 (*bna2Δ PfnadB PfnadA*) on SMDΔnic was not significantly different from that of the reference strain on complete SMD [51], indicating full complementation of the anaerobic nicotinate auxotrophy of *S. cerevisiae*. The specific growth rate of strain IMX2300-1 (*fms1Δ NcadcA*) on SMDΔpan was only 20 % lower than this benchmark (Table 2). Biomass and ethanol yields of strain IMX2301 grown in anaerobic batch cultures on SMDΔnic and strain IMX2300-1 grown on SMDΔpan were not significantly different from those of the reference strain IMX585 grown on complete SMD (p-value > 0.05, Table 2).

Discussion

This study shows how expression of *PfnadB, PfnadA,* and *NcadcA* genes from Neocallimastigomycetes, as well as corresponding orthologs from other species (*AtNADB, AtNADA,* and *TcPAND*), confer oxygen-independent nicotinate and panthotenate prototrophy to the facultatively anaerobic yeast *S. cerevisiae*. These results also provide insights into how acquisition of these genes by HGT conferred selective advantage to Neocallimastigomycete ancestors under anaerobic conditions.

Genomic analyses previously suggested that genomes of Neocallimastigomycetes encode a putative L-aspartate oxidase (NadB) and quinolinate synthase (NadA) as alternatives to the canonical kynurenine pathway found in other fungi [6]. However, functionality of these Neocallimastigomycete proteins in an oxygen-independent pathway for synthesis of quinolinate from L-aspartate [19, 20] had not been demonstrated until now.

Neocallimastigomycetes appear to have acquired *nadB* from a proteobacterium while a eukaryotic donor was implicated in the acquisition of *nadA* [6]. Our results demonstrate

that expression of *nadB* and *nadA* homologs, either from the Neocallimastigomycete *P. finnis* or from the plant *A. thaliana* [21], suffice to allow anaerobic synthesis of NAD^+ of *S. cerevisiae*. Due to the involvement of the Bna2 and Bna4 oxygenases in NAD^+ synthesis by *S. cerevisiae*, nicotinate is an essential growth factor for this yeast under anaerobic conditions [14, 54, 55]. The present study represents the first demonstration of a metabolic engineering strategy to eliminate oxygen requirements for NAD^+ synthesis in this yeast. A similar strategy was successfully applied to enable oxygen-independent synthesis of pyridine nucleotides in the bacterium *Pseudomonas putida* [56].

Functional expression of heterologous NadA quinolinate synthases in *S. cerevisiae* was observed despite the fact that these enzymes are 4Fe-4S iron-sulfur cluster proteins [57, 58], which are notoriously difficult to functionally express in the yeast cytosol [59-62]. However, earlier studies on functional expression of the 4Fe-4S activating protein of bacterial pyruvate-formate lyase [63, 64] demonstrated that low-levels of expression can occur without modification of the yeast machinery for cytosolic assembly of Fe-S clusters. The inability of *At*NadB and *At*NadA to support NAD^+ synthesis in aerobic cultures may be due to oxygen-sensitivity of the 4Fe-4S cluster in the *At*NadA quinolinate synthase domain [65]. In contrast to *Pf*NadA, *At*NadA carries an N-terminal SufE domain which, in other organisms, has been demonstrated to allow this oxygen sensitive enzyme to remain active under aerobic conditions by reconstituting its Fe-S cluster [65].

Whereas an alternative to the kynurnine pathway for NAD^+ synthesis was previously inferred from genome sequence analysis, the pathway by which Neocallimastigomycetes synthesize Coenzyme A had not previously been explored. Six pathways for synthesis of the essential CoA precursor β-alanine are known: (1) decarboxylation of L-aspartate [66], (2) transamination of malonate semialdehyde with L-glutamate as aminodonor [67] or L-alanine [68], (3) by reduction of uracil followed by hydrolysis of the resulting

dihydrouracil [69], (4) oxidative cleavage of spermine to 3-aminopropanal followed by oxidation of the aldehyde group [16], (5) 2,3-aminomutase of alanine [70], and (6) addition of ammonia to acryloyl-CoA, followed by hydrolysis of the resulting CoA thioester [70]. Of these pathways, the options (1), (2), (3), (5), and (6) can, in principle, occur in the absence of oxygen. Yeasts and other filamentous fungi typically form β-alanine from spermine (pathway 4), but in some species the use of pathway 3 was also reported [71]. While the aspartate decarboxylation route has not previously been demonstrated in wild-type fungi, functional expression of bacterial and *T. castaneum Tc*PanD was used in metabolic engineering of *S. cerevisiae* to boost supply of β-alanine as a precursor for 3-hydroxypropionate production [22, 50]. Phylogenetic analysis of putative members of the pyridoxal-dependent L-aspartate decarboxylase family encoded by genomes of Neocallimastigomycetes allowed for identification of *NcadcA* which complemented a pantothenate-auxotrophic mutant of *S. cerevisiae*.

Amino acid sequence analysis of the characterised *Nc*AdcA (A0A1Y1ZL74) yielded the highest homology with sequences from chytrid fungi and *Clostridium* bacteria. This observation is in agreement with previous research showing that HGT events played a major role in shaping the genomes of Neocallimastigomycota [4, 6, 7], with Clostridiales as important sequence donors [6]. Phylogenetic analysis of Adc sequences (Fig. 2) are consistent with an earlier report on multiple evolutionary origins and variable evolutionary rates of pyridoxal-5'-phosphate-dependent enzymes, including Adcs and glutamate decarboxylases [72, 73]. A separate clade of Neocallimastigomycete sequences show homology with characterised glutamate decarboxylases (e.g. Q04792 from *S. cerevisiae* and K4HXK6 from *Lactobacillus brevis*; Fig 2. These results further support acquisition of an Adc encoding DNA sequence by HGT rather than by neofunctionalization of a glutamate decarboxylase gene.

Wild-type *S. cerevisiae* strains cannot grow in anaerobic environments unless supplemented with pantothenate. Expression of either *NcadcA* or *TcPAND* in an *fms1Δ S. cerevisiae* strain, which lacks the native oxygen-dependent pantothenate biosynthesis pathway, enabled growth in panthothenate-free medium under aerobic and anaerobic conditions. Although the different specific growth rates of *S. cerevisiae* strains expressing *NcadcA* or *TcPAND* indicate that changing expression levels and/or origin of ADC encoding genes may be required to achieve optimal growth, these results provide a proof-of-principle for a simple metabolic engineering strategy to eliminate oxygen requirements for pantothenate synthesis.

This work contributes to the understanding of how Neocallimastigomycetes adapted to their anaerobic lifestyle by acquiring genes that enable oxygen-independent synthesis of central metabolic cofactors. Experiments with engineered *S. cerevisiae* strains showed that contribution of the heterologous genes to *in vivo* oxygen-independent cofactor synthesis did not require additional mutations in the host genome. These results indicate how acquisition of functional genes by HGT, even if their expression was initially suboptimal, could have conferred an immediate advantage to ancestors of anaerobic fungi living in cofactor-limited anoxic environments. A similar approach was recently applied to study the physiological impact on *S. cerevisiae* of expressing a heterologous gene encoding squalene-tetrahymanol cyclase, which in Neocallimastigomycetes produces the sterol surrogate tetrahymanol [13]. Functional analysis by heterologous expression in *S. cerevisiae* circumvents the current lack of tools for genetic modification of Neocallimastigomycetes [2], and can complement biochemical studies [3-5] and genome sequence analyses [6, 7].

Pantothenate and nicotinate, together with the other compounds belonging to the B-group of water-soluble vitamins, are standard ingredients of chemically defined media for

546 aerobic and anaerobic cultivation of yeasts [48]. *S. cerevisiae* strains have been shown to
547 contain the genetic information required for *de novo* synthesis of these vitamins and, can
548 even be experimentally evolved for complete prototrophy for individual vitamins by
549 prolonged cultivation in single-vitamin depleted media [74, 75]. In large-scale processes,
550 addition of nutritional supplements increases costs, reduces shelf-life of media and
551 increases the risk of contamination during their storage [48]. Therefore, metabolic
552 engineering strategies for enabling oxygen-independent synthesis of NAD^+ and
553 pantothenate are of particular interest for the development robust yeast strains with
554 minimal nutritional requirements that can be applied in anaerobic biofuels production
555 [48]. Further studies of the unique evolutionary adaptations of Neocallimastigomycetes
556 may well provide additional inspiration for engineering robust fungal cell factories that
557 operate under anaerobic conditions.

558 ## *References*

559 1. Gruninger RJ, Puniya AK, Callaghan TM, Edwards JE, Youssef N, Dagar SS, et al.
560 Anaerobic fungi (phylum Neocallimastigomycota): advances in understanding their
561 taxonomy, life cycle, ecology, role and biotechnological potential. *FEMS Microbiol Ecol*.
562 2014;**90**:1-17.
563 2. Wilken SE, Seppala S, Lankiewicz TS, Saxena M, Henske JK, Salamov AA, et al.
564 Genomic and proteomic biases inform metabolic engineering strategies for anaerobic
565 fungi. *Metab Eng Commun*. 2020;**10**:e00107.
566 3. Kemp P, Lander DJ, Orpin CG. The lipids of the rumen fungus *Piromonas communis*.
567 *J Gen Microbiol*. 1984;**130**:27-37.
568 4. Garcia-Vallve S, Romeu A, Palau J. Horizontal gene transfer of glycosyl hydrolases
569 of the rumen fungi. *Mol Biol Evol*. 2000;**17**:352-61.

570 5. Harhangi HR, Akhmanova AS, Emmens R, van der Drift C, de Laat WT, van Dijken
571 JP, et al. Xylose metabolism in the anaerobic fungus *Piromyces sp.* strain E2 follows the
572 bacterial pathway. *Arch Microbiol*. 2003;**180**:134-41.
573 6. Murphy CL, Youssef NH, Hanafy RA, Couger MB, Stajich JE, Wang Y, et al. Horizontal
574 gene transfer as an indispensable driver for evolution of Neocallimastigomycota into a
575 distinct gut-dwelling fungal lineage. *Appl Environ Microbiol*. 2019;**85**:e00988-19.
576 7. Youssef NH, Couger MB, Struchtemeyer CG, Liggenstoffer AS, Prade RA, Najar FZ,
577 et al. The genome of the anaerobic fungus *Orpinomyces* sp. strain C1A reveals the unique
578 evolutionary history of a remarkable plant biomass degrader. *Appl Environ Microbiol*.
579 2013;**79**:4620-34.
580 8. Wang Y, Youssef NH, Couger MB, Hanafy RA, Elshahed MS, Stajich JE. Molecular
581 dating of the emergence of anaerobic rumen fungi and the impact of laterally acquired
582 genes. *mSystems*. 2019;**4**.
583 9. Weete JD, Abril M, Blackwell M. Phylogenetic distribution of fungal sterols. *Plos*
584 *One*. 2010;**5**:e10899.
585 10. Summons RE, Bradley AS, Jahnke LL, Waldbauer JR. Steroids, triterpenoids and
586 molecular oxygen. *Philos T R Soc B*. 2006;**361**:951-68.
587 11. Ourisson G, Rohmer M, Poralla K. Prokaryotic hopanoids and other polyterpenoid
588 sterol surrogates. *Annu Rev Microbiol*. 1987;**41**:301-33.
589 12. Takishita K, Chikaraishi Y, Leger MM, Kim E, Yabuki A, Ohkouchi N, et al. Lateral
590 transfer of tetrahymanol-synthesizing genes has allowed multiple diverse eukaryote
591 lineages to independently adapt to environments without oxygen. *Biol Direct*. 2012;**7**.
592 13. Wiersma SJ, Mooiman C, Giera M, Pronk JT. Expression of a squalene-tetrahymanol
593 cyclase enables sterol-independent growth of *Saccharomyces cerevisiae*. *Appl Environ*
594 *Microbiol*. 2020.

595 14. Panozzo C, Nawara M, Suski C, Kucharczyka R, Skoneczny M, Becam AM, et al.
596 Aerobic and anaerobic NAD^+ metabolism in *Saccharomyces cerevisiae*. *FEBS Lett*.
597 2002;**517**:97-102.
598 15. Landry J, Sternglanz R. Yeast Fms1 is a FAD-utilizing polyamine oxidase. *Biochem*
599 *Bioph Res Co*. 2003;**303**:771-6.
600 16. White WH, Gunyuzlu PL, Toyn JH. *Saccharomyces cerevisiae* is capable of *de novo*
601 pantothenic acid biosynthesis involving a novel pathway of β-alanine production from
602 spermine. *J Biol Chem*. 2001;**276**:10794-800.
603 17. White WH, Skatrud PL, Xue ZX, Toyn JH. Specialization of function among aldehyde
604 dehydrogenases: The *ALD2* and *ALD3* genes are required for β-alanine biosynthesis in
605 *Saccharomyces cerevisiae*. *Genetics*. 2003;**163**:69-77.
606 18. Orpin CG, Greenwood Y. Nutritional and germination requirements of the rumen
607 chytridiomycete *Neocallimastix patriciarum*. *T Brit Mycol Soc*. 1986;**86**:103-9.
608 19. Hao JF, Petriacq P, de Bont L, Hodges M, Gakiere B. Characterization of L-aspartate
609 oxidase from *Arabidopsis thaliana*. *Plant Sci*. 2018;**271**:133-42.
610 20. Tedeschi G, Negri A, Mortarino M, Ceciliani F, Simonic T, Faotto L, et al. L-aspartate
611 oxidase from *Escherichia coli* . II. Interaction with C4 dicarboxylic acids and identification
612 of a novel L-aspartate:fumarate oxidoreductase activity. *Eur J Biochem*. 1996;**239**:427-33.
613 21. Katoh A, Uenohara K, Akita M, Hashimoto T. Early steps in the biosynthesis of NAD
614 in *Arabidopsis* start with aspartate and occur in the plastid. *Plant Physiol*. 2006;**141**:851-
615 7.
616 22. Arakane Y, Lomakin J, Beeman RW, Muthukrishnan S, Gehrke SH, Kanost MR, et al.
617 Molecular and functional analyses of amino acid decarboxylases involved in cuticle
618 tanning in *Tribolium castaneum*. *J Biol Chem*. 2009;**284**:16584-94.

619 23. Entian KD, Kotter P. 25 yeast genetic strain and plasmid collections. *Methods in*
620 *microbiology*. 2007;**36**:629-66.
621 24. Verduyn C, Postma E, Scheffers WA, Van Dijken JP. Effect of benzoic acid on
622 metabolic fluxes in yeasts: a continuous-culture study on the regulation of respiration and
623 alcoholic fermentation. *Yeast*. 1992;**8**:501-17.
624 25. Luttik MA, Kotter P, Salomons FA, van der Klei IJ, van Dijken JP, Pronk JT. The
625 *Saccharomyces cerevisiae ICL2* gene encodes a mitochondrial 2-methylisocitrate lyase
626 involved in propionyl-coenzyme A metabolism. *J Bacteriol*. 2000;**182**:7007-13.
627 26. Solis-Escalante D, Kuijpers NG, Bongaerts N, Bolat I, Bosman L, Pronk JT, et al.
628 amdSYM, a new dominant recyclable marker cassette for *Saccharomyces cerevisiae*. *FEMS*
629 *Yeast Res*. 2013;**13**:126-39.
630 27. Dekker WJC, Wiersma SJ, Bouwknegt J, Mooiman C, Pronk JT. Anaerobic growth of
631 *Saccharomyces cerevisiae* CEN.PK113-7D does not depend on synthesis or
632 supplementation of unsaturated fatty acids. *FEMS Yeast Res*. 2019;**19**:foz060.
633 28. Mans R, van Rossum HM, Wijsman M, Backx A, Kuijpers NG, van den Broek M, et al.
634 CRISPR/Cas9: a molecular Swiss army knife for simultaneous introduction of multiple
635 genetic modifications in *Saccharomyces cerevisiae*. *FEMS Yeast Res*. 2015;**15**:fov004.
636 29. Looke M, Kristjuhan K, Kristjuhan A. Extraction of genomic DNA from yeasts for
637 PCR-based applications. *Biotechniques*. 2011;**50**:325-.
638 30. Gietz RD, Woods RA. Transformation of yeast by lithium acetate/single-stranded
639 carrier DNA/polyethylene glycol method. *Method Enzymol*. 2002;**350**:87-96.
640 31. Inoue H, Nojima H, Okayama H. High efficiency transformation of *Escherichia coli*
641 with plasmids. *Gene*. 1990;**96**:23-8.

642 32. Gibson DG, Young L, Chuang RY, Venter JC, Hutchison CA, Smith HO. Enzymatic
643 assembly of DNA molecules up to several hundred kilobases. *Nat Methods*. 2009;**6**:343-
644 U41.
645 33. Mans R, Wijsman M, Daran-Lapujade P, Daran JM. A protocol for introduction of
646 multiple genetic modifications in *Saccharomyces cerevisiae* using CRISPR/Cas9. *FEMS*
647 *Yeast Res*. 2018;**18**.
648 34. Lee ME, DeLoache WC, Cervantes B, Dueber JE. A highly characterized yeast toolkit
649 for modular, multipart assembly. *Acs Synth Biol*. 2015;**4**:975-86.
650 35. DiCarlo JE, Norville JE, Mali P, Rios X, Aach J, Church GM. Genome engineering in
651 *Saccharomyces cerevisiae* using CRISPR-Cas systems. *Nucleic Acids Res*. 2013;**41**:4336-43.
652 36. Verhoeven MD, Lee M, Kamoen L, van den Broek M, Janssen DB, Daran JMG, et al.
653 Mutations in *PMR1* stimulate xylose isomerase activity and anaerobic growth on xylose of
654 engineered *Saccharomyces cerevisiae* by influencing manganese homeostasis. *Sci Rep-Uk*.
655 2017;**7**:46155.
656 37. Medina VG, Almering MJH, van Maris AJA, Pronk JT. Elimination of glycerol
657 production in anaerobic cultures of a *Saccharomyces cerevisiae* strain engineered to use
658 acetic acid as an electron acceptor. *Appl Environ Microb*. 2010;**76**:190-5.
659 38. Tomita H, Yokooji Y, Ishibashi T, Imanaka T, Atomi H. An archaeal glutamate
660 decarboxylase homolog functions as an aspartate decarboxylase and Is involved in β-
661 alanine and coenzyme A biosynthesis. *J Bacteriol*. 2014;**196**:1222-30.
662 39. Camacho C, Coulouris G, Avagyan V, Ma N, Papadopoulos J, Bealer K, et al. BLAST+:
663 architecture and applications. *BMC Bioinformatics*. 2009;**10**:421.
664 40. Mistry J, Finn RD, Eddy SR, Bateman A, Punta M. Challenges in homology search:
665 HMMER3 and convergent evolution of coiled-coil regions. *Nucleic Acids Res*.
666 2013;**41**:e121.

667 41. Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, et al. Fast, scalable
668 generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol*
669 *Syst Biol*. 2011;**7**:539.
670 42. Kozlov AM, Darriba D, Flouri T, Morel B, Stamatakis A. RAxML-NG: a fast, scalable
671 and user-friendly tool for maximum likelihood phylogenetic inference. *Bioinformatics*.
672 2019;**35**:4453-5.
673 43. Letunic I, Bork P. Interactive Tree Of Life (iTOL) v4: recent updates and new
674 developments. *Nucleic Acids Res*. 2019;**47**:W256-W9.
675 44. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler
676 transform. *Bioinformatics*. 2009;**25**:1754-60.
677 45. Salazar AN, Gorter de Vries AR, van den Broek M, Wijsman M, de la Torre Cortes P,
678 Brickwedde A, et al. Nanopore sequencing enables near-complete *de novo* assembly of
679 *Saccharomyces cerevisiae* reference strain CEN.PK113-7D. *FEMS Yeast Res*. 2017;**17**.
680 46. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. The Sequence
681 Alignment/Map format and SAMtools. *Bioinformatics*. 2009;**25**:2078-9.
682 47. Walker BJ, Abeel T, Shea T, Priest M, Abouelliel A, Sakthikumar S, et al. Pilon: an
683 integrated tool for comprehensive microbial variant detection and genome assembly
684 improvement. *PLoS One*. 2014;**9**:e112963.
685 48. Perli T, Wronska AK, Ortiz-Merino RA, Pronk JT, Daran JM. Vitamin requirements
686 and biosynthesis in *Saccharomyces cerevisiae*. *Yeast*. 2020;**37**:283-304.
687 49. Vermeersch L, Perez-Samper G, Cerulus B, Jariani A, Gallone B, Voordeckers K, et
688 al. On the duration of the microbial lag phase. *Curr Genet*. 2019;**65**:721-7.
689 50. Borodina I, Kildegaard KR, Jensen NB, Blicher TH, Maury J, Sherstyk S, et al.
690 Establishing a synthetic pathway for high-level production of 3-hydroxypropionic acid in
691 *Saccharomyces cerevisiae* via β-alanine. *Metab Eng*. 2015;**27**:57-64.

692 51. Papapetridis I, van Dijk M, Dobbe APA, Metz B, Pronk JT, van Maris AJA. Improving
693 ethanol yield in acetate-reducing *Saccharomyces cerevisiae* by cofactor engineering of 6-
694 phosphogluconate dehydrogenase and deletion of *ALD6*. *Microb Cell Fact*. 2016;**15**:67.
695 52. Visser W, Scheffers WA, Batenburg-van der Vegte WH, van Dijken JP. Oxygen
696 requirements of yeasts. *Appl Environ Microbiol*. 1990;**56**:3785-92.
697 53. da Costa BLV, Basso TO, Raghavendran V, Gombert AK. Anaerobiosis revisited:
698 growth of *Saccharomyces cerevisiae* under extremely low oxygen availability. *Appl*
699 *Microbiol Biotechnol*. 2018;**102**:2101-16.
700 54. Ahmad F, Moat AG. Nicotinic acid biosynthesis in prototrophs and tryptophan
701 auxotrophs of *Saccharomyces cerevisiae*. *J Biol Chem*. 1966;**241**:775-80.
702 55. Suomalainen H, Nurminen T, Vihervaara K, Oura E. Effect of aeration on the
703 synthesis of nicotinic acid and nicotinamide adenine dinucleotide by baker's yeast. *J Inst*
704 *Brew*. 1965;**71**:227-31.
705 56. Kampers LFC, van Heck RGA, Donati S, Saccenti E, Volkers RJM, Schaap PJ, et al. In
706 silico-guided engineering of *Pseudomonas putida* towards growth under micro-oxic
707 conditions. *Microb Cell Fact*. 2019;**18**:179.
708 57. Rousset C, Fontecave M, Ollagnier de Choudens S. The [4Fe-4S] cluster of
709 quinolinate synthase from *Escherichia coli*: investigation of cluster ligands. *FEBS Lett*.
710 2008;**582**:2937-44.
711 58. Saunders AH, Griffiths AE, Lee KH, Cicchillo RM, Tu L, Stromberg JA, et al.
712 Characterization of quinolinate synthases from *Escherichia coli*, *Mycobacterium*
713 *tuberculosis*, and *Pyrococcus horikoshii* indicates that [4Fe-4S] clusters are common
714 cofactors throughout this class of enzymes. *Biochemistry*. 2008;**47**:10999-1012.

715 59. Zou R, Zhou K, Stephanopoulos G, Too HP. Combinatorial engineering of 1-deoxy-
716 D-xylulose 5-phosphate pathway using cross-lapping in vitro assembly (CLIVA) method.
717 *PLoS One*. 2013;**8**:e79557.
718 60. Carlsen S, Ajikumar PK, Formenti LR, Zhou K, Phon TH, Nielsen ML, et al.
719 Heterologous expression and characterization of bacterial 2-C-methyl-D-erythritol-4-
720 phosphate pathway in *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol*.
721 2013;**97**:5753-69.
722 61. Partow S, Siewers V, Daviet L, Schalk M, Nielsen J. Reconstruction and evaluation
723 of the synthetic bacterial MEP pathway in *Saccharomyces cerevisiae*. *PLoS One*.
724 2012;**7**:e52498.
725 62. Benisch F, Boles E. The bacterial Entner-Doudoroff pathway does not replace
726 glycolysis in *Saccharomyces cerevisiae* due to the lack of activity of iron-sulfur cluster
727 enzyme 6-phosphogluconate dehydratase. *J Biotechnol*. 2014;**171**:45-55.
728 63. Waks Z, Silver PA. Engineering a synthetic dual-organism system for hydrogen
729 production. *Appl Environ Microbiol*. 2009;**75**:1867-75.
730 64. Kozak BU, van Rossum HM, Benjamin KR, Wu L, Daran JM, Pronk JT, et al.
731 Replacement of the *Saccharomyces cerevisiae* acetyl-CoA synthetases by alternative
732 pathways for cytosolic acetyl-CoA synthesis. *Metab Eng*. 2014;**21**:46-59.
733 65. Murthy NM, Ollagnier-de-Choudens S, Sanakis Y, Abdel-Ghany SE, Rousset C, Ye H,
734 et al. Characterization of *Arabidopsis thaliana* SufE2 and SufE3: functions in chloroplast
735 iron-sulfur cluster assembly and NAD synthesis. *J Biol Chem*. 2007;**282**:18254-64.
736 66. Ramjee MK, Genschel U, Abell C, Smith AG. *Escherichia coli* L-aspartate-α-
737 decarboxylase: preprotein processing and observation of reaction intermediates by
738 electrospray mass spectrometry. *Biochem J*. 1997;**323 (Pt 3)**:661-9.

739 67. Tamaki N, Aoyama H, Kubo K, Ikeda T, Hama T. Purification and properties of β-
740 alanine aminotransferase from rabbit liver. *J Biochem*. 1982;**92**:1009-17.
741 68. Hayaishi O, Nishizuka Y, Tatibana M, Takeshita M, Kuno S. Enzymatic studies on
742 the metabolism of β-alanine. *J Biol Chem*. 1961;**236**:781-90.
743 69. Schnackerz KD, Dobritzsch D. Amidohydrolases of the reductive pyrimidine
744 catabolic pathway purification, characterization, structure, reaction mechanisms and
745 enzyme deficiency. *Biochim Biophys Acta*. 2008;**1784**:431-44.
746 70. Dalluge JJ, Liao H, Gokarn R, Jessen H. Discovery of enzymatic activity using stable
747 isotope metabolite labeling and liquid chromatography-mass spectrometry. *Anal Chem*.
748 2005;**77**:6737-40.
749 71. Gojkovic Z, Sandrini MP, Piskur J. Eukaryotic β-alanine synthases are functionally
750 related but have a high degree of structural diversity. *Genetics*. 2001;**158**:999-1011.
751 72. Sandmeier E, Hale TI, Christen P. Multiple evolutionary origin of pyridoxal-5'-
752 phosphate-dependent amino acid decarboxylases. *Eur J Biochem*. 1994;**221**:997-1002.
753 73. Salzmann D, Christen P, Mehta PK, Sandmeier E. Rates of evolution of pyridoxal-5'-
754 phosphate-dependent enzymes. *Biochem Biophys Res Commun*. 2000;**270**:576-80.
755 74. Perli T, Moonen DPI, van den Broek M, Pronk JT, Daran JM. Adaptive laboratory
756 evolution and reverse engineering of single-vitamin prototrophies in *Saccharomyces*
757 *cerevisiae*. *Appl Environ Microbiol*. 2020;**86**:e00388-20.
758 75. Bracher JM, de Hulster E, Koster CC, van den Broek M, Daran JG, van Maris AJA, et
759 al. Laboratory evolution of a biotin-requiring *Saccharomyces cerevisiae* strain for full
760 biotin prototrophy and identification of causal mutations. *Appl Environ Microbiol*.
761 2017;**83**.
762 76. Papapetridis I, Goudriaan M, Vazquez Vitali M, de Keijzer NA, van den Broek M, van
763 Maris AJA, et al. Optimizing anaerobic growth rate and fermentation kinetics in

764 *Saccharomyces cerevisiae* strains expressing Calvin-cycle enzymes for improved ethanol
765 yield. *Biotechnol Biofuels*. 2018;**11**:17.
766 77. Wronska AK, Haak MP, Geraats E, Slot EB, van den Broek M, Pronk JT, et al.
767 Exploiting the diversity of Saccharomycotina yeasts to engineer biotin-independent
768 growth of *Saccharomyces cerevisiae*. *Appl Environ Microbiol*. 2020;**86**:e00270-20.

769 *Acknowledgments*

770 We thank Dr. Irina Borodina for providing us the codon-optimized *TcPAND* gene and
771 Sabina Shrestha for constructing strain IMK877. TP and J-MD were supported by the
772 European Union's Horizon 2020 research and innovation programme under the Marie
773 Skłodowska-Curie action PAcMEN (grant agreement No 722287). AMV, WJCD, JB, SJW,
774 RAO-M, CM and JTP were funded by an Advanced Grant of the European Research
775 Council to JTP (grant # 694633). Authors declare that they have no conflict of interests.

776 *Author contributions*

777 All authors contributed to the experimental design. TP, AMV, JMD and JTP wrote a first
778 version of the manuscript. All authors critically read this version, provided input and
779 approved the final version. RAO-M, AMV, and TP performed the phylogenetic analysis. TP
780 constructed the *S. cerevisiae* strains and performed the aerobic characterization. AMV,
781 WJCD and TP performed the anaerobic chamber experiments. JB, CM, TP, AMV, and SJW
782 performed and analysed the bioreactor experiments.

Figure legends

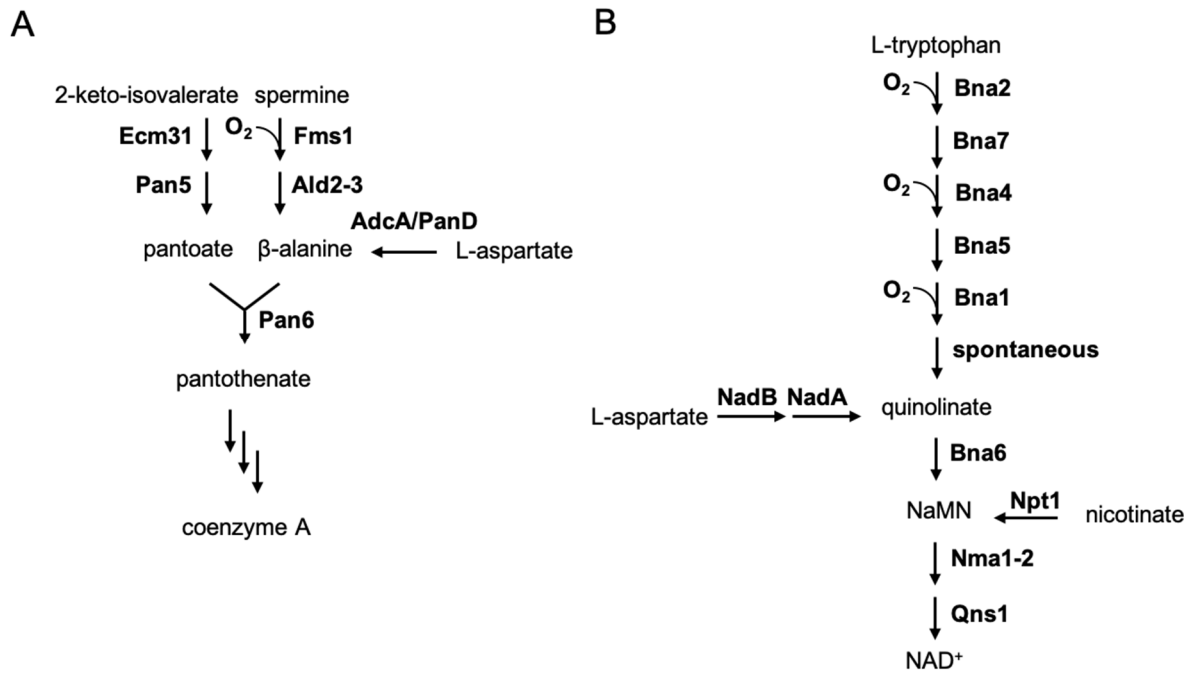
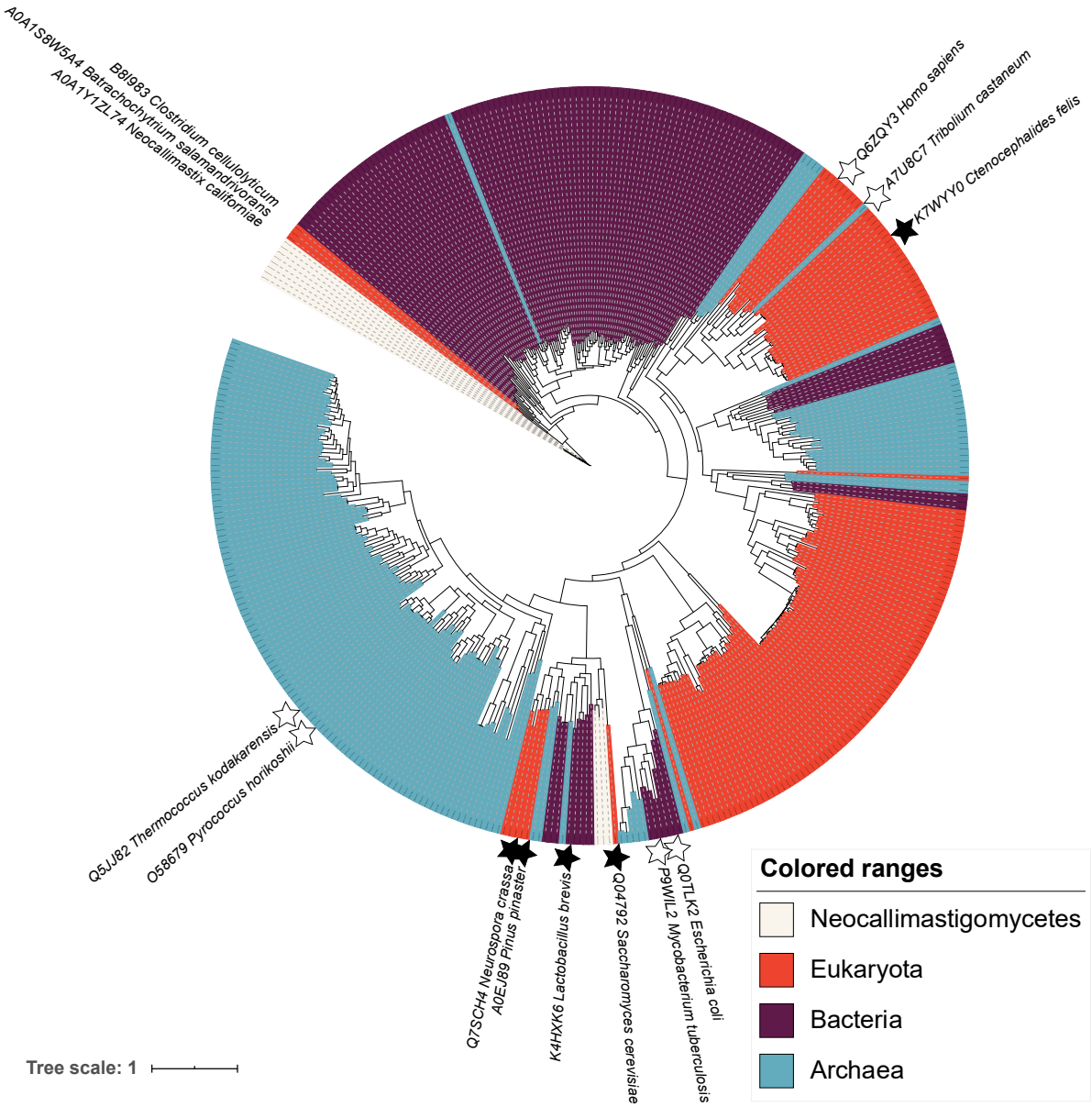


Fig. 1: CoA and NAD⁺ biosynthetic pathways in *S. cerevisiae* and oxygen-independent alternatives. CoA synthesis includes the condensation of pantoate and β-alanine. In *S. cerevisiae* β-alanine is formed from spermine in two steps using the oxygen-dependent poly-amine oxidase Fms1 (A). Other organisms, including archaea, bacteria, and insects, can by-pass this oxygen requirement by synthesizing β-alanine from aspartate using L-aspartate decarboxylase (AdcA/PanD). NAD⁺ is synthesized via the kynurenine pathway in 9 reactions starting from tryptophan, 3 of which require oxygen (B). Other organisms that include plants and bacteria are able to bypass this oxygen requirement by synthesizing quinolinate from aspartate using L-aspartate oxidase and quinolinate synthase (NadB and NadA, respectively)

796



797

798 **Fig. 2: Maximum likelihood phylogenetic tree of aspartate decarboxylase and**
799 **glutamate decarboxylase sequences.** Sequences of proteins with demonstrated
800 enzyme activity are marked with white stars (L-aspartate decarboxylases) or black stars
801 (glutamate decarboxylases). A version of this tree with all sequence identifiers, branch
802 support, distances and bootstrap values, is provided in the Supplementary File 2. An
803 interactive visualisation can be accessed in https://itol.embl.de/shared/rortizmerino

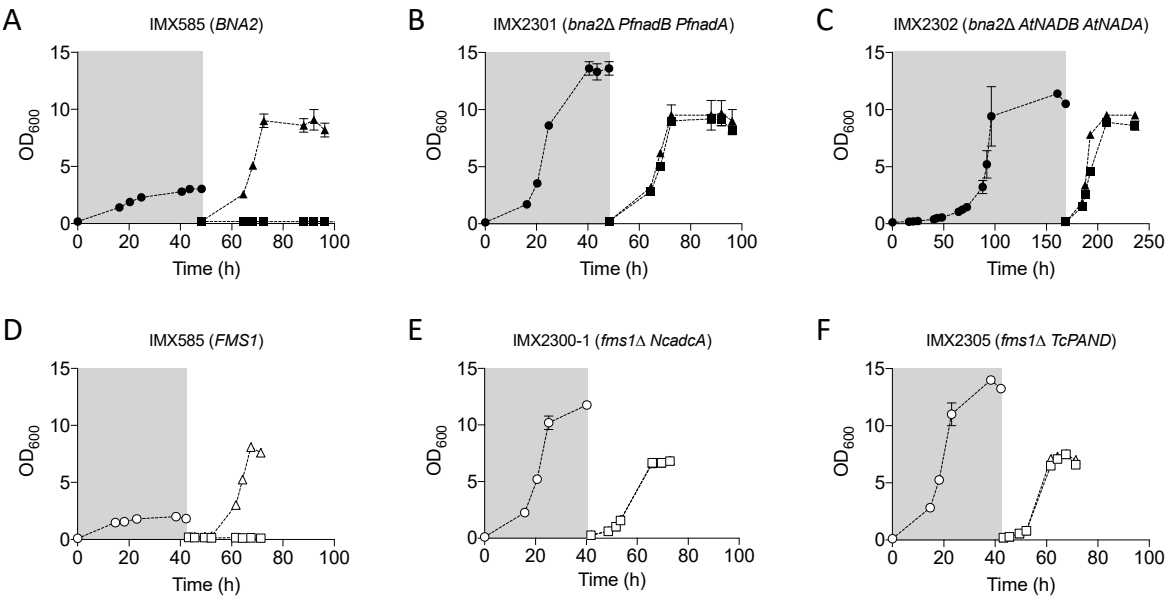
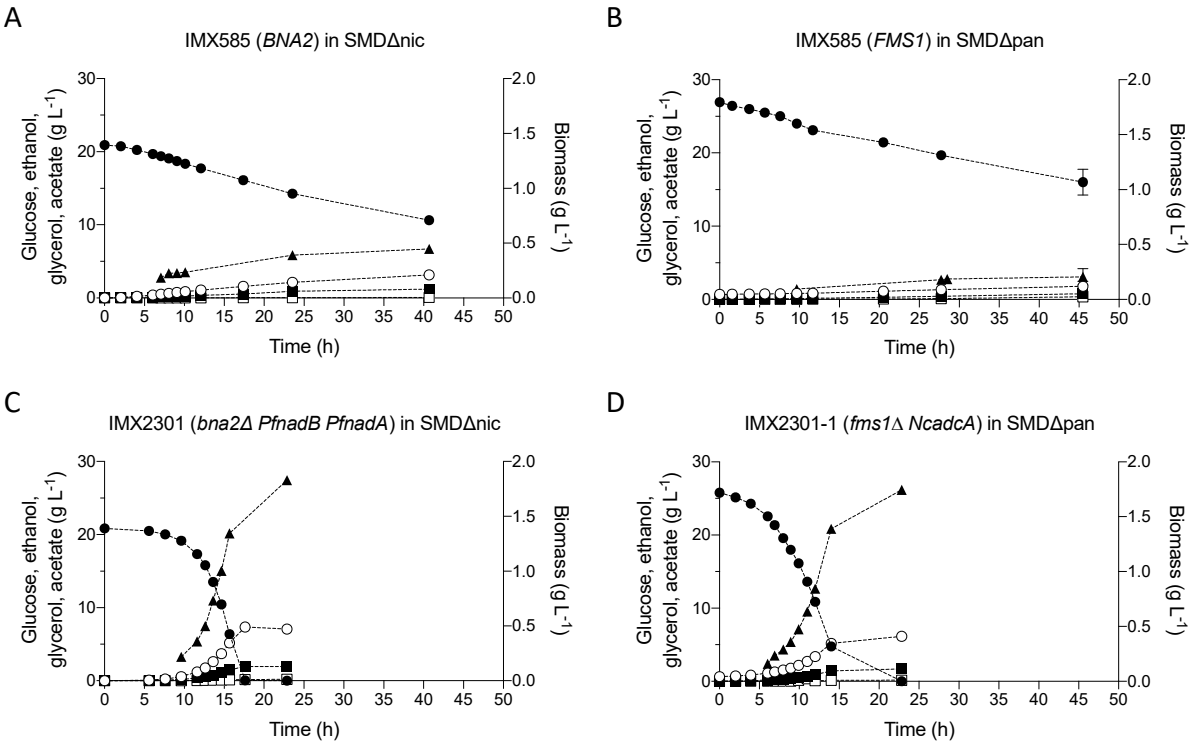


Fig. 3: Anaerobic growth of *S. cerevisiae* strains dependent or independent on supplementation of nicotinic acid (NA) or pantothenic acid (PA) in SMD medium containing Tween 80 and ergosterol. Strains IMX585 (A), IMX2301 (*bna2*Δ *PfnadB PfnadA*) (B), and IMX2302 (*bna2*Δ *AtNADB AtNADA*) (C) transferred to medium with 2 % glucose with (▲) or without (■) nicotinate after a carry-over phase in SMDΔnic containing 4 % glucose (● in grey box). Strains IMX585 (D), IMX2300-1 (*fms1*Δ *NcadcA*) (E), and IMX2305 (*fms1*Δ *TcPAND*)(F) transferred to medium with (△) or without (□) pantothenate after a carry-over phase in SMDΔpan containing 4 % glucose (○ in grey box). Anaerobic condition in the chamber were maintained using a palladium catalyst and a 5 % hydrogen concentration. Error bars represent the mean deviation of independent cultures (n=2)



816

817 **Fig. 4: Anaerobic batch cultivation of IMX585 in SMDΔnic (A) and SMDΔpan (B),**

818 **IMX2301 in SMDΔnic (C) and IMX2300-1 in SMDΔpan (D).** All strains were pre-

819 grown in the corresponding medium lacking one vitamin prior to inoculation in the

820 bioreactor to avoid carry-over effects. Values for glucose (●), ethanol (○), glycerol (■),

821 acetate (□) and biomass (▲) are shown over time. Error bars represent the mean

822 deviation of independent cultures (n=2)

823

Tables

Table 1: *S. cerevisiae* strains used in this study

Name	Relevant genotype	Parental strain	Reference
CEN.PK113-7D	MATa *URA3*	-	[23]
CEN.PK113-5D	MATa *ura3-52*	-	[23]
IMX585	MATa *can1*Δ::*Spycas9*-natNT2 *URA3*	CEN.PK113-7D	[28]
IMX581	MATa *ura3-52 can1*Δ::*Spycas9*-natNT2	CEN.PK113-5D	[28]
IMX2292	MATa *can1*Δ::*Spycas9*-natNT2 *URA3 fms1*Δ	IMX585	[74]
IMK877	MATa *can1*Δ::*Spycas9*-natNT2 *URA3 bna2*Δ	IMX585	This study
IMX2301	MATa *can1*Δ::*Spycas9*-natNT2 *URA3 bna2*Δ *sga1*::p*TDH3-PfnadA*-t*ENO1* p*CCW12-PfnadB*-t*ENO2*	IMK877	This study
IMX2302	MATa *can1*Δ::*Spycas9*-natNT2 *URA3 bna2*Δ *sga1*::p*TDH3-AtNADA*-t*ENO1* p*CCW12-AtNADB*-t*ENO2*	IMK877	This study
IMX2293	MATa *ura3-52 can1*Δ::*Spycas9*-natNT2 *fms1*Δ	IMX581	This study
IMX2300	MATa *ura3-52*::p*TDH3-NcadcA*-t*ENO2 URA3 can1*Δ::*Spycas9*-natNT2 *fms1*Δ	IMX2293	This study
IMX2300-1	MATa *ura3-52*::p*TDH3-NcadcA*-t*ENO2 URA3 can1*Δ::*Spycas9*-natNT2 *fms1*Δ Colony isolate 1	IMX2300	This study
IMX2305	MATa *ura3-52*::p*RPL12b-TcPAND*-t*TDH1 URA3 can1*Δ::*Spycas9*-natNT2 *fms1*Δ	IMX2293	This study

Spy: *Streptococcus pyogenes*; *Pf*: *Piromyces finnis*; *Nc*: *Neocallimastix californiae*; *At*: *Arabidopsis thaliana*; *Tc*: *Tribolium castaneum*.

827 **Table 2: plasmids used in this study**

Name	Characteristics	Reference
pROS10	2µm *bla* ori *URA3* gRNA-*CAN1*.Y gRNA-*ADE2*.Y	[28]
pROS11	2µm *bla* ori amdSYM gRNA-*CAN1*.Y gRNA-*ADE2*.Y	[28]
pROS13	2µm *bla* ori kanMX gRNA-*CAN1*.Y gRNA-*ADE2.Y*	[28]
pUDR119	2µm *bla* ori amdSYM gRNA-*SGA1* gRNA-*SGA1*	[76]
pYTK009	p*TDH3 cat* ColE1	[34]
pYTK010	p*CCW12 cat* ColE1	[34]
pYTK017	p*RPL18B cat* ColE1	[34]
pYTK051	t*ENO1 cat* ColE1	[34]
pYTK055	t*ENO2 cat* ColE1	[34]
pYTK056	t*TDH1 cat* ColE1	[34]
pYTK096	ConLS' *gfp* ConRE'*URA3 ntpII* ColE1 5'*URA3*	[34]
pGGKd017	ConLS' *gfp* ConRE' *URA3* 2 µm *bla* ColE1	[77]
pCfB-361	2µm *bla* ori pTEF1-*TcPAND**-*tCYC1 HIS3*	[50]
pUDR652	*bla* 2µm amdSYM gRNA-*FMS1* gRNA-*FMS1*	[74]
pUD652	*bla PfnadA**	GeneArt, this study
pUD653	*bla PfnadB**	GeneArt, this study
pUD1095	*bla NcadcA**	GeneArt, this study
pUD1096	*bla AtNADA**	GeneArt, this study
pUD1097	*nptII AtNADB**	GeneArt, this study

pUDR315	*bla* 2µm *amdSYM* gRNA-*BNA2* gRNA-*BNA2*	This study
pUDI168	p*RPL18B-TcPAND**-t*TDH1 URA3 ntpII* ColE1 5'*URA3*	This study
pUDI242	p*TDH3-NcadcA**-t*ENO2 URA3 ntpII* ColE1 5'*URA3*	This study
pUDI243	p*TDH3*-Pf*NADA**-t*ENO1 URA3 ntpII* ColE1 5'*URA3*	This study
pUDI244	p*CCW12-PfnadB**-t*ENO2 URA3 ntpII* ColE1 5'*URA3*	This study
pUDI245	p*TDH3-AtNADA**-t*ENO1 URA3 ntpII* ColE1 5'*URA3*	This study
pUDE931	p*CCW12-AtNADB**-t*ENO2 URA3* 2µm *bla* ColE1	This study

Spy: *Streptococcus pyogenes*; *Pf*: *Piromyces finnis*; *Nc*: *Neocallimastix californiae*; *At*: *Arabidopsis thaliana*; *Tc*: *Tribolium castaneum*.

*Codon-optimized for expression in *S. cerevisiae*.

840 **Table 3: Aerobic characterization of engineered strains**. Specific growth rates of *S. cerevisiae* strains grown in SMD, SMDΔnic and

841 SMDΔpan media. The values are average and mean deviation of data from at least two independent cultures of each strain

Strain	**SMD**	**SMDΔnic**	**SMDΔpan**
IMX585 (*FMS1 BNA2*)	0.40 ± 0.01	0.40 ± 0.02	0.11 ± 0.01
IMX2292(*fms1Δ*)	0.39 ± 0.01		< 0.01
IMX2305 (*fms1Δ TcPAND*)	0.39 ± 0.01		0.39 ± 0.01
IMX2300-1 (*fms1Δ NcadcA*)	0.34 ± 0.01		0.34 ± 0.01
IMK877 (*bna2Δ*)	0.40 ± 0.01	< 0.01	
IMX2301 (*bna2Δ PfnadB PfnadA*)	0.37 ± 0.01	0.14 ± 0.01	
IMX2302 (*bna2Δ AtNADB AtNADA*)	0.40 ± 0.01	< 0.01	

842

843

844

Table 4: Maximum specific growth rate (μ_{max}) and yields of glycerol, biomass and ethanol on glucose in anaerobic bioreactor batch cultures of *S. cerevisiae* strains IMX585, IMX2301 and IMX2300-1. Cultures were grown on SMD, SMDΔnic, or SMDΔpan, respectively, with 20 g L^{-1} glucose as carbon source (pH = 5). Growth rates and yields were calculated from the exponential growth phase. The ethanol yield was corrected for evaporation. Values represent average and mean deviation of data from independent cultures (n = 2). Carbon recovery in all fermentations was between 95 and 100%

Strain	**IMX585*** **(*FMS1 BNA2*)**	**IMX2301** **(*bna2Δ PfnadB PfnadA*)**	**IMX2300-1** **(*fms1Δ NcadcA*)**
Medium	SMD	SMDΔnic	SMDΔpan
μ_{max} (h^{-1})	0.32 ± 0.00	0.31 ± 0.01	0.25 ± 0.00
Y glycerol/glucose (g g^{-1})	0.105 ± 0.000	0.103 ± 0.003	0.104 ± 0.000
Y biomass/glucose (g_x g^{-1})	0.094 ± 0.004	0.090 ± 0.002	0.081 ± 0.001
Y EtOH/glucose (g g^{-1})	0.372 ± 0.001	0.372 ± 0.002	0.364 ± 0.003

* data from [51]