

The Evolution of Anaerobic Growth in Saccharomycotina Yeasts

Running Head: Evolution of anaerobic yeasts

David J. Krause¹

¹Department of Biology, University of Wisconsin Oshkosh, Oshkosh, Wisconsin, USA

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Take Away

Only a small subset of budding yeast species can grow under anoxic conditions.

High-throughput genomics has enabled understanding of budding yeast evolution.

Even anaerobic budding yeasts are not likely adapted for long-term anaerobiosis.

Future work must combine high-throughput phenotyping with genomics.

Abstract

Humans rely on the ability of budding yeasts to grow without oxygen in industrial scale fermentations that produce beverages, foods, and biofuels. Oxygen is deeply woven into the energy metabolism and biosynthetic capabilities of budding yeasts. While diverse ecological habitats may provide wide varieties of different carbon and nitrogen sources for yeasts to utilize, there is no direct substitute for molecular oxygen, only a range of availability. Understanding how a small subset of budding yeasts evolved the ability to grow without oxygen could expand the set of useful species in industrial scale fermentations as well as provide insight into the cryptic field of yeast ecology. However, we still do not yet appreciate the full breadth of species that can growth without oxygen, what genes underlie this adaptation, and how these genes have evolved.

Which species grow under anoxic conditions?

To understand the evolution of anaerobic growth in budding yeasts, we must start with identifying which species of budding yeasts are capable of this style of growth. High-throughput growth experiments that assess growth in the more than 1,000 described species of budding yeasts are difficult even in standard aerobic conditions. Such experiments have not yet been performed under anoxic conditions to identify a complete phylogenetic distribution of yeast species capable of anaerobic growth. One of the first attempts to assess the growth of many different yeast species under anoxic conditions measured the growth of up to 75 different species in oxygen-limited shake flasks, oxygen-purged serum flasks, and nitrogen-purged fermenters. The study found that very few species exhibited any signs of anaerobic growth, with only *S. cerevisiae* growing anaerobically without a lag phase of several days (Visser et al., 1990). Another important study assessed anaerobic growth using a single round of growth on agar plates in an anoxic environment as well as batch growth in fermenters. These results found consistent anaerobic growth for species of the genera *Saccharomyces*, *Nakaseomyces*, *Kazachstania*, *Naumovozyma*, and *Tetrapisispora*, with variable growth identified for some species from other genera (Merico et al., 2007). These genera identified as consistent facultative anaerobes comprise the post-whole genome duplication lineage of the Saccharomycetaceae (Kellis et al., 2004; Marcet-Houben and Gabaldón, 2015; Shen et al., 2018; Wolfe and Shields, 1997).

These different methods for testing anaerobic growth demonstrate the difficulty in arriving at a consistent characterization of anaerobic yeast species. Because different anoxic setups allow for different degrees of oxygen contamination, yeast species may be differentially considered obligate aerobes or facultative anaerobes depending on the study. One solution to this could be to accurately measure the oxygen infiltration in a particular setup, and instead of calling yeast species aerobic or anaerobic, determine growth rates over a continuum of oxygen availabilities (da Costa et al., 2018). However, there is a very stringent method available to test for strict anaerobic growth in budding yeasts: measuring growth in an anaerobic chamber over several subsequent rounds of subculturing. This method has been employed in several studies to ensure that the yeast cells are being propagated under anoxic conditions and that they are not utilizing carryover stored molecules from the initial aerobic culture phase (Dekker et al., 2019; Krause and Hittinger, 2022; Wiersma et al., 2020).

This type of rigorous assay for anaerobic growth has not yet been applied in high-throughput to the wide diversity of budding yeast species, so this primer will focus on the species with the best evidence of anaerobic growth: the post-WGD species of Saccharomycetaceae and some species of the *Brettanomyces* genus.

Which genes are responsible for anaerobic growth?

Applying comparative genomics to understand the evolution of a particular phenotype can be as simple as identifying a set of genes that are present only in the group exhibiting the phenotype and absent from those that do not exhibit the phenotype (Do et al., 2022; Hall et al., 2013; Krause et al., 2018). This approach can yield a set of candidate genes for involvement in the phenotype, and their functions can be determined using model genetic organisms. In the case of *S. cerevisiae* and the Saccharomycetaceae, many of the genes known to be involved in the anaerobic growth phenotype have been identified first through genetic studies (Ishtar Snoek and Yde Steensma, 2007). Therefore, the attempt to understand how this phenotype has evolved does not rest solely upon identifying genes using their species distribution, but also on discerning between which anaerobically important genes fundamentally evolved to causally support anaerobic growth and which genes may have served potentiating roles.

During anaerobic growth, budding yeasts must do three things: ferment the available carbon source without respiration; express the necessary anaerobic genetic repertoire; and bypass oxygen-dependent biosynthetic pathways. The genetic basis for the ability to ferment in the absence of respiration is not well understood and may be very complex (Dai et al., 2018; Fekete et al., 2007). The dispensability of respiration is also not directly correlated with anaerobic growth, as shown by the viability of the obligate aerobe *Kluyveromyces lactis* disrupted for the gene encoding for cytochrome c (Chen and Clark-Walker, 1993). *S. cerevisiae* induces a specific set of genes under hypoxic or anoxic conditions (Kwast et al., 2002; Zitomer and Lowry, 1992). However, the lack of parallel studies in other species of budding yeasts under anoxic conditions precludes our understanding of whether the transcriptional response to low oxygen availability is conserved. The final requirement for budding yeasts, the ability to bypass oxygen-dependent biosynthetic pathways, is very well understood in *S. cerevisiae*, with a clear set of genes that can be used to search for homologs in related species.

During anaerobic growth, *S. cerevisiae* cannot synthesize its own ergosterol due to the oxygen dependency of several steps of sterol biosynthesis. To grow and divide, yeasts require specific sterols for proper membrane function (Nes et al., 1993). While *S. cerevisiae* has no oxygen-independent pathways for producing sterols, the species is capable of sterol uptake under anoxic conditions via ABC-type

transporters encoded by *AUS1* and *PDR11* (Wilcox et al., 2002). These genes are induced during anaerobiosis, and their function is facilitated by cell wall mannoproteins that are induced under the same conditions (Abramova et al., 2001; Alimardani et al., 2004). This cell wall mannoprotein family experienced an amplification around the time of the whole genome duplication, and two of these genes, *TIR1* and *TIR3* have been identified as being critical to anaerobic growth (Krause and Hittinger, 2022). A BLAST search of available yeast genome sequences identified at least one homolog of the *PDR11* and *AUS1* genes in nearly all post-WGD genomes, as well as a few non-WGD genomes, but not outside of the Saccharomycetaceae (Figure 1). The ability to uptake sterols is presumed to be one of the most critical factors in anaerobic growth for *S. cerevisiae* (Snoek and Steensma, 2006). Indeed, *S. cerevisiae* fails to grow under anoxic conditions without sterol supplementation and at least one sterol transport protein (Wiersma et al., 2020; Wilcox et al., 2002). The evolution of these sterol transporters and the gene family amplification of the associated cell wall mannoproteins may be critical steps in the ability to grow under anoxic conditions. Indeed the putative sterol importers are most closely related to other ABC-type exporter pumps of the *PDR* gene family, implying that the evolution of sterol import may have required the switching of the direction of solute movement through these proteins (Papay et al., 2020).

The biosynthesis of pyrimidine nucleotides in most budding yeasts utilizes a mitochondrial dihydroorotate dehydrogenase (DHODase) encoded by the gene *URA9* that requires oxygen via the respiratory chain (Wolfe, 2006). However, the Saccharomycetaceae contain an alternative cytosolic DHODase acquired from bacteria via HGT, and it functions independently of the respiratory chain (Hall et al., 2005). While *Saccharomyces* spp. lost the *URA9* gene and now only contain the oxygen-independent *URA1* gene, several species have maintained both versions, and the *URA1* gene is present in several species that cannot grow under anoxic conditions. While *URA1* is useful under anoxic conditions, it is not likely an essential evolutionary adaptation to anaerobic growth due to its presence in *Kluyveromyces* spp. and other obligate aerobic yeast species and its absence in *Candida glabrata*, which can grow well under anoxic conditions (Merico et al., 2007; Wolfe, 2006). A recent study of *URA9* homologs in several anaerobic fungal species identified that *URA9* has evolved into a functional anaerobic uracil biosynthesis gene in some fungal lineages, including the yeast species *Dekkera bruxellensis* (Bouwknegt et al., 2021). In the absence of anaerobic uracil biosynthesis, yeasts can likely use the widely conserved uracil permease encoded by *FUR4* to uptake exogenous uracil (Chevallier, 1982). However, without an anaerobically functional DHODase or supplemented uracil, yeasts fail to grow without oxygen (Bouwknegt et al., 2021).

Under aerobic conditions, budding yeasts utilize the kynurenine pathway for *de novo* NAD⁺ biosynthesis, which requires molecular oxygen at multiple steps, but without oxygen they rely on a salvage pathway (Panozzo et al., 2002). The salvage pathway relies upon the nicotinic acid transporter encoded by *TNA1* and the nicotinate phosphoribosyl transferase encoded by *NPT1* (Llorente and Dujon, 2000; Panozzo et al., 2002). These two genes are widely conserved among budding yeasts, implying that while they are essential to anaerobic growth in *S. cerevisiae*, they are not contributing toward anaerobic growth in most species. Further, several obligately aerobic species use the salvage pathway exclusively as they lack some NAD⁺ biosynthetic genes, implying that the salvage pathway may underlie adaptations toward nicotinic acid auxotrophy, even in aerobic species (Li and Bao, 2007).

While decades of work have identified an exhaustive list of genes underlying anaerobic growth in *S. cerevisiae*, this kind of attention has not been paid to other distantly related yeast species. Even though the facultative anaerobic phenotype has been best demonstrated in yeasts closely related to *S.*

cerevisiae, some species within the distantly related *Brettanomyces/Dekkera* genus have also been found to grow well anaerobically (Rozpędowska et al., 2011). The most parsimonious explanation for the enrichment of anaerobic species in the post-WGD Saccharomycetaceae is the origin of critical genes around the time of the WGD event, while the anaerobic growth in the *Brettanomyces* is likely the result of an independent acquisition of the trait. The number of *Brettanomyces* genomes continues to increase with time, paving the way for comparative genomics among these species to identify gene candidates associated with anaerobic growth (Borneman et al., 2014; Roach and Borneman, 2020). Genetic studies of *Brettanomyces bruxellensis* using CRISPR-Cas9 will also make identifying genes involved in anaerobic growth in this lineage feasible (Varela et al., 2020). With more genetics and genomics, an understanding of how the anaerobic trait evolved in two independent lineages may be on the horizon, with implications for better understanding how two independent yeast lineages may have converged on the same style of growth.

Using comparative genomics to infer ecology

Through “reverse ecology,” information gleaned from comparative genomics can be used to generate hypotheses about the ecology of organisms (Li et al., 2008). This is especially useful in microorganisms for which genomic data is constantly increasing while direct assessment of their ecology is limited (Prosser et al., 2007). For budding yeasts, this approach can be applied to a growing set of sequenced species currently totaling over 330 (Shen et al., 2018). Population genomics is also becoming common for several budding yeast species, which will ultimately enable more detailed assessments of population genetics beyond gene presence and absence, but rather incorporate signatures of selection or identify specific alleles associated with phenotypes (O’Brien et al., 2021; Peris et al., 2016; Peter et al., 2018).

For some anaerobic fungi, our current understanding of their ecology overlaps with the expectations using comparative genomics and reverse ecology. Members of the Neocallimastigomycota are generally isolated from the anoxic environment of the herbivore rumen, and are obligate anaerobes (Gruninger et al., 2014). The genomes of these anaerobic rumen fungi (ARF) contain several genes acquired via HGT from bacteria that replace oxygen-dependent biosynthetic reactions with oxygen-independent versions (Murphy et al., 2019). For example, most fungal cell membranes contain the steroid alcohol ergosterol, the production of which requires molecular oxygen. ARF lack an ergosterol biosynthesis pathway and instead contain a gene encoding for squalene-tetrahymanol cyclase, an enzyme that produces the cyclic triterpenoid tetrahymanol from squalene (Takishita et al., 2012). Expression of a homolog of this gene was shown to enable growth of *S. cerevisiae* in the absence of oxygen and ergosterol biosynthesis (Wiersma et al., 2020). ARF contain bacterial-derived *nadA* and *nadB* genes encoding an oxygen-independent nicotinic acid biosynthesis pathway, and expression of these genes in *S. cerevisiae* has been shown to complement a nicotinic acid biosynthetic mutant (Perli et al., 2021). The acquisition and maintenance of these pathways suggests the need to produce these important molecules in the absence of oxygen. ARF also contain a gene encoding an oxygen-sensitive class III ribonucleotide reductase (Murphy et al., 2019). They also contain hydrogenosomes which are typical in anaerobic eukaryotes (Yarlett et al., 1986). These adaptations imply that ARF consistently live in anoxic environments and may even be sensitive to oxygen, which is consistent with our current understanding of their ecology.

Applying reverse ecology to the genetic repertoire of *S. cerevisiae* does not support the same inference of extreme anaerobic adaptation. As described above, *S. cerevisiae* relies upon conditional auxotrophies during anaerobic growth, meaning that anaerobic growth can only occur when certain nutrients are

available. These conditional auxotrophies include sterols, unsaturated fatty acids, and niacin, as well as potentially biotin, pantothenate, and thiamine (Mooiman et al., 2021). The only prototrophic adaptation under anoxic conditions in *S. cerevisiae* is the bacterial DHODase for biosynthesis of uracil. *S. cerevisiae* also contains a class I RNR gene, which requires trace amounts of oxygen for catalysis (Harder and Follmann, 1990). From a reverse ecology standpoint, the genetic repertoire of *S. cerevisiae* suggests that this yeast does not normally exist under strictly anoxic conditions, but instead may thrive under severely oxygen-limited conditions or brief periods of strict anaerobiosis. While less is known about the evolution of anaerobic growth in the lineage containing *D. bruxellensis*, the ecological inferences to be made are similar. *D. bruxellensis* contains an anaerobically functional DHODase (Bouwknegt et al., 2021), but genomics has not identified other prototrophic adaptations, and the identities of anaerobic genes such as sterol transporters in this organism have yet to be worked out.

Outlook

Understanding how the ability for anaerobic growth evolved in a subset of the budding yeasts will require a thorough accounting of precisely which species are capable of this phenomenon, what genes or mutations differentiate these species from closely related aerobic species, and continued expansion of population genomics to improve our knowledge of budding yeast ecology. This will require broad efforts to attempt to culture many species under anoxic conditions, as well as continued searches for unexplored yeast diversity using anaerobe-specific cultivation methods may also expand our anaerobic species list beyond the close relatives of *Saccharomyces* and *Brettanomyces*. With a growing cohort of researchers interested in studying non-traditional model yeast species, along with rapidly advancing genetic tools for identifying and characterizing gene functions in these yeasts, and continuously increasing access to genomic and transcriptomic sequencing, a greater understanding of many of the diverse phenotypes exhibited by budding yeasts, including anaerobic growth, is on the horizon.

Figure Legend

Maximum likelihood tree of *AUS1/PDR11* homologs in the Saccharomycetaceae. Sequences were identified using a public BLAST database of 332 yeast genomes (<https://y1000plus.wei.wisc.edu/data>) and manually extracted from sequence files. Nucleotide sequences were aligned using the codon-aware translatorX program (Abascal et al., 2010). The resulting alignment was used to compute a phylogenetic tree using RAxML V.8 (Stamatakis, 2014). The phylogenetic tree was visualized in FigTree (<http://tree.bio.ed.ac.uk/software/figtree/>).

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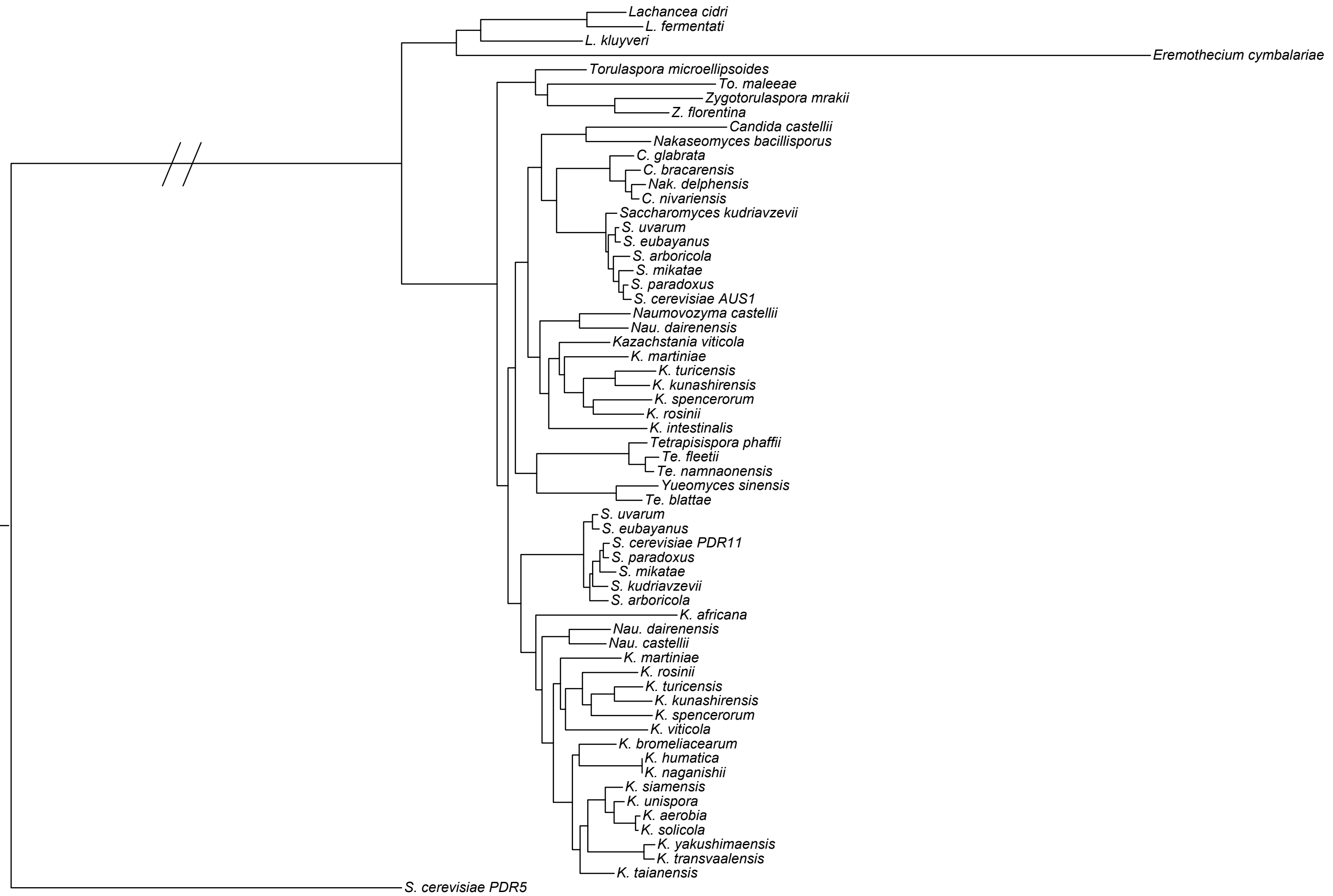
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Table 1 – Genes associated with anaerobic growth

Gene name	RefSeq No.	Description
<i>AUS1</i>	NP_014654.1	Sterol transporter
<i>PDR11</i>	NP_012252.1	Sterol transporter
<i>TIR1</i>	NP_010927.1	Cell wall mannoprotein
<i>TIR3</i>	NP_012254.1	Cell wall mannoprotein
<i>URA1</i>	NP_012706.1	Cytoplasmic DHODase
<i>TNA1</i>	NP_011776.1	Nicotinic acid transporter
<i>NPT1</i>	NP_014852.1	NAD salvage pathway gene
<i>FUR4</i>	NP_009577.1	Uracil permease



0.6