Nitrate assimilation phenotype variability and heterogeneity in *Brettanomyces bruxellensis*

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Contents

Abstract

Brettanomyces bruxellensis is a yeast species associated with industrial fermentation ecosystems. The ability to use alternative nutrient sources, such as nitrate, may be advantageous and allow it to outcompete other microbial species. The assimilation of nitrate is conferred by the expression of structural proteins that facilitate nitrate uptake and reduction. The genes (*YNR1*, *YNI1* and *YNT1*) encoding these structural proteins form the structural nitrate assimilation gene cluster. Expression of these genes is putatively controlled by nitrogen catabolite repression and two $Zn(II)_2Cys_6$ transcription factors, which are encoded by *YNA1* and *YNA2*. These two genes are not collocated with the structural nitrate assimilation gene cluster and form the regulatory nitrate assimilation gene cluster.

This study explores sequence and ploidy variation at these two nitrate assimilation gene clusters in *B. bruxellensis* and relates this to variable nitrate assimilation potential across a cohort of forty-one *B. bruxellensis* strains isolated from a range of industrial fermentative sources. It was found that in some apparent nitrate-negative isolates a subset of the population can switch to a nitrate-positive phenotype, which could be an example of microbial bet-hedging. These strains shared a common haplotype of the structural nitrate assimilation gene cluster. A representative of this cohort of isolates, AWRI1608, was found to switch from nitrate-negative to nitrate-positive at a rate of approximately 1 in 10^5 cells during incubation on solid media with nitrate as the sole nitrogen source for 7 days. Nitrate-positive colonies were isolated from these plates and were shown to retain their nitrate-positive phenotype over 100 generations in non-selective media, but were near-isogenic with the original isolate and showed no mutations in or near nitrate assimilation genes. AWRI1608 and its nitrate-positive variants were used to develop a method for assessing competitive fitness between isolates using a newly-developed transformation protocol to mark strains with antibiotic-resistance. This study is the first to show nitrate assimilation phenotype heterogeneity in *B. bruxellensis*.

Thesis Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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1 Literature Review

Microbial ecosystems are incredibly diverse and there exists a high degree of competition within these ecosystems between resident microbes. The specialization of microbial species via the genetic adaptation to their environment is integral to the survival of species within an ecosystem (Futuyma & Moreno 1988). For example, Cooper & Lenski (2000) described a rapid increase in competitive fitness of *Escherichia coli* populations through adaptation to minimal medium supplemented with glucose. Ecological success is ultimately determined by the fitness of one genotype over others within an environment (Kassen & Rainey 2004). Mechanisms are developed by microbes through evolution that affect their fitness within their niche. These are commonly for better nutrient acquisition or for inhibiting the growth of competitors. One such mechanism that can achieve both these outcomes is fermentation.

1.1 Industrial fermentation ecosystems

Fermentation is a metabolic process employed by some microorganisms in which sugars are broken down to acids, carbon dioxide and/or alcohol. Anaerobic fermentation occurs in a wide range of ecological settings in nature and is an important mechanism for the generation of ATP in the absence of oxygen as an alternative to oxidative phosphorylation (Pfeiffer, Schuster & Bonhoeffer 2001).

In certain yeast species, when glucose levels are high, fermentation is preferred over cellular respiration, even under aerobic conditions (Pfeiffer, Schuster & Bonhoeffer 2001). For example, *Saccharomyces cerevisiae* displays aerobic fermentation to degrade sugars to two-carbon components, resulting ultimately in the production of ethanol (Figure 1). High levels of extracellular glucose trigger increased glycolysis rates, which generate sufficient ATP through substrate-level phosphorylation such that oxidative phosphorylation via the TCA cycle is not necessary (De Deken 1966). This is known as the "Crabtree effect" and allows *S. cerevisiae* to outcompete other microorganisms by quickly metabolising available sugars and producing ethanol to inhibit the growth of competitors at the expense of overall ATP yield (De Deken 1966).

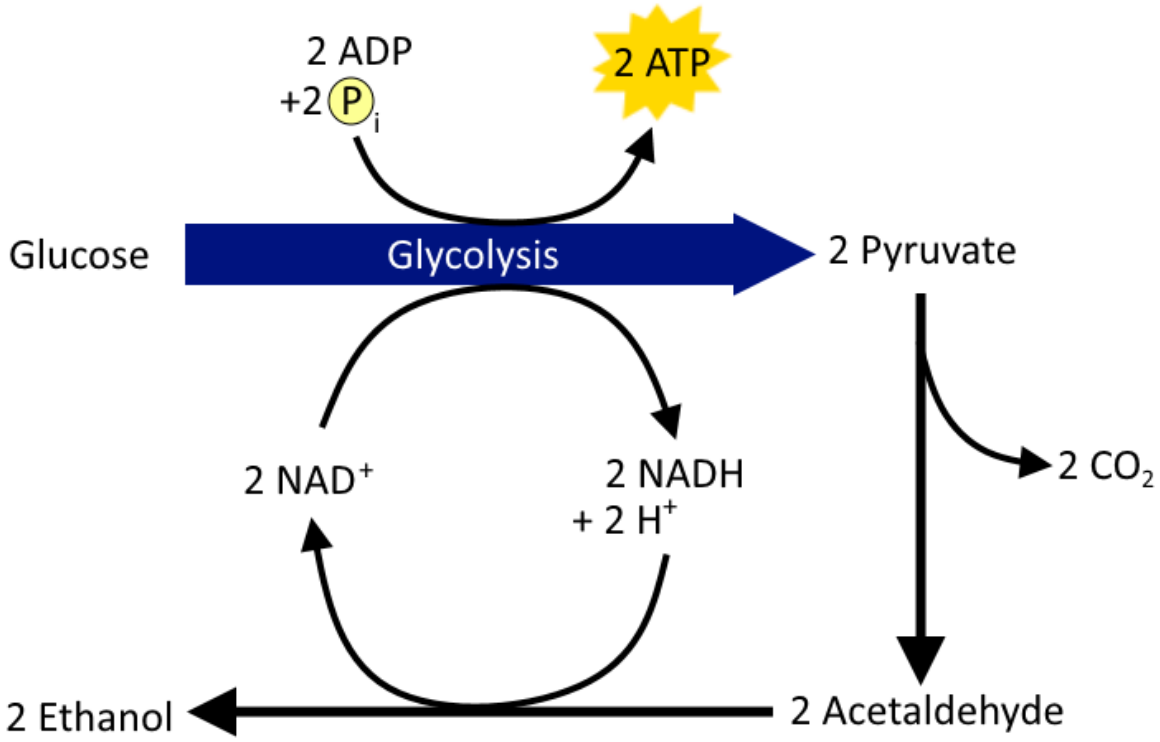


Figure 1. Schematic of the alcoholic fermentation of glucose to produce ethanol.

Glucose is degraded to two pyruvate molecules by glycolysis, a process which generates ATP. Pyruvate is then converted to ethanol via acetaldehyde, giving off carbon dioxide. The accumulation of ethanol balances the $NADH/NAD^+$ ratio in order for glycolysis to continue.

However, under aerobic conditions, more ATP can be produced overall from glucose via the tricarboxylic acid (TCA) cycle than through alcoholic fermentation (36 moles ATP per mole of glucose, compared to 2 mol/mol) (De Deken 1966). Therefore, when glucose becomes limited, fermentative metabolism is inhibited under aerobic conditions in order to maximise the yield of ATP from the available glucose (De Deken 1966). This phenomenon is known as the "Pasteur effect".

Humans have taken advantage of alcoholic fermentation for thousands of years, ultimately resulting in the domestication of *S. cerevisiae* (Martini 1993). The fermentative metabolism of *S. cerevisiae* is integral to the production of alcoholic beverages, such as wine and beer. Recently, this mechanism has also been implemented for the production of biofuels, with the aim of converting base sugars (such as in sugar cane) to a high yield of ethanol. However, even in these industrial fermentative ecosystems there exist a wide range of microbial species, which are in direct competition with one another for available nutrients (Rozpędowska *et al.* 2011). The specialisation of microbial species is an important factor in maintaining competitiveness in such dynamic ecosystems.

1.1.1 *Brettanomyces bruxellensis*

Brettanomyces (formerly designated *Dekkera*) is a genus of the *Saccharomycotina* subphylum that is highly relevant to industrial fermentation processes and species of *Brettanomyces* have been isolated from a wide range of fermentation sources (Table 1). Of particular interest is the species *B. bruxellensis*, which was first isolated from English beer in 1904 (Claussen 1904). Interestingly, despite being isolated from beer, *B. bruxellensis* is the only species of the genus that has been found in wine (Cecchini *et al.* 2013). *B. bruxellensis* has also been observed to succeed *S. cerevisiae* as the dominant microbial species in industrial alcoholic fermentation ecosystems (Nardi, Remize & Alexandre 2010). Although physiologically similar to *S. cerevisiae* (Rozpędowska *et al.* 2011), *B. bruxellensis* is evolutionarily very distant from this yeast and is more closely related to the genus *Ogataea* (Figure 2).

B. bruxellensis is commonly associated with wine spoilage due to its ability to produce unfavourable aroma compounds such as the volatile phenols 4-ethylphenol and 4-ethylguaiacol (collectively termed "Brett" character) (Suárez *et al.* 2007). This spoilage is most commonly observed in red wines during aging in oak barrels (Oelofse, Pretorius & Toit 2008). Wine spoilage by *B. bruxellensis* often devalues wine to the point where it goes to waste, leading to economic losses and increased environmental stress through wasted resources (e.g. water, electricity) and excessive waste production (Fugelsang & Zoecklein 2003).

The production of volatile phenols by *B. bruxellensis* is also a characteristic that has been observed in beer (Gilliland 1961). While low levels of these volatile phenols can be appreciated in some beer styles (Vanbeneden *et al.* 2008; Steensels *et al.* 2015), in high concentrations they can impart phenolic off-flavours that are undesirable, as seen in wine (Vanbeneden *et al.* 2008; Steensels *et al.* 2015).

Table 1. A list of some *Brettanomyces* isolates and their sources.

B. bruxellensis has been isolated from a wide range of sources. While other *Brettanomyces* species have been isolated from some similar sources, *B. bruxellensis* is the only one to have been isolated from wine. The instances where no isolate name is given are indicative of literature in which the detection of *Brettanomyces* is presented, but no isolates are named.

Isolate	Species	Isolation source	Reference
AWRI1499	*B. bruxellensis*	Red wine (McLaren Vale)	(Curtin *et al.* 2007)
AWRI1608	*B. bruxellensis*	Red wine (Margaret River)	(Curtin *et al.* 2007)
AWRI1613	*B. bruxellensis*	Red wine (Barossa Valley)	(Curtin *et al.* 2007)
CBS73	*B. bruxellensis*	Grape must	(Smith 1998)
CBS74	*B. bruxellensis*	Lambic beer	(Smith 1998)
CBS75	*B. bruxellensis*	Beer	(Smith 1998)
CBS4914	*B. bruxellensis*	Tea-beer	(Smith 1998)
CBS5206	*B. bruxellensis*	Grape must	(Smith 1998)
CBS5512	*B. bruxellensis*	Brewery equipment	(Smith 1998)
CBS5513	*B. bruxellensis*	Bantu beer	(Smith 1998)
CBS6055	*B. bruxellensis*	Ginger ale	(Smith 1998)
CBS8027	*B. bruxellensis*	Soft drink	(Smith 1998)
-	*B. bruxellensis*/B. anomala	Cider	(Morrissey *et al.* 2004)
-	*B. bruxellensis*	Coolship ale	(Bokulich, Bamforth & Mills 2012)
-	*B. bruxellensis*	Kefir	(Laureys & De Vuyst 2014)
-	*B. bruxellensis*	Kombucha	(Greenwalt, Steinkraus & Ledford 2000)
-	*B. bruxellensis*	Sourdough	(Meroth, Hammes & Hertel 2003)
CBS1945	*B. nanus*	Bottled beer	(Boekhout *et al.* 1994)
CBS4805	*B. custersianus*	Bantu beer brewery	(van der Walt 1961)
CBS6042	*B. naardenensis*	Soft drink	(Kolfschoten & Yarrow 1970)
CBS8139	*B. anomala*	Spoiled soft drink	(Smith & van Grinsven 1984)

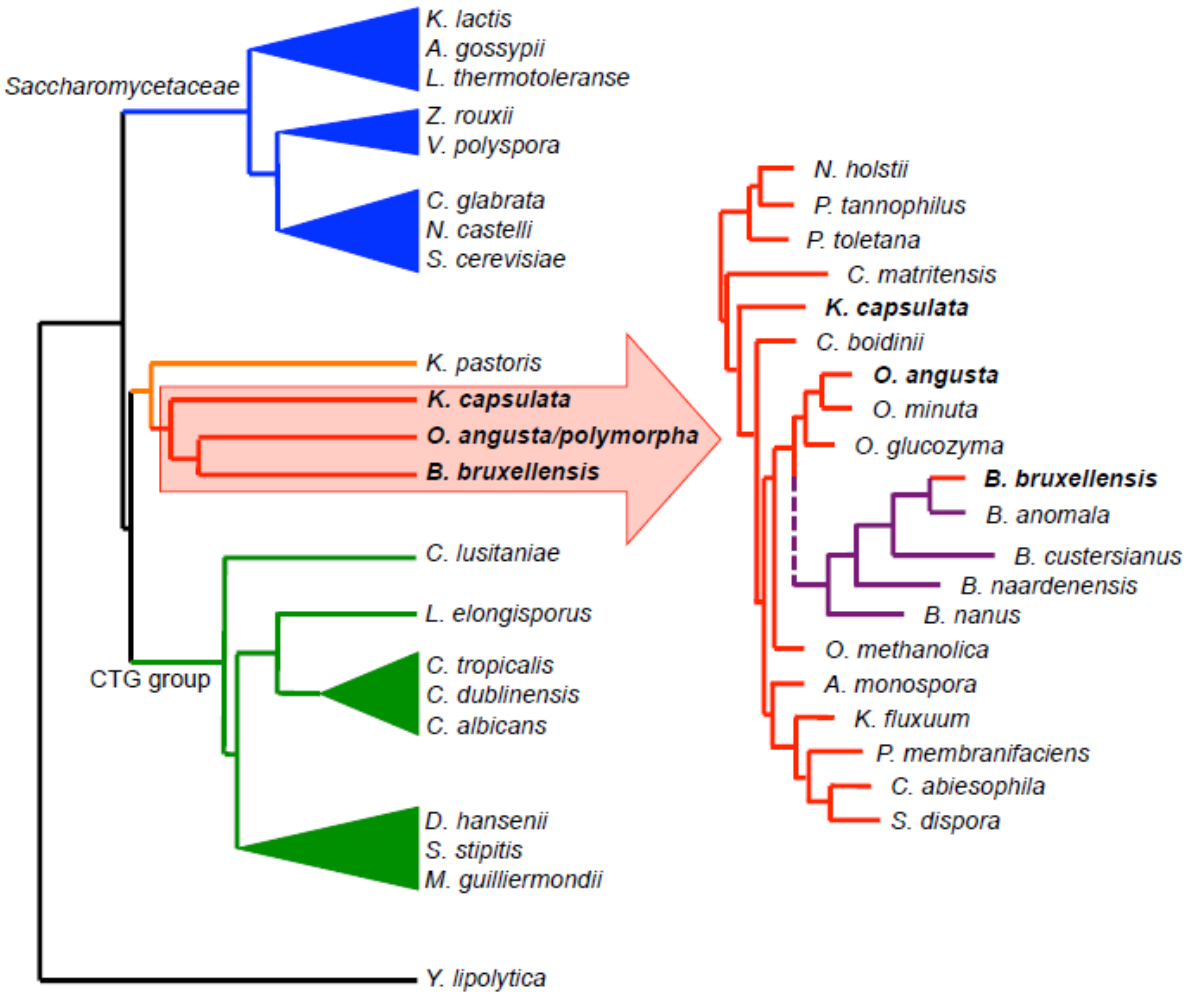


Figure 2. Schematic representation of *B. bruxellensis* phylogenetic relationship with other yeast species.

B. bruxellensis is grouped with methylotrophic yeast species *Komagataela pastoris*, *Kuraishia capsulata* and *Ogataea angusta*/*polymorpha* according to 'whole-genome' based phylogenies (Curtin *et al.* 2012; Piškur *et al.* 2012; Ravin *et al.* 2013) (Left). Other *Brettanomyces* species are predicted to group with *B. bruxellensis* based upon a separate multi-gene phylogeny (Kurtzman 2011) (Right, purple branches). *B. bruxellensis* is the only species in its phylogenic group found in wine. Sourced from Curtin & Pretorius (2014).

The species is also known to contaminate biofuel fermentations, which can result in a decreased ethanol yield from alcoholic fermentation due to the yeast's tendency to favour the production of acetate over ethanol (de Souza Liberal *et al.* 2007). Despite this effect on ethanol yield, *B. bruxellensis* has been identified as a species that could be used as an alternative industrial ethanol yeast (Blomqvist *et al.* 2010). *B. bruxellensis* has been shown to outcompete *S. cerevisiae* during continuous alcoholic fermentations (Blomqvist *et al.* 2010; de Barros Pita *et al.* 2011), and therefore may be better suited to some industrial biofuel fermentations than *S. cerevisiae*. However, the decreased rate of ethanol production and increased formation of acetic acid by *B. bruxellensis* in comparison to *S. cerevisiae* remains problematic.

B. bruxellensis and *S. cerevisiae* have evolved in parallel such that both of these species have converged on similar phenotypes useful for survival and competitiveness in alcoholic fermentations, such as ethanol accumulation and tolerance and acid tolerance (Rozpędowska *et al.* 2011). Both *B. bruxellensis* and *S. cerevisiae* employ a make-accumulate-consume strategy to glucose metabolism, fermenting sugars into ethanol and acetic acid that they can later metabolise further (Rozpędowska *et al.* 2011). However, it is in the nutrient-sparse environment of post-fermentation (e.g. finished wine and biofuel) that *B. bruxellensis* is able to out-compete *S. cerevisiae* and become the predominant species of the ecosystem. This has been attributed to the "scavenging" lifestyle of *B. bruxellensis* (Blomqvist *et al.* 2012). Accordingly, *B. bruxellensis* has been shown to possess genes encoding proteins for the utilisation of alternative carbon sources, such as chitin, *N*-acetylglucosamine, galactose, mannose and lactose (Curtin *et al.* 2012).

Similarly, *B. bruxellensis* is able to use nitrate as a sole source of nitrogen, and this trait has been shown to enable it to outcompete *S. cerevisiae* in continuous industrial fermentations (de Barros Pita *et al.* 2011). Interestingly, some isolates of *B. bruxellensis* are the only known wine-associated yeasts that display the ability to use nitrate (Borneman *et al.* 2014).

1.2 Nitrate assimilation

The availability of a source of nitrogen is a prerequisite for the growth of all organisms. Inorganic nitrogen sources are assimilated by organisms such as plants, algae, yeasts and some bacteria and used to generate organic nitrogen compounds such as amino acids (Magasanik 1982; Miflin & Lea 1976). The requirement for nitrogen results in competition between species within an ecological niche for available nitrogen. When more favourable nitrogen sources, such as ammonium or glutamate, are unavailable some species are able to switch to assimilating a less favourable, but more available, nitrogen source such as nitrate (Geurrero *et al.* 1981; van der Walt 1963).

Nitrate assimilation is common amongst the filamentous fungi, where the genes involved in nitrate assimilation have been well characterised (Takaya 2014). The ability to assimilate nitrate, however, is less common in yeast species. Of the many genera of yeast found within the *Saccharomycotina* subphylum, nitrate assimilation genes have only been identified in the genera *Brettanomyces*, *Ogataea*, *Wickerhamomyces* and *Blastobotrys* (Borneman *et al.* 2014; Morales *et al.* 2013). Until recently, *Ogataea polymorpha* (specifically isolate NCYC495) has been the only model for studying nitrate assimilation in yeast species (Cabrera *et al.* 2014). As such, nitrate assimilation in other yeasts, and the involvement of nitrate in industrial fermentative ecosystems, has not attracted significant research interest. This is mainly due to the fact that *S. cerevisiae*, the pre-eminent industrial yeast species, does not use nitrate. As *Brettanomyces* represents the only yeast genus known to both inhabit fermentative ecosystems and assimilate nitrate, it represents the ideal organism for studying the influence of nitrate assimilation on industrial fermentative ecosystems.

1.2.1 Assimilatory nitrate reduction

Nitrate assimilation in yeasts and fungi is achieved by first transporting nitrate into the cytoplasm via a nitrate transporter protein (Figure 3). Two classes of nitrate transporter have been defined in fungi: one with high-affinity (K_m in µM ranges), found in yeasts, fungi, algae and plants (Crawford & Glass 1998; Daniel-Vedele, Filleur & Caboche 1998; Siverio 2002); and one with low-affinity (K_m in mM ranges), mainly found in plants, however there is some evidence for the existence of low-affinity nitrate transporters in yeasts (Machín *et al.* 2001; Siverio 2002).

The gene *YNT1* in *O. polymorpha* has been cloned and encodes a putative high-affinity nitrate transporter (K_m 2-3 µM) (Machín *et al.* 2004; Perez *et al.* 1997). Ynt1 is the only high-affinity nitrate transporter in *O. polymorpha*, and disruption of the gene encoding the transporter has been shown to cause an inability to grow with nitrate as the sole nitrogen source (Perez *et al.* 1997). It is apparent that Ynt1 also transports nitrite with high affinity, however there is evidence for the existence of a nitrite-specific uptake system, since nitrite uptake is not inhibited by the presence of nitrate in a Δ*ynt1* mutant (Machín *et al.* 2004). Some species have been found to possess multiple nitrate uptake systems, for example, *A. nidulans*, in which the genes *crnA*, *nrtA* and *nrtB* have been characterized to encode nitrate (and nitrite) transporters (Unkles *et al.* 1991; Wang *et al.* 2008).

Following its import into the cell, nitrate is then reduced via nitrite to ammonium by two successive redox reactions catalysed by nitrate reductase and nitrite reductase, respectively (Figure 3). The first (nitrate to nitrite) is catalysed by a nitrate reductase enzyme, which in *O. polymorpha* is encoded by the gene *YNR1* (Avila *et al.* 1995). Ynr1 shares high amino acid sequence similarity with assimilatory nitrate reductases such as those encoded by *Nit-3* (*Neurospora crassa*), *niaD* (*A. nidulans*) and *nia1* (tobacco); particularly within the molybdopterin, heme-iron and FAD cofactor binding regions (Avila *et al.* 1995). Enzymatic function appears to be highly conserved between species for assimilatory nitrate reductases. Ynr1 in *O. polymorpha* preferentially uses NADPH as an electron donor to catalyse the reduction of nitrate, but is also able to use NADH (Pignocchi, Berardi & Cox 1998).

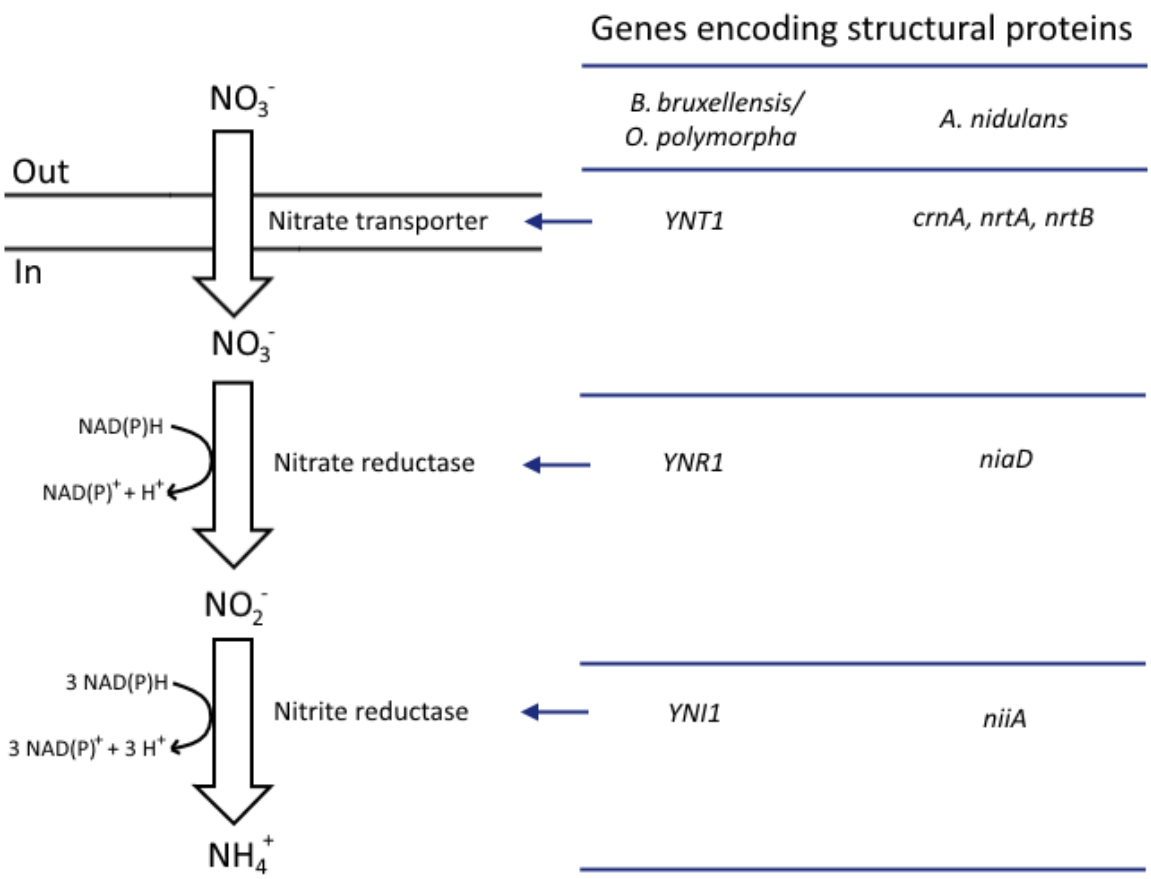


Figure 3. Pathway of nitrate assimilation in yeasts and filamentous fungi.

Nitrate is transported from the medium into the cell cytosol, where it is reduced to ammonium in two successive reactions catalysed respectively by nitrate and nitrite reductase. The table shows the names of genes predicted to encode the structural nitrate assimilation proteins in *B. bruxellensis*/*O. polymorpha* and in *A. nidulans*. Adapted from Siverio (2002).

The second reduction (nitrite to ammonium) is catalysed by nitrite reductase. The gene *YNI1* in *O. polymorpha* encodes a nitrite reductase which, like the nitrate reductase, uses NAD(P)H as an electron donor (Brito *et al.* 1996). In contrast, assimilatory nitrite reductases in plants and algae are known to be dependent on ferredoxin as an electron donor (Geurrero, Vega & Losada 1981). The nitrite reductase putatively encoded by *YNI1* shares approximately 50% identity with nitrite reductases in *A. nidulans* (*niiA*) and *N. crassa* (*nit-6*) (Brito *et al.* 1996).

1.2.2 Regulation of nitrate assimilation

While providing a means to utilise an important source of nitrogen, the nitrate assimilation pathway has a high energy cost, requiring the transfer of 8 electrons to fully reduce nitrate to ammonium. In order to adapt to using the most efficient nitrogen source available, species that can utilise this pathway to assimilate nitrate have complex systems for its regulation. The pathway is typically induced by the presence of nitrate (and/or nitrite) and is repressed in the presence of more favourable nitrogen sources through nitrogen catabolite repression (NCR).

The expression of *YNT1*, *YNR1* and *YNI1* is regulated primarily at the transcriptional level in *O. polymorpha.* Clustered with the three structural nitrate assimilation genes are two additional open reading frames (designated *YNA1* and *YNA2*) (Figure 4), which are predicted to encode Zn(II)2Cys6-type transcription factors and which are indispensable for the induction of the nitrate assimilation pathway (Avila *et al.* 1998, 2002). These transcription factors have high sequence similarity with the *A. nidulans* and *N. crassa* nitrate induction transcription factors NirA and NIT-4, respectively (Avila *et al.* 1998; Avila *et al.* 2002; Burger *et al.* 1991; Yuan *et al.* 1991). When transferred to media containing nitrate as a sole nitrogen source, Δ*yna1* and Δ*yna2* mutants of *O. polymorpha* do not display the increases in transcript levels for *YNT1*, *YNR1* or *YNI1* that are observed in wild-type cells (Avila *et al.* 1998; Avila *et al.* 2002). This suggests that both *YNA1* and *YNA2* play a role in the transcriptional activation of *YNT1*, *YNR1* and *YNI1*.

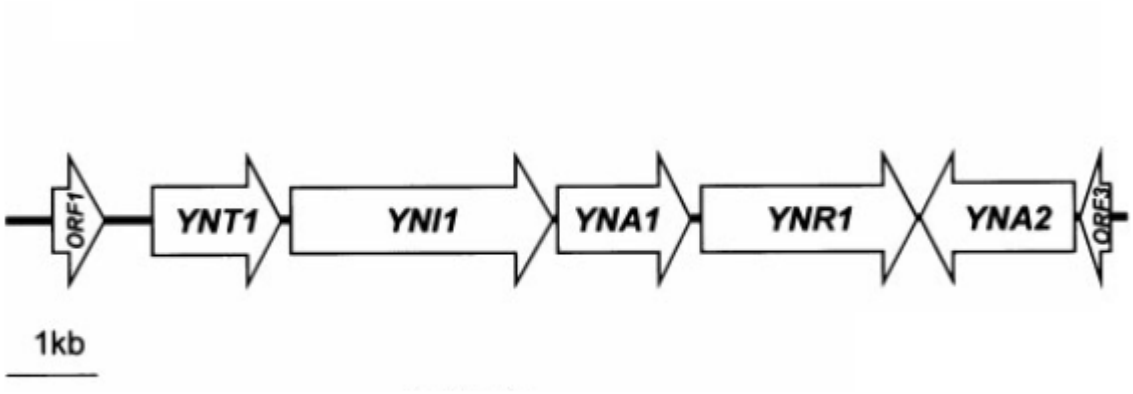


Figure 4. The *O. polymorpha* genomic DNA region containing the cluster of genes involved in nitrate assimilation.

This genomic region contains *ORF1* encoding a protein similar to glutathione-S transferases, not involved in nitrate assimilation (*ORF1*, 618 bp); nitrate transporter (*YNT1*, 1524 bp); nitrite reductase (*YNI1*, 3132 bp); a Zn(II)2Cys6 transcriptional factor regulating nitrate assimilation gene transcription (*YNA1*, 1587 bp); nitrate reductase (*YNR1*, 2577 bp); a second Zn(II)2Cys6 transcriptional factor regulating nitrate assimilation gene transcription (*YNA2*, 1842 bp); and *ORF3* encoding a protein similar to a *S. cerevisiae* Rad3p region, not involved in nitrate assimilation (*ORF3*, 354 bp). The lengths of non-coding regions between ORFs are, from left to right: 565, 220, 45, 113, 7 and 45 bp. The direction of each arrow indicates the direction of transcription of the represented ORF. Sourced from Avila *et al.* (2002).

Interestingly, this is the only nitrate assimilation system to date that has been described to be inducible by two different zinc-finger DNA binding transcription factors. It is possible that a heterodimer of the two transcription factors is necessary for transcriptional activation of the nitrate assimilation structural genes in this yeast, although no experimental evidence exists to sufficiently support or disprove this hypothesis. However it has been shown that in a Δ*yna1* mutant, the levels of *YNA2* transcript do not increase on nitrate as in the wild-type; while in a Δ*yna2* mutant, the levels of *YNA1* transcript increase as per the wild-type on nitrate (Avila *et al.* 2002). This suggests that *YNA1* may have some involvement in the transcriptional activation of *YNA2*, and Avila *et al.* (2002) propose a regulatory cascade of *YNA1*>*YNA2*>[*YNT1*, *YNR1* and *YNI1*].

Experimental characterization of the regulation of this system is limited, however it is clear that nitrate assimilation, under nitrogen catabolite repression, is inducible by the presence of nitrate as a sole nitrogen source and that *YNA1* and *YNA2* play a role in the transcriptional activation of genes encoding structural nitrate assimilation genes.

1.2.3 Nitrate assimilation in *B. bruxellensis*

The sequencing of a *B. bruxellensis* genome revealed the presence of a cluster of genes orthologous to those described in *O. polymorpha* that encode proteins for nitrate assimilation (Woolfit *et al.* 2007): a nitrate transporter (*YNT1*), a nitrate reductase (*YNR1*) and a nitrite reductase (*YNI1*). Woolfit *et al.* (2007) also reported the presence of two Zn(II)2Cys6 transcription factors that are involved specifically in the induction of the nitrate assimilation pathway. While the structural nitrate assimilation genes are clustered in *B. bruxellensis*, the transcription factors are not part of this gene cluster. This is unlike the situation observed in *O. polymorpha* where all five ORFs are closely arranged (Avila *et al.* 2002; Borneman *et al.* 2014; Woolfit *et al.* 2007).

The use of nitrate by *B. bruxellensis* as a nitrogen source may be an important factor in its adaptation to fermentative ecosystems. Indeed, its scavenging lifestyle is particularly beneficial in the nutrient-sparse

conditions typical of the late stages of industrial fermentation processes. Competition experiments have shown that the use of nitrate confers an advantage to *B. bruxellensis* over *S. cerevisiae* in both synthetic medium containing nitrate and low levels of ammonium, and in sugar cane juice (de Barros Pita *et al.* 2011). There is also a correlation between high levels of nitrate (260 ± 30 mg/L) and high cell counts of *B. bruxellensis* (approximately 50% of yeast population) in industrial sugar cane juice fermentations (de Barros Pita *et al.* 2011).

As discussed earlier, *B. bruxellensis* has been isolated from a number of different industrial fermentation ecosystems, including those used for the production of wine, beer and biofuel. While information is somewhat sparse, the presence of nitrate in these industrial fermentative ecosystems has been studied. Ough and Crowell (1980) have determined nitrate to be present in the range of 2.1 - 53.7 mg/L (mean 16.4 mg/L and 0.9 - 41.4 mg/L (mean 8.5 mg/L) in Californian white and red wines respectively (Table 2). Massey *et al.* (1990) found more than 10mg/L nitrate in over half of 172 beers analysed. Sugar cane juice used for biofuel production has been found to have 242 mg/L nitrate (de Barros Pita *et al.* 2011). Childs, Bohlscheid & Edwards (2015) found that ≥6 mg/L yeast assimilable nitrogen (ammonium and amino nitrogen) is sufficient for *B. bruxellensis* growth. Reported nitrate concentrations in wine, beer and sugar cane juice would therefore be sufficient to support the growth of *B. bruxellensis*.

It has not been determined if the ability to use nitrate is advantageous to *B. bruxellensis* in the batch-fermentation of wine or beer as has been shown in continuous alcoholic fermentation of sugar cane juice. Decreases in nitrate concentration in grape must have not been observed. This persistence of nitrate during wine fermentation is presumably due to the inability of other wine associated yeasts to assimilate nitrate (Ough & Crowell 1980). However, it has not been determined if the presence of *B. bruxellensis* in wine or beer fermentations results in a decrease in nitrate levels.

Table 2. Nitrate content (mg/L NO3-) of California wines as reported by Ough and Crowell (1980).

Variety	Number of samples	Mean	Standard deviation	Range
Whites				
Chardonnay	33	18.8	± 13.6	3.0 - 53.7
Chenin blanc	13	20.7	± 13.0	2.7 - 43.9
Sauvignon blanc	12	15.4	± 5.8	5.6 - 24.8
French Colombard	6	14.0	± 9.7	5.0 - 27.0
Riesling	25	13.4	± 7.2	5.3 - 38.4
Gewürztraminer	9	11.3	± 5.8	2.1 - 20.3
Others	24	15.2	± 8.3	2.8 - 36.6
Total	122	16.4		2.1 - 53.7
Reds				
Cabernet Sauvignon	29	6.0	± 7.6	0.9 - 40.4
Pinot noir	13	14.2	± 10.0	2.2 - 36.9
Zinfandel	18	5.11	± 2.7	1.1 - 10.0
Others	15	10.9	± 10.7	2.6 - 41.4
Total	75	8.5		0.9 - 41.4

Effects of nitrate assimilation on the physiology of B. bruxellensis

There is evidence to suggest that nitrate assimilation in *B. bruxellensis* influences the fermentative metabolism of the yeast (Galafassi *et al.* 2013). An important characteristic of fermentative metabolism that has been described in *B. bruxellensis* is that alcoholic fermentation is inhibited under strictly anaerobic conditions (Scheffers 1961). This is known as the "negative Pasteur effect" or "Custers effect". As *B. bruxellensis* emerges as a possible candidate organism for industrial bioethanol production (Blomqvist *et al.* 2010), it is important to understand the physiology of the yeast such that efficient ethanol formation can be reliably attained. More broadly, understanding the physiology of the species is important for understanding the yeast's adaptation to the wide range of fermentative and non-fermentative ecosystems that it is known to inhabit.

Galafassi *et al.* (2013) reported that the use of nitrate in *B. bruxellensis* partially abolishes the "Custers effect" such that the alcoholic fermentation rate is not inhibited under anaerobic conditions. Nitrate assimilation was also shown to trigger *B. bruxellensis* to favour the production of acetic acid over ethanol. The study focused on the growth of *B. bruxellensis* isolate CBS2499 in aerobic and anaerobic conditions with nitrate, ammonium or a mixture of the two nitrogen sources. It was found that induction of the nitrate assimilation pathway, as demonstrated via enzymatic activity assays, resulted in higher growth and alcoholic fermentation rates than when nitrate was not used. It is important to note, however, that the final ethanol yield was unchanged by nitrate assimilation.

Furthermore, a correlation was found between acetic acid production and nitrate consumption under anaerobic conditions. This is particularly interesting as acetic acid production has otherwise not been found under strict anaerobic conditions and its production is normally associated with oxygen concentration (Freer 2002). It was hypothesized that the need of nitrate assimilatory enzymes for NADPH is fuelled by NADP-dependent acetaldehyde dehydrogenase activity (Galafassi *et al.* 2013).

The influence of nitrate on aerobic fermentations is markedly different, as under aerobic conditions nitrate shifts the main product of glucose metabolism to acetic acid, rather than ethanol. It was hypothesized that under these conditions nitrate/nitrite reductases mainly use NADH as electron donors, competing with alcohol dehydrogenase for available NADH, thereby reducing the rate of ethanol production. This then results in the accumulation of acetaldehyde and the formation of acetic acid (Galafassi *et al.* 2013).

A similar study using the *B. bruxellensis* industrial isolate GDB248 found that the use of nitrate resulted in a 45% lower specific growth rate than in ammonium as well as lower and slower ethanol production anaerobically. As the conditions used in these experiments are largely similar to the conditions used by Galafassi *et al.* (2013), the most likely explanation for these contradictory results is that they represent differences in physiology between the CBS2499 and GDB249 isolates. de Barros Pita *et al.* (2013) characterized anaerobic fermentation by a low yield of acetate (0.02 g/g_{sugar}), whereas Galafassi *et al.* (2013) found that acetate formation was characteristic of anaerobic fermentation when nitrate was used. It is possible that Galafassi *et al.* (2013) did not actually observe anaerobic fermentation with nitrate, however this is unlikely as the cultures were maintained under "strict[ly] controlled anaerobic conditions in bioreactor[s]". Further analyses are required to corroborate these data, particularly to get a broader view of possible phenotypic variations in a wider range of *B. bruxellensis* isolates from different ecological backgrounds.

Variation in nitrate assimilation potential between B. bruxellensis isolates

Despite the obvious advantage that nitrate assimilation can confer to *B. bruxellensis*, it is not a defining characteristic of the species (Custers 1940). Nearly one third of *B. bruxellensis* isolates from various sources have been shown to not grow on nitrate (Conterno *et al.* 2006). It is possible that in some ecological settings, nitrate assimilation does not provide a selectable advantage for *B. bruxellensis*. Variability between *B. bruxellensis* isolates in this regard is poorly understood.

Genomic comparisons between *B. bruxellensis* isolates have provided some insight into the genetic mechanisms that underlie the apparent inability of some isolates to use nitrate. For example, a number of *B. bruxellensis* isolates appear to have undergone specific deletion of all, or part of, the nitrate assimilation gene cluster. This group of deletants includes the wine isolate AWRI1613 (Figure 5A) (Borneman *et al.* 2014), eleven different Belgian lambic beer isolates and one isolate from a Dutch soft drink (Crauwels *et al.* 2014). In addition to genomic deletion, other structural variants have also been observed to affect the nitrate cluster. The *B. bruxellensis* wine isolate AWRI1608 appears to have undergone a loss of heterozygosity at the nitrate assimilation gene cluster locus via gene conversion, resulting in three identical alleles of the cluster (Figure 5A) (Borneman *et al.* 2014). CBS2499, however, has apparently undergone a duplication of the cluster, resulting in four copies of the genomic region in a 1:1 ratio of two alleles (Figure 5A) (Borneman *et al.* 2014).

Phenotypicially, the isolates CBS2499 and AWRI1499 were shown to grow normally on nitrate as a sole nitrogen source (Figure 5B), consistent with the presence of complete ORFs for the nitrate assimilation genes (Borneman *et al.* 2014). AWRI1613 did not grow on nitrate (Figure 5B), as expected due to the lack of nitrate and nitrite reductase genes (Borneman *et al.* 2014). Interestingly, while AWRI1608 was shown to possess complete ORFs of each of the nitrate assimilation genes, it was not shown to grow on nitrate as a sole nitrogen source (Figure 5B) (Borneman *et al.* 2014). The genetic mechanism for the apparent inability of AWRI1608 to grow on nitrate could not be explained and warrants further investigation. While the nitrate assimilation genes in AWRI1608 are predicted to encode full-length proteins, it is not known if any of these proteins have reduced activity compared to those in AWRI1499 and CBS2499. It has also not been determined if the expression of these genes is lower in AWRI1608, which would limit the isolate's ability to use nitrate.

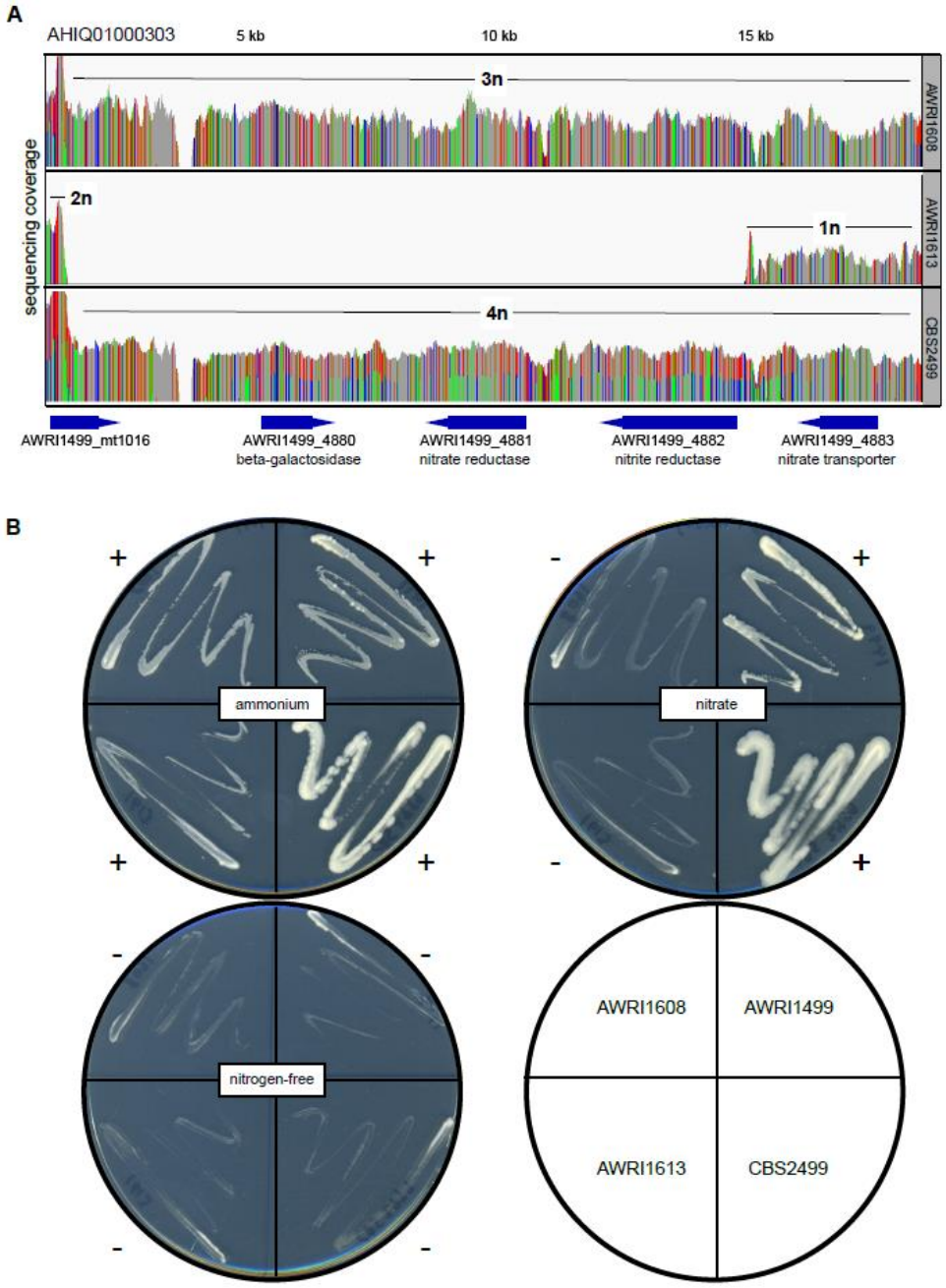


Figure 5. The loss of nitrate assimilation in *B. bruxellensis* isolates.

(A) Sequencing coverage across AWRI1608, AWRI1613 and CBS2499. Bases in disagreement to the AWRI1499 reference are coloured according to their sequence and proportion (blue=adenosine; green=cytosine; brown=guanine; red=thymine). Copy numbers of sequences predicted via the sequence read depth are shown (e.g. 4n). Positions of open reading frames according to the AWRI1499 genome are shown as blue arrows. Sourced from Borneman *et al.* (2014).

(B) Phenotypic analysis of *B. bruxellensis* isolates grown on either ammonium or nitrate. Scores for growth (+ or -) are indicated. Sourced from Borneman *et al.* (2014).

One would assume that it would not be disadvantageous for a yeast species to have the ability to assimilate an alternative source of nitrogen, such as nitrate. It is therefore surprising to see that a number of *B. bruxellensis* isolates display a loss of the phenotype. The selective pressure that has driven the loss of nitrate utlisation is not known but may stem from the high energy demand of the mechanism or the possible effects of nitrate assimilation on fermentative metabolism. It is possible that the use of nitrate is detrimental under some environmental conditions that the species encounters, thereby driving the loss of the phenotype in some isolates.

A key aim of this study was to establish a link between nitrate assimilation genotypes and phenotypes, with the hypothesis that certain nitrate assimilation gene alleles are less effective in conferring the ability to use nitrate. A further aim of this study was to determine a selective disadvantage to nitrate assimilation, as it was hypothesised that nitrate-negative *B. bruxellensis* isolates had lost this phenotype due to such a selective pressure.

2 Variable nitrate assimilation in *Brettanomyces bruxellensis* isolates

2.1 Introduction

Brettanomyces bruxellensis, like *Saccharomyces cerevisiae*, employs a make-accumulate-consume strategy, fermenting glucose in the presence of oxygen (Rozpędowska *et al.* 2011). However, unlike *S. cerevisiae*, *B. bruxellensis* is particularly adept at nutrient scavenging and assimilating alternative sources of nutrition (Conterno *et al.* 2006). A prime example of this is its ability to use nitrate as its sole source of nitrogen, a phenotype rarely observed in the *Saccharomycotina* subphylum. Nitrate assimilation has been shown to confer a distinct competitive growth advantage to *B. bruxellensis* over *S. cerevisiae* in the continuous industrial fermentation of sugar cane juice (de Barros Pita *et al.* 2011). Furthermore, the nitrate assimilation pathway has been shown to improve the anaerobic growth of *B. bruxellensis* and its fermentative metabolism by acting as a redox sink (Galafassi *et al.* 2013).

Despite the competitive benefits of being able to use an alternative source of nutrition, particularly in an environment where nutrients are scarce such as in post-alcoholic fermentation wine, *B. bruxellensis* displays intra-specific variation in nitrate assimilation. In previous studies, at least a quarter of *B. bruxellensis* isolates were found to be nitrate-negative (Conterno *et al.* 2006; Crauwels *et al.* 2014). Several *B. bruxellensis* isolates have also recently been shown to exhibit either full or partial deletions of the nitrate assimilation structural gene cluster, which corresponds with the inability to assimilate nitrate (Borneman *et al.* 2014; Crauwels *et al.* 2014). Specific gene deletion at this locus could be the result of selection against the nitrate-positive phenotype in these isolates. As discussed in Chapter 1, this could be due to the high energy input required for the reduction of nitrate to ammonium or could be related to the effects that it has on redox and subsequent redirection of the fermentation pathway. In addition to deletion of the nitrate assimilation cluster, there have been several other genomic structural variants

observed. For example, the heterozygous triploid isolate, AWRI1608, displays a nitrate-negative phenotype, despite possessing intact coding regions for the entire complement of nitrate assimilation genes (Borneman *et al.* 2014). However, in this heterozygous triploid isolate, the nitrate assimilation structural gene cluster was predicted to have undergone extensive gene conversion, resulting in three identical haplotypes across this large genomic locus (Borneman *et al.* 2014). Conversely, in the heterozygous diploid isolate CBS2499 the structural nitrate assimilation gene cluster has expanded from two to four copies (2 haplotypes, 1:1 ratio) and the isolate was found to grow well with nitrate as a sole nitrogen source. So, there have been observed several occurrences of genetic divergence at the structural nitrate assimilation gene cluster, including gene deletion, gene conversion and changes in copy number; which suggest that the ability to utilise nitrate in *Brettanomyces* may result in complex fitness outcomes. These genetic differences could be indicative of active selection for and against nitrate assimilation in different isolates.

The aim of this chapter was to explore sequence variation in nitrate assimilation structural and regulatory gene clusters and relate this to phenotype variability across a larger group of isolates.

2.2 Methods

2.2.1 Yeast isolates and culture conditions

B. bruxellensis isolates (Table 3) were obtained from the Australian Wine Research Institute Microorganisms Culture Collection and maintained on MYPG agar buffered with calcium carbonate (malt extract, 3.0 g/L; yeast extract, 3.0 g/L; bacteriological peptone, 2.0 g/L; glucose, 10.0 g/L; bacteriological agar, 15.0 g/L; calcium carbonate, 20.0 g/L). Starter cultures were grown from single colonies in liquid YPD (yeast extract, 10.0 g/L; bacteriological peptone, 20.0 g/L; glucose, 20.0 g/L) in test tubes incubated with orbital mixing at 28°C for 48 hours.

Table 3. A list of *B. bruxellensis* isolates used in this study and their source of isolation.

Isolate	AWRI number	Isolation source	Region	Country
CBS5512	1103	Brewing equipment		South Africa
CBS6055	1130	Dry ginger ale		United States
AWRI1499	1499	Wine	McLaren Vale	Australia
AWRI1605	1605	Wine	Yarra Valley	Australia
AWRI1606	1606	Wine	Swan Valley	Australia
AWRI1607	1607	Wine	Murchison	Australia
AWRI1609	1608	Wine	Margaret River	Australia
AWRI1609	1609	Wine	Margaret River	Australia
AWRI1613	1613	Wine	Barossa Valley	Australia
AWRI1615	1615	Wine	Mornington Peninsula	Australia
CBS2499	1626	Wine		France
AWRI1677	1677	Wine	Coonawarra	Australia
UCD615	2800	Wine	California	United States
UCD738	2801	Wine	Missouri	United States
UCD752	2802	Wine		France
UCD2030	2803	Wine	California	United States
UCD2041	2804	Fruit wine		Thailand
UCD2049	2805	Wine		New Zealand
UCD2050	2806	Wine		New Zealand
UCD2053	2807	Wine	California	United States
UCD2054	2808	Wine	California	United States
UCD2060	2809	Wine		Chile
UCD2076	2811	Wine	British Colombia	Canada
UCD2077	2812	Wine		Chile
UCD2082	2813	Wine	California	United States
CBS2797	2814	Wine	Bordeaux	France
UCD2093	2815	Wine	New York	United States
UCD2397	2816	Wine		Portugal
UCD2399	2817	Wine		Portugal
UCD2485	2818	Wine	North Carolina	United States
UCD2493	2819	Wine	Colorado	United States
UCD2494	2820	Wine	California	United States
UCD2504	2821	Wine		South Africa
UCD2506	2822	Wine		South Africa
UCD2841	2823	Kombucha tea		
L0461	2835	Wine	Bordeaux	France
L0505	2836	Wine	Bordeaux	France
L0516	2837	Wine	Bordeaux	France
L0611	2838	Wine	Bordeaux	France
L0620	2839	Wine	Bordeaux	France
L0463	2841	Wine	Bordeaux	France

2.2.2 DNA extraction and sequencing

Genomic DNA was extracted from liquid YPD cultures using the Gentra Puregene Yeast/Bact. Kit B following the protocol: DNA Purification from Yeast Using the Gentra Puregene Yeast/Bact. Kit, with the exception that 7.5 U Zymolyase was added for cell lysis in addition to the Lytic Enzyme Solution from the kit.

Genomic libraries were prepared using the Nextera XT platform (Illumina) and sequenced using Illumina Miseq, paired-end 300 bp chemistry (Ramaciottti Centre for Functional Genomics, University of N.S.W, Australia).

2.2.3 Gene sequence analyses

Raw sequence data was quality trimmed (trimmomatic v0.22 (Bolger, Lohse & Usadel 2014); TRAILING:20 MINLEN:50) and aligned to the AWRI2804 scaffold using novoalign (v3.02.12; -n 300 -i PE 100-1000 -o SAM; www.novocraft.com) and converted to sorted .bam format using samtools (v1.2; (Li *et al.* 2009)) (credit to Anthony Borneman).

Sequence alignments were visualised using Integrative Genomics Viewer v2.3. Nitrate assimilation gene cluster haplotypes were manually assembled for isolates that were heterozygous across the gene cluster, by visually identifying contiguous SNPs within overlapping reads. Maximum-likelihood phylogenies of aligned nucleotide sequences were constructed using PhyML (GTR model, default parameters, bootstrapped with 100 replicates) and visualised using SeaView v4.0.

2.2.4 Nitrate assimilation phenotype screen

YPD starter cultures of each isolate were grown as above. These cultures were then used to inoculate liquid YNB (Difco™ Yeast Nitrogen Base w/o Amino Acids and Ammonium Sulphate, 1.7 g/L; glucose, 5.0 g/L; ammonium sulphate, 5.0 g/L). The liquid YNB cultures were then incubated with orbital mixing at 28°C for 48 hours. The liquid YNB cultures were then centrifuged for 5 minutes at 16,000 g and the supernatant was removed. Each cell pellet was washed with water prior to resuspension in water to

approximately 1x10^8 cells/mL. 1:100 serial dilutions were prepared of each culture down to 1x10^2 cells/mL. 5 µL of each dilution was spotted in an 8x8 grid on solid YNB-A (Difco™ Yeast Nitrogen Base w/o Amino Acids and Ammonium Sulphate, 1.7 g/L; glucose, 5.0g/L; ammonium sulphate, 5.0 g/L; bacteriological agar, 15.0 g/L), YNB-N (Difco™ Yeast Nitrogen Base w/o Amino Acids and Ammonium Sulphate, 1.7 g/L; glucose, 5.0 g/L; sodium nitrate, 6.4 g/L; bacteriological agar, 15.0 g/L) and YNB-neg (Difco™ Yeast Nitrogen Base w/o Amino Acids and Ammonium Sulphate, 1.7 g/L; glucose, 5.0 g/L; bacteriological agar, 15.0 g/L). The YNB plates were incubated at 28°C for 7 days. *B. bruxellensis* isolates AWRI1499, AWRI1608, AWRI1613 and CBS2499 were included on each plate to assess the reproducibility of the assay.

High resolution images were scanned from each plate against a black background. Unprocessed images were used to evaluate growth by determining pixel whiteness intensity for each spot using the ImageJ plug-in: flexible SGA colony detector. Each spotted 5 µL in an image was gated individually and the intensity of whiteness within each gated area was determined by the software (threshold value=55; this equated to the whiteness intensity of the blank space on the agar plates). A ratio of growth on nitrate versus ammonium was determined for each isolate using the values for whiteness intensity on YNB-N (I_N), YNB-A (I_A) and YNB-neg (I_{neg}) plates using Equation 1.

Equation 1. Formula to determine the relative success of *B. bruxellensis* isolates on nitrate.

$$Relative\ growth\ on\ Nitrate = \frac{I_N - I_{neg}}{I_A - I_{neg}}$$

2.3 Results

2.3.1 Allelic diversity in *B. bruxellensis* nitrate assimilation genes

Sequence variation has previously been observed in the nitrate assimilation genes of *B. bruxellensis* isolates that differ in their ability to grow on nitrate media (Borneman *et al.* 2014; Crauwels *et al.* 2014). Taking advantage of the recently sequenced *B. bruxellensis* isolates (Borneman *et al.* 2014), sequence variability across the structural and regulatory nitrate assimilation gene clusters was investigated across an additional cohort of thirty-seven diverse *B. bruxellensis* isolates (Table 3) to provide insight into the association of variant nitrate assimilation gene alleles with nitrate assimilation phenotypes. Short reads were mapped to a scaffold genome sequence of AWRI2804 (credit to Anthony Borneman) and a whole-genome maximum-likelihood phylogeny was constructed to estimate the relatedness of this cohort of *B. bruxellensis* isolates (

Figure 6).

In addition to the whole-genome phylogeny, phased sequence alignments for each of the two nitrate assimilation gene clusters (structural and regulatory) were constructed for all isolates. A total of nine distinct haplotypes were observed for the structural nitrate assimilation gene cluster across thirty-seven isolates (Figure 7), in addition to four isolates with either a full or partial deletion of the gene cluster (AWRI1613, CBS2797, UCD2506 & L0463). Twenty-eight isolates were found to be homozygous across this gene cluster, while nine were heterozygous. The haplotype previously described in *B. bruxellensis* isolate AWRI1608 (Borneman *et al.* 2014) was identified in a total of twenty-five isolates and was thus the most prevalent haplotype across the cohort of isolates investigated in this study (S9) (Figure 7). In five of these isolates, haplotype S9 was present in heterozygosity with a second allele at a 1:1 ratio, while for the remaining twenty isolates, a situation like that observed in AWRI1608 was encountered, where the cluster was homozygous in an otherwise heterozygous genomic background.

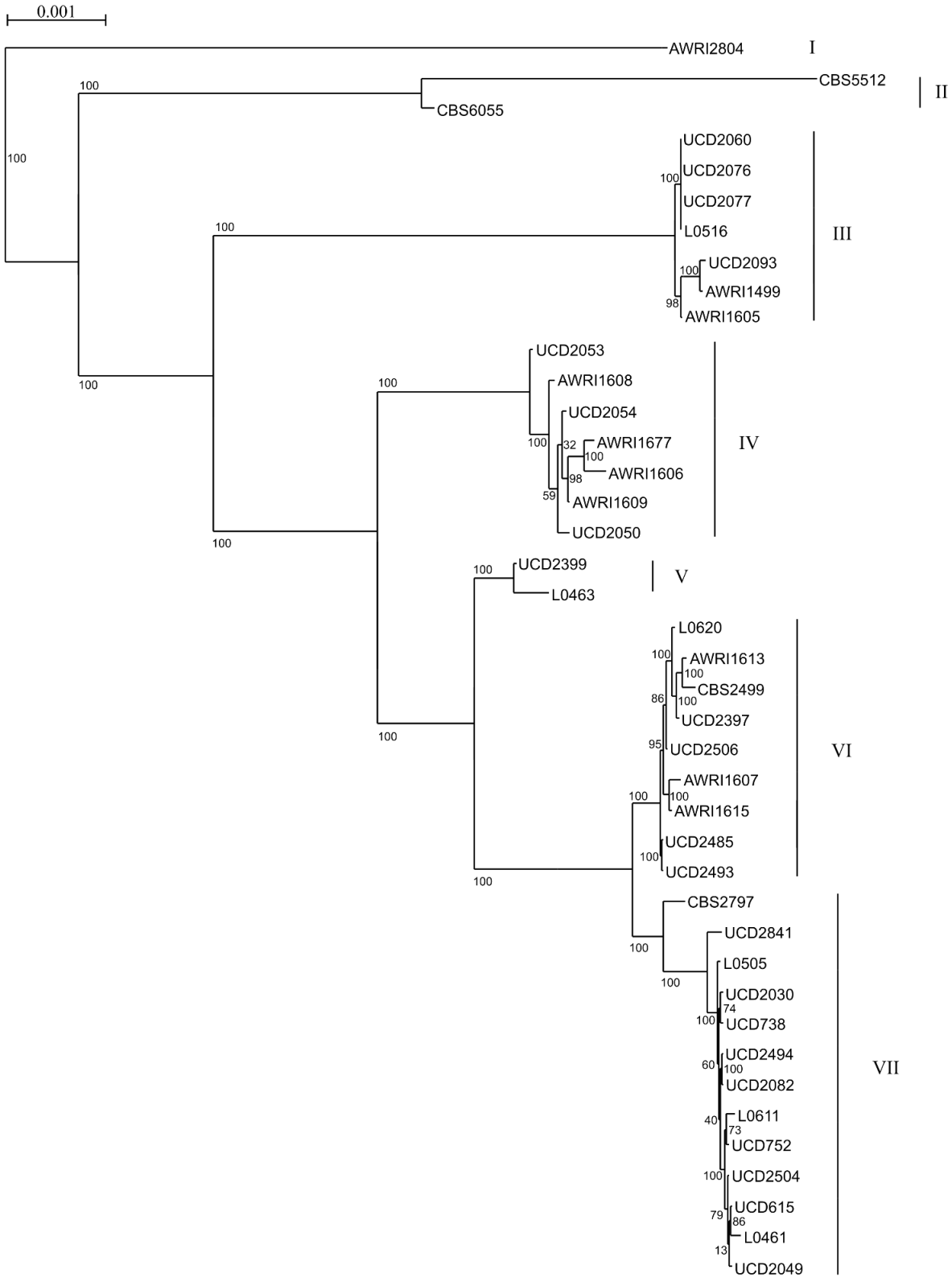

0.001
AWRI2804
I
CBS5512
CBS6055
II
UCD2060
UCD2076
UCD2077
L0516
UCD2093
AWRI1499
AWRI1605
III
UCD2053
AWRI1608
UCD2054
AWRI1677
AWRI1606
AWRI1609
UCD2050
IV
UCD2399
L0463
V
L0620
AWRI1613
CBS2499
UCD2397
UCD2506
AWRI1607
AWRI1615
UCD2485
UCD2493
VI
CBS2797
UCD2841
L0505
UCD2030
UCD738
UCD2494
UCD2082
L0611
UCD752
UCD2504
UCD615
L0461
UCD2049
VII

Figure 6. Whole genome phylogeny of all *B. bruxellensis* isolates used in this study.

Raw sequence data was quality trimmed and aligned to the AWRI2804 scaffold. Regions in these nucleotide alignments exceeding the minimum coverage were then used to construct this maximum-likelihood phylogeny, bootstrapped using 100 replicates (values on branches). Roman numerals are used to define clades. Scale bar represents genetic difference between isolates in substitutions per site. Credit to Anthony Borneman for generating the maximum-likelihood phylogeny

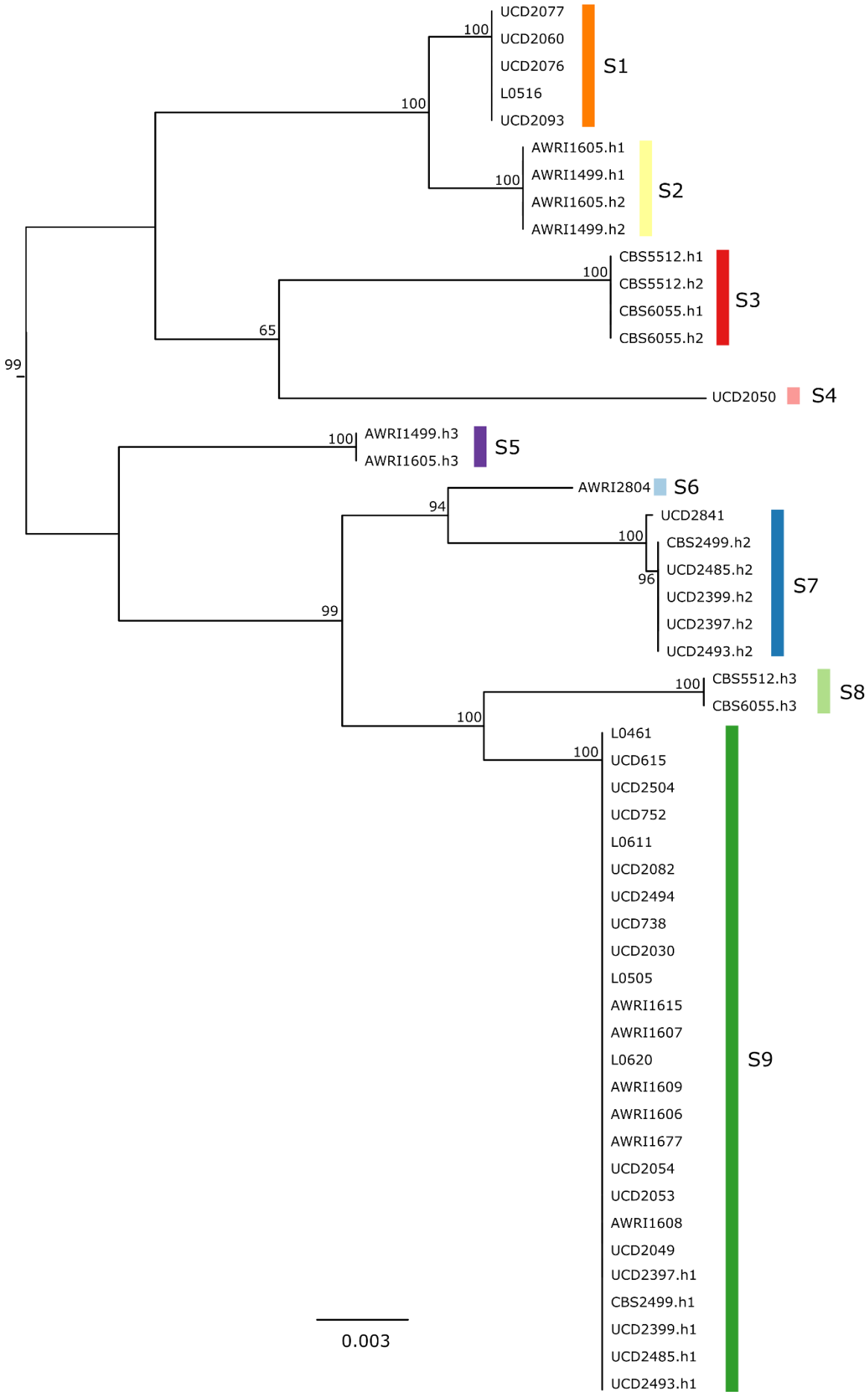
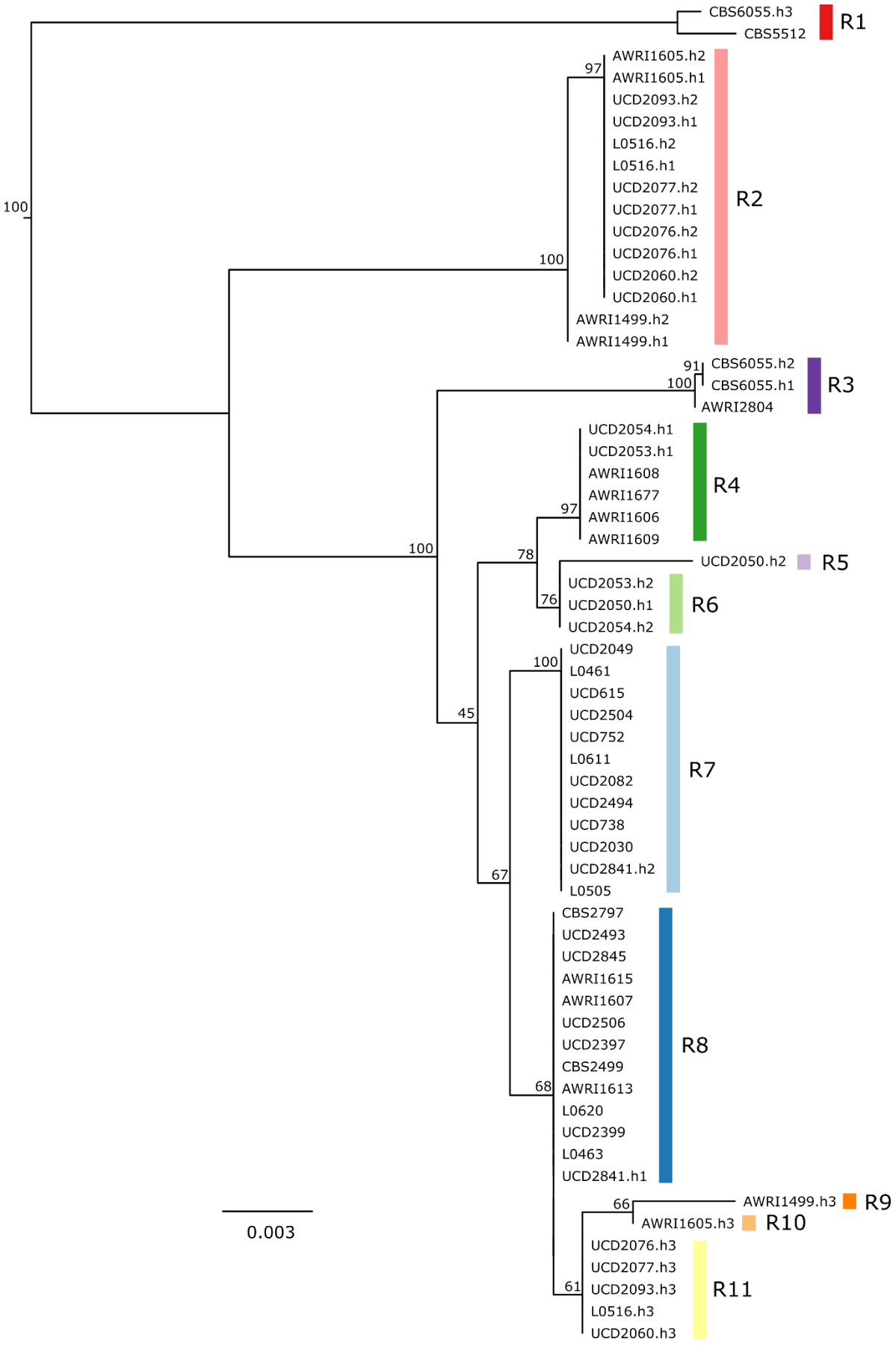

UCD2077
UCD2060
UCD2076
L0516
UCD2093
S1
AWRI1605.h1
AWRI1499.h1
AWRI1605.h2
AWRI1499.h2
S2
CBS5512.h1
CBS5512.h2
CBS6055.h1
CBS6055.h2
S3
UCD2050
S4
AWRI1499.h3
AWRI1605.h3
S5
AWRI2804
S6
UCD2841
CBS2499.h2
UCD2485.h2
UCD2399.h2
UCD2397.h2
UCD2493.h2
S7
CBS5512.h3
CBS6055.h3
S8
L0461
UCD615
UCD2504
UCD752
L0611
UCD2082
UCD2494
UCD738
UCD2030
L0505
AWRI1615
AWRI1607
L0620
AWRI1609
AWRI1606
AWRI1677
UCD2054
UCD2053
AWRI1608
UCD2049
UCD2397.h1
CBS2499.h1
UCD2399.h1
UCD2485.h1
UCD2493.h1
S9
0.003

Figure 7. Maximum-likelihood phylogeny of structural nitrate assimilation gene cluster haplotypes in *B. bruxellensis* isolates of this study.

Haplotypes from the "start" codon of *YNT1* to the "stop" codon of *YNR1* were manually identified in isolates that were heterozygous at this locus. Haplotypes from heterozygous isolates received suffixes ".h1" and ".h2" in cases where the SNP ratio 1:1. In cases where the SNP ratio was close to 2:1 - ".h1", ".h2" and ".h3" were used as suffixes and the sequence represented at the higher proportion was named both ".h1" and ".h2" to reflect this 2:1 ratio. Nucleotide sequences of this region were aligned and used to construct this maximum-likelihood phylogeny, bootstrapped using 100 replicates. The scale bar represents genetic difference between isolates in substitutions per site. Clades are designated numbers S1-9, as indicated to the right of each clade. The coloured bars are used for visual representation of these haplotypes in Figure 9.

The isolates with haplotype S9 were all found within the AWRI1608 and CBS2499 clades (IV & VI, respectively) of the whole-genome maximum-likelihood phylogeny, while three deletion isolates (AWRI1613, CBS2797, UCD2506) resided within clade VI, suggesting that they may have stemmed from a single deletion event. However, AWRI1613 differs from the other two in that it has retained *YNT1*, so that may have been a deletion event independent from the others. Found within clade VII, isolate UCD2841 was found to have a unique homozygous haplotype for the structural gene cluster, which is highly similar to the CBS2499.h2 haplotype S7 (Figure 7). Within the clade IV, isolate UCD2050 was also found to have a unique homozygous haplotype for the structural gene cluster (S4), which is divergent from the haplotypes of closely related isolates (Figure 7). The AWRI1499 clade (III) was found to include one isolate (AWRI1605) with the same heterozygous complement of structural nitrate assimilation genes as AWRI1499 (S2 & S5), while the remaining five isolates were found to have a homozygous complement of the gene cluster (S1) that was closely related to the AWRI1499.h1 and AWRI1499.h2 haplotypes (Figure 7).

A total of fourteen variant haplotypes were identified for the nitrate assimilation regulatory gene cluster (Figure 8). In contrast with what was found at the structural gene cluster, no isolates were identified with deletions at this locus. Thirty isolates were found to be homozygous across the regulatory gene cluster (Figure 8). These are predicted to have undergone loss of heterozygosity (LOH) at this locus, similarly to what has been observed in some isolates at the structural gene cluster, since the homozygous region did not present with decreased read depth and heterozygous reads flanking the homozygous region showed each haplotype contiguous with the homozygous gene cluster. Ten isolates were found to be heterozygous at the regulatory gene cluster (Figure 8). These were typically heterozygous triploids from clade III and the heterozygous triploid CBS6055 presenting two haplotypes each in a 2:1 ratio (Figure 8). Three isolates from the clade IV (UCD2050, UCD2053 and UCD2054) and one from clade VII (UCD2841) were each found to have two closely related haplotypes of the regulatory gene cluster in a 1:1 ratio (Figure 8).


CBS6055.h3
CBS5512
R1
AWRI1605.h2
AWRI1605.h1
UCD2093.h2
UCD2093.h1
L0516.h2
L0516.h1
UCD2077.h2
UCD2077.h1
UCD2076.h2
UCD2076.h1
UCD2060.h2
UCD2060.h1
AWRI1499.h2
AWRI1499.h1
R2
CBS6055.h2
CBS6055.h1
AWRI2804
R3
UCD2054.h1
UCD2053.h1
AWRI1608
AWRI1677
AWRI1606
AWRI1609
R4
UCD2050.h2
R5
UCD2053.h2
UCD2050.h1
UCD2054.h2
R6
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AWRI1615
AWRI1607
UCD2506
UCD2397
CBS2499
AWRI1613
L0620
UCD2399
L0463
UCD2841.h1
R8
AWRI1499.h3
R9
AWRI1605.h3
R10
UCD2076.h3
UCD2077.h3
UCD2093.h3
L0516.h3
UCD2060.h3
R11
100
97
100
91
100
97
78
76
100
45
67
68
66
61
0.003

Figure 8. Maximum-likelihood phylogeny of regulatory nitrate assimilation gene cluster haplotypes in *B. bruxellensis* isolates of this study.

Haplotypes from the "start" codon of *YNA1* to the "stop" codon of *YNA2* were manually identified in isolates that were heterozygous at this locus. Haplotypes from heterozygous isolates received suffixes ".h1" and ".h2" in cases where the SNP ratio was close to 1:1. In cases where the SNP ratio was close to 2:1 - ".h1", ".h2" and ".h3" were used as suffixes and the sequence represented at the higher proportion was named both ".h1" and ".h2" to reflect this 2:1 ratio. Nucleotide sequences of this region were aligned and used to construct this phylogeny, bootstrapped using 100 replicates. The scale bar represents genetic difference between isolates in substitutions per site. Clades are designated numbers R1-11, as indicated to the right of each clade. The coloured bars are used for visual representation of these haplotypes in Figure 9.

2.3.2 Nitrate assimilation phenotype variability amongst *B. bruxellensis* isolates

To determine a link between the sequence variability observed at the nitrate assimilation gene loci and nitrate assimilation potential, phenotyping was performed on all *B. bruxellensis* isolates for which genome data had been analysed. Isolates were spot-plated onto solid YNB media with ammonium, nitrate or no nitrogen source and then scored for growth using image analysis. Twenty-five isolates (58%) showed strong growth on the medium containing nitrate as sole nitrogen source, equivalent to the extent to which they grew on ammonium (Figure 9). Each of the variant haplotypes of the structural and regulatory nitrate assimilation gene cluster was represented within this cohort of nitrate-positive isolates (Figure 9). However, those that were found to be homozygous for haplotypes R4 and R7 were almost exclusively poor performers on nitrate, with only UCD2082 (with R7) displaying close to 100% growth on nitrate (Figure 9). The group of eighteen isolates (43%) that displayed a nitrate-negative phenotype was comprised of the four isolates with nitrate structural gene cluster deletions (AWRI1613, CBS2797, L0463 and UCD2506), in addition to fourteen (33%) with structural nitrate assimilation gene haplotype S9. However, a high degree of inter-isolate variability in growth on nitrate was observed amongst clade IV isolates in this phenotypic screen (Figure 9).

This variation in nitrate utilisation by clade IV and VII isolates was most readily observed in the high cell density spots on the nitrate medium where they manifested as small, distinct colony foci appearing within a non-growing background (Figure 10). As such, these isolates did not typically score highly for growth on nitrate, but it appeared that a very small subpopulation of cells were able to efficiently utilise this nitrogen source. This phenomenon was only observed in isolates that are homozygous for haplotype S9. These isolates not displaying 100% growth on nitrate warranted further investigation, which is explored in the next chapter.

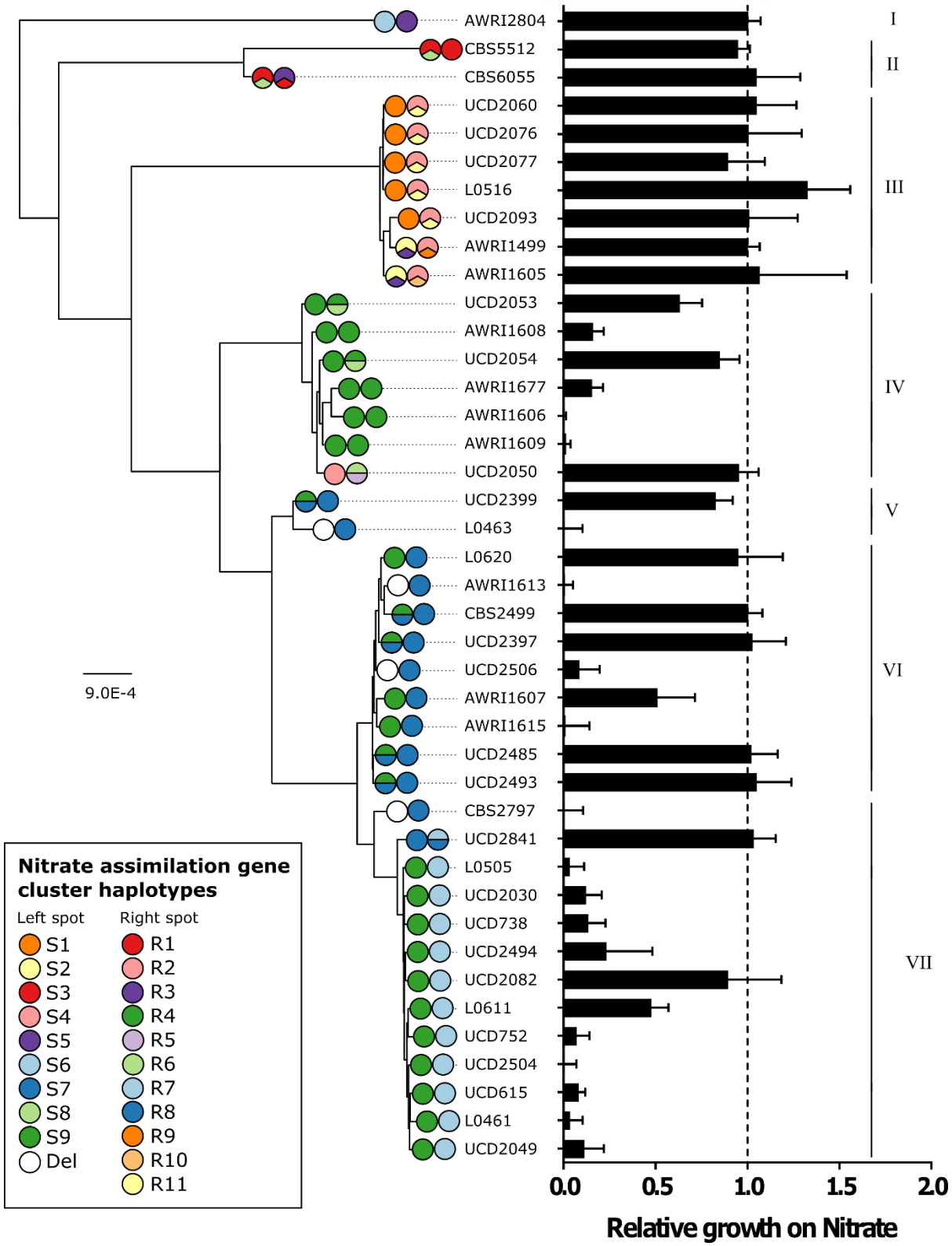
AWRI2804
CBS5512
CBS6055
UCD2060
UCD2076
UCD2077
L0516
UCD2093
AWRI1499
AWRI1605
UCD2053
AWRI1608
UCD2054
AWRI1677
AWRI1606
AWRI1609
UCD2050
UCD2399
L0463
L0620
AWRI1613
CBS2499
UCD2397
UCD2506
AWRI1607
AWRI1615
UCD2485
UCD2493
CBS2797
UCD2841
L0505
UCD2030
UCD738
UCD2494
UCD2082
L0611
UCD752
UCD2504
UCD615
L0461
UCD2049
I
II
III
IV
V
VI
VII
9.0E-4
Nitrate assimilation gene cluster haplotypes
Left spot
S1
S2
S3
S4
S5
S6
S7
S8
S9
Del
Right spot
R1
R2
R3
R4
R5
R6
R7
R8
R9
R10
R11
0.0
0.5
1.0
1.5
2.0
Relative growth on Nitrate

Figure 9. Nitrate assimilation potential amongst *B. bruxellensis* isolates, whole-genome phylogeny and nitrate assimilation gene variants.

The whole genome phylogeny is as per

Figure 6. The coloured circles on the nodes to represent which nitrate assimilation gene cluster haplotypes each isolate possesses with segmented circles indicating heterozygosity. The colours used here are derived from those in the phylogenies of each nitrate assimilation gene cluster in Figure 7 & Figure 8. A white circle is indicative of a deletion of all or, in the case of AWRI1613 only, part of that gene cluster. The left circle represents the structural nitrate assimilation gene cluster (Figure 7), while the right circle represents the regulatory nitrate assimilation gene cluster (Figure 8). The bar graph shows the growth intensity of each isolate on nitrate relative to the growth intensity of each isolate on ammonium as determined by image analysis of independent triplicate spot plates (error bars show standard deviation). A score of 1 indicates that growth on nitrate was the same as was observed on ammonium for that isolate. A score of 0 indicates that growth intensity on nitrate was the same as observed on plates with no nitrogen source (negative control – i.e. background or no growth). Roman numerals show where clades are defined as per

Figure 6.

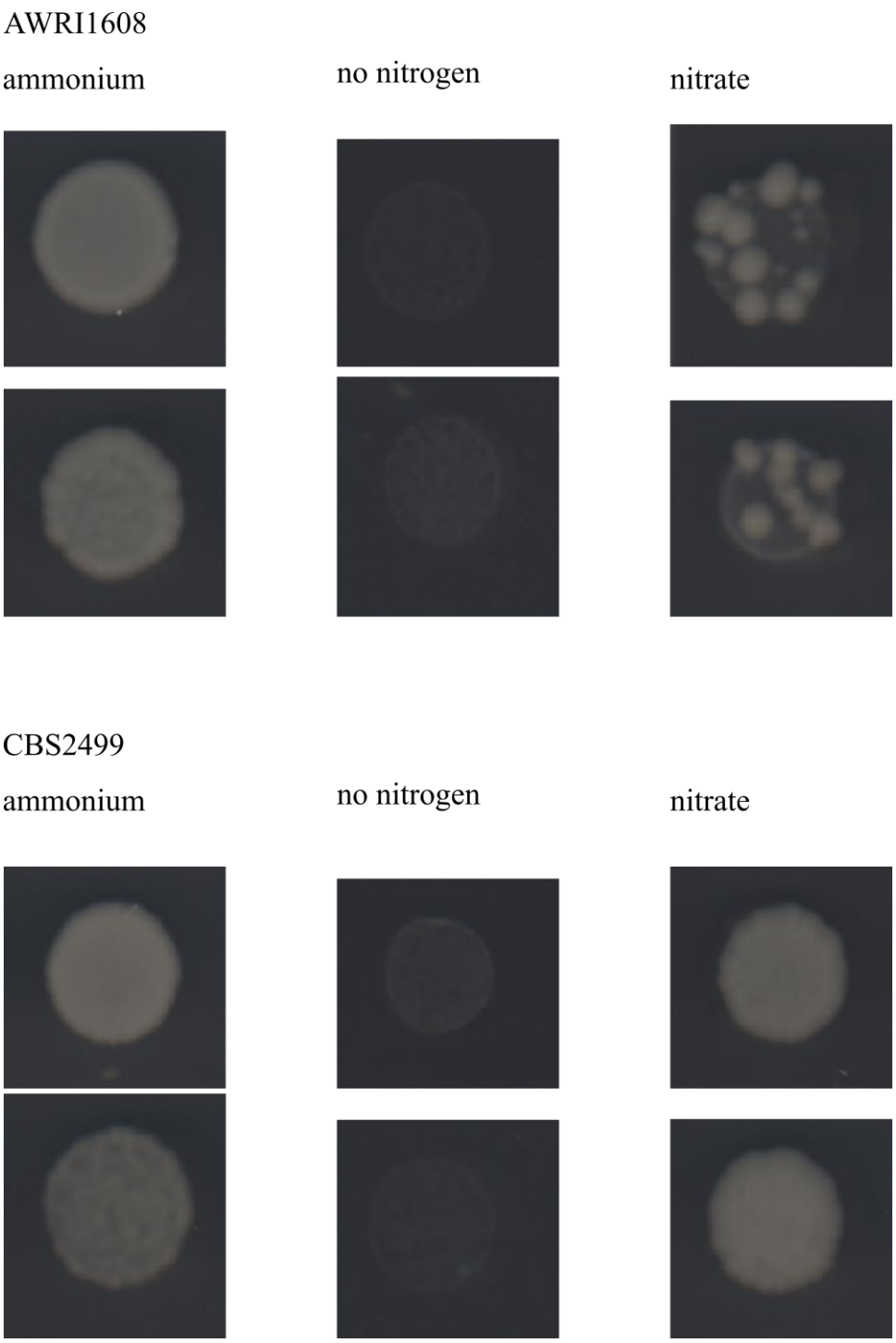

Figure 10. Phenotypic heterogeneity in nitrate assimilation potential in AWRI1608.

The top row (AWRI1608) shows spots on ammonium, no nitrogen source, and nitrate indicating a decreased propensity to grow on nitrate compared to ammonium. Within the negative background of the nitrate spots for AWRI1608, colonies are visible. The bottom row (CBS2499) shows efficient growth on nitrate, comparable to that on ammonium. Each of these spots contained approximately 5×10^5 viable cells.

2.4 Discussion

B. bruxellensis is known to be adept at utilising alternative sources of nutrition, a feature of the species that is likely to have influenced its adaptation to industrial fermentative ecosystems (Conterno *et al.* 2006). Indeed, the assimilation of nitrate has been shown to be an advantageous phenotype in continuous fermentations, allowing *B. bruxellensis* to outcompete *S. cerevisiae* (de Barros Pita *et al.* 2011). Interestingly, it is apparent that several events have occurred in the evolutionary history of *B. bruxellensis* resulting in the specific loss of this phenotype in some isolates (Borneman *et al.* 2014). However, variability in nitrate assimilation potential due to genetic variations other than deletions, such as the loss of heterozygosity (LOH) in the nitrate assimilation structural gene cluster in AWRI1608 (Borneman *et al.* 2014), had not been characterised prior to this study.

A diverse range of haplotypes of the nitrate assimilation gene clusters were identified through the whole-genome analysis of forty-one *B. bruxellensis* isolates. Each variant haplotype of both the structural and regulatory nitrate assimilation gene clusters was represented by at least one isolate that tested positive for nitrate assimilation. There is, therefore, no individual haplotype in this study that can be linked with a nitrate-negative phenotype using this dataset. Some haplotypes (e.g. R2 and R11) only appear in heterozygous isolates. It is therefore not known if one such haplotype does not confer nitrate assimilation.

The most common structural gene cluster haplotype identified across these isolates was identical to the homozygous haplotype of AWRI1608 and these isolates were highly variable in nitrate assimilation potential, with most exhibiting low levels of growth on nitrate. Two haplotypes of the regulatory nitrate assimilation gene cluster (R4 & R7) were identified to have an association with the nitrate-negative phenotype, as isolates that were found to be homozygous for these haplotypes typically did not grow efficiently on nitrate.

It was previously described that AWRI1608 had undergone gene conversion at the nitrate assimilation structural gene cluster, resulting in LOH (Borneman *et al.* 2014) and this has now also been observed in

the other isolates sharing this specific homozygous haplotype. Such an event possibly arose in an ancestor shared with CBS2499, which is heterozygous across the gene cluster but carries one haplotype identical to that found in AWRI1608. Interestingly, UCD2841 was also found to have undergone LOH at this locus, however this occurred in the opposite direction, resulting in multiple copies of the alternate CBS2499 haplotype being present and a nitrate-positive phenotype. It is also possible that the unique UCD2050 haplotype (S4) arose in a similar manner, but through LOH in an isolate that was originally heterozygous for both S4 and S9. A similar LOH event was observed in clade III, with isolates found to be homozygous for a close homologue of the AWRI1499 haplotype S2. A further three isolates were found in this study to have undergone gene deletions at the nitrate assimilation structural gene cluster, similarly to AWRI1613.

B. bruxellensis has been found to have a highly variable genome, commonly displaying structural variations and inter-isolate differences in karyotype (Borneman *et al.* 2014; Hellborg & Piskur 2009). Large duplications, deletions and gene conversion events have commonly been observed in *B. bruxellensis* genome studies (Borneman *et al.* 2014; Crauwels *et al.* 2014). *B. bruxellensis* does not typically go through meiosis, which would likely filter out the vast majority of structural variations to the genome. Such unfiltered plasticity in the genome of this species can readily contribute to the emergence of phenotypic variants (Borneman *et al.* 2014; Crauwels *et al.* 2014), such as the variability observed here in nitrate assimilation phenotypes.

Nitrate assimilation has previously been shown to facilitate *B. bruxellensis* outcompeting *S. cerevisiae* in continuous sugar cane juice fermentation. It is within reason that the ability to use alternative sources of nutrition, such as nitrate, would be advantageous in the sparse environment that is post-fermentation wine. However, selection against high nitrate assimilatory potential can be inferred, with multiple isolates showing either gene deletions or gene conversions that were specifically linked to a decrease in nitrate assimilatory potential. There may be a significant disadvantage in the use of this metabolic pathway under some conditions or in some environment these isolates have been exposed to. This could be due to the

high energy demand of the pathway or previously observed effects on redox balance (Galafassi *et al.* 2013).

Previous studies on nitrate assimilation potential in *B. bruxellensis* isolates have typically used liquid culture methods to determine growth in nitrate (Crauwels *et al.* 2014; Galafassi *et al.* 2013; de Barros Pita *et al.* 2011). This approach is useful for investigating fermentation kinetics and enzyme activity under nitrate assimilation. For this study, nitrate phenotype testing was conducted on solid agar media with the ambition to gain insight into differences in growth potential, rather than growth rate, of *B. bruxellensis* on a nitrate-based medium.

These experiments revealed the potential for phenotypic heterogeneity, seen in isolates such as AWRI1608 where a few colonies formed in an otherwise negative background spot. This could be an example of bet-hedging, where a subset of the population has the potential to continue to grow under possible future conditions, in this case, where nitrate is the favoured nitrogen source. This would represent an interesting adaptation to fermentative ecosystems and the nutrient scarcity that is typical of late/post fermentation. Nitrate assimilation phenotype heterogeneity has not previously been described in *B. bruxellensis* or any other yeast/fungal species.

3 Nitrate assimilation phenotype heterogeneity

3.1 Introduction

In the previous chapter, it was noted that AWRI1608 and closely-related isolates with the same haplotype of nitrate assimilation genes, formed small numbers of discrete colonies within what was considered the background inoculum (i.e. a negative result for growth) on nitrate. This inspired further investigation into the heterogeneous growth of these isolates on solid nitrate media.

Phenotypic heterogeneity within what should be a genetically clonal population can provide the basis for population-scale adaption to rapid fluctuations in environmental conditions (Beaumont *et al.* 2009; Philippi & Seger 1989). Clonal populations can exhibit phenotypic heterogeneity through stochastic fluctuations in cellular components (Veening, Smits & Kuipers 2008). For example, *Candida albicans*, a human fungal pathogen, can stochastically switch between two distinct cell types known as “white” and “opaque” via an epigenetic switch (Lohse & Johnson 2009; Slutsky *et al.* 1987). The two cell types differ in many respects, such as colony morphology, metabolic states and, perhaps most notably, mating behaviour, with mating only occurring between opaque cells (Lohse & Johnson 2009; Slutsky *et al.* 1987). In order to undergo mating, white cells are therefore required to undergo a rare stochastic switch to the opaque cell type, that is normally maintained through the action of a transcriptional feedback loop (Lohse & Johnson 2009; Zordan *et al.* 2007). Each phenotype is heritable due to the conservation of factors involved in the regulation of each cell type through mitosis (Lohse & Johnson 2009).

Phenotypic diversity within a clonal population can also be advantageous by preparing subset of the population for adaptation to new environmental pressures (Hufton, Lin & Galla 2018). In such cases, the majority of the population does not need to bear the fitness cost associated with pre-adapting to a potentially rare change in the environment. For example, Arnoldini *et al.* (2014) have shown that the heterogeneous expression of virulence genes in *Salmonella typhimurium* exhibits the characteristics of a

classical bet-hedging mechanism. While the virulent subpopulation displayed a slower growth rate than the avirulent subpopulation, due to the metabolic cost of virulence gene expression (Sturm *et al.* 2011), the virulent cells were more tolerant to the addition of ciprofloxacin, ensuring that at least some of the total population survives the introduction of the antibiotic (Arnoldini *et al.* 2014).

Brettanomyces bruxellensis and *Saccharomyces cerevisiae* have both been shown to use the [*GAR*$^{+}$] yeast prion in order to switch off their natural state of glucose repression, facilitating the assimilation of alternative carbon sources in the presence of glucose (Jarosz *et al.* 2014). [*GAR*$^{+}$] is induced by a secreted bacterial factor and this state is heritable under favourable conditions. When growth conditions worsen, however, protein-folding is negatively affected, which decreases the effect of yeast prions such as [*GAR*$^{+}$] (Jarosz *et al.* 2014). This kind of epigenetic control over phenotypes is a prime example of microbial bet-hedging, as changes between these two states is much more rapid than relying solely on genetic mutations for adaptation, which is advantageous under fluctuating environmental conditions (Jarosz *et al.* 2014).

The nitrate assimilation screen in the previous chapter indicated that for some *B. bruxellensis* genetic backgrounds only a portion of the population could grow on nitrate. While intra-specific variation in nitrate assimilation had been previously described, phenotypic heterogeneity for nitrate assimilation in isogenic populations of *B. bruxellensis* has not. This chapter explores the use of spread plates to better observe the low rate of colony formation for some *B. bruxellensis* isolates on nitrate. The use of spread plates allowed the quantification of the rate at which these colonies form and the isolation of these colonies for further investigation of their nitrate phenotypes and nitrate gene sequences.

3.2 Methods

3.2.1 Yeast isolates and culture conditions

B. bruxellensis isolates (Table 3) were obtained from the Australian Wine Research Institute Microorganisms Culture Collection and maintained on MYPG agar buffered with calcium carbonate (malt extract, 3.0 g/L; yeast extract, 3.0 g/L; bacteriological peptone, 2.0 g/L; glucose, 10.0 g/L; bacteriological agar, 15.0 g/L; calcium carbonate, 20.0 g/L). Starter cultures were grown from single colonies in liquid YPD (yeast extract, 10.0 g/L; bacteriological peptone, 20.0 g/L; glucose, 20.0 g/L) in test tubes incubated with orbital mixing at 28°C for 48 hours.

3.2.2 Quantification of nitrate-assimilating cells and isolating nitrate revertant colonies

YPD starter cultures of each isolate were grown as above. These cultures were then used to inoculate liquid YNB, which were then incubated with orbital mixing at 28°C for 48 hours. Cell pellets were washed once with water and then resuspended in water. Dilutions of cell suspensions were spread plated onto solid YPD as a positive control for viability, YNB-ammonium as a positive control for viability on YNB media, YNB-negative as a negative control for growth without a supplemented nitrogen source, and YNB-nitrate to assess the proportion of cells in yeast cultures with the ability to grow using nitrate as a sole source of nitrogen. The plates were incubated at 28°C for 7 days before colonies were manually counted.

In testing *B. bruxellensis* isolate AWRI1608 by this method, colonies were counted after 5, 6 and 7 days to track colony formation over that period. Six random colonies of AWRI1608 that formed on YNB-nitrate plates were streaked out onto YNB-nitrate to select single colonies. One single colony from each of these plates was picked and stored at -80°C in a Protect vial. A sample of each was submitted to the Australian Wine Research Institute Culture Collection (AWRI3005 – AWRI3010).

3.2.3 Stability of nitrate-reversion

YPD starter cultures of AWRI1608, CBS2499 and the six nitrate-positive AWRI1608 isolates; AWRI3005, AWRI3006, AWRI3007, AWRI3008, AWRI3009 and AWRI3010; were grown as above and cell counts were performed at stationary phase using a haemocytometer. The liquid cultures were then used to inoculate fresh YPD at ~1x10^6 cells/mL. This process was repeated with the new liquid cultures until one hundred cell doublings had been recorded. These cultures were used in spread plate quantification of nitrate assimilating cells as above.

3.2.4 DNA extraction and sequencing

Genomic DNA was extracted from liquid YPD cultures using the Gentra Puregene Yeast/Bact. Kit B following the protocol: DNA Purification from Yeast Using the Gentra Puregene Yeast/Bact. Kit, with the exception that 7.5 U Zymolyase was added for cell lysis in addition to the Lytic Enzyme Solution from the kit.

Genomic libraries were prepared using the Nextera XT platform (Illumina) and sequenced using Illumina Miseq, paired-end 300 bp chemistry (Ramaciottti Centre for Functional Genomics, University of N.S.W, Australia).

3.2.5 Identification of mutations in nitrate-positive colonies vs. AWRI1608

Raw sequence data was quality trimmed (trimmomatic v0.22 (Bolger, Lohse & Usadel 2014); TRAILING:20 MINLEN:50) and aligned to the extended *Saccharomyces sensu stricto* clade using novoalign (v3.02.12; -n 300 -i PE 100-1000 -o SAM; www.novocraft.com) and converted to sorted .bam format using samtools (v1.2; (Li *et al.* 2009))

Mutations in the revertant isolates were identified against AWRI1608 via the “somatic” function in Varscan using default parameters with a p-value cut-off 10^{-5} to remove what was considered background noise. SNPs that were identified across all six revertants were investigated further by visualisation in IGV and a BLASTn search of the surrounding area to identify potential functions of (putative) genes at those loci.

3.3 Results

3.3.1 Quantification of nitrate-assimilating subpopulations

In the previous chapter it was shown that a small number of discrete colonies were able to form within high cell density inocula of isolates from clades IV and VII on solid nitrate media. It was hypothesised that phenotypic heterogeneity in nitrate assimilatory potential existed within these clonal populations, such that nitrate positive cells exist at low frequency within an otherwise nitrate negative population. A quantitative plating method was therefore employed to determine the rate of colony formation of isolates, and therefore population heterogeneity, in these isolates when growing on nitrate.

When plated into either nitrate or ammonium as the primary nitrogen source, isolates such as CBS2499, display a classical nitrate positive phenotype, with equal CFU on both media (Figure 11). However, when AWRI1608 was plated on the same two media, the nitrate media yielded a CFU count that was 1×10^5 lower than that observed for growth on ammonium. In addition, while colonies of CBS2499 appeared synchronously after around 5 days of incubation (Figure 12), the number of observable colonies of AWRI1608 growing on nitrate slowly increased over time, suggesting that the phenotypic reversion may be an ongoing process (Figure 12).

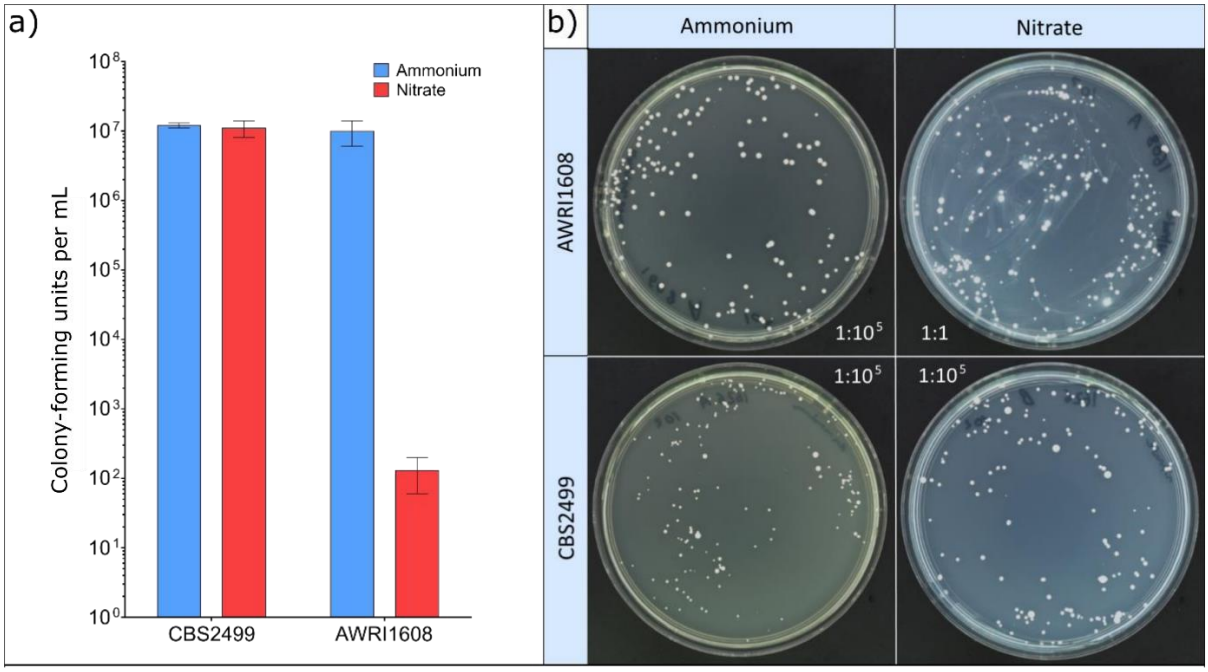

Figure 11. Differences in colony formation on nitrate between *B. bruxellensis* isolates CBS2499 and AWRI1608.

a) Colony-forming units per mL of CBS2499 and AWRI1608 on ammonium (blue) and nitrate (red) as determined from colony counts on spread plates. Error bars indicate standard deviation between triplicate independent assays. b) Photographs of spread plates from this assay. The ratios in the centre of the figure are the dilution factors of the cell solutions that were plated, where 1:1 is a fresh, stationary-phase culture resuspended in sterile water to the same volume.

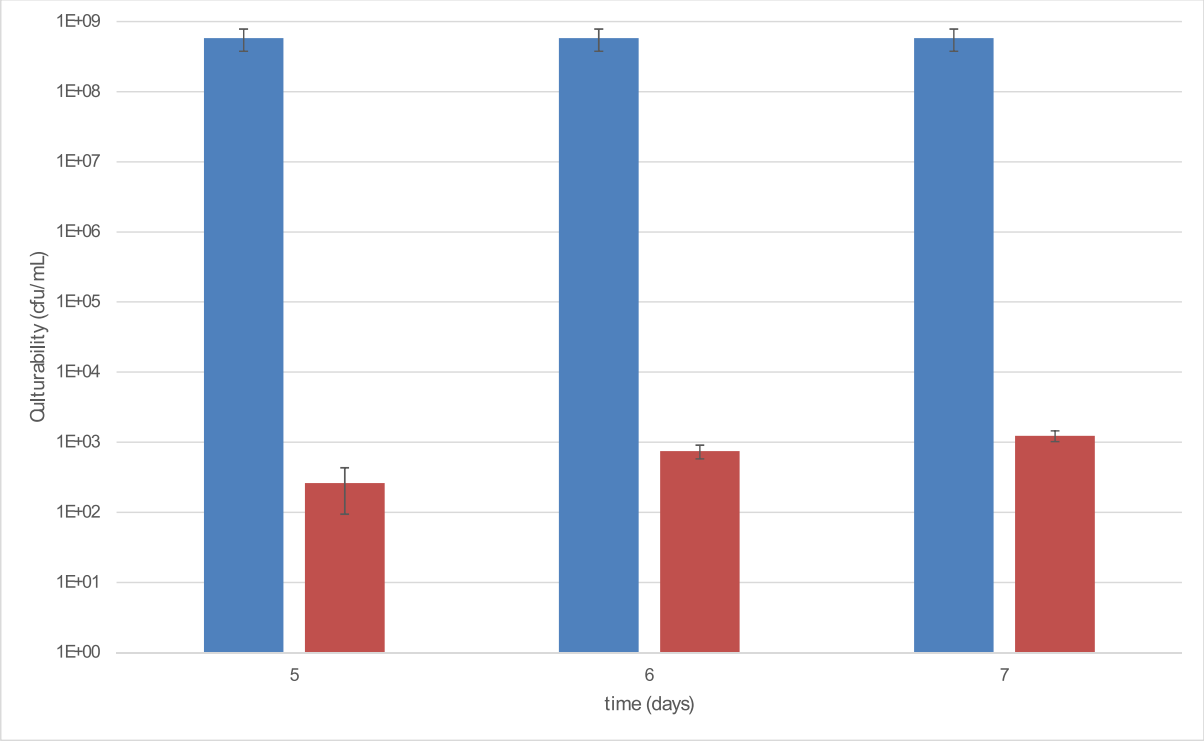

Figure 12. AWRI1608 colony formation over time on YNB supplemented with either ammonium or nitrate.

AWRI1608 was grown on YNB-ammonium (blue) and YNB-nitrate (red) spread plates. Colonies were counted upon first evidence of growth (five days) and subsequently each day until the seventh. No new colonies were formed on ammonium after the initial count at five days, but more colonies appeared on nitrate plates in the subsequent days. Error bars show the standard deviation between independent triplicate plates.

In order to assess the prevalence of phenotypic heterogeneity, the quantitative plating assay was expanded to incorporate all *B. bruxellensis* isolates that displayed the same homozygous complement of structural nitrate assimilation genes as AWRI1608, haplotype S9. In addition, UCD2050 and UCD2841 were also included as each displayed unique nitrate genotypes that were closely related to haplotype S9. While the rate of nitrate reversion was shown to be highly reproducible for each isolate, the magnitude of reversion was highly variable between individual isolates, ranging from $1:10^6$ to 1:1 (Figure 13). UCD2050 and UCD2841 were found to be nitrate-positive using this assay, as was the case for the spot plate assay in the previous chapter. Interestingly, UCD2053 and UCD2054 also exhibited prolific growth on nitrate. These two isolates differ from the other isolates in clade IV in that they are not entirely homozygous at the regulatory nitrate assimilation gene cluster, with two slightly different haplotypes at this locus.

Several isolates failed to grow on YNB-ammonium after numerous attempts to culture them on this medium, hence YPD was used as a positive control medium. It cannot be definitively determined from this assay how these isolates perform on nitrate, since it cannot be determined if the lack of growth on YNB-nitrate is because of failing to use nitrate or simply an incapability to grow on YNB under these conditions. It is possible that the lack of growth on YNB for some of these isolates was due to the media lacking a necessary component that could be sourced from dead yeast cells, since all isolates were able to grow on the spot plates of the same media where cell density was far greater. This could be associated with the reason why isolates in this set do not efficiently grow on YNB-nitrate but provides no definitive answer.

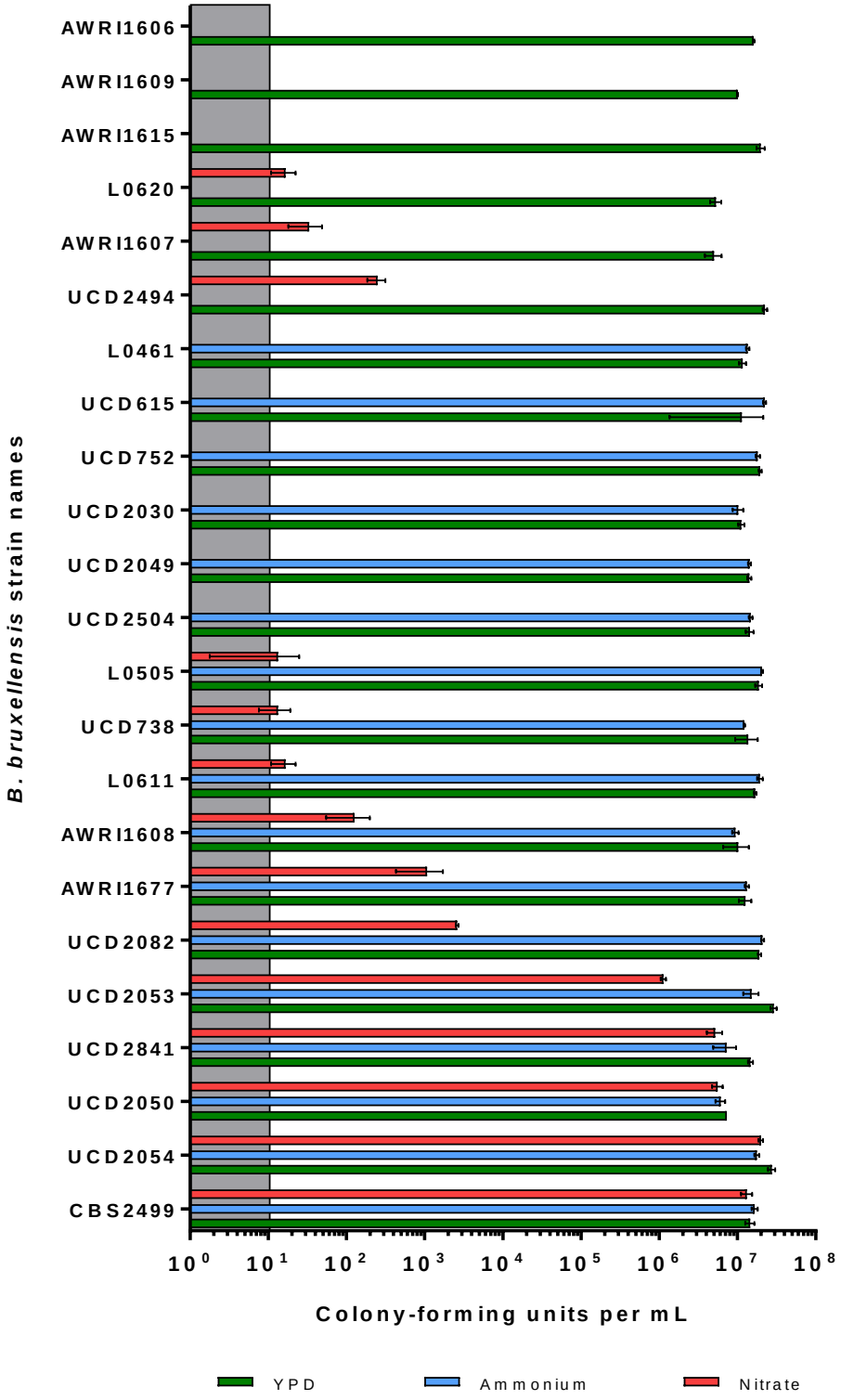

Figure 13. Variable nitrate-assimilation potential across closely-related *B. bruxellensis* isolates.

Colony-forming units per mL of each isolate was determined by counting colonies on spread plates. YPD was used as a positive control for growth since several isolates failed to grow on YNB-ammonium. Error bars indicate standard deviation between triplicate independent assays. The grey box indicates the limit of detection for Colony-forming units per mL on these plates.

3.3.2 Nitrate-positive phenotype stability

The results of the quantitative plate assay clearly showed that phenotypic heterogeneity existed within many of the *B. bruxellensis* isogenic populations. However, it was not known whether the nitrate-positive phenotype would be genetically stable in the absence of selection. To assess the stability of the nitrate-positive phenotype displayed by the AWRI1608 revertants, six random isolates were passaged for 100 generations under non-selective (YPD) conditions. These populations were then subjected to the same quantitative plating assay as performed previously. Unlike initial results with AWRI1608, all six revertant populations were found to display a nitrate-positive phenotype, with a 1:1 ratio of CFU present on media with either nitrate or ammonium as the sole nitrogen source (Figure 14), indicating that the nitrate positive phenotype is not rapidly lost once selective conditions are relaxed.

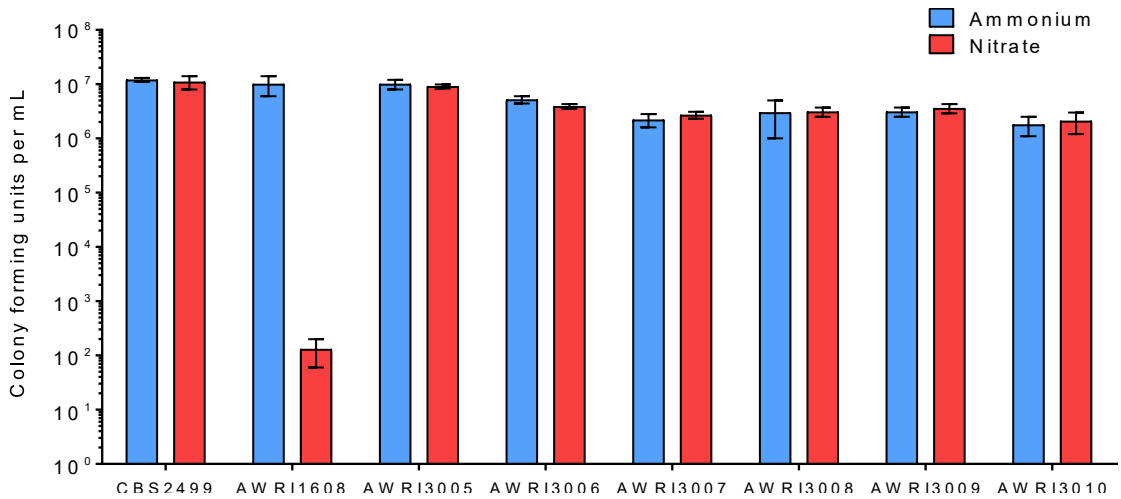

Figure 14. Retention of the nitrate-positive phenotype in isolates from AWRI1608 colony formation on nitrate.

Isolates from the previous assay that formed from AWRI1608 on nitrate were subjected to the same phenotype analysis after 100 generations of growth in liquid YPD (non-selective for nitrate assimilation).

3.3.3 Nitrate-positive AWRI1608 colony sequence comparisons

The switch from nitrate-negative to nitrate-positive might have been due to mutation(s), specifically in the nitrate assimilation genes or potentially elsewhere in the genome. Whole genome sequences of the revertant isolates were compared to AWRI1608 to determine genomic alterations that were consistent across all revertants.

The “somatic” feature of varscan was used to identify any potential SNPs that could have facilitated the observed switch in phenotype from nitrate-negative to nitrate-positive in the colonies isolated from AWRI1608 on nitrate. SNPs that were not found in all six isolates were disregarded. A total of twenty-six SNPs were identified in all six isolates (Table 4). These genomic loci were inspected visually using IGV 2.3 and a BLASTn search was used to identify any potential functions of the area (Table 5). The majority of these SNPs were found to be in repetitive sequences and could not be associated with any particular function. Two LOH mutations were identified within a Flo11 superfamily conserved domain, which is associated with pseudohyphae formation (Lo & Dranginis 1998). Four mutations were identified within a putative ORF (g116.t1), which was not found to be similar to anything via BLASTx. Two mutations were identified within a repeated region of another putative ORF (g4420.t1), which was determined to contain a motif associated fungal adhesins. No mutations were identified in or near either nitrate assimilation gene cluster.

Table 4. Total number of SNPs identified via varscan somatic analysis of nitrate-positive variant isolates of AWRI1608 vs. AWRI1608 itself.

The total number of SNPs that were identified and the number of nitrate-positive AWRI1608 variant isolates they were found in (e.g. 1054 SNPs were found in any one, but only one, of the six isolates).

Number of isolates	Number of SNPs
1	1054
2	226
3	63
4	23
5	18
6	26

Table 5. Details of the SNPs found in all six nitrate-positive AWRI1608 revertants vs. AWRI1608.

The location of each SNP (contig and position on contig), the reference (ref) and variant (var) bases, read percentages that contain the variant base in AWRI1608 (1608 var freq) and the revertant isolates (Mutant var freq), the designation of the type of mutation by varscan (Status) and a description of the genomic locus (Locus description) are listed for each SNP that was identified in all six nitrate-positive AWRI1608 isolates against the wild-type AWRI1608 genome sequence.

contig	position	ref	var	1608 var freq	Mutant var freq	Status	Locus description
1898	10126	C	T	24%	7%	LOH	repetitive sequence
1898	10128	A	G	24%	6%	LOH	
1898	10964	A	G	20%	34%	Somatic	
1898	11366	T	C	3%	44%	Somatic	
1898	11376	C	T	0%	46%	Somatic	
1898	11408	C	T	0%	48%	Somatic	
1898	11432	C	T	0%	48%	Somatic	
2201	232237	T	C	51%	100%	LOH	Flo11 superfamily conserved
2201	232241	C	T	51%	100%	LOH	domain
2201	2084221	T	C	12%	80%	Somatic	no ORFs
2201	2084224	T	C	12%	83%	Somatic	
2201	2084225	T	G	12%	79%	Somatic	
2201	3834376	T	A	51%	86%	LOH	no ORFs
2203	283392	T	C	0%	82%	Somatic	short GOH event ~100bp after stop
2203	283393	G	A	0%	76%	Somatic	codon of putative ORF g116.t1, no
2203	283397	C	A	0%	80%	Somatic	BLAST result
2203	283403	C	T	3%	71%	Somatic	
787	683036	G	A	0%	64%	Somatic	putative ORF g4420.t1, repetitive
787	683038	A	G	0%	68%	Somatic	seq, possibly a fungal adhesin
787	1608083	T	A	12%	40%	Somatic	repetitive sequence
787	1612030	T	C	0%	43%	Somatic	
795	80144	C	A	58%	0%	LOH	repetitive sequence
795	288067	A	G	74%	0%	LOH	repetitive sequence
795	288070	C	A	74%	0%	LOH	
795	288915	C	T	70%	4%	LOH	
795	288946	G	A	34%	1%	LOH	

3.4 Discussion

In previous studies, the assimilation of nitrate by *B. bruxellensis* has typically been studied in liquid media (de Barros Pita *et al.* 2011; de Barros Pita, Tiukova, *et al.* 2013; Galafassi *et al.* 2013; de Barros Pita, Silva, *et al.* 2013; Crauwels *et al.* 2014). While this is useful for investigating the effects of nitrate assimilation on fermentation kinetics and enzyme activity, liquid assays are not able to detect the population-level phenotypic heterogeneity that was displayed by some of the isolates used in this study. Furthermore, previous studies have typically determined growth on nitrate using a +/- scoring system (Conterno *et al.* 2006; Borneman *et al.* 2014; Crauwels *et al.* 2014). In contrast, this study evaluated the intensity of growth on nitrate in comparison with the growth on ammonium.

The appearance of new colonies over time on nitrate plates suggests that there may be a stochastic switch that allows subsets of the population to begin to assimilate nitrate. It was found that once cells from an AWRI1608 culture had switched to using nitrate, the phenotype was retained over one hundred generations, even in the absence of selection. However, it is possible that after the switch to nitrate utilisation, a subset of each population could revert to a nitrate-negative phenotype at a similarly low rate as a very low rate of reversion could not be detected via the plating assay used in this study. A method using chlorate plates could be developed to determine this, since nitrate-positive isolates are also chlorate-sensitive, however preliminary testing of such a method in this study was unsuccessful and further development is required. Interestingly, while the isolates that exhibited this behaviour displayed a consistent switching rate across replicate assays, individual isolates differed significantly in their nitrate-positive sub-population proportions ($1:10^6$ to 1:1). This inter-isolate variation may be due to genetic differences influencing the mechanism that drives the switch. Alternatively, it may be indicative of recent environmental pressures on each isolate. For example, isolates that exhibited a higher proportion of nitrate-positive cells may have more recently been exposed to an environment promoting the assimilation of nitrate. Adverse culture conditions could drive these populations away from their nitrate-positive state via an epigenetic effect like what has been described in the case of the [*GAR*$^+$] prion.

This type of phenotypic bet-hedging is an example of an evolutionary adaptation that facilitates survival in fluctuating environmental conditions. As such, this suggests that there may be some disadvantage or fitness cost to a fully positive nitrate assimilation phenotype. This could simply be due to the energy demand of nitrate assimilation, as the pathway requires the transfer of eight electrons to fully reduce nitrate to ammonium. The effects of nitrate assimilation on redox and redirection of the fermentation pathway as described by Galafassi *et al.* (2013) might also have implications in competitive fitness. For example, Galafassi *et al.* (2013) found that nitrate assimilation under aerobic conditions redirected glucose metabolism from ethanol production towards acetate production. Ethanol and acetate production are important factors to the 'make-accumulate-consume' strategy of glucose metabolism employed by *B. bruxellensis*, and it is possible that changes to the balance of this pathway could affect competitive fitness (Rozpędowska *et al.* 2011). Nitrate assimilation has been shown to improve the growth and fermentative metabolism of *B. bruxellensis* under anaerobic conditions (Galafassi *et al.* 2013), so negative effects of nitrate assimilation might be specific to aerobic conditions. Isolates other than AWRI1608 were identified in this study to express this phenotypic heterogeneity. Further experiments incorporating some of these isolates could provide further insight into the nature of the observed phenotypic heterogeneity.

This study is the first to describe phenotypic heterogeneity in *B. bruxellensis*, and the first to describe this phenomenon with regards to nitrate assimilation. A bet-hedging mechanism in response to environments with rapidly changing nitrogen availabilities may be an important factor in the evolution of nitrate assimilation in *B. bruxellensis*. An important step in better understanding this phenotype heterogeneity is to explore the potential effects of nitrate assimilation on competitive fitness in these *B. bruxellensis* isolates; the next chapter is about developing a method for such investigation.

4 Development of a method for assessing competitive fitness of nitrate-variable *Brettanomyces bruxellensis* isolates

4.1 Introduction

The use of nitrate as a source of nitrogen in *Brettanomyces bruxellensis* is not a phenotype shared across all isolates, as demonstrated in previous chapters. A large proportion of isolates in this study have been shown to be deficient in their propensity to grow using only nitrate, and four isolates have been identified with deletions of the genes that function to acquire and reduce nitrate. It would be expected that the ability to use an alternative source of nutrition would be a favourable trait to have. However, the existence of isolates specifically not using nitrate suggests that there may be some competitive disadvantage to this phenotype. As discussed in Chapter 3, phenotypic heterogeneity and bet-hedging can be an advantageous strategy to employ when there is some fitness cost associated with a certain phenotype.

One study has shown that using nitrate as a source of nitrogen can facilitate *B. bruxellensis* outcompeting *Saccharomyces cerevisiae* in continuous fermentations of sugar cane juice (de Barros Pita *et al.* 2011). However, there has been no study exploring possible effects of nitrate assimilation that could lead to selection against the nitrate-positive phenotype in *B. bruxellensis*. The assimilation of nitrogen from nitrate is known to be energy-demanding, requiring the transfer of eight electrons for the reduction of nitrate to ammonium. A study has shown that the use of nitrate by *B. bruxellensis* under aerobic and anaerobic conditions can result in a higher production of acetate a lower ethanol yield (Galafassi *et al.* 2013). This is thought to occur due to the reduction of nitrate acting as a redox sink, which favours acetate formation over ethanol from acetaldehyde. The implications of the energy demand of nitrate assimilation, the redox effects and fermentation products on the overall fitness of *B. bruxellensis* are not yet known. However, it is unclear as the whether the physiological changes that are associated with the use of nitrate

may have a negative impact on competitive fitness that could result in selection against a nitrate-positive phenotype.

A common strategy for assessing competitive fitness between isolates is through head-to-head competition experiments where the isolates are grown together in co-inoculated cultures, along with the use of genetic markers to distinguish between isolates in the cultures (Razinkov *et al.* 2013; Bell 2010; Delneri *et al.* 2008; Baganz *et al.* 1998). This type of experiment is better suited to exploring competitive fitness than a method where isolates are grown as monocultures and a comparison of their growth curves used to determine fitness defects. Not only is a directly competitive environment more similar to real-world conditions that microbes are faced with, it is more difficult to observe slight differences in fitness when using monocultures. Competition assays are needed to determine some fitness cost associated with the nitrate-positive state in *B. bruxellensis*, which would go some way to explaining the emergence of nitrate-negative *B. bruxellensis* isolates. This type of assay has not previously been used with *B. bruxellensis*.

This chapter explores the development and application of a new method for directly assessing competitive fitness between *B. bruxellensis* isolates. This is through the use of genetic transformation of *B. bruxellensis*, which has been developed relatively recently (Miklenić *et al.* 2013, 2015). These experiments used *B. bruxellensis* isolates AWRI1608 and AWRI3005, an isogenic nitrate-positive variant of AWRI1608 (Chapter 3). Since these isolates are genetically indistinguishable, genetic transformation was used to mark AWRI3005 with a selectable clonNAT-resistance marker in order to be able to determine proportions of each isolate grown competitively in mixed cultures with different nitrogen sources.

4.2 Methods

4.2.1 Yeast isolates and culture conditions

B. bruxellensis isolates (Table 3) were obtained from the Australian Wine Research Institute Microorganisms Culture Collection and maintained on MYPG agar buffered with calcium carbonate (malt extract, 3.0 g/L; yeast extract, 3.0 g/L; bacteriological peptone, 2.0 g/L; glucose, 10.0 g/L; bacteriological agar, 15.0 g/L; calcium carbonate, 20.0 g/L). Starter cultures were grown from single colonies in liquid YPD (yeast extract, 10.0 g/L; bacteriological peptone, 20.0 g/L; glucose, 20.0 g/L) in test tubes incubated with orbital mixing at 28°C for 48 hours.

4.2.2 Transformation protocol

The protocol for transformation of *B. bruxellensis* previously described by Miklenić *et al.* (2015) was used to randomly integrate the clonNAT resistance gene cassette into *B. bruxellensis* isolate AWRI3005, with the following modifications:

1. A modified gene cassette. The cassette contained NatMX for clonNAT resistance under the control of a string *Brettanomyces*-specific promoter. The cassette was amplified by PCR from plasmid TDH1pr-NatMX pMK-T (Figure 15) with primers M13-F/R and the resulting PCR fragment was purified and quantified. The transformation was performed using 55.4ng of the purified PCR product.
2. The initial culturing of cells was performed in four 50 mL falcon tubes, rather than one 200 mL batch.
3. In step 2, cells were washed three times with sterile deionised water in two 50 mL falcon tubes, rather than twice in 100 mL. These were consolidated to one tube in step 3 upon resuspension in cell treatment solution.
4. After electroporation, cell suspensions were incubated overnight at 28°C, rather than for six hours.

5. YPD-NAT agar plates (yeast extract, 10.0 g/L; bacteriological peptone, 20.0 g/L; glucose, 20.0 g/L, bacteriological agar, 15.0 g/L; clonNAT, 100 µg/mL) were used for selection of transformants.

Three independent transformation were performed and plated onto YPD-NAT agar plates, incubated at 28°C for 7 days. A single colony from each plate was isolated and streaked onto a fresh YPD-NAT plate to confirm clonNAT resistance. Each isolate was submitted to the AWRI Culture Collection (AWRI3718, AWRI3719, AWRI3720).

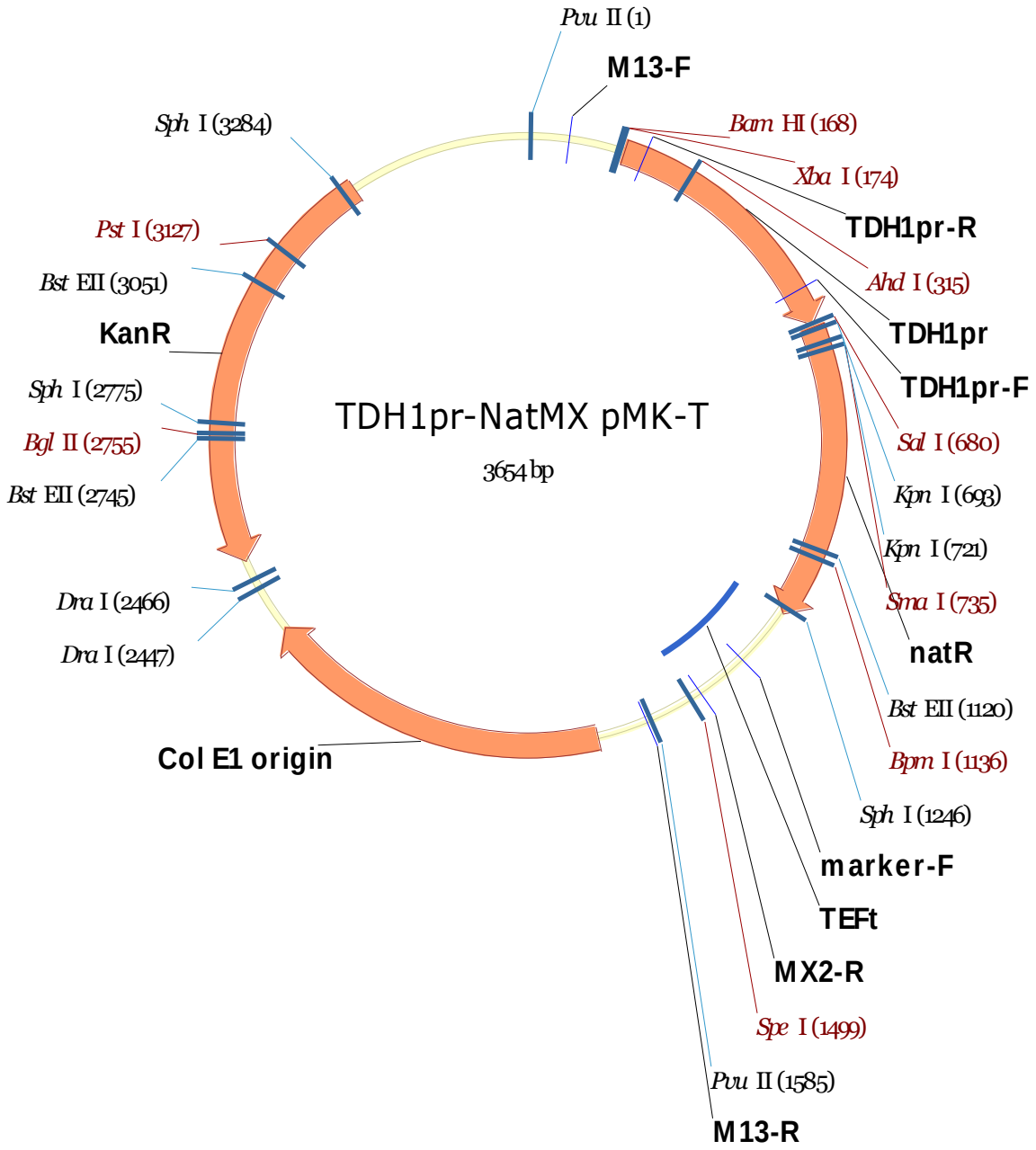

Figure 15. A schematic representation of the TDH1pr-NatMX pMK-T plasmid, from which the gene cassette for transformation was sourced.

The gene cassette was amplified by PCR from M13-F to M13-R (clockwise in this schematic) and contains the TDH1 promoter sequence (TDH1pr), nourseothricin resistance gene NatMX (natR) and the TEF terminator (TEFt). The plasmid contains ColE1 origin for replication in *E. coli* and KanR for selection of *E. coli* carrying the plasmid in the presence of kanamycin.

4.2.3 Competition experiments

Starter cultures of *B. bruxellensis* isolates AWRI1608, AWRI3718, AWRI3719 and AWRI3720 (Table 6) were prepared in liquid YPD as above and grown to stationary phase at 28°C with orbital shaking. Cultures were centrifuged to form cell pellets, which were washed and then resuspended in sterile water. Cell concentrations were determined by cell counting in a haemocytometer. Cell suspensions were transferred into 100mL liquid YPD, YNB-ammonium (Difco™ Yeast Nitrogen Base w/o Amino Acids and Ammonium Sulphate, 1.7 g/L; glucose, 5.0 g/L; ammonium sulphate, 5.0 g/L), YNB-nitrate (Difco™ Yeast Nitrogen Base w/o Amino Acids and Ammonium Sulphate, 1.7 g/L; glucose, 5.0 g/L; sodium nitrate, 6.4 g/L) and YNB-mix (Difco™ Yeast Nitrogen Base w/o Amino Acids and Ammonium Sulphate, 1.7 g/L; glucose, 5.0 g/L; ammonium sulphate, 2.5 g/L; sodium nitrate, 3.2 g/L) as a 50:50 mix of AWRI1608:AWRI3718, AWRI1608:AWRI3719 and AWRI1608:AWRI3720. These liquid cultures were then incubated at 28°C and stirred with a magnetic stirrer bar until they reached stationary phase as determined by OD600. Each culture was then spread plated onto solid YPD and YPD-NAT agar plates, which were incubated at 28°C for 7 days and colonies were counted manually. The next successive batches were then prepared from these stationary phase cultures by going back to washing the cell pellets and resuspending in sterile water, then inoculating fresh media and continuing with the protocol as before (Figure 16).

Table 6. A summary of *B. bruxellensis* isolates relevant to the competition experiments conducted in this study.

AWRI3005 is a nitrate-positive variant isolate from AWRI1608 grown on solid nitrate media (Chapter 2). AWRI3718, AWRI3719 and AWRI3720 are NatMX transformants of AWRI3005.

Isolate	Nitrate assimilation potential	clonNAT resistance	Description
AWRI1608	$1:10^5$	-	wine isolate
AWRI3005	+	-	nitrate-positive variant isolate of AWRI1608
AWRI3718	+	+	NatMX transformed variant of AWRI3005
AWRI3719	+	+	NatMX transformed variant of AWRI3005
AWRI3720	+	+	NatMX transformed variant of AWRI3005

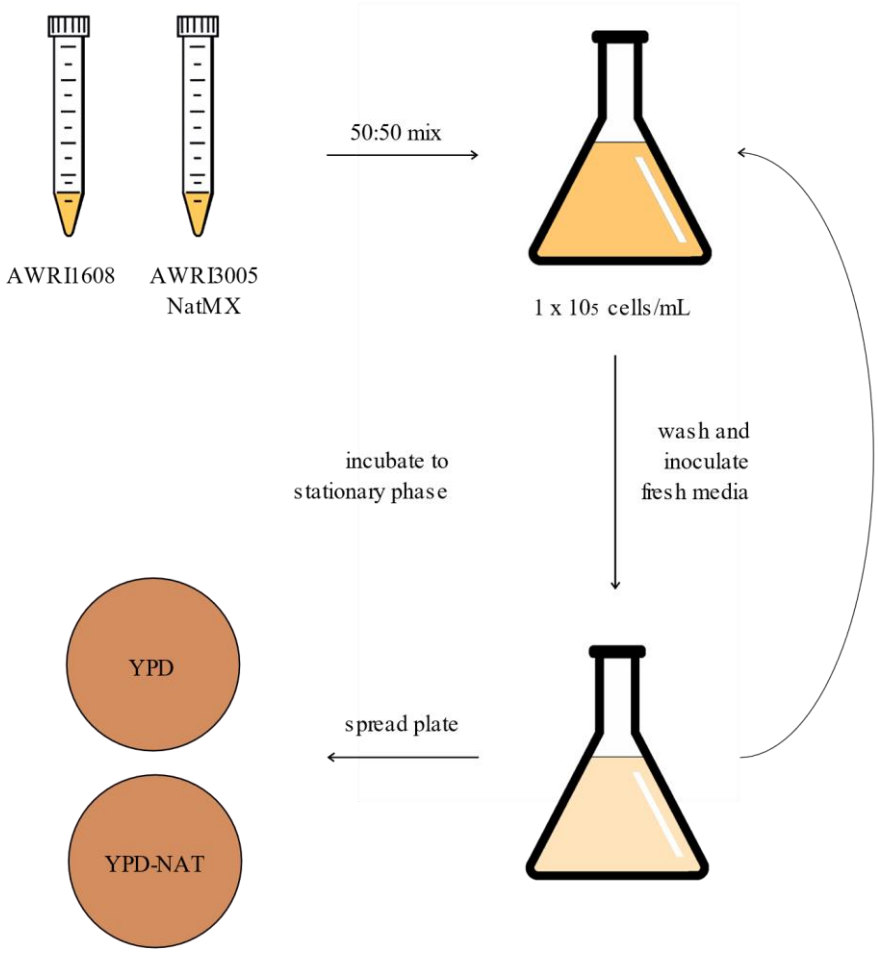

Figure 16. A flowchart of the protocol for the competition experiment.

Cultures of a 50:50 mix of AWRI1608 and one of the AWRI3005 NatMX-marked transformants (AWRI3718, AWRI3719, AWRI3720) were inoculated at 1×10^5 cells/mL and grown to stationary phase. These cultures were spread plated onto YPD and YPD-NAT agar plates to quantify proportions of each isolate within each culture. The cultures were then used to inoculate fresh media for the next successive batch. Data for population proportions of each of AWRI3718, AWRI3719 and AWRI3720 against AWRI1608, as determined by the spread plates, were collated such that each of the transformants in culture with AWRI1608 represented one of a triplicate set of cultures.

4.3 Results

4.3.1 Construction of drug-resistant marker strains

In order to assay competitive fitness between *Brettanomyces* isolates growing in mixed liquid cultures, there needs to be a means to unambiguously identify each isolate in order to quantify its relative proportion in the mixed population over time. There have been a number of methods developed for this, which either make use of existing genetic/phenotypic markers between isolates or use genetic transformation to introduce markers when they do not exist (Razinkov *et al.* 2013; Bell 2010; Delneri *et al.* 2008; Baganz *et al.* 1998).

As it was not feasible to reliably detect genetic difference between the nitrate negative parent isolate AWRI1608 and the nitrate-positive revertants (AWRI3005), a recently developed high-efficiency transformation protocol (adapted from Miklenić *et al.*, 2015) was used to randomly integrate the NatMX gene cassette into *B. bruxellensis* isolate AWRI3005 in order to provide an unambiguous phenotypic selection (clonNAT resistance) between it and AWRI1608 following growth as a mixed culture.

As it is not possible to target genetic material that is introduced into *B. bruxellensis* to a specific neutral location, individual isolates were isolated from three separate transformation reactions (AWRI3718, AWRI3719 & AWRI3720) to ensure against unwanted phenotypic effects due to location-specific integration of the marker. The efficiency for each transformation was higher than that achieved originally by Miklenić *et al.* (2015) (Figure 17) and, more importantly, each transformant retained its nitrate-positive phenotype following the process.

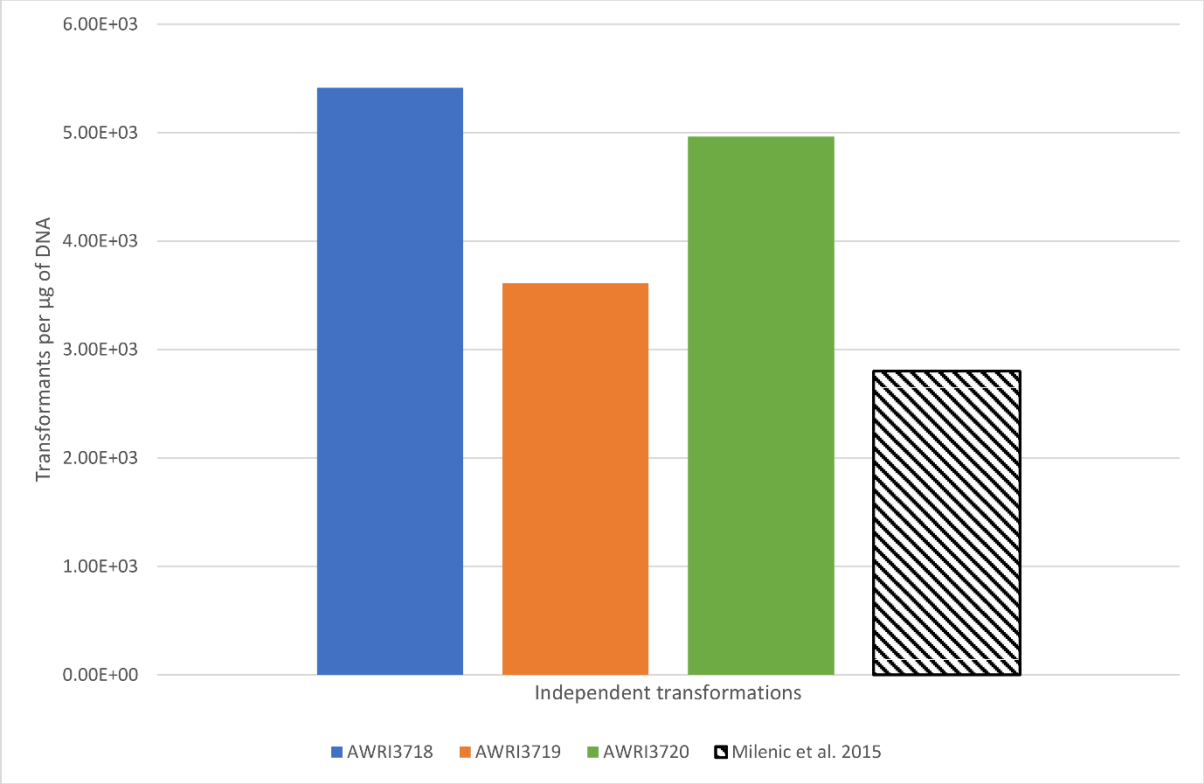

Figure 17. The efficiency of transformations of AWRI3005 with NatMX.

Three independent transformations of AWRI3005 were performed with the NatMX gene cassette. The transformation efficiency as the number of transformants per µg of DNA is given here with the average transformation efficiency originally published by Miklenić *et al.* (2015).

4.3.2 Competition assay

Once it had been established that the cloNAT resistant revertant isolates could be reliably quantified from mixed cultures of cloNAT resistant and sensitive cells, it was possible to directly monitor long-term competitive fitness between AWRI1608 and its nitrate-positive phenotypic revertant.

Replicate liquid cultures of AWRI1608 and each of the NatMX-marked revertants (AWRI3718, AWRI3719 & AWRI3720) were established (50:50 mix of both isolates) in six different media; YPD and YNB supplemented with ammonium, nitrate and a mixture of both ammonium and nitrate. These cultures were successively passaged through three batch fermentations (approximately 18 generations), with the proportions of each subpopulation quantified via differential plate counting using cloNAT resistance to specifically count AWRI3718, 3719 and 3720 (Figure 18). Control cultures of AWRI1608 were maintained in each medium throughout the experiment and none of these produced any colonies on clonNAT plates at any point indicating that the cloNAT marker was not providing false-positive colony counts (Figure 18).

As AWRI3005 displays a positive nitrate assimilation phenotype and AWRI1608 does not, it was expected that the clonNAT resistant isolates would strongly outcompete AWRI1608 in YNB supplemented only with nitrate. This was indeed the outcome, with populations in this medium shifting to an average of approximately 98% clonNAT resistant by the end of the first batch (Figure 18). By the end of the third batch, however, the clonNAT resistant subpopulation comprised 84% of the total population (Figure 18). This might have been due to a subset of AWRI1608 beginning to use nitrate by the end of the first batch.

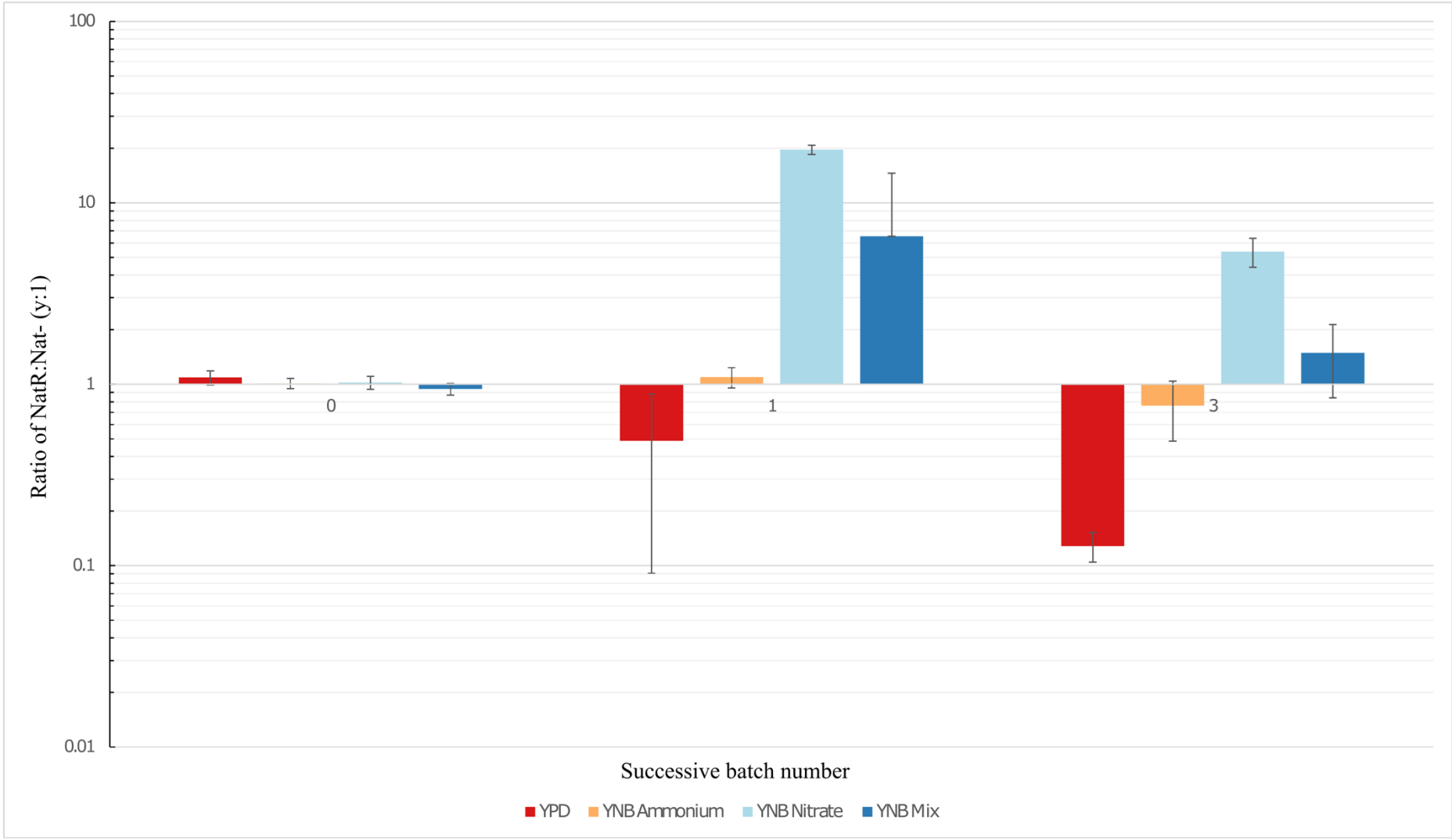
100
10
1
0.1
0.01
Ratio of NatR:Nat- (y:1)
0
1
3
Successive batch number
YPD
YNB Ammonium
YNB Nitrate
YNB Mix

Figure 18. Population proportions through the competition assay.

50:50 mixed cultures of AWRI1608 and each of the NatMX-marked AWRI3005 transformants (AWRI3718, AWRI3719, AWRI3720) were prepared in liquid YPD, YNB-ammonium, YNB-nitrate and YNB-mix. These were spread plated onto solid YPD and YPD-NAT prior to incubation to check population proportions (batch number = 0). Cultures were used to inoculate fresh media to generate successive batches, which were spread plated again onto solid YPD and YPD-NAT after stationary phase was reached in the first and third batches. Population proportions are represented as a ratio of clonNAT-resistant to clonNAT-sensitive colonies on the spread plates (NatR:Nat-). A result of 1 indicates a 50:50 mix of each subpopulation, >1 indicates a higher proportion of clonNAT-resistant cells, <1 indicates a higher proportion of clonNAT-sensitive cells. Error bars indicate the standard deviation between triplicates, where each transformant is represented in one of the replicate cultures.

A medium containing a mixture of nitrate and ammonium was also tested in this assay, where the molar concentration of nitrogen was the same as the other YNB media with half coming from ammonium and half from nitrate. The clonNAT resistant subpopulation out-competed AWRI1608 in this medium, but to a lesser extent than observed with nitrate as sole nitrogen source. At the end of the first batch, these cultures averaged approximately 74% clonNAT resistant (Figure 18). While there was a high degree of variability between these batches, they did all shift towards favouring the clonNAT resistant subpopulations.

In YNB supplemented only with ammonium, the population was maintained at ~50:50 mix by the end of the first batch, (Figure 18). The replicates were more widely spread and two of the three shifted towards a higher proportion of AWRI1608 by the end of the third batch, with 33%, 43% and 51% clonNAT resistant cells at this stage of the experiment (Figure 18).

Populations in YPD shifted towards favouring AWRI1608 strongly, with 11% clonNAT resistant cells at the end of the third batch (Figure 18). The standard deviation between replicates at the end of the first batch was high (17%) due to one of the replicates presenting 48% clonNAT resistant cells, while the other two were much lower at 16% and 25% (Figure 18).

4.4 Discussion

The aim of the work in this chapter was to lay the foundations in developing a method to explore competitive fitness between *B. bruxellensis* isolates AWRI1608 and AWRI3005: two 'near isogenic' isolates, differing in nitrate assimilation phenotypes. A commonly used approach to explore competitive fitness is through head-to-head growth assays using genetically-marked isolates. Newly developed methods for the genetic transformation of *B. bruxellensis* (Miklenić *et al.* 2013, 2015) have facilitated the use of selective markers for this type of experiment. The average transformation efficiency in this study was approximately double what was reported by Miklenić *et al.* (2015). This is likely due to the use of the *Brettanomyces*-specific TDH1 promoter in the gene cassette, rather than the TEF promoter used by Miklenić *et al.* (2015).

The clonNAT cassette was integrated into the genome of *B. bruxellensis* isolate AWRI3005 in a random manner. As such, this could have directly or indirectly affected nitrate metabolism or, indeed, any mechanism to have some impact on competitive fitness. Independent replicate transformations were performed in order to limit the impact of any such occurrence to just one isolate.

The data obtained from the growth experiment shows varying degrees of error throughout. Some points, for example the YPD cultures after the first batch, show dramatic variation between triplicate cultures. This may be related to the random nature of the integration of the clonNAT cassette used in this experiment. However, without further study, this variation cannot be explained.

An alternative method for comparing fitness in yeasts is to grow each isolate independently and compare their growth curves. Such a method would be much simpler to implement but there are draw-backs. Chiefly, independent growth is not representative of competitive fitness as it would occur in a real-world environment. These isolates would not normally be found as a monoculture and would have to compete for nutrients with other *B. bruxellensis* isolates and other microbial species. This is a particularly important point to note in the context of these experiments, since they are specifically aimed to explore competitive

fitness regarding the ability to assimilate a source of nitrogen that would normally only be used in a nutrient-scarce environment.

At the time of writing, there is no published work that similarly explores competitive fitness in this manner in *B. bruxellensis*. This is likely because methods for genetic transformation of *B. bruxellensis* have only been published relatively recently (Miklenić *et al.* 2013, 2015). These methods could also be used to mark different isolates with fluorescence markers, as opposed to antibiotic-resistance markers, for differentiation using fluorescence-activated cell sorting. That strategy would likely be more precise than the use of antibiotic-resistance and counting colonies on plates, removing random error from plating. The prospective future of coupling genetic transformation and assessing competitive fitness in *B. bruxellensis* looks to be promising.

The data from this experiment shows that AWRI3005 is able to strongly outcompete AWRI1608 when nitrate is the sole source of nitrogen. This shows that once nitrate assimilation has been induced in AWRI1608, that subpopulation is competitive under such conditions. However, populations in the later batches of the experiment were found to contain a higher proportion of AWRI1608 than the earlier batches when nitrate was present. This might be due to a subset of the AWRI1608 population having adapted to use nitrate in these cultures.

The nitrate-positive subpopulation was outcompeted by AWRI1608 when nitrate was not present and ammonium was available as a source of nitrogen. It is not clear from these experiments what the specific cause of this decline in competitive fitness of AWRI3005 is. However, this data suggests that there is some negative effect(s) associated with expressing a nitrate-positive phenotype where nitrate is not present. Nitrate assimilation might have been lost in some isolates (Chapter 2) because of a lack of requirement to use nitrate combined with such an effect on competitive fitness.

5 Concluding discussion and future perspectives

Nitrate assimilation is a phenotype that is not always expressed in *Brettanomyces bruxellensis* isolates. In this study, 58% of a cohort of forty-three isolates were found to efficiently assimilate nitrate and grow with nitrate as their sole source of nitrogen (Chapter 2). This result is in line with those from previous studies that have similarly explored nitrate assimilation phenotypes in *B. bruxellensis*, each finding less than three quarters of *B. bruxellensis* isolates with nitrate-positive phenotypes (Conterno *et al.* 2006; Crauwels *et al.* 2014). The phenotype screen in this study differs in the use of solid media, rather than liquid cultures, for nitrate assimilation phenotype determination. It is this use of solid media that provided the first glimpse at phenotypic heterogeneity in nitrate assimilatory potential in clonal *B. bruxellensis* cultures.

Previous studies have typically used a +/- scoring system for growth with nitrate as the sole source of nitrogen (Conterno *et al.* 2006; Borneman *et al.* 2014; Crauwels *et al.* 2014). In this study, however, the intensity of growth on nitrate was compared to that on ammonium. While most isolates were found to grow equally well on both media, the remainder were not all definitively negative in nitrate assimilation potential, typically falling somewhere in the 'grey' area between nitrate-positive and negative. Since solid media were used for this phenotype screen, it was possible to see that these isolates were not simply growing more slowly on nitrate, but had individual colonies growing efficiently on nitrate within a largely nitrate-negative background. This was the first indication of the possibility of a nitrate-positive subpopulation within a clonal *B. bruxellensis* population.

Phenotypic heterogeneity is an example of an evolutionary adaptation that facilitates survival in rapidly changing environmental conditions (Beaumont *et al.* 2009; Philippi & Seger 1989). This form of adaptation has been described as bet-hedging (Philippi and Seger, 1989). While a subset of the population can, at some cost to fitness, be primed for a certain change in environmental conditions, the majority can forego that cost to thrive in the conditions prior to the change. Some isolates in this study were found to have a

subset of their population expressing an ability to use nitrate as their sole source of nitrogen. However, it is unclear why the majority of cells in those populations did not display a propensity to grow on nitrate-based media. In order to better understand this phenotypic heterogeneity, it is important to determine what the cause is, i.e. how nitrate assimilation impacts competitive fitness such that nitrate-negative phenotypes are selected for. A method for genetic transformation of *B. bruxellensis* (Miklenić *et al.* 2015) was adapted to differentiate isolates based on antibiotic resistance. This allowed for the use of co-inoculated cultures such that the comparative fitness of two isolates in direct competition with one another could be determined (Chapter 4). An important direction for future research in *B. bruxellensis* nitrate assimilation phenotype heterogeneity is in developing an understanding of why some isolates express nitrate-negative phenotypes. The methods used in this study for assessing competitive fitness between isolates provides a good starting point for determining this (Chapter 4).

There are a number of culture conditions that could be tested via competitive growth analysis, for example: a broader scope of ammonium/nitrate mixed media at different concentrations, especially a medium that more closely resembles the nitrogen and carbon availability that these *B. bruxellensis* isolates would encounter in wine, around ten-fold lower than what was used in this study. On the subject of more realistic culture conditions, it would also be pertinent to assess competitive fitness under added stresses, such as with higher ethanol, lower pH and/or the addition of sulphite. Nitrate assimilation has been shown to improve *B. bruxellensis* growth under anaerobic conditions and improve fermentative metabolism (Galafassi *et al.* 2013). Since nitrate assimilation has differing effects on *B. bruxellensis* physiology under aerobic and anaerobic conditions, there might also be differences in competitive fitness under each condition and, as such, future assays should be conducted both aerobically and anaerobically. Such experimentation could elucidate some form of selection against the use of nitrate in *B. bruxellensis* and provide insight into a bet-hedging nature regarding nitrate assimilation phenotypes.

Furthermore, the mechanism behind nitrate assimilation phenotype heterogeneity should also be explored. It is apparent from this study that it is not due to genetic mutation, because nitrate-positive

variant isolates from AWRI1608 did not present with any realistic candidate mutations for such a change in phenotype (Chapter 3). The variation in phenotype may be caused by changes to gene expression. Gene expression analyses, for example using quantitative reverse-transcription PCR, could be used to show differences in nitrate gene expression. It could be that the nitrate-positive variants have a higher basal expression of the nitrate assimilation genes. Higher basal expression of the nitrate transporter gene *YNT1* could, for instance, allow for a better initial influx of nitrate into the cell that could then stimulate nitrate gene expression and, therefore, nitrate assimilation. Nitrate-negative variants might down-regulate nitrate gene expression due to some associated fitness cost, and thus find it more difficult to switch to nitrate assimilation. Little is known about the role of the two transcription factors, Yna1 and Yna2, in the regulation of the nitrate assimilation pathway other than that they are both required to induce expression of the pathway in *O. parapolymorpha* (Avila *et al.* 1998, 2002). Nitrate assimilation phenotype heterogeneity was found to be associated with two distinct haplotypes of the regulatory gene cluster in this study, and these may be affecting expression of the nitrate assimilation pathway.

Developing an understanding of the physiology of *B. bruxellensis* is important for industries relying on alcoholic fermentation, from controlling spoilage in wine to the use of *B. bruxellensis* for fermentation in beer and bioethanol production. Since *B. bruxellensis* is such a competitive species in late/post fermentation, it is particularly important to understand how it scavenges for nutrients and uses alternative sources of nutrition, such as nitrate, that help it adapt to these environments. This study provides unique insight into inter-isolate variability in nitrate assimilation potential in the discovery of phenotypic heterogeneity. Further exploration of this phenomenon would help develop a better understanding of this species' adaptation to industrial alcoholic fermentative ecosystems.

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