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Review

Kluyveromyces marxianus: A yeast emerging from its sister's shadow

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ARTICLE INFO

Article history:
Received 31 August 2009
Received in revised form
5 January 2010
Accepted 8 January 2010

Keywords:
Biotechnology
Ethanol production
Kluyveromyces marxianus
Metabolism
Yeast

ABSTRACT

Yeasts have a long tradition of application in biotechnology and a more recent history of use as research models for biochemistry, metabolism, genetics and cell biology. Without doubt, *Saccharomyces cerevisiae* has been the dominant representative in all these aspects. There is tremendous diversity among yeasts, however, and the application of modern microbiological and molecular approaches has resulted in renewed focus on the biology and industrial potential of other yeasts. The dairy yeast *Kluyveromyces marxianus* is of particular interest in this regard because of traits that render it especially suitable for industrial application. These include the fastest growth rate of any eukaryotic microbe, thermotolerance, the capacity to assimilate a wide range of sugars, secretion of lytic enzymes, and the production of ethanol by fermentation. Despite the importance of these traits, and significant exploitation by the biotechnology sector, fundamental research with *K. marxianus* is just emerging from the shadow of its sister species, *Kluyveromyces lactis*. The availability of new molecular tools and resources for *K. marxianus*, its interesting metabolic and cellular traits, and the potential to become the leading yeast for many biotechnological processes, argue strongly for increased research into this particular species.

1. Introduction

Yeasts are widely used in both traditional and modern biotechnology for the production of foods, beverages, enzymes, fine chemicals and pharmaceutical reagents. *Saccharomyces cerevisiae* and related species are particularly well-known because of their importance in making fermented beverages (beer, wine, cider), but there is a wide diversity of yeasts, for example within the *Kluyveromyces*, *Pichia*, *Debaryomyces* and *Yarrowia* genera, that have roles in biotechnology. Yeasts have also been to the forefront of research in modern genetics, molecular biology and cell biology, although it is true to say that the majority of research has focused on two species, *S. cerevisiae* and *Schizosaccharomyces pombe*, and more latterly, because of its medical significance, on *Candida albicans*. As applications in the biotechnology sector continue to develop, however, there is increasing interest in applying modern molecular tools to understand and improve some of the so-called ''non-conventional'' yeasts. The particular subject of this review is one of these yeasts, *Kluyveromyces marxianus*.

K. marxianus is described as a homothallic, hemiascomycetous yeast, is phylogenetically related to *S. cerevisiae*, and is a sister species to the better-known *Kluyveromyces lactis* (Lachance, 1998; Llorente *et al.*, 2000). The major common feature of *K. lactis* and *K. marxianus* is the capacity to

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doi:10.1016/j.fbr.2010.01.001

assimilate lactose and to use this sugar as a carbon source. This trait, which is absent in *S. cerevisiae*, leads to the frequent isolation of these yeasts from dairy sources, for example fermented milks, yoghurt and cheeses. The long history of safe association with food products helped these two yeasts achieve GRAS (Generally Regarded As Safe) and QPS (Qualified Presumption of Safety) in the United States and European Union, respectively. This designation means that there are few restrictions on application and greatly enhances their potential in the biotechnology sector. *K. lactis* has been the predominant research species within *Kluyveromyces*, initially for studies on lactose metabolism, but then as a model for non-conventional yeasts (Fukuhara, 2006; Schaffrath and Breunig, 2000). In contrast, the sister species *K. marxianus*, has been more widely adopted by industry, mainly because it possesses traits that are desirable for biotechnology applications (Fig 1). These include the capacity to assimilate key sugars, namely lactose and inulin; an extremely rapid growth rate, with typical generation times of ~70 min; thermotolerance, with the ability to growth up to 52 °C; and a high secretory capacity (reviewed recently by Fonseca *et al.*, 2008).

2. Taxonomy and phylogeny of *K. marxianus*

Prior to the development of molecular biology, classification of yeasts was primarily based on morphology and physiology, in particular the capacity to assimilate particular carbon sources. The obvious limitations of these methods encouraged the application of molecular approaches, but different techniques often generated conflicting or inconclusive findings. The work of Kurtzman and colleagues, however, has definitively established variation in the D1/D2 region of the large subunit (25S) rDNA as the benchmark for categorizing yeast and understanding the relatedness between strains (Kurtzman and Robnett, 1998). Application of this sequence-based taxonomy resulted in major re-organisation and reclassification within the *Saccharomyces* complex/clade, with yeasts previously classified as part of the genus *Kluyveromyces* strongly affected. The highly polyphyletic nature of the original *Kluyveromyces* genus required the renaming of most of its species and the genus now retains just six species (Kurtzman, 2003; Lachance, 2007). Interestingly, some well-known ''*Kluyveromyces*'' species such as *K. thermotolerans* and the original type species, *K. polysporus*, are no longer part of the *Kluyveromyces* genus and the type species is now *K. marxianus*, a species that incorporates many synonyms. *K. dobzhanskii* is the species most closely related to *K. marxianus* but there are relatively few studies on this yeast, which appears to be phenotypically quite different to *K. marxianus* (Fig 1).

An appreciation of the phylogenetic relationship between the *Kluyveromyces* and *Saccharomyces* genera is important when considering the genetics and metabolism of yeast within these genera. Both genera are part of the ''*Saccharomyces*'' complex, itself a subclade within the Saccharomycotina or hemiascomycetes. Other hemiascomycete yeasts that are important medically, such as *Candida*, or industrially, such as *Debarromyces* and *Yarrowia*, are outside the *Saccharomyces* complex and so could be expected to be more different to both *Saccharomyces* and *Kluyveromyces* (Scannell *et al.*, 2007). The *Saccharomyces* complex is itself divided, however, by a whole-genome duplication (WGD) event that occurred 100 million years ago (mya) ago (Wolfe and Shields, 1997). Genera and species in the *Saccharomyces* complex are defined by whether they emerged pre- or post- the WGD, with *Saccharomyces sensu stricto* species post-WGD and *Kluyveromyces*

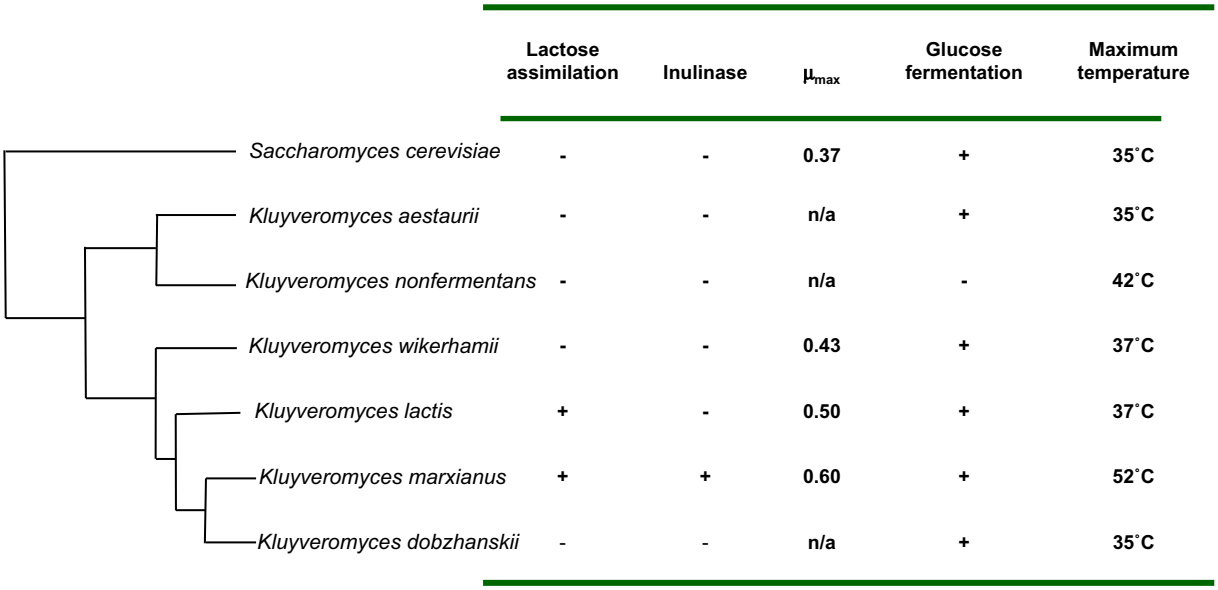

Fig. 1 – Relationship of *K. marxianus* to other yeasts. The tree shows the phylogenetic relationships between the species in the genus *Kluyveromyces* with *Saccharomyces cerevisiae* included for comparison. Some key traits are also presented, highlighting notable features of *K. marxianus* that differentiate this yeast from other *Kluyveromyces* species. Individual strains within a species may show some variation but the general traits listed apply to the species. With the exception of growth rates, which were sourced from the scientific literature (see main text), details were taken from the *Centraalbureau voor Schimmelcultures* (CBS) culture collection database (http://www.cbs.knaw.nl/).

species, pre-WGD. Because genes were present in additional copy, genes in yeasts that underwent the WGD had the flexibility to evolve new functions, a feature that is believed to have been very important in the evolution of *S. cerevisiae*. It follows that genes that are homologous between *Saccharomyces* and *Kluyveromyces* may have functionally diverged and, in some cases, functional conservation may be higher with ''less-related'' yeasts outside the *Saccharomyces* complex. This can be important when trying to understand metabolic pathways and processes in the cell.

Genome structure and ploidy is variable within yeasts. Some species like *C. albicans* are diploid, whereas other such as *Saccharomyces* can be found in haploid, diploid or polyploid forms. In *Saccharomyces*, most molecular genetics is carried out with haploid *S. cerevisiae*, whereas traditional strains used in biotechnology are often diploid or even hybrids (Mortimer, 2000; Nguyen and Gaillardin, 2005). The pre-eminence of *S. cerevisiae* as a eukaryotic model organism is directly linked to the powerful genetic tools that can be applied. In particular, haploid, heterothallic strains that readily mate to form stable diploids are available, and these can easily be induced to undergo meiosis to re-form haploid progeny. Classical genetics and molecular biology integrate seamlessly with *S. cerevisiae*, as it is possible to test gene function by targeted knockouts in haploid or diploid strains, and by combining mutations *via* mating. The ploidy status of the species within the *Kluyveromyces* genus is not fully resolved though it was established that most species are homothallic and can be divided into mating groups (Johannsen, 1980). Like *S. cerevisiae*, *K. lactis* is haploid and present in two mating types that can mate to form an unstable diploid that reverts quickly to the haplophase. This trait does, however, allow genetic crosses and it is sometimes used to combine mutants and check the effect of double mutations. The status of *K. marxianus* is less clear. Traditionally, the species was considered to be haploid (Johannsen, 1980; Steensma *et al.*, 1998) but recent molecular work indicates that this may not always be true and it is suggested, for example, that the widely used strain *K. marxianus* CBS 6566 may be a diploid (Ribeiro *et al.*, 2007). In contrast, molecular approaches indicate that other strains are haploid and it therefore seems likely that both haploid and diploid forms of *K. marxianus* are prevalent in research and industrial settings (Hong *et al.*, 2007; Nonklang *et al.*, 2009; Pecota *et al.*, 2007). It would certainly be of interest to determine the ploidy of the most commonly used strains as this has direct implications for the application of molecular methodologies.

3. Sugar metabolism and physiology

Commencing with the work of Louis Pasteur in the nineteenth century, yeast research placed a major emphasis on growth and utilization of sugars. Following a long history of biochemical approaches, molecular genetics opened up new prospects for research, and indeed the original reason that *K. lactis* was chosen as a research model was to compare genetic regulation of the *lac/LAC* genes in bacteria and eukarya (Fukuhara, 2006). The ability to utilize particular sugars, and the pathways used to generate energy from sugars, are defining features of different yeasts and, until the advent of molecular tools, was a major classification tool for yeasts (see above). Detailed knowledge of these pathways is therefore of fundamental importance to the understanding of any particular yeast. It is fascinating that although components of the core pathways of glycolysis and the TCA cycle are largely conserved, regulation differs dramatically between yeast species (Flores *et al.*, 2000). These differences in regulatory mechanisms give rise to a number of peculiarities that are commonly referred to as the Crabtree, Kluyver, Pasteur and Custer effects, reflecting the scientists who first described them. It is true to say that these effects are still not completely understood, but an overview of research in yeast sugar metabolism, and up to date explanations of these effects based on the most recent data, is provided in a comprehensive recent review article in Barnett's excellent ''history of research on yeast'' series (Barnett and Entian, 2005). When considering *Kluyveromyces*, the relatively close phylogenetic relationship with *Saccharomyces* might be expected to facilitate direct comparisons, whereas, when it comes to sugar metabolism, *Saccharomyces* is quite unique and has evolved a whole series of idiosyncrasies not found in other yeasts. Following the WGD in the lineage leading to *Saccharomyces*, most genes returned to single copy, but duplicate copies of many of the genes involved in glycolysis and sugar metabolism were retained. It is postulated that this enabled *Saccharomyces* to develop an increased glycolytic flux and the capacity to rapidly utilize glucose by fermentation to ethanol, conferring a competitive advantage in its particular ecological niche (Conant and Wolfe, 2007; van Hoek and Hogeweg, 2009). Clearly, yeasts that did not undergo the WGD were under different selective pressure and have evolved particular regulatory mechanisms suited to their own niches. Caution must therefore be exercised when extrapolating from studies with *S. cerevisiae* to *K. marxianus* or other pre-WGD yeasts and, as has been noted elsewhere, perhaps *S. cerevisiae* is the true ''non-conventional'' yeast (Blank *et al.*, 2005).

Yeasts can be classified as aerobic, facultative or respiro-fermentative, or fermentative depending on their status *vis a vis* oxygen (Merico *et al.*, 2007). *K. marxianus*, like *S. cerevisae*, is a respiro-fermentative yeast, and it can generate energy either *via* the TCA cycle by oxidative phosphorylation or by fermentation to ethanol. In the case of *S. cerevisiae*, when the sugar concentration is high, the bias is strongly towards fermentation, meaning that the cell preferentially directs pyruvate to the production of ethanol although the net energy yield is lower than that which could be achieved from the TCA cycle. This phenomenon is termed the Crabtree effect and is postulated to deliver a competitive advantage is certain ecological niches (Piskur *et al.*, 2006). The mechanistic basis of the Crabtree effect is still not fully understood but probably arises from a combination of enzyme saturation under high glycolytic flux, glucose repression of TCA enzymes and expression of specific redox balancing metabolic reactions (Merico *et al.*, 2007). Although *K. lactis* and *K. marxianus* are generally classified as Crabtree negative, both species do carry the genes necessary for ethanol production by fermentation and under certain conditions will adopt the fermentation lifestyle. It should be noted that there are conflicting reports in the literature of the ''Crabtree status'' of *K. marxianus* and it has been referred to as ''Crabtree-effect positive'' in some studies. This apparent conflict almost certainly reflects strain

variability, a problem compounded by the fact that most yeast comparative studies choose a single strain as a representative of each species. A study of 40 yeasts within the ''*Saccharomyces* complex'' demonstrated that the widespread ability to ferment in the presence of oxygen is often restricted by the capacity of the yeast to achieve NAD(P)/NAD(P)H (redox) balance in the cell (Merico *et al.*, 2007). Based on the available evidence, the authors speculate that a preference for a fermentative lifestyle may represent the ancestral state for this yeast clade, including *Kluyveromyces*. It was interesting that the particular strain of *K. marxianus* (CBS 712) used in that study grew significantly better by fermentation than the *K. lactis* (CBS 2359) strain, probably both a reflection of underlying differences between the species, and also of variation within both species. A later study specifically on *K. lactis* found that oxygen levels strongly influence the cell's fermentative capacity (Merico *et al.*, 2009), a phenomenon that is possibly linked to the ''Pasteur effect'', which is manifest as a reduction in glycolytic flux in presence of oxygen (Barnett and Entian, 2005). It can be concluded that *Kluyveromyces* are essentially Crabtree negative yeasts and preferentially direct metabolism towards the TCA cycle and optimum energy generation. Nevertheless, *K. marxianus* is capable of carrying out simultaneous fermentation and respiration and the precise balance between these pathways is strain specific. There is a very clear trade-off between production of ethanol or biomass: for example, Merico and colleagues report figures of a biomass yield of 0.13 gg^{-1} (grams biomass per gram of sugar) for *S. cerevisiae* and 0.40 gg^{-1} for *K. lactis* (Merico *et al.*, 2007). For applications where biomass is important, this difference between yeast species is certainly relevant. It must be considered also, however, that the Crabtree effect results from several related factors/effects and these may not exert themselves equally in all species. Thus, there is a spectrum between extreme Crabtree positive and Crabtree negative yeasts, and the strength of the effect can be influenced by extrinsic factors. As mentioned, variation in the strength of the effect even occurs within species, which explains why some, but not all, strains of *K. marxianus* are very effective producers of ethanol (Hong *et al.*, 2007; Nonklang *et al.*, 2008). When considering metabolism, it is notable that *K. marxianus* has one of the fastest growth rates of any eukaryotic microbe, for example one chemostat study recorded the specific growth rate (μ_{max}) of *K. marxianus* CBS 6566 as 0.6 h^{-1} (doubling time of 70 min), and an even faster growing variant with a μ_{max} of 0.8 h^{-1} could be selected (Groeneveld *et al.*, 2009). The particularly high metabolic flux of *K. marxianus* was also noted in a comparison of fourteen yeast species but the underlying reasons for the faster growth, especially relative to its sister species, *K. lactis* remain to be determined (Blank *et al.*, 2005).

It was mentioned already that a defining feature of *K. lactis* and *K. marxianus* is their capacity to utilize lactose as a carbon source, a trait that is absent in *S. cerevisiae*. Lactose utilization has been extensively studied in *K. lactis* and it is assumed that the fundamental aspects are conserved in *K. marxianus* (Rubio-Texeira, 2006; Schaffrath and Breunig, 2000). The ability to utilize lactose is conferred by two genes, *LAC12*, which encodes a lactose permease required for lactose uptake into the cell, and *LAC4*, which encodes a β-galactosidase that hydrolyses lactose to the monomers glucose and galactose. As in *S. cerevisiae*, galactose is further metabolised to glucose-6-P *via* the Leloir pathway. The evolutionary history of the *LAC12–LAC4* gene pair is not clear but their regulation is integrated with the Gal4p/Gal80p system that is well-studied in *S. cerevisae*. There are, however, differences between the species, for example, basal expression of the *LAC/GAL* genes is higher in *Kluyveromyces*, probably because of a *GAL4* positive autoregulatory loop, and the extent to which glucose repression controls expression also seems quite strain specific. Mechanistically, there are also differences, for example, *Kluyveromyces* lacks a specific galactose permease, encoded by *GAL2* in *Saccharomyces*, and retains enzymatic and regulatory functions in Gal1p, whereas these are split between Gal1p and Gal3p in *Saccharomyces* (Rubio-Texeira, 2005; Traven *et al.*, 2006).

4. Industrial exploitation

The underlying interest in *K. marxianus* is undoubtedly driven by applications in the biotechnology industry. This is reflected in the scientific literature, where there are more papers dealing with biotechnological applications than metabolism, molecular biology, or other fundamental aspects of this yeast. The industrial potential of *K. marxianus* was comprehensively covered in a recent review and some key applications are listed in Table 1 (Fonseca *et al.*, 2008). Commercially, the most important current application is production of native enzymes, such as inulinase, β-galactosidases and pectinases. Inulinase, which hydrolyses a plant fructan called inulin, is of particular interest as this enzyme is not commonly found in other yeasts or fungi. Apart from some novel enzymes, rapid growth and ease of handling make *K. marxianus* a preferable system to filamentous fungi, which are often an alternative source of fungal enzymes. Future developments will certainly include the construction of recombinant strains producing additional enzymes, a point illustrated by the construction of a strain expressing cellulose-hydrolysis genes (Hong *et al.*, 2007). The β-galactosidase activity of *K. marxianus* has been exploited for some time, where the yeast was used to treat lactose-containing waste from the cheese industry. In fact, whey permeate, formerly a problematic waste product, has become a key inexpensive substrate for the growth of *K. marxianus*, either to produce biomass or ethanol. The biomass is used as animal feed or converted to extract and used in the food processing industry. The interest in ethanol is driven by the development of biofuels, though the costs associated with distillation mean that the production of bioethanol from whey permeate is a marginal commercial activity at present. Apart from the treatment of dairy waste streams, the biosorption and bioaccumulative properties of *K. marxianus* are utilized in bioremediation of textile dyes and copper from wastewaters, and the yeast is used to treat waste and paper sludge. As more tools become available, it is likely that the intrinsic advantages of *K. marxianus* over other yeasts will see increasing applications in the biotechnology and biopharmaceutical sectors.

5. Molecular and genetic tools

Traditional biotechnology has always applied physiological and biochemical methods to derive knowledge to improve

Table 1 – Overview of key industrial applications of *K. marxianus*. Examples of the range of biotechnological applications of *K. marxianus*. The diversity of strains studied for applications is illustrated. Representative studies are reported and a more detailed list can be found in Fonseca *et al.* (2008)

Application	Strains where specified	References
Production of ethanol from whey or lactose		
Immobilised and suspended cells	*K. marxianus* NBRC 1963	Oda and Nakamura (2009)
Continuous and batch fermentations	*K. marxianus* DSMZ-7239	Ozmihci and Kargi (2007)
	K. marxianus MTCC 1288	Zafar and Owais (2006)
	n/a	Kourkoutas *et al.* (2002)
	K. marxianus IMB3	Brady *et al.* (1997)
Production of biomass or single cell protein		
Continuous, batch and fed batch fermentation	*K. marxianus* ZIM 1867	Pas *et al.* (2007)
Iron and nucleotide rich biomass	*K. marxianus* CBS 6556	Schultz *et al.* (2006)
Comparable autolysate composition to *S. cerevisiae*	*K. fragilis* NRS 5790	Ghaly *et al.* (2005)
	K. marxianus FII 510700	Lukondeh *et al.* (2005)
	K. marxianus CBS 6556	Revillion *et al.* (2003)
	K. marxianus ATCC10022; CBS 7894	Pinheiro *et al.* (1998)
Production of endogenous enzymes		
β-xylosidase	n/a	Rajoka (2007)
β-galactosidase	*K. marxianus* CBS 7894	Pinheiro *et al.* (2003)
β-glucosidase	*K. fragilis* IpF1	Szczodrak (2000)
Inulinase	*K. marxianus* CDBB-L278	Cruzguerrero *et al.* (1995)
Heterologous expression of enzymes		
Thermostable cellulases	*K. marxianus* NBRC 0219; NBRC 0541; NBRC 0617; NBRC 1777	Hong *et al.* (2007)
Lactate dehydrogenase	*K. marxianus* KM1	Pecota *et al.* (2007)
Endopolygalacturonase	*K. marxianus* BKM Y-719	Siekstele *et al.* (1999)
α-galactosidase	*K. marxianus* CBS 6556	Bergkamp *et al.* (1993a,b)
Food industry		
Natural emulsifier – mannoprotein	n/a	Vasallo *et al.* (2006)
	n/a	Lukondeh *et al.* (2003)
Aroma compounds	*K. marxianus* ATCC10024	Medeiros *et al.* (2000)
Bakers yeast	*K. marxianus* NRRL-Y-2415; NRRL-Y-1109	Caballero *et al.* (1995)
Environmental applications		
Treatment of paper wastes and sludge	*K. marxianus* Y01070	Kadar *et al.* (2004)
Removal of lactose/other sugars from wastewater	*K. marxianus* NRRL Y-610	Hang *et al.* (2003)
Biosorption of dyes	*K. marxianus* IMB3	Meehan *et al.* (2000)
Recovery of heavy metals from wastewater	n/a	Pal *et al.* (2009)

processes and to identify and select more efficient strains. Notwithstanding the large body of fundamental knowledge that has been gained, it is apparent that future advances, in both understanding and application, require molecular approaches. Although molecular research with *K. marxianus* lags behind *K. lactis*, tools are now becoming available that will facilitate strain engineering. The current status of these tools is illustrated in Fig 2. One major limitation is the lack of comprehensive genome sequence information and there is still a strong reliance on a partial genome sequence of strain CBS 712 (Llorente *et al.*, 2000), and comparison to the completed *K. lactis* genome sequence (Dujon *et al.*, 2004).

Introduction of DNA into *K. marxianus* can be achieved by electroporation or by LiAc transformation using protocols adapted from *S. cerevisiae* and *K. lactis* (Iborra, 1993; Zhang *et al.*, 2003). Construction of stable strains requires the integration of DNA into the chromosome, either to make defined mutants lacking a specific gene, or to introduce a new trait into a strain. The main issues with this approach in any species are gene targeting and marker availability. Integration of a fragment of DNA into the genome exploits the DNA repair processes in the cell and can be random or targeted (Aylon and Kupiec, 2004; Daley *et al.*, 2005). When a linear DNA fragment flanked by sequences with homology to a region of genomic DNA is introduced into a cell, the process of homologous recombination (mediated by the Rad52p system) can direct allele replacement and introduction of the new DNA fragment into that specific genomic locus. The second mechanism, mediated by the Ku70/Ku80 heterodimer and termed non-homologous end joining (NHEJ), results in random integration of DNA into the genome. The frequency at which any species or strain integrates DNA into a specific target site is largely determined by the relative efficiency of the HR and NHEJ systems, which compete for binding to free DNA ends. *S. cerevisiae* is unusual among eukaryotes in that HR is highly effective and efficiencies of close to 100 % can be obtained (Baudin *et al.*, 1993). In contrast, efficiency can be extremely low in *K. lactis* and *K. marxianus* and construction of mutants therefore requires either screening of large number of candidate transformants, or improvements in gene-targeting efficiency. A study with *K. lactis* has identified a number of strategies that can be taken to improve gene-targeting efficiencies (Kooistra

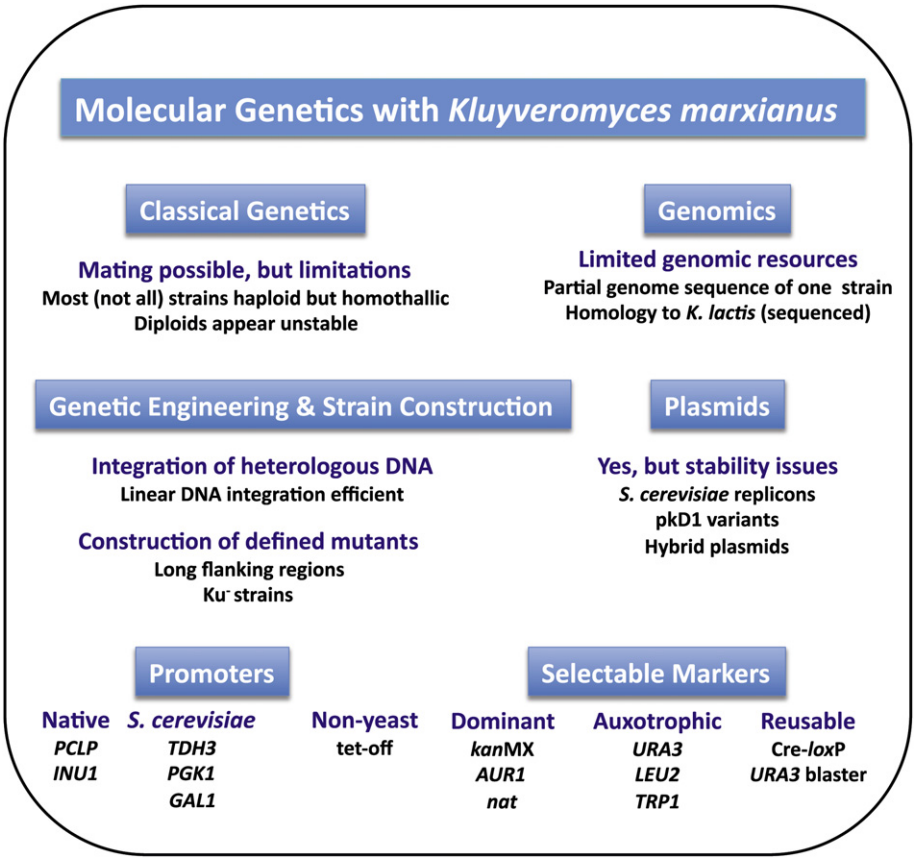

Fig. 2 – Molecular tools and resources for *K. marxianus*. A summary is presented of the status of the key molecular reagents that are available, or required, to facilitate molecular genetics and strain improvement in *K. marxianus*. Further details are provided in the main text.

et al., 2004). The first is to increase the length of homologous flanking sequence – increasing the length from 50 bp to 600 bp led to a progressive increase in HR from 0 % to 88 %. Manipulating the concentration of DNA ends by the addition of salmon sperm carrier DNA also improved targeting efficiencies, presumably by altering the dynamic between HR and NHEJ. The most striking results, however, were obtained when NHEJ was inactivated by deletion of the *KU80* gene. This resulted in targeting efficiencies of close to 100 %, regardless of the length of the flanking sequence. A key benefit is that flanking sequences can be of oligonucleotide length, removing the requirement to clone large stretches of flanking DNA before making mutants. Despite the benefit of being able to now carry out one-step PCR disruption as with *S. cerevisiae* by using a *ku* mutant strain, most workers with *K. lactis* have not adopted this approach, probably because of strain legacies in each research group. In contrast, with *K. marxianus*, molecular biology is at a much earlier stage of development and there is a clear opportunity for the development of *ku* strains as the work-horses for the species, as is being done with other fungi, for example *Aspergillus* (Chang, 2008; da Silva Ferreira *et al.*, 2006). An additional point that is very important here is the ploidy of the strains that are currently used. This issue was mentioned in an earlier section above, and haploid strains are certainly more tractable for almost all types of molecular analysis.

Autonomously replicating plasmids are common in manipulation of yeasts for biotechnology applications but options within *Kluyveromyces* are generally quite limited. Most replicating vectors for *K. lactis* use the replication origin and *cis* stabilising locus of the natural plasmid pKD1 found in *Kluyveromyces drosophilarum*. This is a 2 μm – like vector and is the only natural circular plasmid found in *Kluyveromyces* to date (Bianchi *et al.*, 1991). pKD1 can be transformed into *K. marxianus* but stability is poor (Chen *et al.*, 1989). Modified versions of pKD1 with improved stability have been developed (Bartkeviciute *et al.*, 2000), and ''artificial'' vectors using autonomously replicating sequence (ARS) elements isolated from the *K. marxianus* genome have been constructed (Ball *et al.*, 1999; Iborra and Ball, 1994). It is also possible to transform *S. cerevisiae* vectors into *K. marxianus* but long-term vector stability remains an issue and chromosomal integration of DNA is a more reliable approach. Nonetheless, a number of heterologous enzymes have been successfully expressed from vectors in *K. marxianus* (Almeida *et al.*, 2003; Ball *et al.*, 1999; Bartkeviciute *et al.*, 2000; Hong *et al.*, 2007; Nonklang *et al.*, 2008; Nonklang *et al.*, 2009).

Selectable markers are required to identify transformants and, as with other yeasts, dominant markers such as *kan*MX, *AUR1-C* or *nat*, for resistance to G418, aureobasidin A and nurseothricin, respectively, can be used (Hashida-Okado *et al.*, 1998; Ribeiro *et al.*, 2007). Wild-type strains are typically

prototrophic but auxotrophic strains can be created, for example, uracil, leucine, tryptophan, lysine and adenine auxotrophs of different *K. marxianus* strains have been constructed (Bergkamp *et al.*, 1991; Bergkamp *et al.*, 1993b; Hong *et al.*, 2007; Nonklang *et al.*, 2008; Pecota *et al.*, 2007; Siekstele *et al.*, 1999). Multiple selectable markers are required if one wants to construct sequential gene knockouts or to complement mutants. This is being tackled by the adaption to *K. marxianus* of systems for recycling markers. The marker most amenable to this is *URA3* as it is possible to also counter-select this gene using 5-fluoro orotic acid (5-FOA). This trait was exploited initially in *S. cerevisiae* with a construct termed the ''*URA3* blaster'' (Alani *et al.*, 1987). This construct contains the *URA3* gene flanked by direct repeats (from *Salmonella hisG*) and following integration and selection for uracil prototrophy, spontaneous recombinants that have excised the *URA3* gene between the *hisG* repeats can be selected by their ability to grow on medium containing 5-FOA. A *K. marxianus* version of the *URA3* blaster was constructed and used to select for transformants carrying integrated DNA (Pecota *et al.*, 2007). Recombinants that lost the *URA3* gene were selected and a second round of integration was carried out, demonstrating the capacity to use the marker more than once. The second marker-recycling system that has been adapted for *K. marxianus* is the Cre–*lox*P system (Ribeiro *et al.*, 2007). This is also a system first developed in *S. cerevisiae* and adapted for other yeasts including *K. lactis* (Gueldener *et al.*, 2002; Sauer, 1996; Steensma and Ter Linde, 2001). Like the *URA3* blaster, the Cre–*lox*P system works by recombination, though in this case it is the Cre recombinase that mediates recombination between two *lox*P sites. These sites flank a selectable marker (typically *kan*MX) and in turn are flanked by homologous sequences to direct gene targeting. Following successful integration of the *lox*P-*kan*MX-*lox*P fragment into the target site, the Cre recombinase is then introduced into the transformed strain, catalyzing recombination between the *lox*P sites and excision and loss of the *kan*MX marker. This approach was successful in sequential disruption of two copies of the *LAC4* gene in *K. marxianus* CBS 6556 (Pecota *et al.*, 2007).

One of the real strengths of *S. cerevisae* is the range of well-characterized promoters that are available to drive expression of heterologous genes. This facilitates regulation of the timing and level of expression of genes of interest. The limited numbers of studies of heterologous gene expression in *K. marxianus* have used a variety of promoters. Several genes have been expressed using *S. cerevisiae* promoters such as *PGK1* (Ball *et al.*, 1999; Pecota *et al.*, 2007), *TDH3* (Nonklang *et al.*, 2009) or *GAL1* (Almeida *et al.*, 2003). Obviously, native *K. marxianus* promoters are also active, for example the inulinase promoter *INU1* (Bergkamp *et al.*, 1993a). An interesting approach was to screen a library of *K. marxianus* genomic fragments to identify candidate promoters (Ball *et al.*, 1999). A promoter, termed *PCPL3*, that showed a high level of constitutive expression, was cloned and characterized. A non-yeast promoter system that is of interest is the tetracycline promoter, which was shown to give very good on/off regulation in *S. cerevisiae* (Gari *et al.*, 1997). The basis of this ''tet-off'' promoter is that the presence of tetracycline prevents expression and this can be relieved by transferring the cells to tetracycline-free medium. This system was assessed in *K. marxianus* and found to work well, with an added advantage that expression was growth phase-dependent with high levels of expression only in the late-log and stationary phase (Pecota and Da Silva, 2005). This is potentially very useful for driving expression of heterologous secreted proteins for industrial applications.

To date, there are relatively few reports of the construction of engineered strains with new industrially relevant functionalities as most molecular studies focused on proof of concept and the development of tools. This is likely to change as there is greater take-up of the new molecular reagents described above. Already, strains expressing fungal enzymes have been constructed and shown to give *K. marxianus* the ability to grown on additional substrates that are of industrial significance (Bartkeviciute *et al.*, 2000; Bergkamp *et al.*, 1993a; Hong *et al.*, 2007; Nonklang *et al.*, 2008; Siekstele *et al.*, 1999). The study by Hong and co-workers was particularly significant for two reasons. First, it involved the simultaneous expression of several heterologous genes involved in cellulose degradation. Second, the genes involved encoded thermostable enzymes and so advantage was taken of the capacity of *K. marxianus* to grow and produce ethanol at higher temperatures (45 °C). Another recent study exploited the fact that *S. cerevisiae* genes typically function in *K. marxianus* to construct a strain expressing *S. cerevisiae* flocculation genes (Nonklang *et al.*, 2009). This illustrates the capacity to improve other aspects of a strain that are important for the industrial process.

6. Conclusions and future directions

The relative paucity of scientific publications on fundamental aspects of *K. marxianus* stands in contrast to its biotechnological potential and applications. It seems very likely that a greater depth of knowledge exists from research conducted in proprietary settings. The freedom of academic researchers to ask questions that are perhaps only loosed linked to short-term application, however, is very important to increase the knowledge base and the longer-term exploitability of this yeast. Since so many biotechnological products are linked to primary and secondary metabolism, an enhanced understanding of metabolic pathways in what is in reality more of a ''conventional'' yeast than *S. cerevisiae*, is certainly required. It is equally important to continue to develop molecular tools to facilitate genetic studies and to construct strains with new functionalities. One interesting question is whether the research field would be best served by choosing a ''model'' strain or strains on which to focus research, a strategy recently proposed (Fonseca *et al.*, 2008). There are some very clear advantages to this, especially the rapid progress that can be made when all studies can be integrated in a systems way without fear of strain variations confounding interpretations. In contrast perhaps, an alternative view is to consider *K. marxianus* primarily as a ''family of functional strains'', and to undertake studies on those strains that are or might be used for biotechnological applications. The advantage of that approach is that the knowledge gained is one step closer to application and it becomes easier to blur the interface

between basic and applied research, ironically perhaps returning yeast research to the ethos that inspired the work of Louis Pasteur.

Acknowledgements

We thank Pat Higgins, Paddy O'Reilly, colleagues in our group, and colleagues at Carbery Group, for supporting our research on *Kluyveromyces marxianus*. Yeast research in JM's group is funded by grants from Science Foundation Ireland (08-RFP-GEN1319), The Irish Department of Agriculture (06/RDC/506 and 06/RDC/459), and Enterprise Ireland (IP/2008/0559).

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