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Galactose utilization sheds new light on sugar metabolism in the sequenced strain Dekkera bruxellensis CBS 2499.

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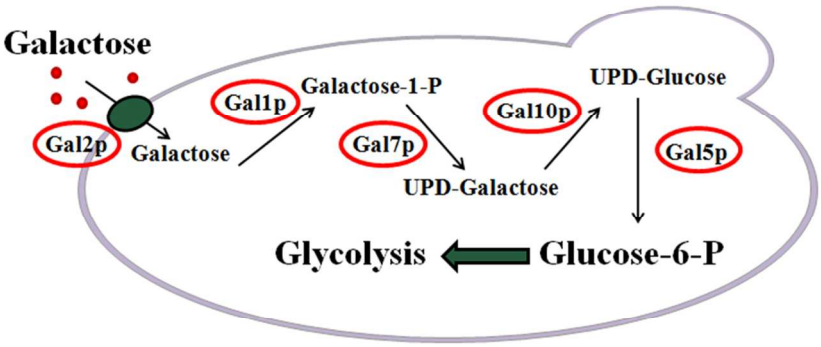

Dekkera bruxellensis and *Saccharomyces cerevisiae* share several industrially relevant traits, such as the ability to produce ethanol and to grow without oxygen. We show here that in *D. bruxellensis* CBS 2499 galactose is a not-fermentable carbon source and its metabolism is affected by the nitrogen source. The expression of genes involved in galactose utilization and in respiratory metabolism in *D. bruxellensis* is repressed by glucose, similarly to what occurs in *S. cerevisiae*.

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Galactose utilization sheds new light on sugar metabolism in the sequenced strain *Dekkera bruxellensis* CBS 2499.

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Abstract

Dekkera bruxellensis and *Saccharomyces cerevisiae* are considered two phylogenetically distant relatives, but they share several industrial relevant traits such as the ability to produce ethanol under aerobic conditions (Crabtree effect), high tolerance towards ethanol and acids, and ability to grow without oxygen. Beside a huge adaptability, *D. bruxellensis* exhibits a broader spectrum in utilization of carbon and nitrogen sources in comparison to *S. cerevisiae*. Whit the aim to better characterize its carbon source metabolism and regulation, the usage of galactose and the role that glucose plays on sugar metabolism was investigated in *D. bruxellensis* CBS 2499. The results indicate that in this yeast galactose is a non-fermentable carbon source, in contrast to *S. cerevisiae* that can ferment it. In particular, its metabolism is affected by the nitrogen source. Interestingly, *D. bruxellensis* CBS 2499 exhibits also the "short-term Crabtree effect", and the expression of genes involved in galactose utilization and in respiratory metabolism is repressed by glucose, similarly to what occurs in *S. cerevisiae*.

Introduction

Sugar metabolism provides an essential source of energy and metabolites for most organisms. To develop industrial strategies and processes based on cell as a factory the understanding of the metabolic pathways and their regulation is mandatory. Although glucose is the preferred sugar by microorganisms and the most abundant component of natural polysaccharides, the use of other sugars is becoming more and more attractive at industrial level to obtain cost efficient bioprocesses and to avoid interfering with the use of food crops for the production of chemicals. Nowadays, research efforts are in fact focused both to obtain monosaccharides from alternative sources than food and to develop microorganisms able to use all of them, hexoses and pentoses as well. In this regard, *Saccharomyces cerevisiae*, which is one of the most frequently used cell factory in industrial biotechnology, prefers glucose as carbon and energy source and is unable to use pentoses. In recent years, yeast strains capable of overcoming this limitation have been developed by metabolic

modifications (Van Vleet & Jeffries, 2009). Together with glucose, galactose is a component of hemicellulose and it is the major sugar in the red seaweed biomass, representing an attractive industrial carbon source (Packer, 2009). It utilization has been extensively studied in *S. cerevisiae* where it occurs through the Leloir pathway. In particular, galactose is phosphorylated to galactose-1-phosphate by galactokinase (Gal1p) and then isomerized to glucose-1-phosphate by galactose-1-phosphate uridyltransferase (Gal7p). Phosphoglucomutase (Gal5p) converts glucose-1-phosphate into glucose-6-phosphate which can be shunted to glycolysis and pentose phosphate pathway (Bhat & Murthy, 2001). Gal4p and Gal80p are the main transcriptional regulators of GAL genes that are repressed in presence of glucose by the Mig1-Tup1-Ssn6 complex (Nehlin *et al.*, 1991; Traven *et al.,* 2006). *S. cerevisiae* consumes galactose much more slowly compared to glucose and its metabolism is respire-fermentative, ethanol being one of the final products (Sierkstra *et al.,* 1993; Ostergaard *et al.,* 2000).

Another approach to extend the range of carbon sources that can be used at industrial level for biotech processes could be the exploration of the huge yeast biodiversity, in order to find out new species which already possess a wider range of carbon source utilization. The *Brettanomyces/Dekkera* complex is composed of five species, *Dekkera bruxellensis*, *D. anomala, Brettanomyces naardenensis, B. nanus* and *B. custersianus*. *D. bruxellensis* can be isolated from extreme environments like wine and beer (Fugelsang, 1997; Loureiro, 2003; Vanbeneden *et al.,* 2008), but its presence has been reported also in the continuous fermentation systems for bioethanol production, due to its ability to grow under anaerobic conditions and at high ethanol concentrations (de Souza Liberal *et al.,* 2007; Passoth *et al.,* 2007). Moreover, several *Brettanomyces/Dekkera* strains have been reported to be able to utilize pentose like xylose and arabinose (Toivola *et al.,* 1984; Galafassi *et al.,* 2011), possibly resulting from the high genetic variability observed in the species (Vigentini et al. 2012). Although *D. bruxellensis* and *S. cerevisiae* are considered two phylogenetically distant relatives they share several peculiar traits, such as the ability to produce ethanol under aerobic conditions (Crabtree effect), high tolerance towards ethanol and acids, and ability to grow without oxygen (van Dijken & Scheffers, 1986; Rozpędowska *et al.,* 2011; Blomqvist *et al.,* 2012). Apparently, these traits have evolved in parallel in both groups, but the molecular mechanisms involved could be very different (Rozpędowska *et al.,* 2011). Recently, it has been shown that *D. bruxellensis* can use also nitrate as nitrogen source, and this characteristic can render *D. bruxellensis* able to overcome *S. cerevisiae* populations, which is unable to use it, in industrial fermentations (de Barros Pita *et al.,* 2011; Galafassi *et al.,* 2013). All these metabolic

features have been leading to the idea that *D. bruxellensis* could become a new industrial producing organism (Blomqvist *et al.,* 2010; Galafassi *et al.,* 2011). The present work aimed to investigate how *D. bruxellensis* CBS 2499 can use galactose and the role that glucose plays on carbon source utilization. We show here that under controlled aerobic conditions galactose is a not-fermentable carbon source. Depending on the available nitrogen source (ammonium or nitrate), the pattern of final products obtained from galactose can change. We also show that the addition of glucose to cells growing through a respiratory galactose metabolism triggers the so called "short-term Crabtree effect". The expression of genes involved in different metabolic pathways linked to respiratory metabolism was also investigated.

Materials and Methods.

Yeast strains

The yeast used in this work is the sequenced strain of *D. bruxellensis* CBS 2499. Stocks of the strain were stored at -80 °C in 15% v/v glycerol.

Media and growth conditions

Shake flask cultures in the synthetic medium reported in Merico *et al.* 2007 incubated at 30 °C in a rotary shaker at 200 rpm were used to test the growth with specific carbon sources (glucose 20 g L^{-1} or galactose 20 g L^{-1} or ethanol 2% v/v). Aerobic batch cultivations were performed in a Biostat-Q system bioreactor (B-Braun) with a working volume of 0.8 L. The temperature was set at 30 °C, the stirring speed at 500 rpm, and the pH, measured by a Mettler Toledo pH electrode, was adjusted to 5.0 by automatic addition of 5M KOH. The fermenters were continuously sparged with air, 1 L min^{-1}, and the dissolved oxygen concentration (always higher than 30% of air saturation) was measured by a Mettler Toledo polarographic oxygen probe. For batch cultivations the synthetic medium was that reported above. Carbon sources were, as specified, glucose 20 g L^{-1}, galactose 20 g L^{-1} or ethanol 2% v/v. Nitrogen sources were ammonium sulfate 5 g L^{-1} or sodium nitrate 6.43 g L^{-1}, as specified. All the experiments were performed in duplicate. The growth was monitored by OD_{600nm} measurement.

Glucose "pulse" experiments

Glucose "pulse" experiments were performed in duplicate using batch cultures of cells growing on synthetic medium reported in Merico *et al.* 2007, and containing ammonium sulfate 5 g L^{-1} as nitrogen source. To obtain a population of cells growing in exponential phase, with a respiratory galactose metabolism and in presence of a lower concentration of residual galactose at the moment of the glucose addition, a concentration of 5 g L^{-1} galactose (instead of 20 g L^{-1}) was used for the pulse trials. When cells reached the exponential growth phase (approx. 6 OD_{600nm}), glucose was added to give a final concentration of 10 g L^{-1}.

Dry weight and metabolites assays

Samples collected at several points during the cultivation were submitted to the dry weight determination after removal of the medium by filtration (0.45 µm glass microfiber GF/A filter; Whatman). The filters were washed with three volumes of de-ionized water and dried overnight at 105 °C. Supernatants were used for glucose, acetic acid, ethanol and nitrate quantification using commercial enzymatic kits (Roche, cat. numb. 1 0716251 035, 1 0148261 035, 1 0176290 035 and 1 0905658 035). All the assays were performed in triplicate.

Enzymatic assays

Cell extracts for enzymatic assays were obtained by extraction with acid-washed glass beads (SIGMA) as described previously (Postma *et al.,* 1989), and total protein concentrations were determined by Bio-Rad kit no. 500-002 (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as standard. The specific activities of pyruvate decarboxylase (PDC), acetaldehyde dehydrogenase (ALD) and alcohol dehydrogenase (ADH) in cells extract were determined at room temperature in a spectrophotometer at 340 nm as previously described (Postma *et al.,* 1989).

RNA extraction and cDNA synthesis.

Pellets for RNA extraction were collected when cultures reached the exponential phase, which ensured that the gene expression analyses were performed from cells in the same physiological state. Harvested cells were centrifuged and pellets were immediately frozen with liquid nitrogen prior to store them at -80 °C until RNA extraction. RNA extraction was performed according to Presto™ Mini RNA Yeast Kit manufacturer's protocol with few changes. Pellets were disrupted with acid washed glass beads (500 µl RB Buffer, 5 µL β-mercaptoethanol and equal volume of glass beads) in a mini-bead beater (Precellys™ 24 homogenizer) instead of using zymolyase

enzymatic breakdown. The setting chosen for cell disruption was a run of three repetitions lasting 30 s at high speed. The RNA was quantified by means of a spectrophotometric method, and its integrity was evaluated by running an electrophoresis agarose gel in denaturing conditions. RNA was stored at -80 °C until cDNA synthesis. The cDNA was synthesized with QuantiTect® Reverse Transcription Kit (Qiagen) following the manufacturer's instructions. An amount of 1 µg total RNA was used for each reverse transcription reaction. Synthesized cDNA was stored at -20 °C until RT-qPCR assays.

Primers design

The sequences of target genes were obtained from the *D. bruxellensis* CBS 2499 database (DOE Joint Genome institute database, JGI; http://genome.jgi-psf.org/Dekbr2/Dekbr2.home.html) after BLASTx analysis using the corresponding potential orthologous sequences in *S. cerevisiae* genome retrieved from the SGD database (http://www.yeastgenome.org/), except for *AOX1* orthologous sequence that was obtained from the *Candida* genome database (http://www.candidagenome.org/). A list of the primers is reported in Table S1.

RT-qPCR analysis

RT-qPCR analyses were performed using SsoFast™ EvaGreen® Supermix (BIO-RAD). 96-well plates were used in a BioRad C1000™ Thermal Cycler machine, and each amplification reaction was composed of 7.5 µl of EvaGreen master mix, 1 µL of each primer (333 nmol L^{-1}, final), 2 µL of cDNA (5 times diluted cDNA synthesized from RNA), and 3.5 µL of MQ water. Cycling parameters were 98 °C for 30 s as hot-start, followed by 39 cycles of 95 °C for 3 s and 60 °C for 5 s, and melting curve was included at end of each run. Negative PCR control (for unspecific amplification) and negative RT control (for genomic DNA contamination) were run in parallel as internal control. Standard curves were created for each couple of primers by plotting CT (threshold cycle) values of the real-time PCR performed on dilution series of cDNA. From the standard curve, the amplification efficiency (E) was estimated by BioRadCFXManager software (BIO-RAD). The reaction specificity was evaluated by analyzing the melting curve profile. The α-tubulin gene was used as endogenous reference, previously used by Rozpędowska (Rozpędowska *et al.,* 2011), and cDNA samples from cells grown on glucose or ethanol were considered as the reference condition. For each growth condition, a total of 3 independent cDNA samples were prepared from two biological replicates. Each cDNA sample was run in technical triplicates during RT-qPCR assays.

Statistically significant differences of each gene expression among three growth conditions were analyzed by ANOVA test. The level of statistical significance was set at $p \leq 0.05$.

Promoter motif presence

The 1000 nt sequence upstream the translation start site of each gene was considered as the promoter sequence of the gene. A home-made python script was used to search the motif sequences presents in the promoters.

Phylogenetic analysis

Protein sequences of homologous genes among different species were aligned by MUSCLE Version 3.8.31. Trimal was used to trim the sequence alignment with the following parameter: -cons 50 –gt 0.5. PhyML was used to build the phylogenetic tree of each group of genes.

Results

Galactose metabolism is affected by nitrogen source

Preliminary cultivation on shake-flasks indicated the ability of several *D. bruxellensis* strains to grow on galactose-based media (data not shown). In the aim to study galactose metabolism in detail, aerobic cultures of the sequenced strain *D. bruxellensis* CBS 2499 (Piškur *et al.*, 2012) were performed in batch at controlled concentration of dissolved oxygen. Moreover, due to the fact that *D. bruxellensis* is able to use nitrate as nitrogen source (Galafassi *et al.,* 2013), we tested if the galactose metabolism can be affected by the nitrogen source. When *D. bruxellensis* was cultivated on media containing ammonium salts, galactose metabolism was respiratory, as indicated by the biomass level and by the failed production of metabolites normally resulting from fermentative metabolisms, such as ethanol or acetic acid (Fig. 1 A, Table 1). The growth kinetic showed a long lag phase when the pre-inoculum was performed on glucose-containing media (not shown), that was not present when the pre-inoculum grew on galactose-containing media (Fig. 1 A) This fact could indicate that in *D. bruxellensis* CBS 2499 the genes for galactose metabolism are repressed by glucose (see below). The growth rate was lower than on glucose (Table 1), and galactose was utilized at a lower specific rate (Table 1). On the other hand the biomass yield was higher, as

expected due to the respiratory metabolism exhibited in this condition, in comparison with the respire-fermentative one occurring on glucose-based media (Table 1).

Interestingly, when the medium contained nitrate as sole nitrogen source, the final products of galactose metabolism changed: together with biomass also acetic acid and a very low amount of ethanol were produced (Fig. 1 B, Table 1). As a consequence, a lower biomass yield than on ammonium-based media was obtained (Table 1). The rate at which galactose was consumed on nitrate-based media was higher than on the ammonium-based ones (Table 1), nevertheless the growth rates were very similar. Notably, acetic acid and ethanol were produced at lower rates and yields than on glucose. In conclusion, these results indicated that when cells use ammonium as nitrogen source the galactose metabolism is respiratory. Nevertheless, when nitrate is the nitrogen source a partial redirection of pyruvate occurred: some pyruvate was in fact converted by pyruvate decarboxylase to acetaldehyde, which was in turn converted to acetic acid and, at a low level, to ethanol.

Gene expression and enzyme activities for galactose and respiratory metabolism

The availability of the complete genome sequence of the *D. bruxellensis* CBS 2499 strain (Piškur *et al.*, 2012) allowed the identification of genes encoding all the enzymes that in *S. cerevisiae* are required for galactose assimilation (Table 2). Interestingly, *DbGAL7*, *DbGAL10* and *DbGAL1* are clustered, as reported in other *Ascomycota* (Martchenko *et al.,* 2007). A phylogenetic tree obtained using sequences identified by similarity with *S. cerevisiae* HXT and *C. albicans* HGT protein (Fig. 2) showed that the majority of *D. bruxellensis* putative sugar transporters map within the *C. albicans* group, but four of them are in the group containing also *S. cerevisiae* HXT. Two sequences, HXTA and HXTD, showed a high similarity to *Sc*Gal2p (53 – 56%). In *S. cerevisiae* galactose is sensed in the cytoplasm via Gal3p (Johnston, 1987), but no *GAL3* orthologue was found in *D. bruxellensis* CBS 2499 genome, as reported also in *C. albicans* (Martchenko *et al.,* 2007). On the other hand, in the analyzed genome, a gene encoding a putative protein which showed a 54% identity with CaHgt4p (Table S2) was found; this gene has been demonstrated to sense both glucose and galactose in *C. albicans* (Brown *et al.,* 2009).

The expression of genes involved in galactose and respiratory metabolism was analyzed, in order to understand the role that the carbon source plays on their transcriptional regulation. In *S. cerevisiae*

these genes are repressed by glucose (Johnston, 1999; Schüller, 2003; Daran-Lapujade *et al.,* 2004). The same pattern of regulation seems to work in *D. bruxellensis* CBS 2499 as well. Results from genes encoding putative sugar transporters showed that the expression of *HXTA* and *HXTD* was galactose-induced (Fig. 3 A). Also the expression of *DbGAL1, DbGAL7* and *DbGAL10* was strongly induced by galactose (Fig. 3 B). These latter genes were expressed even on ethanol and, by comparison, repressed by glucose (Fig. S1). The transcriptional analysis of genes for respiratory metabolism, such as those encoding TCA enzymes, cytochrome components, glyoxylate cycle and gluconeogenesis enzymes, showed that also in this yeast glucose can repress these pathways (Fig. 3 C, D, E, F). In the genome of *D. bruxellensis* CBS 2499 we identified a sequence with a high similarity to *C. albicans AOX1,* which encode an oxidase involved in mechanisms of alternative respiration (Huh & Kang, 1999). Interestingly, in the strain under study the expression of this gene was lower on galactose than on glucose (Fig. 3 C), despite the respiratory metabolism exhibited on the former sugar.

In order to understand if the respiratory galactose metabolism was due to a lack of enzymes involved in the fermentative pathway, the activity of pyruvate decarboxylase (PDC), acetaldehyde dehydrogenase (ACDH) and alcohol dehydrogenase (ADH) was tested. We found that PDC activity was lower on galactose than on glucose (0.25 U mg^{-1} and 0.48 U mg^{-1}, respectively). The activity of ACDH was higher on galactose than on glucose (1.2 U mg^{-1} and 0.51 U mg^{-1}, respectively), on the contrary ADH exhibited the same activity on both the carbon sources (2.9 U mg^{-1}).

Glucose addition triggers the "short-term Crabtree effect"

The observation that *D. bruxellensis* CBS 2499 metabolizes galactose by a respiratory way prompted us to study in this yeast the presence of the so called "short-term Crabtree effect", the immediate production of ethanol upon the addition of glucose to a culture growing through a respiratory metabolism. This phenomenon has been reported to occur in *S. cerevisiae*, but in some other Crabtree-positive yeasts the production of ethanol is delayed (Pronk *et al.,* 1996; Møller *et al*., 2002; Hagman *et al.,* 2013). To analyze this effect, cells growing in exponential phase on galactose were "pulsed" with glucose, and samples were collected in order to detect if the addition of glucose triggered aerobic fermentation. Ethanol production was detected just after 15 minutes from the glucose addition, and it continued to be produced all along the glucose consumption phase (Fig. 4).

Interestingly, also galactose continued to be slowly consumed for at least four hours, then stopped (Fig. 4). Acetic acid production started but delayed in respect to ethanol (Fig. 4). Glucose consumption resulted also in biomass production (Fig. 4). The calculated parameters like specific consumption/production rates and yields gave interesting information about the dynamic of glucose utilization during the pulse. Glucose consumption rate was in fact high in the first 30 minutes from the pulse (3.5 mmol g^{-1} h^{-1}), then decreased, resulting in a value of 2 mmol g^{-1} h^{-1}. This means that glucose was consumed at an higher rate than galactose, as observed also in the batch cultures performed on the single sugar (see Table 1). During the glucose pulse the ethanol production rate and yield increased, from 0.3 mmol g^{-1} h^{-1} in the first 30 minutes to 0.8 mmol g^{-1} h^{-1} after five hours and from 0.013 to 0.12 respectively, indicating that the fermentative pathway could start early (after 15 minutes), but other factors were required to reach a higher fermentative capacity.

Discussion

Recent studies have highlighted that *D. bruxellensis* shares with *S. cerevisiae* the ability to survive in challenging environments such as the industrial bioethanol production processes. Beside the adaptability to conditions of low pH and high ethanol concentration, *D. bruxellensis* exhibits a broader spectrum of consumable carbon and nitrogen sources in comparison to *S. cerevisiae*, having cellobiose and lactose as well as nitrate-assimilating capacity (Conterno *et al.,* 2006*;* Galafassi *et al.,* 2013), and this can contribute also to its ecological distribution. Here we showed that in *D. bruxellensis* CBS 2499 galactose is a not-fermentable carbon source (Table 1). This is in contrast to what occurs in *S. cerevisiae,* which produces ethanol even from galactose (Sierkstra *et al.,* 1993; Ostergaard *et al.,* 2000; Martinez *et al.,* 2014). Another interesting aspect of the galactose metabolism in *D. bruxellensis* CBS 2499 is that it is affected by the nature of the nitrogen source. When the cells used nitrate as nitrogen source galactose was metabolized leading to the production of biomass and acetic acid. The enzymes involved in nitrate assimilation, nitrate and nitrite reductases, in *D. bruxellensis* have been shown to use, *in vitro*, NADH and NADPH as electron donors (Galafassi *et al.,* 2013), and an increased production of acetic acid has been observed to occur on glucose-based media when nitrate is the sole nitrogen source (Galafassi *et al.,* 2013). This means that acetic acid synthesis can generate the NADPH necessary for nitrate assimilation, on glucose as well as on galactose. The presence of nitrate determined an increase in the galactose consumption rate (Table 1), that could be due to the higher energetic cost for the utilization of this

nitrogen source compared to the ammonium sulphate (Siverio, 2002). We suppose that the increased galactose consumption rate can also "push" pyruvate toward acetaldehyde and then to acetic acid and ethanol (this last produced in a very low amount).

The scenario that comes out from these new observations is that when the strain of *D. bruxellensis* under study is cultivated on galactose-based media it behaves like a Crabtree-negative yeast, not producing ethanol, in contrast to *S. cerevisiae*, that behaves like a Crabtree-positive yeast both on glucose and on galactose. This could be related to the galactose consumption rate: in *D. bruxellensis* we observed in fact that galactose was consumed at a slower rate than glucose (Table 1). We think that this factor could determine an insufficient pyruvate overflow, which is necessary to trigger aerobic ethanol production, as occurs in *S. cerevisiae* (Ostergaard *et al.,* 2000). The ability to produce ethanol aerobically can start when the glycolytic flow increase. The addition of glucose to a galactose-based culture determined a faster sugar consumption (glucose consumption rate 3.5 mmol g^{-1} h^{-1}) and a quick production of ethanol (Fig. 4), causing a shift in the metabolism from respiratory toward aerobic fermentative, the so-called "short-term Crabtree effect". The occurrence of the Crabtree effect has been already demonstrated in *D. bruxellensis* in batch cultures (van Dijken & Scheffers, 1986; Rozpędowska *et al.,* 2011), as well as by glucose pulse to glucose-limited continuous cultures growing at low rate with a respiratory metabolism (Leite *et al.,* 2013). In this study we show that the "short-term Crabtree effect" resulted from a high glycolytic flow occurring when the cells use glucose, which can in turn determine a pyruvate overflow. This is known to be one of the main causes that can activate the "short-term Crabtree effect" in *S. cerevisiae* (Postma *et al.*, 1989; Daran-Lapujade *et al.*, 2004; Huberts *et al.,* 2012). Furthermore, it has been demonstrated that by increasing the galactose consumption rate the ethanol production rate increases (Ostergaard *et al.,* 2000).

Glucose repression is the regulatory mechanism that has been shown to determine the "long-term Crabtree effect" in *S. cerevisiae* (Pronk *et al.,* 1996). The transcription of genes essential for respiratory metabolism and mitochondrial functions, as well as for the utilization of other carbon sources, is repressed when *S. cerevisiae* is cultivated on rapidly fermentable sugars like glucose, fructose and mannose (Verstrepen *et al.,* 2004). In particular, we focused our attention on the expression of genes containing in their promoters putative consensus sequences related to the transcription factor Mig1p (Table S3), which is known to be one of the key elements in glucose repression in *S. cerevisiae* (Lutfiyya *et al.,* 1998; Klein *et al.,* 1998; Westholm *et al.,* 2008). In the

genome of *D. bruxellensis* CBS 2499 two sequences that show 39-49% similarity with *S. cerevisiae MIG1* (notice that in *S. cerevisiae* genome the presence of three genes, *MIG1*, *MIG2* and *MIG3*, has been reported, Westholm *et al.,* 2008) were identified. In *C. albicans* CaMig1p has been shown to play a role in the regulation of carbon source utilization and energy production (Murad *et al.,* 2001). Our results indicated that genes involved in galactose utilization, TCA, cytochromes structure, glyoxylate cycle, and gluconeogenesis are repressed by glucose (Fig. 3). On the other hand, a sequence encoding a protein with a 44% identity to *Ca*Cph1p, which in *C. albicans* acts as a transcriptional factor required for the regulation of galactose metabolism (Martchenko *et al.,* 2007; Brown *et al.,* 2009), was present also in *D. bruxellensis* CBS 2499 genome (Table S2). Moreover, putative regulatory motifs recognized by this transcriptional factor were found in all the promoters of the galactose-related genes in the analyzed strain. (Table S2). Although the structural genes for galactose metabolism in *D. bruxellensis* CBS 2499 seems well conserved by evolution, the regulatory components appear to be also related to other species like *C. albicans* (Ihmels *et al.,* 2005). The molecular mechanisms operating in *D. bruxellensis* are still far to be elucidated, and it will be interesting in next future to demonstrate the specific role of different transcriptional factors.

In conclusion, the present work demonstrates that several metabolic implications are linked to the galactose metabolism in *D. bruxellensis*. Contrarily to *S. cerevisiae*, galactose is respired by cells. However, as far the glucose metabolism, this work suggests that a regulation operated by this sugar occurs. In particular, by proving the "short-term Crabtree effect" in *D. bruxellensis*, here is shown that this species behaves like *S. cerevisiae*, since to obtain aerobic ethanol production from sugars a glycolytic "threshold flow" needs to be reached.

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Figure 1: Growth kinetics of cultures on galactose-based media (one example of kinetic is showed) containing ammonium sulphate (A) and sodium nitrate (B) as nitrogen source. Symbols: ■, galactose; ♦, biomass; ●, acetate; ▲, ethanol.

Figure 2: A phylogenetic tree of the *S. cerevisiae* HXTp and the HGTp homologs of *C. albicans* and *D. bruxellensis.* The HXT homologs in *D. bruxellensis* are indicated by red box. Note that some *C. albicans* transporters map within the HXT group and that a majority of *D. bruxellensis* transporters map within the *C.albicans* HGT group.

Figure 3: Expression levels of *D. bruxellensis* CBS 2499 genes involved in different metabolic pathways. The transcription level on each carbon source is relative to its expression level on glucose. A: genes involved in hexoses (glucose and galactose) transport; B: genes involved in galactose metabolism; C: genes involved in respiratory pathway; D: TCA genes; E: genes involved in glyoxylate cycle; F: gene involved in gluconeogenesis. Errors bars are the standard deviation of three replicates. The level of statistical significance was set at $p \leq 0.05$.

Figure 4: Kinetic of a glucose "pulse" to a galactose-based culture (one experiment is showed). Time starts after glucose addition. Symbols: *, glucose; ■, galactose; ♦, biomass; ●, acetate and ▲, ethanol.

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Table 1. Growth parameters of cultivations on galactose-based media containing ammonium sulphate and sodium nitrate as nitrogen source. For comparison, the growth parameters calculated during cultivations on glucose-based media are indicated in brackets.

	q [mmol g DW^{-1} h^{-1}]				Yield [g g^{-1}]			μ [h^{-1}]
	Galactose (Glucose)	Ethanol	Acetate	Nitrate	Biomass	Ethanol	Acetate	
Ammonium	0.82-0.86* (3.6-3.7)[a]	n.d. (3.9 – 4.4)[a]	n.d. (0.62 – 0.070)[a]	- (-)[a]	0.497-0.501* (0.17 – 0.18)[a]	n.d. (0.320 – 0.335)[a]	n.d (0.058 – 0.060)[a]	0.0752-0.0755* (0.11 – 0.12)[a]
Nitrate	1.20-1.3* (2.94 ± 0.006)[b]	0.011-0.011* (1.650 ± 0.007)[b]	0.491-0.496* (1.83 ±0.009)[b]	0.466* (-)[b]	0.374-0.379* (0.19 ± 0.004)[b]	0.002* (0.133 ± 0.006)[b]	0.068-0.072* (0.216 ± 0.006)[b]	0.0714-0.073* (0.92 ± 0.006)[b]

*experiments performed in duplicate (the range of values reported)

[a] Data from Rozpędowska *et al.,* 2011

[b] Data from Galafassi *et al.,* 2013

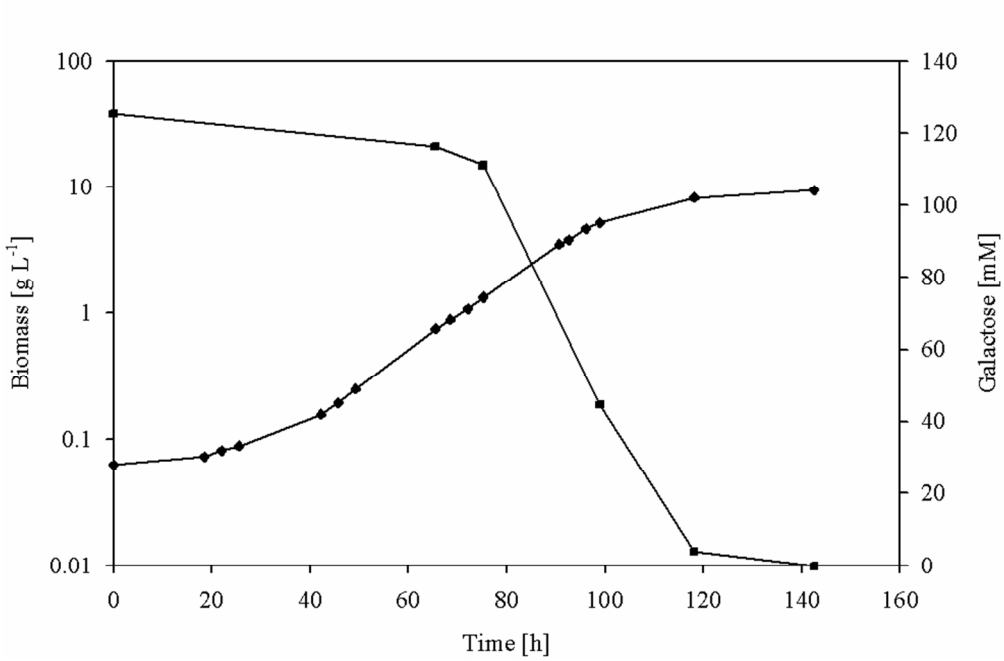
q: specific consumption/production rate

DW: dry weight

n.d.: not detectable

Table 2: Genes involved in galactose metabolism. For each protein analyzed, the function (Rubio-Texeira, 2005), the ID number (in http://genome.jgi-psf.org/Dekbr2/Dekbr2.home.html database), the identity with the related protein in *S. cerevisiae* (http://www.ncbi.nlm.nih.gov/) and the presence or absence of regulative motifs in promoter sequences of *D. bruxellensis* are indicated. Mismatches with the reference motif are underlined.

Gene	Function	ID number in *D.bruxellensis*	Identity (%)	***MIG1*** (C/G)(C/T)GG(G/A)G	***CPH1*** TGTAACGTT
ScGAL1	Galactokinase/Bifunctional sensor inducer	jgi|Dekbr2|8686|	61%	GTGGGG GCGGAG GTGGGG	**GA**TA**C**CGTT TT**T**TA**A**CGT**G**TGT**TG**C**T**TT TG**C**A**G**C**T**TT
ScGAL2	Galactose permease	jgi|Dekbr2|66929| jgi|Dekbr2|25434	56-57%	-	**G**GTAACGT**A**
ScGAL3	Galactose Sensor	no hit found	-	-	-
ScGAL4	Transcriptional activator	no hit found	-	-	-
ScGAL7	Galactose-1-phosphate uridylyltransferase	jgi|Dekbr2|26690|	66%	CTGGGG CCGGAG CCGGGG	TGTAA**A**GTT
ScGAL10	Uridine diphosphoglucose 4-epimerase	jgi|Dekbr2|26691|	63%	CTGGAG GTGGGG CCGGGG	**G**GTAAAGT**T**
ScGAL80	Gal4p repressor	no hit found	-	-	-



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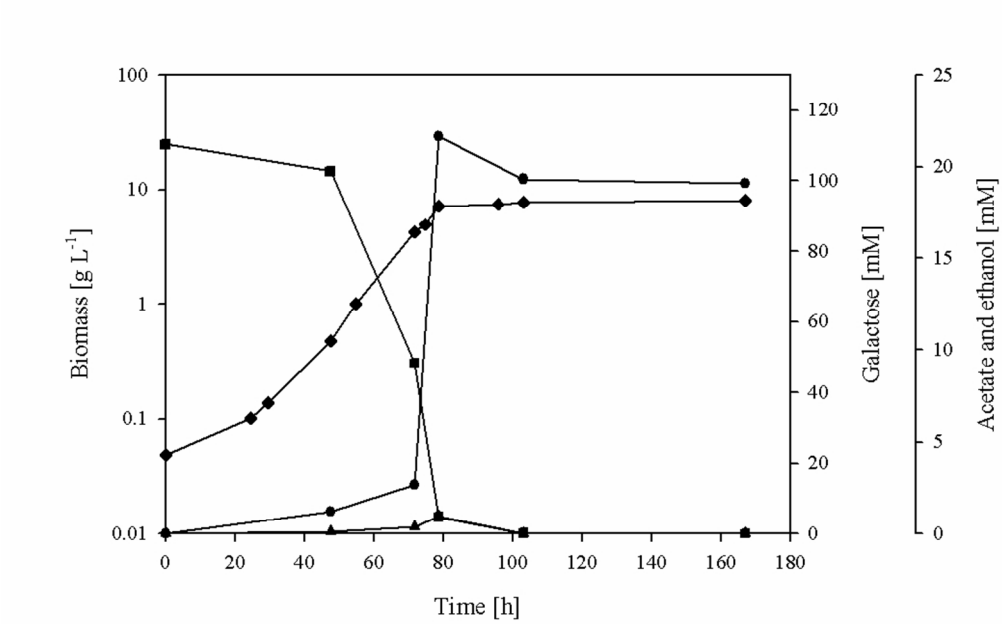

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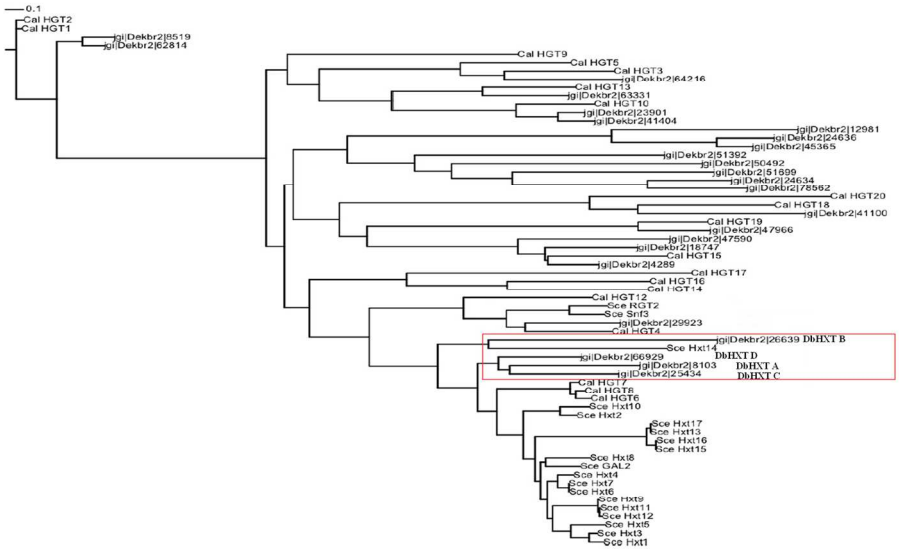

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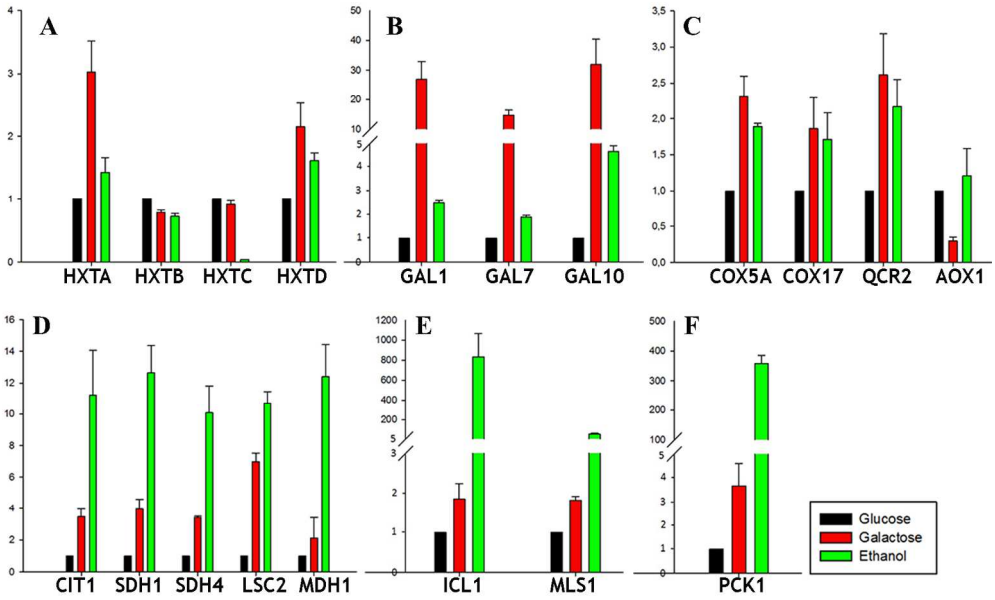

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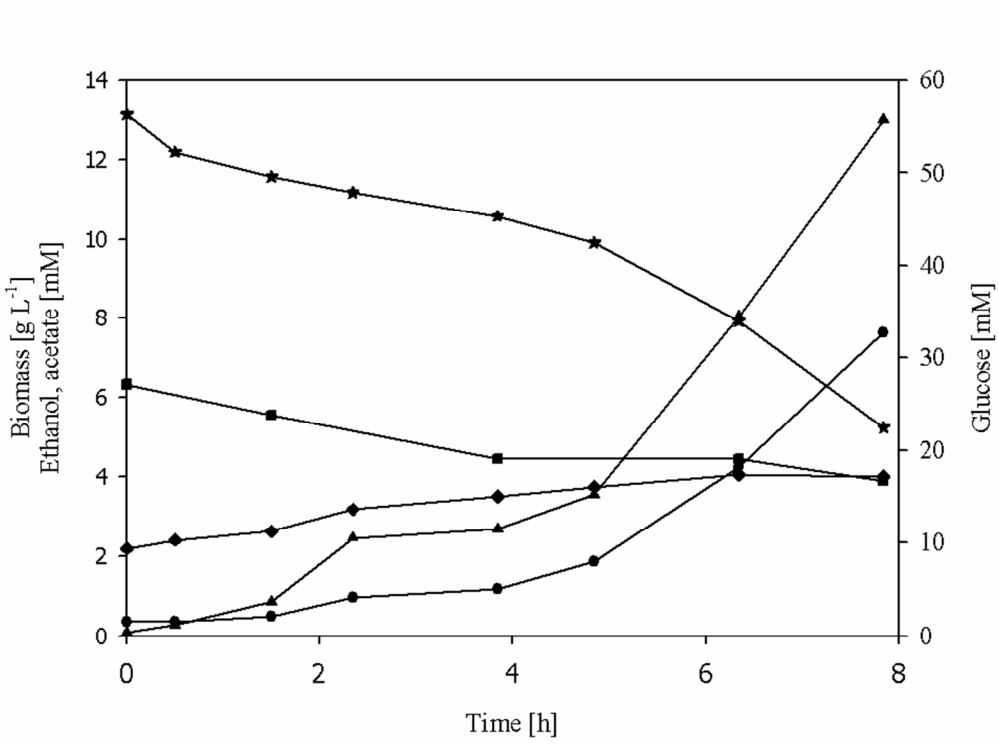

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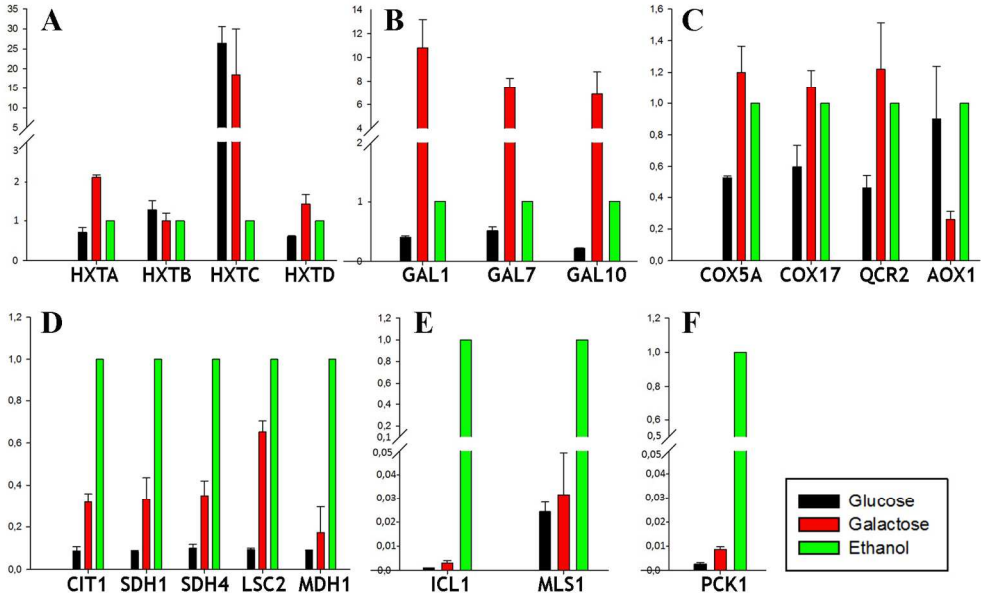
Supporting information list

Table S1: Primers used in this study

Table S2: Genes involved in galactose metabolism in *C. albicans*

Table S3: Protein ID numbers in *D. bruxellensis* CBS 2499 database.

Figure S1: Expression levels of *D. bruxellensis* CBS 2499 genes involved in different metabolic pathways. The transcription level on each sugar is relative to its expression level on ethanol.



335x206mm (150 x 150 DPI)

Table S1: Primers used in this study

Name	Sequence	Name	Sequence
CIT1 F	5′-CCACTTTGCATCCAATGGCAC-3′	*HXT*-B F	5′-CAAAACTGTTGGAGTGAACAACCC-3′
CIT1 R	5′-AGAGTTGGCAACTTCGCAAGC-3′	*HXT*-B R	5′-GTAAACACACACATTGTAAGAGCC-3′
SDH1 F	5′-ATGCGGTGAAAGTGCATGTGC-3′	*HXT*-C F	5′-GGAGCTGCACCAAACGATGC-3′
SDH1 R	5′-GACTCAAAGCCAATGTCTGCG-3′	*HXT*-C R	5′-ACACATCACACCAGCAAAAGCG-3′
SDH4 F	5′-TCACAGGGTCCAGACATTGG-3′	*HXT*-D F	5′-CAACTTCGCCTCTTCATTTATTGC-3′
SDH4 R	5′-CGATAATGCAACTTTCCCAGCC-3′	*HXT*-D R	5′-CTTGCTGATTAGGTTTGACACCG-3′
LSC2 F	5′-CTCCACCCCAAAGACCATCG-3′	*GAL1* F	5′-CTTTGGTTCATTTCACGACTGAGG-3′
LSC2 R	5′-GATGACAGGAACCTTCAAGCC-3′	*GAL1* R	5′-TTCACAATACAACTTCCAACTGCG-3′
MDH1 F	5′-GTGTTGCTGCTGATCTTTCGC-3′	*GAL7* F	5′-TGGATTGTTCCTGGTTCCACG-3′
MDH1 R	5′-CGTCTCTCGTCATTCCTGGC-3′	*GAL7* R	5′-CCAACTGTTGAAGTTTTGCAGCG-3′
COX5A F	5′-GTTGCTGGTCTTGGTATCTGC-3′	*GAL10* F	5′-AAATTGTTGGCCGTCGGAGTGG-3′
COX5A R	5′-CTGGGAGTAATGGCTGAATGG-3′	*GAL10* R	5′-CCTTGTGGATTCTTTGTGGTCC-3′
COX17 F	5′-TATGAAGAAAGATGACGGAAAGCC-3′	*ICL1* F	5′-TGATTTGCATAAGGGCATCTGC-3′
COX17 R	5′-CTTGAATCCATAACCAGCCATGC-3′	*ICL1* R	5′-GATGTCAGGATTCGTGCTTCC-3′
QCR2 F	5′-TCCCATACGAGGAAGCAGG-3′	*MLS1* F	5′-GATTCATGCTCTCCAACCTGG-3′
QCR2 R	5′-GGAAATTGCCGCCAAATAGCC-3′	*MLS1* R	5′-GCACAATCAGAACTGGAACACG-3′
AOX1 F	5′-CCTTACTTACACAAGATGCTTGC-3′	*PCK1* F	5′-GAAGTCGGAGTCACAGAACC-3′
AOX1 R	5′-CTTCTCTGTGCTTGGCCTCG-3′	*PCK1* R	5′-ATCCAGTATTGAGCAGCCAGG-3′
HXT-A F	5′-ATCACAGCCGCATTCTAGCAG-3′		
HXT-A R	5′-CCAAAGCGTTTAACAAAATCGTCC-3′		

Table S2: Genes involved in galactose metabolism in *C. albicans*. For each protein analyzed, the function (Rubio-Texeira, 2005), the ID number (in *D. bruxelesis* http://genome.jgi-psf.org/Dekbr2/Dekbr2.home.html database), the identity with the related protein in *C.albicans* (http://www.ncbi.nlm.nih.gov/) and the presence or absence of regulative motifs in the promoter sequences of *C. albicans* are indicated. Mismatches with the reference motif are underlined.

Gene	**Function**	**ID number in *D.bruxellensis***	**Identity (%)**	***MIG1*** (C/G)(C/T)GG(G/A)G	***CPH1*** TGTAACGTT
CaGAL1	Galactokinase	jgi|Dekbr2|8686|	51%	GTGGGG-CTGGAG-CCGGGG	TGTAACGTT
CaGAL2	Hexose transporter	jgi|Dekbr2|66929|	53%	GTGGAG	TGT**T**A**T**GTT -T**T**TA**T**CGTT
CaGAL3	Sensor	No hit found	-	-	-
CaGAL4			DNA binding site	-	T**T**T**TC**CGTT TGTAA**GA**T**A** TGTA**C**CG**AC** TGTAA**TTG**T **AAC**AACGTT T**T**T**T**AC**T**TT **AC**TAA**A**GTT - **CAC**AACGTT TATAA**TT**TT **C**GT**C**AC**C**TT
CaGAL7	Galactose-1-phosphate uridylyltransferase	jgi|Dekbr2|26690|	64%	-	TGTAACGTT
CaGAL10	Uridine diphosphoglucose 4-epimerase	jgi|Dekbr2|26691|	56%	CTGGAG	TGTAACGTT- TGTAACGTT
CaCPH1	Transcription factor	jgi|Dekbr2|29960|	44%	-	-

Table S3: Protein ID numbers in *D. bruxellensis* database (http://genome.jgi-psf.org/Dekbr2/Dekbr2.home.html) related to genes in *S. cerevisiae* (*AOX1* sequence from *C. albicans*) are listed and the presence or absence of regulative motifs in the promoter sequences is indicated. The percent of identity matrix was created by ClustalW2.

Gene (s)	Functions	Protein ID in *D. bruxellensis* database	Identity (%)	*MIG1* motif(s) (C/G)(C/T)GG(G/A)G
ScCIT1	Citrate synthase activity	7923	66	GCGGAG
ScSDH1	Flavin adenine dinucleotide binding, Succinate dehydrogenase (ubiquinone) activity	25329	70	CCGGGG; CTGGGG; GTGGGG;
ScSDH4	Contributes to succinate dehydrogenase (ubiquinone) activity	26333	57	CCGGAG; GTGGAG
ScLSC2	Succinate-CoA ligase (ADP-forming) activity	8258	65	CCGGGG; CTGGAG; GTGGAG
ScMDH1	L-malate dehydrogenase activity; mRNA binding	8132	65	CCGGAG; GCGGAG
CaAOX1	Alternative oxidase	4905	62	No hit
ScCOX5A	Contributes to cytochrome-c oxidase activity	40584	61	CTGGGG; CTGGAG; GCGGGG
ScCOX17	Copper chaperone activity	123777	67	GCGGGG
ScQCR2	Contributes to ubiquinol-cytochrome-c reductase activity	3933	55	CCGGGG; GCGGGG;
ScGAL1	Galactokinase activity	8686	61	GTGGGG; GCGGAG
ScGAL7	UDP-glucose:hexose-1-phosphate uridylyltransferase activity	26690	66	CCGGGG; CTGGGG; CCGGAG
ScGAL10	Aldose 1-epimerase activity; UDP-glucose 4-epimerase activity	26691	63	CCGGGG; CTGGAG; GTGGGG
ScICL1	Isocitrate lyase activity	29922	671	CCGGGG
ScMLS1	Malate synthase activity	3634	63	GTGGGG; GTGGAG
ScPCK1	Phosphoenolpyruvate carboxykinase (ATP) activity	34770	67	CCGGAG; GTGGGG; GTGGAG
ScGAL2	Galactose and glucose transmembrane transporter activity	DbHXT A 25434	52	GTGGGG
		DbHXT B 26639	68	GCGGGG
		DbHXT C 8103	62	CCGGGG; CTGGAG; GTGGGG
		DbHXT D 66929	57	No hit
ScHXT1	Glucose, galactose, fructose, mannose, pentose	DbHXT A 25434	56	GTGGGG

transmembrane transport activity	DbHXT B 26639	53	GCGGGG
	DbHXT C 8103	80	CCGGGG; CTGGAG; GTGGGG
	DbHXT D 66929	61	No hit