Gene Responses to Oxygen Availability in *Kluyveromyces lactis*: an Insight on the Evolution of the Oxygen-Responding System in Yeast

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Abstract

The whole-genome duplication (WGD) may provide a basis for the emergence of the very characteristic life style of *Saccharomyces cerevisiae*—its fermentation-oriented physiology and its capacity of growing in anaerobiosis. Indeed, we found an over-representation of oxygen-responding genes in the ohnologs of *S. cerevisiae*. Many of these duplicated genes are present as aerobic/anaerobic pairs, and form a specialized system responding to changing oxygen availability. *HYP2/ANB1* and *COX5A/COX5B* are such gene pairs, and their unique orthologs in the ‘non-WGD’ *Kluyveromyces lactis* genome behaved like the aerobic versions of *S. cerevisiae*. ROX1 encodes a major oxygen-responding regulator in *S. cerevisiae*. The synteny, structural features and molecular function of putative *KlROX1* were shown to be different from that of ROX1. The transition from the *K. lactis*-type ROX1 to the *S. cerevisiae*-type ROX1 could link up with the development of anaerobes in the yeast evolution. Bioinformatics and stochastic analyses of the Rox1p-binding site (YYYATTGTTCTC) in the upstream sequences of the *S. cerevisiae* Rox1p-mediated genes and of the *K. lactis* orthologs also indicated that *K. lactis* lacks the specific gene system responding to oxygen limiting environment, which is present in the ‘post-WGD’ genome of *S. cerevisiae*. These data suggested that the oxygen-responding system was born for the specialized physiology of *S. cerevisiae*.

Introduction

The yeast *Saccharomyces cerevisiae*, as well as other *sensu stricto* *Saccharomyces* species and a few of their close relatives, has a characteristic physiology that is obviously different from that of other eukaryotic cells and even most yeast species. One of the specialized features of *S. cerevisiae* is its regulatory network responding to oxygen availability, which allows this species to live on an exclusively fermentative mode even in fully aerated conditions, and also to grow vigorously in the complete absence of oxygen. Such a particular life style must rely on rapid and efficient gene responses to oxygen availability. The regulation of the oxygen-responding system, including both aerobic and hypoxic(anaerobic) genes that function either in presence or in absence of oxygen molecules, has been extensively investigated in *S. cerevisiae*, and the major transcription activators and repressors such as Hap1p, Rox1p and Mot3p have been identified and characterized [1–6].

It has been proposed that a whole-genome duplication (WGD) event occurred after the separation of *S. cerevisiae* and *Kluyveromyces lactis* from a common ancestral ‘pre-WGD’ yeast [7–9]. Some recent analyses suggested that the WGD could provide the basis for the evolution that led to the special physiological properties of the modern *S. cerevisiae* yeast containing a ‘post-WGD’ genome [10,11]. For example, an increase in the number of some genes resulted in an enhanced glycolytic flux, which is necessary for the fermentation-oriented metabolism of *S. cerevisiae* [12]. This change was regarded as an outcome of the WGD event followed by a selection in glucose-rich environments [13].

Among massive duplicated genes of *S. cerevisiae* (most of which were formed by whole-genome duplication, and called ohnologs—a subset of paralogs), many gene pairs were combined into a network system specially involved in oxygen response. *HYP2/ANB1* and *COX5A/COX5B* are thought to be such gene pairs. While *HYP2* and *COX5A* work in aerobic condition, *ANB1* and *COX5B* function in hypoxic and anaerobic conditions [14–16]. This oxygen-dependent alternative expression pattern of aerobic versus hypoxic(anaerobic) genes can also be found in many other cases of the duplicated gene pairs, such as *CYC1/CYC7* [17], *PET9/AAAC3* [18], and *HMGI1/HMGI2* [19]. A trait shared with these gene pairs is that their hypoxic(anaerobic) partners are commonly under the repression of Rox1p in an oxygen-dependent
way [6]. Some hypoxic(anaerobic) genes such as ANB1 and HEM13 are also regulated by another repressor Mot3p [2,20–22].

As opposed to S. cerevisiae, K. lactis shows a quite different oxygen response. It is Crabtree effect-negative [10] and Kluyver effect-positive [23], like many other yeast species, whilst S. cerevisiae is not. Further, K. lactis contains a ‘non-WGD’ genome [24] in which there are sets of genes comparable to those of S. cerevisiae [25]. The genomic and physiological features of K. lactis suggest that this yeast may have retained some major characteristics of the oxygen response patterns of the ancestral genome that gave rise to S. cerevisiae and K. lactis. The regulation of a few genes involved in respiration and haem synthesis in K. lactis has been reported to be dependent on oxygen [26–28]. Our previous work revealed that the target genes and regulatory modes of the oxygen-dependent regulator KlHap1p in K. lactis were different from those of the S. cerevisiae Hap1p [29]. However, the information is still limited about the gene response to oxygen availability and, in particular, about its regulators in K. lactis. We therefore selected some K. lactis genes, which appeared equivalent to the well-known oxygen-responding genes in S. cerevisiae such as HEM13, HYPO/ANB1 and COX5A/COX5B, and investigated the gene response to oxygen in K. lactis, in an attempt to understand how the oxygen-responding system has evolved from a ‘pre-WGD’ to a ‘post-WGD’ genome.

Results

Oxygen-responding genes are over-represented in ohnologs of S. cerevisiae

It has been reported that hundreds of genes respond to oxygen in S. cerevisiae [30]. In this ‘post-WGD’ genome, 554 ohnolog pairs have been identified (http://wolfe.gen.tcd.ie/ygob/) [9,31–33]. To estimate the role of the ohnologs in oxygen response, we checked the expression of these genes under aerobic and anaerobic conditions according to the published microarray survey including 6020 ORFs [30] (also see http://transcriptome.ens.fr/ymgv/publi_desc.php?pub_id=34). The results are included in Supplementary Table S1. With respect to a factor of 1.5, 2 or 3 in the change of the gene expression level between aerobic and anaerobic conditions, a significantly higher proportion of genes responding to oxygen were always found in ohnologs than the genes scattered over the whole genome. This is the case for both up- and down-regulated genes (Supplementary Table S2-A). The results indicated that oxygen-responding genes arose from ohnologs more frequently than from non-ohnologs (Supplementary Table S2-B) in the evolution of the S. cerevisiae genome. For example, considering a factor of 2, 18.7% of ohnologs were oxygen responsive, but only 10.4% in non-ohnologs (Figure 1). The former is 78.9% higher than the latter (P<0.05). There are 174 ohnolog pairs among 554, in which at least one gene has oxygen response, indicating that nearly one third of genes (31.4%) became involve in response to oxygen availability after the WGD event. These data suggested a connection between the formation of efficient oxygen-responding system in S. cerevisiae and the whole-genome duplication event, which has been previously discussed [34,35].

Aerobic/hypoxic(anaerobic) gene pairs in S. cerevisiae usually appear unique in K. lactis

There are many aerobic/hypoxic(anaerobic) paralog pairs in S. cerevisiae, such as HYPO/ANB1 and COX5A/COX5B, [3,30,36]. Either gene in each pair functions differently when environmental oxygen concentration changes, constituting an optimized oxygen response network. Most of these paralogs are in the list of ohnolog pairs, and might be formed as an outcome of selection for oxygen response after the whole-genome duplication. There are also a few exceptions among the aerobic/hypoxic(anaerobic) paralog pairs. For example, OYE3/OYE2 [37] and AAC1/AAC3 (a triplet including PET9/AAC2) [18] could derive from individual gene duplication. Sequence similarity search revealed that such aerobic/hypoxic(anaerobic) pair mostly has only one ortholog in K. lactis (Table 1). We may ask how these singular genes behave to respond to oxygen availability in K. lactis and question whether K. lactis and S. cerevisiae share similar gene categories involved in oxygen response.

KIHME13, KIHYP2(ANB1) and KICOX5A(SB) show differential responses to oxygen availability in K. lactis

HEM13, HYPO/ANB1 and COX5A/COX5B are well-known genes responding to oxygen in S. cerevisiae [14,16,20,38–42]. These genes play important roles in heme synthesis, translational initiation and mitochondrial respiratory chain biogenesis, respectively. We therefore investigated the expression of their K. lactis orthologs under both aerobic condition and during the shift to hypoxic condition. The results of Northern hybridization are shown in Figure 2A and 2B.

HEM13 of S. cerevisiae encodes coproporphyrinogen III oxidase [43]. KIHME13, its counterpart in K. lactis, is a singleton gene. The transcript level was slightly increased under hypoxic condition, though not as much as that of HEM13 in S. cerevisiae. This response of KIHME13 was the same as that reported previously [29], in which cells were grown under hypoxic condition instead of here a shift from aerobic to hypoxic conditions.

Both HYPO/ANB1 and COX5A/COX5B, encoding translation initiation factor elf-5A [44] and subunit V of cytochrome c oxidase [45] respectively, are ohnolog pairs in S. cerevisiae [9,31–33]. Each of these two gene pairs has only one unique ortholog, KIHYP2(ANB1) and KICOX5A(SB) respectively, in the K. lactis genome. Analyses of sequence similarity (Table 1) and genomic context (http://wolfe.
expression of \[15\]. Therefore the singular \(c_{\text{e}}\) double deletion mutants of \(\text{cox}_{5\text{a}}\text{cox}_{5\text{b}}\) \(K.\, lactic\) may be a reason for its inability to grow in anaerobiosis. The necessary for viability of \(c_{\text{er}}\) unlike the hypoxic(anaerobic) which gene in the pair was closest to the \(\text{tcd}_{\text{e}}\) gene, however, could not decide \(Kl_{\text{HYP2}}\) like the aerobic genes \(Kl_{\text{HYP2}}\) by presence of an intron. The situation is the same for \(c_{\text{er}}\) sequence, but is more related to \(c_{\text{er}}\) example, \(Kl_{\text{COX5A}}\) was also reported for \(c_{\text{er}}\) meaning of this observation is unknown, but a similar phenomenon \(\text{NADPH oxidoreductase containing flavin mononucleotide (FMN)}\) \(\text{OYE3}\) \(\text{acyl-CoA:sterol acyltransferase (ARE2)}\) \(\text{POR1}\) \(\text{transcription factor of sterol biosynthesis or sterol uptake (ECM2)}\) \(\text{acyl-CoA:sterol acyltransferase (ARE2)}\) \(\text{NADPH oxidoreductase containing flavin mononucleotide (FMN) (OYE3)}\)

Table 1. Some examples of aerobic/hypoxic(anaerobic) paralog pairs in \(S.\, ceder\)ia and their orthologs in \(K.\, lacttis\).

| Function (annotation in \(S.\, ceder\)ia) | \(S.\, ceder\)ia | \(K.\, lacttis\) | Score bits | \(E\) value |
|----------------------------------------|-----------------|-----------------|-----------|-----------|
| cytochrome c                           | \(\text{CYC1}^a\) | \(KLLA0F16940g\) | 202       | \(7e^{-54}\) |
| translation initiation factor elf-5A    | \(\text{HYP2}^a\) | \(KLLA0E22286g\) | 281       | \(3e^{-77}\) |
| cytochrome c oxidase chain V            | \(\text{COX5}^a\) | \(KLLA0F3641g\) | 221       | \(4e^{-59}\) |
| mitochondrial inner membrane ADP/ATP translocator | \(\text{AAC1}^a\) | \(KLLA0E12353g\) | 429       | \(e^{-121}\) |
| 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA reductase) | \(\text{HMG1}^a\) | \(KLLA0B04642g\) | 1250      | \(0.0\) |
| mitochondrial matrix protein, scaffold of iron-sulfur cluster assembly | \(\text{ISU1}^a\) | \(KLLA0D07161g\) | 233       | \(9e^{-63}\) |
| ceramide synthase component             | \(\text{LAG1}^a\) | \(KLLA0B13497g\) | 482       | \(e^{-137}\) |
| suppressor of DNA polymerase mutations  | \(\text{PSF1}^a\) | \(KLLA0CD1716g\) | 427       | \(e^{-120}\) |
| protein disulfide isomerase             | \(\text{PDI1}^a\) | \(KLLA0CD1111g\) | 500       | \(e^{-142}\) |
| phosphoglucomutase                     | \(\text{PGM1}^a\) | \(KLLA0B12694g\) | 797       | \(0.0\) |
| mitochondrial porin (voltage-dependent anion channel) | \(\text{POR1}^a\) | \(KLLA0F03553g\) | 369       | \(e^{-103}\) |
| transcription factor of sterol biosynthesis or sterol uptake | \(\text{ECM2}^a\) | \(KLLA0A04169g\) | 423       | \(e^{-119}\) |
| acyl-CoA:sterol acyltransferase         | \(\text{ARE2}^a\) | \(KLLA0C09152g\) | 516       | \(e^{-147}\) |
| NADPH oxidoreductase containing flavin mononucleotide (FMN) | \(\text{OYE3}^a\) | \(KLLA0A09075g\) | 563       | \(e^{-161}\) |

1: BLAST search was carried out at Genolevures http://cbi.labri.fr/Genolevures/.
2: aerobic paralog; 3: hypoxic(anaerobic) paralog in \(S.\, ceder\)ia.

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genes.coldie/cgi/browser/ygob.pl?gene), however, could not decide which gene in the pair was closest to the \(K.\, lacttis\) ortholog. For example, \(KCOX5A(5B)\) shows more similarity to \(COX5A\) in sequence, but is more related to \(COX5B\) in terms of synten and by presence of an intron. The situation is the same for \(KHYC\). Northern hybridization showed that the transcription of both \(KHYC(ANBI)\) and \(KCOX5A(5B)\) was markedly reduced under hypoxic condition (Figure 2A and 2B), very much like the aerobic genes \(HYP2\) and \(COX5A\) in the \(S.\, ceder\)ia pairs, but unlike the hypoxic(anaerobic) \(ANBI\) and \(COX5B\). It is worth noting that \(KCOX5A(5B)\) showed two transcripts of different size. The meaning of this observation is unknown, but a similar phenomenon was also reported for \(KROX1\) [46,47] and \(HGT1\) [48].

Simultaneous deletion of both \(HYP2\) and \(ANBI\) is lethal for \(S.\, ceder\)ia [15]. Therefore the singular \(KHYC(ANBI)\) could be also necessary for viability of \(K.\, lacttis\). In this sense, the decreased expression of \(KHYC(ANBI)\) under oxygen limited conditions in \(K.\, lacttis\) may be a reason for its inability to grow in anaerobiosis. The \(cox_{5acox_{5b}}\) double deletion mutants of \(S.\, ceder\)ia are completely non-respiratory and only grow by fermentation [49]. Inactivation of respiration in \(K.\, lacttis\) (for example disruption of the single cytochrome c gene \(KCYC1\)) leads to severe growth defect even on glucose because of its limited fermentation capacity [50]. Thus, \(KCOX5A(5B)\) must be important for growth of \(K.\, lacttis\), even under aerobic condition. Therefore, the growth impact of a reduced \(KCOX5A(5B)\) expression under hypoxic condition could be another possible reason of the distinct oxygen response of \(K.\, lacttis\).

Analysis of putative \(ROX1\) orthologs suggests that there was a transition from the \(K.\, lacttis\)-type \(ROX1\) to the \(S.\, ceder\)ia-type \(ROX1\).

\(ROX1\) encodes a major repressor of the expression of many oxygen-responding genes such as \(HEM13\), \(ANBI\) and \(COX5B\) in \(S.\, ceder\)ia [6,51]. The differential oxygen responses of \(KHEM13\), \(KHYC(ANBI)\) and \(KCOX5A(5B)\) led us to question whether there exists a \(ROX1\) ortholog in \(K.\, lacttis\). By a BLAST search using the amino acid sequence of \(ROX1\), we found in the \(K.\, lacttis\) genome (Genolevures: http://www.
another gene that may be orthologous to ROX1 to oxygen availability in K. lactis. Cells were grown to OD_{600} = 4 at 28°C in complete YP glucose medium supplemented with ergosterol and Tween 80 under aerobic (O_2) condition. A half of the culture was used for hypoxic treatment (N_2) as described in the "Materials and Methods", and then incubated at 28°C for a further 6 hours. Total RNA was extracted and Northern hybridization was performed to probe KIHEM13, KIHYP2(ANB1) and KICOX5A(SB) transcripts (see Materials and Methods). Panel A: the K. lactis wild type MW270-7B and its isogenic mutant Δkrox1. Panel B: MW270-7B and its isogenic mutant Δkurno2. The RNA samples of MW270-7B in panels A and B were independent preparations. The rRNA was used for quantifying sample loads. 

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Figure 2. Response of KIHEM13, KIHYP2(ANB1) and KICOX5A(SB) to oxygen availability in K. lactis. The analysis of possible ROX1 orthologs found in various yeast species has raised the question whether the changes from the K. lactis-type ROX1 to the S. cerevisiae-type ROX1 accompany a functional alteration of these HMG-encoding genes. In order to see whether KIROX1 has a transcriptional repression function similar to that of the S. cerevisiae ROX1, a Δkrox1 null mutant was constructed and used in the investigation of oxygen response in K. lactis.

Northern hybridization showed that the krox1 mutation had no obvious effect in the expression of KIHEM13, KIHYP2(ANB1) and KICOX5A(SB), neither in aerobic nor hypoxic conditions (Figure 2A). Therefore, KIROX1 is not involved in the transcriptional repression of these three K. lactis genes that are orthologous to the well-known oxygen-responsing genes of S. cerevisiae.

KIROX1p is not functionally equivalent to Rox1p in the repression of HEM13

In S. cerevisiae, the repression of Rox1p on the HEM13 expression has been extensively investigated [6,38,51]. As reported, deletion of ROX1 derepressed the expression of HEM13 under aerobic growth (Figure 4A). The KIROX1 gene on a 2μ-based vector was then transformed into the S. cerevisiae Rox1p mutant, and expression of KIROX1p was confirmed by Western blot (data not shown). As compared to the empty vector, introduction of KIROX1 led to an activation rather than to a repression on HEM13 expression (Figure 4B), suggesting different molecular functions between KIROX1p and Rox1p.

Search for the Rox1p-binding site YYYATTGTTCTC in the upstream regions of the K. lactis genes orthologous to the S. cerevisiae Rox1p target genes

In S. cerevisiae, Rox1p controls its target genes through binding to cis-acting elements with consensus sequence YYYATTGTTCTC [6]. The HMG domain is responsible for the protein-DNA interaction [52,53] and its binding induces a topological change of 90° bending of DNA [54]. Up to now, other genes coding for HMG-containing proteins [Figure 3C] have been characterized in Schiz. pombe [55,56] and C. albicans [57–59]. They encode either a mating-type M-specific polypeptide or a repressor of filamentous growth, the functions of which are completely divergent from that of Rox1p in S. cerevisiae. Although not involved in oxygen response, both proteins of Schiz. pombe and C. albicans could bind specifically to the YYYATTGTTCTC site, and the binding was dependent on the HMG domain [56,59,60].
Since the HMG domain is well conserved in KlRox1p (Figure 3D), we may expect that it would possess some of the Rox1p functions. KlRox1p could activate, but not repress, the expression of HEM13 in the S. cerevisiae Δrox1 mutant (Figure 4). We therefore supposed that the K. lactis protein, like the HMG proteins of other yeast species, could bind the YYYYATTGTTCTC
were included. The rRNA was used for sample loading quantification. The plasmid carrying KlROX1 was used for the transformation of S. cerevisiae wild type W303-1B and its isogenic mutant.

Total RNA was extracted and Northern hybridization was performed to detect the difference in the expression of repressed genes mediated by Rox1p according the one-line sensitization to oxygen-responding genes. We then investigated the possible site in the upstream regions of the K. lactis genes, which are orthologous to the S. cerevisiae oxygen-responding genes mediated by Rox1p according the one-line sensitization to oxygen. When 1 mismatch was allowed, only 2 upstream regions were detected 1 site each in YGR035C and YGR035C. When 1 mismatch was allowed, only 2 upstream regions were detected. Seven sites perfectly matched with the YYYATTG-TTCTC motif present in 6 promoters of the K. lactis genes. When 1 mismatch was allowed, only 2 upstream regions were detected 1 site each in K. lactis, much less than in S. cerevisiae (13 promoters containing total 15 sites). The situation was similar when 2 mismatches were allowed (K. lactis 13 sites in 11 upstream regions, S. cerevisiae: 43 in 27) (see Table S3 and Figure 5A).

**KimOT3** is dispensable for the regulation of the **KlHEM13, KlHIP2**, and **KlCOX5A** in K. lactis

Our data above suggested that the Rox1p-mediated oxygen-responding system might not exist in K. lactis. Besides ROX1, MOT3 has also been reported to be involved in repressing a subset of hypoxic genes such as HEM13 and ANB1 in S. cerevisiae [20–22,51]. By BLAST searching the amino-acid sequence of the S. cerevisiae Mot3p, we found a candidate ortholog KLLA0E18645g (designated KlMOT3) in K. lactis, with a moderate E-value (7.6e−28). The similarity between KlMot3p and Mot3p is concentrated in the C-terminal part containing two Cys2-His2 zinc fingers. But the gene is not affected in the KlMOT3 null mutant was constructed to investigate its possible role in oxygen response of K. lactis. Northern hybridization indicated that the expression of KlHEM13, KlHIP2, and KlCOX5A was not affected in the KlMOT3 null mutant, under either aerobic or hypoxic conditions (Figure 2B). The result suggested that KlMOT3, as well as KlROX1, is not involved in the transcriptional repression of these three K. lactis genes expression and that KlMOT3 and Mot3p are not functional equivalents in their role of oxygen response.

**Discussion**

The pre-WGD genome of K. lactis might lack a specific gene system responding to oxygen-limiting environment. In S. cerevisiae, the transcriptional repressor Rox1p and its target genes such as **ANB1** and **COX5B** form a network devoted to respond to low oxygen environments. This repressor seems absent in K. lactis and the genes orthologous to **ANB1** and **COX5B** showed only a down-regulation response to low oxygen condition. Our results suggested that the Rox1p-mediated response to oxygen is not operating in this non-WGD yeast species. This proposition is consistent with a recent observation in which the hypoxic **AAC3**
**Figure 5. Occurrence of the Rox1p-binding site.** Panel A: Comparison of the upstream regions (1 kb) containing the site YYYATTGTTCTC between the Rox1p-mediated hypoxic genes of *S. cerevisiae* (green circle) and their orthologs of *K. lactis* (red square). Panel B: stochastic model about the presence of the YYYATTGTTCTC site in a system containing 37 1-kb DNA sequences. Series white, prefect match; series yellow, 1 mismatch allowed; series blue, 2 mismatches allowed. Green bars represent the probabilities corresponding to the situations in *S. cerevisiae* and red in *K. lactis*.

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gene of S. cerevisiae was expressed constitutively in K. lactis under both aerobic and hypoxic conditions [28].

The upstream YYYATTGTTCTC site connects the Rox1p target genes to the oxygen response in S. cerevisiae. We found that this site is much less abundant in the upstream regions of the forty-one K. lactis genes orthologous to the thirty-seven Rox1p target genes of S. cerevisiae. To evaluate the significance of such difference in the site occurrence, we calculated the stochastic probabilities for 5’-YYYATTGTTCTC site, suggesting that these K. lactis genes have not associated into a regulatory network which can respond synergistically to oxygen availability.

Appearance of the S. cerevisiae-type ROX1 gene could be a hallmark that links the ability of anaerobic growth in some yeasts

Some metabolites can be produced through either oxygen-dependent pathway or oxygen-independent pathway. Utilization of oxygen independent or less-dependent pathway is the reasonable choice for growth under oxygen limiting conditions. For example, NAD is synthesized from both tryptophan and niacin by the oxygen-requiring kynurenine pathway and the alternative oxygen-independent salvage pathway in S. cerevisiae. The salvage pathway has been proved to be necessary for the anaerobic growth for S. cerevisiae [61], but the variation in the modes of NAD acquisition in yeasts seems to have no direct link to the ability of anaerobic growth [62], since the salvage pathway for NAD synthesis exists in all yeast species including strict aerobes such as Y. lipolytica and Schiz. pombe (Table 2). However, it has been found that some metabolic processes are reconfigured to avoid dependence on oxygen-requiring reactions or respiration in the evolution towards anaerobic or hypoxic growth. For example, the enzyme dihydroorotate dehydrogenase (DHODes) in pyrimidine synthesis pathway was converted from a mitochondrial component into a cytoplasmic protein by horizontal gene transfer [63–66]. Also, birth of D AL gene cluster and loss of genes encoding urate oxidase (UOX) and urate permease (UAP) led a switch from urate to allantoin utilization [67]. These biochemical reorganizations in the yeast evolution would economize oxygen consumption to adapt the life under oxygen-poor or –depleted environments.

Our analyses indicated that the S. cerevisiae-type ROX1 is specific for anaerobes including S. castelli and S. kluveri [64,65,68] and it is absent in aerobes (Table 2). ROX1 and its target hypoxic genes form a specialized network that actively takes challenges of oxygen-limiting or -absent conditions.

Formation of the Rox1p-mediated hypoxic genes and whole-genome duplication

The transcription analyses in this work revealed the expression of some important genes for cell viability such as KlHYP2(ANBI) and KlROX1(5B) was significantly repressed by hypoxic condition in the pre-WGD species K. lactis. Yeast has to devise a gene version capable of hypoxic/anoxic expression in order to survive in the absence of oxygen. Among 37 targets of Rox1p, 16 (43.2%) genes have an homolog and 13 (35.1%) singular genes are located within the duplication blocks (Supplementary Table S3), suggesting that as high as 78% of Rox1p-mediated genes may originate from the whole-genome duplication event. The whole-genome duplication would provide a basis for the construction of a hypoxic/anoxic working system with many genes. After the duplication, acquisition or creation of Rox1p-binding site could render one gene of a duplicated pair to become a hypoxic/anoxic version (Figure 6). Ideally, the paired genes function concertedly under aerobic and hypoxic/anoxic conditions. The capability of anaerobic growth of S. cerevisiae can be understood as a consequence of the whole-genome duplication that allowed acquisition of a new physiological property.

Materials and Methods

Strains and media

Yeast strains are listed in Table 3. Yeast cells were routinely grown at 28°C in a complete YP medium (1% yeast extract, 1% peptone, and 2% glucose), or synthetic minimal medium (0.67% Yeast Nitrogen Base without amino acid, and 2% glucose) supplemented with auxotropic nutrients. The antibiotic G418 was added to the complete medium when required (200 µg ml⁻¹). The media for hypoxic growth were complemented with 30 µg ml⁻¹ of ergosterol and 0.2% Tween 80 (polyoxyethylene sorbitan monooleate), and the hypoxic condition was established through 5-minutes air evacuation and 5-minutes nitrogen filling, repeated three times in sealed flasks.

Gene disruption

Deletion of the S. cerevisiae ROX1 gene was performed by a PCR (polymerase chain reaction)-based one step disruption procedure. A disruption cassette containing a KanMX gene was PCR-amplified (primers: 5’-TACTAATACTTCTTCCACAAAGAGACCCAGTTGACAATTCAGAGTCGGCAGCGAG-GAT-3’ and 5’-ATAGTATATAATATATACGGGAAAGGAAAAATGGAAGGATAGGACGAGCGAGCAGT-3’), and transformed into the S. cerevisiae strain W303-1B. Correct integration was verified by Southern hybridization: the entire open reading frame of ROX1, exactly from the initiation codon ATG to the stop codon TGA, was deleted and replaced by the KanMX gene to result in a Δrox1 mutant. K. lactis Δklrox1 and Δklmot3 null mutants were constructed by “split-marker recombination” [69]. The DNA fragments corresponding to the upstream and downstream flanking regions of KlROX1 or KlMOT3 were amplified by PCR (2 pairs of primers for KlROX1: 5’-CGGGATCCATCGATCTTTCATCTACATCGCCG-3’ and 5’-CGGGATCCATCGGCAAAAATCTGGAATCAGACCATAC-3’ and 5’-CGGGATCCATCGGCAAAAATCTGGAATCAGACCATAC-3’ and 5’-CGGGATCCATCGGCAAAAATCTGGAATCAGACCATAC-3’), and cloned into pKA and pAN vectors [69] respectively. The resulting plasmids were co-transformed into the K. lactis strain MW270-7B. Expected structure of integration was confirmed by Southern hybridization: the sequences from the 7th codon to the 373rd codon of KlROX1 and from the 15th codon to the 429th codon of KlMOT3 were deleted and replaced by a KanMX selection marker respectively, to give a Δklrox1 mutant and a Δklmot3 mutant.
Cloning of the KlROX1 gene into the expression vector

The open reading frame of KlROX1 was amplified by PCR (primers: 5'-GGCGGGCCGCATGACGCTCCTCGGTTTGT-CATAGAC-3' and 5'-GGCTGCAGCTTTATTTTGGGAT-TGCTCT-3') from K. lactis genomic DNA, and inserted into the NotI-PstI site of a 2μ-based multi-copy vector pCM262 [70]. The resulting plasmid pCM262/KlROX1 contained a KlROX1-HA (a haemagglutinin epitope) fusion expression cassette.

Cell-free protein extraction and Western blot

Cells grown to an early stationary phase in minimal medium were harvested, washed and resuspended in a buffer containing 20 mM Tris-HCl, pH 8.0, 50 mM ammonium acetate, 2 mM EDTA and 2 mM phenylmethysulfonyl fluoride (PMSF). One volume of 20% trichloroacetic acid (TCA) was added. The cells were then disrupted with glass beads four times by a vigorous shaking for 1 minute followed by a cooling on ice for 1 minute. The mixture was centrifuged and the protein pellet was washed with acetone and dissolved in gel loading buffer. Proteins were separated in 10% SDS-polyacrylamide gels, transferred to Hybond-C Extra membrane (Amersham), and probed with specific monoclonal antibody 12CA5 for the HA epitope. The reacted protein band was visualized using the ECL chemiluminescence detection system (Amersham).

RNA extraction and Northern hybridization

Total RNA was isolated as described [71], fractionated on an agarose/formaldehyde denaturing gel, and immobilized onto a Hybond-N+ membrane (Amersham). Hybridization was performed at 65°C in a buffer containing 7% SDS, 0.5 M sodium phosphate buffer, pH 7.2, and 10 mM EDTA. Probes were synthesized by PCR (oligonucleotides for HEM13 amplification: 5'-GATCCAAGGAATCTTCCAAT-3' and 5'-TAACCATGAGGCAATGTTCCAG-3'; for KlHEM13: 5'-TTCCATTCGACTCACCAACTG-3' and 5'-TTCAAAAAGCTAACATGAGGGAACG-3'; for KlHYP2[ANBI]: 5'-CCAAACGCATTAAACAAATCA-3' and 5'-CTTCTTTCCATTTATCCAGGG-3'; for KlCOX5A[5B]: 5'-CCACTTTGCAATATGTCTGA-3' and 5'-AGAAGAGGAGGAGAATGCA-3'), and labelled with 32P using the ‘Ready to Go DNA Labelling Kit’ (Pharmacia).

Comparative genome analysis in yeasts

BLAST search was performed using tools implemented at the database websites of the yeast species (S. cerevisiae, Saccharomyces paradoxus, Saccharomyces mikatae, Saccharomyces bayanus, Saccharomyces castellii, Saccharomyces kloekii, and Kluyveromyces waltii: http://db.yeastgenome.org/cgi-bin/FUNGI/showAlign or/and http://seq.yeastgenome.org/cgi-bin/blast-fungal.pl?name; Candida glabrata, K. lactis, Debaryomyces hansenii and Yarrowia lipolytica: http://cbi.labri.fr/Genolevures/blast/index.php; Candida albicans: http://www.candidagenome.org/cgi-bin/unwrap-blast; Schizosaccharomyces pombe http://www.genedb.org/genedb/pombe/blast.jsp).

Synteny comparison was carried out manually or viewed at http://db.yeastgenome.org/cgi-bin/FUNGI/FungiMap?locus (S. cerevisiae, S. paradoxus, S. mikatae and S. bayanus) and http://wolfe.gen.tcd.ie/cgi/browsable/ygbh.pl?gene (S. cerevisiae, S. bayanus, C. glabrata, S. castellii, K. waltii, S. kloekii, and K. lactis).

Mathematical calculation

**Cumulative binomial distribution.** To determine the significance of the difference of the fraction of a certain property between two samples (i.e., datasets), we used the formula below to calculate the cumulative binomial distribution.
Table 3. Yeast strains used in this study.

| Yeast strains | Genotype | Source |
|---------------|----------|--------|
| K. lactis strains |            |        |
| MW270-7B      | MATa uraA1-1, leu2, metA1-1 | M. Wósolowski-Louvel, University of Lyon 1 |
| Yklnx1        | MW270-7B klnx1:KanMX | This work |
| Ykimot3       | MW270-7B klmot3:KanMX | This work |
| S. cerevisiae strains |           |        |
| W303-1B       | MATa ade2-1, leu2-3, ura3-1, trp1-1, his3-11, 3-15, can1-100 | R. Rothstein, Columbia University |
| Arox1         | W303-1B arox1:KanMX | This work |

calculate P-values:

\[ P(c \geq c_0) = \sum_{c = c_0}^{N} \binom{N}{c} \left( \frac{N!}{c!(N-c)!} \right) \frac{p}{N-c} \]

where \( N \) is the total number of genes in the testing sample, \( c \) is the number of genes with a specific property and \( c_0 \) is the number of observed genes with this property, and \( p \) is the probability of finding a gene with the same property randomly [picking from the entire genome] or in the control sample.

Stochastic model of the site YYYTTGGTTCCT.

According to a previous report about the DNA sequence requirements of the consensus Roxlp-binding site Y(Y/T)/(Y/T)G(Y)(T/A)C(Y/T)G(T/A)C(Y/T)G(T/A)C(Y/T)G(T/A)C(Y/T)G(T/A)(C/T), the sequence T1G(T)C11(T)G(T)A8 is absolutely required for the affinity of Roxlp binding; A11 can be relatively tolerated to substitution by T, T9 by C or A and Y3 by A; A11 and T3 can not be permitted to change at the same time. With these limitations of mismatches, we searched for the possible binding sites in the upstream sequences (1 kb) of both the thirty-seven Roxlp-target genes of S. cerevisiae and their forty-one orthologs of K. lactis (see Results). And we also calculated the stochastic probabilities that the site YYYTTGGTTCCTT appears in thirty-seven and forty-one 1-kb DNA sequences as described below:

Lemma 1 [72]: Let \( B_1, B_2, \ldots, B_N \) denote the events in some probability space. Then \( P(\bigcup_{i=1}^{N} B_i) \leq 2^{N-1} \sum_{1 \leq k_1 < \ldots < k_N \leq N} P(\bigcap_{i=1}^{N} B_{k_i}) \leq 2^{N-1} \sum_{1 \leq k_1 < \ldots < k_N \leq N} P(\bigcap_{i=1}^{N} B_{k_i}) \) holds for all \( 2 \leq 2k \leq N \), with the sum \( \sum_{1 \leq k_1 < \ldots < k_N \leq N} \) taken over all nonnegative integers with \( 1 \leq k_1 < \ldots < k_N \leq N \) and \( \sum_{1 \leq k_1 < \ldots < k_N \leq N} \).

Let \( L \) denote the string “Z1Z3Z5Z7Z9TTGGTTCZ1Z3Z5Z7Z9”, where Z1, Z3, Z5, Z7, Z9 ∈ {A, T, C, G}, Z3, Z5, Z7, Z9 ∈ {A, T, C, G}, and Z9 ∈ {A, T}.

Define \( f_1(Z_i) = \begin{cases} 0 & Z_i \in \{T,C\} \\ 1 & Z_i \in \{A,G\} \end{cases} \), \( f_2(Z_i) = \begin{cases} 0 & Z_i = A \\ 1 & Z_i = T \end{cases} \), \( f_3(Z_i) = \begin{cases} 0 & Z_i \in \{A,G\} \\ 1 & Z_i \in \{T,C\} \end{cases} \), \( f_4(Z_i) = \begin{cases} 0 & Z_i = A \\ 1 & Z_i = T \end{cases} \), \( f_5(Z_i) = \begin{cases} 0 & Z_i \in \{A,G\} \\ 1 & Z_i \in \{C,T\} \end{cases} \), \( f_6(Z_i) = \begin{cases} 0 & Z_i = C \\ 1 & Z_i \in \{A,G\} \end{cases} \), and \( f_7(Z_i) = \begin{cases} 0 & Z_i = C \\ 1 & Z_i \in \{A,T\} \end{cases} \).

DNA is double-stranded. For complementary strand, let \( L \) denote the string “Z1Z3Z5Z7Z9ACAATGGTTCZ1Z3Z5Z7Z9”, where Z1, Z3, Z5, Z7, Z9 ∈ {A, T, C, G}, Z3, Z5, Z7, Z9 ∈ {A, T, G} and Z9 ∈ {A, T}.

Define \( g_1(Z_i) = \begin{cases} 0 & Z_i \in \{A,G\} \\ 1 & Z_i \in \{T,C\} \end{cases} \), \( g_2(Z_i) = \begin{cases} 0 & Z_i \in \{A,G\} \\ 1 & Z_i \in \{T,C\} \end{cases} \), \( g_3(Z_i) = \begin{cases} 0 & Z_i \in \{A,G\} \\ 1 & Z_i = T \end{cases} \), \( g_4(Z_i) = \begin{cases} 0 & Z_i \in \{A,G\} \\ 1 & Z_i = T \end{cases} \), \( g_5(Z_i) = \begin{cases} 0 & Z_i \in \{A,G\} \\ 1 & Z_i = A \end{cases} \), \( g_6(Z_i) = \begin{cases} 0 & Z_i = G \\ 1 & Z_i \in \{A,T,C\} \end{cases} \), \( g_7(Z_i) = \begin{cases} 0 & Z_i = G \\ 1 & Z_i \in \{A,T,C\} \end{cases} \), \( g_8(Z_i) = \begin{cases} 0 & Z_i = G \\ 1 & Z_i \in \{A,T,C\} \end{cases} \).

Define \( S_{L_j} = \{ L_j | g_j(Z_i) \leq j, g_4(Z_i) + g_5(Z_i) \leq 1 \} \), \( j = 0, 1, 2 \) (perfect match, 1 mismatch allowed, 2 mismatches allowed).

Let \( S_{L_0} \) be the probability space consisting of all the character strings of length \( n \) on set \( \{A,T,C,G\} \) with equality probability. Now we can choose a string \( L_n \) from \( S_{L_0} \) at random. For \( j = 0, 1, 2 \), let \( B(j)(D(j)) \) be the event that the \( L_n \) contains substring \( S_{L_j} \) (\( S_{L_j} \)) and \( B(j)(D(j)) \) be the event that the substring of \( L_n \) consisting of \( i \) st character to \( (i+1) \) st character is \( S_{L_j} \) (\( S_{L_j} \)). Then \( P(B(j)) = P(B_j(\bigcup_{i=1}^{n} D(i))) \) and \( P(B(j)(D(j))) = P(\bigcup_{i=1}^{n} j(i)) \).

By Lemma 1, we can have that \( \sum_{i=1}^{n} P(B(i)) - \sum_{1 \leq k_1 < \ldots < k_n \leq n} P(\bigcap_{i=1}^{n} B_{k_i}) \leq P(B(j)) \leq \sum_{1 \leq k_1 < \ldots < k_n \leq n} P(\bigcap_{i=1}^{n} B_{k_i}) \),

By symmetry, \( P(B(j)) = P(D(j)) \).

Since \( P(B(j)(D(j))) = P(B(j)) + P(D(j)) \), we can know that \( \sum_{i=1}^{n} P(B(i)) - 2 \sum_{1 \leq k_1 < \ldots < k_n \leq n} P(\bigcap_{i=1}^{n} B_{k_i}) - P(\bigcup_{i=1}^{n} j(i)) \leq P(B(j)(D(j))) \leq 2 \sum_{i=1}^{n} P(B(i)) - P(B(j)) \).
Significance of distribution of oxygen-responding genes in the oohnologs of S. cerevisiae.

Table S1 List of oxygen-responding genes in the oohnologs of S. cerevisiae.

Table S2 Significance of distribution of oxygen-responding genes in the oohnologs of S. cerevisiae.

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4. Let n = 1000. If j = 0, 0.0000427432 ≤ P(B(0)∪D(0)) ≤ 0.0000431839, that is P(B(0)∪D(0)) = 0.0000429635 ± 2.2055e-07. If j = 1, 0.01451393 ≤ P(B(1)∪D(1)) ≤ 0.01461935, that is P(B(1)∪D(1)) = 0.01456684 ± 5.270803e-05. If j = 2, 0.09122358 ≤ P(B(2)∪D(2)) ≤ 0.09573317, that is, P(B(2)∪D(2)) = 0.09347838 ± 0.00225479.

Now we can choose m strings of length n = 1000 from Sn. For j = 0,1,2, let Xj be the number of the strings that contains substring SLj or SLj. Let pj = P(B(0)∪D(0)), then Xj ∼ B(m,pj), that is P(Xj = k) = \frac{m!}{k!(m-k)!} p^k(1-p)^{m-k}, where k = 0,1,2,...,m.

Suppporting Information

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Table S3 Number of YYYATTGTTCTC site in the upstream sequences of Rox1p-mediated genes in S. cerevisiae and of the orthologs in K. lactis.

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Author Contributions

Conceived and designed the experiments: WGB. Performed the experiments: ZAF YTL JPL YYL WGB. Analyzed the data: WGB. Wrote the paper: MFB WGB. Performed the mathematical analyses. Performed the mathematical analyses: AC.
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