1 **Re-oxidation of cytosolic NADH is a major contributor to the high oxygen requirements of the**
2 **thermotolerant yeast *Ogataea parapolymorpha* in oxygen-limited cultures**

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Abstract

Thermotolerance is an attractive feature for yeast-based industrial ethanol production. However, incompletely understood oxygen requirements of known thermotolerant yeasts are incompatible with process requirements. To study the magnitude and molecular basis of these oxygen requirements in the facultatively fermentative, thermotolerant yeast *Ogataea parapolymorpha*, chemostat studies were performed under defined oxygen-sufficient and oxygen-limited cultivation regimes. The minimum oxygen requirements of *O. parapolymorpha* were found to be at least an order of magnitude larger than those of the thermotolerant yeast *Kluyveromyces marxianus*. This high oxygen requirement coincided with absence of glycerol formation, which plays a key role in NADH reoxidation in oxygen-limited cultures of other facultatively fermentative yeasts. Co-feeding of acetoin, whose reduction to 2,3-butanediol can reoxidize cytosolic NADH, supported a 2.5-fold higher biomass concentration in oxygen-limited cultures. The apparent inability of *O. parapolymorpha* to produce glycerol correlated with absence of orthologs of the *S. cerevisiae* genes encoding glycerol-3P phosphatase (ScGPP1, ScGPP2). Glycerol production was observed in aerobic batch cultures of a strain in which genes including key enzymes in mitochondrial reoxidation of NADH were deleted. However, transcriptome analysis did not identify a clear candidate for the responsible phosphatase. Expression of ScGPD2, encoding NAD⁺-dependent glycerol-3P dehydrogenase, and ScGPP1 in *O. parapolymorpha* resulted in increased glycerol production in oxygen-limited chemostats, but glycerol production rates remained substantially lower than observed in *S. cerevisiae* and *K. marxianus*. These results identify a dependency on aerobic respiration for reoxidation of NADH generated in biosynthesis as a key factor in the unexpectedly high oxygen requirements of *O. parapolymorpha*.

Importance

Thermotolerant yeasts hold great potential for anaerobic fermentation processes but their application is so far hampered by incompletely understood oxygen requirements. Based on

quantitative physiological studies in oxygen-limited chemostat cultures, this study shows that the thermotolerant yeast *Ogataea parapolymorpha* has a much higher oxygen requirement than other, previously investigated facultatively fermentative yeasts. The large oxygen requirement of *O. parapolymorpha* was linked to an inability of oxygen-limited cultures to efficiently reoxidize NADH formed in biosynthetic processes by glycerol formation. These results provide a basis for reducing the oxygen requirements of *O. parapolymorpha* by targeted metabolic engineering. In addition, this study shows that diversity of oxygen requirements should be taken into account in selecting yeast species for application in anaerobic or oxygen-limited industrial processes.

Keywords: *Ogataea parapolymorpha*, anaerobic, glycerol metabolism, oxygen requirements, thermotolerance

Introduction

Microbial biotechnology offers promising options for replacing petrochemically produced chemicals by sustainable bio-based alternatives (1, 2). For example, microbial production of ethanol as a renewable transport fuel, based on plant carbohydrates as renewable feedstocks, is already applied on a large scale. The current annual production volume of 87 Mton ethanol (3) is almost exclusively produced with the yeast *Saccharomyces cerevisiae* (4, 5). In well-established ‘first-generation’ ethanol processes, this yeast converts readily fermentable sugars, derived from corn starch or sugar cane, at high rates, titers and yields (6). Use of engineered pentose-fermenting *S. cerevisiae* strains for conversion of pentose-containing lignocellulosic hydrolysates, generated from agricultural residues such as corn stover and sugar cane bagasse, is currently explored at industrial scale (4).

Economic viability of the microbial production of ethanol and other large-product-volume fermentation products requires near-theoretical product yields on carbohydrate feedstocks. These can only be obtained in the absence of respiration, and with minimum processing costs (4, 5). In practice, industrial ethanol fermentation is performed in large tanks, which readily become and remain anaerobic as a consequence of vigorous carbon dioxide production by fermenting yeast cells.

The popularity of *S. cerevisiae* in these processes is related to its high fermentation rates under anaerobic conditions, its high innate ethanol tolerance, its tolerance to low pH and increasingly also to its amenability to modern genome-editing techniques (7–10).

S. cerevisiae is a mesophilic yeast, which grows optimally at temperatures of approximately 35 °C (11). Ethanol fermentation at higher temperatures could reduce cooling costs of large-scale bioreactors and, moreover, enable improved integration of plant-polysaccharide hydrolysis with fermentation of the released monomeric sugars. Integration of enzymatic polysaccharide hydrolysis and fermentation (simultaneous saccharification and fermentation, SSF (12)) in a single unit operation could simplify processing and prevent feedback inhibition of polysaccharide hydrolysis by released monosaccharides (12, 13). Such an integration is especially relevant for second-generation bioethanol production, in which physically and/or chemically pre-treated lignocellulosic plant biomass is hydrolyzed to monomeric sugars by fungal hydrolases, which typically operate at temperatures of 50 – 80 °C (14, 15). However, the efficiency of yeast-based SSF processes is currently constrained by the limited temperature range of *S. cerevisiae*.

Since supra-optimal temperatures potentially affect all proteins in a cell (16), substantial extension of the temperature range of *S. cerevisiae* by metabolic engineering may prove to be an elusive target. Indeed, elegant adaptive laboratory evolution and metabolic engineering studies aimed at improving the thermotolerance of *S. cerevisiae*, e.g. by engineering its sterol composition, have only enabled modest improvements of its maximum growth temperature (17–19). Exploration of naturally thermotolerant, facultatively fermentative yeasts such as *Ogataea* sp. (*Hansenula* sp.)(20) and *Kluyveromyces marxianus*, whose temperature maxima can reach 50 °C (21, 22), appears to offer an attractive alternative approach. As is observed for the large majority of yeast species, these thermotolerant yeasts readily ferment sugars to ethanol under oxygen-limited conditions (23–25). However, with few exceptions, Saccharomycotina yeasts such as *O. parapolyomorpha* and *K. marxianus*, whose evolutionary history did not involve the whole-genome duplication event (WGD)

that shaped the genomes of *S. cerevisiae* and closely related species (26), cannot grow under strictly anaerobic conditions (23, 27). This inability is generally attributed to direct requirements for molecular oxygen and/or requirements for mitochondrial respiration in essential biosynthetic reactions and processes (28).

The molecular basis for the biosynthetic oxygen requirements has not been identified for all facultatively fermentative yeasts and can encompass processes such as sterol synthesis and/or sterol uptake, unsaturated fatty-acid synthesis, pyrimidine biosynthesis and synthesis of cofactors such as coenzyme A and pyridine nucleotides (28). In yeasts, molecular oxygen is required for unsaturated fatty acid synthesis due to involvement of the cytochrome-b5 Δ^9 -desaturase Ole1 (29). Similarly, involvement of multiple mono-oxygenases explains the requirement for synthesis of ergosterol (reviewed in (30)). Anaerobic cultures of *S. cerevisiae* are therefore routinely supplemented with ergosterol and Tween 80, an oleic acid ester that serves as source of unsaturated fatty acids (31). In most non-*Saccharomyces* yeasts, pyrimidine synthesis depends on a mitochondrial, respiration-coupled dihydro-orotate dehydrogenase (DHODase, Ura9). In contrast, *S. cerevisiae* only harbors a cytosolic DHODase (Ura1), which couples an oxygen- and respiration-independent dihydro-orotate oxidation to fumarate reduction (26, 32–34). *Kluyveromyces* sp. represent an evolutionary intermediate containing both cytosolic and mitochondrial DHODase variants, whose expression is regulated in response to oxygen availability (35). Development of metabolic engineering strategies for eliminating oxygen requirements of thermotolerant non-*Saccharomyces* yeasts requires elucidation of the underlying oxygen- and/or respiration-dependent biochemical reactions.

The goal of the present study was to investigate oxygen requirements of the thermotolerant yeast *O. parapolyomorpha*. To this end, we first characterized its physiological and transcriptional responses to oxygen limitation in chemostat cultures and compared previously reported data (35) for *K. marxianus* and *S. cerevisiae*. Co-feeding of acetoin was tested to explore a possible impact of cytosolic NADH oxidation on the observed physiology of *O. parapolyomorpha* under these conditions.

Subsequently, we analyzed the genome of *O. parapolymorpha* for the presence or absence of orthologs of genes that have been implicated in the (in)ability of other yeasts to grow anaerobically and, in particular, in the ability to produce glycerol as ‘redox sink’ for reoxidation of NADH formed in biosynthetic reactions. Glycerol metabolism in *O. parapolymorpha* was further explored by studying growth of a mutant strain in which key genes involved in mitochondrial respiratory-chain linked NADH oxidation had been deleted. Based on the results of these analyses, we explored metabolic engineering of redox metabolism in *O. parapolymorpha* by expressing *S. cerevisiae* genes involved in glycerol production.

Results

Oxygen requirements of *O. parapolymorpha* in oxygen-limited chemostat cultures.

To quantitatively assess biosynthetic oxygen requirements of *O. parapolymorpha*, physiological responses of the wild-type strain CBS11895 (DL-1)(36) were studied under two oxygenation regimes in chemostat cultures that were grown on glucose at a dilution rate of 0.10 h^{-1} (**Fig. 1**). Physiological parameters of *O. parapolymorpha* were compared to results obtained under the same cultivation conditions in a recent study on *S. cerevisiae* CEN.PK113-7D and *K. marxianus* CBS6556 (35).

In fully aerobic chemostat cultures sparged with air (0.5 L min^{-1}), growth of *O. parapolymorpha* was glucose limited and sugar dissimilation occurred exclusively via respiration, as indicated by a respiratory quotient (RQ) close to 1 (**Table 1**). The apparent biomass yield on glucose in these cultures was approximately 10 % higher than previously reported (37) due to co-consumption of ethanol, which was used as solvent for the anaerobic growth factor ergosterol. When oxygen availability was decreased by sparging with a mixture of N_2 and air (0.5 L min^{-1} , oxygen content 840 ppm), the apparent biomass yield on glucose in steady-state cultures was approximately four-fold lower than in fully aerobic cultures (0.15 g g^{-1} and 0.59 g g^{-1} , respectively, **Table 1**). Moreover, a high residual glucose concentration (15.9 g L^{-1}) indicated that growth was limited by oxygen rather than by glucose. Respiro-fermentative glucose metabolism in the oxygen-limited cultures was evident

from a respiratory quotient (RQ) of 10.7 and a specific ethanol-dissimilation rate of 4.8 mmol (g biomass)⁻¹ h⁻¹. In contrast to results obtained in the same cultivation system with *K. marxianus* and *S. cerevisiae* (35), reduction of the O₂ content of the inlet gas to < 0.5 ppm O₂ led to complete wash-out of the *O. parapolymorpha* chemostat cultures.

S. cerevisiae can grow anaerobically in media supplemented with B-type vitamins, sterols and a source of UFA (31, 38, 39). In *K. marxianus*, which lacks a functional sterol-uptake system, sterol synthesis was recently identified as the key oxygen-requiring process during growth in such media (35). Based on UFA and sterol contents of aerobically grown *S. cerevisiae* biomass, the required oxygen uptake rate for synthesis of these lipids at a specific growth rate of 0.10 h⁻¹ corresponds to approximately 0.01 mmol O₂ (g biomass)⁻¹ h⁻¹ (39). This requirement is 60-fold lower than the biomass-specific oxygen consumption rate of 0.60 mmol O₂ (g biomass)⁻¹ h⁻¹ observed in oxygen-limited cultures of *O. parapolymorpha*.

A comparison of oxygen-uptake and ethanol-production rates indicated that, in the oxygen-limited *O. parapolymorpha* cultures, approximately 3 % of the total consumed glucose by the oxygen-limited cultures was respired. In contrast, under the same oxygen-limited regime, chemostat cultures of *S. cerevisiae* and *K. marxianus* showed specific oxygen-consumption rates below 0.25 mmol (g biomass)⁻¹ h⁻¹, RQ values above 50 and very low residual glucose concentrations (**Table 1**)(35). For these two yeasts, the fraction of the total consumed glucose that was respired amounted to only approximately 0.5 %.

In comparison with oxygen-limited cultures of *K. marxianus* and *S. cerevisiae* (**Table 1**), those of *O. parapolymorpha* showed an over ten-fold lower biomass-specific rate of glycerol production. Glycerol formation plays an important role in anaerobic and severely oxygen-limited cultures of several yeasts by reoxidizing a surplus of cytosolic NADH formed in biosynthetic reactions (40–43). In fully anaerobic cultures of *S. cerevisiae* and in severely oxygen-limited cultures of *K. marxianus*, glycerol formation has been reported to be 7-12 mmol (gram biomass)⁻¹(35). At a dilution rate of

0.10 h⁻¹, reoxidation of an equivalent amount of NADH by aerobic respiration would require an uptake rate of 0.4 to 0.6 mmol O₂ g (biomass)⁻¹ h⁻¹, which closely corresponds with the observed oxygen consumption rate of the oxygen-limited *O. parapolymorpha* cultures.

In some yeast species, an insufficient capacity for glycerol production causes an inability to grow under severe oxygen limitation. This phenomenon, which is referred to as the 'Custers effect', can be complemented by supplementation of oxygen-limited cultures with acetoin, whose NADH-dependent reduction to 2,3-butanediol can replace glycerol formation as a cellular 'redox sink' (40, 44). Addition of acetoin to oxygen-limited chemostat cultures of *O. parapolymorpha* led to an increase of the steady-state biomass concentration from 0.62 to 1.57 g (biomass)⁻¹ L⁻¹ and metabolism became more fermentative, as indicated by a higher rate of ethanol production and higher RQ (**Table 3**).

Table 1 | Physiology of yeast strains in glucose-grown chemostat cultures under different aeration regimes. Cultures were grown at pH 6.0 on synthetic medium with urea as nitrogen source, with glucose concentrations in the feed of aerobic and oxygen-limited cultures of 7.5 and 20 g L⁻¹, respectively. Data for *K. marxianus* and *S. cerevisiae* were obtained from a previous study performed under identical conditions (35). Media were supplemented with the anaerobic growth factors ergosterol and Tween 80, except for aerobic cultures of *O. parapolyomorpha*, from which Tween 80 was omitted to prevent excessive foaming. Data are represented as average ± SEM of data obtained from independent chemostat cultures of each strain. Ethanol concentrations were corrected for evaporation (35). Negative and positive biomass-specific conversion rates (q) represent consumption and production rates, respectively; subscript x denotes biomass dry weight. Calculations not applicable due to co-consumption of ethanol (-) and concentrations below detection limit (B.D., concentrations < 0.01 mM) are indicated.

Yeast strain	<i>O. parapolyomorpha</i> CBS11895		<i>K. marxianus</i> CBS6556		<i>S. cerevisiae</i> CEN.PK113-7D	
O ₂ in inlet gas (ppm)	21·10 ⁴	840	21·10 ⁴	840	21·10 ⁴	840
Replicates	2	2	2	5	3	3
Biomass (g _x L ⁻¹)	4.33 ± 0.04	0.62 ± 0.01	3.79 ± 0.02	1.57 ± 0.10	4.22 ± 0.06	2.29 ± 0.04
Residual glucose (g L ⁻¹)	B.D.	15.92 ± 0.01	B.D.	0.10 ± 0.03	B.D.	0.07 ± 0.00
Biomass-specific rates						
Specific growth rate (h ⁻¹)	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00	0.11 ± 0.01	0.10 ± 0.00	0.10 ± 0.00
q _{glucose} (mmol g _x ⁻¹ h ⁻¹)	-0.94 ± 0.02	-3.67 ± 0.14	-1.05 ± 0.00	-7.46 ± 0.30	-0.95 ± 0.03	-4.59 ± 0.10
q _{ethanol} (mmol g _x ⁻¹ h ⁻¹)	-0.48 ± 0.04	4.75 ± 0.23	-0.52 ± 0.00	11.49 ± 0.44	-0.44 ± 0.03	7.48 ± 0.10
q _{glycerol} (mmol g _x ⁻¹ h ⁻¹)	B.D.	0.02 ± 0.00	B.D.	1.12 ± 0.06	B.D.	0.45 ± 0.01
q _{succinate} (mmol g _x ⁻¹ h ⁻¹)	B.D.	0.00 ± 0.00	B.D.	0.02 ± 0.00	B.D.	0.00 ± 0.00
q _{O₂} (mmol g _x ⁻¹ h ⁻¹)	-2.35 ± 0.08	-0.60 ± 0.01	-3.52 ± 0.05	-0.23 ± 0.02	-2.61 ± 0.11	-0.15 ± 0.01
q _{CO₂} (mmol g _x ⁻¹ h ⁻¹)	2.89 ± 0.12	6.42 ± 0.22	3.73 ± 0.03	10.5 ± 0.5	2.82 ± 0.10	7.91 ± 0.53
Stoichiometries						
RQ (qCO ₂ /qO ₂)	1.23 ± 0.09	10.7 ± 0.2	1.06 ± 0.01	49.3 ± 7.5	1.08 ± 0.02	52.2 ± 2.4
Y _{glycerol/x} (mmol g _x ⁻¹)	B.D.	0.24 ± 0.05	B.D.	10.7 ± 0.7	B.D.	4.66 ± 0.08
Y _{X/glucose} (g _x g ⁻¹)	0.59 ± 0.00	0.15 ± 0.01	0.53 ± 0.00	0.08 ± 0.01	0.57 ± 0.01	0.12 ± 0.00
Y _{ethanol/glucose} (g g ⁻¹)	-	0.33 ± 0.03	-	0.39 ± 0.00	-	0.42 ± 0.02
Y _{X/O₂} (g _x mmol ⁻¹)	0.04 ± 0.00	0.17 ± 0.00	0.03 ± 0.01	0.50 ± 0.08	0.04 ± 0.00	0.64 ± 0.03
Recoveries (out/in)						
Carbon (%)	100.3 ± 0.4	94.4 ± 4.4	100.5 ± 0.1	91.1 ± 2.0	99.9 ± 0.7	101.2 ± 3.3
Degree of reduction(%)	97.8 ± 0.5	92.0 ± 4.3	98.8 ± 0.1	98.4 ± 0.7	98.4 ± 0.7	100.1 ± 0.8

Absence of orthologs of *S. cerevisiae* NAD⁺-dependent glycerol-3P phosphatase in *Ogataea* species.

To study the molecular basis for the apparent absence of a functional glycerol pathway in the oxygen-limited *O. parapolymorpha* cultures, we investigated the presence of orthologs of *S. cerevisiae* *GPD1/2* and *GPP1/2*. These genes encode isoenzymes catalyzing the two key reactions of the *S. cerevisiae* glycerol pathway, NAD⁺-dependent glycerol-3P dehydrogenase and glycerol-3P phosphatase, respectively (45–47). A homology search in translated whole genome sequences of 16 strains of *Ogataea* species (48) revealed clear orthologs of Gpd, but no orthologs of Gpp were detected (**Fig. 1**). In this respect, *Ogataea* yeasts resembled the phylogenetically related genus *Brettanomyces* (syn. *Dekkera*) (**Fig. 1**) for which the occurrence of a Custers effect is well documented (49, 50). In the absence of an active glycerol-3P phosphatase, NAD⁺-dependent glycerol-3P dehydrogenase can still be involved in glycerolipid synthesis (51) and in the glycerol-3P shuttle, in which glycerol-3P is reoxidized to dihydroxyacetone phosphate by a mitochondrial, respiratory-chain-coupled glycerol-3P dehydrogenase (Gut2 in *S. cerevisiae*, (52–54), **Fig. 1**).

Figure 1. (A) Possible key reactions in glycerol metabolism and upper glycolysis in yeast. **(B)** Distribution of *S. cerevisiae* orthologs of structural genes encoding glycerol-3P dehydrogenase

(Gpd2), glycerol-3P phosphatase (Gpp1) and FAD-dependent mitochondrial glycerol-3P dehydrogenase (Gut2) in *Ogataea* sp., *Brettanomyces* (syn. *Dekkera bruxellensis*), *K. marxianus* and *S. cerevisiae*. Data represents homology search results with *S. cerevisiae* S288C proteins as queries against whole genome translated sequences, present (black) and absent (white) orthologs are indicated. Strains are mapped to the phylogenetic tree of *Saccharomycotina* yeasts (48).

Transcriptional responses of *O. parapolymorpha* to oxygen limitation.

To further investigate responses of *O. parapolymorpha* to oxygen limitation, transcriptome analyses were performed on chemostat cultures grown under the aerobic and micro-aerobic regimes described above. These transcriptome data were first used to refine the genome annotation of a *de novo* assembled genome sequence of *O. parapolymorpha* CBS11895 obtained from long-read sequence data (**Data availability**). Data analysis focused on *O. parapolymorpha* genes with orthologs in *S. cerevisiae* and *K. marxianus* (**Fig. 2**). Transcriptional responses to oxygen limitation in *O. parapolymorpha* were compared with previously obtained transcriptome datasets (35, 55) of the latter two facultatively fermentative yeasts, grown under aerobic and oxygen-limited conditions. A global comparison indicated large differences in the transcriptional responses to these cultivation conditions (**Fig. 2**). The only shared global transcriptional responses of *O. parapolymorpha*, *K. marxianus*, and *S. cerevisiae* were a downregulation, in the oxygen-limited cultures, of genes involved in metabolism of alternative non-glucose carbon sources and in isocitrate metabolism. These responses are in line with an oxygen dependency for the dissimilation of these substrates and for a key role of the tricarboxylic acid cycle in respiratory glucose metabolism, respectively. At first glance, the different global transcriptional responses of these three yeasts appear to indicate a completely different wiring of their oxygen-responsive transcriptional regulation networks. However, it should be noted that high residual glucose concentrations in the oxygen-limited *O. parapolymorpha* cultures (**Table 1**) may have influenced transcriptional regulation (56).

In contrast to *S. cerevisiae*, which showed downregulation of genes associated with sterol metabolism, *O. parapolymorpha* and *K. marxianus* showed upregulation of genes associated with this GO-term (based on GO-term enrichment analysis). Sterol biosynthesis requires molecular oxygen and, under anaerobic or oxygen-limited conditions, *S. cerevisiae* can acquire ergosterol from the media. *K. marxianus* and several other pre-WGD yeast species lack a functional sterol import system (35, 57). This analysis of the transcriptome response of *O. parapolymorpha* suggested that this is also the case for this yeast.

OpURA9, which encodes the respiratory-chain-linked dihydroorotate dehydrogenase of *O. polymorpha*, showed an upregulation under oxygen-limited conditions, while KmUra9 was downregulated (**Fig. 3**). This response is consistent with the absence of a respiration-independent ScUra1/KmUra1-type dihydroorotate dehydrogenase in *O. parapolymorpha*.

The importance of glycerol production for oxygen-limited growth of *S. cerevisiae* and *K. marxianus* was evident from a strong upregulation of *GPP1*, for which no ortholog was found in *O. parapolymorpha* (**Fig. 3**). This observation and the lack of a transcriptional response of the single *GPD* ortholog in *O. parapolymorpha* to oxygen limitation are in line with the conclusion that this yeast does not use glycerol formation as a redox sink during oxygen-limited growth.

Figure 2. | Genome-wide transcriptional responses of *O. parapolymorpha*, *K. marxianus* and *S. cerevisiae* to oxygen limitation. Chemostat cultures of *O. parapolymorpha* (opar) were grown at pH 6 on synthetic medium with urea as nitrogen source and either aerated with air (aerobic $21 \cdot 10^4$ ppm O_2 , regime 1), or with a mixture of N_2 and 840 ppm O_2 (micro-aerobic, regime 2). Glucose concentrations in the feed of aerobic and oxygen-limited cultures were 7.5 and 20 g L^{-1} , respectively. Data for *K. marxianus* (kmar) and *S. cerevisiae* (scer) were obtained from a previous study performed under identical conditions (35). **(A)** Comparison of gene-set enrichment analysis using GO-terms for biological processes in response to oxygen limitation (micro-aerobic vs. aerobic). Distinct directionalities were calculated with Piano (58) and indicated as distinct directional up (pddup), mixed directional up (pmdup), non-directional (pnd), mixed directional down (pmddn) and distinct directional down (pdddn). Hierarchical clustering was performed based on the frequency of enrichment of GO-terms in the three yeast strains. **(B)** Highlighted gene sets show similar log fold changes of orthologs in the three yeasts, **(C)** both in all three yeasts upregulated **(D)** both downregulated. **(E)** and **(F)** indicate *S. cerevisiae* genes that were found to be consistently **(E)** up-

253 regulated or (F) down-regulated in anaerobic chemostat cultures across four nutrient limitation
254 regimes (55).

are annotated with the corresponding *S. cerevisiae* ortholog name; absence of orthologs is indicated by grey dots. Abbreviations used; glucose-6-phosphate (G6P), fructose-6-phosphate (F6P), fructose-1,6-bisphosphate (F1,6P), dihydroxyacetone phosphate (DHAP), glycerol-3P (G3P), glyceraldehyde phosphate (GAP), inner-mitochondrial membrane (IM), outer-mitochondrial membrane (OM). Respiratory chain complexes are indicated by Roman numerals.

Glycerol production in aerobic cultures of an *O. parapolyomorpha* strain lacking mitochondrial glycerol-3P dehydrogenase and alternative NADH dehydrogenases.

The presence of orthologs of *S. cerevisiae* *GPD1/2* and *GUT2* in *O. parapolyomorpha* suggested possible involvement of a glycerol-3P shuttle (52) to respiratory oxidation of the cytosolic NADH derived from glycolysis and from biosynthetic reactions. To further investigate redox metabolism in *O. parapolyomorpha*, we studied growth and product formation in strain IMX2167. In this *O. parapolyomorpha* strain, *OpGUT2* as well as the genes encoding the three cytosol- and matrix-facing alternative mitochondrial NADH dehydrogenases were deleted (59).

In aerobic chemostat cultures grown at a dilution rate of 0.10 h^{-1} , conversion rates of strain IMX2167 did not substantially differ from those of the wild-type strain CBS11895 (**Table 2**). Apparently, as previously observed in aerobic cultures of corresponding mutant strains of *S. cerevisiae* (43), mechanisms such as an ethanol-acetaldehyde shuttle could compensate for the absence of mitochondrial glycerol-3P dehydrogenase and external NADH dehydrogenases. In strain IMX2167, in which also internal alternative NADH dehydrogenase was absent, such a shuttle mechanism could then couple reoxidation of cytosolic NAHD to the *O. parapolyomorpha* complex-I NADH dehydrogenase (43).

In contrast to the absence of a clear phenotype in the chemostat cultures, aerobic batch cultures of strain IMX2167 showed a lower specific growth rate than the wild-type strain CBS11895 (0.26 h^{-1} and 0.36 h^{-1} , respectively, **Table 2**). Glycerol production suggested that, despite the absence of an

ScGPP1 ortholog, *O. parapolymorpha* contains a phosphatase that can use glycerol-3P as a substrate. In the genome sequence of strain CBS11895, 24 genes were annotated with the GO-term 'phosphatase activity' (GO:0016791). However, although all these genes were transcribed (log Counts per million (CPM) > 3.5), none showed log FC > 2 between the wild-type and strain IMX2167 in either aerobic batch cultures or aerobic chemostat cultures (Table 2).

Table 2 | Physiology of *O. parapolymorpha* wild-type and mutant strains in glucose-grown aerobic batch and chemostat cultures. Aerobic chemostat cultures were grown on synthetic medium with and 20 g L⁻¹ glucose as carbon- and energy substrate. Batch cultures were grown on synthetic medium with a reduced 7.5 g L⁻¹ glucose concentration. Data are represented as average ± SEM of data obtained from independent chemostat cultures of each strain. Ethanol concentrations were corrected for evaporation. Positive and negative biomass-specific conversion rates (q) represent consumption and production rates, respectively; subscript x denotes biomass dry weight. N.D.: not determined values; and B.D.: compounds below detection limit (concentrations < 0.01 mM).

<i>O. parapolymorpha</i> strain	CBS11895	IMX2167	CBS11895	IMX2167
Relevant genotype	wt	<i>ndh1-3Δ gut2Δ</i>	wt	<i>ndh1-3Δ gut2Δ</i>
Replicates	2	2	2	2
Biomass-specific rates				
Specific growth rate (h ⁻¹)	0.36 ± 0.006	0.26 ± 0.00	0.10 ± 0.00	0.10 ± 0.00
q _{glucose} (mmol g _x h ⁻¹)	-3.90 ± 0.15	-2.73 ± 0.06	-1.08 ± 0.03	-1.04 ± 0.00
q _{ethanol} (mmol g _x h ⁻¹)	B.D.	0.22 ± 0.08	B.D.	B.D.
q _{glycerol} (mmol g _x h ⁻¹)	B.D.	0.34 ± 0.01	B.D.	B.D.
q _{O2} (mmol g _x h ⁻¹)	N.D.	-3.65 ± 0.05	-2.69 ± 0.05	-2.14 ± 0.00
q _{CO2} (mmol g _x h ⁻¹)	N.D.	4.34 ± 0.01	2.82 ± 0.03	2.25 ± 0.00
Stoichiometries				
RQ (q _{CO2} /q _{O2})	N.D.	1.19 ± 0.02	1.05 ± 0.01	1.05 ± 0.00
Y _{X/glucose} (g _x g ⁻¹)	0.52 ± 0.01	0.51 ± 0.01	0.51 ± 0.00	0.52 ± 0.00
Y _{X/O2} (g _x mmol ⁻¹)	N.D.	0.07 ± 0.00	0.04 ± 0.00	0.05 ± 0.00
Recoveries (out/in)				
Carbon (%)	N.D.	97.8 ± 1.0	99.3 ± 1.2	98.7 ± 0.3

Metabolic engineering of glycerol metabolism in *O. parapolymorpha*.

Based on analysis of the *O. parapolymorpha* genome (**Fig. 1**), we investigated whether expression of the *S. cerevisiae* glycerol-3P phosphatase ScGpp1 in *O. parapolymorpha* supported glycerol production by oxygen-limited cultures of *O. parapolymorpha*. To this end, an expression cassette in which the coding region of *ScGPP1* was expressed from the *OpPMA1* promoter was integrated into the genome of *O. parapolymorpha* CBS11895. In oxygen-limited chemostat cultures, the resulting engineered strain IMX2119 showed a 9-fold higher biomass-specific rate of glycerol formation than observed in the wild-type strain ($0.18 \text{ mmol (g biomass)}^{-1} \text{ h}^{-1}$ and $0.02 \text{ mmol (g biomass)}^{-1} \text{ h}^{-1}$, respectively, **Table 3, Fig. 4A**). A further increase of the glycerol production rate to $0.22 \text{ mmol (g biomass)}^{-1} \text{ h}^{-1}$ was observed when, in addition to the expression cassette for *ScGPP1*, a second expression cassette was integrated in which *ScGPD2* was expressed from the *OpTEF1* promoter (strain IMX2588, **Table 3, Fig. 4A**). Integration of only the *ScGPD2* cassette (strain IMX2587) did not lead to a significantly higher rate of glycerol production in oxygen-limited cultures than observed in the wild-type strain (**Table 3**).

The higher biomass-specific rates of glycerol production by the *ScGPP1* and *ScGPP1/ScGPD2* expressing *O. parapolymorpha* strains enabled higher biomass yields on oxygen under oxygen-limited conditions (0.24 and $0.32 \text{ g biomass mmol O}_2^{-1}$, respectively, versus $0.17 \text{ g biomass mmol O}_2^{-1}$ for the wild-type strain, **Table 3 and Fig. 3B**). A shift towards a more fermentative metabolism was, in addition, evident from the RQ values of strains IMX2119 and IMX2588 (14.4 and 17.4 , respectively), which were significantly higher than those of corresponding cultures of the wild-type strain (RQ of 10.7 , **Table 3**).

In fully anaerobic chemostat cultures of *S. cerevisiae* CEN.PK113-7D grown at a dilution rate of 0.10 h^{-1} , a biomass-specific rate of glycerol production of $0.67 \text{ mmol (g biomass)}^{-1} \text{ h}^{-1}$ has been reported (**Fig. 4A**) (35, 60). An even higher rate of glycerol production ($1.1 \text{ mmol (g biomass)}^{-1} \text{ h}^{-1}$) has been reported for strains of another *S. cerevisiae* lineage grown under these conditions (41, 61). Assuming that biomass composition and biosynthetic pathways in *S. cerevisiae* CEN.PK113-7D and *O.*

parapolyomorpha CBS11895 lead to a similar net generation of NADH from biosynthetic reactions, the glycerol production rate of the *ScGPP1/ScGPD2* expressing *O. parapolyomorpha* strain IMX2588 was approximately four-fold lower than required for reoxidation of all NADH generated in biosynthetic processes. A limiting capacity of the engineered glycerol pathway in oxygen-limited cultures of strain IMX2588 was also indicated by residual glucose concentrations, which were higher than in cultures of the wild-type strain CBS11895 supplemented with acetoin (Table 3).

Table 3 | Physiology of *O. parapolyomorpha* strains expressing *S. cerevisiae* glycerol pathway genes in glucose-grown oxygen-limited chemostat cultures. Cultures were grown at pH 6.0 on synthetic medium with urea as nitrogen source and 20 g L⁻¹ glucose as carbon and energy substrate, media contained anaerobic growth factors ergosterol and Tween 80. Data are represented as average ± SEM of data obtained from independent chemostat cultures of each strain. Ethanol concentrations were corrected for evaporation (35). Positive and negative biomass-specific conversion rates (q) represent consumption and production rates, respectively; subscript x denotes biomass dry weight. B.D.: concentrations below detection limit (concentration < 0.01 mM).

Yeast strain	CBS11895	IMX2119	IMX2587	IMX2588	CBS11895
Relevant genotype	wt	<i>ScGPP1</i>	<i>ScGPD2</i>	<i>ScGPP1</i> <i>ScGPD2</i>	wt
O ₂ in inlet gas (ppm)	840	840	840	840	840
Acetoin	-	-	-	-	yes
Replicates	2	3	2	3	3
Biomass (g _x L ⁻¹)	0.62 ± 0.01	0.91 ± 0.03	0.66 ± 0.06	0.86 ± 0.05	1.57 ± 0.10
Residual glucose (g L ⁻¹)	15.92 ± 0.01	14.33 ± 0.35	15.71 ± 0.40	14.98 ± 0.37	9.41 ± 0.24
Biomass-specific rates					
Specific growth rate (h ⁻¹)	0.10 ± 0.00	0.11 ± 0.01	0.10 ± 0.01	0.12 ± 0.01	0.11 ± 0.00
q _{glucose} (mmol g _x ⁻¹ h ⁻¹)	-3.67 ± 0.14	-3.69 ± 0.25	-2.97 ± 0.12	-3.92 ± 0.28	-4.11 ± 0.07
q _{ethanol} (mmol g _x ⁻¹ h ⁻¹)	4.75 ± 0.23	4.92 ± 0.30	4.72 ± 0.14	7.26 ± 0.44	5.90 ± 0.23
q _{glycerol} (mmol g _x ⁻¹ h ⁻¹)	0.02 ± 0.00	0.18 ± 0.01	0.03 ± 0.01	0.22 ± 0.00	0.03 ± 0.00
q _{succinate} (mmol g _x ⁻¹ h ⁻¹)	B.D.	B.D.	0.02 ± 0.02	0.01 ± 0.01	0.02 ± 0.00
q _{acetoin} (mmol g _x ⁻¹ h ⁻¹)	-	-	-	-	-7.45 ± 0.18
q _{O₂} (mmol g _x ⁻¹ h ⁻¹)	-0.60 ± 0.01	-0.44 ± 0.02	-0.59 ± 0.04	-0.37 ± 0.02	-0.29 ± 0.01
q _{CO₂} (mmol g _x ⁻¹ h ⁻¹)	6.42 ± 0.22	6.41 ± 0.46	5.33 ± 0.26	6.45 ± 0.24	7.00 ± 0.24
Stoichiometries					
RQ (qCO ₂ /qO ₂)	10.7 ± 0.2	14.4 ± 0.5	9.11 ± 0.16	17.4 ± 1.0	24.2 ± 0.5
Y _{glycerol/x} (mmol g _x ⁻¹)	0.24 ± 0.05	1.71 ± 0.05	0.30 ± 0.09	1.93 ± 0.15	0.32 ± 0.02
Y _{X/glucose} (g _x g ⁻¹)	0.15 ± 0.01	0.16 ± 0.00	0.21 ± 0.01	0.16 ± 0.01	0.14 ± 0.00
Y _{ethanol/glucose} (g g ⁻¹)	0.33 ± 0.03	0.34 ± 0.01	0.41 ± 0.00	0.48 ± 0.03	0.37 ± 0.01

Y_{X/O_2} (g _x mmol ⁻¹)	0.17 ± 0.00	0.24 ± 0.00	0.18 ± 0.02	0.32 ± 0.03	0.37 ± 0.00
Recoveries (out/in)					
Carbon (%)	94.4 ± 4.4	98.4 ± 1.3	102.8 ± 1.2	106.1 ± 3.3	96.1 ± 1.0
Degree of reduction (%)	92.0 ± 4.3	93.5 ± 1.8	102.9 ± 0.3	109.6 ± 3.3	99.5 ± 1.0

Figure 4. Impact of heterologous expression of ScGPP1 and/or ScGPD2 expression in *O. parapolymorpha* CBS11895 on glycerol production in oxygen-limited chemostat cultures. Biomass specific conversion rates (q) of *O. parapolymorpha* strains were measured in oxygen-limited conditions (dilution rate 0.10 h⁻¹), aerated at 500 mL min⁻¹ with a gas mixture containing 840 ppm O₂. Data of anaerobic and oxygen-limited *S. cerevisiae* cultures, grown in the same experimental set-up where derived from (35). Symbols: white circle *S. cerevisiae* CEN.PK113-7D, blue circles *O. parapolymorpha* CBS11895, blue box IMX2119 (ScGPP1), blue triangle up IMX2587 (ScGPD2), blue triangle down IMX2588 (ScGPP1 ScGPD2). Data are represented as average ± SEM of data obtained from independent chemostat cultures of each strain. **(A)** Biomass-specific glycerol production rate versus biomass-specific oxygen consumption rate. The dashed line depicts the stoichiometric relationship between glycerol and oxygen interpolated from the anaerobic rate of glycerol production by *S. cerevisiae* under the assumption that, for NADH reoxidation, consumption of one mol O₂ is equivalent with the production of two mol glycerol. **(B)** Biomass yield on oxygen versus biomass-specific rate of glycerol production.

Discussion

The large majority of facultatively fermentative non-*Saccharomyces* yeasts require molecular oxygen and/or aerobic respiration for essential biosynthetic processes (23, 62). Comparative physiology studies across strains and species, as well as elucidation and differentiation of underlying biochemical mechanisms, require rigorous standardization of cultivation conditions to minimize oxygen contamination and differences in gas-transfer properties. In this study, chemostat cultivation under defined oxygenation regimes revealed surprisingly high oxygen requirements of oxygen-limited cultures of *Ogataea parapolymorpha* (previously *Hansenula polymorpha* (20)). These requirements far exceeded those reported for the facultatively fermentative pre-WGD yeasts *Kluyveromyces marxianus*, *K. lactis* and *Candida utilis* (*Cyberlindnera jadinii*) (41, 63). *O. parapolymorpha* is currently applied in aerobic industrial processes for production of heterologous proteins (64) and based on its thermotolerance and natural ability to metabolize D-xylose, is intensively investigated as a potential platform organism for second-generation ethanol production (21). In anaerobic industrial applications of *Saccharomyces* yeasts, such as beer fermentation, introduction of a brief aeration phase enables yeast cell to synthesize and intracellularly accumulate sterols and unsaturated fatty acids, which are then used during the subsequent anaerobic fermentation phase (65, 66). Since such a process intervention cannot address the large oxygen requirements of *O. parapolymorpha*, reduction or complete elimination of its oxygen requirements should be a priority metabolic-engineering target for development of industrial ethanol-producing strains.

Very low glycerol-production rates and an increased biomass yield upon acetoin co-feeding to oxygen-limited cultures identified reoxidation of NADH formed in biosynthetic reactions as a key contributor to the large oxygen requirements of *O. parapolymorpha*. A substantial oxygen requirement for fermentative growth ('Custers effect'; (67)), absence of glycerol production and a stimulating effect of acetoin on oxygen-limited growth were previously observed in *Brettanomyces* (*Dekkera*) yeasts (40, 49, 67, 68). Further studies on *B. bruxellensis* related the Custers effect to

absence of glycerol-3P phosphatase in cell extracts (49) and of an ortholog of the *S. cerevisiae* *GPP1/GPP2* genes in its genome (69). The genera *Ogataea* and *Brettanomyces* both belong to the Pichiaceae family (70). Our observations on *O. parapolymorpha* and on the absence of *ScGPP1/ScGPP2* orthologs in *Ogataea* species, provide an incentive for further studies into occurrence of this intriguing phenomenon in Pichiaceae. In view of its fast growth in synthetic media (71) and its accessibility to modern genome-editing techniques (72, 73), *O. parapolymorpha* offers an interesting experimental platform to study the regulation networks and ecophysiological significance of the Custers effect. Formation of glycerol in aerobic cultures of a strain in which genes encoding key enzymes of respiratory NADH oxidation, including the mitochondrial flavoprotein NADH dehydrogenase (OpGut2), were deleted, raises the possibility that absence of glycerol formation in oxygen-limited cultures might be related to the operation of a glycerol-3P shuttle for mitochondrial oxidation of cytosolic NADH in this yeast (reviewed in (43)).

While heterologous expression of *S. cerevisiae* *GPD2* and *GPP1* in *O. parapolymorpha* increased glycerol production and biomass yield on oxygen in oxygen-limited cultures, it did not allow for complete fermentation of the supplied glucose. Increased expression of these heterologous genes, possibly combined with expression of a glycerol exporter and/or laboratory evolution under oxygen-limited conditions, is likely to be required for further improvement of oxygen-independent NADH reoxidation. Alternatively, use of other heterologous pathways in *O. parapolymorpha* that enable NADH-dependent reduction of acetyl-CoA to ethanol (74) or NADH oxidation via a pathway involving ribulose-1,5-bisphosphatase and phosphoribulokinase (75, 76) can be explored to reduce its oxygen requirement for NADH reoxidation.

The absence of orthologs of the *S. cerevisiae* Aus1 and Pdr11 sterol transporters indicates that, similar to other pre-WGD yeasts (57), *O. parapolymorpha* is most likely unable to import sterols. Due to the incompletely resolved role of cell wall proteins in sterol import in *S. cerevisiae* (77), functional expression of a heterologous system for sterol import may not be a trivial challenge. Alternatively,

the importance of sterol uptake by oxygen-limited cultures can be investigated by expression of a heterologous squalene-tetrahymanol cyclase. In *S. cerevisiae* and *K. marxianus*, this modification was recently shown to enable synthesis of the sterol surrogate tetrahymanol and sterol-independent anaerobic growth. Genome-sequence data indicate that pyrimidine synthesis in *O. parapolymorpha* depends on a respiratory-chain-linked dihydroorotate dehydrogenase (OpUra9), thus most probably also rendering pyrimidine biosynthesis in this yeast oxygen dependent (78, 79). As previously explored in *Scheffersomyces stipitis* (78), expression of the soluble fumarate-coupled dihydroorotate dehydrogenase from *S. cerevisiae* (ScUra1), may be explored to bypass this oxygen requirement.

We previously showed that, in the thermotolerant yeast *K. marxianus*, an inability to import sterols was the sole feature preventing growth in media supplemented with the known anaerobic growth factors for *S. cerevisiae* (35). The results described in this study on *O. parapolymorpha* underline that oxygen requirements of industrially relevant yeasts, as well as their transcriptional responses to oxygen limitation, show strong quantitative and qualitative differences. In addition to illustrating the need for further fundamental research on oxygen requirements in yeasts and other fungi, this diversity should be considered when selecting host strains for novel anaerobic, yeast-based processes.

Methods

Strain maintenance.

Yeast strains used in this study were derived from the wild-type *Ogataea parapolymorpha* strain CBS11895 (DL-1) obtained from CBS-KNAW (Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands). For propagation and strain maintenance, yeast cultures were grown on yeast peptone dextrose (YPD) media (10 g L⁻¹ Bacto yeast extract, 20 g L⁻¹ Bacto peptone, 7.5 g L⁻¹ glucose) in an Innova shaker incubator (New Brunswick Scientific, Edison, NJ, USA) set at 30 °C and 200 rpm. YP medium was heat sterilized (121 °C for 20 minutes). Stock solutions of concentrated D-glucose were autoclaved separately at 110 °C and added to a concentration of 7.5 g L⁻¹. Cryo-stocks were prepared

from exponentially growing cultures by addition of glycerol to a final concentration of 30 % (v/v) and aseptically stored at -80 °C.

Molecular biology techniques.

PCR amplification for cloning was routinely performed with Phusion High Fidelity polymerase (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. DreamTaq polymerase (Thermo Fisher Scientific) was used for diagnostic PCR reactions. Oligonucleotide primers were ordered either desalted or PAGE-purified (Sigma-Aldrich, St. Louis, MO, USA) and are listed in **Table S3**. DNA fragments obtained by PCR amplification were analysed by gel electrophoresis, and when required, purified from agarose gels with the Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. Prior to purification, template plasmid DNA was removed by FastDigest *DpnI* digestion (Thermo Fisher Scientific). Alternatively, DNA fragments were directly purified from the PCR mix using the GenElute PCR Clean-Up Kit (Sigma-Aldrich). Gibson assemblies were performed with the NEBuilder HiFi DNA Assembly Master mix (New England Biolabs, Ipswich, MA, USA) with the total reaction volume downscaled to 5 µL and a total incubation time of 1 h at 50 °C. *Escherichia coli* XL1-Blue cells were used for transformation, amplification and storage of plasmids. Plasmids were isolated from overnight *E. coli* cultures with the GenElute Plasmid Miniprep kit (Sigma-Aldrich). For diagnostic PCR amplifications, yeast colonies were grown overnight in YPD, after which the genomic DNA was extracted using the LiAc/SDS method (80).

Plasmid construction.

Plasmids used in this study are described in **Table 4**. *O. parapolymorpha* promoters and terminators were chosen based on high transcript levels across a range of specific growth rates (81). Promoter and terminator regions were defined as 800 bp upstream and 300 bp downstream of the coding sequence, respectively. For targeted integration into a genetic locus, 500 bp flanking homology

regions were designed to partially delete the target region, without altering promoters (800 bp) or terminators (300 bp) of adjacent genes.

Plasmid pUD1069 (*ScGPP1*) was constructed by Gibson assembly from fragments with 20 bp terminal sequence overlaps. The *GPP1* coding sequence was PCR amplified from genomic DNA of *S. cerevisiae* CEN.PK113-7D with primers 15183 and 15814 thereby introducing 20 bp overlaps. The promoter and terminator of the endogenous *O. parapolymorpha* *PMA1* were amplified from genomic DNA using primers 15185/15186 and, 15191/15193 respectively. The natNT2 marker (*AgTEF1p-nat1-ScADH1t*) from *Streptomyces noursei* (82–84) was PCR amplified from pUD803 (59) with primers 15187 and 15188. Up- and downstream 500 bps homology flanks to the *gbu1* locus were PCR amplified from genomic DNA of *O. parapolymorpha* using primers 15192/15196 and 15195/15198 respectively. The pUG6 backbone was PCR amplified using primers 15189 and 15190. Fragments were mixed in equimolar amounts and Gibson assembled resulting in plasmid pUD1069. Correct plasmid assembly was verified by restriction digestion and diagnostic PCR amplification with primers 15224, 15225, 15226, 15227, 15228, 15229, 15230, 15231 and 15232.

For heterologous expression of *ScGPD2*, its coding sequence was PCR amplified from genomic DNA of CEN.PK113-7D with primers 15745 and 15744, resulting in the addition of 20 bp homology flanks for Gibson assembly. The *OpTEF1* promoter and *OpTEF1* terminator were PCR amplified from genomic DNA of *O. parapolymorpha* CBS11895 with primers 15749/15748 and 15746/15747, respectively. Upstream and downstream 800-bp recombination flanks for integration of the *SGA1* integration locus were PCR amplified with primers 15740/15739 and 15735/15736, respectively. A *Klebsiella pneumoniae* *hph* (Hyg^R) marker cassette, including the *AgTEF1* promoter and *AgTEF1* terminator from *Ashbya gossypii*, was PCR amplified from pUDP002 (72) with primers 1312 and 15743. The pUC19 backbone was PCR amplified with primers 15738 and 15737. The resulting 6 fragments were mixed in in equimolar amounts and Gibson assembled. The resulting plasmid pUD1082 (*ScGPD2*) was verified by restriction digestion.

Table 4. Plasmids used in this study. Superscripts indicate restriction sites, subscripts indicate gRNA target sequences. Sc: *Saccharomyces cerevisiae*; Op: *Ogataea parapolymorpha*, Ag: *Ashyba gossypii*, Aa: *Arxula adenivorans*, Sp: *Streptococcus pyogenes*.

Plasmid	Characteristics	Source
pUC19	ori ampR	(85)
pUD803	HR _{NDH2-3} AaTEF1p-nat1-ScPHO5t HR _{NDH2-3}	(59)
pUD1069	ori amp ^R <i>gbu1</i> (HR) OpPMA1p-ScGPP1-OpPMA1t-AgTEF1p-nat1-ScADH1t <i>gbu1</i> (HR)	This study
pUD1082	ori ampR <i>sga1</i> (HR) OpTEF1p-ScGPD2-OpTEF1t AgTEF1p-hph-AgTEF1t <i>sga1</i> (HR)	This study
pUDP002	panARS AgTEF1p-hph-AgTEF1t ScTDH3p ^{BsaI BsaI} ScCYC1t AaTEF1p-Spcas9-ScPHO5t	(72)
pUG6	ori ampR loxP-kanMX-loxP	(86)

Table 4. Yeast strains used in this study. Sc: *Saccharomyces cerevisiae*; Op: *Ogataea parapolymorpha*

Genus	Strain	Relevant genotype	Reference
<i>S. cerevisiae</i>	CEN.PK113-7D	MATa URA3 HIS3 LEU2 TRP1 MAL2-8c SUC2	(87)
<i>O. parapolymorpha</i>	CBS11895	-	Westerdijk
<i>O. parapolymorpha</i>	IMX2119	<i>gbu1Δ::OpPMA1p-ScGPP1-OpPMA1t natNT2</i>	This study
<i>O. parapolymorpha</i>	IMX2587	<i>sga1Δ::OpTEF1p-ScGPD2-OpTEF1t-hph</i>	This study
<i>O. parapolymorpha</i>	IMX2588	<i>gbu1Δ::OpPMA1p-ScGPP1-OpPMA1t natNT2 sga1Δ::OpTEF1p-ScGPD2-OpTEF1t-hph</i>	This study
<i>O. parapolymorpha</i>	IMX2167	<i>ndh2-1Δ::kanR ndh2-2Δ::hph ndh2-3::natNT2 gut2Δ::pat</i>	(59)

Strain construction

O. parapolymorpha strains were transformed by electroporation of freshly prepared electrocompetent cells (72). Yeast cells were selected after transformation on YPD agar containing hygromycin B (300 µg mL⁻¹) or nourseothricin (100 µg mL⁻¹). Strains IMX2119, IMX2587 and IMX2588 were constructed using the split-marker integration approach (88), with an approximately 480 bp overlapping homology sequences for marker recombination and genome integration. The natNT2 split-marker fragments for integration of a ScGPP1 expression cassette into the OpGBU1 locus were constructed by PCR amplification with primers 15192, 15194, 15196 and 15197 from pUD1069 (ScGPP1) resulting in two integration fragments with a homologous sequence overlap. Similarly, the

hph split-marker fragments for integration of the *ScGPD2* cassette were constructed by PCR amplification of a pUD1082 (*ScGPD2*) fragment with primers 15740, 15741, 15742 and 15736. The chromosomal integration of the split-marker fragment was verified by diagnostic PCR. Diagnostic PCR of the *OpGBU1* integration locus was performed with primers 15192, 15197, 15233 and 15234, and integration in the *OpSGA1* integration locus was verified with primers 15894, 15748, 15895.

Bioreactor cultivation.

Aerobic batch and chemostat cultures for physiological characterization of *O. parapolymorpha* strains CBS11895 and IMX2167 strains were grown in 2 L bioreactors (Applikon Biotechnology, Delft, the Netherlands) with 1.0 L working volume. Cultures were grown on synthetic medium with vitamins (89), supplemented with glucose and with sterile antifoam pluronic acid 6100 PE (0.2 g L⁻¹, BASF, Ludwigshafen, Germany). Mineral salt solutions were sterilized by autoclaving at 121 °C for 20 min. Glucose solutions were prepared and autoclaved separately at 110 °C for 20 min and added to a final concentration of 7.5 g L⁻¹.

Inocula for bioreactor cultures were prepared by harvesting an exponential growing shake-flask culture by centrifugation and washing once with sterile demineralized water. Cultures were continuously stirred at 800 rpm, maintained at 30 °C and sparged with air (O₂ 21·10⁴ ppm) at a volumetric rate of 0.5 L min⁻¹ (0.5 vvm) to maintain a high dissolved oxygen concentration. After CO₂ emission in the batch cultivation phase had reached a maximum and started to decline sharply, a constant medium feed and continuous effluent removal were initiated to maintain a constant working volume.

Oxygen-limited chemostat cultures of *O. parapolymorpha* strains were grown in 2 L bioreactors (Applikon) with 1.2 L working volume on synthetic medium with vitamins and urea as nitrogen source (90). A stock solution of urea was filter sterilized (0.2 µm) and added to the sterile media. Before autoclaving, bioreactors were checked for gas leakage by submersion in water while applying a 0.3 bar overpressure. Chemostats were started as aerobic batch cultures to obtain a sufficiently

high initial cell density. The glucose concentration in the aerobic batch phase was 1.5 g L⁻¹ while the chemostat feed media contained 20 g L⁻¹ glucose for the oxygen-limited and 7.5 g L⁻¹ for the aerobic reference cultures.

The anaerobic growth factors ergosterol (10 mg L⁻¹, Sigma Aldrich), Tween 80 (84 mg L⁻¹, Sigma Aldrich) were solubilized in pure ethanol (671 µg L⁻¹, Sigma Aldrich). Sterile anaerobic growth factors ergosterol and Tween 80 were prepared as described previously (39), to prevent excessive foam formation while still supporting maximal growth the Tween 80 concentration was reduced 5 fold compared to anaerobic *S. cerevisiae* reference cultures. For aerobic chemostat cultures, Tween 80 was omitted to prevent excessive foam formation and the glucose concentration was adjusted to 7.5 g L⁻¹. Dissolved gas concentrations in the bioreactor cultures were maintained by sparging with either air (21·10⁴ ppm O₂) or a gas mixture of N₂/Air (840 ppm O₂) at a volumetric rate of 0.5 L min⁻¹ (0.4 vvm). Bioreactors were equipped with Fluran tubing and viton O-rings to minimize oxygen diffusion. The glass medium reservoir was equipped with Norprene tubing and continuously sparged with pure nitrogen gas.

Analytical methods

Off-gas analysis, biomass dry weight measurements, optical density measurements, metabolite HPLC analysis of culture supernatants and correction for ethanol evaporation in bioreactor experiments were performed as described previously (35). Rates of substrate consumption and metabolite production were determined from glucose and metabolite concentrations in steady-state cultures, analyzed after rapid quenching of culture samples (91). Carbon and degree of reduction balances were calculated based on concentrations of relevant components in medium feed, culture samples and in- and out-going gas streams. Calculations were based on an estimated degree of reduction of biomass as described in (92).

RNA extraction, isolation, sequencing and transcriptome analysis

Biomass samples from bioreactor batch and chemostat cultures were directly sampled into liquid nitrogen to prevent mRNA turnover (93) and stored at -80 °C before extraction and isolation. Batch cultures were sampled in mid-exponential phase when approximately 75 % of the initial glucose concentration was still unused (81). Processing of RNA for long-term storage and isolation was performed as described previously (35). The quality of isolated RNA was analyzed with an Agilent Tapestation (Agilent Technologies, CA, USA) using RNA screen tape (Agilent). RNA concentrations were measured with a Qubit RNA BR assay kit (Thermo Fischer Scientific). The TruSeq Stranded mRNA LT protocol (Illumina, San Diego, CA, USA) was used to generate RNA libraries for paired-end sequencing by Macrogen (Macrogen Europe, Amsterdam, the Netherlands) with a read-length of 151 bp on a NovaSeq sequencer (Illumina).

To refine the genome annotation of *O. parapolymorpha* CBS11895, pooled RNAseq libraries were first used to perform *de novo* transcriptome assembly using Trinity (v2.8.3)(94) and the used for the PASA pipeline (95) as implemented in funannotate (96). RNA reads from *O. parapolymorpha* wild-type batch and chemostat (81) were downloaded from NCBI (www.ncbi.nlm.nih.gov) with the gene expression omnibus accession GSE140480.

RNA reads were mapped to the genome of strain CBS11895 (81) using bowtie (v1.2.1.1) (97) and alignments were filtered and sorted using samtools (v1.3.1)(98) as described previously (35). Reads were counted with featureCounts (v1.6.0)(99) of which both pairs of the paired-end reads were aligned to the same chromosome. edgeR (v3.28.1)(100) was used to perform differential gene expression and genes with fewer than 10 reads per million in all conditions were eliminated from subsequent analysis. Counts were normalized using the trimmed mean of M values (TMM)(101) method and the dispersion was estimated using generalized linear models. Differential expression was calculated using a log ratio test adjusted with the Benjamini-Hochberg method. Absolute log₂ fold-change values (>2), false discovery rate (< 0.5) and *p*-value (<0.05) were used a significance cutoffs.

Gene set analysis (GSA) based on gene ontology (GO) terms with Piano (v2.4.0)(58) was used for functional interpretation of differential gene expression profiles. Interproscan (102) was used to assign GO terms to the genome annotation of *O. parapolymorpha*. Co-ortholog groups of genes were generated with ProteinOrtho5 (103) as implemented in the funannotate pipeline and used to homogenize GO terms for co-ortholog groups as described previously (35). Gene set analysis was performed with Piano (v2.4.0)(58) and gene statistics were calculated with Stouffer, Wilcoxon rank-sum test and reporter methods as implemented in Piano. Consensus gene level statistics were obtained by *p*-value and rank aggregation and considered significant based on absolute log 2 fold-change values > 1. ComplexHeatmap (v2.4.3)(104) was used to visualize the differentially expressed genes, including those shared with a previous study (55).

To interpret the GO-term based gene set analysis between three yeast species in response to oxygen limitation, hierarchical clustering (complete method and Euclidian distance) in R (105) was performed on GO-terms from biological process category. Clustering was based on the number of overlapping distinct directionality *p*-values in the three yeast species with a significance *p*-value cut-off of 0.01.

Sequence homology search

S. cerevisiae protein sequences were used as query for searching whole genome sequences of 16 *Ogataea* species, *K. marxianus*, *Candida arabinofermentans* and *B. bruxellensis* with tblastn (blast.ncbi.nlm.nih.gov)(106). Significance was based on alignment criteria with an e-value ($< 10^{-7}$), alignment coverage (>70%) and nucleotide percent identity of (>50%). Blast results were mapped to a subtree of selected yeast species in the fungal phylum Ascomycota (48) using Treehouse (107) to subset the phylogenetic tree.

Data availability

Data presented in all figures in this work are available at the www.data.4TU.nl repository doi: 10.4121/14270138.

599 Raw sequencing data that supports this study are available from the NCBI website
600 (www.ncbi.nlm.nih.gov/geo/) with the BioProject PRJNA717220.

601 **Code availability**

602 The codes that were used to generate the results obtained in this study are archived in a Gitlab
603 repository (https://gitlab.tudelft.nl/rortizmerino/opar_anaerobic).

604 **Author Contributions**

605 WD, and JTP designed the study and wrote the manuscript. WD performed molecular cloning with
606 the support of MG and RB. WD, HJ and CM performed the bioreactor experiments with the support
607 of AK. RO contributed the transcriptome analysis and genome annotation.

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611 **Conflict of Interest**

612 The authors declare no conflict of interest.

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904 Supplementary Material

905 Supplementary Figure 1. Consensus biological process GO term enrichment for *S. cerevisiae* contrast

906 21. GO terms are clustered according to their rank. See legend of Fig. 3 for experimental details.

Supplementary Figure 2. Consensus biological process GO term enrichment for *K. marxianus* contrast 21. GO terms are clustered according to their rank. See legend of **Fig. 3** for experimental details.

910 **Supplementary Figure 3.** Consensus biological process GO term enrichment for *O. parapolymorpha*
 911 contrast 21. GO terms are clustered according to their rank. See legend of **Fig. 3** for experimental
 912 details.

Supplementary Figure 4. Different transcriptional regulation of NADH reduction in *O. parapolymorpha*, *K. marxianus*, *S. cerevisiae*. (A) For each aeration regime (1 to 3) RNAseq was performed of chemostat cultures (dilution rate 0.10 h⁻¹) for *O. parapolymorpha* CBS11895 and, *K. marxianus* CBS6556 and *S. cerevisiae* CEN.PK113-7D where possible (contrast 31 was not possible for *O. parapolymorpha*). RNAseq data of *S. cerevisiae* and *K. marxianus* was reanalyzed from (35) which included anaerobic-growth factor supplementation regimes (4 to 5). RNAseq was performed on independent replicate batch and chemostat cultures of *O. parapolymorpha* CBS11895 and IMX2167 (*gut2Δ ndi/eΔ*) in aerobic conditions (condition/regime 6 to 9). (B) Biochemical reactions are

921 represented by arrows and multiple reactions by dashed arrows. Boxes with colors indicate up-
 922 (blue-green) or downregulation (brown) with color intensity indicating the log₂ fold change (logFC)
 923 with color range capped to a maximum value of 5. Reactions are annotated with the corresponding
 924 *S. cerevisiae* ortholog name and grey dots indicate lack of an ortholog in the respective yeast.
 925 Abbreviations used; pentose-phosphate pathway (PPP), glucose-6-phosphate (G6P), fructose-6-
 926 phosphate (F6P), fructose-1,6-bisphosphate (F1,6P), dihydroxyacetone phosphate (DHAP), glycerol-
 927 3P (G3P), glyceraldehyde phosphate (GAP), inner-mitochondrial membrane (IM), OM outer-
 928 mitochondrial membrane, respiratory chain complex's annotated with corresponding roman
 929 numerals.

933 **Supplementary Table 1.** Transcriptional response of phosphatases in *O. parapolymorpha* wild-type
 934 to *gut2Δ nde/i1-3Δ* knockout in aerobic batch cultures. Log fold-change (logFC), log counts per
 935 million (logCPM), gene identifiers used in the genome annotations and *S. cerevisiae* S288C orthologs
 936 and three-letter abbreviation (standard name).

logFC	logCPM	PValue	FDR	Geneid	DL.1	S288C	Standard Name
-1.61	5.74	2.06E-19	1.11E-18	OPAR_004591	HPODL_02465	YGR208W	SER2
-1.56	3.52	1.10E-08	2.56E-08	OPAR_004515	HPODL_01991		
1.39	7.90	1.51E-23	1.07E-22	OPAR_002912	HPODL_03896	YKL212W	SAC1
-1.37	6.68	2.73E-18	1.36E-17	OPAR_005066	HPODL_02910	YOR283W	
1.36	5.25	9.20E-12	2.80E-11	OPAR_002432	HPODL_03461	YJR110W	YMR1
1.29	6.58	6.85E-21	4.09E-20	OPAR_000036	HPODL_00031	YFR028C	OAF3
1.19	5.46	2.48E-06	4.74E-06	OPAR_004387	HPODL_01871	YDL230W	PTP1
-0.99	7.74	1.28E-12	4.11E-12	OPAR_003314	HPODL_04287	YDL236W	PHO13
0.93	5.77	2.55E-09	6.27E-09	OPAR_001025	HPODL_04916	YBR204C	LDH1
0.73	6.09	5.08E-06	9.40E-06	OPAR_002585	HPODL_03597	YBR276C	PPS1
0.64	5.10	0.011907	0.01586	OPAR_000879	HPODL_04782	YDL057W	
-0.64	6.01	0.000114	0.000186	OPAR_005045	HPODL_02888	YNL128W	TEP1
0.63	5.72	0.000104	0.00017	OPAR_001327	HPODL_00279	YDR481C	PHO8
0.58	7.20	1.47E-05	2.60E-05	OPAR_001974	HPODL_00894	YBR092C	PHO3
0.52	7.69	8.32E-05	0.000139	OPAR_003977	HPODL_01484	YLR019W	PSR2
0.48	6.67	0.001628	0.002382	OPAR_002901	HPODL_03887	YOR155C	ISN1
0.45	5.71	0.003449	0.004877	OPAR_004178	HPODL_01675	YNL217W	PPN2
-0.43	8.41	0.00185	0.002689	OPAR_005130	HPODL_02972	YNL010W	PYP1
0.38	6.44	0.004739	0.006577	OPAR_001369	HPODL_00320		
-0.28	4.14	0.256548	0.289337	OPAR_002548	HPODL_03567	YMR087W	PDL32
-0.28	6.41	0.066792	0.081355	OPAR_001685	HPODL_00615	YHR004C	NEM1
0.18	6.02	0.220151	0.25152	OPAR_000296	HPODL_02098		
0.12	7.52	0.372061	0.408252	OPAR_002280	HPODL_03321	YLR377C	FBP1
0.12	6.55	0.366906	0.402931	OPAR_002299	HPODL_03339	YNL108C	

937

938 **Supplementary Table 2. Primers used in this study.**

Number	Sequence (5'-3')
1312	GACATGGAGGCCAGAAATAC
15183	TGCTAGAATAGAGACTCATCACCATCGCAATGCCTTTGAC
15184	AACCCATTTCAAGTGCTAGCTTACCATTCAACAAGTCATCCTTAG
15185	CGGTGGATGCGGAATGAATG
15186	GATGAGTCTCTATTCTAGCAATTGAACTGAAAG
15187	TAGTGATCTCCCTCGAGATG
15188	CTATATTCAGCGGTGCGGGTGGCATGCCAGTTCTTTGAG
15189	GAAGAATCCGCGAGTTGGCGTCTGCCGGTCTCCCTATAGTG
15190	CAAACGGTCAGTGGTCCACGCCGCGTTCTATAGTGTACC
15191	GCTAGCACTTGAAATGGGTTAAT
15192	ACGCCAACTGCGGATTCTTC
15193	CCGCCGACCGCTGAATATAG
15194	GGCCTGTTCACCGTCAGATG
15195	CATCTCGAGGGAGATCACTAATCTCTTTGCCAACGGGAAC
15196	CATTCATTCCGCATCCACCGGAGACGGGCTGGAAAAAAG
15197	AGTCCCTGGTGATGCTGAAG
15198	CGTGGACCACTGACCGTTTG
15224	GCAACACGTGCAAGGCGAAAC
15225	TGTTTCGCCTGCACGTGTTG
15226	ACGGCTGAAACTCGGGATAC
15227	TCGGCAATGTCCCGGTATCC
15228	TTCTTCGTGCCACGACAG
15229	CCGCCGACCGCTGAATATAG
15230	GGTGTCGGTGGTGAATGAAC
15231	CCTGATTGAACGGCGTATG
15232	GCAGATCCGAGCCTTTATCC
15233	GCGGAACCTTTGAGCACAG
15234	GATGAACCTGATGGATACG
15735	GTATTCTGGGCTCCATGTGCTGCTGATGGGTTAGAAAGC
15736	GGGAGCCTTGCCCTTGTTTG
15737	CAAACAAGGGCAAGGCTCCCGCGGTCTATAGTGTACC
15738	TCGCAAGCGTTTGCCGACACTCTGCCGGTCTCCCTATAGTG
15739	GATTTTTGTGACCTGTGTTTGACGCGGGCACTATAATTGGG
15740	GTGTCGGCAAACGCTTGCGAAC
15741	GCTGCGCCGATGGTTTCTAC
15742	TTATGCGGCCATTGTCCGTC
15743	AGGGTTCTCGAGAGCTCCAGTATAG
15744	ATTATTCAAAGCTAGCTTGCGGGAGAGTGTCTATTCTCATC
15745	CAAAGACTGAATACTATAAAATGCTTGCTGTGAGAAGATTAAC
15746	GCAAGCTAGCTTTGAATAATTTC
15747	CTGGAGCTCTCGAGAACCTGACTGTAAATTCACAAAACACTGTTTC
15748	TTTATAGTATTCTAGTCTTTGAAAAAC
15749	CCGTGTACTGTGGTAAATCC
15894	CGCGTGCTCCTTGATAATAG
15895	TCCAGTCACCTGAAATTCG

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