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Oxygen Consumption by Anaerobic *Saccharomyces cerevisiae* under Enological Conditions: Effect on Fermentation Kinetics

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The anaerobic growth of the yeast *Saccharomyces cerevisiae* normally requires the addition of molecular oxygen, which is used to synthesize sterols and unsaturated fatty acids (UFAs). A single oxygen pulse can stimulate enological fermentation, but the biochemical pathways involved in this phenomenon remain to be elucidated. We showed that the addition of oxygen (0.3 to 1.5 mg/g [dry mass] of yeast) to a lipid-depleted medium mainly resulted in the synthesis of the sterols and UFAs required for cell growth. However, the addition of oxygen during the stationary phase in a medium containing excess ergosterol and oleic acid increased the specific fermentation rate, increased cell viability, and shortened the fermentation period. Neither the respiratory chain nor de novo protein synthesis was required for these medium- and long-term effects. As de novo lipid synthesis may be involved in ethanol tolerance, we studied the effect of oxygen addition on sterol and UFA auxotrophs (*erg1* and *ole1* mutants, respectively). Both mutants exhibited normal anaerobic fermentation kinetics. However, only the *ole1* mutant strain responded to the oxygen pulse during the stationary phase, suggesting that de novo sterol synthesis is required for the oxygen-induced increase of the specific fermentation rate. In conclusion, the sterol pathway appears to contribute significantly to the oxygen consumption capacities of cells under anaerobic conditions. Nevertheless, we demonstrated the existence of alternative oxygen consumption pathways that are neither linked to the respiratory chain nor linked to heme, sterol, or UFA synthesis. These pathways dissipate the oxygen added during the stationary phase, without affecting the fermentation kinetics.

The physiological constraints that limit the fermentative activity of the yeast *Saccharomyces cerevisiae* are not fully understood. Several studies have shown that the lipids or molecular oxygen that are required for lipid biosynthesis are essential for growth (3, 4), plasma membrane integrity (1, 59), and the maintenance of high glycolytic and ethanol production rates (16, 52). In fact, fermentative efficiency and resistance to ethanol are generally linked to: (i) an increase in the ergosterol/phospholipid ratio and (ii) a decrease in the saturation index of the fatty acids in yeast cells (17, 53). Molecular oxygen is required for the conversion of squalene into ergosterol: 1 O_2 molecule is required for the conversion of squalene to lanosterol, 9 O_2 molecules are required for the conversion of lanosterol to zymosterol, and 2 O_2 molecules are required for the conversion of zymosterol to ergosterol (6, 7, 11, 23, 34, 44, 45). However, only one acyl coenzyme A Δ^9-desaturase is responsible for the biosynthesis of unsaturated fatty acids (UFAs) (palmitoleate and oleate): 1 O_2 molecule is required for the formation of each double bond (47). During enological and brewery fermentations, the addition of air or oxygen is legally permitted as an often common practice. During brewing fermentations, oxygen is generally added during the growth phase to improve biomass synthesis (36). However, under enological conditions, oxygen is used to increase the fermentation rate in the case of sluggish fermentation (52). This is only efficient at the end of the cell growth phase (49, 51). The oxygen requirement is low and has been estimated to be 1.5 to 3.5 mg per g (dry mass) of yeast for enological fermentations (14, 49). Sterol biosynthesis requires 0.1 to 0.3 mg of dissolved oxygen per g (dry mass) of yeast (38), and UFA synthesis requires about 0.35 mg of oxygen per g (dry mass) of yeast (37) during high-gravity brewing fermentations. In contrast, during enological fermentations, the oxygen consumption linked to the sterol biosynthesis pathway can reach 0.5 mg of oxygen per g (dry mass) of yeast, whereas a negligible amount of oxygen is required for UFA synthesis (54).

However, even if ergosterol and UFAs are added to the medium, yeast cells can consume much more oxygen during alcoholic fermentation without affecting the ethanol yield (42, 43, 54). The oxygen consumption capacity of anaerobically grown cells has not been yet characterized under these conditions. Nevertheless, it is partially sensitive to KCN (0.5 to 1 mM) and represents 1 to 5% of the respiratory capacity of fully derepressed aerobically grown cells (28, 48, 54, 62). Some authors believe that the oxygen consumption observed in anaerobically grown repressed cells is mainly due to the classical respiratory chain (25, 43). As the respiratory enzymes, which are subject to glucose catabolite repression, exhibit basal activity (about 1% of the derepressed activity) (25, 30), such ambiguities were reinforced by the fact that some genes (*CYC7* and *COX5b*) encoding respiratory chain components (iso-2

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cytochrome *c* and the cytochrome *c* oxidase subunit Vb, respectively) are induced under anaerobic conditions. This suggests the existence of an electron flux across cytochrome *c* oxidase following the addition of oxygen (2, 15, 18). However, recent studies have demonstrated that, despite the fact that they are sensitive to cyanide, promitochondria do not contain any classical respiratory chain activity and do not contribute significantly to the overall oxygen consumption capacities of anaerobic cells (E. Rosenfeld, J. Schaeffer, B. Beauvoit, and J. M. Salmon, submitted for publication). The apparent consumption of excess oxygen by yeast cells is correlated with the hypoxic induction of several genes (*HEM13*, *ERG11*, *CPR1*, and *OLE1*) (39) and with the cytochrome b_5 and P450 contents of microsomal fractions (28, 33, 64). These observations support the partial functioning of heme and microsomal lipid synthesis. Sterol and UFA synthesis may exhibit a temporal priority during the respiratory adaptation to oxygen for mitochondrial membrane biogenesis (19, 28). Nevertheless, the roles of the hypoxic genes that are involved in lipid metabolism remain unknown in the absence of respiratory chain induction (i.e., under hypoxic conditions, following oxygen pulses or following oxygenation of the respiratory-deficient [*rho*^{0}] mutant strain). This is especially true during the stationary phase (nitrogen starvation) and when the lipid content of the medium is not limiting for growth.

The aim of this work was thus to determine which pathways are the main oxygen-consuming pathways during anaerobic growth during alcoholic fermentation. The fermentation kinetics and the oxygen consumption capacity of the *erg1* and *ole1* mutant strains were measured to determine how sterol and UFA synthesis may contribute to the response of anaerobic cells to oxygen pulse. Finally, we investigated the potential effect of this oxygen consumption on yeast physiology during anaerobic growth.

MATERIALS AND METHODS

Strains, vectors, and culture conditions. (i) Yeast strains. The *S. cerevisiae* strain used in this study was V5 (*MAT***a** *ura3*), which was derived from a diploid industrial wine strain. The *ura3* genotype was introduced into the V5 strain at the haploid stage. This strain was obtained from the Collection Nationale de Cultures de Microorganismes (Institut Pasteur, Paris, France), reference no. I-1222. The V5-[*rho*^{0}] respiratory-deficient mutant was obtained by ethidium bromide induction (57). We ensured that strain V5-[*rho*^{0}] completely lacked mitochondria by checking for a lack of fluorescence inside the cytoplasm after staining with the fluorescent dye DAPI (4′-6′-diamidino-2-phenylindole) (63). Both the V5 and V5-[*rho*^{0}] strains are *ura3* auxotrophs.

(ii) Culture media. All media were heat sterilized (110°C, 20 min). Unless otherwise specified, yeast strains were grown in the standard nutrient medium (YPD), which contained 1% yeast extract (Difco), 2% Bacto peptone (Difco), and 5% glucose. YNB medium contained 0.17 g of yeast nitrogen base (YNB without amino acids and ammonium sulfate; Difco) liter^{-1}, 5 g of glucose liter^{-1}, 1.15 g of NH_4Cl liter^{-1}, and 20 mg of uracil liter^{-1}. A synthetic fermentation medium (Medium Synthetic [MS]) that was strongly buffered to pH 3.3 was used to simulate a standard grape juice (13). This medium contained 200 g of glucose liter^{-1}, 6 g of citric acid liter^{-1}, 6 g of D,L-malic acid liter^{-1}, 20 mg of uracil liter^{-1}, 750 mg of KH_2PO_4 liter^{-1}, 500 mg of K_2SO_4 liter^{-1}, 250 mg of $MgSO_4 \cdot 7H_2O$ liter^{-1}, 155 mg of $CaCl_2 \cdot 2H_2O$ liter^{-1}, 200 mg of NaCl liter^{-1}, 4 mg of $MnSO_4 \cdot H_2O$ liter^{-1}, 4 mg of $ZnSO_4$ liter^{-1}, 1 mg of $CuSO_4 \cdot 5H_2O$ liter^{-1}, 1 mg of KI liter^{-1}, 0.4 mg of $CoCl_2 \cdot 6H_2O$ liter^{-1}, 1 mg of H_3BO_3 liter^{-1}, 1 mg of $(NH_4)_6Mo_7O_{24} \cdot 2H_2O$ liter^{-1}, 20 mg of *myo*-inositol liter^{-1}, 2 mg of nicotinic acid liter^{-1}, 1.5 mg of calcium pantothenate liter^{-1}, 0.25 mg of thiamine-HCl liter^{-1}, 0.25 mg of pyridoxine-HCl liter^{-1}, 0.003 mg of biotin liter^{-1}, and 300 mg of ammoniacal nitrogen (NH_4Cl) liter^{-1}. Unless otherwise specified, 15 mg of ergosterol liter^{-1} dissolved in 1 ml of Tween 80-pure ethanol (50:50, vol/vol) was added to the medium. Approximately 71% of the molar mass of Tween 80 consists of oleic acid (32). When required, Tergitol NP-40 was used to dissolve ergosterol instead of Tween 80 (1 ml of Tergitol NP-40–pure ethanol [50:50, vol/vol]).

(iii) Growth conditions. In the absence of anaerobic growth factors (i.e., when oxygen was required for growth), yeast cells were precultured at 28°C under hypoxic conditions without agitation. They were grown in small Erlenmeyer flasks with a liquid-to-air ratio of 9/10. In the presence of anaerobic growth factors, yeast cells were precultured in small penicillin flasks (125-ml volume) containing 50 ml of fermentation medium. After inoculation, flasks were tightly closed with a penicillin septum, and pure sterile argon (NP; Air-liquide) (O_2 contamination, <2 ppm) was flushed through a needle into the fermentation medium for 5 min. In control experiments (cell-free systems), the decolorization of resazurin (2 mg liter^{-1}) was monitored to ensure that the conditions were indeed anaerobic. Yeast cells were cultured anaerobically in handmade glass fermentors (1.25-liter working volume) fitted with fermentation locks (CO_2 bubbling outlets filled with water) (51, 54). The fermentation medium was strongly deoxygenated by bubbling pure sterile argon for 30 min before inoculation. The medium was always inoculated with 10^6 cells ml^{-1}. After inoculation, the flasks were bubbled with pure sterile argon (NP; Air-liquide) for 40 min to restore anaerobic conditions. In control experiments (cell-free systems), the decolorization of resazurin (2 mg liter^{-1}) was monitored to check anaerobiosis. Fermentations were carried out under isothermal conditions (28°C) with continuous magnetic stirring (500 rpm). A disposable test (Anaerotest; Merck, Darmstadt, Germany) was fitted in the fermentor headspace to ensure that anaerobic conditions were maintained. When required, oxygen was added through a silicone membrane tube as described previously (14). During oxygenation, the culture medium was pumped by way of a peristaltic pump (flow rate, 10 liters h^{-1}) through a silicone tube (length = 2.2 m) set in a jar previously saturated with pure oxygen. The dissolved oxygen transfer rate to the culture medium was 0.8 mg min^{-1} liter^{-1} (14). Oxygen additions were repeated several times without significant fouling or changing of silicone membrane properties.

Genetic methods. (i) Disruption of the *OLE1* gene. *OLE1* was disrupted by deleting the open reading frame (ORF) as described by Güldener et al. (26). A 1.7-kb fragment containing a dominant resistance module (*kanMX*) was amplified by PCR with plasmid pUG6-kanMX4 as a template and two oligonucleotides (5′-CTT TAA TGG GCT CCA AGG AAA TGG TTT CCG TGG AAT TCG ACA AGA TTC GTA CGC TGC AGG TCG AC-3′ and 5′-ATC AGC CAA GAC ATT TTG AGC GGC ATT TGA GTG ACG GTA GAC ACC GCA TAG GCC ACT AGT GGA TCT G-3′) that are homologous to the pUG6-kanMX4 multicloning site. These primers also contained 45 nucleotide extensions that were homologous to regions downstream of the start codon (nucleotides 161 to 205) or surrounding the stop codon (nucleotides 1396 to 1440) of the *OLE1* ORF (1,533 nucleotides). The PCR product was used to directly transform strain V5 by the lithium acetate method (56). Cells were incubated at 28°C in YPD medium for 14 h and then plated out on YPD medium containing 150 mg of G418 (geneticin) liter^{-1}.

(ii) Disruption of the *ERG1* gene. *ERG1* was disrupted by a similar method: the two oligonucleotides 5′-ATG TCT GCT GTT AAC GTT GCA CCT GAA TTG ATT AAT GCC GAC AAC TTC GTA CGC TGC AGG TCG AC-3′ and 5′-TTA ACC AAT CAA CTC ACC AAA CAA AAA TGG GGT GAA TAC TCT AAT GCA TAG GCC ACT AGT GGA TCT G-3′ were homologous to the pUG6-kanMX4 multicloning site. These primers had 45 nucleotide extensions that were homologous to regions downstream of the start codon (nucleotides 1 to 45) or surrounding the stop codon (nucleotides 1447 to 1491) of the *ERG1* ORF (*YGR175C*, 1,491 nucleotides). Due to the aerobic exclusion of sterols (41), the *erg1* mutant was unable to grow under aerobic conditions. This phenotype of the *erg1* mutant was systematically monitored before use, as mutations in the heme synthesis pathway (spontaneous *hem* mutants) can suppress this growth phenotype by allowing the efficient uptake of sterols under aerobic conditions (41).

Correct replacement of the *OLE1* and *ERG1* ORFs by the *kanMX4* cassette was checked by PCR on lysed cells (31).

Analytical methods. (i) Number and volume of cells. Cell numbers and volume were determined by using an electronic particle counter (model ZB2; Beckman-Coulter, Margency, France) fitted with a 100-μm-diameter probe after sonication (30 s, 10 W).

(ii) Cell dry mass. The cell dry mass was determined by filtering 50 ml of culture medium through membrane filters (pore size, 1.2 μm). Filters were rapidly rinsed three times with 1 ml of distilled water and desiccated at 105°C until reaching a constant weight (24 h).

(iii) Cell viability. Cell viability was determined by plating out about 250 cells on petri dishes containing YPD agar medium (20 g of agar liter^{-1}, 10 g of yeast

extract [Difco] liter^{-1}, 20 g of Bacto peptone [Difco] liter^{-1}, 50 g of glucose liter^{-1}, and anaerobic growth factors). Petri dishes were then incubated at 28°C for 60 h. When necessary, the cells were grown under anaerobic conditions by storing the plates in anaerobic jars fitted with Anaerocult A (Merck). When specified in the text, viability was directly determined on harvested cells by an epifluorescent method with the magnesium salt of 1-anilino-*o*-naphthalene sulfonic acid (35). For each assay, at least 300 cells were numbered in random microscopic fields.

(iv) Fermentation kinetics. The amount of CO_2 released was determined by automatic measurement of fermentor weight loss every 20 min (50). Loss of ethanol and water due to CO_2 stripping represented less than 2% of the total weight loss. The CO_2 production rate was calculated by polynomial smoothing of the last ten measurements of fermentor weight loss. The frequent acquisition of CO_2 release and the precision of the balance (0.01 g) allowed calculation of the CO_2 production rate with good precision and reproducibility [coefficient of variation for $d(CO_2/dt)_{max} = 0.8\%$] (13). The fermentation progress (FP) was calculated from the amount of CO_2 released from the culture medium according to the following equation: $FP = CO_{2,t}/CO_{2,max}$, where $CO_{2,t}$ is the cumulative amount of CO_2 released at any time t, and $CO_{2,max}$ is the total observed amount of CO_2 released at the end of complete fermentation. The use of fermentation progress instead of fermentation time allows a normalization of the fermentation kinetics, which is closely linked to the disappearance of the substrate from the medium. Fermentation was considered to be completed when the medium contained less than 2 g of residual glucose liter^{-1}. In the present study, no fermentation stopped before 99% completion.

(v) Measurement of oxygen consumption. Oxygen consumptions were measured at 28°C by using a high-resolution oxygraphic system (Oroboros, Innsbruck, Austria). Data were recorded at sampling intervals of 1 s (Datlab Acquisition software; Oroboros, Innsbruck, Austria) (27). After harvesting, cells were quickly washed twice in cold physiological saline (9 g of NaCl liter^{-1}). Oxygen consumption by yeast cells (about 90×10^6 cells ml^{-1}) was then rapidly measured under normoxic conditions (between 3.75 and 7.5 mg of O_2 liter^{-1}) in a buffer containing 31 mM citric acid, 45 mM D,L-malic acid, 10 mM KH_2PO_4 (pH 3.3), and 10% (wt/vol) glucose.

RESULTS

Determination of lipid and oxygen requirements of *S. cerevisiae* V5 for anaerobic growth. First, we studied the effect of anaerobic growth factors (ergosterol and Tween 80) on the anaerobic growth of *S. cerevisiae* V5. As expected, the presence of sterols and fatty acids in the medium resulted in normal fermentation and a final biomass of 170×10^6 cells ml^{-1} (Fig. 1). In their absence, anaerobic cells only divided 2.8 to 3 times (7×10^6 to 8×10^6 cells ml^{-1}) in 30 h (Fig. 1A), whereas the yeast population remained viable at this time (92% ± 3%). This residual growth has previously been observed by several other authors (3, 24, 32) and has been attributed to the transfer of sterols and UFAs from the inoculum (24). Under these conditions (45 h of culture, 87% ± 4% cell viability), the addition of 5 mg of oxygen liter^{-1} restored a normal fermentation (Fig. 1B) and the cells divided about four more times (final population, 120×10^6 cells ml^{-1}) (Fig. 1A). At the time of oxygen addition, the oxygen consumption rate (resistant to antimycin A) of the cells was about 2.9 mg of O_2 h^{-1} per 10^{10} viable cells, which corresponds to 8.4 mg of O_2 h^{-1} g (dry mass) of yeast^{-1}. From the total amount of oxygen added and the cell biomass, we estimated that all of the added oxygen was consumed between 20 min and 5 h before growth resumed. We used this experimental approach (addition of growth factors after 45 h of culture in a synthetic lipid-depleted medium, after cells had entered a true stationary phase) to quantify the oxygen, oleic acid, and ergosterol requirements for strain V5 growth (Fig. 2). About 5 to 7.5 mg of oxygen liter^{-1} was required for the optimal growth of V5 (Fig. 2A). These values are compatible with previous data obtained under enological

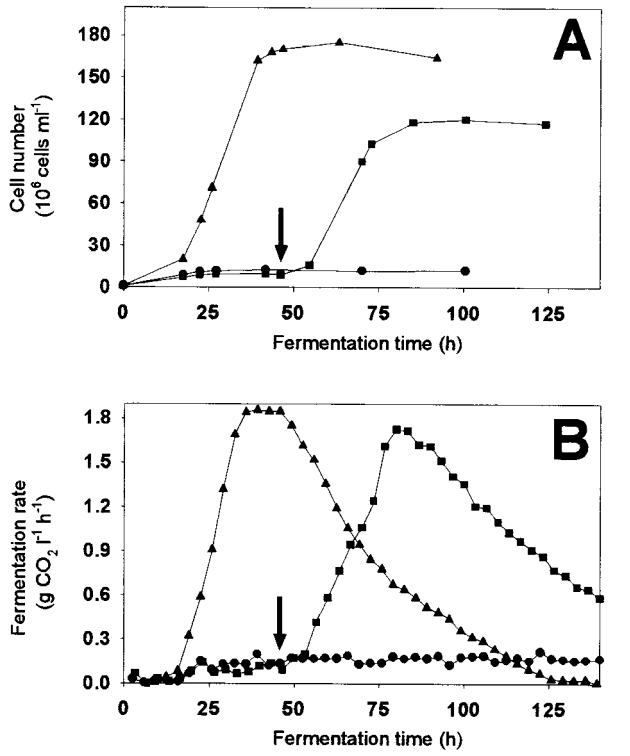

FIG. 1. Variations in cell population (A) and CO_2 production rate (B) as a function of the lipid composition of MS medium. The V5 strain was anaerobically cultured in the presence (▲) or absence (●, ■) of anaerobic growth factors (1 ml liter^{-1} of 15-mg/ml ergosterol dissolved in Tween 80-pure ethanol [50:50, vol/vol]). The arrows indicate the addition of 5 mg of dissolved oxygen liter^{-1} in the absence of anaerobic growth factors (■). For clarity, only 50 of the 500 data points are shown on the graph in panel B.

(49) or brewery (21) conditions. Similar values were obtained in the presence of antimycin A, suggesting that the respiratory chain is not induced during oxygen pulses (data not shown). In the presence of excess oleic acid (1 ml of Tween 80-pure ethanol [50:50, vol/vol] liter^{-1}, corresponding to 74 mg of total oleic acid liter^{-1}), maximal growth was achieved with 5 mg of ergosterol liter^{-1}, which is in agreement with previous results (32) (Fig. 2B). As all the supplied ergosterol is incorporated into yeast cells (22), ergosterol accounted for about 0.18% of the final biomass (148×10^6 cells ml^{-1}, about 2.8 g [dry mass] of yeast liter^{-1}). This is the average ergosterol content found in anaerobically grown glucose-repressed *S. cerevisiae* cells (8, 9, 20, 46). When the medium contained excess ergosterol, 15 mg of oleic acid liter^{-1} sustained maximal growth (Fig. 2C). This value is very similar to that obtained by Barber and Lands (10) but lower than that obtained by Verduyn et al. (61). The oxygen equivalence of ergosterol and oleic acid requirements were calculated and are listed in Table 1. The oxygen required for the growth of V5 exactly matched the cellular requirements for both ergosterol and UFA under anaerobic conditions. Moreover, 75% of the oxygen requirements accounted for sterol synthesis. However, the potential accumulation of sterol intermediates would probably lower the total amount of oxygen required for sterol biosynthesis. This was not considered in

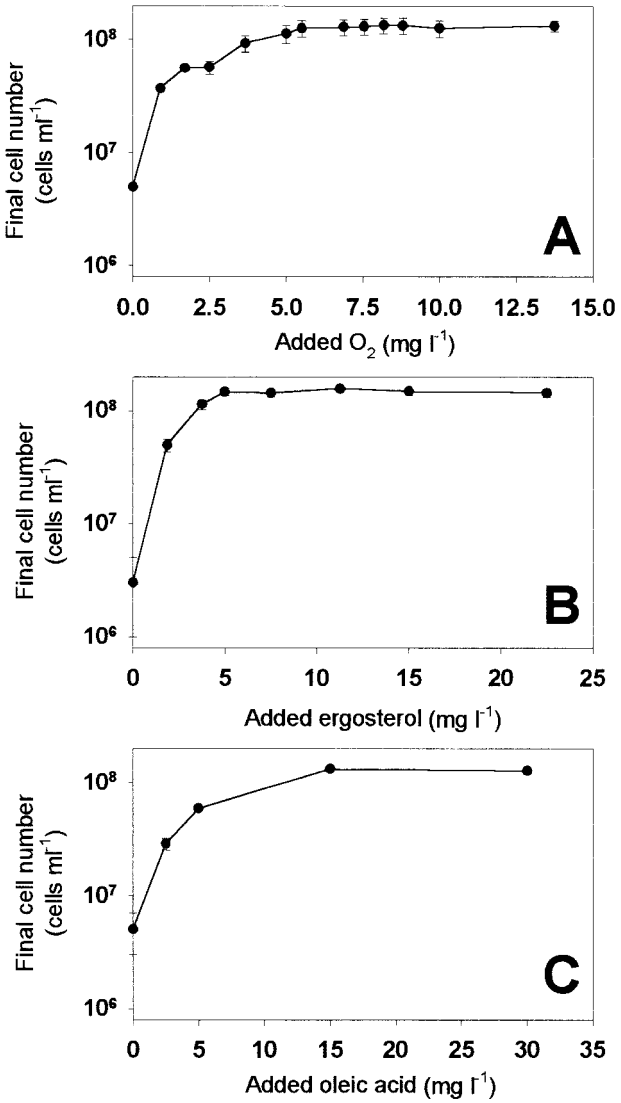

FIG. 2. Effect of adding oxygen (A), ergosterol (B), or oleic acid (C) to wild-type strain V5 grown as described in the legend to Fig. 1 for 50 h under anaerobic conditions in MS synthetic lipid-depleted medium without anaerobic growth factors. The final cell density was measured after a further 50 h. For panel B, 1 ml of Tween 80-pure ethanol (50:50, vol/vol) liter^{-1} was added to dissolve ergosterol and act as a source of excess oleic acid. For panel C, ergosterol (1 ml liter^{-1} of 15-mg/ml ergosterol dissolved in Tergitol NP-40–pure ethanol [50:50, vol/vol]) was added. The means and ranges from two different experiments are given.

our calculations. Nevertheless, these results appear to contradict previous results obtained under brewery conditions, which suggested that only 20 to 30% of the available oxygen was used for the synthesis of lipids (37, 43). As a consequence, we further studied the effect of oxygen addition during alcoholic fermentation in a synthetic medium containing an excess of all of the anaerobic factors essential for the growth of V5 (15 mg of ergosterol liter^{-1} and 1 ml of Tween 80-pure ethanol [50:50, vol/vol] liter^{-1}).

Effect of oxygen on fermentation of *S. cerevisiae* V5 in the presence of anaerobic growth factors. The punctual addition of oxygen (5 mg liter^{-1}) during the growth phase (at 26 or 44 h) led to a low but reproducible increase in both biomass synthesis (Table 2) and fermentation rates (Fig. 3). Similar results were obtained with oxygen additions in the range 1 to 10 mg liter^{-1} (data not shown). However, when oxygen was added during the stationary phase (50.5 and 61 h), an increase in the fermentation rate was observed (Fig. 3), but the cell population was not affected (Table 2). The specific fermentation rate was more increased when oxygen was added during the stationary phase than when it was added during the growth phase (Table 2). This was accompanied by a long-term increase in cell viability during the stationary phase (Fig. 4A). This phenomenon has also been observed during industrial fermentations but never explained. This is especially true in the presence of excess ergosterol and oleic acid (49, 51). Identical results were obtained following the addition of antimycin A (5 μM) or when the respiratory-deficient V5-[*rho*^{0}] mutant was used, indicating that this effect is not due to the function of the classical respiratory chain (data not shown). We also confirmed that the oxygen pulses (1 to 10 mg liter^{-1}) did not induce any respiratory chain activity, as revealed by the full antimycin A resistance of oxygen consumption by [*rho*$^+$] cells measured under normoxic conditions (data not shown). Moreover, the stimulation of fermentation by oxygen pulse (5 mg liter^{-1}) during the stationary phase was still observed in the presence of cycloheximide (data not shown). This suggests that such stimulation of the fermentation rate by oxygen does not require de novo protein synthesis. As this phenomenon may be of great importance for industrial fermentations with regards to the acquisition of ethanol tolerance mechanisms, we further studied the effect of oxygen during the stationary phase.

TABLE 1. Comparison of the amounts of oxygen, ergosterol, and oleic acid required for the anaerobic growth of *S. cerevisiae* V5

Growth-limiting nutrient	Minimal requirement for maximal growth (μmol/10^9 cells)[a]	Equivalent oxygen requirement (μmol/10^9 cells)
Oxygen	1.300	1.300
Ergosterol	0.084	1.008[b]
Oleic acid	0.310	0.310[c]

[a] Nutrient requirements were calculated by dividing the minimal amount of nutrient required to reach the maximum biomass (Fig. 2) by the biomass (cell number).

[b] The equivalent oxygen requirement was calculated by assuming that the biosynthesis of 1 mol of ergosterol requires 12 mol of oxygen (1 O_2 molecule is required to convert squalene to lanosterol, 9 O_2 molecules are required to convert lanosterol to zymosterol, and 2 O_2 molecules are required to convert zymosterol to ergosterol).

[c] The equivalent oxygen requirement was calculated by assuming that the biosynthesis of 1 mol of Δ^9 -monounsaturated fatty acid required 1 mol of oxygen.

Involvement of de novo lipid synthesis in *S. cerevisiae* strain V5 during oxygen addition in the presence of anaerobic growth factors. To determine the importance of sterol and UFA neosynthesis in the observed response to oxygen addition, two mutant strains were derived from the wild-type V5 strain. First, we disrupted *ERG1*, which encodes squalene epoxidase, the first enzyme involved in the oxygen-dependent parts of the ergosterol biosynthesis pathway (29, 55). Due to the aerobic exclusion of sterols (41), the resulting *ERG1* null allele strain

TABLE 2. Effect of adding oxygen (5 mg liter^{-1}) during anaerobic fermentation of *S. cerevisiae* V5 in the presence of excess anaerobic growth factors[a]

Culture time (h) (fermentation progress)	Mean no. of cells ± SD (10^6 cells ml^{-1})	Oxygen added	Specific fermentation rate[b] (g of CO_2 h^{-1}/10^{10} cells)		Total fermentation time (h)	Mean final no. of cells ± SD (10^6 cells ml^{-1})
			At the time of oxygen addition	After 10 h		
26 (0.05)	67 ± 2	No	0.132	0.132	131	172 ± 2
		Yes	0.129	0.132	107	209 ± 5
44 (0.37)	167 ± 3	No	0.111	0.092	131	172 ± 2
		Yes	0.108	0.091	104	185 ± 3
50.5 (0.48)	170 ± 4	No	0.102	0.075[c]	131	172 ± 2
		Yes	0.105	0.087[c]	107	175 ± 2
61 (0.65)	172 ± 1	No	0.078	0.050[c]	131	172 ± 2
		Yes	0.080	0.063[c]	107	172 ± 1

[a] From the data presented in Fig. 3.
[b] Specific fermentation rate was calculated by dividing the instantaneous fermentation rate by cell biomass (measured as cell number) at the same fermentation time.
[c] No significant changes in the average cell volume were observed when oxygen addition exerted no detectable effect on cell growth.

(V5 *erg1*::*kan*) was unable to grow under aerobic conditions and required the presence of both oleic acid and ergosterol for anaerobic growth (Fig. 5). Second, we disrupted the *OLE1* gene, which encodes the microsomal Δ^9 fatty acid desaturase (58). The resulting mutant strain (V5 *ole1*::*kan*) clearly required oleic acid for growth (Fig. 5). Under anaerobic conditions, both mutant strains reached a similar final cell number (about 160 × 10^6 cells ml^{-1}) when grown in a complete synthetic medium containing excess oleic acid and ergosterol. The fermentation kinetics of the two mutants were similar to those of the wild-type strain, except that both mutants exhibited an additional 8-h lag phase. The addition of oxygen (5 mg liter^{-1}) during the stationary phase did not have any effect on the fermentation kinetics of the *erg1* strain, showing that the *ERG1* gene product is essential for the effect of oxygen on fermentation rates (Fig. 6B). On the contrary, the *OLE1* gene product is not involved in this response (Fig. 6A). Similar results were obtained in terms of cell viability (Fig. 4). Indeed, when oxygen was added during the early stationary phase, a high proportion

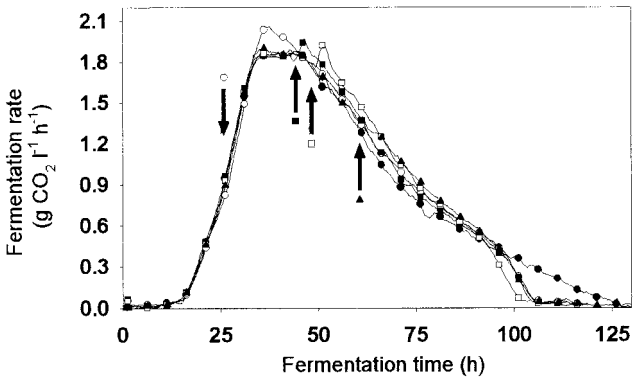

FIG. 3. Changes in the CO_2 production rate by V5 cultured anaerobically in MS synthetic medium in the presence of anaerobic growth factors. For the control experiment (●), arrows indicate the addition of dissolved oxygen (5 mg liter^{-1}) at 26 h (○), 44 h (■), 50.5 h (□), and 61 h (▲). For clarity, only 40 of the 450 data points are shown. Under all culture conditions, about 96 g of CO_2 liter^{-1} and 92 g of ethanol liter^{-1} were produced during the fermentation of 200 g of glucose liter^{-1}. This experiment was performed twice with good reproducibility. For clarity, only one set of experiments is represented.

(about 60%) of wild-type and *ole1* mutant cells remained viable at the end of the fermentation period (Fig. 4A and B). Considerably fewer *erg1* mutant cells were viable under the same conditions (Fig. 4C) (36% ± 8%). These data are consistent with the hypothesis that sterols play a specific role in ethanol resistance and in the increase in the specific fermentation rate following the addition of oxygen (17, 22, 60).

Although *erg1* cell viability (estimated by the epifluorescence method) was not affected by a 5-mg liter^{-1} oxygen addition during the stationary phase (Fig. 4C), the viability of resting cells was progressively affected in response to exposure to normoxia (data not shown). In fact, the strict anaerobe *erg1* strain only seems able to tolerate oxygen when low quantities are added during fermentation.

Contribution of lipid biosynthesis to overall oxygen consumption. In parallel to the analysis of fermentation kinetics, a high-resolution oxygraph was used to determine the overall oxygen consumption capacities of the wild-type and mutant strains. Oxygen consumption capacities were measured on washed cells in the absence of ergosterol throughout the entire fermentation period (Fig. 7). It is noteworthy that these capacities are not related to the classical respiratory chain functionality, as they were not inhibited by specific inhibitors of the bc1 complex (5 to 50 μM antimycin A or 1 to 4 μM myxothiazol) (data not shown). Moreover, the contribution of heme biosynthesis to oxygen consumption is probably negligible because cells grown on glucose have low heme and porphyrin requirements (about 50 nmol g [dry mass] of yeast^{-1}) (40).

Interestingly, the impairment of sterol synthesis in the *erg1* mutant strain resulted in a 40% decrease in oxygen consumption (Fig. 7). On the contrary, the oxygen consumption capacity of the *ole1* mutant and of the wild type were similar over the entire fermentation period. This result is consistent with the fermentation kinetics data, which suggest a strong involvement of de novo sterol biosynthesis and the dispensability of the OLE1 gene in the cellular response to moderate oxygen pulses during nitrogen starvation. This last observation was reinforced by the fact that the hypoxic induction of *OLE1* does not occur in respiration-deficient strains (as shown by Kwast et al. [39]), whereas the V5-[*rho*^{0}] strain exhibits an oxygen consumption capacity similar to that of the wild type (about 2

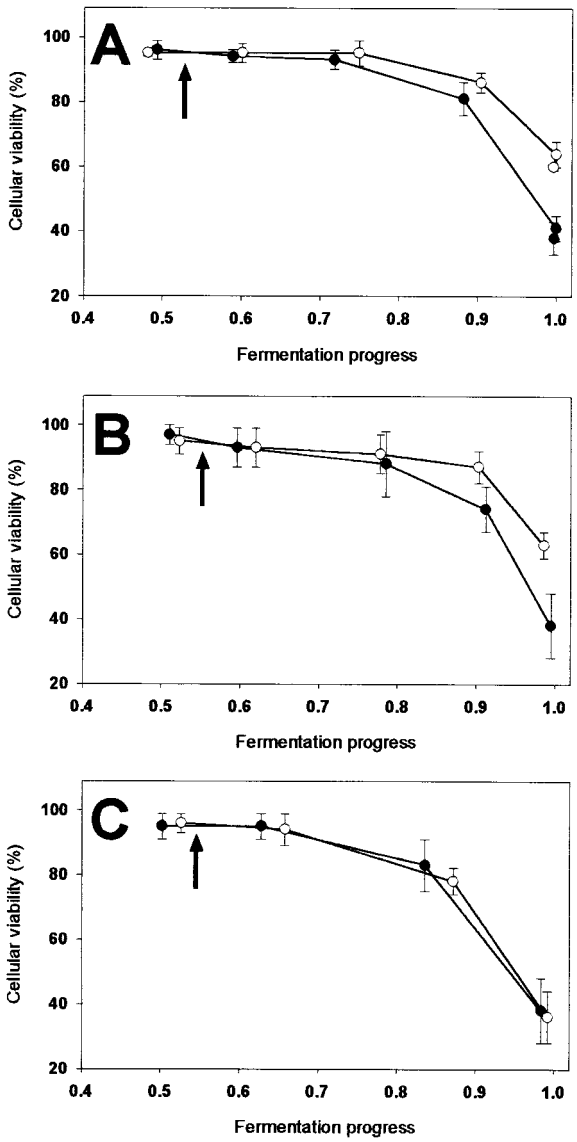

FIG. 4. Changes in percent cell viability as a function of fermentation progress. The wild-type V5 (A) and *ole1*::*kan* (B) and *erg1*::*kan* (C) mutant strains were anaerobically cultured in MS synthetic medium in the presence of anaerobic growth factors. Cell viability was either determined by the plating method (A) or estimated by the epifluorescence method (B and C) (see text). Data were obtained from one of two independent reproducible fermentations. Mean values and standard deviations of three to four determinations are represented. For the control experiment (●), the black arrow indicates the addition of dissolved oxygen (5 mg liter^{-1}) (○) at 50.5 h (A) and 61 h (B and C). The epifluorescent method gives cell viability results similar to those of the plating method but with greater standard deviations.

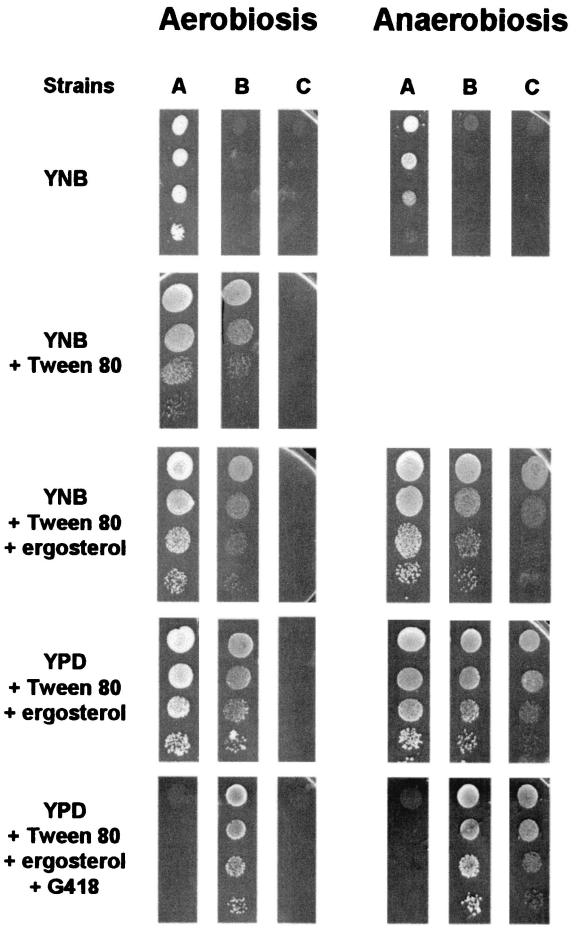

FIG. 5. Comparison of the phenotypes of wild-type V5 (A) and the *ole1*::*kan* (B) and *erg1*::*kan* (C) mutant strains on different types of solid media. Strains were grown for 96 h at 28°C. When required, 1 ml of Tween 80 liter^{-1}, 15 mg of ergosterol liter^{-1}, or 200 mg of geneticin (G148) liter^{-1} was added.

μmol of O_2 min^{-1} g [dry mass] yeast^{-1} during the early stationary phase).

The contribution made by the sterol pathway in the oxygen consumption capacities of the wild-type and mutant strains was also investigated by measuring the effects of several inhibitors. As expected, this contribution was considerable in the wild type and the *ole1* mutant (Table 3). The oxygen consumption capacity of the *erg1* mutant was not inhibited by terbinafine or fenpropimorph, which inhibited several crucial steps of the sterol pathway. This unusual oxygen consumption capacity of the *erg1* mutant might be explained by hydroxylation-desaturation reactions (dependent or not on cytochrome P450) from intermediate sterols to ergosterol. However, this is unlikely to occur, as the added ergosterol did not contain significant amounts of other sterols (22) and the oxygen consumption rates of viable *erg1* washed cells (suspended in lipid-free media) were almost constant for several hours (data not shown). Therefore, although the *erg1* mutant is a strict anaerobe, it is still able to transiently consume oxygen, probably by unknown oxygen consumption pathways. These pathways (which account for a significant amount of the oxygen consumption abilities of the wild-type, [*rho*^{0}], and *ole1* strains) are partially sensitive to cyanide (Table 3). However, the interpretation of this effect is difficult because cyanide probably has several targets. Finally, we postulate that the uncharacterized oxygen consumption retained by anaerobically grown cells may dissipate the added oxygen (moderate pulse) without having any significant effect on fermentation kinetics (as shown in Fig. 6B).

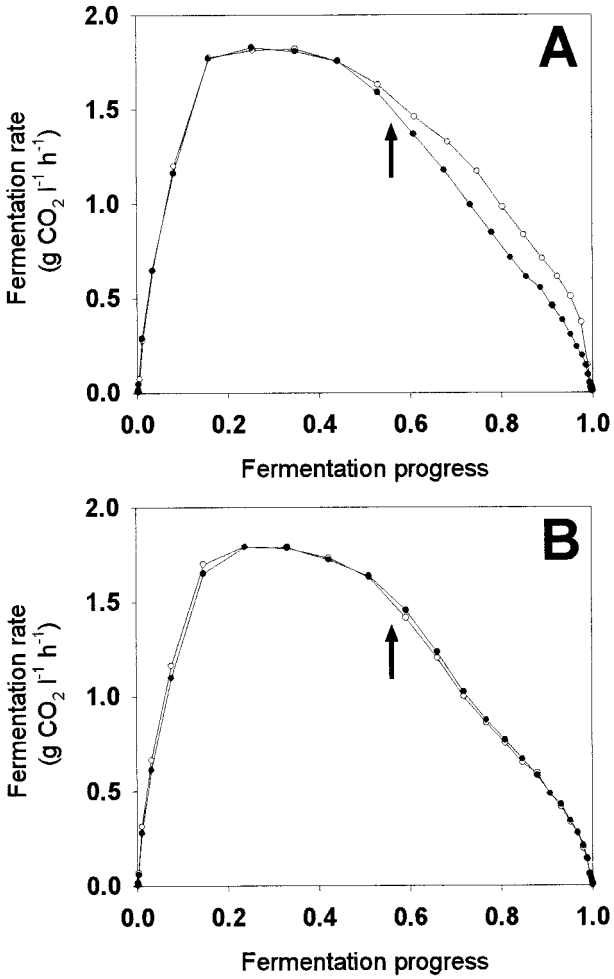

FIG. 6. Effect of oxygen addition on the fermentation of *ole1*::*kan* (A) and *erg1*::*kan* (B) mutant strains grown under anaerobic conditions in MS synthetic culture medium in the presence of anaerobic growth factors. Filled circles denote the control experiment. The arrows indicate the addition of dissolved oxygen (5 mg liter^{-1}) at 61 h (○). The times needed for complete fermentation (95.6 ± 0.8 g of CO_2 liter^{-1}) were 143 h (A) (●), 124 h (A) (○), 140 h (B) (●), and 140 h (B) (○). For clarity, one of three independent reproducible experiments is represented and only 40 of the 450 data points are shown.

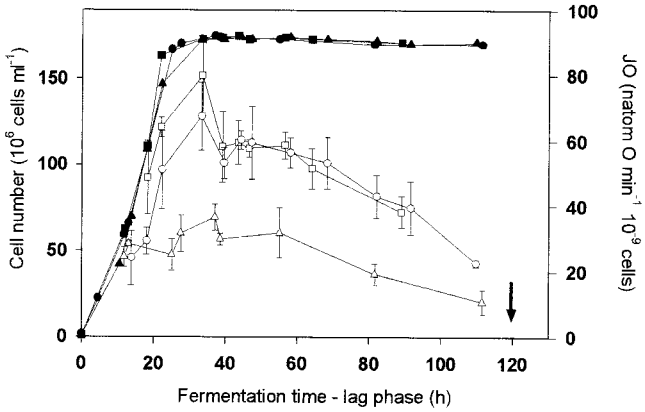

FIG. 7. Changes in the cell number (filled symbols) and specific oxygen consumption capacities (JO) (open symbols) of V5 (circles), *ole1*::*kan* (squares), and *erg1*::*kan* (triangles) strains that had been anaerobically cultured in MS synthetic culture medium in the presence of anaerobic growth factors. The means and standard deviations from three different JO determinations are represented. The lag phase was subtracted from the fermentation time to make it possible to compare the mutant strains with the wild-type strain. The arrow indicates the end of fermentation. Ethanol (50 mM) had no effect on the observed oxygen consumption rates.

DISCUSSION

Anaerobic cells retain a capacity to consume oxygen. This capacity may be related to the anaerobic induction of several genes encoding enzymes involved in oxygen utilization (39). However, the relative oxygen fluxes passing through the numerous pathways implicated (e.g., sterol, UFA and heme synthesis, classical or alternative respiratory chain, and soluble oxidases, etc.) have yet to be described. Moreover, whereas the addition of oxygen stimulates enological fermentation (52), the oxygen consumption pathways involved in these phenomena had not been identified.

The first part of the present study looked at the effect of oxygen addition on anaerobic batch fermentation kinetics (enological conditions) as a function of the lipid composition of the medium. We demonstrated that a pulse of dissolved oxygen (1 to 10 mg liter^{-1}) can stimulate alcoholic fermentation, even in the presence of excess ergosterol and oleic acid and without the functioning or induction of the mitochondrial respiratory chain. This phenomenon appeared to contradict outwardly the well-known Pasteur effect (i.e., inhibition of glycolysis by mitochondrial respiration). However, such an oxygen pulse stimulates fermentation only moderately but finally has an influence on long-term fermentative capacities. We showed that de novo sterol synthesis (in the presence of excess ergosterol) is required for the increase of the specific fermentation rate, whereas de novo UFA or protein synthesis is not required. The sterol molecule responsible for this effect has yet to be identified. The use of other *erg* mutants may lead to the identification of this molecule rather than the use of the anaerobic addition of sterols, which also implies the addition of

TABLE 3. Effect of sterol biosynthesis inhibitors on oxygen consumption of anaerobically grown cells[a]

Inhibitor (concn)[b]	Target(s) in sterol biosynthesis pathway	% Inhibition of oxygen consumption by V5 strain[g]:		
		Wild type	*ole1*::*kan*	*erg1*::*kan*
Terbinafine[c] (1 μM)	ERG1[f]	27 ± 6	25 ± 5	0
Fenpropimorph[d] (3 μM)	ERG24, ERG2, ERG4	23 ± 5	26 ± 4	0
KCN[e] (2.5 mM)	ERG25[f], ERG3[f]	53 ± 3	57 ± 6	33 ± 4

[a] Yeast cells were anaerobically grown in the presence of anaerobic growth factors and harvested during the early stationary phase (54 h for wild-type V5 and 62 h for the V5 *erg1*::*kan* mutant). Oxygen consumption rates were rapidly measured on washed cells.
[b] Optimal concentrations of the inhibitors were added from stock solutions prepared in methanol.
[c] See reference 29.
[d] Fenpropimorph acts in the same way as other morpholines (12).
[e] See references 5 and 43.
[f] Enzymes directly involved in hydroxylation-desaturation reactions.
[g] The means and standard deviations of three different determinations are shown.

detergents. The major effect of moderate oxygen additions was on yeast survival (long-term response). Therefore, we hypothesize that a de novo sterol synthesis increased the cellular tolerance to ethanol. However, the short-term response to the oxygen pulse suggests that endogenous sterols have a specific role in the maintenance of the high fermentative capacities of viable cells. Newly synthesized sterols may incorporate plasmalemma microdomains, which may in turn enhance glucose uptake, especially during the stationary phase.

The strong involvement of sterol synthesis during oxygenation of stationary-phase anaerobically grown cells was also quantified in terms of oxygen consumption capacity. By using terbinafine and fenpropimorph, we showed that the sterol pathway accounts for about 30% of the overall oxygen consumption capacity developed in the presence of anaerobic growth factors (but measured in their absence). As anaerobic cells seem to retain a high apparent affinity for oxygen, the sterol pathway may play a crucial role both in hypoxic conditions and during the early stages of normoxic growth. However, it is important to remember that normoxia and the addition of moderate amounts of oxygen exert two distinct and cumulative long-term effects on glucose metabolism, namely the induction of respiratory chain activity despite the strong glucose repression (Rosenfeld et al., submitted) and the stimulation of fermentation rate (this study). These two effects required distinct mechanisms of sterol distribution among cellular membranes. Our study also demonstrated that anaerobically grown cells retain a relatively high oxygen consumption capacity, which seems linked to neither (i) classical respiratory chain activity, (ii) heme biosynthesis, nor (iii) sterol and UFA synthesis. The corresponding alternative cellular pathways also seem to retain high oxygen affinities and likely permit the dissipation of the added oxygen. The subcellular localization, characterization, and analysis of the physiological role of these unusual oxygen consumption pathways are currently under way in our laboratory.

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