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Survival of the anaerobic fungus *Orpinomyces* sp. strain C1A after prolonged air exposure

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Anaerobic fungi are efficient plant biomass degraders and represent promising agents for a variety of biotechnological applications. We evaluated the tolerance of an anaerobic fungal isolate, *Orpinomyces* sp. strain C1A, to air exposure in liquid media using soluble (cellobiose) and insoluble (dried switchgrass) substrates. Strain C1A grown on cellobiose survived for 11, and 13.5 hours following air exposure when grown under planktonic, and immobilized conditions, respectively. When grown on switchgrass media, strain C1A exhibited significantly enhanced air tolerance and survived for 168 hours. The genome of strain C1A lacked a catalase gene, but contained superoxide dismutase and glutathione peroxidase genes. Real time PCR analysis indicated that superoxide dismutase, but not glutathione peroxidase, exhibits a transient increase in expression level post aeration. Interestingly, the C1A superoxide dismutase gene of strain C1A appears to be most closely related to bacterial SODs, which implies its acquisition from a bacterial donor via cross kingdom horizontal gene transfer during Neocallimastigomycota evolution. We conclude that strain C1A utilizes multiple mechanisms to minimize the deleterious effects of air exposure such as physical protection and the production of oxidative stress enzymes.

Anaerobic fungi (Phylum Neocallimastigomycota) are inhabitants of the rumen and alimentary tract of mammalian and reptilian herbivores^{1,2}. Within these ecosystems, anaerobic fungi rapidly and extensively colonize ingested plant materials. Anaerobic fungi are highly fibrolytic organisms and produce a wide array of plant cell-wall degrading enzymes. These enzymes allow for the simultaneous saccharification of the cellulosic and hemicellulosic fractions of plants, and the fermentation of the resulting hexose and pentose sugars to a mixture of volatile fatty acids and ethanol³. Collectively, these capabilities render anaerobic fungi promising agents that could be utilized in a variety of industrial applications⁴, including consolidated bioprocessing schemes for biofuel production from cellulosic biomass³.

Anaerobic microorganisms that are used for biotechnological applications such as ethanol production from lignocellulosic biomass, routinely encounter incidents of accidental oxygen exposure during industrial fermentations⁵. Therefore, if anaerobic fungi are to be adopted for various biotechnological applications, an assessment of the effect of air exposure on their viability for various durations (minutes to hours) is essential. Previous studies examining the survival of anaerobic fungi following air exposure are available, but have focused primarily on the enumeration of native populations of anaerobic fungi in biological samples such as feces, saliva, and rumen digesta^{6–9}. Currently, very few reports exist on the ability of pure cultures of anaerobic fungi to withstand air exposure and these studies have reported widely variable results. Various isolates have been shown to survive anywhere from 5 minutes (*Piromyces* and *Caecomycetes* isolates H1–H3 in¹⁰) to between 9 and 13 hours (*Neocallimastix* isolate R1 in⁷) after air exposure, depending on the culturing conditions employed⁷.

We are currently evaluating the suitability of an anaerobic fungal isolate (*Orpinomyces* sp. strain C1A) for the production of biofuels from cellulosic biomass. This strain is easy to maintain and has survived greater than 400 subcultures in a cellobiose medium supplemented with rumen fluid. As such, it does not exhibit senescence, previously observed in multiple anaerobic fungal strains¹¹. Strain C1A can also efficiently metabolize several types of biomass that have been touted as biofuel crops including switchgrass, corn stover, sorghum, and energy cane³. Here, we examined its tolerance to atmospheric air exposure for various time intervals and under different culturing conditions. We also analyzed the recently published genome sequence of strain C1A to identify genes putatively involved in protection against oxidative stress and quantified gene expression patterns post exposure.



The results clearly indicate that strain C1A can survive exposure to atmospheric air for time intervals that vary depending on the culturing conditions, and suggest a combined mechanism of physical shielding, prompt sporulation, and production of enzymes protective against oxidative stress.

Results

Survival of strain C1A post aeration. A. In liquid medium. Nearly identical numbers of fungal colonies were recovered in aliquots of liquid cellobiose medium that were collected at different time intervals up to 6 hours post oxygen exposure (Table 1). The first negative effects of oxygen exposure on strain C1A were observed after 7.5 hours (Table 1). The number of viable thallus forming unit (TFU) declined substantially after this 7.5 hours time point and were no longer recoverable from liquid cellobiose medium after 11 hours of oxygen exposure (Table 1). No growth inhibition was observed in tubes that were not subjected to aeration, as well as those that were subjected to aeration using CO₂ instead of oxygen at all sampled time intervals (Table 1).

B. In liquid medium amended with a solid agar surface. Similar to C1A in liquid medium, no significant difference was observed in the number of colonies recovered between 0 and 6 hours after aeration. The first negative effects of oxygen were observed 7.5 hours after aeration (Table 1). The decline in viable TFU observed after the 7.5 hours time point in microcosms with agar was not as steep as the decline observed in microcosms that only contained liquid media. This observation, coupled with the fact that C1A colonies were detectable for longer periods of time after oxygen exposure in the presence of agar (13.5 hours) than in the absence of agar (11.5 hours), suggests that oxygen tolerance was slightly enhanced when strain C1A was immobilized (Table 1). No growth inhibition was observed in tubes that were not subjected to aeration, as well as those that were subjected to aeration using CO₂ instead of oxygen at all sampled time intervals (Table 1).

C. In switchgrass-amended medium. No significant difference was observed in the number of colonies recovered between 0 and 8 hours after aeration (Table 2). Only very slight declines in the numbers of

viable colonies were observed in aliquots taken from the switchgrass-grown cultures of strain C1A following either 25.5 or 31.5 hours of oxygen exposure (Table 2). While the number of fungal colonies recovered from the switchgrass-grown cultures of strain C1A began to decline significantly after 48.5 hours of oxygen exposure, viable fungi were still recovered from aliquots of the switchgrass-grown cultures of strain C1A following 55 to 168 hours of oxygen exposure (Table 2). No viable fungal colonies were recovered from aliquots of the switchgrass grown cultures of strain C1A following either 192 or 216 hours of oxygen exposure (Table 2). Thus, it appears that strain C1A was able to survive for at least 168 hours in liquid medium that contained switchgrass as the sole carbon source. No growth inhibition was observed in tubes that were not subjected to aeration, as well as those that were subjected to aeration using CO₂ instead of oxygen at all sampled time intervals (Table 2).

Identification, phylogenetic analysis, and expression profiles of oxidative stress genes in strain C1A.

We queried the genome and transcriptome of C1A for the presence of oxidative stress enzymes. The C1A transcriptome and genome contain genes encoding for superoxide dismutase (SOD) (IMG accession number 2510868704), and glutathione peroxidase enzymes (IMG accession number 2510864274). Both the genome and transcriptome lack catalase-encoding genes. SOD catalyzes the dismutation of oxygen free radicals (O) to oxygen and hydrogen peroxide. Glutathione peroxidase (GTH) is an oxidative stress enzyme present in multiple strict anaerobes that catalyzes the reduction of hydrogen peroxide to water. Strain C1A superoxide dismutase belongs to the Fe/Mn family, a family of SODs that has been widely encountered in prokaryotes and eukaryotic mitochondria¹². Similar to all bacterial, fungal, and algal GTH enzymes, C1A GTH belongs to group II GTH cluster¹³. Interestingly, homology search and detailed analyses of the phylogenetic affiliation of C1A SOD gene sequence revealed its close similarity to SODs from bacterial genomes, which implies its acquisition from bacterial counterparts via horizontal gene transfer (HGT) (Fig. 1A). On the other hand, analysis of the phylogenetic affiliation of C1A GTH suggests its close affiliation to GTH from fungi and early-diverging eukaryotes (Fig. 1B).

Expression patterns and activity of SOD and GTH. We used RT-PCR analysis to examine the patterns of gene expression of both SOD

Table 1 | Oxygen tolerance of *Orpinomyces* strain C1A when grown on cellobiose medium in the presence and absence of an agar attachment matrix

Time of sampling post Oxygen Exposure (hours)*	Growth of strain C1A on cellobiose without agar**	Growth of strain C1A on cellobiose with agar**
0	+++++	+++++
1	+++++	+++++
2.5	+++++	+++++
3.5	+++++	+++++
5	+++++	+++++
6	+++++	+++++
7.5	++++	++++
9	++	+++
11	++	+++
13.5	-	+
15.5	-	-
16.5	-	-
17.5	-	-
18.5	-	-

*TFU counts $>9.5 \times 10^3$ (i.e. +++) were observed in positive control tubes that were not subjected to aeration at all sampled time intervals, as well as in tubes aerated with 100% CO₂. **Values are indicative of the number of thallus forming units (TFU) recovered on roll tubes at different sampling time: +++++ indicates $>9.5 \times 10^3$, ++++ indicates 3.9×10^3 – 9.5×10^3 , +++ indicates 1.1×10^3 – 3.9×10^3 , ++ indicates 1.1×10^2 – 1.1×10^3 colonies, and + indicates 1.0×10^2 – 1.1×10^3 TFUs/0.5 ml sampled aliquots. - indicates no growth.

Table 2 | Oxygen tolerance of *Orpinomyces* strain C1A when grown on switchgrass as a carbon source

Time of sampling post Oxygen Exposure (hours)*	Growth of strain C1A on switchgrass**
0	+++++
8	+++++
25.5	++++
31.5	++++
48.5	++
55	+
64	+
72	+
88	+
99	+
120	+
147.5	+
168	+
192	-
216	-

*TFU counts $>9.5 \times 10^3$ (i.e. +++) were observed in positive control tubes that were not subjected to aeration, as well as in tubes aerated with 100% CO₂ at all sampled time intervals. **Values are indicative of the number of thallus forming units (TFU) recovered on roll tubes at different sampling time: +++++ indicates $>9.5 \times 10^3$, ++++ indicates 3.9×10^3 – 9.5×10^3 , +++ indicates 1.1×10^3 – 3.9×10^3 , ++ indicates 1.1×10^2 – 1.1×10^3 colonies, and + indicates 1.0×10^2 – 1.1×10^3 TFUs/0.5 ml sampled aliquots. - indicates no growth.

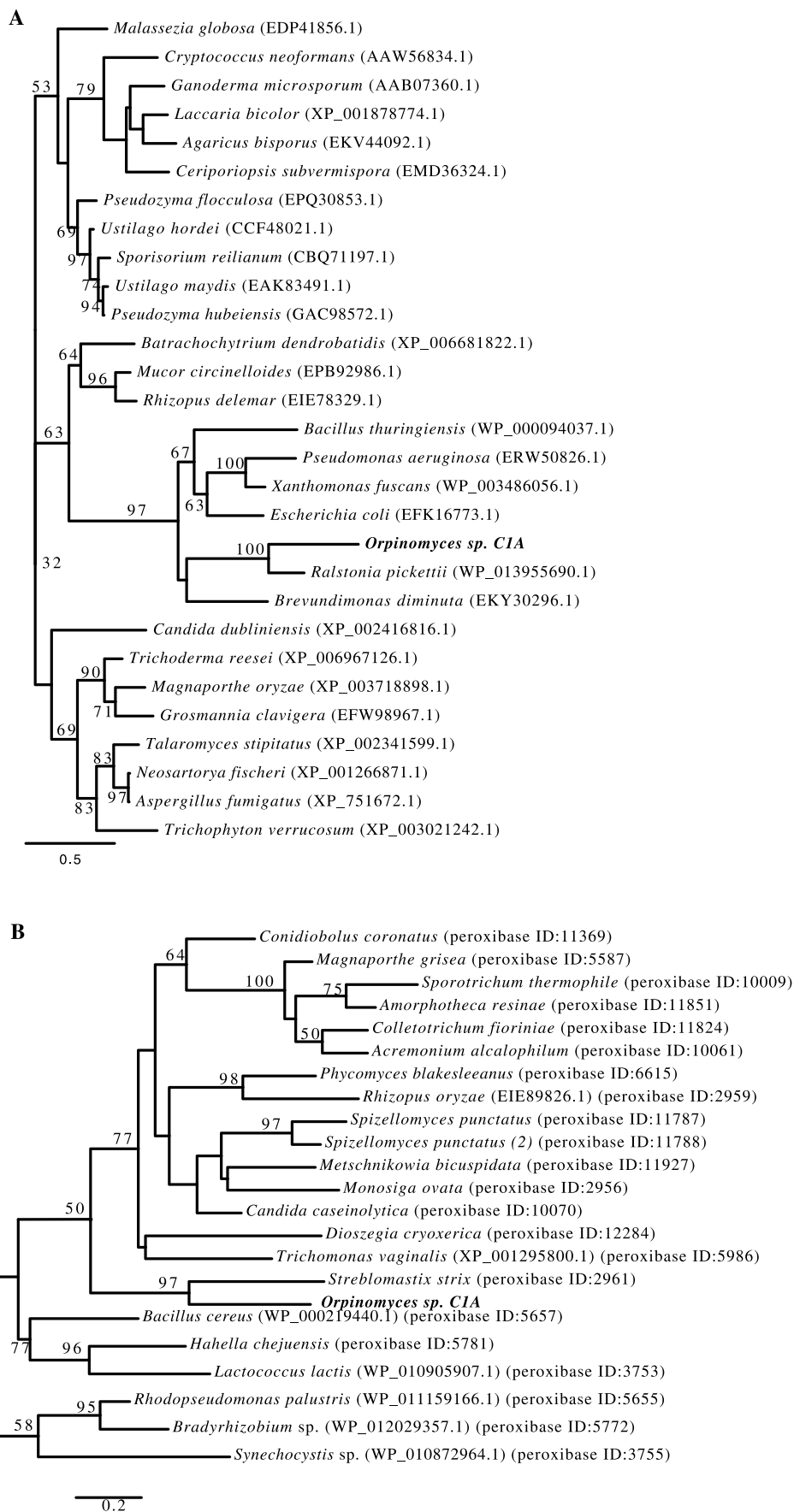


Figure 1 | Phylogeny of *Orpinomyces* sp. CIA (A) superoxide dismutase, and (B) glutathione peroxidase. Maximum likelihood phylogenetic trees were constructed using RaxML²⁷. Bootstrap support values are shown for nodes with > 50% support.


Table 3 | Transcription levels of SOD and GTH relative to β -tubulin in strain C1A prior to and following exposure to air

Duration of air exposure	SOD transcription level compared to β -tubulin*	GTH transcription level compared to β -tubulin*
0	66.6 \pm 14.9	4.12 \pm 1.6
2	75.6 \pm 1.48	3.34 \pm 1.06
5	79.7 \pm 21.6	3.22 \pm 0.84
20	54 \pm 2.38	1.11 \pm 0.34
60	20 \pm 2.44	0.58 \pm 0.13
24 h	11.9 \pm 1.05	1.06 \pm 0.38

*Values are averages \pm standard deviation from three independent measurements.

and GTH genes in response to air exposure. SOD was expressed in anaerobic cultures prior to aeration (Table 3), indicating that it is constitutively expressed in strain C1A (Table 3). Upon aeration, SOD transcription levels transiently increased, peaking at five minutes before decreasing again, presumably due to the overall metabolic inhibition of strain C1A due to oxygen exposure. The transient increase of mRNA levels of this cytosolic enzyme suggests that it acts as a fast protective mechanism to shield intracellular components against transient exposure to atmospheric oxygen in strain C1A, which frequently occurs with the introduction of newly ingested materials to the herbivorous gut. On the other hand, expression levels of GTH were much lower than SOD (Table 3). Further, the expression levels of GTH did not show any significant change following air exposure (Table 3).

While SOD in strain C1A is cytosolic, and appears to protect intracellular metabolism from transient air exposure; the fact that anaerobic fungi undergo slow autolysis and spore release post air exposure could render the release of this enzyme into the culture medium a useful protective mechanism for shielding free anaerobic fungal zoospores for longer periods of air exposure. Prior studies have indicated that superoxide dismutase enzymes are stable and retain activity under aerobic conditions for prolonged periods of time. To examine the stability of C1A SOD, we assayed SOD activity in C1A protein extracts obtained from a growing C1A cultures and maintained under aerobic conditions. Results show strain C1A SOD was stable in C1A protein extracts maintained at room temperatures for prolonged periods of time (Table 4).

Discussion

In this study, we examined the ability of *Orpinomyces* sp. strain C1A to survive prolonged periods of air exposure under different culturing conditions. We show that its survival time was dependent on the type of culturing conditions employed (11, 13, and 168 hours when grown on liquid cellobiose medium alone, liquid cellobiose medium with agar, and liquid medium with switchgrass, respectively). We also identified the presence of superoxide dismutase and glutathione peroxidase genes in the C1A genome, and demonstrated the active transcription and enzymatic activity of SOD genes at different times post aeration. We argue that these results suggest the interplay of multiple factors in conferring air tolerance in this strict anaerobe,

including physical protection from oxygen in culture media, hyphal production of zoospores, and the production of oxidative stress enzymes.

The observed differences in the survival times of strain C1A following air exposure under several different growth conditions suggest a role for physical protection from air in prolonging survival times. Our observations indicated that when subjected to air exposure, the strict anaerobic fungal strain C1A undergoes prompt release of its zoospores, which remains motile for approximately up to 4–5 minutes (Structemeyer et al., personal observation). Within this time interval, zoospores attach to solid material, encyst, and their germination could occur when optimal redox conditions and optimal substrates are provided¹⁴. When grown on a liquid substrate, the growth of strain C1A is characterized by a mixture of free floating cell aggregates, cell aggregates that were bound to the walls of the serum bottle, and cell aggregates that settled to the bottom of the serum bottle. Under these conditions, protection from atmospheric air is minimal, and restricted to the aggregates of biomass the fungus forms. When grown on cellobiose in the presence of an agar surface, much lower concentrations of free floating aggregates of strain C1A were observed (compared to liquid cellobiose medium without agar). This formation of more orderly and structured biofilm reduces the extent of air penetration into lower layers of the culture, hence postponing the deleterious effects of oxygen on C1A zoospores. Switchgrass provided the maximum protection from the deleterious effects of oxygen, since it serves as an attachment matrix into which spores can form cysts (similar to what occurs during anaerobic fungal life cycle in the rumen). Further, when anaerobic fungi grow on plant materials, their hyphae are able to physically penetrate into the plant cell wall, and encysted zoospores would be further protected from atmospheric oxygen as well.

Cells have developed multiple enzymatic-based defense mechanisms to overcome oxidative stress, which occurs as a result of the deleterious effects of reactive oxygen species (ROS) including the superoxide radical, hydrogen peroxide, and the hydroxyl radical, on cellular proteins, lipids, and nucleic acids. The major anti-oxidative enzymes in aerobes are catalase, superoxide dismutase, and peroxidases. In spite of their lowered ability to thrive in the presence of molecular oxygen, anaerobes possess some of the mechanisms found in aerobes to counter the effects of minimal oxygen and/or ROS exposure that may occur in their environments. The roles of such enzymes in protection from air exposure under anaerobic conditions have mostly been studied in prokaryotes. Superoxide dismutase, and catalase activities were overexpressed upon exposure to oxidative stress factors in several anaerobic microorganisms, and SOD activity was detected in *Clostridium acetobutylicum*, *C. perferingens*, *C. glycolyticum*, *Bifidobacterium*, *Lactobacillus lactis*, the majority of *Bacteroides* species, *Desulfovibrio vulgaris*, and *Methanosarcina barkeri* (^{15,16} and references within). In addition to anaerobic prokaryotes, SOD activity was also detected in the anaerobic ciliate, *Metopus* sp.¹⁷, and the anaerobic amitochondriate parasites *Entamoeba histolytica*¹⁸, and *Trichomonas vaginalis*¹⁹. We here show that this enzyme is constitutively expressed in C1A, and that its relative expression levels are elevated shortly after air exposure,

Table 4 | Enzymatic activity of SOD prior to and following exposure to air

Time (h) following air exposure	SOD activity (mU/mg protein)*
0	4.55 \pm 0.21
1	9.26 \pm 0.61
4	4.07 \pm 0.16
24	4.67 \pm 0.31
48	6.39 \pm 0.33

*1 Unit of SOD is defined the amount of the enzyme that will inhibit the rate of reduction of WST-1 dye (2-[4-iodophenyl]-3-[4-nitrophenyl]-5-[2,4-disulfo-phenyl]-2H-tetrazolium, monosodium salt) by 50% in a coupled system, using xanthine and xanthine oxidase at pH 7.8 at 25°C.



before being completely inhibited due to general arrest of growth and transcription. This is in accordance with prior transcriptomic analysis³ in which SOD was constitutively expressed in active cultures grown under different conditions (Transcripts per million of SOD ranged from 38–170). Within its natural habitat, the expression of the cytosolic SOD in anaerobic fungi could be regarded as a defense mechanism to guard its intracellular organelles and metabolic processes against short-term, transient introduction of atmospheric air to the herbivorous guts during food ingestion. However, within our experimental setting, we argue that intracellular oxidative stress enzymes, such as SOD, still play an important role in longer exposures to atmospheric air *ex-vivo*. In addition to zoospore release, air-exposure in strain C1A results in slow hyphal autolysis and the release of cytosolic macromolecules. These released components include intracellular oxidative stress enzymes such as SOD, in addition to numerous compounds possessing reduced moieties e.g. sulfhydryl groups that aid in scavenging oxygen from the culture media.

Interestingly, phylogenetic analysis suggests a distinct prokaryotic origin for strain C1A SOD. The SOD enzyme in strain C1A belongs to the Fe/Mn family of SOD, one of the three known SOD families in addition to Cu/Zn and NiSOD²⁰. Evolutionary, within the Fe/Mn family of SODs, the switch from the ancestral Fe-SOD to Mn-SOD appears to reflect earth history and the lack of global availability of Fe that was associated with the evolution of oxygenic photosynthesis¹². Currently Fe-SODs are encountered in prokaryotes and algae, while Mn-SOD is present in prokaryotes and eukaryotic mitochondria¹². Phylogenetic analysis demonstrates that C1A SOD is monophyletic with prokaryotic Mn-SODs (Fig. 1A), which suggests a possible role of HGT in conferring this activity on C1A. This is in accordance with our current understanding of the importance of HGT in conferring ecologically beneficial traits to recipient species within the microbial world. Indeed, the role of HGT in enhancing lignocellulolytic capabilities of anaerobic fungi has widely been recognized^{13,21}. However, its role in shaping the other aspects of anaerobic fungal biology still needs further study.

In conclusion, this study demonstrates that strain C1A is capable of surviving prolonged exposure to atmospheric air, and suggests that it utilizes a combination of physical shielding and molecular mechanisms to survive such exposures. This trait, coupled to its fast growth rate, ease of maintenance and excellent lignocellulolytic capabilities render scaling-up its growth in industrial settings for various biotechnological applications potentially feasible.

Methods

***Orpinomyces* sp. strain C1A.** Strain C1A was isolated in our laboratory from the feces of an Angus steer in 2009 and maintained as described previously (Fig. 2)^{3,22}.



Figure 2 | Phase contrast micrograph of rhizoidal growth of strain C1A.

Experimental setup. The oxygen tolerance of strain C1A was evaluated under three different aeration and growth conditions (Fig. 3). The decline in the number of anaerobic fungal thallus forming unit (TFU) was monitored under these conditions as well as in an unaerated control. Control experiments included a no aeration control, as well as a CO₂ exposure control, in which the cultures were flushed using 100% CO₂ for identical time and flow rate to that applied in air-exposure experiment. TFU counts $>9.5 \times 10^3$ (i.e. + + + + +) were observed in bottles that were not subjected to aeration as well as in those flushed by 100% CO₂ instead of air at all sampled time intervals, indicating that declines observed are due to the effect of aeration.

1. In defined liquid medium using a soluble substrate. Strain C1A was cultured in forty five milliliters of a basal medium in 120 ml serum bottles²³ with cellobiose (3.75 g/L) as the sole source of carbon.

2. In defined liquid medium with a solid agar surface for immobilization of strain C1A. Previous work demonstrated enhanced plant biomass utilization when strain C1A was immobilized relative to planktonic controls (Struchtemeyer et al., unpublished results). Here, we sought to determine whether immobilization would result in a similar enhancement of air tolerance in strain C1A. Serum bottles were amended with 10 ml of agar, which was dispensed under a stream of 100% N₂ gas using anaerobic techniques²². The serum bottles were then stoppered, sealed, and autoclaved at 121 °C for 20 minutes. After autoclaving, the agar in the serum bottles was allowed to solidify. The agar surface was then overlaid with 45 ml of a sterile anaerobic basal medium inside an anaerobic glove bag (Coy Laboratory Products Inc., Ann Arbor, MI). The media that was used for these amendments contained cellobiose (3.75 g/L) as the sole source of carbon. Following the addition of medium, the serum bottles were removed from the glove bag and their headspace was replaced by repeated vacuuming and pressurization with 100% CO₂²².

3. In defined medium with switchgrass as the sole carbon source. Serum bottles were amended with 45 ml of basal medium and 0.5 g of Kanlow Switchgrass (*Panicum virgatum* var. Kanolow), obtained from Oklahoma State University experimental

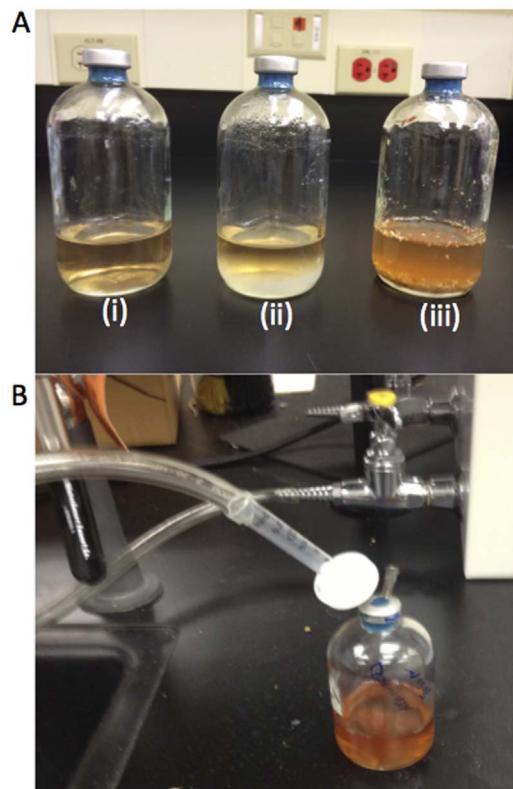


Figure 3 | (A). Media utilized in this study: i. defined liquid media with cellobiose, ii. defined liquid media with cellobiose and a solid agar surface for immobilization, and iii. Defined media with switchgrass as the sole carbon source. (B). Basal setting for aeration of strain C1A. Aeration was conducted by attaching one end of a piece of tygon tubing to a compressed air line and the other end to a sterile 3 ml syringe fitted with a sterile 20 μm filter and a 22 gauge needle that penetrated the butyl rubber stopper of the serum bottles. A second sterile 22 gauge needle was used to vent each microcosm during the aeration process.



Table 5 | Primers used in this study

Primer	Target gene (accession number)	Primer Sequence	Expected size
SOD-1F SOD-1R	Superoxide dismutase (IMG accession number 2510868704)	CAACGCTGCTCAAGTTT CTGAACCAAACGACCAG	176 bp
GTH-1F GTH-1R	Glutathione peroxidase (IMG accession number 2510864274)	AGCAGCAGTTTCAGCTGGT GGCTATGAAGGCTGTCAAGG	211 bp
TUB-1F TUB-1R	β -Tubulin (IMG accession number 2518723601)	GCTTGCCTTTAATGATG CGGTTTTCCATCCTTCT	115 bp

plots in Stillwater, OK. The basal medium used in these serum bottles was dispensed under strictly anaerobic conditions as previously described. The bottles were then stoppered, sealed, and autoclaved at 121 °C for 20 minutes. After autoclaving, the bottles were cooled to room temperature and placed in anaerobic glove bag where switchgrass was added. Following switchgrass addition, the bottles were stoppered, sealed, and removed from the glove bag. The headspace of these bottles was then replaced by repeated vacuuming and pressurization with 100% CO₂. Triplicate serum bottles were prepared for each of the conditions described above. Experiments were initiated by adding 5 ml of an actively growing culture of *Orpinomyces* strain C1A to each serum bottle. After inoculation, the microcosms were incubated at 39 °C for 2 days, which corresponds with mid log phase growth of strain C1A, prior to air exposure.

Evaluation of air tolerance of strain C1A. Serum bottles were aerated using a stream of compressed air (approximately 15 liters/minute) for 5 minutes. Aeration was conducted by attaching one end of a piece of tygon tubing to a compressed air line and the other end to a sterile 3 ml syringe fitted with a sterile 0.2 μ m filter and a 22 gauge needle that penetrated the butyl rubber stopper of the serum bottles (Fig. 3). A second sterile 22-gauge needle was used to vent each microcosm during the aeration process (Fig. 3). Cultures were immediately placed back in a 39 °C incubator following aeration. In all incubations the redox indicator resazurin changed to a pink color upon aeration and remained this color for the duration of these experiments. This observation indicated that the medium was oxygenated. To assess C1A viability, triplicate 0.5 ml aliquots were collected from each microcosm at different time intervals post aeration. These aliquots were used to inoculate triplicate Balch tubes, which contained 9 ml of an anaerobic rumen fluid medium that was amended with cellobiose (3.75 g/L) and agar (15 g/L). After inoculation, these tubes were mixed by hand inversion, then rolled in ice cold water until agar solidification occurred on the sides of the tubes. The agar roll tubes were incubated at 39 °C for 7 days. The number of colonies was counted after incubation to estimate the number of viable thallus-forming units (TFUs) per ml of culture after various air exposure times as previously described²⁴.

Identification of oxidative stress enzymes in strain C1A. Recently published genome and transcriptome of *Orpinomyces* sp. strain C1A were queried for the presence of catalase, superoxide dismutase, and peroxidases³. Phylogenetic analysis was conducted by aligning protein sequences of C1A oxidative stress proteins identified (superoxide dismutase and glutathione peroxidase) with reference sequences using ClustalX²⁵, and the best protein substitution model was predicted using ModelTest 3.7²⁶. The predicted model was applied in maximum likelihood tree construction in RaxML²⁷, with bootstrap support calculated using 100 bootstrapping events.

RNA extraction, cDNA preparation, and real time PCR analysis. Strain C1A was grown on cellobiose media as described above, and RNA was extracted from 50-ml cultures in mid log phase under anaerobic conditions (i.e. prior to aeration), as well as after 2, 5, 10, 30, and 60 minutes, and 24 h post aeration. RNA extraction protocol involved biomass harvesting by vacuum filtration followed by immersion in a mortar containing liquid nitrogen and grounding the biomass with a pestle to fine particles. RNA was extracted using Epicentre MasterPure Yeast RNA Purification kit (Epicentre, Madison, WI, USA). The RNA extracted was quantified using Qubit[®] fluorometer (Life Technologies, Carlsbad, CA, USA). Total RNA reverse transcription (cDNA construction) was conducted on 1 μ g of RNA preparations using Superscript III first strand synthesis kit with random hexamers (Life Technologies, Carlsbad, CA, USA). Quantitative reverse transcription PCR (RT-PCR) was conducted on the cDNA obtained and levels of expression of different genes at different time intervals post aeration were normalized using the C_s obtained for the *tubC* (β -tubulin) gene as suggested before²⁸. Primers were designed using Oligoperfect[™] designer online tool (Life Technologies, Carlsbad, CA, USA). The primer sequences and amplicon sizes are shown in Table 5.

Enzyme assay for SOD. Protein was extracted from C1A log-phase cultures using the protocol described in²⁹. Total protein concentration was determined using Qubit[®] fluorometer (Life Technologies, Carlsbad, CA, USA). Superoxide dismutase enzyme activity in the protein extract was measured colorimetrically using EnzyChrom[™] Superoxide Dismutase Assay Kit (Bioassay Systems, Hayward, CA, USA) directly after extraction. Extracts were then incubated aerobically, and at time intervals,

superoxide dismutase enzyme activity was measured again to quantify the effect of aeration on SOD activity.

- Theodorou, M. K., Davies, D. R. & Orpin, C. G. Nutrition and survival of anaerobic fungi [D.O. Mountfort and C.G. Orpin (eds)] (Marcel Dekker, Inc., New York, NY, 1994).
- Trinci, A. P. J. *et al.* Anaerobic fungi in herbivorous animals. *Mycol. Res.* **98**, 129–152 (1994).
- Youssef, N. H. *et al.* Genome of the anaerobic fungus *Orpinomyces* sp. C1A reveals the unique evolutionary history of a remarkable plant biomass degrader. *Appl. Environ. Microbiol.* **79**, 4620–4634 (2013).
- Ljungdahl, L. G. The cellulase/hemicellulase system of the anaerobic fungus *Orpinomyces* PC-2 and aspects of its use. *Ann. N. Y. Acad. Sci.* **1125**, 308–321 (2008).
- Giannattasio, S., Guaragnella, N. & Marra, E. in *Microbial Stress Tolerance for Biofuels* (ed Z.L. Liu) 58–70 (Springer-Verlag, Berlin, Germany, 2012).
- Lowe, S. E., Theodorou, M. & Trinci, A. Growth and fermentation of an anaerobic rumen fungus on various carbon sources and effect of temperature on development. *Appl. Environ. Microbiol.* **53**, 1210–1215 (1987).
- Milne, A., Theodorou, M. K., Jordan, M. G. C., King-Spooner, C. & Trinci, A. P. J. Survival of anaerobic fungi in feces, in saliva, and in pure culture. *Exp. Mycol.* **13**, 27–37 (1989).
- McGranaghan, Davies, J. C., Griffith, G. W., Daview, D. R. & Theodorou, M. K. The survival of anaerobic fungi in cattle faeces. *FEMS Microbiol. Lett.* **29**, 293–300 (1999).
- Davies, D. R., Theodorou, M. K., Lawrence, M. I. & Trinci, A. P. Distribution of anaerobic fungi in the digestive tract of cattle and their survival in faeces. *J. Gen. Microbiol.* **139**, 1395–1400 (1993).
- Orpin, C. G. Isolation of cellulolytic phycomycete fungi from the caecum of the horse. *J. Gen. Microbiol.* **123**, 287–296 (1981).
- Ho, Y. W. & Barr, D. J. S. Classification of anaerobic gut fungi from herbivores with emphasis on rumen fungi from malaysia. *Mycologia* **87**, 655–677 (1995).
- Miller, A.-F. Superoxide dismutases: Ancient enzymes and new insights. *FEBS Lett.* **586**, 585–595 (2012).
- Margis, R., Dunand, C., Teixeira, F. K. & Margis-Pinheiro, M. Glutathione peroxidase family – an evolutionary overview. *FEBS J.* **275**, 3959–3970 (2008).
- Orpin, C. G. Anaerobic fungi: Taxonomy, biology, and distribution in nature. [D.O. Mountfort and C.G. Orpin (eds)] (Marcel Dekker, Inc., New York, NY, 1994).
- Brioukhanov, A. L. & Netrusov, A. I. Catalase and superoxide dismutase: distribution, properties, and physiological role in cells of strict anaerobes. *Biochemistry (Mosc)* **69**, 949–962 (2004).
- Brioukhanov, A. L. & Netrusov, A. I. Aerotolerance of strictly anaerobic microorganisms and factors of defense against oxidative stress: A review. *Appl. Biochem. Microbiol.* **43**, 567–582 (2007).
- Narayanan, N., Krishnakumar, B. & Manilal, V. B. Oxygen tolerance and occurrence of superoxide dismutase as an antioxidant enzyme in *Metopus*. *Res. Microbiol.* **161**, 227–233 (2010).
- Wassmann, C., Hellberg, A., Tannich, E. & Bruchhaus, I. Metronidazole resistance in the protozoan parasite *Entamoeba histolytica* is associated with increased expression of iron-containing superoxide dismutase and peroxiredoxin and decreased expression of ferredoxin 1 and flavin reductase. *J. Biol. Chem.* **274**, 26051–26056 (1999).
- Leitsch, D., Kolarich, D. & Duchêne, M. The flavin inhibitor diphenyleneiodonium renders *Trichomonas vaginalis* resistant to metronidazole, inhibits thioredoxin reductase and flavin reductase, and shuts off hydrogenosomal enzymatic pathways. *Mol. Biochem. Parasitol.* **171**, 17–24 (2010).
- Fink, R. C. & Scandalios, J. G. Molecular evolution and structure–function relationships of the superoxide dismutase gene families in angiosperms and their relationship to other eukaryotic and prokaryotic superoxide dismutases. *Arch. Biochem. Biophys.* **399**, 19–36 (2002).
- García-Vallve, Romeu, A. & Palau, J. Horizontal gene transfer of glycosyl hydrolases of the rumen fungi. *Mol. Biol. Evol.* **17**, 352–361 (2000).
- Balch, W. E. & Wolfe, R. S. New approach to the cultivation of methanogenic bacteria: 2-mercaptoethanesulfonic acid (HS-CoM)-dependent growth of *Methanobacterium ruminantium* in a pressurized atmosphere. *Appl. Environ. Microbiol.* **32**, 781–791 (1976).



23. Marvin-Sikkema, F. D., Richardson, A. J., Stewart, C. S., Gottschal, J. C. & Prins, R. A. Influence of hydrogen-consuming bacteria on cellulose degradation by anaerobic fungi. *Appl. Environ. Microbiol.* **56**, 3793–3797 (1990).
24. Theodorou, M. K., Gill, M., King-Spooner, C. & Beever, D. E. Enumeration of anaerobic Chytridiomycetes as thallus-forming units: Novel method for quantification of fibrolytic fungal populations from the digestive tract ecosystem. *Appl. Environ. Microbiol.* **56**, 1073–1078 (1990).
25. Larkin, M. A. *et al.* Clustal W and Clustal X version 2.0. *Bioinformatics* **23**, 2947–2948 (2007).
26. Posada, D. ModelTest Server: a web-based tool for the statistical selection of models of nucleotide substitution online. *Nucl. Acids Res.* **34**, W700–W703 (2006).
27. Stamatakis, A. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* **22**, 2688–2690 (2006).
28. Semighini, C. P., Marins, M., Goldman, M. H. S. & Goldman, G. H. Quantitative analysis of the relative transcript levels of ABC transporter Atr genes in *Aspergillus nidulans* by real-time reverse transcription-PCR Assay. *Appl. Environ. Microbiol.* **68**, 1351–1357 (2002).
29. Bridge, P., Kokubun, T. & Simmonds, M. J. in *Protein Purification Protocols* Vol. **244** [Paul Cutler (ed)] (Humana Press, 2004).

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Author contributions

C.G.S. and M.S.E. designed research, C.G.S., M.B.C., A.R., A.S.L. and N.H. conducted research. C.G.S. and M.S.E. wrote manuscript. QAll authors reviewed the manuscript.

Additional information

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