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A fast and reliable procedure for spore collection from anaerobic fungi:

Application for RNA uptake and long-term storage of isolates.

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Abstract

Anaerobic gut fungi (AGF) represent a basal fungal lineage (Phylum Neocallimastigomycota) that resides in the rumen and alimentary tracts of herbivores. The AGF reproduce asexually, with a life cycle that involves flagellated zoospores released from zoosporangia followed by encystment, germination and the subsequent development of rhizomycelia. A fast and reliable approach for AGF spores collection is critical not only for developmental biology studies, but also for molecular biological (e.g. AMT-transformation, RNAi) approaches. Here, we developed and optimized a simple and reliable procedure for the collection of viable, competent, and developmentally synchronized AGF spores under strict anaerobic conditions. The approach involves growing AGF on agar medium in serum bottles under anaerobic conditions, and flooding the observed aerial growth to promote spore release from sporangia into the flooding suspension. The released spores are gently collected using a wide bore sterile needle. Process optimization resulted in the recovery of up to 7×10^9 spores per serum bottle. Further, the released spores exhibited synchronized development from flagellated spores to encysted spores and finally to germinating spores within 90 minutes from the onset of flooding. At the germinating spore stage, the obtained spores were competent, and readily uptook small interfering RNA (siRNA) oligonucleotides. Finally, using multiple monocentric and polycentric AGF isolates, we demonstrate that AGF grown on agar surface could retain viability for up to 16 weeks at 39°C, and hence this solid surface growth procedure represents a simple, cryopreservative- and freezing temperatures-free approach for AGF storage.

Introduction

The anaerobic gut fungi (AGF) constitute a basal fungal lineage (phylum Neocallimastigomycota) that is encountered in the rumen and alimentary tract of herbivores. The anaerobic gut fungi play an important role in breaking down ingested plant materials in the herbivorous gut by possessing a complete arsenal of lignocellulosic enzymes that enable the degradation of a wide range of plant polysaccharides. The AGF has a life cycle that involves the asexual production and release of flagellated zoospores from sporangia. These zoospores attach and encyst on solid surfaces, leading to the development of extensive rhizomycelia that function as an anchor for the production of zoospore-laden sporangia [1].

Due to their strict anaerobic nature [2], the propensity of many strains for senescence upon continuous subculture [3], and the lack of reliable long-term storage and maintenance procedures [4], progress on various aspects of AGF research has lagged behind their aerobic counterparts. For example, development of molecular biological approaches for genetic manipulations in the AGF (e.g. gene silencing, insertion, deletion, and mutation) is missing. Currently, there are no established procedures for genetic manipulations of AGF using any of the routinely utilized approaches in their aerobic counterparts (e.g. AMT transformation, RNAi). Similarly, apart from microscopic-based observations [5-12], developmental biology studies seeking to understand the physiological, structural, regulatory, and gene expression patterns associated with various stages of development within the AGF complex life cycle have been extremely sparse. The paucity in molecular biological and developmental biological studies is in stark contrast to the rich body of knowledge available on genetic manipulations [13-18] and

developmental biological [19-27] aspects of various aerobic fungal lineages.

Central to achieving progress on the molecular biological and developmental biological fronts in AGF is the ability to collect intact, viable developmentally synchronized spores (i.e. all spores at the same stage of development). Such collection is crucial, since molecular biological protocols e.g. RNAi approaches ([13, 15, 18], and AMT transformation [14, 16, 17] are conducted on spores due to their amenability to nucleic acids uptake. Similarly, the ability to collect viable developmentally synchronized spores will allow for synchronized growth experiments enabling detailed structural, behavior, and physiological investigation of spores at various stages of development, as well as studies on gene expression during spore encystment and germination e.g. RT-PCR and transcriptomics studies [21, 28].

In aerobic fungi, harvesting viable, developmentally synchronized spores is straightforward, and involves flooding aerial sporangia grown on solid surface to induce spore release [28-31]. However, given their strict anaerobic nature, the process is challenging in AGF and requires extensive adaptations and safeguards to allow for aerial sporangial development under anaerobic conditions, as well as spore retrieval from anaerobic containers without transient oxygen exposure, or compromising spores' structural integrity via syringe-based manipulations.

Here, we present a simple and reliable approach for collection of viable developmentally synchronized spores from the AGF isolate *Orpinomyces* sp. strain C1A (henceforth referred to as C1A). The approach is based on multiple modifications of earlier procedures utilized for spore collection from the aerobic basal fungus *Phytophthora megasperma* [31]. We show that this protocol recovers almost exclusively (>90%) spores

94 compared to other life forms (e.g. sporangia, hyphal mats), and that the obtained spores
95 are viable, synchronized with respect to their development stage (swimming, encysted, or
96 germinating), and are capable of uptaking small interfering RNA (si-RNA)
97 oligonucleotides. Moreover, using multiple AGF monocentric and polycentric strains, we
98 demonstrate the value of the developed solid surface growth procedure as a method for
99 storage of AGF without the need for cryopreservative addition or subjecting cultures to
100 sub-freezing temperatures.

Materials and Methods

Microorganisms and culture maintenance. Strain C1A was isolated from the feces of an Angus steer [32] and maintained by routine subculturing in an anaerobic, rumen-fluid-cellobiose medium (RFC) that was reduced by cysteine hydrochloride and dispensed under a stream of 100% CO₂. Media composition was as follows (per liter): 150 ml of minerals solution I (K₂HPO₄ 3 g.l⁻¹), 150 ml of mineral solution II (g.l⁻¹: KH₂PO₄, 3; (NH₄)₂SO₄, 6; NaCl, 6; MgSO₄.7H₂O, 0.6, and CaCl₂.2H₂O, 0.6), 10 ml Balch vitamins solution (mg.l⁻¹: biotin, 2; folic acid, 2; pyridoxine-HCl, 10; thiamine-HCl, 5; riboflavin, 5; nicotinic acid, 5; calcium pantothenate, 5; vitamin B12, 0.1; p-aminobenzoic acid, 5; lipoic acid, 5), 1 ml of Wolin's metal solution (g.l⁻¹: EDTA, 0.5; MgSO₄.6H₂O, 3.0; MnSO₄.H₂O, 0.5; NaCl, 1; CaCl₂.2H₂O, 0.1; FeSO₄.7H₂O, 0.1; ZnSO₄.7H₂O, 0.1; CuSO₄.7H₂O, 0.01; AlK(SO₄)₂, 0.01; Na₂MoO₄.2H₂O, 0.01; boric acid, 0.01; Na₂SeO₄, 0.005; NiCl₂.6H₂O, 0.003; CoCl₂.6H₂O, 0.1), cellobiose 3.75 g, sodium bicarbonate 6 g, and 150 ml of clarified sterile rumen fluid. Medium pH was adjusted to 6.6. Following autoclaving, the medium was amended with kanamycin, penicillin, streptomycin, and chloramphenicol from an anaerobic stock solution in order to provide final concentrations of 50 µg/ml, 50 µg/ml, 20 µg/ml, and 50 µg/ml, respectively. Strain C1A was used for the development and optimization of the approach described below for spore collection. In addition to C1A, five additional strains were used to examine the utility of the developed approach in long term storage of AGF isolates. These isolates are: strains G3, a monocentric species putatively identified as *Neocallimastix* and isolated from goat feces; G3G, C3G and C3J, polycentric species isolated from goat (G3G) and cow (C3G, C3J) feces and putatively identified as *Anaeromyces*; and S4B, a monocentric strain isolated

from sheep feces and putatively identified as belonging to a novel genus that is phylogenetically most closely related to *Orpinomyces*.

A procedure for the recovery of viable, developmentally synchronized AGF spores.

i. Growth on agar media. C1A was grown in 160-ml serum bottles containing 45 mL of RFC medium supplemented with 2% agar (RFC-A) by transferring a 10% inoculum of an actively growing liquid culture. Three inoculation strategies were assessed, with the goal of identifying the strategy that yields the highest number of aerial colonies: 1. Seed inoculum i.e. inoculating C1A while the RFC-A medium was still in a liquid state allowing C1A to grow throughout the agar, 2. Inoculating C1A while the agar was partially solidified in a ‘jelly-like’ state allowing for growth throughout the top to middle layers of the agar, and 3. Surface inoculation of C1A after the agar completely solidified where the inoculum created a culture overlay on the agar surface. In all cases, following inoculation, the agar cultures in the serum bottles were placed on ice laying flat on their side until the agar was completely solidified (Figure 1).

ii. Flooding procedure for spore release and collection.

Following growth of C1A on RFC-A with visible numerable colonies on the agar surface, 10 ml of a solution of sterile anoxic water with L-cysteine hydrochloride (0.05 g/l final concentration) as a reductant, and resazurin (0.0001% final concentration) as a redox indicator (SAW solution) was introduced to the surface of the agar culture by slow injection through the butyl rubber stopper while keeping the serum bottle on its side. The flooded serum bottle was then incubated statically on its side, and special care was taken in order not to disturb the culture so that no other life cycle stages will be dislodged from the agar surface into the flooding solution. We reasoned that such exposure to SAW will

induce spore release from aerial sporangia, and hence only viable, newly released spores that are at the same development stage will be obtained, as previously suggested for *Phytophthora*, *Blastocladiella*, and *Allomyces* strains [28-31]. Following incubation, the flooding solution (SAW plus the released spores) was gently collected anaerobically using a sterile syringe and a wide-bore needle into a pre-sterilized serum bottle with 100% CO₂ headspace. The procedure is outlined in Figure 1.

iii. Process optimization. Various operational parameters were evaluated to determine the optimal conditions that produce (i) the highest number of spores/ml, (ii) the highest percentage of spores as opposed to other life cycle stages, and (iii) viable spores capable of starting new cultures when inoculated in fresh media tubes. The numbers of obtained

spores were counted using a hemocytometer counting chamber and a phase contrast Olympus BX51 microscope (Olympus, Center Valley, PA). Microscopic examinations were also utilized to visually determine whether other life cycle stages, e.g. sporangia, hyphal mats, were co-released with spores into the flooding solution. The spore: other life cycle stages ratio was determined empirically after examining multiple (at least 10) fields of vision. The viability of obtained spores was assessed by inoculating 9 mL of anaerobic RFC medium with 1 mL of the flooding suspension. Tubes were incubated at 39°C, and growth was scored every day (see the footnote to Table 1) for a week and compared to the growth of an active subculture of strain C1A.

The following parameters were evaluated in a single factorial design from a starting empirical condition of 7-day old culture of C1A on RFC-A media with 2% agar flooded with 10 ml SAW and incubated at 39°C for 60 minutes in the dark: (1) Concentration of agar in the serum bottles (1%, 1.5%, 2%, 2.5%), (2) culture age at the onset of flooding (5-9 days of growth on solid agar media), (3) Flooding solution composition (SAW versus a previously suggested sporulation solution of 1mM CaCl₂, 1mM Tris-Maleate with pH adjusted to 6.7 [33]), (4) incubation temperatures (i.e. temperature of incubation between flooding and recovery of spores into a sterile bottle (i.e. between steps 2 and 3 in Figure 1). The following temperatures were tested: 4°C, 22°C, and 39°C. (5) light vs. dark incubation between flooding and recovery, and (6) duration of incubation between flooding and recovery. Incubation times of 2.5, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 minutes were tested.

RNA oligomers uptake by collected spores. Dikarya spores in the germinating stage were shown to be amenable for RNAi and AMT-based transformation approaches

involving nucleic acids uptake. Prior studies have clearly shown that viable spores of aerobic fungi are amenable for nucleic acids scavenging in a development-dependent manner, where germinating spores were found to accumulate the highest amount of exogenously added nucleic acids [34]. We hypothesized that the AGF spores released by flooding would be amenable to nucleic acids uptake at the germinating spore stage. To this end, we monitored the progress of C1A spore development during incubation with SAW solution on the agar surface. We observed that as the incubation time with SAW increases, the spores developed from swimming (<30 minutes), to encysted (50-60 minutes), to swollen elongated germinating spores (90-100 minutes).

Guided by C1A transcriptomic data [35], we designed a Cy3-labeled 21-nucleotide si-RNA (Sense: 5' UCGUUGGCGUGAGCUUCCAUU 3', and antisense 5' UGGAAGCUCACGCCAACGAUU 3'. The overhangs on each sequence are underlined) that theoretically does not anneal to any of the mRNA transcripts in C1A. The sense si-RNA was labeled with Cy3 at the 5' end to facilitate tracking of the si-RNA uptake by the germinating spores. The annealed (double stranded) si-RNA was purchased from Dharmacon (Lafayette, CO). We added the annealed siRNA to the flooding solution after 75 minutes of the onset of flooding (where spores were observed to be at the onset of germinating), and allowed it to incubate at 39°C for an additional 15 minutes. The spores were recovered as described above, and transferred to tubes containing fresh RFC medium such that the final concentration of the annealed siRNA in the culture media was 20 nM. Tubes were incubated at 39°C, and at intervals samples were obtained, stained with DAPI, and examined for the uptake of the fluorescent si-RNA using an Olympus BX51 microscope (Olympus, Center Valley, PA), equipped with Brightline fluorescein

isothiocyanate (FITC) filter set for Cy3, as well as a Brightline DAPI high-contrast filter set for DAPI fluorescence. Photomicrographs were taken with a DP71 digital camera (Olympus). The ratio of Cy3-labeled spores to the total number of spores (DAPI-labeled) was evaluated. The goal here was not only to study the ability of C1A germinating spores to uptake exogenously added si-RNA, but also to examine the effect of the uptake on the normal development of C1A when inoculated in fresh media.

Viability of anaerobic fungal cultures grown on agar surfaces. The spore recovery protocol described above starts with growth of C1A in serum bottles on agar media (step 1 in Figure 1). Fungal growth on solid media has been known to maintain a state of low metabolic activity for prolonged periods of time [36, 37]. As such, we reasoned that this growth procedure could lead to longer viability of AGF cultures compared to liquid media and could be used as a simple freezing- and cryopreservative-free approach for storing AGF. To this end, we inoculated C1A in agar-serum bottles with RFC-A media as described above, and incubated these cultures at 39°C in the dark for extended periods of time (ranging from 2-16 weeks). Serum bottles were flooded at various time intervals (weeks 2, 4, 6, 8, 10, 12, and 16) and the released spores were collected as described above and used to inoculate fresh liquid RFC medium. Growth was monitored and scored in comparison to actively growing, routinely subcultured C1A cultures. The procedure was also tested on other anaerobic fungal isolates (strains G3, G3G, C3G, C3J, and S4B) described above to assess its suitability for long-term storage of a wide range of AGF isolates.

Results

Growth of AGF on agar surface in serum bottle. Strain C1A readily grew on agar media using the setting described above (Figure 1). Preliminary experiments demonstrated that the inoculation strategy employed greatly influences the number of colonies formed on the agar surface. Surface inoculation of agar resulted in patchy, uneven, growth that was loosely attached to the surface (Figure S1A). Seed inoculation of agar prior to solidification resulted in the majority of colonies residing deep within the agar block and hence inaccessible through the surface (Figure S1B). However, inoculation of agar during solidification, i.e. while it is still in a “jelly-like” state, yielded the largest number of colonies on the agar surface (similar to the one shown in Figure 1B-C), and hence was utilized in all subsequent experiments.

Flooding Process Optimization.

• *Effect of flooding conditions on the number of spores released.* All of the six parameters examined, were shown to have a significant effect on spore recovery numbers, 1. Agar concentration: cultures grown on 2% agar produced 8 to 16-fold more spores compared to cultures grown on lower (1-1.5%) and higher (2.5%) agar concentrations (Fig 2A, p-value=0.00015). 2. Culture age: 7 day-old culture produced 10 to 16-fold more spores compared to younger cultures (5-day (p-value=0.00012), 6-day old (p-value=0.00014)), and 43-fold more spores compared to older cultures (9-day old (p-value=0.0001)) (Fig. 2B). 3. Dark versus light incubation: Dark incubations during flooding produced 57-fold more spores compared to flooding in the ambient light (Fig 2C, p-value=0.0001). 4. Incubation temperature: Incubation during flooding at temperatures lower than 39°C resulted in 94- to 117-fold decrease in the number of

spores released (Fig 2D, p-value=0.0001) compared to cultures incubated at 39°C during flooding, 5. Flooding solution composition: SAW released 12.4-fold more spores than did the sporulation solution (Fig 2E, p-value=0.0002) previously used for maintaining *Blastocladiella* spores in the swimming stage [33], and 6. Duration of incubation: 60-minute incubation resulted in the largest number of spores obtained (Figure 2F, p-value=0.0001-0.0016 for number of spores obtained with 60 minutes flooding versus shorter and longer incubation times).

• *Effect of flooding conditions on the ratio of spores released to other life cycle stages:*

Table 1 shows the effect of changing flooding conditions on the percentage of spores obtained in the flooding suspension in relation to other life cycle stages including sporangia and hyphal mats. In the majority of conditions tested, the flooding procedure yielded >90% spores compared to other life forms. However, under few conditions, the flooding process yielded a relatively lower proportion (<90%) of spores. These include flooding 5-day old culture, and flooding for very short (2.5 minutes) or very long times (>90 minutes).

• *Effect of flooding conditions on viability of spores.* To test the viability of spores released, we used the obtained flooding suspension as an inoculum in fresh RFC media and evaluated the growth compared to a regular subculture of actively growing C1A. All conditions tested resulted in the release of viable spores capable of germinating and development into active C1A cultures. When spores obtained from flooding under the optimal conditions detailed above were used to inoculate fresh CBM media tubes, fungal biomass was similar to routine sub-cultures. On the other hand, specific sub-optimal flooding condition, while they produced viable spores, resulted in lower fungal biomass (Table 1).

Collectively, these results suggest that the optimal conditions for obtaining the highest number and percentage of viable spores involve flooding 7-day old C1A cultures grown on 2% RFC-agar with 10 ml of SAW followed by incubation for 1h at 39°C in the dark.

C1A spores are capable of RNA oligonucleotide uptake. Interestingly, the duration of the flooding procedure (duration of incubation with SAW on the agar surface) not only impacted the number of recovered spores as shown above, but also their developmental stage. Microscopic examination revealed the exclusive release of very active swimming spores in incubations shorter than 30 minutes. As the incubation time increases, the proportion of resting (encysted) to motile spores steadily increase. In incubations longer than 80 minutes, the absolute majority of collected spores were in the resting stage, with few spores becoming elongated and some spores showing germ tube emergence. The 90

and 100 minutes incubation flooding produced exclusively germinating spores (Figure 3).

Prior research has demonstrated that germinating spores of *Aspergillus* were capable of uptaking oligonucleotides, and more specifically si-RNA molecules, and hence could be utilized for RNAi-based studies [13-15, 18]. In an attempt to test the feasibility of using the described flooding procedure with longer incubation time (90 minutes to obtain exclusively germinating spores) for future RNAi-based studies in anaerobic fungi, we added a Cy3-labeled double stranded siRNA (20 nM) that bears no sequence similarity to any of mRNA transcripts identified in C1A [35] during a 90-minute incubation flooding of C1A cultures. At the end of the flooding duration, the solution containing the released spores and the Cy3-labeled siRNA was used to inoculate fresh RFC tubes. Cy3-labeled spores were observed starting 5 minutes following transfer to fresh media. The ratio of Cy3-labeled spores to the total number of spores reached its maximum (>90%) at 2 hours following transfer (Figure 4). These results clearly demonstrate the ability of germinating C1A spores to uptake si-RNA, and that the

exogenous addition of si-RNA to the spores had no effect on their development. RFC tubes inoculated with released spores in presence of the Cy3-labeled siRNA showed normal growth (data not shown).

Suitability of AGF solid surface growth as a long-term storage mechanism. Prior research has suggested that AGF colonies maintained on roll tubes could remain viable for extended periods of time [6, 38], although the process was often dependent on addition of plant biomass (sisal), and recovery required the addition of a carbon source and further incubation. We sought to determine whether the solid surface growth procedure utilized here could provide a long-term storage that is independent of sub-freezing temperatures, and the addition of cryopreservatives. To this end, we incubated strain C1A cultures on agar surface for various periods of time (2-16 weeks), as described above. At the end of these periods, we used the optimized flooding procedure to recover spores and used the obtained flooding suspension (water + spores) to inoculate fresh RFC tubes (Table 2). C1A cultures 2-16 weeks old were revived from the agar surface by flooding with water. Cultures older than 4 weeks did not produce enough zoospores in the flooding suspension to start a new culture. However, when the flooding solution was vigorously shaken, pieces of the mycelial mats dislodged from the agar surface and

released in the suspension were sufficient to start a new culture upon inoculating fresh RFC tubes.

To demonstrate the putative broad applicability of this storage process, we applied the same technique to five anaerobic fungal isolates (monocentric strains G3 and S4B putatively identified as *Neocallimastix* and a novel genus most closely related to *Orpinomyces*, and polycentric strains G3G, C3G, and C3J putatively identified as *Anaeromyces*). All cultures were successfully recovered after 4-16 weeks incubations on solid surface by flooding and inoculation into RFC media. The ease of the described technique plus the fact that it does not involve exposure to air or a change in temperature, both of which have proven detrimental for some isolates before (Table 3), makes it ideal for long-term storage of anaerobic fungal isolates.

Discussion.

Here, we report on the feasibility of using surface agar cultures for spore collection within the AGF. We show the feasibility of recovery of up to 10^9 spores/50 ml culture under optimal conditions and that the obtained spores are viable and develop in a synchronized fashion from swimming spores to encysted spores to germinating spores within a reasonable timespan. Obtaining viable and synchronized spores is a prerequisite for conducting developmental biology and genetic manipulations in fungi. Similar efforts in basal fungal aerobic counterparts have enabled studies on physiology [26, 27, 39], pathogenicity [19, 40], global gene expression patterns during sporulation and germination [21, 28], immunolocalization of proteins [41], as well as *Agrobacterium*-mediated transformations [42, 43]. We hope that this approach opens the door for similar advances in AGF developmental biology and molecular biology research.

Prior Studies on AGF spore collection and cultural synchronization have been relatively sparse (summarized in Table 4). Approaches for inducing sporogenesis and spore release have been reported for *Neocallimastix* by adding hemin to the culture media [44]. Most studies that reported spore collection from anaerobic fungi mainly utilized low speed centrifugation or filtration [45, 46]. While straightforward, these approaches would not be suitable for separation of the different developmental stages of spores (i.e. swimming spores, encysted spores, and germinating spores). In addition, most previous reports did not conduct any viability measurements on the released spores. More importantly, the above approaches depend on the presence of free spores in the culture supernatant [7, 8, 10-12, 47]. Such dependency on the presence of free spores could be problematic in monocentric species (e.g. genera *Piromyces*, *Neocallimastix*) where the

number of spores in the culture supernatant greatly depends on the culture age, as well as in polycentric species (e.g. genera *Anaeromyces* and *Orpinomyces*), where the paucity or complete absence of sporangial development and zoosporogenesis with repeated subculturing has been noted [5]. Our approach enabled the release and collection of a large number of viable spores under optimal conditions.

We show that the released spores are developmentally synchronized, and, when at the germinating stage, could readily uptake small-interfering RNAs. This is significant, because, while it does not demonstrate gene silencing, it opens the door for genetic manipulations in AGF using RNAi approaches, similar to what have previously been reported in *Aspergillus* [13, 15, 18], as well as *Agrobacterium*-mediated transformation similar to what was reported before for the basal fungal genera *Blastocladiella* and *Batrachochytrium* [42, 43], as well as for *Aspergillus* [14]. To our knowledge, apart from a single attempt of biolistic transformation in *Neocallimastix* [48], genetic manipulations of AGF have not been actively pursued so far.

Finally, the proposed protocol builds on the observation that AGF can readily be cultured on solid agar surface under strict anaerobic conditions to allow for aerial sporangial development. Our preliminary observation was that AGF isolates could be revived, even after prolonged incubations under anaerobic conditions. Long-term storage of aerobic fungi on solid media at ambient temperatures has long been applied, albeit with the addition of mineral oil or water to prevent culture dryness [36, 37]. This storage technique keeps fungi in a low metabolic state until revived [36]. Addition of mineral oil or other liquids to prevent dryness would be dispensable for AGF since they are cultured in tightly sealed serum bottles. One previous study suggested that AGF cultures on roll

tubes in presence of sisal fibers could survive for up to 7 months [6]. We set to examine and quantify the feasibility of growth of AGF on a solid surface as a method of long-term storage that does not require subjecting cultures to ultra low temperatures. We show that this approach represents an excellent method for storing cultures up to 16 weeks, with easy routine sub-culturing by flooding and re-inoculating fresh media tubes. The simplicity and ease of this growth pattern renders it an interesting alternative and valued addition to prior reported methods for AGF long-term storage that required cryopreservative addition, multiple transfers and manipulations which result in transient air-exposure risks, and the exposure to ultra-low (-80°C) temperatures (Table 3).

In conclusion, we show that: 1) under the most optimal conditions, more than 10^9 spores are released from anaerobic fungal colonies growing on a solid agar surface, 2) variation in the incubation time with the flooding solution can be adapted to obtain either swimming or germinating spores, both of which were shown to be viable and resulted in growth when inoculated in fresh media, 3) the released germinating spores can uptake 21-nucleotide small-interfering RNA molecules, and 4) the growth on solid surface can be easily adapted for long-term storage of both polycentric and monocentric anaerobic fungal isolates with no incidental O₂ exposure or a decrease in temperature to the ultra-low values shown before to be detrimental for some isolates [49].

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Table 1. Flooding optimization for strain C1A.

Condition		% Spores in the flooding suspension ^a	Viability ^b
Temperature	4°C	> 95%	++
	22°C	> 95%	+
	39°C	> 95%	+++
Dark vs. Light	Dark	> 95%	+++
	Light	> 95%	+++
Age of culture (days)	5	> 80%	++
	6	> 90%	++
	7	> 95%	+++
	9	> 95%	+++
Agar conc (% w/v)	1%	> 95%	+
	1.5%	> 95%	++
	2%	> 95%	+++
	2.5%	> 90%	+++
Length of incubation with SAW at 39°C (min)	2.5	> 85%	++
	5	> 95%	++
	10	> 90%	++
	20	> 90%	++
	30	> 95%	++
	40	> 90%	++
	50	> 90%	++
	60	> 95%	+++
	70	> 90%	++
	80	> 95% ^c	++
	90	> 90% ^c	++
	100	> 80% ^c	++
Flooding solution	Water	> 95%	+++
	SS ^d	> 90%	++

a: % of spores was determined during counting by recording the number of other life cycle stages (e.g. early sporangia, late sporangia, hyphal mats) per examined field of vision.

b: Viability scores: (-) no visible growth; (+) little growth; (++) moderate growth; (+++) excellent growth (examples shown in Figure S2).

c: spores were all resting with no flagella. Some have started producing a germ tube.

d: SS, sporulation solution (1mM CaCl₂, 1mM Tris-Maleate, pH 6.7)

Table 2. Application of growth on solid media for AGF long-term storage.

Isolate	Culture age (weeks) ^a	Viability post flooding and inoculation ^b
C1A	2	+++ (2)
	4	+++ (2)
	6	+++ (1)
	8	+++ (1)
	10	++ (1)
	12	++ (1)
	16	+++ (1)
<i>Anaeromyces</i> -like (3)	4	+++ (1)
	8	+++ (1)
	16	+++ (1)
<i>Neocallimastix</i> (1)	4	+++ (1)
	8	+++ (1)
	16	+++ (1)
Novel genus (1)	4	+++ (1)
	8	+++ (1)
	16	+++ (1)

a: cultures were grown on solid RFC media for the indicated periods of time (under culture age). For culture revival of 2-4 week-old cultures, SAW was anoxically added to the surface followed by incubation for 60 min at 39°C. The flooding suspension was then used to inoculate fresh RFC media (10% v/v). Post 6 weeks, the serum bottles were flooded with SAW and incubated undisturbed at 39°C for 60 minutes then were shaken vigorously to dislodge pieces of the mycelial mats before transfer of the flooding suspension to fresh RFC media (30% v/v) and recording growth.

b: growth was scored after inoculating the flooding suspension into tubes with fresh media. Viability scores: (-) no visible growth; (+) little growth; (++) moderate growth; (+++) excellent growth. The number in parenthesis refers to the number of days following inoculation until visible growth was seen in the tubes. Upon subculturing, growth was observed in all tubes and was comparable to the routine subculture.

Table 3. Prior methods for long-term storage of the anaerobic gut fungi.

Method of preservation	Fungal species	Survival
Adding DMSO to the growth media followed by gradual decrease in temperature and storage in liquid N ₂ [50].	<i>Neocallimastix patricarium</i>	Several months to 1 year
Cryopreservation in presence of ethylene glycol, cell-free rumen fluid, and a zoospore density of at least 5 x 10 ⁴ zoospores/ml, followed by gradual decrease in temperature [45].	<i>Piromyces communis</i> strain OTS1	More than 80% recovery was observed after 10 weeks of storage.
Cryopreservation with glycerol at -70°C [51].	Several <i>Ceacomycetes</i> species	More than 90 days
Cryopreservation with glycerol at -80°C [49]	<i>Anaeromyces robustus</i> , <i>Neocallimastix californiae</i> , and <i>Piromyces finnis</i>	Up to 23 months with the exception of the <i>Piromyces</i>
Cryopreservation with 5% DMSO followed by centrifugation and freezing the pellet at -80°C [52].	<i>Neocallimastix</i> strains TU1 and TU2, <i>Piromyces</i> strain TU3, an unidentified polycentric fungus strain TU4, and <i>Ceacomycetes sp.</i> TU5.	All strains remained viable after storage at -80°C for at least 4 months.
Storage on sisal agar roll tubes followed by incubation at 39°C. Revival by addition of glucose, vigorous shaking and incubation for 2-3 days [6].	Three unidentified anaerobic fungal isolates strains K1, K2, and K3.	Up to 7 months

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599 **Table 4. Prior methods for spore collection and germination induction for the anaerobic gut fungi**

Method	Fungal species	Reference	Notes
A. Methods for spore collection or release from active sporangia			
Filtering using nylon cloth followed by high speed centrifugation ^a	<i>Neocallimastix frontalis</i> EB188	[46]	Viability of spores questionable, suitable only for nucleic acids extraction
Collection of hyphal-free culture supernatant (spores of monocentric species are abundant in the culture supernatant) ^a .	<i>Neocallimastix</i> sp. R1	[7, 8]	Number of spores released is heavily dependent on the growth phase in monocentric species
	<i>Neocallimastix</i> sp. MC-2 <i>Neocallimastix</i> sp. MC-2, <i>N. frontalis</i> sp. PN1 and PN2, and <i>N. patriciarum</i>	[11, 12]	
	<i>Neocallimastix</i> sp. N1, and <i>Piromyces</i> sp. R1	[10]	
Low speed centrifugation (1000-1500 xg) of actively growing culture to pellet the zoospore present in the culture supernatant ^a .	<i>Piromyces communis</i> strain OTS1	[45]	Difficulty of maintaining anoxic conditions
	<i>Neocallimastix frontalis</i> MCH3	[47]	
Transferring pieces of mycelial mats with abundant sporangia into fresh liquid medium to induce zoosporogenesis and zoospore liberation.	Three polycentric species; <i>Anaeromyces elegans</i> , and <i>Orpinomyces</i> sp. LL and LC2	[5]	While this method allowed collection of spores from polycentric species, the method depends on the stage of sporangial development where only newly developed sporangia differentiated and released spores as opposed to older sporangia.
B. Methods for induction of spore germination			

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Incubating spores in the presence of agar cellobiose blocks followed by washing to remove non-adhering spores.	<i>Neocallimastix frontalis</i> MCH3	[47]	The encysted or germinating spores will adhere to the agar blocks and will be difficult to remove.
Addition of glucose to stimulate encystment and germination (within 30 min to 6h of C source introduction).	<i>Neocallimastix frontalis</i>	[9]	The process of germination was not synchronized and so it would be difficult to obtain a germinating spores-only sample.

600 a: These methods cannot be applied for spore collection from polycentric species due to the scarcity or outright absence of sporangia
601 that has been observed with these species [5].

Figure legends

Figure 1. (A) Cartoon depicting the flooding technique utilized for AGF spore collection. (B) A serum bottle with RFC-agar media inoculated with *Orpinomyces* C1A while the agar was partially solidified and incubated at 39°C for 1 week. Note that the colonies developed on the agar surface (arrows) as well as throughout the top to middle agar layers. A bottom view of a serum bottle with RFC-agar showing the plentiful colonies on the surface (arrows) is shown in C.

Figure 2. Results of flooding optimization showing the number of spores released per ml of the flooding suspension as agar concentration (A), culture age (B), light intensity (C), temperature (D), the nature of the flooding solution used (E), and the duration of flooding (F) were changed. The Y-axis is shown in a logarithmic scale and depicts 10^7 times the number of spores obtained per ml of the flooding solution. Error bars represent standard deviation from two counts.

Figure 3. Phase contrast (A-C) and negative stain TEM (D-F) pictures of C1A spores obtained during flooding. (A, D) spores obtained by flooding agar surface with SAW followed by a 5-minute incubation at 39°C, arrows in A depict swimming spores, the flagella of which are shown in D (bar=100 nm). Longer incubation times (e.g. > 60 minutes) produced resting spores (B, E) that have started swelling (arrows pointing to swollen spores). Note the different sizes of spores and the absence of flagella in E (bar=500 nm). Incubation times longer than 90 minutes resulted in the production of swollen germinating spores (C, F) that have considerably increased in size compared to

A. Arrows in F point to the germ tube starting to form on some of the spores (bar=500 nm).

Figure 4. Germinating spores uptake of si-RNA. A 21-nucleotide double stranded si-RNA was added to the flooding solution 75 minutes after the onset of flooding followed by incubation for 15 more minutes at 39°C. The flooding solution was then used to inoculate 45 mls of fresh RFC medium and a sample was taken at regular intervals for visualization. The same field is shown for DAPI (A), and Cy3 (B)-labeled germinating spores 2 hours post inoculation (arrows point to the spores concurrently stained with DAPI and fluorescing green indicating the uptake of the Cy3-labeled siRNA) (bar=20 μ m).

Figure 1

Figure 2

Figure 3

Figure 4

Figure S1

Figure S2