cryptogamie
Mycologie
2024 • 45 • 6

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art. 45 (6) — Published on 29 April 2024
www.cryptogamie.com/mycologie

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Extrait de la Figure 3 / Extract of Figure 3

Cryptogamie, Mycologie est indexé dans / Cryptogamie, Mycologie *is indexed in*:
- Biological Abstracts
- Current Contents
- Science Citation Index
- Publications bibliographiques du CNRS (Pascal)

Cryptogamie, Mycologie est distribué en version électronique par / Cryptogamie, Mycologie *is distributed electronically by*:
- BioOne® (http://www.bioone.org/loi/crym)

Cryptogamie, Mycologie est une revue en flux continu publiée par les Publications scientifiques du Muséum, Paris
Cryptogamie, Mycologie *is a fast track journal published by the Museum Science Press, Paris*

Les Publications scientifiques du Muséum publient aussi / *The Museum Science Press also publish*: ***Adansonia, Geodiversitas, Zoosystema, Anthropozoologica, European Journal of Taxonomy, Naturae, Comptes Rendus Palevol, Cryptogamie*** **sous-sections** ***Algologie, Bryologie.***

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Tél. : 33 (0)1 40 79 48 05 / Fax : 33 (0)1 40 79 38 40
diff.pub@mnhn.fr / http://sciencepress.mnhn.fr
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ISSN (imprimé / *print*) : 0181-1584 / ISSN (électronique / *electronic*) : 1776-100

Fungal/bacterial syntrophy of glycerol utilization

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Submitted on 5 September 2023 | Accepted on 7 November 2023 | Published on 29 April 2024

Gautier V., Nguyen T.-S. & Silar P. 2024. — Fungal/bacterial syntrophy of glycerol utilization. *Cryptogamie, Mycologie* 45 (6): 53-69. https://doi.org/10.5252/cryptogamie-mycologie2024v45a6. http://cryptogamie.com/mycologie/45/6

ABSTRACT

Glycerol uptake as a carbon source has been investigated in a few fungi, mainly in *Saccharomyces cerevisiae* Meyen ex E.C.Hansen, *Aspergillus* spp., and *Neurospora crassa* Shear & B.O.Dodge. In the present study, we aimed at understanding glycerol use as food source in another fungus, the model *Podospora anserina* (Rabenh.) Niessl. Gene deletion is easy in this ascomycete and it has been used to study various biological phenomena including biomass degradation. We show that *P. anserina* is unable to use glycerol as a sole carbon source to fuel its vegetative growth; glycerol is even toxic to the fungus. However, *P. anserina* is able to use glycerol during perithecium maturation, albeit inefficiently. Genome mining identified possible glycerol uptake and catabolic pathways of *P. anserina*, and the two genes coding for enzymes of the glycerol-3-phosphate pathway were deleted. Deletions resulted in a lack of perithecium production on glycerol media, but not on optimal media containing dextrin as a carbon source. Intriguingly, the presence of bacteria in co-culture with the fungus greatly helps *P. anserina* to use glycerol both during vegetative growth and perithecium production. This offers new perspectives for improved biotransformation of glycerol into high-value products.

KEY WORDS
Carbon source,
glycerol,
syntrophy.

RÉSUMÉ

Syntrophie fongique/bactérienne de l'utilisation du glycérol.

L'utilisation de glycérol comme source de carbone a été étudiée chez peu de champignons, la plupart des études portant sur *Saccharomyces cerevisiae* Meyen ex E.C.Hansen, *Aspergillus* spp. et *Neurospora crassa* Shear & B.O.Dodge. Dans la présente étude nous proposons d'analyser l'utilisation du glycérol chez une autre espèce, le champignon modèle *Podospora anserina* (Rabenh.) Niessl. La délétion de gène est facile chez cet ascomycète et a été utilisée pour étudier divers phénomènes biologiques, en particulier la dégradation de la biomasse. Nous montrons que *P. anserina* est incapable d'utiliser le glycérol comme seule source de carbone durant sa phase végétative. Le glycérol est même toxique pour le champignon. Cependant, *P. anserina* peut utiliser le glycérol durant la maturation des périthèces. L'analyse du génome a permis d'identifier des voies potentielles d'incorporation et de métabolisation du glycérol. Les deux gènes codant pour la voie du glycérol-3-phosphate ont été délétés. Leur délétion entraîne une incapacité à produire des périthèces en présence de glycérol comme seule source de carbone, mais n'entraîne aucun phénotype sur le milieu optimal qui contient de la dextrine comme source de carbone. De manière intrigante, la présence de bactéries en coculture aide de manière très importante le champignon à utiliser le glycérol à la fois durant la croissance végétative et la production de périthèces. Ceci offre de nouvelles perspectives pour améliorer la biotransformation du glycérol en des produits à haute valeur ajoutée.

MOTS CLÉS
Source de carbone,
glycérol,
syntrophie.

INTRODUCTION

Glycerol is a chemical compound with multiple and clashing roles in biological systems. On the one hand, it is a precursor of membrane lipids in all three domains of life and as such, is essential for all living organisms (Gull & Pasek 2021). It is synthesized in stressful conditions by many organisms and protects cells from damages (Oren 2017; Blomberg 2022), and is thus used as a cryoprotectant (Pegg 2007). It may be used as a carbon source by bacteria (Lin 1976; Doi 2019), archaea (Williams *et al.* 2017), fungi (Papanikolaou *et al.* 2002; Liu *et al.* 2013; Swinnen *et al.* 2013) and animals (Khattab 2015). It is even added under the name E422 as a texturing agent for human consumption. On the other hand, glycerol can be toxic (Roger *et al.* 1992); especially, its catabolism is prone to produce noxious compounds (Applebee *et al.* 2011; Trifunović *et al.* 2021).

Fungal glycerol uptake as carbon source (reviewed in Nicol *et al.* 2012; Klein *et al.* 2017) (Fig. 1) is well studied in *Saccharomyces cerevisiae* Meyen ex E.C.Hansen. In this species, although glycerol may be used as a sole carbon source by most strains, the addition of metabolites such as amino acids or bases often improves glycerol metabolization (Merico *et al.* 2011; Swinnen *et al.* 2013). Glycerol is imported into the cell by STL1 (Ferreira *et al.* 2005) and FSP1 (Luyten *et al.* 1995; Oliveira *et al.* 2003), as well as by passive diffusion (Oliveira *et al.* 2003). It is then metabolized by the so-called G3P pathway into glycerol-3-phosphate by the glycerol kinase (GK) GUT1 (Pavlik *et al.* 1993), which is then converted into dihydroxy-acetone-phosphate by the FAD-dependent glycerol-3-phosphate dehydrogenase (G3PDH) GUT2 (Rønnow & Kielland-Brandt 1993). GUT1 and GUT2 expressions are under the direct control of the ADR1 transcription factor (Tachibana *et al.* 2005). In *S. cerevisiae*, another glycerol catabolism pathway, the DHA pathway, present in *Schizosaccharomyces pombe* Lindner and involving the Gld1 NAD^+-dependent glycerol dehydrogenase (NDGD) along with the Dak1 and Dak2 dihydroxyacetone kinases (DHAK) (Kimura *et al.* 1998; Matsuzawa *et al.* 2010), does not appear to be operational. Indeed, although *S. cerevisiae* has a NDGD activity dependent upon the GCY1 protein (Norbeck & Blomberg 1997; Jung *et al.* 2012) and possesses DHAK homologues, it appears to lack a *bona fide* DHAK activity (Klein *et al*. 2017). Interestingly, GCY1 does not show any similarity with Gld1, although both enzymes display NDGD activity. Similarly, a third pathway present in some fungi, the GA pathway involving the transformation of glycerol into glyceraldehyde by a glycerol oxidase (GO) (Uwajima *et al.* 1980), has not been evidenced in *S. cerevisiae* (Klein *et al.* 2017). Nevertheless, *S. cerevisiae* likely possesses part of this pathway, i.e., an aldehyde dehydrogenase (ADH) encoded by the *ALD6* gene (Meaden *et al.* 1997) and a glycerate-3 kinase (G3K) encoded by TDA10 (Boldt *et al.* 2005), but seemingly no glyceraldehyde kinase (GDHK).

Glycerol import and metabolism appear to be "moderately" conserved in the different fungal phyla (Kayingo *et al.* 2004; Li *et al.* 2022). Especially, glycerol catabolism appears to be slightly different from the one of *S. cerevisiae* in the few Pezizomycotina in which studies have been carried out, i.e., mainly in a few *Aspergillus* P.Micheli ex Link species and in *Neurospora crassa* Shear & B.O.Dodge.

In Aspergilli, glycerol is also metabolized principally through the G3P pathway (Hondmann *et al.* 1991; Li *et al.* 2022), and metabolic network reconstruction in *A. nidulans* G.Winter identified the GK as encoded by AN5589.2 and the G3PDH by AN1396.2 (David *et al.* 2006); they are orthologues of GUT1 and GUT2, respectively. Upregulation on glycerol of the putative NDGHD gene AN7193.2 (homologous to GCY1) and putative DHAK gene AN0034.2 (homologous to DAK1 and DAK2) suggested that the DHA pathway is operational in this fungus (David *et al.* 2006). Similarly, the GA pathway may also be used since a GO enzyme oxidizing glycerol into glyceraldehyde has been detected in several Aspergilli (Uwajima *et al.* 1980). To the best of our knowledge, this enzyme has not been connected to a coding sequence (CDS), although a GO activity has been purified from several fungi (Uwajima & Terada 1980; Uwajima *et al.* 1984; Lin *et al.* 1996; Gayda *et al.* 2006; Linke *et al.* 2014). Nonetheless, an alcohol oxidase with a weak activity towards glycerol has been described in the basidiomycete *Phanerochaete chrysosporium* Burds. (Nguyen *et al.* 2018); it belongs to the glucose-methanol-choline (GMC) oxidoreductase family. Similarly, although classical mutants affected by their glycerol catabolism are available in Aspergilli (Uitzetter *et al.* 1986; Visser *et al.* 1988; Arst *et al.* 1990; Witteveen *et al.* 1990), none have been connected to a mutated CDS except in *Aspergillus fumigatus* Fresen. (Zhang *et al.* 2018; Zhang *et al.* 2021). In this species, deletion of the GK GlcA (homologous to AN5589.2 of *A. nidulans* and encoded by AFUB_068560 in *A. fumigatus*) results as expected in growth and conidiation defects on glycerol as sole carbon source (Zhang *et al.* 2018), while deletion of the *GlcB* paralogous gene (encoded by AFUB_074430) does not (Zhang *et al.* 2021). Little is known about glycerol import in Aspergilli and filamentous fungi in general since only one transporter from *Aspergillus glaucus* (L.) Link (called AgglpF) and related to FSP1 has been characterized as involved in glycerol import (Liu *et al.* 2015). Transcriptomic analyses identified a transcription factor having the ADR1 consensus binding site as the primary significant regulator of genes involved in glycerol catabolism of Aspergilli, arguing for conservation of the regulation of glycerol utilization between *S. cerevisiae* and *A. nidulans* (Salazar *et al.* 2009).

Neurospora crassa, the other fungal species extensively studied for glycerol catabolism is able to grow with glycerol as sole carbon source on minimal medium, although glycerol is not as efficient in supporting growth as hexoses and other sugars (Ryan *et al.* 1943; Tom *et al.* 1978). The GK and G3PDH activities of the G3P pathways have been detected, and this pathway is supposed to be the main one in *N. crassa* (Courtright 1975c). Both enzymes are induced by glycerol (Courtright 1975b), and GK is also induced by low temperatures (North 1973, 1974). As in Aspergilli and although classical mutants affected in their glycerol uptake by the G3P pathway are available in *N. crassa* (Courtright 1975a; Denor & Courtright 1978;

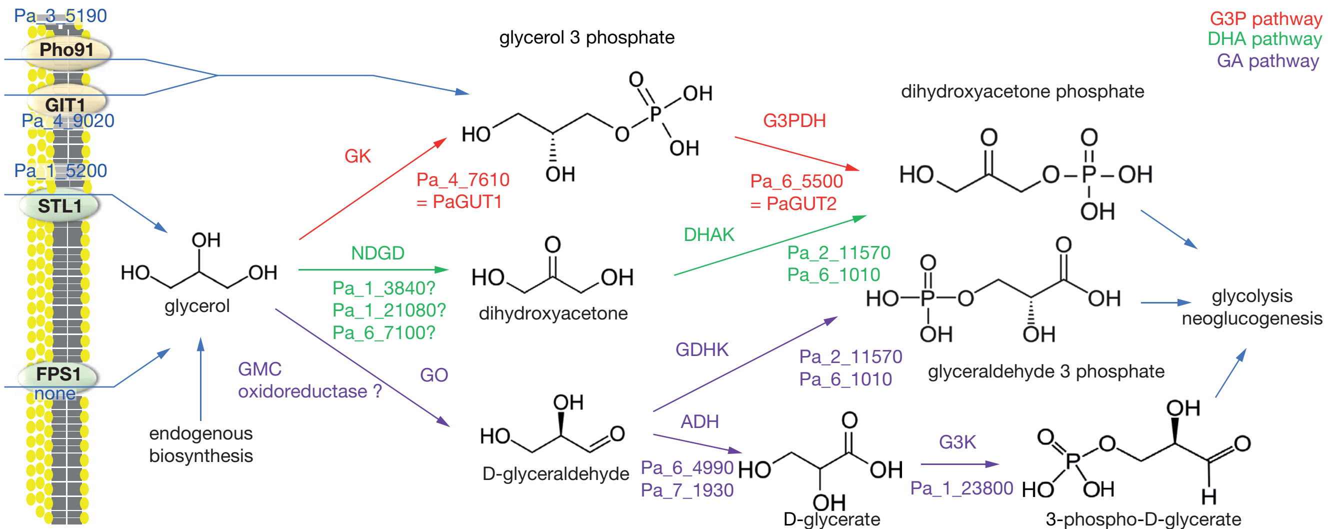

Fig. 1. — Scheme of glycerol import and catabolism in *Podospora anserina* (Rabenh.) Niessl as transposed from pathways known in *Saccharomyces cerevisiae* Meyen ex E.C.Hansen, *Schizosaccharomyces pombe* Lindner, and *Aspergillus nidulans* G.Winter.

Tom *et al.* 1978; Denor & Courtright 1982; Pyle & Howe 1983), none appears to have been connected to mutation(s) in known CDS. Interestingly, analyses of mutants of *Neurospora tetrasperma* Shear & B.O.Dodge, a species closely related to *N. crassa* and that interbreed with it, indicated that both species likely have the GA pathway but not the DHA one (Viswanath-Reddy *et al.* 1977). Accordingly, an enzyme oxidizing glycerol into glyceraldehyde has been detected in both *N. crassa* and *N. tetrasperma* (Uwajima *et al.* 1980).

Podospora anserina is another well-studied fungal model (Silar 2020); yet more is needed to know about the glycerol metabolism in this species. *P. anserina* belongs like *N. crassa* to the Sordariales order and as such, it would be expected to have a glycerol metabolism similar to the one of *N. crassa*. Here, we show on the contrary that unlike *N. crassa*, which can use glycerol as a sole carbon source in minimal medium, *P. anserina* cannot use glycerol to fuel its vegetative growth even in the presence of additional metabolites. Glycerol is moreover toxic to the fungus. *Podospora anserina* can, however use glycerol to promote maturation of its fruiting bodies. Interestingly, the presence of bacteria greatly helps *P. anserina* to use glycerol as a carbon source for vegetative growth, underlining a possible syntrophic utilization of this carbon source *in vitro* and possibly in nature.

MATERIAL AND METHODS

Strains and growth conditions

All the mutant strains used in this study derived from the "S" (uppercase S) wild-type strain that was used for sequencing (Espagne *et al.* 2008; Grognet *et al.* 2014). The *N. crassa* strain was OR74A. Standard culture conditions, media and genetic methods for *P. anserina* have been described in Silar (2020). The M0 medium has the following composition: 0.25 g/L KH2PO4, 0.3 g/L K2HPO4, 0.25 g/L MgSO4-7H2O, 0.5 g/L urea, 0.05 mg/L thiamine, 0.25 µg/L biotine, 2.5 mg/L citric acid, 2.5 mg/L ZnSO4, 0.5 mg/L CuSO4, 125 µg/L MnSO4, 25 µg/L boric acid, 25 µg/L sodium molybdate, 25 µg/L iron alum, 10 g/L agar. M2 has the same composition as M0 except that a carbon source is added as 4.5 g/L of potato dextrin.

Deletions of the genes *PaGut1* and *PaGut2*

Deletions were made using the split marker method (Silar 2013, 2020) with the primers reported in Appendix 4. *PaGut1* (Pa_4_7610) was deleted using the hygromycin B resistance marker, and *PaGut2* (Pa_6_5500) was deleted using the geneticin resistance marker (Appendix 1). Potential independent candidates obtained after transformation were crossed to the wild type and in the progeny, strains carrying the proper resistance marker were verified by Southern blot analyses (Appendix 1). For both genes, strains having the proper replacements were recovered. These were called *PaGut1*$^{\Delta}$ and *PaGut2*$^{\Delta}$ for deletion of *PaGut1* and *PaGut2*, respectively.

The *PaGut1*$^{\Delta}$ *PaGut2*$^{\Delta}$ double mutant was constructed by crossing the single mutants. In the progeny, we identified the double mutant as being resistant to both hygromycin B and geneticin.

Gene complementation

To complement the *PaGut2*$^{\Delta}$ mutant, a 3.2 kb DNA fragment encompassing the gene was amplified using the primers 5'-Pa_4_5500A bis and 3'-Pa_4_5500D bis (Appendix 4) and the wild-type genomic DNA as template. The 3.2 kb fragment was cloned into pBC-phleo (Silar 1995). The resulting plasmid carrying the wild-type *PaGut2* gene was transformed into *PaGut2*$^{\Delta}$. Fifty phleomycin resistant transformants were obtained. Four were selected for further studies. Among those, three regained the ability to produce perithecia on medium with glycerol as a sole carbon source, indicating that the wild-type *PaGut2* gene complemented the *PaGut2* deletion

Table 1. — Putative glycerol genes of *Podospora anserina* (Rabenh.) Niessl.

Enzyme	Enzyme abbreviation	*S. cerevisiae*	*S. pombe*	*A. nidulans*	*P. anserina*
Transport					
Glycerol symporter	–	STL1	–	–	Pa_1_5200
Glycerol channel	–	FPS1	–	–	none
Glycerol 3-phosphate transporter	–	GIT1	–	–	Pa_4_9020
Glycerol 3 phosphate transporter	–	Pho91	–	–	Pa_3_5190
Metabolism					
Glycerol kinase (EC2.7.1.30)	GK	GUT1	–	AN5589.2	Pa_4_7610
FAD-dependent glycerol-3-phosphate dehydrogenase (EC1.1.5.3)	G3PDH	GUT2	Gut2	AN1396.2	Pa_6_5500
NAD+-dependent glycerol dehydrogenase (EC1.1.16)	NDGD	GCY1	SPBC8E4.04	AN7193.2	Pa_1_3840
		YPR1	SPAC26F1.07		Pa_1_21080
		–	–		Pa_6_7100
		–	–		Pa_3_2850
		–	–		Pa_3_4920
		–	GLD1	–	none
Dihydroxyacetone kinases (EC2.7.1.29)	DHAK	DAK1 & DAK2	Dak1 & Dak2	AN0034.2	Pa_2_11570 Pa_6_1010
Glycerol oxidase (EC1.1.1.B4)	GO	–	–	Enzyme present sequence not available	GMC oxidoreductases?
Glyceraldehyde kinase (EC2.7.1.28)	GDHK	–	–	–	Pa_2_11570? Pa_6_1010?
Aldehyde dehydrogenase (EC1.2.1.3)	ADH	ALD6	–	–	Pa_6_4990 Pa_7_1930
Glycerate-3 kinase (EC2.7.1.31)	G3K	TDA10	–	–	Pa_1_23800
Regulation					
Transcription factor	–	ADR1	–	–	none

as expected. One of these three transformants was crossed to the *PaGut2*$^{\Delta}$ mutant, and in the progeny, we observed a co-segregation of the resistance to phleomycin and the ability to produce perithecia on glycerol medium, indicating the resistance marker and the *PaGut2*$^{+}$ wild-type allele integrated at the same location in the genome.

Phenotypic analyses

Growth and fertility were assayed on mat+/mat- heterokaryotic mycelia generated by grinding in the "TeSeE precess 24" apparatus from Biorad. Two mycelium plugs of 1 mm^3, one from a fresh mat+ mycelium and the other from a fresh mat- one, were added to 500 µL of sterile distilled water and shaken for 20 seconds at 5000 rpm. 10 µL of the mix was inoculated at the center of the plates.

Isolation and identification of bacteria

The first bacterium spontaneously contaminated some M0 + glycerol Petri plates containing mat+/mat- wild-type heterokaryons. To identify the bacterium, PCR amplified its 16S rDNA gene with primers U1 (5'-ccagcagccgcggtaatacg-3') and U2 (5'-atcggytaccttgttacgacttc-3') and the resulting PCR product was sequenced using the same primers. Blast analysis of GenBank of the recovered sequence showed that the contaminating bacterium likely was *Panaebacillus validus* (Nakamura) Ash *et al.* since the sequence of the PCR product was identical to the 16S sequence of this bacterium. Additional bacteria were then isolated by incubating M0 + 0.1 M glycerol plates without their cover or inoculating some soil. Six additional bacterial strains that grew well on M0 + glycerol were recovered, and identified by sequencing a PCR product obtained with the U1 and U2 primers. Two were shown to be *Pseudomonas putida* Trevisan, and the four others were *Microbacterium proteolyticum* Alves *et al.*, *Massilia* sp., *Paracoccus* sp., and *Sphingobacterium kitahiroshimense* Matsuyama *et al.*

Bioinformatic analyses

The proteome of *P. anserina* was searched with BLASTp using the proteins of *S. cerevisiae*, *S. pombe*, and *A. nidulans* indicated in Table 1 as queries. When the best hit(s) consisted of one or two proteins, these were, in turn used to search by BLAST the proteomes of *S. cerevisiae*, *S. pombe*, and *A. nidulans*. If the first hit of this second BLASTp corresponded to the original queried protein, the *P. anserina* protein was considered to be the ortholog of the protein used in the first BLAST analysis (BEST-BEST hit analysis). The human protein was used in the Glyceraldehyde kinase (EC2.7.1.28) since no sequence is available for fungi. In all instances, we could clearly define the *P. anserina* orthologues of the queried proteins, except for NDGDs. For NDGD, all proteins similar to AN7193 present in the genome of *A. nidulans*, *P. anserina*, *S. pombe* and *S. cerevisiae* were retrieved from GenBank, aligned with MAFFT using the default parameters (Katoh *et al.* 2005). The resulting alignment was manually cleaned for poorly aligned regions and used to construct a phylogenetic tree using the maximum likelihood method with 100 bootstrap replicates

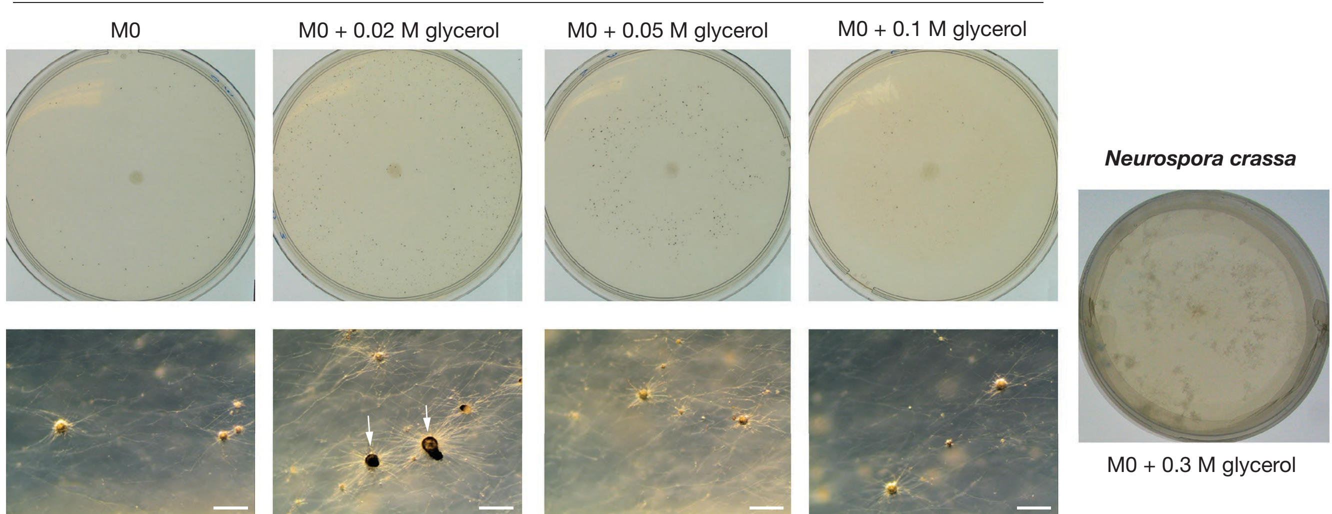

Fig. 2. — Growth and fertility of *Podospora anserina* (Rabenh.) Niessl on glycerol media. The plates were inoculated at the center with a mat+/mat- heterokaryon and incubated for three weeks at 27°C in the presence of constant light, at which time pictures were taken. **Arrowheads** point toward ascospore-containing perithecia. See also Figure 4 for a dark field picture of the wild-type strain growing on M0 and M0 + 0.05 M glycerol. **Top**, Petri plate (Ø = 8 cm). Scale bars: 200 µm.

(PhyML software using the default parameters) (Guindon & Gascuel 2003) and transferred to FigTree for visualization (Rambaut 2007).

The reads per kilobase per million mapped reads (RPKM) of the genes of Appendix 5 were calculated with Artemis after mapping the RNAseq data onto the *P. anserina* genome with TopHat2.

RESULTS

Growth of *P. anserina* with glycerol as carbon source

As seen in Figure 2, *P. anserina* appears unable to use glycerol as a sole carbon source for its vegetative growth as mycelium growth speed and density was not different on M0 medium containing 0.01 M to 0.04 M (= 0.9 g/L to 3.7 g/L) of glycerol compared to M0 medium lacking a carbon source. With higher glycerol concentrations (0.05 M to 0.3 M; i.e., 4.5 g/L to 27.6 g/L), growth of *P. anserina* was inhibited and even completely abolished at the highest concentrations. At 0.05 M glycerol, growth speed was inhibited by 15% (6.5 mm/day instead of 7.5 mm/day on M0 without glycerol) and at 0.1 M by 66% (2.5 mm/day instead of 7.5 mm/day). On the same media containing glycerol with concentrations up to 0.3 M, *N. crassa* could grow and form a profuse mycelium (Fig. 2).

Interestingly, the plates containing 0.01 to 0.04 M of glycerol developed mature perithecia producing ascospores (Fig. 2), while the ones lacking glycerol did not (Fig. 2). These matured slowly and required three weeks to produce ripe ascospores instead of one week on the standard M2 medium. They were mainly located within the medium, were small and contained few ascospores. At higher concentrations, perithecium production was inhibited and mostly small protoperithecia were observed on the M0 medium. This indicated that *P. anserina*, while unable to use glycerol for its vegetative growth could use it to produce mature fruiting bodies, albeit inefficiently.

The presence of small amounts of metabolites (0.1 g/L of glucose, galactose, glutamine, peptone and yeast extract) did not improve the growth of *P. anserina* on M0 medium containing 0.05 M glycerol unlike what has been reported for other fungi. Indeed, the cultures with 0.05 M glycerol displayed the same growth speed, spindly mycelium morphology, and fertility as the cultures without glycerol and having only 0.1 g/L of added metabolites.

Overall, the data showed that *P. anserina* was unable to use glycerol as a carbon source for its vegetative growth even in the presence of metabolites, unlike what had been reported for other fungi, including its close relative *N. crassa*. Nevertheless, albeit inefficiently, *P. anserina* could use glycerol to make mature fruiting bodies.

Glycerol is toxic to *P. anserina*

Growth and fertility of *P. anserina* on medium with glycerol as sole carbon source was inhibited at high concentrations. We thus explored whether these inhibitions were also observed on the standard M2 medium containing dextrin as a carbon source that is routinely used to grow *P. anserina*. As a control, we tested sorbitol at the same osmolarity as glycerol to check whether inhibition was due to high osmolarity. As seen in Figure 3, glycerol inhibited the growth of *P. anserina* in M2 medium like it did in M0 medium (by 15% at 0.05 M and 69% at 0.1 M), while sorbitol did not even at the highest concentrations. We tested whether sorbitol could be used as sole carbon source and whether in these conditions high amounts of sorbitol had an inhibitory effect on *P. anserina* growth and fertility (Fig. 3). Sorbitol was used by *P. anserina* as sole carbon source and, even at the highest concentration,

sorbitol did not impair the growth of the fungus and was efficiently used as a sole carbon source. This indicated that glycerol was toxic to *P. anserina* and that toxicity was not due to an effect on osmolarity.

Mining the genome of *P. anserina* for genes involved in glycerol catabolism

The *P. anserina* genome was mined to identify CDS possibly involved in glycerol uptake and catabolism (Table 1; Fig. 1). We could detect by "BEST-BEST hit" using BLAST an orthologue of the *S. cerevisiae* STL1 transporter. However, BLAST analyses failed to uncover homologues for *S. cerevisiae* FPS1 or *A. glaucus* AgglpF channels. We could detect orthologues of GUT1/AN5589.2 encoding GK and GUT2/AN1396.2 encoding G3PDH (encoded by Pa_4_7610 and Pa_6_5500, respectively), indicating that *P. anserina* likely has a functional G3P pathway.

The presence of the DHA pathway in *P. anserina* needed to be clarified. Indeed, five proteins with significant levels of similarity to *S. cerevisiae* GCY1 and *A. nidulans* AN7193.2 NDGDs were encoded in the *P. anserina* genome. To identify which one was a potential NDGD, a phylogenetic tree was constructed with the five *P. anserina* potential NDGDs, the *S. cerevisiae* GCY1 protein and its paralogue resulting from the duplication of the *S. cerevisiae* genome YPR1, the putative *S. pombe* NDGDs SPBC8E4.04 and SPAC26F1.07, as well as all the proteins of *A. nidulans* similar to AN7193.2 (Appendix 2). As seen in Appendix 2, GCY1 and AN7193.2 did not appear to have true orthologues in *P. anserina*. However, most nodes are poorly supported owing to the small sizes of the proteins and the poor conservation of their primary sequences, making it difficult to draw a final decision as to the presence of actual NDGD in *P. anserina*. We tentatively propose that Pa_1_3840, Pa_1_21080, and Pa_6_7100 may be NDGDs owing to their similarity to AN7193.2 or GCY1, while no conclusion may be reached for Pa_3_2850 and Pa_3_4920. Note that a homologue for the Gld1 NDGD of *S. pombe* could not be found in the genomes of *P. anserina*, (nor in the genomes of *S. cerevisiae* and *A. nidulans*). On the contrary, two clear homologues of the Dak1 and Dak2 DHAK of *S. pombe*, DAK1 and DAK2 DHAK of *S. cerevisiae*, and AN0034.2 DHAK of *A. nidulans* were encoded in the *P. anserina* genome (Pa_2_11570 and Pa_6_1010).

As stated in the introduction, the key GO enzyme of the GA pathway has yet to clearly be identified in fungi. The only known enzyme endowed with a weak GO activity has been identified in *P. chrysosporium* (Nguyen *et al.* 2018) and belongs to the GMC oxidoreductase family. The genome of *P. anserina* contains 28 genes encoding such enzymes, none of which has been characterized to date (Ferrari *et al.* 2021). Similarly, no GDHK sequence appears available for fungi, only for mammals (https://www.brenda-enzymes.org/enzyme.php?ecno=2.7.1.28). Using sequences of GDHK from mammals, BLAST analysis identified Pa_2_11570 and Pa_6_1010 putative DHAKs as closely related to these enzymes from animals; due to the close chemical structure of dihydroxyacetone and D-glyceraldehyde (Fig. 1), both may be used as substrates by Pa_2_11570 and Pa_6_1010. Finally, *P. anserina* has two potential ADHs encoded by Pa_6_4990 and Pa_7_1930 and one potential G3K encoded by Pa_1_23800.

Regarding a potential regulator of glycerol metabolism, we could not detect in the genome of *P. anserina* an obvious orthologue of the *S. cerevisiae* ADR1 transcription factor involved in the regulation of GUT1 and GUT2 expression. Mining the genome of *A. nidulans* and *N. crassa* also failed to retrieve an orthologue of ADR1, suggesting that, even though *A. nidulans* glycerol genes are controlled by a transcription factor binding a consensus similar to the one bound by ADR1, a different transcription factor is likely involved in glycerol gene regulation in the Pezizomycotina.

We also mined the transcriptomic data generated by Silar *et al.* (2019), which provided an estimate of the expression of the genes in non-germinated ascospores, in ascospores eight hours after germination trigger, in 1-day-old and 4-day-old mycelia and in 2-day-old and 4-day-old perithecia (Appendix 5). All glycerol genes were expressed at all stages of the lifecycle, including the glycerol symporter encoded by Pa_1_5200, the GK encoded by Pa_4_7610, and the G3PDH encoded by Pa_6_5500.

Overall, genome and transcriptome mining suggested that *P. anserina* can import glycerol and metabolize it through the G3P pathway. The presence of the DHA and GA pathways is more dubious; however, *P. anserina* appears to have enzymes from both pathways.

Deletion of the *P. anserina PaGut1* and *PaGut2* genes

Because the G3P pathway appears to be the major pathway for glycerol uptake in the Pezizomycotina, including for *P. anserina*, we deleted the genes encoding the putative GK Pa_4_7610 and G3PDH Pa_6_5500; these were renamed *PaGUT1* and *PaGUT2*, respectively, and the deletion mutants *PaGUT1*$^{\Delta}$ and *PaGUT2*$^{\Delta}$. We also retrieved the *PaGUT1*$^{\Delta}$ *PaGUT2*$^{\Delta}$ double mutant in the progeny of the cross between the *PaGUT1*$^{\Delta}$ and *PaGUT2*$^{\Delta}$ single mutants.

The mutants exhibited no phenotype on M0 and M2 media, i.e., their growth speed, mycelium morphology and fertility were indistinguishable from that of the wild type (Fig. 4). On the contrary, on M0 and M2 medium in the presence of 0.05 M of glycerol, the vegetative growth of *PaGUT1*$^{\Delta}$, *PaGUT2*$^{\Delta}$ and *PaGUT1*$^{\Delta}$ *PaGUT2*$^{\Delta}$ was slightly more severely affected than that of the wild type because growth of the mutants was reduced by 20% instead of 15% at 0.05 M glycerol in the medium (speed of *c.* 6 mm/day instead of *c.* 6.5 mm/day for the wild type; see Figure 4 for pictures of the growth plates). However, at 0.1 M glycerol in M0 or M2, the growth of the mutants was as much impaired as that of the wild type (Fig. 4). Importantly, unlike the wild type, *PaGUT1*$^{\Delta}$, *PaGUT2*$^{\Delta}$ and *PaGUT1*$^{\Delta}$ *PaGUT2*$^{\Delta}$ did not produce mature perithecia after three weeks incubation on medium containing 0.02 M glycerol (Fig. 5). Longer incubation time did not result in the production of mature perithecia by these mutants. Finally, their fertility was like that of the wild type on an M2 medium containing glycerol. Also, on M0 and M2 media with sorbitol, the mutants grew and were fertile like the wild type.

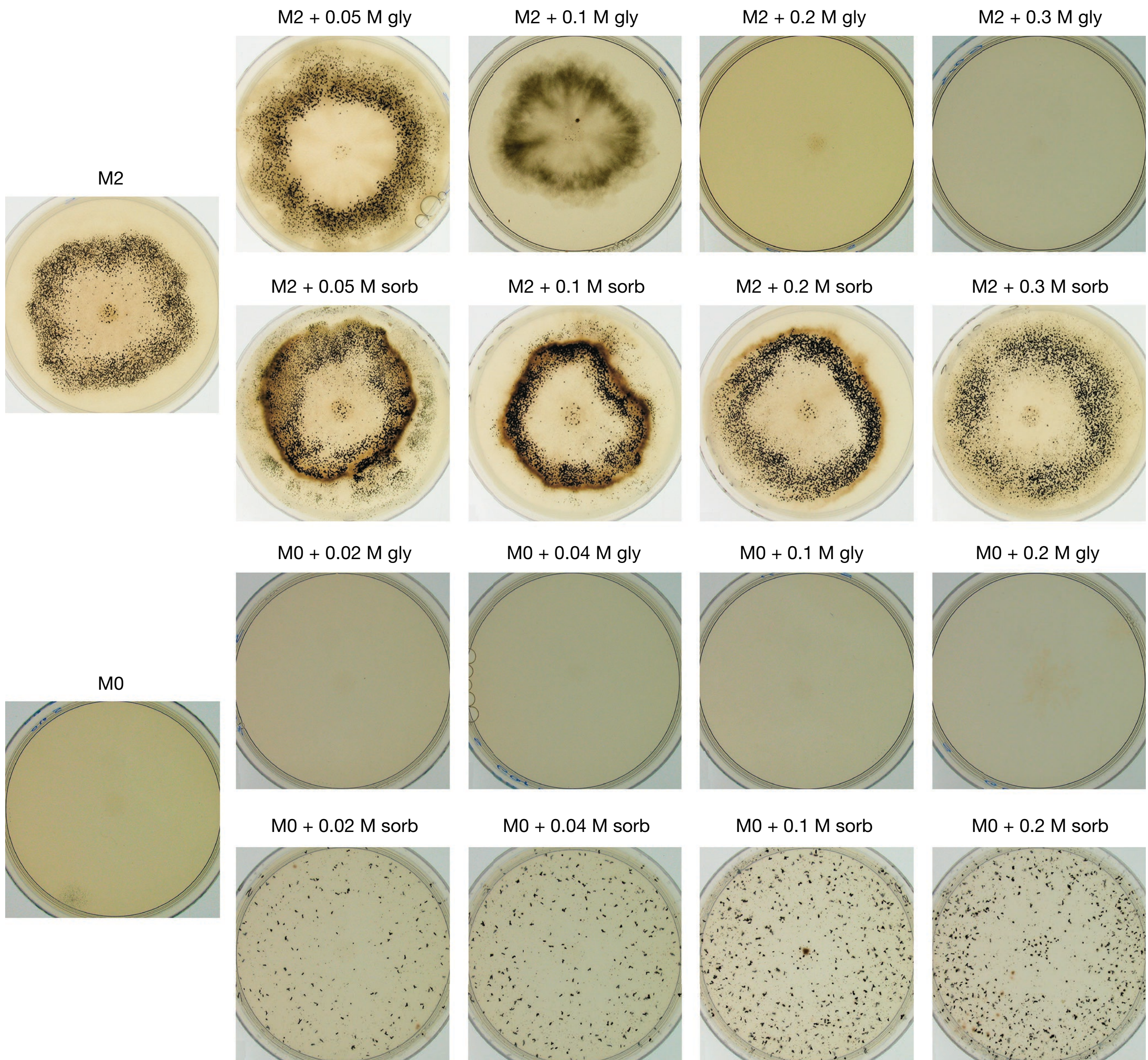

Fig. 3. — Toxicity of glycerol. M2 and M0 Plates containing the indicated concentration of glycerol (**gly**) and sorbitol (**sorb**) were inoculated at the center with a mat+/mat- heterokaryon and incubated for two weeks at 27°C in the presence of constant light, at which time pictures were taken. Perithecia are visible as small black dots. On the M2 medium, the presence of 0.1 M of glycerol inhibited growth and abolished fertility; higher concentrations of glycerol resulted in no growth. On the contrary, *Podospora anserina* (Rabenh.) Niessl grew and was fertile even in M2 medium containing the highest concentration of sorbitol. *Podospora anserina* was also fertile on medium containing sorbitol as sole carbon source at all concentrations, while it produced no visible perithecia on a medium with the same glycerol concentrations after two weeks.

Reintroduction of the *PaGut2* wild-type allele into the mutant *PaGUT2*Δ restored a wild-type phenotype (i.e., the production of perithecia on M2 + 0.02 M glycerol). Unfortunately, we could not complement the *PaGUT1*Δ mutant because we failed to recover in bacteria a plasmid carrying the *PaGut1* wild-type allele without sequence alteration (a recombination event eliminated systematically the end of the gene) and direct co-transformation with the *PaGut1* wild-type allele PCR amplification product with a resistance marker yielded few transformants for an unknown reason despite several attempts; none of these had a wild-type phenotype. Nonetheless, in crosses between *PaGUT1*Δ and the wild type the lack of perithecium production on glycerol medium co-segregated with the resistance used for deleting *PaGut1*, arguing that this phenotype was actually due to the deletion of *PaGut1* and not to an additional unlinked mutation.

The presence of bacteria greatly improves the growth and fertility of *P. anserina* on glycerol media

We serendipitously discovered that the presence of bacteria in co-culture with *P. anserina* significantly improves growth and

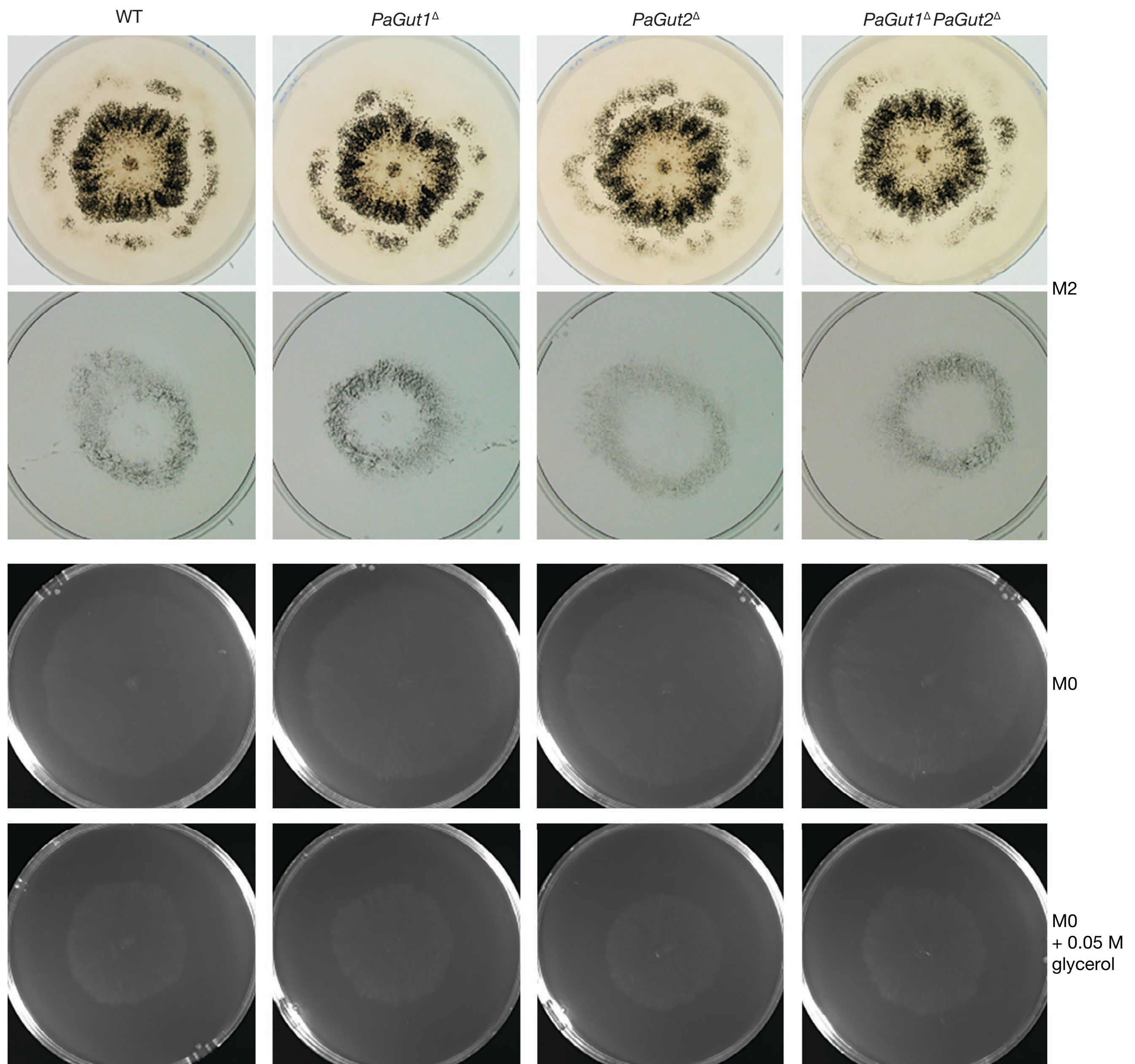

Fig. 4. — Phenotype of the G3P mutants. Growth and fertility on M2 were evaluated eight days after inoculation of mat+/mat- heterokaryons of the indicated strains. For M2, the **top** plates are cultures on the M2 medium; the **bottom** ones are the cover of the same plates onto which ascospores seen as tiny black dots were expelled. Growth on M0, M0 + 0.05 M glycerol, and M0 + 0.1 M glycerol was evaluated after four days of growth.

fertility of the fungus on M0 medium added with glycerol as sole carbon source. Indeed, we observed that, in a series of plates contaminated by bacteria, the bacterial colonies were surrounded by what looked like a denser hyphal thallus bearing mature perithecia (Fig. 6). We identified the contaminating bacterium by sequencing its 16S barcode and found it to be *Paenibacillus validus* (Bacillota formerly Firmicutes). To check whether the improved growth and fertility were specific to *P. validus* or could be achieved by other bacteria, we tested several other species including *Microbacterium proteolyticum* (Actinomycetota), *Massilia* sp. (Pseudomonadota – Betaproteobacteria), *Paracoccus* sp. (Pseudomonadota – Alphaproteobacteria), *Sphingobacterium kitahiroshimense* (Bacteroidota – Sphingobacteriia), *Pseudomonas putida* (Pseudomonadota – Gammaproteobacteria) and *Escherichia coli* T.Escherich DH5α (Pseudomonadota – Gammaproteobacteria). All were found to improve the growth and fertility of *P. anserina* on glycerol as sole carbon source (see Figures 6 and 7 for improvement of growth and fertility of *P. anserina* by *E. coli*).

As it is a well-known model and grows well on high glycerol concentrations (Appendix 3), we chose *E. coli* DH5α for further testing. We especially tested whether the growth and fertility of the *PaGUT1*Δ, *PaGUT2*Δ and *PaGUT1*Δ *PaGUT2*Δ mutants were also improved in the presence of the bacteria. As

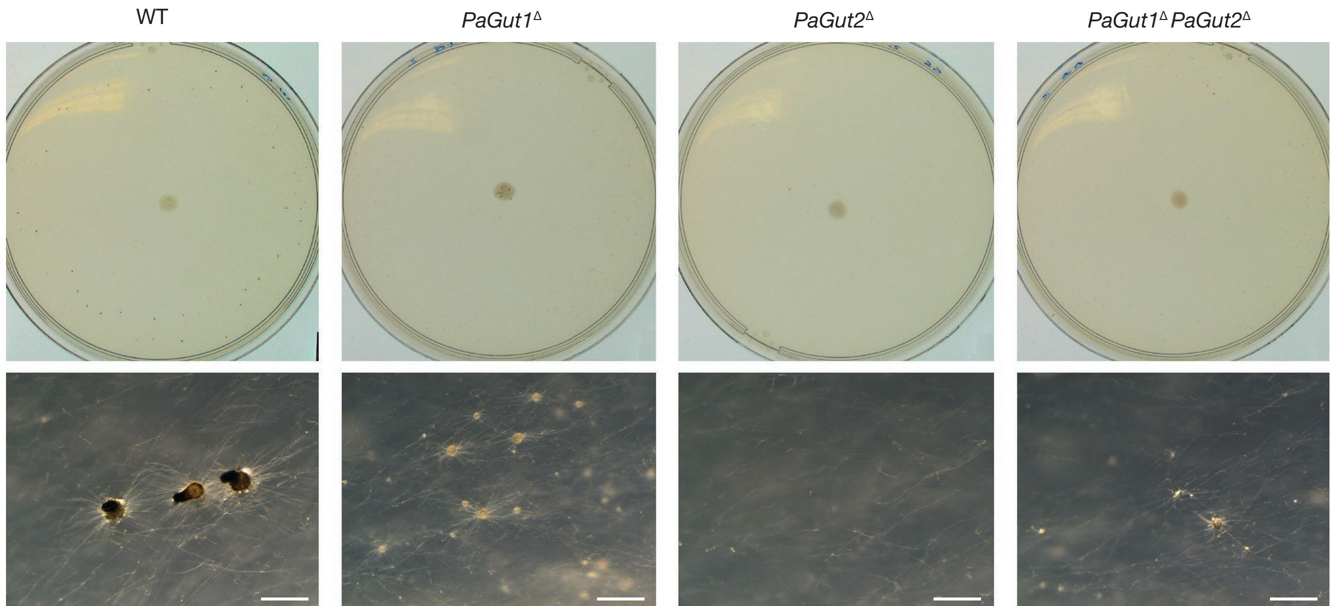

Fig. 5. — The *PaGut1*Δ and *PaGUT2*Δ mutants are sterile on glycerol media. Small but mature perithecia can be seen at the periphery of the colony when the wild type is incubated for 20 days on media containing 0.02 M glycerol, while they cannot be seen in the *PaGut1*Δ, *PaGUT2*Δ, and *PaGut1*Δ *PaGUT2*Δ mutants. **Bottom**, enlargement of representative fruiting bodies. Scale bars: 250 μm.

seen in Figure 7, *E. coli* improved the vegetative growth of all mutants and the fertility of all mutants except for *PaGUT2*Δ, for which only growth was improved, as *PaGUT2*Δ did not produce perithecia in the presence of bacteria.

DISCUSSION

Unlike what would be expected from its close evolutionary relatedness to *Neurospora crassa*, a species able to use glycerol as a carbon source during vegetative growth, *P. anserina* is unable to use glycerol to fuel its vegetative growth. *Podospora anserina* is most often found growing on herbivore dung and less often in soils, thus in biotopes that are expected to have low content of readily-available simple sugars and alcohol such as glucose and glycerol. It is thus possible that PaGut1 and PaGut2 enzymes are not active in the apical hyphae that are responsible for the bulk of *P. anserina* mycelium growth. Indeed, although we detected in the RNAseq data the expression of both genes, as well as that of the putative glycerol transporter in these hyphae involved in mycelium growth, additional levels of regulation may prevent the expression of active enzymes and/or transporters. Especially, the glycerol symporter Pa_1_5200 appears less expressed in 1-day-old mycelium than in 4-day-old one (Appendix 5). *Neurospora* species are abundant in soils and frequently proliferate after wildfires on burnt vegetation, in situations where the fungus also likely encounters low levels of glycerol. It needs to be clarified why *Neurospora* can use glycerol and *P. anserina* cannot.

As seen in Figure 1, glycerol may also be produced endogenously during either triglyceride biosynthesis or de-esterification (Gull & Pasek 2021). Endogenous production is also required as glycerol is a precursor of some metabolic pathways that require oxidized or phosphorylated derivatives of glycerol (Gull & Pasek 2021). It may also be produced by fermentation (Rehm 1996; Zhang *et al.* 2002) and photosynthesis (Ben-Amotz *et al.* 1982). Finally, glycerol is also synthesized during stress conditions (Gull & Pasek 2021). The G3P pathway of glycerol metabolization may rather be tailored in *P. anserina* for using the endogenously produced glycerol, especially during perithecium maturation, as seen by the production of perithecia on media with glycerol as sole carbon source. Indeed, in *P. anserina* and related fungi, perithecium production occurs in the stationary phase, i.e., in a situation where the mycelium must mobilize carbon-based molecules to produce energy and building blocks for fruiting body maturation. Glycerol may be one of these molecules that could be produced during lipid reserve breakdown. It may not be a preferred carbon/energy source, as seen by the inefficient production of mature fruiting bodies on media with glycerol as sole carbon source. Nonetheless, endogenous glycerol consumption through the G3P seems not crucial for proper perithecium maturation since the mutants are as fertile as the wild type on media containing an optimal carbon source (i.e., dextrin).

Glycerol is toxic to *P. anserina*. The reason for this toxicity is not clear, however, the mutants of the G3P pathway were slightly more sensitive than the wild type, arguing for the role of the pathway in resistance to exogenous glycerol. Interestingly, in the presence of bacteria, both mycelium growth and perithecium production are improved in the wild type and in the *PaGut1* mutant, while only mycelium growth is promoted in the *PaGut2* mutant. Moreover, the double mutant, affected for both *PaGut1* and *PaGut2*, is also able to produce many perithecia in the presence of bacteria. Among these strains only *PaGut2*Δ should accumulate large

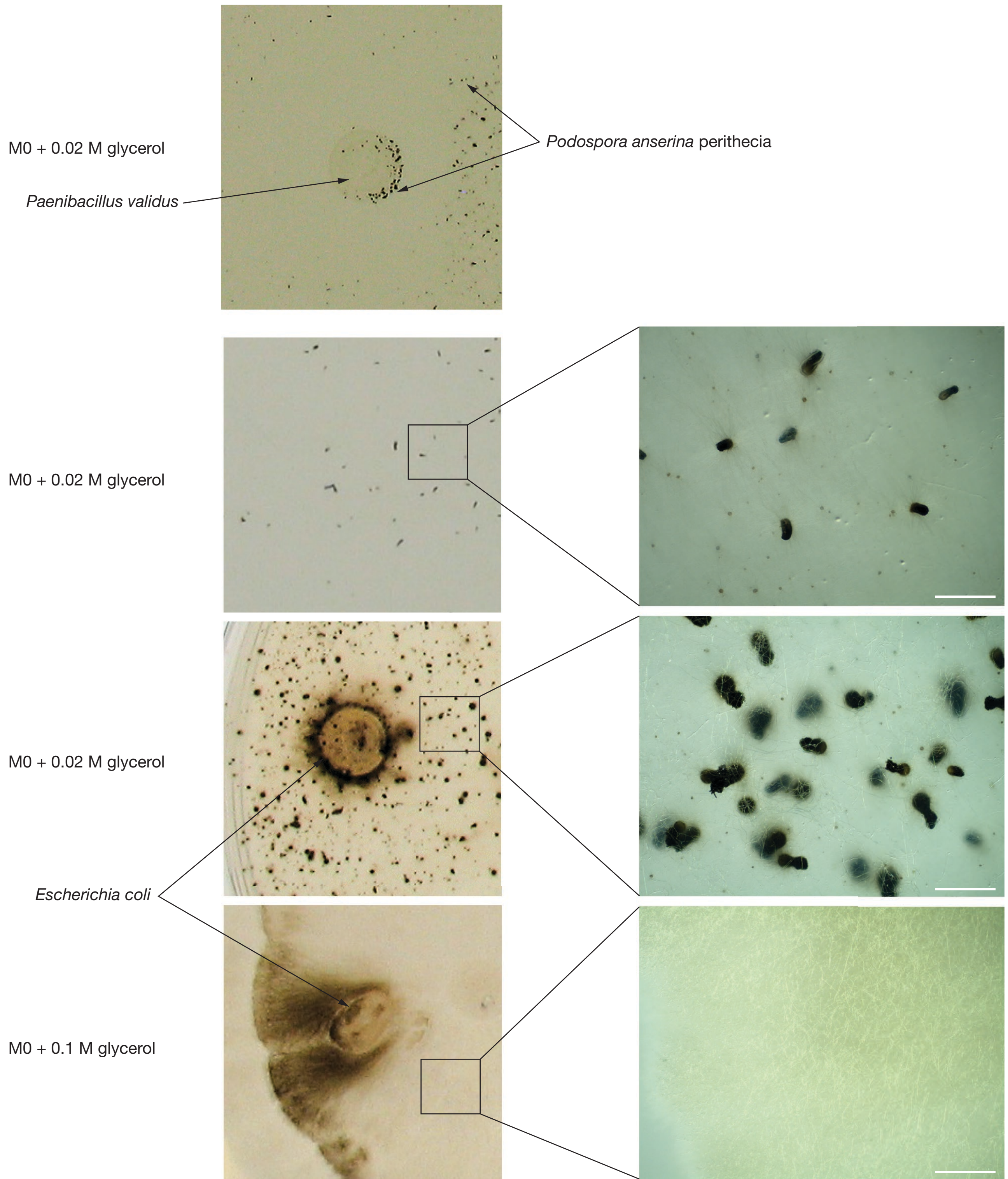

Fig. 6. — Production of perithecia by *Podospora anserina* (Rabenh.) Niessl in the presence of *Paenibacillus validus* (Nakamura) Ash *et al*. and *Escherichia coli* T.Escherich. The presence of large amounts of mature perithecia (the black dots) are seen around and farther away from the bacterial colony on media containing 0.02 M glycerol. The fungus formed a dense mycelium on a plate containing 0.1 glycerol and the bacteria. Note that at 0.1 M glycerol, *P. anserina* remained sterile even in the presence of bacteria. Scale bars: 250 µm.

amounts of glycerol-3-phosphate. Indeed, it should not be produced in *PaGut1*$^{\Delta}$ and *PaGut1*$^{\Delta}$ *PaGut2*$^{\Delta}$ and be rapidly transformed into dihydroxyacetone phosphate in the wild type. Possibly large amounts of glycerol-3-phosphate may repress fruiting body formation. This molecule may be toxic or be a precursor of toxic molecules that would impair perithecium maturation; alternatively, glycerol-3-phosphate may have a role as a signaling agent repressing development since such

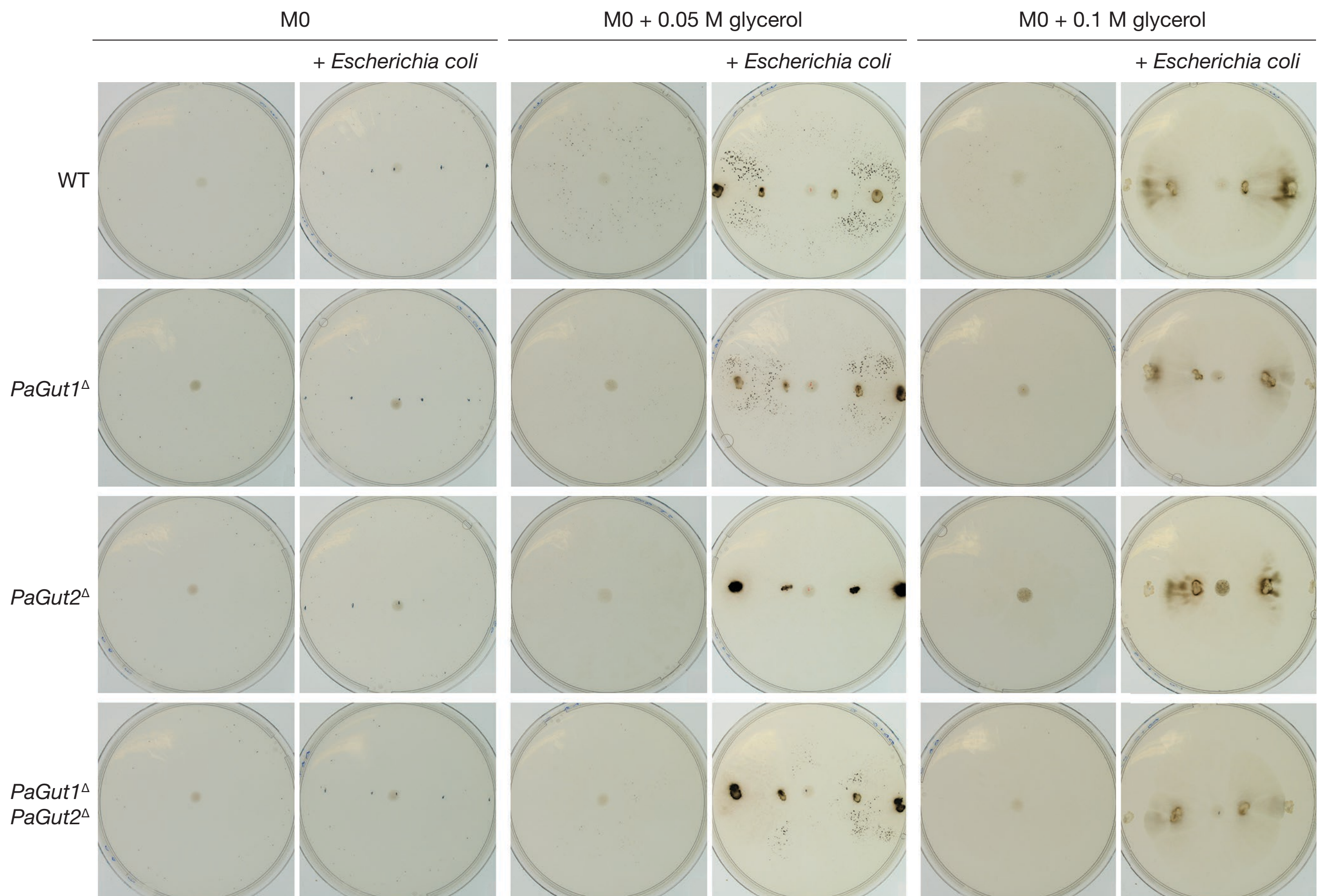

Fig. 7. — Production of mycelium and perithecia in the presence of *Escherichia coli* T.Escherich by *Podospora anserina* (Rabenh.) Niessl wild-type and mutant strains. The plates were inoculated at the same time at the center by a mat+/mat- heterokaryon of the fungus with the indicated genotypes, alone or with *E. coli* DH5α at the position indicated by the blue dots on the right and left of the center (visible on the M0 plates). The plates were incubated for 20 days at 27°C and constant illumination, after which they were photographed. *Escherichia coli* promoted the growth of the mycelium (clearly visible on plates containing 0.1 M glycerol) for all strains, and development of perithecia (clearly visible as small black dots on plates containing 0.05 M glycerol) in all strains except for the *PaGut2*Δ mutant.

a role in signaling has been evidenced in plants (Venugopal *et al.* 2009). This could in part account for glycerol toxicity.

How bacteria improve the growth and fertility of *P. anserina* in the presence of glycerol as sole carbon source is presently still being determined. One possibility is that the presence of bacteria activates a *P. anserina* signaling cascade that results in the increased efficiency of the G3P pathway through elevated expression or activation of PaGut1, PatGut2, and the Pa_1_5200 (= SLT1-like) transporter. Alternatively, bacteria could transform their surrounding glycerol into product(s) more edible for the fungus. A candidate could be glycerol-3-phosphate. *Podospora anserina* has orthologues for the Pho91 and GIT1 transporters involved in glycerol-3-phosphate uptake in *S. cerevisiae* (Fig. 1; Table 1; Popova *et al.* 2010) and it may thus be efficiently imported. This fits the fact that only growth is restored in the *PaGut2* mutant. In both the wild type and the mutants, it would be used to fuel vegetative growth. Still, it could only be used through its transformation into dihydroxyacetone phosphate by the PaGut2 enzyme for perithecium production and maturation. Whatever the mechanism, it seems to be promoted by all the bacteria that we have tested suggesting that this syntrophy may exist in nature where *P. anserina* encounters many different bacterial species in large amounts. Although metabolic syntrophy is known between bacteria (see Morris *et al.* 2013 for a review), it has not been often described between bacteria and fungi (see Deveau *et al.* 2018; Weiland-Bräuer 2021 for reviews). Carbon flow from fungi to bacteria has been shown in the rhizosphere (Ballhausen & de Boer 2016), and metabolic exchanges have been evidenced in yeast/bacteria biofilms (Swinnen *et al.* 2016; Du *et al.* 2022; Sadiq *et al.* 2022). The syntrophy presented here is to the best of our knowledge the first described one in which bacteria help a filamentous fungus to use a carbon source that is non-edible in their absence.

Pursuing the study of this syntrophy may be of technological importance. Indeed, glycerol is now produced in large amounts as a by-product of biodiesel production and other technologies. Many studies aimed at improving the growth of *S. cerevisiae* and different yeasts to efficiently biotransform glycerol into added-value products (see for example Ochoa-Estopier *et al.* 2011; Swinnen *et al.* 2016). The use of mixed cultures of fungi and bacteria instead of pure cultures of yeasts may help to achieve this goal.

CONCLUSION

We confirm that the ability to use glycerol as a food source appears loosely conserved during fungal evolution even between fairly close species. Indeed, unlike the related *Neurospora* spp., *P. anserina* is unable to use glycerol to fuel its vegetative growth. Glycerol is even toxic to the fungus. However, *P. anserina* is able to use inefficiently glycerol during perithecium maturation and utilization relies on the G3P pathway. Finally, presence of bacteria from a wide array of taxa greatly enhanced the ability of *P. anserina* to use glycerol during both vegetative growth and sexual reproduction.

Acknowledgements

We would like to thank Aurélien Renault for his expert technical assistance. This work was supported by intramural funding of University Paris Cité.

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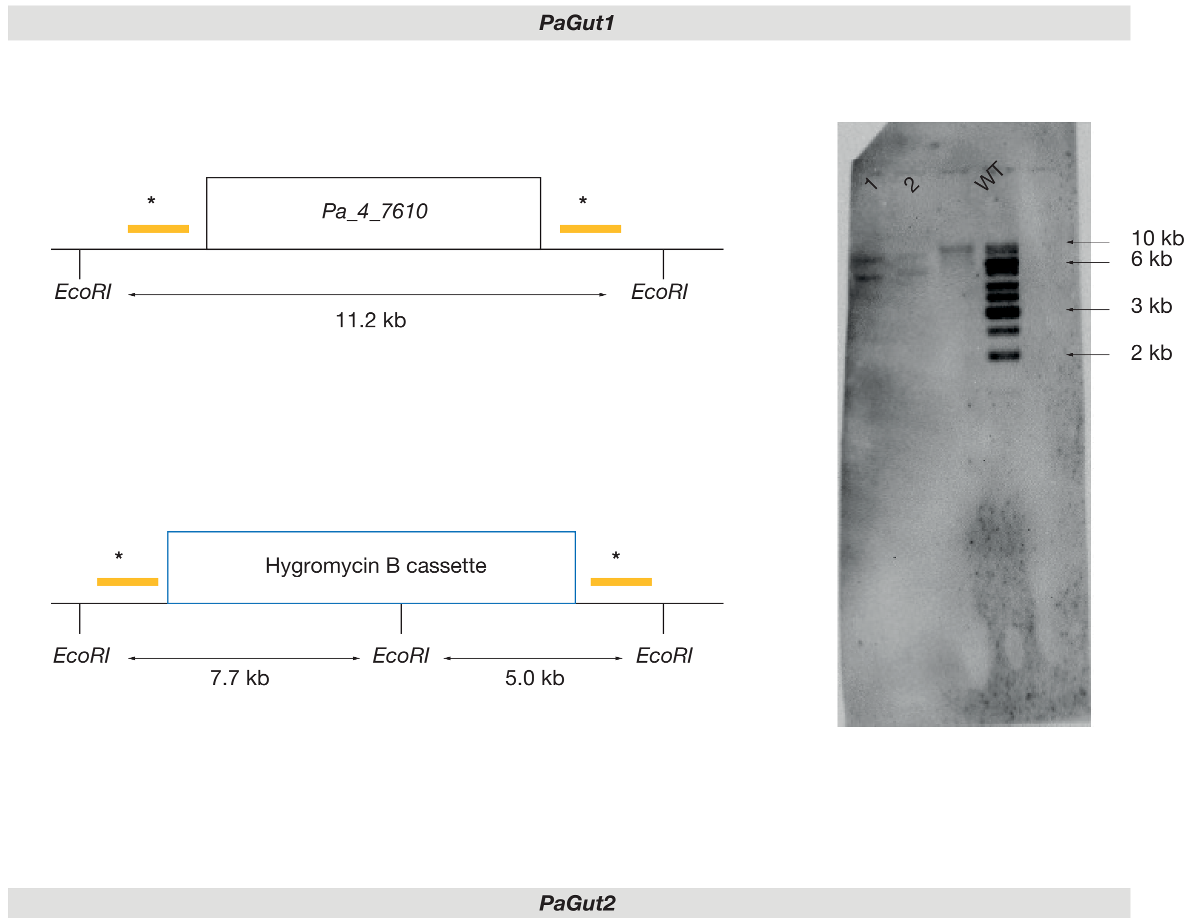
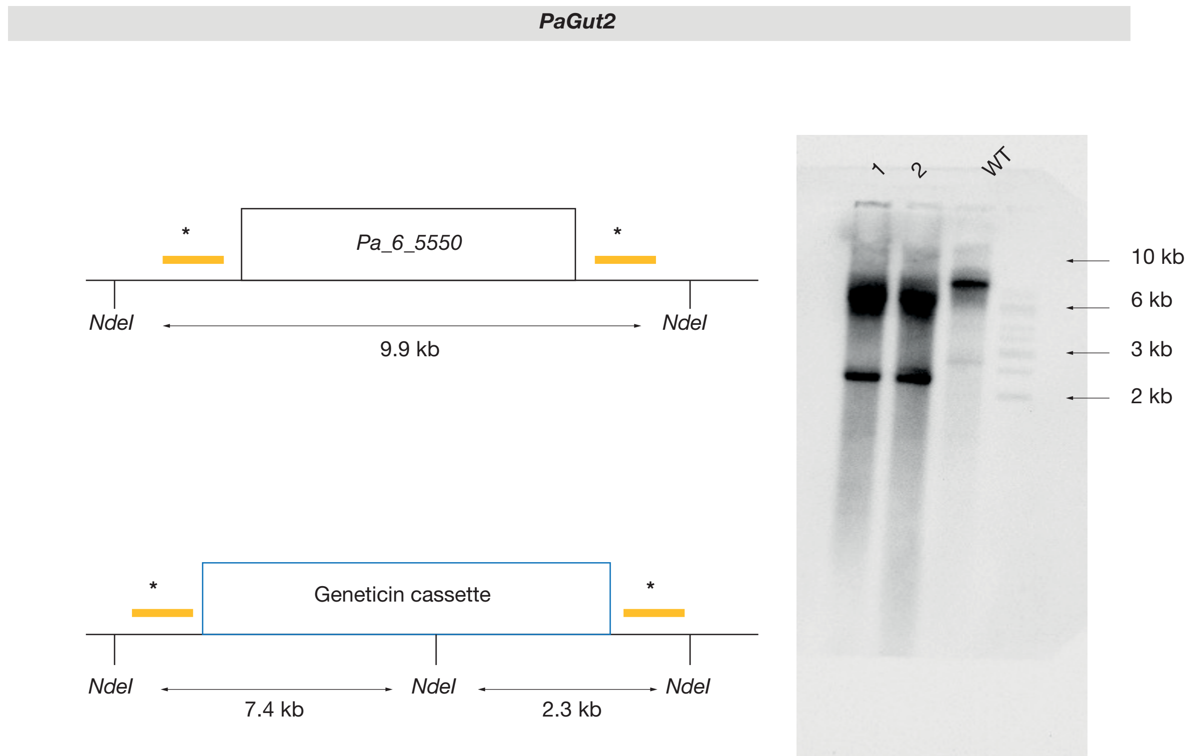
Submitted on 5 September 2023;
accepted on 7 November 2023;
published on 29 April 2024.

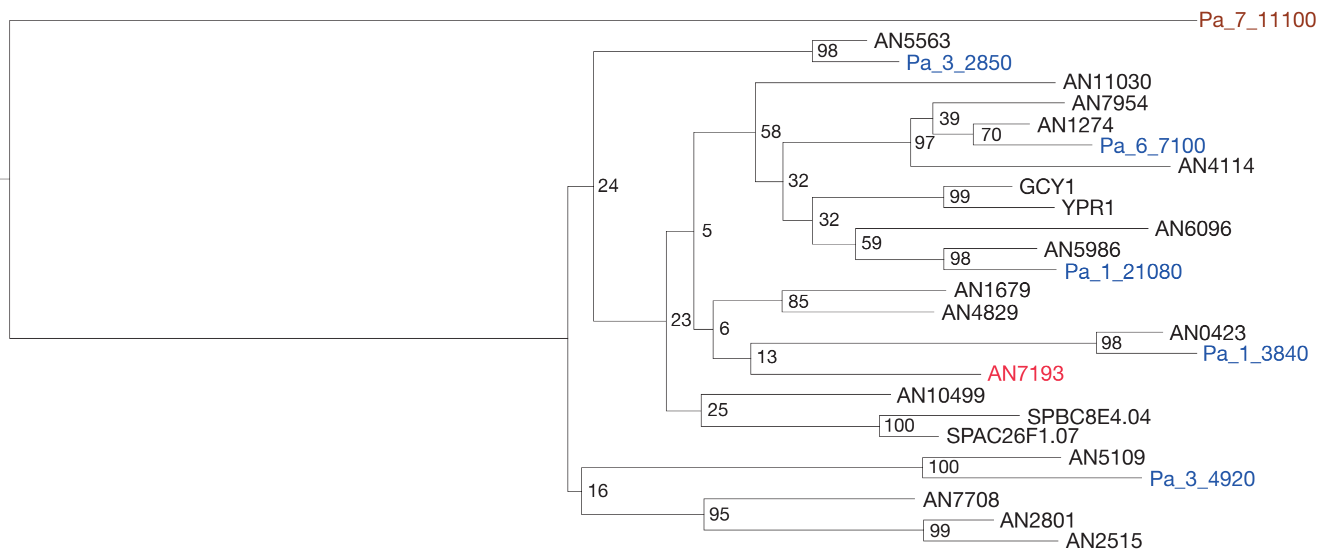
APPENDICES

Appendix 1. — Southern blot validation of the deletions. For each gene, on the left, schematic representation of the wild-type and deleted locus. Enzymes and probes (in **yellow** with *) used for Southern blots are indicated; on the right, corresponding autoradiograms. In both cases, the two tested candidates had the correct replacement; WT = wild type

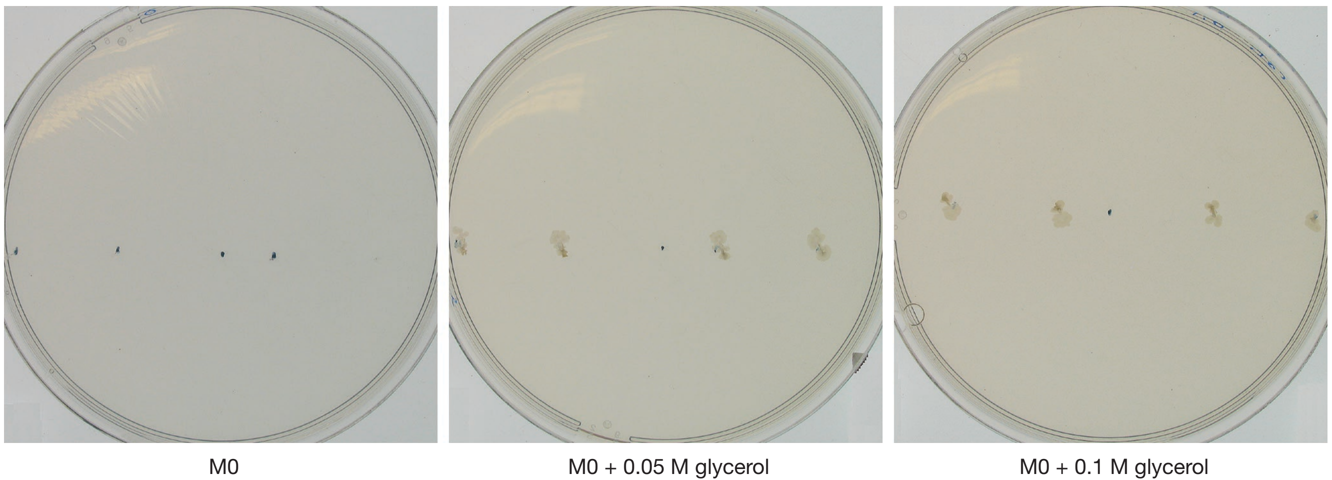




Appendix 2. — Phylogenetic analysis of NDGD. The tree was rooted with the divergent protein encoded by Pa_7_11100. The AN7193 protein defined as a NDGD is in **red** and the *Podospora anserina* (Rabenh.) Niessl proteins are in **blue**.



Appendix 3. — Growth of *Escherichia coli* T.Escherich on M0 medium supplemented with glycerol. *Escherichia coli* DH5α was inoculated at position indicated by the blue dots (except at the one on the center where *Podospora anserina* (Rabenh.) Niessl would be inoculated when the plates contain the fungus, e.g. see Fig. 7) on the indicated media and the plates were incubated for 20 days at 27°C under constant illumination at which point photographs were taken. In these conditions, the growth of the *E. coli* colonies is readily visible after one week of incubation and starts to mask the blue dots, except on M0 that does not permit the growth of *E. coli*.



Appendix 4. — Primers used for deletion and complementation.

Genes	Primer names	Sequences
Pa_4_7610	5'-Pa_4_7610A	tcctggtgagctgtatgtaggctggacacg
	5'-Pa_4_7610B	ctatttaacgaccctgccctgaaccgaccaggcagtagagtgaaaaggtcgaaggc
	mk_4_7610E	gccttcgaccttttcactctactgcctggtcggttcagggcagggtcgttaaatag
	mk_4_7610F	atcggcccaaacccaaacaaaccatgatgccatcgaactggatctcaacagcggtaag
	3'-Pa_4_7610C	cttaccgctgttgagatccagttcgatggcatcatggtttgtttgggtttgggccgat
	3'-Pa_4_7610D	tatccaacgcagcaaaccgacgaagcatct
Pa_6_5500	5'-Pa_6_5500A	tgtagcctcgtgtgtgtgtgtgtgtgtgag
	5'-Pa_6_5500B	ctatttaacgaccctgccctgaaccgcaagagtgagagagacagttcagacgacggc
	mk_6_5500E	gccgtcgtctgaactgtctctctcactcttgcggttcagggcagggtcgttaaatag
	mk_6_5500F	aaaccagccctctcatgacaagaacccatctcatcgaactggatctcaacagcggtaag
	3'-Pa_6_5500C	cttaccgctgttgagatccagttcgatgagatgggttcttgtcatgagagggctggttt
	3'-Pa_6_5500D	tgggcttgatatcgtgctatattggcggcc
Pa_6_5500 complementation	5'-Pa_4_5500A bis	aagatcgacgctagaggtgg
	3'-Pa_4_5500D bis	tatattggcggccgggttaa

Appendix 5. — Transcriptomic analysis of *Podospora anserina* (Rabenh.) Niessl glycerol genes.

Enzyme	CDS number	Non-germinated ascospores	Ascospores 8 hours after germination trigger	Mycelium 1-day-old	Mycelium 4-day-old	Perithecium 2-day-old	Perithecium 4-day-old
Glycerol symporter	Pa_1_5200	12.184	0.646	7.136	49.364	68.761	41.700
Glycerol 3 phosphate transporter	Pa_4_9020	3.517	0.000	3.302	5.499	52.820	125.993
Glycerol 3 phosphate transporter	Pa_3_5190	37.568	107.991	1.270	1.215	0.701	0.391
Glycerol kinase (EC2.7.1.30)	Pa_4_7610	533.454	307.021	353.911	488.853	579.858	463.980
FAD-dependent glycerol-3-phosphate dehydrogenase (EC1.1.5.3)	Pa_6_5500	84.694	56.787	41.655	29.787	14.441	44.365
	Pa_1_3840	436.134	179.289	2584.046	612.126	242.839	673.340
	Pa_1_21080	6.622	0.749	252.057	84.861	4.536	105.881
NAD+-dependent glycerol dehydrogenase (EC1.1.16)	Pa_6_7100	249.347	63.674	408.709	160.759	144.929	278.570
	Pa_3_2850	122.757	320.125	0.355	1.275	3.757	1.059
	Pa_3_4920	90.541	586.413	0.926	0.686	3.553	1.011
Dihydroxyacetone kinases (EC2.7.1.29)/ Glyceraldehyde kinase (EC2.7.1.28)	Pa_2_11570	27.621	2.234	49.856	23.465	27.426	36.895
	Pa_6_1010	1.547	0.556	1322.087	115.621	321.642	184.978
Aldehyde dehydrogenase (EC1.2.1.3)	Pa_6_4990	2428.975	4321.565	2105.613	3620.179	1316.311	2693.604
	Pa_7_1930	199.285	224.121	301.229	1422.921	643.561	591.603
Glycerate-3 kinase (EC2.7.1.31)	Pa_1_23800	391.960	17.192	17.018	57.265	49.584	86.601