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STUDY ON THE GLUTATHIONE METABOLISM OF THE FILAMENTOUS FUNGUS *ASPERGILLUS NIDULANS*

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Yeast protein sequence-based homology search for glutathione (GSH) metabolic enzymes and GSH transporters demonstrated that *Aspergillus nidulans* has a robust GSH uptake and metabolic system with several paralogous genes. In wet laboratory experiments, two key genes of GSH metabolism, *gcsA*, and *glrA*, encoding γ-L-glutamyl-L-cysteine synthetase and glutathione reductase, respectively, were deleted. The gene *gcsA* was essential, and the Δ*gcsA* mutant required GSH supplementation at considerably higher concentration than the *Saccharomyces cerevisiae gsh1* mutant (8–10 mmol l^{-1} vs. 0.5 μmol l^{-1}). In addition to some functions known previously, both genes were important in the germination of conidiospores, and both gene deletion strains required the addition of extra GSH to reach wild-type germination rates in liquid cultures. Nevertheless, the supplementation of cultures with 10 mmol l^{-1} GSH was toxic for the control and Δ*glrA* strains especially during vegetative growth, which should be considered in future development of high GSH-producer fungal strains. Importantly, the Δ*glrA* strain was characterized by increased sensitivity toward a wide spectrum of osmotic, cell wall integrity and antimycotic stress conditions in addition to previously reported temperature and oxidative stress sensitivities. These novel phenotypes underline the distinguished functions of GSH and GSH metabolic enzymes in the stress responses of fungi.

Keywords: glutathione, glutathione metabolism, environmental stress, *in silico* analysis, *Aspergillus nidulans*

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Introduction

Glutathione (γ-L-glutamyl-L-cysteinyl-glycine, GSH) is the most abundant thiol in aerobic prokaryotic and eukaryotic organisms, which plays essential roles in many biological processes, e.g., in the defense against harmful xenobiotics, heavy metals, and reactive oxygen species as well as in the redox stabilization of membranes, membranous cellular structures like mitochondria and the nuclei [1, 2]. GSH also fulfills important redox-independent functions like in the maturation of iron–sulfur clusters [3, 4] and also serves as an important nitrogen and sulfur reserve under starvation [1].

The synthesis of GSH is catalyzed in two consecutive steps by γ-L-glutamyl-L-cysteine synthetase (γ-GCS, or glutamate–cysteine ligase) and glutathione synthetase [1, 5]. In the baker's yeast *Saccharomyces cerevisiae*, the disruption of γ-GCS resulted in GSH auxotrophy [6], which could be rescued by dithiothreitol, cysteine, β-mercaptoethanol, and N-acetylcysteine [7, 8]. γ-GCS deficiency also results in severe clinical symptoms in humans including hemolytic anemia [9].

Besides *de novo* biosynthesis, the regeneration of GSH from its oxidized form glutathione disulfide (GSSG) by the NADPH-consuming enzyme glutathione reductase (GR) is also of pivotal importance in the stabilization of the GSH/GSSG redox balance within the cells [1, 5]. In *S. cerevisiae*, the GR-encoding gene *GLR1* is not essential [10] but it is a key element of the oxidative stress defense system of yeast [7]. Importantly, the GR-encoding gene *pgr1* was essential in the fission yeast *Schizosaccharomyces pombe* to maintain growth and spore viability [11]. Although the deletion of *glrA* coding for GR in *Aspergillus nidulans* resulted in a temperature-sensitive phenotype it was not essential [12] and, hence, the overall view on the role of GR in *A. nidulans* was more budding yeast-like than fission yeast-like. It is noteworthy that reductions in growth, oxidative stress defense, and cephalosporin C production caused by the disruption of the GR-encoding gene could be reversed by the addition of exogenous methionine [13].

Although the anabolic and catabolic pathways as well as the transport processes influencing intracellular GSH concentrations are well understood [1, 5, 14], future studies may shed light on the fine-tuning of cellular (and sub-cellular) redox control by GSH and also on further, not-yet-known biosynthetic, physiological, and developmental functions attributable to this sulfur-containing tripeptide [1, 3, 4]. For example, more recent studies demonstrated the involvement of GSH in the biosynthesis of the important *Aspergillus fumigatus* virulence factor gliotoxin [15].

Hence, to gain a deeper insight in the importance and functions of GSH in the stress defense and development of *A. nidulans*, we deleted the genes *glrA* and

gcsA in this filamentous fungus model organism and phenotypically characterized the Δ*gcsA* and Δ*glrA* strains. In this study, we collected and discussed new data on the important roles played by GcsA and GlrA in supporting germination of conidiospores and also in the defense against various types of environmental stress, e.g., hyperosmotic stress, cell wall integrity stress and exposures to antimycotics. The foreseeable biomedical and industrial significances of the new observations are also discussed in this paper.

Materials and Methods

In silico reconstruction of GSH metabolic pathways and identification of putative GSH transporters in A. nidulans

Putative elements of the GSH metabolic pathways as well as presumptive GSH transporters were identified in *A. nidulans* by extracting information from the *Aspergillus* Genome Database (AspGD, http://www.aspgd.org/) and by homology search following the protocols previously described by Miskei et al. [16]. Concisely, homology search was carried out with the sequences of *S. cerevisiae* GSH metabolic proteins and GSH transporters (downloaded from the *Saccharomyces* Genome Database, SGD, http://www.yeastgenome.org/) using the BLASTP search program in AspGD. Following that another round of BLAST homology search was carried out in SGD using the sequences of the candidate *A. nidulans* proteins. If the highest homology yeast protein was identical to that we started the homology search with the *A. nidulans* protein was regarded as part of the GSH metabolism and transport of the fungus and was discussed [16].

Strains, culture media, and production of conidia

The following strains were used in our study: rJMP1.59 (*pyrG89*; *pyroA4*; *veA*$^{+}$), rRAW16 (*pyrG89*; *yA2*; *veA*$^{+}$), THS30.3 [17], Δ*glrA* (*pyrG89*; Δ*glrA*::*AfupyrG*$^{+}$; *veA*$^{+}$), and Δ*gcsA* (*pyrG89*; Δ*gcsA*::*AfupyrG*$^{+}$; *veA*$^{+}$). For cultivation of the *A. nidulans* strains, minimal nitrate medium (MNM) was used with appropriate nutritional supplements [18]. The GSH supplementations of the Δ*glrA* and the Δ*gcsA* mutants were optimized (Supplementary Figure S1) and, as a result, culture media (both MNM agar plates and MNM liquid media) were supplemented with 10 mmol l^{-1} GSH unless otherwise indicated, which was in line with the protocol of Sato et al. [12]. Conidiospores were also produced usually on MNM agar supplemented with 10 mmol l^{-1} GSH, and these sporulation cultures were

incubated at 37 °C for 6 days [19]. All cultures were supplemented with GSH dissolved in sterile distilled water at temperatures lower than 50 °C.

Construction of the ΔglrA and ΔgcsA gene deletion strains

Two genes encoding key players in the maintenance of the intracellular GSH concentration and the GSH/GSSG redox balance, *glrA* (encoding GR, locus ID: AN0932, AspGD; [12]) and *gcsA* (coding for γ-GCS, locus ID: AN3150, AspGD), were deleted by the DJ-PCR method of Yu et al. [20] using the primers listed in Supplementary Table S-I [18, 20]. The amplified deletion cassettes were used to transform RJMP1.59 strain using the Vinoflow FCE lysing enzyme [21]. Single copy transformants were selected after Southern blot analysis and crossed with rRAW16 to get prototrophic strains. All progenies of the independent crosses proved to be single-copy deleted mutants by Southern analyses.

Growth studies with the ΔgcsA strain on MNM agar plates in the presence of various nutritional supplements

A series of MNM agar growth assays were carried out to screen for possible nutritional supplement(s) complementing GSH auxotrophy in the Δ*gcsA* mutant. The following supplements were added at the concentrations indicated: 24 mmol l^{-1} Na-glutamate (Na-Glu), 4 mmol l^{-1} cysteine (Cys), 20 mmol l^{-1} glycine (Gly), 20 mmol l^{-1} methionine (Met), 24 mmol l^{-1} Na-Glu + 4 mmol l^{-1} Cys + 20 mmol l^{-1} Gly, 24 mmol l^{-1} Na-Glu + 20 mmol l^{-1} Met + 20 mmol l^{-1} Gly, 0.5% yeast extract, and 2.0% (58 mmol l^{-1}) lactose [15, 22]. In these assays, agar plates were point-inoculated by pipetting 10^5 freshly grown (6 days) conidia suspended in 5 μl aliquots of 0.9% NaCl, 0.01% Tween 80 solution onto MNM agar plates, and were incubated for 5 days at 37 °C [19].

Determination of germination rates in submerged liquid cultures

To determine germination rates, 10^8 freshly grown conidia were inoculated into 100 ml MNM without any nutritional supplements, or supplemented with 10 mmol l^{-1} GSH or the mixture of 20 mmol l^{-1} Met, 20 mmol l^{-1} Gly, and 4 mmol l^{-1} Na-Glu as required. All submerged cultures were grown at 37 °C with shaking at 3.3 Hz frequency for 6, 10, and 24 h incubation times [19]. Germination rates (number of germinated spores/total number of spores) were determined *via* counting conidia and also germinated conidia under a phase contrast microscope (Euromex, Arnhem, The Netherlands).

Production of reactive species (RS) and determination of specific intracellular enzyme activities

In these experiments, the strains were sporulated either on GSH-free MNM agar (the THS30.3 control strain) or on MNM agar supplemented with 10 mmol l^{-1} GSH as required (all tested strains including the THS30.3 control strain). Following that all strains were pre-cultured in 100 ml aliquots of MNM with (the THS30.3 control and both the Δ*glrA* and Δ*gcsA* gene deletion strains) or without (only the THS30.3 control strain) 10 mmol l^{-1} GSH supplementation. All culture media were inoculated and incubated as described above for 20 h. Mycelia were always harvested (filtered and washed on sintered glass) and were transferred to 100 ml aliquots of freshly prepared MNM without any GSH supplementation. The intracellular RS levels were characterized by the formation of 2′,7′-dichlorofluorescein (DCF) from 2′,7′-dichlorofluorescin diacetate according to Yin et al. [23]. Changes in the specific activities of antioxidant enzymes were also recorded in separate experiments as described before by Emri et al. [24].

Stress sensitivity studies

To study the stress sensitivity of the Δ*glrA* mutant, the agar plate assays of Balázs et al. [19] were adapted with small modifications. The following stress-generating agents were tested: hyperosmotic stress: 1.0 mol l^{-1} KCl, 1.0 mol l^{-1} NaCl, 2.0 mol l^{-1} sorbitol; cell wall integrity stress: 54 μmol l^{-1} Congo Red; antimycotic stress: 200 μg ml^{-1} fluconazole and 75 μg ml^{-1} amphotericin B. Stress plates were also point-inoculated with 10^5 conidia and were incubated at 30 °C for 5 days [19, 23].

Statistical analysis of experimental data

All experiments were performed in three independent sets, and mean ± SD values were calculated and are presented. Statistical significances were calculated using Student's *t*-test, and *p*-values less than 0.05 were considered as statistically significant.

Results

GSH metabolic enzymes and GSH transporters in A. nidulans

As summarized in Figure 1 and Supplementary Table S-II, all elements of GSH metabolism and GSH transport, which are well-characterized in budding

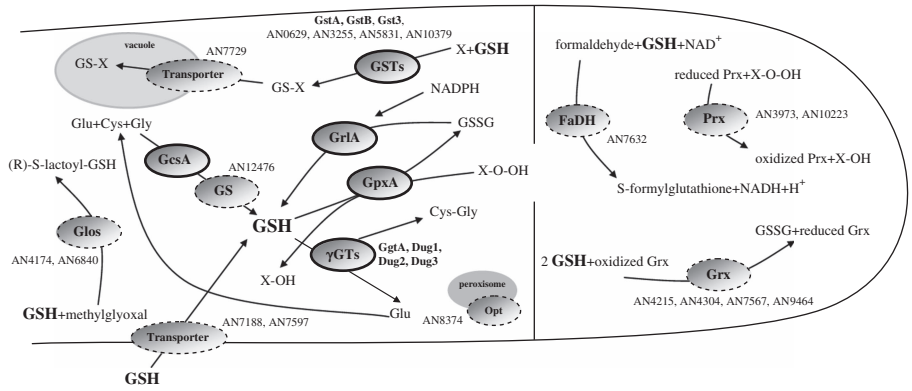

Figure 1. A schematic model of GSH metabolism in *A. nidulans*. Locus IDs were exported from the AspGD (http://www.aspergillusgenome.org/). Note that enzymes marked with dashed lines have not been characterized yet in *A. nidulans*. For further details, see Supplementary Table S-II

yeast [1], are also present in *A. nidulans.* These proteins are associated with the biosynthesis and degradation of GSH, the maintenance of redox balance within the cells, the detoxification of toxic endogenous and exogenous metabolites as well as the uptake and intracellular transport of GSH (Figure 1, Supplementary Table S-II; [1]). Importantly, this filamentous fungus seems to possess two putative homologs of the following *S. cerevisiae* proteins: Prx1 peroxiredoxin (AN3973, AN10223), Ure2 glutathione peroxidase/glutathione S-transferase (GPx/GST; AN3255, AN4905/GstA), and Opt1 cell surface GSH transporter (AN7597, AN7188).

*Optimization of the GSH supplementation of the Δ*glrA *and Δ*gcsA *mutant strains*

Both strains required GSH supplementation when they were grown on MNM agar at 37 °C for 5 days, and optimal growths were recorded at 8–10 mmol l^{-1} GSH concentrations in both cases (Supplementary Figure S1). It is worth noting that the temperature sensitivity of the Δ*glrA* strain observable at 37 °C [12] was fully complemented by the addition of 10 mmol l^{-1} GSH. Nevertheless, the supplementation of MNM agar with 8–10 mmol l^{-1} GSH still resulted in a high but still partial (75%–80%) complementation of the no growth phenotype of the Δ*gcsA* strain, which was observed at lower GSH concentrations (Supplementary Figure S1).

*Growth of the Δ*gcsA *strain on MNM agar supplemented with various nutrients*

As shown in Supplementary Figure S2, the GSH auxotrophy of the Δ*gcsA* mutant could not be complemented with any nutritional supplements tested

including yeast extract, lactose, or the amino acids Na-Glu, Gly, Cys, Met, and their combinations.

Germination of the conidiospores of the ΔglrA and ΔgcsA mutants

As expected, both mutants germinated well in GSH supplemented MNM culture medium (Figure 2). The Δ*glrA* mutant also germinated in MNM medium supplemented with 20 mmol l^{-1} Met, 20 mmol l^{-1} Gly, and 24 mmol l^{-1} Na-Glu instead of GSH owing to *de novo* GSH biosynthesis. Not surprisingly, the germination rates of both mutant strains were rather low in the absence of GSH (10.7% for the Δ*glrA* and 8.1% for the Δ*gcsA* strains) because these spores could only rely on their intracellular GSH reserves, and the Δ*gcsA* mutant was not able to use the combination of the amino acids Met, Gly, and Glu for *de novo* GSH

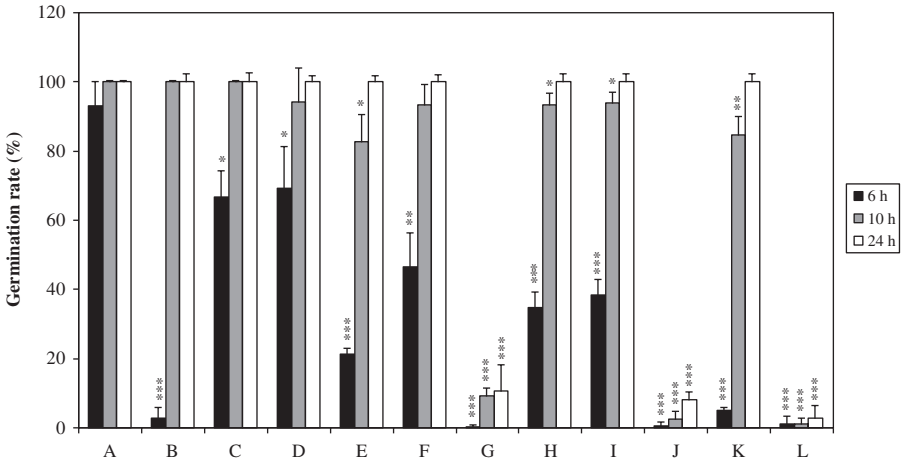

Figure 2. Germination rates of conidiospores produced by the control THS30.3 and the gene deletion mutant Δ*glrA* and Δ*gcsA* strains under various culture conditions. Freshly grown (harvested after 6 days incubation) conidia (10^8 spores in each experiment) were inoculated into 100 ml aliquots of MNM, and the submerged cultures were grown at 37 °C and at 3.7 Hz shaking frequency for 6, 10, and 24 h. In Parts A–C, the THS30.3 control strain was sporulated on MNM without any GSH supplementation. In all other cases (the THS30.3 control strain in Parts D–F, the Δ*glrA* strain in Parts G–I and the Δ*gcsA* strain in Parts J–L), conidiospores were produced on MNM supplemented with 10 mmol l^{-1} GSH. Conidia were inoculated into one of the following culture media: MNM (Parts A, D, G, and J), MNM supplemented with 10 mmol l^{-1} GSH (Parts B, E, H, and K), and MNM supplemented with 20 mmol l^{-1} methionine, 20 mmol l^{-1} glycine, and 24 mmol l^{-1} Na-glutamate (Parts C, F, G, and L). Note that the germination rates reached 100% after 24 h incubation in all cases with the exception of Parts G, J, and L. In this figure, mean ± SD values calculated from three independent experiments are presented. Asterisks indicate significant differences in comparison to the appropriate controls (Part A) at $p < 5\%$ (*), $p < 1\%$ (**), and $p < 0.1\%$ (***) as calculated by the Student's *t*-test

synthesis either (Figure 2). Importantly, the addition of 10 mmol l^{-1} GSH to MNM hindered considerably the germination of THS30.3 conidia (6 h incubation time; Part D in Figure 2) when the control strain was sporulated on GSH-free MNM agar. In addition, when conidia of the control strain were produced on GSH-supplemented MNM agar the sporulation rates measured in GSH-free or in Met–Gly–Glu supplemented MNM media (Parts D and F in Figure 2) were significantly lower than those found in the appropriate controls, where THS30.3 conidia were produced on GSH-free MNM agar (Parts A and C in Figure 2).

RS productions and GSH-dependent and GSH-independent antioxidant enzyme activities

To characterize the nature of stress sensed by *A. nidulans* cells pre-cultured in the presence of 10 mmol l^{-1} GSH and transferred to GSH-free MNM in liquid cultures, increases in DCM, changes in RS levels (given by specific DCF productions) as well as alterations in the specific activities of some antioxidative enzymes were measured at 6 and 12 h incubation times (Table I).

Unexpectedly, the growth of the THS30.3 control strain was almost completely inhibited during pre-culturing in MNM supplemented with 10 mmol l^{-1} GSH (Table I). When the physiological conditions of THS30.3 control cultures pre-cultured in the presence and absence of GSH were compared, mycelia from the GSH-supplemented cultures possessed decreased specific GPx and GR activities (3.9 times and 2.6 times lower, respectively) with remarkably high (about 100 times higher) RS levels although the specific catalase activity was about eleven times higher (Table I). After 12 h incubation in GSH-free MNM, biomass gains by the GSH pre-cultured control were approximately 7-fold with improving physiological parameters and recovery from oxidative stress (e.g., RS levels decreased 150-fold concomitantly with a 6.5-fold increase in the specific GPx activity). Meanwhile there were no significant differences left in specific RS productions and GPx activities of the GSH-MNM and MNM pre-cultured mycelia the differences between the specific GR and catalase activities remained still high, approximately 3.7 times lower and 7.8 times higher for the GSH pre-cultured mycelia, respectively (Table I).

As far as the gene deletion mutants are concerned, the Δ*glrA* and Δ*gcsA* strains grew considerably better in the presence of 10 mmol l^{-1} GSH than the GSH pre-cultured THS30.3 control strain (Table I). Nevertheless, the mycelia of the Δ*glrA* mutant with no GR activity accumulated 2.5 times more RS than those of the Δ*gcsA* strain although the specific catalase activity in the Δ*glrA* strain was 3.8 times higher (Table I). Importantly, the oxidative stress was completely

Table I. Changes in the specific DCM (dry cell mass) and RS (intracellular reactive species) productions and in the specific GPx (glutathione peroxidase), GR (glutathione reductase), and catalase activities

	DCM [g (l culture)$^{-1}$]			RS [(mmol DCF) (kg DCM)$^{-1}$]			GPx [mkat (kg protein)$^{-1}$]			GR [mkat (kg protein)$^{-1}$]			Catalase [kat (kg protein)$^{-1}$]		
Strains	0 h	6 h	12 h	0 h	6 h	12 h	0 h	6 h	12 h	0 h	6 h	12 h	0 h	6 h	12 h
THS30.3[a]	2.5 ± 0.4	4.2 ± 0.4††	4.5 ± 0.9†	0.21 ± 0.05	0.41 ± 0.02††	0.23 ± 0.04	3.0 ± 0.3	1.5 ± 0.3††	4.4 ± 0.4††	9.5 ± 0.5	8.6 ± 0.5	12.8 ± 0.9††	0.13 ± 0.05	0.14 ± 0.05	0.20 ± 0.01
THS30.3[b,c]	0.17 ± 0.05	0.50 ± 0.02†††	1.2 ± 0.4†	22 ± 6***	2.4 ± 0.9**,††	0.15 ± 0.07††	0.77 ± 0.02***	0.7 ± 0.2**	5.0 ± 0.4†††	3.7 ± 0.7***	4.4 ± 0.8***	3.5 ± 0.7***	1.4 ± 0.1***	1.51 ± 0.04***	1.56 ± 0.02***
Δ*glrA*[b,d]	0.6 ± 0.2	0.9 ± 0.3	1.8 ± 0.7†	4.7 ± 1.9^{+++}	0.8 ± 0.1$^{++,\ \dagger}$	0.25 ± 0.09†	3.9 ± 0.4^{+++}	4.8 ± 1.1^{+++}	4.1 ± 0.4	n.d.	n.d.	n.d.	1.40 ± 0.01	0.8 ± 0.2$^{++,\ \dagger\dagger}$	1.9 ± 0.1$^{++,\ \dagger\dagger}$
Δ*gcsA*[b,d]	1.00 ± 0.08$^{\#}$	1.50 ± 0.06$^{\#,\dagger\dagger}$	1.7 ± 0.4†	1.9 ± 0.1^{+++}	4.9 ± 1.1$^{++,\#\#,\dagger\dagger}$	4.5 ± 0.9$^{+++,\#\#,\dagger\dagger}$	4.4 ± 0.8^{+++}	0.7 ± 0.3$^{\#\#,\dagger\dagger}$	0.12 ± 0.02^{+} $^{++,\#\#\#,\dagger\dagger\dagger}$	9.2 ± 1.3^{+++}	5.5 ± 1.3†	1.2 ± 0.1$^{++,\dagger\dagger\dagger}$	0.37 ± 0.04$^{+++,\ \#\#\#}$	0.23 ± 0.04^{+} $^{++,\#\#,\dagger}$	0.6 ± 0.1$^{+++,\#\#\#,\dagger}$

Note: In this table, mean ± SD values calculated from three independent experiments are presented. Significant differences between THS30.3 cultures grown with and without 10 mmol l^{-1} GSH in the pre-cultures are indicated by asterisks (**$p < 1\%$ and ***$p < 0.1\%$, calculated by the Student's *t*-test). Meanwhile, DCF values and enzyme activities of the gene deletion mutants (Δ*gcsA*, Δ*glrA*) were compared to those measured in MNM cultures of the THS30.3 control strain, inoculated with mycelia harvested from GSH-supplemented pre-cultures ($^{++}p < 1\%$ and $^{+++}p < 0.1\%$). Significant differences between the Δ*gcsA* and Δ*glrA* cultures ($^{\#}p < 5\%$, $^{\#\#}p < 1\%$, and $^{\#\#\#}p < 0.1\%$) and between 6, 12 h vs. 0 h cultures are also shown ($^{\dagger}p < 5\%$, $^{\dagger\dagger}p < 1\%$, and $^{\dagger\dagger\dagger}p < 0.1\%$). "n.d." stands for "not detectable."

[a]THS30.3 was sporulated on MNM agar, was pre-cultured in 100 ml MNM with no GSH supplementation for 20 h at 37 °C, and mycelia were harvested and transferred to 100 ml aliquots of MNM.

[b]Strains were sporulated on MNM agar supplemented with 10 mmol l^{-1} GSH, were pre-cultured and cultured as described under footnote ([a]) with the exception that MNM used during the 20 h pre-incubation period also contained 10 mmol l^{-1} GSH.

[c]Because the THS30.3 control strain hardly grew in submerged MNM cultures supplemented with 10 mmol l^{-1} GSH, mycelia from 4 × 100 ml pre-cultures were harvested and transferred to 100 ml MNM, which did not contain any extra GSH. Hence DCM values recorded in these cultures were not compared to those measured in any other cultures.

[d]Mycelia from 2 × 100 ml cultures were harvested and transferred to 100 ml MNM, which did not contain any extra GSH. Only DCM values measured in cultures of the Δ*glrA* and Δ*gcsA* strains were compared.

eliminated in the Δ*glrA* cultures after 12 h incubation in the absence of GSH (RS production decreased 18.8-fold) similar to the THS30.3 control strain. Meanwhile an accumulating oxidative stress was clearly observable in the Δ*gcsA* cultures with increased RS productions (a 2.4-fold increase between 0 and 12 h incubation times) and specific catalase activities (increased 1.35-fold) as well as with significantly decreased specific GPx and GR activities (decreased 36.7-fold and 7.7-fold, respectively) (Table I).

Stress sensitivity phenotypes of the ΔglrA mutant

Temperature sensitivity and oxidative sensitivity phenotypes of a Δ*glrA* gene deletion mutant have been reported before by Sato et al. [12]. The Δ*glrA* mutant constructed and characterized in this study by us also showed increased temperature and oxidative stress sensitivities similar to those described previously (data not shown; [12]). In addition, we also demonstrated for the first time the increased hyperosmotic stress (1.0 mol l^{-1} KCl, 1.0 mol l^{-1} NaCl, and 2.0 mol l^{-1} sorbitol), cell wall integrity stress (54 μmol l^{-1} Congo Red) and antimycotic (200 μg ml^{-1} fluconazole and 75 μg ml^{-1} amphotericin B) sensitivity of the Δ*glrA* strain (Figure 3).

Discussion

GSH possesses a number of redox-dependent and redox-independent functions in eukaryotic organisms including fungi [1, 3, 4]. It is important to note that the elucidation of the remarkably versatile physiological functions of GSH needs further research. In this study, we primarily aimed at screening for new GSH-metabolism-related phenotypes especially concerning conidiospore germination and also for GSH-dependent stress defenses. Any new information on the physiological and developmental functions of GSH and GSH metabolic enzymes in fungi may help us to gain a deeper insight in the pathogeneses of human pathogenic fungi [15], to set up new antifungal drug development strategies [25, 26] and also to develop new GSH overproducer [27–29] as well as more stress tolerant and more stable [1, 2, 17, 30] industrial fungal strains.

A. nidulans *possesses a robust GSH uptake and GSH metabolic system*

The aspergilli, which represent an outstandingly important group of filamentous fungi, have a complex and robust stress response system, which is

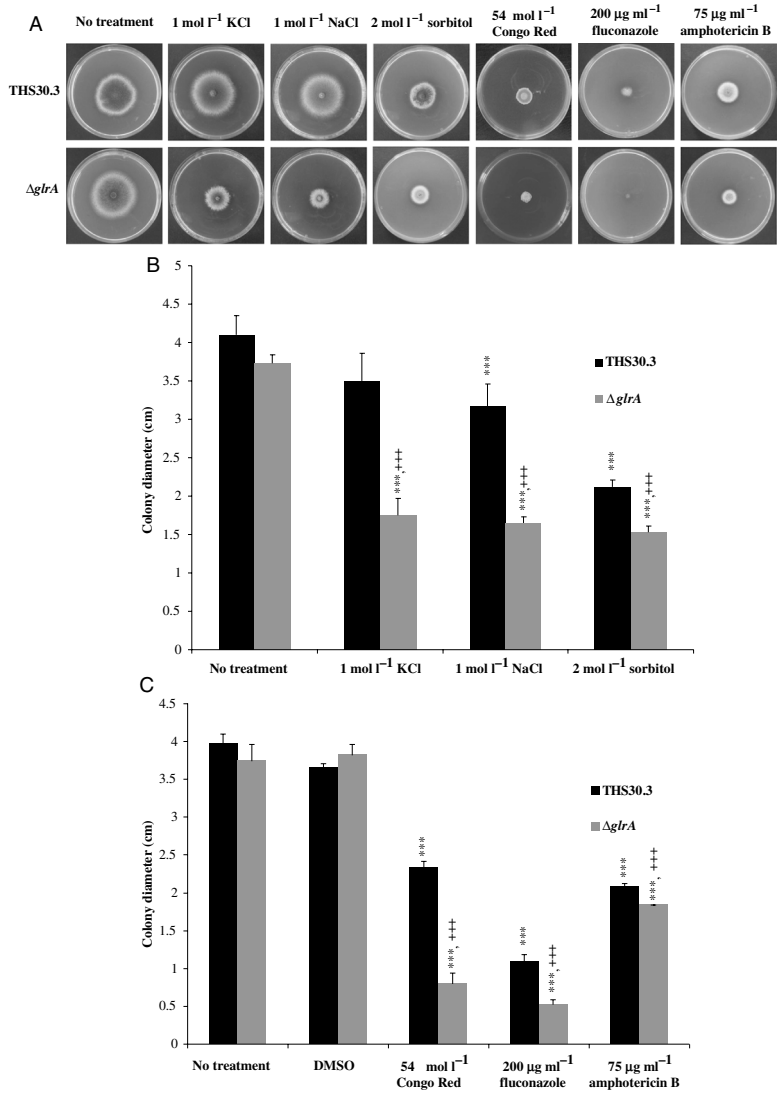

Figure 3. Stress sensitivities of the THS30.3 control and the Δ*glrA* gene deletion mutant strains exposed to various types of stress. (A) Typical stress sensitivities observed in surface cultures on MNM agar plates are shown. (B) and (C) Mean colony diameters of the control (black columns) and the mutant (gray columns) strains are presented with SD values calculated from three independent experiments. In Part B, cultures were exposed to ionic (NaCl and KCl) and non-ionic (sorbitol) hyperosmotic stress, meanwhile in Part C, growth inhibitions caused by cell wall integrity stress (Congo Red) and antimycotics (fluconazole and amphotericin B) are shown. Conidiospores (10^5 in 5 µl suspension) were point-inoculated on MNM agar and the agar plates were incubated for 5 days. Since growth of Δ*glrA* was comparable to the control strain without GSH supplementation at 30 °C, all plates were incubated at this temperature. Because amphotericin B was dissolved and added in DMSO, suitable controls for this antifungal were always prepared. The concentration of DMSO in these MNM agar plates was 2% (v/v). Asterisks and plus symbols indicate significant differences in comparison to non-treated controls (treated THS30.3 vs. non-treated THS30.3 and treated Δ*glrA* vs. non-treated Δ*glrA*) and to treated control strain (treated Δ*glrA* vs. treated THS30.3), respectively, at $p < 0.1\%$ (***, +++). p values were calculated using the Student's t-test

suitable to cope with a wide spectrum of environmental stress [23]. In good accordance with this, some genes encoding important GSH metabolic enzymes in *S. cerevisiae* like Prx1 mitochondrial peroxiredoxin (possessing thioredoxin peroxidase activity and its regeneration requires thioredoxin reductase and GSH in yeast [22]) and Ure2 protein with GPx activity (but also can mutate to gain GST activity [31]) as well as Opt1 cell surface GSH transporter (also known as Gsh11 or Hgt1 [32]) each brought up two paralogs in *A. nidulans* (Figure 1, Supplementary Table S-II).

Although *A. nidulans* is a widely used filamentous fungus model organism with its whole genome sequenced and annotated [33] the number of functionally characterized proteins related to GSH metabolism and transport is surprisingly low (Figure 1, Supplementary Table S-II). For example, the GlrA enzyme with GR activity plays an important role in the oxidative stress defense system of the fungus [12] and also in the reduction of cytotoxic elemental sulfur [34]. Interestingly, meanwhile GstA and GstB GSTs take part in the degradation of various toxic xenobiotics [19, 34] and even in the detoxification of metals (GstA, [35]), GgtA γ-glutamyl transpeptidase is not necessary for the bulk degradation of GSH [36]. Instead, the DUG pathway relying on Dug1–3 proteins is likely to be responsible for the cytosolic degradation of GSH [34]. It is worth mentioning that some genes coding for important GSH metabolism-related enzymes have only been characterized through transcriptional (*dug1-3* [36]; *gpxA* [37]) or translational (*gst3* [38]) changes. Therefore, a deeper characterization of GSH metabolic enzymes and GSH transporters (Figure 1, Supplementary Table S-I) using molecular genetic tools are urgently needed in *A. nidulans*.

A. nidulans *needs high concentration of GSH for growth and germination of conidia*

In this study, the deletion of *gcsA* encoding γ-GCS resulted in a lethal phenotype unless culture medium was supplemented with exogenous GSH similarly to previous observations with the Δ*GSH1* mutant of *S. cerevisiae* [6–8]. Unlike in the case of budding yeast [7, 8], the Δ*gcsA* mutant required exogenous GSH at much higher concentration (at 8–10 mmol l^{-1} at least) than GSH-depleted *S. cerevisiae* Δ*gsh1* cells (0.5 μmol^{-1} [8]). These findings indicate that GSH may possess further crucially important physiological functions in *A. nidulans* other than the stabilization of the redox milieu of the fungal cells [7, 8]. The essential redox-independent functions of GSH, e.g., in the maturation of iron-sulfur clusters, have been summarized more recently by several authors [3, 4]. Of course, we cannot underestimate the significance of the versatile redox-dependent

functions played by GSH either, e.g., in the stabilization of various cellular membranous structures [1–3]. It is remarkable that other attempts to rescue the growth of γ-GCS-deficient *A. nidulans* cells e.g., by sulfur-containing amino acids like Cys or Met and by their combinations with Na-Glu and Gly, or by the addition of yeast extract or lactose (a carbon source, which stimulates GSH-synthesis in *Penicillium chrysogenum* [24]) also failed in this filamentous fungus. These observations shed light on the minimal GSH requirements of filamentous fungi, which seem to well exceed those of saccharomycetes yeasts [7, 8].

The maturation, survival during storage, stress tolerance, and germination of conidiospores highly depend on the availability of antioxidants like catalase [39], mannitol [40], GSH [41], and also osmolytes like trehalose [42]. The remarkable importance of GSH in germination of *A. nidulans* conidiospores was demonstrated in this study by the decreased germination rates of the Δ*glrA* and Δ*gcsA* strains, although the GR deficient mutant was able to re-synthesize GSH effectively from the combined amino acids of Na-Glu+Met+Gly (Figure 2). Any disturbances of the GSH metabolic system of the aspergilli seem therefore suitable tools to control the germination of the asexual spores of these ascomycetous fungi, which include well-known opportunistic fungal parasites of humans like *A. fumigatus* [43–45].

Overdosing GSH is toxic for A. nidulans

Interestingly, the germination of the THS30.3 control strain was delayed by exogenous GSH added at 10 mmol l^{-1} concentration, and the same strain showed decreased germination rate at 6 h incubation time, when conidiospores produced on GSH-supplemented MNM agar were inoculated into GSH-free or Na-Glu+Met+Gly containing MNM (Figure 2). On the other hand, the same spores germinated better in GSH-supplemented MNM than those which were produced in the absence of exogenously added GSH (Figure 2). These observations together with the findings that the THS30.3 and the Δ*glrA* strains pre-cultured in MNM in the presence of GSH accumulated high concentrations of RS suggested that GSH added at high, 10 mmol l^{-1} concentration was toxic for *A. nidulans* cells (Table I). The severe toxicity of GSH on the THS30.3 control strain (almost no gain in biomass after 20 h incubation at 37 °C) was attributed to the profoundly decreased specific activities of GPx and GR. Meanwhile significant increases in the specific GPx and catalase activities helped *A. nidulans* THS30.3 and Δ*glrA* strains, respectively, to eliminate residual oxidative stress fast after transferring mycelia to GSH-free MNM (Table I). In the Δ*glrA* strain, the reduction of GSSG by the thioredoxin–thioredoxin reductase–NADPH system [12, 39] may also contribute to the neutralization of RS. Increases in the specific catalase, thioredoxin reductase

and cytochrome peroxidase activities in the Δ*glrA* strain were regarded as parts of the compensatory mechanisms being set into operation by the deletion of *glrA* [12]. All these observations may be valuable in the biotechnologist's point of view when the reduction of the toxic effects of GSH on GSH-overproducer industrial fungal strains [26] is aimed at in future strain development projects.

After transferring Δ*gcsA* mycelia into GSH-free MNM, the depletion of the GSH reserves stimulated RS production with the concomitant decreases in the specific GPx and GR activities (Table I). Under these conditions, compensatory mechanisms like a significant increase in the specific catalase activity could not hinder the onset of oxidative stress in the fungal cells (Table I).

GSH is needed for general stress tolerance in A. nidulans

GR enzymes regenerate GSH by reducing GSSG at the expense of NADPH [1, 5]. The elimination of GR-encoding genes resulted in various phenotypes in fungi including reduced growth [13] or even lethality [11], temperature sensitivity [12] as well as decreased oxidative stress [10–13, 46] and sulfur [22] tolerances. Lee et al. [11] reported on the role of GR in the heat and hyperosmotic stress defense systems of *S. pombe* and, in this study, we demonstrated the importance of GlrA in the osmotic, cell wall integrity, and antimycotic stress responses of *A. nidulans* (Figure 2). It is important to note that a series of *tert*-butyl hydroperoxide stress tolerant mutants of *Candida albicans* was developed by Fekete et al. [47, 48], and each mutant possessed significantly increased specific GR, GPx, and glucose-6-phosphate dehydrogenase activities. A more recent study by Jakab et al. [49] demonstrated that a selection of these mutants also showed decreased sensitivities to 19 different stress conditions including various types of oxidative, hyperosmotic, heavy metal, cell wall and membrane integrity, unfolded protein response, pH, and thermal (heat and cold) stress. These observations strengthen the view that "altruistic" GSH and GSH metabolic enzymes play a distinguished role in the general stress tolerance of fungi, which is of primary importance when these microorganisms are employed in various bioprocesses [1, 2].

Conclusions

A. nidulans has a robust GSH metabolic and transport system, the elements of which need a thorough functional analysis (Figure 1). GSH itself and GSH metabolic enzymes play a crucially important role in the germination of conidiospores, which observation may be useful when future strategies for the development of new-type antifungals are designed and elaborated. On the other

hand, any overdose of GSH can be disadvantageous during both spore germination and vegetative growth of hyphae, which should be considered in the development of high-GSH-producer industrial fungal strains. Experimental data collected in this study and also in various fungal species by others have shed light on the remarkable contribution of GSH and GSH metabolic enzymes to the general stress tolerance of fungi, which may be equally interesting for experts working on different fungal growth control and fungal strain development projects.

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Conflict of Interest

The authors declare no conflict of interest.

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