Zsolt Spitzmüller[1], Nak-Jung Kwon[2$], Melinda Szilágyi[1], Judit Keserű[3], Viktória Tóth[1], Jae-Hyuk Yu[2], István Pócsi[1] and Tamás Emri[1,*]

γ-Glutamyl transpeptidase (GgtA) of *Aspergillus nidulans* is not necessary for bulk degradation of glutathione

1 Department of Microbial Biotechnology and Cell Biology, Faculty of Science and Technology, University of Debrecen, Debrecen, Hungary

2 Departments of Bacteriology and Genetics, University of Wisconsin, Madison, WI, USA

3 Department of Human Genetics, Faculty of Medicine, University of Debrecen, Debrecen, Hungary

* Correspondence to: Tamás Emri, Department of Microbial Biotechnology and Cell Biology, Faculty of Science and Technology, University of Debrecen, Egyetem tér 1, H4032 Debrecen, Hungary; e-mail: emir.tamas@science.unideb.hu, +36-52-512900 /62305ext

$ Present address: Macrogen Korea, Seoul Korea.

Abstract

Aspergillus nidulans exhibited high γ-glutamyl transpeptidase (γGT) activity in both carbon starved and carbon limited cultures. Glucose repressed, but casein peptone increased γGT production. Null mutation of *creA* did not influence γGT formation but the functional *meaB* was necessary for the γGT induction. Deletion of the AN10444 gene (*ggtA*) completely eliminated the γGT activity and the mRNA levels of *ggtA* showed strong correlation with the observed γGT activities. While *ggtA* does not contain a canonical signal sequence, the γGT activity was detectable both in the fermentation broth and the hyphae. Deletion of the *ggtA* gene did not prevent the depletion of glutathione observed in carbon starved and carbon limited cultures. Addition of casein peptone to carbon starved cultures lowered the formation of reactive species (RS). Deletion of *ggtA* could hinder this decrease and resulted in elevated RS formation. This effect of γGT on redox homeostasis may explain the reduced cleistothecia formation of *ΔggtA* strains in surface cultures.

Key words: γ-glutamyl transpeptidase, *Aspergillus nidulans*, meaB, glutathione, carbon starvation, carbon limitation, sexual development

Running title: γ-Glutamyl transpeptidase of *A. nidulans*

Introduction

Glutathione (GSH, γ-L-glutamyl-L-cysteinyl-glycine) is one of the most common γ-glutamyl compounds in the Nature occurring in many prokaryotes and almost all the eukaryotes (Penninckx and Elskens 1993, Pócsi *et al.* 2004, Smirnova and Oktyabrsky 2005). Until the biosynthesis of GSH by glutamate-cysteine ligase (EC 6.3.2.2) and glutathione

synthase (EC 6.3.2.3) is well described and documented (Penninckx and Elskens 1993, Pócsi *et al.* 2004, Smirnova and Oktyabrsky 2005), the biochemical pathway of GSH degradation is still unclear. γ-Glutamyl transpeptidase (γGT; EC 2.3.2.2) was described as the enzyme responsible for GSH degradation (Penninckx and Elskens 1993, Pócsi *et al.* 2004, Smirnova and Oktyabrsky 2005), but emerging data in this field suggest that there are alternative pathways. γ-Glutamyl cyclotransferase (EC 2.3.2.4) can eliminate the γ-glutamyl group from γ-glutamyl-peptides forming 5-oxoproline (pyroglutamate) and free peptides. The products of this enzyme, 5-oxoproline and peptides, are hydrolyzed forward to glutamate and other amino-acids by 5-oxoprolinase and different peptidases, respectively. Although GSH is not a substrate of several γ-glutamyl cyclotransferases (Bodnaryk and McGirr 1973, Orlowski *et al.* 1969), the cytosolic ChaC γ-glutamyl cyclotransferase of mammals acts specifically on GSH but not on other γ-glutamyl compounds (Kumar *et al.* 2012). A γ-glutamyl cyclotransferase and 5-oxoprolinase dependent GSH degradation also occurs in the plant *Arabidopsis thaliana* (Ohkama-Ohtsu *et al.* 2008). In *Saccharomyces cerevisiae*, the DUG (Deficient in Utilization of Glutathione) system represents an alternative pathway for degradation of GSH (Kumar *et al.* 2003, Ganguli *et al.* 2006). The genes *dug2* and *dug3* encode the two subunits of a glutamine amidotransferase complex which remove the γ-glutamyl moiety from GSH, while *dug1* encodes a cysteinyl-glycine metallo-di-peptidase (Kaur *et al.* 2012). The DUG system is also important in the degradation of GSH in *Candida albicans* (Desai *et al.* 2011).

Due to these recent observations, the primary physiological role of γGT has been questionable. In mammalian cells, the membrane bound γGT degrades the extracellular GSH and other γ-glutamyl compounds (Heisterkamp *et al.* 2008). The products of hydrolysis (*e.g.* cysteine) can enter the cells and serve substrate for cellular GSH biosynthesis, which is particularly important during oxidative stress (Zhang *et al.* 2005, 2009, Heisterkamp *et al.* 2008). In *Saccharomyces cerevisiae*, the vacuolar membrane bound γGT is important in the

degradation of GSH stored in vacuoles. The released amino-acids are utilized as nitrogen sources during nitrogen starvation (Mehdi and Penninckx 1997, Springael and Penninckx 2003) or support GSH synthesis *e.g.* during Cd^{2+} stress (Adamis *et al.* 2009). Transcription of *Schizosaccharomyces pombe ggt1* and *ggt2* genes is stress sensitive; they are induced by not only carbon and nitrogen limitation but both genes are up-regulated during oxidative or nitrosative stress (Park *et al.* 2004, Kang *et al.* 2005). The γGT of *Bacillus subtilis* participates in the utilization of capsular poly-γ-glutamic acid as glutamate source in stationer phase cultures (Kimura *et al.* 2004). In the human pathogen bacterium *Helicobacter pylori*, the main physiological function of periplasmic γGT is to metabolize extracellular GSH and glutamine (Shibayama *et al.* 2007). In *Colletotrichum graminicola,* γGT is involved in the utilization of (exogenous) GSH as nitrogen source (Bello *et al.* 2013), while in another plant pathogen fungus, *Sclerotinia sclerotiorum*, γGT is important in the maintenance of redox homeostasis during development of sclerotia and compound appressoria (Li *et al.* 2012). γGT can also participate in the degradation of oxidized glutathione (GSSG) in plant apoplasts (Ohkama-Ohtsu *et al.* 2008) and in the degradation of glutathione-S-conjugates (Ohkama-Ohtsu *et al.* 2008, Wünschmann *et al.* 2010, Agblor and Josephy 2013).

Previously, we found that γGT is induced by carbon starvation and this induction was accompanied with the bulk degradation of GSH both in *Penicillium chrysogenum* (Sámi *et al.* 2001) and *Aspergillus nidulans* (Emri *et al*. 2004). Here, we would like to elucidate the physiological significance of the elevated γGT activities in carbon stressed *A. nidulans* cultures. We demonstrate that the induction of AN10444 gene, encoding a γGT-domain containing protein was responsible for the increased specific γGT activities in carbon starved and carbon limited (Winderickx *et al*. 2003) cultures. We also show that γGT was accumulated both in the fermentation broth and mycelia and this activity was not necessary for the observed decrease in GSH pools. We demonstrate that utilization of extracellular

peptides was important to prevent the accumulation of reactive species (RS) in carbon starved cultures and we suggest that γGT contributed in this process. This effect of γGT on redox homeostasis may explain the decreased cleistothecia formation observed in *ΔggtA* strains.

Materials and methods

Strains and culturing conditions

Strains used in this study are presented in Table 1. Fungi were grown in shake flasks (500 ml) containing 100 ml minimal-nitrate medium, pH 6.5 supplemented with 5 g/l yeast extract and with appropriate nutritional supplements (Barratt *et al.* 1965). Culture media were inoculated with $5x10^7$ spores and were incubated at 37 ºC, 200 rpm for 18 h (glucose growing late exponentially phase cultures). Mycelia from these cultures were washed and transferred into either glucose free minimal medium (GD - glucose deprived, carbon starving cultures) or lactose (20 g/l) containing medium (LC - lactose containing, carbon limited cultures) and were further cultivated at 37 ºC, 200 rpm. The starting dry cell mass was always approximately 6 g/l. In some experiments the glucose free minimal medium and the lactose containing medium was supplemented with 4 g/l casein peptone, 4 g/l yeast extract, 4 g/l bovine serum albumin (BSA), 4 g/l glycyl-glycine (Gly-Gly), 4 g/l Na-glutamate or 4 g/l Na-glutaminate.

Determination of conidia and cleistothecia produced in surface cultures

Minimal-nitrate media (pH 6.5) with appropriate nutritional supplements (Barratt *et al.* 1965) were point inoculated with 5 µl freshly made conidia suspension containing 10^5 conidia and were incubated at 37 ºC for 6 d. Conidia were collected from the entire colony with

distilled water containing 0.1 v/v % Tween 20 and were counted using a hemocytometer (Hagiwara *et al.* 2007). The amount of produced conidia was expressed in conidia/cm^2.

Cleistothecia formation was induced by oxygen limitation as described by Kawasaki *et al.* (2002). Plates were incubated at 37 °C for 6 d. The number of cleistothecia were counted under a stereo microscope (both the dark pigmented, fully developed cleistothecia and the immature cleistothecia initiates were counted) and given in cleistothecia/cm^2.

Generation of AN10444 (ΔggtA) and AN5658 deletion and complemented strains

To generate the tNJ190 (ΔAN10444) and tNJ188 (ΔAN5658) strains, the double-joint PCR method was used (Yu *et al*., 2004). Both flanking regions of AN10444 were amplified using the primer pairs of oNK-1068 (5'-TGCTCGATCTTCATCATGCTCTGTG-3'), oNK-1069 (5'-GCTTTGGCCTGTATCATGACTTCA ACTGGACAGCCTCGTCTTCTACTG-3') and oNK-1070 (5'-ATCGACCGAACCTAGGTAGGGTA TCACACTTAAGGTGAATCTAGAGGC-3'), oNK-1071 (5'-TCTCCTTGTATCCTAGCTCACCAG-3') from the WT genomic DNA as a template, respectively. For the amplification of AN5658 flanking regions, the primer pairs of oNK-1062 (5'-TCGTGAGCTGTCCAGAATTCAGAG-3'), oNK-1063 (5'-GCTTTGGCCTGTATCATGACTTCA TGATTGAGGTTGAGTAGAGAATGTG-3') and oNK-1064 (5'-ATCGACCGAACCTAGGTAGGGTA ATCTGGGTGAAATCCATGTCCGTC-3'), oNK-1065 (5'-TCCACAGCCTCAATATCTTACTCAC-3') were used. The *AfupyrG* marker was amplified with the primer pairs oJH-83 and oJH-86. The final deletion constructs of AN10444 and AN5658 were amplified with oNK-1072 (5'-TGCGCGTTCCTCTTTGAGCAGCTAG-3'), oNK-1073 (5'TGATGTCACCACCACGTCCTTCATG-3') and oNK-1066 (5'-ACGTAGTGTCTGTTTGGCGAGCTG-3'), oNK1067

(TCTCGAGTCATCCTGCGTTACAC-3'), respectively. The final PCR products were introduced into *A. nidulans* rJMP1.59 (parental strain) (Kwon *et al*. 2010).

For the generation of AN10444 and AN5658 complemented strains (tNJ151 and tNJ189, respectively), the genomic DNA fragments of both genes were PCR-amplified by the primer pairs oNK-1076 (5'-atat GGATCC TGCTCGATCTTCATCATGCTCTGTG), oNK-1077 (5'-atat GCGGCCGC TCTCCTTGTATCCTAGCTCACCAG-3') and oNK-1074 (5'-atat CAGCTG TCGTGAGCTGTCCAGAATTCAGAG-3'), oNK-1075 (5'-atat GCGGCCGC TCCACAGCCTCAATATCTTACTCAC-3') from each genomic DNA, and digested with *Bam*HI·Klenow/*Not*I (*AN10444*) and P*vu*II/*Not*I (*AN5658*), then cloned between P*vu*II and *Not*I of pHS3 (Kwon *et al*., 2010) containing ¾*AnipyroA* (Osmani *et al*., 1999), respectively. Each construct was introduced into the recipient ΔAN10444 or ΔAN5658 strain, where preferentially a single copy inserted into the *pyroA* locus. Deletion strains were checked by PCR-amplification followed by restriction enzyme digestion. Complemented strains were confirmed by PCR amplification and by Northern-blot.

Determination of reactive species (RS) as well as GSH and GSSG content of cultures

Intracellular RS production (Halliwell and Gutteridge, 2007) was characterized by the formation of 2',7'-dichlorofluorescein (DCF) from 2',7'-dichlorofluorescin diacetate. 2',7'-dichlorofluorescein was detected by spectrofluorimeter from cell free extracts prepared by 5-sulfosalicylic acid treatment as described earlier (Emri *et al*. 1997).

GSH and GSSG contents were measured from cell free extracts prepared by 5-sulfosalicylic acid treatment (Emri *et al*. 1997) and from fermentation broth using the DTNB-glutathione reductase assay according to Anderson (1985).

Enzyme activity assays

Intracellular γGT activity (intra-γGT)

Mycelia from submerged cultures were filtrated and washed with distilled water. Cell free extracts were prepared by x-pressing. For determining γGT activities 0.1 mol/l Tris-HCl (pH = 8.0) buffer containing 1 mmol/l γ-glutamyl-*p*-nitroanilide (γGpNA; as γ-glutamyl donor) and 20 mmol/l Gly-Gly (as γ-glutamyl acceptor) was added to equal volume of cell free extract. After 1 h incubation at room temperature samples were centrifuged (10000 G, 4 ºC, 10 min) and the released p-nitroanilide was detected in the supernatant at 410 nm using a spectrophotometer (Emri *et al.* 1997). Protein content of cell free extracts was measured with Bradford-reagent (Bradford 1976) and specific intra-γGT activities were expressed in nkat/mg protein.

Extracellular (secreted) γGT activity (extra-γGT)

Mycelia from cultures were removed by filtration and the cell free fermentation broth was used for measurements. Extra-γGT activities were determined as described above, but in this case 5 % (v/v) filtered fermentation broth was used as sample and the incubation time was 20 min. Specific extra-γGT activities were expressed in nkat/ml fermentation broth.

The amount of the released p-nitroanilide was also measured in the absence of the γ-glutamyl acceptor Gly-Gly (hydrolase activity). This γGpNA hydrolase activity was measured from both the cell free extracts and the fermentation broth and was always less than 10 % of the transpeptidase activity.

Extracellular protease activity

Protease activity of the cell free fermentation broth was measured with azocasein substrate as described earlier (Szilágyi *et al.* 2012).

RT-qPCR assay

Total RNA was isolated from lyophilized mycelia using Trisol reagent according to Chomczynski (1993). Samples were taken at 6h after transferring late exponentially phase mycelia into GD, GD + casein peptone, LC, LC + casein peptone media. Changes in the relative transcription of AN10444 were measured in reverse transcription quantitative real-time PCR (RT-qPCR) experiments as described earlier (Kovács *et al.* 2013) with the following primer pairs at 51 °C as annealing temperature:

AN10444 (*ggtA*): F: 5´-GTCGCCATCGCTGTCGTTATC-3´, R: 5´-CGAGTCCAACGGTGACGGAAG-3´.

AN6700 (*eEF-3*; as housekeeping gene): F: 5'- CCTATTCCCGAGCAAGTTC-3', R: 5'-TGATGTTCCTGACGATGGC-3'

AN6542 (*actA*; as housekeeping gene): F: 5′-GAAGTCCTACGAACTGCCTGATG-3, R: 5′-AAGAACGCTGGGCTGGAA-3'

Relative transcription levels were quantified with $\Delta\Delta CP = \Delta CP_{treated} - \Delta CP_{control}$, where $\Delta CP_{treated} = CP_{housekeeping\ gene} - CP_{AN10444}$ measured in GD, GD + casein peptone, LC, LC + casein peptone cultures, $\Delta CP_{control} = CP_{housekeeping\ gene} - CP_{AN10444}$ measured in control (glucose containing late exponentially phase) cultures. CP values stand for the RT-qPCR cycle numbers of crossing points recorded for the AN10444 target and AN6700 (Szilágyi *et al.* 2013) or AN6542 (Kovács *et al.* 2013) "housekeeping" genes.

2-D gel electrophoresis and identification of extracellular proteins

Proteins present in the fermentation broths were precipitated with trichloroacetic acid and washed with acetone as described earlier (Pusztahelyi *et al.* 2011). Proteins (100 mg) were redissolved in 125–125 ml of rehydration buffer (IEF) containing 7 mol/l urea, 2 mol/l thiourea, 2 w/v % CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate), 0.2 w/v % 3/10 IPG buffer (Bio-Rad Laboratories), 50 mmol/l dithiothreitol and 0.002 w/v %

Bromophenol Blue. For first dimension isoelectric focusing, 7 cm pH 3–10 IPG strips (Bio-Rad Laboratories) were rehydrated in IEF buffer containing the sample overnight at room temperature. Isoelectric focusing was performed using Bio-Rad Protean IEF Cell. Proteins on the IPG strips were focused at 20 °C at 4000 V to a total of 10,000 Vh initiated by a slow voltage gradient from 0 to 250 V in 20 min, then from 250 to 4000 V in 2 h. After isoelectric focusing, IPG strips were processed for the second-dimension separation as described previously (Keserű *et al.* 2011). Strips were applied to a Mini-Protean 2-D Electrophoresis Cell (Bio-Rad Laboratories, Paris) with a 5 % stacking gel, 13 % separating gel and electrophoresis was performed at constant voltage of 200 V.

Selected spots were cut from the gels manually, distained and throughout washed three times with 50 % acetonitrile in 25 mmol/l ammonium bicarbonate solution. After stain removal trypsin digestion was performed using stabilized MS grade bovine trypsin at 37 ^{o}C overnight. The digested peptides were extracted, concentrated with speed-vac and redissolved in 10 µl 1 w/v % formic acid. The mass spectrometry analysis was carried out on a 4000 QTRAP (ABSciex) mass spectrometer coupled to an Easy nLC II nanoHPLC (Bruker). The peptides were desalted on a Zorbax 300SB-C18 precolumn (Agilent) and separated on a reverse phase Zorbax 300SB-C18 analytical column (Agilent) using a 90 min water/acetonitril gradient and 300 nl/min flow rate. Information Dependent Acquisition was performed starting with a positive mode survey scan (440-1400 amu) followed by an enhanced resolution scan in order to determine the charge state of the two most intensive ions. Using these information the proper collision energies were calculated, the selected parent ions were fragmented and the product ions were scanned (100-1900 amu). During analyses the spray voltage was 2800 V, the nebulizig gas 50, the curtain gas 10, the source temperature 70 ^{o}C and the declustering potential was 50V. The acquired LC-MS/MS data were used for protein identification with the help of ProteinPilot 4.5 (ABSciex) search engine searching the SwissProt database and

using the Biological modification table included in the ProteinPilot 4.5. For protein identification minimum two peptides with 99% confidence were required.

Results

Both depletion of glucose (GD cultures) and growth on lactose (LC cultures) induced the production of γGT (Fig. 1). The produced γGT accumulated both in the hyphae (intra-γGT; Fig. 1a) and in the extracellular space (extra-γGT; (Fig. 1b). Casein peptone (Fig 1) as well as yeast extract, BSA, Gly-Gly, glutamine or glutamate (data not shown) could elevate the specific γGT activities in the absence of glucose, however this difference was not significant for extra-γGT activities in the case of GD cultures (Student's t-test, $n = 4$, $p < 0.05$).

Involvement of the CreA and MeaB transcription factors, both influence carbon starvation stress response in *A. nidulans* (Emri *et al.* 2006, Szilágyi *et al.* 2010), in γGT production was studied. Null mutation of the CreA transcription factor, responsible for carbon catabolite repression in *A. nidulans* (Shroff *et al.* 1997), had minor effect on γGT formation (Table 2). Loss-of-function mutation of *meaB*, encoding a transcription factor involved in nitrogen repression (Wong *et al*. 2007), inhibited the production of both intra- and extra-γGT (Table 2). In the presence of glucose none of these mutations had significant effect on the formation of γGT (data not shown).

The genome of *A. nidulans* encodes two genes containing γGT-like domain: AN10444 and AN5658 (Bello and Epstein, 2013; AspGenome Database http://www.aspergillusgenome.org). In order to find which gene(s) are responsible for the γGT activities detected in our cultures deletion strains were created. Deletion of the AN10444 (*ggtA*) gene completely eliminated both the extra-γGT and intra-γGT activities and

complementation of *ΔggtA* strains with the *ggtA* gene restored the γGT production (Table 3). In addition, the relative transcription of the *ggtA* gene showed strong correlation with the observed enzyme activities (Table 2). It was increased in GD or LC media (Table 2) and it was reduced in the *meaB6* but not in the *creA*-null mutants (Table 2). Deletion of the AN5658 gene had no significant effect on γGT activities (Table 3).

In order to elucidate the role of γGT, we compared certain physiological properties of the *ΔggtA* and the control strains. Deletion of the *ggtA* gene did not affect the intracellular GSH degradation observed in GD or LC cultures (Fig. 2). It is important to note that no significant amount of GSH or GSSG was detected in the fermentation broth of either the *ΔggtA* or the control cultures. The extracellular GSH+GSSG contents were always less than 0.5 nmol/ml. Addition of casein peptone to carbon starved cultures inhibited the formation of RS in cells (Fig. 3). Deletion of the *ggtA* gene markedly decreased the RS decreasing effect of casein peptone (Fig. 3). Deletion of the *ggtA* gene also decreased the protease production both in GD and LC cultures (Fig. 4).

A functioning *ggtA* gene was not necessary for efficient conidia formation but was important during sexual development in surface cultures. The number of produced conidia was between 5-7 10^7 conidia/cm^2 with all the deletion, complementation and control strains tested. The number of immature cleistothecia was higher, while the number of mature cleistothecia was less in the mutants than in the control strain (Fig. 5). The GSH and GSSG contents of these surface cultures were independent of the *ggtA* deletion (Fig. 5).

Despite the extracellular presence of GgtA, *ggtA* gene does not encode signal peptide according to the SignalP 4.1 software (Petersen *et al.* 2011). To support the view that cell lysis was not significant in LC cultures we analyzed the protein content of the fermentation broth. Using 2-D gel electrophoresis and nanoHPLC-MS we could identify a putative aminopeptidase (AN8445), CelB cellulase (AN3418), CatB catalase (AN9339) GelA putative

1,3-ß-transglycosidase (AN7657), AbnC endoarabinase (AN8007), PepJ deuterolysin-type metallo-protease (AN7962) and SodA Cu/Zn superoxide dismutase (AN0241) from the fermentation broth. All the 7 proteins are known secreted proteins of *A. nidulans* (Holdom *et al.* 1996, Emri *et al.* 2009, Saykhedkar *et al.* 2012). We also compared the predicted amino acid sequences of certain orthologues of GgtA. Multiple protein alignment demonstrated that some of the γGT genes do not contain signal sequence (Afu7g04760), some have classical signal sequence at the N-terminus (ACLA_006420 and ATEG_04362), while some have "hidden signal sequence" (*ggtA* and AO090023000537) (Fig. 6).

Discussion

The genome of *A. nidulans* contains two genes AN10444 and AN5658 encoding putative γGTs which belong to the "*Pezizomycotina*-only clade (GGT1 sub-clade)" and to the *Pezizomycotina-Saccharomycotina* (GGT3) clade, respectively (Bello and Epstein, 2013). Deletion of the AN10444 (*ggtA*) gene, but not that of the AN5658 gene, completely eliminated the γGT activity in *A. nidulans* and complementation of the mutants with *ggtA* restored their γGT activity (Table 3). These data together with the observed correlation between the transcription of *ggtA* gene and the measured γGT activities (Table 2) clearly demonstrate that *ggtA* was responsible for the detected γGT activities. Our data do not exclude that AN5658 also encodes a γGT showing only small activity with the used γGpNA substrate Table 3).

Localization of γGT is of paramount importance, when we want to understand its physiological role. γGT can be bound to the membrane of cytoplasm or vacuole in mammals (Heisterkamp *et al.* 2008) and yeasts (Springael and Penninckx 2003, Matsuyama *et al.* 2006), respectively but it can also be detected in the endoplasmic reticulum membrane of *S. pombe*

(Matsuyama *et al.* 2006), in plant cell wall (Ohkama-Ohtsu *et al.* 2008), in bacterial periplasmic space (Shibayama *et al.* 2007) or in the fermentation broth of *Histoplasma capsulatum* (Zarnowski *et al.* 2008). In our experiments both intracellular (from hyphal cells) and extracellular (from fermentation broth) γGT activities were measured (Fig. 1). The extracellular presence of the *A. nidulans* GgtA protein was demonstrated by Saykhedkar *et al.* (2012) in cultures grown on sorghum, but this protein was not part of the secretom when the fungus was grown on cork (Martins *et al.* 2014). The extracellular appearance of GgtA is interesting since the *ggtA* gene does not encode a classical signal peptide according to the SignalP 4.1 software (Petersen *et al.* 2011). A passive release of γGT by autolysing cells in carbon starved culture may be a possible explanation. However, extra-γGT production was also observed in carbon limited, growing cultures (LC cultures; Fig. 1). All the 7 proteins (CelB, CatB, GelA, AbnC, PepJ, a putative aminopeptidase and SodA) we could detect from the fermentation broth of carbon limited cultures, are known secreted proteins of *A. nidulans* (Holdom *et al.* 1996, Emri *et al.* 2009, Saykhedkar *et al.* 2012) and all of them (except SodA) contains signal sequence according to the SignalP software. This observation support the view that the lysis of hyphae was not significant in LC cultures and GgtA was secreted actively. Although the *ggtA* gene does not contain classical signal sequence, we could detect signal sequences in several genes among its orthologues including AFL2G_00188 (*A. flavus*), ACLA_006420 (*A. clavatus*), ATEG_04362 (*A. terreus*), Pc21g09300 (*Penicillium chrysogenum*) or EAS30515.2 (*Coccidioides immitis*). Extracellular formation of an enzyme without detectable signal sequence in its gene is not restricted to γGT in *A. nidulans*. The *chiB* gene encoding an extracellular chitinase and the *sodA* gene coding for Cu/Zn superoxide dismutase do not contain detectable signal sequences either (Erdei *et* al. 2008, Szilágyi *et* al. 2012, Holdom *et al.* 1996, Saykhedkar *et al.* 2012). These genes may encode unpredicted N-terminal residues or contain a novel signal sequence. According to a multiple protein

alignment analysis (Fig. 5) the *ggtA* gene – in contrast to the *chiB* or *sodA* genes - contains a "hidden" signal sequence. A similar signal sequence was found in the AO090023000537 gene (an orthologue of *ggtA* in *A. oryzae*) (Fig. 5). The existence of these unusual signal sequences is open to speculation. They can be the consequences of mistakes made during the prediction of the translated sequences or they may have special biological function: *e.g.* they can be responsible for the dual intra- and extracellular accumulation of γGT.

Regarding the function of GgtA, the physiological features of the *ΔggtA* strains suggest that the γGT of *A. nidulans* was not necessary in the bulk degradation of intracellular GSH induced by carbon depletion or carbon limitation (Fig. 2). Similarly to our findings, γGT does not contribute to the cytosolic degradation of GSH in *Saccharomyces cerevisiae* (Ganguli *et al.* 2006), in *Arabidopsis thaliana* (Ohkama-Ohtsu *et al.* 2008) or in mammalian cells (Kumar *et al.* 2012). Moreover, deletion or overexpression of *Colletotrichum graminicola* GGT1 (an orthologue of *ggtA*) gene had no significant effect on intracellular GSH levels (Bello *et al.* 2013). In contrast, deletion of *Sclerotinia sclerotiorum* GGT1 resulted in elevated total glutathione (GSSG+GSH) concentration (Li *et al.* 2012). Since it was accompanied with accumulation of hydrogen-peroxide, it assumed that deletion of GGT1 (directly or indirectly) resulted in increased GSSG level in this case (Li *et al.* 2012). It is worth mentioning that degradation of GSSG by cell wall bound γGT is important in the maintenance the redox status of apoplasts in *Arabidopsis thaliana* (Ohkama-Ohtsu *et al.* 2008). In our case, the intracellular GSSG levels were constant and we could not detect GSH or GSSG from the fermentation broth either even in the *ΔggtA* strain. Therefore, it is unlikely that γGT of *A. nidulans* was involved in the degradation of extracellular or intracellular GSSG in submerged cultures. The observed decrease in cellular GSH contents during carbon stress may be explained by the activity of an alternative pathway. Orthologues of *S. cerevisiae* DUG genes, responsible for cytosolic degradation of GSH (Kaur *et al.* 2012), can be found in

almost all yeasts and fungi including *A. nidulans* (Desai *et al.* 2011). On the other hand, it is also possible that more than one enzyme (including GgtA, AN5658 or different γ-glutamyl cyclotransferases) are responsible for GSH degradation and they can substitute one and other.

γGTs of different species can degrade extracellular or vacuolar GSH, GSSG, GSH S-conjugates or γ-glutamyl compounds (*e.g.* glutamine or poli-γ-glutamate) (Kimura *et al.* 2004, Pócsi *et al.* 2004, Shibayama *et al.* 2007, Suzuki *et* al. 2007, Heisterkamp *et al.* 2008, Ohkama-Ohtsu *et al.* 2008, Adamis *et al.* 2009, Bello *et al.* 2013). These biochemical processes are important in the utilization of γ-glutamyl derivatives as carbon, nitrogen or sulfur sources, in the maintenance of redox status, detoxification of harmful compounds (Kimura *et al.* 2004, Pócsi *et al.* 2004, Shibayama *et al.* 2007, Heisterkamp *et al.* 2008, Ohkama-Ohtsu *et al.* 2008, Adamis *et al.* 2009, Bello *et al.* 2013) or even in iron uptake (Zarnowski *et al.* 2008). Several experimental data demonstrate that γGT and extracellular protease formation is co-regulated in *A. nidulans*:

1. Production of γGT (Fig. 1, Table 2) and extracellular proteases (Szilágyi *et al.* 2010) were induced by organic nitrogen sources under carbon stress, when the degradation of these weak energy sources is particularly important for the fungus.

2. γGT induction was depended on MeaB (Table 2), a bZIP-type transcription factor which is involved in nitrogen catabolite repression of *A. nidulans* (Wong *et al*. 2007). MeaB – in contrast to the AreA nitrogen catabolite repressor (Todd *et al.* 2005) – also contributes to the up-regulation of extracellular protease production in carbon starved cultures (Szilágyi *et al.* 2010).

3. Deletion of the *ggtA* gene resulted in reduced protease production (Fig. 4). Mutations in the FadA/FlbA or GanB/RgsA signalings altered the γGT production which was accompanied with altered protease formation (Molnar *et al.* 2004, 2006).

4. Casein peptone could decrease efficiently RS formation in carbon starved cultures only if the ggtA gene was functional (Fig. 3). It can be the consequence of the reduced utilization of this weak energy source due to the missing γGT activity and/or the decreased protease activity of *ΔggtA* strains.

According to these data we suggest that one physiological function of γGT is contributing to the utilization of extracellular peptides. It is not obvious how γGT is involved in this process.

In microbes, which are unable to uptake glutamine directly (*e.g. Helicobacter pylori*) γGT releases glutamate from this substrate, which is than taken up by cells (Shibayama *et al.* 2007). The absolute glutamine requirement of *A. nidulans* glutamine synthetase mutants (Margelis *et al.* 2001) suggests that this case is quite unlikely in this species.

γGT may have regulatory function. Its substrates and/or products (*e.g.* glutamate, glutamine and γ-glutamyl derivatives) may influence the regulation of extracellular protease synthesis. The regulatory role of glutamine in nitrogen metabolism is well documented (Krappmann and Braus 2005) and a similar function of γ-glutamyl compounds produced by γGT has also been suggested by Viña *et al.* (1985).

Another hypothesis is based on the following observations: γGTs are suitable for *in vitro* production of γ-glutamyl compounds (Suzuki *et* al. 2007). In these enzymatic reactions glutamine as γ-glutamyl donor and different amino-acids as γ-glutamyl acceptors were tested (Suzuki *et* al. 2007). Alkali pH and high γ-glutamyl acceptor/γ-glutamyl donor ratio often enhanced the formation of γ-glutamyl compounds (Suzuki *et* al. 2007). γ-Glutamyl amino-acids have some beneficial properties: γ-glutamylation can increase the solubility of certain amino-acids and peptides (*e.g.* γ-glutamylation of cysteine increased its solubility in water with three orders of magnitude; Hara *et al.* 1992), γ-glutamylation can increase the stability of certain amino-acids (*e.g.* γ-glutamylation decrease the formation of pyroglutamic acid from

glutamine; Suzuki *et* al. 2007) and γ-glutamyl compounds are resistant to several peptidases (Hara *et al.* 1992). According to these data we hypothesize that extra-γGT forms γ-glutamyl peptides during the extracellular degradation of proteins using glutamine as γ-glutamyl donor. The alkali pH of carbon starved cultures (as a consequence of utilization of organic nitrogen compounds; Emri *et al.* 2004) as well as the high concentration of the γ-glutamyl acceptors support this process. Some of the formed γ-glutamyl compounds are more stable and more soluble than the amino-acids and peptides released from protein degradation and due to their γ-glutamyl bound they can preserve these beneficial properties even at high peptidase activity. Fungi can take up the γ-glutamyl compounds (together with other peptides and amino-acids) and they use their intra-γGT activity to liberate the amino-acids intracellularly.

Utilization of weak energy sources (*e.g.* peptides) is crucial under carbon stress (Szilágyi *et al.* 2013). It can moderate the accumulation of RS (Fig. 3) and determines the viability of cultures. A well controlled RS accumulation is also important in sexual development of carbon stressed *A. nidulans* cultures (Thön *et al.* 2007). γGT had an indirect negative effect on RS accumulation in our experiments (Fig. 3), which is a possible explanation why *ggtA* deletion decreased the number of matured cleistothecia in surface cultures (Fig. 5). In *Sclerotinia sclerotiorum*, deletion of GGT1 increased hydrogen-peroxide levels and caused the overproduction of sclerotial initials that were arrested in further development and failed to produce apothecia (Li *et al.* 2013). The increased total glutathione content of sclerotia in the *Δggt1* mutants suggest that ScGGT1 is important in recycling of glutathione during development. The GSH and GSSG contents of deletion strains were similar to the control strain in our surface culture experiments (Fig. 5), which do not support the existence of a similar γGT function in *A. nidulans*.

Acknowledgements

This project was supported financially by the Hungarian Research Found OTKA-K100464 and TÁMOP-4.2.2.A-11/1/KONV-2012-0045 project, which is co-financed by the European Union and the European Social Fund. The work at UW-Madison was supported by the Intelligent Synthetic Biology Center of Global Frontier Project (2011-0031955) funded by the Ministry of Education, Science and Technology, Republic of Korea. Protein identification was carried out at the BMBI Proteomics Core Facility, University of Debrecen, Department of Biochemistry and Molecular Biology and was supported in part by KMA 0149/3.0 grants from the Research Fund Management and Research Exploitation.

References

Adamis PD, Mannarino SC, Eleutherio EC (2009) Glutathione and gamma-glutamyl transferases are involved in the formation of cadmium-glutathione complex. FEBS Lett 583:1489-1492

Agblor AA, Josephy PD (2013) Donor substrate specificity of bovine kidney gamma-glutamyltransferase. Chem Biol Interact 203:480-485

Anderson ME (1985) Determination of glutathione and glutathione disulfide in biological samples. Methods Enzymol 113:548-555

Barratt RW, Johnson GB, Ogata WN (1965) Wild-type and mutant stocks of *Aspergillus nidulans*. Genetics 52:233-246

Bello MH, Epstein L (2013) Clades of γ-glutamyltransferases (GGTs) in the ascomycota and heterologous expression of *Colletotrichum graminicola* CgGGT1, a member of the pezizomycotina-only GGT clade. J Microbiol 51:88-99

Bello MH, Morin D, Epstein L (2013) γ-Glutamyltransferases (GGT) in *Colletotrichum graminicola*: mRNA and enzyme activity, and evidence that CgGGT1 allows glutathione utilization during nitrogen deficiency. Fungal Genet Biol 51:72-83

Bodnaryk RP, McGirr L (1973) Purification, properties and function of a unique gamma-glutamyl cyclotransferase from the housefly, *Musca domestica*. Biochim Biophys Acta 315:352-362

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 2:248-254

Chomczynski P (1993) A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. Biotechniques 15:532-537

Desai PR, Thakur A, Ganguli D, Paul S, Morschhäuser J, Bachhawat AK (2011) Glutathione utilization by *Candida albicans* requires a functional glutathione degradation (DUG) pathway and OPT7, an unusual member of the oligopeptide transporter family. J Biol Chem 286:41183-41194

Emri T, Pócsi I, Szentirmai A (1997) Glutathione metabolism and protection against oxidative stress caused by peroxides in *Penicillium chrysogenum*. Free Radical Biol Med 23:809-814

Emri T, Molnár Zs, Pusztahelyi T, Pócsi I (2004) Physiological and morphological changes in autolysing *Aspergillus nidulans* cultures. Folia Microbiol 49:277-284

Emri T, Molnár Zs, Veres T, Pusztahelyi T, Dudás G, Pócsi I (2006) Glucose mediated repression of autolysis and conidiogenesis in *Emericella nidulans*. Mycol Res 110:1172-1178

Emri T, Szilágyi M, László K, M-Hamvas M, Pócsi I (2009) PepJ is a new extracellular proteinase of *Aspergillus nidulans*. Folia Microbiol 54:105-109

Erdei E, Pusztahelyi T, Miskei M, Barna T, Pócsi I (2008) Characterization and heterologous expression of an age-dependent fungal/bacterial type chitinase of *Aspergillus nidulans*. Acta Microbiol Immunol Hung 55:351-361

Ganguli D, Kumar C, Bachhawat AK (2006) The alternative pathway of glutathione degradation is mediated by a novel protein complex involving three new genes in *Saccharomyces cerevisiae*. Genetics 175:1137-1151

Hagiwara D, Asano Y, Marui J, Furukawa K, Kanamaru K, Kato M, Abe K, Kobayashi T, Yamashino T, Mizuno T (2007) The SskA and SrrA response regulators are implicated in oxidative stress responses of hyphae and asexual spores in the phosphorelay signaling network of *Aspergillus nidulans*. Biosci Biotechnol Biochem. 71:1003-1014

Halliwell B, Gutteridge JMC (2007). Measurement of Reactive Species. In: Halliwell B, Gutteridge JMC (edd) Free Radicals in Biology and Medicine, 4th edn. Oxford University Press, Oxford, pp 268–330

Hara T, Yokoo Y, Furukawa T (1992) Potential of γ-L-glutamyl-L-cystine and bis-γ-L-glutamyl-L-cystine as a cystine-containing peptide for parental nutrition. In: Takai K (ed) Frontiers and new horizons in amino acid research, Elsevier, Amsterdam, pp 607–611

Heisterkamp N, Groffen J, Warburton D, Sneddon TP (2008) The human gamma-glutamyltransferase gene family. Hum Genet 123:321-332

Holdom MD, Hay RJ, Hamilton AJ (1996) The Cu,Zn superoxide dismutases of *Aspergillus flavus, Aspergillus niger, Aspergillus nidulans*, and *Aspergillus terreus*: purification and biochemical comparison with the *Aspergillus fumigatus* Cu,Zn superoxide dismutase. Infect Immun 64:3326-3332

Kang HJ, Kim BC, Park EH, Ahn K, Lim CJ (2005) The gene encoding gamma-glutamyl transpeptidase II in the fission yeast is regulated by oxidative and metabolic stress. J Biochem Mol Biol. 38:609-618

Kaur H, Ganguli D, Bachhawat AK (2012) Glutathione degradation by the alternative pathway (DUG pathway) in *Saccharomyces cerevisiae* is initiated by (Dug2p-Dug3p)2

complex, a novel glutamine amidotransferase (GATase) enzyme acting on glutathione. J Biol Chem 287:8920-8931

Kawasaki L, Sánchez O, Shiozaki K, Aguirre J (2002) SakA MAP kinase is involved in stress signal transduction, sexual development and spore viability in *Aspergillus nidulans*. Mol Microbiol 45:1153-1163

Keseru JS, Szabó I, Gál Z, Massidda O, Mingoia M, Kaszanyitzky E, Jánosi S, Hulvely J, Csorba A, Buzás K, Hunyadi-Gulyás E, Medzihradszky KF, Biró S (2011) Identification of β-lactamases in human and bovine isolates of *Staphylococcus aureus* strains having borderline resistance to penicillinase-resistant penicillins (PRPs) with proteomic methods. Vet Microbiol 147:96-102

Kimura K, Tran LS, Uchida I, Itoh Y (2004) Characterization of *Bacillus subtilis* γ-glutamyltranspeptidase and its involvement in the degradation of capsule poly-gammaglutamate. Microbiol 150:4115-4123

Kovács Zs, Szarka M, Kovács Sz, Boczonádi I, Emri T, Abe K, Pócsi I, Pusztahelyi T (2013) Effect of cell wall integrity stress and RlmA transcription factor on asexual development and autolysis in *Aspergillus nidulans*. Fungal Genet Biol 54:1-14

Krappmann S, Braus GH (2005) Nitrogen metabolism of *Aspergillus* and its role in pathogenicity. Med Mycol 43 Suppl 1:S31-40.

Kumar A, Tikoo S, Maity S, Sengupta S, Sengupta S, Kaur A, Bachhawat AK (2012) Mammalian proapoptotic factor ChaC1 and its homologues function as γ-glutamyl cyclotransferases acting specifically on glutathione. EMBO Rep 13:1095-1101

Kumar C, Sharma R, Bachhawat AK (2003) Utilization of glutathione as an exogenous sulfur source is independent of gamma-glutamyl transpeptidase in the yeast *Saccharomyces cerevisiae*: evidence for an alternative gluathione degradation pathway. FEMS Microbiol Lett 219:187-194

Kwon NJ, Garzia A, Espeso EA, Ugalde U, Yu JH (2010) FlbC is a putative nuclear C2H2 transcription factor regulating development in *Aspergillus nidulans.* Mol Microbiol 77:1203–1219

Margelis S, D'Souza C, Small AJ, Hynes MJ, Adams TH, Davis MA (2001) Role of glutamine synthetase in nitrogen metabolite repression in *Aspergillus nidulans*. J Bacteriol 183:5826-5833.

Martins I, Garcia H, Varela A, Núñez O, Planchon S, Galceran MT, Renaut J, Rebelo LP, Silva Pereira C (2014) Investigating *Aspergillus nidulans* secretome during colonisation of cork cell walls. J Proteomics 98:175–188

Matsuyama A, Arai R, Yashiroda Y, Shirai A, Kamata A, Sekido S, Kobayashi Y, Hashimoto A, Hamamoto M, Hiraoka Y, Horinouchi S, Yoshida M (2006) ORFeome cloning and global analysis of protein localization in the fission yeast *Schizosaccharomyces pombe*. Nat Biotechnol 24:841-847

McCluskey K (2003) The Fungal Genetics Stock Center: from molds to molecules. Adv Appl Microbiol 52:245-262

Mehdi K, Penninckx MJ (1997) An important role for glutathione and γ-glutamyltranspeptidase in the supply of growth requirements during nitrogen starvation in the yeast *Saccharomyces cerevisiae*. Microbiology 143: 885-1889

Meister A, Tate SS (1976) Glutathione and related gamma-glutamyl compounds: biosynthesis and utilization. Annu Rev Biochem 45:559-604

Molnár Zs, Mészáros E, Szilágyi Zs, Rosén S, Emri T, Pócsi I (2004) Influence of *fadA*^{G203R} and *ΔflbA* mutations on the morphology and physiology of submerged *Aspergillus nidulans* cultures. Appl Biochem Biotech 118:349-360

Molnár Zs, Emri T, Zavaczki E, Pusztehelyi T, Pócsi I (2006) Effects of mutations in the GanB/RgsA G protein mediated signaling on the autolysis of *Aspergillus nidulans*. J Basic Microbiol 46:495-503

Ohkama-Ohtsu N, Oikawa A, Zhao P, Xiang C, Saito K, Oliver DJ (2008) A gamma-glutamyl transpeptidase-independent pathway of glutathione catabolism to glutamate *via* 5-oxoproline in *Arabidopsis*. Plant Physiol 148:1603-1613

Orlowski M, Richman PG, Meister A (1969) Isolation and properties of gamma-L-glutamylcyclotransferase from human brain. Biochemistry 8:1048-1055

Osmani AH, May GS, Osmani SA (1999) The extremely conserved *pyroA* gene of *Aspergillus nidulans* is required for pyridoxine synthesis and is required indirectly for resistance to photosensitizers. J Biol Chem 274:23565–23569

Park HJ, Lim HW, Kim K, Kim IH, Park EH, Lim CJ (2004) Characterization and regulation of the γ-glutamyl transpeptidase gene from the fission yeast *Schizosaccharomyces pombe*. Can J Microbiol 50:61-66

Penninckx MJ, Elskens MT (1993) Metabolism and functions of glutathione in micro-organisms. Adv Microb Physiol 34:239-301

Petersen TN, Brunak S, von Heijne G, Nielsen H (2011) SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat Methods 8:785-786

Pócsi I, Leiter E, Kwon NJ, Shin KS, Kwon GS, Pusztahelyi T, Emri T, Abuknesha RA, Price RG, Yu JH (2009) Asexual sporulation signalling regulates autolysis of *Aspergillus nidulans* via modulating the chitinase ChiB production. J Appl Microbiol 107:514-523

Pócsi I, Prade RA, Penninckx MJ (2004) Glutathione, altruistic metabolite in fungi. Adv Microb Physiol 49:1-76

Pusztahelyi T, Klement E, Szajli E, Klem J, Miskei M, Karányi Z, Emri T, Kovács S, Orosz G, Kovács KL, Medzihradszky KF, Prade RA, Pócsi I (2011) Comparison of transcriptional and translational changes caused by long-term menadione exposure in *Aspergillus nidulans*. Fungal Genet Biol 48:92-103

Sámi L, Emri T, Pócsi I (2001) Autolysis and aging of *Penicillium chrysogenum* cultures under carbon starvation: III: glutathione metabolism and formation of reactive oxygen species. Mycol Res 105:1246-1250

Saykhedkar S, Ray A, Ayoubi-Canaan P, Hartson SD, Prade R, Mort AJ (2012) A time course analysis of the extracellular proteome of *Aspergillus nidulans* growing on sorghum stover. Biotechnol Biofuels 5:52

Shibayama K, Wachino J, Arakawa Y, Saidijam M, Rutherford NG, Henderson PJ (2007) Metabolism of glutamine and glutathione via gamma-glutamyltranspeptidase and glutamate transport in *Helicobacter pylori*: possible significance in the pathophysiology of the organism. Mol Microbiol 64:396–406

Shroff RA, O'Connor SM, Hynes MJ, Lockington RA, Kelly JM (1997) Null alleles of *creA*, the regulator of carbon catabolite repression in *Aspergillus nidulans*. Fungal Genet Biol 22:28-38

Smirnova GV, Oktyabrsky ON (2005) Glutathione in bacteria. Biochemistry (Mosc) 70:1199-1211

Springael JY, Penninckx MJ (2003) Nitrogen-source regulation of yeast gamma-glutamyl transpeptidase synthesis involves the regulatory network including the GATA zinc-finger factors Gln3, Nil1/Gat1 and Gzf3. Biochem J 371:589-595

Suzuki H, Yamada C, Kato K (2007) Gamma-glutamyl compounds and their enzymatic production using bacterial gamma-glutamyltranspeptidase. Amino Acids 32:333-340

Szilágyi M, Pócsi I, Forgács K, Emri T (2010) MeaB dependent nutrition sensing regulates autolysis in carbon starving *Aspergillus nidulans* cultures. Indian J Microbiol 50:104-108

Szilágyi M, Anton F, Forgács K, Yu JH, Pócsi I, Emri T (2012) Antifungal activity of extracellular hydrolases produced by autolysing *Aspergillus nidulans* cultures. J Microbiol 50:849-854

Szilágyi M, Miskei M, Karányi Z, Lenkey B, Pócsi I, Emri T (2013) Transcriptome changes initiated by carbon starvation in *Aspergillus nidulans*. Microbiology 159:176-190

Thön M, Al-Abdallah Q, Hortschansky P, Brakhage AA (2007) The thioredoxin system of the filamentous fungus *Aspergillus nidulans*: impact on development and oxidative stress response. J Biol Chem 282:27259-27269

Todd RB, Fraser JA, Wong KH, Davis MA, Hynes MJ (2005) Nuclear accumulation of the GATA factor AreA in response to complete nitrogen starvation by regulation of nuclear export. Eukaryot Cell 4:1646–1653

Viña JR, Puertes IR, Montoro JB, Saez GT, Viña J (1985) Gamma-glutamyl-amino acids as signals for the hormonal regulation of amino acid uptake by the mammary gland of the lactating rat. Biol Neonate 48:250-256

Winderickx J, Holsbeeks I, Lagatie O, Giots F, Thevelein J, de Winde H (2003) From feast to famine; adaptation to nutrient availability in yeast. Top Curr Genet 1:305-386

Wong KH, Hynes MJ, Todd RB, Davis MA (2007) Transcriptional control of *nmrA* by the bZIP transcription factor MeaB reveals a new level of nitrogen regulation in *Aspergillus nidulans*. Mol Microbiol 66:534–551

Wünschmann J, Krajewski M, Letzel T, Huber EM, Ehrmann A, Grill E, Lendzian KJ (2010) Dissection of glutathione conjugate turnover in yeast. Phytochemistry 71:54-61

Yu JH, Hamari Z, Han KH, Seo JA, Reyes-Domínguez Y, Scazzocchio C (2004) Double-joint PCR: a PCR-based molecular tool for gene manipulations in filamentous fungi. Fungal Genet Biol 41:973–981

Zarnowski R, Cooper KG, Brunold LS, Calaycay J, Woods JP (2008) *Histoplasma capsulatum* secreted gamma-glutamyltransferase reduces iron by generating an efficient ferric reductant. Mol Microbiol 70:352-368

Zhang H, Forman HJ, Choi J (2005) Gamma-glutamyl transpeptidase in glutathione biosynthesis. Methods Enzymol 401:468-483

Zhang H, Forman HJ (2009) Redox regulation of gamma-glutamyl transpeptidase. Am J Respir Cell Mol Biol 41:509-515

Legends to figures

Fig. 1 Formation of intracellular (a) and extracellular (b) γGT activities of *A. nidulans* tNJ36 in glucose free media.

GD (■), GD + casein peptone (□), LC (●), LC + casein peptone (○) media were inoculated with late exponentially phase mycelia grown up on glucose. Similar results were found when casein peptone was replaced with yeast extract, BSA, Gly-Gly, glutamine or glutamate. Mean ± SD calculated from 4 experiments are presented. Lactose was consumed by 120 h of cultivation; therefore data from cultures older than 100 h were not presented.

Experiments with the *A. nidulans* FGSC A26 and tNJ151 strains resulted in similar data to those obtained with tNJ36 strain.

Fig. 2 Degradation of intracellular GSH.

Late exponentially phase mycelia (tNJ190-1 *ΔggtA* strain – black symbols; tNJ36 control strain – white symbols) grown up on glucose were used to inoculate GD (●, ○) and GD + casein peptone (■, □) (a) as well as LC (●, ○) and LC + casein peptone (■, □) (b) media. Mean ± S.D. values calculated from 4 experiments are presented. The GSSG contents varied between 0.3-0.5 nmol/mg DCM in each measurement. The tNJ190-2 and tNJ190-3 strains behaved very similarly to the tNJ190-1 strain, therefore data are not shown for clarity.

Significant differences (Student's t-test, n=4, $p < 0.05$) between the casein peptone containing and casein peptone free cultures are indicated with asterisks. No significant difference was found between the appropriate tNJ190-1 and tNJ36 cultures.

Fig. 3 Effect of casein peptone on RS production

Late exponentially phase mycelia (tNJ190-1, tNJ190-2, tNJ190-3 *ΔggtA* strains – grey; tNJ36 control strain – white) grown up on glucose were used to inoculate GD and GD + casein peptone media. Mean ± S.D. values calculated from 4 experiments are presented. Samples were taken at 25h and 50 h.

The RS levels detected at 50 h were significantly higher (Student's t-test, $n = 4$, $p < 0.05$) than those measured at 25 h with all strains. Significant differences (Student's t-test, $n = 4$, $p < 0.05$) between the tNJ190 and the appropriate tNJ36 cultures are indicated with asterisks.

Fig. 4 Formation of extracellular proteases

Late exponentially phase mycelia (*ΔggtA* strain – black symbols; control – white symbols) grown up on glucose were used to inoculate GD + casein peptone (■, □) or LC + casein peptone (●, ○) media. Mean ± S.D. values calculated from 4 experiments are presented. Since lactose was consumed by 120 h of cultivation, data from cultures older than 100 h was not presented. The tNJ190-2 and tNJ190-3 strains behaved very similarly to the tNJ190-1 strain, therefore data are not shown for clarity. Significant differences (Student's t-test, $n = 4$, $p < 0.05$) in compare to the appropriate tNJ36 cultures are indicated with asterisks.

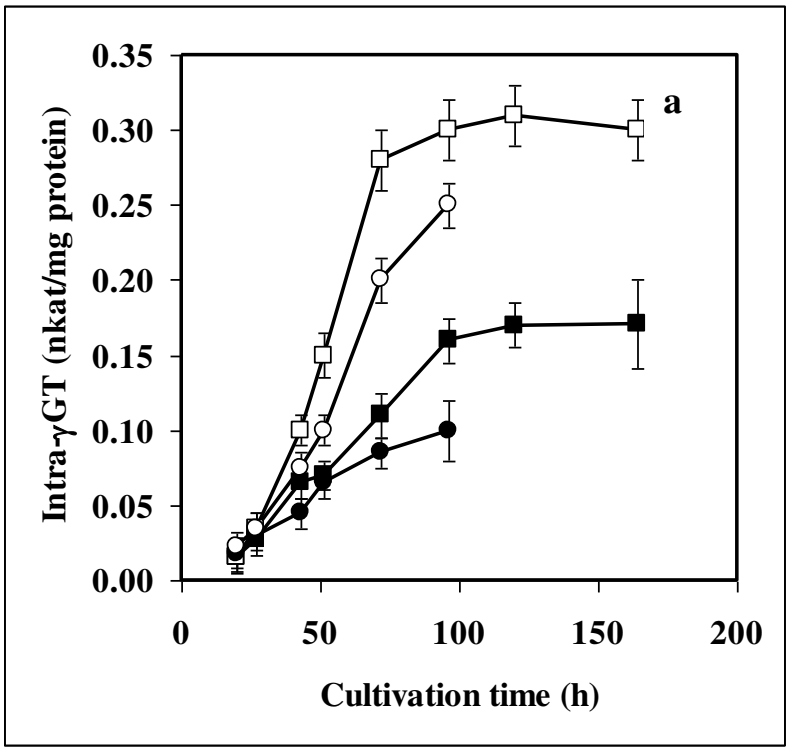
Fig. 5 Cleistothecia formation and glutathione contents in surface cultures

Control (tNJ36; white) and *ΔggtA* strains (tNJ190-1, tNJ190-2, tNJ190-3; grey) were grown in surface cultures according to Kawasaki *et al.* (2002). The number of all cleistothecia (immature + mature; a), the number of fully developed mature cleistothecia (b) as well as the GSH (plain) and GSSG (striped) content (c) of the cultures were determined at 7 d. Mean ± S.D. values calculated from 3 independent experiments are presented. Significant differences (Student's t-test, $n = 3$, $p < 0.05$) in compare to the tNJ36 cultures are indicated with asterisks.

Fig. 6 Multiple protein alignment of the *ggtA* gene and some of its orthologues

Aligment was made by Cobalt Constraint-based Multiple Protein Alignment Tool (http://www.ncbi.nlm.nih.gov/tools/cobalt/) using the translated sequence of AN10444 (*A. nidulans*), Afu7g04760 (*A. fumigatus*), ACLA_006420 (*A. clavatus*), ATEG_04362 (*A. terreus*) and AO090023000537 *(A. oryzae*). Only the N-terminals of the proteins are presented. Identical and similar amino acids are marked with black and gray background colors, respectively. Signal sequences were identified with the SignalP 4.1 software and are framed on the figure.

Fig. 1


a
Intra-γGT (nkat/mg protein)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
50
100
150
200
Cultivation time (h)

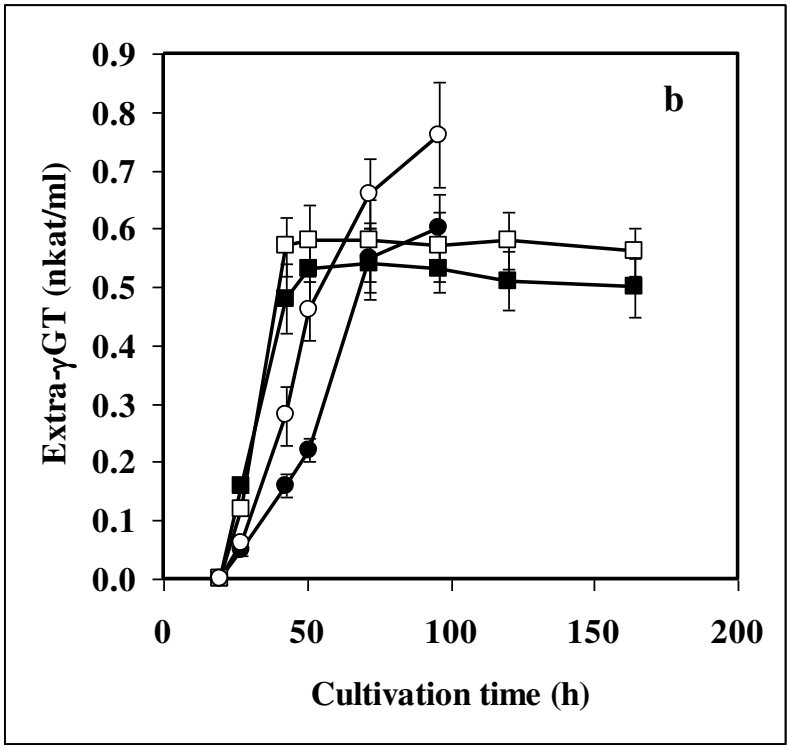
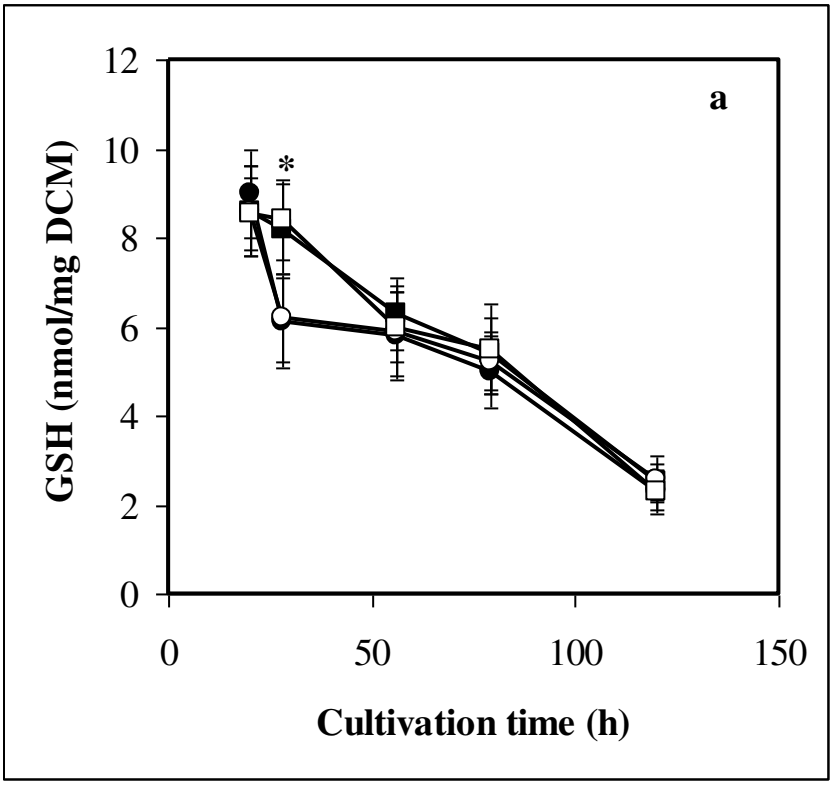

b
Extra-γGT (nkat/ml)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
50
100
150
200
Cultivation time (h)

Fig. 2


a
GSH (nmol/mg DCM)
Cultivation time (h)
0
2
4
6
8
10
12
0
50
100
150
*

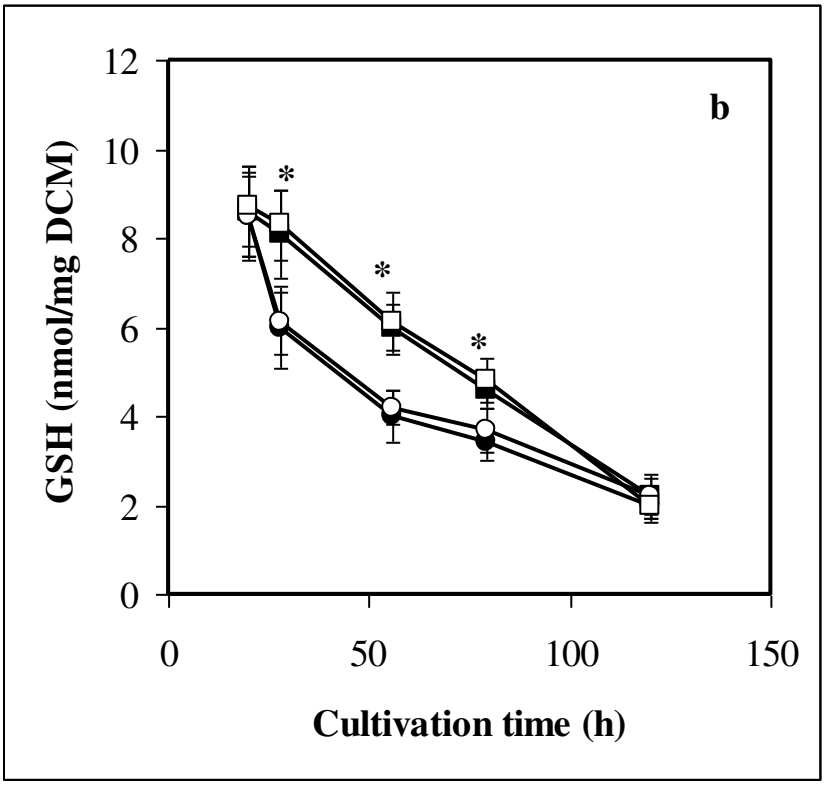

b
GSH (nmol/mg DCM)
Cultivation time (h)
0
2
4
6
8
10
12
0
50
100
150
*
*
*

Fig. 3

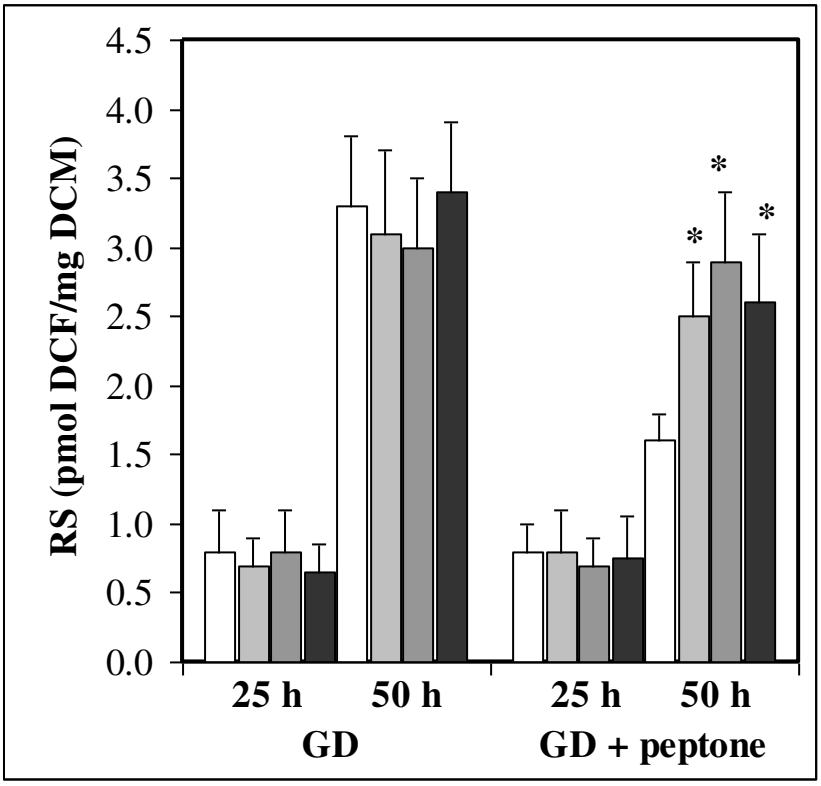

RS (pmol DCF/mg DCM)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
*
25 h
50 h
25 h
50 h
GD
GD + peptone

Fig. 4

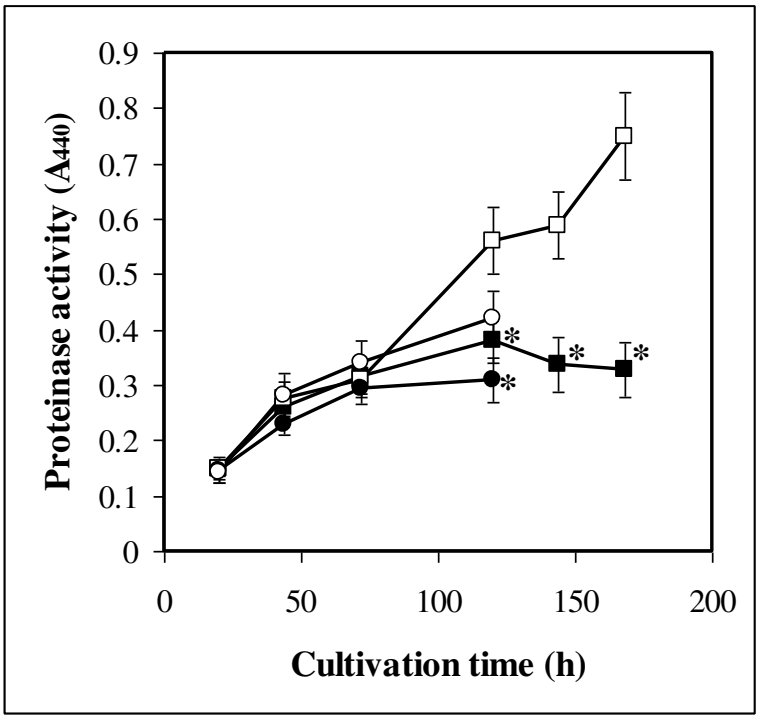
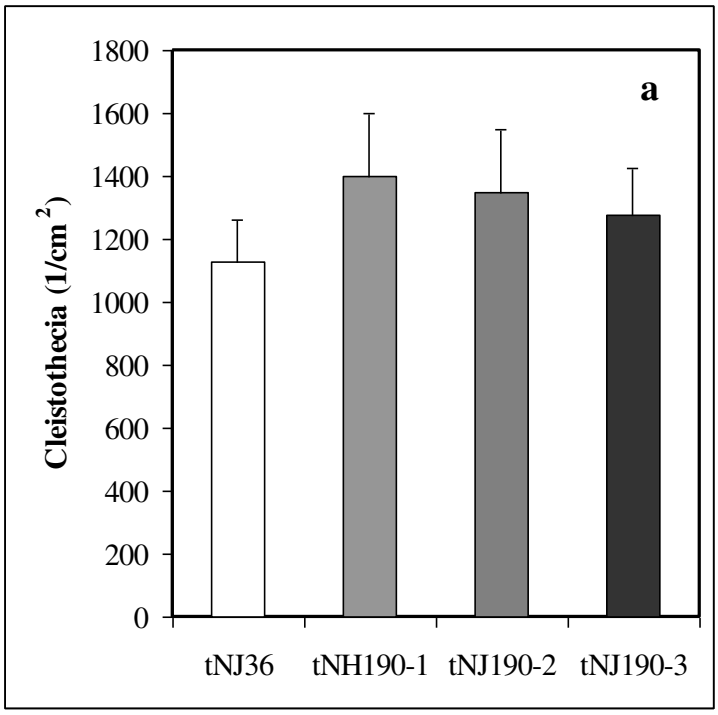
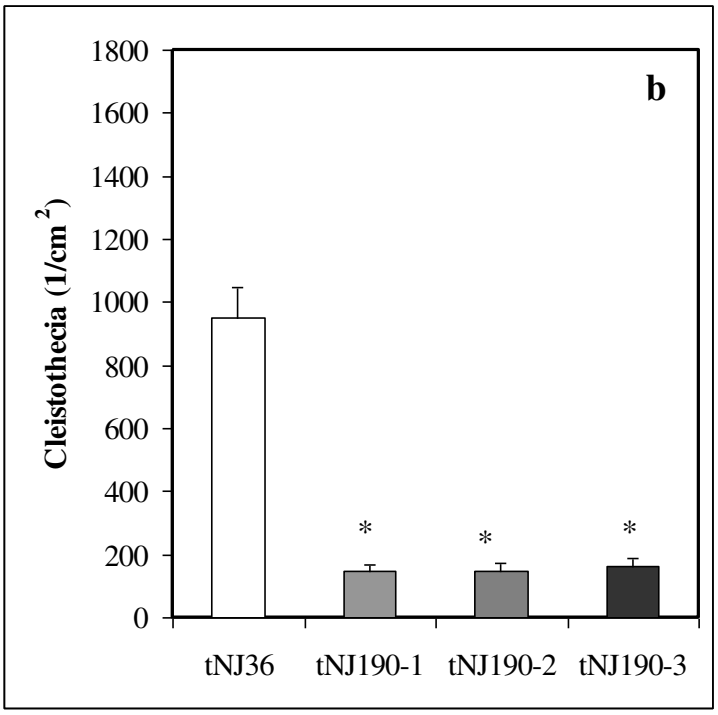

Proteinase activity (A440)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
50
100
150
200
Cultivation time (h)
*
*
*
*

Fig. 5


a
Cleistothecia (1/cm 2)
1800
1600
1400
1200
1000
800
600
400
200
0
tNJ36
tNH190-1
tNJ190-2
tNJ190-3


b
Cleistothecia (1/cm 2)
1800
1600
1400
1200
1000
800
600
400
200
0
*
*
*
tNJ36
tNJ190-1
tNJ190-2
tNJ190-3

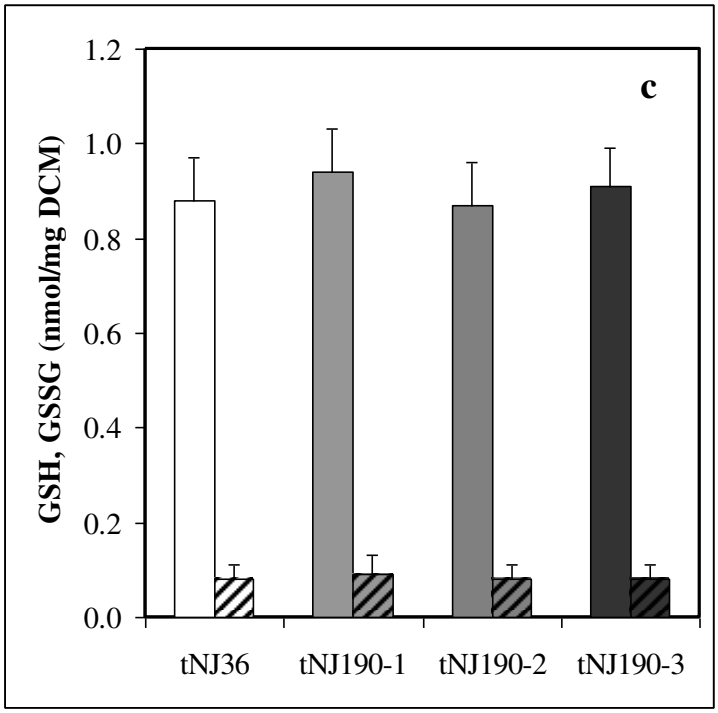
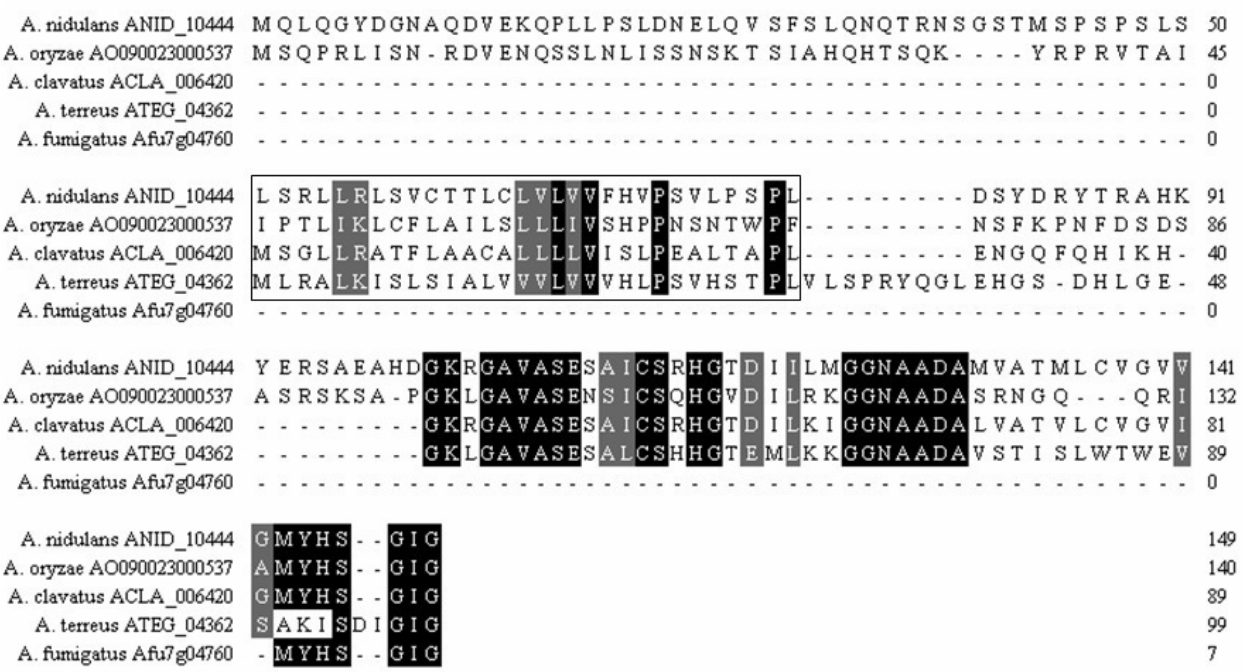

c
GSH, GSSG (nmol/mg DCM)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
tNJ36
tNJ190-1
tNJ190-2
tNJ190-3

Fig. 6


A. nidulans ANID_10444 MQLQGYDGNAQDVEKQPLLPSLDNELQVSFSLQNQTRNSGSTMSPSPSLS 50
A. oryzae AO090023000537 MSQPRLISN-RDVENQSSLNLISSNSKTSIAHQHTSQK----YRPRVTAI 45
A. clavatus ACLA_006420 ---------- 0
A. terreus ATEG_04362 ---------- 0
A. fumigatus Afu7g04760 ---------- 0
A. nidulans ANID_10444 LSRLLRLSVCTTLCLVLVVFHVPSVLPSPL---------DSYDRYTRAHK 91
A. oryzae AO090023000537 IPTLIKLCFLAILSLLLIVSHPPNSNTWPF---------NSFKPNFDSDS 86
A. clavatus ACLA_006420 MSGLLRATFLAACALLLLVISLPEALTAPL---------ENGQFQHIKH- 40
A. terreus ATEG_04362 MLRALKISLSIALVVVLVVVHLPSVHSTPLVLSPRYQGLEHGS-DHLGE- 48
A. fumigatus Afu7g04760 ---------- 0
A. nidulans ANID_10444 YERSAEAHDGKRGAVASESAICSRHGTDIILMGGNAADAMVATMLCVGVV 141
A. oryzae AO090023000537 ASRSKSA-PGKLGAVASENSICSQHGVDILRKGGNAADASRNGQ---QRI 132
A. clavatus ACLA_006420 ---------GKRGAVASESAICSRHGTDILKIGGNAADALVATVLCVGVI 81
A. terreus ATEG_04362 ---------GKLGAVASESALCSHHGTEMLKKGGNAADAVSTISLWTWEV 89
A. fumigatus Afu7g04760 ---------- 0
A. nidulans ANID_10444 GMYHS--GIG 149
A. oryzae AO090023000537 AMYHS--GIG 140
A. clavatus ACLA_006420 GMYHS--GIG 89
A. terreus ATEG_04362 SAKISDIGIG 99
A. fumigatus Afu7g04760 -MYHS--GIG 7

Strain	Genotype	Origin/Reference
Parental strain used for mutant construction:		
rJMP1.59	*pyrG89; pyroA4; veA*$^{+}$	Kwon *et al*. (2010)
ΔggtA deletion strains		
tNJ190-1	*pyrG89, ΔAN10444::A. fumigatus pyrG*$^{+}$*, pyroA4, veA*$^{+}$	this study
tNJ190-2	*pyrG89, ΔAN10444::A. fumigatus pyrG*$^{+}$*, pyroA4, veA*$^{+}$	this study
tNJ190-3	*pyrG89, ΔAN10444::A. fumigatus pyrG*$^{+}$*, pyroA4, veA*$^{+}$	this study
ΔAN5658 deletion strains		
tNJ188-1	*pyrG89, ΔAN5658::A. fumigatus pyrG*$^{+}$*, pyroA4, veA*$^{+}$	this study
tNJ188-2	*pyrG89, ΔAN5658::A. fumigatus pyrG*$^{+}$*, pyroA4, veA*$^{+}$	this study
tNJ188-3	*pyrG89, ΔAN5658::A. fumigatus pyrG*$^{+}$*, pyroA4, veA*$^{+}$	this study
ΔggtA deletion strains complemented with *ggtA*		
tNJ151-1	*pyrG89, ΔAN10444::A. fumigatus pyrG*$^{+}$*, 3/4pyroA4::AN10444:pyroA*$^{+}$*, veA*$^{+}$	this study
tNJ151-2	*pyrG89, ΔAN10444::A. fumigatus pyrG*$^{+}$*, 3/4pyroA4::AN10444:pyroA*$^{+}$*, veA*$^{+}$	this study
ΔAN5658 deletion strains complemented with AN5658		
tNJ189-1,	*pyrG89, ΔAN5658::A. fumigatus pyrG*$^{+}$*, 3/4pyroA4::AN5658:pyroA*$^{+}$*, veA*$^{+}$	this study
tNJ189-2	*pyrG89, ΔAN5658::A. fumigatus pyrG*$^{+}$*, 3/4pyroA4::AN5658:pyroA*$^{+}$*, veA*$^{+}$	this study
Control strain		
tNJ36	*pyrG89, pyrG89::A. fumigatus pyrG*$^{+}$*, pyroA4, veA*$^{+}$	Szilágyi *et al.* (2010)

Other strains		
JMK1	*pabaA1, riboB, yA1, ΔcreA, veA1*	Shroff *et al.* (1997)
FGSC A24	*sC12, veA1*	McCluskey (2003)
FGSC A26	*biA1, veA1*	McCluskey (2003)
FGSC A41	*biA1, sB3, veA1*	McCluskey (2003)
FGSC A451	*pabaA1, meaB6, cnxH3, sC12, veA1*	McCluskey (2003)
FGSC A553	*biA1, cnxH3, veA1*	McCluskey (2003)

Table 1 Strains used in this study

Strain	Additives	Relative transcription (ΔΔCP)	Extra-γGT (nkat/ml)	Intra-γGT (nkat/mg protein)
Control	GD	4.1 ± 0.5[b]	0.53 ± 0.05[b]	0.16 ± 0.02[b,c]
Control	GD + casein peptone	4.4 ± 0.6[b]	0.58 ± 0.06[b]	0.30 ± 0.03[a,b]
Control	LC	3.0 ± 0.4[a,c]	0.22 ± 0.03[a,c]	0.10 ± 0.02[a,c]
Control	LC + casein peptone	3.7 ± 0.4[b]	0.46 ± 0.05[b,c]	0.25 ± 0.03[a,b]
creA-null	GD + casein peptone	4.8 ± 0.5[b]	0.60 ± 0.05[b]	0.36 ± 0.05[a,b]
meaB6	GD + casein peptone	1.7 ± 0.2[a,b,c]	0.15 ± 0.02[a,b,c]	0.12 ± 0.02[a,c]

Table 2 Formation of γGT and relative transcription (ΔΔCP) of the AN10444 gene in certain *A. nidulans* strains

Late exponentially phase mycelia grown up on glucose were used to inoculate GD, GD + casein peptone, LC or LC + casein peptone media. The *A. nidulans* tNJ36 strain was used as control. We got essentially the same results with the FGSC A26 and tNJ151 control strains (data not shown). Samples were taken at 6h (relative transcription), 48 h (extra-γGT) and 100 h (intra-γGT) after inoculation of media with late exponentially phase mycelia. Mean ± S.D. calculated from 4 experiments are presented. Relative transcription levels were quantified with $\Delta\Delta CP = \Delta CP_{treated} - \Delta CP_{control}$, where $\Delta CP_{treated} = CP_{AN6700} - CP_{AN10444}$ measured in the glucose free cultures, $\Delta CP_{control} = CP_{AN6700} - CP_{AN10444}$ measured in glucose containing late exponentially phase cultures. CP values stand for the RT-qPCR cycle numbers of crossing points recorded for the AN10444 target and AN6700 ”housekeeping” genes. Similar results were found using the AN6838 gene as ”housekeeping” gene.

a - Significant difference in compare to GD cultures (Student’s t-test, n=4, $p < 0.05$)

b - Significant difference in compare to LC cultures (Student's t-test, n=4, $p < 0.05$)

c - Significant difference in compare to GD +casein peptone cultures (Student's t-test, n=4, $p < 0.05$)

Strain	Glucose	GD + casein peptone	
	Intra-γGT (nkat/mg protein)	Extra-γGT (nkat/ml)	Intra-γGT (nkat/mg protein)
tNJ36 (control)	0.016 ± 0.004	0.58 ± 0.05	0.30 ± 0.04
tNJ190-1 (*ΔggtA*)	< 0.005[a]	< 0.006[a]	< 0.005[a]
tNJ190-2 (*ΔggtA*)	< 0.005[a]	< 0.006[a]	< 0.005[a]
tNJ190-3 (*ΔggtA*)	< 0.005[a]	< 0.006[a]	< 0.005[a]
tNJ151-1 (complemented with *ggtA*)	0.014 ± 0.004	0.58 ± 0.05	0.37 ± 0.04
tNJ151-2 (complemented with *ggtA*)	0.012 ± 0.005	0.43 ± 0.06[a]	0.31 ± 0.03
tNJ188-1 (ΔAN5658)	0.015 ± 0.004	0.57 ± 0.05	0.33 ± 0.03
tNJ188-2 (ΔAN5658)	0.012 ± 0.004	0.51 ± 0.05	0.29 ± 0.05
tNJ188-3 (ΔAN5658)	0.013 ± 0.003	0.52 ± 0.05	0.36 ± 0.03
tNJ189-1 (complemented with AN5658)	0.013 ± 0.005	0.55 ± 0.05	0.34 ± 0.004
tNJ189-2 (complemented with AN5658)	0.012 ± 0.004	0.51 ± 0.06	0.33 ± 0.005

Table 3 Specific γGT activities in the deletion and complemented strains

Late exponentially phase mycelia grown up on glucose were used to inoculate GD + casein peptone media. Samples were taken at 0 h (before mycelia were transferred into the GD + casein peptone media; glucose containing cultures), at 48 h (extra-γGT) and at 100 h (intra-γGT). No extracellular γGT activities were detected in glucose containing cultures. Mean ± S.D. calculated from 4 experiments are presented. The detection limit (LOD) of intra-, and extra-γGT activities were 0.005 nkat/mg protein and 0.006 nkat/ml, respectively.

a - Significant difference in compare to the control strain (Student's t-test, n=4, $p < 0.05$)

Covering Letter

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