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

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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. \(\gamma\)-Glutamyl transpeptidase (\(\gamma\)GT; 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. \(\gamma\)-Glutamyl cyclotransferase (EC 2.3.2.4) can eliminate the \(\gamma\)-glutamyl group from \(\gamma\)-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 \(\gamma\)-glutamyl cyclotransferases (Bodnaryk and McGirr 1973, Orlowski et al. 1969), the cytosolic ChaC \(\gamma\)-glutamyl cyclotransferase of mammals acts specifically on GSH but not on other \(\gamma\)-glutamyl compounds (Kumar et al. 2012). A \(\gamma\)-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 \textit{dug2} and \textit{dug3} encode the two subunits of a glutamine amidotransferase complex which remove the \(\gamma\)-glutamyl moiety from GSH, while \textit{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 \(\gamma\)GT has been questionable. In mammalian cells, the membrane bound \(\gamma\)GT degrades the extracellular GSH and other \(\gamma\)-glutamyl compounds (Heisterkamp et al. 2008). The products of hydrolysis (\textit{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 \(\gamma\)GT 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 \emph{et al.} 2009). Transcription of \textit{Schizosaccharomyces pombe ggt1} and \textit{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 \emph{et al.} 2004, Kang \emph{et al.} 2005). The \(\gamma\)GT of \textit{Bacillus subtilis} participates in the utilization of capsular poly-\(\gamma\)-glutamic acid as glutamate source in stationer phase cultures (Kimura \emph{et al.} 2004). In the human pathogen bacterium \textit{Helicobacter pylori}, the main physiological function of periplasmic \(\gamma\)GT is to metabolize extracellular GSH and glutamine (Shibayama \emph{et al.} 2007). In \textit{Colletotrichum graminicola}, \(\gamma\)GT is involved in the utilization of (exogenous) GSH as nitrogen source (Bello \emph{et al.} 2013), while in another plant pathogen fungus, \textit{Sclerotinia sclerotiorum}, \(\gamma\)GT is important in the maintenance of redox homeostasis during development of sclerotia and compound appressoria (Li \emph{et al.} 2012). \(\gamma\)GT can also participate in the degradation of oxidized glutathione (GSSG) in plant apoplasts (Ohkama-Ohtsu \emph{et al.} 2008) and in the degradation of glutathione-S-conjugates (Ohkama-Ohtsu \emph{et al.} 2008, Wünschmann \emph{et al.} 2010, Agblor and Josephy 2013).

Previously, we found that \(\gamma\)GT is induced by carbon starvation and this induction was accompanied with the bulk degradation of GSH both in \textit{Penicillium chrysogenum} (Sámi \emph{et al.} 2001) and \textit{Aspergillus nidulans} (Emri \emph{et al.} 2004). Here, we would like to elucidate the physiological significance of the elevated \(\gamma\)GT activities in carbon stressed \textit{A. nidulans} cultures. We demonstrate that the induction of AN10444 gene, encoding a \(\gamma\)GT-domain containing protein was responsible for the increased specific \(\gamma\)GT activities in carbon starved and carbon limited (Winderickx \emph{et al.} 2003) cultures. We also show that \(\gamma\)GT 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 $\gamma GT$ contributed in this process. This effect of $\gamma GT$ on redox homeostasis may explain the decreased cleistothecia formation observed in $\Delta ggtA$ 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 $5 \times 10^7$ spores and were incubated at 37 °C, 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 starved cultures) or lactose (20 g/l) containing medium (LC - lactose containing, carbon limited cultures) and were further cultivated at 37 °C, 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 °C 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/cm².

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/cm².

**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’-TGCTCGATCTTCATCTGCTCCTG-3’), oNK-
1069 (5’-GCTTTGGCCTGTATCATGACCTCTCA ACTGGACAGCCTCCTCTACTG-3’)
and oNK-1070 (5’-ATCGACCGAACCTAGGTAGGGTA
TCACACTTAAGGTGAATCTAGAGGC-3’), oNK-1071 (5’-
TCTCCTGTATCCTAGCTCACCAG-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’-
GCTTTGGCCTGTATCATGACCTCTCA TGATTGAGGGTACTAGAATGTG-3’) and
oNK-1064 (5’-ATCGACCGAACACTAGGTAGGGTA
ATCTGGTGAATCCATGCCTGC-3’), oNK-1065 (5’-
TCCACAGCCTCAATATCTTACTCAGC-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’TGATGTCACCACGTCCTCTCATG-3’) and oNK-1066 (5’-
ACGTAGTGTCTTTGCGAGCTG-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 TCTCCTTGATCCTAGCTCACCAG-3’) and oNK-1074 (5’-atat CAGCTG TCGTGAGCTGTCCAGAATTCAGAG-3’), oNK-1075 (5’-atat GCGGCCGC TCCACAGCCTCAATCTTACTCAC-3’) from each genomic DNA, and digested with BamHI-Klenow/NotI (AN10444) and PvuII/NotI (AN5658), then cloned between PvuII and NotI of pH3 (Kwon et al., 2010) containing ¾AnipyroA (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’-dichlorofluorescein 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 $\gamma$GT activity (intra-$\gamma$GT)

Mycelia from submerged cultures were filtrated and washed with distilled water. Cell free extracts were prepared by x-pressing. For determining $\gamma$GT activities 0.1 mol/l Tris-HCl (pH = 8.0) buffer containing 1 mmol/l $\gamma$-glutamyl-$p$-nitroanilide ($\gamma$GpNA; as $\gamma$-glutamyl donor) and 20 mmol/l Gly-Gly (as $\gamma$-glutamyl acceptor) was added to equal volume of cell free extract. After 1 h incubation at room temperature samples were centrifuged (10000 G, 4 °C, 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-$\gamma$GT activities were expressed in nkat/mg protein.

Extracellular (secreted) $\gamma$GT activity (extra-$\gamma$GT)

Mycelia from cultures were removed by filtration and the cell free fermentation broth was used for measurements. Extra-$\gamma$GT 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-$\gamma$GT activities were expressed in nkat/ml fermentation broth.

The amount of the released p-nitroanilide was also measured in the absence of the $\gamma$-glutamyl acceptor Gly-Gly (hydrolase activity). This $\gamma$GpNA 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:


AN6700 (eEF-3; as housekeeping gene): F: 5´-CCTATTCCCGAGCAAGTTGATC-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 °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 nebulizing gas 50, the curtain gas 10, the source temperature 70 °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 \( \Delta ggtA \) strains with the \( ggtA \) gene restored the \( \gamma \)GT 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 \( \gamma \)GT activities (Table 3).

In order to elucidate the role of \( \gamma \)GT, we compared certain physiological properties of the \( \Delta ggtA \) 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 \( \Delta ggtA \) 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 \textit{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) \( \gamma \)GT 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 \( \gamma \)GT by autolysing cells in carbon starved culture may be a possible explanation. However, extra-\( \gamma \)GT 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 \( \gamma \)GT 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.


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 (Thôn 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 Agtl 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.

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

Alignment 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) vs. Cultivation time (h)

(b) Extra-γGT (nkat/ml) vs. Cultivation time (h)
Fig. 2

**a**

![Graph showing GSH (nmol/mg DCM) over cultivation time (h).](image-a)

**b**

![Graph showing GSH (nmol/mg DCM) over cultivation time (h).](image-b)
Fig. 3

![Graph showing RS (pmol DCF/mg DCM) for GD and GD + peptone treatments over 25 h and 50 h. The graph includes error bars indicating variability. Asterisks denote statistical significance.](image-url)
Fig. 4

![Graph showing Proteinase activity (A440) vs. Cultivation time (h).](image)
Fig. 5

(a) Cleistothecia (1/cm²) for tNJ36, tNH190-1, tNJ190-2, tNJ190-3.
(b) Cleistothecia (1/cm²) with significant differences indicated by asterisks for tNJ36, tNJ190-1, tNJ190-2, tNJ190-3.
(c) GSH, GSSG (mmol/mg DCM) for tNJ36, tNJ190-1, tNJ190-2, tNJ190-3.
Fig. 6

A. nuchalans ANID_10446 MQLQYGDNQVKEQPLPLLFLSNLQEVSLSLQNTNRNSGSTMSPSPSLS 30
A. oryzae AC009220000537 MSQPRILSH-RDNQSLNLSRNHRQSKK...YRPRVTAI 45
A. clavatus ACLA_005420 - - - - - - - - - - - - - - - - - - - - - - - - - - - 0
A. teresus ATBD_04602 - - - - - - - - - - - - - - - - - - - - - - - - - - - 0
A. fumigatus Afu7g04760 - - - - - - - - - - - - - - - - - - - - - - - - - - - 0

A. nuchalans ANID_10446 LSRLLLSVLCTTLCGETVYTFWYVSWSWSFL 66
A. oryzae AC009220000537 IPTIKLCLFLAILLDETVHPSWNTWFF 86
A. clavatus ACLA_005420 MSQLRATFLAACDILIEVLIRALTAFL 40
A. teresus ATBD_04602 MLRAISLSIALWTVYVHLSVHTSLPSRYQGQLEOS...DHLQE 48
A. fumigatus Afu7g04760 - - - - - - - - - - - - - - - - - - - - - - - - - - - 0

A. nuchalans ANID_10446 YERSAEAKDGKQGAVASESALSCHRHTDIILMGNADAMVATMLCVYY 141
A. oryzae AC009220000537 ARSKSA...FGKQGAVASESALSCHRHTDIILKGNADASSNGQ...QR 132
A. clavatus ACLA_005420 - - - - - - - - - - - - - - - - - - - - - - - - - - - 81
A. teresus ATBD_04602 - - - - - - - - - - - - - - - - - - - - - - - - - - - 89
A. fumigatus Afu7g04760 - - - - - - - - - - - - - - - - - - - - - - - - - - - 0

A. nuchalans ANID_10446 GMYHS...GIG 149
A. oryzae AC009220000537 GMYHS...GIG 140
A. clavatus ACLA_005420 GMYHS...GIG 39
A. teresus ATBD_04602 SKIKGDIOIIO 99
A. fumigatus Afu7g04760 - - - - - - - - - - - - - - - - - - - - - - - - - - - 7
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<th>Strain</th>
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<td>rJMP1.59</td>
<td>pyrG89; pyroA4; veA⁺</td>
<td>Kwon et al. (2010)</td>
</tr>
<tr>
<td></td>
<td><strong>ΔggtA deletion strains</strong></td>
<td></td>
</tr>
<tr>
<td>tNJ190-1</td>
<td>pyrG89, ΔAN10444::A. fumigatus pyrG⁺, pyroA4, veA⁺</td>
<td>this study</td>
</tr>
<tr>
<td>tNJ190-2</td>
<td>pyrG89, ΔAN10444::A. fumigatus pyrG⁺, pyroA4, veA⁺</td>
<td>this study</td>
</tr>
<tr>
<td>tNJ190-3</td>
<td>pyrG89, ΔAN10444::A. fumigatus pyrG⁺, pyroA4, veA⁺</td>
<td>this study</td>
</tr>
<tr>
<td></td>
<td><strong>ΔAN5658 deletion strains</strong></td>
<td></td>
</tr>
<tr>
<td>tNJ188-1</td>
<td>pyrG89, ΔAN5658::A. fumigatus pyrG⁺, pyroA4, veA⁺</td>
<td>this study</td>
</tr>
<tr>
<td>tNJ188-2</td>
<td>pyrG89, ΔAN5658::A. fumigatus pyrG⁺, pyroA4, veA⁺</td>
<td>this study</td>
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<tr>
<td>tNJ188-3</td>
<td>pyrG89, ΔAN5658::A. fumigatus pyrG⁺, pyroA4, veA⁺</td>
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<td><strong>ΔggtA deletion strains complemented with ggtA</strong></td>
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<tr>
<td>tNJ151-1</td>
<td>pyrG89, ΔAN10444::A. fumigatus pyrG⁺, 3/4pyroA4::AN10444::pyroA⁺, veA⁺</td>
<td>this study</td>
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<tr>
<td>tNJ151-2</td>
<td>pyrG89, ΔAN10444::A. fumigatus pyrG⁺, 3/4pyroA4::AN10444::pyroA⁺, veA⁺</td>
<td>this study</td>
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<td><strong>ΔAN5658 deletion strains complemented with AN5658</strong></td>
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<tr>
<td>tNJ189-1</td>
<td>pyrG89, ΔAN5658::A. fumigatus pyrG⁺, 3/4pyroA4::AN5658::pyroA⁺, veA⁺</td>
<td>this study</td>
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<tr>
<td>tNJ189-2</td>
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<td>this study</td>
</tr>
<tr>
<td></td>
<td><strong>Control strain</strong></td>
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<tr>
<td>tNJ36</td>
<td>pyrG89, pyrG89::A. fumigatus pyrG⁺, pyroA4, veA⁺</td>
<td>Szilágyi et al. (2010)</td>
</tr>
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</table>
### Other strains

<table>
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<th>Strain</th>
<th>Mutations</th>
<th>Reference</th>
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</thead>
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<tr>
<td>JMK1</td>
<td>pabaA1, riboB, yA1, ΔcreA, veA1</td>
<td>Shroff et al. (1997)</td>
</tr>
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</table>

**Table 1** Strains used in this study
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 ΔΔCP = ΔCP\text{treated} - ΔCP\text{control}, where ΔCP\text{treated} = CP_{AN6700} - CP_{AN10444} measured in the glucose free cultures, ΔCP\text{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.

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

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)
<table>
<thead>
<tr>
<th>Strain</th>
<th>Intra-γGT (nkat/mg protein)</th>
<th>Extra-γGT (nkat/ml)</th>
<th>Intra-γGT (nkat/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>tNJ36 (control)</td>
<td>0.016 ± 0.004</td>
<td>0.58 ± 0.05</td>
<td>0.30 ± 0.04</td>
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<tr>
<td>tNJ190-1 (ΔggtA)</td>
<td>&lt; 0.005&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt; 0.006&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt; 0.005&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>tNJ190-2 (ΔggtA)</td>
<td>&lt; 0.005&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt; 0.006&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt; 0.005&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>tNJ190-3 (ΔggtA)</td>
<td>&lt; 0.005&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt; 0.006&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt; 0.005&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>tNJ151-1 (complemented with ggtA)</td>
<td>0.014 ± 0.004</td>
<td>0.58 ± 0.05</td>
<td>0.37 ± 0.04</td>
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<td>tNJ151-2 (complemented with ggtA)</td>
<td>0.012 ± 0.005</td>
<td>0.43 ± 0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.31 ± 0.03</td>
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<td>tNJ188-1 (ΔAN5658)</td>
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<td>tNJ188-3 (ΔAN5658)</td>
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<td>tNJ189-2 (complemented with AN5658)</td>
<td>0.012 ± 0.004</td>
<td>0.51 ± 0.06</td>
<td>0.33 ± 0.005</td>
</tr>
</tbody>
</table>

**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.

<sup>a</sup> - Significant difference in compare to the control strain (Student’s t-test, n=4, p< 0.05)
Covering Letter

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