MeaB-dependent nutrition sensing regulates autolysis in carbon starved *Aspergillus nidulans* cultures

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Abstract Carbon starvation induced autolysis is an active process of self-digestion and is under complex regulation in *Aspergillus nidulans*. In this study we investigated how autolysis depends on the composition of the culture medium, especially on the presence of yeast extract. We demonstrated that the rate of autolytic cell wall degradation as well as the extracellular chitinase and proteinase productions significantly decreased in the presence of this nutrient. The effect of yeast extract on carbon starved cultures was independent of loss-of-function mutations in the carbon and nitrogen regulatory genes *creA* and *areA* and in the heterotrimeric G protein signalling genes *fadA* and *ganB*. In contrast, the nitrogen regulating transcription factor MeaB was involved in the yeast-extract-mediated repression of autolysis. Reverse transcriptase – polymerase chain reaction (RT-PCR) experiments demonstrated that MeaB affects the FluG-BrlA sporulation regulatory pathway by affecting transcription of *brlA*, a gene also initiating the autolytic cell wall degradation in this fungus.

Keywords MeaB · *Aspergillus nidulans* · Autolysis · Carbon starvation · Chitinase

Autolysis can be defined as a natural process of self-digestion of ageing hyphal cultures, occurring as a result of hydrolase activity and causing vacuolization and disruption of cell organelles and cell wall structures [1]. Autolysis of filamentous fungi is thought to be an active and well-regulated process, which helps these microorganisms to survive in a continuously changing, hostile environment [2–4]. Emerging regulatory elements of autolysis provide us with promising tools to control and manipulate this process for several practical purposes in the fermentation industry [2, 4]. Moreover, a deeper understanding of the underlying mechanism of fungal autolysis may also be exploited in future antifungal drug research [5–7].

In the filamentous fungus model organism *Aspergillus nidulans*, the last step of carbon starvation triggered autolysis is the degradation of the cell wall of empty hyphae (autolytic cell wall degradation). The regulation of this process is independent of concomitantly progressing cell death [4]. In previous experiments, we studied the glucose-mediated repression of autolysis in *A. nidulans*. We found that glucose repressed the autolytic cell wall degradation via the carbon catabolite repressor CreA as well as independently of CreA, via repressing the conidiogenesis regulatory gene *brlA* and activating the heterotrimeric G protein FadA-dependent signalling [4, 8].

Here, we studied the effect of yeast extract on the autolysis of *A. nidulans*. We demonstrated that the bZip-type transcription factor MeaB, an activator of *nmrA* expression and a co-repressor of *areA* expression [9], was involved in the yeast-extract-mediated repression of autolytic cell wall degradation.

A. nidulans FGSC 24 (*sC12*), FGSC 26 (*biA1*, *veA1*), FGSC 41 (*biA1*, *sB3*), FGSC 451 (*pabaA1*, *meaB6*, *cnxH3*, *sC12*), FGSC 553 (*biA1*, *cnxH3*) and FGSC 1056 (*yA2*,

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ΔfadA::argB) strains were purchased from the Fungal Genetics Stock Center (University of Kansas Medical Center, Kansas, USA [10]). The RMDgB03 (*pabaA1*, *yA2*, *ΔargB::trpC*⁺, *ΔGanB::argB*⁺, *trpC801*), *creA* null (*pabaA1*, *yA1*, *ΔcreA::argB*⁺, *riboB2*) and *areA600* (*areA600*, *biA1*, *sB43*) strains were kind gifts of Drs. J. H. Yu (University of Wisconsin), J. M. Kelly (University of Adelaide) and A. Dzikowska (Warsaw University), respectively.

Strains were grown in shake flasks (500 ml) containing 100 ml defined minimal-nitrate medium (pH 6.5) [11] supplemented with 4 g/l yeast extract, 200 μg/l p-aminobenzoic acid, 25 μg/l biotin and 20 μg/l riboflavin as required. Culture media were inoculated with 5×10^7 spores and were incubated at 37°C, 200 rpm for 20 h. Mycelia from the 20 h old cultures were washed and transferred into 100 ml glucose-free minimal-nitrate medium (pH 6.5) either supplemented (“rich medium”) or not (“minimal medium”) with 4 g/l yeast extract and were further incubated at 37°C, 200 rpm for 134 h. The starting dry cell mass (DCM) were always approximately 6 g/l. In some experiments, these media were supplemented with 1 g/l 2-deoxy-D-glucose or instead of yeast extract contained 5 g/l bovine serum albumin (BSA), casein peptone or amino acids (Glu, Asp, Lys or Met).

To test the effect of 2-deoxy-D-glucose on sporulation, strains were cultivated on minimal-nitrate medium supplemented with 20 g/l agar, 4 g/l yeast extract and 0–5 g/l 2-deoxy-D-glucose at 37°C for 168 h.

DCMs were determined as described previously [12]. Extracellular chitinase activities were determined in culture filtrates using CM-chitin-RBV (Loewe Biochemical GmbH, Sauerlach, Germany) as substrate according to the manufacturer's protocol [8]. One unit of chitinase activity was defined as an amount of enzyme resulting in 0.001/min increase in the absorbance at 550 nm in compare to the blank. Extracellular proteinase activities of the filtrates were characterized by the velocity constant of the enzyme reaction according to Tomarelli et al. [13]. One unit of proteinase activity was defined as an amount of enzyme resulting in 0.001 increase of velocity constant.

Total RNA was extracted from freeze-dried mycelial mats with TRISOL reagent (Invitrogen, Lofer, Austria) as recommended by Chomczynski [14]. RNA was DNase treated and used in RT-PCR mRNA quantification assays. RNA samples (7.5 μg) were run in 1% agarose gels for quality control. Only RNA preparations with consistent optical density and no indication of RNA degradation were used in the RT-PCR reactions. In ageing cultures, RNA samples could only be extracted from up to 70 h of growth, after this time there was degradation and streaking of RNA samples. RT-PCR reactions were carried out using QuantiTect SYBR Green RT-PCR kit (Qiagen, Hilden,

Germany) according to the manufacturer's recommendations with 400 ng total RNA per reaction, 2.5 mM/l Mg²⁺ and 0.5 mM/l gene-specific primers. The steps for the RT-PCR reaction were as follows: (1) reverse transcription, 50°C, 30 min; (2) PCR initial activation step, 95°C, 15 min; (3) DNA denaturation, 94°C, 15 sec; (4) annealing, 49°C, 30 sec; and (5) extension, 72°C, 30 sec and 40 cycles. The following oligonucleotide PCR primers were employed through this study to amplify *brlA* specific gene transcript F: 5'-CGACTTTCTCTCTGGATACGATG-3' and R: 5'-CTGGTGACGGTAGTTGTTGTTG-3'. The initial amounts of *brlA* specific cDNA in the samples were estimated by calculating the number of cycles needed for the accumulation of the PCR product (cycle threshold; CT). To test the homogeneity of the RT-PCR products, the melting temperature was determined by heating the products up to 95°C and decreasing the temperature of the PCR system block stepwise to 55°C in 0.5°C steps; the fluorescence was read at each step. The same PCR products were also subjected to 1% agarose gel electrophoresis in 1× Tris-acetate-EDTA buffer (TAE; 0.4 mol/l Tris/acetic acid, 0.001 mol/l Na₂-EDTA, pH 8.5) buffer. All cDNA amplification products evaluated and presented in this paper were found homogeneous by both methods.

Results are expressed as mean ± standard deviation, and the statistical significance of changes in physiological parameters was determined using the Student's 't'-test. Only probability levels of $p \leq 5\%$ were regarded as statistically significant.

Carbon starved FGSC 26 cultures showed significant decrease in DCM and increased extracellular chitinase and proteinase production when cultured in minimal medium (Fig. 1). Addition of 4 g/l yeast extract to the medium hindered both DCM declination and extracellular hydrolase productions (Fig. 1). The specific extracellular chitinase production (extracellular chitinase activity factored with DCM), which is a good marker of autolytic cell wall degradation [4], was also higher in minimal medium than those found in yeast-extract-supplemented nutrient-rich medium (Fig. 1). Beside of yeast extract, other organic nitrogen compounds including BSA, casein peptone, Glu, Asp, Lys or Met could efficiently inhibit the autolysis of *A. nidulans*. The specific extracellular chitinase productions at the 90 h were 60 ± 6% (yeast extract; Fig. 1), 65 ± 6% (BSA), 67 ± 6% (casein peptone), 69 ± 5% (Glu), 70 ± 6% (Asp), 70 ± 6% (Lys) and 75 ± 7% (Met) of the value measured in minimal medium.

Transferring *A. nidulans* mycelia into glucose-free yeast-extract-rich medium instead of glucose-free minimal medium resulted in considerably slower autolytic cell wall degradation and extracellular hydrolase productions in all strains tested including *ΔcreA*, *ΔganB*, *ΔfadA* and

Fig. 1 Autolysis and hydrolase production of *Aspergillus nidulans* FGSC 26 (control) and FGSC 451 (*meaB6*) strains. Figures present changes in the DCM (Part A), extracellular proteinase (Part B) and chitinase (Part C) productions as well as in the specific chitinase productions (Part D). Symbols represent the observed changes with FGSC 26 in glucose-free minimal ($\square$) and yeast-extract-rich ($\blacksquare$) media as well as with FGSC 451 in glucose-free minimal ($\circ$) and yeast-extract-rich ($\bullet$) media. Mean values calculated from four independent experiments are presented. Standard deviation values were always <12% of the means.

areA600 mutants (data not shown; for genotypes see section “Materials and methods”) with the exception of the strain *meaB6*. The loss-of-function *meaB6* mutant (FGSC 451) showed as dynamic autolytic cell wall degradation and chitinase (but not proteinase) production in yeast-extract-supplemented medium as in minimal medium (Fig. 1).

The loss-of-function *meaB* mutation also affected the asexual sporulation of the fungus including the transcription of the *brlA* gene, the product of which, BrlA, is a key regulator of both conidiogenesis [15] and autolytic cell wall degradation [4]. Elevated expression of *brlA* was detected in both minimal and yeast-extract-rich medium in the *meaB6* mutant, meanwhile yeast-extract significantly hindered *brlA* transcription in the FGSC 26 control strain

(Fig. 2). Addition of 2-deoxy-D-glucose (1 g/l) to submerged cultures of *meaB6* did not repress the expression of *brlA*, which was clearly seen in controls (Fig. 2).

In surface cultures, 2-deoxy-D-glucose added in a concentration of 1 g/l did not inhibit the sporulation of the *meaB6* mutant, which was typical in all other strains tested (Fig. 3). In addition, the *meaB6* strain was characterized with a conidiophore formation phenotype in submerged yeast-extract-rich cultures (Fig. 3).

In carbon-depleted cultures of *A. nidulans*, organic nitrogen compounds including yeast extract, BSA, casein peptone and amino acids (Glu, Asp, Lys, Met) inhibited effectively the autolysis, although they did not induce growth. Since yeast extract proved to be the most efficient inhibitor

Fig. 2 Expression of the *brlA* gene in *Aspergillus nidulans* FGSC 26 (control; ■ and □) and FGSC 451 (*meaB6*; ● and ○) strains. Figures show *brlA* transcription levels determined with RT-PCR in glucose-free minimal (□ and ○) and yeast-extract-rich (■ and ●) media (Part A) as well as in yeast-extract-supplemented media in the presence (■ and ●) or absence (□ and ○) of 2-deoxy-D-glucose (Part B). Transcription levels are expressed as the number of cycles needed for the accumulation of the PCR product. Symbols represent mean values calculated from six independent experiments. Standard deviation values always were <10% of the means.

we studied its effects on carbon-depleted, non-growing cultures. Yeast extract inhibited the DCM declination and chitinase production – well-known markers of progressing autolysis in submerged cultures of filamentous fungi (Fig. 1) [4]. Moreover, the release of extracellular proteinase was also higher in minimal medium than in yeast-extract-supplemented medium (Fig. 1), questioning the need for any exogenic proteins, peptides or amino acids to trigger autolytic proteinase production in ageing autolytic cultures of filamentous fungi.

The autolysis-hindering effect of yeast extract on carbon starved cultures was independent of *creA*, *fadA* and *ganB* genes (data not shown), however, these genes had been demonstrated to influence autolysis and extracellular hydrolase production [4]. The AreA transcription factor regulates the utilization of several nitrogen compounds in growing cultures [16–18]. Since carbon starvation inhibits its nuclear accumulation [19] the loss-of-function *areA600* mutant behaved very similarly to control strains in terms of the appearance of autolytic markers (data not shown).

More recently, Wong et al. [9] reported that the bZip regulatory protein MeaB was involved in nitrogen repression by inducing the *nmrA* gene, the product of which represses AreA activity. The pleiotrop effect of *meaB* mutation suggests that the physiological function of MeaB is not exclusively in the fine-tuning of *areA* transcription according to the nitrogen status of the cells [9, 20]. Our results underline the importance of MeaB in the regulation of autolytic cell wall degradation and hydrolase (e.g. chitinase) production under carbon starvation (Fig. 1). Moreover, MeaB seems to be an important factor in

the hindrance of conidiogenesis in submerged cultures (Fig. 3) and in the glucose repression of sporulation in surface cultures (Fig. 3) [8].

As shown in Fig. 2, MeaB is likely to effect conidiogenesis and autolysis by modulating the expression of *brlA*, a major key player in the regulation of these physiological processes [4, 15], because neither yeast extract nor 2-deoxy-D-glucose inhibited *brlA* transcription in the loss-of-function *meaB6* mutant. It is noteworthy, that 2-deoxy-D-glucose still repressed extracellular chitinase and proteinase productions in the *meaB6* mutant (data not shown) most likely via the CreA-dependent carbon repression pathway [4].

Our results suggest that carbon starving cultures can sense nutrients (proteins, peptides or amino acids presented in the medium or released by autolysis) in their environment and can regulate autolytic cell wall degradation (i.e. the liberation and use of cell wall biopolymers) as a function of the availability of these nutrients. Transcription factor MeaB seems to be important in the coordination of nutrient uptake and degradation as well as disassembly of cell wall constituents under carbon starvation. The target of MeaB activity seems to be the FluG-BrlA sporulation/autolysis signalling pathway.

Because the chitinase production of the loss-of-function *meaB6* mutant was not repressed by the supplementation of the fermentation broths of carbon starved autolytic cultures with yeast extract, i.e. was independent of the nutrient status of culture media, *meaB*-defected strains can be considered in future industrial enzyme-producer strain development projects.

Fig. 3 Asexual sporulation of *Aspergillus nidulans* FGSC 451 (*meaB6*; Part A) and FGSC 26 (control; Part B) strains on yeast-extract-rich media containing 2-deoxy-D-glucose (1 g/l). Part C shows conidiophores of the FGSC 451 (*meaB6*) mutant appearing in submerged cultures (no conidiophore formation was observed in the cultures of the FGSC 26 control strain). Bars = 1 cm (Parts A and B) and 6 µm (Part C).

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