0273-2289/04/118/0349/$25.00

Influence of *fadA*^{G203R} and Δ*flbA* Mutations on Morphology and Physiology of Submerged *Aspergillus nidulans* Cultures

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Received April 28, 2003; Accepted August 30, 2003

Abstract

Morphologic and physiologic changes taking place in carbon-limited submerged cultures of *Aspergillus nidulans* Δ*flbA* and *fadA*^{G203R} strains were studied. Loss-of-function mutation of the *flbA* gene resulted in an altered germination with unusually thick germination tubes, "fluffy" pellet morphology, as well as a reduced fragmentation rate of hyphae during autolysis. In the *fadA*^{G203R} mutant strain, conidiophores formed in the stationary phase of growth, and the size of pellets shrank considerably. There were no significant differences in the generation of reactive oxygen species (ROS) and in the specific catalase and superoxide dismutase activities by the tested mutants and the appropriate parental strains. Therefore, the participation of ROS or antioxidative enzymes in FadA/FlbA signaling pathways seems to be unlikely in submerged cultures. On the other hand, earlier increases in the extracellular protease and ammonia production were recorded with the Δ*flbA* strain, whereas the protease and ammonia production of the *fadA*^{G203R} mutant lagged behind those of the wild-type strains. Similar changes in the time courses of the induction of γ-glutamyltranspeptidase and the degradation of glutathione were observed. These results suggest that FadA/FlbA signaling may be involved in the mobilization of protein and peptide reserves as energy sources during carbon starvation.

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Index Entries: *Aspergillus nidulans*; *fadA*; *flbA*; autolysis; chitinase; glutathione.

Introduction

Fungal autolysis, which is regarded as a dynamic phase of cell death, influences numerous biotechnological processes including secondary metabolite and heterologous protein productions *(1)*. In earlier studies, we used holistic approaches to characterize the morphologic and physiologic events taking place in autolyzing and postautolytic *Penicillium chrysogenum (2)* and *Aspergillus nidulans (3)* cultures. Many of the observed changes, such as accumulation of reactive oxygen species (ROS), induction of antioxidant enzymes, and decrease in vitality, were in good accordance with the propositions of the Free Radical Theory of Aging *(4)*. Therefore, we used the term *aging* to describe the physiologic changes characteristic of postautolytic *P. chrysogenum* cultures *(2,5)*. Other literature data support the idea that fungal autolysis shares similarities with the apoptosis of higher eukaryotes *(2,6)*; for example, autolysis was not only energy dependent *(6)*, but the biosynthesis and processing of age-related hydrolases were highly regulated in deceleration and stationary-phase cultures *(2,7–9)*. Currently, very little is known about the signals, signal transduction pathways, transcriptional regulations, and genomic programs that initiate and govern autolysis and cell death processes in filamentous fungi *(2)*.

The balance between growth (autolysis) and sporulation is tightly controlled by the products of the *fadA* and *flbA* genes in *A. nidulans*, a widely used model organism for filamentous fungi. FlbA is an RGS (regulator of G protein signaling) domain protein that is required to suppress growth signaling via FadA, the α-subunit of a G protein and, concomitantly, to initiate sporulation *(10–12)*. Loss-of-function mutations in *flbA* or dominant activating mutations in *fadA* ($fadA^{G42R}$, $fadA^{R178C}$, $fadA^{Q204L}$) cause a block in both asexual and sexual sporulation accompanied by uncontrolled growth and autolysis in mature colonies in surface cultures. Both loss of function and a dominant interfering allele of *fadA* ($fadA^{G203R}$) resulted in reduced growth and hyperactive asexual sporulation and suppressed the developmental defects observable in *flbA* deletion mutants *(10–12)*. Although an abrupt formation of an air/water interface at the hyphal surface is a prerequisite for the onset of conidiation in liquid-grown *A. nidulans* hyphae, the sporulation block observed in submerged cultures is not absolute; for example, the $fadA^{G203R}$ mutant strain develops conidia under such circumstances *(11)*.

A. nidulans mutants defected in sporulation provided us with a superb opportunity to investigate the involvement of certain signaling elements in the regulation of autolysis in submerged cultures. We characterized the cell morphology and physiology of the Δ*flba* and $fadA^{G203R}$ G protein-signaling mutants. In this article, we present data on the possible role of FadA/FlbA signaling in the regulation of cell morphology and cell physiology in submerged carbon-limited autolyzing cultures of *A. nidulans*.

Materials and Methods

Chemicals

Unless otherwise indicated, all chemicals were purchased from Sigma-Aldrich (Budapest, Hungary).

Organism, Growth Conditions, Sample Preparation, and Analytical Procedures

A. nidulans FGSC26 (*biA1, veA1*), RJH046 (*argB2, biA1, pyroA4 veA1, Δflba::argB*), FGSC33 (*biA1, pyroA4, veA1*), FGSC1035 (*yA2, fadA^{G203R}*), and FGSC 116 (*yA2*) strains were purchased from the Fungal Genetic Stock Center (University of Kansas Medical Center, Kansas City, KS).

The strains were grown in shake flasks (500 mL) containing 100 mL of minimal-nitrate medium (pH 6.5) supplemented with 0.5% yeast extract and, as required, with 25 μg/L of biotin and 1 μg/L of pyridoxine *(13)*. Culture media were inoculated with 5×10^7 spores and were incubated at 37°C, 200 rpm for 168 h.

For glutathione (GSH) and glutathione disulfide (GSSG) determinations, mycelia were separated from 5- to 10-mL aliquots of the cultures by filtration on sintered glass. Cells were washed with distilled water and resuspended in ice-cold 5% (w/v) 5-sulfosalicylic acid by vigorous mixing and were left at 0°C for 20 min *(14)*. After centrifuging at 10,000*g* for 10 min, the supernatants were neutralized with triethanolamine at 0°C. The intracellular GSH and GSSG concentrations were determined according to Anderson *(15)*.

The intracellular peroxide and superoxide levels were characterized in separate experiments by the formation of 2',7'-dichlorofluorescein (DCF) from 2',7'-dichlorofluorescin diacetate and ethidium (Et) from dihydroethidium, respectively, as described earlier *(5)*.

Changes in the specific activities of some antioxidative enzymes were also followed in separate experiments. In these cases, mycelia harvested by filtration on sintered glass were washed with distilled water and resuspended in ice-cold 0.1*M* K-phosphate buffer (pH 7.5). Cell-free extracts were prepared by X-pressing and centrifugation *(14,16)*. Specific γ-glutamyltranspeptidase (γGT) *(16)*, catalase *(17)*, superoxide dismutase (SOD) *(18)*, glucose-6-phosphate dehydrogenase (G6PD) *(19)*, and NADP-specific isocitrate dehydrogenase (NADP-ID) *(20)* activities were measured according to the methods in the cited references.

Glucose consumption and ammonia production were measured by the rate assay of Leary et al. *(21)* and using an OP-NH3-7113-S type of ammonia-sensitive electrode (Radelkis, Budapest, Hungary) *(7)*, respectively. Extracellular protease activities of the filtrates were characterized by the velocity constant of the enzyme reaction (K) according to Tomarelli et al. *(22)*. Extracellular chitinase activities were determined using carboxy methylchitin-Remayol Brilliant Violet (Loewe Biochimica GmbH, Sauerlach, Germany) as substrate.

A slightly modified method of Lee et al. *(23)* was used to measure the specific methylthiazoletetrazolium (MTT)-reducing activity of the cells, a marker of cell vitality. Mycelia from 1-mL aliquots of cultures were transferred into test tubes containing 2 mL of fresh medium supplemented with 5 mg/mL of MTT. Cultures were incubated for 4 h at 37°C, and then 0.6-mL aliquots of 20 mmol/L HCl solution also containing 20% (w/v) sodium dodecyl sulfate were added. After incubating the cultures for another 24 h, the samples were centrifuged (10,000*g*, 5 min), and the MTT-formazan content of the supernatant was measured spectrophotometrically ($\lambda = 550$ nm). Specific MTT-reducing activities were calculated by factoring the A_{550} values with the dry cell mass (DCM) of the samples.

DCMs were determined as described in previous publications *(7,8)*, and protein contents of the cell-free extracts were measured using a modification of the Lowry method *(24)*.

Microscopy

Cell morphology was examined under an Olympus BH-2 microscope equipped with an SPlan 20NH phase-contrast objective *(25)*.

Statistical Analysis

Variations among experiments were estimated by standard deviations (SDs), and the statistical significance of changes in physiologic parameters was estimated by the student's *t*-test. Only probability levels of $p \leq 5\%$ were regarded as indicative of statistical significance.

Results

Morphology of ΔflbA *and* fadA^{G203R} *Strains in Submerged Cultures*

With the Δ*flbA* strain, we observed altered germination with unusually thick germination tubes after 5 h of inoculation (Fig. 1). Later, at the end of the exponential phase of growth, the pellet size of the mutant strain was similar to that of the controls (FGSC26, FGSC33), but the pellets showed "fluffy" morphology in this case (Fig. 2). These pellets were surrounded by a region of long, unchained, intensively growing hyphae (Fig. 2). In the stationary phase, no conidiophore formation was observed with either the mutant or the wild-type strains, and the cultures preserved their pelleted morphology for a long time in the autolytic phase of growth. The fragmentation of hyphae progressed rather slowly and, as a consequence, only a few (1×10^4/mL) "yeastlike" cells (one- to two-celled hyphal fragments) were observed (Fig. 3).

In the case of the *fadA*^{G203R} strain, the mutation did not influence the morphology of the germination tube. The pellet size in the late exponential phase of growth was less than half that of the control (Fig. 4), but, in contrast to the Δ*flbA* strain, the pellet morphology was not altered (Fig. 2). In the stationary phase, concomitant formation of conidiophores and large bulbous cells was observed (Fig. 5). Wild-type strains never produced bulbous cells in these experiments, and conidiophores developed only when the

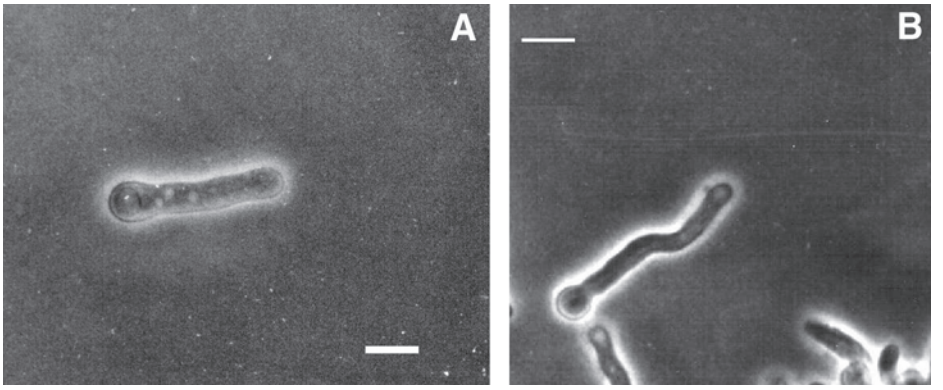

Fig. 1. Germination of *A. nidulans* conidia at 7 h after inoculation: **(A)** Δ*flbA* mutant; **(B)** FGSC26 wild-type strain (7 h). Bars = 5 µm.

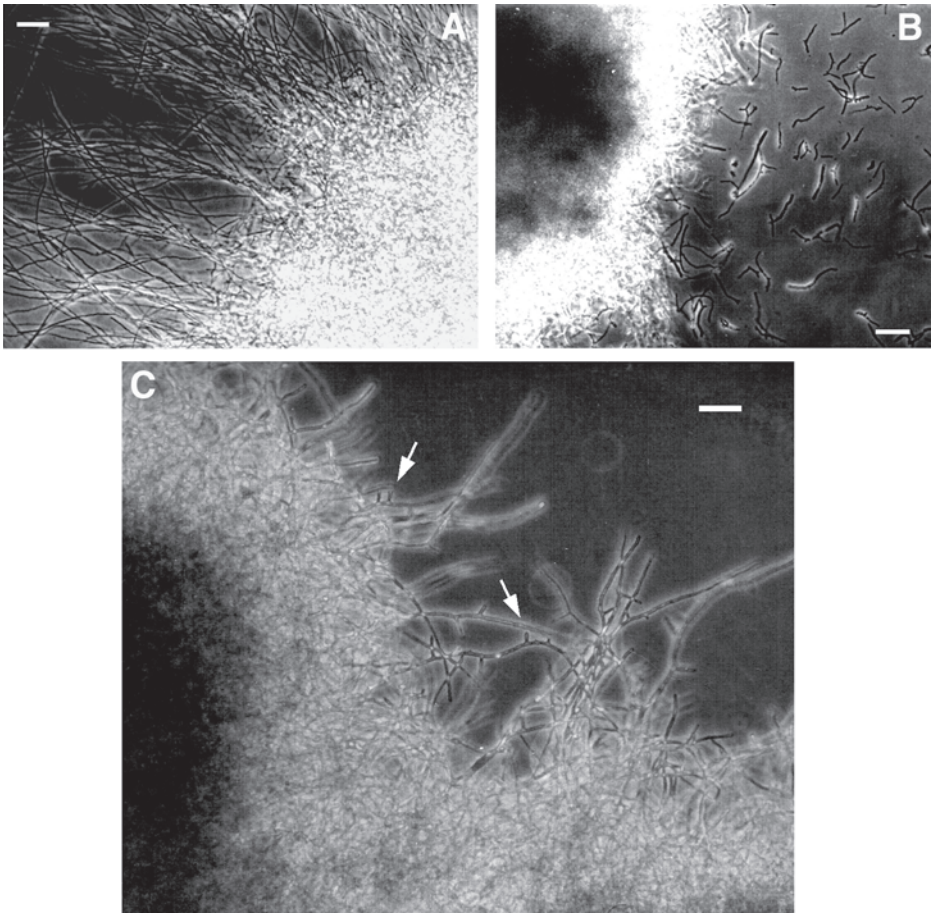

Fig. 2. Pellet morphology in late exponential phase cultures: **(A)** Δ*flbA* strain at 24-h incubation time; **(B)** wild-type FGSC26 strain (24 h); **(C)** *fadAG*^{203R} mutant (20 h). Arrows indicate the short side chains typical of the *fadAG*^{203R} pellets. Bars = 20 µm.

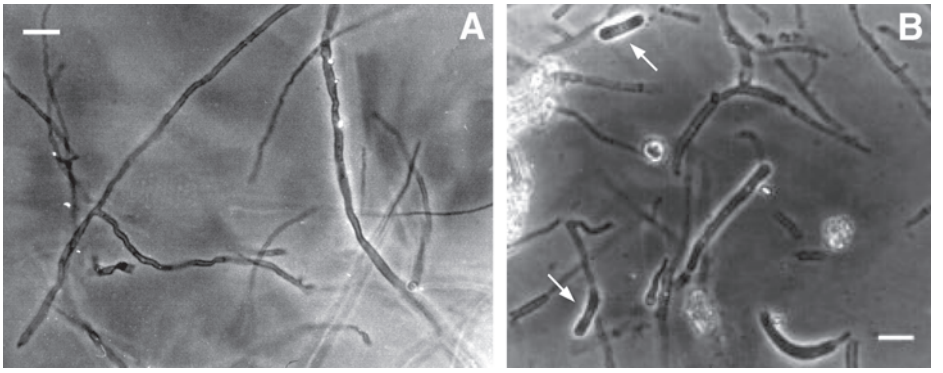

Fig. 3. Cell morphology in autolytic phase of growth: **(A)** long, unfragmented hyphae of Δ*flbA* mutant (160-h incubation time); **(B)** fragmentation of wild-type FGSC26 hyphae (160 h). Arrows indicate "yeastlike" fragments.

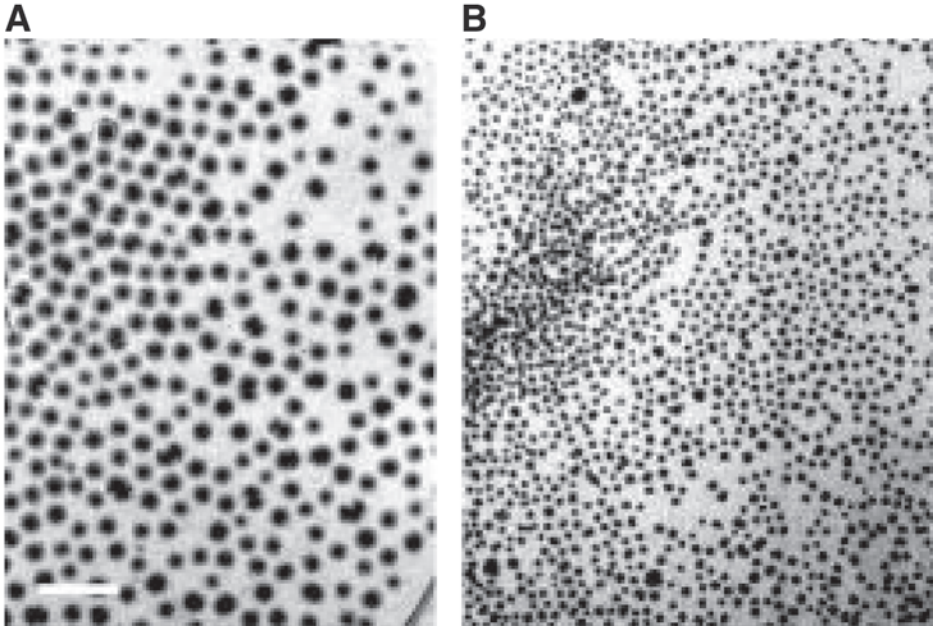

Fig. 4. Comparison of pellet sizes: **(A)** *fadAG*^{203R} mutant (24-h incubation time); **(B)** FGSC26 wild-type (24-h) strains. Bar = 10 mm. Average pellet diameters and standard deviations were 2.9 ± 0.3 (n = 30) and 1.2 ± 0.2 (n = 30), respectively.

media were inoculated with less then 0.1×10^6 conidia/mL. During the autolytic phase, the fragmentation of mycelia resulted in "yeastlike" fragments, and the number of these hyphal elements ($0.3–0.7 \times 10^6$/mL) was similar to that observed in control cultures ($0.1–0.5 \times 10^6$/mL).

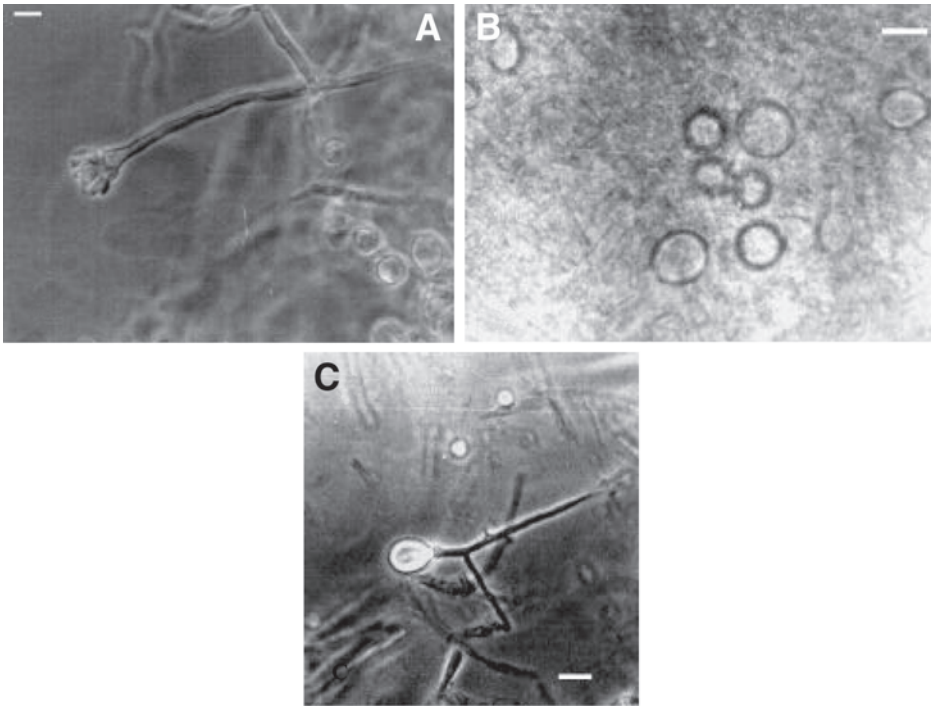

Fig. 5. Stationary-phase morphology of *fadAG*^{203R} strain at 140-h incubation time: **(A)** conidiophore with large bulbous cells; **(B)** bulbous cells in pellet; **(C)** bulbous cell attached to fragment. Bars = 5 μm.

Physiologic Changes in Mutant Strains

In most cases, the physiologic properties of the parental strains (FGSC26, FGSC116, and FGSC33) were indistinguishable and, therefore, unless the differences among the parental strains were significant, we present data solely obtained with the FGSC26 strain.

Changes in DCM and specific chitinase activities were not affected by the Δ*flbA* and *fadA*^{G203R} mutations (Fig. 6). However, the vitality of the Δ*flbA* strain characterized by MTT reduction activity declined much faster than that of the wild-type strains (Fig. 6). The glucose consumption rates were also very similar in all the tested strains (data not shown), and all the starting glucose was consumed by 21 h of incubation, independently of the FadA/FlbA signaling.

Extracellular protease production and ammonia accumulation started in the cultures in the following order: Δ*flbA* mutant, wild-type strain, *fadA*^{G203R} mutant (Fig. 7). Similar tendencies were recorded in the induction of γGT activity and the degradation of GSH (Fig. 8). However, we did not find any significant differences between the intracellular GSSG levels (Fig. 8).

Although the time course of the specific G6PD and NADP-ID activities was quite different in the wild-type and mutant strains (Fig. 9), no similar tendencies were observed for those in the case of the GSH degradation or extracellular protease production (Figs. 7 and 8).

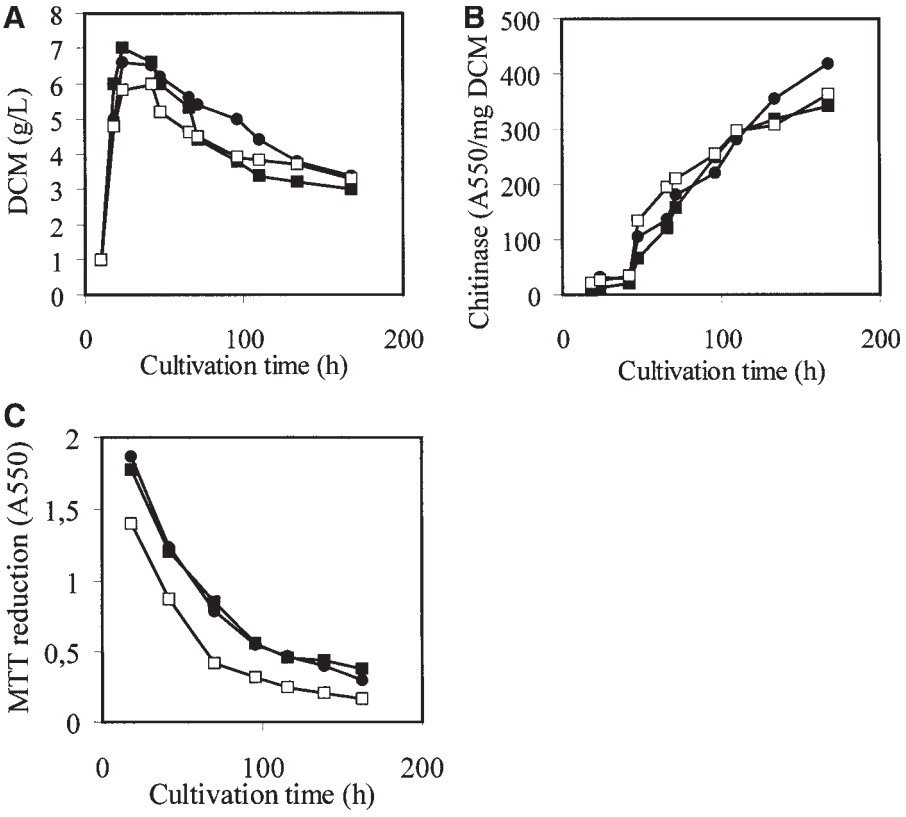

Fig. 6. Changes in **(A)** DCM; **(B)** extracellular chitinase activities; and **(C)** MTT reduction activity of (●) FGSC26, (□) Δ*flbA*, and (■) *fadAG*^{203R} strains in submerged cultures. Symbols represent mean values calculated from three to four independent experiments. SDs were <12%.

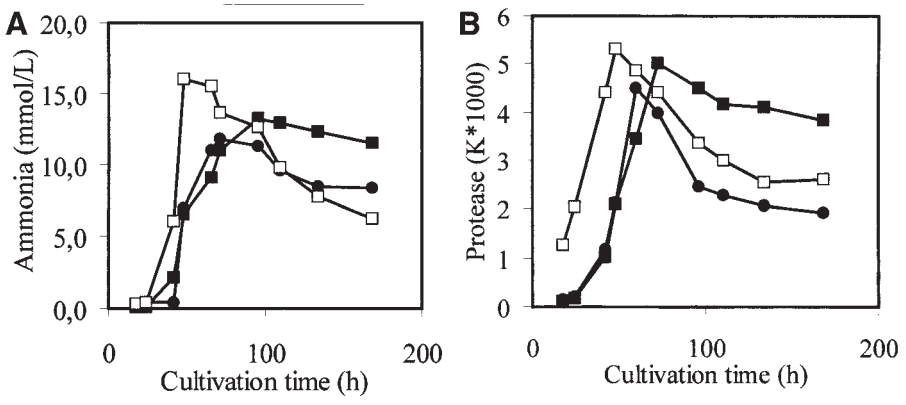

Fig. 7. **(A)** Extracellular protease activities and **(B)** ammonia production of (□) Δ*flbA*, (●) FGSC26, and (■) *fadAG*^{203R} strains in submerged cultures. Symbols represent mean values calculated from four independent experiments. SDs were <10%.

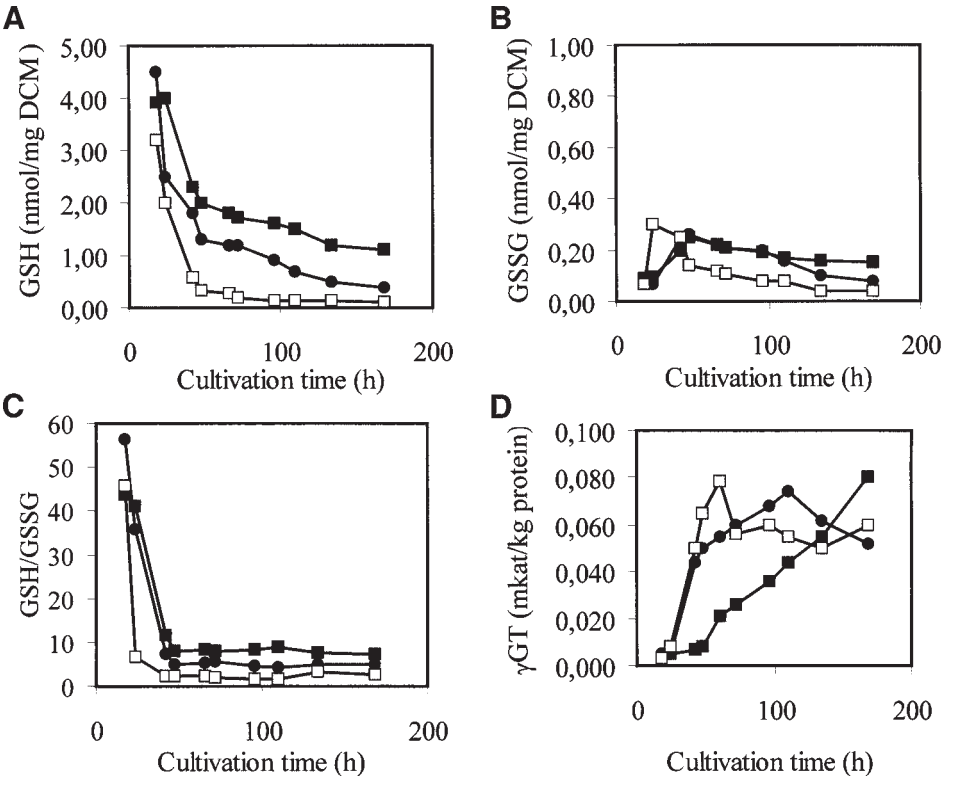

Fig. 8. Intracellular **(A)** GSH and **(B)** GSSG levels; **(C)** GSH/GSSG ratios; and **(D)** specific γGT activities in submerged cultures of (●) FGSC26, (□) Δ*flbA*, and (■) *fadAG*^{203R}. Symbols represent mean values calculated from four independent experiments. SDs were <15%.

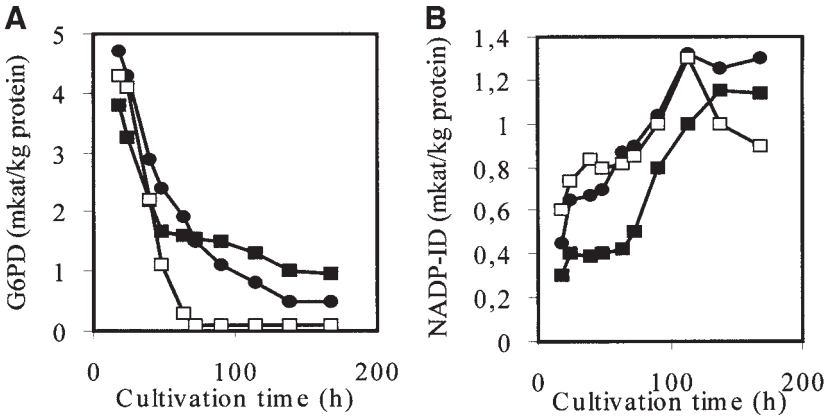

Fig. 9. Changes in specific **(A)** G6PD and **(B)** NADP-ID activities in submerged cultures of (●) FGSC26, (□) Δ*flbA*, and (■) *fadAG*^{203R}. Symbols represent mean values calculated from four independent experiments. SDs were <10%.

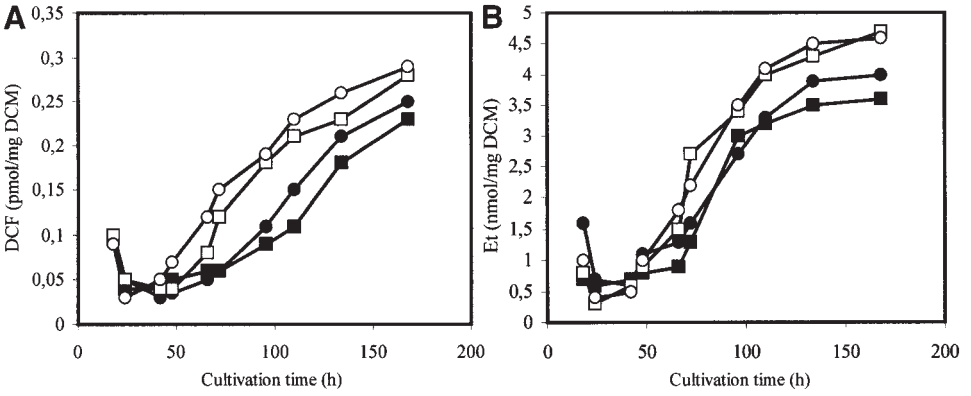

Fig. 10. Changes in specific **(A)** DCF and **(B)** Et production of (●) FGSC26, (○) FGSC33, (□) Δ*flbA*, and (■) *fadAG*^{203R} strains in submerged cultures. FGSC26 and FGSC33 are the parent strains the *fadAG*^{203R} and Δ*flbA* (□) mutants, respectively. Symbols represent mean values calculated from four independent experiments. SDs were <14%. The specific DCF and Et productions are indicative of the intracellular peroxide and superoxide levels, respectively.

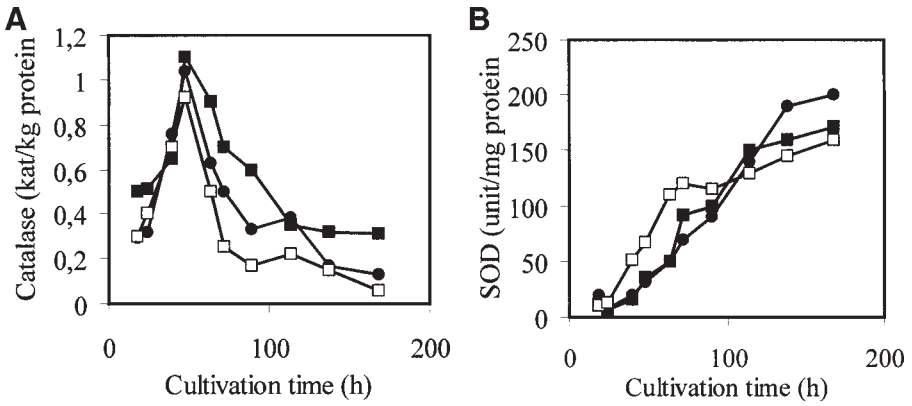

Fig. 11. Specific **(A)** catalase and **(B)** SOD activities in submerged cultures of (●) FGSC26, (□) Δ*flbA*, and (■) *fadAG*^{203R}. Symbols represent mean values calculated from four independent experiments. SDs were <12%.

Interestingly, there were no significant differences in the generation of ROS by the mutants tested and their appropriate wild-type strains (*fadAG*^{203R} vs FGSC26 and Δ*flbA* vs FGSC33) during the whole period of observation (Fig. 10), and the mutations did not affect the specific antioxidant enzyme (catalase, SOD) activities either (Fig. 11). In addition, the ROS production of the pyridoxine and biotin double auxotrophs (Δ*flbA* and FGSC33) was higher than that observed in the biotin auxotrophs *fadAG*^{203R} and FGSC26 independently of the changes in the FadA/FlbA signaling (Fig. 10).

Discussion

The balance between sporulation and growth (autolysis) is maintained by FadA/FlbA signaling in *A. nidulans (11)*. As a consequence, mutations affecting this signaling pathway are expected to cause profound morphologic changes. In fact, the dominant interfering allele of *fadA* (*fadA*^{G203R}) resulted in reduced growth, intensive sporulation, and Hülle-cell formation on surface cultures *(10–12)*. In submerged cultivation, this mutant showed a quite similar phenotype with small pellets (Fig. 4) and with extensive conidiophore and bulbous cell formation during the stationary phase of growth (Fig. 5). The Δ*flbA* mutant showed an autolytic phenotype in surface cultures without any asexual or sexual sporulation *(11,12)*. This strain also showed an altered cell morphology in submerged cultures including the emergence of unusually thick germination tubes (Fig. 1); the formation of "fluffy" pellets, indicating uncontrolled growth of hyphae (Fig. 2); a lack of conidiophore formation even when the cultures were inoculated with less than 0.1×10^6 conidia/mL; and hindered fragmentation of hyphae during autolysis (Fig. 5). The weak fragmentation counterbalanced the accelerated autolytic loss of biomass (Fig. 6), which is a phenotypic feature of this mutant in surface cultures. Nevertheless, declining cell vitality and progressing cell death were not reversed by the submerged cultivation, as indicated by the fast decrease in the MTT reduction capacity after glucose depletion (Fig. 6).

There were no significant differences in either the generation of ROS or the production of antioxidant enzymes (SOD, catalase) by the tested mutants and the appropriate wild-type strains and, therefore, the involvement of ROS or antioxidant enzymes in FadA/FlbA signaling seems to be unlikely in submerged *A. nidulans* cultures. The intracellular accumulation of ROS is a typical event taking place in carbon-depleted autolytic and postautolytic cultures (Fig. 10) and is responsible for the induction of antioxidant enzymes (Fig. 11) *(3,5)* and the initiation of complex physiologic processes such as aging and programmed cell death *(26–28)*. Of course, a FadA/FlbA-independent redox regulation of fungal autolysis cannot be totally excluded, as discussed elsewhere *(29)*. In general, the genomic expression programs of fungi governing morphologic transitions seems to be activated by several different signals and controlled by different regulatory systems according to the culture conditions and the stress factors present in the environment *(2)*.

The effect of the Δ*flbA* and the *fadA*^{G203R} mutations on the degradation of GSH (Fig. 8) and the formation of extracellular proteases (Fig. 7) suggests that the FadA/FlbA signaling is involved in the mobilization of intracellular and extracellular protein and peptide reserves as energy sources during carbon starvation *(8)*. In fact, these weak carbon sources provide the surviving fragments with energy under harsh environmental conditions *(8)* and may even defray the energy requirement of programmed cell death processes in aging fragments or "yeastlike" cells *(5,6)*.

Acknowledgments

This work was supported by a Széchenyi Scholarship, awarded by the Hungarian Ministry of Education and a Bolyai János Scholarship. This work was also supported financially by the Office for Higher Education Programmes (grant no. 0092/2001) and by the OTKA (grant nos. T034315 and T037473).

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