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Genetics

Developmental repression of growth and gene expression in *Aspergillus*

(development/filamentous fungi/sporulation/starvation)

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ABSTRACT **Asexual reproductive development can be initiated in *Aspergillus nidulans* in the presence of excess nutrients through artificial induction of the developmental regulatory genes *brlA* or *abaA* by fusing the genes to the promoter from the alcohol dehydrogenase I gene (*alcA*) and culturing cells in the presence of an inducing alcohol. Artificially induced development completely inhibits growth and represses expression of the endogenous *alcA* gene and the coordinately controlled aldehyde dehydrogenase gene (*aldA*). Repression of *alcA* and *aldA* expression probably occurs at both the transcriptional and posttranslational levels. We propose that developmental induction results in a generalized metabolic shutdown, leading to an inability of cells to acquire nutrients from the growth medium. Self-imposed nutrient limitation could reinforce the primary developmental stimulus and ensure progression through the asexual reproductive pathway.**

Many microorganisms, including the filamentous fungus *Aspergillus nidulans*, produce spores for surviving unfavorable growth conditions and for dispersal to new environments. Sporulation is often triggered by nutrient limitation. However, the precise extracellular requirements for *A. nidulans* sporulation are poorly understood. Even when nutritional conditions favor growth, some vegetative cells (hyphae) from *A. nidulans* colonies grown on surfaces will differentiate to form multicellular reproductive structures, called conidiophores, and asexual spores, called conidia (1). Even continual replacement of the growth medium does not inhibit sporulation (2). Conversely, sporulation by surface cultures of wild-type strains is inhibited by nutrient limitation, and poor supplementation of auxotrophic strains often makes them nearly asporogenous (3). Thus, nutrient depletion may neither be required for nor induce conidiation in *A. nidulans*. Alternatively, it is possible that vegetative cells giving rise to conidiophores suffer nutrient limitation due to competition from neighboring cells. This view is supported by the observation that conidiation, which is normally completely repressed in submerged cultures, can occur to a limited extent if available nutrients limit growth (4, 5).

Three *A. nidulans* genes, *brlA*, *abaA*, and *wetA*, form a linear dependent pathway that regulates conidiophore development and spore differentiation (6–8). Remarkably, hyphal expression of the first gene in the pathway, *brlA*, induced from an alcohol dehydrogenase 1 promoter gene fusion [*alcA*(p)::*brlA*], transcriptionally activates *abaA* and *wetA* and transforms the hyphal tips into reduced conidiophores that differentiate viable spores (7). Hyphal expression of *abaA* has similar effects but does not lead to spore differentiation (8).

Artificial induction of sporulation in the presence of excess nutrients provides an opportunity to distinguish between alterations in cellular metabolism induced by starvation and those that occur as a result of development *per se*. In this paper, we show that *brlA*- or *abaA*-induced development leads to complete cessation of growth and to generalized losses of protein and RNA. In addition, we have found that expression of the coordinately regulated *alcA* and *aldA* (aldehyde dehydrogenase) genes is completely repressed during artificially induced sporulation, even though developmental induction is achieved by activation of the *alcA*(p) fused to either *brlA* or *abaA*. Repression probably occurs at the transcriptional and posttranslational levels. These observations lead us to propose that a self-imposed inability to acquire nutrients from the environment reinforces the initial stimuli that induce vegetative cells to enter the sporulation pathway. Thus, starvation of some cells probably follows developmental induction, regardless of the external environment.

MATERIAL AND METHODS

Fungal Strains and Growth Conditions. *A. nidulans* strains TTAARG (*biA1*; *methG1*; *veA1*), TTA292 (*biA1*; *alcA*(p)::*brlA*; *methG1*; *veA1*), and TPM1 (*biA1*; *alcA*(p)::*abaA*; *methG1*; *veA1*) have been described (7, 8). FGSC752 (*pabaA*; *sB43*; *alcR125 amdA7*; *amdl66 amdS1005*) was obtained from the Fungal Genetics Stock Center. All strains were maintained on appropriately supplemented minimal medium (9). Development was induced as described (7) by transferring 12-hr germlings from medium containing 50 mM glucose to medium containing 100 mM L-threonine. Dry weight measurements were made by harvesting 30 ml of growing cultures onto preweighed filter papers and drying under vacuum. Three samples were taken for each time point, and the dry weights were averaged. Less than 10% variability was observed between samples.

Enzyme Assays. Alcohol dehydrogenase 1 (ADH1) and aldehyde dehydrogenase (AldDH) activities were determined as described (10–12), except that cell-free protein extracts were made by using lyophilized cells. Protein concentrations were determined by the Bradford procedure (13).

RNA Preparation and Analysis. Cultures for RNA isolation were grown as described (7), except that 100 μM 4-thiouridine (Sigma) and 5 μCi (0.15 nmol) (1 Ci = 37 GBq) of [5,6-^{3}H]uridine per ml of culture were added to each sample 30 min before harvesting. Samples were frozen in liquid nitrogen and lyophilized; total RNA was isolated as described (14). Thiol-containing poly(A)$^+$ RNA was isolated as described by Stetler and Thorner (15) by using phenylmercury agarose (16) (Affi-Gel 501, Bio-Rad), except that binding was done in batches rather than on columns. Poly(A)$^+$ RNA (≈100 μg) was dissolved in 50 ml of 0.1% $NaDodSO_4$/0.15 M

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Abbreviations: ADH1, alcohol dehydrogenase 1; AldDH, aldehyde dehydrogenase.

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NaCl/50 mM NaOAc, pH 5.5, heated to 65°C, mixed with 600 ml of Affi-Gel 501 resin, and incubated with shaking for 12 hr at 25°C. Samples were washed three times with an equal volume of buffer, and bound RNA was eluted four times with 100 μl of binding buffer containing 10 mM 2-mercaptoethanol.

Typically ≈5% of the total RNA was recovered as poly(A)$^+$ RNA, and this RNA contained ≈20% of the incorporated radioactivity. Typically ≤30% of the poly(A)$^+$ RNA bound to the Affi-Gel 501, but this RNA contained ≥75% of the radioactivity in the poly(A)$^+$ fraction, indicating that a substantial enrichment for newly synthesized RNA was achieved.

Hybridization Analysis. ^{32}P-labeled cDNA probes were synthesized by priming 1 μg of the poly(A)$^+$ and 4-thiouridine RNA fractions with 40 ng of oligo(dT)$_{12-18}$ and extending with 40 units of Moloney murine leukemia virus reverse transcriptase. Radioactive probes (10^6 dpm; 5×10^6 dpm/μg of RNA template) were hybridized with slot blots containing 500 ng of DNA from plasmids pJA1 or pJA4. pJA1 contains a 600-base pair (bp) *Sal* I–*Eco*RI fragment from *alcA* (17) cloned into Bluescript(−). pJA4 contains a 400-bp *Eco*RV–*Bam*HI fragment from *aldA* (18) cloned into Bluescript(+). Membranes were hybridized and washed as recommended by the membrane supplier (Hybond N, Amersham).

RESULTS

Developmental Induction Inhibits Vegetative Growth. We had shown (7, 8) that *brlA* or *abaA* activation in hyphae induces differentiation and inhibits apical extension of hyphae. To determine whether or not actual growth was inhibited by regulatory gene induction, we grew strains TTAARG (isogenic control), TTA292 [*alcA*(p)::*brlA*], TPM1 [*alcA*(p)::*abaA*], and FGSC752 (*alcR*$^-$) in glucose-containing medium for 12 hr, transferred the cells to medium containing L-threonine as sole carbon source, and determined total cell dry mass at given intervals. *alcA* and *aldA* are completely repressed by glucose and strongly induced by L-threonine (10). L-Threonine induction of *alcA* and *aldA* is mediated by and requires the positive regulatory gene *alcR* (10, 19). Fig. 1 shows that TTAARG and FGSC752 grew after transfer to inducing medium; cell masses doubled within 16 hr. By contrast, growth of TTA292 and TPM1 was completely inhibited. No developmental changes were seen with strains TTAARG or FGSC752, whereas TTA292 and TPM1 underwent characteristic morphological changes (data not shown) (7, 8).

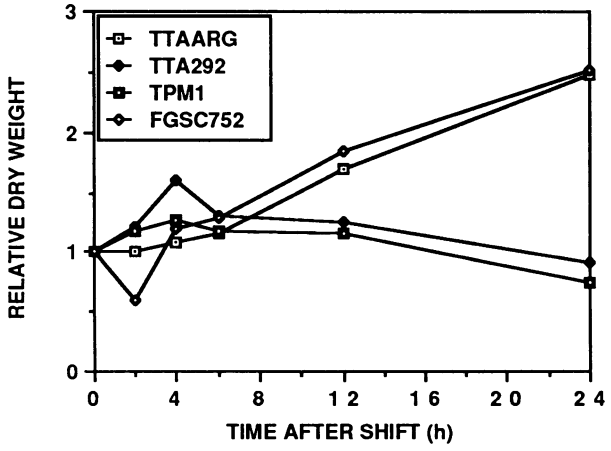

FIG. 1. *brlA* and *abaA* expression in vegetative cells inhibits growth. Strains TPM1, TTA292, and TTAARG were transferred from medium containing glucose as carbon source to medium containing L-threonine as carbon source at 0 hr. Samples were taken at the indicated hour (h), and dry weights were determined. Relative dry weight is expressed as dry weight per initial dry weight.

Developmental Induction Leads to Loss of RNA and Protein. Microorganisms faced with starvation typically use existing protein and RNA to supply the substrates required for spore differentiation or execution of other survival strategies (20–23). To determine whether development occurring in the presence of excess nutrients led to turnover of macromolecules, we measured protein and RNA levels in strains TTAARG, TTA292, and TPM1 after transfer from glucose-containing medium to L-threonine-containing medium. Fig. 2 shows that the ratio of soluble protein to total cell mass (primarily cell-wall mass) remained fairly constant in strain TTAARG. By contrast, in strains TTA292 and TPM1 protein-to-dry weight ratios declined from 5 μg of protein per 50 μg of dry mass at time of transfer to <1 μg of protein per 50 μg of dry mass 12 hr later. Total RNA levels remained relatively constant for strain TTAARG but declined by 50% in strain TTA292 and by 80% in strain TPM1 by 12 hr after induction (data not shown).

Developmental Induction Represses *alcA* and *aldA* Expression. We had shown that transcription of the *alcA*(p)::*brlA* and *alcA*(p)::*abaA* fusion genes was induced by L-threonine (7, 8). To determine whether the *alcA* and *aldA* genes were similarly induced, we measured ADH1 and AldDH activities in strains TTAARG, TTA292, and TPM1 after transfer to L-threonine-containing medium. Fig. 3 shows that both enzymes accumulated to high levels in the control strain. By contrast, the enzymes accumulated only transiently in the strains undergoing development, and neither enzyme was detectable after 4 hr of growth in L-threonine-containing media.

This result was surprising because developmental induction was achieved through activation of an ectopic copy of the *alcA* promoter attached to a developmental regulatory gene. We therefore estimated the transcription rates and steady-state mRNA levels for *alcA* and *aldA*. Cultures were induced to develop, and at various times 4-thiouridine was added for 30 min to label newly synthesized RNA (15). Total and newly synthesized poly(A)$^+$ RNAs were then used to prepare radiolabeled cDNA probes that were hybridized to excess amounts of filter-immobilized plasmid DNAs corresponding to the *alcA* or *aldA* genes. Fig. 4 shows that transcription of both *alcA* and *aldA* began within 1 hr of transfer of cells to L-threonine-containing medium for all three strains. However, the estimated transcription rates and total mRNA levels were significantly higher for both genes in the TTAARG control than in the strains expressing *brlA* (TTA292) or *abaA* (TPM1).

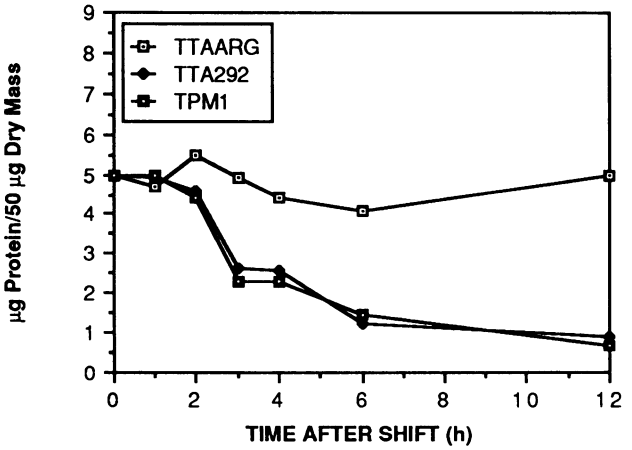

FIG. 2. Induction of *brlA* or *abaA* reduces total cellular protein. Strains TPM1, TTA292, and TTAARG were grown in glucose-containing medium for 12 hr and then transferred to L-threonine-containing medium; at the indicated hour (h) samples were taken, and protein content per μg of dry weight was determined.

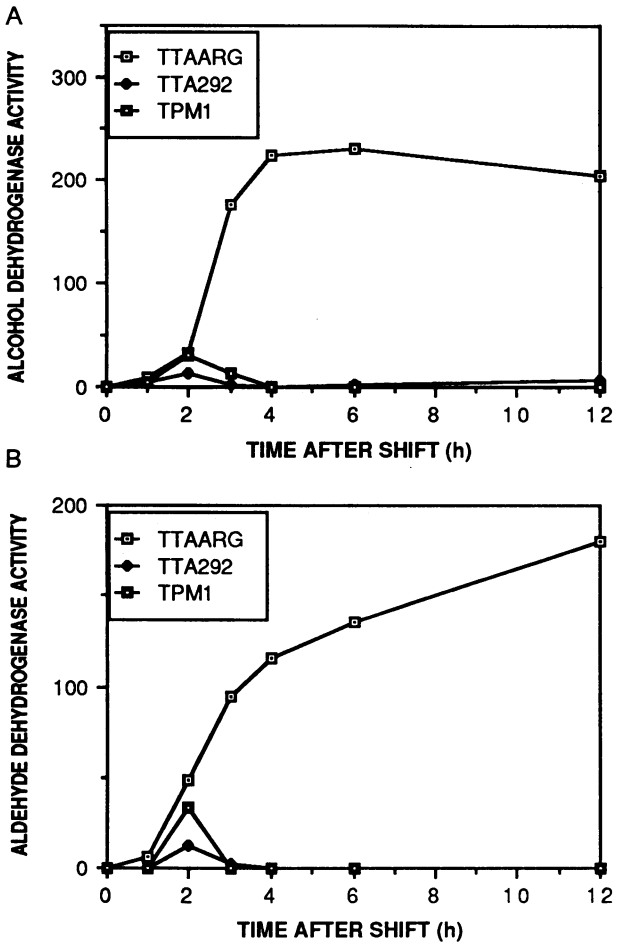

FIG. 3. *brlA* and *abaA* expression inhibits *alcA* and *aldA* enzyme accumulation. Strains TPM1, TTA292, and TTAARG were grown in glucose-containing medium for 12 hr (h) and transferred to L-threonine-containing medium (0 hr). DH1 (11) and AldDH activity (12) were determined at the indicated hour and are reported as change in absorbance per min per mg of protein.

To determine whether *alcA* and *aldA* transcripts were being translated, we isolated polyribosomes (24) from cultures of TTAARG, TTA292, and TPM1 4 hr after transfer to inducing medium. Sucrose gradient fractions were hybridized with DNA probes corresponding to *alcA* or *aldA*. With all three strains the great majority of *alcA* and *aldA* mRNAs were present in >100S polyribosomes (data not shown), indicating that these mRNAs were active in translation.

To determine whether ADH1 and AldDH polypeptides accumulated during development, we subjected protein extracts to SDS/PAGE analysis. Fig. 5 shows that proteins migrating appropriately for ADH1 and AldDH (17, 18) accumulated to high levels during L-threonine induction of TTAARG and to lower levels with TTA292. The patterns of proteins present in both strains were qualitatively highly similar at all times. A similar result was obtained with TPM1 (data not shown). Thus, with both TTA292 and TPM1 presumptive ADH1 and AldDH polypeptides were present even after enzyme activities were no longer detectable.

DISCUSSION

Nutrient deprivation is the primary signal-inducing sporulation in many microorganisms. The signals inducing sporulation in *A. nidulans* are poorly understood, in part, because sporulation is difficult to study in heterogeneous surface colonies and reliable procedures for inducing homogeneous sporulation in submerged culture have not been worked out. The observation that direct induction of either the *brlA* or *abaA* developmental regulatory genes activates the conidiation pathway (7, 8) means that developmental events can be studied in the absence of the environmental cues that normally initiate sporulation. Development can be synchronously initiated in cells grown in the presence of high levels of nutrients. In this study, we have shown that activation of either *brlA* or *abaA* by fusion to and induction of the *alcA* promoter leads to a complete cessation of growth and to major reductions in total cellular protein and RNA levels. Thus, nutrient depletion is not a prerequisite for two of the primary cellular events associated with microbial development. This result implies that growth and development are not mutually exclusive simply because they can occur only under different environmental conditions that preclude one or the other pathway. Instead, metabolic alterations occurring as a consequence of developmental induction must stop growth and inhibit synthesis or stimulate degradation of macromolecules.

Numerous aspects of metabolism could be inhibited during development and lead to growth cessation. One possibility is that expression of genes needed for nutrient acquisition is inhibited by developmental induction. Consistent with this idea is our observation that the *alcA* and *aldA* genes are

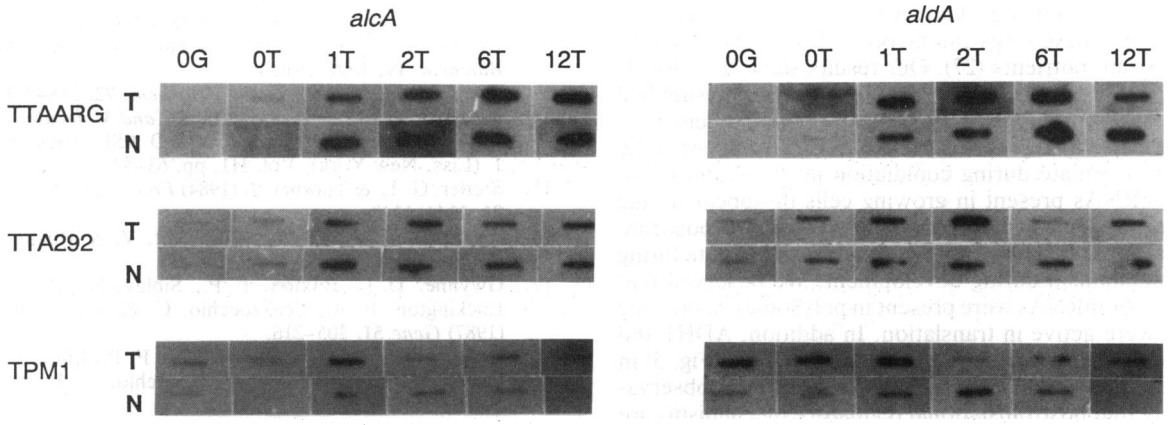

FIG. 4. Inhibition of *alcA* and *aldA* enzyme accumulation is, in part, transcriptional. Plasmid DNA (500 ng) corresponding to the *A. nidulans aldA* or *alcA* genes was denatured, fixed to a nylon membrane, and probed with ^{32}P-labeled cDNA made from either newly synthesized (N) or total (T) poly(A)$^+$ RNA isolated from cultures of TPM1, TTA292, and TTAARG as described. RNAs were isolated after 12 hr of growth in glucose-containing media (0G) or at 30 min (0T), 1 hr and 30 min (1T), 2 hr and 30 min (2T), 6 hr and 30 min (6T), and 12 hr and 30 min (12T) after transferring cultures to L-threonine-containing medium.

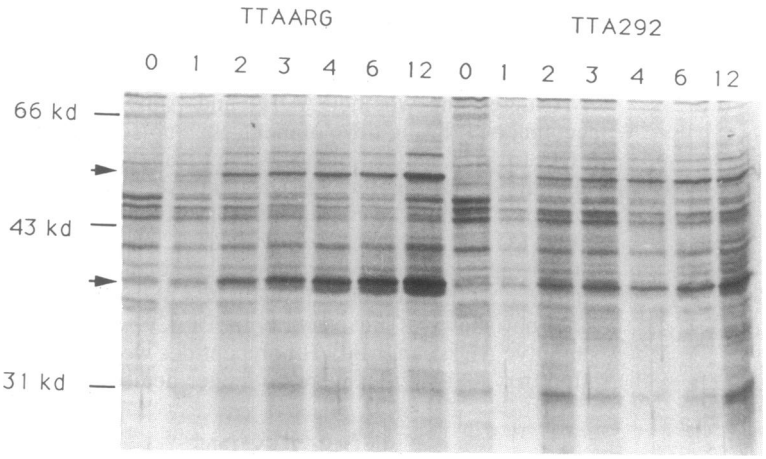

FIG. 5. *brlA*-induced development inhibits ADH1 and AldDH protein accumulation. Ten μg of total soluble protein obtained from *A. nidulans* strains TTA292 and TTAARG at various times after transfer to L-threonine-containing medium were separated by electrophoresis on a 10% polyacrylamide/SDS gel. Lanes correspond to 1, 2, 3, 4, 6, and 12 hr after transfer to L-threonine medium. The arrows indicate the predicted positions for migration of AldDH (upper) and ADH1 (lower), respectively. kd, kDa.

expressed only transiently in developmentally induced strains. If ethanol had been used to induce the *alcA*(p)::*brlA* or *alcA*(p)::*abaA* fusion genes, growth stoppage would have been expected from the lack of enzymes needed to metabolize the only available carbon source. Although L-threonine is a strong inducer of *alcA* and *aldA*, its use does not require the products of these genes, as shown by the ability of an *alcR*$^-$ strain (FGSC752) to grow with L-threonine as sole carbon source (Fig. 1). *alcR*$^-$ strains are unable to induce either *alcA* or *aldA* and cannot grow on ethanol (10, 11). The mechanism of L-threonine use has not been elucidated for *A. nidulans* but probably proceeds by threonine aldolase-mediated cleavage to yield glycine and the *alcR* coinducer acetaldehyde (10). Acetaldehyde would then be converted to acetate by the *aldA*-encoded acetaldehyde dehydrogenase, whereas glycine would be converted to pyruvate. The lack of L-threonine toxicity in *alcR*$^-$ strains implies that an *aldA*-independent mechanism exists at least to detoxify, if not to utilize, acetaldehyde. As we have not directly determined the ability of developmentally induced cells to metabolize L-threonine, it is possible that failed induction of enzymes required for carbon-source use is the primary cause of growth cessation. The idea that developmental induction leads to a more generalized metabolic shutdown is supported by the observed decreases in both total protein (Fig. 2) and RNA levels.

Whether or not the primary stimulus for initiating development by *A. nidulans* cultures is nutrient limitation, starvation induced by development could play a major role in commitment to the conidiation pathway. Such commitment steps, providing irreversibility to microbial sporulation after initiation, have long been known (25, 26), and it has been suggested that these steps can involve a loss in the ability to take up certain nutrients (27). Our results show that, for *A. nidulans*, changes in the ability to use exogenously supplied nutrients can be a direct consequence of development.

Timberlake (28) reported that hundreds of stage-specific mRNAs accumulate during conidiation in *A. nidulans*, but that few mRNAs present in growing cells disappear during development. This result raised the possibility that posttranscriptional mechanisms could be important in restructuring cellular metabolism during development. We observed that *alcA* and *aldA* mRNAs were present in polysomes, indicating that they were active in translation. In addition, ADH1 and AldDH polypeptides apparently remained in cells (Fig. 5) in which we could detect no enzyme activity. These observations imply that posttranslational regulatory mechanisms are used in the developmental control of expression of these two genes. Similar controls could be important in modulating other metabolic pathways. The ability to induce development under a variety of conditions by direct activation of the *brlA* and *abaA* genes will facilitate studies of these regulatory mechanisms. In addition, this capability should allow the distinction between cellular changes that result from environmental perturbations but are not directly related to development and those that play a direct role in the metabolic alterations required for sporulation.

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