Starvation stress modulates the expression of the *Aspergillus nidulans* *brlA* regulatory gene

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Expression of the *Aspergillus nidulans* *brlA* gene plays a fundamental role in the switch from vegetative growth to asexual reproduction. Using a media-shifting protocol to induce submerged sporulation and *brlA-lacZ* as an expression marker, it was shown that carbon and nitrogen starvation stress induced *brlA* transcription to different degrees. Glucose starvation induced *brlA* rapidly to high levels and resulted in spore formation on reduced conidiophores, whereas nitrogen starvation induced *brlA* gradually to lower levels and sporulation occurred to a lesser extent but from more complex conidiophores. β -Galactosidase activity paralleled *brlA α* and *brlA β* mRNA. No clear qualitative differences between the two *brlA* transcripts were found in these starvation conditions, suggesting that the different patterns of sporulation could be explained by quantitative expression differences. Since *brlA* mRNA did not accumulate in the presence of a high glucose concentration, we investigated the role of other carbon sources on *brlA* expression. Non-repressing carbon sources such as glycerol, acetate and arabinose were as effective as glucose in preventing *brlA* mRNA accumulation, suggesting that the glucose effects on *brlA* expression could be explained as a response to nutrient starvation, rather than by carbon catabolite repression. Despite similar low levels of *brlA* transcripts being detected during growth in glucose or non-repressing carbon sources, conidiophores were formed only in medium containing glycerol, acetate or arabinose. When mycelia were not shifted to starvation conditions, sporulation was not observed in standard minimal medium even after glucose was exhausted, unless the medium was buffered. This and other results suggest that strong deviation from external neutral pH partially prevented full induction and/or function of *brlA*.

Keywords: *Aspergillus nidulans*, *brlA*, starvation stress, glucose regulation, submerged sporulation

INTRODUCTION

Development in many micro-organisms has often been shown to be induced by nutrient limitation and other conditions which have a detrimental effect on growth, but very little is known about how these environmental signals lead to development (for a discussion see Hansberg & Aguirre, 1990; Strauch & Hoch, 1992). Asexual reproduction (conidiation) of the Ascomycete fungus *Aspergillus nidulans* is an excellent experimental system to study these signals, since the different conditions able to induce sporulation will converge upon expression of the *brlA* (Bristle) gene. This is an early regulatory gene involved in the formation of the conidiophores of *A. nidulans*. These multicellular, spore-producing structures

are composed of a basal foot cell, an aerial stalk ending in a multinucleate globose vesicle, a layer of uninucleate primary sterigmata or metulae, and a layer of uninucleate secondary sterigmata or phialides, from which spores are produced by repeated division (Clutterbuck, 1969; Oliver, 1972; Mims *et al.*, 1988). Mutants in *brlA* form aerial stalks that fail to differentiate vesicles, metulae, phialides or spores (Clutterbuck, 1969). They also fail to express many genes associated with later stages of conidiophore development (Zimmermann *et al.*, 1980; Boylan *et al.*, 1987; Adams *et al.*, 1988; Mirabito *et al.*, 1989; Clutterbuck, 1990; Stringer *et al.*, 1991; Timberlake, 1991).

The *brlA* gene encodes two mRNAs, designated *brlA α* and *brlA β* . The *brlA β* mRNA encodes a polypeptide

which is a putative Zinc-finger transcription factor identical to *brlA α* , except that 23 additional amino acids are present at the amino terminus (Adams *et al.*, 1988; Prade & Timberlake, 1993). *brlA α / β* transcripts are present at barely detectable levels in hyphae, and begin to accumulate about 5 h after induction of development by exposure to air (Boylan *et al.*, 1987; Prade & Timberlake, 1993), primarily in conidiophore vesicles, metulae and phialides (Aguirre *et al.*, 1990). Under conditions where sporulation does not normally occur, expression of either *brlA α* or *brlA β* from the strongly inducible *alcA* promoter results in spore production from hyphal tips (Adams *et al.*, 1988; Han *et al.*, 1993).

Since expression of *brlA* leads to sporulation, it is important to understand how this gene is regulated. In *A. nidulans* the ability to conidiate, and therefore to express *brlA*, is not reached until a certain amount of mycelial growth is completed, a phenomenon described as competence (Axelrod *et al.*, 1973). Competent mycelia, however, will not differentiate until sporulation is induced by exposure to air (Axelrod *et al.*, 1973; Champe & Simon, 1992), or as shown in this paper, by nutrient limitation.

It has been reported that other *A. nidulans* developmental regulatory genes (such as *stuA*, *medA* and *abaA*; Mirabito *et al.*, 1989) control the pattern of expression of the *brlA* gene, including *brlA* itself (Aguirre, 1993). However, half of the total induction of *brlA* transcription is *brlA*-independent (Aguirre, 1993), and nothing is known about the signals that initiate *brlA* expression. The study of such signals has been limited by the fact that sporulation in *A. nidulans* is typically induced by exposure to air in solid media, a condition where environmental factors such as the nutritional status of the cells are difficult to evaluate and manipulate.

In this paper, we show that the *brlA* gene can be induced in submerged liquid culture, either by carbon or nitrogen starvation, or both carbon and nitrogen starvation and that the rate and amount of *brlA* mRNA correlates inversely with the morphological complexity of the conidiophore. We propose that *brlA* is induced as a response to a general starvation stress condition.

METHODS

Aspergillus strain and growth conditions. *A. nidulans* strain TJA22 [*biA1*; *brlA*(-2913 *p/h*)/*lacZ*(*argB*⁺/*argB*::*CAT*); *metG1*] (Aguirre, 1993) was grown in appropriately supplemented minimal medium (C, glucose 1%; N, NaNO₃ or NH₄Cl; Käfer, 1977) at 37 °C for 18 h and 300 r.p.m. (culture volume was kept to one-fifth of the flask volume).

Freshly collected 5-d-old spores were filtered through Miracloth (Calbiochem), washed four times with 10 ml sterile water, and used immediately to inoculate a single culture at a density of 5×10^5 conidia ml⁻¹. The inoculated medium was then divided into 50 ml samples per 250 ml Erlenmeyer flask and incubated at 37 °C for 18 h and 300 r.p.m. Fresh spores were used because variability in results was observed when using spore suspensions stored at 4 °C for different times.

For starvation experiments, 50 ml cultures incubated for 18 h were filtered through Miracloth. Mycelia were briefly rinsed

with minimal glucose/nitrate-free medium, and resuspended in 50 ml of either glucose-free or nitrogen-free medium (250 ml flask) and incubation was continued under the same conditions. For shifts to media with different glucose concentrations or with different carbon sources, filtered cultures were washed with 50 ml pre-warmed sterile water, then with 50 ml supplemented minimal medium with no glucose, and finally resuspended in 50 ml of the appropriate medium. Care was taken to control the amount of biomass transferred to the starvation media, since this can affect both the kinetics and the absolute levels of *brlA*/*lacZ* induction.

β -Galactosidase activity, glucose determination and microscopy. β -Galactosidase activity was determined as previously reported (Miller, 1972; Aguirre *et al.*, 1990).

Glucose was measured directly from fresh or frozen culture filtrates using a quantitative, enzymic (glucose oxidase) determination kit from Sigma.

Mycelia from different culture conditions were examined under a microscope for conidiophore formation and spore counting, and were photographed using a green filter and Kodak Tri-X-Pan 400 film.

RNA preparation and analysis. Total mRNA was extracted as described by Timberlake (1980), fractionated by electrophoresis in formaldehyde-agarose gels (10–15 μ g per lane), transferred to Hybond-N membranes (Amersham), and hybridized to ³²P-labelled random-primed specific probes (BRL) according to the membrane manufacturer's instructions. A *brlA* *Bam*HI–*Eco*RI fragment from pBS2:5 (Boylan *et al.*, 1987) was used as the *brlA α / β* -specific probe, and an *argB* *Hind*III fragment from pDGH25 (Gems *et al.*, 1991) was used as a probe to confirm equal loading of mRNA samples. For dot-blot analysis, 15 μ g total mRNA was applied to a Hybond-N membrane as recommended by the manufacturer, and hybridized to ³²P-labelled random-primed specific probes for *wA* (*Sal*I–*Xba*I fragment from pNK15; Mayorga & Timberlake, 1990), *yA* (*Xba*I–*Eco*RI from pRA6; R. Aramayo, unpublished) and *CAN5* (Boylan *et al.*, 1987).

RESULTS AND DISCUSSION

brlA can be induced in submerged cultures by carbon or nitrogen starvation, resulting in different patterns of sporulation

Martinelli (1976) reported submerged sporulation by *A. nidulans* in liquid cultures using different carbon sources. Since the high surface area/volume ratio (20 ml medium in 250 ml flasks) used in her experiments could have induced air interface sporulation, we produced submerged sporulation using lower surface area/volume ratios (50 ml medium in 250 ml flasks), as well as by media shifting, to test the ability of carbon and nitrogen starvation to induce the *brlA* gene.

A. nidulans strain TJA22, which contains a single copy of a *brlA α / β -lacZ* gene fusion integrated at the *argB* locus (Aguirre, 1993) was grown in liquid culture for 18 h at 37 °C and then the mycelia were shifted to glucose-free medium, nitrate-free medium, or standard medium (Käfer, 1977). Samples were taken at different times and processed for β -galactosidase assay, mRNA extraction, and microscopic examination. The β -galactosidase assays showed that both glucose- and nitrate-starvation induced

Fig. 1. *brlA* induction and conidiophore morphology in submerged cultures of *A. nidulans* TJA22 during glucose or nitrate starvation. (a) Accumulation of β -galactosidase in *A. nidulans* grown for 18 h as described in Methods and shifted to media with no glucose (●) or no nitrate (○), or to standard medium (□) as a control. Incubation was continued under the same conditions and mycelia from entire cultures were harvested at the indicated times, frozen and lyophilized. Part of the samples were processed for β -galactosidase determination;

the *brlAα/β-lacZ* gene; glucose starvation induced *brlA* expression more rapidly and to a higher level than nitrate starvation (Fig. 1a). Shifting to standard glucose-nitrate medium caused a slight induction of *brlA* at 20 h. Endogenous β -galactosidase activity was barely detectable in all conditions tested (not shown).

Distinctive morphological changes were observed at 20 h following shifting to the different media. Glucose starvation induced spore formation from highly reduced conidiophores, bypassing the vesicle and metulae stages (Fig. 1c, top). This morphology was similar to that produced when either *brlAα* or *brlAβ* are induced rapidly to high levels from the inducible *alcA* promoter (Adams *et al.*, 1988; Han *et al.*, 1993). By contrast, nitrate starvation gave rise to more elaborate conidiophores, similar to those produced at an air interface (Fig. 1c, middle). No obvious signs of sporulation were observed when shifting was to standard medium (Fig. 1c, bottom).

These two different morphological patterns of sporulation may have resulted from differential induction of either *brlAα* or *brlAβ* transcripts. To address this question, the presence of both transcripts was assayed by Northern blotting. The results presented in Fig. 1(b) show that both *brlAα* and *brlAβ* mRNAs had accumulated by 10 and 20 h after shifting to glucose- or nitrate-free medium. Accumulation paralleled β -galactosidase activity and no clear differences, other than quantitative ones, were observed. In fact, densitometric analysis of Fig. 1(b) showed that the *brlAβ/brlAα* ratio was around 1.4 in all cases (*brlAβ* is usually more abundant than *brlAα*; Prade & Timberlake, 1993). Longer autoradiographic exposure of this Northern blot showed a slight accumulation of *brlA* mRNA when mycelia were transferred to standard 1% (w/v) glucose medium, but no obvious morphological changes were detected under those conditions (Fig. 1c, bottom). Other starvation experiments, shifting from glucose-NH₄Cl to glucose-free or NH₄Cl-free media, produced similar results to those shown in Fig. 1(a) and b, i.e. *brlA/lacZ* was expressed less when mycelia were nitrogen-starved than when they were glucose-starved, and complex and reduced conidiophores were formed, respectively. Cultures starved of both glucose and nitrate gave results similar to cultures starved of glucose only (some of

specific activity corresponds to U (mg protein)⁻¹ (Miller, 1972). About 300 U is reached 35 h after induction of sporulation in an air interface (Aguirre, 1993). A representative experiment is shown. (b) *brlAα/β* mRNA accumulation induced by glucose or nitrate starvation. Total mRNA was isolated from samples of the experiment shown in (a), Northern blotted and probed with a *brlAα/β*-specific probe. The lanes correspond to different times after shifting to the indicated media: 0 h after shifting corresponds to 18 h growth in standard minimal medium. Equal loading of mRNA was confirmed by stripping the membrane and reprobing with an *argB*-specific probe. (c) Conidiophore morphology 20 h after shifting to media with no glucose (top) or no nitrate (middle), or to standard medium (bottom). The long arrow in the top panel indicates a typical reduced conidiophore; arrowheads indicate spores. Short arrows in the middle panel point to complex conidiophores. The bottom panel shows non-differentiated hyphae. Bars, 50 μ m.

Fig. 2. *brlA* expression in *creA*-repressing and non-repressing carbon sources. (a) *A. nidulans* was grown for 18 h in glucose medium and shifted to media containing either no glucose (●), 0.1% glucose (▲), 1% glucose (■), 1% arabinose (○), 1% glycerol (△) or 100 mM acetate (□). Samples were isolated at the times indicated and β -galactosidase was assayed as in Fig. 1. The results represent mean values from two independent experiments with a maximum variation of 16% about the mean. (b) Total mRNA was isolated from 24 h samples, Northern blotted and probed with a *brlA* α/β -specific probe. Lanes: 1, no glucose; 2, 0.1% glucose; 3, 1% glucose; 4, 1% arabinose; 5, 1% glycerol; 6, 100 mM acetate. Equal loading of mRNA was confirmed by stripping the membrane and reprobing with an *argB*-specific probe.

these results are shown in Table 2). *brlA1* and *abaA2* null mutants failed to sporulate under these conditions, indicating that submerged sporulation employs the same basic developmental pathway as described previously by Clutterbuck (1969) and Adams *et al.* (1988). In fact, transcripts from the late sporulation genes *yA*, *wA* and *CAN5* (Adams *et al.*, 1988; Mayorga & Timberlake, 1990; Boylan *et al.*, 1987) were detected in 24 h samples starved of glucose. These results are important because in *A. nidulans*, sporulation has been defined as a process triggered not by starvation, but rather by a programmed life-cycle event (Axelrod *et al.*, 1973; Adams *et al.*, 1988, 1992; Mooney & Yager, 1990; Champe & Simon, 1992; Han *et al.*, 1993; Lee & Adams, 1994).

In addition to their relevance for development, we believe that these results have practical consequences for research involving *A. nidulans* as a model system, since it can no

longer be assumed that development is prevented by submerged culture conditions. Pertinent to this is the use of the *alcA* promoter to ectopically express developmental genes (Adams *et al.*, 1988), since this involves growth for 12 h in glucose and then shifting of mycelia to a poor carbon source such as threonine. As reported by Adams *et al.* (1988), we did not find sporulation after 24 h in threonine medium using our strain TJA22. However, modification of this protocol by growing 18 h in glucose before shifting gave rise to the formation of complex conidiophores after 24 h in threonine medium. This implies that care should be taken when modifying the reported protocol (i.e. using ethanol or other inducers instead of threonine, growing for different times before shifting, etc.). In any case, appropriate controls of gene expression have to be included when media manipulations are used to alter the expression of developmental genes.

The observed correlation between the rate and level of *brlA* induction and the different conidiophore morphologies, suggests that conidiophore cell-types could result from gradual increases in *brlA* activity, the lowest dosage corresponding to vesicle formation and the highest to phialide- and spore-development. This proposal is elaborated upon below.

Non-repressing carbon sources can also prevent *brlA* accumulation

To investigate the effect of glucose on *brlA* expression, *A. nidulans* TJA22 was grown as before and shifted to media containing glucose (0, 0.1 or 1.0%, w/v), or the *creA*-mediated non-repressing carbon sources arabinose (1%, w/v), glycerol (1%, w/v) (Arst & Bailey, 1977) or acetate (100 mM). Cultures were sampled at different times to determine β -galactosidase activity and *brlA* mRNA accumulation (Fig. 2a, b), morphology (Fig. 3) and spore numbers (Table 1). β -Galactosidase activity was higher when cultures were shifted to 0.1% glucose than when shifted to glucose-free medium, whereas acetate, arabinose or glycerol were as good as 1% glucose in preventing *brlA* α/β -*lacZ* accumulation (Fig. 2a), allowing a low level of *brlA* expression. The *brlA* α/β -*lacZ* activity also correlated with the actual *brlA* mRNA accumulation (Fig. 2b) and, within the limitations of the technique (low *brlA* transcription is not easily detectable in Northern blots, probably because the *brlA* mRNA is unstable; Prade & Timberlake, 1993), no obvious difference in the relative abundance of *brlA* α or *brlA* β transcripts was observed between 1% glucose and the non-repressing carbon sources (Fig. 2b). The high levels of *brlA* expression with 0.1% glucose could be related to the fact that this glucose concentration is low enough not to repress *brlA*, but high enough to fuel the sporulation process. However, glucose was exhausted from the medium by 18 h after shifting to 1% glucose (not shown), and the low *brlA* induction observed under this condition was probably due to partial carbon starvation.

The results obtained with arabinose, acetate and glycerol make it unlikely that *brlA* glucose repression could be directly mediated by a *creA*-dependent carbon catabolite

Fig. 3. Conidiophore morphologies 24 h after shifting of glucose-nitrate grown mycelia to different carbon sources: (a), 0.1 % glucose; (b), 1.0 % arabinose; (c), 1.0 % glycerol; and (d), 100 mM acetate. The long arrow in (a) indicates a reduced conidiophore and arrowheads indicate spores. The thick arrows in (b)–(d) indicate complex conidiophores. Bars, 20 μ m.

repression mechanism (Arst & Bailey, 1977; Dowzer & Kelly, 1991). Instead, we propose that *brlA* is regulated by nutrient limitation and that starvation for carbon, nitrogen, carbon and nitrogen, or other nutrients can lead to a similar kind of metabolic stress (see Hansberg & Aguirre, 1990).

Rate and level of *brlA* expression correlates with the complexity of conidiophore development

Figure 3 shows the morphology observed in some of the 24 h samples exposed to different carbon sources from the experiment presented in Fig. 2. Again, rapidly increasing levels of β -galactosidase activity and *brlA* mRNA corresponded with high spore numbers and reduced conidiophore morphology (Fig. 1c, top, Fig. 3a, and Table 1), whereas low but gradually increasing levels corresponded with either the absence of sporulating structures (as in Fig. 1c, bottom), or the presence of few conidiophores with more complex morphology and low spore production (Fig. 3b–d; Table 1). As in the experiments shown in Fig. 1, these different conidiophore morphologies could not be correlated with differential expression of either *brlA α* or *brlA β* transcripts, as judged from

Northern blots (Fig. 2b). These results support a quantitative model for *brlA* regulation of conidiophore development, which would be consistent with the following findings. Firstly, high induction of either of the two *brlA* transcripts (α or β) from the strongly inducible *alcA* promoter causes spore production directly from hyphal tips (Adams *et al.*, 1988; Han *et al.*, 1993), a morphology virtually identical to that observed in the glucose starvation experiments (Figs 1c and 3a). Secondly, *brlA α* and *brlA β* seem to perform similar functions, since multiple copies of either *brlA α* or *brlA β* can overcome the need for the other transcript during normal development (Prade & Timberlake, 1993). Thirdly, developmental mutants such as *medA* and *stuA* (Clutterbuck, 1969; Miller *et al.*, 1992) present aberrant conidiophore morphologies that are consistent with their abnormal temporal or spatial transcription of *brlA* (Aguirre, 1993).

Several *cis*-acting regulatory elements could regulate *brlA* transcription rates through multiple inputs such as carbon and nitrogen starvation. It is important to note that *brlA* regulatory sequences, up to –2913 bp, are required for full *brlA* expression, and progressive deletion of these sequences in a *brlA α / β -lacZ* fusion gradually decreases

Table 1. Spore production and external pH after shifting to different carbon sources

Mycelia grown in minimal nitrate-glucose (1%) media for 18 h were shifted to the indicated carbon sources and sampled for spore production after 24 h and for external pH after 6 and 24 h. Spore numbers shown are mean values from duplicates with a maximum variation of 13% about the mean.

Minimal medium plus:	$10^{-3} \times$ Spores per culture	External pH	
		6 h	24 h
No glucose	50000	6.8	7.2
0.1% glucose	130000	7.1	7.6
1% glucose	8	7.6	8.4
1% arabinose	20	7.5	8.3
1% glycerol	14	6.9	8.1
100 mM acetate	14	8.5	8.8

maximum β -galactosidase levels but does not alter the developmental timing or cellular specificity of expression (J. Aguirre & W. E. Timberlake, unpublished). Miller (1993) has pointed out that several *brlA*-dependent genes contain more than one copy of *brlA*-responsive elements (Chang & Timberlake, 1992), providing a mechanism for a *brlA* dosage control of development. In microorganisms, incremental regulation of gene expression has been shown for the *Mycococcus xanthus csgA* gene, which encodes an extracellular protein whose steady increases are regulated at the transcriptional level and determine the proper ordering of developmental steps (Li *et al.*, 1992).

We cannot explain why some conidiophore development occurs in non-repressing carbon sources (Fig. 3b–d), but it does not occur in 1% glucose (Fig. 1c), despite similar moderate levels of *brlA* expression being detected in all these conditions. It is possible that although *brlA* itself does not seem to be repressed by glucose through a *creA*-dependent mechanism, other genes necessary for sporulation could be repressed by the glucose-*creA* system. The different carbon sources could also have diverse effects on *brlA* at the translational level (i.e. by stabilizing the *brlA* messages), since there is evidence showing that *brlA* β , but not *brlA* α , is regulated by translational repression (Han *et al.*, 1993). Further work is necessary to answer these questions.

pH changes during starvation-induced *brlA* expression

Espejo *et al.* (1993) have shown that the *ipnA* gene, which encodes isopenicillin N-synthase, is carbon repressed by a *creA*-independent mechanism that can be overcome by external alkaline pH. We monitored pH changes during growth and liquid sporulation in different carbon sources. The pH values in Table 1 show that incubation in the different carbon sources resulted in different degrees of medium alkalization. The largest differences occurred during the first 6 h of incubation, and acetate and 1%

Table 2. Spore production, conidiophore morphology and external pH changes induced by different types of starvation

Experimental conditions were as indicated for Table 1, except that shifting was to media lacking either glucose, nitrate or ammonium, or both glucose and nitrate. When shifting was to media with or without ammonium, the 18 h pre-growth was done in glucose-ammonium medium. Spore production and conidiophore morphology were determined on 24 h samples. pH was determined in medium samples from 6 and 24 h. Representative results are shown.

Type of starvation	$10^{-3} \times$ Spores per culture	Conidiophore morphology	External pH	
			6 h	24 h
– Glucose	50000	Reduced	6.8	7.2
+ Nitrate				
– Glucose	3100	Reduced	6.0	6.2
+ Ammonium				
+ Glucose	64900	Complex	6.2	6.1
– Nitrate				
+ Glucose	52800	Complex	6.3	6.2
– Ammonium				
– Glucose	107000	Reduced	6.5	6.8
– Nitrate				

glucose resulted in the strongest alkalization after 24 h. When mycelia were shifted to 1% glucose containing NH_4Cl rather than NaNO_3 , *brlA* was fully repressed and, in this case, external pH became acidic instead of alkaline (not shown), indicating that glucose prevented *brlA* expression in both alkaline and acidic conditions.

These results argue against external pH changes as the sole explanation for the different morphologies observed in repressing and non-repressing carbon sources. However, we observed that submerged sporulation induced either by starvation of glucose, nitrate, ammonium, or by simultaneous glucose and nitrate starvation, always resulted in external pH values close to neutrality (Table 2); in fact, sporulation was reduced, but not prevented, when mycelia were starved of both glucose and nitrate in medium previously adjusted to pH 4 or 8.

Without media shifting, submerged sporulation does not occur, even after glucose exhaustion

All the experiments reported above were done by growing mycelia for 18 h in standard minimal nitrate-glucose medium (Käfer, 1977), and then transferring to different media. The same kind of experiment was performed without media shifting and the resulting culture was sampled at different times. It was found that external pH became strongly alkaline and *brlA/lacZ* was induced to moderate levels after glucose was exhausted (Fig. 4a). However, no signs of sporulation were observed even after prolonged incubation (morphology was similar to that shown in Fig. 1c; bottom). Most of the reluctance to

Fig. 4. *brlA/lacZ* induction (●), glucose consumption (■) and external pH (△) during growth of *A. nidulans* in minimal nitrate-glucose liquid medium without (a) or with (b) 0.1 M sodium phosphate buffer. Minimal nitrate-glucose (1%) medium with or without buffer was inoculated with *A. nidulans* and grown for 42 h. At the indicated times, mycelial samples were harvested and assayed for β -galactosidase. Medium samples were used for glucose and pH determinations (Methods). A representative experiment is shown.

consider submerged sporulation as a common occurrence, and starvation as a triggering factor, could be related to this finding, since Käfer's medium is used extensively.

Considering that the buffering capacity of Käfer's medium is very limited (11 mM KH_2PO_4), and that our previous results suggested that strong deviation from external neutral pH could partially prevent full induction and/or function of *brlA*, we decided to include 0.1 M sodium phosphate buffer in Käfer's medium and follow external pH, *brlA/lacZ* induction and morphological changes during growth. Fig. 4(b) shows that external pH was maintained around 6.5 and that *brlA/lacZ* was induced to higher levels (≈ 200 U) than those shown in Fig. 4(a)

(≈ 70 U). Abundant sporulation (complex conidiophores) was observed under these conditions by 42 h, showing that reproducible submerged sporulation can be obtained in standard buffered media without media shifting. Even in this case, starvation (glucose exhaustion) seems to be a necessary step. It should be noted that previously reported submerged sporulation was observed in media that included 0.1 M phosphate buffer (Martinelli, 1976).

Concluding remarks

The results presented in this paper show that the *brlA* gene and sporulation can be induced under defined liquid culture conditions by different environmental stimuli. This could be an important tool to study the signals that initiate development in *A. nidulans*. Further studies are needed to elucidate the molecular mechanisms by which nutrient starvation induces *brlA* and how this is affected by external pH. Particularly relevant to this understanding would be the study of starvation effects on genes known to affect *brlA* expression, such as *stuA* and *medA* (Clutterbuck, 1969; Miller, 1993; Aguirre, 1993), as well as the roles that the carbon and nitrogen catabolite regulator genes *creA* and *areA* (Arst & Bailey, 1977; Kudla *et al.*, 1990) could have in this process. Developmental competence (Axelrod *et al.*, 1973) is necessary for air-interface-induced conidiation. Thus, it is relevant to address the question of whether or not competence is required for nutrient stress induced sporulation, and we are currently working on this.

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