Carbon Source Dependence and Photostimulation of Conidiation in *Hypocrea atroviridis*^{∇†}

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Hypocrea atroviridis is frequently used as a photomorphogenetic model due to its ability to conidiate upon exposure to light. Light is thereby believed to be the primary trigger for spore formation. In contrast, we show here that conidiation is primarily carbon source dependent and that illumination plays a catalytic role; of a total of 95 tested carbon sources, only a small set of carbohydrates, polyols, and sugar acids allowed conidiation in darkness, and on most of them, conidiation was significantly more strongly expressed in light. In addition, there are also a number of carbon sources on which *H. atroviridis* conidiates in darkness, but light does not further stimulate the process. Yet on another small set of carbon sources (L-sorbitol, D-fucose, D- and L-arabinose, and erythritol), *H. atroviridis* shows better sporulation in darkness than in light. No sporulation was observed on organic acids and amino acids. Mutants with deletions in the two blue-light receptor proteins BLR-1 and BLR-2 generally showed weaker conidiation on a smaller number of carbon sources than did the parental strain, yet they clearly sporulated on 15 and 27 of the 95 carbon sources tested, respectively. Of the carbon sources supporting sporulation, only 11 supported the conidiation of both mutants, suggesting that the BLR-1 and BLR-2 receptors are variously involved in the carbon source-dependent regulation of spore formation. The addition of cyclic AMP, which has been reported to lead to conidiation in darkness, both positively and negatively affected sporulation and resulted in different effects in the parental strain and the two Δblr mutants. Our data show that the carbon source is the prime determinant for conidiation and that it influences the organism's regulation of conidiation by means of BLR-1 and BLR-2 and their cross talk with cyclic AMP.

Hypocrea atroviridis (anamorph *Trichoderma atroviride*, which was previously and is still elsewhere frequently misnamed *Trichoderma viride*) is able to conidiate upon exposure to light and can thus be used as a photomorphogenetic model organism (2). In complete darkness, *H. atroviridis* has been reported to grow infinitely as a mycelium but, upon nutrient deprivation, initiates the development of specialized asexual reproductive structures, conidiophores, and conidia (18). Similarly, a pulse of blue light (450 to 495 nm) induces sporulation (14).

In *Neurospora crassa*, the response to blue light is controlled by the zinc finger transcription factors white collar 1 and white collar 2 (WC-1 and WC-2, respectively) (19, 21). WC-1 and WC-2 interact through PAS domains to form the functional white collar complex (WCC) that binds to the promoters of photoregulated genes to rapidly activate transcription in response to light (29). The corresponding orthologues in *H. atroviridis*, blue-light regulators 1 and 2 (BLR-1 and BLR-2), have recently been shown to fulfill a similar function (5). Yet the mechanisms by which the WCC transduces the signal of light to initiate sporulation in *H. atroviridis* are not clear yet; following a pulse of blue light and changes in the plasma

membrane potential and in the concentration of intracellular ATP, the activation of adenylate cyclase (20) and a transient biphasic oscillation in intracellular cyclic AMP (cAMP) levels are the first detectable events (12). Several lines of evidence suggest that this rise in the concentration of cAMP is important for photoinduced sporulation. The addition of exogenous cAMP promotes sporulation in darkness (1, 22), whereas atropine, a compound known to reduce cAMP levels in fungal cells, prevents sporulation even after photoinduction (22). The expression of an antisense version of the *pkrl* gene, encoding the regulatory subunit of protein kinase A (PKA), resulted in a nonsporulating phenotype, and the overexpression of this gene produced colonies that conidiated even in darkness, whereas Casas-Flores et al. showed that overexpression of *pkrl* blocked the induction of early light response genes (6). Interestingly, Casas-Flores et al. also demonstrated that the WCC is necessary for conidiation even when conidiation is induced by carbon starvation instead of illumination but that blue-light-dependent activation of PKA was found to be independent of the WCC. These data suggest cross talk between the WCC and cAMP signaling and between light and carbon sensing.

Interestingly, in all of these studies, little attention has been paid to the possible regulatory effect of carbon metabolism, which can, however, be expected from the fact that carbon catabolite repression is known to influence sporulation in bacteria and fungi (9, 26). Chovanec et al. (7) previously reported that photoconidiation of *H. atroviridis* is differently influenced by a small number of carbon sources. The objectives of the

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present study were to examine these findings on a broader scale and to investigate the roles of the blue-light receptor proteins BLR-1 and BLR-2 and signaling by cAMP in this process.

MATERIALS AND METHODS

Strains. *H. atroviridis* strain IMI 206040 (wild type; initially deposited as *Trichoderma harzianum* IMI 206040 but reidentified by means of sequence analysis of internal transcribed spacers 1 and 2 of the rRNA gene cluster, NCBI GenBank accession no. AF278795) and the two $\Delta blr-1$ and $\Delta blr-2$ mutants derived from it (5) were used in this study. All cultures were maintained on plates containing 3% potato dextrose agar (PDA) and subcultured monthly.

PM analysis. The Biolog Phenotype MicroArray (PM) technique (Biolog Inc., Hayward, CA), as optimized for *Trichoderma* previously (8), was used to investigate the effect of constant darkness and light on carbon assimilation patterns. To prepare the inoculum, the strains were precultivated on 3% PDA plates for 5 days. Conidiation of the *H. atroviridis* $\Delta blr-1$ and $\Delta blr-2$ mutant strains was stimulated by mechanical injury of the mycelium on PDA with a sterile scalpel (5). Inocula were prepared by rolling a sterile, wetted cotton swab over sporulating areas of the plates. The conidia were then suspended in sterile Biolog FF inoculating fluid (0.25% Phytigel, 0.03% Tween 40), gently mixed, and adjusted to a transmission of 70% at 590 nm. Ninety microliters of this conidial suspension was then dispensed into each of the wells of the PM plates, and the plates were incubated either with alternating illumination (12 h of 1,800-lx white light) and darkness or with constant darkness at 28°C. The optical density at 750 nm (OD₇₅₀) (mycelial growth) and OD₄₉₀ (mitochondrial activity) were measured after 12, 18, 24, 36, 42, 48, 60, 66, 72, 96, and 168 h using a microplate reader (Biolog Inc., Hayward, CA). In order to avoid contact of the plates incubated in darkness with light, each data point was assayed with a single plate. Each assay was repeated three to six times in a series of independent experiments. Due to a generally decreased growth rate of *blr* mutants, the OD₇₅₀ at 66 h was chosen as a reference time point for the growth assays, since it allowed a comparison of biomass formation on all carbon sources when the majority of growth curves were in the linear phase.

To study the effects of the addition of dibutyl-cAMP, stock solutions in sterile, distilled water were prepared separately and added to the corresponding inoculation fluids at a concentration of 1 mM.

To quantify conidiation, we developed a nominative scale which differentiates between the complete absence of conidiophores and conidia (0), the formation of immature pustules without mature conidia (1), and four levels of conidiation intensity (2 to 5) from weak diffuse sporulation (2) up to the development of a thick conidial mat covering the whole well (5). The levels were distinguished by visual inspection of the plate images at a high resolution and cross-checked by plate examination under a light microscope. An example is given in the inset of Fig. 1.

Statistical data evaluations. Data from all experiments were combined into a single matrix and analyzed with the STATISTICA 6.1 software package (StatSoft, Inc., Tulsa, OK). All data were subjected to descriptive statistical evaluations (mean, minimum, maximum, and standard deviation values) and checked for outliers.

Cluster analysis (16, 31) was used to detect groups in the data set. This method was used to simultaneously group both carbon sources and strains/conditions in a two-way joining analysis according to their sporulation profile. In most cases, the cluster-joining analysis was made with Euclidian distance and complete linkage as the amalgamation rule; i.e., distances between clusters were determined by the greatest distance between any two objects in the different clusters. We used a discrete counter plot, which is a graphical representation of two-way

joining results, to obtain a carbon source-specific sporulation map based on the OD₇₅₀ at 168 h. In the merged sporulation map, each data point is represented as a color-coded rectangular region. The carbon source order remained intact, but the positions of strains/conditions on the combined map were manually modified according to the logic of the experiment.

When needed, one-way or main-effect analyses of variance were used to compare the levels of growth of selected strains on individual carbon sources. Tukey's honestly significant difference test as implemented in STATISTICA 6.1 was used for post hoc comparisons to detect the contribution of each variable to the main effect of the *F* test resulting from the analyses of variance.

RESULTS

Carbon source-specific conidiation of *H. atroviridis* in darkness. The species-specific carbon source utilization of *H. atroviridis* based on its Biolog PM profile has been characterized by Seidl et al. (with strain ATCC 74058 or P1 [25]) and M. A. Friedl et al. (with strain IMI 206040 [submitted for publication]). In these two studies, all carbon sources were divided by joining cluster analysis into four standard groups based on the corresponding dynamics of the resulting growth curves. Clusters I, II, and III thereby contained carbon sources which allow fast, moderate, and slow growth, respectively, and cluster IV contained carbon sources on which *H. atroviridis* grows very poorly or does not grow at all. In order to check whether the specific setup of the Biolog system would influence any of these results, selected carbon sources from each group were also tested on agar plates and on standing liquid cultures. Consistent data were obtained (data not shown), thus ruling out any specific interference.

Using the nominative six-step scale developed in this study to evaluate conidiation intensity on microplates, we first tested the effect of carbon sources on spore production in darkness. After 168 h of incubation under these conditions, conidiation was noted on only 48 out of 95 tested carbon sources (the first track in Fig. 1). These included almost all of the carbohydrates, polyols, and sugar acids but, with the exception of *N*-acetyl- β -D-glucosamine, not the other sugar amines. Conversely, it did not occur on any amino acids, aliphatic acids, or alcohols, with the exception of β -hydroxybutyric acid. In order to learn whether the growth rates achieved on these carbon sources were determinative for conidiation, we compared the relative abundances of conidiation with the growth rates on these carbon sources (Fig. 2). From this, it is evident that the growth rate itself does not correlate with conidiation. Among the 48 carbon sources which supported conidiation in darkness, 9 supported fast growth, 10 supported moderate growth, and 15 supported only slow growth (clusters I, II, and III, respectively). The remaining 14 carbon sources which supported conidial development provided very poor mycelial growth of

FIG. 1. Summed map of conidiation of *H. atroviridis* wild-type strain IMI 206040 and two *blr* deletion mutants after 168 h of incubation on 95 carbon sources and water under conditions of darkness and light or darkness with elevated levels of cAMP. The map was composed after two-way joining cluster analysis applied to (i) the carbon sources and (ii) the fungal strains cultivated under different conditions as two groups of variables. The nine-pointed stars, five-pointed stars, and pentagons correspond to carbon sources providing fast (cluster I), good (cluster II), and decreased (cluster III) mycelial growth of all three strains in darkness as estimated after 48 h of incubation. The respective intensities of sporulation (thumbnail images in the inset) are given by a corresponding color as indicated in the color scale. Examples of conidiation grades are estimated according to the nominative scale, where the complete absence of conidiophores and conidia corresponds to 0, the formation of immature pustules without mature conidia corresponds to 1, and true conidiation is measured by one of four levels of intensity, from weak diffuse sporulation (2) up to the development of a thick conidial mat covering the whole well (5). "light" corresponds to an alternating 12-h-light/12-h-darkness cycle; "cAMP" indicates 1 mM of exogenous cAMP.

FIG. 2. Relation between conidiation of *H. atroviridis* wild-type strain IMI 206040 and its biomass density at 72 h of incubation on 95 carbon sources and water. Shaded circles indicate mycelial growth rates in complete darkness, and open squares correspond to growth rates in alternating 12-h-light/12-h-darkness cycles. The conidiation grades are indicated in Fig. 1. The biomass density (OD_{750}) for 72 h is given (instead of a growth rate), because at this time all cultures are still in the linear phase of growth and the respective values directly correlate with the growth rates (25).

the fungus (cluster IV). Therefore, conidiation in darkness is strongly carbon source dependent.

Photostimulation of conidiation is also carbon source dependent. In order to investigate whether the well-known photostimulation of conidiation would overrule the above-noted carbon source regulation of sporulation or be confined to those 48 carbon sources where conidiation was already present in darkness, we subjected the microplates to illumination. The data are shown in Fig. 1 (second track). Those carbon sources on which *H. atroviridis* did not conidiate in darkness also did not enable conidiation under illumination. The presence of light enabled sporulation only on those carbon sources which also empowered conidiation in darkness by increasing the intensity and speed of conidial development such that the appearance of conidiophores and conidia were detected earlier during cultivation (data not shown) and more intensely at 168 h. However, a couple of exceptions were also noted: in contrast to the situation in darkness, conidiation under illumination was not observed on D-arabinose or D-gluconic acid.

The regulatory role of the blue-light receptor proteins BLR-1 and BLR-2 in conidiation is carbon source dependent. Light-induced stimulation of conidiation in *H. atroviridis* has been demonstrated to be dependent on the function of the blue-light receptor proteins BLR-1 and BLR-2 (5). In order to test whether their function would also be carbon source dependent, we made use of the $\Delta blr-1$ and $\Delta blr-2$ mutant strains. As shown in Fig. 1, conidiation in the dark is indeed significantly impaired in the two mutants on most carbon sources. On average, both mutants produced only diffuse pustules or single conidiophores and rarely conidiated to grade 3 or 4 of our scale. However, strong conidiation clearly occurred on sorbitol with the $\Delta blr-1$ strain and on glucuronic acid with the $\Delta blr-2$

strain. In addition, the $\Delta blr-1$ and $\Delta blr-2$ strains were able to moderately produce spores on 15 and 27 carbon sources, respectively. Therefore, the carbon sources supporting the low level of conidiation in the two mutants were nearly always from the pool of carbon sources that favor the conidiation of the parent strain. A notable exception, however, was α -methyl-D-glucoside, on which no spores were detected in the parent strain IMI 206040; however, both mutants formed mature conidia. A comparison of the two mutants showed that only 11 carbon sources supported conidiation in both mutants, suggesting that the BLR-1 and BLR-2 receptors were variously involved in the carbon source-dependent regulation of spore formation.

The addition of cAMP alters the carbon source dependence of conidiation. Casas-Flores et al. (5) have described that the addition of cAMP induces conidiation in *H. atroviridis* in darkness. Our study confirmed this finding but also showed cAMP to be carbon source dependent. The addition of cAMP induced conidiation in the parent and both *blr* mutant strains on some but not all carbon sources (see the Biolog data in the supplemental material). The most notable effects were (i) the complete reduction of conidiation on D-xylose (cluster I) and on L-rhamnose and β -hydroxybutyric acid (both from cluster IV) and (ii) the stimulation of sporulation on D-saccharic acid, on which IMI 206040 did not sporulate without cAMP addition (Fig. 1).

The addition of cAMP to the cultivation medium of both Δblr mutants also essentially changed the carbon source dependence of sporulation, and these changes were different from those seen with the parent strain IMI 206040. Thus, while the addition of cAMP reduced the conidiation of the last strain on D-xylose, the $\Delta blr-1$ mutant formed mature conidia on this

compound and the $\Delta blr-2$ mutant did not sporulate on D-xylose either with or without the addition of cAMP.

DISCUSSION

Conidiation serves a dual function in fungi. It produces reproductive structures for dispersal and allows survival under the adverse conditions of environmental stress. Obviously, these two functions overlap because dispersal from a given habitat is mostly associated with one or another type of stress (UV light, starvation, altered humidity, and other stresses). Consequently, it would not be surprising for conidiation to be triggered by a variety of such conditions, of which, so far, only macronutrient limitation and light have received major attention. The results of this study add to this list the type of carbon source as a dominant parameter. Previous studies investigated photoconidiation on common fungal solid media, which contain mostly glucose, sucrose, or maltose as the carbon source. Our data on these carbon sources essentially confirm the results of others, i.e., the stimulation of conidiation by light (2). However, contrary results were obtained with other carbon sources (D-fucose, erythritol, L-arabinose, and D-arabinose), and light was essentially without effect on, e.g., rhamnose, glucosamine, and xylitol. Also, strong sporulation was obtained on a number of carbon sources in the absence of light. It is important to note in this context that we have observed conidiation under every condition tested (darkness, light, the addition of cAMP, the loss of function of BLR-1/BLR-2) only on carbohydrates and their derivatives (polyols and sugar acids) and, in contrast to Chovanec et al. (7), never on amino acids, aliphatic acids, or aliphatic alcohols. A plausible explanation for these findings would be that different carbon sources enable different growth rates and that conidiation would therefore be observed with those sources on which the fungus has, at the time of analysis, already reached the point of starvation. Alternatively, the fungus may grow on some carbon sources at a very low rate for maintenance metabolism and therefore may start to sporulate. If this were the case, however, we should have seen a correlation between conidiation and either high or low growth rates. However, although a weak relationship between growth rate and conidiation was seen, it was not sufficient to use this relationship as the entire explanation, thus indicating that it is the nature of the carbon source that determines whether or not the conidiation of the fungus can be triggered by light, thus making it a parameter of prime importance. Unfortunately, the lack of knowledge about the biochemistry and regulation of catabolism of many of the carbon sources employed in this study prevents a more detailed discussion of the individual results obtained. Yet we would nevertheless like to emphasize that it is well known that light provokes severe changes in carbon metabolism, such as protein phosphorylation and low glucose uptake in *Aspergillus orneatus* (17) and the inhibition of glucose consumption in *Physarum polycephalum* (24). Recent work, for which global expression analysis was used for *H. atroviridis* (23) and *Hypocrea jecorina* (24a), revealed that the expression of genes involved in the transport, signaling, and catabolism of monosaccharides and related components, and in mitochondrial energy production, is stimulated by light. We therefore speculate that light stimulates the expression of genes involved in some metabolic

networks but that it does not affect (or even inhibit [24a]) that of others. The different responses to light on different carbon sources would therefore be due to the relative levels of dependence of their catabolism on these light-affected metabolic networks.

A similar explanation may be used to explain the different effects of the addition of cAMP. Data available in the literature suggest that cAMP is involved in blue-light-induced conidiation in *Trichoderma* (12, 13, 20), and the addition of exogenous dibutyryl-cAMP to *Trichoderma* cultures growing on rich medium even triggers conidiation in darkness (1, 6). Again, we confirmed these data with the major carbon sources used in the literature but obtained divergent and even contrary results with others. This could be related to the fact that, unlike what is expected from stimulation by cAMP, conidiation in *H. atroviridis* and other fungi is regulated by a pathway which is negatively controlled by PKA (4, 6). Casas-Flores et al. (6) suggested that *H. atroviridis* uses separate signaling pathways for stimulation by external and intracellular cAMP, i.e., the classical G protein/PKA pathway for the former and an alternate pathway that uses a membrane receptor for exogenous cAMP like the one which has been reported for *N. crassa* (3). We could confirm the presence of such a receptor gene in the *H. atroviridis* genome sequence (C. P. Kubicek, unpublished data). Thus, if this receptor is not expressed on one or more of these carbon sources, the fungus will not be able to respond to the stimulus. As for the involvement of the G protein/PKA pathway, it is clear that this pathway leads to strongly different internal cAMP pools, depending on whether or not the respective carbon source involves a G protein-coupled receptor or transporter.

While the $\Delta blr-1$ and $\Delta blr-2$ mutant strains exhibited strongly decreased conidiation, our results contribute two novel findings: first, the mutations do not completely block sporulation but only strongly decrease it and, second, they still show (light-insensitive) sporulation on some carbon sources (notably glucuronic acid and 2-ketogluconic acid). Casas-Flores et al. (6) hypothesized that the BLR proteins are not only involved in photosensing but may also perceive or transduce the signal originating from the lack of glucose (e.g., by redox and oxygen sensing via their PAS domains). This explanation may be further adapted to explain the results of the present study, thereby using recent findings on the mechanism of apoptosis for comparison. Sporulation and apoptosis share a number of common physiological events, such as mycelium vacuolization and protein degradation (10, 10a, 11). Thrane et al. (30) recently emphasized the role of a caspase-like activity, a central marker for apoptosis (32), in *Aspergillus nidulans* during sporulation. Apoptosis is influenced by the cellular redox potential, i.e., the ratio of oxidized to reduced glutathione (15), which coincides with the necessity of NADPH (27) and glutathione (28) for conidiation. Although we are not aware of any work comparing the glutathione and NADPH pools and their turnover in fungi on different carbon sources, their stoichiometric involvements in the catabolism of, e.g., hexoses and pentoses are different. It can therefore be expected that the intracellular pools of glutathione and of NADPH/NADP and NADH/NAD will also differ. We therefore extend the model of Casas-Flores et al. (6), i.e., that the BLR proteins act as redox and oxygen sensors, by hypothesizing that the observed differences in levels of

conidiation on different carbon sources are due to different redox potentials in the cell during growth on them.

Apart from these theoretical considerations, our findings may also offer some practical hints. First, strains of *H. atroviridis* are used as biocontrol agents, and for this purpose, a formulation of conidia is used. Our study may help to design optimal conditions for the conidiation of these strains. Second, many fungal mutant strains have no conidiating phenotype or only poorly conidiate. Our data suggest that this phenotype may not be universal, but a careful investigation may identify carbon sources on which the respective strain can still form conidia.

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