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CHARACTERIZATION OF *HDC1* AND *HDC2*, TWO HISTONE DEACETYLASES FROM *COCHLIOBOLUS CARBONUM*, A FUNGAL PATHOGEN OF MAIZE

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CHARACTERIZATION OF *HDC1* AND *HDC2*, TWO HISTONE DEACETYLASES FROM *COCHLIOBOLUS CARBONUM*, A FUNGAL PATHOGEN OF MAIZE

By

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ABSTRACT

CHARACTERIZATION OF *HDC1* AND *HDC2*, TWO HISTONE DEACETYLASES FROM *COCHLIOBOLUS CARBONUM*, A FUNGAL PATHOGEN OF MAIZE

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Histone deacetylases (HDAC) are enzymes, predominantly nuclear, known to help regulate transcription in eukaryotes. In order to understand the function of HDACs in the filamentous fungus *Cochliobolus carbonum*, a genetic approach was undertaken. Targeted gene disruptions of *HDC1* and *HDC2*, via homologous recombination, were attempted in *C. carbonum*. Whereas *HDC1* was mutated successfully, *HDC2* was not despite various attempts. These results suggest that *HDC2* is either essential for survival in *C. carbonum* or the chromatin structure surrounding *HDC2* is refractory to recombination. Disruption of *HDC1* revealed a novel phenotype in *C. carbonum*. The *hdc1* mutant of *C. carbonum* displayed delayed growth on polysaccharides and complex carbohydrates. The *hdc1* conidia were smaller and less septate than the wild type. On maize plants, the *hdc1* mutant caused only small lesions and was unable to develop full disease symptoms as the wild type, which ultimately kills the plant. Overexpression of *HDC2* in *C. carbonum* had no apparent phenotype. There was no difference in virulence levels between the *HDC2* over-expressor and the wild type. Biochemical analysis of the *HDC2* over-expressor provided evidence that the *HDC2* product contains HDAC activity in *C. carbonum*.

I would very much like to dedicate this work, however insignificant, to the native inhabitants of my beautiful homeland, Colombia. To the Colombian indians, those forgotten by time and put into oblivion by an invading culture that never knew how to appreciate who they are and, because of ignorance, has tried at all costs to obliterate them. To the Pijaos, the Guambianos, the Chibchas, the Huitotos, and many, many more who have been there since the inception of time and cared for the land they inhabited with respect and love. This is nothing but a small token of appreciation, nothing but a small way to apologize for taking their rights away and exploiting them, as the white man did and still does. For trying to deprive their people from living off the land that once belonged only to them, although I believe there is no way we can be forgiven. Because you were the one and only rightful owners and landlords. For the profound admiration that I feel towards these natives of Colombia, and all the respect that you deserve, today and always. To you and for you.

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LITERATURE REVIEW

Among all plant diseases, the ones caused by fungal pathogens are the most devastating type. Fungi can be defined as small, generally microscopic spore-bearing organisms that lack chlorophyll and have cell walls that contain chitin, cellulose, or both. Most of the 100,000 fungal species known are strictly saprophytic, living on dead organic matter, which they help decompose. About 50 species cause diseases in humans, and about as many cause diseases in animals, most of them superficial diseases of the skin or its appendages. More than 8,000 species of fungi, however, can cause diseases in plants. All of the species of flowering plants (over 300,000) are attacked by pathogenic fungi. However, a single plant species can be host to only a few fungal species, and similarly, most fungi usually have a limited host range. Throughout history, the vast majority of important crop diseases have been caused by phytopathogenic fungi. Significant annual crop yield losses have made fungal pathogens of plants a serious economic factor. For instance, 15% ($33 billion) of the total rice production between 1988 and 1990 were lost to fungal diseases, 12.4% ($14 billion) in the case of wheat, and 10.9% ($7.8 billion) in the case of maize (Oerke et al., 1994).

When a fungal spore encounters a plant, it must be able to either penetrate the host tissue or tap an external source of the host's nutrients if it is to survive. Penetration, the method employed by most phytopathogenic fungi, can occur either enzymatically or mechanically. Some fungi secrete a large variety of enzymes that can break down the plant cell wall, including cutinases, cellulases, pectinases, and proteases. Cell wall degrading enzymes (CWDE) probably did not evolve particularly as pathogenicity

factors. All fungal species that live in a saprophytic fashion can also secrete enzymes necessary for the digestion of plant cell wall polymers even though they are not pathogenic. It is believed that CWDEs contribute to the virulence of the fungal pathogen but are not essential pathogenicity factors. Removal of a regulatory factor that controls CWDE expression in the fungus *Cochliobolus carbonum* shut off their transcription and the mutant displayed a significant reduction in virulence (Tonukari et al., 2000). Other fungi exert tremendous amounts of pressure and penetrate by sheer mechanical force. *Magnaporthe grisea*, the causal agent of rice blast disease, requires formation of an appressorium for plant infection. During penetration, *M. grisea* appressoria can generate as much as 8.0MPa of turgor pressure. This is the result of synthesis of large quantities of glycerol, a compatible solute, in the appressorium (deJong et al., 1997). Turgor is translated into mechanical force and this forces a thin penetration hypha through the plant cuticle. Other fungal species, including some rusts, have not evolved a direct penetration mechanism and instead bypass the plant cuticle and outer cell wall by entering through stomates. Stomates are the small openings on the leaf epidermis important for gas exchange in the plant. The bean rust *Uromyces appendiculatus*, for instance, uses a thigmo-responsive mechanism that allows it to find its way into the stomates (Correa and Hoch, 1995).

After penetration, many fungi secrete toxins or plant hormone-like compounds that manipulate the plant's physiology to the pathogen's benefit. The end result may simply be host cell death for the purpose of nutrient uptake, or a more subtle redirecting of the cellular machinery via the production of phytotoxins with varying degrees of specificity toward different plants. Some toxins are host selective, whereas others are

active in a wide range of plant species. Host-selective toxins (HST) can be generally described as low molecular weight compounds with diverse structures that act as positive agents of virulence or pathogenicity (Walton, 1996). HSTs can help determine host range or specificity in that plant species, such that genotypes sensitive to an HST are found to be susceptible to the producing pathogen. All HSTs known are produced by fungi and most of them can be classified as secondary metabolites. They are low molecular weight compounds of diverse structure that are restricted in their taxonomic distribution and are not necessary for normal survival and reproduction. HSTs are active at concentrations ranging from 10 pM to 1μM, and their degree of specificity (host selectivity) ranges from 100-fold to $>10^6$ fold (Walton et al., 1985). Despite the diverse array of secondary metabolites produced by fungi, they are all thought to be synthesized from a limited number of primary metabolites modified in unique ways. The major biosynthetic pathways for secondary metabolites include the isoprenoid pathway, the polyketide pathway, the shikimate pathway, and the use of amino acids as precursors (Bentley, 1999). In addition, some compounds are derived from carbohydrates, intermediates of the tricarboxylic acid cycle, and combinations of multiple pathways.

The Model System

The Walton laboratory is studying the interaction between the filamentous fungus *Cochliobolus carbonum* and the maize plant (*Zea mays* L.), causing the disease commonly known as northern leaf spot and ear mold. On a sensitive corn variety (Pr), race 1 of *C. carbonum* will give rise to well-defined, zonate, rapidly spreading lesions on the foliage and pronounced black mycelium on the kernels of infected ears. Race 2, the

other race of *C. carbonum*, is much less virulent when compared to race 1, unable to colonize much beyond the site of penetration, and causing only mild chlorotic-necrotic flecks on the leaves. This fungus was originally called *Helminthosporium carbonum* until 1959, when its sexual stage was discovered. It was then determined that this pathogen was an ascomycete belonging to the genus *Cochliobolus* (Nelson, 1959). Genetic crosses between race 1 and race 2 isolates of *C. carbonum* revealed that virulence is determined by a single genetic locus, *TOX2,* which also confers the ability to produce the secondary metabolite (HC)-toxin, where HC stands for *Helminthosporium carbonum.* HC-toxin was proven to be a bona-fide HST as it can inhibit root growth in susceptible maize but not in other related plant species (Scheffer and Ullstrup, 1965). HC-toxin is a cyclic tetrapeptide with the structure cyclo (D-Pro-L-Ala-D-Ala-L-Aeo), where Aeo stands for 2-amino-9,10-epoxi-8-oxodecanoic acid (Walton et al., 1982).

Molecular analysis of the *TOX2* locus led to the discovery of some of the genes required by race 1 isolates of *C. carbonum* to produce HC-toxin. The genes of the *TOX2* locus are loosely clustered within ~540 Kb of DNA and are unique to race 1 isolates (Ahn and Walton, 1996); (Ahn et al., 2002). This large and complex locus contains multiple copies of all the genes that appear to be needed for HC-toxin biosynthesis. The cyclic peptide synthetase, encoded by *HTS1*, is a non-ribosomal peptide synthetase required for the synthesis of HC-toxin. The open reading frame is 15.7 Kb in size, there are two copies in race 1 isolates of *C. carbonum*, and the enzyme encoded has a molecular weight of 570 KD (Scott-Craig et al., 1992). Located upstream of *HTS1* there is another gene, *TOXA*, which is also present only in HC-toxin producing isolates. The predicted product of *TOXA* exhibits a high degree of similarity to members of the major

facilitator superfamily (MFS), which encode membrane-localized antibiotic efflux pumps (Pitkin et al., 1996). Three other genes, *TOXC*, *TOXF*, and *TOXG*, are also unique to race 1 isolates and have been shown to be essential for HC-toxin biosynthesis. The predicted product of *TOXC* is highly similar to the β-subunit of fatty acid synthases from several lower eukaryotes, and contains domains predicted to encode acetyl transferase, enoyl reductase, dehydratase, and malonyl-palmityl transferase (Ahn and Walton, 1997). *TOXF* is predicted to encode a protein with moderate homology to many known or putative branched-chain-amino-acid transaminases (Cheng et al., 1999). Genetic analyses demonstrated that both *TOXC* and *TOXF* are required for HC-toxin production and *C. carbonum* virulence. Another gene, *TOXG*, was found to encode an alanine racemase, based on sequence comparisons and biochemical evidence (Cheng and Walton, 2000). Also within the *TOX2* locus are *TOXD* and *TOXE*. Targeted disruption of *TOXD* did not unveil any change in either HC-toxin synthesis or pathogenicity (Y.Q. Cheng and J.D. Walton, unpublished results). Interestingly, *TOXE* seems to be the regulatory factor for at least part of the *TOX2* locus. Deletion of *TOXE* resulted in loss of HC-toxin production and reduced virulence. In addition, transcripts of *TOXA*, *TOXC*, *TOXD*, *TOXF*, and *TOXG* are down-regulated in the *TOXE* mutant (Ahn and Walton, 1998). *TOXE* has four ankyrin repeats and a basic region similar to those found in basic leucine zipper (bZIP) proteins, but lacks any apparent leucine zipper. It was demonstrated that *TOXE* is a DNA-binding protein that recognizes a ten-base motif (the "tox-box") without dyad symmetry that is present in the promoters of all of the known genes present in the *TOX2* locus (Pedley and Walton, 2001).

In maize, a single dominant gene, *HM1*, governs resistance and confers complete protection at all stages of growth against *C. carbonum* (Nelson and Ullstrup, 1964). Using a cell-free extract from a resistant maize genotype (*Hm1/hm1*), it was shown that HC-toxin could be inactivated via a reduction of the 8-carbonyl group at the Aeo group by means of an enzymatic activity that was NADPH dependent (Meeley and Walton, 1991). Subsequently, this HC-toxin reductase activity (HCTR) was shown to be present in all maize extracts from resistant genotypes (*Hm1/hm1, Hm1/Hm1*) tested, but absent in susceptible ones (*hm1/hm1*) (Meeley et al., 1992). The *HM1* gene was cloned and proven to be similar to known NADPH-dependent reductases (Johal and Briggs, 1992). Furthermore, the cloning of *HM1* revealed that HCTR activity alone is sufficient to prevent severe infection by *C. carbonum* race 1 (Meeley et al., 1992). HCTR activity is detectable in extracts of several other grasses (e.g., barley, oats, and wheat) and hence may represent an ancient resistance strategy within the *Poaceae* against HC-toxin and similar compounds. The strong DNA sequence similarities between various HCTRs supports the idea that the function may be the same among different plant species (Han et al., 1997).

The biological role of HC-toxin in *C. carbonum* that allows the fungus to colonize maize is not yet known. HC-toxin is unique because when compared with other phytotoxins, it appears to be cytostatic rather than cytotoxic (Wolf and Earle, 1991). Instead of killing the host cells ahead of the growing hyphae, HC-toxin is thought to suppress the active defense responses that will typically be mounted by the host against pathogen attack (Cantone and Dunkle, 1990). A bioassay for testing HC-toxin activity consists of root growth inhibition in germinating maize seedlings. However, unlike most

other phytotoxins, HC-toxin only weakly promotes ion leakage (Yoder, 1980). HC-toxin is soluble in chloroform and water but not in solvents of intermediate polarity such as ether. The biological significance of this is that HC-toxin should be able to move readily through both the hydrophobic and hydrophilic domains of plant and fungal tissues. HC-toxin is also active against mammalian cells (Walton et al., 1997).

The first major advance in understanding the mode of action of HC-toxin came from studies on the mode of action of trapoxin, an Aeo-containing fungal secondary metabolite (Itazaki et al., 1990). Trapoxin was shown to induce morphological reversion from transformed to normal in *sis*-transformed NIH-3T3 fibroblasts (Kijima et al., 1993). In addition, trapoxin was found to cause accumulation of highly acetylated histones by binding irreversibly to histone deacetylases (HDAC), a family of enzymes primarily localized to the nucleus. Based on this knowledge, Schreiber and colleagues synthesized *in vitro* a trapoxin affinity matrix and used it to isolate and later clone the gene for the first mammalian HDAC, a human ortholog of the yeast transcriptional regulator *RPD3*. This gene, initially called *HD1*, is now called *HDAC1*, and its product has intrinsic HDAC activity (Taunton et al., 1996).

Histone Deacetylases

In all eukaryotes, the nuclear genetic material is arranged in a highly complex structure made up of histones and DNA called chromatin. The basic unit of structure in chromatin is called the nucleosome. Each nucleosome contains 147 base pairs of DNA and a histone octamer. The histone octamer is composed of a dimer of each of the core histone proteins: H3, H4, H2A, and H2B (Kornberg and Lorch, 1999). Histones are small

basic proteins consisting of a globular domain and a more flexible and charged amino terminus (histone "tail") that protrudes from the nucleosome. The function of HDACs is to remove the acetyl moieties from the ε-amino group of specific lysine residues present at histone tails previously acetylated. The acetylation of histone tails is enzymatically mediated by a family of enzymes known as histone acetyl-transferases (HAT). HATs take Acetyl-CoA as substrate and attach the acetyl moiety to the ε-amino group of lysine residues. This type of reaction has been estimated to have a large negative ΔG value (-7.5 kcal/mol), which makes it thermodynamically favorable and able to occur spontaneously (Stryer, 1995). Because HDACs are part of nuclear complexes known to repress gene transcription, they are called "co-repressors." HATs, on the other hand, are called "co-activators" as they have been implicated in activation of gene expression.

Because HC-toxin, like trapoxin, contains an Aeo tail, it was inferred that HC-toxin could be also an HDAC inhibitor. Walton and collaborators tested this hypothesis and found that HC-toxin can inhibit HDAC activity not only from maize but also chicken, the myxomycete *Physarum polycephalum*, and the yeast *Saccharomyces cerevisiae* (Brosch et al., 1995). In addition, they found that HC-toxin is an noncompetitive inhibitor and its binding is reversible. In a follow-up study, Ransom and Walton (1997) discovered that treatment *in vivo* of maize embryos and tissue cultures with HC-toxin leads to an accumulation of hyperacetylated forms of histones H3 and H4, but not H2A and H2B, in Pr, the sensitive genotype. Further, the fact that accumulation of hyperacetylated histones began 24 hours after inoculating maize leaves with *C. carbonum,* which is before development of visible disease symptoms, argues that

inhibition of HDACs by HC-toxin is necessary for *C. carbonum* pathogenesis (Ransom and Walton, 1997).

Histone Deacetylase Families

HDACs have been organized into three distinct classes. Class I includes mammalian *HDAC* 1, 2, 3, and 8, which are related to *RPD3* from *S. cerevisiae.* These HDACs have been found to be part of similar repressor complexes, have similar sensitivity levels to trichostatin A (TSA, an HDAC inhibitor), are similar in size, and share a well-conserved catalytic domain (Rundlett et al., 1996). Class II includes mammalian *HDAC* 4, 5, 6, and 7, which are related to *HDA1*, *HOS1*, *HOS2*, and *HOS3* from *S. cerevisiae*, respectively (Grozinger et al., 1999). Members of class II are much greater in size than members from class I and also exhibit much greater sensitivity to TSA (Khochbin et al., 2001). Class III, which is composed of HDACs related to *SIR2* from *S. cerevisiae*, is involved in heterochromatin silencing at silent mating loci, telomeres, and ribosomal DNA (Moazed, 2001a). Unlike the other two classes, SIR2 has *in vitro* NAD^+-dependent HDAC activity as well as ADP-ribosyltransferase activity (Imai et al., 2000). Heterochromatin can be defined as the densely staining regions of the nucleus that generally contain condensed, trancriptionally inactive regions of the genome. Euchromatin, on the other hand, contains the decondensed, transcriptionally active regions of the genome. A novel plant-specific class of HDACs has been discovered recently. *HD2*, a member of this new class, was isolated from maize embryos and immunologically localized to the nucleolus (Lusser et al., 1997). *HD2* is a nucleolar phosphoprotein that might regulate ribosomal chromatin structure and function. In

Arabidopsis thaliana, two genes with high similarity to *HD2* from maize were identified. It was shown that these *HD2*-like HDACs not only are expressed in various *Arabidopsis* organs but also appear to be important in its reproductive development (Wu et al., 2000).

Histone Deacetylase Function

The action of HDACs, in theory, causes a stronger attraction between the histone tails and DNA phosphate backbone via stronger ionic interactions. At promoter regions, this process could potentially tighten up chromatin structure so that genomic DNA becomes less accessible. Therefore, the function of HDACs could contribute to repression of gene expression (Ng and Bird, 2000). The first association between histone deacetylation and transcriptional repression came from genetic studies on nuclear repressor complexes. The yeast SIN3/RPD3 complex, for instance, has been studied in detail. The complex is about 600 KD in size. SIN3 contains several paired amphipathic helix (PAH) domains postulated to be involved in protein-protein interactions (Halleck et al., 1995). SIN3 is thought to act as a molecular scaffold for assembly of the other proteins involved in the complex (Kasten et al., 1997). Struhl and colleagues showed that localized histone deacetylation on a repressed yeast promoter depends on the recruitment of the SIN3/RPD3 complex by UME6, a DNA-binding transcriptional repressor (Kadosh and Struhl, 1997, 1998). More recent work has now shed light on the mechanism by which histone deacetylation may lead to repression of transcription. The evidence presented suggests that Rpd3-dependent repression is associated with decreased occupancy by TATA binding protein (TBP), the Swi/Snf nucleosome-remodeling complex, and the SAGA histone acetylase complex (Deckert and Struhl, 2002). The

authors concluded that the domain of localized histone deacetylation generated by recruitment of Rpd3 might mediate repression by inhibiting recruitment of chromatin-modifying activities and TBP.

Another well-characterized complex is the Ssn6/Tup1 repressor complex. TUP1 interacts directly with the amino-terminal tail domains of histones H3 and H4 *in vitro* (Edmondson et al., 1996). These tail domains are both necessary and sufficient for Tup1 binding. Moreover, the region of Tup1 that interacts with the histones closely coincides with a domain that can confer repression independently when fused to LexA (Tzamarias and Struhl, 1994), indicating that the function of Tup1 *in vivo* depends on its interactions with histones. Indeed, mutations in these histone domains synergistically reduce repression of multiple classes of Tup1-regulated genes *in vivo* (Huang et al., 1997). Tup1 binds poorly to highly acetylated forms of H3 and H4 *in vitro* but interacts very well with unacetylated isoforms. In addition, genes repressed by Tup1 in yeast are associated with unacetylated forms of histones H3 and H4 *in vivo* (Bone and Roth, 2001). Roth and coworkers demonstrated that histone hyperacetylation caused by combined mutations in the HDAC genes *RPD3*, *HOS1*, and *HOS2* abolishes Ssn6/Tup1 repression in yeast (Watson et al., 2000). Further, they showed that the Ssn6/Tup1 complex can interact with at least two different HDAC proteins, Rpd3 and Hos2. Tup1 was also found to interact with Hda1, which brings specific deacetylation to histones H3 and H2B *in vivo* (Wu et al., 2001), and this interaction is required for gene repression. Neither Ssn6 nor Tup1 can bind DNA directly. They are recruited to individual promoters through interactions with DNA-bound repressors, such as α2/Mcm1 for repression of *STE2* and *STE6*, two mating-specific genes (Komachi et al., 1995), and Mig1 and Sko1 for repression of *ENA1*, which

encodes a membrane ATPase involved in sodium efflux from the cytoplasm (Proft and Serrano, 1999).

Other Histone Modifications

In addition to acetylation, histones undergo a variety of post-translational modifications. Histones may be phosphorylated, methylated, ubiquitinated, and nucleosomes may be remodeled in an ATP-dependent fashion (Berger, 2001). Phosphorylation of histones is critical for regulation of several genes involved in the cell cycle control. Rsk2, for instance, is an H3 kinase that when mutated is associated with Coffin-Lowry syndrome in humans (Sassone-Corsi et al., 1999). Transcriptional activation in response to mitogenic and other stimuli are altered in Coffin-Lowry cells, indicating an important role for H3 phosphorylation in regulating gene transcription (De Cesare et al., 1998).

Histone methylation is regarded as a more long-term epigenetic mark than other histone modifications, which is consistent with the relatively low turnover of the methyl group (Jenuwein, 2001). This histone modification, which occurs on arginine and lysine residues, is very stable and can be maintained from one generation to the next. Bulk histone methylation steadily increases during the S and G2 phases of the cell cycle, consistent with a role in preparing chromatin for mitotic condensation (Byvoet et al., 1972). As a chemical modification, methylation is not significant in the sense that the overall charge of a lysine residue remains unchanged. However, methylation creates a binding site for heterochromatic proteins such as HP1 that contain a chromodomain (Lachner et al., 2001). HP1 is involved in propagation of heterochromatic subdomains

(Nakayama et al., 2001). All histone methyltransferases (HMT) contain the SET motif, a highly conserved domain 130 amino acids in length responsible for the catalysis of this reaction (Jenuwein, 2001). It has now become evident that specific methylation patterns are correlated with gene activity. H3-K9 methylation seems to be primarily associated with heterochromatin (Noma et al., 2001), whereas H3-K4 methylation (in higher eukaryotes) is observed in transcriptionally active regions (Strahl et al., 1999). DNA methylation has recently been shown to be dependent on histone methylation in some fungi. Methylation of DNA at CpG sites is also an epigenetic mark, which can be inherited through mitosis, and often through meiosis. In *Neurospora crassa*, it was shown that all DNA methylation is dependent on H3-K9 methylation, suggesting that methylation of H3-K9 occurs prior to DNA methylation (Tamaru and Selker, 2001).

Nucleosome remodeling complexes use energy from ATP to modify chromatin structure in a noncovalent manner. Their function is to increase accessibility of nucleosomal DNA, a fundamental requirement for several steps in transcription. Each remodeling complex contains a central ATPase subunit which can alter chromatin structure in the absence of the remaining subunits (Kingston and Narlikar, 1999). The role of the remaining subunits is thought to be targeting and modulation of the activity of the ATPase subunit. There are two main families of nucleosome remodeling complexes: Swi/Snf and Iswi. Swi/Snf can remodel nucleosomes in the absence of histone tails whereas Iswi can not (Langst and Becker, 2001). Swi/Snf complexes can be stimulated similarly by nucleosomes and naked DNA, while Nurf (an Iswi complex) is stimulated significantly better by nucleosomes than by naked DNA (Tsukiyama and Wu, 1995). Two main mechanisms have been proposed as to how chromatin remodeling may occur.

Sliding is the translational repositioning of histone octamers, so that the DNA that was originally interacting with histones becomes non-nucleosomal. However, this mechanism can not explain all scenarios of nucleosome displacement, such as the way in which substantial tracts of DNA can be made accessible in regions of tightly spaced nucleosomes. Hence, a second mechanism must exist, whereby the conformation of the histone octamer is changed without displacement, facilitating DNA exposure in regions of closely packed nucleosomes by bringing DNA to the surface of the histone octamer. In addition, it has been shown that RSC, an abundant chromatin remodeling complex, can transfer a histone octamer from a nucleosome core particle to naked DNA (Lorch et al., 1999).

Histone Code Hypothesis

Histones are integral and dynamic components of the machinery responsible for regulating gene transcription. The histone tails, which protrude from the surface of the chromatin polymer, are subject to a diverse array of covalent modifications. The combinations of these modifications are specifically recognized by individual transcriptional regulatory protein modules. These combinations act as a code or "language" that somehow must be deciphered or "read" by the corresponding protein module or modules (Strahl and Allis, 2000). The histone code hypothesis fits well in instances where research results previously seemed conflicting. For instance, as mentioned before, H3-K9 HMTases such as the Su(var)3-9 methylate histones generating an affinity for HP1, which leads to heterochromatin-induced gene silencing in *Drosophila* (Schotta et al., 2002). By immunological co-localization, it was shown that Su(var)3-9

and HP1 have the same site of action. Set-9/Set-7, a H3-K4 HMTase, on the other hand, stimulates transcriptional activation both by competing with HDACs as well as precluding H3-K9 methylation by Suv39h1 (Nishioka et al., 2002). The major point to be drawn from these and similar results is that the specificity of histone residues modified and their combinations serve as platforms for binding transcriptional regulators that will in turn determine whether genes will be silenced or highly expressed. The histone code hypothesis also predicts that histone modifications can influence one another in either a synergistic or antagonistic way, providing a mechanism to generate and stabilize specific imprints (Jenuwein and Allis, 2001). Further support for the hypothesis was provided by Struhl and colleagues. They showed that an increase in acetylation levels at various promoters is not necessarily associated with an increase in transcriptional activation (Deckert and Struhl, 2001).

Histone Deacetylases in *C. carbonum*

The genome of *C. carbonum* contains a total of four HDACs, excluding the ones that are NAD-dependent. This information was inferred by examination of a very closely related species of *Cochliobolus* called *C. heterostrophus*. The genome of *C. heterostrophus* was fully sequenced by the company Syngenta Biotechnology and found to contain four HDACs. Each one of the HDACs has its counterpart in *C. carbonum* and no other HDACs were found in *C. heterostrophus*. Hence, it is assumed from these data that there are no extra HDACs in *C. carbonum*. The *C. carbonum* HDACs all have counterparts in the yeast *S. cerevisiae* (Figure1). HDA1 is part of HDA, a 350KD complex that exhibits greater sensitivity to TSA than the HDB complex

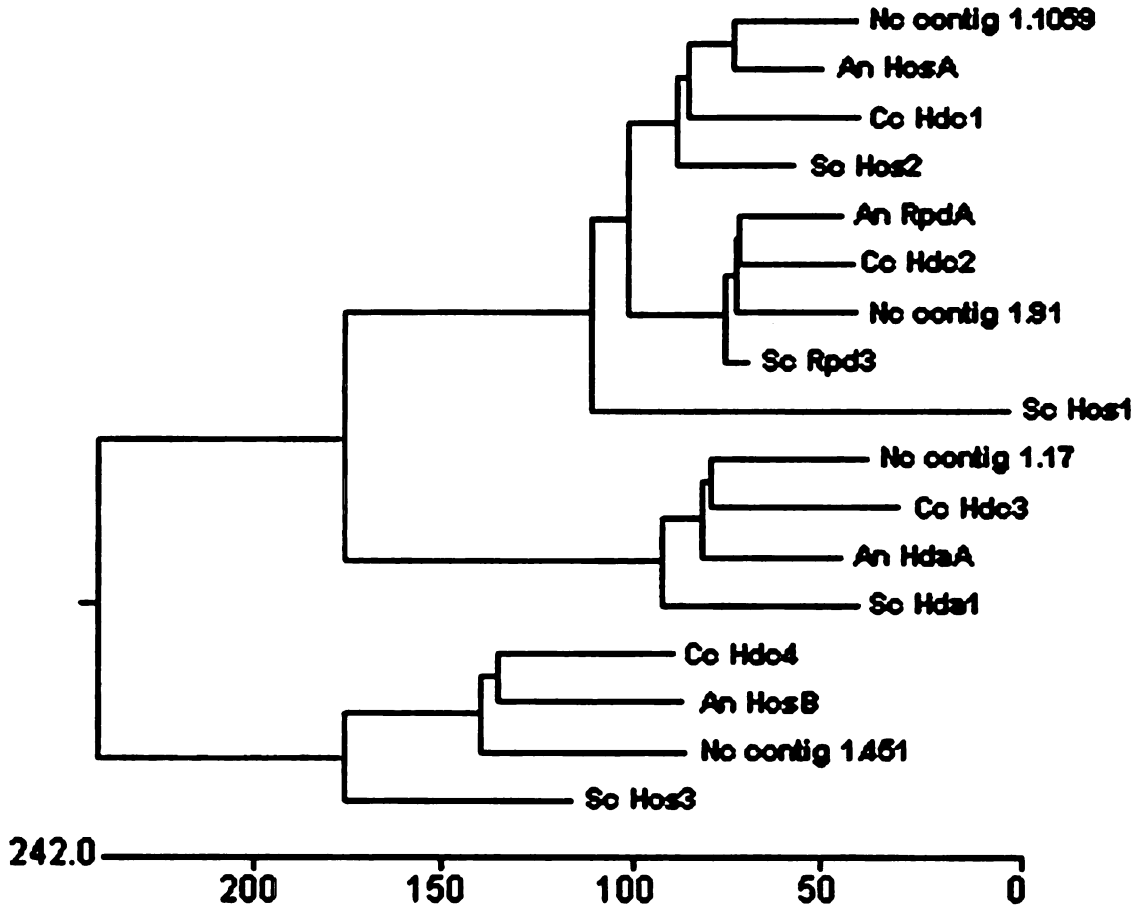

Figure 1. Unrooted Cladogram showing relatedness among histone deacetylases from different fungi. An, *Aspergillus nidulans*; Sc, *Saccharomyces cerevisiae*; Cc, *Cochliobolus carbonum*; Nc, *Neurospora crassa*. All Nc Contigs represent predicted proteins from the eponymous genomic DNA sequences of *N. crassa*. The units on the scale at bottom are numbers of substitution events. Cladogram was created using MegAlign by the DNAStar software package (version 5.03).

(Rundlett et al., 1996). The HDA complex contains three HDAC proteins, Hda1, Hda2, and Hda3. When the *HDA1* gene is disrupted in yeast, the nucleosomes of Tup1-regulated genes are hyperacetylated specifically at histones H3 and H2B. It was also shown that Tup1 interacts with Hda1 *in vitro*, suggesting that Tup1 recruits Hda1 to promoters of Tup1-regulated genes (Wu et al., 2001). *HDC3* is the *C. carbonum* ortholog of *HDA1*. *HDC3* was disrupted in *C. carbonum* but there was no detectable phenotype (Baidyaroy and Walton, unpublished results). The yeast *HOS3* gene has 38.9% similarity to RPD3 over 271 amino acids and its disruption was found to increase histone H4 acetylation in yeast cell extracts (Carmen et al., 1999). Rather than being part of a large complex, Hos3 was purified as a homo-dimer, and its activity is relatively insensitive to TSA, unlike Hda1 and Rpd3 (Carmen et al., 1999). *HDC4* is the *C. carbonum* ortholog of *HOS3*. Disruption of HDC4 also results in no detectable phenotype (Baidyaroy and Walton, unpublished results). The other two HDAC genes from *C. carbonum, HDC1* and *HDC2*, are the subjects of this dissertation work. *HDC1* is the ortholog of the yeast *HOS2* gene. *HOS2* stands for HDA One Similar and was discovered when the histone deacetylase-A (HDA) complex was first purified (Rundlett et al., 1996). The Hda1 protein sequence was compared with sequences from GenBank and the best scores obtained included three newly-sequenced open reading frames (ORF) that Grunstein and coworkers termed hos1, and hos2, and hos3. Despite having no visible phenotype, the *hos2* yeast strain was shown, by microarray analysis, to be hyperacetylated specifically at the ribosomal protein genes (Robyr et al., 2002). In addition, Hos2 was shown to be required for deacetylation of histone H4 K12. *HDC2* is the *C. carbonum* ortholog of *RPD3*. *RPD3* stands for Reduced Potassium Dependency and the *rpd3* yeast strain was

found in a screen for mutants that were able to grow well under low potassium concentrations (Vidal et al., 1990; Vidal and Gaber, 1991). Microarray analyses have shown that genes up-regulated by *RPD3* deletion correspond to cell cycle-regulated genes (Bernstein et al., 2000). However, deletion of *RPD3* also down-regulated certain genes, indicating that Rpd3 may also activate transcription. In fact, 40% of endogenous genes located within 20 Kb of telomeres are down-regulated by *RPD3* deletion. Unlike Hos2, it was demonstrated that Rpd3 affects the regulation of genes in virtually all cellular pathways, with a modest over-representation of genes that take part in sporulation, germination, and meiosis (Robyr et al., 2002).

C. carbonum, as any other eukaryote, contains HDACs to modify its own histones. This raises the question: why are the HDACs of *C. carbonum* not affected by its own toxin? How does *C. carbonum* protect itself against its own toxin? *TOXA* is one of the genes found at the *TOX2* locus in the genome of *C. carbonum*. The function of *TOXA* is unknown but based on its amino acid sequence it appears to encode a putative HC-toxin efflux pump. The inability to recover *toxA* knockouts of *C. carbonum* race 1 isolates supports the idea that ToxA may secrete HC-toxin out of the cytoplasm and is essential for the survival of *C. carbonum* (Pitkin et al., 1996). This kind of efflux carriers belongs to the Major Facilitator Superfamily (MFS) of transporters (Del Sorbo et al., 2000). MFS transporters do not hydrolyze ATP. Transport of compounds by MFS transporters through membranes is driven by an electrochemical proton gradient. It is now clear that MFS transporters are involved in secretion of HSTs and non-HSTs in several species of plant pathogens. For instance, the soybean pathogen *Cercospora*

kikuchii produces the toxin cercosporin. Callahan and colleagues identified and cloned *CFP*, a gene from *C. kikuchii* with similarity to several MFS transporters. Targeted disruption of *CFP* (Cercosporin Facilitator Protein) resulted in drastic reduction in cercosporin production, greatly reduced virulence, and increased sensitivity to exogenous cercosporin (Callahan et al., 1999). Hence, *CFP* most likely encodes a cercosporin transporter that contributes resistance to cercosporin by actively exporting the toxin and maintaining low cellular concentrations. This and other results—e.g. (Alexander et al., 1999)—support the hypothesis that active secretion of toxins by MFS transporters in fungi is likely to be a common virulence factor.

Other self-protection mechanisms seem plausible in addition to *TOXA*. The *C. carbonum* HDACs may be intrinsically resistant to HC-toxin, having slight changes in amino acid composition that lead to changes in protein folding. Then, HC-toxin would be unable to cause inhibition because its binding site has been altered. In addition, it is conceivable that resistance may be extrinsic. A protein factor unique to *C. carbonum* could bind HC-toxin and thus prevent it from inhibiting *C. carbonum* HDACs. Alternatively, to abrogate HDAC inhibition, such protein factor could bind HDACs and cause a conformational change such that HC-toxin can no longer bind. Some evidence supporting the "intrinsic" hypothesis was presented previously (Brosch et al., 2001). Brosch and collaborators have partially purified and characterized two HDAC complexes from *C. carbonum.* One of them is 60 KD in size with HDAC activity resistant to high concentrations of HC-toxin and TSA. This HDAC activity unique to *C. carbonum* appears to be dependent on HC-toxin production. More recent work has shown evidence for the "extrinsic" hypothesis. Isolates of *C. carbonum* that do not produce HC-toxin are

sensitive to the toxin when applied exogenously, whereas all toxin-producing isolates are resistant. HDAC extracts from resistant strains were found to protect sensitive extracts when mixed together (Baidyaroy et al., 2002). This protection was specific to *C. carbonum* and could not be achieved with other *Cochliobolus* species or *Neurospora crassa.* In conclusion, it appears that *C. carbonum* has multiple mechanisms of self-protection against HC-toxin.

The objective of this study is to better understand the function of HDACs in filamentous fungi in general and in *C. carbonum* in particular. It is also important to better understand the differences and similarities between the *C. carbonum* HDACs and their yeast counterparts. The work presented here undertook both a biochemical and genetic approach to studying specifically two HDACs from *C. carbonum*, *HDC1* and *HDC2*. These two genes are orthologs of the yeast HDACs *HOS2* and *RPD3*, respectively. Targeted disruption of *HDC1* was found to cause a significant reduction in *C. carbonum* virulence against maize, altered conidia morphology, and poor growth on complex polysaccharides when compared to the parental wild type. When placed on maize leaves, the *hdc1* strain was unable to penetrate the cell wall. Indeed, expression of various CWDE transcripts was highly reduced. *HDC2*, on the other hand, could not be disrupted despite many attempts, most likely because this gene is essential for the survival of *C. carbonum.* Overexpression of Hdc2, instead, proved that it has intrinsic HDAC activity, just as Hdc1. However, characterization of this strain showed no significant difference from its parental wild type.

CHAPTER ONE

CHARACTERIZATION OF THE HISTONE DEACETYLASE *HDC1* FROM *COCHLIOBOLUS CARBONUM*

Introduction

The gene *HDC1* from *C. carbonum* most closely resembles the sequence of *HOS2* from yeast. As it is the case with other histone deacetylases (HDAC), *HOS2* plays a role in repression of transcription (Watson et al., 2000). *HOS2* is part of large complexes that can remodel chromatin at promoter regions leading to repression of gene expression. For instance, Hos2 was recently found to be a member of the Set3 complex, named after Set3, a histone methyltransferase (Pijnappel et al., 2001). The Set3 complex also includes Hst1, a member of the Sir2 class of NAD-dependent deacetylases (Smith et al., 2000). When Hos2 was removed from the Set3 complex, repression of meiosis-dependent genes was abolished. Both the *hos2* and *set3* strains underwent normal premeiotic DNA synthesis but showed a faster progression through meiosis. Hence, Hos2 is essential for the normal function of the Set3 repressor complex.

Genome-wide expression studies have proven important to unveil the molecular role of HDACs in yeast. Using acetylation microarrays, Grunstein and coworkers showed that *HOS* deacetylase genes (*HOS1, HOS2,* and *HOS3*) are required for the deacetylation of histone H4 K12 preferentially at a very limited number of intergenic sites mainly on chromosome XII-R (Robyr et al., 2002). Further, using the *hos2* strain, they showed that *HOS2* is required for the preferential deacetylation of ribosomal protein genes.

To understand the biological role of HDACs in filamentous fungi, the structure and function of HDAC genes in *C. carbonum* were investigated. Using *C. carbonum* as a model organism makes this a unique study since *C. carbonum* produces a potent HDAC inhibitor. The report presented here focuses on the mutational analysis of *HDC1*, a gene from *C. carbonum* whose ortholog is the yeast *HOS2*.

Results

HDC1 was the second gene encoding an HDAC to be identified in *C. carbonum*. HDC1 was originally isolated using polymerase chain reaction (PCR) primers based on amino acid sequences that are conserved in known HDAC genes from other organisms (Hassig et al., 1998). These PCR primers were used to amplify a putative *HDC1* fragment using *C. carbonum* genomic DNA as template (Baidyaroy et al., 2001). The PCR product was radiolabeled and used as a probe to screen a genomic library of C. carbonum (Scott-Craig et al., 1990). The gene contains no introns based on comparison of its genomic and cDNA sequences. The closest matches of Hdc1 to proteins in the public databases were the products of *HOSA*, a gene encoding for a HDAC in *Aspergillus nidulans* (Graessle et al., 2000), followed by *HOS2* of yeast (Rundlett et al., 1996). Hdc1 has an overall amino acid identity of 46% to HosA from *A. nidulans*, 44% to Hos2, and 38% to Rpd3 from yeast (Figure 2). The predicted molecular masses and pI values of Hdc1, HosA, and Hos2 are similar: 56.9 KD and 5.7, 53.4 KD and 5.9, and 51.4 KD and 5.1, respectively. As expected, Hdc1 contains all seven of the motifs characteristic of class I HDACs, but lacks the characteristic features of class II HDACs.

Figure 2. Amino acid sequence alignment of three HDAC proteins using CLUSTAL W (Thompson et al., 1994). Hos2 from *S. cerevisiae*, HosA from *A. nidulans*, and Hdc1 from *C. carbonum*. Amino acids that are identical in all three proteins are indicated by asterisks. Sixteen of the seventeen amino acids that are highly conserved in all Rpd3-like HDACs and related bacterial proteins are underlined.

Strains of *C. carbonum* that were specifically mutated in *HDC1* were constructed by targeted gene replacement. Two fragments of *HDC1* flanking the cassette encoding resistance to the drug hygromycin were cloned into the shuttle vector pSP72 and the resultant plasmid (see Materials and Methods) was used to transform *C. carbonum* wild type strains 367-2 and 164R1 (Figure 3A). Whereas 367-2 is a toxin-producing strain (Tox+), 164R1 is a naturally occurring toxin-non-producing strain (Tox-). 164R1 lacks the genes required for HC-toxin biosynthesis (Panaccione et al., 1992). The rationale behind the disruption of *HDC1* in a Tox- background is that Hdc1 may be the only toxin-resistant HDAC that binds most of the toxin, and its deletion may cause self-inhibition as the toxin no longer binds Hdc1 but will inhibit the other *C. carbonum* HDACs. If this hypothesis is correct, deleting *HDC1* in 164R1 should not cause any phenotype, whereas there will be a phenotype in 376-2. Otherwise, the hypothesis is incorrect and any phenotype observed in 367-2 can be attributable specifically to the mutation of *HDC1* and not to a secondary effect caused by self-inhibition of other HDACs by HC-toxin. The *HDC1* mutational analysis on 367-2 has been described elsewhere (Baidyaroy et al., 2001). The results presented here focus primarily on 164R1. Putative mutants were screened for their ability to grow in the presence of the drug hygromycin. Five independent transformants were obtained and purified by two rounds of single-spore isolation to nuclear homogeneity. The aim of this process is to ensure that the genotype of every mutant spore is identical. These putative mutants were confirmed by Southern hybridization (Figure 3B and Figure 3C). All five isolates showed the expected pattern of DNA hybridization for single gene replacement.

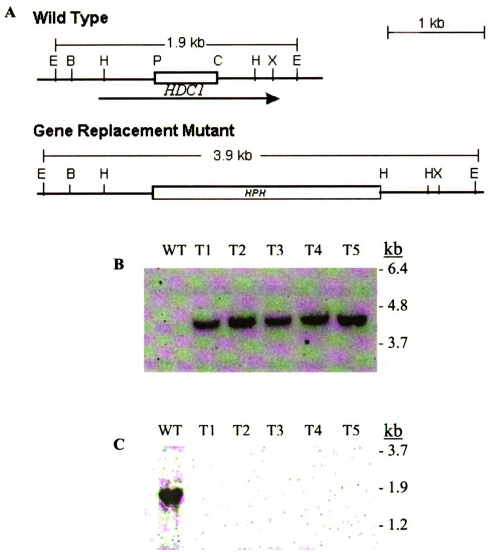

Figure 3. Construction and analysis of the *hdc1* disruption transformants. **A.** Restriction map of the genomic region of wild-type *HDC1* and predicted map of the gene replacement mutant. The open box in the wild-type map indicates the fragment of *HDC1* DNA replaced by the *HPH* gene encoding hygromycin phosphotransferase in the mutant. The arrow indicates the location of the *HDC1* coding region. E, *Eco*RI; B, *Bam*HI; H, *Hin*dIII; P, *Pst*I; C, *Cla*I; X, *Xho*I. **B.** DNA gel blot probed with a fragment of *HPH*. WT, Wild Type; T1, T717-1; T2, T717-2; T3, T717-3; T4, T717-4; T5, T717-5. **C.** The same blot was stripped and then probed with the deleted segment of *HDC1*. Sizes of DNA markers in kilo-bases are shown at right. DNA was digested with *Eco*RI.

The phenotypes observed for the mutants in the Tox+ background (T702) were identical to those observed in the Tox- background (T717). Mutation of *HDC1* resulted in a striking developmental defect. Filamentous ascomycetes such as *C. carbonum* multiply by dispersing non-motile asexual spores called conidia. The conidia in the *hdc1* mutant were reduced in size and septum number (Figure 4). However, the germination rates *in vitro* were the same as in the wild type. Furthermore, the degree of curvature present in the wild type conidia is reduced in the conidia of *hdc1*.

HDAC activity in crude extracts of *hdc1* is approximately half of that in the wild type (Figure 5). This result remained consistent after the assays were repeated several times. This finding strongly suggests that *HDC1* encodes a functional HDAC. However, because HDACs associate with each other in mammals and yeast, an indirect effect of disrupting HDC1 on other HDAC activities can not be excluded (Grozinger et al., 1999). As further assurance that the HDAC activity phenotype was attributable specifically to the mutation of *HDC1*, HDAC activity was measured in the *ccsnf1* mutant of *C. carbonum.* As it turns out, the *ccsnf1* strain shares many of the same phenotypes found in *hdc1* as described below (Tonukari et al., 2000). HDAC activity in *ccsnf1* (strainT688) was not significantly different from that in the wild type (Figure 5). This result indicates that the reduced HDAC activity in *hdc1* was not a side effect of any of the phenotypic abnormalities shared by the two mutants.

To test the ability of *hdc1* to grow on complex carbohydrates, growth rates were measured against the wild type as a control. The *C. carbonum* strains were grown on agar plates containing one of the following carbon sources: sucrose, glucose, arabinose, xylan,

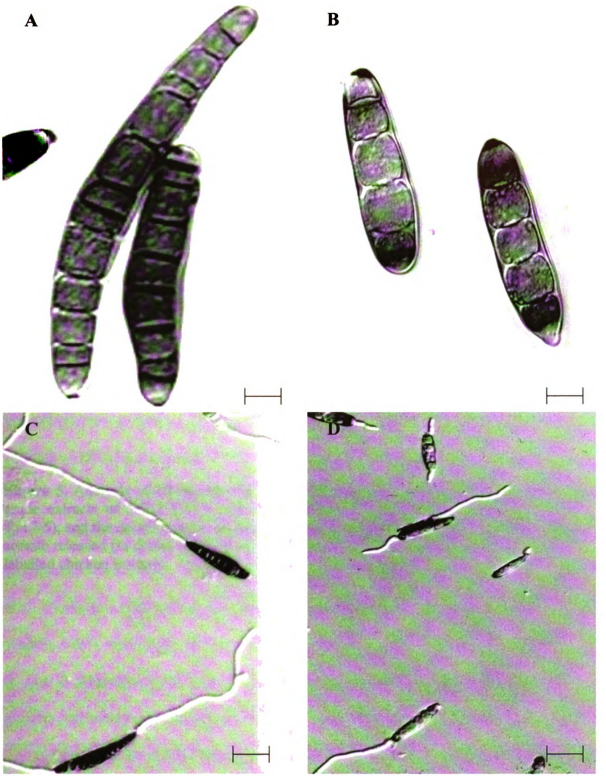

Figure 4. Microscopical analysis of the *hdc1* conidia. **A.** Ungerminated wild type conidia. **B.** Ungerminated conidia of T717-1. **C.** Wild type conidia 6 hours after germination on glass slides. **D.** Conidia of T717-1 6 hours after germination on glass slides. Bar in **A.** and **B.** is 12.5 μm; bar in **C.** and **D.** is 50 μm.

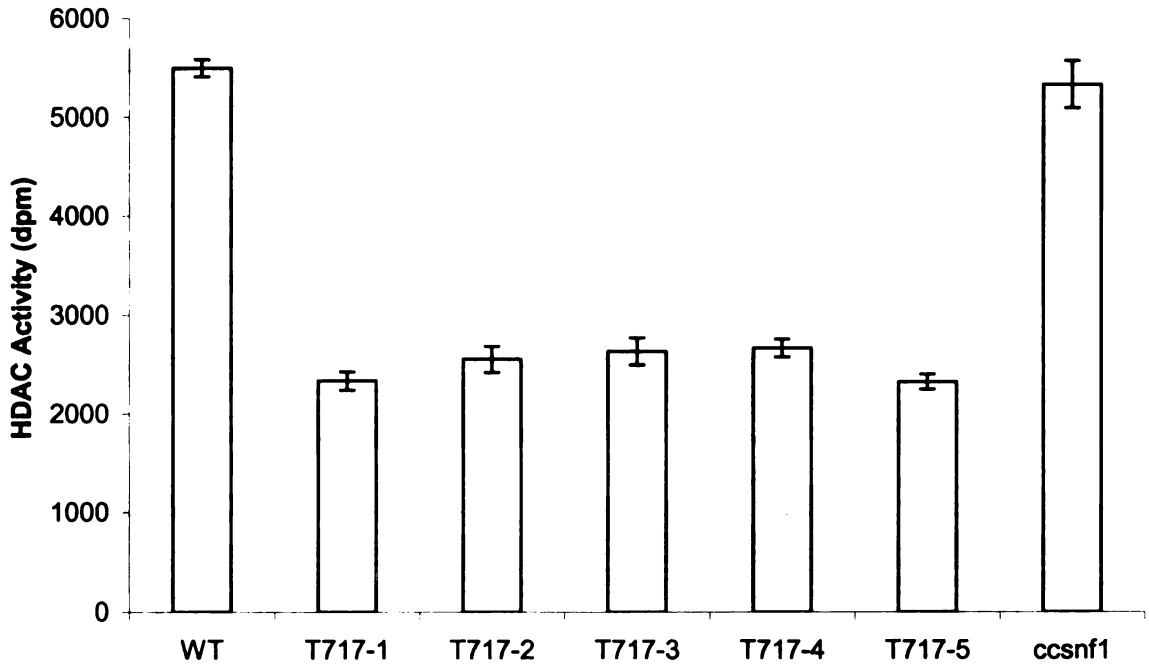

Figure 5. Analysis of HDAC activity in the *hdc1* mutant. HDAC activity measured in crude extracts of 367-2 (WT), five *hdc1* mutants (T717-1, T717-2, T717-3, T717-4, T717-5), and the *ccsnf1* mutant. HDAC activity is measured by the amount of ^{3}H-labelled acetate released (in dpms) by the enzymatic activity of HDACs when incubated with ^{3}H-labelled chicken histones.

pectin, or corn cell walls. Growth of the *hdc1* mutant on sucrose and glucose was very similar to that of the wild type. However, growth of the *hdc1* mutant on corn cell walls, pectin, and arabinose was reduced by at least 50% when compared with the growth of the wild type (Figure 6). Growth of the *hdc1* mutant on xylan was reduced by about 30% when compared to the wild type. The degree of growth reduction on the various substrates tested was similar for the *ccsnf1* and *hdc1* mutants. For instance, the growth of both mutants was severely affected on arabinose, pectin, and maize cell walls (Figure 6) (Tonukari et al., 2000), while on glucose and sucrose there were no significant differences.

One possible explanation for the reduced growth of *hdc1* on complex carbohydrates and not on glucose is that *HDC1* is required for the expression of cell-wall-degrading genes, of which *C. carbonum* contains an abundant variety. To test this possibility, RNA was extracted from the *hdc1* mutant and wild type after growing on various liquid media for 7 days. The RNA samples were blotted and probed for several different transcripts. The steady-state levels of mRNA of *EXG1*, encoding exo-β-1,3-glucanase (Vanhoof et al., 1991), *PGN1,* encoding endo-polygalacturonase (Scott-Craig et al., 1998), and *XYL1*, *XYL2*, and *XYL3*, encoding endo-xylanases (Apel-Birkhold and Walton, 1996), were either decreased or entirely down-regulated in the *hdc1* mutant (Figure 7). Therefore, *HDC1*, like *SNF1*, is required for the expression of at least some glucose-repressed genes in *C. carbonum.*

The virulence in T717 was not tested because the parental line does not produce HC-toxin. There is no significant virulence in natural isolates of *C. carbonum* that do not

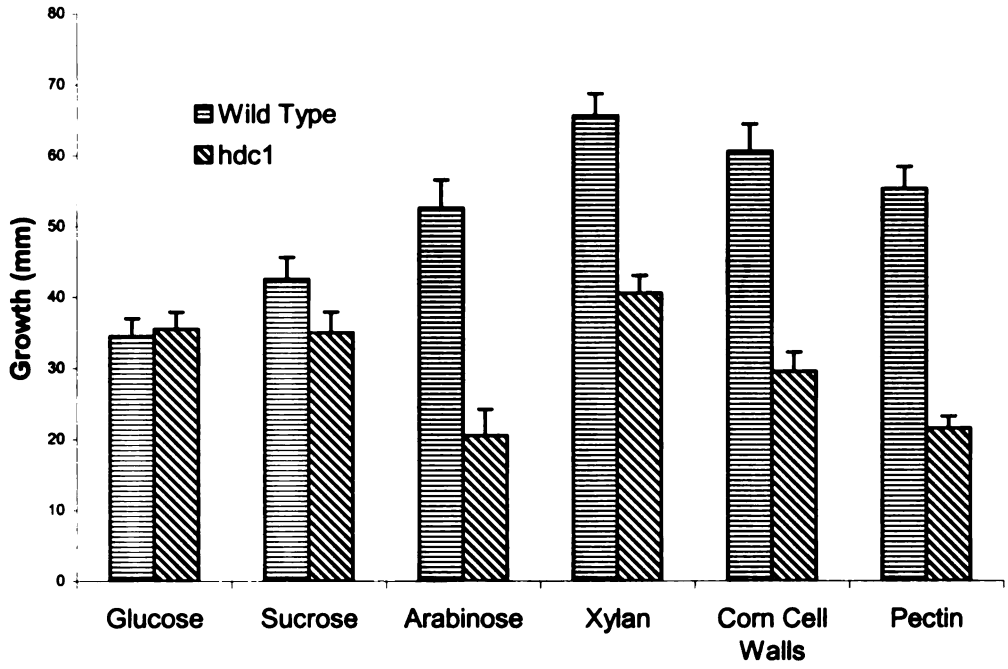

Figure 6. Growth comparisons between the *hdc1* mutant and wild type. Various carbon sources were used: simple sugars such as glucose and sucrose, or complex carbohydrates such as arabinose, xylan, corn cell walls, and pectin. Growth was measured in millimeters as a radius, by day 7, from the spot of inoculation at one extreme of the petri plate.

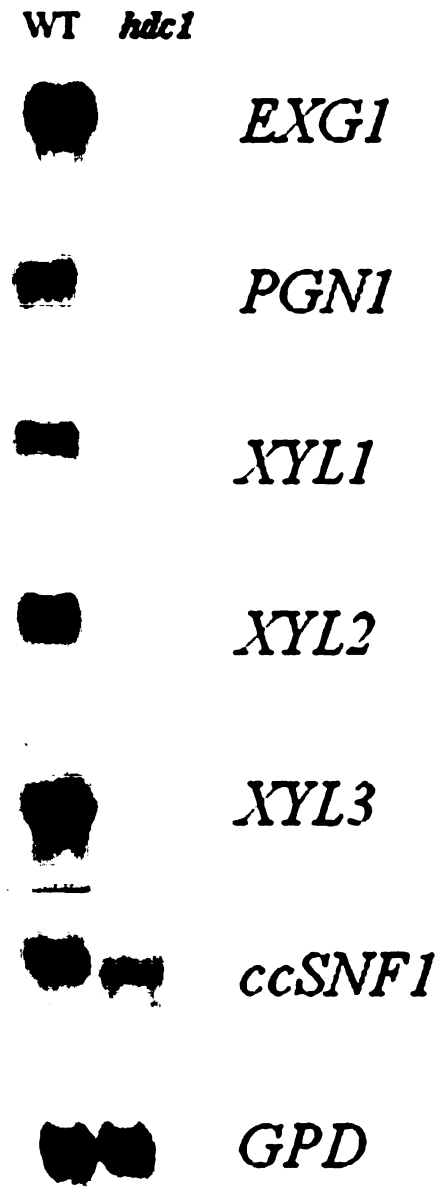

Figure 7. RNA expression profiles of selected genes in the *hdc1* mutant versus wild type. Total RNA was extracted from wild type and T717 strains. The fungus was grown on maize cell walls as the sole carbon source for *EXG1* expression, on pectin for *PGN1*, on xylan for *XYL1*, *XYL2*, and *XYL3*, and on sucrose for *ccSNF1* and *GPD*. *EXG1* encodes exo-β-1,3-glucanase; *PGN1* encodes endo-α-1,4-polygalacturonase; *XYL1*, *XYL2*, and *XYL3* encode endo-β-1,4-xylanases; *ccSNF1* encodes a protein kinase; *GPD* encodes glyceraldehyde 3-phosphate dehydrogenase and is used here as a loading control.

synthesize HC-toxin. Virulence of T702 was tested as its parental line makes HC-toxin and is fully virulent on maize. Virulence of T702 was greatly reduced as indicated by a reduction in the number of lesions formed on maize leaves (Baidyaroy et al., 2001). Lesions that developed had similar morphology and rates of expansion, as did those lesions caused by the wild type. Even at high inoculation densities (10^5 conidia/mL) and extended periods of disease development (more than 14 days), T702 never killed plants, unlike the wild type, which eventually colonized and killed seedlings (Baidyaroy et al., 2001). Conidia of T702 could be seen microscopically to adhere efficiently to maize leaves, indicating that there is no defect on appressorium formation. However, Baidyaroy and coworkers found that all lesions formed by T702 were associated with clumps of conidia. These results suggest that T702 is deficient in the production of a virulence factor or factors and only many T702 conidia in close proximity can compensate for such deficiency. Therefore, the product of *HDC1* may be considered a virulence factor necessary for successful penetration of the maize epidermis.

Is the reduced virulence of T702 caused by a reduced efficiency of germination or appressorium formation on leaves? Baidyaroy and coworkers answered this question by examining inoculated leaves using scanning electron microscopy. They found no differences between the germination rates of T702 and wild type conidia. The T702 hyphae were able to develop appressoria of wild type morphology preferentially at the junctions between leaf epidermal cells, as has been reported for wild-type *C. carbonum* and other species of *Cochliobolus* (Jennings and Ullstrup, 1957; Murray and Maxwell, 1975). This finding indicates that the defect in virulence of T702 is at a stage after germination and appressorium formation. Confocal microscopy with reconstructed cross-

sectional views also indicated that although *hdc1* conidia germinated and grew along the surface of the maize leaf, they did not penetrate efficiently (Baidyaroy et al., 2001).

HC-toxin is an essential virulence determinant for *C. carbonum* (Walton, 1996). In vitro, the *hdc1* mutant T702 is able to synthesize HC-toxin at concentrations similar to those in the wild type (Baidyaroy et al., 2001). Therefore, the reduction of virulence by T702 can not be attributed to a decrease in HC-toxin production, and *HDC1* does not regulate HC-toxin biosynthesis.

Discussion

The *HDC1* gene product is related to many characterized HDAC proteins and is most closely related to the yeast Hos2. Reduction in HDAC activity by as much as 50% in total extracts of the *hdc1* strain of *C. carbonum* when compared to the wild type indicates that Hdc1 is likely to be a histone deacetylase. Further biochemical studies are needed for full confirmation of this hypothesis. Viability of *hdc1* mutants is surprising considering that HDAC function is expected to have critical effects on global gene expression. However, Grunstein and colleagues have recently used chromatin immunoprecipitation and intergenic microarrays to demonstrate that Hos2 preferentially affects ribosomal protein genes (Robyr et al., 2002). Hence, it is possible that *HDC1* is not essential for the survival of *C. carbonum*, unlike other HDACs (e.g. *RPD3*) that may affect a much wider variety of genes (Kurdistani et al., 2002). Further, at least in the case of yeast, it was found that resulting phenotypes due to *HOS2* gene disruption are dependent on strain background (Bilsland et al., 1998). As in the case of *C. carbonum*, one yeast *hos2* strain in particular displayed a slow-growth phenotype.

Strains of the plant pathogenic fungus *C. carbonum* mutated in the *HDC1* gene encoding a putative HDAC are viable but have several significant phenotypes. The *hdc1* mutants originated from a Tox+ background (T702) are severely reduced in virulence. Evidence suggests that the reduced virulence is attributable not to reduced conidial germination or appressorium formation *in vitro* or *in vivo*, but to a decreased efficiency in penetration of the maize leaf epidermis (Baidyaroy et al., 2001). Since the lesions caused by T702 have normal morphology, *HDC1* does not appear to be important for ramification within the maize leaf.

C. carbonum probably breaches the maize epidermis by enzymatic action and not mechanical force (Horwitz et al., 1999) and, as shown here, *HDC1* is required for expression of at least some of the genes encoding glucose-repressed extracellular enzymes that can break down the cell wall. Hence, the inability of T702 to penetrate leaves might be due to its lack of synthesis of some of these enzymes. The fact that clustering of T702 conidia causes small lesions may mean that extracellular depolymerases are virulence factors in *C. carbonum.* However, it is possible that *HDC1* controls other kinds of virulence factors.

The decreased growth of the *hdc1* mutant on complex polysaccharides can be accounted for by decreased production of the polysaccharide depolymerases and/or enzymes needed for uptake or metabolism of alternative sugars, which in turn can be attributed to decreased expression of the encoding genes. No strict correspondence was found between mRNA levels and growth on the corresponding substrates in the *hdc1* mutant (Figures 6 and 7). That is, no sound correlation was observed between the inability of the *hdc1* mutant to grow on a certain carbohydrate and expression in the *hdc1*

mutant of the gene presumed to be involved in degradation of such carbohydrate. It is possible that the utilization of a complex carbohydrate requires the uptake and metabolism of the released sugars. For instance, complete down-regulation of the major xylose uptake carrier would have a severe effect on the growth on xylan regardless of the expression levels of xylanase. All known depolymerases are redundant in *C. carbonum*. Hence, other xylanase genes in addition to *XYL1, XYL2,* and *XYL3* might permit some amount of growth on xylan despite down-regulation of *XYL1, XYL2,* and *XYL3* in the *hdc1* mutant (Figure 7).

Interestingly, there is overlap between the phenotypes of the *C. carbonum hdc1* and *snf1* mutants. The similarities found suggest that the two gene products, Hdc1 and Snf1, may be part of the same signaling cascade in *C. carbonum*. The yeast Tup1 has been shown to recruit Hda1 to promoters of Tup1-regulated genes (Wu et al., 2001). Tup1 itself is recruited to promoters via Mig1, a zinc-finger protein, in association with Ssn6 (Treitel and Carlson, 1995; Smith and Johnson, 2000). Filamentous fungi have orthologs of *TUP1* and *MIG1*, although *MIG1* goes by the name of *CREA* (Ebbole, 1998). Hence, when yeast is grown on glucose as carbon source, genes required for growth on alternative sugar sources are repressed by the Mig1/Tup1-Ssn6/Hda1 complex. Mig1 itself is regulated by Snf1, a protein kinase (Treitel et al., 1998). In the absence of glucose, Snf1 phosphorylates Mig1, thereby causing it to dissociate from the promoters of glucose-repressed genes (Carlson, 1999). Recently, it was shown that Snf1 can also activate transcription, in response to glucose limitation, by directly interacting with the RNA polymerase II holoenzyme in yeast (Kuchin et al., 2000).

The yeast regulatory circuit logically predicts that the disruption of *HOS2*, an HDAC gene whose product interacts with Tup1, should cause de-repression of glucose-repressed genes. Indeed, it was observed that mutation of *HOS2* (in an *rpd3/hos1* background) causes de-repression of *SUC2*, which encodes a sucrose invertase, even under repressing (high-glucose) conditions (Watson et al., 2000). In contrast, the yeast logic is not consistent with the results obtained from mutational analysis of *HDC1*. If *HDC1* encodes a co-repressor, its disruption would be predicted to result in the de-repression of glucose-repressed genes. In fact, the exact opposite is observed. This finding suggests that the biological role of *HDC1* is markedly different from that of *HOS2*.

Materials and Methods

Fungal Cultures, Media, and Growth Conditions: The wild type HC-toxin producing isolate of *C. carbonum*, 367-2A, was derived from isolate SB111 (ATCC 90305) and maintained on V8 juice-agar plates. The wild-type Tox- isolate was 164R1, a progeny of SB111 (Walton, 1987). The fungus was grown in liquid media or agar plates containing mineral salts, 0.2% yeast extract, and trace elements (van Hoof et al., 1991). Carbon sources were 2% (w/v) glucose, sucrose, oat spelt xylan (fluka, Buchs, Switzerland), citrus pectin (catalog no. P-9135; sigma), or maize cell walls (Sposato et al., 1995). For quantifying growth on agar plates, 5 μL of a conidial suspension (10^4 conidia per mL) in 0.1% Tween 20 was spotted on one extreme end of the plate. Plates were incubated under fluorescent lights at 21°C. Growth was measured in millimeters every day as a linear progression from the point that was initially spotted.

Disruption of *HDC1* and Nucleic Acid Manipulations: To construct the replacement vector pAJ63, pSP72 (Promega) was cut with *Sph*I, blunted, and cut again with *Eco*RV to eliminate the multiple cloning sites between *Sph*I and *Eco*RV. The resulting plasmid was cut with *Hin*dIII and ligated with a 1.3-kb *Hin*dIII fragment of *HDC1*.This plasmid was then cut with *Pst*I and *Cla*I, and the deleted fragment was replaced with a *Pst*I-*Cla*I fragment from pHYG4, which contains the *HPH* gene for hygromycin resistance from pCB1004 (Carroll et al., 1994) sub-cloned into the *Sma*I site of pBluescript (KS) +. pAJ63 was then linearized at the unique *Pst*I site prior to transformation of *C. carbonum* wild type strains 367-2A and 164R1. Transformation was performed exactly as described previously (Apel et al., 1993). Transformants were then purified to nuclear homogeneity by two rounds of single-spore isolation. The *C. carbonum* genomic and cDNA libraries have been described previously (Scott-Craig et al., 1990). DNA and total RNA were extracted from lyophilized mats after 7 days of growth in still liquid culture (Apel et al., 1993). The methods used for DNA and RNA electrophoresis, gel blotting, probe labeling, and hybridization have been described elsewhere (Apel-Birkhold and Walton, 1996).

HDAC Assay: HDAC activity was measured using ^{3}H-acetate-labeled chicken reticulocyte histones (Kölle et al., 1998). Freeze-dried tissue (0.5g) from mycelial mats grown in still culture for 7 days was ground in liquid nitrogen and re-suspended by vortexing in 4.0 mL of extraction buffer (15 mM Tris-HCl, pH 7.3, 10 mM NaCl, 0.25 mM EDTA, 10% [v/v] glycerol, and 1 mM β-mercaptoethanol) containing one protease inhibitor tablet (Roche, Mannheim, Germany) per 30 mL of buffer. After centrifugation

at 11,000*g* for 15 min, 3 mL of the supernatant was de-salted by gel filtration (Econo-Pak 10 DG; Bio-Rad, Richmond, CA). Fifty micro-liters of protein extract and 5 μL of tritiated histones (40,000 dpm) were incubated for 2 hr at 23°C, 35 μL of 1N HCl was added, and the released acetate was extracted twice with ethyl acetate, first with 0.8 mL (removing 0.6 mL) and then with 0.6 mL (removing 0.7 mL). The ethyl acetate fractions were combined and counted by scintillation spectroscopy.

CHAPTER 2

STUDIES ON THE HISTONE DEACETYLASE *HDC2* FROM *COCHLIOBOLUS CARBONUM*

Introduction

A fundamental aspect of eukaryotic gene regulation is the ability of DNA-binding activators and repressors to recruit chromatin-modifying activities to specific promoters. Once recruited, such modifying activities generate local domains of altered chromatin structure that influence the level of gene activity. The yeast repressor Ume6, for instance, specifically binds DNA sequences (URS1) in a variety of promoters and inhibits transcription by recruiting the Rpd3 HDAC complex (Kadosh and Struhl, 1997). Recruitment occurs through an interaction between the Ume6 repression domain and Sin3, a component of the Rpd3 complex (Washburn and Esposito, 2001). Targeted recruitment of Rpd3 leads to localized deacetylation of the N-terminal tails of histones H3 and H4 over a range of one to two nucleosomes (Kadosh and Struhl, 1998; Rundlett et al., 1998).

Genome-wide binding maps of Rpd3 and its associated factor Ume1 have shown that this HDAC complex is common to a large and diverse set of promoters (Kurdistani et al., 2002). While Rpd3 affects the acetylation of promoters of genes that are part of many cellular pathways, there is a modest over-representation of genes involved in sporulation, germination, and meiosis (Robyr et al., 2002). There is also a significant preference for genes throughout the genome that are involved in carbohydrate utilization. These include genes involved in carbohydrate transport and metabolism, as well as energy reserves.

Although many intergenic regions that are under the regulation of Rpd3 contain URS1 (the Ume6 binding site), just as many have been found not to contain the Ume6 recognition site (Robyr et al., 2002). Hence, many of the promoters affected by Rpd3 must use other mechanisms to recruit the deacetylase.

Microarray studies in yeast have shown a significant overlap in the genes that are regulated by Rpd3 and Sin3. At least 107 genes in common are up-regulated, and 198 genes in common are down-regulated (at least two-fold) (Bernstein et al., 2000). Loss of *RPD3* and *SIN3* results in the two-fold down-regulation of 264 and 269 transcripts, respectively. Two conclusions can be drawn from these results. First, Rpd3 and Sin3 functions are linked and loss of one protein results in complete loss of the linked function. Second, Rpd3 may also activate transcription in addition to being a co-repressor. Treatment of yeast cells with the HDAC inhibitor TSA results in down-regulation of many of the same genes as those found in the *rpd3* strain, thus indicating that HDACs may function as direct transcriptional activators (Bernstein et al., 2000). Transcription profiles have also demonstrated that 40% of endogenous genes located within 20 kb of yeast telomeres are down-regulated by *RPD3* deletion. One possible model is that Rpd3 might activate telomeric genes repressed by SIR proteins directly by deacetylating H4 K12 (Hecht et al., 1995; Strahl-Bolsinger et al., 1997; Moazed, 2001b). H4 K12 is acetylated at silenced loci in yeast, and this appears to facilitate interaction with Sir3, which leads to silencing (Braunstein et al., 1996). Since Rpd3 is known to deacetylate at H4 K12, this action in certain cases may activate transcription simply by preventing binding of the repressive SIR complex.

Recently, it was shown that the domain of localized histone deacetylation generated by recruitment of Rpd3 mediates repression by inhibiting recruitment of chromatin-modifying activities and the TATA binding protein (TBP) (Deckert and Struhl, 2002). Further, Struhl and colleagues showed that repression by Rpd3 depends on the activator and the level of activation, not the extent of histone deacetylation. They were able to abolish repression by direct recruitment of TBP, but not Pol II, to the *HIS3* promoter.

In *C. carbonum*, the gene *HDC2* is the ortholog of the yeast *RPD3*. The first gene encoding an HDAC found in *C. carbonum*, *HDC2* has been studied using genetic as well as biochemical strategies. The results of this work are presented here.

Results

The gene *HDC2* from *C. carbonum* was isolated using degenerate PCR primers that were designed from the DNA sequence of the yeast *RPD3* gene. The product generated was radioactively labelled and used to screen a genomic library of *C. carbonum* in a similar fashion as it was done for *HDC1* described in chapter one (S. Wegener and J.D. Walton, unpublished results). Disruption of *HDC2* was attempted several times by several scientists and all attempts were unsuccessful. These results indicate that *HDC2* may be required for the survival of *C. carbonum* or, alternatively, the chromatin surrounding the *HDC2* locus is refractory to integration of foreign DNA.

In order to find out whether HC-toxin can inhibit purified Hdc2, in the absence of other *C. carbonum* HDACs, the gene *HDC2* was cloned and expressed in *Escherichia coli*. The *HDC2* open reading frame was cloned into plasmid pQE30 and the resulting

construct was transformed into *E. coli* M15 cells. High levels of stably expressed Hdc2 protein were obtained by inducing actively growing cells with IPTG (see Materials and Methods). Expression of Hdc2 was determined by denaturing polyacrylamide gel electrophoresis on a time course, using the pQE30 vector without insert as a control (Figure 8). High levels of accumulation of Hdc2, a protein of 75 kD, were found two hours after induction with IPTG. To maintain HDAC activity in recombinant Hdc2, zinc was added to the growth media. Zinc is known to be a co-factor required for optimal HDAC activity (Hassig et al., 1998). Most of Hdc2 was found in the soluble fraction (data not shown). This fraction was assayed for HDAC activity but none could be found. It is possible that the recombinant Hdc2 protein is quickly degraded since it has been found to be sensitive to proteases (Brosch et al., 2001). Alternatively, Hdc2 may have specific pH, salt, or temperature requirements for optimal function that are not yet known.

A different strategy to study the function of *HDC2* is to over-express the gene *in vivo.* The objective was to insert a second copy of *HDC2* and then remove the native *HDC2* from this new *C. carbonum* strain. The second copy of *HDC2* would be under the control of a promoter that can be easily induced and regulated. Since it is likely to be an essential gene, the only effective way to delete the native copy of *HDC2* requires the ability to control efficiently the levels of expression of the chimeric copy of *HDC2*. The levels of expression of *HDC2* may be modulated by the kind and concentration of carbon source added to the growth medium. Only by this strategy will it be feasible to unveil any phenotypes that may be a consequence of a deficiency in *HDC2* expression, while maintaining *C. carbonum* viable.

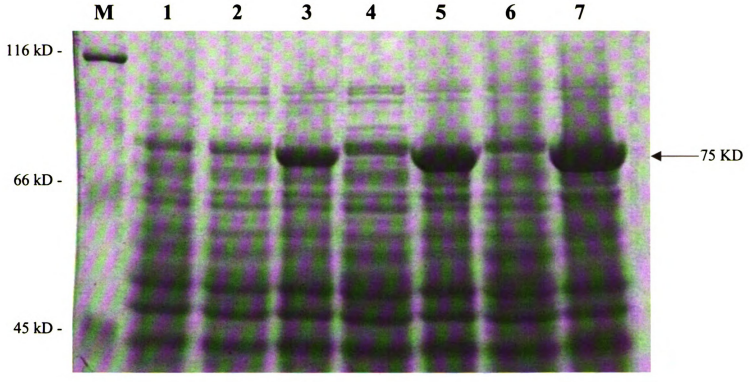

Figure 8. Time course study of Hdc2 synthesis in *E. coli.* Protein extracts were induced with IPTG and run on a 7.5% SDS-PAGE. **M.** Markers; **1.** Hdc2 no induction; **2.** Control (expression vector without insert) at 0.5 hr; **3.** Hdc2 at 0.5 hr after induction; **4.** Control at 1 hr after induction; **5.** Hdc2 at 1 hr after induction; **6.** Control at 2 hr after induction; **7.** Hdc2 at 2 hr after induction.

For this purpose, the *HDC2* open reading frame was fused to the β-xylosidase (*XYP1)* promoter and the resulting chimera was cloned into the pKP5 vector. pKP5 is a vector for *C. carbonum* transformation, which contains the hygromycin resistance cassette and the *PGN1* target locus. This target locus allows homologous recombination to occur, by a single cross-over event, at the genomic *PGN1* site, thereby integrating the foreign DNA into the *PGN1* locus at high efficiency. Disruption of the *PGN1* locus does not cause any apparent phenotype in *C. carbonum* (Scott-Craig et al., 1990), so it is deemed safe to use this locus as an integration site. The promoter of *XYP1* was chosen because, as any other glucose-repressed gene, *XYP1* is easily induced by growing the fungus on either corn cell walls or pure xylose as a carbon source (Wegener et al., 1999). From this transformation event, four independent transformants were obtained and two of these four (T709-2 and T709-3) had the correct DNA pattern expected for true transformants (Figure 9). These *C. carbonum* mutants (strains T709-2 and T709-3) contain two copies of *HDC2,* one under the control of the native *HDC2* promoter and the other (chimera) under the control of the *XYP1* promoter.

Reverse transcriptase PCR (RT-PCR) reactions were performed to determine the expression pattern of *HDC2* in T709 (strain T709-3), using the wild type parent as a control. Sets of primers were used to amplify specifically three different cDNA strands: the native *HDC2*, the native *XYP1*, or the *XYP1-HDC2* chimera. The RT-PCR results demonstrate the non-quantitative nature of the RT-PCR technique (Figure 10). The control lane, with no reverse transcriptase added, shows that there was no DNA contamination. As expected, there was no amplification product present in the chimera lane of the wild type. A chimera product is present in T709, both when grown on sucrose

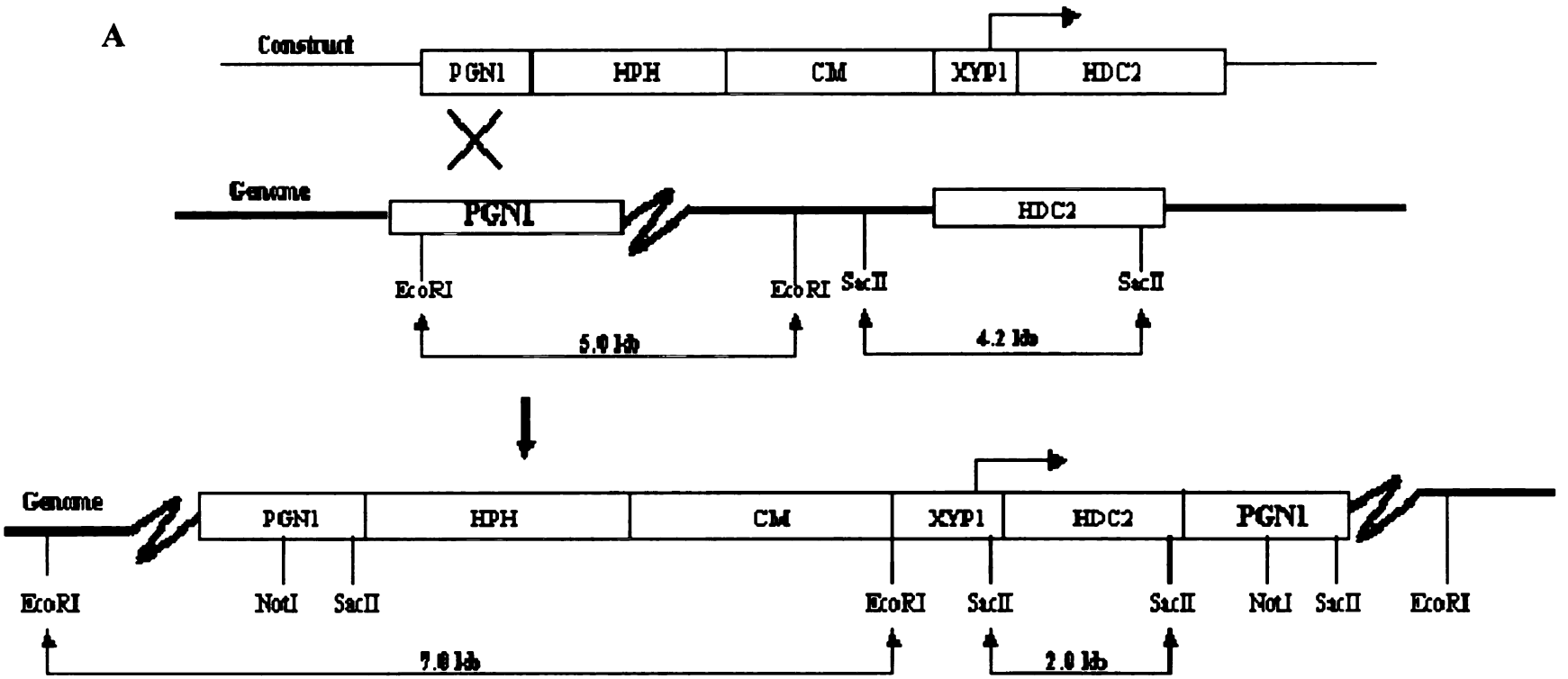

Figure 9. Integration of *HDC2* at the genomic *PGN1* locus. This process occurs via homologous recombination, by a single cross-over event. **A.** Map of the disruption construct and what it looks like after integration at the *PGN1* locus. Note that *PGN1* becomes split, half from the construct and half genomic, thus no longer functional.

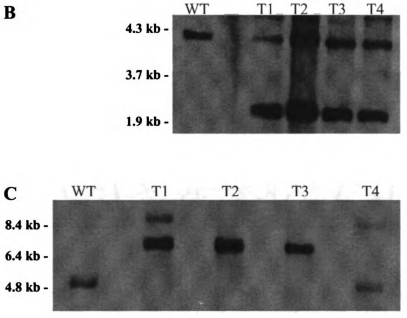

Figure 9, Continued. B. Southern blot hybridized with a fragment of the *HDC2* cDNA. Genomic DNA was digested with *Sac*II. Two bands are expected: the native *HDC2* and the chimeric *HDC2* that is only present in the transformants. **C.** Southern blot hybridized with a piece of *PGN1* that only recognizes the plasmid *PGN1* fragment. Genomic DNA was digested with *Eco*RI. T1, T709-1; T2, T709-2; T3, T709-3; T4, T709-4.

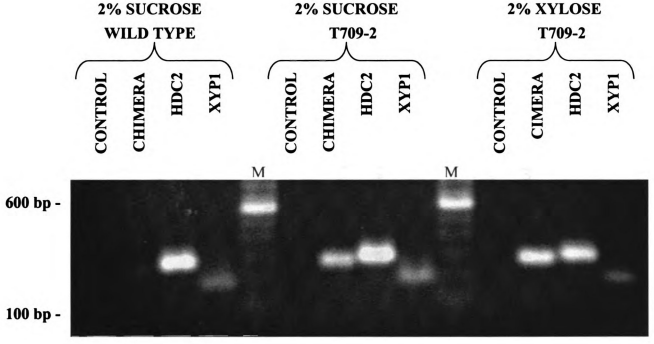

Figure 10. RT-PCR expression analysis of *HDC2* in the T709 and wild type strains. For control reaction, no RT enzyme was added. For chimera amplification, the two primers used could amplify only the border of the *XYP1-HDC2* chimera. Thus, no band was present in the wild type, chimera lane. A chimera band of expected size was seen in T709-2 both when grown in sucrose and xylose. Note that RT-PCR is not a quantitative technique. For amplification of *HDC2*, two primers amplified a segment of the *HDC2* cDNA in the 5' region. For amplification of *XYP1*, the two primers used amplified a segment of the *XYP1* cDNA.

and xylose. This result was unexpected because *XYP1* is not supposed to be actively transcribed when *C. carbonum* is grown on sucrose. The drawback with RT-PCR is that even only one *XYP1-HDC2* RNA molecule present can be reverse transcribed, and the product after the amplification steps will appear as a prominent band just as if the *XYP1-HDC2* message were abundant. It seems that the native *HDC2* is expressed under sucrose and xylose with no significant differences, both in T709 and in the wild type. A similar pattern of expression is observed for the native *XYP1*. A PCR product is present when T709 is grown on both sucrose and xylose. In conclusion, the *XYP1-HDC2* chimera is expressed at normal levels in T709, but the *XYP1* promoter is not nearly as tightly regulated as previously thought.

Northern blot analysis was performed also to examine the levels of expression of *HDC2* in T709. Both T709 and wild type strains were grown for seven days on various carbon sources. Total RNA was extracted and blotted onto nitro-cellulose membranes. The carbon sources used include sucrose, xylose, glucose, and a combination of sucrose and xylose. Blots were probed with a piece of the *HDC2* cDNA as well as the untranslated region (5'-UTR) of the *XYP1* promoter (Figure 11A). Because one of the project goals was to precisely modulate *HDC2* expression, it was critical to determine how well regulated the *XYP1* promoter was under various carbon sources. The expression level of the *XYP1-HDC2* chimera was highest when the fungus was grown in a mixture of sucrose and xylose (Figure 11B). However, the chimera was also expressed, at lower levels, when the fungus was grown in glucose and sucrose. Therefore, the *XYP1* promoter is not under tight regulation, confirming the RT-PCR results. Interestingly, the behavior of the *XYP1* promoter appeared to be different in the chimera gene from that of the native

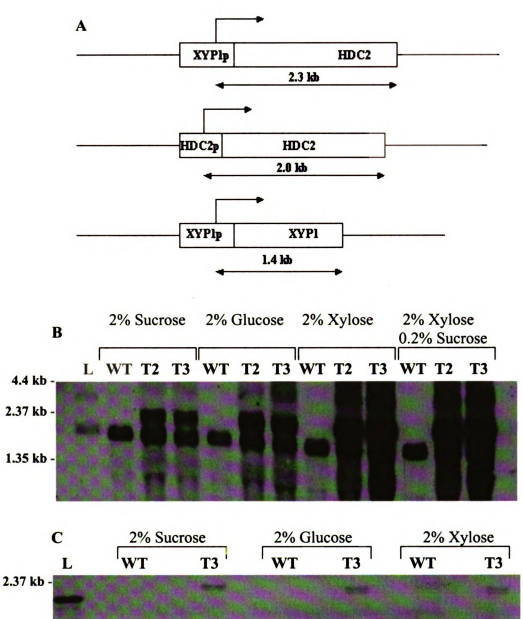

Figure 11. RNA gel blot analysis of expression of *HDC2* in T709 versus wild type strains. **A.** Map describing the sizes (in kb) of the expected transcripts. **B.** RNA blot probed with a segment of *HDC2* (500 bp). As expected, the wild type has one transcript, the native *HDC2*, whereas the transformants have an additional, chimera transcript. **C.** RNA blot probed with the 5'-UTR of *XYP1*. Being a much smaller probe (70 bp), the signal was not as strong. As expected, the native *XYP1* was most highly expressed on xylose as the carbon source. L, ladder; WT, wild type; T2, T709-2; T3, T709-3.

XYP1 gene. The chimeric promoter could not be strongly induced under xylose, but the native one was (Figure 11C). There are two possible explanations for this phenomenon. First, the chimeric *XYP1* promoter may be missing some regulatory elements that are present in the native *XYP1* promoter. The *XYP1* promoter used in the chimera is 500 base pairs in length and there could be other regulatory elements further upstream in the native *XYP1* promoter. Second, the site of integration for the chimera might be different from that of the native *XYP1* locus, and thus the new chromatin environment may alter the pattern of expression of the *XYP1* promoter.

Does the *C. carbonum* strain T709 have more Hdc2 enzyme activity? Total HDACs were extracted from T709 and the wild type (367-2), and their HDAC activity was assayed. As shown on Figure 12, after 5 days of growth on xylose, there is no significant difference in total HDAC activity between T709 and the wild type. After 10 days of growth, total HDAC activity actually diminished by 50% in T709 when compared with the wild type (Figure 12). However, after only 3 days of growth, the total HDAC activity of T709 appears to be more resistant to HC-toxin than the wild type. The HDAC activity in T709 after 3 days is only inhibited by about 20% in the presence of HC-toxin, while the HDAC activity in the wild type is reduced by about 80% due to HC-toxin inhibition (Figure 12). Resistance to the toxin in the wild type develops at 5 days of growth and is maintained thereafter. In T709, a significant amount of resistance is already present at 3 days of growth and maintained thereafter as well. In conclusion, it seems that over-production of Hdc2 renders *C. carbonum* resistant to exogenous HC-toxin at an earlier growth stage. The results also indicate that the total levels HDAC activity may not be necessarily higher in T709, but indeed, the level of toxin-resistant HDAC activity is

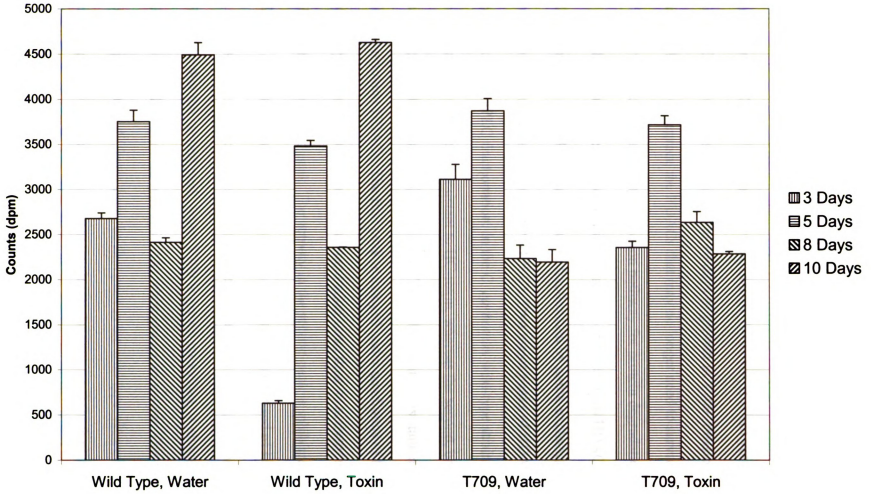

Figure 12. The effect of HC-toxin on HDAC activity comparing T709 versus wild type strains as a function of growth. Fungi were grown for 3, 5, 8, and 10 days of still culture using xylose as the carbon source. Total HDAC activity was measured from crude HDAC extracts. Each assay was performed in replicates, two sets with water (control) and two with HC-toxin.

higher. This phenomenon might be explained by a feedback regulatory mechanism that would hypothetically control the total amount of HDAC activity in *C. carbonum.* More production and activity of Hdc2 in *C. carbonum* thus would lead to lower activity levels by other HDACs as a compensatory mechanism. However, it is not yet understood why total HDAC activity is diminished at 10 days of growth in T709. Possibly, as the fungus ages, it begins to degrade the HDACs that may no longer need to survive, targeting them for the proteasome via the ubiquitination pathway for protein degradation.

For a more precise biochemical dissection of Hdc2, total HDAC extracts of the T709 and wild type (367-2) strains were fractionated by anion-exchange high-performance liquid chromatography (HPLC). Two major peaks of HDAC activity are obtained after HPLC fractionation. In the *C. carbonum* wild-type strain SB111, the first peak is almost completely resistant to HC-toxin, whereas the second, broader peak is almost completely sensitive (Figure 13A). This HPLC pattern is standard in fractionation of *C. carbonum* HDACs. The first peak is assayed under conditions of higher substrate and enzyme concentration and longer incubation time, thus its activity is much weaker than that of the second peak. The activity in the second peak is inhibited by salt, so the dilution increases its apparent activity. The first peak is comprised of HPLC fractions 9, 10, 11, and 12; the second peak is comprised of fractions 13, 14, 15, and 16 (Figure 13B). It is clear that the second peak, when T709 is grown on xylose, is almost twice the size of the second peak in the wild type (367-2A) when grown on xylose. The first peak in T709 is not significantly different from the first peak in the wild type. However, the wild type's second peak, when grown on sucrose, is almost the same as that when grown on xylose. On the other hand, there is a very drastic difference in the size of T709's second peak

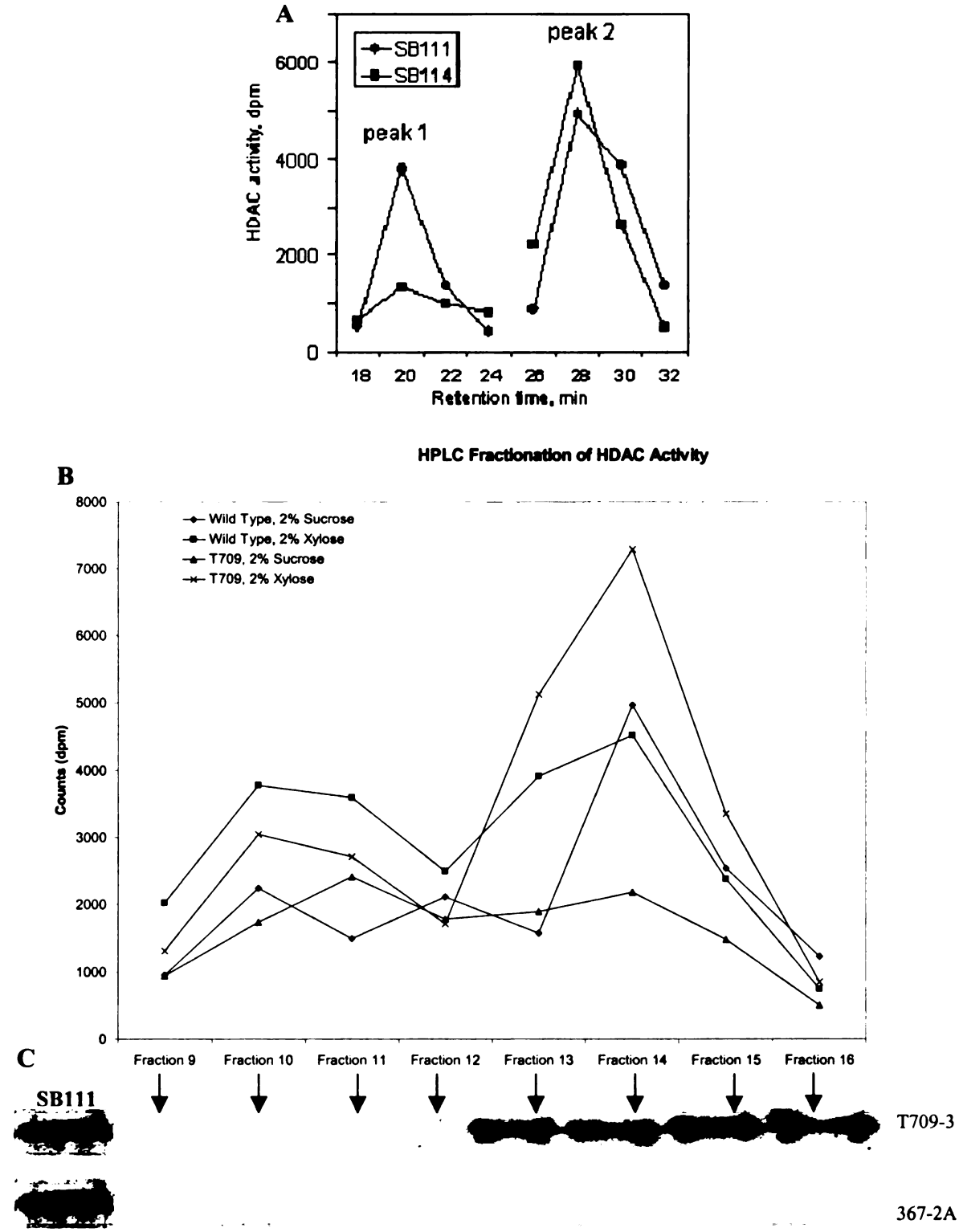

Figure 13. HPLC separation of HDAC activity comparing T709 and wild type strains. **A.** Standard pattern of HPLC separation of HDACs (from Baidyaroy et al., 2002). 367-2A, SB111, and SB114 are wild-type isolates of *C. carbonum*. **B.** HPLC fractionation of HDACs followed by assays of each fraction, strains 367-2A and T709-3 grown in sucrose versus xylose. **C.** Western blot analysis of the HPLC fractions, hybridized with an antibody raised against the C-terminus of Hdc2. SB111= control, total HDAC extracts. 367-2A and T709-3 were grown on xylose.

when grown on xylose, as compared to sucrose (Figure 13B). Under xylose, T709's second peak is about twice as large as the second peak when T709 is grown on sucrose.

Western analysis indicates that the majority of the Hdc2 protein seems to be contained within the second HPLC peak in the T709 strain (Figure 13C). Polyclonal antibodies raised against the unique carboxyl terminus of Hdc2 (~200 amino acids) were obtained from Brosch and collaborators at the University of Innsbruck, Austria. HPLC fractions 9 through 16 were concentrated with TCA and loaded on gradient SDS-PAGE gels (Biorad). The western blot was hybridized with the α-Hdc2 antibodies (1:3,000) overnight. Hdc2 could be detected quite well in fractions 13 through 16 on T709, but it is absent in fractions 9 through 12. There is no strict correlation between the amount of protein in the fractions and the corresponding HDAC activity. The reason may be that Hdc2 in fraction 16, for instance, could be inactive, yet still recognized by the α-Hdc2 antibodies. Surprisingly, Hdc2 could not be detected in any fraction in the wild type strain 367-2A. Total HDAC extracts from the wild type strain SB111 were used as a control. As a way to ensure that the fraction samples contained a similar concentration of proteins, the HPLC traces were found to contain areas under the major peaks of similar size, meaning that most likely there was not over-loading of any one sample (data not shown). Interestingly, SB111 shows a prominent band indicating that adequate amounts of protein were loaded. However, it is not yet understood why the α-Hdc2 antibody recognizes Hdc2 in the wild type SB111 but not in the wild type 367-2A strains. In conclusion, the antibody used indicates that Hdc2 is present abundantly in the second peak of T709 but is unable to detect Hdc2 in the first peak. In the wild type, Hdc2 can not

be detected by this particular antibody in any of the two peaks. It appears that the antibody is only recognizing the recombinant Hdc2 while the native protein is not being recognized. It is possible that the native Hdc2 is bound by other proteins such that the antibody can not bind to it. Alternatively, a post-translational modification (e.g. phosphorylation) occurs uniquely to the native Hdc2 and not the recombinant one, so the antibody is unable to bind the native Hdc2.

The two HDAC peaks were analyzed for sensitivity to exogenous HC-toxin. The first peak in both wild type and T709 was almost completely resistant to the toxin (Figure 14). The second peak, on the other hand, was inhibited by almost 80% in both the wild type and T709. These results indicate that Hdc2 is present mostly in the second peak and this HDAC is particularly sensitive to HC-toxin.

Does over-expression of an HDAC lead to more virulence in *C. carbonum*? To answer this question, three-week-old maize seedlings were inoculated with spore suspensions (10^4/mL in 0.1% Tween 20) of T709-3 and wild type as a control. Maize leaves were analyzed 48 hours after inoculation. As seen on Figure 15, there is no significant distinction between the damage caused by wild type versus that caused by T709. Therefore, it appears that over-expression of an HDAC, *HDC2*, has no effect on virulence in *C. carbonum.*

Discussion

The filamentous ascomycete *C. carbonum*, a maize pathogen, probably has multiple mechanisms to protect itself against HC-toxin. One of these mechanisms of self-protection may involve its own HDAC activity being insensitive to HC-toxin when

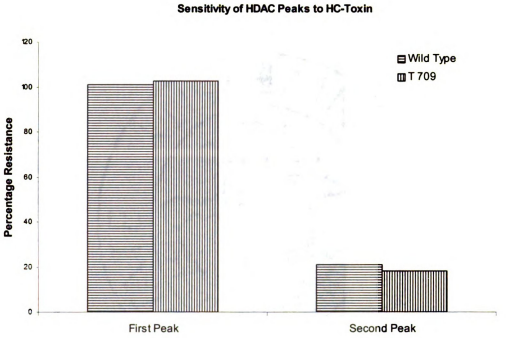

Figure 14. Sensitivity of the T709 and wild type major HDAC activity peaks to HC-toxin. The two fractions that represent the two major peaks were tested for sensitivity to HC-toxin. Both fractions, in both wild type (367-2A) and T709 strains, seem to have similar levels of sensitivity to the toxin. The first peak is mostly resistant while the second peak is mostly sensitive.

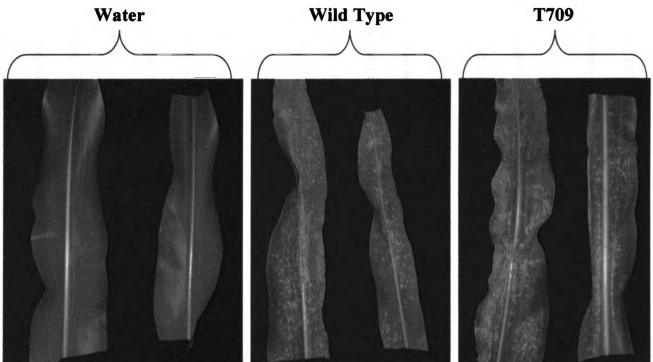

Figure 15. Virulence assays comparing wild type versus T709 strains. Three-week-old maize seedlings were inoculated with *C. carbonum* spores of either the wild type (367-2A) or T709. Pictures were taken 48 hours after inoculation.

compared with the HDAC activity of other fungi (Baidyaroy et al., 2002). This insensitivity appears to have two components. One is intrinsic, where certain HDACs cannot be inhibited by HC-toxin. The second is extrinsic, where there may be a factor that renders sensitive HDACs resistant to HC-toxin. One of the *C. carbonum* HDAC genes, *HDC2*, appears to be essential for survival. To date, no deletion mutants have been generated despite many attempts. Initially, the project's objectives included the generation of a conditional knock-out of *C. carbonum*. This strain would have a second copy of *HDC2* introduced, followed by the disruption of the native *HDC2*. This second copy of *HDC2* would be under the control of an inducible promoter and by removing the native *HDC2*, it would be feasible to determine if *HDC2* is indeed critical for the survival of *C. carbonum*. The chimeric *HDC2* was successfully introduced but the native copy could not be disrupted despite various attempts. Hence, the work that was described here focused only on the over-expression of *HDC2*.

Over-expression of *HDC2* has shown that this HDAC in particular is sensitive to HC-toxin. Further, Hdc2 is found mostly in the second peak after HPLC fractionation. If Hdc2 is sensitive to HC-toxin, why might it be essential in *C. carbonum*? Cross-protection experiments using sensitive and resistant HDAC extracts from different *C. carbonum* isolates have indicated that the resistant HDAC extracts have a heat-labile, proteinaceous factor or factors that can protect sensitive extracts in a species-specific manner in *trans* (Baidyaroy et al., 2002). This protection factor may bind Hdc2 to render the enzyme HC-toxin resistant. Such factor is probably separated from Hdc2 during anion-exchange chromatography, thus Hdc2 in the second peak is sensitive to HC-toxin. There are at least two hypotheses that could explain why Hdc2 is essential. First, as an

HDAC, Hdc2 plays a much more active, versatile, and important role in the cell. This has been well documented with acetylation as well as general microarray studies on the *rpd3* mutant in yeast (Kurdistani et al., 2002; Robyr et al., 2002). Therefore, disruption of *HDC2*, the *RPD3* ortholog, leads to cellular havoc, which in turn leads to cell death. A second hypothesis is that a complex formed by Hdc2 and the protection factor traps all the HC-toxin that makes it to the nucleus. The absence of Hdc2 means that the protection factor is no longer functional and therefore all the other *C. carbonum* HDACs can be inhibited by HC-toxin, leading to cell death.

Materials and Methods

Nucleic Acid Manipulations: Please see Materials and Methods, Chapter 1. Reverse Transcriptase PCR (RT-PCR) reactions were carried out using the "Superscript One-Step RT-PCR System" kit and protocols available from Life Technologies (Rockville, MD).

Expression of *HDC2* in *E. coli*: The *HDC2* cDNA was cloned into the bacterial expression vector pQE30 obtained from Qiagen as part of the "QIAexpressionist" kit (Valencia, California). This construct was transformed into *E. coli* strain M15 (pREP4) and grown overnight with shaking at 37°C in 5 mL of Luria-Bertani medium containing ampicillin (200 μg/mL) and kanamycin (25 μg/mL). A sample of the overnight culture (2.5 mL) was used to inoculate 50 mL of 2XYT medium with the same concentrations of ampicillin and kanamycin, grown with vigorous shaking at 37°C. When the O.D. reached 0.6 at 600 nm of wavelength, expression of *HDC2* was induced with IPTG addition to a final concentration of 1mM. The cultures were grown for an additional two hours and the

cells were then collected by centrifugation at 4,000 x *g* for 20 min. Samples were run using standard SDS-PAGE procedures.

Purification of HDACs: Anion-exchange chromatography of HDACs was performed on a Waters HPLC using a TSK DEAE-5PW column (TosoHaas, Montogomeryville, Pa.). Typically, 2-4 mL of desalted crude extract were injected per run. Proteins were eluted with a linear gradient from 10 to 500 mM NaCl in the same buffer used for extraction (omitting the protease inhibitors) in 30 min at a flow rate of 1 mL/min. Fractions of 2 mL were collected.

Chromatographic fractions were assayed as follows. For the first peak of activity eluted from an anion-exchange column, 50 µL of each fraction and 10 µL of [^{3}H]-histones (~70,000 dpm) were incubated for 4 hr at 21°C. For the second peak of activity, 20 µL of each fraction, 30 µL of extraction buffer, and 5 µL of [^{3}H]-histones were incubated at 21°C for 2 hr.

HDAC Extractions and Assays: Please see Materials and Methods, Chapter 1. HC-toxin was extracted with chloroform from culture filtrates of *C. carbonum* grown for 14 days in still culture. The extraction protocol followed has been described in detail elsewhere and was performed exactly (Walton et al., 1982).

Western Analysis: Protein samples were precipitated with TCA overnight at 20°C. The next day, SDS-PAGE gradient gels (Biorad) were run for 2-3 hours at 100 Volts at room temperature. Gels were then blotted for one hour in the cold room at 200 Volts and pre-hybridized in 2% milk for 2 hours in the cold room. Antibody was then added and allowed to hybridize overnight in the cold room with mild shaking. The Hdc2 antibody was used at a titer of 1:3,000. Secondary antibody was added the next day and incubated

for one hour at room temperature. Blots were exposed for 1-5 minutes and developed by standard chemiluminescence procedure.

Pathogenicity Assay: Tests were done by spray-inoculating 3-week-old plants of the susceptible inbred maize line Pr (genotype *hm1/hm1*), with a suspension of conidia (10^4/mL) in 0.1% Tween 20. After inoculation in the afternoon, plants were covered with plastic bags overnight. Plants were grown in a greenhouse and monitored daily until death.

CONCLUSIONS AND PERSPECTIVES

The first hints that alterations in chromatin structure accompany changes in gene expression came from now classic nuclease digestion studies (Gross and Garrard, 1988). These investigations were predicated upon findings that chromatin could be cleaved into mono- and oligo-nucleosome-sized particles by micrococcal nuclease (MNase), which preferentially cleaves linker DNA between nucleosomes (Noll and Kornberg, 1977). Subsequently, it became clear that genes were more sensitive to digestion by MNase, DNAase I, or DNAase II in tissues where they were transcribed than in tissues where they were not transcribed, and that, within a cell, transcribed genes were more sensitive to digestion than were non-transcribed genes (Levy and Noll, 1981). For instance, the globin gene cluster is present in a nuclease-sensitive region in chick erythrocyte nuclei but is nuclease resistant in other tissues, such as liver or oviduct, where globin is not expressed (Stalder et al., 1980). Currently, there is little doubt that chromatin plays an active part in the regulation of gene expression. The challenge now is to understand what types of structures formed by chromatin actually exist *in vivo*, how they are regulated, and how they affect the functions of the transcription machinery.

Histone deacetylases (HDAC) are one member of a family of eukaryotic enzymes involved in modification of the chromatin structure. A recent paradigm shift has led to a new understanding in the role of HDACs in gene regulation. HDACs appear to act as co-repressors as well as co-activators depending on the promoter and surrounding chromatin environment. Current evidence indicates that for example, Rpd3, an actively-studied HDAC, can repress transcription by at least two distinct mechanisms. One repression

mechanism involves inhibition of activator-dependent recruitment of Swi/Snf and SAGA to promoters (Deckert and Struhl, 2002). Repression by this mechanism should also result in decreased TBP occupancy, because Swi/Snf and SAGA recruitment is often required for and precedes TBP association (Agalioti et al., 2000). A second repression mechanism involves inhibition of TBP/TFIID binding to the TATA element by localized histone deacetylation. Rpd3-dependent repression was found to be alleviated when TBP/TFIID, but not Pol II holoenzyme, is directly recruited to the promoter (Deckert and Struhl, 2002).

Genome-wide studies have proven particularly useful to elucidate the role of HDACs as transcriptional co-activators. Results obtained from acetylation microarrays have recently demonstrated that the yeast HDAC Hos2 preferentially associates with the coding regions of genes with high transcriptional activity genome-wide (Wang et al., 2002). Particularly, Hos2 was found to be important for activation of *GAL1* and *INO1* genes in vivo. Further, as a component of the Set3 complex, Hos2 is now thought to function as an activator of *GAL1* gene expression. Studies from Schreiber's group showed that loss of *RPD3* in yeast results in the two-fold down-regulation of as many as 264 transcripts (Bernstein et al., 2000). Further, they showed that 40% of endogenous genes located within 20 kb of telomeres are down-regulated by *RPD3* deletion. Rpd3 appears to activate telomeric genes sensitive to histone depletion indirectly by repressing transcription of histone genes. Rpd3 also appears to activate telomeric genes repressed by the SIR complex directly, possibly by deacetylating lysine 12 of histone H4.

Recent evidence indicates that different histone sites of acetylation have different functions in gene regulation. In particular, acetylation of H4 lysines K5 and K12 in the

yeast *rpd3* strain correlates better with increased transcription than acetylation of H4 K16 (Robyr et al., 2002). These results agree and serve as evidence for the histone code hypothesis. This hypothesis predicts that specific combinations of histone modifications provide regulatory information through changes in the structure of chromatin and in the association of non-histone proteins with particular nucleosomes (Strahl and Allis, 2000; Jenuwein and Allis, 2001). Inherent in this hypothesis is the idea that modification of one residue in a histone may affect the type and frequency of modifications at other sites. One of the first examples of such cross-regulation was the discovery that phosphorylation of serine 10 in H3 augments recognition of the H3 amino-terminal tail by Gcn5, leading to increased acetylation of lysine 14 (Cheung et al., 2000). Recently, evidence has been presented that supports *in vivo* the histone code hypothesis. Phosphorylation or mutation of serine 10 was shown to have an opposite effect on acetylation of lysine 9 (Edmondson et al., 2002). These results indicate that acetylation of each lysine within a histone tail is independently regulated and each lysine probably has unique functions. The role of HDACs as co-activators is also supported by the histone code hypothesis because this hypothesis also predicts that not all histone methylation marks correspond with gene silencing, and some histone acetylation events may repress rather than stimulate the readout of the genetic information. In conclusion, the histone code hypothesis, for which the scientific evidence has greatly increased in the past few years, predicts that one histone modification can influence another in either a synergistic or an antagonistic way, providing a mechanism to generate and stabilize specific genome imprints.

In comparison to yeast HDACs, our knowledge on the structure and function of HDACs from filamentous fungi is incipient. The organism of study in this thesis,

Cochliobolus carbonum, is a filamentous fungus, a pathogen of maize, and secretes copious amounts of HC-toxin, an inhibitor of HDACs (Ransom and Walton, 1997). This toxin is required for causing disease, but how does HDAC inhibition allow for pathogenesis? For a long time, it was thought that HC-toxin acts as a suppressor of induced plant defense responses through inhibition of HDACs (Ciuffetti et al., 1995). In the past, HDACs were thought to play a role only in gene repression as co-repressors. Hence, maize defense or other genes controlled directly by HDAC activity would be expected to be over-expressed during infection, not repressed. However, as described earlier and as reported in chapter one of this thesis, HDACs seem to also have a role in activation of transcription. Here we have shown that in particular, *HDC1*, the HDAC gene orthologous to the yeast *HOS2* gene, is required for the induction of a set of strongly induced genes. In *C. carbonum*, these genes encode extracellular depolymerases, but perhaps a similar requirement for HDAC activity exists for the strongly induced defense genes of maize. As a result, presence of HC-toxin inside the maize cell could result in repression of defense response genes by inhibition of HDACs.

C. carbonum, as any other eukaryote, contains HDACs to modify its own histones. How does *C. carbonum* protect its HDACs from its own toxin? As discussed in chapter 2, there seem to be multiple mechanisms of self-protection. One of these mechanisms includes the intrinsic properties of the *C. carbonum* HDACs making one or more of them insensitive to HC-toxin, by having distinct conformations that do not allow binding of the toxin. Another mechanism may be the production of a protection factor that renders otherwise sensitive HDACs insensitive to the toxin. All the evidence available to date is in favor of the second mechanism. As seen on chapter 2, total HDAC

activity from toxin-producing strains of *C. carbonum* is resistant to exogenous HC-toxin. However, when the HDACs are fractionated by HPLC, at least one of the two peaks reveals sensitivity to the toxin. This is because the protection factor's bondage to the HDACs is probably broken by the anion-exchange HPLC conditions. Also in favor of this mechanism are the results from cross-protection experiments (Baidyaroy et al., 2002). When total HDAC extracts from sensitive and resistant *C. carbonum* strains are mixed at various ratios, protection can be conferred from the resistant to the sensitive extract against HC-toxin. This phenomenon is species specific and works against HDAC inhibitors other than HC-toxin that are chemically unrelated. Full cross-protection requires a ratio of resistant extract to sensitive extract of at least 3:7. Hence, the protection factor probably acts stoichiometrically with the sensitive HDAC rather than catalytically; that is, the protection factor could be a protein that binds to HDACs in a 1:1 ratio rather than enzymatically altering them.

In order to more effectively answer important questions in the biology of *C. carbonum* HDACs, we believe that microarray studies are necessary. A look at the genes that are either up- or down-regulated in the *hdc1* strain as compared to the wild type could be revealing. It could tell us, among other things, which genes are targets for regulation by this particular HDAC. It could also unveil one or more of the components that may be acting upstream of Hdc1 in some kind of regulatory network. The *snf1* strain can also be studied by microarray technology. Compared to the wild type, it is possible to determine what components may be controlling Snf1 function upstream in the regulatory network. Ultimately, we could determine whether Snf1 and Hdc1 are part of the same regulatory network or their signaling pathways share any elements in common.

Chromatin immunoprecipitation (ChIP) is another technique that could be useful in studying the function of *C. carbonum* HDACs. We could discover how the histone acetylation levels in *C. carbonum* are affected in *hdc1* and learn in more detail about the molecular function of Hdc1 in *C. carbonum.* In a similar manner, ChIP studies could be conducted for the other HDACs in *C. carbonum* for which mutants are available (i.e. *HDC3* and *HDC4*) and learn about their molecular function.

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