博士論文

Functional analysis of histone deacetylase of *Aspergillus oryzae*

（麹菌 histone deacetylase の機能解析）

河内　護之

広島大学大学院先端物質科学研究科

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主論文

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SUMMARY

The filamentous fungus *Aspergillus oryzae* is used in a number of industries including the manufacturing of pharmaceuticals and the production of traditional Japanese fermented foods. Because histone deacetylases (HDACs) play key roles in the regulation of fundamental cellular processes such as development, metabolism, genome integrity, and the stress response in higher model organisms, I hypothesized that they would also play diverse roles in *A. oryzae.* In this thesis, therefore, I performed a comprehensive analysis of HDACs in *A. oryzae* with the aim of elucidating their function.

I used BLAST analysis to identify HDACs homologs in the *A. oryzae* genome (*A. oryzae* histone deacetylases: AoHDACs) and found 11 AoHDACs. I successfully disrupted 10 of the 11 AoHDACs, but only heterokaryon transformants were obtained in the case of *hdaB/Aorpd3*.

I tested the basic phenotypes of the disruptants such as growth, conidiation. I also tested secondary metabolite (SM) production of AoHDAC disruptants. The *hdaD/Aohos2* and *hstD/Aohst4* disruptants showed defects in conidia formation and a high level of kojic acid production, which is a major SM of *A. oryzae*. I also found that *hstD/Aohst4* coordinates the secondary metabolism and development through the regulation of *laeA*, which is key coordinator of secondary metabolism and development in filamentous fungi.

I also examine the various kinds of phenotypes included stress resistance, drug resistance, and rice-*koji* making using these AoHDAC disruptant. From these results, four AoHDACs, *hdaA/Aohda1*, *hdaB/Aorpd3*, *hdaD/Aohos2*, and *hstD/Aohst4*, were shown to be involved in diverse *A. oryzae* cellular processes such as the stress response,

cell wall synthesis, protein secretion, and genome integrity.

This thesis identified several functions of HDACs in *A. oryzae*. Because many types of HDACs are conserved in diverse organisms, these findings will be useful not only in understanding the function of HDACs in *A. oryzae*, but also in other filamentous fungi and other organisms. In contrast to these conserved HDACs, I showed fugal-specific role of fungal-specific HDAC like *hstD/AohstD*. This finding suggests the importance of fungal-specific HDACs in the fungal specific phenotypes.

INTRODUCTION

Eukaryotic DNA is packaged into chromatin, limiting transcriptional activity (Cairns 2009). Histone acetylation is one of the most important modifications to regulate chromatin accessibility. It is controlled by two opposing enzymes, histone acetyltransferases (HATs) and HDACs. These are conserved in a wide range of organisms from yeast to humans and also filamentous fungi (Brosch et al. 2008; Frye 2000; Gregoretti et al. 2004; Nishida 2009; Shahbazian and Grunstein 2007). Acetylation is usually associated with transcriptional activation, while, by contrast, histone deacetylation controls transcriptional repression.

HDACs remove the acetyl moiety from the lysine residue of a histone tail, and also deacetylate many non-histone substrates (Yang and Seto 2008). Protein deacetylation affects diverse cellular processes such as development, metabolism, and stress responses in eukaryotic cells. In mammalian and yeast cells, HDACs are divided into two major families, the sirtuins and the classical HDACs, which are phylogenetically classified into four classes. The sirtuins constitute class III, and the classical HDACs are grouped into classes I, II, and IV (Ekwall 2005; Yang and Seto 2008). With the exception of mammalian specific class IV HDACs, these enzymes are also conserved in the genome of filamentous fungi (Borkovich et al. 2004; Brosch et al. 2008).

Filamentous fungi include a diverse range of species, many of which are important to human life. Some are used in industry to produce fermented foods, commercial enzymes, and useful chemicals, while others are human and plant pathogens (Hoffmeister and Keller 2007; Iwashita 2002; Pagiotti et al. 2011; Raffaele and Kamoun 2012). Recently, the role of HDAC in fungal development, conidiation, the stress response, secondary metabolism, and virulence has been demonstrated in filamentous

fungi (Ding et al. 2010; Gacek and Strauss 2012; Li et al. 2010; Shimizu et al. 2012; Tribus et al. 2010; Tribus et al. 2005).

A. oryzae is one of useful filamentous fungi. It has been used for more than 1,000 years in the traditional food industries. Because of its long history in food production, it is determined to be generally recognized as safe (GRAS) by the US Food and Drug Administration and its safety is also documented by the World Health Organization (Machida et al. 2008). *A. oryzae* can produce high levels of enzymes and beneficial secondary metabolites (SMs) such as kojic acid and WYK-1 (Christensen et al. 1988; Imamura et al. 2012; Terabayashi et al. 2010). Such commercial importance has resulted in many investigations into its molecular biology and physiology. However, while the regulation of genes involved in *A. oryzae* metabolite or enzyme production is an attractive topic of study, its epigenetic regulation, including histone acetylation, has been not investigated.

In this thesis, therefore, I studied the function of *A. oryzae* HDACs. In Chapter I, I describe the identification of 11 HDAC homologs in the *A. oryzae* genome. I attempted to disrupt these AoHDACs and succeeded for 10 of the AoHDACs and heterokaryon transformants were obtained in the case of *hdaB/Aorpd3*. Using these disruptants, their basic phenotypes, including growth and conidiation, were studied. The SM production of AoHDAC disruptants was also tested, because regulation of SM production is often associated with fungal development (Bayram and Braus 2012). As the result, *hdaD/Aohos2* and *hstD/Aohst4* disruptants were shown to have defective conidia formation and a high production of kojic acid, which is a major SM of *A. oryzae*. Recently, it was reported that *laeA* regulates kojic acid production in *A. oryzae*, so the genetic interaction between *laeA* and *hstD/AohstD* was also focused. As the result, I

concluded that *hstD/Aohst4* regulate SM production and development through the control of *laeA*.

In the higher eukaryote, HDACs play key roles in the regulation of fundamental cellular process such as development regulation, stress response and genome integrity. Therefore, AoHDACs have more divergent role indicated in Chapter I. In the Chapter II, thus, I examine various phenotypes including stress resistance, drug resistance, and rice koji production using the AoHDAC disruptants. This analysis showed that the four AoHDACs *hdaA/Aohda1*, *hdaB/Aorpd3*, *hdaD/Aohos2*, and *hstD/Aohst4* are involved in diverse cellular process such as the stress response, cell wall synthesis, protein secretion, and genome integrity in *A. oryzae*.

CHAPTER I
Phylogenetic analysis of AoHDACs and its role in growth, conidiation and secondary metabolism in *A. oryzae*

I.1 ABSTRACT

The filamentous fungus *A. oryzae* has been used in a number of industries such as the production of traditional Japanese foods and pharmaceutical manufacturing. In higher model organisms, HDACs are concerned with multiple cellular processes such as development, metabolism, and the stress response, so can also be expected to be important in *A. oryzae*. In this chapter, I describe the identification of 11 HDACs homologs in the *A. oryzae* genome (AoHDACs) using BLAST analysis. Ten of these AoHDACs were successfully disrupted, but only heterokaryon transformants were obtained for *hdaB/Aorpd3*.

Basic phenotypes of these disruptants were investigated; including growth and conidiation, as well as SM production because its regulation is often associated with fungal development. The *hdaD/Aohos2* and *hstD/Aohst4* disruptants were defective in conidia formation and produced high levels of the SM kojic acid. I also showed that the gene expression of *laeA*, which is the most studied fungal-specific coordinator for the regulation of secondary metabolism and fungal development, was induced in the *ΔhstD* strain. Genetic interaction analysis of *hstD/Aohst4* and *laeA* clearly indicated that *hstD/Aohst4* works upstream of *laeA*. Thus, I concluded that the fungal-specific sirtuin *hstD/Aohst4* coordinates fungal development and secondary metabolism via the regulation of *laeA* in filamentous fungi.

I.2 INTRODUCTION

Histone acetylation plays key roles in the control of chromatin structure and function (Shahbazian and Grunstein 2007). The acetylation state is controlled by two histone modification enzymes with opposing actions, HATs and HDACs. Acetylation is generally associated with transcriptional activation. In contrast, histone deacetylation is generally associated with transcriptional repression. These enzymes are highly conserved from yeast to humans, and they are also conserved in filamentous fungi (Borkovich et al. 2004; Brosch et al. 2008; Ekwall 2005; Nishida 2009).

HDACs remove the acetyl moiety from the lysine residue of a histone tail. In addition to histones, these enzymes deacetylate many non-histone substrates (Yang and Seto 2008). Protein deacetylation affects diverse cellular process such us development, metabolism, and stress responses in eukaryotic cells (Yang and Seto 2008). In mammalian and yeast cells, HDACs are divided into two major families called the sirtuins and the classical HDACs. The HDACs are phylogenetically classified to four classes. The sirtuins constitute class III, and the classical HDACs are grouped into classes I, II, and IV (Ekwall 2005; Yang and Seto 2008). Except for mammalian specific class IV HDACs, these enzymes are also conserved in the genome of filamentous fungi (Borkovich et al. 2004; Brosch et al. 2008).

Recently, HDACs in some filamentous fungi have been investigated for their role in the regulation of histone modification, developmental processes, stress resistance, pathogenesis, metabolism, and other such processes (Brosch et al. 2008; Ding et al. 2010; Izawa et al. 2009; Li et al. 2010; Smith et al. 2008). For example, the homolog of yeast *rpd3* is required for growth and conidiation in several filamentous fungi (Tribus et al. 2010). The class II HDAC *hdaA/Afhda1,* a homolog of yeast *hda1*, is involved in

germination and the oxidative stress response in *Aspergillus fumigates* (Lee et al. 2009; Tribus et al. 2005). The yeast *hos2* homolog is required for conidial development, invasive growth, and the production of virulence factors in some plant-pathogenic filamentous fungi (Baidyaroy et al. 2001; Ding et al. 2010; Li et al. 2010).

Filamentous fungi produce wide varieties of SMs, which are small bioactive molecules that include both beneficial medicines and cosmetics, and toxins that are harmful for animals and plants (Hoffmeister and Keller 2007). Therefore, because of the importance of fungal SMs, there has been much research into the mechanisms that regulate their production. *LaeA*, a putative fungal-specific methyltransferase, is implicated in the global regulation of SM production (Bayram and Braus 2012; Sanchez et al. 2012). *LaeA* also has an important role in coordinating fungal development and SM production (Bayram and Braus 2012). Recent studies have shown that histone modification plays key roles in the regulation of SM biosynthetic genes expression (Gacek and Strauss 2012). In *Aspergillus nidulans*, the HDACs *hdaA/Anhda1* and *sirA/Ansir2* regulate carcinogenic sterigmatocystin and antibiotic penicillin production (Shimizu et al. 2012; Shwab 2007). The loss of *hdf1/Fghos2* reduces conidial development and the production of deoxynivalenol, which is the most characterized virulence factor in *Fusarium graminearum* (Li et al. 2010).

However, the importance of fungal HDACs in the regulation of secondary metabolism and fungal development is still not known; studies of these HDACs are limited even though several types of HDACs are found in fungal genomes (Brosch et al. 2008). Moreover, the relationship between the global regulator *laeA* and histone modification is still poorly understood.

In the Chapter I, I examined the phenotypes caused by the disruption of all HDACs

using *A. oryzae*, which is an important filamentous fungus in industry and has potential for the production of pharmaceutical and cosmetic SMs (Abe et al. 2006; Imamura et al. 2012; Machida et al. 2008). My observations indicated that the fungal-specific sirtuin *hstD/Aohst4* regulates conidial development and kojic acid production, which is an important cosmetic material for preventing melanogenesis in skin, as well as antimicrobial penicillin production (Terabayashi et al. 2010). I also performed microarray analysis of *ΔhstD* to examine the global function of this sirtuin and found that the disruption of this gene affects the expression of many metabolite genes. As described above, *laeA* is an important coordinator for the regulation of secondary metabolism and development. In this context, I also analyzed the genetic interaction between *hstD/Aohst4* and *laeA* and found that *hstD/Aohst4* regulates *laeA* expression. I first describe the function of fungal-specific sirtuin *hstD/Aohst4* in SM production and then on conidial development through the regulation of *laeA* gene expression.

I.3MATERIALS AND METHODS

I.3.1 Strains, media, physiological tests

The strains used in the Chapter I are listed in Table I.6.1. *A. oryzae* RIB40 was used as the DNA donor. The *A. oryzae NSR-ΔLD2* strain was used as the host for AoHDACs disruption (Maruyama and Kitamoto 2008). M + Met medium or M + Ade medium (M + Ade medium containing 0.5 g of adenine sulfate dihydrate instead of L-methionine was used as the selectable medium for *A. oryzae adeA*$^+$ transformants and *A. oryzae sC*$^+$ transformants, respectively (Maruyama and Kitamoto 2008). TS medium (6 g of $NaNO_2$, 0.52 g of KCl, 1.52 g of KH_2PO_4, 0.52 g of $MgSO_4 \cdot 7H_2O$, 10 g of glucose, 1 ml of trace elements, pH 6.5, in 1 L) was used as the selectable medium for *A. oryzae adeA*$^+$ *sC*$^+$ transformants. M + Met or M + Ade or TS medium with 0.8 M NaCl added was used for transformation. KAS medium (15 g of Tryptone, 1.52 g of K_2HPO_4, 1.5 g of L-methionine, 0.5 g of $MgSO_4 \cdot 7H_2O$, 100 g of glucose, 1 ml of trace elements, pH 6.5, in 1 L) was used for screening HDAC-affected kojic acid productivity. KA medium (1 g of yeast extract, 1 g of K_2HPO_4, 1.5 g of L-methionine, 0.5 g of $MgSO_4 \cdot 7H_2O$, 100 g of glucose, 0.5 g of adenine sulfate dihydrate, pH 6.0, in 1 L) was used to test for KA productivity and RNA preparation. N medium (3 g of L-glutamic acid, 0.52 g of KCl, 1.52 g of K_2HPO_4, 0.52 g of $MgSO_4 \cdot 7H_2O$, 30 g of glucose, 1.5 g of L-methionine, 1 ml of trace elements, pH 6.5, in 1 L) was used for morphological analysis. TSB medium (30 g of Tryptic soy broth, 1.5 g of L-methionine, 3 g of L-glutamic acid, 0.5 g of adenine sulfate dihydrate, pH 7.5, in 1 L) was used for the penicillin bioassay and RNA preparation.

Morphological analysis was performed in 20 ml of 2% agar N plate or 100 ml of N

liquid medium. Three independent disruptants were used for each experiment. For the spore count, conidia suspensions of each strain were point inoculated (1×10^5 conidia) on the center of each plate, and the strain was grown for 5 days at 30°C. Colony diameters were measured at this time, and the spores were harvested in suspension solution (0.025% Tween-80/0.5% NaCl), vortexed vigorously, and counted using a TC10 automated cell counter (Bio-Rad). The conidiation rate was calculated by the conidia number/ radial growth area (cm^2). For biomass analysis in N liquid medium, 4 cm^2 of full-growth colonies in plate cultures of each strain were cut and homogenized in 1 ml of suspension solution and then used to inoculate each flask. Flasks were incubated for 2 days at 30°C with shaking at 100 rpm. Then, mycelia were harvested, dried at 105°C for 2 h, and weighed.

I.3.2 Protein identification, domain prediction, and phylogenetic analysis

HDAC sequences of *Saccharomyces cerevisiae* were obtained from the *Saccharomyces* genome database (http://www.yeastgenome.org/). HDAC sequences of *H. sapiens*, *Neurospora crassa*, and *A. nidulans* were obtained from the NCBI proteins database (http://www.ncbi.nlm.nih.gov/guide/proteins/), the *Neurospora crassa* database (http://www.broadinstitute.org/annotation/genome/neurospora/MultiHome.html), and AspGD (http://www.aspgd.org/), respectively. HDAC genes of *A. oryzae* were identified from the Comparative fungal genome database (CFGD; http://nribf2.nrib.go.jp/) by BLAST searching using HDAC sequences of *S. cerevisiae* and *H. sapiens* as the query. The sequence of these HDACs in *A. oryzae* was verified by RNA sequencing using SOLiD3 (Applied Biosystems). The gene structure

of all AoHDACs was confirmed by RNA sequence data (details of my RNA-seq data are available in AspGD) (Arnaud et al. 2010). The mapping data of all reads are also available in CFGD. The protein sequence of the HDAC homolog in *A. oryzae* was analyzed for recognizable domains using Interproscan (Quevillon et al. 2005). The protein sequence of the *hstD* homolog in filamentous fungi was identified using NCBI blast with the pezizomycotina genomes (http://www.ncbi.nlm.nih.gov/sutils/genom_tree.cgi) using the amino acid sequence of *hstD* as a query.

For the classification of HDACs in *A. oryzae*, protein sequences of HDACs in *S. cerevisiae*, *N. crassa*, *A. nidulans*, and *A. oryzae* were aligned with ClustalW software in the Molecular Evolutionary Genetic Analysis 5 (MEGA5) program (Tamura et al. 2011). Phylogenetic analysis was carried out using the neighbor-joining method with 1000 bootstrap replicates by the MEGA5 program. For the classification of *hstD/Aohst4* in filamentous fungi, alignment and phylogenetic analysis was performed as described above. A list of sequence accession numbers used for AoHDACs analysis is in Table I.6.2, and sequence accession numbers of each *hstD* homolog are described in Fig. I.6.6.

I.3.3 RNA preparation

KA culture was performed in 20 ml of KA liquid medium inoculated with 200 µl of 1×10^8 conidia /ml suspension and incubated at 30°C for 4 or 7 days with shaking at 130 rpm. TSB culture was performed in 40 ml of TSB liquid medium inoculated with 400 µl of 1×10^8 conidia /ml suspension and incubated at 30°C for 1 day with shaking at 200 rpm. After cultivation, mycelia were harvested using Miracloth (Merck). Then, mycelia were immediately frozen in liquid N_2 and ground to a fine powder. Total RNA

was isolated from mycelia from KA or TSB liquid media using Isogen (Nippon Gene) according to the manufacturer's instructions.

I.3.4 Northern hybridization

Denatured total RNA (20 μg) was electrophoresed on a formaldehyde-agarose gel and transferred in 20 × SSC onto a Hybond N^+. Northern analysis was performed with a Detection starter kit II (Roche) according to the manufacturer's instructions. DIG-labeled probes were prepared using a PCR digoxigenin probe synthesis kit (Roche) with genomic *A. oryzae* RIB40 DNA as the template and the primers X-probe-F and X-probe-R. The letter X means the respective gene for northern analysis. Each blot was imaged using Luminescent Image Analyzer LAS1000plus (FUJIFILM). A list of the primers used for these PCRs is shown in Table I.6.3.

I.3.5 Construction of the disruption cassette

Each disruption cassette was constructed by fusion PCR of three mutually primed DNA fragments, the 5' and 3' flanking region of the target genes and the *adeA* fragment (Szewczyk et al. 2007). About 1 kb of the 5' and 3' flanking region of the target genes and the *adeA* gene were amplified from genomic *A. oryzae* RIB40 DNA with primers X-A and X-B, X-C and X-D, and adeA-F and adeA-R, respectively. Only for the construction of the *ΔhstDΔlaeA*, *A. nidulans sC* gene, amplified from pUSA with sC-F and sC-R, was the gene fused to the flanking region of *laeA* (Yamada et al. 2003). The letter 'X' in the primer names represents the name of each target gene. Each region was amplified by KOD Plus DNA polymerase (TOYOBO). These fragments were combined by a second PCR with KOD Plus DNA polymerase and the primers X-A and X-D or

X-A2 and X-D2. The amplified fragment was purified by the QIAquick PCR purification kit (QIAGEN) and then used as a disruption cassette. A list of the primers used for these PCRs is shown in Table I.6.3.

I.3.6 Complementation of *hstD/Aohst4*

To recover the native locus of *hstD/Aohst4*, I first amplified the same 5' flanking region of the disruption construct of the *hstD/Aohst4* and *adeA* fragments from RIB40 DNA with the primers hstD-A and hstD-compB or adeA fusion sC-F and adeA-R, respectively. I also amplified the *A. nidulans* sC gene from pUSA with sC-F and sC-R as autotrophic markers (Yamada et al. 2003). Then, these fragments were combined by fusion-PCR using nested-adeA-R and hstD-A2 primers (Szewczyk et al. 2007). The amplified fragment was purified by a QIAquick PCR purification kit and used as a complementation cassette. A list of the primers used for these PCRs is shown in Table I.6.3.

I.3.7 Construction of overexpression plasmids

The ORFs of *laeA* or *hstD/Aohst4* were amplified from genomic *A. oryzae* RIB40 with Fusion-laeA-F and Fusion-laeA-R or Fusion-hstD-F and Fusion-hstD-R, respectively. The resulting fragments were fused into *Sma*I-cut pUSA using an In-Fusion HD cloning kit (TAKARA) (Yamada et al. 2003). The *amyB* promoter of pUSA was used to drive the overexpression of *laeA* and *hstD/Aohst4,* respectively (Jin et al. 2011). The resulting plasmids, pUSlaeA and pUShstD*,* were linearized with one cut restriction enzyme of *Bgl*II and *Eco*T22I on *laeA* or *hstD/Aohst4* ORF, respectively. The resulting linearized fragments were used as overexpression cassettes. A list of

primers used for these PCRs is shown in Table I.6.3.

I.3.8 Transformation of *A. oryzae*

Transformation of *A. oryzae* strains was performed using the protoplast-polyethylene glycol method (Kitamoto 2002). To verify the disruption of the target gene, direct colony PCR was performed using primers (X-F and X-G; X-A and X-D), KOD-FX (TOYOBO), and a crude DNA sample of each transformant. Primers X-F and X-G were designed at the region of each target gene. The crude DNA sample was prepared as follows: conidia and hyphae from each transformant culture were suspended in 100 μl Buffer A [100 mM Tris-HCl (pH 9.5), 1 M KCl, 10 mM EDTA]. This mycelia suspension was vortexed vigorously and incubated at 95°C for 10 min. Immediately thereafter, this hot solution was vortexed vigorously and centrifuged at 5,000 rpm for 1 min. A total of 1 μl of supernatant was used as the crude DNA sample. A list of primers used for these assays is shown in Table I.6.3.

I.3.9 Time-lapse imaging

For time-lapse imaging, conidiophores were germinated in 1.5% agar N medium in 35 mm glass-bottom dishes. Cells were imaged using a real-time cultured cell monitoring system (ASTEC) controlled by CCM software. DIC images of each disruptant were taken every 20 min for approximately 84 h. All imaging was carried out at 30°C. Pictures and movies were edited with CCM software (ASTEC).

I.3.10 Secondary metabolite analysis

For the plate assay of kojic acid production, conidia suspensions of each strain

were point inoculated (1×10^5 conidia) on the center of 2% agar KAS and KA medium containing 5 mM $FeCl_3$ and grown for 5 days at 30°C. KAS and KA medium were used for screening and genetic interaction analysis, respectively. Then, a red halo, which indicates the existence of kojic acid, was observed.

For the quantification of kojic acid, 20 ml of KA liquid medium inoculated with 200 µl of 1×10^8 conidia /ml suspension was incubated at 30°C with shaking at 130 rpm. After cultivation for the appropriate period, mycelia were filtered by Miracloth (Merck), and then the filtrate was collected. Harvested mycelia were dried at 105°C for 1 h and weighed. The collected KA medium was filtered by MillexHV (Millipore), and then the kojic acid concentration was quantified by colorimetric methods.

I.3.11 Microarray analysis

The *A. oryzae* GeneChip (AoDNAChip; NCBI GEO platform GPL16184) was designed by Affymetrix to refer to the entire genome sequence of *A. oryzae*, and predicted ORFs are published at the Comparative fungal genome database (http://nribf2.nrib.go.jp/, *A. oryzae* RIB40 Ace33v2). The AoDNAchip covered 13,765 ORFs and 6143 promoters of *A. oryzae*. In the Chapter I, I performed transcriptome analysis using probes of 13,765 ORFs set on this microarray.

Total RNA using microarray analysis was purified by using an RNeasy mini kit (QIAGEN). RNA quality was determined by using a BioAnalyzer 2100 (Agilent Technology), and the quantity was determined by using an Ultrospec 3300 pro (Amersham Pharmacia Biotech). Fragmentated biotin labeled cRNA was prepared by using a GeneChip One-cycle Target Labeling and Control Reagent Kit (Affymetrix) according to manufacturer's instructions. The fragmentated cRNA was hybridized to an

AoDNAchip. Then, this GeneChip was washed, stained, and scanned by using a GeneChip Fluidics Station FS-450 (fluidics protocol FS450_001) and a GeneChip Scanner 3000.

Scanned probe array images were converted into CEL files and normalized by using GCOS v.1.4 (Affymetrix). Calculations of signal intensity and detection *p*-values were also performed using GCOS v.1.4. The trimmed mean signal of the array was scaled to the target signal of 500 with the All Probe Sets scaling option. Detection call was used in detection of a particular transcript, with a detection $p < 0.04$ as present (P), $0.04 \leq p < 0.06$ as marginal (M), and $0.06 \leq p$ as absent (A). These calculation data were exported as CHP files. For microarray data analysis, CHP files were imported into GeneSpring7.3 (Agilent Technologies). Expression data were normalized per chip to the 50th percentile. In the Chapter I, I analyzed genes detected as P or M flags. Genes with statistically significant changes in transcript abundance were identified using a cutoff value of 2-fold and Welch's *t*-test value of less than 5%. FungiFun software was used for FunCat categorization (Priebe et al. 2011; Ruepp et al. 2004). Significantly enriched FunCat categories were extracted using FungiFun software (cut-off $p < 0.05$; the *p*-value indicates the significance of the number of hits for each category in the dataset taking the number of hits for the whole genome of *A. oryzae* as a background. The calculation is based on a two-tailed Fisher's exact test). The distribution of whole *A. oryzae* genes was indicated in Fig. I.6.8. and I.6.9, which was used as a reference of FunCat enrichment analysis. Two biological replicates were used for the microarray analysis. The microarray data have been deposited in NCBIs Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO series accession number GSE41612.

I.4 RESULTS

I.4.1 Phylogeny and morphology of AoHDACs

A total of 11 HDACs (AoHDACs: *A. oryzae* histone deacetylases) were found in the *A. oryzae* genome based on BLAST analysis. I classified AoHDACs according to a previous phylogenetic study of HDACs. These AoHDACs were phylogenetically divided into class I to III HDACs, but the mammal-specific class IV HDACs were not found in the genome. Class III HDACs are generally described as sirtuin-type HDACs, and 6 AoHDACs belonged to this class. The AoSirtuins (*A. oryzae* sirtuins) were classified into classes I to III, but class IV sirtuins were not found in the genome. The class I sirtuins were further categorized into three sub-classes, including the fungal-specific HDACs of sirtuin sub-class C (Fig. I.6.1). I attempted to disrupt these 11 AoHDACs and succeeded for 10 of the AoHDACs. However, only heterokaryon transformants were obtained in the case of *hdaB/Aorpd3* over several trials (data not shown). This result suggests that *hdaB/Aorpd3* is essential in *A. oryzae,* but further experiments are required to confirm this hypothesis. Thus, in this report, the heterokaryon disruptant of *hdaB/Aorpd3* and AoHDACs disruptants were used for subsequent experiments.

I first observed the growth and conidia generation of AoHDAC disruptants and *hdaB/Aorpd3* heterokaryon transformants on the plate culture (Figs. I.6.1-5). Observation of the growth of AoHDACs disruptants revealed an obvious defect of *ΔhstD* and *ΔhdaD* in morphogenesis (Fig. I.6.2A). The conidial formation of these disruptants was significantly decreased and a slight growth effect was observed in plate cultivation (Fig. I.6.2B, C). I additionally observed the growth of AoHDACs disruptants and the *hdaB/Aorpd3* heterokaryon transformant in liquid culture (Fig. S2D). A growth

defect of *ΔhdaD* was observed in submerged cultivation (Fig. I.6.4D). Thus, *hdaD/Aohos2* is required for the growth integrity of *A. oryzae*. These two disruptants were further examined using time-lapse imaging for more detailed observation. As expected, *ΔhdaD* showed slow growth but exhibited more crowded aerial hyphae than the wild-type strain (Fig. I.6.4A). However, a few invasive hyphae were detected in the *ΔhstD* strain but not in *ΔhdaD*. In this time-lapse imaging analysis, a significant defect of conidial development was also found in both *ΔhdaD* and *ΔhstD* strains.

These results indicate that both *hdaD/Aohos2* and *hstD/Aohst4* play an important role in the growth and development of *A. oryzae,* especially in asexual development.

I.4.2 Fungal-specific sirtuin regulates SM production

I further examined SM production of AoHDAC disruptants and the *hdaB/Aorpd3* heterokaryon transformant using a plate assay for kojic acid productivity (Fig. I.6.3). High production of kojic acid was observed in *ΔhdaB* and *ΔhstA* (Fig. I.6.3), and significant overproduction was observed in *ΔhdaD* and *ΔhstD* (Fig. I.6.2A). I quantified the kojic acid production of these two HDACs disruptants, and the *ΔhstD* strain showed a 200-fold increased productivity in a 7-day culture (Fig. I.6.2D). In contrast, the *ΔhdaD* strain showed a 30-fold overproduction compared with the control strain (Fig. I.6.2D). The *ΔhstD* strain started to produce kojic acid by 4 days in culture, while kojic acid was not detected in the wild-type strain.

I further analyzed kojic acid production at the gene expression level by examining three key genes in the kojic acid gene cluster (Terabayashi et al. 2010). Northern analysis of 4-day cultures showed extremely high expression of these genes in the

ΔhstD strain, but no expression was observed in the wild-type strain (Fig. I.6.2E). The *kojA* gene was also expressed in the *ΔhdaD* strain, but the expression was not as high as in the *ΔhstD* strain. This earlier and higher expression in both disruptants is consistent with the earlier and higher production of kojic acid.

These results indicate the importance of *ΔhdaD* and *ΔhstD* in the regulation of kojic acid production. On the basis of phylogenetic classification, *hstD/Aohst4* belongs to the fungal-specific class of sirtuins, and this protein is widely conserved in filamentous fungi (Frye 2000) (Fig I.6.6). Interestingly, *nst3*/*Nchst4* is involved in the silencing mechanism of *N. crassa* (Smith et al. 2010). Moreover, the phenotype of *ΔhstD* was reminiscent of the global use of HSTD/AOHST4 for the production of various SMs in *A. oryzae*.

In this context, I examined the regulation of penicillin biosynthesis in the *ΔhstD* strain. As expected, a higher production of penicillin was found in the *ΔhstD* strain, and a higher expression of the penicillin biosynthetic gene was confirmed (Fig. I.6.2F, G). Along with the morphogenetic defect and kojic acid production, these phenotypes were rescued by the complementation of *hstD/Aohst4* (Fig. I.6.5).

These results suggest that *hstD/Aohst4* is required for the global regulation of SMs biosynthesis. Thus, I examined *ΔhstD* by microarray analysis to investigate the expression of other SM-related genes. The expression of 388 genes were significantly affected by *hstD/Aohst4* deletion (absolute fold change >2, $p < 0.05$) (Table I.6.4). These genes were spread across the whole genome; 299 of 388 genes were up-regulated in the *ΔhstD* strain. To reveal the functional distribution of *hstD/Aohst4* affected genes, FunCat enrichment analysis was carried out using FungiFun software (Priebe et al.

2011; Ruepp et al. 2004) (Fig. I.6.7). The “C-compound and carbohydrate metabolism” and “Secondary metabolism” categories were significantly enriched in down-regulated genes. Most of the genes categorized as “Secondary metabolism” overlapped with “C-compound and carbohydrate metabolism” categorized genes. Genes categorized as “C-compound and carbohydrate metabolism” were mainly related to polysaccharide degradation or glycolysis. This result suggested that carbon source degradation related genes were down-regulated in the *ΔhstD* strain. “Secondary metabolism” and “detoxification” categories were significantly enriched in genes up-regulated by *hstD* deletion. Genes categorized “Secondary metabolism” and “detoxification” were mainly constituted by cytochrome P450 (CYP) genes. Interestingly, three non-ribosomal peptide synthetases (NRPS) and one polyketide synthase (PKS) were also up-regulated in the *ΔhstD* strain. One of three NRPSs encoded *wykN* (AO080501000008), which is involved in WYK-1 production in *A. oryzae* (Imamura et al. 2012). PKS and NRPS enzymes generate the general structural scaffolds of most secondary metabolites (Brakhage 2013). Additionally, fungal CYPs have PKS and NRPS associated functions, and generate structural variation of fungal SMs. Thus, *hstD/Aohst4* affects many kinds of SM production (at least 6 different SM genes clusters) (Kelly et al. 2009; Podust and Sherman 2012).

I.4.3 *hstD/Aohst4* regulates the expression of *laeA*

The LAEA complex coordinates the development and SM biosynthesis in *A. nidulans*, and is conserved in numerous fungal genomes (Bayram and Braus 2012). Recently, it was reported that deleting *laeA* diminishes kojic acid production and gene expression in *A. oryzae* (Oda et al. 2011). Additionally, penicillin production in *A.*

oryzae is diminished after deletion of *veA*, which is a member of the LAEA complex (Bayram and Braus 2012; Marui et al. 2010). These reports suggest that the LAEA complex also plays a general role in the induction of the SM genes cluster in *A. oryzae*. Thus, the SM overproduction phenotype of the *hstD/Aohst4* disruptant should be associated with the LAEA complex, and HSTD/AoHST4 may play a role under the control of LAEA. According to this hypothesis, the expression of *laeA* should not be altered in the *hstD/Aohst4* disruptant. Thus, I examined the expression of *laeA* in the *hstD/Aohst4* disruptant. Surprisingly, *laeA* was highly expressed in the *hstD/Aohst4* disruptant, even in the 4-day culture, but remained unexpressed in the wild-type strain (Fig. I.6.10A). This result suggests that HSTD/AoHST4 is involved in *laeA* gene repression. Thus, both SM overproduction phenotypes and the altered morphological phenotype of the *ΔhstD* strain are caused by the high expression of *laeA*.

I.4.4 Genetic interaction of *hstD/Aohst4* and *laeA*

To confirm the above hypothesis, I prepared a *ΔlaeA* strain and a *ΔhstDΔlaeA* double disruptant, and then examined the SM production and conidial development. At first, these strains exhibited the phenotype of the *ΔlaeA* strain, including a lack of kojic acid production (Fig. I.6.10A), similar to previous reports (Oda et al. 2011). The production of penicillin and conidial development was also lost in the *ΔlaeA* strain (Fig. I.6.10B, C). Next, I observed the effect of *laeA* disruption in the *ΔhstD* background and found that the SM overproduction phenotype of *ΔhstD* was abolished by *laeA* disruption (Fig. I.6.10B, D). I further observed conidial development in the double disruptant, which exhibited a *ΔlaeA*-like fluffy phenotype (Fig. 1.6.10B, C). These results clearly

indicate a genetic interaction between *hstD/Aohst4* and *laeA* and that HSTD/AoHST4 plays a role upstream of *laeA*.

To confirm this epistatic relationship, I examined the effect of *laeA* and *hstD/Aohst4* overexpression using *amyB* promoter, respectively. The *laeA*-overexpressed strain exhibited *ΔhstD*-like phenotypes such as SM overproduction and low conidial formation (Fig. I.6.11). The effects of *laeA* overexpression were also observed in the *ΔhstD* background (Fig. I.6.11). Additionally, both the *OE::laeA* strain and *ΔhstD OE::laeA* strain showed high expression of *laeA*, *kojA*, and *ipnA*, respectively (Fig. I.6.12). These results indicated that *laeA* is downstream of *hstD/Aohst4*. Therefore, I overexpressed *hstD/Aohst4* in a *laeA* disruption background. As expected, the overexpression of *hstD/Aohst4* resulted in a *ΔlaeA*-like phenotype, such as no SM production and a fluffy morphology, in the *ΔlaeA* background (Fig. I.6.12). Overexpression of *hstD/Aohst4* in the wild-type *laeA* background had no measurable effect on SM production and development (Fig. I.6.12.).

From these results, I suggest that the fungal-specific sirtuin HSTD/AoHST4 controls SM production and fungal development through the regulation of *laeA* gene expression (Fig. I.6.13).

I.5 DISCUSSION

In the past decade, the importance of the role of classical HDACs in filamentous fungi in the regulation of some fungal phenotypes has been revealed (Brosch et al. 2008). For example, *F. graminearum hdf1/fghos2* is important for conidial development and SM production (Li et al. 2010). In the Chapter I, I also determined the importance of *hdaD/Aohos2* in the regulation of hyphal growth, conidiation, and SM production. Compared with the study of classical HDACs, only a few reports have been published for sirtuin-type HDACs in filamentous fungi (Shimizu et al. 2012; Smith et al. 2010). Recently, the *A. nidulans sirA/Ansir2*, which is a homolog of yeast *sir2* and mammalian *sirt1*, was reported to function in secondary metabolism regulation, but no effects on growth, conidiation, and morphogenesis were reported (Shimizu et al. 2012).

The fungal-specific putative methytransferase 'LAEA' coordinates fungal development and SMs in several filamentous fungi (Bayram and Braus 2012). Previously, it was reported that kojic acid production is regulated by LAEA, but I found that *laeA* coordinates both SM production and conidial development (Oda et al. 2011). In general, the production of fungal SMs is coordinated with fungal development, and the LAEA complex coordinates these (Bayram and Braus 2012). In higher eukaryotes, sirtuin affects various physiological functions, such as differentiation, metabolism, and the stress response (Horio et al. 2011; Imai and Guarente 2010; Zhang and Kraus 2010). In the Chapter I, I found that the fungal-specific sirtuin HSTD/AoHST4 affects both fungal development and SM production. Furthermore, the epistatic study revealed that HSTD/AoHST4 is involved in the coordination of fungal development and SM production via *laeA* expression. These results indicate that HSTD/AoHST4 plays a *sirt1*-like central role that coordinates the developmental state and metabolism in

filamentous fungi.

The yeast HST4, which is a homolog of *A. oryzae*'s *hstD/Aohst4*, is a NAD^+-dependent histone H3K56 deacetylase (Yang et al. 2008). In the northern analysis, deletion of *hstD/Aohst4* affected the gene expression level of *laeA*. Therefore, HSTD/AoHSTD may be involved in the epigenetic regulation of *laeA* gene expression. However, the overexpression of *hstD/Aohst4* did not affect SM production and conidial development. In general, histone acetyltransferase (HAT) is required for gene activation of silenced genes. In budding yeast, it has been reported that the fungal-specific HAT of RTT109 catalyzes H3K56 acetylation and restores the silencing defects of the *Δhst3Δhst4* mutant (D'Arcy and Luger 2011; Yang et al. 2008). I found one *rtt109* homologue of AO090020000581 (*Aortt109*) in the *A. oryzae* genome. This suggests that AoRTT109 is required for the expression of the silenced *laeA* by H3K56 deacetylation and overcomes even *hstD/Aohst4* overexpression. In this context, I will investigate histone H3K56 acetylation of the *laeA* locus and the effect of *Aortt109* on the acetylation and expression of *laeA* in future studies.

In the overexpression analysis of *laeA*, several different phenotypes of kojic acid production were observed compared with the *hstD/Aohst4* mutant. The *ΔhstD* strain showed high kojic acid production in a 4-day liquid culture while the same phenotype was not observed in the *OE::laeA* strain. Compared with the *OE::laeA* strain, interestingly, higher kojic acid production and higher expression of *kojA*, *ipnA,* and *laeA* were observed in the *ΔhstD OE::laeA* strain. These results suggested that HSTD/AoHST4 has some LAEA-independent role in the regulation of SM production.

I developed a model of the regulatory system of HSTD/AoHST4 (Fig. I.6.13). The expression of *hstD/Aohst4* may be induced or suppressed by unknown signals. As

described above, an unknown factor like AoRTT109 may compete with HSTD/AoHST4 activity in *hstD/Aohst4*-inducing conditions. In the *hstD/Aohst4* suppressed condition, *laeA* expression was induced. This in turn led to conidiation and secondary metabolism production being induced. However, it is possible that HSTD/AoHST4 directly regulates fungal development and secondary metabolism independent of *laeA*.

The *hstD/Aohst4* gene is fungal-specific but is conserved in the vast family of filamentous fungi (Fig. I.6.6). Furthermore, this gene plays a role in the coordination of fungal development and SM production. These results indicate that *hstD/Aohst4* has great potential as a target to improve the productivity of useful SMs. It is also important in the development of an attractive host for the production of several heterogeneous metabolites.

I.6 FIGURES AND TABLES

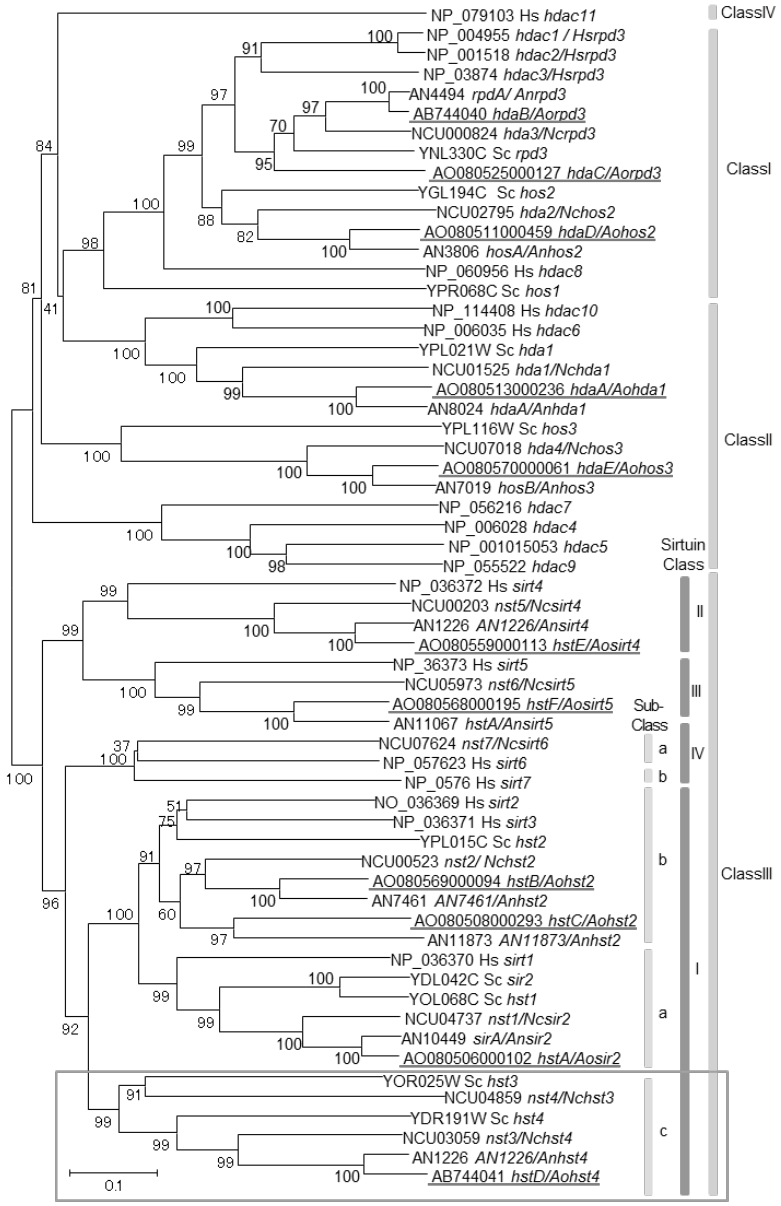

Figure I.6.1. Phylogenetic analysis of histone deacetylase in *A. oryzae*.
Accession numbers and HDAC names are indicated for each branch. HDAC names of *S. cerevisiae* or *H. sapiens* with the species name indicated followed by a slash. The numbers at the nodes are bootstrap values obtained from 1000 replicates and are indicated as percentages. The scale bar indicates a distance corresponding to 0.2 amino acid substitutions per site. The class or sub-class of HDACs is shown on the right. These classes of HDACs are referred to in previous phylogenetic studies (Ekwall 2005; Frye 2000; Yang and Seto 2008). AoHDACs are indicated by underlines. Abbreviations of AoHDACs gene names are as follows: Hda, Histone deacetylase; Hst, Homolog of sirtuin. The class to which *hstD* belongs is surrounded by a grey border. The gene names and their accession numbers are identified in Table I.6.2. Sc, *Saccharomyces cerevisiae*; An, *Aspergillus nidulans*; Nc, *Neurospora crassa*; Hs, *Homo sapiens*; Ao, *Aspergillus oryzae*.

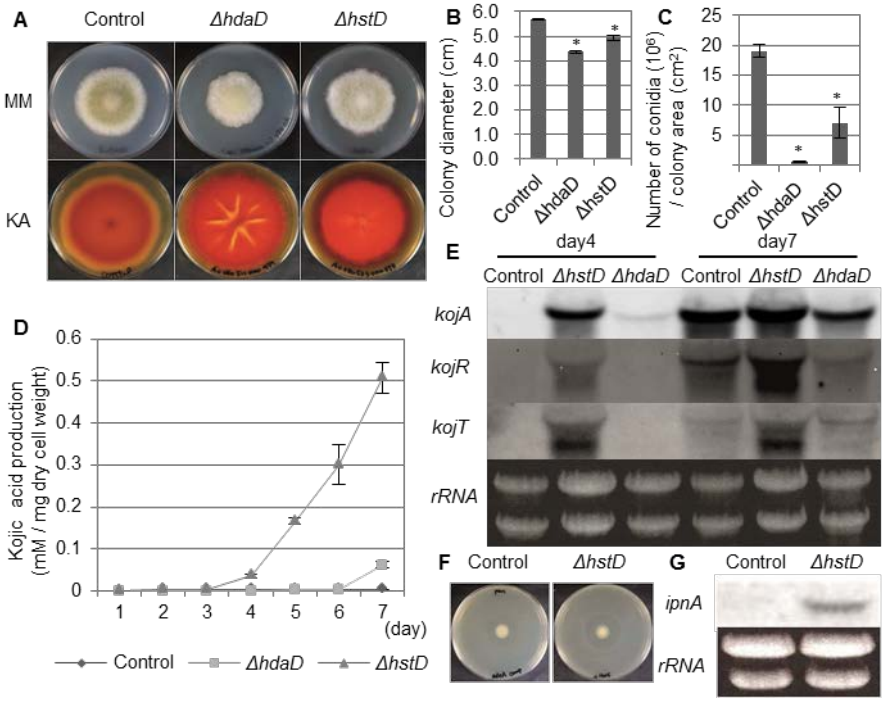

Figure I.6.2. *hstD/Aohst4* and *hdaD/Aohda1* regulate SM production and development.

(A) The panels for MM and KA show the morphological phenotype and kojic acid production plate assay of the indicated strain, respectively. **(B, C)** Radial growth and conidiation on MM of the indicated strains. **(D)** Time-course characteristics of kojic acid production of the indicated disruptants. **(E)** Expression profiles of kojic acid cluster genes represented by northern hybridization. The culture times of the indicated strains are shown at the top of the panel. The analyzed gene is indicated on the left side of each blot. *rRNA* is shown as the loading control. **(F)** Penicillin production bioassay of the *ΔhstD* strain. **(G)** Northern hybridization of the penicillin biosynthetic gene *ipnA* in the *ΔhstD* strain. *rRNA* is shown as the loading control. The *adeA*$^{+}$ strain was used as a control in this figure. All data are represented as means ± s.d. (n=3); *$p < 0.01$, t-test.

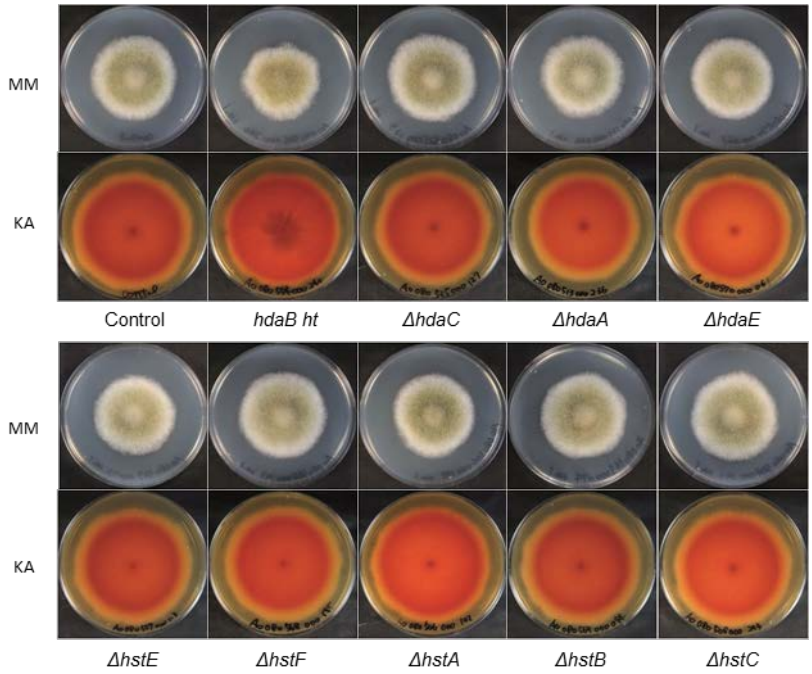

Figure I.6.3. Kojic acid production and developmental phenotype of AoHDACs disruptants and a heterokaryon strain.
MM and KA show the morphological phenotype and kojic acid production plate assays of the indicated strains, respectively.

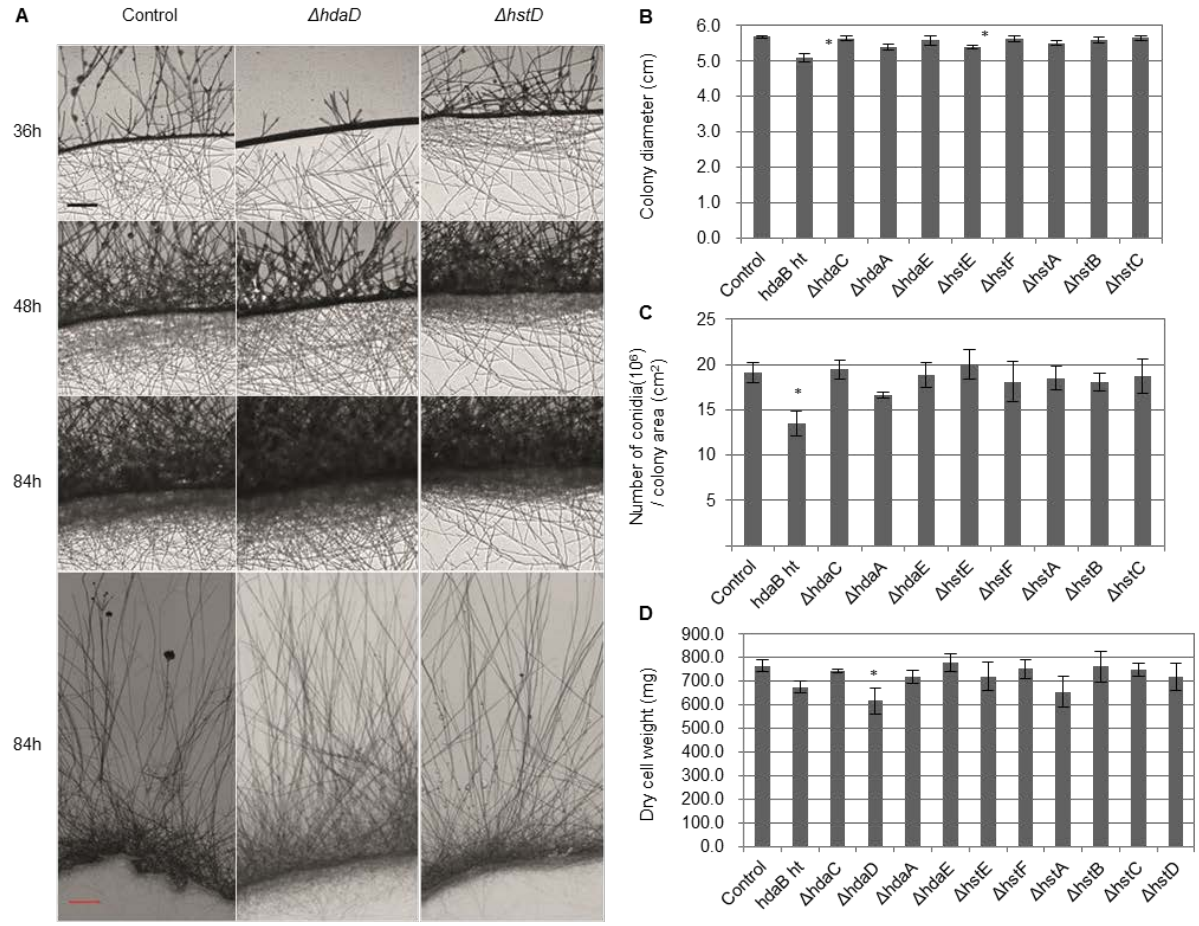

Figure I.6.4. Phenotypic analysis of AoHDACs disruptants and a heterokaryon strain on plates and submerged cultures.
(A) Time-lapse microscopic analysis of *ΔhstD* and *ΔhdaD*. The pictures were taken at 36, 48, and 84 hr of incubation (black scale bar, 200mm; red scale bar, 500mm). **(B-D)** Quantification of colony diameter, conidiation rate and mycelial dry cell weight of AoHDACs disruptants or a heterokaryon strain, respectively. The colony diameter and conidiation rate were measured on plate cultures. Dry cell weight was measured on submerged cultures. All data represented as mean ±s.d. (n=3); **p*< 0.01 t-test.

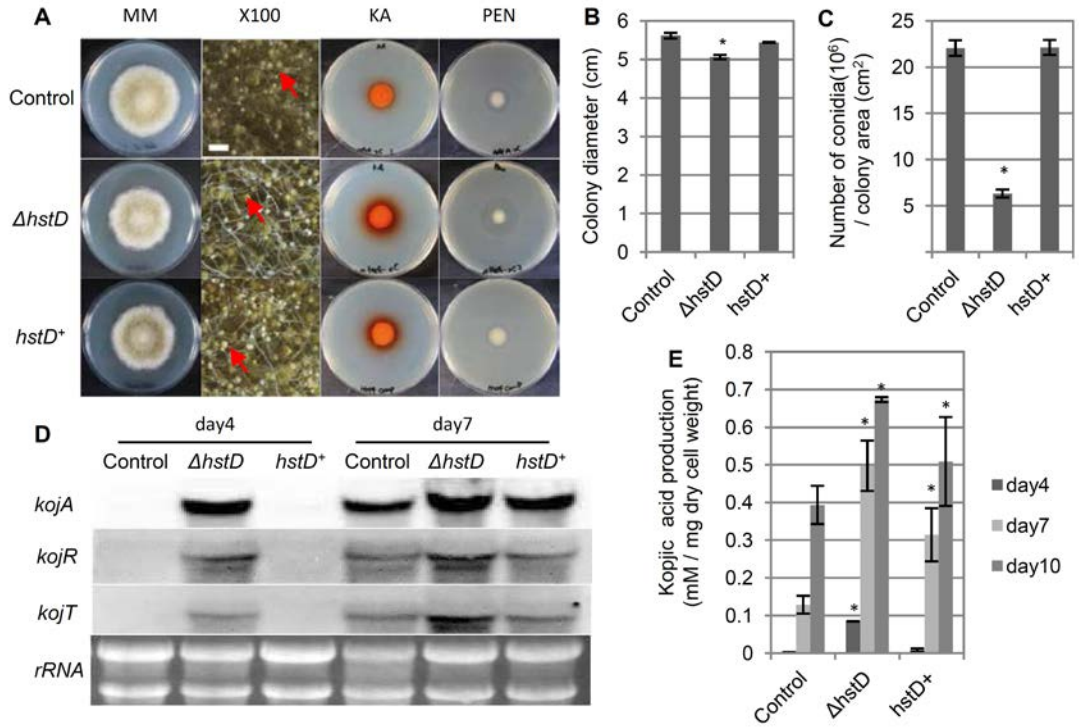

Figure I.6.5. Complementation analysis of *hstD/Aohst4*.
(A) Analysis of the morphology and SM production of the *ΔhstD* and *hstD*$^+$ strains. The panel for MM shows the morphological phenotype of the indicated strain, and the close-up stereomicroscopic images of the strains on MM are shown in adjoining panels (scale bar, 500 μm). Red arrows indicate examples of conidia. The panels for KA and PEN show the plate assay or bioassay of kojic acid and penicillin, respectively. **(B, C)** Quantification of colony diameter and conidiation rate of *ΔhstD* and *hstD*$^+$ strains. **(D)** Expression profiles of the kojic acid cluster genes were determined by northern hybridization. The culture time of the indicated strain is shown at the top of the panel. The analyzed gene is indicated on the left side of each blot. *rRNA* is shown as the loading control. **(E)** Quantification of kojic acid production. The *adeA*$^+$*sC*$^+$ strain was used as the control, and the *ΔhstDsC*$^+$ strain represents *ΔhstD* in this figure. All data are represented as means ± s.d. (n=3); **p* < 0.01, t-test.

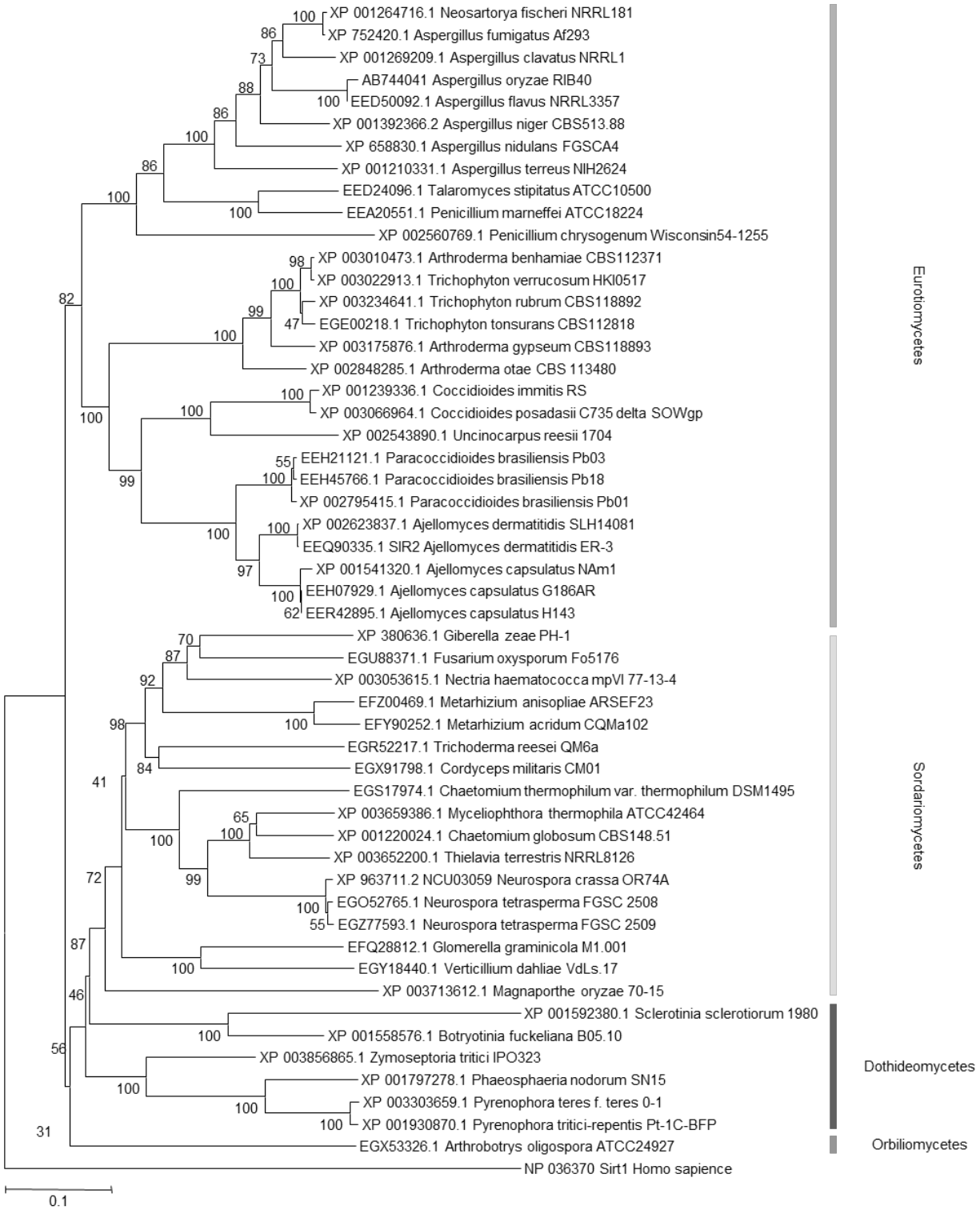

Figure I.6.6. Phylogenetic analysis of *hstD* in filamentous fungi.
The Genebank accession number and species name was indicated each blanches. The number at the nodes is bootstrap values obtained from 1000 and indicated percentage replicates. The scale bar indicates a distance corresponding to 0.1 amino acid substitutions per site. Each class of ascomycetes are shown in the right. The *sirt1* is used us out group of this phylogenetic analysis.

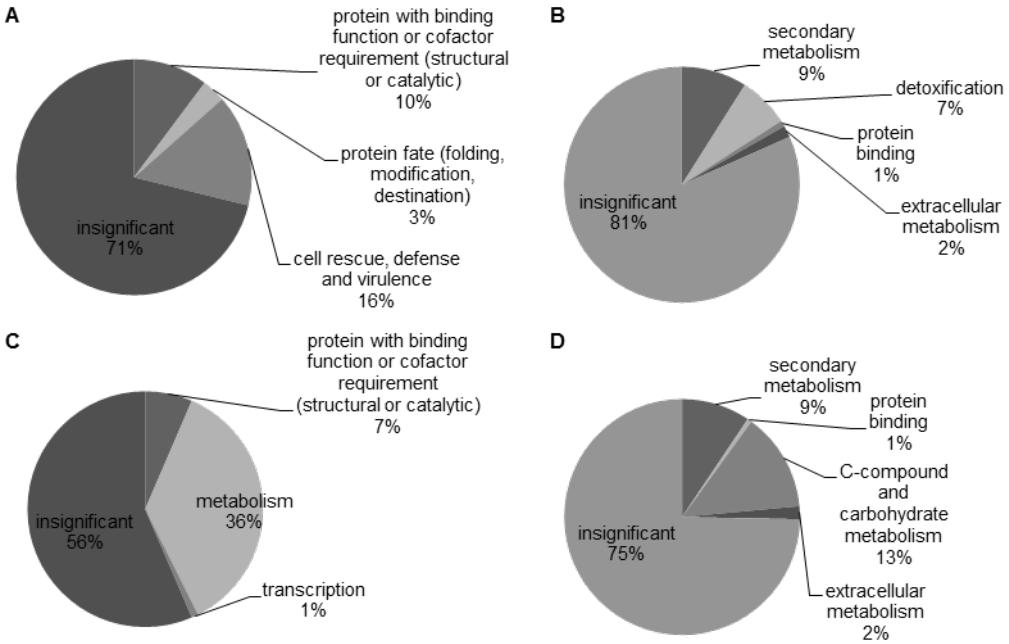

Figure I.6.7. Enrichment analysis of the FunCat categorization of the microarray analysis.
Significantly enriched FunCat level 1 and level 2 categories of genes up-regulated **(A, B)** or down-regulated **(C, D)** by *hstD/Aohst4* deletion, respectively. The Functional Catalogue (FunCat) is the organism independent functional description of proteins (Ruepp et al. 2004). FunCat consists of 28 main functional categories (level 1). The level 1 is the most general one, whereas level 2 shows much more detail. The percentage indicated for each category contributes to total mapping. Insignificant FunCat categories are indicated as insignificant in the pie-charts. Significantly enriched categories were extracted by FungiFun software (cut-off $p < 0.05$, Fisher's exact test) (Priebe et al. 2011). Details of the enrichment analysis are available at the FungiFun website (https://sbi.hki-jena.de/FungiFun/FungiFunHelp.html).

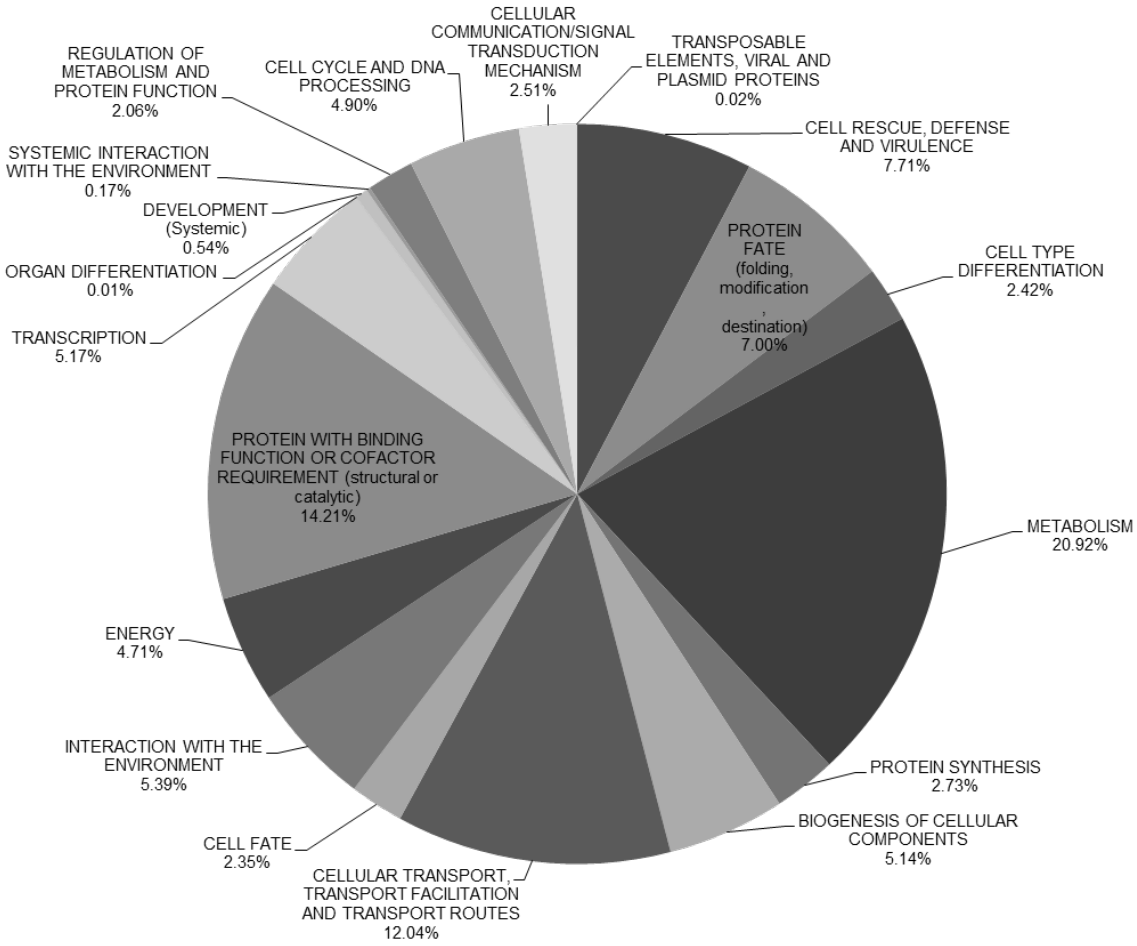

Figure I.6.8. Whole genome distribution of FunCat level 1 categories.
The circular chart indicates the whole genome distribution of indicated categories FunCat level 2. Miner categories (Whole genome distribution < 1%) were indicated as other in pie chart. Percentage indicated each category contributes to total mapping. The data of FunCat categorization of all genes of *A.oryzae* were imported from FungiFun software (https://sbi.hki-jena.de/FungiFun/FungiFun.cgi). Details of each category were available at FunCat Databases (http://mips.helmholtzmuenchen.de/proj/funcatDB/search_main_frame.html). This data was referance of FunCat level 2 enrichment analysis of *A. oryzae*.

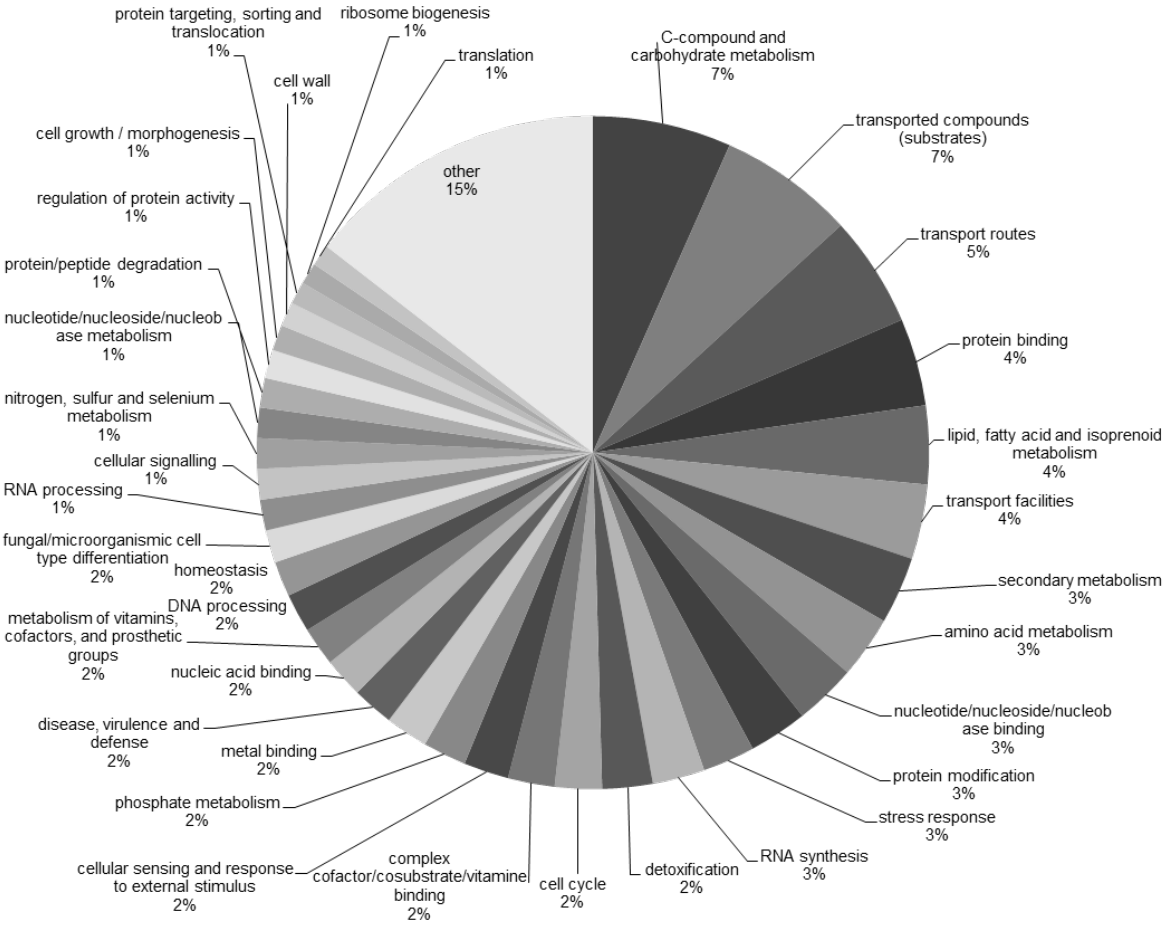

Figure I.6.9. Whole genome distribution of FunCat level 2 categories.
The circular chart indicates the whole genome distribution of indicated categories FunCat level 2. Miner categories (Whole genome distribution < 1%) were indicated as other in pie chart. Percentage indicated each category contributes to total mapping. The data of FunCat categorization of all genes of *A.oryzae* were imported from FungiFun software (https://sbi.hki-jena.de/FungiFun/FungiFun.cgi). Details of each category were available at FunCat Databases (http://mips.helmholtzmuenchen.de/proj/funcatDB/search_main_frame.html). This data was referance of FunCat level 2 enrichment analysis of *A. oryzae*.

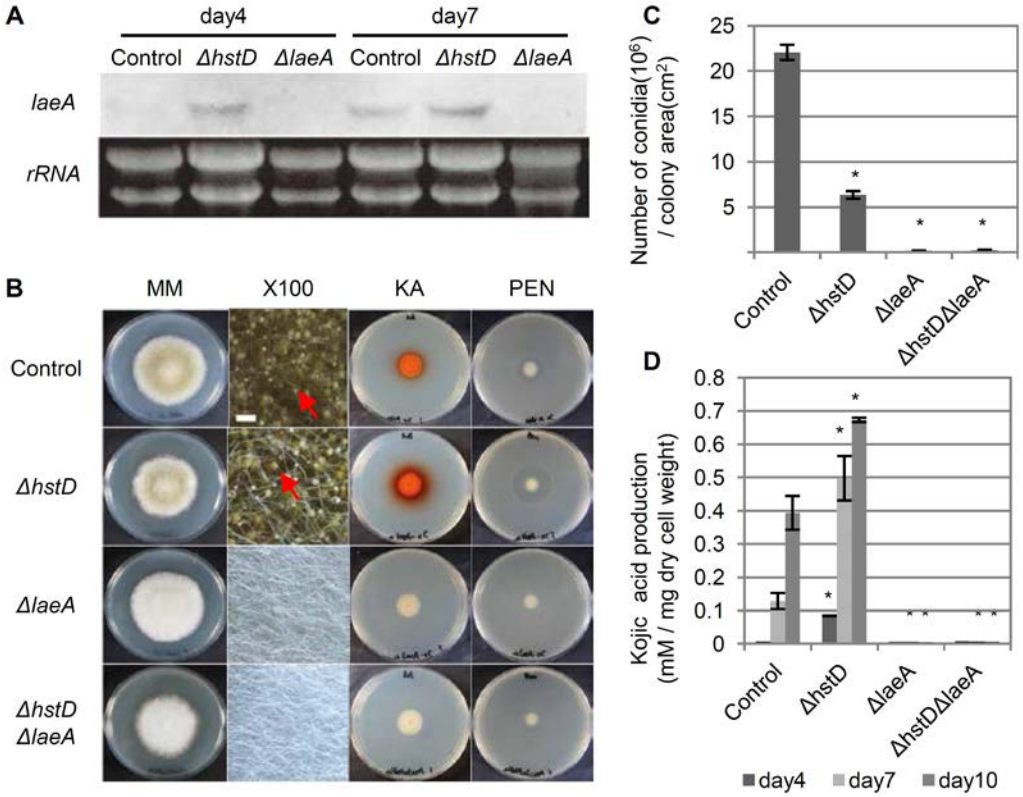

Figure I.6.10. Genetic interaction between *hstD/Aohst4* and *laeA*.
(A) Expression profile of *laeA* in the KA-producing condition. The *adeA*$^{+}$ strain was used as a control. The culture time of the indicated strain is shown at the top of the panel. *rRNA* is shown as the loading control. **(B)** Analysis of morphology and SM production of the *ΔhstD*, *ΔlaeA*, and *ΔhstDΔlaeA* strains. The panel for MM shows the morphological phenotype of the indicated strain, and the close-up stereomicroscopic images of the strains on MM are shown in adjoining panels (scale bar, 500 μm). Red arrows indicate examples of conidia. The panels for KA and PEN show the plate assay or bioassay of kojic acid and penicillin, respectively. **(C, D)** Quantification of the conidiation rate and kojic acid production of the *ΔhstD*, *ΔlaeA,* and *ΔhstDΔlaeA* strains. Except for panel **(A)** the *adeA*$^{+}$*sC*$^{+}$ strain was used as the control, and the *ΔhstD sC*$^{+}$ and *ΔlaeA sC*$^{+}$ strains represent *ΔhstD* and *ΔlaeA* in this figure, respectively. All data are represented as means ± s.d. (n=3); **p* < 0.01, t-test.

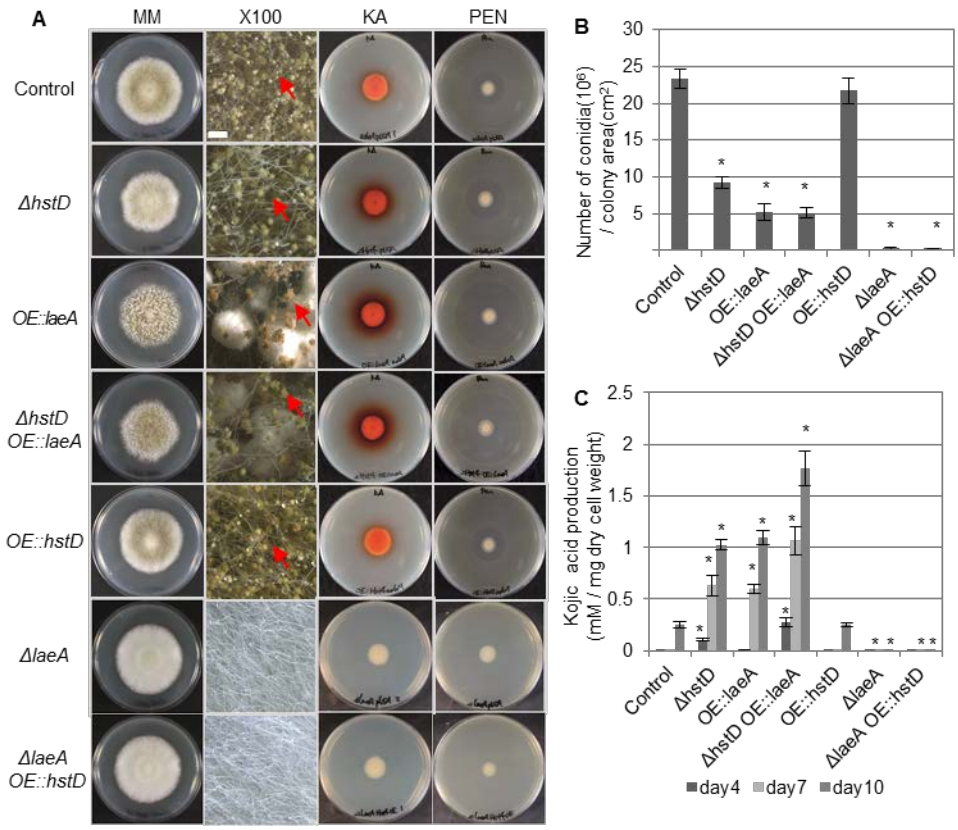

Figure I.6.11. Epistatic relationship between *hstD/Aohst4* and *laeA*.
(A) Analysis of the morphology and SM production of the *ΔhstD OE::laeA* and *ΔlaeA OE::hstD* strains. The MM panel shows the morphological phenotype of the indicated strain, and the close-up stereomicroscopic images of the strains on MM are shown in the adjoining panels (scale bar, 500 μm). Red arrows indicate examples of conidia. The panels for KA and PEN show the plate assay or bioassay of kojic acid and penicillin, respectively. **(B, C)** Quantification of the conidiation rate and kojic acid production of the *ΔhstD OE::laeA* and *ΔlaeA OE::hstD* strains. The $adeA^{+}$ $pUSA^{+}$ strain was used as the control, and *ΔhstD* $pUSA^{+}$, *OE::laeA* $adeA^{+}$, *ΔlaeA* $pUSA^{+}$, and *OE::hstD* $adeA^{+}$ strains represent *ΔhstD, OE::laeA*, *ΔlaeA,* and *OE::hstD* in this figure, respectively. The *amyB* promoter was used to drive overexpresion of *laeA* and *hstD/Aohst4*. All data are represented as means ± s.d. (n=3); $*p < 0.01$, t-test.

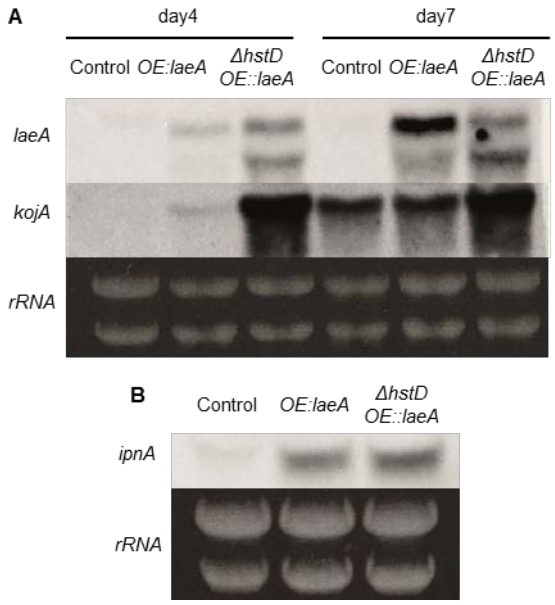

Figure I.6.12. Expression profiling of *laeA*, *kojA*, and *ipnA* in *OE::laeA* and *ΔhstD OE::laeA* strains, respectively.

(**A**) Northern hybridization of *laeA* and *kojA*, respectively. The culture time of the indicated strain is shown at the top of the panel. *rRNA* is shown as the loading control. The analyzed gene is indicated on the left side of each blot. **(B)** Northern hybridization of the penicillin biosynthetic gene *ipnA*. *rRNA* is shown as the loading control. The $adeA^{+}$ $pUSA^{+}$ strain was used as the control, and the *OE::laeA* $adeA^{+}$ strain represents *OE::laeA* in this figure.

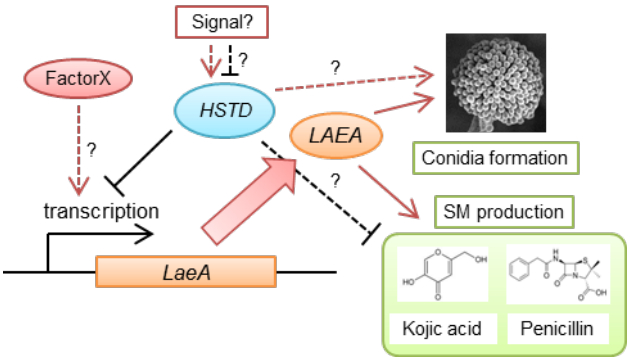

Figure I.6.13. Schematic model of the regulation of SM production and development by *hstD/Aohst4* through *laeA*.
An unknown signal induces or suppresses the function of HSTD/AoHST4. Suppression of *hstD/Aohst4* leads to expression of *laeA*. This activation stimulates fungal development and SM production. However, there is the possibility of an *hstD/Aohst4* competitive mechanism by an unknown factor (described as FactorX in this figure). SM production: secondary metabolite production.

Table I.6.1. Strains used in the Chapter I

Name	Parental strain	Genotype[b]	Reference
RIB40		wild type	NRIB[a]
NSR-ΔLD2	*RIB40*	*niaD⁻ sC⁻ adeA⁻ argB::adeA⁻ ΔligD::argB*	Maruyama et al. 2008
adeA⁺	*NSR-ΔLD2*	*niaD⁻ sC⁻ adeA⁻ argB::adeA⁻ ΔligD::argB adeA⁺*	this study
pUSA⁺	*NSR-ΔLD2*	*niaD⁻ sC⁻ adeA⁻ argB::adeA⁻ ΔligD::argB pUSA⁺*	this study
adeA⁺ sC⁺	*adeA⁺*	*niaD⁻ sC⁻ adeA⁻ argB::adeA⁻ ΔligD::argB adeA⁺ AnsC⁺*	this study
adeA⁺ pUSA⁺	*adeA⁺*	*niaD⁻ sC⁻ adeA⁻ argB::adeA⁻ ΔligD::argB adeA⁺ pUSA⁺*	this study
hdaB ht[c]	*NSR-ΔLD2*	*niaD⁻ sC⁻ adeA⁻ argB::adeA⁻ ΔligD::argB ΔhdaB::adeA hdaB*	this study
ΔhdaC	*NSR-ΔLD2*	*niaD⁻ sC⁻ adeA⁻ argB::adeA⁻ ΔligD::argB ΔhdaC::adeA*	this study
ΔhdaD	*NSR-ΔLD2*	*niaD⁻ sC⁻ adeA⁻ argB::adeA⁻ ΔligD::argB ΔhdaD::adeA*	this study
ΔhdaA	*NSR-ΔLD2*	*niaD⁻ sC⁻ adeA⁻ argB::adeA⁻ ΔligD::argB ΔhdaA::adeA*	this study
ΔhdaE	*NSR-ΔLD2*	*niaD⁻ sC⁻ adeA⁻ argB::adeA⁻ ΔligD::argB ΔhdaE::adeA*	this study
ΔhstA	*NSR-ΔLD2*	*niaD⁻ sC⁻ adeA⁻ argB::adeA⁻ ΔligD::argB ΔhstA::adeA*	this study
ΔhstB	*NSR-ΔLD2*	*niaD⁻ sC⁻ adeA⁻ argB::adeA⁻ ΔligD::argB ΔhstB::adeA*	this study
ΔhstC	*NSR-ΔLD2*	*niaD⁻ sC⁻ adeA⁻ argB::adeA⁻ ΔligD::argB ΔhstC::adeA*	this study
ΔhstD	*NSR-ΔLD2*	*niaD⁻ sC⁻ adeA⁻ argB::adeA⁻ ΔligD::argB ΔhstD::adeA*	this study
ΔhstE	*NSR-ΔLD2*	*niaD⁻ sC⁻ adeA⁻ argB::adeA⁻ ΔligD::argB ΔhstE::adeA*	this study
ΔhstF	*NSR-ΔLD2*	*niaD⁻ sC⁻ adeA⁻ argB::adeA⁻ ΔligD::argB ΔhstF::adeA*	this study
ΔlaeA	*NSR-ΔLD2*	*niaD⁻ sC⁻ adeA⁻ argB::adeA⁻ ΔligD::argB ΔlaeA::adeA*	this study
ΔhstD sC⁺	*ΔhstD*	*niaD⁻ sC⁻ adeA⁻ argB::adeA⁻ ΔligD::argB ΔhstD::adeA AnsC⁺*	this study
ΔhstD pUSA⁺	*ΔhstD*	*niaD⁻ sC⁻ adeA⁻ argB::adeA⁻ ΔligD::argB ΔhstD::adeA pUSA⁺*	this study
ΔhstD OE::laeA	*ΔhstD*	*niaD⁻ sC⁻ adeA⁻ argB::adeA⁻ ΔligD::argB ΔhstD::adeA pUSlaeA⁺*	this study
ΔlaeA sC⁺	*ΔlaeA*	*niaD⁻ sC⁻ adeA⁻ argB::adeA⁻ ΔligD::argB ΔlaeA::adeA AnsC⁺*	this study
ΔlaeA pUSA⁺	*ΔlaeA*	*niaD⁻ sC⁻ adeA⁻ argB::adeA⁻ ΔligD::argB ΔlaeA::adeA pUSA⁺*	this study
ΔlaeA OE::hstD	*ΔlaeA*	*niaD⁻ sC⁻ adeA⁻ argB::adeA⁻ ΔligD::argB ΔlaeA::adeA pUShstD⁺*	this study
OE::laeA	*NSR-ΔLD2*	*niaD⁻ sC⁻ adeA⁻ argB::adeA⁻ ΔligD::argB pUSlaeA⁺*	this study
OE::hstD	*NSR-ΔLD2*	*niaD⁻ sC⁻ adeA⁻ argB::adeA⁻ ΔligD::argB pUShstD⁺*	this study
ΔhstD ΔlaeA	*ΔhstD*	*niaD⁻ sC⁻ adeA⁻ argB::adeA⁻ ΔligD::argB ΔhstD::adeA ΔlaeA::AnsC*	this study
hstD⁺	*ΔhstD*	*niaD⁻ sC⁻ adeA⁻ argB::adeA⁻ ΔligD::argB ΔhstD::adeA::hstD::AnsC*	this study
OE::laeA adeA⁺	*OE::laeA*	*niaD⁻ sC⁻ adeA⁻ argB::adeA⁻ ΔligD::argB pUSlaeA⁺ adeA⁺*	this study
OE::hstD adeA⁺	*OE::hstD*	*niaD⁻ sC⁻ adeA⁻ argB::adeA⁻ ΔligD::argB pUShstD⁺ adeA⁺*	this study

[a]National Research institute of Brewing

[b]*AnsC: Aspergillus nidulans sC*

[c]*ht:* heterokaryon

Table I.6.2. List of sequence accession numbers used in the Chapter I

Saccharomyces cerviciae		*Homo sapience*		*Neurospora crassa*		*Aspergillus nidulans*		*Aspergillus oryzae*	
Gene[a]	Accession number	Gene[a]	Accession number	Gene[a]	Accession number	Gene[a]	Accession number	Gene[a]	Accession number
Classical HDACs familly									
rpd3	YNL330C	*hdac1 / Hsrpd3*	NP_004955	*hda3 / Ncrpd3*	NCU00824	*rpdA / Anrpd3*	AN4493	*hdaB / Aorpd3*	AB744040
		hdac2 / Hsrpd3	NP_001518					*hdaC / Aorpd3*	AO080525000127
		hdac3 / Hsrpd3	NP_003874						
hos2	YGL194C			*hda2 / Nchos2*	NCU02795	*hosA / Anhos2*	AN3806	*hdaD / Aohos2*	AO080511000459
hos1	YPR068C								
hda1	YRL021W	*hdac6 / Hshda1*	NP_006035	*hda1 / Nchda1*	NCU01525	*hdaA / Anhda1*	AN8024	*hdaA / Aohda1*	AO080513000236
		hdac10 / Hshda1	NP_114408						
hos3	YPL116W			*hda4 / Nchos3*	NCU07018	*hosB / Anhos3*	AN7019	*hdaE / Aohos3*	AO080570000061
		hdac4	NP_006028						
		hdac5	NP_001015053						
		hdac7	NP_056216						
		hdac8	NP_060956						
		hdac9	NP_055522						
		hdac11	NP_079103						
TheSirtuin familly									
sir2	YDL042C	*sirt1 / Hssir2*	NP_036370	*nst1 / Ncsir2*	NCU04737	*sirA / Ansir2*	AN10449	*hstA / Aosir2*	AO080506000102
hst1	YOL068C								
hst2	YPL015C	*sirt2 / Hshst2*	NP_036369	*nst2 / Nchst2*	NCU00523	*AN7461/ Anhst2*	AN7461	*hstB / Aohst2*	AO080569000094
		sirt3 / Hshst2	NP_036371			*AN11873/ Anhst2*	AN11873	*hstC / Aohst2*	AO080508000293
hst3	YOR025W			*nst4 / Nchst3*	NCU04859				
hst4	YDR191W			*nst3 / Nchst4*	NCU03059	*AN1226 / Anhst4*	AN1226	*hstD / Aohst4*	AB744041
		sirt4	NP_036372	*nst5 / Ncsirt4*	NCU00203	*hstA / Ansirt4*	AN11067	*hstE / Aosirt4*	AO080559000113
		sirt5	NP_036373	*nst6 / Ncsirt5*	NCU05973	*AN1782 / Ansirt5*	AN1782	*hstF / Aosirt5*	AO080568000195
		sirt6	NP_057623	*nst7 / Ncsirt6*	NCU07624				
		sirt7	NP_057622						

[a] HDAC of *Saccharomyces cerevisiae* or *Homo Sapiens* with the species name indicated followed by a slash.

[b] Hda : Histone DeAcetylase

[c] Hst : Homolog of SirTuins

Table I.6.3. PCR primers used in the Chapter I

Primer name	Sequence(5' to 3')[a,b]	Region or purpose
adeA-F	CCGTCATGTCCAGGAAGATAGGTCAG	*adeA* amplification
adeA-R	CTGCGCAACAGCATACGAGTCCACAG	
hdaB-A	CAATGGCATGACAAAGAACC	5' flanking region of *hdaB*
hdaB-B	CTGACCTATCTTCCTGGACATGACGGCTGTTCCTGCAACATGAGATACA	
hdaB-C	CTGTGGACTCGTATGCTGTTGCGCAGAAGATCCGTGCGCAAGTT	3'flanking region of *hdaB*
hdaB-D	CCATGGTGAATTAGGGCTCA	
hdaB-A2	CAATAGAATATTCCCCGCGT	fusion PCR for *hdaB*
hdaB-D2	CCCTTGGGATTAGAGTGCTT	
hdaB-F	GTTGATCGGGATGTCAAAGG	*hdaB* ORF
hdaB-G	AATTCTCGGTTCTGCTGGTG	
hdaC-A	TCTGTGCAAGCCTTATGTGC	5' flanking region of *hdaC*
hdaC-B	CTGACCTATCTTCCTGGACATGACGGTCCGTCGAGGTTAGTGACAA	
hdaC-C	CTGTGGACTCGTATGCTGTTGCGCAGTCACTTTGACTACGGAGGGCT	3'flanking region of *hdaC*
hdaC-D	GCCTCGAAATCATGGTCCTA	
hdaC-F	TGAGTGCCTCGTAATGCTTG	*hdaC* ORF
hdaC-G	GTGGGCAGGTTGAAACTCTT	
hdaD-A	TAACTGGCGCAGACCCATAA	5' flanking region of *hdaD*
hdaD-B	CTGACCTATCTTCCTGGACATGACGGCCCTTCTTCTCTTTCCTTATTGC	
hdaD-C	CTGTGGACTCGTATGCTGTTGCGCAGCTGTACGGTAAATGAAGGTCAGC	3'flanking region of *hdaD*
hdaD-D	AAGGGGTCAGATCCACAATG	
hdaD-A2	CGCAGACCCATAAGAAGGAA	fusion PCR for *hdaD*
hdaD-D2	CTGTGTCCACAACTGCCATT	
hdaD-F	GTTGTTTGGTCAGCGTCAGA	*hdaD* ORF
hdaD-G	CCCAAAGGTGACAAGACGAT	
hdaA-A	AACAAAGTGCCCTGTTGACC	5'flanking region of *hdaA*
hdaA-B	CTGACCTATCTTCCTGGACATGACGGCATTGCTATGGCTAGCACCA	
hdaA-C	CTGTGGACTCGTATGCTGTTGCGCAGTTTAGGACGTTCAGATGGGG	3'flanking region of *hdaA*
hdaA-D	TAGGTTTTCTGATGGCCCAG	
hdaA-F	GTGATGCCTATTGCACAGGA	*hdaA* ORF
hdaA-G	GCTTTCGGGTACATGCAACT	
hdaE-A	TCCGAAGTCCACTTTCTTGC	5' flanking region of *hdaE*
hdaE-B	CTGACCTATCTTCCTGGACATGACGGCAAATAGTAGGTTCATTTGGGGG	
hdaE-C	CTGTGGACTCGTATGCTGTTGCGCAGAGTCTATCGGACTTTTGGTCG	3'flanking region of *hdaE*
hdaE-D	AGATCCGGAGTCGTTCCTTT	
hdaE-A2	AGTCTCTTTTCTTTGGCCGC	fusion PCR for *hdaE*
hdaE-D2	ATTGTTCACGTTCTCCACCC	
hdaE-F	GGACCTTTACGCATCCAAGT	*hdaE* ORF
hdaE-G	GATGCGTGTTGGACATTAGC	
hstA-A	ATTACCTGGCGTTCTTGTGG	5'flanking region of *hst A*
hstA-B	CTGACCTATCTTCCTGGACATGACGGGGGTTCTTGTTTGGAGGGTT	
hstA-C	CTGTGGACTCGTATGCTGTTGCGCAGCGGATTCTTCAACGAAGAGC	3'flanking region of *hstA*
hstA-D	ACAGCTGCGAACTGATGATG	
hstA-F	GATTTCCGGCTCTTGTGTGT	*hstA* ORF
hstA-G	GGTATTCCCGATTTTCGGTC	
hstB-A	CTTTGCTTTGAGTTCCTGCC	5'flanking region of *hstB*
hstB-B	CTGACCTATCTTCCTGGACATGACGGTCTAACCTGGCGGAGAGAAA	
hstB-C	CTGTGGACTCGTATGCTGTTGCGCAGTACCGCGAAAAGGAGAGAGA	3'flanking region of *hstB*
hstB-D	AACAGTCGGCGATGTATTCC	
hstB-F	CTTTTTCAGGGAGAATCCGC	*hstB* ORF
hstB-G	CGCTCCATGTTAATGAGCAC	
hstC-A	AGTCATGGAAAAGACTGCGG	5'flanking region of *hstC*
hstC-B	CTGACCTATCTTCCTGGACATGACGGATTGGACTCAGCCTGATTGG	
hstC-C	CTGTGGACTCGTATGCTGTTGCGCAGAGTGCCCGAATAGGTTTCTG	3'flanking region of *hstC*
hstC-D	AGCCATCGCTGTCAGTTTCT	
hstC-F	GCCTTGCTGGCTAAGAAGAA	*hstC* ORF
hstC-G	TCACACGACCCAAGGATACA	
hstD-A	TGCGGAAATGGGTTGTTT	5'flanking region of *hstD*
hstD-B	CTGACCTATCTTCCTGGACATGACGGATGGCACTTGTCGCATGTC	
hstD-C	CTGTGGACTCGTATGCTGTTGCGCAGGGCGTGGTGTAATTCTTCGT	3'flanking region of *hstD*
hstD-D	TAACCGTGACATGACCCTTG	
hstD-A2	TGAAAGGATTACCTCCTCCC	fusion PCR for *hstD*
hstD-D2	ACGTCCGGGATATTATGGGT	

hstD-F	ACCATCAAGTCCCAGCAATC	*hstD* ORF
hstD-G	AGACATCCATGCCTCCCTTA	
hstE-A	TCCATCTGATAAGGTTCGGC	5'flanking region of *hstE*
hstE-B	CTGACCTATCTTCCTGGACATGACGGAGATAATGGGTACGGCGAGA	
hstE-C	CTGTGGACTCGTATGCTGTTGCGCAGGGCGCTGTCCGAATGTATTA	3'flanking region of *hstE*
hstE-D	CCAGTGTACAATTCCGCCAT	
hstE-A2	ATAAGGTTCGGCATGAGTGG	fusion PCR for *hstE*
hstE-D2	GCCATGATACATCCAGCAGA	
hstE-F	AATATCTGGGGCAGACGAGA	*hstE* ORF
hstE-G	GGCACATTCTCAAGAGCACA	
hstF-A	ATTGACGAACCCTTGGACTG	5'flanking region of *hstF*
hstF-B	CTGACCTATCTTCCTGGACATGACGGGCGGGGTGTTGATAATGACT	
hstF-C	CTGTGGACTCGTATGCTGTTGCGCAGGGTATTATGGGGATTGTCGG	3'flanking region of *hstF*
hstF-D	TCTTCGTCTTCAAAGGCTCC	
hstF-A2	CGTCAGCTCACGGATTATGA	fusion PCR for *hstF*
hstF-D2	TCTCGTTTGCCTTAGCTGTG	
hstF-F	AGACATATGGCGCTGAAAGC	*hstF* ORF
hstF-G	GCAATGTGGAAGGACATCAG	
Fusion-hstD-F	tcgagctcggtacccATGGTGCGGTCGCTGTCCGAAGAGG	vector construction for *pUShstD*
Fusion-hstD-R	ctctagaggatccccTCATGCGGCCGGCTGATTTAACAAC	
Fusion-laeA-F	tcgagctcggtacccATGTTTGGAAACGGCCAGACTGGAC	vector construction for *pUSlaeA*
Fusion-laeA-R	ctctagaggatccccTCAGTTCGCAGGTTTCCGTGCTTGG	
laeA-A	CCGGCTGTTCAAGATCCATGGATAG	5'flanking region of *laeA*
laeA(adeA)-B	CTGACCTATCTTCCTGGACATGACGGTCGATGGCGACAGGCTGATG	
laeA(adeA)-C	CTGTGGACTCGTATGCTGTTGCGCAGAGAGCTCCATTACTGGGTATTCGG	3'flanking region of *laeA*
laeA-D	GAACCCGCCAACATCAAGCTTC	
laeA-A2	GGGATACCAACCACAACACCT	fusion PCR for *laeA*
laeA-D2	TTACGTTTGGGAACGGAGTCA	
laeA-F	CAGCCCTCAAACCACCCAAA	*laeA* ORF
laeA-G	TTGAACGCCTCCGACTTGAC	
laeA(sC)-B	GAACGAGACGAACGAGGAGCCATATTCGATGGCGACAGGCTGATG	disruption casette for *laeA* in *ΔhstD* background
laeA(sC)-C	CATACGGGCAGCTATTGCCAAGAGAAGAGCTCCATTACTGGGTATTCGG	
sC-F	ATATGGCTCCTCGTTCGTCTCGTTC	*sC* amplification
sC-R	TCTCTTGGCAATAGCTGCCCGTATG	
P-amyB-F	GGCAACTCGCTTACCGATTAC	confirmation of transformation
hstD-comp-B	GAACGAGACGAACGAGGAGCCATATTAACCGTGACATGACCCTTG	Complementation of *hstD*
adeA fusion sC-F	CATACGGGCAGCTATTGCCAAGAGAACCGTCATGTCCAGGAAGATAGGTCA	
nested adeA-R	GCCTTGGTCTGGGAGTGT	
kojA-F	GGTTTCCAGGGCCTCATCAG	probe for *kojA*
kojA-R	GAGAAATCCGGGCCAGAACC	
kojR-F	CGGCCAGCTATGACCCCATT	probe for *kojR*
kojR-R	GGCGTCATGGGAGAGTGTGA	
kojT-F	CGAGGTGTCTCTTGCAAACC	probe for *kojT*
kojT-R	GTTCTGGGATAGGCGAACCA	
ipnA-F	CACCTACTCACGAGGTCAAC	probe for *ipnA*
ipnA-R	GTTGACCTCGTGAGTAGGTG	
laeA-F	AGCCCTCAAACCACCCAAAG	probe for *laeA*
laeA-R	TTGAACGCCTCCGACTTGAC	

[a] Additional nucleotides for fusion PCR are indicated by underlines

[b] Additional nucleotides for In-Fusion reaction are indicated in small letters

Table I.6.4. Significantly changed genes in *ΔhstD*

Gene ID	Description	Fold change (*ΔhstD*/ Control)	FunCat categorization[a,b] level 1	FunCat categorization[a,b] level 2
AO080553000121	KojT, putative transporter; present in the kojic acid biosynthetic gene cluster	105.5	[20][32]	[20.01][20.03][20.09][32.05][32.07]
AO080550000061	Hydroxyindole-O-methyltransferase and related SAM-dependent methyltransferases	102.5	[01]	[01.05][01.20]
AO080501000196	fleA fucose-specific lectin	61.82	#	#
AO080563000007	CpaD, O-dimethylallyltransferase (DMAT); dimethylallylates cAATrp to form beta-cyclopiazonic acid	49.39	#	#
AO080554000441	Uncharacterized membrane protein, predicted efflux pump	38.39	[20][32]	[20.01][32.07]
AO080563000004	Predicted protein	32.27	[20][32][34]	[20.01][20.03][20.09][32.05][32.07][34.11]
AO080525000393	Predicted protein	29.96	[01][11]	[01.02][11.02]
AO080563000005	Cytochrome P450 CYP3 CYP5 CYP6 CYP9 subfamilies	27.59	[01]	[01.06][01.20]
AO080563000006	Predicted protein	25.97	#	#
AO080508000254	Predicted protein	24.93	[01]	[01.05]
AO080511000457	Predicted protein	21.4	#	#
AO080522000026	Catalase (peroxidase I)	20.19	[32]	[32.07]
AO080536000097	Predicted protein	19.6	#	#
AO080542000015	Predicted protein	19.4	[32]	[32.05]
AO080515000039	Predicted protein	17.93	#	#
AO080550000056	Proteins containing the FAD binding domain	14.42	[01]	[01.05][01.20]
AO080508000512	mleA lectin	12.87	#	#
AO080515000015	Uncharacterized protein, possibly involved in utilization of glycolate and propanediol	12.31	#	#
AO080513000111	Predicted protein	11.85	#	#
AO080529000071	Predicted protein	10.43	#	#
AO080508000290	Predicted protein	10.11	#	#
AO080523000388	Predicted protein	9.984	#	#
AO080554000006	Predicted protein	9.951	#	#
AO080550000062	Dehydrogenases (flavoproteins)	9.86	#	#
AO080525000250	Predicted protein	9.747	#	#
AO080553000119	KojA, FAD-dependent oxidoreductase; present in the kojic acid biosynthetic gene cluster	9.711	#	#
AO080541000467	Predicted protein	9.668	#	#
AO080550000076	Predicted protein	9.323	#	#
AO080539000068	Predicted protein	9.028	#	#
AO080510000132	Predicted protein	8.772	#	#
AO080529000068	Predicted protein	8.053	#	#
AO080554000051	Predicted protein	7.73	#	#
AO080550000193	RNA 3 -terminal phosphate cyclase	7.67	#	#
AO080551000140	Polyketide synthase modules and related proteins	7.028	[01][32]	[01.20][32.05]
AO080511000259	Predicted protein	6.937	#	#
AO080523000239	TPR repeat	6.617	#	#
AO080537000053	Predicted hydrolases or acyltransferases (alpha beta hydrolase superfamily)	6.315	#	#
AO080527000194	Predicted xylanase chitin deacetylase	6.251	[01][16]	[01.05][01.25][16.05]
AO080551000190	Beta-lactamase class C and other penicillin binding proteins	6.175	#	#
AO080508000358	Predicted protein	5.923	#	#
AO080515000064	Multicopper oxidases	5.855	[01][32]	[01.05][01.07][01.25][32.07]
AO080527000284	Predicted protein	5.77	#	#
AO080549000421	Predicted protein	5.727	#	#
AO080550000107	Predicted protein	5.723	#	#
AO080523000444	Predicted protein	5.71	#	#
AO080567000074	Predicted protein	5.689	#	#
AO080541000077	Predicted protein	5.673	#	#
AO080523000389	Cytochrome P450 CYP4 CYP19 CYP26 subfamilies	5.589	[32]	[32.07]
AO080521000213	Predicted protein	5.575	#	#
AO080547000015	Predicted protein	5.394	#	#
AO080523000405	Predicted protein	5.171	[32][42]	[32.01][42.01][42.04]
AO080527000412	Predicted protein	5.107	#	#
AO080525000701	Alkaline phosphatase	5.07	[01]	[01.04][01.06][01.07]
AO080523000550	Predicted protein	4.932	#	#
AO080562000080	aflJ Predicted protein	4.922	#	#
AO080554000398	Predicted protein	4.746	#	#
AO080525000625	Predicted protein	4.693	#	#
AO080541000440	Predicted protein	4.676	#	#
AO080530000095	Predicted protein	4.602	#	#
AO080554000103	Cytochrome P450 CYP3 CYP5 CYP6 CYP9 subfamilies	4.597	[01][20][32]	[01.01][01.02][01.06][01.20][20.01][32.07]
AO080547000014	Ankyrin	4.512	#	#
AO080549000406	Predicted dehydrogenases and related proteins	4.472	[01][11]	[01.05][11.02]
AO080508000357	Chitin synthase hyaluronan synthase (glycosyltransferases)	4.45	#	#
AO080523000006	Predicted protein	4.401	#	#
AO080525000059	Cytochrome P450 CYP2 subfamily	4.37	[01][16][20][32]	[01.06][01.07][01.20][16.17][16.21][20.01][32.07]
AO080523000500	Predicted protein	4.32	#	#
AO080527000186	Oxidosqualene-lanosterol cyclase and related proteins	4.272	[01]	[01.06][01.20]
AO080523000718	Carboxylesterase type B	4.266	#	#
AO080529000067	Cytochrome P450 CYP11 CYP12 CYP24 CYP27 subfamilies	4.247	[01][32]	[01.01][01.20][32.07]
AO080515000065	Predicted protein	4.223	#	#
AO080532000090	Predicted protein	4.183	#	#
AO080508000361	Predicted protein	4.159	#	#
AO080551000170	Predicted protein	4.114	[01][16]	[01.01][01.20][16.17]
AO080525000706	Predicted protein	4.098	#	#
AO080532000546	Cytochrome P450 CYP2 subfamily	4.046	[01][16][20]	[01.20][16.17][16.21][20.01]
AO080508000305	Vesicular amine transporter	4.008	#	#
AO080527000377	Glucose dehydrogenase choline dehydrogenase mandelonitrile lyase (GMC oxidoreductase family)	4.008	[01][16][20]	[01.01][01.05][01.20][16.21][20.01]
AO080531000104	Predicted protein	3.951	#	#
AO080525000269	Predicted protein	3.848	#	#
AO080557000082	Cofilin; actin depolymerisation factor	3.847	#	#
AO080532000337	Chaperone-dependent E3 ubiquitin protein ligase (contains TPR repeats)	3.838	#	#
AO080523000512	Predicted protein	3.774	#	#
AO080523000573	Predicted protein	3.77	#	#
AO080536000086	ATPases of the AAA$^+$ class	3.719	#	#
AO080515000151	Predicted protein	3.707	[43]	[43.01]
AO080525000687	Predicted transporter (major facilitator superfamily)	3.648	#	#
AO080557000045	Predicted protein	3.624	[32]	[32.05]
AO080515000071	Predicted protein	3.604	#	#
AO080501000080	Predicted protein	3.559	#	#
AO080566000093	Ankyrin	3.555	#	#
AO080525000026	SAM-dependent methyltransferases	3.536	#	#
AO080515000098	Predicted protein	3.503	#	#
AO080554000362	Predicted protein	3.493	#	#
AO080503000171	Phosphoenolpyruvate carboxykinase (ATP)	3.468	[01][02]	[01.04][01.05][02.01]
AO080523000572	Predicted protein	3.423	#	#
AO080533000376	Multidrug resistance-associated protein mitoxantrone resistance protein, ABC superfamily	3.391	[01][16][20][32]	[01.04][16.19][20.03][32.07]
AO080508000531	Predicted protein	3.39	#	#
AO080549000304	Carboxylesterase type B	3.38	[01]	[01.06]
AO080506000286	Predicted protein	3.315	#	#
AO080523000216	Predicted protein	3.303	#	#
AO080538000066	Acyl-CoA synthetase	3.298	[01]	[01.01][01.05][01.20]
AO080523000032	Amino acid transporters	3.296	[01][20][32][34]	[01.06][20.01][20.03][20.09[32.01][34.01][34.11]
AO080510000108	ATPases of the AAA$^+$ class	3.199	#	#

AO080513000093	Jacalin-like lectin domain-containing protein	3.158	#	#
AO080525000640	Predicted protein	3.156	#	#
AO080527000200	Chitinase	3.085	[01]	[01.05][01.25]
AO080553000120	KojR, Zn(II)2Cys6 transcription factor; induced by kojic acid; present in the kojic acid biosynthetic gene clus	3.083	[11][34]	[11.02][34.11]
AO080525000062	Acetylcholinesterase Butyrylcholinesterase	3.076	[01][32]	[01.05][01.06][01.20][01.25][32.05][32.07][32.10]
AO080541000359	Integral membrane ankyrin-repeat protein Kidins220 (protein kinase D substrate)	3.076	#	#
AO080541000097	Predicted transporter (major facilitator superfamily)	3.063	[20][32]	[20.01][32.07]
AO080546000223	Predicted protein	3.039	#	#
AO080542000014	Cytochrome P450 CYP4 CYP19 CYP26 subfamilies	3.037	[01][32]	[01.01][32.05][32.07]
AO080539000029	Predicted protein	3.016	[11]	[11.02]
AO080547000070	Predicted protein	2.985	#	#
AO080523000242	Predicted protein	2.977	#	#
AO080502000014	Uncharacterized conserved protein	2.976	#	#
AO080503000338	NAD^+ ADP-ribosyltransferase Parp, required for poly-ADP ribosylation of nuclear proteins	2.959	[01][10][14][16]	[01.05][10.01][14.07][16.01][16.03]
AO080506000124	Predicted protein	2.951	#	#
AO080542000029	Conserved protein domain typically associated with flavoprotein oxygenases, DIM6 NTAB family	2.943	#	#
AO080521000317	Predicted protein	2.904	#	#
AO080536000058	Predicted protein	2.901	#	#
AO080518000104	Predicted protein	2.888	#	#
AO080551000149	Predicted protein	2.88	#	#
AO080550000101	Predicted protein	2.853	#	#
AO080501000088	3-polyprenyl-4-hydroxybenzoate decarboxylase and related decarboxylases	2.843	#	#
AO080509000188	Predicted aminoglycoside phosphotransferase	2.835	#	#
AO080554000077	Monodehydroascorbate ferredoxin reductase	2.835	[02]	[02.11]
AO080536000060	Predicted protein	2.826	#	#
AO080506000051	Predicted short chain-type dehydrogenase	2.821	[01][32]	[01.05][01.20][32.05]
AO080549000353	ATP adenylyltransferase (5 ,5 -P-1, P-4-tetraphosphate phosphorylase II)	2.812	[01][11][16][20]	[01.04][01.06][11.04][16.17][16.19][20.01][20.03][20.09]
AO080523000207	Predicted protein	2.766	#	#
AO080536000143	Tellurite resistance protein and related permeases	2.743	#	#
AO080505000118	Predicted protein	2.726	#	#
AO080546000372	Ornithine aminotransferase	2.705	[01][16]	[01.01][01.02][01.20][16.21]
AO080505000202	Methionyl-tRNA formyltransferase	2.701	#	#
AO080522000033	Predicted protein	2.694	#	#
AO080509000132	Predicted protein	2.686	#	#
AO080546000363	Predicted protein	2.676	#	#
AO080534000002	Non-ribosomal peptide synthetase modules and related proteins	2.663	#	#
AO080561000015	Predicted protein	2.659	#	#
AO080532000416	Threonine dehydrogenase and related Zn-dependent dehydrogenases	2.657	[01][02][16]	[01.01][01.05][02.16][16.17]
AO080515000061	Uncharacterized conserved protein	2.644	#	#
AO080503000025	Predicted protein	2.631	#	#
AO080525000209	Dihydrolipoamide succinyltransferase (2-oxoglutarate dehydrogenase, E2 subunit)	2.617	#	#
AO080541000475	Glutathione S-transferase	2.593	[32][40]	[32.01][32.07][40.10]
AO080512000001	Transposon-encoded proteins with TYA, reverse transcriptase, integrase domains in various combinations	2.561	#	#
AO080523000247	Predicted acyl-CoA transferases carnitine dehydratase	2.553	#	#
AO080508000270	RNA polymerase II transcription termination factor TTF2 lodestar, DEAD-box superfamily	2.541	#	#
AO080523000391	Cytochrome P450 CYP3 CYP5 CYP6 CYP9 subfamilies	2.519	[16][32]	[16.21][32.05][32.07]
AO080527000504	Predicted protein	2.503	#	#
AO080525000318	Predicted protein	2.501	#	#
AO080566000091	Predicted protein	2.493	#	#
AO080503000216	Predicted protein	2.492	#	#
AO080523000079	Beta-lactamase class C and other penicillin binding proteins	2.491	#	#
AO080532000061	Uncharacterized conserved protein	2.483	[20]	[20.01][20.03][20.09]
AO080505000203	Nucleoside-diphosphate-sugar epimerases	2.467	[01][11][42]	[01.05][11.02][42.10]
AO080513000020	Predicted protein	2.459	#	#
AO080505000159	Alcohol dehydrogenase, class V	2.446	[01][02][16][42]	[01.05][01.20][02.01][02.16][16.17][42.01]
AO080546000050	Protein tyrosine serine phosphatase	2.442	[14]	[14.07]
AO080531000027	Dimethylglycine dehydrogenase precursor	2.426	[01][16][20]	[01.01][01.02][01.05][16.21][20.01]
AO080501000014	wykB, FAD-dependent oxidoreductase	2.412	[14]	[14.07]
AO080567000034	Predicted protein	2.409	#	#
AO080536000139	7-keto-8-aminopelargonate synthetase and related enzymes	2.406	#	#
AO080523000390	Cytochrome P450 CYP4 CYP19 CYP26 subfamilies	2.401	[01]	[01.01]
AO080563000003	Synaptic vesicle transporter SVOP and related transporters (major facilitator superfamily)	2.394	[20][32][34]	[20.01][20.03][20.09][32.05][32.07][34.11]
AO080520000010	Reverse transcriptase	2.393	#	#
AO080527000001	Predicted protein	2.392	#	#
AO080546000314	Predicted protein	2.368	#	#
AO080537000005	Predicted flavin-nucleotide-binding protein structurally related to pyridoxine 5 -phosphate oxidase	2.363	#	#
AO080568000203	Predicted protein	2.359	#	#
AO080521000212	Predicted protein	2.35	#	#
AO080515000262	ATP-dependent RNA helicase	2.337	#	#
AO080506000125	ADP-ribose pyrophosphatase	2.331	[01][16][32]	[01.03][16.17][32.01]
AO080548000022	Zn-finger	2.326	#	#
AO080533000254	Inositol monophosphatase	2.32	[01][30]	[01.04][01.05][01.06][01.20][30.01]
AO080554000301	Predicted protein	2.319	#	#
AO080509000068	Predicted protein	2.315	#	#
AO080521000046	Putative dehydrogenase domain of multifunctional non-ribosomal peptide synthetases and related enzymes	2.299	[01][20][34]	[01.20][20.01][34.01]
AO080561000093	Predicted protein	2.298	#	#
AO080503000246	Predicted transporter (major facilitator superfamily)	2.293	[01][20][34]	[01.05][20.01][20.03][20.09][34.01][34.11]
AO080523000538	Predicted glutamine synthetase	2.287	#	#
AO080528000042	Permease of the major facilitator superfamily	2.284	[20]	[20.01][20.03][20.09]
AO080523000723	Acetyltransferases, including N-acetylases of ribosomal proteins	2.282	#	#
AO080570000066	Permease of the major facilitator superfamily	2.263	[20]	[20.01][20.03][20.09]
AO080569000133	Predicted protein	2.255	[10][11][42]	[10.03][11.04][11.06][42.16]
AO080523000499	Predicted protein	2.238	#	#
AO080527000070	Lactoylglutathione lyase and related lyases	2.231	#	#
AO080553000127	Predicted protein	2.231	#	#
AO080567000088	Predicted protein	2.219	#	#
AO080551000005	Spt6 ortholog, DNA-binding subunit of a DNA-dependent protein kinase (Ku70 autoantigen)	2.214	[10][11]	[10.01][11.02]
AO080515000013	Predicted protein	2.212	#	#
AO080537000052	Predicted protein	2.212	#	#
AO080550000146	Exopolyphosphatase	2.207	[01][11][30]	[01.05][11.02][30.01]
AO080527000050	Predicted protein	2.19	#	#
AO080511000186	Predicted protein	2.175	#	#
AO080509000038	Predicted protein	2.172	[43]	[43.01]
AO080515000014	Predicted protein	2.168	#	#
AO080532000091	Cysteine desulfurase NFS1	2.162	[01]	[01.20]
AO080511000049	Predicted protein	2.161	#	#
AO080511000294	Predicted protein	2.16	#	#
AO080523000130	Predicted protein	2.157	#	#
AO080536000059	Predicted protein	2.152	#	#
AO080508000090	Predicted protein	2.151	#	#
AO080527000162	Predicted protein	2.147	[30][34][43]	[30.01][34.11][43.01]
AO080501000155	Predicted transporter (major facilitator superfamily)	2.145	[01][20]	[01.05][20.01][20.03][20.09]
AO080505000279	Predicted protein	2.142	#	#
AO080508000097	3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthase	2.139	[01]	[01.01][01.05][01.20]
AO080523000024	Glycerol uptake facilitator and related permeases (Major Intrinsic Protein Family)	2.129	#	#
AO080501000008	wykN, Non-ribosomal peptide synthetase modules and related proteins	2.117	#	#

AO080539000074	Predicted protein	2.097	#	#
AO080518000037	Predicted transporter (major facilitator superfamily)	2.073	[20][32]	[20.01][20.03][20.09][32.05][32.07]
AO080541000154	Carboxypeptidase C (cathepsin A)	2.062	[14]	[14.13]
AO080567000053	Predicted protein	2.062	#	#
AO080550000102	Predicted acyl esterases	2.055	#	#
AO080505000097	Transthyretin and related proteins	2.053	[01]	[01.02][01.03]
AO080546000347	Catalase (peroxidase I)	2.051	[20][32][42]	[20.01][32.01][32.07][42.16]
AO080533000188	Predicted protein	2.046	#	#
AO080551000123	Predicted protein	2.039	#	#
AO080562000073	Predicted protein	2.036	#	#
AO080551000142	Predicted protein	2.034	#	#
AO080532000609	Predicted protein	2.032	#	#
AO080527000203	Isocitrate isopropylmalate dehydrogenase	2.021	[01][02][16][20]	[01.01][01.05][02.01][02.10][16.21][20.01]
AO080525000648	Predicted protein	2.02	#	#
AO080571000012	Molecular chaperone (DnaJ superfamily)	2.014	#	#
AO080523000419	Predicted protein	2.007	#	#
AO080531000318	2-polyprenyl-6-methoxyphenol hydroxylase and related FAD-dependent oxidoreductases	2.004	[01]	[01.05][01.20]
AO080504000029	Phospholipase C	2.002	#	#
AO080533000108	Protein kinase PCTAIRE and related kinases	2.001	[01][02][10][11][14][16][18][30][34][40][42][43]	[01.04][01.05][02.19][10.01][10.03][11.02][14.07][16.01][16.03][16.19][18.01][18.02][30.01][30.05][34.05][34.11][40.01][42.10][43.01]
AO080505000181	Glycosyltransferase	0.498	[01][02][32][34][43]	[01.05][02.19][32.01][34.11][43.01]
AO080503000128	Predicted protein	0.496	#	#
AO080521000172	Predicted membrane protein	0.496	#	#
AO080514000015	Transcription factor PRD and related proteins, contain PAX and HOX domains	0.495	#	#
AO080531000179	Predicted protein	0.494	#	#
AO080503000252	Predicted protein	0.492	#	#
AO080546000219	Predicted protein	0.492	#	#
AO080560000080	Predicted protein	0.492	#	#
AO080513000270	Predicted protein	0.491	#	#
AO080508000004	Predicted protein	0.487	#	#
AO080503000123	Predicted protein	0.486	#	#
AO080513000060	Alpha-amylase	0.485	[01]	[01.05]
AO080532000307	Predicted protein	0.481	#	#
AO080554000482	NADPH:quinone reductase and related Zn-dependent oxidoreductases	0.481	[01][16]	[01.05][01.20][16.17]
AO080523000408	Dehydrogenases with different specificities (related to short-chain alcohol dehydrogenases)	0.48	#	#
AO080550000156	Predicted protein	0.479	#	#
AO080513000204	Predicted protein	0.478	#	#
AO080560000087	Predicted protein	0.478	#	#
AO080505000104	Predicted protein	0.476	#	#
AO080568000096	Cytochrome P450 CYP4 CYP19 CYP26 subfamilies	0.474	[01][02]	[01.06][01.20][02.45]
AO080523000182	Acetylcholinesterase Butyrylcholinesterase	0.47	#	#
AO080532000189	Predicted protein	0.469	[11][14]	[11.06][14.07][14.13]
AO080570000072	Cytochrome P450 CYP4 CYP19 CYP26 subfamilies	0.469	[01][32]	[01.01][32.05][32.07]
AO080561000074	Endopolygalacturonase	0.468	#	#
AO080511000067	Predicted protein	0.466	#	#
AO080533000118	Predicted protein	0.464	#	#
AO080508000310	Ketopantoate hydroxymethyltransferase	0.463	[01][42]	[01.07][42.01]
AO080523000640	Sorbin and SH3 domain-containing protein	0.463	[10]	[10.03]
AO080536000108	Predicted protein	0.459	#	#
AO080508000430	Predicted protein	0.457	#	#
AO080521000200	Predicted protein	0.456	[20][34]	[20.01][34.11]
AO080522000004	Cytochrome P450	0.456	#	#
AO080506000272	Predicted protein	0.455	#	#
AO080557000027	Predicted protein	0.455	#	#
AO080555000231	Predicted protein	0.454	#	#
AO080523000128	Predicted protein	0.453	#	#
AO080533000173	Predicted protein	0.453	#	#
AO080508000082	Predicted protein	0.451	#	#
AO080515000125	Permeases of the major facilitator superfamily	0.449	[20]	[20.01][20.03][20.09]
AO080542000184	Cytochrome P450 CYP4 CYP19 CYP26 subfamilies	0.448	#	#
AO080533000250	Predicted protein	0.447	#	#
AO080549000362	Endonuclease III	0.447	[01][10][16][32]	[01.03][10.01][16.03][16.17][16.21][32.01]
AO080523000306	Predicted protein	0.442	#	#
AO080532000098	beta-1,6-N-acetylglucosaminyltransferase, contains WSC domain	0.442	#	#
AO080536000028	Predicted protein	0.442	#	#
AO080550000149	Predicted protein	0.441	#	#
AO080513000148	Amidases	0.44	[01]	[01.02]
AO080508000396	Acetylornithine aminotransferase	0.438	[01]	[01.07][01.20]
AO080501000092	Nucleoside phosphorylase	0.437	#	#
AO080521000357	Predicted protein	0.437	#	#
AO080536000070	Predicted transporter (major facilitator superfamily)	0.437	[01][20][32][34]	[01.07][20.01][20.09][32.07][34.01]
AO080532000519	Predicted protein	0.435	#	#
AO080503000049	Serine threonine protein kinase	0.434	#	#
AO080508000367	Predicted protein	0.433	[01]	[01.04]
AO080541000303	Predicted protein	0.433	#	#
AO080525000618	Predicted protein	0.432	#	#
AO080529000044	Predicted Zn-dependent hydrolases of the beta-lactamase fold	0.432	#	#
AO080550000111	Predicted protein	0.432	#	#
AO080508000204	Predicted dehydrogenase	0.431	#	#
AO080523000678	Predicted protein	0.431	[10][16]	[10.01][16.03]
AO080515000221	Aspartyl protease	0.43	[01][14]	[01.25][14.13]
AO080521000254	1-Acyl dihydroxyacetone phosphate reductase and related dehydrogenases	0.427	[01][43]	[01.05][01.06][43.01]
AO080523000411	Predicted protein	0.426	#	#
AO080541000075	Predicted protein	0.426	#	#
AO080501000186	Predicted protein	0.424	#	#
AO080538000052	Uncharacterized conserved protein	0.419	#	#
AO080508000165	FAD FMN-containing dehydrogenases	0.418	[01]	[01.20]
AO080501000074	nucS nuclease S1 precursor	0.416	[01]	[01.03]
AO080523000712	Permeases of the major facilitator superfamily	0.416	[01][20]	[01.05][20.01][20.03]
AO080530000037	Signal transduction histidine kinase	0.413	#	#
AO080515000068	Carboxylesterase and related proteins	0.412	#	#
AO080515000315	Fructose tagatose bisphosphate aldolase	0.412	[01][02]	[01.05][02.01][02.07]
AO080539000047	Predicted protein	0.412	#	#
AO080558000032	Permeases of the major facilitator superfamily	0.41	#	#
AO080550000154	Amino acid transporters	0.409	#	#
AO080531000003	SAM-dependent methyltransferases	0.402	#	#
AO080502000037	NADPH:quinone reductase and related Zn-dependent oxidoreductases	0.399	#	#
AO080523000067	Predicted metal-dependent hydrolase with the TIM-barrel fold	0.399	#	#
AO080525000576	Predicted protein	0.398	#	#
AO080515000209	Uncharacterized protein conserved in bacteria	0.395	#	#
AO080553000093	Predicted protein	0.394	#	#
AO080549000130	Predicted transporter (major facilitator superfamily)	0.393	[01][20][34]	[01.05][20.01][20.03][20.09][34.01]
AO080530000032	Predicted protein	0.385	#	#
AO080533000073	Predicted protein	0.379	#	#
AO080503000122	Predicted protein	0.371	#	#
AO080513000198	Cytochrome P450 CYP2 subfamily	0.369	#	#

AO080521000073	Aldo keto reductase family proteins	0.369	[01][02][16][32][34]	[01.01][01.05][01.06][01.07][01.20][02.01][02.16][16.21][32.01][32.10][34.11]
AO080521000107	Flavonol reductase cinnamoyl-CoA reductase	0.369	[01][02][32][42]	[01.05][01.07][01.20][02.01][32.01][32.07][42.01]
AO080567000015	Predicted protein	0.369	#	#
AO080505000144	Predicted protein	0.368	#	#
AO080503000326	Predicted protein	0.367	#	#
AO080508000394	Predicted protein	0.366	#	#
AO080515000088	Dehydrogenases with different specificities (related to short-chain alcohol dehydrogenases)	0.366	[01][02][43]	[01.05][01.06][01.20][02.25][43.01]
AO080508000236	Predicted protein	0.363	#	#
AO080531000178	Predicted protein	0.362	#	#
AO080522000018	Fatty acid desaturase	0.356	[01]	[01.06]
AO080533000203	Cytochrome P450 CYP3 CYP5 CYP6 CYP9 subfamilies	0.355	[01][20][32]	[01.06][01.20][20.01][32.05][32.07]
AO080508000166	Synaptic vesicle transporter SVOP and related transporters (major facilitator superfamily)	0.354	[20]	[20.01]
AO080541000328	1-aminocyclopropane-1-carboxylate synthase, and related proteins	0.353	[01][36][40]	[01.02][01.05][01.20][36.02][40.02]
AO080610000005	Predicted protein	0.343	#	#
AO080502000006	Endo-1,4-beta-glucanase IV	0.34	[01]	[01.05][01.25]
AO080554000005	Predicted protein	0.338	#	#
AO080539000025	Predicted protein	0.332	[10][14][20][32][42]	[10.01][10.03][14.07][20.01][32.01][32.07][42.25]
AO080515000066	Permease of the major facilitator superfamily	0.331	[20]	[20.01][20.03][20.09]
AO080523000164	Predicted protein	0.328	[32]	[32.07]
AO080525000600	Mg^{2+} and Co^{2+} transporters	0.325	#	#
AO080530000035	Predicted protein	0.325	#	#
AO080536000027	manD, Endo-beta-mannanase	0.319	[01]	[01.05]
AO080541000084	Predicted protein	0.316	[01]	[01.05]
AO080521000262	Predicted protein	0.314	#	#
AO080525000417	Predicted protein	0.314	#	#
AO080551000032	Predicted protein	0.314	#	#
AO080541000430	Dehydrogenases with different specificities (related to short-chain alcohol dehydrogenases)	0.309	#	#
AO080503000237	Predicted protein	0.308	#	#
AO080525000729	Probable taurine catabolism dioxygenase	0.308	[01]	[01.02]
AO080521000084	2,4-dihydroxyhept-2-ene-1,7-dioic acid aldolase	0.302	[01]	[01.20]
AO080513000199	Predicted protein	0.299	#	#
AO080523000583	Predicted protein	0.299	#	#
AO080523000582	Predicted transporter (major facilitator superfamily)	0.289	[01][20][34][41]	[01.05][20.01][20.03][20.09][34.01][34.11][41.01]
AO080523000410	Predicted protein	0.288	#	#
AO080528000012	Predicted protein	0.283	#	#
AO080532000211	Ca^{2+} Na^{+} antiporter	0.28	#	#
AO080503000406	Dehydrogenases with different specificities (related to short-chain alcohol dehydrogenases)	0.276	[01][16]	[01.05][01.20][16.21]
AO080513000058	Predicted protein	0.275	#	#
AO080562000011	Amino acid transporters	0.274	[20]	[20.01][20.03][20.09]
AO080525000086	Predicted protein	0.271	[01]	[01.05][01.25]
AO080508000345	Dehydrogenases with different specificities (related to short-chain alcohol dehydrogenases)	0.269	[01][02][10][16][20][30][32][34]	[01.05][01.06][01.07][01.20][02.07][10.03][16.01][16.21][20.01][30.05][32.05][34.01]
AO080549000389	Sorbitol dehydrogenase	0.269	[01][02][16]	[01.01][01.05][01.07][01.20][02.16][16.17][16.21]
AO080536000095	Predicted protein	0.266	#	#
AO080532000017	Phenylpropionate dioxygenase and related ring-hydroxylating dioxygenases, large terminal subunit	0.263	#	#
AO080554000001	H^{+} oligopeptide symporter	0.259	[20]	[20.01][20.03][20.09]
AO080532000023	Predicted protein	0.258	#	#
AO080525000588	1-aminocyclopropane-1-carboxylate synthase, and related proteins	0.251	[01]	[01.02][01.05][01.20]
AO080510000163	Predicted protein	0.244	#	#
AO080541000142	WD40 repeat	0.243	#	#
AO080513000202	Chitinase	0.24	#	#
AO080531000064	Predicted protein	0.24	#	#
AO080531000061	Predicted protein	0.238	#	#
AO080541000150	Predicted protein	0.228	#	#
AO080549000322	Protocatechuate 3,4-dioxygenase beta subunit	0.225	#	#
AO080525000018	Predicted protein	0.224	#	#
AO080532000465	Predicted protein	0.221	#	#
AO080532000197	Synaptic vesicle transporter SVOP and related transporters (major facilitator superfamily)	0.216	[20][32][34]	[20.01][20.03][20.09][32.05][32.07][34.11]
AO080530000022	Predicted protein	0.208	#	#
AO080523000427	Permease of the major facilitator superfamily	0.193	#	#
AO080513000203	Predicted protein	0.19	#	#
AO080521000112	Dehydrogenases with different specificities (related to short-chain alcohol dehydrogenases)	0.172	[01]	[01.05][01.06][01.20]
AO080501000148	Hydroxyindole-O-methyltransferase and related SAM-dependent methyltransferases	0.162	[01]	[01.05][01.20]
AO080501000144	Glutaminyl cyclase	0.155	[01][14]	[01.01][14.07][14.13]
AO080501000147	Predicted protein	0.151	#	#
AO080532000466	Predicted protein	0.142	#	#
AO080554000025	Amino acid transporters	0.14	[20]	[20.01][20.09]
AO080501000146	Predicted protein	0.136	#	#
AO080501000145	Predicted protein	0.135	#	#
AO080561000089	Endo-1,4-beta-xylanase G2	0.111	[01]	[01.05]
AO080536000049	Predicted protein	0.0778	#	#
AO080508000347	Dehydrogenases with different specificities (related to short-chain alcohol dehydrogenases)	0.0748	#	#
AO080522000002	Predicted protein	0.0479	#	#
AO080513000200	Predicted protein	0.0336	#	#
AO080501000141	Predicted protein	0.0322	#	#

[a] FunCat (http://www.webcitation.org/getfile?fileid=be4936ae25ebb5dfb89b687842ea640f8acf7790) is the organism independent functional description of proteins. FunCat consists of 28 main functional categories (level 1). The level 1 is the most general one, whereas level 2 shows much more detail.

[b] #: Unclassified gene

[c] Detailed descriptions of each category are available at the MIPS Functional Catalogue Database (http://mips.helmholtz-muenchen.de/proj/funcatDB/)

CHAPTER II
Comprehensive phenotypic analysis of AoHDAC disruptants

II.1 ABSTRACT

In the eukaryotic cell, HDACs play key roles in the regulation of fundamental cellular process such as development regulation, stress response, secondary metabolism and genome integrity. Thus, it was expected that AoHDAC also have divergent functions in *A. oryzae*. In the Chapter II, I provide a comprehensive phenotypic analysis using HDAC disruptants in *A. oryzae*. My study revealed that four AoHDACs, *hdaA/Aohda1*, *hdaB/Aorpd3*, *hdaD/Aohos2* and *hst4/AohstD* were involved in stress response, cell wall synthesis and chromatin integrity in *A. oryzae*. Osmotic stress sensitivity of HDAC disruptants differed between plate cultures and liquid cultures, suggesting that HDACs adapt to the difference environmental conditions. Using a common *A.oryzae* fermentation medium, rice-*koji*, I also characterized HDACs related to growth and enzyme production to investigate which HDACs will be required for adaptation to environmental conditions and stress resistances. Because HDACs are widely conserved, my study has broad applications and may inform work with filamentous fungi and other eukaryote.

II.2 INTRODUCTION

In the past decade, the importance of HDACs have been recognized as important to multiple cellular processes, including development, stress response and genome integrity (Horio et al. 2011; Yang and Seto 2008).

The filamentous fungi are a diverse group with important economical applications, including the production of fermented foods, commercial enzymes and useful chemicals (Hoffmeister and Keller 2007; Iwashita 2002). Others are human or plant pathogens and virulence has even been reported in some filamentous fungi (Ding et al. 2010; Gacek and Strauss 2012; Pagiotti et al. 2011; Raffaele and Kamoun 2012). Recently, attention has been drawn to HDACs and their industrial applications as regulators of fungal development, conidiation, stress response and secondary metabolite production (Li et al. 2010; Shimizu et al. 2012; Tribus et al. 2010; Tribus et al. 2005). As described in introduction of Chapter I, HDACs have been suggested their role in multiple fungal processes in some filamentous fungi. Thus, AoHDACs will concern with diverse cellular processes in *A. oryzae*.

The filamentous fungus *A. oryzae* has been used for more than 1000 years in the traditional food industry and is listed as a GRAS species (i.e., generally recognized as safe) by the Food and Drug Administration in the United States. Its safety is also confirmed by the World Health Organization (Machida et al. 2008). *A. oryzae* has the ability to produce high quantities of enzymes and beneficial secondary metabolites, such as kojic acid and WYK-1 (Christensen et al. 1988; Imamura et al. 2012; Terabayashi et al. 2010). Thus, it was attractive to understand the relationship between these beneficial phenotypes of *A. oryzae* and functions of AoHDACs.

In the chapter II, I more closely examine the phenotypic expression of HDAC in *A.*

oryzae, specifically in response to several types of stress and drugs and its role in growth and production of protein in rice-*koji*.

II.3 MATERIALS AND METHODS

II.3.1 Strains and media

All *A. oryzae* strains used in the Chapter II are listed in Table I.6.1 (Kawauchi et al. 2013). N medium was used as the basic medium for all stress and drug-resistance analyses (Kawauchi et al. 2013). For rice-*koji* making, 15g α-rice (70% polished *Akihikari*) was used. Distilled water suspending the conidia of disruptants was added to the α-rice as 30% initial water content and 1×10^5 conidia/g α-rice. The inoculum was incubated at 35 °C for 42h in 100% humidity.

II.3.2 Environmental stress resistance assay

For the stress test on plates, 1 μl of conidia suspension (1×10^5 conidia) of each strain was point inoculated on the center of the plate and grown for five days at 30°C. N medium containing 1.6 M NaCl was used for the osmotic stress resistance test and 20mM H_2O_2 was used for the oxidative stress resistance test. For the heat resistance test, the plate was incubated at 37°C. For hypoxic growth test, the plate was incubated in a 2% O_2 concentration using the Multi Gas Incubator APM-50DR (Astec, Fukuoka, Japan). After these incubations, Colony diameter was measured after the incubation period. For biomass analysis in the liquid culture, a 4 cm^2 plug of each strain were cut from the full-growth plate cultures and homogenized in 1 ml of suspension solution and then inoculated with 100 ml of N liquid medium containing 0.8 M NaCl. Flasks were incubated for two days at 30°C with shaking at 100 rpm. Mycelia were harvested, dried at 105°C for 1 h and weighed.

II.3.3 Drug resistance test

For drug resistance tests, conidia suspensions of each strain were point inoculated an incubated as outlined in the stress test above. The following chemicals were tested: calcofluor white (300 μg/ml), Congo red (50 μg/ml), micafungin (2 ng/ml), hydroxy urea (10 mM), camptothecin (1 μM), methyl methane sulfonate (MMS; 0.1%), tunicamycin (5 μg/ml), nocodazole (500 ng/ml), dithiothreitol (10mM), brefeldin A (5 μg/ml). Congo red, dithiothreitol, camptothecin, MMS, brefeldin A and nocodazole were purchased from Wako Chemicals (Osaka, Japan); tunicamycin were purchased from Calbiochem (La Jolla, CA, USA); and the hydroxy urea and calcofluor white were purchased from Sigma Aldrich (St. Louis, MO, USA). The micafungin (Astellas, Tokyo, Japan) was gifted. A stock solution of was prepared by dissolving 100 mg/ml calcofluor white, 10mg/ml Congo red, 1mg/ml micafungin, 1M hydroxyl urea, and 1M dithiothreitol (1M) in water. The camptothecin (10 mM), nocodazole (1 mg/ml), brefeldin A (1 mg/ml) and tunicamycin (2 mg/ml) were dissolved in dimethyl sulfoxide.

II.3.4 Measurement of enzyme activity and total proteins production

Enzymes were extracted from 5 g of rice-*koji* after incubation with 25 ml acetate buffer (10 mM, pH 5.0, 0.5% NaCl) at 4°C for 3 h with shaking at 80 rpm followed by filtration. Enzyme activities of α-amylase, glucoamylase and acid carboxypeptidase were measured using enzyme assay kits (Kikkoman, Chiba, Japan). Acidic protease activity was assayed according to a previous report (Iemura et al. 1999). Total protein was measured by using the Bio-Rad Protein Assay Kit II.

II.3.5 Measurement of N-acetylglucosamine content in rice-*koji*

The rice-*koji* (5g) was dried at 105°C for 1h, then homogenized in 12.5 ml

phosphate buffer (50 mM, pH 6.8) using physcotron (Microtec, Chiba, Japan),and centrifuged. The pellet was washed with a 10-ml phosphate buffer more than five times then suspended in a 20-ml phosphate buffer. A 2-ml of suspension was mixed with 7 ml phosphate buffer and 1 ml Yatalase (TaKaRa, Kyoto, Japan) solution (10 mg /ml in phosphate buffer). This mixture was shaking at 80rpm at 37°C for 3 h. The N-acetylglucosamine composition was determined with a pulse high-performance anion-exchange chromatography with a pulse electrochemical detector (DX500 chromatography system, Dionex, Sunnyvale, CA, USA) and an anion exchange column (Carbo PAC PA-1, 4×250 mm, Dionex) at a flow rate of 1 mL/min. Isocratic elution was performed with 16mM NaOH. The column was stabilized for 20min before injection and washed with 100mM NaOH / 600mM CH_3COONa for 10 min after elution. To quantify the N-acetylglucosamine, I used N-acetyl-D (+)-glucosamine (Wako) as a standard and D-fucose as an internal standard.

II.4 RESULTS

II.4.1 Stress resistance of AoHDACs

In the previous chapter, I identified 11 HDACs homologs in *A. oryzae* genome and construct 10 AoHDAC disruptants and one AoHDAC heterokaryon disruptant as listed in Table I.6.1 (Kawauchi et al. 2013).

The osmotic, oxidative, heat and hypoxia stress tolerances of all AoHDAC disruptants were quantified to analyze the importance of HDACs in environmental adaptations (Figs. II.6.1-3). Three AoHDAC disruptants showed sensitivity against these stresses (Fig. II.6.1A, B). The *ΔhstD* strain was sensitive to the osmotic stress; the *hdaB/Aorpd3* heterokaryon disruptant (*hdaB ht* strain) showed significant sensitivity against osmotic, oxidative and heat stress; the *ΔhdaD* strain showed significant sensitivity against the low oxygen and osmotic stress. These results suggest that *hdaB/Aorpd3*, *hdaD/Aohos2* and *hstD/Aohst4* are required for stress tolerance in *A. oryzae*.

In the liquid culture of the osmotic-stress test, I found a unique phenotype of *ΔhdaA* strain (Fig. II.6.1C). This strain showed significant sensitivity against osmotic stress in liquid culture, but not in plate culture. This result suggests that *hdaA/Aohda1* plays a role in liquid culture specific osmotic stress response. I also found that the *hdaB ht* and *ΔhdaD* strain were more osmo-sensitive in liquid culture (Fig. II.6.1C). These findings suggest that different osmotic stress resistance mechanisms between the plate and liquid culture conditions, and *hdaB/Aorpd3*, *hdaA/Aohda1 and hdaD/Aohos2* will be included in these mechanisms.

II.4.2 Drug (inhibitor) resistance of AoHDAC disruptants

I examined AoHDAC disruptants' resistance to several chemicals on plate culture and, at first, the cell wall integrity was tested using cell wall synthesis inhibitors (Figs. II.6.4-5). The *hdaB ht* strain was significantly sensitive to micafungin, a *β*-1,3-glucan synthesis inhibitor (Free 2013). The deletion of *hdaD/Aohos2* showed the greatest sensitivity against the calcofluor white, which is a chitin synthesis inhibitor. The *ΔhstA* strain also showed the slight sensitivity to calcofluor white (Fig. II.6.4.5). These results suggest that there is variation in how different AoHDACs affect cell wall integrity, and *hdaB/Aorpd3*and *hdaD/Aohos2* will be concerned with these mechanisms.

HDACs are also relevant to genome integrity (Huertas et al. 2009); thus, I examined the genotoxin tolerances of all AoHDAC disruptants (Figs. II.6.6-7). As expected, the *hdaB ht, ΔhdaD* and *ΔhstD* strains showed defects against methyl methane sulfonate, which methylates DNA predominantly on N7-deoxyguanosine and N3-deoxyadenosine and is believed to cause double-stranded DNA breaks. Additionally, the *ΔhstD* strain showed the strongest sensitivity to camptothecin, a topoisomerase inhibitor. These results suggested *hdaB/Aorpd3*, *hdaD/Aohos2* and *hstD/Aohst4* are involved in genome integrity.

No obvious sensitivity to protein secretion inhibitors was observed when tested dithiothreitol, tunicamycin or brefeldin A (Fig. II.6.8). A slight sensitivity was observed with nocodazole, which depolymerizes microtubules, in the *ΔhstD* and *hdaB ht* strain.

II.4.3 Effect of AoHDACs disruption on the rice-*koji*

A distinctive feature of the *koji* fermentation process is the use of solid-state culture where *A. oryzae* is grown on steamed cereals such as rice and soybean. The resulting material contains abundant hydrolytic enzymes and metabolites that are

important for the quality of the final products (Kitamoto 2002). I analyzed the effect of AoHDACs disruption on the growth and protein production of rice-*koji* (Figs. II.6.9-10).

It is difficult to separate the mycelia from rice-*koji* to measure exact fungal biomass, so instead I measured N-acetylglucosamine, which has been shown strongly correlated with biomass (Arima and Uozumi 1967). I found significant decreases in N-acetylglucosamine on rice-*koji* made by the *hdaB ht* and *ΔhdaD* strains (Fig. II.6.9A). The *ΔhdaA* strain also decreased N-acetylglucosamine, but the results were not significant. The disruptant strains also decreased total protein production, but enzyme activity differed by disruptant (Fig. II.6.9B, C). The *hdaB ht* strain showed significant decreases in all measured enzyme activity, while the *ΔhdaA* and *ΔhdaD* strains only showed a significant decrease in glycoside hydrolase activity and a slight increased acid carboxypeptidase activity. In these strains, the low glycoside hydrolase productivity or growth defect might cause low protein productivity. Compared with these three strains, the *ΔhstD* strain showed no decrease in N-acetylglucosamine but had lowered protein production. I also found significant decreased in glycoside hydrolase and acid protease. These results suggest that *hstD/Aohst4* can affect many types of protein production even when it does not affect growth.

II.5 DISCUSSION

HDACs play diverse roles in higher eukaryote development, stress response, and genome integrity (Horio et al. 2011; Yang and Seto 2008). In some filamentous fungi, HDACs are important to growth, conidia formation, and secondary metabolism (Brosch et al. 2008). The results of ChapterII strongly suggest that functional divergence of HDACs in *A. oryzae* with important industrial implications for HDAC deletion.

The modes of development and enzyme production in *A. oryzae* are significantly different when it grows in solid-state or liquid culture conditions (Biesebeke et al. 2002). This supports recent omics studies of *A. oryzae* that revealed how the transcriptome and proteome were altered depending on culture type (Oda et al. 2006; Wang et al. 2010). However, a detailed molecular investigation is needed to understand the underlying mechanisms.

HDACs widely affected gene expression through the structural modification of chromatin by the deacetylation of histones. I found phenotypic differences between disruptants grown on the liquid culture as opposed to the plate culture. The *ΔhdaA* strain did not show any significant effect against osmotic stress on the plate culture, but an obvious defect was observed in liquid culture. Additionally, the *hdaB ht* strain and *ΔhdaD* strains had a more sensitive phenotypic response to osmotic stress in the liquid culture. These results indicated some difference between the liquid and plate culture on the osmotic adaptation and these three HDACs would play a role in the adaptation mechanisms in *A. oryzae*.

I also found a significant decrease in the amount of N-acetylglucosamine in rice-*koji* produced by the *hdaB ht*, *ΔhdaD* and *ΔhdaA* strains. N-acetylglucosamine is a key constituent saccharide of chitin, the major glycan composing fungal cell walls and a

good surrogate for measuring fungal growth on rice-*koji* (Arima and Uozumi 1967). The lack of growth effects in these three HDACs in the solid-state cultures suggests the importance of the role of other compounds stabilizing the fungal cell wall. In this experiment I only measured the amount of N-acetylglucosamine whereas fungal cell walls also contain β- and α-1,3-glucan, chitin and galactomannan (Latgé 2010). Thus, it is not clear whether the disruption of *hdaB/Aorpd3* and *hdaD/Aohos2* affects the overall glycan construction of cell wall. Future work should examine the detailed role of AoHDACs on the cell wall synthesis mechanisms and the relationship of AoHDAC disruption to fungal biomass.

The *hstD/AoHst4* HDAC has been phylogenetically classified in the fungal specific sirtuin class but its function is still poorly understood (Frye 2000). I indicated that *hstD/Aohst4* coordinates fungal specific phenotypes of secondary metabolite production and conidia formation and a separate study using *S. cerevisiae*, found that *hst4* (a homolog of *hstD* in *A.oryzae*) was important for genome integrity and resistance to genotoxin (Kawauchi et al. 2013; Miller et al. 2006). In my work, I reveal an additional function. The deletion of *hstD/Aohst4* led to MMS- and CPT-sensitive phenotypes suggests that this family of proteins plays a conserved role in the genome integrity among fungi.

I also found a novel effect on enzyme production in rice-*koji* produced by the disruptant strains. The *ΔhstD* strain decreased enzyme production despite the growth of mycelia being unchanged. This was surprising because, in general, alteration of the protein secretion pathway affects filamentous growth of *A. oryzae* (Shoji et al. 2008). The lack of sensitive phenotypes when I tested protein secretion pathway inhibitors suggests that *hstD/Aohst4* may not affect the protein secretion pathway. In general,

HDACs affect transcriptional regulation, so it is possible *hstD/Aohst4* affects extracellular enzyme expression. This would have significant industrial applications and future work should investigate the expression, histone modification and chromatin structure of these genes.

This work reveals novel phenotypes of four AoHDAC disruptants in *A. oryzae*: *hdaA/Aohda1*, *hdaB/Aorpd3*, *hdaD/Aohos2*, and *hstD/Aohst4* with diverse cellular process that include stress response, cell wall synthesis, protein secretion, and genome integrity. Because HDACs are highly conserved among filamentous fungi and other eukaryote, my study is broadly applicable to understanding the function of HDACs.

II.6 FIGURES

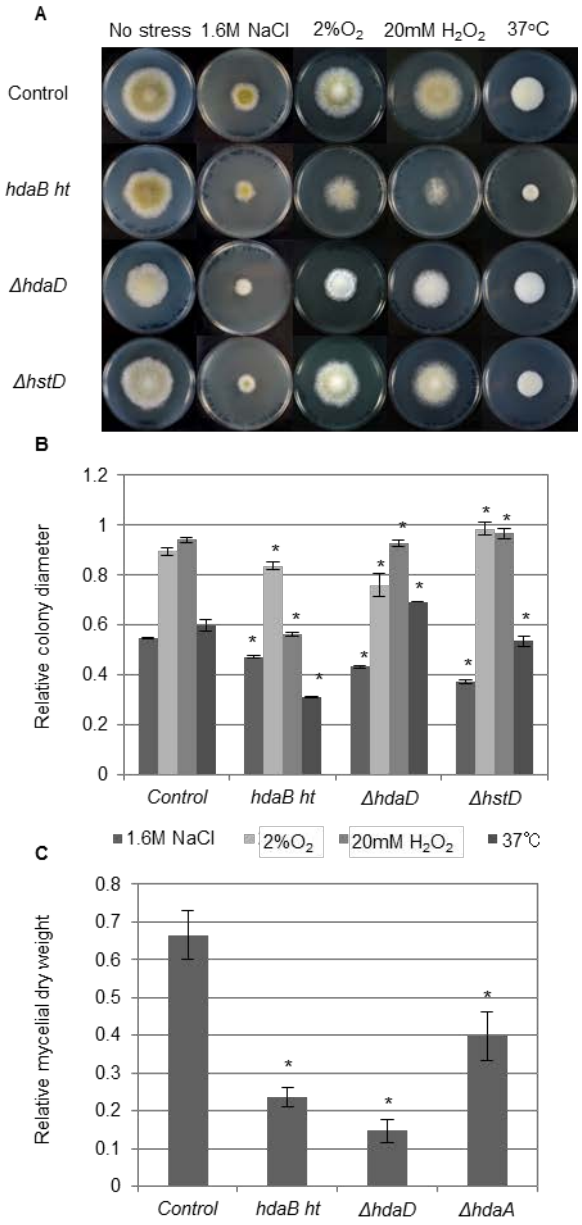

Figure II.6.1. Stress sensitivity test of AoHDAC disruptants.
(A) Morphological phenotypes of disruptants on stress plate culture on day five. **(B)** Comparison of stress sensitivity on plate culture. Graphs represent radial growth relative to the no-stress plate culture (corresponding to 1.0). **(C)** Comparison of osmotic stress sensitivity on liquid culture. Graphs represent mycelia dry weight relative to the no-stress liquid culture (corresponding to 1.0). The *adeA*$^{+}$ strain was used as a control in this figure. All data are represented as means ± s.d. (n=3); * $p < 0.05$, t-test.

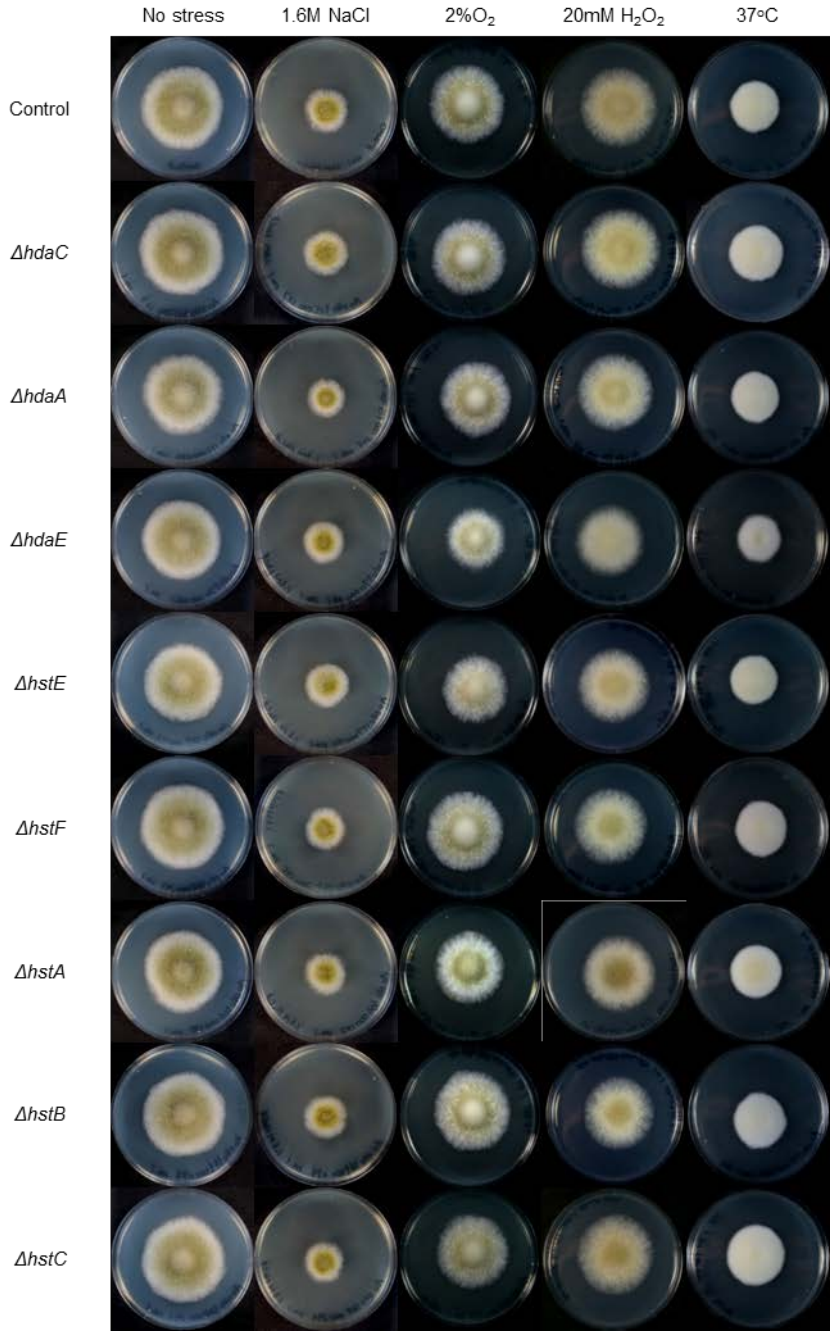

Figure II.6.2. Phenotypes of AoHDAC disruptants on stress plate culture at day five.
The *adeA*$^{+}$ strain was used as a control.

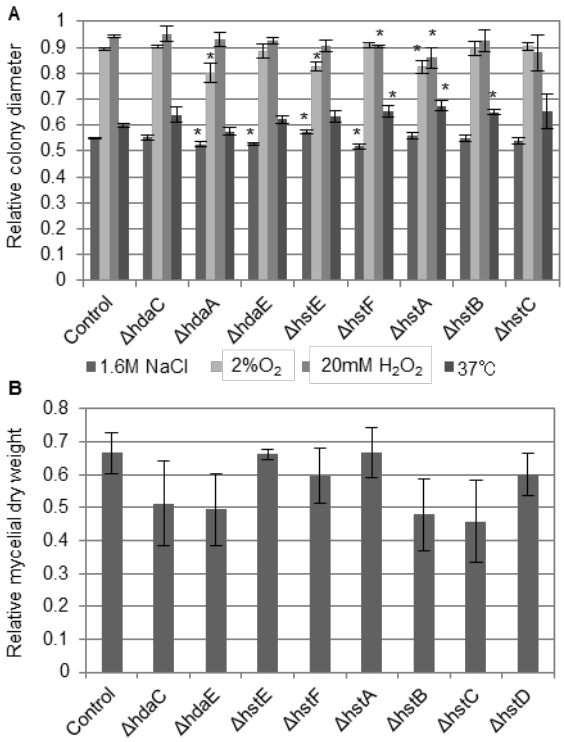

Figure II.6.3. Stress sensitivity test of AoHDAC disruptants.
(A) Comparison of stress sensitivity on plate culture. Graphs represent radial growth relative to the no stress plate culture (corresponding to 1.0). **(B)** Comparison of osmotic stress sensitivity on liquid culture. Graphs represent mycelia dry weight relative to the no stress liquid culture (corresponding to 1.0). The *adeA*$^{+}$ strain was used as a control. All data are represented as means ± s.d. (n=3); * $p < 0.05$, t-test.

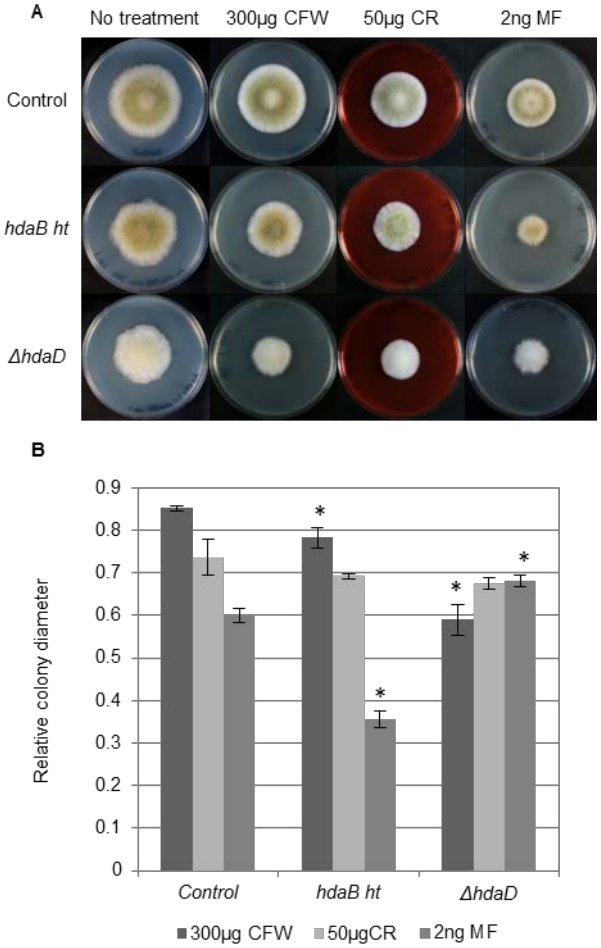

Figure II.6.4. Cell wall synthesis inhibitor sensitivity of AoHDAC disruptants.
(A) Morphological phenotypes of indicated strain on cell wall synthesis inhibitor treated culture on day five. **(B)** Comparison of inhibitors sensitivity on plate culture. Graphs represent radial growth relative to the plate culture lacking an inhibitor (corresponding to 1.0). The *adeA*$^{+}$ strain was used as a control. All data are represented as means ± s.d. (n=3); * $p < 0.05$, t-test. Abbreviations: CFW, calcofluor white; CR, Congo red; MF, micafungin.

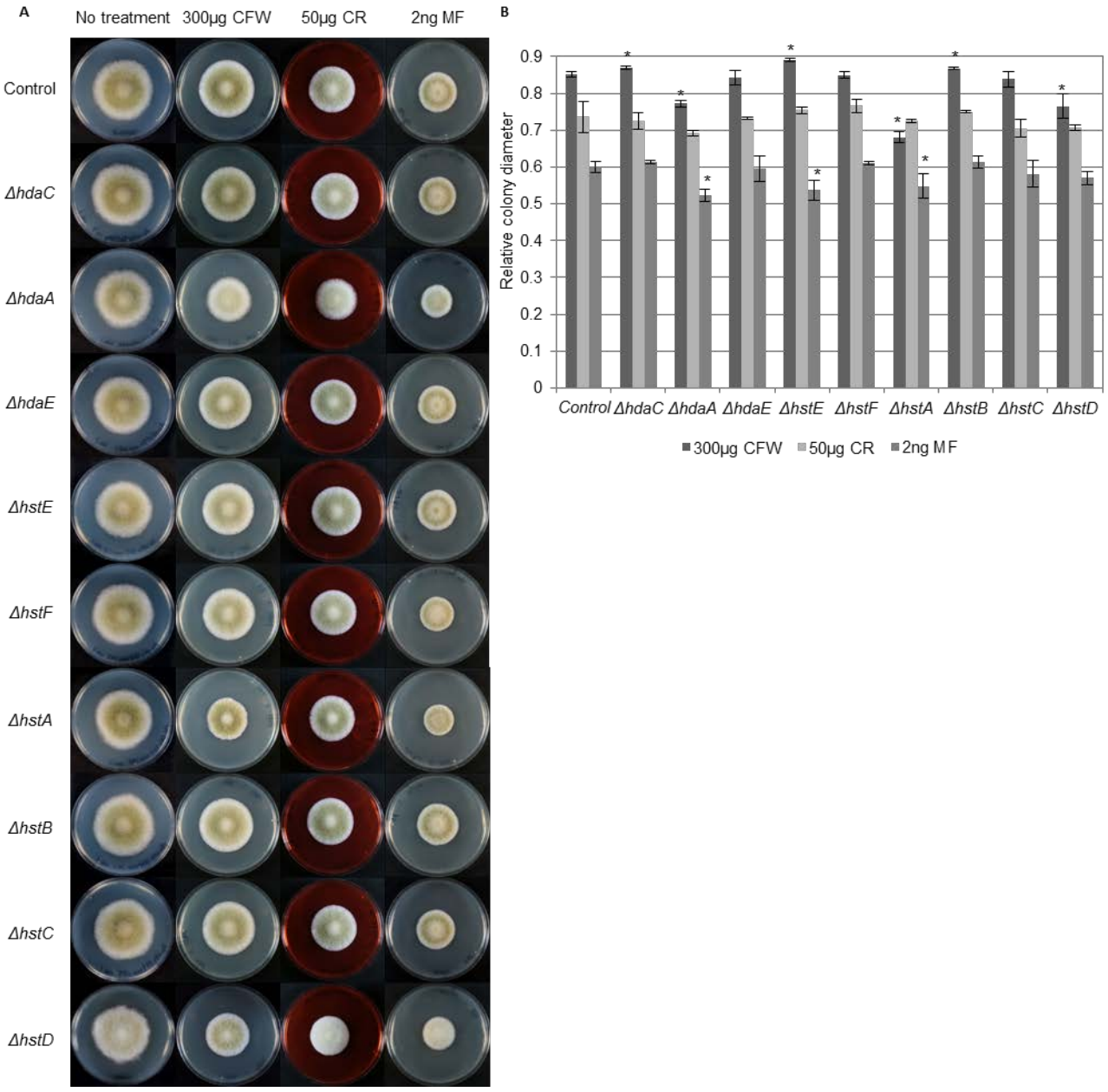

Figure II.6.5. Cell wall synthesis inhibitor sensitivity of AoHDAC disruptants.
(A) Morphological phenotypes of indicated strain on cell wall synthesis inhibitor treated culture on day five. **(B)** Comparison of inhibitor sensitivity on plate culture. Graphs represent radial growth relative to the plate culture lacking an inhibitor (corresponding to 1.0). The *adeA*$^{+}$ strain was used as a control. All data are represented as means ± s.d. (n=3); * $p < 0.05$, t-test. Abbreviations: CFW, calcofluor white; CR, Congo red; MF, micafungin.

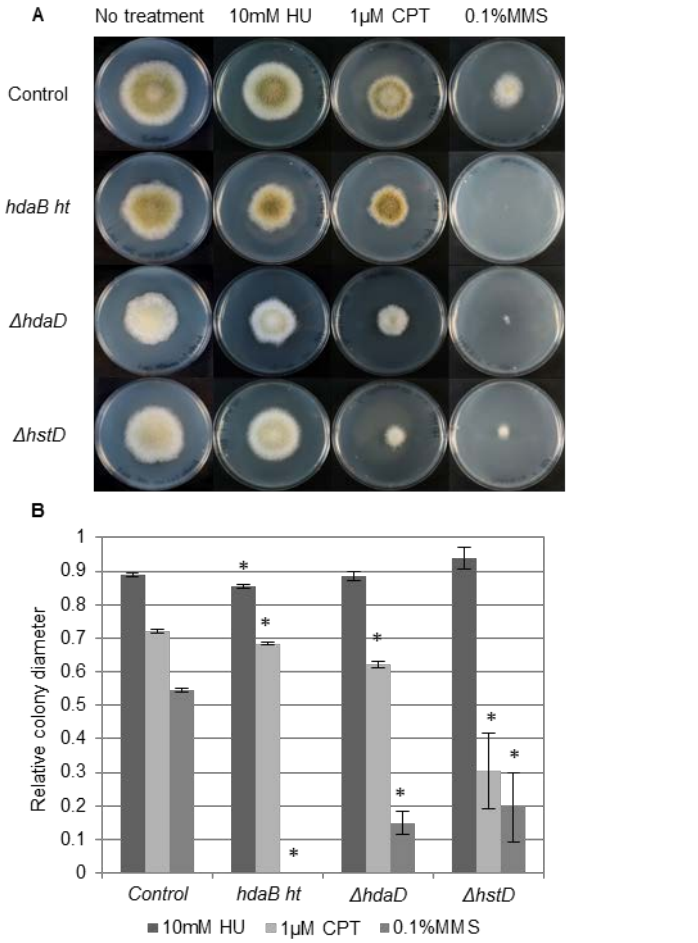

Figure II.6.6. Genotoxin sensitivity of AoHDAC disruptants.
(A) Morphological phenotypes of indicated strain on genotoxin treated culture. The picture of 5 days colony was shown. **(B)** Comparison of genotoxin sensitivity on plate culture. In order to minimize the effect of different growth on no genotoxin treated plate culture among the strain, each graph represent relative radial growth to that on no genotoxin treated plate culture (corresponding to 1.0). The *adeA*$^{+}$ strain was used as a control in this figure. All data are represented as means ± s.d. (n=3); * $p < 0.05$, t-test. Abbreviations: HU, hydroxy urea; CPT, Camptothecin; MMS, Methyl methane sulfonate.

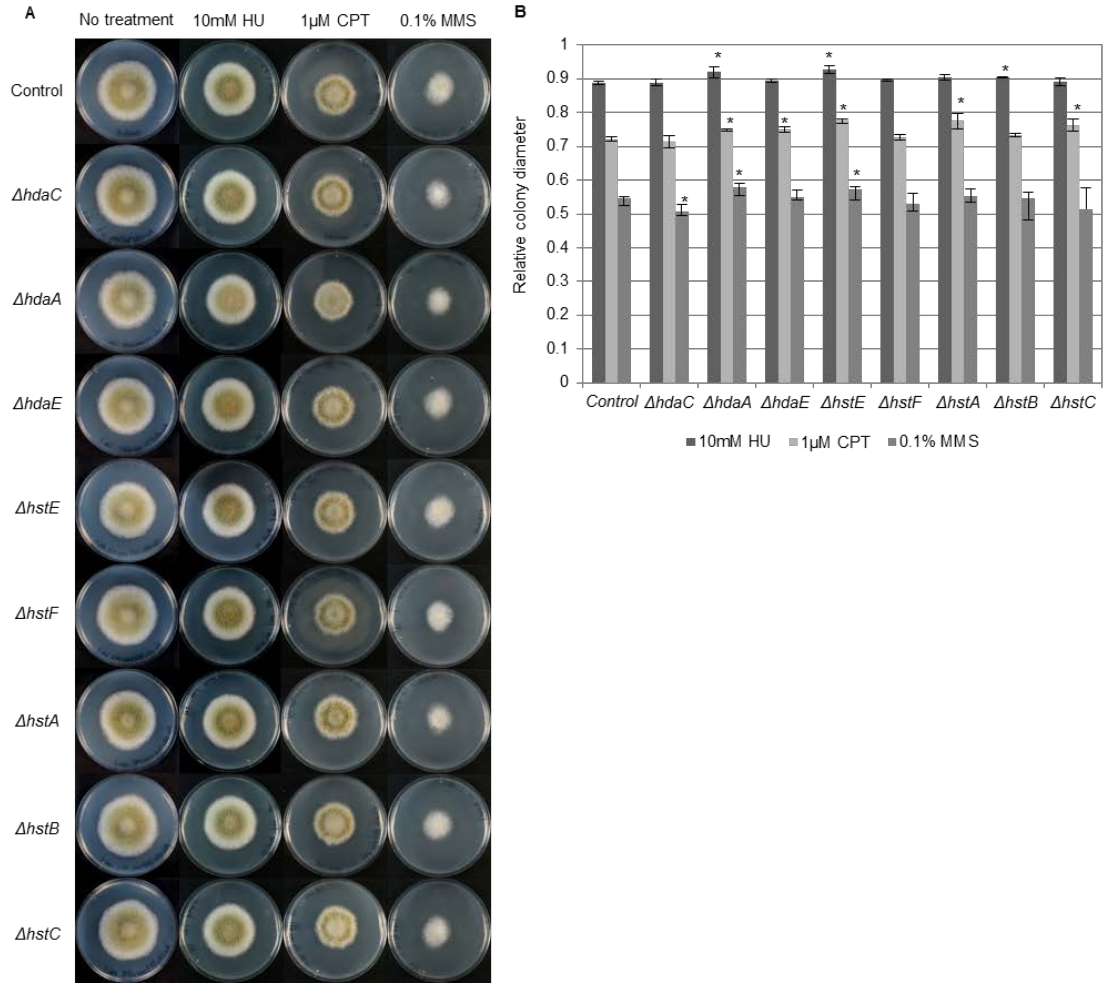

Figure II.6.7. Genotoxin sensitivity of AoHDAC disruptants.
(A) Morphological phenotypes of indicated strain on cell wall synthesis genotoxin treated culture on day five. **(B)** Comparison of inhibitors sensitivity on plate culture. Graphs represent radial growth relative to the no genotoxin treated plate culture (corresponding to 1.0). The *adeA*$^{+}$ strain was used as a control. All data are represented as means ± s.d. (n=3); * $p < 0.05$, t-test. Abbreviations: HU, hydroxy urea; CPT, camptothecin; MMS, methyl methane sulfonate.

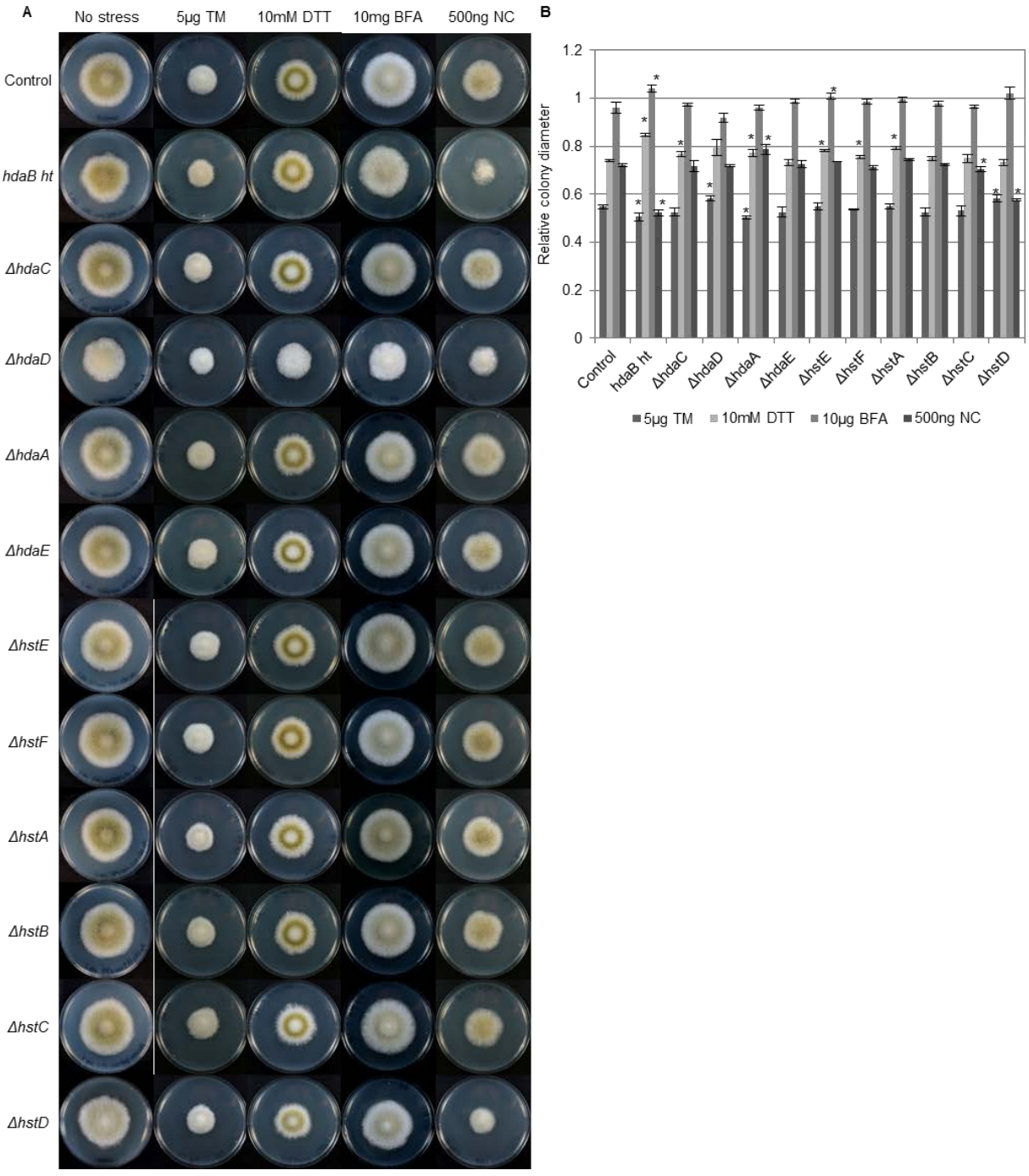

Figure II.6.8. Protein secretion inhibitor sensitivity of AoHDAC disruptants.
(A) Morphological phenotypes of indicated strain on protein synthesis or secretion treated culture on day five. **(B)** Comparison of inhibitors sensitivity on plate culture. Graphs represent radial growth relative to the plate culture lacking an inhibitor (corresponding to 1.0). The *adeA*$^{+}$ strain was used as a control. All data are represented as means ± s.d. (n=3); * $p < 0.05$, t-test. Abbreviations: TM, tunicamycin; DTT, dithiothreitol; BFA, brefeldin A; NC, nocodazole.

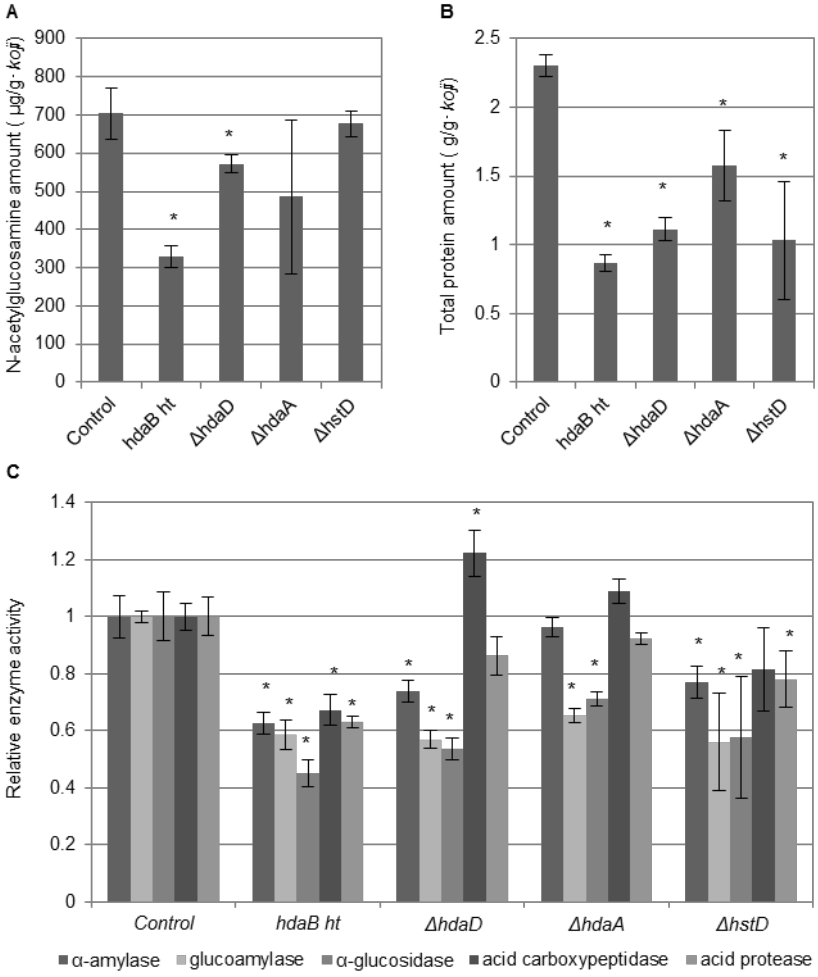

Figure II.6.9 Phenotypes of AoHDACs on rice-*koji*.
(A) N-acetylglucosamine amounts in rice-*koji*. **(B)** Total protein equivalent in rice-*koji*. **(C)** Activity of enzymes in rice-*koji*. Enzyme activities represented as the activity relative to the control strain (corresponding to 1.0). The *adeA*$^{+}$ strain functions as a control. All data are represented as means ± s.d. (n=3); * $p < 0.05$, t-test.

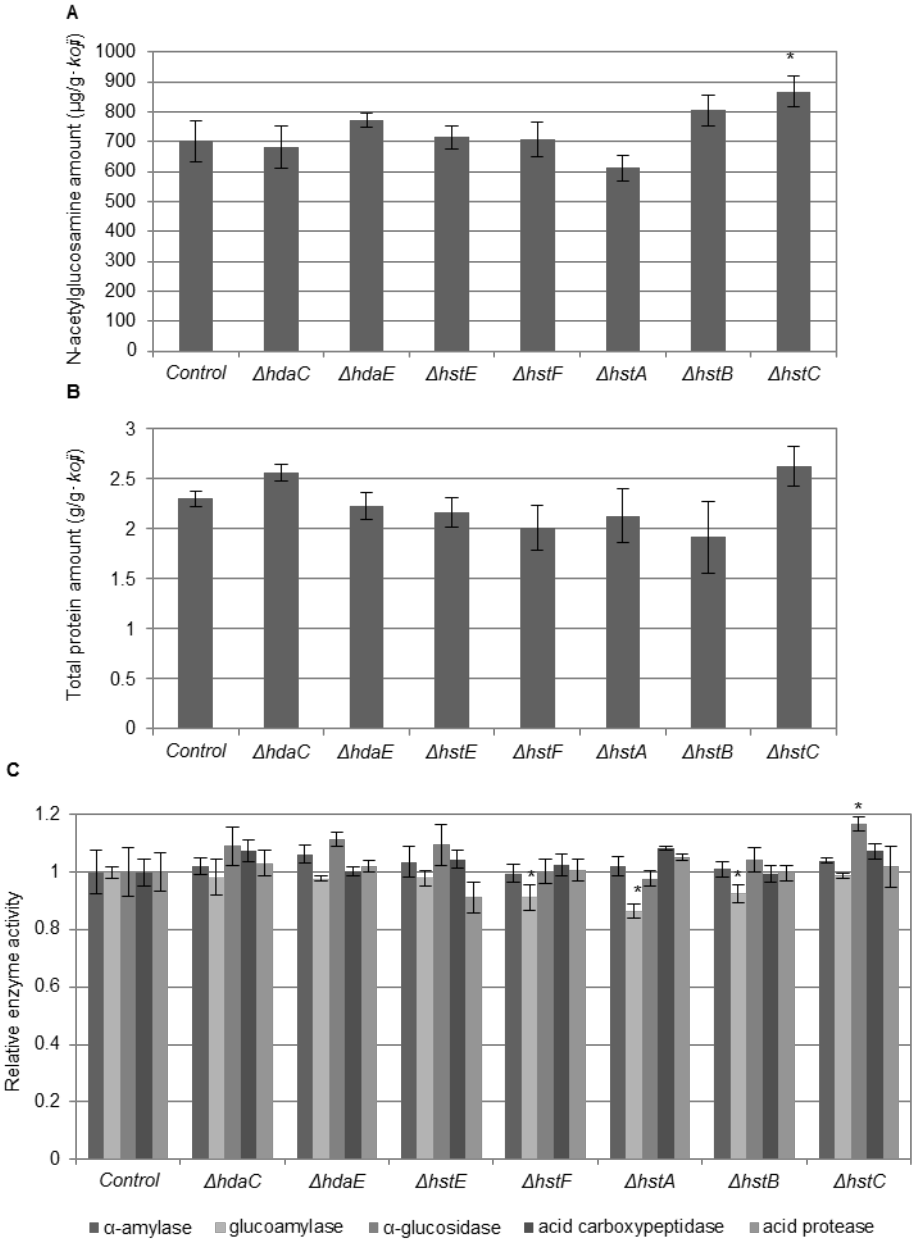

Figure II.6.10 Phenotypes of AoHDAC disruptants on rice-*koji*.
(A) N-acetylglucosamine amounts in rice-*koji*. **(B)** Total protein equivalent in rice-*koji*. **(C)** Activity of indicated enzyme in rice-*koji*. Enzyme activities represent activity relative to the *adeA*$^{+}$ control strain (corresponding to 1.0). All data are represented as means ± s.d. (n=3); * $p < 0.05$, t-test.

CONCLUSION REMARKS

HDACs are concerned with multiple cellular process (Horio et al. 2011; Yang and Seto 2008), and the importance of the role of classical HDACs in the regulation of filamentous fungal phenotypes has been appreciated during the past decade (Brosch et al. 2008). While the molecular biology of *A. oryza*e has been widely studied because of its industrial importance in many fields (Machida et al. 2008), an investigation of its epigenetic regulation has yet to be performed. In this work, we showed the diverse functions of AoHDACs.

In the Chapter I, I identified 11 AoHDACs and constructed these disruptants. In the case of *hdaB/Aorpd3* disruption, we only obtained a heterokaryon disruptant, suggested that *hdaB/Aorpd3* is essential in *A. oryzae*. The *hdaD/Aohos2* and *hstD/Aohst4* disruptants were shown to have defective conidia formation and to produce high levels of the SM kojic acid. The *hstD/Aohst4* was also found to coordinate secondary metabolism and development through the regulation of *laeA*, which is a key coordinator in filamentous fungi. The *hstD/Aohst4* gene is fungal-specific and is conserved throughout the filamentous fungi. It also has great potential to be a target for the improvement of SM productivity, and could be useful in the development of an attractive host for the production of heterogeneous metabolites.

In the Chapter II, we also examine the various kinds of phenotypes included stress resistance, drug resistance, and rice-*koji* making using these AoHDAC disruptant. As the result, I suggest that 4 AoHDACs named *hdaA/Aohda1*, *hdaB/Aorpd3*, *hdaD/Aohos2*, and *hstD/Aohst4* will relate diverse cellular process such as stress

response, cell wall synthesis, protein secretion, and genome integrity.

To my knowledge, this is the first study of *A. oryzae* HDACs, and also the first comprehensive analysis of HDACs in filamentous fungi. This work will be of great value in helping to understand HDACs in all filamentous fungi because of the high level of HDAC conservation. It will also contribute to the improvement of SMs and enzymes production of filamentous fungi, and the development of drugs against pathogenic filamentous fungi.

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REFERENCE

Abe K, Gomi K, Hasegawa F, Machida M (2006) Impact of *Aspergillus oryzae* genomics on industrial production of metabolites. *Mycopathologia* **162**:143-153

Arima K, Uozumi T (1967) A new method for estimation of the mycelial weight in *koji*. *Agri. Biol. Chem.* **31**:119-123

Arnaud MB, Chibucos MC, Costanzo MC, Crabtree J, Inglis DO, Lotia A, Orvis J, Shah P, Skrzypek MS, Binkley G, Miyasato SR, Wortman JR, Sherlock G (2010) The *Aspergillus* Genome Database, a curated comparative genomics resource for gene, protein and sequence information for the *Aspergillus* research community. *Nucleic Acids Res.* **38**:D420-427

Baidyaroy D, Brosch G, Ahn J-h, Graessle S, Wegener S, Tonukari NJ, Caballero O, Loidl P, Walton JD (2001) A gene related to yeast HOS2 histone deacetylase affects extracellular depolymerase expression and virulence in a plant pathogenic fungus. *Plant Cell* **13**:1609-1624

Bayram Ö, Braus GH (2012) Coordination of secondary metabolism and development in fungi: the velvet family of regulatory proteins. *FEMS Microbiol. Rev.* **36**:1-24

Biesebeke R, Ruijter G, Rahardjo YSP, Hoogschagen MJ, Heerikhuisen M, Levin A, Driel KGA, Schutyser MAI, Dijksterhuis J, Zhu Y, Weber FJ, Vos WM, Hondel KAMJJ, Rinzema A, Punt PJ (2002) *Aspergillus oryzae* in solid-state and submerged fermentations. *FEMS Yeast Res.* **2**:245-248

Borkovich KA, Alex LA, Yarden O, Freitag M, Turner GE, Read ND, Seiler S, Bell-Pedersen D, Paietta J, Plesofsky N, Plamann M, Goodrich-Tanrikulu M, Schulte U, Mannhaupt G, Nargang FE, Radford A, Selitrennikoff C, Galagan JE, Dunlap JC, Loros

JJ, Catcheside D, Inoue H, Aramayo R, Polymenis M, Selker EU, Sachs MS, Marzluf GA, Paulsen I, Davis R, Ebbole DJ, Zelter A, Kalkman ER, O'Rourke R, Bowring F, Yeadon J, Ishii C, Suzuki K, Sakai W, Pratt R (2004) Lessons from the genome sequence of *Neurospora crassa*: tracing the path from genomic blueprint to multicellular organism. *Microbiol. Mol. Biol. Rev.* **68**:1-108

Brakhage AA (2013) Regulation of fungal secondary metabolism. *Nat. Rev. Microbiol.* **11**:21-32

Brosch G, Loidl P, Graessle S (2008) Histone modifications and chromatin dynamics: a focus on filamentous fungi. *FEMS Microbiol. Rev.* **32**:409-439

Cairns BR (2009) The logic of chromatin architecture and remodelling at promoters. *Nature* **461**:193-198

Christensen T, Woeldike H, Boel E, Mortensen SB, Hjortshoej K, Thim L, Hansen MT (1988) High level expression of recombinant genes in *Aspergillus oryzae*. *Nat. Biotechol.* **6**:1419-1422

Ding S-L, Liu W, Iliuk A, Ribot C, Vallet J, Tao A, Wang Y, Lebrun M-H, Xu J-R (2010) The *tig1* histone deacetylase complex regulates infectious growth in the rice blast fungus *Magnaporthe oryzae*. *Plant Cell* **22**:2495-2508

D'Arcy S, Luger K (2011) Understanding histone acetyltransferase *Rtt109* structure and function: how many chaperones does it take? *Curr. Opin. Struct. Biol.* **21**:728-734

Ekwall K (2005) Genome-wide analysis of HDAC function. *Trends Genet.* **21**:608-615

Free SJ (2013) Chapter Two - Fungal Cell Wall Organization and Biosynthesis. P.32-82 In: Freedman T, Dunlap JC, Goodwin SF(ed.) *Adv. Genet.*, vol. 81. Academic

Press, Waltham, MA

Frye RA (2000) Phylogenetic classification of prokaryotic and eukaryotic *Sir2*-like proteins. *Bioche. Biophys. Res. Commun.* **273**:793-798

Gacek A, Strauss J (2012) The chromatin code of fungal secondary metabolite gene clusters. *Appl. Microbiol. Biotech.* **95**:1389-1404

Gregoretti I, Lee Y-M, Goodson HV (2004) Molecular evolution of the histone deacetylase family: Functional implications of phylogenetic analysis. *J. Mol. Biol.* **338**:17-31

Hoffmeister D, Keller NP (2007) Natural products of filamentous fungi: enzymes, genes, and their regulation. *Nat. Prod. Rep.* **24**:393-416

Horio Y, Hayashi T, Kuno A, Kunimoto R (2011) Cellular and molecular effects of sirtuins in health and disease. *Clin. Sci.(Lond.)* **121**:191-203

Huertas D, Sendra R, Muñoz P (2009) Chromatin dynamics coupled to DNA repair. *Epigenetics* **4**:31-42

Iemura Y, Yamada T, Takahashi T, Furukawa K, Hara S (1999) Properties of the peptides liberated from rice protein in *Sokujo-moto*. *J. Biosci. Bioeng.* **88**:276-280

Imai S-i, Guarente L (2010) Ten years of NAD-dependent SIR2 family deacetylases: implications for metabolic diseases. *Trends. Pharmacol. Sci.* **31**:212-220

Imamura K, Tsuyama Y, Hirata T, Shiraishi S, Sakamoto K, Yamada O, Akita O, Shimoi H (2012) Identification of a gene involved in the synthesis of a Dipeptidyl Peptidase IV Inhibitor in *Aspergillus oryzae*. *Appl. Environ. Microbiol.* **78**:6996-7002

Iwashita K (2002) Recent studies of protein secretion by filamentous fungi. *J. Biosci. Bioeng.* **94**:530-535

Izawa M, Takekawa O, Arie T, Teraoka T, Yoshida M, Kimura M, Kamakura T

(2009) Inhibition of histone deacetylase causes reduction of appressorium formation in the rice blast fungus *Magnaporthe oryzae*. *J. Gen. Appl. Microbiol.* **55**:489-498

Jin FJ, Takahashi T, Matsushima K, Hara S, Shinohara Y, Maruyama J, Kitamoto K, Koyama Y (2011) SclR, a basic helix-loop-helix transcription factor, regulates hyphal morphology and promotes sclerotial formation in *Aspergillus oryzae*. *Eukaryotic Cell* **10**:945-955

Kawauchi M, Nishiura M, Iwashita K (2013) Fungal-specific sirtuin HstD coordinates secondary metabolism and development through control of LaeA. *Eukaryotic Cell* **12**:1087-1096

Kelly DE, Kraševec N, Mullins J, Nelson DR (2009) The CYPome (Cytochrome P450 complement) of *Aspergillus nidulans*. *Fungal Genet. Biol.* **46**:S53-S61

Kitamoto K (2002) Molecular biology of the Koji molds. p. 129-153, *Adv. Appl. Microbiol.*, vol. 51. Academic Press, Waltham, MA

Latgé J-P (2010) Tasting the fungal cell wall. *Cell. Microbiol.* **12**:863-872

Lee I, Oh J-H, Keats Shwab E, Dagenais TRT, Andes D, Keller NP (2009) *HdaA*, a class 2 histone deacetylase of *Aspergillus fumigatus*, affects germination and secondary metabolite production. *Fungal Genet. Biol.* **46**:782-790

Li Y, Wang C, Liu W, Wang G, Kang Z, Kistler HC, Xu J-R (2010) The *HDF1* histone deacetylase gene is important for conidiation, sexual reproduction and pathogenesis in *Fusarium graminearum*. *Mol. Plant Microbe Interact.* **24**:487-496

Machida M, Yamada O, Gomi K (2008) Genomics of *Aspergillus oryzae*: Learning from the history of *koji* mold and exploration of its future. *DNA Res.* **15**:173-183

Marui J, Ohashi-Kunihiro S, Ando T, Nishimura M, Koike H, Machida M (2010) Penicillin biosynthesis in *Aspergillus oryzae* and its overproduction by genetic

engineering. *J. Biosci. Bioeng.* **110**:8-11

Maruyama J, Kitamoto K (2008) Multiple gene disruptions by marker recycling with highly efficient gene-targeting background (*ΔligD*) in *Aspergillus oryzae*. *Biotech. Lett.* **30**:1811-1817

Miller K, Maas N, Toczyski D (2006) Taking it off: regulation of H3 K56 acetylation by *Hst3* and *Hst4*. *Cell Cycle* **5**:2561-2565

Nishida H (2009) Evolutionary conservation levels of subunits of histone-modifying protein complexes in fungi. *Comp. Funct. Genomics.* **2009**:379317

Oda K, Kakizono D, Yamada O, Iefuji H, Akita O, Iwashita K (2006) Proteomic analysis of extracellular proteins from *Aspergillus oryzae* grown under submerged and solid-state culture conditions. *Appl. Environ. Microbiol.* **72**:3448-3457

Oda K, Kobayashi A, Ohashi S, Sano M (2011) *Aspergillus oryzae laeA* regulates kojic acid synthesis genes. *Biosci. Biotechnol. Biochem.* **75**:1832-1834

Pagiotti R, Angelini P, Rubini A, Tirillini B, Granetti B, Venanzoni R (2011) Identification and characterisation of human pathogenic filamentous fungi and susceptibility to *Thymus schimperi* essential oil. *Mycoses* **54**:e364-e376

Podust LM, Sherman DH (2012) Diversity of P450 enzymes in the biosynthesis of natural products. *Nat. Prod. Rep.* **29**:1251-1266

Priebe S, Linde J, Albrecht D, Guthke R, Brakhage AA (2011) FungiFun: a web-based application for functional categorization of fungal genes and proteins. *Fungal Genet. Biol.* **48**:353-358

Quevillon E, Silventoinen V, Pillai S, Harte N, Mulder N, Apweiler R, Lopez R (2005) InterProScan: protein domains identifier. *Nucleic Acids Res.* **33**:W116-120

Raffaele S, Kamoun S (2012) Genome evolution in filamentous plant pathogens:

why bigger can be better. *Nat. Rev. Microbiol.* **10**:417-430

Ruepp A, Zollner A, Maier D, Albermann K, Hani J, Mokrejs M, Tetko I, Güldener U, Mannhaupt G, Münsterkötter M, Mewes HW (2004) The FunCat, a functional annotation scheme for systematic classification of proteins from whole genomes. *Nucleic Acids Res.* **32**:5539-5545

Sanchez JF, Somoza AD, Keller NP, Wang CCC (2012) Advances in *Aspergillus* secondary metabolite research in the post-genomic era. *Nat. Prod. Rep.* **29**:351-371

Shahbazian MD, Grunstein M (2007) Functions of site-specific histone acetylation and deacetylation. *Annu. Rev. Biochem.* **76**:75-100

Shimizu M, Masuo S, Fujita T, Doi Y, Kamimura Y, Takaya N (2012) Hydrolase controls cellular NAD, sirtuin and secondary metabolites. *Mol. Cell. Biol.* **32**:3743-3755

Shoji J, Arioka M, Kitamoto K (2008) Dissecting cellular components of the secretory pathway in filamentous fungi: insights into their application for protein production. *Biotech. Lett.* **30**:7-14

Shwab EK (2007) Histone deacetylase activity regulates chemical diversity in *Aspergillus*. *Eukaryotic Cell* **6**:1656-1664

Smith K, Kothe G, Matsen C, Khlafallah T, Adhvaryu K, Hemphill M, Freitag M, Motamedi M, Selker E (2008) The fungus *Neurospora crassa* displays telomeric silencing mediated by multiple sirtuins and by methylation of histone H3 lysine 9. *Epigenetics Chromatin* **1**:5

Smith KM, Dobosy JR, Reifsnyder JE, Rountree MR, Anderson DC, Green GR, Selker EU (2010) H2B- and H3-specific histone deacetylases are required for DNA methylation in *Neurospora crassa*. *Genetics* **186**:1207-1216

Szewczyk E, Nayak T, Oakley CE, Edgerton H, Xiong Y, Taheri-Talesh N,

Osmani SA, Oakley BR (2007) Fusion PCR and gene targeting in *Aspergillus nidulans*. *Nat. Protoc.* **1**:3111-3120

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: Molecular Evolutionary Genetics Analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* **28**:2731-2739

Terabayashi Y, Sano M, Yamane N, Marui J, Tamano K, Sagara J, Dohmoto M, Oda K, Ohshima E, Tachibana K, Higa Y, Ohashi S, Koike H, Machida M (2010) Identification and characterization of genes responsible for biosynthesis of kojic acid, an industrially important compound from *Aspergillus oryzae*. *Fungal Genet. Biol.* **47**:953-961

Tribus M, Bauer I, Galehr J, Rieser G, Trojer P, Brosch G, Loidl P, Haas H, Graessle S (2010) A novel motif in fungal Class 1 histone deacetylases is essential for growth and development of *Aspergillus*. *Mol. Biol. Cell* **21**:345-353

Tribus M, Galehr J, Trojer P, Brosch G, Loidl P, Marx F, Haas H, Graessle S (2005) *HdaA*, a major class 2 histone deacetylase of *Aspergillus nidulans*, affects growth under conditions of oxidative stress. *Eukaryotic Cell* **4**:1736-1745

Wang B, Guo G, Wang C, Lin Y, Wang X, Zhao M, Guo Y, He M, Zhang Y, Pan L (2010) Survey of the transcriptome of *Aspergillus oryzae* via massively parallel mRNA sequencing. *Nucleic Acids Res.* **38**:5075-5087

Yamada O, Na Nan S, Akao T, Tominaga M, Watanabe H, Satoh T, Enei H, Akita O (2003) *dffA* gene from *Aspergillus oryzae* encodesl-ornithineN5-oxygenase and is indispensable for deferriferrichrysin biosynthesis. *J. Biosci. Bioeng.* **95**:82-88

Yang B, Miller A, Kirchmaier AL (2008) HST3/HST4-dependent deacetylation of lysine 56 of histone H3 in silent chromatin. *Mol. Biol. Cell.* **19**:4993-5005

Yang X-J, Seto E (2008) The *Rpd3/Hda1* family of lysine deacetylases: from bacteria and yeast to mice and men. *Nat. Rev. Mol. Cell. Biol.* **9**:206-218

Zhang T, Kraus WL (2010) SIRT1-dependent regulation of chromatin and transcription: Linking NAD^+ metabolism and signaling to the control of cellular functions. *Biochim. Biophys. Acta* **1804**:1666-1675

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Fungus-Specific Sirtuin HstD Coordinates Secondary Metabolism and Development through Control of LaeA

Moriyuki Kawauchi,[a,b] Mika Nishiura,[a,b] Kazuhiro Iwashita[a,b]

Department of Molecular Biotechnology, Graduate School of Advanced Science of Matter, Hiroshima University, Kagamiyama, Higashi-Hiroshima, Hiroshima, Japan[a]; National Research Institute of Brewing, Higashi-Hiroshima, Hiroshima, Japan[b]

The sirtuins are members of the NAD$^+$-dependent histone deacetylase family that contribute to various cellular functions that affect aging, disease, and cancer development in metazoans. However, the physiological roles of the fungus-specific sirtuin family are still poorly understood. Here, we determined a novel function of the fungus-specific sirtuin HstD/*Aspergillus oryzae* Hst4 (AoHst4), which is a homolog of Hst4 in *A. oryzae* yeast. The deletion of all histone deacetylases in *A. oryzae* demonstrated that the fungus-specific sirtuin HstD/AoHst4 is required for the coordination of fungal development and secondary metabolite production. We also show that the expression of the *laeA* gene, which is the most studied fungus-specific coordinator for the regulation of secondary metabolism and fungal development, was induced in a Δ*hstD* strain. Genetic interaction analysis of *hstD/Aohst4* and *laeA* clearly indicated that HstD/AoHst4 works upstream of LaeA to coordinate secondary metabolism and fungal development. The *hstD/Aohst4* and *laeA* genes are fungus specific but conserved in the vast family of filamentous fungi. Thus, we conclude that the fungus-specific sirtuin HstD/AoHst4 coordinates fungal development and secondary metabolism via the regulation of LaeA in filamentous fungi.

Histone acetylation plays key roles in the control of chromatin structure and function (1). The acetylation state is controlled by two histone modification enzymes with opposing actions, histone acetyltransferases (HATs) and deacetylases (HDACs). Acetylation is generally associated with transcriptional activation. In contrast, histone deacetylation is generally associated with transcriptional repression. These enzymes are highly conserved from yeasts to humans, and they are also conserved in filamentous fungi (2–5).

HDACs remove the acetyl moiety from the lysine residue of a histone tail. In addition to histones, these enzymes deacetylate many nonhistone substrates (6). Protein deacetylation affects diverse cellular processes, such us development, metabolism, and stress responses in eukaryotic cells (6). In mammalian and yeast cells, HDACs are divided into two major families called the sirtuins and the classical HDACs. The HDACs are phylogenetically classified into four classes. The sirtuins constitute class III, and the classical HDACs are grouped into classes I, II, and IV (4, 6). Except for mammal-specific class IV HDACs, these enzymes are also conserved in the genome of filamentous fungi (2, 5).

Recently, histone deacetylases in some filamentous fungi have been investigated for their role in the regulation of histone modification, developmental processes, stress resistance, pathogenesis, metabolism, and other such processes (2, 7–10). For example, the homolog of yeast Rpd3 is required for growth and conidiation in several filamentous fungi (11). The class II HDAC HdaA/*Aspergillus fumigatus* Hda1 (AfHda1), a homolog of yeast Hda1, is involved in germination and the oxidative stress response in *Aspergillus fumigatus* (12, 13). The yeast Hos2 homolog is required for conidial development, invasive growth, and the production of virulence factors in some plant-pathogenic filamentous fungi (7, 8, 14).

Filamentous fungi produce wide varieties of secondary metabolites (SMs), which are small bioactive molecules that include both beneficial medicines and cosmetics and toxins that are harmful for animals and plants (15, 16). Therefore, because of the importance of fungal SMs, there has been much research into the mechanisms that regulate their production. LaeA, a putative fungus-specific methyltransferase, is implicated in the global regulation of SM production (16, 17). LaeA also has an important role in coordinating fungal development and SM production (17). Recent studies have shown that histone modification plays key roles in the regulation of SM biosynthetic gene expression (18). In *Aspergillus nidulans*, the HDACs HdaA/*Aspergillus nidulans* Hda1 (AnHda1) and SirA/*A. nidulans* Sir2 (AnSir2) regulate carcinogenic sterigmatocystin and production of the antibiotic penicillin (19, 20). The loss of Hdf1/*Fusarium graminearum* Hos2 (FgHos2) reduces conidial development and the production of deoxynivalenol, which is the most characterized virulence factor in *Fusarium graminearum* (7).

However, the importance of fungal HDACs in the regulation of secondary metabolism and fungal development is still not known; studies of these HDACs are limited, even though several types of HDACs are found in fungal genomes (2). Moreover, the relationship between the global regulator *laeA* and histone modification is still poorly understood.

In this study, we examined the phenotypes caused by the disruption of all HDACs using *Aspergillus oryzae*, which is an important filamentous fungus in industry and has potential for the production of pharmaceutical and cosmetic SMs (21–23). Our observations indicated that the fungus-specific sirtuin HstD/*Aspergillus oryzae* Hst4 (AoHst4) regulates conidial development

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and production of kojic acid (KA), which is an important cosmetic material for preventing melanogenesis in skin, as well as production of the antimicrobial penicillin (24). We also performed microarray analysis of the Δ*hstD* strain to examine the global function of this sirtuin and found that the disruption of this gene affects the expression of many metabolite genes. As described above, *laeA* is an important coordinator for the regulation of secondary metabolism and development. In this context, we also analyzed the genetic interaction between *hstD*/*Aohst4* and *laeA* and found that *hstD*/*Aohst4* regulates *laeA* expression. We describe the function of the fungus-specific sirtuin HstD/AoHst4 first in SM production and then in conidial development through the regulation of *laeA* gene expression.

MATERIALS AND METHODS

Strains, media, and physiological tests. The strains used in this study are listed in Table S1 in the supplemental material. *Aspergillus oryzae* RIB40 was used as the DNA donor. The *A. oryzae NSR*-Δ*LD2* strain was used as the host for *A. oryzae* HDAC (AoHDAC) disruption (25). M+Met medium [2 g NH_4Cl, 1 g $(NH_4)2SO_4$, 0.5 g KCl, 1 g KH_2PO_4, 0.5 g $MgSO_4{\cdot}7H_2O$, 0.02 g $FeSO_4$, 20 g glucose, 1.5 g L-methionine, pH 5.5, in 1 liter] or M+Ade medium (M+Met medium containing 0.5 g of adenine sulfate dihydrate instead of L-methionine) was used as the selection medium for *A. oryzae adeA*$^+$ transformants and *A. oryzae sC*$^+$ transformants, respectively (25). TS medium (6 g of $NaNO_2$, 0.52 g of KCl, 1.52 g of KH_2PO_4, 0.52 g of $MgSO_4{\cdot}7H_2O$, 10 g of glucose, 1 ml of trace elements, pH 6.5, in 1 liter) was used as the selectable medium for *A. oryzae adeA*$^+$ *sC*$^+$ transformants. M+Met, M+Ade, or TS medium with 0.8 M NaCl added was used for transformation. KAS medium (10 g of tryptone, 1.52 g of K_2HPO_4, 1.5 g of L-methionine, 0.5 g of $MgSO_4{\cdot}7H_2O$, 1 ml of trace elements, pH 6.5, in 1 liter) was used for screening for HDAC-affected kojic acid productivity. KA medium (1 g of yeast extract, 0.5 g of KCl, 1 g of K_2HPO_4, 1.5 g of L-methionine, 0.5 g of $MgSO_4{\cdot}7H_2O$, 100 g of glucose, 0.5 g of adenine sulfate dihydrate, pH 6.0, in 1 liter) was used to test for KA production and RNA preparation. N medium (3 g of L-glutamic acid, 0.52 g of KCl, 1.52 g of K_2HPO_4, 0.52 g of $MgSO_4{\cdot}7H_2O$, 30 g of glucose, 1.5 g of L-methionine, 1 ml of trace elements, pH 6.5, in 1 liter) was used for morphological analysis. TSB medium (20 g of tryptic soy broth, 1.5 g of L-methionine, 3 g of L-glutamic acid, 0.5 g of adenine sulfate dihydrate, pH 7.5, in 1 liter) was used for the penicillin bioassay and RNA preparation.

Morphological analysis was performed in 20 ml of N 2% agar medium or 100 ml of N liquid medium. Three independent disruptants were used for each experiment. For the spore count, suspensions of conidia of each strain were point inoculated (1×10^5 conidia) on the center of each plate, and the strain was grown for 5 days at 30°C. Colony diameters were measured at this time, and the spores were harvested in suspension solution (0.025% Tween 80, 0.5% NaCl), vortexed vigorously, and counted using a TC10 automated cell counter (Bio-Rad). The conidiation rate was calculated by the conidium number/radial growth area (cm^2). For biomass analysis in N liquid medium, 4 cm^2 of full-growth colonies in plate cultures of each strain were cut out, homogenized in 1 ml of suspension solution, and then used to inoculate each flask. Flasks were incubated for 2 days at 30°C with shaking at 100 rpm. Then, mycelia were harvested, dried at 105°C for 2 h, and weighed.

Protein identification, domain prediction, and phylogenetic analysis. HDAC sequences of *Saccharomyces cerevisiae* were obtained from the *Saccharomyces* genome database (http://www.yeastgenome.org/). HDAC sequences of *Homo sapiens*, *Neurospora crassa*, and *A. nidulans* were obtained from the NCBI proteins database (http://www.ncbi.nlm.nih.gov/guide/proteins/), the *Neurospora crassa* database (http://www.broadinstitute.org/annotation/genome/neurospora/MultiHome.html), and AspGD (http://www.aspgd.org/), respectively. The HDAC genes of *A. oryzae* were identified from the Comparative Fungal Genome Database (CFGD; http://nribf2.nrib.go.jp/) by BLAST searching using the HDAC sequences of *S. cerevisiae* and *H. sapiens* as the query. The sequences of these HDACs in *A. oryzae* were verified by RNA sequencing using the SOLiD3 system (Applied Biosystems). The gene structures of all AoHDACs were confirmed from the RNA sequence data (details of our RNA sequence data are available in AspGD [accession no. ASPL0000367586; http://www.aspergillusgenome.org/download/large_scale_data/Iwashita_2012/]) (26). The mapping data for all reads are also available in CFGD. For recognizable domains, the protein sequence of the HDAC homolog in *A. oryzae* was analyzed using the InterProScan tool (27). The protein sequence of the HstD homolog in filamentous fungi was identified using an NCBI blast search with Pezizomycotina genomes (http://www.ncbi.nlm.nih.gov/sutils/genom_tree.cgi) and the amino acid sequence of HstD as a query.

For the classification of HDACs in *A. oryzae*, protein sequences of HDACs in *S. cerevisiae*, *N. crassa*, *A. nidulans*, and *A. oryzae* were aligned with the ClustalW software in the Molecular Evolutionary Genetic Analysis, v.5 (MEGA5), program (28). Phylogenetic analysis was carried out by the MEGA5 program using the neighbor-joining method with 1,000 bootstrap replicates. For the classification of HstD in filamentous fungi, alignment and phylogenetic analysis were performed as described above. A list of sequence accession numbers used for AoHDAC analysis is provided in Table S2 in the supplemental material, and the sequence accession numbers of each HstD homolog are provided in Fig. S3 in the supplemental material.

RNA preparation. KA culture was performed in 20 ml of KA liquid medium inoculated with 200 μl of 1×10^8 conidia/ml suspension, and incubation was at 30°C for 4 or 7 days with shaking at 130 rpm. TSB culture was performed in 40 ml of TSB liquid medium inoculated with 400 μl of 1×10^8 conidia/ml suspension, and incubation was at 30°C for 1 day with shaking at 200 rpm. After cultivation, mycelia were harvested using Miracloth (Merck). Then, mycelia were immediately frozen in liquid N_2 and ground to a fine powder. Total RNA was isolated from mycelia from KA or TSB liquid medium using the Isogen reagent (Nippon Gene) according to the manufacturer's instructions.

Northern hybridization. Denatured total RNA (20 μg) was electrophoresed on a formaldehyde-agarose gel and transferred in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015M sodium citrate) onto a Hybond N$^+$ membrane. Northern analysis was performed with a Detection starter kit II (Roche) according to the manufacturer's instructions. Digoxigenin-labeled probes were prepared using a PCR digoxigenin probe synthesis kit (Roche) with genomic *A. oryzae* RIB40 DNA as the template and the primers X-probe-F and X-probe-R. The letter X means the respective gene used for Northern analysis. Each blot was imaged using an LAS1000plus luminescent image analyzer (Fujifilm). A list of the primers used for these PCRs is shown in Table S3 in the supplemental material.

Construction of the disruption cassette. Each disruption cassette was constructed by fusion PCR of three mutually primed DNA fragments, the 5′ and 3′ flanking regions of the target genes and the *adeA* fragment (29). About 1 kb of the 5′ and 3′ flanking regions of the target genes and the *adeA* gene was amplified from genomic *A. oryzae* RIB40 DNA with primers X-A and X-B, X-C and X-D, and adeA-F and adeA-R, respectively. Only for the construction of the Δ*hstD* and Δ*laeA* genes and the *A. nidulans sC* gene, amplified from pUSA with primers sC-F and sC-R, was the gene fused to the flanking region of *laeA* (30). The letter X in the primer names represents the name of each target gene. Each region was amplified by KOD Plus DNA polymerase (Toyobo). These fragments were combined by a second PCR with KOD Plus DNA polymerase and the primers X-A and X-D or X-A2 and X-D2. The amplified fragment was purified by a QIAquick PCR purification kit (Qiagen) and then used as a disruption cassette. A list of the primers used for these PCRs is shown in Table S3 in the supplemental material.

Complementation of *hstD*. To recover the native locus of *hstD*, we first amplified the same 5′ flanking region of the disruption construct of the *hstD* and *adeA* fragments from RIB40 DNA with primers hstD-A and

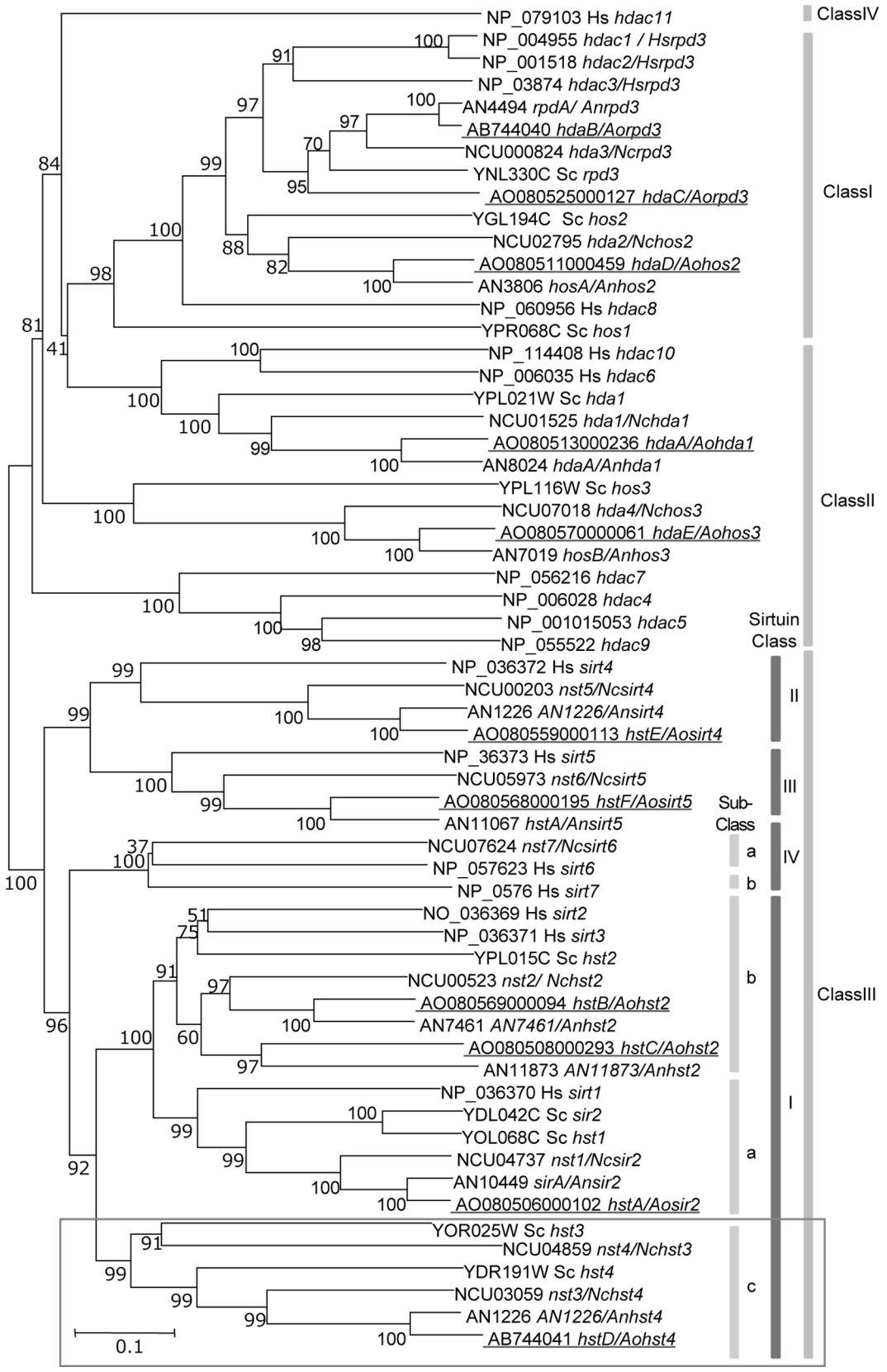

FIG 1 Phylogenetic analysis of histone deacetylase in *A. oryzae*. Accession numbers and HDAC names are indicated for each branch. The HDAC names of *S. cerevisiae* or *H. sapiens* with the species name indicated are followed by a slash. The numbers at the nodes are bootstrap values obtained from 1,000 replicates and are indicated as percentages. Scale bar, a distance corresponding to 0.2 amino acid substitution per site. The class or subclass of HDACs is shown on the right. These classes of HDACs are referred to in previous phylogenetic studies (4, 6, 34). AoHDACs are indicated by underlines. Abbreviations of AoHDAC gene names are as follows: *hda*, histone deacetylase; *hst*, homolog of sirtuin. The class to which *hstD* belongs is surrounded by a gray border. The gene names and their accession numbers are identified in Table S2 in the supplemental material. Sc, *Saccharomyces cerevisiae*; An, *Aspergillus nidulans*; Nc, *Neurospora crassa*; Hs, *Homo sapiens*; Ao, *Aspergillus oryzae*.

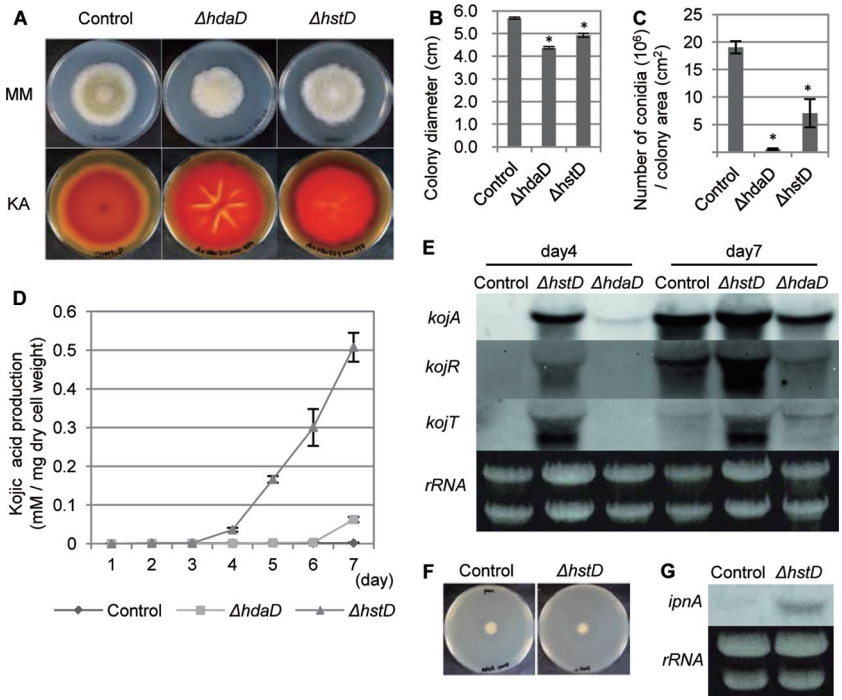

FIG 2 *hstD*/*Aohst4* and *hdaD*/*Aohos2* regulate SM production and development. (A) The morphological phenotype on N agar medium (MM) and results of the kojic acid production plate assay (KA) are provided for the indicated strains. (B, C) Radial growth and conidiation of the indicated strains on N agar medium, respectively. (D) Time course characteristics of kojic acid production of the indicated disruptants. (E) Expression profiles of kojic acid cluster genes represented by Northern hybridization. The culture times of the indicated strains are shown at the top of the panel. The analyzed gene is indicated on the left side of each blot. The results for rRNA, used as the loading control, are shown. (F) Bioassay of penicillin production of the Δ*hstD* strain. (G) Northern hybridization of the penicillin biosynthetic gene *ipnA* in the Δ*hstD* strain. The results for rRNA, used as the loading control, are shown. The *adeA*$^+$ strain was used as a control in the experiment whose results are presented in this figure. All data are represented as means ± SDs ($n = 3$); *, $P < 0.01$, *t* test.

hstD-compB or *adeA* fusion primers sC-F and adeA-R, respectively. We also amplified the *A. nidulans sC* gene from pUSA with sC-F and sC-R as autotrophic markers (30). Then, these fragments were combined by fusion PCR using nested primers adeA-R and hstD-A2 (29). The amplified fragment was purified by a QIAquick PCR purification kit and used as a complementation cassette. A list of the primers used for these PCRs is shown in Table S3 in the supplemental material.

Construction of overexpression plasmids. The open reading frames (ORFs) of *laeA* or *hstD*/*Aohst4* were amplified from the genomic DNA of *A. oryzae* RIB40 with Fusion-laeA-F and Fusion-laeA-R or Fusion-hstD-F and Fusion-hstD-R, respectively. The resulting fragments were fused into SmaI-cut pUSA using an In-Fusion HD cloning kit (TaKaRa) (30). The *amyB* promoter of pUSA was used to drive the overexpression of *laeA* and *hstD*/*Aohst4*, respectively. The resulting plasmids, pUSlaeA and pUShstD, were linearized with one cut of restriction enzyme BglII and EcoT22I on the *laeA* ORF and *hstD*/*Aohst4* ORF, respectively. The resulting linearized fragments were used as overexpression cassettes. A list of the primers used for these PCRs is shown in Table S3 in the supplemental material.

Transformation of *A. oryzae*. Transformation of *A. oryzae* strains was performed using the protoplast-polyethylene glycol method (31). To verify the disruption of the target gene, direct colony PCR was performed using primers (X-F and X-G, X-A and X-D), KOD-FX (Toyobo), and a crude DNA sample of each transformant. Primers X-F and X-G were designed at the region of each target gene. The crude DNA sample was prepared as follows: conidia and hyphae from each transformant culture were suspended in 100 μl buffer A (100 mM Tris-HCl [pH 9.5], 1 M KCl, 10 mM EDTA). This mycelial suspension was vigorously vortexed and incubated at 95°C for 10 min. Immediately thereafter, this hot solution was vigorously vortexed and centrifuged at 5,000 rpm for 1 min. A total of 1 μl of supernatant was used as the crude DNA sample. A list of the primers used for these assays is shown in Table S3 in the supplemental material.

Time-lapse imaging. For time-lapse imaging, conidiophores were germinated in 1.5% agar N medium in 35-mm glass-bottom dishes. Cells were imaged using a real-time culture cell monitoring system (Astec) controlled by CCM software. Differential interference contrast images of each disruptant were taken every 20 min for approximately 84 h. All imaging was carried out at 30°C. Pictures and movies were edited with CCM software (Astec).

Secondary metabolite analysis. For the plate assay of kojic acid production, KAS medium and KA medium containing 5 mM $FeCl_3$ were used for screening and genetic interaction analysis, respectively. Suspensions of conidia of each strain were point inoculated (1×10^5 conidia) on the center of the 2% agar medium and grown for 5 days at 30°C. Then, a red halo, which indicates the existence of kojic acid, was observed.

For the quantification of kojic acid, 20 ml of KA liquid medium inoculated with 200 μl of 1×10^8 conidia/ml suspension was incubated at 30°C with shaking at 130 rpm. After cultivation for the appropriate period, mycelia were filtered through Miracloth (Merck), and then the filtrate was collected. Harvested mycelia were dried at 105°C for 1 h and weighed. The collected KA medium was filtered through a MillexHV filter (Millipore), and then the kojic acid concentration was quantified by colorimetric methods.

Microarray analysis. The *A. oryzae* GeneChip microarray (AoDNAChip; NCBI Gene Expression Omnibus [GEO] platform GPL16184) was designed by Affymetrix to refer to the entire genome sequence of *A. oryzae*, and predicted ORFs are published at the Comparative Fungal Genome Database (http://nribf2.nrib.go.jp/, accession number *A. oryzae* RIB40 Ace33v2). The AoDNAChip covered 13,765 ORFs and 6,143 promoters of *A. oryzae*. In this

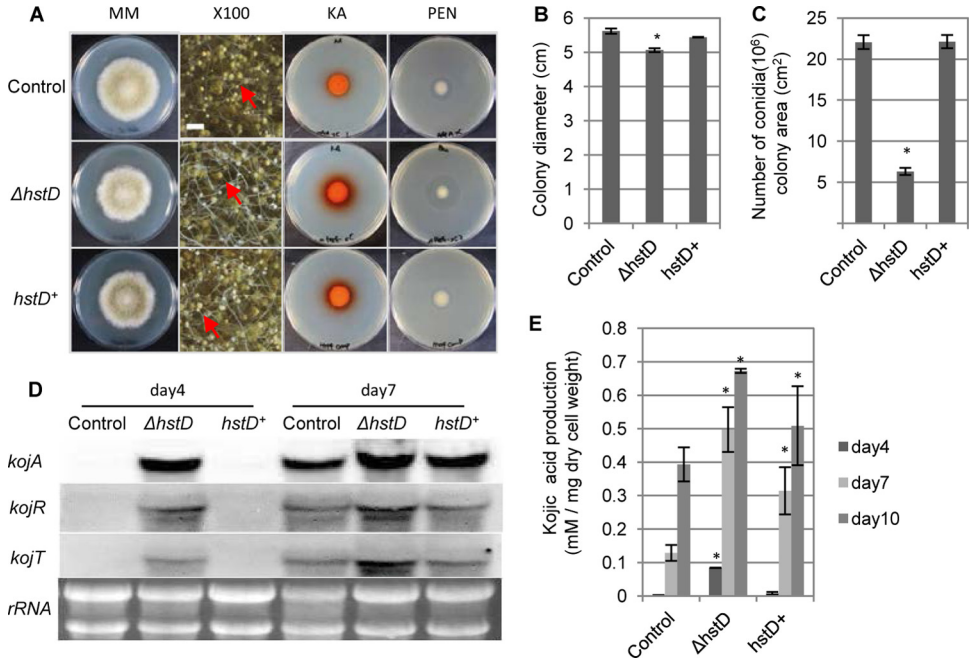

FIG 3 Complementation analysis of *hstD*. (A) Analysis of the morphology and SM production of the Δ*hstD* and *hstD*$^+$ strains. MM, morphological phenotype of the indicated strain on N agar medium; ×100, closeup stereomicroscopic images of the strains on N agar medium (magnification, ×100; bar, 500 μm; red arrows, examples of conidia); KA and PEN, plate assay or bioassay of kojic acid and penicillin, respectively. (B, C) Quantification of colony diameter and rate of conidiation of Δ*hstD* and *hstD*$^+$ strains, respectively. (D) The expression profiles of the kojic acid cluster genes were determined by Northern hybridization. The culture time of the indicated strain is shown at the top. The analyzed gene is indicated on the left side of each blot. The results for rRNA, used as the loading control, are shown. (E) Quantification of kojic acid production. The *adeA*$^+$ *sC*$^+$ strain was used as the control, and the Δ*hstD sC*$^+$ strain represents the Δ*hstD* strain. All data are represented as means ± SDs ($n = 3$); *, $P < 0.01$, *t* test.

study, we performed transcriptome analysis using probes of 13,765 ORFs set on this microarray.

Total RNA was purified for microarray analysis by using an RNeasy minikit (Qiagen). RNA quality was determined by using a BioAnalyzer 2100 system (Agilent Technology), and the quantity was determined by using an Ultrospec 3300 pro spectrophotometer (Amersham Pharmacia Biotech). Fragmented biotin-labeled cRNA was prepared by using a GeneChip one-cycle target labeling and control reagent kit (Affymetrix) according to the manufacturer's instructions. The fragmented cRNA was hybridized to an AoDNAChip. Then, this GeneChip was washed, stained, and scanned by using a GeneChip FS-450 fluidics station (fluidics protocol FS450_001) and a GeneChip 3000 scanner.

Scanned probe array images were converted into CEL files and normalized by using the GCOS v.1.4 program (Affymetrix). Calculations of signal intensity and detection *P* values were also performed using GCOS v.1.4. The trimmed mean signal of the array was scaled to the target signal of 500 with the all probe sets scaling option. The detection call was used for detection of a particular transcript, with a detection *P* value of <0.04 considered present (P), $0.04 \leq P < 0.06$ considered marginal (M), and a *P* value of ≥ 0.06 considered absent (A). These calculation data were exported as CHP files. For microarray data analysis, CHP files were imported into GeneSpring v.7.3 (Agilent Technologies). Expression data were normalized per chip to the 50th percentile. In this study, we analyzed genes detected as P or M flags. Genes with statistically significant changes in transcript abundance were identified using a cutoff value of 2-fold and a Welch's *t* test value of less than 5%. FungiFun software was used for functional catalogue (FunCat) categorization (32, 33). Significantly enriched FunCat categories were extracted using FungiFun software (cutoff *P* value, <0.05; the *P* value indicates the significance of the number of hits for each category in the data set, taking the number of hits for the whole genome of *A. oryzae* as the background). The calculation is based on a two-tailed Fisher's exact test). The distribution of whole *A. oryzae* genes is indicated in Fig. S4 and S5 in the supplemental material and was used as a reference for FunCat enrichment analysis. Two biological replicates were used for the microarray analysis.

Accession numbers. The microarray data have been deposited in the NCBI Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO series accession number GSE41612. The coding sequences of *hdaB* and *hstD* were deposited in DDBJ under accession no. AB744040 and AB744041, respectively.

RESULTS

Phylogeny and morphology of AoHDACs. A total of 11 AoHDACs were found in the *A. oryzae* genome on the basis of a BLAST analysis. We classified the AoHDACs according to a previous phylogenetic study of HDACs. These AoHDACs were phylogenetically divided into class I to III HDACs, but the mammal-specific class IV HDACs were not found in the genome. Class III HDACs are generally described as sirtuin-type HDACs, and 6 AoHDACs belonged to this class. The *A. oryzae* sirtuins (AoSirtuins) were classified into classes I to III, but class IV sirtuins were not found in the genome. The class I sirtuins were further categorized into three subclasses, including the fungus-specific HDACs of sirtuin subclass C (Fig. 1). We attempted to disrupt these 11 AoHDACs and succeeded for 10 of the AoHDACs. However, only heterokaryon transformants were obtained in the case of *hdaB/A. oryzae rpd3* (*Aorpd3*) over several trials (data not shown). This result suggests that *hdaB/Aorpd3* is essential in *A. oryzae*, but further experiments are required to confirm this hypothesis. Thus, in this report, the heterokaryon disruptant of *hdaB/Aorpd3* and AoHDAC disruptants were used for subsequent experiments.

We first observed the growth and generation of conidia of AoHDAC disruptants and *hdaB*/*Aorpd3* heterokaryon transfor-

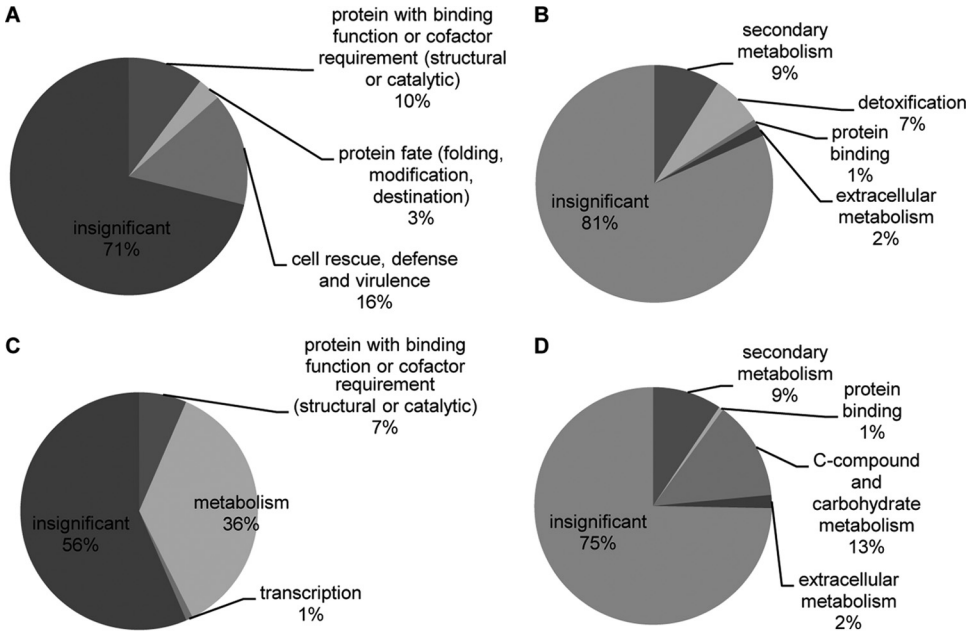

FIG 4 Enrichment analysis of the FunCat categorization of the microarray analysis. Significantly enriched FunCat level 1 and level 2 categories of genes upregulated (A, B) or downregulated (C, D) by *hstD*/*Aohst4* deletion are shown. The FunCat is the organism-independent functional description of proteins (33). FunCat consists of 28 main functional categories (level 1). Level 1 is the most general one, whereas level 2 shows much more detail. The percentage indicated for each category contributes to the total mapping. Insignificant FunCat categories are indicated as insignificant in the pie charts. Significantly enriched categories were extracted by FungiFun software (cutoff *P* value, <0.05; Fisher's exact test) (32). Details of the enrichment analysis are available at the FungiFun website (https://sbi.hki-jena.de/FungiFun/FungiFunHelp.html).

mants on the plate culture (see Fig. S1 and S2B and C in the supplemental material). Observation of the growth of AoHDAC disruptants revealed an obvious defect of the Δ*hstD* and Δ*hdaD* strains in morphogenesis (Fig. 2A). The conidial formation of these disruptants was significantly decreased, and a slight growth effect was observed in plate cultivation (Fig. 2B and C). We additionally observed the growth of AoHDAC disruptants and the *hdaB*/*Aorpd3* heterokaryon transformant in liquid culture (see Fig. S2D in the supplemental material). A growth defect of the Δ*hdaD* strain was observed in submerged cultivation (see Fig. S2D in the supplemental material). Thus, *hdaD*/*Aohos2* is required for the growth integrity of *A. oryzae*. These two disruptants were further examined using time-lapse imaging for more detailed observation. As expected, the Δ*hdaD* strain showed slow growth but exhibited more crowded aerial hyphae than the wild-type strain (see Fig. S2A in the supplemental material). However, a few invasive hyphae were detected in the Δ*hstD* strain but not in the Δ*hdaD* strain. In this time-lapse imaging analysis, a significant defect of conidial development was also found in both the Δ*hdaD* and Δ*hstD* strains.

These results indicate that both HdaD/AoHos2 and HstD/AoHst4 play an important role in the growth and development of *A. oryzae*, especially in asexual development.

Fungus-specific sirtuin regulates SM production. We further examined SM production of AoHDAC disruptants and the *hdaB*/*Aorpd3* heterokaryon transformant using a plate assay for kojic acid productivity (see Fig. S1 in the supplemental material). High levels of production of kojic acid were observed in the Δ*hdaB* and Δ*hstA* strains (see Fig. S1 in the supplemental material), and significant overproduction was observed in the Δ*hdaD* and Δ*hstD* strains (Fig. 2A). We quantified the kojic acid production of these two HDAC disruptants, and the Δ*hstD* strain showed a 200-fold increased productivity in a 7-day culture (Fig. 2D). In contrast, the Δ*hdaD* strain showed a 30-fold overproduction compared with the level of production for the control strain (Fig. 2D). The Δ*hstD* strain started to produce kojic acid by 4 days in culture, while kojic acid was not detected in the wild-type strain culture.

We further analyzed kojic acid production at the gene expression level by examining three key genes in the kojic acid gene cluster (24). Northern analysis of 4-day-old cultures showed extremely high levels of expression of these genes in the Δ*hstD* strain, but no expression was observed in the wild-type strain (Fig. 2E). The *kojA* gene was also expressed in the Δ*hdaD* strain, but the expression was not as high as that in the Δ*hstD* strain. This earlier and higher expression in both disruptants is consistent with the earlier and higher production of kojic acid.

These results indicate the importance of Δ*hdaD* and Δ*hstD* in the regulation of kojic acid production. On the basis of phylogenetic classification, HstD/AoHst4 belongs to the fungus-specific class of sirtuins, and this protein is widely conserved in filamentous fungi (34) (see Fig. S3 in the supplemental material). Interestingly, *nst3*/*N. crassa hst4* (*Nchst4*) is involved in the silencing mechanism of *N. crassa* (35). Moreover, the phenotype of the Δ*hstD* strain was reminiscent of the global use of HstD/AoHst4 for the production of various SMs in *A. oryzae*.

In this context, we examined the regulation of penicillin biosynthesis in the Δ*hstD* strain. As expected, a higher level of production of penicillin was found in the Δ*hstD* strain, and a higher level of expression of the penicillin biosynthetic gene was confirmed (Fig. 2F and G). Along with the morphogenetic defect and kojic acid production, these phenotypes were rescued by the complementation of *hstD*/*Aohst4* (Fig. 3).

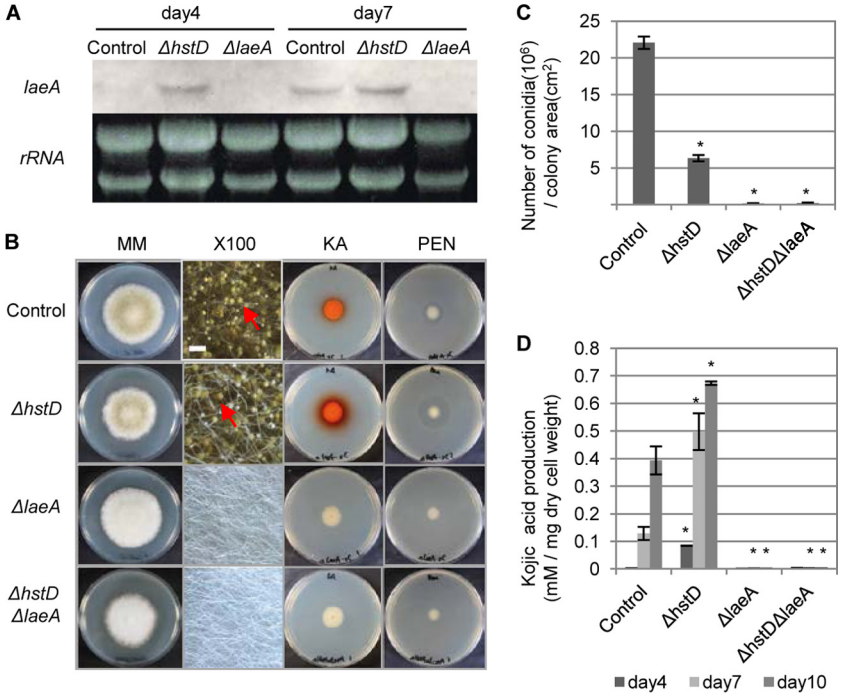

FIG 5 Genetic interaction between *hstD* and *laeA*. (A) Expression profile of *laeA* under the KA-producing condition. The *adeA*$^+$ strain was used as a control. The culture time of the indicated strain is shown at the top of the panel. The results for rRNA, used as the loading control, are shown. (B) Analysis of morphology and SM production of the Δ*hstD*, Δ*laeA*, and Δ*hstD* Δ*laeA* strains. MM, morphological phenotype of the indicated strain on N agar medium; ×100, closeup stereomicroscopic images of the strains on N agar medium (magnification, ×100; bar, 500 μm; red arrows, examples of conidia); KA and PEN, plate assay or bioassay of kojic acid and penicillin, respectively. (C, D) Quantification of the conidiation rate and kojic acid production of the Δ*hstD*, Δ*laeA*, and Δ*hstD* Δ*laeA* strains, respectively. Except for panel A, the *adeA*$^+$ *sC*$^+$ strain was used as the control, and the Δ*hstD sC*$^+$ and Δ*laeA sC*$^+$ strains represent the Δ*hstD* and Δ*laeA* strains, respectively, in this figure. All data are represented as means ± SDs ($n = 3$); *, $P < 0.01$, *t* test.

These results suggest that *hstD*/*Aohst4* is required for the global regulation of SM biosynthesis. Thus, we examined the Δ*hstD* strain by microarray analysis to investigate the expression of other SM-related genes. The expression of 388 genes was significantly affected by *hstD*/*Aohst4* deletion (absolute fold change, >2; $P <$ 0.05) (see Table S4 in the supplemental material). These genes were spread across the whole genome; 299 of 388 genes were upregulated in the Δ*hstD* strain. To reveal the functional distribution of genes affected by *hstD*/*Aohst4*, FunCat enrichment analysis was carried out using FungiFun software (32, 33) (Fig. 4). The C-compound and carbohydrate metabolism and the secondary metabolism categories were significantly enriched in downregulated genes. Most of the genes categorized as secondary metabolism overlapped with the genes categorized as C-compound and carbohydrate metabolism. Genes categorized as C-compound and carbohydrate metabolism were mainly related to polysaccharide degradation or glycolysis. This result suggests that carbon source degradation-related genes are downregulated in the Δ*hstD* strain. The secondary metabolism and detoxification categories were significantly enriched in genes upregulated by *hstD* deletion. Genes categorized as secondary metabolism and detoxification were mainly constituted by cytochrome P450 (CYP) genes. Interestingly, three nonribosomal peptide synthetases (NRPSs) and one polyketide synthase (PKS) were also upregulated in the Δ*hstD* strain. One of three NRPSs encoded *wykN* (*AO080501000008*), which is involved in Wyk-1 production in *A. oryzae* (23). PKS and NRPS enzymes generate the general structural scaffolds of most secondary metabolites (36). Additionally, fungal CYPs have PKS- and NRPS-associated functions and generate structural variation of fungal SMs. Thus, *hstD*/*Aohst4* affects many kinds of SM production (at least 6 different SM gene clusters) (37, 38).

***hstD*/*Aohst4* regulates expression of *laeA*.** The LaeA complex coordinates the development and SM biosynthesis in *A. nidulans* and is conserved in numerous fungal genomes (17). Recently, it was reported that deleting *laeA* diminishes kojic acid production and gene expression in *A. oryzae* (39). Additionally, penicillin production in *A. oryzae* is diminished after deletion of *veA*, which is a member of the LaeA complex (17, 40). These reports suggest that the LaeA complex also plays a general role in the induction of the SM gene cluster in *A. oryzae*. Thus, the SM overproduction phenotype of the *hstD*/*Aohst4* disruptant should be associated with the LaeA complex, and HstD/AoHst4 may play a role under the control of LaeA. According to this hypothesis, the expression of *laeA* should not be altered in the *hstD*/*Aohst4* disruptant. Thus, we examined the expression of *laeA* in the *hstD*/*Aohst4* disruptant. Surprisingly, *laeA* was highly expressed in the *hstD*/*Aohst4* disruptant, even in the 4-day culture, but remained unexpressed in the wild-type strain (Fig. 5A). This result suggests that HstD/AoHst4 is involved in *laeA* gene repression. Thus, both SM overproduction phenotypes and the altered morphological phenotype of the Δ*hstD* strain are caused by the high level of expression of *laeA*.

Genetic interaction of *hstD* and *laeA*. To confirm the hypothesis presented above, we prepared a Δ*laeA* strain and a Δ*hstD* Δ*laeA* double disruptant and then examined the strains for SM production and conidial development. At first, these strains ex-

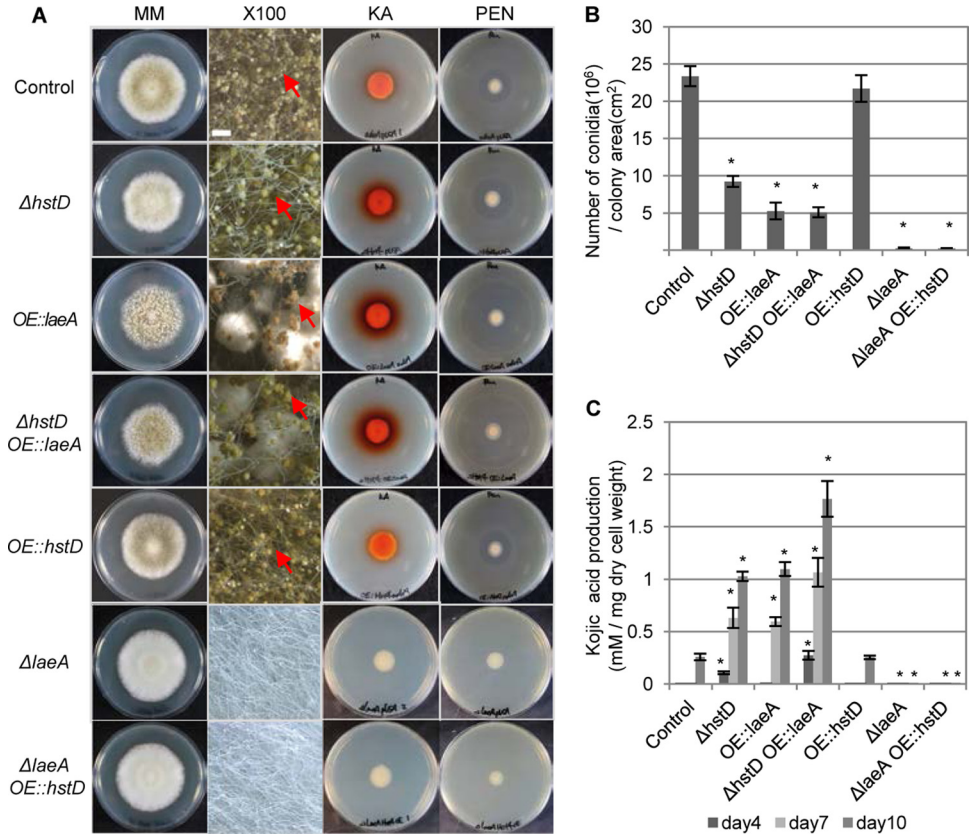

FIG 6 Epistatic relationship between *hstD* and *laeA*. (A) Analysis of the morphology and SM production of the Δ*hstD OE*::*laeA* and Δ*laeA OE*::*hstD* strains. MM, morphological phenotype of the indicated strain on N agar medium; ×100, closeup stereomicroscopic images of the strains on N agar medium (magnification, ×100; bar, 500 μm; red arrows, examples of conidia); KA and PEN, plate assay or bioassay of kojic acid and penicillin, respectively. (B, C) Quantification of the conidiation rate and kojic acid production of the Δ*hstD OE*::*laeA* and Δ*laeA OE*::*hstD* strains, respectively. The *adeA*$^+$ *pUSA*$^+$ strain was used as the control, and the Δ*hstD pUSA*$^+$, *OE*::*laeA adeA*$^+$, Δ*laeA pUSA*$^+$, and *OE*::*hstD adeA*$^+$ strains represent the Δ*hstD*, *OE*::*laeA*, Δ*laeA*, and *OE*::*hstD* strains, respectively, in this figure. The *amyB* promoter was used to drive overexpression of *laeA* and *hstD*. All data are represented as means ± SDs ($n = 3$); *, $P < 0.01$, *t* test.

hibited the phenotype of the Δ*laeA* strain, including a lack of kojic acid production (Fig. 5B and D), similar to previous reports (39). The production of penicillin and conidial development were also lost in the Δ*laeA* strain (Fig. 5B and C). Next, we observed the effect of *laeA* disruption in the Δ*hstD* strain background and found that the SM overproduction phenotype of the Δ*hstD* strain was abolished by *laeA* disruption (Fig. 5B and D). We further observed conidial development in the double disruptant, which exhibited a Δ*laeA* strain-like fluffy phenotype (Fig. 5B and C). These results clearly indicate a genetic interaction between *hstD*/*Aohst4* and *laeA* and that HstD/AoHst4 plays a role upstream of LaeA.

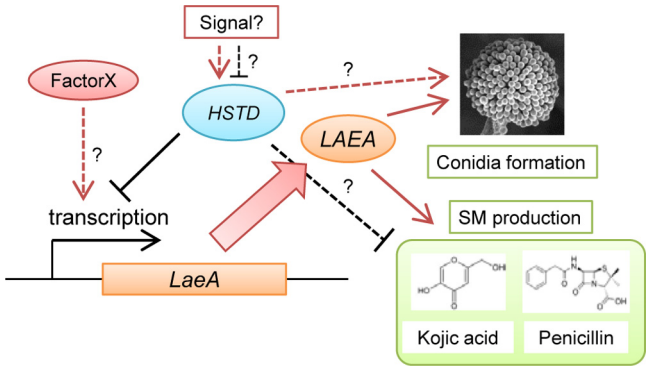

FIG 7 Schematic model of the regulation of SM production and development by HstD/AoHst4 through LaeA. An unknown signal induces or suppresses the function of HstD/AoHst4. Suppression of HstD/AoHst4 leads to expression of LaeA. This activation stimulates fungal development and secondary metabolite production. However, there is the possibility of an HstD/AoHst4 competitive mechanism by an unknown factor (described as factor X in this figure).

To confirm this epistatic relationship, we examined the effect of *laeA* and *hstD*/*Aohst4* overexpression using the *amyB* promoter. The strain overexpressing *laeA* exhibited a Δ*hstD* strain-like phenotype, such as SM overproduction and low levels of conidial formation (Fig. 6). The effects of *laeA* overexpression were also observed in the Δ*hstD* strain background (Fig. 6). Additionally, both the *OE*::*laeA* strain and Δ*hstD OE*::*laeA* strain showed high levels of expression of *laeA*, *kojA*, and *ipnA* (see Fig. S6 in the supplemental material). These results indicate that *laeA* is downstream of *hstD*/*Aohst4*. Therefore, we overexpressed *hstD*/*Aohst4* in a *laeA* disruption background. As expected, the overexpression of *hstD*/*Aohst4* resulted in a Δ*laeA* strain-like phenotype, such as no SM production and a fluffy morphology, in the Δ*laeA* background (Fig. 6). Overexpression of *hstD*/*Aohst4* in the wild-type *laeA* background had no measurable effect on SM production and development (Fig. 6).

From these results, we suggest that the fungus-specific sirtuin HstD/AoHst4 controls SM production and fungal development through the regulation of *laeA* gene expression.

DISCUSSION

In the past decade, the importance of the role of classical HDACs in filamentous fungi in the regulation of some fungal phenotypes has been revealed (2). For example, *F. graminearum hdf1*/*F. graminearum hos2* is important for conidial development and SM production (7). In this study, we also determined the importance of *hdaD*/*Aohos2* in the regulation of hyphal growth, conidiation, and SM production. Compared with the study of classical HDACs, only a few reports have been published for sirtuin-type HDACs in filamentous fungi (20, 35). Recently, *sirA*/*A. nidulans sir2* (*Ansir2*), which is a homolog of yeast *sir2* and mammalian *sirt1*, was reported to function in secondary metabolism regulation, but no effects on growth, conidiation, or morphogenesis were reported (20).

The fungus-specific putative methyltransferase LaeA coordinates fungal development and SMs in several filamentous fungi (17). It was previously reported that kojic acid production is regulated by LaeA, but we found that *laeA* coordinates both SM production and conidial development (39). In general, the production of fungal SMs is coordinated with fungal development, and the LaeA complex coordinates these (17). In higher eukaryotes, sirtuin affects various physiological functions, such as differentiation, metabolism, and the stress response (41–43). In this study, we found that the fungus-specific sirtuin HstD/AoHst4 affects both fungal development and SM production. Furthermore, the epistatic study revealed that HstD/AoHst4 is involved in the coordination of fungal development and SM production via LaeA expression. These results indicate that HstD/AoHst4 plays a Sirt1-like central role that coordinates the developmental state and metabolism in filamentous fungi.

The yeast Hst4, which is a homolog of *hstD/Aohst4* of *A. oryzae*, is a NAD^+-dependent histone H3K56 deacetylase (44). In the Northern analysis, deletion of *hstD*/*Aohst4* affected the level of *laeA* gene expression. Therefore, HstD/AoHstD may be involved in the epigenetic regulation of *laeA* gene expression. However, the overexpression of *hstD*/*Aohst4* did not affect SM production or conidial development. In general, histone acetyltransferase (HAT) is required for activation of silenced genes. In budding yeast, it has been reported that the fungus-specific HAT of RTT109 catalyzes H3K56 acetylation and restores the silencing defects of the Δ*hst3* Δ*hst4* mutant (44, 45). We found one *rtt109* homologue of *AO090020000581* (*A. oryzae rtt109* [*Aortt109*]) in the *A. oryzae* genome. This suggests that *Aortt109* is required for the expression of the silenced *laeA* by H3K56 deacetylation and overcomes even *hstD*/*Aohst4* overexpression. In this context, we will investigate histone H3K56 acetylation of the *laeA* locus and the effect of *Aortt109* on the acetylation and expression of *laeA* in future studies.

In the overexpression analysis of *laeA*, several different phenotypes of kojic acid production were observed compared with the phenotype of the *hstD*/*Aohst4* mutant. The Δ*hstD* strain showed high levels of kojic acid production in a 4-day liquid culture, while the same phenotype was not observed in the *OE*::*laeA* strain. Compared with the *OE*::*laeA* strain, interestingly, higher levels of kojic acid production and higher levels of expression of *kojA*, *ipnA*, and *laeA* were observed in the Δ*hstD OE*::*laeA* strain. These results suggested that HstD/AoHst4 has some LaeA-independent role in the regulation of SM production.

We developed a model of the regulatory system of HstD/AoHst4 (Fig. 7). The expression of *hstD/Aohst4* may be induced or suppressed by unknown signals. As described above, an unknown factor like AoRtt109 may compete with HstD/AoHst4 activity under *hstD*/*Aohst4*-inducing conditions. Under the *hstD*/*Aohst4*-suppressed condition, *laeA* expression was induced. This in turn led to conidiation and induction of secondary metabolite production. However, it is possible that HstD/AoHst4 directly regulates fungal development and secondary metabolism independently of LaeA.

The *hstD*/*Aohst4* gene is fungus specific but is conserved in the vast family of filamentous fungi (see Fig. S3 in the supplemental material). Furthermore, this gene plays a role in the coordination of fungal development and SM production. These results indicate that HstD/AoHst4 has great potential as a target to improve the productivity of useful SMs. It will also be important in the development of an attractive host for the production of several heterogeneous metabolites.

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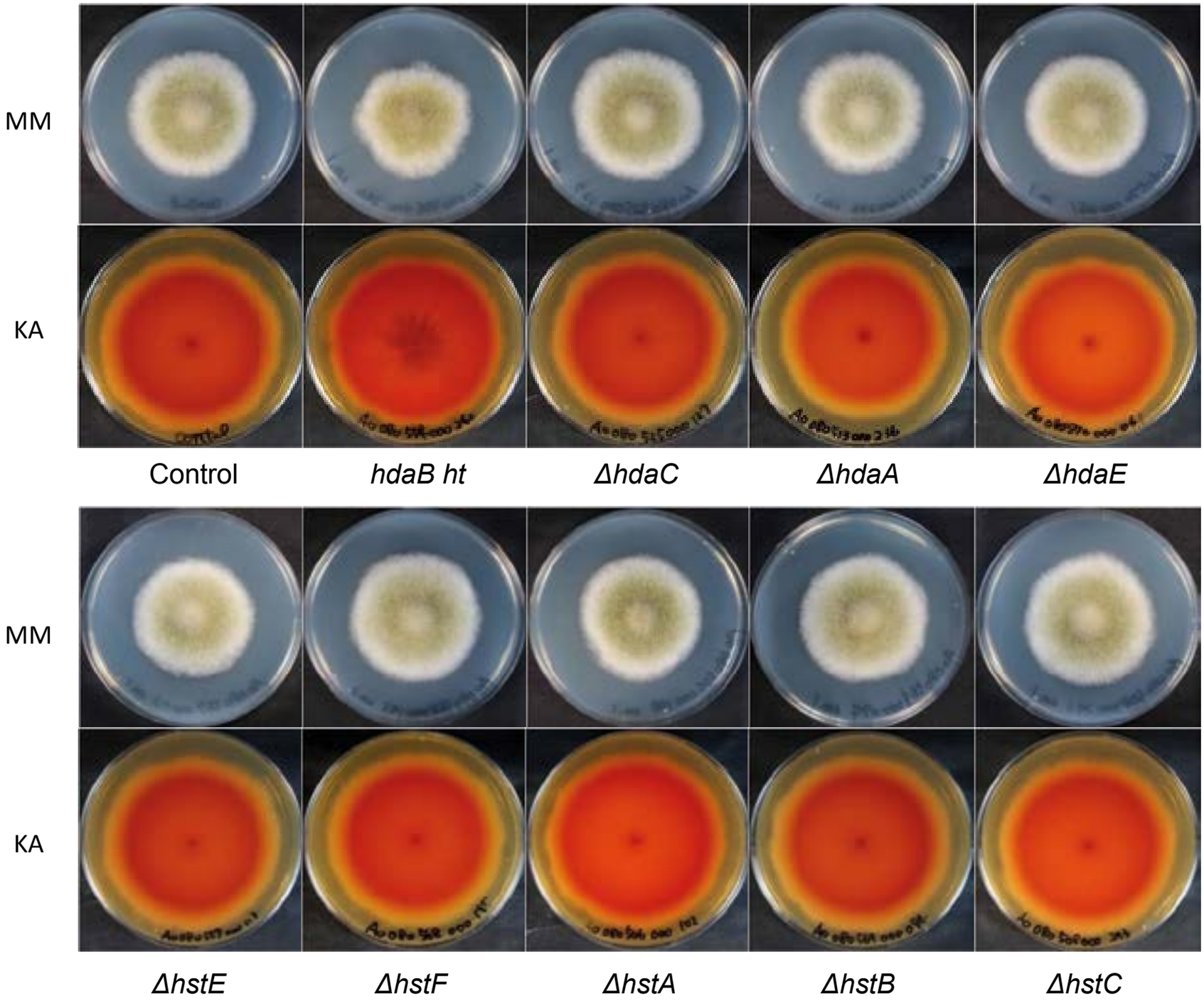
We thank Ken Oda for great discussions, Katsuhiko Kitamoto for the gift of the *A. oryzae NSR*-Δ*LD2* strain, Ryoko Hamada for technical help with the microarray analysis, and Kanae Koike (Electron Microscopy Service, Center for Gene Science, Hiroshima University) for technical help with electron microscopic analysis.

REFERENCES

1. **Shahbazian MD, Grunstein M.** 2007. Functions of site-specific histone acetylation and deacetylation. Annu. Rev. Biochem. **76:**75–100.
2. **Brosch G, Loidl P, Graessle S.** 2008. Histone modifications and chromatin dynamics: a focus on filamentous fungi. FEMS Microbiol. Rev. **32:** 409–439.
3. **Nishida H.** 2009. Evolutionary conservation levels of subunits of histone-modifying protein complexes in fungi. Comp. Funct. Genomics **2009:** 379317. doi:10.1155/2009/379317.
4. **Ekwall K.** 2005. Genome-wide analysis of HDAC function. Trends Genet. **21:**608–615.
5. **Borkovich KA, Alex LA, Yarden O, Freitag M, Turner GE, Read ND, Seiler S, Bell-Pedersen D, Paietta J, Plesofsky N, Plamann M, Goodrich-Tanrikulu M, Schulte U, Mannhaupt G, Nargang FE, Radford A, Selitrennikoff C, Galagan JE, Dunlap JC, Loros JJ, Catcheside D, Inoue H, Aramayo R, Polymenis M, Selker EU, Sachs MS, Marzluf GA, Paulsen I, Davis R, Ebbole DJ, Zelter A, Kalkman ER, O'Rourke R, Bowring F, Yeadon J, Ishii C, Suzuki K, Sakai W, Pratt R.** 2004. Lessons from the genome sequence of *Neurospora crassa*: tracing the path from genomic blueprint to multicellular organism. Microbiol. Mol. Biol. Rev. **68:**1–108.
6. **Yang X-J, Seto E.** 2008. The Rpd3/Hda1 family of lysine deacetylases: from bacteria and yeast to mice and men. Nat. Rev. Mol. Cell Biol. **9:**206–218.
7. **Li Y, Wang C, Liu W, Wang G, Kang Z, Kistler HC, Xu J-R.** 2011. The *HDF1* histone deacetylase gene is important for conidiation, sexual reproduction, and pathogenesis in *Fusarium graminearum*. Mol. Plant Microbe Interact. **24:**487–496.
8. **Ding S-L, Liu W, Iliuk A, Ribot C, Vallet J, Tao A, Wang Y, Lebrun M-H, Xu J-R.** 2010. The tig1 histone deacetylase complex regulates infectious growth in the rice blast fungus *Magnaporthe oryzae*. Plant Cell **22:** 2495–2508.
9. **Izawa M, Takekawa O, Arie T, Teraoka T, Yoshida M, Kimura M, Kamakura T.** 2009. Inhibition of histone deacetylase causes reduction of appressorium formation in the rice blast fungus *Magnaporthe oryzae*. J. Gen. Appl. Microbiol. **55:**489–498.
10. **Smith K, Kothe G, Matsen C, Khlafallah T, Adhvaryu K, Hemphill M, Freitag M, Motamedi M, Selker E.** 2008. The fungus *Neurospora crassa* displays telomeric silencing mediated by multiple sirtuins and by methyl-

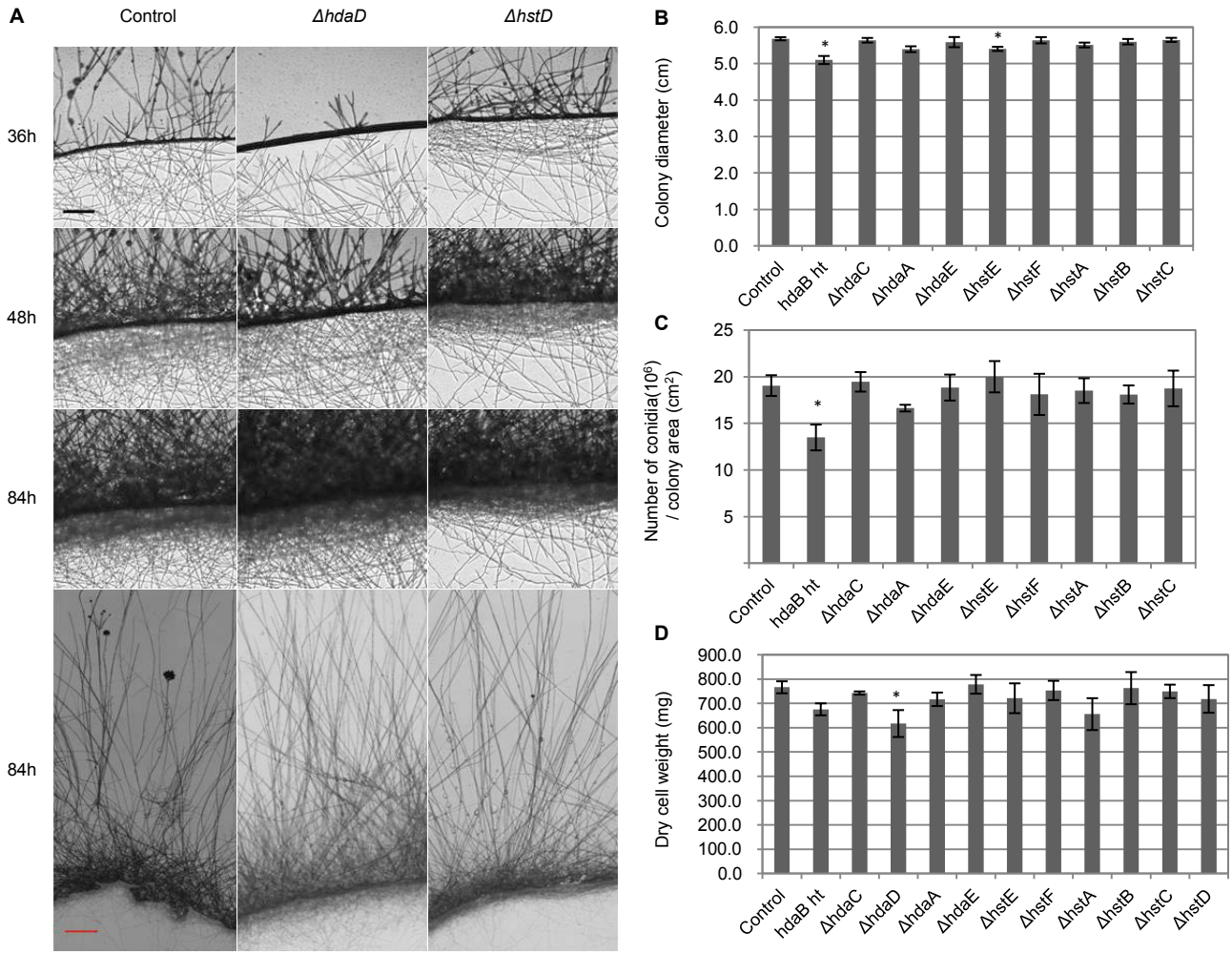
ation of histone H3 lysine 9. Epigenetics Chromatin **1:**5. doi:10.1186/1756-8935-1-5.
11. **Tribus M, Bauer I, Galehr J, Rieser G, Trojer P, Brosch G, Loidl P, Haas H, Graessle S.** 2010. A novel motif in fungal class 1 histone deacetylases is essential for growth and development of *Aspergillus*. Mol. Biol. Cell **21:** 345–353.
12. **Tribus M, Galehr J, Trojer P, Brosch G, Loidl P, Marx F, Haas H, Graessle S.** 2005. HdaA, a major class 2 histone deacetylase of *Aspergillus nidulans*, affects growth under conditions of oxidative stress. Eukaryot. Cell **4:**1736–1745.
13. **Lee I, Oh J-H, Keats Shwab E, Dagenais TRT, Andes D, Keller NP.** 2009. HdaA, a class 2 histone deacetylase of *Aspergillus fumigatus*, affects germination and secondary metabolite production. Fungal Genet. Biol. **46:**782–790.
14. **Baidyaroy D, Brosch G, Ahn J-H, Graessle S, Wegener S, Tonukari NJ, Caballero O, Loidl P, Walton JD.** 2001. A gene related to yeast *HOS2* histone deacetylase affects extracellular depolymerase expression and virulence in a plant pathogenic fungus. Plant Cell **13:**1609–1624.
15. **Hoffmeister D, Keller NP.** 2007. Natural products of filamentous fungi: enzymes, genes, and their regulation. Nat. Prod. Rep. **24:**393–416.
16. **Sanchez JF, Somoza AD, Keller NP, Wang CCC.** 2012. Advances in *Aspergillus* secondary metabolite research in the post-genomic era. Nat. Prod. Rep. **29:**351–371.
17. **Bayram Ö, Braus GH.** 2012. Coordination of secondary metabolism and development in fungi: the velvet family of regulatory proteins. FEMS Microbiol. Rev. **36:**1–24.
18. **Gacek A, Strauss J.** 2012. The chromatin code of fungal secondary metabolite gene clusters. Appl. Microbiol. Biotechnol. **95:**1389–1404.
19. **Shwab EK, Bok JW, Tribus M, Galehr J, Graessle S, Keller NP.** 2007. Histone deacetylase activity regulates chemical diversity in *Aspergillus*. Eukaryot. Cell **6:**1656–1664.
20. **Shimizu M, Masuo S, Fujita T, Doi Y, Kamimura Y, Takaya N.** 2012. Hydrolase controls cellular NAD, sirtuin, and secondary metabolites. Mol. Cell. Biol. **32:**3743–3755.
21. **Machida M, Yamada O, Gomi K.** 2008. Genomics of *Aspergillus oryzae*: learning from the history of koji mold and exploration of its future. DNA Res. **15:**173–183.
22. **Abe K, Gomi K, Hasegawa F, Machida M.** 2006. Impact of *Aspergillus oryzae* genomics on industrial production of metabolites. Mycopathologia **162:**143–153.
23. **Imamura K, Tsuyama Y, Hirata T, Shiraishi S, Sakamoto K, Yamada O, Akita O, Shimoi H.** 2012. Identification of a gene involved in the synthesis of a dipeptidyl peptidase IV inhibitor in *Aspergillus oryzae*. Appl. Environ. Microbiol. **78:**6996–7002.
24. **Terabayashi Y, Sano M, Yamane N, Marui J, Tamano K, Sagara J, Dohmoto M, Oda K, Ohshima E, Tachibana K, Higa Y, Ohashi S, Koike H, Machida M.** 2010. Identification and characterization of genes responsible for biosynthesis of kojic acid, an industrially important compound from *Aspergillus oryzae*. Fungal Genet. Biol. **47:**953–961.
25. **Maruyama J-I, Kitamoto K.** 2008. Multiple gene disruptions by marker recycling with highly efficient gene-targeting background (Δ*ligD*) in *Aspergillus oryzae*. Biotechnol. Lett. **30:**1811–1817.
26. **Arnaud MB, Chibucos MC, Costanzo MC, Crabtree J, Inglis DO, Lotia A, Orvis J, Shah P, Skrzypek MS, Binkley G, Miyasato SR, Wortman JR, Sherlock G.** 2010. The *Aspergillus* Genome Database, a curated comparative genomics resource for gene, protein and sequence information for the *Aspergillus* research community. Nucleic Acids Res. **38:**D420–D427. doi:10.1093/nar/gkp751.
27. **Quevillon E, Silventoinen V, Pillai S, Harte N, Mulder N, Apweiler R, Lopez R.** 2005. InterProScan: protein domains identifier. Nucleic Acids Res. **33:**W116–W120. doi:10.1093/nar/gki442.
28. **Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S.** 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. **28:**2731–2739.
29. **Szewczyk E, Nayak T, Oakley CE, Edgerton H, Xiong Y, Taheri-Talesh N, Osmani SA, Oakley BR.** 2007. Fusion PCR and gene targeting in *Aspergillus nidulans*. Nat. Protoc. **1:**3111–3120.
30. **Yamada O, Na Nan S, Akao T, Tominaga M, Watanabe H, Satoh T, Enei H, Akita O.** 2003. *dffA* gene from *Aspergillus oryzae* encodes L-ornithine N5-oxygenase and is indispensable for deferriferrichrysin biosynthesis. J. Biosci. Bioeng. **95:**82–88.
31. **Kitamoto K.** 2002. Molecular biology of the koji molds. Adv. Appl. Microbiol. **51:**129–153.
32. **Priebe S, Linde J, Albrecht D, Guthke R, Brakhage AA.** 2011. FungiFun: a web-based application for functional categorization of fungal genes and proteins. Fungal Genet. Biol. **48:**353–358.
33. **Ruepp A, Zollner A, Maier D, Albermann K, Hani J, Mokrejs M, Tetko I, Güldener U, Mannhaupt G, Münsterkötter M, Mewes HW.** 2004. The FunCat, a functional annotation scheme for systematic classification of proteins from whole genomes. Nucleic Acids Res. **32:**5539–5545.
34. **Frye RA.** 2000. Phylogenetic classification of prokaryotic and eukaryotic Sir2-like proteins. Biochem. Biophys. Res. Commun. **273:**793–798.
35. **Smith KM, Dobosy JR, Reifsnyder JE, Rountree MR, Anderson DC, Green GR, Selker EU.** 2010. H2B- and H3-specific histone deacetylases are required for DNA methylation in *Neurospora crassa*. Genetics **186:** 1207–1216.
36. **Brakhage AA.** 2013. Regulation of fungal secondary metabolism. Nat. Rev. Microbiol. **11:**21–32.
37. **Podust LM, Sherman DH.** 2012. Diversity of P450 enzymes in the biosynthesis of natural products. Nat. Prod. Rep. **29:**1251–1266.
38. **Kelly DE, Kraševec N, Mullins J, Nelson DR.** 2009. The CYPome (cytochrome P450 complement) of *Aspergillus nidulans*. Fungal Genet. Biol. **46:**S53–S61.
39. **Oda K, Kobayashi A, Ohashi S, Sano M.** 2011. *Aspergillus oryzae laeA* regulates kojic acid synthesis genes. Biosci. Biotechnol. Biochem. **75:** 1832–1834.
40. **Marui J, Ohashi-Kunihiro S, Ando T, Nishimura M, Koike H, Machida M.** 2010. Penicillin biosynthesis in *Aspergillus oryzae* and its overproduction by genetic engineering. J. Biosci. Bioeng. **110:**8–11.
41. **Zhang T, Kraus WL.** 2010. SIRT1-dependent regulation of chromatin and transcription: linking NAD^+ metabolism and signaling to the control of cellular functions. Biochim. Biophys. Acta **1804:**1666–1675.
42. **Imai S-I, Guarente L.** 2010. Ten years of NAD-dependent SIR2 family deacetylases: implications for metabolic diseases. Trends Pharmacol. Sci. **31:**212–220.
43. **Horio Y, Hayashi T, Kuno A, Kunimoto R.** 2011. Cellular and molecular effects of sirtuins in health and disease. Clin. Sci. **121:**191–203.
44. **Yang B, Miller A, Kirchmaier AL.** 2008. HST3/HST4-dependent deacetylation of lysine 56 of histone H3 in silent chromatin. Mol. Biol. Cell **19:**4993–5005.
45. **D'Arcy S, Luger K.** 2011. Understanding histone acetyltransferase Rtt109 structure and function: how many chaperones does it take? Curr. Opin. Struct. Biol. **21:**728–734.

Figure S1. Kojic acid production and developmental phenotype of AoHDACs disruptants and heterokaryon strain



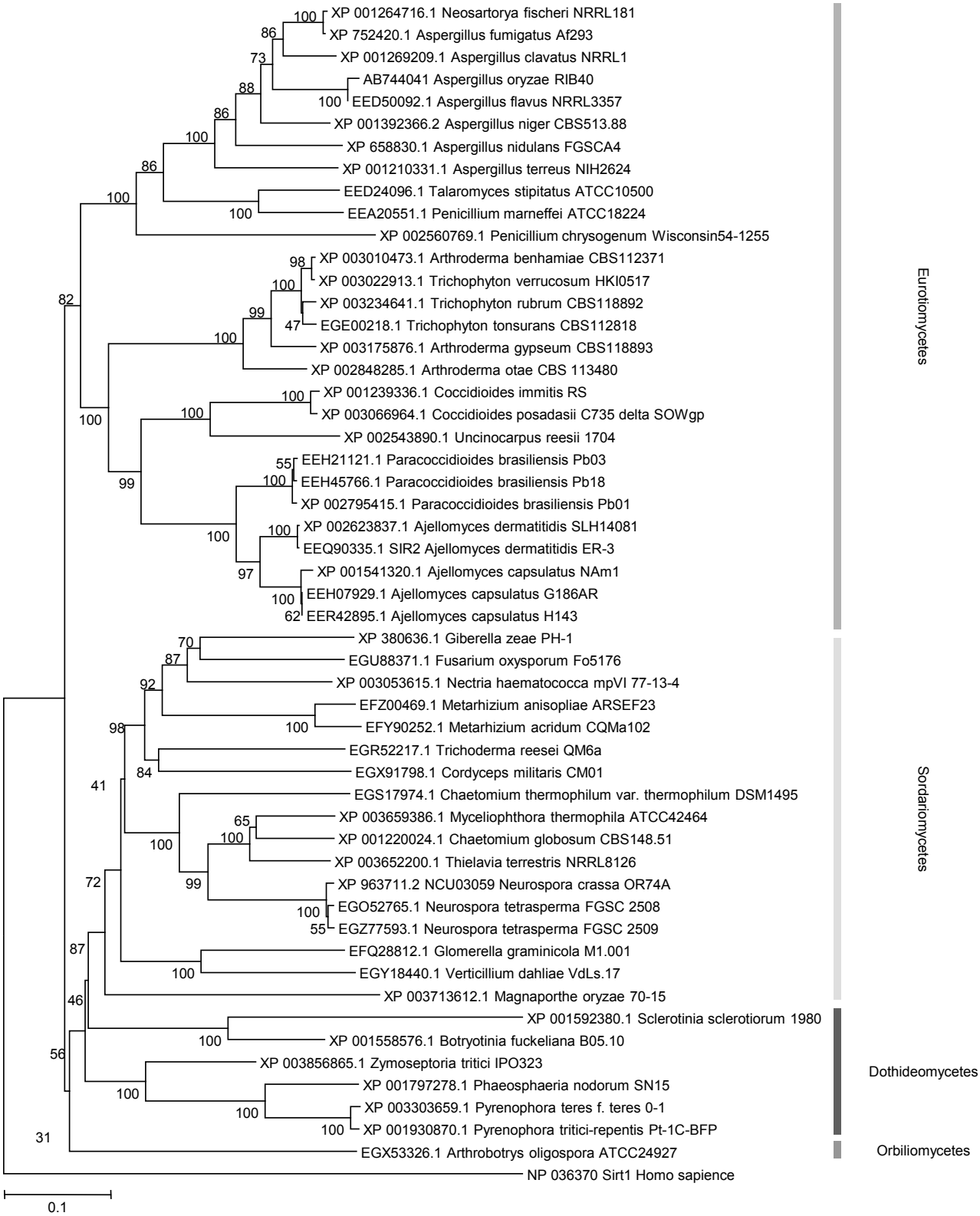
MM and KA show the morphological phenotype and kojic acid production plate assays of the indicated strains, respectively.

Figure S2. Phenotypic analysis of AoHDACs disruptants and hetrokaryon strain on plates and submerged cultures.



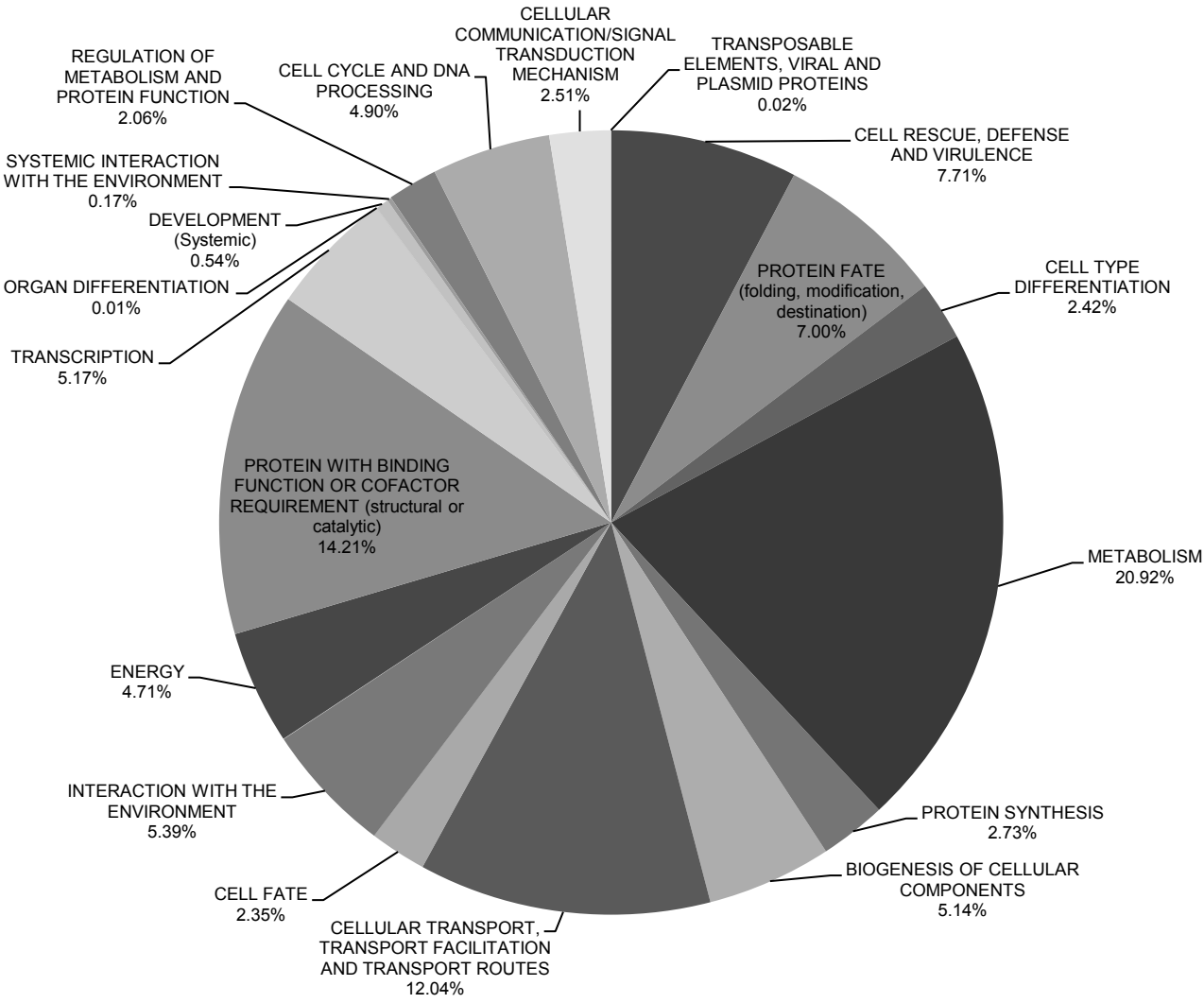
(A) Time-lapse microscopic analysis of *ΔhstD* and *ΔhdaD*. The pictures were taken at 36, 48, and 84 hr of incubation (black scale bar, 200 μm; red scale bar, 500 μm). **(B-D)** Quantification of colony diameter, conidiation rate, and mycelial dry cell weight of AoHDACs disruptants or the heterokaryon strain, respectively. The colony diameter and conidiation rates were measured on plate cultures. Dry cell weight was measured on submerged cultures. All data are represented as means ± s.d. (n=3); **p* < 0.01, t-test

Figure S3. Phylogenetic analysis of *hstD* in filamentous fungi



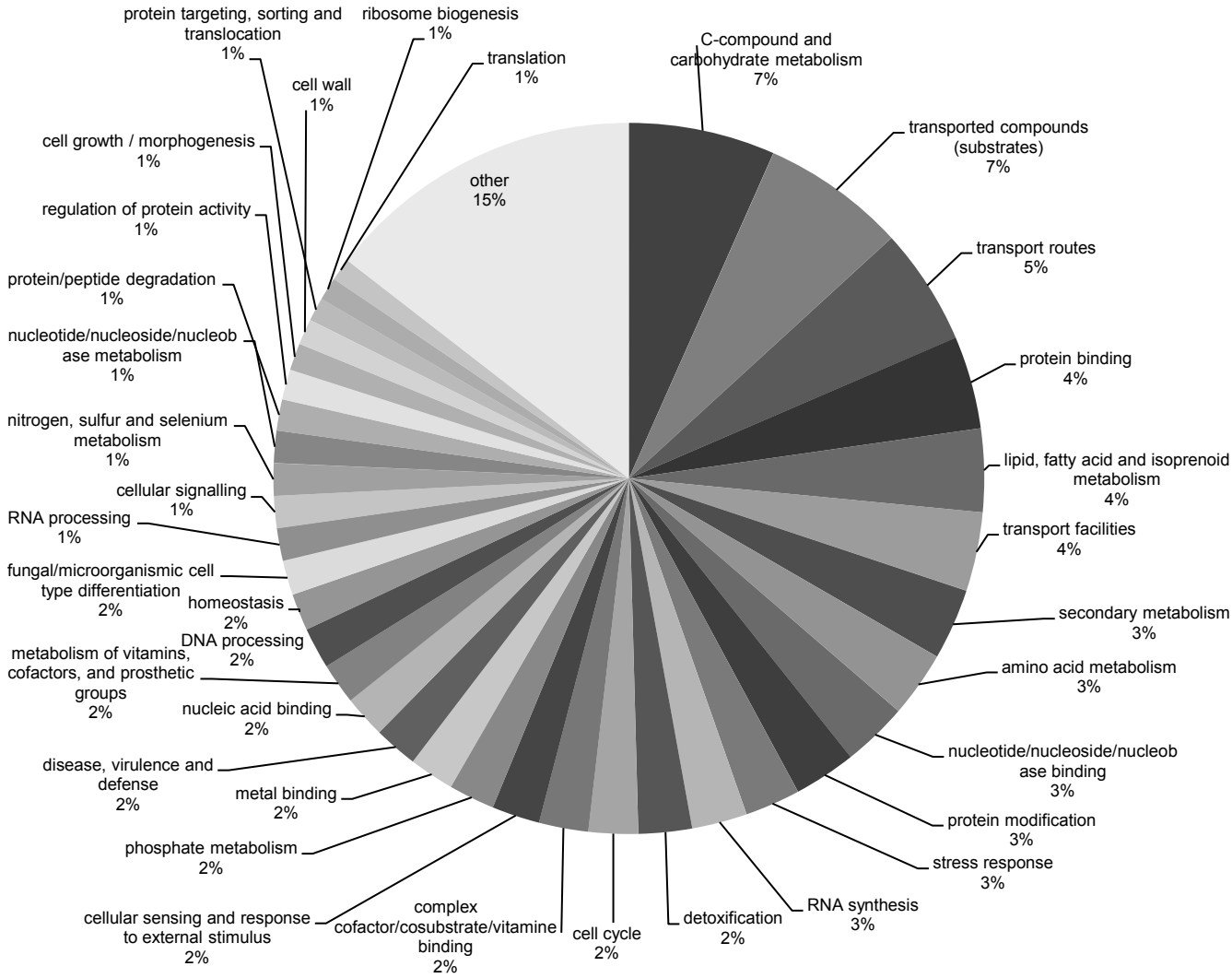
The Genebank accession numbers and species names are indicated for each branch. The numbers at the nodes are bootstrap values obtained from 1000 replicates and are indicated as percentages. The scale bar indicates a distance corresponding to 0.1 amino acid substitutions per site. Each class of ascomycetes is shown at the right. *Sirt1* is used as the out-group in this phylogenetic analysis.

Figure S4. Whole genome distribution of FunCat level 1 categories



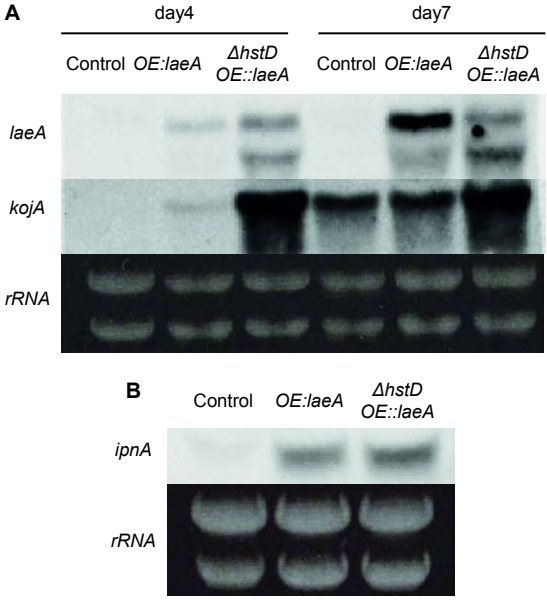
The pie-chart indicates the whole genome distribution of the indicated categories of FunCat level 1. Percentages indicate each category that contributes to total mapping. The data of FunCat categorization of all genes of *A. oryzae* were imported from FungiFun software. Details of each category are available at the FunCat Database (http://mips.helmholtzmuenchen.de/proj/funcatDB/search_main_frame.html). This data was reference of FunCat level 1 enrichment analysis of *A. oryzae.*

Figure S5. Whole genome distribution of FunCat level 2 categories



The circular chart indicates the whole genome distribution of indicated categories FunCat level 2. Miner categories(Whole genome distribution < 1%) were indicated as other in pie chart. Percentage indicated each category contributes to total mapping. The data of FunCat categorization of all genes of *A.oryzae* were imported from FungiFun software (https://sbi.hki-jena.de/FungiFun/FungiFun.cgi). Details of each categories were available at FunCat Databases (http://mips.helmholtzmuenchen.de/proj/funcatDB/search_main_frame.html). This data was referance of FunCat level 2 enrichment analysis of *A. oryzae*.

Figure S6. Expression profiling of *laeA*, *kojA*, and *ipnA* in *OE::laeA* and *ΔhstD OE::laeA* strains, respectively



(A) Northern hybridization of *laeA and kojA*, respectively. The culture time of the indicated strain is shown at the top of the panel. *rRNA* is shown as the loading control. The analyzed gene is indicated on the left side of each blot
(B) Northern hybridization of the penicillin biosynthetic gene *ipnA*. *rRNA* is shown as the loading control. The *adeA*$^{+}$ *pUSA*$^{+}$ strain was used as the control, and the *OE:laeA adeA*$^{+}$strain represents *OE::laeA* in this figure.

Table S1. Strain used in this study

Name	Parental strain	Genotype[b]	Reference
RIB40		wild type	NRIB[a]
NSR-ΔLD2	*RIB40*	*niaD*$^-$ *sC*$^-$ *adeA*$^-$ *argB::adeA*$^-$ *ΔligD::argB*	(25)
adeA$^+$	*NSR-ΔLD2*	*niaD*$^-$ *sC*$^-$ *adeA*$^-$ *argB::adeA*$^-$ *ΔligD::argB adeA*$^+$	this study
pUSA$^+$	*NSR-ΔLD2*	*niaD*$^-$ *sC*$^-$ *adeA*$^-$ *argB::adeA*$^-$ *ΔligD::argB pUSA*$^+$	this study
adeA$^+$ *sC*$^+$	*adeA*$^+$	*niaD*$^-$ *sC*$^-$ *adeA*$^-$ *argB::adeA*$^-$ *ΔligD::argB adeA*$^+$ *AnsC*$^+$	this study
adeA$^+$ *pUSA*$^+$	*adeA*$^+$	*niaD*$^-$ *sC*$^-$ *adeA*$^-$ *argB::adeA*$^-$ *ΔligD::argB adeA*$^+$ *pUSA*$^+$	this study
hdaB ht[c]	*NSR-ΔLD2*	*niaD*$^-$ *sC*$^-$ *adeA*$^-$ *argB::adeA*$^-$ *ΔligD::argB ΔhdaB::adeA hdaB*	this study
ΔhdaC	*NSR-ΔLD2*	*niaD*$^-$ *sC*$^-$ *adeA*$^-$ *argB::adeA*$^-$ *ΔligD::argB ΔhdaC::adeA*	this study
ΔhdaD	*NSR-ΔLD2*	*niaD*$^-$ *sC*$^-$ *adeA*$^-$ *argB::adeA*$^-$ *ΔligD::argB ΔhdaD::adeA*	this study
ΔhdaA	*NSR-ΔLD2*	*niaD*$^-$ *sC*$^-$ *adeA*$^-$ *argB::adeA*$^-$ *ΔligD::argB ΔhdaA::adeA*	this study
ΔhdaE	*NSR-ΔLD2*	*niaD*$^-$ *sC*$^-$ *adeA*$^-$ *argB::adeA*$^-$ *ΔligD::argB ΔhdaE::adeA*	this study
ΔhstA	*NSR-ΔLD2*	*niaD*$^-$ *sC*$^-$ *adeA*$^-$ *argB::adeA*$^-$ *ΔligD::argB ΔhstA::adeA*	this study
ΔhstB	*NSR-ΔLD2*	*niaD*$^-$ *sC*$^-$ *adeA*$^-$ *argB::adeA*$^-$ *ΔligD::argB ΔhstB::adeA*	this study
ΔhstC	*NSR-ΔLD2*	*niaD*$^-$ *sC*$^-$ *adeA*$^-$ *argB::adeA*$^-$ *ΔligD::argB ΔhstC::adeA*	this study
ΔhstD	*NSR-ΔLD2*	*niaD*$^-$ *sC*$^-$ *adeA*$^-$ *argB::adeA*$^-$ *ΔligD::argB ΔhstD::adeA*	this study
ΔhstE	*NSR-ΔLD2*	*niaD*$^-$ *sC*$^-$ *adeA*$^-$ *argB::adeA*$^-$ *ΔligD::argB ΔhstE::adeA*	this study
ΔhstF	*NSR-ΔLD2*	*niaD*$^-$ *sC*$^-$ *adeA*$^-$ *argB::adeA*$^-$ *ΔligD::argB ΔhstF::adeA*	this study
ΔlaeA	*NSR-ΔLD2*	*niaD*$^-$ *sC*$^-$ *adeA*$^-$ *argB::adeA*$^-$ *ΔligD::argB ΔlaeA::adeA*	this study
ΔhstD sC$^+$	*ΔhstD*	*niaD*$^-$ *sC*$^-$ *adeA*$^-$ *argB::adeA*$^-$ *ΔligD::argB ΔhstD::adeA AnsC*$^+$	this study
ΔhstD pUSA$^+$	*ΔhstD*	*niaD*$^-$ *sC*$^-$ *adeA*$^-$ *argB::adeA*$^-$ *ΔligD::argB ΔhstD::adeA pUSA*$^+$	this study
ΔhstD OE::laeA	*ΔhstD*	*niaD*$^-$ *sC*$^-$ *adeA*$^-$ *argB::adeA*$^-$ *ΔligD::argB ΔhstD::adeA pUSlaeA*$^+$	this study
ΔlaeA sC$^+$	*ΔlaeA*	*niaD*$^-$ *sC*$^-$ *adeA*$^-$ *argB::adeA*$^-$ *ΔligD::argB ΔlaeA::adeA AnsC*$^+$	this study
ΔlaeA pUSA$^+$	*ΔlaeA*	*niaD*$^-$ *sC*$^-$ *adeA*$^-$ *argB::adeA*$^-$ *ΔligD::argB ΔlaeA::adeA pUSA*$^+$	this study
ΔlaeA OE::hstD	*ΔlaeA*	*niaD*$^-$ *sC*$^-$ *adeA*$^-$ *argB::adeA*$^-$ *ΔligD::argB ΔlaeA::adeA pUShstD*$^+$	this study
OE::laeA	*NSR-ΔLD2*	*niaD*$^-$ *sC*$^-$ *adeA*$^-$ *argB::adeA*$^-$ *ΔligD::argB pUSlaeA*$^+$	this study
OE::hstD	*NSR-ΔLD2*	*niaD*$^-$ *sC*$^-$ *adeA*$^-$ *argB::adeA*$^-$ *ΔligD::argB pUShstD*$^+$	this study
ΔhstD ΔlaeA	*ΔhstD*	*niaD*$^-$ *sC*$^-$ *adeA*$^-$ *argB::adeA*$^-$ *ΔligD::argB ΔhstD::adeA ΔlaeA::AnsC*	this study
hstD$^+$	*ΔhstD*	*niaD*$^-$ *sC*$^-$ *adeA*$^-$ *argB::adeA*$^-$ *ΔligD::argB ΔhstD::adeA::hstD::AnsC*	this study
OE::laeA adeA$^+$	*OE::laeA*	*niaD*$^-$ *sC*$^-$ *adeA*$^-$ *argB::adeA*$^-$ *ΔligD::argB pUSlaeA*$^+$ *adeA*$^+$	this study
OE::hstD adeA$^+$	*OE::hstD*	*niaD*$^-$ *sC*$^-$ *adeA*$^-$ *argB::adeA*$^-$ *ΔligD::argB pUShstD*$^+$ *adeA*$^+$	this study

[a]National Research institute of Brewing

[b]*AnsC: Aspergillus nidulans sC*

[c]*ht:* heterokaryon

Table S2. List of sequence accession numbers used in this study

Saccharomyces cerviciae		*Homo sapience*		*Neurospora crassa*		*Aspergillus nidulans*		*Aspergillus oryzae*	
Gene[a]	Accession number	Gene[a]	Accession number	Gene[a]	Accession number	Gene[a]	Accession number	Gene[a]	Accession number
Classical HDACs familly									
rpd3	YNL330C	*hdac1 / Hsrpd3*	NP_004955	*hda3 / Ncrpd3*	NCU00824	*rpdA / Anrpd3*	AN4493	*hdaB / Aorpd3*	AB744040
		hdac2 / Hsrpd3	NP_001518					*hdaC / Aorpd3*	AO080525000127
		hdac3 / Hsrpd3	NP_003874						
hos2	YGL194C			*hda2 / Nchos2*	NCU02795	*hosA / Anhos2*	AN3806	*hdaD / Aohos2*	AO080511000459
hos1	YPR068C								
hda1	YRL021W	*hdac6 / Hshda1*	NP_006035	*hda1 / Nchda1*	NCU01525	*hdaA / Anhda1*	AN8024	*hdaA / Aohda1*	AO080513000236
		hdac10 / Hshda1	NP_114408						
hos3	YPL116W			*hda4 / Nchos3*	NCU07018	*hosB / Anhos3*	AN7019	*hdaE / Aohos3*	AO080570000061
		hdac4	NP_006028						
		hdac5	NP_001015053						
		hdac7	NP_056216						
		hdac8	NP_060956						
		hdac9	NP_055522						
		hdac11	NP_079103						
TheSirtuin familly									
sir2	YDL042C	*sirt1 / Hssir2*	NP_036370	*nst1 / Ncsir2*	NCU04737	*sirA / Ansir2*	AN10449	*hstA / Aosir2*	AO080506000102
hst1	YOL068C								
hst2	YPL015C	*sirt2 / Hshst2*	NP_036369	*nst2 / Nchst2*	NCU00523	*AN7461/ Anhst2*	AN7461	*hstB / Aohst2*	AO080569000094
		sirt3 / Hshst2	NP_036371			*AN11873/ Anhst2*	AN11873	*hstC / Aohst2*	AO080508000293
hst3	YOR025W			*nst4 / Nchst3*	NCU04859				
hst4	YDR191W			*nst3 / Nchst4*	NCU03059	*AN1226 / Anhst4*	AN1226	*hstD / Aohst4*	AB744041
		sirt4	NP_036372	*nst5 / Ncsirt4*	NCU00203	*hstA / Ansirt4*	AN11067	*hstE / Aosirt4*	AO080559000113
		sirt5	NP_036373	*nst6 / Ncsirt5*	NCU05973	*AN1782 / Ansirt5*	AN1782	*hstF / Aosirt5*	AO080568000195
		sirt6	NP_057623	*nst7 / Ncsirt6*	NCU07624				
		sirt7	NP_057622						

[a] HDAC names of *Saccharomyces cerevisiae* or *Homo Sapiens* with the species name indicated followed by a slash.

[b] Hda : Histone DeAcetylase

[c] Hst : Homolog of SirTuins

Table S3. PCR primers used in this study

Primer name	Sequence(5' to 3')[a,b]	Region or purpose
adeA-F	CCGTCATGTCCAGGAAGATAGGTCAG	*adeA* amplification
adeA-R	CTGCGCAACAGCATACGAGTCCACAG	
hdaB-A	CAATGGCATGACAAAGAACC	5' flanking region of *hdaB*
hdaB-B	CTGACCTATCTTCCTGGACATGACGGCTGTTCCTGCAACATGAGATACA	
hdaB-C	CTGTGGACTCGTATGCTGTTGCGCAGAAGATCCGTGCGCAAGTT	3'flanking region of *hdaB*
hdaB-D	CCATGGTGAATTAGGGCTCA	
hdaB-A2	CAATAGAATATTCCCCGCGT	fusion PCR for *hdaB*
hdaB-D2	CCCTTGGGATTAGAGTGCTT	
hdaB-F	GTTGATCGGGATGTCAAAGG	*hdaB* ORF
hdaB-G	AATTCTCGGTTCTGCTGGTG	
hdaC-A	TCTGTGCAAGCCTTATGTGC	5' flanking region of *hdaC*
hdaC-B	CTGACCTATCTTCCTGGACATGACGGTCCGTCGAGGTTAGTGACAA	
hdaC-C	CTGTGGACTCGTATGCTGTTGCGCAGTCACTTTGACTACGGAGGGCT	3'flanking region of *hdaC*
hdaC-D	GCCTCGAAATCATGGTCCTA	
hdaC-F	TGAGTGCCTCGTAATGCTTG	*hdaC* ORF
hdaC-G	GTGGGCAGGTTGAAACTCTT	
hdaD-A	TAACTGGCGCAGACCCATAA	5' flanking region of *hdaD*
hdaD-B	CTGACCTATCTTCCTGGACATGACGGCCCTTCTTCTCTTTCCTTATTGC	
hdaD-C	CTGTGGACTCGTATGCTGTTGCGCAGCTGTACGGTAAATGAAGGTCAGC	3'flanking region of *hdaD*
hdaD-D	AAGGGGTCAGATCCACAATG	
hdaD-A2	CGCAGACCCATAAGAAGGAA	fusion PCR for *hdaD*
hdaD-D2	CTGTGTCCACAACTGCCATT	
hdaD-F	GTTGTTTGGTCAGCGTCAGA	*hdaD* ORF
hdaD-G	CCCAAAGGTGACAAGACGAT	
hdaA-A	AACAAAGTGCCCTGTTGACC	5'flanking region of *hdaA*
hdaA-B	CTGACCTATCTTCCTGGACATGACGGCATTGCTATGGCTAGCACCA	
hdaA-C	CTGTGGACTCGTATGCTGTTGCGCAGTTTAGGACGTTCAGATGGGG	3'flanking region of *hdaA*
hdaA-D	TAGGTTTTCTGATGGCCCAG	
hdaA-F	GTGATGCCTATTGCACAGGA	*hdaA* ORF
hdaA-G	GCTTTCGGGTACATGCAACT	

hdaE-A	TCCGAAGTCCACTTTCTTGC	
hdaE-B	CTGACCTATCTTCCTGGACATGACGGCAAATAGTAGGTTCATTTGGGGG	5' flanking region of *hdaE*
hdaE-C	CTGTGGACTCGTATGCTGTTGCGCAGAGTCTATCGGACTTTTGGTCG	3'flanking region of *hdaE*
hdaE-D	AGATCCGGAGTCGTTCCTTT	
hdaE-A2	AGTCTCTTTTCTTTGGCCGC	fusion PCR for *hdaE*
hdaE-D2	ATTGTTCACGTTCTCCACCC	
hdaE-F	GGACCTTTACGCATCCAAGT	*hdaE* ORF
hdaE-G	GATGCGTGTTGGACATTAGC	
hstA-A	ATTACCTGGCGTTCTTGTGG	
hstA-B	CTGACCTATCTTCCTGGACATGACGGGGGTTCTTGTTTGGAGGGTT	5'flanking region of *hst A*
hstA-C	CTGTGGACTCGTATGCTGTTGCGCAGCGGATTCTTCAACGAAGAGC	3'flanking region of *hstA*
hstA-D	ACAGCTGCGAACTGATGATG	
hstA-F	GATTTCCGGCTCTTGTGTGT	*hstA* ORF
hstA-G	GGTATTCCCGATTTTCGGTC	
hstB-A	CTTTGCTTTGAGTTCCTGCC	
hstB-B	CTGACCTATCTTCCTGGACATGACGGTCTAACCTGGCGGAGAGAAA	5'flanking region of *hstB*
hstB-C	CTGTGGACTCGTATGCTGTTGCGCAGTACCGCGAAAAGGAGAGAGA	3'flanking region of *hstB*
hstB-D	AACAGTCGGCGATGTATTCC	
hstB-F	CTTTTTCAGGGAGAATCCGC	*hstB* ORF
hstB-G	CGCTCCATGTTAATGAGCAC	
hstC-A	AGTCATGGAAAAGACTGCGG	
hstC-B	CTGACCTATCTTCCTGGACATGACGGATTGGACTCAGCCTGATTGG	5'flanking region of *hstC*
hstC-C	CTGTGGACTCGTATGCTGTTGCGCAGAGTGCCCGAATAGGTTTCTG	3'flanking region of *hstC*
hstC-D	AGCCATCGCTGTCAGTTTCT	
hstC-F	GCCTTGCTGGCTAAGAAGAA	*hstC* ORF
hstC-G	TCACACGACCCAAGGATACA	
hstD-A	TGCGGAAATGGGTTGTTT	
hstD-B	CTGACCTATCTTCCTGGACATGACGGATGGCACTTGTCGCATGTC	5'flanking region of *hstD*
hstD-C	CTGTGGACTCGTATGCTGTTGCGCAGGGCGTGGTGTAATTCTTCGT	3'flanking region of *hstD*
hstD-D	TAACCGTGACATGACCCTTG	
hstD-A2	TGAAAGGATTACCTCCTCCC	fusion PCR for *hstD*
hstD-D2	ACGTCCGGGATATTATGGGT	
hstD-F	ACCATCAAGTCCCAGCAATC	*hstD* ORF
hstD-G	AGACATCCATGCCTCCCTTA	

hstE-A	TCCATCTGATAAGGTTCGGC	5'flanking region of *hstE*
hstE-B	CTGACCTATCTTCCTGGACATGACGGAGATAATGGGTACGGCGAGA	
hstE-C	CTGTGGACTCGTATGCTGTTGCGCAGGGCGCTGTCCGAATGTATTA	3'flanking region of *hstE*
hstE-D	CCAGTGTACAATTCCGCCAT	
hstE-A2	ATAAGGTTCGGCATGAGTGG	fusion PCR for *hstE*
hstE-D2	GCCATGATACATCCAGCAGA	
hstE-F	AATATCTGGGGCAGACGAGA	*hstE* ORF
hstE-G	GGCACATTCTCAAGAGCACA	
hstF-A	ATTGACGAACCCTTGGACTG	5'flanking region of *hstF*
hstF-B	CTGACCTATCTTCCTGGACATGACGGGCGGGGTGTTGATAATGACT	
hstF-C	CTGTGGACTCGTATGCTGTTGCGCAGGGTATTATGGGGATTGTCGG	3'flanking region of *hstF*
hstF-D	TCTTCGTCTTCAAAGGCTCC	
hstF-A2	CGTCAGCTCACGGATTATGA	fusion PCR for *hstF*
hstF-D2	TCTCGTTTGCCTTAGCTGTG	
hstF-F	AGACATATGGCGCTGAAAGC	*hstF* ORF
hstF-G	GCAATGTGGAAGGACATCAG	
Fusion-hstD-F	tcgagctcggtacccATGGTGCGGTCGCTGTCCGAAGAGG	vector construction for *pUShstD*
Fusion-hstD-R	ctctagaggatccccTCATGCGGCCGGCTGATTTAACAAC	
Fusion-laeA-F	tcgagctcggtacccATGTTTGGAAACGGCCAGACTGGAC	vector construction for *pUSlaeA*
Fusion-laeA-R	ctctagaggatccccTCAGTTCGCAGGTTTCCGTGCTTGG	
laeA-A	CCGGCTGTTCAAGATCCATGGATAG	5'flanking region of *laeA*
laeA(adeA)-B	CTGACCTATCTTCCTGGACATGACGGTCGATGGCGACAGGCTGATG	
laeA(adeA)-C	CTGTGGACTCGTATGCTGTTGCGCAGAGAGCTCCATTACTGGGTATTCGG	3'flanking region of *laeA*
laeA-D	GAACCCGCCAACATCAAGCTTC	
laeA-A2	GGGATACCAACCACAACACCT	fusion PCR for *laeA*
laeA-D2	TTACGTTTGGGAACGGAGTCA	
laeA-F	CAGCCCTCAAACCACCCAAA	*laeA* ORF
laeA-G	TTGAACGCCTCCGACTTGAC	
laeA(sC)-B	GAACGAGACGAACGAGGAGCCATATTCGATGGCGACAGGCTGATG	disruption casette for *laeA* in *Δ hstD* background
laeA(sC)-C	CATACGGGCAGCTATTGCCAAGAGAAGAGCTCCATTACTGGGTATTCGG	
sC-F	ATATGGCTCCTCGTTCGTCTCGTTC	*sC* amplification
sC-R	TCTCTTGGCAATAGCTGCCCGTATG	

P-amyB-F	GGCAACTCGCTTACCGATTAC	confirmation of transformation of over expression construct
hstD-comp-B	GAACGAGACGAACGAGGAGCCATATTAACCGTGACATGACCCTTG	
adeA fusion sC-F	CATACGGGCAGCTATTGCCAAGAGACCGTCATGTCCAGGAAGATAGGTCA	Complementation of *hstD*
nested adeA-R	GCCTTGGTCTGGGAGTGT	
kojA-F	GGTTTCCAGGGCCTCATCAG	probe for *kojA*
kojA-R	GAGAAATCCGGGCCAGAACC	
kojR-F	CGGCCAGCTATGACCCCATT	probe for *kojR*
kojR-R	GGCGTCATGGGAGAGTGTGA	
kojT-F	CGAGGTGTCTCTTGCAAACC	probe for *kojT*
kojT-R	GTTCTGGGATAGGCGAACCA	
ipnA-F	CACCTACTCACGAGGTCAAC	probe for *ipnA*
ipnA-R	GTTGACCTCGTGAGTAGGTG	
laeA-F	AGCCCTCAAACCACCCAAAG	probe for *laeA*
laeA-R	TTGAACGCCTCCGACTTGAC	

[a] Additional nucleotides for fusion PCR are indicated by underlines

[b] Additional nucleotides for In-Fusion reaction are indicated in small letters

Table S4. Significantly changed genes in *ΔhstD*

Gene ID	Description	Fold change (*ΔhstD* / Control)	FunCat categorization[a,b] level 1	FunCat categorization[a,b] level 2
AO080553000121	KojT, putative transporter; present in the kojic acid biosynthetic gene cluster	105.5	[20][32]	[20.01][20.03][20.09][32.05][32.07]
AO080550000061	Hydroxyindole-O-methyltransferase and related SAM-dependent methyltransferases	102.5	[01]	[01.05][01.20]
AO080501000196	fleA fucose-specific lectin	61.82	#	#
AO080563000007	CpaD, O-dimethylallyltransferase (DMAT); dimethylallylates cAATrp to form beta-cyclopiazonic acid	49.39	#	#
AO080554000441	Uncharacterized membrane protein, predicted efflux pump	38.39	[20][32]	[20.01][32.07]
AO080563000004	Predicted protein	32.27	[20][32][34]	[20.01][20.03][20.09][32.05][32.07][34.11]
AO080525000393	Predicted protein	29.96	[01][11]	[01.02][11.02]
AO080563000005	Cytochrome P450 CYP3 CYP5 CYP6 CYP9 subfamilies	27.59	[01]	[01.06][01.20]
AO080563000006	Predicted protein	25.97	#	#
AO080508000254	Predicted protein	24.93	[01]	[01.05]
AO080511000457	Predicted protein	21.4	#	#
AO080522000026	Catalase (peroxidase I)	20.19	[32]	[32.07]
AO080536000097	Predicted protein	19.6	#	#
AO080542000015	Predicted protein	19.4	[32]	[32.05]
AO080515000039	Predicted protein	17.93	#	#
AO080550000056	Proteins containing the FAD binding domain	14.42	[01]	[01.05][01.20]
AO080508000512	mleA lectin	12.87	#	#
AO080515000015	Uncharacterized protein, possibly involved in utilization of glycolate and propanediol	12.31	#	#
AO080513000111	Predicted protein	11.85	#	#
AO080529000071	Predicted protein	10.43	#	#
AO080508000290	Predicted protein	10.11	#	#
AO080523000388	Predicted protein	9.984	#	#
AO080554000006	Predicted protein	9.951	#	#
AO080550000062	Dehydrogenases (flavoproteins)	9.86	#	#
AO080525000250	Predicted protein	9.747	#	#
AO080553000119	KojA, FAD-dependent oxidoreductase; present in the kojic acid biosynthetic gene cluster	9.711	#	#
AO080541000467	Predicted protein	9.668	#	#
AO080550000076	Predicted protein	9.323	#	#
AO080539000068	Predicted protein	9.028	#	#
AO080510000132	Predicted protein	8.772	#	#
AO080529000068	Predicted protein	8.053	#	#
AO080554000051	Predicted protein	7.73	#	#
AO080550000193	RNA 3 -terminal phosphate cyclase	7.67	#	#
AO080551000140	Polyketide synthase modules and related proteins	7.028	[01][32]	[01.20][32.05]
AO080511000259	Predicted protein	6.937	#	#
AO080523000239	TPR repeat	6.617	#	#
AO080537000053	Predicted hydrolases or acyltransferases (alpha beta hydrolase superfamily)	6.315	#	#
AO080527000194	Predicted xylanase chitin deacetylase	6.251	[01][16]	[01.05][01.25][16.05]
AO080551000190	Beta-lactamase class C and other penicillin binding proteins	6.175	#	#
AO080508000358	Predicted protein	5.923	#	#
AO080515000064	Multicopper oxidases	5.855	[01][32]	[01.05][01.07][01.25][32.07]
AO080527000284	Predicted protein	5.77	#	#

AO080549000421	Predicted protein	5.727	#	#
AO080550000107	Predicted protein	5.723	#	#
AO080523000444	Predicted protein	5.71	#	#
AO080567000074	Predicted protein	5.689	#	#
AO080541000077	Predicted protein	5.673	#	#
AO080523000389	Cytochrome P450 CYP4 CYP19 CYP26 subfamilies	5.589	[32]	[32.07]
AO080521000213	Predicted protein	5.575	#	#
AO080547000015	Predicted protein	5.394	#	#
AO080523000405	Predicted protein	5.171	[32][42]	[32.01][42.01][42.04]
AO080527000412	Predicted protein	5.107	#	#
AO080525000701	Alkaline phosphatase	5.07	[01]	[01.04][01.06][01.07]
AO080523000550	Predicted protein	4.932	#	#
AO080562000080	aflJ Predicted protein	4.922	#	#
AO080554000398	Predicted protein	4.746	#	#
AO080525000625	Predicted protein	4.693	#	#
AO080541000440	Predicted protein	4.676	#	#
AO080530000095	Predicted protein	4.602	#	#
AO080554000103	Cytochrome P450 CYP3 CYP5 CYP6 CYP9 subfamilies	4.597	[01][20][32]	[01.01][01.02][01.06][01.20][20.01] [32.07]
AO080547000014	Ankyrin	4.512	#	#
AO080549000406	Predicted dehydrogenases and related proteins	4.472	[01][11]	[01.05][11.02]
AO080508000357	Chitin synthase hyaluronan synthase (glycosyltransferases)	4.45	#	#
AO080523000006	Predicted protein	4.401	#	#
AO080525000059	Cytochrome P450 CYP2 subfamily	4.37	[01][16][20][32]	[01.06][01.07][01.20][16.17][16.21] [20.01][32.07]
AO080523000500	Predicted protein	4.32	#	#
AO080527000186	Oxidosqualene-lanosterol cyclase and related proteins	4.272	[01]	[01.06][01.20]
AO080523000718	Carboxylesterase type B	4.266	#	#
AO080529000067	Cytochrome P450 CYP11 CYP12 CYP24 CYP27 subfamilies	4.247	[01][32]	[01.01][01.20][32.07]
AO080515000065	Predicted protein	4.223	#	#
AO080532000090	Predicted protein	4.183	#	#
AO080508000361	Predicted protein	4.159	#	#
AO080551000170	Predicted protein	4.114	[01][16]	[01.01][01.20][16.17]
AO080525000706	Predicted protein	4.098	#	#
AO080532000546	Cytochrome P450 CYP2 subfamily	4.046	[01][16][20]	[01.20][16.17][16.21][20.01]
AO080508000305	Vesicular amine transporter	4.008	#	#
AO080527000377	Glucose dehydrogenase choline dehydrogenase mandelonitrile lyase (GMC oxidoreductase family)	4.008	[01][16][20]	[01.01][01.05][01.20][16.21][20.01]
AO080531000104	Predicted protein	3.951	#	#
AO080525000269	Predicted protein	3.848	#	#
AO080557000082	Cofilin; actin depolymerisation factor	3.847	#	#
AO080532000337	Chaperone-dependent E3 ubiquitin protein ligase (contains TPR repeats)	3.838	#	#
AO080523000512	Predicted protein	3.774	#	#
AO080523000573	Predicted protein	3.77	#	#
AO080536000086	ATPases of the AAA^{+} class	3.719	#	#
AO080515000151	Predicted protein	3.707	[43]	[43.01]
AO080525000687	Predicted transporter (major facilitator superfamily)	3.648	#	#
AO080557000045	Predicted protein	3.624	[32]	[32.05]

AO080515000071	Predicted protein	3.604	#	#
AO080501000080	Predicted protein	3.559	#	#
AO080566000093	Ankyrin	3.555	#	#
AO080525000026	SAM-dependent methyltransferases	3.536	#	#
AO080515000098	Predicted protein	3.503	#	#
AO080554000362	Predicted protein	3.493	#	#
AO080503000171	Phosphoenolpyruvate carboxykinase (ATP)	3.468	[01][02]	[01.04][01.05][02.01]
AO080523000572	Predicted protein	3.423	#	#
AO080533000376	Multidrug resistance-associated protein mitoxantrone resistance protein, ABC superfamily	3.391	[01][16][20][32]	[01.04][16.19][20.03][32.07]
AO080508000531	Predicted protein	3.39	#	#
AO080549000304	Carboxylesterase type B	3.38	[01]	[01.06]
AO080506000286	Predicted protein	3.315	#	#
AO080523000216	Predicted protein	3.303	#	#
AO080538000066	Acyl-CoA synthetase	3.298	[01]	[01.01][01.05][01.20]
AO080523000032	Amino acid transporters	3.296	[01][20][32][34]	[01.06][20.01][20.03][20.09[32.01] [34.01][34.11]
AO080510000108	ATPases of the AAA^{+} class	3.199	#	#
AO080513000093	Jacalin-like lectin domain-containing protein	3.158	#	#
AO080525000640	Predicted protein	3.156	#	#
AO080527000200	Chitinase	3.085	[01]	[01.05][01.25]
AO080553000120	KojR, Zn(II)2Cys6 transcription factor; induced by kojic acid; present in the kojic acid biosynthetic gene cl	3.083	[11][34]	[11.02][34.11]
AO080525000062	Acetylcholinesterase Butyrylcholinesterase	3.076	[01][32]	[01.05][01.06][01.20][01.25][32.05] [32.07][32.10]
AO080541000359	Integral membrane ankyrin-repeat protein Kidins220 (protein kinase D substrate)	3.076	#	#
AO080541000097	Predicted transporter (major facilitator superfamily)	3.063	[20][32]	[20.01][32.07]
AO080546000223	Predicted protein	3.039	#	#
AO080542000014	Cytochrome P450 CYP4 CYP19 CYP26 subfamilies	3.037	[01][32]	[01.01][32.05][32.07]
AO080539000029	Predicted protein	3.016	[11]	[11.02]
AO080547000070	Predicted protein	2.985	#	#
AO080523000242	Predicted protein	2.977	#	#
AO080502000014	Uncharacterized conserved protein	2.976	#	#
AO080503000338	NAD^{+} ADP-ribosyltransferase Parp, required for poly-ADP ribosylation of nuclear proteins	2.959	[01][10][14][16]	[01.05][10.01][14.07][16.01][16.03]
AO080506000124	Predicted protein	2.951	#	#
AO080542000029	Conserved protein domain typically associated with flavoprotein oxygenases, DIM6 NTAB family	2.943	#	#
AO080521000317	Predicted protein	2.904	#	#
AO080536000058	Predicted protein	2.901	#	#
AO080518000104	Predicted protein	2.888	#	#
AO080551000149	Predicted protein	2.88	#	#
AO080550000101	Predicted protein	2.853	#	#
AO080501000088	3-polyprenyl-4-hydroxybenzoate decarboxylase and related decarboxylases	2.843	#	#
AO080509000188	Predicted aminoglycoside phosphotransferase	2.835	#	#
AO080554000077	Monodehydroascorbate ferredoxin reductase	2.835	[02]	[02.11]
AO080536000060	Predicted protein	2.826	#	#
AO080506000051	Predicted short chain-type dehydrogenase	2.821	[01][32]	[01.05][01.20][32.05]
AO080549000353	ATP adenylyltransferase (5 ,5 -P-1, P-4-tetraphosphate phosphorylase II)	2.812	[01][11][16][20]	[01.04][01.06][11.04][16.17][16.19] [20.01][20.03][20.09]

AO080523000207	Predicted protein	2.766	#	#
AO080536000143	Tellurite resistance protein and related permeases	2.743	#	#
AO080505000118	Predicted protein	2.726	#	#
AO080546000372	Ornithine aminotransferase	2.705	[01][16]	[01.01][01.02][01.20][16.21]
AO080505000202	Methionyl-tRNA formyltransferase	2.701	#	#
AO080522000033	Predicted protein	2.694	#	#
AO080509000132	Predicted protein	2.686	#	#
AO080546000363	Predicted protein	2.676	#	#
AO080534000002	Non-ribosomal peptide synthetase modules and related proteins	2.663	#	#
AO080561000015	Predicted protein	2.659	#	#
AO080532000416	Threonine dehydrogenase and related Zn-dependent dehydrogenases	2.657	[01][02][16]	[01.01][01.05][02.16][16.17]
AO080515000061	Uncharacterized conserved protein	2.644	#	#
AO080503000025	Predicted protein	2.631	#	#
AO080525000209	Dihydrolipoamide succinyltransferase (2-oxoglutarate dehydrogenase, E2 subunit)	2.617	#	#
AO080541000475	Glutathione S-transferase	2.593	[32][40]	[32.01][32.07][40.10]
AO080512000001	Transposon-encoded proteins with TYA, reverse transcriptase, integrase domains in various combinations	2.561	#	#
AO080523000247	Predicted acyl-CoA transferases carnitine dehydratase	2.553	#	#
AO080508000270	RNA polymerase II transcription termination factor TTF2 lodestar, DEAD-box superfamily	2.541	#	#
AO080523000391	Cytochrome P450 CYP3 CYP5 CYP6 CYP9 subfamilies	2.519	[16][32]	[16.21][32.05][32.07]
AO080527000504	Predicted protein	2.503	#	#
AO080525000318	Predicted protein	2.501	#	#
AO080566000091	Predicted protein	2.493	#	#
AO080503000216	Predicted protein	2.492	#	#
AO080523000079	Beta-lactamase class C and other penicillin binding proteins	2.491	#	#
AO080532000061	Uncharacterized conserved protein	2.483	[20]	[20.01][20.03][20.09]
AO080505000203	Nucleoside-diphosphate-sugar epimerases	2.467	[01][11][42]	[01.05][11.02][42.10]
AO080513000020	Predicted protein	2.459	#	#
AO080505000159	Alcohol dehydrogenase, class V	2.446	[01][02][16][42]	[01.05][01.20][02.01][02.16][16.17][42.01]
AO080546000050	Protein tyrosine serine phosphatase	2.442	[14]	[14.07]
AO080531000027	Dimethylglycine dehydrogenase precursor	2.426	[01][16][20]	[01.01][01.02][01.05][16.21][20.01]
AO080501000014	wykB, FAD-dependent oxidoreductase	2.412	[14]	[14.07]
AO080567000034	Predicted protein	2.409	#	#
AO080536000139	7-keto-8-aminopelargonate synthetase and related enzymes	2.406	#	#
AO080523000390	Cytochrome P450 CYP4 CYP19 CYP26 subfamilies	2.401	[01]	[01.01]
AO080563000003	Synaptic vesicle transporter SVOP and related transporters (major facilitator superfamily)	2.394	[20][32][34]	[20.01][20.03][20.09][32.05][32.07][34.11]
AO080520000010	Reverse transcriptase	2.393	#	#
AO080527000001	Predicted protein	2.392	#	#
AO080546000314	Predicted protein	2.368	#	#
AO080537000005	Predicted flavin-nucleotide-binding protein structurally related to pyridoxine 5 -phosphate oxidase	2.363	#	#
AO080568000203	Predicted protein	2.359	#	#
AO080521000212	Predicted protein	2.35	#	#
AO080515000262	ATP-dependent RNA helicase	2.337	#	#
AO080506000125	ADP-ribose pyrophosphatase	2.331	[01][16][32]	[01.03][16.17][32.01]
AO080548000022	Zn-finger	2.326	#	#
AO080533000254	Inositol monophosphatase	2.32	[01][30]	[01.04][01.05][01.06][01.20][30.01]

AO080554000301	Predicted protein	2.319	#	#
AO080509000068	Predicted protein	2.315	#	#
AO080521000046	Putative dehydrogenase domain of multifunctional non-ribosomal peptide synthetases and related enzymes	2.299	[01][20][34]	[01.20][20.01][34.01]
AO080561000093	Predicted protein	2.298	#	#
AO080503000246	Predicted transporter (major facilitator superfamily)	2.293	[01][20][34]	[01.05][20.01][20.03][20.09][34.01] [34.11]
AO080523000538	Predicted glutamine synthetase	2.287	#	#
AO080528000042	Permease of the major facilitator superfamily	2.284	[20]	[20.01][20.03][20.09]
AO080523000723	Acetyltransferases, including N-acetylases of ribosomal proteins	2.282	#	#
AO080570000066	Permease of the major facilitator superfamily	2.263	[20]	[20.01][20.03][20.09]
AO080569000133	Predicted protein	2.255	[10][11][42]	[10.03][11.04][11.06][42.16]
AO080523000499	Predicted protein	2.238	#	#
AO080527000070	Lactoylglutathione lyase and related lyases	2.231	#	#
AO080553000127	Predicted protein	2.231	#	#
AO080567000088	Predicted protein	2.219	#	#
AO080551000005	Spt6 ortholog, DNA-binding subunit of a DNA-dependent protein kinase (Ku70 autoantigen)	2.214	[10][11]	[10.01][11.02]
AO080515000013	Predicted protein	2.212	#	#
AO080537000052	Predicted protein	2.212	#	#
AO080550000146	Exopolyphosphatase	2.207	[01][11][30]	[01.05][11.02][30.01]
AO080527000050	Predicted protein	2.19	#	#
AO080511000186	Predicted protein	2.175	#	#
AO080509000038	Predicted protein	2.172	[43]	[43.01]
AO080515000014	Predicted protein	2.168	#	#
AO080532000091	Cysteine desulfurase NFS1	2.162	[01]	[01.20]
AO080511000049	Predicted protein	2.161	#	#
AO080511000294	Predicted protein	2.16	#	#
AO080523000130	Predicted protein	2.157	#	#
AO080536000059	Predicted protein	2.152	#	#
AO080508000090	Predicted protein	2.151	#	#
AO080527000162	Predicted protein	2.147	[30][34][43]	[30.01][34.11][43.01]
AO080501000155	Predicted transporter (major facilitator superfamily)	2.145	[01][20]	[01.05][20.01][20.03][20.09]
AO080505000279	Predicted protein	2.142	#	#
AO080508000097	3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthase	2.139	[01]	[01.01][01.05][01.20]
AO080523000024	Glycerol uptake facilitator and related permeases (Major Intrinsic Protein Family)	2.129	#	#
AO080501000008	wykN, Non-ribosomal peptide synthetase modules and related proteins	2.117	#	#
AO080539000074	Predicted protein	2.097	#	#
AO080518000037	Predicted transporter (major facilitator superfamily)	2.073	[20][32]	[20.01][20.03][20.09][32.05][32.07]
AO080541000154	Carboxypeptidase C (cathepsin A)	2.062	[14]	[14.13]
AO080567000053	Predicted protein	2.062	#	#
AO080550000102	Predicted acyl esterases	2.055	#	#
AO080505000097	Transthyretin and related proteins	2.053	[01]	[01.02][01.03]
AO080546000347	Catalase (peroxidase I)	2.051	[20][32][42]	[20.01][32.01][32.07][42.16]
AO080533000188	Predicted protein	2.046	#	#
AO080551000123	Predicted protein	2.039	#	#
AO080562000073	Predicted protein	2.036	#	#
AO080551000142	Predicted protein	2.034	#	#
AO080532000609	Predicted protein	2.032	#	#

AO080527000203	Isocitrate isopropylmalate dehydrogenase	2.021	[01][02][16][20]	[01.01][01.05][02.01][02.10][16.21][20.01]
AO080525000648	Predicted protein	2.02	#	#
AO080571000012	Molecular chaperone (DnaJ superfamily)	2.014	#	#
AO080523000419	Predicted protein	2.007	#	#
AO080531000318	2-polyprenyl-6-methoxyphenol hydroxylase and related FAD-dependent oxidoreductases	2.004	[01]	[01.05][01.20]
AO080504000029	Phospholipase C	2.002	#	#
AO080533000108	Protein kinase PCTAIRE and related kinases	2.001	[01][02][10][11][14][16][18][30][34][40]	[01.04][01.05][02.19][10.01][10.03][11.02][14.07][16.01][16.03][16.19][18.01][18.02][30.01][30.05][34.05][34.11][40.01][42.10][43.01]
AO080505000181	Glycosyltransferase	0.498	[01][02][32][34][43	[01.05][02.19][32.01][34.11][43.01]
AO080503000128	Predicted protein	0.496	#	#
AO080521000172	Predicted membrane protein	0.496	#	#
AO080514000015	Transcription factor PRD and related proteins, contain PAX and HOX domains	0.495	#	#
AO080531000179	Predicted protein	0.494	#	#
AO080503000252	Predicted protein	0.492	#	#
AO080546000219	Predicted protein	0.492	#	#
AO080560000080	Predicted protein	0.492	#	#
AO080513000270	Predicted protein	0.491	#	#
AO080508000004	Predicted protein	0.487	#	#
AO080503000123	Predicted protein	0.486	#	#
AO080513000060	Alpha-amylase	0.485	[01]	[01.05]
AO080532000307	Predicted protein	0.481	#	#
AO080554000482	NADPH:quinone reductase and related Zn-dependent oxidoreductases	0.481	[01][16]	[01.05][01.20][16.17]
AO080523000408	Dehydrogenases with different specificities (related to short-chain alcohol dehydrogenases)	0.48	#	#
AO080550000156	Predicted protein	0.479	#	#
AO080513000204	Predicted protein	0.478	#	#
AO080560000087	Predicted protein	0.478	#	#
AO080505000104	Predicted protein	0.476	#	#
AO080568000096	Cytochrome P450 CYP4 CYP19 CYP26 subfamilies	0.474	[01][02]	[01.06][01.20][02.45]
AO080523000182	Acetylcholinesterase Butyrylcholinesterase	0.47	#	#
AO080532000189	Predicted protein	0.469	[11][14]	[11.06][14.07][14.13]
AO080570000072	Cytochrome P450 CYP4 CYP19 CYP26 subfamilies	0.469	[01][32]	[01.01][32.05][32.07]
AO080561000074	Endopolygalacturonase	0.468	#	#
AO080511000067	Predicted protein	0.466	#	#
AO080533000118	Predicted protein	0.464	#	#
AO080508000310	Ketopantoate hydroxymethyltransferase	0.463	[01][42]	[01.07][42.01]
AO080523000640	Sorbin and SH3 domain-containing protein	0.463	[10]	[10.03]
AO080536000108	Predicted protein	0.459	#	#
AO080508000430	Predicted protein	0.457	#	#
AO080521000200	Predicted protein	0.456	[20][34]	[20.01][34.11]
AO080522000004	Cytochrome P450	0.456	#	#
AO080506000272	Predicted protein	0.455	#	#
AO080557000027	Predicted protein	0.455	#	#
AO080555000231	Predicted protein	0.454	#	#
AO080523000128	Predicted protein	0.453	#	#

AO080533000173	Predicted protein	0.453	#	#
AO080508000082	Predicted protein	0.451	#	#
AO080515000125	Permeases of the major facilitator superfamily	0.449	[20]	[20.01][20.03][20.09]
AO080542000184	Cytochrome P450 CYP4 CYP19 CYP26 subfamilies	0.448	#	#
AO080533000250	Predicted protein	0.447	#	#
AO080549000362	Endonuclease III	0.447	[01][10][16][32]	[01.03][10.01][16.03][16.17][16.21][32.01]
AO080523000306	Predicted protein	0.442	#	#
AO080532000098	beta-1,6-N-acetylglucosaminyltransferase, contains WSC domain	0.442	#	#
AO080536000028	Predicted protein	0.442	#	#
AO080550000149	Predicted protein	0.441	#	#
AO080513000148	Amidases	0.44	[01]	[01.02]
AO080508000396	Acetylornithine aminotransferase	0.438	[01]	[01.07][01.20]
AO080501000092	Nucleoside phosphorylase	0.437	#	#
AO080521000357	Predicted protein	0.437	#	#
AO080536000070	Predicted transporter (major facilitator superfamily)	0.437	[01][20][32][34]	[01.07][20.01][20.09][32.07][34.01]
AO080532000519	Predicted protein	0.435	#	#
AO080503000049	Serine threonine protein kinase	0.434	#	#
AO080508000367	Predicted protein	0.433	[01]	[01.04]
AO080541000303	Predicted protein	0.433	#	#
AO080525000618	Predicted protein	0.432	#	#
AO080529000044	Predicted Zn-dependent hydrolases of the beta-lactamase fold	0.432	#	#
AO080550000111	Predicted protein	0.432	#	#
AO080508000204	Predicted dehydrogenase	0.431	#	#
AO080523000678	Predicted protein	0.431	[10][16]	[10.01][16.03]
AO080515000221	Aspartyl protease	0.43	[01][14]	[01.25][14.13]
AO080521000254	1-Acyl dihydroxyacetone phosphate reductase and related dehydrogenases	0.427	[01][43]	[01.05][01.06][43.01]
AO080523000411	Predicted protein	0.426	#	#
AO080541000075	Predicted protein	0.426	#	#
AO080501000186	Predicted protein	0.424	#	#
AO080538000052	Uncharacterized conserved protein	0.419	#	#
AO080508000165	FAD FMN-containing dehydrogenases	0.418	[01]	[01.20]
AO080501000074	nucS nuclease S1 precursor	0.416	[01]	[01.03]
AO080523000712	Permeases of the major facilitator superfamily	0.416	[01][20]	[01.05][20.01][20.03]
AO080530000037	Signal transduction histidine kinase	0.413	#	#
AO080515000068	Carboxylesterase and related proteins	0.412	#	#
AO080515000315	Fructose tagatose bisphosphate aldolase	0.412	[01][02]	[01.05][02.01][02.07]
AO080539000047	Predicted protein	0.412	#	#
AO080558000032	Permeases of the major facilitator superfamily	0.41	#	#
AO080550000154	Amino acid transporters	0.409	#	#
AO080531000003	SAM-dependent methyltransferases	0.402	#	#
AO080502000037	NADPH:quinone reductase and related Zn-dependent oxidoreductases	0.399	#	#
AO080523000067	Predicted metal-dependent hydrolase with the TIM-barrel fold	0.399	#	#
AO080525000576	Predicted protein	0.398	#	#
AO080515000209	Uncharacterized protein conserved in bacteria	0.395	#	#
AO080553000093	Predicted protein	0.394	#	#
AO080549000130	Predicted transporter (major facilitator superfamily)	0.393	[01][20][34]	[01.05][20.01][20.03][20.09][34.01]

AO080530000032	Predicted protein	0.385	#	#
AO080533000073	Predicted protein	0.379	#	#
AO080503000122	Predicted protein	0.371	#	#
AO080513000198	Cytochrome P450 CYP2 subfamily	0.369	#	#
AO080521000073	Aldo keto reductase family proteins	0.369	[01][02][16][32][34]	[01.01][01.05][01.06][01.07][01.20][02.01][02.16][16.21][32.01][32.10][34.11]
AO080521000107	Flavonol reductase cinnamoyl-CoA reductase	0.369	[01][02][32][42]	[01.05][01.07][01.20][02.01][32.01][32.07][42.01]
AO080567000015	Predicted protein	0.369	#	#
AO080505000144	Predicted protein	0.368	#	#
AO080503000326	Predicted protein	0.367	#	#
AO080508000394	Predicted protein	0.366	#	#
AO080515000088	Dehydrogenases with different specificities (related to short-chain alcohol dehydrogenases)	0.366	[01][02][43]	[01.05][01.06][01.20][02.25][43.01]
AO080508000236	Predicted protein	0.363	#	#
AO080531000178	Predicted protein	0.362	#	#
AO080522000018	Fatty acid desaturase	0.356	[01]	[01.06]
AO080533000203	Cytochrome P450 CYP3 CYP5 CYP6 CYP9 subfamilies	0.355	[01][20][32]	[01.06][01.20][20.01][32.05][32.07]
AO080508000166	Synaptic vesicle transporter SVOP and related transporters (major facilitator superfamily)	0.354	[20]	[20.01]
AO080541000328	1-aminocyclopropane-1-carboxylate synthase, and related proteins	0.353	[01][36][40]	[01.02][01.05][01.20][36.02][40.02]
AO080610000005	Predicted protein	0.343	#	#
AO080502000006	Endo-1,4-beta-glucanase IV	0.34	[01]	[01.05][01.25]
AO080554000005	Predicted protein	0.338	#	#
AO080539000025	Predicted protein	0.332	[10][14][20][32][42]	[10.01][10.03][14.07][20.01][32.01][32.07][42.25]
AO080515000066	Permease of the major facilitator superfamily	0.331	[20]	[20.01][20.03][20.09]
AO080523000164	Predicted protein	0.328	[32]	[32.07]
AO080525000600	Mg^{2+} and Co^{2+} transporters	0.325	#	#
AO080530000035	Predicted protein	0.325	#	#
AO080536000027	manD, Endo-beta-mannanase	0.319	[01]	[01.05]
AO080541000084	Predicted protein	0.316	[01]	[01.05]
AO080521000262	Predicted protein	0.314	#	#
AO080525000417	Predicted protein	0.314	#	#
AO080551000032	Predicted protein	0.314	#	#
AO080541000430	Dehydrogenases with different specificities (related to short-chain alcohol dehydrogenases)	0.309	#	#
AO080503000237	Predicted protein	0.308	#	#
AO080525000729	Probable taurine catabolism dioxygenase	0.308	[01]	[01.02]
AO080521000084	2,4-dihydroxyhept-2-ene-1,7-dioic acid aldolase	0.302	[01]	[01.20]
AO080513000199	Predicted protein	0.299	#	#
AO080523000583	Predicted protein	0.299	#	#
AO080523000582	Predicted transporter (major facilitator superfamily)	0.289	[01][20][34][41]	[01.05][20.01][20.03][20.09][34.01][34.11][41.01]
AO080523000410	Predicted protein	0.288	#	#
AO080528000012	Predicted protein	0.283	#	#
AO080532000211	Ca^{2+} Na^{+} antiporter	0.28	#	#
AO080503000406	Dehydrogenases with different specificities (related to short-chain alcohol dehydrogenases)	0.276	[01][16]	[01.05][01.20][16.21]
AO080513000058	Predicted protein	0.275	#	#

AO080562000011	Amino acid transporters	0.274	[20]	[20.01][20.03][20.09]
AO080525000086	Predicted protein	0.271	[01]	[01.05][01.25]
AO080508000345	Dehydrogenases with different specificities (related to short-chain alcohol dehydrogenases)	0.269	[01][02][10][16][20][30][32][34]	[01.05][01.06][01.07][01.20][02.07][10.03][16.01][16.21][20.01][30.05][32.05][34.01]
AO080549000389	Sorbitol dehydrogenase	0.269	[01][02][16]	[01.01][01.05][01.07][01.20][02.16][16.17][16.21]
AO080536000095	Predicted protein	0.266	#	#
AO080532000017	Phenylpropionate dioxygenase and related ring-hydroxylating dioxygenases, large terminal subunit	0.263	#	#
AO080554000001	H^+ oligopeptide symporter	0.259	[20]	[20.01][20.03][20.09]
AO080532000023	Predicted protein	0.258	#	#
AO080525000588	1-aminocyclopropane-1-carboxylate synthase, and related proteins	0.251	[01]	[01.02][01.05][01.20]
AO080510000163	Predicted protein	0.244	#	#
AO080541000142	WD40 repeat	0.243	#	#
AO080513000202	Chitinase	0.24	#	#
AO080531000064	Predicted protein	0.24	#	#
AO080531000061	Predicted protein	0.238	#	#
AO080541000150	Predicted protein	0.228	#	#
AO080549000322	Protocatechuate 3,4-dioxygenase beta subunit	0.225	#	#
AO080525000018	Predicted protein	0.224	#	#
AO080532000465	Predicted protein	0.221	#	#
AO080532000197	Synaptic vesicle transporter SVOP and related transporters (major facilitator superfamily)	0.216	[20][32][34]	[20.01][20.03][20.09][32.05][32.07][34.11]
AO080530000022	Predicted protein	0.208	#	#
AO080523000427	Permease of the major facilitator superfamily	0.193	#	#
AO080513000203	Predicted protein	0.19	#	#
AO080521000112	Dehydrogenases with different specificities (related to short-chain alcohol dehydrogenases)	0.172	[01]	[01.05][01.06][01.20]
AO080501000148	Hydroxyindole-O-methyltransferase and related SAM-dependent methyltransferases	0.162	[01]	[01.05][01.20]
AO080501000144	Glutaminyl cyclase	0.155	[01][14]	[01.01][14.07][14.13]
AO080501000147	Predicted protein	0.151	#	#
AO080532000466	Predicted protein	0.142	#	#
AO080554000025	Amino acid transporters	0.14	[20]	[20.01][20.09]
AO080501000146	Predicted protein	0.136	#	#
AO080501000145	Predicted protein	0.135	#	#
AO080561000089	Endo-1,4-beta-xylanase G2	0.111	[01]	[01.05]
AO080536000049	Predicted protein	0.0778	#	#
AO080508000347	Dehydrogenases with different specificities (related to short-chain alcohol dehydrogenases)	0.0748	#	#
AO080522000002	Predicted protein	0.0479	#	#
AO080513000200	Predicted protein	0.0336	#	#
AO080501000141	Predicted protein	0.0322	#	#

[a] FunCat (http://www.webcitation.org/getfile?fileid=be4936ae25ebb5dfb89b687842ea640f8acf7790) is the organism independent functional description of proteins. FunCat consists of 28 main functional categories (level 1). The level 1 is the most general one, whereas level 2 shows much more detail.

[b] #: Unclassified gene

[c] Detailed descriptions of each category are available at the MIPS Functional Catalogue Database (http://mips.helmholtz-muenchen.de/proj/funcatDB/)

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Title: Functional analysis of histone deacetylase and its role in stress response, drug resistance and solid-state cultivation in *Aspergillus oryzae*

Running title: Function of histone deacetylase in *A. oryzae*

Moriyuki Kawauchi[1, 2] and Kazuhiro Iwashita[1, 2*]

Department of Molecular Biotechnology, Graduate School of Advanced Science of Matter, Hiroshima University, 1-3-1 Kagamiyama, Higashi-hiroshima, Hiroshima 739-8530, Japan[1] and National Research Institute of Brewing, 3-7-1 Kagamiyama, Higashi-hiroshima, Hiroshima 739-0046, Japan[2]

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*Corresponding author. E-mail: iwashitact@nrib.go.jp

Phone: +81-(0)82-420-0824, Fax: +81-(0)82-420-0808

Abstract

In the eukaryotic cell, histone deacetylases (HDACs) play key roles in the regulation of fundamental cellular process such as development regulation, stress response, secondary metabolism and genome integrity. Here, we provide a comprehensive phenotypic analysis using HDAC disruptants in *Aspergillus oryzae*. Our study revealed that four HDACs, *hdaA/Aohda1*, *hdaB/Aorpd3*, *hdaD/Aohos2* and *hst4/AohstD* were involved in stress response, cell wall synthesis and chromatin integrity in *A. oryzae*. Osmotic stress sensitivity of HDAC disruptants differed between plate cultures and liquid cultures, suggesting that HDACs adapt to the difference environmental conditions. Using a common *A.oryzae* fermentation medium, rice-*koji*, we also characterized HDACs related to growth and enzyme production to investigate which HDACs will be required for adaptation to environmental conditions and stress resistances. Because HDACs are widely conserved, our study has broad applications and may inform work with filamentous fungi and other eukaryote.

Introduction

Eukaryotic DNA is packaged into chromatin, limiting transcriptional activity (1). Histone acetylation is one of the most important modifications to regulate chromatin accessibility. It is controlled by two opposing enzymes, histone acetyltransferases (HATs) and histone deacetylases (HDACs), both of which were conserved in wide range of taxa. Deacetylated histones that are intermediated by HDACs lead to transcriptional inactivation (2-5). HDACs are divided into two major types, sirtuins and the classical HDACs. Sirtuins are NAD^+-dependent, while classical HDACs are zinc-dependent HDAC family (6). In the past decade, the importance of sirtuins and classical HDACs have been recognized as important to multiple cellular processes, including development, stress response and genome integrity (6).

The filamentous fungi are a diverse group with important economical applications, including the production of fermented foods, commercial enzymes and useful chemicals (7, 8). Others are human or plant pathogens and virulence has even been reported in some filamentous fungi (9-13). Recently, attention has been drawn to HDACs and their industrial applications as regulators of fungal development, conidiation, stress response and secondary metabolite production (12-16).

The filamentous fungus *Aspergillus oryzae* has been used for more than 1000 years in

52 the traditional food industry and is listed as a GRAS species (i.e., generally recognized
53 as safe) by the Food and Drug Administration in the United States. Its safety is also
54 confirmed by the World Health Organization (17). *A. oryzae* has the ability to produce
55 high quantities of enzymes and beneficial secondary metabolites, such as kojic acid and
56 WYK-1 (18-20).
57 In a previous study, we identified 11 HDACs homolog (AoHDACs) in *A. oryzae*
58 genome and attempt the disruption of all of them (21). We isolated 10 AoHDAC
59 disruptants, and one heterokaryon disruptant for *hdaB*/*Aorpd3* disruption. Using, these
60 disruptants, we revealed the effects of HDACs on the growth, conidiation, and
61 secondary metabolite production. In the present study, we more closely examine the
62 phenotypic expression of HDAC in *A. oryzae*, specifically in response to several types
63 of stress and drugs and its role in growth and production of protein in rice-*koji*.
64

Materials and methods

Strains and media

All *A. oryzae* strains used in this study were derived from previous work and are listed in Table 1 (21). N medium was used as the basic medium for all stress and drug-resistance analyses (21). For rice-*koji* making, 15g α-rice (70% polished *Akihikari*) was used. Distilled water suspending the conidia of disruptants was added to the α-rice as 30% initial water content and 1×10^5 conidia/g α-rice. The inoculum was incubated at 35 °C for 42h in 100% humidity.

Environmental stress resistance assay

For the stress test on plates, 1 μl of conidia suspension (1×10^5 conidia) of each strain was point inoculated on the center of the plate and grown for five days at 30°C. N medium containing 1.6 M NaCl was used for the osmotic stress resistance test and 20mM H_2O_2 was used for the oxidative stress resistance test. For the heat resistance test, the plate was incubated at 37°C. For hypoxic growth test, the plate was incubated in a 2% O_2 concentration using the Multi Gas Incubator APM-50DR (Astec, Fukuoka, Japan). After these incubations, Colony diameter was measured after the incubation period. For biomass analysis in the liquid culture, a 4 cm^2 plug of each strain were cut

from the full-growth plate cultures and homogenized in 1 ml of suspension solution and then inoculated with 100 ml of N liquid medium containing 0.8 M NaCl. Flasks were incubated for two days at 30°C with shaking at 100 rpm. Mycelia were harvested, dried at 105°C for 1 h and weighed.

Drug resistance test

For drug resistance tests, conidia suspensions of each strain were point inoculated an incubated as outlined in the stress test above. The following chemicals were tested: calcofluor white (300 μg/ml), Congo red (50 μg/ml), micafungin (2 ng/ml), hydroxy urea (10 mM), camptothecin (1 μM), methyl methane sulfonate (MMS; 0.1%), tunicamycin (5 μg/ml), nocodazole (500 ng/ml), dithiothreitol (10mM), brefeldin A (5 μg/ml). Congo red, dithiothreitol, camptothecin, MMS, brefeldin A and nocodazole were purchased from Wako Chemicals (Osaka, Japan); tunicamycin were purchased from Calbiochem (La Jolla, CA, USA); and the hydroxy urea and calcofluor white were purchased from Sigma Aldrich (St. Louis, MO, USA). The micafungin (Astellas, Tokyo, Japan) was gifted. A stock solution of was prepared by dissolving 100 mg/ml calcofluor white, 10mg/ml Congo red, 1mg/ml micafungin, 1M hydroxyl urea, and 1M dithiothreitol (1M) in water. The camptothecin (10 mM), nocodazole (1 mg/ml),

101 brefeldin A (1 mg/ml) and tunicamycin (2 mg/ml) were dissolved in dimethyl sulfoxide.

102

103 ***Measurement of enzyme activity and total proteins production***

104 Enzymes were extracted from 5 g of rice-*koji* after incubation with 25 ml acetate buffer

105 (10 mM, pH 5.0, 0.5% NaCl) at 4°C for 3 h with shaking at 80 rpm followed by

106 filtration. Enzyme activities of α-amylase, glucoamylase and acid carboxypeptidase

107 were measured using enzyme assay kits (Kikkoman, Chiba, Japan). Acidic protease

108 activity was assayed according to a previous report (22). Total protein was measured by

109 using the Bio-Rad Protein Assay Kit II.

110

111 ***Measurement of N-acetylglucosamine content in rice-koji***

112 The rice-*koji* (5g) was dried at 105°C for 1h, then homogenized in 12.5 ml phosphate

113 buffer (50 mM, pH 6.8) using physcotron (Microtec, Chiba, Japan),and centrifuged. The

114 pellet was washed with a 10-ml phosphate buffer more than five times then suspended

115 in a 20-ml phosphate buffer. A 2-ml of suspension was mixed with 7 ml phosphate

116 buffer and 1 ml Yatalase (TaKaRa, Kyoto, Japan) solution (10 mg /ml in phosphate

117 buffer). This mixture was shaking at 80rpm at 37°C for 3 h. The N-acetylglucosamine

118 composition was determined with a pulse high-performance anion-exchange

chromatography with a pulse electrochemical detector (DX500 chromatography system, Dionex, Sunnyvale, CA, USA) and an anion exchange column (Carbo PAC PA-1, 4×250 mm, Dionex) at a flow rate of 1 ml/min. Isocratic elution was performed with 16mM NaOH. The column was stabilized for 20min before injection and washed with 100mM NaOH / 600mM CH_3COONa for 10 min after elution. To quantify the N-acetylglucosamine, we used N-acetyl-D (+)-glucosamine (Wako) as a standard and D-fucose as an internal standard.

Results

Stress resistance of AoHDACs

In the previous study, we identified 11 HDACs homologs in *A. oryzae* genome and construct 10 AoHDAC disruptants and one AoHDAC heterokaryon disruptant as listed in Table 1 with the phylogenetic classification (21).

The osmotic, oxidative, heat and hypoxia stress tolerances of all AoHDAC disruptants were quantified to analyze the importance of HDACs in environmental adaptations (Figs. 1, S1 and S2). Three AoHDAC disruptants showed sensitivity against these stresses (Fig. 1A and B). The *ΔhstD* strain was sensitive to the osmotic stress; the *hdaB/Aorpd3* heterokaryon disruptant (*hdaB ht* strain) showed significant sensitivity against osmotic, oxidative and heat stress; the *ΔhdaD* strain showed significant sensitivity against the low oxygen and osmotic stress. These results suggest that *hdaB/Aorpd3*, *hdaD/Aohos2* and *hstD/Aohst4* are required for stress tolerance in *A. oryzae*.

In the liquid culture of the osmotic-stress test, we found a unique phenotype of *ΔhdaA* strain (Fig. 1C). This strain showed significant sensitivity against osmotic stress in liquid culture, but not in plate culture. This result suggests that *hdaA/Aohda1* plays a role in liquid culture specific osmotic stress response. We also found that the *hdaB ht*

and *ΔhdaD* strain were more osmo-sensitive in liquid culture (Fig. 1C). These findings suggest that different osmotic stress resistance mechanisms between the plate and liquid culture conditions, and *hdaB/Aorpd3*, *hdaA/Aohda1 and hdaD/Aohos2* will be included in these mechanisms.

Drug (inhibitor) resistance of AoHDACs **disruptants**

We examined AoHDAC disruptants' resistance to several chemicals on plate culture and, at first, the cell wall integrity was tested using cell wall synthesis inhibitors (Figs. 2 and S3). The *hdaB ht* strain was significantly sensitive to micafungin, a *β*-1,3-glucan synthesis inhibitor (23). The deletion of *hdaD/Aohos2* showed the greatest sensitivity against the calcofluor white, which is a chitin synthesis inhibitor. The *ΔhstA* strain also showed the slight sensitivity to calcofluor white (Fig. S3). These results suggest that there is variation in how different AoHDACs affect cell wall integrity, and *hdaB/Aorpd3*and *hdaD/Aohos2* will be concerned with these mechanisms.

HDACs are also relevant to genome integrity (24); thus, we examined the genotoxin tolerances of all AoHDAC disruptants (Figs. 3 and S4). As expected, the *hdaB ht, ΔhdaD* and *ΔhstD* strains showed defects against methyl methane sulfonate, which methylates DNA predominantly on N7-deoxyguanosine and N3-deoxyadenosine and is

163 believed to cause double-stranded DNA breaks. Additionally, the *ΔhstD* strain showed
164 the strongest sensitivity to camptothecin, a topoisomerase inhibitor. These results
165 suggested *hdaB/Aorpd3*, *hdaD/Aohos2* and *hstD/Aohst4* are involved in genome
166 integrity.
167 No obvious sensitivity to protein secretion inhibitors was observed when tested
168 dithiothreitol, tunicamycin or brefeldin A (Fig. S5). A slight sensitivity was observed
169 with nocodazole, which depolymerizes microtubules, in the *ΔhstD* and *hdaB ht* strain.
170

171 ***Effect of AoHDACs disruption on rice-koji***

172 A distinctive feature of the *koji* fermentation process is the use of solid-state culture
173 where *A. oryzae* is grown on steamed cereals such as rice and soybean. The resulting
174 material contains abundant hydrolytic enzymes and metabolites that are important for
175 the quality of the final products (25). We analyzed the effect of AoHDACs disruption on
176 the growth and protein production of rice-*koji* (Figs. 4 and S6).
177 It is difficult to separate the mycelia from rice-*koji* to measure exact fungal biomass, so
178 instead we measured N-acetylglucosamine, which has been shown strongly correlated
179 with biomass (26). We found significant decreases in N-acetylglucosamine on rice-*koji*
180 made by the *hdaB ht* and *ΔhdaD* strains (Fig. 4A). The *ΔhdaA* strain also decreased

181 N-acetylglucosamine, but the results were not significant. The disruptant strains also
182 decreased total protein production, but enzyme activity differed by disruptant (Fig. 4B
183 and C). The *hdaB ht* strain showed significant decreases in all measured enzyme activity,
184 while the *ΔhdaA* and *ΔhdaD* strains only showed a significant decrease in glycoside
185 hydrolase activity and a slight increased acid carboxypeptidase activity. In these strains,
186 the low glycoside hydrolase productivity or growth defect might cause low protein
187 productivity. Compared with these three strains, the *ΔhstD* strain showed no decrease in
188 N-acetylglucosamine but had lowered protein production. We also found significant
189 decrease in glycoside hydrolase and acid protease. These results suggest that
190 *hstD/Aohst4* can affect many types of protein production even when it does not affect
191 growth.
192
193 **Discussion**
194 HDACs play diverse roles in higher eukaryote development, stress response, and
195 genome integrity (6, 27). In some filamentous fungi, HDACs are important to growth,
196 conidia formation, and secondary metabolism (28). This study, strongly suggests that
197 functional divergence of HDACs in *A. oryzae* with important industrial implications for
198 HDAC deletion.

199 The modes of development and enzyme production in *A. oryzae* are significantly
200 different when it grows in solid-state or liquid culture conditions (29, 30). This supports
201 recent omics studies of *A. oryzae* that revealed how the transcriptome and proteome
202 were altered depending on culture type (30, 31). However, a detailed molecular
203 investigation is needed to understand the underlying mechanisms.
204 HDACs widely affected gene expression through the structural modification of
205 chromatin by the deacetylation of histones. We found phenotypic differences between
206 disruptants grown on the liquid culture as opposed to the plate culture. The *ΔhdaA* strain
207 did not show any significant effect against osmotic stress on the plate culture, but an
208 obvious defect was observed in liquid culture. Additionally, the *hdaB ht* strain and
209 *ΔhdaD* strains had a more sensitive phenotypic response to osmotic stress in the liquid
210 culture. These results indicated some difference between the liquid and plate culture
211 on the osmotic adaptation and these three HDACs would play a role in the adaptation
212 mechanisms in *A. oryzae*.
213 We also found a significant decrease in the amount of N-acetylglucosamine in rice-*koji*
214 produced by the *hdaB ht*, *ΔhdaD* and *ΔhdaA* strains. N-acetylglucosamine is a key
215 constituent saccharide of chitin, the major glycan composing fungal cell walls and a
216 good surrogate for measuring fungal growth on rice-*koji* (26). The growth effects of

217 these three HDACs in the solid-state cultures suggests the importance of the compound
218 stabilizing the fungal cell wall. In this experiment we only measured the amount of
219 N-acetylglucosamine whereas fungal cell walls also contain β- and α-1,3-glucan, chitin,
220 galactomannan (32). Thus, it is not clear whether the disruption of *hdaB/Aorpd3* and
221 *hdaD/Aohos2* affects the overall glycan construction of cell wall. Future work should
222 examine the detailed role of AoHDACs on the cell wall synthesis mechanisms and the
223 relationship of AoHDAC disruption to fungal biomass.
224 The *hstD/AoHst4* HDAC has been phylogenetically classified in the fungal specific
225 sirtuin class but its function is still poorly understood (4). We previously reported that
226 *hstD/Aohst4* coordinates fungal specific phenotypes of secondary metabolite production
227 and conidia formation, and a separate study using *Saccharomyces cerevisiae* found that
228 *hst4* (a homolog of *hstD* in *A.oryzae*) was important for genome integrity and resistance
229 to genotoxin (21, 33). In our work, we reveal an additional function. The deletion of
230 *hstD/Aohst4* led to MMS- and CPT-sensitive phenotypes suggests that this family of
231 proteins plays a conserved role in the genome integrity among fungi.
232 We also found a novel effect on enzyme production in rice-*koji* produced by the
233 disruptant strains. The *ΔhstD* strain decreased enzyme production despite the growth of
234 mycelia being unchanged. This was surprising because, in general, alteration of the

protein secretion pathway affects filamentous growth of *A. oryzae* (34). The lack of sensitive phenotypes when we tested protein secretion pathway inhibitors suggests that *hstD/Aohst4* may not affect the protein secretion pathway. In general, HDACs affect transcriptional regulation, so it is possible *hstD/Aohst4* affects extracellular enzyme expression. This would have significant industrial applications and future work should investigate the expression, histone modification and chromatin structure of these genes. This work reveals novel phenotypes of four AoHDAC disruptants in *A. oryzae*: *hdaA/Aohda1*, *hdaB/Aorpd3*, *hdaD/Aohos2*, and *hstD/Aohst4* with diverse cellular process that include stress response, cell wall synthesis, protein secretion, and genome integrity. Because HDACs are highly conserved among filamentous fungi and other eukaryote, our study is broadly applicable to understanding the function of HDACs.

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Reference

1. **Cairns, B. R.:** The logic of chromatin architecture and remodelling at promoters, Nature, **461**,193-198 (2009).

2. **Shahbazian, MD. and Grunstein, M.:** Functions of site-specific histone acetylation and deacetylation, Annu. Rev. Biochem., **76**, 75-100 (2007).

3. **Nishida, H.:** Evolutionary conservation levels of subunits of histone-modifying protein complexes in fungi, Comp. Funct. Genomics, 379317 (2009).

4. **Frye, R. A.:** Phylogenetic Classification of prokaryotic and eukaryotic Sir2-like proteins, Biochem. Biophys. Res. Commun., **273**, 793-798 (2000).

5. **Gregoretti, I., Lee. Y-M., and Goodson, H. V.:** Molecular evolution of the histone deacetylase family: Functional implications of phylogenetic analysis, J. Mol. Biol., **338**, 17-31 (2004).

6. **Yang, X-J. and Seto, E.:** The Rpd3/Hda1 family of lysine deacetylases: from bacteria and yeast to mice and men, Nat. Rev. Mol. Cell. Biol., **9**, 206-218 (2008).

7. **Iwashita, K.:** Recent studies of protein secretion by filamentous fungi, J. Biosci. Bioeng., **94**, 530-535 (2002).

8. **Hoffmeister, D. and Keller, N. P.:** Natural products of filamentous fungi:

269 enzymes, genes, and their regulation, Nat. Prod. Rep., **24**, 393-416 (2007).

270 9. **Raffaele, S. and Kamoun, S.:** Genome evolution in filamentous plant
271 pathogens: why bigger can be better, Nat. Rev. Micro., **10**, 417-430 (2012).

272 10. **Pagiotti, R., Angelini, P., Rubini, A., Tirillini, B., Granetti, B., Venanzoni, R.,**
273 Identification and characterisation of human pathogenic filamentous fungi and
274 susceptibility to *Thymus schimperi* essential oil, Mycoses, **54**, e364-e376 (2011).

275 11. **Gacek, A. and Strauss, J.:** The chromatin code of fungal secondary metabolite
276 gene clusters, Appl. Microbiol. Biotechnol., **95**, 1389-1404 (2012).

277 12. **Ding, S-L., Liu, W., Iliuk, A., Ribot, C., Vallet, J., Tao, A., Wang, Y., Lebrun,**
278 **M-H., and Xu, J-R.,** The Tig1 histone deacetylase complex regulates infectious
279 growth in the rice blast fungus *Magnaporthe oryzae*, Plant Cell, **22**, 2495-2508
280 (2010).

281 13. **Li, Y., Wang, C., Liu, W., Wang, G., Kang, Z., Kistler, H. C., and Xu, J-R.:**
282 The *HDF1* histone deacetylase gene is important for conidiation, sexual
283 reproduction, and pathogenesis in *Fusarium graminearum*, Mol. Plant Microbe
284 Interact, **24**, 487-496 (2010).

285 14. **Tribus, M., Bauer, I., Galehr, J., Rieser, G., Trojer, P., Brosch, G., Loidl, P.,**
286 **Haas, H., and Graessle, S.:** A novel motif in fungal class 1 histone deacetylases

287 is essential for growth and development of *Aspergillus*, Mol. Biol. Cell., **21**,
288 345-353 (2010).
289 15. **Tribus, M., Galehr, J., Trojer, P., Brosch, G., Loidl, P., Marx, F., Haas, H.,**
290 **and Graessle, S.:** HdaA, a major class 2 histone deacetylase of *Aspergillus*
291 *nidulans*, affects growth under conditions of oxidative stress, Eukaryotic Cell, **4**,
292 1736-1745 (2005).
293 16. **Shimizu, M., Masuo S., Fujita, T., Doi, Y., Kamimura, Y., and Takaya, N.:**
294 Hydrolase controls cellular NAD, sirtuin, and secondary metabolism, Mol. Cell.
295 Biol., 32, 3743-3755 (2012).
296 17. **Machida, M., Yamada, O., and Gomi, K.:** Genomics of *Aspergillus oryzae*:
297 learning from the history of koji mold and exploration of its future. DNA Res.,
298 **15**, 173-183 (2008).
299 18. **Christensen, T., Woeldike, H., Boel, E., Mortensen, S. B., Hjortshoej, K.,**
300 **Thim, L., and Hansen, M. T.:** High level expression of recombinant genes in
301 *Aspergillus oryzae*, Nat. Biotechnol., **6**, 1419-1422 (1988).
302 19. **Imamura, K., Tsuyama, Y., Hirata, T., Shiraishi, S., Sakamoto, K., Yamada,**
303 **O., Akita, O., and Shimoi, H.:** Identification of a gene involved in the synthesis
304 of a dipeptidyl peptidase IV inhibitor in *Aspergillus oryzae*, Appl. Environ.

Microbiol., **78**, 6996-7002 (2012).

20. **Terabayashi, Y., Sano, M., Yamane, N., Marui, J., Tamano, K., Sagara, J., Dohmoto, M., Oda, K., Ohshima, E., Tachibana, K., and other 4 authors:** Identification and characterization of genes responsible for biosynthesis of kojic acid, an industrially important compound from *Aspergillus oryzae*, Fungal Genet. Biol., **47**, 953-961, (2010).

21. **Kawauchi, M., Nishiura, M., and Iwashita, K.:** Fungas-specific sirtuin *HstD* coordinates secondary metabolism and development through control of *LaeA*, Eukaryotic Cell, **12**, 1087-1096 (2013).

22. **Iemura, Y., Yamada, T., Takahashi, T., Furukawa, K., and Hara S.:** Properties of the peptides liberated from rice protein in *Sokujo-moto*, J. Biosci. Bioeng., **88**, 276-280 (1999).

23. **Free, S. J.:** Chapter Two - Fungal Cell Wall Organization and Biosynthesis, p. 33-82. In Freedman, T., Dunlap, J. C., and Goodwin S. F. (ed.), Advances in Genetics, vol. 81. Academic Press, Waltham, MA. (2013).

24. **Huertas, D., Sendra, R., and Muñoz, P.:** Chromatin dynamics coupled to DNA repair, Epigenetics, **4**, 31-42 (2009).

25. **Kitamoto, K.:** Molecular biology of the Koji molds, Adv. Appl. Microbiol., **51**,

129-153 (2002) Academic Press., Waltham, MA.

26. **Arima, K., and Uozumi, T.:** A new method for estimation of mycelial weight in koji, Agr. Biol. Chem., **31**, 119-123 (1967)

27. **Horio, Y., Hayashi, T., Kuno, A., and Kunimoto, R.:** Cellular and molecular effects of sirtuins in health and disease, Clin. Sci.(Lond.), **121**, 191-203 (2011).

28. **Brosch, G., Loidl, P., and Graessle, S.:** Histone modifications and chromatin dynamics: a focus on filamentous fungi, FEMS Microbiol. Rev., **32**, 409-439 (2008).

29. **Biesebeke, R., Ruijter, G., Rahardjo, Y. S. P., Hoogschagen, M. J., Heerikhuisen, M., Levin, A., Driel, K. G. A. , Schutyser, M. A. I., Dijksterhuis, J., Zhu, Y., and other 4 authors.:** *Aspergillus oryzae* in solid-state and submerged fermentations, FEMS Yeast Res., **2**, 245-248 **(**2002).

30. **Oda, K., Kakizono, D., Yamada, O., Iefuji, H., Akita, O., Iwashita, K.:** Proteomic Analysis of Extracellular Proteins from *Aspergillus oryzae* Grown under Submerged and Solid-state Culture Conditions, Appl. Environ. Microbiol., **72**, 3448-3457 (2006).

31. **Wang, B., Guo, G., Wang, C., Lin, Y., Wang, X., Zhao, M., Guo, Y., He, M., Zhang, Y., Pan, L.:** Survey of the transcriptome of *Aspergillus oryzae* via

massively parallel mRNA sequencing, Nucleic Acids Res., **38**, 5075-5087 (2010).

32. Latgé J.P.: Tasting the fungal cell wall., Cell Microbiol., 12, 863-72 (2010)

32. **Miller, K., Maas, N., Toczyski, D.:** Taking it off: regulation of H3 K56 acetylation by *Hst3* and *Hst4*, Cell Cycle, **5**, 2561-2565 (2006).

33. **Shoji, J., Arioka, M., Kitamoto, K.:** Dissecting cellular components of secretory pathway in filamentou fungi: insights into their application for protein production, Biotecnol. Lett., **30**, 7-14 (2008).

351 **Figure legends**

352 FIG. 1. Stress sensitivity test of AoHDAC disruptants.

353 (A) Morphological phenotypes of disruptants on stress plate culture on day five. (B)

354 Comparison of stress sensitivity on plate culture. Graphs represent radial growth

355 relative to the no-stress plate culture (corresponding to 1.0). (C) Comparison of osmotic

356 stress sensitivity on liquid culture. Graphs represent mycelia dry weight relative to the

357 no-stress liquid culture (corresponding to 1.0). The *adeA*$^{+}$ strain was used as a control in

358 this figure. All data are represented as means ± s.d. (n=3); * $p < 0.05$, t-test.

359

360 FIG. 2. Cell wall synthesis inhibitor sensitivity of AoHDAC disruptants.

361 (A) Morphological phenotypes of indicated strain on cell wall synthesis inhibitor treated

362 culture on day five. (B) Comparison of inhibitors sensitivity on plate culture. Graphs

363 represent radial growth relative to the plate culture lacking an inhibitor (corresponding

364 to 1.0). The *adeA*$^{+}$ strain was used as a control. All data are represented as means ±

365 s.d. (n=3); * $p < 0.05$, t-test. Abbreviations: CFW, calcofluor white; CR, Congo red; MF,

366 micafungin.

367

368 FIG. 3. Genotoxin sensitivity of AoHDAC disruptants.

(A) Morphological phenotypes of indicated strain on genotoxin treated culture on day five. (B) Comparison of genotoxin sensitivity on plate culture. Graphs represent radial growth relative to the no genotoxin treated plate culture (corresponding to 1.0). The *adeA*$^{+}$ strain was used as a control. All data are represented as means ± s.d. (n=3); * $p <$ 0.05, t-test. Abbreviations: HU, hydroxy urea; CPT, camptothecin; MMS, methyl methane sulfonate.

FIG. 4. Phenotypes of AoHDACs on rice-*koji*.

(A) N-acetylglucosamine amounts in rice-*koji*. (B) Total protein equivalent in rice-*koji*. (C) Activity of enzymes in rice-*koji*. Enzyme activities represented as the activity relative to the *adeA*$^{+}$ control strain (corresponding to 1.0). All data are represented as means ± s.d. (n=3); * $p < 0.05$, t-test.

382 **Supplementary figure legends**

383

384 FIG. S1. Phenotypes of AoHDAC mutants on stress plate culture at day five.

385 The *adeA*$^{+}$ strain was used as a control.

386

387 FIG. S2. Stress sensitivity test of AoHDAC disruptants.

388 (A) Comparison of stress sensitivity on plate culture. Graphs represent radial growth

389 relative to the no stress plate culture (corresponding to 1.0). (B) Comparison of osmotic

390 stress sensitivity on liquid culture. Graphs represent mycelia dry weight relative to the

391 no stress liquid culture (corresponding to 1.0). The *adeA*$^{+}$ strain was used as a control.

392 All data are represented as means ± s.d. (n=3); * $p < 0.05$, t-test.

393

394 FIG. S3. Cell wall synthesis inhibitor sensitivity of AoHDAC disruptants.

395 (A) Morphological phenotypes of indicated strain on cell wall synthesis inhibitor treated

396 culture on day five. (B) Comparison of inhibitor sensitivity on plate culture. Graphs

397 represent radial growth relative to the plate culture lacking an inhibitor (corresponding

398 to 1.0). The *adeA*$^{+}$ strain was used as a control. All data are represented as means ± s.d.

399 (n=3); * $p < 0.05$, t-test. Abbreviations: CFW, calcofluor white; CR, Congo red; MF,

400 micafungin.

401

402 FIG. S4. Genotoxin sensitivity of AoHDAC disruptants.

403 (A) Morphological phenotypes of indicated strain on cell wall synthesis genotoxin

404 treated culture on day five. (B) Comparison of inhibitors sensitivity on plate culture.

405 Graphs represent radial growth relative to the no genotoxin treated plate culture

406 (corresponding to 1.0). The *adeA*$^{+}$ strain was used as a control. All data are represented

407 as means ± s.d. (n=3); * $p < 0.05$, t-test. Abbreviations: HU, hydroxy urea; CPT,

408 camptothecin; MMS, methyl methane sulfonate.

409

410 FIG. S5. Protein synthesis or secretion inhibitor sensitivity of AoHDAC mutants.

411 (A) Morphological phenotypes of indicated strain on protein synthesis or secretion

412 treated culture on day five. (B) Comparison of inhibitors sensitivity on plate culture.

413 Graphs represent radial growth relative to the plate culture lacking an inhibitor

414 (corresponding to 1.0). The *adeA*$^{+}$ strain was used as a control. All data are represented

415 as means ± s.d. (n=3); * $p < 0.05$, t-test. Abbreviations: TM, tunicamycin; DTT,

416 dithiothreitol; BFA, brefeldin A; NC, nocodazole.

417

418 FIG. S6. Phenotypes of AoHDAC disruptants on rice-*koji*.

419 (A) N-acetylglucosamine amounts in rice-*koji*. (B) Total protein equivalent in rice-*koji*.

420 (C) Activity of indicated enzyme in rice-*koji*. Enzyme activities represent activity

421 relative to the *adeA*$^{+}$ control strain (corresponding to 1.0). All data are represented as

422 means ± s.d. (n=3); * $p < 0.05$, t-test.

TABLE 1. Strains used in this study.

Name	Genotype	Disrupted gene (accession)[b]	HDAC type (HDAC Class)	Reference
adeA^{+}	*niaD*$^{-}$ *sC*$^{-}$ *adeA*$^{-}$ *argB::adeA*$^{-}$ *ΔligD::argB adeA*$^{+}$			(22)
hdaB ht [a]	*niaD*$^{-}$ *sC*$^{-}$ *adeA*$^{-}$ *argB::adeA*$^{-}$ *ΔligD::argB ΔhdaB::adeA hdaB*	*hdaB* / *Aorpd3*(AO080554000240)	Classical HDAC (Class 1)	(22)
ΔhdaC	*niaD*$^{-}$ *sC*$^{-}$ *adeA*$^{-}$ *argB::adeA*$^{-}$ *ΔligD::argB ΔhdaC::adeA*	*hdaC* / *Aorpd3*(AO080525000127)	Classical HDAC (Class 1)	(22)
ΔhdaD	*niaD*$^{-}$ *sC*$^{-}$ *adeA*$^{-}$ *argB::adeA*$^{-}$ *ΔligD::argB ΔhdaD::adeA*	*hdaD* / *Aohos2*(AO080511000459)	Classical HDAC (Class 1)	(22)
ΔhdaA	*niaD*$^{-}$ *sC*$^{-}$ *adeA*$^{-}$ *argB::adeA*$^{-}$ *ΔligD::argB ΔhdaA::adeA*	*hdaA* / *Aohda1*(AO080513000236)	Classical HDAC (Class 2)	(22)
ΔhdaE	*niaD*$^{-}$ *sC*$^{-}$ *adeA*$^{-}$ *argB::adeA*$^{-}$ *ΔligD::argB ΔhdaE::adeA*	*hdaE* / *Aohos3* (AO080570000061)	Classical HDAC (Class 2)	(22)
ΔhstA	*niaD*$^{-}$ *sC*$^{-}$ *adeA*$^{-}$ *argB::adeA*$^{-}$ *ΔligD::argB ΔhstA::adeA*	*hstA* / *Aosir2* (AO080506000102)	Sirtuin (Class3)	(22)
ΔhstB	*niaD*$^{-}$ *sC*$^{-}$ *adeA*$^{-}$ *argB::adeA*$^{-}$ *ΔligD::argB ΔhstB::adeA*	*hstB* / *Aohst2* (AO080569000094)	Sirtuin (Class3)	(22)
ΔhstC	*niaD*$^{-}$ *sC*$^{-}$ *adeA*$^{-}$ *argB::adeA*$^{-}$ *ΔligD::argB ΔhstC::adeA*	*hstC* / *Aohst2* (AO080508000293)	Sirtuin (Class3)	(22)
ΔhstD	*niaD*$^{-}$ *sC*$^{-}$ *adeA*$^{-}$ *argB::adeA*$^{-}$ *ΔligD::argB ΔhstD::adeA*	*hstD* / *Aohst4* (AO080533000358)	Sirtuin (Class3)	(22)
ΔhstE	*niaD*$^{-}$ *sC*$^{-}$ *adeA*$^{-}$ *argB::adeA*$^{-}$ *ΔligD::argB ΔhstE::adeA*	*hstE* / *Aosirt4* (AO080559000113)	Sirtuin (Class3)	(22)
ΔhstF	*niaD*$^{-}$ *sC*$^{-}$ *adeA*$^{-}$ *argB::adeA*$^{-}$ *ΔligD::argB ΔhstF::adeA*	*hstF* / *Aosirt5*(AO080568000195)	Sirtuin (Class3)	(22)

[a] *ht:* heterokaryon

[b] HDAC homolog of *Saccharomyces cerevisiae* or *Homo Sapiens* with the species name (Ao: *Aspergillus oryzae*) indicated followed by a slash.

Fig.1

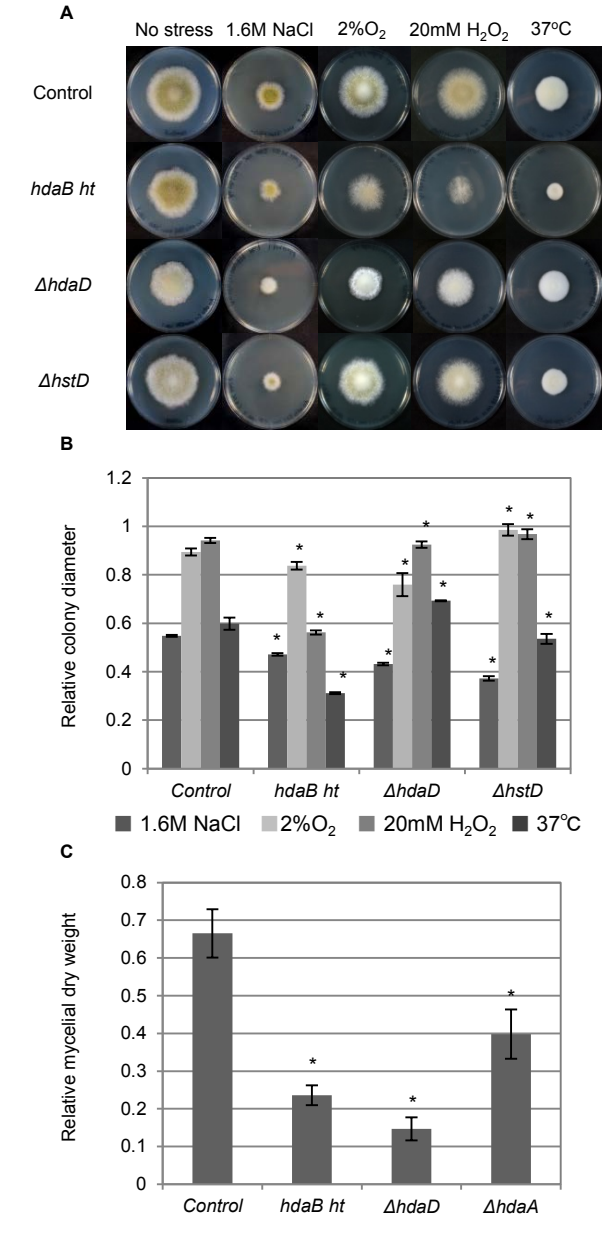
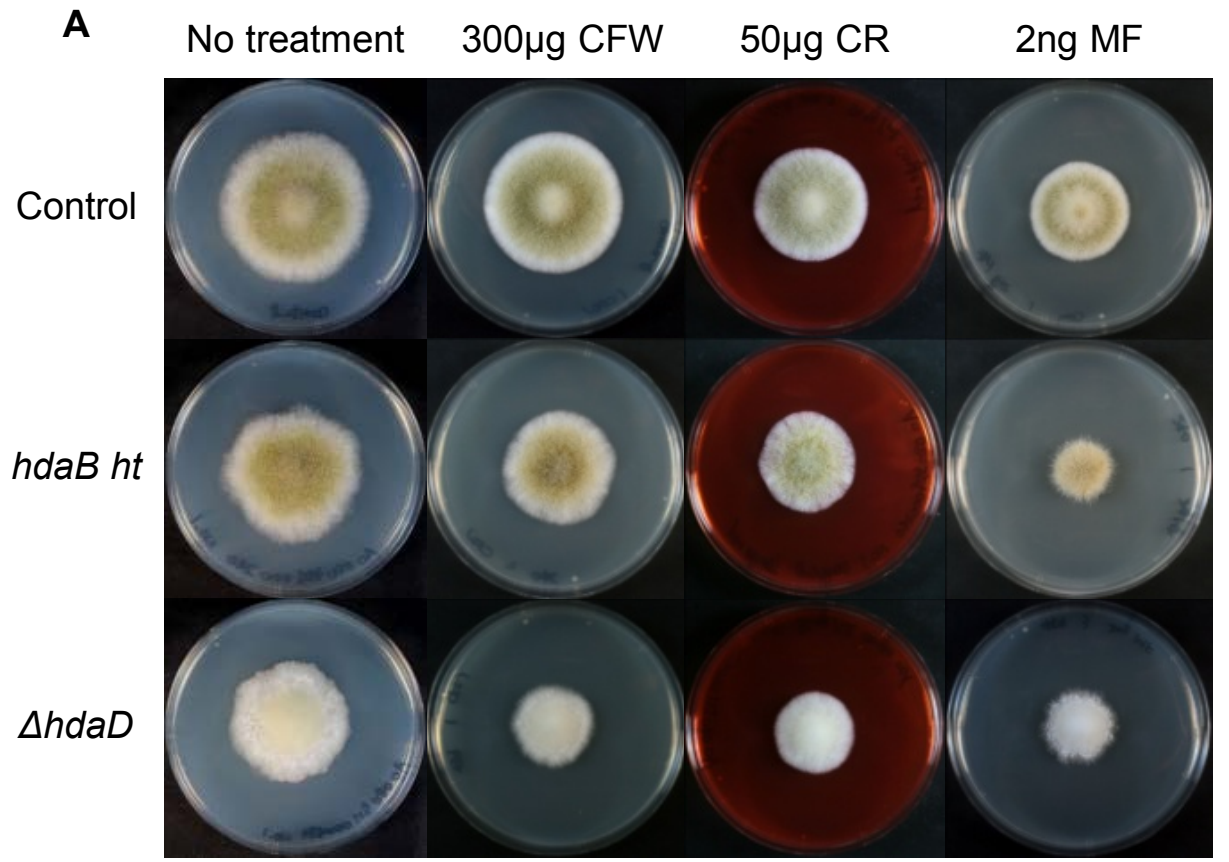

A
No stress
1.6M NaCl
2%O2
20mM H2O2
37°C
Control
hdaB ht
ΔhdaD
ΔhstD
B
Relative colony diameter
1.2
1
0.8
0.6
0.4
0.2
0
Control
hdaB ht
ΔhdaD
ΔhstD
1.6M NaCl
2%O2
20mM H2O2
37°C
C
Relative mycelial dry weight
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Control
hdaB ht
ΔhdaD
ΔhdaA

Fig.2


A
No treatment
300μg CFW
50μg CR
2ng MF
Control
hdaB ht
ΔhdaD

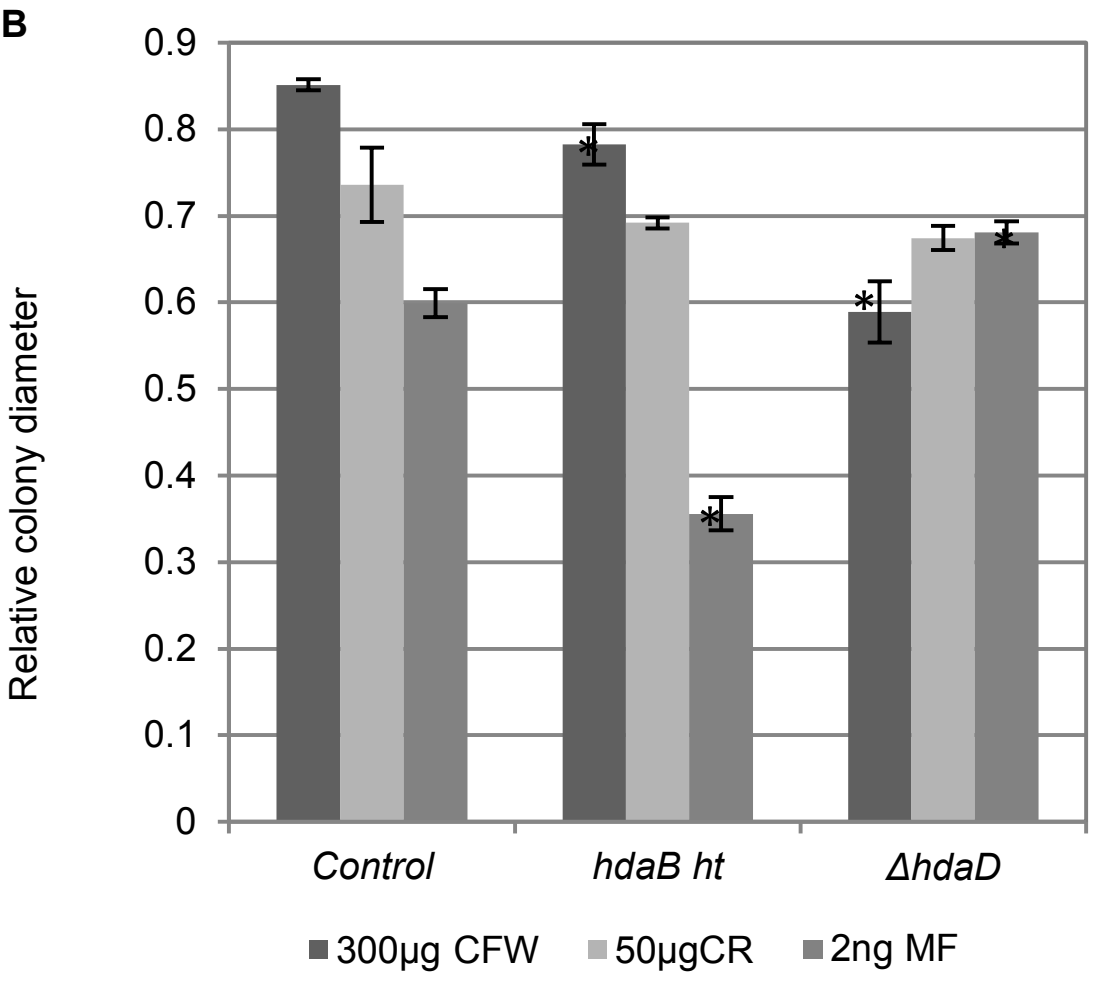
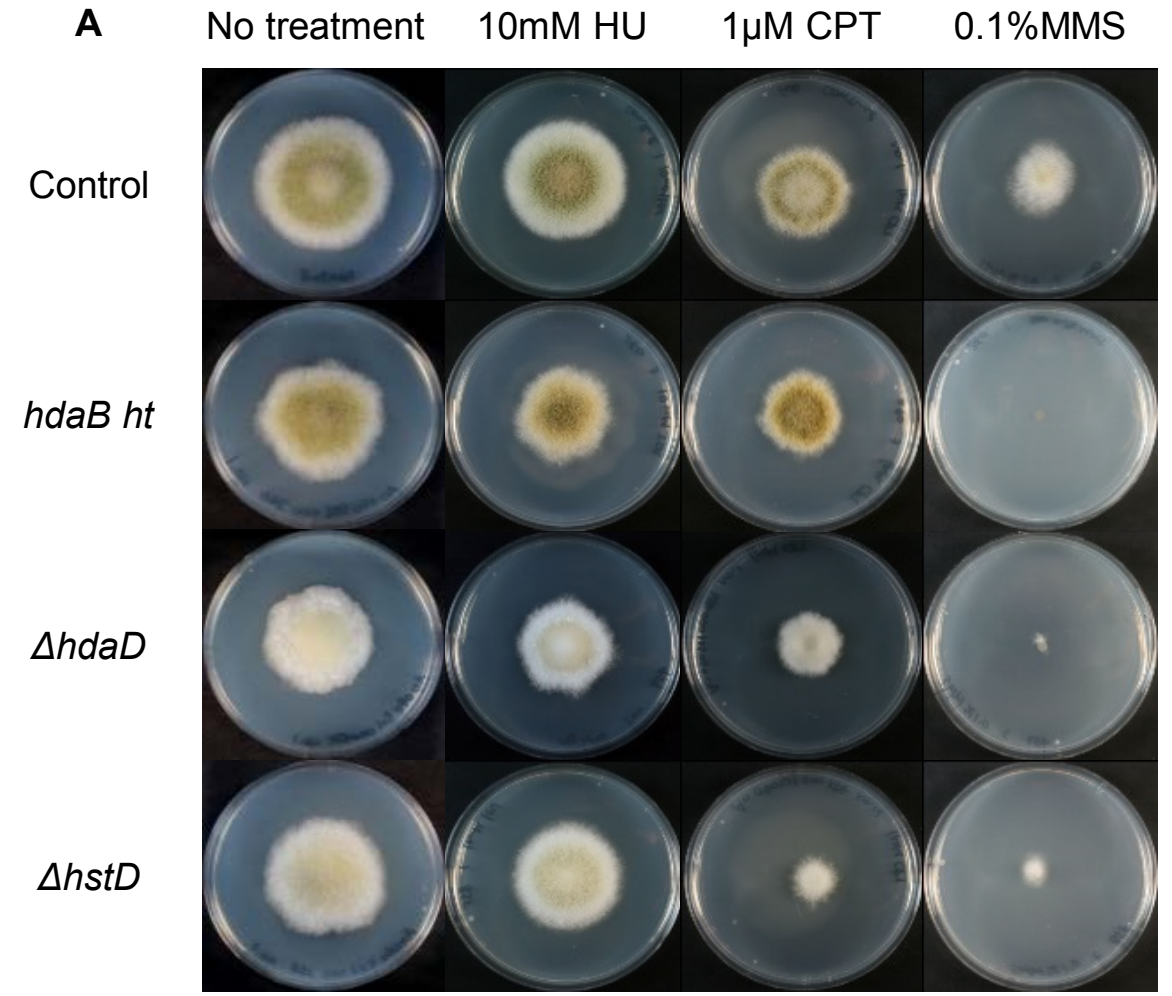

B
Relative colony diameter
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Control
hdaB ht
ΔhdaD
300μg CFW
50μgCR
2ng MF

Fig.3


A
No treatment
10mM HU
1μM CPT
0.1%MMS
Control
hdaB ht
ΔhdaD
ΔhstD

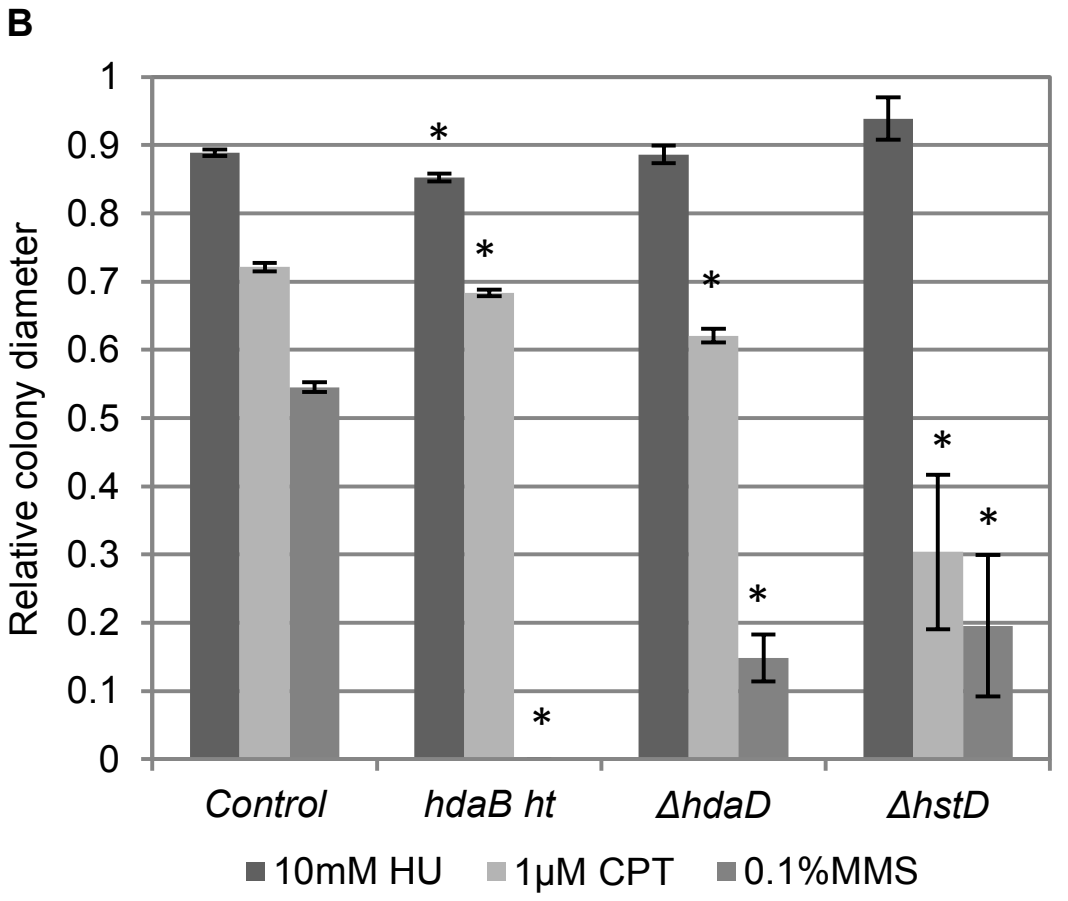

B
Relative colony diameter
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Control
hdaB ht
ΔhdaD
ΔhstD
10mM HU
1μM CPT
0.1%MMS

Fig.4

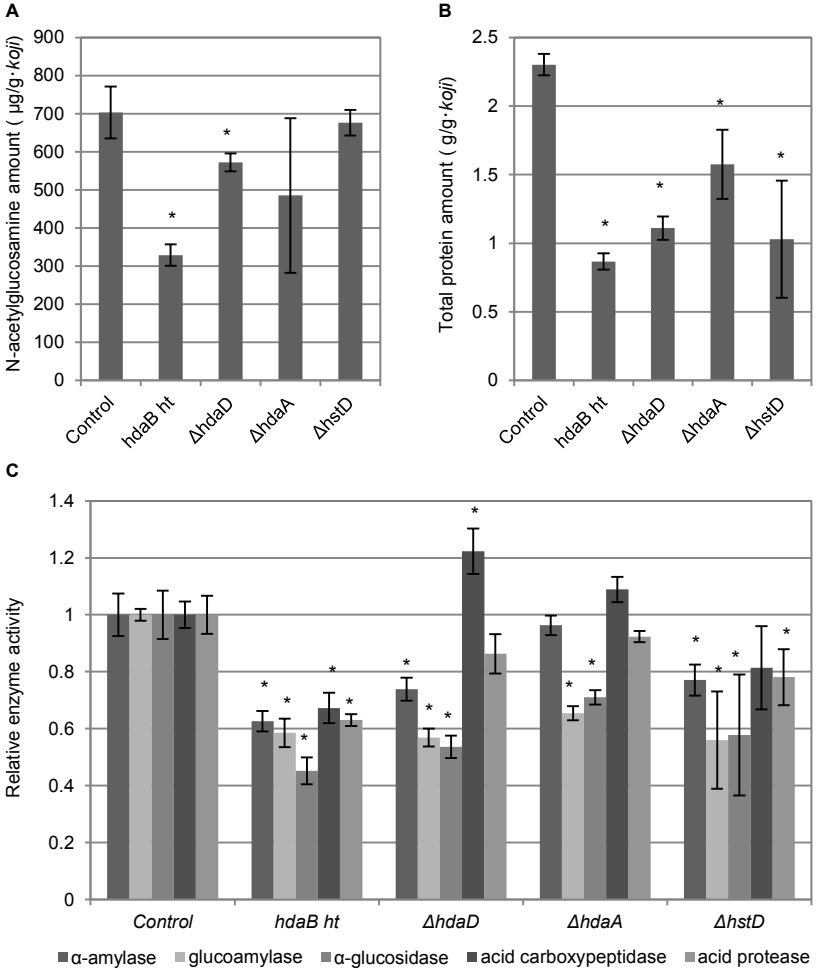

A
N-acetylglucosamine amount (μg/g･koji)
900
800
700
600
500
400
300
200
100
0
Control
hdaB ht
ΔhdaD
ΔhdaA
ΔhstD
B
Total protein amount (g/g･koji)
2.5
2
1.5
1
0.5
0
Control
hdaB ht
ΔhdaD
ΔhdaA
ΔhstD
C
Relative enzyme activity
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control
hdaB ht
ΔhdaD
ΔhdaA
ΔhstD
α-amylase
glucoamylase
α-glucosidase
acid carboxypeptidase
acid protease

Fig. S1

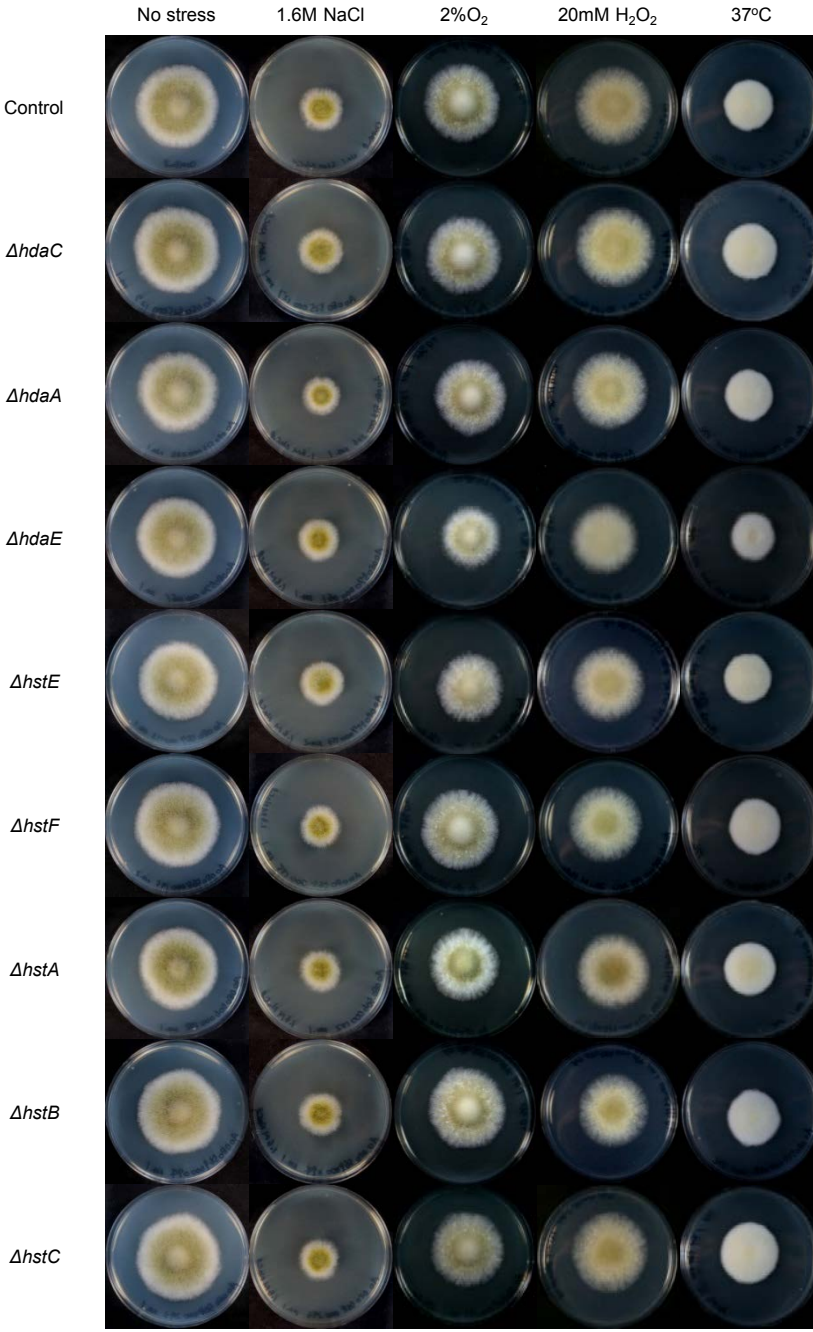
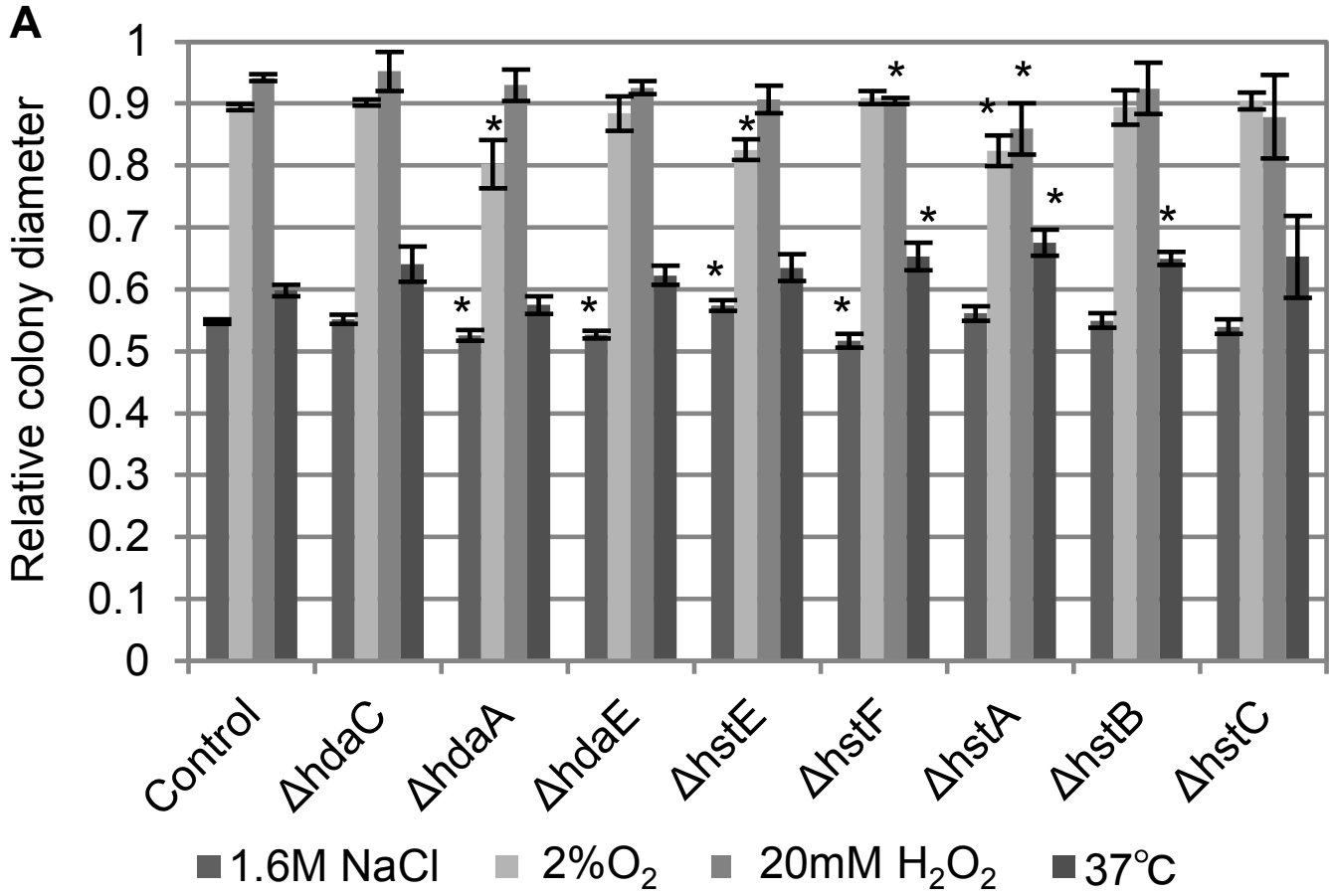

No stress
1.6M NaCl
2%O_2
20mM H_2O_2
37°C
Control
ΔhdaC
ΔhdaA
ΔhdaE
ΔhstE
ΔhstF
ΔhstA
ΔhstB
ΔhstC

Fig. S2


A
Relative colony diameter
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Control
ΔhdaC
ΔhdaA
ΔhdaE
ΔhstE
ΔhstF
ΔhstA
ΔhstB
ΔhstC
1.6M NaCl
2%O2
20mM H2O2
37℃

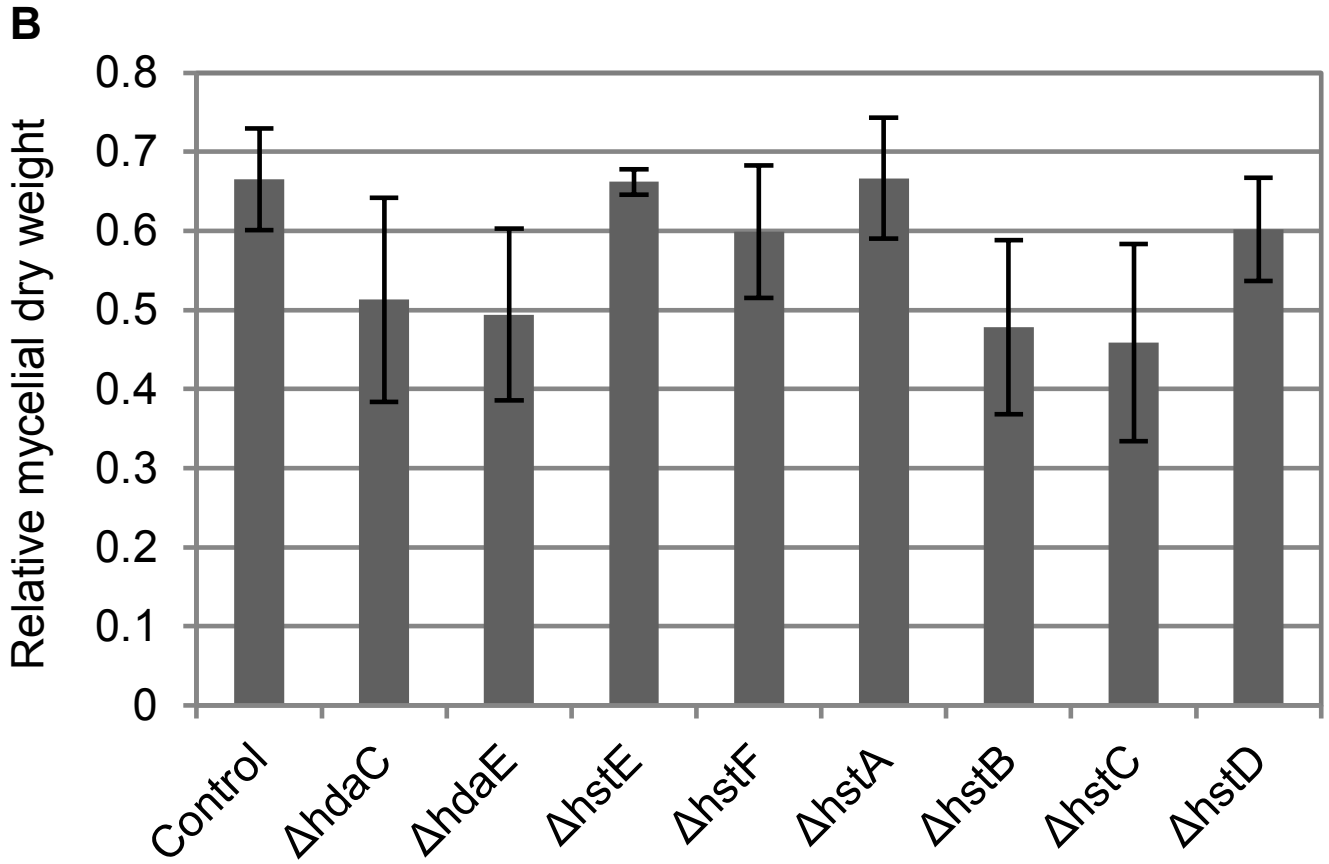
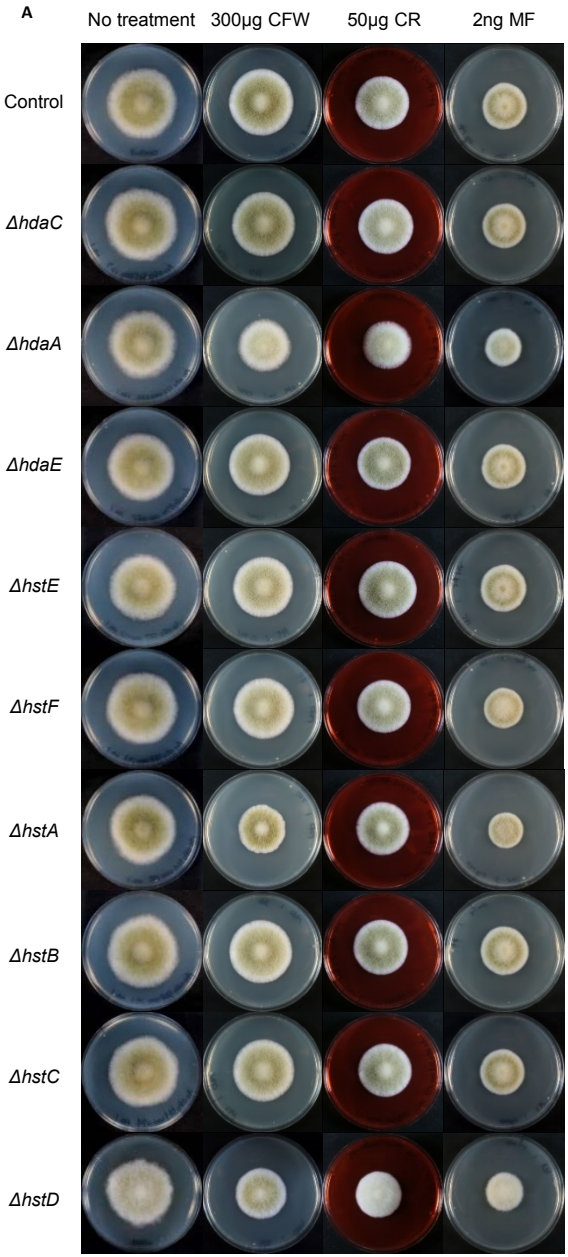

B
Relative mycelial dry weight
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Control
ΔhdaC
ΔhdaE
ΔhstE
ΔhstF
ΔhstA
ΔhstB
ΔhstC
ΔhstD

Fig. S3


A
No treatment
300μg CFW
50μg CR
2ng MF
Control
ΔhdaC
ΔhdaA
ΔhdaE
ΔhstE
ΔhstF
ΔhstA
ΔhstB
ΔhstC
ΔhstD

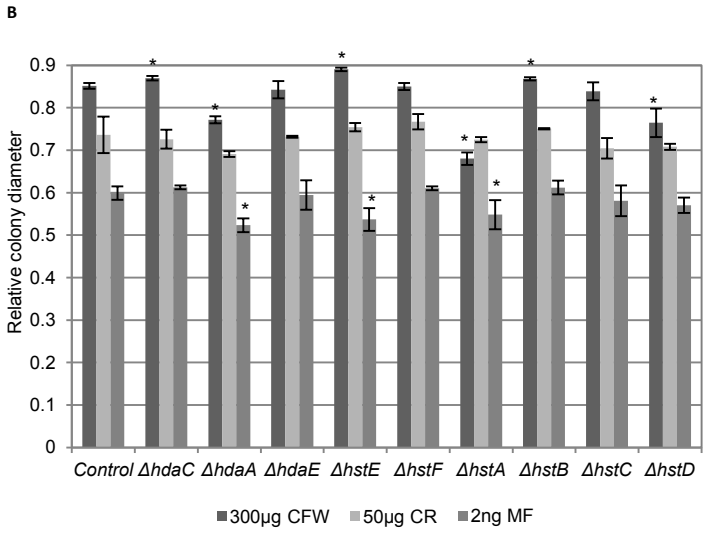

B
Relative colony diameter
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
Control
ΔhdaC
ΔhdaA
ΔhdaE
ΔhstE
ΔhstF
ΔhstA
ΔhstB
ΔhstC
ΔhstD
300μg CFW
50μg CR
2ng MF

Fig. S4

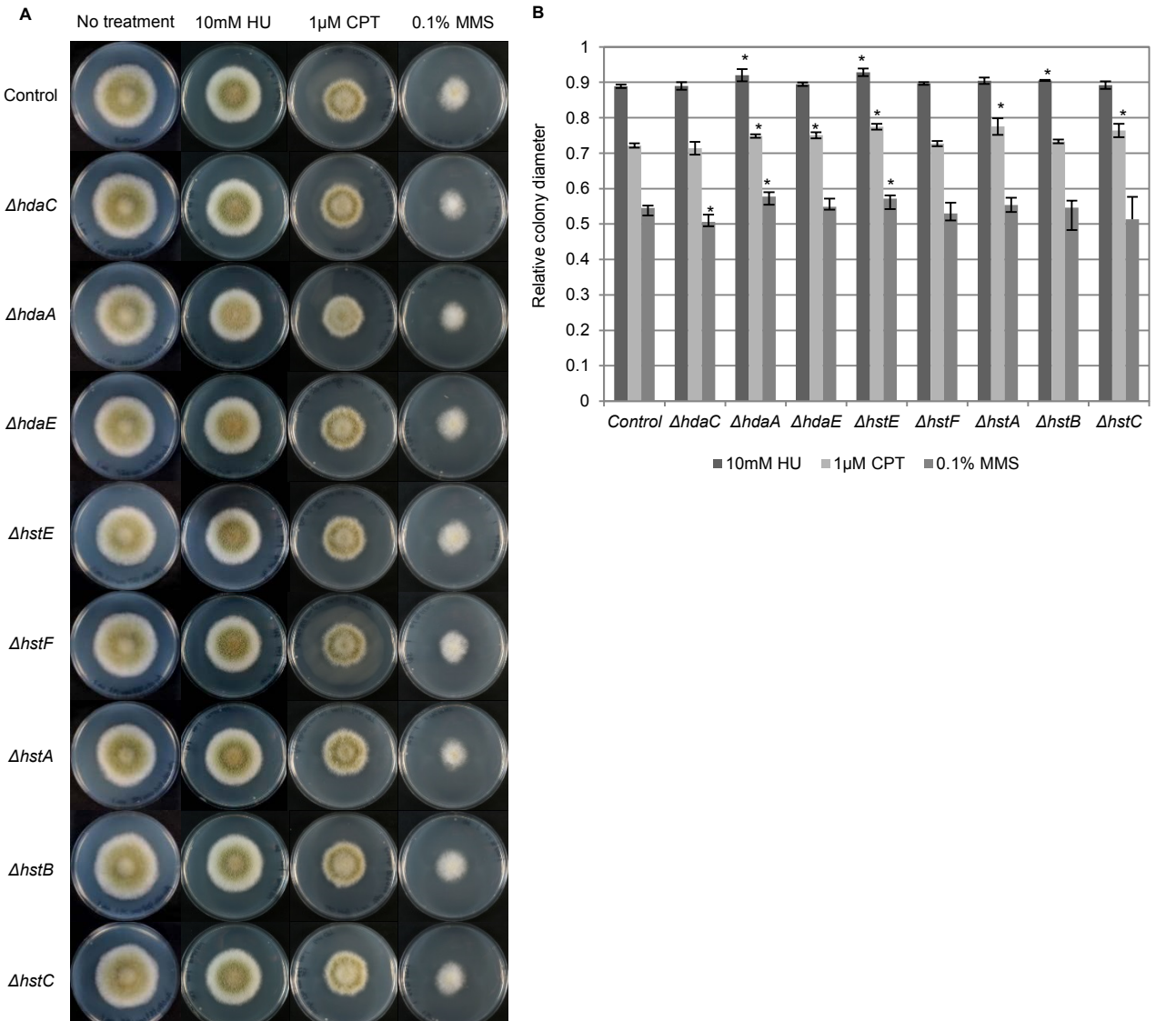
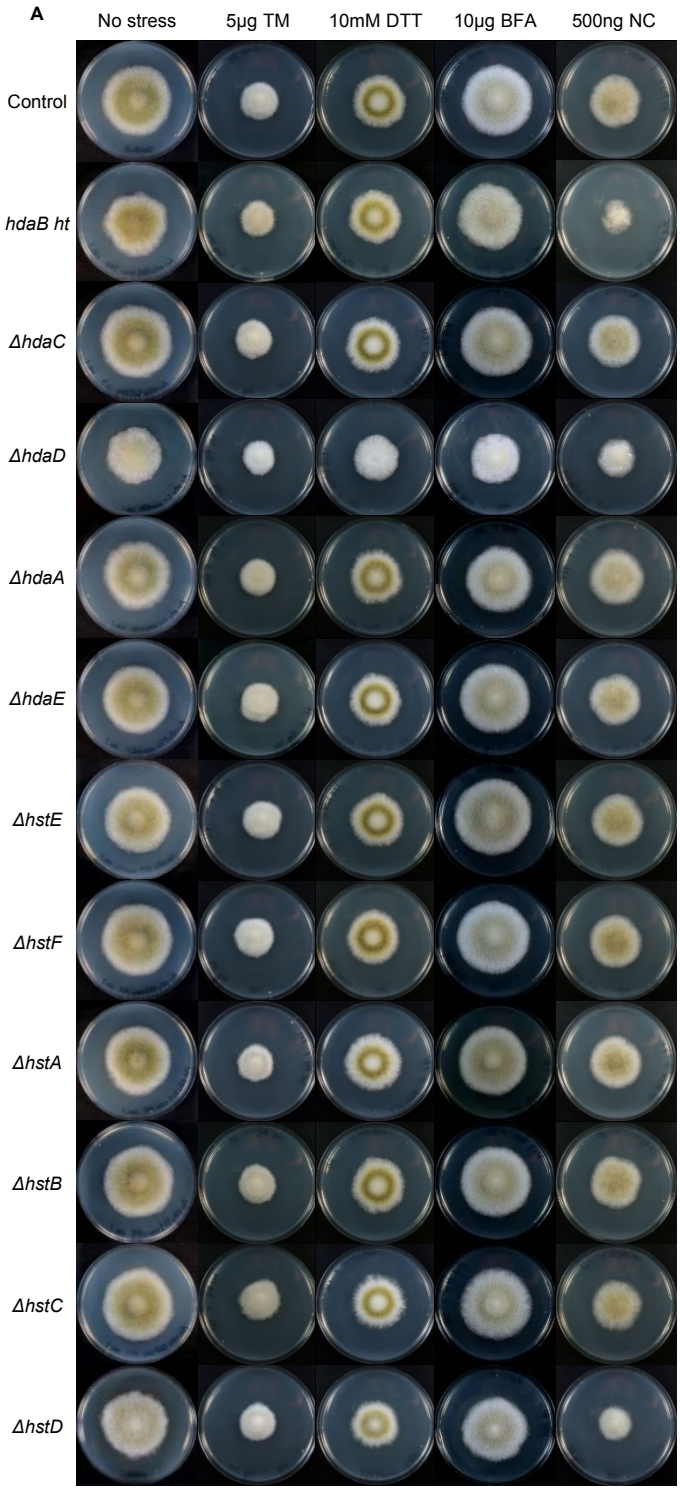

A
No treatment
10mM HU
1μM CPT
0.1% MMS
Control
ΔhdaC
ΔhdaA
ΔhdaE
ΔhstE
ΔhstF
ΔhstA
ΔhstB
ΔhstC
B
Relative colony diameter
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
Control
ΔhdaC
ΔhdaA
ΔhdaE
ΔhstE
ΔhstF
ΔhstA
ΔhstB
ΔhstC
10mM HU
1μM CPT
0.1% MMS

Fig. S5


A
No stress
5μg TM
10mM DTT
10μg BFA
500ng NC
Control
hdaB ht
ΔhdaC
ΔhdaD
ΔhdaA
ΔhdaE
ΔhstE
ΔhstF
ΔhstA
ΔhstB
ΔhstC
ΔhstD

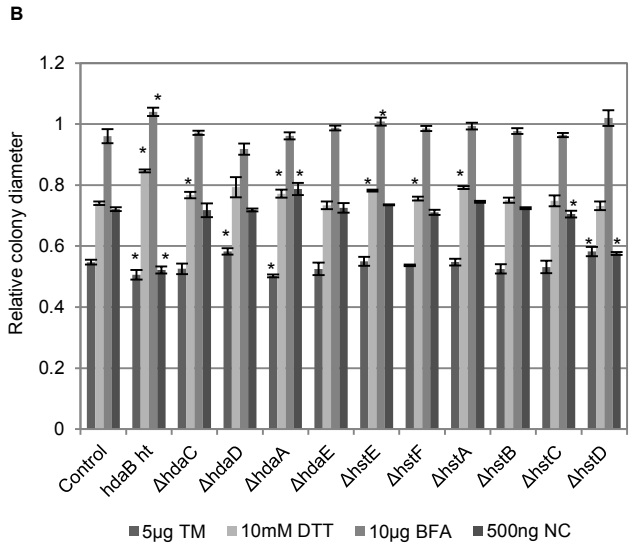
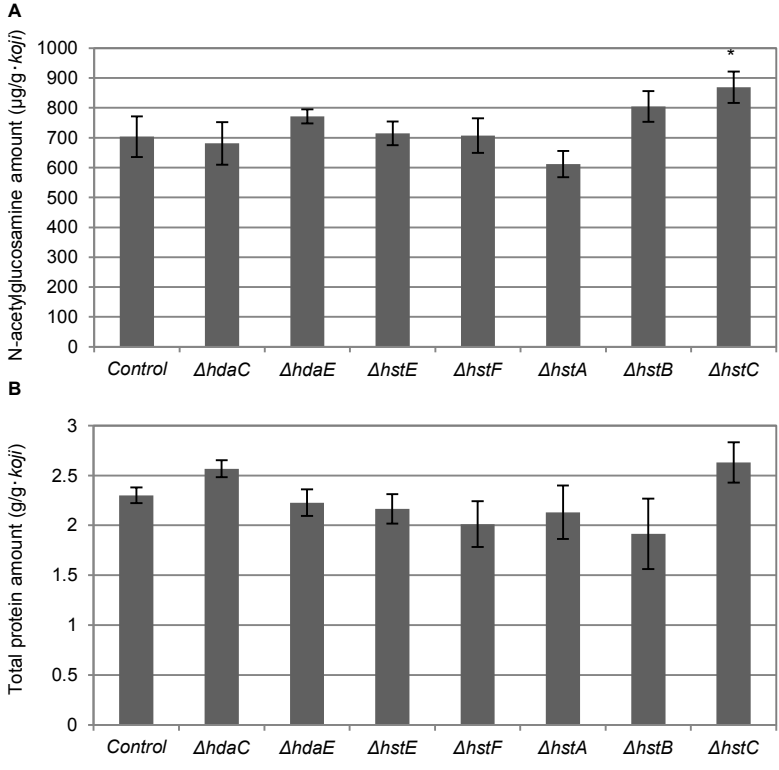
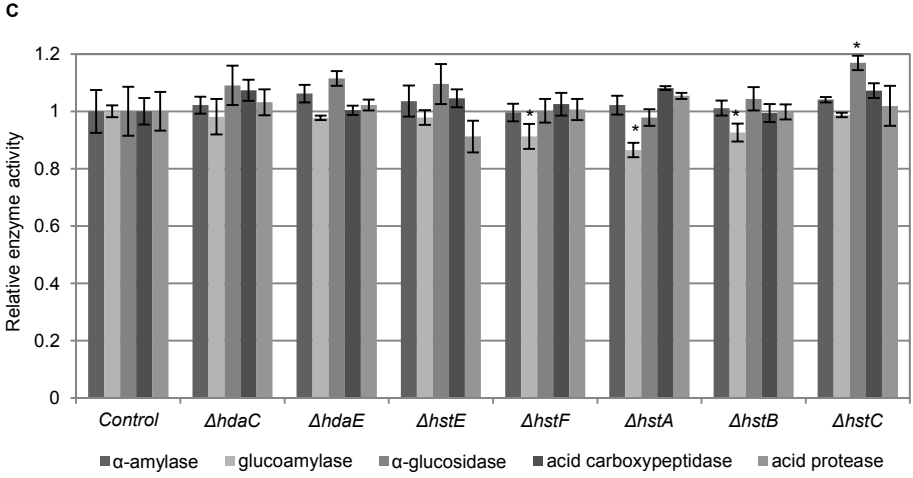

B
Relative colony diameter
1.2
1
0.8
0.6
0.4
0.2
0
Control
hdaB ht
ΔhdaC
ΔhdaD
ΔhdaA
ΔhdaE
ΔhstE
ΔhstF
ΔhstA
ΔhstB
ΔhstC
ΔhstD
5μg TM
10mM DTT
10μg BFA
500ng NC

Fig. S6


A
N-acetylglucosamine amount (μg/g·koji)
1000
900
800
700
600
500
400
300
200
100
0
*
Control
ΔhdaC
ΔhdaE
ΔhstE
ΔhstF
ΔhstA
ΔhstB
ΔhstC
B
Total protein amount (g/g·koji)
3
2.5
2
1.5
1
0.5
0
Control
ΔhdaC
ΔhdaE
ΔhstE
ΔhstF
ΔhstA
ΔhstB
ΔhstC


C
Relative enzyme activity
1.2
1
0.8
0.6
0.4
0.2
0
*
Control
ΔhdaC
ΔhdaE
ΔhstE
ΔhstF
ΔhstA
ΔhstB
ΔhstC
α-amylase
glucoamylase
α-glucosidase
acid carboxypeptidase
acid protease