Curr Genet (2010) 56:75–85
DOI 10.1007/s00294-009-0281-7

RESEARCH ARTICLE

Histone modifying proteins Gcn5 and Hda1 affect flocculation in *Saccharomyces cerevisiae* during high-gravity fermentation

Judith Dietvorst · Anders Brandt

Received: 29 September 2009 / Revised: 18 November 2009 / Accepted: 24 November 2009 / Published online: 13 December 2009

Abstract The performance of yeast is often limited by the constantly changing environmental conditions present during high-gravity fermentation. Poor yeast performance contributes to incomplete and slow utilization of the main fermentable sugars which can lead to flavour problems in beer production. The expression of the *FLO* and *MAL* genes, which are important for the performance of yeast during industrial fermentations, is affected by complex proteins associated with Set1 (COMPASS) resulting in the induction of flocculation and improved maltose fermentation capacity during the early stages of high-gravity fermentation. In this study, we investigated a possible role for other histone modifying proteins. To this end, we tested a number of histone deacetylases (HDACs) and histone acetyltransferases and we report that flocculation is induced in absence of the histone deacetylase Hda1 or the histone acetyltransferase Gcn5 during high-gravity fermentation. The absence of Gcn5 protein also improved utilization of high concentrations of maltose. Deletion of *SIR2* encoding the HDA of the silent informator regulator complex, did not affect flocculation under high-gravity fermentation conditions. Despite the obvious roles for Hda1 and Gcn5 in flocculation, this work indicates that COMPASS mediated silencing is the most important amongst the histone modifying components to control the expression of the *FLO* genes during high-gravity fermentation.

Communicated by S. Hohmann.

J. Dietvorst (✉) · A. Brandt
Carlsberg Laboratory, Gamle Carlsberg Vej 10,
2500 Copenhagen Valby, Denmark
e-mail: jd@crc.dk

Keywords Sir proteins · Set1-complex · Flocculation · Histone deacetylase Hda1 · Histone acetyltransferase Gcn5 · Maltose fermentation

Introduction

High-gravity fermentation is applied in the brewing industry to enhance the brewing capacity by diluting the produced beers with higher alcohol content to beers with the desired alcohol content (about 5%) (Almeida et al. 2001; Reilly et al. 2004). Due to the constantly changing environmental conditions during these industrial fermentations, the yeast performance is negatively affected. Consequently, utilization of the fermentable sugars in wort is often incomplete resulting in aberrant flavour profiles and a lower yield of ethanol (Rautio et al. 2007; Dietvorst et al. 2005; Rautio and Londesborough 2003). Two sets of genes, the *FLO* and the *MAL* genes, are important during high-gravity fermentation and these are located at the subtelomeric regions. The *FLO* genes are important for the sedimentation of yeast after fermentation to improve product clarification (Govender et al. 2008; Verstrepen and Klis 2006; Teunissen and Steensma 1995) while the *MAL* genes are important for the utilization of maltose during fermentation (Dietvorst et al. 2007; Vidgren et al. 2005; Kodama et al. 1995). Premature flocculation negatively affects efficient utilization of fermentable sugars and co-regulation of *MAL* and *FLO* can thus be detrimental to optimal fermentation performance. In the budding yeast *Saccharomyces cerevisiae*, telomeric regions as well as the HML/HMR silent mating-type loci and the ribosomal DNA (rDNA) repeats are subjected to silencing. Silencing is a gene-independent form of repressed chromatin structure that affects specific domains of the yeast genome. Regions of repressed chromatin are

often hypoacetylated, whereas actively transcribed chromosomal regions contain hyperacetylated histones (reviewed in Perrod and Gasser 2003). Complex proteins associated with Set1 (COMPASS) mediated silencing of the expression of the subtelomerically located *FLO* and *MAL* genes occurs during high-gravity fermentation (Houghton-Larsen and Brandt 2006; Dietvorst and Brandt 2008). In the present study, we investigated whether the absence of other histone modifying proteins than the subunits of COMPASS, that have been reported to be involved in (sub)telomeric silencing, also affected the performance of yeast during high-gravity fermentation.

The silent information regulator (SIR) complex, which comprises the histone modifying Sir2, Sir3 and Sir4 proteins, is required for repressing transcription at the HMR and HML silent mating type loci (Rine and Herskowitz 1987) and at the telomeres (Gasser and Cockell 2001). The SIR complex is recruited to the chromosome via interaction with specific DNA sequences termed silencers. Telomeric silencers are composed of an array of Rap1 binding sites embedded in the telomeric $(TG_{1-3})_n$ repeats (reviewed in Rusche et al. 2003). Once recruited to the telomeric silencer, a complex formed by Sir2, Sir3 and Sir4, involving yKU70/80 for its formation, spreads along the chromosome via interaction with histone tails (Luo et al. 2002).

Histone deacetylases (HDACs) are transcriptional repressors that reduce acetylation of histones to create regions of repressed chromatin. In addition to *SIR2*, the yeast genes *RPD3*, *HDA1* and *HST2* are also known to encode HDACs in *S. cerevisiae*. HDACs Rpd3 and Hda1 share significant sequence similarity but are found in two different complexes involved in transcriptional silencing (Rundlett et al. 1996). Hda1, Hda2 and Hda3 are subunits of a histone deacetylase (HDA) complex that is structurally distinct from the complex formed by the Rpd3, Sin3 and Sap30 proteins (Rundlett et al. 1996; Sun and Hampsey 1999; Wu et al. 2001). Disruption of *HDA1* or *RPD3* increases acetylation of histone H3 and H4 resulting in fewer cells expressing the *URA3* reporter gene integrated at the telomeric end of chromosome V R (Rundlett et al. 1996).

Many other histone modification enzymes such as Hat1, Gcn5 and Dot1 have been shown to be involved in silencing. The Hat1 protein is the catalytic subunit of the histone acetyltransferase complex Hat1–Hat2 that is involved in telomeric silencing and DNA double-strand break repair (Kelly et al. 2000; Qin and Parthun 2002). Significant silencing was observed when deletion of *HAT1* or *HAT2* was combined with mutations of specific lysine residues in the histone H3 NH_2-terminal tail, while there was no change in the level of silencing on a telomeric gene in the absence of *HAT1* or *HAT2* (Kelly et al. 2000). Another histone acetyltransferase (HAT), Gcn5, is the catalytic subunit of three chromatin-modifying complexes, Spt–Ada–Gcn5 acetyltransferase (SAGA), ADA and SLIK/SALSA. It has been shown that numerous genes are transcriptionally regulated by these complexes (Eberharter et al. 1999; Sterner et al. 2002; Pray-Grant et al. 2002). Strikingly, deletion of *GCN5* is reported to enhance silencing of the *URA3* reporter gene integrated at the telomeric end of chromosome V R (Sun and Hampsey 1999). The Dot1 protein was isolated as a high copy disruptor of telomeric silencing and has been linked to methylation of histone H3 lysine 79 (van Leeuwen et al. 2002; Ng et al. 2002; Lacoste et al. 2002). In the absence of the Dot1 protein, silencing of telomeres is reduced (Singer et al. 1998). Moreover, mislocalization of silencing regulators Sir2 and Sir3 have been reported to be a result of deletion of *DOT1* (San-Segundo and Roeder 2000).

In this study, the influence of the histone modifying proteins introduced above on flocculation and maltose consumption during high-gravity fermentation were analysed in three different maltose fermenting yeast strains. Strain 192 was used for studying flocculation behaviour under high-gravity fermentation conditions since in this strain, absence of the COMPASS components Spp1, Swd3 or Set1 induces flocculation after approximately 50 h of maltose fermentation (Dietvorst and Brandt 2008). Strain 99R displays flocculation even in media containing low concentrations of sugar and was therefore used to study flocculation induced by 10% glucose or 5% ethanol (Dietvorst and Brandt 2008). Strain Y55a was used for analysing maltose fermentation since absence of COMPASS significantly improved the capacity of strain Y55a to ferment high concentrations of maltose (Houghton-Larsen and Brandt 2006). We now report that HDAC Hda1 as well as HAT Gcn5 plays a role in regulating expression of the *FLO* genes preventing flocculation under high-gravity fermentation conditions. The absence of HAT Gcn5 also improved utilization of high concentrations of maltose.

Materials and methods

Yeast strains

The yeast strains used in this study are listed in Table 1. Those yeast strains that contain a single *MAL1* locus are based on the prototrophic *Saccharomyces cerevisiae* Y55 (McCusker and Haber 1988) derivative JT20149 (*MAT*a *MAL1*) that was obtained from Prof. J. M. Thevelein (KU Leuven, Belgium) and will be referred to as strain Y55a. Strain Y55a is a non-flocculent strain. Strain 192 contains a single *MAL4* locus and is only flocculent in the absence of COMPASS. Strain 99R is a maltose fermenting and flocculent strain. The *kanMX*-cassette was removed from strain

Table 1 *Saccharomyces cerevisiae* strains used in this study

Strain	Relevant genotype	Source or reference
192	*MAT*a *MAL4*	Stewart and Russell (1977)
M5845	192 *swd3::kanMX*	Dietvorst and Brandt (2008)
M5993	192 *hda1::kanMX*	This study
M6002	M5993 *swd3::natMX*	This study
M6022	192 *rpd3::kanMX*	This study
M5853	192 *sir2::kanMX*	This study
M5854	192 *sir3::kanMX*	This study
M6007	192 *sir4::kanMX*	This study
M5994	192 *hst2::kanMX*	This study
M6021	192 *gcn5::kanMX*	This study
M6034	M6021 *swd3::natMX*	This study
M6008	192 *hat1::kanMX*	This study
M5852	192 *dot1::kanMX*	This study
99R	*MAT*α *MAL*	Yeast Genetic Stock Collection
M6013	99R *swd3::kanMX*	Dietvorst and Brandt (2008)
M6025	99R *hda1::kanMX*	This study
M6024	99R *rpd3::kanMX*	This study
M6014	99R *sir2::kanMX*	This study
Y55a	*MAT*a *MAL1*	McCusker and Haber (1988)
M5675	Y55a *swd3::kanMX*	Houghton-Larsen and Brandt (2006)
M5758	Y55a *swd3::loxP*	This study
M6011	Y55a *hda1::kanMX*	This study
M6020	Y55a *rpd3::kanMX*	This study
M5764	Y55a *sir2::kanMX*	This study
M5765	Y55a *sir3::kanMX*	This study
M5773	Y55a *sir4::kanMX*	This study
M6001	Y55a *hst2::kanMX*	This study
M6019	Y55a *gcn5::kanMX*	This study
M6037	M5758 *gcn5::kanMX*	This study
M5774	Y55a *hat1::kanMX*	This study
M5776	Y55a *dot1::kanMX*	This study

M5675 by the lox-out procedure with *Cre* recombinase (Houghton-Larsen and Brandt 2006), yielding strain M5758. A PCR-based strategy (Wach et al. 1994) was used to delete *SIR2*, *SIR3*, *SIR4*, *HAT1*, *DOT1*, *GCN5*, *RPD3*, *HDA1* or *HST2* from the haploid yeast strains Y55a and 192. In addition, *SIR2*, *HDA1* or *RPD3* were deleted from the haploid yeast strain 99R. The same PCR-based strategy was used to delete *GCN5* from yeast strain M5758 and *SWD3* from yeast strains M5993 and M6021. Standard methods were used for yeast transformation (Gietz et al. 1995). Chromosomal DNA constituting the open reading frames of these genes was replaced by the *kanMX*-cassette flanked by two *loxP* sites or by the *nat1*-gene. The *kanMX*-cassette was amplified from plasmid pUG6 (Gueldener et al. 2002) and the *nat1*-gene from plasmid pAG36 (Goldstein and McCusker 1999). The oligonucleotides used for amplification of these fragments contain sequences that allow integration by homologous recombination and are listed in Table 2. All deletion strains were checked by PCR analysis as described previously (Dietvorst and Brandt 2008).

Media

Yeast cells were cultivated for 24 h in YPD (1% yeast extract, 2% peptone, 2% glucose) at 30°C. When required, 300 µg/ml G418 or 150 µg/ml nourseothricin was added. Fermentations were carried out with complex medium, containing 1% yeast extract, 2% peptone and one of the sugars maltose or glucose as described previously (Houghton-Larsen and Brandt 2006).

Fermentations

Stirred miniaerobic fermentations, containing 200 ml medium, were performed at 22°C as described previously (Dietvorst and Brandt 2008). Densities of the fermentation media were determined with a Density Meter (Anton Paar DMA35n) and expressed in degrees Plato (°P) corresponding to the percentage of sucrose by weight.

Stirred microaerobic fermentations at room temperature, containing 4 ml medium, were inoculated with 60 µl from a pre-culture grown aerobically for 72 h in YPD at 30°C.

Flocculation assays

Fermentation cultures were sampled from the middle of the tubes at the beginning and at the end of fermentation. Samples were used directly for analysis or stored on ice for maximum 1 h. Flocculation was quantified by following the absorbance during floc settling in a yeast suspension after agitation as described previously (Dietvorst and Brandt 2008). The extent of flocculation was calculated by the following formula: Flocculation (%) = $100 \times (A_{600}(\text{control}) - A_{600}(\text{sample}))/A_{600}(\text{control})$.

Semi-quantitative RT-PCR

Cells were harvested from 4 ml stirred fermentations performed in complex medium containing 20% maltose at room temperature. RNA was prepared as described previously (Houghton-Larsen and Brandt 2006) and RNA samples were treated with RNase-free DNase (USB). RT-PCR was performed using the FideliTag RT-PCR Master mix

Table 2 Oligonucleotides

Name	Sequence (5′→3′)
JD04fw_SIR2	AGA CAC ATT CAA ACC ATT TTT CCC TCA TCG GCA CAT TAA AGC TGG GCT TCG TAC GCT GCA GGT CGA C
JD04rv_SIR2	ATT GAT ATT AAT TTG GCA CTT TTA AAT TAT TAA ATT GCC TTC TAC GGC CAC TAG TGG ATC TGA TAT CAC
JD05fw_SIR3	GGG GTT TAA GAA AGT TGT TTT GTT CTA ACA ATT GGA TTA GCT AAA GCT TCG TAC GCT GCA GGT CGA C
JD05rv_SIR3	GTA CAT AGG CAT ATC TAT GGC GGA AGT GAA AAT GAA TGT TGG TGG GGC CAC TAG TGG ATC TGA TAT CAC
JD11fw_SIR4	AGG AAG CTT CAA CCC ACA ATA CCA AAA AAG CGA AGA AAA CAG CCA GCT TCG TAC GCT GCA GGT CGA C
JD11rv_SIR4	AAC AGG GTA CAC TTC GTT ACT GGT CTT TTG TAG AAT GAT AAA AAG GGC CAC TAG TGG ATC TGA TAT CAC
JD13fw_HAT1	CAG CAA AAT TAT GCT TAA GCT ATA ACT ATA GTG AGA ATC AAG AAT GCT TCG TAC GCT GCA GGT CGA C
JD13rv_HAT1	GGC TTG TTA AAC AAA TAA ATA TGT TAT TAT ATA TTT AAT AAA CAG GGC CAC TAG TGG ATC TGA TAT CAC
JD14fw_DOT1	GGT CAC CAG TAA TTG TGC GCT TTG GTT ACA TTT TGT TGT ACA GTA GCT TCG TAC GCT GCA GGT CGA C
JD14rv_DOT1	CTA CTT AGT TAT TCA TAC TCA TCG TTA AAA GCC GTT CAA AGT GCC GGC CAC TAG TGG ATC TGA TAT CAC
JD67fw_HST2	TCA CTG AGC TAC CAA CCA GAC GTA CCG CGA TCT CTC CGT GTT TCG GCT TCG TAC GCT GCA GGT CGA C
JD67rv_HST2	AAT AAG GAA AAA AAA AGG GGG ACG GAA AAC ATT GCA ACC AAC ACA GGC CAC TAG TGG ATC TGA TAT CAC
JD70fw_HDA1	GAG AAA GGG AAA GTT GAG CAC TGT AAT ACG CCG AAC AGA TTA AGC GCT TCG TAC GCT GCA GGT CGA C
JD70rv_HDA1	GGC ATG AAG GTT GCC GAA AAA AAA TTA TTA ATG GCC AGT TTT TCC GGC CAC TAG TGG ATC TGA TAT CAC
JD74fw_GCN5	CCG CCC AAA AGT CTT CAG TTA ACT CAG GTT CGT ATT CTA CAT TAG GCT TCG TAC GCT GCA GGT CGA C
JD74rv_GCN5	TTT CTT CTT CGA AAG GAA TAG TAG CGG AAA AGC TTC TTC TAC GCA GGC CAC TAG TGG ATC TGA TAT CAC
JD75fw_RPD3	TGC GCC ATA CAA AAC ATT CGT GGC TAC AAC TCG ATA TCC GTG CAG GCT TCG TAC GCT GCA GGT CGA C
JD75rv_RPD3	TTG TTT CAC ATT ATT TAT ATT CGT ATA TAC TTC CAA CTC TTT TTT GGC CAC TAG TGG ATC TGA TAT CAC
JD47fw_SWD3	TTG AGC GTA GGC TCA TTC ATT TGC A AG TGG CCA TTA TAT GCT AAC CGG GTT AAT TAA GGC GCG CCA GA
JD47rv_SWD3	GTT ACA CCT TAA GGG AAT GTC TGT CGT GTA CAT AAC GCA GAT TCT GAT ATC ATC GAT GAA TTC GAG CTC G
AB_flo1_F	AGT TGT AGT CAT GGC AGT AGT GGC TGT TGT TGG TGT TCT GAT GAC
AB_flo1_R	TCC TAT GGA AAC TGG GGA TGC AAA GGA ATG GGT GCT TGT TCT AAT
AB_flo9_F	GTT GTC TCT GCG ACT ACA GCG GCA TGC CTG CCA GCA AAC TCA AGG
AB_flo9_R	AGT AGA GGT AAA AGT GCT GTT CCA TGG CTC AGT TGT AGT TAT GAT
ACT1_F	AGA CCA AGA CAC CAA GGT ATC ATG
ACT1_R	AGC AGT GGT GGA GAA AGA GTA AC

(USB) according to the manufactures instructions. Briefly, 50 ng of DNase treated RNA and 10 μM of forward and reverse primers (Table 2) were incubated at 42°C for 30 min with the FideliTag mix containing reverse transcriptase. The resulting cDNA was linearly PCR amplified for 20 cycles of 30 s at 95°C, 30 s at 55°C and 2 min at 68°C. PCR products were analysed on 1% agarose gel in 1× TAE buffer. *ACT1* transcript for actin was used as a control. Standard PCR did not amplify DNA fragments using similar RNA preparations as templates.

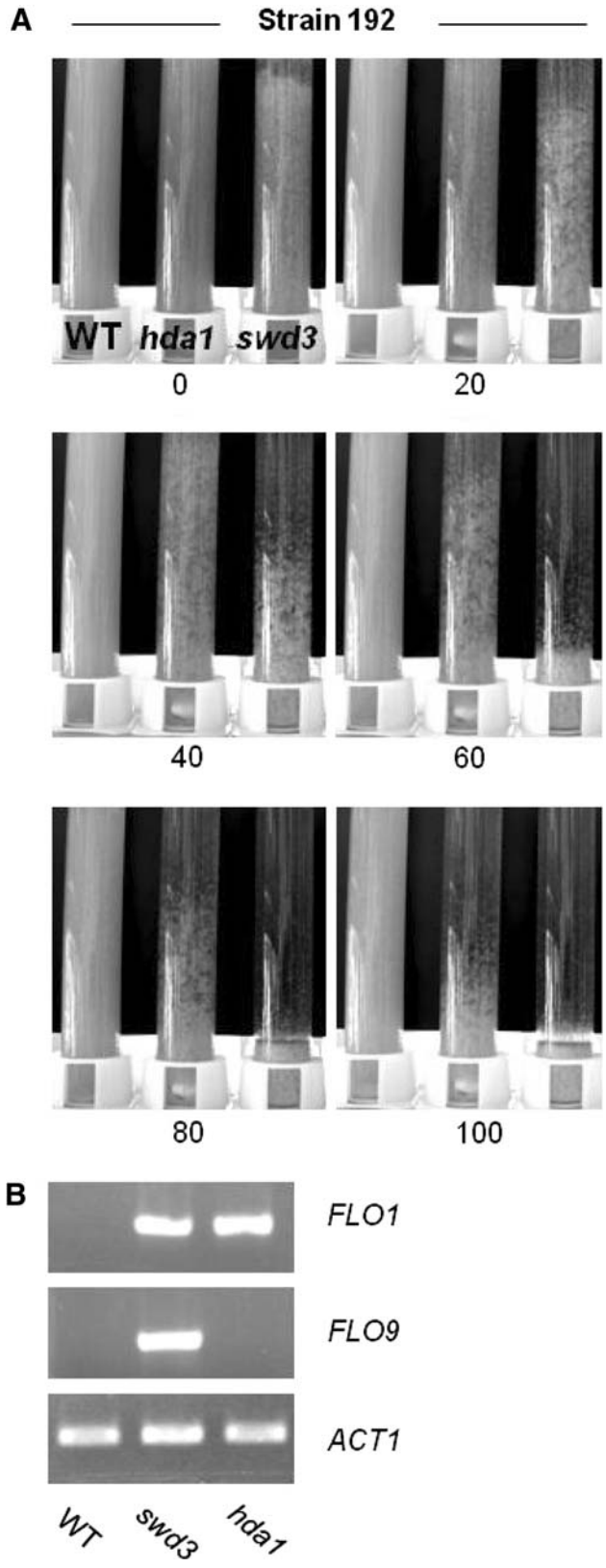


Fig. 1 Flocculation of the mutant strain 192 deleted for *HDA1* during high-gravity maltose fermentation. After 72 h fermentation of complex medium containing 21.8°P maltose, the flocculation phenotype was photographed for the wild-type strain 192 (WT) and strain 192 deleted for *HDA1* (M5993) or *SWD3* (M5845). Photographs were taken immediately after switching off the stirring-device and at 20 s intervals. The time in seconds is indicated under each photograph (**a**). Primers specific for the yeast genes *FLO1*, *FLO9* and *ACT1* were used for semi-quantitative RT–PCR amplification using 50 ng total RNA extracted from wild-type strain 192 and strain 192 deleted for *SWD3* or *HDA1* (**b**)

Results

Influence of histone deacetylases Hda1, Rpd3 and Sir2 on flocculation and fermentation during high-gravity maltose fermentation

Methylation of lysine 4 of histone H3 by COMPASS leads to subtelomeric silencing, as does deacetylation of lysine residues in the tail of histone H3 or H4 by HDACs Hda1, Rpd3 or Sir2 in *S. cerevisiae* (Rundlett et al. 1996; Krogan et al. 2002; Rusche et al. 2003; Mueller et al. 2006). Therefore, we asked if deletion of *HDA1*, *RPD3* or *SIR2* also could affect the flocculation behaviour of strain 192 or the maltose fermentation capacity of strain Y55a.

HDACs Hda1 and Rpd3 are encoded by the highly similar *HDA1* and *RPD3* genes (Rundlett et al. 1996) but only Hda1 prevented flocculation in strain 192 during high-gravity conditions. After 50 h of fermentation, large aggregates of cells were present in cultures of strain 192 deleted for *SWD3* and after approximately 70 h, cultures of strain 192 deleted for *HDA1* displayed a similar pronounced flocculation (Fig. 1a). Cells with both *SWD3* and *HDA1* deleted, showed a similar flocculation profile as strain 192 deleted for *SWD3* only (Table 3). Semi-quantitative RT-PCR analyses suggested that increased expression of *FLO1* in the *hda1* mutant, like in the *swd3* mutant, caused the flocculation (Fig. 1b). Interestingly, the expression of *FLO9* was low in the *hda1* mutant, while the expression of both *FLO1* and *FLO9* was elevated in the *swd3* mutant (Fig. 1b). The *swd3 hda1* double mutant showed a similar expression level for *FLO1* and *FLO9* as the *swd3* mutant (data not shown). The low expression of *FLO9* in the *hda1* mutant may indicate that Hda1 exerts a locus specific effect on *FLO1* expression.

Strain Y55a deleted for *HDA1* showed a similar fermentation profile as the wild-type strain Y55a (Table 3). The mutant strain deleted for *RPD3* fermented complex medium with 22.2°P maltose less complete (about 1.5°P) than the wild-type strain Y55a (Table 3) indicating that Rpd3 function to some extent influences utilisation of maltose. These results are in line with previous findings that HDACs are transcriptional repressors and deletion of *RPD3* resulted in an overall increase in the acetylation of histones H3 and H4 (Zhou et al. 2009) and an increase in silencing of reporter genes inserted near telomeres (Rundlett et al. 1996; Sun and Hampsey 1999).

Table 3 Overview of different phenotypes analysed in this study

Type of histone modifying protein	Genotype	Flocculation phenotype in strain 192	Change in maltose fermentation capacity in strain Y55a compared to wild-type (°P)	Other phenotypes
	Wild-type	+[a]	None	
	swd3	++++	+4	
HDAC	*hda1*	+++	None or −1	
	hda1 swd3	++++	+2	
	rpd3	+	−1.5	
	sir2	+	None, +1.5 or −2[b]	Abolishment of flocculation induced in strain 99R by 2% glucose plus 5% EtOH or 10% glucose
	sir3	+	None	
	sir4	+	None	
	hst2	+	None	
HAT	*gcn5*	+++	+2.5	Slower growth in YPD medium in strains Y55a, 192 and 99R
	gcn5 swd3	++++	−2	
	hat1	+	None	Improvement of maltose fermentation capacity with 2°P in strain 192
Other	*dot1*	+	None	

[a] The number of + symbols indicates the strength of flocculation

[b] Improvement or reduction of the fermentation capacity is indicated with + or − symbols, respectively

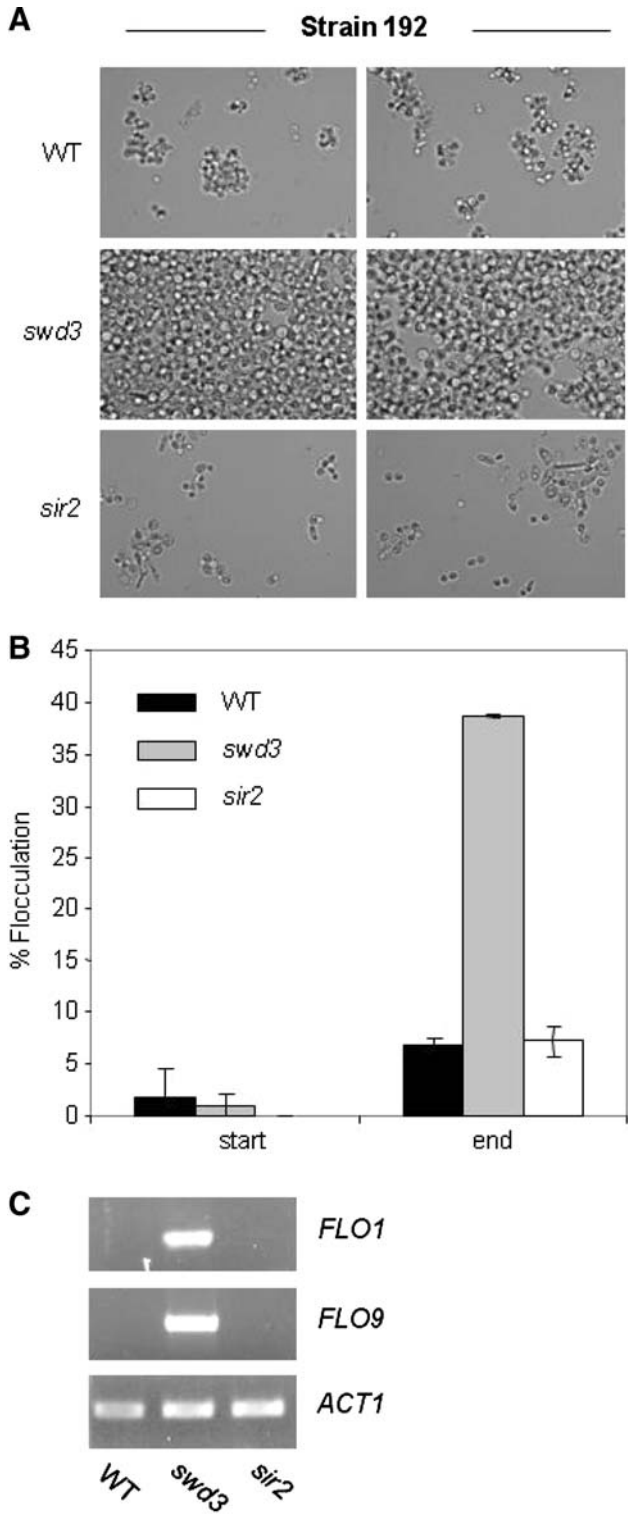
Flocculation was never detected in strain 192 deleted for *SIR2* during the high-gravity maltose fermentations. At the end of fermentation, flocs consisting of many thousands of cells were present in the *swd3* mutant, while both wild-type and *sir2* cells only formed micro-clumps of about 10–20 cells (Fig. 2a). Flocculation was also quantified using a photometric assay (Fig. 2b). The absence of a functional COMPASS resulted in a strong flocculation phenotype at the end of fermentation, while flocculation was barely detectable in the wild-type strain or the *sir2* mutant (Fig. 2b). Semi-quantitative RT-PCR analyses showed elevated expression levels of *FLO1* and *FLO9* in the *swd3* mutant while expression levels in the *sir2* mutant were similar as in the wild-type strain 192 (Fig. 2c).

Strain Y55a deleted for *SIR2* displayed both slightly enhanced and impaired high-gravity maltose fermentations (Table 3). More precisely, in two fermentation experiments the *sir2* mutant fermented maltose faster and more complete compared to wild-type Y55a while a slower and less complete fermentation of the *sir2* mutant was observed in four experiments. In addition, the *SIR2*-deleted Y55a strain often showed variation in the duplicates of stirred fermentations. Together, this suggests that the ability to ferment high concentrations of maltose is inconsistent in cells deleted for *SIR2*. Absence of the Sir2 protein has been reported to result in decreased chromosome stability and decreased lifespan (Holmes et al. 1997), which might explain the observed variations in fermentation capacity. Strain 192 deleted for *SIR3*, *SIR4*, *HST2* (homologue of Sir2) or *DOT1* (Disruptor of Telomeric silencing) did not flocculate during high-gravity fermentation nor did the deletion of these genes improve maltose fermentation capacity in strain Y55a (Table 3).

Previously, we reported that *S. cerevisiae* maltose-fermenting strain 99R displays flocculation when the glucose concentration in the growth medium is increased to 10%. Strain 99R deleted for *SWD3* even displayed a Ca^{2+}-dependent flocculation after growth in YP containing 2% glucose for 18 h at 30°C (Dietvorst and Brandt 2008). The characteristics of strain 99R were used to analyse the effect of deleting the HDACs Hda1, Rpd3 or Sir2 on flocculation induced by 10% glucose or 5% ethanol. Flocculation was induced in wild-type 99R cells when grown in 10% glucose or 2% glucose with 5% ethanol (Fig. 3). Cells deleted for *HDA1* or *RPD3* displayed similar flocculation behaviour as the wild-type while cells deleted for *SIR2* failed to flocculate (Fig. 3). This result suggests that the flocculation of strain 99R induced by high glucose concentration or ethanol, is dependent on the Sir2 protein.

Influence of histone acetyl transferases Gcn5 and Hat1 on flocculation and fermentation capacity during high-gravity maltose fermentation

The steady state level of acetylation of histones is a balance between the action of the HDACs and the histone acetyl-



◀ **Fig. 2** Lack of the silent informator regulator complex component Sir2 has no effect on flocculation of strain 192 during high-gravity maltose fermentation. Fermentation of complex medium with 20.8°P maltose with wild-type strain 192 (WT) and strain 192 deleted for *SWD3* (M5845) or *SIR2* (M5853) was carried out in duplicate, and the error bars indicate the range between duplicate experiments. Microscopy was performed on cells harvested at the end of fermentation to visualise floc formation in the wild-type strain 192 and strain 192 deleted for *SWD3* or *SIR2* (**a**). Flocculation was quantified by measuring the residual absorbance of the yeast suspension after agitation and subsequent undisturbed standing for 15 min (see "Materials and methods"). Relative flocculation levels were determined at the start and at the end of fermentation (**b**). Primers specific for the yeast genes *FLO1, FLO9* and *ACT1* were used for semi-quantitative RT–PCR amplification using 50 ng total RNA extracted from wild-type strain 192 and strain 192 deleted for *SWD3* or *SIR2* (**c**)

transferases (HATs). Parallel to the HDACs described in the previous section, the effect of the absence of HATs Gcn5 and Hat1 on the flocculation behaviour of strain 192 or the maltose fermentation capacity of strain Y55a during high-gravity maltose fermentation was investigated.

Cultures of strain 192 deleted for *GCN5* displayed pronounced flocculation after approximately 70 h of fermentation (Fig. 4a). In addition, strain 192 deleted for both *SWD3* and *GCN5* showed similar flocculation as strain 192 deleted for *SWD3* and *HDA1* or *SWD3* alone (Table 3). Semi-quantitative RT-PCR analyses showed that the expression of both *FLO1* and *FLO9* was elevated in the *gcn5* mutant compared to the levels in the wild-type strain at time points when flocculation was visible (Fig. 4b).

Strain Y55a lacking Gcn5 fermented high concentrations of maltose more complete (about 2.5°P) than the wild-type (Fig. 5a) while Y55a deleted for both *GCN5* and *SWD3* fermented high concentrations of maltose less complete (about 1.5 P) than the wild-type (Fig. 5b). However, deletion of both *SWD3* and *GCN5* resulted in severe defects in cell growth. Growth in YPD liquid medium of strain Y55a deleted for *GCN5* was slower than the wild-type strain (doubling times were 87 ± 5 min and 74 ± 1 min, respectively) while the doubling time was even longer (103 ± 2 min) for strain Y55a deleted for both *SWD3* and *GCN5*. This is in line with previously published observations (Zhang et al. 1998). From these analyses, it is not possible to determine if the reduced completeness and slower speed of high-gravity maltose fermentation resulted from these defects in cell growth or that Gcn5 is directly regulating maltose utilisation (Fig. 5b).

Deletion of *HAT1* in strain 192 did not affect flocculation and strain Y55a lacking Hat1 fermented high-gravity maltose similar as the wild-type (Table 3), indicating that the role of the histone modifying Hat1 protein is insignificant during high-gravity maltose fermentation. However, lack of Hat1 improved high-gravity fermentation performance in *S. cerevisiae* strain 192 (Table 3). COMPASS-mediated improvement of fermentation of high concentrations of maltose in strain 192 was previously found to be insignificant, possibly hidden by the heavy premature flocculation of the COMPASS mutants (Dietvorst and Brandt 2008). The other histone modifying proteins analysed in this study did not affect high-gravity maltose fermentation of strain

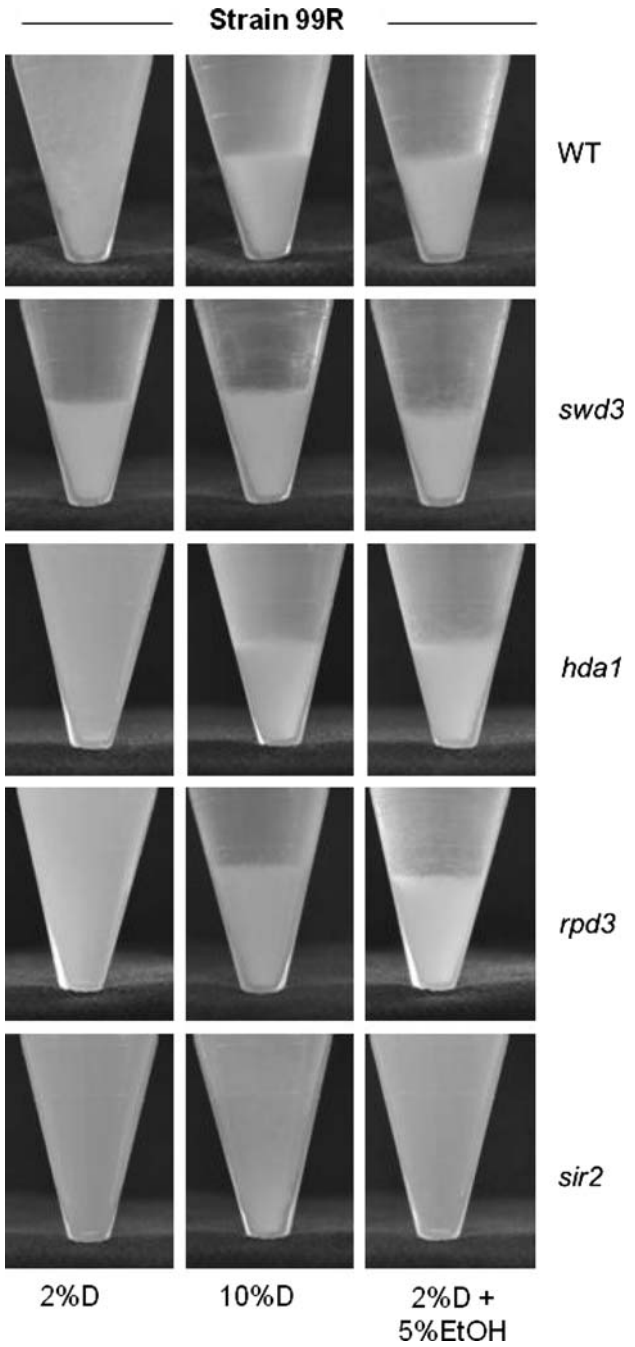


Fig. 3 Flocculation induced by high glucose concentration or ethanol is Sir2 dependent in strain 99R. Cells of wild-type strain 99R (*WT*) and strain 99R deleted for *SWD3* (*M6013*), *HDA1* (*M6025*), *SIR2* (*M6014*) or *RPD3* (*M6024*) were grown for 18 h at 30°C in 4 ml complex medium containing 2% glucose (*2%D*), 2% glucose with ethanol (*EtOH*) or 10% glucose (*10%D*). Flocculation was photographed after allowing the yeast to settle at room temperature for 5 min

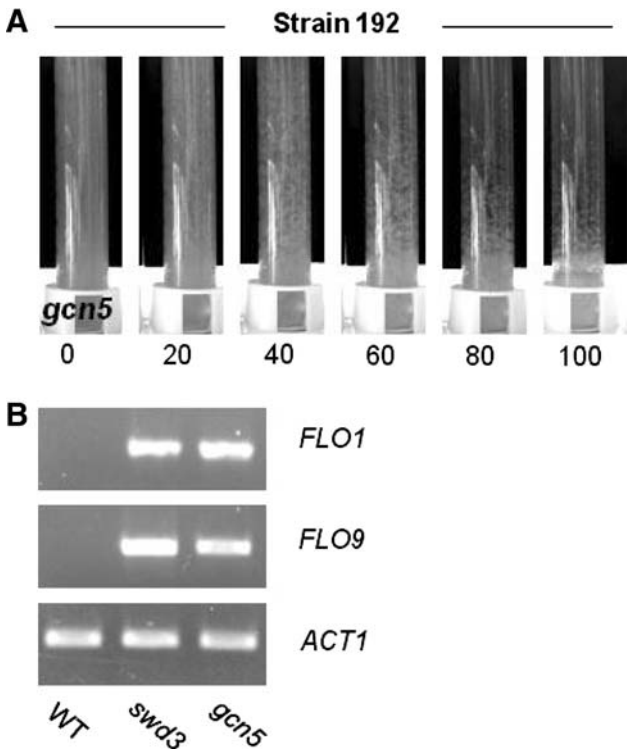


Fig. 4 Flocculation phenotype of the *gcn5* mutant in strain 192 during high-gravity maltose fermentation. After 72 h fermentation of complex medium containing 21.8°P maltose, the flocculation phenotype was photographed for strain 192 deleted for *GCN5* (*M6021*). Photographs were taken immediately after switching off the stirring-device and at 20 s intervals. The time in seconds is indicated under each photograph (**a**). Primers specific for the yeast genes *FLO1*, *FLO9* and *ACT1* were used for semi-quantitative RT–PCR amplification using 50 ng total RNA extracted from wild-type strain 192 (*WT*) and strain 192 deleted for *SWD3* (*M5845*) or *GCN5* (*M6021*) (**b**)

192 (Table 3). Together this indicates a strain specific role for the Hat1 protein in strain 192 during high-gravity fermentation, as was previously found for COMPASS-mediated silencing of the *MAL* genes that also appears to be strain-dependent (Dietvorst and Brandt 2008).

Discussion

Efficient beer fermentation requires the rapid and complete utilisation of the main fermentable sugars in wort. Due to decreased yeast performance because of environmental stresses imposed during high-gravity conditions, fermentations of complex medium containing high concentrations of maltose are often incomplete. The subtelomerically located *FLO* and *MAL* genes are important for the performance of yeast during these fermentations. An important control on the expression of these genes is COMPASS-mediated silencing of the *FLO* and the *MAL* genes. Absence of COMPASS mediated silencing leads to induced flocculation and improved maltose fermentation, respectively (Dietvorst and Brandt 2008; Houghton-Larsen and Brandt 2006).

In the present work, we analysed the effect of several other genes encoding histone-modifying proteins on flocculation and fermentation capacity (Table 3). We show in

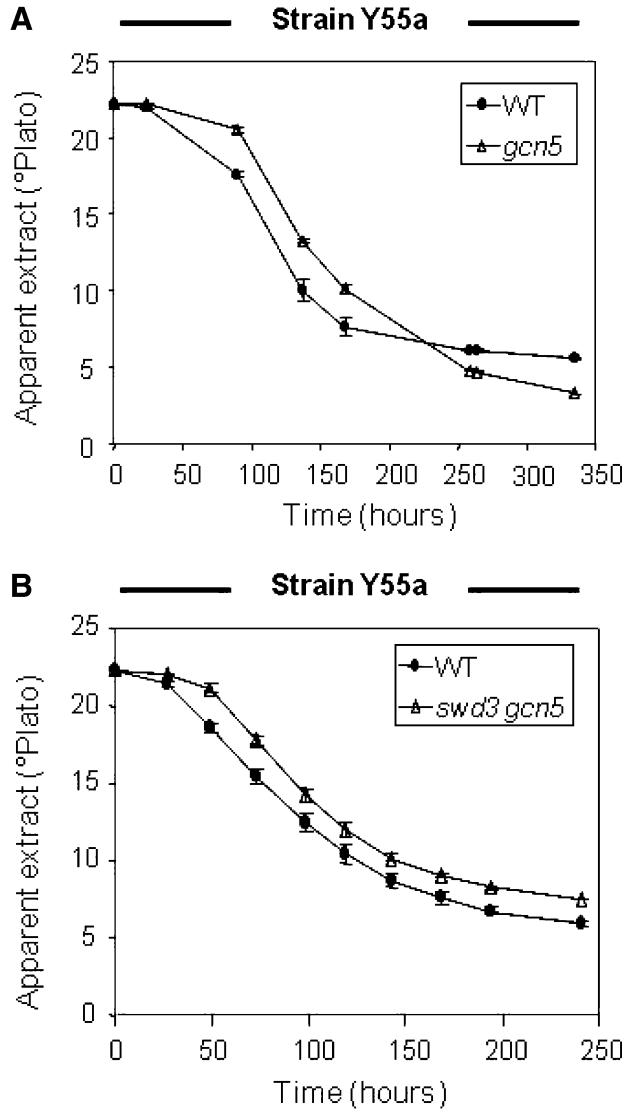


Fig. 5 Histone acetyltransferase Gcn5 affects the fermentation capacity of strain Y55a during high-gravity maltose fermentation. Fermentation of complex medium with 22°P (**a**, **b**) maltose with the wild-type strain Y55a (*WT*) and strain Y55a deleted for *GCN5* (*M6019*) (**a**) or deleted for *SWD3* as well as *GCN5* (*M6037*) (**b**). Fermentations were done in duplicate, and the *error bars* indicate the range between duplicate experiments

addition to COMPASS that the Hda1 and Gcn5 proteins are required to repress flocculation during high-gravity fermentation (Figs. 1a, 4a). Expression of *FLO1* only is induced in the absence of Hda1 (Fig. 1b) whereas the expression of both *FLO1* and *FLO9* is induced in the absence of Gcn5 (Fig. 4b). Whether these two histone-modifying proteins directly affect the transcription of the *FLO* genes is not known. However, Hda1 affects acetylation at a distance of 10–25 kb from the telomeres (Robyr et al. 2002), while Gcn5 as the catalytic subunit of three chromatin-modifying complexes, regulates transcription of numerous genes (Sterner et al. 2002; Pray-Grant et al. 2002). Subtelomeric regions affected by Hda1 appear to be continuous and both the *MAL* and the *FLO* genes are located in domains affected by Hda1 (Robyr et al. 2002). In addition to the effect on flocculation under high-gravity conditions, the absence of Gcn5 also improved efficient high-gravity maltose utilisation of strain Y55a (Fig. 5a) while Hda1 had no effect on maltose consumption. Gcn5 is required for the repression of ARG1 in rich medium (Ricci et al. 2002). These data together indicate that Gcn5 possibly affects expression of multiple genes located over regions extending 20 kb from the telomeres whereas Hda1 possibly is involved in gene specific regulation. The Flo1 protein in particular is thought to be involved in the formation of large aggregates of cells (Verstrepen and Klis 2006) and can thus alone be responsible for the strong flocculation phenotype observed during high-gravity maltose fermentations (Figs. 1, 4).

Gcn5 is a conserved histone acetyltransferase that functions as a catalytic subunit of multiple HAT complexes that primary acetylates lysine 9, 14, 18, 23, 27 and 36 of histone H3 but also lysine 11 and 16 of histone H2B and lysine 8 and 16 of histone H4 (Kuo et al. 1996; Grant et al. 1999; Suka et al. 2001; Morris et al. 2007). Both acetylation and methylation of histone lysine residues have been shown to play key roles in the regulation of chromatin structure and function, with the majority of these modifications occurring on histone H3 (Felsenfeld and Groudine 2003). The involvement of COMPASS (Dietvorst and Brandt 2008) and Gcn5 in silencing of the *FLO* and the *MAL* genes during high-gravity conditions may then be explained by a more specific role for histone H3 in silencing of genes located approximately 20 kb or more from the telomeres. Alternatively, a functional connection between histone acetylation and methylation as reported recently (Kuo et al. 2009), could also explain our data. Enhanced methylation of arginine 3 (R3) of histone H3 as a result of acetylation at any of the four acetylatable lysine residues of histone H4 by Gcn5 has been reported (Kuo et al. 2009). Thus, acetylation of histone H3 could supposedly result in more efficient methylation. In this hypothetic situation, deletion of *GCN5* will reduce acetylation of histone H3 that consequently results in reduced K4H3 methylation by COMPASS, hence derepression of the *FLO* and the *MAL* genes.

Heterochromatin-like regions, formed by the actions of the Sir proteins, are also present in subtelomeric regions. However, there is virtually no overlap between these regions affected by Sir2 and the domains affected by Hda1 (Wyrick et al. 1999; Bernstein et al. 2000; Robyr et al. 2002). In absence of the Sir2, Sir3 or Sir4 proteins, strain Y55a was only marginally affected in its ability to ferment high concentrations of maltose (Table 3) and the absence of Sir function did not induce flocculation nor induce the expression of *FLO1* and *FLO9* (Fig. 2). Since gene silencing at the telomeres requires the silencing proteins Sir2,

Sir3 and Sir4 (Gasser and Cockell 2001) and complete loss of transcriptional silencing due to absence of deacetylase Sir2 has been reported (Smith et al. 2000; Cuperus et al. 2000), a more dramatic effect of deleting *SIR2* in our study was anticipated. Possibly other HDACs maintain telomeric regions in their hypoacetylated state. Since it has been reported that global histone acetylation levels were unchanged when all five members of the *SIR2* gene family (*SIR2* and *HST1-4*) were disrupted (Smith et al. 2000), it is even more likely that the hypoacetylated histones, located approximately 20 kb from the chromosome ends, have been shielded from the action of acetylases. Since *FLO1* and *FLO9* are located 25 up to 40 kb away from the telomere ends and *MAL11*, *MAL12* and *MAL13* are located 10 up to 20 kb away, respectively, a SIR-independent form of silencing in regions stretching further away from the telomere end could be likely. That Sir possibly indirectly may influence the expression of genes located that far from the telomere as the *FLO* genes was underlined by the finding that flocculation induced by 2% glucose plus 5% ethanol or by 10% glucose in strain 99R was dependent on the presence of Sir2 (Fig. 3).

This study identified the Hda1 and Gcn5 proteins, in addition to COMPASS, as being important for regulating the *FLO* and the *MAL* genes located at a distance from the telomere. As indicated in this study caution should be exercised when analysing regulation of gene expression by histone modifying activities, in particular when analysing industrial yeast strains under high-gravity conditions. Both the complexity and the possible interplay of the different histone modifying activities are perhaps reflected in the strain specific expression of phenotypes related to maltose utilisation and flocculation. The consequences of modifying these activities appear to be highly dependent on the yeast strain background and may involve both regulated gene expression and heterochromatic silencing of genes. Thus, regulation of histone modifying activities as a tool to improve the performance of industrial yeast requires careful analyses of the particular yeast strain to prevent unpredictable phenotypic consequences and to assure that the strategy indeed will be successful in a particular industrial yeast strain.

Acknowledgments We thank Lisbeth Faldborg for excellent technical assistance.

References

Almeida RB, Almeida e Silva JB, Lima UA, Silva DP, Assis AN (2001) Evaluation of fermentation parameters during high-gravity beer production. Braz J Chem Eng 18:459–465

Bernstein BE, Tong JK, Schreiber SL (2000) Genomewide studies of histone deacetylase function in yeast. Proc Natl Acad Sci 97:13708–13713

Cuperus G, Shafaatian R, Shore D (2000) Locus specificity determinants in the multifunctional yeast silencing protein Sir2. EMBO J 19:2641–2651

Dietvorst J, Brandt A (2008) Flocculation in *Saccharomyces cerevisiae* is repressed by the COMPASS methylation complex during high-gravity fermentation. Yeast 25:891–901

Dietvorst J, Londesborough J, Steensma HY (2005) Maltotriose utilization in lager yeast strains: *MTT1* encodes a maltotriose transporter. Yeast 22:775–788

Dietvorst J, Blieck L, Brandt R, Van Dijck P, Steensma HY (2007) Attachment of *MAL32*-encoded maltase on the outside of yeast cells improves maltotriose utilization. Yeast 24:27–38

Eberharter A, Sterner DE, Schieltz D, Hassan A, Yates JR, Berger SL, Workman JL (1999) The ADA complex is a distinct histone acetyltransferase complex in *Saccharomyces cerevisiae*. Mol Cell Biol 19:6621–6631

Felsenfeld G, Groudine M (2003) Controlling the double helix. Nature 421:448–453

Gasser SM, Cockell MM (2001) The molecular biology of the SIR proteins. Gene 279:1–16

Gietz RD, Schiestl RH, Willems AR, Woods RA (1995) Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. Yeast 11:355–360

Goldstein AL, McCusker JH (1999) Three new dominant drug resistance cassettes for gene disruption in *Saccharomyces cerevisiae*. Yeast 15:1541–1553

Govender P, Domingo JL, Bester MC, Pretorius IS, Bauer FF (2008) Controlled expression of the dominant flocculation genes *FLO1*, *FLO5*, and *FLO11* in *Saccharomyces cerevisiae*. Appl Environ Microbiol 74:6041–6052

Grant PA, Eberharter A, John S, Cook RG, Turner BM, Workman JL (1999) Expanded lysine acetylation specificity of Gcn5 in native complexes. J Biol Chem 274:5895–5900

Gueldener U, Heinisch J, Koehler GJ, Voss D, Hegemann JH (2002) A second set of *loxP* marker cassettes for Cre-mediated multiple gene knockouts in budding yeast. Nucleic Acids Res 30:1–8

Holmes SG, Rose AB, Steuerle K, Saez E, Sayegh S, Lee YM, Broach JR (1997) Hyperactivation of the silencing proteins, Sir2p and Sir3p, causes chromosome loss. Genetics 145:605–614

Houghton-Larsen J, Brandt A (2006) Fermentation of high concentrations of maltose by *Saccharomyces cerevisiae* is limited by the COMPASS methylation complex. Appl Environ Microbiol 72:7176–7182

Kelly TJ, Qin S, Gottschling DE, Parthun MR (2000) Type B histone acetyltransferase Hat1p participates in telomeric silencing. Mol Cell Biol 20:7051–7058

Kodama Y, Fukui N, Ashikari T, Shibano Y (1995) Improvement of maltose fermentation efficiency: constitutive expression of *MAL* genes in brewing yeasts. J Am Soc Brew Chem 53:29

Krogan NJ, Dover J, Khorrami S, Greenblatt JF, Schneider J, Johnston M, Shilatifard A (2002) COMPASS, a histone H3 (Lysine 4) methyltransferase required for telomeric silencing of gene expression. J Biol Chem 277:10753–10755

Kuo MH, Brownell JE, Sobel RE, Ranalli TA, Cook RG, Edmondson DG, Roth SY, Allis CD (1996) Transcription-linked acetylation by Gcn5p of histones H3 and H4 at specific lysines. Nature 383:269–272

Kuo MH, Xu XJ, Bolck HA, Guo D (2009) Functional connection between histone acetyltransferase Gcn5p and methyltransferase Hmt1p. Biochim Biophys Acta 1789:395–402

Lacoste N, Utley RT, Hunter JM, Poirier GG, Côté J (2002) Disruptor of telomeric silencing-1 is a chromatin-specific histone H3 methyltransferase. J Biol Chem 277:30421–30424

Luo K, Vega-Palas MA, Grunstein M (2002) Rap1-Sir4 binding independent of other Sir, yKu, or histone interactions initiates the

assembly of telomeric heterochromatin in yeast. Genes Dev 16:1528–1539

McCusker JH, Haber JE (1988) Cycloheximide-resistant temperature-sensitive lethal mutations of *Saccharomyces cerevisiae*. Genetics 119:303–315

Morris SA, Rao B, Garcia BA, Hake SB, Diaz RL, Shabanowitz J, Hunt DF, Allis CD, Lieb JD, Strahl BD (2007) Identification of histone H3 lysine 36 acetylation as a highly conserved histone modification. J Biol Chem 282:7632–7640

Mueller JE, Canze M, Bryk M (2006) The requirements for COMPASS and Paf1 in transcriptional silencing and methylation of histone H3 in *Saccharomyces cerevisiae*. Genetics 173:557–567

Ng HH, Feng Q, Wang H, Erdjument-Bromage H, Tempst P, Zhang Y, Struhl K (2002) Lysine methylation within the globular domain of histone H3 by Dot1 is important for telomeric silencing and Sir protein association. Genes Dev 16:1518–1527

Perrod S, Gasser SM (2003) Long-range silencing and position effects at telomeres and centromeres: parallels and differences. Cell Mol Life Sci 60:2303–2318

Pray-Grant MG, Schieltz D, McMahon SJ, Wood JM, Kennedy EL, Cook RG, Workman JL, Yates JR 3rd, Grant PA (2002) The novel SLIK histone acetyltransferase complex functions in the yeast retrograde response pathway. Mol Cell Biol 22:8774–8786

Qin S, Parthun MR (2002) Histone H3 and the histone acetyltransferase Hat1p contribute to DNA double-strand break repair. Mol Cell Biol 22:8353–8365

Rautio JJ, Londesborough J (2003) Maltose transport by brewer's yeasts in brewer's wort. J Inst Brew 109:251–261

Rautio JJ, Huuskonen A, Vuokko H, Vidgren V, Londesborough J (2007) Monitoring yeast physiology during very high gravity wort fermentations by frequent analysis of gene expression. Yeast 24:741–760

Reilly DI, O'Cleirigh C, Walsh PK (2004) Laboratory-scale production of high-gravity wort suitable for a broad variety of research applications. J Am Soc Brew Chem 62:23–28

Ricci AR, Genereaux J, Brandl CJ (2002) Components of the SAGA histone acetyltransferase complex are required for repressed transcription of *ARG1* in rich medium. Mol Cell Biol 22:4033–4042

Rine J, Herskowitz I (1987) Four genes responsible for a position effect on expression from *HML* and *HMR* in *Saccharomyces cerevisiae*. Genetics 116:9–22

Robyr D, Suka Y, Xenarios I, Kurdistani SK, Wang A, Suka N, Grunstein M (2002) Microarray deacetylation maps determine genome-wide functions for yeast histone deacetylases. Cell 109:437–446

Rundlett SE, Carmen AA, Kobayashi R, Bavykin S, Turner BM, Grunstein M (1996) *HDA1* and *RPD3* are members of distinct yeast histone deacetylase complexes that regulate silencing and transcription. Proc Natl Acad Sci USA 93:14503–14508

Rusche LN, Kirchmaier AL, Rine J (2003) The establishment, inheritance, and function of silenced chromatin in *Saccharomyces cerevisiae*. Annu Rev Biochem 72:481–516

San-Segundo PA, Roeder GS (2000) Role of the silencing protein Dot1 in meiotic checkpoint control. Mol Biol Cell 11:3615

Singer MS, Kahana A, Wolf AJ, Meisinger LL, Peterson SE, Goggin C, Mahowald M, Gottschling DE (1998) Identification of high-copy disruptors of telomeric silencing in *Saccharomyces cerevisiae*. Genetics 150:613–632

Smith JS, Brachmann CB, Celic I, Kenna MA, Muhammad S, Starai VJ, Avalos JL, Escalante-Semerena JC, Grubmeyer C, Wolberger C, Boeke JD (2000) A phylogenetically conserved NAD^+-dependent protein deacetylase activity in the Sir2 protein family. Proc Natl Acad Sci USA 97:6658–6663

Sterner DE, Belotserkovskaya R, Berger SL (2002) SALSA, a variant of yeast SAGA, contains truncated Spt7, which correlates with activated transcription. Proc Natl Acad Sci 99:11622–11627

Stewart GG, Russell I (1977) The identification, characterization and mapping of a gene for flocculation in *Saccharomyces* species. Can J Microbiol 23:441–447

Suka N, Suka Y, Carmen AA, Wu J, Grunstein M (2001) Highly specific antibodies determine histone acetylation site usage in yeast heterochromatin and euchromatin. Mol Cell 8:473–479

Sun Z, Hampsey M (1999) A general requirement for the Sin3–Rpd3 histone deacetylase complex in regulating silencing in *Saccharomyces cerevisiae*. Genetics 152:921–932

Teunissen AW, Steensma HY (1995) Review: the dominant flocculation genes of *Saccharomyces cerevisiae* constitute a new subtelomeric gene family. Yeast 11:1001–1013

van Leeuwen F, Gafken PR, Gottschling DE (2002) Dot1p modulates silencing in yeast by methylation of the nucleosome core. Cell 109:745–756

Verstrepen KJ, Klis FM (2006) Flocculation, adhesion and biofilm formation in yeasts. Mol Microbiol 60:5–15

Vidgren V, Ruohonen L, Londesborough J (2005) Characterization and functional analysis of the *MAL* and *MPH* loci for maltose utilization in some ale and lager yeast strains. Appl Environ Microbiol 71:7846–7857

Wach A, Brachat A, Pöhlmann R, Philippsen P (1994) New heterologous modules for classical or PCR-based gene disruption in *Saccharaomyces cerevisiae*. Yeast 10:1793–1808

Wu J, Carmen AA, Kobayashi R, Suka N, Grunstein M (2001) HDA2 and HDA3 are related proteins that interact with and are essential for the activity of the yeast histone deacetylase HDA1. Proc Natl Acad Sci 98:4391–4396

Wyrick JJ, Holstege FC, Jennings EG, Causton HC, Shore D, Grunstein M, Lander ES, Young RA (1999) Chromosomal landscape of nucleosome-dependent gene expression and silencing in yeast. Nature 402:418–421

Zhang W, Bone JR, Edmondson DG, Turner BM, Roth SY (1998) Essential and redundant functions of histone acetylation revealed by mutation of target lysines and loss of the Gcn5p acetyltransferase. EMBO J 17:3155–3167

Zhou J, Zhou BO, Lenzmeier BA, Zhou JQ (2009) Histone deacetylase Rpd3 antagonizes Sir2-dependent silent chromatin propagation. Nucleic Acids Res 37:3699–3713