Title

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Author(s)

Yasuhiro Matsuo 1, Katsunori Tanaka, Hideyuki Matsuda, Makoto Kawamukai

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cda1^{+}, encoding chitin deacetylase is required for proper spore formation in *Schizosaccharomyces pombe*

Yasuhiro Matsuo, Katsunori Tanaka, Hideyuki Matsuda and Makoto Kawamukai*

Department of Applied Bioscience and Biotechnology, Faculty of Life and Environmental Science, Shimane University, 1060 Nishikawatsu, Matsue 690-8504, Japan

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*Corresponding author. Mailing address: Department of Applied Bioscience and Biotechnology, Faculty of Life and Environmental Science, Shimane University, 1060 Nishikawatsu, Matsue 690-8504, Japan. Phone and fax: +81-852-32-6587. E-mail: kawamuka@life.shimane-u.ac.jp.

Abstract

In *Schizosaccharomyces pombe*, a major role of chitin is to build up a complete spore. Here, we analyzed the *cda1*$^{+}$ gene (SPAC19G12.03), which encodes a protein homologous to chitin deacetylases, to know whether it is required for spore formation in *S. pombe*. The homothallic Δ*cda1* strain constructed by homologous recombination was found to form a little amount of abnormal spores that contained one, two, or three asci, similar to (but not as strong as) the phenotype observed in a deletion mutant of *chs1* encoding chitin synthase. This phenotype is reversed by expression of *S. cerevisiae* chitin deacetylase *CDA1* or *CDA2*, suggesting that *cda1* encodes a chitin deacetylase. To support the role of Cda1 in sporulation, the timing of expression of *cda1*$^{+}$ mRNA increased during sporulation process. We also found that the Cda1 protein self-associated when its binding was tested both by two-hybrid system and immuno-precipitation. Thus, these data indicated that *cda1*$^{+}$ is required for proper spore formation in *S. pombe*.

1. Introduction

Chitosan is produced by chitin deacetylase from chitin, a polymer of *N*-acetyl glucosamine, in microorganisms. Some fungi, such as *Saccharomyces cerevisiae* and *Mucor rouxii,* possess chitin deacetylase whose genes were cloned and analyzed [1-3]. Chitin deacetylases from those fungi were similar in their amino acid sequences with rizobial NodB protein [4]. *S. cerevisiae* has two chitin deacetylases called chitin deacetylase I (Cda1p) and II (Cda2p) [2, 3]. Both Cda1p and Cda2p have been shown to retain activities to synthesize chitosan from chitin to build up the proper ascospore wall [5]. Especially, Cda2p was purified and found to be a glycoprotein with optimum temperature for activity is 50ºC [6]. While chitin is an essential component for vegetative growth in *S. cerevisiae,* chitosan is not [2,3]. However, in spore wall formation, both chitin synthesis and chitin deacetylation are required. Chitin is synthesized by three chitin synthases Chs1, Chs2 and Chs3 in *S. cerevisiae*, among them Chs3p plays a major role [7]. Conversion of chitin to chitosan by either Cda1 or Cda2 allowed to make the second layered structure of the spore wall next to the outer dityrosine layer [3]. This chitosan based structure is believed to be important for spores to retain its structural rigidity and resistance to various stresses [3].

While many studies on chitin in *S. cerevisiae* has been conducted, only few studies on chitin of *S. pombe* were reported. It was reported that a major role of chitin in *S. pombe* is to build up a proper spore from the observation that chromosomal deletion of *chs1*$^{+}$ encoding chitin synthase I caused the cells to make aberrant spore formation [8, 9]. *S. pombe* has second chitin synthase called Chs2, but its role is not yet established. Chs2 was assumed to be involved in septum formation, but the activity as a chitin synthase in Chs2 was not detected enzymatically [10]. Genes for four putative regulatory factors (Chr1-4) of chitin synthase were analyzed, but the relevance with

chitin synthase with those was only limited [11]. The role of chitin as a cell wall component in *S. pombe* is probably minor, but the observation that expression of exogenous chitinase in *S. pombe* induced cell elongation may not exclude the role of chitin in cell shape [12].

Because it was not known the existence and function of chitin deacetylase in *S. pombe,* in this study, we aimed the analysis of *cda1*$^{+}$ which encodes chitin deactylase in *S. pombe*. We here show that spore formation of a *cda1* disruptant was abnormal and expression of *cda1* mRNA increased during sporulation process.

2. Materials and methods

2.1. Strains, media, and genetic methods

Yeast strains used in this study are listed in Table 1. Fission yeast cell was grown in YEA medium or MM minimal medium [13]. Supplements (adenine, leucine and uracil) were added to MM medium when it was required [13]. Malt extract medium MEA was used for mating and sporulation [13]. Plasmid pREP1 was used as a shuttle vector [14]. Plasmid pDS472a [15] and pSLF172L [16] were used as a tag fusion vector. Fission yeast cells were transformed by a high-efficiency protocol, as described previously [16].

Budding yeast L40 [17] was grown in YPD medium or the minimal medium (SC). pBTM116 [16] and pGAD424 [16] were used for two-hybrid analysis. *E. coli* DH5α was used as the host strain for construction of various plasmids. Plasmid pBluescript II KS+ (Stratagene), and pT7Blue-T (Novagen) were used as the cloning vector. Standard methods were used for DNA manipulations [18].

2.2. Construction of the cda1^{+} *gene disrupted strains and plasmids*

To clone *cda1*$^{+}$, the sense primer ym61 (5'-CAGAGGTACCTCTCTACATTC-3'; *Kpn*I site is underlined) and the antisense primer ym62 (5'-

CCCTGAGCTCCTTTAGCC-3'; *Sac*I site is underlined) were used for PCR cloning. The amplified fragment containing the *cda1*$^+$ gene was cloned into pBluescript II KS+, and the resulting clone was named pKS-cda1. To obtain a disruptant of *cda1*, pKS-cda1 was digested with *Bam*HI, ligated with the *S. pombe ura4*$^+$ [19] or *S. cerevisiae LEU2* marker [19]. *S. pombe* SP870 [19] was transformed by these fragments, and the resulting strains were named YM802 (*cda1::ura4*$^+$) and YM510 (*cda1::LEU2*). To construct double disruptant of the *chs1* and *cda1* genes, YM625 (Δ*chs1::ura4*$^+$) [9] was mated with YM510, and double disrupted strain was isolated by selecting proper nutritional requirement. The resulting strain was named YM815 (Δ*chs1::ura4*$^+$, Δ*cda1::LEU2*).

To construct pREP1-cda1, the *cda1*$^+$ gene was amplified by PCR using ym63 (5'-AAAGTCGACTTATGTATGAAACACGCGAT-3'; *Sal*I site is underlined) and ym62 primers. The PCR product was first cloned in pBluescript II KS+ and the fragment from this pBluescript II KS+ derivative was ligated with *Sal*I and *Sma*I digested pREP1. To express *S. cerevisiae CDA1* or *CDA2* in *S. pombe*, the ScCDA1 sense primer ym64 (5'-AAAGTCGACATGAGAATACAACTAAATAC-3'; *Sal*I site is underlined) and the ScCDA1 antisense primer ym65 (5'-TTTGGATCCTTAGGACAAGAATTCTTTTAT-3'; *Bam*HI site underlined), the ScCDA2 sense primer ym66 (5'-AAAGTCGACATGAAAATTTTCAATACAA-3'; *Sal*I site is underlined) and the ScCDA2 antisense primer ym67 (5'-TTTGGATCCCTAGTCGTAGCGTTCGATG-3'; *Bam*HI site underlined) were used for PCR cloning. The amplified fragments were first cloned into pT7Blue-T and the fragments from these pT7Blue-T derivatives were ligated with *Sal*I and *Bam*HI digested pREP1.

To analyze protein-protein interaction using two-hybrid system, pKS-cda1orf was

digested with *Sal*I and *Pst*I, and ligated into *Sal*I and *Pst*I sites of pBTM116 and pGAD424, thus creating pBTM116-cda1 and pGAD424-cda1, respectively. To analyze protein-protein interaction in *S. pombe*, ORF encoding the *cda1*$^{+}$ gene was amplified by PCR with ym63 and ym68 (5'-AAAGCGGCCGCACGCTTTGTAAGGATGC-3'; *Not*I site is underlined) primers. The PCR product was digested with *Sal*I and *Not*I, and ligated into the *Sal*I and *Not*I site of pBluescript II KS+. Constructed plasmid pKS-cda1orf2 was digested with *Xho*I and *Not*I, and was ligated with *Xho*I and *Not*I digested pDS472a, thus creating pDS472a-cda1. pSLF172L-cda1 was constructed in the same way as pDS472a-cda1.

2.3. Sporulation

To analyze sporulation efficiency, the colonies were patched on sporulation medium (MEA) plates at 30°C for 30 h or MMA (MM+Adenine) plate at 25 °C for 4 days. Spore formation was estimated by counting the approximately 1,000 cells under the microscope.

2.4. Northern blot analysis

Total RNAs were prepared from cells grown under different conditions using ISOGEN (Nippon gene). An approximately equal amount of total RNA (10 μg) was loaded in each lane, electrophoresed, and transferred to Hybond-N+ membranes (Amersham Pharmacia biotech). Hybridization was carried out as described previously [20] using the 1.0 kb *Sal*I-*Sma*I fragment (*cda1*$^{+}$) from pBluescript II KS-cda1orf or the 1.6 kb *Sty*I-*Sna*BI fragment (*leu1*$^{+}$) from pJK148 [21] as a probe. DNA probes were labeled with [a-^{32}P]dCTP (Amersham Pharmacia biotech) by *Bca* BEST^{TM} Labeling Kit (TaKaRa). For quantitative analysis, Northern blots were detected with the image analyzer, BAS1500-Mac (Fuji Film Co.).

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2.5. Yeast two-hybrid analysis

S. cerevisiae L40 cells were co-transformed with bait (pBTM116) and prey (pGAD424) plasmids or their derivative that contain the *cda1* fragment by the lithium acetate and plated on the synthetic dextrose medium (SC) lacking leucine and tryptophan. Transformants were tested for positive interactions by plating on SC medium lacking leucine, tryptophan, and histidine but containing 5 mM 3-amino-1,2,4-triazole (3AT) and incubated at 30°C.

2.6. Immunoprecipitation

S. pombe diploid, TP4 cells were co-transformed with pSLF172L and pDS472a plasmids or their derivative that contain the *cda1* fragments by the lithium acetate method and plated on MM+thiamine medium. Immuno-precipitation was carried out as described previously [22]. Anti-HA monoclonal antibody sc-7392 (Santa Cruz Biotechnology) against HA was used for immunoprecipitation of HA fusion. 40 μl of protein G Sepharose beads (50% slurry; Amersham Pharmacia) and 1 μl of anti-HA antibody were added and incubated at 4 ˚C for 1hr. This mixture was added into the cell extracts and incubated at 4 ˚C for 1hr. The beads was washed three times with 0.5 ml lysis buffer and resuspended in 20 μl lysis buffer. To pull down using glutathione beads, the extracts were incubated with 40 μl of glutathione Sepharose beads (50% slurry; Amersham Pharmacia) for 1 hr, washed three times with 0.5 ml lysis buffer and resuspended in 20 μl lysis buffer.

One volume of 4 x loading buffer was added to each sample. Samples were heated at 90 °C for 5 min. An approximately equal amount of each sample were analyzed by

SDS-polyacrylamide gel electrophoresis using 12% polyacrylamide gels and then transferred to Immobilon transfer membranes (Millipore) by using a wet-type transfer system. Anti-HA monoclonal antibody sc-7392 and anti-GST polyclonal antibody sc-459 were purchased from Santa Cruz Biotechnology. Horseradish peroxidase conjugated goat anti-mouse IgG and anti-rabbit IgG secondary antibodies were purchased from Santa Cruz and Bio-Rad, respectively. Detection of secondly antibodies were done with the ECL system as described by the manufacturer (Amersham Pharmacia Biotech).

3. Results

3.1. Cloning of SPAC19G12.03 gene encoding a chitin deacetylase homolog

While chitin deacetylase is known to be involved in spore wall formation in *S. cerevisiae* [3,5], nothing is known about chitin deacetylase in *S. pombe*. Then, we aimed to analyze the role of chitin deacetylase in *S. pombe*. We first searched for the gene encoding putative chitin deacetylase of *S. pombe* in Sanger center database (http://www.sanger.ac.uk/Projects/S_pombe/) and found one gene (SPAC19G12.03), which we named *cda1*, has homology with several NodB proteins which are already known to function as chitin deacetylase [23-25]. The *cda1* gene codes for a protein of 320 amino acids with a predicted molecular mass of 36.2 kDa. The c*da1* gene has the putative polysaccharide deacetylase domain which is about 27% identical with *Rhizobium* genus NodB (Fig. 1).

3.2. Spore morphology of cda1 disruptant

To know the relevance of putative chitin deacetylase with spore formation in *S. pombe*, we first examined the effect of the *cda1* deletion on the sporulation process.

Deletion strains of *cda1* and *cda1* with *chs1* were derived from its parental wild type strain as described in Material and Methods (Fig. 1). Proper disruption was verified by southern blot analysis and they were used for analysis of their phenotypes. Four strains SP870 (wild-type), YM625 (Δ*chs1*), YM802 (Δ*cda1*), and YM815 (Δ*chs1*Δ*cda1*) were pre-grown on the rich medium and transferred to sporulation inductible medium (MEA) to count for spore formation. YM625 (Δ*chs1*) is known to form abnormal spores as previously described [8,9]. Comparing to the ratio of abnormal spores of YM625 (Δ*chs1*) formed, YM802 (Δ*cda1*) formed a lower amount of abnormal spores which contain asci of one, two, or three, but apparently it was different from wild type strain (Fig. 2). YM802 (Δ*cda1*) formed about 46% normal spores, each of which contained four asci but only a small numbers of cells (about 3% of cells) formed abnormal spores which contain asci of one, two, or three (Table 2). The *chs1 cda1* double disruptant (YM815) formed abnormal spores with the same extent as YM625 (Δ*chs1*) did, indicating the phenotype of YM802 (Δ*cda1*) is totally depend on chitin synthase I (Fig. 2D and Table 2). Thus it indicates that the $cda1^{+}$ gene is required for normal sporulation process but requirement of $cda1^{+}$ is less stronger than $chs1^{+}$.

To know Cda1 is the functional equivalent of chitin deacetylase, we next tested whether the phenotype of the *cda1* disruptant is reversed by exogenous *S. cerevisiae CDA1* or *CDA2* that encode chitin deacetylase [2, 3]. While YM802 (Δ*cda1*) harboring pREP1 (vector alone) still formed the same extent of abnormal spores, equal to YM802 alone, YM802 harboring pREP1-ScCDA1, pREP1-ScCDA2, and pREP1-cda1, which express *ScCDA1*, *ScCDA2*, and $cda1^{+}$, respectively, formed normal spores comparable to those of wild type strain SP870 (Table 2). Thus the genes for *S. cerevisiae* chitin deacetylase can functionally complement *S. pombe cda1* disruptant, suggesting chitin deacetylase from both yeasts have similar functions.

3.3. Expression of cda1⁺ changed during sporulation process

Since the *cda1* gene is required for normal spore formation, we next analyzed the timing of the expression of *cda1⁺* during sporulation process. The diploid wild-type strain TP4 were cultured and induced to enter meiosis by shifting the culture to MMLU-N medium at 25 ˚C. As shown in Fig. 3, the expression of *cda1⁺* mRNA increased from 12 hr after meiosis was induced under the condition when about 20 % cells were sporulated, and became maximum at 21 hr when 40 % cells were sporulated (Fig. 3). This expression profile is reasonable to think that *cda1⁺* is required only for sporulation process.

3.4. Cda1 interacted with itself

During our analysis to find out the interacting protein with Cda1, we found the Cda1 protein interacted with Cda1 itself by two-hybrid system. *S. cerevisiae* L40 cells harboring pBTM116-cda1 and pGAD424, or pBTM116 and pGAD424-cda1 could not grow on the SC-L-W-H containing 3AT, but *S. cerevisiae* L40 harboring pBTM116-cda1 and pGAD424-cda1 grew on the same plate (Fig. 4A). To confirm this two-hybrid interaction, we next tested whether Cda1 protein associate itself within *S. pombe* cells. To do so, we constructed plasmids pDS472a-cda1 and pSLF172L-cda1 for making two differently tagged Cda1 as described in Materials and Methods. Both tagged proteins were detected as calculated molecular weight by western blotting (Fig. 4B). Immunoprecipitations were performed with cells containing a pair of plasmids (pDS472a or pDS472a-cda1 and either pSLF172L or pSLF172L-cda1) using either an anti-HA antibody or GSH-beads (see Materials and Methods), and coprecipitants were immunoblotted with anti-HA and anti-GST antibodies. Western blotting with anti-HA

antibodies detected Cda1-HA, which was precipitated with Cda1-GST using GSH-beads (Fig. 4B). At the same time, Cda1-GST was detected using anti-GST after it was precipitated with Cda1-HA using anti-HA (Fig. 4B). These results indicate that the Cda1 protein self-associates in *S. pombe* cells.

4. Discussion

The requirement of chitin in proper sporulation process of fission yeast was reported by two different groups [8, 9]. But, the role of chitosan in fission yeast was not studied yet. It has been known that chitosan is produced from chitin by chitin deactylase in several fungi including *S. cerevisiae* [1-3]. In this study, we showed that chitin deacetylase of fission yeast is required for proper formation of spores although the requirement of it was not as strong as chitin synthase I (Chs1). In contrast to the fact that chitin is essential for growth and deacetylation of chitin is required for proper spore formation in *S. cerevisiae* [3, 26, 27], chitin is not essential for growth and is only required for proper spore formation in *S. pombe* [8, 9]. A major difference between the two yeasts regarding with cell wall structure is that a-glucan is not found in *S. cerevisiae*. It is known that the major cell wall structural components of *S. pombe* are ß-glucan, α-glucan, and galactomannan [28], and the cells can not survive when α-glucan or ß-glucan synthesis during vegetative growth is absent [29, 30]. The difference of cell wall components in two yeasts presumably affects the different requirement of chitin in cell growth in two yeasts.

We showed that the expression of *cda1* is induced in the sporulation process 12 hr after meiosis was inducted. This result also supported the role of chitosan in spore formation. The data of the microarray expression profile of *cda1* during meiosis indicated the sharp peak at the start of meiosis

(http://www.sanger.ac.uk/Projects/S_pombe/). This peak may correspond to the band we observed at 0-2hr (Fig. 3), but there is no microarray data 12hr after meiosis was induced, which we need to compare with our result.

We showed the self interaction of Cda1 in fission yeast, but its significance is not clear. Since monomeric chitin deactylase from *S. cerevisiae* was shown to be active when expressed in *E. coli* [6], it may not be necessary for the dimeric form of Cda1 in *S. pombe* to be active. But we believe some benefit must exist for self interaction of Cda1 in *S. pombe,* which need to be clarified by a future study.

Our finding that chitin deacetylase is required for proper spore formation in *S. pombe* is consistent with the role of chitin deacetylase found in *S. cereviase*. On the contrary, the role of chitin in mitotic cell growth was not found in *S. pombe* as i) no mitotic growth defect were found in a *chs1, chs2* double disruptant in *S. pombe*[9], ii) no obvious mitotic growth defect were found in the quadruple mutant of four putative chitin regulatory factors (Chr1-Chr4) [11], and iii) deletion of putative chitinase gene *chi1* (SPAPB1E7.04c) in *S. pombe* did not give any clear phenotype in cell growth and cell separation (our observation), while chitinase plays the role of cell separation in *S. cerevisiae* [26]. Thus, our results combined with the previous results indicated that the roles of chitin as well as chitosan in fission yeast is limited to spore formation process, but not in the mitotic cell growth. Since *S. cerevisiae* and *S. pombe* are thought to diverge one billion years ago, the analysis of both yeasts are useful to think the divergence and common property of cell wall among lower eukaryotes.

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Figure Legends

Fig. 1. (A) Alignments of putative polysaccharide deacetylase domain. The black regions indicate identical residues between *S. pombe* Cda1 and the other proteins. Dashes indicate gaps introduced to optimize alignment. The number indicates amino acid numbers for each protein sequence. RvNodB; chitooligosaccharide deacetylase (Nodulation protein B) from *R. leguminosarum bv. viciae* (Accession No. P04339) [23], RoNodB; chitooligosaccharide deacetylase (Nodulation protein B) from *Mesorhizobium loti* (Accession No. Q52845) [24], RpNodB; chitooligosaccharide deacetylase (Nodulation protein B) from *R. leguminosarum bv. phaseoli* (Accession No. P24150) [25], ScCDA1; chitin deacetylase from *S. cerevisiae* (Accession No. Q06703) [3], ScCDA2; chitin deacetylase from *S. cerevisiae* (Accession No. Q06702) [3], Spcda1; chitin deacetylase from *S. pombe* (Accession No. CAB10114). (B) Schematic depiction of various strains used in this study to analyze the function of *cda1*$^{+}$.

Fig. 2. Spore morphology of wild-type and *cda1* disruptants. SP870 (wild-type) (A), YM625 (Δ*chs1*) (B), YM802 (Δ*cda1*) (C), and YM815 (Δ*chs1cda1*) (D) were incubated on sporulation medium (MEA) at 30˚C for 30 h. Spores were observed by microscopy with a Zeiss Axioskop microscope at a magnification of 1,000. Arrows showed abnormal asci.

Fig. 3. Expression of *cda1*$^{+}$ mRNA during sporulation process. Diploid TP4 cells were cultured in minimal medium (MM+Leu+Ura) at 30˚C for 16hr, then washed by water once and minimal medium once, cells were grown in the minimal medium (MM+Leu+Ura-N) at 25˚C for 34hr. Total RNAs were extracted from cells as described in Materials and Methods. The sporulation index (percentage of spore

formation) was calculated by counting the number of cells and the number of spore. The *cda1*$^{+}$ gene was used as the probe and the *leu1*$^{+}$ gene was used as the loading control. Northern blotting was done as described in Materials and Methods. 802 indicate YM802 (Δ*cda1::ura4*) which was used as a negative control of the *cda1*$^{+}$ mRNA.

Fig. 4. Cda1 protein associated itself. (A) The vector pGAD424 expressing the *GAL4*-activation domain alone or fused to *cda1*$^{+}$, was co-transformed with the vector pBTM116 expressing the *lexA* DNA-binding domain fused to *cda1*$^{+}$ into *S. cerevisiae* strain L40 in the indicated combinations. Protein-protein interactions were detected by assaying the ability of cells to grow in the absence of histidine. (B) Self-interaction of Cda1 in *S. pombe* was detected. Immunoprecipitation were performed with either GSH-beads (lane1, 2, and 3) or anti-HA antibody (lane4, 5, and 6), and were analyzed by western blotting with either anti-GST antibody (a) or anti-HA antibody (b). Asterisks indicate the IgG.

Table 1 Yeast strains used in this study

Strain	Relevant genotype	Reference
S. pombe		
SP870	h^{90}, *ade6-M210*, *ura4-D18*, *leu1-32*	[19]
TP4	h^{+}/h^{-}, *ade6-M216/ade6-M210, ura4-D18/ura4-D18, leu1-32/leu1-32, his2/his2*$^{+}$	lab.stock
YM162	h^{90}, *ade6-M216*, *ura4-D18*, *leu1-32*, Δ*chi1*::*kanMX6*	This study
YM625	h^{90}, *ade6-M210*, *ura4-D18*, *leu1-32*, Δ*chs1::ura4*$^{+}$	[9]
YM802	h^{90}, *ade6-M210*, *ura4-D18*, *leu1-32*, Δ*cda1::ura4*$^{+}$	This study
YM510	h^{90}, *ade6-M210*, *ura4-D18*, *leu1-32*, Δ*cda1::LEU2*	This study
YM815	h^{90}, *ade6-M210*, *ura4-D18*, *leu1-32*, Δ*chs1::ura4*$^{+}$, Δ*cda1::LEU2*	This study
S. cerevisiae		
L40	*MATa, ade2, his3, leu2, trp1, LYS2::lexA-HIS3, URA3::lexA-lacZ*	[17]

Table 2 Sporulation of *cda1* disruptant

SP870, YM625, YM802, and YM815 were patched on sporulation medium (MEA) at 30˚C for 30 h. YM802 was transformed with pREP1, pREP1-ScCDA1, pREP1-ScCDA2, or pREP1-cda1 and was patched on MM-Thiamine at 25˚C for 4 days. Spore formation was estimated by counting under the microscope. At least 1,000 cell were counted in each case. Values were the means and standard deviations calculated from three independent experiments. Tetra, tri, di, and mono refer to spores with 4, 3, 2, and 1 asci, respectively. Total refers to the sum of all sporulated cells.

Strain	Plasmid	tetra	tri	di	mono	total (%)
SP870	-	65.25±2.35	-	-	-	65.25±2.35
YM625	-	4.93±2.46	4.40±1.34	3.67±0.25	1.87±1.39	14.87±2.45
YM802	-	46.60±5.16	2.43±0.29	1.13±0.09	0.13±0.19	51.50±5.99
YM815	-	6.13±2.20	5.87±2.60	6.27±0.83	3.33±1.40	21.60±6.04
YM802	vector	54.90±5.73	3.60±2.11	1.07±0.83	0.60±0.53	60.17±7.40
YM802	ScCDA1	56.20±9.87	-	-	-	56.20±9.87
YM802	ScCDA2	47.67±7.94	-	-	-	47.67±7.94
YM802	cda1	61.04±8.53	-	-	-	61.04±8.53

A

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RvNodB   38  QILDVLAEHR  VPATFFAIGS  YVKDHPELIR  RLVAEG-HDV   76
RoNodB   38  QILDVLAQNR  VPATFFVIGA  YAAEHPELIQ  RMIAEG-HEV   76
RpNodB   38  EILDILAEHR  VPATFFVIGE  FLADQSKLIQ  RMIAEGHHEV   77
ScCDA1  104  DCHHCTEHDD  VYTCSKLSQT  FDDGPSASTT  KLLDRLKHNS  143
ScCDA2   94  DCYNCIDVDD  VTSCFKLSQT  FDDGPAPATE  ALLKKLRQRT  133
Spcda1   85  RILNLFKKHK  VPFTCWAIGQ  AVEKNPVVVG  AMEEAG-CEV  123

RvNodB   77  ANHTMTHPDL  ATCDPKDVKR  EIDEAHQAIV  SACPQALVRH  116
RoNodB   77  GNHTMSHPDL  SKCGLGEVQR  EVFEANQAIM  LACPQASIRY  116
RpNodB   78  ANHTMTHPDL  SDCEPRRVQR  QILETNRAIK  MASPGGGAAH  117
ScCDA1  144  TFFNLG---V  NIVQHPDIYQ  RMQKEGHLIG  SHTWSHVYLP  180
ScCDA2  134  TFFVLG---I  NTVNYPDIYE  HILERGHLIG  THTWSHEFLP  170
Spcda1  124  GSHSHRWINY  EGVPPETEYE  HIKKSVQAIQ  KASPSNSAPR  163
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B

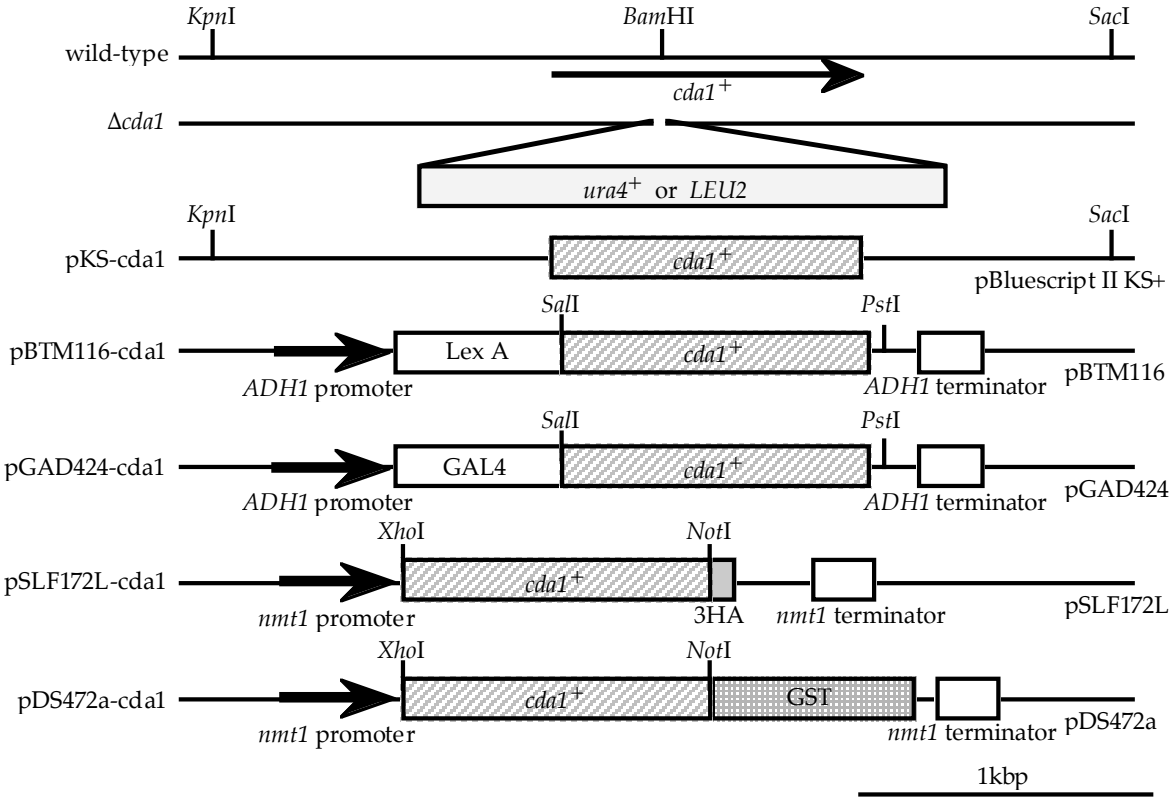

Fig. 1 Y. Matsuo *et al.*

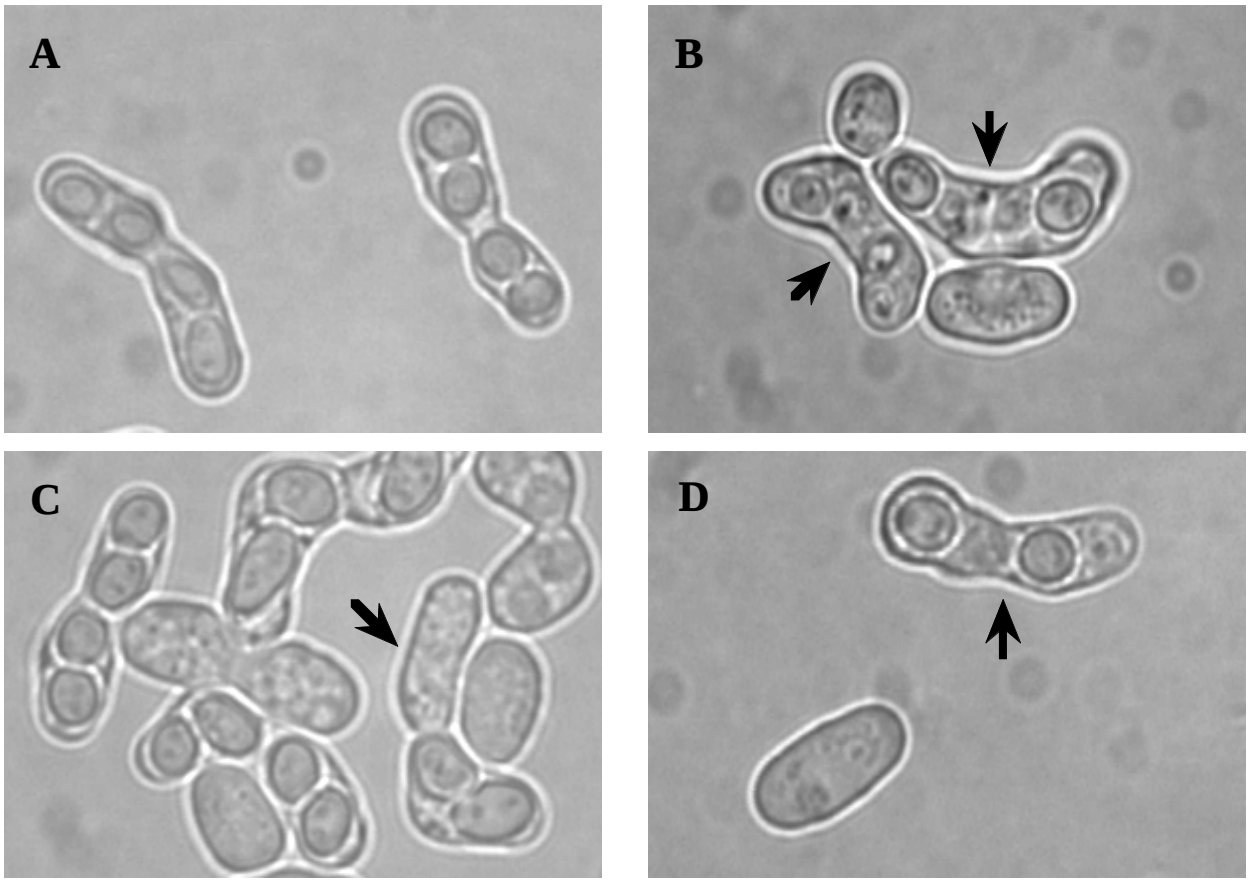

Fig. 2 Y. Matsuo *et al.*

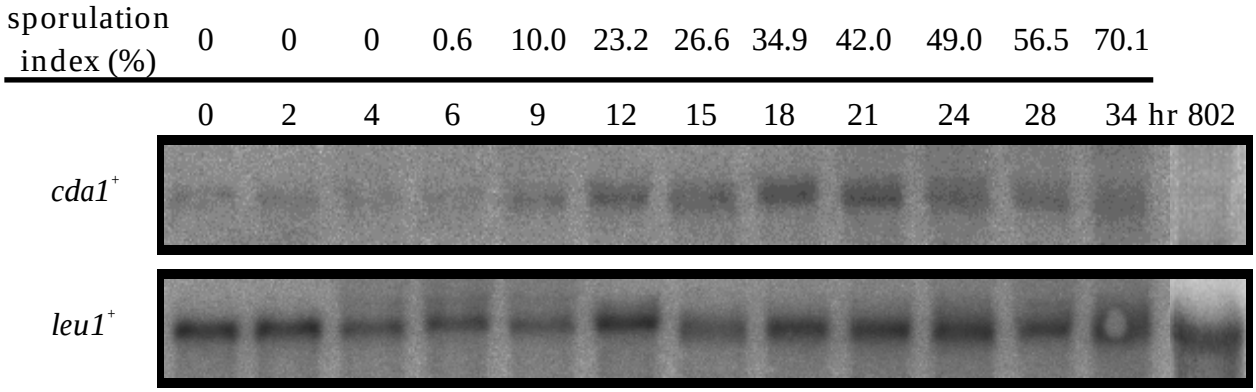

Fig. 3 Y. Matsuo *et al.*

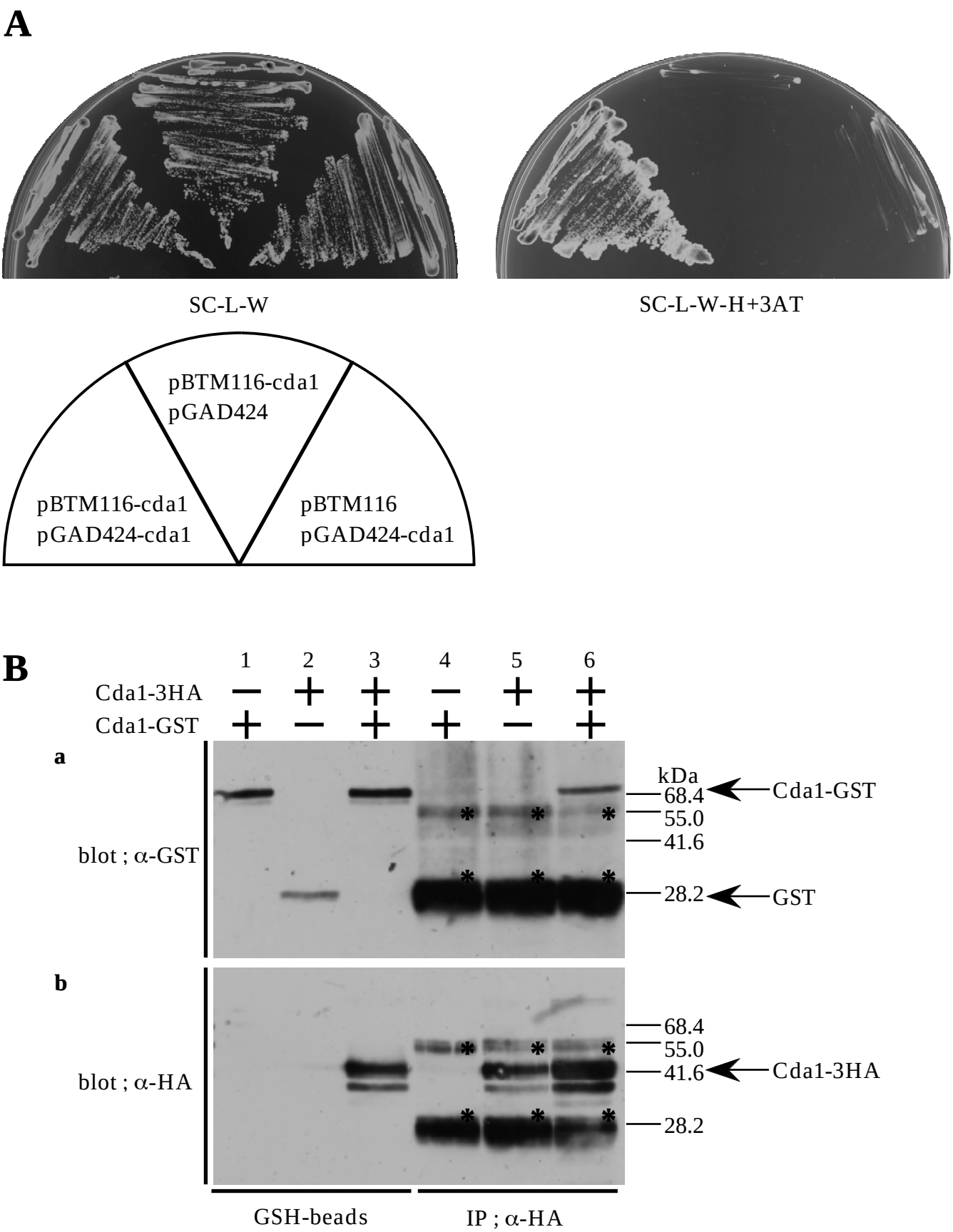

Fig. 4 Y. Matsuo *et al.*