Journal of Biotechnology 151 (2011) 194–203

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Identification of genes that enhance cellulase protein production in yeast

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ARTICLE INFO

Article history:
Received 29 March 2010
Received in revised form 2 December 2010
Accepted 7 December 2010
Available online 15 December 2010

Keywords:
Cellulase
Endoglucanase
β-Glucosidase
Yeast gene deletion strain collection
Saccharomyces cerevisiae

ABSTRACT

In order to enhance heterologous cellulase protein production in yeast, a plasmid harboring the endoglucanase gene from *Clostridium thermocellum* (*Ctcel8A*) was used to systematically transform a homozygous diploid yeast deletion strain collection. We identified 55 deletion strains that exhibited enhanced endoglucanase activity compared with that of the wild-type strain. Genes disrupted in these strains were classified into the categories of transcription, translation, phospholipid synthesis, endosome/vacuole function, ER/Golgi function, nitrogen starvation response, and cytoskeleton. The *vps3*Δ and *vps16*Δ strains, which have deletion in genes encoding components of the class C core vacuole/endosome tethering (CORVET) complex, also exhibited enhanced β-glucosidase activity when *Ctcel8A* was heterologously expressed. Moreover, multiple gene deletion strains were constructed by using the *vps3*Δ strain. Endoglucanase activity of the resulting *rav1*Δ *vps3*Δ double deletion strain was exhibited higher than that of the *rav1*Δ or *vps3*Δ strains. Our genome-wide analyses using the yeast deletion strain collection identified useful genes that allow efficient expression of cellulase.

1. Introduction

In order to solve the worldwide problems of fossil fuel depletion and increased CO_2 concentration, sustainable energy sources are desired. Among the potential alternative energy sources, bioethanol has been commercially produced from sugar cane and corn. However, bioethanol production from feedstocks may lead to a food crisis by competing with production of food biomass. Cellulose and hemicellulose derived from lignocellulosic biomass are sustainable materials, and will not compete with food biomass. To achieve a sustainable society by means of the renewable biomass, utilization of unused lignocellulosic biomass is necessary.

Saccharification of cellulose or hemicellulose is one of the most important steps for converting lignocellulosic biomass to ethanol. Acid or enzymatic saccharification methods have been used to hydrolyze polysaccharides. However, acid saccharification produces by-products due to the hyper-oxidation of sugars, which results in fermentation inhibition. On the other hand, enzymatic saccharification is preferred because it is a mild process that does not produce substances that inhibit fermentation. However, it requires large amounts of saccharification enzymes to hydrolyze polysaccharides. Hence, to reduce the cost of bioethanol production by enzymatic saccharification, it is important to reduce the amount of saccharification enzymes used.

To resolve this problem, consolidated bio-processing (CBP) has been suggested (Lynd et al., 2002). CBP is a process involving simultaneous biomass saccharification and fermentation of hexose and pentose by a single microorganism. If CBP could be achieved, the cost of bioethanol production from lignocellulosic biomass would be dramatically reduced. However, ethanologenic yeast does not have any saccharification enzymes that hydrolyze cellulose and hemicellulose.

Recombinant *Saccharomyces cerevisiae* expressing β-glucosidase has been reported to convert cellobiose to ethanol (McBride et al., 2005; Rajoka, 2007; Shen et al., 2008). The thermotolerant yeast *Kluyveromyces marxianus* expressing heterologous β-glucosidase also converts cellobiose to ethanol (Hong et al., 2007). In addition, some authors have reported direct ethanol production from cellulosic materials. Recombinant *S. cerevisiae* with β-glucosidase and endoglucanase on the cell surface can convert β-glucan to ethanol (Fujita et al., 2002; Kotaka et al., 2008). Recombinant *S. cerevisiae* with β-glucosidase, endoglucanase and cellobiohydrolase genes can convert amorphous cellulose to ethanol (Den Haan et al., 2007; Fujita et al., 2004). Approaches for ethanol production from cellulosic materials are expected to be promising for achieving the goal of CBP through genetic engineering in yeast.

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doi:10.1016/j.jbiotec.2010.12.002

However, the most important problem is that yeast cannot secrete large amounts of heterologous protein. Numerous recombinant yeasts expressing cellulase enzymes have been constructed (van Zyl et al., 2007). The known secretion level of a cellulase enzyme is at maximum 100 mg/l of cellobiohydrolase II from *Trichoderma reesei* in yeast (Penttila et al., 1988). For efficient degradation of cellulosic materials, it is necessary to enhance heterologous protein production in yeast. It has been assumed that heterologous proteins induced the unfolded protein response (UPR), and are degraded by inducing the endoplasmic reticulum-associated degradation machinery (ERAD). Overexpression of UPR genes including *PDI1*, *ERO1*, *SSO2*, *KAR2*, and *HAC1* had a positive effect on heterologous protein production in the yeast *Pichia pastoris* (Gasser et al., 2007). However, it is not known whether these genes are effective for cellulase protein production.

Previous studies have attempted to identify genes that enhance heterologous protein production (Idiris et al., 2010). Some genes that enhance heterologous protein production have been identified in *S. cerevisiae*. The *PMR1* gene, which encodes a Ca^{2+}/Mn^{2+} P type-ATPase in the Golgi fraction, was the first identified gene that enhances heterologous protein production (Rudolph et al., 1989; Smith et al., 1985). In *Kluyveromyces lactis*, deletion of the *PMR1* gene enhanced the amount of secreted recombinant human serum albumin (Uccelletti et al., 2004). Deletion of the *MON2* gene, the product of which plays a role in endocytosis and vacuole maintenance, enhanced *Cypridina noctiluca* luciferase (Cluc) activity (Kanjou et al., 2007). Deletion of the *CYM1* gene, which encodes a lysine-specific metalloprotease, enhanced human holecystokinin protein secretion (Jonson et al., 2004). On the other hand, overexpression of the *SOD1* gene, which encodes a superoxide dismutase, the *PDI1* gene, which encodes a protein disulfide isomerase, or the *RPP0* gene, which encodes a ribosomal protein, also enhanced heterologous protein production (Raimondi et al., 2008; Robinson et al., 1994; Wentz and Shusta, 2008). In addition, mutant strains that exhibited enhanced endoglucanase II activity from *T. reesei* have been isolated, but the genes responsible for the enhanced cellulase protein production were not identified (Aho et al., 1996). Consequently, genes that enhance cellulase protein production have not been identified.

A yeast deletion strain collection, in which each of approximately 4800 non-essential genes was disrupted, has been constructed (Giaever et al., 2002; Winzeler et al., 1999). Synthetic genetic array analyses (Tong et al., 2001) and phenotypic analyses (Scherens and Goffeau, 2004) have been performed using this collection. In addition, genome-wide transformation of the collection led to novel genetic technology, resulting in a large amount of genome-wide data (Kitagawa et al., 2007a,b; Kushner et al., 2003; Neklesa and Davis, 2009; Proszynski et al., 2005; Willingham et al., 2003). Some genome-wide analyses to enhance heterologous protein production have been performed. A high-throughput screen using yeast cDNA libraries in *S. cerevisiae* and *P. pastoris* were performed to identify genes that enhance cell-surface display of a heterologous protein (Stadlmayr et al., 2010; Wentz and Shusta, 2007). However, genome-wide analyses of genes responsible for enhancement of heterologous protein production using a yeast deletion strain collection have not been performed. We introduced a cellulase gene into a yeast deletion strain collection by transformation, and identified genes that enhanced heterologous cellulase protein production.

2. Materials and methods

2.1. Strains and media

S. cerevisiae strains BY4741 (*MAT***a** *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0) (Openbiosystems, AL, USA) and BY4743 (*MAT***a**/α *his3*Δ1/*his3*Δ1 *leu2*Δ0/*leu2*Δ0 *ura3*Δ0/*ura3*Δ0 *lys2*Δ0/*LYS2* *met15*Δ0/*MET15*) (Openbiosystems) were used as parental strains. The homozygous diploid non-essential deletion strain collection (Openbiosystems) was used for this screen, and part of the haploid *MAT***a** non-essential deletion strain collection (Openbiosystems) was used for chromosomal integration of the cellulase gene. N435-1A (*MAT***a** *his7 lys7 met6 arg1 gal4 MAL2 SUC*) and N435-2A (*MAT*α *his7 lys7 met6 arg1 gal4 MAL2 SUC*) were used for mating tests.

YPD (1%, w/v yeast extract; 2%, w/v peptone; 2%w/v glucose) and synthetic dextrose (SD) medium (0.67%, w/v yeast nitrogen base without amino acids; 2%, w/v glucose; amino acids) were used for the selection of transformants. Transformants carrying a hygromycin or phleomycin resistance marker were selected on YPD containing 200 μg/ml hygromycin B (Wako Pure Chemical Industries, Osaka, Japan) or 20 μg/ml phleomycin (Invivogen, CA, USA), respectively.

2.2. Plasmids

The glucoamylase signal peptide from *Rhizopus oryzae* was used to provide the signal sequence for secretion of the cellulase enzymes. *Clostridium thermocellum cel8A* (DDBJ/EMBL/GenBank accession no. K03088) under the control of the *TDH3* promoter was constructed in the pRS436GAP vector (DDBJ/EMBL/GenBank accession no. AB304862) (Ohto et al., 2009) harboring the 2μm replication origin and the *URA3* marker gene, resulting in pRS436GAP-celA. For integration into the chromosome, *Clostridium thermocellum cel8A* (*Ctcel8A*), *Clostridium cellulolyticum cel5A* (*Cccel5A*) (DDBJ/EMBL/GenBank accession no. M32362), *Clostridium cellulolyticum cel9M* (*Cccel9M*) (DDBJ/EMBL/GenBank accession no. AF316823), and *Phanerochaete chrysosporium cel12A* (*Pccel12A*) (DDBJ/EMBL/GenBank accession no. DM474579) were constructed in a plasmid harboring the upstream sequence of the *AAP1* gene and the hygromycin resistance marker, resulting in pAH-HOR7p-Ctcel8Asec, pAH-HOR7p-Cccel5Asec, pAH-HOR7p-Cccel9Msec, and pAH-HOR7p-Pccel12Asec (Fig. 1A). In addition, the *Ctcel8A* gene was also constructed in plasmid harboring the upstream sequence of the *AAP1* gene and the *LEU2* marker gene, resulting in pAL-HOR7p-Ctcel8Asec (Fig. 1A). *Aspergillus aculeatus bgl1* (DDBJ/EMBL/GenBank accession no. D64088) was constructed in plasmid harboring the upstream sequence of the *ADH3* gene and the *URA3* marker, resulting in pDU-HOR7p-AaBGLsec (Fig. 1B).

2.3. Yeast transformation

Systematic yeast transformation was performed using the 96-well transformation method (Kitagawa et al., 2007a). Each of the homozygous diploid deletion strains in the collection was grown in 25 μl of YPD liquid medium at 30 °C for 24 h using 96-well format microplates without shaking. After mixing the cultures, 45 μl of the transformation solution (35 μl of 60%, w/v polyethylene glycol 3350 (PEG3350), 2.5 μl of 4 M lithium acetate, 2.5 μl of 10 mg/ml DNA sodium salt from salmon testes (carrier DNA) (Sigma–Aldrich, MO, USA), 2.5 μl of 1 M dithiothreitol, and 2.5 μl of plasmid DNA) was transferred to each well of a 96-well microplate. The plates were mixed again, followed by incubation at 42 °C for 2 h without shaking. The transformed cells were stamped several times onto SD-uracil plates using the bottom of a 96-well format PCR tube, and then incubated for several days at 30 °C. Chromosome integration of linearized DNA derived from plasmid DNA was performed by the following methods. All plasmid DNA vectors were linearized with Sse8387I restriction enzyme (Takarabio, Shiga, Japan). Yeast cells were grown in YPD liquid medium for 18–24 h at 30 °C. One milliliter of the culture was transferred to 10 ml of YPD medium, followed by growth for 5 h at 30 °C. Yeast cells were harvested by

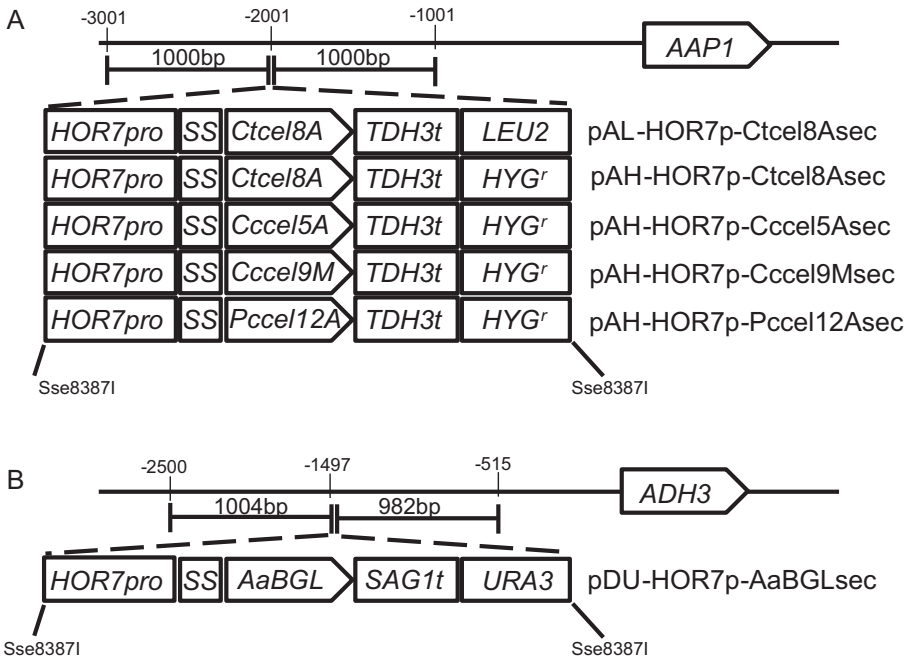

Fig. 1. Plasmids used in this study. The plasmids used for integration into the chromosome were linearized with the Sse8387I restriction enzyme, and gene products were induced to be secreted to the outside of the cell by means of a signal peptide from *Rhizopus oryzae* (SS). (A) Genes under the control of the *HOR7* promoter (*HOR7 pro*) were inserted upstream of the *AAP1* coding region of the chromosome. Other symbols show the *cel8A* mature coding region from *C. thermocellum* (*Ctcel8A*), *cel5A* mature coding region from *C. cellulolyticum* (*Cccel5A*), *cel9M* mature coding region from *C. cellulolyticum* (*Cccel9M*), *cel12A* mature coding region from *P. chrysosporium* (*Pccel12A*), *TDH3* terminator (*TDH3t*), *LEU2* marker gene (*LEU2*), and hygromycin resistance gene (*HYG*^{r}). (B) The *bgl1* gene from *A. aculeatus* (*AaBGL*) under the control of *HOR7 pro* was inserted upstream of the *ADH3* coding region of the chromosome. Other symbols show the *SAG1* terminator (*SAG1t*) and *URA3* marker gene (*URA3*).

centrifugation, washed, and re-harvested. Approximately 50 μl of the yeast cell mixture was transferred to 150 μl of transformation solution (120 μl of 60%, w/v PEG3350; 5 μl of 4 M lithium acetate; 10 μl of 1 M dithiothreitol, 10 μl of 10 mg/ml carrier DNA; 5 μl of linearized DNA). The mixture was incubated for 40–60 min at 42 °C, and then spread onto SD-leucine medium. Confirmation of chromosome integration was performed by a colony PCR method. First, yeast cells on the plates were transferred to 5 μl of 20 mM sodium hydrate, followed by boiling for 10 min at 100 °C. Next, the boiled samples were transferred to 15 μl of distilled water. The PCR reaction was performed with Extaq DNA polymerase (Takarabio).

2.4. Enzyme assay

Systematic screening of deletion strains that exhibit enhanced endoglucanase activity was performed using carboxymethyl cellulose (CMC) as a substrate. Transformants of the deletion strains harboring pRS436GAP-celA in 96-well format microplates were inoculated into 50 μl of SD-leucine liquid medium without buffering the pH, and then incubated for 24 h at 30 °C with shaking using a deep-well maximizer (MBR-022UP, TAITEC, Saitama, Japan). Cultures were centrifuged, and the resulting supernatants, each 5 μl, were spotted onto CMC plates (0.1%, w/v CMC; 2%, w/v agar; 50 mM sodium acetate (pH 6)). The plates were incubated for 24 h at 40 °C, stained with 0.1% (w/v) Congo red in 1 M Tris base (pH 9) for 30 min, and then destained with 1 M sodium chloride. Deletion strains that exhibited enhanced endoglucanase activity were identified based on the halo sizes of the discolored spots.

To quantify endoglucanase activity, the following procedure was performed. Transformants expressing endoglucanase were grown in YPD medium with shaking for 24 h at 30 °C, and then divided into supernatants and yeast cell pellets by centrifugation. The enzyme reaction of the supernatants was performed for 2 h at 40 °C with shaking in a solution comprising 10 μl of a supernatant, 250 μl of 1% (w/v) CMC, 100 μl of 250 mM sodium acetate buffer (pH 5), and 140 μl of deionized water. Five microliter aliquots of each solution were transferred to 100 μl of TZ buffer (Jue and Lipke, 1985), followed by color reaction for 3 min at 100 °C, and then the absorbance values were measured at 660 nm. Enzyme units were calculated from a standard glucose concentration curve. One unit was defined as the amount that released 1 μmol of glucose from the substrate in 1 min.

Quantitative assays of β-glucosidase activity were performed with *p*-nitrophenyl glucopyranoside (pNPG) (Sigma–Aldrich) as a substrate. Transformants expressing β-glucosidase were grown in YPD liquid medium for 24 h at 30 °C, and then the cultures were harvested by centrifugation. Five microliter aliquots of the supernatants were transferred to a reaction solution comprising 695 μl of deionized water, 100 μl of 20 mM pNPG, and 200 μl of 250 mM sodium acetate (pH 5), and then the mixed solutions were incubated for 2 h at 30 °C with shaking. One hundred microliter aliquots of the sample solution were transferred to 50 μl of a 1 N sodium carbonate solution, followed by centrifugation. The supernatants were subjected to absorbance measurement at 400 nm. Enzyme units were calculated from a standard *p*-nitrophenol curve. One unit was defined as the amount that released 1 μmol of *p*-nitrophenol from the substrate in 1 min.

3. Results

3.1. Determination of screening conditions

One of the cellulosomic cellulase enzymes, *C. thermocellum cel8A* (*Ctcel8A*), which belongs to glycoside hydrolase family 8 (Demain et al., 2005), was used for our screening, because its activity is stronger compared with those of other cellulosomic enzymes in yeast and the high-throughput enzymatic assay can be applied by monitoring the halo size of degraded CMC (data not shown). We transformed the pRS436GAP-celA vector harboring the *Ctcel8A* gene under the control of the *TDH3* promoter in the BY4741 strain,

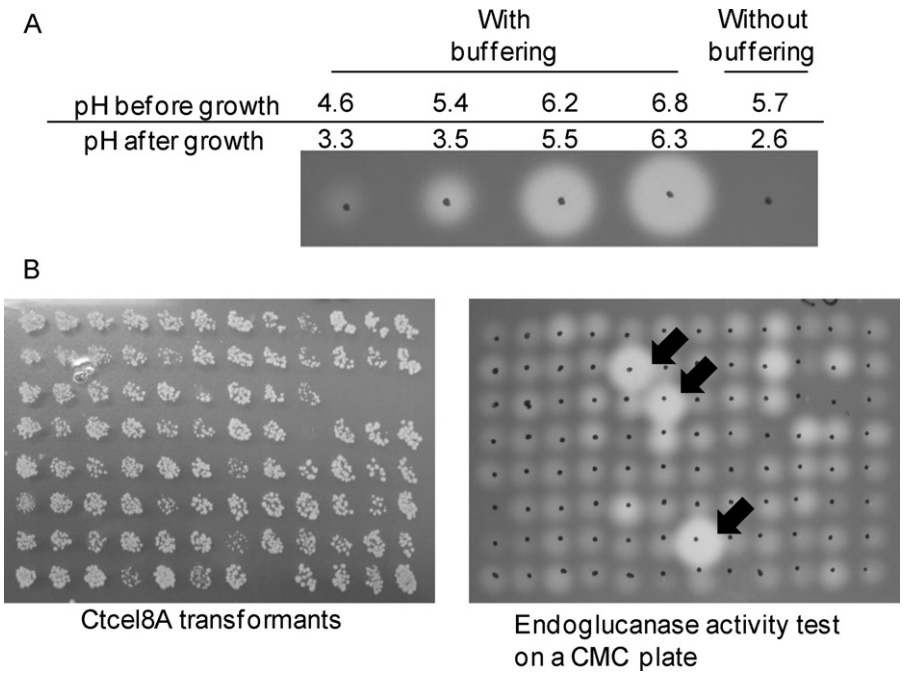

Fig. 2. Screening for enhancement of the endoglucanase activity of Ctcel8A using a diploid homozygous deletion strain collection. (A) Transformants harboring the pRS436GAP-celA vector were grown in SD-uracil medium with or without a buffering agent. Supernatants of the cultures were spotted onto a CMC plate, followed by incubation at 40 °C for a day and then staining with Congo red. (B) (Left) Transformation plate of deletion strains harboring the pRS436GAP-celA vector on SD-uracil medium. (Right) Transformants were grown in SD-uracil medium for 24 h, and then 5 μl aliquots were spotted onto a CMC plate. Arrows indicate the halos of deletion strains that exhibited enhanced endoglucanase activity.

the parental strain for the deletion strain. The halo size of endoglucanase activity on the plate was dramatically smaller when the pH of the medium was lower (Fig. 2A). In particular, endoglucanase activity of the wild-type strain was almost undetectable in the case of the culture of SD medium without a buffering agent. We screened the deletion strains that exhibited enhanced activity by means of SD medium lacking a buffering agent in order to detect larger clear halos.

3.2. Systematic screen of strains that enhance the endoglucanase activity of Ctcel8A with a yeast homozygous deletion strain collection

In order to isolate genes that enhance the endoglucanase activity of the Ctcel8A enzyme, a yeast homozygous gene deletion strain collection was used. For the screen, a diploid collection rather than a haploid collection was chosen, because haploid deletion strains may have unpredictable mutations. Systematic transformations into the collection were performed by the 96-well transformation protocol (see Section 2). Transformation manipulations of the pRS436GAP-celA vector were performed for all 54 collection plates, and the successful transformation of 5197 deletion strains was achieved. These transformants were grown in SD medium without a buffering agent, and then 5 μl aliquots of the supernatants of the cultures were spotted onto CMC plates (Fig. 2B). The first screen isolated 226 candidate deletion strains that enhanced endoglucanase activity (data not shown). To confirm this phenotype, the 226 candidates were individually grown in test tubes, and 147 deletion strains had reproducible activities. In a previous study, haploid strains contaminated the diploid strain collection (Kitagawa et al., 2007b). To exclude contaminating haploid strains, two tests were conducted. Haploid contaminants in this collection exhibit four auxotrophic phenotypes (*his3*Δ1 *leu2*Δ0 *ura3*Δ0 *met15*Δ0 or *his3*Δ1 *leu2*Δ0 *ura3*Δ0 *lys2*Δ0), while diploid strains exhibit three auxotrophic phenotypes (*his3*Δ1/*his3*Δ1 *leu2*Δ0/*leu2*Δ0 *ura3*Δ0/*ura3*Δ0 *lys2*Δ0/*LYS2 met15*Δ0/*MET15*). Hence, haploid contaminants cannot grow on SD supplemented with leucine, histidine, and uracil because they also require either methionine or lysine. The 147 candidates harboring the pRS436GAP-celA vector (containing the *URA3* gene) were streaked onto SD medium supplemented with leucine and histidine, on which the diploid strains can grow. Of the 147 candidates, 117 deletion strains grew. Second, a mating test of the candidates was performed. Mating tester strain N435-1A (*MAT***a**) or N435-2A (*MAT*α) was cross-streaked with the 117 candidates on YPD medium, followed by incubation at 30 °C for a day, and then replica-plated onto SD minimal medium. Seven deletion strains were capable of mating, and therefore were excluded. The remaining 110 candidates were checked again, and 96 deletion strains were shown to have enhanced endoglucanase activity. In our screen, we used an episomal plasmid DNA vector. Our screening candidates possibly included strains that had increased plasmid DNA copy number. To exclude such strains, the 96 deletion strains that were identified from the diploid deletion collection were picked from a haploid *MAT***a** non-essential deletion collection. Thirteen deletion strains were not used because the correct gene deletions could not be confirmed by PCR. Subsequently, the *Ctcel8A* gene was integrated into the chromosomes of 83 deletion strains with the pAL-HOR7p-Ctcel8Asec vector (Fig. 1A). The *Ctcel8A* gene was expressed under the control of the *HOR7* promoter but not the *TDH3* promoter used for the screen, in order to exclude promoter-specific enhancement of endoglucanase activity. Two clones of the *Ctcel8A* transformants were assessed to determine whether or not they enhanced endoglucanase activity on CMC plates. Finally, 55 deletion strains were identified as deletion strains that enhanced endoglucanase activity in yeast (Fig. 3A and Table 1).

3.3. Molecular functions of genes that enhance the endoglucanase activity of Ctcel8A enzymes

The fifty-five genes that enhanced endoglucanase activity were classified into eight groups. The genes were categorized as transcription, translation, phospholipid synthesis,

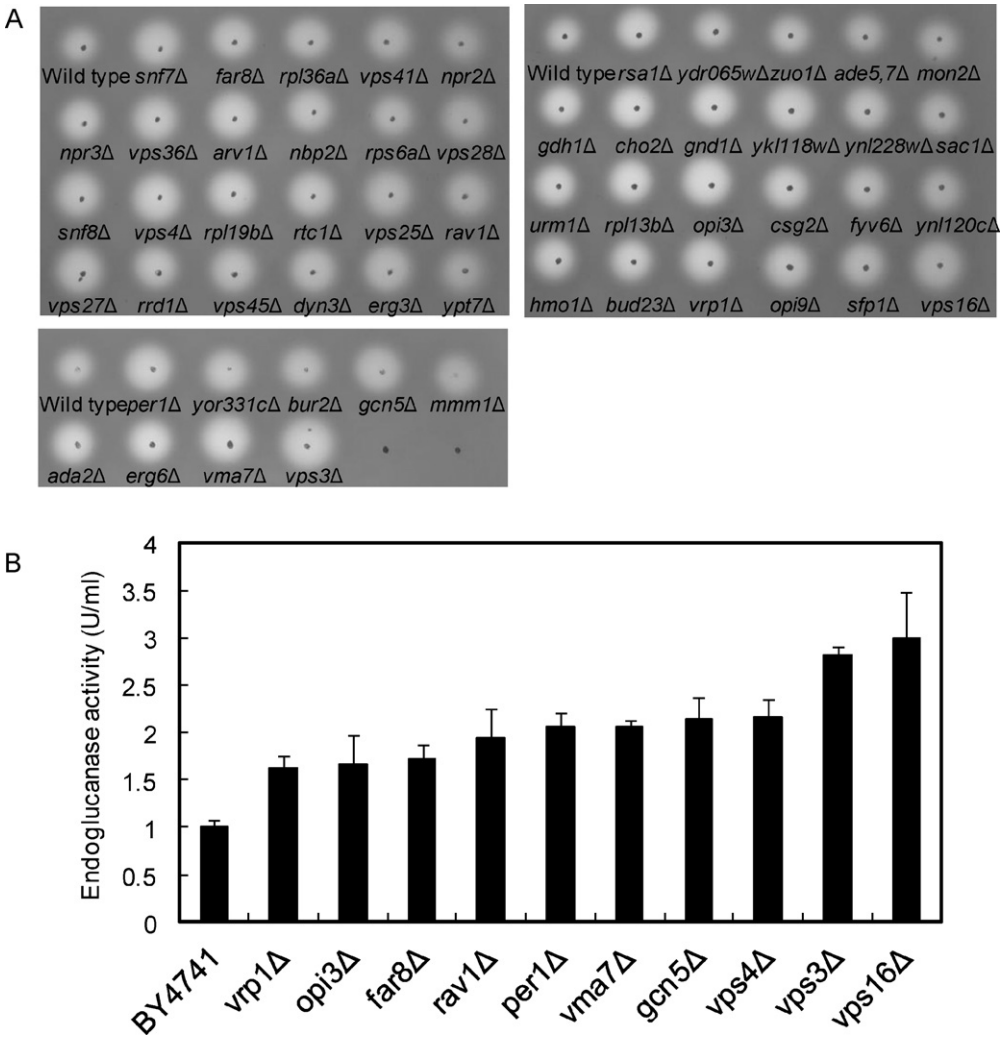

Fig. 3. Deletion strains that exhibit enhanced endoglucanase activity of the Ctcel8A enzyme. (A) The *HOR7pro-Ctcel8A* gene DNA fragment was integrated into the chromosome of haploid deletion strains by means of a restriction enzyme digestion of the pAL-HOR7p-Ctcel8Asec vector. Transformants were grown in YPD medium for 24 h at 30 °C. Supernatants of the cultures were diluted 8-fold, and then 5 μl aliquots were spotted onto a CMC plate. (B) Enzyme activity of Ctcel8A. Ctcel8A transformants were grown in YPD medium for 24 h at 30 °C, and then supernatants of the cultures were subjected to activity measurements with CMC. All error bars indicate ±S.D.; $n = 3$.

endosome/vacuole function, ER/Golgi function, nitrogen starvation response, cytoskeleton, and others (Table 1). Among the fifty-five deletion strains, 18 deletion strains belonged to the endosome/vacuole function group (*mon2*Δ, *rav1*Δ, *snf7*Δ, *snf8*Δ, *vma7*Δ, *vps3*Δ, *vps4*Δ, *vps16*Δ, *vps25*Δ, *vps27*Δ, *vps28*Δ, *vps36*Δ, *vps41*Δ, *vps45*Δ, *ydr065w*Δ, *ykl118w*Δ, *yor331c*Δ, and *ypt7*Δ). Of these strains, 9 were involved in named for vacuole protein sorting (*VPS*). In these categories, the *CHO2* and *OPI3* genes responsible for phosphatidylcholine synthesis were included. In addition, the *ERG3* and *ERG6* genes responsible for ergosterol synthesis were also included in the list. The *GCN5* and *ADA2* genes, which encode part of a histone acetyltransferase complex, were also included. The Gcn5 and Ada2 proteins have been shown to associate with the Ngg1 protein (Grant et al., 1997). Our screen also revealed the *SFP1* gene, which controls ribosomal biogenesis. The Sfp1 protein, which encodes a transcriptional regulator, binds promoters of ribosomal protein genes. The *sfp1* mutant has a reduced TOR pathway response and reduced responses of other stress response pathways (Marion et al., 2004). We also identified the *NPR2* and *NPR3* genes, which were shown to regulate complexes of the TOR pathway in response to amino acid limitation (Neklesa and Davis, 2009).

We quantified endoglucanase activity in culture supernatants of ten deletion strains that exhibited the most enhanced activity out of the 55 deletion strains (Fig. 3B). These deletion strains exhibited 1.5–3-fold enhanced endoglucanase activity compared with that of the wild-type strain. In particular, the *vps3*Δ and *vps16*Δ strains exhibited 3-fold enhanced activity.

3.4. Effects on other types of cellulases in gene deletion strains

We assessed the alteration of activity due to gene deletion for endoglucanase enzymes other than the Ctcel8A enzyme. Three different types of endoglucanase enzymes, *Clostridium cellulolyticum* glycoside family 5 *cel5A* (*Cccel5A*), *Clostridium cellulolyticum* glycoside family 9 *cel9M* (*Cccel9M*), and *Phanerochaete chrysosporium* glycoside family 12 *cel12A* (*Pccel12A*), were integrated into the chromosome of the *vps3*Δ strain (Fig. 4A). All of these endoglucanases enhanced endoglucanase activity in the *vps3*Δ strain. These results suggest that *VPS3* depletion has an effect on any endoglucanase from bacteria to fungi, or any glycoside hydrolase family.

In order to determine whether or not our isolated strains exhibit enhanced activity of a β-glucosidase enzyme that converts cellobiose to glucose, the β-glucosidase from *Aspergillus aculeatus* (*AaBGL*) was used. Nine deletion strains comprising the *gcn5*Δ, *opi3*Δ, *per1*Δ, *vma7*Δ, *vps3*Δ, *vps4*Δ, *vps16*Δ, *vps27*Δ, and

Table 1
The enhanced genes of Ctcel8A enzymes activities.

Standard name	Systematic name	Description
Endosome/vacuole function		
MON2	YNL297C	Peripheral membrane protein with a role in endocytosis and vacuole integrity
RAV1	YJR033C	Subunit of the RAVE complex (Rav1p, Rav2p, Skpip)
SNF7	YLR025W	One of four subunits of the ESCRT-III complex
SNF8	YPL002C	Component of the ESCRT-II complex
VMA7	YGR020C	Subunit F of the eight-subunit V1 peripheral membrane domain of V-ATPase
VPS3	YDR495C	Component of the CORVET-tethering complex
VPS4	YPR173C	AAA-ATPase involved in multivesicular body (MVB) protein sorting
VPS16	YPL045W	A subunit of the class C *VPS* complex
VPS25	YJR102C	Component of the ESCRT-II complex
VPS27	YNR006W	Endosomal protein that forms a complex with Hse1p
VPS28	YPL065W	Component of the ESCRT-I complex
VPS36	YLR417W	Component of the ESCRT-II complex
VPS41	YDR080W	A subunit of the HOPS complex
VPS45	YGL095C	Protein of the Sec1p/Munc-18 family
YDR065W	YDR065W	Protein of unknown function, required for vacuolar acidification
YKL118W	YKL118W	Dubious open reading frame; partially overlaps with VPH2
YOR331C	YOR331C	Dubious open reading frame; partially overlaps with VMA4
YPT7	YML001W	GTPase required for the homotypic fusion event in vacuole inheritance
ER/Golgi function		
ARV1	YLR242C	Protein functioning in transport of glycosylphosphatidylinositol
CSG2	YBR036C	Required for mannosylation of inositolphosphorylceramide
FAR8	YMR029C	Protein involved in G1 cell cycle arrest in response to pheromones
PER1	YCR044C	Protein required for GPI-phospholipase A2 activity that remodels the GPI anchor
SAC1	YKL212W	Phosphatidylinositol phosphate (PtdInsP) phosphatase
Phospholipid synthesis		
CHO2	YGR157W	Phosphatidylethanolamine methyltransferase
ERG3	YLR056W	C-5 sterol desaturase
ERG6	YML008C	Delta(24)-sterol C-methyltransferase
OPI3	YJR073C	Phospholipid methyltransferase (methylene-fatty-acyl-phospholipid synthase)
Transcription		
ADA2	YDR448W	Transcription coactivator of the ADA and SAGA transcriptional adaptor
GCN5	YGR252W	Histone acetyltransferase of the ADA and SAGA
HMO1	YDR174W	Chromatin-associated high mobility group (HMG) family member
SFP1	YLR403W	Transcription factor that controls the expression of many ribosome biogenesis genes
Translation		
BUD23	YCR047C	Methyltransferase, methylates residue G1575 of 18S rRNA
RPL13B	YMR142C	Protein component of the large (60S) ribosomal subunit
RPL19B	YBL027W	Protein component of the large (60S) ribosomal subunit
RPL36A	YMR194W	N-Terminally acetylated protein component of the large (60S) ribosomal subunit
RPS6A	YPL090C	Protein component of the small (40S) ribosomal subunit
RSA1	YPL193W	Protein involved in the assembly of the 60S ribosomal subunit
RTC1	YOL138C	Protein of unknown function; may interact with ribosomes
YNL228W	YNL228W	Dubious open reading frame; partially overlaps with JJJ1
ZUO1	YGR285C	Cytosolic ribosome-associated chaperone
Nitrogen source response		
GDH1	YOR375C	NADP(+)-dependent glutamate dehydrogenase
NPR2	YEL062W	Component with Npr3 that mediates downregulation of the TOR complex
NPR3	YHL023C	Component with Npr2 that mediates downregulation of the TOR complex
Cytoskeleton		
OPI9	YLR338W	Dubious open reading frame of VRP1ORF proline-rich actin-associated protein
VRP1	YLR337C	Proline-rich actin-associated protein
Others		
ADE5,7	YGL234W	Bifunctional enzyme of the 'de novo' purine nucleotide biosynthetic pathway
BUR2	YLR226W	Cyclin for the Sgv1 p (Bur1 p) protein kinase
DYN3	YMR299C	Dynein light intermediate chain (LIC)
FYV6	YNL133C	Protein of unknown function
GND1	YHR183W	6-Phosphogluconate dehydrogenase
MMM1	YLL006W-A	Mitochondrial outer membrane protein
NBP2	YDR162C	Protein involved in the HOG (high osmolarity glycerol) pathway
RRD1	YIL153W	Peptidyl-prolyl cis/trans-isomerase
URM1	YIL008W	Ubiquitin-like protein with weak sequence similarity to ubiquitin
YNL120C	YNL120C	Dubious open reading frame; partially overlaps with NCS2

*ykl118w*Δ strains were selected for enzyme assays, and then the *AaBGL* gene was integrated into the chromosome of the deletion strains using the construct in Fig. 1B. The β-glucosidase (BGL) activity of the deletion strains was measured not only in supernatants but also on the cell surface because AaBGL enzymes stayed attached to the yeast cell surface after washing with deionized water. The *gcn5*Δ, *opi3*Δ, *per1*Δ, *vma7*Δ, and *vps16*Δ strains exhibited 1.2–1.4-fold enhanced BGL activities in the supernatants compared with that of the wild-type strain, whereas the *vps3*Δ, *vps4*Δ, *vps27*Δ, and *ykl118w*Δ strains showed no change in activity or had decreased activity (Fig. 4B). On the other hand, all strains exhibited 1.3–2.3-fold enhanced endoglucanase activity on the cell

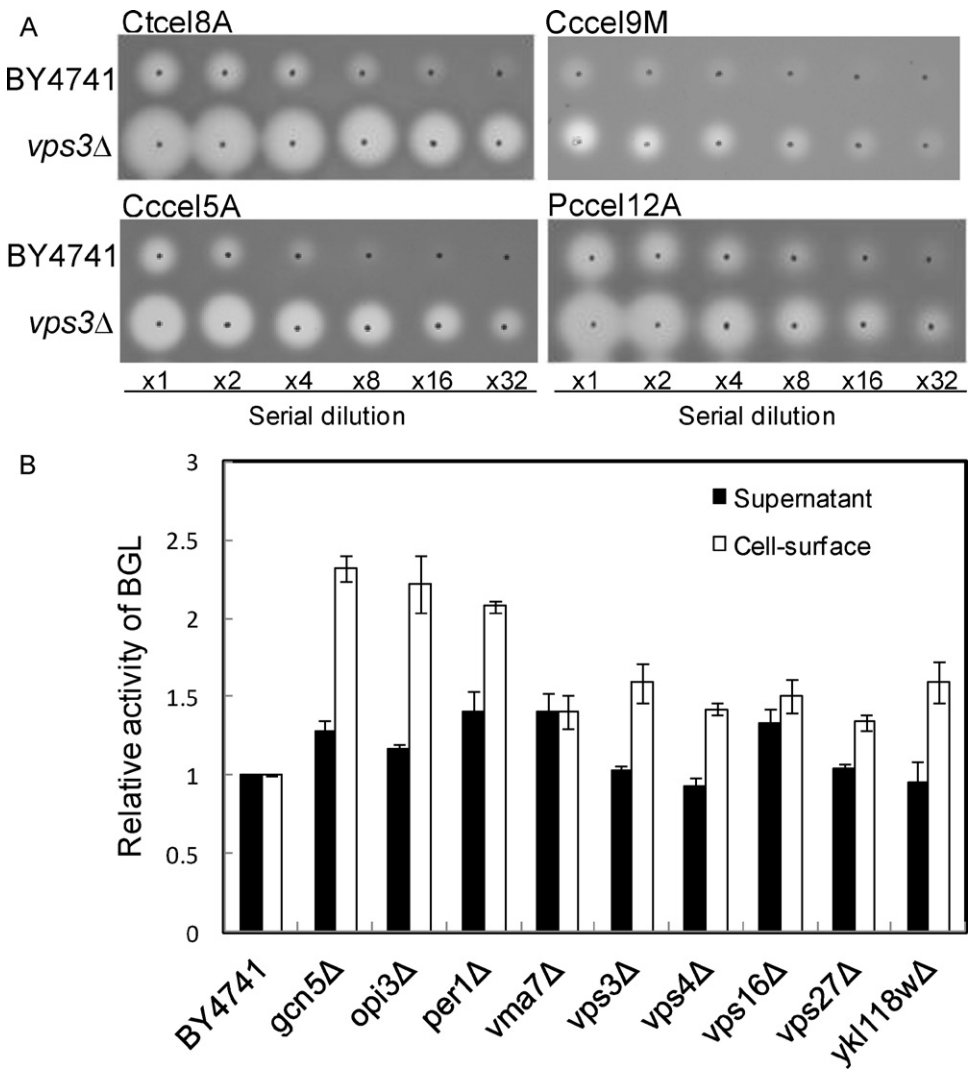

Fig. 4. Effects of cellulase genes other than *Ctcel8A*. (A) pAH-HOR7p-Ctcel8Asec, pAH-HOR7p-Cccel5Asec, pAH-HOR7p-Cccel9Msec, and pAH-HOR7p-Pccel12Asec including four endoglucanase genes (*Ctcel8A*, *Cccel5A*, *Cccel9M*, and *Pccel12A*) were integrated into the haploid *vps3*Δ strain, and then aliquots of supernatants of the transformants were spotted onto a CMC plate. (B) pDU-HOR7p-AaBGLsec including the *AaBGL* gene was integrated into nine haploid deletion strains. AaBGL transformants were grown in YPD medium for 24 h at 30 °C, and then the BGL activity in supernatants and on the cell surface was measured with pNPG. BGL activities relative to the wild-type strain are shown. All error bars indicate ±S.D.; $n = 3$.

surface. In particular, the *gcn5*Δ, *opi3*Δ, and *per1*Δ strains showed efficient enhancement in both cases.

3.5. The effects of multiple gene deletion on endoglucanase activity

We screened for deletion strains that exhibited enhanced endoglucanase activity in the yeast deletion strain collection, and isolated the *vps3*Δ and vps*16*Δ strains. To construct strains that exhibited more endoglucanase activity, multiple gene deletions were performed using our isolated strains. From among fifty-five genes, we selected the *RAV1* gene, which is required for assembly of the V-ATPase holoenzyme (Smardon et al., 2002), as a target gene. Of the ten strains in Fig. 3B, two deletion strains (the *far8*Δ and *vps3*Δ strains) were selected as target strains. The *far8*Δ and *vps3*Δ strains had the *RAV1* gene disrupted by means of the *MET15* marker gene, which resulted in the construction of double deletion strains (the *rav1*Δ *far8*Δ strain and *rav1*Δ *vps3*Δ strains; Fig. 5). The *far8*Δ strain, *rav1*Δ strain, and *vps3*Δ strain exhibited 1.7, 2.1, and 2.9-fold enhanced endoglucanase activity, respectively. Meanwhile, the *rav1*Δ *far8*Δ and *rav1*Δ *vps3*Δ double deletion strains

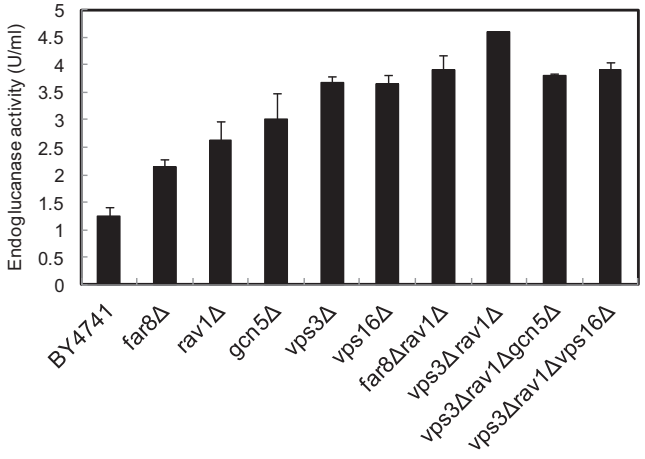

Fig. 5. Effect on endoglucanase activity in multiple gene deletion strains. The Ctcel8A gene was integrated into the chromosome for each of the haploid deletion strains. Ctcel8A transformants were grown in YPD medium, and then aliquots of supernatants of the cultures were subjected to activity measurements with CMC. All error bars indicate ±S.D.; $n = 3$.

exhibited 3.1 and 3.7-fold enhanced endoglucanase activity compared with that of the wild-type strain, respectively. Furthermore, the *rav1*Δ *vps3*Δ strain had the *GCN5* or *VPS16* gene disrupted with a bleomycin resistance marker, which resulted in the construction of two triple deletion strains (the *rav1*Δ *vps3*Δ *gcn5*Δ and *rav1*Δ *vps3*Δ *vps16*Δ strains; Fig. 5). However, these triple deletion strains exhibited decreased endoglucanase activity compared with that of the *rav1*Δ *vps3*Δ strain. As a result, we identified the *rav1*Δ *vps3*Δ strain as a strain that exhibited the most enhanced endoglucanase activity.

4. Discussion

To conduct bioethanol production with the goal of CBP, a genetically modified yeast is necessary because large amounts of saccharification enzymes are required. However, *S. cerevisiae* cannot produce and secrete large amounts of proteins to the outside of the cell. Many researchers have reported heterologous protein production by *S. cerevisiae*, and several genes that enhanced heterologous protein production were isolated. However, a systematic survey of genes required for higher heterologous protein production using a yeast gene deletion strain collection had not been performed. We screened for genes required for cellulase protein production using a yeast gene deletion strain collection.

From the screen, nine *vps* deletion strains (*vps3*Δ, *vps4*Δ, *vps16*Δ, *vps25*Δ, *vps27*Δ, *vps28*Δ, *vps36*Δ, *vps41*Δ, and *vps45*Δ) were found (Table 1). The *vps* strains were identified by systematic genetic screening for mis-sorting of carboxypeptidase Y (CPY) to the outside of the cell (Bonangelino et al., 2002). Our screening included 23 strains (*gcn5*Δ, *mon2*Δ, *per1*Δ, *rav1*Δ, *rpl6A*Δ, *rpl13B*Δ, *rpl36A*Δ, *sac1*Δ, *snf7*Δ, *snf8*Δ, *vps3*Δ, *vps4*Δ, *vps16*Δ, *vps25*Δ, *vps27*Δ, *vps28*Δ, *vps36*Δ, *vps41*Δ, *vps45*Δ, *vma7*Δ, *ypt7*Δ, and *urm1*Δ) among the systematically screened *vps* mutants. Of these 23 strains, 13 (*mon2*Δ, *per1*Δ, *rpl6A*Δ, *vps3*Δ, *vps4*Δ, *vps16*Δ, *vps25*Δ, *vps27*Δ, *vps28*Δ, *vps36*Δ, *vps41*Δ, *vps45*Δ, and *ypt7*Δ) were shown to have a strong or moderate *VPS* phenotype (Bonangelino et al., 2002). These data suggest that the Ctcel8A protein may be recognized as an unfolded or abnormal protein, and as a result it may be sorted into the vacuole in the wild-type strain for degradation. Indeed, our identified strains included the *per1*Δ strain, which does not induce the UPR (Ng et al., 2000). However, no protease-deficient mutants were included on the list. This indicates that multiple proteases may be involved in Ctcel8A protein production. It has been hypothesized that overexpression of UPR genes may enhance Ctcel8A protein production, possibly by efficiently regulating folding of the Ctcel8A protein.

Among the fifty-five deletion strains, the *vps3*Δ and *vps16*Δ strains are the strains that showed the highest endoglucanase activity (Fig. 3B). The Vps3 protein is necessary for vacuole acidification (Rothman et al., 1989), and is a part of the CORVET complex (Vps3, Vps8, Vps21, and class C *VPS* complex), which is required for vesicle transport between the endosome and vacuole (Peplowska et al., 2007). The Vps16 protein is necessary for vacuole biogenesis, and is part of the class C *VPS* complex (Pep3, Pep5, Vps16, and Vps33) (Banta et al., 1988). In addition, the class C *VPS* complex associates with the Ypt7, Vps41, and Vam6 proteins, the resulting complex being called the homotypic fusion and vacuole protein sorting (HOPS) complex (Seals et al., 2000). We also isolated the *ypt7*Δ and *vps41*Δ strains as deletion strains that enhance endoglucanase activity (Table 1). We also identified some of the endosomal sorting complexes required for the transport (ESCRT) complex, including the Vps27 complex (Vps27), ESCRTI (Vps28), ESCRTII (Snf8, Vps25, Vps36), and ESCRTIII (Snf7) (Table 1). The ESCRT complex is required for sorting of ubiquitin-modified cargo proteins into the internal vesicles of the multivesicular body (Hurley and Emr, 2006). These mutants also show the *VPS* phenotype (Bonangelino et al., 2002). These results indicate that inhibition of vesicle sorting to vacuoles may be important for heterologous cellulase protein production. However, we have not yet tested whether all of the components of the complexes are effective for enhancing cellulase protein production.

We isolated the *hmo1*Δ strain, which has a deletion of a gene encoding a high-mobility group DNA protein binding (Table 1). The Hmo1 protein associates with promoters of ribosomal protein genes and the ribosomal RNA (rRNA) gene locus (Berger et al., 2007; Hall et al., 2006). We also isolated the *sfp1*Δ strain, which has a deletion of a transcription factor for ribosomal protein genes, as an enhanced strain (Table 1). The Sfp1 protein regulates cell size (Jorgensen et al., 2002) and is translocated from the nucleus to the cytoplasm in response to various stresses including rapamycin treatment, DNA lesion agents, high osmolarity, reducing agents, and oxidizing agents (Marion et al., 2004). The *sfp1*Δ strain reduced the levels of ribosomal protein gene expression under normal conditions (Jorgensen et al., 2002). These data indicate that the ribosomal protein expression machinery may be inhibited by cellulase protein production. Indeed, we also isolated strains with deletion in genes encoding ribosomal protein subunits including the *rsa1*Δ, *rps6a*Δ, *rpl13b*Δ, *rpl19b*Δ, and *rps36a*Δ strains (Table 1). Of these mutant strains, the *rps6a*Δ, *rpl13b*Δ, *rps36a*Δ strains are known to have the *VPS* phenotype, which results in mis-sorting of CPY to the outside of the cell (Bonangelino et al., 2002). Hence, deletion of some ribosomal proteins may indirectly enhance heterologous cellulase production by inhibiting vesicle transport from the Golgi to the vacuole. On the other hand, other studies have revealed that overexpression of Rrp0, which is one of the components of ribosomal subunit, enhanced heterologous protein synthesis (Wentz and Shusta, 2008). We did not test whether our strains with deletions in the ribosomal protein subunit genes enhanced heterologous protein synthesis. It was assumed that our identified deletion strains enhanced cellulase protein production by a different mechanism.

Double deletion strains including the *rav1*Δ *far8*Δ strain and *rav1*Δ *vps3*Δ strain exhibited enhanced endoglucanase activity compared with each single deletion strain (Fig. 5). The Rav1 protein is required for transport of the Kex2 protease from the trans-Golgi network to the endosome/prevacuolar compartment (Sipos et al., 2004). The Far8 protein was shown to be localized in the ER based on global protein localization analysis (Huh et al., 2003). These results suggest that multiple gene deletions result in a synergistic phenotype. However, a triple deletion strain involving *vps16*Δ or *gcn5*Δ did not show a synergistic phenotype (Fig. 5). The reason was assumed to be that these strains may exhibit decreased growth rates or cell homeostasis compared with the host strain.

We confirmed that our identified strains also exhibited enhanced β-glucosidase activity (Fig. 4B). We performed ethanol fermentation tests with cellobiose as the sole carbon source using our identified strains, and succeeded in increasing the ethanol fermentation rate (data not shown). This result suggests that genetic improvement of the saccharification potential can increase the ethanol production rate. Our identified strains are presumed to have an effect on proteins other than the Ctcel8A enzyme. Indeed, the *vps3*Δ strain exhibited enhanced activity of four endoglucanases and one β-glucosidase (Fig. 4). The *VPS3* gene may be a key gene for heterologous protein production because its deletion had an effect not only in yeast but also in mammalian cells.

In conclusion, we identified 55 deletion strains that exhibited enhanced endoglucanase activity of the Ctcel8A enzyme using a yeast gene deletion strain collection. Systematic transformation of the cellulase gene revealed novel genes responsible for heterologous protein production, and the responsible gene products were identified. Cellulase protein production will be efficiently enhanced with the identified genes, and the resulting improvement in the

ethanol production rate will likely lead to achievement of the goal of CBP.

Acknowledgments

We wish to thank Mayu Mohri, Tomomi Motoyama, and Risa Nagura for their excellent technical assistance.

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