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Identification of genes that enhance cellulase protein production in yeast

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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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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