Short Communication

A new platform host for strong expression under GAL promoters without inducer in Saccharomyces cerevisiae

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ABSTRACT

The gal80 mutant of yeast Saccharomyces cerevisiae is used for the constitutive expression under strong GAL promoters without galactose induction. To enhance productivity of gal80 mutant, an alternative strain, allgal, was developed by removing all galactose-utilizing genes that consume significant cellular resources in the gal80 strain when cultured in non-galactose conditions. The efficacy of the allgal mutant (gal80, gal1, gal2, gal7, and gal10) was verified by assessing the secretory expression of three recombinant proteins, Candida antarctica lipase B (CalB), human serum albumin (HSA), and human epidermal growth factor (hEGF), using the GAL10 promoter. The growth of the allgal mutant was enhanced by 15–38% compared to the gal80 mutant, and the secretion of recombinant proteins also increased by 16–22% in fed-batch fermentation. Thus, the expression of recombinant proteins using GAL10 promoter in the allgal mutant is suitable for the economical production of recombinant proteins in S. cerevisiae.

The yeast Saccharomyces cerevisiae is widely used as a host for recombinant protein production due in part to its high cell density culture and excellent secretion of higher eukaryotic proteins compared to other expression systems [1]. Selection of an appropriate promoter is one of the most important factors affecting recombinant protein production. Not only the constitutive promoters (GAPDH, PGK, ADH, ENO, and TEF) from the genes of the glycolytic pathway and translational elongation factor, but also the inducible promoters (GAL1 and GAL10) of genes related to galactose utilization are widely used in S. cerevisiae. The transcription of GAL genes is induced by more than 1000-fold when galactose is the preferred carbon source [2,3]. When the strength of these promoters was compared using green fluorescent protein (GFP), the GAL promoter showed highest expression of GFP in the induced state [4]. Therefore, the GAL1 and GAL10 promoters are preferred for recombinant protein production in S. cerevisiae [5–7].

The expression of GAL genes is regulated by the transcription activator Gal4p and the transcription repressor Mig1p, which bind to the upstream activator sequence and the upstream repressor sequence of the GAL promoters, respectively. In non-galactose conditions, the negative regulator Gal80p directly binds to Gal4p and inhibits Gal4p function. This inhibition of Gal4p by Gal80p is suppressed by Gal3p in the presence of galactose [2]. Therefore, to induce GAL genes, a certain amount of galactose should be maintained as an inducer. However, the concentration of galactose continuously decreases during fermentation because it is consumed by cells as a carbon source. This is not economical for large-scale fermentation because galactose is much more expensive than glucose. Therefore, a gal1 strain unable to metabolize galactose was developed for the production of recombinant proteins using a minimal amount of galactose, which can induce GAL promoters [8–10]. Similarly, a gal80 mutant that expresses GAL promoters without galactose was also developed and successfully employed for the production of recombinant proteins using minimal amount of galactose, which can induce GAL promoters [11,12]. In the gal80 mutant, the expression under GAL1 promoter increased approximately seven-fold under non-inducing conditions, whereas the expression under GAL1 promoter increased 2.4-fold by deletion of GAL1 under inducing conditions, compared to the wild-type strain [11]. The expression level of recombinant lipase B from Candida antarctica (CalB) of the gal80 mutant without galactose was 1.6-fold higher than that of the gal1 mutant in the presence of a minimal amount of galactose [13]. Thus, the gal80 mutant is a promising host for the production of recombinant proteins using GAL promoters without using expensive galactose. However, the gal80 mutant showed approximately 20%–30% retarded cell growth in fed-batch fermentation compared to that of the wild-type strain. It has been reported that the amount of induced GAL mRNA is between 1% and...
2.5% of the total mRNA within the cell \cite{14} and the proteins for the galactose metabolism comprise ~5% of the total cellular mass \cite{15}. Although the expression of GAL genes in the gal80 mutant under non-inducing conditions was lower than that under inducing conditions, a significant amount of GAL mRNA was detected \cite{16}. In fact, the products of GAL genes are of no use, and so are burdens for the gal80 mutant in a non-galactose medium. Therefore, we assumed that growth retardation might be caused by the exhaustion of resources for the unnecessary expression of GAL metabolism genes.

In the present study, we constructed a mutant strain (allgal) in which GAL80 and all GAL structural genes were disrupted and verified its effect on the growth and protein expression by GAL10 promoter using three recombinant proteins as reporters by fed-batch fermentation. As the GAL1, GAL10, and GAL7 genes are contiguously located on chromosome II, these genes, including promoter regions, are disrupted by the co-transformation of a pCAS-gGAL7 vector (Addgene plasmid # 60,847) \cite{17} and a disruption cassette in one fell swoop (Fig. 1A). The pCAS vector expresses the Cas9 nuclease and sgRNA containing a 20-nucleotide guide sequence targeted to GAL7 (5’-GATTG-TAAGCTCTATGGGAA-3’), and the disruption cassette contains 400 bp of GAL1 and GAL7 fragments to act as a template for homology-directed repair. The GAL2 gene was disrupted separately using a pCAS-gGAL2 vector that expresses sgRNA containing a guide sequence targeting the GAL2 promoter (5’-CAATTG-GAAACGTCTCCTTC-3’) and a linear disruption cassette containing 400 bp of GAL2 promoter and GAL2 ORF fragments. gal1, gal7 and gal2 represent inactive GAL1, GAL7 and GAL2 gene.

![Fig. 1. Schematic diagram for targeted disruption of all GAL genes using CRISPR-Cas9 system. (A) One-step disruption of GAL1, GAL10 and GAL7, (B) Disruption of GAL2. The pCAS-gGAL7 vector expresses the Cas9 nuclease and sgRNA containing a 20-nucleotide guide sequence targeted to GAL7 (5’-GATTG-TAAGCTCTATGGGAA-3’), and the disruption cassette contains 400 bp of GAL1 and GAL7 fragments to act as a template for homology-directed repair. The GAL2 gene was disrupted separately using a pCAS-gGAL2 vector that expresses sgRNA containing a guide sequence targeting the GAL2 promoter (5’-CAATTG-GAAACGTCTCCTTC-3’) and a linear disruption cassette containing 400 bp of GAL2 promoter and GAL2 ORF fragments. gal1, gal7 and gal2 represent inactive GAL1, GAL7 and GAL2 gene.

![Fig. 2. Comparison of cell growth and lipase activity between gal mutants in batch culture. (A) Cell growth, (B) lipase activity. The gal80, allgal strains were cultured in YPD medium (1% yeast extract, 2% peptone, and 2% glucose). The wild-type was cultured in YPDG medium (1% yeast extract, 2% peptone, 1% glucose, and 1% galactose) and gal1, and gal1/10/7 mutants were cultured in YPDg medium (1% yeast extract, 2% peptone, 2% glucose, and 0.05% galactose) to induce the GAL10 promoter. The lipase activity of the culture supernatants was determined using p-nitrophenyl palmitate as a substrate. Mean values and standard deviations of triplicates are shown. *p < 0.03.](image-url)

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**gal1/gal10/gal7** mutants were cultivated under inducing conditions. Although the composition of the carbon source (glucose and galactose) in the media differed, the total amount of carbon sources was identical. The growth and secreted lipase activity of gal1 and gal1/gal10/gal7 mutants were inferior to those of the wild-type; however, the gal80 and allgal mutants surpassed the wild-type strain in growth and lipase activity after 48-h cultivation (Fig. 2A, B). Although the medium used for cultivation of the gal80 and allgal strains contained twice as much glucose as that used for cultivation of the wild-type, glucose repression did not occur in the gal80 and allgal strains, unlike other strains (Fig. 2B). Because the **GAL10** promoter (500 bp) used in YGaT3-CalB1422 vector contains only one putative Mig1p binding site (Fig. S1), contrary to the **GAL1** promoter that contains two Mig1p binding sites [18], glucose repression mediated by Mig1p does not affect the expression of CalB1422 in the gal80 and allgal strains. We confirmed this by comparison of **GAL10** promoter and **GAL1** promoter with deleted putative Mig1p binding site (Fig. S1). The secreted lipase activities of gal80 and allgal mutants were 20% higher than that of the wild-type (Fig. 2B). However, there was no significant difference between the gal80 and allgal mutants in batch cultures because expression of **GAL4** is repressed 4-5 fold by glucose mediated Mig1p, eventually lowering the expression of **GAL** genes [19]. Therefore the effect of **GAL** genes disruption on the growth of the host strain appear to not be prominent in batch cultures.

To minimize glucose repression of **GAL4** expression, the gal80 and allgal strains harboring the YGaT3CalB1422 vector were compared by fed-batch fermentation maintaining low concentration of glucose. Growth of the allgal mutant expressing CalB1422 was found to be increased by approximately 20% (OD<sub>600</sub>=159 vs. 132) compared to the gal80 strain (Fig. 3A). Accordingly, the amount of total secreted proteins was also enhanced by approximately 16% (Fig. 3A). When the transcription of CalB1422 of 36-h culture was compared by qPCR, the allgal mutant showed 2.39 fold higher mRNA level than the gal80 strain and the difference was further increased to 3.37 fold after 48-h fermentation (Fig. 3B). To confirm the effects of allgal mutation on the secretion of CalB1422, the culture supernatants and the intracellular fractions were analyzed using SDS-PAGE and western blot (Fig. S2) and the intracellular and extracellular lipase activity of the 48-h culture were compared (Fig. 3C). Because the culture broth contains various proteins in addition to CalB1422, the increase in lipase activity of the allgal strain compared to the gal80 mutant was higher than that of the total secreted proteins (36% vs. 16%). With the enhanced expression of CalB1422 in allgal strain, intracellular lipase activity of the allgal strain was more than two-fold higher than that of the gal80 mutant (31% vs. 14%).

To exclude recombinant protein-specific effects, human serum albumin (**HSA**) and human epidermal growth factor (**hEGF**) were also tested as passenger proteins. YGaHSA and YEG-HL28-EGF vector [6] that express **HSA** and **hEGF** fusion proteins under the control of the **GAL10** promoter were transformed into gal80 and allgal strains, respectively.

Cell growth was found to be affected by the type of recombinant protein, but the final cell mass and the total secreted proteins of the allgal strains were higher than those of the gal80 mutants, regardless of recombinant proteins (growth: 15%-38% and proteins: 18%-22%) (Fig. 4). When we tested other proteins, the allgal mutant always showed higher performance than the gal80 mutant as a recombinant protein expression host.

In conclusion, the allgal strain developed in this study demonstrated improved cell growth and protein secretion compared to the gal80 mutant during fed-batch fermentation; this difference is likely due to the removal of the burden of expression of galactose-metabolizing genes. Consequently, the allgal strain can be used for the economical production of recombinant proteins with **GAL** promoters in S. cerevisiae.

**Author contributions**

J.H.B. and J.H.S. designed the experiments. M.J.K engineered the yeast strains. H.J.P did the fermentation. B.H.S analyzed the data. J.H.B, M.J.K and J.H.S. drafted the manuscript, which was edited by all authors.

**Data statement**

All data reported in the paper are available from the corresponding author upon reasonable request. Materials and Methods in this study are described in the Supplementary information.

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Declaration of Competing Interest

The authors have no competing interests to declare.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.btre.2022.e00763.

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Fig. 4. Comparison of gal80 and allgal strains by fed-batch fermentation of various proteins. Cell growth (A) and total secreted proteins (B) of gal80 and allgal harboring YGalHSA. Cell growth (C) and total secreted proteins (D) of gal80 and allgal harboring YEGaHL28-EGF. Mean values and standard deviations of triplicates are shown. *p < 0.03.
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