Characterization of Two GAS1 Genes and Their Effects on Expression and Secretion of Heterologous Protein Xylanase B in Kluyveromyces lactis

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Introduction

The yeast Kluyveromyces lactis has been extensively studied and used for development of heterologous protein expression systems because of its strong heterologous protein secretory ability [37]. It has been classified as a GRAS (generally recognized as safe) organism by the US Food and Drug Administration, and utilized for industrial production of a variety of proteins, including serum albumin and interleukin-1β. K. lactis expression systems can be induced by lactose and do not require explosion-proof fermentation equipment like that necessary for large-scale growth of methylotrophic yeasts such as Pichia pastoris [7]. Thus, K. lactis is a safer host strain than P. pastoris and others for industrial production.

The yeast cell wall is typically a physical barrier to movement of large molecules into or out of the cell, and a limiting factor for heterologous protein secretion. It consists of an inner layer containing the polysaccharides β-1,3-glucan, β-1,6-glucan, and chitin, and an outer layer containing mannoproteins. β-1,3-glucan functions as the backbone of a branched network structure that covers large areas of the yeast cell surface [4]. The enzyme β-1,3-glucanyltransferase plays an important role in β-1,3-glucan remodeling processes during yeast cell wall synthesis [23].

The yeast Saccharomyces cerevisiae has five homologs of β-1,3-glucanyltransferase genes (Gas family genes) [27].
GASI, GAS3, and GAS5 are expressed mainly during the vegetative growing phase, and GAS2 and GAS4 during the sporulation phase [17, 28]. We identified six putative β-1,3-glucanoyltransferase genes in K. lactis, termed KGas family genes: KIGAS1-1, KIGAS1-2, KIGAS2, KIGAS3, KIGAS4, and KIGAS5. KIGAS1-1 and KIGAS1-2 are homologs of GASI in S. cerevisiae. Secreted proteome and cell wall composition analyses demonstrated that the products of KGas family genes are localized in the K. lactis cell wall [2, 21, 34].

We previously constructed recombinant strain GXX31 from K. lactis GG799 by inserting a thermostable xylanase B gene (xynB, cloned from Thermotoga maritima MSB8) [43]. xynB has been expressed highly in E. coli [14], P. pastoris [42], and Aspergillus niger [45], but expression was lower in K. lactis. Following medium optimization, the transcription level of KIGAS1-1 was significantly reduced and xylanase B secretion was enhanced, as indicated by RT-qPCR analysis (unpublished data). We hypothesized that these two events were related. To evaluate this possible relationship, and to improve heterologous protein secretion and transport were examined. KIGAS1-1 and KIGAS1-2 engineering will be useful for improving heterologous protein secretion in K. lactis.

Materials and Methods

Strains, Plasmids, and Culture Conditions

The strains and plasmids used in this study are listed in Table 1. K. lactis strains were grown on YPD (2% yeast extract, 1% tryptone, 2% glucose) or YPL (2% yeast extract, 1% tryptone, 2% lactose) liquid or solid (1.5% agar) media at 30°C in a shake flask (200 rpm). Geneticin (G-418) (final concentration 300 µg/ml) or hygromycin B (final concentration 150 µg/ml) was added for transformant selection.

Sequence Alignment and Structure Prediction of KGas1-1 and KGas1-2

Multiple-sequence alignment was performed using the Easy Sequencing in the Postscript (ESPript) online program (http://espirpt.ibcp.fr/ESPript/ESPript/index.php) [30]. The KGas1-1 and KGas1-2 structures were predicted using the SWISS-MODEL program (http://swissmodel.expasy.org) [1, 10, 32].

Construction of KGas1s Deletion Mutants

Gene disruption by homologous replacement in K. lactis requires long DNA sequences (preferably 1,000 bp or more) [15, 18]. Gene-disruption cassettes were prepared by double-joint PCR [40, 44], using the construction method illustrated in Fig. S1A. In the first round of double-joint PCR, the upstream and downstream flanking fragments of target genes for homologous regions and the resistance gene kanMX6 (cloned from pFA6a-kanMX6) or hph (cloned from pCSN44) were amplified using DNA polymerase KOD-plus-Neo (Toyobo, Japan). In the second round, three fragments were purified, mixed (molar ratio 1:3:1) in the reaction mixture, and joined by PCR without primers. Thermocycling conditions: 94°C for 2 min; 15 cycles of 94°C for 30 sec, 58°C for 10 min, and 68°C for 2 min; 68°C for 4 min. In the third round, a pair of nested primers was used for final gene-disruption cassette amplification.

Gene-disruption cassettes were transformed into K. lactis GXX31 by electroporation as described previously [31]. Genomic DNA of transformants was prepared as PCR templates by the DNA-containing cell envelope method [6]. Transformants were confirmed by four rounds of PCR using specific primers. Then the integration regions of positive transformants were sequenced. The primer design method is illustrated in Fig. S1B, and primer sequences are listed in Table S1.

Drug Sensitivity

Cells of each strain were grown in YPD medium until

<table>
<thead>
<tr>
<th>Table 1. Strains and plasmids used in this study.</th>
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<tbody>
<tr>
<td><strong>Strains</strong></td>
</tr>
<tr>
<td>K. lactis GG799</td>
</tr>
<tr>
<td>K. lactis GXX31</td>
</tr>
<tr>
<td>K. lactis K1-1</td>
</tr>
<tr>
<td>K. lactis K1-2</td>
</tr>
<tr>
<td>K. lactis KD</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
</tr>
<tr>
<td>pFA6a-kanMX6</td>
</tr>
</tbody>
</table>

logarithmic phase and then diluted to OD_{600} = 1. Pure sample and 1:10 serial dilutions (5 µl) of each culture were spotted on YPD plates with no agent addition (control) or with 50 µg/ml osmotic destabilizing agent SDS (Amresco, USA) or 50 µg/ml cell wall-perturbing agent Calcofluor White M2R (CFW; Sigma, USA). Plates were incubated for 48 h at 30°C and growth parameters were recorded.

Phase Contrast Microscopy and Transmission Electron Microscopy (TEM)
For phase contrast microscopy, cells were cultured in YPL until logarithmic phase (48 h) and harvested. Samples were viewed using a microscope (model Eclipse 50i; Nikon; Japan) equipped for phase contrast observation. For accurate evaluation of morphological changes in the mutant strains, minor and major axes were measured for 50 cells in each sample, and the ratio of minor to major axis was calculated.

For TEM, each strain was sampled in the logarithmic phase (38 h) and stationary phase (96 h). Sections (thickness 90 nm) were obtained by centrifugation of the medium. Xylanase B activity was determined by the RBB-xylan method as described in our previous study [43], with reaction at 90°C, the optimal temperature of xylanase B [45].

Xylanase B Activity
Cells of each strain were cultured in YPL, and crude proteins were obtained by centrifugation of the medium. Xylanase B activity was determined by the RBB-xylan method as described in our previous study [43], with reaction at 90°C, the optimal temperature of xylanase B [45].

Western Blotting
A 12% gel was used for western blotting, with 10 µl of each sample added to the lanes. Samples were electrophoresed onto PVDF membrane (Roche, UK) and treated with rabbit anti-xylanase B polyclonal antibody (Beijing Protein Institute, China; dilution 1:1,500) and anti-rabbit IgG HRP-conjugated secondary antibody (cat no. CW0103, CWBIO; China; dilution 1:10,000). Data were analyzed using the ImageJ software program (http://imagej.nih.gov/ij).

Total Released Proteins
Cells were cultured in YPL, harvested, and centrifuged. Total proteins in supernatants were determined by Bradford’s dye-binding method (Super-Bradford Protein Assay Kit; CWBIO), using BSA as the standard. Data were normalized according to OD_{595} value, and biomass was estimated on the basic of a linear plot of OD_{595} vs. weight.

Real-Time RT-qPCR
Cells of each strain were grown in YPL and harvested in logarithmic phase (38 h) and in stationary phase at the time of maximal xylanase B activity (96 h). Total RNA was isolated using an RNAsimple Total RNA kit (Tiangen Biotech, China), and extracted RNA was reverse-transcribed into cDNA using M-MLV reverse transcriptase (Promega, USA). Quantitative PCR was performed using a Light Cycler 480 RT-qPCR system with the Light Cycler 480 SYBR Green I Master Kit (Roche, Germany). The relative expression of each gene was calculated by the comparative crossing point (C_{T}) method and presented as 2^{-\Delta\Delta C_{T}} [19]. Values shown are means of three replicates. KIGAS1-1, KIGAS1-2, KIGAS2, KIGAS3, KIGAS4, and KIGAS5 (respective NCBI Gene IDs 2892386, 2892599, 2897360, 2891804, 2894064, and 2892754) in K. lactis were assayed. KIACT1 (NCBI Gene ID 2893427), which encodes the single essential gene for actin, was used as the internal control and reference. Sixteen secretion-related and transport-related genes, reported to be involved in heterologous protein secretion and transport pathways in other yeast species [13], were also assayed in GXX31 and the mutant strains, to evaluate the effect of target gene deletion on heterologous protein secretory pathways. Primers used for real-time RT-qPCR are listed in Table S2.

Results
Identification of Two Homologous Proteins, KIGas1-1 and KIGas1-2
In K. lactis, the KIGAS1-1 gene is composed of 1,668 bp and encodes 555 amino acid residues; the KIGAS1-2 gene is composed of 1,626 bp and encodes 541 amino acid residues. The K. lactis KIGas1-1 and KIGas1-2 proteins are classified as β-1,3-glucanosyltransferases (NCBI Accession No. CAH101681 and CAH101682) and show 67% and 64% homology with β-1,3-glucanosyltransferase Gas1 from S. cerevisiae S288c (NCBI Accession No. NP_014038). Comparative amino acid sequence alignments and predicted protein structures of KIGas1-1, KIGas1-2, and Gas1 are shown in Figs. S2 and S3. Each of these proteins contains a Glyco_hydro_72 domain (glucanosyltransferase domain [24]) and X8 domain (involved in carbohydrate binding [12]). We conclude that the products of KIGAS1-1 and KIGAS1-2 are β-1,3-glucanosyltransferases.

Mutant Strains and Their Growth Curves
Three gene deletion mutant strains were generated: KIGAS1-1 single deletion strain K1-1 (Klgas1-1::kanMX6), KIGAS1-2 single deletion strain K1-2 (Klgas1-2::hph), and double deletion strain KD (Klgas1-1::kanMX6 Klgas1-2::hph).

To assess the effects of KIGAS1-1 and KIGAS1-2 deletion on cell growth, the mutant strains were cultured in YPL.
and growth curves were constructed. Maximal OD_{600} values for GKX31, K1-1, and K1-2 were 44.4, 45.6, and 45.5, respectively (Fig. 1A). The growth curves of K1-1 and K1-2 were not notably affected by the gene deletion. For double deletion strain KD, the cell number increase rate at the beginning of the logarithmic phase, maximal OD_{600} value at the stationary phase (31.7 OD_{600}), and overall growth curve were lower in comparison with K1-1 and K1-2.

Cell Morphology and Drug Sensitivity

The cell morphology of GKX31 and the mutants was observed by phase contrast microscopy. Morphological and physiological phenotypes differed for the KlGAS1-1 and KlGAS1-2 single deletion mutants, K1-1 and K1-2. They did not have a rounded shape like that of S. cerevisiae GAS1 mutant, but rather an oval shape like that of GKX31. In double deletion strain KD, the cell shape was spherical, and altered vacuoles were visible. The ratio of minor to major axis width indicated that the shape of KD was nearly a perfect sphere. Average major and minor axis widths were greater for KD than GKX31 (Table 2).

In experiments with five concentration conditions (pure sample and four serial dilutions) of cells cultured with 50 µg/ml SDS for 48 h at 30°C, K1-1 colonies appeared under all five conditions, but were smaller than in the control (YPD culture without SDS). KD colonies appeared under only one concentration condition, and displayed a growth delay phenotype. Growth of K1-2 and GKX31 was essentially the same in both SDS and YPD cultures (Fig. 1B). Thus, KD and K1-1 were sensitive to SDS, whereas K1-2 was resistant.

In corresponding experiments with cells cultured in 50 µg/ml CFW, GKX31 colonies appeared under all five concentration conditions, but were smaller than YPD culture controls. K1-1 colonies and K1-2 colonies appeared under two and three concentration conditions, respectively. No KD colonies were able to survive in CFW culture (Fig. 1B). Thus, the three deletion mutants were more sensitive to CFW than was GKX31, and K1-2 had greater resistance to CFW than did K1-1 or KD.

Cell Wall Morphology

Cell wall ultrastructure was observed by TEM. Line segments in Fig. 2G indicate the points where cell wall measurements were taken. For each strain, 40 cells in logarithmic phase (38 h) and stationary phase (96 h) were measured. The data are summarized in Table 3. Effects on cell wall structure were quite different for deletion of GAS1 homologs KlGAS1-1 vs. KlGAS1-2. In the logarithmic phase, the cell wall thickness of K1-1 (143.22 nm) was less than that of GKX31 (173.29 nm) (Figs. 2A, 2B, 2E, 2F), whereas the thickness of K1-2 (271.59 nm) was more than that of GKX31 (Figs. 2C, 2D). In the double deletion mutant (KD), cell wall thickness (126.47 nm) was less than that of K1-1 (Figs. 2D, 2H). Cell wall thickness values in the stationary phase showed the same trend: K1-2 (302.39 nm) > GKX31 > K1-1.

Table 2. Minor and major axis widths of the four strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Minor axis width (µm)</th>
<th>Major axis width (µm)</th>
<th>Ratio of minor to major axis width</th>
</tr>
</thead>
<tbody>
<tr>
<td>GKX31</td>
<td>140.055 ± 0.064</td>
<td>184.510 ± 0.080</td>
<td>0.806 ± 0.008</td>
</tr>
<tr>
<td>K1-1</td>
<td>149.423 ± 0.058</td>
<td>174.278 ± 0.076</td>
<td>0.864 ± 0.012</td>
</tr>
<tr>
<td>K1-2</td>
<td>168.109 ± 0.095</td>
<td>197.877 ± 0.117</td>
<td>0.854 ± 0.009</td>
</tr>
<tr>
<td>KD</td>
<td>226.769 ± 0.169</td>
<td>227.970 ± 0.172</td>
<td>0.996 ± 0.008</td>
</tr>
</tbody>
</table>

Values shown are the mean ± SD from 50 cells.
Effects of Two GAS1 Genes on Xylanase B Secretion in *K. lactis*

Protein Secretory Phenotypes

Strains were cultured in YPL, and xylanase B activity curves were plotted (Fig. 3A). Maximal xylanase B activity values for all strains were observed at 96 h, and were in the order K1-1 (28.86 U/ml) > KD (27.34 U/ml) > GKX31 (24.52 U/ml) > K1-2 (15.78 U/ml).

Heterologous protein secretory ability was evaluated as xylanase B activity per OD$_{600}$ (Fig. 3B). Values obtained were in the order KD (0.86 U/ml per OD$_{600}$) > K1-1 (0.68 U/ml per OD$_{600}$) > GKX31 (0.58 U/ml per OD$_{600}$) > K1-2 (0.35 U/ml per OD$_{600}$).

Extracellular and intracellular amounts of xylanase B were measured by western blotting (Fig. 3C). A higher ratio reflected greater xylanase B secretory ability. Values of xylanase B secretion ratio were in the order KD (57.6%) > K1-1 (50.0%) > GKX31 (43.8%) > K1-2 (35.5%).

For measurement of total released proteins, samples were harvested at 48 h for the logarithmic phase and at 96 h for the stationary phase, based on the growth curves. Values for released proteins were expressed per OD$_{600}$ (Fig. 3D). The trends observed were the same as for xylanase B secretory ability. In the logarithmic phase, values of total released proteins were in the order KD (9.54 µg/ml per OD$_{600}$) > K1-1 (4.52 µg/ml per OD$_{600}$) > GKX31 (4.03 µg/ml per OD$_{600}$) > K1-2 (3.92 µg/ml per OD$_{600}$). In the stationary phase, the order was the same: KD (8.11 µg/ml per OD$_{600}$) > K1-1 (5.18 µg/ml per OD$_{600}$) > GKX31 (4.85 µg/ml per OD$_{600}$) > K1-2 (4.28 µg/ml per OD$_{600}$).

In summary, KlGAS1-1 gene deletion (K1-1) resulted in the highest xylanase B activity value: 28.86 U/ml, 17.7% more than the value for GKX31 (Fig. 3A). Double gene deletion (KD) resulted in the highest xylanase B secretion ratio (57.6%), 13.8% more than the value for GKX31 (Fig. 3C). There was some inconsistency between the enzymatic activity of XynB in K1-2 (Fig. 3A) and western blot band of XynB in K1-2 (Fig. 3C). It can be speculated that although the concentration of XynB was lower, the specific activity of XynB increased in K1-2 as a result of conformation change of XynB.

Transcription Levels of KlGas Family Genes

Transcription patterns of six KlGas family genes were evaluated in GKX31 and the deletion mutants. *KlGAS1-1, KlGAS1-2, and KlGAS3* are KlGas family genes most highly expressed in *K. lactis*. Compared with the transcription level of *KlGAS1-1*, that of *KlGAS1-2* was 3.87-fold higher in logarithmic phase and 1.28-fold higher in stationary phase. In the logarithmic phase, *KlGAS1-1* deletion resulted in 1.15-fold increase of *KlGAS1-2* and 0.48-fold decrease of *KlGAS3* transcription levels. *KlGAS1-2* deletion resulted in 2.32-fold increase of *KlGAS1-1* and 0.26-fold decrease of the *KlGAS3* transcription level. *KlGAS1-1* and *KlGAS1-2* double deletion resulted in 6.51-fold increase of the *KlGAS5* transcription level (Fig. 4A).

In the stationary phase, *KlGAS1-1* deletion resulted in 2.22-fold increase of *KlGAS1-2* and 0.58-fold decrease of *KlGAS3* transcription levels. *KlGAS1-2* deletion resulted in 3.86-fold increase of *KlGAS1-1* and 2.26-fold increase of *KlGAS3* transcription levels. *KlGAS1-1* and *KlGAS1-2* double deletion resulted in 5.67-fold increase of *KlGAS4* and 1.45-fold increase of *KlGAS5* transcription levels (Fig. 4B).

**Table 3.** Cell wall thickness of the four strains.

<table>
<thead>
<tr>
<th>Time</th>
<th>Cell wall thickness (nm)</th>
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<tbody>
<tr>
<td></td>
<td>GKX31</td>
</tr>
<tr>
<td>38 h</td>
<td>173.29 ± 2.19</td>
</tr>
<tr>
<td>96 h</td>
<td>178.55 ± 1.71</td>
</tr>
</tbody>
</table>

Values shown are the mean ± SD from 40 cells. Cell wall thickness was determined at 10 different points of the mother cell (see Fig. 2G).
In the logarithmic phase, the transcription level of \( KIGAS1-2 \) in K1-1 was 1.91-fold higher than that of \( KIGAS1-1 \) in K1-2 (Fig. 4A), whereas the cell wall thickness of K1-1 (143.22 nm) was less than that of K1-2 (271.59 nm) (Table 3). No significant differences were observed among transcription levels of the other four KIGas family genes (\( KIGAS2, \ldots \).)
KIGAS3, KIGAS4, and KIGAS5). Based on these findings, we hypothesized that the β-1,3-glucanosyltransferase specific activity of the KIGAS1-1 product is higher than that of the KIGAS1-2 product.

**Transcription Levels of Secretion-Related and Transport-Related Genes**

Information (class, product localization, and function) for the assayed genes is summarized in Table 4. Transcription levels of the assayed genes in GXX31 and the gene deletion mutants, in the logarithmic (38 h) and stationary (96 h) phases, are shown in Fig. 5.

All strains had faster growth and lower xylanase B activity in the logarithmic phase than in the stationary phase. In most cases, transcription levels of secretion- and transport-related genes were lower in the deletion strains than in GXX31. The exceptions were KISEC31 (3.45-fold higher in K1-1, 3.41-fold higher in K1-2, and 3.25-fold higher in KD compared with GXX31) and KIERO1 (1.89-fold higher in KD) (Fig. 5A). These findings suggest that the heterologous protein secretory pathway in the logarithmic phase was more active in GXX31 than in the deletion strains. The deletion strains had higher KISEC31 transcription levels and higher efficiency of protein transport from the endoplasmic reticulum (ER) to Golgi.

All strains had maximal xylanase B activity in the stationary phase. Heterologous protein secretory ability showed the greatest difference in GXX31 vs. the deletion strains in this phase. These differences were reflected in the transcription levels of secretion- and transport-related genes (Fig. 5B). In double deletion strain KD, which had the highest heterologous protein secretion ratio (Fig. 3), transcription levels were higher than in GXX31 for KIHAC1 (13.42-fold), KISSA3 (5.07-fold), KIGCN4 (12.07-fold), KICUP5 (8.06-fold), KIERO1 (9.76-fold), KIKIN2 (2.93-fold), KISSE1 (7.31-fold), and KIMON2 (8.49-fold). These genes are involved in unfolded protein response, protein folding, vacuolar acidification, endocytosis, and vacuole integrity. These genes also showed higher transcription level in deletion strain K1-1, which had high heterologous protein secretory ability, than in GXX31.

**Discussion**

**Two β-1,3-Glucanosyltransferase Genes Display Transcription Complementation That Affects Transcription Levels of Secretion- and Transport-Related Genes**

When yeast cells are challenged by stress conditions that affect the cell wall, salvage mechanisms such as the cell wall integrity pathway are activated to promote cell survival through remodeling of the extracellular matrix [16, 26]. These mechanisms are reflected by specific

<table>
<thead>
<tr>
<th>Class</th>
<th>Gene</th>
<th>Product localization</th>
<th>Function</th>
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<tbody>
<tr>
<td>Folding and QC system</td>
<td>KIERO1</td>
<td>ER membrane</td>
<td>Thiol oxidase required for oxidative protein folding in ER</td>
</tr>
<tr>
<td></td>
<td>KIKAR2</td>
<td>ER lumen</td>
<td>Codes Bip, a major Hsp70 chaperone in ER</td>
</tr>
<tr>
<td></td>
<td>KIPDI1</td>
<td>ER lumen</td>
<td>Protein disulfide bond formation</td>
</tr>
<tr>
<td></td>
<td>KIHAC1</td>
<td>Nucleus</td>
<td>Regulates unfolded protein response via UPRE binding</td>
</tr>
<tr>
<td></td>
<td>KISSA3</td>
<td>Cytoplasm</td>
<td>ATPase involved in protein folding and stress response</td>
</tr>
<tr>
<td></td>
<td>KISSE1</td>
<td>Cytoplasm</td>
<td>Chaperone in Hsp70 family; binds unfolded protein</td>
</tr>
<tr>
<td>Transcription, translation, and signaling</td>
<td>KIGCN4</td>
<td>Nucleus</td>
<td>Activator that responds to amino acid starvation; expression is tightly regulated at both the transcriptional and translational levels</td>
</tr>
<tr>
<td>Trafficking</td>
<td>KIKIN2</td>
<td>Plasma membrane</td>
<td>Serine/threonine protein kinase involved in regulation of exocytosis</td>
</tr>
<tr>
<td></td>
<td>KIBFR2</td>
<td>Nucleus</td>
<td>ER-to-Golgi transport, rDNA processing</td>
</tr>
<tr>
<td></td>
<td>KIMBH1</td>
<td>Cytoplasm</td>
<td>Regulates many cell processes, including exocytosis and vesicle transport</td>
</tr>
<tr>
<td></td>
<td>KICOG6</td>
<td>Golgi apparatus</td>
<td>Mediates fusion of transport vesicles to Golgi compartments</td>
</tr>
<tr>
<td></td>
<td>KICUP5</td>
<td>Vacuole membrane</td>
<td>Required for vacuolar acidification, and important for copper and iron metal ion homeostasis</td>
</tr>
<tr>
<td></td>
<td>KIMH1</td>
<td>Cytoplasm</td>
<td>Mediates transport between endosomal compartment and Golgi</td>
</tr>
<tr>
<td></td>
<td>KIMON2</td>
<td>Endosome</td>
<td>Involved in vacuole integrity</td>
</tr>
<tr>
<td></td>
<td>KISEC31</td>
<td>Vesicle</td>
<td>Component of COPII coat of ER-Golgi vesicles</td>
</tr>
<tr>
<td></td>
<td>KISSO2</td>
<td>Plasma membrane</td>
<td>Involved in fusion of secretory vesicles at the plasma membrane</td>
</tr>
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</table>
transcriptional responses such as enhanced expression of putative cell wall assemblies and potential cross-linking cell wall proteins. In our *K. lactis* model, two single gene deletions and a double gene deletion increased transcription

**Fig. 5.** Transcription levels of secretion- and transport-related genes in the four strains, in the logarithmic phase (38 h) (A) and stationary phase (96 h) (B).

The 16 genes were divided into four groups in each of the two phases according to transcription levels.
Levels of various KlGas family genes (Fig. 4). Cells thereby increased β-1,3-glucanosyltransferase levels to ensure continued cell wall synthesis and protect themselves against lysis. Differing complementary effects of other KlGas family genes in the three gene deletion strains resulted in differences in cell morphology and cell wall thickness (Fig. 2; Tables 2 and 3). In comparison with original strain GKX31, cell wall thickness was greater in K1-2 and less in the other two deletion mutants (Figs. 2C, 2G). Based on transcription levels of KlGas family genes in GKX31 (Fig. 4), KlGas1-1 and KlGas1-2 appeared to be the most highly expressed KlGas family genes in the logarithmic and stationary phases (Fig. 4). In the logarithmic phase, the transcription level of KlGas1-2 in K1-1 was higher than that of KlGas1-1 in K1-2 (Fig. 4A), whereas the cell wall thickness of K1-1 was less than that of K1-2 (Figs. 2B, 2C). Transcription levels of the other four KlGas family genes (Klgas2, KlGas3, KlGas4, and KlGas5) did not differ significantly between K1-1 and K1-2. These findings indicate that the KlGas1-1 product had greater cell wall synthesis activity than did the KlGas1-2 product. KlGas1-2 deletion increased the complementary effect of KlGas1-1, and also increased the effect of KlGas3 and KlGas4 in the stationary phase (Figs. 4B, 6), resulting in a thicker cell wall in K1-2.

In the present study, two β-1,3-glucanosyltransferase genes affected transcription levels of secretion- and transport-related genes. Previous studies showed that the β-1,3-glucanosyltransferase Gas1 is located in not only the cell wall (where it is involved in cell wall synthesis) but also the nuclear periphery (where it affects transcription levels of genes in genomic regions silenced by Sir2, and modulates Sir2-mediated rDNA stability) [11, 17]. We found that deletion of two β-1,3-glucanosyltransferase genes (KlGas1-1 and KlGas1-2) in K. lactis affects transcription levels of genes related to protein secretion and transport (Fig. 5). In the stationary phase, the protein secretory ability of all our strains was maximal (Fig. 3B), and differences among the strains in transcription levels of secretion- and transport-related genes were most pronounced (Fig. 5B). The effects of our three deletion treatments on transcription levels of these genes in the stationary phase, using the Cytoscape program (ver. 3.2.0) [5], are summarized in Fig. 6. Only significant changes (≥2-fold) in transcription are shown in this figure. Enhancement of transcription level was observed for 10 secretion- and transport-related genes by KlGas1-1 and KlGas1-2 double deletion, for six genes by KlGas1-1 single deletion, and for four genes by KlGas1-2 single deletion. The strain having the highest heterologous protein secretion ratio also showed increased transcription of the greatest number of secretion- and transport-related genes (Figs. 3B, 3C, and 6).

In S. cerevisiae, increased transcription of protein secretion- and transport-related genes enhanced heterologous protein secretion [13]. In K. lactis, overexpression of molecular chaperone gene Kiero1 led to increased yield of a
heterologous protein, human serum albumin [20]. We therefore assume that the differences in protein secretion ratio among our deletion strains resulted mainly from changes in transcription levels of secretion- and transport-related genes. Transcription of secretion- and transport-related genes was higher in the strain with lower β-1,3-glucanoyltransferase function (less cell wall thickness) (Figs. 2, 6). We conclude that the two β-1,3-glucanoyltransferase genes KlGAS1-1 and KlGAS1-2 in K. lactis display transcription complementation that affects transcription levels of secretion- and transport-related genes.

**KlGAS1-1 Deletion Is an Effective Engineering Strategy for Enhancing Heterologous Protein Secretion**

Structural modification of the cell wall in yeast can improve heterologous protein secretion [8]. Some studies aimed at increasing heterologous protein secretion in K. lactis have focused on genes involved in cell wall synthesis, including α1,6-mannosyltransferase gene OCH1 [36], α1,3-mannosyltransferase genes MNN1 and MNN10 [3, 18], and apyrase gene KYND1 [35]. Studies on β-1,3-glucanoyltransferase gene GAS1 have shown that its deletion increases heterologous protein secretion and inhibits cell growth in S. cerevisiae and other nonconventional yeasts such as P. pastoris, and Zygosaccharomyces bailii [22, 25, 37]. It is worth noting that the improvements of heterologous protein secretion in GAS1 deletion strains are protein dependent, for the secretion of human trypsinogen and human serum albumin in P. pastoris, human IL-1β in Z. bailii, and Trichoderma reesei endoglucanase I in Ashbya gossypii [29] were not increased after GAS1 deletion.

In this study, we constructed two single deletion mutants (K1-1 and K1-2) and one double deletion mutant (KD) of β-1,3-glucanoyltransferase genes KlGAS1-1 and KlGAS1-2 in K. lactis. Only KD had a phenotype similar to that of the GAS1 deletion mutant in S. cerevisiae, including an abnormal round cell shape and larger size (Table 2), reduced cell growth (Fig. 1A), and increased sensitivity to osmotic destabilizing agents SDS and cell wall-perturbing agent CFW (Fig. 1B). Of the three mutants, KD had the highest heterologous protein secretion ratio (Figs. 3B, 3C) but lower cell growth and cell density, resulting in lower xylanase B activity than that of K1-1 (Figs. 1A, 3A).

Xylanase B activity was highest for K1-1, and transcription of secretion- and transport-related genes was increased by KlGAS1-1 deletion. The genes in K1-1 that showed increased transcription were involved in protein folding (KIERO1, KISSA3, KISSE1), unfolded protein response (KIHACI), vacuole integrity (KJMON2), and vacuolar acidification (KICUP5) (Fig. 6; Table 4). In a study of P. pastoris, increased transcription of HAC1, ERO1, and SSE1 led to enhanced heterologous protein secretion [9]. We observed that increase of the KIERO1 transcription level in GXX31 promoted xylanase B secretion (data not shown). Our findings indicate that the increased transcription of secretion- and transport-related genes in K. lactis similarly enhances heterologous protein secretion, and K1-1 is a “super-secretor” of heterologous protein xylanase B. KlGAS1-1 deletion was a more effective cell wall engineering approach than the other two deletion treatments for increasing heterologous protein secretion in K. lactis.

In conclusion, the findings presented here demonstrate that modification of the cell wall synthesis gene KlGAS1-1 is a useful strategy for enhancing heterologous protein secretion in K. lactis. The next step in our studies is to examine whether heterologous protein secretion can also be improved by increasing the transcription levels of secretion- and transport-related genes such as KIHAC1, KISSE1, KISSA3, and KICUP5. Only xylanase B secretion was examined in the present study. Future functional screening of secretion- and transport-related genes using other secreted-protein models will allow us to further understand the secretory ability of K1-1.

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