Improved Production of Heterologous Proteins by a Glucose Repression-Defective Mutant of *Kluyveromyces lactis*

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The secreted production of heterologous proteins in *Kluyveromyces lactis* was studied. A glucoamylase (GAA) from the yeast *Arxula adeninivorans* was used as a reporter protein for the study of the secretion efficiencies of several wild-type and mutant strains of *K. lactis*. The expression of the reporter protein was placed under the control of the strong promoter of the glyceraldehyde-3-phosphate dehydrogenase of *Saccharomyces cerevisiae*. Among the laboratory strains tested, strain JA6 was the best producer of GAA. Since this strain is known to be highly sensitive to glucose repression and since this is an undesired trait for biomass-oriented applications, we examined heterologous protein production by using glucose repression-defective mutants isolated from this strain. One of them, a mutant carrying a dgr151-1 mutation, showed a significantly improved capability of producing heterologous proteins such as GAA, human serum albumin, and human interleukin-1β compared to the parent strain. dgr151-1 is an allele of RAG5, the gene encoding the only hexokinase present in *K. lactis* (a homologue of *S. cerevisiae* **HXK2**). The mutation in this strain was mapped to nucleotide position +527, resulting in a change from glycine to aspartic acid within the highly conserved kinase domain. Cells carrying the dgr151-1 allele also showed a reduction in N- and O-glycosylation. Therefore, the dgr151 strain may be a promising host for the production of heterologous proteins, especially when the hyperglycosylation of recombinant proteins must be avoided.

Yeasts are very useful hosts for the production of heterologous proteins. The yeast *Kluyveromyces lactis* presents several advantages over other yeast species. It is positive for lactose fermentation, is able to grow on cheap substrates such as residual whey from dairy industries, and has competitive secretory properties, excellent large-scale fermentation characteristics, and food grade status; also, both episomal and integrative expression vectors are available for it (for reviews, see references 20, 40, and 50). Its ability to secrete heterologous proteins into the medium at a concentration higher than that secreted by *Saccharomyces cerevisiae* was demonstrated previously (50), although the secretory and glycosylation processes and their regulation are still poorly understood for *K. lactis* (1, 42, 43).

For *K. lactis*, the regulation of primary carbon metabolism differs markedly from that for *S. cerevisiae* and reflects the dominance of respiration over fermentation that is typical for the majority of yeast species (7). In *K. lactis*, respiration is not repressed by glucose, and fermentative and oxidative metabolism can take place simultaneously. Glucose repression, however, does exist: several enzymes that are required for alternate carbohydrate metabolism have been shown to be subject to glucose repression (6, 13, 17, 25, 30). The *K. lactis* genes involved in glucose repression include RAG1, encoding a low-affinity glucose permease (23, 48); DGR151 (or RAG5), encoding the single hexokinase of this yeast (34), and KIMIG1, encoding a component of the repressor complex acting on glucose-repressed genes (11).

The **rag1** and **dgr151-1** mutants are both non-glucose-repressible pleiotropic mutants (25, 47). They are also impaired in fermentative metabolism and require respiration for growth on glucose (22, 34, 49); the **Kimig1** mutant is instead impaired in glucose repression only for the Lac/Gal pathway (14).

Notwithstanding the available knowledge on *K. lactis* physiology, no connections between the secretion and glycosylation pathways and between energy and carbon metabolism in this yeast have been explored.

We have therefore analyzed the secretory capabilities of *K. lactis* mutants affected in the genes mentioned above. Among these mutants, a notable phenotype was observed for the **dgr151-1** (or **rag5** mutant): this mutation resulted in a slight defect in glycosylation and a significantly improved capability in the secreted production of heterologous proteins.

**MATERIALS AND METHODS**

**Strains and growth conditions.** The yeast strains used and their relevant genotypes are listed in Table 1. The medium contained 5 g of Difco yeast extract and 10 g of Difco Bacto Peptone per liter. Minimal medium contained 0.7 g of yeast nitrogen base without amino acids (Difco) liter–1 supplemented with appropriate amino acids and bases as required to a final concentration of 40 μg ml–1. Media were solidified with 2% Bacto agar. Cells were grown on YP agar supplemented with 2% glucose (YPD) or 2% galactose or on low-phosphate medium as previously described (33). The antibiotic G418 was added to a final concentration of 100 μg ml–1. Fivefold serial dilutions from concentrated suspensions of exponentially growing cells (5 × 10⁵ cell ml–1) were spotted onto
TABLE 1. List of K. lactis strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source or Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>JA6</td>
<td>MATαΔade1-600 adeT-600 trp1-11 uraA1-1</td>
<td>H. Fukuhara</td>
</tr>
<tr>
<td>PM6-7A</td>
<td>MATαΔade1-600 adeT-600 uraA1-1</td>
<td>M. Wesołowski-Louvel</td>
</tr>
<tr>
<td>MW270-7B</td>
<td>MATαmetA1-1 leu2 uraA1-1</td>
<td></td>
</tr>
<tr>
<td>PM4-4B</td>
<td>MATαΔade1-600 adeT-600 uraA1-1 dgr151-1</td>
<td></td>
</tr>
<tr>
<td>JA6/151</td>
<td>MATαΔade1-600 adeT-600 trp1-11 uraA1-1 dgr151-1</td>
<td></td>
</tr>
<tr>
<td>yIG2</td>
<td>MATαΔade1-600 adeT-600 trp1-11 uraA1-1 KIM1G::URA3</td>
<td></td>
</tr>
<tr>
<td>JA6/112</td>
<td>MATαΔade1-600 adeT-600 trp1-11 uraA1-1 rag1kht2</td>
<td></td>
</tr>
</tbody>
</table>

synthetic YPD agar plates containing either no drug, 1 μg of tunicamycin (Sigma) ml⁻¹, or 15 μg of hygromycin B (Calbiochem) ml⁻¹, and the plates were incubated at 30°C for 48 h. Batch experiments were conducted under selective conditions in YP medium supplemented with 2% galactose as described previously (45). Escherichia coli strain JM83 [Δlac-proAB] rpm (ΔstrA) [f80 lacZ D4M] was grown in Tryptone-Bertani medium (38). Ampicillin was added to a final concentration of 100 μg ml⁻¹ for plasmid maintenance.

Plasmids and transformations. The plasmids used for this study were pYG107, carrying a cassette for the secreted expression of recombinant human serum albumin (HSA) driven by the native prepro signal sequence under the control of the LAC4 promoter, as described by Fleer et al. (18), and pYG81, carrying the secretion cassette of interleukin-1β (IL-1β) with the K. lactis killer toxin signal sequence under the control of the PHO5 promoter (19). The K. lactis E. coli shuttle vector pGM-GAM contained the glucose isomerase (GAA) gene from the yeast Arthula adeninivorans (33). Plasmid DNA was prepared from E. coli by use of standard techniques (38). Yeast transformation was carried out by electroporation as described previously (50).

Sequencing of mutation site in dgr151 mutant. The oligonucleotides used for dgr151-1 amplification were as follows: KRag5F (5'-GAGCTAACGCAAAAGCTAAAC-3') and KRag5R (5'-TGGATTGTATGAGGGAAATCA-3'). The product of the PCR was cloned into the pCR2.1-TOPO vector (Invitrogen) according to the manufacturer's instructions and was sequenced (MWG Biotech) by using the same primers.

Detection method for GAA. GAA activity was determined by the measurement of starch hydrolysis. The starch-hydrolyzing activity of the culture medium was assayed by measuring the rate of decrease of Abs at 405 nm (33).

Northern blot analysis. Total RNAs were prepared by extraction with hot 0.3 M sodium citrate, 7% sodium dodecyl sulfate (SDS), 10% glycerol, and 25 mg ml⁻¹ of proteinase K. The pellets were washed with 4 M sodium acetate, air dried, and dissolved in 20 μl of loading buffer (0.1 M Tris-HCl [pH 7.4], 20% glycerol, 4% sodium dodecyl sulfate [SDS], 5% β-mercaptoethanol, 0.02% bromophenol blue), and examined by SDS–10% PAGE. The secreted production of IL-1β was evaluated by Coomassie blue R-250 staining of proteins from the culture supernatant.

Analysis of GAA production. The yeast A. adeninivorans secretes high amounts of GAA into culture media. The GAA gene encoding this enzyme has been cloned, and the cloned gene was fused to the strong GAPDH promoter of S. cerevisiae glyceraldehyde-3-phosphate dehydrogenase for use as a tool to study protein secretion (8, 31, 36). This construct, introduced in a K. lactis multicopy vector, was named pGM-GAM (33) and was used for this study as a reporter. The cloned GAA gene retains the coding information for the 16 N-terminal amino acid residues and this native signal peptide is correctly cleaved during transport to the K. lactis cell surface (8).

The relevance of the host genetic background to heterologous protein production is well known; we therefore used four K. lactis laboratory strains, namely JA6, PM4-4B, PM6-7A, and MW270-7B, that were transformed with the plasmid pGM-GAM. The secretion efficiencies of the transformants, grown on 2% glucose, were compared by measuring the activity of GAA released into the culture medium and were normalized by cell number (Fig. 1A). Strain JA6 was found to be the best secretor strain.

On the other hand, the JA6 strain is known to be one of the most sensitive K. lactis strains for glucose repression (25, 47), a drawback for biomass-dependent industrial applications. We next analyzed the GAA production by JA6 grown on different glucose concentrations. The secretion of GAA was affected by the glucose concentration in the medium: the activity released into the culture medium by JA6 grown on 5% glucose was only one-third that obtained on 0.2% glucose (Fig. 1B).

We then asked whether JA6 mutants that were relieved from glucose repression would have further improvements of the secretory capabilities found in the parent strain.

Analysis of GAA production in non-glucose-repressible mutants. We analyzed whether mutations in the RAG1, DGR151, and RAG1 kht2 genes, present in the JA6 background, could also affect GAA production, since these genes are involved in glucose repression. The RAG1 locus is polymorphic within K. lactis species; in strain JA6, this locus encompasses RAG1 and a second gene, KHT2, which is highly similar and contiguous to RAG1 (7). The double mutant rag1 kht2 was thus analyzed in the JA6 background. The mutants were transformed with the pGM-GAM plasmid and grown on 2% glucose as a carbon source in the presence of G418 (Geneticin).

RESULTS

GAA production is strain dependent. The yeast A. adeninivorans secretes high amounts of GAA into culture media. The GAA gene encoding this enzyme has been cloned, and the cloned gene was fused to the strong GAPDH promoter of S. cerevisiae glyceraldehyde-3-phosphate dehydrogenase for use as a tool to study protein secretion (8, 31, 36). This construct, introduced in a K. lactis multicopy vector, was named pGM-GAM (33) and was used for this study as a reporter. The cloned GAA gene retains the coding information for the 16 N-terminal amino acid residues and this native signal peptide is correctly cleaved during transport to the K. lactis cell surface (8).

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We analyzed the efficiency of secretion by comparing the amount of GAA activity released into the growth medium normalized by cell number (Fig. 2A). The maximum amount of GAA was obtained when dgr151-1 cells were used as the host, whereas the Klmig1/H9004 and rag1 kht2 mutants secreted smaller amounts of GAA than did their parental counterparts. The dgr151-1 mutant remained the best producer of GAA in the medium when cells were also grown on 2% lactose or galactose (not shown).

We then analyzed the levels of GAA mRNA from dgr151-1, Klmig1Δ, and rag1 kht2 cells and from the isogenic parental strain JA6 to determine whether the observed differences could be attributed to a transcriptional alteration of the GAA gene. In the wild-type strain, the level of GAA transcript was higher than that measured in all of the mutants (Fig. 2B). It is worth noticing that, although the amount of GAA mRNA from dgr151-1 cells was lower than that from wild-type cells grown under the same conditions (compare lanes 3 and 1), the secreted GAA activity from the mutant strain was much higher than that from the wild type.

**dgr151-1 as host for recombinant protein production.** The increased production of GAA suggested that alterations in protein synthesis or release may occur when RAG5 is mutated. We therefore analyzed the secretory capabilities of the dgr151-1 strain by utilizing two other reporter proteins, IL-1β and HSA. Wild-type and dgr151-1 cells were transformed with the plasmid pYG81, which carries the IL-1β cDNA fused to the secretion signal of the K. lactis killer toxin (α subunit) (19). The

![FIG. 1. Secretion of A. adeninivorans GAA from four laboratory strain of K. lactis.](image)

![FIG. 2. GAA production by rag1 kht2, Klmig1Δ, and dgr151-1 mutants and the isogenic parental JA6 strain.](image)
amount of IL-1β in the culture medium was analyzed by SDS-PAGE after 3 days of growth on YPD medium deprived of phosphate to induce transcription controlled by PHO5. Each lane was loaded with an amount of medium corresponding to 10⁶ cells (Fig. 3). Densitometric measurements of the IL-1β bands indicated that the amount of IL-1β secreted from dgr151-1 cells was nearly twice that released from the wild-type cells. We then performed a batch production experiment with wild-type and mutant cells transformed with plasmid pYG107, which carries the HSA cDNA, including the native prepro signal sequence that is known to be correctly processed in *K. lactis* (18). The cultures were maintained for 11 days, and the HSA secreted into the culture medium was detected by Western blot analysis. The data, normalized by cell mass and by a comparison with known amounts of commercial HSA used as a standard, are graphically reported in Fig. 4. Again, the dgr151-1 cells secreted about two times more HSA than did the parental cells over the entire batch extension. The cell mass yields were highly similar for the mutant and wild-type strains. Since we used three different reporter proteins, or three different secretion signals, the enhanced secretory capabilities of dgr151-1 mutant cells do not seem to be related to a particular protein.

In order to clarify the nature of the dgr151-1 mutation, we determined the nucleotide change in the dgr151-1 allele (Fig. 5). The products of two independent PCR amplifications of the gene were directly sequenced, and the dgr151-1 allele was also cloned from a third independent amplification in the pCRII vector (pCRII-dgr151) and then sequenced. The obtained sequences were compared with the wild-type *RAG5* coding sequence (EMBL accession number X61680). For all three amplifications, the same single nucleotide change was found, namely a G→A transition at nucleotide 527 causing a change from glycine 176 to aspartic acid (G176D). This change is located in the highly conserved kinase domain, and it is worthwhile to remember that this mutant only retains about 5% of the parental hexokinase activity (25).

**Altered glycosylation in dgr151-1 cells.** Hexokinase II, which converts hexoses to hexose phosphates, plays a central role in carbohydrate metabolism; a strong reduction in its activity may affect the supply of D-mannose derivatives, which are needed for glycosylation reactions as well as cell wall morphogenesis (9). We therefore looked for cell wall- and glycosylation-related phenotypes of the dgr151-1 mutant. We first analyzed the tunicamycin sensitivity of the mutant; this drug is an inhibitor of the initial step of biosynthesis of the core oligosaccharide in the N-linked glycosylation pathway (41). dgr151-1 cells were found to be hypersensitive to this inhibitor compared to their wild-type counterpart (Fig. 6). Since the glycosylation mutants of *S. cerevisiae* have often been reported to be hypersensitive to aminoglycosides (12), we also tested the resistance of the mutant cells to hygromycin B. We indeed found that dgr151-1 cells were hypersensitive to this drug compared to the wild type (Fig. 6). In order to investigate the glycosylation defects of this mutant, we used invertase as a reporter protein that is glycosylated along the secretory pathway. The invertase extracted from the *K. lactis* wild-type strain appeared, upon native gel electrophoresis, as a diffuse band in the upper part of the gel due to the heterogeneous N-linked glycosylation of the protein (Fig. 7A, lane 1). For the dgr151-1 strain, the diffuse band showed a faster mobility, suggesting a partial defect of N-linked glycosylation (Fig. 7A, lane 2). This interpretation was confirmed by treating the protein extracts with endo H; no difference in the migration of invertase produced by the two strains was detected (Fig. 7A, lanes 3 and 4).

We then analyzed the O-linked glycosylation of the dgr151-1 strain, employing chitinase as a reporter. This protein is secreted from yeast cells into the growth medium and is exclusively O-mannosylated (28). Chitinases from the mutant and the wild type were purified from the culture medium by selec-
tive chitin binding. The subsequent analysis by SDS-PAGE was based on the notion that the electrophoretic mobility of chitinase depends on the amount of O-linked mannose (28). The data obtained showed that the chitinase secreted by mutant cells had a faster mobility than that produced by their wild-type counterparts, suggesting that the amount of O-linked mannose is also reduced in the dgr151-1 strain (Fig. 7B).

Defects in glycosylation processes are expected to affect the assembly of cell walls by yeasts. We tested the cell wall integrity by analyzing the sensitivity of the dgr151-1 mutant to cell lysis induced by a treatment with Zymolyase, a commercial preparation of β-1,3-gluconan. Mutant cells were sensitive to enzymatic lysis to the same extent as their wild-type counterparts, indicating that no significant cell wall alterations occurred in dgr151-1 cells (data not shown).

**DISCUSSION**

The yeast _K. lactis_ is one of the model systems utilized for heterologous protein production in the food and pharmacology industries (18, 19, 35, 39, 44, 51). In this work, we reported the characterization of a non-glucose-repressible mutant of _K. lactis_ to be used as a host for recombinant protein production.

The _A. adeninivorans_ GAA was chosen as a tool to study protein secretion. Strain JA6 was the best producer among the wild-type strains analyzed and was chosen for detailed analyses. This strain has been known to be particularly glucose sensitive, which is generally regarded as a negative trait for industrial applications. We therefore examined the external release of heterologous proteins by the available glucose repression-defective mutants of this strain. Among them, only the dgr151-1 mutant produced a higher amount of GAA under all conditions analyzed than did the parental JA6 strain. This result may suggest that the release from glucose repression per se is not sufficient to improve the secretory performance of _K. lactis_ strains; on the other hand, an unexpected link between the secretory process and the activity of hexokinases was pointed out. The increased secretory capability was not restricted to GAA secretion, as it was in fact also observed for human IL-1β and HSA under different secretion signals. The mutation in dgr151-1 cells resides in the RAG5 gene coding for the single hexokinase of _K. lactis_ (25, 34). This mutation resulted in an amino acid change within the kinase domain of the enzyme, thus explaining the severely reduced kinase activity reported for this mutant (25). The enhanced secretory capability of the dgr151-1 mutant is not related to cell wall alterations since this mutant was not affected in Zymolyase sensitivity and did not show any lysis defects (our unpublished results).

It has been known that mutations in _DGR151/RAG5_ affect glucose repression in _K. lactis_ (25), and in _S. cerevisiae_, a mutation of hexokinase II led to a deficiency of glucose repression (16). Indeed a rag5 mutation in _K. lactis_ can be complemented by the hexokinase II gene of _S. cerevisiae_ (34).

Recent reports have demonstrated that, for _S. cerevisiae_, mutants with altered relative levels of glucose-6-phosphate (Glc-6-P) and glucose-1-phosphate (Glc-1-P) (2) or that are unable to convert glucose to Glc-6-P (32) show alterations in cellular Ca²⁺ homeostasis. Also, in the case of our study, a strongly reduced hexokinase activity could conceivably diminish the availability of Glc-6-P, thus altering the relative balance with Glc-1-P in the cell. Calcium signaling plays a crucial role in many cell processes; the relevance of Ca²⁺ to the functioning of the secretory machinery is a feature that is conserved from yeasts to human cells (10, 29). Alterations in cell calcium homeostasis have been observed in an _S. cerevisiae PMR1_-disrupted mutant; _PMR1_ encodes a Ca²⁺-ATPase that is localized in the Golgi apparatus (3, 15). The inactivation of this gene also resulted in a significant increase in the secretion of several heterologous proteins (26, 37). We could thus speculate that the oversecretion phenotype of the dgr151-1 mutant could also be linked to alterations in cell Ca²⁺ homeostasis that, in turn, originate from a perturbed balance of Glc-1-P and Glc-6-P.

The availability of the dgr151-1 mutant is relevant for biomass-directed industrial applications, since in contrast with the rag5Δ mutant (34), dgr151-1 cells are able to utilize different carbon sources, such as glucose and lactose. This ability could be due to the residual hexokinase activity, which might dimin-
lish the intracellular accumulation of unphosphorylated glucose that is regarded as deleterious for the cells (35).

The reduced mannose extension of N- and O-glycoproteins observed for dgr151-1 cells could be ascribed to a reduced availability of GDP-mannose, the substrate for the glycosylation reactions that occur in the Golgi apparatus (46). The substrate for glycosyltransferases is in fact synthesized in the cytosol, starting from mannose-6-phosphate (Man-6-P), which is obtained by transforming fructose-6-phosphate, which in turn originates from the Glc-6-P produced by the hexokinase (9). The S. cerevisiae phosphomannomutase, the product of the SEC53 gene, converts Man-6-P to mannose-1-phosphate (Man-1-P), the direct substrate for the formation of GDP-mannose (27). In S. cerevisiae, three enzymes, namely Hxk2p, and Glk1p, are able to phosphorylate the hexoses at C6, while in K. lactis, Rag5p is regarded as the unique hexokinase (34). The dgr151-1 mutation, by strongly impairing the hexokinase activity (25), would result in a reduced supply of Man-1-P that is not sufficient for a wild-type level of glycosylation. The reduced glycosylation level, however, seems not to have a direct relationship with the increase in secretory capabilities we observed for dgr151-1 cells, since the HSA and IL-1B that we used as reporters are not glycosylated proteins and their transport, therefore, should not be affected by the glycosylation process.

Taken together, our data point out the dgr151-1 mutant strain as a useful host for heterologous protein production, as it provides the possibility not only for obtaining a higher amount of recombinant proteins but also for avoiding the hyperglycosylation of secreted proteins.

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