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Kluyveromyces lactis gene *KlBIM1* in high copy state suppresses heterologous protein secretion

K. Vagonytė[1, 2]**,**

D. Bartkevičiūtė[1]**,**

K. Sasnauskas[1, 2]

[1] *Institute of Biotechnology, Vilnius, Lithuania*

[2] *Vilnius University, Department of Botany and Genetics, Vilnius, Lithuania*

The aim of this work was to investigate the genes that under over-expression in the yeast *Kluyveromyces lactis* decrease the yield of secretory proteins. The MD2/1-9 mutant strain with an enhanced ability to secrete different proteins was used. The genes suppressing the protein secretion process were detected by using the α-amylase gene. The gene *KlBIM1* was found to suppress mutation of the MD2/1-9 mutant strain in yeast *K. lactis*. To test the influence of the chromosomal gene on protein secretion, the *BIM1* gene was disrupted in both yeast *K. lactis* and *S. cerevisae*, and the influence of disruption on the protein secretion process was evaluated. The disruption of the gene *KlBIM1* had no influence on the protein secretion process in *K. lactis*, however, disruption of the *BIM1* gene in *S. cerevisiae* elevated protein secretion by approximately 24%. Our experiment has shown that *K. lactis* strain lacking the gene *KlBIM1* is not suitable for elevated heterologous protein secretion. The *K. lactis* mutant strain MD2/1-9 was transformed by the *S. cerevisiae* gene *BIM1* in order to evaluate the influence of this gene in a high-copy state to the α-amylase secretion process. The *S. cerevisiae* gene *BIM1* in a high-copy-number state significantly decreased secretion of α-amylase in *K. lactis* MD2/1-9. This fact suggests that the homology between the gene *KlBIM1* in *K. lactis* and *BIM1* in *S. cerevisiae* is rather high and the biological function of those genes in both yeasts is quite similar.

Key words: yeast, *Kluyveromyces*, secretion, heterologous proteins, *KlBIM1*

INTRODUCTION

K. lactis is a biotechnologically significant yeast which has already been exploited as a host for the production of heterologous proteins due to its secretory performance [1]. *K. lactis* is related to *S. cerevisiae* and has a distinctive capacity for extracellular secretion of recombinant proteins [2]. Production of a heterologous protein via the eukaryotic secretory pathway ensures a high fidelity of the folding, assembly and modification processes required for biological activity. Although high-level transcription of heterologous genes is attainable in eukaryotic systems, often only a small fraction of the expressed protein is secreted from the yeast cell. A heterologous protein may become inclined to fold incorrectly, because the level of the necessary soluble folding or modification factor is too low to cope up with the elevated throughput of secretory proteins. Alternatively, the protein may be just unable to fold correctly, because the factors required for authentic post-translational modification may be absent or have a low impact [1]. Several publications have provided an approach to overcome such problems in *S. cerevisiae* by modulating the cellular levels of different proteins in all steps of the secretory pathway [3–7]. Non-*Saccharomyces* yeast species have gained more prominence in biotechnology. In this respect, *Kluyveromyces* has been shown to have significant advantages over traditional baker's yeast in production of certain proteins. A high level of secreted correctly folded and processed recombinant serum albumin, chymosin, interleukine-1β can be achieved by using the *K. lactis* expression system [8, 9].

Previously we described the isolation of *K. lactis* mutants over-secreting heterologous proteins in the medium. One of the recessive over-secreting mutant strains was used for the isolation of genes conferring a super-secreting phenotype [10, 11]. In this paper we present the molecular and functional characterization of one of the isolated genes, namely, *KlBIM1*, which in a high copy number suppress the elevated secretion of heterologous proteins.

MATERIALS AND METHODS

Strains. *Escherichia coli* strain *DH5*α *F*$^-$(f*80dlacZ* Δ*M15*) *recA1 endA1 gyrA96 thi-1 hsdR17*($r_k m_k^+$)*supE44*

Correspondence to: K. Vagonytė, Vilnius University, Department of Botany and Genetics, M. K. Čiurlionio 21, LT-03101, Vilnius, Lithuania E-mail: kristina.vagonyte@gf.vu.lt

relA1 deoR Δ(*lacZYA-argF*) *U169*) was used for plasmid construction. The *K. lactis* strain MD2/1 (MATα *argA1 lysA1 ura3*) was kindly provided by Dr. M. Bianchi. The *K. lactis* MD2/1-9 super-secreting mutant has been described previously [11]. *Saccharomyces cerevisiae* CTY182 (MATa *his3-200 lys2-801 ura3-52*) was used for the disruption experiments of the gene *BIM1*. *Saccharomyces cerevisiae* AH 22–214 (MATa/MAT a *his3/his3 leu2/leu2* cir$^+$) was used for multiplying the genes *BIM1*, *YLL079w*.

Media. *E. coli* strain was grown in Luria–Bertani broth. All yeast cultures were incubated at 30 ˚C. Growth media YEPD (1% yeast extract, 2% peptone, 2% glucose, Difco) and YNB (0.67% yeast nitrogen base, 1% casamino acids, 2% glucose, Difco) were used. YPS (0.67% yeast nitrogen base, 0.2% casamino acids, 0.1% glucose, 0.2% starch and 2% agar plates were used for halo assays of a-amylase activity. 5-FOA (0.7% YNB with $(NH_4)_2SO_4$, 2% glucose, 0.1% 5-FOA, 0.002% uracil, 2% agar) plates were used for *K. lactis* transformants.

Recombinant DNA methods. Most recombinant DNA experiments were performed as described [12]. All enzymes were obtained from Fermentas UAB (Vilnius, Lithuania) and used according to the manufacturer's recommendations. Plasmid pBoriAMY used for a-amylase secretion has been described earlier [11, 13]. The 3.8 kb URA3 cassette with the *S. cerevisiae URA3* gene flanked by direct repeats of *Salmonella hisG* DNA [14] was used for the disruption experiments of both *S. cerevisiae* and *K. lactis* genes.

Primers used for selection of clones with the disrupted *BIM1* and *BIM1* genes by PCR analysis:

URA1 5'-ATGATTTATCTTCGTTTCCTGCAG-3'
URA2 5'-TAAATGCATGTATACTAAACTCAC-3'
P197 5'-CGCTATGTCTGGCCAAGTCTGTCCTATTTC-3'
P198 5'-TCTGTAGGCCGGATAAGGCGGAACCCTG-3'
BIM1 5'-GTGTTGCAATGTATGCCG-3'
BIM2 5'-TGCAAGACACTCTGACGC-3'
SCBIM1 5'-TTCTAGAGGATGCGGCGACAGAGA-3'
SCBIM2 5'-ACCAAGAACGGCAAAATAAGATCTT-3'

α-Amylase halo assay. For halo assay, the yeast cultures were diluted to equal OD and 50 μl of each culture were spotted onto YPS plates and incubated at 30 ˚C for 24 h. The halos surrounding transformants were detected by the absence of iodine staining (3 mg ml^{-1} J2, 15 mg ml^{-1} KJ) of the digested starch as earlier described [11].

Invertase assays. Invertase secretion was performed as described [11].

RESULTS AND DISCUSSION

Previously we described the isolation of *K. lactis* mutants conferring elevated secretion of heterologous proteins. Both dominant and recessive mutations were obtained. One of the recessive mutant strains, *K. lactis* MD2/1–9, which secreted heterologous proteins in a five-fold excess compared to the wild-type strain, was used for the cloning of genes conferring a super-secreting phenotype [10]. A set of genes conferring a super-secretion phenotype in *K. lactis* MD2/1-9 has been isolated.

Sequence analysis of the plasmid isolated from the clone B30 revealed three ORF encoding genes with a high homology to *S. cerevisiae* genes *AFG3*, *BIM1* and *FAA*. In order to identify the gene responsible for the suppression of super-secretion phenotype, all three genes separately were subcloned into the pBoriAMY plasmid, and the resulting transformants were tested for starch hydrolyses on starch agar plates as described previously [10] (Fig. 1). This analysis revealed that yeast transformants containing a gene homologous to *S. cerevisiae BIM1* has formed significantly lesser starch hydrolysis zones in comparison to *K. lactis* MD2/1-9. The *K. lactis* gene homologous to the *S. cerevisiae BIM1* gene was named *KlBIM1*.

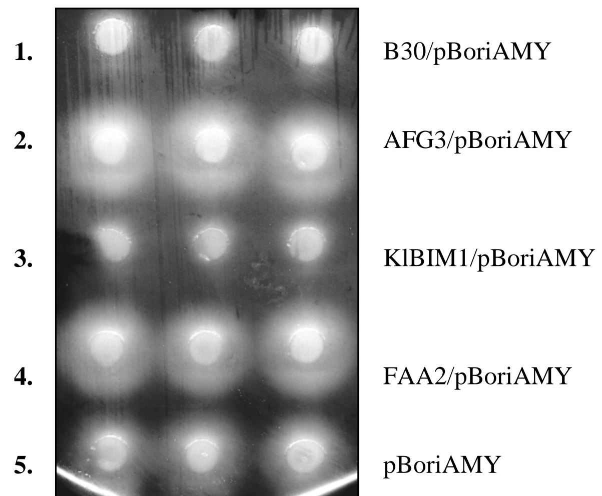

Fig. 1. Influence of gene *KlBIM1* on a-amylase secretion. *K. lactis* MD2/1-9 mutant strain transformed with: 1 – B30/pBoriAMY, 2 – AFG3/pBoriAMY, 3 –BIM1/pBoriAMY, 4 – FAA2/pBoriAMY, 5 – pBoriAMY are shown

The *KlBIM1* gene in a high-copy state suppressed the super-secretion phenotype but had no noticeable influence on super-secretion in the state of a low copy number centromeric plasmid (data not shown). The amino acid sequence alignment of the predicted gene products of *S. cerevisiae BIM1* and *K. lactis KlBIM1* are presented in Fig. 2. Both predicted proteins share 52% aa identity. A high similarity to the predicted Bim1p of *S. cerevisiae* allowed predicting the function of the *KlBIM1* gene to be the same as of *S. cerevisiae*. In *S. cerevisiae* yeast it was demonstrated that protein Bim1p encoded by the *BIM1* gene interacts with α-tubulin and participates in cell cycle control [15]. The process of secretion is closely related to the cell cycle [16]. This fact suggests that Bim1p may influence the protein secretion process.

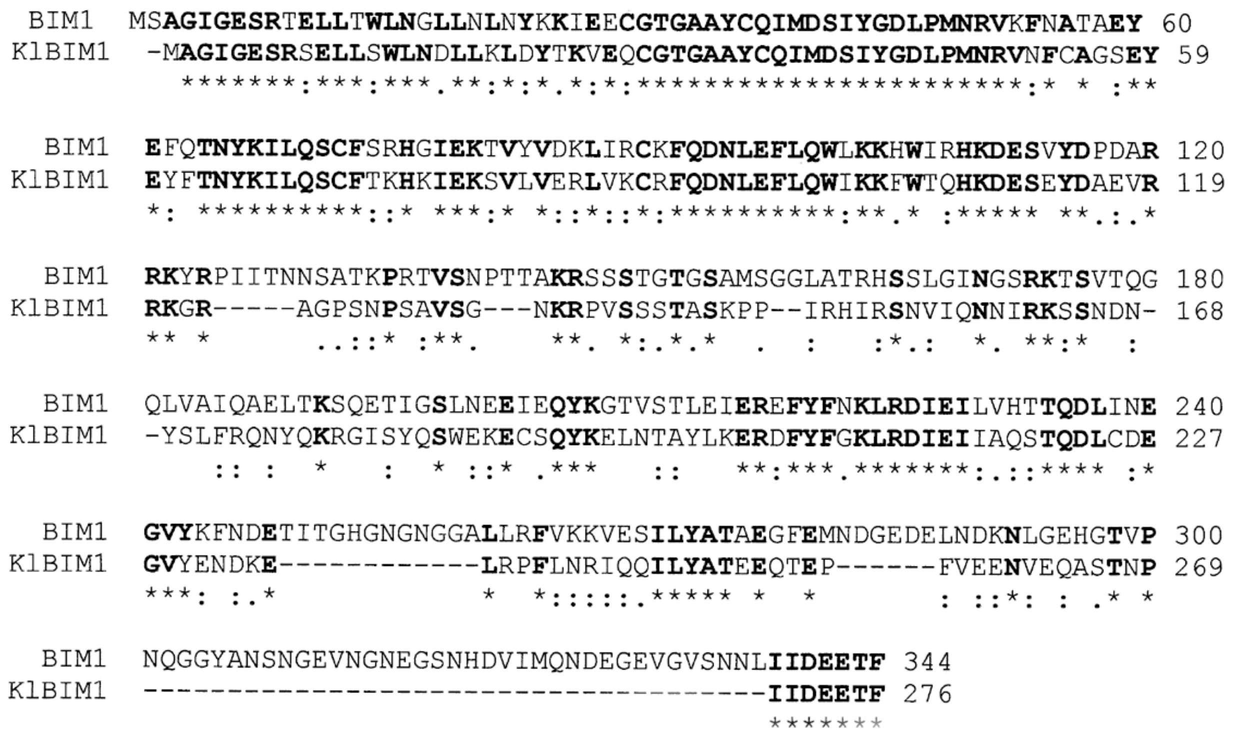

Fig. 2. Alignment of *S. cerevisiae BIM1* and *K. lactis KlBIM1* predicted amino acid sequences (BLAST, **http://www.ncbi.nlm.nih.gov/**). Identical amino acids for both proteins are indicated by asterisks, colons stand for similar amino acids

To confirm this prediction and further investigate the functions and characterize the null allele phenotype related to heterologous protein secretion, disruption of *K. lactis KlBIM1*, *S. cerevisiae BIM1* genes was carried out by the conventional techniques [10, 11].

For gene disruption a special construction was prepared, in which the 2.1 kb BglII-BcuI DNA fragment from *KlBIM1* sequencing was replaced by the 3.8 kb BamHI-XbaI DNA fragment containing the *hisG-URA3-hisG* sequence [14]. The resulting plasmid was digested with BglII and BcuI restriction endonucleases to obtain the *hisG-URA3-hisG* cassette flanked by the *KlBIM1* gene surrounding sequences necessary for homologous recombination, and was used for *K. lactis* MD2/1 transformation. After transformation, the *URA3* gene was removed from the transformants by growing on 5-fluoro-orotic acid agar. *K. lactis* Ura^- clones were analysed by PCR for the selection of clones with the deletion of a targeted gene. *K. lactis klbim1* strains obtained in such a way were tested for invertase secretion (data not shown). Our experiments demonstrated that *KlBIM1* in a high-copy state could suppress the super-secreting phenotype; however, the disruption of this gene did not affect the protein secretion level.

For the disruption of *S.cerevisae BIM1*, the 2431-bp DNA fragment of *S. cerevisiae* AH 22-214 encoding *BIM1* was amplified by using a pair of primers.

Left primer: 5’-TTCTAGAGGATGCGGCGACAGAGA-3’.

Right primer: 5’-ACCAAGAACGGCAAAATAAGATCTT-3’.

The amplified fragment was inserted into the bacterial vector pUC57T. For *BIM1* gene deletion a special construction was prepared. In the resulting plasmid p57SCBIM1, the NcoI-XhoI DNA fragment from gene *BIM1* sequencing was replaced by the 3.8 kb SalI-Ecl136II DNA fragment containing a *hisG-URA3-hisG* sequence [14]. The resulting plasmid, p57NKY-BIMSc, was hydrolysed with BspTI and PsyI to obtain a *hisG-URA3-hisG* cassette flanked with *BIM1* coding sequences for homologous recombination. The hydrolysis product was used for the transformation of *S. cerevisiae* strain CTY182. The isolation and characterization of mutants was carried out as described for *K. lactis*. *S. cerevisiae bim1* strains obtained in such a way were tested for invertase secretion (Fig. 3). Our experiment demonstrated that the disruption of the gene *BIM1* in yeast *S. cerevisiae* elevated protein secretion approximately by 24%.

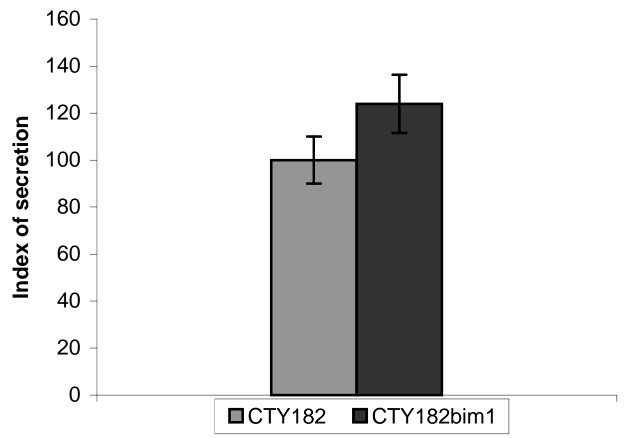

Fig. 3. Influence of *BIM1* gene on invertase secretion. Column 1 – CTY182, column 2 – CTY182*bim1*

Fig. 4. Influence of *BIM1* gene on a-amylase secretion in *K. lactis* mutant strain MD2/1-9. *K. lactis* MD2/1-9 mutant strain transformed with: 1 – pBoriAMY, 2 –BIM1/pBoriAMY, 3 – KlBIM1/pBoriAMY are shown

In order to evaluate the influence of *S. cerevisiae* gene *BIM1* in a high-copy state on heterologous protein secretion, the genes *KlBIM1* and *BIM1* separately were subcloned into the pBoriAMY plasmid. These plasmids were used for transformation of *K. lactis* MD2/1-9 and the starch hydrolyses zones were tested in transformants (Fig. 4). This analysis revealed that yeast transformants containing either the *KlBIM1* or *BIM1* gene secret a significantly less quantity of α-amylase. This fact suggests that a homology between the *K. lactis* gene *KlBIM1* and *S. cerevisiae* gene *BIM1* is rather high and the biological function of these genes is quite similar in both yeasts.

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K. Vagonytė, D. Bartkevičiūtė, K. Sasnauskas

K. LACTIS KLBIM1 GENO DIDELIO KOPIJŲ SKAIČIAUS POVEIKIS HETEROLOGINIŲ BALTYMŲ SEKRECIJAI

Santrauka

Kluyveromyces lactis MD2/1-9 mutantinis kamienas, pasižymintis didesne baltymų sekrecija, buvo panaudotas genų, susijusių su baltymų sekrecija, klonavimui. Nustatyta, kad B30 klono DNR fragmente esantis *KlBIM1* genas slopina MD2/1-9 kamieno mutaciją, nulemiančią didesnę baltymų sekreciją *K. lactis* mielėse. *KlBIM1* genas, slopinanis heterologinių baltymų sekreciją *K. lactis* mielėse, nustatytas panaudojus a-amilazės geną. Siekiant nustatyti chromosominio geno įtaką baltymų sekrecijos procesui, buvo nutarta suardyti *KlBIM1* geną *K. lactis* mielėse ir *BIM1* geną *S. cerevisiae* mielėse. *KlBIM1* geno suardymas *K. lactis* mielėse sekrecijos procesui įtakos neturėjo, o *BIM1* geno suardymas *S. cerevisiae* mielėse padidino baltymų sekreciją apytiksliai 24%. *K. lactis klbim1* kamienas nepasižymi didesne heterologinių baltymų sekrecija. Siekiant įvertinti *BIM1* geno didesnio kopijų skaičiaus įtaką a-amilazės sekrecijai, MD2/1-9 mutantinis *K. lactis* mielių kamienas buvo transformuotas *S. cerevisiae BIM1* genu. Eksperimentas rodo, kad *S. cerevisiae BIM1* genas slopina MD2/1-9 kamieno mutaciją, nulemiančią didesnę baltymų sekreciją *K. lactis* mielėse. Tai leidžia manyti, kad homologija tarp šių dviejų genų yra pakankamai didelė.