Kluyveromyces lactis LAC12 gene in a high copy state suppresses heterologous protein secretion

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The aim of this work was to search for proteins that under over-expression in the yeast *Kluyveromyces lactis* decrease the rate and yield of secretory proteins. *K. lactis* MD2/1 strain was UV-mutagenised and the secretion level of α-amylase was tested. Four strains (9, 6', 9', 22') with the enhanced ability to secrete different proteins were isolated. The 9th mutant strain was used for further investigations. To clone genes that suppress the super-secretion phenotype, the gene bank of *K. lactis* MD2/1 was constructed on the multicopy plasmid. This bank was used for transformation of the 9th strain. Ten different genes suppressing the super-secreting phenotype in a high copy number were isolated. One of the genes appeared to be *LAC12* gene encoding inducible lactose permease. LAC12p is involved in protein secretion processes not directly, but as a membrane protein localized in the cytoplasm membrane and able to influence the secretion. Our experience confirmed that *LAC12* in a high copy number state suppressed the super-secreting phenotype, but the disruption of this gene did not affect the protein secretion level.

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

INTRODUCTION

Production of a foreign protein via the eukaryotic secretory pathway ensures a high reliability of folding, assembly and modification processes required for biological activity. Although high-level transcription of genes is attainable in eucaryotic systems, secretion of the corresponding protein products often does not increase proportionally. Heterologous proteins may become inclined to fold incorrectly, because the level of a 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, 2]. Several publications have provided an approach to overcome such problems in *Saccharomyces 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. The high level of secretion of correctly folded and processed recombinant serum albumin, chymosine, interleukine-1β can be achieved by using the *K. lactis* expression system [8, 9]. This work was aimed at the identification of *K. lactis* genes suppressing the super-secretion phenotype. A set of genes – suppressors of the super-secreting phenotype can be attractive for the further analysis of gene functions, super-secreting mechanisms and the construction of new strains. This collection can be useful also for the expedient construction of reduced yeast genomes optimized for heterologous protein secretion.

MATERIALS AND METHODS

Strains. *Escherichia coli* strain DH5α (F^- *gyrA*96 (NaI^r) *recA1 relA1 endA1 thi*-1 *hsdR*17(r^-_k m^+_k) *glnV44 deoR* Δ(*lacZYA-argF)U169*[ϕ80dΔ*(lacZ)*M15]) was used for plasmid construction. *K. lactis* strain MD2/1 (MATα, *ura3, argA1, lysA1*) was kindly provided by Dr. M. Bianchi.

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Media. *E. coli* strain was grown in Luria–Bertani broth. YEPD (1% yeast extract, 2% peptone, 2% glucose) and YNB (0.67% yeast nitrogen base, 1% casamine acid, 2% glucose) growth media, were used. An α-amylase assay was performed as described previously [10].

DNA methods. Most recombinant DNA experiments were performed as described [11]. All enzymes were obtained from Fermentas AB (Vilnius, Lithuania) and used according to the manufacturer's recommendations.

The gene bank of the *K. lactis* MD2/1 was prepared on the basis of plasmid pBoriAMY [10] by cloning partially filled chromosomal DNA *Bsp*143I fragments into partially filled *Sal*I site of pBoriAMY.

Primers used for selection by PCR analysis of clones with the disrupted *LAC12* gene

URA1 5'-ATGATTTATCTTCGTTTCCTGCAG-3'
URA2 5'-TAAATGCATGTATACTAAACTCAC-3'
LAC12-5 5'-CTCTCGAGGCCAAAGGACTATCGC-3'
LAC12-3 5'-AACTCGAGGCAAGAGCACCTAATC-3'

were used for selection by PCR analysis of clones in which *LAC12* gene was disrupted

DNA sequencing. Double-stranded DNA sequencing was performed on both strands using dideoxy termination method on Exonuclease III nested deletions. The CycleReader™ Auto DNA Sequencing KIT with Cy5 labeled M13/pUC sequencing primer (Fermentas AB, Vilnius, Lithuania) was used.

Invertase secretion. Invertase secretion was performed as described [12].

RESULTS AND DISCUSSION

K. lactis MD2/1 strain was UV-mutagenised and the secretion level of α-amylase was tested. Four strains (9, 6', 9', 22') with the enhanced ability to secrete different proteins were isolated [10]. The 9th mutant strain was used for further investigations. In order to clone the genes that suppress the supersecreting phenotype, the gene bank of *K. lactis* MD2/1 was constructed on the plasmid pBoriAMY. This bank was used for transformation of strain No. 9 which possessed a recessive mutation as shown previously [10]. Transformants that lost the capability to form halos on starch agar were selected. Plasmid DNA from each selected transformant was isolated and used for a repeated transformation of the same mutant strain to ensure that a single plasmid possessed a DNA-suppressing super-secretion phenotype. Afterwards 10 different recombinant plasmids containing different DNA inserts were isolated for further characterization. To investigate whether the cloned DNA possessed the ability to suppress the super-secreting phenotype in a low copy state, the inserts from the 10 different plasmids described above were recloned into the centromeric plasmid KCp491. The resulting *URA3* marker gene containing centromeric plasmids was co-transformed with the G418 genetic marker and α-amylase gene containing the plasmid pBoriG418. Transformants, grown on G418 medium without uracil were selected and tested for halo formation on starch agar plates. None of the 10 different DNA inserts tested in centromeric plasmid suppressed the super-secreting phenotype in centromeric plasmid.

The 6 kb *Bam*HI DNA fragment from the recombinant plasmid of B11 clone was cloned into the pUC57 plasmid digested with the *Bam*HI restrictase. The resulting plasmid p11A3 was used for constructing a library of nested deletions and sequencing. Sequence analysis of cloned DNA revealed that this fragment contained the gene *LAC12* encoding inducible lactose permease [13]. Lactose permease is involved in the protein secretion processes not directly, but as a membrane protein localized in the cytoplasm membrane and able to influence secretion.

To test the influence of *LAC12* chromosomal gene on protein secretion, the *LAC12* gene was deleted by using homologous recombination. For deletion a special construction was prepared, in which 1.9 kb *Bcu*I–*Nco*I DNA fragment from *LAC12* coding sequence in the plasmid p11A3 was replaced by 3.8 kb *Xba*I–*Ecl*136II DNA fragment containing *hisG-URA3-hisG* sequence [14]. The resulting plas-

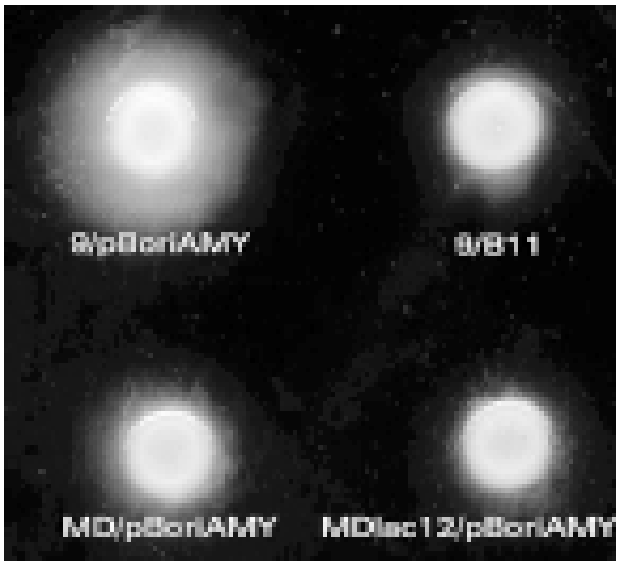

Fig. 1. Influence of *LAC12* gene on α-amylase secretion. On the top the mutant 9th strain transformed with pBoriAMY and pBoriAMY-LAC12 (B11) plasmids respectively, on the bottom MD2/1*wt* and MD2/1*lac12* strains transformed with pBoriAMY plasmid are shown

mid pLAC12URA3 was linearized with *Eco*RI and used for *K. lactis* MD2/1 transformation. Ura^+ transformants were replica plated on media with lactose. Clones that were not growing on lactose were tested in PCR for the confirmation of the replacement of *LAC12* gene. *K. lactis lac12* strains obtained in such a way were transformed with plasmid pBoriAMY to test the influence of *LAC12* deletion on the secretion of α-amylase (Fig. 1). The secretion of invertase was tested also (Fig. 2).

Our experience confirmed that *LAC12* in a high copy number state could suppress the super-secreting phenotype, but the disruption of this gene did not affect the protein secretion level.

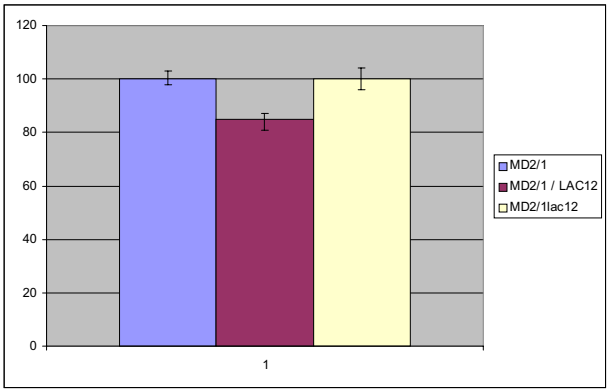

Fig. 2. Influence of *LAC12* gene on invertase secretion. Column 1 – MD2/1*wt* / pBoriAMY, column 2 – MD2/1*wt* / pBoriAMY-LAC12, column 3 – MD2/1*lac12* / pBoriAMY

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DIDESNIO *K. LACTIS LAC12* GENO KOPIJŲ SKAIČIAUS POVEIKIS HETEROLOGINIŲ BALTYMŲ SEKRECIJAI

S a n t r a u k a

Kluyveromyces lactis sekreciniai mutantai buvo panaudoti genų, susijusių su baltymų sekrecija, klonavimui. Tam tikslui sukonstruotas *K. lactis* laukinio kamieno DNR bankas ir, panaudojant fenotipinės supresijos metodą, buvo klonuota 10 skirtingų *K. lactis* DNR fragmentų, turinčių genus, kurie daugiakopijinėje būsenoje mažina baltymų sekreciją. Vienas iš klonuotų genų *LAC12* koduoja laktozės permeazę. Nustatyta, kad *LAC12* geno kopijų didesnis kiekis sumažina heterologinių ir homologinių baltymų sekreciją *K. lactis* mielėse, tuo tarpu *LAC12* geno suardymas neturi įtakos sekrecijai.