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# Acetamide Selection of *Kluyveromyces lactis* Cells Transformed with an Integrative Vector Leads to High-Frequency Formation of Multicopy Strains[▽]

Jeremiah D. Read, Paul A. Colussi, Mehul B. Ganatra, and Christopher H. Taron*

*New England BioLabs, 240 County Road, Ipswich, Massachusetts 01938-2723*

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**The yeast *Kluyveromyces lactis* has been extensively used as a host for heterologous protein expression. A necessary step in the construction of a stable expression strain is the introduction of an integrative expression vector into *K. lactis* cells, followed by selection of transformed strains using either medium containing antibiotic (e.g., G418) or nitrogen-free medium containing acetamide. In this study, we show that selection using acetamide yields *K. lactis* transformant populations nearly completely comprised of strains bearing multiple tandem insertions of the expression vector pKLAC1 at the *LAC4* chromosomal locus, whereas an average of 16% of G418-selected transformants are multiply integrated. Additionally, the average copy number within transformant populations doubled when acetamide was used for selection compared to G418. Finally, we demonstrate that the high frequency of multicopy integration associated with using acetamide selection can be exploited to rapidly construct expression strains that simultaneously produce multiple heterologous proteins or multisubunit proteins, such as Fab antibodies.**

The yeast *Kluyveromyces lactis* was first developed as a host for heterologous protein expression over 15 years ago. To date, *K. lactis* has been used to produce greater than 50 proteins originating from a diverse range of organisms (see reference 18 for a review). *K. lactis* is generally regarded as safe (1), is easily genetically manipulated, has a fully sequenced genome (3), and is capable of producing heterologous proteins in high titer (18), making it an attractive expression host. Additionally, *K. lactis* produces heterologous proteins in simple growth medium, making it especially well suited for industrial-scale protein production, and it does not require the addition of methanol, often needed with methylotrophic yeasts (e.g., *Pichia pastoris*). *K. lactis* has been used for over 15 years for 100-$m^3$ scale fermentation of recombinant bovine prochymosin for the food industry (17).

Expression of a protein in *K. lactis* typically involves the assembly of an expression vector containing the desired heterologous gene cloned downstream of a strong yeast promoter and the introduction of the vector into a host *K. lactis* strain. For many industrial applications, integration of a linearized expression vector into the *K. lactis* genome is used to increase the genetic stability of expression strains that require long periods of bioreactor growth in the absence of selection. One common expression strategy employs transformation of *K. lactis* cells with an integrative expression vector that is targeted to insert into the promoter region of the *LAC4* chromosomal locus (13). In general, integrative transformation schemes sometimes generate strains having multiple copies of the vector tandemly inserted into a target locus. Typically, multicopy integration occurs at a low frequency and large populations of transformed strains must be screened to identify those having multiple vector copies. which often produce more heterologous protein than single-copy integrants.

Selectable markers commonly used in *K. lactis* vectors are antibiotic resistance genes, such as those for neomycin or hygromycin B, or genes that complement common auxotrophic markers used in yeast genetics (e.g., *ura3*, *his3*, and *trp1*). An alternative selection strategy involves the use of acetamide in nitrogen-free medium to impose a nitrogen source selection upon transformed cells (11, 12). In this method, a gene encoding an *Aspergillus* acetamidase (*amdS*), present on an expression vector (2), confers the ability of a transformed cell to process acetamide to ammonia for use as a source of nitrogen. Furthermore, cells that have lost or have been cured of the *amdS* gene can be counterselected on medium containing fluoroacetamide. Thus, *amdS* can be used in a dominant selection/counterselection strategy that permits modification of wild-type strains and subsequent removal of the selectable marker (18). This strategy has been used to construct selection marker-free strains for use in food industry processes (11, 12). Acetamide selection has been used to transform various filamentous fungi, including *Aspergillus*, *Neurospora*, *Penicillium*, *Fusarium*, and *Trichoderma* species, as well as the yeasts *K. lactis* and *Saccharomyces cerevisiae* (2, 5, 9–12, 18–20).

In this study, we examined the effect the selection method (antibiotic resistance versus acetamide selection) has on the frequency of forming multicopy strains and on copy numbers within populations of *K. lactis* cells transformed with integrative expression vectors. We show that acetamide selection nearly completely enriches transformant populations for strains harboring multiple tandem-vector integrations and increases vector copy numbers compared to antibiotic selection with Geneticin (G418). Furthermore, we demonstrate that this phenomenon can be exploited to simultaneously transform cells with multiple independent expression vectors using a sin-

* Corresponding author. Mailing address: New England BioLabs, 240 County Road, Ipswich, MA 01938-2723. Phone: (978) 927-5054. Fax: (978) 921-1350. E-mail: taron@neb.com.

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gle round of transformation and selection. We also show that stably integrated strains that simultaneously secrete multiple different heterologous proteins or multidomain proteins, including active Fab antibodies, can easily be constructed.

## MATERIALS AND METHODS

**Strains, vectors, and culture media.** The wild-type industrial *K. lactis* strain GG799 (2) was used as a host for protein expression. It was routinely grown in YPGal medium (1% yeast extract, 2% peptone, 2% galactose) or YPGlu medium (1% yeast extract, 2% peptone, and 2% glucose) at 30°C.

The *K. lactis* integrative expression vector pKLAC1, which has been previously described (2), contains the *Aspergillus nidulans* acetamidase gene (*amdS*), which permits cell growth on medium containing acetamide. To create pGBN19, the neomycin gene was excised from pGBN1 (2) with a SmaI-BamHI digest and cloned into the same sites of pKLAC1. Thus, vector pGBN19 is identical to pKLAC1 except that the *amdS* gene has been replaced with the bacterial neomycin gene derived from Tn*901* that confers resistance to the antibiotic G418.

**Transformation of *K. lactis*.** Transformation of *K. lactis* with expression vectors was performed using chemically competent *K. lactis* GG799 cells (New England BioLabs, Ipswich, MA) as directed by the manufacturer. Each transformation reaction contained approximately $2.3 \times 10^8$ *K. lactis* cells. For single-vector transformation reactions, 2 μg of vector was digested with SacII and purified using a PCR spin column (QIAGEN, Valencia, CA). A total of 1 μg of linearized vector was used to transform *K. lactis* GG799 cells. Cotransformation with multiple expression vectors was performed by separately digesting 2 μg of each vector with SacII, pooling the digested DNA, and copurifying the DNA using a PCR spin column. A volume of DNA containing a final concentration of 1 μg of each vector was used to cotransform *K. lactis* GG799 cells. Colonies of strains transformed with pKLAC1-based vectors were obtained by growth on YCB agar medium (New England BioLabs) supplemented with 5 mM acetamide at 30°C for 3 to 4 days, whereas strains transformed with pGBN19-based vectors were obtained on YPGlu agar medium supplemented with 200 μg G418 $ml^{-1}$ (Sigma, St. Louis, MO) at 30°C for 2 days.

**Expression vector construction.** The genes encoding human serum albumin (HSA), bovine enterokinase light chain ($EK_L$), *Gaussia princeps* luciferase (Gluc), and *Escherichia coli* maltose binding protein (MBP) were amplified by PCR using primers 4 to 11 (Table 1). Each amplified product was digested with endonucleases corresponding to the restriction sites engineered into the primers and cloned into the corresponding sites of both pKLAC1 and pGBN19.

DNA encoding Fab fragments of anti-MBP and anti-transferrin monoclonal antibodies was amplified by reverse transcription-PCR using degenerate primers (Table 1). Primers 12 and 13 were used to amplify DNA encoding light-chain fragments of both antibodies. DNA fragments encoding anti-transferrin and anti-MBP heavy chains were amplified using primers 14 and 15 and primers 16 and 17, respectively. Total RNA isolated from mouse hybridoma cell lines expressing either anti-MBP or anti-transferrin monoclonal antibodies was used as a template. Amplified DNA was digested with EcoRI, cloned into the EcoRI-SmaI restriction sites of plasmid pNEB193 (New England BioLabs), and sequenced. To clone the genes into pKLAC1 in frame with the α-factor secretion leader, forward primers lacking DNA encoding the native secretion leaders of heavy and light chains were designed. Light chains of both antibodies were amplified using primers 18 and 19, the anti-transferrin heavy chain Fab fragment was amplified with primers 20 and 21, and the anti-MBP heavy chain Fab fragment was amplified with primers 22 and 23 using appropriate recombinant DNA fragments in vector pNEB193 as PCR templates. The amplified DNAs were digested with XhoI-NotI and cloned into the same restriction sites of pKLAC1.

**PCR.** Amplification of DNA for use in cloning or as a hybridization probe was performed using PCR with Deep Vent DNA polymerase (New England BioLabs). Typical PCR mixtures consisted of 1× ThermoPol Buffer [20 mM Tris, pH 8, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100] containing 0.2 mM deoxynucleoside triphosphates, 0.5 μg of each primer, and 100 ng template DNA in a total reaction volume of 100 μl. Thermocycling typically consisted of incubation at 95°C for 5 min, followed by 30 cycles of successive incubations at 94°C for 30 s, 52°C for 30 s, and 72°C (for 1 min per kb of DNA). After the thermocycling, a final extension was performed at 72°C for 10 min.

To determine the frequency of multicopy integration in cell populations, whole-cell PCR was performed simultaneously on 96 *K. lactis* transformants. Individual *K. lactis* transformants were patched onto YCB agar plates containing 5 mM acetamide and incubated overnight at 30°C. Sterile pipette tips were used to scrape approximately 1 $mm^2$ of cells from each patch into 25 μl of a 1-mg Zymolyase $ml^{-1}$ (The Associates of Cape Cod, East Falmouth, MA) solution (in 30 mM $NaPO_4$) in each well of a 96-well TempPlate III PCR plate (USA Scientific, Ocala, FL). The PCR plates were incubated at 25°C for 1 hour to allow cell wall digestion, after which the cells were lysed and the DNA was denatured by incubation at 98°C for 10 min. The temperature was lowered to 80°C, and 75 μl 1× ThermoPol Buffer containing 0.2 mM deoxynucleoside triphosphates, 0.5 μg of primers 2 and 3, and 5 U *Taq* DNA polymerase (New England BioLabs) was added to each well. The thermocycling conditions consisted of 30 cycles of successive incubations at 94°C for 30 s, 50°C for 30 s, and 72°C for 3 min. After the cycling, a final incubation was performed at 72°C for 10 min.

**Pulsed-field gel electrophoresis.** To create agarose plugs of embedded cells, 55 pKLAC1-HSA and 54 pGBN19-HSA multicopy strains were each cultured in 2 ml YPGlu medium at 30°C for 15 h with shaking. Cells from each culture were harvested by centrifugation, washed with 10 ml 50 mM EDTA (pH 8), and suspended in 100 μl 1 M sorbitol containing 20 mM EDTA (pH 8), 14 mM β-mercaptoethanol, and 2 mg Zymolyase $ml^{-1}$. A 1% molten solution of Seakem LE low-melting-point agarose (BMA, Rockland, ME) made in 0.5× Tris-borate-

TABLE 1. Oligonucleotide primers used in this study

| No. | Sequence (5′ to 3′)[a] | Engineered site |
|---|---|---|
| 1 | ACACACGTAAACGCGCTCGGT | |
| 2 | ATCATCCTTGTCAGCGAAAGC | |
| 3 | ACCTGAAGATAGAGCTTCTAA | |
| 4 | CCGCTCGAGAAAAGAGATGCACACAA GAGTGAGGTTGCT | XhoI |
| 5 | ATAAGAATGCGGCCGCTTATAAGCCT AAGGCAGC | NotI |
| 6 | CCGCTCGAGAAAAGAATTGTTGGTGG TTCTGATTCTAGA | XhoI |
| 7 | GGAAGATCTCTAATGTAGAAAACTTTG TATCC | BglII |
| 8 | GCGCTCGAGAAAAGAAAGCCCACCGA GAACAACGAA | XhoI |
| 9 | ATAAGAATGCGGCCGCTTAGTCACCA CCGGCCCCCTTGAT | NotI |
| 10 | CCGCTCGAGAAAAGAATGAAAACTGA AGAAGGTAAAC | XhoI |
| 11 | ATAAGAATGCGGCCGCTTACGAGCTC GAATTAGTCTGCGC | NotI |
| 12 | GGGAATTCCACCATGGASACAGACAC ACTCCTGCTATGG | EcoRI |
| 13 | GCGCCGGTCGACATTAACACTCATTCC TGTTGAAGC | |
| 14 | GGGAATTCCACCATGRACTTCGGGYT GAGCTKGGTTTT | EcoRI |
| 15 | AGGCTTGTCGACACAATCCCTGGGCA CAATTTTCTTG | |
| 16 | GGGAATTCCACCATGGRATGSAGCTG KGTMATSCTC | EcoRI |
| 17 | GTTCTGAGATCTGGGCACTCTGGGCTC | BglII |
| 18 | GCGCTCGAGAAAAGAGACATTGTGAT GACACAGTCTCCA | XhoI |
| 19 | ATAAGAATGCGGCCGCTTAACACTCAT TCCTGTTGAAGCT | NotI |
| 20 | GCGCTCGAGAAAAGAGAGGTGCAGCT GATGGAGTCTGGG | XhoI |
| 21 | ATAAGAATGCGGCCGCTTAACAATCCC TGGGCACAATTTTCTT | NotI |
| 22 | GCGCTCGAGAAAAGACAGGTCCAACT GCAGCAACCTGGG | XhoI |
| 23 | ATAAGAATGCGGCCGCTTAGGGCACT CTGGGCTCAATTTTCTT | NotI |
| 24 | Biotin-TCGGGGATCCTTTCAGAGGCC | |
| 25 | Biotin-ACCGGCTTTGCGGAGCATGGT | |
| 26 | Biotin-AGGCTTCGTCTGCCAAACAGA | |
| 27 | Biotin-TTCATCGAACACTTTGGCATA | |

[a] Engineered restriction sites are underlined. Degenerate bases are indicated as follows: R = A or G, S = C or G, K = G or T, and M = A or C.

EDTA (89 mM Tris base, 89 mM boric acid, and 2 mM EDTA, pH 8) supplemented with 3 mM β-mercaptoethanol was cooled to 55°C, and 800 μl was added to each tube of cells and immediately dispensed into plug molds (Bio-Rad, Hercules, CA) in 100-μl aliquots. The plugs were cooled at 4°C and stored in 50 mM EDTA (pH 8).

Prior to electrophoresis, embedded cells were spheroplasted and lysed as previously described (4). Embedded chromosomal DNA was digested by soaking the plugs twice in 1 ml NEBuffer 2 (10 mM Tris, pH 7.9, containing 50 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol; New England BioLabs) for 30 min and then immersing each plug in 900 μl of NEBuffer 2 containing, 200 U AflII, 200 U SpeI, and 100 μg bovine serum albumin (BSA) $ml^{-1}$ and incubating it at 37°C overnight. The digestions were stopped by the addition of EDTA to a final concentration of 25 mM on ice. The plugs were washed twice for 10 min each time in 0.5× Tris-borate-EDTA and sealed in the wells of a 1% SeaPlaque agarose (BMA) gel with molten agarose (40°C). Pulsed-field gel electrophoresis was performed for 24 h at 15°C with a CHEF-DR III System (Bio-Rad) set at 6 V $cm^{-1}$ with 1.5- to 4-second switch intervals and a 120° field angle. The MidRange I DNA marker (New England BioLabs) was used as a size reference.

For determination of copy number by Southern analysis, electrophoresed DNA fragments were transferred to a Hybond-$N^+$ membrane (Amersham Biosciences Ltd., United Kingdom) using a standard Southern analysis protocol (8). Probes were prepared by PCR using biotinylated primer pairs 24 and 25 or 26 and 27 to amplify segments of the *amdS* or HSA gene, respectively. Biotinylated *amdS* and HSA probes were used to determine copy number in strains harboring integrated pKLAC1-HSA or pGBN19-HSA, respectively. Hybridization was performed at 55°C for 12 h. After hybridization, the blot was washed twice at room temperature with 2× SSC (0.6 M NaCl, 60 mM sodium citrate, pH 7.0) containing 0.1% sodium dodecyl sulfate (SDS) and then twice with 1× SSC containing 1% SDS at 55°C. Hybridized probe was detected using the NEB Phototope-Star Detection Kit (New England BioLabs) as directed, and the blot was exposed to Hyperfilm (Amersham Biosciences).

**Detection of secreted proteins.** To detect secretion of HSA or MBP, *K. lactis* transformants were cultured in 600 μl YPGal in 96-well plates (Nalge Nunc International, Rochester, NY) for 48 h at 30°C with shaking. Each plate also contained two control cultures of reference strains that produce only HSA or MBP. Spent medium (3 μl) from each well was spotted onto a nitrocellulose membrane (Schleicher & Schuell, Keene, NH) and air dried for 1 h. The membranes were blocked in 20 ml phosphate-buffered saline containing 0.05% Tween 20 (PBS-T) and 5% (wt/vol) nonfat milk for 1 h at 4°C. The membranes were probed with either a horseradish peroxidase-coupled anti-MBP monoclonal antibody (New England BioLabs) or an anti-HSA monoclonal antibody (US Biological, Swampscott, MA) diluted 1:1,000 in PBS-T containing 5% nonfat milk. Protein-antibody complexes were detected using LumiGlo detection reagents (Cell Signaling Technology, Beverly, MA) and exposure to Hyperfilm (Amersham Biosciences).

For strains secreting Gluc or $EK_L$, enzyme activity was measured directly in spent medium cleared of cells. $EK_L$ activity was quantitated by measuring cleavage of fluorogenic peptide substrate as previously described (2). Gluc activity was measured by mixing 25 μl of spent culture medium and 50 μl of 1× *Gaussia* luciferase assay buffer (New England BioLabs) in a Microfluor black flat-bottom microtiter plate (Thermo Labsystems, Franklin, MA). Luminescence was measured in an LMax luminometer (Molecular Devices, Sunnyvale, CA).

Secretion of active Fab antibodies was measured by enzyme-linked immunosorbent assay (ELISA) in triplicate reactions. Transformants (95 colonies) were grown in 600-μl YPGal medium cultures in separate wells of a 96-deep-well microtiter plate, along with a single culture of untransformed GG799 cells as a negative control. The plates were incubated at 30°C on a shaking platform for 4 days. The wells of 96-well polystyrene flat-bottom ELISA plates (Corning Life Sciences, Acton, MA) were coated with 100 μl of antigen solution (10 mg MBP $ml^{-1}$ or 10 mg human transferrin $ml^{-1}$ in PBS) overnight at 4°C. The plates were washed once with water and twice with PBS-T and were blocked with 200 μl of 1% BSA in PBS for 1 h, after which the washes were repeated. Spent medium (100 μl) from each culture plate well was added to each corresponding ELISA plate well and incubated for 1 h at room temperature. The wells were washed as described above, and 100 μl of horseradish peroxidase-linked Fab-specific anti-mouse antibody (Sigma) diluted 1:1,000 in PBS containing 1% BSA was added to each well and incubated for 1 h. The wells were washed again, and protein antibody complexes were detected using 1-Step Ultra TMB ELISA reagent (Pierce, Rockford, IL) as directed. Absorbance at 450 nm was measured using a VersaMax microplate reader (Molecular Devices).

## RESULTS

**Acetamide selection increases multicopy integration frequency.** To determine if the selection method influences the frequency of targeted tandem integration of a vector into the chromosome of *K. lactis* cells, two integrative expression vectors that differed only in their selectable marker genes were compared. Vectors pKLAC1 and pGBN19 both contain a variant of the strong *K. lactis* lactase promoter ($P_{LAC4\text{-}PBI}$), which drives expression of a heterologous gene (2) and directs targeted vector insertion into the promoter region of the chromosomal *LAC4* locus (Fig. 1A). However, pKLAC1 contains the *A. nidulans* acetamidase gene (*amdS*), which permits growth of transformed strains on nitrogen-free medium containing acetamide, whereas pGBN19 contains a bacterial neomycin gene that renders transformed strains resistant to the antibiotic G418.

Two whole-cell PCR strategies were devised to detect targeted integration of pKLAC1 or pGBN19 into the *K. lactis* chromosome. The first strategy used a forward primer (primer 1) that anneals to chromosomal *LAC4* promoter DNA lying upstream of the vector integration site and a reverse primer (primer 2) that anneals to either expression vector. A 2.4-kb amplicon is generated only if targeted integration of pKLAC1 or pGBN19 constructs has occurred correctly at the *LAC4* locus (Fig. 1B). The second PCR strategy exploited a unique genomic architecture that is created when tandem integration of two or more pKLAC1 or pGBN19 vector copies (termed "multicopy integration") occurs. In this strategy, only strains having multiple vector integrations produce a 2.3-kb amplicon (Fig. 1C). However, this analysis does not indicate the number of integrated vector copies.

Used in combination, these whole-cell PCR strategies allow analysis of vector integration patterns in populations of cells transformed with pKLAC1 or pGBN19 constructs. For example, transformed strains that do not generate the 2.4-kb amplicon have integrated the vector ectopically at a locus other than the *LAC4* target locus. Thus, the frequency of correctly targeted vector integration at *LAC4* (termed the "targeting efficiency") can be assessed by determining the percentage of a sample transformant population that successfully amplifies the 2.4-kb amplicon. Additionally, generation of the 2.4-kb amplicon, but not the 2.3-kb amplicon, indicates that a strain contains only a single vector copy integrated at *LAC4* (Fig. 1B), whereas production of both amplicons confirms that a strain contains at least two tandem vector integrations at the *LAC4* locus (Fig. 1C). The frequency of tandem vector integration in a transformant population (termed the "multicopy integration frequency") can be determined by first identifying a sample population of strains that produce the 2.4-kb amplicon and then determining the percentage of that population that also produce the 2.3-kb amplicon.

The multicopy integration frequencies were determined for sample populations of strains transformed with various pKLAC1 and pGBN19 constructs. Four heterologous genes encoding HSA, MBP, Gluc, and $EK_L$ were separately cloned into pKLAC1 and pGBN19 and used to individually transform *K. lactis* GG799 cells. Transformants were selected by growth on medium containing either 5 mM acetamide (for pKLAC1 constructs) or 200 μg G418 $ml^{-1}$ (for pGBN19 constructs). For

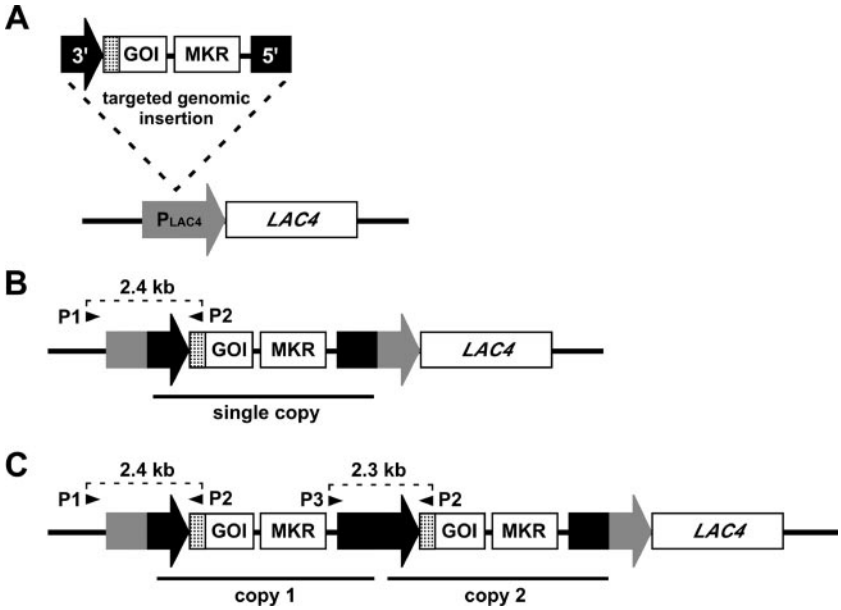


FIG. 1. Targeted vector integration at the *LAC4* chromosomal locus. (A) A SacII- or BstXI-linearized expression vector containing a gene of interest (GOI) is targeted for insertion into the *LAC4* promoter region of the *K. lactis* chromosome by the 3′ and 5′ ends of the $P_{LAC4\text{-}PBI}$ promoter (black arrow and box). pKLAC1 and pGBN19 vectors have *amdS* and neomycin-selectable marker genes (MKR), respectively. Protein secretion is directed by the *K. lactis* α-factor leader sequence (stippled box). (B and C) Whole-cell PCR with primers P1 and P2 amplifies a 2.4-kb diagnostic amplicon if single- or tandem-vector integration has occurred at the target *LAC4* locus. Multicopy integration also creates a unique genomic arrangement that yields a 2.3-kb diagnostic amplicon using primers P2 and P3.

each construct, the multicopy integration frequency (see above) was determined for a sample population of >80 transformants. For transformed strains isolated using growth on medium containing acetamide (pKLAC1-based constructs), the multicopy integration frequencies were 98 to 100%, indicating that nearly the entire sample population was comprised of strains having multiple vector integrations (Fig. 2A). The targeting efficiencies of pKLAC1-HSA, pKLAC1-MBP, pKLAC1-Gluc, and pKLAC1-$EK_L$ were 99%, 98%, 97%, and 91%, respectively, indicating that ectopic integration of these vectors occurred infrequently. In contrast, only 7 to 23% of strains transformed with pGBN19-based constructs contained multiple vector copies when selected on 200 μg G418 $ml^{-1}$ (Fig. 2A). The targeting efficiencies of vectors pGBN19-HSA, pGBN19-MBP, pGBN19-Gluc, and pGBN19-$EK_L$ were 89%, 97%, 85%, and 99%, respectively.

To determine if transformant selection with higher concentrations of G418 could increase the frequency of formation of multicopy strains, cells transformed with pGBN19-HSA were plated on growth media containing 200, 300, 400, or 1,000 μg G418 $ml^{-1}$. Transformation efficiency decreased about fivefold, from 165 ± 5.0 CFU μg of $vector^{-1}$ on medium containing 200 μg G418 $ml^{-1}$ to 30 ± 8.0 CFU μg of $vector^{-1}$ on medium containing 1,000 μg G418 $ml^{-1}$, suggesting that a more stringent selection was being imposed on the transformant population at higher G418 concentrations. The multicopy integration frequency was determined for sample populations of at least 80 transformants selected on media containing 200, 300, 400, and 1,000 μg G418 $ml^{-1}$. Only 7 to 29% of these sample populations were multiply integrated (Fig. 2B), indicating that increasing the G418 concentration during transformant selection did not increase the frequency of formation of multiply integrated strains.

These data demonstrate that both methods of selection permit highly efficient vector integration at the target *LAC4* locus. However, transformant selection by growth on medium containing acetamide dramatically enriches transformant populations for strains having multiple vector integrations compared to G418 selection. Additionally, this phenomenon occurs irrespective of the heterologous gene present in the expression vector.

**Distribution of copy numbers in multicopy strains.** The average integrated vector copy numbers were determined for populations of transformants formed by growth on medium containing 5 mM acetamide (for pKLAC1-HSA) or 200 μg ml $G418^{-1}$ (for pGBN19-HSA). Transformants were screened by whole-cell PCR to identify sample populations of strains having multiple vector integrations at the *LAC4* locus (generation of both 2.4-kb and 2.3-kb amplicons). Of 55 randomly chosen acetamide-selected pKLAC1-HSA transformants tested in this manner, all 55 were multiply integrated and were further subjected to copy number determination (see below). Due to the low multicopy integration frequency observed with G418 selection, numerous pGBN19-HSA transformants had to be tested by PCR to identify a statistically significant sample population of multiply integrated strains. Of 288 pGBN19-HSA transformants, all generated the 2.4-kb amplicon and therefore had at least one vector copy integrated at *LAC4*. However, of these, only 54 strains were multicopy strains that also produced the 2.3-kb amplicon. The remaining 234 strains were deemed to be single-copy pGBN19-HSA integrants. All 54 multicopy pGBN19-HSA strains were further subjected to copy number determination.

To determine the number of vector integrations in multicopy

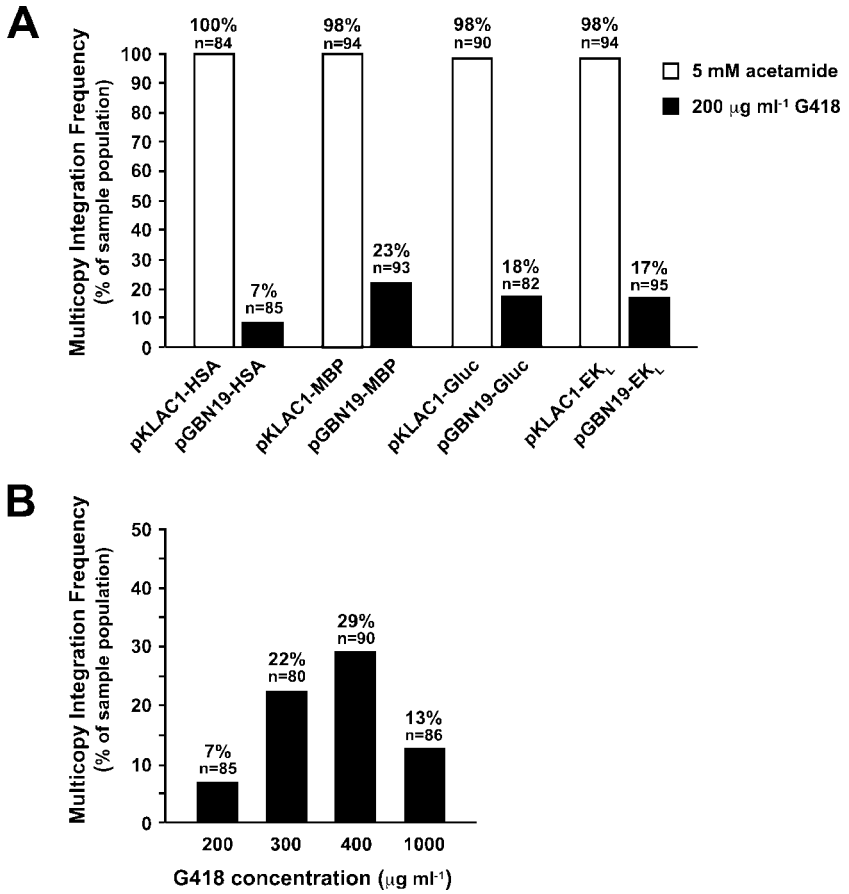


FIG. 2. Multicopy integration frequencies and distribution of copy numbers in transformed *K. lactis* cells. (A) *K. lactis* cells were transformed with pKLAC1 (white bars) or pGBN19 (black bars) containing four different heterologous genes (for HSA, MBP, Gluc, and $EK_L$). (B) Cells were transformed with pGBN19-HSA, and transformants were selected on 200 to 1,000 μg G418 $ml^{-1}$. In both panels A and B, the values indicate the percentages of colonies in a sample population of *n* transformants that tested positive for multicopy integration by whole-cell PCR.

strains, a strategy was used that involved digestion of genomic DNA with restriction endonucleases that flank the vector integration site (in the *LAC4* promoter), followed by separation of large digested DNA fragments by pulsed-field gel electrophoresis and Southern analysis (Fig. 3A). In this method, the vector copy number is indicated by the size of the hybridized DNA fragment (Fig. 3B). The populations of 55 acetamide-selected multicopy transformants and 54 G418-selected multicopy transformants were analyzed in parallel. The distribution of copy numbers within these populations is shown in Fig. 3C. The mean copy numbers were $3.8 \pm 0.45$ and $1.5 \pm 0.16$ at the 95% confidence interval for acetamide- and G418-selected sample populations, respectively. The difference in average copy numbers was confirmed with a *t* test ($\alpha = 0.05$). Thus, the average copy number doubled in transformant populations selected by growth on medium containing 5 mM acetamide.

**Simultaneous coexpression of multiple heterologous proteins.** The high multicopy integration frequency observed with acetamide selection was exploited to construct strains that simultaneously secrete multiple heterologous proteins. In this approach, two or more pKLAC1-based expression constructs, each harboring a different heterologous gene, were linearized and simultaneously introduced into *K. lactis* cells by cotransformation, followed by colony formation on growth medium containing acetamide. The high multicopy integration frequency associated with acetamide selection increases the probability that two different expression vectors will become tandemly integrated in the same cell, leading to coexpression of the two heterologous proteins.

Expression vectors that direct high-level production of HSA (pKLAC1-HSA) and MBP (pKLAC1-MBP) were each linearized and used to cotransform *K. lactis* cells using growth on medium containing acetamide for transformant selection. A sample population of 93 multiply integrated transformants was identified by whole-cell PCR, and each transformant was tested for its ability to secrete HSA and MBP by Western dot blotting. Surprisingly, 70% of the multicopy strains (65 of 93) in the sample population secreted both proteins (Fig. 4A). Similar data were obtained by cotransforming cells with three pKLAC1-based vectors containing DNA encoding HSA, MBP, or luciferase. A sample population of 96 multiply integrated transformants was identified by whole-cell PCR and tested for secretion of HSA and MBP by Western dot blotting and for secretion of luciferase activity into the growth medium. The majority of multicopy integrants tested (60 of 96, or 63%) showed secretion of all three heterologous proteins (Fig. 4B). The remaining 37% of transformants (35 of 96) produced all pairwise combinations of two proteins, and only one strain

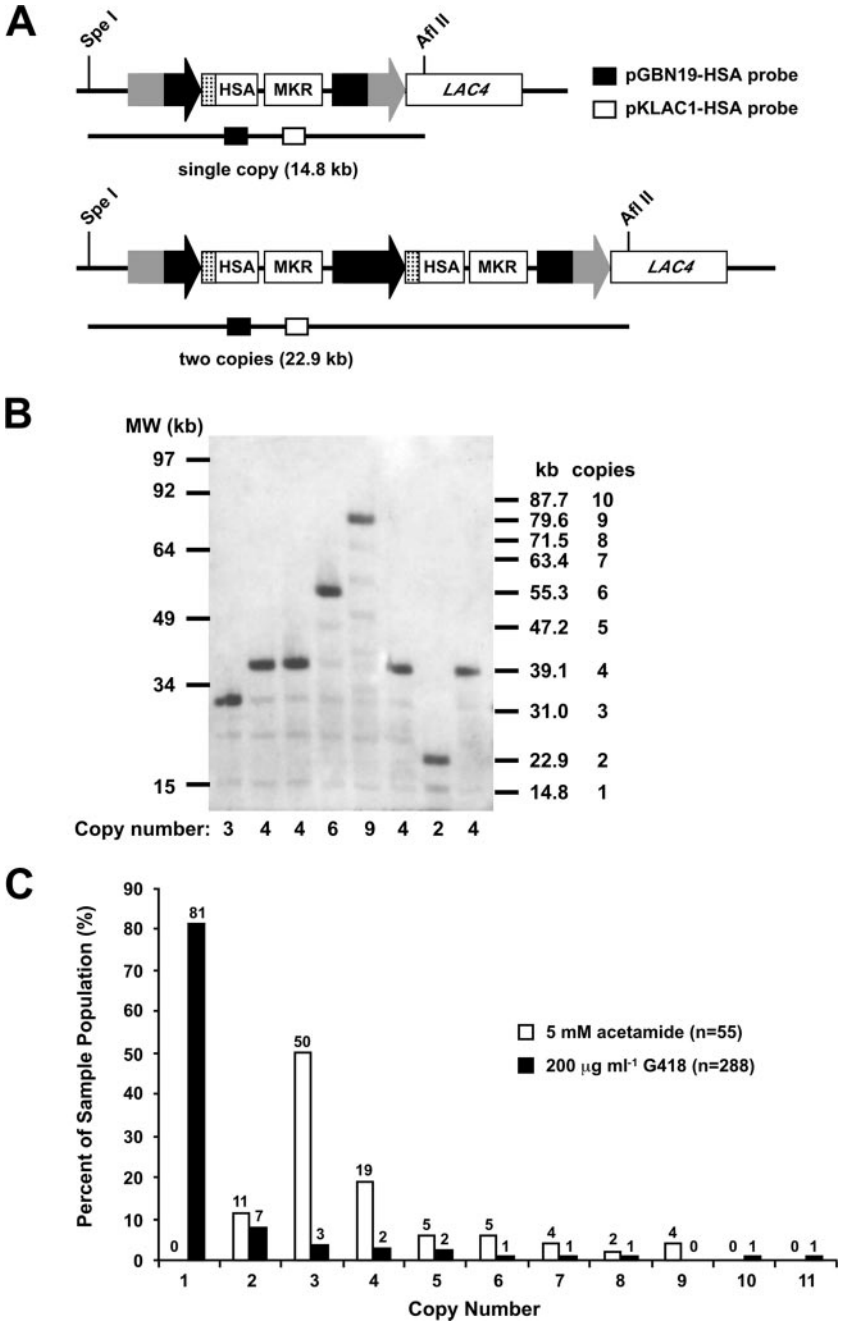


FIG. 3. Vector copy number determination in multiply integrated strains. (A and B) The vector copy number was determined by digestion of genomic DNA with SpeI and AflII (restriction sites that flank the insertion site) and separation of large DNA fragments by pulsed-field gel electrophoresis and Southern blotting. The locations of hybridization probes are shown with black (pGBN19-HSA) or open (pKLAC1-HSA) bars. Single-copy integrants yield a 14.8-kb fragment. This fragment increases in size by 8.1 kb per additional inserted vector (e.g., 22.9 kb for two copies, 31 kb for three copies, etc). (B) A sample Southern blot showing copy number determination for eight strains. (C) Distribution of integrated vector copy numbers determined by both PCR and Southern blotting in sample populations of *K. lactis* transformants selected by growth on acetamide (white bars) or G418 (black bars). The values indicate the percentages of colonies in each sample transformant population that contained each copy number.

produced a single protein (MBP). However, when four expression constructs were used to cotransform cells, a significant drop in the percentage of multicopy transformants secreting all four proteins was observed (25 of 95, or 26%), although strains producing all four proteins were still easily identified (Fig. 4C).

The yields of two cosecreted proteins were also compared. Cleared spent culture media of nine transformants cosecreting MBP and HSA were examined by SDS-polyacrylamide gel electrophoresis separation and Coomassie staining. Qualitatively, all nine transformants produced both proteins (Fig. 5, lanes 4 to 12) in quantities comparable to those obtained with characterized reference strains that secrete HSA (YCT384)

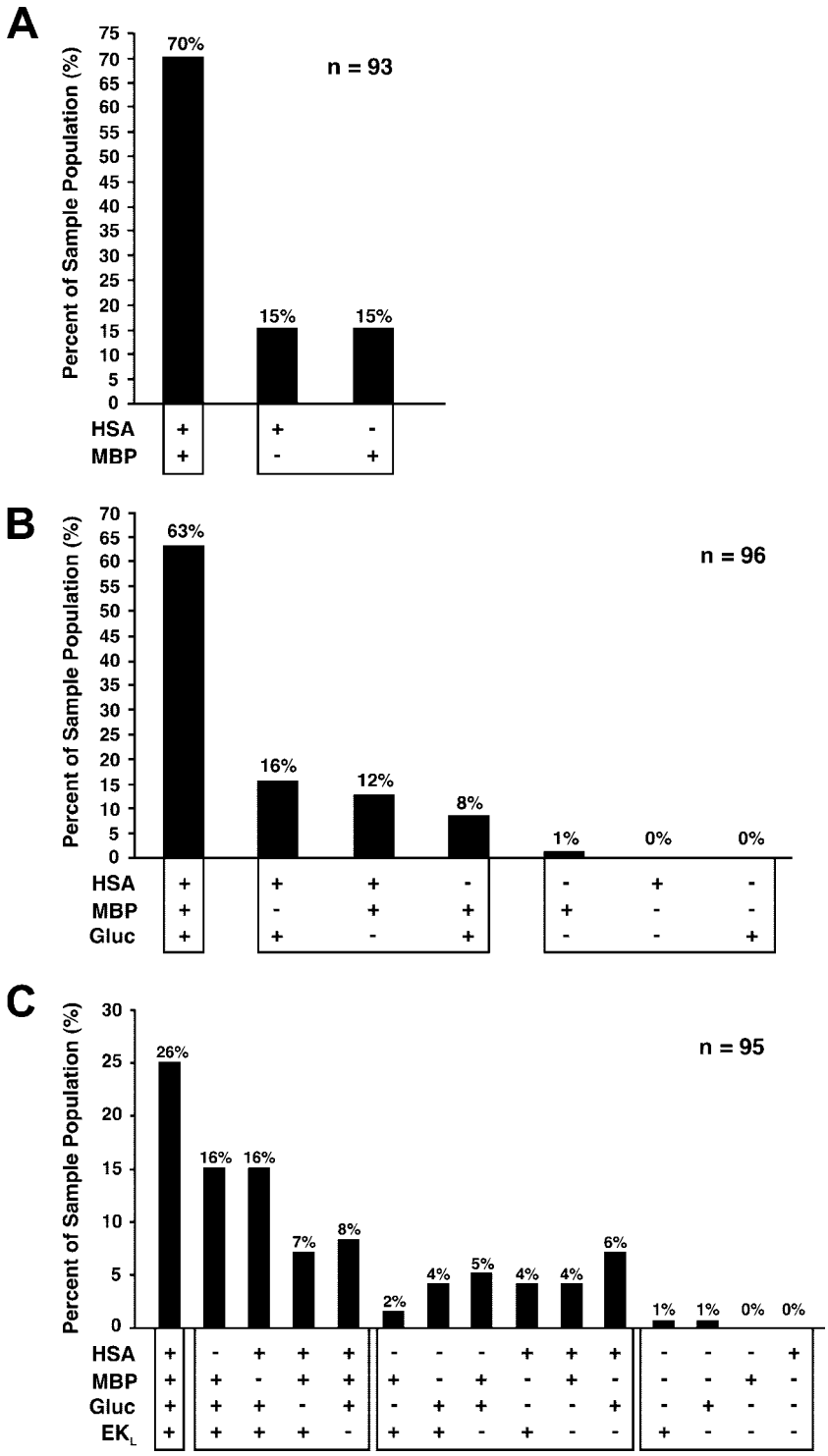


FIG. 4. Frequencies of formation of strains coexpressing multiple heterologous proteins. *K. lactis* cells were cotransformed with two (A), three (B), or four (C) pKLAC1 vectors containing various heterologous genes using growth on 5 mM acetamide for selection. Shown are the percentages of strains in sample transformant populations (n) that produced each protein (HSA, MBP, Gluc, and $EK_L$).

(Fig. 5, lane 2) or MBP (YCT463) (Fig. 5, lane 3) to approximately 75 mg liter$^{-1}$ and 65 mg liter$^{-1}$, respectively.

**Expression of multisubunit proteins.** Numerous eukaryotic secretory proteins are comprised of more than one polypeptide. Some examples are antibodies; cell surface receptors, like the major histocompatibility complexes; and various proteases (e.g., enterokinase and blood-clotting factors). From a biotechnology standpoint, antibodies represent an important class of

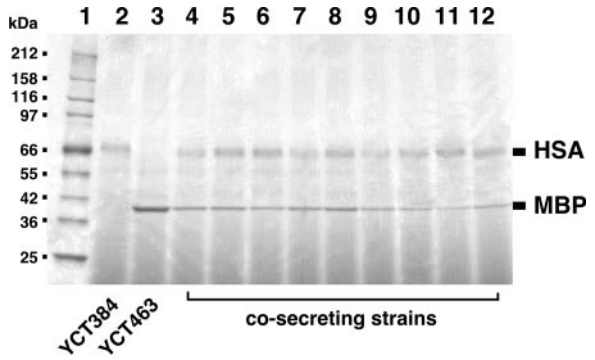


FIG. 5. Cosecretion of HSA and MBP proteins. Shown is a Coomassie-stained SDS-polyacrylamide gel electrophoresis gel with resolution of 13 μl of spent culture medium from nine random transformants producing both HSA and MBP proteins (lanes 4 to 12). Spent medium (13 μl) from cultures of reference strains that produce only HSA (YCT384) or MBP (YCT463) are shown in lanes 2 and 3, respectively. Lane 1 contains a broad-range protein standard (New England BioLabs).

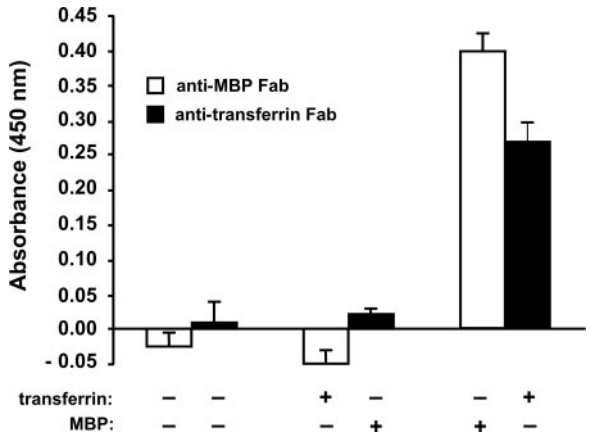


FIG. 6. Specificities of two secreted Fab antibody fragments. Two representative transformed strains that secrete either anti-MBP (open bars) or anti-transferrin (black bars) Fabs were grown, and cleared medium from each was culture incubated in the presence (+) or absence (−) of purified MBP and human transferrin antigens. Immunocomplexes were detected by ELISA. The error bars indicate standard deviations.

two-subunit proteins that are often expressed in yeasts. Therefore, we sought to determine if strains producing active antibody Fab fragments could be easily constructed using cotransformation and acetamide selection.

The genes encoding Fab fragments of monoclonal antibodies that recognize *E. coli* MBP and human transferrin were each amplified from RNA isolated from mouse hybridoma cell lines using reverse transcription-PCR and subsequently subcloned into separate pKLAC1 vectors. For each antibody, vector pairs containing DNA encoding a Fab heavy chain and a light chain were linearized and used to cotransform *K. lactis* cells using growth on medium containing acetamide for transformant selection. For both anti-MBP and anti-transferrin, sample populations of 95 multiply integrated transformants were identified by PCR. These strains were microcultured in 96-deep-well microtiter plates, and spent culture medium from each well was assayed for the presence of an active antibody fragment, using ELISA. A large percentage of each sample transformant population expressed a Fab antibody fragment (82% and 93% of anti-MBP and anti-transferrin transformants, respectively). Additionally, both secreted Fab antibodies specifically recognized their respective antigens, indicating that the secreted proteins were properly assembled (Fig. 6).

## DISCUSSION

In the present study, we examined the effect of the selection method on the frequency of multicopy expression vector integration at the *LAC4* chromosomal locus in populations of transformed *K. lactis* cells. We showed that selection on acetamide medium nearly completely enriched transformant populations for cells harboring at least two tandem copies of an integrated expression vector, whereas selection on medium containing 200 μg G418 $ml^{-1}$ produced strains with multiple integrations in less than 29% of transformants. Additionally, the average copy number increased in acetamide-selected transformant populations. Finally, we demonstrated that the high multicopy integration frequency associated with acetamide selection could be exploited to easily create strains that simultaneously express multiple heterologous proteins or multisubunit proteins using a single round of transformation and selection.

Our data suggest that under the conditions used in this study, acetamide selection is more stringent than G418 selection for *K. lactis* transformant formation. The observation that acetamide-selected transformant populations are almost completely comprised of multiply integrated strains suggests that cells having at least two vector integrations (and therefore at least two copies of the *amdS* gene) have a significant growth advantage over single integrants. Furthermore, our finding that 80% of strains transformed with pKLAC1-HSA and selected on acetamide contain two to four copies of the vector (Fig. 3C) suggests that strains harboring two to four copies of the *amdS* gene are best equipped to produce the level of acetamidase required for *K. lactis* cells to survive acetamide selection. Thus, it might be possible to achieve formation of transformants with higher copy numbers by reducing expression of the *amdS* gene in pKLAC1. In this scenario, more vector copies would be needed to produce acetamidase at the levels required for transformants to survive on acetamide medium. This could be accomplished by expressing *amdS* from a weaker yeast promoter or an attenuated form of the *ADH1* promoter (which drives expression of *amdS* in pKLAC1).

Because multicopy integration in antibiotic-selected transformants is typically an infrequent event, yeast expression strategies that involve integrative coexpression of multiple polypeptides are most often accomplished by expressing genes encoding each protein from two separate promoters on a single expression vector. For example, in the methylotrophic yeast *P. pastoris*, expression of Fab antibody fragments has been accomplished using a single integrative vector containing two yeast promoters (often two copies of the *AOX1* promoter) that concurrently drive expression of DNA encoding Fab heavy and light chains (6, 14). While this approach is viable, it typically involves the assembly of a complex expression vector and

would be very cumbersome for simultaneous expression of more than two polypeptides.

Simultaneous cointegration of multiple independent vectors is a more simplistic approach to strain construction that also offers greater genetic flexibility for strain design. For example, cointegration of separate vectors encoding a target protein and various enhancer proteins could be used to rapidly generate novel expression strains. Various studies have shown that overexpression of certain endogenous proteins can increase the secretion of a target protein in *K. lactis* and other yeasts. For example, increased expression of the Pdi1 and Ero1 chaperone proteins enhanced heterologous protein secretion in *K. lactis* by facilitating more efficient protein folding (7). Furthermore, this effect was dependent upon the copy numbers of the *KlPDI1* and *KlERO1* genes. Additionally, increased expression of certain secretory proteins has enhanced the throughput of the secretory pathway. For example, overexpression of the *K. lactis* Seb1 and Sso1 proteins (involved in translocation of proteins into the endoplasmic reticulum and exocytosis, respectively) in *S. cerevisiae* resulted in increased secretion of proteins (15). Finally, increased expression of GDP-mannose pyrophosphorylase (KlPsa1p) enhanced protein secretion in *K. lactis* (16). It is therefore plausible that various combinations of vectors encoding chaperones or "secretory enhancer" proteins could be cointegrated at the *LAC4* locus, along with a vector encoding a target heterologous protein to rapidly generate novel expression strains. Furthermore, our findings suggest that transformant populations would likely consist of a library of cells containing a random copy number distribution of each vector, which in turn may lead to varying levels of expression of each enhancer protein. Thus, the best secreting strains would be predicted to have the optimal arrangement of all of the integrated vectors and could be identified by screening the transformant population for strains that best produce the target protein.

In summary, acetamide selection of *K. lactis* cells transformed with the integrative vector pKLAC1 yields high-frequency multicopy vector integration and increased vector copy numbers in transformant populations. This high-frequency multicopy integration can be used to generate strains that coexpress multiple proteins. This method not only is a simple strain construction approach, but may offer additional genetic flexibility with implications for optimal protein production.

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