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Improved Fermentation Performance of a Lager Yeast after Repair of Its *AGT1* Maltose and Maltotriose Transporter Genes▽†

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The use of more concentrated, so-called high-gravity and very-high-gravity (VHG) brewer's worts for the manufacture of beer has economic and environmental advantages. However, many current strains of brewer's yeasts ferment VHG worts slowly and incompletely, leaving undesirably large amounts of maltose and especially maltotriose in the final beers. α-Glucosides are transported into *Saccharomyces* yeasts by several transporters, including Agt1, which is a good carrier of both maltose and maltotriose. The *AGT1* genes of brewer's ale yeast strains encode functional transporters, but the *AGT1* genes of the lager strains studied contain a premature stop codon and do not encode functional transporters. In the present work, one or more copies of the *AGT1* gene of a lager strain were repaired with DNA sequence from an ale strain and put under the control of a constitutive promoter. Compared to the untransformed strain, the transformants with repaired *AGT1* had higher maltose transport activity, especially after growth on glucose (which represses endogenous α-glucoside transporter genes) and higher ratios of maltotriose transport activity to maltose transport activity. They fermented VHG (24° Plato) wort faster and more completely, producing beers containing more ethanol and less residual maltose and maltotriose. The growth and sedimentation behaviors of the transformants were similar to those of the untransformed strain, as were the profiles of yeast-derived volatile aroma compounds in the beers.

The main fermentable sugars in brewer's wort are maltose (ca. 60% of the total), maltotriose (ca. 25%), and glucose (ca. 15%). In traditional brewery fermentations, worts of about 11° Plato (°P) are used, corresponding to a total fermentable sugar concentration of about 80 g · liter^{-1}. Many modern breweries ferment high-gravity worts (15 to 17°P), and there are efforts to raise the concentration to 25°P, corresponding to a total sugar concentration of about 200 g · liter^{-1}. Industrial use of such very-high-gravity (VHG) worts is attractive because it offers increased production capacity from the same-size brew house and fermentation facilities, decreased energy consumption, and decreased labor, cleaning, and effluent costs (34, 35).

Whereas glucose, which is used first, is transported into yeast cells by facilitated diffusion, the α-glucosides maltose and maltotriose are carried by proton symporters (2, 26, 39). Maltose transport seems to have a high level of control over the fermentation rate. Thus, during the early and middle stages of fermentation of brewer's wort by a lager yeast, the specific rate of maltose consumption was the same as the specific zero-*trans* maltose uptake rate measured off line with each day's yeast in each day's wort spiked with [^{14}C]maltose (27). Furthermore, introducing a constitutive *MAL61* (maltose transporter) gene into a brewer's yeast on a multicopy plasmid accelerated the fermentation of high-gravity worts (17). Maltotriose is the last sugar to be used in brewing fermentations, and significant amounts of residual maltotriose sometimes remain in beer, causing economic losses (lower yield of ethanol on wort carbohydrate) and possibly undesirable organoleptic effects. The problem of residual sugars in beer is more serious when high-gravity and VHG worts are used. Some, but not all, maltose transporters can also carry maltotriose. The *MALx1* genes (x = 1 to 4 and 6) encode transporters that carry maltose efficiently but are generally believed to have little or no activity toward maltotriose (1, 3, 13, 30), although substantial activity toward maltotriose was reported by Day et al. (4). Some yeast strains contain a gene 57% identical to *MAL11* that is usually known as *AGT1* but is recorded in the *Saccharomyces* Genome Database (SGDB) as *MAL11*. The Agt1 transporter has relatively high activity toward maltotriose, as well as maltose (13), and similar K_m values (4 to 5 mM) for these two substrates (4). Alves et al. (1) found that the specific deletion of *AGT1* from several *Saccharomyces cerevisiae* strains also containing at least one *MALx1* gene (*MAL21*, *MAL31*, and/or *MAL41*) abolished their ability to transport maltotriose but did not decrease their maltose transport activity. These results supported the belief that the Mal21, Mal31, and Mal41 transporters cannot carry maltotriose, though it remains possible that there are differences between Malx1 transporters from different strains. The same group has also shown (33) that overexpression of *AGT1* on a multicopy plasmid in an industrial yeast strain with a very limited ability to ferment maltotriose provided the strain with increased maltotriose uptake activity and the ability to ferment maltotriose efficiently. In 2005, a novel kind of α-glucoside transporter was independently found by two groups (6, 30) in some industrial strains of brewer's, baker's, and distiller's yeasts. These transporters are coded by *MTT1* (also called *MTY1*) genes, which are 90 and 54% identical to the *MAL31* and *AGT1* genes, respectively. The Mtt1 transporters have

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† Dedicated to the memory of Isabel Spencer-Martins, a yeast scientist who made a great contribution to our knowledge of sugar transport.

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TABLE 1. PCR primers used in this study

Name[a]	Primer sequence[b]	Sequence(s) detected[c]
ChrWalk1oligo1	5′-GGCACTATCCTTTTTCCCTTC-3′	127 to 147 of *AGT1* gene
ChrWalk1nested	5′-CATTTTTTGAGGCAGCCTTC-3′	32 to 52 of *AGT1* gene
ChrWalk2oligo2	5′-TTAAAATTGGGTACACTC-3′	−146 to −163 of *AGT1* promoter
ChrWalk2nested	5′-AGTACGCATCAACGGAGT-3′	−220 to −237 of *AGT1* promoter
AGT1-F	5′-CGAGATCTCGATGAAAAATATCATTTCATTGGT-3′	1 to 23 of *AGT1* gene
AGT1-R	5′-GCAGATCTGCTTAACATTTATCAGCTGCATTT-3′	1831 to 1851 of *AGT1* gene (ale), 1832 to 1852 of *AGT1* gene (lager)
AGT1Sek1	5′-GAAATGAAGCTAACAGCG-3′	251 to 268 of *AGT1* gene
AGT1Sek2	5′-GATGATTGGTTTGCAAAT-3′	501 to 518 of *AGT1* gene
AGT1Sek3	5′-TGTTGGTTATTTGGTCAA-3′	751 to 768 of *AGT1* gene
AGT1Sek4	5′-AAAGCAGATTGAATTGAC-3′	1011 to 1028 of *AGT1* gene
AGT1Sek5	5′-GGGTACACTTTGCTCCTG-3′	1251 to 1268 of *AGT1* gene (ale), 1252 to 1269 of *AGT1* gene (lager)
AGT1Sek6	5′-TGCTGGCCCGTATTTGCT-3′	1502 to 1519 of *AGT1* gene (ale), 1503 to 1520 of *AGT1* gene (lager)
AGT1p-705F	5′-CGGAATTCCAGCGGCAAGTCAAGAGAAGAT GGAAC-3′	−684 to −705 of *AGT1* promoter
AGT1p-387F	5′-CGGAATTCCAGCGGGCGAGGAACAAG GTTTTTTTC-3′	−367 to −387 of *AGT1* promoter
AGT1p-1R	5′-CGGAATTCCGATTATAATATTTTTTAGTTGT-3′	−1 to −22 of *AGT1* promoter
AGT1 Southern F	5′-TTGCTTTACAATGGATTTGGC-3′	842 to 862 of *AGT1* gene
AGT1 Southern R	5′-CTCGCTGTTTTATGCTTGAGG-3′	1805 to 1825 of *AGT1* gene
IntegScreenAGT1	5′-GCCTAAATATTTGCCTTTGGG-3′	−342 to −362 of A15 *AGT1* promoter
IntegScreenA15AGT1	5′-CGTTCATCTCATTAATCAT-3′	238 to 219 of A15 *AGT1* gene
IntegScreenA60AGT1	5′-TGTTCGTCTCATTAATCAC-3′	238 to 219 of A60 *AGT1* gene
IntegScreenPGK1	5′-GCTTCCAATTTCGTCACACA-3′	−411 to −431 of *PGK1* promoter

[a] F, forward; R, reverse.
[b] BglII restriction sites are underlined, EcoRI sites are underlined and italicized, and MspA1I sites are in bold italics.
[c] The numbering is from the first nucleotide of the translational start.

high activity toward maltotriose and are the only known α-glucoside transporters with lower K_m values for maltotriose than for maltose (30).

Before the discovery of the *MTT1* genes, Vidgren et al. (36) sequenced *AGT1* genes from two apparently unrelated lager strains and two apparently unrelated ale strains of brewer's yeast. Surprisingly, at that time (because other maltotriose transporters were not known), the *AGT1* genes from the lager strains contained an insertion of one nucleotide, resulting in a premature stop codon, and encoded a truncated, nonfunctional 394-amino-acid polypeptide, whereas those from the ale strains encoded full-length 616-amino-acid transporters. This premature stop codon was later shown (37) to be present in *AGT1* genes from all eight of the lager strains tested but was not in any of the four ale strains tested, whereas *MTT1* genes were present in all of the lager strains tested but in none of the ale strains tested.

In the present work, we have tested whether lager fermentations can be accelerated and residual maltotriose levels decreased by repairing the defective *AGT1* genes of lager strains with appropriate DNA sequences from ale strains. Furthermore, the *MALx1* and *AGT1* genes are repressed by glucose and induced by α-glucosides (9, 16, 19, 25), so that replacing the native *AGT1* promoter with a constitutive *S. cerevisiae* promoter might also increase α-glucoside transport activity and accelerate wort fermentations. The objectives of the present work were to confirm that α-glucoside transport has a high level of control over the rate and extent of wort fermentation and to create a genetically modified lager yeast strain that has improved fermentation performance but contains only *Saccharomyces* DNA.

MATERIALS AND METHODS

Materials. Maltose syrup (Cerestar C Sweet M 015S8) was from Suomen Sokeri (Jokioinen, Finland). [U-^{14}C]maltose was Amersham CFB182 from GE Healthcare Ltd. (Bucks, United Kingdom). [U-^{14}C]maltotriose was ARC627 from American Radiolabeled Chemicals, Inc. (St. Louis, MO), and was repurified before use (6). Nucleotides, enzymes, and antimycin A were from Sigma-Aldrich (Helsinki, Finland) or Roche (Espoo, Finland).

Strains and vectors. Strain A63015 (A15) is a lager yeast from the VTT Culture Collection. Like other lager strains, it is alloploid and probably aneuploid. It contains at least two, probably nonidentical, copies of chromosome VII carrying *MAL1* loci containing both *AGT1* and *MAL11* (36). Strain A60 is an ale yeast. Working stocks were suspensions containing 200 mg of fresh yeast mass · ml of 30% glycerol^{-1} stored in ca. 1-ml portions at −80°C. Plasmid pKX34, for selection of G418 resistance, was kindly provided by C. Lang-Hinrichs (18).

Sequencing of the *AGT1* promoter of lager strain A15. Ligation-mediated PCR amplification was based on the work of Mueller and Wold (24). Portions (50 ng) of strain A15 total chromosomal DNA were digested with blunt-end-generating restriction enzyme DraI or HaeIII for 1 h at 37°C. The restriction enzymes were then removed with the Qiagen QIAquick PCR purification kit, and DNA was eluted in 30 μl of elution buffer (Qiagen). Digested DNA was ligated in a linker mixture (24) containing the PCR linker I (5′-GCGGTGACCCGGGAGATCT GAATTC-3′) and PCR linker II (5′-GAATTCAGATCT-3′) primers with T4 DNA ligase for 3 h at room temperature. After ligation, ligase and primer nucleotides were removed with the Qiagen QIAquick PCR purification kit and DNA was eluted with 50 μl of elution buffer. The first PCR was performed with 5 μl of the ligation mixture, 5 μl of PCR linker I primer (1 pmol/μl), and 5 μl of a specific primer (ChrWalk1oligo1) from the *AGT1* gene (10 pmol/μl; the specific primers used in this study are listed in Table 1). The PCR conditions were 94°C for 3 min; 30 cycles of 94°C for 1 min, 60°C for 2 min, and 72°C for 2 min; and 72°C for 10 min. For the second PCR, 1 to 10 μl of a 1/50 dilution of the previous PCR product, 2.5 μl of the PCR linker I primer (1 pmol/μl), and 2.5 μl of a nested primer (ChrWalk1nested; Table 1) from the *AGT1* gene (10 pmol/μl) were used. Conditions for the second PCR were the same as for the first, except that an annealing temperature of 55°C was used. PCR fragments were resolved on 1% agarose gels. In chromosome walking round 1, ca. 500- and 800-nucleotide fragments were obtained with HaeIII-digested DNA as the template and ca. 500- and 350-nucleotide fragments were obtained with DraI-digested DNA as the

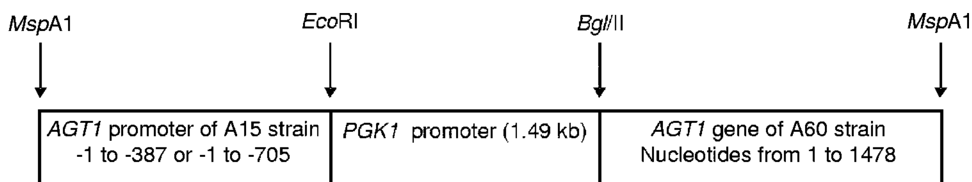

FIG. 1. Diagram of the integration cassettes used in this study. The short and long cassettes contain, respectively, 387 and 705 nucleotides of the *AGT1* promoter from strain A15.

template. The PCR fragments were resolved on 1% preparative agarose gels, cut out, and purified with a Qiagen gel purification kit. The larger fragment of each pair was sequenced directly, without cloning, by using the AGT1p-1R primer (Table 1).

For the second round of chromosome walking, the specific and nested primers were planned according to the sequences obtained from the first round. Specific primer ChrWalk2oligo2 and nested primer ChrWalk2nested were used. Otherwise, chromosome walking was performed as in the first round except that annealing temperatures of 53 and 58°C were used with the specific and nested primers, respectively. After the secondary (nested) PCR, the PCR fragments were resolved on 1% agarose gels. With DraI-digested DNA as the template, fragments of ca. 250 nucleotides and a faint, slightly smaller band were formed. With HaeIII-digested DNA, fragments of ca. 400 and 700 nucleotides were obtained. The 700-bp HaeIII fragments were cloned into the TOPO vector, and two independent clones were sequenced with universal M13 forward and reverse primers. Sequencing was done with the model 3100 Genetic Analyzer sequencer (Applied Biosystems, Foster City, CA).

Integration cassettes. Integration cassettes were constructed in the pBluescript II SK(−) vector (Stratagene, La Jolla, CA). The 1.49-kbp *PGK1* promoter and 0.37-kbp *PGK1* terminator cassette originating from expression vector pMA91 (23) in the YEplac195 multicopy vector (10, 29) was the source of the promoter-terminator cassette. There is a BglII site located between the *PGK1* promoter and terminator. The promoter-terminator cassette was detached with HindIII from the YEplac195 vector and ligated into the pBluescript II SK(−) vector at its HindIII site.

A gene that encodes a functional (36) Agt1 maltose-maltotriose transporter was amplified by PCR with DNA from the ale strain, A60, as the template. PCR primers AGT1-F and AGT1-R (Table 1), for the sense and antisense directions, respectively, were used. To facilitate the next cloning step, BglII restriction sites (underlined in Table 1) were introduced by PCR. The PCR product was cloned into a pCR-TOPO vector (Invitrogen, Espoo, Finland), and the sequence of the *AGT1* gene was verified by using eight sequencing primers, i.e., universal M13 forward and reverse primers, to sequence the start and end of the pCR-TOPO plasmid-ligated *AGT1* gene and six internal primers (AGT1Sek1 to AGT1Sek6; Table 1) from the coding strand. The *AGT1* gene was then excised from the pCR-TOPO plasmid with the BglII enzyme and ligated between the *PGK1* promoter and terminator at the BglII site in the pBluescript II SK(−) plasmid described above.

AGT1 promoter fragments of two different lengths were amplified by PCR with DNA from lager strain A15 as the template. Two primer pairs were used: AGT1p-705F and AGT1p-1R to amplify an *AGT1* promoter fragment from −705 to −1 and AGT1p-387F and AGT1p-1R to amplify an *AGT1* promoter fragment from −387 to −1. To facilitate the next cloning steps and the final detachment of the integration cassette, EcoRI and MspA1I restriction sites were introduced by PCR into the 5′ ends of these fragments and an EcoRI site was introduced into their 3′ ends, as shown in Table 1.

The *AGT1* promoter fragments were ligated to pCR-TOPO plasmids, and their sequences were verified. The promoter fragments were excised from the pCR-TOPO plasmids with EcoRI and separately ligated into the pBluescript II SK(−) vector already possessing the *PGK1* promoter-*AGT1*(A60)-*PGK1* terminator cassette. Ligation was carried out at the EcoRI site, which is located next to the 5′ end of the *PGK1* promoter. Thus, short and long integration cassettes were constructed with, respectively, 387- and 705-nucleotide *AGT1* promoter fragments flanking the *PGK1* promoter on the 5′ side. The cassettes were excised from the pBluescript II SK(−) vector by using MspA1I. It detaches the 3.37-kbp (possessing 387 bp of the *AGT1* promoter flank) and 3.70-kbp (possessing 705 bp of the *AGT1* promoter flank) integration cassettes. Detachment takes place at the 5′ end of the ligated *AGT1* promoter fragments at the newly introduced MspA1I site and on the other side of the cassette at the MspA1I site located in the *AGT1* gene at nucleotide 1478; i.e., the cassettes lack the 3′-terminal 373 nucleotides of the *AGT1* open reading frame (ORF).

Transformation of A15 and selection of *AGT1*-expressing transformants. The A15 lager strain was transformed with the short or long integration cassette together with plasmid pKX34, which contains the kanamycin marker gene giving resistance to the antibiotic G418. A15 cells were inoculated into 2% yeast extract–1% peptone medium (YP) containing 2% glucose, grown overnight at 30°C to 2×10^7 cells/ml (optical density at 600 nm [OD_{600}] of about 1), and transformed by the lithium acetate transformation procedure (11, 14). Transformed cells were resuspended in 1 ml of YP–2% glucose and incubated for 17 h at 30°C with shaking (250 rpm). The cells were pelleted for 15 s and resuspended in 1.0 ml of sterile water. Portions of 500 μl were spread on selective plates of YP–2% glucose containing G418 (200 μg/ml) or YP–2% maltotriose containing antimycin A (3 μg/ml) and G418 (200 μg/ml). The plates were incubated at 30°C for up to 6 days. Transformation frequencies, determined by counting the colonies appearing on the YP–2% glucose–G418 plates, were around 50 to 110 transformants/μg of total transforming DNA (cassette plus selection plasmid DNA).

Southern analyses. Chromosomal DNA was restricted with EcoRI, XbaI, or XmnI. Hybridization was performed with a 984-bp *AGT1* probe prepared with primers AGT1 Southern F and AGT1 Southern R (Table 1). According to the sequence of SGDB laboratory strain S288C, EcoRI cutting sites are 2,425 bp upstream and 1,993 bp downstream from the *AGT1* ORF, respectively, so that an EcoRI fragment of 6,270 bp was expected (the defective *AGT1* ORF of strain A15 is 1,852 bp). A 6.3-kb fragment was detected, indicating that EcoRI sites are in similar locations in the A15 and S288C strains. When integration of the cassette has taken place, a new EcoRI site is introduced at the beginning of the *PGK1* promoter (Fig. 1) and an EcoRI fragment of 5.3 kb is expected. XbaI restriction was used to investigate whether more than one cassette copy integrated into the *AGT1* locus. The SGDB gives XbaI sites 1,753 bp upstream and 1,244 bp downstream of the *AGT1* ORF, so that native loci should give a 4.8-kb fragment (recognized by the above-described *AGT1* probe). The cassette contains no XbaI site, so that after integration of one cassette copy a 6.3-kb fragment is expected, whereas multiple integration will give a larger fragment. XmnI sites are 1,528 bp upstream and 683 bp downstream of the *AGT1* ORF (according to the SGDB), and there is also an XmnI site at position −592 of the *PGK1* promoter in the cassette. XmnI restriction is therefore expected to give a 4.1-kb fragment from the native *AGT1* locus and a 3.1-kb fragment (recognized by the *AGT1* probe) and a 2.4-kb fragment (not recognized) after a single integration event. Multiple tandem integration would give different results, depending on the orientations and sizes of the different cassette copies.

DNA from agarose gels was blotted onto nylon filters (Hybond N; Amersham Biosciences, Espoo, Finland) by standard procedures (31). Probes were labeled with digoxigenin-11-dUTP (Roche, Germany), and hybridization signals were detected with the chemiluminescent (CSPD; Roche, Germany) or colorimetric (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside–nitroblue tetrazolium; Promega, Madison, WI) method by using alkaline phosphatase activity according to the manufacturer's instructions (Roche, Germany).

PCR analyses. PCR was performed by standard procedures. The primers used are listed in Table 1. To verify the integration of the cassette, two PCRs were used. In the first, primers binding to the *AGT1* promoter and the *AGT1* ORF were used. Specific primers which differed from each other in three nucleotides were used for the A15 and A60 *AGT1* ORFs, respectively. The IntegScreenAGT1 primer, which binds to the *AGT1* promoter, and the IntegScreenA15AGT1 primer are expected to produce a 0.6-kb fragment when endogenous *AGT1* is present. The IntegScreenAGT1 and IntegScreenA60AGT1 primers are expected to produce a 2.1-kb fragment when the intact cassette has been inserted.

In the other PCR, a primer in the *PGK1* promoter (IntegScreenPGK1; Table

1) and a primer in the *AGT1* ORF 3′ to the integration cassette (AGT1 Southern R) were used. With these primers, a 2.3-kb PCR fragment is expected after integration of the cassette as planned into the *AGT1* locus of A15 and no product is expected from A15 DNA.

To confirm that integration had occurred 3′ to the frameshift of the *AGT1* gene of A15, a PCR was performed with the IntegScreenPGK1 and AGT1 Southern R primers. The PCR products were cloned into the pCR-TOPO vector (Invitrogen, Espoo, Finland) and sequenced with the AGT1Sek3, AGT1Sek4, and AGT1Sek5 primers (Table 1).

Transcription analyses. Strain A15 and integrants 1, 2, and 14 were pregrown in YP containing 20 g · liter^{-1} glucose (duplicate 50-ml portions in 250-ml flasks) for 30 h at 24°C to OD_{600}s between 16 and 18 and then diluted into YP containing 20 g · liter^{-1} glucose, maltose, or maltotriose (duplicate 100-ml portions in 500-ml flasks) to an initial OD_{600} of 0.5 and shaken at 18°C. Samples were taken when about half of the initial sugar was consumed (at 13 h) and at stationary phase (36 h). Samples containing 50 to 150 mg of fresh yeast were quickly filtered, and the yeast was washed with RNase-free water, frozen in liquid nitrogen, and stored at −80°C as previously described (28). The cells were then lysed, and specific mRNAs were quantitated by TRAC analyses (28); for the mRNA-specific, fluor-labeled probes used, see Table 3. Probes were designed and TRAC assays were performed by PlexPress (Helsinki, Finland).

Genetic stability. A15 and integrants 1, 2, and 14 (after curing of plasmid pKX34) were repeatedly grown in YP containing 20 g · liter^{-1} glucose at 24°C. Each cycle, the yeasts were grown for about 24 h to an OD_{600} of about 12 (or on weekends for 72 h to an OD_{600} of about 21) and then diluted into fresh medium to an OD_{600} of 0.05. After 14 cycles (corresponding to 110 to 112 cell divisions), appropriate dilutions were spread on agar plates containing YP and either 20 g · liter^{-1} glucose or 20 g · liter^{-1} maltotriose and 200 mg · liter^{-1} antimycin A. Plates were incubated at 24°C, and the colonies were counted daily. Ten colonies of each strain were transferred from the glucose plates to liquid YP containing 20 g · liter^{-1} glucose and grown into early stationary phase, and DNA was extracted and analyzed.

Maltose and maltotriose transport assays. Zero-*trans* rates of α-glucoside uptake were determined with 5 mM substrate in 0.1 M tartrate–Tris (pH 4.2, 20°C) as described earlier (21, 32), with minor modifications (12). One unit catalyzes the uptake of 1 μmol of maltose (or maltotriose) per min under these conditions.

Cell viability. Cell viabilities were determined by staining with methylene blue in phosphate buffer, pH 4.6, according to Analytica-Microbiologica-EBC method 3.2.1.1 (7).

Tall-tube wort fermentations. Two fermentation series, A and B, were performed 10 months apart. 25°P worts were made in the VTT pilot brewery from malt with high-maltose syrup as an adjunct (accounting for 40% of the total extract) and contained glucose at 24 g · liter^{-1}, maltose at 110 g · liter^{-1}, maltotriose at 42 g · liter^{-1}, total fermentable sugars at 180 g · liter^{-1}, free amino nitrogen at 380 mg · liter^{-1}, zinc at 0.2 mg · liter^{-1}, magnesium at 180 mg · liter^{-1}, and calcium at 60 mg · liter^{-1}. The pH was 5.2. A 16°P wort was made by diluting 25°P wort with water containing enough $ZnSO_4$ to give a final zinc concentration of 0.2 mg · liter^{-1} in series A and 0.4 mg · liter^{-1} in series B. In series B, extra $ZnSO_4$ was also added to the 25°P wort to give a final Zn concentration of 0.4 mg · liter^{-1}. The 16 and 25°P worts were oxygenated immediately before use to 10 and 12 mg of oxygen · liter^{-1}, respectively.

Pitching yeasts were grown and fermentations were performed essentially as described earlier (27), except that main yeast growths and 24°P fermentations were at 18°C. The precultures of 100 ml of autoclaved YP containing 4% maltose in 250-ml Erlenmeyer flasks were inoculated with 500 μl of glycerol stock (100 mg yeast) and grown overnight at 24°C to an OD_{600} between 6 and 10 (series A) or 20 (series B). For main growths, 3-liter lots of 16°P wort in 5-liter Erlenmeyer flasks (series A) or 1.2-liter lots in 3-liter Erlenmeyer flasks (series B) were inoculated with 50 ml of preculture and shaken on an orbital shaker at 18°C for 2 days and the stationary-phase culture was then allowed to settle at 0°C for 16 to 24 h. Most of the supernatant was decanted, and the settled yeast was mixed into a smooth slurry and diluted with supernatant to 20 g of centrifuged yeast mass/100 g of slurry. Static fermentations were carried out in stainless steel tall tubes designed to mimic industrial cylindroconical fermentors. Yeast was pitched into 2.0 liters of the 16 and 25°P worts at 10°C to concentrations of 5.0 and 8.0 g of centrifuged yeast mass · liter^{-1}, respectively, equivalent to about 20 and 32 million cells · ml^{-1}. Dilution of worts with the yeast slurry led to calculated extracts of 15 and 24°P immediately after pitching. Samples (about 30 ml) withdrawn daily through sampling ports 23 cm above the cones were centrifuged, the pellets were washed twice with water, and their dry mass was determined overnight at 105°C. The pH of the supernatants was measured, and after degassing, their densities were determined with an Anton Paar DMA58 density meter.

At the end of the B fermentation series, yeast was cropped from some of the tall tubes. To do this with minimal exposure to air, the entire contents of each tall tube were transferred to a 2-liter screw-cap glass bottle and kept at 0°C for 2 days. During this time, the yeast settled. Most of the supernatant beer was decanted, and the settled yeast was mixed into a slurry, diluted with its own beer to 20 g of centrifuged mass/100 g of slurry, and pitched into fresh wort as described above.

Chemical analyses of beers. The ethanol concentrations of the final beers were determined by quantitative distillation according to Analytica-EBC method 9.2.1 (8). Volatile aroma compounds were measured by gas chromatography as previously described (38), and residual sugars were measured by high-performance liquid chromatography (Waters, Milford, MA).

Fermentation calculations. Apparent extracts were calculated from the densities by Analytica-EBC method 9.4 (8). "Extract" is a measure of the sum of fermentable sugars and nonfermentable soluble carbohydrate in wort: a solution with an extract of x°P has the same density as a water solution containing x g of sucrose in 100 g of solution. "Apparent extracts" measured during fermentations are uncorrected for the effect of ethanol on the density. Apparent attenuations are the difference between the original extract and the current apparent extract divided by the original extract ([original extract − current apparent extract]/original extract). The apparent attenuation limit is the apparent attenuation measured after exhaustive fermentation with excess yeast and is a measure of the total amount of fermentable carbohydrate in a wort.

Nucleotide sequence accession number. The sequence of the *AGT1* promoter of strain A15 has been deposited under accession no. EU864227 in the NCBI database.

RESULTS

Characterization of transformants. The intention was to repair one or more copies of the defective *AGT1* gene in the alloploid lager strain, A15, and at the same time place the gene(s) under the control of a constitutive *PGK1* promoter of *S. cerevisiae*. Figure 1 shows the two integration cassettes planned to achieve this aim. Between nucleotides −1 and −317, the sequence of the *AGT1* promoter was identical to that of the *MAL11* promoter in the SGDB, except for two changes (A for C at −8 and A for G at −152). However, between −318 and −807, the level of identity fell to 35%.

Lager strain A15 was cotransformed with each of these integration cassettes together with plasmid pKX34, which confers resistance to G418. After transformation, the cells were plated onto either YP containing 20 g of glucose · liter^{-1} and G418 (200 mg · liter^{-1}) or YP containing 20 g of maltotriose · liter^{-1}, antimycin A (3 mg · liter^{-1}), and G418 (200 mg · liter^{-1}). The rationale for the selection procedure was that pregrowth on glucose represses endogenous genes for α-glucoside transporters, causing long lag phases before growth on maltotriose when respiration is inhibited with antimycin A (6, 20). Transformants containing a constitutively expressed maltotriose transporter gene were expected to start growing sooner. Within 2 days at 30°C, 170 to 470 colonies appeared on the glucose-G418 plates. On the maltotriose-antimycin A-G418 plates, in contrast, only one colony appeared after 2 days and after 6 days the total number of colonies was between 4 and 13% of the number appearing on glucose-G418 (Table 2). There was no clear difference between the long and short forms of the cassette in the number of colonies recovered.

Southern blot analysis with the *AGT1* probe was applied to 36 of the 119 colonies recovered on maltotriose-antimycin A-G418 plates. For 30 colonies, both the 6.3-kb EcoRI fragment expected from the native A15 strain and the 5.3-kb EcoRI fragment expected after the integration of either cassette into the correct (*AGT1*) locus (see Materials and Methods) were

TABLE 2. Selection of transformants on glucose or on maltotriose-antimycin A[a]

Transformation	No. of colonies					
	Glucose, day 2	Maltotriose-antimycin A				
		Day 2	Day 3	Day 4	Day 5	Day 6
1	171			2	7	22
2	473		1	7	14	37
3	434	1	2	8	18	38
4	460			1	4	22

[a] Cells (1×10^8) were transformed with 0.6 μg of pKX34 DNA and (transformation 1) 6.4 or (transformations 2 to 4) 8.0 μg of the short (transformations 1 and 2) or long (transformations 3 and 4) form of the cassette DNA. After transformation, the cells were incubated overnight in YP containing 20 g of glucose · liter^{-1} and washed with water. Half were plated onto YP containing glucose (20 g · liter^{-1}) and G418 (200 mg · liter^{-1}), and half were plated onto YP containing maltotriose (20 g · liter^{-1}), G418 (200 mg · liter^{-1}), and antimycin A (3 mg · liter^{-1}). Plates were incubated at 30°C, and total colonies were counted each day.

detected, so these cells contained both an endogenous *AGT1* gene and a transformed *AGT1* gene with a *PGK1* promoter. Six of the colonies tested showed only the 5.3-kb fragment, so that these cells contained only the transformed *AGT1* gene. These 36 colonies and another 19 were tested by PCR with primers in the *AGT1* promoter and the A60-type *AGT1* ORF, and all gave the 2.1-kb product expected after the insertion of an intact cassette with the *PGK1* promoter between the *AGT1* promoter and ORF (see Materials and Methods). Both primers recognize sequences in the cassette, so this result shows that these cells contained the cassette somewhere in their genomes. For A15, the analogous PCR but with primers in the *AGT1* promoter and the A15-type *AGT1* ORF gave the expected native 0.6-kb fragment. Seven transformant colonies (including integrants 1, 2, and 14) that gave both native and recombinant EcoRI fragments in Southern analyses were also tested with this PCR, and all gave the 0.6-kb fragment, confirming that they contain both native and repaired *AGT1* loci.

For 17 of the above-described 36 clones, another PCR was performed with primers in the *PGK1* promoter and the *AGT1* ORF 3′ to the integration cassette (primers IntegScreenPGK1 and AGT1 Southern R; Table 1). All 17 colonies (including integrants 1, 2, and 14) gave the expected 2.3-kb fragment (see Materials and Methods), verifying that the cassette had integrated as planned into the endogenous *AGT1* locus.

To confirm that integration had occurred 3′ to the frameshift of *AGT1* of A15, 20 independent PCR clones of this 2.3-kb fragment were isolated from seven transformants and sequenced from nucleotide 768. After nucleotide 768, the ORFs of the *AGT1* genes in strains A60 and A15 differ (36) at nucleotides 827, 997, 1123, 1183 (where the frameshift in A15 *AGT1* occurs), 1465 and 1647, whereas the transformation cassettes contained A60 sequence up to nucleotide 1478. In 18 of the 20 independent clones, the variant nucleotides at positions up to and including nucleotide 1183 were derived from the A60 sequence, showing that the second crossover had occurred 3′ to the frameshift in the A15 sequence. The exceptions were one of the seven independent PCR clones from integrant 1 (where the crossover had apparently occurred between nucleotides 827 and 997 and nucleotides 997, 1123, 1183, and 1465 were all derived from A15) and one of the seven independent PCR clones from integrant 14 (where the crossover was between nucleotides 1123 and 1183, but we did not identify nucleotide 1465). In this second case, the A15 nucleotide found at position 1183 might be a PCR error, but for the exceptional clone from integrant 1, this is very unlikely, since four nucleotides within the region of the cassette DNA (997, 1123, 1183, and 1465) were all derived from A15. This suggests that, in addition to at least one copy of repaired *AGT1* where crossover has occurred 3′ to the frameshift, integrant 1 also contains a copy of modified *AGT1* where crossover has occurred 5′ to the frameshift. Nucleotide 1465 was derived from A15 in 6 cases and from A60 in 10 cases (undetermined in 4 cases), showing variability in the crossover site at the 3′ end of the cassette.

Transcriptional analysis and α-glucoside transport capacity of the integrants. Integrant 1 (the first to appear during selection; Table 2), integrant 2, and integrant 14 were chosen for studies of α-glucoside transport and fermentation performance. They were cured of plasmid pKX34 by cultivation in the absence of the antibiotic G418. Single-cell colonies were isolated that were unable to grow in the presence of G418 and therefore presumably lacked the plasmid. To check that these cured integrants contained no bacterial DNA from the plasmid, their chromosomal DNAs were digested with either EcoRI or HincII and Southern blots were probed with, respectively, a ca. 1.2-kb EcoRI fragment or a ca. 3-kb HincII fragment of the pKX34 plasmid. These fragments together cover most of the bacterial DNA in the pKX34 plasmid (see reference 18). No hybridization bands were observed from integrants 1, 2, and 14, but the expected strong signals were seen with linearized plasmid pKX34 (not shown).

These three integrants each yielded both 6.3-kb and 5.3-kb EcoRI fragments and so contained both native and repaired *AGT1* loci. The 5.3-kb bands were markedly more intense than the 6.3-kb bands (Fig. 2), suggesting that each integrant contains more repaired loci than native loci, so that A15 must contain at least three *AGT1* loci. Restriction with XbaI yielded the expected 4.8-kb band from A15. Each integrant gave both a 4.8-kb band and the 6.3-kb band expected after the integration of a single cassette copy at each repaired *AGT1* locus (see Materials and Methods). No larger band was observed, showing that multiple integration had not occurred. The 6.3-kb bands were stronger than the 4.8-kb bands, in agreement with the EcoRI results, again suggesting that A15 must contain at least three *AGT1* loci. Results obtained with XmnI were similar, integrants yielding weaker 4.1-kb bands corresponding to the native loci and stronger 3.1-kb bands corresponding to repaired *AGT1* loci.

To test whether the repaired *AGT1* genes were expressed, strain A15 and integrants 1, 2, and 14 were grown at 18°C in shake flasks in YP containing 200 g · liter^{-1} glucose, maltose, or maltotriose. Figure 3 shows the apparent expression levels of the actin gene *ACT1* (as a control), the maltose transporter genes *MTT1*, *AGT1*, and *MALx1*; and the maltase gene *MALx2* in yeast samples taken after 13 h of growth (at OD_{600}s of 5.3 ± 0.5, 4.2 ± 0.5, and 4.0 ± 0.6 on glucose, maltose, and maltotriose, respectively, and residual glucose at 7.0 ± 0.3 g · liter^{-1}; residual maltose and maltotriose were not measured but were presumably similar) or in stationary phase at 36 h (at an OD_{600} of 23 ± 2). The TRAC probes used are shown in Table 3, and the fluorescence signals from these specific probes are assumed to

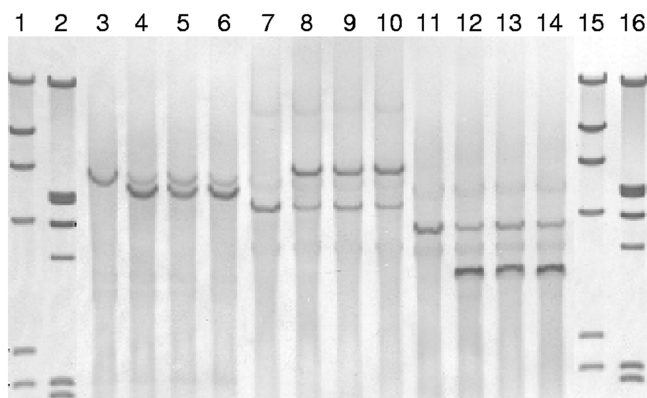

FIG. 2. Southern analyses of strain A15 and integrants 1, 2, and 14. Lanes 1 and 15, molecular weight marker II (23,130, 9,416, 6,557, 4,361, 2,322, and 2,027 bp); lanes 2 and 16, molecular weight marker III (21,226, 5,148, 4,973, 4,268, 3,530, and 2,027 bp); lanes 3, 7, and 11, strain A15; lanes 4, 8, and 12, integrant 1; lanes 5, 9, and 13, integrant 2; lanes 6, 10, and 14, integrant 14. Chromosomal DNA was restricted with EcoRI (lanes 3 to 6), XbaI (lanes 7 to 10), or XmnI (lanes 11 to 14); separated in 0.8% agarose gel; blotted onto a nylon filter; and probed with a 984-bp *AGT1* PCR fragment. The band sizes (in kilobases) predicted from the SGDB sequence of native *AGT1* loci are as follows: EcoRI, 6.3; XbaI, 4.8; XmnI, 4.1. For repaired loci, they are as follows: EcoRI, 5.3; XbaI, 6.3; XmnI, 3.1. Tandem integration of cassette DNA would give a 9.6-kb or larger XbaI band(s).

be proportional to the expression levels. Results in Fig. 3 are normalized to the total amounts of mRNA in each sample. This resulted in similar *ACT1* signals from the different yeasts, so that normalization to *ACT1* levels, instead of total mRNA, would not alter the interpretation of the results. At 13 h, the apparent expression level of the resident *AGT1* gene of A15 was independent of the sugar type and at 36 h it was only slightly higher after growth on maltose or maltotriose than after growth on glucose. Compared to strain A15, all three integrants showed increased expression of *AGT1* (2.6- to 5.8-fold increases at 13 h; 3.8- to 12-fold increases at 36 h). Thus, the repaired *AGT1* genes under the control of *PGK1* promoters were expressed more strongly than the resident gene. In contrast to *AGT1*, the other α-glucoside transporter genes, *MTT1*, *MALx1*, and *MALx2*, were not expressed during growth on glucose and were only weakly expressed in stationary-phase cells after growth on glucose. These genes were expressed during and after growth on maltose and maltotriose at similar levels in strain A15 and the three integrants. Maltose and maltotriose transport activities were measured in cells of each strain harvested at 13 h from one of the two replicate growths on glucose. Strain A15 cells exhibited little or no activity (<0.2 U · g of dry yeast^{-1}), which was expected, as their *AGT1* genes do not encode functional transporters and their other α-glucoside transporter genes were not expressed.

In another experiment, yeast cells were grown on YP containing 20 g of glucose · liter^{-1} at 24°C and harvested at 8.5 ± 0.4 g of glucose · liter^{-1} (strain A15 and integrants 1 and 2) or 10.7 g of glucose · liter^{-1} (integrant 14). The maltose transport capacity of strain A15 was 1.6 U · g of dry yeast^{-1}, whereas integrants 1, 2, and 14 exhibited 10.3, 6.6, and 5.1 U · g of dry yeast^{-1}. Addition of 50 mM unlabeled maltotriose to the assay mixtures inhibited the uptake of 5 mM radiolabeled maltose by 13% for strain A15 but by 59, 50, and 41%, respectively, for integrants 1, 2, and 14. The greater inhibition of the integrants by maltotriose was consistent with their maltose

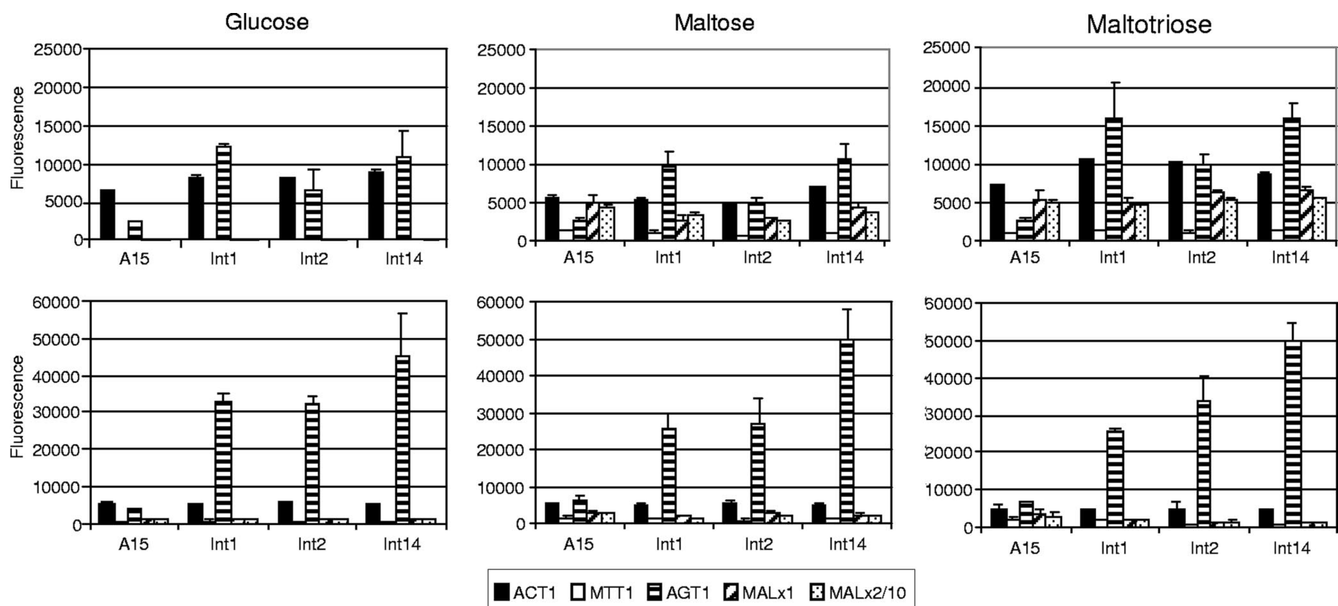

FIG. 3. Expression of *AGT1* and other α-glucoside transporter and maltase genes during batch growth of strain A15 and integrant 1 (Int1), Int2, and Int14 on 20 g · liter^{-1} glucose, maltose, or maltotriose at 18°C. Yeast samples were collected after 13 h (upper row), while sugars were still present at about 7 g · liter^{-1}, or at 36 h (lower row), when cells were in stationary phase. Samples were lysed and analyzed by TRAC with probes specific for *ACT1* (actin control); the transporter genes *MTT1*, *AGT1*, and *MALx1*; and the maltase gene *MALx2*. Shown are the fluorescence signals from specifically bound probes. The signals from the *MALx2* probe were divided by 10 (*MALx2*/10). Results are averages ± ranges of data from two replicate growths of each yeast strain on each sugar.

TABLE 3. TRAC probes used in this study

Gene	Probe sequence	Fluor[a]	Target nucleotides[b]
ACT1	CGGTTTGCATTTCTTGTTCGAAGTCCAAGGCGA	NED	656–688
MTT1	ACTGTTTGTATAGCCAATCCAAATGCGTAAAGGTCAAAC	NED	1278–1316
AGT1	GAGTTTTTCCCTTTCCGAATGGATCAACCAC	HEX	1738–1768
MALx1	GGTTTCTGGTAAATCGACAACAGCCCAAGCTAA	FAM	1615–1647
MALx2	ACCGGGCTTGATCGTGATTCTCGAT	FAM	1033–1057

[a] Fluorescent labels attached to the probes: FAM, 6-carboxyfluorescein; HEX, hexachloro-6-carboxyfluorescein; NED, 2,7′,8′-benzo-5′-fluoro-2′,4,7-trichloro-5-carboxyfluorescein.
[b] Numbering is from the translational start according to the SGDB.

transport activity being caused by the broad-specificity Agt1 transporters (for which maltotriose is necessarily a competitive inhibitor of maltose transport). After growth on YP containing 20 g of maltose · liter^{-1}, strain A15 exhibited relatively high maltose transport activity (18 U · g of dry yeast^{-1}) and integrants 1, 2, and 14 showed only modest (10 to 20%) increases in activity (to, respectively, 20, 21, and 22 U · g of dry yeast^{-1}). However, the transport activity of maltose-grown cells toward maltotriose increased from 3.4 U · g of dry yeast^{-1} for strain A15 to 8.5, 5.1, and 5.4 U · g of dry yeast^{-1}, respectively, for integrants 1, 2, and 14. Thus, compared to the host strain, maltose-grown integrants had an improved capacity to transport maltotriose in particular.

Genetic stability. Strain A15 and integrants 1, 2, and 14 were cycled 14 times through growth on YP containing 20 g of glucose · liter^{-1}. This corresponded to 110 to 112 cell divisions for the different strains. The final yeast suspensions were diluted and spread on nonselective (20 g of glucose · liter^{-1}) and selective (20 g of maltotriose plus 200 mg antimycin A · liter^{-1}) plates. Colonies appearing were counted daily (Table 4). The numbers of colonies on nonselective plates did not increase between 2 and 4 days, and 10 colonies of each strain were picked at random. DNA from these colonies was restricted with EcoRI, and Southern analyses were run as in Fig. 2. The same results were obtained as in Fig. 2; i.e., all 10 A15 colonies gave only the native 6.3-kb band and all 10 colonies of each integrant gave both this band and the 5.3-kb band expected after integration of the cassette DNA (not shown). Thus, the integrant genotypes were stable through 110 generations of nonselective growth. Growth of A15 on the selective plates started after a long lag: no colonies had appeared by day 9, but by day 12 the numbers reached 65 to 88% of the numbers on nonselective plates. In contrast, by day 5 the numbers of integrant colonies on selective plates were 85 to 109% of the numbers on nonselective plates (except 55% for the 25-μl sample of integrant 14). Thus, the phenotype of fast adaptation to growth on maltotriose-antimycin A after growth on glucose was preserved through 110 generations. It is also notable that for integrant 1 (the first to appear during selection; Table 2), the numbers of colonies on selective plates on day 4 were already 58 to 84% of the numbers of colonies on nonselective plates, whereas this proportion was only 19 to 61% for integrants 2 and 14, suggesting that the phenotypic difference between integrant 1 and the others was also preserved.

Wort fermentations with the parent strain and integrants. The cured yeasts were propagated in 16°P wort, and their fermentation performance was tested in static tall-tube fermentors that imitate industrial cylindroconical fermentation tanks. All three integrants fermented both 15°P wort (at 14°C) and 24°P wort (at 18°C) faster and more completely than did the parent, A15, lager strain (Fig. 4 and Table 5; see also Fig. 6), resulting in time savings and increased ethanol yields. For all three fermentation series (one at 15°P, two at 24°P), time savings were estimated as the differences between the time required by A15 and that required by each integrant to reach an apparent attenuation of 80%. The apparent attenuation limits of these worts were close to 86%. Brewers would want to reach an apparent attenuation as close as possible to this limit, but in practice some fermentable sugars always remain in the final beer (and have desired organoleptic effects in some beers). For the two fermentation series (15°P and series B at 24°P) where the control strain, A15, reached a higher apparent attenuation than 80%, we also calculated the time savings at the highest attenuation reached by all of the strains (Table 5).

For the 15°P wort, the differences in final apparent extracts (a measure of residual carbohydrate) were relatively small (0.14 to 0.19°P lower for integrants than for A15) and increases in ethanol yield were close to the experimental error (Table 5).

TABLE 4. Genetic stability of integrants[a]

Strain and vol (μl)	No. of colonies						
	Glucose plates		Maltotriose/antimycin A plates				
	Day 2	Day 4	Day 2	Day 4	Day 5	Day 9	Day 12
A15							
25	57	57	0	0	0	0	50
50	123		0	0	0	0	80
100	213		0	0	0	0	
Integrant 1							
25	45	47	0	26	43		
50	95		0	80	104		
100	170		0		186		
Integrant 2							
25	48	46	0	9	43		
50	109		0	44	99		
100	177		0		150		
Integrant 14							
25	56	58	0	22	31		
50	98		0	60	101		
100	171		0		142		

[a] Each strain was cycled through growth on glucose for 110 to 112 cell divisions and diluted to an OD_{600} of 2.5×10^{-4}, and portions of the indicated volumes were spread onto agar plates containing glucose or maltotriose-antimycin A and incubated at 24°C for up to 12 days. No entry indicates that the colonies were not counted.

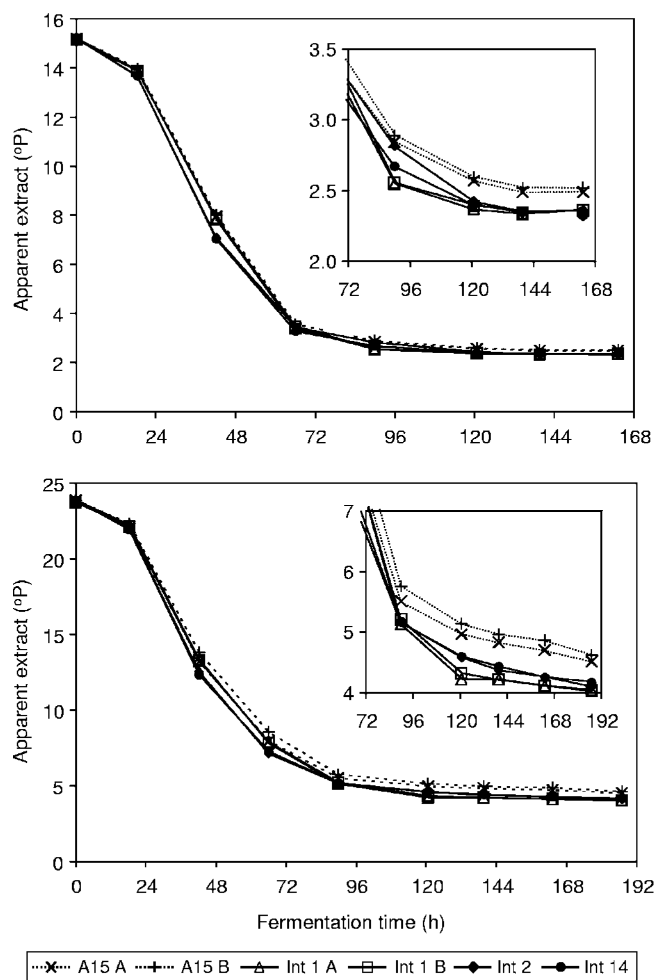

FIG. 4. Attenuation profiles during the fermentation of 15°P (upper panel) and 24°P (lower panel) worts by duplicate growths of strain A15 (A15A, A15B), duplicate growths of integrant 1 (Int 1 A, Int 1 B), and single growths of integrants 2 and 14 (Int 2, Int 14). The 15 and 24°P worts were pitched with, respectively, 5.0 or 8.0 g of fresh yeast mass · liter^{-1} at 10°C, and fermentations were continued at 14°C. The 24°P fermentations were shifted to 18°C at 20 h. Insets show detail during the last 4 or 5 days.

Time savings were also small (2 to 8 h) at an apparent attenuation of 80% but were significant (13 to 32 h, corresponding to 8 to 20% decreases in fermentation time) at 83% apparent attenuation.

For the 24°P worts, larger differences in final apparent extract occurred, 0.40 to 0.53°P in the series A fermentations and 0.25 to 0.39°P in the series B fermentations, which were performed 10 months later (Table 5). Time savings at an apparent attenuation of 80% were 13 to 57 h, corresponding to 10 to 30% decreases in fermentation time. Greater proportional decreases in fermentation time (14 to 37%) were observed in the B series at an apparent attenuation of 81%. Final ethanol production from the 24°P worts rose from 93 g · liter^{-1} for A15 to 95 to 96 g · liter^{-1} for the integrants, and the residual maltose and maltotriose levels were, respectively, about 1.5 and 4 to 7 g · liter^{-1} lower in beers made by the integrants than in those made by A15. Time savings and increases in ethanol production were greatest for integrant 1, which was also the first integrant to appear on the maltotriose-antimycin A plates during the selection of transformants.

Of yeast cells sampled after 20 h from the 24°P fermentations of series A, the integrants exhibited markedly higher maltose transport activity under standard assay conditions (5 mM maltose, pH 4.2, 20°C), but this difference was smaller at 72 h (Table 5) and at 120 and 168 h (data not shown). At 20 h, all of these fermenting worts still contained glucose (with the different yeast strains, the level of glucose fell from 13 ± 3 g · liter^{-1} at 16 h to 3.6 ± 1.0 g · liter^{-1} at 25 h), but it had disappeared by 42 h. In these series A 24°P fermentations, there was little difference between the rates of glucose and maltose consumption by the different yeasts (data not shown) but maltotriose was consumed more rapidly by the integrants. This difference was already evident at 42 h, when maltotriose had fallen from 42 g · liter^{-1} to 32.2 ± 0.7 g · liter^{-1} for strain A15 and 26.7 ± 0.1, 29.2, and 28.1 g · liter^{-1}, respectively, for integrants 1, 2, and 14. By 92 h, maltotriose was at 13.2 ± 0.5 g · liter^{-1} for strain A15 and 5.6 ± 0.3, 9.1, and 10.1 g · liter^{-1} for integrants 1, 2, and 14.

In both 15 and 24°P worts, integrants 1, 2, and 14 grew similarly to strain A15 (Fig. 5). Strain A15 is a so-called "dusty yeast" that sediments poorly at the end of wort fermentations, and this pattern was retained by the integrants. Integrants 2 and 14 sedimented more slowly than strain A15 at the end of fermentation.

During yeast handling, the viability of the integrants was essentially as good as that of strain A15 (Table 5). After propagation, strain A15 had a slightly higher viability (99.4% and 98.6% in series A and B) than the integrants (ca. 97.6 to 98.3%), but all of the strains cropped from the 15°P fermentations at 99% viability. Integrants cropped from the 24°P fermentations at higher viabilities (94.7 to 96.4%) than strain A15 (92.5 ± 2.5%) in series A, but no difference was observed in series B. Final beer pH values were consistently slightly (ca. 0.03 U) lower for the integrants than for strain A15, but these differences were small compared to the differences between beers from 24°P worts (4.29 ± 0.03 for all of the yeast strains) and 15°P worts (3.95 ± 0.03).

Most brewery fermentations are performed with yeast cropped from an earlier fermentation and used again. Figure 6 shows the result obtained when strain A15 and integrant 1 were cropped from a 24°P fermentation and repitched into a new batch of 24°P wort. As expected, tall-tube fermentations of 24°P wort by recycled yeast were much slower than when laboratory-grown yeast was used (this is because the recycled yeast contains smaller amounts of sterols and unsaturated fatty acids and therefore needs more oxygen than can be easily added to 24°P worts under laboratory conditions). However, the difference between strain A15 and integrant 1 was, at least qualitatively, preserved through the cropping and repitching: after 210 h of fermentation, the integrant 1 fermentations had an apparent extract 0.85 ± 0.23°P (mean ± range of duplicates) lower than that of the strain A15 fermentations.

Figure 7 shows the profiles of yeast-derived volatile aroma compounds in beers collected at the end of the series A 24°P fermentations. These esters and secondary alcohols and acetaldehyde are important components of beer flavor. In most cases, differences between the aroma profiles of beers made

TABLE 5. Wort fermentations by A15 and three integrants[a]

Parameter	A15	Integrant 1	Integrant 2	Integrant 14
Series A pitching yeast viability (%)	99.4[c]	98.2 ± 0.2	97.9	98.3
Series A 15°P fermentation				
Final AE (°P)	2.50 ± 0.02	2.36 ± 0.00	2.31	2.36
Extra AE (°P)		0.14	0.19	0.14
Time to 80% AA (AE = 3.0°P) (h)	83.1 ± 1.5	76.7 ± 0.9[b]	81.1	75.2
Time saved at 80% AA (h)		6.4	2.0	7.9
Time to 83% AA (AE = 2.6°P) (h)	121 ± 2	89 ± 0[f]	108	100
Time saved at 83% AA (h)		32	13	21
Final ethanol concn (g · liter^{-1})	57.3 ± 0.2	57.7 ± 0.2	57.7	57.1
Crop viability (%)	99.0 ± 0.2	99.2 ± 0.1	99.0	99.2
Series A 24°P fermentation				
Transport at 20 h (U · g DY^{-1})[d]	12.3 ± 0.2	17.2 ± 1.7	18.2	20.9
Transport at 72 h (U · g DY^{-1})[d]	6.7 ± 0.7	7.2 ± 0.8	7.9	7.7
Final AE (°P)	4.57 ± 0.05	4.04 ± 0.01	4.10	4.17
Extra AE (°P)		0.53	0.47	0.40
Time to 80% AA (AE = 4.8°P) (h)	161 ± 11	104 ± 2[b]	110	111
Time saved at 80% AA (h)		57	51	50
Final ethanol concn (g · liter^{-1})	93.3 ± 0.3	95.4 ± 0.1[b]	95.6	95.0
Final maltose concn (g · liter^{-1})	2.7[e]	1.1 ± 0.0	1.2	1.2
Final maltotriose concn (g · liter^{-1})	7.1[e]	0.0 ± 0.0	3.2	3.4
Crop viability (%)	92.5 ± 2.5	96.4 ± 1.6	95.3	94.7
Series B 24°P fermentation				
Pitching yeast viability (%)	98.6 ± 0.4	97.6 ± 0.2	98.3	97.7
Final AE (°P)	4.41 ± 0.03	4.02 ± 0.00	4.02 ± 0.01	4.16 ± 0.02
Extra AE (°P)		0.39	0.39	0.25
Time to 80% AA (AE = 4.8°P) (h)	126 ± 4	88 ± 1[f]	98 ± 2	113 ± 1
Time saved at 80% AA (h)		38	28	13
Time to 81% AA (AE = 4.6°P) (h)	154 ± 3	97 ± 5[b]	112 ± 2	133 ± 4
Time saved at 81% AA (h)		57	42	21
Final ethanol concn (g · liter^{-1})	93.1 ± 1.1	95.2 ± 0.8[b]	95.4 ± 0.4	94.9 ± 0.6
Crop viability (%)	96.0 ± 0.3	95.7 ± 1.1	95.0	96.3

[a] Series A fermentations are shown in Fig. 2 and 3, and series B fermentations for A15 and integrant 1 are shown in Fig. 6. AE, apparent extract; AA, apparent attenuation. Final AE, ethanol concentration, crop viability, and (where shown) maltose and maltotriose concentrations were measured after 165, 187, and 187 h, respectively, in the series A 15°P, series A 24°P, and series B 24°P fermentations. For A15 and integrant 1, results are means ± ranges from duplicate fermentations pitched with independently grown lots of yeast. For integrants 2 and 14, results are from single fermentations or, where shown, averages ± ranges of duplicate fermentations pitched with the same lot of yeast.

[b] The difference between integrant 1 and A15 duplicates is significant at $P < 0.1$ (two-tailed paired t test).

[c] Viability was measured for only one of the duplicate A15 growths.

[d] Transport shows the maltose transport activity as units per gram of dry yeast (DY) measured in yeast sampled at 20 and 72 h.

[e] Final sugar concentrations were measured in only one A15 duplicate fermentation; assays of both duplicates 18 h earlier gave 2.9 ± 0.1 and 8.1 ± 0.4 g of maltotriose · liter^{-1}.

[f] The difference between integrant 1 and A15 duplicates is significant at $P \leq 0.05$ (two-tailed paired t test).

with the integrants and strain A15 were small. However, integrants 1 and 2 produced ca. 30% more 3-methylbutyl acetate, which gives a banana-like flavor generally considered beneficial (taste threshold, 0.6 to 6 mg · liter^{-1}). Integrant 14 apparently produced 2.5-fold as much acetaldehyde as strain A15. Acetaldehyde (apple-like flavor) at this level would be undesirable in a lager beer.

DISCUSSION

Vidgren et al. (36, 37) found that all eight of the lager strains tested contained defective *AGT1* genes that cannot encode functional maltose and maltotriose transporters because of an extra thymidine residue at nucleotide 1183 which introduces a premature stop codon before the last four transmembrane domains. The present work shows that correcting this sequence defect and, at the same time, placing the *AGT1* genes under the control of a constitutive promoter increases the transport activity of lager strain A15 toward maltose and, especially, maltotriose and improves its fermentation performance in high-gravity and VHG worts. Transformants of strain A15 (integrants 1, 2, and 14) engineered in this way fermented 15 and 24°P worts faster and more completely than did strain A15, producing beers containing more ethanol and lower levels of residual maltose and maltotriose. This result confirms earlier findings (17, 27) that the rate of α-glucoside uptake is a major factor limiting the rate of wort fermentation. The size of the changes was economically significant (Table 5): primary fermentation times for 15 and 24°P worts were decreased by 8 to 20% and 11 to 37%, respectively, which represents a marked increase in annual output from the same-size brew house and fermentor facilities, and for 25°P worts the final ethanol concentrations were increased by 2% (from about 93 to 95 g of ethanol · liter^{-1}), giving a similar increase in yield from raw materials. Residual maltose and maltotriose in the final beers were markedly decreased (Table 5). These improvements were

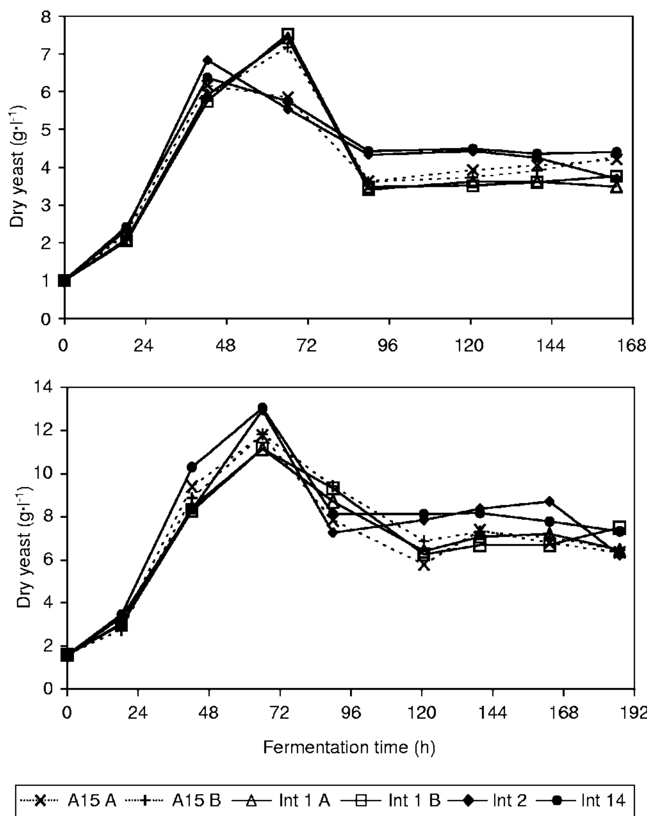

FIG. 5. Yeast in suspension during the fermentations shown in Fig. 4 of 15°P (upper panel) and 24°P (lower panel) worts by strain A15 and integrant 1 (Int 1), Int 2, and Int 14. The plots show the increases in yeast mass in suspension caused by growth during the first 2 to 3 days, followed by decreases as the yeast sediments in the static fermentations. Experimental details are the same as in the legend to Fig. 4.

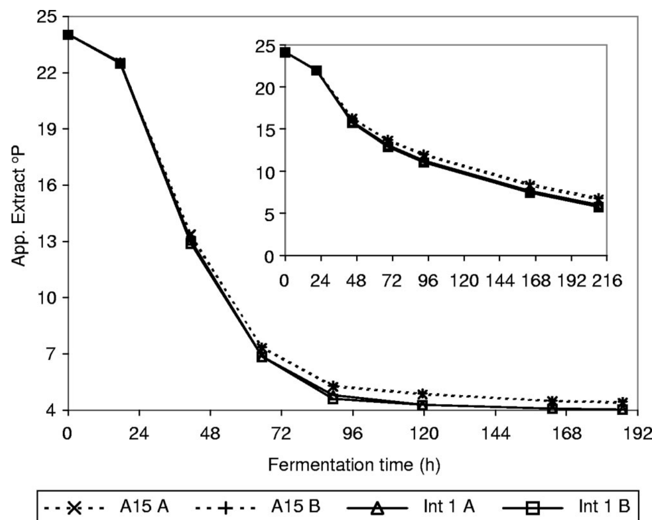

FIG. 6. Effect of cropping and repitching strain A15 and integrant 1. Duplicate growths of strain A15 (A15A, A15B) and integrant 1 (Int 1 A, Int 1 B) were pitched at 8.0 g of fresh yeast mass · liter^{-1} into 24°P wort, each fermentation being performed in duplicate. At 89 h, one of each duplicate pair was stopped and the yeast was cropped as described in Materials and Methods. The attenuation profile of the other duplicate is shown in the main part of the figure. The cropped yeasts were again pitched at 8.0 g of fresh yeast mass · liter^{-1} into 24°P wort, and the attenuation profiles of these fermentations are shown in the inset. App., apparent.

obtained without a change in yeast handling performance (propagation, growth, and sedimentation behavior; Table 5 and Fig. 5) and with little or no change in the levels of yeast-derived volatile aroma compounds (Fig. 7). For at least one transformant (integrant 1), improved fermentation performance compared to the parent strain was still observed when both yeast strains were recycled from one fermentation to the next (Fig. 6), as in normal brewery practice.

The integrants are not expected to contain any non-*Saccharomyces* DNA, and Southern analyses did not reveal any integration of bacterial DNA from the marker plasmid, pKX34, into the chromosomal DNA of integrant 1, 2, or 14. This test cannot exclude the adventitious integration of bacterial sequences too short for hybridization with the probes used. In any case, because the integrants were constructed by methods involving the use of restriction enzymes, it is probably a legal requirement in many countries that beers manufactured with these recombinant yeast strains (or analogous strains constructed by the same method from other brewer's yeast strains) must be labeled to indicate the use of genetically modified organisms.

During selection after transformation, integrant 1 already formed an observable colony on day 2, before integrants 2 and 14 (Table 2). Faster adaptation of integrant 1 to growth on maltotriose in the presence of antimycin was retained after nonselective growth of integrants 1, 2, and 14 through 110 generations (Table 4), so this appears to be a stable phenotype. After growth on glucose, integrant 1 had markedly higher maltose transport activity than integrants 2 and 14 and after growth on maltose it had markedly higher maltotriose transport activity than integrants 2 and 14. Integrant 1 showed somewhat greater improvements in VHG wort fermentation

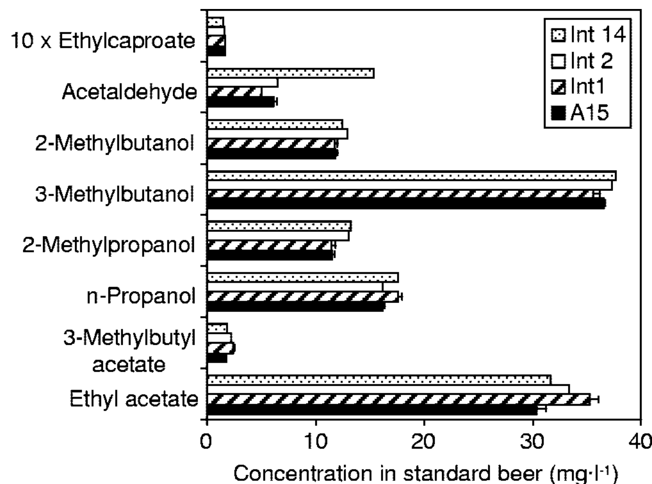

FIG. 7. Volatile aroma compounds in beers from series A 24°P fermentations. Results are normalized to a standard beer containing 35 g of ethanol · liter^{-1}. Error bars for integrant (Int) 1 and strain A15 show the range between the average and highest values for duplicate fermentations with independently grown yeast lots.

behavior than integrants 2 and 14 (Table 5). All three integrants contained both native and recombinant versions of *AGT1* and apparently more copies of the recombinant version than the native version (Fig. 2). Furthermore, none of these integrants contained tandem insertions of the cassette DNA (Fig. 2). Thus, whereas the physiological properties of these three integrants during the selection procedure and during wort fermentation seemed to correlate with their α-glucoside transport activities after growth on glucose or maltose, these transport activities did not, apparently, correlate with the number of repaired copies of *AGT1*. We have no clear explanation for the superior performance of integrant 1 compared to that of integrants 2 and 14. We cannot exclude the possibility that some of the selected clones, including integrant 1, carry other mutations in addition to repair of one or more copies of *AGT1*. Furthermore, if, as seems likely, strain A15 has at least three nonidentical chromosomes VII, the repaired *AGT1* genes of integrants 1, 2, and 14 may be on different versions of chromosome VII and these versions may exhibit different transcription behavior, perhaps resulting from different chromatin structures. *MAL* loci are located in telomeric regions that are known to be sensitive to chromatin modifications (15). It may also be relevant that sequencing of seven independent 2.3-kb PCR fragments extending from the new *PGK1* promoter to nucleotide 1825 of the *AGT1* ORF of integrant 1 suggested that this integrant contains a copy of *AGT1* driven by the *PGK1* promoter but still containing the premature stop codon at nucleotide 1183, in addition to two or more copies of repaired *AGT1*. However, there is evidence that the truncated Agt1 protein encoded by the native *AGT1* gene of strain A15 is not itself an active transporter (37).

Transcription analyses (Fig. 3) showed that integration of the cassette DNA did not alter the expression of other maltose transporter genes (*MTT1* and *MALx1*) or the maltase gene (*MALx2*) but caused increased expression of *AGT1* in integrants 1, 2, and 14 compared to that in strain A15 both during batch growth in the presence of glucose, maltose, or maltotriose and in the subsequent stationary phase. Possible differences between integrants in the expression level of *AGT1* were observed (expression was lower in integrant 2 than in integrants 1 and 14 in samples harvested at about 7 g of sugar $\cdot$ liter^{-1} and higher in integrant 14 than in integrants 1 and 2 in stationary-phase samples) but were close to the experimental error. A low level of expression of *AGT1* was observed in strain A15, which was independent of the sugar used (glucose, maltose, or maltotriose). In earlier work, expression of *AGT1* could not be detected in strain A15 by Northern analysis during batch growth on glucose, maltose, or glucose-maltose mixtures (36). With a different TRAC probe (complementary to nucleotides 1358 to 1388 of the *AGT1* ORF), constant but weak expression of *AGT1* was detected during wort fermentations by another lager strain (28).

After growth on glucose (which represses the endogenous α-glucoside transporter genes), the integrants showed an absolute increase in maltose transport capacity, compared to that of strain A15, of up to 8 U $\cdot$ g of dry yeast^{-1} (for integrant 1), but after growth on maltose (which induced the endogenous genes), the absolute increase was smaller (only 2 U $\cdot$ g of dry yeast^{-1} for integrant 1). Also during wort fermentations, although higher maltose transport activity was observed in integrants than in strain A15 at 20 h, while glucose was still present, the absolute difference between integrants and the parent strain was smaller later in the fermentation (Table 5). Several mechanisms intervene between α-glucoside transporter genes and α-glucoside transport activity. According to the classical model (9, 16, 19, 25), these genes are induced by maltose and maltotriose and repressed by glucose in laboratory yeasts growing at relatively low sugar concentrations (ca. 20 g $\cdot$ liter^{-1}), and similar observations have been reported for lager strains (including A15) under such conditions (see, e.g., reference 36). However, Rautio et al. (28) found that during VHG wort fermentations by lager strains, expression of the *MALx1*, *MALx2*, and *MTT1* genes started to be induced while glucose concentrations were still high (25 g $\cdot$ liter^{-1}), reached a maximum at about the time maltose started to be used, and then rapidly decreased while maltose and maltotriose levels were still high. The cause of this decrease in expression is not known. The recombinant *AGT1* genes in integrants 1, 2, and 14 are driven by the constitutive *PGK1* promoter, and so *AGT1* mRNA is expected to be expressed in the presence of glucose. With glucose-grown cells harvested at about 7 g of glucose $\cdot$ liter^{-1}, we found much higher expression of *AGT1* in integrants 1, 2, and 14 than in host strain A15 (but no expression of *MTT1*, *MALx1*, or *MALx2* in any strain; Fig. 3). In laboratory strains, maltose transporters are subject to glucose-triggered catabolite inactivation and this proteolytic process is related to the presence of PEST sequences in the N-terminal cytoplasmic domains of some maltose transporters (22). Such sequences are not found in Agt1 and Mphx transporters (5). However, nothing is known about how catabolite inactivation affects the stability of Agt1 transporters in brewer's yeast strains. It is also not known how the expression of these recombinant genes might impact on mechanisms regulating the expression of the endogenous genes, such as the above-mentioned decrease in the expression of these genes while maltose and maltotriose are still present. A further relevant factor is that space in the plasma membrane is limited, so that when the recombinant *AGT1* genes and endogenous transporter genes are simultaneously expressed, the transporters synthesized may compete with each other for membrane space. In this case, some molecules of endogenous maltose transporters might be replaced by Agt1 molecules without much increase in the total number of maltose transporter molecules per unit of membrane. This hypothesis is supported by the finding that after growth on YP containing 20 g $\cdot$ liter^{-1} maltose, there was only a modest (ca. 10% for integrant 1) increase in maltose transport activity but a larger increase in maltotriose transport activity (2.5-fold for integrant 1), which is consistent with the replacement of transporters with high specificity for maltose (e.g., Malx1 transporters) by the broader-specificity Agt1 transporters. Similar findings have been reported by other workers: overexpression of *AGT1* increased maltotriose transport activity threefold but slightly decreased maltose transport activity (33), whereas specific deletion of *AGT1* abolished maltotriose transport activity but caused a nearly 20% increase in maltose transport activity (1). To improve our understanding of how to increase transport activities (which is relevant to most biotechnological operations), it is evidently necessary to learn more about the processes that may limit the successful insertion of transporters into the plasma

membrane, including possible competition between different proteins for space in the membrane.

The selection strategy (growth of glucose-repressed cells on maltotriose in the presence of the respiratory inhibitor antimycin A) was exceptionally effective; e.g., all 36 selected clones that were tested contained a *PGK1* promoter between the promoter and ORF of the *AGT1* gene. Both lager and baker's yeast strains have difficulty adapting from growth on glucose to growth on maltotriose when respiration is inhibited (20, 40), and for strain A15 this difficulty seems to be greater than for some other lager strains (6). In principle, the integration cassettes used to transform lager strain A15 should work in the same way with other lager strains, since all of the lager strains studied contain the same premature stop codon in the ORFs of their *AGT1* genes (36, 37) and both of the lager strains studied have identical *AGT1* promoter sequences (V. Vidgren, M. Kankainen, J. Londesborough, and L. Ruohonen, unpublished results). The *AGT1* promoters of ale yeasts are essentially identical to those of lager yeasts from nucleotide −1 to nucleotide −564 (Vidgren et al., unpublished), so that transformation of ale yeasts with the short cassette is expected to put their *AGT1* genes (which do not contain the premature stop codons found in lager strains) under the control of the constitutive *PGK1* promoter. This tactic might increase the maltose and maltotriose transport capacity and fermentation performance of ale yeasts, but this has not yet been tested.

This work was started before the discovery of the *MTT1* genes. These genes encode α-glucoside transporters, for which maltotriose is the preferred substrate rather than maltose (30). They have been found in all of the lager yeasts tested but not, so far, in ale yeasts (30, 37). The Mtt1 transporters function better than Agt1 transporters at the low temperatures (≤10°C) characteristic of traditional lager fermentations (37), whereas in present industrial practice lager fermentations with high-gravity worts are often conducted at higher temperatures (14 −18°C), as was also done in this work. The success of the present work, however, suggests that putting also the *MTT1* genes of lager strains under the control of constitutive promoters would be likely to improve their fermentation performance.

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