

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.

Useful terminology

- **Wort** (wert)- un-fermented beer, mostly maltose, maltotriose and glucose (60, 25, and 15%) and traditionally obtained from malted barley.
- **Plato (°P)**- expresses concentration of extracts (sugars) in wort as percent by weight. 11 °P (100 g wort contains 11 g sugar). 11 °P \approx 4.5 ABV, 15 °P \approx 7 ABV, and 24 °P \approx 11 ABV
- **Apparent attenuation**- describes the percent of malt sugar that is converted by yeast (most yeast attenuate 65 - 80%) and is determined by comparing the original and final gravities of the beer.
- **Organoleptic**- refers to any sensory properties of a product, involving taste, color, odor and feel.
- **Ale yeast (*Saccharomyces cerevisiae*)**- original brewer's yeast, top fermenting, warmer temps. 65-70 °F (18-21 °C). Think Sierra Nevada American Ale
- **Lager yeast (*Saccharomyces pastorianus*)**- natural hybrid of *S. cerevisiae* and *S. bayanus*, bottom fermenting, cooler temps. 50-55 °F (10-13 °C). Think Sam Adams Boston Lager
- **Allopolyploid**- organism with two or more genomes
- **Aneuploid**- unusual number of genes in the same organism

Introduction

- In traditional brewing 11°P wort is used. 17 °P worts are being used, and there is efforts to use very high gravity (VHG) wort.
- Advantages: increased production capacity (fermentation, storage). Saves energy, labor, water, and cleaning, therefore capital costs. Final beer diluted to a gravity of 7-11°P.

Introduction

- Glucose is used first, and is transported by facilitated diffusion
- 85 % of sugars in wort are maltose and maltotriose, which are transported by proton symporters and expression of these transporters is tightly regulated
- Maltotriose is the last one to be consumed and residual maltotriose is sometimes undesirable

Introduction

- *MALx1* genes (x=1 to 4 and 6) encode maltose transporters
- *AGT1* a gene 57% identical to *MAL11*. High activity towards maltotriose as well as to maltose (K_m 4-5)
- If *AGT1* is deleted, maltose is transported, but maltotriose is not
- Overexpression of *AGT1* in multicopy plasmid increase maltotriose uptake and fermentation
- Sequence of *AGT1* from eight lager strains contain a one nucleotide insertion resulting in a premature stop codon encoding a truncated non-functional 394 aa polypeptide vs. a full length 616 aa transporter
- In all ale yeast strains *AGT1* encodes for the full length transporter

Objectives

- Confirm that maltose and maltotriose transport has high level of control over rate and extent of wort fermentation
- Create a GM lager strain with improved fermentation performance containing only *Saccharomyces* DNA

Hypothesis

- Repairing defective *AGT1* genes of lager strains with DNA sequences from ale strains will accelerate fermentation and decrease residual maltotriose levels

Characterization of transformants

- Integration cassettes to repair one or more copies of *AGT1* gene in the lager strain, A15, under the control of a constitutive *PGK1* promoter of *S. cerevisiae*
- *AGT1* promoter was sequenced from A15
- *PGK1* promoter and terminator came from a plasmid
- *AGT1* from ale strain was PCR amplified

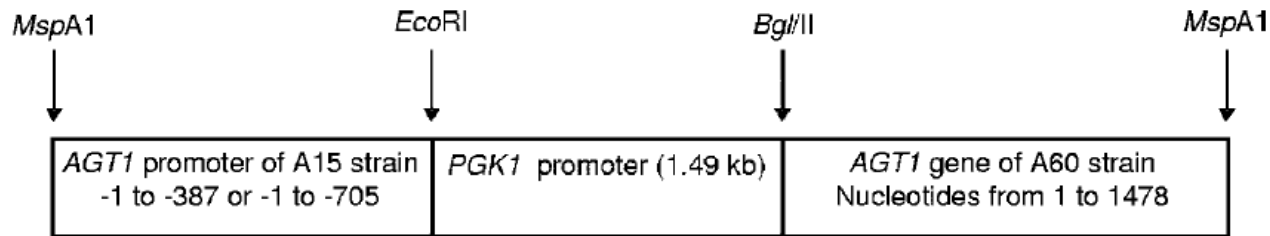


FIG. 1. Diagram of the integration cassettes used in this study. The short and long cassettes contain, respectively, 387 and 705 nucleotides of the *AGT1* promoter from strain A15.

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%

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 \cdot liter⁻¹ and washed with water. Half were plated onto YP containing glucose (20 g \cdot liter⁻¹) and G418 (200 mg \cdot liter⁻¹), and half were plated onto YP containing maltotriose (20 g \cdot liter⁻¹), G418 (200 mg \cdot liter⁻¹), and antimycin A (3 mg \cdot liter⁻¹). Plates were incubated at 30°C, and total colonies were counted each day.

- 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 . Transformants containing a constitutively expressed maltotriose transporter gene were expected to start growing sooner.
- There was no clear difference between the long and short forms of the cassette in the number of colonies recovered.

Confirmation of integration

- Southern blot analysis with the *AGT1* probe was applied to 36 colonies recovered on maltotriose-antimycin AG418 plates
- In 30 colonies, contained both an endogenous *AGT1* gene and a transformed *AGT1* gene with a *PGK1* promoter
- Six colonies tested contained only the transformed *AGT1* gene (and *PGK1* promoter?)
- Verified by PCR that the cassette had integrated into the endogenous *AGT1* locus
- Confirmed by that integration had occurred 3' to the frameshift of *AGT1* of A15

Confirmation of integration

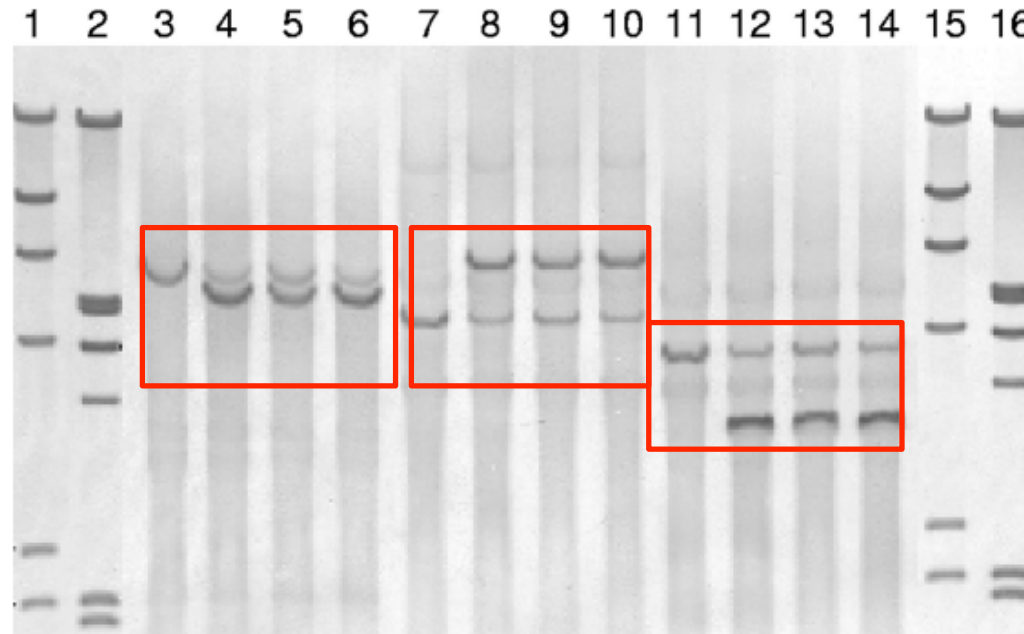
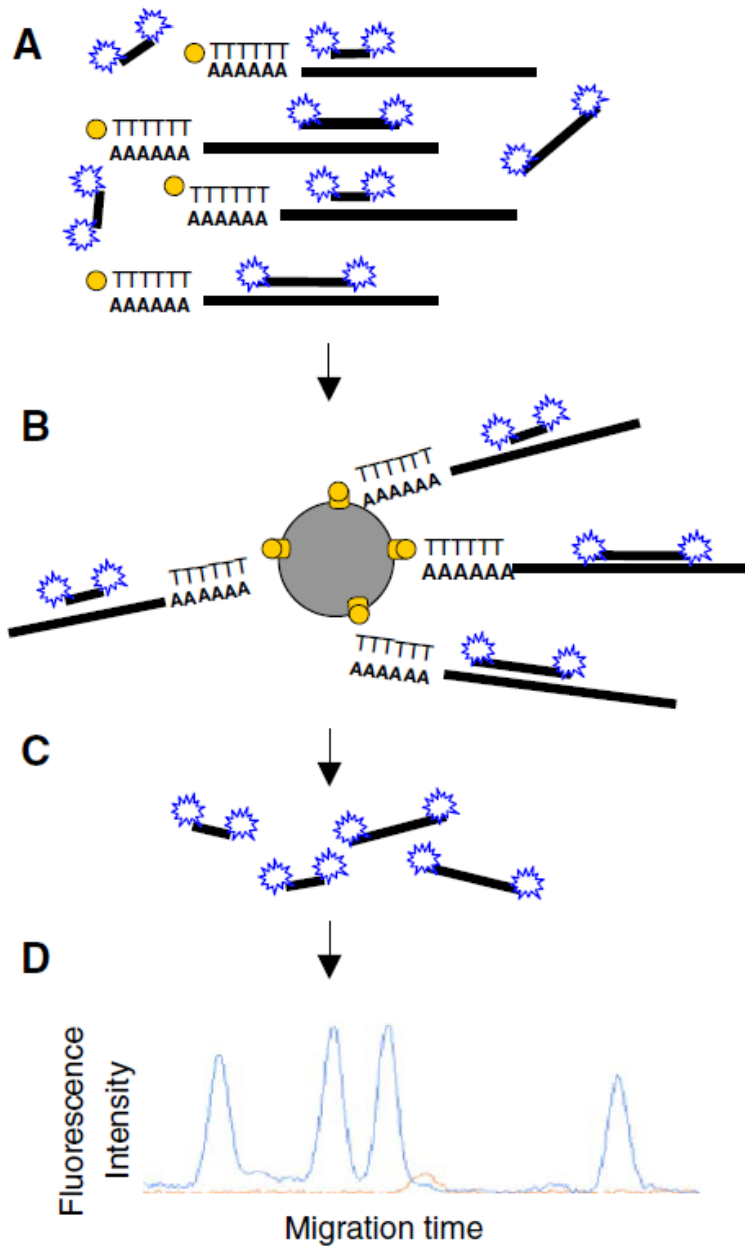


FIG. 2. Southern analyses of strain A15 and integrants 1, 2, and 14. Lanes 1 and 15, molecular weight marker II (23,130, 9,416, 6,557, 4,361, 2,322, and 2,027 bp); lanes 2 and 16, molecular weight marker III (21,226, 5,148, 4,973, 4,268, 3,530, and 2,027 bp); lanes 3, 7, and 11, strain A15; lanes 4, 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 *AGTI* PCR fragment. The band sizes (in kilobases) predicted from the SGDB sequence of native *AGTI* 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).

Transcriptional analysis

- Three integrants were selected (1, 2 and 14)
- Cured of plasmid pKX34
- Absence of bacterial DNA confirmed by Southern blot
- All integrants had at least one native and one (maybe more) repaired *ATG1* loci
- Expression of genes of interest was measured using TRAC (transcript analysis with aid of affinity capture)



- total RNA preparation or cell lysate is hybridized simultaneously with a biotin-labeled oligo(dT) and a pool of gene-specific detection probes labeled with a fluorescent dye at 3' and 5' ends (Fig. 1A)
- mRNA bound to the oligo(dT) is captured with streptavidin coated magnetic beads and nonspecifically bound detection probes with other sample material are washed off (Fig. 1B)
- The specifically bound detection probes are eluted from the beads (Fig. 1C) and detected in capillary electrophoresis (Fig. 1C)
- Each detection probe, i.e. mRNA under study, is distinguished among the pool by its size and quantified based on the area of the peak (Fig. 1D)

Rautio, et al., 2006

Apparent expression levels

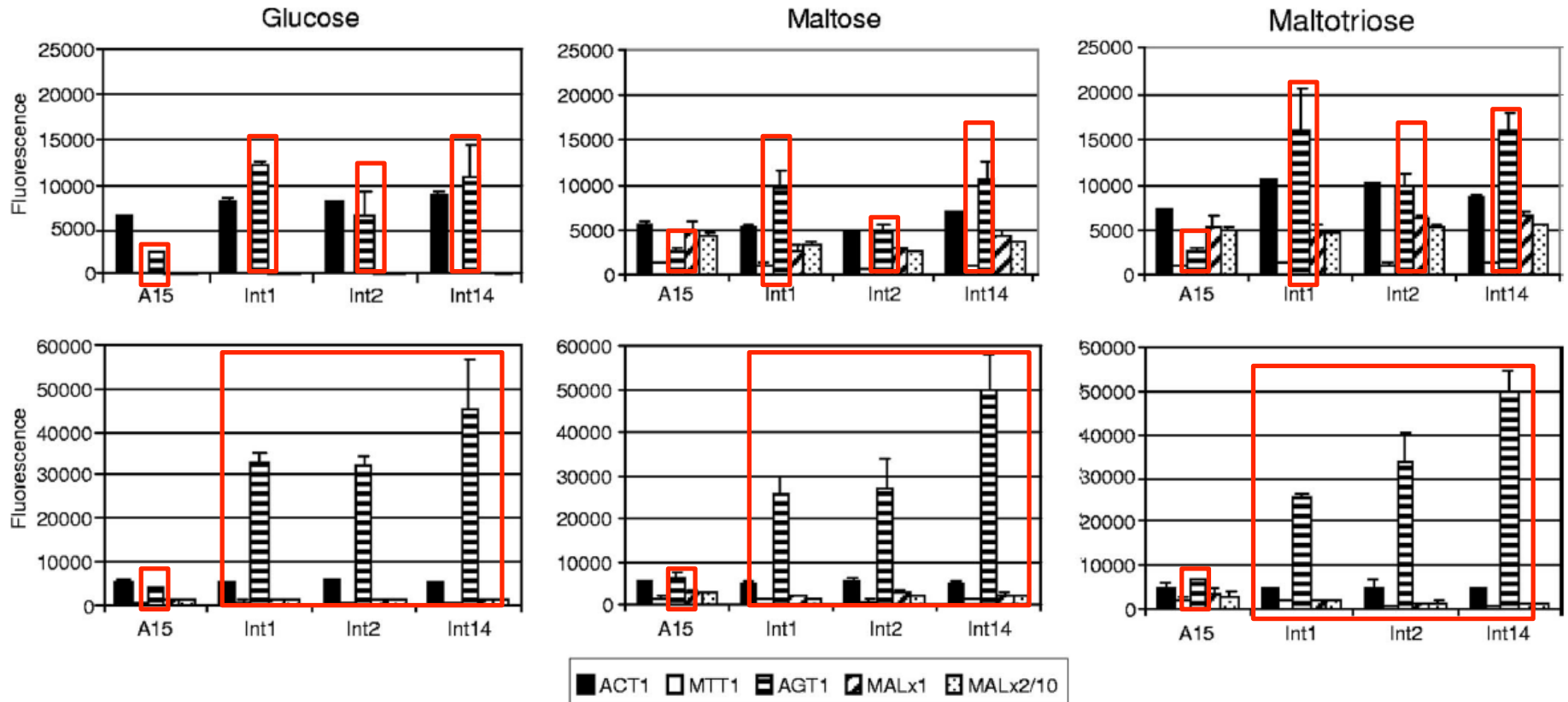


FIG. 3. Expression of *AGT1* and other α -glucoside transporter and maltase genes during batch growth of strain A15 and integrant 1 (Int1), Int2, and Int14 on $20 \text{ g} \cdot \text{liter}^{-1}$ glucose, maltose, or maltotriose at 18°C . Yeast samples were collected after 13 h (upper row), while sugars were still present at about $7 \text{ g} \cdot \text{liter}^{-1}$, 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 \pm ranges of data from two replicate growths of each yeast strain on each sugar.

α -glucoside transport capacity of integrants

- Maltose and maltotriose transport activities were measured in cells of each strain (grown on glucose) harvested at 13 h
- Strain A15 cells exhibited little or no activity (expected, no functional *AGT1*) (they don't say about the integrants)
- Grown on glucose, harvested at near stationary: The maltose transport capacity of strain A15 was 1.6 U/g of dry yeast, whereas integrants 1, 2, and 14 exhibited 10.3, 6.6, and 5.1 U/g of dry yeast
- If grown on maltose, no difference in maltose transport (18, 20, 21, 22 U respectively)
- But maltotriose transport increased from 3.4 U (A15) to 8.5, 5.1, and 5.4 U in integrants 1, 2, and 14

Genetic stability

- Strain A15 and integrants 1, 2, and 14 were cycled 14 times on glucose
- Yeast suspensions were diluted and spread on nonselective (20 g of glucose/liter¹) and selective (20 g of maltotriose plus 200 mg antimycin A/liter¹) plates
- Colonies picked, Southernns performed, obtaining same results as before, therefore integrants were genetically stable through 110 generations in non-selective media

Genetic stability

TABLE 4. Genetic stability of integrants^a

Strain and vol (μ l)	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	
Integrand 1							
25	45	47	0	26	43		
50	95		0	80	104		
100	170		0		186		
Integrand 2							
25	48	46	0	9	43		
50	109		0	44	99		
100	177		0		150		
Integrand 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 OD₆₀₀ 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.

Fermentation of High Gravity Wort

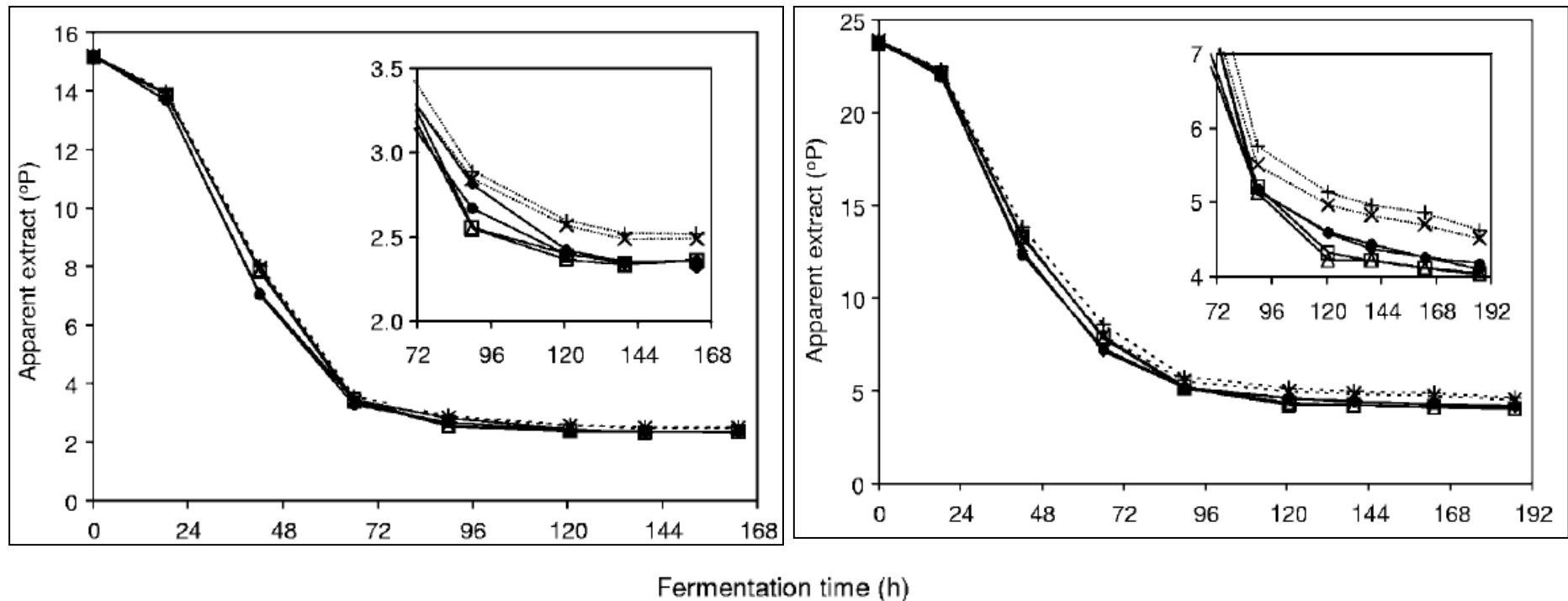
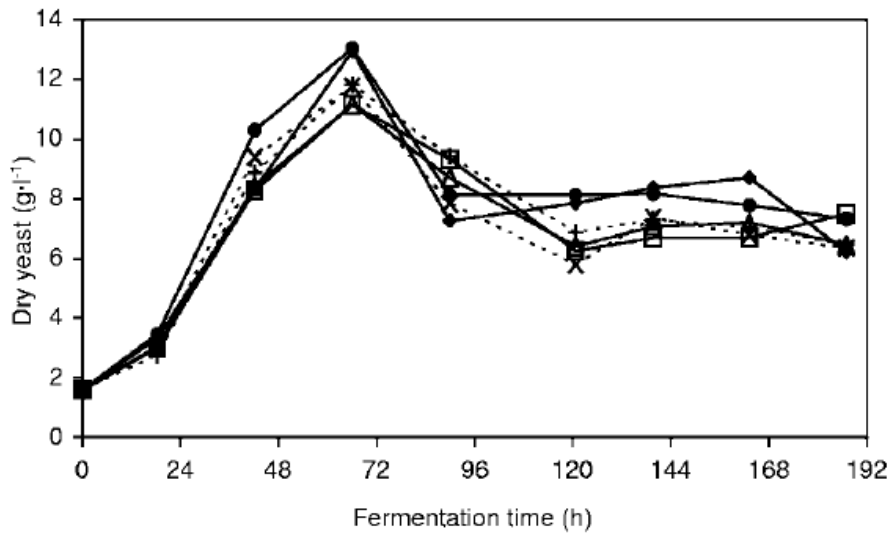
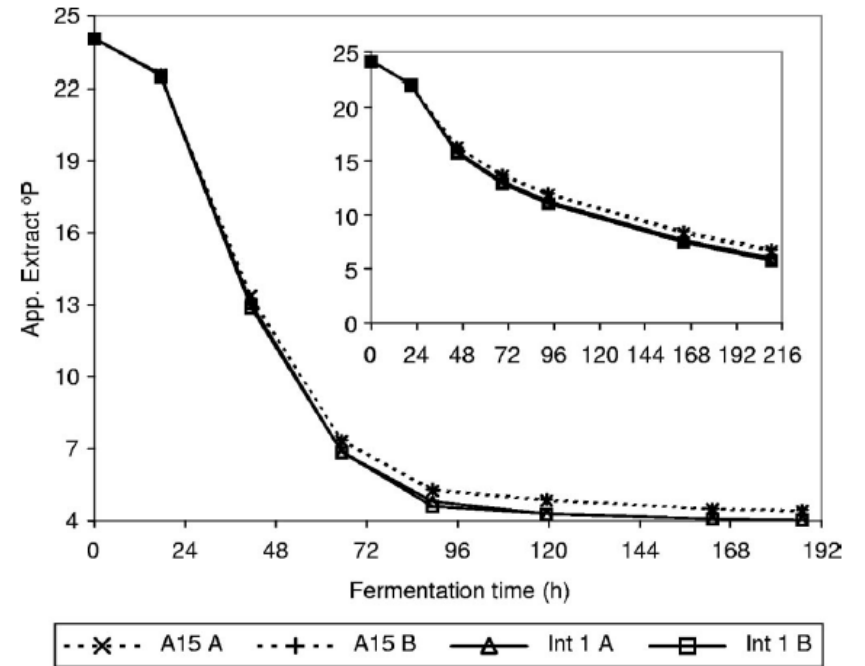
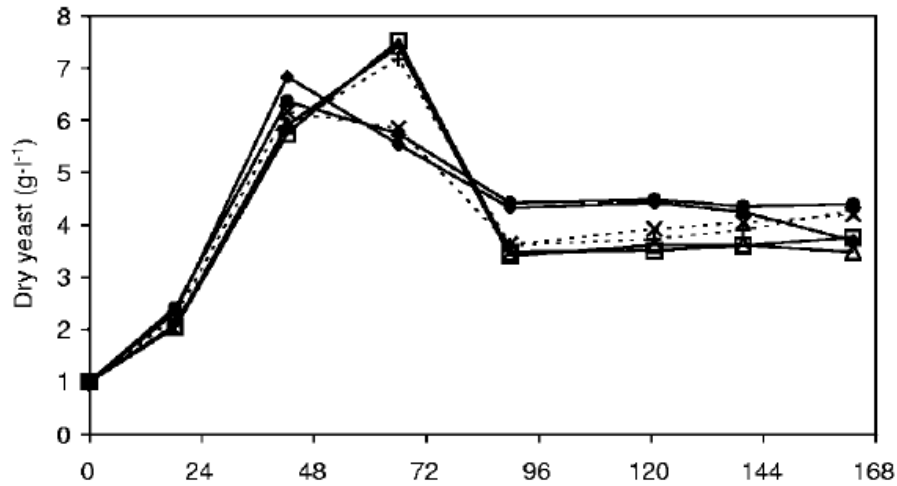


FIG. 4. Attenuation profiles during the fermentation of 15°P (upper panel) and 24°P (lower panel) worts by duplicate growths of strain A15 (A15A, A15B), duplicate growths of integrant 1 (Int 1 A, Int 1 B), and single growths of integrants 2 and 14 (Int 2, Int 14). The 15 and 24°P worts were pitched with, 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.

TABLE 5. Wort fermentations by A15 and three integrants^a

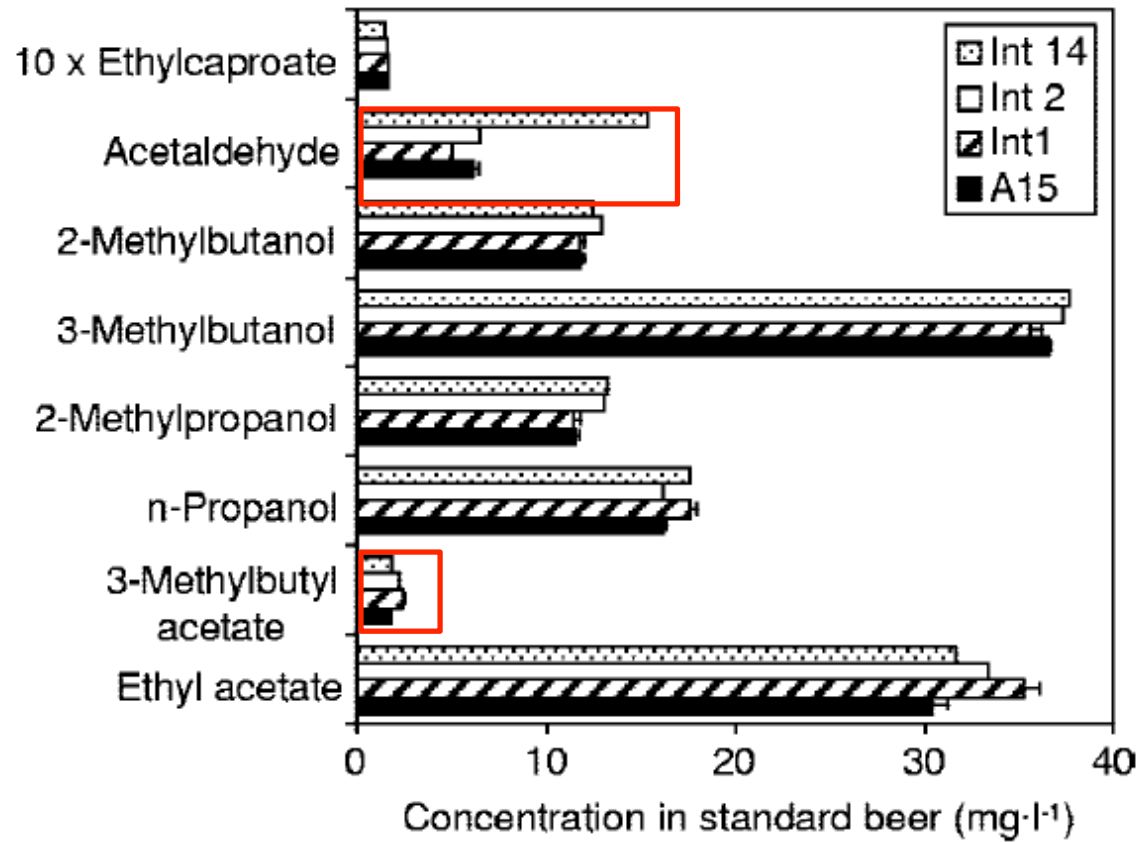
Parameter	A15	Integrant 1	Integrant 2	Integrant 14
Series A pitching yeast viability (%)	99.4 ^c	98.2 ± 0.2	97.9	98.3
Series A 15°P fermentation				
Final AE (°P)	2.50 ± 0.02	2.36 ± 0.00	2.31	2.36
Extra AE (°P)		0.14	0.19	0.14
Time to 80% AA (AE = 3.0°P) (h)	83.1 ± 1.5	76.7 ± 0.9 ^b	81.1	75.2
Time saved 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

Growth Profiles



---x--- A15 A ---+--- A15 B —△— Int 1 A —□— Int 1 B —◆— Int 2 —●— Int 14

Organoleptic Profiles



Conclusions

- Ale-constitutive *ATG1* maltose transporter improves maltose and maltotriose transport and utilization/fermentation
- Speeds up fermentation time
- Improves ethanol yields and lower apparent final extract
- Does not affect organoleptic qualities in beer
- No bacterial DNA is used
- Lager Mtt1 has higher affinity for maltotriose than maltose. Putting *MTT1* under constitutive promoter may improve maltotriose fermentation in high gravity worts

Beer is proof that God loves us and
wants us to be happy!- Ben Frankin

Questions?

Effect of alcohols on inactivation of the maltose and galactose transporters Luceroetal1997.

Cells were harvested during exponential growth with maltose or galactose (about 0.7 mg [dry weight]/ml), washed, and suspended in 3 volumes of an ammoniumfree medium as described previously (2), with 2% glucose and 250 mg of tetracycline hydrochloride per ml to avoid bacterial contamination and in the absence or presence of alcohols at the indicated concentrations. After incubation at 30°C in a rotatory shaker (200 rpm) for the indicated times, inactivation was monitored by measuring the activity of the transporters by using their labeled substrates (4, 28). The cells were harvested, washed, and suspended to a cellular density of 40 mg (dry weight) of yeast/ml in 0.1 M tartaric acid adjusted to pH 4.2 with Tris in the case of the maltose transporter or 50 mM K₂HPO₄ (pH 6.0) in the case of the galactose transporter. Aliquots of 50 ml, containing 2 mg (dry weight) of yeast, were added to 5 ml of 45 mM labeled maltose or galactose (0.5 mCi/mmol), respectively, and incubated at 20°C for 15 s. Sugar uptake was stopped by the addition of 10 ml of chilled water. After rapid filtration, the cells and filters were washed with 10 ml of chilled water and immediately submerged in liquid scintillation cocktail, and the radioactivity was counted.