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Cloning of a New Allelic Variant of a *Saccharomyces diastaticus* Glucoamylase Gene and Its Introduction into Industrial Yeasts

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ABSTRACT

A new allelic variant of the *STA2* gene, designated as $STA2^K$, coding for a secreted glucoamylase, was cloned. Differences were revealed both in the structural gene and in the promoter region, as compared to other *STA* genes. The most peculiar structural features of $STA2^K$ are 1. a 1.1-kb natural deletion in its promoter located 189 nucleotides upstream of the translation start codon; and 2. an Asn→Asp single amino acid change within the putative active site of the encoded glucoamylase. Neither the presence of glucose in the medium nor the host cell's mating type constellation affected the expression level of $STA2^K$ in *S. cerevisiae.*

Self-replicating yeast plasmids containing $STA2^K$ were constructed and used to transform a laboratory yeast strain and various brewing strains. Pilot brewing tests with glucoamylase-secreting transformants of a brewing strain produced superattenuated beers at accelerated fermentation rates.

Index Entries: Gene expression; starch hydrolysis; glucoamylase; *Saccharomyces diastaticus; STA* genes; promoter; engineering of brewing yeast.

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INTRODUCTION

Conventional brewing yeast strains are capable of fermenting glucose, fructose, sucrose, maltose, and maltotriose, leaving oligosaccharides larger than maltotriose, known as dextrins, unfermented (*1*). The dextrins that remain in solution comprise a large fraction of the caloric content of beer produced by the traditional process (*2*). Currently there is high demand for low-calorie or "light" beer, in which the carbohydrate content has been substantially reduced. In current practice this is usually accomplished by adding fungal or bacterial glucoamylase to the mash to increase the degree of dextrin hydrolysis. An alternative to this procedure is to introduce a gene for glucoamylase secretion directly into the genome of brewing yeast strains. Such a transformed amylolytic brewing strain can ferment residual wort dextrins directly, sparing or eliminating the cost of adding microbial enzymes during the mashing process.

The yeast *Saccharomyces diastaticus,* first described by Andrews and Gilliland (*3*), was isolated from superattenuated beer. Superattenuation, or lowering of specific gravity, was found to be caused by dexrin hydrolysis resulting from action of glucoamylase secreted by the yeast. A number of *S. diastaticus* strains have been isolated. (*See review* by Laluce and Mattoon (*4*)). Because *S. cerevisiae* and *S. diastaticus* strains can be hybridized readily (*4,5*) they can be considered to be varieties of the same species. Glucoamylase secretion by *S. diastaticus* or hybrid *Saccharomyces* strains is determined by the presence of one or more unlinked glucoamylase structural genes in *S. diastaticus* (*5–9*). Three such genes, *STA1, STA2,* and *STA3* have been identified and cloned (*5–7,10,11*). The nucleotide sequences of the *STA1* and *STA2* genes have been determined (*11,12*). The structural features of the *STA* genes are compiled in Fig. 1.

Because brewing yeasts (*Saccharomyces cerevisiae* or *S. uvarum*) are also genetically very closely related to *S. diastaticus,* the latter yeast was chosen as a source of glucoamylase structural gene for introduction into brewing strains. By employing a *STA* gene of *Saccharomyces* instead of a glucoamylase gene from some other fungal or bacterial source, the modified brewing strains described here contain DNA derived exclusively from yeast. In fact, this approach can be further developed to exclude bacterial vector sequences completely (*13*).

The present work describes the cloning of a *STA* gene from a genomic library of *S. diastaticus* DNA in the yeast-*E. coli* shuttle vector YEp13. A particular *S. diastaticus* strain (CL9) was selected as the source of the *STA* gene by virtue of its unusually high level of secreted glucoamylase production. The physical map and DNA sequence of the isolated gene, designated as $STA2^K$, exhibits differences from other *STA* genes. These differences, in turn, might contribute to the very high level of glucoamylase production by the particular *S. diastaticus* strain CL9. In *S. cerevisiae* transformants the presence of glucose in the medium had virtually no effect on the expression level of secreted glucoamylase from $STA2^K$,

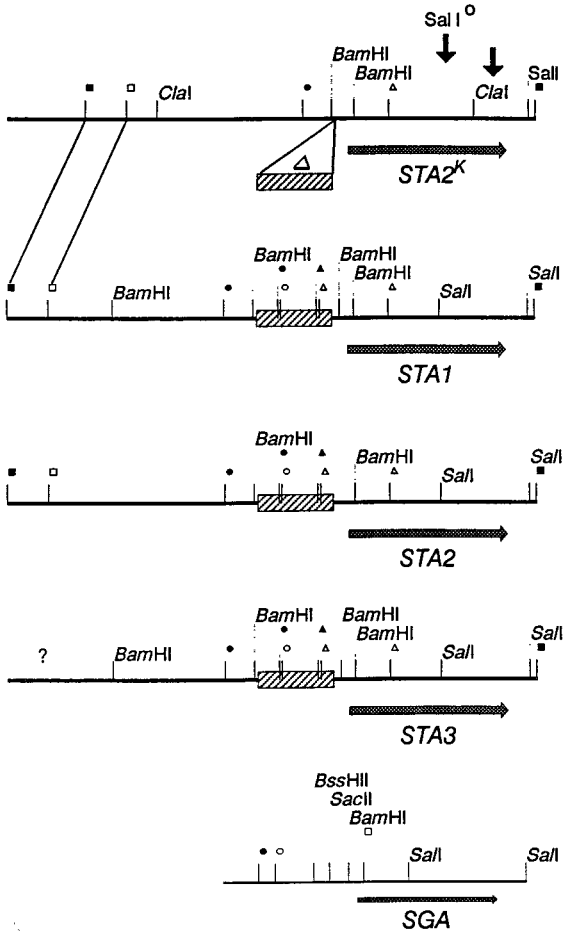

Fig. 1. Structural features of the *STA2*^{K} locus compared to other *STA* genes of *Saccharomyces diastaticus*. Distinctive restriction sites (*SalI*, *ClaI*) are marked with arrows; the hatched region of the upstream sequence of *STA1*, *STA2*, and *STA3* corresponds to a deletion in the promoter of *STA2*^{K} (Δ). (The map data have been compiled from refs. *7*, *11*, *12*, *27*, *30*). Symbols used for restriction sites: ■-*Bgl*II, Δ-*Bst*EII, ○-*Hpa*I, ●-*Nhe*I, ▲-*Xba*I, □-*Xho*I. (The map positions of all indicated restriction sites in *STA2*^{K} have been confirmed by restriction analysis.)

whereas a significant repression of *STA2* expression was observed under similar conditions. In diploid (MAT**a**/α) cells the level of *STA2*^{K} expression was also higher than that of *STA2*.

The *CUP1* gene (*14–16*) was introduced into the self-replicating *STA2*^{K} expression vectors and used as a dominant marker to transform several brewing strains. It has been shown that *CUP1* had no effect on fermentation characteristics or beer quality (*17,18*). Stable yeast transformants producing high levels of the secreted glucoamylase were obtained using a "nonshuttle" YEp vector lacking bacterial plasmid sequences. In pilot

brewing tests a glucoamylase secreting transformant was shown to produce high quality beer at an accelerated fermentation rate and acceptable levels of plasmid retention.

MATERIALS AND METHODS

Strains

E. coli C600SF8 (F⁻ *thr-1 leuB6 thi-1 lacY1 supE44 rfbD1 fhuA21 mcrA1*) and DH5αF′ (*F′*/endA1 hsdR17 ($r_K^- m_K^+$) *supE44 thi-1 recA1 gyrA* (*Nal*^{r}) *relA1*Δ(*lacZYAargF*)*U169*(ϕ*90dlac*Δ(*lacZ*)*M15*) of Bethesda Research Laboratories (Gaithersburg, MD) were used for propagation and amplification of plasmid DNA. *S. cerevisiae* strains: MA24-RS21 (*MAT***a** *ade1 leu2-3 leu2-112 hem1 can*^{r}) was from our collection, RH17/3d (*MAT***a** *leu2-3 leu2-112 his2 cup1*), that contains a single copy of the copper methallothionein gene *CUP1*, and is sensitive to inhibition by low concentrations of Cu^{++} (0.3 m*M*) in a minimal medium, was provided by T. Butt (Smith, Kline and French, Philadelphia, PA), and strain 2180 (*MAT***a** *CUP1*), containing multiple copies of the *CUP1* gene, being consequently resistant to relatively high concentrations of Cu^{++} (3 m*M*) in minimal media, was a gift from S. Fogel (University of California, Berkeley, CA). Yeast strain DBY-746 (*MATα his3-dl leu2-3, 2-112 ura3-52 trpl-289a*) was from ATCC (No. 44773). The **a**/α diploid strain YGB-27 was constructed in our laboratory by crossing two haploid strains:

MATα his3 leu2 ura3 trp1 + *MAT***a** + *leu2 ura3 trp1 met1*

The brewing yeast strains *S. uvarum* BY1-BY7 and were from Sun Y. Lee (Coors Brewing Co., Golden, CO), brewing strain SB was from the Schaeffer's Brewery, and strain KPM (*S. uvarum*) was from our collection. Strains CL9 (*STA1, STA2*^{K}) and J3120 (*STA1, STA2, STA3*) of *Saccharomyces diastaticus* were received from C. Laluce.

Plasmids

YEp13 is an *E. coli*-yeast shuttle vector carrying *LEU2*, the replication origin of the yeast 2μ plasmid and pBR322 sequences (*19*). YEp36, a YEp13 derivative that carries the copper chelatin gene *CUP1* (*15*), was kindly supplied by T. Butt of Smith, Kline and French Laboratories, Philadelphia, PA. Plasmid pUC1813:*CUP1* containing a 2-kb *Kpn*I fragment of the *CUP1* locus (*14,16*) was constructed by K. Josvay and G. Bajszar (Vepex-Biotechnika Ltd., Szeged, Hungary). The plasmid pGEM7Zf$^+$ is from Promega, Madison, WI. A plasmid carrying the *STA2* gene was kindly provided by Marius Lambrechts (Albert Einstein College of Medicine, Bronx, NY). The construction of all *STA*-gene-containing vectors used or cited in this work is shown in Fig. 3 later in this article.

Media

Complete yeast growth medium contained 1% Difco yeast extract (Y) 2% Difco peptone (P), and an appropriate carbon source. YPD medium contained 2% dextrose (D). YP-D1S3 medium contained 1% dextrose and 3% Lintner soluble potato starch (Sigma Chemical Co.). Buffered starch medium BYPS contained 0.1*M* succinic acid, 1% yeast extract (Y), 2% peptone (P), and 2% Lintner starch (S). The pH of buffered media was adjusted to 4.2. NEP medium (10) contained 0.2% $MgSO_4 \cdot 7H_2O$, 0.2% $(NH_4)_2SO_4$, 0.3% KH_2PO_4, 0.025% $CaCl_2 \cdot 2H_2O$, 0.2% Difco yeast extract, 0.3% Difco peptone and an appropriate carbon source. NEPD1 medium contained 1% dextrose; NEP-D1S3 medium contained 1% dextrose and 3% Lintner starch; and NEP-S3 medium contained 3% Lintner starch. The transformants of brewing strains harboring a plasmid containing both *STA* and *CUP1* genes were grown on NEP-D1S3 plus 1 m*M* copper. Selective minimal medium contained 0.67% Yeast Nitrogen Base (Difco, without amino acids), 2% dextrose, and nutritional supplements, as required. YNBCA medium (0.67% Yeast Nitrogen Base, 1% Difco Casamino Acids, 0.01% tryptophan, 1% Na-succinate, and appropriate concentratoins of dextrose and/or Lintner starch) was used to select for and maintain *Ura*$^+$ transformants.

Molecular Cloning and Yeast Transformation

All the procedures involved in plasmid cloning and bacterial transformations were performed as described by Sambrook et al. (*20*) and Asubel et al. (*21*). Yeast cells were transformed according to the method of Finlayson et al. (*22*). For the cloning of a *STA* gene into *S. cerevisiae*, a quasirandom genomic library from *S. diastaticus* strain CL9, known to contain at least 2 *STA* genes (*4*) was constructed using 5–20 Kb DNA fragments, which were obtained from partial digestion with restriction endonuclase *Sau*3A, and ligated into the *Bam*HI site of YEp13. The library, consisting of a population of hybrid vectors, was then used to transform *leu2* auxotrophic *S. cerevisiae* strain MA24-RS21. Transformants carrying the cloned *STA* gene were selected for their ability to complement the *leu2* mutation as well as to hydrolyze starch. The transformants producing glucoamylase formed halos on plates of minimal medium containing 1% dextrose and 3% starch after incubation at 30°C for 4 d followed by refrigeration at 4°C for 2 d. Plasmid DNA from yeast transformants was prepared according to Nasmyth and Reed (*23*).

Mitotic Stability

A diluted sample of cell suspension of a transformed yeast strain harboring a plasmid containing both the cloned *STA2*^{K} and *CUP1*, grown aerobically in 100 mL of BYPS medium, was plated on NEP-D1S3 medium

containing or lacking 1 m*M* copper. After 8 d of incubation, the mitotic stability was calculated according to the following equation:

$$\% = 100 \times \text{No. of colonies on NEPD1S3 + 1 m}M\text{ copper} / \text{No. of colonies on NEPD1S3}$$

Only cells carrying both *STA* and *CUP1* will grow and form halos in the medium containing 1 m*M* copper.

DNA Sequencing

Fragments of the *STA2*^{K} structural gene were subcloned in the plasmid pGEM7Zf$^+$, and DNA sequence determination was carried out using the dideoxy chain-termination method of Sanger et al. (*24*), modified for plasmid sequencing (*25*). The Sequenase™ kit from US Biochemicals was used according to the manufacturer's instructions.

Southern-Type DNA Hybridizations

Chromosomal DNA from *S. diastaticus* was prepared essentially as described by Carle and Olson (*26*). Total nuclear DNA was digested with an appropriate restriction endonuclease (*Bgl*II in these experiments) followed by electrophoretic separation of the digestion products and transfer onto nylon filters (Bio-Rad).

Individual chromosomes of *S. diastaticus* were separated by contour-clamped homogenous electric-field (pulse-field) gel electrophoresis (CHEF-PFGE) in agarose gels using the Hex-A-Field apparatus of BRL. The gels were run in 0.5X TBE buffer (45 m*M* Tris, 45 m*M* boric acid, 1 m*M* EDTA) followed by transfer of the DNA onto Nylon filters (Bio-Rad). Three different *STA2*^{K} probes were prepared by using Boehringer's GENIUS™ chemiluminescent labelling kit, and used for hybridization essentially as described in (*8*).

Glucoamylase Assay

The cell concentration in the yeast culture fluid was adjusted before the preparation of the glucoamylase assay mixture to be 10^{11} cells/mL. The reaction mixture for the enzyme assay contained 0.2 mL of 1.6% Lintner starch, 0.1 mL sodium acetate buffer (1*M*, pH 5.0) and 0.7 mL of centrifuged culture fluid as crude enzyme solution. After a 30-min incubation at 55°C, the reaction was stopped by immersing the reaction tube in a boiling water bath for 10 min. The tube was then cooled, and the glucose content was measured by a glucose oxidase/peroxidase method using a PGO-enzyme kit suppied by Sigma (St. Louis, MO). A reaction mixture containing culture fluid that had been previously heat-treated for 10 min in a boiling water bath, was used as a blank. One unit of enzyme is defined as the amount that liberates 1.0 μmole of glucose/min/mL of enzyme sample.

Aroma Test

Beers resulting from fermentations by transformant and control yeasts were evaluated for aroma characteristics by an experienced flavor profile panel.

Nucleotide Sequence Accession Number

EMBL/GenBank, M90490 (March 23, 1992).

RESULTS

Cloning of a *STA* Gene in a Laboratory Yeast Strain

When *S. creevisiae* strain MA24-RS21 was transformed with the *S. diastaticus* CL9 genomic library (constructed in YEp13/*Bam*HI), 20 halo-forming transformants were obtained from about 45,000 *Leu*$^+$ colonies. The transformant phenotypes of leucine prototrophy and halo-forming ability were always coinherited or were lost coincidentally under non-selective conditions. Total DNA was extracted from the transformed MA24-RS21 cells and used to transform *E. coli* C600SF8. One of the plasmids, YEp(STA)K6, isolated from the *E. coli* transformants contains an insert of 9.9 Kb within the *Bam*HI site of vector YEp13. The restriction map of the 9.9-Kb *STA*-containing fragment cloned into plasmid YEp(STA)K6 exhibits both similarities to and differences from maps reported for other *STA* genes. The obtained *STA* gene variant was subsequently designated as *STA2*^{K}. A compilation of physical maps of the *STA* and *SGA* genes, including that of *STA2*^{K}, is presented in Fig. 1. Restriction maps of coding regions of *STA1* and *STA3* (*11,27*), and of *STA2* (*12*) are almost identical to the map of the coding region of *STA2*^{K}, except that the latter lacks a centrally located *Sal*I restriction site, and contains a *Cla*I site. In contrast, the map of the 3.8-Kb region located upstream of this putative coding region is quite different from the corresponding regions of fragments bearing *STA1, STA2,* or *STA3* or the related sporulation-specific *SGA* gene (*10*). Compared to other *STA* genes, the most distinctive feature of *STA2*^{K} is a 1.1-kbp deletion in its promoter region.

DNA Sequence of the *STA2*^{K} Gene

Sequencing of *STA2*^{K} was carried out on subclones constructed in pGEM7Zf$^+$ vector (the same subclones were used as probes for hybridization to blotted chromosomal DNA; *see below*). The analysis of the DNA sequence (Fig. 2) has revealed an ORF, coding for a protein, which is almost identical to other selected glucoamylases of *S. diastaticus* (*11,12*). As

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-677    GCTAGCG TATCAACCCA TCTCCAGATT TGCCCAGCAT TTCTGTAGAA CACCCAATGC

-620 GAATGAAGAC TAATGAATAT TTTAAAGACA AAAAATAGGA AAAGTTTAAT TATTGATGGT

-560 TCTCACCACC GCAAAACGGT CACTTGCATG CATTGAGGAC ATGCACTGCA GTACTTGTTT

-500 TTCTCGAATG TTTTTTACCA TCTCTTTTTT CCTTATCTGA GCTGCTGACG CAGACGGACA

-440 TTGTTTCATA TTTCGAAAGC TGTGCGGGAA AACGGACCCT AACACGAATG TGGATGCGCT

-380 AATCGGTATT TCCACCTCAT GAAACCTGCT ACTTTTTCAG TGCTCTTCGT ATGCATTTCC

-320 CAAAATTCAT TCGTAGCCTT GTCAACTTAG ACTCAGTCCA CGGCGTGCAG GACGGGGTAT

-260 TATGAATAAA GGATCCACGG GTAAGATTTG TTCTATGTTT TAGGTATGGA GTTTTGTACC

-200 ACAAAACTTT GCAAGAACGA TAACTCATAG ACTTACCTGT ACAAGTTGTT GAAGGGTTCT
                 ↑
-140 CAATTGATAA AAAAGGATCT TTTGCTTCCT AAACTAAACC TATAAAAAGC ACCCTATTCA

 -80 TCAGTTATAA TCTCTTGTCA TGTTGTGGTT CTAATTGAAA ATATACTATG GTAGGCCTCA

                            1
 -20 AAAATCCATA TACGCACACT ATG CAA AGA CCA TTT CTA CTC GCT TAT TTG GTC
                          Met Gln Arg Pro Phe Leu Leu Ala Tyr Leu Val

  34 CTT TCG CTT CTA TTT AAC TCA GCT TTG GGT TTT CCA ACT GCA CTA GTT
     Leu Ser Leu Leu Phe Asn Ser Ala Leu Gly Phe Pro Thr Ala Leu Val

  82 CCT AGA GGA TCC TCC TCT AGC AAC ATC ACT TCC TCC GGT CCA TCT TCA
     Pro Arg Gly Ser Ser Ser Ser Asn Ile Thr Ser Ser Gly Pro Ser Ser

 130 ACT CCA TTC AGC TCT GCT ACT GAA AGC TTT TCT ACT GGC ACT ACT GTC
     Thr Pro Phe Ser Ser Ala Thr Glu Ser Phe Ser Thr Gly Thr Thr Val

 178 ACT CCA TCA TCA TCC AAA TAC CCT GGC AGT AAA ACA GAA ACT TCT GTT
     Thr Pro Ser Ser Ser Lys Tyr Pro Gly Ser Lys Thr Glu Thr Ser Val

 226 TCT TCT ACA ACC GAA ACT ACC ATT GTT CCA ACT ACA ACT ACG ACT TCT
     Ser Ser Thr Thr Glu Thr Thr Ile Val Pro Thr Thr Thr Thr Thr Ser

 274 GTC ATA ACA CCA TCA ACA ACC ACT ATT ACC ACT ACG GTT TGC TCT ACA
     Val Ile Thr Pro Ser Thr Thr Thr Ile Thr Thr Thr Val Cys Ser Thr

 322 GGA ACA AAC TCT GCC GGT GAA ACT ACT TCT GGA TGC TCT CCA AAG ACC
     Gly Thr Asn Ser Ala Gly Glu Thr Thr Ser Gly Cys Ser Pro Lys Thr

 370 ATT ACA ACT ACT GTT CCA TGT TCA ACC AGT CCA AGC GAA ACC GCA TCG
     Ile Thr Thr Thr Val Pro Cys Ser Thr Ser Pro Ser Glu Thr Ala Ser

 418 GAA TCA ACA ACC ACT TCA CCT ACC ACA CCT GTA ACT ACA GTT GTC TCA
     Glu Ser Thr Thr Thr Ser Pro Thr Thr Pro Val Thr Thr Val Val Ser

 466 ACC ACC GTC GTT ACT ACT GAG TAT GCG TCT ACT AGT ACA AAA CAA GGT
     Thr Thr Val Val Thr Thr Glu Tyr Ala Ser Thr Ser Thr Lys Gln Gly

 514 GGT GAA ATT ACA ACT ACA TTT GTC ACC AAA AAC ATT CCA ACC ACT TAC
     Gly Glu Ile Thr Thr Thr Phe Val Thr Lys Asn Ile Pro Thr Thr Tyr
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Fig. 2 (*through page 170*).

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562 CTA ACT ACA ATT GCT CCA ACT TCA TCA GTC ACT ACG GTT ACC AAT TTC
    Leu Thr Thr Ile Ala Pro Thr Ser Ser Val Thr Thr Val Thr Asn Phe

610 ACC CCA CCA CTA TTA CTA CTA CTG GTT TGC TCT TAC AGG ACC AAA TCT
    Thr Pro Thr Thr Ile Thr Thr Thr Val Cys Ser Thr Gly Thr Asn Ser

658 GCC GGT GAA ACT ACC TCT GGA TGC TCT CCA AAG ACT GTC ACA ACA ACT
    Ala Gly Glu Thr Thr Ser Gly Cys Ser Pro Lys Thr Val Thr Thr Thr

706 GTT CCT TGT TCA ACT GGT ACT GGC GAA TAC ACT ACT GAA GCT ACC GCC
    Val Pro Cys Ser Thr Gly Thr Gly Glu Tyr Thr Thr Glu Ala Thr Ala

754 CCT GTT ACA ACA GCT GTC ACA ACC ACC GTT GTT ACC ACT GAA TCC TCT
    Pro Val Thr Thr Ala Val Thr Thr Thr Val Val Thr Thr Glu Ser Ser

802 ACG GGT ACT AAC TCC GCT GGT AAG ACG ACA ACT AGT TAC ACA ACA AAG
    Thr Gly Thr Asn Ser Ala Gly Lys Thr Thr Thr Ser Tyr Thr Thr Lys

850 TCT GTA CCA ACC ACC TAT GTA TTT GAC TTT GGC AAG GGC ATT CTC GAT
    Ser Val Pro Thr Thr Tyr Val Phe Asp Phe Gly Lys Gly Ile Leu Asp

898 CAA AGC TGC GGC GGT GTA TTT TCA AAC AAC GGC TCT TCG CAA GTG CAG
    Gln Ser Cys Gly Gly Val Phe Ser Asn Asn Gly Ser Ser Gln Val Gln

946 CTG CGG GAT GTA GTC TTG ATG AAT GGG ACA GTG GTA TAC GAT TCA AAC
    Leu Arg Asp Val Val Leu MET Asn Gly Thr Val Val Tyr Asp Ser Asn

994 GGC GCT TGG GAC AGT AGT CCG CTG GAG GAG TGG CTC CAG CGA CAG AAA
    Gly Ala Trp Asp Ser Ser Pro Leu Glu Glu Trp Leu Gln Arg Gln Lys

1042 AAA GTT TCC ATC GAA AGA ATA TTT GAA AAT ATT GGG CCC AGC GCC GTG
     Lys Val Ser Ile Glu Arg Ile Phe Glu Asn Ile Gly Pro Ser Ala Val

1090 TAT CCG TCT ATT TTG CCT GGG GTC GTG ATT GCG TCA CCA TCG CAA ACG
     Tyr Pro Ser Ile Leu Pro Gly Val Val Ile Ala Ser Pro Ser Gln Thr

1138 CAT CCA GAC TAC TTC TAC CAA TGG ATA AGG GAC AGC GCG TTG ACG ATA
     His Pro Asp Tyr Phe Tyr Gln Trp Ile Arg Asp Ser Ala Leu Thr Ile

1186 AAC AGT ATT GTC TCT CAT TCT GCG GAC CCG GCA ATA GAG ACG TTA TTG
     Asn Ser Ile Val Ser His Ser Ala Asp Pro Ala Ile Glu Thr Leu Leu

1234 CAG TAC CTG AAC GTT TCA TTC CAC TTG CAA AGA ACC AAC AAC ACA TTG
     Gln Tyr Leu Asn Val Ser Phe His Leu Gln Arg Thr Asn Asn Thr Leu

1282 GGC GCT GGC ATT GGT TAC ACT AAC GAT ACA GTG GCT TTG GGA GAC CCT
     Gly Ala Gly Ile Gly Tyr Thr Asn Asp Thr Val Ala Leu Gly Asp Pro

1330 AAG TGG AAC GTT GAC AAC ACG GCT TTC ACG GAA CCT TGG GGT CGT CCT
     Lys Trp Asn Val Asp Asn Thr Ala Phe Thr Glu Pro Trp Gly Arg Pro

1378 CAA AAC GAT GGC CCT GCT CTT CGA AGC ATT GCC ATC TTA AAA ATC ATC
     Gln Asn Asp Gly Pro Ala Leu Arg Ser Ile Ala Ile Leu Lys Ile Ile

1426 GAC TAC ATC AAG CAA TCT GGC ACT GAT CTG GGG GCC AAG TAC CCA TTC
     Asp Tyr Ile Lys Gln Ser Gly Thr Asp Leu Gly Ala Lys Tyr Pro Phe

1474 CAG TCC ACC GCA GAT ATC TTT GAT GAT ATT GTA CGT TGG GAC CTG AGG
     Gln Ser Thr Ala Asp Ile Phe Asp Asp Ile Val Arg Trp Asp Leu Arg
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Fig. 2 (*through page 170*).

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1522 GTT CAT TAT TAC CAC TGG AAT TCT TCC GGA TTT GAT CTA TGG GAG GAA
     Phe Ile Ile Asp His Trp Asn Ser Ser Gly Phe Asp Leu Trp Glu Glu

1570 GTC AAT GGC ATG CAT TTC TTT ACT TTA CTG GTA CAA CTG TCT GCA GTG
     Val Asn Gly MET His Phe Phe Thr Leu Leu Val Gln Leu Ser Ala Val

1618 GAC AGG TCG CTG TCG TAT TTT AAC GCC TCA GAA CGG TCG TCT CCC TTT
     Asp Arg Ser Leu Ser Tyr Phe Asn Ala Ser Glu Arg Ser Ser Pro Phe

1566 GTT GAA GAA TTG CGT CAG ACA CGC CGG GAC ATC TCC AAG TTT TTA GTG
     Val Glu Glu Leu Arg Gln Thr Arg Arg Asp Ile Ser Lys Phe Leu Val

1614 GAC CCT GCG AAT GGG TTT ATC AAC GGC AAG TAC AAT TAT ATT GTT GAG
     Asp Pro Ala Asn Gly Phe Ile Asn Gly Lys Tyr Asn Tyr Ile Val Glu

1662 ACA CCC ATG ATT GCC GAC ACA TTG AGA TCC GGA CTG GAC ATA TCC ACT
     Thr Pro MET Ile Ala Asp Thr Leu Arg Ser Gly Leu Asp Ile Ser Thr

1710 TTA TTA GCT GCG AAC ACC GTC CAC GAT GCG CCA TCT GCT TCC CAT CTT
     Leu Leu Ala Ala Asn Thr Val His Asp Ala Pro Ser Ala Ser His Leu

1758 CCG TTC GAT ATC GAT GAC CCT GCC GTC CTG AAC ACG TTG CAC CAT TTG
     Pro Phe Asp Ile Asp Asp Pro Ala Val Leu Asn Thr Leu His His Leu

1806 ATG TTG CAC ATG CGT TCG ATA TAC CCC ATC AAC GAT AGC TCC AAA AAT
     MET Leu His MET Arg Ser Ile Tyr Pro Ile Asn Asp Ser Ser Lys Asn

1854 GCA ACG GGT ATT GCC CTG GGC CGG TAT CCT GAG GAC GTA TAT GAT GGA
     Ala Thr Gly Ile Ala Leu Gly Arg Tyr Pro Glu Asp Val Tyr Asp Gly

1902 TAT GGC GTT GGC GAG GGA AAT CCC TGG GTC CTG GCC ACG TGT GCC GCT
     Tyr Gly Val Gly Glu Gly Asn Pro Trp Val Leu Ala Thr Cys Ala Ala

1970 TCA ACA ACG CTT TAT CAG CTC ATT TAC AGA CAC ATC TCT GAG CAG CAT
     Ser Thr Thr Leu Tyr Gln Leu Ile Tyr Arg His Ile Ser Glu Gln His

1998 GAC TTG GTT GTC CCA ATG AAC AAC GAT TGT TCG AAC GCA TTT TGG AGC
     Asp Leu Val Val Pro MET Asn Asn Asp Cys Ser Asn Ala Phe Trp Ser

2046 GAG CTG GTA TTC TCC AAC CTC ACG ACT TTG GGA AAT GAC GAA GGC TAT
     Glu Leu Val Phe Ser Asn Leu Thr Thr Leu Gly Asn Asp Glu Gly Tyr

2094 TTG ATT TTG GAG TTC AAT ACA CCT GCC TTC AAT CAA ACC ATA CAA AAA
     Leu Ile Leu Glu Phe Asn Thr Pro Ala Phe Asn Gln Thr Ile Gln Lys

2142 ATC TTC CAA CTA GCT GAT TCA TTC TTG GTC AAG CTG AAA GCC ACG TGG
     Ile Phe Gln Leu Ala Asp Ser Phe Leu Val Lys Leu Lys Ala Thr Trp

2190 GAA CAG ACG GGG AAC TAA      GTG AACAATTTAA CAAATACACA GGGTTTATGC
     Glu Gln Thr Gly Asn ---
2241 AGGGTGCCCA ACACCTTACC TGGTCCTATA CTTCATTCTG GGATGCCTAT CAAATAAGAC

2301 AAGAAGTTTT ACAGAGTTTG TAGACAAAAA AAAATAAAAG AAAAGCGAGA AGTATACACA

2361 AGTGTATTTC CTAGATATTT ACATCAAATA TATATATATA TACTTATTTA CAAAACTCTG

2421 ATATTATAAA TTAATTAGAT ACTATGTCGG AACGTCCAGC CCAACCACGT TTGCAGTTCT

2481 TTTCACTTTC TCATCCTGTG TCAACTTGTT GCCAGGATTG TATCTGTCGA C
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Fig. 2. The DNA sequence of the *STA2*^{K} gene. The site of the 1.1-kb deletion (at -189) is indicated by the arrow. Core promoter elements are underlined. The transcription start sites (*see accompanying paper*) are indicated by asterisks. Nucleotides different from those in the published sequences of *STA1* and *STA2* are typed in bold letters.

a result of a C → T conversion (a silent replacement at *nt* No. 1340 downstream of the ATG), *STA2*^{K} lacks a *Sal*I site common to all reported *STA* (and the *SGA*) genes. An A → G conversion (at *nt* No. 1869) creates a new *Cla*I site, and, at the same time, results in a single amino acid change: Asn → Asp, as compared to either *STA1*, or *STA2*-encoded glucoamylases.

Chromosomal Localization of *STA2*^{K} as Determined by Southern Hybridization

The chromosomes of *S. diastaticus* CL9 were separated by pulse-field gel electrophoresis (CHEF-PFGE), blotted onto nylon filters and hybridized with various labeled " *STA*" probes. Two of the DNA probes were subcloned fragments of the *STA2*^{K} gene: *Probe 1* contained part of the promoter and the 5'-terminal sequence of the coding region (Fig. 3A), *Probe 2* was a "far-away upstream" sequence, i.e., the 0.6 kbp *Bgl*II-*Xho*I fragment, located at about 3.2 kb upstream of the ATG codon, and *Probe 3* was a *STA2* fragment corresponding to the sequence of the deletion in the *STA2*^{K} promoter. This sequence is present in either *STA1* or *STA2*, but is missing from *STA2*^{K}. Figure 3B demonstrates the Southern hybridizations to the separated *S. diastaticus* chromosomes. The two bands correspond to chromosome IV and II (*8*), indicating that the *STA* probes hybridize to the loci corresponding to *STA1* and *STA2*, respectively. *Probe 3* (the "promoter deletion" fragment), on the other hand, hybridized only to chromosome IV (*STA1*). The lack of hybridization of this probe to the *STA2* locus on chromosome II, in turn, strongly suggests that *STA2*^{K} is an allelic variant of *STA2*, and that is located on chromosome II.

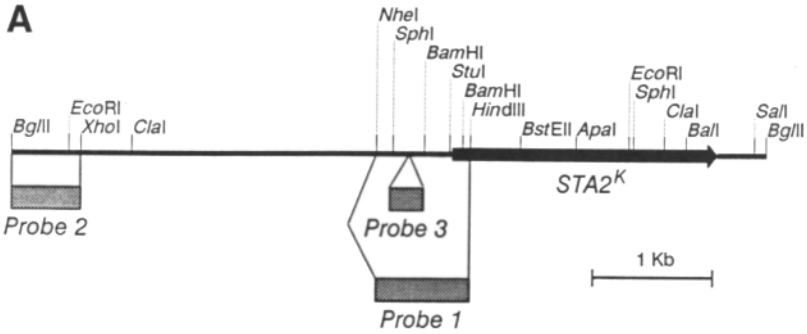
Detection of the *STA2*^{K} Allelic Variant by Southern Hybridization

Purified samples of total nuclear DNA of *S. diastaticus*, strains CL9 and J3120 were digested to completion with *Bgl*II, blotted onto a nylon filter and hybridized with the *STA*-specific DNA *Probe 1* (Fig. 3A). In contrary to strain CL9, which contains two *STA* genes, the strain J3120 was shown to contain three *STA* genes (*4*), and was used in these experiments as a control.

The southern hybridization of the *Bgl*II fragments revealed a *STA*-specific genomic fragment of 6.5 kbp in both strains CL9 and J3120. In addition, a second, 5.4-kbp long fragment was detected in the strain CL9 indicating the presence of a shorter *Bgl*II fragment containing a *STA* gene. The size of the shorter *Bgl*II fragment was in a good correlation with the promoter deletion of the *STA2*^{K} gene.

Expression of *STA2*^{K} and *STA* Genes in *S. cerevisiae* Hosts

In order to assess the extent to which the natural deletion in the *STA2*^{K} promoter affects regulated promoter functions, the expression of


A
NheI
SphI
BamHI
StuI
BamHI
HindIII
EcoRI
SphI
ClaI
SalI
BglII
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XhoI
ClaI
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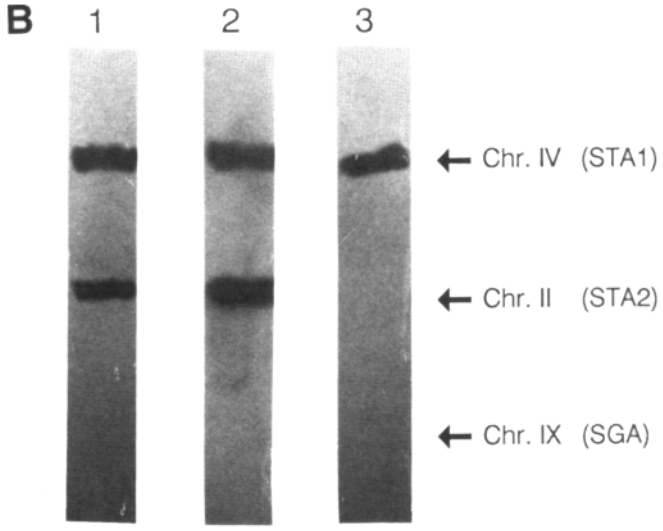
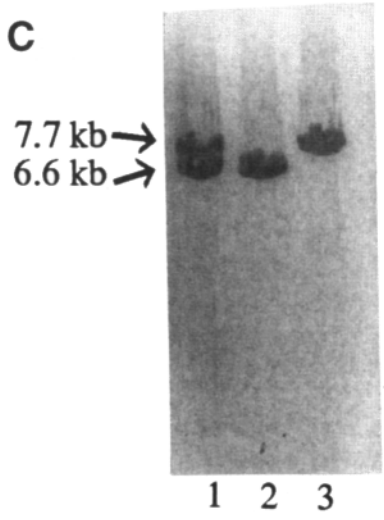
ApaI
BalI
BglII
STA2K
Probe 2
Probe 3
1 Kb
Probe 1


B
1
2
3
Chr. IV (STA1)
Chr. II (STA2)
Chr. IX (SGA)


C
7.7 kb
6.6 kb
1 2 3

STA2^K was compared to that of *STA2* in haploid and **a**/α diploid laboratory strains. The host strains were transformed by single-copy self-replicating (CEN-ARS) plasmids (Fig. 4) carrying an *URA3* selection marker and either the *STA2*^{K} or *STA2* gene. The production of secreted GA was monitored as a function of the glucose concentration in the medium and of the host cell's mating type constitution (Table 1). The highest level of secreted GA was produced by the *STA2*-expressing haploid transformants grown in low-glucose medium. The GA expression was lower by about 12% in *STA2*^{K}-expressing transformants grown under similar conditions, exhibiting variations only within the range of the experimental error even under different growth conditions (low glucose concentration) in both the haploid (DBY-746) and the MAT**a**/α diploid (YGB-27) transformants. The expression of *STA2*, on the other hand, was significantly affected by either high glucose concentration (2%, causing $\approx$37% inhibition of GA production) and/or by the **a**/α constellation in the diploid transformants, with the lowest level of *STA* expression in the diploid host grown in the presence of high glucose concentration. These data indicate the absence of target sites for repression of GA expression by both glucose and the MAT**a**/α protein in the mutant promoter of the *STA2*^{K}. In fact, the absence of the MAT**a**/α protein target sequence TCRTGTNNWNANNTACATCA (*28*) has been confirmed by sequencing, whereas the sequence of the glucose-repression element of the *STA* promoter(s) has not yet been defined (*see* ref. *7* for review).

Construction of Yeast Vectors YEpK4 and YEpC2 Bearing both *STA2*^{K} and *CUP1*

STA2^K expression vectors containing *CUP1* as a selection marker gene were constructed for transformation of brewing yeast strains. The plasmid cloning steps are demonstrated in Fig. 4.

Fig. 3. (*opposite page*) Chromosomal localization and size of the *STA2*^{K} gene. A. Map of the *STA2*^{K} locus indicating the localization of the DNA probes 1, 2, and 3 used for Southern hybridization to the chromosomes of *S. diastaticus* (strain CL9) separated by pulse-field gel electrophoresis, using the CHEF system. *Probe 3* is a 566-bp *Nhe*I-*Xha*I *STA2* promoter fragment (*12*) (cloned at the *Xba*I site of pGEM7Zf$^+$), which is deleted from the *STA2*^{K} promoter; B. Southern blot of separated chromosomes using digoxygenin-dUTP-labeled DNA probes *1*, *2*, and **3**. The arrows indicate the localization of separated chromosomes IV, II, and IX, carrying the genes *STA1*, *STA2*, and *STA3*, respectively, as resolved on an ethidium bromide-stained gel (not shown) according to (*8*). C. Southern blot hybridization of the *Bgl*II restriction fragments of genomic DNA of *S. diastaticus* strains CL9 (*STA1*, *STA2*^{K}; lane 1) and J3120 (*STA1*, *STA2*, *STA3*; lane 2). Lane 3: *Bgl*II digest of total DNA of *S. cerevisiae* transformed with plasmid YEp(STA)K6. *Probe 1* (*see* Fig. 3A) was used for hybridization to immobilized DNA samples. The size of hybridizing DNA fragments (indicated by arrows) was determined by coelectrophoresis of *Hind*III fragments of bacteriophage λ DNA.

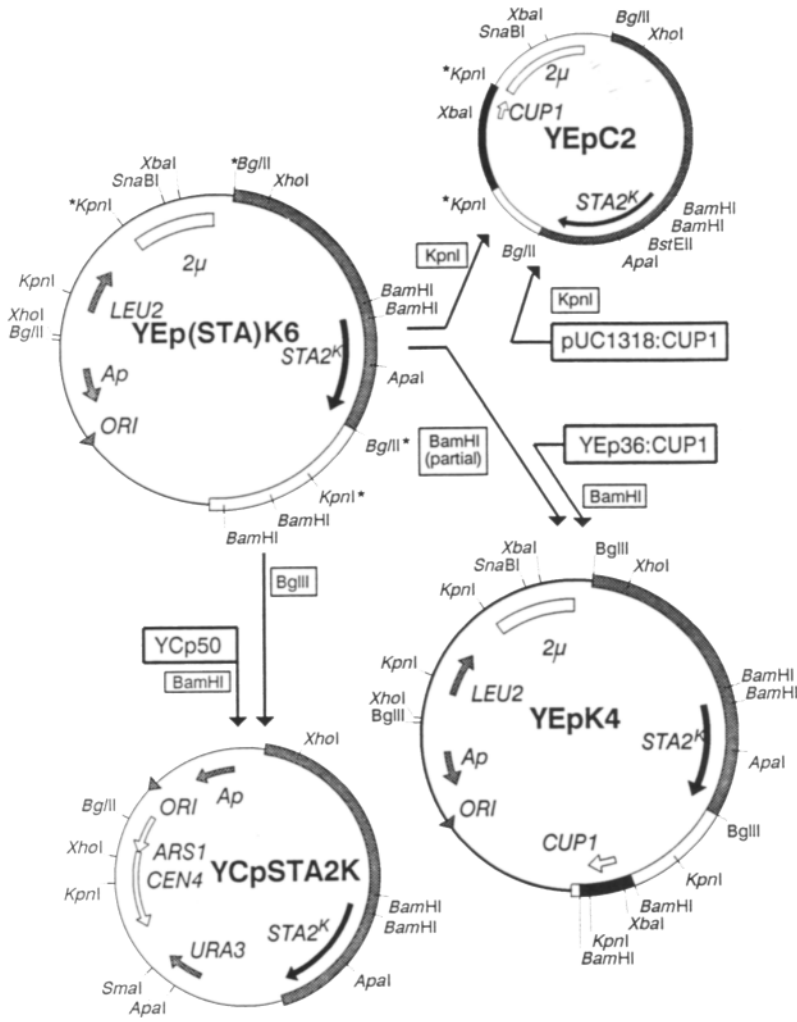


Fig. 4. Construction of the self-replicating *STA2*^{K}-expression vectors. YEpC2. The *CUP1* gene was excised from the vector pUC1813:*CUP1* as a 2-Kb *Kpn*I fragment and ligated with the ≈11kb *Kpn*I fragment of YEp(STA)K6 comprising the cloned *STA2*^{K} gene (hatched), the replication origin of the 2-μm plasmid and the *LEU2* gene. YEpK4. A *CUP1* fragment was obtained from YEp36 (*15*) by *Bam*HI digestion, that was ligated with the YEp(STA)K6 DNA linearized by partial digestion with *Bam*HI. Plasmids containing *STA2*^{K} and *CUP1* in the same and opposite orientation were obtained. YEpK4 was used in all subsequent experiments. YCpSTA2K. The 6.5-kb *Bgl*II fragment (hatched) of YEp(STA)K6 containing *STA2*^{K} was ligated into the unique *Bam*HI site of YCp50 (*35*).

Table 1
Comparison of Secreted Glucoamylase Production[a] by Yeast Strains
Transformed by Single-Copy *STA* Expression Vectors YCpSTA2K and YCpSTA2

Plasmid	Host strain	Glucose in medium, %[b]	Cell growth, mg dry wt/mL; 72-h culture	Glucoamylase activity, $\times 10^3$ U/mg dry wt	Relative GA activity, %
YCpSTA2K	DBY-746 (*MAT*α...)	2.00	8.11	3.38	83.2
		0.02	0.98	3.46	85.2
	YGB-27 (*MAT***a**/α...)	2.00	10.32	3.56	87.7
		0.02	1.36	3.44	84.7
YCpSTA2	DBY-746 (*MAT*α...)	2.00	7.94	2.57	63.3
		0.02	0.86	4.06	100.0
	YGB-27 (*MAT***a**/α...)	2.00	9.43	1.95	48.0
		0.02	1.40	2.98	73.4

[a]The results are shown as mean values of three independent experiments.
[b]The concentration of glucose and starch in YNBCA medium was 2% glucose with no starch, and 0.02% + 1% starch, respectively. The glucose level was maintained by adjusting the glucose concentration in the "2%"-samples every six hours during the first 60 h, and every 2 h during the last 12 h of the shake-flask experiment.

YEpC2

A 10-kb *Kpn*I fragment of YEp(STA)K6 containing the $STA2^K$ gene and the replication origin of the 2μ plasmid was ligated to a 2-kb *Kpn*I fragment carrying the *CUP1* gene (*16*). YEpC2 is a "nonshuttle" yeast plasmid, in which the orientation of the *CUP1* insert has not been determined. The resulting mixture of ligation products (approx 2.5 μg DNA) were used directly to transform the copper-sensitive yeast strain RH17/3d (*see* Table 2), and, in a separate experiment, the brewing yeast strain BY6. The transformation mixtures were plated on NEP-minimal medium containing 3% starch plus 0.3 m*M* copper. The yeast transformants harboring YEpC2 were selected for copper resistance and the ability to form halos on starch-containing plates.

YEpK4

The plasmid YEp(STA)K6 was linearized by partial digestion with *Bam*HI following by the insertion of a 2-kb *Bam*HI fragment from YEp36 (*15*) carrying the *CUP1* gene. The ligation mixture (approx 4 μg DNA) was used directly to transform the copper-sensitive yeast strain RH17/3d. Copper-resistant GA-expressing transformant colonies were chosen at random to prepare total yeast DNA used subsequently to transform *E. coli*. Mapping of restriction sites in YEpK4 revealed that in each of the three hybrid plasmids the fragment containing the *CUP1* gene had been inserted into the same *Bam*HI site located in the 3′ flanking DNA downstream of the *STA*-coding region. YEpK4 contains $STA2^K$ and *CUP1* in the same orientation.

Transformation of Brewing Strains with Plasmids YEpK4 and YEpC2

Prior to transformation with the plasmids YEpK4 and YEpC2, copper sensitivities of various brewing yeast strains in minimal and enriched media were tested. The copper-sensitive control laboratory strain RH17/3d was sensitive to 0.3 m*M* Cu^{++} in minimal medium, whereas the copper-resistant control strain 2180 exhibited growth at 6.0 m*M* Cu^{++} in the same medium. Yeast cells were generally more resistant to Cu^{++} in the complex NEP-D1S3 medium than in minimal medium; e.g., yeast strain KPM was sensitive to 0.3 m*M* copper in minimal medium, whereas it was sensitive to 1.0 m*M* copper in NEP-D1S3 medium. A preliminary experiment also showed that addition of starch to a medium decreased the toxic effect of Cu^{++} (data not shown), The sensitivity to Cu^{++} was a graded response rather than an all-or-nothing response. Twelve out of 18 brewing strains were sensitive to relatively low concentrations Cu^{++} (0.3 m*M*), and therefore, considered suitable for transformation using copper-resistance as a selection marker.

Table 2
Growth and Glucoamylase Production by Cultures
of Strain RH17/3d Transformed with Multicopy *STA2*^{K} Expression Vectors

Plasmid	GA activity, $\times 10^{-3}$ U/mg dry wt	Cell growth, mg dry wt/mL	Glucose in culture broth, μg/mL	Relative halo size[c]
YEp(STA)K6[a]	5.0	4.5	27.6	Large
YEpK4[a]	10.4	7.7	43.7	Small
YEpK4[b]	13.5	8.6	47.5	Medium
YEpC2[b]	16.4	9.2	50.3	Large

[a]Shaken cultures of each transformant were incubated in BYPS lacking leucine for 4 d.
[b]Cultures of transformants were grown for 4 d in YNBCA medium containing 0.3 m*M* $CuSO_4$.
[c]The relative halo sizes were determined on minimal agar plats containing 3% starch and 1% glucose after 6 d incubation at 30°C followed by refrigeration at 4°C for 3 d.

By lowering the concentrations of copper in the medium to slightly below the concentrations needed for 100% inhibition, it was possible to identify YEpK4 and YEpC2 transformants as individual halo-forming colonies against a background of about 2000 small colonies produced from partially inhibited, untransformed cells. Although the average transformation frequencies were very low (about 0.1/μg DNA), copper-resistant, glucoamylase-producing YEpK4 transformants of several brewing strains (KPM, SB, BY3, BY4, BY5, BY6, and BY7) were successfully isolated in NEP-D1S3 medium containing different concentrations of copper. BY6 was the only brewing strain used for transformation with the "nonshuttle" plasmid YEpC2 containing *CUP1* as the only selection marker. The results of the experiments monitoring cell growth, glucoamylase production, and mitotic stability of various BY6 transformants are shown in Table 3. One can see that mitotic stability and secreted glucoamylase production and cell growth parameters were higher in transformants harboring the "nonshuttle" plasmid YEpC2, containing only yeast DNA sequences, than those harboring the YEp13-based large shuttle vector YEpK4. A peculiar variant was found, moreover, among the YEpK4 transformants, as a single, fast-growing colony exhibiting also enhanced glucoamylase production and mitotic stability. This strain designated as BY6-A6 produced almost three times as much glucoamylase as the parental BY6/YEpK4 transformant, and grew much more rapidly. However, copper resistance of strain BY6-A6 did not appear to be significantly higher than that of the parental strain. This particular transformant (BY6-A6) was used in pilot-scale fermentation experiments.

Fermentation Characteristics of the Brewing Strain Transformant BY6-A6 Harboring Plasmid YEpK4

The sugar (fermentable carbohydrate) content of wort during alcoholic fermentation by the *STA2*^{K}-expressing brewing yeast transformant BY6-A6 was examined, and the results are shown in Fig. 5. Relative to the untransformed control strain BY6, the transformant exhibited a rate of decrease in carbohydrate content (attenuation) that was consistently higher.

Beer Quality

Beer quality was judged to be good by an experienced flavor panel comparing standard aroma characteristics of experimental brews made with transformed and nontransformed brewing strains (Table 4). The quality of the beer produced by the engineered brewing strain BY6-A6 was comparable to beer brewed with untransformed strain BY6 as judged by the standard aroma test panel.

Table 3
Glucoamylase Production, Culture Growth, and Mitotic Stability of GA-Producing Transformants of Brewing Strain BY6

Yeast strain[a]	Culture time,[b] d	Glucoamylase activity,[d] × 10^3 U/mg dry wt	Cell growth,[d] mg dry wt/mL	Mitotic stability, %[d]
BY6 (control)	4	0.0	0.68	N/A
	7	0.6	0.71	N/A
BY6/YEpK4	4	13.3	1.47	54.7
	7	23.7	3.99	26.1
Transformant	4	19.7	2.91	60.3
BY6-A6 (YEpK4)[c]	7	83.8	6.84	N/D
BY6/YEpC2	4	17.9	1.80	69.3
	7	53.5	5.34	55.2

[a]The stock cultures of untransformed and transformed strains were maintained in NEPS3D1 and NEPS3D1 + 1 m*M* Cu^{++}, respectively.
[b]The cells of each strain were inoculated into BYPS, and the glucoamylase activity, cell growth and mitotic stability were determined after 4 and 7 d.
[c]Transformant BY6-A6 was isolated as a spontaneous, fast-growing mutant from a 7-d plate culture of BY6/pK4 transformants grown on NEPS3 medium (lacking dextrose).
[d]Values represent averages of two separate experiments.
N/A = not applicable; N/D = not determined.

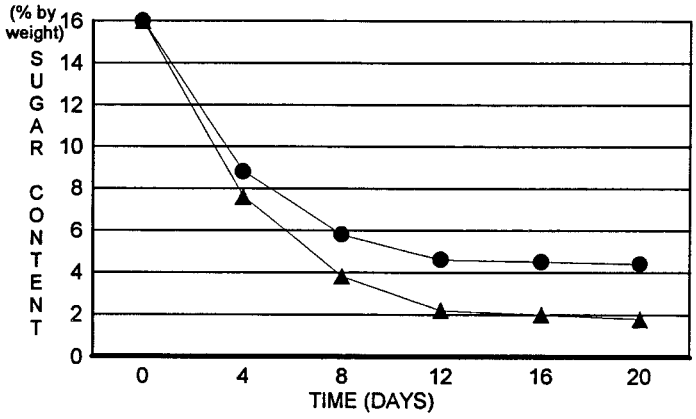


Fig. 5. Fermentation characteristics of brewing strain BY6 (-●-) and transformant BY6-YEpK4 (-▲-). The brews were maintained at 15°C. Sugar content refers to fermentable carbohydrate as determined by change in specific gravity.

Table 4
Beer Aroma Test Panel Results
for Fermenter-Drop Beers Produced by Regular (BY6)
and Transformed (BY6-A6) Brewing Yeast[a]

	Aroma rating[b]		
Yeast strain	Sulfur	Diacetyl	Esters
BY6 (control)	1.0	0.4	2.3
BY6-A6 (test)	1.0	0.5	2.5

[a]The results are the average of ratiangs by panelists.
[b]Ratings: 0 (none)–5 (most intense).

DISCUSSION

An extremely efficient glucoamylase producer strain of *S. diastaticus* was chosen as the source of a cloned *STA* gene, designated subsequently as $STA2^K$. The chromosomal location of the $STA2^K$ gene was determined by Southern hybridization to separated chromosomes of the parent *S. diastaticus* strain CL9. The fact that the labeled *STA* probes hybridized to two separate chromosomes (IV and II; Fig. 3B) supports the result of previous genetic analyses (*4*), namely that the *S. diastaticus* strain CL9 contains two *STA* genes: one corresponding to *STA1* (on chromosome IV), another to *STA2* (on chromosome II). The hybridization probes used in our experiments did not hybridize to the *SGA* gene (located on chromosome IX), as they were derived from upstream sequences that do not have sequence

homology with *SGA*. The Southern hybridizations suggests that the *STA2*^{K} gene was derived and cloned from a chromosomal locus which represents an allelic variant of the *STA2* locus. It is unlikely that the different promoter region of *STA2*^{K} could have arisen from a fortuitous ligation of *Sau*3A fragments during genomic library construction, because different *STA* clones from the same *S. diastaticus* strain CL9, obtained in independent cloning experiments, yielded a *STA* gene of similar promoter structure in which the site of the deletion did not coincide with a *Sau*3A site. As compared to several *S. cerevisiae* promoters (*GAPDH, ADH1, TRP1*), the *STA2*^{K} promoter was found to be rather strong, being capable of directing expression levels of a reporter gene in *S. cerevisiae* hosts nearly comparable to those by the *GAPDH* or *ADH1* promoters (*29*). It might be possible that the large deletion in the *STA2*^{K} promoter is responsible for the increased expression of glucoamylase by strain CL9 compared to other *S. diastaticus* strains. This possibility, however, has not been tested experimentally as it would be difficult to establish assay conditions to exclude the "background" activity of *STA1* in this particular *S. diastaticus* strain. An appropriate experimental approach is in preparation now employing *sta1*$^-$ or *sta1*$^\circ$ mutants of strain CL9 and other *S. diastaticus* strains to compare the expression of *STA2*^{K} to that of its *STA2* counterpart.

The coding region of *STA2*^{K} is almost identical to that of any of the published genes *STA1* (*11*) and *STA2* (*12*). Therefore, it is assumed that the subunit structure of the encoded glucoamylase enzyme is quite similar to that of, for instance GAI, the product of the *STA1* gene (*31*). Figure 5 shows the summarized subunit/domain structure of the *STA2*^{K}-encoded glucoamylase based on the compilation of published sequences, subunit analysis of the GAI enzyme as well as on computer analyses of *STA2*^{K} and of the derived protein sequence. Regarding the postulated location of the active site of the GA enzyme at amino acid No. 624 (*32,33*), it is therefore rather curious that the A → G conversion (at *nt* No. 1869; creating a new *Cla*I site) resulted in an Asn → Asp replacement of amino acid No. 624. It should be noted that, based on the protein's sequence, the Asn residue in question is not a target site for asparagine-linked glycosylation, and at this moment it may be only a matter of speculation how the enzyme function is affected by this amino acid change. The comparison of enzyme characteristics (kinetics, K_m, thermal stability, and so on) of *purified* glucoamylases encoded by the *STA2* and *STA2*^{K} genes is expected to provide initial explanation on the effect of this amino acid replacement. On the other hand, from the point of view of a protein engineering approach to studying the structure-function relationship in the catalytic domain of the glucoamylase enzyme, the *Cla*I site in *STA2*^{K} represents a useful natural target for introducing purposedly designed modifications into the coding DNA.

In order to compare how the expression levels of *STA2*^{K} and *STA2* in haploid and diploid *S. cerevisiae* laboratory strains are influenced by

medium composition, particularly by glucose concentration, single-copy (CEN-ARS) expression vectors (YCpSTA2K and YCpSTA2; Fig. 4) were constructed. The use of single-copy vectors was expected to minimize experimental errors caused by variations in plasmid copy number, and, as a possible consequence, by "overloading" the secretion pathways (*29*). The finding that the expression of the secreted glucoamylase is practically unaffected by either different glucose levels in the medium, or by the **a**/α constellation of the host cells (*28,32,34*) suggests that *STA2*^{K} apparently lacks the corresponding glucose-repression element(s) from its promoter region and provides a biological significance for the DNA sequence data demonstrating the lack of the **a**/α protein target sequence TCRTGTNNWNANNTACATCA (*12,28*). It should be pointed out that the glucoamylase activity secreted by transformants of strain RH17/3d or brewing strains carrying plasmid YEpK4 or YEpC2 was only 14–21% of that produced by our stock cultures of *S. diastaticus* (data not shown). The lower glucoamylase activities of transformants may reflect the presence of an inhibitory gene in the brewing yeast hosts, such as the *STA10* gene described by Polaina and Wiggs (*30*), or *INH1* found by Yamashita and Fukui (*9*). The molecular mechanism(s) of action and/or possible target sites of these inhibitory genes have not yet been defined (*see* ref. *7* for review). We assume, on the other hand, that the lack of repression by glucose and by the host cell's mating type constellation makes *STA2*^{K}, this mutant allelic variant of *STA2*, an especially attractive source of a heterologous glucoamylase gene for industrial applications.

In this work *STA*-expressing transformants of brewing yeast strains were successfully isolated using *CUP1* as a selection marker on self-replicating plasmids (YEpK4; YEpC2; Fig. 4) and by employing the double selection of halo formation and resistance to partial inhibition by relatively low concentrations of copper ions. In our hands polyploid brewing yeast strains seemed to be more difficult to transform with a *STA2*^{K} expression vectors containing *CUP1* than the haploid laboratory strain RH17/3d: The average transformation efficiency for the seven brewing strains tested was only 0.1/μg DNA using plasmid YEpK4. The *STA2*^{K} gene was expressed in the cells of all the brewing strains (seven strains) tested. Attempts to use growth on starch as primary carbon source to provide the sole pressure for transformant selection were not successful (data not shown). The use of the *STA2*^{K}-*CUP1* ("nonshuttle") yeast vector YEpC3 lacking bacterial plasmid DNA sequences was made possible using high-efficiency transformation techniques (electroporation). Using an essential cell-cycle gene as a selection marker, a yeast host-vector system of very high mitotic stability has been developed using self-replicating *STA*-expression vectors entirely lacking bacterial plasmid DNA sequences (*13*). Transformants harboring YEpC2 exhibited not only enhanced mitotic stability, but also higher levels of secreted glucoamylase expression compared to trans-

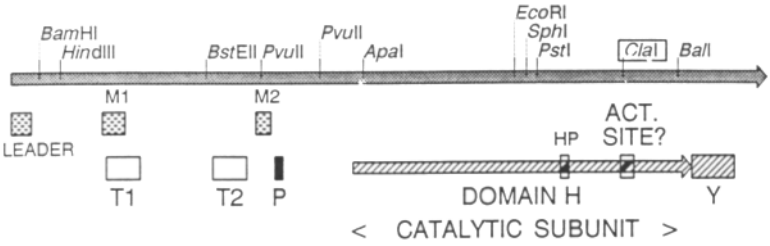


Fig. 6. Sites and domains of the *STA2*^{K}-encoded glucoamylase of *S. diastaticus* strain CL9. The analysis of the membrane-buried regions and protein sites was carried out using the software package *PC/Gene* by IntelliGenetics Inc. (Mountain View, CA); the functional subunits H and Y and the postulated active site are compiled from data published by the Yamashita group(ref. (*31*)), *see also* GenBank, accession NO. X0 2649 and K0 2840).

Symbol	Amino acid no.	Site or structural/functional region
LEADER	1- 21	SECRETION SIGNAL
T1	97–132	CYSTEINE-THREONINE-RICH REPEAT
T2	205–240	CYSTEINE-THREONINE-RICH REPEAT
M1	93–117	MEMBRANE-BOUND REGION
M2	249–264	MEMBRANE-BOUND REGION
P	268–277	POSSIBLE ATP/GTP-BINDING (P-) LOOP
DOMAIN H	348–691	H SUBUNIT (43 kDa, unglycosylated)
HP	560–565	HYDROPHILIC REGION
DOMAIN Y	692–767	Y SUBUNIT (3.4 kDa, unglycosylated)
ACTIVE SITE	624	(PUTATIVE; ref. *31*)

formants harboring the shuttle vector YEpK4 (Table 2). On the other hand, a variant (BY6-A6) exhibiting enhanced levels of both *STA* expression and mitotic stability appeared among transformants harboring YEpK4. The nature of possible alterations (or mutations?) in either the host or the structure or copy number of the transforming plasmid resulting in this peculiar transformant phenotype has not yet been defined.

The introduction of the glucoamylase gene *STA2*^{K} from *Saccharomyces diastaticus* into brewing yeast increased both rate and degree of attenuation (Fig. 5). The relatively high mitotic stability made possible the maintenance of the plasmid-harboring transformants without the presence of copper in the fermentation medium (wort). Moreover, no undesirable aroma characteristics were detected by an experienced profile panel in the superattenuated product (Table 4). It is likely, therefore, that *STA2*^{K}-containing brewing yeast strains could be efficiently used in making low-calorie beers.

SUMMARY

A new allelic variant of a *STA* gene, designated as *STA2^K*, coding for a secreted glucoamylase, was cloned from a YEp13-based genomic library of a *Saccharomyces diastaticus* strain exhibiting an exceptionally high level of glucoamylase production. The pattern of Southern hybridizations to separated *S. diastaticus* chromosomes indicates that *STA2^K* represents an allelic variant of *STA2*. Differences were revealed by restriction mapping and DNA sequencing both in the structural gene and in the promoter region, as compared to other *STA* genes. The most peculiar structural features of *STA2^K* are 1. A 1.1-kb natural delection in its promoter located 189 nucleotides upstream of the translation start codon; and 2. An Asn → Asp single amino acid change within the putative active site of the encoded glucoamylase.

In *S. cerevisiae* transformants the presence of glucose in the medium had virtually no effect on the expression level of secreted glucoamylase from *STA2^K*, whereas a significant repression of *STA2* expression was observed under similar conditions. The level of *STA2^K* expression was also higher in **a**/α diploid cells than that of *STA2*.

Self-replicating yeast plasmids containing *STA2^K* and the dominant selection marker gene *CUP1* were constructed and used to transform a laboratory yeast strain and various brewing strains. *STA2^K-CUP1* YEp plasmids containing virtually no bacterial plasmid DNA sequences exhibited high levels of glucoamylase production accompanied by increased plasmid stability. Pilot brewing tests with glucoamylase-secreting transformants of a brewing strain produced superattenuated beers at accelerated fermentation rates.

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