Cloning of Wheat LTP1500 and Two Fusarium culmorum Hydrophobins in Saccharomyces cerevisiae and Assessment of Their Gushing Inducing Potential in Experimental Wort Fermentation

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Cloning of Wheat LTP1500 and Two *Fusarium culmorum* Hydrophobins in *Saccharomyces cerevisiae* and Assessment of Their Gushing Inducing Potential in Experimental Wort Fermentation

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ABSTRACT

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The potential of surface active proteins to affect gushing upon their formation *in situ* during fermentation was investigated. This was achieved by cloning the genes of two hydrophobins of *F. culmorum* and of a wheat lipid transfer protein (LTP1500) in *Saccharomyces cerevisiae,* with expression of these genes under control of the constitutive TPI (triose phosphate isomerase) promoter. The transgenic yeast clones were used for fermentation of wort. The resulting beers were bottled and examined for the occurrence of gushing. Gushing was induced by the class II hydrophobin FcHyd5p of the fungus *F. culmorum,* found in naturally occurring cases of gushing worldwide.

Key words: beer, *Fusarium,* gushing, hydrophobins, LTP, yeast.

INTRODUCTION

Infection of barley or wheat with *Fusarium* spp. is the cause of *Fusarium* head blight (FHB) and of mycotoxin contamination of cereals[3,4,18,20]. Apart from the decreased crop yield and the hazard connected with mycotoxin infestation, infection of cereal grain with *Fusarium* spp., especially *F. culmorum* and *F. graminearum,* is known to be the source of primary or malt-induced gushing of beer[9,19,23]. Yet, the mechanisms leading to malt-induced gushing are not fully understood.

Gushing factors produced by various genera of filamentous fungi have been the subject of extensive research during the past decades[1,8,14,15,24]. Gushing factors and their possible modes of action have recently been reviewed by Hippeli and Elstner[11] and physical prerequisites of primary gushing, e.g. microbubbles, were described by Fischer[5]. The substances which were found to act as gushing factors in previous studies, range from peptides and peptidoglycans to extremely surface active proteins, like hydrophobins. Hydrophobins have been connected with primary gushing of beer, since hydrophobins, purified from cultures of a *Trichoderma reesei* strain (VTT D-74075, ATCC 26921), caused gushing when added to beer. Hydrophobin levels in malts could be related with gushing volume[10,16,22]. Differences in the gushing inducing capacity were observed with hydrophobins produced by various fungi (*Trichoderma reesei, Fusarium poae* and *Nigrospora* sp.). Among these, the smallest activity was observed with the hydrophobin prepared from *F. poae*[22]. Actually, hydrophobins are produced by all mycelial fungi, regardless, whether or not they are gushing inducers. Hydrophobins of gushing inducers, such like *F. culmorum* and *F. graminearum,* may have special structural properties resulting in a higher activity in the induction of gushing. However, hydrophobins of *F. culmorum* or *F. graminearum,* as the main causal agents of primary gushing, have not yet been subject of brewing experiments in order to evaluate their gushing activity.

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MATERIALS AND METHODS

Organisms and culture conditions

Saccharomyces cerevisiae DSM 3820 (= ATCC 38626, NRRL Y-12843), purchased from DSMZ (Braunschweig, Germany), was used as the acceptor organism for constructs derived from plasmid pYX242. *S. cerevisiae* sp. *uvarum* var. *carlsbergensis,* strain W66/70 was obtained from Hefebank Weihenstephan (Freising, Germany). *Escherichia coli* DH5α was used as the recipient for constructs with vector pSTBlue-1 Acceptor. *Fusarium culmorum* BBA 62182 was obtained from Biologische Bundesanstalt (Berlin, Germany).

LB-Amp-Medium for selective growth of transformed *E. coli* carrying ampicillin resistance mediating plasmids contained 1% tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7; 100 µg/mL Ampicillin. LB-XGal-Agar for blue-white selection with vector pSTBlue-1 contained 1% tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7; with 100 µg/mL ampicillin, 100 µmol/L X-Gal (GERBU Biochemicals, Gaiberg, Germany), 170 µmol/L IPTG (GERBU). XGal and IPTG were added after autoclaving. SC-DO-

Table I. Oligonucleotide primers used in the current study.

Designation	Sequence
LTP1f	5′-ATG CGG CGC GTC CAG GTA AT-3′
LTP1r	5′-TTA CAC CCT GCT GCA GTC GA-3′
LTP2r	5′-TTA CAC CCT GCT GCA GTC GAT GTT GAG-3′
LTP1f-*Eco*RI	5′-G GAA TTC ATG GCC CGC GCT CAG GTA AT-3′
LTP1r-*Xho*I	5′-CCG CTC GAG TTA CAC CCT GCT GCA GCT GA-3′
pYX242f	5′-GGC TCG GCT GCT GTA ACA GGG-3′
pYX242r	5′-CAG TTA GCT AGC TGA GCT CGA G-3′
hyd30f	5′-AAC CAA TCA CTC TAT TCA TC-3′
hyd31r	5′-GGT CAC AAA CTC GTG ACA CT-3′
hyd32f	5′-ATG AAG TTC TCA CTC GCC GCC GT
hyd33f	5′-TTA GRC CTG GAC ACC AGT AGG AG
hyd42f	5′-ATG CAG TTC TCT ACT CTC ACC ACT G-3′
hyd43r	5′-CTA GAG GAG CTT GAC ACA GTT GAG-3′
hyd46f	5′-ATG AAA TTT TCT TTG GCT GCA C
hyd47r	5′-TTA ATC TTG AAC ACC TGT TGG AG
hyd48f	5′-GGA ATT CAA AAA TGC AAT TTT CTA CTT TG-3′
hyd49r	5′-CCC TCG AGT CAT TAC AAC AAT TTA ACA C-3′

Table II. PCR conditions used in the current study.

Primer pair	Denaturation	Annealing	Elongation	Number of cycles
LTP1f/LTP1r	94°C/45 s	60°C/45 s	72°C/60 s	36
hyd42f/hyd43r	94°C/45 s	66°C/45 s	72°C/60 s	36
Hyd46f/hyd47r	94°C/45 s	62°C/45 s	72°C/60 s	36
hyd48f/hyd49r	94°C/45 s	62°C/45 s	72°C/60 s	36
hyd30f/hyd31r	94°C/45 s	56°C/45 s	72°C/60 s	36
Hyd32f/hyd33r	94°C/45 s	66°C/45 s	72°C/60 s	36
pYX242f/pYX242r	94°C/45 s	60°C/45 s	72°C/60 s	36

Medium (*synthetic complete drop out*) without leucine (SC-DO Leu$^-$) was used as the selective medium for *S. cerevisiae* DSM3820 transformed with pYX242 derived plasmids. SC-DO Leu$^-$ medium contained per litre: Adenine-hemisulphate 67 mg; L-arginine 135 mg; L-aspartic acid 400 mg; L-histidine 67 mg; isoleucine 200 mg; L-lysine 270 mg; L-methionine 67 mg; L-phenylalanine 400 mg; L-threonine 335 mg; L-tryptophan 270 mg; L-tyrosine 100 mg; uracil 67 mg, Yeast Nitrogen Base (Difco) 6.7 g; glucose-monohydrate 22 g; additionally for plating after transformation: 182 g sorbitol. *E. coli* was incubated at 37°C, *S. cerevisiae* DSM 3820 was incubated on plates at 30°C. Shaken cultures of *S. cerevisiae* DSM 3820 were incubated at 25°C. Media were solidified with 15 g/L agar (European Agar, Difco, BD Biosciences, Heidelberg, Germany) if used for plates. Untransformed *S. cerevisiae* DSM 3820 and *F. culmorum* BBA 61182 were grown on malt extract medium, containing 3% malt extract, 0.2% soy peptone, pH 5.6 at 25°C.

The transgenic yeast clones *S. cerevisiae* DMS3820 [pYX242-LTP1500] #961, *S. cerevisiae* DMS3820 [pYX-242-FcHyd3] #1064 and *S. cerevisiae* DMS3820 [pYX-242-FcHyd5] #858 were used in brewing experiments. *S. cerevisiae* DMS3820 [pYX242] #970 and commercial yeast *S. cerevisiae* ssp. *uvarum* var. *carlsbergensis* strain W66/70 were used as controls. For propagation of these yeast clones, 50 mL of SC-DO Leu$^-$ medium was inoculated from stock cultures and shaken (120 rpm) at 25°C for seven days. Cultures were transferred into 500 mL of SC-DO Leu$^-$ medium containing 25 g/L of glucose and incubated at 25°C and 90 rpm. Four days after inoculation, 50 mL of sterile glucose solution (50% w/v) was added to each culture. On day six, cultures were cooled to 4°C for sedimentation of the yeast cells. After three days, supernatants were decanted, leaving sedimented cells and liquid remainders of 150 mL in the culture flasks. 20 mL of sterile glucose solution (50% w/v) was added to each culture and cultures were shaken for five hours at 25°C and 90 rpm. These yeast suspensions were used to inoculate hopped wort. Hopped 12°P wort from pale barley malt was kindly provided by Hofbrauhaus Freising (Freising, Germany).

Extraction of genomic and plasmid DNA

Wheat leaves were crushed after freezing with liquid nitrogen prior to DNA extraction. Mycelium was freeze dried and ground using sterile sand. Extraction of genomic DNA from wheat leaves and fungal mycelium, respectively, was carried out according to Raeder and Broda[21]. Plasmid DNA was purified in analytical scale from cultures of *E. coli* transformants using the EZNA Plasmid Miniprep Kit I (S-Line) (peqlab Biotechnologie GmbH, Erlangen, Germany) according to the manufacturer's directions. Plasmids were purified in preparative scale from cultures of *E. coli* transformants using PhoenIX Midiprep Kit (MP Biomedicals, Illkirch, France) according to the manufacturer's directions.

Conditions for PCR reactions and gel electrophoresis of PCR products and restricted templates

PCR reactions were set up in 25 µL volumes by mixing 50 ng of target DNA per reaction with 2.5 µL of 10× amplification buffer provided with the taq polymerase (MP Biomedicals, Eschwege, Germany), 1.25 µL per reaction of dNTP mix (10 mM each dATP, dTTP, dGTP, dCTP, MP Biomedicals), 1.25 µL per reaction of each oligonucleo-

tide primer from 50 µM stock (MWG Biotech, Ebersberg, Germany) and sterile chromatography grade water (Merck, Darmstadt, Germany) to the final volume. PCR conditions for primer pairs used in the current study (Table I), were as described in Table II. Each PCR was preceded by an initial denaturation step at 94°C for 4 min and followed by final elongation at 72°C for 7 min.

Following PCR, samples were separated on TBE gels containing 45 mM Tris-borate, 1 µM EDTA, pH 8.0 and 1.5% (w/v) agarose (SeaKem LE Agarose, Cambrex Bio Science Rockland Inc., Rockland, ME, USA). For preparative purposes, samples were run on TAE gels containing 40 mM Tris-acetate, 1 µM EDTA, pH 8.0 and 1.5% (w/v) agarose (SeaKem LE Agarose, Cambrex Bio Science Rockland Inc., Rockland, ME, USA). Following gel electrophoresis, bands were stained by incubation of the gel in aqueous ethidium bromide solution (1 mg/L), washing and visualisation under short wave UV light. PCR products separated by electrophoresis in TAE gels were briefly stained with ethidium bromide. Bands were visualized under UV light and fragments of interest were cut out. PCR products contained in excised bands were purified using EZNA Gel Extraction Kit (peqlab Biotechnologie GmbH, Erlangen, Germany) according to the manufacturer's directions.

Cloning procedures

High copy-plasmid pSTBlue-1 Acceptor, used for the cloning of PCR products prior to sequencing, was purchased in linearized form from Novagen (Schwalbach, Germany). Cloning of PCR products into pSTBlue-1 and transformation of *E. coli* DH5α was performed as follows: PCR products were purified by electrophoresis in TAE gels. Following electrophoresis, gels were briefly stained with ethidium bromide and the bands were visualized using UV light. Bands containing the PCR fragment were excised and purified using the EZNA Gel Extraction Kit (peqlab Biotechnologie GmbH, Erlangen, Germany) according to the manufacturer's directions. Purified PCR fragments were ligated into the linearized vector pSTBlue-1 using T4 DNA Ligase (Fermentas, St. Leon-Rot, Germany) with supplied buffer. Ligation was performed at 14°C for 17 h. The ligation product was used to transform *E. coli* DH5α according to the method using calcium chloride described by Inoue et al.[12]. Positive transformants were selected using LB-XGal-Agar.

For transformation of *S. cerevisiae* DSM3820, insert DNA and vector pYX242 were restricted for 20 h at 37°C using the endonucleases *Eco*RI (Fermentas) and *Xho*I (Promega, Freiburg, Germany) in Orange "O" buffer (Fermentas). Restriction reactions were stopped by thermal treatment at 65°C for 10 min. Restricted products were purified by gel electrophoresis in TAE gels and briefly stained using ethidium bromide. Bands were visualized under UV light, excised and the products contained were further purified from excised gel bands as described above. Inserts were subsequently ligated in frame between *Eco*RI and *Xho*I restriction sites of pYX242 vector using T4 DNA Ligase (Fermentas) with supplied buffer. Ligation was performed at 14°C for 17 h. Resulting vectors were used to transform *S. cerevisiae* DSM3820 according to Gietz and Schiestl[7], using herring sperm DNA (Roche) as carrier nucleic acid. Transformed cells were plated onto selective SC-DO Leu$^-$ agar containing sorbitol. *E. coli*–yeast shuttle-Vector pYX242 (Novagen) was kindly provided by Prof. Manfred Schmitt, Saarland University, Saarbrücken, Germany.

Integrity and correct orientation of inserts were checked by sequencing of the vector and insert using standard sequencing oligonucleotide primers. T7 and SP6 oligonucleotide primers were used for pSTBlue-1 derived plasmids, oligonucleotide primers pYX242f and pYX242r were used for pYX242 derived constructs. T7 and SP6 oligonucleotide primers were provided by MWG Biotech AG (Ebersberg, Germany). Sequencing of plasmids and PCR products were carried out by the same company. *In-vitro* synthesis of genes was carried out by GENEART GmbH (Regensburg, Germany).

RESULTS

Cloning of *Triticum aestivum* LTP1500 (AF551849) into *S. cerevisiae*

PCR reaction was carried out with genomic DNA prepared from wheat leaves using oligonucleotide primers LTP1f and LTP1r as forward and reverse primers, respectively. Primer LTP1r was designed to bind to the terminal 20 nt of the first exon and had the second exon (7 nt) as 3′-appendix, thereby bridging the 90 nt intron of the gene. Gene and amino acid sequence of LTP1500 are depicted in Fig. 1. Purified PCR product was used as template for PCR with oligonucleotide primers LTP1f-*Eco*RI/LTP1r-*Xho*I. The purified PCR fragment was restricted using the endonucleases *Eco*RI and *Xho*I as described above and subsequently ligated between *Eco*RI and *Xho*I restriction sites of pYX242 vector, previously cut with the same enzymes. The resulting plasmid was designated as pYX242-LTP1500. Plasmid pYX242-LTP1500 was used for transformation of *E. coli* DH5α cells and *E. coli* DH5α [pYX-242-LTP1500] #881 was the clone selected for yeast transformation. Plasmid pYX242-LTP1500 was purified from *E. coli* clone DH5α [pYX242-LTP1500] #881. Presence of the insert in the plasmid was checked by PCR using the oligonucleotide primers LTP1f and LTP1r as forward and reverse primers, respectively. The correct orientation was checked by sequencing of the plasmid using oligonucleotide primers pYX242f and pYX242r as forward and reverse primers, respectively. The plasmid was used for the transformation of *S. cerevisiae* DMS3820 as described above. Several positive transformants were selected from SC-DO Leu$^-$ plates. Clone *S. cerevisiae* DMS3820 [pYX-242-LTP1500] #961 was selected for brewing experiments.

Cloning of class I hydrophobin FcHyd3p of *Fusarium culmorum* into *S. cerevisiae*

Fuchs et al.[6] published a partial sequence of a class I hydrophobin of *F. verticillioides* (*Gibberella moniliformis*), designated as Hyd3p. This sequence of the Hyd3 gene coding for the protein was used to search the corresponding gene within the *G. zeae* PH-1 genome (anamorph: *F. graminearum*) available from Fungal Genetics Stock Centre (*www.fgsc.net*). The corresponding sequence

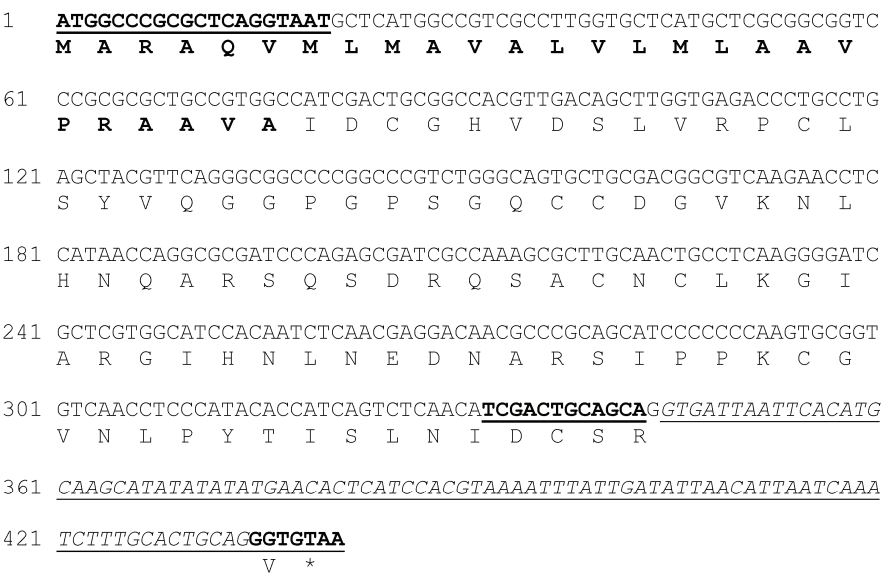

Fig. 1. DNA- and deduced amino acid sequence of LTP1500 of *Triticum aestivum* (AF-551849). The sequence of the intron is typed in italics and underlined. Binding sites for primers LTP1f and LTP1r are given in bold and underlined. The signal peptide is typed in bold.

Fig. 2. DNA sequence of FcHyd3 and deduced protein sequence of FcHyd3p from *F. culmorum* BBA 62182. The sequence of the intron is typed in italics and underlined. Binding sites for primers hyd42f and hyd43r are given in bold and underlined. The signal peptide was predicted using SignalP Server 3.0 and is typed in bold.

was annotated as hypothetical protein with the locus tag FG09066.1 and the protein ID XM 389242.1. The nucleotide sequence of locus FG09066.1 has a length of 299 bp, containing one intron of 50 bp. It shared 69.9% homology with the nucleotide sequence of *F. verticillioides* Hyd5 (GenBank accession no. AY155498). Translation of both DNA sequences into amino acid sequence showed that both proteins shared 55.4% homology when the signal peptide was included. Oligonucleotide primers hyd42f and hyd43r were designed to fit with the N-terminus and the C-terminus of the encoded protein, respectively. PCR reaction with hyd42f and hyd43r oligonucleotide primers and genomic DNA of *F. culmorum* BBA 62182 revealed a band of approx. 300 bp.

The PCR fragment was purified from an excised band and ligated into pSTBlue-1 AccepTor vector as described above. The plasmid pSTBlue-1-FcHyd3 was used to transform *E. coli* DH5α. Positive transformants were selected using LB-XGal medium. Plasmids were purified from selected transformants and were sequenced using T7 and SP6 standard primers. The insert encoded a hydrophobin gene, referred to in the following as FcHyd3. The sequence of the FcHyd3 gene (GenBank accession no. DQ-449531) and the deduced protein sequence of FcHyd3p are given in Fig. 2. Comparison of the DNA sequence of FcHyd3 with the DNA sequences of FgHyd3 and Hyd3 from *F. verticillioides* showed 93.3% and 69.4% of sequence homology, respectively. Based on translation of the DNA sequences to amino acid sequences, homology between FcHyd3p and FgHyd3p was 85.5% with signal peptides included. FcHyd3p shared 61.1% homology with Hyd3p in *F. verticillioides.*

Unlike the LTP1500 gene, the intron in FcHyd3 could not easily be bridged to result in one open reading frame,

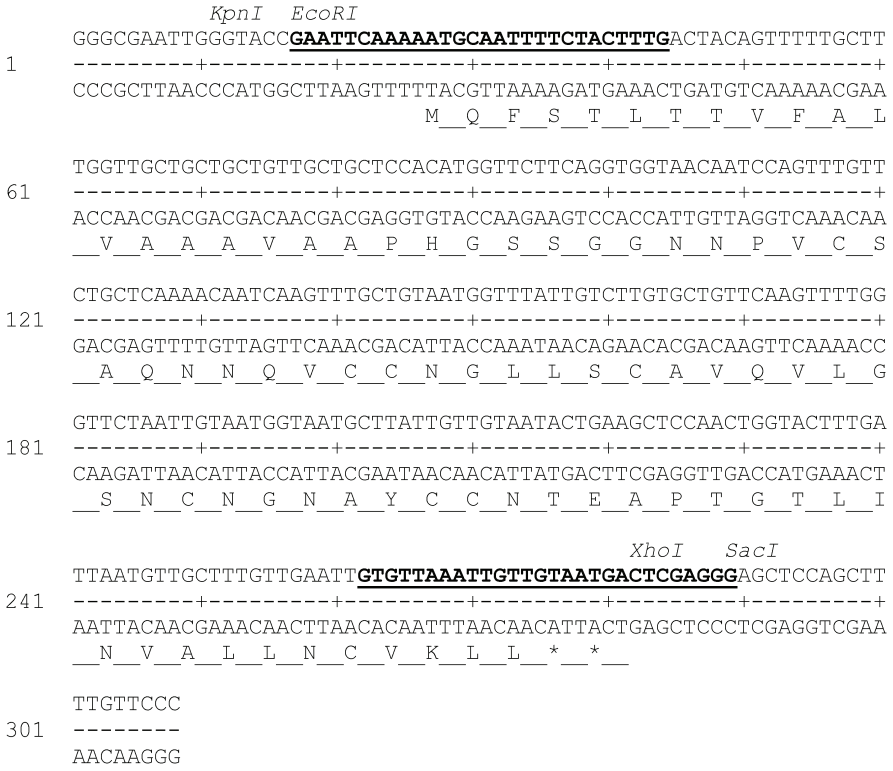

Fig. 3. Sequence of synthesized FcHyd3 gene, optimized for codon usage of *S. cerevisiae.* Binding sites for primers hyd48f and hyd49r are given in bold and underlined.

as exon 2 was considerably longer than in the first gene. Therefore, the FcHyd3 gene sequence was transmitted to GENEART GmbH (Regensburg, Germany) for adaptation of the codon usage to that of *S. cerevisiae* and subsequent *in-vitro*-synthesis of an intron-free and sequence optimized gene. The synthesized fragment was delivered as pPCR-Script vector, designated as 052549pPCR-Script. The sequence of the synthesized gene is depicted in Fig. 3. Vector 052549pPCR-Script was used to transform *E. coli* DH5α, as described above. Positive transformants *E. coli* DH5α [052549pPCR-Script] #975 and #980 were selected from LB-AMP plates. Plasmid from clone *E. coli* DH5α [052549pPCR-Script] #980 was purified as described above. The FcHyd3 gene was amplified from 052549pPCR-Script plasmid by PCR reaction using hyd48f and hyd49r oligonucleotide primers. The PCR product was separated by gel electrophoresis and purified from excised gel bands as described above. The purified PCR product was restricted using *Eco*RI and *Xho*I endonucleases as described above and subsequently ligated between *Eco*RI and *Xho*I restriction sites of the multiple cloning site of vector pYX242, also restricted with the said enzymes. The resulting vector was designated as pYX242-FcHyd3 and used for the transformation of *E. coli* DH5α, as described above. After establishing presence and correct orientation of the insert, vector pYX242-FcHyd3 was purified from transformed *E. coli* DH5α [pYX242-FcHyd3] and used for the transformation of *S. cerevisiae* DMS3820. The resulting clones were checked for the presence of the FcHyd3 gene by PCR with primers hyd48f/hyd49r after plasmid isolation using the Zymoprep II Yeast Plasmid Miniprep Kit (HISS Diagnostics, Freiburg, Germany). All tested clones were demonstrated to carry the FcHyd3 gene. *S. cerevisiae* DMS3820 [pYX-242-FcHyd3] #1064 was randomly chosen for the brewing experiments described below.

Cloning of class II hydrophobin FcHyd5 of *Fusarium culmorum* into *S. cerevisiae*

Fuchs et al.[6] published a partial sequence of a class II hydrophobin of *F. verticillioides,* designated as Hyd5p. This sequence was used to search the corresponding gene within the *Gibberella zeae* PH-1 genome (anamorph: *F. graminearum*) available from Fungal Genetics Stock Centre. A highly homologous sequence was annotated as hypothetical protein under locus tag FG01831.1 with protein ID XM 382007. The gene sequence had a length of 392 bp, containing two introns. Nucleotide sequence of locus FG01831.1 shared 37.9% homology with the Hyd5 sequence of *F. verticillioides* (GenBank accession no. AY158024). On the protein level, sequence homology was found to be 48.4% when sequences including leader peptides were compared. Based on the genome sequence of *F. graminearum,* oligonucleotide primers hyd30f and hyd31r were designed to fit with regions just upstream of the 5′-start codon and downstream of the 3′-stop codon of locus FG01831.1. PCR reaction with hyd30f and hyd31r oligonucleotide primers and genomic DNA of *F. culmorum* BBA 62182, followed by gel electrophoresis and staining with ethidium bromide revealed a band of approx. 450 bp. The size of the obtained fragment was consistent with an approximate gene size of 392 bp plus 40 bp added by the framing primers. The PCR product was purified and ligated into pSTBlue-1 AccepTor vector. The resulting vec-

Fig. 4. DNA sequence of FcHyd5 and deduced protein sequence of FcHyd5p from *F. culmorum* BBA 62182. The sequence of the intron is set in italics and underlined. Binding sites for primers hyd32f and hyd33r are given in bold and underlined. The signal peptide was predicted using SignalP Server 3.0 and is typed in bold.

tor pSTBlue-1-FcHyd5 was used for the transformation of *E. coli* DH5α, as described above. The plasmid was purified from transformants *E. coli* DH5α [pSTBlue-1-FcHyd5] and sequenced using T7 and SP6 standard primers, revealing a hydrophobin gene, referred to as FcHyd5. Gene sequence of FcHyd5 (GenBank accession no. DQ-449530) and the deduced amino acid sequence of FcHyd5p are given in Fig. 4. The gene shared 93.9% identity with the nucleotide sequence of the homologous sequence of locus FG01831.1 in *F. graminearum* and 36.3% with the Hyd5 gene in *F. verticillioides* (GenBank accession no. AY158024) Comparison of translated amino acid sequences revealed 83.8% homology of *F. culmorum* FcHyd5p with *F. verticillioides* Hyd5p, but only 54.4% homology with *F. graminearum* FgHyd5p when the leader peptides were included.

Primers hyd32f/hyd33r were used to screen genomic DNA of three isolates of *F. culmorum* and seven isolates of *F. graminearum* for the presence of a Hyd5 homolog. Moreover, genomic DNA of *F. camptoceras, F. equiseti, F. fujicuroi,* and *F. reticulatum* were tested with the same pair of primers. Results obtained showed that all isolates of *F. culmorum* and *F. graminearum* had the gene. Morover, also testing of *F. camptoceras, F. equiseti,* and *F. reticulatum* gave positive results.

Like in FcHyd3, introns could not readily be removed for heterologous production of the protein in yeast. The FcHyd5 gene sequence was transferred to GENEART GmbH (Regensburg, Germany) for adaptation of the codon usage to that of *S. cerevisiae* and subsequent *in-vitro*-synthesis of an intron-free gene. The synthesized fragment was delivered as pPCR-Script vector, designated as 042849pPCR-Script. The sequence of the synthesized gene is depicted in Fig. 5. Vector 042849pPCR-Script was used to transform *E. coli* DH5α, as described above. Positive transformant *E. coli* DH5α [042849pPCR-Script] #730 was selected from LB-AMP plates. Plasmid 042849-pPCR-Script was purified from clone #730 in preparative scale. The FcHyd5 gene was obtained from purified 042849pPCR-Script plasmid DNA by restriction digestion using *Eco*RI and *Xho*I endonucleases with 'Orange' buffer. The fragment was further purified by gel electrophoresis, extracted from excised gel bands and subsequently ligated between *Eco*RI and *Xho*I restriction sites of the pYX242 vector. The resulting vector pYX242-FcHyd5 was used for the transformation of *E. coli* DH5α as described above, resulting in clone *E. coli* DH5α [pYX242-FcHyd5] #820. Vector pYX242-FcHyd5 was purified from *E. coli* DH5α [pYX242-FcHyd5] #820 and used for the transformation of *S. cerevisiae* DMS3820. Clones obtained were checked for the presence of the FcHyd5 gene by PCR with primers hyd46f/hyd47r after plasmid isolation using the Zymoprep II Yeast Plasmid Miniprep Kit (HISS Diagnostics, Freiburg, Germany). All tested clones were demonstrated to carry the FcHyd5 gene. One of the resulting yeast clones, *S. cerevisiae* DMS3820 [pYX242-FcHyd5] #858, was randomly chosen for brewing experiments as described below. Additionally, empty circular vector pYX242 was used to transform *S. cerevisiae* DMS3820. Clone *S. cerevisiae* DMS3820 [pYX242] #970 served as control in brewing experiments.

Brewing experiments

S. cerevisiae DMS3820 [pYX242-LTP1500] #961, *S. cerevisiae* DMS3820 [pYX242-FcHyd3] #1064, *S. cerevisiae* DMS3820 [pYX242-FcHyd5] #858 carrying plasmids encoding for proteins LTP1500, FcHyd3p and FcHyd5p, respectively, were used for brewing experiments. *S. cerevisiae* DMS3820 [pYX242] #970 carrying empty plasmid pYX242 and commercial yeast *S. cerevisiae* ssp. *uvarum* var. *carlsbergensis* strain W66/70 served as controls. The yeast suspensions were used to inoculate 15 L of aerated, hopped wort per yeast isolate, using Cornelius containers as fermentation vessels. Fermentation was carried out at 20°C for one week. Hoses were attached to the gas valves of the Cornelius vessels

Fig. 5. Sequence of synthesized FcHyd5 gene, optimized for codon usage of *S. cerevisiae.* Binding sites for primers hyd46f and hyd47r are given in bold and underlined.

and the hoses were bent downwards to ensure sterile discharge of the CO_2 developing during fermentation. After one week, no more CO_2 production was observed. Containers were subsequently stored at 4°C and carbonized using oil-free, sterile filtered CO_2. Pressure was maintained at 1 bar until saturation to a final CO_2 content of 5 g/L was reached. Saturation with CO_2 was accelerated by daily shaking. After saturation, vessels were left undisturbed for one week at 4°C. Then beers were filled under counter pressure into new, sterile 0.5 L NRW beer bottles and capped using crown corks. Filled bottles were stored at 4°C for 14 days to allow the yeast to settle down. The presence of the plasmids in the yeasts after fermentation of wort was controlled by plating of serial dilutions on non-selective malt extract agar and selective SC-DO Leu$^-$ agar and comparison of the cell counts. Percentage of plasmid containing cells was calculated as the ratio between cell numbers growing under selective conditions versus the cell numbers growing under non selective conditions. After fermentation, 77%, 65% and 50% of living cells still contained the plasmid in yeasts transformed with genes LTP1500, FcHyd5, and FcHyd3, respectively. Thus the majority of yeast cells had maintained their plasmids during fermentation in all experiments.

Bottles were adapted to room temperature (20°C) and weighed prior to the assessment of gushing volumes. Since shaking of beer is known to enforce gushing, four bottles of each beer were tumbled at 28 rpm for 22 h. Bottles were left to rest for one hour and immediately prior to opening, they were slowly tumbled five times and left standing for one more minute. Crown corks were lifted and the gushing volumes were determined by weighing of bottles and comparison with the original weight. Assuming a density of approx. 1 g/cm³, difference in weight was equivalent to gushing volume.

Beers prepared with *S. cerevisiae* ssp. *uvarum* var. *carlsbergensis* strain W66/70, *S. cerevisiae* DMS3820 [pYX242-FcHyd3] #1064, and *S. cerevisiae* DMS3820 [pYX242-LTP1500] #961 displayed no gushing. Beer produced with *S. cerevisiae* DMS3820 [pYX242] #970 produced a small head of foam on one bottle. Consistent gushing was observed with *S. cerevisiae* DMS3820 [pYX242-FcHyd5] #858. All bottles gushed with volumes varying between 10.1 and 26.2 mL. Gushing volumes of this experiment are given in Table III. A photograph of gushing mediated by the fermentation with *S. cerevisiae* DMS3820 [pYX242-FcHyd5] #858 is depicted in Fig. 6.

DISCUSSION

Lipid transfer protein LTP1500 of wheat and two hydrophobins of *F. culmorum* BBA 62182 were successfully cloned into *S. cerevisiae.* The hydrophobins represent both classes of hydrophobins. FcHyd3 is member of class I hydrophobins, which tend to form extremely stable aggregates. FcHyd5 is a class II hydrophobin. The class II members usually form less stable aggregates. Thus an aggregate once formed may redissolve and hydrophobin molecules are available for re-aggregation at a new interface.

Table III. Gushing volumes determined from beers produced with transgenic yeasts.

Yeast clone	Gushing volumes [mL]				Mean [mL]	Standard dev. [mL]
	Bottle 1	Bottle 2	Bottle 3	Bottle 4		
S. cerevisiae ssp. *uvarum* var. *carlsbergensis* strain W66/70	0	0	0	0	0	0
S. cerevisiae DMS3820 [pYX242] #970	0	0.3	3.5	0.5	1.1	1.6
S. cerevisiae DMS3820 [pYX242-LTP1500] #961	0	0	0	0.5	0.125	0.25
S. cerevisiae DMS3820 [pYX242-FcHyd3] #1064	0	0	0	0	0	0
S. cerevisiae DMS3820 [pYX242-FcHyd5] #858	14.2	25.0	10.1	26.2	18.8	7.9

Fig. 6. Photograph of gushing of beer mediated by fermentation with yeast clone *S. cerevisiae* DMS3820 [pYX242-FcHyd5] #858 containing the hydrophobin gene FcHyd5.

The genes were cloned into multi-copy plasmid pYX-242. In this plasmid, genes are under control of the constitutive TPI (triose phosphate isomerase) promoter. As long as glycolysis takes place in the yeast cell, TPI is active and expression of the proteins is ensured. The proteins carry eukaryotic signal peptides, which mediate the secretion of the protein from the cell into the medium. Signal peptides are cut off enzymatically at the time of membrane passage, so the proteins are available in mature state after secretion.

In beers from both control fermentations, using either *S. cerevisiae* ssp. *uvarum* var. *carlsbergensis* strain W66/70 or *S. cerevisiae* DMS3820 [pYX242] #970, no significant over foaming occurred. From that, we concluded that the wort used for this brewing experiment was stable against gushing, regardless of the type of yeast, commercial strain or laboratory type. Any kind of gushing, occurring after fermentation with one the transgenic yeasts must have been caused by the protein produced by the respective yeast clone.

The beer produced with *S. cerevisiae* DMS3820 [pYX-242-LTP1500] #961 did not show any signs of gushing. This is consistent with the fact, that proteins of the LTP class are important stabilizers of beer foam. Their foam stabilizing properties improve by Maillard reactions and unfolding during the boiling of wort[13,17]. A role of LTP as causal agent at too high concentrations has never been shown, whereas some results suggest, that a reduced content of wheat LTP can be related with primary gushing of wheat beers[25].

Beer produced with FcHyd5p hydrophobin producing yeast *S. cerevisiae* DMS3820 [pYX242-FcHyd5] #858 displayed gushing. This finding was consistent with the results obtained by Kleemola et al.[16] and Sarlin et al.[22], who demonstrated that the addition of purified hydrophobins to beer can cause gushing. However, the hydrophobins used in the cited studies were purified from cultures of fungi, which have no general relevance as gushing inducers in central Europe (*T. reesei, F. poae* and *Nigrospora* sp.). Yet one additional parallel can be drawn between the findings of the study presented here and results of Kleemola et al.[16] and Sarlin et al.[22]. Just as FcHyd5p, the *Trichoderma* hydrophobins used in the studies cited above, were class II hydrophobins[2]. In this respect, gushing caused by class II hydrophobin FcHyd5p is the first proof for the role of *F. culmorum* hydrophobins in gushing, with *F. culmorum* being one of two *Fusarium* species found in naturally occurring cases of gushing worldwide. On the other hand, class I hydrophobins like FcHyd3p did not cause any sign of gushing in this approach indicating their different role with respect to foam stabilizing/destabilizing properties in beer.

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