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The *Saccharomyces cerevisiae EHT1* and *EEB1* Genes Encode Novel Enzymes with Medium-chain Fatty Acid Ethyl Ester Synthesis and Hydrolysis Capacity*

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Fatty acid ethyl esters are secondary metabolites produced by *Saccharomyces cerevisiae* and many other fungi. Their natural physiological role is not known but in fermentations of alcoholic beverages and other food products they play a key role as flavor compounds. Information about the metabolic pathways and enzymology of fatty acid ethyl ester biosynthesis, however, is very limited. In this work, we have investigated the role of a three-member *S. cerevisiae* gene family with moderately divergent sequences (*YBR177c/EHT1*, *YPL095c/EEB1*, and *YMR210w*). We demonstrate that two family members encode an acyl-coenzymeA:ethanol *O*-acyltransferase, an enzyme required for the synthesis of medium-chain fatty acid ethyl esters. Deletion of either one or both of these genes resulted in severely reduced medium-chain fatty acid ethyl ester production. Purified glutathione *S*-transferase-tagged Eht1 and Eeb1 proteins both exhibited acyl-coenzymeA:ethanol *O*-acyltransferase activity *in vitro*, as well as esterase activity. Overexpression of Eht1 and Eeb1 did not enhance medium-chain fatty acid ethyl ester content, which is probably due to the bifunctional synthesis and hydrolysis activity. Molecular modeling of Eht1 and Eeb1 revealed the presence of a α/β-hydrolase fold, which is generally present in the substrate-binding site of esterase enzymes. Hence, our results identify Eht1 and Eeb1 as novel acyl-coenzymeA:ethanol *O*-acyltransferases/esterases, whereas the third family member, Ymr210w, does not seem to play an important role in medium-chain fatty acid ethyl ester formation.

The synthesis of fatty acid ethyl esters (FAEEs)[3] is widely distributed in microorganisms, higher plants, and mammals. In mammals, FAEEs are the result of the nonoxidative pathway for the metabolism of ethanol, after ethanol intake (1, 2). In higher plants and microorganisms, FAEEs are formed as secondary metabolites. Because of their strong fruit flavor, ethyl esters of short- and medium-chain fatty acids (MCFAs) constitute a large group of flavor compounds particularly important in the food, beverage, cosmetic, and pharmaceutical industries. The biosynthesis of FAEEs proceeds by two different enzymatic mechanisms, esterification or alcoholysis (3). Esterification is the formation of esters from alcohols and carboxylic acids and is catalyzed by FAEE synthases/carboxylesterases. Alcoholysis is the production of esters from alcohols and acylglycerols or from alcohols and fatty acyl-CoAs derived from metabolism of fatty acids. Alcoholysis is essentially a transferase reaction in which fatty acyl groups from acylglycerols or acyl-CoA derivatives are directly transferred to alcohols. The formation of FAEEs by alcoholysis is catalyzed by acyl-CoA:ethanol *O*-acyltransferases (AEATases) (4).

Ester biosynthesis is very common in microorganisms, especially in bacteria and yeasts that are used in the fermentation of alcoholic beverages and food products. Information about the metabolic pathways and enzymology of ester biosynthesis in these microorganisms, however, is still very limited (3). In *Saccharomyces cerevisiae*, however, significant progress has recently been made. *S. cerevisiae* cells produce a broad range of esters during fermentation, which greatly affect the complex flavor of food and fermented alcoholic beverages (5). *S. cerevisiae* produces not only ethyl esters of short- to medium-chain fatty acids but also acetate esters of different alcohols (6). The enzymes responsible for acetate ester formation are already well defined, in contrast to enzymes involved in the formation of ethyl esters of short- and medium-chain fatty acids. Acetate esters are formed intracellular, in an enzyme-catalyzed condensation reaction between acetyl-CoA and ethanol or a higher alcohol. The reaction is catalyzed by alcohol *O*-acetyltransferases (EC 2.3.1.84). At present, three different alcohol *O*-acetyltransferases have been identified in yeast: Atf1, its closely related homologue Lg-Atf1, and Atf2 (for a review, see Ref. 6). Atf1 and Atf2 are present in both *S. cerevisiae* var. *cerevisiae* and *S. cerevisiae* var. *pastorianus*, whereas Lg-Atf1 is found only in *S. cerevisiae* var. *pastorianus*. Homology-based searches of the *S. cerevisiae* genome have not revealed any other gene encoding a putative ester-synthesizing enzyme with sequence similarity to Atf1 and/or Atf2. Of all known ester synthases, Atf1 is the most

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[3] The abbreviations used are: FAEE, fatty acid ethyl ester; AEATase, acyl-CoA:ethanol *O*-acyltransferase; MCFA, medium-chain fatty acid; GC-FID, headspace gas chromatography coupled with a flame ionization detector; GC-MS, purge-and-trap gas chromatography coupled with mass spectrometry; *p*NP, *p*-nitrophenyl; ORF, open reading frame; GST, glutathione *S*-transferase.

important for the production of acetate esters. Deletion analysis has shown that Atf1 is responsible for 80% of isoamyl acetate formation, 75% of phenyl ethyl acetate production, and about 40% of ethyl acetate synthesis. In addition, overexpression of the *ATF1* gene results in a more than 100-fold increase in isoamyl acetate production, as well as a 10–200-fold increase in the production of other esters, such as ethyl acetate, phenyl ethyl acetate, and C_3-C_8 acetate esters (7). The deletion and overexpression analysis also showed that Atf1 is only involved in acetate ester synthesis and not in FAEE synthesis.

Recently, a possible alcohol acyltransferase, designated Eht1 (ethanol hexanoyl transferase I) has been suggested as a candidate ethyl ester synthase (5). However, this putative alcohol acyltransferase has not been studied in any detail, and there are no experimental data to confirm the role of this protein in fatty acid ethyl ester synthesis. Here, we show that *EHT1* belongs to a three-member gene family, also containing *YPL095c* and *YMR210w*, and we demonstrate an enzymatic role for Eht1 and Ypl095c in the synthesis and hydrolysis of MCFA ethyl esters in yeast. Because Ypl095c seems to be the most important enzyme for the synthesis of MCFA ethyl esters, we propose to call the *YPL095c* gene, *EEB1*, for *e*thyl *e*ster *b*iosynthesis gene 1. On the other hand, our results do not reveal an important role for Ymr210w in the synthesis of MCFA ethyl esters. Its precise function therefore remains currently unclear.

EXPERIMENTAL PROCEDURES

Microbial Strains and Culturing Conditions—All plasmids, bacterial strains, and yeast strains used in this study are listed in Table 1. Yeast cultures were routinely grown at 30 °C in YPD medium (4% [w/v] glucose (Merck), 2% peptone (Difco), and 1% yeast extract (Difco) (8). Cultures were shaken with an orbital shaker at 50 rpm for test tubes or a horizontal shaker at 150 rpm for Erlenmeyer flasks. For selection of yeast overexpression transformants, minimal synthetic defined medium was used, containing 1.7 g liter^{-1} yeast nitrogen base without amino acids and without ammonium (Difco), 2.5 g liter^{-1} $(NH_4)_2SO_4$ and 2% glucose (Merck), supplemented with 0.69 g liter^{-1} complete supplement mixture-Leu (Bio 101, Inc. Systems). For selection of yeast deletion mutants, YPD medium was used, supplemented with 150 mg liter^{-1} Geneticin (G418, Duchefa Biochemie). *Escherichia coli* was grown in Luria-Bertani medium containing 1% Bacto tryptone (Difco), 1% NaCl, and 0.5% yeast extract (Difco).

DNA Manipulations—Standard procedures for the isolation and manipulation of DNA were used (9). Restriction enzymes, T4 DNA ligase, and Expand high fidelity DNA polymerase (Roche Applied Science) were used for enzymatic DNA manipulations as recommended by the supplier. Yeast transformation was carried out using the lithium acetate method (10).

Construction of the Deletion Strains—Original deletions of *EHT1*, *EEB1*, and *YMR210w* were constructed by integrative transformation of strain BY4741, using *KANMX* from pUG6 (see Table 1) as selection cassette (11). The following primers were used for the amplification of DNA fragments by PCR: for the *EHT1* ORF, *EHT1*-ORF-F (ATTAATATGAGCGTTTTTTAAGTTCTATTATTACATTGATAGTAGTTGCGTAAAAAACAAAGCTCATAAAAGTTTCCGATCAGCTGAAGCTTCGTACGC) and *EHT1*-ORF-R (AAAAATACATAACTTAAAATAAGGGGCATTGATCCAATGTTGTATAAATACATAGGAAAGTGGTTTGAAAATTGTGTGACAGCATAGGCCACTAGTGGATC); for the *EEB1* ORF, *EEB1*-ORF-F (GGTTGCCTACTTATTTTCGGTATTTTTGAAGATTAGCAAAAGTCAAGATATCAAGTATTTTCATATTTGTCATTTTACAGCTGAAGCTTCGTACGC) and *EEB1*-ORF-R (AGCACAGCGTGGGGAGGATGTAAATAGAGAAATAAAAGAACAGATTATTATGTGTAAGGAATTTTATTAAGAACAATGATAGGCCA CTAGTGGATC); and for the *YMR210w* ORF, *YMR210w*-ORF-F (TATTTGAATTCGATAAAAACCAACTACTTTGTTATTTTAAACTGTATTATACAAACGCTGGTAAACTTCCAGAGACGATCAGCTGAAGCTTCGTACGC) and *YMR210w*-ORF-R (TTTCATTCAGAAAAATGATGTCGAACATCAAAAAAAAAATTAGGTTACACATCTAAAAAGTTGACTTATTTACAAAGCATAGGCCACTAGTGGATC).

Double deletants were constructed by crossing single deletants in all three pair wise combinations (Table 1), followed by sporulation. Haploid double deletants were isolated from non-parental ditypes (two $G148^R$ spores/two $G418^S$ spores) or from tetratypes (three $G418^R$ spores/one $G418^S$spore). The triple deletant was constructed by crossing the two haploid double deletants BY4741 (*eht1*Δ *eeb1*Δ) and BY4741 (*eht1*Δ *ymr210w*Δ) and sporulation of the diploid. All deletant strains used in this work were verified by PCR and sequencing to confirm the replacement of the genomic *EHT1*, *EEB1*, or *YMR210w* gene with the kanamycine gene. Sequencing was performed by the dideoxy chain-method with an Applied Biosystems (Foster City, California) model 3100 Avant sequencer according to the supplier's instructions. All sequencing reactions were performed at least twice. Sequences were analyzed with ABI Prisma and vector NTI Advance (Informax/Invitrogen, Merelbeke, Belgium) software.

Construction of the Overexpression Strains—The plasmids p*EHT1*s, p*EEB1*s, and p*YMR210w*s were constructed by insertion of the respective ORFs into the XhoI restriction site in the *PGK1* overexpression cassette of the ps vector (see Table 1) (the *EHT1*, *EEB1*, and *YMR210w* PCR products were cut with XhoI). The following primers were used for the amplification of DNA fragments by PCR: for the *EHT1* ORF, XhoI-*EHT1*-ORF-F (TTGCCTCGAGATGTCAGAAGTTTCCAAAGCC, the XhoI restriction site is underlined) and XhoI-*EHT1*-ORF-R (TTGCCTCGAGTCATACA TATTCATCA AAC); for the *EEB1* ORF, XhoI-*EEB1*-ORF-F (TTGCCTCGAGATGTTTCGCCGTACTATC) and XhoI-*EEB1*-ORF-R (TTGCCTCGAGTTATAAAACTAACTCATCAAAG) and for the *YMR210w* ORF, XhoI-*YMR210w*-ORF-F (TTGCCTCGAGATGCGTTAAGAATTGTTAC) and XhoI-*YMR210w*-ORF-R (TTGCCTCGAGCTAATTCGCGCGAAAGGTGTG). Before transformation, the vectors were linearized in the inserted gene: p*EEB1*s was linearized with Bstz17I, and p*EHT1*s and p*YMR210w*s were linearized with SmaI. The empty vector was linearized in the *SMR1–410* marker gene, which is a single base mutant of the *ILV2* gene, with BlpI. The overexpression strains were verified using PCR and sequencing to confirm the correct genomic integration of the respective *PGK1* overexpression constructs.

Construction of the pSSE1 and pSSE2 Plasmids—The plasmids pSSE1 and pSSE2 were constructed by insertion of the respective ORFs into the SalI/NotI restriction site of pGEX-4T-1 (Table 1) (the *EHT1* and *EEB1* PCR products were cut with SalI and NotI). The following primers were used for the amplification of DNA fragments by PCR: for the *EEB1* ORF, *EEB1*-GST-F (AGTTGCCGTCGACTTCGCTCGGGTTACTATCCAAC; the SalI restriction site is underlined) and *EEB1*-GST-R (ATCAACGGGCGGCCGCATAAAACTAACTCATCAAA; the NotI restriction site is underlined); for the *EHT1* ORF, *EHT1*-GST-F (AGTTGCCGTCGACCAGAAGTTCCAAATGGCCAGC) and *EHT1*-GST-R (ATCAACGGGCGGCCGCATACGACTAATTCATCAAA). Plasmids were constructed in *E. coli* strain DH5α and transformed in *E. coli* strain BL21(DE3) for the purification of Eht1 and Eeb1.

Fermentation Experiments—Yeast precultures were shaken overnight at 28 °C in test tubes containing 5 ml of YPD medium. After 16 h of growth, 1 ml of the overnight culture was used to inoculate 50 ml of YPD medium in 250-ml Erlenmeyer flasks, and this second preculture

was shaken at 28 °C until stationary growth phase (A_{600} = 2) was reached. Cells were washed with sterile, distilled water and used to inoculate 350 ml of fresh, prewarmed (28 °C) YPD medium containing 8% glucose to an A_{600} of 0.4. Static fermentation was carried out at 20 °C in flasks with water locks placed on top, to create semi-anaerobic conditions to maximize ester production. Samples for chromatographic analysis were taken after 96 h of fermentation and immediately cooled on ice in an airtight container.

Headspace Gas Chromatography Coupled with Flame Ionization Detection (GC-FID) Analysis—Headspace gas chromatography coupled with flame ionization detection (GC-FID) was used for the measurement of ethyl hexanoate in the fermentation products of the deletion and overexpression strains. Samples of 5 ml were collected in 15-ml precooled glass tubes, which were immediately closed and cooled on ice. The GC-FID was also calibrated for ethyl butanoate, ethyl hexanoate, and ethyl octanoate for the enzyme tests. In this case, 200-μl samples were used. Samples were then analyzed with a calibrated Autosystem XL gas chromatograph with a headspace sampler (HS40; PerkinElmer Life Sciences) and equipped with a CP-Wax 52 CB column (length, 50 m; internal diameter, 0.32 mm; layer thickness, 1.2 μm; Chrompack, Varian, Palo Alto, CA). Samples were heated for 16 min at 60 °C in the headspace autosampler. The injection block and flame ionization detector temperatures were kept constant at 180 and 250 °C, respectively; helium was used as the carrier gas. The oven temperature was 75 °C held for 6 min and then increased to 110 °C at 25 °C min^{-1} and held at 100 °C for 3.5 min. Results were analyzed with PerkinElmer Life Sciences Turbochrom Navigator software.

Purge-and-Trap GC-MS Analysis—Purge-and-trap gas chromatography coupled with mass spectrometry (GC-MS) was used for the measurement of ethyl butanoate, ethyl octanoate, and ethyl decanoate in the fermentation products of the deletion and overexpression strains. Samples (25 ml) were collected in airtight tubes and centrifuged (5 min; 5000 × *g*; 2 °C). The supernatant was poured into precooled 25 ml airtight tubes, and 100 μl of a 10% antifoam reagent (Sigma) was added to the sample. In addition, 100 μl of a 250 mg $liter^{-1}$ solution of 2-ethyl hexanal (Sigma) in distilled water was added as an internal standard. Five milliliters of this sample was transferred into a Tekmar Dohrman 3000 (Emerson, Mason, OH) purge-and-trap sampler unit with following characteristics: helium carrier gas; 10 min purge at 120 °C; 15 min dry purge; cold trap temperature, −100 °C; 6-min desorption at 250 °C. A Fisons GC 8000 + MFA 815 cold-trap/control unit (Thermofinnigan, San Jose, CA) contained a Chrompack CP-Wax 52 CB column (length, 50 m; internal diameter, 0.32 mm; layer thickness, 1.2 μm; Varian). The oven program was as follows: 1 min at 50 °C, 4 °C min^{-1} to 120 °C, 2.5 °C min^{-1} to 165 °C, 15 °C min^{-1} to 240 °C, and 5 min at 240 °C. Total ion mass chromatograms were obtained in a Fisons MD 800 apparatus and analyzed with the masslab software program.

Protein Purification—400-ml cultures of *E. coli* BL21(DE3) cells expressing the appropriate GST fusion were grown to an $A_{600\,nm}$ = 1, induced with isopropyl β-D-thiogalactopyranoside (0.6 mM final) for 3 h at 30 °C, collected by centrifugation, and washed once in lysis buffer A (125 mM NaCl, 0.5% Triton-X-100, 1 mM dithiothreitol, 1 mM EDTA, and 50 mM sodium phosphate, pH 7.5). Washed cells were resuspended in 5 ml of lysis buffer A containing protease-inhibitor mix (Complete EDTA-free, Roche Applied Science) and 0.2 mg/ml lysozyme, incubated on ice for 15 min, and then lysis was completed by two 15 s pulses of sonication (model 450 sonifier, Branson). Lysates were clarified at 4 °C by centrifugation at 12,000 × *g*. The resulting supernatant fraction was mixed with 200 μl of a 50:50 slurry of glutathione-agarose beads (Amersham Biosciences) that had been pre-equilibrated in lysis buffer A and incubated for 1 h at 4 °C on a rollerdrum. Beads were collected by centrifugation for 1 min at 500 × *g*, washed five times with 1 ml of wash buffer (125 mM NaCl, 0.05% Triton X-100, and 50 mM sodium phosphate, pH 7.5), and GST fusion proteins were eluted with 200 μl of elution buffer (125 mM NaCl, 0.1% Triton-X-100, 20 mM glutathione, and 50 mM sodium phosphate, pH 7.5). 16 μl of the GST fusion proteins were boiled with 4 μl of sample buffer for SDS-PAGE and were analyzed by staining with Coomassie Brilliant Blue and by immunoblotting with an appropriate antibody.

In Vitro AEATase Enzyme Assay—Ethyl butanoate, ethyl hexanoate, and ethyl octanoate synthase activity was measured by headspace gas chromatography. The method described here is a modified version of the method described by Malcorps and Dufour (12). Ethyl butanoate, ethyl hexanoate and ethyl octanoate synthase assays were carried out for 1 h at 30 °C in a medium (200 μl) containing 200 mM KH_2PO_4, pH 7.8, 0.513 M ethanol, and 100 μM butyryl-CoA, hexanoyl-CoA, and octanoyl-CoA, respectively. Butyryl-CoA, hexanoyl-CoA, and octanoyl-CoA were purchased from Sigma. The specific activity is expressed as nmol of ester formed s^{-1} mg^{-1} protein. Total amount of protein in the samples was determined using a standard method (13). Under all conditions, the enzyme activities were proportional to the amount of protein added and to the incubation time.

Esterase Assay—Esterase activity with *p*-nitrophenyl esters as substrates was determined by measuring the amount of *p*-nitrophenol released by esterase catalyzed hydrolysis (14). Substrate specificity against *p*-nitrophenyl (*p*-NP) esters was determined using *p*-nitrophenyl esters with a chain length between C2 (*p*-nitrophenyl acetate) and C18 (*p*-nitrophenyl stearate). Stock solutions of 100 mM *p*-nitrophenyl ester were made in CH_2Cl_2. All *p*-nitrophenyl esters were purchased from Sigma. Immediately prior to initiation of the assay, 10 μl of the stock solution was diluted into 10 ml of buffer containing 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.01% Triton-X-100. Protein samples of up to 20 μl were incubated with 0.2 ml of substrate solution in 96-well clear microtiter plates. After incubation for 1 h at 30 °C, the liberation of *p*-nitrophenol was measured as the increase in absorbance at 410 nm in an ultraviolet-visible spectrophotometer against a blank without enzyme. The specific activity is expressed as nmol of *p*-nitrophenol released per s^{-1} μg^{-1} protein. The total amount of protein in the samples was determined using a standard method (13). Under all conditions, the enzyme activities were proportional to the amount of protein added and to the incubation time.

RESULTS

S. cerevisiae Contains a Family of Putative Ethyl Ester Biosynthesis Genes—The genes *EHT1*, *YPL095c*, and *YMR210w* constitute a three-gene family of moderately divergent sequences. We have named *YPL095c EEB1* for "ethyl ester biosynthesis" gene 1 (see further). Pair wise comparisons of *EHT1* with *EEB1* showed 58 and 63% identity at the amino acid and DNA levels, respectively, and comparison with *YMR210w* showed 32 and 58% identity at the amino acid and DNA levels, respectively. The comparison of *EEB1* and *YMR210w* gives 31 and 55% identity at the amino acid and DNA levels, respectively. Fig. 1*A* shows an alignment of Eht1, Eeb1, and Ymr210w.

Sequence comparisons of *EHT1*, *EEB1*, and *YMR210w* with annotated data bases revealed sequence similarity with orthologues of various fungi (Fig. 1*B*). Eht1 and Eeb1 share common orthologues, while Ymr210w clearly shows similarity with a different set of orthologues. The sequence similarity of Eht1 and Eeb1 with common orthologues suggests that one of these two genes is a duplicated gene copy possibly arising from the ancient genome duplication of *S. cerevisiae*. According

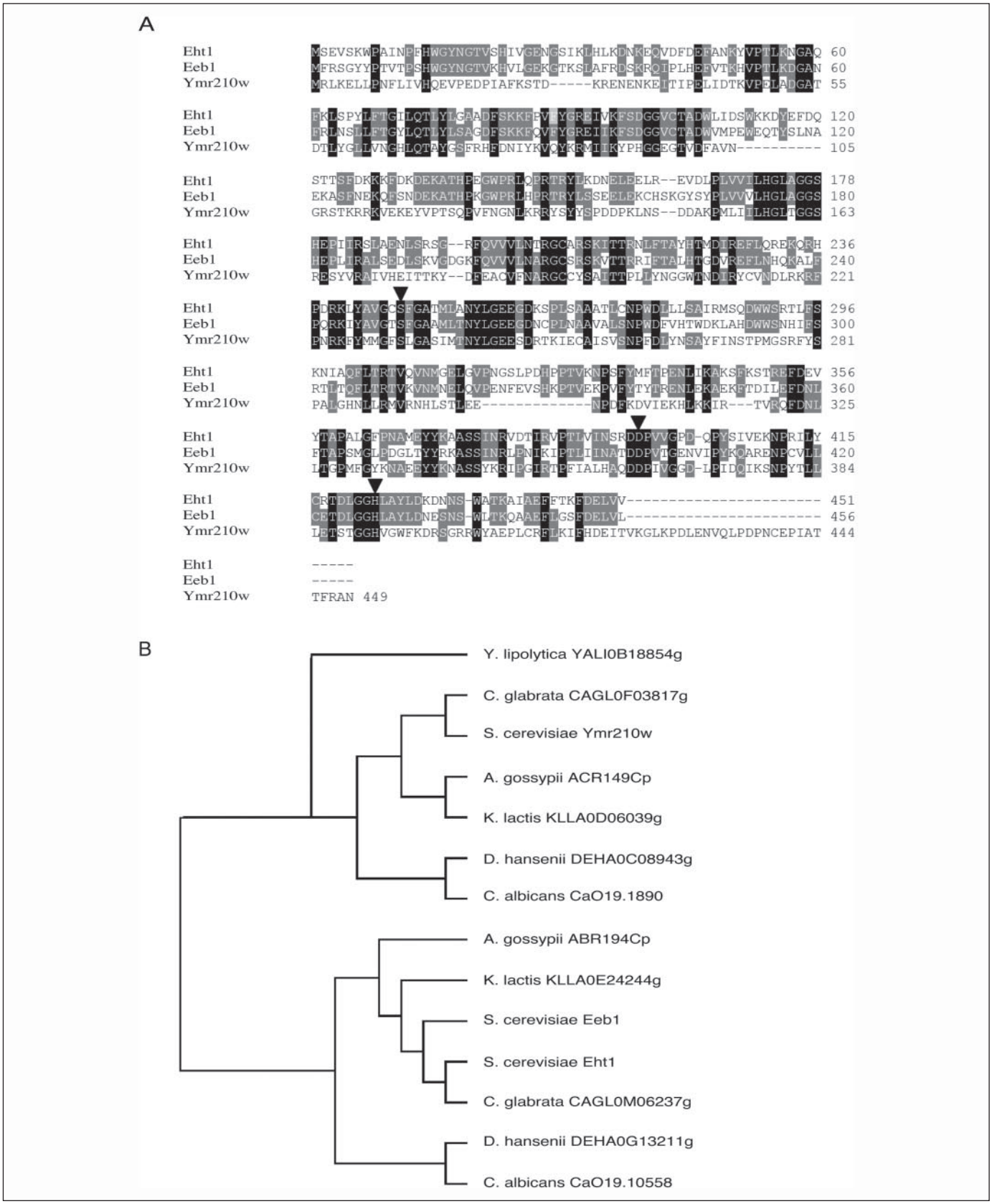

FIGURE 1. *A*, alignment of the amino acid sequences of Eht1, Eeb1, and Ymr210w from *S. cerevisiae*, made using the ClustalX software (29). Identical residues are on a *gray background*, and residues conserved in all sequences are on a *black background*. The conserved residues, potentially essential for the catalytic activity, are marked with a *triangle*; these are Ser-247, Asp-395, and His-423 for Eht1; Ser-251, Asp-399, and His-428 for Eeb1; and Ser-232, Asp-364, and His-392 for Ymr210w. *B*, fungal phylogenetic tree of the Eht1, Eeb1, and Ymr210w family, making use of Fungal BLAST on Saccharomyces Genome Data Base (30) and ClustalX and Treeview (31). Sequence information was available from Dujon *et al.* (32), Dietrich *et al.* (33), and Jones *et al.* (34).

TABLE 1
Strains and plasmids used in this study

Strains and plasmids	Genotype or description	Source or Ref.
S. cerevisiae		
BY4741 (wt)	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	ResGen/Invitrogen Belgium
BY4741 Δ*eht1*	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 eht1Δ0::KAN*^{r}	This study
BY4741 Δ*eeb1*	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 eeb1Δ0::KAN*^{r}	This study
BY4741 Δ*ymr210w*	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ymr210wΔ0::KAN*^{r}	This study
BY4741 Δ*eht1* Δ*eeb1*	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 eht1Δ0::KAN*^{r} *eeb1Δ0::KAN*^{r}	This study
BY4741 Δ*eht1* Δ*ymr210w*	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 eht1Δ0::KAN*^{r} *ymr210wΔ0::KAN*^{r}	This study
BY4741 Δ*eeb1* Δ*ymr210w*	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 eeb1Δ0::KAN*^{r} *ymr210wΔ0::KAN*^{r}	This study
BY4741 Δ*eht1* Δ*eeb1* Δ*ymr210w*	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* *eht1Δ0::KAN*^{r} *eeb1Δ0::KAN*^{r} *ymr210wΔ0::KAN*^{r}	This study
BY4741 p*EHT1*s	*MAT***a** *his3Δ1 leu2Δ0/LEU2 met15Δ0 ura3Δ0* *EHT1::PGK1*$_p$*-EHT1-PGK1*$_t$ *SMR1–410*	This study
BY4741 p*EEB1*s	*MAT***a** *his3Δ1 leu2Δ0/LEU2 met15Δ0 ura3Δ0* *EEB1::PGK1*$_p$*-EEB1-PGK1*$_t$ *SMR1–410*	This study
BY4741 p*YMR210w*s	*MAT***a** *his3Δ1 leu2Δ0/LEU2 met15Δ0 ura3Δ0* *YMR210w::PGK1*$_p$*-YMR210w-PGK1*$_t$ *SMR1–410*	This study
E. coli		
DH5α	*F′ end A1 hsdR17 supE44 thi-1 recA gyrA relA1* Δ (*lacZYA-argF*) *U169 deoR* [Φ80d*lac* DE(*lacZ*)M15]	GIBCO-BRL/Life technologies
BL21(DE3)	F$^-$ *ompT hsdS*$_B$ (*r*$_B^-$ *m*$_B^-$) *gal dcm rne131* (DE3)	ResGen/Invitrogen Belgium
Plasmids		
pUG6	*bla TEF2*$_p$*-KANMX-TEF2*$_t$	11
ps (empty vector, Yip)	*bla LEU2 SMR1–410 PGK1*$_p$*-PGK1*$_t$	35
p*EHT1*-s	*bla LEU2 SMR1–410 PGK1*$_p$*-EHT1-PGK1*$_t$	This study
p*EEB1*-s	*bla LEU2 SMR1–410 PGK1*$_p$*-EEB1-PGK1*$_t$	This study
p*YMR210w*-s	*bla LEU2 SMR1–410 PGK1*$_p$*-YMR210w-PGK1*$_t$	This study
pSSE1[a]	GST-*EEB1*	This study
pSSE2[a]	GST-*EHT1*	This study

[a] *EEB1* and *EHT1* were cloned into pGEX-4T-1 (Amersham Biosciences).

to Manolis *et al.* (15), *EHT1* and *EEB1* are located on duplicated regions of the *S. cerevisiae* genome, which could mean that they share a similar function. It is interesting to note that all the orthologues of Eht1 and Eeb1 and also Ymr210w have an as yet unknown function. This means that Eht1, Eeb1 and Ymr210w belong to yet uncharacterized gene families. With the functional investigation of these three enzymes, we will have a first clue of the possible functional role of the members of these two gene families.

Deletion Analysis of EHT1, EEB1, and YMR210w and Medium-chain Fatty Acid Ethyl Ester Synthesis—To determine the possible function of *EHT1*, *EEB1*, and *YMR210w* in MCFA ethyl ester synthesis, we first constructed all the single, double, and triple deletion mutants for those three genes (see "Experimental Procedures" and Table 1). All single and multiple deletion strains are haploid viable. They were tested for possible growth defects by growing them in complete medium with glucose at 30 °C. The strain *eht1*Δ *eeb1*Δ showed a little delayed lag phase but reached the same $A_{600\,nm}$ as the other strains in stationary phase (data not shown).

To determine the effect of the deletion of *EHT1*, *EEB1*, and *YMR210w* on the synthesis of MCFA ethyl esters, the strains *eht1*Δ, *eeb1*Δ, *ymr210w*Δ, *eht1*Δ *eeb1*Δ, *eht1*Δ *ymr210w*Δ, *eeb1*Δ *ymr210w*Δ, and *eht1*Δ *eeb1*Δ *ymr210w*Δ were tested in batch culture fermentations for MCFA ethyl ester production. After 5 days of fermentation, samples for volatile compound determination were taken. They were analyzed by headspace GC-FID and purge-and-trap GC-MS. Fermentations and chromatographic analysis were performed as described under "Experimental Procedures." The results of the GC-FID and GC-MS analyses are given in Fig. 2. Each fermentation experiment and the subsequent analysis were repeated three times for each strain. Fig. 2 shows that the levels of ethyl butanoate, ethyl hexanoate, ethyl octanoate, and ethyl decanoate produced during fermentation with the *eeb1*Δ strain were reduced in comparison with those produced by the wild type strain by, respectively, 36, 88, 45, and 40%. Compared with the *eeb1*Δ strain, deletion of *EHT1* did not affect the production of ethyl butanoate and ethyl decanoate and resulted in only minor decreases in ethyl hexanoate formation (36%) and ethyl octanoate formation (20%). Deletion of *YMR210w* did not affect the production of MCFA ethyl esters, suggesting that Ymr210w has no role in the production of MCFA ethyl esters or that its role is redundant with that of other gene products. The double deletion strain *eht1*Δ *eeb1*Δ produced similar levels of ethyl butanoate, ethyl hexanoate, and ethyl decanoate as the *eeb1*Δ single deletion strain and a lower level of ethyl octanoate (82% reduction in comparison to the wild type), indicating that Eht1 plays only a minor role in MCFA ethyl ester synthesis, while Eeb1 is the most important enzyme for MCFA ethyl ester synthesis. The double deletion of *EEB1* and *YMR210w* produced similar levels of ethyl butanoate and ethyl hexanoate in comparison to the *eeb1*Δ strain but lower levels for ethyl octanoate and ethyl decanoate. The double deletion strain *eht1*Δ *ymr210w*Δ showed no significant difference in the production of MCFA ethyl esters in comparison with the wild type strain. The *eht1*Δ *eeb1*Δ *ymr210w*Δ strain produced similar levels of ethyl butanoate and ethyl hexanoate as the *eht1*Δ *eeb1*Δ strain but showed a further 65% decrease in the production of ethyl octanoate and a further 88% decrease in the production of ethyl decanoate in comparison with the *eht1*Δ *eeb1*Δ strain. This confirms that Ymr210w plays no significant role in ethyl butanoate and ethyl hexanoate production but indicates that it does contribute to ethyl octanoate and ethyl decanoate formation. In summary, Eht1 and Eeb1 seem to be the most important enzymes for the formation of MCFA ethyl ester synthesis, while the minor role of Ymr210w in the production of ethyl octanoate and ethyl decanoate is only revealed in the absence of Eht1 and Eeb1.

Overexpression Analysis of EHT1, EEB1, and YMR210w Confirms Their Role in Medium-chain Fatty Acid Ethyl Ester Synthesis—To further support the role of Eht1 and Eeb1 in the synthesis of MCFA ethyl esters, and evaluate the contribution of Ymr210w, overexpression strains were constructed (see "Experimental Procedures" and Table 1).

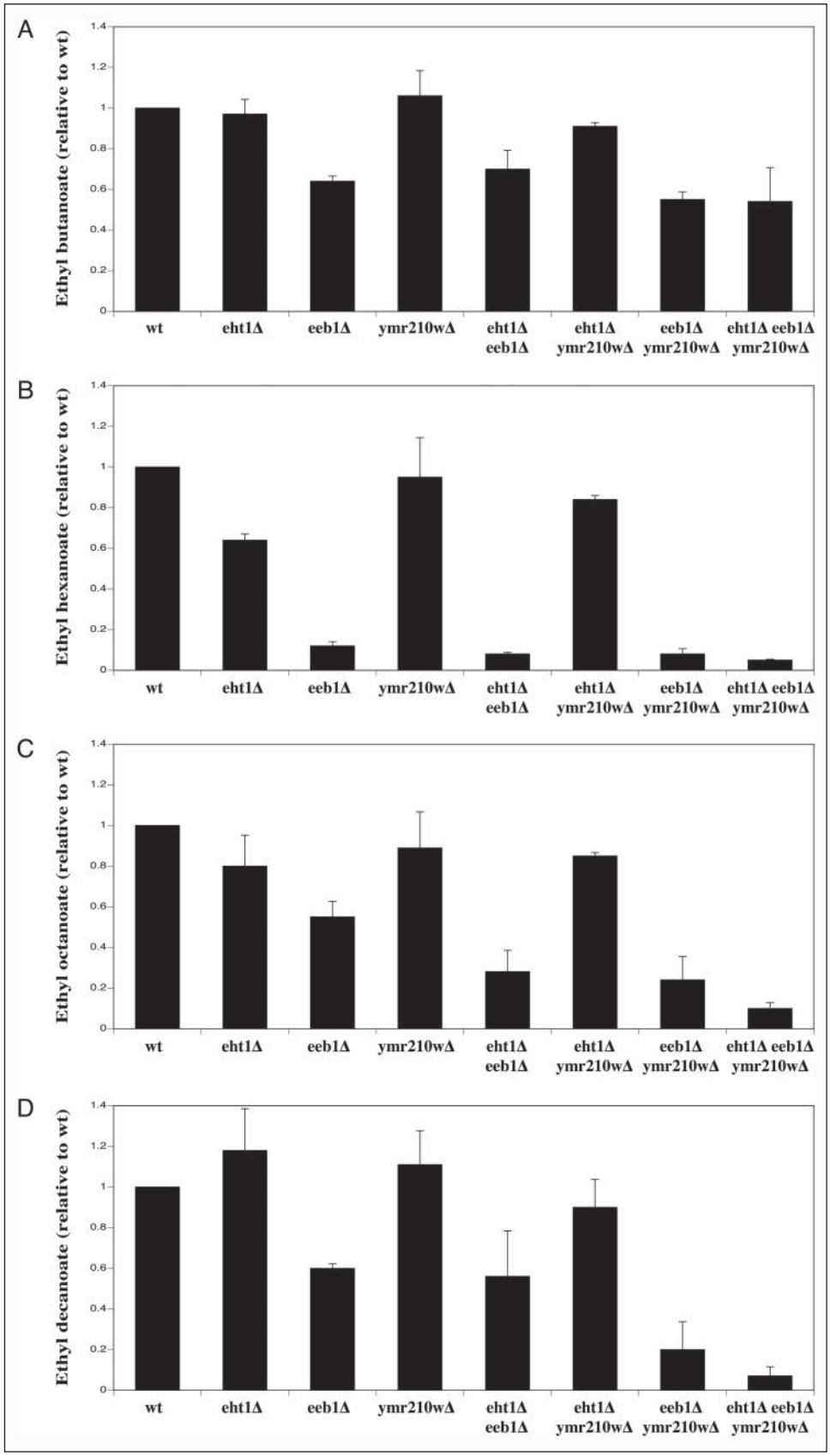

FIGURE 2. **Ethyl ester production in *eht1*Δ, *eeb1*Δ, and *ymr210w*Δ, single and multiple mutants.** Gas chromatographic measurement of ethyl butanoate (*A*), ethyl hexanoate (*B*), ethyl octanoate (*C*), and ethyl decanoate (*D*) produced by the wild type (*wt*) and the deletion strains *eht1*Δ, *eeb1*Δ, *ymr210w*Δ, *eht1*Δ *eeb1*Δ, *eht1*Δ *ymr210w*Δ, *eeb1*Δ *ymr210w*Δ, and *eht1*Δ *eeb1*Δ *ymr210w*Δ after 96 h of fermentation.

The overexpression strains BY4741 + p*EHT1*s, p*EEB1*s, or p*YMR210w*s were tested in batch culture fermentations for MCFA ethyl ester production. Fermentations and chromatographic analysis were performed in the same way as for the deletion strains. Unexpectedly, overexpression of *EEB1* and *EHT1* did not result in a significant increase in MCFA ethyl ester formation (results not shown). This can be explained by

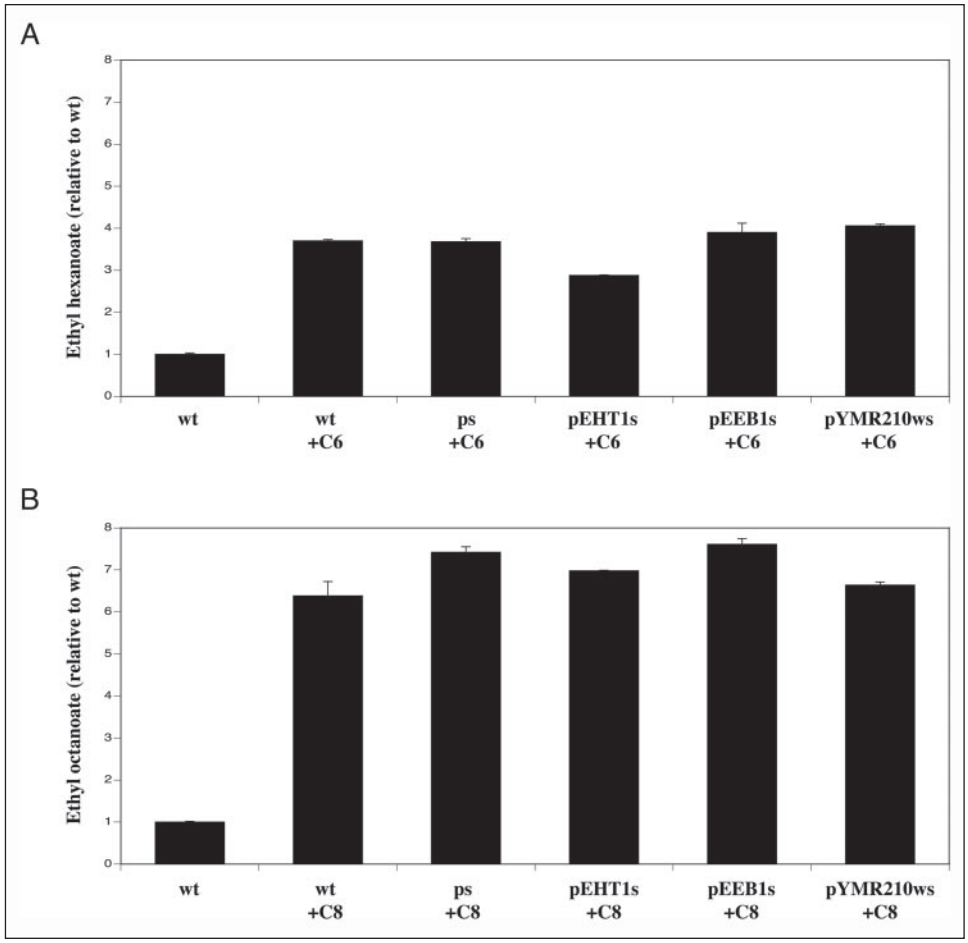

FIGURE 3. **Ethyl ester production in BY4741 + p*EHT1*s, p*EEB1*s, or p*YMR210w*s with addition of substrates.** Gas chromatographic measurement of ethyl hexanoate (*A*) and ethyl octanoate (*B*) produced by the wild type (*wt*), the empty vector control strain (*ps*), and the overexpression strains p*EHT1*s, p*EEB1*s, and p*YMR210w*s after 96 h of fermentation. +C6 = 5 mM hexanoic acid added to the medium; +C8 = 1 mM octanoic acid added to the medium.

several hypotheses: (i) the substrate levels are the limiting factor for MCFA ethyl ester synthesis, (ii) the enzymes also possess hydrolyzing activity, (iii) a combination of i and ii, or (iv) Eht1 and Eeb1 are only indirectly required for MCFA ethyl ester production and they do not have intrinsic MCFA ethyl ester synthesis capacity.

To evaluate whether the substrate levels are a limiting factor for the formation of MCFA ethyl esters, the wild type strain, the empty vector control strain, and the overexpression strains BY4741 + p*EHT1*s, p*EEB1*s or p*YMR210w*s were tested in fermentations with addition of hexanoic acid or octanoic acid. If substrate levels are indeed limiting for the production of MCFA ethyl esters, an increase in MCFA ethyl esters is expected when the respective acid is added to the medium. This increase should be higher for the overexpression strains, compared with the empty vector control strain. The fermentation of the wild type strain BY4741 without addition of hexanoic and octanoic acid was used as a control. Addition of 5 mM hexanoic acid to the wild type strain resulted in a 4-fold increase in ethyl hexanoate concentration (Fig. 3*A*). However, no significant difference could be seen between the empty vector control strain and overexpression strains when hexanoic acid was added to the fermenting medium. Addition of 1 mM octanoic acid to the wild type strain resulted in an 8-fold increase in ethyl octanoate concentration (Fig. 3*B*). Again, the production of ethyl octanoate was not significantly different between the empty vector control strain and the overexpression strains BY4741 + p*EHT1*s, p*EEB1*s, and p*YMR210w*s. These results indicate that in a wild type strain the endogenous substrate level is limiting for the formation of MCFA ethyl esters, since addition of 5 mM hexanoic acid or 1 mM octanoic acid strongly increased the synthesis of ethyl hexanoate or ethyl octanoate, respectively. However, the production of MCFA ethyl esters was not significantly different between the overexpression strains BY4741 + p*EHT1*s, p*EEB1*s, or p*YMR210w*s and the empty vector control strain in the presence of exogenously added hexanoic or octanoic acid. Hence, the failure to observe an increase in the overexpression strains is apparently not due to substrate limitation.

GST-tag-purified Eht1 and Eeb1 Have the Capacity to Synthesize and Hydrolyze Medium-chain Fatty Acid Ethyl Esters—To evaluate whether Eht1 and Eeb1 possess intrinsic MCFA ethyl ester synthesis and/or hydrolysis activity, we have purified the two proteins using GST-tag affinity chromatography after expression in *E. coli.* After binding of the GST fusion proteins to GSH Sepharose, GST-Eht1 and GST-Eeb1 were eluted. The purity of the GST fusion proteins was determined by loading these proteins on an SDS-PAGE gel and staining the gel with Coomassie Brilliant Blue. As only one band was visible for each GST fusion protein (result not shown), both GST-Eht1 and GST-Eeb1 were subsequently used for *in vitro* assays of enzymatic activity.

The ability of Eht1 and Eeb1 to synthesize fatty acid ethyl esters was tested in AEATase assay (see "Experimental Procedures"). As is apparent from Fig. 4, Eht1 and Eeb1 were capable of synthesizing ethyl butanoate, ethyl hexanoate, and ethyl octanoate from ethanol and butyryl-CoA, hexanoyl-CoA, and octanoyl-CoA, respectively (Fig. 4). The results demonstrate that both Eht1 and Eeb1 possess intrinsic MCFA ethyl ester synthesis capacity but with different substrate specificity. Eht1 and Eeb1 did not display FAEE synthase capacity, since the enzymes were not able to synthesize fatty acid ethyl esters from ethanol and the acids butyric acid, hexanoic acid, or octanoic acid (results not shown). Eeb1 showed a preference for octanoyl-CoA as substrate, com-

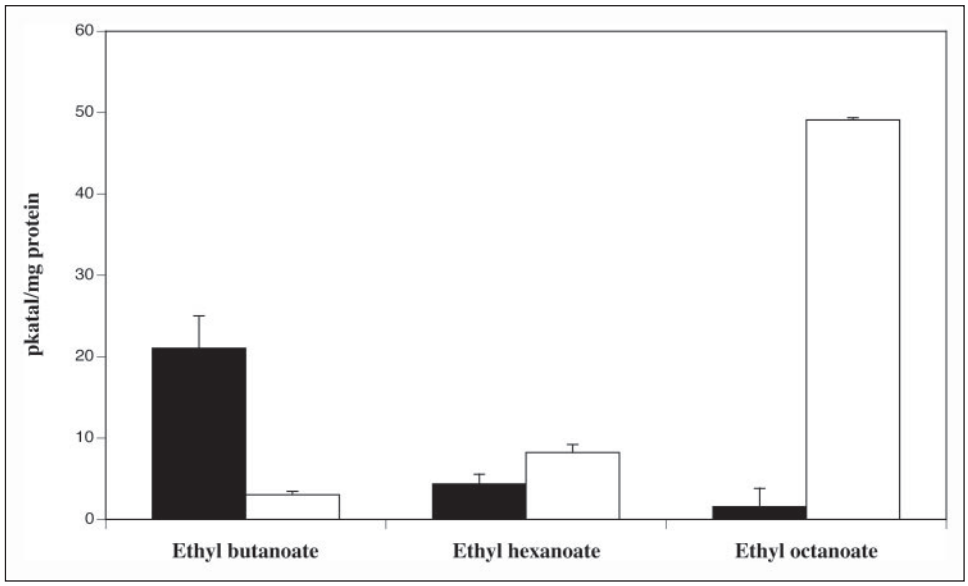

FIGURE 4. **Ethyl ester production by GST-tag-purified Eht1 and Eeb1.** Gas chromatographic measurement of ethyl butanoate, ethyl hexanoate, and ethyl octanoate produced *in vitro* by Eht1 (*black*) and Eeb1 (*white*). Substrates added were 0.5 M ethanol and 100 μM butyryl-CoA, hexanoyl-CoA, or octanoyl-CoA for the measurement of ethyl butanoate, ethyl hexanoate, or ethyl octanoate, respectively.

pared with hexanoyl-CoA and butyryl-CoA, as 10 times more ethyl octanoate than ethyl hexanoate or ethyl butanoate was synthesized with the same concentration of substrate. On the other hand, Eht1 preferred short-chain acyl-CoAs as substrates, as it produced four times more ethyl butanoate from ethanol and butyryl-CoA than ethyl hexanoate from ethanol and hexanoyl-CoA.

In addition to AEAT activity, Eht1 and Eeb1 were also evaluated for intrinsic esterase activity using nine different *p*NP esters as substrate. The results clearly show that both enzymes also possess esterase activity (Fig. 5). Eht1 and Eeb1 seem to be real short-chain esterases, as they can hydrolyze efficiently *p*NP acetate, *p*NP butyrate, *p*NP hexanoate, and *p*NP octanoate, whereas the activity with *p*NP decanoate and especially *p*NP dodecanoate drops sharply. Eht1 and Eeb1 do not posses lipase activity, since they do not hydrolyze long-chain *p*NP esters. Eeb1 had an optimum esterase activity with *p*-nitrophenyl acetate (C2). Its activity with longer substrates (C4 until C18) gradually decreased toward the longer chain *p*-nitrophenyl esters and became virtually undetectable with C14, C16 and C18. Eht1 showed a different pattern of esterase activity. It increased with increasing substrate chain length from C2 until C6. Optimum esterase activity was observed with *p*-nitrophenyl caproate (C6). It decreased then gradually for the longer chain *p*-nitrophenyl esters to become virtually undetectable with C14, C16, and C18, as was the case with Eeb1.

Molecular Modeling of the Three-dimensional Structure of Eht1 and Eeb1—The amino acid sequence of Eht1, Eeb1, and Ymr210w, taken from the NCBI protein data base, did not show sufficient similarity with other structures in the Protein Data Bank data base to make a direct prediction of the three-dimensional structure. The sequences were therefore submitted to the mgentraeder fold recognition server for alignment with a suitable template (16, 17). For the first 145 amino acids no fold could be identified. This part of the sequence, which is highly similar for Eht1 and Eeb1, but clearly different from Ymr210w, lacks a recognizable secondary structure. For the C-terminal part of Eht1 and Eeb1 a clear α/β hydrolase fold was identified. The two common highest ranked templates (Protein Data Bank 1UK6 (18)) and Protein Data Bank 1C4X (19)) were taken for further use and the alignments were merged for modeling with Modeler 8v1.4. The resulting models were optimized in brugel (20) and minimized using the dead end elimination method (21).

The three-dimensional structure model obtained for Eht1 is shown in Fig. 6*A*. The model for Eeb1 is highly similar (data not shown). Also for Ymr210w a similar structure was predicted, but for this protein we do not have a clear function identified yet. The α/β-hydrolase fold family of enzymes is one of the largest groups of structurally related enzymes with diverse catalytic functions (22). The enzymes all have a nucleophile-His-acid catalytic triad with its residues positioned in loops that are the best conserved of the fold. For Eht1 the catalytic triad was identified as Ser-247, Asp-395, and His-423, while for Eeb1 the catalytic triad is composed of Ser-251, Asp-399, and His-428. These residues are indicated in the alignment of the Eht1, Eeb1, and Ymr210w amino acid sequences in Fig. 1*A*. They are also conserved in Ymr210w, but the adjacent sequences are less well conserved in this protein, so that its function remains speculation.

Eht1 and Eeb1 are predicted to have a tunnel-shaped catalytic site in which the substrates can be positioned for hydrolysis (Fig. 6*B*). The tunnel is constructed by conserved residues, but the ending of the tunnel is made up of a helix of divergent residues (the *red* and *blue* colored β-sheet in Fig. 6*B*). These divergent residues could be responsible for the different substrate range of Eht1 and Eeb1 with respect to the length of the fatty acid chain that can be esterified or hydrolyzed. The distance between the catalytic site and these amino acids corresponds approximately with the length of the fatty acid tails.

The serine of the catalytic triad plays a critical role in the transesterification reaction mechanism. For the esterification reaction, the serine attacks the carbon of the thio-ester bond of the CoA in the activated fatty acids (acyl-CoA). The CoASH leaves the enzyme and in the second step the fatty acid is transferred to ethanol, resulting in an ester molecule. For the ester hydrolysis reaction, the second step takes place in reverse order after which the fatty acid is transferred to a water molecule.

DISCUSSION

EHT1 and EEB1 Encode Ethyl Ester Synthase/Hydrolase Enzymes—In this work we have explored a possible function for the *EHT1*, *EEB1*, and *YMR210w* gene family in ester biosynthesis in yeast. Single and multiple deletion analysis hinted at a role especially for Eht1 and Eeb1 in MCFA ethyl ester biosynthesis and in the further analysis we have therefore concentrated on these two gene products. The results obtained

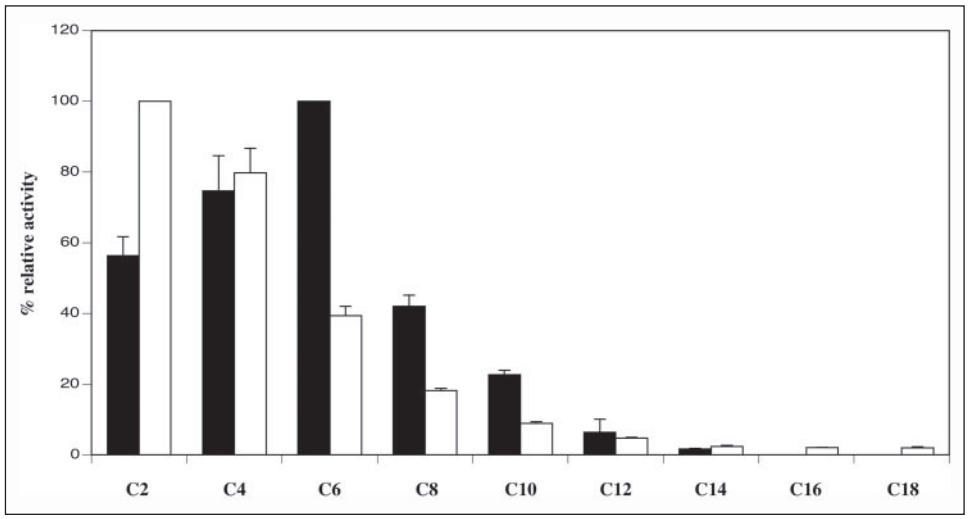

FIGURE 5. **Relative esterase activity of Eht1 (*black*) and Eeb1 (*white*) with nine different *p*-nitrophenyl esters with increasing chain length.** For each enzyme, the highest activity with a particular substrate was taken as 100% (for Eht1 with *p*-nitrophenyl caproate and for Eeb1 with *p*-nitrophenyl acetate).

with the purified GST-Eht1 and GST-Eeb1 fusion proteins clearly indicate that these proteins display enzymatic activity both for MCFA ethyl ester synthesis and hydrolysis. This is in agreement with the results of the MCFA ethyl ester analysis in the single and multiple deletion strains. Deletion of *EHT1* or *EEB1*, and especially the double deletion, caused a significant decrease in the production of most MCFA ethyl esters. The Eeb1 enzyme appears to have the largest contribution, followed by Eht1, while Ymr210w seems to have only a minor contribution in the production of MCFA ethyl esters.

There was no clear correlation between the effect of *EHT1* or *EEB1* deletion on accumulation of the different MCFA ethyl esters *in vivo* (Fig. 2) and the enzymatic activity of the pure proteins as determined *in vitro* (Fig. 4). *In vitro*, Eht1 preferred short-chain substrates (highest production was for ethyl butanoate), whereas Eeb1 preferred longer chain substrates (highest production was for ethyl octanoate). The difference between the *in vitro* preference and the results of the *in vivo* deletion analysis might be due to differences in substrate availability for the synthesis of the different MCFA ethyl esters *in vivo*. Our results (Fig. 3) clearly show that substrate availability is a major factor determining MCFA ethyl ester accumulation *in vivo*. The intracellular localization of the enzymes can also strongly influence substrate availability and activity. Eht1 is known to be located in lipid particles (23). These are composed of a highly hydrophobic core formed from neutral lipids (triacylglycerols and steryl esters) surrounded by a phospholipid monolayer in which only a few proteins are embedded. Lipid particles serve as an energy source and/or as a source of fatty acids and sterols needed for membrane biogenesis. In 1999, Athenstaedt *et al.* (23) discovered that an *eht1*Δ strain contains higher amounts of triacylglycerols and steryl esters, compared with a wild type strain. These results fit with our discovery that Eht1 is involved in MCFA ethyl ester biosynthesis. The localization of Eeb1 is unknown.

The presence of both MCFA ethyl ester synthase and esterase activity in the purified Eht1 and Eeb1 proteins explains why we did not observe an increase in the production of MCFA ethyl esters in the strains with overexpression of these proteins. Since the provision of hexanoic or octanoic acid caused a strong increase in the formation of the corresponding ethyl ester (Fig. 3), substrate availability cannot have been a limiting factor in the overexpression strains. The provision of hexanoic or octanoic acid did not cause any further increase in ethyl ester formation in the overexpression strains compared with the empty vector control strain. In the case of acetate ester synthesis, overexpression of the *ATF1* gene caused a strong increase in acetate ester production, indicating that in this case enzymatic synthase activity rather than substrate availability is a major limiting factor (7). The combined presence of MCFA ethyl ester synthase and esterase activity in the Eht1 and Eeb1 proteins raises questions as to the precise regulation of the balance between MCFA ethyl ester synthesis and hydrolysis *in vivo*.

The three-dimensional structure prediction of Eht1 and Eeb1 provided further support for their enzymatic function. The structure prediction allowed us to identify the catalytic triad (Ser-Asp-His), which is known to be responsible for the transesterification reaction, and is thus involved both in ester synthase activity and esterase activity (22). The substrate preference of Eht1 and Eeb1 *in vitro* was different and might be explained at the molecular level by the different amino acid residues at the end of the tunnel-shaped catalytic site in the predicted three-dimensional structure of the proteins.

S. cerevisiae May Contain Additional Medium-chain Fatty Acid Ethyl Ester Synthase Enzymes besides Eht1 and Eeb1—The results obtained for MCFA ethyl ester production in the double deletion strains indicate that Eht1 and Eeb1 are responsible for the majority of MCFA ethyl ester synthesis in yeast (Fig. 2). On the other hand, although the double deletion of *EHT1* and *EEB1* caused a pronounced drop in the production of all MCFA ethyl esters, only the production of ethyl hexanoate was virtually eliminated and that of ethyl octanoate was reduced with 70%. For ethyl butanoate and ethyl decanoate the reduction was less than 50%. Hence, yeast cells must contain one or more additional enzymes responsible for MCFA ethyl ester synthesis. In the case of ethyl octanoate and ethyl decanoate production, additional deletion of *YMR210w* in the *eht1*Δ *eeb1*Δ strain produced a further drop in their level, but in the case of ethyl butanoate there was little effect. Hence, at least in the case of ethyl butanoate, one would expect the existence of one or more additional enzymes that can support its synthesis. On the other hand, non-enzymatically catalyzed chemical synthesis of ethyl butanoate might also occur. Although the *YMR210w* gene product displays more limited sequence similarity to Eht1 and Eeb1, the results with the triple deletion mutant suggest that it might contain residual MCFA ethyl ester synthesis activity. As opposed to *EHT1* and *EEB1*, however, single deletion of *YMR210w* had no significant effect on the production of any of the four MCFA ethyl esters. Hence, at present, the evidence to link the gene with MCFA ethyl ester biosynthesis is weak.

Previously, the genes *ATF1*, Lg-*ATF1* (only present in lager yeast), and *ATF2* have been identified as encoding acetate ester synthase enzymes. Double deletion of *ATF1* and *ATF2*, however, does not affect MCFA ethyl ester synthesis (7). Hence, given the residual activity in the

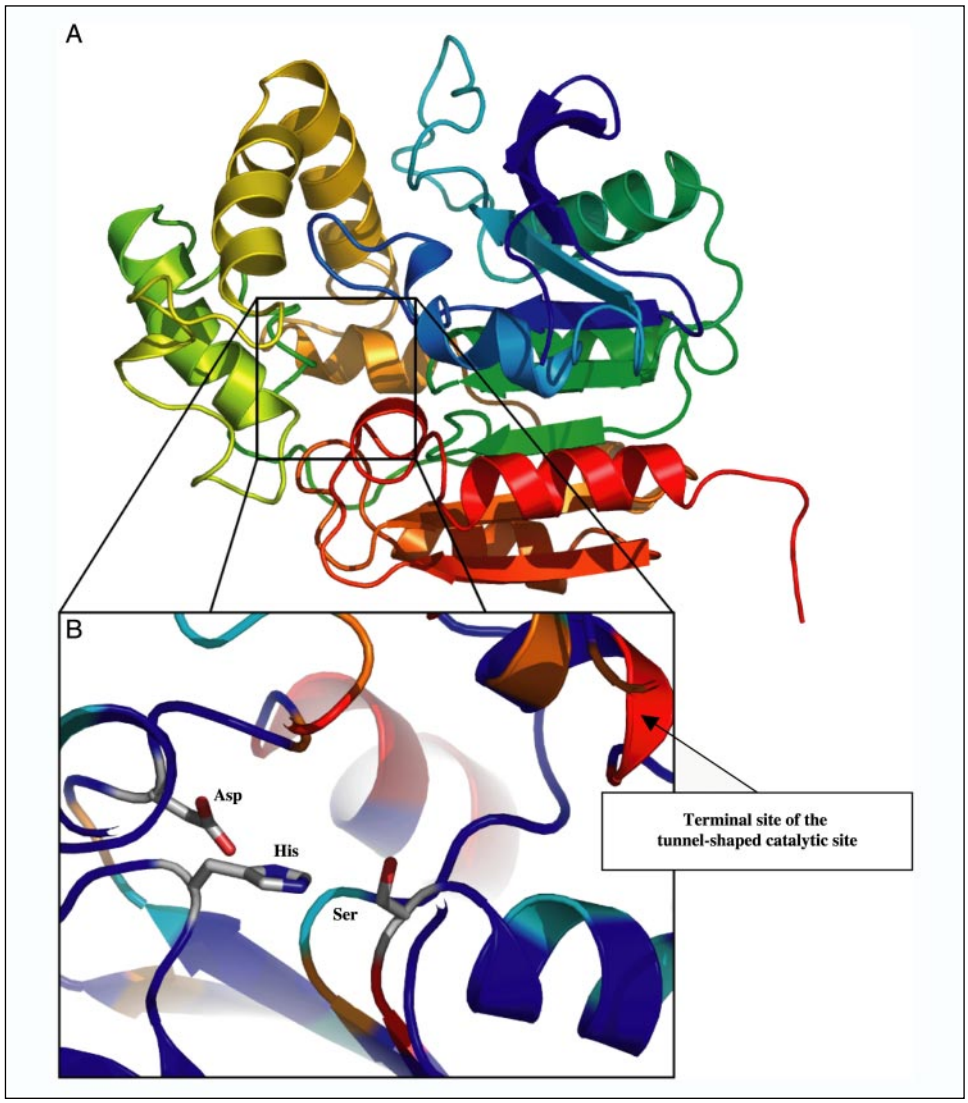

FIGURE 6. **Modeling of the three-dimensional structure of Eht1.** *A*, the tertiary structure of Eht1 colored by spectrum *blue* to *red* (N- to C-terminal part, respectively). *B*, enlargement of a view (*top* of *A*) of the catalytic triad (Ser-Asp-His) typically present in esterases and a top view on the tunnel (coloring *blue* to *red* by homology: *dark blue* for identical residues to *red* for residues completely different between Eht1 and Eeb1).

*eht1*Δ *eeb1*Δ double deletion mutant, there must still exist unknown genes involved in ester biosynthesis in yeast.

Physiological Role of MCFA Ethyl Ester Biosynthesis—Our results suggest a possible role of Eht1 and also Eeb1 in cellular lipid metabolism and detoxification processes. Indeed, we show that these two enzymes are involved in the esterification of MCFAs, which are toxic compounds for yeast (24). Hence Eht1 and Eeb1 could thus be involved in their detoxification by esterification. In mammals, FAEE synthases synthesize FAEEs from endogenous fatty acids and alcohol (25). Despite the wide distribution of this enzyme in mammals, most of its known substrates are foreign compounds that are not normally involved in intermediary metabolism. Some of their known substrates are analogs of physiologically or pharmacologically important compounds. Therefore, FAEEs may play a role in the detoxification system of the body by conjugating compounds with ester and amide bonds (4). Fatty acid conjugation appears one of the pathways for the disposition of many xenobiotic compounds (26). It is possible that also in yeast, endogenous produced MCFAs are toxic, as observed for exogenously added MCFAs. In yeast, MCFAs can be released during fatty acid synthesis under certain conditions. Upon release from the fatty acid synthase complex, these MCFAs rapidly dissociate and are thus unable to cross cellular membranes (27). Esterification allows (partial) diffusion of the fatty acid residues and could thus serve as a strategy to remove these toxic substrates. The fact that Eht1 and other ester synthases have been localized in the cellular lipid particles further supports this hypothesis (23, 28). Our identification of Eht1 and Eeb1 as two novel enzymes involved in MCFA ethyl ester biosynthesis creates new approaches to explore the true physiological function of MCFA ethyl ester formation in yeast and use this system as a model for similar processes in higher eukaryotes.

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