Applied and Environmental Microbiology, May 2003, p. 2512–2520 Vol. 69, No. 5
0099-2240/03/$08.00+0 DOI: 10.1128/AEM.69.5.2512–2520.2003
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The *ftsH* Gene of the Wine Bacterium *Oenococcus oeni* Is Involved in Protection against Environmental Stress

Jean-Paul Bourdineaud,* Benjamin Nehmé, Sonia Tesse, and Aline Lonvaud-Funel

Faculté d'Oenologie, Université de Bordeaux II, 33405 Talence Cedex, France

Received 30 July 2002/Accepted 25 January 2003

The wine bacterium *Oenococcus oeni* has to cope with harsh environmental conditions, including an acidic pH, a high alcoholic content, nonoptimal growth temperatures, and growth-inhibitory compounds such as fatty acids, phenolic acids, and tannins. We describe the characterization and cloning of the *O. oeni ftsH* gene, encoding a protease belonging to the ATP binding cassette protein superfamily. The *O. oeni* FtsH protein is closest in sequence similarity to the FtsH homologue of *Lactococcus lactis*. The *O. oeni ftsH* gene proved to be stress-responsive, since its expression increased at high temperatures or under osmotic shock. *O. oeni* FtsH protein function was tested in an *Escherichia coli ftsH* mutant strain, and consistent with the *O. oeni ftsH* gene expression pattern, the *O. oeni* FtsH protein provided protection for the *E. coli ftsH* mutant against heat shock. *O. oeni* and *Bradyrhizobium japonicum* FtsH proteins also triggered *E. coli* resistance to wine toxicity. Genes homologous to *O. oeni ftsH* were detected in many other lactic acid bacteria found in wine, suggesting that this type of gene constitutes a well-conserved stress-protective molecular device.

The winemaking process consists of two main steps: alcoholic fermentation by yeasts, followed by a process whereby lactic acid bacteria transform malic acid into lactic acid. This so-called malolactic fermentation is favored by winemakers because it improves the organoleptic properties of wines, decreases acidity, and improves microbial stability. Although many lactic acid bacterial species are present in grape must, a natural selection occurs throughout alcoholic fermentation, and *Oenococcus oeni* finally becomes the dominant species among those triggering the malolactic fermentation (14). *O. oeni* is indeed the bacterium best adapted to such an aggressive ecological medium as wine, and its growth is necessary in order to achieve a high sensory quality of wine by means of malolactic fermentation. However, it may happen that growth of *O. oeni* stops, thereby preventing the malolactic fermentation. This problem is attributed to various toxic compounds contained in wine and to inhibitory environmental parameters (13). Indeed, the pH of wine is low (between 2.5 and 3), and the temperature in the fermentation tanks is not always optimal and varies throughout the whole process. In addition, the wine contains around 12% alcohol, which constitutes the main toxic compound, and several other bacterial growth inhibitors, such as free fatty acids (decanoic and dodecanoic acids are powerful inhibitors of lactic acid bacterial growth) (4, 12), some phenolic acids, and oak wood ellagitannins (24). Thus, an understanding of the adaptation and resistance of *O. oeni* bacteria to environmental stress would be most valuable.

In addition to the lactic acid bacteria occurring naturally in wine, starter culture strains of *O. oeni* are often used during the winemaking process to improve the efficiency of the malolactic fermentation. Differences between starter culture strains are related to their inherent stress resistance. Exposure to a mild stress can result in improved resistance to subsequent exposures, either to more extreme forms of the same stress or to other stresses. These phenomena are referred to as acquired stress resistance and cross protection. The choice of an inherently stress-tolerant strain, as well as the specific preparation procedure used during the production of commercial active dried wine starter cultures, can therefore affect the degree of viability and vitality, as well as the subsequent fermentation performance of the cultures. For instance, it has been observed that an acidic treatment gives rise to a subsequent increase in sulfite tolerance in *O. oeni* (7). The better and faster a wine starter culture strain is able to adapt to changes in the environment, the faster fermentation will be completed. Since an efficient alcoholic and malolactic fermentation process is of great importance to wine quality, it is essential to understand the basic physiology and genetics associated with enhanced stress tolerance in wine starter culture strains.

Bacteria have evolved sophisticated mechanisms to enable them to survive a variety of environmental stresses. Among the stress genes whose expression changes in response to various stimuli in *Escherichia coli*, the *ftsH* gene appears to be necessary for bacterial life and also appears to be heat inducible (8, 22). The *E. coli ftsH* (for filamentation temperature sensitive) gene encodes a 71-kDa membrane-anchored ATP-dependent metalloprotease. FtsH belongs to the AAA family (ATPases associated with diverse cellular activities), members of which are found universally in prokaryotes and in mitochondria and chloroplast eukaryotes. A regulatory function of FtsH in *E. coli* is the degradation of the heat shock factor σ^{32} under normal growth conditions, where only minute amounts of this alterna-

* Corresponding author. Present address: Laboratoire d'Ecophysiologie et Ecotoxicologie des Systèmes Aquatiques, CNRS (UMR EPOC 5805), Université de Bordeaux I, Place du Docteur Peyneau, 33120 Arcachon, France. Phone: (33) 556 223 926. Fax: (33) 556 549 383. E-mail: jp.bourdineaud@ecotox.u-bordeaux.fr.

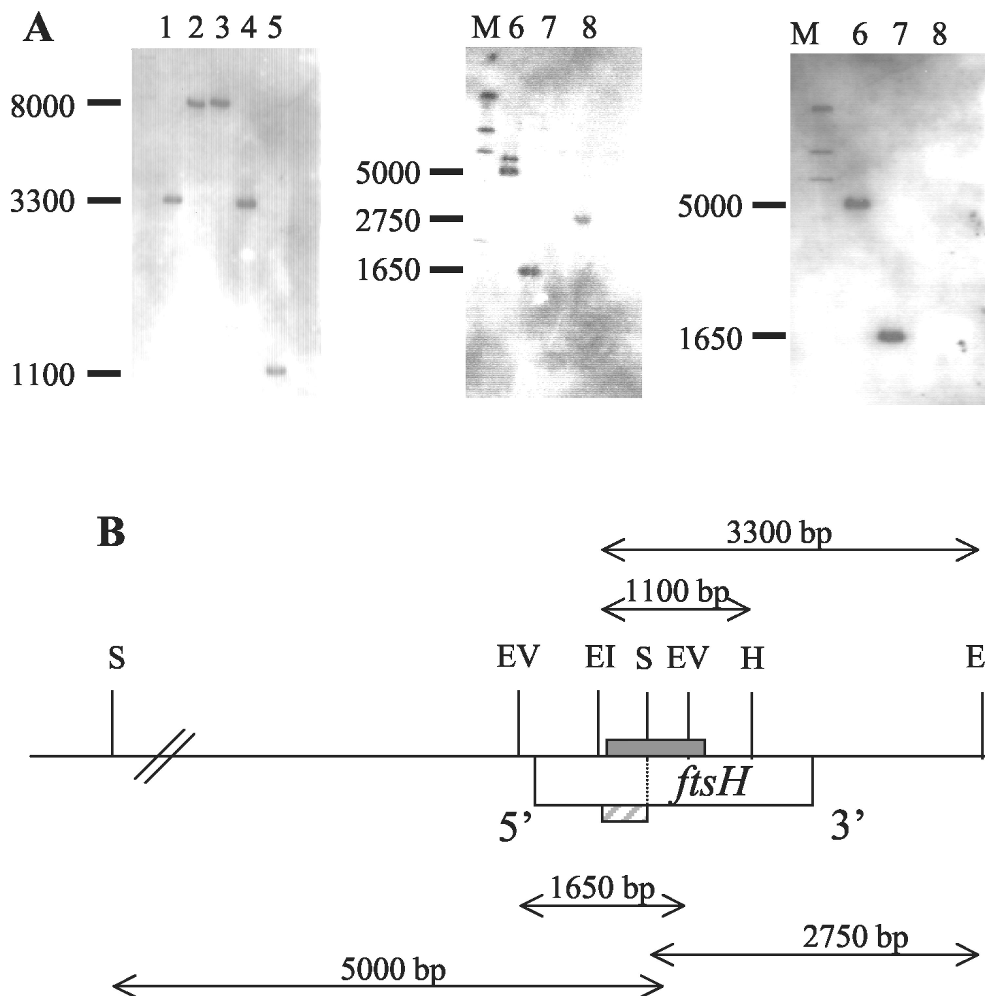

FIG. 1. Restriction mapping of the *O. oeni ftsH* gene. (A) Southern blots were hybridized with either the ftsH probe (left and middle panels) or probe 2 (right panel). The *O. oeni* genomic DNA was digested with the following enzymes: lane 1, *Eco*RI; lane 2, *Bam*HI; lane 3, *Hin*dIII; lane 4, *Eco*RI and *Bam*HI; lane 5, *Eco*RI and *Hin*dIII; lanes 6, *Sal*I; lanes 7, *Eco*RV; lanes 8, *Eco*RI and *Sal*I. Lanes M, molecular size markers (in base pairs). (B) Physical map of the *O. oeni ftsH* gene. Restriction enzyme sites: EI, *Eco*RI; EV, *Eco*RV; H, *Hin*dIII; S, *Sal*I. The location of the ftsH probe is indicated by a grey box above the *ftsH* gene; the location of probe 2 is indicated by a hatched box below the *ftsH* gene. Distances between restriction sites are indicated.

tive sigma factor are necessary (8, 23). FtsH serves other functions in addition to being a protease. It participates in the assembly of proteins into the membrane and in the translocation of exported proteins (1, 18).

Genes homologous to *E. coli ftsH* have been discovered in various other bacteria and were associated with stress resistance. A *Lactococcus lactis ftsH* mutant showed growth defects either with 4% NaCl or at 38°C (17). Growth of *Bacillus subtilis* during osmotic shock required an increased expression of the *ftsH* gene (3), and disruption of the *ftsH* gene led to the loss of viability of *Helicobacter pylori* (5) and of *Bradyrhizobium japonicum* (16).

The objective of the present study was to clone and characterize the *O. oeni ftsH* gene and study its role in the stress response. We here describe the cloning of the *O. oeni ftsH* gene and show that the expression of this gene responds to stress such as that caused by heat and salt. In addition, we show that the survival rate of an *E. coli* Δ*ftsH* mutant when exposed to wine toxicity improves when it is transformed with the *ftsH* gene from *O. oeni* or *B. japonicum*.

MATERIALS AND METHODS

Materials. The wine used was red wine from the Bordeaux region, consisting of a combination of Cabernet Franc, Cabernet Sauvignon, and Merlot musts. The pH was 3.4, the ethanol content was 12%, and the sulfite content was 20 mg/liter.

Strains and growth conditions. The *O. oeni* strain used was *O. oeni* IOEB 8406. This strain was grown at 25°C in medium adjusted to pH 5 and containing the following (per liter): yeast extract, 4 g; beef extract, 8 g; Bacto Peptone, 10 g; glucose, 10 g; fructose, 10 g; malic acid, 10 g; KH_2PO_4, 2 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $MnSO_4 \cdot H_2O$, 0.1 g; and Tween 80, 1 ml. *E. coli* strain XL1Blue (Stratagene) was used for cloning procedures. The Δ*ftsH E. coli* strain AR3291 (W3110 *zad220*::Tn*10 sfhC21* Δ*ftsH3*::*kan*) (20) was used to study *O. oeni* FtsH protein function. The *E. coli* strains used were grown in Luria-Bertani (LB) medium supplemented with appropriate antibiotics at the following concentrations when necessary: ampicillin, 50 μg/ml; chloramphenicol, 10 μg/ml; and kanamycin, 50 μg/ml.

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                  *  +          +   +         +  *        +       +  + +
oeni       (1)  MK-N---KNRGFFRSSLSYAFVILAVIFLIYSFFGRSD-GSVKHLSTTTFLKELKNNKIK
lactis     (1)  MNNNkqpKQGNFVKNILMWVILAIVVVVGFNFFFSSNQ-SSVDKISYSQLMTKLDGNKIE
japonicum  (1)  MNAN--------LRNFALWVIIVLLLLALFTLFQNPGQrASSQDIAFSQLLSEVDRGNVR
coli       (1)  MA-----------KNLILWLVIAVVLMSVFQSF-GPSE-SNGRKVDYSTFLQEVNNDQVR

                   ++        *                              +  *  + +  +
oeni       (56) DFTIQPGDSgVYTIAGDFKKAQKsSSSSSSTTLLSGYQSSVTKFTAYVLPNNSSLKQITT
lactis     (60) NVTMQPSDS-LITVTGEYKEPVK-VKGTNNFPLLGNSSSEVKNFQAYIIPTDSVVKDIQN
japonicum  (53) DVVIQ----------G---------------PDIHGTFTNGSSFQTYA-PNDPTL--VKR
coli       (48) EARIN----------G---------------REINVTKKDSNRYTTYIPVQDPKL--LDN

                     *    +     +  +   +   * +   +   *       ++   + ** *  *
oeni      (116) AAQKAGVAVNPKPAASN-FW-GSM-LTLILPTLIMFAlLYWMLIGSQRGQGGSGGPGGIM
lactis    (118) AAKSNDVKLSVVQASSSGMW-VQI-LSYIIPMLLFVG-IFWLMMGG-MGARGGGGGGNPM
japonicum  (85) LYDSK-VQITAKPPGDNVPWfVSLLVSWL-PFIALIG--VWIFLSRQMQ----GGAGKAM
coli       (81) LLTKN-VKVVGEPPEEPSLL-ASIFISWF-PMLLLIG--VWIFFMRQMQ--GGGGKG-AM

                +**+*+ *        * *+**** * *+++ *+*++*  * ++  ** +** ***+ *
oeni      (173) SFG-SKAKPADPKQNKIRFADVAGEEEEKQELVEVVEFLKDPKKFTKLGARIPKGVLLEG
lactis    (174) SFGKSRAKQQDGKTSKVRFADVAGSEEEKQELVEVVDFLKNPKKYHDLGARIPAGVLLEG
japonicum (137) GFGKSRAKMLTEAHGRVTFEDVAGVDEAKQDLQEIVEFLRDPGKFQRLGGRIPRGVLLVG
coli      (133) SFGKSKARMLTEDQIKTTFADVAGCDEAKEEVAELVEYLREPSRFQKLGGKIPKGVLMVG

                Walker A                                      Walker B
                ********+*+*+**** +**+ ******************* ** *** ** ******
oeni      (232) PPGTGKTLLAKAVAGEAKTPFFSISGSDFVEMFVGVGASRVRDLFENAKKSAPSIIFIDE
lactis    (234) PPGTGKTLLAKAVAGEAGVPFFYISGSDFVEMFVGVGASRVRDLFENAKKTAPSIIFIDE
japonicum (197) PPGTGKTLIARAVAGEANVPFFTISGSDFVEMFVGVGASRVRDMFEQAKKNAPCIIFIDE
coli      (193) PPGTGKTLLAKAIAGEAKVPFFTISGSDFVEMFVGVGASRVRDMFEQAKKAAPCIIFIDE

                                                         SRH Motif
                ****** ****+***+*********+*+*****  ++++++*++*+***+*********+
oeni      (292) IDAVGRRRGAGMGGGNDEREQTLNQILIEMDGF--EGSEGVIVLASTNRSDVLDPALLRS
lactis    (294) IDAVGRQRGAGLGGGNDEREQTLNQLLVEMDGFqdDGN-SVIVIAATNRSDVLDPALLRP
japonicum (257) IDAVGRHRGAGLGGGNDEREQTLNQLLVEMDGF--EANEGVILIAATNRPDVLDPALLRP
coli      (253) IDAVGRQRGAGLGGGHDEREQTLNQMLVEMDGF--EGNEGIIVIAATNRPDVLDPALLRP

                ***** + *+ *** *** +*+**     *** * ++  +*  ***+ **** *+ *****
oeni      (350) GRFDRKILVGAPDVKGREAILRVHAKNKPLAADVDLKVIAQQTPGFVGADLENLLNEAAL
lactis    (353) GRFDRKVLVGAPDVKGREAVLKVHAKNKPLASDVDLHNVATQTPGYVGADLENVLNEAAL
japonicum (315) GRFDRQVVVPNPDVVGREQILKVHVRKVPLAPDINLKTIARGTPGFSGADLMNLVNEAAL
coli      (311) GRFDRQVVVGLPDVRGREQILKVHMRRVPLAPDIDAAIIARGTPGFSGADLANLVNEAAL

                 *** ++  +  +   ++ *  + *   +     +  +  +  ****+****+*+
oeni      (410) LAARNDEKAVTAADIDEAEDRVIAGPAKKDRKTTQDERETVAYHEAGHAIVGLVLNDAQV
lactis    (413) VAARQNKKEINAADIDEGMDRAMAGPAKKDRIQSMREREIVAYHEAGHAIVGLVLENGST
japonicum (375) TAARRNKRMVTQAEFEEAKDKVMMGAERKSLVMTEEEKLLTAYHEGGHAIVGLNVPATDP
coli      (371) FAARGNKRVVSMVEFEKAKDKIMMGAERRSMVMTEAQKESTAYHEAGHAIIGRLVPEHDP

                + *+*+ ****+ *      +*         +        ++ ++***  ++*+  +      **+
oeni      (470) VRKVTIVPRGRAGGYALMMPKDERYLMSEKDAKEELAGLMGGRAA-EILIN-HVASSGAS
lactis    (473) VRKVTVVPRGRIGGYMLALPDEEIMQPTNFHLQDQLASLMGGRLGEEIVFG--VATPGAS
japonicum (435) IHKATIIPRGRALGMVMQLPERDKLSMSLEQMTSRLAIMMGGRVAEELIFGREKVTSGAA
coli      (431) VHKVTIIPRGRALGVTFFLPEGDAISASRQKLESQISTLYGGRLAEEIIYGPEHVSTGAS
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                 +*+  **  ** ***  * * +** + + *   +** *         *  *   ** *++
oeni       (528) NDFQQATQIAREMVTQYGMSDKLGMVQL-EGSSNVFVGDPNNPNPPYSQKTSELIDEEVR
lactis     (531) NDIEKATHIARSMVTEYGMSKKLGMVSY-EGDHQVFIGRDYGQTKTYSEATAVMIDDEVR
japonicum  (495) SDIEQATRLARMMVTRWGLSEELGTVSYGENQDEVFLGMSVSRTQNASEATVQKIDSEIR
coli       (491) NDIKVATNLARNMVTQWGFSEKLGPLLYAEEEGEVFLGRSVAKAKHMSDETARIIDQEVK

                 ++  + * +*            * + +*+++**++  +*  *   +  +         *
oeni       (587) RLTNEAYKRAVDIIKSHPKQHKAIAEALLKYETLDEAQIRSLFETGEIPsDLVKDSQRPA
lactis     (590) RILGEAYDRAKEAIETHREQHKAIAEALLKYETLDAKQIMSLFKTGKMP-DEAAAAEVP-
japonicum  (555) RLVEEGYKEATRILTEKHGDLEALAKGLLEFETLSGDEIVDLLK-GKKP--NRESVLEPT
coli       (551) ALIERNYNRARQLLTDNMDILHAMKDALMKYETIDAPQIDDLMA--------RRDV-RP-

                  *   ++        ++       *
oeni       (647) RPLSYEEskAALKKNGAvdNKEAEDELkrtKMIrKMKMIPSRVPIQLQKRRQPRRLQLL-
lactis     (648) EPKTFEE---SLKDANA-----NVDDF---SNI-NIYNGDEKTDSKPEENKEKSEDETA-
japonicum  (612) TPRA-----SAVPPAGK--SRPRPDP-------------DPGLEPQPQA-----------
coli       (601) -PAGWEE-------PGA--SNNSGDN------------GSPKAPRPVDEPRTPNPGNTMs

oeni       (706) DAVNNKfd
lactis     (695) E-------
japonicum  (641) --------
coli       (639) EQLGDK--
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FIG. 2. Comparison of the amino acid sequences of FtsH proteins from *O. oeni*, *L. lactis*, *B. japonicum*, and *E. coli*. The first residue in each row is numbered. A asterisk above the sequences indicates a perfect consensus between the four protein sequences. A cross indicates three matching residues among the four sequences. The predicted transmembrane α-helices of FtsH proteins are underlined. The Walker A and B motifs are indicated. The SRH motif is indicated and appears in boldface. The boxed HEAGH domain is the zinc binding motif. Dashes indicate gaps introduced to optimize the alignment.

Plasmids used. Plasmids pBluescript II KS(+) (Stratagene) and pGEM-T (Promega) were used for cloning and sequencing. Both harbor the ampicillin resistance marker. Plasmid pCR-XL-TOPO (Invitrogen) was used as an expression vector in *E. coli*. This plasmid harbors the kanamycin resistance marker. Plasmids pBAD18-Cm (6) and pRJ5188 (harboring the *B. japonicum ftsH* gene) (16) were used to test the ability of the *B. japonicum ftsH* gene to enhance bacterial resistance to a wine shock. Both of these plasmids harbor the chloramphenicol resistance marker.

Construction of a probe specific to the *ftsH* gene of *O. oeni*. To obtain a probe for an *O. oeni* gene homologous to previously characterized bacterial *ftsH* genes, redundant primers were deduced from an alignment of the FtsH protein sequences from *H. pylori*, *L. lactis*, *E. coli*, and *B. subtilis*. The chosen primers were specific to the Walker A motif and the zinc binding motif, respectively. PCR was performed on *O. oeni* IOEB 8406 genomic DNA (100 ng) in a final volume of 100 μl with a 1 μM concentration of each primer, a 0.2 mM concentration of each of the four deoxynucleoside triphosphates (dNTPs), 8 mM $MgCl_2$, and 5 U of *Taq* polymerase in the appropriate commercial buffer. The PCR program consisted of 40 cycles of 1 min at 94°C, 1 min at 45°C, and 1.5 min at 72°C, followed by 10 min at 72°C. The primers F1 (5′-CGGAATTCCGGICCICCIGGIACIGGIAA[A,G]AC-3′) and F2 (5′-CGAAGCTTGC[A,G,T]ATIGC[A,G]TGICCIGC[C,T]TC[A,G]TG [A,G]TAIGC-3′) produced an amplicon of 710 bp. This amplified product was digested with *Bam*HI and *Eco*RI, since these sites were included in the design of primers F1 and F2, respectively. The digested fragment was cloned in pBluescript II KS(+) and sequenced. Similarities between this fragment and various bacterial genes homologous to *ftsH* confirmed that we had amplified the internal portion of a homologous gene from *O. oeni*. This probe was called ftsH.

Southern blot analysis. Chromosomal DNA was isolated from the *O. oeni* IOEB 8406 strain as follows. A 1-g cell pellet from an exponential-phase culture was washed with 10 mM EDTA–25 mM Tris-HCl (pH 8) and resuspended in 10 ml of this buffer containing 10% glycerol. Solid lysozyme (10 mg) was added, and the cells were allowed to digest overnight at 37°C. Proteinase K was added to a final concentration of 0.2 mg/ml, and incubation was continued for 12 h at 50°C. One milliliter of 10% *N*-laurylsarcosinate was added, and incubation was continued for an additional 12 h at 50°C. Twenty milliliters of 95% ethanol

FIG. 3. The *O. oeni ftsH* gene is a stress-responsive gene. The induction of *O. oeni ftsH* gene expression after heat or osmotic shocks was analyzed by RT-PCR. The RT-PCR patterns were obtained with cDNA from the RNA of *O. oeni* IOEB 8406 extracted after 3 h of application of the indicated thermal or osmotic stress. The gene-specific primers that were used are indicated on the bottoms of the panels.

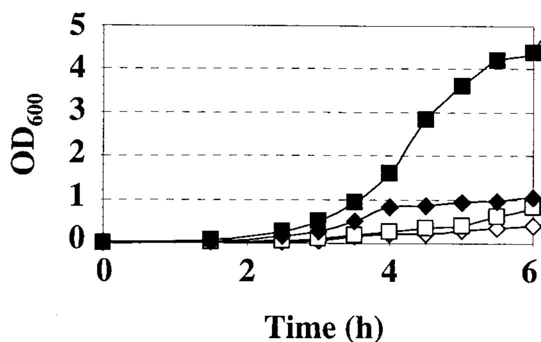

FIG. 4. Growth complementation of an *E. coli* Δ*ftsH* mutant by the *O. oeni ftsH* gene. The *E. coli* Δ*ftsH* strain AR3291 transformed with pCR-XL-TOPO (diamonds) or with pJPB65 (squares) was grown in LB medium at either 25°C (open symbols) or 37°C (closed symbols). OD_{600}, optical density at 600 nm.

was added, and the high-molecular-weight DNA was spooled out on a glass rod. This was rinsed twice in 70% ethanol, air dried briefly, and then redissolved in 4 ml of 1 mM EDTA–10 mM Tris-HCl (pH 8). *O. oeni* IOEB 8406 genomic DNA was digested with appropriate restriction enzymes and transferred onto a Hybond-N$^+$ membrane (Amersham) under vacuum. Hybridizations (overnight at 65°C) were performed with probes labeled with digoxigenin-labeled 11-dUTP by using the DIG-DNA labeling and detection kit (Roche). Detection was carried out by chemiluminescence, using an anti-digoxigenin antibody and CDP-star (Roche), as recommended by the manufacturer.

Cloning of the *O. oeni ftsH* gene by inverse PCR. A precise restriction map was first established by hybridization of digested *O. oeni* DNA with the ftsH probe and a probe generated by restriction of the ftsH probe with the *Sal*I enzyme (probe 2). Probe 2 allowed the orientation of the *Eco*RI and *Eco*RV restriction sites with respect to the *Sal*I site, internal to the region hybridizing with the ftsH probe (Fig. 1).

The 5′ side of the *ftsH* gene was then cloned by inverse PCR (19) from genomic *O. oeni* DNA made of closed circular fragments generated by *Eco*RV enzyme restriction and ligation with T4 DNA ligase. Primers FTSH1 (5′-CGA CGGCTTTAGCGAGCAAA-3′) and FTSH2 (5′-AAAAAGCTGTTACAGCA GCT-3′) were selected from the ftsH probe sequence, and the amplification of a 1,200-bp fragment was then able to take place. The polymerase used was the Expand enzyme (a blend of *Taq* and *Pwo* polymerases) (Boehringer). PCR was performed with 200 ng of DNA, prepared as described above, in a final volume of 100 μl with a 2 μM concentration of each primer, a 0.2 mM concentration of each of the four dNTPs, and 1.5 μl of Expand enzyme in the appropriate commercial buffer. The PCR program used consisted of 39 cycles of 1 min at 94°C, 1 min at 50°C, and 3 min at 72°C, followed by 10 min at 72°C. The fragment was purified and cloned in plasmid pGEM-T to yield pJPB60. The 3′ side of the *ftsH* gene was cloned by inverse PCR from genomic *O. oeni* DNA made of closed circular fragments generated by *Eco*RI enzyme restriction and ligation with T4 DNA ligase. PCR with the Expand enzyme was carried out with FTSH1 and FTSH2 primers, and a 2,750-bp fragment encompassing the 3′ side of the *ftsH* gene was amplified. This fragment was purified and cloned in plasmid pCR-XL-TOPO to yield pJPB62. The fragment inserted in pJPB62 was sequenced.

Next, the complete *O. oeni ftsH* gene was cloned in pCR-XL-TOPO to take advantage of the ability to express the FtsH protein through the control of the *lac* promoter. PCR was carried out with the FTSH3 (5′-GTTAACCCCGATAATT TATTCATAATG-3′) and FTSH4 (5′-GCGACCAGCTAAAGTCGCGTTTT G-3′) primers. PCR was performed with 200 ng of genomic *O. oeni* DNA in a final volume of 100 μl with a 1 μM concentration of each primer, a 0.2 mM concentration of each of the four dNTPs, and 1.5 μl of Expand enzyme in the appropriate commercial buffer. The PCR program used consisted of 39 cycles of 1 min at 94°C, 1 min at 50°C, and 1.5 min at 72°C, followed by 10 min at 72°C. The FTSH3 primer encompasses the region from position −61 to −35 of the *ftsH* promoter sequence. This oligonucleotide therefore allowed cloning of the *O. oeni ftsH* gene with its own promoter and ribosome binding site. The FTSH4 primer represents the region from position +2229 to +2206 of the *ftsH* gene. Thus, its 5′ extremity ends 81 nucleotides after the *ftsH* stop codon. A 2,290-bp fragment was amplified, purified, and cloned in pCR-XL-TOPO to yield pJPB65. We expected to find a 50:50 distribution in the orientation of inserted fragments. However, none of the 24 positive recombinant clones that were tested contained the cloned *ftsH* gene in the correct orientation (under the control of the *lac* promoter), and all contained the *ftsH* gene in the incorrect orientation. This is compatible with a *lac* promoter-monitored toxic level of *ftsH* gene expression (even under noninduced conditions) and is in keeping with the observation that overproduction of the *H. pylori ftsH* gene considerably reduced the growth rate of *E. coli* host cells (5). Nevertheless, the plasmid pJPB65, containing the *ftsH* gene in the opposite orientation with respect to the *lac* promoter, allowed the study of the FtsH protein function. In this construct the *ftsH* gene is not expressed under its own promoter and benefits only from its own ribosome binding site. Despite this, a low level of *ftsH* expression was possible, as observed by reverse transcription-PCR (RT-PCR) (data not shown).

RNA isolation and RT-PCR analysis. The mRNAs from the *O. oeni* IOEB 8406 strain were extracted after 3 h of incubation under various conditions. We tested the following environmental factors: heat shock (37°C), osmotic shock (0.5 M NaCl), alcohol addition (20%), sulfite addition (100 mg/liter), and ethidium bromide addition (20 μg/ml). The control culture was grown at 25°C at pH 5.0 in modified MRS medium. RNA preparation was carried out with the StrataPrep Total RNA Miniprep kit (Stratagene), which includes a DNase (RNase-free) treatment. Bacterial cells were disrupted with glass beads (0.1-mm diameter) by shaking on a vortex mixer. The quality of the RNA samples was checked on a 1% (wt/vol) agarose gel, and the concentration was determined by measuring absorbance at 260 nm. The corresponding cDNAs were synthesized by reverse transcription with murine leukemia virus reverse transcriptase and the ProSTAR first-strand RT-PCR kit (Stratagene). The reverse transcriptase reaction used random primers from the kit with 10 to 15 μg of total RNA, in a final volume of 50 μl. Three microliters of the first-strand cDNA synthesis reaction product was used as a template for PCR amplifications with specific primers. Thirty-five cycles were carried out, each

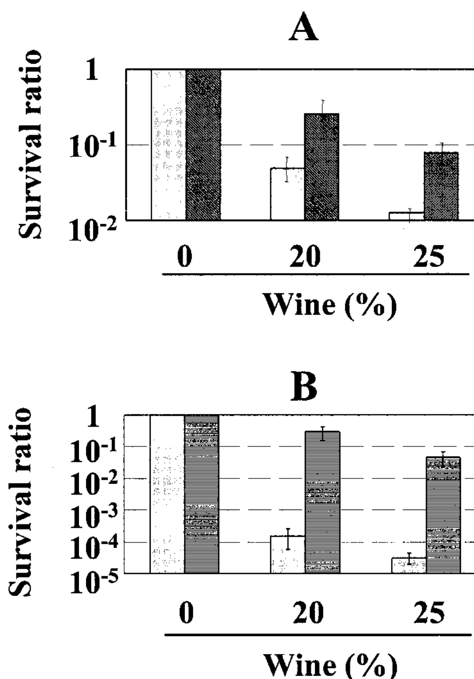

FIG. 5. The *O. oeni* and *B. japonicum ftsH* genes protect *E. coli* from wine toxicity. The *E. coli* Δ*ftsH* strain AR3291 was transformed with control plasmids (pCR-XL-TOPO [A] or pBAD18-Cm [B]) (light-gray bars) and *O. oeni* or *B. japonicum ftsH* gene-containing plasmids (pJPB65 [A] and pRJ5188 [B], respectively) (dark-gray bars). After 3 h of incubation at 25°C with the indicated concentrations of wine, the bacterial cultures were serially diluted and plated. Colonies were counted, and the survival ratios (ratios of CFU observed at a given concentration of wine to those observed without added toxic compounds) were calculated.

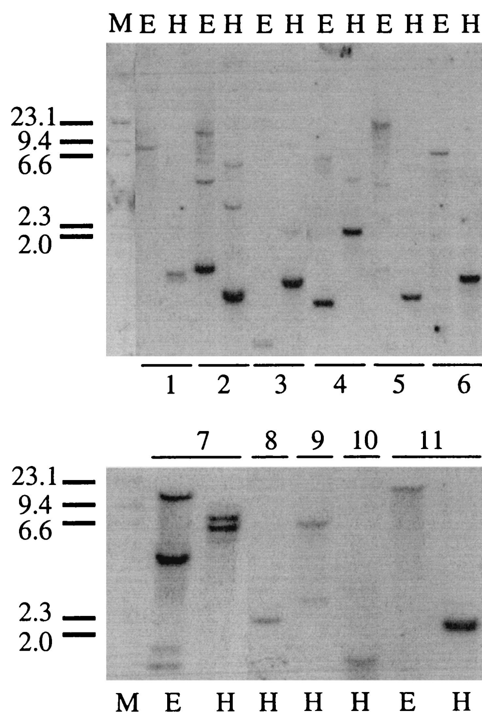

FIG. 6. *ftsH* homologues in other wine lactic acid bacteria. Southern hybridization of the ftsH probe to chromosomal DNAs from various bacteria is shown. The genomic DNAs were digested before transfer with either the *Eco*RI (lanes E) or *Hin*dIII (lanes H) enzymes. Lanes M, molecular size markers (in kilobase pairs). Lanes: 1, *Lactobacillus fructivorans* ATCC 8288; 2, *P. pentosaceus* ATCC 33326; 3, *P. dextrinicus* ATCC 33087; 4, *Lactobacillus plantarum* IOEB 9106; 5, *P. parvulus* ATCC 19371; 6, *Lactobacillus buchneri* ATCC 11305; 7, *P. damnosus* ATCC 25248; 8, *Lactobacillus hilgardii* IOEB 9101; 9, *Lactobacillus brevis* ATCC 14869; 10, *Lactobacillus delbrueckii* ATCC 9649; 11, *Leuconostoc mesenteroides* IOEB 8293.

consisting of 30 s of denaturation at 95°C, 30 s of annealing at 48°C, and 30 s of enzymatic primer extension at 72°C. We verified that these amplification conditions resulted in signals belonging to the exponential phase of the PCR and not to the saturation portion of the experiment. PCR fragments were visualized on a 1% (wt/vol) agarose gel. Negative controls included PCR with either water instead of reverse transcriptase mix or 3 μl of crude RNA. None of the negative controls resulted in DNA amplification. Genomic DNA was used as a positive control. Primers 5′-GGTCCTCCGGGAA CCGGTAAAAC-3′ and 5′-AATTGCATGGCCGGCTTCGTGATATGC-3′ were used to amplify a 687-bp fragment specific to the *O. oeni ftsH* gene. They hybridize to the same regions as do the F1 and F2 primers, respectively. The *O. oeni* 23S rRNA gene (14) was used as a constitutive expression control, since it is a housekeeping gene which is not induced by environmental stress.

Resistance assays. Overnight cultures at 25°C of the Δ*ftsH E. coli* strain AR3291 transformed with either pBAD18-Cm (control plasmid) or pRJ5188 (harboring the *B. japonicum ftsH* gene) were grown in LB medium supplemented with chloramphenicol. They were diluted to an optical density at 600 nm of 0.1 into fresh LB medium, supplemented with antibiotic and containing 0.1% arabinose instead of glucose to induce FtsH biosynthesis through the arabinose PBAD promoter (6), and grown for 2 h at 37°C. Next, 20 μl of cell cultures was added to 180 μl of LB medium devoid of antibiotic but containing 0.1% arabinose and supplemented with various concentrations of wine (0, 20, and 25%, making 0, 2.4, and 3% ethanol, respectively). Cultures were incubated for 3 h at 25°C. Serial 10-fold dilutions were then plated on solid LB medium, and colonies were counted on the following day. Survival ratios of CFU observed in the presence of wine to those observed without wine were calculated.

Overnight cultures at 25°C of the Δ*ftsH E. coli* strain AR3291 transformed with either pCR-XL-TOPO (control plasmid) or pJPB65 (harboring the *O. oeni ftsH* gene) were grown in LB medium supplemented with kanamycin. They were diluted to an optical density at 600 nm of 0.1 into fresh LB medium, supplemented with antibiotic and containing glucose, and grown for 2 h at 37°C. Resistance assays were then carried out as described above, except that the LB medium contained glucose instead of arabinose during the wine shock.

Nucleotide sequence accession number. The GenBank accession number for the *O. oeni ftsH* gene is AY196466.

RESULTS

Cloning of the *O. oeni ftsH* gene. PCR with redundant primers designed from the conserved ATP binding motifs and ATPase module of several *ftsH*-homologous genes resulted in the amplification of an expected 710-bp DNA fragment from *O. oeni* IOEB 8406. Cloning and DNA sequence analysis revealed that the amplified fragment encoded a highly conserved amino acid sequence characteristic of proteins containing an ATP binding cassette (25). This fragment was used as a probe (the ftsH probe) during Southern analysis of genomic DNA of *O. oeni* IOEB 8406 digested with different restriction enzymes. This analysis demonstrated that the ftsH probe was represented by a single-copy gene (Fig. 1A) and that this probe was specific to a single putative *ftsH* gene, since no additional fragment containing other genes belonging to the AAA family could be hybridized. Using the Southern analysis, we were able to construct a restriction map (Fig. 1B) and then select appropriate restriction enzymes in order to clone the putative *O. oeni ftsH* gene by an inverse PCR-based approach. The cloned gene was then sequenced. Since the amino acid sequence deduced from the predicted open reading frame was highly homologous to various bacterial FtsH proteins, as described below, this open reading frame was designated the *O. oeni ftsH* gene.

The *O. oeni ftsH* gene is made of an open reading frame of 2,145 bp and encodes a polypeptide of 715 amino acids with a calculated molecular mass of 75 kDa. Comparison of *O. oeni* FtsH protein with members of the AAA protein family revealed a high sequence identity within an ATPase module of around 200 amino acids (2). Among these AAA proteins, the *O. oeni* FtsH protein displayed the highest overall sequence similarity to the bacterial FtsH proteins, most notably the *L. lactis* homologue (Fig. 2). The *O. oeni* and *L. lactis* FtsH proteins share 59% identical residues (as given by the Clustal program), and *O. oeni* FtsH exhibits 52% identity to *E. coli* FtsH and 51% identity to *B. japonicum* FtsH (Fig. 2).

A model of the membrane topologies of the *E. coli* and *L. lactis* FtsH proteins was generated (16, 21), and according to the model, a hydropathy analysis of the *O. oeni* FtsH protein revealed an N-terminal domain with two putative α-helical transmembrane segments (residues 12 to 31 and 134 to 157). The cytoplasmic domain carried the ATP binding motifs (Walker A and B motifs), the conserved region of the AAA protein family, and the putative zinc binding site (Fig. 2).

The *O. oeni ftsH* gene is a stress-responsive gene. The influence of typical wine stresses upon *O. oeni ftsH* gene expression was tested. We did not observe any significant changes in *ftsH*

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                                                                **Walker B**
oeni       LLAKAVAGEAKTPFFSISGSDFVEMFVGVGASRVRDLFENAKKSAPSIIFIDEIDAVGRR
mesen      LLAKAVAGEASVPFFSMSGSDFVEMFVGVGASRVRDLFENAKKSAPAIIFIDEIDAVGRR
delbru     LLAKAVAGEAAVPFYSISGSDFVEMSVGVGASRVRDSFDQAKKNSPSIIFIDEIDAVGRQ
buch       LLAKAVAGEAAVPFYSISGSDFVEMFVGVGASRVRDLFDQAKKNSPSIIFIDEIDAVGRQ
hilg       LLAKAVAGEAAVPFFSISGSDFVEMFVGVGASRVRDLFEQAKKAAPSIIFIDEIDAVGRQ
acidi      LLAKAVAGEAGVPFYSISGSDFVEMFVGVGASRVRDLFDQAKKNAPSIIFIDEIDAVGRR
pento      LLAKAVAGEAGVPFYSISGSDFVEMFVGVGASRVRDLFDQAKKNAPSIIFIDEIDAVGRR
damno      LLAKAVAGEAGVPFYSISGSDFVEMFVGVGASRVRDLFDQAKKNSPSIIFIDEIDAVGRK
fruct      LLAKAVAGEAGVPFYSISGSDFVEMFVGVGASRVRDLFDQAKKAAPAIIFIDEIDAVGRR
plant      LLAKAVAGEAGVPFFSISGSDFVEMFVGVGASRVRDLFEQAKKNAPSIIFIDEIDAVGRQ
saliv      LLAKAVAGEAGVPFFSISGSDFVEMFVGVGASRVRDLFENAKKNAPSIIFIDEIDAVGRR
dextri     LLARAVAGEAGVPFFSISGSDFVEMFVGVGASRVRDLFENAKKNAPAIIFIDEIDAVGRQ
lactis     LLAKAVAGEAGVPFYSISGSDFVEMFVGVGASRVRDLFENAKKTAPSIIFIDEIDAVGRQ
           ***:****** .**:*:******** ********** *::*** :*:************:

                                                                 **SRH**
                                                   VIVMAATNRSDVLDPALLRPGRFDRKIL
oeni       RGAGMGGGNDEREQTLNQILIEMDGFEGS-EGVIVLASTNRSDVLDPALLRSGRFDRKIL
mesen      RGTGMGGGNDEREQTLNQILIEMDGFEGS-EGVIILASTNRSDVLDPALLRSGRFDRKIL
delbru     RGAGMGGGHDEREQTLNQLLVEMDGFTGN-EGVIVMAATNRSDVLDPALLRPGRFDRKIL
buch       RGAGMGGGHDEREQTLNQLLVEMDGFTGN-EGVIVMAATNRSDVLDPALLRPGRFDRKIL
hilg       RGAGMGGGHDEREQTLNQLLVEMDGFTGS-EGVIVMAATNRPDVLDPALLRPGRFDRKIL
acidi      RGNGTGGGHDEREQTLNQLLVEMDGFTGN-EGVIVMAATNRSDVLDQALLRPGRFDRKIL
pento      RGNGTGGGHDEREQTLNQLLVEMDGFTGN-EGVIVMAATNRSDVLDPALLRPGRFDRKIL
damno      RGSGMGGGHDEREQTLNQLLVEMDGLTGN-EGVIVMAATNRSDVLDPALLRPGRFDRKIL
fruct      RGAGMGGGHDEREQTLNQLLVEMDGFSGN-EGVIVIAATNRADVLDPALTRPGRFDRKIL
plant      RGNGMGGGHDEREQTLNQLLVEMDGFTGN-EGVIVMAATNRSDVLDPALLRPGRFDRKIL
saliv      RGAGMGGGHDEREQTLNQMLVEMDGFEGD-EGVIVMAATNRSDVLGPALLRPGRFDRKIL
dextri     RGAGMGGGHDEREQTLNQLLVEMDGFQGN-EGVIVIAATNRSDVLDPALLRPGRFDRKIL
lactis     RGAGLGGGNDEREQTLNQLLVEMDGFQDDGNSVIVIAATNRSDVLDPALLRPGRFDRKVL
           ** * ***:*********:*:****: .. :.**::*:***.***. ** *.******:*

oeni       VGAPDVKGREAILRVHAKNKPLAADVDLKVIAQQTPGFVGADLENLLNEAALLAARNDEK
mesen      VGAPDVKGREAILNVHAKNKPLADNVDLKAIAQQTPGYVGADLENLLNEAALLAARRNKS
delbru     VGRPDVKGREAILRVHAKNKPLAGDVDLREIAKQTPGFVGADLENLLNEAALLAARRNKT
buch       VGRPDVKGREAILRVHAKNKPLAGDVDLKEISKQTPGFVGADLENLLNEAALLAARRNKT
hilg       VGRPDVKGREAILKVHAKNKPLANDVDLKEISKQTPGFVGADLENLLNEAALLAARRNKT
acidi      VGRPDVNGREAILHVHAKNKPIASDVDLAEIAKQTPGFVGADLENLLNEAALLAARRNKK
pento      VSRPDVNGREAILKVHAKNKPIDSDVDLAEIAKQTPGFVGADLENLLNEAALLAARRNKK
damno      VGRPDVRGREAILKVHSKNKPLDKDVDLNEIAKQTPGFVGADLENLLNEAALLAARRNKK
fruct      VGRPDINGREAILKVHSKNKPFANDVDLHEIAKQTPGFVGADLANLLNEAALLAARRNKT
plant      VGRPDVKGREAILKVHAKNKPLAADVDLKEIAKQTPGFVGADLENLLNEAALLAARRNKK
saliv      VGRPDVKGREAILKVHAKNKPLAKDVDSKMIAKQTPGFVGADLENLLNEAALQAARRDKK
dextri     VGRPDVKGREAILKVHAKDKPLAPDVDLKAVARETPGFVGADLENVLNEAALVAARRNKT
lactis     VGAPDVKGREAVLKVHAKNKPLASDVDLHNVATQTPGYVGADLENVLNEAALVAARQNKK
           *. **:.****:*.**:*:**:  :**   :: :***:***** *:****** ***.::.

oeni       AVTAADIDEAEDRVIAGPAKKDRKTTQDERETV
mesen      KVDAADIDEAEDRIFQGPAKTNHNMSESERRTT
delbru     AIDASDLDEAEDRVIAGPAKRDRVMSKQERNTV
buch       AVDASDLDEAEDRVIAGPAKRDRVISKQERNTV
hilg       QIDASDLDEAEDRVIAGPAKKDRVVSPQERKTV
acidi      VIDAADLDEAEDRVIAGPAKKNAVVSKKERQTV
pento      TIDAADLDEAEDRVIAGPAKKNAVVSKKERQTV
damno      VIDASDLDEAEDRVIAGPAKKNAVVNPLERKTV
fruct      KIDASDIDEAEDRVIAGPAKRNRVISKEERETV
plant      QVDAADLDEAEDRVIAGPAKHDRVVNKHERETV
saliv      EIDASDIDEAEDRVIAGPAKRDRVISKKERETV
dextri     QIDAKDVDEAEDRVIAGPAKKDRVISVKERNTV
lactis     EINAADIDEGMDRAMAGPAKKDRIQSMRERETV
            : * *:**. ** : **** :   .  **. .
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expression when *O. oeni* was subjected to alcohol, sulfite, or ethidium bromide addition. In contrast, increased *ftsH* gene expression was observed at high temperatures and under osmotic shock (Fig. 3).

The *O. oeni* and *B. japonicum* FtsH proteins protect bacteria against wine stress. Since there is no known transformation method for *O. oeni*, knockout constructions and complementation tests are impossible. Therefore, the function of *O. oeni* FtsH was explored in the Δ*ftsH E. coli* strain AR3291. This strain is a viable Δ*ftsH* mutant, due to a suppressor mutation in *sfhC* (*fabZ*) that allows cells to survive although showing slowed growth at 37°C (20). The same strain had been used to study the function of *B. japonicum* FtsH (15). When *E. coli* AR3291/pCR-XL-TOPO was grown at 37°C, it showed a clear growth defect, whereas the expression of *O. oeni* FtsH significantly improved the growth of *E. coli* AR3291/pJPB65 at 37°C (Fig. 4). Thus, *O. oeni* FtsH is able to confer a growth advantage upon the *E. coli ftsH* mutant, indicating that the heterologous protein can compensate for the loss of at least some of the important FtsH functions in *E. coli* AR3291.

We then analyzed whether the *O. oeni* and *B. japonicum ftsH* genes could confer protection on bacteria when grown in a wine-containing medium. When *E. coli* Δ*ftsH* cells harboring pJPB65 (*O. oeni ftsH*) or vector without *ftsH* (pCR-XL-TOPO) were exposed to wine in liquid medium, it was readily apparent that *O. oeni* FtsH confers resistance to this toxic medium (Fig. 5). The survival ratios were significantly higher when cells cultured with wine harbored the *O. oeni* and *B. japonicum ftsH* expression plasmids. The expression of *O. oeni ftsH* resulted in a 10- to 100-fold relative resistance, and that of *B. japonicum ftsH* resulted in a 100- to 10,000-fold relative resistance. We define relative resistance as the ratio of the survival rate of the bacteria transformed with *O. oeni* or *B. japonicum ftsH* to that of the bacteria transformed with the corresponding vectors in a given environmental condition.

Homologous genes in other lactic acid bacteria. The ftsH probe (Fig. 1) was used in Southern hybridization analysis with either *Eco*RI- or *Hin*dIII-digested chromosomal DNAs from various wine lactic acid bacteria (Fig. 6). Single chromosomal bands could be detected from the bacteria *Lactobacillus fructivorans*, *Pediococcus pentosaceus*, *Pediococcus dextrinicus*, *Lactobacillus plantarum*, *Pediococcus parvulus*, *Lactobacillus buchneri*, *Pediococcus acidilactici*, *Pediococcus damnosus*, *Lactobacillus hilgardii*, *Lactobacillus brevis*, *Lactobacillus delbrueckii*, and *Leuconostoc mesenteroides*.

The FtsH1 and FtsH2 primers, which allow amplification of the *O. oeni* ftsH probe, were used in an attempt to amplify sequences that would be homologous to this probe. All of the genomic DNAs tested from the bacteria mentioned above generated a 710-bp PCR fragment that proved to be homologous to the ABC and SRH (second region of homology) domains of the previously known FtsH proteins (Fig. 7).

DISCUSSION

The *ftsH* gene of *O. oeni* was cloned, sequenced, and shown to functionally replace the *ftsH* requirement in an *E. coli* Δ*ftsH* mutant when grown at 37°C. The salt- and temperature-induced *ftsH* gene expression in *O. oeni* is in keeping with the reported observation that in *L. lactis* most of the salt-induced proteins were also induced by heat shock (10). We have shown here that the *ftsH* gene is one of the molecular devices in *O. oeni* that might enable this species to cope better with high fermentation temperatures. In addition, the *O. oeni* FtsH protein conferred protection against wine toxicity to the *E. coli* Δ*ftsH* mutant. This ability is shared not only by the *O. oeni* homologue but also by the *B. japonicum* counterpart. Since this ability was tested in a foreign host, *E. coli*, one can speculate that it is likely to be shared by the FtsH proteins of many other species.

FtsH proteins belong to the AAA protein family (11), which constitutes a distinct subfamily of the Walker-type ATPases. In addition to the two consensus motifs, Walker A and B, in the Walker-type ATPases, AAA proteins have another highly conserved amino acid sequence within their ATPase domain, the SRH. The SRH plays an important role in ATP hydrolysis, and some highly conserved amino acid residues within the SRH were found to be essential for the in vivo protease activity of FtsH (9).

Alignment of the 13 sequences of the FtsH ATPase domains from various lactic acid bacteria defined an SRH consensus (Fig. 7). This consensus differs in only two positions from the SRH consensus obtained by comparison of 54 representative AAA proteins (9). However, these two differences, I297 M and P303S (the numbering is that of the *E. coli* FtsH protein), affect only poorly conserved residues of the SRH, which are not essential for FtsH activity. Indeed, the activity of a P303A mutant of the *E. coli* FtsH protein is almost the same as that of a wild-type FtsH protein (9). The highly conserved residues N301, D307, L310, R312, and R315, on the other hand, which are critical for *E. coli* FtsH activity, belong to the lactic acid bacterial consensus sequence. In conclusion, the lactic acid bacterial sequences collected are likely to be part of authentic and active FtsH proteins. Thus, FtsH is probably a universal molecular device, at least throughout the prokaryotic kingdom.

FIG. 7. PCR amplification of sequences from various lactic acid bacteria, homologous to the ABC domain of *ftsH*. The amplified sequences were aligned and compared. oeni, *O. oeni*; mesen, *Leuconostoc mesenteroides*; delbru, *Lactobacillus delbrueckii*; buch, *Lactobacillus buchneri*; hilg, *Lactobacillus hilgardii*; acidi, *P. acidilactici* ATCC 8042; pento, *P. pentosaceus*; damno, *P. damnosus*; fruct, *Lactobacillus fructivorans*; plant, *Lactobacillus plantarum*; saliv, *Lactobacillus salivarius* ATCC 11740; dextri, *P. dextrinicus*; lactis, *L. lactis*. An asterisk below the sequences indicates a perfect consensus between the protein sequences. Dots indicate conservative substitutions. The Walker B motif and the SRH domain are indicated. The consensus SRH sequence is outlined above the black bar. Dashes indicate gaps introduced to optimize the alignment.

ACKNOWLEDGMENTS

We thank Franz Narberhaus for the gifts of plasmid pRJ5188 and the *E. coli* Δ*ftsH* strain AR3291. We also thank Amélie Vallet for technical assistance.

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