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# Molecular Characterization of the Gene Encoding an 18-Kilodalton Small Heat Shock Protein Associated with the Membrane of *Leuconostoc oenos*

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**In *Leuconostoc oenos*, different stresses such as heat, ethanol, and acid shocks dramatically induce the expression of an 18-kDa small heat shock protein called Lo18. The corresponding gene (*hsp18*) was cloned from a genomic library of *L. oenos* constructed in *Escherichia coli*. A 2.3-kb DNA fragment carrying the *hsp18* gene was sequenced. The *hsp18* gene encodes a polypeptide of 148 amino acids with a calculated molecular mass of 16,938 Da. The Lo18 protein has a significant identity with small heat shock proteins of the alpha-crystallin family. The transcriptional start site was determined by primer extension. This experiment allowed us to identify the promoter region exhibiting high similarity to consensus promoter sequences of gram-positive bacteria, as well as *E. coli*. Northern blot analysis showed that *hsp18* consists of a unique transcription unit of 0.6 kb. Moreover, *hsp18* expression seemed to be controlled at the transcriptional level. This small heat shock protein was found to be peripherally associated with the membrane of *L. oenos*.**

*Leuconostoc oenos* is a gram-positive acidophilic bacterium that is able to grow in a hostile environment such as wine. Reclassification of *L. oenos* as *Oenococcus oeni* has been proposed (5). This bacterium is of economic interest because it is responsible for an important step in the vinification process: malolactic fermentation (MLF). MLF consists of the decarboxylation of L-malic acid to L-lactic acid, which decreases the total acidity and improves the stability and quality of wine (23). The ethanol resistance and especially the acid tolerance of *L. oenos* are considered to be the crucial properties for its survival in wine. Three mechanisms appear to play an important role in the acid tolerance of *L. oenos*: (i) the proton motive force generated by L-malate fermentation (31), (ii) the stress protein synthesis as part of a typical acid shock response (17), and (iii) the activation of proton-extruding ATPase (7).

Until now, many studies on MLF have been concerned with L-malate transport (32), the energetics aspects of the reaction (31), and the purification of the malolactic enzyme (29). Recently, a genetic study was initiated to clone and characterize the genes encoding the malolactic enzyme and the malate permease of *L. oenos* (24). However, knowledge about the stress response in *L. oenos* is at a much more elementary stage. Nevertheless, we demonstrated that the survival of *L. oenos* in wine and its ability to perform malolactic fermentation were improved after direct inoculation with cells pretreated at 42°C. At this temperature, the synthesis of stress proteins was induced (11).

One of the stress proteins induced by multiple stresses (heat, low pH, and ethanol) in *L. oenos*, an 18-kDa polypeptide named Lo18, was purified by two-dimensional polyacrylamide gel electrophoresis and its N-terminal sequence was determined (13). The synthesis of Lo18 was also induced in the stationary growth phase. A significant identity was found between the N-terminal part of Lo18 and the Hsp18 from *Clostridium acetobutylicum* (35). These low-molecular-weight Hsp (smHsp) are related to the alpha-crystallin family of small heat shock proteins. In vitro studies indicated that the alpha-crystallins possess a chaperone-like function (19). Moreover, previous studies have demonstrated that the C-terminal region of the alpha-crystallin molecule is important for the ability of this protein to protect other polypeptides against heat-induced aggregation (39). The exact function of many other prokaryotic and eukaryotic smHsp remains unclear (36, 38). However, a chaperone-like activity was recently revealed for a 16-kDa alpha-crystallin-like small heat shock protein from *Mycobacterium tuberculosis* (43).

We report here the cloning and nucleotide sequence of *hsp18*, the gene encoding Lo18. The transcription start site and the size of the transcript were determined. An association of Lo18 with the cell membrane in *L. oenos* was demonstrated.

## MATERIALS AND METHODS

**Bacterial strains, media, and plasmids.** The bacterial strains and plasmids used in this study are listed in Table 1. *L. oenos* Lo84.13 was grown at 30°C in FT80 medium (pH 5.3) (3) modified by the addition of meat extract instead of Casamino Acids. *Escherichia coli* TG1 was grown in Luria-Bertani broth or agar at 37°C. Erythromycin was used at a final concentration of 200 $\mu g \cdot ml^{-1}$.

**Preparation of polyclonal antibodies and screening of the genomic bank.** Lo18 was purified from heat-shocked *L. oenos* cells, and polyclonal antibodies were obtained by direct immunization of a rabbit with the purified protein, as previously described (13). An *L. oenos* genomic bank was constructed as described by Labarre et al. (24). This bank was transferred to nylon filters (pore size, 0.45 μm; Schleicher & Schuell, Dassel, Germany) overlaid on Luria-Bertani plates. Bacteria that grew on the filter were then lysed by adding 5% sodium dodecyl sulfate (SDS) and baking for 15 min at 95°C. The proteins were fixed to the filters by electrotransfer with a Trans-Blot SD electrophoretic transfer cell (Bio-Rad, Richmond, Calif.) as recommended by the manufacturer. These filters were screened with the polyclonal antiserum diluted at 1:1,000, as previously described (12).

**DNA manipulations and sequence determination.** Molecular procedures were carried by using standard methods (33). Double-stranded plasmid DNA was purified by using a Qiagen plasmid kit (tip 100; Qiagen, Hilden, Germany) and was sequenced by the dideoxy chain termination method (34) with a T7 sequencing kit (Pharmacia Biotech, Uppsala, Sweden). Both strands were sequenced with synthetic oligonucleotide primers. Computer analysis of nucleotide and amino acid sequences was carried out with PC/GENE software.

**Isolation of total RNA and Northern blot analysis.** *L. oenos* cells were harvested by centrifugation for 5 min at 10,000 × *g* and resuspended in 1 ml of Tri

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TABLE 1. Bacterial strains and plasmids used in this study

| Strain or plasmid | Description | Source or reference |
|---|---|---|
| Strains | | |
| *E. coli* TG1 | *supE hsd*Δ*5 thi* Δ(*lac-proAB*) F′ (*traD36 proA*$^+$*B*$^+$ *lacI*$^q$ *lacZ*ΔM15) | 9 |
| *L. oenos* Lo84.13 | MLE$^+$ | Oenological Institute of Bordeaux |
| Plasmids | | |
| pJDC9 | Em$^r$ Δ*lacZ* | 4 |
| pGID023 | Shuttle vector for *E. coli* and gram-positive bacteria; derivative of pJDC9 containing the pE194 replication function; Em$^r$ | 18 |
| pJMP1 | pJDC9 containing the 4.5-kb *Sau*3A fragment from *L. oenos* | This study |
| pJMP2 | pGID023 containing the 2.3-kb *Hin*dIII fragment of pJMP1 | This study |

Reagent (Sigma, St. Louis, Mo.). The cells were mechanically disrupted at 4°C with a mini-beadbeater (Polylabo, Strasbourg, France) with 200 mg of glass beads (diameter, <50 μm). The procedure consisted of four 1-min periods of homogenization at 5,000 rpm, each separated by 1-min pauses. Samples were then treated as recommended by the manufacturer (Sigma).

Total RNA (40 μg) was separated under denaturing conditions and subjected to Northern blot analysis as described elsewhere (8). Probes A, B, and C (see Fig. 1) were obtained by PCR amplification from recombinant plasmid pJMP1 (Table 1) with oligonucleotide primers used for the sequence determination. The probes were radiolabelled with [α-$^{32}$P]dATP, with a random-primer labelling kit (GIBCO-BRL, Gaithersburg, Md.).

**Primer extension analysis.** The transcriptional start site of *hsp18* was determined with oligonucleotide primers PE1 (5′-CAAGTTGCCCATCATTACGG-3′) and PE2 (5′-TATGCCGGGCACTTTCCCAC-3′) (see Fig. 2). RNA (30 μg) isolated from heat-shocked (15 min at 42°C) *L. oenos* cells was mixed with 40 pmol of each primer. The mixture was denatured at 100°C for 5 min followed by an annealing step of 1 min at 50°C. A 33-μl volume of a solution containing 6 μl of 5× reverse transcriptase buffer, 5 μl of 0.1 M dithiothreitol, 0.5 μl of 100 mM dCTP, 0.5 μl of 100 mM dGTP, 0.5 μl of 100 mM dTTP, 0.5 μl of [α-$^{32}$P]dATP, 1 μl of RNasin (40 U; Boehringer, Mannheim, Germany), and 0.25 μl of reverse transcriptase (10 U; Appligene, Hillkirch, France) was added to the RNA-primer mix. Polymerization was performed at 37°C for 10 min, and then 0.5 μl of 100 mM dATP was added prior to another incubation period of 50 min. The mixture was precipitated with 3 volumes of ethanol and 1/10 volume of 3 M sodium acetate. The pellet was resuspended in 2 μl of TE (10 mM Tris, 1 mM EDTA [pH 7.4]) and 3 μl of formamide loading buffer (Appligene). The samples were denatured at 85°C for 5 min and loaded onto a 6% polyacrylamide sequencing gel containing 8 M urea. Sequencing reactions with the same primers and an appropriate plasmid DNA template were also performed to allow determination of the endpoints of the extension products.

**Gel electrophoresis and Western blot analysis.** SDS-polyacrylamide gel electrophoresis (PAGE) (14% polyacrylamide) was performed by the method of Laemmli (25). Equal amounts of proteins (10 μg) were loaded on the SDS-PAGE gel. The protein concentration in cellular extracts was estimated by the method of Bradford (2) with a Bio-Rad assay kit. Immunoblot analyses were performed as described previously (12) with the polyclonal antiserum diluted at 1:1,000.

**Cellular fractionation.** Heat-shocked cells were washed with 10 mM Tris-HCl buffer (pH 8.0) resuspended in 15 ml of the same buffer with 250 μg of RNase per ml and lysed in a French pressure cell at $1.2 \times 10^5$ kPa. Then the suspension was centrifuged at 4,000 × *g* for 10 min to remove unbroken cells. After a second centrifugation at 4,000 × *g* for 15 min, the supernatant was subjected to ultracentrifugation at 68,600 × *g* for 1 h. The supernatant, containing cytoplasmic proteins, was collected, and the pellet, containing the membranes, was washed with 10 mM Tris-HCl buffer, (pH 8.0), ultracentrifuged at 68,600 × *g* for 1 h, and resuspended in the same buffer. As a control for the cellular fractionation experiment, lactate dehydrogenase was assayed.

**Membrane extraction.** The membrane pellet was incubated in 0.1 M $Na_2CO_3$ (pH 11.5) for 1 h at 4°C or in 5 M urea for 30 min at 4°C and then subjected to ultracentrifugation at 68,600 × *g* for 1 h. The supernatants and the pellets were analyzed by SDS-PAGE (14% polyacrylamide) and immunoblotting.

**Nucleotide sequence accession number.** The nucleotide sequence data reported in this paper will appear in the EMBL, GenBank, and DDBJ nucleotide sequence databases under accession number X99468.

## RESULTS

**Cloning and sequencing of *hsp18* of *L. oenos*.** A DNA genomic library of *L. oenos* Lo84.13 was screened by colony blotting for Lo18 production since the Lo18 antiserum did not cross-react with *E. coli* proteins (data not shown). Immunodetection revealed 1 clone of 2,880 screened. To confirm that the clone was carrying the structural gene encoding *L. oenos* Lo18, the DNA fragment was expressed in *E. coli* and the proteins were revealed by SDS-PAGE and immunoblotting (data not shown). This isolated clone, named pJMP1, consisted of pJDC9 carrying a 4.5-kb insert (Fig. 1). A 2.3-kb *Hin*dIII fragment from plasmid pJMP1 was subcloned in pGID023, yielding pJMP2. *E. coli* cells that were transformed with pJMP2 were able to express Lo18. The complete insert of pJMP2 was sequenced on both strands. To verify that the cloned genes originated from *L. oenos*, PCR was performed with oligonucleotides LO18U (5′-ATGGATAGAAATGATGGATT-3′) and LO18R (5′-CGTCAGATTTTGGTAGAGTC-3′). No amplification was observed when *E. coli* genomic DNA was used as a template (data not shown).

**Sequence analysis.** The region of pJMP2 corresponding to *hsp18* was sequenced (Fig. 2). The *hsp18* coding region is 447 nucleotides long and starts at a putative ATG codon preceded by a putative ribosome binding site, 5′-AGGAGGT-3′, that is complementary to the 3′ end of *Leuconostoc mesenteroides* 16S rRNA 5′-CACCTCCTT-3′ (complementary nucleotides are underlined) (42). The 3′ end of *L. mesenteroides* 16S rRNA was used as a reference since the 3′ end of *L. oenos* 16S rRNA has not been described yet. The adjacent open reading frames are *orfC*, which is localized upstream of *hsp18* and in the same orientation, and *orfD*, which is localized downstream of *hsp18* and in the reverse orientation. Furthermore, inspection of the 3′-side noncoding region of *hsp18* revealed an inverted-repeat sequence that could form a stem-and-loop structure in mRNA. This structure has a calculated $\Delta G_f$ of −17.2 kcal/mol (71.9

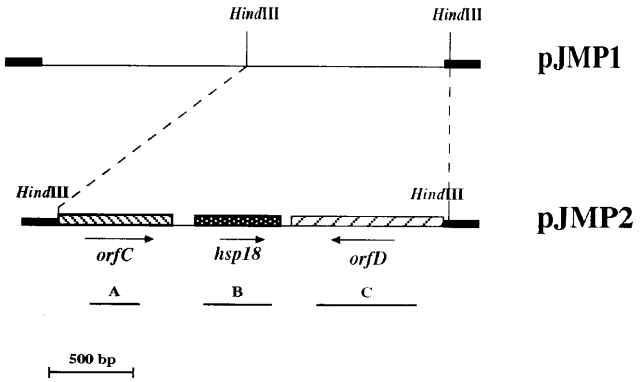


FIG. 1. Physical map of the *L. oenos hsp18* cluster. Restriction sites for *Hin*dIII are shown. The positions of *orfC*, *hsp18*, and *orfD* are indicated by boxes. The direction of transcription is indicated by arrows below the line. The probes used for Northern blotting (A, B, and C) are indicated by bold lines under the map.

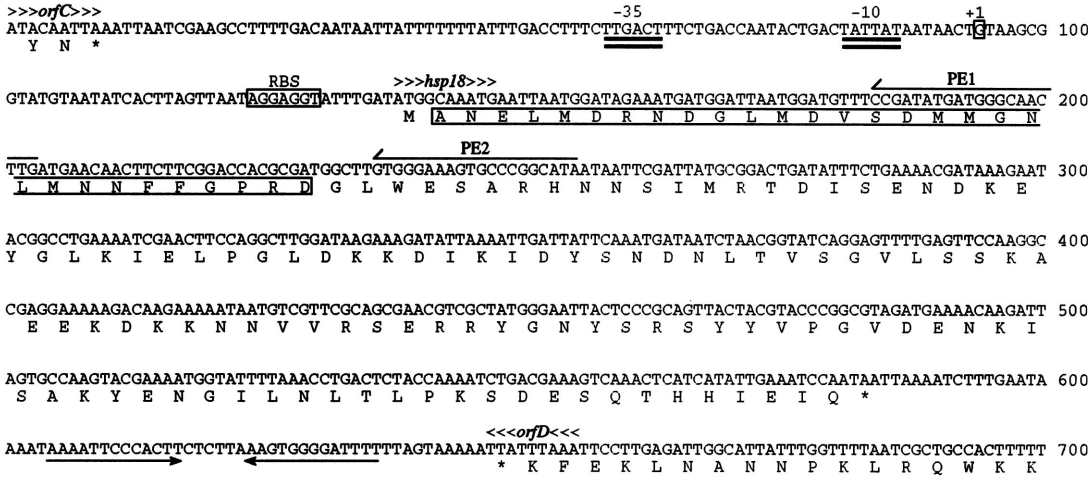


FIG. 2. Nucleotide and deduced amino acid sequences of *hsp18* and flanking DNA from *L. oenos*. The putative promoter hexamers −10 and −35 are doubly underlined. The transcriptional start site (+1) is indicated. The box indicates the putative ribosome binding site (RBS), and a putative terminator (sequence of dyad symmetry downstream of the UAA stop codon) is indicated by convergent arrows. Arrows indicate oligonucleotides (PE1 and PE2) used for the primer extension experiment. The C-terminal amino acid sequences of the two adjacent open reading frames (*orfC* and *orfD*) are indicated by arrowheads. Amino acids confirmed by protein sequencing are boxed. Asterisks indicate the C terminus of each sequence.

kJ/mol) (40) and is likely to function as a rho-independent transcriptional terminator (30).

The protein sequence deduced from *hsp18* has a calculated molecular mass of 16,938 Da, which corresponds to the apparent molecular mass, and has a predicted pI of 4.85. The sequence previously obtained by Edman degradation of the purified protein (13) is found at the N terminus of the predicted sequence of Lo18 (Fig. 2). The amino acid sequence deduced from *hsp18* was compared to sequences available in the Swissprot database. It appears that Lo18 has significant identity to a number of proteins belonging to the large family of smHsp (Fig. 3). The smHsp exhibit much more diversity in size and amino acid sequence than do the high-molecular-weight Hsp (35). Then, the higher identities were observed with the *Clostridium acetobutylicum* Hsp18 (32.4% identity with the amino acid sequence) and the *Stigmatella aurantiaca* SP21 (15) (29.7% identity), especially in the C-terminal region.

**mRNA analysis of *hsp18*.** Total RNA was isolated from *L. oenos* cells grown at 30°C (control cells) or heat shocked at 42°C for 15 min. At first, this RNA was used for Northern blot analysis (Fig. 4A). Hybridization with the radiolabelled PCR-generated probe B, which is internal to *hsp18* (Fig. 1), revealed a single transcript of 600 nucleotides (Fig. 4B, lane 2) that was detectable only in heat-shocked cells. Hybridizations with the radiolabelled PCR-generated probe from *orfC* (Fig. 1, probe A) revealed a minor transcript of 1.4 kb that was detectable only in heat-shocked cells. The probe generated from *orfD* (Fig. 1, probe C) yielded no detectable signal, in control cells as well as in heat-shocked cells. The RNA isolated from heat-shocked cells was also subjected to primer extension analysis to determine the 5′ end of the *hsp18* transcript (Fig. 5). This was done by using oligonucleotides PE1 and PE2, which are complementary to the beginning of *hsp18* (Fig. 2). A single transcription start point was obtained with either primer, allowing location of the 5′ end of the *hsp18* transcript 44 nucleotides upstream of the initiation codon (Fig. 2). This transcription start point allowed location of the promoter to the sequence TTGACT-17 bp-TATTAT (Fig. 2). The *hsp18* promoter shows a high similarity to the consensus sequence found in vegetative promoters from gram-positive bacteria as well as *E. coli* (10).

**Induction of *hsp18* expression.** We investigated the expression of *hsp18* after heat shocks at different temperatures. Heat shocks were induced by transferring an exponentially growing culture of *L. oenos* from 30°C to 37 or 42°C. At 5 min after the shift at 37 or 42°C, Lo18 protein was detected by immunoblotting (Fig. 6A) and was still present 30 min after the beginning


FIG. 3. Alignment of the C-terminal sequences of the *L. oenos* Lo18 (LO18-LEUCO) and the sequences of *Clostridium acetobutylicum* Hsp18 (HS18-CLOAB) (35), *Chenopodium rubrum* Hsp23 (HS2C-CHERU) (21), *Psium sativum* Hsp21 (HS2M-PEA) (26), *Triticum aestivum* Hsp21 (HS2C-WHEAT) (41), *Zea mays* Hsp18 (HS23-MAIZE) (1), *Pharbitis nil* Hsp22 (HS22-PHANI) (22), *Stigmatella aurantiaca* Sp21 (SP21-STIAU) (15), and *Streptomyces albus* Hsp18 (HS18-STRAL) (36). The sequences were aligned with the Clustal program. The numbers on the right are the distance to the C-terminal end. The percentages are the levels of identity between Lo18 and the sequences of the previously described proteins for the C-terminal regions, and the percentages in parentheses are for the entire sequence of the protein. Amino acids identical to those of *L. oenos* Lo18 are shown in black boxes. Asterisks indicate residues that are identical in all sequences.

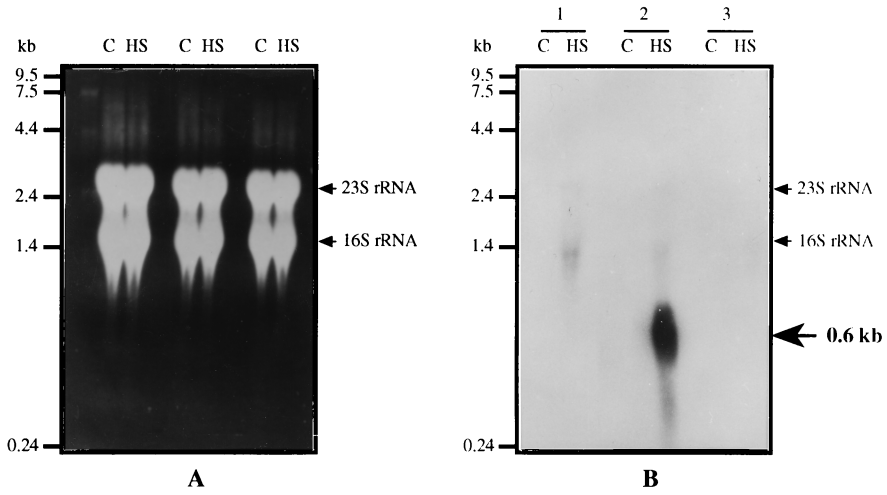


FIG. 4. Northern blot analysis. The same total RNA preparations (40 μg) from *L. oenos* cells grown at 30°C (lanes C) and from shocked cells at 42°C (lanes HS) were loaded onto a formamide-agarose gel. The gel was then blotted onto three nylon membranes. Each membrane was hybridized with one of the three different $^{32}$P-labelled probes. (A) Ethidium bromide-stained gel. (B) Autoradiogram of the Northern blots after hybridization with probe A (lane 1), probe B (lane 2), and probe C (lane 3). Arrows indicate the positions of 23S and 16S rRNAs and the 0.6-kb *hsp18* transcript. Numbers on the left margin indicate the positions of molecular size markers (in kilobases).

of the stress. This result led us to conclude that either Lo18 is a stable protein or its synthesis is still induced after 30 min at high temperature. Another result supports the first hypothesis: in heat-shocked cells returned to normal growth conditions (30°C), the level of Lo18 detected by immunoblotting remained stable for 3 h (data not shown). To gain information about the level of regulation of Lo18 induction, we added 40 μg of actinomycin D ml$^{-1}$ to the culture medium prior to heat shock at 42°C. After 15 min in the presence of actinomycin D at 42°C, Lo18 was not detected in *L. oenos* heat-shocked cells (Fig. 6B), suggesting that the synthesis was completely prevented in the presence of actinomycin D.

**Cellular location of Lo18.** In the course of Lo18 protein purification for N-terminal sequence determination (13), a fraction of the protein was found to be membrane associated. Therefore, the location of the protein in the cell was investigated with Lo18 antiserum. Heat-shocked cells of *L. oenos* were lysed in a French pressure cell. The membrane and cytoplasmic fractions were separated as described in Materials and Methods. The membrane purity was proved by the faint lactate dehydrogenase activity in this fraction (1.8% of the total activity). The presence of Lo18 was determined in both fractions by SDS-PAGE and immunoblotting. Even if Lo18 was shown to have a deduced sequence similar to that of soluble proteins, the membrane fraction contained a significant amount of Lo18 after a wash with Tris-HCl buffer (Fig. 7A). On the basis of this immunoblotting experiment, it is clear that Lo18 was found in the membrane fraction in addition to the cytoplasmic fraction. The nature of the protein, relatively hydrophilic, and its detection in several subcellular locations allowed us to propose a peripheral association of Lo18 with the membrane. To check this hypothesis, we attempted to extract Lo18 from the membrane fraction by using denaturants. We took advantage of the alkaline solution ($Na_2CO_3$) and urea that are known to solubilize proteins peripherally associated with the membrane (20). Thus, the membrane fraction was treated with 5 M urea or with 0.1 M $Na_2CO_3$ (pH 11.5) as described in Materials and Methods. The soluble and insoluble fractions were separated by centrifugation and analyzed by SDS-PAGE and immunoblotting. Lo18 was found exclusively in the soluble fractions (Fig. 7B). Therefore, these results led

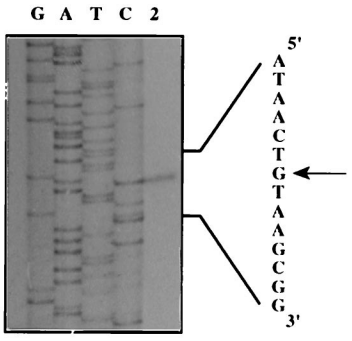


FIG. 5. Primer extension mapping of the 5′ end of the *hsp18* transcript with oligonucleotide PE2 (see Materials and Methods). Total RNA isolated from heat-shocked cells was used. Primer-extended product (lane 2) was electrophoresed in parallel with sequence ladder generated with the same primer on the noncoding strand of pJMP2. The arrow points to the base representing the 5′ end of the *hsp18* transcript.

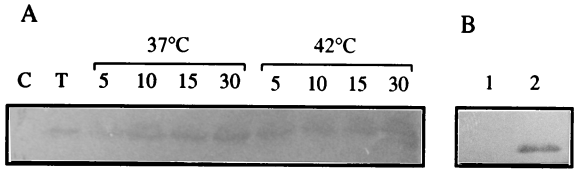


FIG. 6. Western blot analysis of heat shock-induced *hsp18* expression. (A) Induction delay of *hsp18* expression. The heat shock response of *L. oenos* cells was induced at 37 or 42°C, and samples were withdrawn at the time points (minutes) indicated. Cells were washed in 10 mM Tris-HCl buffer (pH 8.0) and disrupted in the same buffer as described for the RNA isolation (see Materials and Methods). The protein extracts were analyzed by SDS-PAGE (14% polyacrylamide) and immunoblotting. Lane C, unshocked cells (grown at 30°C); lane T, heat-shocked cells (1 h at 42°C). (B) Effect of actinomycin D addition on *hsp18* expression. Actinomycin D (40 μg · ml$^{-1}$) was added 5 min prior to heat shock (42°C). After 15 min at 42°C in the presence of actinomycin D, the cellular extracts were prepared as described above and analyzed by Western blotting. Lane 1, cells treated with actinomycin D; lane 2, untreated cells.

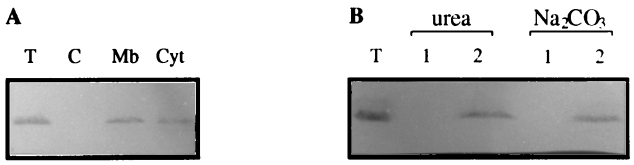


FIG. 7. Cellular location of Lo18. (A) Detection of Lo18 in cellular compartments by immunoblotting. Exponentially growing *L. oenos* cells were transferred to 42°C for 15 min to induce Lo18 expression. The cytoplasmic fraction and the membrane fraction were separated by ultracentrifugation, and the pellet containing the membrane was washed with Tris-HCl buffer as described in Materials and Methods. Lanes: C, total cellular extract of unshocked cells; T, total cellular extract of heat-shocked cells; Mb, membrane fraction; Cyt, cytoplasmic fraction. (B) Release of membrane-associated Lo18 with urea or at alkaline pH. The membrane preparation of heat-shocked cells was extracted with 5 M urea or 0.1 M $Na_2CO_3$ (pH 11.5) as described in Materials and Methods. The soluble and insoluble fractions were subjected to Western blot analysis. Lanes: T, total cellular extract from heat-shocked cells; lanes 1, insoluble fractions; lanes 2, soluble fractions.

us to conclude that Lo18 is peripherally associated with the membrane in *L. oenos*.

## DISCUSSION

We isolated from *L. oenos* a DNA fragment harboring *hsp18*, the gene encoding a smHsp with an apparent molecular mass of 18 kDa. Analysis of the protein sequence derived from the structural gene showed that Lo18 belongs to the small Hsp family found in prokaryotes and eukaryotes. In bacteria, two Hsp18 from *Streptomyces albus* and *Mycobacterium leprae* exhibit high identity (50%) (36). A comparison of the total protein sequence of Lo18 with these smHsp revealed low identity scores. In contrast, higher identity scores were obtained for Lo18 and the smHsp from *C. acetobutylicum*, which is an acetic acid bacterium (35). In fact, our data support the idea of heterogeneity among the bacterial smHsp sequences. As observed in previous studies (15), higher identities were observed in the C-terminal region, in which the consensus motif of smHsp is present. For eukaryotic smHsp, the C-terminal region appears to be involved in chaperone-like activities (19).

Northern blot analysis revealed that *hsp18* was clearly a heat shock gene whose transcription was enhanced upon temperature upshift. Considering the absence of detection of *hsp18* mRNA and the corresponding protein (13) under normal conditions, we can suggest that no or very low constitutive expression occurred. Many major heat shock genes are still efficiently expressed in the absence of stresses (37), and such encoded stress proteins are often essential for cell growth. In the case of Lo18, it seems that the *hsp18* gene is not expressed in *L. oenos* under normal growth conditions, and therefore that Lo18 protein has no essential housekeeping function. However, under stress conditions, the presence of this protein would become crucial for *L. oenos* survival. The disruption of *hsp18* in *L. oenos* would clarify this point. Unfortunately, no usable DNA transfer techniques are available at this time for such a genetic approach in *L. oenos* (44).

The *hsp18* gene forms a unique transcription unit as described for *C. acetobutylicum* and *S. albus* (35, 36). A 0.6-kb mRNA was clearly detected, but only after temperature upshift. Moreover, the addition of actinomycin D prior to temperature elevation completely prevented the induction of Lo18 synthesis. Taken together, these results suggest that the induction of Lo18 synthesis is regulated at the transcription level, as reported for most of the genes encoding Hsp (28).

Analysis of the transcription initiation site of *hsp18* revealed a putative promoter structure homologous to the consensus promoter sequence of vegetative genes from gram-negative and gram-positive bacteria (10). The existence of a vegetative promoter goes against the hypothesis of a control of *hsp18* expression by a $\sigma^{32}$-type sigma factor as is known to occur for the heat shock regulon of *E. coli* (28) or for the class II stress proteins of *B. subtilis* (14). However, an RpoS-like factor (16) may be involved in the expression of *hsp18*, since Lo18 synthesis is induced in the stationary growth phase. Otherwise, the class I heat shock genes from *B. subtilis* involve a CIRCE element, which is a *cis*-acting stem-loop structure generally located between the transcriptional and translational start sites. The interaction between a repressor and the CIRCE element, the operator, prevents the expression of these genes at low temperature. However, no CIRCE element was observed upstream of *hsp18*. The *hsp18* gene from *L. oenos* appears to involve other regulatory mechanisms. Apparently, the *hsp18* gene can respond to different stress factors independently of an alternative $\sigma^{32}$ factor or CIRCE element. This is the case for genes encoding smHsp of bacteria (36) and for a few genes of *B. subtilis* including *lon* and *clp* (14). Expression of these genes occurs at a promoter presumably recognized by the vegetative sigma factor and probably involves additional regulatory elements which remain to be defined.

Lo18 belongs to the family of bacterial smHsp for which the function remains unclear (43). Nevertheless, it has been shown that the homologous eukaryotic smHsp form high-molecular-weight complexes and possess a chaperone activity (19). Our data show that Lo18 is associated with the cytoplasmic membrane and sediments with the membrane fraction due to this association. Membrane-bound Lo18 can be solubilized by alkali or urea treatment, suggesting a protein-protein interaction with other membrane-associated proteins. Lo18 is also detected in the cytoplasmic fraction. The presence of smHsp in the cytoplasm as well as in the cell membrane has already been reported for the SP21 smHsp of *Stigmatella aurantiaca* (27). The cytoplasmic membrane is a primary site for the expression of an adaptative response to stress factors such as heat, ethanol, and low pH (6). Based on the stress-specific high expression of Lo18 and its cellular location, the smHsp Lo18 could be involved in an adaptive response allowing the maintenance of membrane integrity.

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## REFERENCES

1. **Atkinson, B., M. Raizada, R. Bouchard, R. Frappier, and D. Walden.** 1993. The independent stage-specific expression of the 18-kDa heat shock protein genes during microsporogenesis in *Zea mays*. Dev. Genet. **14:**15–26.
2. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
3. **Cavin, J.-F., H. Prevost, J. Lin, P. Schmitt, and C. Diviès.** 1989. Medium for screening *Leuconostoc oenos* strains defective in malolactic fermentation. Appl. Environ. Microbiol. **55:**751–753.
4. **Chen, J. D., and D. A. Morrisson.** 1988. Construction and properties of a new insertion vector, pJDC9, that is protected by transcriptional terminators and useful for cloning of DNA from *Streptococcus pneumoniae*. Gene **64:**155–164.
5. **Dicks, L. M., F. Dellaglio, and M. D. Collins.** 1995. Proposal to reclassify *Leuconostoc oenos* as *Oenococcus oeni* (corrig.) gen. nov. comb. nov. Int. J. Syst. Bacteriol. **45:**395–397.
6. **Dombek, K. M., and O. L. Ingram.** 1984. Effects of ethanol on *Escherichia coli* plasma membrane. J. Bacteriol. **157:**233–239.
7. **Drici-Cachon, Z., J. Guzzo, J.-F. Cavin, and C. Diviès.** 1996. Acid tolerance in *Leuconostoc oenos*. Isolation and characterization of an acid-resistant

mutant. Appl. Microbiol. Biotechnol. **44:**785–789.
8. **Garmyn, D., T. Ferain, N. Bernard, P. Hols, and J. Delcour.** 1995. Cloning, nucleotide sequence, and transcriptional analysis of the *Pediococcus acidilactici* L-(+)-lactate dehydrogenase gene. Appl. Environ. Microbiol. **61:**266–272.
9. **Gibson, T. J.** 1984. Studies on the Epstein-Bar virus genome. Ph.D. thesis. Cambridge University, Cambridge England.
10. **Graves, M. C., and J. C. Rabinowitz.** 1986. *In vivo* and *in vitro* transcription of the *Clostridium pasteurianum* ferredoxin gene. Evidence for "extended" promoter elements in gram-positive organisms. J. Biol. Chem. **261:**11409–11415.
11. **Guzzo, J., J.-F. Cavin, and C. Diviès.** 1994. Induction of stress proteins in *Leuconostoc oenos* to perform direct inoculation of wine. Biotechnol. Lett. **16:**1189–1194.
12. **Guzzo, J., M. Murgier, A. Filloux, and A. Lazdunski.** 1990. Cloning of the *Pseudomonas aeruginosa* alkaline protease gene and secretion of the protease into the medium by *Escherichia coli*. J. Bacteriol. **172:**942–948.
13. **Guzzo, J., F. Pierre, M.-P. Jobin, B. Samyn, J. Van Beeumen, F. Delmas, J.-F. Cavin, and C. Diviès.** A small heat shock protein induced by multiple stresses and during stationary growth phase from *Leuconostoc oenos*. Lett. Appl. Microbiol., in press.
14. **Hecker, M., W. Schumann, and U. Völker.** 1996. Heat-shock and general stress response in *Bacillus subtilis*. Mol. Microbiol. **19:**417–428.
15. **Heidelbach, M., H. Skladny, and H. U. Schairer.** 1993. Heat shock and development induce synthesis of a low-molecular-weight stress-responsive protein in the myxobacterium *Stigmatella aurantiaca*. J. Bacteriol. **175:**7479–7482.
16. **Hengge-Aronis, R.** 1993. Survival of hunger and stress: the role of *rpoS* in early stationary phase gene regulation in *Escherichia coli*. Cell **72:**165–168.
17. **Heyde, M., and R. Portalier.** 1990. Acid shock proteins of *Escherichia coli*. FEMS Microbiol. Lett. **69:**19–26.
18. **Hols, P., T. Ferain, D. Garmyn, N. Bernard, and J. Delcour.** 1994. Use of homologous expression-secretion signals and vector-free stable chromosomal integration in engineering of *Lactobacillus plantarum* for α-amylase and levanase expression. Appl. Environ. Microbiol. **60:**1401–1413.
19. **Horwitz, J.** 1992. α-Crystallin can function as a molecular chaperone. Proc. Natl. Acad. Sci. USA **89:**10449–10453.
20. **Kerppola, R. E., V. K. Shyamala, P. Klebba, and G. F.-L. Ames.** 1991. The membrane-bound proteins of periplasmic permeases form a complex. J. Biol. Chem. **266:**9857–9865.
21. **Knack, G., Z. Liu, and K. Kloppstech.** 1992. Low molecular mass heat-shock proteins of a light-resistant photoautotrophic cell culture. Eur. J. Cell Biol. **59:**166–175.
22. **Krishna, P., R. F. Felsheim, J. C. Larkin, and A. Das.** 1992. Structure and light-induced expression of a small heat shock gene of *Pharbitis nil*. Plant. Physiol. **100:**1772–1779.
23. **Kunkee, R. E.** 1991. Some roles of malic acid in the malolactic fermentation in wine making. FEMS Microbiol. Rev. **88:**55–72.
24. **Labarre, C., J. Guzzo, J.-F. Cavin, and C. Diviès.** 1996. Cloning and characterization of the genes encoding the malolactic enzyme and the malate permease of *Leuconostoc oenos*. Appl. Environ. Microbiol. **62:**1274–1282.
25. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
26. **Lenne, C., M. A. Block, J. Garin, and R. Douce.** 1995. Sequence and expression of the mRNA encoding Hsp22, the mitochondrial small heat-shock protein in pea leaves. Biochem. J. **311:**805–813.
27. **Lünsdorf, H., H. U. Schairer, and M. Heidelbach.** 1995. Localization of the stress protein SP21 in indole-induced spores, fruiting bodies, and heat-shocked cells of *Stigmatella aurantiaca*. J. Bacteriol. **177:**7092–7099.
28. **Mager, W. H., and A. J. J. de Kruijff.** 1995. Stress-induced transcriptional activation. Microbiol. Rev. **59:**506–531.
29. **Naouri, P., P. Chagnaud, A. Arnaud, and P. Galzy.** 1990. Purification and properties of malolactic enzyme from *Leuconostoc oenos* ATCC23278. J. Basic Microbiol. **30:**577–585.
30. **Rosenberg, M., and D. Court.** 1979. Regulatory sequences involved in the promotion and termination of RNA transcription. Annu. Rev. Genet. **13:**319–353.
31. **Salema, M., J. S. Lolkema, M. V. San Romao, and M. C. Loureiro Dias.** 1996. The proton motive force generated in *Leuconostoc oenos* by L-malate fermentation. J. Bacteriol. **178:**3127–3132.
32. **Salema, M., B. Poolman, J. S. Lolkema, M. C. Loureiro Dias, and W. N. Konings.** 1994. Uniport of monoanionic L-malate in membrane vesicles from *Leuconostoc oenos*. FEBS Lett. **124:**1–7.
33. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
34. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
35. **Sauer, U., and P. Dürre.** 1993. Sequence and molecular characterisation of a DNA region encoding a small heat shock protein of *Clostridium acetobutylicum*. J. Bacteriol. **175:**3394–3400.
36. **Servant, P., and P. Mazodier.** 1995. Characterization of *Streptomyces albus* 18-kilodalton heat shock-responsive protein. J. Bacteriol. **177:**2998–3003.
37. **Straus, D. B., W. A. Walter, and C. A. Gross.** 1987. The heat shock response of *E. coli* is regulated by changes in the concentration of σ32. Nature (London) **329:**348–351.
38. **Suzek, R. E., and S. L. Lindquist.** 1989. hsp26 of *Saccharomyces cerevisiae* is related to the superfamily of small heat shock proteins but is without a demonstrable function. Mol. Cell. Biol. **9:**5265–5271.
39. **Takemoto, L., T. Emmons, and J. Horwitz.** 1993. The C-terminal region of α-crystallin: involvement in protection against heat-induced denaturation. Biochem. J. **294:**435–438.
40. **Tinoco, I., Jr., P. N. Borer, B. Dengler, M. D. Levine, O. C. Uhlenbeck, D. M. Crothers, and J. Gralla.** 1973. Improved estimation of secondary structure in ribonucleic acids. Nature New Biol. **246:**40–41.
41. **Weng, J., Z. F. Wang, and H. T. Nguyen.** 1991. Nucleotide sequence of a *Triticum aestivum* cDNA clone which is homologous to the 26 kDa chloroplast-localized heat shock protein gene of maize. Plant Mol. Biol. **17:**255–258.
42. **Yang, D., and C. R. Woese.** 1989. Phylogenetic structure of the *Leuconostoc*: an interesting case of a rapidly evolving organism. Syst. Appl. Microbiol. **12:**145–149.
43. **Yuan, Y., D. D. Crane, and C. E. Barry III.** 1996. Stationary phase-associated protein expression in *Mycobacterium tuberculosis*: function of the mycobacterial α-crystallin homolog. J. Bacteriol. **178:**4484–4492.
44. **Zuniga, M., I. Pardo, and S. Ferrer.** 1996. Transposons Tn916 and Tn925 can transfer from *Enterococcus faecalis* to *Leuconostoc oenos*. FEMS Microbiol. Lett. **135:**179–185.