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The Antisense RNA Approach: a New Application for In Vivo Investigation of the Stress Response of Oenococcus oeni, a Wine-Associated Lactic Acid Bacterium

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Oenococcus oeni is a wine-associated lactic acid bacterium mostly responsible for malolactic fermentation in wine. In wine, O. oeni grows in an environment hostile to bacterial growth (low pH, low temperature, and ethanol) that induces stress response mechanisms. To survive, O. oeni is known to set up transitional stress response mechanisms through the synthesis of heat stress proteins (HSPs) encoded by the hsp genes, notably a unique small HSP named Lo18. Despite the availability of the genome sequence, characterization of O. oeni genes is limited, and little is known about the in vivo role of Lo18. Due to the lack of genetic tools for O. oeni, an efficient expression vector in O. oeni is still lacking, and deletion or inactivation of the hsp18 gene is not presently practicable. As an alternative approach, with the goal of understanding the biological function of the O. oeni hsp18 gene in vivo, we have developed an expression vector to produce antisense RNA targeting of hsp18 mRNA. Recombinant strains were exposed to multiple stresses inducing hsp18 gene expression: heat shock and acid shock. We showed that antisense attenuation of hsp18 affects O. oeni survival under stress conditions. These results confirm the involvement of Lo18 in heat and acid tolerance of O. oeni. Results of anisotropy experiments also confirm a membrane-protective role for Lo18, as previous observations had already suggested. This study describes a new, efficient tool to demonstrate the use of antisense technology for modulating gene expression in O. oeni.

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tigating L018 function in vivo. Over the three last decades, the characterization of extrachromosomal DNA and genetic transformation techniques in O. oeni have received considerable attention. O. oeni transformation is presently possible by electroporation (19). Nevertheless, an efficient expression vector in O. oeni is still missing, and site-directed mutagenesis is presently not achievable because transformation efficiencies are lower than the frequency of recombination events (19, 20). Due to the lack of genetic tools for mutagenesis in O. oeni, we have focused our research on the antisense mechanism to modulate expression of target genes.

Antisense RNA (asRNA) was defined by Good as “natural or synthetic polymers that specifically recognize and inhibit target sense sequences, with mRNA being the usual target” (21). Diverse antisense mechanisms exist, and the term designates all mechanisms using sequence-specific mRNA recognition leading to reduced or altered expression of a transcript or a sense RNA (22). Antisense mechanisms are very common in nature and can affect mRNA transcription by inducing destruction and repression. Based on base pairing, the asRNA strategy is exploited for protein synthesis inhibition. Because asRNA action does not require modification of the organism’s chromosomal DNA, it has a higher throughput than traditional gene inactivation. Moreover, gene deletion strategies are feasible only if the genes are not essential for bacteria survival. While permanent directed inactivation is termed gene knockout, antisense RNA-based gene silencing is described as gene knockdown. Indeed, expression of unmodified RNA targeting the exact antiparallel mRNA sequence is the simplest use of asRNA. Translation inhibition is mainly due either to steric hindrance blocking the translation machinery or to rapid degradation of double-stranded RNA by specific RNases (22). Several studies using asRNA have already been carried out in LAB, demonstrating RNA base-pairing gene inhibition. Baouazzaoui and Lapointe modified the molecular mass of exopolsaccharide by modulating glycosyltransferase gene (wleE) expression using asRNA in Lactobacillus rhamnosus (23). In Lactococcus lactis, an antisense strategy was used to prevent proliferation of four phages (24). More recently, Oddone and collaborators adapted the nisin-controlled expression (NICE) system to express asRNA targeting the clpP protease gene in L. lactis (25). Several strategies can be considered to develop antisense RNA attenuation. Antisense RNA can be designed to target the entire open reading frame (ORF) of the gene, complementary to the mRNA (23–25). The ribosome binding site (RBS) or a ribosome target at a sequence nearby can also constitute an antisense strategy (21, 26). In this study, the target for antisense expression, hsp18, was chosen because it is one of the most well-studied hsp genes in O. oeni. This study reports the development of an asRNA strategy in O. oeni to investigate L018 function under stress conditions. This approach was completed by developing the first stable and efficient expression vector in O. oeni. We used two targeting strategies: (i) the hsp18 gene was cloned in the reverse orientation under the control of a LAB constitutive promoter to give rise to a nontranslatable double-stranded RNA molecule, and (ii) the 5′ untranslated region (5′ UTR) of hsp18 was cloned in the reverse orientation, complementary to the RBS.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. Oenococcus oeni strain ATCC BAA-1163 is an acidophilic strain isolated from red wine. O. oeni was grown at 30°C in FT80m medium (pH 5.3) (27). In preparation for the experiments, O. oeni was cultured from glycerol stocks in FT80m medium, which contained 20 μg·mL·1 of vancomycin, 20 μg·mL·1 of lincomycin, and 20 μg·mL·1 of erythromycin when required. For survival tests, cells were used from the O. oeni culture to the end of exponential phase (A\textsubscript{600} = 0.8) and then transferred at 48°C or into an acid FT80m medium adjusted to pH 3.5 or pH 3 and incubated at 30°C for 90 min. Escherichia coli EC101 strain [supE λod-5 thi Δ(lac-proAB) F′(traD36 proAB lacP·lacZΔM15) repA·] was used for cloning and maintenance of

| TABLE 1 Strains and plasmids used in this study |
|-----------------|-----------------|-----------------|
| **Strain or plasmid** | **Genotype or characteristics** | **Source or reference** |
| **Escherichia coli strains** | | |
| EC101 | JM101 [supE thi (lac proAB) F′ traD36 proAB lacP· ΔM15] with repA from pWV01 integrated in chromosome | Promega (Lyon, France) |
| EcAS18 | EC101 harboring pSIPSYNhs18-ORF | This study |
| EcAS18-UTR | EC101 harboring pSIPSYNhs18-5′ UTR | This study |
| Ecsyn | EC101 harboring pSIPSYN | This study |
| **Oenococcus oeni strains** | | |
| ATCC BAA-1163 | Wild-type strain, Van′ | Laboratory stock |
| OoS18 | ATCC BAA-1163 harboring pSIPSYNhs18-ORF | This study |
| OoS18-UTR | ATCC BAA-1163 harboring pSIPSYNhs18-8BS | This study |
| Oosyn | ATCC BAA-1163 harboring pSIPSYN | This study |
| **Plasmids** | | |
| pGID052 | Ery′ Linco′; 3.38-kb HindIII DNA fragment of pLC22R, oriR of pLC22R | 36 |
| pSYN3 | Ery′ Linco′; derived from pGID052, P\textsubscript{SYN} promoter cloned between SwaI and PvuII sites | This study |
| pSIP409 | Ery′ Ori′ Rep\textsubscript{SPO} sppK sppR gusA, under control of P\textsubscript{oriSPO} promoter from pSIP401 | 41 |
| pSIPSYN | Ery′ Linco′; derived from pSIP409, deletion of sppK, sppR, and gusA, under control of P\textsubscript{SYN} promoter | This study |
| pSIPSYNAS18 | Ery′ Linco′; derived from pSIPSYN, ORF/hsp18 in antisense orientation under control of P\textsubscript{SYN} promoter | This study |
| pSIPSYNAS18-UTR | Ery′ Linco′; derived from pSIPSYN, 5′ UTR hsp18 in antisense orientation under control of P\textsubscript{SYN} promoter | This study |

supervision of JMB and GM (INRA).
TABLE 2 Primers used in this study

<table>
<thead>
<tr>
<th>Gene or targeted region</th>
<th>Primer or probe</th>
<th>Primer sequence (5' to 3')a</th>
<th>Restriction site or DIG labeling</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsp18</td>
<td>ASHsp181</td>
<td>CGTCCCGGGATGCGCAATAGATTAGTATAGTAGAT</td>
<td>Smal</td>
</tr>
<tr>
<td></td>
<td>ASHsp182</td>
<td>GGAGTCTATTGATTGCAGTCGCGAGTTCTGACT</td>
<td>Ncol</td>
</tr>
<tr>
<td>5' UTR hsp18</td>
<td>ASHsp18R</td>
<td>GGGCCCGGTTAAGGCTGTAATATACCTCAGT</td>
<td>Smal</td>
</tr>
<tr>
<td></td>
<td>ASHsp18F</td>
<td>GGAGGCTGTAGTACATCTAATCTTAAAC</td>
<td>Ncol</td>
</tr>
</tbody>
</table>
| Synthetic promoter      | psynC           | GATGACCTGTCGTCAGCGAGGTACCGGCTCGGCAGCTGTAAGTT | Sall |}
|                         | psynD           | CGGATTTAATTTGACTGGGTTACATTTACATAAAT | Ncol                           |
|                         | Olcg305         | CCCGTCGACGCGCAACGTTGCGGAGGG | HindIII                        |
|                         | Olcg302         | GGCGCAGTGGCAAACACGCTTGAGTCAAAG | HindIII                        |
|                         | Olcg303         | CCCCGTCGACGCGCAACGTTGCGGAGGG | HindIII                        |
| mhs18 target            | DIG-mhs18 probe | GGAAGCGCATATTATTTTCTCTGTTCCTGCCC | 3' end-dUTP DIG                |
| ashp18 target           | DIG-ashp18 probe| TGAAAGCGATAAAGAATAGGGGTCGGAAG | 3' end-dUTP DIG                |

a Restriction sites are underlined, and the RBS is in bold.

plasmids. E. coli strains were grown at 37°C in Luria-Bertani (LB) medium supplemented with erythromycin (250 µg·mL⁻¹) when necessary.

DNA manipulation and bacterial transformation. O. oeni genomic DNA was extracted using the InstaGene matrix (Bio-Rad, Hercules, CA, USA). PCR was performed with the Expand high-fidelity PCR system (Roche, Meylan, France). Plasmids from E. coli were prepared with the GeneJet mini-prep kit (Thermo Scientific, Illkirch, France). DNA fragments were purified with the GeneJet PCR purification kit (Thermo Scientific, Illkirch, France). T4 DNA ligase and other restriction endonucleases were purchased from New England BioLabs Inc. ( Ipswich, USA).

Plasmids and ligation products were transfected by electroporation into E. coli EC101 by the method of Taketo (28). Briefly, cells in early exponential phase (A₆₀₀ of 0.5) were collected from 500 ml of LB culture, washed twice in 250 ml of ice-cold ultrapure water, and concentrated 100-fold in 2.5 ml of 25% glycerol. Aliquots of 0.1 ml were stored at -80°C. Aliquots of 0.1 ml were mixed on ice with plasmid DNA or ligation mixture and then subjected to an electroporation pulse of 25 μF, 200 Ω, and 12.5 kV/cm, followed by addition of 1 ml of LB medium. Cell suspensions were incubated for 20 min at 37°C, followed by plating of 0.1 ml on solid LB medium containing erythromycin (250 μg·mL⁻¹). Plasmids were transferred by electroporation into O. oeni as previously described by Assad-Garcia et al. (19). Recombinant strains were selected on FT80m plates supplemented with erythromycin, vancomycin, and lincomycin (20 μg·mL⁻¹ each) (19).

Plasmid constructions and cloning strategy. A synthetic consensus promoter region was designed by aligning the following O. oeni gene promoter sequences: melB-mela (accession number X82326) (29), clpX (Y15953) (30), hsp18 (X94968) (8), alsS-alsD (X93091) (31), clpB-clpL (AJ600644) (3), groES (CAI65392) (6), grpE (CAI68011) (6), dnaG (EAY39431.1) (C. Grandvalet, unpublished data; direct submission), and ctsR-clpC (AJ890338.1) (6). Complementary oligonucleotides psynC and psynD (50 mM each) (Table 2) were annealed by heating (95°C, 5 min) and gently cooling (95°C to 20°C), and the DNA duplex was restricted with SmaI and NcoI restriction enzymes and then subcloned between the SmaI and NcoI sites of plasmid pGID052 (32). The resulting plasmid, named pSYNS3 (Table 1), was then used as a DNA matrix for PCR amplification of the promoter region using oligonucleotides olcg302 and olcg303 (Table 2). An RBS was added by insertion in the sequence of primer olcg302. The resulting PCR product, corresponding to a synthetic promoter called P_syn (KTI76344) (this work), was finally cloned between the Sall and NcoI sites of plasmid pIP409, replacing the sakacin P-based inducible expression system with the synthetic consensus promoter to obtain the plasmid pSI PSYN (33).
Membranes were exposed to Amersham Hyperfilm ECL (GE Healthcare, Little Chalfont, United Kingdom). Signal intensity was measured by Image Lab 4.1 (Bio-Rad) to assess the relative quantity of Lo18 protein in each sample. Values are the means of triplicates and were calculated relative to the signal intensity of the control Oosyn strain grown at 30°C, which was arbitrarily set as 100%.

**Total RNA extraction and detection by dot blot hybridization.** The cell pellet from 25 ml of culture was suspended in 100 μl of TE buffer (Tris-HCl [10 mM], EDTA [1 mM], pH 8.0) containing 40 mg · ml⁻¹ of lysozyme and incubated for 30 min at 37°C for cell lysis. The RNA was then purified on a NucleoSpin RNA column according to the manufacturer’s recommendations (Macherey Nalgene, Hoerdt, France). The RNA samples were then treated with 10 μl of rDNase (Macherey Nalgene, Hoerdt, France) at 37°C for 1 h, followed by purification on NucleoSpin RNA with elution in a final volume of 20 μl. The absence of genomic DNA was tested by PCR amplification using universal bacterial primers for the 16S rRNA gene (27F [5′-AGAGTTTGATC[A/C]TGGCTCAG] and 1492R [5′-TACGG[A/T/C]TACCTGTTACGACTT]) (34). Then, 10 μg of purified total RNA from each sample was loaded on positively charged nylon membranes (Roche, Laval, Québec, Canada). Oligonucleotide probes were purchased 3′ end labeled with digoxigenin (DIG) dideoxy-UTP (Eurolgentec, Liège, Belgium). After prehybridization for 2 h at 50°C in Dig EasyHyb solution (5 × SSC [1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.1% N-laurylsarcosine, 0.02% SDS, 2% blocking solution), the probe (10 pmol) was added and hybridization was carried out 15 h at 50°C. After washing the membranes twice for 5 min in 2 × SSC–0.1% SDS at room temperature, two 15-min washes was carried out in 0.5 × SSC–0.1% SDS at 50°C. To bind the DIG-labeled probes, the membrane was incubated at 25°C for 30 min with antibody solution (alkaline phosphatase-conjugated antidigoxigenin antibody [1:10,000] in blocking solution). Detection of chemiluminescence was performed with disodium 3-(4-methoxyspiro[1,2-dioxetane-M,2′-(5′-chloro)tricyclo[3.3.1.1³7]decan]-4-yl phenyl phosphate) (CSPD) as the chemiluminescent substrate according to the manufacturer’s recommendations (Roche). Membranes were exposed to Kodak BioMax MS film (Kodak, Mandel Scientific, Guelph, Canada).

**Fluorescence anisotropy measurements.** The method for membrane fluidity assays was previously validated in our laboratory (35). Cell energization with a 2-(N-morpholino)ethanesulfonic acid (MES)–glucose buffer (50 mM MES, 10 mM glucose, KOH buffer [pH 5.5]) was necessary to prevent a decrease of membrane fluidity caused by bacterial death independent of the type of stress. Exponentially growing O. oeni cells (20-ml culture at an optical density at 600 nm [OD₆₀₀] of 0.8) were harvested by centrifugation at 6,300 × g for 10 min and washed once in 20 ml of MES-glucose. Pelleted cells were suspended in the same buffer, and the OD₆₀₀ was adjusted to 0.6 for each sample. Hydrophobic 1,6-diphenyl-1,3,5-hexatriene (DPH) (Molecular Probes) was used as a probe, and fluorescence anisotropy measurements were performed immediately on the prepared samples as follows: DPH solution (3 μM) was added to the cell suspension, and samples were then placed in the stirred and thermostated spectrofluorometer cuvette holder (Fluorolog-3; Jobin Yvon, Inc., USA). Anisotropy values were calculated as described by Shinitzky and Barenholz (36). To ensure optimal anisotropy determinations, the probe was stabilized at 30°C for 5 min before shock. Ethanol shock was applied to the labeled cells directly during the measurement. Excitation and emission wavelengths were 352 and 402 nm, respectively. Anisotropy values were automatically calculated by the spectrofluorometer as described by Shinitzky and Barenholz (36) as previously reported (4, 35). Fluorescence anisotropy (inversely proportional to membrane fluidity) was measured every 7 s for 45 min. Each experiment was performed at least two times from independent cultures.

**Statistical analysis.** The significance of the differences among fluorescence anisotropy values and survival tests was determined with a two-tailed Student t test. The confidence interval for a difference in the means was set at 95% (P ≤ 0.05) for all comparisons.
RESULTS
Cloning strategy: construction of expression vector to produce hsp18 antisense RNAs. The pSIPSYN vector constructed in this work derived from the Gram-negative/Gram-positive shuttle expression vector pSIP409 (Fig. 1; Table 1). Previous work has shown that the sakacin P-based vector pSIP409 was the most promising for inducible expression of gusA in lactobacilli such as Lactobacillus plantarum and Lactobacillus sakei (33). Thus, the pSIP409 vector was used as a starting point for studying gene expression in LAB. This vector is a low-copy-number plasmid and stems from the pSIP vector series (33). Previously, our laboratory developed an expression vector in O. oeni called pGID052. This vector is a 5.7-kb Gram-positive/Gram-negative shuttle vector which contained a pLC22R region required for replication, measuring 3.38 kb, from Leuconostoc citreum and a polylinker from the pUC18 vector. However, this vector does not have any transcription signals and has a substantial size, limiting transformation efficiency. Therefore, we developed a new expression vector from pSIP409 adapted to O. oeni. Inducible expression systems, including the sppR, sppK, and sppIP genes and P_oeni promoter, appear to be nonfunctional in O. oeni (data not shown). Consequently, the new vector was derived from pSIP409 by removing and replacing the sakacin P-inducible expression system with a synthetic promoter designed from alignment of promoter sequences from O. oeni genes (see Materials and Methods). The resulting plasmid was named pSIPSYN (Fig. 1B). Using the pSIPSYN vector, two different antisense RNAs of hsp18 (asRNA-hsp18) were produced to modulate hsp18 gene expression and investigate the knockdown of Lo18 protein levels (Fig. 2). First, the noncoding RNA of the hsp18 gene 5’ UTR was designed by cloning the 5’ UTR in antisense orientation to yield pSIPSYNAS18-UTR (Fig. 1B and 2A). Second, the full-length ashsp18 construction was designed by inserting the open reading frame of the hsp18 gene in antisense orientation to obtain plasmid pSIPSYNAS18 (Fig. 1B and 2B). Recombinant vectors, including pSIPSYN, were transferred to O. oeni strain ATCC BAA-1163, and recombinant strains were designated Oosyn, OoAS18-UTR, and OoAS18 for strains carrying plasmids pSIPSYN, pSIPSYNAS18-UTR, and pSIPSYNAS18, respectively.

Detection of Lo18 by Western blotting: validation of asRNA function. For each strain and condition, 5 μg of protein was deposited on membranes and revealed with polyclonal antibodies directed against Lo18. Relative quantities of Lo18 protein were calculated relative to the signal intensity of strain Oosyn grown at 30°C, which was arbitrarily designated 100%. Values are the means of duplicates. Between spot signals of Oosyn, the reference strain, and OoAS18-UTR, no differences in relative protein quantities were observed with (263% and 244%) or without (100% and 114%) thermal shock, indicating that there is no difference in the protein level when the 5’ UTR of the hsp18 gene is targeted by asRNA (Fig. 3). In contrast, the relative quantity of Lo18 in the recombinant strain OoAS18 at 30°C is 32%, indicating that the protein level of Lo18 is lowered 3.1-fold when the whole gene sequence is targeted by asRNA. After a heat shock, the same observation was made, with a 59% relative quantity of Lo18 protein in strain OoAS18 and 263% in strain Oosyn, meaning a 4.5-fold decrease. Having observed a 3.1-fold decrease at 30°C and a 4.5-fold decrease after a heat shock in Lo18 levels by expressing asRNA targeting the whole mRNA of hsp18 and no differences in the Lo18 protein level by targeting the 5’ UTR of hsp18, we focused our research on strain OoAS18.

Detection of hsp18 asRNA and mRNA by Northern blotting: validation of asRNA expression. For each strain and each condition, 10 μg of RNA was deposited on a nylon membrane. Digoxigenin-labeled RNA probes were used to detect hsp18 mRNA and...
<p>Based on survival tests during stress treatments were performed on recombinant strains Oosyn and OoAS18. In contrast, strain OoAS18 lost 95.0% of its population (Fig. 5A). After a 15% decrease of the initial anisotropy value, indicating an instantaneous increase in membrane fluidity (Fig. 6). The fluidifying effect of ethanol was transient. For Oosyn, the reference strain, membrane fluidity returned to its initial level 40 min after a 12% ethanol shock. After 40 min, the anisotropy value was higher than the initial level, indicating a membrane rigidification. The reference strain recovered its initial membrane fluidity with a speed of 0.31% anisotropy unit · min⁻¹. The recombinant strain OoAS18 was able to counteract the effect of ethanol shock at a speed comparable to that of the reference strain. Nevertheless, membrane fluidity did not return to the initial level and remained at 96% of the initial value, with no membrane rigidification. The kinetics of membrane fluidity restoration differed significantly between the reference strain Oosyn and the recombinant strain OoAS18.</p>

**DISCUSSION**

Natural control of gene expression by the antisense strategy has been characterized in many bacteria and eukaryotes (37, 38). This regulatory system, by base pairing using small RNA, has been applied to the investigation of gene function in eukaryotes and is used more and more to study gene function or to modulate phase and plasmid replication (23–25, 39–41). Due to the lack of genetic tools for directed mutagenesis in <i>O. oeni</i>, the asRNA production approach is presently a highly promising approach to investigate the stress response in this bacterium. By targeting <i>hsp18</i> for knockdown, we undertook the first <i>in vivo</i> approach with the aim of studying the only described <i>O. oeni</i> sHSP. The expression of a complementary asRNA targeting the full-length <i>hsp18</i> gene reduced the levels of <i>O</i>.<i>OAS18</i> 3.1-fold during growth at 30°C and 4.5-fold after a heat shock at 42°C. In contrast, the targeting by asRNA of the 5′ UTR of the <i>hsp18</i> gene was not effective. This observation is in agreement with studies performed in LAB using the antisense strategy to target the whole sequences of genes (23, 25, 42, 43). Indeed, the expression of antisense RNA covering the whole gene sequence appeared to be the most effective strategy to inhibit gene expression (43). Moreover, interestingly, reported effective antisense RNA approaches in LAB used 500-bp-length asRNAs, as in our study (23, 25, 43). The 68% decrease in <i>Lol18</i> protein level at 30°C validated the usefulness and efficiency of the pSIPSYN vector to produce asRNA targeting mRNA and thus to attenuate gene expression in <i>O. oeni</i>.</p>

Our Northern dot-blotting analysis allowed validation of the new expression vector by highlighting the presence of <i>hsp18</i>
asRNA. Even though quantities of hsp18 transcripts were not affected by antisense hsp18 expression, the Lo18 protein level and the survival capability of O. oeni under stress conditions were clearly affected. This result seems to indicate a posttranscriptional action of hsp18 antisense RNA expression, probably by preventing translation through steric hindrance. The 68% decrease in Lo18 protein detected by Western blotting validates this hypothesis. For asRNA-based regulatory techniques, the physical interaction of asRNA with the targeted mRNA is an essential condition (44). However, the interaction between asRNA and its target does not necessarily lead to the rapid degradation of the double-stranded RNA duplex. Thus, hybridization by base paring of asRNA with its target can cause the generation of a processing site leading to a translationally inactive or a stabilized form of the targeted mRNA (44). While it is difficult to conclude that the duplex asRNA-mRNA is not targeted by a degradation system, this hypothesis could explain why mRNA levels were only slightly affected in strain OoAS18.

Lo18 is the sole sHSP described in O. oeni and is encoded by the best-characterized in vitro hsp gene of O. oeni. Therefore, we selected it as a primary target for antisense RNA in order to unravel its potential in vivo role. Expression of the hsp18 gene is controlled by CtsR, the master transcriptional regulator of hsp gene expression in O. oeni (6). Lo18 synthesis is induced during the stationary growth phase and is also induced in response to various stresses, such as ethanol, heat, and acid stress (10, 18). Thus, Lo18 seems to have a cytoplasmic localization, which may allow it to exert a ATP-independent molecular chaperone activity (18). During our investigation of the potential of the asRNA approach, we have demonstrated that asRNA expression under the control of the synthetic promoter gave rise to phenotypic differences between the reference strain Osyn and the recombinant strain OoAS18. This phenotypic analysis highlighted the role of Lo18 in counteracting stress effects on O. oeni whole cells. Thus, the attenuation of hsp18 expression led to a significant loss of viability after heat shock or acid shock. As previously shown in E. coli using a heterologous system (45), our findings finally confirm in O. oeni the involvement of Lo18 in the acidophilic capability of bacteria. Indeed, the attenuation of Lo18 levels reduces the stress response ability of O. oeni under the acid stress conditions tested. Moreover, Lo18 had an effect on the ability of cells to counteract heat stress. This observation confirms the in vivo implication of Lo18 in high-temperature thermoprotection and acid tolerance of stressed cells. The implications of sHSP have been previously explored in Lactobacillus plantarum, a wine-related LAB (46). Three small heat shock protein-encoding genes have been previously reported in L. plantarum strain WCFS1, hsp18.5, hsp18.55, and hsp19.3 (47, 48). The deletion of the hsp18.55 gene affects cell recovery following short intense heat stress, as well as cell morphology and membrane fluidity of L. plantarum cells (46). In addition, the hsp18 gene of Streptomyces albus encodes an sHSP that plays a role in thermotolerance (49), and HSP17 of Synechocystis spp. is essential for tolerance to high temperatures (49, 50). In O. oeni, Lo18 was shown to be a membrane-associated sHSP displaying a possible lipochaperone activity (5, 8, 17, 18).

Indeed, after addition of benzyl alcohol, a membrane fluidifier, Lo18 was found at the periphery of the membrane, suggesting a role in membrane stabilization during environmental stress and a potential molecular lipochaperone activity (5). Therefore, we assessed the lipochaperone activity of Lo18 by using fluorescence anisotropy analysis with a DPH probe, a method previously used in O. oeni (35). Attenuation of hsp18 gene expression affects the membrane fluidity of O. oeni recombinant strains, which immediately decreases after ethanol shock. Our findings demonstrate involvement of Lo18 in modulation of membrane fluidification caused by ethanol. These observations are consistent with previous studies in O. oeni during combined and single cold, acid, and ethanol shocks, highlighting a 15% decrease of anisotropy (4, 11, 35). After an ethanol shock, the reference strain Osyn managed to restore its initial membrane fluidity in 45 min, while the OoAS18 strain failed to restore membrane fluidity to its initial...
value. Therefore, the attenuation of Lo18 protein level by asRNA targeting prevents the full restoration of membrane fluidity, which remained at 96% of the initial value. The anisotropy results were highly similar to those obtained by Török and coworkers in *Synchocystis* sp. strain PCC6803, where deletion of a small heat shock protein-encoding gene (*hsp17*) was characterized (13). Differences in membrane fluidity were detected when the fluorescence anisotropies of the wild-type and the *hsp17* deletion strains were compared (13). The deletion of *hsp17* showed that membrane fluidity of *hsp17*-deficient cells was remarkably higher than that of the parental cells suggesting that HSP17 may function primarily as a membrane-stabilizing factor. Our results clearly illustrate the membrane-protective role of sHSP during ethanol shock. In fact, our study using the asRNA strategy shows that Lo18 is required for *O. oeni* survival under environmental stress conditions causing fluidification of the membrane and aggregation of cellular proteins.

In summary, our findings indicate that knockdown by the antisense RNA system is presently the most relevant strategy to understand the role of *hsp18*. Moreover, besides technical restrictions, permanent knockout of *hsp18* by directed mutagenesis may not be achievable due to the possibly lethal character of this mutation. We have reported an efficient technique available to investigate the function of stress genes in *O. oeni* and thus to study the stress response ability of this bacterium.

In addition, we can conclude that the pSIPSYN vector designed for this work is an efficient expression vector used to produce asRNA. This new expression vector opens two main perspectives in order to pursue the study of stress response mechanisms in *O. oeni*. This could enable the opening of a real *in vivo* investigation in *O. oeni*. Indeed, the synthetic promoter of the pSIPSYN vector enables constitutive expression in *O. oeni*, which could be a dual potential use of expressing candidate genes or targeting other genes of interest with the antisense approach. Thus, the pSIPSYN vector has already been exploited for gene overexpression to study the impact of *O. oeni* esterase genes on the aromatic quality of wine, opening the doors for wine-associated LAB engineering (M. Darsonval, unpublished data). Regarding the targeting of other genes by the antisense RNA approach, Sturino and Klaenhammer have already stated that the efficiency of the asRNA approach seems to be highly variable and dependent on the choice of the candidate gene targeted for this purpose (24, 43). These observations imply that some key criteria are needed to select an ideal asRNA target. Thus, the asRNA approach will certainly need further adjustments according to the targeted gene. To further study the stress response in *O. oeni*, we plan to target the ctsR gene, encoding the unique regulator of stress response gene expression identified in *O. oeni*, which should be ideally suited for this strategy (6).

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