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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*.**

Oenococcus oeni is a wine-associated lactic acid bacterium mostly responsible for wine malolactic fermentation (MLF) (1). In wine medium, *O. oeni* evolves under extreme physicochemical conditions: low pH (3.2 to 3.5), low temperature (18°C), and the presence of biological competitors such as yeast, producing inhibitory compounds, i.e., fatty acids, ethanol, 2-phenylethanol, and sulfites. The winemaking process itself may also constitute a hostile factor for bacterial growth. All these hostile environmental factors can cause a delay in MLF initiation. Thus, this fermentation step is currently not yet fully managed. However, the gradual increase in the ethanol level in wine during the alcoholic fermentation performed by yeasts and the production of substances inhibiting growth of lactic acid bacteria (dodecanoic acid or decanoic acid) led to the selection of the species most suited and adapted to wine conditions: *O. oeni* (1, 2). A better understanding of the mechanisms involved in stress-adaptive responses is essential to improve *O. oeni* development in wine and to perfect industrial processes of preparing wine malolactic starter. Because of its acidophilic properties, *O. oeni* is an interesting model for investigating stress response mechanisms in lactic acid bacteria (LAB). Like any organism undergoing an environmental stress, *O. oeni* tries to restore a metabolic profile beneficial for survival. Over the past decades, several mechanisms involved in adaptation to wine were described, including genes related to the general stress response, membrane composition and fluidity, pH homeostasis, the oxidative stress response, and DNA damage (3–8). 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 single small HSP (sHSP) named Lo18 (3, 6, 9, 10), whose synthesis is induced by multiple stresses such as heat, ethanol, low pH, or addition of benzyl alcohol membrane fluidifier (5, 11–13). sHSPs are ubiquitous proteins found throughout all biological domains and characterized by a sequence of about 100 amino acids residues called the α-crystallin (14). These proteins have a cytoplasmic molecular chaperone activity, and some of them were shown to be membrane associated and to play a role in membrane quality control (15).

Several studies have investigated Lo18 function *in vitro* using either heterologous (11, 16) or *in vitro* (17) systems. Under stress conditions, Lo18 is produced in large amounts (10), notably during ethanol stress (5, 10). Lo18 was shown to interact with the cytoplasmic membrane under stress conditions, suggesting that Lo18 could be involved in stress adaptation as a lipochaperone (5). Possible lipochaperone activity and molecular chaperone activity of Lo18 have been examined *in vitro*. Purified Lo18 protein was able to stabilize liposome fluidity (5) and to prevent protein aggregation (18). Since *O. oeni* is not a genetically tractable bacterium and has no available efficient expression plasmid for genetic studies, mutant construction is not presently practicable for inves-

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Genotype or characteristics[a]	Source or reference
Escherichia coli strains		
EC101	JM101[*supE thi* (*lac proAB*) F′ *traD36 proAB lacI*^{q} *Z*ΔM15] with *repA* from pWV01 integrated in chromosome	Promega (Lyon, France)
EcAS18	EC101 harboring pSIPSYN*hsp18*-ORF	This study
EcAS18-UTR	EC101 harboring pSIPSYN*hsp18*-5′ UTR	This study
Ecsyn	EC101 harboring pSIPSYN	This study
Oenococcus oeni strains		
ATCC BAA-1163	Wild-type strain, Van^r	Laboratory stock
OoS18	ATCC BAA-1163 harboring pSIPSYNAS*hsp18*-ORF	This study
OoAS18-UTR	ATCC BAA-1163 harboring pSIPSYNAS*hsp18*-RBS	This study
Oosyn	ATCC BAA-1163 harboring pSIPSYN	This study
Plasmids		
pGID052	Ery^r Linco^r; 3.38-kb HindIII DNA fragment of pLC22R, *oriR* of pLC22R	36
pSYNS3	Ery^r Linco^r; derived from pGID052, P_{SYN} promoter cloned between SwaI and PvuII sites	This study
pSIP409	Ery^r Ori$^+$ Rep_{256} *sppK sppR gusA*, under control of P_{orfX} promoter from pSIP401	41
pSIPSYN	Ery^r Linco^r; derived from pSIP409, deletion of *sppK*, *sppR*, and *gusA*, under control of P_{SYN} promoter	This study
pSIPSYNAS18	Ery^r Linco^r; derived from pSIPSYN, ORF*hsp18* in antisense orientation under control of P_{SYN} promoter	This study
pSIPSYNAS18-UTR	Ery^r Linco^r; derived from pSIPSYN, 5′ UTR *hsp18* in antisense orientation under control of P_{SYN} promoter	This study

[a] Van^r, vancomycin resistance; Ery^r, erythromycin resistance; Linco^r, lincomycin resistance.

tigating Lo18 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 exopolysaccharide by modulating glycosyltransferase gene (*welE*) 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 Lo18 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 $\mu g \cdot ml^{-1}$ of vancomycin, 20 $\mu g \cdot ml^{-1}$ of lincomycin, and 20 $\mu g \cdot ml^{-1}$ of erythromycin when required. For survival tests, cells were used from the *O. oeni* culture to the end of exponential phase ($A_{600} = 0.8$) 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 hsd-5 thi* Δ(*lac-proAB*) F′(*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15) *repA*$^+$] was used for cloning and maintenance of

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TABLE 2 Primers used in this study

Gene or targeted region	Primer or probe	Primer sequence (5′ to 3′)[a]	Restriction site or DIG labeling
hsp18	ASHsp181	CGTCCCGGGATGGCAAATGAATTAATGGATAGAAATGAT	SmaI
	ASHsp182	GGGCCATGGTTATTGGATTTCAATATGATGAGTTTGACT	NcoI
5′ UTR *hsp18*	ASHsp18R	GGGCCCGGGTAAGCGGTATGTAATATCACT	SmaI
	ASHsp18F	GGGCCATGGCATATCAAATACCTCCTATTAAC	NcoI
Synthetic promoter	psynC	GATCAGCTGCCATGGCTGCAGGGATCCCCGGGTACCTCGAGCTCTGAATTCA TTATAGTGATTATTGGTACTAAAGTCAAATTTAAATCCG	
	psynD	CGGATTTAAATTTGACTTTAGTACCAATAATCACTATAATGAATTCAGAGCTC GAGGTACCCGGGGATCCCTGCAGCCATGGCAGCTGATC	
	Olcg305	CCCGTCGACGCGCAACTGTTGGGAAGGG	SalI
	Olcg302	GGGCCATGGCAAATA**CCTCCT**GATTCATTAATGCAGGGGTAC	NcoI
	Olcg303	CCCAAGCTTGCGCAACTGTTGGGAAGGG	HindIII
mhsp18 target	DIG-mhsp18 probe	CGAACGACATTATTTTTCTTGTCTTTTTCCTCGGCCTTGG	3′ end-dUTPDig
ashsp18 target	DIG-ashsp18 probe	TGAAAACGATAAAGAATACGGCCTGAAAATCGAACTTCCA	3′ end-dUTPDig

[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 $\mu g \cdot ml^{-1}$) 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 plasmid miniprep 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 transferred by electroporation into *E. coli* EC101 by the method of Taketo (28). Briefly, cells in early exponential phase (A_{600} 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 $\mu g.ml^{-1}$). 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 $\mu g \cdot ml^{-1}$ each) (19).

Plasmid constructions and cloning strategy. A synthetic consensus promoter region was designed by aligning the following *O. oeni* gene promoter sequences: *mleR-mleA* (accession number X82326) (29), *clpX* (Y15953) (30), *hsp18* (X99468) (8), *alsS-alsD* (X93091) (31), *clpP-clpL* (AJ606044) (3), *groES* (CAI65392) (6), *grpE* (CAI68011) (6), *dnaG* (EAV39431.1) (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 SwaI and SmaI restriction enzymes and then subcloned between the SwaI and PvuII 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 olcg305 (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} (KT716344) (this work), was finally cloned between the SalI and NcoI sites of plasmid pSIP409, replacing the sakacin P-based inducible expression system with the synthetic consensus promoter to obtain the plasmid pSIPSYN (33).

Plasmids pSIPSYNAS18-UTR and pSIPSYNAS18 were constructed by inserting the amplified 5′ UTR *hsp18* region containing the RBS or the full-length coding sequence of the *hsp18* gene, respectively, into the multiple-cloning site of pSIPSYN. The antisense *hsp18* gene inserts were amplified by PCR from *O. oeni* ATCC BAA-1163 genomic DNA using oligonucleotide pairs ASHsp181/ASHsp182 and ASHsp18R/ASHsp18F, respectively (Table 2) and cloned between the NcoI and SmaI restriction sites of pSIPSYN. The resulting plasmids were transferred into *O. oeni* by electroporation (19). The pSIPSYN vector alone was also introduced into *O. oeni* as a control. Positive clones were selected on FT80m plates supplemented with erythromycin, vancomycin, and lincomycin (20 $\mu g \cdot ml^{-1}$ each). The presence of plasmids was confirmed by colony PCR amplification with specific primer pair Olcg303/Olcg302 or ASHsp181/ASHsp18R (Table 2).

DNA sequencing and sequence analysis. The plasmid DNA sequences were confirmed by nucleotide sequencing of both strands by Beckman Coulter Genomics (Essex, United Kingdom) using oligonucleotide Olcg303, ASHSP181, or ASHPS18R (Table 2). Nucleotide sequencing results were analyzed using LALIGN software (www.ch.embnet.org/software/LALIGN_form.html).

Total cellular protein extraction and detection by dot blot hybridization. The cell pellet from 50 ml of culture was suspended in 800 μl of lysis buffer (Tris-HCl [10 mM], NaCl [200 mM], pH 8.8), glass beads (0.5 μm) were added, and cells were lysed by two consecutive 60-s treatments with a Precellys homogenizer (Paris, France) at 6,500 rpm. The suspension was centrifuged at 13,200 × *g* for 15 min at 4°C to remove intact cells and cellular debris. Supernatants containing total cellular proteins were collected and assayed with the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA). Protein concentrations were adjusted to 1 $\mu g \cdot \mu l^{-1}$, and 5 μg of from each sample was spotted on a polyvinylidene difluoride (PVDF) membrane activated by a 15-min wash in methanol (GE Healthcare, Little Chalfont, United Kingdom). After drying, the membrane was washed twice in 1× phosphate-buffered saline (PBS). After saturation in 1× PBS–5% milk and three washes in 1× PBS–0.2% Triton X-100, the membrane was first hybridized with anti-Lo18 antibody solution (antibody serum from rabbit [1:100] in 1× PBS–0.2% Triton X-100–5% milk) for 1 h at room temperature with gentle shaking (8). The membrane was then washed three times in 1× PBS–0.2% Triton X-100 and incubated for 1 h at room temperature with peroxidase-conjugated goat anti-rabbit antibody solution (1:5,000 in 1× PBS–0.2% Triton X-100–5% milk) (GE Healthcare, Little Chalfont, United Kingdom). Before detection, the membrane was washed in 1× PBS–0.2% Triton X-100 for 15 min and four times for 5 min. Chemiluminescence was detected using the ECL kit (Clarity ECL Western blotting substrate; Bio-Rad, Hercules, CA, USA).

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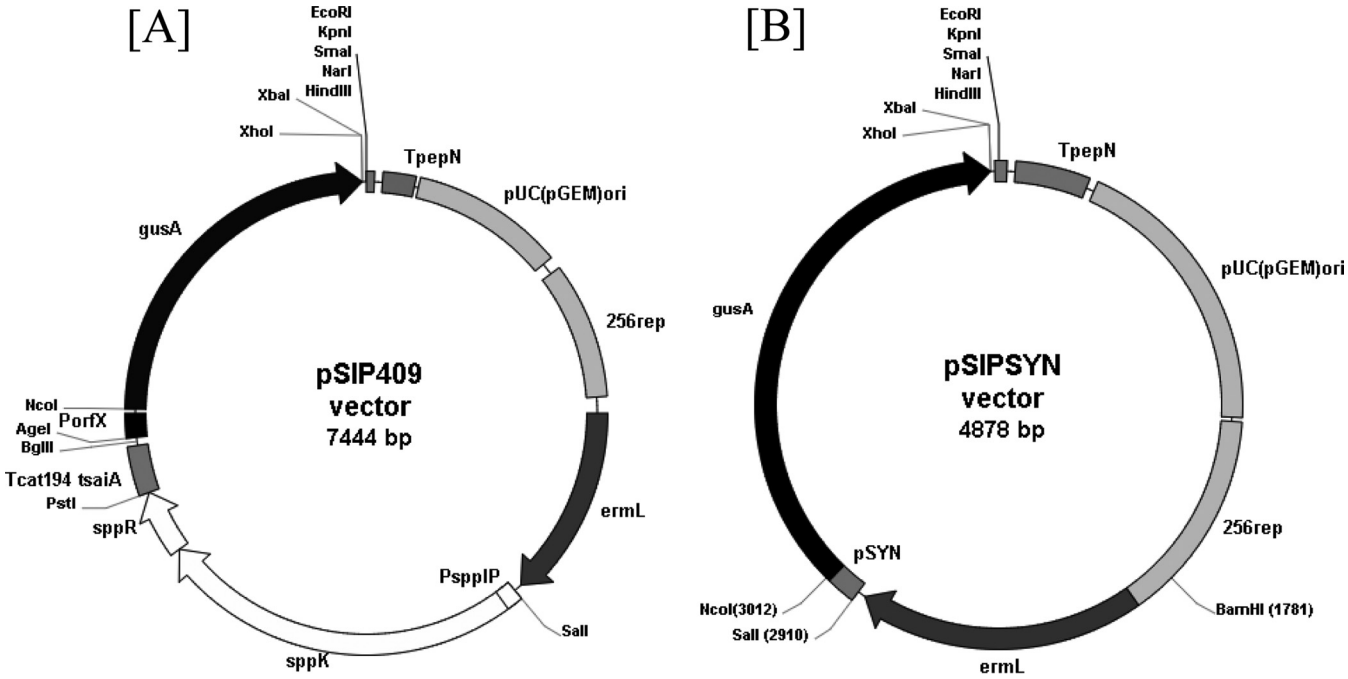


FIG 1 Restriction map of plasmids pSIP409 and pSIPSYN. (A) pSIP409. pSIP409 is a Gram-positive/Gram-negative shuttle vector with the Gram-negative replication origin pUC(GEM)ori, Gram-positive origin of replication 256rep, erythromycin selection (*ermL*), and expression of the reporter gene encoding β-glucuronidase (*gusA*) inducible by addition of a 19-amino-acid peptide pheromone (SppIP, MAGNSSNFIHKIKQIFTHR). (B) pSIPSYN. pSIPSYN is a derivative of pSIP409 with removal of the inducible expression system (*sppK*, *sppR*) and replacement of the P_{orfX} inducible promoter with a synthetic promoter from *O. oeni* strain ATCC BAA-1163 upstream of the gene *gusA* between the SalI and NcoI restriction sites.

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[C/A]TGGCTCAG] and 1492R [5′-TACGG[A/T/C]TACCTTGTTACGACTT]) (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 (Eurogentec, 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 were 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[3.3.1.1^{3,7}]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 [OD_{600}] 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 OD_{600} 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% ($P \leq 0.05$) for all comparisons.

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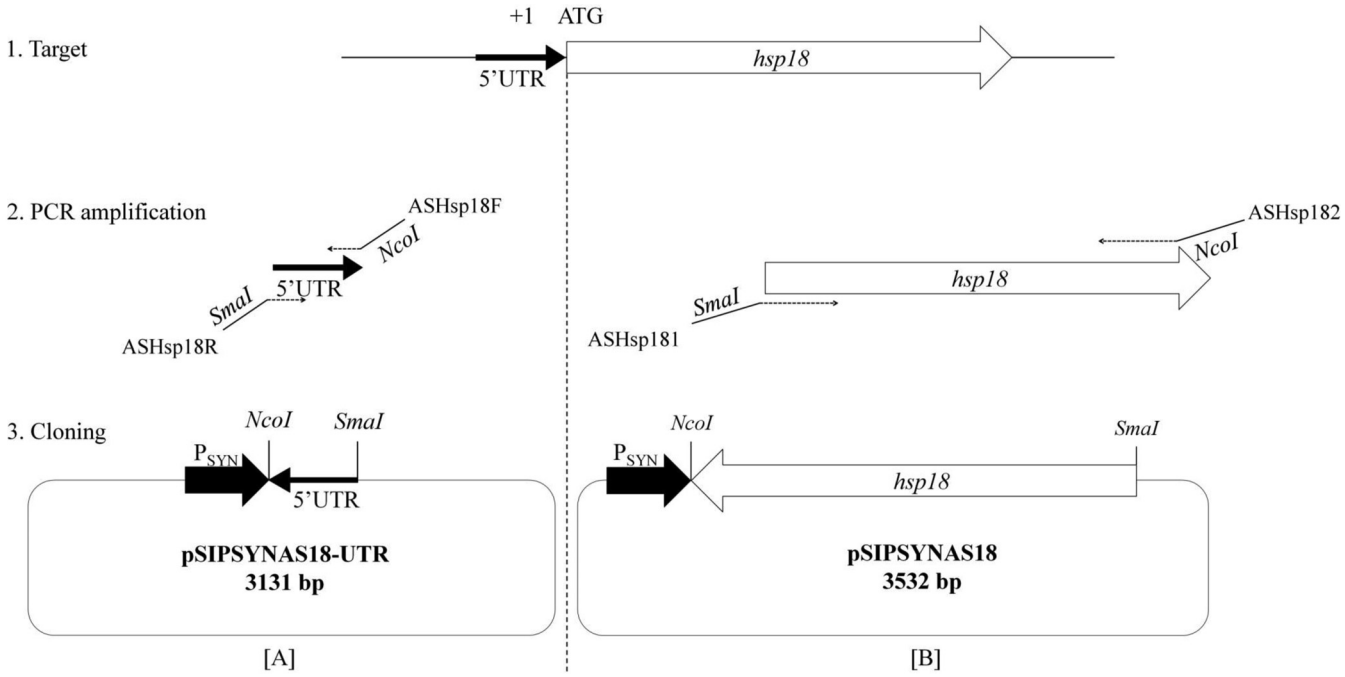


FIG 2 Cloning strategy for antisense *hsp18* expressed under control of the P_{SYN} promoter. Primer sequences are listed in Table 2. (A) Cloning strategy targeting the 5′ UTR of *hsp18*. (B) Cloning strategy targeting the full-length *hsp18* gene.

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_{orfX} 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 as*hsp18* 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

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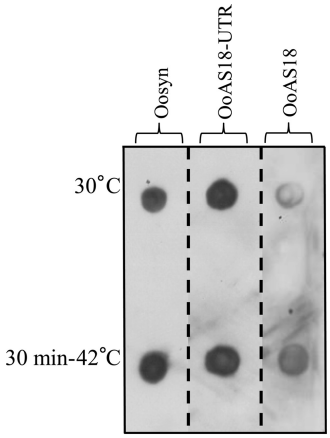


FIG 3 Immunodetection of Lo18 in the *O. oeni* recombinant strains. Strains carrying pSIPSYN (Oosyn), pSINSYNAS18-UTR (OoAS18-UTR), or pSIPSYNAS18 (OoAS18) vectors were cultivated at 30°C in FT80m medium until the end of the exponential growth phase. Total cellular proteins were extracted immediately (30°C) or following a thermal treatment (30 min at 42°C). For each strain and each condition, 5 μg of protein was loaded on a PVDF membrane activated with methanol. After drying the membrane, the *O. oeni* protein Lo18 was detected by immunodetection with polyclonal antibodies directed against Lo18 and anti-rabbit antibody conjugated with peroxidase. Signal intensity was measured by Image Lab 4.1 (Bio-Rad) to assess relative quantities of the Lo18 protein in each spot.

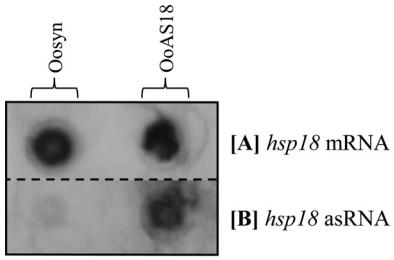


FIG 4 Detection of *hsp18* asRNA and *hsp18* mRNA in the *O. oeni* recombinant strains. Dot blot hybridization with oligonucleotide probe DIG-mhsp18 complementary to mRNA (A) or oligonucleotide probe DIG-ashsp18 complementary to antisense RNA (B) was performed. Recombinant strains carrying the native plasmid (Oosyn) or carrying the plasmid expressing *hsp18* asRNA (OoAS18) were cultivated at 30°C in FT80m medium supplemented with appropriate antibiotics until the end of the exponential growth phase. Total RNA was isolated, and 5 μg of RNA from each strain and each condition was loaded on a nitrocellulose membrane.

hsp18 asRNA (Fig. 4). A signal corresponding to *hsp18* mRNA was detected in the Oosyn and OoAS18 strains, and no difference in *hsp18* mRNA levels was observed between the two strains (Fig. 4A). In contrast, a signal corresponding to *hsp18* asRNA was detected in strain OoAS18 but not in Oosyn (Fig. 4B). This observation validates the pSIPSYN vector for expression of asRNA and target genes of interest. Based on this observation, we investigated the physiological effects of attenuation of *hsp18* expression in *O. oeni*.

What is the effect of lower Lo18 levels on *O. oeni* survival under stress conditions? To address the *in vivo* function of *hsp18*, survival tests during stress treatments were performed on recombinant strains Oosyn and OoAS18. Based on *in vitro* studies of Lo18, we focused our physiological study on stresses causing membrane fluidity changes or protein aggregation, such as heat shock or acid shock. Cells were cultivated under optimal growth conditions to the middle of the exponential growth phase and then subjected to different stress treatments. Following CFU counts on agar plates, the cultivability rate was calculated and normalized to that of the reference strain Oosyn (Fig. 5). After a heat shock or acid shock induced by switching the temperature from 30°C to 48°C or the medium pH from 5.3 to 3 or 3.5 for 90 min, no loss of cultivability of strain Oosyn was observed. In contrast, strain OoAS18 lost 95.0% of its population (Fig. 5A). After a pH 3.5 acid shock, the attenuation of Lo18 levels induced a 59.6% loss of cultivability of strain OoAS18 (Fig. 5B). The loss of cultivability was higher when the acid shock was increased to pH 3, with strain OoAS18 losing 98.1% viability (Fig. 5C).

Effect of ethanol shock on membrane fluidity determined by fluorescence anisotropy. Ethanol shock (12 [vol/vol]) gave rise to 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.

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 phage and plasmid replication (23–25, 39–41). Due to the lack of genetic tools for directed mutagenesis in *O. oeni*, the asRNA production approach is presently a highly promising approach to investigate the stress response in this bacterium. By targeting *hsp18* for knockdown, we undertook the first *in vivo* approach with the aim of studying the only described *O. oeni* sHSP. The expression of a complementary asRNA targeting the full-length *hsp18* gene reduced the levels of Lo18 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 *hsp18* 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 Lo18 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 *O. oeni*.

Our Northern dot-blotting analysis allowed validation of the new expression vector by highlighting the presence of *hsp18*

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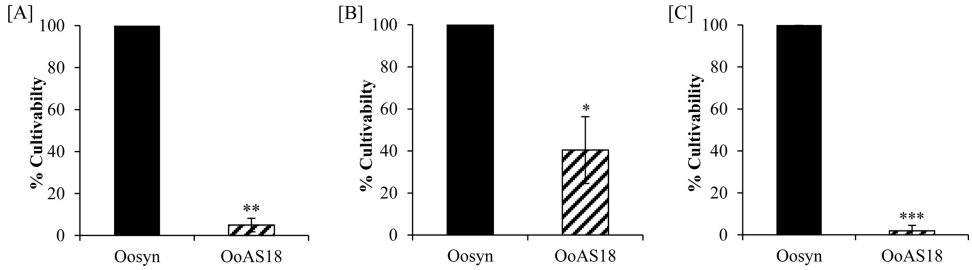


FIG 5 Cultivability tests after stress treatments. Recombinant strains carrying native plasmid (Oosyn) or the plasmid expressing *hsp18* asRNA (OoAS18) were grown at 30°C in FT80m medium until the end of the exponential phase (A_{600} = 0.8). Cultures were incubated at 48°C (A), or cells were transferred into FT80m medium adjusted to pH 3.5 (B) or pH 3 (C) and incubated at 30°C for 90 min. Bacterial cultivability was estimated (CFU · ml^{-1}). Significant differences are based on unilateral and paired *t* tests. ***, $P < 0.0005$; **, $P < 0.005$; *, $P < 0.05$.

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 Oosyn 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).

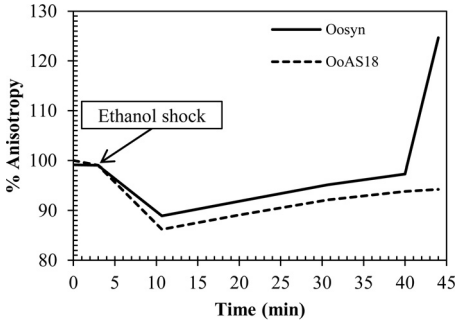


FIG 6 Evolution of membrane fluidity (anisotropy percentage) in *O. oeni* cells during ethanol shock (30°C, pH 5.3, and 12% [vol/vol] ethanol). The reference strain Oosyn (solid line) and the recombinant strain OoAS18 (dashed line) were grown at 30°C in FT80m pH 5.3 medium until the end of the exponential phase (A_{600} = 0.8). After an MES-glucose buffer wash, the OD_{600} was adjusted to 0.6 for each sample. Results are expressed as anisotropy percentage as a function of time.

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 Oosyn managed to restore its initial membrane fluidity in 45 min, while the OoAS18 strain failed to restore membrane fluidity to its initial

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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 *Synechocystis* 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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