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CtsR, the Master Regulator of Stress-Response in Oenococcus oeni, Is a Heat Sensor Interacting With ClpL1

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Oenococcus oeni is a lactic acid bacterium responsible for malolactic fermentation of wine. While many stress response mechanisms implemented by O. oeni during wine adaptation have been described, little is known about their regulation. CtsR is the only regulator of stress response genes identified to date in O. oeni. Extensively characterized in Bacillus subtilis, the CtsR repressor is active as a dimer at 37°C and degraded at higher temperatures by a proteolytic mechanism involving two adapter proteins, McsA and McsB, together with the ClpCP complex. The O. oeni genome does not encode orthologs of these adapter proteins and the regulation of CtsR activity remains unknown. In this study, we investigate CtsR function in O. oeni by using antisense RNA silencing in vivo to modulate ctsR gene expression. Inhibition of ctsR gene expression by asRNA leads to a significant loss in cultivability after heat shock (58%) and acid shock (59%) highlighting the key role of CtsR in the O. oeni stress response. Regulation of CtsR activity was studied using a heterologous expression system to demonstrate that O. oeni CtsR controls expression and stress induction of the O. oeni hsp18 gene when produced in a ctsR-deficient B. subtilis strain. Under heat stress conditions, O. oeni CtsR acts as a temperature sensor and is inactivated at growth temperatures above 33°C. Finally, using an E. coli bacterial two-hybrid system, we showed that CtsR and ClpL1 interact, suggesting a key role for ClpL1 in controlling CtsR activity in O. oeni.

Keywords: Oenococcus oeni, stress response, CtsR, RNA silencing, heterologous expression system, two-hybrid system

INTRODUCTION

Oenococcus oeni is an acidophilic wine-associated lactic acid bacterium (LAB), mainly responsible for malolactic fermentation (MLF) of wine, usually following yeast-driven alcoholic fermentation (Lonvaud-Funel, 1999). Wine and the winemaking process form a harsh and challenging environment combining stresses such as low pH (3–3.5), low temperatures (14–18°C), the presence of ethanol, nutrient starvation and competing organisms (yeasts) generating abiotic growth...
inhibitors (ethanol, sulfites, decanoic, and dodecanoic acids). Like most microorganisms facing stress conditions, *O. oeni* must adapt to survive and deciphering the molecular mechanisms involved in responding to stress is an important step to improve *O. oeni* MLF performance and design future malolactic starter strains. Because of its acidophilic profile and its unique genome organization, *O. oeni* is an intriguing and challenging model to investigate stress response mechanisms in LAB (Bartowsky, 2017; Grandvalet, 2017). Over the past decades, several genetic responses adopted by *O. oeni* during wine adaptation have been described, including genes involved in general stress response, membrane composition and fluidity, pH homeostasis, oxidative stress response, presence of sulfites and DNA damage (Salema et al., 1996; Guzzo et al., 1997, 1998; Jobin et al., 1997, 1999; Tourdot-Maréchal et al., 2000; Da Silveira et al., 2003; Beltramo et al., 2004; Chu-Ky et al., 2005; Coucheny et al., 2005b; Grandvalet et al., 2005, 2008; Da Silveira and Abee, 2009; Maître et al., 2014; Daronval et al., 2016; Margalef-Catalá et al., 2016).

*Oenococcus oeni* stress response mechanisms involve the synthesis of Heat Shock Proteins (HSPs), a universal stress response with several regulatory pathways described in *Firmicutes*. Indeed, *hsp* genes can be induced by the alternative sigma factor σ^B^ or repressed by transcriptional repressors such as CtsR or HrcA (Benson and Haldenwang, 1993; Schulz and Schumann, 1996; Derré et al., 1999; Schumann, 2003). In Streptococci, HrcA and CtsR control two partially overlapping regulons that include most *hsp* genes (Chastanet et al., 2001; Chastanet and Msadek, 2003; Grandvalet et al., 2005; Spano and Massa, 2006; Frees et al., 2007). In contrast, in *Bacilli*, the two regulons are entirely distinct while in *Staphylococci* the HrcA regulon is completely embedded within the CtsR regulon (Chastanet et al., 2003). Both transcriptional repressors control expression of their regulon by specifically binding to their operator sequences in the promoter region, preventing RNA polymerase recruitment. HrcA specifically recognizes the CIRCE (“Controlling inverted repeat of chaperone expression”) palindromic sequence while CtsR binds a tandemly repeated hepta-nucleotide operator sequence (Zuber and Schumann, 1994; Derré et al., 1999). CtsR is the only regulator of stress response gene expression identified and studied in *O. oeni* so far (Grandvalet et al., 2005). The sequence analysis of the scaffold *O. oeni* ATCC BAA-1163 genome allowed the identification of the *ctsR* gene sequence and CtsR operator sequences, but no *hrcA* gene, CIRCE sequences or other genes encoding known stress response regulators have been found. Likewise in *O. oeni* ATCC PSU-1 (NC_008528), the only complete sequenced strain, no gene encoding known regulators of stress response have been identified, except *cstR*. In contrast, six two-component systems (TCS), some of which may be involved in stress response, have been identified in the *O. oeni* genome (Zúñiga et al., 2011). In *Firmicutes*, most molecular chaperone genes (*dnaK, groEL*) and *clp* genes, encoding either ClpATPases and ClpP protease, have CtsR operator sites in their promoter region. To date, *O. oeni* is the only *Firmicutes* where expression of *dnaK* and *groEL* is controlled exclusively by CtsR and not by HrcA (Grandvalet et al., 2005). In *Bacillus subtilis*, CtsR is active as a dimer under optimal growth conditions and represses transcription of its regulon by binding its operator sequence (Derré et al., 1999, 2000). Under stress conditions, the CtsR dimer is phosphorylated by McsA and McsB and then recognized and degraded by the ClpCP proteolytic complex (Derré et al., 2000; Kirstein et al., 2005, 2007; Elsholz et al., 2010, 2011). LAB are mcsAB-deficient *Firmicutes* and alternative mechanisms for regulating the CtsR activity have been described. In *Lactococcus lactis*, ClpE is required to restore repression by CtsR after heat shock. Indeed, replacement of *clpE* by *mcsA* was shown to restore *hsp* gene repression suggesting that ClpE in *L. lactis* has the same function as McsA in *B. subtilis* by interacting with CtsR through its zinc finger motif (Varmanen et al., 2003). More recently, Tao and Biswas (2013) showed that the ClpCP complex is not required for specific degradation of CtsR in *Streptococcus mutans* but that ClpL displays a chaperone protective role helping CtsR to bind its operator sequence (Tao et al., 2012; Tao and Biswas, 2013). In addition, in *L. lactis*, *Geobacillus stearothermophilus*, and *B. subtilis*, CtsR has been shown to act directly as a heat sensor with distinct species-specific thermal derepression temperature thresholds (Elsholz et al., 2010). The *O. oeni* genome does not contain *clpE*, *mcsA*, and *mcsB* genes, however two *clpL* genes are present: *clpL1*, in an operon with *clpF*, and *clpL2* (Beltramo et al., 2006; Assad-García et al., 2008). This strongly suggests a likely involvement of ClpL1 and/or ClpL2 in the regulation of CtsR activity in *O. oeni*. *O. oeni* is not readily genetically tractable, few genetic tools are available and none for directed mutagenesis or gene deletion. Because of these technical barriers, the in vivo function of CtsR and the regulatory mechanisms controlling its activity in *O. oeni* remain unknown.

In this study, we first investigated the in vivo role of CtsR in *O. oeni* using antisense RNA silencing, a technique we recently used to show the first modulation of gene expression in *O. oeni* and confirm the molecular chaperone role of the small Hsp Lo18 (Daronval et al., 2016). Using this approach, we inhibited *ctsR* gene expression by producing a full-length antisense RNA (asRNA) of the *ctsR* mRNA. We then used *B. subtilis* 168 as a tool to explore regulation of CtsR activity. We combined a xylose-inducible heterologous expression system and a β-galactosidase reporter system based on a transcriptional fusion with the *O. oeni* hsp18 promoter to measure repression by *O. oeni* CtsR at different temperatures. Finally, we tested protein–protein interactions between CtsR and target Clp ATPases using an *E. coli* two-hybrid system (Karimova et al., 1998) to identify direct partners of CtsR.

### MATERIALS AND METHODS

#### Bacterial Strains and Growth Conditions

Bacterial strains used in this study are listed in Table 1.

*Oenococcus oeni* ATCC BAA-1163 is an acidophilic strain isolated in Aquitaine (France) from red wine (Lonvaud-Funel, 1999). *O. oeni* was grown at 28°C in FEBom medium (pH 5.3) (Cavin et al., 1989) supplemented with 20 µg · ml⁻¹ of vancomycin, 20 µg · ml⁻¹ of lincomycin and 20 µg · ml⁻¹ of erythromycin when required. For stress survival tests, cells
TABLE 1 | Bacterial strains or plasmids used in this study.

| Strain or Plasmid | Relevant genotype or description | Source or reference |
|-------------------|----------------------------------|---------------------|
| **Escherichia coli** | | |
| EC101 | E. coli JM101puEpEti lacproAB (F' traD36 proABlacR Z :: ΔM15) with repA from pWW01 integrated in chromosome | Laboratory stock (Law et al., 1995) |
| C2992I | E. coli DH5α F' proA^B^ lacQ Δ(lacZ)M15 zff-Tn10 hsdR2 Δ(argF-lacZ)U169 phoA glnIV44 F' Δ(lacZ)M15 gmrA96 recA1 relA1 endA1 thi-1 hsdR17, tet | New England Biolabs Inc., |
| EcAScTsR | E. coli EC101 carrying pSiPSYNAsCtsR | This study |
| EcSyn | E. coli EC101 carrying pSiPSYN | (Darsonval et al., 2016) |
| BL21 | E. coli F' Δdcm ompT Δ(hsdS (rB - mB -) gal [malB^R - lac-12 (α)]) | Thermo Fisher Scientific |
| BL21-CtsR | BL21 carrying pETcTsR25 | This study |
| BL21-Lo18 | BL21 carrying pETrap18 | This study |
| DHT1 | F- glnV44 (AS) recA1 endA1 gyrA96 (raf') thi-1 hsdR17 spoT1 rfbD1 cya -854w-691::Tn10 | (Dautin et al., 2000) |
| DHT1 zip-zip | E. coli DHT1 harboring pT25-zip and pT18-zip encoding the GCN4 leucine zipper | (Karimova et al., 2000) |
| **Oenococcus oeni** | | |
| O. oeni ATCC BAA-1163 | wild type strain, var' | Laboratory stock |
| OoAScTsR | O. oeni ATCC BAA-1163, pSiPSYNAsCtsR | This study |
| OoSyn | O. oeni ATCC BAA-1163, pSiPSYN | (Darsonval et al., 2016) |
| **Bacillus subtilis** | | |
| 168 | Wild type, trpC2 | Laboratory stock (Derré et al., 1999) |
| QB4991 | trpC2 amyE::lacZ, apfA3) ΔctsR | pDLasp18 → 168 (Grandvalet et al., 2005) |
| WT | trpC2 amyE::Δ(hps18'-bgaB cat) | pDLasp18 → QB4991 (Derré et al., 2000; Grandvalet et al., 2005) |
| ΔctsR | trpC2 ΔctsR amyE::Δ(hps18'-bgaB cat) | |
| ΔctsR-XTOoCtsR pXTOoCtsR | trpC2, ΔctsR amyE::Δ(hps18'-bgaB cat), thrC::(pxyA-4-ctsR-O. oeni::spc) | pXTcTsR25 → ΔctsR-hsp18' |
| **Plasmids** | | |
| pSiPSYN | Replicative and low-copy number plasmid allowing gene expression in O. oeni under the control of PSYN promoter, ery^R | (Darsonval et al., 2016) |
| pSiPSYNAsCtsR | pSiPSYN derivative encoding the ctsR ORF in antisense orientation under the control of PSYN promoter | This study |
| pET28a (+) | Vector for expression of N-terminal His-tagged proteins in BL21 under the control of pT7 promoter, kan^R | Novagen |
| pETcTsR | pET28a (+) derivative carrying the O. oeni ctsR coding sequence, kan^R | This study |
| pETrap18 | pET28a (+) derivative carrying the O. oeni hsp18 coding sequence. | This study |
| pXT | pDG1728 derivative allowing transcriptional fusion with P_{xyl} xylode inducible promoter and integration into the B. subtilis thrC locus, spec^R, ampr^R | (Derré et al., 2000) |
| pXTcTsR25 | pXT derivative carrying the O. oeni ctsR coding sequence under the control of P_{xyl} xylode inducible promoter | This study |
| pDL | Integrative plasmid for constructing transcriptional fusions with the G. stearothermophilus bgaB gene and integration into the B. subtilis amyE locus, cm^R | (Yuan and Wong, 1995) |
| pDLasp18 | pDL derivative with transcriptional fusion hsp18'-bgaB | (Grandvalet et al., 2005) |
| pKT25 | BACTH vector designed to express in DHT1 a given polypeptide fused in frame at its N-terminal end with T25 fragment, kan^R | (Karimova et al., 2000) |
| pKNT25 | BACTH vector designed to express in DHT1 a given polypeptide fused in frame at its C-terminal end with T25 fragment, kan^R | (Karimova et al., 2000, 2005) |
| pUT18C | BACTH vector designed to express in DHT1 a given polypeptide fused in frame at its N-terminal end with T18 fragment, ampr^R | (Karimova et al., 2003) |
| pUT18 | BACTH vector designed to express in DHT1 a given polypeptide fused in frame at its C-terminal end with T18 fragment, ampr^R | (Karimova et al., 2003) |
| pKT25-ctsR | Full-length ctsR ORF cloned into pKT25 | This study |
| pKT25-cIcP | Full-length cIcPORF cloned into pKT25 | This study |
| pKT25-cIcL1 | Full-length cIcL1ORF cloned into pKT25 | This study |
were harvested during late exponential phase (OD_{600} = 0.8 corresponding to 1.10^{18} CFU \cdot ml^{-1}) and directly transferred to 48°C for heat stress or into acidified FT80m medium (pH 3.5) then incubated at 28°C during 90 min. Bacterial cultivability was estimated on FT80m agar plates (CFU \cdot ml^{-1}) supplemented with relevant antibiotics. Growth was monitored in liquid culture by following OD_{600} over time.

*Escherichia coli* EC101 and C2992I were used as host strains for cloning and plasmid maintenance. Bacterial adenylate cyclase two-hybrid (BACTH) assays were carried out with the *E. coli* cya strain DHT1, kindly provided by MPSDM. *E. coli* strains were grown at 37°C (except DHT1, 28°C) in Luria-Bertani (LB) medium supplemented with erythromycin (250 \mu g \cdot l^{-1}), kanamycin (50 \mu g \cdot ml^{-1}), or/and ampicillin (100 \mu g \cdot ml^{-1}) when necessary.

*Bacillus subtilis* 168, ctsR-deficient QB4991 and derivative strains were grown at 37°C in LB medium supplemented with chloramphenicol (5 \mu g \cdot ml^{-1}) and spectinomycin (100 \mu g \cdot ml^{-1}) when required.

**DNA Manipulation and Bacterial Transformation**

*Oenococcus oeni* genomic DNA was extracted using the InstaGene™ Matrix (Bio-rad, Hercules, CA, United States), PCR amplifications for cloning constructions were performed with Expand High Fidelity PCR System (Roche, Meylan, France) and colony PCR amplifications were performed with GoTaq®DNA polymerase (Promega, Charbonnières-les-bains, France). Plasmids from *E. coli* were prepared using a GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific, Illkirch, France). PCR DNA fragments were purified with GeneJET PCR Purification Kit (Thermo Fisher Scientific). T4 DNA ligase and restriction endonucleases were purchased from New England Biolabs Inc. (NEB, Evry, France). Plasmids and ligation products were transferred by electroporation into *E. coli* strains (Taketo, 1988). Briefly, *E. coli* cells in early exponential phase (OD_{600} = 0.5) were collected from 500 ml LB culture, washed twice in 250 ml of sterile ice-cold ultrapure water and concentrated 100-fold in 10% glycerol. Aliquots of 0.1 ml were mixed on ice with plasmid DNA or ligation mixture, and then submitted to an electroporation pulse of 25 \mu F, 200 \Omega, and 12.5 kV/cm. After the pulse, cells were directly mixed with 1 ml fresh LB medium, incubated for 20 to 40 min at 37°C and then plated on agar LB medium supplemented with suitable antibiotic. Plasmids were transferred by electroporation into *O. oeni* as previously described (Assad-Garcia et al., 2008). *O. oeni* recombinant strains were selected on FT80m plates supplemented with erythromycin, vancomycin and lincomycin (20 \mu g \cdot ml^{-1} each). *B. subtilis* was transformed with recombinant vectors as previously described by Msadek et al. (1998). Transformants were selected on agar LB medium supplemented with suitable antibiotics.

**Plasmid Constructions and Cloning Strategy**

Plasmids and primers used in this study are respectively listed in Tables 1, 2.

The plasmid pSIPSYNAsctsR was constructed by inserting in reverse orientation the amplified full-length coding sequence of the *O. oeni* ctsR gene downstream from the synthetic P_{SYN} promoter of plasmid pSIpSYN. The ctsR gene was amplified by PCR from *O. oeni* ATCC BAA-1163 genomic DNA using ASCtsR3 and ASCtsR6 primers (Table 2) generating Ncol (3′-end of ctsR) and Smal (5′-end of ctsR) restriction sites. The resulting plasmid named pSIPSYNAsctsR{\textsuperscript{c}}, carrying the complete coding sequence of the ctsR gene in reverse orientation under the control of the synthetic P_{SYN} promoter, was transferred by electroporation into *O. oeni*. The native pSIpSYN vector, without any insert, was introduced into *O. oeni* to serve as a control. The corresponding *O. oeni* recombinant strains were respectively designated by OoASCtsR and Oosyn. The presence of the vector was confirmed by colony PCR amplification with specific primers Olcg303, olg302, or ASCtsR. One colony was suspended in 50 \mu l of lysis buffer (100 mM EDTA, [pH8], 20 mg \cdot ml^{-1} Proteinase K)

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**TABLE 1 | Continued**

| Strain or Plasmid | Relevant genotype or description | Source or reference |
|-------------------|----------------------------------|---------------------|
| pKT25-clpL2       | Full-length clpL2 ORF cloned into pKT25 | This study          |
| pKNT25-csrR       | Full-length ctsR ORF cloned into pKNT25 | This study          |
| pKNT25-clpC       | Full-length clpC ORF without stop codon cloned into pKNT25 | This study          |
| pKNT25-clpL1      | Full-length clpL1 ORF without stop codon cloned into pKNT25 | This study          |
| pKNT25-clpL2      | Full-length clpL2 ORF without stop codon cloned into pKNT25 | This study          |
| pUT18-csrR        | Full-length ctsR ORF cloned into pUT18 | This study          |
| pUT18-clpC        | Full-length clpC ORF cloned into pUT18 | This study          |
| pUT18-clpL1       | Full-length clpL1 ORF cloned into pUT18 | This study          |
| pUT18-clpL2       | Full-length clpL2 ORF cloned into pUT18 | This study          |

van^r^, vancomycin resistance; ery^r^, erythromycin resistance; cm^r^, chloramphenicol resistance; spec^r^, spectinomycin resistance; amp^r^, ampicillin resistance; kan^r^, kanamycin resistance; tet^r^, tetracyclin resistance.
## TABLE 2 | Primers used in this study.

| Primer 1 | Sequence (5′–3′) 2,3 | Plasmid construction and function | Restriction site | Source or reference |
|----------|---------------------|----------------------------------|-----------------|-------------------|
| RNA silencing in *O. oeni* |
| ASctsR3 | CGTCCCCGGATGCGAAGCTAATATTCAGAT | pSIPSYNASctsR: ctsR coding sequence for antisense cloning in pSIPSYN | Smal | This study |
| ASctsR6 | GGGCCATGGCGATGCTGTGTATTGATTATCCA | | Ncol | This study |
| Heterologous expression and reporter system in *B. subtilis* |
| ctsR2 | CCGAGCTAAGCTTATCCATGAATGTTTGATCTCT | pXTctsR25: *O. oeni* ctsR coding sequence into pXT for heterologous expression in *B. subtilis* pETctsR: *O. oeni* CtsR production | HindIII | This study |
| ctsR5 | CCGGGAGGATGCAAGGAGGCGGTTGAATG | | BamHI | This study |
| Olgc16 | ATCGGCGGAGCTATCAAATACCTCCTATATTAACTAA | pDLhsp18: hsp18 promoter region for transcriptional fusion with bgaB in *B. subtilis* | BamHI | (Grandvalet et al., 2005) |
| Olgc20 | GGGCCATGCGCAATACCTCGACTTCAATATGGCGG | pSIPSYN: Synthetic promoter *P<sub>SYN</sub>* amplification | EcoRI | (Darsonval et al., 2016) |
| Olgc302 | CCCAAGCTTGCGCGAACTGTGGGGAAAGG | | Ncol | |
| Olgc303 | CCCAAGCTTGCGCGAACTGTGGGGAAAGG | | HindIII | |
| Over-expression in *E. coli* BL21 |
| Olgc1 | ATGCGAGGCTATGCGAGAAGCTAATATTCAG | pETctsR : *O. oeni* ctsR coding sequence into pET28a(+) for overexpression in *E. coli* BL21 | Ncol | This study |
| Olgc2 | GGGCTCGAGTCTCCATGAATGTTTTGATCTCTCA | | XhoI | |
| hsp18N | GGGCCATGCGCAATACCTCCTGATTAATGGATAGA | pEThsp18 : *O. oeni* hsp18 coding sequence into pET28a(+) for overexpression in *E. coli* BL21 | Ncol | This study |
| hsp18S | GGGGAGCTCCTATGGGATTATCAATATGGATAGA | | SacI | |
| Bacterial Two-Hybrid system in *E. coli* |
| CtsR21 | GGAGGATCCGCGAGAAGCTAATATTCAGATT | ctsR coding sequence, T18-ctsR and T25-ctsR fusions | BamHI | This study |
| CtsR20 | GGAGGATCCGCGAGAAGCTAATATTCAGATT | ctsR coding sequence, T18-ctsR and T25-ctsR fusions | EcoRI/Bsal | This study |
| CtsR25 | AAAGAGGTTATTGAGAGGATCCTCAATATGGCAAGAAGCTAATA | ctsR coding sequence, ctsR-T18 and ctsR-T25fusions | HindIII | This study |
| CtsR22 | GGAGGATCCGCGAGAAGCTAATATTCAGATT | ctsR coding sequence, T18-ctsR and T25-ctsR fusions | BamHI | This study |
| ClpL1-21 | GGAGGATCCGCGAGAAGCTAATATTCAGATT | ctsR coding sequence, T18-ctsR and T25-ctsR fusions | BamHI | This study |
| ClpL1-23 | GGAGGATCCGCGAGAAGCTAATATTCAGATT | ctsR coding sequence, T18-ctsR and T25-ctsR fusions | BamHI | This study |
| ClpL1-20 | GGAGGATCCGCGAGAAGCTAATATTCAGATT | ctsR coding sequence, T18-ctsR and T25-ctsR fusions | EcoRI | This study |
| ClpL1-21 | GGAGGATCCGCGAGAAGCTAATATTCAGATT | ctsR coding sequence, T18-ctsR and T25-ctsR fusions | HindIII/Bsal | This study |
| ClpL1-22 | GGAGGATCCGCGAGAAGCTAATATTCAGATT | ctsR coding sequence, T18-ctsR and T25-ctsR fusions | BamHI | This study |
| ClpL2-21 | GGAGGATCCGCGAGAAGCTAATATTCAGATT | ctsR coding sequence, T18-ctsR and T25-ctsR fusions | BamHI | This study |
| ClpL2-20 | GGAGGATCCGCGAGAAGCTAATATTCAGATT | ctsR coding sequence, T18-ctsR and T25-ctsR fusions | EcoRI/Bsal | This study |
| ClpL2-27 | GGAGGATCCGCGAGAAGCTAATATTCAGATT | ctsR coding sequence, T18-ctsR and T25-ctsR fusions | HindIII/Bsal | This study |

(Continued)
and then mixed by vortex. The resulting mix was heated 15 min at 65°C then 5 min 95°C and 5 μl of this mix were used as a DNA template in the PCR mix.

The pET28a(+) plasmid (Novagen, VWR International S.A.S, Fontenay-sous-Bois, France) was used for ctsR and hsp18 overexpression in E. coli BL21 λ(DE3) strain. The ctsR and hsp18 coding sequences were amplified by PCR using primer pairs olcg1 and olcg2 and hsp18N and hsp18S, respectively. PCR products coding sequences were amplified by PCR using primer pairs olcg1 and olcg2 and hsp18N and hsp18S, respectively. PCR products were respectively cloned between the NcoI and XhoI sites of the pET28a(+) (+). The resulting plasmids, pETcTsR1 and pEThsp18, were then introduced into E. coli BL21 λ(DE3) by electroporation.

The pXT integrative plasmid was used to express the O. oeni ctsR in the B. subtilis ΔctsR mutant strain (Derré et al., 2000). The full-length coding sequence was amplified by PCR from O. oeni ATCC BAA-1163 genomic DNA using primers CtsR2 and CtsR5 (Table 2). The PCR-generated fragment was cloned between the BamHI and HindIII sites of the plasmid pXT under the control of a xylose inducible promoter (pXylA). The resulting plasmid, pXTcTsR25, was integrated at the B. subtilis thrC locus of the ΔctsR-hsp18′-OoctsR strain, corresponding to the ΔctsR mutant strain (QB4991) carrying a transcriptional fusion between the promoter region of the O. oeni hsp18 gene and the bgaB gene of Geobacillus stearothermophilus (pDLhsp18′→QB4991) (Grandvalet et al., 2005). The recombinant strains were first selected for resistance to spectinomycin and screened for susceptibility to erythromycin and colony PCR amplifications were performed with primers CtsR2 and CtsR5 to confirm successful chromosomal integration.

To construct recombinant plasmids used in the BACTH complementation assays, genes coding for the O. oeni proteins were amplified by PCR using appropriate primers pairs (Table 2) and genomic DNA from O. oeni ATCC BAA-1163 as the template. The resulting PCR products were respectively cloned between the BamHI and EcoRI sites of the pKT25 and pUT18C vectors (except for the clpC gene, cloned between the PsiI and XbaI sites). The resulting plasmids expressed hybrid proteins, in which the proteins of interest were fused to the C-terminus of the T25 or T18 fragment of adenylate cyclase (AC) from Bordetella pertussis. For the reciprocal combinations, PCR-generated fragments (without the stop codon, see Table 2) were digested with appropriate enzymes and cloned between the HindIII and BamHI sites of the pKNT25 and pUT18 vectors (except for clpC which was cloned between HindIII and PsiI sites). This second set of recombinant plasmids expressed hybrid proteins in which the proteins of interest were fused to the N-terminus of the T25 or T18 fragment of AC.

DNA Sequencing and Sequence Analysis
Nucleotide sequences of recombinant vector inserts were verified on both strands by nucleotide sequencing with labeling and capillary separation on the AB3730xl performed by GeneWiz Europe (Essex, United Kingdom). Nucleotide sequencing results were analyzed using Clustal Omega software.

Production and Purification of CtsR and CtsR Polyclonal Antibody Production
CtsR was purified from E. coli BL21-CtsR strain, as described previously (Assad-García et al., 2008). Briefly, E. coli BL21-CtsR was grown aerobically in LB medium supplemented with 50 μg·ml⁻¹ kanamycin at 37°C until OD600 = 0.7–1. Isopropyl-β-D-thiogalactopyranoside (IPTG, 1 mM) was added to the culture to induce recombinant CtsR synthesis. After 15 h aerobic incubation (150 rpm) at 21°C, cells were harvested by centrifugation (6,500 × g for 10 min). The pellet was suspended in cold lysis buffer (50 mM Na2HPO4/NaH2PO4 [pH 8], 300 mM NaCl) and disrupted in a constant cell disruption system (Cell- D, Constant Systems Ltd., Roquemaure, France) with glass beads (0.5 μm). The suspension was centrifuged at 6,300 × g for 20 min at 4°C to remove unbroken cells and cell debris. The supernatant was loaded onto a 1-ml Ni-Nitritolriacetic acid column (Qiagen, Courtaboeuf, France) equilibrated with lysis buffer. The column was washed 10 times with 1 column volume of lysis buffer supplemented with 20 mmol·l⁻¹ imidazole. The recombinant CtsR protein was then eluted with lysis buffer supplemented with 250 mmol·l⁻¹ imidazole. The eluate

TABLE 2 | Continued

| Primer | Sequence (5′−3′) | Plasmid construction and function | Restriction site | Source or reference |
|--------|-----------------|----------------------------------|-----------------|---------------------|
| ClpL2-22 | GGTGGTCTCGGATCTTCTTCTTTTGATTCGTCGCGG | clpC coding sequence, T25-clpC fusions | BamHI/Bsal | This study |
| ClpC21 | GGTGGTCTCCTGCAGATACTAAATACAGGCATCTG | clpC coding sequence (with clpC20), T18-clpC fusions | PsiI | This study |
| ClpC20 | GGTGGTCTCCTGCAGATACTAAATACAGGCATCTG | clpC coding sequence (with clpC20), T25-clpC fusions | PsiI | This study |
| ClpC21C | GGTGGTCTCCTGCAGATACTAAATACAGGCATCTG | clpC coding sequence, clpC-T18 and clpC-T25 fusions | HindIII | This study |
| ClpC21KT | GGTGGTCTCCTGCAGATACTAAATACAGGCATCTG | clpC coding sequence, clpC-T18 and clpC-T25 fusions | HindIII | This study |
| ClpC27 | CCCAGGGTTAGGAGGAAATCCATGGATAATACATACACAGCATCG | clpC coding sequence, T25-clpC fusions | HindIII | This study |
| ClpC24 | GGTGGTCTCCTGCAGTTTTTTGTACTTTTTGATTTGGTG | clpC coding sequence, T25-clpC fusions | HindIII | This study |

1Forward primers are numbered with odd numbers and Reverse primers with even numbers; 2Restriction sites are underlined; and 3ATG codons are in italic and STOP codon appears in bold.

2https://www.ebi.ac.uk/Tools/msa/clustalo/
was dialyzed against lysis buffer and protein purification was monitored by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS–PAGE, 12.5% polyacrylamide) as described by Laemmli (1970). Polyclonal antibodies were obtained by direct immunization of SPF-rabbits with purified CtsR protein (Eurogentec, Liège, Belgium). Antiserum was used for CtsR immunodetection.

**Whole Cell Extracts and Western Blotting**

*Oenococcus oeni* Lo18 and CtsR protein levels were detected using rabbit antiserum raised against Lo18 (laboratory stock) or CtsR (obtained as described above). In both cases, the pellet from 50 ml of culture was washed twice in saline (9 g l⁻¹ NaCl) and adjusted to 30 OD Unit ml⁻¹ in lysis buffer (10 mM Tris–HCl [pH 8]). Cells were disrupted by two consecutive treatments using a Precellys homogenizer with glass beads (0.5 µm) at 6,500 × g (Precellys, Paris, France; 60 s–20 s pause–60 s). The suspension was centrifuged at 13,200 × g for 5 min. Detection was performed with Odyssey Fc Western Blot Imaging System (LiCor Biosciences-GmbH) in blocking buffer. The membrane was washed again in 1X PBS-T at room temperature three times and incubated one hour at room temperature with conjugated antisem, containing polyclonal antibodies directed against *Lo18* (1:1,000) or CtsR (1:1,000), for one night at room temperature, OD₅₉₀ was monitored by sodium dodecylsulfate-polyacrylamide gel electrophoresis. Total cellular proteins were collected and assayed with a Bio-Rad Protein Assay. Experiments were performed three times.

**BACTH Assays**

For BACTH assays, recombinant vectors (pKT25, pKNT25, pUT18C, and pUT18) carrying the studied *O. oeni* genes were co-transformed in all possible combinations into *E. coli* DHT1 cells (Dautin et al., 2000). Co-Transformants were plated on LB agar medium supplemented with 40 µg ml⁻¹ X-Gal and 0.5 mM IPTG (isopropylthio-β-galactoside) and incubated at 28°C for 24 to 36 h. Interaction efficiencies between different hybrid proteins were quantified by measuring β-galactosidase activity in a 96-well microtiter plate after cell permeabilization. For each co-transformation combination, six independent clones were tested. *E. coli* co-transformed clones were grown in 300 µl LB broth supplemented with 0.5 mM IPTG, 50 µg ml⁻¹ kanamycin and 100 µg ml⁻¹ ampicillin in 5 ml hemolysis tube and then incubated at 28°C for 16 to 24 h under aerobic conditions (150 rpm). Cultures were diluted 1:5 into M63 medium (Sambrook et al., 1989) into a final volume of 200 µL and OD₅₉₅ was measured by GENios reader (Tecan, Lyon, France). Cell suspensions were replaced by LB medium diluted 1:5 into M63 medium for control wells. Cells were permeabilized by adding 7 µl SDS (0.05%, w/v) and 10% chloroform per well with vigorous mixing with a multichannel pipette and incubated 30 to 40 min at room temperature. For enzymatic assays, 20 µl of permeabilized cells were added to 105 µl of reaction mixture: 70 mM Na₂HPO₄, 30 mM NaH₂PO₄, 1 mM MgSO₄, 0.2 mM MnSO₄ [pH7.0], 100 mM β-mercaptoethanol and 0.1% ONPG (o-nitrophenyl-β-D-galactopyranoside). After 30 min at room temperature, OD₄₀₅ was measured using a GENios reader (Tecan). Enzymatic activities, A, were calculated in relative units using the following formula:

\[
A_{R,u} = 1,000 \times \frac{OD_{405} - OD_{405\text{control well}}}{OD_{595} - OD_{595\text{control well}}} / \text{Incubation time}_{\text{min}}
\]

**Statistical Analysis**

The significance of the difference among percentage of cultivability values was determined by 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**

**Antisense RNA Approach in O. oeni to Characterize CtsR Function in vivo**

The antisense RNA approach is the only method currently available to modulate gene expression in *O. oeni* (Darsonval et al., 2016). Investigation of ctsR gene function in *O. oeni* was therefore performed by producing antisense RNA (asRNA) targeting ctsR mRNA. *O. oeni* was transformed with the recombinant plasmid encoding asRNA targeting the full-length ctsR mRNA.

**β-Galactosidase Assays in B. subtilis**

Overnight cultures of *B. subtilis* grown in LB medium supplemented with chloramphenicol (5 µg ml⁻¹) were diluted to OD₅₉₀ = 0.05 in fresh LB medium and grown at different temperatures 28, 37, or 42°C under aerobic conditions (140 rpm). At the mid-exponential phase (OD₅₉₀ = 1.5), xylose was added at a final concentration of 20 mM to induce ctsR gene expression during 12 h (DO₆₀₀ = 3). For each sample, β-galactosidase activity was determined as previously described (Miller, 1972; Grandaude et al., 2005) and expressed as Miller units per mg cellular protein. Protein concentrations were determined using the Bio-Rad protein assay. Experiments were performed three times on three independent cultures.

Repression of hsp18'-bgaB expression by CtsR was tested at six different temperatures by following β-galactosidase activity
E. coli smaller than Lo18 are detected in both OoASctsR and ASctsR RNA. Protein bands with an apparent molecular mass of the Lo18 gene are detected in both conditions, with and without thermal treatment (Derré et al., 1999). This result suggests that derepression of the CtsR regulon in the absence of stress interferes with cell survival under stress conditions, indicating that CtsR plays a key role in the O. oeni stress response.

CtsR of O. oeni Represses hsp18 Expression

Bacillus subtilis was used as a tool for the functional analysis of O. oeni CtsR. The B. subtilis Q4991 ΔctsR mutant strain (Darsonval et al., 2005a) was used to express the O. oeni ctsR gene in single copy from a xylose-inducible promoter (strain Q4991-$\beta$-galactosidase; see Materials and Methods). In order to assess the functionality of O. oeni CtsR in B. subtilis, a transcriptional fusion between the promoter region of O. oeni hsp18 and the $\beta$-galactosidase gene (hsp18'-bgaB) was integrated in single copy in the B. subtilis wild type and Q4991-XTOctsR strains, respectively designated WT and ΔctsR-XTOctsR.

Expression of the O. oeni CtsR protein in B. subtilis was verified by Western blot (Figure 3). B. subtilis wild-type (WT), Oosyn control strain 90 min after temperature shifting from 28 to 48°C (Figure 2A) or a shift in pH from 5.3 to 3.5 (Figure 2B). In contrast, 56% of the OoASctsR cells are lost following heat stress (Figure 2A) and 58% after pH 3.5 acid stress (Figure 2B). This result suggests that derepression of the CtsR regulon in the absence of stress interferes with cell survival under stress conditions, indicating that CtsR plays a key role in the O. oeni stress response.

Since this antisense RNA approach leads to derepression of the CtsR regulon, we tested survival of the bacteria following stress treatment (Figure 2). No loss of cultivability was observed for the OoASctsR strain 90 min after temperature shifting from 28 to 48°C (Figure 2A) or a shift in pH from 5.3 to 3.5 (Figure 2B). In contrast, 56% of the OoASctsR cells are lost following heat stress (Figure 2A) and 58% after pH 3.5 acid stress (Figure 2B). This result suggests that derepression of the CtsR regulon in the absence of stress interferes with cell survival under stress conditions, indicating that CtsR plays a key role in the O. oeni stress response.

**FIGURE 1** Immunodetection of Lo18 in O. oeni wild-type strain (ATCC BAA-1163) and recombinant strains carrying the control pSIPSYN plasmid (Oosyn) or pSIPSYNAstcsR plasmid allowing expression of asRNA targeting ctsR (OoASctsR) were cultivated at 28°C in FT80m medium up to the end of the exponential growth phase. Total cellular proteins were extracted immediately (28°C) or following a sub-lethal thermal treatment (30 min at 42°C). For each strain and each condition, 15 µg of protein were separated by SDS-PAGE. The O. oeni Lo18 protein was detected by immunodetection with polyclonal antibodies directed against Lo18 as secondary antibody. Total protein extract from E. coli BL21(DE3) carrying pET-hsp18 plasmid was used as a positive control (BL21-Lo18).

**FIGURE 2** Cell cultivability following stress treatment. Recombinant strains carrying the control empty vector (Oosyn; black bars) or plasmid with asRNA targeting ctsR (OoASctsR; hatched bars) were grown at 28°C until mid-exponential phase. Cultures were transferred to 48°C (A) or into acidified FT80m medium (pH 3.5) (B). Cultivability was estimated on agar plates (CFU·ml$^{-1}$) after 90 min treatment. After CFU counting on agar plates, the cultivability rate is calculated by dividing the number of cells following treatment by total number of cells before stress treatment, normalized to the reference strain Oosyn set at 100%. Significant differences are based on a unilateral and paired t-test (**P < 0.005; *P < 0.05).
is fully functional in

The results indicate that the O. oeni CtsR of

acts as a heterologous host. Expression of

BACTH Analysis of

O. oeni hsp18 (Grandvalet et al., 2005). As expected, in wild-type B. subtilis strain (Figure 4, lane [1], WT), the CtsR regulator is active and represses hsp18'-bgaB expression during growth at temperatures ranging from 28 to 42°C. Following incubation at 50°C, expression of hsp18'-bgaB is induced due to inactivation of CtsR. As previously reported, these results confirm that the B. subtilis CtsR is active at temperatures up to 42°C (Elsholz et al., 2010). In the strain expressing O. oeni CtsR (ΔctsR-XTOctsR), hsp18'-bgaB is expressed at all growth temperatures in the absence of xylose, (Figure 4, lane [2]) due to absence of CtsR repression. When the O. oeni ctsR gene is expressed by adding xylose (Figure 4, lane [3] ΔctsR-XTOctsR + Xylose) the hsp18'-bgaB fusion is repressed during growth at 28°C. However, repression by O. oeni CtsR no longer occurs during growth at all temperatures tested above 28°C (33, 35, 37, 42, and 50°C) indicating that O. oeni CtsR is active at 28°C, the optimal growth temperature for O. oeni, and totally inactive at 33°C and above. These results strongly indicate that the O. oeni CtsR transcriptional repressor is an intrinsic heat sensor with a specific temperature threshold adapted to the natural habitat of O. oeni.

ΔctsR mutant (ΔctsR) and ΔctsR mutant with the xylose-inducible O. oeni ctsR gene (ΔctsR-XTOctsR) strains were grown at 28°C in LB with xylose added in the mid-log phase. No signal was detected in whole cell extracts from the wild type or ΔctsR mutant strains whereas a band with a 17 kDa apparent molecular mass, corresponding to that of the CtsR positive control (BL21-CtsR), was detected in the strain carrying the O. oeni ctsR gene under the control of PxyIA promoter, confirming correct heterologous expression.

Expression of the hsp18'-bgaB fusion in B. subtilis strain ΔctsR-XTOctsR was followed by β-galactosidase activity assays during growth at 28°C in LB broth medium with or without xylose. In the absence of xylose, the hsp18'-bgaB fusion is strongly expressed (approximately 350,000 Miller units - mg⁻¹). In the presence of xylose, expression of the hsp18'-bgaB fusion is repressed more than 10-fold (34,000 Miller units - mg⁻¹). Taken together, these results indicate that the O. oeni CtsR repressor is fully functional in B. subtilis and acts as a repressor of hsp18 expression.

CtsR of Oenococcus oeni Acts as a Thermosensor

As previously suggested by Derré et al. (2000) and confirmed in L. lactis, G. stearothermophilus, and B. subtilis by Elsholz et al. (2010), CtsR is an intrinsic heat sensor with a species-specific temperature threshold. We tested thermostability of hsp18 by O. oeni CtsR using B. subtilis as a heterologous host. Expression of hsp18'-bgaB was followed by measuring β-galactosidase activities of the wild type (WT) and ΔctsR-XTOctsR B. subtilis strains during growth at different temperatures (28, 33, 35, 37, 42, and 50°C) with blue/white screening on X-Gal containing LB-agar plates with or without added xylose (Figure 4). White colonies correspond to the transcriptional repression of the hsp18'-bgaB fusion by CtsR. Blue colonies indicate thermostability of hsp18'-bgaB expression following CtsR inactivation. We have previously shown that CtsR of B. subtilis is able to repress expression of O. oeni CtsR (Karimova et al., 1998). Upon interaction of the hybrid proteins, AC activity is restored due to the spatial proximity of the T25 and T18 fragments, leading to cAMP synthesis and transcriptional activation of catabolic operons such as the well-known lactose operon.

To characterize physical interactions, the four proteins (CtsR, ClpL1, ClpL2, and ClpC) were methodically tested for pairwise interactions using the BACTH complementation assay. The DNA sequences encoding the proteins of interest were cloned into appropriate BACTH vectors to generate hybrid proteins fused either at the N-terminal (pKT25 or pUT18C) or C-terminal extremities (pKNT25 or pUT18) of either the T25 or the T18 fragment of the B. pertussis AC (see Materials and Methods). To analyze putative physical associations between the resulting hybrid proteins, the E. coli DHT1 cya− strain was cotransformed with pairs of recombinant plasmids (Table 1) expressing the T25 and the T18 hybrid proteins (pKT25/pUT18C, pKT25/pUT18, pKNT25/pUT18C, or pKNT25/pUT18). The efficiency of functional complementation between the different hybrid proteins was determined by β-galactosidase assays and the results of the different pairwise combinations are summarized in Table 3.

Based on the characteristic features of CtsR and ClpATPases in the Firmicute phylum, we would anticipate homodimerization of CtsR and the oligomerization of the three ClpATPases.
combinations of the hybrid proteins with β-galactosidase activities ranging from 203 to 458 units (Table 3). The BACTH analysis data also confirmed self-association of the ClpL1 and ClpC ATPases, but for ClpL2 self-association was less obvious. Among the four pairwise combinations of the hybrid proteins, a single one led to β-galactosidase activity indicating a possible self-association, but reaching only 54 units. These observations suggest either weak physical interactions or that ClpL2 may require additional partners to form proper oligomers.

The BACTH results suggest that ClpC is likely to form symmetric homo-oligomers. Indeed, the ClpC-T25 hybrid protein, in which ClpC is C-terminally fused with T25, interacts efficiently with the ClpC-T18 hybrid protein, with a free ClpC N-terminus (140 units) but not with T18-ClpC (49 units). Our analysis also revealed that the CtsR repressor associates with ClpL1. For CtsR and ClpL1, four out of eight possible pairwise combinations of the hybrid proteins led to β-galactosidase activities ranging from 62 to 92 units, indicating efficient physical interaction between these two proteins.

The BACTH analysis also highlights that ClpL1 is also able to interact with ClpL2. As shown in Table 3, T25-ClpL2 associates with T18-ClpL1, leading to β-galactosidase levels 10 times higher than with ClpL1-T18 (111 and 11 units, respectively) suggesting a possible symmetric hetero-oligomer formation. Taken together, these results suggest that ClpL1 may play a crucial role in regulation of CtsR activity during stress response in O. oeni.

**DISCUSSION**

Due to poor transformability and the lack of efficient genetic tools, O. oeni is not readily genetically tractable. Directed mutagenesis in O. oeni remains impossible to date and little is known about its genetic regulatory mechanisms. The asRNA production approach is presently the only approach to investigate stress response in this bacterium (Darsonval et al., 2016). By targeting ctsR for knockdown, we undertook an *in vivo* approach with the aim of broadening our knowledge on O. oeni CtsR, the only stress response regulator described to date. In this work, ctsR inhibition by asRNA affects expression of ctsR regulon genes leading to derepression under optimal growth conditions, as shown by an increased Lo18 protein levels at 28°C (Figure 1). This confirms for the first time *in vivo* CtsR-dependent regulation of *hsp18* gene expression in O. oeni. We also highlighted that expression of a complementary asRNA targeting the full-length of ctsR mRNA reduced cultivability of O. oeni by 50 % following sub-lethal temperature or acid stress exposure (Figure 2). Our data indicate that CtsR plays an essential role in the stress response process with a crucial involvement in coping with prolonged heat and acid stress. Regulation by CtsR may extend beyond a specific role in thermoprotection or acid-tolerance since its inactivation in *Lactobacillus plantarum* leads to pleiotropic effects correlated with its obvious housekeeping function as a central stress response regulator in O. oeni (van Bokhorst-van de Veen et al., 2013). We show here that ctsR inhibition conferred a heat- and acid-sensitive phenotype to the recombinant strain. Interestingly, we previously observed this phenomenon by inhibiting *hsp18* expression with the same RNA silencing approach (Darsonval et al., 2016). These results suggest that in LAB, stress response gene expression must be carefully fine-tuned: levels that are too low under stress conditions or too high under optimal conditions would be deleterious. Indeed, whereas in most Gram-positive bacteria described so far, ctsR deletions lead to an increase in heat resistance and general stress tolerance (Nair et al., 2000; Chastanet et al., 2001; Karatzas and Bennik, 2002; Hüfner et al., 2007; Zotta et al., 2009), in *L. plantarum*, incubation at 40°C strongly inhibited the growth of the mutant strain without affecting the wild type (Fiocco et al., 2010). These unexpected acid- and heat-sensitive phenotypes observed in O. oeni OoASCtsR (Figure 2),
are in agreement with the observation in _L. plantarum_, where inactivation of _ctsR_ causes derepression of _hsp_ genes including proteases and molecular chaperones. Excessive levels of Clp proteases and Hsp proteins would be detrimental to the cell, similar to the activity of the acyldepsipeptide antibiotic ADEP4 which has been shown to activate the ClpP protease, resulting in death of growing cells (Brötz-Oesterhelt et al., 2005). For example, accumulation of Lo18, known to be addressed to the membrane under environmental stress conditions, would provoke deleterious effects on physical state of the bacterial cell leading to a stress sensitive phenotype (Fiocco et al., 2010). Indeed, the heat-sensitive phenotype reported for the _L. plantarum ΔctsR_ mutant by Fiocco et al led to an intriguing impairment in the cell envelope (Fiocco et al., 2010). Taken together these results raise the possibility that CtsR might control other activities including cell envelope integrity. The partial impact on the cultivability observed suggests the involvement of other regulators of stress response that have not yet been described. In addition, modifications of _ctsR_ expression seem to have pleiotropic effects as observed in _L. plantarum_ (Fiocco et al., 2010). Further studies will be needed to shed light on this aspect. In _O. oeni_, the combination of Lo18 immune-localization by electron microscopy observation and fluorescence anisotropy could be investigated to consider the impact of CtsR regulon dysregulation on the cell wall and membrane integrity (Coucheney et al., 2005a; Daronval et al., 2016). Our findings confirm that gene knockdown by antisense RNA is a powerful strategy to study the role of _O. oeni_ genes _in vivo_. Obviously, even if this antisense RNA approach is currently the only available technique to modify gene expression in such a genetically intractable organism such as _O. oeni_, it is not a perfect solution.

We began studying the regulation of CtsR activity using _B. subtilis_ and _E. coli_ as heterologous hosts. In _B. subtilis_, thermo-induction of a _hsp18β-bgaB_ transcriptional fusion at different temperatures allowed us to show that CtsR can be inactivated in a temperature-dependent manner. These results are in agreement with Derré et al., 1999) and with results obtained in _L. lactis_, _G. stearothermophilus_, and _B. subtilis_ by Elsholz et al. (2010) showing that CtsR is an _in vivo_ intrinsic heat sensor with specific temperature thresholds according to the bacterial species. Indeed, CtsR-dependent gene expression is induced at temperatures above 42°C in _L. lactis_ but repressed at temperatures up to 50°C in _G. stearothermophilus_ (Elsholz et al., 2010). We show here that _O. oeni_ CtsR-dependent gene expression is induced at growth temperatures of 33°C and above, acting as a molecular thermometer. CtsR sequences are highly conserved across the phylogenetic group of low-GC% Gram-positive bacteria. Two regions, the HTH and the winged HTH domains, are both conserved and crucial for CtsR activity, Derré et al., 2000) described two point mutants for _B. subtilis_ CtsR (V16M and G65S), suppressing CtsR inactivation during heat stress, while Elsholz substituted the Glycine residue 64 by a proline at the tip of the hairpin in the highly conserved glycine-rich loop of the CtsR winged HTH domain and showed that this residue is essential for _B. subtilis_ CtsR activity _in vivo_ and responsible for CtsR thermosensor ability in several low-GC% Gram-positive bacteria (Derré et al., 2000; Fuhrmann et al., 2009; Elsholz et al., 2010). A piezotolerant strain of _Listeria monocytogenes_ resistant to heat, acid and oxidative treatments had a single codon deletion in this conserved glycine-rich hairpin (Karatzas and Bennik, 2002; Karatzas et al., 2003). Nevertheless, while this glycine-rich domain appears to be responsible for CtsR thermosensitivity, it does not seem to be responsible for species-dependent temperature thresholds. Indeed, the glycine-rich domain is highly conserved across _Firmicutes_ (Derré et al., 1999). Differences in temperature thresholds may be due to the Hsp machinery specific to each species rather than the glycine-rich region. Regulatory mechanisms of stress response genes have been well investigated in low-GC Gram-positive bacteria and revealed a diversity of actors involved in regulation of CtsR activity (Derré et al., 2000; Nair et al., 2000; Chastanet et al., 2003; Varmanen et al., 2003; Elsholz et al., 2010; Fiocco et al., 2010; Tao et al., 2012; Tao and Biswas, 2013). Namely, _B. subtilis_ CtsR is
addressed to ClpCP by McsAB complex for degradation above 50°C, whereas in L. lactis, ClpE replaces the McsB adaptor and addresses CtsR for degradation above 37°C while the S. mutans CtsR is folded and stabilized by ClpL even at room temperature (Derré et al., 2000; Varmanen et al., 2003; Elsholz et al., 2010; Tao and Biswas, 2013). In O. oeni, the absence of ClpE, McsA and McsB orthologs may in part explain the difference in CtsR threshold temperature.

Our BACTH analysis indicates an interaction between ClpL1 and CtsR suggesting for the first time in the LAB the involvement of a ClpL family member in regulation of CtsR activity. These findings are consistent with the work of Tao and Biswas in S. mutans showing ClpL–CtsR interaction via the ClpL D2-small domain at the amino-terminal extremity, a domain conserved in the O. oeni ClpL1 sequence (Tao and Biswas, 2013). However, our BACTH analysis did not reveal an interaction between CtsR and ClpL2, which also carries a D2-small region. We noticed that ClpL2 could not form strong homo-oligomers but can interact strongly with ClpL1, possibly by forming hetero-oligomers. ClpATPase proteins usually form hexameric rings of homo- or hetero-oligomers (Ogura and Wilkinson, 2001). This could suggest that ClpL2 might require another partner such as ClpL1 to form stable oligomers in our BACTH model and by extension in O. oeni. In L. lactis and B. subtilis, ClpE and McsA present CtsR to a proteolytic complex by interacting with CtsR through their zinc finger motifs, which is absent in ClpL1 (Varmanen et al., 2003; Fuhrmann et al., 2009). One explanation for our observations might be that CtsR and ClpL1 in O. oeni interact via another domain than D2-small or a Zinc-finger motif. A possible role of ClpX, which has a zinc-finger pattern at its amino-terminal region, cannot be excluded.

The carboxy-terminal region of CtsR may be involved in physical interaction with the chaperone since three out of the four possible BACTH combinations with a free CtsR C-terminal showed interactions with ClpL1. This is the first study to suggest a role for C-terminal domain of CtsR. Indeed, while the amino-terminal region including the dimerization domain, the DNA binding-domain and the thermo-sensing domain, is well-characterized, the role of the C-terminal region is still unknown. It was suggested by Derré et al. that without the C-terminal region, CtsR is sensitive to protease activity and is unstable in vivo (Derré et al., 2000). In O. oeni, the C-terminal domain of CtsR would be recognized by Clp ATPases to be stabilized, as shown for ClpL in S. mutans (Tao and Biswas, 2013).

We propose the following model as a model of CtsR activity regulation in O. oeni (Figure 5). In agreement with the results of Tao and Biswas showing that ClpL stabilizes CtsR bound to its operator sequence in S. mutans, ClpL1 would act similarly under optimal conditions (Figure 5A). Under heat stress conditions, above 33°C, the CtsR–ClpL1 complex would dissociate from the operator site, allowing stress gene expression (Figure 5B). ClpL1 would address inactive CtsR to a proteolytic complex for degradation, such as ClpL2ClpP by interacting with ClpL2 or ClpCP by interacting with ClpC. The involvement of ClpCP in CtsR degradation was previously proposed in a CtsR regulation model (Derré et al., 2000; Elsholz et al., 2010).

Taken together, our results confirmed in vivo the central involvement of CtsR in stress response in O. oeni, extending an earlier laboratory study using B. subtilis as a heterologous host (Grandvalet et al., 2005). Antisense inactivation of ctsR expression impacted stress survival of O. oeni, confirming CtsR as a master coordinator of general stress response. In addition, we showed that CtsR-dependent gene expression fully induced at 33°C by O. oeni CstR identifying CtsR as an intrinsic heat sensor. Furthermore, interaction of CtsR with ClpL1 suggests it is a likely player involved in controlling CtsR activity.

AUTHOR CONTRIBUTIONS

MD, FJ, CG, and TM designed the study. MD, FJ, and CG performed the experiments. MD performed the statistical analysis. MD, CG, and TM drafted the manuscript. FJ and HA contributed to the interpretation.
of the results and writing of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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