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CtsR Is the Master Regulator of Stress Response Gene Expression in *Oenococcus oeni*

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Although many stress response genes have been characterized in *Oenococcus oeni*, little is known about the regulation of stress response in this malolactic bacterium. The expression of eubacterial stress genes is controlled both positively and negatively at the transcriptional level. Overall, negative regulation of heat shock genes appears to be more widespread among gram-positive bacteria. We recently identified an ortholog of the *ctsR* gene in *O. oeni*. In *Bacillus subtilis*, CtsR negatively regulates expression of the *clp* genes, which belong to the class III family of heat shock genes. The *ctsR* gene of *O. oeni* is cotranscribed with the downstream *clpC* gene. Sequence analysis of the *O. oeni* IOB 8413 (ATCC BAA-1163) genome revealed the presence of potential CtsR operator sites upstream from most of the major molecular chaperone genes, including the *clp* genes and the *groES* and *dnaK* operons. Using *B. subtilis* as a heterologous host, CtsR-dependent regulation of *O. oeni* molecular chaperone genes was demonstrated with transcriptional fusions. No alternative sigma factors appear to be encoded by the *O. oeni* IOB 8413 (ATCC BAA-1163) genome. Moreover, apart from CtsR, no known genes encoding regulators of stress response, such as HrcA, could be identified in this genome. Unlike the multiple regulatory mechanisms of stress response described in many closely related gram-positive bacteria, this is the first example where *dnaK* and *groESL* are controlled by CtsR but not by HrcA.

In order to respond to stressful situations, all organisms have developed adaptive networks, including stress responses. Organisms undergo complex programs of differential gene expression, involving a rapid increase in the concentrations of specific sets of proteins, such as heat shock proteins (HSPs). Some HSPs, such as GroEL, DnaK, small HSP, and several Clp ATPases, are molecular chaperones that facilitate the proper folding of cellular proteins. Others, such as the Clp ATP-dependent protease, degrade incorrectly folded proteins (25). In *Escherichia coli*, HSP synthesis is controlled at the transcriptional level by two alternative sigma factors: σ^{32} and σ^{24} (58). In contrast to *E. coli*, the heat shock response in the model gram-positive bacterium *Bacillus subtilis* involves at least three different classes of heat-inducible genes distinguished by their regulatory mechanisms (28). Genes of class I (*groESL* and *dnaK*) are regulated by the HrcA repressor, which binds to the palindromic operator sequence CIRCE (for "controlling inverted repeat of chaperone expression"). Expression of class II heat shock genes requires the alternative sigma factor σ^{B} (43, 44). Class III genes are controlled by the class three stress gene repressor, CtsR, which recognizes a tandemly repeated heptad operator sequence (16). Class IV genes comprise heat shock genes of unknown regulation, suggesting the existence of other thermoregulatory mechanisms. However, the model organism *B. subtilis* does not reflect all of the mechanisms of stress response regulation in gram-positive bacteria. Thus, in *Streptomyces albus*, a mycelium-forming gram-positive soil bacterium, two negative regulators in addition to HrcA, HspR (for "heat shock protein repressor") and RheA (for "repressor of *hsp18*"), control HSP synthesis. The HspR repressor binds to an inverted-repeat sequence called HAIR (for "HspR-associated inverted repeat") and represses the *dnaK* operon (27). The *hsp18* gene is controlled by the negative regulator RheA, which binds to an inverted-repeat sequence (53). The *groESL* operon is controlled by HrcA (26). Although the induction of *hsp* genes is a universal response, organisms have diverse regulatory mechanisms for controlling HSP synthesis. Comparative genomics allows us to predict the regulation of heat shock genes by CtsR and/or HrcA. Thus, the *dnaK* and *groESL* operons of the lactic acid bacterium *Lactococcus lactis* contain CIRCE elements in their promoter regions, suggesting that these genes may be regulated by HrcA, whereas CtsR regulates *clp* gene expression (56). Some bacteria simultaneously use more than one strategy to ensure the well-adjusted production of heat shock proteins under harsh conditions. For example, heat shock regulation processes mediated by CIRCE and σ^{32} coexist in some bacteria, including *Agrobacterium tumefaciens*, *Bordetella pertussis*, *Caulobacter crescentus*, and *Zymomonas mobilis* (2, 3, 20, 39, 40, 45, 46, 50, 51). Dual heat shock regulation by HrcA and CtsR has been demonstrated for the *Staphylococcus aureus dnaK* and *groESL* operons and for the *Streptococcus salivarius clpP* gene (10, 11). These dual regulatory mechanisms are probably not redundant but may act together synergistically to maintain low levels of expression in the absence of stress and to ensure that synthesis of different HSPs is tightly coordinated under adverse environmental conditions.

The lactic acid bacterium *Oenococcus oeni*, mostly responsible for malolactic fermentation in wine, is able to survive and grow under very harsh conditions. Malolactic fermentation, occurring after the completion of alcoholic fermentation, low-

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ers wine acidity by converting malic acid into lactic acid and improves the taste (18, 36). After alcoholic fermentation, wine is a hostile medium for bacterial growth because of nutritional starvation, low pH, and the presence of sulfites and of high ethanol concentrations. Because of its ability to grow in such a hostile medium, *O. oeni* is a good model for studies of stress response in lactic acid bacteria. Among *O. oeni* stress response genes, *hsp18*, *trxA*, *clpX*, and *clpP* have been previously characterized (4, 30, 31). Expression of these genes was followed during growth and under several stress conditions. All these genes are heat inducible, but differential expression was observed during the growth phase. *clpX* is preferentially expressed at the beginning of the exponential phase, *clpP* was expressed during all stages of growth at a high basal level and reached its maximum in the exponential phase, and *hsp18* mRNA was detected only at the end of the exponential phase. The *trxA* gene was expressed during all stages of growth with no significant difference in the level of expression. An understanding of the regulatory mechanisms controlling stress gene expression is therefore essential in studying the ability of *O. oeni* to survive and grow under unfavorable environmental conditions. The determination of the complete genome sequence of *O. oeni* strain IOB 8413 (ATCC BAA-1163) was carried out by our laboratory in collaboration with the Laboratoire de Biotechnologie et Microbiologie Appliquée (UMR 1219, INRA-Université Victor Segalen Bordeaux 2), the Centre de Bioinformatique de Bordeaux (CbiB, Université Victor Segalen Bordeaux 2), and GENOME Express (Grenoble, France) (33). The genome assembly of *O. oeni* currently consists of 33 contigs. This project has revealed numerous gene systems that are likely to be important for our understanding of the physiology of this lactic acid bacterium. Here, we report the identification of a CtsR ortholog in *O. oeni*. Potential CtsR operator sites were found upstream from the *clp* genes and the *groESL* and *dnaK* operons, and we show that CtsR controls the expression of most of the *O. oeni* molecular chaperone genes. In contrast to the diversity of stress response mechanisms described in many gram-positive bacteria, no gene encoding an alternative sigma factor or any other known regulator of stress response, such as HrcA, could be identified in the *O. oeni* IOB 8413 (ATCC BAA-1163) genome. This is the first example of *dnaK* and *groESL* operons under the exclusive control of CtsR without dual regulation by both HrcA and CtsR.

MATERIALS AND METHODS

Bacterial strains, growth media, and transformation conditions. *O. oeni* strain IOB 8413 (ATCC BAA-1163) was grown at 30°C in FT80 medium (pH 5.3) (9). For stress experiments, cells grown to the mid-exponential phase were shocked for half an hour by transfer to 42°C and addition of ethanol (11% [vol/vol]) or HCl (1 M) to pH 3.6. *B. subtilis* 168 (*trpC2*) and QB4991 [*trpC2 amyE*::(*'lacZ aphA3*)Δ*ctsR*] (16) were used as heterologous hosts to measure the activities of transcriptional fusions. *E. coli* ER2738 [F′ *lacI*^{q} Δ(*lacZ*)*M15 zzf*::Tn*10*(Tet^r)/*fhuA2 supE thi* Δ(*lac-proAB*) Δ(*hsdMS-mcrB*)] (New England Biolabs) was used for cloning experiments. *E. coli* was grown at 37°C in Luria-Bertani (LB) medium. Electroporation was used for *E. coli* transformation, with selection on LB plates supplemented with ampicillin (100 μg/ml). *B. subtilis* was grown at 37°C in LB medium and transformed as described previously (42). Transformants were selected on LB plates supplemented with chloramphenicol (5 μg/ml).

RNA extraction and analysis. RNA extraction was performed using Tri Reagent (Sigma) according to the manufacturer's instructions and 0.4 g of glass beads (70 to 100 μm) to disrupt cells with a FastPrep cell disintegrator (Bio 101, Inc.). Samples were then treated as recommended by the manufacturer and used for Northern blotting, primer extension analysis, reverse transcription-PCR (RT-PCR), or quantitative RT-PCR (QRT-PCR) experiments. Northern blotting was carried out as described by Sambrook et al. (48). A DNA fragment corresponding to the *O. oeni ctsR* gene was amplified by PCR using oligonucleotides olcg1 and ctsR1 (Table 1). This fragment was radiolabeled with [α-^{32}P] dATP (Perkin-Elmer) using a random-primer DNA-labeling kit (Invitrogen) and used as a probe in Northern hybridization experiments. Primer extensions were performed by incubating 5 μg of RNA, 20 pmol of oligonucleotide, 92 GBq of [α-^{32}P]dATP (111 TBq/mmol; Perkin-Elmer), and 100 U of SuperScript II reverse transcriptase (Invitrogen). For each primer extension experiment, two oligonucleotides were chosen to hybridize approximately 100 bp downstream from the translation initiation codon (Table 1). The corresponding DNA-sequencing reactions were carried out by using the same oligonucleotides and PCR-amplified DNA fragments carrying the respective promoter regions. RT-PCR and QRT-PCR were performed with *O. oeni* RNA (2 μg) treated with 2 units of DNase (Invitrogen). cDNAs were synthesized with random hexamers (50 ng) using the Superscript II reverse transcriptase. PCRs were performed using cDNAs with appropriate primers (Table 1). *hsp* mRNA levels were quantified by QRT-PCR assays using qPCR Mastermix and a SYBR Green I kit (Eurogentec). The *ldhD* gene (AJ831540) was chosen as an internal control gene (4, 17). Amplifications were performed on a Bio-Rad I-cycler with the SYBR Green system. Thermal-cycling conditions included the following steps: initial denaturation at 95°C for 10 min, followed by 45 cycles at 95°C for 15 s and 60°C for 30 s. Fluorescence measurements were recorded during each annealing step. Four dilutions of cDNAs were performed. The specificity of each primer pair was determined with a melting curve. The efficiency of real-time amplification was determined by running a standard curve with serial dilutions of cDNA. A PCR that amplifies the target sequence with 100% efficiency (E) can double the amount of PCR products in each cycle. The efficiency, E, is calculated by the formula $E = [10^{-(1/s)} - 1] \times 100$, where s is the slope of the standard curve. The results were analyzed using the comparative critical-threshold method, in which the amount of target RNA is adjusted to a reference relative to an internal calibrated target RNA.

DNA manipulations and analysis. Molecular biology techniques were carried out using standard methods (48). Double-stranded plasmid DNA was purified with the QIAprep spin miniprep kit (QIAGEN). Nucleotide sequences were determined by the dideoxy chain termination method using the DNA sequencing cycle Reader kit (MBI Fermentas). PCR products and DNA restriction fragments were purified using the QIAquick PCR purification kit (QIAGEN). Restriction endonucleases (Invitrogen), DNA ligase (Invitrogen), and *Taq* polymerase (Qbiogen) were used according to the manufacturers' specifications.

Plasmids and plasmid constructions. Plasmid pDL (57) was used to construct transcriptional fusions with the *Bacillus stearothermophilus bgaB* gene, encoding a thermostable β-galactosidase (29), with subsequent integration at the *amyE* locus. *bgaB* transcriptional fusions were constructed using EcoRI-BamHI (or EcoRI-HindIII) fragments generated by PCR using the oligonucleotide pairs olcg17/olcg18, olcg11/olcg12, olcg16/olcg20, olcg9/olcg40, olcg7/olcg8, clpE1/clpE2, olcg3/olcg4, and olcg14/olcg15 (Table 1), corresponding to the *O. oeni mleA* (X82326), *ctsR* (AJ890338), *hsp18* (AJ250422), *clpX* (Y15953), *clpP* (AJ606044), *clpL2* (AJ890337), *grpE* (AJ890339), and *groES* (AJ890340) promoter regions, respectively. These fragments were digested with EcoRI and BamHI (or HindIII) and inserted into EcoRI- and BamHI (or HindIII)-digested pDL to generate pDL*mleA*, pDL*ctsR*, pDL*hsp18*, pDL*clpX*, pDL*clpP*, pDL*clpL2*, pDL*grpE*, and pDL*groES*, respectively. To examine the effect of site-directed mutagenesis of the CtsR operator sequence on the expression of a *groES′-bgaB* fusion, a PstI site was introduced between nucleotides −22 and −27 of the *groES* regulatory region in pDL*groES* to generate pDL$groES_{mut}$ through PCR using the oligonucleotide pairs olcg15/groES4 and groES5/olcg14 (Table 1).

β-Galactosidase assays. Overnight cultures of *B. subtilis* grown on LB medium supplemented with chloramphenicol (5 μg/ml) were diluted to an optical density at 600 nm of 0.05 in fresh medium and grown at 37°C until an optical density at 600 nm of 0.5 was reached, after which one-half of the culture was shifted to 48°C. Samples were taken each hour to determine β-galactosidase activity as described previously (41, 42), and the activity was expressed as Miller units per mg cellular protein. Protein concentrations were determined using the Bradford method (Bio-Rad [Richmond, Calif.] reagent) with bovine serum albumin as the standard (8).

Database comparisons and sequence analysis. Sequence comparisons with the GenBank database were accomplished using the National Center for Biotechnology Information BLAST (1) network service. Multiple alignments were performed with the CLUSTAL W program (13). A systematic search using regular expressions was performed to find CtsR-binding sites (GTCAANNNNGGTC) in the *O. oeni* genome. *O. oeni* sequences were from the *O. oeni* IOB 8413 (ATCC BAA-1163) sequencing project (CONSRTM Laboratoire de Microbiologie-Uni-

TABLE 1. Oligonucleotides used in this study

Name	Sequence (5′ → 3′)[a]	Created restriction site	Plasmid construction and function
Transcriptional fusion constructions			
olcg11	GGGGAATTCAATTGAGTGACGGACAAAAGAT	EcoRI	pDL*ctsR*
olcg12	GGGGGATCCCGCTTCGTTTAATCTCGGCG	BamHI	pDLctsR and primer extension on *ctsR*
olcg16	ATCGGCGGATCCTATCAAATACCTCCTATTAACTAA	BamHI	pDL*hsp18*
olcg20	GCCCGAATTCTAAATTAATCGAAGCCTTTTGAC	EcoRI	pDL*hsp18*
olcg3	GGGGAATTCCCATTGGGGAGAGGCCGGC	EcoRI	pDL*grpE*
olcg4	GGGAAGCTTGCTTGGATCCTTTCACTGCT	HindIII	pDL*grpE* and primer extension on *grpE*
olcg14	CCCGAATTCTTTCGTTGATAATTTTGAAAACATT	EcoRI	pDL*groES* and pDL$groES_{mut}$
olcg15	CCCGGATCCTATGTAAAACCTCCTTTTAATTTG	BamHI	pDL*groES* and pDL$groES_{mut}$
groES4	CTACGGCTGCAGAAAAGGTCAAACTTTTTGACAAAG	PstI	pDL$groES_{mut}$
groES5	CGGACTCTGCAGAATTGCGTTATAATTTTACGCGG	PstI	pDL$groES_{mut}$
clpE1	CCCGAATTCGACAATTTGAGAATCTCTGACCA	EcoRI	pDL*clpL2*
clpE2	CCCGGATCCCTGAACCGTTATTTGCTTGTTG	BamHI	pDL*clpL2*, QRT-PCR and primer extension on *clpL2*
olcg7	GGGGAATTCCCCGGAGGCCAGTTTAAGC	EcoRI	pDL*clpP*
olcg8	GGGGGATCCCCTTGAACCAAGATAATTCGG	BamHI	pDL*clpP*
olcg9	GGGGAATTCGGATGCTTTTGAAGATGCCG	EcoRI	pDL*clpX*
olcg40	CCCGGATCCTGATTCTACGTCCATGCTTTTT	BamHI	pDL*clpX*
olcg17	ATCGGCGAATTCATAAGATAATTTTATCTCTTTTATAAG	EcoRI	pDL*mleA*
olcg18	ATCGGCGGATCCATTTTCTCCTCAAGAACCACAT	BamHI	pDL*mleA*
RNA analysis			
olcg1	ATGCATGCCATGGCAGAAGCTAATATTTCAG		RT-PCR, QRT-PCR on *ctsR* and DNA probe
ctsR1	AAACGGGTGTTGATTACATAATT		RT-PCR, QRT-PCR, primer extension on *ctsR* and DNA probe
clpCtsR2	TTTCATTTCTAATATCATCGTCG		RT-PCR on *ctsR-clpC*
clpE3	ATTATAATGACGATCCCTTCGT		QRT-PCR on *clpL2*
clpE4	TGTCCAAACGGATAACCTCC		Primer extension on *clpL2*
groES2	TTCCGCCAACCTTTTCTTCTT		Primer extension on *groES*
groES3	TAACAGACTTGGGAGCTTTTG		Primer extension on *groES*
groES6	GCCACAACAGAACCCATCACTGGTT		QRT-PCR on *groES*
groES7	GGCGATCGAATTGTTCTTAGTAT		QRT-PCR on *groES*
grpE7	CGCAGGCAGAAAAGAACAATC		QRT-PCR on *grpE*
grpE8	GCTGAAGACGAAGCAGTTGC		QRT-PCR and primer extension on *grpE*
ldhD1	GCCGCAGTAAAGAACTTGATG		QRT-PCR on *ldhD*
ldhD2	TGCCGACAACACCAACTGTTT		QRT-PCR on *ldhD*

[a] Specific restriction sites are underlined.

versité de Bourgogne, GENOME Express, Institut National de la Recherche Agronomique [INRA], Laboratoire de Microbiologie et Biotechnologique Appliquée-Faculté d'Oenologie de Bordeaux, and Centre de Bioinformatique de Bordeaux-Université Bordeaux 2). The genome sequence data will be available though a dedicated website.

Nucleotide sequence accession numbers. The DNA sequence data described in this work have been deposited in GenBank with the following accession numbers: AJ890337 (*clpL2*), AJ890340 (*groES*), AJ890339 (*grpE*), and AJ890338 (*ctsR-clpC*).

RESULTS

Characterization of a *ctsR*-like gene in *O. oeni*. Analysis of the *O. oeni* IOB 8413 (ATCC BAA-1163) genome revealed a 480-bp open reading frame (ORF) encoding an 18-kDa protein sharing 50% amino acid sequence identity with CtsR of *B. subtilis*, which negatively regulates the transcription of several stress-regulated genes (16, 28). Thus, this *O. oeni* ORF was named *ctsR*. The *O. oeni* CtsR protein contains the predicted helix-turn-helix DNA binding motif characterized by Derré et al. (16). Analyzing the DNA region downstream of *ctsR*, we identified a 2.47-kb ORF encoding an 823-amino-acid polypeptide with a predicted molecular mass of 91.5 kDa. The amino acid sequence of this predicted protein has strong amino acid sequence identity with members of the HSP100/Clp ATPase family. The similarity is particularly striking in the ATP binding domains. Comparison with the microbial genome database (http://www.ncbi.nlm.nih.gov/BLAST/) revealed that the *O. oeni* Clp ATPase shares 58%, 51%, and 50% identical amino acid residues with the ClpC proteins of *Enterococcus faecalis* (NP 816879), *B. subtilis* (P37571), and *Listeria innocua* (NP 469609), respectively, and it was therefore designated ClpC. The stop codon (TAA) of the *O. oeni ctsR* gene overlaps the probable start site of *clpC* by 8 nucleotides, as previously described in *Lactococcus lactis* (56). Downstream of *ctsR*, we did not find an inverted-repeat structure typical of Rho-independent transcriptional terminators, suggesting that *ctsR* and *clpC* are organized as an operon. The 5′-end mRNA of *ctsR* was determined by primer extension analysis using RNA extracted from an exponentially growing bacterial culture either before

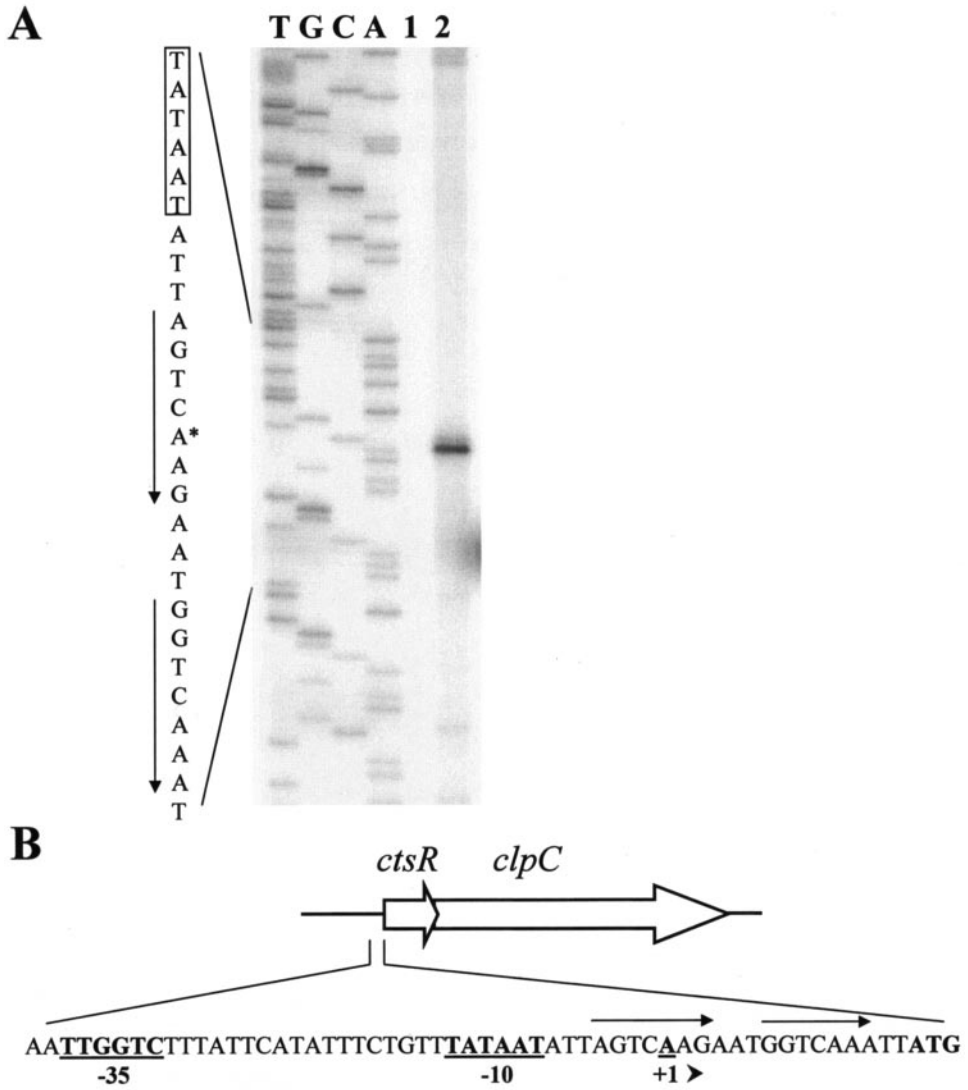


FIG. 1. (A) Determination of the transcription initiation site of the *ctsR* gene by primer extension analysis. Total RNA was extracted from *O. oeni* cells harvested in the exponential phase before (lane 1) or after (lane 2) a 30-min shift to 42°C. Primer extension products corresponding to the *ctsR* gene are shown alongside DNA-sequencing reaction products (lanes T, G, C, and A). The corresponding nucleotide sequence is shown on the left. The transcriptional start site is indicated by an asterisk, the −10 sequence is boxed, and arrows indicate the likely CtsR operator sites. (B) Organization of the *ctsR-clpC* operon and nucleotide sequence of the *ctsR* promoter region. The putative −10 and −35 sequences are underlined and boldface, arrows indicate the likely CtsR operator sites, and the initiation codon (ATG) is in boldface.

or after heat shock treatment (Fig. 1). The transcriptional start site was identified at nucleotide position −15, with reference to the presumed translational start codon. A −35 (TTGGTC) and a −10 (TATAAT) hexamer separated by 18 nucleotides, highly similar to the consensus of *O. oeni* housekeeping promoters, were identified at an appropriate distance from the transcriptional start site. No extension signal was detected using RNA extracted from cells harvested before stress treatment. These results show that *ctsR* expression is induced by heat shock. Three nucleotides upstream of the transcriptional start site, we found a 17-bp sequence (AGTCAAGAATGGT CAAA) closely resembling the consensus sequence A/GGTC AAANANA/GGTCAAA of the CtsR-binding site in gram-positive bacteria (16). Expression of *O. oeni ctsR* was investigated by Northern blotting using a 150-bp *ctsR*-specific PCR fragment as a probe. A 3.6-kb transcript, likely corresponding to a *ctsR* and *clpC* cotranscript, was detected in RNA extracted from the shocked culture (data not shown). Additionally, RT-PCR analysis using appropriate primers confirmed the presence of a *ctsR-clpC* cotranscript (data not shown).

Stress induction of the *O. oeni ctsR-clpC* operon. To examine whether expression of *ctsR* is induced by stress in *O. oeni*, we exposed exponential-phase cultures to various stress conditions: upshift from 30°C to 42°C, addition of ethanol (11% [vol/vol]), or shift to pH 3.6. Total RNAs were isolated before or 30 min after stress treatment. A quantitative RT-PCR experiment was set up using the constitutive *ldhD* gene as an internal control (4, 17). Intragenic fragments of *ldhD* and *ctsR* were amplified using the ldhD1/ldhD2 and olcg1/ctsR1 oligo-

TABLE 2. Relative mRNA expression of *O. oeni* stress genes as determined by QRT-PCR

Gene	Increase (*n*-fold) in ratio of stress gene expression[a]		
	42°C	Ethanol (11% [vol/vol])	pH 3.6
ctsR	5	5	4
clpL2	12	75	5
grpE	16	5	3
groES	6	5	1

[a] Thirty minutes after stress treatment. Induction ratios were calculated relative to the level of transcripts detected in unstressed cells.

nucleotide pairs, respectively (Table 1). As expected, higher levels of *ctsR* mRNA were detected in stressed cells than in unstressed cells (Table 2, row 1). The amount of *ctsR* transcript increased fivefold after heat shock or the addition of ethanol and fourfold after downshift to pH 3.6.

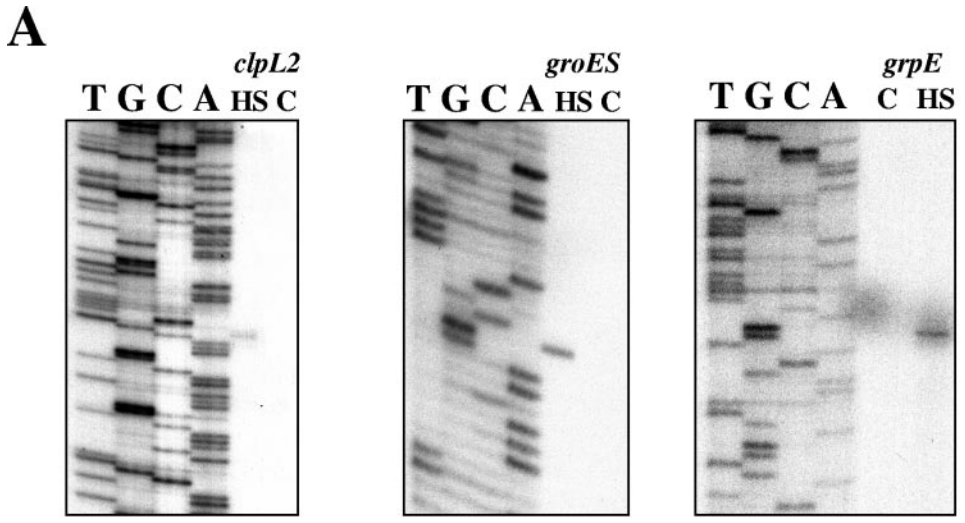
Prediction of CtsR regulon members by scanning the IOB 8413 (ATCC BAA-1163) genome. Invariance of the DNA recognition helix motif of the CtsR sequence is in agreement with the high conservation of the target nucleotide sequence (16). A similar sequence was identified in the promoter regions of the *hsp18*, *clpX*, and *clpP* genes of *O. oeni* (4, 30, 32). To identify members of the *O. oeni* CtsR regulon, a detailed DNA motif analysis of the *O. oeni* IOB 8413 (ATCC BAA-1163) genome was carried out using the CtsR operator consensus sequence. Additional CtsR operator sites were found upstream from the *clpL2* gene, as well as the *dnaK* and *groESL* operons encoding the major cell chaperones. No CtsR operator site was found upstream from the temperature-induced *ftsH* gene, encoding an AAA-type metalloprotease (7). Alignment of the seven potential CtsR-binding sites identified in the *O. oeni* genome produced the following consensus sequence: (A/G)GTCAA (A/G)AANGGTCAA(A/G) (Fig. 2), which is very similar to the consensus sequence defined by Derré et al., (A/G)GTCA AAAN(A/G)GTCAAA (16). As previously pointed out by Jobin et al. (32), although a potential binding site is also found upstream from the *clpX* gene (32), the number of nucleotides between the repeats is not conserved, suggesting that this sequence may be vestigial. We note that three direct heptanucleotide sequences are found upstream from the *grpE* gene, the first gene of the *dnaK* operon, where the second repeat is complementary to the −35 sequence of the promoter. Three

FIG. 2. Alignment of CtsR-binding sites identified upstream from *ctsR*, *hsp18*, *clpX*, *grpE*, *clpP*, *groES*, and *clpL2* genes of *O. oeni* IOB 8413 (ATCC BAA-1163). Identical nucleotides are shaded. The numbers indicate positions relative to the transcriptional start site. GenBank accession numbers for database sequences are as follows: *clpL2* (AJ890337), *groES* (AJ890340), *grpE* (AJ890339), *ctsR* (AJ890338), *hsp18* (AJ250422), *clpX* (Y15953), and *clpP* (AJ606044).

motifs have previously been reported upstream from the *clpP* gene and four motifs upstream from the *hsp18* gene (4, 16, 30).

Expression of the *clpL2* gene and the *groESL* and *dnaK* operons is induced by stress. The *clpL2* gene of *O. oeni* encodes a second ClpL protein showing 48% identical amino acid residues with the product of the previously characterized *clpL* gene (4). The *O. oeni dnaK* operon is comprised of three genes, *grpE*, *dnaK*, and *cbpA*, whereas the *groES* and *groEL* genes constitute the *groESL* operon. The organization of the major cell chaperone genes in *O. oeni* did not differ significantly from that described in many gram-positive bacteria (52). However, instead of *dnaJ*, the *O. oeni dnaK* operon presents a *cbpA* gene. The CbpA protein is an analog of the DnaJ molecular chaperone, which lacks the 69-amino-acid cysteine-rich zinc finger domain of DnaJ (55). Mapping of the transcriptional start sites was investigated for the *clpL2* gene and for the *groES* and *dnaK* operons (Fig. 3A). Transcription start points are preceded by appropriately spaced −10 sequences and −35 sequences, which share strong similarity with sequences previously described for *O. oeni* promoters (Fig. 3B). Moreover, putative CtsR operator sequences overlap or are near the −35 and −10 sequences of the identified transcriptional start sites, which strongly suggests CtsR-dependent expression of these genes. The induction of *hsp18*, *clpX*, and *clpP* expression under stress conditions has previously been reported (4, 30, 32). Characterization of the *clpL2* gene and the *groESL* and *dnaK* operons was investigated to determine if these genes are also stress response genes. In order to determine the level of induction of the *clpL2* gene and the *dnaK* and *groESL* operons after stress treatment, a quantitative RT-PCR experiment was set up. For the *dnaK* and *groESL* operons, expression of the first gene of each operon, *grpE* and *groES*, respectively, was followed. Induction ratios were calculated relative to the level of transcripts detected in unstressed cells (Table 2). Intragenic fragments of the *clpL2* (AJ831552), *groES*, and *grpE* genes were amplified using the clpE2/clpE3, groES6/groES7, and grpE7/grpE8 oligonucleotide pairs, respectively. As shown in Table 2, the amounts of *clpL2*, *grpE*, and *groES* transcripts were increased 12-fold, 16-fold, and 6-fold, respectively, 30 min after a temperature upshift from 30 to 42°C. After addition of ethanol (11% [vol/vol]), the induction factors were 75 for *clpL2* and 5 for *grpE* and *groES*. After a shift to pH 3.6, the amounts of *clpL2* and *grpE* transcripts increased fivefold and threefold, respectively. No increase in the amount of *groES* transcripts was observed under these conditions. These results show that the expression of genes preceded by a likely CtsR operator site is stress inducible and probably depends on the CtsR repressor.

Apart from *clpX*, all *O. oeni* genes preceded by a likely CtsR operator site are derepressed in a *B. subtilis* Δ*ctsR* mutant. Because no genetic tool adapted to carry out gene inactivation in *O. oeni* is yet available, CtsR-dependent regulation of *O. oeni* genes was examined using *B. subtilis* as a heterologous host. Transcriptional fusions between the promoter regions of the *O. oeni ctsR*, *hsp18*, *clpX*, *clpP*, *clpL2*, *grpE*, and *groES* genes (Table 1) and the *bgaB* gene of *B. stearothermophilus*, which encodes a thermostable β-galactosidase, were integrated as single copies at the *amyE* locus of *B. subtilis*. The *mleA* gene, whose promoter region contains no CtsR operator site, was used as a negative control. The β-galactosidase activities of



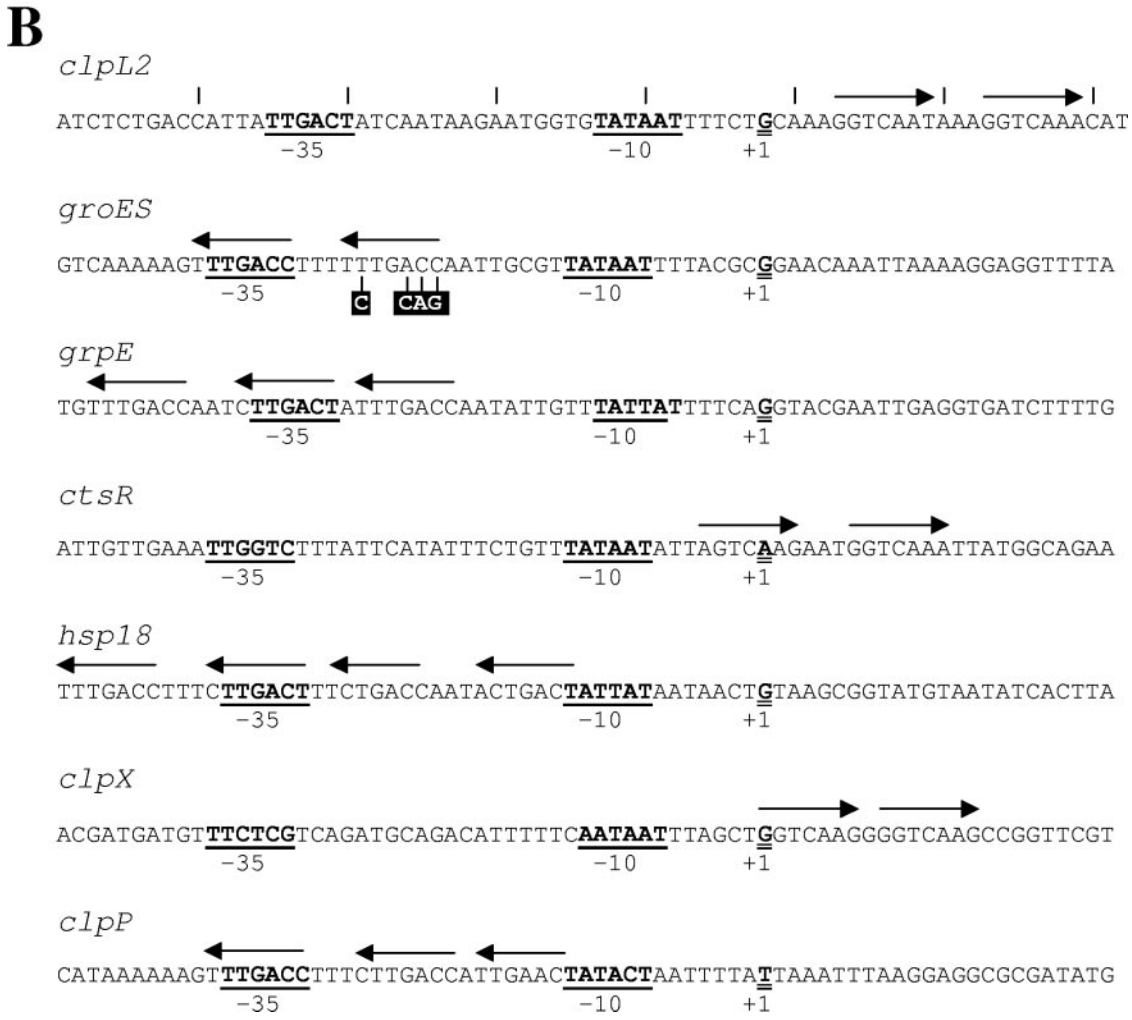


FIG. 3. (A) Primer extension analysis of *clpL2*, *groES*, and *grpE* mRNAs. Total RNA was extracted from *O. oeni* cells harvested in the exponential phase (lane C) or after a 30-min shift to 42°C (lane HS). Primer extension products are shown alongside DNA-sequencing reaction products (lanes T, G, C, and A). (B) Nucleotide sequences of the *clpL2*, *groESL*, *grpE*, *ctsR*, *hsp18*, *clpX*, and *clpP* promoter regions. Potential −35 and −10 sequences are underlined and boldface, transcriptional start points are indicated by +1, and CtsR heptad direct-repeat operator sequences are indicated by arrows. Mutation of the *groES* promoter performed by insertion of a PstI site in the repeated sequence of the CtsR operator site is indicated.

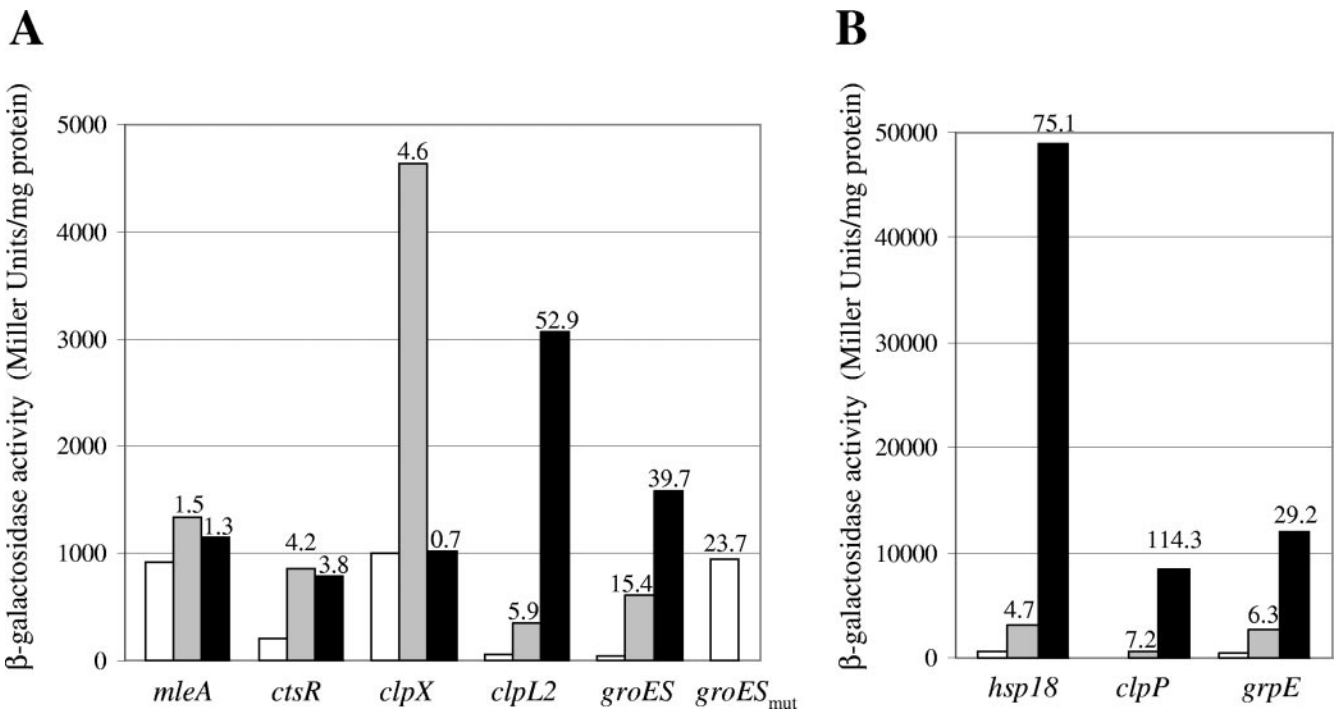


FIG. 4. CtsR negatively regulates *hsp* gene expression. Expression of the *mleA′-bgaB*, *ctsR′-bgaB*, *clpX′-bgaB*, *clpL2′-bgaB*, *groES′-bgaB*, and *groES$_{mut}$′-bgaB* (A) and the *hsp18′-bgaB*, *clpP′-bgaB*, and *grpE′-bgaB* (B) transcriptional fusions was measured in the wild-type strain (white and gray bars) or in the Δ*ctsR* mutant (QB4991) (black bars). Cells were grown in LB medium at 37°C. Expression of *bgaB* fusions was compared in the mid-exponential phase before (white and black bars) or after (gray bars) transfer to 48°C. The numbers indicate induction factors, which were calculated relative to the expression level of each fusion measured in the wild-type strain grown at 37°C.

strains carrying *bgaB* transcriptional fusions were monitored during growth in Luria broth medium. As expression of fusions followed similar patterns (data not shown), expression levels were compared in the mid-exponential phase. As shown in Fig. 4, expression of transcriptional fusions was low in the wild-type strain at 37°C and expression was increased 4- to 15-fold when cells were shifted to 48°C. As expected, no increase in the expression of the *mleA′-bgaB* fusion was observed. These results indicate that the tested expression of *O. oeni* stress response genes is inducible under heat shock conditions in the heterologous host, *B. subtilis*. To test whether CtsR does indeed play a role in controlling expression of *O. oeni* genes, the expression of these fusions was tested in *B. subtilis* strain QB4991, in which the entire *B. subtilis ctsR* gene is deleted (16) (Fig. 4). In a Δ*ctsR* background, expression of *ctsR′-bgaB*, *clpL2′-bgaB*, *groES′-bgaB*, *hsp18′-bgaB*, *clpP′-bgaB*, and *grpE′-bgaB* fusions was derepressed, whereas expression of *mleA′-bgaB* and *clpX′-bgaB* fusions was unchanged. Apart from expression of the *clpX′-bgaB* fusion, expression levels of fusions were not significantly different at 37°C and 48°C in the Δ*ctsR* mutant (data not shown). These results strongly suggest that the *O. oeni hsp18* and *clpL2* genes and the *ctsR-clpC*, *dnaK*, and *groESL* operons are negatively controlled by CtsR in *O. oeni*, whereas *clpX* expression does not seem to depend on this regulator. We note that, apart from *clpX* and *ctsR* genes, the derepressed expression levels are higher in the Δ*ctsR* mutant than in the wild-type strain after a temperature upshift from 37°C to 48°C. Similar results were reported by Derré et al. in experiments to follow heat shock induction of the *B. subtilis clpP* gene (16). The residual inductions observed in the Δ*ctsR* mutant could be the consequence of a complex pleiotropic role played by the CtsR regulator. Moreover, in a heterologous host, the expression of fusions may not be completely derepressed because of differences in the affinities of the *B. subtilis* CtsR protein for the *O. oeni* direct-repeat CtsR operator sites.

Site-directed mutagenesis of the *groESL* promoter. To confirm that the CtsR operator site of the *hsp* genes is the key element recognized by CtsR in *B. subtilis*, site-directed mutagenesis of the *groES* promoter was performed. The mutated promoter sequence was obtained by insertion of a PstI site in the repeated sequence of the CtsR operator site. In this way the TTGACC sequence (located at positions −22 to −27) was mutated to CTGCAG (Fig. 3B). This mutated promoter sequence was cloned upstream from the *bgaB* gene, and the *groES$_{mut}$′-bgaB* transcriptional fusion was then integrated as a single copy at the *amyE* locus of *B. subtilis*. The activity of the *groES$_{mut}$′-bgaB* fusion was measured in the wild-type *ctsR* background. Compared to that of *groES′-bgaB*, expression of the *groES$_{mut}$′-bgaB* fusion was 24-fold higher. These data demonstrated that *B. subtilis* CtsR negatively regulates *O. oeni hsp* expression by specific recognition of the predicted direct-repeat CtsR operator site located upstream from these genes.

DISCUSSION

Little is known about the regulation of stress response in *O. oeni*, although many stress response genes have been characterized in the bacterium. Analysis of the complete *O. oeni* IOB

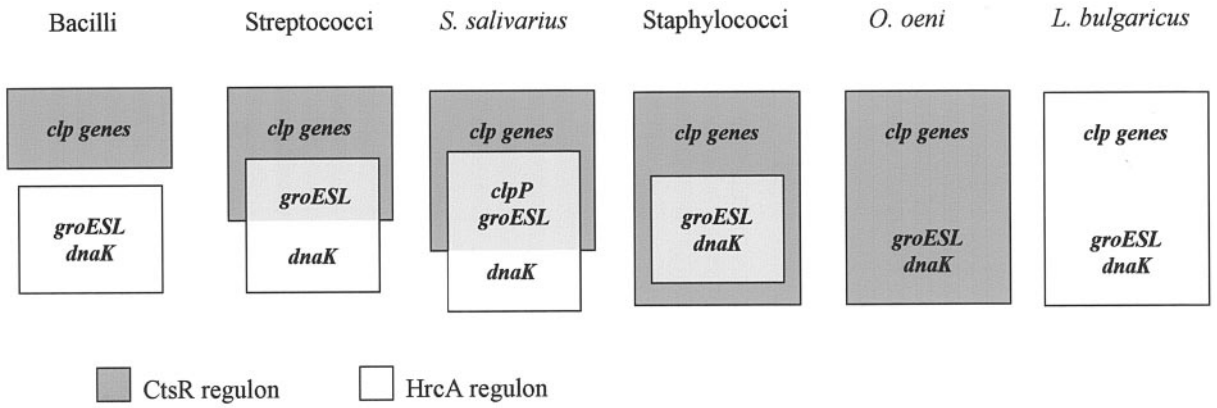


FIG. 5. Dual regulation by CtsR and HrcA in different gram-positive bacteria. In many gram-positive bacteria, the CtsR and the HrcA regulons coexist. In *B. subtilis* and closely related bacilli (*B. anthracis*, *B. stearothermophilus*, *B. halodurans*, *C. acetobutylicum*, *C. difficile*, *C. perfringens*, *L. monocytogenes*, and *L. innocua*), the two regulons are distinct, whereas in the streptococcal group (*S. pneumoniae*, *L. lactis*, and *S. salivarius*), they partially overlap, and the HrcA regulon is entirely embedded within the CtsR regulon in *S. aureus*. *O. oeni* and *L. bulgaricus* have original heat shock gene regulation with a predominant control of molecular chaperone genes by either CtsR or HrcA, respectively (adapted from Chastanet et al. [11]).

8413 (ATCC BAA-1163) genome sequence indicates the existence of an ortholog of the CtsR regulator, as well as several potential target genes. In this work, we have characterized the *ctsR* gene of *O. oeni* IOB 8413 (ATCC BAA-1163). As in most low-G+C% gram-positive bacteria studied so far, *ctsR* is the first gene of the *clpC* operon in *O. oeni*. Expression of the *ctsR-clpC* locus is induced by stress, and the transcription start site, located upstream from *ctsR*, appears to be dependent on CtsR. In *S. aureus* and the gram-positive rod-shaped bacteria (*Bacillus*, *Clostridium*, and *Listeria*), the *ctsR* gene is the first gene of a four-cistron operon in which the last gene encodes the ClpC protein and the *mcsA* and *mcsB* genes encode modulators of CtsR (10, 34, 35, 47, 49). In *L. lactis*, three genes, *ctsR*, *clpC*, and *orf*55, constitute the operon (56), whereas in the *Streptococcus* group there are only two genes, *ctsR* and *clpC*, expressed via the same transcript driven from the promoter upstream of *ctsR* (49). Furthermore, genome sequence analysis allowed us to predict that the *ctsR* and *clpC* genes are organized as an operon in *Lactobacillus plantarum* (NC_004567). The absence of *mcsA*-like and *mcsB*-like genes appears to be specific to the *Streptococcus* group and lactic acid bacteria and suggests a mechanism to modulate CtsR different from those described for the model organism *B. subtilis*. Initial attempts to complement the *B. subtilis* Δ*ctsR* mutant by expressing the heterologous *O. oeni ctsR* gene in *B. subtilis* were unsuccessful. The *O. oeni ctsR* gene, integrated at the *thrC* locus under the control of the P*xylA* xylose-inducible promoter, was transcribed in the heterologous host in the presence of xylose. Nevertheless, repression of transcriptional fusions was not observed. A similar result was obtained with constructions including the ribosome binding site motif of the *B. subtilis ctsR* gene. This result could be explained by a positive effect of a Clp ATPase, such as the *O. oeni* ClpL protein, on CtsR. Indeed, previous work suggests a positive role of the *B. subtilis* ClpC protein on CtsR by activating CtsR and promoting DNA binding (15). The *O. oeni* CtsR protein may be rapidly degraded in the *B. subtilis* Δ*ctsR* mutant, because of the absence of a specific cochaperon.

In addition to the likely CtsR operator sites previously identified upstream from the *clpX* and *hsp18* genes, analysis of the *O. oeni* IOB 8413 (ATCC BAA-1163) genome database allowed us to predict CtsR-binding sites upstream from the *clpL2* gene, as well as the *ctsR-clpC*, *groESL*, and *dnaK* operons. The CtsR-binding sites overlap or are near the σ^{70}-dependent promoters, suggesting that CtsR probably acts by competing or interfering with RNA polymerase E-σ^{70} binding. Repression may also occur throughout a roadblock mechanism preventing RNA polymerase progression along the DNA. Using *B. subtilis* as a heterologous host, we showed that the *O. oeni hsp18*, *clpP*, and *clpL2* genes and *ctsR-clpC*, *dnaK*, and *groESL* operons are repressed by CtsR from *B. subtilis*. Moreover, site-directed mutagenesis of the CtsR operator site located in the *groESL* promoter region led to derepression of the expression of the *groESL* operon. Taken together, these results indicate that CtsR acts as the master regulator of molecular chaperone gene expression in *O. oeni*.

Heat shock gene regulation in gram-positive bacteria is mediated by the alternative sigma factor σ^{B} and/or by transcriptional repressors, such as CtsR, HrcA, or HspR (10–12, 16, 21, 23, 56). However, the numbers of sigma factors found in bacterial genomes differ greatly, ranging from 18 in *B. subtilis* (37) to only 3 in the *L. lactis* genome (6), emphasizing the complexity and diversity of genetic regulatory mechanisms in bacteria. The *O. oeni* IOB 8413 (ATCC BAA-1163) genome contains only one housekeeping sigma factor gene (*rpoD*), and to the best of our knowledge no genes for alternative sigma factors, such as σ^{B}. Moreover, the promoters of stress genes characterized in this study are preceded by typical σ^{70} −35 and −10 boxes, suggesting that they all depend on the housekeeping form of RNA polymerase for expression. Regulation of *clp* genes of gram-positive bacteria is often described as CtsR dependent, whereas *groESL* and *dnaK* operons are often controlled by HrcA. In *B. subtilis* and closely related species (*Bacillus anthracis*, *B. stearothermophilus*, *Bacillus halodurans*, *Clostridium acetobutylicum*, *Clostridium difficile*, *Clostridium perfringens*, *Listeria monocytogenes*, and *L. innocua*), the two regulons are clearly distinct (11), whereas in the streptococcal

group (*Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Streptococcus mutans*, *Streptococcus agalactiae*, *L. lactis*, and *S. salivarius*), the HrcA and CtsR regulons partially overlap (Fig. 5). In the latter group, the *groESL* operon presents both the CtsR target site and the CIRCE sequence organized in tandem (12). In *S. aureus*, HrcA and CtsR act together to control the expression of the *dnaK* and *groESL* operons (10). In this work, we demonstrate that the CtsR repressor is the major regulator of molecular chaperone gene expression in *O. oeni*. Interestingly, we have been unable to identify either an *hrcA* gene or CIRCE operator sequences in the *O. oeni* IOB 8413 (ATCC BAA-1163) genome sequence so far. In contrast, genome sequence analysis indicates the existence of a predominant HrcA control of stress gene expression in *Lactobacillus bulgaricus* (S. Penaud and E. Maguin, personal communication), suggesting the absence of combined regulation by CtsR and HrcA in both of these lactic acid bacteria. Chastanet et al. (12) have shown that CtsR and HrcA act together synergistically to maintain low-level expression of the *dnaK* and *groESL* operons in the absence of stress, suggesting that this dual regulation is probably not redundant. In fact, the predominant CtsR control of molecular chaperone gene expression in *O. oeni*, like the probable major role of HrcA in *L. bulgaricus*, may play a role in coordinating synthesis of HSPs during stress response. Under optimal growth conditions, CtsR would prevent the synthesis of unnecessary stress proteins until environmental changes (ethanol, acid, nutritional stresses, etc.) strongly induce transcription of stress genes, including the *clp* genes and major cell chaperone operons (*groESL* and *dnaK*), thus enhancing the adaptability of these lactic acid bacteria under adverse environmental conditions by coordinating the synthesis of HSPs.

Apart from the *clpX* gene, all of the tested *O. oeni* molecular chaperone genes are dependent on CtsR. The expression of *clpX* has been shown to be heat inducible in different organisms (22, 24, 32, 54). The *clpX* gene of *B. subtilis* belongs to class IV heat shock genes, controlled by an unknown mechanism (23). In this work, we have noted the presence of a vestigial CtsR-binding site upstream from the *clpX* gene promoter of *O. oeni*, since the spacing between the direct repeats is not conserved and CtsR is not able to repress *clpX*. This site may be an evolutionary remnant, suggesting that this gene was once under CtsR regulation. Furthermore, quantitative RT-PCR analysis of genes under the control of CtsR revealed that induction factors are different depending on the gene and type of stress treatment. There may be other mechanisms, perhaps at the posttranscriptional level, such as mRNA stability, to ensure regulation of molecular chaperone synthesis under adverse environmental conditions. Previous studies have identified a long untranslated sequence at the 5′ end of *clpX* mRNA (5′ untranslated region) and suggested that it could be involved in stability and/or control of translation (32), as described previously in different organisms (14, 19, 38). Future work will involve studying the role of this 5′ untranslated region in the stability of mRNA under stress conditions, which could constitute an additional level of heat shock gene regulation in *O. oeni*.

The identification of members of the CtsR regulon is an essential step toward a more comprehensive understanding of the role of this regulon in stress adaptation. Analysis of the effects of mutations in individual CtsR-dependent genes on stress adaptation is required to address whether and to what extent CtsR-dependent genes contribute to survival in wine. These studies will provide an essential contribution to the understanding of the cell physiology of *O. oeni*. In order to carry out gene inactivation in *O. oeni*, future studies will focus on the construction of an integrative vector for gene disruption derived from the conjugative plasmid pGID052 (5).

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