

Evidence for Multiple Levels of Regulation of *Oenococcus oeni* *clpP-clpL* Locus Expression in Response to Stress

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A locus containing the *clpP* and *clpL* genes in the lactic acid bacterium *Oenococcus oeni* was studied. Real-time reverse transcription-PCR analysis revealed different induction factors involved in expression of these genes during stress. According to the conditions, *clpP* and *clpL* genes could be transcribed as two distinct transcripts or cotranscribed. The *clpP* promoter depended on the CtsR regulator, but surprisingly the *clpL* promoter did not. The amount of the *clpL* transcript depended on mRNA stability. This *clp* ATPase gene is at least controlled at the posttranscriptional level.

Stress response plays a key role in the cell adaptation of all organisms to environmental conditions. To survive environmental changes, cells synthesize proteins, including both chaperones and proteases, above all to prevent accumulation of abnormal proteins (20). These stress proteins are also involved in various cellular regulations during growth (28). Recent studies have focused on a large family of proteins, named Clp (for caseinolytic protein), which is well conserved in both eukaryotic and prokaryotic organisms. Many Clp proteins are ATPases and may play a part in determining the half-life of regulatory proteins (29, 32, 33, 41).

The Clp ATPase proteins are divided into two classes. Members of the first class, ClpA, ClpB, ClpC, ClpD, ClpE, and ClpL, also known as HSP100 proteins, contain two ATP nucleotide-binding domains (NBDs). The second class includes smaller proteins containing only one NBD, such as ClpX (31). It is widely accepted that Clp ATPases can function either as molecular chaperones or as regulator components of the proteolytic complex (38). This complex consists of two types of subunit: the proteolytic ClpP subunit and the Clp ATPase subunit. When ClpP is associated with a Clp ATPase, it can degrade larger specific substrates (7, 11, 23, 39). In *Bacillus subtilis* or *Lactococcus lactis*, ClpP is required for growth at high temperature (8, 10). The *clpP* gene of *B. subtilis* and *Listeria monocytogenes* is induced after several stresses (10, 21, 35). Besides stress, ClpP and associated Clp ATPases have been linked to many cellular development processes in bacteria (9, 15, 19, 25, 27).

The majority of *clp* genes are heat inducible, and their regulation is often described as CtsR dependent in gram-positive bacteria. In *B. subtilis*, *clpP* and *clpC* genes are class III heat shock genes, whose transcription is repressed by CtsR. The CtsR repressor recognizes a directly repeated heptanucleotide operator (4). In many cases, a gene similar to *ctsR* and a CtsR

operator located upstream from the *clp* genes have been found in several gram-positive bacteria (19, 34).

Oenococcus oeni, a gram-positive bacterium most often responsible for malolactic fermentation in wine, is tolerant to various stresses. *O. oeni*, which is introduced into starter cultures, is able to grow after alcoholic fermentation under acidic conditions and in the presence of high concentrations of ethanol. Among the *O. oeni* stress genes, the expression of *hsp18*, *trxA*, and *clpX* has been explored (16–18). These studies made it possible to characterize the pattern of expression of these genes during growth or under stress conditions. The *clpX* gene, which is preferentially expressed at the beginning of the exponential phase, is heat inducible, but its regulation remains unknown. However, it has been suggested that the long 5'-untranslated region could be involved in stability of mRNA (18). The present work focuses on an *O. oeni* locus containing the *clpP* and *clpL* genes. The expression of this *clp* locus was characterized during growth and following different stress conditions. Moreover, elements of transcriptional regulation of this locus were investigated.

The *clpP* gene is located on the same locus as the *clpL* gene. Based on knowledge about the *O. oeni* I.O.B 8413 genome, thanks to a sequencing project involving our laboratory, the Genome Express S.A. (France) and the Genological Institut of Bordeaux (France), the annotation step revealed the presence of a gene product that had 70 and 65% identities with ClpP of *B. subtilis* and *L. lactis*, respectively. This *clpP* gene (609 bp in length) encodes a protein of 202 amino acid residues with a calculated molecular mass of 21.5 kDa and a predicted pI of 6.88. A typical putative ribosome-binding site (RBS) is located upstream from the putative ATG codon at an appropriate distance. The residues Ser, His, and Asp (at positions 96, 121, and 172, respectively), which constitute the catalytic triad of the serine protease ClpP in *Escherichia coli*, are conserved in the ClpP sequence of *O. oeni* (24, 37).

No typical sequence of the Rho-independent transcriptional terminator sequence was found downstream from the *clpP* gene. Nevertheless, a putative open reading frame (ORF) gene (2,163 bp in length) encoding a 720-amino-acid protein was predicted 303 nucleotides downstream from the *clpP* gene. This ORF is preceded by a putative RBS, 8 bases upstream of the putative start codon. The encoded protein has a predicted molecular mass

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TABLE 1. Primers used in this study

Name	Sequence (5'→3')	Function ^a
P5	CGGTACCAAAGGCAAGCGTTTTAT	Probe P, QRT-PCR, RT-PCR
P6	CTCTCCGAGTCTTCAAAAGTTGAT	Probe P, QRT-PCR
L4	GCGCGACAAGGCAAAGTAGAT	Probe L, QRT-PCR
L5	GCCGGAACATTTCCATTGAT	Probe L, QRT-PCR
L1	AATTATTAAGAAGCCGTTTGACT	RT-PCR
P8	GGCAAACCTTAATTACTTCACA	Probe I
P9	GGCCTTTATGTCAATTTCTAT	Probe I
L2	CCCGAATTCGAATGGATCATTATTACTGTTATT	Primer extension with <i>clpL</i> , RT-PCR
P7	TTTGAGTGGCTTGTGCGAGAGC	Primer extension with <i>clpL</i> , RT-PCR
Pi2	CCATTTGATCTTCGACTTCTC	Primer extension with <i>clpP</i>
P10	CGACTTGGAAGATCATAACT	Primer extension with <i>clpP</i>
Ldh1	GCCGCAGTAAAGAAGCTTGATG	QRT-PCR
Ldh2	TGCCGACAACACCAACTGATG	QRT-PCR
16S1L	TCGTAAAGCACTGTTGTAAGG	QRT-PCR
16S2R	CCTATTGCGAGCCCTGTATGC	QRT-PCR
Olcg7	GGGGAATTCGGGAGGCCAGTTTAAG	pDL <i>clpP</i> construction
Olcg8	GGGGATCCCCTTGAACCAAGATAATTCGG	pDL <i>clpP</i> construction
ClpPL1	CTACGGGAATTCCTTATTAAGAAGCCGTTTGACTTC	pDL <i>clpL</i> construction
ClpPL2	CTACGGGGATCCGATTGAATCCTCTATCAATCT	pDL <i>clpL</i> construction

^a QRT-PCR, quantitative real-time RT-PCR.

of 79 kDa and a predicted pI of 6.14. Blastprot analysis against the SwissProt databank revealed identities of 57% with the ClpL of *Lactococcus lactis* and 68% with the ClpL of *Streptococcus pneumoniae*. A likely Rho-independent transcription terminator stem-loop sequence (AAAAAATCCCTAAAAATTATTTTTGGGG ATTTTTT; $\Delta G = -12.5 \text{ kcal} \cdot \text{mol}^{-1}$) is located downstream from the stop codon (TAA) of *clpL*. To our knowledge, *O. oeni* is the first microorganism discovered to have such a genetic organization between these two genes. In general, the organization *tig-clpP-clpX* is found in gram-negative bacteria (26). In the case of several gram-positive bacteria, the *clpP* gene is located in a chromosomal region with no *clp* ATPase gene nearby (6, 8, 10, 30). Until now, very little information about the physiological role of ClpL has been available.

Differential expression of the *clpP* and *clpL* genes was observed during the growth phase. Clp proteins are known for being implicated in regulatory mechanisms during growth. The ClpP protease especially affects the stability of key regulators during growth phases (28). Thus, Northern blot analyses with specific intragenic probes for *clpP* and *clpL* (Table 1) were performed with total RNAs extracted from cells in different growth phases. Probes were radiolabeled by using a random-primer DNA labeling kit (Invitrogen), and their position was given in Fig. 1. The strain *O. oeni* IOB84.13 (2) was grown at 30°C in modified FT80 medium (pH 5.3) (1). Then RNAs were

purified with Tri-reagent (Sigma) after disruption of the cells in a Fastprep cell disintegrator FP120 Instrument Savant (BIO 101) for $6 \times 20 \text{ s}$ at $6,000 \times g$.

A 0.7-kb transcript was detected with the *clpP* probe, whereas the *clpL* probe allowed us to detect a larger transcript of 2.4 kb (data not shown). The *clpP* and *clpL* mRNAs had different sizes, and in this way, they could be detected by the same Northern blot analysis. Differences in the relative amounts of the *clpP* and the *clpL* transcript during growth are shown in Fig. 2. Contrary to *clpP*, the *clpL* transcript was very weakly detected during growth, and its level increased dramatically at entry into the stationary phase. Curiously, as found by Ingmer et al. (14) and Lemos and Burne (19) with *clp* ATPase probes, Northern blot analyses showed that with the *clpL*-specific probe, more than one transcript size was detected, contrary to the results obtained with the *clpP*-specific probe (Fig. 2, 3B, and 4C).

The *clpP* gene was expressed during all stages of growth at a high basal level, but its level reached its maximum in the exponential phase. The *clpL* transcript was highly detected in the stationary phase, as previously described for *S. pneumoniae* (30). Jobin et al. found a high level of *clpX* mRNA in the early log phase (18). The differential expression of *clpX* and *clpL* genes may reflect the possibility of different complexes between ClpP and Clp ATPase subfamilies.

A larger transcript was detected under stress conditions. The transcriptional analyses of *clpP* and *clpL* were performed under two stress conditions for 30 min: heat at 42°C or the presence of 10% (vol/vol) ethanol. Northern blotting was performed with *clpP* and *clpL* probes. As shown in Fig. 3, the signals of the 0.7- and 2.4-kb transcripts, corresponding to *clpP* and *clpL* transcripts, respectively, were more intense after stress treatments. Thus, both genes are induced by stress. A stronger increase in *clpP* transcript level was obtained after heat stress compared to that obtained after ethanolic stress, whereas signal corresponding to *clpL* transcript was more intense after ethanolic stress. Moreover, under heat stress conditions, a larger transcript of 3.1 kb was detected with *clpL* and

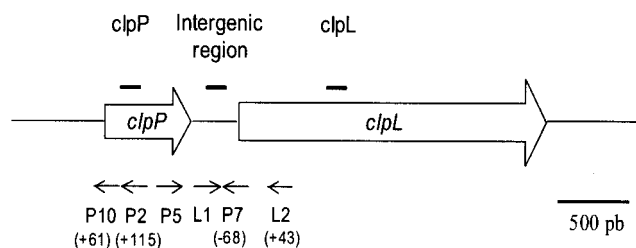


FIG. 1. Schematic diagram of the *clpP-clpL* locus and positions of probes (black lines) and primers (arrows) used in this study. Positions relative to ATG are indicated in parentheses. pb, base pairs.

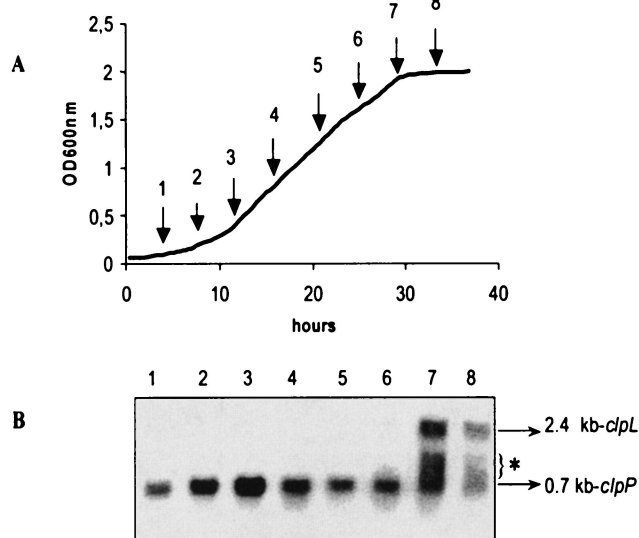


FIG. 2. Expression of *clpP* and *clpL* as a function of growth phase. (A) Growth curve of *O. oeni*. Arrows indicate cells samples for RNA extraction. (B) Northern blot analysis of RNA samples 1 to 8 using the *clpP*- and *clpL*-specific probes P and L (at each of the eight sampling times indicated in panel A). In all cases, 20 μ g of RNA was applied per lane. This experiment was carried out two times. Note that with longer time exposure, a 2.4-kb signal was weakly detected during the mid-exponential phase. The sizes of the transcripts detected are indicated, with 0.7 kb corresponding to *clpP* and 2.4 kb corresponding to *clpL*. An asterisk indicates transcript detected with the *clpL* probe, the size of which did not correlate with the entire *clpL* gene.

clpP probes (Fig. 3A and B). This transcript was better detected when a mix of specific probes of the intergenic region *clpP-clpL*, *clpL*, and *clpP* genes was used (Fig. 3C). The size of this transcript corroborates the hypothesis of the cotranscription of both genes *clpP* and *clpL*. A Northern blot analysis with probes of the regions located upstream from *clpP* and downstream from *clpL* and reverse transcription-PCR (RT-PCR) analysis with appropriate primers (Table 1) confirmed the presence of a cotranscript (data not shown). This finding differs from previous works on *clpP* gene expression in other gram-

positive bacteria, which have shown that *clpP* transcription was monocistronic (8, 9, 30).

***clpL* and *clpP* transcripts were differentially induced under stress conditions.** In order to measure the stress induction factor of the *clp* genes, a quantitative RT-PCR experiment was set up. *O. oeni* cDNAs were synthesized by using the Superscript II RT-PCR system (Invitrogen) using random hexamers as recommended. Specific cDNAs were amplified by real-time PCR with appropriate primers (Table 1) using the PCR Master mix SYBR Green I (Invitrogen). The presence of intact cellular mRNAs and the uniform efficiency of each RT reaction have been checked. Amplifications were performed on a Bio-Rad I cycler. The specificity of real-time PCR for each primer pair was determined with a melting curve. The efficiency of real-time amplification is calculated by the formula $E = [10^{(1/s)} - 1] \times 100$, where s is the slope of standard curve. The results were calculated by the comparative critical threshold ($\Delta\Delta C_T$) method, in which the amount of target RNA is adjusted to a reference relative to an internal calibrated target RNA. The constitutive *ldhD* gene was chosen as an internal control for these experiments. The intragenic region of *ldhD*, *clpP*, and *clpL* was amplified using Ldh1/Ldh2, P5/P6, and L4/L5 oligonucleotide pairs, respectively (Table 1). The comparison of the ΔC_T values between *ldhD* in unstressed and stressed cells confirmed that the transcription of this gene is not influenced by heat shock or ethanolic shock. As expected, higher levels of *clpP* and *clpL* messengers were detected in stressed cells compared to unstressed cells. After several calculations, we concluded that the amounts of *clpP* and *clpL* transcripts increased by 16- and 38-fold, respectively, after 30 min at 42°C. After 30 min in the presence of ethanol (10% [vol/vol]), the induction factors were 4 for *clpP* and 53 for *clpL*. As observed by Northern blot analysis, the folds of induction depended on the kind of stress. The difference between *clpP* and *clpL* induction rates after stress implied differential stability of transcripts and/or the presence of an initiation transcriptional site upstream from the *clpL* gene.

Stability of transcripts modulate the expression level of the *clpL* gene. To examine the possibility that environmental conditions affected the stability of *clp* transcripts and particularly *clpL* mRNA, the rate of these transcripts was investigated under three conditions: the mid-exponential phase (optical density at 600 nm [OD₆₀₀] of 0.7), part of the mid-exponential-phase culture subjected to heat shock (30 min, 42°C), and the stationary phase (OD₆₀₀ of 1.6). Total RNAs were extracted from cells treated with rifampin (final concentration of 250 μ g \cdot ml⁻¹) to prevent the initiation of transcription. Northern blot analysis was performed with RNAs extracted at different times after rifampin addition (Fig. 4). To compare the amounts of the three transcripts directly, Northern blotting was performed with a mix of the three specific probes of *clpP*, *clpL*, and the intergenic region. To calculate half-lives, the amount of *clp* transcripts is adjusted to the amount of 16S rRNA transcript. A linear regression analysis of the data is given in Fig. 4D. As shown in Fig. 4C and D, under stress conditions, *clpP* transcript presented a half-life of approximately 6 min, whereas *clpL* transcript and *clpPL* cotranscript had half-lives of 3 and 1.5 min, respectively. On the other hand, the half-lives of *clpP* mRNA were approximately the same in the exponential phase and after heat shock. The increase in the amount of *clpP* mRNA under heat shock conditions was weakly influenced by

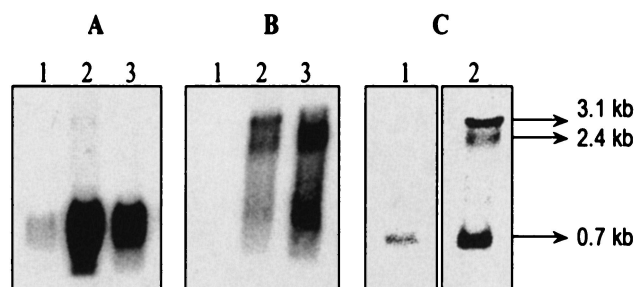


FIG. 3. Expression of *clpP* and *clpL* genes under stress conditions. Total RNAs were isolated from *O. oeni* cells grown at 30°C (lane 1) and submitted to heat shock (30 min at 42°C) (lane 2) or ethanolic shock (30 min at 10% [vol/vol]) (lane 3). Northern blot analyses of RNAs were performed with *clpP* probe (A); *clpL* probe (B); or a mixture of *clpP*, *clpL*, and intergenic region-specific probes (C). The sizes of the transcripts detected are indicated. In all cases, 15 μ g of RNA was applied per lane.

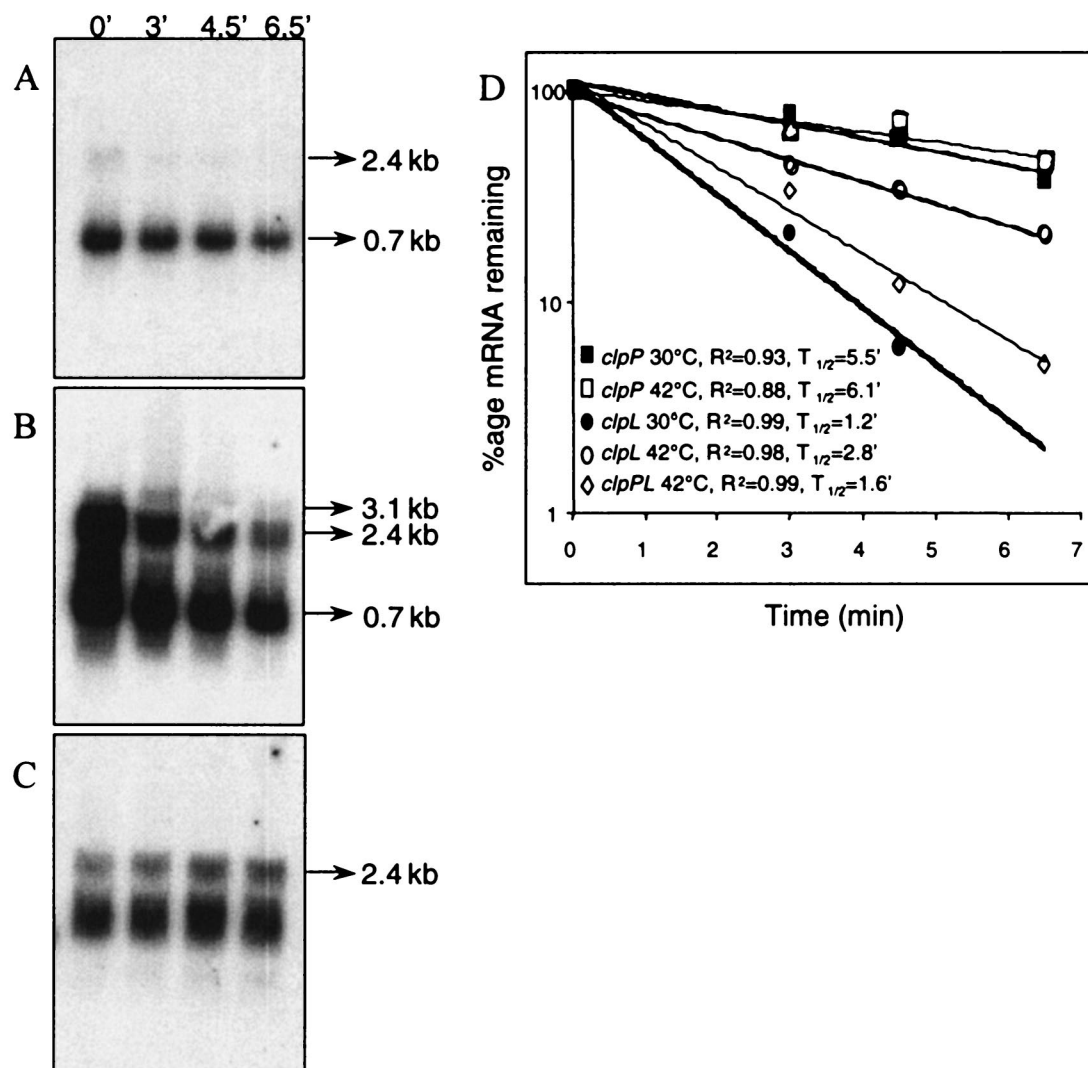


FIG. 4. Northern blotting performed to determine the relative rates of degradation of transcripts produced from the *clp* locus. The decay was measured in the exponential phase (A) at an OD_{600} of 0.7, with a heat stress at 42°C during 30 min (B) at an OD_{600} of 0.7, and in the stationary phase (C) at an OD_{600} of 1.8. The RNAs were prepared after 0, 3, 4.5, or 6.5 min after rifampin addition ($250 \mu\text{g ml}^{-1}$). Northern blot analyses of RNAs were performed with a mixture of the three *clpP*, *clpL*, and intergenic region-specific probes. In all cases, $15 \mu\text{g}$ of RNA was applied per lane. This experiment was carried out two times. (D) Semilogarithmic plot of (i) *clpP* mRNA decay at OD_{600} of 0.7 (solid squares) or after heat-shock (open squares), (ii) *clpL* mRNA decay at an OD_{600} of 0.7 (solid circles) or after heat shock (open circles), and (iii) cotranscript mRNA decay at an OD_{600} of 0.7 after heat shock (open diamonds). The correlation coefficients (R^2) and half-life ($T_{1/2}$) were determined for each regression analysis.

the rate of mRNA decay and strongly influenced by RNA synthesis. In contrast, the increase in the amount of *clpL* under heat shock conditions and especially in the stationary phase appeared to be affected more by the rate of mRNA decay (Fig. 4C and D). Indeed, *clpL* transcript seemed very stable during the stationary phase (Fig. 4C) compared to the exponential phase (Fig. 4A). However, the Northern blot analysis was not sensitive enough to determine precisely the half-life of this transcript during the exponential phase. Thus, the half-life of *clpL* transcript was calculated thanks to real-time PCR. For this experiment, the 16S rRNA transcript was chosen as an internal control. The results confirmed that the half-lives of *clpL* transcript were about 1 min in the exponential phase, 3 min under heat shock, and at least 10 min in the stationary

phase. Thus, the stability of the *clpL* mRNA acts as a form of posttranscriptional regulation.

The *clpP* promoter depended on CtsR but the *clpL* promoter did not. In order to characterize the promoter region of *clpP* and to highlight potential transcription initiation in the intergenic region, primer extension analyses were carried out with specific primers for *clpP* or *clpL* (Table 1). Primer extensions were performed from RNA extracted from an exponential culture either before or after heat shock treatment. Total RNAs ($5 \mu\text{g}$) were mixed with 2 pmol of each primer (Table 1). RT was performed with 1 U of Superscript II RNase H⁻ reverse transcriptase (Invitrogen) as recommended by the manufacturer in the presence of [α -³²P]dATP (Perkin Elmer). The 5'-end mRNA of the *clpL* gene was located 256 bp up-

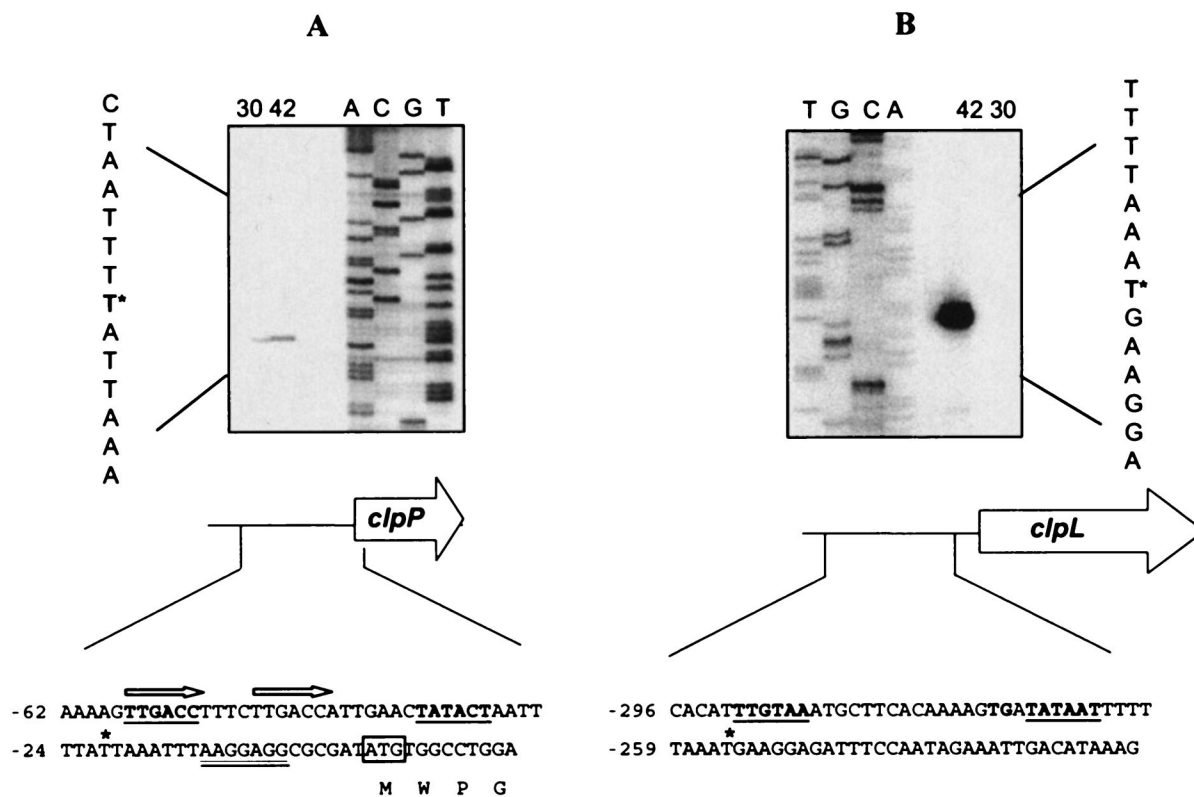


FIG. 5. Determination of the 5' end of *clp* transcripts. The RT reaction was performed with 5 μ g of total RNA isolated from *O. oeni* grown at 30°C or heat shocked for 1 h at 42°C. Lanes A, C, G, and T show the dideoxy sequencing ladder obtained with the same primer as that used for the reverse transcriptase reaction. The asterisk indicates the base representing the 5' end of *clpP* mRNA or *clpL* mRNA in the sequence shown to the left. The RBS is double underlined. The putative -10 and -35 sequences are underlined and in boldface. An asterisk indicates the transcription start site. The ATG start codon was boxed. Arrows indicate the probable operator sites for CtsR. Positions relative to the translation initiation site are indicated to the left. (A) Determination of the 5' end of the *clpP* transcript with oligonucleotide Pi2. (B) Determination of the 5' end of the *clpL* transcript with oligonucleotide L2.

stream from the putative start codon (Fig. 5B). This signal was detected only from RNA extracted from heat-shocked cells. Two hexanucleotides (TTGTAA and TATAAT) separated by 17 nucleotides presented high similarities to the -35 and -10 sequences of several promoters of *O. oeni* (13). The -10 sequence could be extended, since it is preceded by TGN (36). The transcription of *clpL* could be initiated from this site, supporting the hypothesis of an independent transcription of *clpL* gene. The long untranslated sequence at the 5' end of *clpL* mRNA is not uncommon in other bacteria, but its role remains obscure. Nevertheless, this sequence could be involved in stability as described previously (5, 18, 22).

A 5'-end mRNA was located 22 bp upstream from the putative start codon of *clpP* (Fig. 5A). Promoter sequence with significant similarity to the housekeeping promoter recognition sequence was found upstream from this transcription signal. Analysis of this promoter region revealed a directly tandem heptanucleotide sequence (5'-TTTGACCTTCTTGACC-3') overlapping the potential -35 sequence and showing high similarities to the operator sequence recognized by the repressor *ctsR* (4). Analysis of the genome sequence of *O. oeni* has shown the presence of a gene product having 52% identity to the CtsR of *B. subtilis*. These findings led us to think about the possibility of CtsR-dependent regulation.

However, no CtsR consensus sequence was found in the intergenic region *clpP-clpL*. Differences observed in profile expression between *clpP* and *clpL* could be explained by several mechanisms of regulation. To investigate the CtsR-dependent regulation of *clpP* suggested by the CtsR consensus sequence in the promoter region, transcriptional fusions were built in pDL vector (40). Promoter regions of *clpP* and *clpL* amplified by PCR with appropriate primers (Table 1) were cloned upstream of the *bgaB* gene encoding the *Bacillus stearothermophilus* β -galactosidase in pDL vector. Recombinant vectors were transferred in *B. subtilis* strain 168 and *B. subtilis* strain QB 4991, which had the *ctsR* gene deleted (3), and β -galactosidase activity was measured as described by Grandvalet et al. (12). A high level of activity (8,000 Miller units \cdot mg $^{-1}$) was measured for *clpL'*-*bgaB*, which confirmed the functionality of this promoter. The *clpP'*-*bgaB* fusion was expressed at a low level in the wild-type strain (74 Miller units \cdot mg $^{-1}$). In the Δ *ctsR* genetic background, the level of *clpL'*-*bgaB* expression remained the same (7,300 Miller units \cdot mg $^{-1}$), whereas *clpP'*-*bgaB* fusion increased 200-fold (15,800 Miller units \cdot mg $^{-1}$). These results clearly indicated that CtsR of *B. subtilis* negatively regulated the *clpP* gene, whereas transcription from *clpL* promoter was not dependent on CtsR. It is interesting to note that if CtsR regulates the *clpP* gene in *O.*

oeni, its repression was only partial, since a high basal expression of *clpP* was detected by Northern blot analysis. The CtsR-dependent repression in *O. oeni* could be modulated, and in this case, it is presumably due to the preponderant role of ClpP during the growth phase.

Taken together, these results give evidence for the presence of multiple levels of control of the *clpP-clpL* locus in *O. oeni*. We would like to point out the fact that findings strongly suggest the existence of a *clpL* promoter. (i) Analysis by primer extension allowed us to identify a potential transcription start site upstream from *clpL*. (ii) A transcription event involving the *clpL* gene was demonstrated in *B. subtilis*. (iii) The *clpL* level highly increased in the stationary phase, while *clpP* transcript decreased in this phase. However, this promoter did not appear to be dependent on CtsR. Moreover, neither an alternative sigma factor nor the *hrcA* gene has been identified in the *O. oeni* genome so far. Thus, future work will be focused on identification of the mechanism involved in *clpL* gene regulation.

Nucleotide sequence accession number. The nucleotide sequence has been submitted to the EMBL nucleotide sequence database under accession no. AJ606044.

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