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Characterization of the CtsR Stress Response Regulon in *Lactobacillus plantarum*▽†

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***Lactobacillus plantarum ctsR* was characterized. *ctsR* was found to be cotranscribed with *clpC* and induced in response to various abiotic stresses. *ctsR* deletion conferred a heat-sensitive phenotype with peculiar cell morphological features. The transcriptional pattern of putative CtsR regulon genes was examined in the Δ*ctsR* mutant. Direct CtsR-dependent regulation was demonstrated by DNA-binding assays using recombinant CtsR and the promoters of the *ctsR-clpC* operon and *hsp1*.**

In order to respond to stressful conditions, microorganisms have evolved multiple adaptive mechanisms. Indeed, external signals can elicit dramatic changes in the expression pattern of a variety of stress-related genes encoding proteins thought to improve adaptation to the changing environment. Heat shock induces the synthesis of a specific set of proteins, known as heat shock proteins (HSPs). Some of these, such as GroEL, DnaK, small HSP, and several Clp ATPases, are molecular chaperones that facilitate the proper folding of cellular proteins (32). Others, such as the Clp ATP-dependent protease, degrade incorrectly folded proteins (14).

In *Bacillus subtilis*, the model organism of Gram-positive bacteria, the heat shock response involves at least six different classes of heat-inducible genes that are distinguished by their regulatory mechanisms (25). Class III genes are transcriptionally controlled by the class III stress gene repressor, CtsR, which binds to a specific heptanucleotide direct repeat (5′ RGTCADN NAN RGTCADN 3′) referred to as the CtsR box (6).

Among Gram-positive bacteria, CtsR mainly regulates the expression of genes encoding the Clp ATPases and the ClpP protease (25). However, in staphylococci, the CtsR regulon also comprises the *groESL* and *dnaK* operons (3) and, in *Oenococcus oeni*, even a small-HSP gene (15). Recently, we have reported that the *Lactobacillus plantarum ftsH* gene, encoding a membrane-bound metalloprotease, is a novel member of the CtsR stress response regulon, representing an exception with respect to other Gram-positive species (11).

Lactobacillus plantarum, a facultative heterofermentative lactic acid bacterium (LAB), is extremely widespread in the environment. It is a natural inhabitant of the human gastrointestinal tract and widely used as a starter and probiotic in many food processes for which stress conditions such as heat, cold, and acidity are common. The survival of *L. plantarum* under harsh and diverse conditions indicates that it has developed several tolerance and resistance mechanisms (20, 28). Although some stress genes have been characterized in this species (9, 27, 29, 30), little is known about their transcriptional regulation.

Here we characterize *ctsR* in relation to stress response in *L. plantarum*.

***ctsR* is contranscribed with *clpC* and induced by heat and osmotic stress.** *ctsR* of *L. plantarum* WCFS1 (20) was studied by DNA sequence analysis, 5′ rapid amplification of cDNA ends (RACE), and reverse transcription-PCR (RT-PCR). The genomic organization of *ctsR* is shown in Fig. S1 in the supplemental material. The *ctsR* open reading frame encodes a 155-amino-acid (aa) protein with a predicted molecular mass of 17.6 kDa. The protein shares 45% aa sequence identity with CtsR from *B. subtilis* and displays even higher similarity with respect to CtsRs from other *Lactobacillus* species. The highest levels of identity are found in the HTH (helix-turn-helix) motif region (residues 25 to 48; 56 to 92% identity), which is responsible for specific DNA sequence recognition and binding; in the amino-terminal domain (residues 6 to 15; 80 to 90% identity); and in the central glycine-rich region (residues 59 to 70; 92 to 100% identity), playing a putative heat-sensing function (7).

The transcriptional start site was determined by 5′-RACE analysis of total RNA extracted from exponentially growing bacterial cells (optical density at 600 nm [OD_{600}] = 0.6) following incubation at 40°C for 10 min. The initiation site was mapped to position −31 relative to the translational start codon (see Fig. S1 in the supplemental material). Oligonucleotides used for 5′-RACE analysis and other experiments in this study are listed in Table S1 in the supplemental material. Putative −35 and −10 hexameric boxes were identified at proper distances. The promoter contains a 17-bp sequence (AGTCAAT ATT AGTCAAA) closely resembling the suggested consensus heptanucleotide direct-repeat CtsR binding

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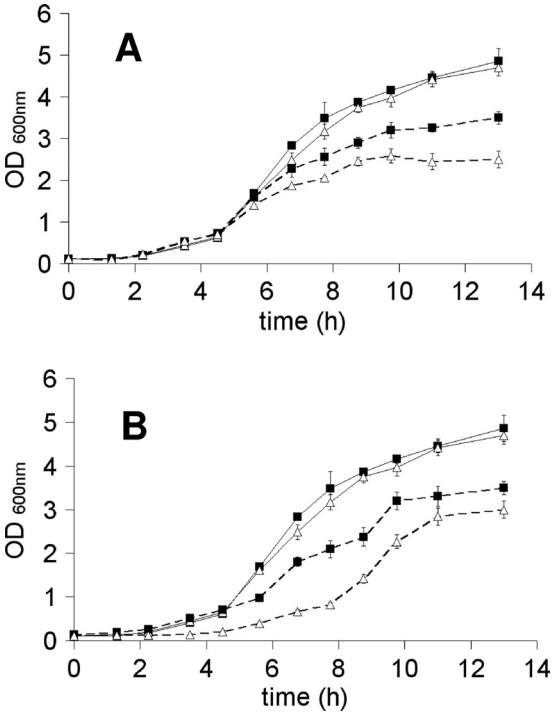

FIG. 1. Growth of wild-type *L. plantarum* and Δ*ctsR* mutant strains at optimal temperature and under heat and ethanol stress conditions. Cells were cultivated in MRS broth at 28°C (solid lines) or a suboptimal temperature of 40°C (dashed lines) (A) or at 28°C in MRS broth containing ethanol (2% [vol/vol]) (dashed lines) (B). The increase in OD_{600} is shown as a function of time (hours) and was monitored over 13 h for both the wild-type (■) and Δ*ctsR* mutant (△) strain. Data shown are means ± standard deviations of the results from three independent experiments.

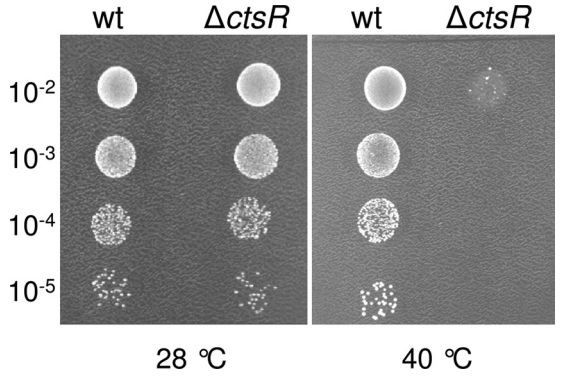

FIG. 2. CtsR is required for growth at high temperatures. Cultures of wild-type (wt) and Δ*ctsR* mutant strains were grown exponentially in MRS broth at 28°C. At an OD_{600} of 0.5, cultures were serially diluted (10^{-2}-, 10^{-3}-, 10^{-4}-, and 10^{-5}) and 10 μl of each dilution was spotted on an MRS plate and incubated at the indicated temperatures of 28°C (control temperature) and 40°C (heat stress). Pictures were taken after 20 h of growth.

site RGTCADN NAN RGTCADN, so far defined in several Gram-positive bacteria (6). This potential CtsR operator overlaps the transcriptional start site, consistent with a role for CtsR as a repressor and in agreement with previously characterized σ^A-dependent promoters under CtsR-mediated negative control (6, 25). RT-PCR analysis, using appropriate primers spanning *ctsR* and the downstream *clpC* coding region, demonstrated that *ctsR* and *clpC* are cotranscribed (data not shown). A canonical Rho-independent terminator was found downstream of *clpC*, indicating a bicistronic operon organization. In contrast to that of *B. subtilis* but similar to those of streptococci (26) and LAB, such as *Lactococcus lactis* (35) and *O. oeni* (15), *L. plantarum*'s *ctsR-clpC* operon lacks genes encoding potential CtsR modulators (McsA and McsB [*m*odulators of *C*t*s*R repression]) (19), thus suggesting that regulation of CtsR might require alternative pathways.

ctsR mRNA levels were analyzed by quantitative RT-PCR (qRT-PCR) following the exposure of exponential-phase cultures ($OD_{600\ nm} = 0.6$) to a range of abiotic stresses, mimicking conditions commonly encountered by this bacterial species (for details, see Table S2 in the supplemental material). Total RNAs were extracted from control and stressed cultures, reverse transcribed, and subjected to qRT-PCR with appropriate primers (see Table S1 in the supplemental material) on an Applied Biosystems 7300 real-time PCR instrument, using SYBR green I detection and the *ldhD* and *gyrA* genes as internal controls (10, 11, 12). Strong induction of *ctsR* expression was observed in response to heat shock (35-fold increase) and osmotic stress (14-fold increase); more marked induction resulted from combined heat and osmotic stress (120-fold increase). A weaker increase (2-fold) in mRNA level was also observed in ethanol-stressed cultures. This finding substantiates the results of previous studies demonstrating that the *ctsR-clpC* operon is induced by stresses other than heat shock (1, 23).

Deletion of *ctsR* confers a heat-sensitive phenotype and peculiar morphological features. To understand the relevance of CtsR in *L. plantarum*, the phenotype of a previously generated *ctsR* deletion mutant strain was investigated (11). Growth rates of wild-type and mutant strains were analyzed by OD_{600} monitoring and direct plate counting under the following different culture conditions: at 28°C (control temperature); at 40°C (heat stress); and at 28°C in MRS broth containing 2% ethanol (vol/vol), 0.05% (wt/vol) porcine bile, or 0.8 M NaCl or in HCl-acidified MRS broth (pH 5). In comparison to the wild type, the Δ*ctsR* strain displayed a similar growth rate when cultivated under optimal temperature and medium conditions. However, a growth impairment became evident in cultures of the mutant strain growing either under heat stress or in the presence of ethanol (Fig. 1A and B), suggesting the involvement of *ctsR* in adaptation to these kinds of stress. By spotting serial dilutions of exponentially growing cultures on MRS plates, we noted that incubation at 40°C strongly inhibited the growth of the mutant strain without affecting the wild type (Fig. 2).

To learn more about the role of CtsR under heat shock conditions, the ability of the mutant to tolerate a short intense heat stress and to induce thermotolerance was examined. When exponentially growing cultures were challenged for 15 min at 50°C, the number of surviving cells, as determined by CFU counting, was not significantly different between the wild type and the Δ*ctsR* strain (data not shown). Similarly, no significant differences in recovery and growth capacity rates were observed when cells were pre-exposed to 40°C for 30 min and

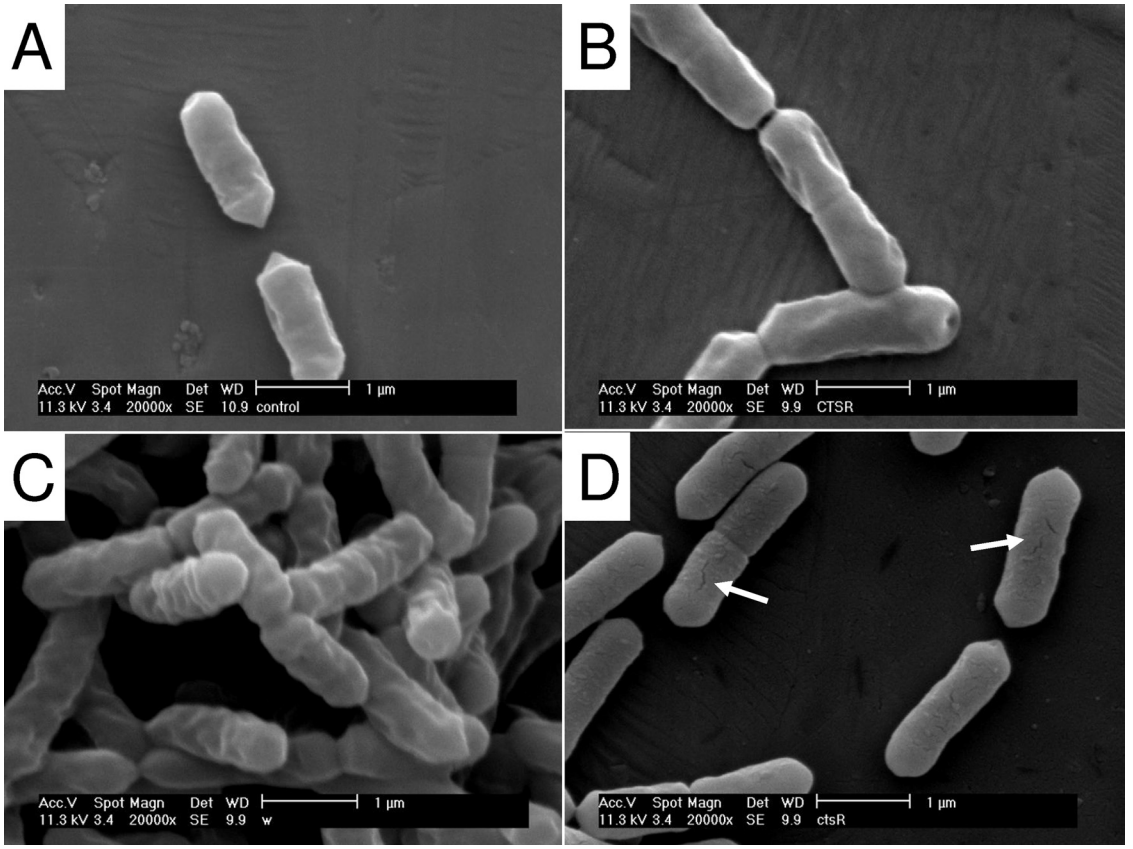

FIG. 3. SEM analysis of wild-type and CtsR-deficient strains. Exponentially growing cells (OD_{600} = 0.6) from wild-type and Δ*ctsR* mutant strains were imaged by SEM before (A and B, respectively) and after (C and D, respectively) a 30-min temperature upshift to 40°C. Arrows show fissures in mutant cell envelopes. Bars, 1 μm.

subsequently shifted to 50°C for 15 min, suggesting that *ctsR* inactivation does not affect the ability of *L. plantarum* to induce thermotolerance. It is well known that pre-exposure at a sublethal temperature enables *L. plantarum* to better survive for several minutes at the lethal temperature of 50°C (32). Our data indicate that CtsR does not play an essential role in this adaptation process; however, they highlight a crucial role of CtsR for coping with prolonged heat stress. This is a novel, unexpected finding, since CtsR inactivation generally causes derepression of heat stress genes, thus rendering cells more prepared to face stressful conditions. Indeed, in most Gram-positive bacteria analyzed so far, *ctsR* mutations increased heat resistance and/or general stress tolerance (4, 16, 17, 18, 24, 36).

The cell morphology of wild-type *L. plantarum* and Δ*ctsR* mutant strains was analyzed before and after heat stress exposure by scanning electron microscopy (SEM) (Fig. 3), using a Philips XL30 ESEM scanning electron microscope as reported before (5). Under optimal growth temperature conditions, cells exhibited the characteristic rod-shaped, smooth-surface morphology of *L. plantarum*, and no relevant difference between wild-type and Δ*ctsR* strains could be observed. In contrast, after heat shock, compared to those of the wild type, the mutant cell envelopes looked stiffer and presented somewhat fissured surfaces. These intriguing features suggest that the cell wall might be critically damaged in the Δ*ctsR* strain, pointing to a novel function of CtsR for cell wall integrity control in *L. plantarum*. Indeed, in *B. subtilis*, key enzymes of cell wall biosynthesis have been proven to be specific substrates of Clp proteolytic complexes (21), which, in turn, are transcriptionally regulated by CtsR.

Prediction and transcriptional analysis of putative CtsR regulon members. To identify members of the *L. plantarum* CtsR regulon, a detailed DNA motif analysis of the *L. plantarum* WCFS1 genome (20) was carried out using the CtsR operator consensus sequence (6) and Artemis version 9 software (Sanger Institute; http://www.sanger.ac.uk/Software/Artemis). Potential CtsR binding sites were found in the promoter regions of 10 *L. plantarum* genes (see Fig. S2 in the supplemental material), including *ftsH*, for which involvement of CtsR in transcriptional regulation was previously reported (11). Putative CtsR boxes were detected upstream from *ctsR-clpC*, *clpB*, *clpE*, *clpP*, and *hsp1*, encoding one of the three small heat shock proteins (HSP20 family) of *L. plantarum* (20, 29, 34). Likely CtsR operators were also found in the promoters of four genes annotated as lp_0836 (*nrpR1*), lp_1995, lp_2090, and lp_2942 (20). Except for *nrpR1*, these latter genes do not seem to encode heat stress-related functions, as confirmed by the lack of appreciable transcriptional induction following heat shock (data not shown). Conversely, *hsp1* and *clp* genes were substantially induced by thermal upshift (data not shown), confirming their involvement in heat stress response mechanisms.

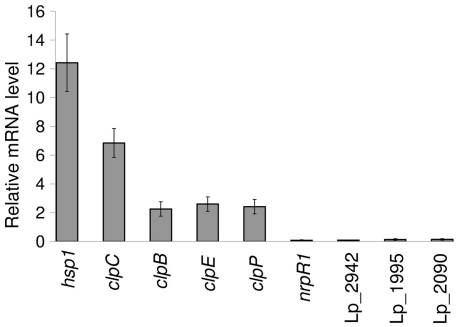

FIG. 4. Relative mRNA expression of putative CtsR regulon genes in the Δ*ctsR* mutant strain of *L. plantarum*. mRNA levels were calculated relative to the transcript levels detected in the wild-type strain. Total RNA from mid-exponential-phase cultures (OD_{600} = 0.6) growing under optimal temperature conditions (28°C) was extracted and analyzed. *ldhD* was used as the internal control gene. Data shown are means ± standard deviations of the results of three independent experiments.

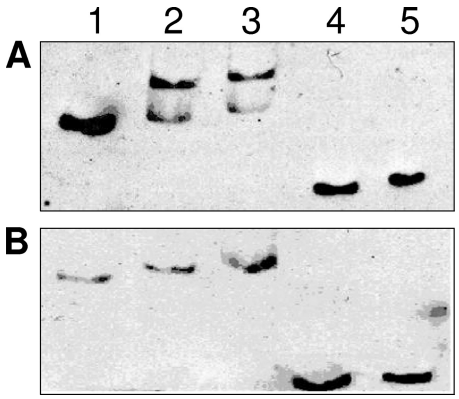

FIG. 5. CtsR binding to *hsp1* and *ctsR-clpC* promoter regions. Electrophoretic mobility shift assay was performed with purified *L. plantarum* CtsR. Biotin-labeled DNA fragments corresponding to the promoter regions of the *ctsR-clpC* operon (A) and *hsp1* (B) were incubated with increasing amounts of purified recombinant CtsR. Lanes: 1, no CtsR; 2 and 3, 100 and 250 ng of CtsR, respectively; 4 and 5, promoter fragments lacking the CtsR box preincubated with or without 250 ng of CtsR protein, respectively.

The transcript levels of all the putative CtsR-controlled genes in both the Δ*ctsR* and wild-type strain were investigated by qRT-PCR (Fig. 4). Total RNA was purified from exponentially growing cultures (cultivated at 28°C), retrotranscribed, and analyzed using appropriate oligonucleotides (see Table S1 in the supplemental material) and the real-time PCR procedure described above. The transcription of five of the genes investigated, namely, *hsp1*, *clpC* (examined as part of the *ctsR-clpC* operon), *clpB*, *clpE*, and *clpP*, was significantly derepressed in the mutant relative to their transcription in the wild-type control, with transcript levels increasing 12-, 7-, 2-, 2.6-, and 2.5-fold, respectively. Conversely, the expression of the other four genes was considerably repressed (approximately 10-fold lower). These results strongly suggest a negative regulation exerted by CtsR on its own operon, as well as on *hsp1* and *clp* genes, in agreement with previously characterized CtsR regulons (2, 4, 8, 15, 24, 35). On the other hand, since the transcription of *nrpR1*, lp_1995, lp_2090, and lp_2942 appears affected by CtsR inactivation, we cannot rule out that the expression of these genes might be controlled by CtsR activity either directly or indirectly.

CtsR binds specifically to promoter regions of *hsp1* and the *ctsR-clpC* operon. An *in vitro* approach was used to demonstrate the direct interaction of CtsR with its putative target sites in the *hsp1* and *ctsR-clpC* promoters. CtsR of *L. plantarum* was cloned, overexpressed, and purified as described elsewhere (6, 11) and used in gel shift assays with biotin-labeled, PCR-generated DNA fragments corresponding to the promoter regions of the *ctsR-clpC* operon and *hsp1* (Fig. 5A and B, respectively). Recombinant CtsR was able to bind specifically to the promoters, with progressive displacement of the free DNA fragment as the protein concentration increased. The binding was specific for both promoter regions, as shown by the absence of any protein-DNA complex when CtsR was incubated with promoter fragments lacking the CtsR target sequence.

These results confirm the quantitative transcriptional analysis of *hsp1* and *clpC* in the Δ*ctsR* strain, indicating that CtsR controls the expression of *hsp1* and that of its own operon by interacting directly with their promoters. A small heat shock gene named *Lo18* was also reported to be controlled by CtsR in *O. oeni* (15); therefore, *L. plantarum hsp1* would represent an additional example of *ctsR*-mediated regulation of small HSPs.

Concluding remarks. Regulatory mechanisms of stress response genes have been investigated in several Gram-positive bacteria and studied in detail in the genetic model *B. subtilis*. Conversely, very little is known about the transcriptional regulation of stress genes in *L. plantarum*, a versatile LAB which is likely to have evolved multiple and complex adaptive mechanisms. Comparative genomics revealed that CtsR and its target sequences are highly conserved among Gram-positive bacteria with low G+C content (6, 26). However, the CtsR-dependent stress response may vary significantly in terms of the composition of the regulon, combination of regulatory patterns, and control of CtsR activity (2, 3, 15, 35).

The results of this work indicate, for the first time, that *L. plantarum* CtsR is necessary for survival under stress conditions and, most importantly, plays a critical role for growth at high temperature. Since the heat sensitivity of the Δ*ctsR* strain became detectable only after long incubation periods and not after short heat shocks, CtsR seems to be involved in coping with a prolonged heat stress regimen. Interestingly, our phenotypic analyses suggest that CtsR does not contribute to the induction of thermotolerance. The growth impairment and the intriguing morphological changes observed in the Δ*ctsR* cells under heat stress might derive from a transcriptional dysregulation consequential to the *ctsR* inactivation and point to a novel protective function of CtsR for cell wall homeostasis.

Similarly to CtsRs of other Gram-positive bacteria (6, 15, 22, 23, 35), *L. plantarum* CtsR autoregulates its own transcription together with that of the cotranscribed *clpC*. However, the lack of McsA and McsB homologues in the *ctsR-clpC* operon, as well as in the rest of the *L. plantarum* genome, suggests that, in this species, the regulation of CtsR activity might differ significantly from that in *B. subtilis*.

Sequence analysis of the *L. plantarum* WCFS1 genome al-

lowed us to identify potential CtsR regulon members. The results of transcriptional analysis indicated that *clp* genes are under CtsR control, thus confirming the regulatory pattern observed for *clp* in most Gram-positive bacteria (6, 13, 15, 35) but in contrast to that in several closely related *Lactobacillus* species in which *clp* gene transcription has been suggested to be CtsR independent (31, 33). Besides *clp*, CtsR controls the expression of a *shs* gene in *L. plantarum*: thus far, only one other small HSP gene, *Lo18* of *O. oeni*, had been included in a *ctsR* regulon (15). The occurrence of likely CtsR operators upstream from four apparently heat stress-unrelated genes of *L. plantarum* raises the possibility that CtsR might control other activities. However, further studies are needed and will be undertaken to shed light on this aspect.

Overall, our results confirm the high conservation of the CtsR regulatory system among Gram-positive bacteria but hint at possible differences in the regulation of CtsR activity and suggest that control by CtsR might extend to other major cellular functions.

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