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ORIGINAL PAPER

The CtsR regulator controls the expression of *clpC*, *clpE* and *clpP* and is required for the virulence of *Enterococcus faecalis* in an invertebrate model

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Abstract The intrinsic ruggedness of *Enterococcus faecalis* is responsible for its widespread distribution in nature and is often viewed as an important virulence determinant. Previously, we showed that the ClpB ATPase is negatively regulated by CtsR and is required for thermotolerance and virulence in a *Galleria mellonella* invertebrate model. Here, we used in silico, Northern blot and quantitative real-time PCR analyses to identify additional members of the CtsR regulon, namely the *clpP* peptidase and the *clpC* and *clpE* ATPases. When compared to the parent strain, virulence of the Δ*ctsR* strain in *G. mellonella* was significantly attenuated.

Keywords *clp* genes · ctsR regulon · *Enterococcus faecalis* · Thermotolerance · Virulence

Ana Paula Vaz Cassenego and Naira Elane Moreira de Oliveira have contributed equaly for the development of this work.

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Introduction

Protein quality control, which includes the refolding or degradion of damaged proteins, plays an indispensable role in bacterial homeostasis. Under stress conditions, the synthesis of molecular chaperones and proteases is rapidly induced to minimize aggregation of denatured proteins. In addition to protein quality control, bacterial proteases also play important roles in other cellular processes by controlling the stability of central regulatory proteins (Frees et al. 2007). In low-GC Gram-positive bacteria (Firmicutes), the ClpP protease is the main system involved in protein degradation. Functional ClpP proteases have a bipartite molecular architecture, with two heptameric rings of the ClpP serine protease forming a central proteolytic chamber and one or two hexameric rings of Clp ATPases docked to either one or both apical sides of the ClpP chamber, a structure that is remarkably similar to that of the eukaryotic proteosome (Frees et al. 2007). The genomes of Gram-positive cocci typically encode up to five Clp ATPases, namely ClpB, ClpC, ClpE, ClpL and ClpX, but only ClpC, ClpE and ClpX have the recognition tripeptide that permits interaction with ClpP (Frees et al. 2007). The ClpB and ClpL proteins lack the ClpP recognition peptide and function as independent chaperones (Woo et al. 1992). Given their central role in stress reponse and cell homeostasis, it is not surprinsing that several *clp* genes are required for virulence and the progression of disease of many bacterial pathogens including

Enterococcus faecalis, *Streptococcus pneumoniae* and *Staphylococcus aureus* (Frees et al. 2003; Ibrahim et al. 2005; Oliveira et al. 2011). In low-GC Gram positive bacteria, the levels of ClpP and selected Clp ATPases are controlled by the CtsR repressor that binds to a consensus heptad sequence located in the regulatory region of these genes (Derré et al. 1999). In *Lactobacillus platarum,* the CtsR regulon was characterised and CtsR is essential for stress response and growth at elevated temperatures. Moreover, the *ctsR* gene is co-transcribed with *clpC* and CtsR binds to the promoter region of the *ctsR–clpC* operon (Fiocco et al. 2010).

The genome of *E. faecalis*, a major nosocomial pathogen, encodes orthologs of the ClpB, ClpC, ClpE and ClpX ATPases and a single copy of the ClpP peptidase (Paulsen et al. 2003). In a previous study, we showed that the *E. faecalis clpB* gene was induced by a variety of stress conditions and negatively regulated by CtsR (Oliveira et al. 2011). In this report, we use in silico, transcriptional and mutational approaches to show that the *clpC, clpE* and *clpP* genes belong to the CtsR regulon. In addition, we showed that virulence of the Δ*ctsR* strain is significantly attenuated in a *Galleria mellonella* invertebrate model.

Materials and methods

Bacterial strains and general culture conditions

The *E. faecalis* OG1RF and Δ*ctsR* (Oliveira et al. 2011) strains were routinely grown in brain heart infusion (BHI) medium at 37 °C under aerobic conditions. When required for selective growth of strains erythromycin (10 μg/ml) was added to the growth medium. For Northern blot analyses, cells were grown in BHI to mid-log phase ($OD_{600} \sim 0.5$) and subjected to heat-shock at 42, 45, 48 or 50 °C for 10 min, whereas for quantitative real-time reverse-transcripatse PCR (qRT-PCR) experiments cultures were subjected to 48 °C for 30 min.

RNA methods

For each growth condition, cell pellets were collected by centrifugation and immediately treated with the RNA protect reagent (Qiagen). Total RNA was isolated by the hot acid–phenol method as described previously (Abranches et al. 2006). The crude RNA was treated with DNase I (Ambion) and then further purified by using the RNeasy mini kit (Qiagen), including an on-column DNase I digestion (Qiagen). For qRT-PCR analysis, cDNA was generated from three independent RNA samples using the Super Script first-strand synthesis system kit (Invitrogen) and gene specific primers (Table 1). The primers used for qRT-PCR were designed using the Beacon designer 2.0 software (Premier Biosoft International). qRT-PCR was carried out following the a protocol described elsewhere (Ahn et al. 2005). A Student's *t* test was performed to verify significance of the qRT-PCR results. For Northern analysis, 5 μg per lane of RNA was separated on a 1.2 % agarose–formaldehyde denaturing gel and blotted to a nitrocellulose membrane as described (Oliveira et al. 2011). The membrane was probed with a fragment of the *E. faecalis clpC*, *clpE* and *clpP* genes labelled with [α^{32}P] dCTP and the ready-to-go DNA labelling beads system according to the manufacturer's recommendations (Amersham Pharmacia Biotech).

Galleria mellonella infection

For the *G. mellonella* infection model, 5-μl aliquots containing 1×10^6 CFU ml^{-1} of overnight-grown cultures of *E. faecalis* in sterile saline were injected into the hemocoel of each larva via the last left proleg, as described previously (Oliveira et al. 2011). Larvae injected with heat-inactivated bacteria os saline were used as controls. After injection, larvae were kept in the dark at 37 °C, and survival was recorded at selected intervals. Experiments were performed in triplicate. Kaplan–Meier killing curves were plotted and estimations of differences in survival were compared using the log rank test. P values of ≤ 0.05 were considered significant Experiments were performed independently three times with similar results.

Results and discussion

Promoter region and northern blot analysis of *clp* gene transcription

Previously, we conducted in silico, transcriptional and mutational analyses of the *clpB* chaperone from *E. faecalis* (Oliveira et al. 2011). Here, in silico analysis

Table 1 Oligonucleotides used in real-time PCR quantitative

Oligonucleotídeos	Sequência	T*m* (°C)
clpB–D	GAT GCT GGT TTA GAT GTT GAC G	54.0
clpB–R	CGA AGT GAA TCA GCT TCT TGC	54.3
clpC–D	TCA CAA TGG ACA GGC GTT C	55.4
clpC–R	CGA CCC GTT GAT GTA AAA TGG	54.1
clpE–D	GCG GAC AAT TTG AAG AAC GG	54.7
clpE–R	CAT CAC CAG CAG CAC CAG	56.5
clpP–D	TGA AGT GAT TGA ACG TGA TAC C	52.7
clpP–R	AGG CGC TAC TAT TTT CCA TTA C	52.7
clpX–D	AAT GTT GAA CGT GCG GAA AAG G	56.6
clpX–R	CAC CCT CAC CTG AAA CAT CTC G	57.6
16SrDNA- D	CGC TAG TAA TCG TGG ATC AGA ATG	55.2
16SrDNA – R	TGT GAC GGG CGG TGT GTA	59.3

of the remaining *clp* genes present in the *E. faecalis* genome (*clpC*, *clpE*, *clpP* and *clpX*) identified potential binding sites for the CtsR repressor, the so-called CtsR box (Derré et al. 1999), upstream the start codon of *ctsR* (co-transcribed with *clpC*), *clpE* and *clpP* (Fig. 1). However, no CtsR box was found in the upstream region of *clpX*. These findings are in line with observations made with related low-GC Gram-positive bacteria whereby *clpX* is the only *clp* gene that is not under CtsR regulation in *Streptococcus mutans* and *S. pneumoniae* (Chastanet et al. 2001; Lemos and Burne 2002).

Northern blot analysis was performed using specific probes for the *clpC, clpE* and *clpP* genes. Analysis with the specific *clpC* DNA probe revealed a transcript of approximately 3.0 kb, which corresponds in size to a bicistronic message containing the *ctsR* and *clpC* genes (Fig. 2a). The *clpE* transcript (approximately 2.6 kb) was larger than expected (Fig. 2b), suggesting that *clpE* is co-transcribed with a small gene (EF707) encoding for a small hypothetical protein. The detected 0.6 kb *clpP* mRNA was consistent with the expected size of a *clpP* monocistronic mRNA (Fig. 2c). Transcription of *clpE* and *clpP* was strongly induced when the growth temperature was shifted from 37 to 45 °C, with expression levels peaking between 48 and 50 °C. Transcription of *clpC* was induced at a lower temperature, 42 °C, but remained steady at higher temperatures (up to 50 °C). In general, qRT-PCR analysis using RNA isolated from cells grown to mid-log phase at 37 °C and shifted to 48 °C for 15 and 30 min confirmed the heat-inducibility of *clpC*, *clpE* and *clpP* (Fig. 3).

clpC, *clpE* and *clpP* are under CtsR regulation

Previously, an *E. faecalis* strain bearing a complete deletion of the *ctsR* gene (Δ*ctsR*) was isolated and used to demonstrate that CtsR is a repressor of *clpB* (Oliveira et al. 2011). We surveyed the available *E. faecalis* genomes for the presence of additional CtsR binding sites in non-coding regions and could only find conserved CtsR sites upstream of the *clpB*, *ctsR* (co-transcribed with *clpC*), *clpE* and *clpP* genes. As expected, analysis by qRT-PCR of the Δ*ctsR* strain revealed that transcription of *clpC, clpE* and *clpP* at 37 °C was at least twofold higher when compared to the wild-type strain (Fig. 3). When transcription of the *clpC*, *clpE* and *clpP* genes in the Δ*ctsR* strain was compared to the parent strain exposed to the same 48 °C heat stress, we could still observe a twofold increase in mRNA levels of *clpC* and *clpP* and fivefold increase in *clpE* transcription (Fig. 3). These findings suggest that there is a second, yet-to-be-identified, heat shock-responsive regulator controlling the expression of *clp* genes. Given that the *clpX* promoter region does not possess a CtsR-binding box, it was not surprising that transcription of *clpX* was not affected in the Δ*ctsR* grown at 37 °C. Moreover, *clpX* transcription was not induced by heat shock, which indicated that *clpX* is not a member of the heat stress regulon. Unexpectedly, *clpX* transcription was strongly induced in the Δ*ctsR* strain when compared to the parent strain subjected to the same stress, or against the Δ*ctsR* strain grown at 37 °C (Fig. 3d). Thus, while *clpX* transcription was not directly regulated by CtsR, loss of CtsR indirectly affects its expression under heat

Fig. 1 Nucleotide sequences of the promoter regions of the **a** *clp*C, **b** *clp*E, **c** *clp*P, and **d** *clpX* genes from *E. faecalis*. Regulatory sequences underlined, are as follows: one putative CtsR element (consensus sequence GGTCAAANANGGT CAAA) indicated in *bold*, the putative −35 and −10 regions, the initiation codon ATG and ribosome binding site or SD (Shine-Dalgarno)

stress conditions. It is tempting to speculate that this induction may be a compensatory mechanism that allows *clpX* to compete with the other highly expressed ClpC and ClpE proteins for binding with the ClpP peptidase. Future studies to address the underlying mechanisms controlling hyperinduction of *clpC*, *clpE*, *clpP* as well as the induction of *clpX* in the Δ*ctsR* mutant upon heat shock will be necessary for a complete understanding of the regulatory mechanisms controlling *clp* gene expression.

In addition to CtsR, HrcA is another major transcriptional regulator of heat shock proteins (Narberhaus 1999). The HrcA repressor recognizes a highly conserved DNA element known as controlling invereted repeat of chaperone expression (CIRCE) and is mainly involved in the transcriptional repression of the *dnaK* and *groE* heat shock operons. In some cases, these heat shock genes can be under dual regulation by CtsR and HrcA. Specifically, the *dnaK* operon of *S. aureus*, the *clpP* gene from *Streptococcus salivarius* and the *groE* operon from a number of *streptococci* are regulated by both CtsR and HrcA (Chastanet et al. Chastanet and Msadek 2003; Lemos and Burne 2002). Sequence analysis of the promoter regions of *ctsR*–

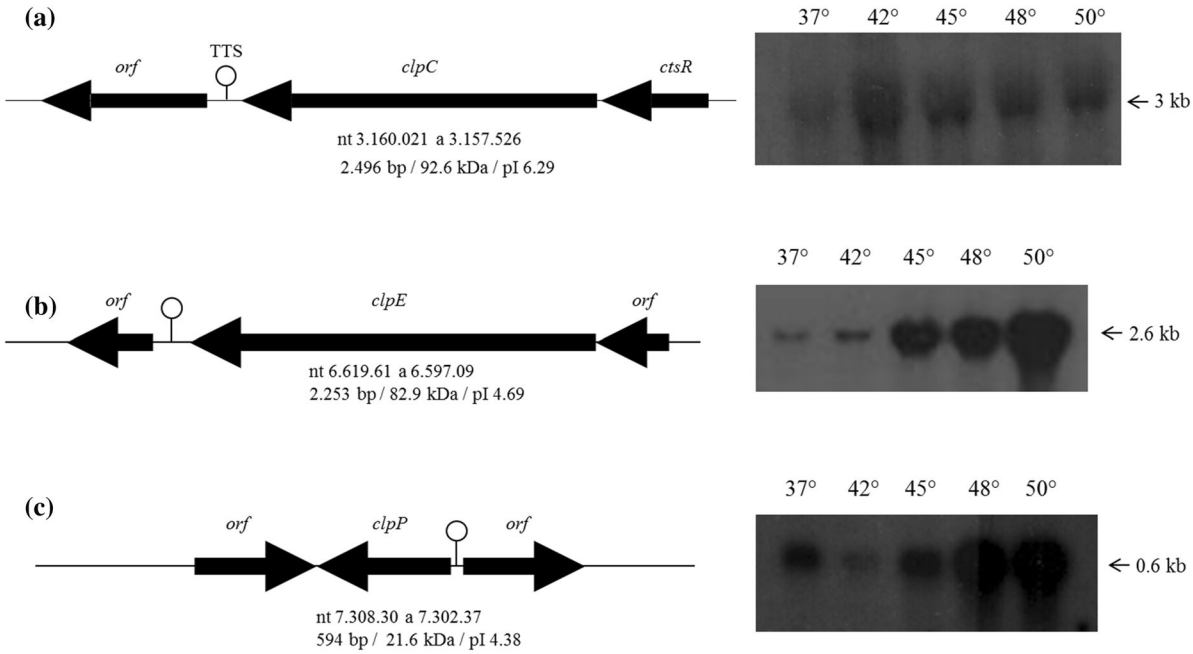

Fig. 2 Analysis of genomic regions and *clp* genes expression with schematic representation of the genomic region of *clp* genes from *E. faecalis* V583. The *arrows* indicate the direction of transcription. The diagram presented is noted in the genome and shows the location and size of genes in the genome of this microrganism (www.tigr.org), molecular weight (kDa) of probable amino acid sequence and isoelectric point (p*I*). Northern blot analysis of *clpC, clpE and clpP* under conditions of thermal stress in *E. faecalis* growth at 37 °C (control) and subjected to heat stress at 42, 45, 48 and 50 °C for 10 min. The signal observed in autoradiograph represents hybridization with a specific probe for the indicated gene. TTS, transcription termination site

clpC, *clpE* and *clpP* failed to identify a CIRCE-like element, which suggests that these genes are not under HrcA regulation. Further in silico analysis using the Multiple Em for Motif Elicitation (MEME) suite also failed to identify conserved *cis*-acting elements within the promoter regions of these genes. Thus, the molecular factor responsible for the heat stress activation of *clpC*, *clpE* and *clpP* in the absence of the CtsR repressor remains to be identified.

Role of CtsR during induction of thermotolerance

Acquired thermotolerance due to prior adaptation at sub-lethal temperatures is a common phenomenon that has been described in *E. faecalis* (Flahaut et al. 1997). Previously, we showed that the ClpB chaperone plays a major role in thermotolerance (Oliveira et al. 2011). To test the involvement of CtsR in thermotolerance, cultures of the wild-type and Δ*ctsR* strains grown at 37 °C were incubated at 45 °C (sub-lethal heat stress) for 30 min and then transferred to 60 °C (lethal heat stress). Alternatively, control cultures were transferred from 37 to 60 °C. There were no significant differences in the numbers of survivors between wild-type and Δ*ctsR* strains in cultures subjected to the 60 °C lethal stress (data not shown). Pre-incubation at 45 °C conferred nearly full protection towards a lethal treatment at 60 °C to both wild-type and Δ*ctsR* strains (data not shown). These results indicate that CtsR repression is not required for heat stress survival or thermotolerance acquisition in *E. faecalis*.

Virulence of the Δ*cts*R is attenuated in the *G. mellonella* invertebrate model

By using a *G. mellonella* systemic infection model, we found that virulence of the Δ*ctsR* is significantly attenuated when compared to the parent strain ($p \leq 0.001$) (Fig. 4). After 30 h of infection, approximately 65 % of larvae infected with the parent strain were dead compared to 25 % death in larvae infected with the Δ*ctsR*. Larvae used as controls were

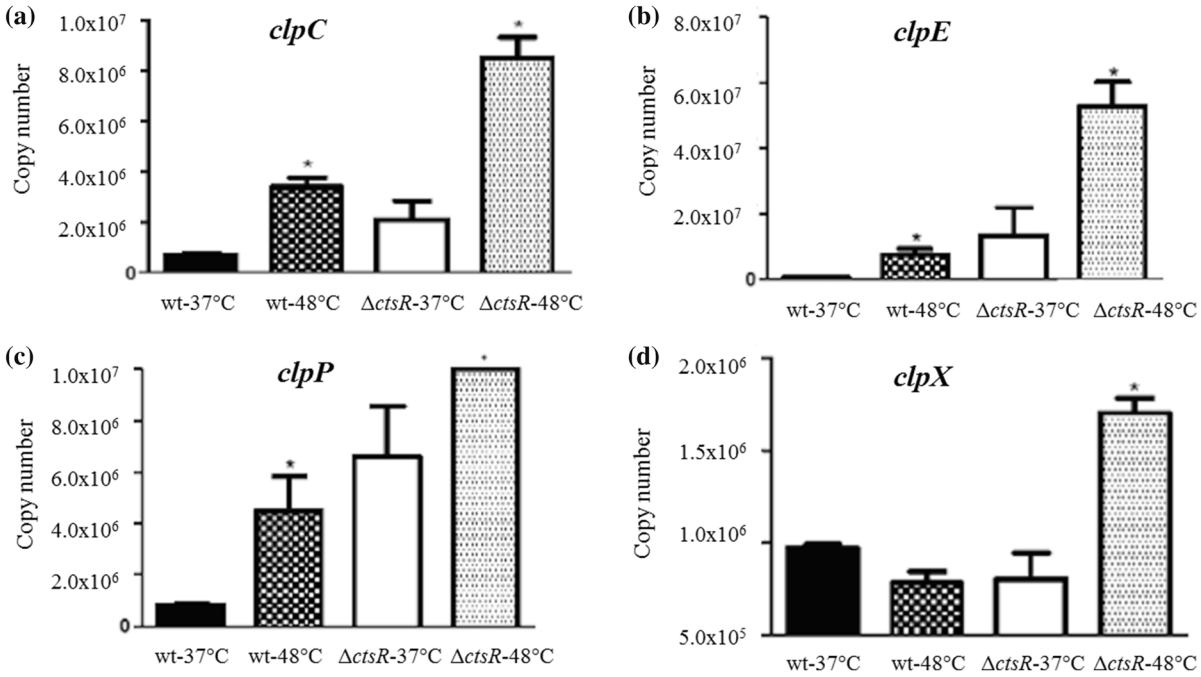

Fig. 3 qRT-PCR analysis of the *clpC, clpE, clpP* and *clpX* genes.. Analysis expression of mRNAs from *clp*C (**a**), *clp*E (**b**), *clp*P (**c**) and *clp*X (**d**) genes in wild type cells (wt) from *E. faecalis* OG1RF (*first two bars—black* and *chess*), or Δ*ctsR* strain (*last two bars—white* and *dotted*) subjected to heat shock at 48 °C for 30 min (*chess* and *dotted bars*). As a control, cells were used at 37 °C (*black* and *white bars*). (*$p \leq 0.05$ student's *t* test) compared to the control at 37 °C

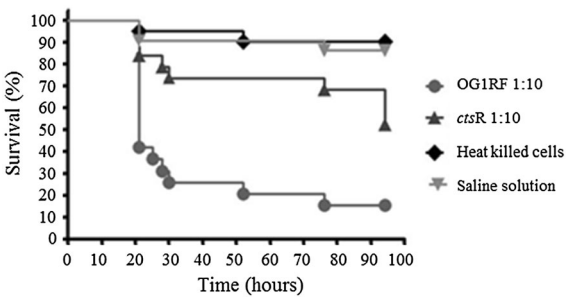

Fig. 4 Attenuated virulence of the Δ*ctsR* strain in *G. mellonella*. Larvae survival after infection with OG1RF wild type cells and Δ*ctsR* strains. Negative control: Larvae were infected with saline solution, or with heat-killed OG1RF cells (75 °C for 20 min)

inoculated with saline solution or heat-killed cells and showed approximately 95 % survival after five days of infection. In this in vivo experiment we observed that mutant cells Δ*ctsR* showed a significantl lower virulence when compared to wild type cells of *E. faecalis* OG1RF suggesting that CtsR mediates virulence of *E. faecalis*. This result is in agreement with, to our knowledge, the only other studies that have evaluated the virulence potential of a *ctsR* mutant. More specifically, a strain of *Listeria monocytogenes* harboring a single amino acid substitution at a conserved residue that rendered CtsR inactive showed attenuated virulence in a murine peritoneal infection model (Karatzas et al. 2003). Conversely, another group showed that their *L. monocytogenes* Δ*ctsR* strain was as virulent as the parent strain but, virulence of a wild-type strain constitutively expressing CtsR was significantly attenuated, presumably because of repression of the *clp* genes (Nair et al. 2000). It has been shown that stability and proper folding of CtsR is mediated by the ClpC, ClpE, ClpL ATPases (Derré et al. 2000; Miethke et al. 2006; Varmanen et al. 2003; Tao and Biswas 2013). In addition to degradation of irreversibly damaged proteins, ClpP proteases perform essential roles by controlling the cellular levels of other transcriptional regulators such as the oxidative stress Spx and the DNA damage HdiR regulators (Frees et al. 2007). Thus, it appear that any imbalance in the expression levels of the CtsR and Clp proteins can have profound effects on the pathophysiology of *E. faecalis* and related organisms.

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