

clpB, a class III heat-shock gene regulated by CtsR, is involved in thermotolerance and virulence of *Enterococcus faecalis*

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Here, we transcriptionally and phenotypically characterized the *clpB* gene from *Enterococcus faecalis*. Northern blot analysis identified a monocistronic mRNA strongly induced at 48 and 50 °C. *In silico* analysis identified that the *clpB* gene encodes a protein of 868 aa with a predicted molecular mass of approximately 98 kDa, presenting two conserved ATP-binding domains. Sequence analysis also identified a CtsR-binding box upstream of the putative –10 sequence, and inactivation of the *ctsR* gene resulted in an approximately 2-log increase in *clpB* mRNA expression, confirming ClpB as a member of the CtsR regulon. While expression of *clpB* was induced by heat stress, a $\Delta clpB$ strain grew relatively well under many different stressful conditions, including elevated temperatures. However, expression of ClpB appears to play a major role in induced thermotolerance and in pathogenesis, as assessed by using the *Galleria mellonella* virulence model.

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INTRODUCTION

Enterococcus faecalis inhabits the gastrointestinal tract of humans and animals and can also colonize the oral cavity, genito-urinary tract and skin. This bacterium is also an important opportunistic pathogen, particularly as an aetiological agent of nosocomial infections accounting for approximately 12% of the hospital-acquired infections in the USA (Murray, 1990; Fisher & Phillips, 2009). *E. faecalis* can cause a variety of diseases including bacteraemia, endocarditis, intra-abdominal, urinary tract and soft tissue infections (Murray, 1990). The strong association of this organism with infections in hospital settings has been attributed to its inherent capacity to withstand harsh environmental conditions, and to its innate and acquired resistance to multiple antibiotics. The ability of *E. faecalis* to survive under adverse conditions is particularly important, and many of its stress-resistant characteristics have been used to distinguish enterococci from other Gram-positive cocci (Murray, 1990). For example, *E.*

faecalis can grow at temperatures ranging from 5 to 50 °C and in a wide pH range (4.6–9.9), and can tolerate and grow in the presence of 6.5% NaCl or 40% bile salts (Murray, 1990).

The stress responses of *E. faecalis* have been the subject of extensive research. It has been demonstrated that pre-exposure to sublethal stresses, oligotrophic conditions or glucose starvation leads to an overall increase in stress resistance (Flahaut *et al.*, 1996a, b, 1997; Giard *et al.*, 1997; Hartke *et al.*, 1998). A correlation has been made between the capacity to adapt to stress and the cross-protection phenomenon with the increased synthesis of many proteins, but, with a few exceptions such as the general stress protein Gls24 and the molecular chaperones GroEL and DnaK, the identity of the majority of these proteins remains unknown (Flahaut *et al.*, 1996a, b, 1997; Hartke *et al.*, 1998; Giard *et al.*, 2000; Laport *et al.*, 2004, 2006). In many bacteria, Clp ATPases and ClpP proteolytic complexes are protein machines that play crucial roles in promoting folding, assembly or degradation of proteins during normal growth and, in particular, under stress-inducing conditions (Houry, 2001; Frees *et al.*, 2004). Two

Abbreviation: qRT-PCR, quantitative real-time reverse-transcriptase PCR.

classes of Clp ATPases have been identified based on the presence of one or two ATP-binding domains and the occurrence of specific signature sequences (Wawrzynow *et al.*, 1996). Class 1 consists of relatively large proteins, such as ClpA, ClpB, ClpC, ClpE and ClpL, with two distinct ATP-binding domains, whereas class 2 proteins, such as ClpX and ClpY, display a single ATP-binding domain (Schirmer *et al.*, 1996). In low-GC Gram-positive bacteria, transcription of *clpP* and selected *clp* ATPase genes is negatively regulated by CtsR (for class three stress repressor), which recognizes a repeated heptanucleotide (A/GGTCAAANANA/GGTCAA) that usually overlaps with the transcription initiation site or the -35 and -10 sequences of the promoter of the corresponding gene (Derré *et al.*, 1999). In addition to repression by CtsR, additional mechanisms involved in control of *clp* expression have been identified in low-GC Gram-positive bacteria, including dual repression of *clpP* by CtsR and HrcA in *Streptococcus salivarius* (Chastanet & Msadek, 2003).

Given the variety of environmental stresses that *E. faecalis* withstands, it is likely that the Clp proteins may play a role in stress survival and virulence. The genome of *E. faecalis* encodes a homologue of the gene encoding the ClpP peptidase, three orthologues of class 1 Clp ATPases (ClpB, ClpC and ClpE), and one class 2 Clp ATPase (ClpX) (Paulsen *et al.*, 2003). Among the class 1 Clp ATPases found in *E. faecalis*, ClpB is distinguished from the other large Clp ATPases by a relatively longer spacer region between the two ATP-binding domains (Houry, 2001), and by the absence of the ClpP recognition tripeptide. In *Escherichia coli*, ClpB has been shown to act synergistically with the DnaK system to remodel or dissolve protein aggregates that arise from heat stress (Doyle *et al.*, 2007), but it is not known whether ClpB–DnaK interactions also occur in Gram-positive bacteria. In both Gram-positive and Gram-negative bacteria, inactivation of *clpB* normally results in reduced thermotolerance (Chastanet *et al.*, 2004; Frees *et al.*, 2004; Yuan *et al.*, 2007). ClpB has also been associated with multiplication or survival of *Staphylococcus aureus* and *Porphyromonas gingivalis* within mammalian

cells (Frees *et al.*, 2004; Capestany *et al.*, 2008). Moreover, animal models have been used to demonstrate that ClpB is important for virulence of bacterial pathogens, including *Listeria monocytogenes* and *P. gingivalis* (Chastanet *et al.*, 2004; Yuan *et al.*, 2007).

In this study, we report the genetic and physiological characterization of the *clpB* gene in *E. faecalis*. Transcriptional analysis revealed that *clpB* expression is induced by a variety of stress conditions and negatively regulated by CtsR. Although inactivation of *clpB* had no major impact on the ability of *E. faecalis* to grow under different stress conditions, induced thermotolerance and virulence in the *Galleria mellonella* insect model were significantly reduced in the absence of ClpB.

METHODS

Bacterial strains and general culture conditions. Bacterial strains used in this study are listed in Table 1. *Escherichia coli* strains were routinely grown in Luria–Bertani medium and used for plasmid construction and propagation. *E. faecalis* strains and its derivatives were grown in brain heart infusion (BHI) medium at 37 °C. When required for selective growth of strains, erythromycin (10 µg ml⁻¹ for *E. faecalis*, 300 µg ml⁻¹ for *Escherichia coli*), spectinomycin (1000 µg ml⁻¹), rifampicin (200 µg ml⁻¹) or fusidic acid (25 µg ml⁻¹) was added to the growth medium.

Construction of *E. faecalis* Δ*clpB* or Δ*ctsR* strains. A markerless genetic exchange system was used for construction of the *clpB* and *ctsR* deletion mutants in *E. faecalis* OG1RF (Kristich *et al.*, 2007). To create each strain, two PCR fragments (each approximately 1 kb in size) flanking the target gene were obtained with the primers listed in Table 2. After digestion with the appropriate restriction enzymes, the two PCR products flanking the gene of interest were simultaneously cloned into pGEM7 (Promega) to give plasmids pG*clpB* and pG*ctsR* in *Escherichia coli* DH10B. The 2 kb fragments containing the *clpB* (pG*clpB*) and *ctsR* (pG*ctsR*) up–down fragments were subcloned into pCJK47 using *Escherichia coli* EC1000 as the host strain. The resulting plasmids, pCJK-*clpB* and pCJK-*ctsR*, were electroporated into competent *E. faecalis* CK111 (donor strain). The *E. faecalis* CK111 strains containing the pCJK-*clpB* and pCJK-*ctsR* plasmids were conjugated with wild-type *E. faecalis* OG1RF and transformants were selected on BHI agar medium containing rifampicin and erythromycin. Single colonies were subjected to the PheS* negative counter-selection

Table 1. Bacterial strains used in this study

Fus, Fusidic acid; Rif, rifampicin; Sp, spectinomycin.

Strains	Relevant characteristic	Source
<i>E. faecalis</i>		
OG1RF	Laboratory strain; Rif ^r Fus ^r	Lab stock
CK111	OG1Sp <i>upp4::P₂₃repA4</i>	Kristich <i>et al.</i> (2007)
EF- <i>clpB</i>	<i>clpB</i> deletion mutant of OG1RF, Δ <i>clpB</i>	This study
EF- <i>ctsR</i>	<i>ctsR</i> deletion mutant of OG1RF, Δ <i>ctsR</i>	This study
cΔ <i>clpB</i>	EF- <i>clpB</i> harbouring pTG55 (pMSP3535 expressing <i>clpB</i>)	This study
<i>Escherichia coli</i>		
DH10B	Cloning host	Grant <i>et al.</i> (1990)
EC1000	Host for cloning RepA-dependent plasmids	Leenhouts <i>et al.</i> (1996)

Table 2. Oligonucleotide primers and probes used in this study

The underlined bases correspond to restriction sites included to aid in the subsequent cloning of the PCR products.

Primer name	Sequence (5'–3')	Application
FW <i>clpB</i> UP	GGAGGATGG AAT <u>TCTCGCTTTTCT</u>	<i>clpB</i> deletion
RV <i>clpB</i> UP	TGAGCTT <u>CACCCGGGGCCTCTTGA</u>	<i>clpB</i> deletion
FW <i>clpB</i> DOWN	GAAGGTGT <u>GCCCGGGAAGGAAC</u>	<i>clpB</i> deletion
RV <i>clpB</i> DOWN	AACCCTTGGGCATGCGACAGACAA	<i>clpB</i> deletion
FW <i>ctsR</i> UP	CTTTGCAAAA <u>GGATCCGCG</u>	<i>ctsR</i> deletion
RV <i>ctsR</i> UP	AGCCTCAAGAAT <u>TCTGACGTATT</u>	<i>ctsR</i> deletion
FW <i>ctsR</i> DOWN	GGAAGGCAAGAAT <u>TCGCTAGCTG</u>	<i>ctsR</i> deletion
RV <i>ctsR</i> DOWN	CCA <u>ACTACTGGATCCAAAC</u>	<i>ctsR</i> deletion
<i>clpB</i> forw 3	CAA <u>AGGATCCAAGAGTTGGTCAAAC</u>	$\Delta clpB$ complementation
<i>clpB</i> rev 3	GAAT <u>TCCGTCTCGAGTTACACTTC</u>	$\Delta clpB$ complementation
<i>clpB</i> forw 2	GATGCTGGTTTAGATGTTGACG	qRT-PCR
<i>clpB</i> rev 2	CGAAGTGAATCAGCTTCTTGC	qRT-PCR
16S RNA-F	CGCTAGTAATCGTGGATCAGAATG	qRT-PCR
16S RNA-R	TGTGACGGGCGGTGTGTA	qRT-PCR
<i>clpB</i> forw 1	ATCATTGGTCTGTGACGAA	<i>clpB</i> probe
<i>clpB</i> rev 1	ATTCACTCATGTCAATCC	<i>clpB</i> probe

system to isolate double crossover deletions as described elsewhere (Kristich *et al.*, 2007). The *clpB* and *ctsR* gene deletions were confirmed by PCR sequencing of the insertion site and flanking sequences.

Construction of complemented *clpB* strain. To express the *clpB* gene *in trans*, the full-length *clpB* gene including the ribosome-binding site was amplified by PCR with primers containing *Bam*HI restriction sites (Table 2) and ligated into pMSP3535 (Bryan *et al.*, 2000), which had been digested with *Bam*HI and *Xho*I. The ligation mixture containing the pMSP3535 expressing *clpB* was electroporated onto CK111 and subsequently conjugated with *E. faecalis* $\Delta clpB$. The resulting plasmid (pTG55) contains the intact *clpB* gene under the control of the lactococcal nisin promoter. Expression of *clpB* from pTG55 was induced by adding 5 ng nisin ml⁻¹ to exponentially grown cultures.

Stress challenges. To investigate the role of the *E. faecalis* ClpB in stress responses, cells of the wild-type OG1RF and its isogenic *clpB* mutant were subjected to a variety of stress challenges. For Northern blot analysis, cells were grown in BHI to mid-exponential phase (OD₆₀₀~0.5) and subjected to heat shock at 42, 45, 48 or 50 °C. To evaluate the capacity of *E. faecalis* strains to grow under stress conditions (5% NaCl, pH 5, pH 9 and 2 mM H₂O₂), mid-exponential phase cultures grown in BHI were diluted 1:100 into fresh BHI medium adjusted to each specific condition and growth was monitored using a Bioscreen C growth monitor (Oy Growth Curves Ab). Disc inhibition assays were used to assess the sensitivity of the strains to H₂O₂, HCl or NaOCl. Briefly, a uniform layer of exponentially grown cells was spread onto BHI agar plates using a sterile swab, and filter paper discs (1 mm diameter) saturated with each solution (5% H₂O₂, 3 M HCl or 10% NaOCl) were placed onto the agar. All plates were incubated at 37 °C for 48 h after which the halo of inhibition was recorded. To test the involvement of ClpB in induced thermotolerance, cultures were grown in BHI or BHI containing erythromycin for the complemented mutant. Briefly, cultures were grown exponentially at 37 °C to an OD₆₀₀~0.4 and split in two aliquots; one aliquot remained at 37 °C while the other was incubated at the sublethal temperature of 50 °C. After 30 min incubation at 37 or 50 °C, both cultures were incubated at a lethal temperature of 60 °C for 30 and 60 min. ClpB expression in the complemented $\Delta clpB$ strain ($\Delta clpB$) was induced by adding nisin

(5 ng ml⁻¹) to exponentially grown cultures (OD₆₀₀~0.1). Cell survival was evaluated by plating serially diluted aliquots on BHI plates that were incubated at 37 °C for 48 h prior to counting c.f.u. The capacity of the $\Delta clpB$ mutant to tolerate oxidative stress was further investigated in an H₂O₂ killing assay. Briefly, exponentially grown cultures were incubated at 37 °C in the presence of 30 mM H₂O₂. Every 30 min for up to 2 h, aliquots were taken and serially diluted, plated on BHI plates and incubated at 37 °C for 48 h before colonies were counted.

Biofilm assay. Biofilm formation on polystyrene microtitre plates was quantified essentially as described previously (Loo *et al.*, 2000). Briefly, biofilms were grown in a semi-defined biofilm medium (BM) containing glucose as the carbohydrate source for 24 h at 37 °C before adherent bacteria were stained with 0.1% crystal violet. The bound dye was extracted from the stained cells with 33% acetic acid solution, and the biofilms were quantified by measuring OD₅₇₅.

Metabolic labelling of proteins and Western blotting. Overnight cultures were diluted in BHI to 10⁷ c.f.u. ml⁻¹ and incubated for 30 min at 37 °C. For continuous labelling at different temperatures, cells were concentrated by centrifugation to 10⁸ c.f.u. ml⁻¹ in a methionine-free essential medium (MEM; Difco) containing 200 μ Ci ml⁻¹ (7.4 MBq ml⁻¹) [³⁵S]methionine. Control cells were labelled at 37 °C, whereas heat-shocked cells were labelled at 42, 45, 48, 50 or 52 °C. After 30 min incubation at the different temperatures, protein lysates from each set of samples obtained by lysozyme treatment were separated by 7.5% SDS-PAGE. After separation, the gel was stained with Coomassie blue, destained, dried and exposed to an X-ray film for detection of radiolabelled proteins. Alternatively, a duplicate gel was transferred to a nitrocellulose membrane for Western blot analysis using a polyclonal antibody raised against the *Synechococcus* spp. ClpB (a generous gift from Dr Adrian K. Clarke) (Eriksson & Clarke, 1996). Immune reactivity was visualized using the Western blotting luminol reagent according to the manufacturer's recommendations (Santa Cruz Biotechnology).

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 Northern analysis, RNA was separated on a 1.2% agarose-formaldehyde denaturing gel and blotted to a nitrocellulose membrane as described elsewhere (Sambrook & Russell, 2001). The membrane was probed with a fragment of the *E. faecalis* *clpB* gene labelled with [α - 32 P]dCTP and the ready-to-go DNA labelling beads system according to the manufacturer's recommendations (Amersham Pharmacia Biotech). To confirm the integrity and the amount of RNA analysed in the Northern blot, a replica gel was stained with ethidium bromide. For quantitative real-time reverse-transcriptase PCR (qRT-PCR) analysis, cDNA was generated from three independent RNA samples using the SuperScript first-strand synthesis system kit (Invitrogen) and gene-specific primers. The primers used for qRT-PCR were designed using the Beacon designer 2.0 software (Premier Biosoft International). qRT-PCR was carried out by following the protocols described elsewhere (Ahn *et al.*, 2005). A Student's *t* test was performed to verify significance of the qRT-PCR results.

Galleria mellonella infection. For the *G. mellonella* killing assays, insects in the final larval stage were purchased from Vanderhorst Inc., stored at 4 °C in the dark and used within 5 days of shipment. Groups of 20 larvae, ranging from 200 to 300 mg in weight and with no signs of melanization, were randomly chosen and used for subsequent infection. A 10 μ l Hamilton syringe was used to inject 5 μ l aliquots of bacterial inoculum (5×10^5 c.f.u. ml $^{-1}$) into the haemocoel of each larva via the last left proleg. Bacterial colony counts on BHI plates were used to confirm the initial inoculum. Groups injected with saline solution or heat-inactivated (20 min at 75 °C) cells were used as controls in each experiment. After injection, larvae were incubated at 37 °C, and survival was recorded at selected intervals. Larvae were scored as dead when they displayed no movement in response to touch. Kaplan–Meier killing curves were plotted and estimation of differences in survival were compared by using the log-rank test. A *P*-value ≤ 0.001 was considered significant. All data were analysed with GraphPad Prism 4.0 software. Experiments were performed independently three times with similar results.

RESULTS AND DISCUSSION

In silico analysis of *E. faecalis* *clpB*

Analysis of the *E. faecalis* V583 genome sequence (Paulsen *et al.*, 2003) identified the gene encoding the ClpB ATPase. The *clpB* gene encodes a protein of 868 aa with a predicted molecular mass of approximately 98 kDa and an estimated pI of 4.78. The protein presents two conserved nucleotide-binding domains (NBD) separated by a relatively long spacer (154 aa). The NBDs present the consensus sequences Walker-A and Walker-B (Fig. 1a). Analysis of the amino acid sequence confirmed the presence of the N-terminal region and the two C-terminal signature sequences characteristic of ClpB (Fig. 1b). The predicted start codon is preceded by a putative σ^A -type promoter (TTGACC-N₁₇-TATAAT) (Fig. 2), and a putative transcription terminator [$\Delta G = -45.3$ kcal mol $^{-1}$ (189.5 kJ mol $^{-1}$)] was identified downstream of the predicted stop codon. In addition, a CtsR-binding sequence (GGTCAAA-N₃-GGTCAAT) was identified starting 30 bp upstream of the -10 sequence. A putative ribosome-binding site (GGGAGG) was found

8 bp upstream of the ATG start codon. Of note, sequence analysis identified a second initiation codon (GTG) and a putative ribosome-binding site (AGGAGG) downstream of the ATG start codon (Fig. 2). This potential internal translation initiation site suggests that a smaller form of ClpB, with a theoretical molecular mass of approximately 80 kDa, may be produced. Of note, sequence analysis of the *clpB* gene from several other *E. faecalis* strains with complete genomes available at the Broad Institute (http://www.broadinstitute.org/annotation/genome/enterococcus_faecalis/MultiHome.html) indicated that the second putative translational initiation region inside *clpB* is fully conserved in other *E. faecalis* strains.

Induction of *clpB* mRNA and ClpB in response to heat shock

To determine whether ClpB expression is modulated by heat stress, Northern blot, 35 S-pulse protein labelling and Western blot analyses were performed. Northern analysis revealed a single stress-inducible transcript of 2.6 kb, compatible with a *clpB* monocistronic transcript (Fig. 3a). No induction was observed when the temperature was shifted from 37 to up to 45 °C. However, at 48 and 50 °C, a significant increase in induction of 10- and 35-fold, respectively, was observed (Fig. 3a). The result of the autoradiogram of 35 S-labelled cells and Western blotting further confirmed ClpB as a heat-shock protein. Autoradiograms of polypeptides labelled at temperatures ranging from 37 to 55 °C revealed two heat-inducible bands of approximately 95 and 80 kDa (Fig. 3b), as well as the induction of the molecular chaperones DnaK and GroEL that have been identified previously (Laport *et al.*, 2001). All heat-inducible bands reached a maximum induction at temperatures between 48 and 50 °C. Western blotting using a polyclonal antibody against ClpB identified two bands of approximately 95 and 80 kDa, suggesting that ClpB may be present in two isoforms (Fig. 3c). The predicted molecular mass of ClpB is 98 kDa, whereas *in silico* analysis suggested the presence of a putative internal translational initiation site that could potentially yield a smaller 80 kDa protein. Although we cannot rule out that the smaller band is a result of enzymic cleavage of the 98 kDa ClpB, it is tempting to speculate that the 80 kDa protein is a product from a second and internal translational initiation site. Of note, similar observations have been reported in *Escherichia coli* and in the unicellular cyanobacterium *Synechococcus* sp., in which *clpB* was shown to contain two initiation sites for translation, resulting in the synthesis of ClpB polypeptides of ~95 and ~80 kDa (Park *et al.*, 1993; Clarke & Eriksson, 2000). Interestingly, in the cyanobacterium *Synechococcus* sp., the smaller ClpB displayed the same capacity as the larger ClpB for the acquisition of thermotolerance (Eriksson & Clarke, 2000). Moreover, a potential internal translation initiation site preceded by a typical ribosome binding site has also been identified in *L. monocytogenes* (Chastanet *et al.*, 2004), suggesting that the existence of

(a)

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MNI EKMTTTLQEAIAEAQKVAVTRQHQEIDIAHLWKIFLQPNHFGRNFYTDAGLDVDAFE 60
  KFT----ALA-A--LA----H-H-----PHHLA-ALH  N-Terminal
REV DNALDEYPSVAGGNVQYQNLNSQNLFHLLQEADSLREEFQDEFSLSTEIVLLALMKLK 120
PIIGRDEEIRDVIRILSRKTKNNPVLI GEPGVGKTAIVEGLAQRIVRKDV PENLKDKTIF 180
  ATP-1 WalkerA  G---GKT
PIIGRDEEIRDVIRILSRKTKNNPVLI GEPGVGKTAIVEGLAQRIVRKDV PENLKDKTIF 240
SLDMGALIAGAKFRGEFEERLKA VLKEVKKSDGKIILFIDEIHNIVGAGKTEGSM DAGNL 300
LKPMLARGELHLIGATTLDEYRQYMEKDKALERRFQKVLVKEPTVEDTISILRGLKERFE 360
  WalkerB1  K----
EIHGVMNIHDNALVAAATLSDRYITDRFLPKAIDLVD EASATIRVEMNSMPTELDQVTR 420
--HHH---HD
LMQLEIEEAALKKESDDASKRRLANLQEEELADL REEANSMKMQWETEKEEVNAVSNKRAE 480
  WalkerB2  R-----AIDLHD
IDKAKHELEDAENNYDLERA AVL RHGTIPQLEHELKELEEKNAKDNV KMVQESVTENEIA 540
Middle II  R-GDLAR-SELQYG-IP
QVVGRLTGIPVTKLVEGEREKLMLKNETLHKRVIGQDEAVDAVSDAVIRSRAGLQDPNRP 600
  RWTGIPV-KH  Middle III
LGSFFLGPTGVGKTELAKALAE DLFDSEDHMVRIDMSEYMEKHAVSRLVGAPPGYGYE 660
  G---GKT  ATP-2 WalkerA
EGGQLTEAVRRNPYTVLLDEIEKAHPDVFNILLQVLD DGRRLTDSKGRVVD FKNTVLIMT 720
WalkerB  R-----HHHD
SNIGSQLLEGVTPG TIPEEVENQVMN ILKGHFPEFLNRIDDTLF TPLSLDNVKGII 780
  C-Terminal IV  FRPEFLNRLEIIVFL
GKMTAQLAHRLEQQEIVLEITDEAKTWIAENGYEPAY GARPLKRFITRE VETPLAKEIVS 840
  C-Terminal V  GARPLRR
GRVMPKTKVTISLLDNQLVFENEPIEEV 868
  
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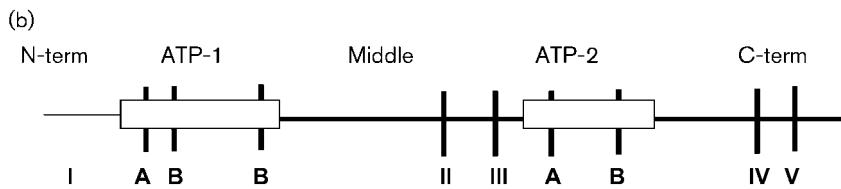


Fig. 1. Structural features of the ClpB proteins of *E. faecalis*. (a) Amino acid sequence deduced from the *E. faecalis clpB* gene indicating the consensus sequences. (b) Consensus motifs of the ClpB proteins. ATP-1 and ATP-2 represent the Walker-type nucleotide-binding sites: Walker-A is a glycine-containing segment and Walker-B represents hydrophobic segment motifs. I, N-terminal; II and III, middle; and IV and V, C-terminal consensus sequences.

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ACAGTGATATTTAGTTGTTTTAGGTTTTTTTTATCAAAAAGGTCAAAAGGTCAATTTA 60
TTCATTGACCTTTACTGACCAATGAGGTATAATAAATCTATAAGGTTTACGAATACCTTA 120
ACTTTTGAAGAAATCCATCAAAAAGTCGGATAATAATAATTGTAAGAATGTTAAAATAAA 180
AAAGACAAAACAATAAACGGGAGGGATCGTTTATGAATATTGAAAAAATGACAACCACGC 240
TACAAGAGGCGATTGCTGAAGCTCAAAAAGTTGCGGTGACACGTC AACATCAAGAAATTG 300
ATATCGCACATTTATGAAAATTTTTCTTCAACCAACCATTTTGGACGTAATTTTACA 360
CAGATGCTGGTTTAGATGTTGACGCATTTGAACGAGAAGTAGATAACGCGTTAGATGAAT 420
ATCCAAGTGTGGCAGGCGGTAATGTGCAATATGGTCAAAATTTAAGTCAAAATTTGTTC 480
ATTTATTGCAAGAAGCTGATTCACCTCGAGAAGAATCCAAGATGAATTTTATCAACCG 540
AAATTGTACTTTTAGCTTTAATGAAATGAAAAATATCGTTTAACAAAATTTTAAATGC 600
ACAAGGCATTACGAAAAAGAGTTAAGAAAAATATTGAAGAGATGAGAGGAGGAGATC 660
GTGTGACTTCTCAAACCAGGAAGAACAATATAAAGCACTAGAAAAATACGGTGTGACT 720
  
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Fig. 2. Nucleotide sequence of the promoter region and the 5' coding region of the *E. faecalis clpB* gene. Regulatory sequences, in bold type and underlined, are as follows: one putative CtsR element (consensus sequence GGTCAANANGGTCAAAA) indicated with an arrow below, the putative -35 and -10 regions, the initiation codon ATG and its ribosome-binding site (RBS), and a second putative initiation codon GTG and its related RBS (boxed).

two different-sized polypeptides may be a common feature of ClpB proteins. Finally, Western blot analysis with the $\Delta clpB$ strain confirmed that the 98 kDa polypeptide was ClpB. The 80 kDa band was still observed in the $\Delta clpB$ strain albeit not as strongly as it was detected in the parent strain (Fig. 3d). Initially, this finding indicates that the

80 kDa band is not the predicted ClpB internal product but another heat-inducible protein that cross-reacts with the ClpB antibody. However, given the precedents from the ClpB literature and our own findings, we believe that ClpB is in fact produced in two different isoforms and that another protein, which co-migrates with the smaller

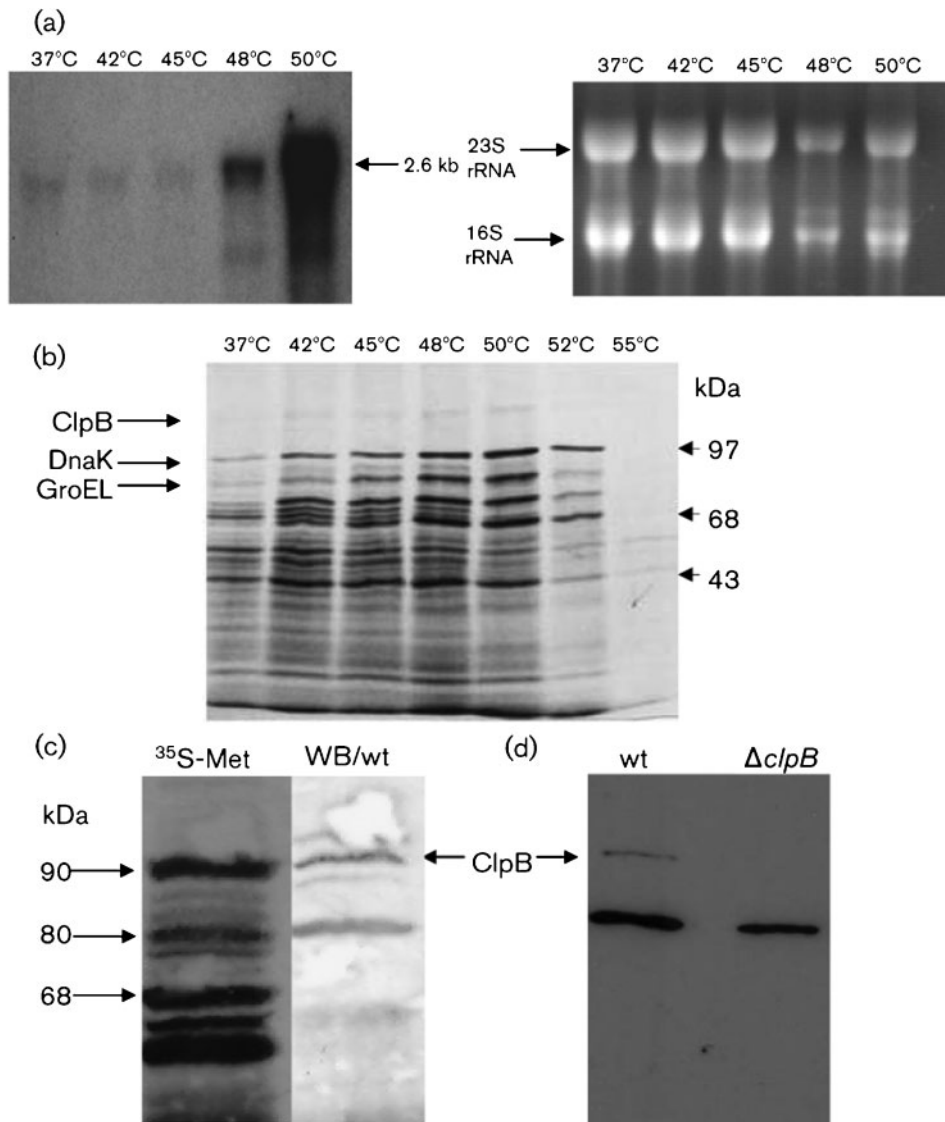


Fig. 3. Induction of *clpB* mRNA and ClpB in response to heat shock. (a) Northern blot analysis of the *E. faecalis clpB* gene. In order to stimulate the production of the heat-shock mRNA, the cells were either cultivated at 37 °C (control cells) or stressed by upshift to 42, 45, 48 or 50 °C for 10 min. Total RNA was separated under denaturing conditions, transferred to a nitrocellulose membrane, and hybridized to a *clpB*-specific probe (left). A parallel gel was stained with ethidium bromide to show equal loading of the slots (right). (b) Influence of supra-optimum temperatures on protein synthesis. Wild-type cells were either incubated at 37 °C (control) or treated at 42, 45, 48, 50, 52 or 55 °C in the presence of [³⁵S]methionine. Labelled proteins were separated by SDS-PAGE and analysed by autoradiography. The ClpB protein is indicated. DnaK and GroEL, which were previously identified, are also indicated (Laport *et al.*, 2001). (c) Identification of the ClpB protein. Wild-type (wt) cells were subjected to heat shock at 45 °C in the presence of [³⁵S]methionine. Labelled proteins were separated by SDS-PAGE and analysed by either autoradiogram (³⁵S-Met) or Western blotting using polyclonal antibodies raised against *Synechococcus* spp. ClpB diluted 1 : 1000. (d) Western blotting of wild-type (wt) or $\Delta clpB$ cells subjected to heat shock at 45 °C showing the cross-reaction of the ClpB polyclonal antibody.

ClpB, cross-reacted with the ClpB antibody. Considering that Clp ATPases are relatively conserved, it is possible that the cross-reacting protein is one of the two other large class 1 Clp ATPases, ClpC and ClpE, encoded in the *E. faecalis* genome. Of note, ClpC has a predicted molecular mass of 92 kDa and shares 61 % identity with the *E. faecalis* ClpB, whereas ClpE has a predicted molecular mass of 82.9 kDa and shares 55 % identity with ClpB. Work is underway to identify the 80 kDa heat-inducible protein(s).

clpB is a member of the CtsR regulon

Due to the identification of a CtsR consensus-binding motif in the promoter region of the *clpB* gene, a Δ *ctsR* strain was constructed to investigate the role of CtsR in controlling *clpB* expression. qRT-PCR analysis revealed that, under non-stressful conditions, CtsR is indeed responsible for *clpB* repression since an approximate 2-log increase in *clpB* expression was observed in the Δ *ctsR* strain compared with the wild-type strain (Table 3). Expression of the transcript of the housekeeping gene 16S rDNA was used as an internal standard and no difference was observed between samples (data not shown). Interestingly, induction after exposure to 48 °C was also observed in the Δ *ctsR* strain, suggesting that the *clpB* gene may have an additional layer of regulation. In Gram-positive bacteria, HrcA and CtsR are two major transcriptional repressors that regulate expression of distinct heat-shock genes (Narberhaus, 1999). The HrcA repressor recognizes a DNA element called CIRCE (for controlling inverted repeat of chaperone expression) and is mainly involved in the transcriptional regulation of the *groE* and *dnaK* operons. In some cases, however, heat-shock genes are under dual negative control by the HrcA and CtsR repressors. This is the case for the *dnaK* operon in *Staphylococcus aureus*, *clpP* in *Streptococcus salivarius* and the *groE* operon in several streptococci (Chastanet *et al.*, 2001, 2003; Lemos & Burne, 2002; Chastanet & Msadek, 2003). Sequence analysis of the promoter regions of the *clpB* gene did not indicate the presence of a CIRCE element, indicating that HrcA does not play a role in the regulation of *clpB* in *E. faecalis*.

Growth characteristics, stress tolerance of and biofilm formation by the Δ *clpB* strain

Analysis of the growth rates and doubling time of the Δ *clpB* strain under normal growth conditions (i.e. aerobic conditions at 37 °C) indicated that the mutant grew slightly slower than the parent strain (Table 3; $P \leq 0.05$). The capacity of the Δ *clpB* strain to grow under a variety of stress conditions was also examined. No significant differences in growth between the mutant and parent strains were observed when cells were grown at different temperatures (45 and 48 °C), at pH 5 or in the presence of NaCl or H₂O₂. However, the slower growth of Δ *clpB* at pH 9 compared with its parent was statistically significant

(Table 4; $P \leq 0.05$). We also tested the susceptibility of the mutant strain to HCl, H₂O₂ and NaOCl using disc diffusion assays. Based on the diameter of the zone of inhibition for each compound tested, no significant differences were observed between strains. Finally, the capacity of the mutant to form biofilms on polystyrene microtitre plates was also assessed. Quantitative analysis of biofilms formed by the Δ *clpB* mutant did not differ from biofilms formed by the parent OG1RF strain (data not shown).

ClpB contributes to thermotolerance

While expression of *E. faecalis clpB* was highly induced upon heat shock, the Δ *clpB* strain did not show any inherent growth defect at elevated temperatures. Here, we tested whether ClpB is necessary for the acquisition of thermotolerance in *E. faecalis* (Fig. 4). In the wild-type strain, pre-incubation of the cells at 50 °C for 30 min prior to exposure to a lethal temperature (60 °C) resulted in full protection for up to 60 min (Fig. 4). In contrast with the wild-type strain, pre-incubation of Δ *clpB* at 50 °C resulted in only an intermediate level of protection against lethal 60 °C treatment. The introduction of pTG55 (*clpB* plasmid) partially restored thermotolerance in Δ *clpB* (*c* Δ *clpB* strain). Notably, after 60 min at 60 °C, the cells grown in the presence of nisin (induced) showed an approximately 15-fold increased heat tolerance compared with the Δ *clpB* strain, whereas *c* Δ *clpB* grown in the absence of nisin displayed fivefold increased survival (Fig. 4). This result showed that ClpB plays an important role in the induction of thermotolerance and this fact might contribute to the persistence of *E. faecalis* in different environments. Notably, ClpB has been implicated in the acquisition of thermotolerance in *Escherichia coli* and in other Gram-positive pathogens, such as *L. monocytogenes* and *Staphylococcus aureus* (Thomas & Baneyx, 1998; Chastanet *et al.*, 2004; Frees *et al.*, 2004). In *Escherichia coli*, the ClpB requirement for induction of thermotolerance is

Table 3. qRT-PCR-based expression profiles of *clpB*

Total RNA was isolated from mid-exponential phase cultures that were kept at 37 °C (control) or incubated at 48 °C for 30 min. The results were obtained using qRT-PCR as described in Methods. Data are means \pm SD of three independent experiments performed with RNA isolated from three independent cultures. All samples were assayed in triplicate. ND, Not done.

Strain	Relative <i>clpB</i> mRNA (copies μ l ⁻¹)
OG1RF (37 °C)	$8.53 \times 10^4 \pm 1.33 \times 10^4$
OG1RF (48 °C)	$1.14 \times 10^6 \pm 4.41 \times 10^5$ *
Δ <i>ctsR</i> (37 °C)	$4.37 \times 10^6 \pm 1.99 \times 10^6$
Δ <i>ctsR</i> (48 °C)	$1.00 \times 10^7 \pm$ ND*

*Significant difference compared with the control ($P \leq 0.05$).

Table 4. Growth characteristics of the OG1RF and $\Delta clpB$ strains grown under different conditions

Doubling time data are means \pm SD of three independent experiments.

Strain	Doubling time (min) when grown in/at:						
	pH 7	pH 5	pH 9	5% NaCl	2 mM H ₂ O ₂	45 °C	48 °C
OG1RF	71 \pm 6.1	209.3 \pm 17.4	60.1 \pm 3.6	144.3 \pm 4.7	82.3 \pm 9.6	227 \pm 49	458.3 \pm 58.9
$\Delta clpB$	80.8 \pm 4.6	234.8 \pm 13.1	72.2 \pm 2.4*	150 \pm 5.2	80.7 \pm 2.6	203 \pm 20	334.8 \pm 31.6

*Significant difference compared with OG1RF ($P \leq 0.05$).

related to its ability to act as a molecular chaperone rescuing protein aggregates formed during heat-shock treatment (Thomas & Baneyx, 1998; Doyle *et al.*, 2007). This aggregation-reversing activity of ClpB requires cooperation with the DnaK chaperone machinery (Doyle *et al.*, 2007) but evidence of ClpB–DnaK interactions in Gram-positive bacteria are yet to be demonstrated.

Virulence of the $\Delta clpB$ strain is attenuated in the *G. mellonella* model

Killing of the larvae of *G. mellonella* has been routinely used as a virulence model for several pathogenic bacteria, including *E. faecalis* (Park *et al.*, 2007; Lebreton *et al.*, 2009). Therefore, *G. mellonella* was tested as a model to verify whether *E. faecalis* ClpB is involved in virulence. As shown in Fig. 5, the rates of killing were significantly lower in larvae infected with $\Delta clpB$ than in those infected with the wild-type strain ($P \leq 0.001$). To confirm that killing was due to an infectious process, we also assessed the survival of larvae injected with heat-killed *E. faecalis* or saline with minimal killing observed within the two groups during the course of the experiment. Of note, the ClpB protein has

been implicated in the expression of virulence by other Gram-positive pathogens. In *Staphylococcus aureus* and *P. gingivalis*, ClpB was associated with intracellular survival and multiplication (Frees *et al.*, 2004; Capestany *et al.*, 2008), and virulence of *L. monocytogenes* and *P. gingivalis* lacking *clpB* was significantly attenuated in murine models of infection (Chastanet *et al.*, 2004; Yuan *et al.*, 2007).

In *G. mellonella*, the production of superoxide and other reactive oxygen species (ROS) via a respiratory burst by the insect specialized phagocytic cells, known as haemocytes, is an important line of defence against microbial infections (Bergin *et al.*, 2005). Thus, we hypothesized that the reduced virulence of the $\Delta clpB$ strain in *G. mellonella* could be due to increased sensitivity to ROS. To verify this possibility, we tested the ability of the wild-type and mutant strains to survive a lethal treatment with H₂O₂. Our results indicated that both strains exhibited similar sensitivity to H₂O₂ killing (data not shown), suggesting

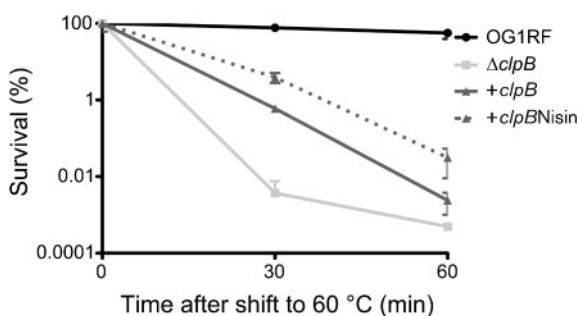


Fig. 4. ClpB is important for development of thermotolerance. Cultures of wild-type OG1RF, $\Delta clpB$, the complemented $c\Delta clpB$ and nisin-induced $c\Delta clpB$ strains were grown exponentially at 37 °C to OD₆₀₀ ~0.4. At this point, cultures were pre-incubated at 50 °C for 30 min prior to exposure to 60 °C (a lethal temperature) for 30 and 60 min. Cell survival was evaluated by plating diluted samples on BHI plates. The result presented is a representative of five independent experiments; error bars, SD.

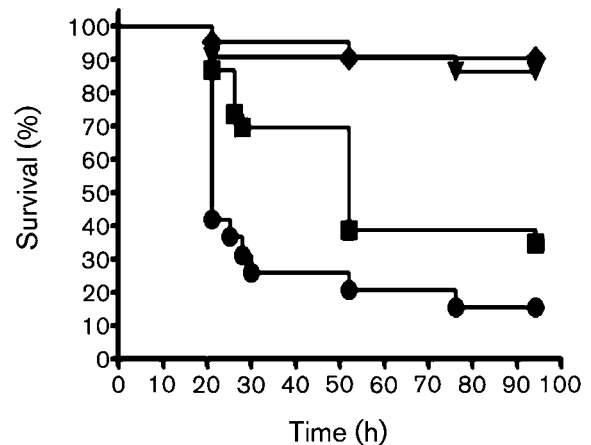


Fig. 5. Killing of *G. mellonella* larvae infected with *E. faecalis* OG1RF and $\Delta clpB$ at 37 °C. Survival (Kaplan–Meyer) plots of larvae injected with saline solution (▼) or heat-killed OG1RF (◆) were used as controls with minimum killing. The experiments were repeated three times, and the results are representative of a typical experiment. Compared with the wild-type OG1RF strain, $\Delta clpB$ showed attenuated virulence ($P \leq 0.001$).

that other host-derived factors may be responsible for the attenuated virulence of $\Delta clpB$ in this particular model.

In conclusion, we report the identification and characterization of the ClpB ATPase. We demonstrate that ClpB is a member of the CtsR regulon and, even though it is induced by heat stress, it is not required for growth at high temperatures. However, expression of ClpB appears to play a major role in induced thermotolerance and in pathogenesis, as assessed using the *G. mellonella* virulence model. Future efforts will be devoted to the identification of proteins that interact with ClpB, which will help to disclose the underlying mechanisms by which ClpB affects virulence traits in *E. faecalis*.

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