clpP of Streptococcus salivarius Is a Novel Member of the Dually Regulated Class of Stress Response Genes in Gram-Positive Bacteria

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Streptococcus salivarius is a widespread human commensal colonizing the oral cavity and upper respiratory tract. A member of the Streptococcus viridans group, this gram-positive bacterium is also an opportunistic pathogen responsible for endocarditis and was recently diagnosed as the pathogen in several cases of meningitis (12, 29).

Stress resistance genes play an important role in the virulence of several pathogens. clpP, clpC, and clpE of Listeria monocytogenes were shown to participate in intracellular parasitism (6), cell adhesion (20), and virulence (19); clpX of Staphylococcus aureus (16) and clpE of Streptococcus pneumoniae (24) were identified by signature-tagged mutagenesis; and the synthesis of DnaK and GroESL in S. aureus was shown to be induced during infection of human epithelial cells (25).

Stress-induced proteins are mainly molecular chaperones or proteases acting to refold or degrade misfolded or denatured proteins (9). Among these, the Clp ATP-dependent protease is composed of Clp ATPase subunits, which confer substrate specificity to the proteolytic subunit ClpP. ClpP has been shown to play a central role in stationary-phase adaptive responses of Bacillus subtilis (18), in the degradation of SsrA-tagged proteins in Escherichia coli (8) and B. subtilis (28), in the modulation of virulence gene expression in Yersinia enterocolitica (23), and in the biofilm formation of Pseudomonas fluorescens (22).

In gram-positive bacteria, stress genes have been grouped into four regulatory classes (4, 26). Class I genes, encoding classical chaperones (DnaK, GroES, and GroEL), are controlled by the HrcA repressor, which recognizes the controlling classical chaperones (DnaK, GroES, and GroEL) are controlled by the HrcA repressor, which recognizes the controlling...
expression. In order to test whether repression by CtsR was relieved during heat shock, strains were grown at 48°C in the presence of xylose. Expression levels of clpP-bgaB were found to be fully derepressed in the presence of CtsR during growth at 48°C (Fig. 1B). These results strongly suggest that the S. salivarius clpP gene is subjected to negative regulation by an ortholog of CtsR and that this repression no longer occurs during growth at high temperature, leading to heat shock induction of clpP.

Purified CtsR of B. subtilis binds specifically to the heptad direct repeat. An in vitro approach was used to confirm the direct interaction of CtsR with the target site of S. salivarius. For this purpose, the CtsR protein of B. subtilis was overproduced in E. coli with a carboxy-terminal histidine tag and purified on a Ni-nitrilotriacetic acid agarose (Qiagen) column as previously described (4). The purified protein was then used in gel mobility shift DNA-binding assays. A radiolabeled DNA fragment, corresponding to the S. salivarius clpP promoter region (positions -142 to +44 with respect to the position of the translation initiation codon), was incubated with increasing amounts of CtsR of B. subtilis in the presence of an excess of nonspecific competitor DNA. DNA binding, and gel electrophoresis mobility shift assays were performed as previously described (4). As shown in Fig. 2A, CtsR bound specifically, forming a single protein-DNA complex, with a complete displacement of the DNA fragment occurring at the highest CtsR concentration.

A DNase I footprinting assay was performed on the S. salivarius DNA fragment carrying the clpP promoter region to determine the extent of the protected region and the precise location of the potential CtsR binding site (Fig. 2B). Labeling and DNase I treatment were performed as previously described (4). When the nontemplate strand of the clpP DNA fragment was end labeled, CtsR protected a region extending from positions -63 to -39 relative to the translational start site (Fig. 2B and C). The region protected by CtsR contains the tandem heptanucleotide repeat recognition sequence and the −35 sequence of the promoter in agreement with sequence analysis predictions (Fig. 2C). These results indicate that clpP of S. salivarius is controlled directly by CtsR.

Analysis of the S. salivarius clpP promoter region reveals a potential CIRCE sequence. During the analysis of the published S. salivarius clpP nucleotide sequence (7), we noted the existence of a 9-bp palindromic sequence upstream from the CtsR binding site, sharing 100% identity with the CIRCE consensus sequence. Surprisingly, the two repeats were separated by 8 bp, whereas in a compilation of 70 CIRCE sequences, the spacer region was invariably 9 nucleotides (10). Since all CIRCE sequences described to date are exclusively associated with the dnaK or groEL operon, it was therefore tempting to consider this noncanonical CIRCE sequence as a cryptic sequence.

In order to test whether HrcA does indeed play a role in controlling expression of the clpP gene, we first sequenced the clpP promoter region of S. salivarius ATCC 9758. As described above, a DNA fragment generated by PCR with chromosomal DNA from the reference strain of S. salivarius ATCC 9758 was cloned into plasmid pDK. The nucleotide sequence of two DNA fragments resulting from independent PCRs was determined (GenBank accession number AY137346), revealing nu-
merous differences with the nucleotide sequence of strain ATCC 25975 (indicated in Fig. 2C). Interestingly, in strain ATCC 9758, the inverted repeat sequences of the CIRCE motif are separated by the consensus 9-bp spacer instead of 8 bp. This reinforced the hypothesis of a potential role for HrcA in the regulation of clpP and suggests that the reported 8-nucleotide spacer sequence from strain ATCC 25975 (7) may be due to a sequencing error.

**clpP of S. salivarius is controlled by HrcA.** To evaluate the role of HrcA and this potential CIRCE sequence in *S. salivarius clpP* regulation, we used *B. subtilis* as a heterologous host as detailed above.

A DNA fragment corresponding to the coding sequence of *B. subtilis hrcA* was generated by PCR and cloned between the HindIII and EcoRI sites of plasmid pXT (3), placing *hrcA* under the control of the xylose-inducible promoter (PxyA). The construct was then integrated as a single copy at the thrC locus of *B. subtilis*. The resulting strain, QB8083 [prpC1 ΔctsR amyE:(clpP-bgaB aphA3) ΔhrcA::cat thrC::(PxyA-B. subtilis hrcA spec)], carries the *S. salivarius clpP*-bgaB transcriptional fusion integrated as a single copy at the *amyE* locus, as well as a deletion-replacement of the endogenous *hrcA* gene (17).

β-Galactosidase assays were performed during growth at 37°C in the presence or absence of xylose. Expression of *clpP*-bgaB was repressed twofold in the presence of HrcA (Fig. 3A), whereas expression in the same background was repressed up to 16-fold by CtsR (Fig. 1A). When strain QB8083 was grown at 48°C in the presence of xylose, expression levels of the bgaB fusion were fully derepressed (Fig. 3B). These results indicate that the *S. salivarius clpP* gene is also repressed by HrcA, albeit weakly but reproducibly, and that this repression is abolished during heat shock, leading to induction of this gene.

The small effect of HrcA may be due to a weak interaction between the *B. subtilis* protein and the *S. salivarius* promoter since HrcA proteins of different bacteria are poorly conserved (10). However, it is worth noting in this case that the CIRCE operator is placed 17 bp upstream from the transcriptional start site (21, 27), which may explain the weak repression by HrcA. Indeed, it was shown with *B. subtilis* that increasing the distance between the transcriptional start site and a downstream CIRCE motif progressively decreased the negative regulatory effect (30), and expression of groE′-bgaB was totally abolished when the distance reached 21 bp. Although we cannot exclude the possibility of the existence of a second promoter upstream from the CIRCE sequence, we suggest that HrcA contributes to the repression of *clpP* expression by interfering with RNA polymerase binding to the downstream promoter.

Although the large majority of HrcA-controlled promoters have a CIRCE motif located downstream from the transcriptional start site (21, 27), we note that in the streptococcal group, the CIRCE operator sequence is present twice within the promoter region of the dnaK operon. One CIRCE sequence is found a couple of bases downstream from the transcriptional start site, which has been characterized in some cases (13-15). The second CIRCE sequence is located 17 to 18 bp upstream from the −35 promoter sequence in *Streptococcus mutans*, *Streptococcus agalactiae*, *S. pneumoniae*, *Streptococcus pyogenes*, and *Enterococcus faecalis* at the same position as the single CIRCE sequence upstream from the *clpP* gene in *S. salivarius*. In *Lactococcus lactis*, there is only a single CIRCE
sequence upstream from the dnaK operon and it is located upstream from the potential promoter (5). Although these upstream HrcA recognition sites have not been characterized, their perfect conservation strongly suggests that these sequences are not cryptic and that they must play a role in the regulation of stress gene expression.

clpP of S. salivarius is the first example of a clp gene controlled by both CtsR and HrcA. Using both in vivo and in vitro approaches, we have shown that the S. salivarius clpP gene is efficiently repressed by CtsR from B. subtilis, strongly suggesting the existence of a CtsR regulon in this bacterium. Furthermore, we have presented evidence indicating a likely repression of clpP expression by the transcriptional regulator HrcA as well. Although the genome of S. salivarius has not been sequenced, the incomplete genome sequence of the closely related bacterium Streptococcus thermophilus is available (http://www.biol.ucl.ac.be/gene/genome), and we were able to identify potential genes encoding orthologs of both HrcA and CtsR. Inspection of the nucleotide sequence preceding the S. thermophilus clpP gene revealed the same tandem arrangement of conserved CIRCE and CtsR binding sites, suggesting that dual regulation of clpP by both HrcA and CtsR also occurs in S. thermophilus. We recently reported the existence in S. aureus of a significant regulatory overlap between class I and class III stress response genes, since the entire HrcA regulon (consisting of the dnaK and groESL operons) is embedded within the CtsR regulon (Fig. 4), with both operons being preceded by tandemly arranged operator sites for the two repressors (1). Furthermore, comparative genome analysis allowed us to predict the existence in many gram-positive bacteria of a partial overlap between class I and class III genes, particularly in the streptococcal group (S. pneumoniae, S. pyogenes, S. mutans, S. agalactiae, and L. lactis) (Fig. 4), in which only the groESL operon presents both the highly conserved CIRCE-HrcA recognition sequence and the CtsR target site organized in tandem (2). This dual regulation is probably not redundant, since we have shown that in S. aureus, CtsR and HrcA act together synergistically to maintain low levels of expression of the dnaK and groESL operons in the absence of stress (1).

The S. salivarius clpP promoter has an original structure with a classical CtsR binding site present in most clpP promoters of low-G+C-content gram-positive bacteria and an upstream CIRCE motif more characteristic of the dnaK promoters of streptococci (Fig. 2C and 4). Nevertheless, clpP of S. salivarius is the first example of a clp gene associated with a CIRCE sequence. Dual regulation by both repressors may play a role in the fine-tuning of clpP expression in S. salivarius as well as closely coordinating synthesis of the classical chaperones (GroESL and DnaK) with that of the Clp ATP-dependent protease during the stress response.

FIG. 3. (A) The expression of clpP of S. salivarius is repressed by HrcA. Levels of expression of clpP-bgaB (QB8083) in the presence (○, ●) or absence (□, ■) of xylose were monitored as indicated in the legend to Fig. 2A. (B) The repression of S. salivarius clpP expression by HrcA is abolished by heat shock. Levels of expression of clpP-bgaB (QB8081) were measured in cells expressing hrcA during growth at 37°C (□, ■) and 48°C (○, ●) as indicated in the legend to Fig. 2B. ONP, o-nitrophenyl.

FIG. 4. Dual regulation by CtsR and HrcA in different gram-positive bacteria. In bacilli the two regulons are distinct, whereas in streptococci they partially overlap, and the HrcA regulon is entirely embedded within the CtsR regulon in staphylococci. clpP of S. salivarius is the first example of a clp gene that is dually regulated by both HrcA and CtsR.
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REFERENCES


