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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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Nucleotide sequence analysis of the *Streptococcus salivarius clpP* locus revealed potential binding sites for both the CtsR and HrcA repressors. Dual regulation by HrcA and CtsR was demonstrated by using *Bacillus subtilis* as a heterologous host, and CtsR was shown to bind directly to the *clpP* promoter sequence. This is the first example of a *clpP* gene under the control of HrcA.

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 inverted-repeat chaperone expression (CIRCE) operator sequence. This highly conserved element is composed of a well-conserved 9-bp inverted repeat sequence separated by 9 bp (TTAGCACTCX₉GAGTGCTAA) and is always found in association with *dnaK* or *groEL* genes (21). Class II genes encode general stress proteins, and their expression requires the σ^B stress sigma factor. Class III heat shock genes are controlled by

the CtsR repressor, a DNA-binding protein which recognizes a tandemly repeated heptad operator sequence (GGTCAAAXA XGGTCAA) (4). Class IV genes are defined as those devoid of the CIRCE or CtsR operator sequences and whose induction by heat shock or general stress conditions is σ^B independent.

Sequencing of the upstream and downstream regions of the *fff* gene of *S. salivarius* ATCC 25975 (GenBank accession number LO7793) revealed a gene whose product is highly similar to ClpP of *E. coli* (7). In this work, we report an analysis of the *clpP* promoter of *S. salivarius*, showing evidence for a direct control at the transcriptional level of this gene by a putative ortholog of CtsR. We also demonstrate that HrcA negatively controls *clpP* expression as well, which is the first example of a *clp* gene under dual heat shock repression.

Expression of the *S. salivarius clpP* gene is controlled by CtsR. Analysis of the *S. salivarius clpP* promoter region revealed a potential CtsR binding site (4). In order to demonstrate regulation by CtsR, we used *B. subtilis* as a heterologous host, since the *S. salivarius* genome sequence is not available. A transcriptional fusion between the *S. salivarius* ATCC 9758 *clpP* promoter region and the *Bacillus stearothermophilus bgaB* gene, which encodes a thermostable β -galactosidase (11), was first constructed by cloning a PCR-generated fragment (positions –295 to –12 with respect to the position of the translation initiation codon) into plasmid pDK (2) and then introduced into *B. subtilis*.

Derivatives of strain QB8068, in which the endogenous *ctsR* gene was deleted (2), were then constructed in several steps. The final strain, QB8081 [*trpC2* Δ *ctsR* *amyE*::(*clpP'*-*bgaB* *aphA3*) Δ *hrcA*::*cat* *thrC*::(*P*_{*XylA*}-*B. subtilis* *ctsR spec*)], carries the *S. salivarius clpP'*-*bgaB* transcriptional fusion integrated as a single copy at the *amyE* locus, a deletion-replacement of the endogenous *hrcA* gene (17), and a copy of the *B. subtilis* *ctsR* gene integrated at the *thrC* locus under the control of the *P*_{*XylA*} xylose-inducible promoter.

Strain QB8081 was grown in Luria-Bertani medium until an optical density at 600 nm (OD₆₀₀) of 0.3 was achieved, and β -galactosidase activities were assayed during growth at 37°C in the presence or absence of 20 mM xylose and expressed as

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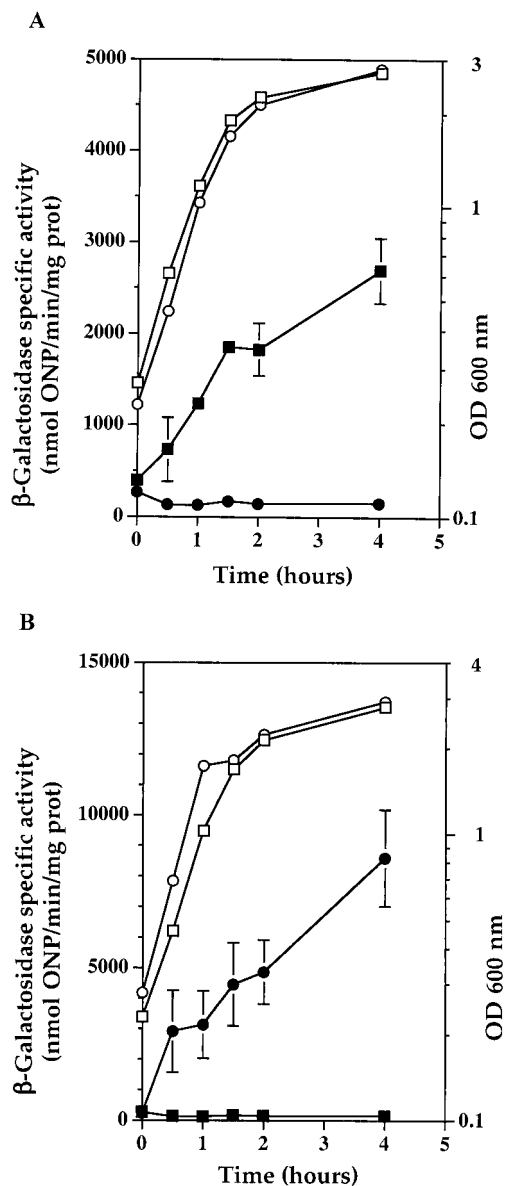


FIG. 1. (A) Expression of *S. salivarius clpP* is repressed by CtsR. Levels of expression of *clpP'-bgaB* (strain QB8081) in the presence (○, ●) or absence (□, ■) of xylose are shown. Cultures were grown in Luria-Bertani medium at 37°C until the OD₆₀₀ was 0.3, and xylose was added to one-half of the culture at a final concentration of 20 mM. (B) Repression of *S. salivarius clpP* expression by CtsR is abolished by heat shock. The expression of *clpP'-bgaB* (QB8081) was measured in cells expressing *ctsR* during growth at 37°C (□, ■) or 48°C (○, ●). Cells were grown in Luria-Bertani medium at 37°C until the OD₆₀₀ was 0.3, 20 mM xylose was added, and one-half of the culture was shifted to 48°C. Open symbols indicate OD₆₀₀s. Solid symbols indicate β-galactosidase specific activities expressed as nanomoles of *o*-nitrophenyl per minute per milligram of protein, and values are the means ± standard deviations of results from two independent assays. ONP, *o*-nitrophenyl.

nanomoles of *o*-nitrophenyl per minute per milligram of protein.

As shown in Fig. 1A, induction by xylose of the *B. subtilis ctsR* gene led to an up to 16-fold repression of *clpP'-bgaB*

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 [1 μg of poly(dI-dC)]. Radiolabeling, 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-

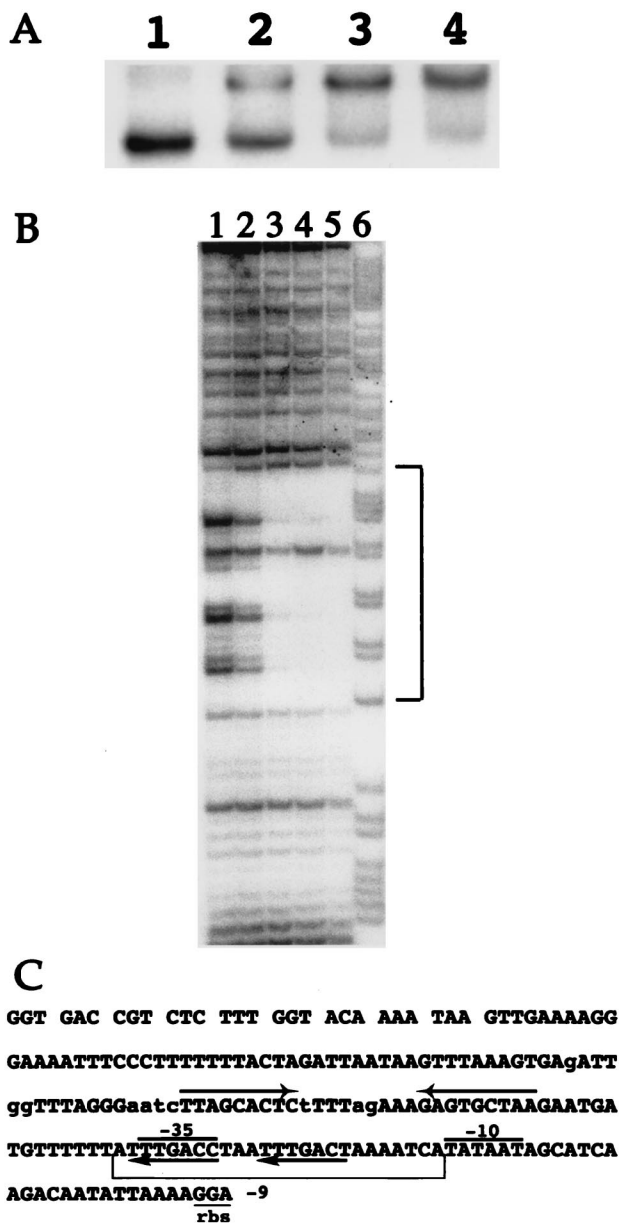


FIG. 2. (A) CtsR binds specifically to the *S. salivarius* *clpP* promoter region. In gel mobility shift experiments, a radiolabeled DNA fragment (10,000 cpm) corresponding to the *S. salivarius* *clpP* promoter region (positions -142 to +44 with respect to the translation initiation codon) was incubated with increasing amounts of purified *B. subtilis* CtsR protein. Lanes 1 to 4, 0, 20, 40, and 80 ng of CtsR, respectively. (B) CtsR protects the directly repeated heptad sequence. In DNase I footprinting analyses of CtsR binding, a radiolabeled DNA fragment (50,000 cpm) corresponding to the *S. salivarius* *clpP* promoter region (positions -142 to +44 with respect to the translation initiation codon) was incubated with increasing amounts of purified CtsR. Lanes 1 to 5, 0, 100, 200, 400, and 800 ng of CtsR, respectively; lane 6, G+A Maxam and Gilbert reaction of the corresponding DNA fragment. The region protected by CtsR is shown by the bracket. (C) Nucleotide sequence of the *S. salivarius* ATCC 9758 *clpP* promoter region. Potential -35 and -10 promoter sequences are overlined; the CtsR heptad direct-repeat operator sequences are indicated by arrows; the CIRCE operator sequence is indicated by facing arrows; the region protected by CtsR in DNase I footprint experiments is indicated by a bracket; lowercase letters indicate differences in the nucleotidic sequence from that of the ATCC 25975 strain. Positions are numbered relative to the translation initiation codon.

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 *Hind*III and *Eco*RI sites of plasmid pXT (3), placing *hrcA* under the control of the xylose-inducible promoter (*P_{xyL}*). The construct was then integrated as a single copy at the *thrC* locus of *B. subtilis*. The resulting strain, QB8083 [*trpC2* Δ *ctsR* *amyE*::(*clpP'*-*bgaB* *aphA3*) Δ *hrcA*::*cat* *thrC*::(*P_{xyL}*-*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 -35 sequence of the promoter (Fig. 2C), 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

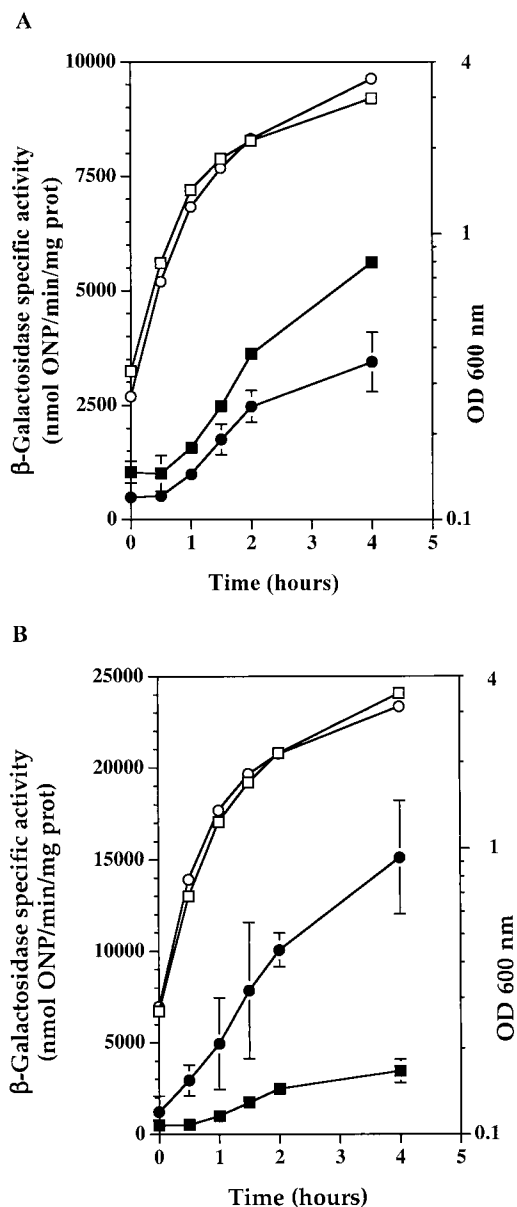


FIG. 3. (A) The expression of *clpP* of *S. salivarius* is repressed by HrcA. Levels of expression of *clpP*-*bgaB* (QB8083) in the presence (○, ●) or absence (□, ■) of xylose were monitored as indicated in the legend to Fig. 2A. (B) The repression of *S. salivarius clpP* expression by HrcA is abolished 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.

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

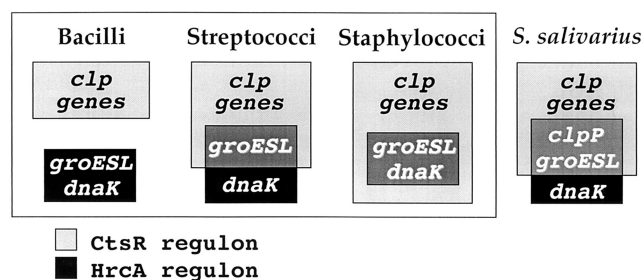


FIG. 4. Dual regulation by CtsR and HrcA in different gram-positive bacteria. In bacilli the two regulons are distinct, whereas in streptococci they partially overlap, and the HrcA regulon is entirely embedded within the CtsR regulon in staphylococci. *clpP* of *S. salivarius* is the first example of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

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.

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