Transcription of *clpP* Is Enhanced by a Unique Tandem Repeat Sequence in *Streptococcus mutans*[▽]

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Streptococcus mutans, the primary causative agent of human dental caries, contains a single copy of the gene encoding ClpP, the chief intracellular protease responsible for tolerance to various environmental stresses. To better understand the role of ClpP in stress response, we investigated the regulation of *clpP* expression in *S. mutans*. Using semiquantitative reverse transcription-PCR analysis, we observed that, under nonstressed conditions, *clpP* expression is somewhat constant throughout the growth phases, although it gradually decreases as cells enter the late stationary phase. The half-life of the *clpP* transcript was found to be less than 1 minute. Sequence analysis of the *clpP* locus reveals the presence of a 50-bp tandem repeat sequence located immediately upstream of the *clpP* promoter (*PclpP*). PCR and DNA sequence analyses suggest that the number of tandem repeat units can vary from as few as two to as many as nine, depending on the particular *S. mutans* isolate. Further analysis, using a transcriptional reporter fusion consisting of *PclpP* fused to a promoterless *gusA* gene, indicates that the presence of the repeat sequence region within *PclpP* results in an approximately fivefold increase in expression from *PclpP* compared to the repeat-free transcriptional reporter fusion. CtsR, a transcriptional repressor that negatively regulates *clpP* expression, has no effect on this repeat-mediated induction of *clpP* transcription. Furthermore, the repeat sequence is not necessary for the induction of *clpP* under stress conditions. Database searches indicate that the region containing the tandem repeats is absent in the *clpP* loci in other bacteria, including other closely related *Streptococcus* spp., suggesting that the repeat sequences are specific for the induction of *clpP* expression in *S. mutans*. We speculate that a host-specific transcriptional activator might be involved in the upregulation of *clpP* expression in *S. mutans*.

Streptococcus mutans has been strongly implicated as the principal etiological agent in human dental caries (36). In addition to having this role in dental caries, *S. mutans* is also an important agent of infective endocarditis (2, 57). *S. mutans* colonizes the oral cavity through the formation of diverse, multispecies biofilms on the tooth surface, commonly known as dental plaque. This pathogenic microorganism has developed a variety of mechanisms to adapt and to flourish in the hostile environment of the oral cavity. The presence of a high proportion of *S. mutans* in the cariogenic flora is due to its ability to respond rapidly and efficiently to various environmental fluxes, including severe nutrient limitation, fluctuations in pH and temperature, and changes in oxidative and osmotic tensions (32).

In order to survive adverse environmental conditions, bacteria transiently induce expression of a subset of genes encoding proteins that protect the cell from the damages caused by various environmental stresses. Most of these proteins are either molecular chaperones or proteases that play crucial roles in the refolding or degradation of unfolded or misfolded proteins (for recent reviews, see references 16, 21, 25, 57, and 61).

Like other bacteria, *S. mutans* also possesses proteins that belong to the Clp (caseinolytic protease) family, which is composed of a protease, ClpP, and various ATPases belonging to the AAA+ family of proteins. The ClpP protease, which is a relatively small cytoplasmic serine protease composed of two heptameric rings (37, 59), is associated with a partner Clp ATPase, forming a functional complex that specifically targets damaged or misfolded proteins for degradation or translocation (for recent reviews, see references 21 and 61). *S. mutans* possesses five such Clp ATPases, as follows: ClpB, ClpC, ClpE, ClpL, and ClpX (1). While the ATPase subunits determine the substrate specificity, it is ClpP that actually degrades the damaged protein.

ClpP plays an indispensable role in cellular protein quality control, by degrading denatured proteins in both stressed and nonstressed cells (19, 28, 30, 33, 40). ClpP is also important for maintaining the stability and activity of several transcriptional regulators and, therefore, has a significant role in the regulation and maintenance of cellular physiology. In various microorganisms, targets for ClpP-mediated proteolysis include several global regulators, including the following: Spx, which is involved in oxidative stress (62); RsiW, which is required during alkaline stress (60); and several regulators involved in bacterial development, such as ComK (competence development) (29, 41, 56), Sda (sporulation) (53), and σ^H alternate sigma factor (34, 35, 44). Since ClpP is involved in protein quality control, inactivation of *clpP* leads to various biological changes, such as stress sensitivity, aberrant cell morphology, developmental defects, and severely attenuated virulence (20, 22, 26,

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TABLE 1. List of *S. mutans* strains used in this study

Strain	Genotype or description	Source
UA159	Wild type, serotype c	R. A. Burne, University of Florida, Gainesville, FL
NG8	Wild type	A. S. Bleiweis, University of Florida, Gainesville, FL
V100	Wild type, serotype c	A. S. Bleiweis, University of Florida, Gainesville, FL
8Vs3	Wild type	M. Duncan, The Forsyth Institute, Boston, MA
IBS514	UA159::PclpP-gusA	This study
IBS515	UA159::PclpPΔRS-gusA	This study
IBS516	UA159 PclpP::loxP-Km-loxP	This study
IBS517	UA159 PclpPΔRS::loxP	This study
IBS938	IBS514 ΔctsR	This study
IBS939	IBS515 ΔctsR	This study

43). Thus, ClpP protease, along with the Clp ATPases, is central in the coordination of developmental decisions and stress response in prokaryotic microorganisms.

In bacteria, production of *clpP* transcript is strongly induced after exposure to thermal or other stress conditions (18, 19, 23, 43, 49). Furthermore, in low-G+C-content gram-positive bacteria, including *S. mutans*, regulation of the *clpP* expression is primarily controlled by the CtsR repressor, which binds to a heptanucleotide repeat, A/GGTCAA/T (15, 42). This sequence is generally located near the −35 region of the *clpP* promoters. In addition to CtsR-mediated repression, *clpP* expression is also subject to other regulatory controls, which vary significantly depending on the organism. In the cases of *Bacillus subtilis* and *Listeria monocytogenes*, *clpP* expression is also under the regulation of σ^B , the alternate stress sigma factor specifically involved in general stress response (21, 47). On the other hand, *clpP* expression in *Streptococcus salivarius* and *Streptococcus thermophilus* is negatively regulated by HrcA, a transcriptional repressor. HrcA binds to a well-conserved sequence known as CIRCE, which contains a 9-bp inverted repeat sequence separated by a 9-bp spacer sequence (TTAGCACTCN₉GAGTGCTAA) (13). CIRCE elements are found immediately upstream of the −35 region of the *clpP* promoters in low-G+C-content gram-positive bacteria. In contrast to low-G+C-content gram-positive bacteria, high-G+C-content gram-positive bacteria, such as *Streptomyces lividans* and *Corynebacterium glutamicum*, contain multiple copies of *clpP* genes that are regulated by two transcriptional activators, ClgR and PopR (4, 17, 58).

In this report, we studied the regulation of *clpP* expression in *S. mutans*, which contains a single-copy *clpP* gene. During the course of this study, we identified a tandem repeat sequence which is present immediately upstream of the *clpP* promoter. The number of repeat units varies depending on the strains under study. The functional significance of this repeat sequence in the regulation of *clpP* expression was investigated. Our results suggest that in *S. mutans*, in addition to CtsR-mediated regulation, expression of the *clpP* gene is also under the control of an additional regulator.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Escherichia coli* strain DH5 α was grown in Luria-Bertani medium, supplemented when necessary with ampicillin (100 μ g $\cdot$ ml^{−1}), kanamycin (Km; 50 μ g $\cdot$ ml^{−1}), and erythromycin (400

μ g $\cdot$ ml^{−1}). *S. mutans* strains used in this study are listed in Table 1. Unless otherwise stated, *S. mutans* strains were routinely grown in Todd-Hewitt medium (BBL; Becton Dickinson) supplemented with 0.2% yeast extract (THY). When necessary, Km (300 μ g $\cdot$ ml^{−1}) or erythromycin (10 μ g $\cdot$ ml^{−1}) was included in the growth medium.

Isolation of RNA from bacterial cultures. Total RNA was isolated from bacterial cultures according to the protocol described earlier (6). Briefly, *S. mutans* cultures were grown until the desired optical density was reached, and the bacterial cell pellet was resuspended in 5 ml of RNeasy lysis buffer (Qiagen). Total RNA was isolated using an RNeasy minikit (Qiagen) with a modified bacterial lysis step (6). The supernatant obtained after bacterial cell lysis was loaded onto an RNeasy mini column, and DNA contaminants were removed by on-column DNase I treatment following the manufacturer's instructions. The quality and quantity of the RNA samples were verified using an RNA 6000 Nano Chip kit with an Agilent 2100 bioanalyzer (Agilent Technologies) according to the manufacturer's instructions.

Stability of mRNA. *S. mutans* cultures were grown to mid-exponential phase (70 Klett units), and rifampin was added to the bacterial cultures at a final concentration of 300 μ g $\cdot$ ml^{−1}. At different time intervals, metabolic processes of the bacterial cultures were terminated by addition of 100 mM sodium azide, followed by freezing of the cultures on dry ice. A rifampin-free culture was used as a control to demonstrate that the levels of *clpP* mRNA were in a steady state during the period of study. Total RNA was isolated according to the protocol described above, and stability of the *clpP* transcript was determined by Northern analysis.

Northern blotting. Assays were done as described previously, with the following modifications (6). Total RNA (4 μ g), isolated from *S. mutans* cultures, was denatured, separated by electrophoresis in a 1.0% agarose gel, and transferred to a nylon membrane (Zeta probe; Bio-Rad) according to the NorthernMax-Gly protocol (Ambion). A DNA probe was prepared by PCR amplification using the BamHI-clpP-F2 and HindIII-clpP-R2 primers (Table 2) and *S. mutans* UA159 chromosomal DNA as a template. PCR fragments were labeled with [α -³²P]dATP by random priming using a DECAprime II kit (Ambion). Blots were hybridized with the radiolabeled *clpP* probe in ULTRAhyb buffer (Ambion), washed according to the manufacturer's instructions. RNA blots were analyzed using a PhosphorImager (Molecular Dynamics).

Determination of initiation of transcription. Transcription initiation of *clpP* mRNA was determined by primer extension analysis, which was performed as described previously (9). Briefly, RNA samples isolated from mid-exponential-phase cultures were incubated with 0.5 pmol of a 5'-end-labeled Smu-clpP-Rout1 primer at 70°C for 5 min. Primer extension was accomplished using Superscript II RNase H reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Samples were analyzed on an 8% sequencing gel using sequencing reaction mixture (SequiTherm Excel II; Epicenter) as markers, followed by phosphorimaging.

Semiquantitative RT-PCR (sqRT-PCR). RNA samples isolated from *S. mutans* cultures were used for semiquantitative analysis of the *clpP* and the *gyrA* transcript levels using the Titan one-tube reverse transcription-PCR (RT-PCR) system (Roche) as described previously (6). The primers specific for the *clpP* transcript (BamHI-clpP-F2 and HindIII-clpP-R2) produce a 630-bp PCR product, while primers specific for the *gyrA* transcript (*gyrA*-For and *gyrA*-Rev) generate a 470-bp PCR product. Totals of 5 ng and 25 ng of RNA were used for each RT-PCR, followed by separation of PCR products on 1% agarose gel electrophoresis and quantitation using Doc-It-LS (UVP) software. Expression of the *gyrA* gene served as an internal control to ensure that equal amounts of RNA were being used in all RT-PCR mixtures.

Construction of PclpP-gusA reporter strains and GusA assays. For construction of a transcriptional reporter fusion strain, we selected pIB107, which contains a promoterless *gusA* gene. Plasmid pIB107 can be used for integration at the smu.1405 locus for single-copy reporter fusion, as previously described (9). The *clpP* promoter region, with (PclpP) and without (PclpPΔRS) the tandem repeats, was amplified using BamHI-clpP-F1/XhoI-clpP-Rout1 and BamHI-clpP-F6/XhoI-clpP-Rout1 primer pairs, respectively (Table 2), and was cloned into BamHI-XhoI-digested pIB107 to create pIB521 and pIB522, respectively. pIB521 and pIB522 were linearized with BglI and transferred to *S. mutans* UA159 by natural transformation to create strains IBS514 and IBS515, respectively.

Similarly, *clpP* promoters containing different numbers of repeats were amplified from NG-8, V-100, and 8Vs3 chromosomal DNA by using BamHI-clpP-F1/XhoI-clpP-Rout1 and were cloned into pIB107 to construct pIB931, pIB932, and pIB930, respectively. These plasmids were linearized by BglI and transferred to *S. mutans* UA159.

TABLE 2. List of oligonucleotides used in this study

Primer	Sequence (5' to 3') ^a	Purpose ^b
BamHI-clpP-F2	GATCAGGATCCAAGGAGGAATACAGTATGATTCTCTG	Probe for Northern analysis and RT-PCR
HindIII-clpP-R2	GCGCGAAGCTTTTATTTTAATTCATTATTTT	Probe for Northern analysis and RT-PCR
gyrA-For	GACTATGCTATGAGTGTTATTGTTGCTCGGGC	RT-PCR
gyrA-Rev	GGCCATTCCAACAGCAATACCTGTCGCTCC	RT-PCR
Smu-clpP-Rout-1	GCTCGTTTGTTCATAACTACAGGAATCAT	Primer extension
BamHI-clpP-F1	GATCAGGATCCATCGCTTGTGGGCACAA	<i>PclpP-gusA</i> fusion
BamHI-clpP-F6	GAGCTGGATCCACGAGGTAAAGTCTGATAAAT	<i>PclpPΔRS-gusA</i> fusion
XhoI-clpP-R-out-1	AGTCGCTCGAGTGTTCAATAACTACAGGAATCAT	<i>PclpP-gusA</i> , <i>PclpPΔRS-gusA</i> fusion
Smu-clpP-F2	CTGAAGATATTGACCAACGTCA	Amplification of 5' IGS of <i>clpP</i>
Smu-clpP-R-out2	TATAAAGATAAATGTCTTTGGTATTATCTT	Amplification of 5' IGS of <i>clpP</i>
Smu-clpP-F1	GTTCCAGGTCTTGGGGATGCAGGCGATCGCTTG	Sequencing
Smu-clpP-R2	GACGTGAATAAATATCATAAGAGCGTTTAC	Sequencing
loxP-Km-F	CGATAACTTCGTATAATGTATGCTATAGCAAGTTATGAGGATGAA GAGGATGAGGAGGCAG	Amplification of Km ^r cassette with <i>loxP</i>
loxP-Km-R	CGATAACTTTGTATAGCATACATTATACGAAGTTATGCTTTTTAG ACATCTAAATCTAGG	Amplification of Km ^r cassette with <i>loxP</i>
L-ClpPF	GGCTTCATCGATGAAATCATGGAAAATAATGAATTA	Amplification of 3' IGS of <i>clpP</i>
L-Smu1523R	CATCAAGAGTATCTTCAATTGCAAATAATTGACTGTTA	Amplification of 3' IGS of <i>clpP</i>
CtsR-F2	CTAGATTTCTTTTGGCTTTCC	Amplification of 5' region of <i>ctsR</i>
Sp-CtsR-R1	GATATGATCTTTTCATTTCATAAAACGCATAACCCACGGCTCTCTGT GAAAC	Amplification of 5' region of <i>ctsR</i> for fusion with <i>aad</i> gene
Sp-CtsR-F2	TAAAAAATTTGAAAAAATGGTGGAACGTTGAGAGCAAACGTGG TGGTGG	Amplification of 3' region of <i>ctsR</i> for fusion with <i>aad</i>
CtsR-R2	CTACCATTGCAAGAAGGACATGC	Amplification of 3' region of <i>ctsR</i>
CtsR-Sp-F1	GTTTCACAGAGAGCCGTGGTTATGCGTTTTATGGAATGAAAGAT CATATC	Amplification of <i>aad</i> for fusion with the <i>ctsR</i> upstream region
CtsR-Sp-R2	CCACCACCAGTTTGCTCTCAACGTTTCCACCATTTTTTCAATTTT TTTA	Amplification of <i>aad</i> for fusion with the <i>ctsR</i> downstream region

^a Overlapping sequences in the primers used for fusion PCR are underlined.

^b IGS, intergenic region.

β-Glucuronidase (Gus) assays were performed after the bacterial cultures reached mid-exponential phase (70 Klett units) as described earlier (8).

Deletion of the *ctsR* locus. A *ctsR* deletion mutant was obtained by allelic replacement using a fusion PCR technique, and the primers used for this purpose are listed in Table 2. A DNA fragment containing a 0.26-kb region upstream of the 64th codon of the *ctsR* gene was amplified from *S. mutans* UA159 chromosomal DNA by using primers CtsR-F2 and Sp-CtsR-R1. The 0.41-kb region downstream of the 74th codon was amplified using primers Sp-CtsR-F2 and CtsR-R2. To replace the *ctsR* gene, a spectinomycin resistance gene was also amplified using primers CtsR-Sp-F1 and Sp-CtsR-R1. A fusion PCR product was then produced using an equal molar ratio of the three PCR fragments as a template and CtsR-F2 and CtsR-R2 as the primers. This PCR fragment was directly transformed into *S. mutans* strains IBS514 and IBS515 to generate IBS938 and IBS939, respectively. A gene replacement event was confirmed by PCR on the chromosomal DNA isolated from IBS938 and IBS939 strains.

Deletion of repeat sequences using Cre-*loxP* method. Deletion of the repeat sequences upstream of the *clpP* gene was achieved by adopting the Cre-*loxP* methodology, as described earlier (3). Briefly, the intergenic region between *upp* and *clpP*, including the repeat sequences was amplified using primers Smu-clpP-F2 and Smu-clpP-Rout2, and the PCR product was cloned into the pGEMT-Easy TA cloning vector (Promega), resulting in pIB523. A kanamycin resistance (Km^r) cassette amplified from pUC40Km2 (45) using loxP-Km-F and loxP-Km-R primers was then cloned into *Ava*I-digested/T4 DNA polymerase-blunted pIB523, yielding pIB524. Plasmid pIB524 was linearized with *Not*I and used for the transformation of *S. mutans* UA159, as previously described (7), with transformants being selected on THY plates containing Km. One such double crossover mutant, designated as IBS516, was transformed with pCrePA (48). The chromosomally integrated *loxP*-Km^r cassette was excised by transient expression of Cre recombinase from pCrePA at a restrictive temperature (30°C). After successful excision of the *loxP*-Km^r cassette, cells were cured of pCrePA by elevation of the growth temperature to 37°C, resulting in a mutant strain con-

taining a deletion in the repeat sequences upstream of *clpP* gene (IBS517). The deletion was verified by PCR using the Smu-ClpP-F2/Smu-ClpP-R2 primers.

Exposure of bacterial cells to thermal shock. *S. mutans* cells were grown up to mid-exponential phase (70 Klett units) and exposed to heat shock treatment by incubating the cells at 45°C for 40 min. Cultures were processed for Gus activity or for RNA isolation for Northern blot and RT-PCR analyses, depending upon the nature of the experiments.

RESULTS

Analysis of the *clpP* locus. A genomic map of the *clpP* locus including the genes in the vicinity of *S. mutans* UA159 is shown in Fig. 1A. The gene immediately upstream of the *clpP* locus, *upp*, encodes uracil phosphoribosyltransferase and is transcribed in the same direction as *clpP*. The length of the intergenic region between *upp* and *clpP* is 531 bp; this region contains a putative σ^A-type promoter (TTGACC-N₁₇-TATAAT) that maps a 34-bp region upstream of the translational start codon. Overlapping with the putative −35 region is a tandem heptanucleotide repeat, GGTCAAA-N₄-GGTCAAA, which is known to be the target binding motif for the CtsR repressor (15). Further analysis of the intergenic sequence revealed the presence of yet another sequence of 49 to 50 bp in length that is located approximately 192 bp upstream from the promoter and that is present seven times as tandem repeats (Fig. 1B). Each tandem repeat unit contains a central core of 29 bp that is invariant among the repeat units (RS1 to RS3), with flanking

FIG. 1. Characterization of the *clpP* locus in *S. mutans* UA159. (A) Schematic representation of the *clpP* locus and the surrounding genes. Open reading frames are represented by block arrows, and their orientations indicate the transcriptional direction. (B) The intergenic region between *upp* and *clpP*. The sequence of the *clpP* promoter region is also shown with putative -35 , -10 , and Shine-Dalgarno (SD) sequences. The transcription start site determined by primer extension assay is indicated by an asterisk. Underlined sequences denote putative CtsR repressor binding sites. Symbols: bent arrow, promoter region; lollipop, transcription termination site; shaded triangles, repeat sequences. (C) Multiple-sequence alignment of the different repeat sequences. The intergenic region of *upp* and *clpP* from *S. mutans* UA159 was PCR amplified by the Smu-*clpP*-F2 and Smu-*clpP*-Rout2 primers, and the PCR products were sequenced using the Smu-*clpP*-F1 and Smu-*clpP*-Rout1 primers (Table 1). Repeat sequences derived from DNA sequence analysis were aligned by ClustalW software. At least three different types of repeat sequences were observed (RS1, RS2, and RS3). (D) Detection of the *clpP* transcript. Northern blot analysis of total RNA isolated from *S. mutans* UA159 for detection of the *clpP* transcript. (E) RT-PCR analysis of the *upp* and *clpP* genes. RNA, isolated from the UA159 strain, was used as a template to produce cDNA. PCR was then performed on RNA (control mRNA), cDNA, and chromosomal DNA (K-DNA), using the primer pairs as described in Table 1. M, marker.

sequences that vary slightly (Fig. 1C). Analysis of the region downstream of the *clpP* stop codon revealed two open reading frames, SMU.1671 and SMU.1670, that show homology with the *B. subtilis* *ylbF* and *ylbG* genes, respectively (35% and 43% identities, respectively). These two genes are induced during cold shock response (27) and are involved in sporulation and competence development (55). The intergenic region between *clpP* and SMU.1671, which is 115 bp long, encodes a putative ρ -independent terminator located between bp 85 and 109 that was identified by the FindTerm program (Softberry, Inc.).

Characterization of the *clpP* transcript. To confirm that the putative promoter is indeed used for *clpP* expression, we used a primer extension assay using RNA extracted from exponentially growing *S. mutans* UA159 cells to map the transcription start site. We found that the start of the transcript, which is located 30 nucleotides (nt) upstream from the start codon, matches with that of the putative σ^A -type promoter identified in the upstream region (Fig. 1B) (data not shown). The results of the primer extension assay also indicated the presence of some minor bands that are 5 to 11 nt shorter than the major band corresponding to the transcript from the σ^A -type pro-

motor (data not shown); these bands are probably the products of RNA degradation.

In order to obtain insight into the transcriptional organization of the *clpP* locus, Northern blot analysis of RNA isolated from UA159 was performed as described in Materials and Methods. Northern blot analysis revealed two distinct transcripts (Fig. 1D). The major transcript, which is about 0.67 kb in size and $\sim 80\%$ of the total, corresponds to the length of *clpP*, suggesting that *clpP* is transcribed as a monocistronic message. In addition, a relatively low-abundance transcript ($\sim 20\%$ of the total) of about 1.9 kb in length was also observed. The length of the larger transcript suggests that the origin of the transcript is from the promoter region of the upstream *upp* gene. In order to confirm that *upp* and *clpP* genes are cotranscribed, we performed an RT-PCR from RNA isolated from the UA159 strain. As shown in Fig. 1E, RT-PCR analysis demonstrates that the *upp* and *clpP* genes are cotranscribed, despite the presence of a long intergenic region.

The stability of the *clpP* transcript and expression of *clpP* during various growth phases. The stability of both the 1.9-kb and the 0.67-kb *clpP* transcripts produced by the UA159 strain

FIG. 2. Stability of the *clpP* transcript in *S. mutans*. The stability of the *clpP* transcript was measured from cultures at mid-exponential-growth phase (70 Klett units). RNA was extracted from UA159 at the times (min) indicated above each lane, following the addition of rifampin to block the synthesis of new mRNA. Northern blot analyses (A) were performed with 4 μ g of total RNA, using the *clpP* sequence as a probe. Decay kinetics (B) for *clpP* transcripts were determined from the Northern blot. Half-lives of the transcripts were estimated from the curve. Experiments were repeated at least twice with two different amounts of RNA, and a representative Northern blot and quantitation are shown.

was measured using RNA extracted from cultures grown to the mid-exponential growth phase. Just prior to RNA extraction, rifampin was added to the cell culture to prevent de novo synthesis of mRNA. RNA was then extracted from the cultures at 0, 1, 2, 4, 6, 10, 15, 20, 30, and 60 min, and the stabilities of the *clpP* transcripts were determined using Northern blot analysis (Fig. 2). Decay kinetics of the *clpP* transcripts were measured, and the chemical half-life of the *clpP* transcripts was determined. As shown in Fig. 2, both the *clpP* transcripts appeared to be very unstable, with the half-life of each being less than 1 min; the 1.9-kb transcript was less stable than the 0.67-kb transcript (Fig. 2B).

sqRT-PCR was used to quantify the level of expression of *clpP* during various growth phases. RNA was isolated from *S. mutans* UA159 grown at 37°C at 10 different time points, spanning early exponential phase to late stationary phase (12 h) (Fig. 3A), and sqRT-PCRs were performed as described in Materials and Methods. The level of expression of *gyrA* was included as an internal control to ensure that equivalent amounts of RNA were used for the sqRT-PCR analysis. Two different amounts of RNA samples (5 ng and 25 ng) were used for sqRT-PCR analysis. As shown in Fig. 3B, expression of *clpP* remained constant throughout the growth phase; there were no differences between the two amounts of RNA samples. However, a slight decrease in the level of *clpP* expression was observed (from both the RNA samples) when the cells were at the late stationary phases (Fig. 3B, lanes 9 and 10).

FIG. 3. Expression of *clpP* at different stages of growth. (A) Total RNA was isolated from *S. mutans* UA159 cells grown in THY broth at the indicated time points. (B) sqRT-PCR analysis of RNA isolated at various time points. Two different amounts of RNA, 5 ng and 25 ng, were subjected to RT-PCR with primers specific for the *clpP* gene as described in the text. RT-PCR results with the 25 ng RNA are shown here. The growth points are as follows: 1, 31 Klett units; 2, 47 Klett units; 3, 63 Klett units; 4, 89 Klett units; 5, 120 Klett units; 6, 140 Klett units; 7, 140 Klett units (6 h); 8, 140 Klett units (8 h); 9, 140 Klett units (10 h); and 10, 139 Klett units (12 h). The *gyrA* gene was included to ensure that equal amounts of RNA were used for all reactions.

Presence of a unique repeated sequence in the *clpP* promoter region. When we analyzed the promoter regions of the *clpP* locus from various streptococci whose genomes have been completely sequenced, we did not find the unique 50-bp tandem repeat sequence. Therefore, we wanted to verify whether this repeat sequence is present in other *S. mutans* isolates. For that, we performed PCR analysis using the primers Smu-clpP-1 and Smu-clpP-Rout1 (Table 1) with the genomic DNA isolated from 13 *S. mutans* isolates (109c, 8Vs3, GS-5, NG-8, OMZ175, SJ32, SM3209, SP-2, T8, UA130, UA159, V100, and V403), two *Streptococcus rattus* isolates (BHT and FA-1), and one *Streptococcus cristatus* isolate (ATCC 51100). A single PCR product of various lengths of about 350 bp to 650 bp was observed for all the strains except those of *S. rattus*, for which no PCR product was seen (Fig. 4B) (data not shown). The largest PCR product was obtained with the 8Vs3 strain, while the smallest product was seen with the V100 isolate (Fig. 4B). The lengths of the PCR products of two commonly studied *S. mutans* isolates, GS-5 and NG-8, were slightly larger than the PCR product of V100. The size of the PCR product of the *S. cristatus* strain was similar to that of the NG-8 strain (data not shown).

To analyze the upstream *clpP* intergenic region further, the PCR products isolated from four *S. mutans* strains, UA159, 8Vs3, NG-8, and V100, were sequenced. We found that the number of the repeat units varied from two (V100) to nine

FIG. 4. (A) Schematic representation of the intergenic regions flanking the *clpP* locus in different *S. mutans* strains. The intergenic region between *upp* and *clpP* was PCR amplified from different *S. mutans* strains using Smu-clpP-F2 (arrowhead 1) and Smu-clpP-Rout2 primers (arrowhead 2). The intergenic region between *clpP* and SMU.1671 was PCR amplified from different *S. mutans* strains using L-ClpPF (arrowhead 3) and L-Smu1523R primers (arrowhead 4). (B) PCR amplification of the 5' intergenic region (5'IGS) of *clpP*. The *S. mutans* strains used were UA159, 8Vs3, NG8, and V100. Length variation in the regions is due to different numbers of repeat sequences. At least three different types of repeat sequences were observed—RS1, RS2, and RS3. (C) PCR amplification of the 3' intergenic (3'IGS) region of *clpP*.

(8Vs3), while the rest of the nucleotide sequence between the last repeat unit at the *clpP* locus and the ClpP start codon was invariant (Fig. 4A).

To analyze whether the downstream intergenic region between *clpP* and SMU.1671 is also variable, the intergenic region was amplified by PCR using L-ClpPF and L-Smu1523R primers, with the genomic DNA extracted from the same four isolates described above. We obtained a single PCR product of about 300 bp for all the strains, indicating that the intergenic region downstream of *clpP* is invariable (Fig. 4C).

Presence of the repeat sequence stimulates *clpP* expression.

In order to study the role of the repeat sequence in *clpP* expression, a transcriptional reporter strain was constructed. The promoter region of *clpP*, with or without the repeat sequences, along with the sequence encoding the first 8 amino acids of ClpP, was fused to a *gusA* reporter gene. The *PclpP-gusA* reporter constructs were inserted into the UA159 chromosome at the smu.1405 locus (which is not linked to the *clpP* locus). Strain IBS514 contains the wild-type *PclpP* promoter with the tandem repeat sequence (*PclpP*), while strain IBS515 contains the *PclpP* promoter without the tandem repeat sequence (*PclpP* Δ RS). Expression from the *PclpP* promoter was

measured in IBS514 and IBS515; as shown in Fig. 5A, the *gusA* expression was about fivefold higher than expression from *PclpP* Δ RS when the tandem repeat sequence was present, suggesting that the tandem repeat sequence is essential for the induction of *clpP* expression.

In several bacteria, including *S. mutans*, expression of *clpP* is induced when the cells are exposed to heat shock and other stresses. To study the role of the repeat sequence in stress-induced induction, we used IBS514 and IBS515 cells for further analysis. Cells were grown to mid-exponential-growth phase at 37°C and then divided into two aliquots. One aliquot was incubated at 37°C for 40 min, while the other aliquot was subjected to heat shock at 45°C for 40 min, followed by measurement of Gus activity from each culture. As shown in Fig. 5A, Gus activity was increased by about twofold in both strains IBS514 and IBS515, indicating that the tandem repeat does not play a role in thermal-stress-induced *clpP* expression.

To further confirm our results, we deleted the tandem repeats from the native *clpP* locus on the *S. mutans* UA159 chromosome. A Cre-*loxP*-mediated markerless gene deletion system that was recently developed in our laboratory for manipulating the *S. mutans* chromosome was used for deletion of the upstream region. This deletion procedure removed all of the repeats from the upstream *clpP* locus except for one, which was disrupted by the insertion of *loxP* site. The newly constructed strain, designated IBS517, along with the wild-type strain UA159, was grown at 37°C until mid-exponential-growth phase. Each culture was then divided into two aliquots; one aliquot was incubated at 37°C for 40 min, while the other aliquot was subjected to heat shock at 45°C for 40 min (Fig. 5B). RNA samples were then isolated and subjected to Northern blot analysis. As shown in Fig. 5C, *clpP* expression was approximately twofold lower in IBS517 than in the wild-type strain UA159. On the other hand, *clpP* expression was induced about threefold higher in both IBS517 and UA159 following heat shock. This observation is consistent with our Gus assay results, indicating that the presence of the tandem repeat sequences stimulates *clpP* expression independent of thermal stress. However, when both IBS517 and wild-type UA159 were incubated at 42°C in THY broth, there was no significant difference in growth kinetics between IBS517 and UA159, indicating that loss of the repeat sequence is not sufficient to display any growth defect (data not shown). Furthermore, both IBS517 and UA159 were able to form sucrose-dependent biofilm equally well on polystyrene surfaces (data not shown), suggesting that the repeat sequence units are not involved in thermal stress or biofilm formation, but rather, they may be involved in other biological processes.

Since the number of repeat sequence units varies among the *S. mutans* isolates, we wanted to study stimulation of *clpP* expression with respect to the number of repeats. Toward this end, we selected three extra isolates, 8Vs3 (nine repeats), NG-8 (three repeats), and V100 (two repeats), in addition to UA159 (seven repeats). Transcriptional reporter strains were constructed in the UA159 strain by cloning the individual promoter regions of *clpP* from the strains mentioned above, with various numbers of repeat sequences, to a *gusA* reporter gene. Cells containing reporter constructs were grown to mid-exponential phase, and Gus activities were measured. There was no

FIG. 5. Role of the repeat sequences in *clpP* expression. (A) Gus activity. Reporter strains IBS514 and IBS515, which contain the promoter region of *clpP* with (*PclpP*) or without (*PclpP* Δ RS) the repeat sequences, respectively, fused to the *gusA* gene, were grown in THY medium. Expression from *PclpP* or *PclpP* Δ RS from cultures grown to mid-exponential phase was quantified by determining the level of Gus activity, as described in the text. (B) Deletion of repeat sequences. Repeat sequences upstream of *clpP* were deleted by the Cre-*loxP* method. PCR amplification was performed with Smu-*clpP*-F2 (arrowhead 1) and Smu-*clpP*-Rout2 (arrowhead 2) primers to verify the deletion. (C) Induction of *clpP* transcription under thermal stress in UA159. *S. mutans* strains UA159 and IBS517 were grown to mid-exponential phase and exposed to heat shock (45°C) treatment, as described in the text. RNA was isolated from the bacterial cells and subjected to RT-PCR with primers specific for *clpP* gene. The *gyrA* gene was included to ensure that equal amounts of RNA were used for all reactions. Control samples refer to RNA isolated from bacterial cells without any thermal shock (37°C).

FIG. 6. Tandem repeat sequence-mediated induction is CtsR independent. Gus activity was measured in both the IBS938 and IBS939 strains (lacking functional CtsR) and compared with the Gus activities of IBS514 and IBS515, respectively. The values shown are the Miller units of Gus activity, with standard errors of the mean of the results from experiments repeated thrice.

significant difference in Gus activity among the various reporter strains (data not shown). Similar levels of *clpP* induction were seen in reporter strains with two repeats (V100) or reporter strains with nine repeats (8Vs3) (data not shown). This suggests that as few as two repeats can be sufficient for the induction of *clpP*.

Expression of *clpP* is also modulated by the CtsR repressor that binds to a well-defined tandem heptanucleotide, which is present at the -35 box of the *PclpP*; in *S. mutans*, CtsR was also shown to function as a transcriptional repressor (33). To study whether CtsR is involved in the repeat-mediated induction of *clpP* expression, we constructed *ctsR* deletion mutants IBS938 and IBS939, which are the *ctsR* deletion derivatives of the IBS514 and IBS515 strains, respectively. These strains were grown to mid-exponential phase, and Gus activities were measured. As expected, when *ctsR* was deleted, Gus activity was increased by nearly sevenfold compared to the wild-type *PclpP*-*gusA* fusion, indicating that CtsR does indeed repress expression from the *PclpP* promoter (Fig. 6, IBS514 versus IBS938). The increase was nearly the same (about eightfold) between IBS515 and IBS939 (*PclpP* Δ RS). When the Gus activities of IBS938 (*PclpP*) and IBS939 (*PclpP* Δ RS) were compared, the activity was about fourfold lower in the *PclpP* Δ RS construct, consistent with observations with the strains containing wild-type CtsR. Thus, the repeat-mediated induction of *PclpP* expression appears to be independent of CtsR.

To study whether other *cis*-acting elements in the *PclpP* promoter sequence are necessary for the observed stimulation of transcription by the presence of repeat sequence units, a heterologous promoter, *Pami*, which is distinct from the native *PclpP* promoter, was selected for analysis. The *Pami* promoter is derived from *Streptococcus pneumoniae* and is active in *S. mutans* (8). To determine whether the presence of repeat sequences can stimulate expression from *Pami*, we cloned the repeat sequences, in both orientations, immediately upstream of the *Pami* promoter in plasmid pIB188, which contains a *gusA* gene fused to the promoter. Insertion of the repeat sequence units in front of the promoter did not stimulate expres-

sion from *Pami* (data not shown), suggesting that the repeat-mediated stimulation effect is promoter specific and requires other *cis*-acting elements.

DISCUSSION

In low-G+C-content gram-positive bacteria, such as streptococci, the intracellular protease ClpP plays important roles in many cellular processes, including stress resistance, biofilm formation, and pathogenesis (12, 18, 31, 33, 38, 43, 49). Although, the importance of ClpP in various biological processes is well documented, regulation of its expression has not been thoroughly investigated. Bacteria belonging to the *Streptococcus* genus possess a single-copy *clpP* gene whose expression appears to vary depending on the species. While CtsR is the major repressor of *clpP* expression in all low-G+C-content gram-positive bacteria, another repressor, HrcA, also appears to regulate *clpP* expression in certain *Streptococcus* species, such as *S. thermophilus* and *S. salivarius* (13, 15, 42). To better understand the expression of *clpP* in *S. mutans*, we initiated this study, in which several new findings have emerged.

Our Northern blot analysis results suggest that in *S. mutans*, *clpP* is apparently expressed from the following two transcripts: a minor (20%) transcript of 1.9 kb and a major (80%) transcript of 0.67 kb. The size of the larger transcript correlates with a transcript originating from the promoter of the *upp* gene, which lies upstream of the *clpP* gene. The cotranscription of *clpP* and *upp* was later confirmed by RT-PCR analysis. Previous studies on *clpP* gene expression in other gram-positive bacteria, including streptococci, have shown that *clpP* transcription is monocistronic and that the length of its product correlates with the length of the smaller transcript observed here (19, 23, 33, 38, 43, 49). It is very surprising to see that the cotranscription of the *clpP* gene with *upp* is observed only in *S. mutans*, since the *upp* locus is topologically linked to the *clpP* locus in all streptococci. The *upp* gene encodes uracil phosphoribosyl transferase, a key enzyme in the microbial pyrimidine salvage pathway. This enzyme catalyzes the conversion of uracil and 5-phosphoribosyl- α -1-pyrophosphate to UMP and pyrophosphate. Cotranscription of *clpP* and *upp* suggests a link between ClpP-mediated protein degradation and the nucleotide biosynthesis pathway, which may be important for the lifestyle of *S. mutans*, an obligate biofilm former. Interestingly, in *B. subtilis*, aspartate transcarbamoylase (PyrB), which catalyzes the first committed step of pyrimidine biosynthesis, and several other purine biosynthetic enzymes are bona fide ClpP substrates that are degraded by ClpP upon entry of cells into stationary phase (24, 28). This indicates that under conditions of stress, ClpP downregulates de novo biosynthetic pathways and probably stimulates alternate salvage pathways for efficient recycling of cellular resources. Further experiments are required to establish a definite link between ClpP-mediated protein degradation and activation of salvage pathways in biofilm.

The size (0.67 kb) of the major *clpP* transcript that we observed correlates with the location of the transcriptional start site that lies 30 nt upstream of the *clpP* start codon, which was mapped by primer extension. This major *clpP* transcript is reported to be regulated by CtsR (33), which presumably binds to the CtsR consensus sequence present near the -35 region (Fig. 1). The stability of the 0.67-kb transcript was found to be

very short lived, with a half-life of less than 1 min; the half-life of the 1.9-kb transcript was even shorter than that of the smaller transcript (Fig. 2). Different half-lives of these two transcripts suggest that different RNases may degrade them. Although the stability of the *clpP* transcript has not been studied in any streptococcal species, in *B. subtilis* and *Oenococcus oeni*, the respective *clpP* transcripts have also been shown to have short half-lives (5, 23). Thus, it appears that the turnover of the *clpP* transcript is very rapid in gram-positive bacteria.

We observed that under nonstressed conditions, the *clpP* gene was expressed during all stages of growth. However, *clpP* expression begins to decline after 10 to 12 h of growth. Although not measured precisely, it appears that the basal level of the *clpP* transcript was relatively high compared to the level of the *gyrA* transcript (Fig. 3), suggesting that despite CtsR-mediated repression, significant levels of the *clpP* transcript are being produced in *S. mutans*. Our observation is very similar to that of *O. oeni clpP* expression, where a high basal level of *clpP* transcription is also observed, with the maximal level reached at the exponential phase. However, in *B. subtilis*, Gerth et al. have observed that the ClpP protein level is maximal during stationary phase (23). These apparent differences between *B. subtilis* and *S. mutans* or *O. oeni* could be because the level of ClpP protein produced may not be correlated with the transcript level or may be due to a different regulatory mechanism operating in *B. subtilis*.

Here, we identified a unique tandem repeat sequence at upstream of the *clpP* promoter region that stimulates *clpP* transcription. This repeat sequence, which is 50 bp in length, was not found anywhere else on the *S. mutans* chromosome and is absent in other bacterial genomes for which sequence information is available. However, a part of this repeat sequence, about 30 bp, with four mismatches, has been found upstream of the *dnaJ* gene in *S. mutans*. Long repeats are not frequently present in the bacterial genome. However, a recent bioinformatic analysis by Rocha et al. (51) has suggested that, in contrast to the common belief, large repeat sequences, including tandem repeats, are widely present in bacteria. The number of repeats can vary depending on the organism; over 500 repeats are found in *Mycoplasma pneumoniae*, while only 170 repeats are found in *B. subtilis* (51). The mean repeat length in bacteria also varies, ranging from 50 to 100 bp, and the maximum repeat length can reach up to 2.0 kb (51).

A recent study with *E. coli* suggests that repeat sequences are more frequently present in stress response-related genes (52). However, these repeats are small simple sequence repeats and are generally present within the coding region (52). Repeat sequences are often substrates for replication slippage, illegitimate recombination, or homologous recombination, and lead to genomic instability and phenotypic variability (11, 39, 50, 54). In *S. pneumoniae*, short direct repeats are present in genes encoding autolysins and neuraminidases, and a large amount of hydrogen peroxide can lead to resistance to autolysis due to genetic rearrangement (46). Thus, there is a strong association between the presence of tandem repeat sequences and the stress tolerance response in bacteria. However, nothing is known about the simple sequence repeats and their role in *S. mutans* stress response. We have performed an in silico analysis of the *S. mutans* genome and found that there are nine different tandem repeat sequences with a minimum length of

20 bp, whose copy number varies between two and seven (data not shown). If the length of the repeat is decreased to 8 bp, 34 different repeat sequences can be found in the *S. mutans* chromosome (data not shown). The majority of these repeats, including the repeat studied here, are present within intergenic regions; therefore, rearrangement, such as deletion or expansion, of the repeats in the intergenic regions can lead to altered levels of gene expression. Indeed, analysis of 13 different *S. mutans* strains shows that the number of units of the tandem repeat studied here varies from two to nine, depending on the isolates under study; this suggests that rearrangement does occur in the *S. mutans* chromosome with high frequency, at least at the *clpP* locus. However, the consequence of rearrangement at this locus needs to be explored further.

Transcription induction from *PclpP* by the tandem repeats may be due to some specific DNA structure that facilitates transcription, such as DNA bending or looping, or may be due to some transcriptional activator. If it is indeed a transcriptional activator, it would appear to be specific to *S. mutans*, as the reporter construct carrying the repeat sequence did not lead to transcription stimulation when tested in *E. coli* (data not shown). Since the stimulation appears to be promoter specific, it appears that binding of a host-encoded transcriptional activator to the repeat is not sufficient for transcription stimulation; other parameters, such as upstream sequence elements or promoter spacing, may also be critical. However, proteins from a crude cell extract of *S. mutans* UA159 failed to bind to the *PclpP* promoter in a gel shift assay (data not shown). We speculate that the transcriptional activator appears to be present in very low abundance in the cell. Alternatively, our in vitro DNA binding conditions may not have been optimal for the transcriptional factor binding. We are currently evaluating the role of promoter context also in the observed stimulation.

In summary, this study identified a novel mechanism of regulation of *clpP* expression in *S. mutans*. ClpP plays a critical role in thermal stress response as well as in biofilm formation (33), which are crucial to the lifestyle of *S. mutans*. Both multispecies and single-species biofilm formation are drastically affected by ClpP deficiency in oral bacteria (12, 33). The level of ClpP inside the cell is regulated at both the transcriptional and posttranscriptional levels and is precisely maintained during various biological processes (23). Uncontrolled ClpP activity can lead to impaired cell division and cell death (10). Moreover, ClpP is becoming an important target for the development of antimicrobial compounds. Compounds that inhibit ClpP activity (14) as well as compounds that cause increased ClpP proteolysis (10) are currently being developed to be used as antimicrobial agents.

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