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Transcriptome analysis reveals that ClpXP proteolysis controls key virulence properties of *Streptococcus mutans*

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The ClpXP proteolytic complex is critical for maintaining cellular homeostasis, as well as expression of virulence properties. However, with the exception of the Spx global regulator, the molecular mechanisms by which the ClpXP complex exerts its influence in *Streptococcus mutans* are not well understood. Here, microarray analysis was used to provide novel insights into the scope of ClpXP proteolysis in *S. mutans*. In a Δ*clpP* strain, 288 genes showed significant changes in relative transcript amounts ($P \leqslant 0.001$, twofold cut-off) as compared with the parent. Similarly, 242 genes were differentially expressed by a Δ*clpX* strain, 113 (47 %) of which also appeared in the Δ*clpP* microarrays. Several genes associated with cell growth were downregulated in both mutants, consistent with the slow-growth phenotype of the Δ*clp* strains. Among the upregulated genes were those encoding enzymes required for the biosynthesis of intracellular polysaccharides (*glg* genes) and malolactic fermentation (*mle* genes). Enhanced expression of *glg* and *mle* genes in Δ*clpP* and Δ*clpX* strains correlated with increased storage of intracellular polysaccharide and enhanced malolactic fermentation activity, respectively. Expression of several genes known or predicted to be involved in competence and mutacin production was downregulated in the Δ*clp* strains. Follow-up transformation efficiency and deferred antagonism assays validated the microarray data by showing that competence and mutacin production were dramatically impaired in the Δ*clp* strains. Collectively, our results reveal the broad scope of ClpXP regulation in *S. mutans* homeostasis and identify several virulence-related traits that are influenced by ClpXP proteolysis.

INTRODUCTION

Streptococcus mutans is a member of the oral microbiome known for its close association with dental caries and, occasionally, infective endocarditis. The niche in which *S. mutans* thrives is the biofilm that forms on the enamel surface of teeth (Loesche, 1986). The dental biofilm environment is constantly and unpredictably changing due to the eating habits of the human host, resulting in large fluctuations in nutrient source and availability, pH, and oxygen tension, among other stresses (Lemos & Burne, 2008). The remarkable ability of *S. mutans* to tolerate and thrive during stressful conditions, particularly low pH, is closely linked to its virulence in the oral cavity.

The Clp proteolytic complex is critical in maintaining cellular homeostasis, particularly for organisms that must continually endure environmental fluctuations (Frees *et al.*, 2004; Gottesman, 2003; Jenal & Hengge-Aronis, 2003; Kajfasz *et al.*, 2009). In *S. mutans*, Clp proteases are the result of the association of the ClpP peptidase with one of several Clp ATPases (ClpC, ClpE or ClpX), forming barrel-shaped complexes that will target proteins for degradation (Kajfasz *et al.*, 2009; Lemos & Burne, 2002). Although *S. mutans* also encodes ClpB and ClpL ATPases, these proteins do not contain the recognition tripeptide that permits interaction with ClpP, and are believed to function mainly as molecular chaperones (Frees *et al.*, 2004; Kajfasz *et al.*, 2009). When Clp ATPase subunits associate with ClpP, the resulting protease performs an important protein quality control role by targeting damaged or misfolded proteins, threading them through its barrel for degradation (Butler *et al.*, 2006; Jenal & Hengge-Aronis, 2003). This

Abbreviations: CSP, competence-stimulating peptide; Erm, erythromycin; IPS, intracellular polysaccharide; Kan, kanamycin; MLF, malolactic fermentation; qRT-PCR, real-time quantitative reverse transcriptase polymerase chain reaction.

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Three supplementary figures and two supplementary tables are available with the online version of this paper.

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process is particularly important during stressful conditions that increase the likelihood of misfolded or damaged cellular proteins. Notably, Clp proteases also target regulatory proteins, thereby keeping their numbers in check, and providing a link between Clp proteolysis and regulation of gene expression (Frees *et al.*, 2004; Zuber, 2004).

Previously, we and others have demonstrated that ClpP plays an important role in the expression of key virulence attributes of *S. mutans*, including biofilm formation, cell viability and acid tolerance (Chattoraj *et al.*, 2010; Deng *et al.*, 2007; Kajfasz *et al.*, 2009; Lemos & Burne, 2002; Zhang *et al.*, 2009). We also uncovered some surprising phenotypic characteristics shared by strains bearing deletions in *clpP* or *clpX*, including enhanced survival under short- and long-term acidic conditions and increased sucrose-dependent biofilm formation (Kajfasz *et al.*, 2009). Additionally, we showed that the Spx global regulator accumulates in *S. mutans* strains lacking functional ClpXP proteolysis and that inactivation of either one of the two Spx orthologues, SpxA and SpxB, caused a reversion of many phenotypes observed in *S. mutans* Δ*clpX* and Δ*clpP* strains (Kajfasz *et al.*, 2009). Thus, the underlying mechanisms by which ClpXP affects virulence traits in *S. mutans* are intimately associated with accumulation of the SpxA and SpxB proteins. However, not all phenotypes associated with the *clpP* or *clpX* mutant strains are expected to be linked to Spx accumulation, as several distinct biological traits and regulatory circuits controlled by Clp proteolysis in other bacterial species are known to be Spx-independent (Frees *et al.*, 2007).

Although ClpP proteolysis has been consistently implicated in virulence (Frees *et al.*, 2003; Gaillot *et al.*, 2000; Ibrahim *et al.*, 2005), has shown promising results as a vaccine candidate (Kwon *et al.*, 2004) and is the target of two new classes of antibiotics (Böttcher & Sieber, 2008; Brötz-Oesterhelt *et al.*, 2005), a complete picture of the biological role of Clp-dependent proteolysis in bacterial pathogenesis has yet to emerge. As our previous study established a firm connection between ClpXP proteolysis and cellular accumulation of Spx proteins, our goals in this study were to unveil the scope of the ClpXP regulon and to identify and characterize virulence traits linked to functional ClpXP proteolysis in both Spx-dependent and Spx-independent manners. Microarray analysis provided insights into the pleiotropic effects of deletions of *clpP* and *clpX* in *S. mutans*, with expression of more than 10 % of the genome altered in each case. A follow-up physiological characterization of selected virulence traits of *S. mutans* identified in the microarrays validated the transcriptomic data and revealed that ClpXP proteolysis is involved in intracellular polysaccharide (IPS) production, malolactic fermentation (MLF), competence development and bacteriocin production.

METHODS

Bacterial strains and growth conditions. The strains used in this study are listed in Table 1. *S. mutans* UA159 and its derivatives were routinely grown in brain heart infusion (BHI) medium at 37 °C in a 5 % CO_2 atmosphere. When appropriate, kanamycin (Kan, 1 mg ml^{-1}) or erythromycin (Erm, 10 μg ml^{-1}) was added to the growth medium. For microarray analysis, *S. mutans* UA159 (wild-type) and its Δ*clp* derivatives were grown in BHI medium to mid-exponential phase (OD_{600} 0.5).

RNA extraction. RNA from *S. mutans* cells was isolated as described previously (Abranches *et al.*, 2006). Briefly, *S. mutans* cells grown to the desired OD_{600} were homogenized by repeated hot acid phenol/chloroform extractions. The nucleic acid was precipitated with 1 vol. cold 2-propanol and 0.1 vol. 3 M sodium acetate (pH 5) at −20 °C overnight. RNA pellets were resuspended in nuclease-free H_2O and treated with DNase I (Ambion) at 37 °C for 30 min. The RNA was repurified using an RNeasy mini-kit (Qiagen), including a second on-column DNase treatment as recommended by the supplier. RNA concentrations were determined in triplicate and samples were run on an agarose gel to verify RNA integrity.

Microarray experiments. *S. mutans* UA159 version 1 microarray slides were provided by the J. Craig Venter Institute Pathogen Functional Genomics Resource Center (PFGRC; http://pfgrc.jcvi.org/index.php/microarray). The microarray experiments and analysis were as previously described (Abranches *et al.*, 2006; Kajfasz *et al.*, 2010). Briefly, reference RNA was prepared from *S. mutans* UA159 cells that were grown in BHI medium to an OD_{600} of 0.5, and used in all hybridizations. cDNA samples generated from 2 μg RNA originating from four independent cultures of each strain studied were hybridized to the microarray slides, as was cDNA derived from the reference culture. cDNA was coupled to Cy3-dUTP (test samples) or Cy5-dUTP (reference samples; GE Healthcare). Mixtures of test and reference cDNA were hybridized to the microarray slides for 16 h at 42 °C in a MAUI (MicroArray User Interface) hybridization chamber (BioMicro Systems). Hybridized slides were washed and scanned using a GenePix scanner (Axon Instruments). Data were analysed using the T4 software suite available at the PFGRC website. Statistical analysis was carried out with BRB Array Tools (http://linus.nci.nih.gov/BRB-ArrayTools.html) with a cut-off *P*-value of 0.001. Additional details regarding array protocols are available at http://pfgrc.jcvi.org/index.php/microarray/protocols.html. Microarray data have been deposited in the NCBI Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo) under GEO Series accession number GSE29871.

Real-time quantitative PCR. A subset of genes was selected to validate the microarray data by real-time quantitative reverse transcriptase PCR (qRT-PCR). Gene-specific primers (see Supplementary Table S1, available with the online version of this paper) were designed using Beacon Designer, version 2.0, software (Premier Biosoft International). Reverse transcription and qRT-PCR were carried out according to previously described protocols (Abranches *et al.*, 2006; Lemos *et al.*, 2008). A Student's *t*-test was performed to verify significance of the PCR quantifications.

Construction of *glg* mutant strains. Standard DNA manipulation techniques were used as previously described (Lemos & Burne, 2002; Sambrook *et al.*, 1989). The primers used to isolate the mutants are listed in Supplementary Table S1. *S. mutans* strains lacking an approximately 2.4 kb region of the genome encompassing 1160 bases of the 3′ end of *glgB*, all 1146 bases of *glgC* and 100 bases of the 5′ end of *glgD* were constructed using a PCR ligation mutagenesis approach (Lau *et al.*, 2002). Briefly, PCR fragments flanking the 5′ end of *glgB* and the 3′ end of *glgD* were obtained and ligated to an erythromycin resistance (Erm^r) marker, and the ligation mix was used to transform *S. mutans* UA159. Mutant strains were isolated on BHI plates supplemented with Erm. Double Δ*clpP* (or Δ*clpX*) Δ*glg* strains were constructed by transformation of the single Δ*clp* strains with the *glg*

Table 1. Strains used in this study

Strain	Relevant genotype	Source or reference
S. mutans		
UA159	Wild-type	Laboratory stock
JL1 (Δ*clpP*)	*clpP*: : NP (non-polar) Kan	Kajfasz *et al.* (2009)
JL2 (Δ*clpX*)	*clpX*: : NPKan	Kajfasz *et al.* (2009)
JL29	UA159 + pMSP3535	This study
JL30 (Δ*clpP* complement)	JL1 + pMSP3535 expressing *clpP*	This study
JL31 (Δ*clpX* complement)	JL2 + pMSP3535 expressing *clpX*	This study
JL26 (Δ*glg* region)	*glgB glgC glgD*: : Erm	This study
JL27 (Δ*clpP*/Δ*glg* region)	*clpP*: : NPKan; *glg*: : Erm	This study
JL28 (Δ*clpX*/Δ*glg* region)	*clpX*: : NPKan; *glg*: : Erm	This study
Other species		
L. lactis ATCC 11454	Wild-type	Laboratory stock
S. gordonii DL-1	Wild-type	Laboratory stock

deletion PCR fragment obtained from the Δ*glg* strain. The deletions were confirmed as correct by PCR sequencing of the insertion site and flanking sequences.

IPS determination. Accumulation of stored IPS was evaluated by using a colorimetric assay that relies on the formation of an iodine–polysaccharide complex (Busuioc *et al.*, 2009). Briefly, bacteria were streaked on Todd–Hewitt agar plates containing 2 % glucose or 2 % sucrose and incubated for 48 h. The plates were then flooded with 5 ml iodine solution [0.2 % (w/v) iodine in 2.0 % (w/v) potassium iodide solution]. IPS was detected by the formation of a brown pigment, which was visible almost immediately upon adding the iodine solution.

Glycolytic pH minima. The ability of *S. mutans* strains to continue to undergo glycolysis in an increasingly acidic environment was evaluated by pH drop experiments (Belli & Marquis, 1991). Cultures of *S. mutans* strains grown to exponential phase were harvested by centrifugation, washed in ice-cold distilled water and resuspended in 10 % culture volume of 50 mM KCl, 1 mM $MgCl_2$ salt solution. KOH was used to titrate the suspensions to pH 7.2. Glycolysis was initiated with the addition of 55.6 mM glucose, and the resulting fall in pH of the suspension was monitored over a 30 min period. To study differences in sugar storage, changes in pH were also monitored immediately after cells were resuspended in salt solution, without addition of glucose.

Long-term survival. The ability of the *S. mutans* Δ*glg* strains to survive a period of several days at low pH was assessed via long-term survival assays, in which an overnight culture of cells was diluted 1 : 20 in tryptone-yeast extract (TY) medium containing excess glucose (50 mM) (Kajfasz *et al.*, 2009, 2010). The growth and pH of the cultures were monitored at 37 °C and 5 % CO_2 until stationary phase was reached, at which point an aliquot was removed for serial dilution and plating on BHI agar. Bacterial survival was assessed by plating the cultures daily until growth was no longer detected.

Malolactic fermentation assay. The MLF assay was performed as previously described (Martinez *et al.*, 2010; Sheng & Marquis, 2007). Briefly, overnight cultures were grown in BHI medium buffered to pH 7 with 25 mM KPO_4, then cells were harvested by centrifugation, washed with 50 mM KCl, 1 mM $MgCl_2$ salt solution, and starved for 1 h at 37 °C in half the original culture volume of salt solution. After the starvation period, cells were harvested by centrifugation and resuspended in half the culture volume of potassium phosphate buffer (20 mM, pH 7). To assay for MLF activity, the suspension was adjusted to pH 4.0 with HCl to achieve the optimal pH for MLF activity in *S. mutans* (Sheng & Marquis, 2007), and MLF was initiated by the addition of L-malic acid to a final concentration of 50 mM. Aliquots were removed immediately and 90 min following the addition of L-malic acid, and assayed for the presence of L-malate in the supernatant by using a L-malic acid detection kit (R-Biopharm). The values of L-malic acid metabolized were normalized to cell dry weight of the samples.

Assay of genetic competence. Cultures were diluted 1 : 20 in 500 µl BHI medium containing 10 % horse serum, and grown to an OD_{600} of 0.15 at 37 °C in a 5 % CO_2 atmosphere, at which point 200 ng plasmid pMSP3535 (Bryan *et al.*, 2000) was added. When desired, 5 µl (at 1 mg ml^{-1}) of synthetic competence-stimulating peptide (CSP) (Li *et al.*, 2001) was added to the cultures. The cultures were incubated until stationary phase was reached (≈3 h for UA159, ≈4.5 h for Δ*clpX* and ≈5 h for Δ*clpP*). Transformants and total c.f.u. were enumerated by plating appropriate dilutions on BHI agar plates with and without Erm, respectively. The numbers of c.f.u. were counted after 72 h of incubation, and transformation efficiency was expressed as the percentage of transformants among the total viable cells.

Deferred antagonism assay. Bacteriocin production was measured by assaying the ability of *S. mutans* to inhibit the growth of mutacin IV- and mutacin V-sensitive species, *Streptococcus gordonii* and *Lactococcus lactis*, respectively. Briefly, *S. mutans* cultures grown to an OD_{600} of 0.3 were spotted onto BHI agar and incubated for 24 h. Following incubation, 500 µl of an overnight culture of *S. gordonii* or *L. lactis* was added to 5 ml soft (0.75 %) BHI agar, spread as an overlay and incubated for another 24 h before zones of growth inhibition around the *S. mutans* spots were measured.

RESULTS

Microarray analysis provides novel insights into the scope of ClpXP proteolysis in *S. mutans*

To gain a better understanding of the phenotypes previously observed in the Δ*clpP* and Δ*clpX* strains (Kajfasz *et al.*, 2009; Lemos & Burne, 2002), microarray analysis was performed to compare the transcriptome of mid-exponential-phase cultures of each mutant with that of the parent strain. Prior to the microarray analysis, both Δ*clpP* and Δ*clpX* were complemented by providing,

respectively, the full-length *clpP* and *clpX* genes *in trans* using the nisin-inducible plasmid pMSP3535 (Bryan *et al.*, 2000). Several phenotypes characteristic of the Δ*clpP* and Δ*clpX* strains (Kajfasz *et al.*, 2009), including slow growth rates and aggregation in broth, were fully reversed by the complementation (Supplementary Figs S1, S2 and S3). At an assigned *P*-value of $\leqslant 0.001$ and applying a twofold change cut-off, there were 288 genes in the Δ*clpP* strain and 242 in Δ*clpX* that showed significant changes in relative transcript amounts as compared with the parent strain. The complete list of genes with altered expression in the Δ*clp* strains is provided in Supplementary Table S2. Of the 242 genes differentially expressed in Δ*clpX*, 113 (47 %) also appeared, following the same trend, in the Δ*clpP* microarrays. The genes found to be differentially expressed in only one of the two mutant strains are probably due to a ClpP-independent function of ClpX, or to ClpP interactions with another Clp ATPase partner (ClpC or ClpE). To facilitate data interpretation, the genes that appeared on these microarray analyses were grouped into functional categories (Fig. 1). A subset of the differentially expressed genes was selected and used for qRT-PCR analysis (Supplementary Table S1) for validation of the microarray data, and the results were consistent with the expression trends observed in the microarrays.

Several genes encoding ribosomal proteins, translation initiation factors and elongation factors were downregulated in both mutants, consistent with the slow-growth phenotype previously described for the two Δ*clp* mutant strains (Kajfasz *et al.*, 2009). Interestingly, an overall upregulation of genes involved in energy metabolism (*acoB*, *adh*, *mle*, *pckA*, *pfl*, among others) and in sugar uptake and metabolism (*glg*, *gtfB*, *mal*, *msm* and *scr*) was observed in the mutants, suggesting an increased need for ATP by these strains. Genes encoding putative peptidases or endopeptidases (*gcp*, *pepO*, *pepB*, *pepT*) were found to be upregulated in both Δ*clpP* and Δ*clpX* strains, a finding that suggests an attempt by the cells to compensate for the loss of ClpXP 'housekeeping' proteolysis.

Previous studies showed an increased capacity of the Δ*clpP* and Δ*clpX* strains to form biofilms when grown with sucrose (Deng *et al.*, 2007; Kajfasz *et al.*, 2009), suggesting that glucan production is enhanced in these strains. Our microarrays showed that expression of *gtfB*, encoding the glucosyltransferase B enzyme responsible for establishing the extracellular polysaccharide matrix along with *gtfC* and *gtfD* (Ooshima *et al.*, 2001), was upregulated more than fourfold in Δ*clpP*, and more than sixfold in Δ*clpX*. Western blot analysis using a polyclonal antibody against the *S. mutans* GtfB protein (Wunder & Bowen, 2000) confirmed that expression of GtfB was higher in Δ*clpP* and Δ*clpX* (data not shown).

The Δ*clpP* and Δ*clpX* strains exhibit enhanced accumulation of IPS

A common trend revealed by the microarray data was the enhanced expression of the *glg* genes, coding for the

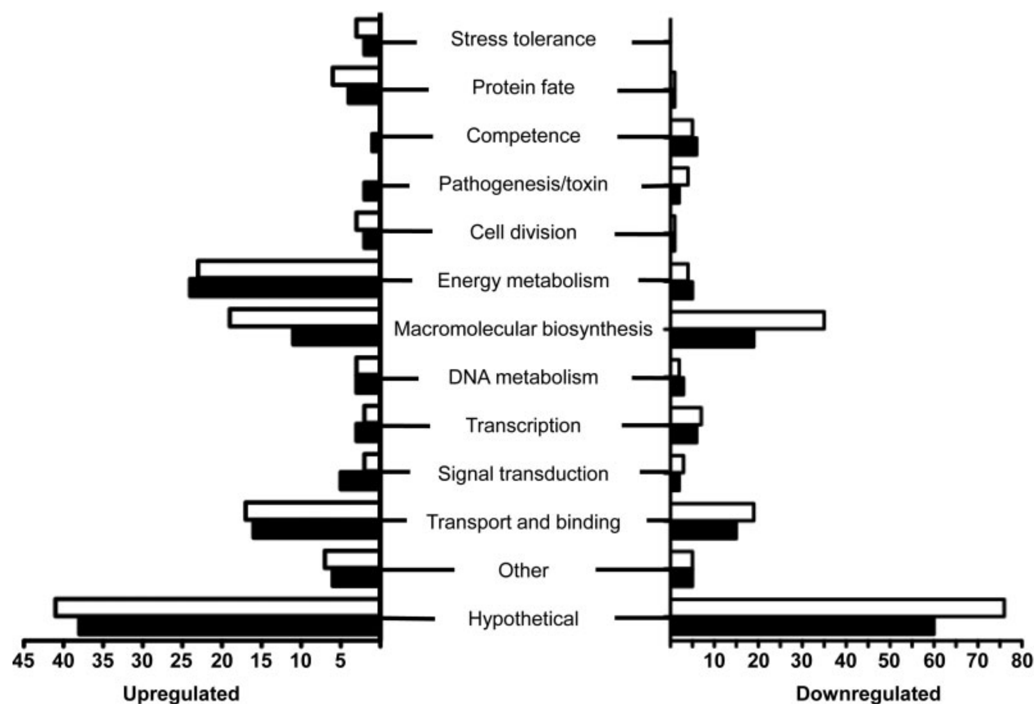

Fig. 1. Numbers of genes separated by functional categories that were differentially expressed by *S. mutans* Δ*clpP* (open bars) and Δ*clpX* (solid bars) as compared with the parent strain UA159 with a twofold cut-off ($P \leqslant 0.001$).

enzymes responsible for the production of glycogen-like IPS. To investigate whether the Δ*clpP* and Δ*clpX* strains have enhanced capacity to store IPS, we used an iodine-staining colorimetric assay to visualize IPS accumulation. Both Δ*clpP* and Δ*clpX* strains indeed showed enhanced accumulation of IPS, as demonstrated by a darker brown colour compared to the light brown colour observed in the parent strain (Fig. 2). To investigate whether these phenotypes in the Δ*clp* strains would revert when the *glg* operon was disrupted, we created double mutants by inserting the *glg* mutation (constructed by deleting a 2.4 kb region encoding all of *glgC*, as well as parts of *glgB* and *glgD*) in the Δ*clpP* and Δ*clpX* strains. As expected, the Δ*glg*, Δ*clpP*/Δ*glg* and Δ*clpX*/Δ*glg* strains showed no apparent incorporation of the iodine solution (Fig. 2). Similar results were observed with plates containing 2 % sucrose (data not shown).

To further demonstrate that the *clpP* and *clpX* mutants have enhanced IPS storage, we performed pH drop experiments to evaluate the ability of these strains to reduce the extracellular pH through glycolysis. When the pH drop experiment was performed by the addition of glucose to cells resuspended in salt solution, no differences in kinetics and final pH were observed between the parent, Δ*clpP* and Δ*clpX* strains (data not shown). To evaluate the consumption of endogenous sugars, the pH drop experiments were repeated without pH titration or the addition of exogenous glucose, so that any drop in the extracellular pH could be attributed to IPS utilization. In this case, both the Δ*clpP* and the Δ*clpX* strains were able to reduce the pH faster and to values considerably lower than those of the parent strain (over 0.5 pH units lower) (Fig. 3). As expected, the Δ*glg* as well as the Δ*clp*/Δ*glg* double mutants were unable to lower the pH without addition of exogenous glucose.

Increased production of IPS by the Δ*clp* strains does not appear to account for the enhanced survival phenotype

To test the hypothesis that enhanced IPS storage contributes to the prolonged survival of *S. mutans*, we repeated the long-term survival assay with the Δ*clpP* and Δ*clpX* strains (Kajfasz *et al.*, 2009), including the Δ*glg* and Δ*clp*/Δ*glg* strains that were unable to accumulate IPS. When grown in TY medium containing 50 mM glucose at 37 °C in a 5 % CO_2 atmosphere, all strains attained a similar final pH of approximately 4.2 within 18–24 h of initiating the experiment. However, we did not see a reversion of the enhanced-survival phenotype when a deletion in the *glg* operon was added to the Δ*clp* strains (data not shown), suggesting that enhanced IPS storage plays a minor role in the enhanced survival of the Δ*clpP* and Δ*clpX* strains.

MLF activity is significantly enhanced in the absence of ClpXP proteolysis

Our microarray analysis also revealed that the genes responsible for MLF, *mleS* (SMU.0137) and *mleP* (SMU.0138), were highly upregulated in both Δ*clpP* and Δ*clpX* strains (>10-fold induction, Supplementary Table S2). Recently, MLF has been identified as an important buffering system in *S. mutans* and shown to protect the cells against acid, oxidative and starvation stresses (Lemme *et al.*, 2010; Sheng & Marquis, 2007; Sheng *et al.*, 2010). The *mle* genes and enzyme activity are positively regulated

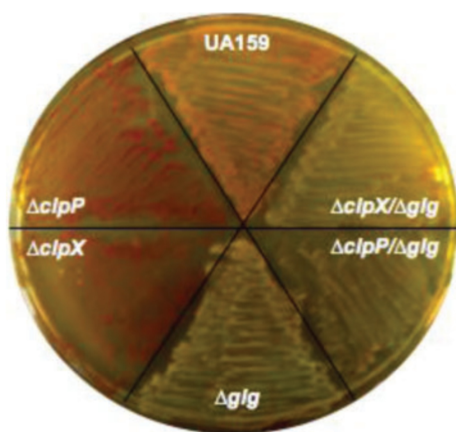

Fig. 2. Vizualization of IPS stores in *S. mutans* UA159 and its derivatives. Agar plates inoculated with *S. mutans* UA159, Δ*clpP*, Δ*clpX*, Δ*glg*, Δ*clpP*/Δ*glg* and Δ*clpX*/Δ*glg* were flooded with iodine solution. IPS was visualized by the formation of a brown pigment. The image shown is a representative of three independent experiments.

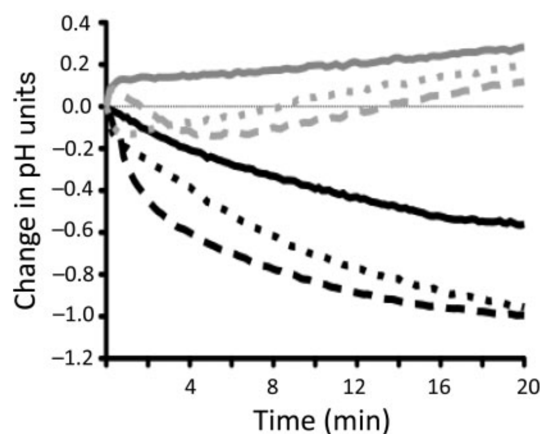

Fig. 3. Glycolytic pH minima of *S. mutans* UA159 (black solid line), Δ*clpP* (black dashed line), Δ*clpX* (black dotted line), Δ*glg* (grey solid line), Δ*clpP*/Δ*glg* (grey dashed line) and Δ*clpX*/Δ*glg* (grey dotted line). pH drop experiments were performed without addition of glucose to cells. The curves shown are representatives of at least three independent experiments.

by addition of external L-malate and by low pH (Lemme *et al.*, 2010; Martinez *et al.*, 2010; Sheng & Marquis, 2007). In agreement with our microarray data, MLF activity was minimal in the wild-type strain grown without addition of L-malate at pH 7 but a >10-fold increase in activity was seen in Δ*clpP* and Δ*clpX* strains grown under the same conditions (mean MLF activity reported as μmol L-malate decarboxylated during a 90 min period per mg cell dry weight: *S. mutans* UA159, 1.196; Δ*clpP*, 12.64, $P \leqslant 0.005$; Δ*clpX*, 13.53, $P \leqslant 0.0005$). When cultures were grown in the presence of 25 mM L-malate, the Δ*clpP* and Δ*clpX* strains also showed significantly enhanced ability to metabolize L-malic acid at both neutral and acidic (pH 5.5) pH values (data not shown).

ClpXP proteolysis regulates competence development and bacteriocin production

The microarray data also revealed that transcription of a number of genes known or predicted to be involved in competence development and bacteriocin production were downregulated in the Δ*clp* strains (Table 2). Among the known competence genes found to be downregulated were the sigma factor *comX*, several genes of the late competence *comY* operon and the newly described *comR* transcriptional regulator (Mashburn-Warren *et al.*, 2010). We assessed the transformation efficiency of Δ*clpP* and Δ*clpX* with and without the addition of exogenous CSP. In the absence of CSP, we were unable to obtain a single transformant colony of the Δ*clpP* and Δ*clpX* strains, whereas UA159 was readily transformable (3.05×10^{-5} % transformation efficiency). Addition of exogenous CSP re-established competence in Δ*clpP* and Δ*clpX* (1.2×10^{-4} and 2×10^{-5} % transformation efficiencies, respectively), but dramatically improved transformation efficiencies of the parent strain (8.7×10^{-3} %). Fig. 4 shows a typical example of the number of transformants obtained for each strain at the same serial dilution.

Among the bacteriocin-related genes that appeared in the microarray analysis were *nlmA* and *nlmB*, encoding the non-lantibiotic mutacin IV (Qi *et al.*, 2001), and *cipB* (*nlmC*), encoding mutacin V (Hale *et al.*, 2005; Matsumoto-Nakano & Kuramitsu, 2006). We performed a deferred antagonism assay, which revealed that the Δ*clpP* and Δ*clpX* strains have lost the ability to antagonize growth of *S. gordonii* or *L. lactis* by producing, respectively, mutacin IV or mutacin V (Fig. 5).

DISCUSSION

In the oral cavity, *S. mutans* must cope with large and constant environmental fluctuations, including changes in pH, oxygen tension, and nutrient source and availability (Lemos & Burne, 2008). In such an environment, cytoplasmic proteases that control the stability of regulatory proteins and prevent the accumulation of damaged proteins are central to physiological homeostasis and virulence. Clp-dependent proteolysis is of particular relevance in *Streptococcus*, as this genus lacks other known cytoplasmic proteases, such as Lon and ClpYQ (Kajfasz *et al.*, 2009). In fact, proteolysis mediated through ClpXP is an important trait for stress tolerance, gene regulation and virulence in several Gram-positive pathogens (Chastanet *et al.*, 2001; Chattoraj *et al.*, 2010; Frees *et al.*, 2003, 2004; Gaillot *et al.*, 2000; Ibrahim *et al.*, 2005; Kwon *et al.*, 2004). Previously, we demonstrated that a number of virulence properties of *S. mutans*, including stress tolerance, biofilm formation and colonization in a rodent caries model, were influenced by ClpXP proteolysis (Kajfasz *et al.*, 2009). Although we have shown that accumulation of the global regulators SpxA and SpxB was associated with a number of the phenotypes observed in the Δ*clpP* and Δ*clpX* strains (Kajfasz *et al.*, 2009), we reasoned that ClpXP would probably control a number of additional physiological properties of *S. mutans* in an Spx-independent manner.

Here, we used microarrays to better understand the global effects of ClpXP proteolysis in *S. mutans*. The large number of genes that were differentially expressed as compared with the parent strain supports the hypothesis that ClpXP is critical for *S. mutans* homeostasis, as the number of differentially expressed genes represents over 12 % of the *S. mutans* genome (Ajdić *et al.*, 2002). The large number of genes following the same trend in both mutants confirmed the cooperative nature of ClpP and ClpX, and is in agreement with the nearly identical phenotypes previously observed for the Δ*clpP* and Δ*clpX* strains (Kajfasz *et al.*, 2009). Surprisingly, the genes found to be differentially expressed in our Δ*clpP* arrays showed limited overlap with a previous microarray analysis that used an *S. mutans clpP* mutant generated using a markerless gene deletion system (Chattoraj *et al.*, 2010). Despite differences in growth conditions, microarray platform and mutant construction, the reasons for the small number of genes following the same trend in the current and previous study are not entirely clear.

Some of the trends that were uncovered by the microarray analysis support the relationship that we have previously demonstrated between ClpXP proteolysis and the transcriptional regulators SpxA and SpxB (Kajfasz *et al.*, 2010), which are targets of ClpXP proteolysis. For example, the genes encoding Gor and TrxB, two enzymes involved in oxidative stress responses regulated by Spx, were upregulated in the Δ*clp* strains. These same genes were downregulated in Δ*spxA* and Δ*spxAB* microarrays (Kajfasz *et al.*, 2010). Although this relationship between regulatory mechanisms of ClpXP and Spx is clearly important, the analysis described here also identified new genes and pathways that are under ClpXP proteolytic control in an Spx-independent fashion.

Our microarray analysis identified a large number of genes involved in sugar uptake and metabolism as being upregulated in the Δ*clp* strains. Among these were the genes encoding enzymes responsible for the biosynthesis of

Table 2. Competence- and bacteriocin-related genes differentially expressed in the Δ*clpP* and Δ*clpX* strains

Locus tag	Gene name	Definition	Expression relative to UA159	
			Δ*clpP*	Δ*clpX*
SMU.0061	*comR*	Rgg family, transcriptional regulator	0.119	
SMU.0150	*nlmA*	Non-lantibiotic mutacin IV A	0.121	
SMU.0151	*nlmB*	Non-lantibiotic mutacin IV B, GG-motif-containing peptide	0.223	
SMU.0259	*oppF*	Oligopeptide ABC transporter	0.246	
SMU.0283		GG-motif-containing peptide	0.226	0.129
SMU.0286	*nlmT*	ABC transporter, bacteriocin	0.309	
SMU.0287	*nlmE*	ABC transporter, bacteriocin	0.244	0.209
SMU.0423	*bsmC*	Putative bacteriocin	0.142	0.327
SMU.0499	*comFC*	Late competence gene		7.392
SMU.1811	*scnF*	ABC transporter, bacteriocin		0.192
SMU.1855	*hrdM*	Histidine kinase		0.377
SMU.1889		GG-motif-containing peptide	5.492	
SMU.1895		GG-motif-containing peptide	0.174	0.314
SMU.1896		GG-motif-containing peptide	0.133	0.284
SMU.1903		Hypothetical protein	0.095	
SMU.1904		Hypothetical protein	0.100	
SMU.1905	*bsmL*	GG-motif-containing peptide	0.102	
SMU.1906	*bsmB*	Putative bacteriocin, GG-motif-containing peptide	0.168	
SMU.1908		Hypothetical protein	0.047	
SMU.1909		Hypothetical protein	0.054	
SMU.1910		Hypothetical protein	0.027	
SMU.1912		Hypothetical protein	0.078	
SMU.1913	*blpL*	Putative immunity protein	0.068	
SMU.1914	*cipB*, *nlmC*	Mutacin V, GG-motif-containing peptide	0.183	
SMU.1916	*comD*	Histidine kinase	0.286	0.346
SMU.1981	*comG*	Late competence gene		0.216
SMU.1982		Hypothetical protein		0.096
SMU.1984	*comYC*	Late competence gene		0.354
SMU.1985	*comYB*	Late competence gene	0.354	0.094
SMU.1987	*comYA*	Late competence gene		0.220
SMU.1997	*comX*	Sigma factor ComX	0.227	

IPS, a glycogen-like polymer that serves as a storage form of carbohydrate (DiPersio *et al.*, 1978). The synthesis of IPS has been implicated in enhanced survival of *S. mutans* and has been shown to contribute to caries formation (Gibbons & Socransky, 1962; Spatafora *et al.*, 1995; van Houte *et al.*, 1970). Previous work revealed that production of IPS via *glgA* (encoding glycogen synthase) significantly extended the survival of *S. mutans* during starvation, especially when cells were grown in the presence of glucose (Busuioc *et al.*, 2009). The upregulation of several *glg* genes led us to hypothesize that the enhanced long-term survival of Δ*clpP* and Δ*clpX* (Kajfasz *et al.*, 2009) could be associated with increased IPS storage. Although we were able to demonstrate that the Δ*clpP* and Δ*clpX* strains indeed accumulate larger amounts of IPS when compared with the parent strain, no phenotypic reversion was seen when the Δ*clp*/Δ*glg* strains were subjected to long-term survival experiments. These results do not rule out that increased IPS stores play a role in the enhanced survival of Δ*clpP* and Δ*clpX* but indicate that other factors may participate in the enhanced survival of the Δ*clp* strains. By comparing the transcriptome of the Δ*clp* strains with the previously published Δ*spx* strains (Kajfasz *et al.*, 2010), we found no indication that the upregulation of the *glg* genes in the Δ*clpP* microarrays was linked to Spx regulation.

In a previous report, we showed that expression of *mleS* and *mleP* (encoding the malolactic enzyme and malate permease, respectively) was downregulated in the Δ*spxA*/Δ*spxB* double mutant (Kajfasz *et al.*, 2010). Here, we showed that transcription of *mleS* and *mleP* was highly upregulated (>10-fold) in both Δ*clp* strains, suggesting that the enhanced MLF activity of Δ*clpP* and Δ*clpX* may be associated with the accumulation of Spx in the absence of ClpXP proteolysis. Work is under way to specifically address the possibility that the *mle* operon is under Spx transcriptional control.

Although L-malic acid is not an energy source for *S. mutans*, MLF is a major process for alkali generation and for ATP synthesis by means of F-ATPase acting in the synthase mode (Sheng & Marquis, 2007), a process that confers protection against acid, oxidative and starvation

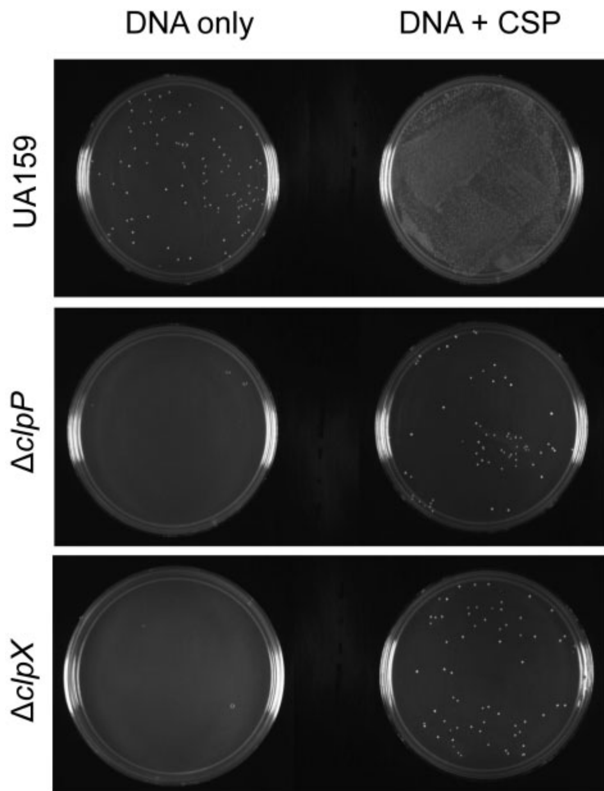

Fig. 4. Genetic competence of *S. mutans* UA159, Δ*clpP* and Δ*clpX*. Plasmid (pMSP3535) was added to early exponential-phase (OD_{600} of 0.15) cells with or without the addition of CSP, then plated at stationary phase. Images shown are representatives of three independent experiments.

stresses (Lemme *et al.*, 2010; Sheng & Marquis, 2007; Sheng *et al.*, 2010). Thus, it is possible that the enhanced acid tolerance of the Δ*clp* strains *in vitro* (Kajfasz *et al.*, 2009) may be linked to the increased MLF activity observed in these strains.

Another interesting finding was that a large number of genes involved in competence development and bacteriocin production were downregulated in the Δ*clp* strains. Coordinated competence development and production of bacteriocin have been well documented, suggesting that *S. mutans* utilizes competence-induced cell lysis to eliminate competition and acquire DNA from neighbouring species (Kreth *et al.*, 2005, 2007; Steinmoen *et al.*, 2003; van der Ploeg, 2005). The repression of competence genes in the *S. mutans* Δ*clpP* strain supports previous studies reporting reduced genetic competence of an *S. mutans clpP* mutant (Lemos & Burne, 2002). We assessed competence in Δ*clpP* and Δ*clpX* and found that transformation efficiency of both mutants was drastically reduced. Although only a few competence- and bacteriocin-related genes were differentially expressed in our Δ*spx* microarrays (Kajfasz *et al.*, 2010), studies with *Bacillus subtilis* and *Streptoccocus pneumoniae* show a link between Spx regulation and competence development. For example, the *B. subtilis* Spx negatively regulates competence development by disrupting interactions between the ComA~P response regulator and the C-terminal domain of the RNA polymerase (Nakano *et al.*, 2003). Likewise, the *S. pneumoniae* SpxA1, a protein sharing 75 % homology with the *S. mutans* SpxA, was shown to regulate competence in *S. pneumoniae* by repressing transcription of early competence genes (Turlan *et al.*, 2009). Thus, current evidence points towards a global model in which Spx negatively regulates competence development in Gram-positive bacteria, suggesting that the reduced transformation efficiencies of the *S. mutans* Δ*clpP* and Δ*clpX* strains may be linked to Spx accumulation.

Peptide bacteriocins are produced to inhibit the growth of other microbes sharing the same ecological niche. The *S. mutans* UA159 strain used in this study produces at least two types of bacteriocins: mutacin IV, encoded by *nlmA* and *nlmB*, and mutacin V, encoded by *cipB* (also known as *nlmC*). Mutacin IV is active against members of the mitis group of streptococci (Hale *et al.*, 2005; Qi *et al.*, 2001), whereas mutacin V is active mostly against non-streptococcal species (Hale *et al.*, 2005). In addition to the genes encoding mutacin IV and mutacin V, several other bacteriocin-related genes, including immunity factors and the locus encoding the ABC transporter required for

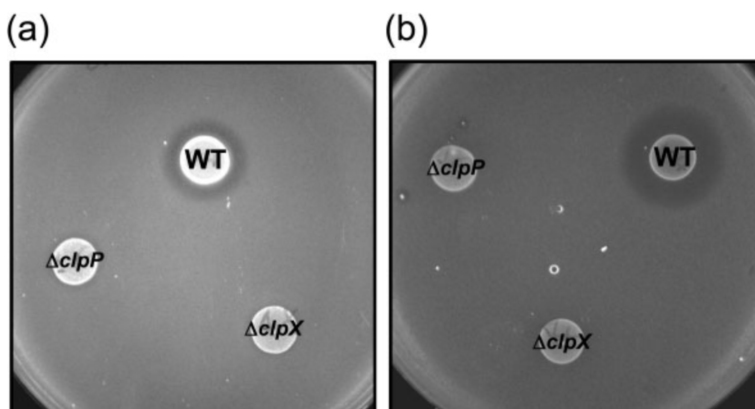

Fig. 5. Deferred antagonism assay. *S. mutans* UA159 (wild-type, WT), Δ*clpP* and Δ*clpX* were spotted onto agar plates and allowed to grow for 24 h before an overlay with the test strain was introduced. (a) Overlay with *S. gordonii* DL-1 (mutacin IV sensitive). (b) Overlay with *L. lactis* ATCC 11454 (mutacin V sensitive). The images shown are representatives of three independent experiments.

mutacin export, *nlmET* (Hale *et al.*, 2005), were downregulated in both Δ*clp* strains. Deferred antagonism assays confirmed the microarray results and revealed that both Δ*clpP* and Δ*clpX* were unable to inhibit growth of mutacin IV- and mutacin V-sensitive strains. Despite the limited overlap of genes found to be differentially expressed in our Δ*clpP* microarrays and in a previous report (Chattoraj *et al.*, 2010), it is interesting to note that the data regarding expression of bacteriocin-related genes and bacteriocin production between both studies were in full agreement.

A general characteristic of secreted peptides in Gram-positive bacteria is the presence of a highly conserved double glycine (GG) motif preceding the site where specific proteolytic cleavage of the signal peptide occurs during peptide export (Håvarstein *et al.*, 1995). The *S. mutans* UA159 genome encodes several putative peptides containing the conserved GG motif, including mutacin IV and V, and CSP. Notably, several other putative GG-motif-containing peptides were downregulated in the Δ*clp* strains, suggesting that ClpXP proteolysis may have a broad role in biosynthesis or maturation of secreted peptides. Collectively, these results identify ClpXP proteolysis as a participant in the regulation of competence development and bacteriocin production in *S. mutans*.

The coordinated activation of competence and bacteriocin production has been linked to *S. mutans* virulence via different mechanisms that are not mutually exclusive. For example, in addition to killing bacterial competitors, bacteriocin production has been linked to biofilm formation through extracellular DNA release and autolysis (Perry *et al.*, 2009a, b). Perry *et al.* (2009a) showed that intracellular accumulation of unprocessed CipB (mutacin V) mediates autolysis and proposed that CipB may be involved in altruistic cell death of a subset of the population (fratricide) during stressful conditions. It is tempting to speculate that the unexpected enhanced survival of Δ*clpP* and Δ*clpX* during acid stress (Kajfasz *et al.*, 2009) could, at least partially, be explained by a reduction in normal levels of mutacin V-mediated autolysis in these strains. In fact, we have previously shown that the Δ*clpP* and Δ*clpX* strains undergo autolysis at a slower rate than the parental strain (Kajfasz *et al.*, 2009). Our previous microarray data with *S. mutans* Δ*spx* strains did not offer additional insight regarding a direct role of Spx with bacteriocin production. However, given the relationship of Spx regulation with competence in other organisms (Nakano *et al.*, 2003; Turlan *et al.*, 2009), and the tight linkage between competence development and bacteriocin production in *S. mutans*, we cannot rule out that Spx may participate in the regulation of the genes involved in bacteriocin production in a manner that has yet to be determined.

In summary, the results presented here confirm the broad scope of ClpXP regulation in *S. mutans* homeostasis and identify virulence-related traits that are influenced by ClpXP proteolysis, including IPS storage, MLF activity and competence/bacteriocin production. Our data further confirm the importance of Spx removal by Clp proteolysis during unstressed conditions, as some of the identified virulence traits affected by ClpXP are probably regulated in an Spx-dependent manner. At the same time, it is clear that ClpXP can also influence the expression of virulence traits, such as IPS storage, in an Spx-independent manner. Considering the high degree of conservation among Clp proteins and given that ClpP-mediated proteolysis is an established virulence factor for several Gram-positive organisms (Frees *et al.*, 2003; Gaillot *et al.*, 2000; Ibrahim *et al.*, 2005; Kajfasz *et al.*, 2009), our findings are likely to have broad implications.

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