The putative autolysin regulator LytR in *Streptococcus mutans* plays a role in cell division and is growth-phase regulated

Christa H. Chatfield, Hyun Koo and Robert G. Quivey, Jr

Center for Oral Biology in the Aab Institute for Biomedical Sciences and Department of Microbiology and Immunology and Eastman Department of Dentistry, University of Rochester, Rochester, NY 14642, USA

*Streptococcus mutans* is the primary odontopathogen present in supragingival plaque and causes the oral disease known as dental caries. Colonization of the oral cavity by *S. mutans* requires the bacteria to adhere to the tooth surface and occurs by both sucrose-dependent and-independent mechanisms. Sucrose-independent adherence of *S. mutans* in vitro has been shown to involve an ORF (ORF0317) encoding a homologue (39 %) to LytR, a regulator of autolysin activity in *Bacillus subtilis*. The protein encoded by ORF0317, LytR, belongs to the LytR/CpsA/Psr protein family. This family has a putative role in cell-wall structural maintenance, possibly through autolysin regulation. Autolysins have also been shown to be important in surface adhesion in *Lactococcus lactis* and in the pathogenic properties of *Streptococcus pneumoniae*. To investigate the role of autolysins in the adhesion and pathogenesis of *S. mutans*, a LytR mutant was constructed. The mutant grows in long chains, which may indicate a defect in cell division. Further experiments with the mutant strain show increased autolytic activity, indicating that LytR attenuates *S. mutans* autolytic activity, possibly through regulation of the expression of autolytic enzymes. No defect in cell-to-surface adherence or biofilm growth was seen in the LytR mutant. However, a connection between cell growth phase and transcription of *lytR* was found.

INTRODUCTION

As a primary bacterial agent of dental caries, the mechanisms by which *Streptococcus mutans* adheres to tooth surfaces are important potential targets for anti-cariogenic intervention. Sucrose-dependent mechanisms of adherence, as mediated by extracellular enzymes (glucosyl- and fructosyltransferases) and glucan-binding proteins, have well-established roles in the virulence of *S. mutans* (Kuramitsu, 2001). Sucrose-independent mechanisms are less well-understood, including the potential modification/ regulation of the surface proteins of the organism.

Recently, an ORF, ORF0371 (http://www.oralgen.lanl.gov/) in the *S. mutans* genome (Ajdic et al., 2002), was shown to have an important role in sucrose-independent attachment to polystyrene surfaces (Wen & Burne, 2002; Yoshida & Kuramitsu, 2002). The protein encoded by ORF0371, LytR (BrpA; Wen & Burne, 2002) is a member of the LytR/CpsA/Psr protein family, a group of proteins that have putative cell-wall maintenance properties. LytR has 39 % homology to *Bacillus subtilis* LytR, a protein that attenuates the expression and activity of autolysin enzymes (Lazarevic et al., 1992). Although *lytR* is not located adjacent to a locus containing putative autolysin genes, mutations in *lytR* increase the lysis of *S. mutans* in the presence of Triton X-100 (Wen & Burne, 2002). Autolysins, enzymes with peptidoglycan hydrolase activity, are known to be part of the cell-wall maintenance of bacteria and must be carefully regulated to avoid cell lysis. Autolysin activity is important in the bacterial processes of cell division, peptidoglycan turnover, cell-to-surface adhesion, antibiotic resistance and spore development (Groicher et al., 2000; Mercier et al., 2002).

A requirement for autolysin activity to initiate cell-to-surface adherence has been seen in *Staphylococcus epidermidis* and *Lactococcus lactis*, suggesting that peptidoglycan breaks created by autolysin activity can function as adhesion molecules (Heilmann et al., 1997; Mercier et al., 2002). Autolysin activity and regulation is also involved in *Staphylococcus aureus* pathogenesis (Fujimoto et al., 2000; Rossi et al., 2003). Despite the importance of autolysin activity, mechanisms for its regulation have not yet been identified in *S. mutans*. The possible connection of autolysin activity to biofilm formation and tooth-surface adherence is unclear.
Recently, it has been shown that cell-density sensing systems, or quorum sensing systems, have an important and necessary role in the cell-to-surface adherence of S. mutans (Li et al., 2002; Wen & Burne, 2002). Two different quorum sensing systems have been found in S. mutans, and both appear to be vital in biofilm growth and cell-surface adherence. However, the genes that are responsible for these quorum sensing signalling responses have yet to be completely identified. Interestingly, the transcription of ORF0371 has been shown to be under the control the luxS gene (Wen & Burne, 2004), with decreased lytR (brpA) transcript present in a luxS mutant. Additionally, transcription of the msrR gene, a member of the LytR/CpsA/Psr family from Staphylococcus aureus, has been shown to be downregulated during late-exponential growth phase (Groicher et al., 2000; Rossi et al., 2003), and thus is possibly regulated by quorum sensing.

Because of these interesting links between growth phase, cell density and expression of the LytR/CpsA/Psr family, the experiments described in this study have included S. mutans cells from different growth phases. In addition, we present experiments to describe the role of LytR in the cell-to-surface adherence and autolysis of S. mutans. We have created a LytR-null strain and have examined the autolysis, adherence and biofilm growth properties of that strain. Additionally, we describe the growth-phase-dependent transcription of LytR.

**METHODS**

**Bacterial strains and growth conditions.** A strain of S. mutans UA159 (Murchison et al., 1986), defective in LytR, was constructed using PCR to amplify a 700 bp fragment of ORF0371 (lytR), using the following primer sequences: upstream primer 5'-ACCAATCTA-TTTCAT-3' (corresponding to +516 to +530 of the coding region) and downstream primer 5'-ATACCAATTCGCCTTCC-3' (corresponding to −1201 to −1219 of the coding region). Amplified fragments were cloned into the pGEM-T vector (Promega) and an erythromycin resistance cassette from pT19E (Aoki et al., 1986) was inserted into a unique NotI restriction site (located 720 bp from the start site of the coding region) within the lytR sequence. Plasmid DNA containing the construct was transformed into naturally competent Streptococcus mutans UA159 and recombinants were selected on BHI (Difco) medium plus erythromycin (5 μg ml⁻¹). Erythromycin insertion into lytR by homologous recombination was confirmed by Southern blot analysis (Southern, 1975) to create strain URLytR. This strain was then used to assess the role of LytR in the physiology of S. mutans.

For all experiments using bacteria from different growth phases, S. mutans was grown in BHI medium (Difco) at 37 °C in 95% air/5% CO₂ (v/v). Growth was monitored using optical density measurements at 600 nm (cuvette path = 10 mm; Beckman Spectrophotometer 640) and samples were collected at three different growth phases: early exponential (OD₆₀₀ = 0·2), mid-exponential (OD₆₀₀ = 0·5) and late exponential (OD₆₀₀ = 1·0).

**Chain length quantification.** Bacteria from each growth phase (as described above) were spotted onto silanized glass microscope slides (DAKO) and air-dried. After fixing for 5 min in 2·5% (w/v) glutaraldehyde, slides were washed with TE (10 mM Tris, 1 mM EDTA, pH 8·0) and visualized using differential interference contrast microscopy on a Nikon E800 upright microscope. Images of the bacteria at x 1000 magnification were digitally recorded with a SPOT2 camera. Three randomly chosen fields were chosen for counting and over 500 bacteria were counted for each growth phase (Iacono et al., 1985). The percentage content of chain lengths was calculated against the total number of chains counted for each of the three growth phase samples.

**Autoysis assay.** Bacteria were grown in BHI medium and collected at three different stages of growth (as described above) to assess the connection between growth phase and autoysis activity. After washing to remove medium, bacteria were resuspended in buffer (50 mM Tris, pH 8·0), sonicated to a single cell suspension and aliquoted to 24-well polystyrene plates (Corning). Bacteria were incubated for 20 h with 20 μg hen egg white lysozyme (HEWL) ml⁻¹, 50 μg (50 U) Streptomyces globisporus mutanolysin (SGM) ml⁻¹, or 0·2% (v/v) Triton X-100 (all Sigma) at 37 °C. Lysis of S. mutans UA159 and URLytR was monitored turbidimetrically after incubation with the cell-wall damaging agents. Percentage lysis was the optical density (OD₆₀₀) after incubation that exceeded the lysis by cells incubated in buffer only. The percentage lysed cells was calculated as 100 − (OD₆₀₀buffer)/ (OD₆₀₀buffer) × 100.

**Hydroxyapatite (HA) adherence assay.** Strains UA159 and URLytR were grown in TSBYE [4% (v/v) tryptic soy broth (American Bioorganics), 0·5% (w/v) yeast extract (Difco)] containing 5 μCi (1·85 × 10⁸ Bq) ml⁻¹ [³H]thymidine (Perkin-Elmer) at 37 °C in 95% air/5% CO₂ (v/v). Samples of bacteria were collected at three stages of growth as described above, washed with sterile water, resuspended to equal optical density and sonicated to a single cell suspension using a Branson Sonifier 450 (twice, each time consisting of three 10 s pulses with 5 s intervals at 50 W) as described elsewhere (Koo et al., 2002). Approximately 1·0 × 10⁹ cells was added to either non-conditioned HA beads, saliva-coated (sHA) or glucan-coated (gsHA) beads (Schilling & Bowen, 1992) and incubated, with shaking, at 37 °C for 1 h. Conditioning of the HA bead surfaces (80 μm diameter beads; Bio-Rad) was included to mimic aspects of the acquired enamel pellicle found on the tooth surface in the oral cavity. The experimental saliva/pellicle (saliva-conditioned HA) was formed on the HA surface by treating the beads with clarified, whole human saliva as described elsewhere (Clark et al., 1978; Schilling & Bowen, 1992). In addition, the saliva-coated beads were exposed to purified glucosyltransferase B, and the surface-adsorbed glucosyltransferase B was incubated with sucrose to deposit insoluble glucans on the bead surfaces (gsHA) (Schilling & Bowen, 1992). The adherence of both S. mutans strains was examined on each of the three distinct surfaces (HA, sHA, gsHA). The radiolabelled S. mutans cells were incubated with each of the HA bead preparations for 30 min. After incubation, the beads were washed three times with adsorption buffer (50 mM KCl, 1 mM KPO₄, 1 mM CaCl₂, 0·1 mM MgCl₂, pH 6·5) to remove non-adherent cells. The number of adherent bacteria on the beads was estimated by liquid scintillation counting as described elsewhere (Schilling & Bowen, 1992). Three independent experiments were performed and datasets were compared using the standard Student’s t-test (two-tailed distribution) at the 95% confidence level (P < 0·05).

**Biofilm growth and analysis.** Biofilms of UA159 and URLytR were grown on saliva-coated HA disks suspended vertically in TSBYE medium containing 1% (w/v) sucrose at 37 °C in 95% air/5% CO₂ (v/v). The disks were transferred into fresh medium daily for 5 days, after which biofilms were removed and sonicated to disperse individual cells, as described by Koo et al. (2003). The suspension was used to determine the biomass (dry weight) and the water-soluble and -insoluble polysaccharide fractions were
quantified colorimetrically as described by Koo et al. (2003). Three replicates were performed for each measurement; the data are presented as means ± SD.

**RNA purification and analysis.** *S. mutans* UA159 was grown as described for the autolysis assay above and total RNA was collected from cells. The procedure for RNA extraction was performed as described previously (Kuhnert & Quivey, 2003), except 5 M LiCl was used to precipitate total RNA. Total RNA samples were diluted 1:100 in TE (10 mM Tris, 20 mM EDTA, pH 6-7) and quantified by observing the A_{260/280} ratio by UV spectrometry (cuvette path = 10 mm; Beckman Spectrophotometer 640). Next, 0.5 μg total RNA was loaded into a slot blot apparatus (Bio-Rad), UV cross-linked to a Nytran membrane (Schleicher & Schuell) and hybridized to a lytR-specific DNA probe so that lytR-specific mRNA levels could be analysed. The lytR-specific DNA probe was prepared by PCR amplification of lytR DNA. To perform a control for RNA loading, the 16S rRNA signal was also measured using a 16S DNA amplicon. The resulting amplicons were then radiolabelled with [α-32P]dATP (Perkin Elmer) using the Random Primer DNA Labelling Kit (Invitrogen). The lytR-specific signal was quantified by using the Bio-Rad Quantity One Software for the Molecular FX phosphorimager (Bio-Rad).

**RESULTS AND DISCUSSION**

**LytR is a member of a conserved protein family**

Bioinformatic analysis of the LytR protein sequence showed that the deduced 407 aa sequence contains a domain (residues 80–238, Fig. 1) that defines the LytR/CpsA/Psr family of proteins (PF03816; http://pfam.wustl.edu). To date, no definite function has been assigned to the shared domain of the LytR/CpsA/Psr family, though it is postulated to be involved in the maintenance and regulation of cell-wall structure. Clearly, the proteins of this family are important in the general physiology of Gram-positive bacteria, since a broad phylogenetic distribution is found in the pfam database. Within the Streptococcaceae family, 76 proteins have been found to have this putative regulatory domain, and four family members are present in the *Mycobacterium tuberculosis* H37Rv genome. Also included in the pfam database are one sequence from the extremophile *Thermotoga maritima*, four sequences from *Bacillus anthracis* and five sequences from *Staphylococcus aureus*. Thus, the LytR/CpsA/Psr domain is vital for bacteria that survive in widely different environments and has been retained in the genomes of both pathogenic and non-pathogenic bacteria. We postulate that LytR is located outside of the cell and involved in the maintenance of cell-wall structure.

Additionally, the C terminus of LytR (residues 282–407, Fig. 1) contains a region high in serine and threonine residues (31 %), a composition believed to be associated with anchoring proteins to cell-wall peptidoglycan (Huard et al., 2003). LytR also has a short sequence in the N terminus (residues 7–29, Fig. 1) (Krogh et al., 2001) that may be a single transmembrane domain that would place the majority of the LytR C terminus outside of the cell membrane. This N-terminal domain structure is also quite common within the LytR/CpsA/Psr family.

---

**The LytR mutant strain has a defect in cell division**

To examine the potential role of LytR in cell-wall homeostasis we constructed a strain interrupted in the coding region of the protein. We found a significant increase in the chain length of the mutant strain, URLytR, when observed microscopically. Quantification of this phenotype shows that 30 % of the mutant chains were over 10 bacteria in length, whereas approximately 1 % of the wild-type chains were of the same length (Fig. 2b). This report quantifies and expands on the similar phenotype of a LytR (BrpA) mutant as described by Wen & Burne (2002). The completion of cell division required to liberate cells from the end of chains is a process that requires autolysin enzymes, which are regulated...
by LytR in B. subtilis (Lazarevic et al., 1992). The long-chain phenotype suggested that the URLytR strain has defects in autolysin activity, leading to defects in the completion of S. mutans division, though no changes in the growth rate of URLytR were observed (Fig. 2a).

**The LytR mutant shows increased autolysin activity**

In B. subtilis, LytR is an attenuator of autolysin transcription, and in Staphylococcus aureus the absence of LytR increases cellular rates of autolysis (Brunskill & Bayles, 1996; Lazarevic et al., 1992). To further examine the extent of the autolysis of S. mutans UA159 versus URLytR, cellular lysis was monitored turbidometrically after incubation with HEWL, Triton X-100 and SGM, agents known to induce cellular lysis, possibly through direct activation of autolysin activity (Cornett & Shockman, 1978). Strains were grown in BHI medium to early-, mid- and late-exponential growth phases to assess connections between growth phase and autolysis activity. We found that HEWL induced up to 80 % lysis of S. mutans URLytR (Fig. 3b) and approximately 36 % lysis of UA159 (Fig. 3a). This may mean LytR is a significant factor in the reported resistance of S. mutans to lysis by HEWL (Goodman et al., 1981a, b).

Triton X-100 is a surfactant that induces bacterial cell lysis through the activation of autolysin enzymes (Cornett & Shockman, 1978). Incubation of S. mutans with 0-2 % (v/v) Triton X-100 also induced more lysis of URLytR (70 %, Fig. 3b) than UA159 (30 %, Fig. 3a). Triton X-100-induced lysis of LytR (BrpA) mutants has been shown by Wen & Burne (2002), though we expand upon that study by conducting this experiment on cells from different growth phases.

SGM is a commercially available mixture of enzymes purified from Streptomyces globisporus that contains protease and endo-N-acetylmuramidase activities that are especially potent against the S. mutans cell wall (Hamada et al., 1978; Yokogawa et al., 1974). Incubation of cells with 50 U SGM ml⁻¹ resulted in a maximum lysis of 99 % of intact URLytR (Fig. 3b) and a maximum lysis of approximately 70 % of UA159 (Fig. 3a). Taken together, the increased sensitivity of URLytR to three different cell-wall-damaging agents might be a result of increased...
autolysin enzyme activity in the strain when responding to cell-wall stressors.

These data suggest LytR is a negative regulator of \emph{S. mutans} autolysins, though no protein homology exists to suggest a mechanism of regulation. No autolysin enzymes have been identified experimentally in \emph{S. mutans} that might be targets of LytR. However, it is known that in \emph{Staphylococcus aureus}, LytR is part of a regulatory system that is responsible for regulating expression of the \textit{lrgA} and \textit{lrgB} genes. These genes are postulated to be necessary for export and activity of autolysin enzymes, perhaps forming holin-like pores in the cell membrane (Fujimoto et al., 2000). Homologues to \emph{Staphylococcus aureus} \textit{lrgAB} are found in the \emph{S. mutans} genome and future experiments will attempt to determine if these are regulatory targets of LytR. Additionally, an increase in lysis due to autolysin activity seems to contradict the chain length data in Fig. 2(b), where autolysin activity leading to cell division seems to have decreased. The downstream regulatory targets of LytR are not known, but the regulon may include genes that lead to inhibition of the cell division process that do not hinder the lytic events measured in Fig. 3.

This autolysis assay also revealed an increase in resistance to lysis as the bacteria moved into the late-exponential growth phase (Fig. 3a, b). A similar effect has been reported in \emph{Enterococcus hirae} (Sapunaric et al., 2003). It may be that as cellular division and cell-wall turnover slow, the abundance of autolysin enzymes or the activity of autolysins decreases. Alternatively, the activation of density-dependent signalling events may lead to altered cell-wall structure or decreased autolysin expression.

**Sucrose-independent adherence is growth-phase-dependent**

It is known that autolysin enzymes are important, and necessary, for cell-to-surface adherence of \emph{L. lactis} (Mercier et al., 2002). To explore the role of \emph{S. mutans} LytR in the initiation of cell-to-surface adherence, we measured the ability of the mutant and wild-type strains to bind to HA beads over a very short time period (1 h). HA is an important surface to include in \emph{S. mutans} adherence studies because it is very similar to the calcium mineral composition of human tooth enamel and thus mimics \textit{in vivo} conditions. This assay tested sucrose-independent mechanisms of adherence by exposing the bacteria to both HA beads alone and salivary-protein-coated HA beads (sHA), two conditions where no sucrose or glucan polymers are present. The use of human salivary proteins provided an experimentally acquired enamel pellicle, a surface conditioning of the tooth surface in the oral cavity that is known to provide targets for adherence of \emph{S. mutans} (Clark & Gibbons, 1977). A third condition in the assay used glucan-conditioned HA beads (gSHA), which included a layer of glucan polymers coating the beads and was, therefore, a test of sucrose-dependent adherence. Interestingly, after full replicate datasets were analysed, URLytR exhibited no significant difference from the wild-type UA159 in initial adherence (Fig. 4a, b, c), regardless of the surface conditioning of the HA beads. These results differed from previous reports where LytR mutants were shown to be defective in adherence to plastic surfaces (Wen & Burne, 2002; Yoshida & Kuramitsu, 2002). The present data suggest that there are fundamental differences in the adherence of \emph{S. mutans} to polystyrene versus HA beads, perhaps due to the difference in surface hydrophobicity of the two surfaces.

The use of cells from three phases of growth allowed us to examine the connection between cell-to-surface attachment and growth phase, something that has not been established previously. We found that sucrose-independent

![Fig. 4. Quantification of the initial adherence of \emph{S. mutans} UA159 (black bars) and URLytR (white bars) to HA surfaces. Bacteria were radiolabelled with [\textsuperscript{3}H]thymidine and collected from different growth phases (early-, mid- and late-exponential). Single cell suspensions were incubated with HA beads for 1 h. Adherent cells were quantified by liquid scintillation counting and normalized to cell number. The first two HA preparations, (a) HA beads alone and (b) sHA beads (human salivary protein-coated), were used to assay sucrose-independent adherence. The final condition, (c) gSHA (glucan-coated), was used to assay sucrose-dependent adherence. Data presented are from a single replicate, representative of trends seen in all three replicates. A standard Student's \textit{t}-test analysis (\(P<0.05\)) showed no significant differences between strains within HA bead preparations across all replicates.](image-url)
adherence (Fig. 4a, b) decreased significantly as the cells entered the mid- and late-exponential growth phases, suggesting that some adhesion molecules functioning in sucrose-independent conditions are downregulated by the changing growth rate or by density-dependent signalling pathways. This was not the case under the sucrose-dependent assay conditions (Fig. 4c), where growth-phase changes had a very small effect on adherence for both strains. These results highlight the already well established fact that sucrose, and the glucan polymers created from it, are important targets for adherence of S. mutans (Schilling & Bowen, 1992), so important that the sucrose-dependent adhesion molecules appeared to be expressed constitutively.

**Biofilm formation is not affected by loss of LytR**

This HA adherence assay only quantified the initial adherence of these strains, a very small portion of the total biofilm growth process. Other studies have shown biofilm formation is a complex process requiring many different gene products (Davey & O’Ttoole, 2000; Sauer & Camper, 2001) and previous studies have shown that global protein expression in S. mutans biofilms is considerably different to that in planktonic suspensions (Svensater et al., 2001). We were interested in the possibility that the URLytR strain might be unable to produce elements necessary for successful mature biofilm growth and survival. Therefore, we grew both strains in a late-stage, single-organism biofilm model, using saliva-coated HA disks suspended in rich medium and continuing the biofilm cultures for 5 days. Fresh medium was supplied to the biofilms once daily and sucrose was included to induce the production of glucans and allow for stable long-term surface adherence. Comparison of the wild-type and the lytR mutant strains for growth yield on saliva-coated HA disks (dry wt) showed no differences in three replicates (UA159, 6.4 ± 2.0 mg cell material per disk; URLytR, 6.2 ± 1.9 mg cell material per disk). The strains were also very similar in their abilities to produce water-soluble (UA159, 6.1 ± 2.7% dry wt; URLytR, 6.7 ± 2.8% dry wt) and water-insoluble polysaccharide (UA159, 25.5 ± 4.6% dry wt; URLytR, 27.4 ± 6.1% dry wt). Thus, LytR does not appear to play a vital role in the general physiology and growth of S. mutans in single-organism biofilms.

**Transcription of lytR is growth-phase-dependent**

Having observed that sensitivity to autolysin degradation was dependent on cell growth phase, we postulated that lytR transcription might also be growth-phase-controlled. Transcriptional regulation of lytR expression was determined by both slot blot (Fig. 5) and Northern blot analyses (data not shown). The results showed clearly that the lytR transcript was regulated in a growth-phase-dependent manner, with highest expression at early- and mid-exponential growth phases, and a decrease of almost an order of magnitude at late-exponential phase (Fig. 5). These results follow from studies showing that lytR transcription is decreased in the absence of the LuxS quorum sensing system (Wen & Burne, 2004), a signalling cascade that could result in downregulation of lytR during late-exponential growth, when cells are at high density. Similar patterns of transcriptional growth phase regulation have been seen with the mssR gene of Staphylococcus aureus and the acmB gene of L. lactis, a regulator of autolysins and an autolysin enzyme, respectively (Huard et al., 2003; Rossi et al., 2003). Thus, the transcriptional regulation of lytR expression suggests that it is part of autolysin regulation and cell-wall-remodelling while the cells are rapidly dividing.

Despite the decreased lytR transcript during late-exponential growth, and thus a predicted increase in autolysin activity due to loss of LytR negative regulation, UA159 showed a decrease in autolysin-induced lysis during the late-exponential growth phase (Fig. 3b). The effects of the many environmental factors (pH, nutrient limitation, dissolved oxygen concentration, etc.) that fluctuate as the cells enter different growth phases are not clear at this point. Indeed, two different quorum sensing systems are known to be active in the life cycle of S. mutans and the downstream targets of these are still being described. Once the targets of LytR regulation are better understood, the reasons for the late-exponential phase autolysis resistance of UA159 may become clear.

**ACKNOWLEDGEMENTS**

The authors wish to thank Dr Robert Marquis for helpful discussion throughout. The authors also thank Ms Roberta Faustoferi for her technical expertise. This work was supported by grants from the NIH,
REFERENCES


