Environmental Virulence Regulation in *Streptococcus mutans*

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Summary: *Streptococcus mutans (S. mutans)* is an inhabitant of the normal flora of human dental plaque, and is considered the major etiological agent of caries. *S. mutans* has the ability modulate its physiology in response to changing environmental conditions. This review discusses the acid tolerance response pathway and quorum sensing systems that are two major conduits by which *S. mutans* accomplishes these physiological modulations.

Key words: mutans, acid, quorum sensing

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INTRODUCTION

Bacteria's ability to sense and respond to their environment is paramount to their survival within a particular ecosystem. Human dental plaque is a complex ecosystem in which the diverse bacterial consortium must withstand wide-ranging environmental conditions including variable nutrient availability, oxidative stress, pH fluctuations, and physical sheer forces. *S. mutans*, a normal plaque inhabitant, is able to sense and respond to the changing environment, and under certain conditions is able to dominate specific ecological niches. The domination of a niche by *S. mutans* and its corresponding acid production leads to the initiation of dental caries. Pivotal to this cariogenicity/virulence are acidogenesis, aciduricity, and the ability to form the biofilm, dental plaque.

In 1890, Miller was the first to recognize that carbohydrate metabolism by oral bacteria resulted in the generation of metabolic acid end products know to have a central role in the process of dental caries. Since that time *S. mutans* has been implicated as the major etiological agent of caries. Because *S. mutans* is highly acidogenic, the introduction of fermentable dietary carbohydrates into the mouth results in a rapid drop in plaque pH down to as low as pH 3 (Jensen et al, 1982). Frequent carbohydrate intake leads to a sustained drop in local pH leading to decalcification of tooth enamel and subsequently the progression of caries. Parallel to this robust acidogenecity, *S. mutans* has evolved a highly elaborate system allowing it to tolerate these large pH decreases. *S. mutans* utilizes both constitutive and acid inducible mechanisms for acid tolerance. The constitutive mechanisms include the F_1F_0-ATPase proton pump, a broad pH optimum of the glycolytic enzymes, and rapid efflux of acid metabolic end products (Bender et al, 1986; Hamilton and Buckley, 1991; Carlsson and Hamilton, 1996; Daspher and Reynolds, 1996).

The acid-inducible mechanisms are collectively referred to as the acid-tolerance response (ATR). The ATR is best visualized as increased survival at apparent sub-lethal pH upon pre-exposure to an adaptive acid environment (pH 5.5) (Svensäter et al, 1997). Several proteins that have shown to be important in acid tolerance have been characterized. Among these proteins are the molecular chaperones GroEL and DnaK (Jayaraman et al, 1997; Lemos et al, 2001). Also showing genetic up-regulation during acid are the DNA repair proteins UvrA (Quivey et al, 1995) and RecA (Hanna et al, 2001). The cell membrane's fatty acid composition is also modified by acid challenge, where the fatty acid profile at pH 7 consists of short-chained, saturated fatty acids compared to that of the long-chained, monounsaturated profile at pH 5 (Fozo and Quivey, 2004).

Work by Svensäter et al (1997) was the first to show that protein synthesis is an essential component of the ATR in *S. mutans*. Other recent work utilizing two dimensional gel-electrophoresis and mass spectroscopic analysis has identified 123 proteins that were up-regulated at least 1.5 fold during chemostatic growth at pH

5 compared to pH 7 (Len et al, 2004a,b). Of these proteins, 70 were associated with *S. mutans* metabolism indicating that metabolic regulation is an important component of the ATR (Len et al, 2004a).

Dental plaque is a complex, highly structured ecosystem composed of over 400 bacterial species. These bacteria are predominantly encapsulated in a polysaccharide matrix (Paster et al, 2001). Central to plaque formation is the ability of the bacteria to attach to the tooth surface or the polysaccharide matrix. Oral bacteria do not bind to the teeth directly but adhere to the acquired pellicle, which is a thin coating of salivary glycoproteins, mucins, and other proteins covering the teeth. *S. mutans* mediates its attachment through three main mechanisms, including antigen I/II, glucosyltransferases (GTFs), and glucan-binding proteins (GBPs) (reviewed in Banas, 2004). The biofilm 'lifestyle' is characterized by modulation in gene expression and cellular physiology compared to planktonic growth. These changes allow *S. mutans* to withstand various environmental stressors, and adjust its physiology accordingly.

Many bacterial species, both Gram-positive and Gram-negative, sense their environment and regulate their physiology via quorum sensing systems. *S. mutans* utilizes its quorum sensing system to modulate the expression of molecular pathways integral to the development of caries. The *S. mutans*' quorum sensing system is typical of other Gram-positive bacteria as it contains three key components. These components include a signal peptide (called competence-stimulating-peptide (CSP) in *S. mutans*), a membrane-bound histidine kinase and an intracellular response regulator (Li et al, 2001a). The histidine kinase and the response regulator are referred as a two-component signal transduction system. These are encoded in the *S. mutans* genome by the *com*CDE locus (Li et al, 2001a). The *S. mutans* quorum sensing system, mediated through CSP, has been linked to many different types of altered cell physiology (Li et al, 2001a,b, 2002a,b).

Bacteria have various mechanisms to sense and respond to the varying conditions of their particular ecological niche. In order to inhabit, and in certain instances dominate, the human plaque ecosystem, *S. mutans* has evolved two broad sensory systems. These are the acid tolerance response and quorum sensing. The goal of this review is to highlight the environmental virulence regulation afforded by the ATR and quorum sensing to *S. mutans*.

THE ACID TOLERANCE RESPONSE

Acid Tolerance Mediated via the DNA-Repair Protein UvrA

A major component of the *S. mutans* ATR is the repair of acid-induced damage to cellular components including protein and DNA. Two protein repair chaperones, DnaK and GroEL, have shown importance in acid survival of *S. mutans* (Jayaraman et al, 1997; Lemos et al, 2001). The DNA damage regulatory-repair protein RecA has also been implicated in *S. mutans* ATR (Quivey et al, 1995).

Recent data have strengthened the link between ART and DNA repair in *S. mutans*. Hanna et al (2001) have, in *S. mutans*, implicated the enzyme UvrA to be responsive for the repair of acid-damaged DNA. The UvrABC protein complex, in which UvrA is a component, is activated by distortions of the DNA helix, and is thought to be responsible for the excision of large DNA fragments (Sancar, 1996). In *S. mutans*, UvrA was shown to be up-regulated at the transcriptional level during acid-adaptation to a pH 5.0 environment relative to pH 7.5 (Hanna et al, 2001). These results were visualized utilizing both differential-display PCR as well as via RNA dot plots where acid induced (pH 5.0) cells were compared to pH 7.5 cells. Further evidence supporting for the involvement of *UvrA* is shown by the diminished survival of *UvrA* -knockout mutants at pH 3.0 following a 2 hour acid adaptation at pH 5.0 (Hanna et al, 2001). Compared to wild-type UA159, the corresponding *UvrA* -strain displayed a ten-fold decrease in acid-adapted survival (Hanna et al, 2001). This mutant also had impaired growth at pH 5.0 compared to that of UA159. A *UvrA* -strain showed large-scale genomic DNA degradation at pH 4.0 compared wild-type proving *UvrA* to be essential for the maintenance of DNA integrity at low pH (Hanna et al, 2001). Interestingly acid-adapted wild-type cells also had increased resistance to ultra-violet light damage indicating the ATR can afford cross-protection against other damaging stresses (Hanna et al, 2001). This work confirms *S. mutans*' UvrA is acid-inducible, involved in the ATR and acid survival, and maintains DNA integrity at low pH.

In order to access whether the mutation in *UvrA* and the resultant *in vitro* phenotype was truly defective in causing caries *in vivo* a rat study was initiated. The effects of infection and colonization of sucrose-containing-diet-fed Osborne-Mendel rats by the *S. mutans UvrA* mutant and the parental strain UA159 were compared for smooth surface plaque, smooth and fissure caries as well as an assessment of recovered oral microbiota essentially as described for the *S. mutans vicK* mutant (Senadheera et al, 2005, see later). Surprisingly a significant increase ($Pf < LSD = 0.001$) in smooth surface plaque extent was observed, while initial and advanced dentinal fissure and smooth surface caries lesions were no different than those observed with the wild type *S. mutans* strain UA159 in rats kept under the same conditions. This data raises several questions as to the relevance of *in vitro* acid tolerance assays in assessing cariogenic potential of *S. mutans* mutants suggest-

ing that a cautious approach must be taken when extrapolating *in vitro* data to a potential decrease in the virulence of the bacterium.

The Role of Citrate in Acid Tolerance

Human saliva is low in carbohydrates and thus *S. mutans* acquires the vast majority of its nutrition from human food consumption. This results in cycles of high and low nutrient availability. *S. mutans* has evolved methods to adapt to these fluctuations. These include a diverse array of sugar transport systems and a highly efficient glycolytic cycle, as well as the ability to produce glucan and glycogen for carbohydrate storage.

The human diet not only subjects members of the oral flora to protein and carbohydrates, but to varying pH environments and differing organic acids. One common dietary organic acid present in fruit juices is citric acid. In lactic acid bacteria citrate transport has also been linked to increased survival in acidic conditions (Aranha et al, 1982). *S. mutans*, like many other bacterial species, including *Bacillus subtilis, Klebsiella pneumoniae*, and *Lactococcus lactis (L. lactis)*, has the ability to transport and metabolize citrate (Carlsson and Hamilton, 1994; Bashir and Lagerlöf, 1996; Daspher and Reynolds, 1996; Korithoski et al, 2005). Unlike the citrate transporters in other Gram-positive, the *S. mutans* citrate transporter, CitM, preferentially utilizes ferric ions as cofactors. *S. mutans* has a nutritional requirement for trace metals including iron (Aranha et al, 1982), therefore citrate and its transporter CitM may play a part in iron acquisition.

Korithoski et al (2005) were the first to describe citrate metabolism by *S. mutans*. This metabolism results in the conversion of citrate into aspartate mediated through the citrate lyase and aspartate transferase enzymes. Citrate does appear to enter central metabolism and does not seem to provide any growth or survival benefit under acidic conditions (Korithoski et al, 2005).

In *S. mutans*, at a physiologically plausible concentration, 10 mM, citrate negatively affects growth and survival at pH 5.0, contrary to the advantages citrate provides other lactic acid bacteria. At pH 5.0 the presence of citrate caused increased killing of the wild-type UA159 compared to the killing induced in the corresponding citrate transport knockout mutant indicating that citrate induces killing via both intracellular and extracellular processes. Interestingly, pre-exposure of *S. mutans* to citrate at neutral pH enhanced both acid survival and citrate survival at pH 5.0. Citrate at neutral pH could induce the ATR and also a citrate tolerance response (Korithoski et al, 2005).

Acid Tolerance and Metabolic Modulation

Recent studies have suggested that the modulation of metabolic processes in *S. mutans* is important for acid tolerance. Two-dimensional gel electrophoresis studies of the *S. mutans* proteome revealed that a number of proteins were differentially expressed when the cells were grown under acidic conditions. Three enzymes that were shown to be up-regulated by these studies were pyruvate dehydrogenase A (PdhA), enolase (Eno), and lactoylglutathione lyase (Lgl), suggesting that these enzymes might be important in *S. mutans* acid tolerance (Wilkins et al, 2002; Len et al, 2004a; Welin et al, 2004).

Currently little is known about the role of Lgl in Gram-positive bacteria. Lgl is believed to be involved in detoxification via formation of *S*-D-lactoylglutathione from the hemimercaptal adduct that forms non-enzymatically between glutathione and the 2-oxoaldehyde methylglyoxal (Frickel et al, 2001). Glyoxalase II then converts *S*-D-lactoylglutathione into reduced glutathione and D-lactate (Frickel et al, 2001). Methylglyoxal modifies both proteins and nucleic acids and therefore its removal is vital to cell survival. Macrophage engulfed *Salmonella* express elevated levels of an Lgl homologue compared to cells grown in liquid culture concentration of methylglyoxal (Eriksson et al, 2003), is also greater in highly metabolically active human red blood cells, and Lgl expression appears to be regulated by the rate of glycolysis (Thornalley, 1988; Phillips and Thornalley, 1993). There is interest in the lactoylglutathione lyase pathway as a potential target for anti-tumour drug. These tumouricidal agents are designed to specifically target this pathway in order to induce elevated concentrations of cytotoxic methylglyoxal in tumour cells (Creighton et al, 2003).

The function of the pyruvate dehydrogenase complex has been extensively studied in *L. lactis*. This complex is composed of 4 subunits, that function to convert pyruvate to acetyl-CoA (Snoep et al, 1992; Jensen et al, 2001). In *L. lactis* it is thought that the pyruvate dehydrogenase complex is involved in shifting metabolism from homofermentation to heterofermentation under aerobic conditions and is regulated by glycolytic flux (Snoep et al, 1992; Jensen et al, 2001). Work completed by our collaborator Dr. G. Svensäter has revealed that the PdhA protein is more abundant under acidic than pH neutral conditions in *S. mutans* (personal communication).

Enolase is an integral enzyme in the central metabolic pathway. Enolase is a 47-kDa cytoplasmic enzyme in the glycolytic pathway responsible for the conversion of 3-phosphoglycerate into phosphoenolpyruvate (PEP). PEP is the first phospho donor of the major glucose uptake system, PEP:PTS, in *S. mutans*. Recent work has shown cell surface associated enolase is involved in *S. mutans* adhesion via binding salivary mucin MG2 (Ge et al, 2004).

Recent research performed in our laboratory has invested the relationship between Lgl, PdhA, and Eno. These investigations have shown that the corresponding

isogenic mutants, designated LGL-, PDHA-, ENO-, had reduced growth capacity at pH 5.0 compared to the wild-type strain. The Lgl enzyme is involved in the detoxification of methylglyoxal as seen by the hypersensitivity of LGL- to methylglyoxal, and by the obliteration of enzyme activity in LGL-. The *lgl, pdhA*, and *eno* genes are up-regulated during growth at pH 5.0 compared to growth at pH 7.5.

QUORUM SENSING

ComCDE

S. mutans regulates many of its physiological processes via quorum sensing. Like other Gram-positive bacteria, the *S. mutans* quorum sensing system contains three key components: a signal peptide (CSP), a histidine kinase, and response regulator. The latter two elements comprise a two-component signal transduction system. The *S. mutans* quorum sensing *com*CDE locus has been extensively studied and is believed to control many physiological processes (Li et al, 2001a, 2002b).

S. mutans can become genetically competent, a state in which exogenous DNA is transported and incorporated. Quorum sensing has been intrinsically linked to competence in *S. mutans*. The addition of synthetic CSP to cells grown in a chemostat resulted in a 100-fold increase in competence (Li et al, 2001a). The inactivation of the individual *com* genes conferred a competence defective phenotype (Li et al, 2001a). Furthermore the addition of a CSP to a *comC* knockout strain, (i.e. a strain unable to produce CSP) resulted in a return to near wild-type transformation efficiency (Li et al, 2001a). These data provide clear evidence that quorum sensing is involved in the genetic competence of *S. mutans*.

Quorum sensing has also been linked to the ATR displayed by *S. mutans*. Knockout mutants of the genes *comC*, *comD*, and *comE* displayed a diminished log-phase ATR (Li et al, 2001a). The addition of CSP to the *comC*- strain resulted in the partial recovery of ATR (Li et al, 2001a). Interestingly with the addition of cell free culture supernatant from acidic grown cell in conjunction with CSP resulted in the compete restoration of wild-type ATR (Li et al, 2001a). This finding indicates that the presence of at least one other signal molecular in addition to CSP is required for the ATR in *S. mutans*. This work by Li et al clearly shows the link between quorum sensing and the *S. mutans* ATR.

As a complement to the other physiological processes, quorum sensing in *S. mutans* influences biofilm formation and the biofilm 'lifestyle'. Studies utilizing the knockouts in the genes of the *com* locus revealed these mutants had altered biofilms. The *comC*- mutant produced biofilms with altered structure, in which cells formed large aggregates of long chains, whereas wild-type biofilms consisted of confluence microcolonies composed of shorter cell chains (Li et al, 2002b). The addition of CSP to *comC*- partially restored wild-type biofilm architecture (Li et al, 2002b). Interestingly *comD*- and *comE*- mutants had differing biofilms from that of *comC*-. Mutations in these genes resulted in biofilms with substantially reduced biomass (Li et al, 2002a,b). The addition of CSP to both *comD*- and *comE*- did not result in the restoration of biofilm biomass, indicating these genes are part of the two-component signal transduction system responsible for detecting CSP (Li et al, 2002b).

A Second Two-Component Signaling System?

The varying biofilm phenotypes of the com locus mutants eluded to the existence of a second receptor. *S. mutans* genome analysis revealed 13 separate two-component signal transduction systems. One pair consisting of the histadine-kinase *hk11* and the response-regulator *rr11* has been investigated. Deletion of either *hk11* or *rr11* results in biofilms with reduced biomass and a sponge-like architecture, that differed from wild-type and the *comC*-, *comD*-, and *comE*- biofilms (Li et al, 2002a,b). The addition of CSP to these mutants did not restore wild-type architecture to the biofilms (Li et al, 2002b). Results show that the *hk11/rr11* system is important to proper biofilms formation in a manner that completely separate from that of *comDE*. Competence was not affected by an *rr11* deletion unlike the reduced competence seen with *comE*- (Li et al, 2002a). This data also indicates that *hk11/rr11* act independently from *comDE*.

The *hk11*- strain also shows significant decreases in both the ability to grow at low pH and the ability to mount an ATR whereas the cognate *rr11*- did not display these insufficiencies (Li et al, 2002a). These data suggest that Hk11 acts as a pH sensor mediating acid-tolerance pathway and 'cross talk' exists between Hk11, Rr11, and another non-cognate response regulator. The presence of cross talk within two-component system has been previously described in *E. coli* (Verhamme et al, 2002).

VicRK Mediated Physiological Modulation

A recent study has investigated another two-component signal transduction system designated VicRK, with VicK, the histidine kinase and VicR, the associated response regulator (Senadheera et al, 2005). This regulatory system impacts a number of virulence characteristics in *S. mutans*. The *vicR* gene knockout was proven to be lethal and therefore a *vicRK* over-expressing mutant was generated as well as a vicK knockout. Both mutants formed aberrant biofilms that were rough and clumpy in appearance (Senadheera et al, 2005). Further examination of the *vicK* mutant showed biofilm cells with a rough surface and forming long chains (Senadheera et al, 2005). Integral to dental plaque formation is adhesion mediated via the production of glu-

cans. The *vicRK* over-expressing mutant diplayed increased mRNA expression of the glucantransferases *gtfB*, *gtfC*, and *gtfD*, the *gbpB* glucan binding protein, and the fructantranferase *ftf* (Senadheera et al, 2005). Utilizing gel shift mobility assays it was established that VicR bound to the promoter regions of *gtfB*, *gtfC* and *ftf*, thus suggesting that these genes are directly regulated by VicR (Senadheera et al, 2005). The over-expressing mutant produced more exopolysaccharide (EPS) whereas the vicK- strain produced less EPS compared to that of wildtype (Senadheera et al, 2005). These results clearly link the *vicRK* two-component system to the regulation of biofilm formation.

To determine if the observed *in vitro* biofilm deficiencies of the *vicRK* mutants translated into *in vivo* deficiencies a pathogen free rat model was utilized. When wild-type and *vicK*- strains separately inoculated into the oral cavity of the rats to colonize for 27 days, the vicK- strain had increased surface plaque formation compared to wild-type (Senadheera et al, 2005). Interestingly, the increase in plaque did not correlate into increased caries incidence over that of wild-type (Senadheera et al, 2005). From these results it can be inferred that *VicK* regulates plaque formation, but does necessary regulate cariogenicity.

The *S. mutans* two-component quorum sensing systems have evolved to allow the bacteria to perceive and response to its surrounding environment. This system plays important roles in genetic competence, acid tolerance, and biofilm formation. Work in our laboratory has identified the signal molecule CSP encoded by the gene *comC* and its importance to virulence regulation. Research thus far has implicated the histidine kinases *comD*, *hk11*, and *vicK* and their corresponding response regulators *comE*, *rr11*, and *vicR* as being integral to the processes involved in biofilm/virulence regulation. More work is currently being undertaken to fully understand the mechanisms of *S. mutans* quorum sensing and its regulation of cariogenic phenotypes.

CONCLUSIONS

Human dental caries caused by oral bacteria, mainly *S. mutans*, remains a fundamental problem in the field of dentistry in spite of the great advances that have been achieved. Effective new treatments against dental caries are necessary, as caries remains a major global health problem.

The ability of *S. mutans* to perceive and respond to changes within the dental plaque environment is pivotal to the progression of carious lesions. As a part of the normal plaque flora, *S. mutans* can modulate its physiology in response to environmental cues including nutrient availability, cell density, and varying pH (Fig 1). Current research is focusing on the molecular architecture of *S. mutans* environmental virulence regulation, paying particular attention to the mechanisms of the acid tolerance response and quorum sensing. The ultimate goal of this work is to discover novel means to control or eliminate *S. mutans* in the hopes of reducing caries.

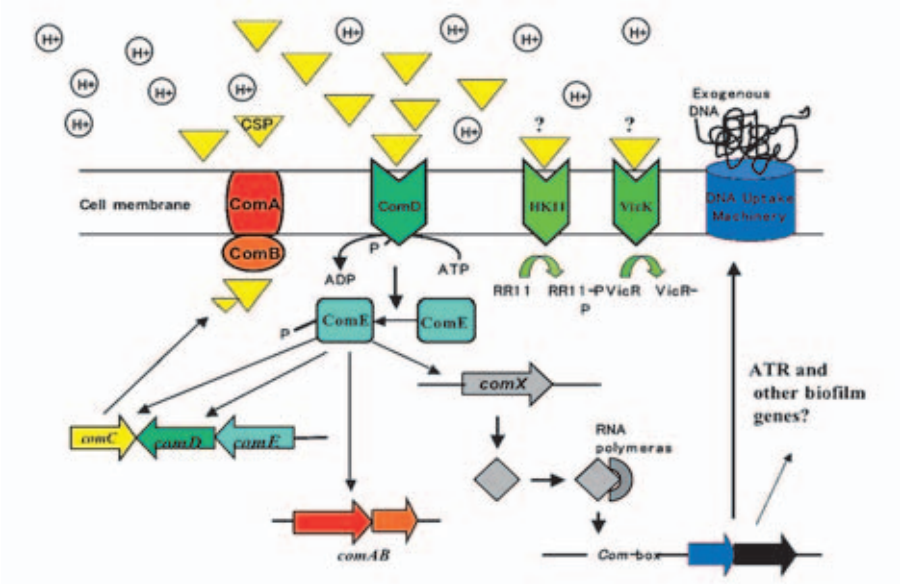

Fig 1 Some known environmentally regulated genes in *Streptococcus mutans*. The competence regulon is comprised of a signal peptide molecule precursor, encoded by *comC*, which produces a peptide that is processed into a 21 amino acid active competence stimulating peptide (CSP) upon export via *comAB*. CSP is detected by a histidine kinase, *comD*, that becomes phosphorylated by ATP. The response regulator ComE is then phosphorylated by ComD and is able to direct transcription of several genes including *comX*, a second transcription factor that then increases transcription of a number of other genes including those involved in DNA uptake and recombination. Other two component signal transduction systems are involved in sensing CSP and other environmental signals such as pH.

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