MOLECULAR MECHANISMS INVOLVED IN THE ACID TOLERANCE RESPONSE OF *STREPTOCOCCUS MUTANS*

by

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DEDICATION

To my wife Danielle and parents Nasr and Wafaa

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Firstly, I would like to thank my parents for the contribution they have made to allow me to pursue this degree. They have given me emotional and financial support over the years, which has made it possible to complete my M.Sc..

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MOLECULAR MECHANISMS INVOLVED IN THE ACID TOLERANCE RESPONSE OF *STREPTOCOCCUS MUTANS*.

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ABSTRACT

The acid tolerance response (ATR) plays a major role in the acid adaptation process in *Streptococcus mutans*. The ATR is an inducible molecular response mechanism activated when the bacterium encounters a moderate pH that enables the cells to tolerate further drops in pH. Differential display PCR (dd-PCR) was used to identify and clone genes that were activated when *S. mutans* was exposed to moderate pH (5.0). One of the dd-PCR products had 67% identity to a *uvrA*/UV repair excinuclease gene in *Bacillus subtilus*. *UvrA* knockouts were constructed in *S. mutans* by PCR-mediated mutagenesis and were shown to be sensitive to UV irradiation and pH (5.0) when compared to the wild-type. Acid-adapted *S. mutans uvrA* mutants were more resistant to UV irradiation than unadapted cells, but were unable to survive exposure to a killing pH of 3.0. Here we suggest that *uvrA* is necessary for the repair of acid-induced DNA damage and is essential for the ATR in *S. mutans*.

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CHAPTER 1: Introduction

Section 1.1: Overview

Dental caries (cavities) is among the most common infections found in humans. The 'mutans streptococci', primarily *Streptococcus mutans* and *Streptococcus sobrinus*, have been isolated most frequently in carious lesions with the infrequent isolation of *Streptococcus rattus* and *Streptococcus cricetus*, all of which are cariogenic in animal models (Hamilton, 2000). However, since *S. mutans* is the etiologic agent most commonly associated with human dental caries (Loesche, 1986) our laboratory has focused on this organism as the paradigm for this disease. Caries production is initiated by the adherence of *S. mutans* to the tooth surface, enabling it to colonize and eventually dominate its particular niche in the oral cavity. Initial attachment of *S. mutans* to tooth surfaces is thought to involve weak reversible forces followed by the influence of the high-affinity adhesion molecule, P1/SpaP, (Bowen *et al.*, 1991) which has been shown to interact with salivary pellicle constituents (McBride *et al.*, 1984; Lee *et al.*, 1988; Koga *et al.*, 1990) and other bacteria (Lamont *et al*, 1994; Jenkinson *et al.*, 1993). Also, this organism produces water-insoluble glucan polymers from sucrose, through the action of glucosyltransferases (Gtfs), and glucan-binding proteins that facilitate its attachment and colonization on the tooth surface (Hamada and Slade, 1979; Yamashita *et al.*, 1993a). Once established, *S. mutans* is able to generate acid end-products from sugar metabolism, which promotes the demineralization of tooth enamel.

S. mutans is able to transport a variety of sugars via a Phospho-enolpyruvate (PEP): phosphotransferase transport system (PTS) and multiple sugar metabolism (*msm*) operon system, and uptake and process high concentrations of glucose via permease and glycolytic activities, respectively. Lastly, *S. mutans* is able to tolerate acid. In this

regard, *S. mutans* is able to carry out glycolysis and other cellular functions at low pH (Bender *et al.*, 1986); extrude protons using a H^+/ATPase that functions to maintain intracellular pH (Dashper and Reynolds, 1992); and induce an adaptive response to low pH, termed the acid tolerance response (ATR) (Belli and Marquis, 1991). It is important to note that *S. mutans* was found to be the most prevalent microorganism, found in largest numbers, in incipient carious lesions (white spots) (Loesche, 1986). Other species such as *Lactobacillus sp.* and *Actinomyces sp.* are also found to be associated with caries, but are usually found in significant numbers after the colonization of *S. mutans* (Boyar and Bowden, 1985). Nevertheless, these infections represent a costly addition to health care, with figures in Canada estimated to be in the billions of dollars each year (Lewis and Ismail, 1994).

Section 1.2: Factors involved in caries formation

The acid produced by dental plaque bacteria results in low pH conditions, which contributes to the caries process via demineralization of the tooth surface. As demonstrated by Stephan (1940), the pH of plaque decreases immediately after sugar intake by the host, but it slowly rebounds back to a neutral resting pH. If the pH drops below the critical pH (defined as pH 5.0-5.5) teeth begin to lose mineral content and destruction of the enamel ensues (Jenkins, 1963). With a consistent acid challenge over time, the resting pH at localized tooth surfaces may eventually be lowered to values closer to the critical pH and periods of demineralization will eventually outlast periods of remineralization, resulting in a carious lesion.

Caries formation is dependent on a variety of factors. Firstly, the availability and concentration of sugar is a major determinant. Under high sugar concentrations, the acid produced by dental plaque bacteria results in low pH conditions that directly influence the demineralization of tooth surfaces. The degree of acid generated by these bacteria can depend on the length of time these bacteria have to utilize the sugars as well as the amount of sugar available for them to metabolize. The metabolic activity of the plaque microflora can also be a determinant since most oral bacteria are only able to grow within a narrow pH range, usually between 6 and 8 (Svensäter, 1997). However, some oral bacteria are aciduric (primarily mutans streptococci, *Lactobacilli sp.*, and some other acid tolerant streptococci) and will grow at significantly lower pH values. These organisms are favored when the plaque environment becomes acidic, and they can eventually dominate localized areas of the tooth and maintain the acidic pH environment required for caries formation.

Flow rate and buffering capacity of saliva have been shown to be factors governing caries formation. Flow rate and buffering capacity aid in maintaining a neutral pH in the oral cavity. Bacterial and acid clearance are accomplished more effectively with increased flow rate and buffering by salivary bicarbonates and peptides in the saliva, all of which help in the recovery from low pH exposure (Kleinberg *et al.* 1982). Patients with reduced salivary flow (xerostomia) have been shown to be more prone to dental caries (Dreizen and Brown, 1976). Evidence also suggests that alkali generation from salivary substrates, urea and arginine, and bacterial metabolism of such peptides could possibly moderate the initiation and progression of dental caries by neutralizing acidic environments (Kleinberg *et al.*, 1982; Burne and Marquis, 2000). Also, arginine and urea

are processed by oral bacteria into ammonia and CO_2, and ornithine (from arginine metabolism), which prevent drops in plaque pH. A study was done where *S. mutans*, which lacks urease activity, was genetically engineered to express the urease genes of *Streptococcus salivarius*. Rats were infected with the alkali-producing *S. mutans* and fed a high sugar diet supplemented with urea. Rats infected with the recombinant strain showed lower incidence of caries compared with the controls infected with non-urease active parent (Clancy *et al.*, 2000). This suggested that alkali generation from oral bacteria could be a significant determinant in caries formation.

Recent evidence suggests that dental plaque architecture also could influence caries formation. *S. mutans* mutants defective in the glucan-binding protein gene, *gbpA*, have been shown to display a densely packed biofilm architecture, which is more resistant to mechanical stress and more virulent than the wild-type strain (Hazlett *et al.*, 1999). The increase in virulence of *gbpA* mutants was hypothesized to be due to tighter diffusion barriers formed between the tooth surface and saliva, resulting in decreased influx of salivary buffering capacity and efflux of acid generated by the bacteria, which essentially traps the acid on the tooth surface. Similarly, tooth architecture can present areas that have reduced salivary flow and buffering capacity such as in the pits and fissures of teeth. Foodstuff also can become trapped in these areas and between teeth, providing extended time for oral bacteria to metabolize the nutrient source and produce acid. Lastly, host genetics is believed to influence caries formation (Mandel, 1994; Hart *et al*, 2000) such as genetic determinants for saliva and immunologic function, tooth morphology, enamel thickness, enamel structure and composition, and behavioral aspects such as food preference.

Section 1.3: Effects of acid on the ecology of dental plaque

The bacterial composition of plaque is relatively stable despite regular exposure to minor environmental disruptions (Wimpenny, 1995). Nevertheless, within this overall environment some factors have significant impact on the ecology of dental plaque including variables such as oxygen, pH, mineral salts, anti-microbial factors, and microbial interactions (Marsh, 1991; Marquis, 1995). During caries formation plaque homeostasis is altered, leading to shifts in the balance of microflora. The frequent exposure of plaque to low pH leads to inhibition of acid-sensitive species and the selection of acid-tolerant species such as mutans streptococci and lactobacilli (Marsh and Bradshaw, 1997).

Early plaque formation consists of the pioneer colonizers *Streptococcus sanguis*, *Streptococcus oralis*, *Haemophilus spp*, *Neisseria spp.* and *Actinomyces spp.*, adhering to the tooth pellicle (Kolenbrander, 2000). These bacteria then become substrates for attachment of late colonizers. Early colonizers tend to be less sensitive to toxic products, such as those derived from oxygen metabolism, than are late colonizers. It has been proposed that this lower sensitivity is important in the early phase of plaque development (van der Hoeven and Camp, 1993).

Upon ingestion of fermentable carbohydrate by the host, plaque pH can drop dramatically due to the production of acids (Stephan, 1940). Repeated acidification of the plaque environment due to frequent consumption of sugar is a major selective force favoring dominance of acid-tolerant bacteria. Acid-tolerant bacteria are able to grow and produce acid at pH values below that required for demineralization of tooth enamel (Bender *et al.*, 1986). Although *S. mutans* is involved in the initiation of dental caries,

subpopulations of bacteria, primarily streptococci and lactobacilli, are found to produce and tolerate acid and coexist in carious lesions (van Houte *et al.*, 1994). Non-mutans streptococci, which are pioneer bacteria, exhibit weaker acid tolerance than mutans streptococci, but may be essential for the initial shift of dental plaque towards an acid environment, which consequently promotes the colonization of more acid–tolerant and acidogenic bacteria (Takahashi and Yamada, 1999).

Acid-resistance by oral bacteria has been linked mainly to the activity and pH optima of the proton extruding ATPase (Bender *et al.*, 1986). The most acid-resistant bacteria are those with the highest levels of ATPase activity and the lowest pH optima for ATPase activity (Sturr and Marquis, 1992). In acid-tolerant bacteria H+/ATPase activity is involved in internal pH (pH_i) homeostasis during acid challenges, whereas the low pH optimum for glucose transport, glycolysis, and ATPase activity allows for survival and acid production at low pH. Furthermore, bacteria such as the mutans streptococci can become adaptively tolerant to acidification by an inducible molecular mechanism termed the acid tolerance response (ATR), which enhances its dominance in the plaque community at low pH.

Section 1.4: Acid tolerance/adaptation

Some acid tolerant bacteria, including *S. mutans*, have evolved an inducible adaptation mechanism to low pH. Acid adaptation was first described in enteric bacteria when the observation was made that neutral pH grown cells were more susceptible to acid killing than those cells that were first exposed to a moderate pH (~pH 5.5) (Foster and Hall, 1990; Bearson *et al.*, 1997). This adaptive mechanism was referred to as the

acid tolerance response (ATR). The ATR has been well studied in *Escherichia coli* (Goodson and Rowbury, 1989) and *Salmonella typhimurium* (Foster and Hall, 1990) where these organisms must endure acidic environments encountered in the stomach and parts of the intestine of the host. Both organisms possess a log phase and stationary phase acid tolerance response system.

More than 50 acid shock proteins (ASPs) are synthesized during adaptation and thought to contribute to acid survival (Foster and Hall, 1990). Internal and/or external pH appears to be an inducing signal for ASPs as well as regulatory genes which include the alternative sigma factor σ^S, encoded by *rpoS*, the iron regulator Fur, the two-component signal transduction system PhoPQ, and the acid/alkaline-induced DNA repair regulator Ada, each of which control the synthesis of a subset of ASP products involved in the log-phase ATR (reviewed in Foster, 1995). Mutations in any of these regulatory genes results in an acid-sensitive phenotype (Foster and Moreno, 1999). Additionally, acid adaptation induces cross-protection against osmotic, heat, and oxidative stresses (Leyer and Johnson, 1993). This result is not surprising considering that about half of the ASPs induced by acid are also induced by these stresses (Foster, 1995).

One of the best understood molecular responses to acid pH in *E. coli* and *S. typhimurium* is the induction of the *cadBA* operon (encoding lysine decarboxylase CadA and lysine/cadaverine antiporter CadB) and the *E. coli* gene for arginine decarboxylase, *adiA* (Meng and Bennett, 1992; Stim and Bennett, 1993; Park *et al.*, 1996). These acid-inducible genes encoding amino acid decarboxylases are involved in pH_i homeostasis by alkalinization of the cytoplasm during low pH exposure. Apart from the synthesis of ASPs in response to growth in moderate pH, other notable changes include a shift in fatty

acid profile in the cell membrane (Leyer and Johnson, 1993), and the secretion of a signaling molecule that induces acid tolerance in other organisms (Rowbury and Goodson, 1998). However, differences in the regulation of the ATR exist between *E. coli* and *S. typhimurium* when log-phase cells are grown in nutrient rich medium and when cells are grown in stationary-phase (Bearson *et al.* 1997; Foster, 1999). Under these conditions *E. coli* becomes more acid resistant than *S. typhimurium* when challenged at low pH (Foster, 1999).

Some oral bacteria (*S. mutans*, *Lactobacillus* sp., and others to a lesser extent) also exhibit an ATR (Svensäter *et al.*, 1997; Takahashi and Yamada, 1999). Work by Svensäter *et al.* (1997) has demonstrated in several oral bacterial species that the terminal pH for log-phase growth was related to the ability of the organisms to induce an ATR, such that those organisms showing the lowest terminal pH had the strongest ATR. As in the ATR of enteric bacteria during log phase growth, *S. mutans* requires *de novo* protein synthesis during acid-adaptation for successful survival to lower pH challenges (Svensäter *et al.*, 1997), with the induction of at least 36 acid-regulated proteins (Hamilton and Svensäter, 1998). Prior acid adaptation did not, however, induce cross-protection to stresses such as heat, salt, oxidation and starvation, although the reciprocal held true (Svensäter *et al.*, 2000). In our lab and elsewhere (Quivey *et al.*, 1995), prior adaptation to acid has been found to induce cross-protection to UV irradiation. As in enteric bacteria, it appears that *S. mutans* may also exhibit a stationary-phase ATR. Although there are no published data proving this, our lab and others (Svensäter *et al.*, 1997; Svensäter *et al.*, 2001) have observed that unadapted cells in stationary-phase demonstrate an increased resistance to acid. Whether the regulatory mechanisms that

control log-phase ATR in *S. mutans* also control stationary-phase ATR is still uncertain, although we have shown in our lab that some of the genes induced during log-phase ATR are also induced during stationary phase.

Genetic studies of acid-protective mechanisms in *S. mutans* have resulted in the identification of genes that, when inactivated, result in acid-sensitive phenotypes. Using transposon mutagenesis (Tn*916*), a deficiency in *S. mutans* GS5 diacylglycerol kinase, which is involved in phospholipid metabolism, was shown to give rise to a mutant sensitive to acid as well as to high osmolarity and temperature (Yamashita *et al.*, 1993b). Also, an acid-sensitive phenotype was found using Tn*917* in *S. mutans* JH1005 when the gene encoding the 54 kDa subunit homologue of the eukaryotic signal recognition particle, *ffh*, was interrupted (Gutierrez *et al.*, 1999). Both Ffh and diacylglycerol kinase are thought to be involved in maintaining cell membrane integrity during acid challenges and both have been shown to be involved in the ATR, whereby mutants revealed a lack of adaptive response to acid pH. The *fhs* (formyl-tetrahydrofolate synthetase) gene in *S. mutans*, involved in purine biosynthesis and formylation of Met-tRNA, was disrupted using Tn*917* and also found to be acid-sensitive, but not linked to the ATR (Crowley *et al.*, 1997).

It has been proposed that the ATR in bacteria can be simplified into two main components (Foster and Hall, 1991). The first involves mechanisms that maintain internal pH homeostasis. In *S. mutans* this primarily involves an increase in H+/ATPase activity and acid end-product efflux (Bender *et al.*, 1986; Hamilton and Buckley, 1991; Dashper and Reynolds, 1992), and a decrease in proton permeability (Hamilton and Buckley, 1991) by changes in membrane fatty acid composition (Quivey *et al.*, 2000) and

increased synthesis of the cell surface component, D-alanyl-lipoteichoic acid (Boyd *et al.*, 2000). The second component of the ATR is thought to involve the repair of cellular components damaged by acidic pH. Previous studies in *S. mutans* have shown the repair of acid-induced cellular damage to consist of the protein repair chaperone DnaK (Jayaraman *et al.*, 1997), a DNA repair enzyme exhibiting AP endonuclease activity (Hahn *et al.*, 1999), and the DNA damage regulatory/repair protein RecA (Quivey *et al.*, 1995). In Chapter 3 of this report we suggest that the activity of the *uvrA* /DNA repair gene in *S. mutans* is involved in the repair of acid-induced DNA damage and is essential in the ATR.

In spite of the general features of the cellular response of *S. mutans* to acid pH, very little is known about the regulatory networks (See Discussion in Chapter 3 for possible regulatory mechanisms involved) and molecular responses regulated by acidic pH. To date, only the genes for the protein repair chaperone, *dnaK*, (Jayaraman *et al.*, 1997) and cell membrane maintenance protein, *ffh* (Gutierrez *et al.*, 1999), in *S. mutans* were shown to be inducible by acidic external pH. Therefore, our lab sought to use the differential display-polymerase chain reaction technique to identify additional genes induced by pH during acid-adaptation.

Section 1.5: Differential Display-Polymerase Chain Reaction (DD-PCR)

Since its development in 1992 (Liang and Pardee, 1992), differential display has quickly overtaken subtractive hybridization to become the method of choice for cloning differentially expressed genes. Other recent methods used for cloning differentially expressed genes include the RNA-arbitrarily primed PCR (RAP-PCR) (Shepard and

Gilmore, 1999), Representational Difference Analysis (RDA) (Hubank and Schatz, 1994), DNA Microarray analysis (Wei *et al.*, 2001), and Serial Analysis of Gene Expression (SAGE) (Velculescu *et al.*, 1995). These methods vary greatly in their methodology and sensitivity for detecting mRNA. Nevertheless, the techniques incorporating PCR, dd-PCR and RAP-PCR, appear to be among the most economical and sensitive methods.

Differential display is a powerful technique for analyzing differences in gene expression that can be useful for identifying novel genes and gene function. Differential gene expression can result from a number of factors including: mutations, viral infections, cellular differentiation, and environmental conditions (*e.g.* pH). Originally used to identify differentially expressed genes in eukaryotes (Liang and Pardee, 1992, Welsh *et al.*, 1992), this system has more recently been applied to prokaryotes (Abu Kwaik and Pederson, 1996; Fislage *et al.*, 1998). In the dd-PCR method, two or more total RNA pools isolated from bacteria under different conditions are first reverse transcribed into cDNA. Subsequently, the cDNA fragments are amplified by PCR using a set of arbitrary primers in the presence of radiolabeled dNTP. After separation by denaturing polyacrylamide gel electrophoresis, the gels are fixed, dried and analyzed for differential expression by autoradiography. Practical problems with the dd-PCR method are presented by the relatively large proportion of structural RNA (rRNA) species in the total RNA pool and the lack of poly (A) tails in most prokaryotic mRNAs (Sarkar, 1997), which prevents the use of the 3' dT primers normally used to reverse transcribe eukaryotic mRNA. In prokaryotes there is also an inherent instability and short half-life of low abundance mRNA species compared to eukaryotes (Higgins *et al.*, 1992). In spite

of these limitations we have shown here that dd-PCR is a highly effective tool to investigate changes in gene expression under different environmental conditions in prokaryotes.

In this report (Chapter 3), we described the use of dd-PCR to identify genes expressed in *S. mutans* in response to low pH. Here we isolated total RNA from cells grown at pH 7.5 (acid-unadapted state) and pH 5.0 (acid-adapted state) and subjected these two pools of RNA to dd-PCR. Using this technique, potential gene candidates that were up-regulated at pH 5.0 could then be mutagenized and tested for their involvement in the ATR (Figure 1.1). From this analysis we hoped to identify acid-inducible genes that code for acid-protective proteins required in the ATR.

Section 1.6: Adherence

The synthesis of extracellular polysaccharides (glucans) by *S. mutans* in the presence of high sucrose concentrations plays a major role in the adherence and pathogenicity of this organism. *In vitro* experiments suggest that extracellular polysaccharides facilitate in both diffusion and tooth demineralization (van Houte *et al.*, 1989; MacPherson *et al.*, 1990). Adherence by *S. mutans* is mediated mainly by sucrose-dependent mechanisms (glucan synthesis) (Schilling and Bowen, 1992), but other mechanisms, independent of sucrose, also influence adherence (involving P1 protein) (Bowen *et al.*, 1991). Both of these mechanisms are reviewed here.

Section 1.6A: Sucrose-dependent Adhesion

Sucrose-dependent adherence is mediated by glucans, the products of the extracellular glucosyltransferase (GTF) enzymes, which have glucan-binding properties

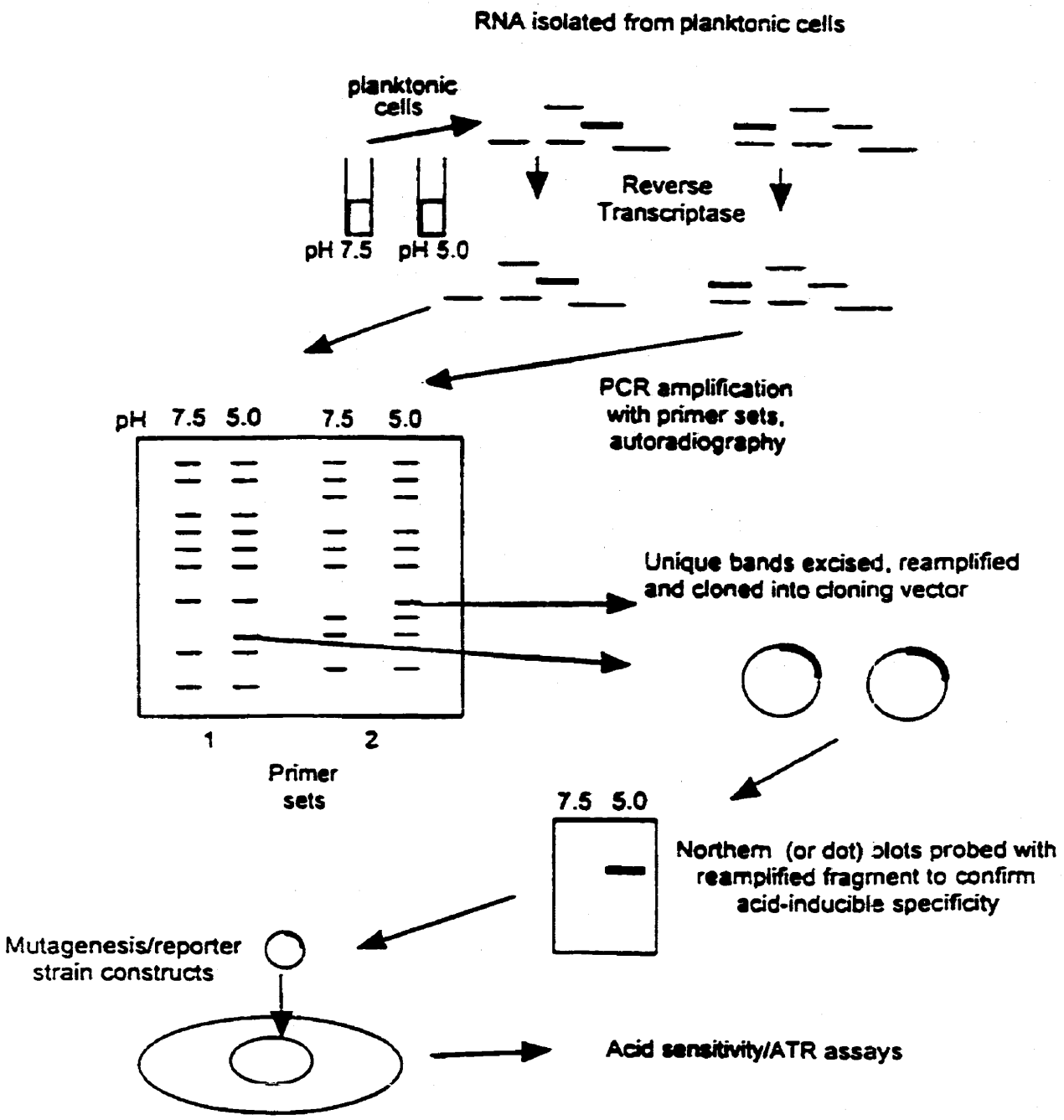

Figure 1. 1: Schematic illustration of protocol used to isolate and identify *S. mutans* acid-inducible genes using dd-PCR.

(Gibbons and Van Houte, 1975). *S. mutans* produces and secretes three different types of GTFs. GTF-I, GTF-SI, and GTF-S ('I'-insoluble/ 'S'-soluble) are produced respectively by the *gtfB*, *gtfC*, and *gtfD* genes. The enzyme activities of these products polymerize the glucose moieties of sucrose into glucans. The products of *gtfB* and *gtfC*, forming water-insoluble glucans, have been implicated in the cariogenicity of *S. mutans* (Yamashita *et al.*, 1993a) and have been shown to aid in the adherence of *S. mutans* to the tooth surface. It is hypothesized that the conformation of the insoluble glucans formed by *gtfB* and *gtfC*, primarily α-1,3 and α-1,6 linkages creates a bacterial binding site not present in the predominately α-1,6 linked soluble GTF-S product (Venkitaraman *et al.*, 1995). *S. mutans* also synthesizes three glucan-binding proteins, GbpA, GbpB, and GbpC. Mutation in the gene encoding GbpA (*gbpA*) resulted in changes in plaque architecture, which was more resistant to mechanical stress and correlated with changes in virulence (Hazlett *et al.*, 1999). This provided the first evidence that biofilm structure could, in fact, influence virulence of *S. mutans* and possibly other pathogens that form biofilms.

Section 1.6B: Sucrose-independent Adhesion

Protein mediated cell adhesion in *S. mutans* primarily involves the cell surface protein P1, also referred to as antigen I/II, antigen B, IF, SR, MSL-1, and PAc (Oho *et al.*, 1998). In the absence of sucrose, P1 is believed to be a major adhesin (Jenkinson and Demuth, 1997). Use of isogenic mutants of *S. mutans* deficient in P1 have demonstrated that the "fibrillary fuzzy layer" described by Ayakawa *et al.* (1987) is absent in these strains, indicating that P1 protein plays an important role in the fibrillar structure of the cell (Lee *et al.*, 1989). Studies have indicated that P1 is involved in adherence (Koga *et al.*, 1990), aggregation (Brady *et al.*, 1991), and bacterial coaggregation (Crowley *et al.*,

1987). P1 has also been shown to be an effective immunogen against dental caries (Russel *et al.*, 1982; Lehner *et al.*, 1985; Ma *et al.*, 1987). However, other binding mechanisms besides P1 may exist in *S. mutans* as illustrated by the fact that deficiency in P1 expression still shows some adhesion both *in vivo* (Crowley *et al.*, 1999) and *in vitro* (Koga *et al.*, 1990; Bowen *et al.*, 1991).

Section 1.7: Sugar transport and metabolism

S. mutans and other oral bacteria must be able to survive the 'feast or famine' nature of available nutrient sources found in dental plaque. For example, sugar levels in the oral cavity can dramatically fluctuate, from minimum levels found when saliva is the only source (~10 μM), to levels 10, 000 fold greater upon food intake (~100 mM) (Carlsson, 1989). Subsequently, mechanisms have evolved in oral bacteria that allow them to adapt to these extreme conditions. Several distinct mechanisms have been discovered in *S. mutans* under periods of low and high nutrient availability.

During times of famine, the high-affinity phosphoenolpyruvate: sugar phosphotransferase system (PTS) is utilized to scavenge available fermentable sugars at micromolar concentrations (Vadeboncoeur and Pelletier, 1997). The PTS system is an 'active' transport carrier that utilizes phosphoenolpyruvate (PEP) from the glycolytic pathway as an energy source and results in the concomitant transport and phosphorylation of the sugar to the inner surface of the cell. The PTS consists of two energy-coupling proteins, enzyme I (EI) and a heat-stable protein (HPr), and a collection of sugar-specific enzyme II (EII) complexes. Each EII complex consists of enzymes IIA, IIB, and IIC. Enzyme I, HPr, IIA, and IIB are involved in phosphoryl transfer, whereas IIC, the

membrane-bound permease, catalyzes the transport of the incoming sugar into the cytoplasm (Saier, *et al.*, 1995). The following sugars or sugar alcohols are known to be transported by the PTS: glucose, mannitol, sucrose, sorbitol, lactose, mannose, maltose, and trehalose (Jacobson *et al.*, 1989). The PTS system also plays a role in the control of carbon metabolism as demonstrated by the selective use of PTS-specific sugars by *S. mutans* and illustrated by diauxic growth on a mixture of sugars (Vadeboncoeur and Pelletier, 1997). This phenomenon is termed catabolite repression, whereby the expression of genes for certain catabolic enzymes is repressed in the presence of readily metabolized carbon sources such as glucose. Catabolite repression, although vaguely characterized, has been described in *S. mutans* (Cvitkovitch *et al.*, 1995; Simpson and Russell, 1998). The mechanism of catabolite repression has been described in other Gram-postive bacteria, whereby exposure to high sugar conditions resulted in increased cellular concentrations of fructose 1,6-bisphosphate and ATP, which results in the phosphorylation of the HPr serine residue as opposed to the histine residue normally phosphorylated under low sugar conditions. The phosphorylated Ser-HPr, a catabolite control protein (CcpA), and fructose 1, 6-bisphosphate then bind to a specific DNA sequence CRE (catabolite repression elements) found in the promoter region of catabolite genes, subsequently preventing the transcription of these catabolite enzymes (Deutscher *et al.*, 1995; Saier *et al.*, 1995). In *S. mutans*, a homologue of CcpA has been indentified and has been shown not to be essential for catabolite repression by a number of carbohydrates (Simpson and Russell, 1998).

During episodes of high sugar availability the PTS system is repressed due to the decrease in pH, sugar (glucose) is thought to be transported by an ATP-dependent

glucose permease (Cvitkovitch *et al.*, 1995), and a shift from heterofermentive (acetate, formate, ethanol, H_2O_2 end-products) to predominately homofermentive (mainly lactic acid end-product) metabolism is observed (Hamilton, 1987). The latter process is a physiologic response to the large influx of sugar and is termed the 'lactate gate' (Carlsson, 1984). Glycolytic activity in *S. mutans* was shown to be optimal at an external pH of 6.0, with activity detectable to as low as pH 3.5 (Dashper and Reynolds, 1992). Another sugar transport system, the multiple sugar metabolism (*msm*) operon system, is responsible for the uptake of sugars such as melibiose, raffinose, and isomaltosaccharides during sugar-excess conditions (Tao *et al.*, 1993) and is regulated by the PTS system (Cvitkovitch *et al.*, 1995).

Section 1.8: Hypotheses

Our goal was to examine the molecular mechanisms involved in the ATR of *S. mutans*. We used the dd-PCR technique to identify genes induced during acid-adaptation, which might provide insight into the possible genes and/or mechanisms that allow *S. mutans* to survive acidic pH challenges. We propose that the dd-PCR products up-regulated at pH 5.0, but not at pH 7.5, represent genes that are involved in the ATR and protect the cells from low pH. From our dd-PCR analysis, we have identified a *uvrA*/DNA repair gene in *S. mutans* that is induced during acid-adaptation (see Chapter 3). We propose that acid-induced DNA damage occurs in *S. mutans* during acid challenges and that induction of the DNA repair enzyme, UvrA, is an essential part of the acid adaptation process.

CHAPTER 2: Materials and Methods

Section 2.1: Insertion Duplication Mutagenesis (IDM) of *uvrA*

Knockout mutants of *uvrA* were created in strains *S. mutans* JH1005, NG8, and UA159 by insertion-duplication mutagenesis. Primer pairs uvrA**F** and uvrA**B** (Table 3.1) were designed to amplify the internal region of *uvrA*. The amplicon was then ligated to the integration plasmid pVA8912 via *Bam*HI and *Eco*RI sites. The recombinant constructs, designated pJHUVRA-KO, pNGUVRA-KO, and pUAUVRA-KO, were individually transformed into Epicurian coli® XL1-Blue supercompetent cells for propagation (Table 2.1). Colonies with pUVRA-KO were selected on LB-erythromycin (300 μg/ml) agar plates, and isolated by precipitation with polyethylene glycol (Applied Biosystems, Foster City, CA). Purified pUVRA-KO from all three strains was used to transform wild-type *S. mutans* strains JH1005, NG8, UA159 with their respective construct. Mutants harboring the integrated plasmids were selected on THYE-erythromycin (10 μg/ml) agar plates.

Confirmation of plasmid insertions causing gene disruption was performed by Southern hybridization or by a rapid protocol involving PCR. Southern blotting was carried out with digoxygenin (DIG)-labeled PCR *uvrA* and Erm cassette probes using the DIG Non-Radioactive Nucleic Acid Detection Kit (Roche Diagnostics, Laval, QC, Canada). For PCR verification, primers previously designed for the internal region of *uvrA* were used in combination with those made for the Erm cassette (Table 3.1) to test whether gene segments could be amplified from wild-type and mutant *S. mutans* chromosomal DNA. Insertion at the desired locus for each mutant was confirmed by PCR using primer pairs uvrA**F** and ERM2-B1 and uvrA**B** and ERM1-F3. The recombinant plasmids used for transformation were employed as PCR templates for

positive controls. Mutants with a pattern of amplification identical to that seen in the positive control showed correct gene disruption (See Figure 2.1).

Section 2.2: PCR-mediated mutagenesis of *pepP*

An aminopeptidase P gene (*pepP*) was found immediately 3' proximal from *uvrA*. We sought to create a mutant in *pepP* to determine if it too had any influence on the ATR. A rapid method for generating mutants in *S. mutans* NG8 and UA159 was employed using PCR. DNA fragments of 500-1000 b.p., which flank the target gene, were fused to an erythromycin resistance cassette [obtained from Dr. Don Morrison (refer to Claverys *et al.*, 1995)]. An 860 b.p. portion of the Erm cassette containing the Erm^r marker expressed from a synthetic promoter was amplified for the fusion. The construct was designed so that its integration would not disrupt the original reading frame, minimizing any downstream polar effects. Primers used to amplify the Erm cassette were ERM CST**P1** and ERM CST**P2**. Pep**PP1/P2** primers were used to amplify the 3' proximal flanking region of *pepP* and pep**PP3/P4** were used to amplify the 5' proximal flanking region (see Table 2.2). Primers P2 and P3 were designed to overlap the target gene within 120 b.p. of the 5' and 3' ends of the gene sequence, respectively. PCR products for each fragment, generated in triplicate, were purified using the PCR Purification Kit (Stratagene), digested with the appropriate restriction enzyme, *FseI* or *AscI* (MBI Fermentas), ligated with T4 DNA Ligase (Promega). Ligated products from the PCR-mediated mutagenesis step (containing the left and right flanking regions and the Erm cassette) were used to transform *S. mutans* using competence stimulating peptide

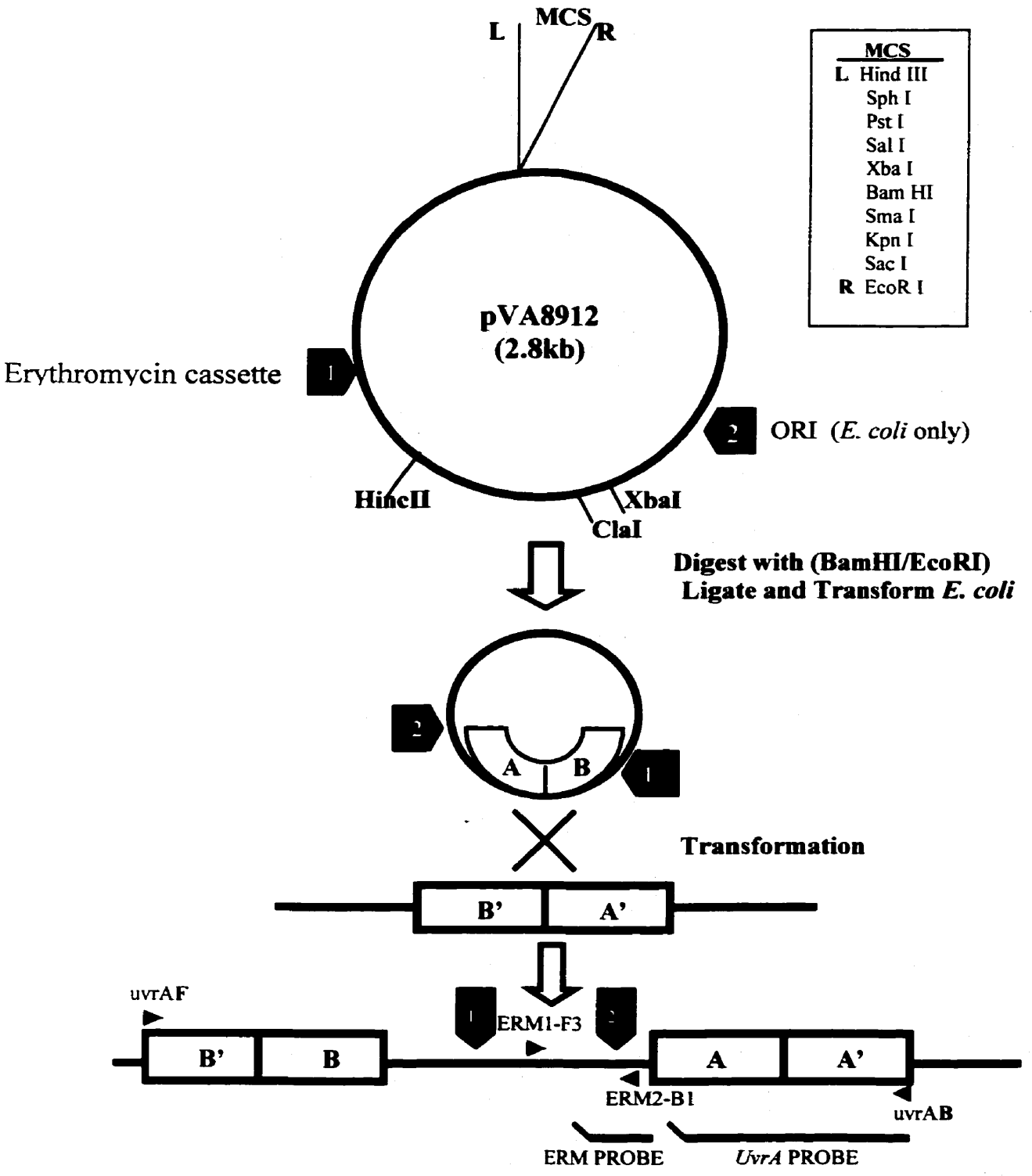

Figure 2. 1: Insertion duplication mutagenesis schematic used for *S. mutans uvrA* knockouts. Probe and PCR primer annealing locations illustrate how the Erm cassette and a fragment of the *uvrA* gene were detected to confirm disruption of the gene locus.

Table 2. 1: Plasmids used in IDM of JH1005 *uvrA*

Plasmid	Relevant Characteristics	Source/Reference
pVA8912	Streptococcal integration plasmid derived from pVA891, Erm^r	Horst Malke, Jena Univ., Germany
pJHUVRA-KO	pVA8912 harboring a 578 bp internal uvrA fragment, Erm^r (tansformed in JH1005)	This study
pNGUVRA-KO	pVA8912 harboring a 578 bp internal uvrA fragment, Erm^r (tansformed in NG8)	This study
pUAUVRA-KO	pVA8912 harboring a 578 bp internal uvrA fragment, Erm^r (tansformed in UA159)	This study

(CSP) (Li *et al.*, 2001). Briefly, 4 ml of TH broth was inoculated with each strain of *S. mutans* and incubated overnight at 37°C. A 20-fold dilution of each culture was made into 5 ml of fresh TH broth and incubated to early log phase. Ligated DNA product (10-20 µl) along with 500 ng/ml of freshly prepared CSP was added to each tube and incubated a further 1.5-2 hours. Cells were then centrifuged at 1,000 rpm for 10 min and resuspended in 200 µl TH broth and plated on THYE plates containing erythromycin. Mutant confirmation was performed by isolating genomic DNA from positive colonies for use as a PCR template with primer sets **P1**/ERM CST**P2** and **P4**/ERM CST**P1**. The resulting products were analyzed by agarose gel electrophoresis and their observed sizes compared to the sizes predicted following successful allelic exchange (See Figure 2.2).

Section 2.3: Cell density-dependent ATR experiment

A previous study in *S. mutans* suggested the idea that cell density may play a role in acid resistance (Svensäter *et al.*, 1997). We sought to confirm this observation in our study. An overnight culture of THYE-grown *S. mutans* JH1005 was diluted 10-fold in fresh THYE pH 7.5 and incubated at 37°C. Cultures were grown to $O.D._{600}$ values of 0.5, 0.65, and 0.9 (representing, early- and mid- log, and stationary phases). Cells were then harvested by centrifugation and resuspended in THYE pH 7.5 and 5.0 for unadapted and adapted conditions, respectively. Cells were incubated for 2 hours at 37°C. A fraction of each sample (1 ml) was taken for total RNA extraction and analyzed for *ffh* and *uvrA* gene expression using reverse transcription-PCR (RT-PCR) (see Chapter 3, Section: 3.3C/G). A second fraction (1 ml) was taken for quantitation of cell survival before

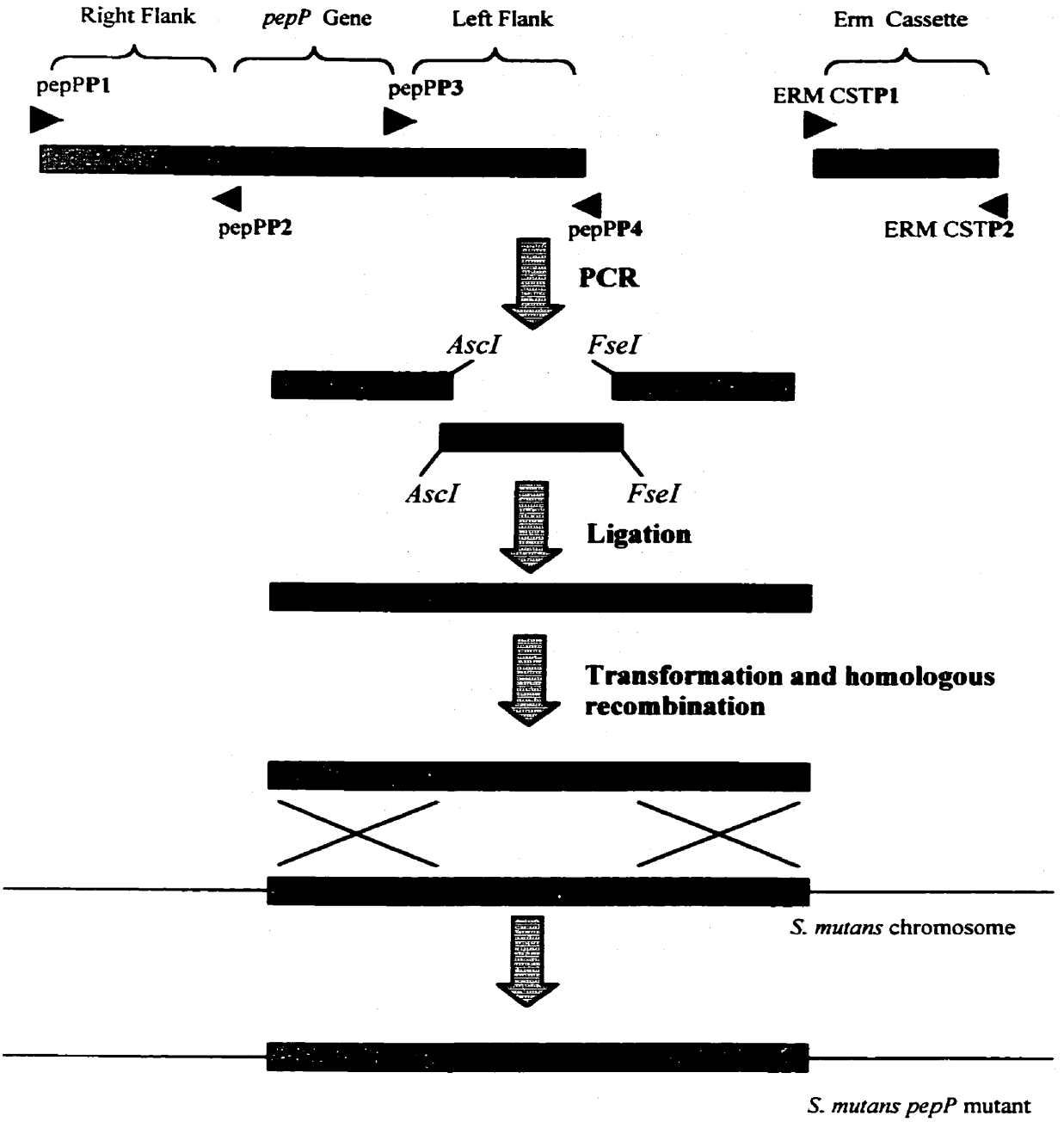

Figure 2. 2: Schematic of PCR-mediated mutagenesis of *S. mutans pepP* knockouts. Using primers P1/P2 and P2/P4 two separate PCR reactions were used to generate ~500 bp fragments which flank the target gene. Both P2 and ERM CSTP1 contained the restriction enzyme linker, *AscI*, and P3 and ERM CSTP2 contained the *FseI* linker. All fragments were digested with the appropriate restriction enzyme, ligated, and used to transform *S. mutans* NG8 and UA159 strains.

Table 2. 2: Oligonucleotides used in this study

Name	Sequence (5' -3')	Description
pepPP1	.....ACACCAAGATCCAATCCTGCC	*pepP* PCR-mediated mutagenesis
pepPP2	.....GGCGCGCCGCTGTTCCCCAAAACCCTGTTAG	"
pepPP3	.....GCTGGCCGGGGATGGTTGTGAAGTGTTGACCC	"
pepPP4	.....GCTATTGTAGCTCTTCCTGCTGGC	"
ERM CSTP1	...GGCGCGCCCCGGGCCCAAAATTTGTTTGAT	Erm Cassette for PCR-mediated mutagenesis (Lee *et al.* 1999)
ERM CSTP2	...GCTGGCCGG ACTCGGCAGCGACTCATAGAAT	
erm1-F3	.....GACTAGTCCAAACAGGTAACGGTTATTGCAGG	Erm Cassette for IDM of *uvrA*
erm2-B1	.....GCTCTAGAGCCCTCTTTAGCTCCTTGGAAGCTGT	"

exposure to the killing pH by diluting the cells (10^{-6}) and plating them on THYE plates using a spiral plater (Model D, Spiral System Inc., Cincinnati, OH). The remaining cell fractions were harvested and resuspended at a killing pH of 3.0 for 3 hours. Cells were then harvested a final time and plated on THYE plates using the spiral platter. All plates (including before- and after- acid killing) were incubated at 37°C for 48 hours followed by enumeration. Phenotypic detection of the ATR was valid only when a proportion of acid-adapted cells were able to withstand the 3-hour, 37°C incubation at the killing pH of 3.0 whereas the unadapted cells could not (refer to Svensäter *et al.*, 1997).

Section 2.4: Competence-stimulating peptide (CSP) and ATR experiment

To determine if induction of competence in *S. mutans* also induced acid tolerance we incubated cultures with synthetic CSP prior to exposure to the killing pH. An overnight culture of THYE-grown *S. mutans* JH1005 was diluted 10-fold in fresh THYE pH 7.5 and incubated at 37°C to an $O.D._{600}$ of 0.5. Cells were then harvested by centrifugation and resuspended in THYE pH 7.5 and 5.0 for unadapted and adapted conditions, respectively. Additionally, synthetic CSP, based on the peptide sequence indentified in *S. mutans* (Li *et al.* 2001), was added to the cell cultures at either 500 ng/ml or 1 µg/ml, with control samples devoid of CSP. The cells were then incubated for 2 hours at 37°C. A fraction (1 ml) from each sample was taken for quantitation of cell survival before exposure to the killing pH by diluting the cells (10^{-6}) and plating them on THYE plates using a spiral plater (Model D, Spiral System Inc., Cincinnati, OH). The remaining cell fractions were harvested and resuspended at a killing pH of 3.0 for 3 hours. Cells were then harvested a final time and plated on THYE plates using the spiral

plater. All plates (including before- and after- acid killing) were incubated at 37°C for 48 hours prior to enumeration.

Section 2.5: Detection of acid-induced DNA damage using TdT-mediated dUTP nick end labeling (TUNEL)

Growth Conditions of cells analyzed for DNA damage using TUNEL

The In Situ Cell Death Detection Kit, Fluorescein (Roche Diagnostics) was used to detect DNA damage caused by acid. The kit uses a terminal deoxynucleotidyl transferase enzyme that adds fluorescein-dUTP at the ends of fragmented DNA strands. Growth conditions for these experiments were as follows: an overnight culture of *S. mutans* NG8 was diluted 10-fold into fresh pH 7.5 THYE and grown to early- log phase. Cells were divided into 10 ml aliquots, harvested and resuspended into 10 ml THYE at pH 7.5, 5.0. 4.0, and 3.0. These cells were incubated for 3 hours at 37°C followed by TUNEL pretreatment.

Pretreatment of cells before TUNEL Labeling

Cells were fixed and permeabilized for TUNEL labeling. Briefly, cells were centrifuged at 13,000 X g for 2 min, resuspended in 1 ml of ice-cold fixing solution (4% paraformaldehyde, pH 7.4) and incubated for 30 min at room temperature. After incubation the cells were again pelleted and resuspended in 500 µl of PBS (pH 7.4) and 0.01% vol. toluene. Cells were permeabilized by vortexing the cells for 3X 1 min and placed on ice between intervals. Again, cells were pelleted and washed once with PBS (pH 7.4).

TUNEL Labeling (for fluorescence microscopy)

The TUNEL labeling protocol was taken from the In Situ Cell Death Detection Kit, Section 6.2 (Roche/Boehringer Mannheim) with modifications. Fixed and permeabilized cells were placed on slides, air-dried, and heat fixed. Cells were then rinsed twice with 1X PBS. 50 µl of TUNEL reaction mixture was added to the slide and a cover slip was placed on the slide to prevent evaporation during incubation. The slide was incubated in a humidified chamber for 60 min at 37°C in the dark. Finally, the slide was rinsed 3X with PBS and analyzed using epifluorescence microscopy.

TUNEL Labeling (for flow cytometry)

Flow cytometry was employed to more accurately quantitate DNA damage by TUNEL using the protocol outlined by Rohwer *et al.* (2000). Briefly, fixed and permeabilized cells were resuspended in 100 µl of TUNEL reaction mix. Negative control samples contained the TUNEL mix with the enzyme omitted. Samples were incubated at 37°C in the dark for 1 hour, washed once with PBS, and resuspended in filter sterilized PBS using a 0.2 µm Millipore® filter. Cells were then stained with propidium iodide at a concentration of 20 µg/ml (PI made in PBS) and analyzed on a FACSort sorter (Becton Dickenson, San Jose, Calif.).

CHAPTER 3: *UvrA* and the acid tolerance response in *S. mutans*

(submitted to Journal of Bacteriology)

UvrA is an acid-inducible gene essential for the adaptive response to low pH in *Streptococcus mutans*

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Running title: *uvrA* and the acid tolerance response in *S. mutans*

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Key words: uvrA, acid tolerance, DNA repair, *Streptococcus mutans*, differential display PCR, DNA damage

Section 3.1: Abstract

The acid tolerance response (ATR) is believed to play a major role in the acid adaptation process in *Streptococcus mutans*. The ATR is an inducible molecular response mechanism activated when the bacterium encounters a moderate pH that enables the cells to tolerate further drops in pH. This mechanism has been shown to require *de novo* synthesis of key proteins, some of which are encoded by acid-inducible genes. To study this phenomenon in *S. mutans* JH1005, differential display PCR was used to identify and clone genes that were activated by cells exposed to moderate pH (5.0). Thirteen cDNA products were identified as having increased expression in response to pH 5.0 when compared to pH 7.5 grown cells. One of these products, confirmed to be pH- inducible by RNA dot blot and reverse transcription PCR analyses, had 67% identity to a *uvrA*/UV repair excinuclease gene in *Bacillus subtilus*. UvrA is part of the uvrABC complex belonging to the nucleotide excision repair (NER) pathway that is involved in the recognition and excision of bulky DNA lesions caused by UV and other damaging agents. Further sequence analysis of the *uvrA* homologue using the *S. mutans* genome database revealed the complete gene was encoded in an ORF of 2829 b.p. (944 amino acids; 104.67 kDa). Immediately 3' proximal from *uvrA* was an ORF encoding a putative aminopeptidase gene (*pepP*). *UvrA* knockouts were constructed in *S. mutans* strains JH1005, NG8, and UA159 using PCR-mediated mutagenesis, replacing the entire gene with an erythromycin-resistance cassette. As with *uvrA* mutants in other bacteria, the *S. mutans uvrA* mutants were extremely sensitive to UV irradiation. The *uvrA* mutant in *S. mutans* JH1005 was also more sensitive than the wild-type to growth at pH 5.0, showing a 2-3 fold reduction in growth rate and a 14 % reduction in final resting

culture density. Acid-adapted *S. mutans* JH1005 *uvrA* mutants were shown to be more resistant to UV irradiation, but were unable to survive exposure to a killing pH of 3.0. Moreover, agarose gel electrophoretic analysis of chromosomal DNA isolated from *uvrA* deficient cells exposed to low pH demonstrated more DNA damage when compared to the wild-type strain. Here we suggest that *uvrA* and the NER pathway is necessary for the repair of acid-induced DNA damage and is essential for successful adaptation of *S. mutans* to low pH.

Section 3.2: Introduction

The oral bacterium *Streptococcus mutans* is able to gain selective advantage over other oral microbes by withstanding extreme fluctuations in plaque pH. In the plaque environment, resident bacteria metabolize dietary carbohydrate which results in the production of organic acids and a decrease in plaque pH. Telemetric measurements of plaque pH indicate that the pH can drop from pH 7.0 to values ranging from 4.0 –3.0 (24). The ability to adapt to moderate pH promotes the survival of *S. mutans* to lower pH conditions that would otherwise be lethal (40). This adaptive response in *S. mutans* is called the acid tolerance response (ATR) and similar mechanisms have been identified in some enteric bacteria (12, 13, 40, 46). Acid adaptation in *S. mutans* requires *de novo* protein synthesis (40) of up to 36 acid-regulated proteins (20) presumably encoded by acid-inducible genes.

Aside from the general features of the cellular response to acid pH, very little is known about the gene expression of key cellular proteins that are regulated by pH and play a role in the ATR. The genes for the protein repair chaperone, DnaK, (23) and the fifty-four kDa subunit homologue of the eukaryotic signal recognition particle, Ffh, (17) have been shown to be acid-inducible in *S. mutans*, but only *ffh* has been linked to the ATR whereby *ffh* mutants revealed a lack of adaptive response to acidic pH (17). To our knowledge, no other genes in *S. mutans* have been reported as being acid- inducible and related to the ATR. To elucidate the molecular mechanisms of the ATR we utilized the dd- PCR technique adapted from Kwaik and Pederson in 1996. Here, total RNA isolated from cells grown at pH 7.5 (unadapted state) and pH 5.0 (adaptive state) were reverse transcribed followed by PCR amplification with abritray primers and seperation by

polyacrylamide gel electrophoresis (PAGE) for visualization of differential expression. Our goal was to identify up-regulated genes in *S. mutans* during acid-adaptation. From this analysis, we have identified a gene with strong homology to the *uvrA* gene belonging to the nucleotide excision repair (NER) pathway involved in DNA repair in *Bacillus subtilis*. This pathway primarily consists of the protein complex, UvrABC, which functions in locating and excising bulky DNA lesions (37).

It has been proposed that the ATR in bacteria can be simplified into two main components (14). The first involves mechanisms that maintain internal pH (pH_i) homeostasis. In *S. mutans* this primarily involves an increase in H+/ATPase activity and acid end-product efflux (4, 10, 19), and a decrease in proton permeability (19) by changes in membrane fatty acid composition (34) and increased synthesis of the cell surface component, D-alanyl-lipoteichoic acid (7). The second component of the ATR is thought to involve the repair of cellular components damaged by acidic pH. Previous studies in *S. mutans* have shown the repair of acid-induced cellular damage to consist of the protein repair chaperone DnaK (23), a DNA repair enzyme exhibiting AP endonuclease activity (18), and the DNA damage regulatory/repair protein RecA (33). However, little more is known about other repair mechanisms in *S. mutans*, specifically those involved in DNA repair.

Several known DNA repair mechanisms in bacteria could potentially be involved in the repair of acid-induced DNA damage, including direct damage reversal repair, recombinational repair (e.g. RecA), mismatch repair, base excision repair (e.g. AP endonuclease), and nucleotide excision repair (e.g. UvrA) (2, 16). This picture is further complicated by the existence of specialized, regulated forms of repair such as those

potentially found in the SOS, heat shock, and adaptive responses (45). The NER pathway, however, is thought to be the major system for repairing damaged DNA because of its capacity to repair essentially all types of DNA lesions (30). DNA repair (including NER) has been implicated in the resistance of bacteria to acidic pH (5, 18, 35, 36, 43). In *E. coli*, mutants defective in the NER constituents *uvrA* and *uvrB* (35, 39) were shown to be more acid sensitive than the parent strain, suggesting that the NER pathway plays a role in the repair of acid-induced DNA damage. Whereas the pH_i of *E. coli* is maintained near neutral during acid challenge (32), *S. mutans* maintains a pH gradient that is only 0.5 –1.0 pH units higher than the extracellular pH (10), indicating an increased likelihood of intracellular acidification in *S. mutans* during low pH exposure. Therefore, the need for several DNA repair mechanisms in *S. mutans*, such as the NER pathway, would be paramount to ensure the integrity of the genome during acid stress and ultimately the survival of the species in its natural habitat.

In this study we have demonstrated that the *S. mutans uvrA* gene is upregulated in response to an acidic environmental pH. We also show that in several strains of *S. mutans uvrA* mutants were not as resilient as the wild-type in surviving UV irradiation and pH challenges to as low as 3.0.

Section 3.3: Materials and Methods

Section 3.3A: *Bacterial strains and cultivation conditions*

S. mutans strains JH1005 (22), NG8 (A.S. Bleiweis, U of Florida), and UA159 (J. Ferretti ACGT, U of Oklahoma) were grown in Todd-Hewitt (TH) broth containing 0.3% yeast extract (THYE) (BBL® ; Becton Dickinson, Cockeysville, MD) at pH 7.5 – 3.0, with the pH adjusted by addition of HCl. THYE media was prepared by autoclaving followed by aseptic titration to the desired pH. Buffering solution (0.1M MOPS, without sodium acetate) was added to pH 7.5 media to prevent significant pH drops during incubation for analysis of the ATR. For DNA damage and growth studies, 40 mM K_2HPO_4/citrate buffer was added to pH 7.5-3.0 media. In liquid media, cultures of *S. mutans* were incubated aerobically in closed screw cap tubes without agitation at 37°C. Cultivation of *S. mutans* on solid media was performed in candle jars to provide a CO_2 enriched environment. Ten µg/ml of erythromycin (Sigma-Aldrich, St. Louis, MO) was incorporated into the media when required. Epicurian coli ® XL-1 Blue supercompetent cells (Stratagene, La Jolla, CA) were used as a cloning host as described by the manufacturer.

Section 3.3B: *Induction of the ATR and phenotypic detection*

An overnight culture of THYE grown *S. mutans* JH1005 was diluted 10-fold in fresh THYE pH 7.5 and incubated at 37°C until the culture reached mid-log phase ($O.D._{600}$ = 0.4-0.5). Cells were harvested by centrifugation and resuspended in THYE pH 7.5 and 5.0 for unadapted and adapted conditions, respectively. Cells were incubated at 37°C for 1 hour for use in dd-PCR and 2 hours for phenotypic assessment of the ATR. Phenotypic detection of the ATR was valid only when a proportion of acid-adapted *S.*

mutans JH1005 wild-type (WT) cells were able to withstand a 3 hour, 37°C incubation at the killing pH of 3.0 whereas the unadapted cells could not (40). This was quantitatively confirmed by plating cells both before and after incubation at the killing pH on THYE plates using a spiral platter (Model D, Spiral System Inc., Cincinnati, OH) and incubating them at 37°C for 48 hours followed by enumeration. ATR studies performed with *S. mutans* UA159 used a killing pH of 3.5.

Section 3.3C: *Total RNA isolation*

S. mutans JH1005 cells were disrupted using the FastPrep™ FP 120 cell disrupter (BIO 101-Savant, Holbrook, NY) and RNA was extracted using the TRIzol® Reagent (Life Technologies Inc., Gaithersburg, MD) according to the manufacturer's instructions with the following modification. The isopropanol precipitation step included the addition of a High Salt Precipitation Solution (Molecular Research Centre, Inc., Cincinnati, OH) to remove polysaccharide and proteoglycans from the preparation. RNA (~ 150-200µg) was dissolved in RNA*secure* Resuspension Solution (Ambion, Austin, TX). One hundred µg aliquots of each isolated RNA preparation was treated with 40 Units of RQ1 DNase (Promega, Madison, WI) for 45 min. at 37°C, extracted with TRIzol® Reagent and chloroform, and precipitated with ethanol. Washed RNA pellets were then resuspended in diethyl pyrocarbonate (DEPC) (Sigma-Aldrich)- water and stored in aliquots at -80°C.

Section 3.3D: *Differential display polymerase chain reaction (dd-PCR)*

The RNA-DNA free samples from *S. mutans* JH1005 unadapted and adapted cells were subjected to reverse transcription using random hexamers (Pharmacia Biotech, Piscataway, NJ) and SuperScript II reverse transcriptase (Life Technologies Inc.) following the manufacturer's preamplification protocol. Reactions containing 5 µg of

RNA, 50 ng random hexamers, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 500 μM dNTPs, 10 mM DTT, and 200 U SuperScript II reverse transcriptase were incubated in a volume of 20 μl for 60 min. at 42°C. Controls without the addition of reverse transcriptase were also performed. All reactions were diluted 5-fold prior to PCR amplification.

PCR reactions (20 μl) were performed using a pair of arbitrary primers from the RNAimage mRNA Differential Display System (GenHunter, Nashville, TN). The reactions included 1 μl or 2 μl of the diluted reverse transcription reaction, 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 μM of each arbitrary primer (GenHunter), 2 μM dNTPs, 2.5 μCi α-[^{33}P]-dATP (2000-4000 Ci/mmol; New England Nuclear, Boston, MA), and 1U AmpliTaq DNA polymerase (Perkin-Elmer Applied Biosystems, Foster City, CA). Primer-pairs consisted of H-AP1/H-AP2, H-AP3/H-AP4, H-AP1/H-AP5, H-AP5/H-AP6, and H-AP6/H-AP7 (Table 3.1). After an initial denaturing step of 94°C for 5 minutes, 40 PCR cycles were run with the following conditions: 94°C for 15 seconds; 40°C for 2 minutes; 72°C for 30 seconds; followed by a 10 minute 72°C extension step. An aliquot of each PCR reaction was heated for 3 minutes at 80°C with DNA sequencing loading dye and separated by electrophoresis on a 5% denaturing Long Ranger (FMC BioProducts, Rockland, ME) gel. The gel was dried under vacuum at 80°C and exposed to Biomax MR X-ray film (Eastman Kodak Company, Rochester, NY) for 18-48 hours.

Selected cDNA PCR fragment bands showing differential gene expression at pH 5.0 were recovered and reamplified. Briefly, cDNA PCR products were excised from the dried sequencing gel, eluted in 100 μl distilled water by boiling, and ethanol precipitated

using glycogen as a carrier. An aliquot of each band was reamplified in a reaction volume of 40 µl using the same primer pair and under the same PCR conditions as the differential display reaction except the final dNTP concentration was 20 µM and radioisotope was not added. Reamplified PCR products were then run on a 1% agarose gel excised, purified using the PCR Purification Kit (Stratagene), and ligated to pCRscript from the PCR-Script™ Amp SK(+) Cloning Kit (Stratagene) according to the manufacturer's instructions. Colonies were screened for inserts using a colony screen-PCR procedure (www.invitrogen.com/expressions/597-12.html) that involved disrupting the cells by heating them to 94°C for 10 minutes prior to PCR and using the T7/T3 promoter primers flanking the inserts. Positive colonies were grown overnight in LB broth containing ampicillin and plasmid DNA was isolated using a commercial plasmid-prep kit (Qiagen Inc., Mississauga, ON, Canada). Plasmids harboring inserts were sequenced from both ends using the original amplification primers with either a Pharmacia ALF or a Perkin-Elmer-ABI Prism 377, using either dye-primer or dye terminator chemistry (DNA Sequencing Facility at The Center for Applied Genomics, The Hospital for Sick Children, Toronto, ON, Canada).

Section 3.3E: *DNA sequence analysis*

The cloned dd-PCR products were compared with other sequences in the NCBI databases using the programs BLASTX and BLASTP (1) in an attempt to identify the representative genes. Cloned fragments showing high identity to known genes were compared to the partially completed *S. mutans* genome database using TBLASTN, available from the University of Oklahoma's Advanced Center for Genome Technology (OU-ACGT at http://www.genome.ou.edu/smutans.html). The Contigs containing

homologous sequence to the gene fragments were analyzed for open reading frames (ORFs) to obtain the complete gene sequences in *S. mutans*. The ORF analysis and sequence alignments (ClustalW) were performed using MacVector 7.0 (Oxford Molecular, Madison, WI).

Section 3.3F: *RNA dot blots*

To confirm that the recovered clones harbored inserts of differentially expressed pH-inducible genes the DNA fragments were digoxygenin(DIG)-labeled by PCR (Table 3.1) using the PCR DIG Probe Synthesis Kit (Roche Diagnostics, Laval, QC, Canada) according to the manufacturer's instructions. The PCR probes were then used to screen RNA dot blots of RNA extracted from *S. mutans* JH1005 cells grown at pH 7.5 and pH 5.0. Five µg of total RNA was serially diluted and fixed onto a nylon membrane (Boehringer Mannheim). The RNA dot blot procedure from the *DIG System User Guide for Filter Hybridization* manual was used (Roche/Boehringer Mannheim). The *ffh* gene was incorporated as a positive control for acid - inducible gene expression as previously demonstrated (17).

Section 3.3G: *Reverse Transcription PCR (RT- PCR)*

As an alternative method to confirm differential expression of the *uvrA* gene, semi -quantitative RT-PCR was performed to confirm the differentially expressed pH-inducible genes. Using the *S. mutans* genome database the complete *uvrA* ORF was retrieved and internal primers to this region were designed and used in the RT- PCR reaction (Table 3.1). The *ldh* gene served as an internal control (Table 3.1). Calypso™ RT-PCR (DNAmp Ltd.- Bio/Can Scientific, Mississauga, ON, Canada), a one-step RT-PCR reaction system, was used as described by the manufacturer. Briefly, 1 µg of DNase

- treated total RNA was added to each reaction mixture. Lactate dehydrogenase (*ldh*) primers along with the primers for the specific target gene were added together at a concentration of 0.3 µg/µl per primer. Standard curves were constructed for each primer set to determine their optimum cycle number. Samples were subjected to RT-PCR as outlined by the manufacturer with an annealing temperature of 52°C with a total of 24 cycles. Controls without the addition of reverse transcriptase were also performed. These reactions contained 10 mM dNTPs (Life Technologies) and Taq DNA polymerase (MBI Fermentas, Burlington, ON, Canada), not provided in the Calypso™ kit. A Biometra ® UNOII thermocycler (Biometra, Inc., Tampa, FL) was used for all amplification procedures. Ten µl of each amplified product was analyzed on a 1% agarose gel containing ethidium bromide.

Section 3.3H: *Genomic DNA isolation and qualitative DNA damage analysis*

Genomic DNA was isolated by the following procedure. An overnight culture of *S. mutans* JH1005 was grown in 10 ml TH broth at 37°C. Cells were divided into two 2 ml microcentrifuge tubes, centrifuged, and washed in 1 ml TE buffer, resuspended in 545 µl of TE (50 mM TRIS and 10 mM EDTA) and incubated at 60°C for 20 min. Mutanolysin (10 µl from 10,000 U/ml stock) and lysozyme (25 µl from 250 mg/ml stock) (Sigma-Aldrich) were added, and cells were further incubated at 37°C with gentle mixing for 1 hour. One hundred µl of 10% SDS was added and the tubes gently inverted until the cells lysed. This lysate was incubated at 65°C for 15 min, cooled to room temperature, followed by the addition of 50 µg proteinase K (Roche Biochemicals) and further incubation at 37°C for 30 min. Then 0.7M NaCl/10% cetyltrimethylammonium bromide (CTAB) was added to the mixture, incubation continued for an additional 20

min. at 65°C, and the mixture was extracted with 900 µl of chloroform. Cells were phenol/chloroform extracted 2-4 times followed by DNA precipitation and resuspension in ddH_2O. Samples were RNase (Promega) treated with 1 Unit of enzyme and incubated for 1 hour at 37°C.

For qualitative DNA damage analysis an overnight culture of *S. mutans* JH1005 was diluted 10-fold into fresh pH 7.5 THYE and grown to mid- log phase. Cells were divided into 10 ml aliquots, harvested and resuspended into 10 ml THYE at pH 7.5, 5.0, and 4.0. These cells were incubated for 3 hours at 37°C followed by genomic DNA isolation omitting the RNase treatment to retain the ribosomal RNA (rRNA). The 23S and 16S rRNA bands were used to standardize the amount of 'total' genomic nucleic acid loaded in each well. Each sample was quantitated using the spectrophotometer ($O.D._{260}$) and 5 µg of each sample was visualized on a 1.0 % agarose gel containing ethidium bromide for comparison.

Section 3.3I: *Construction of mutants by PCR-mediated mutagenesis*

A rapid method for generating mutants in *S. mutans* JH1005, NG8, and UA159 was employed using PCR. DNA fragments of 500-1000 b.p., which flank the target gene were fused to an erythromycin resistance cassette (8). An 860 b.p. portion of the Erm cassette containing the Erm^r marker expressed from a synthetic promoter was amplified for the fusion. The construct was designed so that its integration would not disrupt the original reading frame, minimizing any downstream polar effects. Primers used to amplify the Erm cassette were ERM CSTP1 and ERM CSTP2. UvrAP1/P2 primers were used to amplify the 3' proximal flanking region of *uvrA* and uvrAP3/P4 were used to amplify the 5' proximal flanking region (see Table 3.1). Primers P2 and P3 were

designed to overlap the target gene within 120 b.p. of the 5' and 3' ends of the gene sequence, respectively. PCR products for each fragment, generated in triplicate, were purified using the PCR Purification Kit (Stratagene), digested with the appropriate restriction enzyme, *FseI* or *AscI* (MBI Fermentas), ligated with T4 DNA Ligase (Promega), and directly used for natural transformation into *S. mutans* (see Figure 3.4).

Ligated products from the PCR-mediated mutagenesis step (containing the left and right flanking regions and the Erm cassette) were used to transform *S. mutans* using competence stimulating peptide (CSP) (29). Briefly, 4 ml of TH broth was inoculated with each strain of *S. mutans* and incubated overnight at 37°C. A 20-fold dilution of each culture was made into 5 ml of fresh TH broth and incubated to early log phase. Ligated DNA product (10- 20 µl) along with 500 ng/ml of freshly prepared CSP was added to each tube and incubated a further 1.5-2 hours. Cells were then centrifuged at 1,000 rpm for 10 min and resuspended in 200 µl TH broth and plated on THYE plates containing erythromycin. Mutant confirmation was performed by isolating genomic DNA from positive colonies for use as a PCR template with primer sets P1/ERM CSTP2 and P4/ERM CSTP1. The resulting products were analyzed by agarose gel electrophoresis and their observed sizes compared to the sizes predicted following successful allelic exchange.

Section 3.3J: *Qualitative/Quantitative survival following UV irradiation*

For qualitative assessment of survival, overnight cultures of *S. mutans* WT and *uvrA* mutant strains were evenly streaked onto THYE plates using a sterile cotton swab and incubated at 37°C for 1h 30 min. The plates were then covered by a sheet of aluminum foil and progressively retracted at 10, 5 and 2 seconds approximately 27 inches

below a Phillips G30T8, 30 W UV-C germicidal lamp (254 nM). The irradiated plates were then covered in aluminum foil (dark environment) and incubated at 37°C overnight and photographed. Quantitative assessment of UV irradiated *S. mutans* WT and *uvrA* mutant strains were performed by first inducing the ATR for phenotypic detection (see methods "Induction of the ATR and phenotypic detection"), followed by a 10^6 dilution of the cells prior to spiral plating on THYE plates. Cells were then incubated at 37°C for 1hr 30min., and exposed to ultraviolet light, as stated above, for 0, 10, 20 and 30 seconds. Plates were incubated in the dark at 37°C for 24-48 hrs and counted to determine the percent survival.

Section 3.3K: *Growth Analysis*

The Bioscreen C (Labsystems, Franklin, MA) was employed to continuously grow and measure cell growth at 37°C for 24 hrs. $O.D._{600}$ was measured every 20 min. with shaking every 3 min. to prevent cell aggregation. Briefly, overnight *S. mutans* JH1005 WT and *uvrA* mutant cells were diluted 10 – fold in 1 ml of fresh THYE broth, read at $O.D._{600}$, and adjusted to the same density. Another 10-fold dilution was made into 400 µl of either THYE pH 7.5 or pH 5.0 broth and added to the Bioscreen C plates in triplicate. The growth rates of the cultures were determined by plotting and analyzing their change in $O.D._{600}$ over time using the Bioscreen C BioLink software.

Section 3.4: Results

Section 3.4A: *Differential- display PCR using adapted and unadapted S. mutans JH1005 RNA*

We used dd-PCR to analyze differences in gene expression between unadapted and acid-adapted *S. mutans* cells. Acid induction was initiated by incubating *S. mutans* cells for one hour at pH 7.5 (unadapted) and pH 5.0 (adapted) followed by total RNA isolation to observe early expression of acid-inducible genes. Total RNA from both conditions were reverse transcribed into cDNA using random hexamers. Controls without the addition of reverse transcriptase (RT) enzyme were used to ensure that the cDNA products resulted only from newly synthesized cDNA and not from chromosomal DNA contamination. The two cDNA pools were subjected to PCR using arbitrary primers. Figure 3.1 shows a portion of the entire autoradiograph of the gel resulting from amplification with arbitrary primers H-AP1/H-AP5 and H-AP5/AP6 in lanes A and B, respectively. A total of 13 amplicons were observed to be either upregulated or exclusively present in the pH 5.0 samples, 5 of which are indicated by arrows. Reactions without RT were lacking amplification products, indicating that there was no DNA contamination in the RNA preparations (data not shown). Each primer set reaction was performed in triplicate to confirm the reproducibility of the expression patterns observed. Bands showing upregulation at pH 5.0 were reamplified and cloned into pCRscript. Several ampicillin-resistant colonies were obtained and plasmid DNA from 5-6 of these colonies was isolated and sequenced. For each reamplified band multiple products were cloned since excision of the band was not precise, resulting in other products present in the reamplification reactions. Table 3.2 shows the potential heterogeneity of clones from one reamplified dd-PCR product. Three out of the 5 clones contained DNA representing

a partial ORF with 67% identity to a *uvrA*/DNA repair gene found in *Bacillus subtilis*. Dd-PCR product 4-b from Table 3.2 representing one of the *uvrA* clones was further characterized in this study. The other 12 clones upregulated by acid are still under investigation in our laboratory.

Section 3.4B: *Confirmation and characterization of the uvrA homologue*

RNA dot blots from *S. mutans* JH1005 unadapted and adapted cells were probed with the DIG-labeled *uvrA* homologue cloned insert 4b to confirm the acid- inducible expression observed in the dd-PCR experiment. RT-PCR was employed as a second method to confirm upregulation of the gene at pH 5.0 using *uvrA* primers that amplified an internal region of the entire ORF sequence. Parallel experiments were done using *ffh* as a probe or with *ffh* specific primers as a positive control for acid- inducible gene expression. *Ffh* is known to be upregulated after incubation of cells at pH 5.0 under the same conditions used in this experiment (17). Acid-adapted cells exhibited an approximate 3- fold increase in *uvrA* expression compared to unadapted cells (Fig. 3.2A). There appeared to be an even higher difference in *uvrA* and *ffh* expression using RT-PCR, probably due to the greater sensitivity of the method (Fig. 3.2B). RT-PCR reactions included the addition of primers for the internal control gene, Lactate Dehydrogenate (*ldh*), known to be equally expressed under the two experimental conditions (17) (results not shown).

Section 3.4C: *In silico analysis of the uvrA genetic locus*

Upon confirmation of acid-inducibility of the *uvrA* homologue we began characterization of the gene in *S. mutans*. Using the *S. mutans* genome database the complete gene was found to be encoded in an ORF of 2829 b.p. (Contig 51; Feb. 07/01

file). The complete *uvrA* sequence was translated into protein [~ 944 amino acids (104.67 kDa)] and compared with UvrA sequences from other bacteria (Fig. 3.3). The high degree of similarity observed provided evidence that *uvrA* was highly conserved amongst both Gram-positive and Gram-negative bacteria. Analysis of the deduced *S. mutans* UvrA sequence revealed high structural similarity to common motifs found in other organisms including two zinc finger motif regions and two nucleoprotein -ATP binding sites (2) found at amino acids 252-279/738-764 and 33-40/639-646, respectively. A search for the remaining NER constituents (*uvrB* and *C*) in the *S. mutans* database revealed that putative ORF regions for *uvrB* and *uvrC* were located elsewhere in the genome (Contig 53 and 49, respectively; Feb. 07/01 file). Immediately 3' proximal from *uvrA* was an ORF showing 50% identity to an aminopepidase P (*pepP*) gene in *Lactococcus lactis* (Fig. 3.4B). Other neighboring ORFs surrounding *uvrA* and their putative functions are also shown in Fig. 3.4B.

Section 3.4D: *Survival to UV exposure*

UvrA mutants were constructed in *S. mutans* strains JH1005, NG8, and UA159 (named JHUVRA, NGUVRA, and UAUVRA, respectively) using PCR-mediated mutagenesis. Here, DNA fragments flanking the target gene were amplified (Fig. 3.4B) and fused to an amplified portion of the erythromycin resistance cassette containing the Erm^r marker with a synthetic promoter (Fig. 3.4A). The fused construct was then used to transform *S. mutans* wild-type strains with competence stimulating peptide (CSP) (29) resulting in double-crossover recombination and substitution of the target gene with the Erm cassette. Prior to assessment of the *uvrA* mutant's ability to survive acid challenge, we first determined if a mutant defective in the putative *uvrA* gene was UV sensitive,

since *uvrA* mutants in other bacteria were reportedly extremely sensitive to UV irradiation (9, 11). All three *S. mutans uvrA* mutant strains were streaked on THYE plates and subjected to varying durations of UV-C irradiation with results showing extreme UV-C sensitivity when compared to the wild-type strains (Fig. 3.5). After less than 10 seconds of UV-C irradiation the *uvrA* mutants were unable to survive while the wild-type strains showed survival for up to 30 seconds of exposure. It has been previously demonstrated that an adaptive response to one stress can often lead to cross-protection against other stresses (21). We wanted to determine whether acid-adapted *S. mutans* JH1005 WT and JHUVRA cells were more resistant to UV irradiation when compared to unadapted cells. Results indicated this to be the case (Fig. 3.6) with JH1005 and JHUVRA showing an increase in UV survival when acid-adapted.

Section 3.4E: *Growth of S. mutans JH1005 wild-type and JHUVRA during acid challenge*

S. mutans JH1005 wild-type and JHUVRA strains were grown in THYE broth supplemented with 40 mM K_2HPO_4/citrate buffer adjusted to pH values of 7.5 and 5.0. Using an automated growth reader, we were able to monitor growth of several different cultures simultaneously. This allowed us to perform three independent experiments under each desired condition and to determine the mean for plotting the growth curves. Results showed that both strains grew similarly at pH 7.5 and, apart from the slower growth rate normally observed at pH 5.0 due to the shift in cell's energy towards maintaining internal pH homeostasis (10), JHUVRA showed a two-fold decrease in growth rate at pH 5.0 when compared to the wild-type (Fig. 3.7). Similar results were observed with *S. mutans* strains NG8 and UA159 and their respective mutants (data not

shown). Also, final resting culture density of JHUVRA cells grown at pH 5.0 was shown to be approximately 14% less than the wild-type.

Section 3.4F: *S. mutans JH1005 and UA159 uvrA mutants show deficiency in the ATR*

To determine whether JHUVRA was able to elicit an adaptive response to low pH as shown by the parent, exponential-phase JH1005 WT and JHUVRA cells were incubated at the adaptive pH (5.0) and unadaptive pH (7.5) for 2 hours followed by exposure to the killing pH for 3 hours. Under these condition outlined previously (40), unadapted JH1005 wild-type cells showed no survivors after exposure to the killing pH, whereas adapted cells showed a significant number of survivors (Fig. 3.8A). Unlike the parent, the JHUVRA strain when exposed to the adaptive pH was unable to show enhanced survival when incubated at the killing pH (Fig. 3.8A). *S. mutans* JH1005 is known to have a weak adaptive response (ATR) compared to other mutans strains (40). This is seen in our results by the low percentage of survivors of adapted JH1005 wild-type cells after exposure to the killing pH. As a result, we repeated the ATR experiments with the *uvrA* mutant we had constructed in *S. mutans* UA159. Here, UA159 WT and UAUVRA unadapted and adapted cells were incubated for 3 hours at a killing pH of 3.5. The ATR in UA159 displayed a stronger adaptive response indicated by the significantly larger proportion of survivors found in adapted wild-type cells. Under the conditions tested we also observed that UAUVRA demonstrated a reduced capacity in mounting an ATR as demonstrated by a 10-fold decrease in survivors of adapted cells to the killing pH when compared to the wild-type strain (Fig. 3.8B).

Section 3.4G: *Low external pH (pH_o) causes more DNA damage in JHUVRA than in the wild-type*

To our knowledge there is no direct evidence that demonstrates that DNA damage occurs in *S. mutans* during low external pH (pH_0) exposure. Our observed induction of the DNA repair gene, *uvrA*, in *S. mutans* (by exposure to pH 5.0) suggests that DNA damage may be occurring during low pH_0 exposure. To investigate whether *uvrA* plays a significant role in the repair of acid-induced DNA damage we grew JH1005 wild-type and JHUVRA cells to mid-log phase then exposed them to pH 7.5, 5.0, and 4.0 for 3 hours followed by 'total' genomic isolation whereby chromosomal DNA and ribosomal RNA (rRNA) could be visualized on an agarose gel. Figure 3.9 shows JHUVRA chromosomal DNA to be significantly more degraded at pH 4.0 when compared to the wild type. Also, visual assessment of wild-type DNA indicated that degradation might be occurring at pH 4.0. Both JH1005 and JHUVRA were not killed (100 % survival) after exposure to pH 7.5 and 5.0 for 3 hours. After a 3 hour exposure to pH 4.0 there was a difference between unadapted parent and mutant cells showing 29.4 % (± 2.52) and 19 % (± 6.49) survival, respectively (data not shown).

Section 3.5: Discussion

The differential-display PCR (dd-PCR) technique had been successfully used with prokaryotic cells to evaluate differences in gene expression due to environmental changes (6, 25, 26, 38). We have demonstrated the use of the dd-PCR technique with a gram-positive organism to identify genes differentially expressed due to changes in pH. Further analysis of one gene, *uvrA*, confirmed its role in the acid tolerance response (ATR). The ATR has been defined as the adaptation process to acid, whereby exposure to acidic pH [5.5-4.0 in *S. mutans* (40)] afforded protection to lower pH values that would otherwise be lethal to the cell. By identifying genes induced at pH 5.0 we hoped to discover genes that encode the proteins necessary for acid-adaptation. Using dd-PCR we visualized the increased expression of approximately 13 gene products in response to exposure to pH 5.0 when compared to pH 7.5 grown cells (Fig. 3.1). Each cloned product recovered from the dd-PCR experiment was shown to be heterogeneous since resolving and excising each 'band' separately was sometimes impossible.

The *uvrA* gene in *S. mutans* was identified as being acid-inducible by differential display-PCR and was confirmed by RNA dot blot and reverse transcriptase- PCR analysis (Fig. 3.2). From the *S. mutans* genome database we retrieved the complete *uvrA* ORF and identified immediately 3' proximal from *uvrA* an ORF encoding a putative aminopeptidase gene homologous to *pepP* in *Lactococcus lactic*. This raised the question as to the potential involvement of peptidase activity in DNA repair, specifically involving *uvrA*. Numerous bacterial cellular processes involve proteolysis, but their exact functions are not often known (27). In *E. coli*, the proteolytic activity of RecA is known to cleave the SOS repressor protein LexA, activating DNA repair mechanisms amongst other

things (31, 45). Also, aminopeptidases (*pepA*) have been shown to possibly bind DNA and regulate transcription of virulence genes regulated by pH in *Vibrio cholerae* (3). It is tempting to speculate that PepP activity in *S. mutans* may be involved in regulation of *uvrA* in a pH-dependent manner.

The UvrA protein in *S. mutans* appears similar to other UvrA proteins found in other organisms (Fig. 3.3). The two main DNA binding structural motifs found in UvrA, mainly the two zinc finger and nucleoprotein-ATP binding regions, allow UvrA to preferentially bind to single- and double-stranded DNA breaks which then initiates recruitment of the remaining proteins in the UvrABC complex (2, 42). In bacteria, the UvrABC complex is the principal component of the nucleotide excision pathway (NER)(37) which is the main pathway for the removal of damage caused by UV light. Indeed, in *E. coli* and other organisms, gene knockouts in any of the NER constituents create mutants that are extremely UV sensitive (9, 11, 43) suggesting that any mutation made in the NER pathway completely obliterates the system. We have shown in *S. mutans* that *uvrA* mutants are also extremely sensitive to UV irradiation (Fig. 3.5) presumably because the entire UV repair system of NER is inoperative. However, mutants in *uvrB* and *uvrC* would need to be constructed and tested for UV sensitivity in *S. mutans* to make this statement conclusive.

It has been shown with *S. mutans* UR100 (*recA*$^-$) (33) and *E. coli* K12 (*uvrA*$^-$ and *recA*$^-$) mutants (15) that previous exposure to acidic pH provided cross-protection to UV irradiation. In our present study we have also shown that acid-adapted *S. mutans* JH1005 and JHUVRA strains exhibit the same phenomena of increased resistance to UV irradiation. Here, the majority of UV damage seems to be repaired by the NER system

based on the extreme UV sensitivity of *uvrA* mutants, but we can surmise that other acid-inducible repair system(s) in *S. mutans* are involved in UV damage repair since acid-adapted *uvrA* mutants still exhibit enhanced resistance to UV irradiation relative to unadapted cells (Fig. 3.6). Although not immediately apparent from the graph, there is a 6-fold increase in survival after two seconds UV exposure as seen in adapted JHUVRA cells (0.15 ± 0.006) versus unadapted cells ($0.02 \bullet 0.012$). In addition, other studies in *S. mutans* have demonstrated that prior adaptation to other stresses including salt, oxidation, and starvation, increased resistance to acid challenge (41). Also, in *Lactococcus lactis* previous exposure to UV light has been shown to enhance resistance to acid challenge (21). These findings collectively suggest that there may be an overlap in DNA repair mechanisms involved in repairing both UV- and acid- induced DNA damage.

The importance of DNA repair mechanisms for survival to acid shock (pH 4-3) has been established previously in other bacteria (33, 39, 43). DNA repair-deficient bacteria exposed directly to acid shock have been shown to have poor survival rates when compared to the parent strains. Our visual analysis of DNA damage by gel electrophoresis of chromosomal DNA isolated from *uvrA*-deficient *S. mutans* cells exposed to acid shock (pH 4.0) indicated that there was more DNA damage than the DNA repair proficient JH1005 wild-type (Fig. 3.9). The observed differences seen at pH 4.0 could be as a result of *uvrA*-deficient DNA being less stable and tolerant of the extraction technique. It is interesting to note that although 70 % of the parent cells were dead after 3 hours exposure to pH 4.0 the DNA from these samples appeared rather intact compared to the DNA of *uvrA*-deficient cells under the same conditions. These data suggest that *uvrA* is required for the repair of acid-induced DNA damage. Additionally,

we have shown that disruption of *uvrA* results in a reduced ability to grow at pH 5.0 (Fig. 3.7) suggesting that *uvrA* and possibly other DNA repair systems are essential not only for survival of acid shock, but also for growth at moderately acidic pH.

Evidence in *E. coli* indicates that DNA repair mechanisms are induced during acid-adaptation and are responsible for an apparent decrease in DNA damage occurring during growth at pH 5.0 (35, 36). To account for this increase in DNA repair and decrease in DNA damage during acid-adaptation, one would envision the necessity of DNA repair mechanisms for maintaining the integrity of the DNA template for successful synthesis of proteins essential for acid-adaptation. Without adequate DNA repair from insults such as acidic pH or other damaging agents during the critical adaptation process, the bacterium's ability to successfully adapt and survive at lower pH would be diminished. To evaluate this idea further we measured the ability of the DNA repair-deficient strain JHUVRA to exhibit an acid-adaptive response (ATR). After incubation at pH 5.0 for 2 hours to induce acid-adaptation, both wild-type and *uvrA* mutant strains were exposed to the killing pH 3.0 for 3 hours. Our results showed that adapted JHUVRA cells were unable to survive the killing pH when compared to adapted wild-type cells (Fig. 3.8A). We also tested the *uvrA* mutant we had constructed in UA159 and discovered similar trends (Fig. 3.8B). Here we observed at a killing pH of 3.5 that adapted UAUVRA mutants had a dramatically decreased survival rate when compared to the wild-type. These results emphasize the importance of *uvrA* in survival to acid shock and acid adaptation in *S. mutans*.

The apparent damage done to DNA during growth at low pH, and the subsequent DNA repair essential for cell survival suggests that several repair mechanisms may be

inducible by acidic pH. The activity of a *S. mutans* AP endonuclease, involved in repair of damaged or incorrect bases, was found to be inducible by low pH (18). One could conceive the idea that the base repair activity of the AP endonuclease would be responsible for initiating the DNA repair process of UvrABC which is activated by helical distortions, caused by the displacement of bases, rather than recognition of any particular group (44). Therefore, the role of base excision repair could be to repair minor DNA damage, whereas UvrA and the NER pathway could be responsible for excising larger DNA lesions caused by acid and other agents. It was shown in *S. mutans* that the gene expression and protein levels of the heat shock protein, DnaK, were upregulated in response to acid adaptation and acid shock (23). In *E. coli*, the DnaK protein was also discovered to increase the stability of UvrA during heat stress (47) suggesting that heat shock proteins might indirectly be involved in acid-induced DNA repair by ensuring proper functioning of DNA repair mechanisms in less than optimal conditions. These observations not only support the idea that acid-inducible DNA repair mechanisms exist in *S. mutans*, but suggest that specialized, regulated forms of DNA repair such as those found in the SOS, heat shock, and adaptive responses potentially exist and probably have a significant overlap with the ATR. These responses could either operate as independently regulated systems or overlap in their activities in response to acid-induced DNA damage. Further evidence that these regulatory networks exist in *S. mutans* are supported by 1- and 2-dimensional SDS-polyacrylamide electrophoresis (PAGE) studies comparing protein extracts from acid-induced and -uninduced cells, demonstrating that the synthesis of acid- regulated proteins may include acid-specific proteins and general stress proteins (e.g. heat shock) (20, 41). Obviously, studies exploring the involvement of

these regulatory networks in DNA repair and acid-adaptation in *S. mutans* need to be investigated further.

This study supports earlier observations made in *S. mutans* and other acid-tolerant bacteria as to the importance of DNA repair in survival to low pH. We have confirmed that *S. mutans* mutants defective in *uvrA* are UV sensitive. We have also confirmed that this acid-inducible gene is essential for growth at acidic pH and during acid-adaptation. The differential display-PCR technique was also shown to be effective in identifying potential acid-inducible genes. Future work will involve further characterization of other acid-inducible genes identified in our dd-PCR experiment in combination with 2D-SDS PAGE and microarray techniques to better characterize the genes, proteins, and regulatory networks involved in the adaptation process to low pH.

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Section 3.6: References

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403-410.
2. **Aravind, L., D. R. Walker, and E. V. Koonin.** 1999. Conserved domains in DNA repair proteins and evolution of repair systems. Nucleic Acid Res. **27:**1223-1242.
3. **Behari, J., L. Stagon, and S. B. Calderwood.** 2001. *PepA*, a gene mediating pH regulation of virulence genes in *Vibrio cholerae*. J. Bacteriol. **183:**178-188.
4. **Bender, G. R., S. V. W. Sutton, and R. E. Marquis.** 1986. Acid tolerance, proton permeabilities, and membrane ATPases of oral Streptococci. Infect. Immun. **53:**331-338.
5. **Bijlsma, J. J. E., M. Lie-A-Ling, I. C. Nootenboom, C. M. J. E. Vandenbroucke-Grauls, and J. G. Kusters.** 2000. Identification of loci essential for the growth of *Helicobacter pylori* under acidic conditions. J. Infect. Dis. **182:**1566-1569.
6. **Bonass, W. A., P. D. Marsh, R. S. Percival, J. Aduse-Opoku, S. A. Hanley, D. A. Devine, and M. A. Curtis.** 2000. Identification of *ragAB* as a temperature-regulated operon of *Porphyromonas gingivalis* W50 using differential display of randomly primed RNA. Infect. Immun. **68:**4012-4017.
7. **Boyd, D. A., D. G. Cvitkovitch, A. S. Bleiweis, M. Y. Kiriukhin, D. V. Debabov, F. C. Neuhaus, and I. R. Hamilton.** 2000. Defects in D-alanyl-lipoteichoic acid synthesis in *Streptococcus mutans* results in acid sensitivity. J. Bacteriol. **182:**6055-6065.
8. **Claverys, J. -P., A. Dintilhac, E. V. Pestova, B. Martin, and D. A. Morrison.** 1995. Construction and evaluation of new drug-resistance cassettes for gene

disruption mutagenesis in *Streptococcus pneumoniae*, using an *ami* test platform. Gene **164:**123-128.

9. **Courcelle, J., D. J. Crowley, and P. C. Hanawalt.** 1999. Recovery of DNA replication in UV-irradiated *Escherichia coli* requires both excision repair and RecF protein function. J. Bacteriol. **181:**916-922.

10. **Dashper, S. G., and E. C. Reynolds.** 1992. PH regulation by *Streptococcus mutans*. J. Dent. Res. **71:**1159-1165.

11. **De la Morena, M. L., D. R. Hendrixson, and J. W. St. Geme.** 1996. Isolation and characterization of *Haemophilus influenzae uvrA* gene. Gene **177:**23-28.

12. **Foster, J. W.** 1999. When protons attack: microbial strategies of acid adaptation. Curr. Opin. Microbiol. **2:**170-174.

13. **Foster, J. W., and H. K. Hall.** 1990. Adaptive acidification tolerance response of *Salmonella typhimurium*. J. Bacteriol. **173:**5129-5135.

14. **Foster, J. W., and H. K. Hall.** 1991. Inducible pH homeostasis and the acid tolerance response of *Salmonella typhimurium*. J. Bacteriol. **173:**5129-5135.

15. **Goodson, M. and R. J. Rowbury.** 1991. RecA-independent resistance to irradiation with U.V. light in acid-habituated *Escherichia coli.* J. Appl. Bacteriol. **70:**177-180.

16. **Grogan, D. W.** 2000. The question of DNA repair in hyperthermophilic archaea. Trends Microbiol. **8:**180-185.

17. **Gutierrez, J. A., P. J. Crowley, D. G. Cvitkovitch, L. J. Brady, I. R. Hamilton, J. D. Hillman, and A. S. Bleiweis.** 1999. *Streptococcus mutans ffh*, a gene encoding a homologue of the 54 kDa subunit of the signal recognition particle, is involved in resistance to acid stress. Microbiology **145:**357-366.

18. **Hahn, K., R. C. Faustoferri, and R. G. Quivery, Jr.** 1999. Induction of an AP endonuclease activity in *Streptococcus mutans* during growth at low pH. Mol. Microbiol. **31:**1489-1498.

19. **Hamilton, I. R, and N. D. Buckley.** 1991. Adaptation by *Streptococcus mutans* to acid tolerance. Oral Microbiol. Immunol. **6:**65-71.

20. **Hamilton, I. R., and G. Svensäter.** 1998. Acid-regulated proteins induced by *Streptococcus mutans* and other oral bacteria during acid shock. Oral Microbiol. Immunol. **13:**292-300.

21. **Hartke, A., S. Bouche, X. Gansel, P. Boutibonnes, and Y. Auffray.** 1995. UV-inducible proteins and UV-induced cross protection against acid, ethanol, H_2O_2 or heat treatments of *Lactobacillus lactis* subsp. *Lactis*. Arch. Microbiol. **163:**329-336.

22. **Hillman, J. D., A. L. Dzuback, and S. W. Andrews.** 1987. Colonization of the human oral cavity by a *Streptococcus mutans* mutant producing increased bacteriocin. J. Dent. Res. **66:**1092-1094.

23. **Jayaraman, G. C., J. E. Penders, and R. A. Burne.** 1997. Transcriptional analysis of the *Streptococcus mutans hrcA*, *grpE*, and *dnaK* genes and regulation of expression in response to heat shock and environmental acidification. Mol. Microbiol. **25:**329-341.

24. **Jensen, M. E., P. J. Polansky, and C. F. Schachtele.** 1982. Plaque sampling and telemetry for monitoring acid production on human buccal tooth surfaces. Arch. Oral. Biol. **27:**21-31.

25. **Kullen, M. J., and T. R. Klaenhammer.** 1999. Identification of the pH-inducible, proton-translocating F_1F_0-ATPase (*atpBEFHAGDC*) operon of *Lactobacillus*

acidophilus differential display: gene structure, cloning and characterization. Mol. Microbiol. **33:** 1152-1161.

26. **Kwaik, Y. A., and L. L. Pederson.** 1996. The use of differential display-PCR to isolate and characterize a *Legionella pneumophila* locus induced during the intracellular infection of macrophages. Mol. Microbiol. **21:**543-556.

27. **Lazdunski, A. M.** 1989. Peptidases and proteases of *Escherichia coli* and *Salmonella typhimurium*. FEMS Microbiol. Rev. **63:**265-276.

28. **Lee, M. S., and D. A. Morrison.** 1999. Identification of a new regulator in *Streptococcus pneumoniae* linking quorum sensing to competence for genetic transformation. J. Bacteriol. **181:**5004-5016.

29. **Li, Y. H., P. C-Y Lau, J. H. Lee, R. P. Ellen, and D. G. Cvitkovitch.** 2001. Natural genetic transformation of *Streptococcus mutans* growing in biofilms. J. Bacteriol. **183:** 897-908.

30. **Lin, J. J., and A. Sancar.** 1992. (A)BC excinuclease: the *Escherichia coli* nucleotide excision repair enzyme. Mol. Microbiol. **6:**2219-2224.

31. **Little, J. M., and D. W. Mount.** 1982. The SOS regulatory system of *E. coli*. Cell **29:** 11-22.

32. **Paden, E., D. Zilberstein, and S. Schuldiner.** 1981. PH homeostasis in bacteria. Biochim. Biophys. Acta. **650:**151-166.

33. **Quivey, Jr., R. G., R. C. Faustoferri, K. Anne Clancy, and R. E. Marquis.** 1995. Acid adaptation in *Streptococcus mutans* UA159 alleviates sensitization to environmental stress due to RecA deficiency. FEMS Microbiol. Lett. **126:**257-262.

34. **Quivey, Jr., R. G., R. Faustoferri, K. Monahan, and R. Marquis.** 2000. Shifts in membrane fatty acid profiles associated with acid adaptation of *Streptococcus mutans*. FEMS Microbiol. Lett. **189:**89-92.

35. **Raja, N., M. Goodson, W. C. M. Chui, D. G. Smith, and R. J. Rowbury.** 1991a. Habituation to acid in *Escherichia coli*: conditions for habituation and its effects on plasmid transfer. J. Appl. Bacteriol. **70:**59-65.

36. **Raja, N., M. Goodson, D. G. Smith, and R. J. Rowbury.** 1991b. Decreased DNA damage by acid and increased repair of acid-damaged DNA in acid-habituated *Escherichia coli*. J. Appl. Bacteriol. **70:**507-511.

37. **Sancar, A.** 1996. DNA excision repair. Annu. Rev. Biochem. **65:**43-81.

38. **Shepard, B. D., and M. S. Gilmore.** 1999. Identification of aerobically and anaerobically induced genes in *Enterococcus faecalis* by random arbitrarily primed PCR. Appl. and Environ. Micro. **65:**1470-1476.

39. **Sinha, R. P.** 1986. Toxicity of organic acids for repair-deficient strains of *Escherichia coli*. Appl. and Environ. Micro. **51:**1364-1366.

40. **Svensäter, G., U. –B. Larsson, E. C. G. Greif, D. G. Cvitkovitch, and I. R. Hamilton.** 1997. Acid tolerance response and survival by oral bacteria. Oral. Microbiol. Immunol. **12:**266-273.

41. **Svensäter, G., B. Sjogreen, and I. R. Hamilton.** 2000. Multiple stress responses in *Streptococcus mutans* and the induction of general and stress-specific proteins. Microbiology **146:**107-117.

42. **Thiagalingam S., and L. Grossman.** 1991. Both ATPase sites of *Escherichia coli* UvrA have functional roles in nucleotide excision repair. J. Biol. Chem. **266:**11395-11403.

43. **Thompson, S. A., and M. J. Blaser.** 1995. Isolation of the *Helicobacter pylori recA* gene and involvement of the *recA* region in resistance to low pH. Infect. Immun. **63:** 2185-2193.

44. **Voet, D., and J. Voet.** 1990. *Biochemistry* (pp. 968). Toronto: John Wiley and Sons, Inc.

45. **Walker, G. C.** 1984. Mutagenesis and Inducible Responses to Deoxyribonucleic Acid Damage in *Escherichia coli*. Microbiol. Rev. **48:**60-93.

46. **Yousheng, M., T. M. Curran, and R. E. Marquis.** 1997. Rapid procedure for acid adaptation of oral lactic-acid bacteria and further characterization of the response. Can. J. Microbiol. **43:**143-148.

47. **Zou, Y., D. J. Crowley, and B. Van Houten.** 1998. Involvement of molecular chaperonins in nucleotide excision repair. Dnak leads to increased thermal stability of UvrA, catalytic UvrB loading, enhanced repair, and increased UV resistance. J. Biol. Chem. **273:**12887-12892.

Section 3.7: Figure Legends

Figure 3.1. Differential display PCR autoradiograph of amplified *S. mutans* JH1005 RNA isolated from adapted (pH 5.0) and unadapted (pH 7.5) cells. The cDNAs were amplified with primers H-AP1/H-AP5 **(A)** and H-AP5/H-AP6 **(B)** in the portion of the gel shown. The arrows denote pH-inducible gene fragments present at pH 5.0 and absent or greatly reduced at pH 7.5.

Figure 3.2. Dot blot hybridization and RT-PCR of total RNA isolated from *S. mutans* JH1005 acid-adapted and unadapted cells. The dot blots were probed with the digoxygenin (DIG)-labeled *uvrA* cloned fragment and a DNA fragment representing the internal region of *ffh* **(A)**. RT-PCR reactions amplified the internal regions of *uvrA* and *ffh* **(B)**. The Lactate Dehydrogenase gene (*ldh*) served as the internal control for the RT-PCR (data not shown). All experiments were repeated more than three times to confirm the acid-inducibility of *uvrA* and *ffh*.

Figure 3.3. Alignment of the *S. mutans* UvrA homologue with other UvrA proteins from other bacteria. The sequences were aligned with the program ClustalW. Homologous amino acids are indicated by the shaded area and % identities by the bordered areas. The two zinc finger and nucleoprotein-APT binding site motifs are located at amino acids 252-279/738-764 and 33-40/639-646 in the S.m. sequence. Abbr.: (S.m) *Streptococcus mutans*, (B.s) *Bacillus subtilis*, (M.t) *Mycobacterium tuberculosis*, and (E.c) *Escherichia coli*.

Figure 3.4. PCR construct **(A)**, was made by ligating the amplified Erm cassette (Erm CSTP1/P2) and left (P1/P2) and right (P3/P4) flanking regions of *uvrA*. This was used to transform *S. mutans* wild-type strains resulting in substitution of the *uvrA* gene with the

Erm cassette by double-crossover recombination. Arrowheads mark primer sites used for PCR. The mutagenesis procedure is further described in Materials and Methods. Open reading frame map of *S. mutans* UA159 *uvrA* region and neighboring genes **(B)** and schematic of PCR-mediated mutagenesis of *uvrA*. ORF1= potassium channel protein (32% identity, *Rattus norvegicus*), *pepP*= aminopeptidase P (50% identity, *Lactococcus lactis*), ORF4= probable transport protein/membrane protein (33% identity, *Deinococcus radiodurans*).

Figure 3.5. Survival of wild-type *S. mutans* JH1005, NG8, UA159 and *uvrA* strains following UV irradiation. The plates were placed approximately 27 inches below 30 W UV-C germicidal lamp (254 nM) for 10, 30, and 60 seconds.

Figure 3.6. Survival of unadapted (Un) and adapted (Ad) wild-type *S. mutans* JH1005 and *uvrA* mutant strains after exposure to UV irradiation. Cells were plated at the appropriate dilution after the adaptation period, incubated for 11/2 hours, UV irradiated, and incubated a further 48 hours for enumeration. The results are the means of three experiments.

Figure 3.7. Growth curve of *S. mutans* JH1005 wild-type and *uvrA* mutant strains at pH 5.0 and pH 7.5 (control). Each data point is the mean of three independent experiments.

Figure 3.8. Acid tolerance response of *S. mutans* JH1005 **(A)** and UA159 **(B)** wild-type and *uvrA* mutant strains. Mid-log cells were harvested and resuspended in THYE pH 7.5 and 5.0 for unadapted and adapted conditions, respectively. Cells were incubated at 37°C for 2 hours followed by a 3-hour incubation at the killing pH of 3.0.

Figure 3.9. Agarose gel electrophoresis of total genomic nucleic acid (consisting of DNA, rRNA, and mRNA) of *S. mutans* JH1005 and JHUVRA cells exposed to pH 7.5,

5.0 and 4.0 for 3 hours. An equal amount (5 μg) of total nucleic acid was added in each well. The 23S and16S rRNA seen in each lane provided a means of standardizing the total amount of nucleic acid added in each lane.

Table 3. 1: Oligonucleotides used in this study

Name	Sequence (5' -3')	Description
H-AP1	AAGCTTGATTGCC	GenHunter® arbitrary primers
H-AP2	AAGCTTCGACTGT	"
H-AP3	AAGCTTTGGTCAG	"
H-AP4	AAGCTTCTCAACG	"
H-AP5	AAGCTTAGTAGGC	"
H-AP6	AAGCTTGCACCAT	"
H-AP7	AAGCTTAACGAGG	"
uvrAF	GGCTCCTTGGAATCCTATTTCTTC	*uvrA* probe + RT- PCR
uvrAB	GAATGCGTTGGCTTTCACCTC	"
ffhF	GGATCCATGGCTTTTGAAAGTTTA	*ffh* probe + RT- PCR
ffhB	GCAACATTTGCGGCTTCT	"
ldhF	CGGATGCTGACCTTGTTGTTATC	internal control for RT- PCR
ldhB	AAGTGCTTGACGGAAACGAGC	"
ERM CSTP1[a]	GGCGCGCCCCGGGCCCAAAATTTGTTTGAT	Erm Cassette (28)
ERM CSTP2[b]	GCTGGCCGGACTCGGCAGCGACTCATAGAAT	"
uvrAP1	TCTGCTGTTGCGTTCTTCTGG	*uvrA* PCR-mediated mutagenesis
uvrAP2[a]	GGCGCGCCTTTCCTGAACCCGATAAGCCCG	"
uvrAP3[b]	GCTGGCCGGAGCTGAGATGACGGAAAGCTATAC	"
uvrAP4	CAGAACGGTAACCTGATGCAAC	"

[a] Underlined bases represent *Asc I* restriction sequence
[b] Underlined bases represent *Fse I* restriction sequence

Table 3. 2: Nucleotide sequences of heterogeneous clones obtained from a single reamplified dd-PCR product

Clone	Homolog (BLASTX)	Identity
dd-PCR 4a	*Staphylococcus aureus TraG* (conjugal transfer membrane protein)	45%
dd-PCR 4b dd-PCR 4d dd-PCR 4e	*Bacillus subtilis uvrA* (UV repair excinuclease)	67%
dd-PCR 4c	*Bacillus subtilis YCF24* (hypothetical DNA binding protein)	35%

Section 3.8: Figures

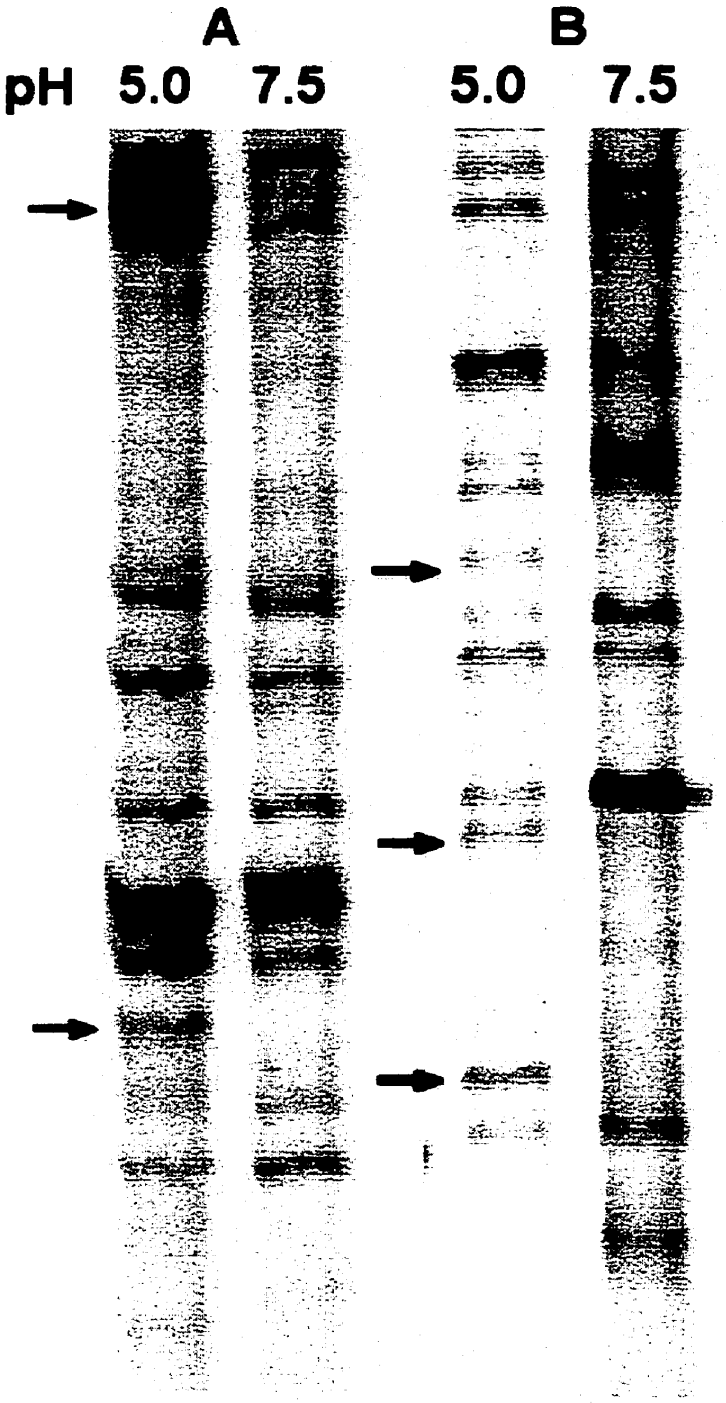

Figure 3. 1

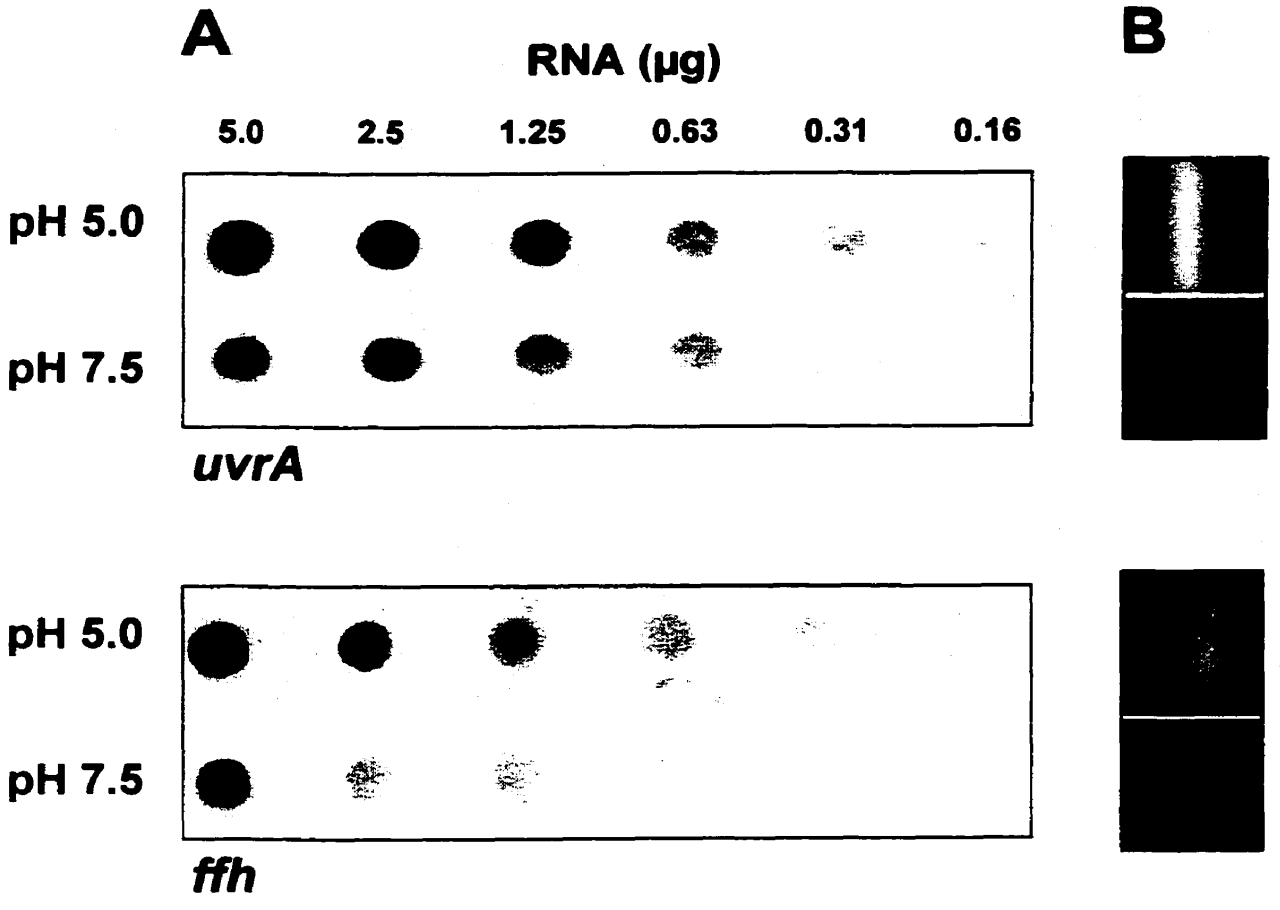

Figure 3. 2

Figure 3. 3

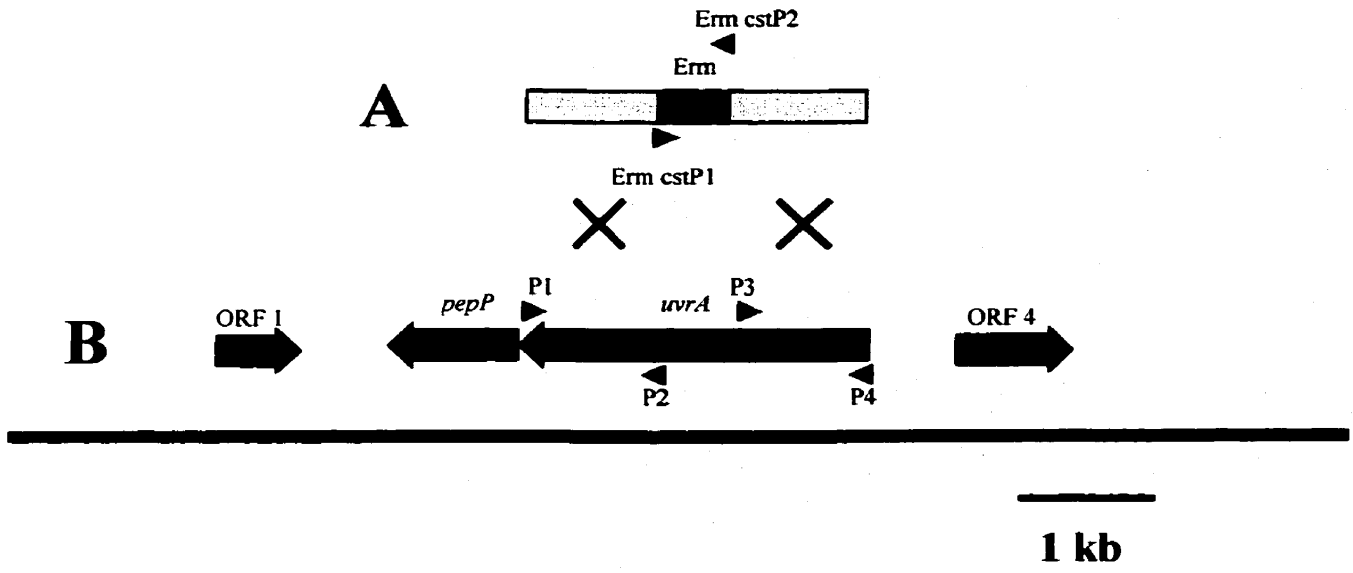

Figure 3. 4

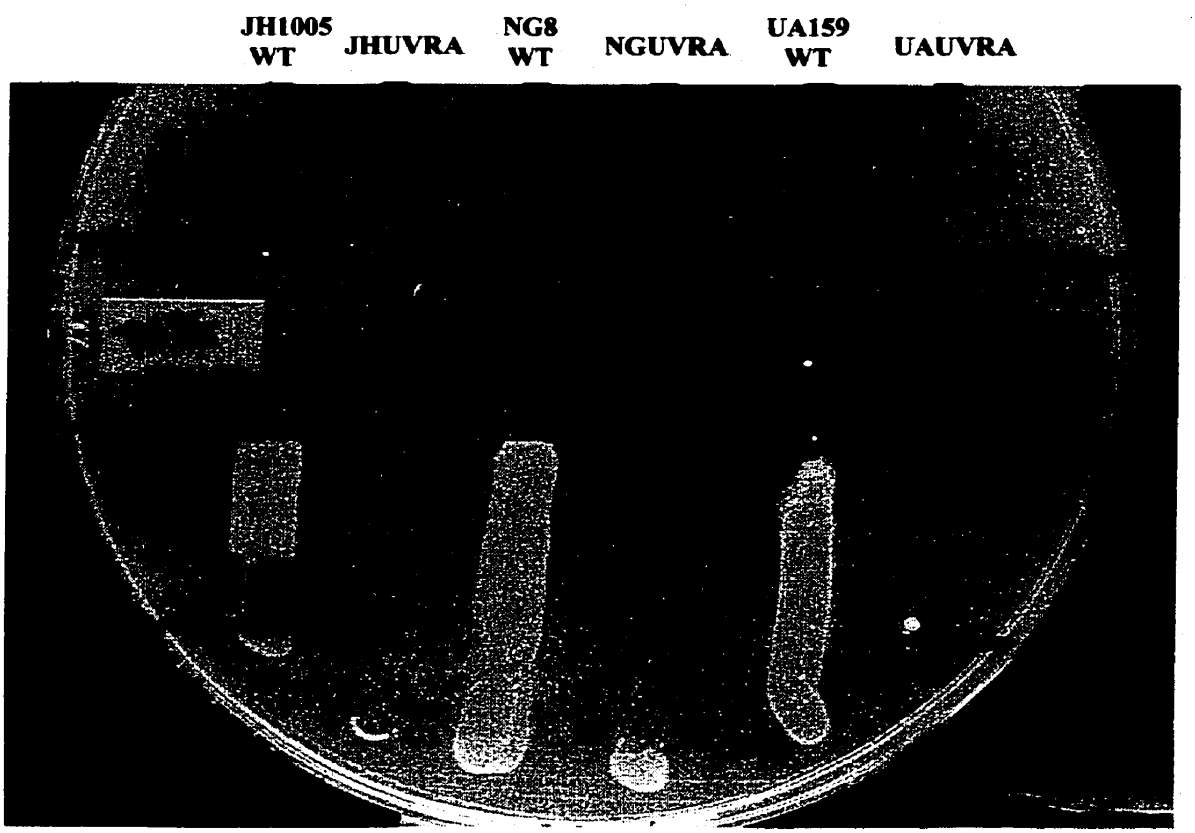

Figure 3. 5

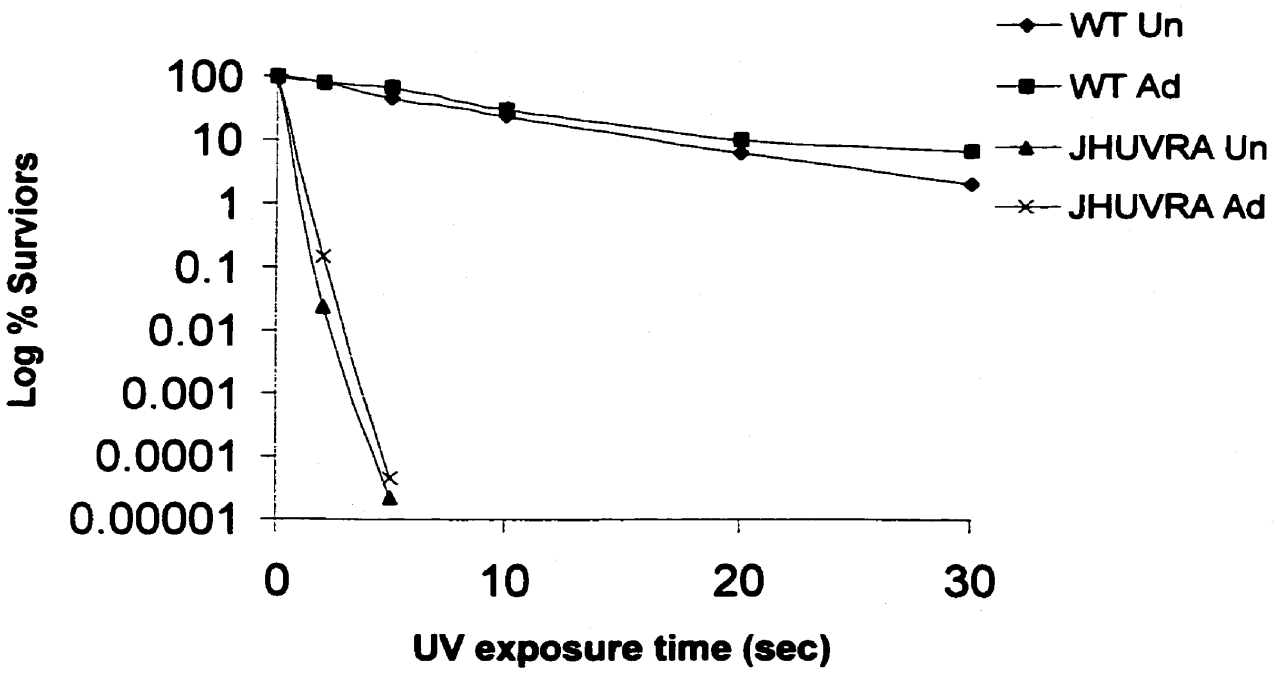
WT Un
WT Ad
JHUVRA Un
JHUVRA Ad
Log % Surviors
100
10
1
0.1
0.01
0.001
0.0001
0.00001
0
10
20
30
UV exposure time (sec)

Figure 3. 6

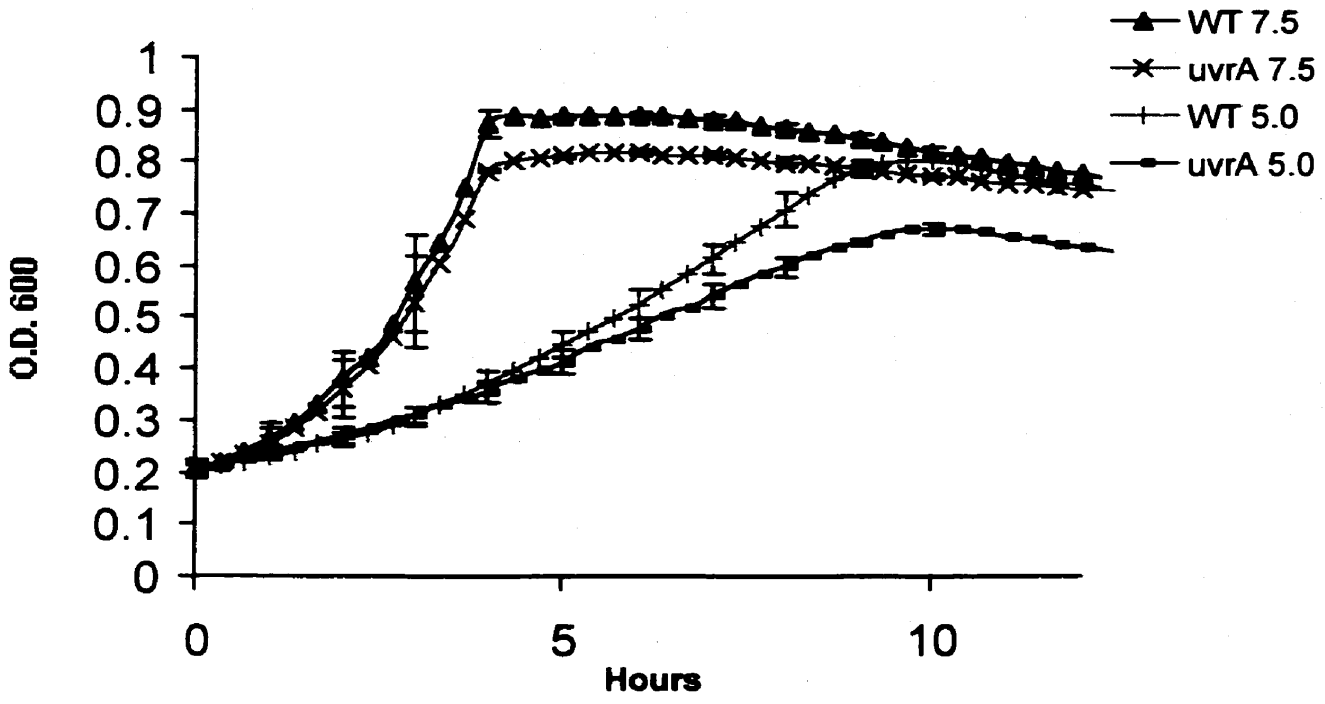
WT 7.5
uvrA 7.5
WT 5.0
uvrA 5.0
O.D. 600
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
5
10
Hours

Figure 3. 7

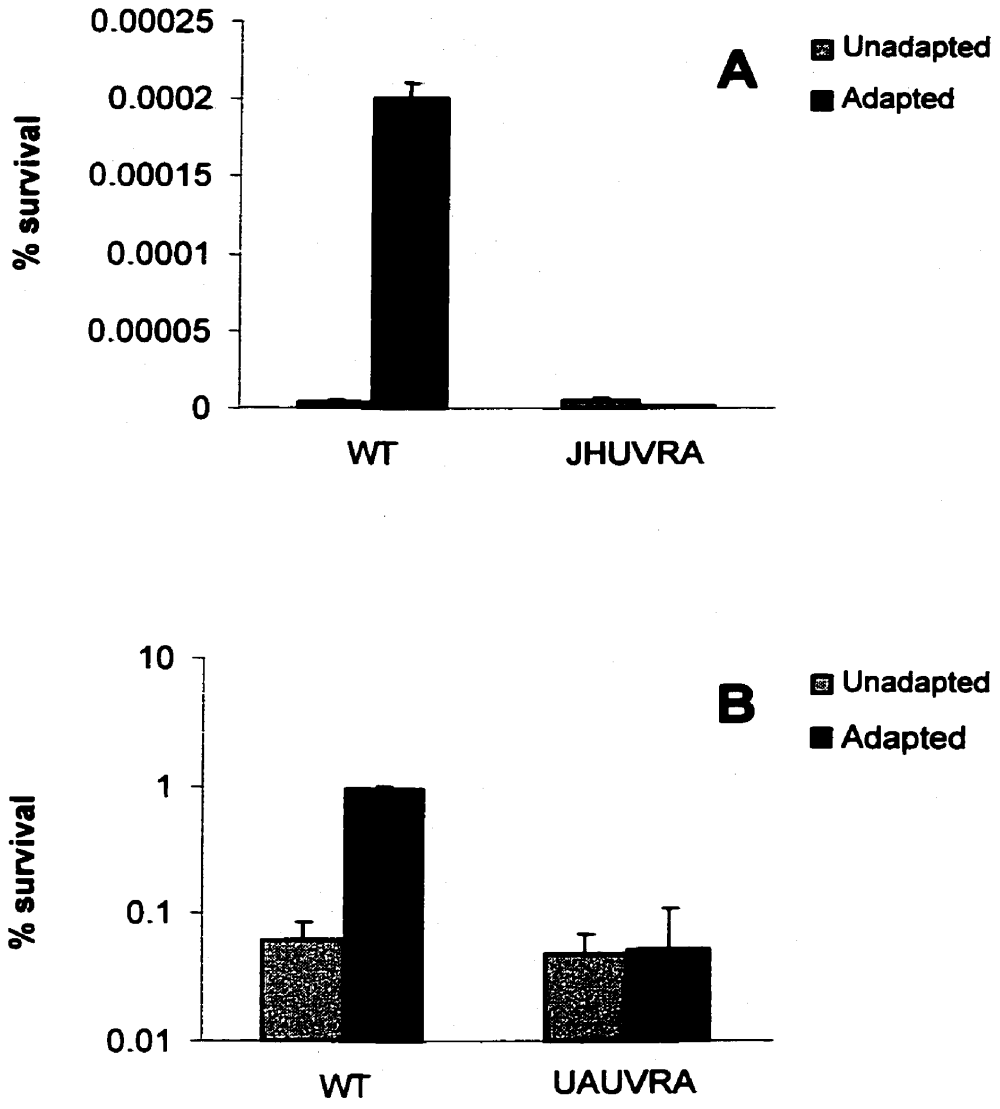
A
Unadapted
Adapted
% survival
0.00025
0.0002
0.00015
0.0001
0.00005
0
WT
JHUVRA
B
Unadapted
Adapted
% survival
10
1
0.1
0.01
WT
UAUVRA

Figure 3. 8

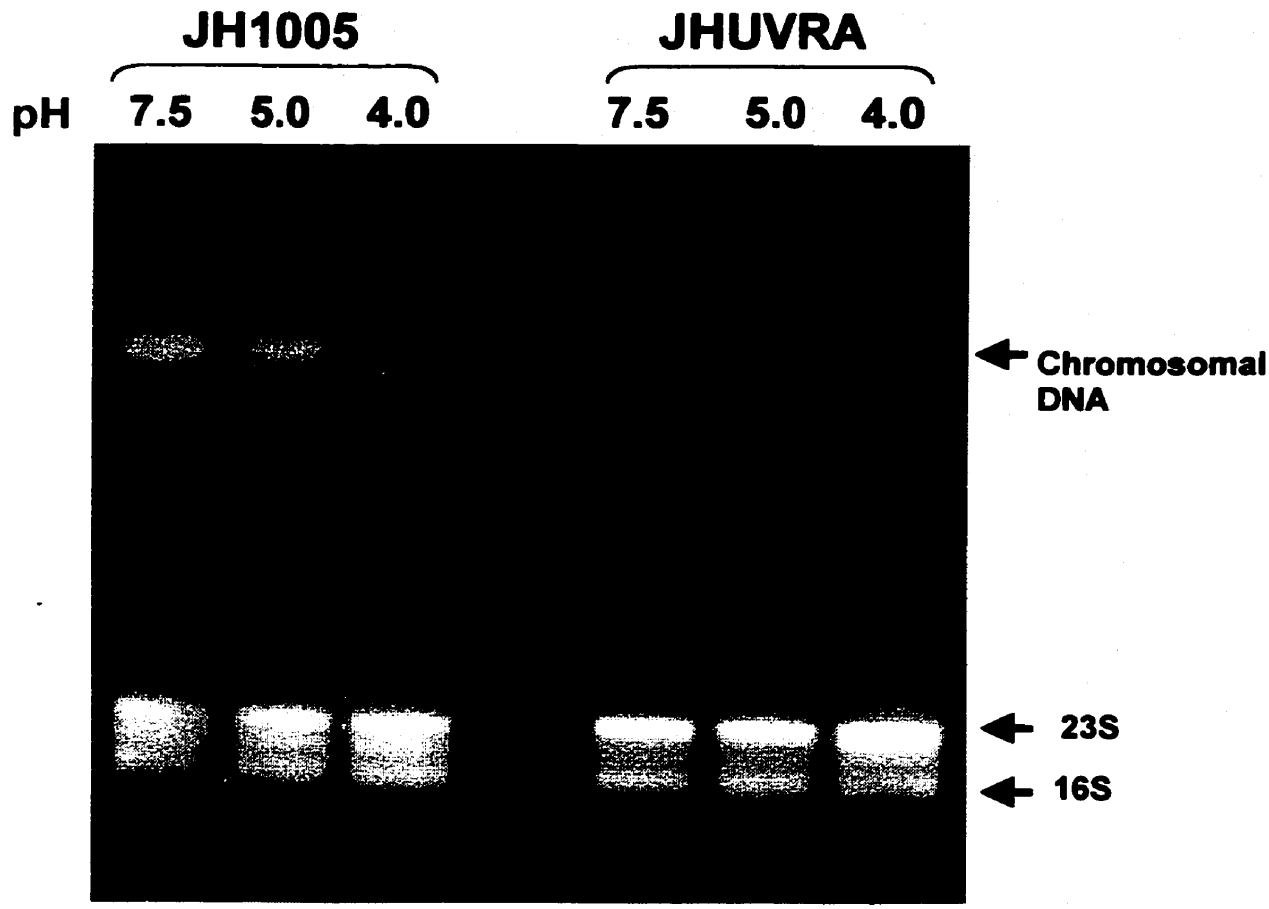

Figure 3. 9

CHAPTER 4: Other variables influencing the ATR: cell density and competence-stimulating peptide (CSP)

Section 4.1: Introduction

Transition into stationary phase growth in *Escherichia coli* and *Salmonella typhimurium* is associated with increased resistance to a variety of environmental stresses, including acid (McCann *et al.*, 1991; Kolter *et al.*, 1993; Bearson *et al.*, 1997). The alternative sigma factor σ^S, encoded by *rpoS*, is thought to be one of the main regulators of this general stress response influenced by cell density (Lee *et al.*, 1995; Hengge-Aronis, 1999). In *S. typhimurium*, RpoS has been shown to be induced during log-phase acid exposure and stationary phase growth (Lee *et al.*, 1995). In *E. coli* RpoS is also found to be induced by stationary-phase growth (Hengge-Aronis, 1999) and is thought to be involved in the stationary-phase ATR (Castanie-Cornet *et al.*, 1999). *E. coli* also possesses two other global gene regulators induced during high density growth, a DNA binding- histone-like H-NS protein and the EnvZ-OmpR two-component regulator involved in membrane permeability by regulation of porin proteins (Pratt *et al.*, 1996). RpoS and H-NS have also been shown to influence the transcription of porin genes *ompF* and *ompC* in *E. coli* (Pratt *et al.*, 1996).

Unlike the general stress response system mentioned above, which is induced when cells enter stationary phase growth, the stationary-phase ATR in these organisms is induced when stationary phase cells are exposed to low pH. The stationary-phase ATR in *S. typhimurium* is RpoS-independent, and is thought to be controlled by an acid-inducible response regulator, OmpR (Bang *et al.*, 2000). In *E. coli*, stationary-phase ATR is RpoS-dependent, requiring glutamate and a putative glutamate/gamma amino butyric acid antiporter (*gadC*), and an arginine-dependent system thought to rely on arginine decarboxylase encoded by *adiA* (Lin *et al.*, 1995; Castanie-Cornet *et al.*, 1999).

E. coli cells during stationary-phase growth in complex media are thought to be significantly more acid resistant than *S. typhimurium* under the same conditions due to the apparently greater number of acid-inducible systems present in *E. coli* (Bearson *et al.*, 1997).

To our knowledge, there is no published work that details similar mechanisms present in *S. mutans*. In 1997, Svensäter and colleagues observed that there were differences between log- and stationary-phase cells when exposed to the killing pH, with the stationary-phase cells being more resistant to acid than the log-phase cells (Svensäter *et al.*, 1997). In this present study we sought to confirm the observations made by Svensäter *et al.*, 1997, and to determine if stationary-phase acid resistance in *S. mutans* was induced by cell density, pH effects (i.e. ATR), or both.

In Gram-negative bacteria, the signal molecule N-acyl-L-homoserine lactones (AHLs) are utilized by the bacteria in cell-cell communication in a process known as quorum sensing (Camara *et al.*, 1995; Hussain *et al.*, 1998;). Recently, *E. coli*, which apparently fails to produce or respond to AHLs, has been shown to secrete extracellular components, mostly proteins, which can modify the stress tolerance of organisms (Hussain *et al.*, 1998; Rowbury and Goodson, 1998). These extracellular induction components appear to be secreted in *E. coli* in response to acid stress. In a study done by Rowbury and Goodson in 1998, culture supernatants from acid adapted cells (grown at pH 5.0 during log-phase) where found to induce acid tolerance in cells grown at neutral pH. The implication of this finding is that *E. coli* could signal tolerance to other unadapted organisms through the secretion of a protein signaling molecule. Further investigation of the acid-adapted culture supernatants in *E. coli* revealed that a protein-

like molecule, termed extracellular induction component (EIC), was required for acid tolerance induction at pH 5.0. The extracellular precursor appeared to convert to the active EIC form by exposure to pH values ranging from 4.5-6.0 (Rowbury and Goodson, 1999).

In Gram-positive bacteria, peptides are the predominant signal molecule for quorum sensing (Dunny and Leonard, 1997). Competence for genetic transformation in *Streptococcus pneumoniae* has been shown to involve a quorum-sensing signal peptide, competence-stimulating peptide (CSP), which is synthesized via the quorum-sensing circuit involving activation of early competence genes (*comAB* and *comCDE*) that encode the proteins for cell-cell signaling (Lee and Morrison, 1999). The mechanism by which the quorum-sensing circuit activates the expression of genes for the machinery of genetic transformation is not understood. It has been proposed that the quorum-sensing transducer, ComE, may act as a transcription factor to induce both the competence machinery genes and those of the CSP circuit (Alloing *et al.*, 1998). The transcriptional regulator for competence in *Bacillus subtilis*, ComA, was also shown to regulate competence machinery genes as well as adaptation genes for starvation (Mueller *et al.*, 1991). Also, the late competence gene in *S. pneumoniae*, *recA*, involved in the DNA uptake recombination process, was shown to be upregulated in response to CSP (Pearce *et al.*, 1995). RecA has been identified in *S. mutans* and, apart from its role in recombination, has been shown to be involved in acid tolerance (Quivey *et al.*, 1995). Substantial evidence suggests that the mechanisms of transformation in competence used by *S. pneumoniae* are similar in oral streptococci including *S. mutans* (Håvarstein *et al.*, 1997; Li *et al.*, 2001). The CSP peptide in *S. mutans* has been identified by our lab (Li *et*

al., 2001). In this study, we proposed that the CSP in *S. mutans* affects the transcriptional regulation of genes involved in acid-adaptation due to a overlap of the competence and acid stress adaptation pathways.

Section 4.2: Results

Section 4.2A: Cell density modulates induction of acid resistance and possibly acid adaptation in *S. mutans* JH1005

A previous study in *S. mutans* suggested the idea that cell density may play a role in acid resistance (Svensäter *et al.*, 1997). Our preliminary results confirm the observations made by this study which demonstrated that stationary-phase cells were more acid resistant than those in log-phase. Our lab has also observed this phenomena in several different strains of *S. mutans* (data not shown). Two genes known to have acid-inducible expression affecting the ATR in *S. mutans*, *ffh* (Gutierrez *et al.*, 1999) and *uvrA* (this study) were evaluated in conjunction with the cell-density experiment. Here, total RNA was isolated from each cell sample prior to exposure to the killing pH of 3.0 and expression of *ffh* and *uvrA* was determined using RT-PCR. Recalling that stationary-phase acid resistance is dependent on cell density only and not by exposure to acid pH, Figure 4.1B shows that pH 7.5 grown cells in stationary phase ($O.D._{600}$ of 0.9) are 16-fold more acid resistant than log-phase cells when exposed to the killing pH. Expression of *ffh* and *uvrA* appears to be inducible in stationary phase cells grown at pH 7.5 (Figure 4.1A), independent of pH, since these cells are incubated in pH 7.5 buffered media which maintains the pH ($\pm$ 0.6) relatively well. A stationary-phase ATR may also be present in *S. mutans* since acid-adapted cells in stationary phase were slightly more acid-resistant than unadapted stationary-phase cells (Figure 4.1B). Furthermore, stationary-phase ATR

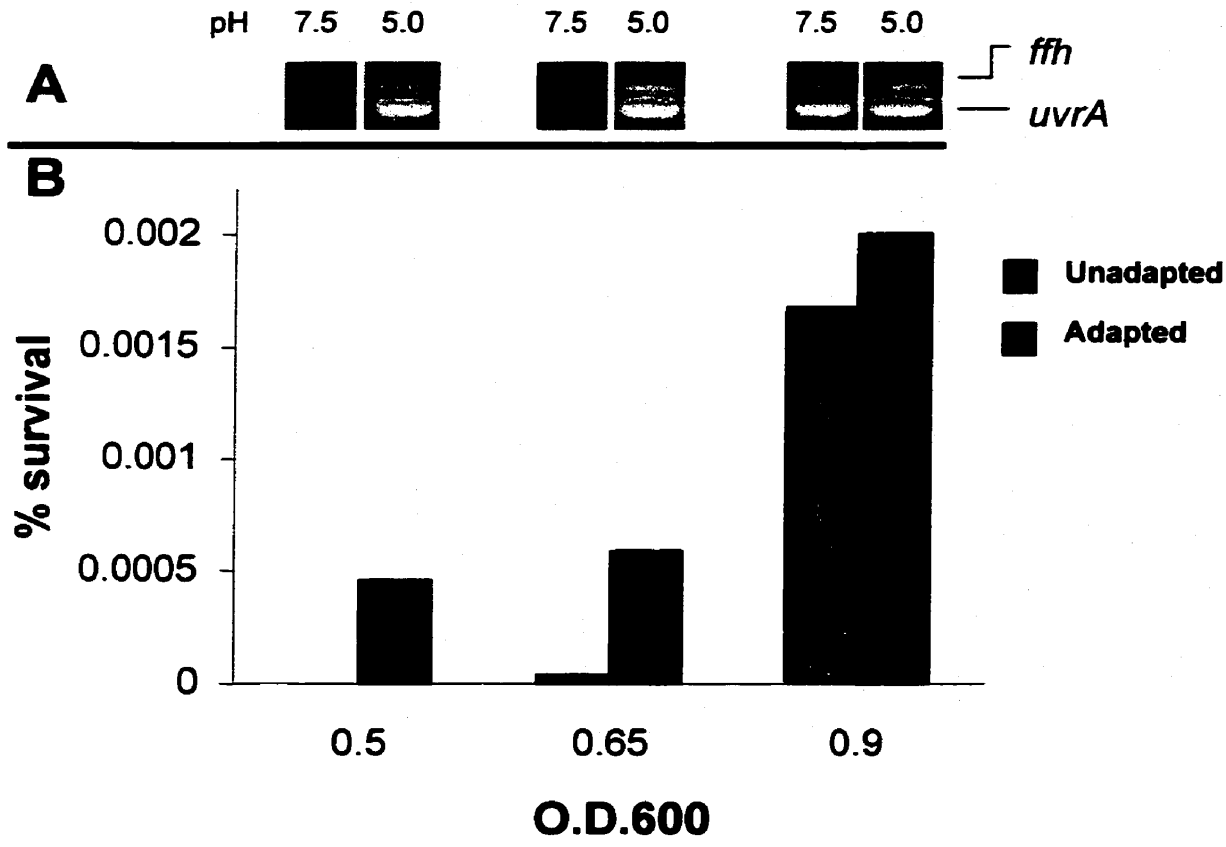

Figure 4. 1: Acid tolerance response of *S. mutans* JH1005 cells grown at early-log $O.D._{600}$= 0.5) through to stationary phase ($O.D._{600}$= 0.9) **(B)**. Cells were harvested and resuspended in either pH 7.5 or pH 5.0 buffered THYE media, incubated for 2 hours at 37°C, followed by exposure to the killing pH of 3.0 for 3 hours. Cells were then plated and calculated for % survival. A fraction of the cells after the 2 hour incubation were extracted for total RNA and used in RT-PCR to quantitate the expression of *ffh* and *uvrA*, which are known to be induced during acid-adaptation **(A)**.

appears considerable more effective than log-phase ATR as shown by a 4-fold increase in % survivors after exposure to the killing pH (Figure 4.1B). *Ffh* and *uvrA* expression appears inducible in unadapted cells during stationary- phase growth (Figure 4.1A).

Section 4.2B: Competence- stimulating peptide (CSP) induces adaptation in *S. mutans* JH1005

To determine if synthetic CSP could act as a 'signal' molecule to activate acid-adaptation in *S. mutans* JH1005, CSP was added to the cultures during incubation at pH 7.5 (unadapted state) and pH 5.0 (acid-adapted state) for two hours followed by exposure of the cells to the killing pH of 3.0. The results showed that unadapted cells incubated with 500 ng/ml and 1 μg/ml CSP were 8- and 60- times more resistant to the killing pH, respectively, than the negative control cells incubated without CSP, apparently exhibiting a dose-dependent response (Figure 4.2). Unadapted cells exposed to 1 μg CSP were also significantly more resistant to acid killing when compared to acid-adapted cells under the same conditions (Figure 4.2). Also, acid-adapted cells with and without CSP show no significant difference in cell survival when exposed to the killing pH.

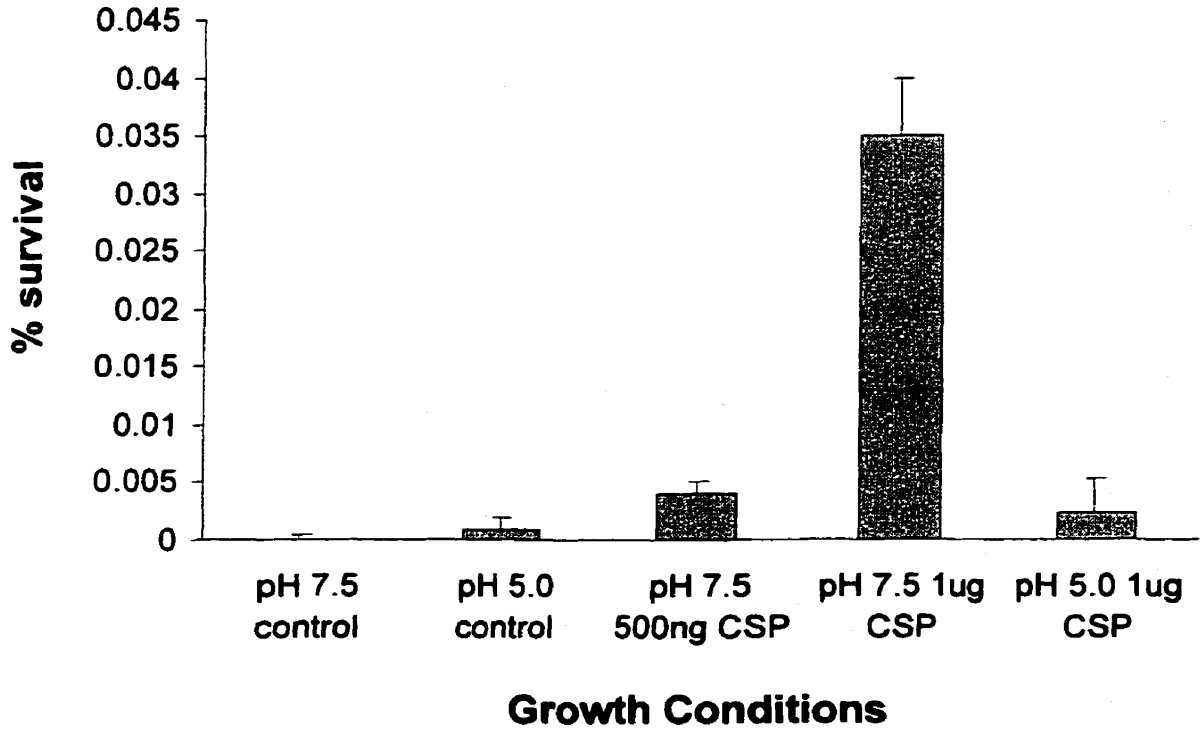

Figure 4. 2: Acid tolerance response of mid-log *S. mutans* JH1005 cells incubated for 2 hours in pH 7.5 or pH 5.0 buffered THYE media with competence-stimulating peptide (CSP). Cells were then harvested and incubated in the killing pH of 3.0 for 3 hours, plated and calculated for % survival. Control cultures were devoid of any CSP.

Section 4.3: Discussion

In bacteria, the regulation of gene expression in response to changes in cell density is called quorum sensing (Fuqua *et al.*, 1994). In this study we observed that two genes involved in acid-adaptation in *S. mutans*, *ffh* and *uvrA*, were induced when cells entered stationary phase, correlating with an increase in acid resistance (Figure 4.1A). These results suggest that a general (or specific) stress response regulator induced by cell density, similar to the sigma factor σ^S (RpoS), may also regulate genes involved in acid-adaptation. Recent evidence has shown that the general stress transcription factors sigma B and H in *Bacillis subtilis* and sigma B in *Listeria monocytogenes* are involved in acid resistance (Wiedmann *et al.*, 1998; Gaidenko and Price, 1998). Investigation of similar general stress global regulators in *S. mutans* is warranted.

In the plaque environment the accumulation of lactate generated by *S. mutans* and other fermentative bacteria and/or nutrient limitation leads to the arrest of cell growth and, consequently, to the onset of stationary phase. A physiologic consequence of stationary-phase growth in streptococci cells is the reduction in electrochemical gradient (proton motive force:PMF) (Ten Brink and Konings, 1982) which contributes to its acid resistance (Svensäter *et al.*, 1997). With a PMF virtually non-existent in stationary cells, *S. mutans* cells are more impermeable to proton attack. These factors as well as other undefined changes most likely account for the acid resistance observed during stationary-phase growth in *S. mutans*. Our results show that unadapted cells in stationary-phase are significantly more resistant to acid killing than acid-adapted cells in log-phase (data not shown). This suggests that growth and accumulation of *S. mutans* to high cell density is paramount for sustained acid survival, whereas log-phase cells, during the accumulation

stage, may depend more on acid-inducible mechanisms, independent of density. To support this theory, our lab has recently shown that the addition of chloramphenicol prior to acid adaptation in stationary-phase cells did not significantly alter the outcome of survival to the killing pH in *S. mutans* BM71 (data not shown) suggesting that *de novo* protein synthesis for the stationary-phase ATR plays a nominal role compared to the acid resistance effects of cell density.

Quorum-sensing bacteria produce and release acyl-homoserine lactone (Gram-negatives) or peptide (Gram-positives) –signaling molecules that accumulate in the environment as the cell density increases. When a threshold concentration of signaling molecule is obtained in the environment, the signal is received by other bacteria, typically of the same species, by specific receptors that then elicit a signal transduction cascade that changes the physiology of the bacterium. From our observation that high cell-density promoted induction of acid resistance and possibly acid adaptation (Figure 4.2), it was hypothesized that *S. mutans* JH1005 might utilize a cell density-dependent mechanism to enhance induction of acid resistance in the population through release of an extracellular signal molecule(s). The quorum sensing peptide, competence stimulating peptide (CSP), has been shown to induce competence in *S. mutans* (Li *et al.*, 2001). In *S. pneumoniae* uptake of CSP by the cell has been shown to initiate a cascade of competence-specific genes (Morrison and Baker, 1979) including the induction of the alternate sigma factor, ComX (Lee and Morrison, 1999). ComX directs RNA polymerase to the promoters of late competence genes (i.e. involved in DNA uptake and processing). Conceivably, ComX could also induce other genes possibly involved in acid tolerance since mutations made in alternate sigma factors in other bacteria have displayed acid

sensitive phenotypes (Wiedmann *et al.*, 1998; Gaidenko *et al.*, 1998). In our study we have shown that CSP added at 500 _g/ml and 1 μg/ml significantly enhanced acid-unadapted cells to survival at the killing pH (Figure 4.2). It appears that the concentration of CSP affects acid resistance in a dose-dependent manner. In streptococci, competence develops in early- to mid- log growth phase (Håvarstein *et al.*, 1997; Li *et al.*, 2001) suggesting the physiologic levels of CSP required for competence are much lower than the concentrations used in this study. Perhaps when the cell population reaches stationary-phase growth a threshold concentration of CSP in the environment triggers different regulons that affect physiologic systems other than competence.

Our results suggest that CSP becomes inactive at pH 5.0, since addition of 1 μg/ml CSP to acid-adapted cells (i.e. cells grown at pH 5.0) did not exhibit the same acid protective effects of CSP when compared to the same cells grown at pH 7.5 (Figure 4.2). Additionally, in another study (Li *et al.*, 2001) external pH was shown to affect competence in *S. mutans*, since cells grown at low pH (6.0) were unable to induce competence as indicated by reduced transformation efficiency. It is yet to be determined whether the consequence of competence (i.e. DNA uptake and processing) in *S. mutans* influences acid resistance or if indirect mechanisms are employed such that the CSP activates other regulons that influence acid resistance.

The implication that cell density and extracellular signaling peptides influence stationary-phase acid resistance and ATR are far-reaching considering that *S. mutans* spends the majority of its life cycle at high density.

CHAPTER 5: Troubleshooting/Partially completed work

This chapter gives a brief summary of the work from this project that is ongoing in our lab. Each topic for the most part is unrelated and therefore is divided into separate sections.

Section 5.1: Unconfirmed/uncharacterized differential display-PCR products

From the dd-PCR experiment that was done in this study, 13 amplicons were observed to be either upregulated or exclusively present in the pH 5.0 samples. One of these products was cloned and sequenced, and was found to be homologous to a *uvrA*/DNA repair gene in *Bacillus subtilis*. We have confirmed in this study that the *uvrA* gene in *S. mutans* is acid-inducible. The others amplicons are still uncharacterized in their function and are unconfirmed for acid-inducibility. As shown in Table 3.2, there is potential heterogeneity of the clones when individual bands are excised since each clone may have different gene products. Table 5.1 shows a list of some of the cloned and sequenced amplicons and their possible homologies to known or unknown bacterial genes.

Section 5.2: Insertion Duplication Mutagenesis (IDM) of *S. mutans uvrA*

IDM was initially employed to create *uvrA* knockouts in *S. mutans* JH1005, NG8, and UA159. Several clones harboring the integrated plasmid from each strain grew on THYE-erythromycin selective plates, but we were unable to confirm that the insertion had taken place in the correct locus for two out of the three strains (NG8 and UA159) using Southern blotting. For the JH1005 strain we were able to detect a reduction in band size in two *uvrA* mutants (#2 and #8) when their chromosomal DNA was digested with

Table 5. 1: List of unconfirmed/uncharacterized dd-PCR cloned products

Clone	Homolog (BLASTX)	Identity
dd-PCR 1a/d	*Bacillus subtilis* Cody protein (nutritional repressor, DNA binding protein)	43 %
dd-PCR 3b	*Lactococcus lactis* RadA homolog (DNA repair protein)	66 %
dd-PCR 3h	*Streptococcus pyogenes* unknown protein	70 %
dd-PCR 4a	*Staphylococcus aureus* TraG (conjugal transfer membrane protein)	45 %
dd-PCR 4c	*Bacillus subtilis* YCF 24 (hypothetical DNA binding protein)	35 %
dd-PCR 5e	*Bacillus subtilis* CTJR hypothetical protein (transcriptional regulator)	45 %
dd-PCR 5b/f/g	*Bacillus subtilis* Thioredoxin Reductase (general stress protein)	60 %
dd-PCR 6a/c	*Bacillus firmus* Orf B (Na^+/H^+ antiporter activity)	32 %
dd-PCR 8d	*Streptococcus mutans* DexA (dextranase precursor)	96 %
dd-PCR 15f	*Bacillus subtilis* SpaF protein (homology to ABC transporter)	55 %

ClaI, run on a gel, and probed with a DIG-labeled *uvrA* internal fragment probe (Figure 5.1A). Another blot containing the same digested DNA was probed with a DIG-labeled erythromycin resistance gene probe that was present in the mutants but not in the wild-type (Figure 5.1B). Southern blot detection was unsuccessful in the other strains because no shift in band size was seen between the mutant and wild-type chromosomal DNA when probed. This may be due to the fact that there was no appropriate restriction enzyme that cut the chromosomal DNA in such a way that allowed for visual confirmation of integration at the desired locus. An alternative verification method was attempted using PCR. Here, insertion at the desired locus was detected by using primer pairs uvrA**F** and ERM2-B1 and uvrA**B** and ERM1-F3. (See Figure 2.1). Successful amplification of these primer pairs ensured that the native *uvrA* gene was disrupted and that the erythromycin cassette was integrated in the chromosome in the correct location. Using this technique we were able to confirm the *S. mutans uvrA* mutant in NG8 only. It is known that integration of the suicide vector into the desired locus is not always a precise event. Sometimes incomplete integration occurs or two copies of the vector may integrate instead of one. These occurrences may explain why it was difficult to confirm some of the mutants.

As a final note to the IDM method, we must consider the possibility that integration of the suicide vector may disrupt or shift the reading frame downstream from the target gene, affecting translation of downstream genes and possibly disrupting their function. Therefore, several gene deletions may contribute to the resultant phenotypic outcome of deleting the target gene. To circumvent this problem we used a novel and efficient method to create in-frame gene knockouts using PCR.

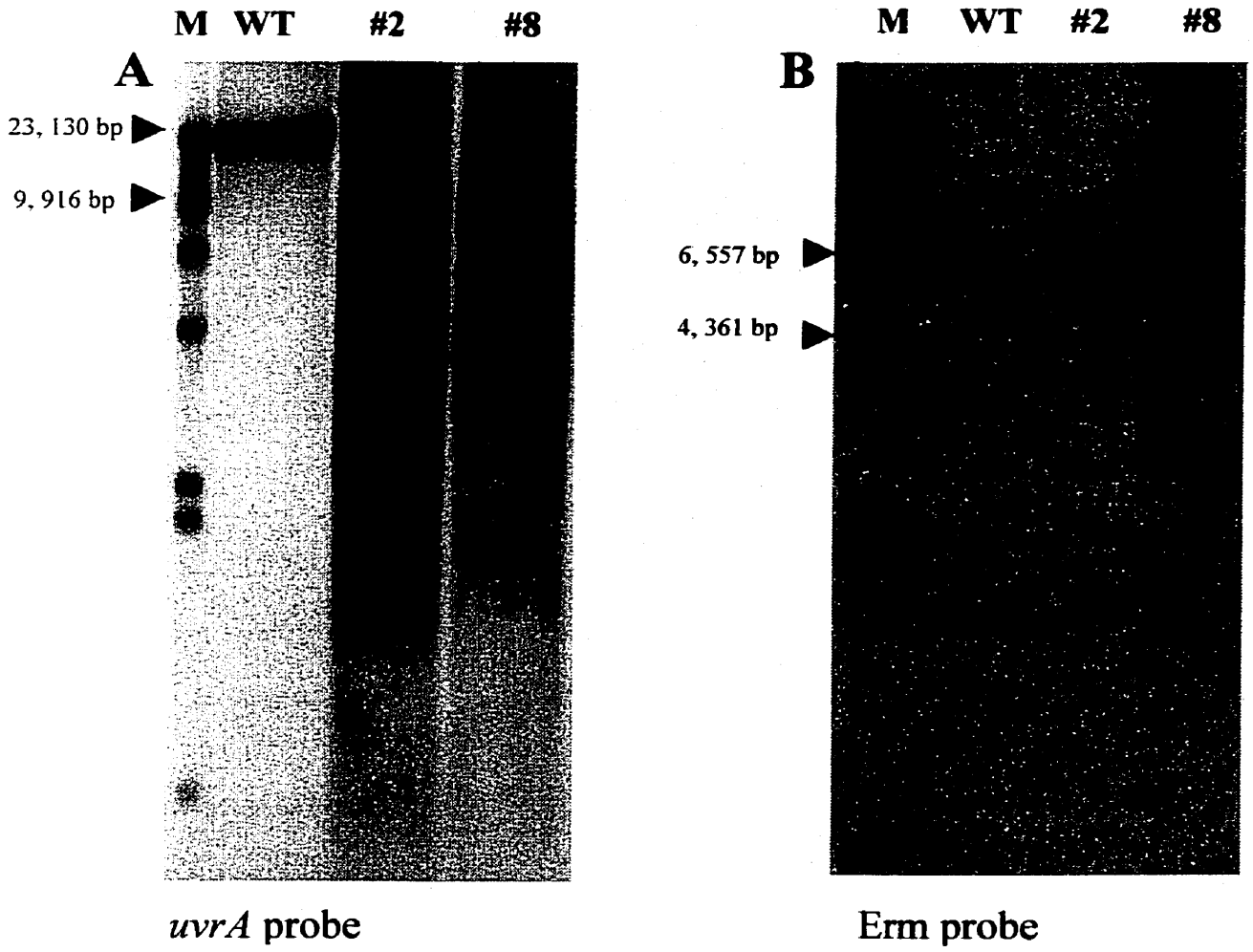

Figure 5. 1: Southern blot confirmation of *S. mutans* JH1005 *uvrA* mutants made with the IDM technique. Colonies # 2 and 8 show a decrease in band size when cut with *ClaI* when compared to the wild-type **(A)**. Mutant colonies also contain the erythromycin cassette as shown by labeling with the Erm probe **(B)**.

Section 5.3: PCR-mediated mutagenesis of *S. mutans pepP*

From the partially completed *S. mutans* UA159 genome database we found an ORF immediately 3' proximal from *uvrA* that had 50 % identity to an aminopeptidase P (*pepP*) gene in *Lactococcus lactis*. We used the PCR-mediated mutagenesis method to create *pepP* gene knockouts in *S. mutans* NG8 and UA159 (called NGPEPP and UAPEPP, respectively). Figure 5.2B shows the strategy for confirming the mutants using PCR. Briefly, genomic DNA was isolated from positive colonies (i.e. those that were Erm resistant) and were used as a PCR template with primer sets pep**P1**/Erm CST**P2** and **P4**/ERM CST**P1**. Each primer set amplified a region of known size that allowed for confirmation based on predicted size (Figure 5.2A). When primers **P4**/ERM CST**P1** were used to confirm the mutant in UA159 the actual band size was unexplainably smaller than the predicted size (Figure 5.2A). Preliminary experiments using NGPEPP and UAPEPP showed that the strains were not sensitive to UV irradiation, but may be sensitive to growth at pH 5.0 (data not shown). Furthermore, growth of these mutant strains at pH 7.5 showed a nutrient limiting response indicated by an approximate 14 % reduction in final resting density when compared to the wild-type strains (data not shown).

Although poorly documented, the proteolytic system of lactic acid bacteria is involved in physiological and universal functions such as protein turnover, protein maturation, signal peptide processing, degradation of abnormal proteins, and inactivation of regulatory proteins (Lazdunski, 1989; Kunji *et al.*, 1996; Matos *et al.*, 1998). Recently in *E. coli* an aminopeptidase (PepA) has demonstrated DNA-binding activity (Charlier *et al.*, 2000) suggesting that aminopeptidases could potentially regulate other genes by

binding to their promoter regions. Indeed, a PepA DNA-binding consensus sequence was found in the *carAB* operon in *E. coli* (Charlier *et al.*, 1995). Investigation of similar consensus sequences in *S. mutans* may yield additional information as to the regulatory function of PepP.

The proteolytic activity of proline aminopeptidases (such as PepP) in *E. coli* specifically releases the N-terminal residue from a peptide where the pen-ultimate residue is a proline (Wilce *et al.*, 1998). Aminopeptidases have been shown to be activated by divalent metal ions such as of Mn^{2+} (Vanhoof *et al.*, 1995). The activity of PepA in *E. coli* is separate from its regulatory function. However, the regulatory role of PepA may depend on peptidase activity to degrade or modify peptides that function as inducers or repressors (McCulloch *et al.*, 1994). Our hypothesis is that PepP in *S. mutans* may be involved in the processing or activation of *uvrA* (NER pathway) via its enzymatic activity or by some other regulatory capacity.

To investigate if the gene expression of *pepP* and *uvrA* are associated, further promoter sequence analysis of these genes needs to be done to determine if they are expressed separately or in one operon. As well, aminopeptidase activity in *S. mutans* could be monitored under different conditions (i.e. pH, different nutritional conditions, growth state etc.) to further characterize its function.

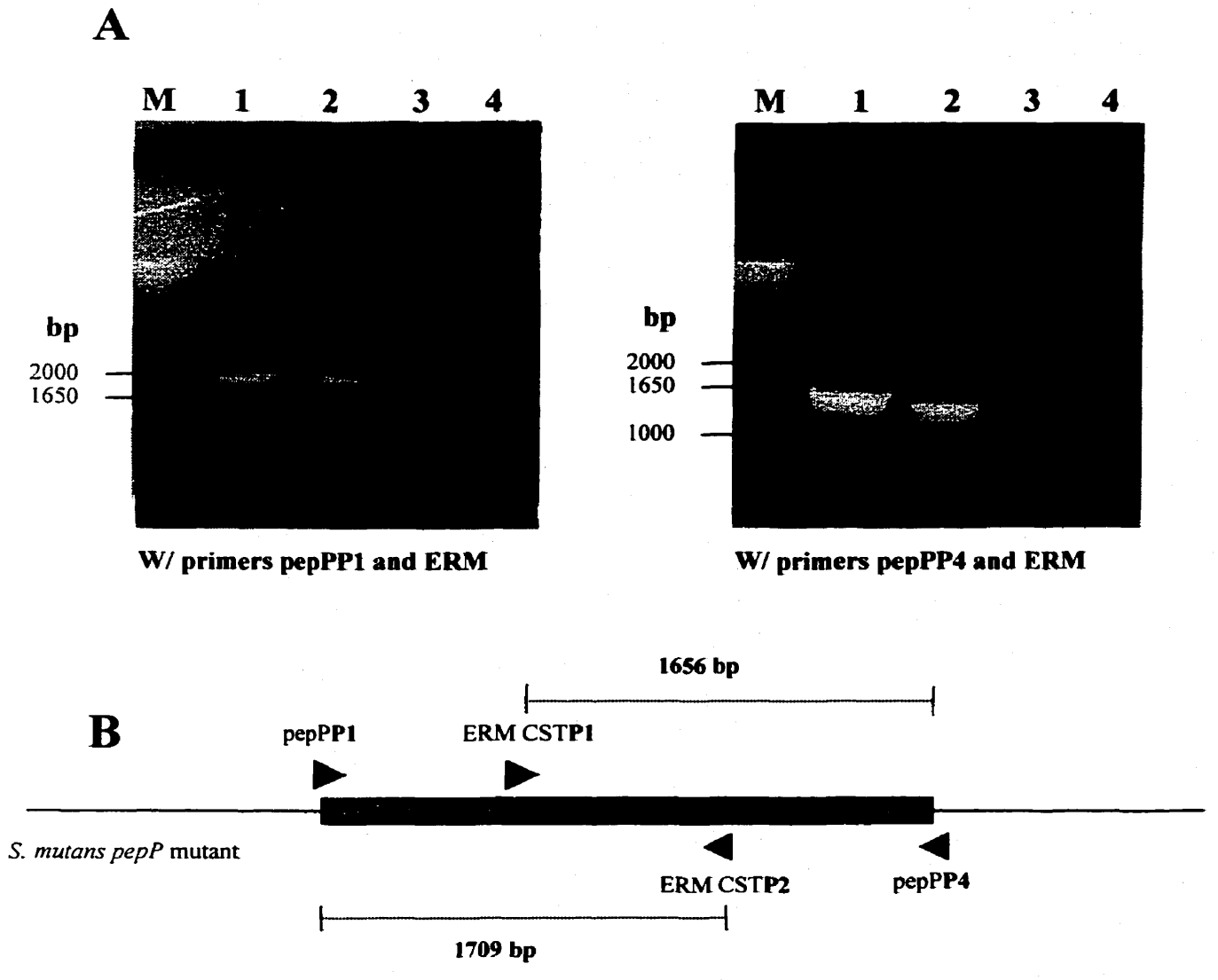

Figure 5. 2: Confirmation of *S. mutans* NG8 and UA159 *pepP* mutants (lanes 1 and 2, respectively) using PCR **(A).** Primers P1/ERM CSTP2 and P4/ERM CSTP1 generated product with a predicated size of 1709 bp and 1656 bp, respectively. Control lanes, 3 and 4, represent NG8 and UA159 wild-type strains, which yielded no amplified products. **(B)** Diagrammatic representation of PCR verification method for mutants generated by PCR-mediated mutagenesis.

Section 5.4: TUNEL detection of acid-induced DNA damage

Since we were able to observe obvious degradation in chromosomal DNA of *S. mutans* cells exposed to acid using agarose gel electrophoresis (Figure 3.9) we used a fluorescein labeled –dUTP and terminal transferase in the TUNEL procedure to more accurately quantitate this acid-induced DNA damage. This method was essentially adapted from Rohwer and Azam (2000) who published the first evidence that the eukaryotic DNA detection kit could be successfully used for prokaryotic studies. In their study they examined DNA damage in *E. coli* and *Haloferax volcanii* by treatment of cells with hydrogen peroxide, UV, and bacteriophage infection and examined them using TUNEL. The TUNEL kit (In Situ Cell Death Detection Kit, Fluorescein: Boehringer Mannheim) used for prokaryotic cells was originally designed to detect apoptosis in eukaryotic cells since DNA damage is an indication of cell death. In eukaryotes, cleavage of genomic DNA during apoptosis may yield double-stranded, as well as single stranded breaks or 'nicks' in high molecular weight DNA. These DNA strand breaks were identified by enzymatically labeling free 3'-OH ends with dUTP-fluorescein isothiocyanate using terminal deoxyribonucleotide transferase (Tdt). Cells labeled by this method could be analyzed by fluorescence microscopy or flow cytometry.

Initial experiments using TUNEL in *S. mutans* NG8 cells exposed to various pH values (pH 7.5, 5.0, 4.0, and 3.0) revealed low fluorescence labeling. A possible cause of this outcome may have been due to the permeabilization method used to allow labeled molecules to enter the cell and access the damaged DNA. Many standard protocols for permeabilization were used including the use of acetone, chloroform, and toluene. A toluene method whereby 0.01 volume toluene was combined with cells followed by 90

seconds vortexing was employed for an extended time since initial experiments using fluorescence microscopy showed promising results. However, further experiments revealed that cell labeling occurred when cells were exposed to low pH without any permeabilization treatment (negative controls). This suggests that either the label was bound to the surface of the cell at low pH or that the cell membrane became leaky, allowing for uptake of the flourescein molecule. Other negative controls, without the addition of Tdt, were also incorporated and revealed that the cells fluoresced non-specifically in several control cells (grown at neutral pH). This again suggested that the label was bound to the surface of the cell. Several DNA damaging agents other than acid treatment were used as positive controls to determine if the TUNEL method was detectable in *S. mutans*. Treatments included H_2O_2, UV, DNaseI, and *SmaI*, all of which revealed low cell labeling. It appears that the problem was probably a result of the permeablization method since labeling of most cells, regardless of damaging treatment, resulted in low levels of fluorescence.

Finally, interpretation of TUNEL results must be made carefully since detectable DNA damage by creation of 3'-OH ends may be generated by direct DNA exposure to the damaging agent or as a result of 3'-OH ends generated by the activity of DNA repair mechanisms. Therefore, fluorescing cells do not discriminate between DNA nicked by repair enzymes actively repairing damaged DNA or by an agent that is directly damaging the DNA.

CHAPTER 6: Summary and Future Directions

The acid tolerance response (ATR) is an inducible molecular response mechanism activated when the bacterium encounters a moderate pH that enables the cells to tolerate further drops in pH. Extensive examination of the ATR in *E. coli* and *S. typhimurium* has resulted in a wealth of information regarding the genes and regulatory networks involved in this adaptive response. The mechanisms of the ATR in *S. mutans* are less defined and substantial research in this area is needed. *S. mutans* occupies a unique niche in the oral cavity which necessitates its survival at low pH. The acidic conditions within plaque create a selective pressure resulting in increased numbers of the acid tolerant *S. mutans* concurrent with a decrease in the proportion of less tolerant bacteria (Staat *et al.*, 1975; McDermid *et al.*, 1986; Hamilton and Buckley, 1991). The ATR has been linked to the ability of these organisms to survive acidic pH conditions (Svensäter *et al.*, 1997) whereby cells capable of withstanding the lowest pH challenges had the strongest ATR. In *S. mutans* the moderate or adaptive pH that induces the ATR ranges from 5.5 – 4.0 (Svensäter *et al.*, 1997). Acid adaptation in *S. mutans* has been shown to require *de novo* protein synthesis of up to 36 acid-regulated proteins (Svensäter *et al.*, 1997; Hamilton and Svensäter, 1998) presumably encoded by acid-inducible genes. However, the ATR in *E. coli* and *S. typhimurium* exhibit more than 50 proteins synthesized during adaptation, indicating that differences do exist between the ATR of enteric bacteria and *S. mutans*. Aside from the obvious differences in cell architecture and environmental habitat, enteric bacteria and *S. mutans* exhibit a marked difference in their Δ pH. Enteric Gram-negatives maintain a near neutral pH_i during acid challenge (Paden *et al.*, 1981) whereas *S. mutans* maintains a pH gradient that is only 0.5-1.0 pH units higher than the

extracellular pH (Dashpur and Reynolds, 1992). This observation strongly supports that the ATRs between these bacteria are very different.

The goal of our work was to examine the molecular mechanisms involved in the ATR in *S. mutans* and to identify key proteins essential in this process by identifying genes up-regulated when cells were exposed to pH 5.0. The approach taken involved using the differential display-PCR technique (dd-PCR), whereby total RNA from cells grown at pH 7.5 (unadapted) and pH 5.0 (acid-adapted) were extracted, randomly amplified, and were visualized for differential gene expression. Also in this study we investigated the possible affects of cell density and extracellular signaling (quorum sensing) molecules on the ATR.

From the dd-PCR analysis we identified 13 amplicons that were potentially up-regulated at pH 5.0 when compared to the pH 7.5 grown cells. One of these products, confirmed to be acid-inducible by RNA dot blot and reverse transcription PCR analyses, had 67 % identity to a *uvrA*/DNA repair excinuclease in *Bacillus subtilis*. The other amplicons which have been cloned and sequenced are still uncharacterized and have not been confirmed as being acid-inducible.

Further sequence analysis of the *uvrA* locus using the *S. mutans* genome database revealed the complete gene was encoded in an ORF of 2829 b.p. (944 amino acids; 104.67 kDa). Immediately 3' proximal from *uvrA* was an ORF encoding a putative aminopeptidase gene (*pepP*) that may be expressed with *uvrA* in the same operon. We propose that PepP in *S. mutans* might be involved in regulation of UvrA (NER pathway) by either modifying UvrA to its active form or by modifying/activating another protein which regulates *uvrA* expression. Another intriguing possibility is that PepP might

regulate *uvrA* by binding to its promoter region since it was shown in *E. coli* that PepA has DNA-binding activity (Charlier *et al.*, 2000). Further characterization of *pepP* (e.g. searching for PepP DNA-binding concensus sequences) as well as ATR studies and aminopeptidase P activity experiments using the *pepP* mutant in *S. mutans* should be done to elucidate the role(s) of this enzyme.

The UvrA protein in *S. mutans* appears structurally similar to UvrA proteins in other bacteria. UvrA consists of two zinc finger and two nucleoprotein-ATP binding regions found at amino acids 252-279/738-764 and 33-40/639-646, respectively. These structures allow the protein to preferentially bind to single- and double-stranded DNA breaks which then initiate recruitment of the other proteins normally associated with UvrA, primarily UvrB and UvrC. The UvrABC complex is the principle component of the nucleotide excision pathway (NER) which is thought to be responsible for repairing large DNA lesions (Sancar, 1996). To evaluate whether up-regulation of the *uvrA* gene and the subsequent increase in UvrA activity was essential for the *S. mutans* ATR, we created *uvrA* knockouts in strains JH1005, NG8, and UA159 using PCR-mediated mutagenesis. *PepP* mutants were also made in strains NG8 and UA159, but still need to be tested for their involvement in *uvrA*/DNA repair and the ATR.

As with *uvrA* mutants in other bacteria, the *S. mutans uvrA* mutants were extremely sensitive to UV irradiation. Also, previously exposed JH1005 and JHUVRA cells to pH 5.0 provided cross-protection to UV irradiation suggesting that other acid-inducible DNA repair mechanisms exist. A previous study in *S. mutans* showed that the activity of an AP endonuclease, involved in repair of damaged or incorrect bases, was found to be inducible by low pH (Hahn *et al.*, 1999). Also, although not confirmed as

being acid-inducible, one of the dd-PCR products (dd-PCR 3b) from our study was shown to have 66% identity to *RadA*/DNA repair protein in *Lactococcus lactis*. The RadA protein is a homologue of the *Escherichia coli* RecA and *Saccharomyces cerevisiae* Rad51 proteins, which are involved in recombinational repair of damaged DNA. Further investigation of these genes in *S. mutans* are warranted to demonstrate their involvement in repair of UV-induced and acid-induced DNA damage.

The *uvrA* mutant in *S. mutans* JH1005 was also shown to be more sensitive than the wild-type to growth at pH 5.0 suggesting that DNA repair mechanisms are essential for growth at acidic pH. Evidence in *E. coli* has shown that DNA repair mechanisms are induced during growth at pH 5.0 and that they account for the decrease in DNA damage seen at this pH (Raja *et al.*, 1991). We hypothesized that induction of DNA repair enzymes at pH 5.0 (including *uvrA*) occurs to ensure that the genes that encode the proteins necessary for acid adaptation are intact so that a successful ATR could be mounted upon further pH drops. To evaluate this hypothesis JH1005 and JHUVRA cells were exposed to either pH 7.5 or 5.0 for two hours to induce acid adaptation, followed by exposure to a lethal pH of 3.5. Our results revealed that JHUVRA was unable to elicit an acid-adaptation response since adapted mutants were unable to survive exposure to the killing pH. Moreover, similar results were seen when UA159 and UAUVRA strains were tested under these conditions. Agarose gel electrophoretic analysis of chromosomal DNA isolated from *uvrA* deficient cells exposed to low pH (4.0) demonstrated more DNA damage than the wild-type strain under similar conditions. These results collectively suggest that *uvrA* (NER pathway) is necessary for the repair of acid-induced DNA damage and is essential for successful adaptation of *S. mutans* to low pH.

The acid tolerance response in *S. typhimurium* and *E. coli* has been shown to involve stationary-phase systems. In this study we proposed that *S. mutans* also possesses a stationary-phase ATR. Transition into stationary phase growth brings upon changes in gene expression that is regulated by cell density in a process known as quorum sensing. We observed that two *S. mutans* genes involved in acid-adaptation, *ffh* and *uvrA*, were induced when cells entered stationary phase, correlating with an increase in acid resistance. These results suggested that a general (or specific) stress response regulator induced by cell density, similar to RpoS, may also regulate genes involved in acid-adaptation. From our observation that high cell-density promoted induction of acid resistance and possibly acid adaptation, it was hypothesized that *S. mutans* JH1005 might utilize a cell density-dependent mechanism to enhance induction of acid resistance in the population through release of an extracellular signal molecule(s). The quorum sensing peptide, competence stimulating peptide (CSP), previously shown in our lab to induce competence in *S. mutans* (Li *et al.*, 2001) was also shown to promote acid resistance in *S. mutans* JH1005. The exact mechanism by which CSP induces acid resistance in *S. mutans* still needs to be elucidated. In *S. pneumonie* CSP turns on the alternate sigma factor, ComX. The possibility then exists that comX may be a potential regulator of acid-tolerance genes similar to the activity of RpoS. Additionally, sigma factors in other organisms have recently been implicated in acid tolerance (Wiedmann *et al.*, 1998; Gaidenko and Price, 1998).

Much work remains to identify the molecular mechanisms involved in the ATR in *S. mutans*. In this study we have established the importance of the DNA repair gene *uvrA* in survival to low pH. We have yet to establish the cariogenic nature of *S. mutans uvrA*

deficient mutants. To do this, a germ free rat (or specific pathogen free) model could be used whereby the animals are infected separately with either the *uvrA* mutant or wild-type strain and fed a cariogenic diet (high sucrose). Caries incidence could then be monitored to see if there is a significant difference between the two strains. Co-infecting the animals with *uvrA* mutant and wild-type strains in competition studies could also be employed. From this we hope to define the contribution of *uvrA* in the cariogenicity of *S. mutans*.

In this study we have demonstrated the effectiveness of the differential display-PCR technique in identifying potential acid-inducible gene candidates. Several cloned and sequenced dd-PCR products are still uncharacterized and unconfirmed for acid-inducibility. These gene products, especially those products that show homologies to stress-related proteins, need to be examined since they may provide additional evidence into the mechanisms of the ATR system in *S. mutans*. Also, collaboration with the Svensäter lab in Sweden, where 2D protein gel analysis is being employed with *S. mutans* protein isolates from acid-induced cells, will complement our work and will yield additional information. Preliminary data suggest that a number of identical proteins are induced by both the CSP and pH 5.0. Finally, with the advent of DNA microarray technology, we hope to use this method to enable a large-scale, systematic approach to the analysis of transcriptional profiles in *S. mutans* upon exposure to low pH.

REFERENCES

Abu Kwaik, Y., and Peterson, L. L. (1996). The use of differential display-PCR to isolate and characterize a *Legionella pneumophila* locus induced during the intracellular infection of macrophages. *Mol. Microbiol.* **21**, 543-556.

Alloing, G., Martin, B., Granadel, C., and Claverys, J. P. (1998). Development of competence in *Streptococcus pneumoniae*: pheromone autoinduction and control of quorum-sensing by the oligopeptide permease. *Mol. Microbiol.* **29**, 75-83.

Aravind, L., Roland Walker, D., and Koonin E.V. (1999). Conserved domains in DNA repair proteins and evolution of repair systems. *Nuc. Acid Res.* **27**, 1223-1242.

Ayakawa, G.Y., Boushell, L.W., Crowley, P.J., Erdos, G.W., McArthur, W.P. and Bleiweis, A.S. (1987). Isolation and characterization of monoclonal antibodies specific for antigen P1, a major surface protein of mutans streptococci. *Infect. Immun.* **55**, 2759-2767.

Bearson, S., Bearson, B., and Foster, J. W. (1997). Acid stress responses in enterobacteria. *FEMS Microbiol. Lett.* **147**, 173-180.

Behari, J., Stagon, L., Calderwood, S. B. (2001). *PepA*, a gene mediating pH regulation of virulence genes in *Vibrio cholerae*. *J. Bacteriol.* **183**, 178-188.

Belli, W. A., and Marquis, R. E. (1991). Adaptation of *Streptococcus mutans* and *Enterococcus hirae* to acid stress in continuous culture. *Appl. Environ. Microbiol.* **53**, 2124-2128.

Bender, G. R., Sutton, S. V. W., and Marquis, R. E. (1986). Acid tolerance, proton permeabilities, and membrane ATPases of oral streptococci. *Infect. Immun.* **53**, 331-338.

Bijlsma, J. J. E., Lie-A-Ling, M., Nootenboom, I. C., Vandenbroucke-Grauls, C. M. J. E., and Kusters J. G. (2000). Identification of loci essential for the growth of *Helicobacter pylori* under acidic conditions. *J. Infect. Dis.* **182**, 1566-1569.

Bonass, W. A., Marsh, P. D., Percival, R. S., Aduse-Opoku, J., Hanley, S. A., Devine, D. A., and Curtis, M. A. (2000). Identification of *ragAB* as a temperature-regulated operon of *Porphyromonas gingivalis* W50 using differential display of randomly primed RNA. *Infect. Immun.* **68**, 4012-4017.

Bowan, W.H., Schilling, K., Giertsen, E., Pearson, S., Lee, S.F., Bleiweis, A. and Beeman, D. (1991). Role of a cell surface-associated protein in adherence and dental caries. *Infect. Immun.* **59**, 4606-4609.

Bowen, W. H., Schilling, K., Giertsen E., Pearson, S., Lee, S. F., Bleiweis, A., *et al.* (1991). Role of a cell surface-associated protein in adherence and dental caries. *Infect. Immun.* **59**, 4604-4609.

Boyar, R. M., and Bowden, G. H. (1985). The microflora associated with the progression of incipient carious lesions in teeth of children living in a water-fluoridated area. *Caries Res.* **19**, 298-306.

Boyd, D. A., Cvitkovitch, D. G., Bleiweis, A. S., Kiriukhin, M. Y., Debabov, D. V., Neuhaus, F. C., and Hamilton, I. R. (2000). Defects in D-alanyl-lipoteichoic acid synthesis in *Streptococcus mutans* results in acid sensitivity. *J. Bacteriol.* **182**, 6055-6065.

Brady, L. J., Piacentini, D. A., Crowley, P. J., and Bleiweis, A. S. (1991). Identification of monoclonal antibody-binding domains within antigen P1 of *Streptococcus mutans* and cross-reactivity with related surface antigens of oral streptococci. *Infect. Immun.* **59**, 4425-4435.

Brady, L. J., Piacentini, D. A., Crowley, P.J., Oyston, P. C. F. and Bleiweis, A. S. (1992). Differentiation of salivary agglutinin-mediated adherence and aggregation of mutans streptococci by use of monoclonal antibodies against the major surface adhesin P1. *Infect. Immun.* **60**, 1008-1017.

Burne, R. A., and Marquis R. E. (2000). Alkali production by oral bacteria and protection against dental caries. *FEMS Micro. Lett.* **193**, 1-6.

Camara, M., Latifa, A., Foglino, M., Chhabra, S. R., Daykin, M., Bally, M., Chapon, V., Salmond, G. P. C, Bycoft, B. W., Stewart, G. S. A. B., and Williams, P. (1995). Multiple N-Acyl homoserine lactone signal molecules regulate production of virulence determinants and secondary metabolites in *Pseudomonas aeruginosa. Proc. Nat. Acad. Sci.* **92**, 9427-9431.

Carlsson, J. (1984). Regulation of sugar metabolism in relation to the "feast- and-famine" existence of plaque. In: Cariology. Guggenheim B, ed.. Basel: Krager, pp. 205-211.

Carlsson, J. (1986). Metabolic activities of oral bacteria. In: Thylstrup, A., Fejerskov, O., eds. Textbook of cariology. Copenhagen: Munksgaard, pp. 74-106.

Castanie-Cornet, M. P., Penfound, T. A., Smith, D., Elliott, J. F., and Foster, J. W. (1999). Control of acid resistance in *Escherichia coli. J. Bacteriol.* **181**, 3525-3535.

Charlier, D., Kholti A., Huysveld, N., Gigot, D., Maes, D., Thia-Toung, T. L., and Glansdroff, N. (2000). Mutational analysis of *Escherichia coli* PepP, a multifunctional DNA-binding aminopeptidase. *J. Mol. Biol.* **302**, 411-426.

Charlier, D., Hassanzadeh, G., Kholti, A., Gigot, D., Peirard, A., and Glansdroff, N. (1995). *CarP*, involved in pyrimidine regulation of the *Escherichia coli* carbamoylphosphate synthetase protein identical to Xer-B and PepA, also required for resolution of ColEI multimers. *J. Mol. Biol.* **250**, 392-406.

Clancy, K. A., Pearson, S., Bowen, W. H., and Burne, R. A. (2000). Characterization of recombinant, ureolytic *Streptococcus mutans* demonstrates an inverse relationship between dental plaque ureolytic capacity and cariogenicity. *Infect. Immun.* **68**, 2621-2629.

Claverys, J.-P., Dintilhac, A, Pestova, E. V., Martin, B., and Morrison, D. A. (1995). Construction and evaluation of new drug-resistance cassettes for gene disruption mutagenesis in *Streptococcus pneumoniae*, using an *ami* test platform. *Gene* **164**, 123-128.

Courcelle, J., Crowley, D. J., and Hanawalt, P. C. (1999). Recovery of DNA replication in UV-Irradiated *Escherichia coli* requires both excision repair and RecF protein function. *J. Bacteriol.* **181**, 916-922.

Crowley, P. J., W. Fischlschweiger, Coleman, S., and Bleiweis, A. S. (1987). Intergeneric bacterial coaggregations involving mutans streptococci and oral actinomyces. *Infect. Immun.* **55**, 2695-2700.

Crowley, P. J., Gutierrez, J. A., Hillman, J. D. and Bleiweis, A. S. (1997). Genetic and physiologic analysis of a formyl-tetrahydrofolate synthetase mutant of *Streptococcus mutans*. *J. Bacteriol.* **179**, 1563-1572.

Crowley, P. J., Brady, L. J., Michalek, S. M. and Bleiweis, A. S. (1999). Virulence of a *spaP* mutant of *Streptococcus mutans* in a gnotobiotic rat model. *Infect. Immun.* **67**, 1201-1206.

Cvitkovitch, D. G., Boyd, D. A., and Hamilton, I. R. (1995). Regulation of sugar transport via the multiple sugar metabolism operon of *Streptococcus mutans* by the phosphoenolpyruvate phosphotransferase system. *J. Bacteriol.* **177**, 5704-5706.

Dashper, S. G., and Reynolds, E. C. (1992). pH regulation by *Streptococcus mutans*. *J. Dent. Res.* **71**, 1159-1165.

De la Morena, M. L., Hendrixson, D. R., and St. Geme, J. W. (1996). Isolation and characterization of *Haemophilus influenzae uvrA* gene. *Gene* **177**, 23-28.

Deutscher, J., Kuster, E., Bergstedt, U., Charrier, V., and Hillen, W. (1995). Protein kinase-dependent HPr/CcpA interaction links glycolytic activity to carbon catabolite repression in Gram-positive bacteria. *Mol. Microbiol.* **15**, 381-395.

Dreizen, S. and Brown, L. R. (1976). Xerostomia and dental caries, p.263-273. Sties, G. M., Loesche, W.J., and O'Brien, T. Microbial aspects of dental caries. Information Retrieval, Washington, D.C.

Dunny, G. M., and Leonard, B. A. B. (1997). Cell-cell communication in Gram-positive bacteria. *Annu. Rev. Microbiol.* **51**, 527-564.

Fislage, R. (1998). Differential display approach to quantitation of environmental stimuli on bacterial gene expression. *Electrophoresis* **19**, 613-616.

Foster, J. W. (1995). Low pH adaptation and the acid tolerance response of *Salmonella typhimurium*. *Crit. Rev. Micrbiol.* **21**, 215-237.

Foster, J. W. (1999). When protons attack: microbial strategies of acid adaptation. *Curr. Opin. Microbiol.* **2**, 170-174.

Foster, J. W., and Hall, H. K. (1990). Adaptive acidification tolerance response of *Salmonella typhimurium*. *J. Bacteriol.* **173**, 5129-5135.

Foster, J. W., and Hall, H. K. (1991). Inducible pH homeostasis and the acid tolerance response of *Salmonella typhimurium*. *J. Bacteriol.* **173**, 5129-5135.

Foster, J. W., and Moreno, M. (1999). Bacterial responses to pH. Novartis Foundation Symposium 221) (pp.57) Chadwick, D. J., and Cardew, G. (eds.). Toronto: John Wiley and Sons, Inc.

Fuqua, W. C., Winans, S. C., and Greenberg, E. P. (1994). Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. *J. Bacteriol.* **176**, 269-275.

Gaidenko, T. A., and Price, C. W. (1998). General stress transcription factor sigma B and sporulation transcription factor sigma H each contribute to survival of *Bacillus subtilis* under extreme growth conditions. *J. Bacteriol.* **180**, 3730-3733.

Gibbons, R. L., and Van Houte, J. (1975). Dental Caries. *Annu. Rev. Med.* **26**, 121-136.

Gill, R. T., Valdes, J. J., and Bentley, W. E. (1999). Reverse transcription-PCR differential display analysis of *Escherichia coli* global gene regulation in response to heat shock. *Appl. Environ. Microbiol.* **65**, 5386-5393.

Goodson, M. and Rowbury, R. J. (1989). Habituation to normally lethal acidity by prior growth at a sub-lethal acid pH value. *Lett. Appl. Microbiol.* **8**, 77-79.

Goodson, M. and Rowbury, R. J. (1991). RecA-independent resistance to irradiation with U.V. light in acid-habituated *Escherichia coli*. *J. Appl. Bacteriol.* **70**, 177-180.

Grogan, D. W. (2000). The question of DNA repair in hyperthermophilic archaea. *Trends Microbiol.* **8**, 180-185.

Gutierrez, J. A., Crowley, P. J., Cvitkovitch, D. G., Brady, L. J., Hamilton, I. R., Hillman, J. D. and Bleiweis, A. S. (1999). *Streptococcus mutans ffh*, a gene encoding a homologue of the 54 kDa subunit of the signal recognition particle, is involved in resistance to acid stress. *Microbiology* **145**, 357-366.

Hahn, K., Faustoferri, R. C. and Quivery Jr., R. G. (1999). Induction of an AP endonuclease activity in *Streptococcus mutans* during growth at low pH. *Mol. Microbiol.* **31**, 1489-1498.

Hamada, S., and Slade H. D. (1979). Synthesis and binding of glucosyletransferase and *in vitro* adherence of *Streptococcus mutans* grown in a synthetic medium. *Arch. Oral Biol.* **24**, 399-402.

Hamilton, I. R. (1987). Effect of changing environment on sugar transport and metabolism by oral bacteria. In: Reizer, J., Peterkofsky, A., eds. Sugar transport and metabolism by gram-positive bacteria. Chichester: Ellis Horwood, 94-133.

Hamilton, I. R, and Buckley, N. D. (1991). Adaptation by *Streptococcus mutans* to acid tolerance. *Oral Microbiol. Immunol.* **6**, 65-71.

Hamilton, I. R., and Svensäter, G. (1998). Acid-regulated proteins induced by *Streptococcus mutans* and other oral bacteria during acid shock. *Oral Microbiol. Immunol.* **13**, 292-300.

Hamilton, I. R. (2000). Oral Bacterial Ecology: The Molecular Basis. Kuramitsu, H. K., and Ellen R. P. (eds.). (pp. 228) Great Britain: Horizon Scientific Press

Hart, T. C., Marazita, M. L., and Wright, J. T. (2000). The impact of molecular genetics on oral health paradigms. *Crit. Rev. Oral. Biol. Med.* **11**, 26-56.

Hartke, A., Bouche, S., Gansel, X., Boutibonnes, P., and Auffray, Y. (1995). UV-inducible proteins and UV-induced cross protection against acid, ethanol, H_2O_2 or heat treatments of *Lactobacillus lactis* subsp. *Lactis*. *Arch. Microbiol.* **163**, 329-336.

Håvarstein, L. S., Hakenbeck, R., and Gaustad, P. (1997). Natural competence in the genus *Streptococcus*: evidence that streptococci can change phenotype by interspecies recombinational exchanges. *J. Bacteriol.* **179**, 6589-6594.

Hazlett, K. R. O., Mazurkievicz, J. E., and Banas, J. A. (1999). Inactivation of the *gbpA* gene of *Streptococcus mutans* alters structural and functional aspects of plaque biofilm which are compensated by recombination of the *gtfB* and *gtfC* genes. *Infect. Immun.* **67**, 3909-3914.

Hengge-Aronis, R. (1999). Interplay of global regulators and cell physiology in the general stress response of *Escherichia coli*. *Curr. Opin. Microbiol.* **2**, 148-152.

Higgins, C. F., Peltz, S. W., and Jacobson, A. (1992). Turnover of mRNA in prokaryotes and lower eukaryotes. *Curr. Opin. Genet. Dev.* **2**, 739-747.

Hillman, J. D., Dzuback, A. L. and Andrews, S. W. (1987). Colonization of the human oral cavity by a *Streptococcus mutans* mutant producing increased bacteriocin. *J. Dent. Res.* **66**, 1092-1094.

Hubank, M., and Schatz, D. G. (1994). Identifying differences in mRNA expression by representational difference analysis of cDNA. *Nucleic Acids Res.* **22**, 5640-5648.

Hussain, N. H., Goodson, M., and Rowbury, R. J. (1998). Intercellular communication and quorum sensing in microorganisms. *Sci. Prog.* **81**, 69-80.

Jacobson, G. R., Lodge, J., and Poy, F. (1989). Carbohydrate uptake in the oral pathogen *Streptococcus mutans*: mechanisms and regulation by protein phosphorylation. *Biochimie* **71**, 997-1004.

Jayaraman, G. C., Penders, J. E., and Burne, R. A. (1997). Transcriptional analysis of the *Streptococcus mutans hrcA*, *grpE*, and *dnaK* genes and regulation of expression in response to heat shock and environmental acidification. *Mol. Microbiol.* **25**, 329-341.

Jenkins, G. N. (1963). Theories on the mode of action of fluoride in reducing dental decay. *J. Dent. Res.* **42**, 444-452.

Jenkinson, H. and Demuth, D.R. (1997). Structure, function and immunogenicity of streptococcal antigen I/II polypeptides. *Mol. Microbiol.* **23**, 183-190.

Jensen, M. E., Polansky P. J., and Schachtele, C. F. (1982). Plaque sampling and telemetry for monitoring acid production on human buccal tooth surfaces. *Arch. Oral. Biol.* **27**, 21-31.

Kleinberg, I., Jenkins, G. N., Chatterjee, R., and Wijeyeweera, L. (1982). The antimony pH electrode and its role in the assessment and interpretation of dental plaque pH. *J. Dent. Res.* **61**, 1139-1147.

Koga, T., Okahashi, N., Takahashi, I., Kanamoto, T., Asakawa, H. and Iwaki, M. (1990). Surface hydrophobicity, adherence, and aggregation of cell surface protein antigen mutants of *Streptococcus mutans* serotype c. *Infect. Immun.* **58**, 289-296.

Kolenbrander, P. E. (2000). Oral microbial communities: biofilms, interactions, and genetic systems. *Annu. Rev. Microbiol.* **54**, 413-437.

Kolter, R., Siegele, D. A., and Tormo, A. (1993). The stationary phase of the bacterial life cycle. *Annu. Rev. Microbiol.* **47**, 855-874.

Kullen, M. J., and Klaenhammer, T. R. (1999). Identification of the pH-inducible, proton-translocating F_1F_0-ATPase (*atpBEFHAGDC*) operon of *Lactobacillus acidophilus* differential display: gene structure, cloning and characterization. *Mol. Microbiol.* **33**, 1152-1161.

Kunji, E. R. S., Mierau, I., Hagting, A., Poolman, B., and Koonings W. N. (1996). The proteolytic system of lactic acid bacteria. *Antonie Leeuwenhoek* **70**, 187-221.

Kwaik, Y. A., and Pederson, L. L. (1996). The use of differential display-PCR to isolate and characterize a *Legionella pneumophila* locus induced during the intracellular infection of macrophages. *Mol. Microbiol.* **21**, 543-556.

Lazdunski, A. M. (1989). Peptidases and proteases of *Escherichia coli* and *Salmonella typhimurium*. *FEMS Microbiol. Rev.* **63**, 265-276.

Lee, I. S., Lin, J., Hall, H. K., Bearson, B., and Foster, J. W. (1995). The stationary-phase sigma factor σ^S (RpoS) is required for a sustained acid tolerance response in virulent *Salmonella typhimurium*. *Mol. Microbiol.* **17**, 155-167.

Lee, M. S., and Morrison, D. A. (1999). Identification of a new regulator in *Streptococcus pneumoniae* linking quorum sensing to competence for genetic transformation. *J. Bacteriol.* **181**, 5004-5016.

Lee, S. F., Progulske-Fox, A., and Bleiweis, A. S. (1988). Molecular cloning and expression of a *Streptococcus mutans* major surface protein antigen, P1 (I/II), in *Escherichia coli*. *Infect. Immun.* **56**, 2114-2119.

Lee, S.F. (1995). Active release of bound antibody by *Streptococcus mutans*. *Infect. Immun.* **63**, 1940-1946.

Lehner, T., Caldwell, J., and Smith, R. (1985). Local passive immunization by monoclonal antibodies against Streptococcal antigen I/II in the prevention of dental caries. *Infect. Immun.* **50**, 796-799.

Lewis, D. W., and Ismail, A. I. (1994). Prevention of dental caries. Canadian Task Force on the Periodic Health Examination. Canadian Guide to Clinical Preventive Health care, Ottawa: Health Canada, 408-417.

Leyer, G. J., and Johnson, E. A. (1993). Acid adaptation induces cross protection against environmental stresses in *Salmonella typhimurium*. *Appl. Environ. Microbiol.* **59**, 1842-1847.

Li, Y. H., Lau, P.C-Y, Lee, J. H., Ellen, R. P. and Cvitkovitch, D. G. (2001). Natural genetic transformation of *Streptococcus mutans* growing in biofilms. *J. Bacteriol.* **183**, 897-908.

Liang, P., and Pardee, A. B. (1992). Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* **257**, 967-971.

Lin, J. J., and Sancar, A. (1992). (A)BC excinuclease: the *Escherichia coli* nucleotide excision repair enzyme. *Mol. Microbiol.* **6**, 2219-2224.

Lin, J., Lee, I. S., Frey, J., Slonczewski, J. L., and Foster, J. W. (1995). Comparative analysis of extreme acid survival in *Salmonella typhimurium*, *Shigella flexneri*, and *Escherichia coli*. *J. Bacteriol.* **177**, 4097-4104.

Lindahl, T., and Nyberg, B. (1972). Rate of depurination of native deoxyribonucleic acid. *Biochemistry* **11**, 3610-3618.

Little, J. M. and Mount, D. W. (1982). The SOS regulatory system of *E. coli*. *Cell* **29**, 11-22.

Loesche, W. J. (1986). Role of *Streptococcus mutans* in human dental decay. *Microbiol. Rev.* **50**, 353-380.

Ma, J. K-C., Smith, R., and Lehner, T. (1987). Use of monoclonal antibodies in local passive immunization to prevent colonization of human teeth by *Streptococcus mutans*. *Infect. Immun.* **55**, 1274-1278.

MacPherson L. M. D., MacFarlane, T. W., and Stephan, K. W. (1990). An intra-oral appliance study of the plaque microflora associated with early enamel demineralization. *J. Dent. Res.* **69**, 1712-1716.

Mandel, I. D. (1994). Nature v.s. nurture in dental caries. *J. Am. Dent. Assoc.* **125**, 1345-1351.

Marquis, R. E. (1995). Oxygen metabolism, oxidative stress and acid-base physiology of dental plaque biofilms. *J. Ind. Microbiol.* **15**, 198-207.

Marsh, P. D. (1991). Sugar, fluoride, pH and microbial homeostasis in dental plaque. *Proc. Finn. Dent. Soc.* **87**, 515-525.

Matos, J., Nardi, M., Kumura, H., and Monnet, V. (1998). Genetic characterization of *pepP*, which encodes an aminopeptidase P whose deficiency does not affect *Lactococcus lactis* growth in milk, unlike deficiency of the X-prolyl dipeptidyl aminopeptidase. *Appl. Environ. Microbiol.* **64**, 4591-4595.

McCann, M. P., Kidwell, J. P., and Matin, A. (1991). The putative sigma factor KatF has a central role in development of starvation-mediated general resistance in *Escherichia coli. J. Bacteriol.* **173**, 4188-4194.

McCulloch, R., Burke, M. E., and Sherratt, D. J. (1994). Peptidase activity of *Escherichia coli* aminopeptidase A is not required for its role in Xer site-specific recombination. *Mol. Microbiol.* **12**, 241-251.

McDermid, A. S., McKee, A. S., Ellwood, D. C., and March, P. D. (1986). The effects of lowering the pH on the composition and metabolism of a community of nine oral bacteria growing in a chemostat. *J. Gen. Microbiol.* **132**, 1205-1214.

Meng, S.,-Y., and Bennett, G. N. (1992). Nucleotide sequence of the *Escherichia coli cad* operon: a system for neutralization of low extracellular pH. *J. Bacteriol.* **174**, 2659-2669.

Morrison, D. A., and Baker, M. F. (1979). Competence for genetic transformation in pneumococcus depends on synthesis of a small set of proteins. *Nature* **282**, 215-217.

Mueller, J. P., Mathiopoulos, C., Slack, F. J. and Sonenshein, A. L. (1991). Identification of *Bacillus subtilis* adaptive response genes by subtractive differential hybridization. *Res. Microbiol.* **142**, 805-813.

Padan, E., Zilberstein, D., and Schuldiner, S. (1981). PH homeostasis in bacteria. *Biochim. Biophys. Acta.* **650**, 151-166.

Park, Y. K., Bearson, B., Bang, S. H, Ban, I. S., and Foster, J. W. (1996). Internal pH crisis, lysine decarboxylase and the acid tolerance response of *Salmonella typhimurium. Mol. Microbiol.* **20**, 605-611.

Pearce, B. J., Naughton, A. M., Campbell, E. A., and Masure, H. R. (1995). The *rec* locus, a competence-induced operon in *Streptococcus pneumoniae. J. Bacteriol.* **177**, 86-93.

Perry, D., and Kuramitsu H. K. (1981). Genetic transformation of *Streptococcus mutans. Infect. Immun.* **32**,1295-1297.

Pratt, L. A., Hsing, W. H., Gibson, K. E., and Silhavy, T. J. (1996). From acids to OsmZ- multiple factors influence the synthesis of the OmpF and OmpC porins in *Escherichia coli. Mol. Microbiol.* **20**, 911-917.

Quivey, R. G. Jr., Faustoferri, R. C., Anne Clancy, K., Marquis, R. E. (1995). Acid adaptation in *Streptococcus mutans* UA159 alleviates sensitization to environmental stress due to RecA deficiency. *FEMS Microbiol. Lett.* **126**, 257-262.

Quivey, R. G. Jr., Faustoferri, R., Monahan, K., and Marquis, R. (2000). Shifts in membrane fatty acid profiles associated with acid adaptation of *Streptococcus mutans*. *FEMS Microbiol. Lett.* **189**, 89-92.

Raja, N., Goodson, M., Chui, W. C. M., Smith, D. G. and Rowbury, R. J. (1991a). Habituation to acid in *Escherichia coli*: conditions for habituation and its effects on plasmid transfer. *J. App. Bact.* **70**, 59-65.

Raja, N., Goodson, M., Smith, D. G. and Rowbury, R. J. (1991b). Decreased DNA damage by acid and increased repair of acid-damaged DNA in acid-habituated *Escherichia coli*. *J. App. Bacteriol.* **70**, 507-511.

Rohwer, F., and Azam, F. (2000). Detection of DNA damage in prokaryotes by terminal deoxyribonucleotide transferase-mediated dUTP nick end labeling. *App. and Env. Micro.* **66**, 1001-1006.

Rowbury, R. J. (1998). Life sciences up-date: Do we need to rethink our ideas on the mechanisms of inducible processes in bacteria? *Sci. Prog.* **81**, 193-204.

Rowbury, R. J., and Goodson, M. (1998). Induction of acid tolerance at neutral pH in log-phase *Escherichia coli* by medium filtrates from organisms grown at acidic pH. *Lett. Appl. Microbiol.* **26**, 447-451.

Rowbury, R. J., and Goodson, M. (1999). An extracellular acid stress-sensing protein needed for acid tolerance induction in *Escherichia coli*. *FEMS Microbiol. Lett.* **174**, 49-55.

Russel, R. R. B., Beighton, D., and Cohen, B. (1982). Immunization of monkeys (*Macaca fascicularis)* with antigens purified from *Streptococcus mutans*. *Brit. Dent. J.* **152**, 81-85.

Saier, M. H. Jr., Chavaux, S., Deutscher, J., Reizer, J., Ye, J. J-. (1995). Protein phosphorylation and regulation of carbon metabolism in Gram-negative versus Gram-positive bacteria. *Trends Biochem. Sci.* **20**, 267-271.

Sancar, A. (1996). DNA excision repair. *Annu. Rev. Biochem.* **65**, 43-81.

Sancar, A., and Tang, M. -S. (1993). Nucleotide excision repair. *Photochem. Photobiol.* **57**, 905-921.

Sarkar, N. (1997). Polyadenylation of mRNA in prokaryotes. *Ann. Rev. Biochem.* **66**, 173-197.

Schilling, K. M., and Bowen, W. H. (1992). Glucans synthesized *in situ* in experimental salivary pellicle function as specific binding sites for *Streptococcus mutans*. *Infect. Immun.* **60**, 284-295.

Shepard, B. D., and Gilmore, M. S. (1999). Identification of aerobically and anaerobically induced genes in *Enterococcus faecalis* by random arbitrarily primed PCR. *Appl. Envir. Micro.* **65**, 1470-1476.

Simpson, C. L., and Russell, R. R. B. (1998). Identification of a homolog of CcpA catabolite repressor protein in *Streptococcus mutans*. *Infect. Immun.* **66**, 2085-2092.

Sinha, R. P. (1986). Toxicity of organic acids for repair-deficient strains of *Escherichia coli*. *App. and Env. Micro.* **51**, 1364-1366.

Staat, R. H., Gawronski, T. H., Cressey, D. E., Harris, R. S., and Folke, L. E. (1975). Effects of dietary sucrose levels on the quantity and microbial composition of human dental plaque. *J. Dent. Res.* **54**, 872-880.

Stephan, R. M. (1940). Changes in hydrogen-ion concentration on tooth surfaces and in carious lesions. *J. Am. Dent. Assoc.* **27**, 718-723.

Stim, K. P., and Bennett, G. N. (1993). Nucleotide sequence of the *adi* gene, which encodes the biodegradative acid-induced arginine decarboxylase of *Escherichia coli*. *J. Bacteriol.* **175**, 1221-1234.

Sturr, M. G., and Marquis, R. E. (1992). Comparative acid tolerances and inhibitor sensitivities of isolated F-ATPases of oral lactic acid bacteria. *Appl. Environ. Microbiol.* **58**, 2287-2291.

Svensäter, G., Larsson, U.-B., Greif, E. C. G., Cvitkovitch, D. G., and Hamilton, I. R. (1997). Acid tolerance response and survival by oral bacteria. *Oral Microbiol. Immunol.* **12**, 266-273.

Svensäter, G., Sjogreen, B. and Hamilton, I. R. (2000). Multiple stress responses in *Streptococcus mutans* and the induction of general and stress-specific proteins. *Microbiology* **146**, 107-117.

Svensäter, G., Björnsson, O., and Hamilton, I. R. (2001). Effect of carbon starvation and proteolytic activity on stationary-phase acid tolerance by *Streptococcus mutans*. Submitted.

Takahashi, N., and Yamada, T. (1999). Acid-induced acid tolerance and acidogenicity of non-mutans streptococci. *Oral Microbiol. Immunol.* **14**, 43-48.

Tao, L., Sutcliffe, I. C., Russel, R. R., and Ferretti, J. J. (1993). Transport of sugars, including sucrose, by the msm transport system of *Streptococcus mutans*. *J. Dent. Res.* **72**, 1386-1390.

Ten Brink, B, and Konings, W. N. (1982). Electro-chemical proton gradient and lactate concentration in *Streptococcus cremoris* cells grown in batch culture. *J. Bacteriol.* **152**, 682-686.

Thiagalingam S., and Grossman L. (1991). Both ATPase sites of *Escherichia coli* UvrA have functional roles in nucleotide excision repair. *J. Biol. Chem.* **266**, 11395-11403.

Thompson, S. A., and Blaser, M. J. (1995). Isolation of the *Helicobacter pylori recA* gene and involvement of the *recA* region in resistance to low pH. *Infect. Immun.* **63**, 2185-2193.

Vadeboncoeur, C., and Pelletier M. (1997). The phosphoenolpyruvate-sugar phosphotransferase system of oral streptococci and its role in the control of sugar metabolism. *FEMS Microbiol. Rev.* **19**, 187-207.

van der Hoeven, J. S., and Camp, P. J. M. (1993). Mixed continuous cultures of *Streptococcus mutans* with *Streptococcus sanguis* or with *Streptococcus oralis* as a model to study the ecological effects of lactoperoxidase system. *Caries Res.* **27**, 26-30.

Vanhoof, G., Goossen, S. F., De Meester, I., Hendriks, D., and Scharpe, S. (1995). Proline motifs in peptidases and their biological processing. *FASEB J.* **9**, 736-744.

van Houte, J., Russo, J., and Prostack, K. S. (1989). Increased pH lowering ability of *Streptococcus mutans* cell masses associated with extracellular glucans rich matrix material. *J. Dent. Res.* **68**, 451-459.

van Houte, J., Lopman, J., and Kent, R. (1994). The predominant cultivable flora of sound and carious human root surfaces. *J. Dent. Res.* **73**, 1727-1734.

Velculescu, V. E., Zhang, L., Vogelstein, B., and Kinzler, K. W. (1995). Serial analysis of gene expression. *Science* **270**, 484-487.

Venkitaraman, A. R., Vacca, S. A., Kopec, L. K., and Bowen, W. H. (1995). Characterization of glucosyltransferase B, GtfC, and GtfD in solution and on the surface of hydroxyapatite. *J. Dent. Res.* **74**, 1695-1701.

Walker, G. C. (1984). Mutagenesis and inducible responses to deoxyribonucleic acid damage in *Escherichia coli. Microbiol. Rev.* **48**, 60-93.

Wei, Y., Lee, J. M., Richmond, C., Blattner, F. R., Rafalski, J. A., and LaRossa, R. A. (2001). High-density microarray-mediated gene expression profiling of *Escherichia coli. J. Bacteriol.* **183**, 545-556.

Welsh, J., Chada, K., Dalal, S. S., Cheng, R., Ralph, D., and McClelland, M. (1992). Arbitrarily primed PCR fingerprinting of RNA. *Nucleic Acids Res.* **20**, 4965-4970.

Wiedmann, M., Arvik, T. J., Hurley, R. J., and Boor, K. J. (1998). General stress transcription factor sigma B and its role in acid tolerance and virulence of *Listeria monocytogenes. J. Bacteriol.* **180**, 3650-3656.

Wilce, M. C. J., Bond, C. S., Dixon, N. E., Freeman, H. C., Guss, J. M., Lilley, P. E., and Wilce, J. A. (1998). Structure and mechanism of a proline-specific aminopeptidase from *Escherichia coli. Proc. Natl. Acad. Sci.* **95**, 3472-3477.

Wimpenny, J. (1995). Biofilms: structure and organization. *Microbial Ecol. Health Dis.* **8**, 305-308.

Yamashita, Y., Bowen, W. H., Burne, R. A., and Kuramitsu, H. K. (1993a). Role of the *Streptococcus mutans gtf* genes in caries induction in the specific-pathogen-free rat model. *Infect. Immun.* **61**, 3811-3817.

Yamashita, Y., Takehara, T., Kuramitsu, H. K. (1993b). Molecular characterization of a *Streptococcus mutans* mutant altered in environmental stress response. *J. Bacteriol.* **175**, 6220-6228.

Yasui, A., and McCready, S. (1998). Alternative repair pathways for UV-induced DNA damage. *BioEssays* **20**, 291-297.

Yousheng, M., Curran, T. M., and Marquis, R. E. (1997). Rapid procedure for acid adaptation of oral lactic-acid bacteria and further characterization of the response. *Can. J. Microbiol.* **43**, 143-148.

Zou, Y., Crowley, D. J., Van Houten, B. (1998). Involvement of molecular chaperonins in nucleotide excision repair. DnaK leads to increased thermal stability of UvrA, catalytic UvrB loading, enhanced repair, and increased UV resistance. *J. Biol. Chem.* **273**, 12887-12892.