Dental Caries and Chemical Warfare Within the Mouth

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ABSTRACT

To date, it appears that the dentists' war against dental caries has no end in sight due to the fact that dentists lack any genuine offensive firepower. Make no mistake, the defense has drastically improved as dentists have shifted the focus more toward the preventive aspects of dental care. But defense by itself cannot defeat the enemy; at best, it can maintain the status quo. In order to defeat the enemy, one must study and understand the enemy, to know its strengths and weaknesses, and to strike at those points of vulnerability. The aim of this research is to identify and characterize genes that are responsible for observed virulence factors in *Streptococcus mutans*, which is the primary pathogen involved in the development of dental caries. Once there is a more defined understanding of the many virulence factors of *S. mutans*, there will be a much more valuable insight into its role in the ecology of the oral cavity. Eventually, this knowledge could enable dentists to convert the bacterial "weaponry" into its own arsenal, which could then be innovatively employed as preventive, diagnostic, and therapeutic agents to treat oral bacterial-related diseases.

Dental caries is a direct result of the localized destruction of tissues of the teeth by acids produced from fermentation of dietary carbohydrates by plaque bacteria on the tooth surface. Teeth are made of calcium phosphate hydroxyapetite together with some organic constituents. Under prolonged and repeated acidic conditions, the hard crystals of the tooth enamel slowly dissolve, resulting in cavitation, which is the clinical manifestation of dental caries.

It is well known that dental caries is one of the most common infectious dis-

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eases afflicting humans. The fact that it tends to remain untreated, particularly in underdeveloped parts of the world, leads to considerable levels of suffering that are often eradicated only by extraction or exfoliation of the infected teeth. The significance of dental caries is well exemplified by the fact that annual expenditures of the United States population on dental services exceed $65 billion, with more than half of these costs attributable to dental caries.[1]

Streptococcus mutans is considered as a primary agent in cariogenesis.[2] Its abilities to adhere to and to form biofilms on the tooth surface, to metabolize carbohydrates to produce acids, and to survive low pH and other environmental insults are believed to be critical in its persistence, and eventual becoming dominant in the dental plaque. The dental plaque consists of a complex bacterial community of more than 500 different species of bacteria.[3] Thus, in addition to the previously mentioned virulence properties, *S. mutans* also possesses the ability to kill other competing species in the dental plaque. This killing ability is conferred by the production of proteinaceous antibiotics called mutacin.[4] Mutacin production gives *S. mutans* an edge over its competitors when nutrients become limited in the dental plaque, as is often the case between meals. Thus, mutacin is, in essence, a "weapon" that *S. mutans* uses in this "chemical warfare" in order to prevent the growth of other competing bacteria and to facilitate its dominance within the dental plaque.[5,6]

Mutacin production is controlled by many factors, some of which are genetic, and others of which are environmental.[7] Environmental factors usually need to interact with genetic factors (regulatory genes) to exert their effects. These environmental factors may range from temperature and pH levels to the presence of certain minerals, buffers, and nutrients within the oral cavity. It is apparent that nutritional availability plays a critical role in modulating the levels of mutacin production. One of the nutritional factors is phosphate, which is present in large quantities in the saliva.[8] Our preliminary studies have demonstrated that in the presence of 5 mM phosphate, mutacin production is severely impaired. This study aims to understand how phosphate regulates mutacin production. Specifically, the authors wanted to find the genes that are involved in this phosphate inhibition of mutacin production. Genetic and molecular techniques were used to carry out this study.

Research Materials and Methods

Bacterial Strains and Culture Conditions

Escherichia coli strain DH5α was used for cloning as well as plasmid amplifications. *E. coli* cells were grown in Luria-Bertani (LB, Fisher) medium aerobically at 37°C. *E. coli* strains carrying plasmids were grown in LB medium containing spectinomycin (300 µg/ml). *S. mutans* wild-type strain UA140 was cultured in brain heart infusion (BHI, Difco) medium or on Todd-Hewitt (TH, Difco) agar plates. For selection of antibiotic resistant colonies, BHI medium was supplemented with spectinomycin (800 µg/ml). TH medium supplemented with potassium phosphate buffer (5 mM) was used to screen for mutants that have overcome phosphate inhibition of mutacin production.

Library Screening

The deferred antagonism assay was employed to screen a random insertional mutagenesis library for mutations that produce mutacin in the presence of inhibitory amounts of phosphate.[9] Briefly, the mutant colonies, which were grown in 96-well plates, were stabbed onto potassium phosphate plates and grown anaerobically for 72 hours. Each plate was then overlaid with a thin layer of soft agar mixed with overnight culture of the mutacin-sensitive indicator strain, *S. sobrinus*. The zone of inhibition was inspected after an overnight incubation under anaerobic conditions. The presence of "zones of growth inhibition" around any of the colonies was considered to be a positive indicator of the presence of a mutant that has overcome inhibition by phosphate (**Figure 1**).

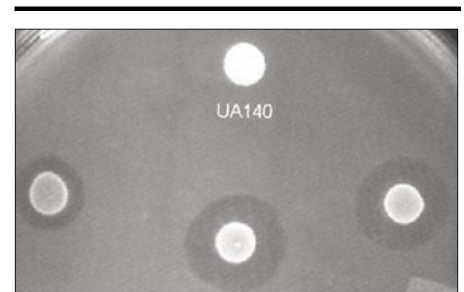

Figure 1. Deferred antagonism assay of mutacin. Mutacin-producing mutants can be identified by the presence of "halos" or zones of inhibition which circumscribe the colonies. The colony labeled UA140 is the wild-type (non-mutant) strain, which the authors would expect to produce no mutacin under experimental conditions. Note the absence of a "halo" around this colony.

Identification of Mutated Genes

Chromosomal DNA from each selected mutant was prepared from 10 ml of cell culture at OD_{600} of 0.8 by standard DNA extraction protocol.[10] Ten µg of chromosomal DNA from each mutant was digested with one of the following restriction enzymes: *Xmn*I, *Sca*I, *Acl*I and *Bst*BI. All of these restriction enzymes do not cut within the p0SC plasmid that was used for constructing the library. DNA fragments

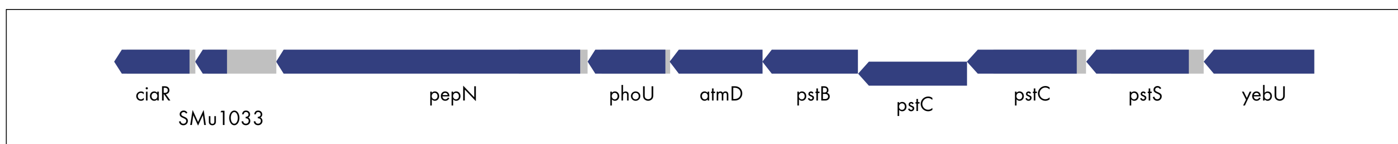

Figure 2. Schematic diagram of the Smu1034 region of the *S. mutans* UA159 chromosome. The operon consists of seven genes in the order of *pstS, pstC, pstB, atmD, phoU,* and *pepN* (Smu1034). *pstS* encodes the phosphate ABC transporter, a periplasmic phosphate-binding protein. *pstC* encodes the permease protein of the phosphate ABC transporter system. *pstB* and *atmD* encode the ATP binding proteins. *phoU* encodes the phosphate transport regulatory protein, and *pepN* is annotated as the peptidase N gene.

were circularized by self-ligation with T4 DNA ligase (Promega). The recircularization of DNA fragments allowed for direct sequencing of the inserts by using M13 primers. The ligated DNA fragments were transformed into *E. coli*, and plasmid was isolated from positive clones. The insert was sequenced by the University of California, Los Angeles Core DNA Sequencing Facility. The sequences obtained were compared to the genomic sequences of *S. mutans* UA159 available at the Los Alamos Oral Pathogen Sequence Databases (http://www.oralgen.lanl.gov) via BLAST.

Table 1

Summary of genes involved in phosphate regulation of mutacin production

Organism	Gene	Function of protein
S. mutans UA 159	Smu1034	Aminopeptidase
S. mutans UA 159	Smu1713	Hypothetical membrane protein
S. mutans UA 159	Smu1848	Hypothetical membrane protein

Results

Screening for Mutants that Produce Mutacin on Phosphate Plates

A random insertional mutagenesis library of 12,000 clones was generated via random insertions of a suicide plasmid into the chromosome of *S. mutans* strain UA140.[11] This library was screened for mutants which demonstrated mutacin production in the presence of phosphate. Screenings for each mutant within the library were performed in triplicates. Overall, 48 mutants were isolated which consistently produced mutacin in the presence of 5 mM phosphate.

Identification of the Mutated Genes

A portion of the 48 mutants was sequenced and their mutated genes were identified as described in Materials and Methods. From these sequenced clones, three unique genes were identified (**Table 1**). Two of these genes (Smu1848 and 1713) are classified as conserved hypothetical proteins, which means they currently have no known function. The third gene, which is of particular interest, is Smu1034, which is in the same transcription unit (an operon) as the phosphate transporter genes.

Discussion and Summary

In this paper, the authors reported the identification of three genes that are possibly involved in phosphate regulation of mutacin production: Smu1034, Smu1848, and Smu1713.

Smu1034 is also known as *pepN*, which is a gene that codes for an aminopeptidase (**Figure 2**). It is involved with other peptidases in the degradation of peptides generated during intracellular protein breakdown.[12] As mentioned previously, *pepN* is in the same transcription unit as the phosphate transporter genes, and the authors speculate that a mutation in this gene could affect the phosphate transporters. The *pepN* gene may be an integral component required for the process of the transporter protein themselves. In this situation, a mutation in *pepN* may result in a loss of high-affinity phosphate transport, thus reducing the intracellular concentration of phosphate. This would consequentially alleviate the toxic effect of excess phosphate on mutacin production.

The other two genes (Smu1848 and Smu1713) are classified as conserved hypothetical proteins and currently have no known function. Nevertheless, it is important to note that both proteins are membrane proteins. Thus, it can be speculated that their inactivation may have disrupted the normal membrane structure, which then indirectly affected phosphate transport. Alternatively, these proteins may be part of a signaling pathway for phosphate mediated gene

regulation. More studies are required to determine their function.

In summary, the authors screened a random mutation library of 12,000 clones and isolated 48 mutants that rescued mutacin production on 5 mM phosphate plates. Some of the clones were sequenced and three unique genes were identified. One of the genes may be involved in phosphate transport, which explains why its mutation alleviated the toxic effect of phosphate on mutacin production. The other genes encode membrane proteins, but their function is currently unknown. In the continuing investigations, the authors will complete sequencing all 48 mutants, which would allow the opportunity to identify all genes involved in phosphate regulation of mutacin production. This information will hopefully help future studies aimed at finding new target or strategies to curb dental caries.

Conclusion

Dentists seem to do a lot of "rescuing" of patients, saving them from the pain and agony of tooth decay and caries caused by *S. mutans*. But the philosophy behind this research is simple: Dentists must fix the problem at its source. Once there is a more defined understanding of the intricate weaponry of the enemy, *S. mutans*, dentists will have much more valuable insight into its role in the ecology of the oral cavity.

Eventually, this knowledge could enable dentists to develop "counterattack" measures that could depress these aggressors for good. On the other hand, this offensive weapon of *S. mutans* can be used for the benefit of the host. For example, a genetically engineered effector strain of *S. mutans* has been constructed, which produces elevated amounts of mutacin but diminished amounts of acids.[13] This strain is currently being used in a limited clinical trial for the prevention of dental caries. This is just one of an infinite number of possible uses of mutacin that could be innovatively employed as preventive, diagnostic and therapeutic agents to treat oral bacterial-related diseases. This may change the face of dentistry as it is known today. CDA

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