Mechanistic, Genomic and Proteomic Study on the Effects of BisGMA-derived Biodegradation Product on Cariogenic Bacteria

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Abstract

Objectives—Investigate the effects of a Bis-phenyl-glycidyl-dimethacrylate (BisGMA) biodegradation product, bishydroxypropoxyphenyl-propene (BisHPPP), on gene expression and protein synthesis of cariogenic bacteria.

Methods—Quantitative real-time polymerase chain reaction was used to investigate the effects of BisHPPP on the expression of specific virulence-associated genes, i.e. gtfB, gtfC, gbpB, comC, comD, comE and atpH in Streptococcus mutans UA159. Possible mechanisms for bacterial response to BisHPPP were explored using gene knock-out and associated complemented strains of the signal peptide encoding gene, comC. The effects of BisHPPP on global gene and protein expression was analyzed using microarray and quantitative proteomics. The role of BisHPPP in glucosyltransferase (GTF) enzyme activity of Streptococcus mutans biofilms was also measured.

Results—BisHPPP (0.01, 0.1 mM) up-regulated gtfB/C, gbpB, comCDE, and atpH most pronounced in biofilms at cariogenic pH (5.5). The effects of BisHPPP on the constructed knockout and complemented strains of comC from quorum-sensing system, implicated this signaling pathway in up-regulation of the virulence-associated genes. Microarray and proteomics identified BisHPPP-regulated genes and proteins involved in biofilm formation, carbohydrate transport, acid tolerance and stress-response. GTF activity was higher in BisHPPP-exposed biofilms when compared to no-BisHPPP conditions.

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Significance—These findings provide insight into the genetic and physiological pathways and mechanisms that help explain *S. mutans* adaptation to restorative conditions that are conducive to increased secondary caries around resin composite restorations and may provide guidance to clinicians’ decision on the selection of dental materials when considering the long term oral health of patients and the interactions of composite resins with oral bacteria.

Keywords
resin composite; dental adhesive; secondary caries; biodegradation; *Streptococcus mutans*; biofilm; gene expression; proteomics; microarray

1. INTRODUCTION

Resin composites are currently the most widely-used restorative materials in dentistry. While providing several beneficial properties over amalgam such as superior esthetics and excellent adhesive strength to dentin and enamel, resin composites experience significant biological breakdown in the oral cavity. These processes result in an increased rate of recurrent/secondary caries and reduced restoration longevity compared to amalgam [1–4]. Furthermore, two most recent systematic reviews suggested that resin composite restorations in posterior teeth have shorter service life and suffer from a higher number of secondary caries when compared to amalgam restorations [5, 6]. Biodegradation of the resin composites and adhesives *in vivo* is one of the major contributors to the cascade of events leading to composite restoration failure associated with recurrent/secondary caries [7], and impacting clinical and economical outcomes [4, 8–10].

Bis-phenyl glycidyl dimethacrylate (BisGMA) is a universal monomer used extensively in dental restorative materials such as dental composites and adhesives [11]. The ester linkages contained within this monomer render the dental composites and adhesives susceptible to hydrolytic degradation, which can be catalysed by salivary and bacterial esterases. The hydrolysis produces the biodegradation by-product (BBP) bishydroxypropoxyphenyl-propane (BisHPPP) [12–16]. Degradation of the tooth-restoration interfaces can facilitate infiltration of cariogenic bacteria into the margins and contribute to the progression of recurrent caries [17].

*Streptococcus mutans* is the most cariogenic of all the oral streptococci and is one of the leading species associated with human dental caries [18]. *S. mutans* has been used in *in vitro* models to identify candidate molecular pathways relevant to resin composite restoration failure [19, 20]. The present study builds upon our previous reports that showed regulation of gene expression for *gtfB* (a known virulence factor) and *yfiV* (a putative transcriptional regulator) by BBPs in *S. mutans* NG8 [19, 20]. The latter studies demonstrated the need to further examine the relationship between BBPs and gene expression in *S. mutans*, and directing further work toward understanding the *S. mutans* genetic and physiological response after exposure to BBPs from dental composite and adhesives. While there is extensive evidence supporting the cytotoxic, genotoxic and estrogenic effects of dental resin monomers on both mammalian and bacterial cells, there are only a few studies reporting the
biological effects of the BBPs from these monomers, despite their long-term release from the bulk of the restoration and from the adhesive within the critical interface [15].

Here the effects of BBPs on S. mutans virulence-associated gene and protein expression were further investigated. Since S. mutans UA159 genome has been fully sequenced and annotated, comprehensive gene expression analysis is possible [21]. Thus it was decided to chose this strain for the present study. First, the effect of the BisGMA-derived universal degradation product, BisHPPP, on the expression of seven established key virulence genes in S. mutans UA159 i.e. gtfB, gtfC, gbpB, comC, comD, comE and atpH was investigated. GtfB and gtfC encode glucosyltransferase (GTF) enzymes involved in the synthesis of water-insoluble glucan that together with glucan binding protein (encoded by gbpB) facilitate bacterial adhesion to the tooth surface. ComCDE are involved in quorum-sensing and atpH encodes subunit C of a multi-subunit enzyme (F1F0-ATPase) involved in intracellular pH regulation and acid tolerance. The selected genes have unique virulence properties in S. mutans and have been linked to its cariogenicity by previous animal and human studies [22–29]. Second, the potential signaling pathways underlying these effects were explored by using knock-out and complemented strains of the key regulatory gene, comC, a component of quorum-sensing system. Quorum-sensing is an integral component of bacterial global gene regulatory networks responsible for bacterial adaptation in biofilms [30]. This system has been shown to have a positive regulatory effect on the expression of biofilm-related genes including gtfB, gtfC, gbpB and acid tolerance in S. mutans [31, 32]. It was also desired to define other candidate pathways involved in S. mutans cariogenic potential after exposure to BisHPPP, using microarray and quantitative proteomics analyses in an in vitro biofilm model. Finally, the effect of BisHPPP on S. mutans GTF enzyme activity was measured to assess the effect of BisHPPP on the synthesis of water-insoluble exopolysaccharide glucan, a known virulence factor in S. mutans [33].

The current study is the first investigation relating possible underlying signaling pathways and their effect on S. mutans virulence factor expression, to the presence of BBPs. This is of importance with regards to unravelling molecular mechanisms associated with recurrent caries around resin composite restorations.

### 2. MATERIALS & METHODS

#### 2.1 Bacterial Strains and Growth Conditions

S. mutans UA159 wild-type strain was obtained from Dr. Arnold Bleiweis (University of Florida) and stored in 15% (v/v) glycerol (3 mL of 50% glycerol in Todd-Hewitt Yeast Extract broth was added to 7 mL of overnight bacterial culture) at −80°C. To construct the comC-deficient mutant strain (SMΔcomC1), a PCR-ligation mutagenesis strategy was used as previously described [34, 35]. The comC complemented strain (SMΔcomC1C) was made using pIB166 plasmid that contained the S. mutans recombinant comC as described previously [23]. The primers used for the comC deletion and complementation constructs are listed in Table 1 (Operon, AL, USA). The S. mutans wild-type strain was sub-cultured on Todd-Hewitt agar plate supplemented with 0.3% yeast extract (THYE) (BBL; Becton Dickenson, Cockeysville, MD, USA), whereas the comC mutant was maintained on THYE agar containing 10 μg of erythromycin/mL [34]. All S. mutans overnight cultures were
routinely grown in THYE broth at 37°C in a 5% CO₂-95% air mixtures. In order to investigate the differential effects of BisHPPP on S. mutans gene/protein expression at neutral (pH 7.0) and cariogenic pH (pH 5.5), the first set of experiments (qRT-PCR) were carried out at both pHs.

2.2 Preparation of Planktonic Cells

Overnight cultures of S. mutans UA159 were diluted (1:10) in TYEG medium containing tryptone (10 g/L), yeast extract (5 g/L) and glucose (5 mM) buffered either at pH 5.5 with 100 mM MES (2-(N-Morpholino)-ethanesulfonic acid, Sigma-Aldrich, St. Louis, MO, USA) or at pH 7.0 with 100 mM MOPS (3-(N-Morpholino) propanesulfonic acid, Bioshop, Burlington, ON, Canada), to control pH, supplemented with 0.1% glucose and 500 μL of the appropriate amount of sterile filtered BisHPPP (99.9% pure, Sigma-Aldrich) yielding final concentrations of 0, 0.001, 0.01, 0.1 mM BisHPPP in the solutions [36]. The culture tubes were incubated at 37°C in a 5% CO₂-95% air mixtures. Bacterial cell density was monitored using a UV spectrophotometer (Ultraspec 3000, Biotech) at 600 nm in order to ensure that the cultures were harvested once they reached mid-logarithmic phase (optical density or OD=0.4). The pH of sample cultures was verified (H135 minilab™ pro, HACH, Germany). This was followed by centrifugation at 2300 x g for 10 minutes (4°C). The supernatants were discarded and the pellets were snap frozen in liquid nitrogen and stored at -80°C until required for RNA isolation.

2.3 Preparation of Biofilm Cells

Overnight cultures of S. mutans UA159 were diluted (1:60) in TYEG medium and added to six-well polystyrene microtiter plates (Fisher Scientific). Each well containing 3 mL of ¼ strength TYEG medium buffered to pH 5.5 or 7.0 and 50 μL of overnight culture were supplemented with 0.1% glucose and 500 μL of BisHPPP stock solutions as described for planktonic conditions above. Cells were then incubated for 18 hours (37°C, 5% CO₂) after which the pH of sample cultures was confirmed. Then, the liquid contents were removed and 3 mL of phosphate buffer (PBS) was slowly added to each well and gently stirred to remove loosely attached cells, leaving only adhered biofilm cells. The remaining PBS was then removed and replaced with 1 mL of fresh PBS. Biofilm cells from each well were scraped and the resulting cell suspensions from each well were transferred to 50 mL tubes and centrifuged at 2300 x g for 10 minutes at 4°C. The supernatants were then discarded and the pellets were snap frozen in liquid nitrogen and stored at −80°C until required for RNA isolation.

2.4 Gene Expression Analysis Using qRT-PCR

Total RNA was isolated by disruption of S. mutans UA159 cells using the 120 cell disrupter (Thermo Savant, Fast-Prep FP 101), followed by DNase treatment of the RNA samples and cDNA synthesis as described before [19]. QRT-PCR was used to quantify the relative gene expression of selected genes: gtfB, gtfC, gbpB, comC, comD, comE and atpH as previously described [19]. The primers used are presented in Table 2 (Operon, AL, USA). Quantitative gene expression data were then normalized to the 16S rRNA, a well-established housekeeping gene [37]. The level of 16S rRNA message was not affected by various
concentrations of BisHPPP (data not shown). QRT-PCR gene expression analysis was also employed to investigate the involvement of the comCDE system in regulation of the seven S. mutans virulence genes in the presence of BisHPPP. Using the comC knock-out (SMΔcomC1) and complemented (SMΔcomC1C) strains, gene expression analysis was repeated for three representative genes, gtfB (biofilm formation), comD (quorum sensing) and atpH (acid tolerance), each representing a functional category of virulence genes. For statistical analysis, one-way analysis of variance (ANOVA) and Tukey post hoc analyses were performed to determine the differences in gene expression between different concentrations BisHPPP and the no-BisHPPP control within each growth mode (P<0.05). Two-way ANOVA and Tukey post-hoc analyses were conducted to validate differences in gene expression between growth modes (biofilm vs. planktonic) at the same concentration (P<0.05). Homogeneity of variance and normality were verified with Leven’s and Shapiro-Wilk tests, respectively. A fold change in gene expression more than 2 (up-regulation) and less than 0.5 (down-regulation) with a P-value cut-off of < 0.05 were considered physiologically significant \[38\]. All qRT-PCR reactions were run in triplicate for each experimental condition and the experiments were reproduced four separate times using four independent cultures.

2.5 Microarray Analysis

Total RNA was isolated by disruption of S. mutans UA159 biofilms from both experimental (0.01, 0.1 and 1.0 mM) and control (no BisHPPP) groups using the 120 Cell disrupter (Thermo Savant, Fast-Prep FP 101) followed by DNase treatment of the RNA samples and cDNA synthesis as described previously \[19\]. S. mutans UA159 microarray slides designed by Dr. Dragana Ajdic, were used (Affymetrix, Santa Clara, CA). The S. mutans microarray processing including hybridization, washing and scanning of microarray slides, were performed according to the procedures described by Affymetrix \[39\] and conducted by The Center for Applied Genomics, (Hospital for Sick Children Research Institute, Toronto, Ontario, Canada). Data was first checked for overall quality using R (v2.15.2) with the Bioconductor framework and the Array Quality Metrics package. Microarray data processing and analysis employed the operating software. Statistically significant genes were then identified using a class comparison analysis (GeneSpring v12.6, Agilent technologies, Santa Clara, CA, USA). An analysis of variance (ANOVA) with the parametric P-value cut off set at <0.05 was used for statistical analysis. Specific comparisons among the different concentrations in each group was done by a post-hoc Tukey’s test. A fold change in gene expression > 1.5 (up-regulation) and < 0.5 (down-regulation) with a P-value cutoff of < 0.05 were considered significant \[40\]. All study groups were run 3 separate times with 4 independent samples (using 4 independent S. mutans cultures) in each group.

2.6 Proteomic Analysis

2.6.1 Sample preparation for Mass Spectrometry—Biofilms from both experimental (0.01, 0.1 and 1.0 mM) and control (no BisHPPP) groups were washed twice in cold PBS and re-suspended in 1 mL of PBS buffer. The cells were disrupted using a homogenizer (Thermo Savant, FastPrep FP 101) for 45 s and then centrifuged at 15,700 X g for 1 min. Supernatant was carefully removed, separated in aliquots of 50 μL and stored at −80°C. The total protein concentration in each sample was assessed by the Micro Bicinchoninic Acid

*Dent Mater. Author manuscript; available in PMC 2018 February 01.*
Equal amounts of protein (20 μg) from both experimental and control groups were dried by a rotary evaporator, denatured and reduced for 2 h by the addition of 200 μL of 4 M urea, 10 mM dithiothreitol (DTT), and 50 mM NH₄HCO₃, pH 7.8. After fourfold dilution with 50 mM NH₄HCO₃, pH 7.8, tryptic digestion was carried out for 18 h at 37°C, following the addition of 2% (w/w) sequencing-grade trypsin (Promega, Madison, WI, USA). All study groups were run 3 separate times to increase coverage of the samples and identify more proteins, with 4 independent samples (using 4 independent S. mutans cultures) in each group.

2.6.2 Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry (LC-ESI-MS/MS) and Relative Proteome Quantitation—Peptide separation and mass spectrometric analyses were carried out as described previously [41]. The obtained MS/MS spectra were searched against a streptococci protein database (Swiss Prot and TrEMBL, Swiss Institute of Bioinformatics, Geneva, Switzerland, http://ca.expasy.org/sprot/) using SEQUEST algorithm in Proteome Discoverer 1.3 software (Thermo Scientific, San Jose, CA, USA). [41].

For quantitative proteome analysis, three MS raw files from each group (control and experimental groups) for a total of 12 MS raw files were analyzed using SIEVE software (Version 2.0 Thermo Scientific, San Jose, CA, USA) [41]. For the alignment step, a single MS raw file belonging to the control group (no BisHPPP) was selected as the benchmark and all of the other files were adjusted to generate the best correlation to this reference file. After alignment, the feature detection and integration (or framing) process was performed using the MS level data. For statistical analyses of protein abundance, peak integrations were summarized into protein-level annotation in SIEVE using a weighted average of intensities of LC-ESIMS/MS for each protein. In addition, a statistical model based on an ANOVA framework with Tukey’s post hoc test was carried out. Relative abundance of an individual protein from different BisHPPP concentration groups was considered significantly different from the control group (no BisHPPP) when the values observed were >1.5 for increased and < 0.5 for decreased abundance with a P-value cut-off of < 0.05 [41, 42].

2.7 Correlation Analysis

For correlation analysis between microarray and proteomics data both data sets were merged by cross-referencing the sequence identifier, which was “Gene Symbol” in gene expression data and “Gene Name” in the protein data. In total 38 unique genes had both gene expression and protein abundance data. A Pearson estimate and a non-parametric estimate (Spearman) were used to detect any correlation between gene expression and proteomics data with the P-value cut-off set at < 0.05.

2.8 GTF Enzyme Activity Assay

GTF enzyme activity was measured by determining the rate that [14C]-sucrose was converted to glucan polymers by GTF, which cleaves the sucrose into fructose and glucose that are added to the growing exopolysaccharide [43]. Briefly, S. mutans UA159 biofilm was grown in a 6-well polystyrene microtiter plate containing ¼ TYEG medium buffered to pH 5.5. Appropriate amounts of BisHPPP were added to the medium to yield the target final
concentrations (0, 0.01, 0.1 and 1.0 mM). Overnight cultures were added to the mixture and incubated for 18 h. Bacterial cells were collected, transferred to tubes and pelleted by centrifugation at 2300 g and 4°C for 10 min. The supernatant was then removed/discarded and the pellets were washed twice in cold PBS and re-suspended in 1 mL of PBS buffer. The cells were disrupted (Thermo Savant, FastPrep FP 101) for 45 s and then centrifuged at 15,700 g for 1 minute. Separate aliquots of the supernatant were carefully removed and stored separately at −80°C. Total protein concentration in each sample was assessed by the Micro BCA assay described above [41]. 15 μg of protein from both experimental and control groups was added to 0.2 M potassium phosphate buffer (pH 6.8) for a total volume of 20 μL, this solution was mixed with 20 μL of 14C-radiolabelled reaction buffer containing 0.2 M KPO4 (pH 6.8), 20 mM sucrose, and 10 μL/mL 14C-sucrose (24.4 GBq/mmol; Amersham). The mixture was incubated at 37°C for 60 min after which the reaction mixtures were adsorbed onto 25 mm filters (0.22 μm GVWP; Millipore). The samples were then air dried for 20 min and washed three times with 2 mL distilled water to remove the water-soluble glucan and serve only as water-insoluble glucan samples. The water-insoluble glucan, mainly synthesized by GtfB and GtfC enzymes. The samples were then placed in 5 mL scintillation fluid (ScintiSafe Econo 2 Cocktail; Fisher) and synthesized [14C]-glucan was measured using a liquid scintillation counter (Beckman LS6500). Homogeneity of variance and normality were verified with Leven’s and Shapiro-Wilk tests, respectively. One way ANOVA and Tukey post hoc analyses were used to determine significant changes in GTF activity between the different groups (P<0.05). All study groups were run 3 separate times with 4 independent samples (using 4 independent S. mutans cultures) in each group.

3. RESULTS

3.1 Effects of BisHPPP on S. mutans UA159 Gene Expression at pH 5.5 and 7.0

Exposure of S. mutans UA159 to different concentrations of BisHPPP at pH 5.5 resulted in a significant up-regulation (P<0.05) of gtfB, gtfC, gbpB, comC, comD and atpH at either 0.001 (gtfB only), or 0.01 and/or 0.1 mM of BisHPPP in biofilms only as demonstrated by qRT-PCR (Fig. 1). ComE was the only gene not affected by BisHPPP in biofilm, but for planktonic cells along with comC the expressions were mildly down-regulated in the presence of BisHPPP compared to the control (Fig. 1). Biofilms showed a significant upregulation of the expression of all genes vs. planktonic cells at several concentrations of BisHPPP (P<0.001) (Fig. 1). In contrast to the findings at pH 5.5, the data at pH 7.0 showed that none of the tested genes were affected by BisHPPP in the biofilm state, while two of them, comD and atpH, were up-regulated at 0.1 and 0.01 mM, respectively only for planktonic cells (Fig. 2). Based on these findings, all genes showed greater up-regulation in biofilms compared to planktonic grown cells at cariogenic pH (5.5) in the presence of BisHPPP. Therefore, the rest of the experiments including knock-out and complemented experiments, microarray, proteomics and GTF enzyme activity were conducted only with S. mutans UA159 biofilms under acidic pH (5.5). Given that 0.001 mM concentration of BisHPPP had very limited effect vs. 0.01 and 0.1 mM on S. mutans gene expression analysis using qRT-PCR, this concentration was substituted for a higher dose (1.0 mM) in the subsequent studies for microarray, proteomics, and GTF enzyme activity experiments.
3.2 Effects of BisHPPP on *S. mutans* UA159 *comC* Knock-out and Complemented Strains’ Gene expression

Exposure of *S. mutans* UA159 *comC* knock-out (SMΔcomC1) biofilms to different concentrations of BisHPPP (0.01 and 0.1 mM) resulted in no significant change (P>0.05) in the expression level of any of the genes that were tested in this study when compared to the no BisHPPP controls. Data are presented for three representative genes, *gtfB* (biofilm formation), *comD* (quorum sensing) and *atpH* (acid tolerance) in Fig. 3. However, the above three representative genes, were up-regulated in *S. mutans* UA159 *comC* complemented strain (SMΔcomC1C) by different concentrations of BisHPPP similarly to what was observed for the wild-type strain (Fig. 3).

3.3 Microarray Gene Expression Analysis of *S. mutans* following Exposure to BisHPPP

Microarray analysis results covered 1911 genes and revealed alteration of transcription of a total number of 115 genes, by at least 1.5 fold (P<0.05), after exposure of *S. mutans* UA159 biofilms to 1.0 mM BisHPPP. Of these genes, 62 genes were up-regulated and 53 genes were down-regulated (Supplemental File 1). BisHPPP at concentrations of 0.01 and 0.1 mM did not show an observable significant change in the *S. mutans* UA159 gene expression (P>0.05). The functional classification for the significantly up- or down-regulated genes is represented in Figure 4. Microarray data showed that exposure to BisHPPP resulted in up-regulation of eight genes involved in biofilm formation, ten were related to the Phosphoenolpyruvate: phosphotransferase system (PEP:PTS) system, six were associated with the acid tolerance mechanism of bacteria, and nine were stress-responsive genes (Fig. 4). Furthermore, genes encoding for proteins involved in cellular processes, such as signal transduction, biosynthetic process, transcription, translation and energy metabolism were also affected by BisHPPP (Supplemental File 1). Attention was focused on investigating a subset of genes belonging to these diverse functional classes, all of which encode products that are important to *S. mutans*-induced caries formation.

3.4 *S. mutans* Global Protein Synthesis in Response to BisHPPP

Using high-throughput quantitative proteomics profiling, a total number of 314 proteins were identified (Supplemental File 3), of which 137 proteins were differentially expressed in *S. mutans* biofilms grown in the presence of different BisHPPP concentrations (0.01, 0.1 and 1.0 mM), among which 90 proteins were more abundant and 47 proteins were less abundant compared to the no-BisHPPP control. Among the more abundant proteins, six were involved in biofilm formation, eight were stress-response proteins (including acid stress-response), and 19 were related to metabolic processes, as well as the glycolysis and the phosphotransferase systems (Fig. 5). The distribution of proteins identified in gene ontology (GO) categories for the specific biological processes showed that the majority of proteins were associated with important functions such as energy metabolism, amino acid biosynthesis, transcription, translation, transport and binding (Fig. 5). The analysis was performed via proteomics website [http://www.uniprot.org](http://www.uniprot.org). Exposure of *S. mutans* UA159 to different concentrations of BisHPPP resulted in altered (P<0.05) abundance of several proteins including proteins involved in biofilm formation (*GtfB*, *GtfC*, *GbpB*, *ComD* and *ComE*) (Table 3), proteins implicated in carbohydrate transport via the
Phosphoenolpyruvate: phosphotransferase system (PEP:PTS), including EI and mannose-specific EII B (Table 4), proteins associated with acid-tolerance, which are different subunits of the F1F0-ATPase proton translocating pump (AtpH, subunit C, AtpA, subunit α, and AtpD, subunit β) as well as two other proteins, fabG and Yidc2, involved in fatty acid biosynthesis and transport (Table 5), and stress response proteins (DnaK, GroL, RecA and ClpX) (Table 6). Other differentially expressed proteins by BisHPPP including sixteen proteins involved in metabolic and glycolysis processes such as pyruvate kinase glycolytic enzyme, along with five proteins involved in transcription, and five proteins involved in RNA binding and processing, twenty-two proteins involved in translation, nineteen proteins involved in amino acid biosynthesis, four proteins involved in transport, and several other uncharacterized or not assigned categories are provided in Tables 1–7 of the Supplemental File 2.

3.5 Effects of BisHPPP on GTF Enzyme Activity

Assays for GTF enzyme activity showed that the total amount of insoluble glucan synthesized by S. mutans UA159 cell-associated GTF grown in the absence or presence of different concentrations of BisHPPP (0.01, 0.1 and 1.0 mM) was significantly higher (P<0.05) for cells grown in the presence of 0.1 and 1.0 mM BisHPPP vs. no BisHPPP (Fig. 6).

3.6 Correlation of Microarray and Proteomics Findings

Correlation analysis between the microarray and proteomics data indicated that there was a significant overall correlation between gene expression and protein abundance regardless of the concentrations P<0.05, (Table 5). Thirty eight genes showed the highest protein-gene correlation including gtfB, gtfC, atpH, atpA, atpD, ClpX, DnaK and RecA (Fig. 7). There was no significant correlation between the individual concentrations of BisHPPP and the control P>0.05 (Table 7).

4. DISCUSSION

The findings of the present study demonstrated that the gene expression effects related to BisHPPP exposure to S. mutans extends far beyond of those of gtfB and yfiV, which the authors had reported on in previous work [19, 20], especially when the expression of all key virulence genes, gtfB, gtfC, gbpB, comCDE and atpH, was pronounced in biofilms at cariogenic pH (5.5) in yet another S. mutans strain, UA159.

The results of the gene knock-out and complementation study confirmed that comCDE quorum-sensing signaling pathway in S. mutans could be a potential molecular pathway involved in BisHPPP mediated S. mutans gene regulation. Considering the involvement of comCDE system in the regulation of the tested genes in this study [31, 32], this finding suggests a role for this system in the formation of secondary caries at the resin-dentin interface and could potentially facilitate the development of a new generation of resin composites that can modulate this signaling pathway to control S. mutans cariogenicity.

In addition, the microarray and proteomics results suggested that BisHPPP can induce a global response in S. mutans UA159 gene and protein expression by affecting multiple
phenotypes including biofilm formation, acid tolerance, optimization/modulation of the PTS system, and stress-response pathways that could affect its survival, persistence and pathogenicity in the oral environment. The findings also showed that these pathways were differentially affected by changes in BisHPPP concentrations (0.01, 0.1 and 1.0 mM).

Another important finding of this study is the stimulatory effect of different concentrations (0.1 and 1.0 mM) of this BBP on GTF enzyme activity in *S. mutans*. The sharp increase in GTF enzyme activity by BisHPPP, a BBP from the universal ingredient of dental adhesives, BisGMA, is of critical importance since this enzyme activity results in increased production of insoluble and sticky glucan polymers that facilitate *S. mutans* adhesion to the tooth surface thereby promoting the formation of cariogenic biofilms [44–46] around the composite restoration-tooth margins. The findings of the current work suggest that BisHPPP can potentially contribute in reduced resin composites restorations longevity by increasing *S. mutans* cariogenic potential via activating specific subsets of genes, leading to the synthesis of their corresponding proteins that could affect the bacteria’s cariogenicity when exposed to the BBP from resin composite and adhesives. This implies that the dental clinical and research community must initiate activities to establish new resin systems that are either more biostable or do not generate BBPs that promote bacterial biofilm activity.

The ability of *S. mutans* to initiate dental caries depends on several significant virulence traits, including: (1) initiation of biofilm formation by adherence and accumulation on the tooth surface that is promoted by the synthesis of insoluble extracellular polysaccharides, (2) high efficiency in metabolizing dietary carbohydrates and producing lactic acid, (3) the ability to grow, survive and tolerate low pH environment [47–50]. *S. mutans* possesses GTF enzymes, GtfB and GtfC that are responsible for producing water-insoluble glucan polymers that in concert with specific glucan binding proteins (GBPs) play key roles in adhesion and accumulation of biofilms [29, 51–53]. The qRT-PCR results demonstrated that BisHPPP up-regulated genes that are involved in biofilm formation including *gtfB, gtfC, gbpB, comC* and *comD* more significantly in biofilms and only at acidic pH (5.5), which are both optimum conditions for development of dental caries. Similarly, the microarray and proteomics findings demonstrated the enhanced expression of the same biofilm-related genes and proteins in the presence of BisHPPP in *S. mutans* biofilms at cariogenic pH (5.5).

The BisHPPP concentrations used in this study (0.01-1.0 mM) corresponds to levels of BBPs found in previously reported in *in vivo* studies [36]. The importance of these concentrations is accentuated by the existing knowledge within the field, as it has been suggested by investigators that the *in vivo* concentrations of BBPs could be even higher than those that were applied in the *in vitro* studies. This is because dental plaque behaves like a diffusion barrier that may increase concentrations in the local microenvironment [6]. BBPs concentration *in vivo* could also be elevated in the space occupied within the restoration-tooth interface, restricting their ability to diffuse [17].

The findings also confirmed the pH dependence effects of BisHPPP on the up-regulation of *S. mutans* virulence genes and more specifically that this was most pronounced at acidic pH *vs* neutral pH. This could be related to unique interactions of bacterial cell membrane fatty acid molecules, FabG and Yidc2, involved in fatty acid biosynthesis, with BisHPPP under
acidic conditions, as these molecules can affect membrane permeability [54, 55], thereby resulting in easier penetration of stressors, such as BisHPPP from the environment. On the other hand, since BisHPPP exhibits properties of a weak acid (pKₐ of 14.6), its undissociated form in acidic pH makes it more likely to penetrate the bacterial membrane when compared to neutral pH [56].

The more significant effect of BisHPPP on the expression of the virulence-associated genes in biofilms vs. planktonic cells could be related to the fact that during biofilm growth bacterial cells are in close proximity to each other, and therefore less exposure time is needed for the stress compounds to induce competence stimulating peptide (CSP) production, and hence less time is required for CSP to reach a critical concentration to activate the quorum-sensing pathway. The comDE two-component signal transduction system (TCSTS) is activated by CSP, which is encoded by comC and together they form a quorum-sensing system in the bacterium [32]. The comCDE TCSTS system plays important roles in regulation of genetic transformation, biofilm formation, acidogenicity, aciduricity, cell viability and bacteriocin production in response to environmental cues in *S. mutans* [30, 57]. The implication of this system are even more compelling when the proteomic results are examined. The significant up-regulation of regulatory gene products, ComD and ComE, which have positive regulatory effects on the expression of *S. mutans* virulence-associated genes, including *gtfB*, *gtfC* and *gbpB* [31, 32] is important and is validated by the observation that GtfB, GtfC and GbpB were up-regulated in the presence of BisHPPP. It should be noted, the high level of biofilm-associated proteins GtfB, GtfC and GbpB, and regulatory proteins, ComCDE, are in agreement with the microarray and qRT-PCR results for the respective genes. Although the fold changes in protein synthesis and gene expression were not at the same magnitude in all cases, the trend was conserved. These differences could be due to several factors including sensitivity of the analytical methods, post-translational modifications of proteins or the status of mRNA stability and degradation [33].

Access to adequate nutrients is a key factor for bacterial survival in oral biofilms. Phosphoenolpyruvate: phosphotransferase system (PEP:PTS) is the major carbohydrate transport system in oral streptococci, especially under carbohydrate limiting conditions [58]. The PEP:PTS system also has a global regulatory function in *S. mutans* and its components influence many other cellular processes such as biofilm development, regulation of acid tolerance and global control of gene expression [59, 60]. The PEP:PTS consists of two proteins that are common to all PTS substrates, Enzyme I (EI) and Hpr, the heat stable phosphocarrier protein, as well as a sugar-specific permease known as enzyme II (EII) [61]. In *S. mutans*, EII can regulate the activity of other sugar-specific enzymes as well as the expression of important virulence genes *gtfB*, *gtfC* and *fit* [59]. The results of proteomics analysis revealed the up-regulation of both EI and EII components (mannose-specific EIIB), encoded by *ptsI* and *SMU1960c* respectively, by BisHPPP. These data indicate contribution of this BBP to the important ability of *S. mutans* to scavenge sugars at low extracellular concentrations. In addition, there were 17 proteins involved in metabolic and glycolysis processes that were up-regulated by BisHPPP. These findings are in agreement with the microarray data, and collectively suggesting that BisHPPP affected *S. mutans* physiology and optimized its metabolism to adapt and survive within the cariogenic biofilm. In the oral environment, *S. mutans* is subjected to rapid pH fluctuations due to periods of variable
availability of carbohydrates. In order to withstand these continual cycles of acid shock, this bacterium has evolved different mechanisms including constitutive and inducible mechanisms such as the acid tolerance response (ATR) [62]. In *S. mutans*, the membrane-bound F1F0-ATPase is the primary mechanism of proton extrusion to maintain pH homeostasis [63]. This is achieved by up-regulation of F1F0-ATPase that extrudes H+ as the external environment becomes more acidic. The proteomics results demonstrated that AtpH, a subunit C of the F1F0-ATPase proton translocating pump, was up-regulated by BisHPPP at 0.01 and 1.0 mM concentrations. Similarly, AtpA and AtpD representing subunits α and β respectively, were both up-regulated by BisHPPP at 0.1 mM, however both of the subunits were down-regulated at 0.01 mM BisHPPP. This degree of BisHPPP sensitivity could be attributed to the heterogeneity of biofilm cells, which might have caused the paradoxical results with different concentrations of BisHPPP in some cases [42]. The higher abundance of AtpH is in agreement with the gene expression analysis using both microarray and qRT-PCR.

There were many other examples in the data which showed how BisHPPP affected bacterial cell function. Two proteins of particular interest, FabG and Yidc2, involved in fatty acid biosynthesis were up-regulated in the presence of BisHPPP. Studies showed that a shift in lipid composition could affect the permeability of the cell to protons by altering the F1F0-ATPase activity [54, 55]. Therefore, proteins encoded by the fatty acid biosynthetic gene cluster such as fabG and yidc2 that are induced by BisHPPP are involved in *S. mutans* acid tolerance. Based on these results, dental composite/adhesive degradation product, BisHPPP could contribute to *S. mutans* ability to enhance acid adaptation and survival in cariogenic biofilm.

In order to survive environmental changes, bacteria synthesize stress responsive proteins including chaperones and proteases to prevent the accumulation of abnormal proteins [64–67]. *S. mutans* uses stress-response pathways to respond to environmental fluctuations with modulation of its virulence in a way that ensures persistence in the oral cavity that can lead to dental caries [68]. The up-regulation of four stress response proteins, DnaK, GroL, RecA and ClpX, by different concentrations of BisHPPP is a strong indication of the effect of BisHPPP on *S. mutans* adaptation. Dnak and GroL affect the expression of key virulence traits in *S. mutans*, including biofilm formation and acid tolerance [69]. Dnak and GroL also regulate signal transduction pathways by controlling the stability and activities of transcriptional regulators and protein kinases [70, 71]. The recombinase A (RecA) protein is required for homologous recombination of GtfB and GtfC in *S. mutans* [72]. Augmented production of molecular chaperones ensures the quality of proteins being expressed, and enables *S. mutans* to thrive in a cariogenic biofilm. Proteins that cannot be folded by molecular chaperones are targeted for degradation. CLP family proteins have dual chaperone/protease roles [73]. Proteolysis by CLP proteins can regulate cellular events by degrading regulatory proteins and they have been consistently implicated in virulence [74]. These findings are also in agreement with the microarray data that demonstrated the up-regulation of nine stress-response proteins by 1.0 mM BisHPPP including, Dnak, GroL, RecA and ClpX, which suggest that *S. mutans* orchestrates the expression of gene products from distinct stress responsive pathways in response to BisHPPP within cariogenic biofilms for its survival and persistence. There was a good general correlation between microarray
and proteomics data, however a direct correlation/dose response between each concentration and control could not be found. The absence of correlation for the individual concentrations of BisHPPP can be explained by the fact that the correlation of mRNA and protein levels in complex samples is notoriously poor and far from perfect [75–78] since there are so many mechanistic steps within the cell machinery that can divert the cell’s ultimate protein synthesis and release. Several parameters that contribute to this poor correlation include mRNA-protein interactions, RNA secondary structure, regulatory proteins, regulatory sRNA, codon bias and codon adaptation index, ribosomal density and ribosomal occupancy, protein half-lives and other biological factors such as untranscribed RNA species, and secreted proteins that have escaped identification by mass spectrometry [76]. Furthermore, many proteins are regulated post-transcriptionally, with no significant corresponding change in mRNA abundance [77]. More than half of the discordance observed between mRNA and protein abundance might be attributed to post-transcriptional regulatory mechanisms [79, 80]. In the current study, the magnitude of gene expression might not match with the expected change in abundance of the respective protein, but the trend was still the same for both data sets. A particularly novel and critically important finding was the association of GTF enzyme activity with the total amount of insoluble glucan synthesized by S. mutans UA159 grown at different concentrations of BisHPPP. Glucan production was significantly higher (P<0.05) for cells grown in the presence of 0.1 and 1.0 mM BisHPPP compared to the control. The elevated amounts of glucan production suggests that the influence of BisHPPP is not limited to the transcriptional and translational levels of gtfB and gtfC in S. mutans, but that this BBP can also affect the activity of the synthesized biomolecules, which results in production of more glucan polymer, a well-known virulence factor in S. mutans [44–46].

S. mutans is a genetically-diverse species that co-exists in the oral cavity with a number of other streptococci and hundreds of additional species of bacteria from a wide range of taxa [81, 82]. In addition, S. mutans natural transformability contributes to its genetic diversity, which may have enhanced the ability of S. mutans to adapt and survive fluctuations in a hostile oral environment [83, 84]. A number of studies demonstrated the genotypic and phenotypic diversity in S. mutans [85–89]. It has been shown that biofilm-forming capacity, stress tolerance, autolysis, and competence development varies widely among genetically diverse S. mutans strains [89]. Western blot analysis revealed differences between 15 different S. mutans strains that expressed different amounts of both gtfB and gtfC, which affected the ability to form biofilm [89]. Also, a high level of diversity of gtf genes, levels of enzymatic activity, and isozyme production was identified in 44 genotypes of S. mutans [88]. These studies indicate that the genetic response of S. mutans to environmental stresses could vary among different strains. This can explain the difference between the response patterns of gtfB expression for S. mutans UA159 used in the current study, and the previous reports for S. mutans NG8 [19, 20].

Although this simplified in vitro study cannot reproduce all of the complexity of the in vivo condition, it suggests a possible mechanistic pathway that may be involved in the increased incidence of secondary caries activity, where synthetic biomaterials are present within the in vivo oral environment, and that has been consistently noted in multiple clinical studies [1, 3, 4, 9]. In addition, this in vitro biofilm model can provide the opportunity to examine direct
effect(s) of specific molecules such as BisHPPP and other resin composite components on bacterial cariogenic potential independent of other complex interactions in vivo. Additional studies are warranted to determine the influence of the resin composites and adhesive degradation products on the pathogenic potential of cariogenic oral bacteria in a multispecies biofilm under both in vitro and in vivo conditions.

5. CONCLUSIONS

The present study revealed a complex interplay between S. mutans UA159 virulence-associated genes and proteins including those involved in biofilm formation, acid tolerance, carbohydrate transport and stress response, which are particularly induced when S. mutans encounters environmental cues such as BBPs. Augmented expression of the above-mentioned genes/proteins in response to BisHPPP is an adaptation response by S. mutans that can affect its pathogenicity at the resin-dentin interface, where the BBP is released, providing a mechanistic contribution to the field of secondary caries, a frequent reason for resin composite restoration failure [3]. Understanding the genetic and physiological processes underlying recurrent caries can lead to innovative therapeutic strategies and improved materials for use at tissue interfaces within the oral cavity. This represent a significant contribution to the field of restorative dental materials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Heliya Ziai, Kirsten Krastel, Eduardo B. Moffa, Yizhi Xiao for their technical assistance. This investigation was funded by National Institute of Dental & Craniofacial Research R01DE021385; Canadian Institute of Health Research MOP115113, # 97577 and Canada Foundation for Innovation grant #25116. This is an independent study free of conflict of interest.

References


Highlights

- A BisGMA-derived degradation product affects gene expression of cariogenic bacteria
- Resin composite degradation product affects gene expression of cariogenic bacteria
- A BisGMA-derived biodegradation product affects virulence of *S. mutans*
- Resin composite biodegradation product affects virulence of *S. mutans*
- A BisGMA-derived biodegradation product regulates biofilms-related proteins/ enzymes
Figure 1.
Relative expression of the S. mutans virulence genes: gtfB, gtfC, gbpB, comC, comD, comE and atpH for planktonic and biofilm growth conditions with different concentrations of BisHPPP (0.001, 0.01 and 0.1 mM) at pH 5.5 relative to the no BisHPPP control. * represents significant difference between individual BisHPPP concentration compared to the control (no BisHPPP) in either growth mode (P<0.05). # represents significant difference between biofilm and planktonic cultures at the same BisHPPP concentration (P<0.001). Data are plotted with standard error of the mean (±SE), n = 4.
Figure 2.
Relative expression of the *S. mutans* virulence genes: *gtfB, gtfC, gbpB, comC, comD, comE* and *atpH* for planktonic and biofilm growth conditions with different concentrations of BisHPPP (0.001, 0.01 and 0.1 mM) at pH 7.0 relative to the no BisHPPP control. * represents significant difference between individual BisHPPP concentration compared to the control (no BisHPPP) in either growth mode (P<0.05). # represents significant difference between biofilm and planktonic cultures at the same BisHPPP concentration (P<0.001). Data are plotted with standard error of the mean (±SE), n = 4.
Figure 3.
Relative expression of *gtfB*, *comD* and *atpH* in wild-type, knock-out (SMΔcomC1) and complemented (SMΔcomC1C) *comC* strains of *S. mutans* UA159 in the presence of different concentrations of BisHPPP (0.01 and 0.1 mM) at pH 5.5. One-way analysis of variance (ANOVA) and Tukey post hoc analyses were performed to determine the differences in gene expression between individual BisHPPP concentration and the no-BisHPPP control (P<0.05). Expression of the related genes in complemented strain was similar to that of wild-type. Data are plotted with standard error of the mean (±SE), n = 4.
Figure 4.
Number of genes in functional categories differentially expressed in the presence of BisHPPP. A total number of 115 genes showed altered expression after exposure of S. mutans UA159 biofilms to 1.0 mM BisHPPP of which 62 genes were up-regulated and 53 genes were down-regulated.
Distribution of *S. mutans* UA159 proteins into specific gene ontology (GO) of biological processes after exposure to different concentrations of BisHPPP (0.01, 0.1 and 1.0 mM). A total number of 137 proteins were differentially expressed in *S. mutans* biofilm grown in the presence of different BisHPPP concentrations, among which 90 proteins were more abundant and 47 proteins were less abundant compared to the no-BisHPPP control.
Effects of different concentrations of BisHPPP (0.01, 0.1 and 1.0 mM) on *S. mutans* UA159 glucosyltransferase activity. The total amount of insoluble glucan synthesized by biofilm cells grown in the presence of 0.1 and 1 mM BisHPPP was significantly increased compared to the no-BisHPPP control (P<0.05). Data are plotted with standard error of the mean (±SE), n = 4.
Figure 7.
### Table 1

Primers used for construction of comC knock-out and complemented strains

<table>
<thead>
<tr>
<th>Primer use and name</th>
<th>Nucleotide Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primers for comC deletion:</strong></td>
<td></td>
</tr>
<tr>
<td>comC-P1</td>
<td>TACAAAGCAAATCTGAACAG</td>
</tr>
<tr>
<td>comC-P2</td>
<td>GCCGCCTGATAATCCAATCCATC</td>
</tr>
<tr>
<td>comC-P3</td>
<td>GCAGCAAGGAACGCTATCAAC</td>
</tr>
<tr>
<td>comC-P4</td>
<td>AACGATGCTGTAAGG</td>
</tr>
<tr>
<td>Erm cst-F</td>
<td>GC^CGCCCCGGCAGAATAATCTTTGAT</td>
</tr>
<tr>
<td>Erm cst-B</td>
<td>GCCGG^CCAGTGGCAAGCATAGAAT</td>
</tr>
<tr>
<td><strong>Primers for comC complementation:</strong></td>
<td></td>
</tr>
<tr>
<td>comC F(ApaI)</td>
<td>GGGGCCCCACTGCTGCAATTCGA</td>
</tr>
<tr>
<td>comC R(ClaI)</td>
<td>GATCGATGAGGAGCCTATCTTAAG</td>
</tr>
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</table>
Table 2

Nucleotide sequence of primers used for qRT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
<th>Primer Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Forward</td>
</tr>
<tr>
<td>gtfB</td>
<td>GTF I, glucan production</td>
<td>ACACTTTGCAGGTTGCTTG</td>
</tr>
<tr>
<td>gtfC</td>
<td>GTF II, glucan production</td>
<td>CCAAAATGGATTTATGCTTGCTG</td>
</tr>
<tr>
<td>gbpB</td>
<td>Glucan binding protein</td>
<td>AGCAACAGAAGCAACACCAGCATG</td>
</tr>
<tr>
<td>comC</td>
<td>Competence-stimulating peptide</td>
<td>GACTTTGAAGAAATTAGACTG</td>
</tr>
<tr>
<td>comD</td>
<td>Two-component regulatory system</td>
<td>CTCGTGATGACCCATTCTTCTG</td>
</tr>
<tr>
<td>comE</td>
<td>Two-component regulatory system</td>
<td>CCTGAAAAAGGCAATCACCAG</td>
</tr>
<tr>
<td>atpH</td>
<td>Acid tolerance</td>
<td>ACCATAAACCTTCCAGCTG</td>
</tr>
<tr>
<td>16S rRNA</td>
<td>Normalizing internal standard</td>
<td>CTTACCAGGTCTTGACATCCCG</td>
</tr>
</tbody>
</table>
### Table 3

Biofilm-related proteins from *S. mutans* UA159 grown in the presence of BisHPPP at pH 5.5

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Protein name</th>
<th>Protein function</th>
<th>Ratio 1mM/0mM</th>
<th>P</th>
<th>Ratio 0.1mM/0mM</th>
<th>P</th>
<th>Ratio 0.01mM/0mM</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>gtfB</em></td>
<td>GtfB</td>
<td>Glucan biosynthetic process</td>
<td>1.92*</td>
<td>0.05</td>
<td>2.55</td>
<td>0.12</td>
<td>1.26</td>
<td>0.04</td>
</tr>
<tr>
<td><em>gtfC</em></td>
<td>GtfC</td>
<td>Glucan biosynthetic process</td>
<td>2.40</td>
<td>0.10</td>
<td>3.21*</td>
<td>0.01</td>
<td>1.83*</td>
<td>0.03</td>
</tr>
<tr>
<td><em>gbpB</em></td>
<td>GbpB</td>
<td>Single-species biofilm formation on inanimate substrate</td>
<td>1.33</td>
<td>0.04</td>
<td>2.92*</td>
<td>0.01</td>
<td>0.84</td>
<td>0.03</td>
</tr>
<tr>
<td><em>comC</em></td>
<td>ComC</td>
<td>Multiorganism process, biological adhesion</td>
<td>1.12</td>
<td>0.06</td>
<td>1.00</td>
<td>0.01</td>
<td>1.33</td>
<td>0.01</td>
</tr>
<tr>
<td><em>comD</em></td>
<td>ComD</td>
<td>Single-organism process, cellular process, metabolic process</td>
<td>0.85</td>
<td>0.01</td>
<td>1.97*</td>
<td>0.03</td>
<td>1.39</td>
<td>0.01</td>
</tr>
<tr>
<td><em>comE</em></td>
<td>ComE</td>
<td>Signaling, biological regulation, single-organism process</td>
<td>1.69*</td>
<td>0.01</td>
<td>1.65</td>
<td>0.10</td>
<td>2.03*</td>
<td>0.01</td>
</tr>
</tbody>
</table>

*Represents P<0.05*
Table 4

Carbohydrate transport proteins from *S. mutans* UA159 grown in the presence of BisHPPP at pH 5.5

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Protein name</th>
<th>Protein function</th>
<th>Ratio 1mM/0mM</th>
<th>P</th>
<th>Ratio 0.1mM/0mM</th>
<th>P</th>
<th>Ratio 0.01mM/0mM</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>ptsI</td>
<td>Phosphoenolpyruvate-protein phosphotransferase</td>
<td>Phosphoenolpyruvate-dependent sugar phosphotransferase system</td>
<td>1.79*</td>
<td>0.05</td>
<td>2.66*</td>
<td>0.04</td>
<td>2.29*</td>
<td>0.03</td>
</tr>
<tr>
<td>SMU_1960c</td>
<td>PTS system protein, mannose-specific IIB</td>
<td>Phosphoenolpyruvate-dependent sugar phosphotransferase system</td>
<td>1.93*</td>
<td>0.01</td>
<td>1.53*</td>
<td>0.05</td>
<td>0.71</td>
<td>0.01</td>
</tr>
</tbody>
</table>

* Represents P<0.05.
<table>
<thead>
<tr>
<th>Gene name</th>
<th>Protein name</th>
<th>Protein function</th>
<th>Ratio 1mM/0mM</th>
<th>P</th>
<th>Ratio 0.1mM/0mM</th>
<th>P</th>
<th>Ratio 0.01mM/0mM</th>
<th>P</th>
</tr>
</thead>
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<tr>
<td><em>atpH</em></td>
<td>F1F0 membrane-bound proton-translocating ATPase c subunit</td>
<td>Hydrogen ion transmembrane transporter activity</td>
<td>2.36 *</td>
<td>0.01</td>
<td>1.24</td>
<td>0.05</td>
<td>1.78 *</td>
<td>0.02</td>
</tr>
<tr>
<td><em>atpA</em></td>
<td>ATP synthase F1, alpha subunit</td>
<td>ATP hydrolysis coupled proton transport, plasma membrane ATP synthesis coupled proton transport</td>
<td>0.98</td>
<td>0.01</td>
<td>2.26 *</td>
<td>0.01</td>
<td>0.37 *</td>
<td>0.01</td>
</tr>
<tr>
<td><em>atpD</em></td>
<td>ATP synthase F1, beta subunit</td>
<td>ATP hydrolysis coupled proton transport, plasma membrane ATP synthesis coupled proton transport</td>
<td>0.96</td>
<td>0.01</td>
<td>1.58 *</td>
<td>0.05</td>
<td>0.53 *</td>
<td>0.02</td>
</tr>
<tr>
<td><em>fabG</em></td>
<td>3-oxoacyl-[acyl-carrier-protein] reductase</td>
<td>Fatty acid biosynthetic process</td>
<td>2.19 *</td>
<td>0.01</td>
<td>2.33 *</td>
<td>0.05</td>
<td>2.33 *</td>
<td>0.01</td>
</tr>
<tr>
<td><em>yidC2</em></td>
<td>Membrane protein insertase YidC 2</td>
<td>Protein insertion into membrane, protein transport</td>
<td>1.80 *</td>
<td>0.01</td>
<td>2.04 *</td>
<td>0.01</td>
<td>1.69 *</td>
<td>0.03</td>
</tr>
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</table>

* Represents P<0.05
Table 6

Stress-response proteins from *S. mutans* UA159 grown in the presence of BisHPPP at pH 5.5

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Protein name</th>
<th>Protein function</th>
<th>Ratio 1mM/0mM</th>
<th>P</th>
<th>Ratio 0.1mM/0mM</th>
<th>P</th>
<th>Ratio 0.01mM/0mM</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>DnaK</td>
<td>Chaperone protein DnaK</td>
<td>Protein folding, response to stress</td>
<td>2.02*</td>
<td>0.01</td>
<td>0.22</td>
<td>0.10</td>
<td>1.01</td>
<td>0.04</td>
</tr>
<tr>
<td>groL</td>
<td>60 kDa chaperonin</td>
<td>Protein refolding</td>
<td>1.47</td>
<td>0.05</td>
<td>2.61*</td>
<td>0.01</td>
<td>1.70*</td>
<td>0.03</td>
</tr>
<tr>
<td>recA</td>
<td>Protein RecA</td>
<td>DNA recombination, DNA repair, SOS response</td>
<td>3.14*</td>
<td>0.02</td>
<td>2.02</td>
<td>0.06</td>
<td>0.96</td>
<td>0.01</td>
</tr>
<tr>
<td>clpX</td>
<td>ATP-dependent Clp protease, ATP-binding subunit</td>
<td>Protein folding</td>
<td>1.80*</td>
<td>0.01</td>
<td>2.17*</td>
<td>0.01</td>
<td>0.69</td>
<td>0.03</td>
</tr>
</tbody>
</table>

*Represents P<0.05
### Table 7

Estimated correlation between gene expression fold change and protein fold changes by BisHPPP concentrations

<table>
<thead>
<tr>
<th></th>
<th>Pearson Correlation</th>
<th>Spearman Correlation</th>
<th>P-value (Pearson)</th>
<th>P-Value (Spearman)</th>
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<tbody>
<tr>
<td>0.01 vs ref</td>
<td>0.03</td>
<td>0.11</td>
<td>0.84</td>
<td>0.50</td>
</tr>
<tr>
<td>0.1 vs ref</td>
<td>0.05</td>
<td>0.09</td>
<td>0.70</td>
<td>0.66</td>
</tr>
<tr>
<td>1 vs ref</td>
<td>0.26</td>
<td>0.21</td>
<td>0.78</td>
<td>0.52</td>
</tr>
<tr>
<td>all</td>
<td>0.20</td>
<td>0.17</td>
<td><strong>0.03</strong>&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0.08</td>
</tr>
</tbody>
</table>

* Represents P<0.05