Published in final edited form as:

Mol Oral Microbiol. 2018 October ; 33(5): 353–363. doi:10.1111/omi.12230.

Deficiency of BrpA in *Streptococcus mutans* Reduces Virulence in Rat Caries Model

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Abstract

Our recent studies have shown that BrpA in *S. mutans* plays a critical role in cell envelope biogenesis, stress response and biofilm formation. In this study, a 10-species consortium was used to assess how BrpA-deficiency influences *S. mutans*' establishment, persistence and competitiveness during growth in a community under conditions typical of the oral cavity. Results showed that like the wild-type, the *brpA* mutant was able to colonize and establish on the surfaces tested. Relative to the wild-type, however, the *brpA* mutant had a reduced ability to persist and grow in the 10-species consortium ($P < 0.001$). A rat caries model was also used to examine the effect of BrpA, as well as Psr, a BrpA paralogue, on *S. mutans* cariogenicity. The results showed no major differences in infectivity between the wild-type and the *brpA* and *psr* mutants. Unlike the wild-type, however, infection with the *brpA* mutant, but not the *psr* mutant, showed no significant differences in both total numbers of caries and caries severity, compared to the control group that received bacterial growth medium ($P > 0.05$). Metagenomic and qPCR analysis showed that *S. mutans* infection caused major alterations in the composition of the rats' plaque flora and that significantly less *S. mutans* was identified in the rats infected with the *brpA* mutant, as compared to those infected with the wild-type and the *psr* mutant. These results further suggest that BrpA

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Conflicts of interest

The authors declare no conflict of interest.

plays a critical role in *S. mutans* pathophysiology and that BrpA can be a potential target in modulation of *S. mutans* virulence.

Keywords

Streptococcus mutans; BrpA; Psr; dental caries; rat caries model

Introduction

Human dental plaque consists of microbial communities of hundreds of different species / phylogenetic groups in high density ^{1,2}. It is believed that different species in the communities of the plaque flora exist in homeostasis with each other and with the host and that balanced plaque flora is generally associated with oral health. However, host immune status, antibiotic therapy, the quantity and quality of saliva, and the conditions in the dynamic oral cavity, such as pH, oxygen tension, and sugar nature and availability can have major effects on the composition and stability of the plaque communities, and ultimately the pathogenicity of the plaque ^{1,3}. Under conditions like continuous consumption of a high sucrose-containing diet, or individuals with poor oral hygiene or saliva deficiency such as Sjögren's syndrome, the proportion of acidogenic and aciduric species including *Streptococcus mutans*, increase significantly in the plaque community, leading to the development of carious lesions. Dental caries is one of the most prevalent infectious diseases and still a major health burden worldwide ^{4,5}.

A major etiologic agent of human dental caries, *S. mutans* is well-documented for its abilities to colonize and persist on the tooth surfaces, to utilize various sugars at micro-concentrations, and to survive low pH and other adverse conditions ^{6,7}. It possesses at least three glucosyltransferases (GTFs) that utilize sucrose as a substrate generating glucose polymers including α -1, 3-linked, water-insoluble adhesive glucans ⁸. These GTFs and their adhesive products play a central role in cariogenicity of this bacterium. When grown in the mixed-species consortium *in vitro*, *S. mutans* can also facilitate several other bacteria, including cariogenic lactobacilli, to colonize and form biofilms on various surfaces tested ^{9,10}. It also produces mutacins that have shown a wide-spectrum of antimicrobial activity against other oral bacteria ¹¹. However, it awaits further investigation how the organism interacts with other species in the plaque community and influences the composition, stability and ultimately, the pathogenicity of the plaque.

The cell envelope plays an essential role in bacterial pathophysiology, including maintenance of structural integrity and characteristic cell shape. The cell envelope of bacteria is also directly involved in bacterial cell-environment, cell-surface and cell-cell interactions, and thus, influences bacterial adherence and biofilm formation. The Gram-positive bacteria also have a thick and highly complex layer of peptidoglycan and attached anionic cell wall polymers, including teichoic acids and capsular polysaccharides ^{12,13}. Wide-spread in Gram-positive bacteria, the LytR-CpsA-Psr (LCP) family of proteins is shown in *B. subtilis* and several other species to function as a ligase, promoting attachment of anionic cell wall polymers to the peptidoglycan, and thus influencing bacterial

pathophysiology, although differences in both structure and functionality exist between different species^{14–19}.

S. mutans possesses two LCP paralogues BrpA and Psr^{14,15,20–22}. Our recent studies have shown that some redundancy in functionality exists between these two LCP paralogues, but BrpA appears to be the predominant player in *S. mutans* pathophysiology and at least one functional BrpA or Psr is required for viability¹⁵. Deficiency of BrpA drastically weakens the ability of the mutant strain to survive low pH, oxidative challenge, and cell envelope stressors¹⁵. *In vitro*, the BrpA-deficient mutant can still bind and establish on a surface, but its ability to accumulate and develop into mature biofilm decreases drastically^{20–23}. In addition, BrpA-deficiency also alters the expression of large groups of genes, including genes known to be involved in cell envelope homeostasis, stress tolerance response and biofilm formation^{15,22,23}. Like BrpA, Psr-deficiency also significantly reduces biofilm formation and weakens the acid tolerance response. However, unlike BrpA, Psr-deficiency does not have any significant effects on cell morphology, oxidative stress tolerance and cell envelope stress response¹⁵. Psr-deficiency also affects expression of genes studied, including those for the glucosyltransferases, although both the scope and the effects differ from the *brpA* mutants¹⁵.

In this study, a 10-species consortium representing oral health and disease was grown under continuous flow conditions in a constant depth film fermenter (CDFF)^{24–26} to further assess the impact of BrpA-deficiency on *S. mutans*' ability to colonize, persist and compete in a community. A rat caries model was also used to examine how BrpA, as well as Psr, affects the cariogenicity of this major cariogenic bacterium. Results have shown that relative to the parent strain, UA159, mutants deficient of BrpA displayed a significant reduction in population when grown in the mixed-species community, suggesting effects of such a deficiency on the ability of the *brpA* mutant to colonize and accumulate in a community. Unlike the wild-type, rats infected with the *brpA* mutant showed no significant differences in both the number and severity of caries, when compared to the uninfected negative control, indicative of reduction in cariogenicity as a result of BrpA-deficiency. However, no such effect was observed with the Psr-deficient mutant.

Materials and Methods

Bacterial strains and growth conditions

All bacterial strains used in this study are listed in Table 1. *S. mutans*, *Streptococcus oralis*, *Streptococcus sanguinis*, *Lactobacillus casei*, *Actinomyces naeslundii*, and *Neisseria subflava* were maintained using Brain Heart Infusion (BHI, Becton, Dickinson and Company, MD). *Veillonella dispar*, *Prevotella nigrescens*, *Fusobacterium nucleatum*, and *Porphyromonas gingivalis* were maintained in Cooked Meat medium (BD Diagnostics, Franklin Lakes, NJ). All solid media were prepared similarly with inclusion of Bacto agar (Difco Laboratories) at the level of 1.5% (w/v). Unless otherwise stated, all cultivations were carried out at 37°C with the anaerobes *V. dispar*, *P. nigrescens*, *F. nucleatum*, and *P. gingivalis* in an anaerobic box (BD Diagnostic), while all others in an aerobic chamber with or without (*N. subflava* only) inclusion of 5% CO₂^{25,27}. When needed, erythromycin (Erm,

Sigma) (10 µg/mL), spectinomycin (Spc, Sigma) (1 mg/mL), and /or kanamycin (Kan, Sigma) (1 mg/mL) were added to the growth medium.

Mixed-species biofilm analysis

Mixed-species biofilms were grown using CDFF, as described previously^{24–26,28}. A mucin-based medium (BMM), containing (grams per liter) porcine gastric mucin (Sigma) (2.5), proteose peptone (2.0), potassium chloride (2.5), yeast extract (1.0), trypticase peptone (1.0), cysteine hydrochloride (0.1), and hemin (0.001) was used to support growth^{25,26,28}. A group of 10 different oral bacteria (Table 1), representing oral health and disease, were used in this study. They were grown individually, and late-exponential phase cultures (with colony-forming-units estimated at 5×10^8 per mL), 10 ml each, were then mixed proportionally (1:1 ratio to each other) and gently with *S. mutans* UA159 and TW14D, respectively. The mixed cultures were then pumped into the system at a flow rate of 0.352 ml/minute over a period of 4 hours. The medium flow was run at 0.542 ml/minute, which resembles the rate of un-stimulated saliva flow. Biofilms were allowed to grow on the polytetrafluoroethylene (PTFE) plugs accessed at a depth of 300 µm in the sample pans. The whole system was maintained in a 37°C incubator supplied with filtered N₂ containing 5% CO₂. Samples were taken from the inocula for microbiological analysis and then from CDFF at 4, 24, 48, 120 and 240-hour post inoculation. For each sample, brief sonication was used to disperse the biofilm⁹, and then serial dilutions were plated in triplicate on a range of selective and nonselective agar media as described previously^{25,28} (Table S1). For characterization of biofilm structure, *S. mutans* wild-type, UA159, and the *brpA* mutant, TW14D, were tagged with green fluorescent protein (GFP). A promoterless *gfp* gene was fused with the promoter of *S. mutans* lactate dehydrogenase gene^{29,30}, and the resulting *gfp* reporter construct was integrated at the glucosyltransferase A (*gftA*) locus in the UA159 and TW14D chromosome by double crossover homologous recombination (Wen et al., unpublished data). Hydroxylapatite (HA) discs that were deposited on the top of the sample pan were used to support biofilm growth^{9,26}. By the end of each time point, biofilms developed on HA discs were treated with SYTO 59 (Invitrogen), which confers red fluorescence to all populations, and analyzed using a confocal laser scanning microscope (Olympus)^{15,31}.

Membrane fatty acid determination

The membrane fatty acid content of the cultures was determined using Gas Chromatographic analysis by MIDI Labs, Inc. (Newark, DE)^{32,33}. Briefly, 100 ml of BHI grown cultures of *S. mutans* UA159, TW14D, TW251, and TW314, which has *psr* deleted and *brpA* expressed at a reduced level¹⁵, were harvested at mid-exponential phase by centrifugation. The cell pellets were washed twice with sterile H₂O, and stored at –80°C. The bacterial samples were first saponified with 1 ml of 1.2 M NaOH in 50% methanol, and fatty acids were methylated in 2 ml of 3.52 N HCl in 45% methanol and then extracted with 1.25 ml of hexane and methyl tert-butyl ether mixture (1: 1, v/v). Following proper washes, the extract containing fatty acid methyl esters (FAMES) were injected into a GC system series II gas chromatograph equipped with a split inlet and flame-ionization detector, automatic sampler 6890 and fused-silica capillary column (Ultra 2, HP 19091 B-102; 25 m×0.2 mm with 5.0 % cross-linked phenyl methyl silicone) as the stationary phase. FAME peak identification and

quantification, FAME profile comparison and data analysis were performed using the system software (part 1300) of the Sherlock software package of MIDI (version 1.06). Two independent cultures for each strain were analyzed.

Cell wall phosphate estimation

Cell wall phosphate was estimated from murein sacculi prepared following the protocols of Chan et al.^{34,35}, with minor modifications. Briefly, a 20 mL overnight culture of each strain was harvested by centrifugation at 3,000xg for 10 minutes, the cell pellet was washed with saline, and then boiled in 4% SDS for 30 minutes. The pellet was washed five times in Tris buffer (50 mM, pH 7.5) and homogenized using a glass beads in a MiniBeadBeater (Biospec, Bartlesville, Okla.) as described previously^{27,36}. The cell debris containing murein sacculi was washed twice in Tris buffer, and then incubated in the same buffer containing 10 mM CaCl₂, 20 mM MgCl₂, 10 µg/mL DNase and 50 µg/mL RNase at 37°C for 2 h. It was followed by incubation in 1% SDS and proteinase K (20 µg/ml) at 37°C overnight. The resulting murein sacculus was boiled and treated in 1% Triton X-100 and 0.1 M EDTA. Following washes with Tris buffer, the sediment was then re-suspended in acetone, and the lipid solubilized murein sacculus was obtained by centrifugation at 10,000xg for 10 minutes, air dried to remove residual acetone, washed once in Tris buffer and re-suspended in 200 µL deionized water. For phosphate estimation, 45 µL of murein sacculus prep was mixed with 5 µL of trichloroacetic acid and incubated at 80°C overnight. The inorganic phosphate released was estimated as described by Chan et al. (33), then quantitated spectrophotometrically at 820 nm using a Synergy 2 plate reader (BioTek, Inc.). The phosphate concentration was determined against a standard curve generated using known concentrations of NaH₂PO₄.

Rat caries model

Cariogenicity of the test strains was determined using a standard protocol³⁷. Briefly, dams of Sprague-Dawley rats along with sufficient pups, aged 17 days, were purchased, and randomly divided into five groups, 10 each. At 21 days of age, the groups of pups were infected with 10⁹ bacterial cells of the *brpA* mutant, the *psr* mutant, the *brpA*-down and *psr*-deficient mutant, or MX804, an erythromycin-resistant derivative of the wild-type³⁸, UA159, as a positive control. For the uninfected control group, pups received bacterial culture medium only. All groups were handled in a blind-fashion using color-coding of bacterial samples, and cage-tagging via a third-party uninvolved with the experiment. All pups were provided with Diet-2000 (56% sucrose, w/w, with rodent nutrients), *ad libitum*, and provided with sterile water containing 5% (w/v) sucrose. By the end of five weeks, animals were sacrificed by carbon dioxide euthanasia followed by decapitation and surgical removal of mandibular hemisections. One half of the lower jaw was sonicated gently to release residing bacteria for metagenomic analysis via deep sequencing of the V4 region of 16S rDNA and qPCR. Dental caries, including the smooth surface and the sulcal caries, were scored using the scoring system for rodent caries, devised by Keyes and Fitzgerald, and as modified by Larson^{39,40}. The protocol was reviewed and approved by the IACUC at LSU Health Sciences Center in New Orleans (IACUC#3267) and by University of Rochester, Rochester, NY (UCAR# 100553 /2006-148), and was performed at the University of Rochester Center of Oral Biology according to established methods^{37,41}.

Plaque bacteria DNA extraction, 16S rDNA sequencing and data analysis

DNA were extracted from the plaque samples of the rats and then sequenced by following our established protocols^{42,43}. Briefly, using isolated DNA, 16S ribosomal DNA hypervariable region V4 was PCR amplified using primers 515F 5'-GTGCCAGCMGCCGCGGTAA-3' and 5'-805R GGACTACHVGGGTWTCTAA-3' gene-specific sequences, Illumina adaptors, and molecular barcodes to produce ~290 base pair (bp) amplicons. Samples were sequenced on an Illumina MiSeq (Illumina) using V2 sequencing kit (250 bp paired end reads). The forward and reverse read files were processed through the DADA2 v1.4 pipeline⁴⁴. The first 20bp of each read were trimmed to remove the 16S specific primers, forward reads were truncated to 210bp and reverse reads to 180bp based on read quality profiles in order to maintain highest quality data. Forward and reverse sequence variants were inferred with the DADA2 algorithm using default parameters and sequence variants were merged to form a sequence variant table. Chimeric sequence variants were removed using removeBimeraDenovo. QIIME v1.9 (open source, www.qiime.org) was used to align sequence variants and build a phylogenetic tree⁴⁵. The Ribosomal Database Project classifier was used to assign a taxonomic classification to each sequence variant. Relative abundance of each sequence variant was examined at phylum, class, order, family, genus, and species levels. Alpha- (within a community) and beta- (between communities) diversity metrics as well as taxonomic community assessments were produced using QIIME 1.9 scripts.

Quantitative polymerase chain reactions (qPCR)

qPCR analysis was used to determine the relative abundance of *S. mutans* in the plaque flora. For *S. mutans* estimation, species-specific primers for *gtfB* were used with 5'-AGCAATGCAGCCATCTACAAAT-3' for forward primer and 5'-ACGAACTTTGCCGTTATTGTCA-3' as the reverse primer. For estimation of the total plaque microbiome, the 16S rRNA universal primers were 5'-GGTTAAGTCCCGCAACGAGC-3' forward and 5'-AGGGGCATGATGATTTGACG-3' reverse. Real Time quantitative PCR was done using SYBR Green Supermix (BioRad) with a Bio-Rad iCycler using procedures detailed elsewhere^{46,47}.

Statistical Analysis

Unless specified otherwise, all quantitative data were further analyzed by either Student's *t*-test or ANOVA and Tukey's pairwise comparison. A difference of $P < 0.05$ is considered as statistically significant.

Results

BrpA-deficiency affects membrane fatty acid composition

Previously, DNA microarray analysis showed that BrpA deficiency affects the expression of genes involved in fatty acid biosynthesis, including reduced expression of SMU.1739 coding for a putative 3-oxoacyl-(acyl-carrier-protein) synthase (KAS-II, FabF) involved in the condensation step of fatty acid biosynthesis²². In this study, gas chromatography (GC) was used to analyze the FAME profile of the *brpA*-deficient mutant, TW14D, *psr*-deficient

mutant, TW251, and the *brpA*-down and *psr*-deficient mutant, TW314. Relative to UA159, iso-C19:1 I (I for a double bond at an unknown position) was reduced by 15.2% ($P=0.066$) in TW14D, with a percent average of 0.494 ± 0.004 for the UA159 and 0.419 ± 0.038 for TW14D, respectively (Table S2). None of the fatty acids showed any differences in TW251 when compared to UA159, but iso-C19:1 I was undetectable in TW314 ($P<0.001$). No other significant differences in the FAME profiles of the mutants were identified.

BrpA and Psr-deficiency affects cell wall phosphate content

Gram-positive bacteria possess a thick layer of peptidoglycan and peptidoglycan-associated anionic polymers including teichoic acids, which are chains of multiple glycerol phosphate (Gro-P) or ribitol phosphate (Rbo-P) repeating units¹³. Like the LCP proteins in *B. subtilis* and several others, BrpA and Psr have recently been shown to play a role in attachment of the cell wall-associated antigen RGP, which also contains P¹⁹. To examine if BrpA and Psr in *S. mutans* play a role in biogenesis of other P-containing anionic polymers, the phosphate content of the cell wall murein sacculi was measured using a colorimetric assay, and the results showed that relative to the parent strain, BrpA-deficiency resulted in phosphate reduction by >2.55 -fold with an average phosphate concentration of $4451\text{ }\mu\text{M/g}$ dry weight for the *brpA* mutant vs $11365\text{ }\mu\text{M/g}$ dry weight for the wild-type ($P<0.01$) (Fig. 2). On the other hand, the phosphate content of the *psr* mutant, TW251, was increased by 1.3-fold relative to its parent strain UA159 ($P<0.01$), compared to the wild-type. In addition, deficiency of RgpG, the first enzyme of the glucose-rhamnose biosynthesis pathway, was also found to have a reduced phosphate content with an average of $639.2\text{ }\mu\text{M/g}$ dry weight ($P<0.05$). Double mutants deficient in RgpG and BrpA or Psr further reduced the cell wall phosphate content by 18.5 and 16.7-fold, respectively ($P<0.001$).

BrpA-deficiency reduces the ability to compete in a community *in vitro*

To examine if BrpA-deficiency affects *S. mutans*' establishment, persistence and competitiveness when grown in a multiple-species community, we used CDFF, a continuously-flowing, multi-species model commonly used in studying population dynamics and examining the effects of individual genetic determinants on the ecology of oral biofilms (Liao and Wen, 2011, ASM Annu. Meet. Abstr. #1746)^{24,25,28}. The group of 10 species used in this study was known to be associated with plaque formation in both healthy individuals and in patients with dental caries and periodontal disease, and to form stable biofilm communities *in vitro* with each individual species being well-represented. The experiments were carried out as consistently as possible, including the age and size (in colony-forming-unit, CFU) of the inocula. When grown in BMM, a mucin-based medium in CDFF, *S. mutans* wild-type, UA159 was able to establish and accumulate quickly, with an increase in CFU per square milliliter of >10 -fold within 24 hours and maintained at an average of $>3.05\times10^6$ over the course of the experiment (Fig. 1). The BrpA-deficient mutant, TW14D, was able to establish on the PTFE surface, albeit in a slightly decreased capacity. Unlike UA159, however, TW14D showed no significant accumulation from day 1 through day 5 with an average CFU of $<1.36\times10^5$, which is >10 -fold less than UA159 in the same time period ($P<0.001$). However, as the experiment continued, TW14D was shown to be able to accumulate slowly, and by the end of the experiment (day 10) reached a level like the

wild-type ($P>0.05$). Similar trends were also observed when analyzed using confocal laser scanning microscopy (data not shown).

S. mutans is known for its ability to enhance biofilm formation by some other oral bacterial species, when grown in a community^{9,10,48}. Some major differences were also observed with some other members of the 10-species consortium, especially, *P. nigrescens* and *P. gingivalis*, when they were grown with *S. mutans* TW14D (Fig. 1A), as compared to growing with *S. mutans* wild-type, UA159 (Fig. 1B). When grown with UA159, *P. nigrescens* increased significantly from day 2 (data not shown) and reached an average CFU of 1.56×10^6 at day 5 and 2.08×10^7 at day 10. It established similarly well when grown with TW14D, but accumulated only ~10-fold over the entire experiment, >100-fold less than levels observed when grown with UA159 ($P<0.001$) (Fig. 1). *P. gingivalis* accumulated an average of 8.78×10^4 CFU within 24 hours when grown with UA159, but decreased continuously thereafter to an average of 1.63×10^3 at day 10. In contrast when grown with TW14D, no significant accumulation was observed until day 5 and at day 10 with an average CFU of 3.33×10^4 and 6.17×10^4 , respectively.

BrpA-deficiency reduces dental caries and caries severity in a rat caries model

To investigate if the deficiency of *brpA* and/or *psr* affects the ability of *S. mutans* to cause dental caries, a well-established rat caries model was employed with groups receiving either the *brpA*- or *psr*-deficient mutant, or the wild-type analog MX804, as a positive control. During the entire course of the study, no differences were observed in the pattern and amount of meal consumption by rats of the different groups. Consistently, no major differences were measured in weight gains between the animals of different groups (data not shown). When assessed using blood agar with proper antibiotics, no significant differences were observed between the mutant strains and the wild-type in infectivity (data not shown).

When examined using Illumina sequencing of 16S rRNA variable regions and qPCR analysis with genomic DNAs extracted from the bacteria residing on the tooth surface, the plaque flora of uninfected negative control (NC) rats was shown to be significantly different from those infected with *S. mutans*, as illustrated by principal component analysis plot of beta diversity (Fig. 3A). Consistent with recent reports by Garcia *et al.* (52–54), the rats' plaque flora is dominated by firmicutes (79.4%), which include *Streptococcus* spp. (26.8%), *Lactobacillus* spp. (17.7%), and *Veillonella* spp. (33.2%) (Fig. 3B&C; Table S3). However, following infection with *S. mutans* MX804, major shifts in relative abundance were shown in the plaque microbiome (Fig. 3B). These include elevations of *Haemophilus parainfluenzae* (from 3.5% to 13%, $P<0.01$) and *Aggregatibacter* spp. (3.1 to 7.3%, $P<0.05$). Among the reduced were *Lactobacillus* spp. (from 17.7 to 7.5% $P<0.1$) including *L. ruminis* (13.2 to 1.8%, $P<0.001$), *Veillonella* spp. (from 33.2% to 22.1%, $P<0.05$), *Bifidobacterium* spp. (4.1 to 2.4%, $P<0.05$), and *Rothia* (from 7.9% to 3.9%, $P<0.05$) (Fig. 3C). Relative to MX804, infection with the *brpA* mutant, TW14D, but not the *psr* mutant, TW251, led to further reduction of *V. dispar* (Table S3). Like MX804, similar reductions of *Lactobacillus* spp. were seen with TW14D, but not with TW251. Infection with MX804 increased *Aggregatibacter* spp., while no such effect was seen with either TW14D or TW251. When

analyzed using species-specific primers, significantly less *S. mutans* was detected in rats infected with TW14D, as compared to those infected with TW251 or MX804 (Fig. 3D).

When carious lesions were examined, significant differences were observed between the groups in both the number of caries and the severity of carious lesions (Table 2). Relative to the un-infected medium control, infection of *S. mutans* wild-type, MX804, was shown to result in significant increases in total caries ($P<0.05$) and lesion severity, especially on smooth surfaces ($P<0.001$). Unlike MX804, however, infection with the *brpA* mutant, TW14D, showed no significant differences from the uninfected control in total caries numbers on smooth surfaces, while moderate increases in carious lesion severity (Ds, Dm and Dx) were also measured ($P<0.01$) (Table 2). No significant differences in either total caries or caries severity on sulcal surfaces were observed between TW14D and the negative control ($P>0.05$). Like MX804, but different from TW14D, infection with TW251, as well as TW314, a *brpA*-down and *psr*-deficient mutant, resulted in significant increases in total caries and lesion severity on both smooth and sulcal surfaces ($P<0.001$) (Table 2).

Discussion

The results presented here showed that like the LCP proteins in *B. subtilis*, *S. aureus* and *S. pneumoniae*, Psr and especially BrpA in *S. mutans*, play important roles in cell envelope biogenesis, although major differences exist between these two LCP paralogues. The results that BrpA-deficiency, and especially the strain carrying *brpA*-down expression and *psr*-deficiency, resulted in major reduction of iso-C19:1 I suggest that BrpA, as well as Psr, albeit to a lesser degree, is involved in fatty acid biosynthesis, which is also consistent with the results of our DNA microarray analysis of the BrpA-deficient mutant²². Long chained iso-fatty acids are abundant in bacteria and play an important role in bacterial physiology⁴⁹. However, it awaits further investigation what the exact nature of the iso-C19:1 I is and what role it plays in the physiology of *S. mutans*. Thick peptidoglycan and the associated anionic polymers such as teichoic acids, which are known to contribute to most of the cell wall phosphates, are features of the Gram positive bacterial cell envelope. Interestingly, *S. mutans* is not shown to produce copious cell wall teichoic acids (data not shown), and some key members of the cell wall teichoic acid biosynthesis pathway have not yet been identified. On the other hand, our recent studies have shown that BrpA and Psr-deficiency affects the attachment of the rhamnose-containing glucose polymers, RGP, which also contains phosphate moieties¹⁹. Therefore, it is likely that part of the cell wall phosphate reduction observed with the BrpA-deficient mutant can be attributed to the defects in attachment of RGP to the cell envelope as a result of BrpA-deficiency. Consistently, deficiency of RgpG, the first enzyme of the RGP biosynthesis pathway, also causes major reduction of cell wall phosphate (by 43.7%, Figure 2), and a BrpA/RgpG double mutant had its cell wall phosphate further reduced (by 94.6%). Psr-deficiency also led to significant reduction of cell wall antigens¹⁹, but interestingly, the cell wall phosphate content of the Psr-deficient mutant was shown to be significantly higher than the wild-type (by 29.3%). These results further suggest that what constitutes the cell wall phosphate in *S. mutans* is more than RGP, although the nature of these phosphate-containing polymers and the exact roles of the two LCP proteins in *S. mutans* await further investigation.

As we, and Yoshida and coworkers, have shown, BrpA-deficiency in *S. mutans* causes major defects in biofilm formation^{20,21,23,50}, and such defects appear to be related to biofilm accumulation and development rather than bacterial adherence and biofilm initiation²³. We have shown that deficiency of BrpA leads to reduced viability, increased susceptibility to cell envelope stressors, weakened tolerance to low pH and oxidative stresses, and alters the expression of genes involved in the related functions, which all could contribute to the defects of the deficient mutant in biofilm formation^{15,22,23}. Besides, the BrpA-deficient mutants also have a high tendency to form long chains and aggregates²⁰, which depending on the system used for the study, will likely affect how the bacterium accumulates on the surface. Consistently, the results of 10-species consortium in CDFF also showed that relative to the wild-type, UA159, the BrpA-deficient mutant, TW14D, was able to establish on the surface tested, albeit to a lesser degree ($P>0.05$). However, unlike the wild-type, the BrpA-deficient mutant was not able to compete efficiently and accumulate in the mixed-species community initially. Interestingly, however, the *brpA* mutant was able to slowly regain its ability to grow in the community and eventually reach to a level similar to the wild-type by the end of the experiment (day 10). This could be in part because *S. mutans* also possesses another LCP homologue in Psr, which partly overlaps with BrpA in functions including roles in biofilm formation (see more details below)¹⁵. With an intact Psr, some of the defects from the *brpA* deficiency were likely compensated by Psr, which could in turn contribute to the slow recovery in the mixed-species community. It is also noteworthy that the mucin-based medium used in this study contains limited readily metabolizable sugars, and as such, acid tolerance response was probably not a major factor under the conditions studied.

When the rats are fed with a high sucrose-containing diet and water, *S. mutans*, a major etiologic agent of human dental caries, catabolizes glucose through homofermentative pathways, yielding primarily lactic acids, the most acidic of all organic acids, leading to rapid reduction in plaque pH. In response to a low pH environment, *S. mutans* can launch a so-called adaptive acid tolerance response that is featured with enhanced tolerance to lower pH⁵¹. The consistent acidic environment also favors aciduric species in the plaque community, leading to formation of cariogenic plaque⁵². It is noteworthy that *Streptococcus rattus*, a member of the mutans group, is also abundant in rodents, which can cause caries themselves, so an uninfected control group was used as the “caries baseline” group (Table 2). The results of the rats’ microbiota analysis also showed that rats’ plaque flora is diverse, consistent with a few other recent studies^{53–55}, and that infection with *S. mutans* can have major impact on its composition, especially when the rats are fed with high concentration of readily metabolizable sugars. Relative to the negative controls that received bacterial growth medium, rats that received *S. mutans* wild-type, MX804, displayed a major shift in the composition of their plaque microbiota, including a reduction in the proportion of *Lactobacillus* spp. and *Veillonella dispar*. This can be in part attributed to the likely acidification and pH shift of the plaque environment as a result of *S. mutans*’ sugar fermentation. On the other hand, the results also suggest that the rats’ indigenous lactobacilli, including *L. ruminis*, are not as acid tolerant as some of the lactobacilli of the human oral cavity¹⁰. Our recent studies have shown that relative to mono-species cultures, *S. mutans*-*L. casei* dual-species cultures also exhibited a reduced culture pH¹⁰. Likely, the

lower pH environment following *S. mutans* infection played a major role in reduction of *Lactobacilli* in the plaque flora. In addition, BrpA-deficiency is also shown to affect the expression of RgpG and the GTF enzymes, especially GTF-D¹⁵, which would likely result in alterations in cell envelope structure and function and influence the production, as well as the composition, of the polysaccharide matrices, and thus become part of the factors that underlie the further reduction of *Lactobacillus* spp. following TW14D infection. *Veillonella* spp. are known for their close association with lactic acid bacteria due to nutrient coupling between these bacteria involving lactic acid. Reduction of lactobacilli in the plaque community will certainly reduce the availability of nutrient sources for *Veillonella* spp., contributing to the observed reduction of *V. dispar* in rats infected with *S. mutans*.

Consistently, rats infected with *S. mutans* wild-type, MX804, led to the development of substantial dental caries with significant increases in both numbers of caries and caries severity, especially on smooth surfaces, when compared to the rats that received bacterial culture medium as the background /negative control. On the other hand, infection with the BrpA-deficient mutant did not catalyze the formation of caries above the background level. This is consistent with the apparent roles of BrpA in cell envelope biogenesis and acid and oxidative stress tolerance responses. As we have recently shown, deficiency of BrpA causes alterations in cell morphology and defects in cell envelope and leads to weakened tolerance to low pH, oxidative stress, and cell envelope antibacterial agents. While growing in a multiple-species consortium in mucin-based medium, which contains limited glucose and no sucrose, the BrpA-deficient mutant was able to slowly accumulate. However, when provided with high concentrations of readily fermentable sugars, such as in the rat caries model, the bacterium shifts its sugar metabolism to the homofermentative pathway yielding a large quantity of lactic acids and resulting in a rapid reduction in environmental pH. *S. rattus*, *Lactobacillus* spp. and probably others in the endogenous microbiota of the rats are also likely contributors to lactic acid production and the quick pH shift when the rats were fed with high concentrations of sucrose. As such, the weakened acid tolerance response could become a major factor modulating the ability of the deficient mutant to compete and persist in the plaque community and contributing to the reduced caries. Besides, alterations in cell envelope biogenesis can also directly influence the cell surface-associated functions, which include cell-surface and cell-cell interactions in biofilm initiation and accumulation. On the other hand, altered cell envelope biogenesis could also be part of the underlying factors explaining why the BrpA-deficient mutant and certain members of the mixed-species consortium, and the rats' plaque flora, interacted differently from the parent strain. Our recent studies have shown that BrpA-deficiency affects the expression of P1 and GTF-B^{15,22}, and both of which have been known to play a role in *S. mutans* mediated inter-species interactions and biofilm formation *in vitro* models^{10,56}.

BrpA and Psr are the only two homologues of the LCP family proteins in *S. mutans*, which we and others have shown to play an important role in regulation of the bacterial pathophysiology, although consistent with our previous findings, the results presented here further suggest that distinctive differences exist in both structure and function between these two proteins^{15,19,22}. The BrpA protein possesses a threonine and serine-rich C-terminus (~30%) that appears to be unique only to *S. mutans*²⁰, which also differs from Psr in the bacterium. While both play a significant role in biofilm formation, BrpA and Psr differ in

their effects on biofilm formation in part due to their different effects on the expression of the GTF enzymes¹⁵. Both BrpA and Psr show similar effects on acid tolerance response, but unlike BrpA, Psr has no effects on the oxidative stress response and cell envelope stress response¹⁵. In a wax worm model, BrpA-deficiency affects *S. mutans* virulence, but no such effects can be seen with the Psr-deficient mutant^{15,22}. Deficiency of BrpA, but not Psr, causes alterations in cell division and morphology. BrpA-deficiency results in major reduction of cell wall antigens and a concurrent accumulation in the growth medium, but no such accumulation was seen with the Psr-deficient mutant¹⁹. As revealed in this study, BrpA-deficiency leads to reduction of fatty acid iso-C19:1 I, but no such effect was detected with the Psr-deficient mutant, TW251. The *brpA* mutant also had significantly reduced cell wall phosphate, while the Psr-deficient mutant had it increased. As expected, deficiency of BrpA, but not Psr, significantly reduced its cariogenicity in rat caries model.

In summary, the results presented here further support our notion that BrpA is the major player of the two LCP paralogues in regulation of *S. mutans* pathophysiology. Relative to the wild-type, the BrpA-deficient mutant displayed major reduction in its ability to compete in a community both *in vitro* and *in vivo*, especially under conditions with high concentrations of readily metabolizable sugars. In addition, a rat caries model study also showed that deficiency of BrpA, but not Psr, significantly weakens the ability of the bacterium to cause dental caries. These results further suggest that BrpA can be used as potential target in modulation of *S. mutans* virulence.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported in part by NIDCR grant DE19452 to ZTW. We also want to thank Ms. Ashton N. Jorgensen for her assistance with some of the experiments.

References

1. Jenkinson HF. Beyond the oral microbiome. *Environmental Microbiology*. 2011; 13(12):3077–3087. [PubMed: 21906224]
2. Wade WG. The oral microbiome in health and disease. *Pharmacological research: the official journal of the Italian Pharmacological Society*. 2013; 69(1):137–143.
3. Jenkinson HF, Lamont RJ. Oral microbial communities in sickness and in health. *Trends Microbiol*. 2005; 13(12):589–595. [PubMed: 16214341]
4. Marcenes W, Kassebaum NJ, Bernabe E, et al. Global burden of oral conditions in 1990–2010: a systematic analysis. *J Dent Res*. 2013; 92(7):592–597. [PubMed: 23720570]
5. Frencken JE, Sharma P, Stenhouse L, Green D, Lavery D, Dietrich T. Global epidemiology of dental caries and severe periodontitis - a comprehensive review. *Journal of clinical periodontology*. 2017; 44(Suppl 18):S94–S105. [PubMed: 28266116]
6. Burne RA. Oral streptococci...products of their environment. *J Dent Res*. 1998; 77(3):445–452. [PubMed: 9496917]
7. Lemos JA, Quivey RG Jr, Koo H, Abranches J. *Streptococcus mutans*: a new Gram-positive paradigm? *Microbiology*. 2013; 159(Pt 3):436–445. [PubMed: 23393147]
8. Bowen WH, Koo H. Biology of *Streptococcus mutans*-Derived Glucosyltransferases: Role in Extracellular Matrix Formation of Cariogenic Biofilms. *Caries Res*. 2011; 45(1):69–86.

9. Wen ZT, Yates D, Ahn SJ, Burne RA. Biofilm formation and virulence expression by *Streptococcus mutans* are altered when grown in dual-species model. BMC Microbiol. 2010; 10:111. [PubMed: 20398271]
10. Wen ZT, Liao S, Bitoun JP, et al. *Streptococcus mutans* Displays Altered Stress Responses While Enhancing Biofilm Formation by *Lactobacillus casei* in Mixed-Species Consortium. Front Cell Infect Microbiol. 2017; 7:524. [PubMed: 29326887]
11. Merritt J, Qi F. The mutacins of *Streptococcus mutans*: regulation and ecology. Mol Oral Microbiol. 2012; 27(2):57–69. [PubMed: 22394465]
12. Navarre WW, Schneewind O. Surface proteins of gram-positive bacteria and mechanisms of their targeting to the cell wall envelope. Microbiology and Molecular Biology Reviews: MMBR. 1999; 63(1):174–229. [PubMed: 10066836]
13. Silhavy TJ, Kahne D, Walker S. The bacterial cell envelope. Cold Spring Harbor perspectives in biology. 2010; 2(5):a000414. [PubMed: 20452953]
14. Hubscher J, Luthy L, Berger-Bachi B, Stutzmann Meier P. Phylogenetic distribution and membrane topology of the LytR-CpsA-Psr protein family. BMC Genomics. 2008; 9:617. [PubMed: 19099556]
15. Bitoun JP, Liao S, McKey BA, et al. Psr is involved in regulation of glucan production, and double deficiency of BrpA and Psr is lethal in *Streptococcus mutans*. Microbiology. 2013; 159(Pt 3):493–506. [PubMed: 23288544]
16. Kawai Y, Marles-Wright J, Cleverley RM, et al. A widespread family of bacterial cell wall assembly proteins. EMBO J. 2011; 30(24):4931–4941. [PubMed: 21964069]
17. Over B, Heusser R, McCallum N, et al. LytR-CpsA-Psr proteins in *Staphylococcus aureus* display partial functional redundancy and the deletion of all three severely impairs septum placement and cell separation. FEMS Microbiology Letters. 2011; 320(2):142–151. [PubMed: 21554381]
18. Hanson BR, Runft DL, Streeter C, Kumar A, Carion TW, Neely MN. Functional analysis of the CpsA protein of *Streptococcus agalactiae*. J Bacteriol. 2012; 194(7):1668–1678. [PubMed: 22287515]
19. De A, Liao S, Bitoun JP, et al. Deficiency of RgpG Causes Major Defects in Cell Division and Biofilm Formation, and Deficiency of LCP proteins Leads to Accumulation of Cell Wall Antigens in Culture Medium by *Streptococcus mutans*. Applied and Environmental Microbiology. 2017
20. Wen ZT, Burne RA. Functional genomics approach to identifying genes required for biofilm development by *Streptococcus mutans*. Appl Environ Microbiol. 2002; 68(3):1196–1203. [PubMed: 11872468]
21. Chatfield CH, Koo H, Quivey RG Jr. The putative autolysin regulator LytR in *Streptococcus mutans* plays a role in cell division and is growth-phase regulated. Microbiol. 2005; 151(Pt 2): 625–631.
22. Bitoun JP, Liao S, Yao X, et al. BrpA is Involved in Regulation of Cell Envelope Stress Responses in *Streptococcus mutans*. Appl Environ Microbiol. 2012; 78(8):2914–2922. [PubMed: 22327589]
23. Wen ZT, Baker HV, Burne RA. Influence of BrpA on critical virulence attributes of *Streptococcus mutans*. Journal of Bacteriology. 2006; 188(8):2983–2992. [PubMed: 16585759]
24. Kinniment SL, Wimpenny JW, Adams D, Marsh PD. Development of a steady-state oral microbial biofilm community using the constant-depth film fermenter. Microbiol. 1996; 142(Pt 3):631–638.
25. Shu M, Browngardt CM, Chen YY, Burne RA. Role of urease enzymes in stability of a 10-species oral biofilm consortium cultivated in a constant-depth film fermenter. Infect Immun. 2003; 71(12): 7188–7192. [PubMed: 14638814]
26. Fan Y, Wen ZT, Liao S, et al. Novel amelogenin-releasing hydrogel for remineralization of enamel artificial caries. J Bioact Compat Polym. 2012; 27(6):585–603. [PubMed: 23338820]
27. Burne RA, Wen ZT, Chen YM, Penders JEC. Regulation of expression of the fructan hydrolase gene of *Streptococcus mutans* GS-5 by induction and carbon catabolite repression. Journal of Bacteriology. 1999; 181:2863–2871. [PubMed: 10217779]
28. Bradshaw DJ, Marsh PD, Schilling KM, Cummins D. A modified chemostat system to study the ecology of oral biofilms. J Appl Bacteriol. 1996; 80(2):124–130. [PubMed: 8642010]

29. Kreth J, Merritt J, Shi W, Qi F. Co-ordinated bacteriocin production and competence development: a possible mechanism for taking up DNA from neighbouring species. *Mol Microbiol.* 2005; 57(2): 392–404. [PubMed: 15978073]
30. Liu C, Worthington RJ, Melander C, Wu H. A new small molecule specifically inhibits the cariogenic bacterium *Streptococcus mutans* in multispecies biofilms. *Antimicrobial Agents and Chemotherapy.* 2011; 55(6):2679–2687. [PubMed: 21402858]
31. Wen ZT, Nguyen AH, Bitoun JP, Abranches J, Baker HV, Burne RA. Transcriptome analysis of LuxS-deficient *Streptococcus mutans* grown in biofilms. *Mol Oral Microbiol.* 2011; 26(1):2–18. [PubMed: 21214869]
32. Ozbek A, Aktas O. Identification of three strains of *Mycobacterium* species isolated from clinical samples using fatty acid methyl ester profiling. *International Journal of Medical Research.* 2003; 31(2):133–140.
33. Vidigal PGMF, Koehling HL, Mueller KD, Buer J, Rath PM, Steinmann J. Delineation of *Stenotrophomonas maltophilia* isolates from cystic fibrosis patients by fatty acid methyl ester profiles and matrix-assisted laser desorption/ionization time-of-flight mass spectra using hierarchical cluster analysis and principal component analysis. *J Medical Microbiology.* 2014; 63(12):1615–1620.
34. Chan YG, Frankel MB, Dengler V, Schneewind O, Missiakas D. *Staphylococcus aureus* mutants lacking the LytR-CpsA-Psr family of enzymes release cell wall teichoic acids into the extracellular medium. *J Bacteriol.* 2013; 195(20):4650–4659. [PubMed: 23935043]
35. Chan YG, Kim HK, Schneewind O, Missiakas D. The capsular polysaccharide of *Staphylococcus aureus* is attached to peptidoglycan by the LytR-CpsA-Psr (LCP) family of enzymes. *Journal of Biological Chemistry.* 2014; 289(22):15680–15690. [PubMed: 24753256]
36. Wen ZT, Burne RA. Analysis of *cis*- and *trans*-acting factors involved in regulation of the *Streptococcus mutans* fructanase gene (*fruA*). *Journal of Bacteriology.* 2002; 184(1):126–133. [PubMed: 11741852]
37. Fozo EM, Scott-Anne K, Koo H, Quivey RG Jr. Role of unsaturated fatty acid biosynthesis in virulence of *Streptococcus mutans*. *Infection and Immunity.* 2007; 75(3):1537–1539. [PubMed: 17220314]
38. Quivey RG Jr, Grayhack EJ, Faustoferri RC, et al. Functional profiling in *Streptococcus mutans*: construction and examination of a genomic collection of gene deletion mutants. *Mol Oral Microbiol.* 2015; 30(6):474–495. [PubMed: 25973955]
39. Larson RM. Merits and modifications of scoring rat dental caries by Keyes' method. In: Tanzer JM, editor *Animal Models in Cariology*. Oxford: IRL Press; 1981. 195–203.
40. Keyes PH. Dental caries in the molar teeth of rats. I. Distribution of lesions induced by high-carbohydrate low-fat diets. *J Dent Res.* 1958; 37(6):1077–1087. [PubMed: 13611122]
41. Bowen WH, Quivey RG Jr, Smith AV. Glucosyltransferase inactivation reduces dental caries. *J Dent Res.* 2001; 80(6):1505–1506.
42. Bruce-Keller AJ, Salbaum JM, Luo M, et al. Obese-type Gut Microbiota Induce Neurobehavioral Changes in the Absence of Obesity. *Biological Psychiatry.* 2014
43. Samuelson DR, Shellito JE, Maffei VJ, et al. Alcohol-associated intestinal dysbiosis impairs pulmonary host defense against *Klebsiella pneumoniae*. *PLoS Pathogens.* 2017; 13(6):e1006426. [PubMed: 28604843]
44. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJ, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods.* 2016; 13(7):581–583. [PubMed: 27214047]
45. Caporaso JG, Kuczynski J, Stombaugh J, et al. QIIME allows analysis of high-throughput community sequencing data. *Nature methods.* 2010; 7(5):335–336. [PubMed: 20383131]
46. Bitoun JP, Nguyen AH, Fan Y, Burne RA, Wen ZT. Transcriptional repressor Rex is involved in regulation of oxidative stress response and biofilm formation by *Streptococcus mutans*. *FEMS Microbiology Letters.* 2011; 320(2):110–117. [PubMed: 21521360]
47. Childers NK, Osgood RC, Hsu KL, et al. Real-time quantitative polymerase chain reaction for enumeration of *Streptococcus mutans* from oral samples. *European Journal of Oral Sciences.* 2011; 119(6):447–454. [PubMed: 22112030]

48. Gregoire S, Xiao J, Silva BB, et al. Role of Glucosyltransferase B in Interactions of *Candida albicans* with *Streptococcus mutans* and with an Experimental Pellicle on Hydroxyapatite Surfaces. *Applied and Environmental Microbiology*. 2011; 77(18):6357–6367. [PubMed: 21803906]
49. Fozo EM, Kajfasz JK, Quivey RG Jr. Low pH-induced membrane fatty acid alterations in oral bacteria. *FEMS Microbiol Lett*. 2004; 238(2):291–295. [PubMed: 15358413]
50. Yoshida A, Kuramitsu HK. Multiple *Streptococcus mutans* genes are involved in biofilm formation. *Appl Environ Microbiol*. 2002; 68(12):6283–6291. [PubMed: 12450853]
51. Burne RA, Quivey RG Jr, Marquis RE. Physiologic homeostasis and stress responses in oral biofilms. *Methods Enzymol*. 1999; 310:441–460. [PubMed: 10547811]
52. Takahashi N, Nyvad B. Caries ecology revisited: microbial dynamics and the caries process. *Caries Res*. 2008; 42(6):409–418. [PubMed: 18832827]
53. Garcia SS, Blackledge MS, Michalek S, et al. Targeting of *Streptococcus mutans* Biofilms by a Novel Small Molecule Prevents Dental Caries and Preserves the Oral Microbiome. *J Dent Res*. 2017; 96(7):807–814. [PubMed: 28571487]
54. Manrique P, Freire MO, Chen C, Zadeh HH, Young M, Suci P. Perturbation of the indigenous rat oral microbiome by ciprofloxacin dosing. *Mol Oral Microbiol*. 2013; 28(5):404–414. [PubMed: 23844936]
55. Hyde ER, Luk B, Cron S, et al. Characterization of the rat oral microbiome and the effects of dietary nitrate. *Free Radical Biology & Medicine*. 2014; 77:249–257. [PubMed: 25305639]
56. Brady LJ, Maddocks SE, Larson MR, et al. The changing faces of Streptococcus antigen I/II polypeptide family adhesins. *Mol Microbiol*. 2010; 77(2):276–286. [PubMed: 20497507]
57. Ajdic D, McShan WM, McLaughlin RE, et al. Genome sequence of *Streptococcus mutans* UA159, a cariogenic dental pathogen. *Proc Natl Acad Sci U S A*. 2002; 99(22):14434–14439. [PubMed: 12397186]

Figure 1.

S. mutans wild-type UA159 (A) and the *brpA* mutant TW14D (B) biofilms formed in CDFF with *A. naeslundii* (An), *S. oralis* (So), *S. sanguinis* (Ss), *L. casei* (Lc), *N. subflava* (Ns), *V. dispar* (Vd), *P. nigrescens* (Pn), *F. nucleatum* (Fn), and *P. gingivalis* (Pg). Biofilms were grown in BMM medium, and samples were taken for enumeration by viable counting on selective media as detailed in the text. Viable counts are expressed as the average ($\pm$ standard deviation in error bars) of the log CFU obtained per mm² surface of organisms that could be recovered from the recessed pockets in the PTFE pans. The data represent results from at least three independent sets of experiments.

Figure 2. Cell wall phosphate estimation

S. mutans wild-type (UA159) and the *brpA* (TW14D), *psr* (TW251), *rgpG* (TW322), *rgpG/brpA* (TW340), *rgpG/psr* (TW341), and *rgpG/brpA/psr* (TW343) mutants, were grown overnight in BHI, and cell wall sacculi were prepared and analyzed for cell wall phosphate content using colorimetric assays. Symbols @, # and * indicate statistical difference at $P < 0.05$, < 0.01 , and 0.001 respectively.

Figure 3. Microbiome and qPCR analysis of the rats' plaque flora

A. Principal component analysis plot of beta diversity; B. Phyla composition; C. Genus-level composition; and D. Relative abundance of *S. mutans* in the plaque flora by qPCR analysis of the plaque microbiome using *S. mutans*-specific primers. Relative to the wild-type (MX804) as well as the *psr* mutant (TW251), the abundance *brpA* mutant (TW14D) in the plaque flora was significantly reduced (*, $P < 0.001$). No significant differences were measured between TW251 and MX804. No significant differences were measured in total flora abundance between the groups. #, no *S. mutans* was detected in the uninfected negative group.

Table 1

Bacterial strains and plasmids used in this study*

Strains /Plasmid	Relevant characteristics	References
<i>S. mutans</i> UA159	wild-type	57
<i>S. mutans</i> TW184	UA159/ <i>gtfA::Pldh::gfp</i> , Kan ^r	This study
<i>S. mutans</i> TW14D	UA159/ <i>brpA</i> (SMU.410), Erm ^r	20,23
<i>S. mutans</i> TW322	<i>rgpG</i> (SMU.246), Spc ^r	15
<i>S. mutans</i> TW340	<i>rgpG/brpA</i> , Spc ^r , Erm ^r	19
<i>S. mutans</i> TW341	<i>rgpG/psr</i> , Spc ^r , Kan ^r	19
<i>S. mutans</i> TW343	<i>rgpG/brpA/psr</i> , Spc ^r , Kan ^r , Erm ^r	19
<i>S. mutans</i> MX804	UA159 derivative, Erm ^r	38
<i>S. oralis</i> 34	wild-type	25
<i>S. sanguinis</i> SK150	wild-type	25
<i>A. naeslundii</i> WVU45	wild-type	25
<i>L. casei</i> 4646	wild-type	25
<i>N. subflava</i> A1078	wild-type	25
<i>V. dispar</i> ATCC17745	wild-type	25
<i>P. nigrescens</i> T588	wild-type	25
<i>P. gingivalis</i> ATCC33277	wild-type	25
<i>F. nucleatum</i> ATCC 10953	wild-type	25

Note: Kan^r, Spc^r and Erm^r for kanamycin, spectinomycin and erythromycin resistance, respectively. The non-polar spectinomycin element contains the *aadA* gene encoding an aminoglycoside-3'-adenylyltransferase.

Table 2

Influence of different strains on development of dental caries in rats

Strain/group	Smooth surface			Sulcal surface		
	Total caries E		Lesion severity		Lesion severity	
			Ds	Dm	Ds	Dm
Uninfected	43.8±13.2		4.3±4.0	1.1±1.6	33.0±2.9	15.2±6.2
TW14D	44.8±9.6		12.9±5.0 [#]	6.9±5.2 ^{**}	34.7±4.1	15.3±5.3
TW251	59.0±9.9 [#]		32.9±11.5 [*]	20.5±8.7 [*]	40.4±3.4	27.3±6.9 [*]
TW314	58.8±15.4 [#]		30.7±12.0 [*]	21.2±11.8 [*]	38.5±2.9	26.5±7.3 [*]
MX804	55.0±14.4 [#]		28.4±12.0 [*]	21.4±10.0 [*]	38.0±4.1	26.1±9.5 [*]
					44.1±5.3	14.6±9.4 [*]

Note: Data are expressed as average ± standard deviation (n=10). E, enamel; Ds, slight dental caries; Sm, moderate dental caries; Dx, extensive dental caries.

[#], ^{*} indicate significant differences at $P<0.05$ and 0.001 , respectively, when compared to TW14D and the uninfected control in the same columns.

^{**} and ^{##} indicate significant differences a $P<0.01$ and 0.001 , respectively, when compared to the uninfected control.

Statistics using Tukey-Kramer honestly significant difference test and Student's *t*-test.