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Deficiency of BrpB causes major defects in cell division, stress responses and biofilm formation by *Streptococcus mutans*

Jacob P. Bitoun,[1] Sumei Liao,[1] Gary G. Xie,[2] Wandy L. Beatty[3] and Zezhang T. Wen[1,4,5]

Correspondence
Zezhang T. Wen
zwen@lsuhsc.edu

[1]Center of Excellence in Oral and Craniofacial Biology, School of Dentistry, Louisiana State University Health Sciences Center, New Orleans, LA 70119, USA

[2]Biology and Bioinformatics, Los Alamos National Laboratory, NM 87545, USA

[3]Department of Molecular Microbiology, Washington University School of Medicine, St Louis, MO 63110 USA

[4]Department of Comprehensive Dentistry and Biomaterials, School of Dentistry, Louisiana State University Health Sciences Center, New Orleans, LA 70119, USA

[5]Department of Microbiology, Immunology and Parasitology, School of Medicine, Louisiana State University Health Sciences Center, New Orleans, LA 70112, USA

Streptococcus mutans, the primary aetiological agent of dental caries, possesses an YjeE-like protein that is encoded by locus SMU.409, herein designated *brpB.* In this study, a BrpB-deficient mutant, JB409, and a double mutant deficient of BrpB and BrpA (a paralogue of the LytR–CpsA–Psr family of cell wall-associated proteins), JB819, were constructed and characterized using function assays and microscopy analysis. Both JB409 and JB819 displayed extended lag phases and drastically slowed growth rates during growth in brain heart infusion medium as compared to the wild-type, UA159. Relative to UA159, JB409 and JB819 were more than 60- and 10-fold more susceptible to acid killing at pH 2.8, and more than 1 and 2 logs more susceptible to hydrogen peroxide, respectively. Complementation of the deficient mutants with a wild-type copy of the respective gene(s) partly restored the acid and oxidative stress responses to a level similar to the wild-type. As compared to UA159, biofilm formation by JB409 and JB819 was drastically reduced ($P<0.001$), especially during growth in medium containing sucrose. Under a scanning electron microscope, JB409 had significantly more giant cells with an elongated, rod-like morphology, and JB819 formed marble-like super cells with apparent defects in cell division. As revealed by transmission electron microscopy analysis, BrpB deficiency in both JB409 and JB819 resulted in the development of low electron density patches and formation of a loose nucleoid structure. Taken together, these results suggest that BrpB likely functions together with BrpA in regulating cell envelope biogenesis/homeostasis in *Strep. mutans*. Further studies are under way to elucidate the mechanism that underlies the BrpA- and BrpB-mediated regulation.

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INTRODUCTION

Streptococcus mutans, a major causative agent of dental caries (Loesche, 1986), lives primarily on the tooth surface in tenacious biofilms commonly known as dental plaque. The oral cavity is a microenvironment that is known for its unpredictable fluctuations in conditions such as carbohydrate source and availability, acid and alcohol concentration, oxygen tension, sheering forces, and the presence of antimicrobial agents (Burne, 1998; Lemos & Burne, 2008). This bacterium has evolved strategies to endure and cope with drastic changes in the environmental conditions (Lemos & Burne, 2008). The cell envelope plays a vital role during these processes as it protects the cell from the environment, acts as a molecular sieve, and provides a platform for components of the cell involved in sensing and transmission of environmental signals. In addition, maintenance

Abbreviations: FE-SEM, field emission-scanning electron microscopy; HA, hydroxylapatite; Kan, kanamycin; TEM, transmission electron microscopy.

Two supplementary figures are available with the online version of this paper.

and turnover of the cell envelope govern the fundamental properties of cell growth, shape and division. Ensuring envelope integrity is therefore crucial for bacterial cells to survive.

Our recent studies have shown that BrpA, for biofilm regulatory protein A, a paralogue of the LytR–CpsA–Psr (LCP) family of cell wall-associated proteins highly conserved in Gram-positive bacteria (Hübscher *et al.*, 2008), plays a major role in cell envelope biogenesis/homeostasis in *Strep. mutans* (Bitoun *et al.*, 2012a, 2013). Relative to the wild-type, *Strep. mutans* strains deficient of BrpA displayed significant increases in susceptibility to cell envelope antimicrobials, and drastic reductions in acid and oxidative stress tolerance responses (Bitoun *et al.*, 2012a; Chatfield *et al.*, 2005; Wen *et al.*, 2006). In addition, the deficient mutants also had an elevated autolysis rate and major defects in cell division, cell morphology and biofilm formation (Bitoun *et al.*, 2012a, 2013; Wen *et al.*, 2006). Similar results were also observed with Psr, the only other LCP paralogue *Strep. mutans* possesses (Bitoun *et al.*, 2013). A Psr-deficient, BrpA-down mutant exists primarily in long chains of dividing, giant cells with multiple septa, and deficiency of both BrpA and Psr appears to be lethal in *Strep. mutans* under the conditions studied.

The YjeE-like proteins are a unique, new family of P-loop ATP/GTPase proteins widespread in virtually all eubacteria, but not in archaea and eukaryotes (Teplyakov *et al.*, 2002), although their exact function in cellular physiology remains unclear (Allali-Hassani *et al.*, 2004; Karst *et al.*, 2009). In *Escherichia coli*, YjeE is required for growth, and strains with decreased expression of YjeE had an elongated lag phase and a decreased growth rate (Allali-Hassani *et al.*, 2004). Repression of YjeE expression also caused alterations of cell morphology, including massive cells with curving and branching, and an unusual distribution of the cell nucleoid (Handford *et al.*, 2009). Unlike *E. coli*, however, *Bacillus subtilis* deficient of YdiB orthologue was shown to be viable, but had a much reduced growth rate, when compared to the wild-type (Karst *et al.*, 2009). Recombinant proteins of YjeE from *E. coli* and *Haemophilus influenzae*, and YdiB from *B. subtilis*, all possessed low ATPase activity (Allali-Hassani *et al.*, 2004; Karst *et al.*, 2009; Teplyakov *et al.*, 2002), and in *B. subtilis* ATPase activity was shown to be essential for the function of YdiB protein *in vivo* (Karst *et al.*, 2009). More recently, an *in vitro* study has also provided evidence that in *E. coli* YjeE is part of a three essential protein complex required for biosynthesis of threonylcarbamoyl adenosine (t^6A), a modified tRNA nucleoside involved in codon decoding (Deutsch *et al.*, 2012). However, certain component(s) of the complex were shown to be species specific, which again suggests diversity in function of the YjeE-like proteins in different species.

Strep. mutans possesses a gene in locus SMU.409, here designated *brpB*, which encodes a protein with homology to YjeE of *E. coli* and YdiB of *B. subtilis* (Allali-Hassani *et al.*, 2004; Karst *et al.*, 2009). Previous studies showed that *brpB* can be co-transcribed with *brpA*, suggesting likely involvement of its product, BrpB, in BrpA-regulated cell envelope biogenesis/homeostasis (Bitoun *et al.*, 2012a, 2013). The objective of this study was to determine the role of BrpB in the cellular physiology and fitness of *Strep. mutans*. Here a BrpB-deficient mutant, JB409, and a BrpB/BrpA-deficient double mutant, JB819, were constructed by allelic replacement. Characterization of these mutants revealed that BrpB deficiency weakened the ability of *Strep. mutans* to cope with both acid and oxidative stress relative to the parent strain; biofilm formation by the deficient mutants was also greatly compromised. In addition, electron microscopy analyses also showed BrpB deficiency in *Strep. mutans* caused alteration in cell size and morphology of the deficient mutants.

METHODS

Plasmids, bacterial strains and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *Strep. mutans* strains were maintained in brain heart infusion (BHI) medium (Difco Laboratories). Solid media were prepared similarly, but Bacto agar (Difco Laboratories) was added at a concentration of 1.5 % (w/v). When needed, kanamycin (Kan, 1 mg ml^{-1}) and/or spectinomycin (Spe) (1 mg ml^{-1}) were added to the growth medium. Unless stated otherwise, cultures were grown aerobically in a 37 °C chamber containing 5 % CO_2 under static conditions. For growth studies, overnight cultures were diluted 1 : 100 into fresh BHI and allowed to continue growing until mid-exponential phase (OD_{600} 0.4–0.5), when they were further diluted 1 : 100 and the optical density of the cultures at 600 nm was continuously monitored using a Bioscreen C (Oy Growth Curves) at 37 °C with and without sterile mineral oil overlay (Bitoun *et al.*, 2012b). For biofilm assays, *Strep. mutans* were grown in modified biofilm medium (BM) with glucose (20 mM, BMG), sucrose (10 mM, BMS) or glucose (18 mM) plus sucrose (2 mM) (BMGS) as the supplementary carbohydrate sources (Li *et al.*, 2002; Loo *et al.*, 2000).

Construction of mutants and complemented strains. To construct the BrpB-deficient mutant, a PCR-ligation-mutagenesis strategy was used as described elsewhere (Lau *et al.*, 2002; Wen & Burne, 2004). Briefly, the 5′ and 3′ regions flanking *brpB* were amplified by PCR using Phusion high-fidelity DNA polymerase (New England Biolabs) with the gene-specific primers shown in Table 2. Following proper restriction enzyme digestions, the flanking regions were ligated to a non-polar Kan-resistance element (Zeng *et al.*, 2006) that was digested similarly. The resulting ligation mixtures were used to directly transform *Strep. mutans* UA159 in the presence of competence stimulating peptide (CSP) (Li *et al.*, 2001). An allelic replacement mutant, JB409, with *brpB* deficiency was isolated on BHI-Kan plates, and further confirmed using PCR and sequencing. For complementation of mutant JB409, the *brpB* coding sequence plus its cognate promoter region were amplified by PCR, digested and cloned directly into shuttle vector pDL278. After sequence confirmation, the resulting construct, pDL:*brpB*, was then used to transform JB409, generating complemented strain, JB409C. To construct the *brpB* and *brpA* double mutant, a similar PCR-ligation-mutagenesis strategy was used with the 5′ region flanking upstream of *brpB* and the 3′ region flanking downstream of *brpA*. The *brpBA* operon deletional mutant resulted in the removal of nucleotide 47 within the ORF of *brpB* through nucleotide 1208 of *brpA*. Allelic replacement mutant, JB819, with *brpB* and *brpA* deficiencies, was isolated on BHI-Kan plates, and further confirmed using PCR and sequencing.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
Strep. mutans UA159	Wild-type, serotype c	Ajdić *et al.* (2002)
Strep. mutans JB409	Derivative of UA159, Δ*brpB* (SMU.409), Kan^r	This study
Strep. mutans JB409C	JB409 carrying pDL278:*brpB*, Spe^r, Kan^r	This study
Strep. mutans JB819	Derivative of UA159, Δ*brpBA* (SMU.409-410), Kan^r	This study
Strep. mutans JB819C	JB819 carrying pDL278:*brpBA*, Spe^r, Kan^r	This study
Plasmids		
pDL278	Shuttle vector, Spe^r	LeBlanc & Lee (1991)
pDL278:*brpB*	Shuttle carrying *brpB*, Spe^r	This study
pDL278:*brpBA*	Shuttle carrying *brpBA*, Spe^r	This study

Kan^r and Spe^r are kanamycin and spectinomycin resistance, respectively.

JB819C, a complemented strain that carried the *brpBA* coding sequence plus its cognate promoter region (Bitoun *et al.*, 2012a) in shuttle vector pDL278, was constructed in a similar manner to that described above.

Induced autolysis assay. For the autolysis assay, *Strep. mutans* strains were grown in BHI overnight. Bacterial cells were harvested by centrifugation at 3250 ***g***, at 4 °C for 10 min, washed once with PBS (100 mM potassium phosphate buffer, pH 7.2, sodium chloride, 0.9 %, w/v), and then resuspended in PBS containing 0.2 % (v/v) Triton X-100 (Sigma) (Bitoun *et al.*, 2013; Wen & Burne, 2002). The optical density of the cell suspension was then monitored automatically using a Bioscreen C at 37 °C every 30 min with moderate shaking (10 s) before measurement.

Acid and hydrogen peroxide killing assays. The impact of *brpB* and *brpBA* deficiencies on the ability of *Strep. mutans* to withstand acid and hydrogen peroxide stress was determined by using acid killing and hydrogen peroxide challenge assays as described elsewhere (Wen & Burne, 2004). In brief, planktonic cultures were grown in BHI until early exponential phase (OD_{600} 0.3) and biofilms cells were grown in BMGS on sterile glass slides in 50 ml Falcon tubes for 24 h (Wen *et al.*, 2010). The cells were then collected by centrifugation at 3250 ***g*** at 4 °C for 5 min, and washed twice with 0.1 M glycine buffer, pH 7.0. For the acid killing assay, the washed cells were resuspended in 0.1 M glycine buffer, pH 2.8, and incubated at room temperature for periods of 15, 30, 45 and 60 min. For preparation of cells that had undergone an adaptive acid tolerance response, cultures with an OD_{600} 0.3 were washed once with 0.1 M glycine buffer, pH 7.0, as described above, and the cell pellets were resuspended in fresh BHI that was adjusted to pH 5.0 with HCl. Following an additional hour of incubation, cells were harvested, washed and subjected to acid killing as described above. For the hydrogen peroxide killing assays, washed cells were resuspended in 0.1 M glycine buffer, pH 7.0, and hydrogen peroxide was added at a final concentration of 0.2 % (58 mM, final concentration). Surviving cells were appropriately diluted, plated on BHI plates in triplicate and incubated in 5 % CO_2 at 37 °C for 24 to 48 h.

Biofilm analysis. *Strep. mutans* biofilms were grown in semi-defined BM (BMG, BMS and BMGS) as described elsewhere (Li *et al.*, 2002; Loo *et al.*, 2000). Overnight cultures were inoculated 1 : 100 into fresh

Table 2. Primers used in this study

Underlined nucleotides are restriction sites engineered for cloning. qPCR, Quantitative PCR.

Name	Nucleotide sequence (5′-3′)	Application
5′-5′ 409 up	5′-TACAGCTAACTCTTCTGCAACACCATC-3′	5′ fragment for *brpB* and *brpBA* mutation
5′-3′ 409 RI	5′-TGGCTCTTGGACAGAGAATTCGTCA-3′	5′ fragment for *brpB* and *brpBA* mutation
3′-5′ 409 RI	5′-TGCGGAATTCGAGCATGACTGA-3′	3′ fragment for *brpB* mutation
3′-3′ 409	5′-AGCTTTAGACAGTGTCAGCAAGTATCGT-3′	3′ fragment for *brpB* mutation
3′-5′ 409-410 RI	5′-TGCTGCAGGAACGGGAATTCGTA-3′	3′ fragment for *brpBA* mutation
3′-3′ 409-410	5′-TCATAGGCCTCAATGGTGCAGGA-3′	3′ fragment for *brpBA* mutation
5′ 409comp RI	5′-GCATATATAGGAATTCAGATAAGGCTGAGCT-3′	*brpB* and *brpBA* complementation
3′ 409comp Hd	5′-CAGTTATATGAGGGCAAGCTTCATCT-3′	*brpB* complementation
3′ 409-410comp Bm	5′-GTATAACTGAAAAACCGGGATCCTTCCA-3′	*brpBA* complementation
brpB Fw	5′- TAAGAGTCCAACCTATACGATT-3′	138 bp, qPCR of *brpB*
brpB Rev	5′-ATCACTGTTACGCCATCT-3′	138 bp, qPCR of *brpB*
brpA Fw	5′-CGTGAGGTCATCAGCAAGGTC-3′	148 bp, qPCR of *brpA*
brpA Rev	5′-CGCTGTACCCCAAAAGTTTAGG-3′	148 bp, qPCR of *brpA*
16S rRNA Fw	5′-CACACCGCCCGTCACACC-3′	160 bp, qPCR of SMU_r01 (16S rRNA)
16S rRNA Rev	5′-CAGCCGCACCTTCCGATACG-3′	160 bp, qPCR of SMU_r01 (16S rRNA)

BHI and allowed to grow until OD_{600} 0.5 when the biofilm media was inoculated by 1 : 100 dilutions. For quantitative and structural analysis, biofilms were allowed to grow on hydroxylapatite (HA) discs that were placed vertically in fabricated orthodontic wire holders made to fit 24-well plates in a 5 % CO_2 incubator at 37 °C under static conditions (Lemos *et al.*, 2010). Biofilm samples were collected after 24 h and were stained with either a *Bac*Light Live/Dead bacterial viability kit (Invitrogen), SYTO 62 (Invitrogen) or SYTO 59/Alexa488 Concanavalin A (Invitrogen). Optical dissections of the biofilms were carried out using a laser scanning confocal microscope (Olympus Fluoview BX61) with pixel size on *x*- and *y*-axis representing 0.414 μm, and the *z*-axis was set at 2.0 microns. At least three independent image stacks were acquired at × 600 magnification on the HA surface using a × 60 water-immersion objective lens for each sample at random positions on the HA surface. The resulting image stacks were analysed using Slidebook 5.0 (Olympus). COMSTAT 2.0 was used to quantify the biovolume, surface area, mean thickness and maximum thickness of the biofilms as described elsewhere (Heydorn *et al.*, 2000; Wen *et al.*, 2011). Biovolume is defined as the biofilm volume (μm^3) per unit area (μm^2) (Heydorn *et al.*, 2000).

Electron microscopy analysis. For field emission-scanning electron microscopy (FE-SEM) analysis, *Strep. mutans* overnight cultures were grown in BMG, BMGS or BMS on HA discs that were placed horizontally in 24-well plates as described elsewhere (Bitoun *et al.*, 2011; Wen & Burne, 2004). After 24 h of growth, the HA discs were removed and washed twice with PBS, pH 7.4, before overnight fixation with 2.5 % glutaraldehyde (Polysciences) at 4 °C. The samples were dehydrated using a graded ethanol series, dried using a critical point dryer and then coated with carbon using a sputter coater. Microscopy analysis was performed with a Hitachi S-4800 high-resolution microscope (at Tulane University, New Orleans, LA, USA).

For transmission electron microscopy (TEM) analysis, *Strep. mutans* strains were grown in BHI until mid-exponential phase, harvested by centrifugation at 3250 ***g*** at 4 °C for 10 min, washed twice with PBS and then fixed in 2.5 % glutaraldehyde/2 % paraformaldehyde (Polysciences) in PBS for 1 h at room temperature (Bitoun *et al.*, 2013). Cells were washed in PBS and post-fixed in 1 % osmium tetroxide (Polysciences) for 1 h. Samples were then rinsed extensively in dH_20 prior to en bloc staining with 1 % aqueous uranylacetate (Ted Pella Inc.) for 1 h. The cells were then washed with dH_20 and dehydrated in a graded series of ethanol solutions and embedded in Eponate 12 resin (Ted Pella Inc.). Sections of 90–100 nm were prepared, stained with uranyl acetate and lead citrate, and viewed under a JEOL 1200 EX transmission electron microscope (JEOL USA) equipped with an AMT 8 megapixel digital camera (Advanced Microscopy Techniques).

Statistical analysis. Quantitative data were analysed using the paired Student's *t*-test.

RESULTS

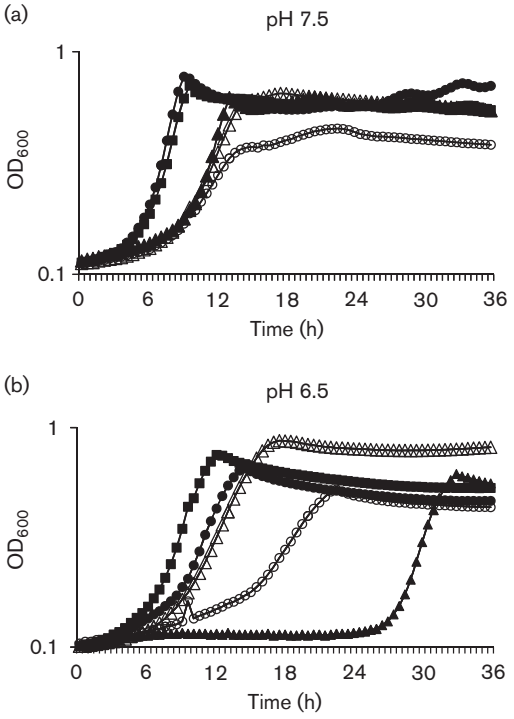
BrpB is a member of YjeE family of P-loop ATPase and the *brpA/brpB* cluster is highly conserved among streptococci

As annotated (Ajdić *et al.*, 2002), the *brpB* gene (locus SMU.409) encodes a polypeptide with high similarity to YjeE of *E. coli*, YdiB of *B. subtilis* and other members of the highly conserved UPF0079 family of uncharacterized P-loop hydrolases (www.expasy.org) (Allali-Hassani *et al.*, 2004; Handford *et al.*, 2009; Karst *et al.*, 2009; Teplyakov *et al.*, 2002). As shown by pairwise sequence alignment analysis, BrpB displays the highest similarity in amino acid composition to the streptococcal homologues, and the least homology was observed between BrpB and YjeE of *E. coli* (data not shown). However, like YjeE of *E. coli*, BrpB and its streptococcal homologues all show conservation of a Walker A motif (GX_4GKTT) that is typical of nucleotide-binding proteins, including ATP/GTPase (data not shown) (Hanson & Whiteheart, 2005).

Our previous studies showed that locus SMU.409 could be co-transcribed with *brpA* (SMU.410) despite the presence of a large (323 bp) intergenic region (Bitoun *et al.*, 2012a). Based upon this genetic linkage and the function relationship as suggested by data presented here, the gene encoded in SMU.409 was designated *brpB*. Indeed, analysis of the genetic structure also revealed that the regions flanking the loci for the YjeE homologues are highly conserved among streptococci, although differences exist between *Strep. mutans* and the other streptococci (Fig. S1, available in *Microbiology* Online). Like *Strep. mutans,* the gene encoding the YjeE homologue in locus SP_1944 of *Streptococcus pneumoniae* is located upstream of *lytR* (locus SP_1942) (Johnsborg & Håvarstein, 2009). Unlike *Strep. mutans*, however, the *yjeE* locus in *Strep. pneumoniae* represents the leading gene of an apparent three gene operon that includes in the middle locus SP_1943 encoding a putative acyltransferase, followed by *lytR*. Similar features were also found in group A *Streptococcus pyogenes*, group B *Streptococcus agalactiae* and all other streptococcal species, including other oral streptococci whose genome sequences are available and accessible (Fig. S1). Interestingly, when compared to other streptococci, *Strep. mutans* species are the only streptococci lacking the acyltransferase locus, a likely result of genetic deletions during the evolutionary processes and an attributing factor to the presence of a large intergenic region between *brpA* and *brpB* (Bitoun *et al.*, 2012a). Unlike streptococci, however, no such linkage could be identified between the loci for YjeE (or YdiB for *B. subtilis*) homologues and the genes for any LCP proteins in *Staphylococcus aureus, B. subtilis* and other Gram-positive bacteria (Eberhardt *et al.*, 2012; Johnsborg & Håvarstein, 2009; Kawai *et al.*, 2011; Over *et al.*, 2011).

Deficiency of BrpB causes major growth defects in *Strep. mutans*

A BrpB-deficient *Strep. mutans* strain, JB409, was constructed with a region of 380 bp from nucleotides 47 to 427 of its coding sequence (435 bp total) relative to the start codon deleted and replaced with a nonpolar Kan-resistance cassette. As compared to the parent strain, UA159, the BrpB-deficient mutant, JB409, took an additional day to form discernible colonies on solid medium (data not shown), suggesting growth defects as a result of BrpB deficiency. During planktonic growth in BHI with pH adjusted to 7.5 (Fig. 1), the maximal culture density (OD_{600}) of JB409 was found to be reduced by more than



Lag phase and doubling time at pH 7.5

Strain	Lag phase	Doubling time
S. mutans UA159	5 h	86.3 min
S. mutans JB409	8 h	212.6 min
S. mutans JB409C	5 h	88.2 min
S. mutans JB819	8 h	149.0 min
S. mutans JB819C	8 h	101.8 min

Lag phase and doubling time at pH 6.5

Strain	Lag phase	Doubling time
S. mutans UA159	5 h	132.6 min
S. mutans JB409	15 h	233.6 min
S. mutans JB409C	8 h	158.5 min
S. mutans JB819	8 h	156.1 min
S. mutans JB819C	28 h	112.7 min

Fig. 1. Growth study of *Strep. mutans*. *Strep. mutans* wild-type, UA159 (■), BrpB-deficient mutant, JB409 (○), BrpB-complemented strain, JB409C (●), BrpB- and BrpA-deficient double mutant, JB819 (Δ), and BrpB- and BrpA-complemented strain, JB819C (▲), were grown in BHI adjusted to pH 7.5 (a) and pH 6.5 (b) in a Bioscreen C at 37 °C with a sterile mineral oil overlay (50 μl), and the optical density of the cultures was continuously monitored at 600 nm. Results showed that BrpB deficiency in JB409 greatly affects bacterial cell growth. Interestingly, the BrpB and BrpA double deficiency in JB819 partially alleviated the slow growth phenotype of the BrpB-deficient mutant, JB409. During growth in BHI adjusted to pH 6.5, the complemented strain, JB819C, showed an extremely delayed lag phase, when compared to the wild-type and the parent strain, JB819. The data presented here are representative of three independent experiments. The lag phase duration and the doubling time of each strain under the conditions tested are expressed as means and presented in the tables to the right of the growth curves.

40 %, when compared to UA159. Also, relative to UA159, JB409 had an extended lag phase and increased its doubling time by more than 2 h (Fig. 1a). Such defects were further magnified during growth in BHI adjusted to pH 6.5 (Fig. 1b). As expected, complementation *in trans* with a wild-type copy of *brpB* plus its cognate promoter in multiple copy shuttle vector pDL278 was able to restore the slow growth phenotype to a level similar to UA159 (Fig. 1a). A BrpB/BrpA-deficient double mutant, JB819, was constructed with the coding sequences of both *brpB* and *brpA* deleted (from nucleotide 47 of *brpB* relative to the start codon through nucleotide 1208 of *brpA*) and replaced with a nonpolar Kan-resistance cassette (Tables 1 and 2). JB819 also showed major defects in growth with both its lag phase and doubling time significantly increased, when compared to the wild-type. Interestingly, however, the doubling time of the BrpA/BrpB-deficient double mutant was significantly faster than that of the BrpB single mutant (Fig. 1). Previous studies showed that BrpA deficiency did not result in any major effect on cell growth when cells were grown in regular BHI (Bitoun *et al.*, 2013; Wen & Burne, 2002; Wen *et al.*, 2006). When complemented with a wild-type copy of the *brpA/brpB* operon plus their cognate promoter(s) in multiple copy shuttle vector pDL:*brpB/A*, the complemented strain, JB819C, showed a further decreased doubling time with a level closer to the wild-type (Fig. 1). Interestingly, during growth in BHI adjusted to pH 6.5, however, the complemented strain, JB819C, demonstrated a severely delayed lag phase although the doubling time was shorter than that of UA159 (Fig. 1b). Paraquat (methyl viologen; Sigma) is commonly used to test for the ability of cultures to adapt to superoxide stress. The addition of paraquat did not further exacerbate the growth deficiencies; albeit, all strains grew at slower, but proportional rates in the presence of paraquat (data not shown).

BrpB deficiency results in exacerbated sensitivity to acid and oxidative stress

It was apparent that during growth in BHI adjusted to pH 6.5, the deficient mutants, JB409 and JB819, were challenged to adapt to the acidic conditions (Fig. 1b). When

analysed by acid killing with mid-exponential phase cultures grown in BHI adjusted to pH 7.4, the survival rate of JB409 and JB819 was decreased by 60- and 11-fold, respectively, after 30 min of incubation in 0.1 M glycine buffer of pH 2.8, as compared to UA159 (Fig. 2a). Similar results were obtained using planktonic cells grown in regular BHI and cells derived from 48 h biofilms grown in BMGS on glass slides (data not shown). As expected, complementation with the wild-type *brpB* and *brpA/brpB* in a multiple copy plasmid in strains JB409C and JB819C, respectively, partly restored the acid-sensitive phenotypes to a level similar to that of UA159 (Fig. 2a).

For adaptive acid tolerance response, *Strep. mutans* strains were allowed to adapt and grow in BHI adjusted to pH 5.0 for 1 h before being subjected to acid killing (Wen & Burne, 2004). The results showed that like wild-type UA159, the BrpB-deficient mutant, JB409, also significantly increased its survival rate after 1 h incubation in BHI adjusted to pH 5.0 (Fig. 2b). Relative to UA159, however, the survival rate of JB409 was still 2 logs less. Unlike UA159 and JB409, the BrpB/BrpA-deficient double mutant, JB819, did not show any adaptive response after initial exposure to low pH. Consequently, the survival rate of JB819 was further reduced by more than 500-fold after 60 min of acid killing (Fig. 2b).

When challenged with hydrogen peroxide (0.2 %, w/v, or 58 mM), the BrpB-deficient strain, JB409, and the BrpB/BrpA-deficient double mutant, JB819, were at least 1 and 2 logs more sensitive than the wild-type, UA159 (Fig. 3). Complementation with a wild-type copy of *brpB* plus its cognate promoter in strain JB409C fully restored the phenotype to the wild-type UA159 (Fig. 3). Interestingly, complementation in strain JB819C, which carries a wild-type copy of both *brpB* and *brpA* plus their promoter(s), showed little effect in reducing the increased sensitivity to hydrogen peroxide observed in JB819.

BrpB deficiency causes drastic reductions in biofilm formation by the deficient mutants

When grown on vertically positioned HA discs, a commonly used *in vitro* tooth model, and analysed using laser scanning confocal microscopy following fluorescent staining using SYTO 62 (Invitrogen), UA159 accumulated robust biofilms after 24 h, with a biovolume of 14.48 ($\pm$5.26) μm^3 μm^{-2} (Fig. 4). In comparison, biofilm formation by the BrpB-deficient mutant, JB409, and BrpB- and BrpA-deficient double mutant, JB819, was drastically reduced, with a biovolume of 0.38 ± 0.24 μm^3 μm^{-2} ($P<0.001$) for JB409 and 0.02 ± 0.01 μm^3 μm^{-2} ($P<0.001$) for JB819, respectively. Further analyses using COMSTAT 2.0 also revealed that JB409 and JB819 had reduced the surface area of the biofilms by 1 log and 2 logs, respectively, when compared to UA159. The mean and maximum thicknesses of the biofilms formed by JB409 and JB819 were also significantly reduced, as compared to UA159 (Fig. S2). As expected, complementation in strains JB409C and JB819C restored biofilm formation to a level similar to that of UA159 (Figs 4 and S2). In addition, fluorescence staining using Concanavalin A-conjugated antibody also revealed that the BrpB-deficient mutant, JB409, possessed reduced glucans as compared to the wild-type UA159 (data not shown).

BrpB deficiency causes defects in cell division and alters cell morphology

Unlike the parent strain, UA159, the BrpB-deficient mutant, JB409, formed aggregates and precipitated to the bottom of

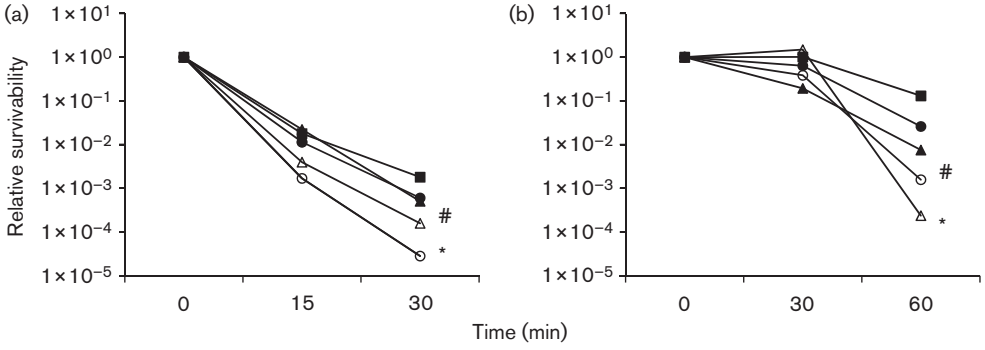


Fig. 2. Acid killing assay of non-adapted (a) and adapted (b) *Strep. mutans* strains. (a) *Strep. mutans* wild-type, UA159 (■), BrpB-deficient mutant, JB409 (○), BrpB-complemented strain, JB409C (●), BrpB- and BrpA-deficient double mutant, JB819 (Δ), and BrpB- and BrpA-complemented strain, JB819C (▲), were grown in BHI until mid-exponential phase (OD_{600} 0.3). Bacterial cells were then harvested by centrifugation and washed twice in 0.1 M glycine, pH 7.0. Acid killing was performed by incubating the bacterial cells in 0.1 M glycine buffer, pH 2.8, for periods of 15, 30 and 45 min. Data presented here are representative of three independent experiments; significant difference is indicated by *, $P<0.001$, and #, $P<0.01$, as compared to the wild-type under similar conditions. (b) For adaptive conditions, bacterial cells were incubated in BHI, pH 5.0, for 1 h prior to acid killing. Other details are the same as for (a).

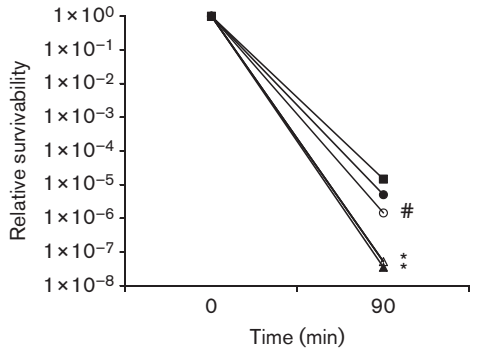


Fig. 3. Hydrogen peroxide killing assay. *Strep. mutans* wild-type, UA159 (■), BrpB-deficient mutant, JB409 (○), BrpB-complemented strain, JB409C (●), BrpB- and BrpA-deficient double mutant, JB819 (Δ), and BrpB- and BrpA-complemented strain, JB819C (▲), were grown in regular BHI broth, harvested during mid-exponential phase (OD_{600} 0.3), and then washed twice with 0.1 M glycine buffer before being subjected to hydrogen peroxide killing by incubating the cells in 0.1 M glycine buffer with 0.2 % (w/v) hydrogen peroxide for a period of 90 and 110 min. Results showed that BrpB deficiency weakened the ability of the mutants, JB409 and JB819, to withstand hydrogen peroxide stress. Data presented here are representative of three independent experiments with significant difference being indicated by *, $P<0.001$, and #, $P<0.01$, as compared to the wild-type under similar conditions.

the culture tubes during growth in BHI broth. FE-SEM analysis revealed that when grown in BM with glucose, sucrose, or glucose plus sucrose, as the supplementary carbohydrate sources, the BrpB-deficient mutant, JB409, displayed elongated, rod-like cell morphology and slightly longer chaining properties (Fig. 5), and such characteristics appeared to be most evident during growth in BMGS. The BrpB- and BrpA-deficient double mutant, JB819, grown in BMG, displayed irregular cell septa as compared to the wild-type UA159 (Fig. 5). The inclusion of 2 mM sucrose in BMGS exacerbated the predominance of irregular cell septa and the overall biofilm architecture of both JB409 and JB819, resulting in the formation of swollen marble-like cell clusters, which is likely a reflection of defects in cell division, and is similar to recent findings with mutants deficient of BrpA (Bitoun *et al.*, 2013). When grown in BM with sucrose only, differences between JB409 and UA159 became less obvious, while the irregular cell septa of JB819 and marble-like cell phenotypic characteristics remained evident, and more cell debris was seen, possibly resulting from increased cell lysis.

Under TEM with cultures grown in regular BHI broth, the BrpB mutant, JB409, and the BrpB/BrpA-deficient double mutant, JB819, displayed striking defects in cell morphology, when compared to the wild-type, UA159. As shown in Fig. 6, both JB409 and JB819 were littered with regions of low electron density in both non-dividing and dividing cells. In addition, the cross-sectional surface areas of the deficient mutants were also increased from 0.13 ($\pm$0.02) μm^2 for UA159 to 0.15 ($\pm$0.03) μm^2 for JB409 ($P<0.01$) and to 0.18 ($\pm$0.04) μm^2 for JB819 ($P<0.01$), consistent with the results of FE-SEM analysis (Fig. 5).The nucleoid also appears very grainy and disorganized in JB409 and JB819, when compared to UA159. As expected, the complementation strains, JB409C and JB819C, all had morphological characteristics that were similar to the wild-type.

BrpB deficiency causes elevation of autolysis rates

As compared to the parent strain, UA159, both the BrpB-deficient strain, JB409, and the BrpB/BrpA-deficient double mutant, JB819, showed increased autolysis rates, especially upon the inclusion of 0.2 % Triton X-100 (Fig. 7).

DISCUSSION

As the outermost barrier between the cell and the environment, the cell envelope is known to play a crucial role in a number of biological processes vital for bacterial growth and survival, including environmental sensing and signal transduction, maintenance of osmotic pressure, cell shape and size, and cell division. As part of a two-gene operon, *brpB* is genetically linked to *brpA*, which encodes a paralogue of a highly conserved LCP family of cell wall-associated proteins. Data presented here have shown that like BrpA, BrpB plays an important role in *Strep. mutans* cellular physiology. BrpB deficiency increases autolysis rates, drastically weakens acid and oxidative stress tolerance responses, and causes major defects in biofilm formation in the deficient mutants. These results further suggest that BrpB and BrpA are linked in genetic structure as well as functionality in the regulation of cell division, stress tolerance response and cell envelope biogenesis/homeostasis.

Like YdiB in *B. subtilis*, but different from YjeE in *E. coli* (Allali-Hassani *et al.*, 2004; Handford *et al.*, 2009; Karst *et al.*, 2009), BrpB deficiency in *Strep. mutans* is viable, but causes an extended lag phase and increased doubling time of the deficient mutants, which could be attributed to defects in stress tolerance response and cell division. Similar to YjeE in *E. coli*, whose depletion was shown to result in unusual cell morphology, including alterations in nucleoid distribution (Handford *et al.*, 2009), *Strep. mutans* strains deficient of BrpB also displayed higher numbers of elongated, giant cells, and seemingly, disorganization of the nucleoid under FE-SEM and TEM, which is also indicative of defects in cell division and cell envelope biogenesis/homeostasis. The increased bacterial cell size seen in JB409 and JB819 also suggests alterations in the osmotic pressure on the cell; therefore, this could in part explain the increased autolysis rates, whereby swelling causes the cells to lyse. Characteristically, all BrpB-deficient mutants possessed low-density patches under TEM, which appears to be unique to BrpB in *Strep. mutans*. It is worth pointing out that the

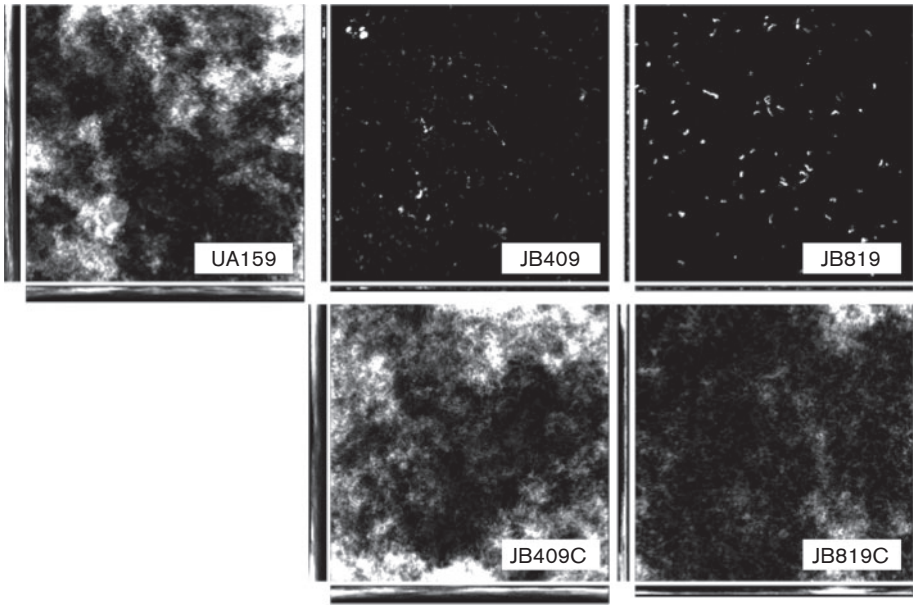


Fig. 4. Confocal laser scanning microscopy analysis of biofilms. *Strep. mutans* wild-type, UA159, BrpB-deficient mutant, JB409, BrpB-complemented strain, JB409C, BrpB- and BrpA-deficient double mutant, JB819, and BrpB- and BrpA-complemented strain, JB819C, were cultivated in BMGS for 24 h with biofilms growing on HA discs deposited vertically in 24-well plates. Following staining using SYTO62, biofilms were dissected using an Olympus laser scanning confocal microscope, and post-acquisition analyses were performed with SLIDEBOOK 5.0 and COMSTAT 2.0. As shown, biofilm formation on vertical HA discs for the BrpB-deficient mutant, and the BrpB- and BrpA-deficient double mutant, was significantly reduced, as compared to the wild-type. Images shown are representative stacks composed of *xyz*, *xz* and *yz* dimensions (512×512). At least five independent fields were dissected at ×600 magnification, and more than three independent experiments were carried out for quantitative analysis using COMSTAT, which is shown in Fig. S2.

low-density patches seem to have a structure/texture different from the nucleoid, although their exact nature awaits further investigation. Nevertheless, defects in cell envelope biogenesis/homeostasis and nucleoid organization would likely affect the ability of the deficient mutants to survive and adapt to harsh environmental conditions, influence cell division and growth, cause alterations in morphology, and induce elevations in autolysis rates as observed with the BrpB-deficient mutants, JB409 and JB819.

Strep. mutans is known to possess an adaptive acid tolerance response, featuring an elevated acid tolerance after initial exposure to low pH conditions. The ability of the BrpB-deficient mutant, JB409, to adapt to low pH and mount an elevated survival rate after 1 h incubation in BHI of pH 5.0 suggests that BrpB deficiency primarily affects the constitutive acid tolerance response, such as changes related to cell envelope biogenesis and integrity, but not the adaptive acid tolerance response. Like several members of the LCP proteins (Hanson *et al.*, 2011, 2012; Lazarevic *et al.*, 1992), BrpA in *Strep. mutans* can also function as a transcriptional regulator (Bitoun *et al.*, 2012a, 2013; Wen *et al.*, 2006). Previous studies showed that deficiency of BrpA caused substantial alterations in the transcriptional profile of the deficient mutant, including changes in transcripts involved in peptidoglycan biosynthesis, DNA repair and stress tolerance response, and biofilm formation (Bitoun *et al.*, 2012a, 2013; Wen *et al.*, 2006). Consistent with our previous findings that BrpA is a key regulator of the acid tolerance response in *Strep. mutans* (Wen *et al.*, 2006), deficiency of both BrpA and BrpB further reduced the survival rate of the deficient mutant after acid killing at pH 2.8. Different from the respective BrpB and BrpA single mutants, however, the BrpB/BrpA-deficient double mutant showed limited capacity to mount an adaptive acid tolerance response after initial incubation in low pH medium. Considering both the BrpA (Bitoun *et al.*, 2013; Wen *et al.*, 2006) and BrpB single mutants were still capable of launching an adaptive acid tolerance response, a logical explanation is that BrpB and BrpA double deficiency imposed an additive effect on the deficient mutant that severely damaged or destroyed the constitutive acid tolerance responses, such as those on cell envelope integrity and nucleoid structure. Even if not directly affected, the adaptive acid tolerance response in JB819 failed to sufficiently compensate the resulting defects in acid tolerance response. When introduced via a multiple copy shuttle vector, pDL278, the higher level of BrpA and BrpB in the complemented strain, JB819C, would likely impose a dominant negative effect, which could be in part attributed to the extremely extended lag phase of the complemented strain during growth in pH 6.5 (Fig. 1b)

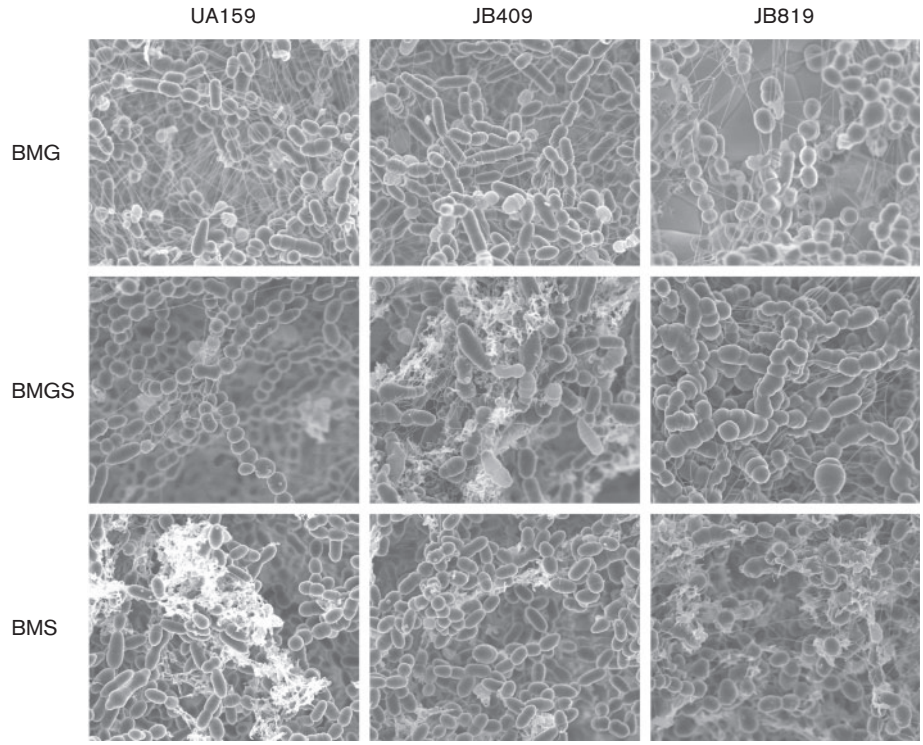


Fig. 5. FE-SEM analysis of biofilms. *Strep. mutans* wild-type, UA159, BrpB-deficient mutant, JB409, and BrpB- and BrpA-deficient double mutant, JB819, were grown in BMG, BMS or BMGS. After 24 h, the biofilms grown on HA discs deposited horizontally in 24-well plates were fixed with 2.5 % glutaraldehyde overnight followed by a series of ethanol dehydrations, and dried at the critical point of CO_2, and then carbon coated and viewed with a Hitachi S-4800 high-resolution microscope. Unlike UA159, JB409 and JB819 formed long chains with multiple sites of cell division when grown in BMG. When grown in BMGS, both JB409 and JB819 showed swollen, giant cells possibly due to incomplete and/or aberrant cell division. However, when grown in BMS, the chaining phenotype was less severe, but relative to UA159, JB819 exhibited more cell debris, which is consistent with the findings that JB409 and JB819 had a higher autolysis rate (Fig. 7). Images shown are representative of three independent experiments at ×10 000 magnification.

and the failure to restore the increased sensitivity to hydrogen peroxide of the double mutant (Fig. 3).

Strep. mutans has adapted biofilms as its primary life style. It produces at least three glucosyltransferases that utilize sucrose to produce adhesive glucans, which function as scaffolding matrix, playing a crucial role in biofilm formation. Accumulating data also suggests that formation of mature biofilms requires coordination of an array of physiological and biochemical reactions in response to environmental conditions. This study showed that BrpB deficiency in *Strep. mutans* affects biofilm formation by the deficient mutants, especially during growth in BM supplemented with glucose and sucrose. In comparison with the wild-type, BrpB-deficient mutants had limited ability to bind to and accumulate on a surface in biofilms, as evidenced by drastic reductions in surface area, thickness and biovolume of the mutant during growth on HA discs. The weakened tolerance responses to acid and oxidative stress, the elevated autolysis rate, and especially the growth defects demonstrated by the extended lag phase and delayed doubling time of the deficient mutants are likely the major contributing factors. Defects in cell envelope as a result of BrpB deficiency will also affect cell-surface and cell–cell interactions, modulating biofilm formation. In addition, fluorescence staining using Concanavalin A-conjugated antibody also revealed that the BrpB-deficient mutants possess reduced glucans. Among others, the growth defects of the deficient mutants are likely the major contributor to the reduced glucan production. Nevertheless, modulation of glucan production in response to BrpB deficiency also explains why the most drastic impact on biofilm formation was seen with cultures grown in medium with sucrose.

While the YjeE-like proteins are highly conserved in eubacteria, many intracellular and symbiotic bacteria do not possess any of these homologues. As shown by data presented here, differences also exist between the YjeE homologues in primary sequence and protein function, as well as the genetic structure of the coding regions. Phylogenetic analysis revealed that in *E. coli* and most

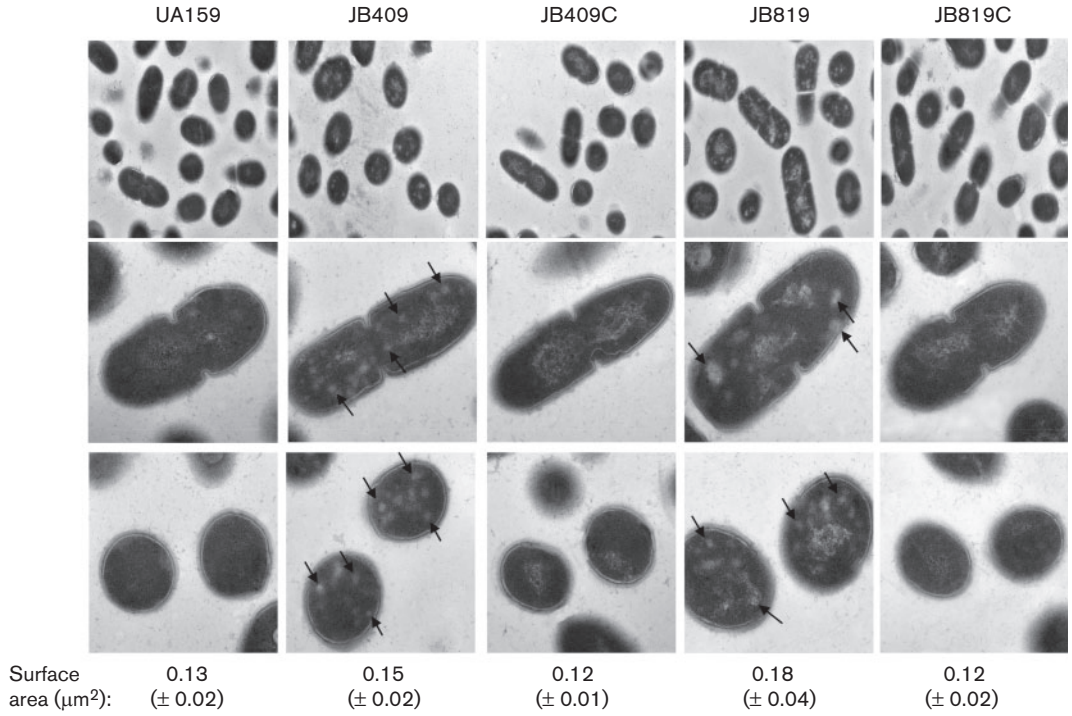


Fig. 6. TEM analysis. *Strep. mutans* wild-type, UA159, BrpB-deficient mutant, JB409, BrpB-complemented strain, JB409C, BrpB- and BrpA-deficient double mutant, JB819, and BrpB- and BrpA-complemented strain, JB819C, were grown in BHI, pH 7.4, until mid-exponential phase. When compared to the parent strain, UA159, both JB409 and JB819 displayed patches of low-density areas (shown by arrows in the middle and bottom panels), as a result of BrpB deficiency. In addition, the nucleoid of JB409 and JB819 also appeared rougher when compared to UA159. Complementation with a copy of the respective wild-type gene plus its cognate promoter(s) in JB409C and JB819C was able to restore the wild-type phenotype. Images in the top panel were taken at a magnification of ×10 000 and those in the middle and bottom panels were taken at ×50 000. Numbers under the image panels are surface areas as means (± SD) of the cell sections.

γ-proteobacteria, the *yjeE* locus is closely linked to genes encoding proteins with potential functions in DNA repair and peptidoglycan remodelling, which is consistent with a role for YjeE in stress tolerance and cell envelope biogenesis (Handford *et al.*, 2009; Teplyakov *et al.*, 2002). All streptococci whose genomes have been sequenced possess a gene encoding a YjeE-like protein. Unlike *E. coli* and the proteobacteria, the loci encoding the YjeE-like proteins in all streptococci are closely linked to genes encoding a member of the LCP family of proteins. This genetic structure appears to be unique to streptococci, as no such linkage could be identified in other Gram-positive bacteria such as *Staph. aureus* and *B. subtilis* that have been shown to possess LCP paralogues. Highly conserved in Gram-positive bacteria, LCP proteins are widely considered as cell wall-associated transcriptional regulators (Hübscher *et al.*, 2008), although there is recent evidence suggesting that LCP proteins are responsible for covalent attachment of anionic cell wall polymers, such as teichoic acid and capsular polysaccharides, to peptidoglycan (Eberhardt *et al.*, 2012; Kawai *et al.*, 2011). BrpA and several other LCP paralogues have been shown to play a major role in cell envelope biogenesis (Bitoun *et al.*, 2012a, 2013; Eberhardt *et al.*, 2012; Johnsborg & Håvarstein, 2009; Kawai *et al.*, 2011; Over *et al.*, 2011; Wen *et al.*, 2006). This highly conserved genetic linkage, along with the characteristic phenotypes of the BrpB-deficient mutants, all support a role for BrpB and its streptococcal homologues in cell wall biogenesis/homeostasis. To assess whether *brpB* is regulated by BrpA, real-time PCR was also used to analyse the transcription of *brpB* in response to BrpA deficiency. The results showed no significant differences between the parent strain and the deficient mutant, TW14D (Bitoun *et al.*, 2012a; Wen *et al.*, 2006), during growth in early exponential phase (OD_{600} 0.3), suggesting that *brpB* is not directly under the control of BrpA under the conditions studied. In *E. coli* and *Salmonella typhimurium*, YjeE was shown to interact with YeaZ (a protease) and YeaZ–YgiD (an atypical DNA-binding protein) complex (Handford *et al.*, 2009; Nichols *et al.*, 2013). Unlike YjeE and YeaZ, *Strep. mutans* and some other streptococci do not seem to possess the YgiD orthologue. The absence of YgiD again suggests differences in the function of BrpB in *Strep. mutans*, and probably the other YjeE-like proteins in other streptococci, from their counterparts in *E. coli* and *Salm. typhimurium*. Considering the drastic differences in the growth and stress responses of the BrpB/BrpA-deficient double mutant, JB819, from the respective single

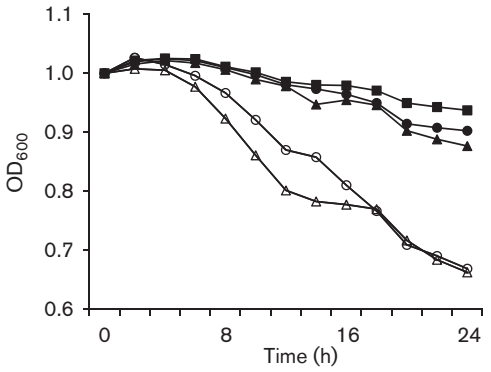


Fig. 7. Autolysis assay. Overnight cultures of *Strep. mutans* wild-type, UA159 (■), BrpB-deficient mutant, JB409 (○), BrpB-complemented strain, JB409C (●), BrpB- and BrpA-deficient double mutant, JB819 (Δ), and BrpB- and BrpA-complemented strain, JB819C (▲), were washed and resuspended in 100 mM PBS, pH 7.2, containing 0.2 % (v/v) Triton X-100, and the optical density at 600 nm was then monitored continuously using a Bioscreen C for 24 h. The autolysis rates of JB409 and JB819 were significantly increased as compared to the wild-type UA159. Complementation strains JB409C and JB819C showed autolysis rates restored to a level similar to the wild-type. Data presented here are representative of three independent experiments.

mutants, JB409 and TW14D (Bitoun *et al.*, 2013; Wen *et al.*, 2006), and especially its complemented strain, JB819C, which possesses a high copy number of both *brpA* and *brpB*, it is highly possible that BrpB and BrpA interact in the regulation of cellular physiology in *Strep. mutans*. Like the two-component system LiaRS of *B. subtilis* (Schrecke *et al.*, 2013), proper stoichiometry of BrpB and BrpA in *Strep. mutans* may be crucial to a functional Brp system. Overexpression of BrpB and BrpA in the complemented strain may cause dysregulation of the Brp system, resulting in an imbalance in cell envelope composition and compromises in cell envelope integrity, which could also be in part attributed to the severely delayed lag phase and the failure to restore the increased susceptibility to hydrogen peroxide.

In summary, the results presented here suggest that while YjeE-like proteins are highly conserved in eubacteria, differences exist in amino acid composition, function and genetic structure between these proteins in different species. In *Strep. mutans*, BrpB is genetically linked to and appears to function in concert with BrpA in the regulation of cell envelope biogenesis/homeostasis. Current effort is being directed to elucidation of the mechanism by which these important regulatory proteins interact in related functions.

ACKNOWLEDGEMENTS

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