INTRODUCTION

One of the most prevalent diseases in the world today, dental caries, is caused by the demineralization of the hard surfaces of the teeth, primarily by acidic species of oral bacteria, most prominently represented by *Streptococcus mutans* (Nyvad et al., 2013; Takahashi & Nyvad, 2011). Despite the fact that developed countries have widespread oral hygiene programmes, the disease is still a problem for large segments of populations, particularly those in low socio-economic groups (Dye & Thornton-Evans, 2010; Dye et al., 2007), and its incidence is rising in developing countries where access to care is limited and consumption of refined carbohydrates has increased substantially (Baehni & Guggenheim, 1996).

*S. mutans* contributes to the formation and accumulation of a multi-species biofilm on tooth enamel, where it is exposed to a dynamic oral environment (Bowen & Koo, 2011; Loesche, 1986). The initiation of biofilm formation begins with the attachment of oral bacteria, generally non-*mutans* streptococci (Whitmore & Lamont, 2011), to host-derived proteins located in the membranous film on teeth referred to as the salivary pellicle (Bowen & Koo, 2011). *S. mutans* is considered a relatively late-colonizing organism that binds to early colonizers, which are bathed in saliva (Bowen & Koo, 2011). When dietary sucrose becomes available, water-insoluble glucans are rapidly formed by extracellular glucosyltransferases (Bowen & Koo, 2011). As glucans are continuously formed, plaque accumulates, enmeshing multiple organisms, salivary proteins and food particles (Bowen & Koo, 2011). Growth within the plaque biofilm promotes differential expression of a number of virulence factors compared with planktonic growth (Biswa & Biaswas, 2005; Huang et al., 2008; Klein et al., 2012a, b; Lemos et al., 2013; Xiao et al., 2012). *S. mutans* competes and survives in the plaque biofilm by fermenting a large variety of sugars to produce organic acids (Belli & Marquis, 1994; Bowen & Koo, 2011; Smith & Spatafora, 2012; Zeng et al., 2013). Furthermore, *S. mutans* is able to store excess carbohydrates as intracellular polysaccharides (IPS), and contains genes for both glycogen and starch synthesis (Ajdic et al., 2002; Harris et al., 1992). In fact, a strain of *S. mutans* that synthesizes IPS at higher levels exhibited elevated virulence (Spatafora et al., 1995).

The nature of the plaque biofilm does not allow for easy diffusion of lactic acid, which becomes concentrated close to the tooth enamel (Jensen & Schachtele, 1983; Jensen...
et al., 1982; Xiao et al., 2012). The concomitant low pH of the microenvironment contributes to the erosion of the tooth surface, leading to demineralization and dental caries (Loesche, 1986). To cope with environmental changes in the oral cavity, S. mutans has evolved a number of stress-response pathways (Lemos et al., 2013; Matsui & Cvitkovitch, 2010), including the ability to alter its membrane fatty acid composition and elevate production of its FpFp-ATPase pump to maintain intracellular pH (Fozo & Quivey, 2004a; Fozo et al., 2004; Kuhnert et al., 2004). Acid tolerance is probably a principal mechanism that facilitates the emergence of S. mutans as a dominant organism among plaque bacteria as caries progresses in humans (Gross et al., 2012).

During physiological screens of single-gene deletion mutant strains of S. mutans for acid-sensitive phenotypes, a strain deleted for SMU.1747c (as designated in the NCBI database, http://www.ncbi.nlm.nih.gov/guide/proteins/) exhibited significant reduction in growth under acidic conditions (our unpublished data). SMU.1747c is annotated as a hypothetical protein of unknown function in the S. mutans genome.

Alignment of the deduced amino acid sequence for SMU.1747c to predicted proteins from the genomes of other Firmicutes revealed a strong homology to the active site of the β-phosphoglucomutase (PgmB) from Lactococcus lactis (Levander et al., 2001). PgmB functions to convert β-D-glucose 1-phosphate (βDG1P) to β-D-glucose 6-phosphate, a more metabolically active sugar phosphate in bacterial cells (Selinger & Schramm, 1961), and should not be confused with the near ubiquitous presence of α-phosphoglucomutase (which converts α-D-glucose 1-phosphate to α-D-glucose 6-phosphate) in lactic acid bacteria.

Here, we report the results of the initial characterization of the role of SMU.1747c in the pathophysiology of S. mutans. Our study revealed a connection between the presence of PgmB and the acid resistance of S. mutans, apparently involving production of membrane fatty acids and fatty alcohols, and that loss of the pgmB gene resulted in reduced virulence in a model of bacterial infection. The results of these studies demonstrate that the protein encoded by SMU.1747c, PgmB, plays a role in the virulence potential of S. mutans.

METHODS

Strains and growth conditions. S. mutans strain UA159 was used as the parental strain for all experiments (Ajdic et al., 2002; Murchison et al., 1986). Strain MU1593 was created by replacement of the SMU.1747c ORF with a non-polar erythromycin-resistance cassette flanked by approximately 400 bp upstream and downstream of the gene using primer pair MU1593P7UPFGA026 (5′-ACATAT-CATCAAATACGGGTGC-3′) and MU1593P8DNRA026 (5′-GTTTCATAAAGCTCTTCTTTCTG-3′) to facilitate homologous recombination, as previously reported (Santiago et al., 2012). The intergenic region preceding the SMU.1747c coding region was left intact upstream of the erythromycin-resistance cassette. S. mutans strains UA159 and MU1593 were grown on brain heart infusion (BHI; BD/Difco) agar at 37°C in a 5% (v/v) CO2/95% air incubator. Antibiotics were added to a final concentration of 5 µg erythromycin ml−1 or 1 mg kanamycin ml−1. Cultures were grown in TY medium (3% tryptone, 0.1% yeast extract, 0.5% KOH, 1 mM H3PO4) for planktonic culture conditions, as well as steady-state culture conditions in a BioFlo 2000 fermenter (New Brunswick Scientific). Planktonic cultures were grown with the addition of 1% (w/v) glucose. Steady-state cultures were grown at a dilution rate of 0.144 l h−1 under glucose-limiting conditions (2.3 mM), with a continuous impeller speed of 200 r.p.m. Steady-state pH levels were maintained by the addition of 2 M KOH. The culture pH was continuously monitored throughout the experiment by using an indwelling pH probe (Mettler Toledo). Cell samples from continuous culture were collected by centrifugation after growth for a minimum of 10 generations. Biofilm cultures were grown on glass slides in TY medium with 1% (w/v) sucrose for 5 days, transferring the slides to fresh medium daily. Cell pellets were either used the same day for cell survival assays, or washed with double distilled H2O and pellets stored at −80°C prior to fatty acid or enzyme analysis.

Escherichia coli DH10B was grown on Luria–Bertani (LB) agar medium at 37°C. Liquid cultures were grown with shaking at 37°C in LB or SOC medium (Hanahan, 1983). Antibiotic was added to a final concentration of 50 µg kanamycin ml−1.

Growth curves were recorded using an automated growth-monitoring device (Biosea C; Growth Curves). Measurement intervals were taken either every 15 min or hourly at 600 nm (OD600) with a 10 s shaking period before each reading, to suspend cultures evenly.

Genetic complementation of the MU1593 mutant strain. A genetic complement strain was created using primers to amplify the SMU.1747c coding region and cognate promoter from UA159 chromosomal DNA. Primer 1593-PROMBGLUP (5′-GACTAG-CTGAAAATTTGACAG-3′) binds 62 bp upstream of the putative SMU.1747c translational start site and contains a BglII restriction site (underlined). Primer 1593BGLDWN (5′-CAGTAG-ATCTTCAAAGTTCTAATCTC-3′) binds downstream of the SMU.1747c coding region and contains a BglII restriction site (underlined). The resulting PCR product was cloned into pCREBlunt (Invitrogen), and transformants were confirmed by nucleotide sequencing. A positive clone was digested with BglII, and the target band was gel purified using a QIAquick gel extraction kit (Qiagen). The recovered DNA fragment was cloned into pSUGK1593 (Derr et al., 2012), transformed into E. coli DH10B, and transformants were screened by colony PCR for proper integration. One positive recombinant plasmid was named pSUGK1593 and verified further by nucleotide sequencing. The MU1593 mutant strain was transformed with pSUGK1593, and colonies were selected on BHII agar medium containing kanamycin. Transformants were confirmed by colony PCR and nucleotide sequencing. One transformant, containing the SMU.1747c coding region, from −45 to +645, in the gfsA locus (SMU.881), was named S. mutans UR286.

β-phosphoglucomutase assays. β-Phosphoglucomutase activity was assayed as previously described (Martin & Russell, 1987; Sjöberg & Hahn-Hägerdal, 1989). Conversion of DG1P (Omicron Biochemicals) to glucose 6-phosphate (G6P) was extrapolated by spectrophotometric measurement of NADPH formation from NADP+ at 340 nm, using S. mutans cell extracts and G6P dehydrogenase (Sigma). Protein concentration was measured by the Bradford assay (Bradford, 1976). Activity is expressed as nmol min−1 (mg protein)−1.

Galleria mellonella (greater wax worm) killing assays. G. mellonella killing assays were performed as described by Kajfazs et al. (2010). Larval insects were purchased from Vanderhorst, stored at 4°C in the dark and used within 4 days of shipment. Groups of 20 larvae, ranging from 220 to 300 mg in weight, and with no signs of melanization, were used for subsequent infection. Groups injected

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with saline solution were used as controls in each experiment. After injection, larvae were incubated at 37 °C, monitored and scored as dead when they displayed no thigmotaxis. Kaplan–Meier killing curves were plotted, and a log-rank test was used to determine significance (P≤0.05) using GraphPad Prism v4.0 (Graphpad Software). Experiments were repeated three times.

Survival assays. Acid survival assays were performed as described by Derr et al. (2012) and Quivey et al. (1995). Cells harvested from steady-state cultures, glass slide biofilms or planktonic cultures of S. mutans UA159 and MU1593 were exposed to an acid challenge of 0.1 M glycine, pH 2.5. Aliquots were sampled at 0, 15, 30 and 60 min, serially diluted and plated on BHI agar medium to obtain c.f.u. Total c.f.u. were counted to estimate cell viability at each time point compared with time zero to obtain per cent survival.

GC fatty acid methyl ester analysis of fatty acid composition. S. mutans cultures were grown either to steady state in a chemostat, as an overnight culture or in glass-slide biofilm conditions. Cells were collected by centrifugation, washed with double distilled H2O and stored at −80 °C. The frozen cell pellets were analysed for fatty acid content using the methyl ester assay of Bligh & Dyer (1959), performed by Microbial ID.

Real-time PCR. Four independent cultures of S. mutans UA159, MU1593 and UR286 (pgmB genetic complement strain) were grown in BHI medium to late exponential phase (OD660 of ~0.7). Cells were harvested and RNA was extracted according to the methods of Abranches et al. (2011) and Santiago et al. (2012). RNA was used along with random primers to synthesize cDNA with the High Capacity cDNA Reverse Transcription kit (Life Technologies). Primer pair 5′-TAATGGTGTCGGGACCTTAC-3′ (FABM RTF) and 5′-AACAGCTCTTACATCTCAACC-3′ (FABM RTR) was used to amplify fabM-specific mRNA using Power SYBR Green Master Mix (Life Technologies) from three replicates of each sample. Primer pair 5′-ACTGGGCCGCAATAAGACAG-3′ (FABQ RTF) and 5′-AGGATGCGGCTGTTCATCAC-3′ (FABQ RTR) was used to amplify pgmB-specific mRNA using Power SYBR Green Master Mix (Life Technologies) from three replicates of each sample. Reactions were carried out in a Step One Plus Real-time PCR System (Life Technologies). The mRNA copy number was quantified based on a standard curve of fabM or pgmB PCR product (Abranches et al., 2011).

Proton permeability. Proton permeability assays were performed as described by Bender et al. (1986) and MacGilvray et al. (2012). Cultures of strain MU1593 were grown in 200 ml TY plus 1 % (w/v) glucose at 37 °C in a 5% (v/v) CO2/95% air atmosphere overnight. Cells were harvested by centrifugation, washed in 5 mM MgCl2, resuspended in 20 mM potassium phosphate buffer (pH 7.2), 50 mM KCl and 1 mM MgCl2, and incubated for 2 h. After incubation, cells were harvested and resuspended in 50 mM KCl and 1 mM MgCl2 to 20 mg ml−1. A 1 ml aliquot was titrated to pH 4.7 by the addition of 10 mM HCl and 50 mM KCl, and the pH was recorded. After 50 min, butanol was added to a final concentration of 10% (v/v), to disrupt the cell membrane and allow the pH of the suspension to equilibrate. At 80 min, end-point pH was recorded.

Minimum glycolytic pH determination. Determination of the minimum glycolytic pH of S. mutans UA159 and MU1593 was accomplished using established protocols (Belli & Marquis, 1994; Santiago et al., 2012). The mutant strains were grown overnight in BHI medium supplemented with 1% (w/v) glucose. Cells were centrifuged, washed, and resuspended in 50 mM KCl and 1 mM MgCl2 to a final concentration of 10 mg cell dry weight ml−1. One 5 ml aliquot of the cell suspension was stirred at room temperature and adjusted to pH 7.2 using 10% (w/v) KOH. Glucose was added to the suspension to a final concentration of 55.5 mM, and the pH was measured every 15 s for 30 min. The experiment was performed in triplicate for three independent cultures.

ATPase assays. Cultures of S. mutans UA159 and MU1593 were grown in BHI medium overnight. ATPase activity, measured as inorganic phosphate released from ATP, was determined according to a previously described protocol (Belli & Marquis, 1991; Santiago et al., 2012). Assays were performed using permeabilized cells from three independent cultures of each strain, assayed in duplicate. Protein concentration was estimated by the bichinchoninic acid assay (Smith et al., 1985) (Sigma). Results are expressed as millimoles PO4− released min−1 (mg protein)−1.

RESULTS

SMU.1747c is orthologous to Pgmb from L. lactis

The predicted protein sequence encoded by the S. mutans SMU.1747c ORF indicated 35 and 59% identity to the proteins denoted as Pgmb in Streptococcus thermophilus and L. lactis, respectively, using the PSI-BLASTP alignment algorithm (Altschul et al., 1997) (Fig. 1b). The pgmB gene of S. thermophilus was deduced from genomic sequence information (STR.0379); however, the pgmB gene of L. lactis has been extensively characterized (Levander et al., 2001; Levander & Rådström, 2001). The S. mutans SMU.1747c protein is predicted to have a molecular mass of approximately 24.5 kDa, similar to other Pgmb proteins.

PgmB activity is abolished in the MU1593 strain of S. mutans

PgmB activity, measured by the reduction of NADP+ in the presence of βDG1P, was quantitatively assayed using cell-free extracts of strains UA159 and MU1593 (Fig. 2). The parental strain UA159 yieldd approximately 100 nmol NADP+ min−1 (mg protein)−1. However, strain MU1593 displayed no detectable Pgmb activity. The complement strain, UR286, showed restoration of PgmB activity, although not to the level of the UA159 parental strain. We ascribe restoration of pgmB activity in the complement to transcription of pgmB in UR286 as confirmed by quantitative real-time PCR (Fig. S1, available in the online Supplementary Material). The results indicated strongly that the SMU.1747c locus encodes a functional orthologue of PgmB in S. mutans, and will be referred to as such throughout.

Loss of PgmB leads to reduced growth rate and sensitivity to low pH conditions

The pgmB mutant strain was first characterized by its reduced ability to grow in media buffered to pH 5.0, compared with the parental strain, UA159 (Fig. 3b). No difference in growth rate was observed between strains UA159 and MU1593 during the exponential growth phase in medium buffered to pH 7.0 (Fig. 3a). The complement strain, UR286, partially restored the growth rate to near
parental levels under acidic conditions (Fig. 3b). Generation time was also determined for growth under oxidative stress conditions, using medium containing 0.5 mM H$_2$O$_2$. No differences between the parental and deletion strain were observed (data not shown). The results suggest that pgmB may play a role in acid stress resistance.

We investigated the possibility that loss of pgmB might affect the ability of S. mutans to adapt to acid stress conditions, as measured by survival in the relatively harsh, acidic conditions of glycine solution, at a pH of 2.5 (Belli & Marquis, 1991). The results showed that, compared with the UA159 parental strain, strain MU1593 exhibited significantly reduced acid resistance following growth at pH 7.0 (Fig. 4a).

S. mutans normally resides in multispecies biofilms on tooth surfaces, with conditions very different from those of a continuous, pure culture. We examined the possibility that growth in model biofilms might affect the ability of strain MU1593 to resist acid. The parental strain and MU1593 were grown on solid supports in medium containing sucrose, to allow biofilms to form, over a period of 5 days. Cells recovered from those cultures were examined for their ability to survive exposure to glycine, at pH 2.5, over time. The recovery data indicated that strain MU1593 was approximately two-logarithmic orders more sensitive to acidic conditions than the parental strain (Fig. 4b).

Deletion of pgmB reduces survival of S. mutans in the G. mellonella model

Infection of the haemocoel in the larvae of G. mellonella has been used to evaluate the virulence contribution of S. mutans genes, among other Gram-positive pathogens and fungi (Brennan et al., 2002; Evans & Rozen, 2012; Gonzalez et al., 2012; Kajfasz et al., 2010). The G. mellonella immune system mimics the mammalian innate immune system by producing specialized phagocytic cells known as haemocytes, secreted proteins that mimic mammalian-complement, and proteins that act as membrane disrupters (Vilmos & Kurucz, 1998). We have shown that loss of
*pgmB* results in diminished growth of the deletion strain in the presence of acid and an inability to tolerate acid stress conditions. As aciduricity is a necessary virulence trait for *S. mutans* (Fozo *et al.*, 2007), we hypothesized that survival of MU1593 in a model for virulence, *G. mellonella*, may be affected. Three groups of 20 larvae each were infected with $1 \times 10^7$ c.f.u. of *S. mutans* UA159, MU1593 and UR286 cultures. The *ΔpgmB* strain had a reduced impact on larval survival as compared with the parental strain (Fig. 5). UR286, the *pgmB*+ strain, displayed an intermediate level of survival as compared with the parental and *ΔpgmB* strains (Fig. 5). As complementation of *pgmB* resulted in partial restoration of virulence, we propose that this is due to expression of *pgmB* from an ectopic locus in the genome, removed from cis-acting regulatory molecules.

**Fig. 3.** *S. mutans* MU1593 is acid-sensitive. Growth curves of the parental strain, *S. mutans* UA159, the Δ*pgmB* strain, MU1593, and the *pgmB*+ strain, UR286. Growth was monitored using a Bioscreen C. (a) Growth in BHI medium buffered to pH 7.0. (b) Growth in BHI medium buffered to pH 5.0. Generation time was determined from the exponential phase of growth. $^*P<0.05$ relative to the value for UA159 (Student’s *t*-test; *n* ≥ 3).

**Fig. 4.** Acid survival curves for *S. mutans* UA159 and MU1593. (a) Survival of strains grown in chemostat cultures. Cultures of *S. mutans* UA159 (squares) or MU1593 (triangles) were grown to steady-state pH values of 7 (black lines, filled symbols) and 5 (grey lines, open symbols) at a dilution rate of 0.144 l h⁻¹. Aliquots were removed and exposed to an acidic challenge (pH 2.5) as described in Methods. Time is represented as minutes after resuspension in 0.1 M glycine, pH 2.5. Results are represented as percentage survival (*n* = 3). (b) Survival of strains grown in biofilm cultures. *S. mutans* UA159 (black line) and MU1593 (grey line) were grown in glass slide biofilm cultures in TY medium supplemented with 1 % (w/v) sucrose. Aliquots of cells were exposed to an acid challenge (pH 2.5) as described in Methods. Time is represented as minutes after resuspension in 0.1 M glycine, pH 2.5. Results are represented as percentage survival (*n* = 3).
Physiological data indicated that strain MU1593 was defective compared with UA159 in all growth conditions tested. The fatty acid profile of MU1593 is altered compared with UA159 in all growth conditions tested. Membrane fatty acid composition was determined from cultures of S. mutans UA159 and MU1593 grown to steady-state pH values of 7 and 5. The fatty acid profile developed from strain MU1593 showed that the organism was able to produce UFAs in response to growth at low pH (Fig. 6a); however, it was unable to produce UFAs in similar abundance to those observed in the parental strain at either pH 5 or pH 7. The percentage of branched-chain fatty acids (bcFAs) was elevated at pH 7, but reduced at pH 5 under steady-state growth. The fatty acid composition of cells grown in biofilm cultures was also determined. The ΔpgmB strain again exhibited a smaller percentage of UFAs and bcFAs in the membrane. Interestingly, as compared with strain UA159 grown under the same conditions, biofilm conditions produced an increase in the long-chain fatty alcohol hexadecanol (Fig. 6b). Hexadecanol was not detected in steady-state cultures, but represented an approximately fourfold increase in the percentage of the total fatty acyl chains in cells grown in biofilms.

Transcription of fabM is unaffected by deletion of pgmB

Given the gene arrangement in the UA159 genome with fabM transcriptionally downstream from pgmB, we postulated that the deletion of pgmB could interfere with downstream transcription of fabM. Real-time PCR was performed to analyse transcription of fabM in the parental strain UA159, the ΔpgmB strain and the pgmB⁺ strain, UR286. The results of the analysis indicated that fabM transcription in the ΔpgmB background was not significantly different from those observed in the UA159 parental strain (Fig. S2). Again, as in the wax worm experiment, we observed that results in strain UR286 differed from the parental strain. As these experiments measured the abundance of transcript, we hypothesize that the ectopic location of the pgmB promoter, in the complement strain, may have affected potential regulatory pathways controlling its expression.

MU1593 exhibits reduced glycolytic output from glucose challenge

It has been previously established that loss of fabM, which is responsible for formation of UFAs, leads to reduced glycolytic output as measured by the ability to acidify cell suspensions in the presence of exogenous glucose (Fozo & Quivey, 2004b). The reduced abundance of UFAs in strain MU1593 suggested that the strain might also have reduced ability to produce lactic acid. Using a suspension of cells in salts and 55 mM exogenous glucose, we show that strain MU1593 had indeed lost the ability to reduce ambient pH below 3.77, which was an approx. 0.4-log decrease in acid production compared with the parental strain (Fig. 7).

Proton permeability is increased in strain MU1593

S. mutans is not impervious to the effects of the lactic acid end product of its own metabolism, but it is able to minimize the negative effects of low pH. One coping strategy exhibited by S. mutans is to maintain an intracellular pH that is higher than external conditions, a state that serves to protect acid-sensitive enzyme functions (Bender et al., 1985, 1986; Dashper & Reynolds, 1992; Sheng & Marquis, 2006; Sturr & Marquis, 1990). Previously, it has been demonstrated that alterations of the cellular fatty acid profile affects the permeability of the S. mutans membrane (Fozo & Quivey, 2004b). We therefore examined the impact of the reduced MU1593 UFA composition on its proton permeability, as compared with the parental strain. The membrane of cells of strain MU1593 was more permeable to protons than the parental strain, as indicated by the slopes of the curves between 50 and 80 min, following addition of butanol to cell suspensions (Fig. 8). The pgmB⁺ strain, UR286, exhibited a virtually identical ΔpH to the parental strain; however, strain UR286 also revealed an elevated terminal pH relative to strain UA159.

Fig. 5. Infection of Galleria mellonella by MU1593 results in attenuated virulence. Kaplan–Meier survival plots from G. mellonella larvae injected with 10⁷ c.f.u. per larva of S. mutans UA159 (■), MU1593 (○) or UR286 (□) at 37 °C. Larvae were also injected with non-lethal saline as a control. The experiments were performed three times, and the results shown are representative of a typical experiment. Statistical comparisons were made to the parental strain, UA159 (*P<0.05).
F<sub>1</sub>F<sub>0</sub>-ATPase activity is not impaired in MU1593

Strain MU1593 demonstrated an inability to maintain the same level of glycolytic output as the parental strain (Fig. 7), as well as an inability to maintain ΔpH across the cell membrane (Fig. 8); we therefore examined the ability of the ΔpgmB strain to expel protons from the cell by measuring F<sub>1</sub>F<sub>0</sub>-ATPase activity, another acid-adaptive mechanism utilized by <i>S. mutans</i> (Belli & Marquis, 1991; Kuhnert et al., 2004). However, loss of <i>pgmB</i> did not significantly alter ATPase activity, as compared with the parental strain (Fig. S3).

## DISCUSSION

Previously, it has been observed that <i>S. mutans</i> is able to utilize a wide variety of carbohydrate sources, with some

![Fig. 6. GC-FAME analysis of steady-state and biofilm cultures of UA159, MU1593, and UR286. Comparison of membrane fatty acid composition from cultures of <i>S. mutans</i> UA159, MU1593 (ΔpgmB), and UR286 (pgmB<sup>+</sup>). Cultures were grown to steady-state pH values of 7 and 5 (a), or in biofilms (b). The membrane fatty acid composition is presented by category: blue, saturated fatty acids; red, unsaturated fatty acids; green, branched-chain fatty acids; yellow; hydroxy fatty acids; purple, long-chain fatty alcohols; cream, aldehydes; orange, methylated; black, unknown fatty-acyl compounds. Student’s t-test was used for statistical evaluations (n=3). All categories of fatty acids were significantly different between, and among, strains, except for bcFAs in the parent strain at pH 7 versus 5.<br><br>![Fig. 7. Strain MU1593 has a higher glycolytic pH minimum than strain UA159. The glycolytic pH minimum was determined as described in Methods. <i>S. mutans</i> UA159 (black line) and MU1593 (grey line) were grown in three independent overnight cultures, and each culture was assayed in triplicate. Means and SD for each time point are shown. Terminal pH values are indicated.<br><br>![Fig. 8. The membrane of strain MU1593 is more permeable to protons than that of the parental strain. Membrane proton permeability assays were performed as detailed in Methods. Means ± SD are shown for three independent cultures of <i>S. mutans</i> UA159 (■) (parental strain), MU1593 (△) (ΔpgmB) and UR286 (●) (pgmB<sup>+</sup>) assayed in duplicate. *P<0.001.](image-url)
being preferred over others (Zeng et al., 2013). These carbohydrate alternatives to glucose and sucrose are taken up through ATP-dependent ABC transporters and a phosphoenolpyruvate-dependent phosphotransferase system (Webb et al., 2007, 2008). In L. lactis, maltose and maltodextrin catabolism is accompanied by the formation of βDG1P, necessitating PgmB activity to convert the metabolite to G6P for further metabolism (Levander et al., 2001). However, there are no previous reports of PgmB enzyme activity in oral streptococci, despite the abundance of maltodextrins in the human diet (Bialostosky et al., 2002). In this study, we show that there is a strong active site homology between SMU.1747c and PgmB of L. lactis and that deletion of the gene results in a loss of PgmB activity in the S. mutans deletion strain, and the data strongly suggest that the ORF designated SMU.1747c encodes a homologous PgmB enzyme.

PgmB activity in S. mutans may not be surprising given the number of carbohydrate the organisms has been demonstrated to effectively metabolize for energy (Abranches et al., 2006, 2008; Ajdic & Pham, 2007; Russell et al., 1992). To survive prolonged periods without food, bacteria, including S. mutans, may rely on stored sources of IPS (Berman & Gibbons, 1966). One precursor of IPS is α-D-glucose 1-phosphate, which can spontaneously epimerize to βDG1P. This conversion may occur often enough to warrant pgmB remaining in the genome. Another potential source of βDG1P is from the breakdown of disaccharides such as maltose, trehalose and nigerose (Andersson et al., 2001; Hüwel et al., 1997; Nihira et al., 2012). These carbohydrates can also be found in plaque biofilms (Abranches & Chen, 2013).

Regardless of the sugar substrates, S. mutans must respond to low pH conditions during metabolically active periods. The pgmB mutant strain exhibited deficiencies in its ability to maintain the same level of aciduricity as the parental strain, UA159. To confirm that S. mutans was negatively affected by the loss of pgmB, we used steady-state chemostat cultures as well as in vitro biofilm cultures to determine low pH survival and cellular membrane fatty acid composition. We determined the glycolytic pH minimum, and showed that the reduced glycolytic output was probably related to cell membrane integrity rather than to the ability of the organism to pump \(H^+\) out of the cell via the \(F_{ATPase}\) (Figs 7 and S3).

The reduced virulence of the ΔpgmB strain in the G. mellonella model provides support for the role of PgmB in survival of S. mutans, and in the acid-adaptive repertoire of S. mutans. Previous reports showed a strong relationship between dental caries in rats and the presence or absence of the fabM enzyme, responsible for UFA synthesis (Fo zo et al., 2007). The fabM deletion strain also showed reduced virulence in the G. mellonella model (our unpublished data). As strain MU1593 had a decreased percentage of UFAs compared with the parental strain, it is consistent that it also exhibited reduced virulence in the larval model, yet not to the same degree as the fabM strain. As there was no complete loss of UFAs in strain MU1593, it suggests that absence of PgmB has its own effect on virulence.

While the percentage of UFAs in the membrane of the ΔpgmB strain was reduced, compared with the parental strain, there was no significant difference in the transcript level of fabM between strains UA159 and MU1593 (Fig. S2). S. mutans MU1593 was able to adapt to acidic conditions, albeit not to the same degree as the parental strain. Further distinguishing it from the ΔfabM strain, MU1593 did not increase ATPase production in relation to the parental strain (Fig. S3) (Fozo & Quivey, 2004b). Taken together, this suggests that the loss of pgmB has an effect on the acid-adaptive shift of S. mutans, although the mechanism is not yet clear. This could potentially be due to cis-regulatory elements that are deleted or interrupted in MU1593, altering fabM translation. Alternatively, we did observe that the ΔpgmB strain produced less IPS than the parental strain (data not shown). A current focus of our efforts is to determine whether loss of pgmB, and reduced IPS abundance, are tied to the acid-adaptive strategies of S. mutans.

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