P1sX deletion impacts fatty acid synthesis and acid adaptation in *Streptococcus mutans*

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*Streptococcus mutans*, one of the primary causative agents of dental caries in humans, ferments dietary sugars in the mouth to produce organic acids. These acids lower local pH values, resulting in demineralization of the tooth enamel, leading to caries. To survive acidic environments, *Strep. mutans* employs several adaptive mechanisms, including a shift from saturated to unsaturated fatty acids in membrane phospholipids. PlsX is an acyl-ACP : phosphate transacylase that links the fatty acid synthase II (FASII) pathway to the phospholipid synthesis pathway, and is therefore central to the movement of unsaturated fatty acids into the membrane. Recently, we discovered that *plsX* is not essential in *Strep. mutans*. A *plsX* deletion mutant was not a fatty acid or phospholipid auxotroph. Gas chromatography of fatty acid methyl esters indicated that membrane fatty acid chain length in the *plsX* deletion strain differed from those detected in the parent strain, UA159. The deletion strain displayed a fatty acid shift similar to WT, but had a higher percentage of unsaturated fatty acids at low pH. The deletion strain survived significantly longer than the parent strain when cultures were subjected to an acid challenge of pH 2.5. The Δ*plsX* strain also exhibited elevated F-ATPase activity at pH 5.2, compared with the parent. These results indicate that the loss of *plsX* affects both the fatty acid synthesis pathway and the acid-adaptive response of *Strep. mutans*.

**INTRODUCTION**

Dental caries affects nearly 100 % of adults and 60–90 % of school-aged children in industrialized countries (Petersen et al., 2005). *Streptococcus mutans* is considered the major cause of dental caries owing to the strong correlation between its presence in dental plaque isolated from carious lesions and its ability to cause caries in the absence of other species (Tanzer et al., 2001). The presence of *Strep. mutans* also influences the oral microbiome, discouraging the growth of less aciduric commensal bacteria and enhancing the growth of other harmful micro-organisms (Koo et al., 2010; Pereira-Cenci et al., 2008; Takahashi & Nyvad, 2011). *Strep. mutans* relies on three general phenotypes to cause caries: adherence to the tooth surface, acidogenicity and aciduricity (Banas, 2004; Quivey et al., 2000b).

*Strep. mutans* rapidly acidifies its local environment by fermenting sugars from the host’s diet and producing organic acids, primarily lactate (Argimón & Caufield, 2011; de Soet et al., 2000). This acidification causes enamel demineralization, leading to caries (Banas, 2004; Tanzer et al., 2001). To survive in the acidic environment it creates, *Strep. mutans* utilizes a range of mechanisms and phenotypic changes collectively known as the acid-adaptive response. Some of these changes include: an increase in the production and activity of F₁F₀-ATPase, which pumps protons out of the cell (Kuhnert et al., 2004); an increase in lactic acid excretion (Banas, 2004); and a shift in the membrane fatty acid composition from a majority of saturated fatty acids to a majority of unsaturated fatty acids (Fozo & Quivey, 2004a, b).

The transition of the cell membrane from saturated to unsaturated fatty acids has been shown to affect the ability of *Strep. mutans* to survive at low pH and also its ability to cause disease (Fozo et al., 2007; Fozo & Quivey, 2004a). The severity of smooth surface caries is reduced by approximately 90 % in a *Strep. mutans* strain unable to produce unsaturated fatty acids. An increase in fatty acid chain length is also associated with growth at low pH, but it is currently unknown if this change is necessary for acid adaptation (Fozo & Quivey, 2004b).
The fatty acid shift is due to changes in the regulation of the fatty acid biosynthesis II pathway (FASII) (Fozo & Quivey, 2004a). The FASII pathway is generally conserved among bacteria, archaea and plants as the main pathway for fatty acid production. The majority of fatty acids produced in bacteria are used to synthesize phospholipids, the main components of bacterial cell membranes (Zhang & Rock, 2008a). Because the membrane is essential for cell survival, the FASII and phospholipid synthesis pathways are active, albeit controversial, targets for rational drug design (Brinster et al., 2009; Grimes et al., 2008; Lu & Tonge, 2008; Parsons et al., 2011). Strep. mutans may resist growth inhibition by FASII inhibitors similarly to Streptococcus pneumoniae (Parsons et al., 2011), but treatment with cerulenin (preventing a shift to unsaturated fatty acids) does have a negative impact on the ability of Strep. mutans to survive at low pH (Fozo & Quivey, 2004b).

The PlsX–PlsY pathway is the most common way for bacteria to initiate phospholipid synthesis. PlsX creates acyl-phosphate, the substrate of PlsY, from acyl-acyl carrier protein (acyl-ACP) and inorganic phosphate. PlsY then attaches the acyl group to glycerol-3-phosphate (G3P) to create lysophosphatidic acid (Lu et al., 2006; Yoshimura et al., 2007). PlsC attaches a second fatty acid to produce phosphatidic acid, the most basic phospholipid, and orthologues of plsC are universal in bacteria (Zhang & Rock, 2008a). There is at least one alternative to the PlsX–PlsY pathway for lysophosphatidic acid synthesis: PlsB, a glycerol-3-phosphate (G3P)-acyltransferase, which uses acyl-ACP directly without the creation of an acyl-phosphate intermediate (Lu et al., 2006). plsB is generally not found in Firmicutes such as Strep. mutans, and, in fact, the genome sequence of Strep. mutans UA159 does not contain an orthologue (Ajdic et al., 2002; Zhang & Rock, 2008b).

Despite the lack of plsB, a recent Strep. mutans genetic deletion library (Quivey et al., 2015) revealed that plsX is not an essential gene. An alternative acyl-phosphate pathway that relies on an acyl-ACP thioesterase and a fatty acid kinase was reported in Strep. pneumoniae (Parsons et al., 2015). The Strep. mutans UA159 genome contains orthologues to the thioesterase and fatty acid kinase genes necessary for this pathway. Experimental confirmation of the role of this pathway in Strep. mutans is under way, but the pathway described by Parsons et al. (2015) is a reasonable explanation for the survival of the Strep. mutans ΔplsX deletion strain. The ΔplsX strain serves as a valuable tool for exploring the role of phospholipid synthesis in Strep. mutans virulence. The present study reports the contribution of PlsX to membrane physiology and the acid-adaptive response of Strep. mutans; specifically, the effects of plsX deletion on growth of the organism, fatty acid synthesis, and aciduricity.

**METHODS**

**Bacterial strains.** Strep. mutans UA159 was the parent strain used in this study (Ajdic et al., 2002). The plsX deletion mutant ΔplsX was constructed by a previously described method that resulted in complete deletion of plsX and replacement with a nonpolymer erythromycin resistance cassette (Buckley et al., 2014; Quivey et al., 2015; Santiago et al., 2012).

To confirm that the observed phenotypes were due to loss of plsX, a plsX complement strain was created. A 1217 bp PCR product was amplified from Strep. mutans UA159 genomic DNA containing the entire plsX gene and the 208 bp upstream intergenic region. The primers both contain BglII restriction sites (underlined) and are designated as follows: 22CompPF (5′-AGACCTAAGGAAAATTTCCAAAAAGAGACATTTTGG-3′) and 22CompR (5′-AGATCTTGTCCTCCCCCGTGTGGAAAAATTC-3′). The amplicon was ligated into pCR-TOPO-XL and the ligated was used to transform Escherichia coli TOP10 according to the Invitrogen TOPO XL PCR Cloning kit protocol (Invitrogen). The plsX gene and upstream sequence were subcloned into the integration vector pSU6K-Bgl, as described previously (Derr et al., 2012). The final construct, pSU6K-Bgl-P22Comp, was used to transform the ΔplsX strain and colonies were selected on BHI agar medium containing kanamycin (1 mg ml⁻¹). Colony PCR was used to screen transformants for the correct insertion of the plsX gene and cognate promoter region in the gfia locus of Strep. mutans, using methods previously described (Buckley et al., 2014; Derr et al., 2012; Santiago et al., 2012). The plsX-complemented strain was named Strep. mutans UR307 (plsX⁺).

**Bioscreen growth curves.** Growth rates were determined using a Bioscreen C (Growth Curves USA). Strains were grown overnight in 5 ml brain–heart infusion (BHI) medium (BD/Difco) at 37°C in a 5 % (v/v) CO₂/95 % air environment. This culture was diluted 1 : 20 for UA159 and plsX⁺, or 1 : 10 for ΔplsX, into 5 ml fresh BHI medium. For growth in 5 ml FMC (Terlecky et al., 1975), all strains were diluted 1 : 10. The subcultures were grown at 37°C in a 5 % (v/v) CO₂/95 % air environment until an OD₆₀₀ of 0.25–0.35 was reached. The strains were assayed for growth in the following types of medium: BHI; BHI buffered with 50 mM potassium phosphate buffer to pH 7.0; BHI titrated to pH 5.2 with HCl; BHI + 1 or 2 % (v/v) ethanol; BHI + 100 or 150 μM SDS (SDS); BHI + 50 or 250 μM myristic acid (C₁₄:0); or FMC + 1 % (w/v) glucose. Growth was assayed at 37°C and the OD₆₀₀ was measured every 30 min, after a 10 s period of medium shaking. Generation times listed in Fig. 1(d) were calculated using the formula 0.3/(ΔN/ΔT₀), where N is the mean OD₆₀₀ at the end of exponential phase and T₀ is the time in minutes that correspond to the OD₆₀₀ values for N and N₀, respectively.

**Chemostat cultures.** Continuous cultures of Strep. mutans UA159 or ΔplsX were grown in a BioFlo 2000 fermenter (New Brunswick Scientific) in TY medium containing 3 % tryptone, 1 % glucose, 0.1 % yeast extract, 0.5 % KOH and 1 mM H₃PO₄, as described previously (Quivey et al., 2000a). Cultures were grown at a dilution rate of 0.144 h⁻¹, were limited by glucose (2.3 mM), and were maintained at steady-state pH values by the addition of 2 N KOH. The culture pH was continuously monitored throughout the experiment by using an in-dwelling pH electrode (Mettler Toledo). After growth at pH 7.0 had been maintained for a minimum of 10 generations, steady-state culture samples were removed. The remaining culture in the chemostat vessel was then supplemented with excess glucose (20 mM). The medium feed, pH control, and waste pump were discontinued until the pH reached 5.2; then the feed, pH control, and waste pump were reactivated. Growth at pH 5.2 was maintained for 10 generations before steady-state culture samples were collected. Growth was maintained at pH 5.2 owing to the inability of ΔplsX to sustain growth at pH 5.0.

**Acid challenge.** Strep. mutans UA159 or ΔplsX was grown in steady-state chemostat cultures (described above), BHI batch cultures, or glass-slide biofilm cultures (Buckley et al., 2014).

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A 20 ml aliquot was centrifuged at 4 °C, 2272 g for 15 min. Cells were resuspended in 0.1 M glycine, pH 2.5. A 0.1 ml aliquot of the cell suspension was immediately removed, serially diluted in 0.1 M glycine (pH 7), plated on BHI agar medium, and incubated for 48 h at 37 °C in 5% (v/v) CO₂/95% air. Samples at subsequent time points were diluted and plated in the same manner. Colonies were counted, and the number of the surviving cells was represented as log(N/N₀), with N₀ being the first time point.

Gas chromatography of fatty acid methyl esters (GC-FAME). Membrane fatty acid composition was determined using GC-FAME performed by Microbial ID (Newark, DE, USA). *Strep. mutans* UA159 and ΔplsX were grown to steady-state in chemostat cultures as described above, and 20 ml samples were removed from cultures at pH 7.0 and 5.2. Cell suspensions were centrifuged at 3452 g for 15 min and washed with sterile deionized H₂O. The cell pellet was stored at −80 °C until it was sent to Microbial ID for analysis.

**ATPase activity assay.** *Strep. mutans* UA159, ΔplsX, and plsX⁺ were assayed for their ability to release inorganic phosphate from ATP, as previously described (Buckley et al., 2014). Briefly, 50 ml cell pellets from the strains were resuspended in membrane buffer (75 mM Tris pH 7, 10 mM MgSO₄) and centrifuged at 2272 g for 15 min. Cells were resuspended and washed in membrane buffer (75 mM Tris pH 7.0, 10 mM MgSO₄) and permeabilized with toluene, by subjecting them to two freeze–thaw cycles in dry ice/ethanol and a 37 °C water bath. Cells were pelleted in a microcentrifuge for 10 min at 16 100 g. Supernatant was decanted and cells were resuspended in ATPase buffer (50 mM Tris maleate pH 6, 10 mM MgSO₄), aliquoted, and stored at −80 °C.

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**Fig. 1.** Growth of ΔplsX is impaired. Cultures of *Strep. mutans* UA159, ΔplsX and plsX⁺ were grown to exponential phase and used to inoculate fresh media. Growth at 37 °C was monitored using a Bioscreen C growth reader. Data points represent the mean of 10–20 individual wells. (a) Growth in BHI medium buffered to pH 7.0 with 50 mM potassium phosphate buffer. (b) Growth in BHI medium titrated to pH 5.2 with HCl. (c) Growth in BHI versus FMC medium. Note the difference in the timescale compared with (a) and (b). (d) Generation times were calculated from the data in (a)–(c) using data points at the beginning and end of exponential phase and expressed in minutes ± SD. Generation times for ΔplsX and plsX⁺ were compared with UA159 grown under the same conditions and statistical significance was determined by Student’s t-test; *P < 0.05.
An aliquot (300 μl) of the toulene extract was added to 9 ml ATPase buffer. ATP (pH 6, 5 mM final concentration) was added to start the reaction, and a sample was immediately removed as the zero time point. Aliquots were removed at time points 0, 5, 10, 20, 30 and 45 min, in triplicate. Reactions were stopped by adding 500 μl aliquots to 2 ml 20 % TCA. Samples were centrifuged for 15 min at 2272 g and 1 ml of supernatant was transferred into tubes containing 1.5 ml H2O and 0.5 ml acid molybdate solution (1.25 g dL-1 in 2.5 N sulfuric acid). Fiske and Subbarow solution (125 μl) (Fiske & Subbarow, 1925) was added to each tube and absorbance was measured at 660 nm. Values were normalized to OD600 measurements at time zero, and the amount of inorganic phosphate released was extrapolated from a phosphate standard curve.

**Quantitative real-time PCR.** Three independent cultures of *Strep. mutans* UA159, ΔplsX and plsX+ were grown in TYG medium to stationary phase in conditions identical to those for stationary phase ATPase assays. Cells were harvested and RNA was extracted according to previously described methods (Abranches *et al.*, 2006). RNA was used to synthesize cDNA with random primers using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Primer pair 5′-ATGCTTGTAAGTGTATGGC-3′ (atpG RTF) and 5′-GACGAAAGCCTATGTTGTCG-3′ (atpG RTR) was used to amplify atpG-specific mRNA using Power SYBR Green Master Mix (Applied Biosystems) from three replicates of each sample. Primer pair 5′-GTCAGAGGCTATGTTGTCG-3′ (plsX RTF) and 5′-TACAAACATCCTGGGACACTG-3′ (plsX RTR) was used to amplify plsX-specific mRNA from three replicates of each sample using Power SYBR Green Master Mix (Applied Biosystems). Reactions were carried out in a Step One Plus Real-time PCR System (Applied Biosystems). The mRNA copy number was quantified based on a standard curve of plsX or atpG DNA produced by standard PCR (Abranches *et al.*, 2006).

## RESULTS AND DISCUSSION

**plsX is not an essential gene in Strep. mutans UA159**

The ORF encoding plsX (GenBank SMU.26; Ajdić *et al.*, 2002) was deleted as part of a study conducted to examine the essential genes in *Strep. mutans* (Quivey *et al.*, 2015). Fig. S1 (available in the online Supplementary Material) shows the results of a PCR amplifying the plsX gene from genomic DNA derived from three isogenic strains of *Strep. mutans*; the parent strain (UA159), the plsX deletion (ΔplsX), and the genetic complement strain (plsX+). The PCR product representing plsX was not present in the reaction using DNA extracted from the ΔplsX strain, but was visible when using DNA derived from either UA159 or plsX+. These data confirm that the coding region for PIsX was successfully deleted. The deletion was further confirmed by Sanger sequencing of the region surrounding the deletion (data not shown).

**Deletion of plsX results in poor growth of Strep. mutans**

The results of growth experiments, shown in Fig. 1, illustrate the effect of the plsX deletion on growth rate of the mutant strain in medium titrated to either pH 7.0 (Fig. 1a) or pH 5.2 (Fig. 1b). In both cases, the plsX deletion strain grew more slowly and had a lower maximum yield (estimated by OD600) compared with the parent strain grown under the same conditions (Fig. 1d). This finding differs from the *Strep. pneumoniae* plsX deletion strain, which demonstrated no reduction in growth rate (Parsons *et al.*, 2015). Disparate methods of regulation for either the alternative acyl-phosphate pathway or the FASII pathway could result in this difference in growth characteristics between the deletion strains (Faustoferri *et al.*, 2015; Jerga & Rock, 2009). The plsX+ strain displayed a partial complementation of the growth defect observed in ΔplsX.

The plsX+ strain exhibited an intermediate phenotype between UA159 and ΔplsX in most of our experiments. This partial complementation can be explained by a 10-fold reduction in plsX transcript in the plsX+ strain, compared with UA159 (Fig. S2). The plsX sequence, along with its cognate promoter, was added back into the genome of ΔplsX; however, the ectopic location resulted in decreased transcription, likely due to the inability of regulatory molecules to interact with the plsX promoter at a different locus.

The ΔplsX strain was also tested for nutritional requirements that would indicate auxotrophy for fatty acids or phospholipids. The deletion strain was capable of growth in both BHI, a rich medium, and FMC, a defined medium containing only amino acids, salts, cofactors and glucose (Fig. 1c) (Terleckyj *et al.*, 1975). The doubling time for the ΔplsX strain was greatly reduced in FMC medium compared with growth in BHI, while the growth rate of UA159 was relatively unaffected (Fig. 1c, d). A plsX deletion strain of *Staphylococcus aureus* has been shown to be a fatty acid auxotroph (Parsons *et al.*, 2015), but data from the present study indicated that plsX is not essential for growth of *Strep. mutans* in medium lacking fatty acids and phospholipids.

**ΔapsX retains acid adaptability**

We wanted to determine if loss of a major enzyme involved in membrane synthesis would alter the ability of the strain to survive acid stress. Cells from steady-state cultures of UA159, ΔapsX, and plsX+ were subjected to an acid challenge (pH 2.5) over 1 h (Fig. 2). As expected, all cultures grown to a steady-state pH value of 5.2 had significantly higher rates of survival than those grown to steady state at pH 7.0 (Fozo & Quivey, 2004a; Quivey *et al.*, 1995). Surprisingly, the ΔapsX strain, grown at both steady-state pH values, survived better than both the parent and plsX complement strains following exposure to the acid challenge. After 15 min of exposure, the UA159 strain, grown at pH 5.2, displayed a 2-log reduction in survival, while survival of the ΔapsX strain, also grown at pH 5.2, did not exhibit a significant change. The plsX+ strain exhibited an intermediate phenotype.
Compared with the parent strain, cultures of the ΔplsX strain grown in a model biofilm culture (Fig. 2b) or in an overnight batch culture (Fig. 2c) were also better able to survive subsequent acid challenge. Resistance to oxidative stress has been associated with acid adaptation (Baker et al., 2014; Derr et al., 2012); however, the ΔplsX strain did not show significant differences in survival, as compared with UA159, when exposed to 16.3 mM hydrogen peroxide (data not shown).

**F1F0-ATPase activity is altered in ΔplsX**

One of the primary mechanisms used by *Strep. mutans* to adapt to a low pH environment is elevated activity of the membrane-bound F1F0-ATPase (Kuhnert et al., 2004); therefore, we hypothesized that this may be a potential mechanism to explain the enhanced survival of the ΔplsX strain, compared with UA159, in the acid-challenge experiment described above. ATPase activity, measured as the ATP-dependent release of inorganic phosphate, was determined using permeabilized cells derived from batch cultures and steady-state chemostat cultures. Results showed that ATPase activity in the ΔplsX strain is elevated, compared with both UA159 and plsX+ in cultures grown to stationary phase (Fig. 3a). The ΔplsX strain also displayed increased ATPase activity following growth in steady-state cultures maintained at pH 5.2, compared with the parent strain examined under the same growth conditions. Yet, the ATPase activity level of the ΔplsX strain was not elevated, compared with the parent strain, at steady-state pH 7.0 (Fig. 3b). A significant increase in ATPase activity at low pH, compared with the parent strain, may contribute to the increased aciduricity of ΔplsX; however, elevated proton extrusion cannot be the only contributing factor. Cytoplasmatic buffering via agmatine deaminase and/or malolactic fermentation are likely candidates for further investigation into the enhanced aciduricity observed in the deletion strain. (Lemos & Burne, 2008; Sheng & Marquis, 2007).

Increased ATPase activity observed at pH 5.2 is at least partially due to elevated ATPase transcription (Kuhnert et al., 2004). Therefore, we examined *atpG* transcription in RNA derived from batch cultures subjected to the same conditions used in the ATPase activity assay shown in Fig. 3(a).
Results from quantitative reverse transcriptase PCR (qRT-PCR), shown in Fig. 3(c), show that the changes in ATPase activity between UA159 and ΔplsX are not due to changes in transcription; that is, the atpG transcript levels in the deletion strain are not significantly different from those measured in the parent strain grown under the same conditions. The physical environment of the F1F0-ATPase enzyme is known to significantly affect enzymatic activity. Specifically, environmental pH and membrane fatty acid composition can alter ATPase activity (MacGilvray et al., 2012; Sturr & Marquis, 1992).

**Dysregulation of fatty acid biosynthesis**

Elevated unsaturated fatty acid content following exposure to an acidic environment has been shown to be essential for *Strep. mutans* adaptation and survival in a low pH environment (Fozo & Quivey, 2004a, b). Since we observed alterations in the acid tolerance of the ΔplsX strain (Fig. 2), we wanted to determine the membrane fatty acid composition. The percentage of unsaturated membrane fatty acids (UFAs) in the ΔplsX strain increases in response to a low pH environment, as for UA159, but the shift is more pronounced when compared with the parent strain (Fig. 4a). At pH 7.0, ΔplsX has a significantly lower percentage of UFAs than UA159. At pH 5.2, ΔplsX has a higher percentage of unsaturated fatty acids than the parent strain.

Previous studies have shown that most of the fatty acids composing the membrane of *Strep. mutans* are 16, 18 or 20 carbons in chain length, and that the ratio of these chain lengths can vary based on environmental conditions (Fozo & Quivey, 2004b; Quivey et al., 2000a). The most common length of fatty acids found in the membrane of *Strep. mutans* UA159 cells grown in a continuous culture at pH 7 is 16 carbons, while at pH 5 the most common length is 18 carbons (Derr et al., 2012; Fozo & Quivey, 2004b; Quivey et al., 2000a). Overall, analysis of the membrane fatty acid composition of ΔplsX, grown in continuous culture, displayed a decrease in fatty acid chain length as compared with the parent strain grown under the same conditions (Fig. 4b). The most abundant fatty acid chain length at both pH 7.0 and pH 5.2 was 16 carbons. During growth at steady-state pH 7.0, the ΔplsX strain was found to have a significantly elevated level of 12- and 14-carbon chains compared with the parent strain.

The most likely explanation for the decrease in fatty acid chain length in ΔplsX is an increase in the acyl-ACP: uncylated-ACP ratio resulting in partial inhibition of fatty acid elongation (Jiang & Cronan, 1994). We hypothesize that plsX deletion results in a build-up of acyl-ACP based on the enzymic function of PLSX and direct measurement of acyl-ACP concentrations in *E. coli* under similar conditions. Experiments in *E. coli* have shown that when phospholipid synthesis is artificially stopped, the rate of fatty acid synthesis is reduced by 80–90% (Jiang & Cronan, 1994). In *Bacillus subtilis*, PlsX is believed to be a key enzyme involved in coordinating the synthesis of both.

**Fig. 3.** ATPase activity is enhanced in the ΔplsX strain. ATPase activity was measured via the method of Fiske and Subbarow, a colorimetric indicator of inorganic phosphate production (Fiske & Subbarow, 1925). Permeabilized cell membranes were prepared as described in methods, and OD660 measurements were taken after 45 min for an end point ATPase activity; *n* = 3. Statistical significance between pairs indicated by brackets was determined by Student’s *t*-test; *P* ≤ 0.05. (a) Activity derived from cultures of *Strep. mutans* UA159, ΔplsX and plsX+ grown in batch cultures in BHI medium. (b) Activity derived from steady-state cultures of *Strep. mutans* UA159 and ΔplsX harvested at fixed pH values of 7.0 and 5.2. (c) Transcription of atpG, the A subunit of F0F1-ATPase, as measured by qRT-PCR. RNA was isolated from three independent 50 ml cultures of each strain, grown in TY medium +1% (w/v) glucose. Each sample was measured in triplicate. Error bars indicate ±SD.
fatty acids and phospholipids, and, upon the depletion of plsX transcript, fatty acid synthesis is halted (Paoletti et al., 2007).

In Strep. pneumoniae, deletion of plsX does not inhibit fatty acid synthesis, but results in longer-chained fatty acids (Parsons et al., 2015). Although we propose that a similar, alternative pathway for acyl-phosphate production is used by Strep. pneumoniae and Strep. mutans, deletion of plsX in Strep. mutans results in shorter-chained fatty acids. The components of the alternative acyl-phosphate pathway (thioesterase and fatty acid kinase) may be regulated differently in the different species. A more active or highly expressed thioesterase would more often cleave acyl-ACP, removing it from the FASII pathway, and lead to shorter-chained fatty acids. Our group has previously shown that the biochemical regulation of FASII in Strep. mutans differs from Strep. pneumoniae, which may also contribute to the observed difference in fatty acid chain length. The presence of acyl-ACP did not enhance Strep. mutans FabT binding to target DNA sequences, as was the case for the Strep. pneumoniae orthologue of FabT (Faustoferri et al., 2015; Jerga & Rock, 2009).

Although changes in membrane fatty acid composition were observed in the ΔplsX strain (Fig. 4a), the permeability of the membrane to protons was not significantly different between ΔplsX and UA159 (data not shown). The altered fatty acid profile of ΔplsX may contribute to the growth defects exhibited in Fig. 1. The efficiency of membrane proteins is affected by surrounding fatty acids, such that the shorter fatty acid chain lengths found in the ΔplsX membrane may inhibit nutrient transport into the cell and other important functions that are dependent on integral membrane proteins (Lundbaek et al., 2010). The altered fatty acid composition, and consequent alterations in phospholipid structure, is also a likely explanation for the enhanced ATPase activity observed in the ΔplsX strain (Fig. 3a, b).

**The ΔplsX strain is sensitive to direct membrane stress, but not C14:0**

Oxidative stress has been shown to increase aciduricity by activating some aspects of the acid-adaptive response (Baker et al., 2014; Derr et al., 2012). We hypothesized that some other type of stress, resulting from the deletion of plsX, may similarly increase aciduricity. Membrane stress was an obvious candidate to investigate, owing to the central role of plsX in membrane synthesis and the observed alterations in the membrane fatty acid composition of ΔplsX (Fig. 4).

Cultures of Strep. mutans UA159, ΔplsX and plsX+ were grown in the presence of two membrane-disrupting agents as a method to indirectly assess the stability of cell membranes (Sikkema et al., 1995). The ΔplsX strain was more sensitive than the parent strain to the presence of both ethanol and SDS in the growth medium (Fig. 5a, b). Ethanol sensitivity was partially alleviated in the plsX+ strain; however, SDS affected the ΔplsX and plsX+ strains in a similar fashion.

A decreased rate of phospholipid synthesis or the shorter-length membrane fatty acids may contribute to increased sensitivity to membrane-disrupting agents (Heipieper et al., 2007; Sardessai & Bhosle, 2002). If ΔplsX is experiencing continuous membrane stress in laboratory growth conditions, this may result in constitutive activation of stress response mechanisms. The specific mechanisms being activated will be the topic of future studies.

**Fig. 4.** Membrane fatty acid composition is altered in the ΔplsX strain. Cells were harvested from three independent chemostat cultures of Strep. mutans UA159 and ΔplsX (used in the acid-challenge assays described in Figs. 2 and 3) and analysed for membrane fatty acid content by GC-FAME; n=3. (a) Percentage of saturated, unsaturated and other membrane fatty acids (±SD) from cells grown in continuous culture. Values in the ΔplsX strain that were significantly different from the parent strain grown under the same condition were determined by Student’s t-test; *P≤0.05. (b) Percentage of each of the fatty acid chain lengths, as measured by number of carbon atoms, from samples harvested from continuous cultures. Data represent all types of fatty acids, i.e. saturated, unsaturated, and others. All pairwise comparisons of the results in panel (b) are significantly different (P≤0.05 using Student’s t-test), except for the comparisons indicated by Ø. Error bars indicate ±SD.
A *plsX* deletion strain of *Strep. pneumoniae* has been shown to resist myristic acid (C14 : 0) toxicity, relative to the parent strain (Parsons et al., 2015). This phenotype is recapitulated in the *D plsX* strain of *Strep. mutans* (Fig. 5c), providing further evidence that the PlsX enzyme, and the alternative pathway, function similarly in *Strep. mutans* and *Strep. pneumoniae*. The mechanism explaining C14 : 0 resistance in *plsX* deletion strains is discussed in detail by Parsons et al. (2015). Briefly, C14 : 0 toxicity depends on incorporation of the short-chained fatty acid into both the sn-1 and sn-2 phospholipid positions. Without PlsX, C14 : 0 is only able to acylate the sn-1 position, and endogenously produced fatty acids occupy the sn-2 position, maintaining membrane stability. The *plsX*+ strain appears to be more sensitive to C14 : 0 than UA159. This is likely due to the decreased transcription of *plsX* (Fig. 3c), which negatively impacts growth (Fig. 1a) while still allowing addition of 14 : 0 to the sn-2 position of phospholipids.

**CONCLUSION**

The observation that the *plsX* deletion strain can survive in defined media, containing no exogenous fatty acids or phospholipids, suggests an alternative pathway for acyl-phosphate or lysophosphatidic acid synthesis. The exact nature of this alternative pathway is currently being investigated, and is most likely homologous to the alternative pathway found in *Strep. pneumoniae* (Parsons et al., 2015).

The results observed in this study clearly indicate that the function of PlsX has an impact on the FASII pathway in *Strep. mutans*. PlsX has a complex effect on acid adaptation, which is an important aspect of the pathogenic capability of *Strep. mutans*. The increased acid tolerance of the *ΔplsX* strain suggests that PlsX, and the phospholipid synthesis pathway, would be poor drug targets. The observed increase in aciduricity is partially explained by an increase in ATPase activity, but other mechanisms of
acid tolerance such as agmatine deaminase and malolactic fermentation could also account for increased survival in cells grown at neutral pH (Lemos & Burne, 2008).

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