Cardiolipin biosynthesis in *Streptococcus mutans* is regulated in response to external pH

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Streptococcus mutans, a causative agent of dental caries in humans, adapts to changing environmental conditions, such as pH, in order to survive and cause disease in the oral cavity. Previously, we have shown that *S. mutans* increases the proportion of monounsaturated membrane fatty acids as part of its acid-adaptive strategy. Membrane lipids function as carriers of membrane fatty acids and therefore it was hypothesized that lipid backbones themselves could participate in the acid adaptation process. Lipids have been shown to protect other bacterial species from rapid changes in their environment, such as shifts in osmolality and the need for long-term survival. In the present study, we have determined the contribution of cardiolipin (CL) to acid resistance in *S. mutans*. Two ORFs have been identified in the *S. mutans* genome that encode presumptive synthetic enzymes for the acidic phospholipids: phosphatidylglycerol (PG) synthase (*pgsA*, SMU.2151c) and CL synthase (*c/s*, SMU.988), which is responsible for condensing two molecules of PG to create CL. A deletion mutant of the presumptive *c/s* gene was created using PCR-mediated cloning; however, attempts to delete *pgsA* were unsuccessful, indicating that *pgsA* may be essential. Loss of the presumptive *c/s* gene resulted in the inability of the mutant strain to produce CL, indicating that SMU.988 encodes CL synthase. The defect in *c/s* rendered the mutant acid sensitive, indicating that CL is required for acid adaptation in *S. mutans*. Addition of exogenous CL to the mutant strain alleviated acid sensitivity. MS indicated that *S. mutans* could assimilate exogenous CL into the membrane, halting endogenous CL incorporation. This phenomenon was not due to repression, as a *c/s* gene transcriptional reporter fusion exhibited elevated activity when cells were supplemented with exogenous CL. Lipid analysis, via MS, indicated that CL is a reservoir for monounsaturated fatty acids in *S. mutans*. We demonstrated that the *c/s* mutant exhibits elevated F-ATPase activity but it is nevertheless unable to maintain the normal membrane proton gradient, indicating cytoplasmic acidification. We conclude that the control of lipid backbone synthesis is part of the acid-adaptive repertoire of *S. mutans*.

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INTRODUCTION

Streptococcus mutans, a major aetiological agent of dental caries in humans, relies on multiple adaptive responses in order to survive the inimical conditions encountered in the oral cavity, including survival in acidic conditions. The acid-adaptive systems that have been reported in *S. mutans*

include an elevated proportion of monounsaturated membrane fatty acids in response to external acidification (Fozo & Quivey, 2004a, b; Fozo *et al.*, 2007).

The role of lipids in the acid response of *S. mutans* is, however, poorly understood at present. It is known that alterations in membrane phospholipid content can provide protection for bacteria subjected to environmental stress. Examples include the osmolality and nutritional deprivation responses in *Escherichia coli* (Romantsov *et al.*, 2007; Hiraoka *et al.*, 1993, respectively), as well as the survival of *Staphylococcus aureus* in high salinity (Tsai *et al.*, 2011).

Abbreviations: ΔpH, proton gradient; CL, cardiolipin; LIC, ligation-independent cloning; PG, phosphatidylglycerol; SFA, saturated fatty acid; UFA, unsaturated fatty acid.

A supplementary figure is available with the online version of this paper.

Genes in the *S. mutans* genome encoding synthetic enzymes for the major acidic lipids are phosphatidylglycerol (PG) synthase (*pgsA*, encoded by SMU.2151c) and cardiolipin (CL) synthase (*cls*, encoded by SMU.988) (Ajdić *et al.*, 2002). PG is a phospholipid containing a glycerol-3-phosphate backbone with two acyl groups attached to carbons 1 and 2 through ester linkages. The other predominant acidic lipid, CL, consists of two PG molecules linked by a central glycerol; thus it contains four acyl groups and two phosphate molecules (Schlame, 2008).

In this report, we show that a putative *cls* gene, SMU.988 (Ajdić *et al.*, 2002), encodes the sole CL synthase in *S. mutans* and that a deletion mutant in *cls* is acid sensitive. We also show that the observed phenotype for the Δ *cls* strain is probably attributable to a decrease in mono-unsaturated fatty acids in the membrane, associated with the loss of CL. Furthermore, the acid sensitivity of the Δ *cls* strain can be rescued by the addition of exogenous bovine CL. Moreover, the presence of exogenous CL resulted in the cessation of endogenous CL incorporation into membranes, suggesting inhibition of the native CL synthase as seen in *E. coli* (Ragolia & Tropp, 1994) or a negative-feedback loop controlling CL biosynthesis at the genetic level.

METHODS

Bacterial strains and growth conditions. Strains and plasmids used in this study are listed in Table 1. Streptococcal strains included, as a parent strain, *S. mutans* UA159, the genomic type strain (Ajdić

et al., 2002; Murchison *et al.*, 1986), and specific deletion strains created from *S. mutans* UA159, designated Δ *cls*, Δ *wapA* and UR297 (Δ *cls_c*). Bacterial strains were maintained on brain heart infusion [(BHI), Becton Dickinson-Difco Laboratories] agar plates and, where appropriate, 5 µg erythromycin ml⁻¹ or 1000 µg kanamycin ml⁻¹. CL and sodium salt from bovine heart (Sigma-Aldrich) was added, as indicated, to a final concentration of 50 or 100 µg ml⁻¹. Streptococcal strains were incubated overnight in BHI or TY [3% (w/v) tryptone and 0.1% (w/v) yeast extract] + 1% (w/v) glucose medium at 37 °C in a 5% (v/v) CO₂-enriched atmosphere. Steady-state cultures were prepared as described previously (Fozo & Quivey, 2004b; Hahn *et al.*, 1999). *E. coli* strains DH10B and One Shot Top 10 (Invitrogen) were used for cloning experiments. *E. coli* strains were maintained on Luria-Bertani agar plates and were supplemented, when appropriate, with 50 µg kanamycin ml⁻¹ (Sigma-Aldrich). Growth curves of *S. mutans* strains were generated using a Bioscreen C system (Growth Curves USA). Wells were inoculated with 10 µl overnight culture and optical density measurements were recorded at 15 min intervals over a 24 h period at 600 nm. Cultures of each strain were grown in ten replicates.

DNA manipulations and strain construction. Plasmid DNA from *E. coli* was isolated with the QIAprep spin miniprep kit (Qiagen). PCR was carried out using platinum *Taq* DNA polymerase, as described by the manufacturer (Invitrogen). *S. mutans* was transformed as described by Perry & Kuramitsu (1981). Mutant strains were generated from *S. mutans* UA159 by deleting the coding region of the gene of interest using ligation-independent cloning (LIC)-mediated mutagenesis, as described by Aslanidis & de Jong (1990) and Lau *et al.*, (2002). Verification of the constructions was accomplished using pairs of oligonucleotide primers, as previously described (Sheng *et al.*, 2010).

Construction of the Δ *cls* complement strain. LIC-PCR was utilized to construct a complement strain containing the following elements: the *cls*-coding region, the intergenic region upstream of *cls*,

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant feature	Source or reference
Strains		
<i>E. coli</i>		
DH10B	Host for cloning vectors	Invitrogen
TOP 10	Host for cloning vectors	Invitrogen
<i>S. mutans</i>		
UA159	Genomic type strain	Murchison <i>et al.</i> (1986), Ajdić <i>et al.</i> (2002)
MU0897 (Δ <i>wapA</i>)	UA159 with deletion of cell wall surface anchor family gene; Erm ^R	This study
MU0898 (Δ <i>cls</i>)	UA159 with deletion of <i>cls</i> ; Erm ^R	This study
UR212	UA159 transformed with pJL84; Kan ^R ; promoterless vector control strain	This study
UR257	Δ <i>cls</i> transformed with pJL84; Kan ^R Erm ^R ; promoterless vector control strain	This study
UR265	Δ <i>wapA</i> transformed with pJL84; Kan ^R Erm ^R ; promoterless vector control strain	This study
UR269	UA159 transformed with pJLwapALong; Kan ^R ; <i>wapA_P</i> fusion in UA159	This study
UR270	Δ <i>wapA</i> transformed with pJLwapALong; Kan ^R Erm ^R ; <i>wapA_P</i> fusion in Δ <i>wapA</i>	This study
UR271	Δ <i>cls</i> transformed with pJLwapALong; Kan ^R Erm ^R ; <i>wapA_P</i> fusion in Δ <i>cls</i>	This study
UR297	Δ <i>cls</i> transformed with a LIC construct containing <i>cls</i> +Kan ^R ; complemented strain, denoted as Δ <i>cls_c</i>	This study
Plasmids		
pJL84	CAT reporter plasmid, Kan ^R	Gift from Jose Lemos, University of Rochester
pJLwapALong	pJL84 containing 370 bp upstream of <i>wapA</i> ; Kan ^R	This study
pCRwapAL	pCRBlunt containing 370 bp upstream of <i>wapA</i> ; Kan ^R	This study

a selectable Kan^R cassette and a partial Erm^R marker. LIC was performed as described by Aslanidis & de Jong (1990). Primers used in the strain constructions are shown in Table 2. The Δ cls strain was transformed with the construct and colonies were selected for Kan^R and screened for the inability to survive on erythromycin. One isolate with this profile was named UR297 (Δ cls₂). PCR was performed on the chromosomal DNA isolated from UR297 to ensure that the construct had integrated as a single copy at the correct location in the genome.

Physiological tests and enzymic assay methods. An acid sensitivity assay (Quivey *et al.*, 1995) was employed to determine the effects of acidic conditions on the survival of *S. mutans* strains. In the case of strains grown in the presence of exogenously added bovine CL, overnight cultures of UA159 and Δ cls were grown with 100 μ g CL ml⁻¹ in TY+1% (v/v) glucose. Cell pellets were washed with PBS and resuspended in 0.1 M glycine-HCl (pH 2.5). Aliquots were removed at pre-determined intervals and plated for the enumeration of survivors. The results of these experiments represent three independent batch cultures or chemostat runs, each assayed in duplicate.

Phospholipid extraction. Phospholipids were isolated by the method described by Bligh & Dyer (1959), with modifications. Cultures of UA159 and Δ cls were grown in TY+1% (w/v) glucose and, where appropriate, 100 μ g CL ml⁻¹. Cultures were harvested, washed three times with deionized water, resuspended in 2.5 ml solvent mixture [1 M acetic acid, 2-propanol, hexane (2:20:30, by vol.)] and vortexed. An equal volume of hexane was added and the samples were again mixed. Lipids were recovered from the upper hexane layer and samples were re-extracted with an equal volume of hexane (Maskrey *et al.*, 2007). Hexane layers were combined and dried under nitrogen. Samples were resuspended for MS analysis in 200 μ l hexane/2-propanol/20 mM ammonium acetate in water, pH 5.5, 30:40:7 (v/v/v) (Sparagna *et al.*, 2005).

MS of membrane lipid composition. Samples for MS analysis were injected (20 μ l) onto a normal phase HPLC column, (Luna 5 μ m silica, 100 Å, 2.0 $\times$ 150 mm (Phenomenex)). In-line separation and analysis was carried out using a micrOTOF-Q II (Bruker Daltonics) in negative ion mode. The elution of CL species was carried out using the gradient profile and solvent conditions described by Sparagna *et al.* (2005). MS-MS was used to determine the side chains present on the

CL species. Parent ions of interest were isolated in the quadrupole of the instrument and fragmented in the collision cell with a collision voltage of 15 eV using argon gas. The mass spectrometer was operated in negative ion mode with a spectral rate of 1 Hz. For quantification of CL species, a calibration curve was established using bovine CL (*m/z* 1448, carrying four 18:2 fatty acyl substituents). Both the peak height and area under the curve were used to validate the quantification for the CL species. Each feature was plotted against the concentration of bovine CL. The results of these experiments represent samples from two independent cultures for UA159 and Δ cls grown in the presence and absence of exogenous CL.

Membrane fatty acid analysis. The membrane fatty acid content of cultures was determined by Microbial ID. Batch and chemostat cultures were grown as described previously, supplemented, where appropriate, with 100 μ g CL ml⁻¹ (Fozo *et al.*, 2004; Fozo & Quivey, 2004b).

F-ATPase activity determinations. Samples were permeabilized and assayed for ATPase-dependent phosphate release as reported previously (Kuhnert & Quivey, 2003; Kuhnert *et al.*, 2004). The level of phosphate was estimated using the method described by Fiske & Subbarow (1925). Results were based on samples from three independent cultures.

Chloramphenicol acetyltransferase (cat) reporter gene assay. Promoter regions from the *pgsA* and *wapA* genes were amplified by PCR using primers listed in Table 2. Amplified DNA sequences were cloned into pJL84, a promoterless *cat* gene integration vector (Santiago *et al.*, 2012). Promoter regions of *pgsA* and *cls* were fused to *cat* and successful clones were named pJLpgsA and pJLcls, respectively. *S. mutans* UA159, Δ cls and Δ wapA were transformed with pJLpgsA, pJLcls or pJL84, containing a promoterless *cat* gene, to yield single-copy chromosomal fusions (Table 1). Using incorporated restriction sites (*SacI*/*BglII*), promoter fragments were subcloned into an integration vector containing a promoterless *cat* gene derived from *Staphylococcus*, pJL84 (gift from J. Lemos, unpublished data). Integration of the promoter-*cat* construction occurred within the intergenic regions *mtlA* (SMu1085)-*glmS* (SMu1086) and *mtlD* (SMu1082)-*phnA* (SMu1081). Colonies were selected on BHI agar medium containing kanamycin (1 mg ml⁻¹). Appropriate genomic integration of the promoter-*cat* construct was verified by colony PCR. CAT activity was estimated using a previously reported

Table 2. Oligonucleotide primers used in this study

Primer name	Nucleotide sequence 5'-3'	Relevant restriction site
Cloning*		
wapA CAT <i>SacI</i> Long	AATATTGAGCTCGAATTCTTTTGG	<i>SacI</i>
wapA CAT <i>BglII</i> Long	TTTCATAGATCTAATAGAATTTCTCC	<i>BglII</i>
1952/53 CAT IR <i>SacI</i>	ATGATCGAGCTCCAAAGGAAATGCAGC	<i>SacI</i>
1952/53 CAT IR <i>BglII</i>	TTGTACAGATCTATTTACCTTACCTCAGTTCC	<i>BglII</i>
Complement strain construction†		
Cls P1	CAACAACCTTCAAAACAAGTAACCAAGC	
Cls P2 LIC	GAGAGAGGGTGAGTGTGC CACAAATCGTCTGAATTGCCAAGCC	
Kan P3 LIC	CACACTCACCTCTCTCG CGTACTGCGGTCGACGAAGGAG	
Kan P4 LIC	CACCTCTCCTCTCCATCG GACAGGACGCCAGCGACTCAT	
Erm P5 LIC	GATGGAGAGGAGGAGTGCC GTGGTTTTTGAAAGCCATGCGTCTGAC	
Erm P6	GACAGGACGCCAGCGACTCAT	

*Restriction sites are underlined.

†LIC sites are underlined.

procedure (Shaw, 1975; Kuhnert & Quivey, 2003). CAT activity is represented as $\text{nmol min}^{-1} (\text{mg protein})^{-1}$ and assays were performed for three replicates per construct in each of the indicated growth conditions.

Proton permeability assay. Permeability of membranes to protons was determined as described previously (Bender *et al.*, 1986; Fozo & Quivey, 2004a). Cell material for the assays was derived from 200 ml cultures of *S. mutans* UA159, Δcls and $\Delta cls + 50 \mu\text{g CL ml}^{-1}$ grown overnight in TY medium supplemented with 1% (w/v) glucose. Measurements were conducted in triplicate for all strains.

RESULTS

Deletion of SMU.988 results in loss of CL in membrane extracts of *S. mutans*

Initially, it was unclear whether *S. mutans* could survive when the genes involved with PG and CL biosynthesis were independently deleted from the chromosome. A viable *cls* mutant had been reported for *Streptococcus pyogenes* (Rosch *et al.*, 2007) and it appeared feasible that *cls* could be deleted from the chromosome of *S. mutans*. Subsequently, we were unable to delete the presumptive PG synthase gene (*pgsA*) under the conditions tested. Given that the organism has one gene encoding each of the presumptive *pgsA* and *cls* genes, loss of *pgsA* would eliminate the formation of PG and the ability to form CL, suggesting that loss of *pgsA* is lethal, as it is in *E. coli* (Raetz & Dowhan, 1990). We were able to isolate a presumptive *cls* deletion mutant in *S. mutans*. We verified that the mutant strain was lacking CL by characterizing the membrane fractions of the parent strain and the presumptive Δcls strain using MS.

Results from mass spectrometric analysis of *S. mutans* UA159 membrane extracts revealed peaks with *m/z* ratios of 1416.0, 1432.0, 1444.0, 1460.0 and 1488.1 (Fig. 1a). The data are consistent with the presence of multiple endogenous CL species in the parent strain. Subsequent spectra revealed that the Δcls strain did not contain lipid species in the 1350–1600 *m/z* range (Fig. 1b), and that the peaks were restored in the genetically complemented strain, UR297 (Fig. 1c). Thus, it was apparent that CL is not produced by the strain with the SMU.988 deletion, which we refer to now as the Δcls strain. The absence of CL also indicated that other genes, with redundant function to *cls*, were not present in the *S. mutans* genome.

The Δcls strain is acid sensitive but can be rescued by the addition of exogenous CL

In order to determine whether CL was required for survival at low pH, growth of the UA159 and Δcls strains was evaluated using medium titrated to pH 5.4 or buffered to pH 7 (Fig. 2a). The parent and mutant strains exhibited similar growth rates and final cell yields when grown in pH 7-buffered medium. However, when the two strains were grown at pH 5.4, the Δcls strain exhibited a marked decrease in the growth rate; an approximately 530 min

doubling time, compared with a doubling time of approximately 260 min for the parent strain UA159. Final cell yield was also diminished in the Δcls strain. *S. mutans* UA159 reached a final OD_{600} of approximately 0.7; whereas the Δcls strain achieved a final OD_{600} of approximately 0.3. The genetic complement of the Δcls strain, UR297 (Δcls_c), exhibited a growth profile similar to UA159 when exposed to medium titrated to pH 5.4, indicating that the complemented strain exhibited growth kinetics similar to the parent strain (Fig. S1, available with the online version of this paper).

The Δcls strain exhibited a growth defect when exposed to moderately acidic pH values and thus it was believed that the Δcls strain could be less effective in mounting an acid stress response. We tested this hypothesis by exposing the mutant and parent strains to an acidic challenge. Cells from UA159 cultures grown to steady state at pH 7 survived 90 min of acid exposure (Fig. 2b). However, the Δcls strain was not viable beyond 30 min. For cultures grown to steady state at pH 5, the parent strain was viable up to at least 90 min post-treatment; however, survivors were only recovered up to 60 min post-treatment for the Δcls strain grown at pH 5. Elevated cell counts were observed for UA159 and the Δcls strain grown at pH 5 versus 7, indicating that both strains were capable of mounting an acid-adaptive response. The data suggested that the absence of CL reduces acid resistance but did not prevent an acid-adaptive response.

Exogenous bovine CL restores the Δcls strain to an acid-resistant phenotype

Since the Δcls strain was incapable of producing CL (Fig. 1b) and the defect resulted in an acid-sensitive phenotype, we investigated the possibility that exogenous CL could rescue the Δcls strain, restoring the strain to acid resistance. We found that when grown at pH 5.4 in the presence of exogenous bovine CL, the Δcls strain reached a higher final cell yield than cultures grown in the absence of CL (Fig. 2c). We also observed that as the concentration of CL increased from 50 to 100 $\mu\text{g ml}^{-1}$, the final cell density increased from OD_{600} 0.62 to 0.82, indicating that CL affected growth in a concentration-dependent manner. Furthermore, exogenous CL had an effect on the growth rate, reducing the doubling time from approximately 590 min for the Δcls mutant to 390 min, if supplemented with 100 $\mu\text{g CL ml}^{-1}$. Not only was the final cell yield elevated in the Δcls strain supplemented with CL, but UA159 grown at pH 5.4 also reached higher final cell densities when treated with CL (data not shown).

We also investigated whether CL could protect cells from extreme acidification of the environment. The presence of exogenous CL correlated with a substantial increase in the number of viable cells for both UA159 and Δcls (Fig. 2d). Although Δcls was more sensitive to acid killing than UA159, both strains exhibited increased survival in comparison to samples grown without treatment. The data strongly indicated a role for CL in acid protection in *S. mutans*.

Fig. 1. Deletion of SMU.988 results in the loss of CL in *S. mutans* membranes. Representative m/z spectra of UA159 and $\Delta c/s$ membrane phospholipids. Phospholipids were extracted from overnight cultures of UA159 and $\Delta c/s$ as described in Methods. (a) CL species endogenous to the parent strain UA159. Negative ions with m/z 1416.0, 1432.0, 1444.0, 1460.0 and 1488.1 are the predominant CL species. (b) Mass spectra for phospholipids extracted from the presumptive $\Delta c/s$ strain. The inset is the amplified spectra. Note: inset intensity scale is $\times 10^{-2}$ units, compared with $\times 10^{-4}$ for the parent strain UA159 in (a). (c) Mass spectra for phospholipids extracted from the genetically complemented strain $\Delta c/s_c$ strain UR297. Representative spectra are shown for the UA159 and $\Delta c/s$ strains.

Loss of the *c/s* gene is correlated with changes in the membrane proton gradient (ΔpH)

The observation that the $\Delta c/s$ strain was acid sensitive suggested that normal membrane function had been disrupted. Previous work had shown that the fatty acid composition of the membrane affects F_1F_0 -ATPase activity and ΔpH in *S. mutans*. Here, we determined the contribution of CL to

F-ATPase activity in *S. mutans*. Following growth in steady-state cultures held at a pH 7, extracts prepared from the $\Delta c/s$ mutant exhibited approximately 1.5-fold more activity than UA159 grown under the same conditions. A similar trend was observed when the strains were grown at pH 5 (Fig. 3a). Regardless of the pH value of the growth medium, the $\Delta c/s$ strain exhibited greater ATP-dependent phosphate release than the parent strain UA159.

Fig. 2. The Δ *cls* mutant is acid sensitive compared with UA159, but can be rescued by the addition of exogenous CL. (a) Growth of UA159, Δ *cls* and UR297 in BHI buffered to pH 7 (open symbols) or titrated to pH 5.4 (closed symbols). Values are means $\pm$ SD of 10 replicates for each strain and growth condition. (b) UA159 and Δ *cls*, grown to steady state at pH 7 (open symbols) or 5 (closed symbols), were subjected to acid challenge as described in Methods. Values are means $\pm$ SD of three separate cultures, each assayed in duplicate, represented as log N/N₀. (c) UA159 and the *cls* mutant were grown in BHI medium pH 5.4 and supplemented, where indicated, with 50 or 100 μ g bovine CL ml⁻¹. Values are means $\pm$ SD of 10 replicates for each strain and growth condition. (d) Acid killing assays were performed on batch cultures of UA159 and the *cls* mutant as previously described. Open symbols represent supplementation with 100 μ g CL ml⁻¹. Values are means $\pm$ SD of three separate cultures, each assayed in duplicate.

Since the Δ *cls* strain exhibited an acid-sensitive phenotype (Fig. 2a, b) and ATPase activity was elevated in cells grown in steady-state conditions (Fig. 3a), it appeared possible that Δ pH of the membrane had been disrupted. We tested this possibility by measuring Δ pH using cultures of UA159 and Δ *cls* strains, grown with or without exogenous CL (Fig. 3b). The data show that the proton gradient across the membrane was modestly reduced in the Δ *cls* mutant by approximately 0.12 pH units, compared with the parent strain, suggesting that CL may be necessary for the maintenance of normal Δ pH in *S. mutans*. The addition of bovine CL was insufficient for restoring the normal Δ pH of the Δ *cls* strain.

The membrane fatty acid profile of the Δ *cls* strain varies from that of the parent strain

The Δ *cls* strain was acid sensitive when grown at pH 5.4 (Fig. 2a), suggesting that the Δ *cls* strain contained reduced proportions of unsaturated fatty acids (UFAs), based on our previous reports (Fozo & Quivey, 2004a, b). GC-fatty acid methyl ester (FAME) analysis indicated a number of

differences in the fatty acid composition of the *cls* deletion mutant as compared with UA159 (Table 3). Regardless of the pH of the growth medium, saturated fatty acids (SFA) were elevated at the expense of UFAs in the Δ *cls* strain (Table 3). At pH 5, the ratio of UFA/SFA in the membrane decreased from approximately 1.8 in UA159 to 1.3 in the *cls* mutant strain, indicating a significant shift in the membrane fatty acid composition of the *cls* mutant.

Differences in specific SFAs and UFAs were observed between UA159 and the *cls* mutant grown at steady state and at pH values of 5 and 7. At pH 5, approximately 27% of all fatty acids isolated from UA159 were *cis*-eicosanoic acid (C_{20:1}); however, this fatty acid constituted less than 19% of fatty acids in the Δ *cls* strain. Changes in individual SFAs in the Δ *cls* strain were also observed. The contribution of palmitic acid (C_{16:0}) to total fatty acids increased by approximately 8% in the Δ *cls* strain when grown at pH 5 (Table 3). Variations in membrane fatty acid content were also observed between UA159 and the Δ *cls* mutant following growth at pH 7 (Table 3). Regardless of the growth condition, the proportion of *cis*-vaccenic acid to total fatty acids was similar in UA159 and the Δ *cls* strain,

Fig. 3. Physiological characterization of the *c/s* mutant. (a) F_1F_0 -ATPase activity is increased in steady-state-grown samples of the *c/s* mutant. Cells were grown to steady state at pH 5 or 7 and ATPase assays were performed as described in Methods. ATPase activity is expressed as a measure of inorganic phosphate released from ATP ($\mu\text{g total protein}^{-1}$). Values are means $\pm$ SD of three separate cultures, each assayed in duplicate. ■, UA159 pH 5; □, UA159 pH 7; ▲, *c/s* mutant pH 5; △, *c/s* mutant pH 7. * and # indicate statistically significant differences between pairs, $P \leq 0.05$. (b) ΔpH is reduced in the *c/s* mutant strain, suggesting that CL is important for regulating the internal pH. Cultures: ■, UA159; ▲, *c/s* mutant; △, *c/s* mutant + $50 \mu\text{g CL ml}^{-1}$ were harvested and titrated to pH 4.7 and the pH was recorded for 50 min. *, Addition of butanol at 50 min. The change in pH was determined by subtracting the pH at 50 min from the terminal pH. Values are means $\pm$ SD of three separate cultures, each assayed in duplicate. Statistically different values between pairs were determined using Student's *t*-test, $P \leq 0.05$.

suggesting that $C_{18:1}$ is not carried exclusively on the CL backbone. Overall, GC-FAME results were consistent with the concept that the $\Delta c/s$ strain is acid sensitive, due in part to reduced production of monounsaturated fatty acids.

Exogenous CL can suppress synthesis or incorporation of endogenous CL

The observation that growth medium supplemented with exogenously supplied CL was apparently protective for the $\Delta c/s$ strain, suggested that it had become incorporated into the cell membrane. It has been well established that exogenous fatty acids can be incorporated into the membrane of *S. mutans* (Fozo & Quivey, 2004a; Sato *et al.*, 1991) and

other bacteria (Parsons *et al.*, 2011; Parsons & Rock, 2011), thus it seemed possible that exogenous lipids could also integrate into the membrane. Phospholipid extractions performed on batch cultures of *S. mutans* UA159 grown in the presence or absence of CL were analysed by MS. Multiple peaks representing CL negative ions were observed within the m/z range 1350–1600. Two of the most prevalent peaks in the spectra were at m/z 1447.9 and 1479.9 (Fig. 4a); neither of these peaks were observed in spectra from cultures of UA159 grown without CL (refer to Fig. 1a), thus, they may have arisen from minor contaminants in the CL preparation. Control spectra showed that the two CL negative ions are, in fact, the major species found in the commercial preparation of the exogenous

Table 3. Membrane fatty acid composition of UA159 and *c/s* strains following growth at pH 5 or 7

The parent strain UA159 and the *c/s* deletion strain were grown in medium at pH 5 or 7. Membrane fatty acids were extracted and quantified as described in Methods. The values shown are percentages of the total areas under the curve as determined by GC-FAME and are means $\pm$ SD of three samples for UA159 and two samples for $\Delta c/s$ strains.

Fatty acid	Total membrane fatty acid composition (%)			
	UA159 pH 5	$\Delta c/s$ pH 5	UA159 pH 7	$\Delta c/s$ pH 7
SFA	35.6 $\pm$ 4.5	41.1 $\pm$ 2.3	61.8 $\pm$ 3.3	66.8 $\pm$ 2.1
UFA	63.4 $\pm$ 4.7	57.6 $\pm$ 2.6	36.9 $\pm$ 3.3	32.3 $\pm$ 1.9
UFA/SFA ratio	1.78	1.21	0.59	0.48
$C_{16:0}$	21.7 $\pm$ 3.5	28.5 $\pm$ 2.0	47.4 $\pm$ 2.5	44.8 $\pm$ 2.6
$C_{18:0}$	10.4 $\pm$ 0.8	9.3 $\pm$ 0.1	5.5 $\pm$ 1.3	3.7 $\pm$ 0.4
$C_{18:1}$	31.3 $\pm$ 0.9	33.6 $\pm$ 0.4	21.6 $\pm$ 1.8	20.7 $\pm$ 0.4
$C_{20:1}$	30.7 $\pm$ 4.1	20.8 $\pm$ 2.2	9.0 $\pm$ 2.1	3.48 $\pm$ 2.9
$C_{20:0} + C_{20:1}$	33.2 $\pm$ 4.5	22.0 $\pm$ 2.3	9.2 $\pm$ 2.4	3.66 $\pm$ 2.9

Fig. 4. UA159 ceases incorporation of endogenous CL into the membrane when grown in the presence of exogenous CL. Representative m/z spectra of UA159 (a) and Δcls (b) membrane phospholipids after growth with $50 \mu\text{g CL ml}^{-1}$. MS was performed on extracted phospholipids as described in Methods. *, CL negative ions derived from exogenously supplied CL. The data shown are from representative spectra.

bovine CL (data not shown). Hence, UA159 can incorporate exogenous CL into the membrane of growing cells.

It was not known if the synthesis and incorporation of endogenous CL into the membrane of the parent strain would be affected by the presence of exogenous CL. We found that the endogenous CL species, 1416.0, 1432.0, 1444.0, 1460.0 and 1488.1 observed in UA159 (Fig. 1a) were no longer detectable when UA159 was supplemented with CL (Fig. 4a). Therefore, *S. mutans* UA159 appears to either halt synthesis or cease incorporation of endogenous CL into the membrane when abundant, exogenous CL is available.

Phospholipids from batch cultures of Δcls treated with CL were also analysed for the presence of CL, which showed the presence of species at m/z peaks 1448.0 and 1479.9 (Fig. 4b). These peaks are representative of the two major CL species known to exist in the commercially available CL. Hence, Δcls , like UA159, was capable of assimilating exogenous CL into the membrane.

The *wapA/cls* promoter is transcriptionally upregulated in *wapA* and Δcls strains and by the addition of CL

The data indicated that CL was not required for growth of *S. mutans* at pH 7, but was beneficial for growth and

survival at low pH. Therefore, we postulated that *cls* could be transcriptionally upregulated in *S. mutans* when cells are exposed to acid stress.

In order to determine how external pH affects Δcls transcription, we first located the start site of transcription by primer extension analysis of the SMU.987 and SMU.988 region. Results from reverse transcriptase experiments revealed that *cls* transcription is driven from the *wapA* promoter, located immediately upstream of *cls*. The start site for *wapA* (and *cls*) was identified as a C residue, 44 bp upstream of *wapA*, SMU.987 (data not shown).

We investigated the relationship of CL to the *wapA/cls* promoter by creating strain UR269, carrying a single-copy *cat* reporter fused to the *wapA/cls* promoter region. CAT activity was elevated approximately 2.2-fold in cells grown at pH 5, compared with samples grown at pH 7, indicating that acidic conditions led to elevated transcription from the *wapA/cls* promoter (Table 4). Minimal CAT activity was observed from the vector control (UR212) grown at pH 5 or 7 (data not shown).

It was of interest to learn what effect the deletion of *wapA* or *cls* would have on the regulation of the *wapA* promoter. Therefore, we constructed *wapA* promoter-*cat* reporter constructs in strains deleted for either *wapA* (UR270) and Δcls (UR271). Extracts prepared from strain UR270 grown

Table 4. Estimates of transcriptional activity in batch cultures of *S. mutans* UA159, $\Delta wapA$ and Δcls containing *S. mutans* *wapA* promoter–*cat* fusion constructs

Strains are as follows: UR269, *wapA_P–cat* fusion in UA159 background; UR270, *wapA_P–cat* fusion in $\Delta wapA$ background; UR271, *wapA_P–cat* fusion in Δcls background. Data represent mean CAT activity $\pm$ SD on cells grown in triplicate with six replicates for each extract. Letters indicate statistical significance between pairs using Student's *t*-test with $P \leq 0.05$.

Strain, growth condition	CAT activity [nmol min ⁻¹ (mg protein) ⁻¹]
UR269, pH 7	59.3 $\pm$ 6.2 ^{a,b,c,d}
UR269, pH 5	130.5 $\pm$ 2.7 ^{a,e,f,g}
UR269, pH 7 + CL	277.0 $\pm$ 12.8 ^b
UR269, pH 5 + CL	143.0 $\pm$ 6.9 ^c
UR270, pH 7	295.8 $\pm$ 11.9 ^c
UR270, pH 5	228.0 $\pm$ 5.5 ^f
UR271, pH 7	258.0 $\pm$ 9.4 ^d
UR271, pH 5	224.0 $\pm$ 6.7 ^g

at pH 7 displayed approximately fivefold greater CAT activity than the parent strain, UR269, grown under identical conditions. UR271 grown at pH 7 exhibited approximately 4.4-fold greater CAT activity than UR269. CAT activity was significantly elevated in the Δcls and $\Delta wapA$ mutant strains compared with UA159, indicating that the *wapA* promoter was upregulated (Table 4). In order to determine whether CAT activity was also elevated at pH 5 in the mutant strain backgrounds, CAT assays were performed on UR270 and UR271 grown at low pH. UR270 and UR271 samples grown at pH 5 exhibited approximately 1.75-fold more CAT activity than was observed in the UA159 background (UR269) under the same conditions (Table 4). Regardless of the growth pH, the *wapA* promoter was transcriptionally upregulated in the $\Delta wapA$ and Δcls backgrounds. It is important to note that CAT activity in the UA159 background (UR269) was also elevated at pH 5, compared with pH 7, indicating that transcription from the *wapA* promoter was elevated at low pH. However, in both UR270 and UR271, CAT activity was elevated at pH 7, indicating transcriptional dysregulation in the absence of *wapA* or *cls*.

MS revealed that UA159, when supplemented with CL, no longer incorporated endogenous CL into the membrane (Fig. 4). It was thought that the *wapA/cls* promoter could be transcriptionally repressed in the presence of exogenous CL, leading to the observed decrease in endogenous CL in the plasma membrane. To test this hypothesis, the *wapA/cls* promoter–fusion strain (UR269) was grown with 50 μ g CL ml⁻¹ and extracts were assayed for CAT activity. Contrary to expectation, UR269 grown with CL exhibited elevated CAT activity in extracts from cells grown at pH 5 and at pH 7, compared with the parent (Table 4). Furthermore, pH-dependence of promoter transcription was essentially abolished in UR269 grown with CL. It appeared that

exogenous CL interferes with normal transcription of the *wapA* promoter.

DISCUSSION

In the present study, we established the identity of the CL synthase gene (*cls*) in *S. mutans* UA159 as SMU.988 and have characterized the role of CL in the acid-adaptive strategy of *S. mutans*. The loss of the presumptive PG synthase gene (*pgs*, SMU.2151c) was lethal in our study, whereas loss of the *cls* gene was viable. The Δcls strain exhibited a number of phenotypic characteristics that associated CL with acid resistance in *S. mutans*.

Results from the physiological characterization of the Δcls strain showed that the loss of the *cls* gene rendered the organism sensitive to both moderate and extreme acid stress. The acid sensitivity of the *cls* mutant strain was not unexpected, as lipids, and their fatty acid constituents, have been implicated in other environmental stress responses; for example, the osmotic stress response in *E. coli* (Romantsov *et al.*, 2007; Hiraoka *et al.*, 1993).

In previous reports, we showed that the loss of unsaturated membrane fatty acids led to extreme acid sensitivity and to elevated levels of F-ATPase activity in *S. mutans* (Fozo & Quivey, 2004b; Kuhnert *et al.*, 2004). Here, we were focused on how the lipid backbone contributed to acid resistance. Somewhat surprisingly, the Δcls strain contained a lower proportion of unsaturated fatty acids, explaining in part, the acid sensitivity of the mutant strains. However, what remains to be learned from future studies, is the mechanism to explain why CL would carry a disproportionate amount of saturated fatty acids. Nevertheless, the physiological and physical data reported here clearly support a role for CL in the overall membrane homeostasis of the organism.

The presence of CL in the growth medium did not restore Δ pH to parent strain levels. This may be attributable to the dominant fatty acid on exogenous bovine CL, which is C_{18:2}. The data suggest that the addition of polyunsaturated fatty acids was insufficient to rescue Δ pH, even though they were adequate for structural complementation. Additional studies with polyunsaturated fatty acids in the *fabM* strain will be necessary before a complete conclusion can be drawn regarding the role of mono- versus polyunsaturated fatty acids on CL backgrounds in *S. mutans* acid resistance.

The observations from the physiological experiments and the mass spectrometric data suggested that transcription from the *cls* promoter could be affected by environmental conditions. The search to identify the *cls* promoter revealed that the *wapA* and *cls* genes are co-transcribed. Previous studies with WapA have suggested that it plays a role in sucrose-independent colonization of tooth surfaces and cell–cell adherence, and that the presence of sucrose in the growth medium resulted in repression of the *wapA* promoter, observed using real-time PCR (Qian & Dao, 1993; Zhu *et al.*,

2006). The relationship between the WapA protein and CL remains unclear; however, here, using promoter–reporter gene fusions, we verified that *wapA* and *cls* transcription levels are elevated during growth at low pH in the parental strain background. Interestingly, in the *cls* and *wapA* mutant backgrounds, transcription was elevated at pH 7. There was also elevated activity in both mutant strain backgrounds compared with UA159, regardless of growth pH. It appeared that there was transcriptional dysregulation in the absence of *cls* or *wapA*, the source of which remains unclear at present. Results from MS indicated that UA159, supplemented with exogenous CL, halted incorporation of endogenous CL into the membrane. It has been shown previously in *E. coli* that CL synthesis is regulated at both the genetic and enzymic level (Ragolia & Tropp, 1994; Tropp, 1997). In the case of *S. mutans*, the most straightforward explanation of our observations is that exogenous bovine CL inhibits endogenous CL synthase, thereby reducing or eliminating the endogenous CL. This would explain the disappearance of monounsaturated fatty acids on CL and the presence of the polyunsaturated fatty acids (C_{18:2}) from the exogenous CL. A mechanism of genetic regulation of CL synthase is, at this point, unclear for *S. mutans*, though a feedback loop to upregulate CL synthesis during growth at acidic pH values would be expected and is the subject of present investigations.

The present study was intended to increase our understanding of how phospholipids contribute to the acid-adaptive process in *S. mutans*. We determined that a *cls* mutant was acid sensitive but could be rescued by exogenous CL of bovine origin, indicating that CL contributes to the acid-adaptive process in *S. mutans*. We also determined that CL carries an elevated proportion of monounsaturated fatty acids, thus enriching the membrane with UFAs required for acid adaptation (Fozo & Quivey, 2004b; Foze *et al.*, 2007). We demonstrated that CL contributes to ΔpH, potentially by influencing the overall abundance of UFAs or their organization in the membrane. The data also show that the *wapA/cls* promoter is upregulated at low pH. The addition of exogenous CL resulted in increased transcription at the *wapA* promoter, indicating that CL does not act as a repressor, though it cannot be excluded that the molecule acts as a co-repressor, as C_{18:1} does for the *fabT* regulator in *Streptococcus pneumoniae* (Jerga & Rock, 2009; Lu & Rock, 2006). Studies are now in progress to further elucidate the role that CL has in the acid resistance of *S. mutans* and how acidic external pH values are signalled to the *wapA–cls* gene cluster, as part of the acid-adaptive response of this universal human pathogen.

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