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Regulation of fatty acid biosynthesis by the global regulator CcpA and the local regulator FabT in *Streptococcus mutans*

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SUMMARY

SMU.1745c, encoding a putative transcriptional regulator of the MarR family, maps to a location proximal to the *fab* gene cluster in *Streptococcus mutans*. Deletion of the SMU.1745c ($fabT_{Sm}$) coding region resulted in a membrane fatty acid composition comprised of longer-chained, unsaturated fatty acids (UFA), compared with the parent strain. Previous reports have indicated a role for FabT in regulation of genes in the *fab* gene cluster in other organisms, through binding to a palindromic DNA sequence. Consensus FabT motif sequences were identified in *S. mutans* in the intergenic regions preceding *fabM*, $fabT_{Sm}$ and *fabK* in the *fab* gene cluster. Chloramphenicol acetyltransferase (*cat*) reporter fusions, using the *fabM* promoter, revealed elevated transcription in a Δ$fabT_{Sm}$ background. Transcription of $fabT_{Sm}$ was dramatically elevated in cells grown at pH values of 5 and 7 in the Δ $fabT_{Sm}$ background. Transcription of $fabT_{Sm}$ was also elevated in a strain carrying a deletion for the carbon catabolite repressor CcpA. Purified $FabT_{Sm}$ and CcpA bound to the promoter regions of $fabT_{Sm}$ and *fabM*. Hence, the data indicate that $FabT_{Sm}$ acts as a repressor of *fabM* and $fabT_{Sm}$ itself and the global regulator CcpA acts as a repressor for $fabT_{Sm}$.

Keywords

acid stress response; fatty acid synthesis; *Streptococcus mutans*

INTRODUCTION

Streptococcus mutans, an etiologic agent of dental caries in humans, colonizes human teeth via the production of an extracellular glucan matrix that acts to bind the organism to oral hard surfaces and initiates the accumulation of glucans with multiple species of oral

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bacteria, leading to the formation of the niche referred to as dental plaque (Bowen & Koo, 2011). As a result of glucan production, *S. mutans* remains in the oral cavity throughout human lifetimes. Following its colonization of teeth, *S. mutans* is confronted with a variety of environmental stressors, including the need to survive the acidic conditions in plaque that arise from the metabolism of dietary carbohydrates to organic acids. The accumulation of acids, without removal of plaque, leads to erosion of tooth enamel and the progression of dental caries (Loesche, 1986; Nyvad *et al*., 2013).

A key acid-adaptive strategy employed by *S. mutans* is the ability to effect substantial changes in membrane fatty acid composition in response to external acidification (Fozo & Quivey, 2004b; Fozo et al., 2004; Lemos & Burne, 2008). As pH values in plaque fall, the membrane fatty acid composition changes from a profile predominant in short-chained, saturated fatty acids (SFA) to a profile containing elevated levels of long-chained, unsaturated fatty acids (UFA) (Quivey *et al*., 2000; Fozo & Quivey, 2004b). Loss of UFAs results in extreme sensitivity to acidic pH values by *S. mutans*, *in vitro*, and to greatly diminished caries in a rat model (Fozo *et al*., 2007).

Clearly, membrane fatty acid composition in S. *mutans* is important to the pathogenesis of the organism. However, little is presently known about the regulatory mechanisms for controlling production of its membrane fatty acid repertoire in response to changes in the external environment. Reports from studies conducted with *Streptococcus pneumoniae* and *Lactococcus lactis* have shown that a transcriptional regulator, referred to as FabT, is encoded at a locus immediately upstream of the *fab* gene cluster (Lu & Rock, 2006; Eckhardt *et al*., 2013). $FabT_{Sp}$ binds to a DNA sequence located upstream of *fabT* and *fabK*, but not upstream of *fabM*. Loss of $FabT_{Sp}$ resulted in a reduced growth rate in medium held at a pH value of 5.5 (Lu & Rock, 2006; Eckhardt *et al*., 2013). Subsequent efforts showed that $FabT_{Sp}$ binds acyl-acyl carrier protein (ACP) as a co-effector for regulation and that $C_{18:1}$, *cis*-vaccenic acid, provided the strongest effects on binding of $FabT_{Sp}$ to its target site (Jerga & Rock, 2009). Similarly, $FabT_{Ll}$ acts to regulate fatty acid biosynthesis in *L. lactis*, via a consensus motif located upstream of $fabT_{Ll}$, *fabD*, *accC* and *fabI* and deletion of $fabT_{Ll}$ led to an increase in saturated acyl chains in the membrane of the organism (Eckhardt *et al*., 2013). *Streptococcus mutans* occupies a very different ecological niche from *S. pneumoniae*, although all of the *fab* genes, including the relative position of the FabM *cis-trans* isomerase-encoding gene, are identically organized in their respective genomes (Tettelin *et al*., 2001; Ajdic *et al*., 2002). Gene organization in *L. lactis* is comparable to the streptococcal arrangement; however, *L. lactis* lacks *fabM* and possesses a *fabI* gene and a second *fabZ* elsewhere in the genome (Wegmann *et al*., 2007).

In the present study, we show that the gene at locus SMU.1745c (as annotated by NCBI) encodes a transcriptional regulator that is orthologous to the *S. pneumoniae* (Lu & Rock, 2006; Jerga & Rock, 2009) and the *L. lactis* FabT (Eckhardt *et al*., 2013). Data from biochemical and physiological experiments revealed significant differences between the *S. mutans* approach to the use of FabT as a regulator of fatty acid production and the regulatory scheme shown in the orthologs.

We report here that loss of *fabT*$_{Sm}$ in *S. mutans* does not exert gross changes in membrane fatty acid composition; however, loss of FabT$_{Sm}$ resulted in broad changes to the *S. mutans* transcriptome following growth at pH values of 5 and 7. Results from experiments using *cat*-reporter fusions indicated an approximately two-log-order dynamic range of fatty acid gene transcription that was highly dependent on external pH values. We also provide evidence for the involvement of the global regulator CcpA in *fab* gene transcription in response to environmental changes. We conclude that relatively subtle changes in membrane composition have dramatic consequences for cellular homeostasis in *S. mutans*, underscoring its central role in the ability of the organism to cope with changes in external pH values.

METHODS

Bacterial strains

Streptococcus mutans UA159 (Murchison *et al*., 1986), the genomic type strain (Ajdic *et al*., 2002), was used in these studies. MU1591, the *fabT*$_{Sm}$ deletion strain, is derived from UA159. Deletion of the *fabT*$_{Sm}$ locus, SMu1591 (GenBank designation: SMU.1745c) was performed essentially as previously described (Lau *et al*., 2002; Sheng *et al*., 2010; Santiago *et al*., 2012). Strain MU1591 was created by replacement of the SMU.1745c open reading frame with a non-polar erythromycin-resistance cassette (Erm^R) flanked by approximately 400 bp upstream and downstream of the gene using primer pair MU1591P7UPF (5'-TAA GAAATAATCAATCGC-3′) and MU1591P8DNR (5'-AAGCCTTTGTGAATGTCG-3′) to facilitate homologous recombination. The intergenic region preceding the SMU.1745c coding region was left intact upstream of the erythromycin-resistance cassette. Strains were maintained on brain–heart infusion (BHI) agar medium (BD/Difco, Franklin Lakes, NJ) and supplemented with erythromycin (Erm) at 5 μg m^{-1}, where necessary. Batch cultures were grown in tryptone–yeast extract (TY) medium supplemented with 1% glucose. Steady-state cultures were grown in a BioFlo2000 (New Brunswick, Edison, NJ) in TY medium at a dilution rate of D = 0.24 h^{-1} as previously described (Fozo & Quivey, 2004b; Kuhnert *et al*., 2004). Cells were limited by glucose (2.3 mM) and pH maintenance was achieved by addition of 2 M KOH. Steady-state cells used in these studies were harvested after a minimum of 10 generations at each pH value.

Growth rate and yield were assessed using a Bioscreen C automated growth reading system (Growth Curves USA, Piscataway, NJ) essentially as described elsewhere (Kajfasz *et al*., 2010; Derr *et al*., 2012).

Complementation of *fabT*$_{Sm}$ locus

Primer pair 1591 PromBglUp2 and 1591 PromBglDwn were used for polymerase chain reaction (PCR) amplification of the *fabT*$_{Sm}$ coding region and cognate promoter fragment from *S. mutans* UA159. The purified amplicon was cloned into pCRBlunt (Life Technologies, Carlsbad, CA) as per the manufacturer's direction, resulting in the plasmid pCRmarRLP8. The *Bgl*II restriction sites introduced by PCR were used to excise the *fabT*$_{Sm}$ plus promoter fragment for subsequent cloning into pSUGK-Bgl, an integration vector (Derr *et al*., 2012), and transformants were selected for Km^R. Proper integration of the *fabT*$_{Sm}$

promoter plus coding region into the *Bgl*II site of pSUGK-Bgl, in the opposite orientation to *gtfA*, was determined by colony PCR with primer pair 1591 PromBglDwn and gtfAseqKan (see Table S4). The appropriate construct, pSUGKmarRLP7, was transformed into MU1591 and colonies were selected on BHI agar medium containing kanamycin (1 mg ml^{-1}). Transformants were screened by colony PCR with the primer pair 1591 PromBglDwn and gtfAseqKan, and also by Southern blotting with a *gtfA*-specific DNA probe (data not shown). One such construct, named *S. mutans* UR294, contained the $fabT_{Sm}$ gene, preceded by its cognate promoter, in the *gtfA* locus.

Stress tolerance assays

Samples were taken from steady-state cultures of *S. mutans* UA159 (parent) and MU1591 (Δ$fabT_{Sm}$ deletion strain) for comparison of sensitivity to acid and hydrogen peroxide (Belli & Marquis, 1991; Quivey *et al*., 1995). Briefly, strains were tested for sensitivity to acid-mediated killing by harvesting steady-state samples from the chemostat at culture pH values of 7 and 5, resuspending cell pellets in 0.1 M glycine, pH 2.5, and stirring at room temperature. Aliquots were removed at 0, 15, 30 and 60-min intervals, serially diluted, and plated on solid BHI medium. Hydrogen peroxide sensitivity assays were also performed on the cells as previously described (Belli & Marquis, 1991; Quivey *et al*., 1995). Briefly, steady-state samples harvested from the chemostat, at culture pH values of 7 and 5, were resuspended in BHI medium, and hydrogen peroxide was added to a final concentration of 16.3 mM. Aliquots were removed at 0, 15, 30 and 60 min, serially diluted, and plated on BHI agar medium. Viable cells from each condition were counted and used to calculate log (N_t/N_o), where N_t = number of colonies obtained at a specific time point, and N_0 = number of colonies at time zero.

Membrane fatty acid determination

Membrane fatty acid composition was analysed in cultures of the parent strain (*S. mutans* UA159), the Δ$fabT_{Sm}$ strain (MU1591), and the $fabT_{Sm}$-complemented strain (UR294) grown in TYG medium in batch conditions overnight in either a 5% [volume/volume (v/v)] CO_2/95% air incubator (batch), in steady-state at pH values of 7 and 5 (described above), and in biofilms. Biofilm cultures of the parent strain and MU1591 (Δ$fabT_{Sm}$ strain) were grown on glass slides in TY medium supplemented with 1% sucrose (TYS). Slides were transferred to tubes containing fresh medium every day for 5 days. At the end of the growth period, biofilms were harvested from glass slides and sonicated to break up chains.

Harvested cell pellets from each growth condition were washed twice with sterile H_2O, and stored at −80°C until the samples were sent to Microbial ID, Inc. (Newark, DE) for analysis. Membrane fatty acid content was determined by GC-FAME as described elsewhere (Bligh & Dyer, 1959; Fozo & Quivey, 2004b).

Transcriptional assays

Reverse transcription (RT-) PCR were performed to determine the extent of co-transcription in the fatty acid biosynthesis cluster. Primer pairs TS12/TS8, TS21/TS10, TS14/TS9, TS22/TS7, TS20/TS11 (detailed in the Table S4) were used to examine transcription across the non-coding regions between *fabM/*$fabT_{Sm}$, *acp/fabK, fabH/acp, fabG/fabF* and *fabF/accB*,

respectively. Total RNA (43 ng) isolated from a steady-state culture of *S. mutans* UA159 grown to a pH value of 7 was incubated with the anti-sense primer in each primer pair with or without SuperScript II Reverse Transcriptase as per the manufacturer's instructions (Life Technologies). Each primer pair was also used in a standard PCR with UA159 genomic DNA as a positive control.

Single-copy reporter gene fusions were constructed using PCR to amplify the promoter fragments for *fabT*$_{Sm}$ (SMU.1745c), *fabM* (SMU.1746c) and *fabK* (SMU.1742c). Transcriptional activity was determined from either an intact promoter fragment or from a truncated promoter fragment lacking the distal FabT$_{Sm}$ binding site (described in Fig. 4). Primer pairs used are detailed (see Table S4). Amplicons were subcloned into pCRBlunt (Life Technologies) and appropriate constructions were verified by nucleotide sequencing. Using incorporated restriction sites (*Sac*I/*Bgl*II), promoter fragments were subcloned into an integration vector containing a promoterless chloramphenicol acetyltransferase (*cat*) gene derived from *Staphylococcus*, pJL84 (Santiago *et al*., 2012). Integration of the promoter-CAT construction occurs within the intergenic regions between *mtlA* (SMU.1185c) – *glmS* (SMU.1187c) and *mtlD* (SMU.1182c) – *phnA* (SMU.1180c). *Streptococcus mutans* UA159 and MU1591 were transformed with the pJL84 constructs and colonies were selected on BHI agar medium containing kanamycin (1 mg ml^{-1}). Proper integration of the specific promoter-*cat* constructs was verified by colony PCR using the primer pair CATJL and ICphnA (see Table S4) and nucleotide sequencing using the CATJL primer. The resulting strains UR211 (*fabM-cat* in UA159), UR221 (*fabK-cat* in UA159), UR222 (*fabT*$_{Sm}$*-cat* in UA159), UR237 (*fabK-cat* in MU1591), UR242 (*fabT*$_{Sm}$*-cat* in MU1591), UR243 (*fabM-cat* in MU1591), UR266 (*fabK* truncated promoter-cat in UA159), UR272 (*fabM* truncated promoter-cat in UA159), UR273 (*fabK* truncated promoter-cat in MU1591) and UR274 (*fabM* truncated promoter-cat in MU1591) are detailed in Table 1.

Reporter constructs for examining the global regulator CcpA were constructed in a similar manner as detailed above. The *fabT*$_{Sm}$*-cat* construct, pJLmarR, and the *fabM-cat* construct, pJLfabM, were transformed into *S. mutans* MU1446, carrying a deletion in *ccpA* (Santiago *et al*., 2013) (Table 1). Colonies were selected on BHI agar medium containing kanamycin (1 mg m^{-1}). Proper integration of the specific promoter-*cat* constructs was verified by colony PCR using the primer pair CATJL and ICphnA (see Table S4) and the strains were named *S. mutans* UR310 and UR313, respectively.

The CAT assays, as modified for oral streptococci, were performed as previously described (Shaw *et al*., 1979; Chen *et al*., 1998; Kuhnert *et al*., 2004). Briefly, all reporter strains were grown in batch culture to a final optical density at 600 nm of approximately 0.5 in a 5% (v/v) CO_2/95% air incubator in BHI medium titrated to pH 5 with HCl or buffered to pH 7 with 50 m$_M$ KPO_4 buffer; or in unbuffered BHI medium or in FMC minimal medium +1% glucose (Terleckyj & Shockman, 1975; Terleckyj *et al*., 1975). Cells were harvested and stored at −80°C until whole cell lysates were prepared. Reactions were initiated by the addition of 20 μl 5 m$_M$ chloramphenicol. Time–course of the reactions was followed by absorbance at 412 nm. Rates were calculated as nmol min^{-1} of product formed. Protein concentration was estimated using the BioRad protein assay reagent (BioRad, Hercules, CA) (Bradford, 1976). CAT activity units were calculated as nmol min^{-1} mg protein^{-1}.

RNA purification

RNA used in the experiments outlined here was isolated from four, independent, steady-state cultures of *S. mutans* UA159 and MU1591 grown to pH values of 7 and 5 and purified as previously described (Abranches *et al*., 2006; Baker *et al*., 2014). Briefly, *S. mutans* cells were homogenized in a MiniBead Beater 8 (BioSpec Products, Bartlesville, OK). Cell lysates were subjected to three hot acid–phenol–chloroform extractions, and the nucleic acid was precipitated overnight at −20°C. RNA pellets were resuspended in nuclease-free H_2O and treated with DNase I (Ambion, Austin, TX) at 37°C for 30 min. The RNA was purified again using an RNeasy minikit (Qiagen, Valencia, CA), including a second on-column DNase treatment, performed as recommended by the supplier. RNA concentrations were determined using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA), and samples were run on a 1.2% FlashGel (Lonza Inc., Allendale, NJ) to verify RNA integrity.

Microarray analysis

Streptococcus mutans UA159 microarray chips were provided by The Institute for Genomic Research (TIGR; JCVI). A reference RNA, isolated from *S. mutans* UA159 cells grown in BHI medium to an optical density at 600 nm of 0.5, was used in every experiment. The reference RNA was purified as above, aliquoted, and stored at −80°C.

All sample RNAs were purified as described above and used to generate cDNA according to the protocol provided by the Pathogen Functional Genomics Resource Center (PFGRC) at the J. Craig Venter Institute (ftp://ftp.jcvi.org/pub/data/PFGRC/MAIN/pdf_files/protocols/M007.pdf) with minor modifications as detailed previously (Abranches *et al*., 2006; Baker *et al*., 2014). Labeled cDNA from four independent cultures of *S. mutans* UA159 and MU1591 grown to steady-state pH values of 7 and 5 were hybridized overnight at 42°C with labeled reference cDNA, generating a total of 16 slides. Hybridization was carried out in a MAUI 4-Bay Hybridization system (BioMicro Systems, Inc., Salt Lake City, UT), washed according to PFGRC protocols, and scanned using a GenePix 4000b Microarray Scanner (Molecular Devices, Inc., Sunnyvale, CA). After scanning, single channel images were gridded using TIGR SPOTFINDER, normalized and analysed using the method previously outlined (Abranches *et al*., 2006) and available from the TM4 MICROARRAY Software Suite (http://www.tm4.org/). Statistical analysis was carried out using BRB array tools (http://linus.nci.nih.gov/BRB-ArrayTools.html) with a cutoff *P*-value of 0.01 and at least two-fold change in expression. The microarray data have been deposited in the NCBI Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo) under GEO accession number GSE36960.

Microarray results were validated by performing real-time PCR on a subset of genes. Primers for the genes tested are listed in Table S4.

Real-time PCR analysis

Real-time quantitative RT-PCR was used to validate microarray experiments. For this, 0.5 μg RNAs isolated (as described above) from four, independent steady-state cultures of *S. mutans* UA159 and MU1591, grown to pH values of 7 and 5, were used to synthesize cDNA using the High Capacity cDNA Reverse Transcription Kit (Life Technologies) containing

random primers. Control reactions were also conducted to determine the contribution of any DNA signal. Gene-specific primers (Table S4) used in all real-time PCR experiments were designed using Beacon Designer 2.0 software (Premier Biosoft International, Palo Alto, CA). Standard curves for each gene were prepared as described elsewhere (Abranches *et al*., 2006; Buckley *et al*., 2014). Briefly, the concentration of the purified amplicon derived from gene-specific primers was determined using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). The template concentration was used to calculate the template copy number, and serial dilutions were performed to create a standard curve. Reactions were carried out in a StepOne Plus Real Time PCR System (Life Technologies) using Power SYBR Green Master Mix (Life Technologies) with three replicates per sample and wells containing dH_2O were used to determine background levels of fluorescence. Melt curves of the amplification reactions were verified to contain a single peak and reaction mixtures from random wells were examined by gel electrophoresis to ascertain that a single band was obtained. The relative quantities for each replicate of a sample were averaged and a standard deviation was calculated.

Expression of FabT$_{Sm}$

The *S. mutans* *fabT*$_{Sm}$ gene was PCR amplified using primer pair MarR Fwd *XhoI* and MarR Rev *BamHI* (Table S4). The resulting amplicon was digested and cloned into the *XhoI* and *BamHI* sites of pET19b (Novagen, Rockland, MA). Proper construction was verified by nucleotide sequencing and a correct clone was transformed into *E. coli* Rosetta competent cells (Novagen) for protein expression. One such transformant was grown at 37°C, with shaking, in LB medium containing ampicillin (100 μg m^{-1}) to an OD_{600} of 0.6, then induced with 1 mM IPTG (isopropyl β-D-galactopyranoside) and allowed to grow overnight, with shaking, at 20°C. Cells were harvested and lysed with addition of lysozyme to 1 mg m^{-1} and mechanical disruption in a Mini BeadBeater (BioSpec Products). Soluble proteins were applied to a Ni-nitrilotriacetic acid (NTA) agarose column (Qiagen) and washed with 50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8 as per the manufacturer's instructions for purification of native protein. His-tagged proteins were eluted with 50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8. Eluant fractions containing recombinant FabT$_{Sm}$ protein, as determined by sodium dodecyl sulfate– polyacrylamide gel electrophoresis, were dialysed against 10 mM Tris–HCl, pH 8; 50 mM NaCl; 10% glycerol overnight in preparation for the electrophoretic mobility shift assay (EMSA).

Protein concentration was determined using the method of Bradford (Bradford, 1976) with the BioRad Protein Assay Reagent. Presence of the 6X His tag was verified by Western blot analysis using α-His IgG primary antibody (GE Healthcare, Piscataway, NJ) (data not shown).

Electrophoretic mobility shift assay

Target DNAs containing the full-length intergenic regions preceding *fabT*$_{Sm}$ (323 bp), *fabM* (163 bp) and *fabK* (300 bp) were PCR amplified from plasmids pJLmarR, pJLfabM, pJLfabK, respectively (Table 1). The truncated *fabM* promoter (79 bp) was PCR amplified from the vector pJLfabMtrunc (Table 1). The gel-purified PCR amplicons were end-labeled

with [γ-^{32}P]ATP (Perkin Elmer, Waltham, MA) using T4 Polynucleotide Kinase as per the manufacturer's instructions (Life Technologies).

The EMSAs involving FabT$_{Sm}$ were performed essentially as described (Lu & Rock, 2006), with modifications. Briefly, the DNA binding reactions containing ^{32}P-labeled probe (approximately 5000 counts min^{-1}) and purified, His-tagged FabT$_{Sm}$ (1000 nM) were incubated for 45 min at room temperature (25°C) in a buffer consisting of 10 mM Tris–HCl, pH 8; 50 mM NaCl; 10 mM EDTA; 10% (v/v) glycerol; 1 mM dithiothreitol in a total volume of 20 μl. For competition experiments, 200-fold excess, cold promoter DNA or 1 μg poly(dG-dC) (Roche Applied Science, Indianapolis, IN) was added to the binding reaction components, including FabT$_{Sm}$, and allowed to preincubate 15 min at room temperature before addition of the labeled probe. Bovine serum albumin (1000 nM) was used as a control for specificity of DNA binding. When used, long-chain acyl-acyl carrier protein, $C_{18:1}$ acyl-ACP (gift of C. Rock), was added at a concentration of 1500 nM. Samples were separated on 6% non-denaturing gels in 1 × Tris–borate EDTA.

EMSAs involving CcpA [gift of R. Burne; (Abranches *et al*., 2008)] were performed as previously described. Briefly, DNA fragments containing the full-length intergenic regions preceding *fabM*, *fabT$_{Sm}$* and *fabK were* created as described above. A representative binding reaction (25 μl) consisted of the following mixture: binding buffer [20 mM Tris–HCl, pH 8; 8.7% (v/v) glycerol, 1 mM EDTA; 5 mM $MgCl_2$; 250 mM KCl; 0.5 mM dithiothreitol; 2 μg bovine serum albumin], radiolabeled DNA probe (3000 counts min^{-1}) and purified recombinant CcpA (3000 nM). Binding reactions were incubated at 37°C for 45 min. CcpA-DNA binding was assessed using an 8% non-denaturing gel in 1 × Tris–glycine buffer.

The gels were exposed to a phosphorimager screen and binding was detected with a Molecular FX phosphorimager and BioRad Quantity One software (BioRad).

RESULTS

Identification of the FabT$_{Sm}$ ortholog in *S. mutans*

The SMU.1745c open reading frame in *S. mutans* UA159, annotated as a transcriptional regulator of the MarR family (Sulavik *et al*., 1995; Ajdic *et al*., 2002) (http://www.ncbi.nlm.nih.gov/gene/1028966), shares homology with the previously characterized regulator of membrane fatty acid biosynthesis in *S. pneumoniae* and *L. lactis*, both denoted as FabT (Lu & Rock, 2006; Jerga & Rock, 2009; Eckhardt *et al*., 2013). The deduced amino acid sequence encoded by SMU.1745c is approximately 60% identical and 79% similar to FabT from *S. pneumoniae* and 36% identical and 57% similar to the *L. lactis* ortholog. Hereafter, we will refer to the *S. mutans* ortholog as FabT$_{Sm}$.

Using a PCR-based approach, the *fabT$_{Sm}$* coding region was deleted, leaving in its place, a non-polar, in-frame erythromycin-resistance marker (Erm^R), transcriptionally driven by the native *fabT$_{Sm}$* promoter. The method of deleting *fabT$_{Sm}$* was accomplished such that its removal did not eliminate transcription of the immediate downstream gene, *fabH* (SMU.1744c), as determined from real-time PCR measurements of *fabH* transcripts in the Δ*fabT$_{Sm}$* strain (see Fig. S2). Deletion of *fabT$_{Sm}$* was verified by colony blotting and real-time PCR

experiments using RNA isolated from the deletion strain, referred to as MU1591 (see Fig. S1).

Stress tolerance of the *fabT*$_{Sm}$ mutant strain

In our previous studies, loss of the *fabM* gene resulted in a greatly diminished growth rate and a high degree of acid-sensitivity in *S. mutans* (Fozo & Quivey, 2004b). In contrast, deletion of *fabT*$_{Sm}$ did not affect the final yield, compared with growth of the parent strain, under various growth conditions, including rich medium (BHI) or in medium adjusted to an acidic pH value (BHI pH 5.4); or, in the presence of H_2O_2 or 8-hydroxyquinoline (see Table S1). In fact, the Δ*fabT*$_{Sm}$ strain exhibited a shorter relative doubling time under acidic (pH 5.4) conditions (Table S1). Previous studies have shown that *S. mutans* mounts a substantial, and effective, response to acid and oxidative stress following growth at low pH (Belli & Marquis, 1991). Accordingly, the Δ*fabT*$_{Sm}$ strain was further tested for its ability to withstand severe acid and oxidative challenges following growth in steady-state cultures held at pH values of 7 or 5 (Fig. 1). The *fabT*$_{Sm}$ deletion strain was more acid-resistant when grown at pH 7 than the parent strain, and slightly more acid-sensitive when grown at pH 5. The *fabT*$_{Sm}$ mutant strain also exhibited a tolerance to peroxide when grown at both pH 7 and pH 5, compared with the parent strain. Hence, overall, loss of FabT$_{Sm}$ resulted in relatively modest physiological effects, under the conditions tested.

Loss of FabT$_{Sm}$ affects membrane fatty acid composition

Under all conditions tested, deletion of *fabT*$_{Sm}$ resulted in a membrane fatty acid composition with an elevated UFA : SFA ratio compared with the parent strain, UA159 (Table 2A; and see Table S3). With the exception of cells grown to steady-state in cultures at pH 5, which nevertheless exhibited the same trend, deletion of *fabT*$_{Sm}$ resulted in a membrane fatty acid composition consisting of a significantly elevated ratio of long-chained (C_{18} and C_{20}) to short-chained (C_{10}, C_{12}, C_{14}, and C_{16}) fatty acids, compared with UA159 (Table 2B). The increase in UFAs observed in the *S. mutans* Δ*fabT* strain is dissimilar to the effect seen in *fabT* mutant strains of *S. pneumoniae* and *L. lactis*, and suggested a role for FabT regulation of *fabM* expression in *S. mutans*.

The *fabT*$_{Sm}$ complement strain, UR294, behaved similarly to the parent strain, with respect to the ratio of long-chained : short-chained fatty acids; yet, the UFA : SFA ratio was more similar to MU1591 (see Table S2). We attribute the intermediate phenotype of the complement strain (between the parent and deletion strain) to the fact that the complementing copy of *fabT*$_{Sm}$ was expressed from an ectopic locus in the genome (*gtfA*, SMU.881) and, therefore, may have been removed from potential native regulatory elements. However, results from quantitative real-time PCR showed that *fabT*$_{Sm}$ expression levels in the complement strain, UR294, were similar to the parent strain, indicating no effect on transcription of the gene (Fig. S1).

Identification of FabT binding motifs in the fab gene cluster

A consensus FabT binding site in Streptococcaceae has been described in the RegPrecise collection of manually curated regulons (http://regprecise.lbl.gov/RegPrecise/regulog.jsp?regulog_id=3571) (Novichkov *et al*., 2010) (Fig. 2A). The putative motif is shared among

various streptococcal species, including *S. mutans*. The predictions included the possibility of FabT binding sites in promoter regions of the *fabM*, *fabK* and *fabT*$_{Sm}$ genes of *S. mutans*, similar to the predicted, and experimentally verified, locations in the *S. pneumoniae* genome (Lu & Rock, 2006) and the *L. lactis* genome (Eckhardt *et al*., 2013). In *S. mutans*, there are two sites predicted upstream of both *fabK* and *fabM*, and one site upstream of *fabT*$_{Sm}$ (Fig. 2B,C), suggesting that FabT$_{Sm}$ may have a role in the expression of these genes.

The presence of small intergenic regions (IGRs), between reading frames in the *S. mutans fab* gene cluster, suggested that the clusters of open reading frames might be co-transcribed. To test this hypothesis, we performed reverse-transcription reactions, using RNA prepared from cells grown at pH 7, between the following loci: *fabM* and *fabT*$_{Sm}$, *fabH* and *acp*, *acp* and *fabK*, *fabG* and *fabF*, and *fabF* and *accB* (Fig. 3). The results revealed co-transcription across all of the junctions tested, except the IGR between *fabM* and *fabT*$_{Sm}$. In addition, the results of primer extension analysis identified a potential start site of transcription between *acp* and *fabK* (data not shown). These data suggested the possibility that multiple transcripts could be involved in *fab* transcription and that, probably, the genes downstream of *fabT*$_{Sm}$ are part of the same transcript.

Loss of FabT$_{Sm}$ affects transcriptional regulation of fatty acid biosynthetic genes

The data so far suggested that FabT$_{Sm}$ might influence expression of the fatty acid biosynthesis genes via consensus motifs, specifically *fabM* and *fabK*, and that transcription of the downstream *fab* genes may be driven from the promoter preceding *fabT*$_{Sm}$. To investigate the level of transcriptional regulation via the putative FabT$_{Sm}$ motifs, we created *cat*-promoter fusion constructs that included potential binding sites for FabT$_{Sm}$ in the promoter regions of *fabT*$_{Sm}$, *fabK* and *fabM* (Figs 2B and 4). Loss of *fabT*$_{Sm}$ led to elevated *fabT*$_{Sm}$ promoter expression in cells grown at pH values of 5 or 7 (Table 3A). Loss of *fabT*$_{Sm}$ also correlated with significantly elevated *fabM* expression in cells grown at pH values of 5 or 7 (Table 3C). These findings support, and provide at least one mechanism for, the increased UFA : SFA ratio seen in the *S. mutans fabT* deletion strain. In contrast, in the *S. pneumoniae* FabT regulon, FabT$_{Sp}$ did not exhibit transcriptional control over *fabM* expression, but did exhibit a reduction in the UFA : SFA ratio in the membrane of the *fabT*$_{Sp}$ mutant strain (Lu & Rock, 2006). The *L. lactis* Δ*fabT* strain also exhibited a higher percentage of SFAs in the membrane; however, the organism does not encode a *fabK* ortholog (Wegmann *et al*., 2007; Eckhardt *et al*., 2013). The authors suggest that *fabI*, a trans-2-enoyl-ACP reductase, may act as a *fabK* substitute, and FabT$_{Ll}$ has been shown to control *fabI* expression. Interestingly, *fabK* transcription in *S. mutans* did not appear to originate from a promoter in the IGR directly upstream of the start site of translation. In fact, promoter-*cat* reporter fusions from this IGR did not yield chloramphenicol acetyl-transferase activity above background in either the parent strain or the *fabT*$_{Sm}$ deletion strain under the conditions tested (data not shown).

The promoter regions of *fabM* and *fabK* revealed two potential binding sites for FabT$_{Sm}$ (Fig. 4A,C, respectively). We wished to determine whether both sites were necessary for binding of the potential regulator. A truncated version of the full-length fabK promoter (indicated by brackets in Fig. 4C) was tested that did not include the distal FabT$_{Sm}$ binding

site. This smaller promoter fragment showed no difference from full-length *fabK*-promoter activity; that is, there was still no activity above background levels even in the absence of one of the motifs (data not shown). However, removal of the distal FabT$_{Sm}$ binding site upstream of the *fabM* coding region, that is, using only the region bracketed in Fig. 4A, abolished *fabM* transcriptional activity, probably as the result of the lack of −10/−35 promoter sequences and the start site of transcription (data not shown).

Local effects of *fabT*$_{Sm}$ deletion

The transcriptional data indicated that the presence of the FabT$_{Sm}$ motifs correlated with expression of *fabT*$_{Sm}$ and *fabM*. We next wanted to examine if the absence of *fabT*$_{Sm}$ would affect transcription of genes flanking *fabT*$_{Sm}$, indicating that the promoter in front of *fabT*$_{Sm}$ may be important for regulation of their expression. In the case of *fabH*, *acp* and *fabK*, the loss of *fabT*$_{Sm}$ resulted in a statistically significant reduction in the copy number from cultures grown to steady-state at pH 7, when compared with copy number from cultures of the parent strain grown under the same conditions (Fig. S2A–C). Expression of *acp* and *fabK* is not influenced by pH value in the parent strain, whereas in the Δ*fabT*$_{Sm}$ strain, the expression of these genes is elevated at low pH (Fig. S2). We infer from the transcriptional data (Fig. S2) and the reverse transcription reactions (Fig. 2) that the promoter in front of *fabT*$_{Sm}$ may play a role in the expression of the *fab* genes from *fabT*$_{Sm}$ through *fabF*. Further, we show an effect on transcription of *fabM*, in the absence of *fabT*$_{Sm}$, that mimics the effects on the downstream genes in the cluster (Fig. S2D).

Differential binding of FabT$_{Sm}$ to *fabT*$_{Sm}$ and *fabM* promoters

To verify that the promoter elements affected by the presence of FabT$_{Sm}$ were the reason for the observed changes in transcription, we performed EMSAs to investigate the ability of FabT$_{Sm}$ to bind directly to DNA fragments derived from the full-length promoter–fusion constructs (illustrated in Fig. 4). The results revealed that the promoter region of *fabM* bound specifically to FabT$_{Sm}$ (Fig. 5A; lane 2), compared with the absence of a shift in the presence of BSA (Fig. 5A; lane 5). The presence of a non-specific competitor, poly(dG-dC), did not compete for FabT$_{Sm}$ binding of the target DNA sequence (Fig. 5A; lane 4). The specificity of FabT$_{Sm}$ binding to the *fabM* promoter was also supported by the observation that non-radiolabelled, *fabM* promoter DNA was an effective competitor for protein binding to labeled DNA (Fig. 5A; lane 3). Similar results were observed when we examined the ability of FabT$_{Sm}$ to bind its cognate promoter (see Fig. S3, lane 2 compared with lane 1), suggesting a role for FabT$_{Sm}$ in auto-regulation supported by transcription assay data (Table 3A).

A previous study in *S. pneumoniae* demonstrated the ability of various length acyl-ACPs to enhance the affinity of FabT$_{Sp}$ for target DNA sequences (Lu & Rock, 2006; Jerga & Rock, 2009). We examined the contribution of $C_{18:1}$ acyl-ACP, from *S. pneumoniae*, to the ability of FabT$_{Sm}$ to bind to the promoters of interest, *fabT*$_{Sm}$, *fabM* and *fabK*. The results indicate that the addition of the long chain-length ACP did not act as a co-effector for FabT$_{Sm}$ and did not contribute to greater specificity of the repressor for its cognate motif when concentrations of FabT$_{Sm}$ are limiting (data not shown).

We observed that *fabM* expression was affected by the presence of the FabT$_{Sm}$ motifs within the promoter. In an effort to determine whether the FabT$_{Sm}$ repressor was able to bind to the proximal FabT$_{Sm}$ motif in the *fabM* promoter (Fig. 4A, bracketed sequence), we performed an EMSA using this *fabM*-truncated promoter as a probe and, indeed, the presence of only one FabT$_{Sm}$ motif was sufficient to allow binding of the probe to the protein (Fig. 5B, lane 2). This DNA–protein affinity was also evident at lower concentrations of FabT$_{Sm}$, but again, the presence of the acyl-ACP did not enhance binding (data not shown). FabT$_{Sm}$ also bound to the *fabT*-motif in the IGR preceding *fabK*, suggesting a potential role for FabT$_{Sm}$ in regulating *fabK* (data not shown).

Global effects of *fabT$_{Sm}$* deletion

Given the evidence so far that *fabM* expression is governed by FabT$_{Sm}$, and that our previous work has shown that the loss of *fabM* resulted in dramatic sensitivity to acidic conditions (Fozo & Quivey, 2004b), we were interested in potential global effects of the loss of the FabT$_{Sm}$ regulator. FabT$_{Sm}$ is also a member of the larger MarR transcriptional regulator family and we hypothesized that deletion of the *fabT$_{Sm}$* locus may impact the transcriptome of the organism. Microarray analysis was performed to ascertain potential global effects of the loss of FabT$_{Sm}$. RNA isolated from steady-state cultures of the Δ*fabT$_{Sm}$* and parent strain, UA159, grown at pH values of 7 and 5, was used examine changes due to environmental pH conditions in the transcriptome of the deletion strain, compared with the parent strain. Using a two-fold cutoff for expression with a *P*-value ≤ 0.01, there were 647 genes, over 30% of the genome, whose transcription was affected. Our observations may reflect the broad ramifications of altering the UFA : SFA ratio in an organism whose membrane is finely tuned to changes in external pH, indicating the ability of *S. mutans* to adjust to stress. We found 230 genes that were upregulated and 305 genes down-regulated at pH 7 in Δ*fabT$_{Sm}$*, and 94 genes upregulated and 55 genes downregulated at pH 5 in Δ*fabT$_{Sm}$*, compared with the parent strain. Furthermore, relative to expression levels in the parent strain, the genes annotated as encoding hypothetical proteins represented the largest functional class of differentially-regulated genes, whether up or down, for both pH 7-grown and pH 5-grown cells. Other highly impacted functional classes, those for which the most genes were either upregulated or downregulated, under each condition tested, included transport and binding proteins, protein synthesis, cellular processes and energy metabolism (Fig. 6). Using the FabT$_{Sm}$ consensus motif (Fig. 2A) to search the genome for potential binding sites, with zero mismatches, via Virtual Footprint (Münch *et al*., 2005), 34 candidate genes were identified, and 17 of these genes appeared in the microarray data set within the parameters that we established.

Coordinated regulation of fatty acid biosynthesis

The number of genes affected by the loss of the FabT$_{Sm}$ regulator, approximately 30% of the reading frames in *S. mutans*, clearly suggested the important role of UFA : SFA content in the physiology of *S. mutans*, as well as the magnitude of the response by the organism to maintain homeostasis in the absence of FabT$_{Sm}$. In an effort to classify the genes included in our array data sets, we used MEME analysis (Bailey & Elkan, 1994; Bailey *et al*., 2009) to predict shared motifs among the intergenic regions preceding open reading frames that were

differentially regulated in the absence of *fabT*$_{Sm}$, at pH 7 and pH 5, looking for commonality of the genes affected.

Allowing for the discovery of a maximum of six motifs, with any number of repetitions, three motifs were predicted with an e-value of $\leq 10^{-9}$. Further analysis of these predicted motifs with TOMTOM (Tool for Motif to Motif comparison; Gupta *et al*., 2007; Bailey *et al*., 2009; Tanaka *et al*., 2011) using the RegTransBase database of manually curated prokaryotic motifs yielded matches to three subfamilies of regulatory proteins: LacI, GntR and AraC/XylS/XylR. All three regulators are part of the helix-turn-helix (H-T-H) family of DNA-binding proteins that differ in their cognate binding sequences and location of the H-T-H domain.

We then sought to determine which of the families of regulators, encoded by streptococci, appeared in the microarray data and were differentially-regulated in response to the loss of *fabT*$_{Sm}$. A single transcript encoding a LacI regulator, two genes encoding GntR regulators, and no AraC/XylS/XylR regulators appeared in the microarray data.

LacI-family regulation

The single LacI regulator that fitted the criteria we set was SMU.1591c, encoding the global regulator CcpA (Simpson & Russell, 1998). SMU.1591c was down-regulated in the Δ*fabT*$_{Sm}$ strain compared with the parent strain during growth at pH 7. CcpA is well established as a negative regulator of metabolism, that acts by binding to catabolite responsive elements (*cre*) and represses transcription of the downstream gene (Hueck & Hillen, 1995; Titgemeyer & Hillen, 2002; Sonenshein, 2007). CcpA has also been demonstrated to play a role in the regulation of branched-chain amino acid production by positively regulating *ilvE* (Santiago *et al*., 2013). Previous work has indicated that *fabT*$_{Sm}$ is differentially transcribed in the absence of ccpA (Abranches *et al*., 2008) and analysis of the *S. mutans* UA159 genome for potential *cre* sites via xBASE (Chaudhuri *et al*., 2008) predicts the motif to be located upstream of the *fabT*$_{Sm}$, *fabM* and *fabK* genes. To test whether CcpA may be involved in fatty acid biosynthesis, we performed transcriptional reporter assays to determine the effect of the loss of *ccpA* on expression of *fabT*$_{Sm}$ and *fabM*. As shown in Table 3A, in a strain carrying a *ccpA* deletion, the amount of CAT activity, as a measure of *fabT*$_{Sm}$ transcription, was significantly elevated, when compared with the levels of activity in the parent strain background, at both a pH value of 5 and 7. The elevated CAT activity was indicative of de-repression of *fabT*$_{Sm}$ by CcpA. As CcpA has been implicated in the regulation of genes involved in growth and homeostasis during low carbohydrate availability, we also tested the *fabT*$_{Sm}$ promoter-*cat* fusion following growth of the reporter strain in FMC minimal media (Terleckyj & Shockman, 1975; Terleckyj *et al*., 1975). As shown in Table 3B, transcription of *fabT*$_{Sm}$ was elevated in the Δ*ccpA* strain grown in either rich or minimal medium, compared with expression levels in the UA159 strain, indicating that low nutrient availability itself does not appear to play a role in *fabT*$_{Sm}$ transcription. Rather, these results are similar to those observed in the Δ*ccpA* strain grown in buffered rich medium to pH values of 5 and 7, where the *fabT*$_{Sm}$ expression levels are increased due to the loss of *ccpA*.

Transcription from the *fabM* promoter was also tested in a Δ*ccpA* background. Here, the loss of CcpA resulted in an intermediate level of *fabM* expression, compared with expression in the parent strain or the Δ$fabT_{Sm}$ strain, at both pH values of 5 and 7. In fact, culture pH had no effect on the expression of *fabM* in the absence of *ccpA* (Table 3C).

Following the transcriptional fusion experiments, we tested the ability of purified, recombinant CcpA to directly bind to the same full-length intergenic regions (shown in Fig. 4) used in the binding assays with $FabT_{Sm}$ and in the *cat*-reporter assays, containing the predicted *cre* sites located upstream of the *fabM*, $fabT_{Sm}$ and *fabK* coding regions. Results from EMSAs show that CcpA bound specifically to all three fragments (Fig. 7), suggesting a direct role for the carbon-catabolite repressor, CcpA, as a participant in the regulation of fatty acid biosynthesis in *S. mutans*.

GntR regulation

The two GntR-encoding regulators identified in the microarrays were SMU.1065c, a hypothetical GntR regulator, and SMU.2040, orthologous to the *Bacillus subtilis* TreR (Helfert *et al*., 1995). Both *S. mutans* genes are annotated as regulatory proteins, based on conserved H-T-H and UTRA (UbiC transcriptional regulator-associated) domains at their N-terminus and C-terminus, respectively; but there are no previous reports on their specific function. It is of note, however, that these predicted proteins are the sole UTRA domain-containing regulators, responsible for modulating activity of bacterial transcription factors in response to a variety of ligands, in the *S. mutans* genome. The association of these conserved domains within the same peptide, mosaic modular association, has been described in *B. subtilis* for the hybrid protein AraR (Mota *et al*., 1999; Rigali *et al*., 2002). Further, these loci appear to be lethal mutations for *S. mutans* (Faustoferri, R.C., Hubbard, C.J. and Quivey, Jr., R.G., unpublished observation), suggesting a vital role in regulation.

DISCUSSION

In the present study, we focused on the regulatory aspects of $FabT_{Sm}$ with respect to production of UFA in *S. mutans*, a well-established member of the complex microbial community found on tooth surfaces (Takahashi & Nyvad, 2011; Nyvad *et al*., 2013). Examination of fatty acid biosynthesis in the gram-positive organisms *S. pneumoniae* and *L. lactis* demonstrated a role for the MarR-family regulator FabT in modulating the proportion of saturated membrane fatty acids. We observed that while there are similarities with the *S. pneumoniae* and *L. lactis* regulatory systems (Lu & Rock, 2006; Jerga & Rock, 2009), there are substantial differences, and additional regulators of production of UFAs in *S. mutans* that indicate responsiveness to a range of environmental stresses confronting the organism in the oral cavity. These studies show that the regulators include, at least the local regulator, $FabT_{Sm}$, and the global regulator, CcpA.

We observed elevated transcription from the $fabT_{Sm}$ promoter in cells grown at low pH, and transcription from the *fabM* promoter that was dependent on $FabT_{Sm}$. No detectable transcription was noted from the putative *fabK* promoter, despite the presence of predicted $FabT_{Sm}$ sites. These results contrast with patterns reported in *S. pneumoniae* and *L. lactis*. We interpret the differences between the organisms, particularly with regard to pH-

responsive transcription of *fabM*, as being an evolutionarily beneficial arrangement for *S. mutans*. Previous work from our group has demonstrated that the membrane composition of the organism changes rapidly, within 20 min, in response to acidification, with the purpose of protecting acid-sensitive glycolytic enzymes (Fozo & Quivey, 2004b). The acid-protective effect maintains ΔpH values (Fozo & Quivey, 2004a), shields glycolytic enzymes from the inimical effects of low pH (Bender *et al*., 1986; Sutton & Marquis, 1987), and elevates proton-removal from cells by concomitant up-regulation of the *atp* operon, producing more membrane-bound F-ATPase (Kuhnert *et al*., 2004). In support of this, the microarray data from the present study indicate that the organism responds aggressively to loss of FabT, with approximately one-third of its genome involved in a survival response, including the genes encoding the membrane subunits of the F-ATPase operon, *atpG*, *atpF* and *atpE*.

Interestingly, the occurrence of FabT binding sites appears to be universal in streptococci, as is the appearance of a *fabM* gene. However, the distribution of FabT binding sites within organisms varies. Existing data indicate that the sites present in *Streptococcus sanguinis* and *Streptococcus gordonii*, oral streptococci generally considered to be non-aciduric, correlate with several observable traits: reduced acid-responsiveness, relatively lower production of UFAs compared with *S. mutans*, and membrane F_1F_0-ATPase activity that is both reduced in specific activity and in abundance of enzyme itself (Sutton & Marquis, 1987; Fozo *et al*., 2004; Sheng & Marquis, 2007). For example, in *S. sanguinis*, two $FabT_{Sm}$ binding sites are predicted upstream of the $fabT_{Ss}$ ortholog, but only one site upstream of *fabK* and *fabM*; whereas, there is a single, predicted, binding site upstream of each of these genes in *S. gordonii* and *S. pneumoniae*. Hence, we hypothesize that the distribution of FabT binding sites in streptococci is part of an overall system contributing to the aciduricity of a given member of the genus. Indeed, our previous studies have shown that loss of UFA-producing capacity results in diminished ΔpH values in *S. mutans* and elevated transcription of the F-ATPase operon, apparently in an attempt to maintain intracellular homeostasis. Non-aciduric microorganisms such as *S. sanguinis*, *S. gordonii*, *S. pneumoniae* and *L. lactis* also have pH optima for their respective F_1F_0-AT-Pase enzymes that are between 1 and 1.5 pH units higher than the optimum pH for the enzyme in *S. mutans* (Sutton & Marquis, 1987; O'Sullivan & Condon, 1999; Martin-Galiano *et al*., 2001).

It is also important to consider that in *S. pneumoniae* and in *L. lactis*, loss of FabT results in a decrease in the UFA : SFA ratio (Lu & Rock, 2006; Eckhardt *et al*., 2013). Here, we demonstrate that $FabT_{Sm}$ appears to interact with, and affect transcription from the *fabM* and $fabT_{Sm}$ promoters, thereby increasing the UFA : SFA ratio. It is well established that loss of UFAs in *S. mutans* results in acid-sensitivity, a reduction in ΔpH across the membrane, and reduction in virulence potential (Fozo & Quivey, 2004a, b; Fozo *et al*., 2007); though the role of membrane unsaturated fatty acids in the acid tolerance response of *S. mutans* is still to be elucidated at the biophysical level.

In addition to the local regulator $FabT_{Sm}$, the presence of *cre* sites upstream of the *fabM*, $fabT_{Sm}$ and *fabK* genes suggested that CcpA, the global regulator of carbon metabolism genes in bacteria (Bruckner & Titgemeyer, 2002), including *S. mutans* (Simpson & Russell, 1998; Abranches *et al*., 2008), may play a role in regulation of these *fab* genes. This

hypothesis is borne out by the data from reporter fusions and EMSAs reported here: loss of CcpA leads to elevated $fabT_{Sm}$ transcription, and as $FabT_{Sm}$ acts to repress *fabM*, transcription of *fabM*, in a Δ*ccpA* background, is also repressed. We interpret the data to indicate that regulation of membrane fatty acids is consistent with global regulation of carbon metabolism in cells. The production of membrane fatty acids, necessarily coordinated with central carbon metabolism, is now firmly established, and has been shown to be a part of the regulons for the global pathways controlled by CcpA and CodY (Santiago *et al*., 2012, 2013).

In summary, we have shown that the regulation of unsaturated fatty acid production in *S. mutans* exhibits a use of the FabT regulator that is different from the *S. pneumoniae* and *L. lactis* systems, which we attribute to the organism's distinct microbiological niche. Wide changes occur in carbohydrate availability and pH values in the oral cavity, in conjunction with the presence of abundant numbers of competing organisms. An increased proportion of membrane UFAs gives *S. mutans* a survival advantage in this niche, potentially providing an altered milieu for membrane proteins involved in the acid-adaptive response (Abranches, J., Faustoferri, R.C., Hubbard, C.J., Courtney, M., and Quivey, Jr., R.G., in preparation). Hence, it is reasonable to propose a model that accounts for the involvement of $FabT_{Sm}$, a local regulator of fatty acid biosynthesis, with specific binding motifs that function as part of a rapid response to decreasing pH values (Fig. 8). The model also incorporates CcpA, a regulator that responds to carbon availability and a key regulator of overall cell growth and metabolism. These two transcriptional regulators act upon the genes responsible for fatty acid biosynthesis, enabling *S. mutans* to endure the decrease in pH resulting from fermentation end products and concurrent depletion of available carbon sources. Future studies will explore other global regulators, such as the GntR orthologs, that may also participate in the modulation of UFA production in *S. mutans* in response to environmental stress.

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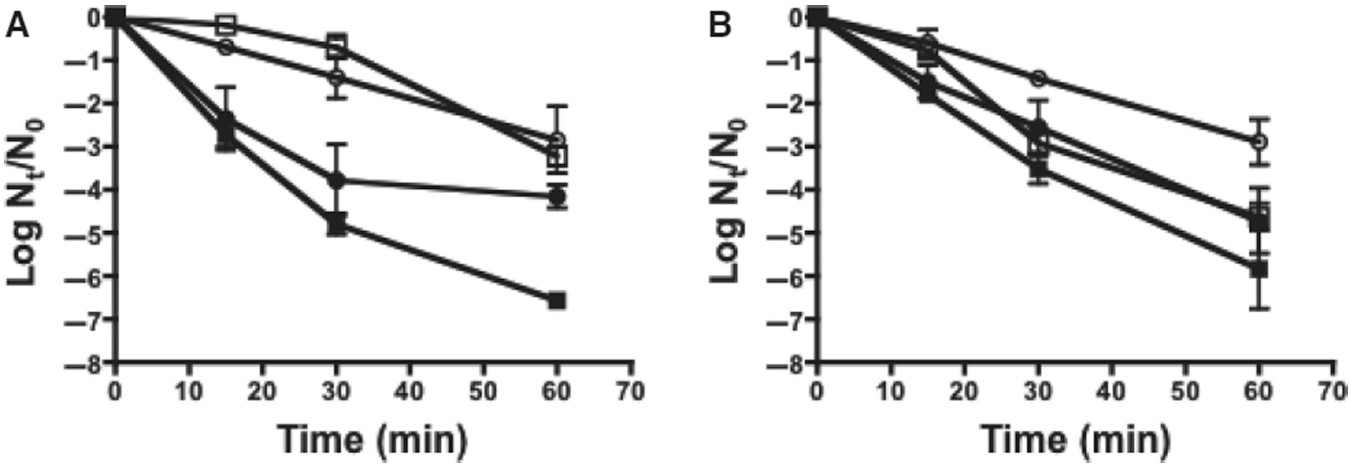

Figure 1.

Stress sensitivity assays. *Streptococcus mutans* UA159 (□) and MU1591 (Δ*fabT*$_{Sm}$) (○) were grown to steady-state in chemostat cultures to pH values of 5 (open symbol) and 7 (filled symbol). Data are from three independent cultures and represented as Log N/N_0. (A) Survival of strains following acid challenge (pH 2.5). (B) Survival of strains following peroxide challenge (16.3 mM).

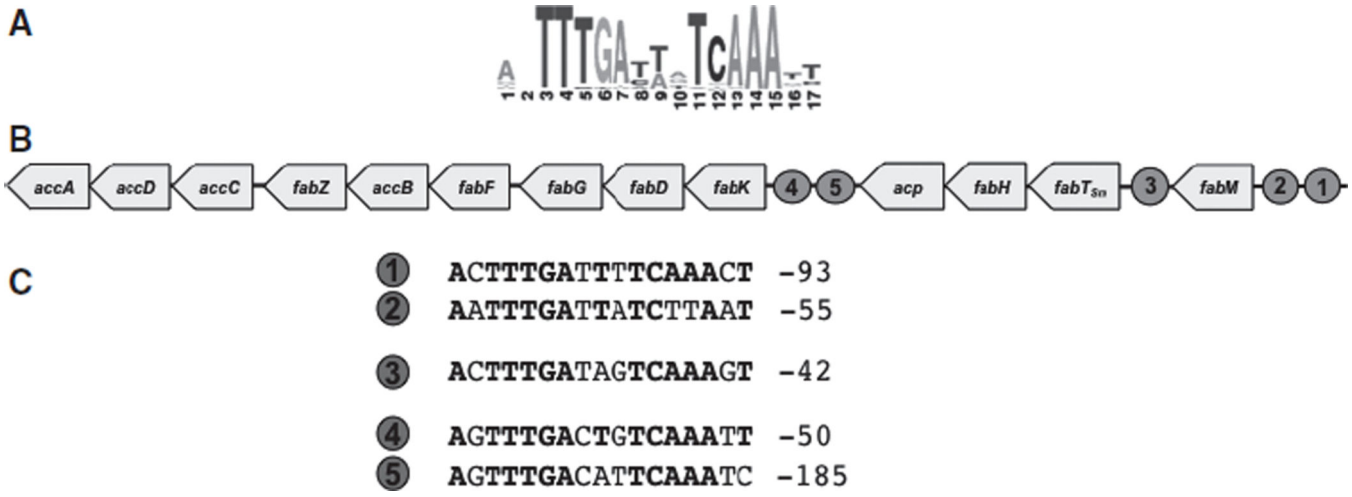

Figure 2.

FabT$_{Sm}$ conserved motifs. (A) Conserved motif for FabT$_{Sm}$ binding depicting probability of nucleotide at each position (from http://regprecise.lbl.gov/RegPrecise/gmregulon.jsp?gmproject_id=6926) (Novichkov *et al*., 2010). (B) Genetic organization of fatty acid biosynthetic genes in *Streptococcus mutans* and locations of putative FabT$_{Sm}$ binding sites adjacent to genes regulated (designated by the numbered circles). (C) Nucleotide sequence of proposed binding site(s) where number on left corresponds to numbered circle in (B) and number on right represents location relative to translational start site. Text in bold indicates conserved sequence.

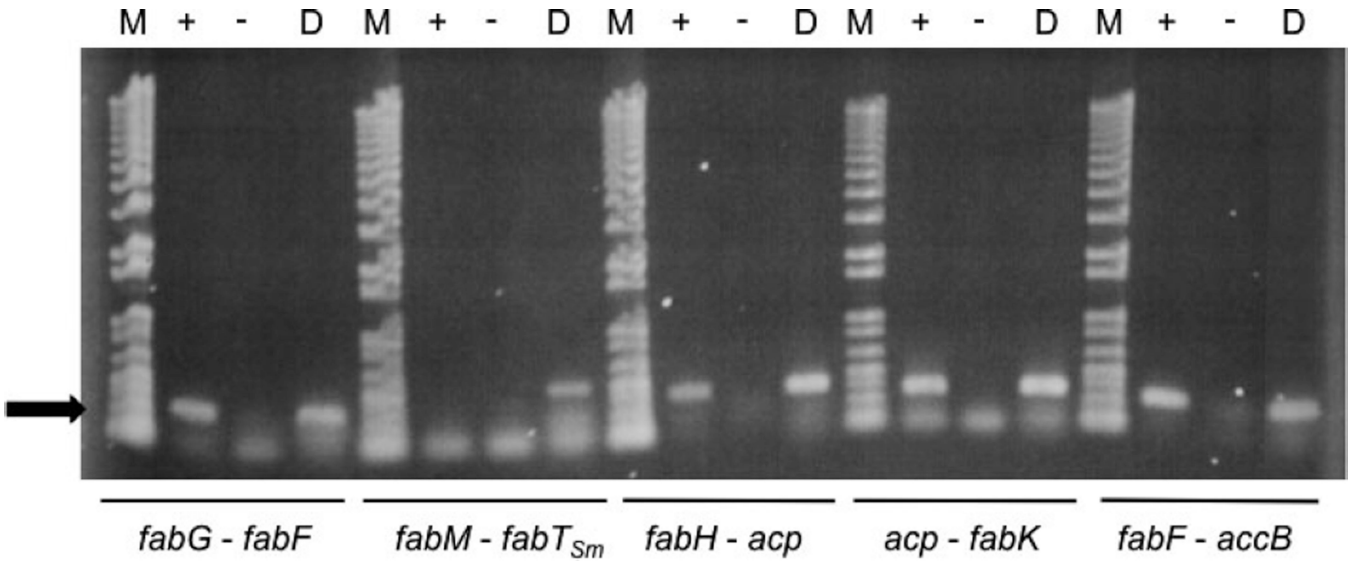

Figure 3.

Co-transcription of genes in *fab* gene cluster. Total RNA (43 ng) isolated from a steady-state culture of *Streptococcus mutans* UA159 grown to a pH value of 7 was incubated with the anti-sense primer in each primer pair with (+) or without (−) SuperScript II Reverse Transcriptase. Each primer pair was also used in a standard PCR with UA159 genomic DNA as a positive control (D). Primers spanned the intergenic spaces between *fabG/fabF*, *fabM/fabT*$_{Sm}$, *fabH/acp*, *acp/fabK* and *fabF/accB* as indicated in the figure. M = 1 kb+ DNA Ladder. RT-PCR or PCR products are indicated by arrow at left of figure.

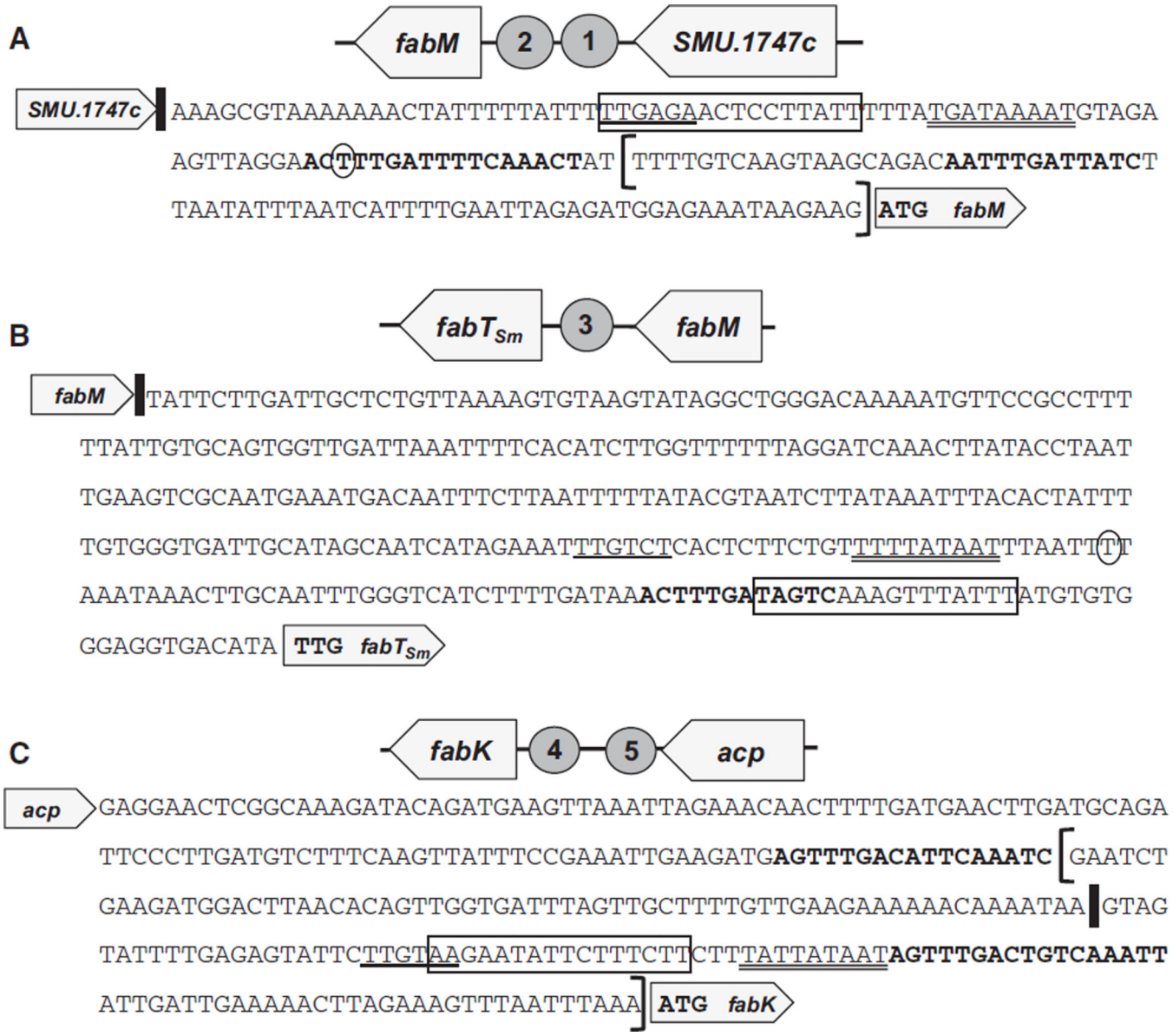

Figure 4.

Intergenic regions (IGRs) containing consensus FabT binding sites. In all panels: the putative −35 sequence (single underline), −10 sequence (double underline), and predicted transcriptional start site (circled nucleotide in A and B) (as predicted by BPROM, Softberry, promoter prediction); vertical bars indicate translational stop sites; sequence in boldface is the predicted FabT$_{Sm}$ binding motif and boxed sequence is the predicted *cre* site (predicted by RegPrecise: Novichkov *et al*., 2010). Brackets in (A) and (C) indicate the shorter, truncated promoters lacking one FabT$_{Sm}$ motif used in transcriptional fusion assays. (A) IGR preceding *fabM*. (B) IGR preceding *fabT$_{Sm}$*. (C) IGR preceding *fabK*.

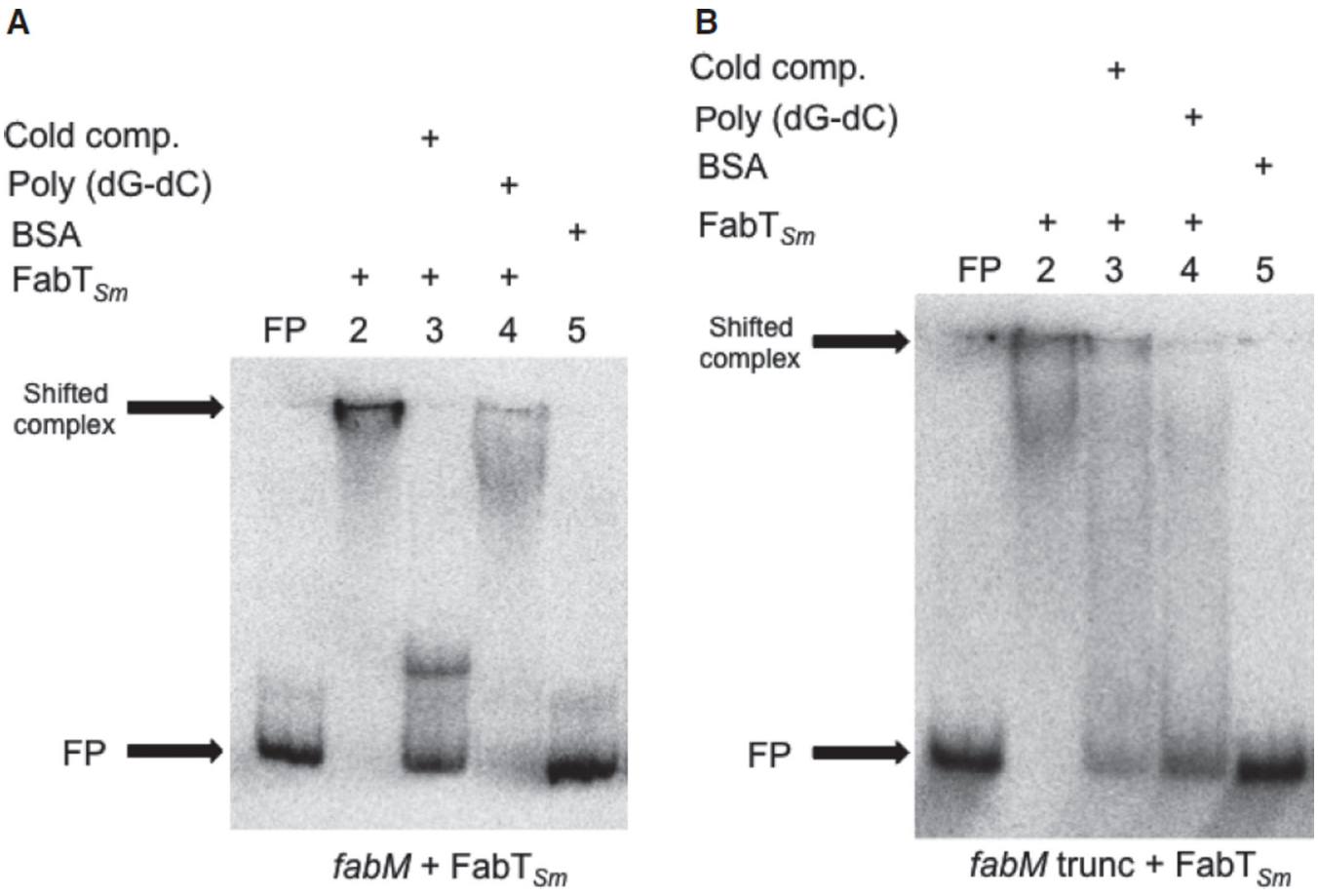

Figure 5.

FabT$_{Sm}$ binds to the *fabM* promoter. Electrophoretic mobility shift assays demonstrating binding of FabT$_{Sm}$ to the *fabM* promoter regions detailed in Fig. 4: the full-length *fabM* promoter region (A), or the truncated *fabM* promoter region lacking the distal FabT$_{Sm}$ motif (B). For each panel, FP = free probe (5000 counts min^{-1}) (no protein added); lane 2 = promoter + FabT$_{Sm}$ (1000 nM) (binding reaction); lane 3 = promoter + FabT$_{Sm}$ (1000 nM) + cold competitor (200-fold excess unlabeled, cognate promoter DNA); lane 4 = promoter + FabT$_{Sm}$ (1000 nM) + unlabeled, non-specific competitor (1 μg poly (dG-dC)); lane 5 = promoter + BSA (1000 nM).

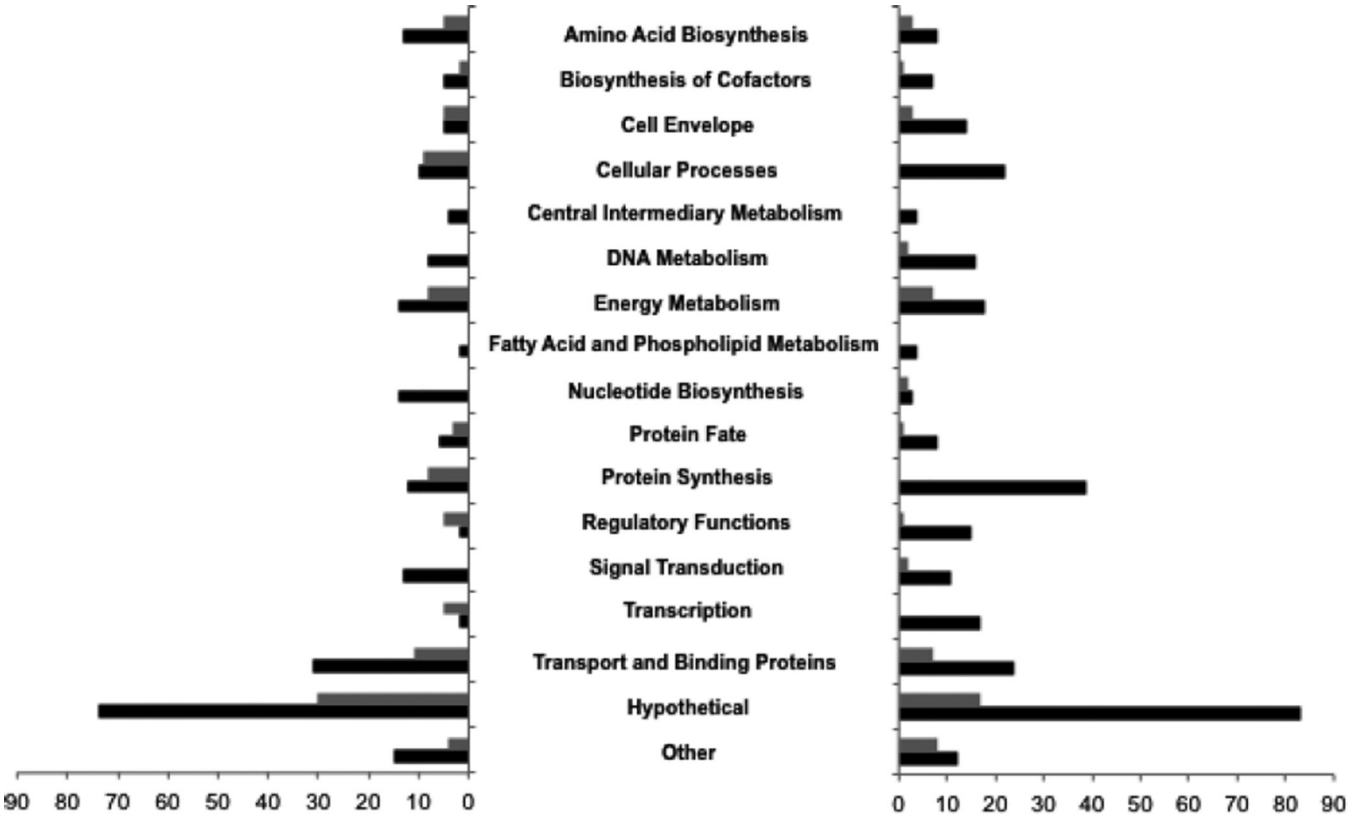

Figure 6.

Loss of FabT$_{Sm}$ results in changes in global transcription. RNA isolated from cultures of *Streptococcus mutans* UA159 and MU1591 (Δ*fabT*$_{Sm}$) grown to steady-state pH values of 5 or 7 were used to synthesize cDNA for microarray analysis. Functional classes down-regulated in the MU1591 strain compared with parent strain are on the left graph, functional classes up-regulated in the deletion strain are on the right graph. Gray bars represent number of genes differentially expressed in steady-state cultures grown to a pH value of 5, black bars represent number of genes differentially expressed in steady-state cultures grown to a pH value of 7. A two-fold cutoff was used for change in expression with a *P*-value ≤ 0.01, as determined by BRB-Array tools; $n = 4$.

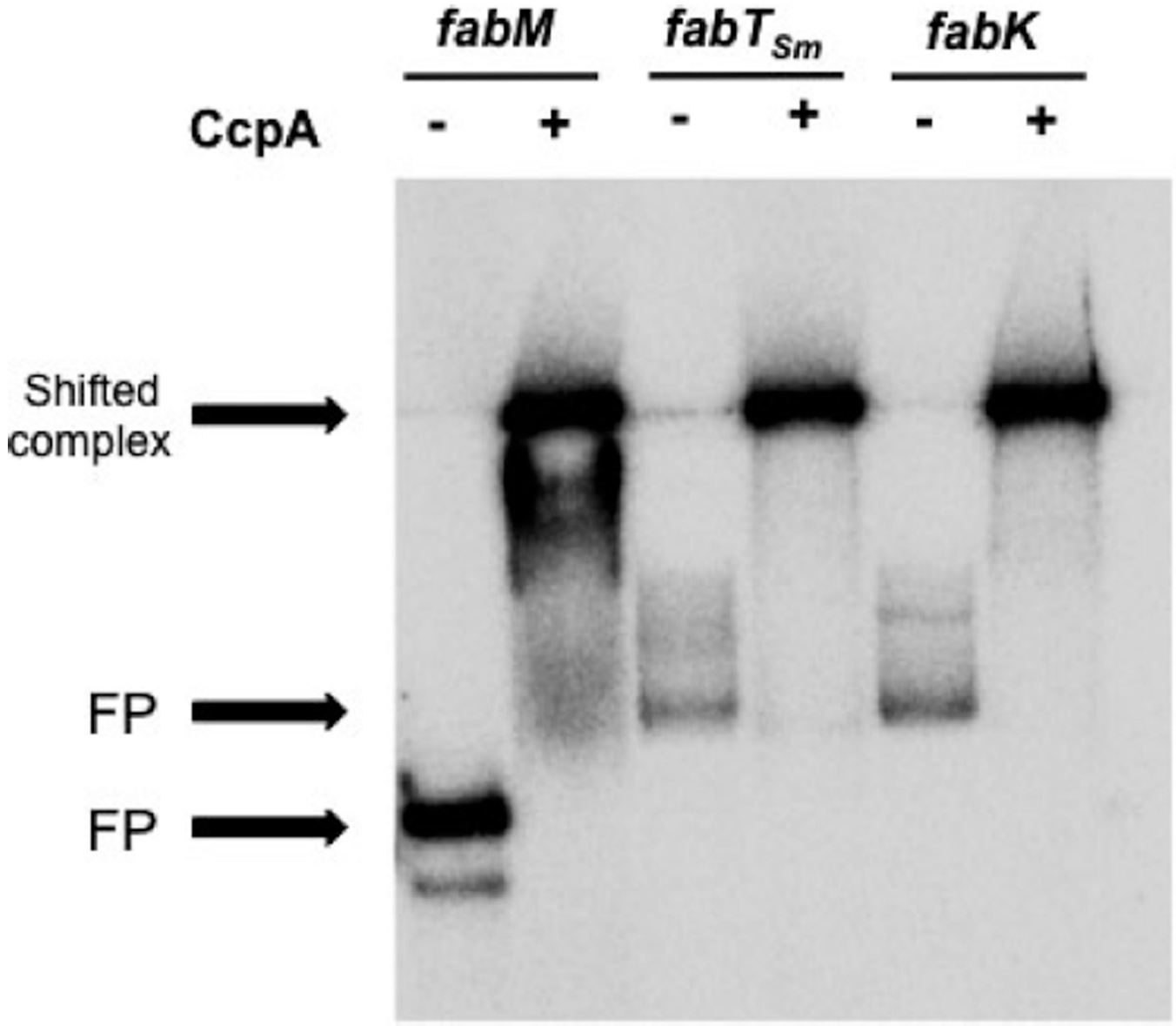

Figure 7.

CcpA binds to intergenic regions preceding *fabM*, *fabT*$_{Sm}$and *fabK*. Electrophoretic mobility shift assay demonstrating binding of CcpA to the intergenic regions preceding *fabM*, *fabT*$_{Sm}$ and *fabK*"—" = free probe (3000 counts min^{-1}) (no protein added); "+" = probe + CcpA (3000 n$_M$).

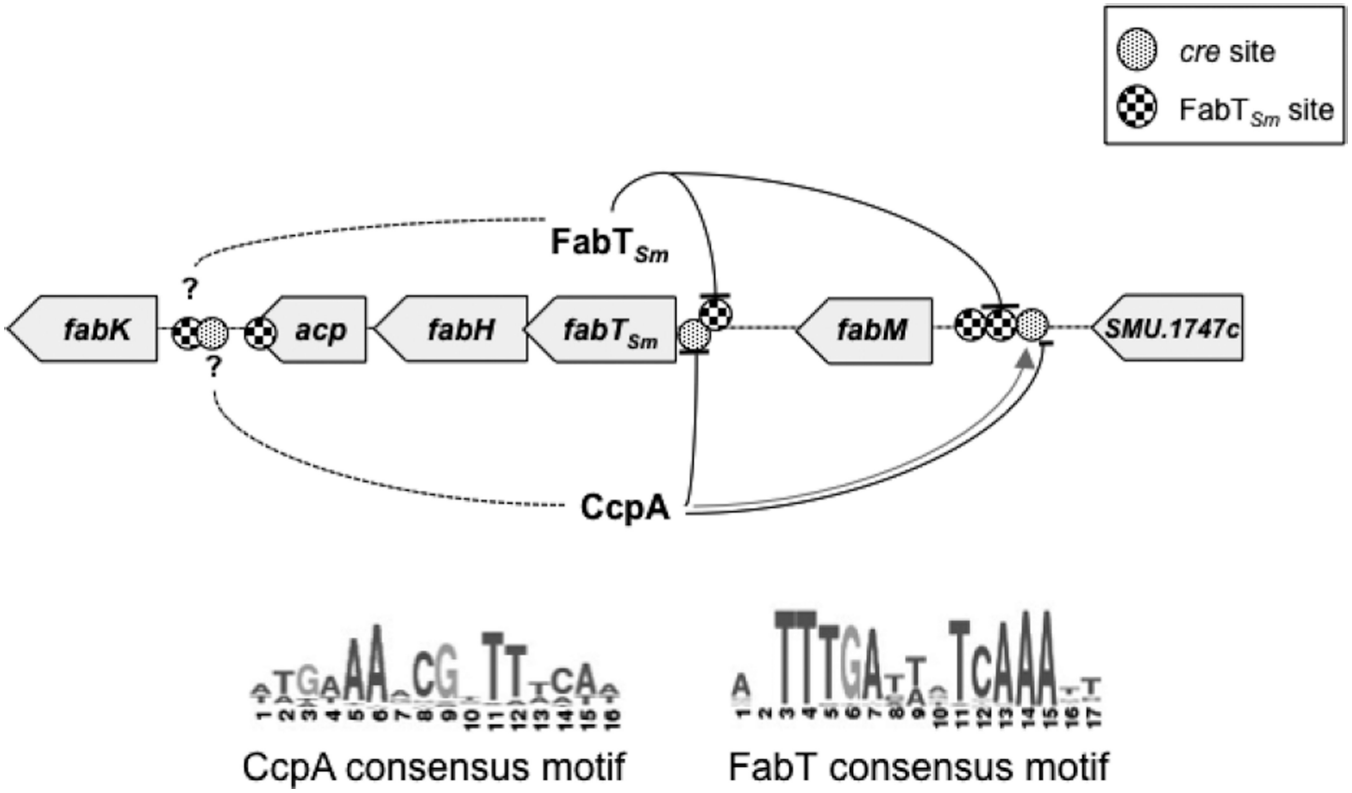

Figure 8.

Proposed model for regulation of fatty acid biosynthesis gene cluster by FabT$_{Sm}$ and CcpA. Location of binding sites for CcpA (*cre* sites) and FabT$_{Sm}$ are shown with respect to their relative position to the coding regions in the fatty acid biosynthetic gene cluster. FabT$_{Sm}$ binds to promoter elements in front of *fabT$_{Sm}$ fabM* and *fabK*. FabT$_{Sm}$ acts as a repressor of *fabT$_{Sm}$* and *fabM* transcription; however, the magnitude of repression is dependent on environmental pH. Under acidic growth conditions (pH 5), CcpA binds to the intergenic region preceding *fabT$_{Sm}$*acting as a repressor. In acidic growth conditions, CcpA also binds to the intergenic region preceding *fabM* thereby activating transcription (gray line with arrow). However, at neutral pH (pH 7), CcpA represses *fabM* expression (black line with bar). CcpA also plays a role in repressing *fabT$_{Sm}$* transcription independent of environmental pH. The role for CcpA and FabT$_{Sm}$ in regulation of *fabK*, if any, is currently unknown.

Table 1

Strains and plasmids

Strains	Genotype description	References
Streptococcus mutans		
UA159	Genomic type strain	Murchison *et al*. (1986), Ajdic *et al*. (2002)
MU1446	*ccpA* deletion strain	Santiago *et al*. (2013)
MU1591	*fabT*$_{Sm}$ deletion strain	This study
UR211	*fabM-cat* in UA159	This study
UR221	*fabK-cat* in UA159	This study
UR222	*fabT*$_{Sm}$*-cat* in UA159	This study
UR237	*fabK-cat* in MU1591	This study
UR242	*fabT*$_{Sm}$*-cat* in MU1591	This study
UR243	*fabM-cat* in MU1591	This study
UR266	*fabK* truncated promoter-cat in UA159	This study
UR272	*fabM* truncated promoter-cat in UA159	This study
UR273	*fabK* truncated promoter-cat in MU1591	This study
UR274	*fabM* truncated promoter-cat in MU1591	This study
UR294	*fabT*$_{Sm}$ complement strain	This study
UR310	*fabT*$_{Sm}$-cat in MU1446	This study
UR313	*fabM-cat* in MU1446	This study
Escherichia coli		
Rosetta(λDE3)	F- *ompT hsdS*$_B$($r_B^- m_B^-$) *gal dcm* (DE3) pRARE (Cm^R)	Novagen
TOP10	F- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80*lacZ*Δ*M15* Δ*lacX74 recA1 araD139* Δ(*ara leu*) 7697 *galU galK rpsL* (Str^R) *endA1 nupG*	Invitrogen
Plasmids		
pCRBlunt	Cloning vector for blunt end PCR products	Invitrogen
pET19b	N-terminal His-tag expression vector	Novagen
pGEM7Zf+	Cloning vector	Promega
pJL84	Promoterless cat integration vector	Santiago *et al*. (2012)
pJLmarR	Promoter of SMU.1745c (*fabT*$_{Sm}$) in pJL84	This study
pJLfabK	Promoter of SMU.1742c (*fabK*) in pJL84	This study
pJLfabKtrunc	*fabK* promoter missing distal *FabT*$_{Sm}$ binding site in pJL84	This study
pJLfabM	Promoter of SMU.1746c (*fabM*) in pJL84	This study
pJLfabMtrunc	*fabM* promoter missing distal FabT$_{Sm}$ binding site in pJL84	This study
pSUGK-Bgl	Low-copy cloning vector derived from pBGK & pSU20 with *BglII* cloning site	Derr *et al*. (2012)
pSUGmarRLP7	pSUGK with *fabT*$_{Sm}$ coding region and native promoter	This study
pCRmarRLP8	pCRBlunt with *fabT*$_{Sm}$ coding region and native promoter	This study

Table 2

The unsaturated fatty acid : saturated fatty acid (UFA : SFA) ratio is increased in cultures of MU1591

Strain	pH	UFA : SFA
(A)		
UA159	7	0.60 ± 0.08[1]
UA159	5	2.00 ± 0.04[2]
Δ*fabT*$_{Sm}$	7	0.78 ± 0.08[1]
Δ*fabT*$_{Sm}$	5	2.19 ± 0.44[2]

Strain	pH	Long-chained : Short-chained
(B)		
UA159	7	0.58 ± 0.10[1]
UA159	5	3.19 ± 0.72
Δ*fabT*$_{Sm}$	7	0.82 ± 0.08[1]
Δ*fabT*$_{Sm}$	5	4.33 ± 0.29

Streptococcus mutans UA159 and MU1591 (Δ*fabT*$_{Sm}$) were grown to steady-state in chemostat culture to pH values of 7 and 5 in TY medium + 1% glucose.

Panel A: Values represent the UFA : SFA ratios for each culture condition ± standard deviation; $n = 3$.

Panel B: Values represent the ratio of long-chained membrane fatty acids (C_{18} or C_{20}) vs. short-chained membrane fatty acids (C_{10} through C_{16}) ± standard deviation; $n = 3$.

In both panels:

[1] $P < 0.05$;

[2] $P < 0.005$ for the pairwise comparisons between parent strain and MU1591, as determined by Student's *t*-test.

Table 3

FabT$_{Sm}$ and CcpA regulate transcription of *fabT*$_{Sm}$ and *fabM*

(A) *fabT*$_{Sm}$ transcription: pH effect		
Background	pH 5	pH 7
UA159	134.83 ± 7.05^{*,†,ø}	93.79 ± 7.68$^{*,\wedge,¥}$
Δ*fabT*$_{Sm}$	224.58 ± 43.47†	203.86 ± 9.14$^{\wedge}$
Δ*ccpA*	140.87 ± 11.17$^{ø,\#}$	157.51 ± 8.09$^{¥,\#}$
(B) *fabT*$_{Sm}$ transcription: minimal media		
Background	BHI	FMC
UA159	143.58 ± 12.78*,†	133.25 ± 15.26$^{*,¥}$
Δ*ccpA*	170.55 ± 5.97†	172.39 ± 29.18$^{¥}$
(C) *fabM* transcription		
Background	pH 5	pH 7
UA159	10.62 ± 1.59$^{*,\dagger,\#}$	2.45 ± 0.68$^{*,\wedge,ø}$
Δ*fabT*$_{Sm}$	18.25 ± 0.99$^{¥,\dagger}$	5.19 ± 0.65$^{¥,\wedge}$
Δ*ccpA*	4.64 ± 1.39$^{\#}$	4.24 ± 1.01^{ø}

Transcriptional activity from promoters of *fabT*$_{Sm}$ and *fabM* was measured using a chloramphenicol acetyltransferase reporter system. Cultures of *Streptococcus mutans* UA159, MU1591 (Δ*fabT*$_{Sm}$), or MU1446 (Δ*ccpA*) carrying promoter-*cat* fusion of interest, were grown in brain–heart infusion (BHI) medium titrated to a pH value of 5 with HCl or buffered to a pH value of 7 with 50 mM KPO_4 buffer (Panel A and C), or in BHI medium or FMC minimal medium + 1% (vol/vol) glucose in a 5% (vol/vol) CO_2/95% air atmosphere (Panel B). Panel A: *fabT*$_{Sm}$ promoter-*cat*; Panel B: *fabT*$_{Sm}$ promoter-*cat*; Panel C: *fabM* promoter-*cat*. Activity from the promoters is represented as nmol chloramphenicol acetylated min^{-1} mg protein^{-1}± standard deviation, $n = 3$. Statistical significance, between pairs, was determined by Student's *t*-test, where pairs of symbols indicate $P < 0.005$.